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Cloned Infectious cDNAs of *Lettuce Chlorosis Virus* (LCV) and Characterization of a *cis*-Acting Element in the 3' Non-Coding Region of LCV RNA 2 Involved in Minus-Strand RNA Synthesis

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Chawin Mongkolsiriwattana

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Dissertation Committee:
Dr. James C.K. Ng, Chairperson
Dr. A.L.N. Rao
Dr. Gregory P. Walker

The Dissertation of Chawin Mongkolsiriwattana is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Cloned Infectious cDNAs of *Lettuce Chlorosis Virus* (LCV) and Characterization of a *cis*-Acting Element in the 3' Non-Coding Region of LCV RNA 2 Involved in Minus-Strand RNA Synthesis

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Dr. James C.K. Ng, Chairperson

For positive-sense single-stranded(ss) RNA virus, determinants that mediate minus-strand RNA synthesis have been often found to reside in 3'-terminal region of the viral genome. Although significant progress has been made in understanding these determinants in several model positive-sense ssRNA viruses, the knowledge of this particular aspect is significantly behind for many economically important viruses including those that belong to the genus *Crinivirus* (family *Closteroviridae*). *Lettuce chlorosis virus* (LCV) is a relatively new member of crinivirus with a bipartite single-stranded positive-sense RNA genome. This dissertation identified and examined determinants that mediate minus-strand RNA synthesis of LCV. Since no molecular tool of LCV was available for examining determinants involved in minus-strand RNA synthesis, Chapter 1 described the construction of infectious cDNA clones of full-length LCV genomic RNAs (1 and 2). Transcripts synthesized from infectious cDNA clones of LCV RNAs were shown to exhibit wild type LCV virion RNAs property in inoculated tobacco protoplasts: similar accumulation kinetics of RNAs 1 and 2, the synthesis of the

major coat protein, and formation of virion-like particles. In addition, several novel LCV RNA 1-derived RNAs were detected, including first reported crinivirus defective RNAs derived from RNA 1. Next, we investigated determinants that mediate minus-strand LCV RNA 2 synthesis in Chapter 2 of this dissertation. First, we found that the exchange of conserved region of 3' non-coding regions (NCRs) of LCV RNAs showed no detrimental effects on minus-strand RNA synthesis, suggesting determinants involved in minus-strand RNA synthesis are similarly present in both 3' NCRs of LCV RNAs 1 and 2. Second, deletion analysis of 3' NCR of LCV RNA 2 showed that minimum sequences required for minus-strand RNA 2 synthesis lie between 25 to 98 nucleotides from the 3' terminus. Third, computer modeling and newly developed SHAPE (Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension) analysis revealed a presence of 3'-terminal stem-loop (SL) structure, and mutational analysis showed that overall structure, but not loop, top stem and internal loop, of SL is required for minus-strand RNA synthesis. In addition, SHAPE analysis of LCV RNA 2 defective RNA showed that SL is a part of a larger Y-shaped structure.

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Introduction

Viruses are non-living pathogens that infect and exploit their host to produce more copies of themselves and have been found to infect organisms in all domain of life. A virus particle or "virion" consists of at least a genetic material in form of nucleic acids and protein that encloses and protects the genetic material. The genetic material of the virus, containing the information necessary to manipulate the host cell machinery for its own use, is one of the main criteria for virus classification as it can be in either form of nucleic acids, DNA or RNA, and can also be either single- (ss) or double-stranded (Baltimore, 1971; International Committee on Taxonomy of Viruses. and King, 2012). For viruses that have single-stranded RNA genome, the genetic material can also further be classified by its ability to directly translate viral encoded protein by host translation machinery. Those genetic materials that can be directly used to translate protein are called positive-sense and those that can't are called negative-sense (Baltimore, 1971; Hull, 2009).

In order to successfully infect a host and continues its survival, the virus will have to propagate more copies of itself by replication, making replication process indispensible step in virus life cycle. Positive-sense single-stranded (ss) RNA virus is the largest group of known viruses (Baltimore, 1971; Hull, 2009; International Committee on Taxonomy of Viruses. and King, 2012). In addition, they are also a majority (65%) of plant-infecting viruses and cause a significant monetary loss worldwide (Hull, 2009). Despite many genomic configurations from single-partite genome to multipartite genome, the positive-

sense ssRNA virus still adheres to the same basic replication strategy. The first step of replication for positive-sense ssRNA is minus-strand RNA synthesis (Baltimore, 1971; Hull, 2009). Like a typical transcription, the template has to be read from 3' to 5' direction and the synthesis will occur in 5' to 3' direction. The viral replicase complex containing both viral and host replications associated proteins (Lai, 1998) will be recognize the template through specific sequences and/or specific higher-order structure (*cis*-acting elements) and initiate the minus-strand RNA synthesis at the 3'-terminal region of viral RNA genome (Buck, 1996; Dreher, 1999; Liu et al., 2009). The newly synthesized minus-sense RNA will then serve as a template for producing more positive-sense RNA genome or subgenomic RNA through specific sequences and/or specific higher-order structures (Buck, 1996; Dreher, 1999).

Initiation of minus-strand RNA synthesis

The replication of RNA virus can be initiated by two major mechanisms: *de novo* initiation and primer-dependent synthesis (Kao et al., 2001). *De novo* initiation is a mechanism of starting viral RNA synthesis with only one single nucleotide complementary to 3'-terminal of RNA genome. In contrast, primer-dependent synthesis requires a longer stretch of complementary nucleotides from other part of viral genome or viral protein to initiate the replication. Majority of RNA virus with an exception of picornavirus use *de novo* initiation as a mechanism for RNA synthesis (Kao et al., 2001; Ogram and Flanegan, 2011).

The initiation site of *de novo* initiation is generally found to be either the ultimate or the penultimate 3'-terminal nucleotide (Chapman and Kao, 1999; Kao et al., 2001; Wu and Kaper, 1994). One of the most well understood initiation site is the penultimate "C" in 3'-terminal CCA motif, which has been found in several positive-sense ssRNA viruses such as *Brome mosaic virus* (BMV), *Tobacco yellow mosaic virus* (TYMV) and *Tobacco mosaic virus* (TMV) (Dreher and Hall, 1988a; Kao et al., 2001; Osman et al., 2000; Singh and Dreher, 1997). The penultimate nucleotide "C" in the 3'-CCA was shown to be initiation site in minus-strand RNA synthesis of both BMV and TYMV by incorporation of [γ-^{32}P]GTP and [γ-^{32}S]Thio-GTP, respectively (Deiman et al., 1998; Miller et al., 1986; Singh and Dreher, 1997). Mutational analyses of the penultimate "C" in BMV, TYMV, and TMV have significant reduction in minus-strand RNA synthesis (Dreher and Hall, 1988a; Osman et al., 2000; Singh and Dreher, 1997). In addition, mutational analyses of nearby initiation site for BMV, TYMV and TMV also showed them to be important determinants in minus-strand RNA synthesis as any nucleotide change in those positions has a detrimental effect in minus-strand RNA synthesis (Dreher and Hall, 1988a; Osman et al., 2000; Singh and Dreher, 1997). However, in case of BMV, those mutational effects of 3'-CCA were confined to only *in vitro* condition as the same mutation can be repaired and rescued the *in vivo* condition (Hema et al., 2005; Rao and Hall, 1993). Interestingly, this particular 3'-CCA motif by itself is sufficient *in vitro* condition for minus-strand RNA synthesis by TYMV, *Turnip crinkle virus* (TCV), and Qβ bacteriophage replicase (Yoshinari et al., 2000). However, the question arose about the origin of the 3'-terminal nucleotide of the progeny plus-strand RNA. Host and viral

proteins, such as tRNA nucleotidyl transferase in case of BMV, and replicase in case of TCV, have been suggested to be responsible for this function (Dreher and Hall, 1988b; Guan and Simon, 2000).

However, not every *de novo* initiation of minus-strand RNA synthesis is found at the 3'-terminal or the 3'-penultimate nucleotide. In case of *Bamboo mosaic potexvirus* (BaMV), the minus-strand RNA synthesis initiates at many different sites within the first 15 adenines of poly(A) tail and not at the viral sequence prior to the poly(A) tail (Cheng et al., 2001). However, in case of another positive-sense ssRNA virus with poly(A) tail, Sindbis virus, the initiation site is not in the poly(A) tail but at the 3'-terminal nucleotide prior to the poly(A) tail (Hardy and Rice, 2005). In addition, initiation site of Sindbis virus can also shift from the nucleotide prior to the poly(A) tail when sequences of 3'-terminal 19 nucleotides conserved sequence element and poly(A) tail were deleted or substituted (Hardy, 2006).

3' *cis*-acting elements involved in minus-strand RNA synthesis

Because viral replicase complex initiates minus-strand RNA synthesis at the 3' terminus of the positive-sense genomic RNA, many critical *cis*-acting elements that regulate minus-strand RNA synthesis generally reside within 3' terminus region of template. The 3'-terminal region of positive-sense ssRNA virus can be categorized into three different types: transfer RNA-like structure (TLS), poly(A) tail, and non-TLS heteropolymeric sequence (Dreher, 1999).

Transfer RNA-like structures (TLS) have been first identified in *Turnip yellow mosaic virus* (TYMV) (Pinck et al., 1970; Yot et al., 1970) and subsequently found in many other genera of positive-sense RNA viruses including those containing well-studied and well-known viruses such as bromoviruses and cucumoviruses (Dreher, 1999). Like transfer RNA, the structure can be aminoacylated and can be categorized into three different types based on specific aminoacylation: valine, histidine, and tyrosine (Dreher, 2009). The structure also have many other functions beside aminoacylation such as acting as telomere by recruiting host CCA tRNA nucleotidyl transferase (Dreher, 2009) or involving in virion encapsidation in case of BMV (Choi et al., 2002). However, the ability to mimic tRNA does not seem to be involved in minus-strand RNA synthesis, but rather only the promoter elements within the TLS itself are associated with minus-strand RNA synthesis (Dreher, 2009). This requirement of minus-strand RNA synthesis, where tRNA mimicry is not needed, has been demonstrated in studies of TYMV and BMV (Chapman and Kao, 1999; Filichkin et al., 2000; Goodwin et al., 1997). A chimeric TYMV mutant with TLS substitution by the 3' NCR of *Erysimum latent tymovirus* (ErLV), which cannot be aminoacylated (Goodwin and Dreher, 1998), has been shown to be able to synthesize minus-strand RNA by TYMV replicase (Filichkin et al., 2000; Goodwin et al., 1997). For BMV, stem C (with both terminal and its internal loop) in the absence of the whole TLS has been shown to bind to BMV replicase and able to direct initiation of minus-strand RNA with an accessible 3'-terminal CCA (Chapman and Kao, 1999).

Another type of 3'-terminal region of positive-sense ssRNA virus is poly(A) tail, which has been found in several genera including potyviruses and potexviruses for plant viruses, and picornavirus and coronavirus for animal viruses (Dreher, 1999). The poly(A) tail has been found to be incorporated into 3'-terminal secondary and tertiary structures, which also found to be involved in minus-strand RNA synthesis. One example is the pseudoknot of Bamboo mosaic potexvirus, where chemical and enzymatic probing revealed at least 13 adenine residues from poly (A) tail in pseudoknot structure (Tsai et al., 1999). Another example is the "kissing" interaction in 3'-terminal region of Poliovirus. The 3' NCR of Poliovirus is 65 nucleotides in length followed by poly(A) tail and folds into two stem loops, stem loops X and Y, with a tertiary "kissing" interaction between nucleotides within loop portions of stem loops X and Y and incorporation of poly(A) tail in the overall structure. Mutational analysis of the "kissing" interaction showed that the interaction is important for viral RNA replication as disruption of the interaction reduced replication activity (Pilipenko et al., 1996). However, deletion of the entire 3' NCR of Poliovirus did not abolish viral RNA replication activity, suggesting that the 3' NCR is not required, but is needed for optimal viral RNA replication (Todd et al., 1997).

3'-terminal region that does not fit the first two categories fall into the third category, non-TLS heteropolymeric sequence, which has been found in many genera including flaviviruses, luteoviruses, tombusviruses, dianthoviruses, and closteroviruses (Dreher, 1999). However, no common secondary and tertiary structure has been found among different genera (Dreher, 1999). One of the well-studied 3'-terminal non-TLS

heteropolymeric sequence is those belong to Hepatitis C virus (HCV). The 3' NCR of HCV consists of three domains with two variable nucleotides length, the variable region and the poly(U/UC) tract, and one highly conserved 98 nucleotides 3' X tail (Blight and Rice, 1997; Ito and Lai, 1997; Tanaka et al., 1996). In addition, the highly conserved 98 nucleotides 3' X tail folds into three putative stem loop structures (SL1, 2, and 3) (Blight and Rice, 1997; Ito and Lai, 1997; Tanaka et al., 1996). Mutational analyses of the 3' NCR of HCV using HCV-N subgenomic replicon system found that both the poly(U/UC) tract and the 3' X tail, but not the variable region, are required for replication as deletion of both the poly(U/UC) tract and the 3' X tail abolished viral RNA replication while deletion of the variable region only reduced the viral RNA replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003a, b). A tertiary "kissing" interaction has also been discovered between loop portions of SL2 of 3' X tail and one of four stem-loop structures within the C-terminal NS5B ORF, SL-V or 5BSL3.2, which is also another essential *cis*-acting element for HCV replication (Friebe et al., 2005; You et al., 2004). The mutation analysis of the interaction showed that disruption of this specific interaction between two stem loops abolished the viral RNA replication (Friebe et al., 2005; You and Rice, 2008).

Another well-studied 3'-terminal non-TLS heteropolymeric sequence is a series of 5 stem-loops with 4 pseudoknot interactions in 3' NCR of *Turnip crinkle virus* (TCV). The analysis of the series of stem-loops showed that the core promoter for satellite TCV (satC) minus-strand RNA synthesis and TCV replication is the 3'-terminal Pr hairpin (Chapman and Kao, 1999; Song and Simon, 1995; Sun and Simon, 2006; Yuan et al.,

2012; Yuan et al., 2010). The structure of Pr hairpin has been found to be important for replication as difference in the structure of Pr hairpin between satC and TCV has shown to have a different promoting strength when subjected to RdRp *in vitro* assay (Zhang et al., 2006b). In addition, Pr hairpin also plays a role in translation as it has been found to enhance a read-through translation of TCV p88 RdRp (Cimino et al., 2011). However, the Pr hairpin is not only minus-strand RNA synthesis promoter as other hairpins and pseudoknots have also found to play roles in TCV replication (Guo et al., 2009; McCormack et al., 2008; Yuan et al., 2009; Zhang et al., 2006a; Zhang et al., 2006b). One of the most interesting structures that are mediating minus-strand TCV RNA synthesis is the satC H5 hairpin with a large asymmetrical internal loop. Although majority of secondary structures of positive-sense ssRNA virusrd have been found to promote the minus-strand RNA synthesis, satC H5 hairpin has been to be a repressor of minus-strand RNA synthesis as the base-parinig interaction between nucleotides in the asymmetrical internal loop and 3'-terminal nucleotides of satC eliminates an access to 3'-terminal nucleotides of satC for initiation of minus-strand RNA synthesis (Zhang et al., 2004). A similar repression of minus-strand RNA synthesis has also been found in defective RNA of tombusvirus (Pogany et al., 2003). In addition to two hairpins, the dynamic of overall complex network of all secondary structures and tertiary interactions within the 3' NCR of TCV has also been suggested to be extremely important in replication as every structure conformation changes in respond to binding to the TCV RdRp (Yuan et al., 2009; Yuan et al., 2010). This conformational shift, which included the disruption of the tRNA-shaped structure (TSS) consisting of hairpins H4a, H4b, H5,

and pseudoknots Ψ 2 and 3, was suggested to be transition from translation to replication (Yuan et al., 2009; Yuan et al., 2010).

Although majority of 3'-terminal region of positive-sense ssRNA viruses presented so far contains a higher-order RNA structure that involved in minus-strand RNA synthesis, not all positive-sense ssRNA viruses utilizes a higher-order structure in 3'-terminal region for promoting minus-stand RNA synthesis as demonstrated by Sindbis virus. The 3' NCR of Sindbis virus is 323 nucleotides in length consisting of 3 copies of a 40 nucleotides long repeat elements and a 3'-terimius 19 nucleotides conserved sequence element (CSE) immediately followed by poly(A) tail with no distinct secondary structure (Liu et al., 2009). Mutation analyses of the 3' NCR of Sindbis virus showed that the long repeat elements are essential, but only the 3'-terminal CSE and specific length of poly(A) tail are required for viral RNA replication (Hardy and Rice, 2005; Kuhn et al., 1990).

Defective RNA

Defective (D) RNA is a class of subviral RNA that derived exclusively or mainly from the helper virus, which differs from satellite RNA that consists of entirely or contains a large segment of unrelated sequences (Hull, 2009). A particular type of DRNA species, called "defective-interfering" (DI) RNA, can be intervene with replication of helper virus, which results in reduction of helper virus accumulation and symptom of the disease (Simon et al., 2004). The reduction of helper virus accumulation has been suggested due to either a competition between the DIRNA and helper virus for replication associated proteins, an interaction between DIRNA and viral-encoded

products, or an activation of host gene silencing (Simon et al., 2004). An example of DIRNA is TBSV DIRNA, which reduces helper virus TBSV accumulation when co-infecting into *Nicotiana benthamiana* protoplasts (Jones et al., 1990). However, a reduction in helper virus accumulation does not always reduce severity of the symptom in all cases as TCV DIRNA has found to reduce TCV accumulation but increases the severity of the symptom (Li et al., 1989). Both DRNA and DIRNA are truncated forms of the helper virus, which generally consists of 5'- and 3'-termini of the helper virus and sometimes internal sequences of virus (Bar-Joseph and Mawassi, 2013; Dalton et al., 2001; Gowda et al., 2001; Mawassi et al., 1995a; Mawassi et al., 1995b; Mawassi et al., 2000; Mongkolsiriwattana et al., 2011; Pogany et al., 2003; Rubio et al., 2000; Yang et al., 1997). The absence of large portion of genomic information indicates DRNA's inability to encode its own replication associated proteins, so both DRNA and DIRNA rely exclusively on the helper virus for propagation. *De novo* replication by helper virus indicates that DRNA and DIRNA contain all necessary *cis*-element for recognize by helper virus' replicase complex so the DRNA and DIRNA has been used to examine replication process for some positive-sense ssRNA virus (Fabian et al., 2003).

Identification of RNA structure

For positive-sense ssRNA virus, RNA secondary structures and tertiary interactions that mediate minus-strand RNA synthesis and also those that is responsible for other functions have been identified through a combination of various methods from both computer modeling and direct RNA modification. Dynamic programming algorithm

has been developed to predict RNA structure by primarily based on the base-paring and base-stacking of RNA sequence and formation of the most stable (lowest free energy) RNA structure (Mathews, 2004; Mathews et al., 2004; Zuker, 2003). In addition, phylogenetic comparative sequence analysis has also been used to predict RNA structure with more accuracy (Gutell et al., 2002; Michel and Westhof, 1990). However, the prediction purely by algorithm has been proven to be not particular accurate (40-70%), especially with a larger RNA segment, due several factors including suboptimal structure formation and different biological states of the structure (Ding et al., 2008; Doshi et al., 2004). Due to inaccuracy of algorithm prediction, structural probing is required to obtain an accurate structure of RNA. The main method of structural probing is chemical and enzymatic RNA modification, where chemical reagent or enzyme modifies specific type of ribose base. For example, dimethyl sulfate (DMS) modifies unpaired adenosine and cytosine through methylation (Tijerina et al., 2007) while RNase V1 digests any base-paired ribonucleotides (Nilsen, 2013). However, combination of several chemical reagents or enzymes that modify unpaired and paired bases is needed to determine the structure as a particular reagent can only modifies specific type of nucleotide. The newly developed method, "Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension" or SHAPE, has proven to be a powerful tool in analyzing complex RNA structures as it bypasses the need to use multiple reagents to obtain accurate structural information (Watts et al., 2009). The analysis is based upon the formation of 2'-O-adducts from acylation of 2'-hydroxyl group of ribonucleotide by electrophilic reagent such as *N*-methylisatoic anhydride (NMIA) (Merino et al., 2005). This formation of 2'-O-adducts is

unbiased to identity of nucleotide and governed by the conformational of each ribonucleotide, where electrophilic reagents react preferentially to a flexible nucleotide rather than a constrained one (Merino et al., 2005). As there is new development in chemical modification of RNA structure, new and updated algorithm has also been developed along with the new chemical modification technique as predicting program *RNAstructure* now has an ability to incorporate information from chemical modification such as SHAPE analysis into the prediction of the RNA structure (Mathews, 2004; Mathews et al., 2004).

Lettuce chlorosis virus

Lettuce chlorosis virus (LCV) is a relatively new plant virus that belonged to the emerging and economically important genus *Crinivirus* in the family *Closteroviridae*.

LCV was discovered in early 1990s when symptomatic lettuce plants with stunted heads brittle leaves and interveinal yellowing was collected from field in Imperial Valley, California and has found to not react with *Lettuce infectious yellows virus* (LIYV) antiserum in indirect enzyme-linked immunosorbent assay (ELISA) (Duffus et al., 1996; McLain et al., 1998).

As LCV has frequent outbreaks and is endemic to the Southwestern USA (J. Ng, unpublished data), a number of studies has been followed up in order to determine the biological properties of this newly discovered virus (Duffus et al., 1996; Liu et al., 2000; McLain et al., 1998; Wisler et al., 1997). Like other criniviruses, LCV induced interveinal yellowing, stunting, and increase in brittleness of the infected leaves and its

infection is limited to the phloem tissues. Virions are long flexuous rod shape with 750-950 nm in length and are transmitted in a semi-persistent manner by *Bemisia tabaci* biotype A and B whiteflies.

However, molecular studies of the virus have only begun in a recent year with sequence and phylogenetic analyses in 2009 (Salem et al., 2009). Sequence analysis has shown that LCV has single-stranded positive-sense bipartite RNA genomes like other members of crinivirus.

Genomic organization of LCV RNA 1

LCV RNA 1 is 8591 nucleotides in length and contains 3 predicted open reading frames (ORFs) including replication module (ORF1a and 1b), one of the two common features among the member of the family *Closteroviridae* (Agranovsky, 1996; Dolja et al., 2006; Karasev, 2000; Martelli et al., 2002). ORF 1a encodes a predicted protein of 227 kDa with conserved domains of a papain-like leader proteinase (P-PRO), a methyltransferase (MTR), and a helicase (HEL).

P-PRO is a protein found to be encoded by many plant viruses and is known for ability to cleave and process polyprotein (Hull, 2009). However, the arrangement of P-PRO in 5'-terminal of ORF1a differs from those of alphaviruses, rubiviruses, and tymoviruses, where P-PRO is located in the between the domains coded for replication (Dolja et al., 2006). This particular arrangement of closterovirus P-PRO has been found in *Beet yellows virus* (BYV) to be responsible for autoprocessing through cleaving itself from polyprotein of ORF1a or ORF1a/1b in *Beet yellows virus* (BYV) (Agranovsky et

al., 1994). Additional studies of BYV P-PRO revealed that P-PRO is involved in long-distance transportation of virus, and plays a crucial role in amplification of viral genome (Peng and Dolja, 2000; Peng et al., 2003).

MTR and HEL along with RNA-dependent RNA polymerase (RdRp), which encodes from ORF1b, are a replication module that is common among alpha-like positive-sense ssRNA viruses (Dolja et al., 2006; Koonin and Dolja, 1993). Despite the conservation of MTR-HEL-RdRp arrangement in member of family *Closteroviridae*, the region in between MTR and HEL domain is highly variable and does not have any similarity with any known proteins (Dolja et al., 2006; Salem et al., 2009).

ORF1b encodes a predicted 59 kDa RNA-dependent RNA polymerase (RdRp) presumably via +1 translational frameshift as the ORF 1b is in a +1 reading frame in comparison to ORF1a (Karasev, 2000). The framshift process is predicted to result in 286.5kDa predicted fusion protein (Salem et al., 2009).

LCV RNA 1 also encodes a putative 22.9 kDa protein (P23) from ORF 3 via a nested set of 3'-coterminal sgRNA. Recent study has been revealed that P23 possesses an ability to suppress RNA silencing (Kubota et al., unpublished). This protein is distinctly differ from the LIYV protein in similar genomic location, which has been shown to function as an enhancer of LIYV RNA 2 replication (Yeh et al., 2000).

Genomic organization of LCV RNA 2

LCV RNA 2 consists of 8556 nucleotides and contains 10 predicted ORFs, which includes the hallmark closterovirus gene array, one of the two common features among

the member in *Closteroviridae*. The hallmark closterovirus gene array consists of five generally conserved ORFs in their amino acid sequences and relative positions on its genome. This unique genetic module encodes a small hydrophobic protein, a heat shock protein homologue (HSP70h), a protein of 50–60 kDa, the major coat protein (CP) and the minor coat protein (CPm).

In LCV, a small hydrophobic protein of the module is encoded by ORF 4, which is predicted to have size of 6.4kDa (P6.4). The protein shows a high level of similarity with proteins in similar position on other crinivirus genome. Even though the function of the protein remains unknown for LCV, BYV P6 has been found to be involved in cell-to-cell movement (Alzhanova et al., 2000; Peremyslov et al., 2004), but not required for virus replication or assembly (Alzhanova et al., 2001; Peremyslov et al., 1998).

A heat shock protein homolog is encoded by ORF 3 in form of a putative 62kDa protein (P62). Heat shock protein is a family of molecular chaperones with many other functions such as protein transport and degradation, and is very common among living organisms (Morano, 2007). In members of family *Closteroviridae*, HSP70h has been shown to be one of the structural proteins along with P60, CP, and CPm in both BYV and LIYV, but only encapsidates tail of the virion (Napuli et al., 2000; Tian et al., 1999). In addition of being a structural protein, HSP70h also plays multiple roles from acting with P60 in assembly of the CPm tail of CTV (Satyanarayana et al., 2004) to being critical part in cell-to-cell movement for BYV and CTV(Alzhanova et al., 2001; Satyanarayana et al., 2000).

ORF 4 encodes a putative protein of 59.8kDa (P60) in size. Like HSP70h, the protein is also one of three structural proteins in the tail component and also plays an essential role in assembling virion tail in both CTV and BYV (Napuli et al., 2003; Satyanarayana et al., 2004).

The major capsid protein has a size of 28.4kDa and is encoded by ORF 7. BYV, CTV, and LIYV CP have been shown to be a major structural protein in virion as they encapsidate almost the entire length of virion and the tip of virion is encapsidated by other structural proteins including HSP70h, P60, and primarily the minor capsid protein (CPm) (Agranovsky et al., 1995; Tian et al., 1999; Zinovkin et al., 1999). This particular pattern of virion encapsidation forms a "rattlesnake"-like structure that is a unique feature of the family *Closteroviridae.* Previous mutational analysis study in BYV revealed that disruption in tail formation allows BYV CP to encapsidate the whole genome (Alzhanova et al., 2001).

LCV CPm (54.7kDa) is encoded by ORF 8 and is confined at the end of the virion. Extensive studies of LIYV have found CPm to be heavily involved in a transmission of virion by whitefly insect vectors as recombinant *E.coli* expressed CPm is only a structural protein that is significantly retained within whitefly foregut (Chen et al., 2011) and purified virion of natural LIYV variant with CPm truncation cannot be transmitted by whitefly (Ng and Falk, 2006a; Stewart et al., 2010).

Numbers of these LCV RNA 2 ORFs (HSP70h, P60, CP, CPm, and P27) are expressed via a nested set of 3′-coterminal sgRNAs (Salem et al., 2009).

5' and 3' non-coding regions (NCRs) of LCV RNAs 1 and 2

The sequencing analysis also revealed several notable characteristics of 5' and 3' NCRs of LCV RNAs 1 and 2. The first five 5'-terminal nucleotides (GAAAT) of LCV RNAs 1 and 2 are identical and are also identical to other criniviruses including *Cucurbit yellow stunting disorder virus* (CYSDV)*, Tomato chlorosis virus* (ToCV), *Sweet potato chlorotic stunt virus* (SPCSV) (Salem et al., 2009). Another notable feature in 5' NCR is a stretch of 14 identical nucleotides located with 220 nucleotides of both RNAs 1 and 2 (Salem et al., 2009), which this similar organization of a stretch of identical nucleotides is also found within 150 5'-terminal nucleotides of LIYV (Klaassen et al., 1995). However, the function of these identical nucleotides at extreme 5'-terminal and within 220 nucleotides remains unknown.

The 3' NCRs of LCV RNAs 1 and 2 share a very high sequence similarity of 81% and this high sequence conservation between RNAs 1 and 2 is a common feature among multipartite RNA viruses (Dreher, 1999), including all sequenced crinivirus excepted for LIYV (Aguilar et al., 2003; Hartono et al., 2003; Klaassen et al., 1995; Kreuze et al., 2002; Lozano et al., 2009; Okuda et al., 2010; Salem et al., 2009; Tzanetakis et al., 2006; Wintermantel et al., 2009; Wintermantel et al., 2005).

Chapter 1: Replication of *Lettuce chlorosis virus* (LCV), a crinivirus in the family *Closteroviridae*, is accompanied by the production of LCV RNA 1-derived novel RNAs

Abstract

Cloned infectious complementary DNAs of the bipartite genomic RNAs of *Lettuce chlorosis virus* (LCV) were constructed. Inoculation of tobacco protoplasts with the *in vitro* produced RNAs 1 and 2 transcripts, or with RNA 1 transcript alone, resulted in viral replication accompanied by the production of novel LCV RNA 1-derived RNAs. They included the abundantly accumulating LM-LCVR1-1 (~0.38 kb) and LM-LCVR1-2 (~0.3 kb), and the lowly accumulating HM-LCVR1-1 (~8.0 kb) and HM-LCVR1-2 (~6.6 kb), all of which reacted with riboprobes specific to the 5' end of RNA 1 in Northern blot analysis. LM-LCVR1-1 and HM-LCVR1-2 accumulated as positive-stranded RNAs that lacked complementary negative strands, while HM-LCVR1-1 and LM-LCVR1-2 accumulated in both polarities. Additional Northern blot, reverse transcription-polymerase chain reaction, cloning, and sequence analyses revealed LM-LCVR1-2 to be an authentic RNA 1-derived defective (D)RNA, suggesting that its synthesis and maintenance are supported *in trans* by an RNA 1 encoded replication machinery.

Introduction

The genus *Crinivirus* in the family *Closteroviridae* contains whitefly transmitted viruses that are emerging and economically important. Criniviruses have single-stranded positive-sense bipartite RNA genomes, and share two common genomic features with other members of the family: 1) a replication module consisting of a papain-like leader proteinase (P-PRO), a methyltransferase (MTR), and a helicase (HEL) encoded in open reading frame 1a (ORF 1a), and an RNA-dependent RNA polymerase (RdRp) encoded in ORF 1b; and 2) a hallmark closterovirus gene array containing ORFs encoding a small hydrophobic protein, a heat shock protein homolog (HSP70h), a protein of 50-60 kDa, the major coat protein (CP) and the minor coat protein (CPm) (Agranovsky, 1996; Dolja et al., 2006; Karasev, 2000; Martelli et al., 2002).

Lettuce chlorosis virus (LCV) is a newly described crinivirus whose genome sequence we have recently determined (Salem et al., 2009). The LCV genome is consists of at least 13 ORFs, with ORFs encoding P23, HSP70h, P60, CP, CPm and P27 being expressed via a nested set of 3'-coterminal subgenomic (sg)RNAs (Salem et al., 2009). The 3' non-coding regions (NCRs) of the LCV genomic RNAs share a relatively high level of sequence identity, a feature that is common among all criniviruses except for *Lettuce infectious yellows virus* (LIYV) (Aguilar et al., 2003; Hartono et al., 2003; Klaassen et al., 1995; Lozano et al., 2009; Okuda et al., 2010; Salem et al., 2009; Tzanetakis et al., 2006; Wintermantel et al., 2009; Wintermantel et al., 2005), and has been hypothesized to contribute to a similar temporal accumulation of both viral genomic RNAs (Salem et al., 2009). In Northern blot analyses of the total RNA extracts of LCV

infected plants, a positive-sense specific riboprobe corresponding to the 5'-proximal end of RNA 1 reacted with a low molecular weight (MW) (~0.3 – 0.4 kb) RNA, while that corresponding to the 5'-proximal end of RNA 2 reacted with several low MW (~0.4 – 1.5 kb) RNAs (Salem et al., 2009). These RNAs resemble the positive-stranded 5'-coterminal sgRNAs, referred to as 'low MW tristeza (LMT) RNAs', produced by *Citrus tristeza virus* (CTV; family *Closteroviridae*) (Che et al., 2001; Gowda et al., 2009; Mawassi et al., 1995a). CTV also produces positive-stranded 5'-coterminal sgRNAs, referred to as 'large MW tristeza (LaMT) RNAs', that are ~10 kb in size (Che et al., 2001). However, negative-stranded LMT and LaMT RNAs have not been observed. In the case of LCV, because it is not known if the low MW RNAs are coterminal in sequence at their 5' ends, they have been referred to as LCV 5'-terminal RNAs. Whether or not these LCV 5'-terminal RNAs are lacking in negative strands and whether the equivalents of LaMT RNAs are produced during LCV infection are unknown.

So far, LIYV is the only crinivirus to have cloned genomic (cDNA) components that are biologically active when delivered to protoplasts and plants (Klaassen et al., 1996; Ng and Falk, 2006a; Wang et al., 2009a). Unlike RNAs 1 and 2 of LCV, those of LIYV exhibit an asynchronous accumulation pattern where the accumulation of RNA 2 lags behind that of RNA 1 in the early stages of infection (Yeh et al., 2000). This phenomenon has been postulated to be partly due to the dissimilarity in sequence and/or structure in the 3' NCRs of both genomic RNAs (Buck, 1996; Dreher, 1999; Yeh et al., 2000).

The differences in temporal accumulation of LIYV and LCV genomic RNAs, as well as the production of the LCV RNA 1 low MW RNAs (Salem et al., 2009; Yeh et al., 2000) prompted us to construct the cloned cDNAs corresponding to the full-length genomic RNAs of LCV for use in studies to understand LCV RNA replication. Inoculation of tobacco protoplasts with the LCV RNA 1 and 2 transcripts demonstrated their biological activity. More importantly, viral replication was accompanied by the production of unique LCV RNA 1-derived RNAs that were either previously not seen, or were observed but not investigated. They included a low MW 5'-terminal RNA that resembled the LMT RNA of CTV, two high MW RNAs (with one resembling the LaMT RNA of CTV), and an authentic RNA 1 defective (D)RNA. The generation of an RNA 1 DRNA is remarkable for it has never been reported for LIYV or any other criniviruses. Furthermore, protoplast inoculation with only the LCV RNA 1 transcript showed that these novel RNAs were produced and accumulated over time. These data demonstrated the complexity of the LCV RNA 1 replication machinery, and provided new evidence that the replication mechanism of LCV differed from that of LIYV.

Materials & Methods

Construction of full-length LCV RNAs 1 and 2 cDNA clones

All oligonucleotide primers used for the construction of full-length LCV RNAs 1 and 2 cDNA clones are indicated in Table 1-1. The first strand cDNA of LCV RNA1 was generated using purified LCV virion RNAs (vRNA), the oligonucleotide primer LCV-55, and ThermoScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Second-strand cDNA synthesis and PCR amplification was performed using the Expand Long Template PCR system (system 1; Roche) with the oligonucleotide primers LCV-55 and LCV-52 according to the manufacturer's instructions. The product was separated by agarose gel electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The resulting product was ligated into the pGEM-T Easy vector (Promega Corp., Madison, WI), to yield the full-length LCV RNA 1 cDNA clone, p101 (Fig. 1-1A).

The bacteriophage T3 promoter was engineered immediately upstream of the 5'-terminus of the LCV sequence in p101 by PCR using p101 as template, and oligonucleotide primers LCV-89-CN and LCV-90-CN. The amplified product was gel-purified, adenylated and cloned into the pGEM-T Easy vector (Promega) to yield the intermediate clone, p102 (not shown). A DNA fragment of p101 was removed by digesting with NcoI and AflII restriction enzymes (New England Biolab) and subsequently replacing it with the similarly digested T3-containing fragment from p102 (not shown), producing the intermediate clone p103 (not shown).

An NgoMIV restriction site was engineered immediately downstream of the LCV RNA 1 3'-terminus in p103 by PCR using p101 as the template and oligonucleotide primers LCV-91-CN and LCV-92-CN. The amplified product was gel-purified, adenylated and cloned into the pGEM-T Easy vector to yield the intermediate clone p104 (not shown). A DNA fragment of p104 was removed by digesting with HpaI and NdeI and subcloned into similarly digested p103, producing pCM1 (Fig. 1-1B).

The development of the full-length LCV RNA 2 cDNA clone began with the synthesis of two RT-PCR fragments (1+2) using vRNA as the template (Fig. 1-1A). Fragment 1, corresponding to LCV RNA 2 nucleotides 1-4096, was generated using the oligonucleotide primers LCV-88-JN and LCV-56-JN. The product was cloned into the pGEM-T Easy vector, resulting in p201.1 (Fig. 1-2A).

The bacteriophage T3 promoter was engineered immediately upstream of the 5'-terminus of the LCV RNA 2 sequence in p201.1 using a PCR-mediated approach essentially similar to the one used for constructing pCM1, except that p201.1 was used as the template for PCR involving the oligonucleotide primers LCV-101-CM and LCV-124-CM. The amplified product was cloned into the pGEM-T Easy vector and digested with KasI and NcoI restriction enzymes. The KasI and NcoI digested fragment was replaced with a similarly digested fragment from p201.1 to yield p202 (Fig. 1-2B).

Fragment 2, corresponding to LCV RNA 2 nucleotide 3964-8556, was generated using the oligonucleotide primers LCV-57-JN and LCV-87-JN. The amplified product was cloned into the pGEM-T Easy vector, resulting in p201.2 (Fig. 1-2A). The product

was subsequently digested with AatII and NcoI restriction enzymes and cloned into similarly digested p202, resulting in p203 (Fig. 1-2B).

Finally, a NgoMIV restriction site was introduced immediately downstream of the LCV RNA 2 3'-terminus in p201.2, similar to the construction of pCM1, except that it involved the oligonucleotide primers LCV-125-CM and LCV-104-CM. The amplified product was gel-purified, adenylated and cloned into pGEM-T Easy vector. The clone was digested with AatII and ApaI restriction enzymes and cloned into similarly digested p203, producing pCM2 (Fig. 1-2C).

Standard molecular procedures were performed according to Sambrook and Russell (Sambrook and Russell, 2001a). All PCR amplifications were performed using Turbo *Pfu* polymerase (Stratagene, La Jolla, CA) unless otherwise stated. All cDNA clones were transformed into *Escherichia coli* DH5α competent cells and grown in Luria-Bertani broth containing ampicillin (100 μg/ml; Sigma-Aldrich, St. Louis, MO). All cloned products derived from PCR-amplification were sequenced in both directions.

Protoplast inoculation and downstream analyses: Northern blot, Immunoblot, virion purification and TEM, cDNA cloning, and sequencing

In vitro transcription of pCM1 and pCM2 followed the procedures as previously described (Ng et al., 2004), except that the plasmids were linearized with NgoMIV restriction enzyme. *Nicotiana tabacum* var. Xanthi (tobacco) protoplast preparation and inoculation with LCV vRNAs, the *in vitro* synthesized transcripts of wild type (WT) pCM1 and pCM2 or of WT pCM1 alone were essentially similar to that previously

described (Ng et al., 2004; Salem et al., 2009) except 2 µg of each of the *in vitro* synthesized transcripts were used for inoculation.

Total RNA extraction of inoculated protoplasts and infected plants were performed as previously described (Ng et al., 2004; Salem et al., 2009). Approximately 2 µg (unless otherwise stated) of total RNA from each sample were analyzed by Northern Blot using DIG-labeled riboprobes I (nts 1 – 482) and II (nts 7813 – 8179), specific to RNA 1, and VIII (nts 7428 – 7817) specific to RNA 2 as previously described (Fig. 1-4) (Salem et al., 2009). Two additional DIG-labeled riboprobes, IX (nts 8331 – 8573) and X (nts 3881 – 4157), specific to RNA 1 were generated in this study following the previously described methods (Fig. 1-4) (Salem et al., 2009). Digoxigenin chemiluminescent signals of RNA-probe hybrids captured on X-ray films were estimated by densitometry using the Scion Image software (Scion Corp.).

Purification of virions from LCV infected *C. murale* plants and from protoplasts inoculated with the pCM1 and pCM2 *in vitro* synthesized transcripts was performed according to the procedures described in Ng et al. (2004). Total soluble proteins were extracted as previously described in Klaaseen et al. (1996). Immunoblot analyses of total soluble protein and purified virions were performed according to Ng and Falk (2006), except that a LCV virion-specific polyclonal IgG was used at a 1:300 fold dilution, and cross absorbed with 2.5%(w/v) *N. benthamiana* leaf extracts prepared in 10%(w/v) non-fat dried milk with 1x PBST (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.3% Tween 20, pH 7.4). The purified virions were subjected to transmission electron microscopy analysis as previously described Tian et al. (1999).

To determine the nucleotide sequences of the DRNAs that were produced during LCV replication, the total RNA extracts of LCV infected *C. murale* plants and tobacco protoplasts (inoculated with the *in vitro* produced transcripts of pCM1 and pCM2, or of pCM1 alone and harvested at 96 hpi) were subjected to reverse transcription (RT) to generate the first strand cDNA. The RT reaction was performed using the oligonucleotide primer LCV-55 and M-MLV Reverse Transcriptase (Promega Corp., Madison, WI) according to the manufacturer's instructions. Second-strand cDNA synthesis and PCR were performed using the oligonucleotide primers LCV-50 [5'-GAAATCAAACTTTCCTTCG-3', corresponding to LCV RNA 1 position 1 to 19] and LCV-55. The above oligonucleotide primers were also used for the RT-PCR and PCR amplification of control templates: pCM1 *in vitro* transcripts and the pCM1 plasmid, respectively. To verify that they were good templates for RT-PCR, the pCM1 *in vitro* produced transcripts were subjected to RT-PCR using two sets of oligonucleotide primers: LCV-25 [5'-GGCATCCTGTTAAATCTGCA-3', complementary to LCV RNA 1 position 482-463] and LCV-50, and LCV-55 and LCV-29 [5'-TACAGGAAGACCTGTTACTGTTACA-3', corresponding to LCV RNA 1 position 8076-8100]. All PCR amplifications were performed using *Taq* DNA polymerase. The cloning of the RT-PCR products into the pGEM-T Easy vector and the subsequent sequencing steps were as described above.

Results

Construction of cloned full-length cDNAs of LCV RNAs 1 and 2

The complete genome sequence information of LCV (Salem et al., 2009) facilitated to generate the cloned cDNAs corresponding to LCV RNAs 1 and 2 to develop a reverse genetics system. We used LCV vRNA as the source of genetic material for reverse transcription and long template PCR, which generated cDNAs with adenylated 3' ends. This allowed the cDNA corresponding to full-length LCV RNA 1 to be directly cloned into a vector with 3' thymidine overhangs (pGEM-T Easy) (Material and Methods; Fig. 1-1A). A similar approach was used to clone the cDNA of LCV RNA 2 but the desired full-length product could not be obtained after several attempts. Consequently, we employed an alternate cloning strategy, where two overlapping cDNA fragments (nucleotides 1 - 4096 and 3964 - 8556) corresponding to LCV RNA 2 were generated and subsequently assembled (Materials and Methods; Figs. 1-2A and 1-2B). Sequence analysis of these cloned cDNAs found nucleotides and deduced amino acids that differed from the published LCV sequences (Salem et al., 2009). In total, there were 12 genomic locations where polymorphisms were observed (Figs. 1-3A and B).

To verify that the cloned cDNAs contained all the genetic information necessary to sustain biological activity, further modifications were made. First, both constructs were designed to contain a modified bacteriophage T3 promoter where two of the three terminal guanine residues at the 3' end were removed (Figs. 1-1B and 1-2C). This ensured that the first nucleotide (guanine) incorporated into the *in vitro* synthesized transcripts corresponded to the first nucleotide in the LCV genomic RNAs. Second, the

cloned cDNA constructs were each modified to contain a NgoMIV restriction site directly downstream of the 3'-terminus of the LCV sequences. This design enabled the first nucleotide in the restriction recognition sequence to correspond to the last nucleotide of the LCV genomic RNAs in both cDNA clones. Thus, digestion with this enzyme would linearize both plasmids at a position immediately after the last nucleotide of the LCV sequence (Figs. 1-1B and 1-2C). Following the above modifications, the final cloned cDNAs, named pCM1 and pCM2 (Figs. 1-1B and 1-2C), were expected to produce transcripts bearing authentic LCV RNAs 1 and 2 5'- and 3'-termini upon *in vitro* synthesis (Figs. 1-1C and 1-2D).

LCV coat protein synthesis and virion formation

We hypothesized that the *in vitro* transcripts synthesized from pCM1 and pCM2 contained all the genomic information required for biological activity in inoculated plant cells. To test this hypothesis, we assessed their ability to encode the major coat protein and determined whether or not virion formation could ensue following protoplast inoculation. Total soluble protein extracts and virions were prepared using pCM1 and pCM2 transcript-inoculated tobacco protoplasts harvested at 96 hours post-inoculation (hpi). Immunoblot analysis of SDS-PAGE-separated proteins from the total protein extract and virion preparation was performed using a LCV virion-specific polyclonal IgG. The approx. 28.4 kDa CP was observed in both the total protein extract and virion preparation samples (Fig. 1-3C, lanes 2 and 3). The virion preparation was also analyzed

by transmission electron microscopy (TEM), which revealed the presence of crinivirus-like particles approx. 600-800 nm in length (Fig. 1-3D).

LCV replication in tobacco protoplasts inoculated with pCM1 and pCM2 transcripts

We next assessed the time course accumulation of LCV RNAs in protoplasts inoculated with the pCM1 and pCM2 *in vitro* synthesized transcripts. Total RNA was extracted from cells at 0, 12, 24, 48, 72, and 96 hpi and analyzed in Northern blots using DIG-labeled single stranded riboprobes II and VIII complementary to the 3'-proximal regions of the positive- and negative-sense LCV RNAs 1 and 2, respectively (Fig. 1-4A). Both positive- and negative-sense LCV genomic RNA 1s were visible as early as 12 hpi (Figs. 1-4B and C, lane 12; insets B1 and C1). Accumulation of both RNAs increased rapidly (approx. 9 fold for positive-sense and 8 fold for negative-sense) from 12 to 24 hpi, and continued to increase gradually until reaching the last time point at 96 hpi (Figs. 1-4B and C, lanes 12 to 96). The subgenomic RNA (sgRNA) of P8 and/or P23 (sg1) was visible at 24 hpi and accumulated rapidly to a maximum at 48 hpi (Fig. 1-4B).

Positive- and negative-sense LCV genomic RNA 2s were observed at 12 hpi (Figs. 1-4D and E, lane 12; insets D1, E1 and E2). The signal at 0 hpi was most likely from the transcript inoculum of pCM2. The accumulation of positive- and negative-sense RNA 2s increased rapidly by approximately 9 fold from 12 to 24 hpi and approximately 20 fold from 24 to 48 hpi (Figs. 1-4D and E, lanes 12, 24, and 48). The increase in accumulation of both RNA species then continued gradually to the last time point at 96 hpi (Figs. 1-4D and E, lanes 12 to 96). All the predicted RNA 2-derived sgRNAs (sg2-6)

were visible as early as 48 hpi (Fig. 1-4D, lanes 48 to 96). Their accumulation increased slightly from 48 to 72 hpi, where the maximum accumulation was reached.

Synthesis and accumulation of novel viral RNAs

Previously, Northern blot analyses using riboprobes complementary to the 5'-proximal ends of positive-sense RNAs 1 and 2 detected several low MW viral RNAs in the total RNA extracts of LCV-infected *Chenopodium murale* plants (Salem et al., 2009). Their hybridization properties suggested that these viral RNAs were potential 5'-coterminal sgRNAs (Che et al., 2001; Gowda et al., 2001; Gowda et al., 2009; Mawassi et al., 1995a; Salem et al., 2009). Here, we focused on RNA 1 and its derivative(s) by first determining if a prominent ~0.3 – 0.4 kb viral RNA previously observed in infected plant samples (Salem et al., 2009) was also produced in vRNA- and transcripts-inoculated protoplasts, and if it was generated in both polarities or was lacking in negative strand.

We isolated total RNA from transcript-, vRNA-, and mock (water)-inoculated protoplasts at 96 hpi and analyzed them by Northern blotting using the RNA 1 positive-sense specific probe I (Fig. 1-4A). The analysis identified genomic RNA 1 and an ~0.38 kb RNA, designated LM-LCVR1-1, in the total RNA extracts of transcript- and vRNA-inoculated protoplasts (Fig. 1-5A, lanes 1 and 2) but not in that of mock (water)-inoculated protoplasts (Fig. 1-5A, lane 3). LM-LCVR1-1 was observed only with probe I (Fig. 1-5A) but not with probe II (complementary to the 3'-proximal region of RNA 1) (Figs. 1-4B and C), suggesting that this was not a conventional 3'-coterminal sgRNA.

The positive-sense specific probe I also hybridized to multiple RNA species with sizes ranging from ~0.4 to ~1.5 kb (indicated as ♦ in Fig. 1-5A, lanes 1, 2 and 4). The positive-sense specific probe I further identified another distinct low MW (~0.3 kb) RNA, LM-LCVR1-2, and specific but low levels of two high MW RNAs, HM-LCVR1-1 (~8.0 kb) and HM-LCVR1-2 (~6.6 kb), from the protoplast and plant samples (Fig. 1-5A, lanes 1, 2 and 4).

We next used the positive- or negative-sense specific probe I to analyze the time course accumulation of LM-LCVR1-1, LM-LCVR1-2, HM-LCVR1-1, and HM-LCVR1-2. Positive-sense LM-LCVR1-1 was visible at 24 hpi and accumulated at amounts higher than genomic RNA 1 (Fig. 1-5B, lane 24; inset B1). LM-LCVR1-1 accumulated approx. 11 fold from 24 to 48 hpi, reaching a maximum at 72 hpi, and was maintained at that level up to the final sampling time of 96 hpi (Fig. 1-5B, lanes 24 to 96). Detectable amounts of negative strand LM-LCVR1-1 were not apparent throughout the sampling period (Fig. 1-5C). Positive strand HM-LCVR1-2 was visible at 48 hpi [and at 24 hpi with a prolonged exposure of the X-ray film (not shown)]; however it accumulated less drastically relative to genomic RNA 1 and LM-LCVR1-1, showing only a slight increase from 24 to 72 hpi, and was maintained at that level up to the final sampling time of 96 hpi (Fig. 1-5B, lanes 48 to 96). The accumulation of negative stranded HM-LCVR1-2 was not apparent throughout the sampling period (Fig. 1-5C, lanes 48 to 96). In contrast, LM-LCVR1-2 and HM-LCVR1-1 were hybridized by both the positive-and negative-sense specific probe I (Figs. 1-5B and C). Positive and negative stranded LM-LCVR1-2 were observed at 48 hpi and accumulated appreciably to a maximum at 96 hpi (Figs. 1-5B and

C), while positive and negative stranded HM-LCVR1-1 were also observed at 48 hpi but accumulated marginally from 48 to 96 hpi compared to genomic RNA 1 and LM-LCVR1-2 (Figs. 1-5B and C). Accumulation of the multiple low MW RNAs seen in Fig 2.5A (indicated as ♦ in Figs. 1-5A and B) was observed when tested using positive-sense specific probe I, but were indistinct when negative-sense specific probe I was used (Fig. 1-5C).

Because we now have biologically active cDNA clones of the LCV genomic RNAs, it was possible to determine whether infection by RNA 1 alone would result in the production of LM-LCVR1-1, LM-LCVR1-2, HM-LCVR1-1, and HM-LCVR1-2. Using the positive- or negative-sense specific probe I in Northern blot analyses of the total RNA extracts from protoplasts inoculated with the pCM1 transcript, our results showed that all of these RNAs were produced and/or accumulated with the same kinetics as they would in cells inoculated with pCM1 and pCM2 transcripts (Figs. 1-5D and E). Thus, these results suggest that the production of the RNA 1-derived novel RNAs is independent of RNA 2.

Identification of an LCV RNA 1-derived defective RNA

We next performed Northern analyses on the pCM1 and pCM2 transcript-inoculated samples using the positive- or negative-sense specific probe IX, complementary to nucleotide 8331 to 8573 at the extreme 3' terminus of LCV RNA 1 (Fig. 1-4A) and observed a specific RNA that corresponded to the size expected of LM-LCVR1-2 (~0.3 kb) (Figs. 1-6A and B). The positive strand was detected from 48 to 96

hpi, similar to the accumulation kinetics of positive strand LM-LCVR1-2. We next conducted a Northern analysis using the positive- or negative-sense specific probe X, complementary to nucleotide 3881 to 4157 in the middle region of RNA 1 (Fig. 1-4A) to further characterize the ~0.3 kb RNA. Neither the positive- nor negative-sense specific probe X hybridized to an RNA species of that size, although they did hybridize to the ~8.0 kb RNA (HM-LCVR1-1), while positive-sense specific probe X also hybridized to the 6.6 kb RNA (HM-LCVR1-2) as anticipated (Figs. 1-6C and D). These data suggested that the ~0.3 kb RNA and LM-LCVR1-2 were likely one and the same RNA species and contained DRNA features i.e. the presence of 5' and 3' termini of RNA 1 but absence of an internal region of the RNA. In an attempt to determine the identity of the ~0.3 kb LM-LCVR1-2, we subjected the total RNA of LCV-infected *C. murale* plants and that of pCM1 and pCM2 transcripts-inoculated protoplasts harvested at 96 hpi to RT-PCR using oligonucleotide primers LCV-50 and LCV-55, complementary to the extreme 5' and 3' termini, respectively, of RNA 1. A discrete ~0.4 kb product was generated from each sample (Fig. 1-7A, lanes 1 and 2) and cloned into the pGEM-T Easy plasmid. Nine to eleven recombinant plasmids containing the cDNA inserts generated from each of the above samples were randomly selected for sequencing. The cDNA inserts ranged in size from 0.3 kb to 0.4 kb and exhibited very similar nucleotide sequence arrangements with DRNA features: the presence of 5' terminal nucleotides from position 1 to between positions 190 and 304, corresponding to the 5' NCR and part of ORF 1a (beginning at position 73) encoding the first 39 to 77 amino acids of the P-Pro, followed immediately by 3' terminal nucleotides from between positions 8391 and 8562 to position 8591 (the

last nucleotide), corresponding to the 3' NCR of RNA 1. 93 - 96 % of RNA 1 nucleotides were absent between these two nucleotide junctions. Two of the cDNA inserts contained additional (non-viral) nucleotides in between the two nucleotide junctions. Their nucleotide sequence arrangements and those of three other representative DRNAs identified from protoplasts or plants are shown in Fig 1-7B. Interestingly, although the terminal regions of RNA 1 that made up these DRNAs were either non-coding sequences or contained only part of an ORF, the resulting DRNAs that formed were found to all contain a predicted small ORF of 123 to 252 nucleotides with the stop codon located in the 3' terminal sequences (Fig. 1-7C). To ensure that any DRNAs identified by this strategy were not artifacts caused by the false priming of the oligonucleotide primers, we also conducted RT-PCR using full-length LCV RNA 1 transcripts as a control. No RT-PCR products were observed from the full-length LCV RNA 1 transcripts (Fig. 1-7A, lane 3), indicating that under similar conditions used for the RT-PCR mediated cDNA synthesis of the DRNAs, neither artifacts nor the full-length cDNA of RNA 1 were generated. As an additional control, the LCV RNA 1 transcripts were used for RT-PCR with two sets of oligonucleotide primers, LCV-50 and LCV-25, and LCV-29 and LCV-55 (see materials and methods), which would amplify products corresponding to nucleotide position 1 to 482 and 8076 to 8591 on RNA 1, respectively. RT-PCR products of 482 and 515 nucleotides, the expected sizes, were obtained (not shown), indicating that the LCV RNA 1 transcripts were amenable to RT-PCR amplification.

We also subjected the total RNA from pCM1 transcript-inoculated protoplasts to RT-PCR using the same oligonucleotide primers and reaction conditions as above and

obtained cloned RT-PCR amplified cDNA products that all contained distinctive DRNA features and a *de novo* produced ORF similar to the ones described above (not shown).

Discussion

We have obtained the cloned full-length cDNAs corresponding to the genomic RNAs of LCV. This is only the second crinivirus for which cloned cDNAs of the viral genomic RNAs are available. While sequence identity between the 3' NCRs of the genomic RNAs is high within most individual criniviruses, there is generally very little sequence conservation in these regions among the different criniviruses. Thus, RT-PCR synthesis of full-length cDNAs corresponding to the LCV genomic RNAs was possible only with the use of LCV-specific oligonucleotide primers, and only after the genome sequence of the virus became available (Salem et al., 2009). Sequence analysis of pCM1 and pCM2 revealed 12 nucleotides and three deduced amino acids that differed from those of the consensus sequences (Fig. 1-3A and B). However, it is clear that these changes did not abolish the biological activity of the pCM1- and pCM2-derived transcripts.

Protoplasts inoculated with these transcripts produced a viral RNA accumulation profile resembling that of protoplasts inoculated with WT LCV vRNA (Salem et al., 2009). First, they supported the accumulation of negative-sense LCV genomic RNAs 1 and 2, as well as the accumulation of all predicted RNAs 1- and 2-derived sgRNAs. The accumulation of sgRNAs 3 and 4 was not readily apparent (Fig. 1-4C and D), although this was consistent with the results of our previous study using LCV-infected plant tissues and LCV vRNA-inoculated tobacco protoplasts (Salem et al., 2009). Thus, it seems likely that sgRNAs 3 and 4 are either synthesized at low amounts or are turned-over quickly in LCV infected cells relative to the other predicted sgRNAs. Second, viral

genomic RNAs exhibited a temporally similar accumulation pattern early in the infection. This was supported by results shown in Fig. 2 wherein both positive- and negative-sense LCV RNAs 1 and 2 were detected within approximately 12 hpi. Third, protoplasts inoculated with pCM1- and pCM2-derived transcripts produced crinivirus-like particles and CPs that were readily detected by TEM and immunoblots, respectively (Figs. 1-3C and D).

A comparison of the viral RNA accumulation profiles in pCM1 and pCM2 transcript-, pCM1 transcript- alone, and vRNA-inoculated protoplasts, as well as of LCV-infected plants revealed that LCV replication is accompanied by the production of RNA 1-derived low MW and high MW RNAs (Figs. 1-5 and 1-6). LM-LCVR1-1 and HM-LCVR1-2, in having only positive stranded forms and accumulating at different levels, clearly possess features that are similar to those of 5'-coterminal sgRNAs/ 5'-terminal RNAs such as CTV LMT and LaMT RNAs, respectively (Che et al., 2001). Synthesis of the CTV LMT RNAs involve the participation of controller elements (CEs) located in the 5' region within ORF 1a of the CTV genome (Che et al., 2001; Gowda et al., 2003; Mawassi et al., 1995a). The CTV CEs are hypothesized to fold into unique stem loops, whose assumed role is to disrupt the progression of the replicase complex, although mutagenesis studies suggested that the secondary structures and primary sequences of these CEs are both required for optimal sgRNA synthesis (Gowda et al., 2003; Gowda et al., 2001). Intriguingly, although a high MW (~7 kb) RNA 1-derived 5' sg-like RNA has been previously observed in the dsRNA preparation of LIYV-infected plants (Rubio et al., 2000), abundantly accumulating low MW 5'-terminal RNAs like LM-LCVR1-1 have

never been reported for LIYV or any other criniviruses. In the case of HM-LCVR1-1, both positive and negative strands were observed. However, neither the mechanism by which they are synthesized nor those for the production of the 5'-terminal RNAs (LM-LCVR1-1 and HM-LCVR1-2) is understood. If CEs regulating the synthesis of the 5'-terminal RNAs exist, then based on our data, they would likely be found within the coding regions of ORFs 1a and 1b in RNA 1.

An important result from this study is the identification of an authentic LCV RNA 1-derived DRNA, LM-LCVR1-2, since DRNAs that are derived entirely from RNA 1 have never before been reported for LIYV or any other criniviruses (Rubio et al., 2000). A previous study reported that DRNAs of the crinivirus *Potato yellow vein virus* (PYVV) were found to contain nucleotide sequences originating from parts of RNA 1. However, these DRNAs also contained nucleotide sequences that originated from other regions of the PYVV genome (Eliasco et al., 2006). RT-PCR results from our study indicated that the ~0.3 – 0.4 kb DRNAs generated from the total RNA of both LCV-infected plants and protoplasts were similar in size to that of the ~0.3 kb LM-LCVR1-2 observed in Northern blot analysis, (Fig. 1-7A, lanes 1 and 2, and Fig. 2.5A, lanes 1, 2 and 4). Several of the RNA 1 DRNAs contained extra nucleotide repeats at the junction sites (Fig. 1-7B), and were consistent with those observed for the DRNAs of CTV (Mawassi et al., 1995a; Mawassi et al., 1995b) as well as for several RNA 2 DRNAs of LIYV (Rubio et al., 2000). Also consistent with the results obtained from studies of CTV DRNAs, was the discovery of a putative *de novo* generated small ORF in the LCV RNA 1 DRNA (Yang et al., 1997). The significance of this putative ORF is unclear; however, as demonstrated by

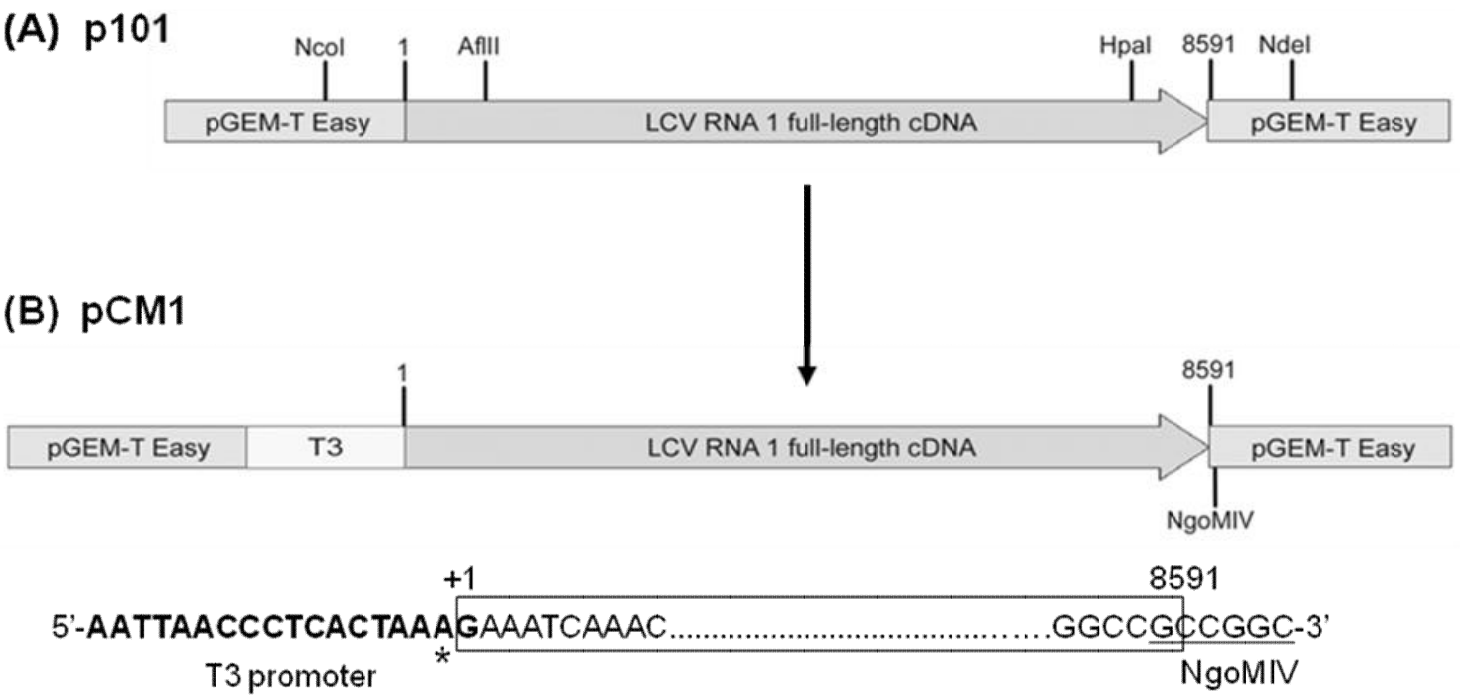
(Mawassi et al., 2000), the putative ORF in the CTV DRNAs was required for their replication and accumulation.

By inoculating protoplasts with only the pCM1 (LCV RNA 1) transcript and still observing the generation of the 5'-terminal RNAs and DRNA, our data have presented clear evidence that their synthesis are coupled to the activities of RNA 1. RNA 1's involvement in the maintenance of the DRNA, LM-LCVR1-2, is especially noteworthy for it has important implications on the replication mechanism of LCV and other criniviruses. In order for it to propagate, the DRNA has to contain all the necessary information as well as sequence and/or structural signals required for replication. However, without any complete sequences (of ORF 1a and 1b) encoding for the replication associated proteins, this function must most likely be supplied *in trans* by the helper WT RNA 1. In striking contrast, LIYV RNA 1 does not appear to support the *trans* replication of any DRNAs, whether they are naturally occurring or artificially constructed (Wang et al., 2009b). The lack of naturally occurring LIYV RNA 1-derived DRNAs, among other lines of evidence such as the observation that RNAs 1 and 2 do not accumulate in a synchronous manner, and the abundance of RNA 2-derived DRNAs have led to the proposal of a *cis* preferential model of replication for LIYV RNA 1 (Rubio et al., 2000; Wang et al., 2009b; Yeh et al., 2000). For LCV, we now know that its RNA 1 supports the replication of a DRNA *in trans*. However, if *cis* preferential replication does exist for LCV RNA 1, based on our data, it is unlikely that it is regulated by a mechanism *similar to that of LIYV. Thus, although LIYV RNA 1 *in cis* replication is the initial step

in the asynchronous replication of the LIYV genomic RNAs (Wang et al., 2009b), this mechanism may not be universally applicable to or identical in all criniviruses.

Figure 1-1. Construction of Lettuce chlorosis virus (LCV) RNA 1 full-length cDNA clone. (A) Full-length LCV RNA 1 cDNA was generated by RT-PCR and cloned into the pGEM-T Easy vector, resulting in p101. Relative positions of the restriction sites (NcoI, AflII, HpaI, and NdeI) used for cloning are indicated. (B) The final cDNA clone, pCM1, was engineered to contain the bacteriophage T3 promoter (open box labeled 'T3') immediately upstream of the LCV RNA 1 5' terminus, and a NgoMIV restriction site immediately downstream of LCV RNA 1 3' terminus. The transcription initiation site (+1), which corresponds to the first nucleotide of LCV RNA 1, begins immediately downstream of the penultimate 3' end nucleotide (*) of the T3 promoter (bold). The genomic sequence of LCV RNA 1 (enclosed in the rectangular box) and the NgoMIV restriction site (underlined) are included as points of reference. (C) The pCM1 *in vitro* synthesized transcript bearing authentic 5' and 3' sequences. Positions of the first (1) and last (8591) nucleotides in LCV RNA 1 are indicated. All diagrams are not drawn to scale. Readers are referred to the Materials and Methods section for details of the cloning procedures.

Figure 1-1



(C) RNA 1 *in vitro* transcript:

+1 8591
5'-GAAATCAAAC.....GGCCG-3'

Figure 1-2. Construction of Lettuce chlorosis virus (LCV) RNA 2 full-length cDNA clone. (A) Two fragments of LCV RNA 2 cDNAs were generated by RT-PCR and cloned into the pGEM-T Easy vector, resulting in p201.1 (fragment #1) and p201.2 (fragment #2). p201.1 was used for the subsequent construction of p202, while p201.2 was used for construction of p203 and pCM2. (B) p202 was engineered with a T3 promoter (open box labeled 'T3') immediately upstream of the LCV RNA 2 5' terminus and digested with NcoI and AatII restriction enzymes. The digested fragment was replaced with a similarly digested fragment from p201.2, resulting in the generation of p203. (C) A NgoMIV restriction site was introduced at the 3' terminus of LCV RNA 2 in p201.2. The resulting construct was digested with AatII and ApaI restriction enzymes and used to replace a similarly digested fragment in p203 to obtain the final cDNA clone pCM2. The transcription initiation site (+1), which corresponds to the first nucleotide of LCV RNA 2, begins immediately downstream of the penultimate 3' end nucleotide (*) of the T3 promoter (bold). The genomic sequence of LCV RNA 2 (enclosed in the rectangular box) and the NgoMIV restriction site (underlined) are included as points of reference. (D) The pCM2 *in vitro* synthesized transcript bearing authentic 5' and 3' sequences. Relative positions of restriction sites (p201.1: NcoI and KasI; p201.2: NcoI and AatII, p202: NcoI and AatII, p203:AatII and ApaI) involved in the cloning steps are indicated. Positions of the relevant nucleotides in LCV RNA 2 are indicated. All diagrams are not drawn to scale. Readers are referred to the Materials and Methods section for details of the cloning procedures.

Figure 1-2

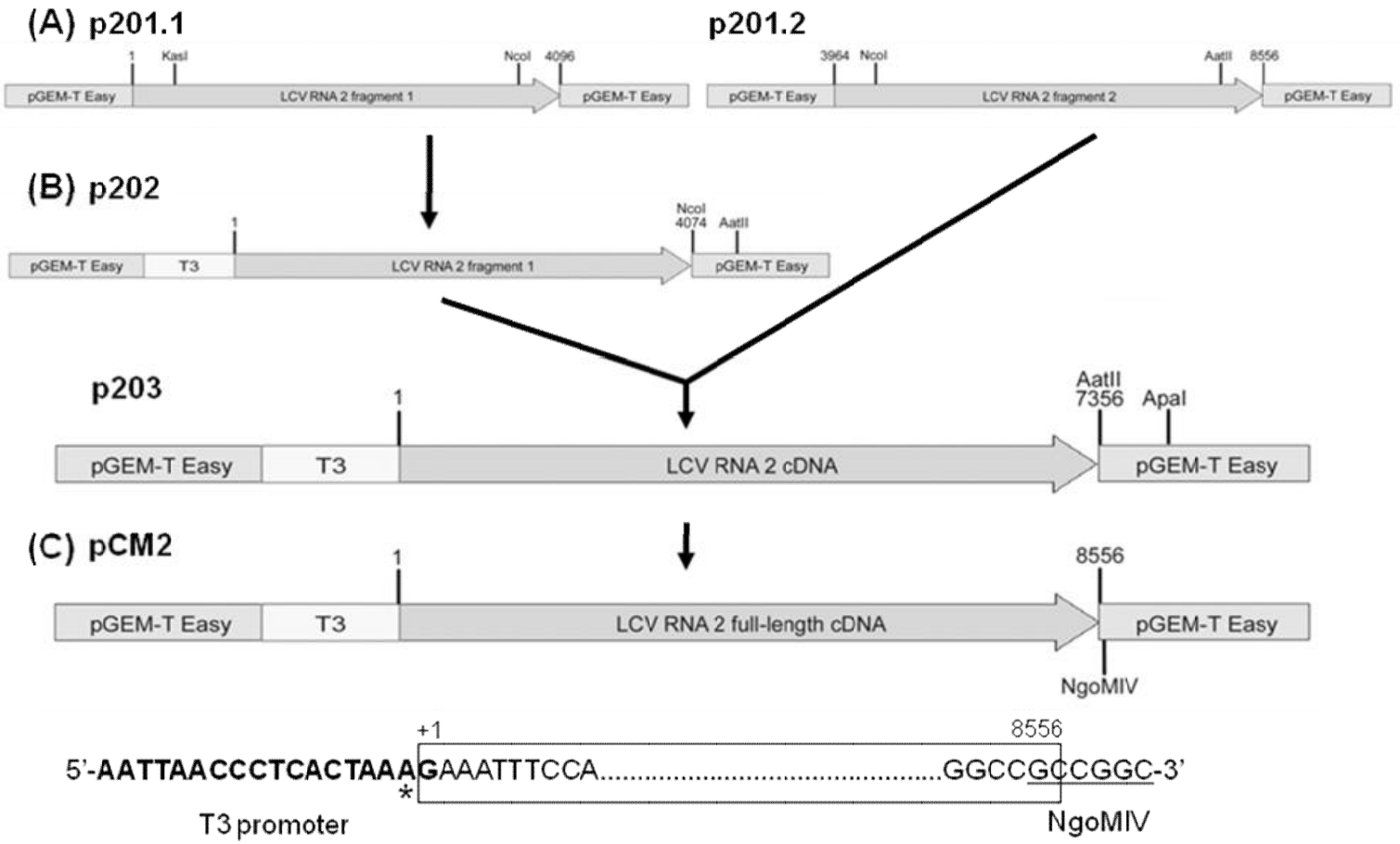

(A) p201.1
1
KasI
NcoI
4096
pGEM-T Easy
LCV RNA 2 fragment 1
pGEM-T Easy
p201.2
3964
NcoI
AatII
8556
pGEM-T Easy
LCV RNA 2 fragment 2
pGEM-T Easy
(B) p202
1
NcoI
4074
AatII
pGEM-T Easy
T3
LCV RNA 2 fragment 1
pGEM-T Easy
p203
1
AatII
7356
ApaI
pGEM-T Easy
T3
LCV RNA 2 cDNA
pGEM-T Easy
(C) pCM2
1
8556
pGEM-T Easy
T3
LCV RNA 2 full-length cDNA
pGEM-T Easy
NgoMIV
+1
8556
5'-AATTAACCCTCACTAAAGAAATTTCCA.....GGCCGCCGGC-3'
*
T3 promoter
NgoMIV
(D) RNA 2 in vitro transcript:
+1
8556
5'-GAAATTTCCA.....GGCCG-3'

Figure 1-3. Organization of LCV RNAs and detection of LCV capsid protein and virion-like particle. Organization of: (A) LCV RNA 1 showing the differences between the consensus and pCM1 (cloned cDNA of RNA 1) sequences; and (B) LCV RNA 2 showing the differences between the consensus and pCM2 (cloned cDNA of RNA 2) sequences. Numbers in the boxes within the genome maps represent open reading frames that encode proteins or protein-domains: P-Pro, papain-like protease; MTR, methyltransferase; HEL, RNA helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, a heat shock protein 70 homolog; CP, major coat protein; CPm, minor coat protein; and proteins that are named according to their relative molecular masses (numbers preceded by 'P'). Numbers and arrows below the genome maps indicate the nucleotide positions where changes (A, U, C, or G) have been observed. Nucleotide positions at which non-synonymous changes have occurred are marked by a *, with the deduced amino acid changes indicated in parentheses. (C) Immunoblot analysis of LCV virions. Virion preparation and total protein extract were separated on a 12% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane, and analyzed using a polyclonal IgG produced against LCV virions. Lane 1, total soluble proteins from water (mock)-inoculated protoplasts; lane 2, total soluble proteins from pCM1 and pCM2 *in vitro* transcripts-inoculated protoplasts; lane 3, virion preparation from pCM1 and pCM2 *in vitro* transcripts-inoculated protoplasts; and lane 4, LCV virions purified from infected *Chenopodium murale* plants. The position of the LCV CP (28.4kDa) is indicated by an arrow. Additional (25kDa and 37kDa) size markers are included as references. (D) Transmission electron microscopy analysis of the LCV virion preparation obtained from pCM1 and pCM2 *in vitro* transcripts-inoculated protoplasts. A 0.2 micron scale bar is provided for size reference.

Figure 1-3

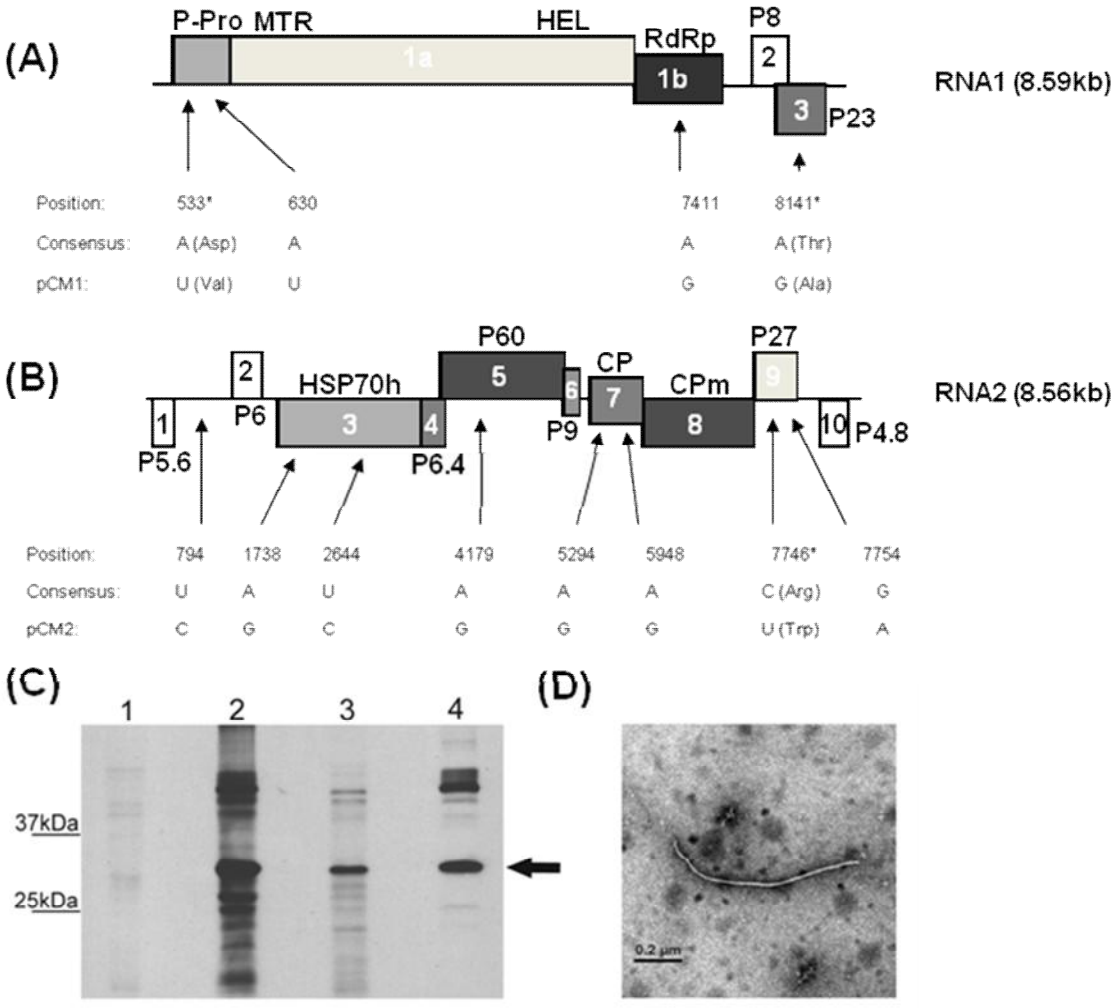

(A)
P-Pro
MTR
HEL
RdRp
P8
1a
1b
2
3
P23
RNA1 (8.59kb)
Position: 533* 630 7411 8141*
Consensus: A (Asp) A A A (Thr)
pCM1: U (Val) U G G (Ala)
(B)
P60
CP
P27
2
HSP70h
5
6
7
CPm
9
1
P6
3
4
P9
8
10
P4.8
P5.6
P6.4
RNA2 (8.56kb)
Position: 794 1738 2644 4179 5294 5948 7746* 7754
Consensus: U A U A A A C (Arg) G
pCM2: C G C G G G U (Trp) A
(C)
1 2 3 4
37kDa
25kDa
(D)
0.2 µm

Figure 1-4. Accumulation of Lettuce chlorosis virus (LCV) RNAs in tobacco protoplast. (A) Genome organization of LCV. Black bars below the genome map (I, II, VIII, IX and X) represent the riboprobes complementary to the corresponding locations in the LCV genomic RNAs. Fine lines beneath the black bars represent the predicted 3' co-terminal subgenomic RNAs: P8 and/or P23 (sg1), P6 and/or HSP70h (sg2), P6.4 and/or P60 (sg3), P9 and/or CP (sg4), CPm (sg5), and P27 (sg6), with sizes (kb) as indicated. (B-E) Viral RNA accumulation in tobacco protoplasts inoculated with the *in vitro* transcripts of pCM1 and pCM2. Total RNA (2 μg each) extracted from the inoculated protoplasts harvested at 0, 12, 24, 48, 72, and 96 hours post-inoculation (hpi) (lanes 0 to 96, respectively), total RNA (2 μg) from water (mock)-inoculated protoplasts harvested at 96hpi (lane W), and LCV virion RNA (10 ng) (lane V) were analyzed using DIG-labeled positive- (B) or negative-sense (C) RNA 1 specific probe II, and positive- (D) or negative-sense (E) RNA 2 specific probe VIII. The polarity (+ or -) of viral RNAs being detected is indicated under each blot. Hybridization signals of positive- and negative-sense genomic RNAs 1 (G1) and 2 (G2), and sgRNAs 1, 2, 3, 4, 5, and 6 are indicated. Insets B1, C1, D1 and E1 show the extended exposures of the hybridization signals for the genomic RNAs (G1 or G2) in lanes 0, 12, and 24. Inset E2 shows the hybridization signals for genomic RNA 2 (G2) in lanes 0, 12, and 24 of a subsequent blot when a 2.5 fold higher amount (5 μg) of total RNA from each sample harvested at 0, 12, and 24 hpi, respectively, were analyzed. Sizes of RNAs were estimated based on methylene blue-stained RNA standards shown on the left of each blot. The methylene blue-stained 25S rRNA of each sample was included to demonstrate the equal loading of total RNA samples.

Figure 1-4

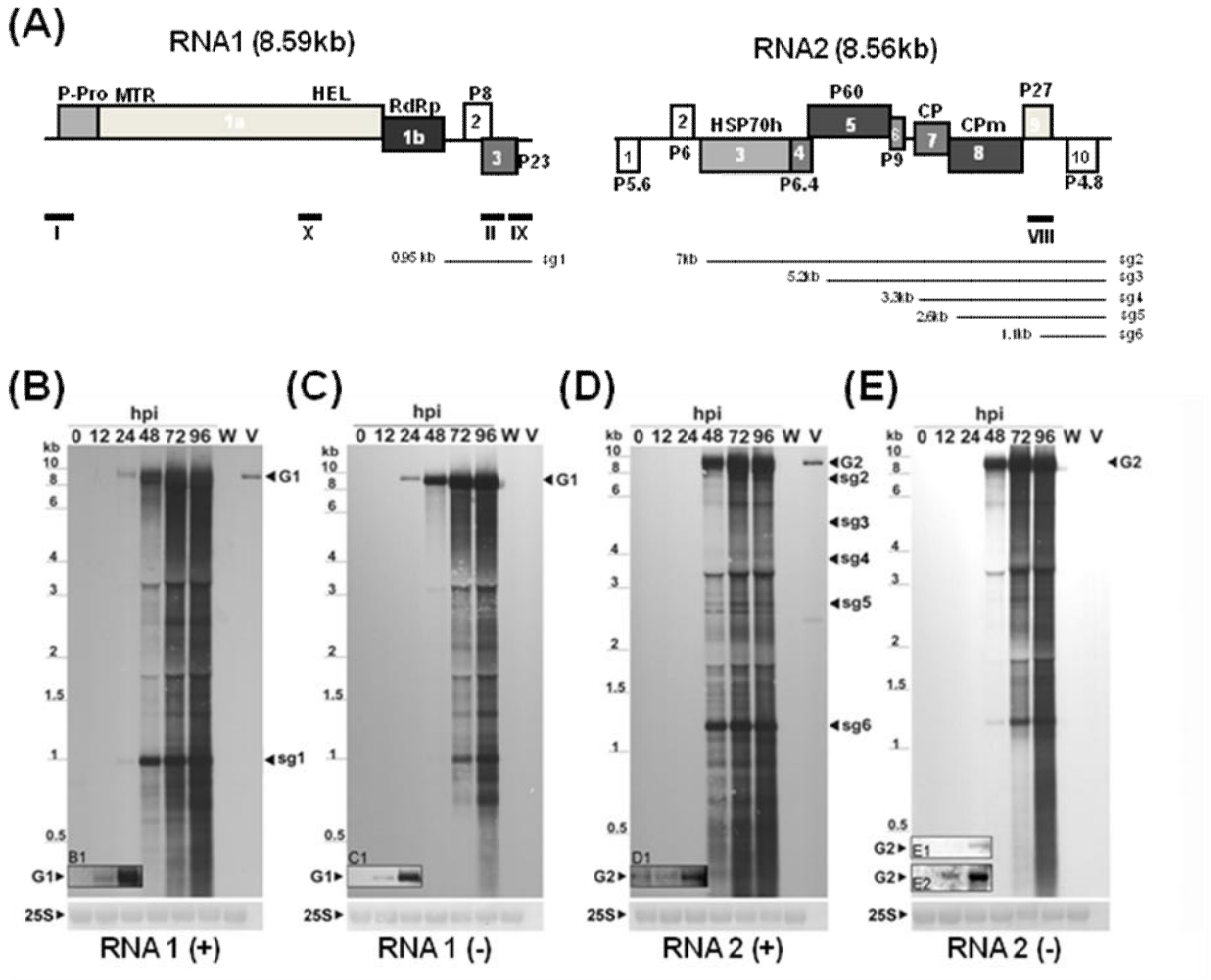

(A)
RNA1 (8.59kb)
P-Pro MTR
HEL
RdRp
P8
1a
1b
2
3
P23
I
X
II IX
0.95 kb
sg1
RNA2 (8.56kb)
P60
CP
P27
HSP70h
CPm
1
2
3
4
5
6
7
8
9
10
P5.6
P6
P6.4
P9
P4.8
VIII
7kb
sg2
5.2kb
sg3
3.3kb
sg4
2.6kb
sg5
1.8kb
sg6
(B)
(C)
(D)
(E)
hpi
0 12 24 48 72 96 W V
kb
G1
sg1
G2
sg2
sg3
sg4
sg5
sg6
B1
C1
D1
E1
E2
25S
RNA 1 (+)
RNA 1 (-)
RNA 2 (+)
RNA 2 (-)

Figure 1-5. Northern analysis of LCV RNA 1-derived novel RNAs. (A) Northern blot using RNA 1 (+)-specific probe I (complementary to nt position 1 – 482). Samples were total RNA of tobacco protoplasts inoculated with: pCM1 and pCM2 transcripts (lane 1), LCV virion RNA (lane 2), and water (mock inoculation) (lane 3). Total RNA from LCV-infected *C. murale* plants (lane 4) and LCV virion RNA (lane 5) were included for comparison. (B) Total RNA of: pCM1 and pCM2 transcripts- (lanes 0 to 96 hpi) and water (mock)-inoculated protoplasts (lane W; harvested at 96 hpi), and LCV virion RNA (V) were analyzed using RNA 1 (+)-specific probe I. (C) Northern blot analysis of the same samples in (B) using the RNA 1 (-)-specific probe I. (D) Total RNA of: pCM1 transcript- (lanes 0 to 96 hpi) and water (mock)-inoculated protoplasts (lane W; harvested at 96 hpi) were analyzed using RNA 1 (+)-specific probe I. (E) Northern blot analysis of the same samples in (D) using the RNA 1 (-)-specific probe I. The polarity (+ or -) of viral RNAs being detected is indicated under each blot. Hybridization signals of positive- and negative-sense genomic RNA 1 (G1), and the RNA 1-derived low MW and high MW RNAs: LM-LCVR1-1 (LM-1), LM-LCVR1-2 (LM-2), HM-LCVR1-1 (HM-1), and HM-LCVR1-2 (HM-2) are indicated. Extended exposures of the signals for LM-1 (inset B1) at 24 hpi, and LM-2 (insets B2 and C1) at 48 hpi, are shown at the bottom of the corresponding lanes. Hybridization signals from less distinct, unidentified RNA species are indicated by ♦. RNA size estimates and methylene blue-stained 25S rRNAs loading controls were as for Fig. 2.

Figure 1-5

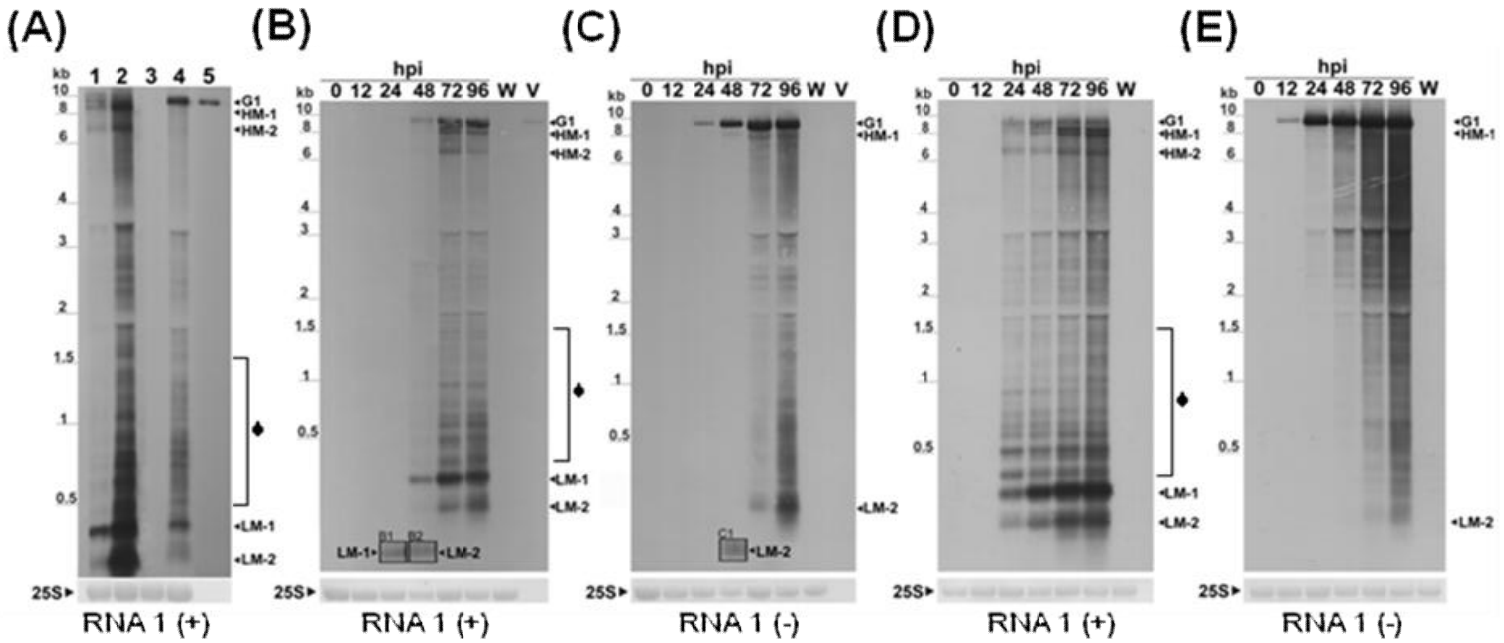

(A)
(B)
(C)
(D)
(E)
kb
1 2 3 4 5
hpi
0 12 24 48 72 96 W V
0 12 24 48 72 96 W
G1
HM-1
HM-2
LM-1
LM-2
B1
B2
C1
25S
RNA 1 (+)
RNA 1 (+)
RNA 1 (-)
RNA 1 (+)
RNA 1 (-)

Figure 1-6. Northern blot characterization of an LCV RNA 1 defective RNA. Total RNA extracted from the pCM1 and pCM2 transcripts-inoculated protoplasts harvested at 0, 12, 24, 48, 72, and 96 hours post-inoculation (hpi) (lanes 0 to 96, respectively), total RNA from water (mock)-inoculated protoplasts harvested at 96 hpi (lane W), and virion RNA (lane V) were analyzed using RNA 1 (+)- (A) or RNA 1 (-)- (B) specific probe IX (nt position 8331 – 8573), and RNA 1 (+)- (C) or RNA 1 (-)- (D) specific probe X (nt position 3881 – 4157). The polarity (+ or -) of viral RNAs being detected is indicated under each blot. Hybridization signals of positive- and negative-sense genomic RNA 1 (G1), and the RNA 1-derived low MW and high MW RNAs: LM-LCVR1-2 (LM-2), HM-LCVR1-1 (HM-1), and HM-LCVR1-2 (HM-2) are indicated. The extended exposure of the hybridization signal for LM-2 (inset B1) at 72 hpi is shown at the bottom of that lane. RNA size estimates and methylene blue-stained 25S rRNAs loading controls were as for Fig. 2.

Figure 1-6

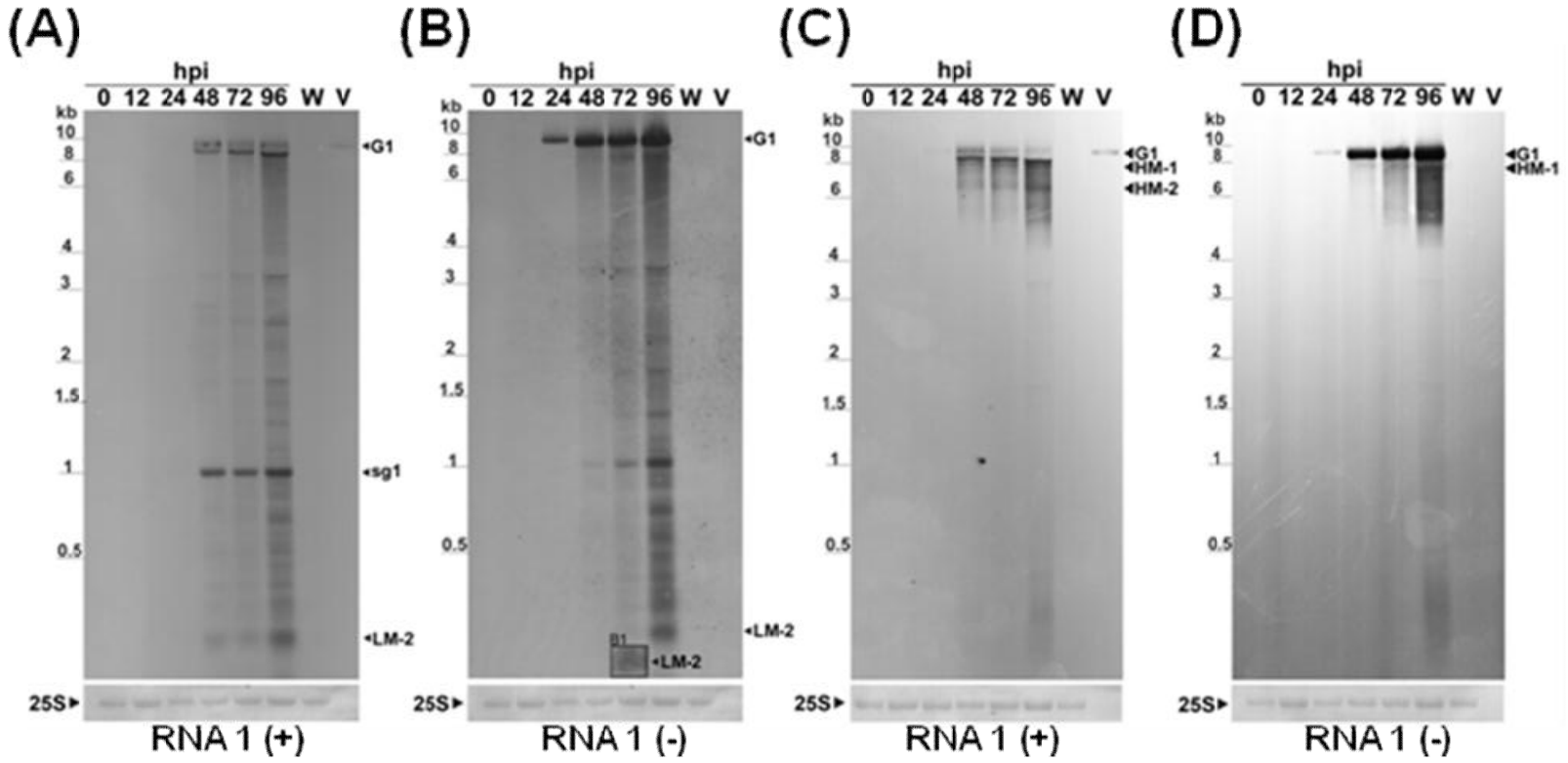

(A)
(B)
(C)
(D)
hpi
0 12 24 48 72 96 W V
kb
10
8
6
4
3
2
1.5
1
0.5
G1
sg1
LM-2
HM-1
HM-2
25S
RNA 1 (+)
RNA 1 (-)

Figure 1-7. Nucleotide sequence analysis of LCV RNA 1 defective (D)RNAs. (A) Ethidium bromide-stained 2 % agarose gel for products amplified from the total RNA of LCV-infected plants (lane 1) and that of pCM1 and pCM2 transcripts-inoculated protoplasts (lane 2) by reverse transcription and polymerase chain reaction (RT-PCR) using oligonucleotide primers specific to the extreme 5' and 3' termini of RNA 1. The same pair of primers and reaction conditions were used for the amplification of pCM1 transcript (lane 3) and the pCM1 plasmid (lane 4). Sizes in base pairs for DNA standards are indicated. (B) Nucleotide sequence arrangements of representative DRNAs isolated from LCV-infected protoplasts (Protos #1, #2, and #3), and LCV-infected plants (Plant #1 and #2). The LCV RNA 1 map is shown at the top. Numbers, 73 and 8362, on the map indicate the nucleotide position of the start codon of open reading frame (ORF) 1a, and the stop codon of ORF 3, respectively. Beneath the map, solid lines represent the nucleotide sequences of DRNAs; dashed lines represent regions of RNA 1 not present in the DRNAs. The numbers preceding the triangles underneath each DRNA indicate the nucleotide positions at the junctions where the loss of RNA 1 nucleotides had occurred. The percentage of RNA 1 missing from each DRNA is indicated in parenthesis. (C) Nucleotide sequences spanning the junction (represented by a vertical line or vertical lines) formed by the union of the LCV RNA 1 5' and 3' terminal sequences in each of the DRNAs described in (B). The nucleotide sequences are shown in triplets to represent codons within the putative ORF. Stop codons are indicated in bold; deduced amino acids are shown on top of each codon triplet. Numbers underneath the triangles represent the nucleotide positions on LCV RNA 1 where union of the 5' and 3' terminal sequences occurs. Dots represent the rest of the nucleotides in the DRNAs. Non-viral sequences occurring at the junctions of DRNAs in (B) and (C) are indicated.

Figure 1-7

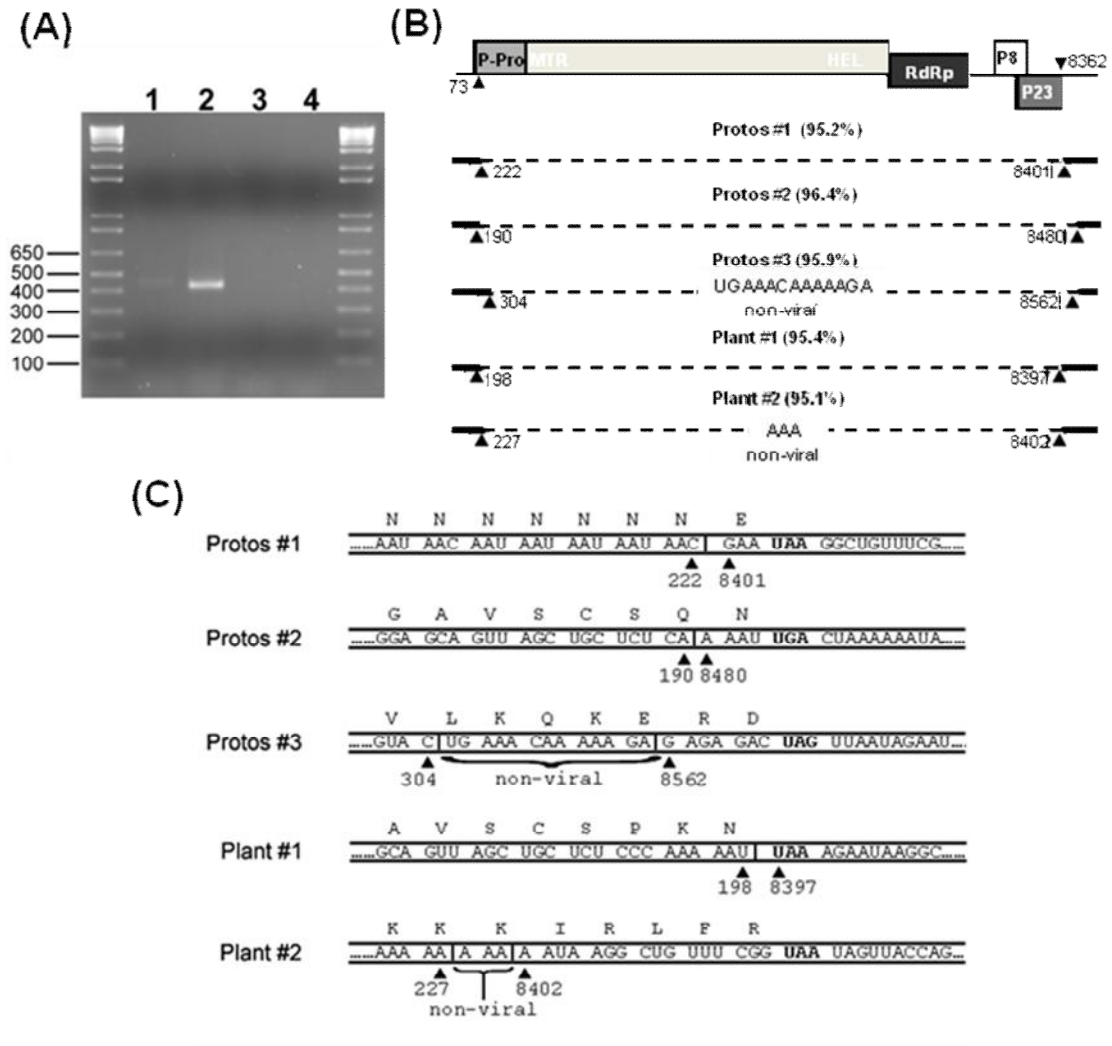

(A)
1 2 3 4
650
500
400
300
200
100
(B)
P-Pro
MTR
HEL
RdRp
P8
P23
73
8362
Protos #1 (95.2%)
222
8401
Protos #2 (96.4%)
190
8480
Protos #3 (95.9%)
UGAAACAAAAAGA
304
non-viral
8562
Plant #1 (95.4%)
198
8397
Plant #2 (95.1%)
AAA
227
non-viral
8402
(C)
Protos #1
N N N N N N N E
AAU AAC AAU AAU AAU AAU AAC GAA UAA GGCUGUUUCG
222 8401
Protos #2
G A V S C S Q N
GGA GCA GUU AGC UGC UCU CA A AAU UGA CUAAAAAAUA
190 8480
Protos #3
V L K Q K E R D
GUA C UG AAA CAA AAA GA G AGA GAC UAG UUAAUAGAAU
304 non-viral 8562
Plant #1
A V S C S P K N
GCA GUU AGC UGC UCU CCC AAA AAU UAA AGAAUAAGGC
198 8397
Plant #2
K K K I R L F R
AAA AA A AA A AUA AGG CUG UUU CGG UAA UAGUUACCAG
227 8402
non-viral

Table 1-1. Oligonucleotide primers used for the construction of the infectious cDNA clones of LCV RNAs 1 and 2.

Primer	Sequence (5' - 3')	Description
LCV-55	ATCTGATT**CATATG**CGGCCTAGTTATTCTATTAACTAGTCTCTC	A NdeI restriction site (bold) and nucleotides complementary to those of LCV RNA 1 position 8591-8562 (underlined)
LCV-52	CATTCGTC**CCATGG**GAAATCAAACTTTCCTTCGTACGAAGAG	A NcoI restriction site (bold) and nucleotides corresponding to LCV RNA1 position 1-28 (underlined)
LCV-89-CN	GGC**AATTAACCCTCACTAAAG**AAATCAAACTTTCCTTCGTACGAAGAG	A T3 promoter sequence (bold) and nucleotides corresponding to those of LCV RNA1 position 1-28 (underlined)
LCV-90-CN	CTTAGGTACTTGGGTGACCTTAAGTTCAGGTTTGGTCACACC	Nucleotides complementary to those of LCV RNA 1 position 1062-1021
LCV-91-CN	TCATTAGTAACGTATCTGTATCAATAAAGATGTGAGATG	Nucleotides corresponding to LCV RNA 1 position 7584-7622
LCV-92-CN	CTGAAT**GCCGG**CGGCCTAGTTATTCTATTAACTAGTCTC	A NgoMIV restriction site (bold) and nucleotides complementary to those of LCV RNA 1 position 8591-8564 (underlined)
LCV-88-JN	TGATCTCTGGACTCTGTACC	Nucleotides complementary to those of LCV RNA 2 position 4096-4076
LCV-56-JN	CAAAGCGTC**GCATGC**GAAATTTCCACGGTTTCCCCGAG	A SphI restriction site (bold) and nucleotides corresponding to LCV RNA2 position 1-23 (underlined)
LCV-101-CM	*cccggg**GCATGC**AATTAACCCTCACTAAAG*AAATTTCCACGGTTTCCCCGAG	XmaI (italic lower case) and SphI (italic upper case) restriction sites; a T3 promoter (bold) and nucleotides corresponding to LCV RNA2 position 1-23 (underlined)
LCV-124-CM	ACATATCCTCAGACACCGCATCGACGCATC	Nucleotides complementary to LCV RNA 2 position 1104-1075
LCV-57-JN	ATCTGATTGC**GGCCGC**CGGCCTAGCTATACTACTAACTAGTCGTTCAAG	A NotI restriction site (bold) and nucleotides complementary to those of LCV RNA 2 position 8556-8524 (underlined)
LCV-87-JN	TTCAGAGAATCGTGGAAGC	Nucleotides corresponding to LCV RNA 2 position 3964-3983
LCV-125-CM	ATTATGATAAGTTGAGAGTGTCCGATCAGG	Nucleotides corresponding to LCV RNA 2 position 7308-7337
LCV-104-CM	*GGGCCC**GCCGG**CGGCCTAGCTATACTAAATC*	ApaI (italic) and NgoMIV (bold) restriction sites, and nucleotides complementary to LCV RNA 2 position 8556-8536 (underlined)

Chapter 2: Structural and functional analyses of the 3' non-coding region of *Lettuce chlorosis virus* RNAs identify unique features of a *cis*-acting element required for minus strand RNA synthesis

Abstract

Our current understanding of elements regulating minus-sense RNA synthesis is significantly behind for economically important viruses in the genus *Crinivirus*. An availability of the cloned infectious cDNAs of *Lettuce chlorosis virus* (LCV), a bipartite (+) single stranded RNA virus in the genus *Crinivirus* (family *Closteroviridae*), allowed us to identify and examine elements involved minus-strand RNA synthesis. We first exchanged a conserved region between the 3' NCR of LCV RNAs 1 and 2 and found that exchanging these regions did not cause any detrimental effects on minus-strand RNA synthesis. We next constructed and tested several LCV RNA 2 mutants engineered with deletions in the 3' NCR and our results indicated that LCV RNA 2 could not tolerate the deletion of more than last 38 nucleotides from 3' terminus, but could can still replicate without the last 24 nucleotides. We also identified a stem-loop structure in the 3' NCR of LCV RNA 2 though the uses of a web server application *Mfold* and SHAPE (Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension) analysis. Mutation analysis showed that any mutations that altered the overall structure abolished minus-strand RNA synthesis, while a compensatory mutation that restored the predicted structure rescued minus-strand RNA synthesis, which strongly suggested that this predicted structure plays an important role in regulating minus-strand RNA synthesis. However, specific parts of the structure (loop, upper stem, and internal loop) are not required for minus-strand RNA synthesis. Furthermore, SHAPE analysis of replication competent LCV RNA 2 defective RNA showed that SL is a part of a larger Y-shaped structure

Introduction

Viruses with single stranded (ss), positive-sense (+)-RNA genomes synthesize minus-strand (-)-RNAs early in the replication process, and use them as templates for the production and propagation of genomic (g)RNAs and, for some viruses, subgenomic (sg)RNAs that serve as messengers for the translation of proteins required for various viral functions (Buck, 1996; Li and Nagy, 2011). (-)-RNA synthesis is a highly regulated affair involving specialized cellular membranes and hinges on the interplay between essential *cis*-acting elements located invariably within the 3'-terminus of the gRNA and the viral replicase complex (VRC; consisting of proteins co-opted from the host, the viral RNA-dependent-RNA polymerase (RdRp), as well as other viral-encoded proteins) (Ahlquist, 2006; Dreher, 1999). Investigations of selected members within the alphavirus-, picornavirus-, and flavivirus-like superfamilies of (+)-RNA viruses have led to the identification and/or characterization of c*is*-acting sequence or structural elements that are important for (-)-RNA synthesis (Dreher, 2009; Ogram and Flanegan, 2011; Pathak et al., 2011). For *Sindbis virus*, sequence elements and specific nucleotides (nts) in the 3' non-coding region (NCR) of the gRNA, the length of its poly(A) tail, as well as the spatial requirement between the poly(A) tail and the sequence elements in the 3' NCR all play significant roles in (-)-RNA synthesis (Hardy and Rice, 2005; Liu et al., 2009). *Poliovirus* (-)-RNA synthesis is dependent on a pseudoknot structure formed by the interactions between two stem loops (SLs) in the 3' untranslated region of the gRNA, and on five conserved nts that separate both these SLs (Jacobson et al., 1993; Melchers et al., 1997; Ogram and Flanegan, 2011; Pilipenko et al., 1992; Pilipenko et al., 1996). Among

plant viruses, *Brome mosaic virus* (BMV) is one of the best characterized with respect to *cis*-acting element-mediated (-)-RNA synthesis. Here, a highly conserved tRNA-like structure (TLS) at the 3' termini of the BMV gRNAs, terminating with a -CCA at the 3' end, plays a defining role in (-)-RNA initiation (Chapman and Kao, 1999; Dreher and Hall, 1988a; Miller et al., 1986). In the case of *Turnip crinkle virus* and its associated satellite (sat-) RNA C, a hairpin situated within the 3' terminal region of the RNA functions as a promoter for (-)-RNA synthesis (Song and Simon, 1995; Yuan et al., 2012; Yuan et al., 2010).

Relative to the well studied (+)-ssRNA viruses described above, very little is known about the 3' *cis*-acting element-mediated (-)-RNA synthesis for many economically important plant viruses, including those in the genus *Crinivirus* (family *Closteroviridae*). The negative impact of criniviruses on plant health is evidenced by the severity of damage inflicted on the wide range of plants that these viruses collectively infect, and by their rapid emergence in different foci around the world, particularly in subtropical areas where their whitefly vectors thrive in abundance (Harris et al., 2001; Navas-Castillo et al., 2011; Tzanetakis et al., 2013). These affiliates of the alphavirus-like supergroup of (+)-ssRNA viruses have large and complex genomes ranging in size from 15.3 – 17.6 kb. Full-length cloned infectious cDNAs of the gRNAs (RNAs 1 and 2) have been developed for the type species of *Crinivirus*, *Lettuce infectious yellows virus* (LIYV), and they have facilitated studies that revealed important information on the biology and molecular biology of this and other related viruses (Chen et al., 2011; Kiss et al., 2013; Ng and Falk, 2006a; Ng et al., 2004; Stewart et al., 2010). LIYV RNAs 1 and 2

are both capped at the the 5' end and not polyadenylated at the 3' end (Karasev et al., 1989; Kiss et al., 2013). Embedded in LIYV RNAs 1 and 2 are distinct features that criniviruses and members of the family *Closteroviridae* all possess. In RNA 1, ORFs 1a and 1b are likely directly translated to encode a replication module consisting of the ORF 1a encoded papain-like leader protease (P-PRO), methyltransferase (MTR), and helicase (HEL); and the RdRp (translated from ORF 1b via a +1 frame shifting event occurring in ORF 1a) (Karasev, 2000; Kiss et al., 2013). As such, RNA 1 is capable of replication on its own (Mongkolsiriwattana et al., 2011; Yeh et al., 2000). In contrast, RNA 2, which contains no known replication-related ORFs, replicates using the RNA 1-encoded replication-associated proteins supplied *in trans* (Mongkolsiriwattana et al., 2011; Yeh et al., 2000). In RNA 2, a hallmark "closterovirus quintuple gene block" encodes a small (5 to 6 kDa) hydrophobic protein, a heat shock protein 70 homolog (HSP70h), a 59 kDa protein (50 – 60 kDa depending on the crinivirus), the major coat protein (CP), and the minor coat protein (CPm) (Kiss et al., 2013; Tian et al., 1999).

LCV (Fig. 2-1A) is the only other crinivirus for which cloned cDNAs of the gRNAs have been found to be infectious at both the cellular and whole plant levels (Chen et al., 2012; Mongkolsiriwattana et al., 2011). Insights gained from studies of LCV stand in good complement and contrast to those of LIYV, most notably in replication, genome expression, and transmission by whitefly vectors (Chen et al., 2011; Mongkolsiriwattana et al., 2011; Ng and Chen, 2011; Salem et al., 2009). The 3' NCR of the LCV gRNAs contains a highly structured non-TLS heteropolymeric sequence that appears to be free of pseudoknots (Salem et al., 2009). In RNA 1, the 3' NCR is 226-nt long and shares 74%

sequence identity with its counterpart in RNA 2. In the latter, the upstream 128 nts belong to the last ORF (encoding P4.8), while the downstream 98 nts constitute the 3' NCR of the RNA. The sequence identity between the 98-nt region (from here on referred to as the "98-nt") of RNAs 1 and 2 is 81% (with 21 non-conserved nts) (Salem et al., 2009) (Fig 2-1B).

The focus of the current work is directed towards original insights on the structure and function of the 3' NCR of RNAs 1 and 2 in regulating (-)-RNA synthesis and accumulation, and it is the first of such studies to be undertaken and reported for a crinivirus. Here we show that the 98-nt of LCV RNAs 1 and 2 can be exchanged without affecting (-)-RNA synthesis and viral RNA accumulation. We further determine by mutational analyses of RNA 2 that progressive deletion of the first 24 nts from the 3' end of the RNA results in a corresponding reduction in its (-)-RNA accumulation, while the deletion of an additional 14nts (i.e. 38 nts from the 3' end of the RNA) cannot be tolerated. These results are consistent with *Mfold* and *RNAstructure* predictions and structural evidence from SHAPE (Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension) footprinting analysis of the 98-nt of LCV RNA 2, indicating the presence of a distinct Stem loop, SL1, positioned upstream (-23 to -61 nts) of the terminal nt at the 3' end of both RNAs. The role of SL1 in (-)-RNA synthesis is substantiated by engineered mutations that either disrupt or restore its structure in LCV RNA 2. SHAPE analysis of a LCV RNA 2-derived, replication-competent defective (D)RNA, which also contains the 98-nt, reveals that SL1 constitutes one arm of a larger secondary (Y-shaped) structure.

The significance and implications of these findings for conceptualizing the role of *cis*-acting elements in (-)-RNA synthesis for LCV and other criniviruses are discussed.

Materials and Methods

Construction of pCM1 and pCM2 mutants

Mutant constructs were engineered by PCR-mediated mutagenesis using the WT LCV cDNA clones, pCM1 (RNA 1) and/or pCM2 (RNA 2), as templates (Mongkolsiriwattana et al., 2011), and Herculase II fusion DNA polymerase with high fidelity proofreading capability (Agilent Technologies). All oligonucleotide primers used for the construction of pCM1 and pCM2 mutants are indicated in Table 2-1.

Mutant pR1-3'R2: To replace the 98-nt of LCV RNA 1 with that of LCV RNA 2, two PCRs were performed. The first reaction was performed using oligonucleotide primers LCV-91-CN and LCV-219-CM (with pCM1 as template), while the second reaction was performed using LCV-220-CM and LCV-164-CM (with pCM2 as template). The PCR amplified products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), and 50 ng of each gel-purified product were subjected to overlapping extension PCR using LCV-91-CN and LCV-164-CM. The resulting product was gel-purified, adenylated and cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI). The recombinant pGEM-T Easy vector was digested with restriction enzymes HpaI and NdeI (New England Biolabs), and the released DNA fragment was subcloned into similarly digested pCM1, resulting in the mutant construct pR1-3'R2.

Mutant pR2-3'R1: replacement of the 98-nt of LCV RNA 2 with that of LCV RNA 1 was achieved in the same manner as for the replacement of the 98-nt of LCV RNA 1 with that of LCV RNA 2 as described above, except that the first two PCRs were

performed by using LCV-161-CM and LCV-221-CM (with pCM2 as template), and LCV-222-CM and LCV-223-CM (with pCM1 as template). The overlapping extension PCR was performed using LCV-161-CM and LCV-223-CM, and restriction digestion of the recombinant pGEMT-T Easy vector and pCM2 was made using AatII and NgoMIV.

Mutant p5'Δ50: the pCM2 mutant with a deletion of 50 nts from the proximal 5'-end of the 98-nt of LCV RNA2, was constructed using pCM2 as template, and essentially in the same manner as pR1-3'R2 except that the first two PCRs were performed using LCV-161-CM and LCV-162-CM, and LCV-163-CM and LCV-164-CM, followed by overlapping extension PCR using LCV-161-CM and LCV-164-CM. Restriction digestion of pCM2 and the recombinant pGEMT-T Easy vector containing the overlapping extension PCR product was made using restriction enzymes AatII and ApaI.

Mutants p3'Δ48, p3'Δ48(4), p3'Δ4, p3'Δ10, p3'Δ24, and p3'Δ38: the pCM2 mutants with various deletions engineered in the proximal 3'-end of the 98-nt of LCV RNA 2 were constructed by using the forward oligonucleotide primer LCV-161-CM and one of the following reverse primers: LCV-167-CM (for constructing p3'Δ48), LCV-165-CM [p3'Δ48(4)], LCV-166-CM (p3'Δ4), LCV-176-CM (p3'Δ10), LCV-175-CM (p3'Δ24), and LCV-188-CM (p3'Δ38). All PCR amplified products were gel-purified, adenylated, and cloned into the pGEM-T Easy vector. Restriction digestion using AatII and ApaI, and AatII and NgoMIV were performed to release the DNA fragments from the resulting recombinant pGEM-T Easy vectors used for the construction of: 1) p3'Δ48, p3'Δ4, p3'Δ10, p3'Δ24, p3'Δ38, and 2) p3'Δ48(4), respectively. The released DNA

fragments were subcloned into similarly digested pCM2, resulting in the respective final products.

Mutants p3'ΔSL, p3'SLD1, p3'SLD2 and p3'SLR: the pCM2 mutants with specific mutations engineered in the stem loop (SL1) located within the 98-nt (-23 to -61 nts) upstream of the final nt at the 3' end of RNA 2, were constructed using pCM2 as template, and in the same manner as pR1-3'R2. The first PCR product was generated using the forward oligonucleotide primer LCV-161-CM and one of the following reverse primers: LCV-189-CM (for constructing p3'ΔSL), LCV-191-CM (p3'SLD1), and LCV-193-CM (p3'SLD2 and p3'SLR). The second PCR product was generated using reverse primer LCV-164-CM and one of the following forward primers: LCV-190-CM (for constructing p3'ΔSL), LCV-192-CM (p3'SLD1), and LCV-194-CM (p3'SLD2 and p3'SLR). Overlapping extension PCR of 50ng of each of the gel-purified form of the above PCR products was performed using oligonucleotide primers LCV-161-CM and LCV-164-CM. The resulting PCR amplified products were gel-purified, adenylated, and cloned into pGEM-T Easy vector. The recombinant pGEM-T Easy vector containing the intermediate product (generated by the overlapping PCR above) to be used for the construction of p3'SLR was used as a template for another PCR to introduce the mutation needed to make this mutant. The first PCR was performed using oligonucleotide primer LCV-161-CM and LCV-191-CM; the second PCR was performed using LCV-164-CM and LCV-192-CM. Overlapping extension PCR (using LCV-161-CM and LCV-164-CM) and cloning of the gel purified products into pGEM-T Easy vector were as described above.

The recombinant pGEM-T Easy vectors containing the PCR products for constructing Mutants p3'ΔSL, p3'SLD1, p3'SLD2 and p3'SLRwere digested with AatII and ApaI, and the released DNA fragments were subcloned into similarly digested pCM2, resulting in the respective final products.

Mutant p3'ΔSL2: the pCM2 mutants engineered with a deletion of the stem loop 2 (SL2) were constructed using pCM2 as template, and in the same manner as pR1-3'R2. The first PCR product was generated using the forward oligonucleotide primer LCV-161-CM and reverse primer LCV-286-CM. The second PCR product was generated using forward primer LCV-287-CM and reverse primer LCV-164-CM. Overlapping extension PCR of 50ng of each of the gel-purified form of the above PCR products was performed using oligonucleotide primers LCV-161-CM and LCV-164-CM. The resulting PCR amplified products were gel-purified, adenylated, and cloned into pGEM-T Easy vector. Restriction digestion of the recombinant pGEMT-T Easy vector was made using AatII and ApaI, and and the released DNA fragments were subcloned into similarly digested pCM2

pDRNA2-6: a defective (D)RNA (DRNA2-6) derived from LCV RNA 2 and cloned into pGEM-T Easy vector (Chen and Ng unpublished data) was engineered by PCR (using oligonucleotide primers LCV-101-CM and LCV-172-AC) to introduce a bacteriophage T3 promoter immediately upstream of the first nt at the 5' end of the DRNA2-6 sequence, and an NgOMIV restriction site immediately downstream of last nt at the 3'-terminus of the DRNA2-6 sequence. The PCR product was gel-purified, adenylated, and cloned into pGEM-T Easy vector.

All standard molecular procedures were performed according to Sambrook and Russell (Sambrook and Russell, 2001b). All cDNA clones were transformed into *Escherichia coli DH5*α competent cells and grown in Luria-Bertani broth containing ampicillin (100 μg/ml; Sigma-Aldrich, St. Louis, MO). All cloned products derived from PCR-amplification were sequenced in both directions.

In vitro transcription, protoplast inoculation, total RNA extraction, and Northern blot analysis

Procedures associated with *in vitro* transcription of pCM1, pCM2, the pCM1- and pCM2-engineered mutants, as well as pDRNA2-6 followed those previously described (Mongkolsiriwattana et al., 2011), except that restriction digestion for the linearization of p3' Δ10, p3' Δ24, and p3' Δ48 was performed using AgeI.

2 μg each of the *in vitro* synthesized transcripts were inoculated to *N. tabacum* var. Xanthi protoplasts following the previously described procedure (Ng et al., 2004; Salem et al., 2009). For mutants p3'Δ4, p3'Δ10, p3'Δ24, pR1-3' R2, pR2-3' R1, and p3' SLR, an additional protoplast inoculation was performed following previous described with the following exception aimed at normalizing any differences arising from potential uneven handling and inoculation of protoplasts: triplicates of the each combination of *in vitro* transcripts were individually inoculated to 0.5 x 10^6 protoplasts, and the protoplasts from all three inoculations were combined into a 100x20 mm petri dish and incubated at 26^0C. Afterwards (16 hpi), the combined protoplasts were redistributed into three 60 x 20 mm petri dishes (10 ml per dish) and incubated at 26^0C until the first harvest at 24 hpi.

The inoculated protoplasts were also harvested at 48 and 96 hpi. Total RNA was extracted by the TRIzol® method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Approximately 2 μg of total RNA from each sample were analyzed by Northern Blot using DIG-labeled riboprobes II and VIII as previously described (Salem et al., 2009) (Fig. 2-1A). Signals of viral RNA accumulation on X-ray films were estimated by densitometry using the Scion Image software (Scion Corp) as previously described.

5'-RACE

To determine whether progenies of pCM1 and/or pCM2 mutants had retained their engineered mutations, 5'-Rapid Amplification of cDNA Ends (RACE) was used to analyze the 5' terminus of the minus-sense progenies. Approximately 5 μg of total RNA sample from protoplasts collected at 96 hpi were subjected to reverse transcription by SuperScript II® reverse transcriptase (Invitrogen) using gene-specific primers LCV-27 and LCV-161-CM (in Table 2-1) for cDNA synthesis specific to LCV RNAs 1 and 2, respectively. The first strand cDNAs were purified using QIAquick PCR purification kit. The resulting purified cDNAs were tailed using 1 μl (15U/μl) terminal deoxynucleotidyl transferase with either dCTP (for analyzing the progenies of p3' Δ4, p3' Δ10, and p3' Δ24), or dTTP (for analyzing the progenies of pCM1 from total RNA of protoplast inoculated with pCM1 and pCM2 transcripts [#5; Fig. 2-2A] , pCM2 from total RNA of protoplast inoculated with pCM1 and pCM2 [#5; Fig. 2-2A], pR1-3' R2 from total RNAs of protoplast inoculated with transcripts of R1-3'R2 and R2-3'R1 [#3; Fig. 2-2A], and

protoplast inoculated with transcripts of R1-3'R2 and R2 [#2; Fig. 2-2A], pR2-3' R1from total RNAs of protoplast inoculated with transcripts of R2-3'R1 and R1 [#1; Fig. 2-2A], and protoplast inoculated with transcripts of R2-3'R1 and R1-3'R2 [#3; Fig. 2-2A]), and p3' SLR). The tailed products were subjected to PCR using oligonucleotide primers AAP(T) and LCV-28 (for amplification of RNA 1-based sequences from pCM1 and mutant pR1-3'R2), AAP(T) and LCV-103 (for amplification of the RNA 2-based sequences from pCM2 and mutants pR2-3' R1,and p3' SLR), or AAP(C) and LCV-103 (for amplification of the RNA 2-based sequences from mutants p3' Δ4, p3' Δ10, and p3' Δ24). The amplified products were diluted (1:100 in 1 x TE [10mM Tris, 1mM EDTA, pH 8]) and used for another round of PCR using oligonucleotide primers AUAP (Invitrogen) and LCV-29, or AUAP and LCV-180 for the amplification of RNA 1- and RNA 2-based sequences, respectively. The cloning of the PCR products into the pGEM-T Easy vector and the subsequent sequencing steps were as described above.

SHAPE analysis

pS-3'R2 (Fig. 2-4), the structure cassette plasmid containing the cDNA sequence of the 98-nt of LCV RNA 2, was constructed using two independent PCRs. In the first reaction, oligonucleotide primers LCV-277-CM and LCV-278-CM (Table 2-1) were used to modify pCM2. The resulting product was gel purified and subsequently used for the second reaction in which further modifications were achieved using oligo-primers LCV-279-CM and LCV-280-CM (Table 2-1). The amplified product was gel-purified, adenylated, and cloned into the pGEM-T Easy vector to create pS-3'R2. 2.5 μg of pS-

3'R2 were linearized by restriction enzyme digestion using XbaI. The linearized DNA then served as a template for the *in vitro* synthesis of RNA transcript (over a 6 hr incubation/reaction duration) using the T3 mMessage mMACHINE kit (Life Technologies). The *in vitro* produced transcript was visualized by ethidium bromide staining following electrophoresis in a 15% polyacrylamide gel under denaturing (7M urea) conditions, and quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 12 µl of 0.5 x TE (5mM Tris, 0.5mM EDTA, pH 8) containing 2 pmol of the transcript was heated at 95^0C for 2 min, and immediately placed on ice for 2 min. 6 µl of 3x RNA folding mix (111 mM HEPES, pH 8.0, 6.67 mM $MgCl_2$, 111 mM NaCl in the final volume) was added to the transcript, and the mixture was divided into two 9 µl portions and incubated at 37^0C for 20 min to allow the RNA to renature (Wilkinson et al., 2006). The first portion of the renatured transcript was treated with 1 µl of 130 mM *N*-methylisatoic anhydride (NMIA) (Molecular Probes, Life Technologies) (13 mM final concentration) made freshly in DMSO, at 37^0C for 50 min. The second portion of the renatured transcript (the control treatment) was treated with 1 µl of DMSO without NMIA under the same conditions. RNA was recovered by ethanol precipitation and resuspended in 10 µl of 0.5x TE (5mM Tris, 0.5mM EDTA, pH 8) (Wilkinson et al., 2006). Primer extension reactions were according to the methods of (McGinnis et al., 2009). Briefly, NMIA- and DMSO (control)-treated RNA was subjected to primer extension (by SuperScript III® reverse transcriptase [Invitrogen], under three consecutive sets of temperature and time conditions: 37°C for 5 min, 52°C for 20 min, 60°C for 5 min), using 5' end-labeled-VIC and -NED 3'-linker primers [5'-

GAACCGGACCGAAGCCCGATTTGC-3', nucleotides complementary to the RT primer binding site of the structure cassette], respectively. Two separate sequencing reactions, each containing 2 pmol of *in vitro* produced transcripts of pS-3'R2, were performed under the same conditions as with the primer extension reaction, except that 1 µl of 5 mM ddTTP was also included in each reaction. In addition, one of the sequencing reactions contained the -VIC 3'-linker primer while the other contained the -NED 3'-linker primer. The Primer extension and sequencing reactions were quenched and combined in the following manner: the NMIA-treated sample with the sequencing sample containing the -NED 3'-linker primer, and the DMSO (control)-treated sample with the sequencing sample containing the -VIC 3'-linker primer. Following ethanol precipitation, cDNAs were resuspended in 10 µl of deionized formamide and then resolved by capillary electrophoresis. The electropherogram data of each reaction was processed and analyzed by the Qushape software (Karabiber et al., 2013). The reactivity of nts to NMIA modification was classified as: unreactive (0-0.4), moderately reactive (0.4-0.85), or highly reactive (>0.85) (Karabiber et al., 2013).

The construction of pSDR2-6, the structure cassette plasmid containing the cDNA of full-length LCV DRNA 2-6 was essentially similar to that of pS-3'R2 except that oligonucleotide primers LCV-283-CM and LCV-284-CM, as well as LCV-283-CM and LCV-285-CM were used for making the necessary modifications to facilitate construction of the clone. In addition, *in vitro* produced transcript of the pSDR2-6 was treated with 1, 7 or 13 mM (final concentration) of NMIA (Wilkinson et al., 2006).

The processed SHAPE reactivities was incorporate as pseudo free energy constraints in *RNAstructure* (Mathews, 2004; Mathews et al., 2004) with slope and intercept value of 2.6 and -0.8, respectively, according to (Low and Weeks, 2010). In addition, base pairing between nucleotides greater than 600 positions was not allowed (Low and Weeks, 2010).

sqPCR

To determine the accumulation levels of miuns-strand progenies of mutant p3'Δ38, p3'ΔSL, p3'SLD1, and p3'SLD2, semi-quantitative PCR was performed. Approximately 5 μg of total RNA sample from protoplasts (inoculated with respective mutants) collected at 96 hpi were subjected to reverse transcription by SuperScript II® reverse transcriptase (Invitrogen) using gene-specific primers LCV-70-PW according to manufacturer's instruction. 1 μl of the amplified cDNAs was then used for PCR with 2.5 μl of 10X Taq buffer, 2.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 0.5 μl of Taq DNA polymerase, and 10 μM of primers LCV-70-PW and LCV-99-AC. The following PCR condition was used: 94°C for 2 min, followed by a different number of cycles (10, 15, 20, 25, and 30 cycles) of 94°C for 45 sec, 54.6°C for 45 sec, and 72°C for 1 min, with a final extension at 72°C for 7 min. For internal control, the same procedure was used as describe above except oligonucleotide primer NtUbiR was used for reverse transcription, and primers NtUbiF and NtUbiR were used for PCR amplification.

Results

Exchanging the 98-nt of LCV RNAs 1 and 2

Non-conserved nts within the 3' NCR of the gRNAs have been found to affect the (-)-RNA synthesis of BMV, a (+)-ssRNA virus with a tripartite genome (Duggal et al., 1992). The 3'-terminal 200 nts of RNAs 1 and 3, and RNAs 2 and 3 differ by 11 nts and 1 nt, respectively. By exchanging these 200 nts among the three gRNAs of BMV, Duggal et al (1992) showed that the strength of their promoter activity for (-)-RNA synthesis differed, with promoter activity of the 3' NCR of RNA 3 being the strongest relative to those of RNAs 1 (weaker) and 2 (weakest) (Duggal et al., 1992). In the case of LCV, 21 nts in the 98-nt of LCV RNAs 1 and 2 are non-conserved. To determine whether or not these non-conserved nts exert any influence on (-)-RNA synthesis, we swapped the cDNAs corresponding to the 98-nt of pCM1 (cDNA clone of RNA 1) and pCM2 (cDNA clone of RNA 2), resulting in the mutants R1-3'R2 and R2-3'R1 (Fig. 2-2A). *In vitro* transcripts of both mutants, WT pCM1 (R1), and pCM2 (R2) were synthesized and inoculated to tobacco protoplasts in various combinations [#1-#5] (Fig. 2-2A). Total RNA was extracted from the inoculated protoplasts 24, 48, and 96 hours post-inoculation (hpi), and subjected to Northern blot analyses using DIG-labeled riboprobes corresponding to the ORFs encoding P23 (LCV RNA 1) or P27 (LCV RNA 2) (Fig. 2-1A). Plus- (not shown) and minus-strand accumulation of R1-3'R2 (whether inoculation was performed using transcripts of R1-3'R2 alone [#4], transcripts of R1-3'R2 and R2-3'R1 [#3], or transcripts of R1-3'R2 and R2 [#2]) were comparable to those of the wild type (R1 and R2) (Figs. 2-2B and C; compare #2, #3, and #4 with #5). Accumulation

levels of plus- (not shown) and minus-strand R2-3'R1 (whether inoculation was performed using transcripts of R2-3'R1 and R1 [#1], or transcripts of R2-3'R1 and R1-3'R2 [#3]) were also comparable to those of wild type (Fig. 2-2D, compare #1 and #3 with #5). In order to ensure that all engineered mutations were retained in the minus-sense progenies of the LCV RNAs 1 and 2 mutants, total RNA of protoplasts harvested at 96 hpi following inoculation with mutant or WT transcripts were subjected to 5'-Rapid Amplification of cDNA Ends (RACE) PCR. The resulting PCR amplified products were cloned and sequenced. Sequence analysis of five randomly selected recombinant plasmids obtained for each queried inoculation showed that in all cases, the original (mutant or WT) nts were retained in the minus-sense progenies. However, there was discrepancy in the identity of the first nucleotide "C" of the minus strand (complementary to last nucleotide "G" of plus strand LCV RNA 2). Majority of the cloned PCR products lacked the 5' -terminal nucleotide "C" of the minus strand (5 clones out of 5 for R1 from total RNA of protoplast inoculated with pCM1 and pCM2 [#5; Fig. 2-2A]; 4 clones out of 5 for R2 from total RNA of protoplast inoculated with pCM1 and pCM2 [#5; Fig. 2-2A]; 5 clones out of 6 for R1-3' R2 from total RNA of protoplast inoculated with transcripts of R1-3'R2 and R2 [#2; Fig. 2-2A]; 5 clones out of 5 for R1-3' R2 from total RNA of protoplast inoculated with transcripts of R1-3'R2 and R2-3'R1 [#3; Fig. 2-2A]; 4 clones out of 6 for R2-3' R1from total RNA of protoplast inoculated with transcripts of R2-3'R1 and R1 [#1; Fig. 2-2A]; 4 clones out of 5 for R2-3' R1from total RNA of protoplast inoculated with transcripts of R2-3'R1 and R1-3'R2 [#3; Fig. 2-2A]). Minority of the cloned PCR products either had "G" as 5'-terminal nucleotide of the minus strand (1

clone out of 6 for R2-3' R1from total RNA of protoplast inoculated with transcripts of R2-3'R1 and R1 [#1; Fig. 2-2A]), “T” as 5’-terminal nucleotide of the minus strand (1 clone out of 6 for R1-3' R2 from total RNA of protoplast inoculated with transcripts of R1-3'R2 and R2-3'R1 [#3; Fig. 2-2A]; 1 clone out of 6 for R2-3' R1from total RNA of protoplast inoculated with transcripts of R2-3'R1 and R1 [#1; Fig. 2-2A]; 1 clone out of 5 for R2-3' R1from total RNA of protoplast inoculated with transcripts of R2-3'R1 and R1-3'R2 [#3; Fig. 2-2A]), or lack the last two 5'-terminal nucleotide “CG” (1 out of 5 R2 from total RNA of protoplast inoculated with pCM1 and pCM2 [#5; Fig. 2-2A]). The variability of the 5' terminal of minus-strand RNA progenies appears to be a natural occurrence associated with ssRNA production in the infected host, and has been reported previously for both LCV and other viruses in the family *Closteroviridae* (Chen et al., 2012; Karasev et al., 1996; Lopez et al., 1998).

These results demonstrated that the exchange between the conserved region (98-nt) of the 3' NCR of LCV RNAs 1 and 2 did not abrogate or have any observable negative effects on minus-strand accumulation for both RNAs, suggesting that the non-conserved nts likely play no significant role in (-)-RNA synthesis. These results also provided the first evidence that the 98nt of both gRNAs likely contain similar sequence or structural elements involved in (-)-RNA synthesis.

Nucleotide determinants in the 98-nt required for minus strand synthesis of LCV RNA 2

We performed deletion mutations of the 98-nt of LCV RNA 2 to identify potential *cis*-acting elements capable of mediating (-)-RNA synthesis. LCV RNA 1 would not be

an ideal reporter template for these mutational analyses since RNA replication would most likely be coupled to translation-related functions needed for the production of the ORFs 1a- and 1b-encoded replication associated proteins (Salem et al., 2009). Thus, introducing mutations to the 98-nt of LCV RNA 1 risked impairing the translation of these proteins, thereby making the interpretation of negative results equivocal. However, given the similarity and interchangeability of the 98-nt of LCV RNAs 1 and 2 (demonstrated above), it seemed likely that structurally and functionally similar *cis*-acting elements are present in the 98-nt of both RNA components; results obtained from the analysis of the 98-nt of RNA 2 would have parallel implications for RNA 1.

We constructed a series of deletion mutants, including those in which nts were progressively removed from the 3' end of the 98-nt in LCV RNA2, made *in vitro* transcripts of each mutant, and inoculated them along with that of WT LCV RNA 1 to tobacco protoplasts. Mutant 5'Δ50 was engineered with a 50-nt deletion from the proximal 5' end of the 98-nt, while mutant 3'Δ48 was designed with 48 nts deleted from its proximal 3' end (Fig. 2-3A). Northern analysis showed that both 5'Δ50 and 3'Δ48 failed to accumulate (-)-RNA (Fig. 2-3B). Previous studies of BMV, *Tobacco mosaic virus* (TMV),and *Tobacco yellow mosaic virus* (TYMV), and *Citrus tristeza virus* (CTV) demonstrated that the 3'-terminal ribonucleotides CCA were critical for the initiation of (-)-RNA synthesis (Dreher, 1999; Dreher and Hall, 1988a; Miller et al., 1986; Osman et al., 2000; Satyanarayana et al., 2002). To test whether or not the lack of (-)-RNA accumulation of 3'Δ48 was due to the absence of an initiation site at the 3' terminus, we constructed and analyzed mutant 3'Δ48(4) (essentially 3'Δ48 except for the presence of

the terminal 4 nts [GCCG]; these 4 nts are found on both LCV RNAs 1 and 2). This mutant also failed to accumulate (-)-RNA (Fig. 2-3B). We also constructed 3'Δ4, a mutant with only the terminal 4 nts deleted, and found that it supported (-)-RNA synthesis at a level comparable to that of the WT (Fig. 2-3C). These results suggest that LCV most likely does not require the terminal 4 nts for (-)-RNA synthesis. To further delineate the minimal nt sequences necessary for (-)-RNA synthesis, mutants with progressive deletions in the 3' terminus of LCV RNA 2: 3'Δ10 and 3'Δ24, and 3'Δ38 (with deletions of 10, 24, and 38 nts from 3' terminus, respectively) were constructed and tested (Fig. 2-3A). (-)-RNA accumulation of 3'Δ10 and 3'Δ24 were detected at lower levels, approx. 71% and 36%, respectively, compared to that of the WT (Figs. 2-3D-E); whereas 3'Δ38 completely failed to accumulate (-)-RNA (Fig. 2-3F). For all of the inoculations involving non-viable mutants, 5'Δ50, 3'Δ48, and 3'Δ38, accumulation of (+)- and (-)-RNA was observed for WT LCV RNA 1 (data not shown).

The 5' -terminal sequences in the minus-sense progenies of the viable mutants, 3'Δ4, 3'Δ10 and 3'Δ24, were determined by sequencing the 5'-RACE PCR products generated using the total RNA extracted from the inoculated protoplasts, and all three were found to contain the engineered nucleotide deletions. However, additional 4 nucleotides were found immediately upstream of the the 5' -terminal nucleotide "T" of the minus strand of 3'Δ10 and 3'Δ24 in all cases (15 out of 15). These additional nucleotide sequences corresponded to the digested AgeI restriction site used for linearlization of p3'Δ10 and p3'Δ24 prior to *in vitro* transcription. So presumably, these additional sequences of AgeI restriction site were transcribed as part of the template of

p3’Δ10 and p3'Δ24. In addition to determining the 5' -terminal sequence in the minus-sense progenies of the viable mutant, we also tried to determine the 5'- terminal sequence of the lethal mutant 3'Δ38. Interestingly, 5'-RACE PCR products were detected and were generated from the protoplast inoculated with *in vitro* transcripts of p3'Δ38 and pCM1. The result showed that the engineered mutation was retained in the minus-strand RNA progenies, but there was still discrepancy in the identity of the first nucleotide “C” of the minus strand progenies. Majority of the cloned PCR products contained an extra nucleotide “G” immediately upstream of the 5' -terminal nucleotide “C” of the minus strand (6 clones out of 8), while minority of the cloned PCR products contained two extra nucleotide “GG” immediately upstream of the 5' -terminal nucleotide “C” of the minus strand (1 clones out of 8) or lacked the 5' -terminal nucleotide “C” of the minus strand (1 clones out of 8).

These data suggested that efficiency of (-)-RNA synthesis of LCV RNA 2 was reduced but not abolished by deletions of up to 24 nts (nt position 8533 to 8556) from the 3' terminus, whereas the removal of 38 (nt position 8519 to 8556) or more (nt position 8507 to 8556) nts from the 3' terminus was detrimental to the virus.

Structural prediction and chemical probing (SHAPE) analysis of the 3' NCR (98-nt) of LCV RNA 2

Substantial studies have demonstrated that secondary structures of (+)-ssRNA viruses participate in various regulatory functions, including (-)-RNA synthesis (An et al., 2010; Buck, 1996; Fabian et al., 2003; Friebe et al., 2005; Koev et al., 2002; Liu et al.,

2009; Lodeiro et al., 2009; McCormack et al., 2008; Tanaka et al., 1996). Here, we first used *Mfold* (version 3.4) to predict the secondary structure in the 98-nt of LCV RNAs 1 and 2 based on folding algorithms that use sequence properties and thermodynamic stability calculations of the RNA (Zuker, 2003). The predicted models generated using the 98-nt as well as the full-length sequences of LCV RNAs 1 and 2 revealed the presence of a prominent and thermodynamically stable stem-loop (SL) structure in as many as 50 conformations examined (data not shown). Further analysis was made to confirm this predicted structure by using the program *RNAstructure* (version 5.4) (Mathews, 2004; Mathews et al., 2004), an RNA structure prediction web server application based on a revised folding algorithm that incorporates constraint from chemical modification. Analysis of the 98-nt and the 2500 3'-terminal nts of LCV RNAs 1 and 2 both reaffirmed the presence of the SL structure predicted above. This predicted SL, from here on referred to as SL1, spans nt position 8495 to 8533 (-23 to -61 nts upstream of the terminal nt at the proximal 3' end of the RNA), and is consists of 9 base pairs (bp) in the lower stem, 5 nts in the internal loop, 5 bp in the upper stem and 4 nts in the loop (Fig. 2-4A).

In order to ascertain the authenticity of SL1, we used Selective 2'-Hydroxyl Acylation Analyzed by Primer Extension (SHAPE) to determine the secondary structure of the 98-nt of LCV RNA 2. We first constructed pS-3'R2, a structure cassette plasmid that contains the cDNA sequence of the 98-nt to facilitate its synthesis by *in vitro* transcription (Fig. 2-4A). The structure cassette contains two linker regions that fold into stable SL structures at the 5' and 3' termini of the transcript upon synthesis (Figs. 2-4A

and C). The 3' linker is designed for the binding of oligonucleotide primer and reverse transcriptase, while the 5' linker is designed to prevent full-length cDNA products (resulting from primer extension) from obscuring the reactivity information at the 5' end of the RNA (Karabiber et al., 2013; Vachon and Conn, 2012). *In vitro* synthesized transcripts were treated with either NMIA (NMIA +) or DMSO (NMIA -), and were subjected to primer extension. The electropherogram of each reaction (red for NMIA +, blue for NMIA -, green and pink for sequencing reactions) was processed and analyzed by the Qushape software (Fig. 2-4B) (Karabiber et al., 2013). The normalized SHAPE reactivity data was then superimposed on the *mfold* predicted secondary structures of the 98-nt of LCV RNA 2 (Fig. 2-4B). The data provided strong evidence in support of the presence of the predicted SL1 structure located at position #37-75 of the 98-nt (position 8495 to 8533 on RNA 2). All but 2 nts in the upper stem, and 1 of the 4 nts in the loop, were unreactive (Karabiber et al., 2013) to NMIA modification (Fig. 2-4C), suggesting that most (31 of 38 or 82%) of these unreactive nts, including the 5 (positions #46-47 and #64-66) located in the internal loop, were constrained (Fig. 2-4C).

SHAPE Analysis of a biologically active LCV RNA 2-derived defective RNA

While SHAPE analysis of the 98-nt of LCV RNA 2 indicating the lack of reactivity to NMIA modification for most of the nts that make up SL1 were consistent with its predicted structure, most of the nts outside SL1 showed moderate (orange) to high (red) NMIA reactivity (Fig. 2-4C) and exhibited weak matches to *Mfold* predicted secondary structures of the 98-nt. The discrepancy was evidently due to the length of the

RNA analyzed (i.e. 98 nts) being insufficient to support the secondary and tertiary interactions needed to generate the types of conformations that are found in the full-length RNA. Therefore, it was of interest to examine the structure of the 98-nt within the context of the full-length sequence of a biologically active RNA (Watts et al., 2009). The task was simplified substantially by using DRNA2-6, a 3 kb defective (D)RNA of LCV RNA 2 that we isolated previously (Ng, unpublished data). DRNA2-6 contains 5' and 3' terminal nts corresponding to positions 1 to 978 and 7664 to 8556, respectively, of LCV RNA 2. The proximal 3'-end of DRNA2-6 has a 98-nt similar to that of LCV RNA 2, except for a missing uridine (U) 10 nt positions upstream of the terminal guanine (G) residue (compare Figs. 2-4C with 2-5D). The intervening region between both termini of DRNA2-6 contains a non-contiguous stretch of nts and nts of the entire CP ORF of LCV (Fig. 2-5A). When *in vitro* produced transcripts of the T3 construct of DRNA2-6 (Fig. 2-5B) were co-inoculated with that of pCM1 (WT LCV RNA 1) to tobacco protoplasts, DRNA2-6 accumulation in both polarities (Fig. 2-5C; not shown) was observed (24-96 hpi) in a Northern blot using riboprobe VIII (Fig 2-1A), indicating that it was replication competent in the presence of LCV RNA1.

We constructed pSDR2-6, the structure cassette plasmid containing the full-length cDNA sequence of DRNA2-6, and used the *in vitro* produced transcripts for SHAPE analysis in the same manner as described for pS-3'R2. NMIA reactivity and sequencing information were obtained for 120 nts located in the proximal 3'-end of DRNA2-6, and they facilitated the identification of several *Mfold*-predicted secondary structures, all with slight variations depending on the concentration of NMIA usedIn addition, the data was

also used as pseudo free-energy constraints in *RNAstructure* to predict secondary structure (Mathews, 2004; Mathews et al., 2004). The best representation among these structures is shown in Figure 2-5D. In all cases, the results were consistent with data obtained from the SHAPE analysis of the 98-nt in regard to the presence and overall structure of SL1 (Fig. 2-5D). In addition, because a much longer (3kb) RNA was analyzed, additional and more precise structural information were revealed. First, all 4 nts in the loop of SL1 as well as 3 of the 5 nts in its internal loop were moderately-highly reactive to NMIA modification. It is interesting to note that in the context of the 98-nt, all 5 nts in the internal loop were unreactive to NMIA modification (Fig. 2-4C). Second, the data revealed that SL1 is part of a larger secondary structure – a Y-shaped configuration spanning nt position 2940 to 3031 (8450 to 8541 on LCV RNA 2) and consisting of two SLs, SL1 (which we have characterized) and SL2. Both SLs are separated by three nts (Fig. 2-5D), and are united at the base of the Y-configuration by the base-paring of nts at positions 2937 and 3006 (8457 and 8534 on LCV RNA 2). The union at the base continues on through 7 base-pairs. SL2 spans nt position 2950 to 2981 (8460 to 8491 of RNA 2) and is consists of 9 bp in the lower stem, 4 nts in the internal loop, 3 bp in the upper stem and 4 nts in the loop (Fig. 2-5D). Nucleotides in the loop and part of the internal loop of SL2 were moderately-highly reactive to NMIA modification, suggesting that these nts are not constrained by interactions with other parts of the RNA.

Targeting SL1 of LCV RNA 2 for mutational analysis

SHAPE data supporting the predicted structure of SL1 in the 98-nt of LCV RNA 2 was revealing since its location was in the path of the nt deletions engineered in mutants 3'Δ48, 3'Δ48(4), 3'Δ38, and 3'Δ24; and (-)-RNA synthesis of LCV RNA 2 was not observed in the first three of these mutants, and was drastically reduced in 3'Δ24 compared to the WT. These observations led us to hypothesize about the importance of SL1 in (-)-RNA synthesis. To test our hypothesis, we subjected SL1 of LCV RNA 2 to a series of engineered mutations, and inoculated the *in vitro* produced transcripts of each mutant, along with those of pCM1 (WT LCV RNA 1), to tobacco protoplasts. Northern blot analyses of total RNA extracted from protoplasts inoculated with mutant 3'ΔSL, engineered with deletion of SL1 (Figs. 2-6A), showed the this mutant failed to accumulate (-)-RNA (Fig. 2-6C) and (+)-RNA (data not shown) at 24 and 96 hpi. To further investigate the basis for the failure to accumulate (-)-RNA when SL1 was deleted, we constructed and tested mutants 3'SLD1, 3'SLD2, and 3'SLR. In 3'SLD1, 6 nts (5'-AACGAC-3') located on the right arm of the lower stem of SL1 were substituted with the complementary sense nts (i.e. 5'-UUGCUG -3') (Figs. 2-6A and B). This mutation disrupted the formation of SL1 as none of the *Mfold*-predicted models of LCV RNA 2 contained this secondary structure in the 98-nt. Northern blot analyses of total RNA extracted from protoplasts inoculated with mutant 3'SLD1 revealed that it failed to accumulate (-)-RNA (Fig. 5D) and (+)-RNA (data not shown) at 24 and 96 hpi. Mutant 3'SLD2 was engineered to substitute the 6 nts (5'-UUGCUG -3') located on the left arm of the lower stem of SL1 with the complementary sense nts (i.e. 5'- AACGAC-3'). As

with 3'SLD1, this mutation disrupted the formation of SL1 as none of the *mfold*-predicted models of LCV RNA 2 contained this secondary structure in the 98-nt. Accordingly, 3'SLD2 showed no evidence of (-)-RNA and (+)-RNA (not shown) accumulation in protoplasts 24-96 hpi. Finally, a compensatory mutation was made to restore SL1 by engineering a complementary 6-nt substitution (5'- AACGAC-3') on the left arm of the lower stem of SL1 in 3'SLD1 (Figs. 2-6A and B). When the resulting mutant, 3'SLR, was inoculated to tobacco protoplasts, it accumulated (-)-RNA (Fig. 2-6E) and (+)-RNA (not shown) to levels comparable to that of the WT (Fig. 2-6E) from 24-96 hpi.

The 5' -terminal sequences in the minus-sense progenies of viable mutant, 3'SLR, were determined by sequencing the 5'-RACE PCR products generated using the total RNA extracted from the inoculated protoplasts and all cloned PCR products were found to contain the engineered mutations. In addition to determining the 5' -terminal sequence in the minus-sense progenies of the viable mutant, we also tried to determine the 3' terminal sequence of the lethal mutants: 3'ΔSL, 3'SLD1, and 3'SLD2. Interestingly, 5'-RACE PCR products were detected and were generated from the inoculated protoplasts. For 3'ΔSL, the engineered mutation was retained except discrepancy in the 5'-terminal sequence of the minus-strand progenies. Majority of the cloned PCR products from 5'-RACE of 3'ΔSL was lacking the first 5'-terminal nucleotide (10 out of 12), while 2 out of 12 cloned PCR products from 5'-RACE of 3'ΔSL had “G” instead of “C” as the first 5'-terminal nucleotide of minus-strand progenies. For 3'SLD1, many of cloned PCR products contained the engineered mutation with heterogeneity of the first 5'-terimal nucleotides of the minus-strand progenies: 2 out of 15 lacked the first two 5'-terminal

nucleotides, 4 out of 15 had an extra “G” upstream of 5’-terminal nucleotide, 2 out of 15 had extra “GG” upstream of 5'-terminal nucleotide, and 1 out of 15 had extra “GGG” upstream of 5'-terminal nucleotide. However, some of cloned PCR products had a significant change in nucleotide sequences in the SL1, which result in rescuing the overall structure of SL1. The result of the change can be categorized into two types: the nucleotide sequences of SL1 is identical to LCV RNA 2 wild type (2 out of 15), and the nucleotide sequences of SL1 is identical to 3'SLR (4 out of 15). For 3'SLD2, the engineered mutation was retained with some discrepancy in the 5'-terminal nucleotide: 5 out of 10 had the 5'-terminal nucleotide, 3 out of 10 lacked the 5'-terminal nucleotide, 1 out of 10 had “T” instead of “C” as 5'-terminal nucleotide, and 1 out of 10 had a digested NgOMIV restriction site upstream of 5'-terminal nucleotide.

Thus, mutations that altered SL1 of LCV RNA 2, including its deletion and the disruption of its structure through nt substitutions in either of both arms of the lower stem, abolished (-)-RNA synthesis of LCV RNA 2; while a compensatory mutation in the opposite arm of the lower stem that restored the SL structure rescued (-)-RNA (LCV RNA 2) synthesis. These results strongly suggest that as a whole, SL1 is dedicated to playing a role in supporting (-)-RNA synthesis of LCV RNA 2.

Targeting SL2 of LCV RNA 2 for mutational analysis

SHAPE data from DRNA2-6 also revealed a larger Y-shaped structure with two stem loop as an arm. SL1 has been shown to be involved in minus-strand RNA synthesis so we hypothesize that SL2 is also important in minus-strand RNA synthesis as a part of

larger Y-shaped structure. To test our hypothesis, we constructed and tested mutant 3'ΔSL, engineered with deletion of SL2, by inoculating the *in vitro* produced transcripts of mutant with those of pCM1 to tobacco protoplasts. Northern blot analyses of total RNA extracted from protoplasts inoculated with mutant 3'ΔSL2 showed that the mutant failed to accumulate (-)-RNA (Fig. 2-7) and (+)-RNA (data not shown). The result suggests that SL2 is involved in minus-strand RNA synthesis.

Semi-quantiative (sq)PCR of LCV RNA 2 lethal mutants

Even though all LCV RNA 2 mutants that alter the overall structure of SL1 (3'Δ38, 3'ΔSL, 3'SLD1, and 3'SLD2) were not detected when total RNAs from inoculated protoplasts were subjected to Northern blot analysis, we were able to detect PCR amplified products when total RNAs from inoculated protoplasts were subjected to 5' RACE. It is possible that minus-strand RNA synthesis of these mutants were not abolished, but greatly reduced to undetectable level in Northern blot analysis. To determine the accumulation levels of miuns-strand progenies of mutants 3'Δ38, 3'ΔSL, 3'SLD1, and 3'SLD2, total RNAs from inoculated protoplasts were subjected to semi-quantitative (sq)PCR. First, the first-strand cDNAs were synthesized from total RNAs of inoculated protoplasts using LCV RNA 2 specific forward primer LCV-70. The synthesized cDNAs were then subjected to PCR amplification using LCV RNA 2 specific primers, LCV-70 and LCV-99, with different number of amplification cycles (10, 15, 20, 25, and 30 cycles). For internal control to ensure that equal amount of total RNAs were used, additional sqPCRs were performed in the same manner as mentioned above except

reverse primer NtUbiR for first strand cDNA synthesis and primers NtUbiF and NtUbiR for PCR amplification.

Amplified products from total RNAs of protoplasts inoculated with mutants 3'Δ38, 3'ΔSL, 3'SLD1, and 3'SLD2 (24 and 96hpi) were detectable after 25x cycles of amplification and rapidly increased after 30x cycles of amplification except those of total RNA of 3'ΔSL-inoculated protoplast harvested at 24hpi, which were detectable after 20x cycles of amplification and rapidly increased with 25x and 30x cycles of amplification (Fig. 2-8). Amplified products from total RNAs of pCM1- and pCM2-inoculated protoplasts were detectable much early comparing those of mutants as they were detectable after 15x and 20x cycles of amplification for protoplast harvested at 96hpi and 24hpi, respectively, and rapidly increased with each increasing cycles of amplification (Fig. 2-8). The result showed that the mutants 3'Δ38, 3'ΔSL, 3'SLD1, and 3'SLD2 were able to synthesize minus-strand RNA progenies, but were synthesized very inefficiently, suggesting that the mutations that alter the overall structure of SL1 significantly reduced minus-strand RNA synthesis.

Discussion

In our previous study, we constructed the cloned infectious cDNAs of LCV genomic RNAs 1 (pCM1) and 2 (pCM2), and demonstrated the replication competence of RNA 1 (R1), consistent with the notion that it alone encodes the necessary proteins required for virus replication (Mongkolsiriwattana et al., 2011). In that regard, this feature is similar between LCV and LIYV (Yeh et al., 2000), and, by inference, common among criniviruses (cloned infectious cDNAs are not yet available for the other criniviruses). In this study, we focused on another feature of LCV that is representative of most (12 of 13) criniviruses (Aguilar et al., 2003; Hartono et al., 2003; Klaassen et al., 1995; Kreuze et al., 2002; Livieratos et al., 2004; Martin et al., 2008; Okuda et al., 2010; Salem et al., 2009; Tzanetakis et al., 2005; Tzanetakis et al., 2006; Tzanetakis et al., 2011; Wintermantel et al., 2009; Wintermantel et al., 2005) – the highly conserved nts in the 3' NCR of its gRNAs. First, we demonstrated that unlike BMV (Duggal et al., 1992), the small number of non-conserved nts within the 3' NCR of LCV RNAs 1 and 2 did not appear to have an influence on the efficiency of (-)-RNA synthesis as exchanging the 98-nt between the two RNAs did not result in any discernible changes in the synthesis and accumulation of the chimeric RNAs relative to those of WT RNAs. These results were analogous to those observed in a previous study of *Alfalfa mosaic virus* (AMV), in which the 3' NCR of all three genomic RNAs were found to be functionally equivalent; upon exchange, they did not have any effect on the replication of the AMV RNAs as long as a secondary structure, hairpin E, present within the exchanged region was not disrupted (van Rossum et al., 1997). The notion that the presence of similar structural element(s)

within the 3' NCR of LCV RNAs 1 and 2 might have enabled the exchangeability of the two without having any ill-effects on (-)-RNA synthesis was also supported by our results. For example, chimeric LCV RNA 1, R1-3'R2, supported its own replication to levels comparable to that of R1 (WT LCV RNA 1) (Fig. 2-2C). These results provided evidence that the 98-nt of LCV RNA 2 is functionally identical to that of LCV RNA 1 with respect to (-)-RNA synthesis.

From the deletion mutation analyses of the 98-nt, it is clear that minus-strand synthesis of LCV RNA 2 does not involve the 3' terminal 4 nts, GCCG (Fig. 2-3C). This is, by contrast, different from BMV, TMV and a number of other positive-sense RNA viruses (Dreher, 1999; Dreher and Hall, 1988a; Satyanarayana et al., 2002; Singh and Dreher, 1997), where the CC(A/G) motif present in the 3' terminus of the gRNA(s) is absolutely required for (-)-RNA synthesis. Furthermore, LCV RNA 2 can tolerate more than 4 nt deletions from the 3' terminus as (-)-RNA accumulation of mutants 3'Δ10 and 3'Δ24, engineered with deletion of the last 10 and 24 nts, respectively, was observed, albeit at reduced efficiencies relative to the wild type (Figs. 2-3D-E). However, LCV RNA 2 cannot tolerate a deletion of 38 nts from the 3' terminus (Fig. 2-3F). These results suggest that the minimum sequence needed for (-)-RNA 2 synthesis lies between 24 to 38 nts from the 3’ terminus, and the terminal 24 nts are not essential, but have important functions for optimal (-)-RNA synthesis.

Our study emphasized the importance of using a combination of computer modeling (*Mfold* and *RNAstructure*), chemical modification/probing analysis (SHAPE) and biological assay of engineered mutants to gain insights on potential *cis*-acting

elements and their roles in (-)-RNA synthesis. SHAPE analysis had been used previously to identify RNA structures in Human immunodeficiency virus (HIV), *Barley yellow dwarf virus*, and satellite tobacco mosaic virus (Archer et al., 2013; Wang et al., 2010; Watts et al., 2009; Wilkinson et al., 2008). Here, it was used for the first time to analyze a crinivirus (LCV); specifically, the 3' NCR of LCV RNA 2 and an LCV RNA 2-derived DRNA (DRNA2-6). Using the above analyses, our experiments clearly identified a single stem loop structure (SL1) within the 3' NCR of LCV RNA 2. This result supported the mutational analyses data indicating that disruption of the overall structure of SL1 by deletion or substitution of the nts in the lower stems completely abolished (-)-RNA accumulation, whereas revival of the SL by a compensatory mutation restored (-)-RNA accumulation to WT levels (Fig. 2-6). Evidence gathered from all of the above analyses of SL1, as well as those from 5' RACE analyses of the viable deletion mutants (3'Δ4, 3'Δ10 and 3'Δ24) all suggest that SL1 is probably an important component in a higher order structure within the 98-nt, and contains all the features of a *cis*-acting element involved in (-)-RNA synthesis as it could not be complemented *in trans* by RNA 1.

The use of a full-length RNA species (DRNA2-6) in SHAPE analysis resulted in additional information that improved the accuracy of the data and prediction of the secondary structures in the 98-nt. Remarkably, the processed data from SHAPE analysis of DRNA2-6 identified SL1 as part of a larger Y-shaped configuration. RNAs bearing the general Y-shaped structure have been identified in riboswitches and ribozymes (de la Pena et al., 2009), as well as in viruses such as the SLA and the 3'-cap-independent translational enhancers of Dengue virus and tombusviruses, respectively (Lodeiro et al.,

2009; Nicholson et al., 2013). The Y-shaped configuration of LCV RNA 2 most closely resembles that seen in RNA 2 of *Red clover necrotic mosaic virus* (RCNMV) in terms of the overall shape and, possibly, role in (-)-RNA synthesis (An et al., 2010). This Y-shaped structure (YRE) of RCNMV RNA 2 is located within the 3' NCR of the RNA and is conserved among other members of the genus *Dianthovirus* (An et al, 2010). Interestingly, a comparison of *mfold*-predicted models of the full-length genomic RNA sequences of criniviruses revealed that the Y-shaped configuration identified in LCV RNA 2 is also conserved among all criniviruses with the exception of LIYV RNA 2. A collection of the Y-shaped configurations predicted for a selected number of criniviruses is shown in Fig. 2-9.

YRE had been suggested to be involved in minus-strand RNA synthesis by acting as recruiter element for replicase binding and membrane localization as the YRE has been found to interact with both RCNMV p27 and 480-kDa RCNMV replicase complex and interaction between p27 and YRE has shown to be involved in recruiting RCNMV RNA 2 to the membrane fraction (Iwakawa et al., 2011). Mutational analysis of YRE revealed that overall structure of both SL7 and SL8, and especially loop portion of SL8 are critical for minus-strand RNA synthesis and interaction with p27 (An et al., 2010; Iwakawa et al., 2011). So the Y-shaped structure LCV RNA 2 may possibly have similar function to that of RCNMV RNA2 YRE since LCV RNA 2 SL-1 and SL-2 is possibly an equivalent of RCNMV RNA 2 SL7 and SL8, respectively. The result from this study showed that only overall stem structure of SL-1 is required for minus-strand RNA accumulation similar to RCNMV RNA 2 SL7, where loop portion can be changed

without effect on accumulation. The result also suggested the function of SL-1 is possibly to provide a structure for overall Y-shaped structure and allow SL-2 to interact with viral or host proteins. In addition, SHAPE analysis of DRNA2 #6 showed that loop portion of SL-2 does not form tertiary interaction with other part of RNA, suggesting possibility of SL-2 loop interact with viral or host proteins in similar manner as RCNMV RNA 2 SL8.

The structurally and functionally similar *cis*-acting elements (SLs 1 and 2) identified in this study are likely the contributing factors for the synchronous accumulation kinetics of LCV RNAs 1 and 2 (Mongkolsiriwattana et al., 2011; Salem et al., 2009). This is possible due to the presence of conserved nts in the 3' NCR of both RNAs (a feature that is common in most of the other criniviruses). By contrast, the 3' NCR of LIYV RNAs 1 and 2 share a low (41%) sequence identity. This is consistent with the *mfold* data showing that the predicted Y-shaped configuration is seen only in LIYV RNA 1, but not in LIYV RNA 2. Thus, the structural differences in the 3' NCR of both RNAs are the likely basis underlying the asynchronous RNA replication-accumulation kinetics of LIYV, where the production and buildup of LIYV RNA 2 is delayed by 24 hrs relative to that of LIYV RNA 1 during the initial stages of infection (Yeh et al., 2000).

Further studies are needed to delineate the regions/nts of the SLs in LCV RNA 2 involved in (-)-RNA synthesis. For example, protoplast inoculation experiments are required to evaluate the viability of mutants engineered with site-specific mutations in the the NIMA reactive loops, and additional SHAPE analyses are needed to assess the potential models of other upstream secondary structures.

Figure 2-1. Lettuce chlorosis virus (LCV) genome organization and nucleotide sequence alignment of the 3' non-coding regions of LCV RNAs 1 and 2. (A) A schematic representation of the LCV genome. Open reading frames (ORFs; 1a – 3 and 1 – 10 in LCV RNAs 1 and 2, respectively) encoding the following predicted viral proteins are as indicated: P-Pro, papain-like protease; MTR, methyltransferase; HEL, RNA helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, heat shock protein 70 homolog; CP, major coat protein; CPm, minor coat protein; and proteins that are named according to their relative molecular masses (indicated by numbers preceded by "P"): P8, P23, P5.6, P6, P6.4, P60, P9, P27, and P4.8. Black bars below the genome map represent DIG-labeled riboprobes (II and VIII) complementary to the corresponding locations in the genomic RNAs. (B) Enlargement of the areas indicated by the dashed-circles (in Fig. 1A) representing the 3' terminal region of LCV RNAs 1 (top) and 2 (bottom).

Figure 2-1

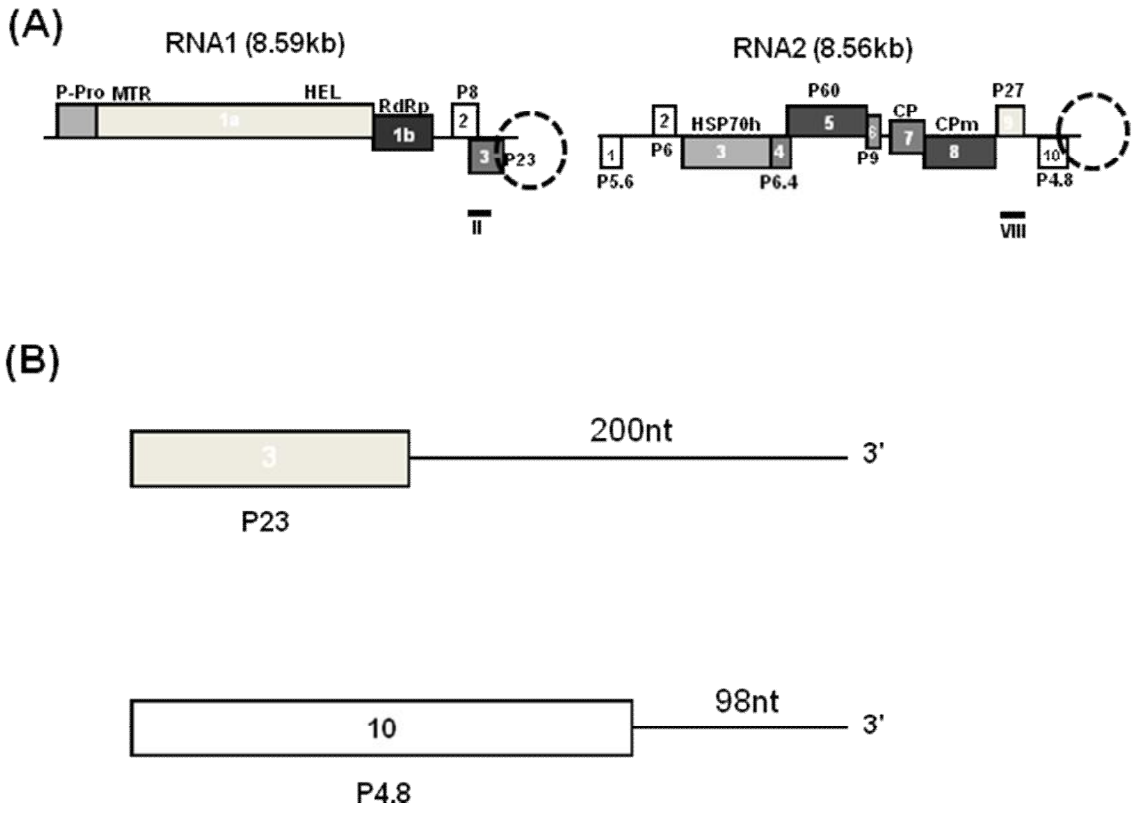

(A)
RNA1 (8.59kb)
P-Pro
MTR
HEL
RdRp
1b
P8
2
3
P23
II
RNA2 (8.56kb)
P60
P27
2
HSP70h
5
CP
CPm
7
8
1
P6
3
4
P9
10
P5.6
P6.4
P4.8
VIII
(B)
3
200nt
3'
P23
10
98nt
3'
P4.8

Figure 2-2. Examination of the effects of exchanging the 3' NCR of LCV RNAs 1 and 2 on minus-strand RNA synthesis in tobacco protoplasts. (A) Schematic diagrams of infectious cDNA clones of wild type LCV RNA 1 (R1; pCM1), wild type LCV RNA 2 (R2; pCM2), mutant LCV RNA 1 containing the 98-nt of RNA 2 (R1-3'R2), and mutant LCV RNA 2 containing 98-nt of RNA 1 (R2-3'R1). Numbers (#1-5) represent different combinations of wild type and/or chimeric mutant *in vitro* transcripts (2 μg each) inoculated into tobacco protoplasts. (B-D) Northern blot analyses of minus-strand RNA accumulation. Northern blots were performed using total RNA (2 μg each) extracted from tobacco protoplasts harvested at 24, 48, and 96 hours (lanes 24, 48, and 96, respectively) following the inoculation of each of the five combinations of *in vitro* transcripts (#1-5); and from water (mock)-inoculated tobacco protoplasts harvested at 96 hours post-inoculation (lane W). Hybridizations were conducted using DIG-labeled negative-sense specific riboprobes II (B and C) and VIII (D) (Fig. 1A). Hybridization signals of negative-sense genomic RNAs 1 and 2 are indicated by G1 and G2, respectively. Sizes of RNAs were estimated based on methylene blue-stained RNA standards shown on the left of each blot. The methylene blue-stained 25S rRNA of each sample was included to demonstrate the equal loading of total RNA samples.

Figure 2-2

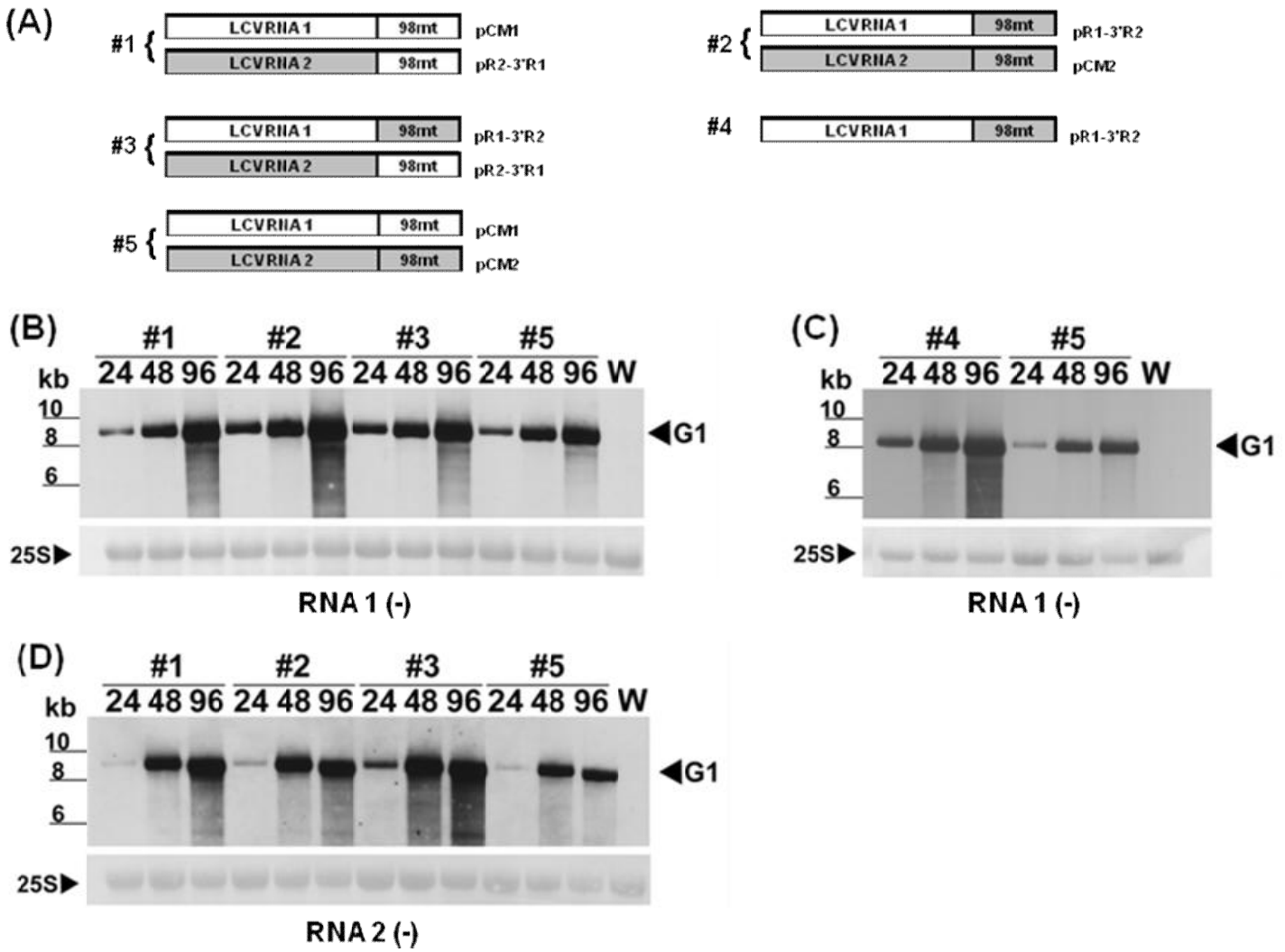

(A)
#1
LCVRNA 1
98mt
pCM1
LCVRNA 2
98mt
pR2-3'R1
#2
LCVRNA 1
98mt
pR1-3'R2
LCVRNA 2
98mt
pCM2
#3
LCVRNA 1
98mt
pR1-3'R2
LCVRNA 2
98mt
pR2-3'R1
#4
LCVRNA 1
98mt
pR1-3'R2
#5
LCVRNA 1
98mt
pCM1
LCVRNA 2
98mt
pCM2
(B)
#1 #2 #3 #5
kb
24 48 96 24 48 96 24 48 96 24 48 96 W
10
8
6
G1
25S
RNA 1 (-)
(C)
#4 #5
kb
24 48 96 24 48 96 W
10
8
6
G1
25S
RNA 1 (-)
(D)
#1 #2 #3 #5
kb
24 48 96 24 48 96 24 48 96 24 48 96 W
10
8
6
G1
25S
RNA 2 (-)

Figure 2-3. Northern analyses of minus-strand accumulation of LCV RNA 2 mutants engineered with deletions in terminal end of the 3' NCR. (A) Nucleotide sequences in the 3' NCR of WT LCV RNA 2 (R2) and 3' NCR mutants of LCV RNA 2 engineered with the following deletions: the first 50 nucleotides (5'Δ50), the last 48 nucleotides (3'Δ48), the last 48 nucleotides without deletion of the 3' terminal 4 nucleotides [3'Δ48(4)], the last 4 nucleotides (3'Δ4), the last 10 nucleotides (3'Δ10), the last 24 nucleotides (3'Δ24), and the last 38 nucleotides (3'Δ38). Deleted nucleotides are indicated by dash lines. Numbers, 8459 and 8556, indicated by arrows, are the nucleotide positions of the LCV RNA 2 3' NCR, while the numbers in parentheses, 1 and 98, indicate the first and last nucleotides of the 3' NCR, respectively. (B-F) Minus-strand LCV RNA 2 accumulation in tobacco protoplasts inoculated with the *in vitro* transcripts of R1 (LCV RNA 1) and each of the following mutants: 5'Δ50 (B), 3'Δ48 (B), 3'Δ48(4) (B), 3'Δ4 (C), 3'Δ10 (D), 3'Δ24 (E), 3'Δ38 (F) or wild type (WT) R2 (B-F). Total RNA (2 μg each) extracted from the inoculated protoplasts harvested at 24, 48, and 96 hpi (lanes 24, 48 and 96, respectively), and total RNA (2 μg) from water (mock)-inoculated protoplasts harvested at 96 hpi (lane W) were analyzed using DIG-labeled RNA 2 negative-sense specific riboprobe VIII (Fig. 1A). Hybridization signals of negative-sense genomic RNA 2 (G2) are indicated. Inset E1 show the extended exposure of the hybridization signals for the genomic RNA (G2) of 3'Δ24 and WT in lanes 24, 48 and 96. RNA size estimates and methylene blue-stained 25S rRNA loading controls are as in Fig 2-2.

Figure 2-3

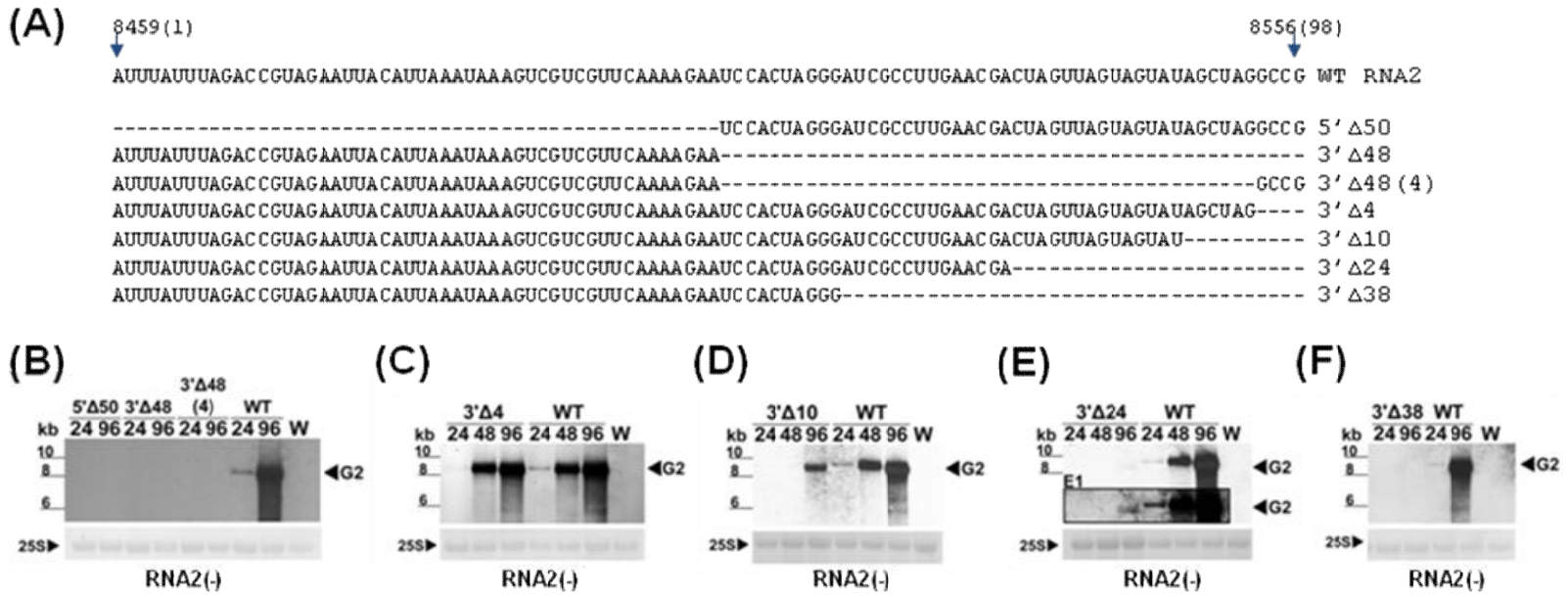

(A)
8459(1)
8556(98)
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCUUGAACGACUAGUUAGUAGUAUAGCUAGGCCG WT RNA2
----------UCCACUAGGGAUCGCCUUGAACGACUAGUUAGUAGUAUAGCUAGGCCG 5′Δ50
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAA---------- 3′Δ48
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAA----------GCCG 3′Δ48(4)
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCUUGAACGACUAGUUAGUAGUAUAGCUAG---- 3′Δ4
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCUUGAACGACUAGUUAGUAGUAU---------- 3′Δ10
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCUUGAACGA---------- 3′Δ24
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGG---------- 3′Δ38
(B)
3′Δ48
5′Δ50 3′Δ48 (4) WT
kb 24 96 24 96 24 96 24 96 W
10
8
6
◀G2
25S▶
RNA2(-)
(C)
3′Δ4 WT
kb 24 48 96 24 48 96 W
◀G2
RNA2(-)
(D)
3′Δ10 WT
kb 24 48 96 24 48 96 W
◀G2
RNA2(-)
(E)
3′Δ24 WT
kb 24 48 96 24 48 96 W
E1
◀G2
◀G2
RNA2(-)
(F)
3′Δ38 WT
kb 24 96 24 96 W
◀G2
RNA2(-)

Figure 2-4. SHAPE analysis of 3' NCR of LCV RNA 2. (A) Organization of the structure cassette plasmid, pS-3'R2, from which the *in vitro* transcribed 3' NCR of LCV RNA 2 was synthesized for SHAPE analysis 2. pS-3'R2contains a bacteriophage T3 promoter for *in vitro* transcription, a 5'-linker, the nt sequence of interest (the 3' NCR of LCV RNA 2 containing SL1), a 3' linker, and an RT primer binding site along with several restriction sites for cloning and linearization for *in vitro* transcription. (B) Electropherograms of: (Top) normalized NMIA reactivities (for positive [+NMIA; red] and negative [-; blue] reactions), and (Bottom) sequencing reactions (using ddTTP [A ladder]) as a function of nucleotide position. Electropherograms of sequencing reactions are shown in both green and pink, and each peak is one nucleotide longer than the corresponding position in the (+) and (-) electropherogram. Black bars above the electropherogram correspond to each section of the predicted SL structure. (C) Superimposition of normalized NMIA reactivity data on a predicted secondary structure of the 3' NCR of LCV RNA 2. Each colored nucleotide corresponds to the level of NMIA reactivity for that particular nucleotide, with black (0-0.4), yellow (0.4-0.85), and red (>0.85) representing unreactive, moderately reactive, and highly reactive, respectively. Numbers above the sequence indicate the nt position within the 3' NCR of the RNA. Gray and blue nucleotides correspond to nucleotides for which information of NMIA reactivity was unavailable and nucleotides of the primer binding site, respectively.

Figure 2-4

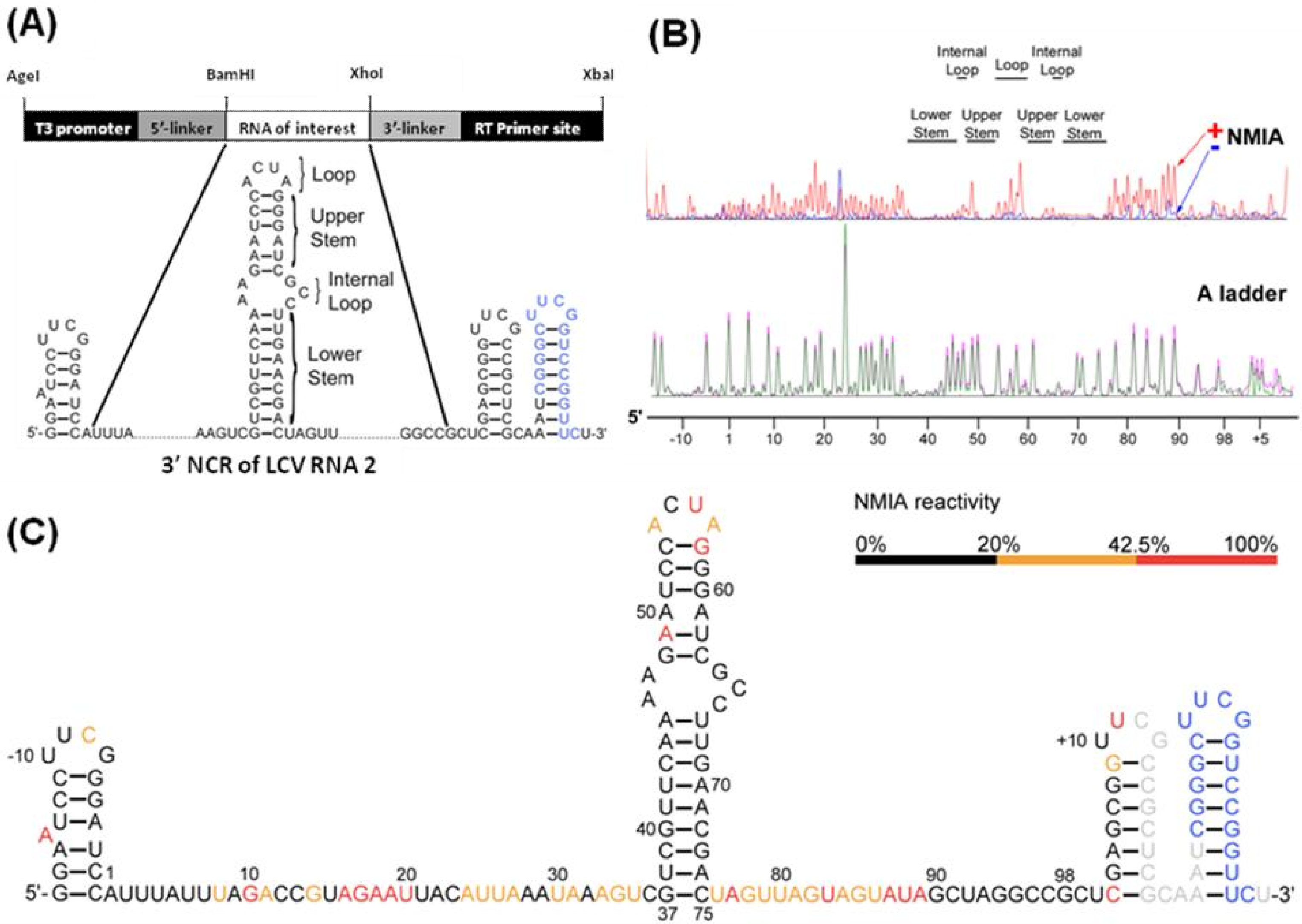

(A)
AgeI
BamHI
XhoI
XbaI
T3 promoter
5'-linker
RNA of interest
3'-linker
RT Primer site
Loop
Upper Stem
Internal Loop
Lower Stem
3' NCR of LCV RNA 2
(B)
Internal Loop
Loop
Internal Loop
Lower Stem
Upper Stem
Upper Stem
Lower Stem
+ NMIA
-
A ladder
5'
3'
(C)
NMIA reactivity
0%
20%
42.5%
100%
5'-G-CAUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCG-CUAGUUAGUAGUAUAGCUAGGCCGCUC-GCAA-UCU-3'

Figure 2-5. SHAPE analysis of LCV RNA 2 defective (D)RNA (DRNA2-6). (A) Genomic organization of DRNA2- relative to that of LCV RNA 2. The LCV RNA 2 map is shown at the top. In DRNA2-6 (bottom), solid lines and dashed lines represent existing nucleotides and missing regions of RNA 2, respectively. The percentage of RNA 2 missing from DRNA is indicated in parenthesis. Numbers next to the triangles indicate the nucleotide positions at the junctions between each of the deleted regions of RNA 2 in DRNA2-6. (B) Top: schematic of the infectious cDNA clone of DRNA2-6 engineered in the pGEM-T easy vector, showing the locations of the bacteriophage T3 promoter (labeled 'T3') and NgoMIV restriction site. Middle: the transcription initiation site (+1), which corresponds to the first nucleotide of DRNA2-6, guanine (G), begins immediately downstream of the penultimate 3' end nucleotide (*) of the T3 promoter (bold). The genomic sequence of DRNA2-6 (enclosed in the rectangular box) and the NgoMIV restriction site (underlined) are included as points of reference. Bottom: *in vitro* synthesized transcript of the infectious cDNA clone of DRNA2-6 bearing authentic 5' and 3' termini, with positions of the first (1) and last (3045) nucleotides indicated. (C) Northern blot analysis of total RNA extracted from tobacco protoplasts inoculated with the *in vitro* produced transcripts of R1 (WT LCV RNA 1) and that of DRNA2-6 or R2 (WT LCV RNA 2) 24 – 96 hpi, using DIG-labeled negative-sense RNA 2-specific riboprobe VIII (Fig. 1A). Total RNA from water-inoculated protoplasts (lane W) was used as a negative control. Hybridization signals of negative-sense genome RNA 2 are indicated as G2. Estimation of RNA sizes and methylene-blue stained 25s rRNA equal loading controls are as in Fig 2. (D) Superimposition of normalized NMIA reactivity data on a predicted secondary structure model of the 3' terminal region of DRNA2-6. Each colored nucleotide corresponds to the level of NMIA reactivity for that particular nucleotide, with black (0-0.4), yellow (0.4-0.85), and red (>0.85) representing unreactive, moderately reactive, and highly reactive, respectively. Numbers above the sequence indicate the nt position within DRNA2-6. Gray and blue nucleotides correspond to nucleotides for which information of NMIA reactivity was unavailable and nucleotides of the primer binding site, respectively.

Figure 2-5

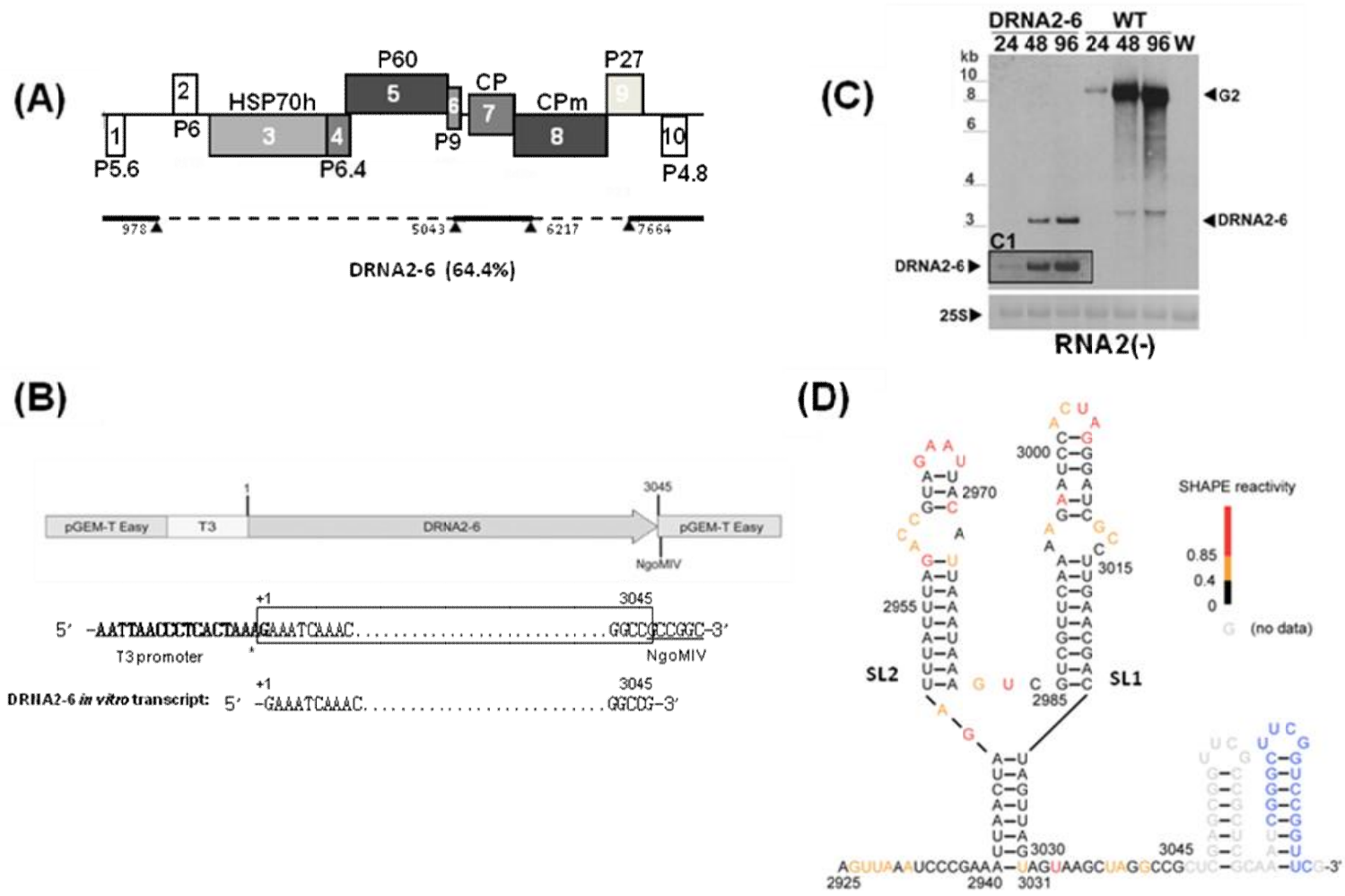

(A)
P60
P27
2
HSP70h
5
CP
CPm
1
P6
3
4
7
8
10
P5.6
P6.4
P9
P4.8
978
5043
6217
7664
DRNA2-6 (64.4%)
(C)
DRNA2-6 WT
24 48 96 24 48 96 W
kb
10
8
6
4
3
G2
DRNA2-6
C1
DRNA2-6
25S
RNA2(-)
(B)
1
3045
pGEM-T Easy
T3
DRNA2-6
pGEM-T Easy
NgoMIV
+1
3045
5′ -AATTAACCCTCACTAAAGAAATCAAAC.....GGCCGCCGGC-3′
T3 promoter
NgoMIV
DRNA2-6 in vitro transcript:
+1
3045
5′ -GAAATCAAAC.....GGCCG-3′
(D)
SHAPE reactivity
0.85
0.4
0
(no data)
SL2
SL1
2970
3000
3015
2955
2985
3030
3045
2925
2940
3031

Figure 2-6. Mutational analyses of a prominent secondary stem-loop (SL1) structure in the 3' NCR of LCV RNA 2 involved in minus-strand RNA synthesis. (A) Nucleotide sequences of the 3' NCR of WT RNA 2 (R2; containing SL1) and the following SL1 mutants: 3'ΔSL (with SL1 deleted), 3'SLD1 (with a 6-nucleotide substitution at the right arm of the lower stem of SL1 engineered to disrupt the SL), 3'SLD2 (with a 6-nucleotide substitution at the left arm of the lower stem of SL1 engineered to disrupt the SL) and 3'SLR (with a compensatory 6-nucleotide substitution at the left arm of the lower stem of 3'SLD1 engineered to restore the SL). Underlined sequences represent the region that is predicted to fold into the SL structure. Dashed lines and bold letters indicate the deleted and substituted nucleotides, respectively. Numbers, 8495 and 8533, above the arrows indicate the nucleotide positions on LCV RNA 2 where SL1 is located. (B) Schematic diagrams of the secondary structure of mutants 3'SLD1, 3'SLD2 and 3'SLR predicted using the web server application *Mfold*. Bold letters represent non-viral nucleotides engineered to substitute the viral nucleotides. (C-F) Tobacco protoplasts were inoculated with the *in vitro* transcripts of R1 (WT) along with those of mutants 3'ΔSL (C), 3'SLD1 (D), 3'SLD2 (E), 3'SLR (F), or R2 (WT). Total RNA (2 μg each) extracted from transcripts-inoculated protoplasts (lanes 24, 48, and 96) and total RNA (2 μg) extracted from water (mock)-inoculated protoplasts (lane W) were analyzed using DIG-labeled negative-sense specific riboprobe VIII (Fig. 1A). Hybridization signals of negative-sense genomic RNA 2 are indicated as G2. Estimation of RNA sizes and methylene-blue stained 25s rRNA equal loading controls are as in Fig. 2-2.

Figure 2-6

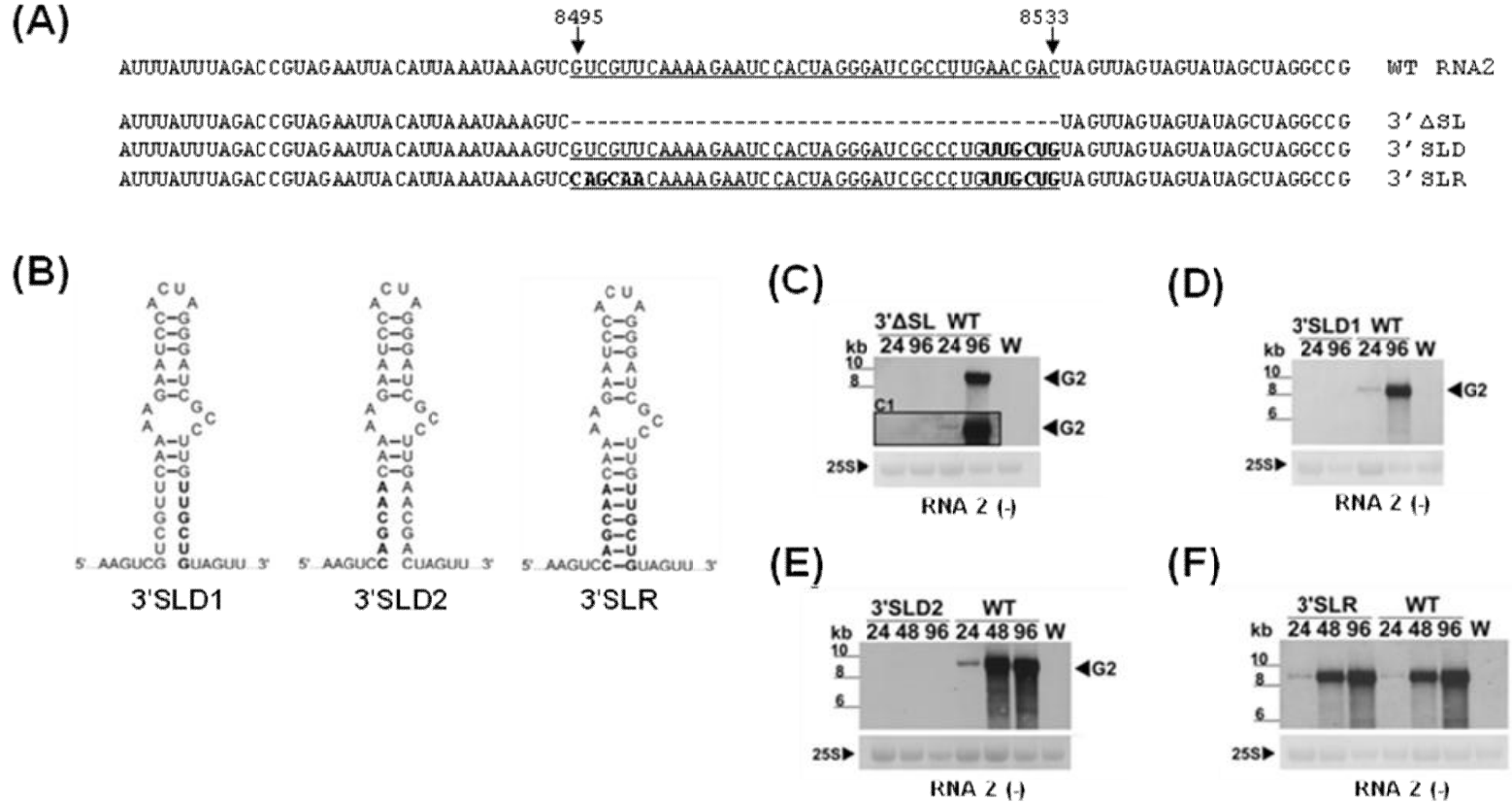

(A)
8495
8533
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCUUGAACGACUAGUUAGUAGUAUAGCUAGGCCG WT RNA2
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUC----------UAGUUAGUAGUAUAGCUAGGCCG 3'ΔSL
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCGUCGUUCAAAAGAAUCCACUAGGGAUCGCCCUGUUGCUGUAGUUAGUAGUAUAGCUAGGCCG 3'SLD
AUUUAUUUAGACCGUAGAAUUACAUUAAAUAAAGUCCAGCAACAAAAGAAUCCACUAGGGAUCGCCCUGUUGCUGUAGUUAGUAGUAUAGCUAGGCCG 3'SLR
(B)
5' AAGUCG GUAGUU 3'
3'SLD1
5' AAGUCC CUAGUU 3'
3'SLD2
5' AAGUCC GUAGUU 3'
3'SLR
(C)
3'ΔSL WT
kb 24 96 24 96 W
10
8
C1
G2
G2
25S
RNA 2 (-)
(D)
3'SLD1 WT
kb 24 96 24 96 W
10
8
6
G2
25S
RNA 2 (-)
(E)
3'SLD2 WT
kb 24 48 96 24 48 96 W
10
8
6
G2
25S
RNA 2 (-)
(F)
3'SLR WT
kb 24 48 96 24 48 96 W
10
8
6
G2
25S
RNA 2 (-)

Figure 2-7. Mutational analyses of a secondary stem-loop (SL2) structure in the 3' terminal region of LCV RNA 2 involved in minus-strand RNA synthesis. (A) Nucleotide sequences of the 3' terminal region of WT RNA 2 (R2; containing SL2) and mutant 3'ΔSL2 (with SL2 deleted). Underlined sequences represent the region that is predicted to fold into the SL2 structure. Dashed lines indicate the deleted nucleotides. Numbers, 8460 and 8491, above the arrows indicate the nucleotide positions on LCV RNA 2 where SL1 is located. (B) Schematic diagrams of the secondary structure of SL2 using the web server application *Mfold*. (C) Tobacco protoplasts were inoculated with the *in vitro* transcripts of R1 (WT) along with those of mutant 3'ΔSL2 or R2 (WT). Total RNA (2 μg each) extracted from transcripts-inoculated protoplasts (lanes 24, 48, and 96) and total RNA (2 μg) extracted from water (mock)-inoculated protoplasts (lane W) were analyzed using DIG-labeled negative-sense specific riboprobe VIII (Fig. 1A). Hybridization signals of negative-sense genomic RNA 2 are indicated as G2. Estimation of RNA sizes and methylene-blue stained 25s rRNA equal loading controls are as in Fig. 2-2.

Figure 2-7

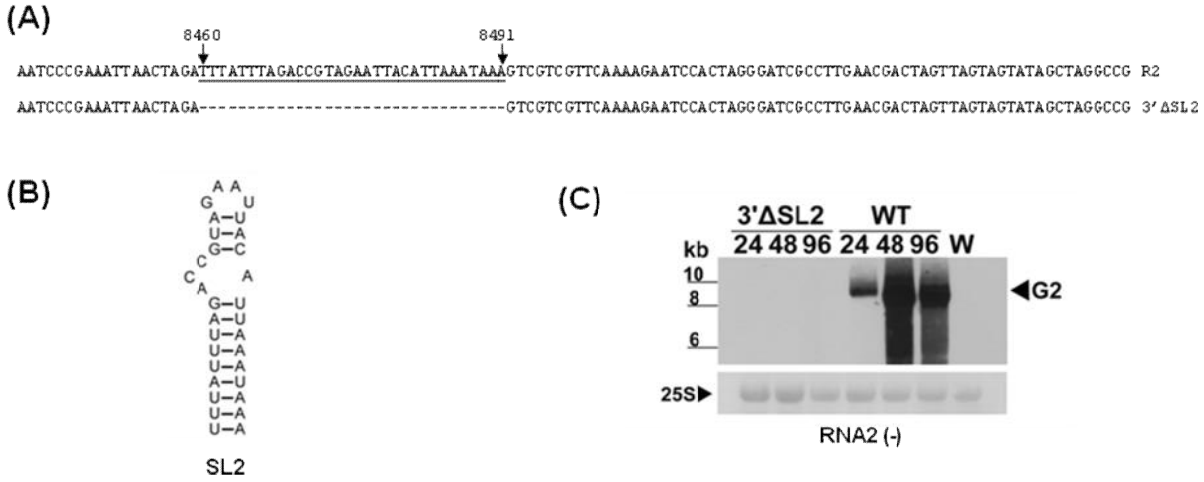

(A)
8460
8491
AATCCCGAAATTAACTAGATTTATTTAGACCGTAGAATTACATTAAATAAAGTCGTCGTTCAAAAGAATCCACTAGGGATCGCCTTGAACGACTAGTTAGTAGTATAGCTAGGCCG R2
AATCCCGAAATTAACTAGA----------GTCGTCGTTCAAAAGAATCCACTAGGGATCGCCTTGAACGACTAGTTAGTAGTATAGCTAGGCCG 3′ΔSL2
(B)
SL2
(C)
3'ΔSL2
WT
kb
24 48 96 24 48 96 W
10
8
6
G2
25S
RNA2 (-)

Figure 2-8. Accumulation levels of minus-strand progenies of LCV RNA 2 and SL1 mutants: 3'ΔSL, 3'SLD1, and 3'SLD2. Total RNA of wild type- and SL1 mutants-inoculated protoplasts harvested at 24 and 96 hpi were subjected to first-strand cDNA synthesis. The cDNAs were then subjected to semi-quantitative PCR with different numbers of cycles of amplification (10, 15, 20, 25, and 30x cycles; indicated on top of 2% agarose gels). For negative control, total RNA of water-inoculated protoplasts were subjected to RT and semi-quantitative PCR in the same manner. Amplification of rRNA gene was used as an internal control in order to ensure equal amount of total RNA used.

Figure 2-8

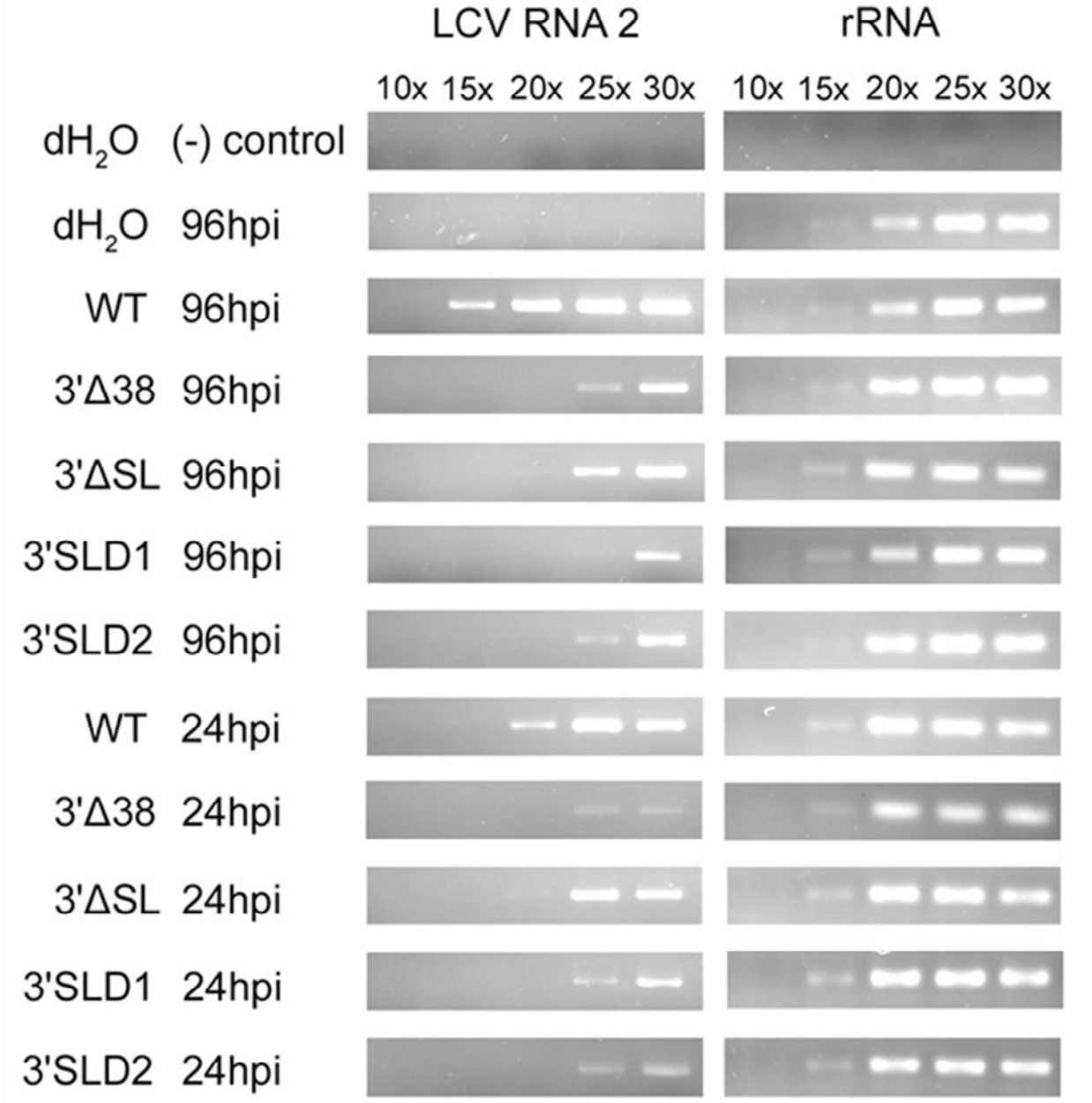

LCV RNA 2
rRNA
10x 15x 20x 25x 30x
10x 15x 20x 25x 30x
dH2O (-) control
dH2O 96hpi
WT 96hpi
3'Δ38 96hpi
3'ΔSL 96hpi
3'SLD1 96hpi
3'SLD2 96hpi
WT 24hpi
3'Δ38 24hpi
3'ΔSL 24hpi
3'SLD1 24hpi
3'SLD2 24hpi

Figure 2-9. 3'-terminal MFOLD-predicted secondary structures of crinivirus: *Lettuce chlorosis virus* (LCV) RNA 1, *Lettuce infectious Yellows Virus* (LIYV) RNA 1, *Bean yellow disorder virus* (BnYDV) RNAs 1 and 2, *Beet pseudo-yellows virus* (BPYV) RNAs 1 and 2, *Blackberry yellow vein associated virus* (BYVaV) RNAs 1 and 2, *Cucurbit chlorotic yellows virus* (CCYV) RNAs 1 and 2, *Cucurbit yellow stunting disorder virus* (CYSDV) RNAs 1 and 2, *Tomato infectious chlorosis virus* (TICV) RNAs 1 and 2, and *Tomato chlorosis virus* (ToCV) RNAs 1 and 2.

Figure 2-9

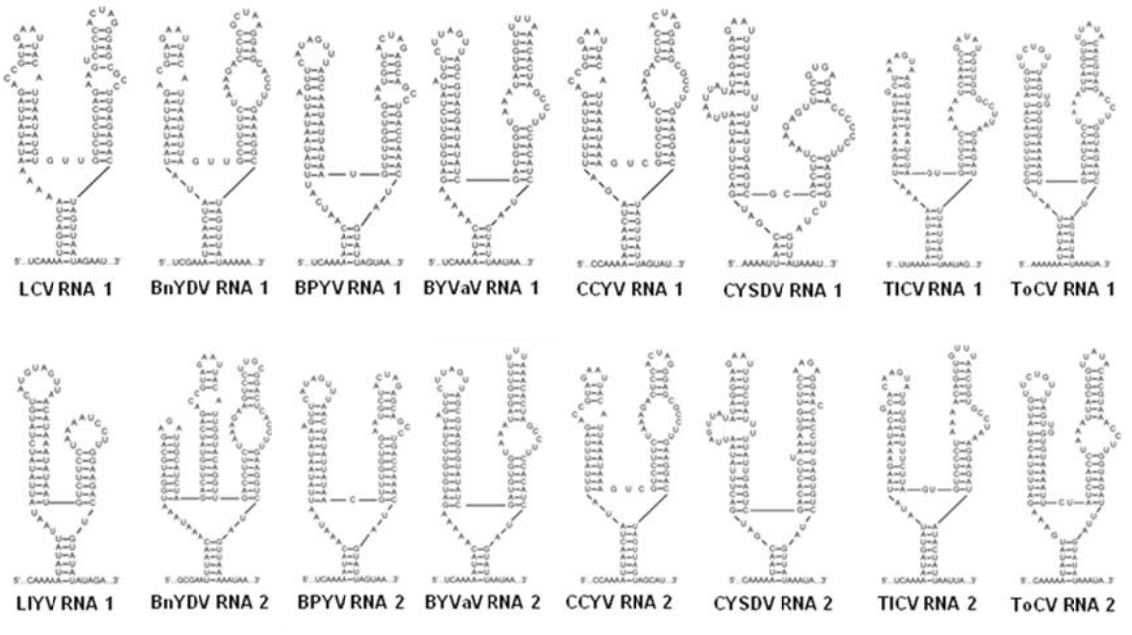

LCV RNA 1
BnYDV RNA 1
BPYV RNA 1
BYVaV RNA 1
CCYV RNA 1
CYSDV RNA 1
TICV RNA 1
ToCV RNA 1
LIYV RNA 1
BnYDV RNA 2
BPYV RNA 2
BYVaV RNA 2
CCYV RNA 2
CYSDV RNA 2
TICV RNA 2
ToCV RNA 2

Table 2-1. Oligonucleotide primers used in Chapter 2

Primer	Sequence (5' - 3') and polarity (+ or -)	Description	Use for
LCV-27	TTGAAACCTGTTCAAGACTGCTAAC (+)	Nucleotides corresponding to LCV RNA1 position 7923-7947	5' RACE
LCV-28	ACGAAGGGGTACACCGAATC (+)	Nucleotides corresponding to LCV RNA1 position 8006-8025	5' RACE
LCV-29	TACAGGAAGACCTGTTACTGTTACA (+)	Nucleotides corresponding to LCV RNA1 position 8076-8100	5' RACE
LCV-70-PW	GTGTCAGGTCTACGTGTCA (+)	Nucleotide corresponding to LCV RNA 2 position 7592-7611	sqPCR of LCV RNA 2
LCV-91-CN	TCATAGTTAACGTATCTGTATCAATAAAGATGTGA GATG (+)	Nucleotides corresponding to LCV RNA 1 position 7584-7622	Construction of pR1-3'R2
LCV-99-AC	TTGAGAAGTCTGCTTGAATGTTACT(-)	Nucleotides complementary to LCV RNA 2 position 7950-7926	sqPCR of LCV RNA 2
LCV-101-CM	*cccgggGCATGC* **AATTAACCCTCACTAAA**GAAATTT CCACGGTTTCCCCGAG (+)	XmaI (italic lower case) and SphI (italic upper case) restriction sites; a T3 promoter (bold) and nucleotides corresponding to LCV RNA 2 position 1-23 (underlined)	Construction of pDRNA2-6
LCV-103-CM	ATGGGTTCTTGAACAGTCACTACAGGTTTG (+)	Nucleotides corresponding to LCV RNA 2 position 7894-7923	5' RACE
LCV-161-CM	TGATAAGTTGAGAGTGTCCGATCAGG (+)	Nucleotides corresponding to LCV RNA 2 position 7312-7337	Construction of various pCM1 and pCM2 mutants and 5' RACE
LCV-162-CM	**CCCTAGTGGA**CTAGTTAATTTCGGGATTTA (-)	Nucleotides complementary to LCV RNA 2 position 8518-8509 (bold) and 8458-8539	Construction of p5'Δ50
LCV-163-CM	TTAACTAG**TCCACTAGGGATCGCCTTG (+)**	Nucleotides corresponding to LCV RNA 2 position 8451-8458 and 8509-8527 (bold)	Construction of p5'Δ50
LCV-164-CM	GGGTTGAGTGTTGTTCCAGTTTG (-)	Nucleotides complementary to position 492-514 of the pGEM-T Easy backbone of pCM2	Construction of various pCM1 and pCM2 mutants[2]
LCV-165-CM	**GCCGGC** *GGC* TTCTTTTGAACGACGA (-)	A NgoMIV restriction site (bold) and nucleotides complementary to LCV RNA 2 positions 8508 to 8493 (underlined) and 8556-8553 (italic)	Construction of p3'Δ48(4)

LCV-166-CM	**GGGCCC**GCCGGC *TAGCTATA* (-)	ApaI (bold) and NgoMIV (underlined) restriction sites, and nucleotides complementary to LCV RNA 2 position 8552-8544 (italic)	Construction of p3'Δ4
LCV-167-CM	**GGGCCC**ACCGGT *TCTTTTGAACGACGACT* (-)	ApaI (bold) and AgeI (underlined) restriction sites, and nucleotides complementary to LCV RNA 2 position 8508-8491 (italic)	Construction of p3'Δ48
LCV-172-AC	**GGGCCC**GCCGGC *GGCCTAGCTTACTACTAAC* (-)	ApaI (bold) and NgoMIV (underlined) restriction sites, and nucleotides complementary to DRNA2-6 position 3045-3026 (italic)	Construction of pDRNA2-6
LCV-175-CM	**GGGCCC**ACCGGT *CGTTCAAGGCGATCC* (-)	ApaI (bold) and AgeI (underlined) restriction sites, and nucleotides complementary to LCV RNA 2 position 8532-8517 (italic)	Construction of p3'Δ24
LCV-176-CM	**GGGCCC**ACCGGT *ACTACTAACTAGTCG* (-)	ApaI (bold) and AgeI (underlined) restriction sites, and nucleotides complementary to LCV RNA 2 position 8545-8530 (italic)	Construction of p3'Δ10
LCV-180-AC	TTACGCGTGTGACTTAATTTGAGAG (+)	Nucleotides corresponding to LCV RNA 2 position 8290-8314	5' RACE
LCV-188-CM	**GGGCCC**GCCGGC *CCTAGTGGATTCTTT* (-)	ApaI (bold) and NgoMIV (underlined) restriction sites, and nucleotides complementary to LCV RNA 2 position 8518-8503 (italic)	Construction of p3'Δ38
LCV-189-CM	**CTACTAACTAG**ACTTTATTTAATGTAATTCTACGG (-)	Nucleotides complementary to LCV RNA 2 position 8543-8534 (bold) and 8494-8470	Construction of p3'ΔSL
LCV-190-CM	AATAAAGTC**TAGTTAGTAGTATAGCTAGGCCG** (+)	Nucleotides corresponding to LCV RNA 2 position 8486-8494 and 8534-8556 (bold)	Construction of p3'ΔSL
LCV-191-CM	*ACTA* **CAGCAA**CAAGGCGATCCCTAGT (-)	Nucleotides complementary to LCV RNA 2 positions 8537-8534 (italic) and 8527-8512 (underlined); and designed for substituting nucleotides from position 8533-8528 with non-viral nucleotides (bold)	Construction of p3'SLD and 3'SLR
LCV-192-CM	CTTG **TTGCTG***TAGTTAGTAGTATAGCTAGG* (+)	Nucleotides corresponding to LCV RNA 2 positions 8524-8527 (underlined) and 8534-8553 (italic);, and designed for substituting nucleotides from position 8533-8528 with non-viral nucleotides (bold)	Construction of p3'SLD and 3'SLR
LCV-193-CM	*TTTG* **TTGCTG**GACTTTATTTAATGTAATT (-)	Nucleotides complementary to LCV RNA 2 positions 8504-8501 (italic) and 8494-8476 (underlined); and designed for the substituting nucleotides from position 8500-8495 with non-viral nucleotides (bold)	Construction of p3'SLR
LCV-194-CM	AGTC**CAGCAA***CAAAAGAATCCACTAGG* (+)	Nucleotides corresponding to LCV RNA 2 positions 8491-8494 (underlined) and 8501-8517 (italic); and designed for substituting nucleotides from position 8494-8500 with non-viral nucleotides (bold)	Construction of p3'SLR
LCV-219-CM	**CGGTCTAAATAAAT**TTAGTCAATTTTGAGATTTT AC (-)	Nucleotides complementary to LCV RNA 2 position 8472-8459 (bold) and LCV RNA 1 position 8491-8470	Construction of pR1-3'R2

LCV-220-CM	AATTGACTAA**ATTTATTTAGACCGTAGAATTAC** (+)	Nucleotides corresponding to LCV RNA 2 position 8459-8481 (bold) and LCV RNA 1 position 8482-8491	Construction of pR1-3'R2
LCV-221-CM	**CTAATATATTT**CTAGTTAATTTCGGGATTTAAC (-)	Nucleotides complementary to LCV RNA 1 position 8503-8492 (bold) and LCV RNA 2 position 8458-8437	Construction of pR2-3'R1
LCV-222-CM	CGAAATTAACTAG**AAAATATATTAGACCGTAG** (+)	Nucleotides corresponding to LCV RNA 1 position 8492-8510 (bold) and LCV RNA 2 position 8446-8458	Construction of pR2-3'R1
LCV-223-CM	GCAGTGAGCGCAACGCAATTA (-)	Nucleotides complementary to position 289-309 of the pGEM-T Easy backbone of pCM1	Construction of pR2-3'R1
LCV-277-CM	**AAAGG**AATCCTTCG*GGATCC* ATTTATTTAGACCG TAGAATTACATTAAATAAAG (+)	Nucleotides of part of the bacteriophage T3 promoter (bold), 5'-linker (underlined) with BamHI restriction site (italic underlined), and nucleotides corresponding to LCV RNA 2 position 8459-8492	Construction of pS-3'R2
LCV-278-CM	ATTTGCGAGCGGCGAACCG*CTCGAG* CGGCCTAGCTA TACTACTAACTAGTCGTTC (-)	3'-linker (underlined) with XhoI restriction site (italic underlined), and nucleotides complementary to LCV RNA 2 position 8556-8527	Construction of pS-3'R2
LCV-279-CM	accggt**AATTAACCCTCACTAAAGG**AATCCTTCG*GG ATCC* ATTTATTTAG (+)	AgeI restriction site (lower case), the bacteriophage T3 promoter (bold), 5'-linker (underlined) with BamHI restriction site (italic underlined), and nucleotides corresponding to LCV RNA 2 position	Construction of pS-3'R2
LCV-280-CM	tctaga**GAACCGGACCGAAGCCCG**ATTTGCGAGCG GCGAACCG*CTCGAG* CGGCC (-)	XbaI restriction site (lower case), RT primer binding site (bold), 3'-linker (underlined) with XhoI restriction site (italic underlined), and nucleotides complementary to LCV RNA 2 position 8556-8552	Construction of pS-3'R2
LCV-283-CM	**AATTAACCCTCACTAAAG**AAATTTCCACGGTTTC CCCGAGGTAATGG (+)	Nucleotides of the bacteriophage T3 promoter (bold) and nucleotides corresponding to position 1-30 of DRNA2-6 (underlined)	Construction of pSDR2-6
LCV-284-CM	ATTTGCGAGCGGCGAACCG*CTCGAG* CGGCCTAGCTT ACTACTAACTAGTCGTTC (-)	3'-linker (underlined) with XhoI restriction site (italic underlined), and nucleotides complementary to position 3045-3017 of DRNA2-6	Construction of pSDR2-6
LCV-285-CM	gccggc**GAACCGGACCGAAGCCCG**ATTTGCGAGCG GCGAACCG*CTCGAG* CGGCC (-)	A NgoMIV restriction site (lower case), RT primer binding site (bold), a 3'-linker (underlined), XhoI restriction site (italic underlined) and nucleotides complementary to LCV RNA 2 position 3045-3041	Construction of pSDR2-6
LCV-286-CM	GAACGACGAC**TCTAGTTAATTTCGGGATTTAAC** (-)	Nucleotides complementary to LCV RNA 2 position 8501-8492 and 8495-8437 (bold)	Construction of p3'ΔSL2
LCV-287-CM	**ATTAACTAGA**GTCGTCGTTCAAAAGAATCCAC (+)	Nucleotides corresponding to LCV RNA 2 position 8450-8459 (bold) and 8492-8513	Construction of p3'ΔSL2
AAP(C)	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG (-)	5' RACE Abridged Anchor Primer with a poly G/I tract	5' RACE

AAP(T)	GGCCACGCGTCGACTAGTACAAAAAAAAAAAAAAA A (-)	5' RACE Abridged Anchor Primer with a poly A tract	5' RACE
AUAP	GGCCACGCGTCGACTAGTAC (-)	5' RACE Abridged Universal Amplification Primer	5' RACE
NtUbiF	TGCTTAACACATGCAAGTCGGA (+)	Nucleotides corresponding to 16SrRNA	sqPCR of 16SrRNA
NtUbitR	AGCCGTTTCCAGCTGTTGTTC (-)	Nucleotides complementary to 16SrRNA	sqPCR of 16SrRNA

Conclusion

As *Lettuce chlorosis virus* (LCV) is a relatively new member of emerging and economically important genus *Crinivirus* in the family *Closteroviridae,* there is very limited knowledge about LCV besides some information on pathological and serological aspect of the virus until the recent sequence and phylogenetic analyses in 2009 (Salem et al., 2009). However, a tool was not available to investigate molecular aspect of the virus.

First part of this dissertation described the making and testing of the molecular tool for further molecular study of LCV, infectious cDNA clones of full-length LCV genomic RNAs. For synthesizing *in vitro* transcripts, a bacteriophage T3 promoter and an NgOMIV restriction site were engineered immediately upstream and downstream of the LCV RNAs sequences, respectively. Tobacco protoplasts were used to assess biological activities of *in vitro* synthesized transcripts from both infectious cDNA clones of LCV RNAs. Transcripts of infectious cDNA clones of LCV RNAs 1 and 2 exhibit wild type LCV virion RNAs property in inoculated tobacco protoplasts: similar accumulation kinetics of RNAs 1 and 2, the synthesis of the major coat protein, and formation of virion-like particles.

While total RNAs from LCV RNAs transcripts-inoculated protoplasts were analyzed by Northern blot using riboprobe specific to 5'-terminus of LCV RNA 1, several novel RNA 1-derived RNA species were detected: two high molecular weight RNA species (HM-LCVR1-1and HM-LCVR1-2) and two low molecular weight RNA species (LM-LCVR1-1and LM-LCVR1-2). HM-LCVR1-1 has a size of approximately 8.0kb with both positive- and negative-stranded forms, while HM-LCVR1-2 has a size of approximately 6.6kb with only positive-stranded form. LM-LCVR1-1 has a size of

approximately 0.38kb with only positive-stranded form while LM-LCVR1-2 has a size of approximately 0.3kb with both positive- and negative-stranded forms. In addition, subsequence examination of LM-2 by both Northern blot analyses and RT-PCR revealed its identity to be defective (D) RNA, which have not been discovered before for LIYV or any other criniviruses (Rubio et al., 2000).

Several questions arise from the study as the function and identity of these RNA species remain largely unknown. Several CTV studies have provided some insight on the mechanism and function of these RNA species (Che et al., 2001; Gowda et al., 2003; Gowda et al., 2001; Gowda et al., 2009). Several 5'-coterminal RNA species with only positive stranded form have been identified in *Citrus tristeza virus* (CTV) such as those CTV LMT and LaMT RNAs, in which similar to LM-LCVR1-1 and HM-LCVR1-2, respectively (Che et al., 2001). Studies of these CTV RNA species have also identified both primary sequences and secondary structures required for production of these RNA species (Gowda et al., 2003; Gowda et al., 2001). However, no specific function has been found to be associated with the CTV 5'-coternimal RNA except for CTV LMT2, which correlates with virion assembly (Gowda et al., 2009). With information from CTV, we can examine in the future whether similar promoter elements also exist in LCV and whether LM-LCVR1-1 has similar function in virion assembly of LCV RNA 1 as those of CTV LMT2.

Discovery of RNA1 DRNAs provides an interesting insight in replication of LCV RNA 1 as the presence of DRNAs derived from RNA 1 suggested that LCV RNA 1 can support replication *in trans*, in contrast with a *cis* preferential model of replication for

LIYV RNA 1 (Rubio et al., 2000; Wang et al., 2009b; Yeh et al., 2000). However, question still need to be address whether RNA 1 DRNAs can be *de novo* generated from DRNAs as it is possible that all DRNAs are simply byproducts generated directly from LCV RNA 1 and DRNAs are not replication competent. Another interesting characteristic of RNA 1 DRNAs is a putative small ORF in DRNAs. A similar type of putative ORF has also been found to play a role in replication and accumulation of CTV DRNAs (Yang et al., 1997); (Mawassi et al., 2000). The importance of this putative ORF is unclear in LCV RNA 1 DRNAs whether this putative ORF is required for replication as in the case of CTV and whether this putative ORF encode any functionally important protein for LCV.

Second part of this dissertation dealt with examination of determinants that mediate minus-strand LCV RNA 2 synthesis. In order to determine whether non-conserved nucleotides in the conserved region of 3' NCRs of LCV RNAs 1 and 2 also have specific function in regulating optimal minus-strand RNA production, we first exchanged the conserved regions 3' NCR of LCV RNAs and showed that the conserved regions of 3' NCRs can be exchanged without any detrimental effect on minus strand RNA synthesis. As the result suggested that the conserved regions of 3' NCRs of LCV RNAs 1 and 2 possess similar sequence/structural elements involved in regulating minus-sense RNA synthesis, we decided to use LCV RNA 2 as a reporter to avoid making any disruption of translation of replication-associated proteins. Through deletion analysis of 3' NCR of LCV RNA 2, we first found the minimum sequence required for minus-strand RNA 2 synthesis lie between 25 to 98 nucleotides from the 3' terminus of LCV RNA 2.

Next, we used folding algorithm *MFOLD* and chemical modification SHAPE analysis to analyze structure within 3' NCR of LCV RNA 2 and found a presence of 3'-terminal stem-loop (SL) structure (Wilkinson et al., 2006; Zuker, 2003). Mutation analyses of the SL by deletion and substitution showed that only overall structure is required for minus-strand RNA 2 synthesis as deletion and/or substitution of loop, top stem and internal loop, of SL did not affect viral RNA 2 accumulation. An addition SHAPE analysis was performed on replication-competent LCV RNA 2 defective RNA to more stringently characterize the SL structure. The result showed that SL is a part of a larger Y-shaped structure.

Even though LCV RNA 2 SL has been found to be involved in minus-strand RNA synthesis, the precise mechanism of how SL involves in minus-strand RNA synthesis and whether other parts of the large Y-shaped element also precipitate in minus-strand RNA synthesis remain unknown. However, studies of a similar Y-shaped structure (YRE) in *Red clover necrotic mosaic virus* (RCNMV) RNA 2 can provide some insight in involvement of LCV RNA 2 SL (An et al., 2010; Iwakawa et al., 2011). The YRE has been showed to be involved in minus-strand RNA synthesis (An et al., 2010) and has been found to interact with both RCNMV p27 and 480-kDa RCNMV replicase complex (Iwakawa et al., 2011). In addition, interaction between RCNMV p27 and YRE has shown to be involved in recruiting RCNMV RNA 2 to the membrane fraction (Iwakawa et al., 2011). So it is possible that LCV RNA 2 Y-shaped structure can have same function as a recruiter element for replicase binding and membrane localization. The

future study can explore whether LCV RNA 2 Y-shaped structure interacts with LCV replicase or replicase complex and what is the requirement of this specific interaction.

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Appendices

Appendix A. Optimization of *Lettuce chlorosis virus* virion (LCV) virion purification from infected plant

Summary:

Procedure of LIYV virion purification from infected plant is adopted for purifying LCV virion from plant as there is no established procedure for LCV virion purification. However, the average yield of plant-purified LCV virion (200-800ng/µl) is significantly lower than those of LIYV (>1000ng/µl). Here, I attempted to optimize the virion purification procedure for a better yield of LCV virion from infected plants by changing several parameters from the original LIYV purification procedure. However, the result showed that any changes from original purification did not increase the yield of LCV virion (Table A-1).

List of parameters changed for optimization:

Standard LIYV purification procedure:	**Change into:**
Purification procedure:	
LIYV	BYV
Plant host:	
Chenopodium Murale	*Nicotiana Benthamiana*
Extraction Buffer:	
0.1M Tris-Cl Buffer pH 7.4	pH 4, 9, or 10
Detergent:	
Day #1 and 2 Triton 100x 2% final concentration	4%
Sucrose cushion:	
Day #1 20% sucrose in 0.1M Tris-Cl Buffer pH 7.4	17% sucrose or pH 4, 9, or 10
Day #2 20% sucrose in 1x TE pH 7.4	17% sucrose or pH 4,9, or 10
Resuspension buffer:	
Day #1 0.1M Tris-Cl Buffer pH 7.4	pH 4, 9, or 10
Day #2 1x TE pH 7.4	pH 4, 9, or 10

Procedure:

A. Preparation before Day 1:

1.) Chill JA-14 and Ti-35 rotors, parafilm-wrapped autoclaved mortar and pestle, and paraflim-wrapped 1000ml beaker in the refrigerator

B. Day 1:

1.) Collect infected tissues (70-100g) from greenhouse

- Keep tissues on ice while collecting

2.) Weight tissues and leave it on ice while preparing extraction buffer

3.) Set up 2L beaker on ice with stir bar under fume hood

4.) Make the extraction buffer: 5x tissues weight

- 0.1M Tris-Cl, pH 7.4, 0.5% Na_2SO_3, 0.5% 2-mercaptoethanol
  - 70g of tissue = 350ml extraction buffer
  - Add ~330ml 0.1M Tris-Cl (pH7.4) into cylinder
  - Dissolve 1.75g Na_2SO_3 (final: 0.5%) in 0.1M Tris-Cl in blue cap tube
  - Add 1.75ml of mercapto-ethanol (final: 0.5%) under fume hood
  - Top up with 0.1M Tris-Cl (pH7.4) to 350ml

5.) Transfer extraction buffer into 2L beaker and stir the buffer

6.) Get liquid nitrogen using yellow liquid Nitrogen bucket and orange autoclave gloves

7.) Get mortar and pestle from refrigerator and spoon

8.) Transfer plant tissues into mortar until full

9.) Pour liquid nitrogen gently and slowly into mortar until close to the brim

10.) Grind leaves using pestle until they become fine powder **(work fast)**

- Hold mortar with left hand and grind leaves with pestle in right hand
- If the tissues become fine powder, do not add more liquid nitrogen

11.) Put clean spoon in liquid nitrogen and transfer the grinded tissue into the buffer

- Make sure the extraction buffer is stirring while adding the grinded tissue

12.) Repeat the grinding until all tissues are grinded and transfer into the buffer

13.) **Continuously Stirring for 10 min**

- Use spoon or glass rod to break up the clump of tissue
- While waiting:
  - Set up1L beaker from refrigerator into ice bucket
  - Put the cheese cloth over the beaker and secure it with rubber band

14.) After 10 min, filter the solution through the prepared beaker with cheese cloth

15.) Close up the cheese cloth and squeeze all the supernatant out into the beaker

16.) Measure the volume of supernatant

17.) Prepare to add Triton 100x for 2% final concentration

- Calculate the amount need to add from the volume
- Microwave Triton 100x in blue cap tube for 5 sec.

18.) Add Triton 100x drop wise while the solution is stirring

- using autoclave glass pipette

19.) Stirring on ice under the hood @ speed #2 for 2 hours

- While waiting:

- Set up Beckman J2-21 High speed centrifuge and set temperature to 4^{o}C
- Chill 2x GSA bottles
    - Check the O ring under the cap
- Prepare a 20% sucrose cushion with extraction buffer without 2-mercaptoethanol (6+ ml for each Ti-35 polycarbonate tubes)
    - For 70g of tissue:
        - 50ml 0.1M Tris-Cl (pH7.4) + 0.25g Na_2SO_3 + 10g sucrose
- Chill 6x Ti-35 polycarbonate tubes with black plastic cap
    - Check the O ring under the cap
- Set up Beckman L9-70M Ultra speed centrifuge
    - Temperature: 4^{o}C
    - Speed: 28000 rpm
    - Time: 1 hour
    - Press vacuum to cool the centrifuge

20.) After stirring for 2 hours, transfer the supernatant into 2x GSA bottles on ice

21.) Weight out the bottle to 0.1g difference by using transfer pipette

- Make sure to wipe bottle dry before weighting
- Always reweight the bottles to make sure the difference is within 0.1g
- Bring the GSA bottles on ice to the centrifuge room

22.) Equip JA-14 rotor into high speed centrifuge

- Check the cleanness of rotor

23.) Load GSA bottles into the rotor

- Always wipe the bottles clean of ice and water before loading

24.) Tighten the rotor cover (make sure it is hand-tight)

25.) Set up J2-21 high speed centrifuge:

- Set the time to 10 min
- Set the speed to 10000xg (10189 rpm)
- Wait until the needle on the vacuum gauge go into green zone
- Press Start
- Wait and observe any sign of abnormality (sound or shaking) until the speed reaches the set rpm

26.) After 10 minutes and rotor stop spinning, take bottles out of rotor

27.) Open bottle gently and pour supernatant into the new beaker

- Avoid pouring the pellet out with supernatant

28.) Transfer the supernatant into 6x Ti-35 polycarbonate bottles evenly

- Have some leftover for balancing the tubes

29.) Get needle, syringe and Kim-wipe tissue

30.) Add 6ml of 20% sucrose cushion to each tubes by following method:

- Pour the prepared sucrose cushion into beaker on ice
- Use syringe to draw up the cushion to ~7ml
- Tap out all the bubble and equip the needle onto the syringe
- Eject some sucrose solution out to get rid of bubble
- Put needle into bottom of the bottle
- Slowly inject the sucrose cushion

- Clean needle with Kim-wipe tissue after done injecting

31.) Balancing each tube (including cap) using transfer pipette to 0.01 g difference

- Always wipe out all ice and water before weighting
- Reweight all of the tubes again before load into the rotor

32.) Check the Ti-35 rotor for the O ring and the break disk at the bottom

- Break disk = white/black circular strip on the bottom

33.) Load all the tubes into the rotor

- Always wipe out all ice and water before loading

34.) Tighten the cover and make sure that it is hand-tight

35.) Turn off the vacuum and wait until the hissing sound is gone

- The vacuum display will display "---" sign

36.) Open the chamber up and equip the rotor inside

37.) Try to spin the rotor and check if the speed detector pick up the spinning

38.) Close the door and press vacuum

- The vacuum display will display chamber pressure

39.) Check all the setting (temp: 4^0C; time: 1 hr; speed: 28k rpm)

40.) Wait until vacuum display pressure of 150-200, then press start

- Wait and observe any sign of abnormality (sound or shaking) until the speed reaches the set rpm

41.) After 1 hour, press vacuum and wait until hissing sound is gone

42.) Sign and fill out the sheet for using the Ultra speed centrifuge

43.) Take out the rotor and turn off the machine

44.) Bring the rotor back to the lab and open up the cover

45.) Use forceps to take out the bottle

46.) Drain out all supernatant and set it upside down on paper towel

47.) Place the tube on ice and drain all the tubes before proceeding to next step

48.) Wrap forceps with Kim-wipe tissue and remove excessive liquid inside the tube

- Do not touch or disturb pellet

49.) Add 5ml of 0.1M Tris Cl pH7.4 buffer to each tube,

- Make sure the buffer cover the pellet

50.) Seal the opening of tube with paraflim (sealing it off at the shoulder of opening)

51.) Set tubes on ice slanting at the angle for the buffer cover the pellet

52.) Put mark the location of pellet

53.) Put it on the ice slanting and store it in refrigerator overnight

C. Preparation before Day 2:

1.) Chill JA-20 and Ti-70 rotors in refrigerator

D. Day 2:

1.) Set up a beaker with stirring magnet on ice

2.) Transfer all the solution from the tubes to the beaker using transfer pipette

- Rinse the pellet with the buffer inside the tube to dislodge the pellet
- Transfer everything including the pellet into the beaker

3.) Measure the resuspension

4.) Prepare to add Triton 100x for 2% final concentration

- Calculate the amount need (600 µl for 30ml)
- Microwave Triton 100x for 5-6 sec.
- Cut p1000 pipette tip with razor blade

5.) Add the Triton 100x drop wise while the solution is stirring using cut pipette tip

6.) Cover the beaker with paraflim

7.) Stirring gently for 2 hours on ice

- While waiting:
  - Set up Beckman J2-21 High speed centrifuge and set temperature to 4^{o}C
  - Chill GSA tube and 50ml blue cap tube
  - Prepare a 20% sucrose cushion with TE buffer (4+ ml for each Ti-35 polycarbonate tubes)
    - 20ml 1x TE buffer pH7.4 + 4g sucrose
  - Chill 2x Ti-70 polycarbonate tubes with red metal cap
    - Check the O ring under the cap
  - Set up Beckman L9-70M Ultra speed centrifuge
    - Temperature: 4^{o}C
    - Speed: 30000 rpm
    - Time: 2 hour
    - Press vacuum to cool the centrifuge

8.) After 2 hours, transfer all the solution to a GSA bottle on ice

9.) Weight out the tube to 0.1g difference by using transfer pipette

- Make sure to wipe bottle dry before weighting
- Always reweight the bottles to make sure the difference is within 0.1g
- Bring the GSA bottles in ice to the centrifuge room

10.) Equip JA-20 onto the centrifuge

- Check the cleanness of rotor

11.) Load GSA tubes into the rotor

- Always wipe the bottles clean of ice and water before loading

12.) Tighten the rotor cover (make sure it is hand-tight)

13.) On J2-21 high speed centrifuge:

- Set the time to 5 min
- Set the speed to 8200rpm
- Wait until the needle on the vacuum gauge go into green zone
- Press Start
- Wait and observe any sign of abnormality (sound or shaking) until the speed reaches the set rpm

14.) After centrifugation, transfer supernatant into the blue cap tube

- Avoid pouring the pellet out with supernatant

15.) Slowly transfer 15 ml using 10ml pipette into Ti-70 polycarbonate tube

- Take up and release 1ml then stop for 3 seconds

16.) Get needle, syringe and Kim-wipe tissue

17.) Adding 4 ml of 20% sucrose cushion to each tubes by following method:

- Pour the prepared sucrose cushion into beaker on ice

- Use syringe to draw up the cushion to ~5ml
- Tap out all the bubble and equip the needle onto the syringe
- Eject some sucrose solution out to get rid of bubble
- Put needle into bottom of the bottle
- Injecting the sucrose solution slowly
- Clean needle with Kim-wipe tissue after done injecting

18.) Fill the tube up to 7/8 to the top

- If supernatant ran out, 0.1M Tris Cl pH7.4 can be used to top up and balance

19.) Balance each tube (including cap) using transfer pipette to 0.01 g difference

- Always wipe out all ice and water before weighting
- Reweight all of the tubes to make sure after balancing all of them

20.) Check the rotor for the O ring and the break disk at the bottom

- Break disk = white/black circular strip on the bottom

21.) Load all the tubes into the rotor

- Always wipe out all ice and water before loading

22.) Tighten the cover and make sure that it is hand-tight

23.) Turn off the vacuum and wait until the hissing sound is gone

- The vacuum display will show "---" sign

24.) Open the chamber up and equip the rotor inside

25.) Try to spin the rotor and check if the speed detector pick up the spinning

26.) Close the door and press vacuum

- The vacuum display will display chamber pressure

27.) Check all the setting (temp: 4^0C; time: 2 hr; speed: 30k rpm)

28.) Wait until vacuum displays pressure of 150-200, then press start

- Wait and observe any sign of abnormality (sound or shaking) until the speed reaches the set rpm

29.) After 2 hours, press vacuum and wait until hissing sound is gone

30.) Sign and fill out the sheet for using the Ultra speed centrifuge

31.) Take out the rotor and turn off the machine

32.) Bring the rotor back to the lab and open up the cover

33.) Use forceps to take out the bottle

34.) Drain out all supernatant and set it upside down on paper towel

35.) Wrap forceps with Kim-wipe tissue and remove excessive liquid inside the tube

- Do not touch and disturb pellet

36.) Carefully rinse the wall of the bottle with dH2O

- Gently release water onto the shoulder of bottle and let it slide into the inside
- Wash one side at the time
- The last wall (side with the pellet), Let the water flow until it slightly touch a pellet for a quick second and quickly drain it out

37.) Clean the wall again with forceps wrapped with Kim-wipe tissue

38.) Add 60-120µl of 1x TE buffer pH7.4 to resuspend the pellet

- Make sure the buffer cover the pellet

39.) Seal the opening with paraflim (sealing it off at the shoulder of opening)

40.) Put mark the location of pellet

41.) Put it on the ice slanting and store it in refrigerator

E. Day 3:

1.) Chill 2x 1.5ml microcentrifuge tubes on ice
   - 1x for supernatant
   - 1x for pellet

2.) Turn on the table-top ace Spin Micro R Refrigerated centrifuge

3.) Flick the tube from Day 2 for 20 seconds and put it on ice for 5 minutes
   - Repeated the process until the pellet go into the solution
     - Recommended around 10 times

4.) Cut p200 pipette tip with razor blade

5.) Using the cut pipette to wash the place where pellet was with the buffer inside and transfer everything into 1.5ml microcentrifuge tube
   - If there are a lot of pellets, an additional TE buffer can be added to wash them out. However, this will decrease the virion concentration.

6.) Flick the tube gently for 10 seconds and put it on ice for 3 minutes
   - Repeated the process 5-6 times

7.) Get the rotor from the refrigerator and equip the rotor into the centrifuge

8.) Load the tubes into the rotor and tighten the cap (make sure it is hand-tight)

9.) Centrifuge 10000rpm for 1.5 minutes at 4^0C

10.) Transfer supernatant into another chilled 1.5ml microcentrifuge tube
   - For storage, add equal volume of glycerol (final: 50%) and store in -20^0C

Buffers used in Appendix A:

0.1M Tris-Cl, pH 7.4:

Dissolve 12.1 g of Tris Base in 900ml dH_2O

Adjust the pH to 7.4 with concentrated HCl

Top up to 1L with dH_2O and autoclave

1x TE buffer, pH7.4:

Final concentration: 10mM Tris, 1mM EDTA

Dissolve 1.21g of Tris Base in 900ml dH_2O

Add 2ml of 0.5M EDTA pH8.0

Adjust the pH to 7.4 with concentrated HCl

Top up to 1L with dH_2O and Autoclave

Table A-1. List of parameters and yield of Lettuce *chlorosis virus* (LCV) virion purification from infected plants

Experiment #	Procedure	Plant	Tissue	Extraction	Titron	Surcose	0.1M Tris	Titron	Surcose	1x TE	Resus.	Concentration	Total amount	Yield	Page #
			(g)	Buffer (pH)		pH (con.)	pH		pH (con.)	pH	(ul)	(ng/ul)	(ng)	(ng/g)	
9/13/07-1	LlYV	C. Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	N/A	0	0	16
9/27/07-1	LlYV	C. Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	415	49800	711.4285714	22
10/4/07-1	LlYV	C. Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	180	0	0	0	24
10/11/07-1	LlYV	N. Ben.	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	200	0	0	0	26
10/25/07-1	BYV	N. Ben.	30	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	140	191	26740	891.3333333	28
11/8/07-1	LlYV	N. Ben.	35	9	2%	9 (20%)	9	2%	9 (20%)	9	70	478	33460	956	31
			35	9	2%	9 (20%)	9	2%	9 (20%)	7.4	70	286	20020	572	
11/22/07-1	LlYV	N. Ben.	35	4	2%	4 (20%)	4	2%	4 (20%)	4	100	0	0	0	36
			35	4	2%	4 (20%)	4	2%	4 (20%)	7.4	100	0	0	0	
12/19/07-1	LlYV	N. Ben.	35	7.4	2%	7.4 (17%)	7.4	2%	7.4 (17%)	7.4	80	251	20080	573.7142857	40
			35	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	80	240	19200	548.5714286	
1/9/08-1	LlYV	N. Ben.	35	10	2%	10 (20%)	10	2%	10 (20%)	10	100	372	37200	1062.857143	45
			35	10	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	100	375	37500	1071.428571	
1/23/08-1	LlYV	N. Ben.	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	160	323	51680	738.2857143	54
2/6/08-1	LlYV	N. Ben.	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	160	0	0	0	61
2/15/08-1	LlYV	N. Ben.	75	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	160	338	54080	721.0666667	66
2/21/08-1	LlYV	N. Ben.	28	7.4	4%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	75	277	20775	741.9642857	68
			28	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	75	364	27300	975	
3/5/08-1	LlYV	N. Ben.	64	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	180	834	150120	2345.625	73
3/19/08-1	LlYV	C. Murale	37	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	100	2350	235000	6351.351351	79
3/27/08-1	LlYV	C. Murale	35	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	100	1130	113000	3228.571429	85
			35	7.4	4%	7.4 (20%)	7.4	4%	7.4 (20%)	7.4	100	889	88900	2540	
5/22/08-1	LlYV	C.Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	150	0	0	0	125
6/5/08-1	LlYV	C.Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	13160	1579200	22560	132
7/3/08-1	LlYV	C.Murale	120	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	240	3000	720000	6000	143
8/28/08-1	LlYV	C.Murale	70	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	461	55320	790.2857143	191
10/9/08-1	LlYV	C.Murale	76	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	2000	240000	3157.894737	240
10/30/08-1	LlYV	C.Murale	66	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	1500	180000	2727.272727	267
11/6/08-1	LlYV	C.Murale	49	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	24700	2964000	60489.79592	273
4/8/09-1	LlYV	C.Murale	60	7.4	2%	7.4 (20%)	7.4	2%	7.4 (20%)	7.4	120	864	103680	1728	406

Appendix B. Localization of *Lettuce infectious yellows virus* (LIYV) within whitefly (*Bemisia tabaci*) foregut by using biarsenical labeling of capsid proteins encoding a Tetracysteine (TC) motif

Summary:

Overall goal of this project was to develop a marker on LIYV virion capsid protein that will allow us to localize the virus within the whitefly foregut. The chosen marker was a Tetracysteine motif or TC-Tag consisting of 6 amino acids (Cys-Cys-Pro-Gly-Cys-Cys) that can be bound by Flourescein Arsenical Hairpin or FlAsH reagent which produces fluorescence upon excitation by blue light. Five different locations across sequences of LIYV capsid proteins (two in major capsid protein [CP] and three in minor capsid protein [CPm]) in infectious cDNA clone of LIYV RNA 2, pR6, were chosen to be substituted by sequences encoding TC motif, resulting in five mutants TC-1 to TC-5 (Fig. B-1). TC-1 (LIYV RNA 2 was engineered to encode TC motif at the N-terminus of the CP), TC-4 (LIYV RNA 2 was engineered to encode TC motif at the N-terminus of the CPm), and TC-5 (LIYV RNA 2 was engineered to encode TC motif in the middle of the CPm) mutants were constructed by SS Cao, while TC-2 (LIYV RNA 2 was engineered to encode TC motif at the C-terminus of the CP) and TC-3 (LIYV RNA 2 was engineered to encode TC motif at the N-terminus of the CPm) mutants were constructed by James Ng. However, TC-1 mutants were not constructed correctly.

Nicotiana tabacum var. Xanthi protoplasts were inoculated with *in vitro* transcripts of LIYV RNA 1 (2µg) and LIYV RNA 2 or TC mutants (6µg). Virion-like

particles (VLP) were purified from inoculated protoplasts after 72 hours incubation, and then were subjected to 3 different analyses: SDS-PAGE in-gel fluorescence analysis, immunoblot analysis, and immunogold labeling transmission electron micrograph (TEM) analysis. For the SDS-PAGE in-gel fluorescence analysis, 10µl of virion-like particle preparation was boiled for 5 min in 5x sample buffer, cooled to room temperature, and incubated with 200µM of FlAsH reagent for 20-30 minutes in the dark. The reaction was then subjected to SDS-PAGE, and the gel then was subjected to fluorescent imaging (excitation and emission at 488nm and 520nm, respectively). For immunoblot analysis, the preparation was separated by SDS-PAGE, transferred onto nitrocellulose membrane, and detected by LIYV virion IgG. For immunogold labeling TEM analysis, the preparation was sent to Tongyan Tian.

Purified VLP preparation from TC-2 inoculated protoplast was subjected to 3 different analyses mentioned above. In the SDS-PAGE in-gel fluorescence imaging, a fluorescent protein with the molecular mass corresponding those of CP (approximately 28kDa) was detected in preparation of TC-2 inoculated protoplast and the same protein was also identified as CP in immunoblot analysis (Fig. B-2A and B, lanes 2). Similarly no fluorescent protein was detected in preparation of protoplast inoculated with wild type LIYV RNAs 1 and 2, but CP was detected in immunoblot analysis (Fig. B-2A and B, lanes 3). Immunogold labeling TEM of both TC-2 and WT preparation showed that both CP and CPm were detected on both VLPs (Fig. B-2C).

Fluorescent proteins with the molecular mass corresponding those of CPm (approximately 52kDa) were detected in VLP preparation of TC-3, TC-4, and TC-5

inoculated protoplasts (Figs. B-3, B-4, B-5 A). However, the intensity of fluorescent protein from TC-3, TC-4, TC-5 preparation was significantly lower than those of TC-2 due to lower abundance of CPm relative to CP as CPm encapsidates only the tip of the virion while CP encapsidates almost the entire length of the virion. In immunogold labeling TEM analysis, only CP and no CPm were detected on VLP of TC-3, TC-4, and TC-5 (Figs. B-3, B-4, B-5 C).

An attempt had been made to visualize labeled VLP within the whitefly, but the experiments had not been successful due to low yield of VLP concentration purified from inoculated protoplasts. An availability of Ti plasmids of LIYV genomic RNAs allowed us to increase the yield of virion concentration by infecting plant through agro-infiltration system. Sequences encoding TC motif from mutants TC-2, TC-3, and TC-5 were substituted sequences in Ti plasmid of LIYV RNA 2 by digesting NheI restriction enzymes of TC mutants and subsequently subcloning into similarly digested Ti plasmid of LIYV RNA 2. Ti plasmids of LIYV RNA 1 WT and RNA 2 TC mutants (TC-2, TC-3, and TC-5) were agro-inflitrated into *Nicotiana benthamiana.* After infection, virions were purified and subjected to SDS-PAGE in-gel fluorescence analysis, immunoblot analysis, and transmission analysis.

Four experiments of agro-inoculation of TC-2 mutants were performed with LIYV WT as positive control. However, no system infection of TC-2 agro-infiltrated *N. benthamiana* was detected (Table B-1). In addition, no presence of CP was detected by immunoblot of quick and dirty virion preparation from agro-infiltrated leaves (4 weeks old) with LIYV virion IgG (Fig. B-6). The failure in systemic infection of TC-2 agro-

infiltrated NB can be possibly due to the mutation in the CP region so an attempt has also been made to rescue TC-2 using heterologous CP expressed transgenic *N. benthamiana* plants. However, no systemic infection was detected (Table B-2).

For TC-3 and TC-5, three and two agro-inoculation experiments were performed, respectively. Both TC-3 and TC-5 mutants caused systemic infection (Table B-3 and B-4). Virion was purified from infected TC-3 agro-inflitrated *N. benthamiana* and was then subjected to SDS-PAGE in-gel fluorescence analysis. Fluorescent CPm was detected, but at low intensity comparing to VLP preparation from TC-2 inoculated protoplasts. In addition, high background was also detected as the other proteins purified along with the TC-3 virion were also labeled by FlAsH reagent. Attempts have also been made to localize FLAsH-labeled TC3 purified virion in whitefly and transmit the TC-3 purified virion into plant via membrane-feeding. However, no fluorescence was detected within whitefly foregut fed by both FLAsH-labeled TC3 purified virion and FLAsH-labeled LIYV WT purified virion. In addition, TC-3 and LIYV WT virion failed to be transmitted by whitefly.

In conclusion, VLPs from TC-2 mutant labeled very strongly and efficiently in the in-gel fluorescence analysis, but infected protoplast did not produce VLPs with both quality and quantity that are sufficient for localization within whitefly. In addition, TC-2 mutant did not systemically infect *N. benthamiana* via agro-inoculation. VLPs from mutants that express TC-motif in the CPm (TC-3, TC-4, and TC5) labeled very poorly or not at all in the in-gel can fluorescence analyses, but mutants that express TC-motif in the CPm (TC-3 and TC5) systemically infect *N.* benthamiana. However, purified TC-3 and TC-5 virion

still labeled very poorly in the in-gel analysis and was not sufficiently labeled to visualize in whitefly.

Result:

pJW168-TC2 #19	4/9/09	pg. 411
pJW168-TC3 #4	5/15/09	pg. 451
pJW168-TC5	7/17/09	pg. 496

Figure B-1. Genomic organization of LIYV RNA 2 and five different locations across sequences of LIYV capsid proteins that were chosen to be substituted by sequences encoding TC motif.

Figure B-1

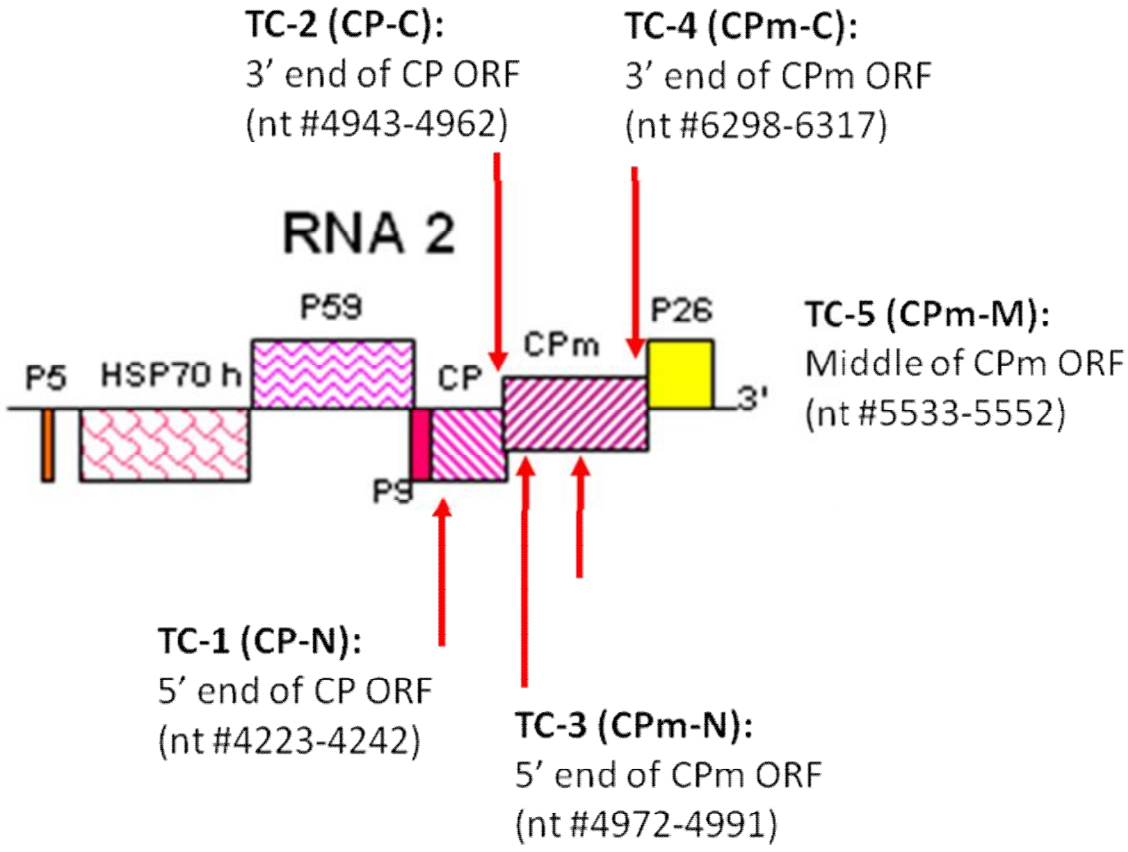
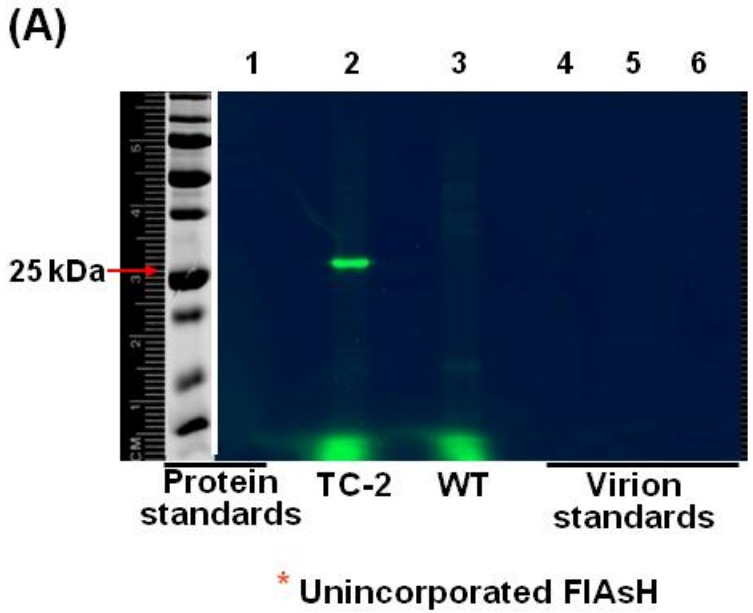
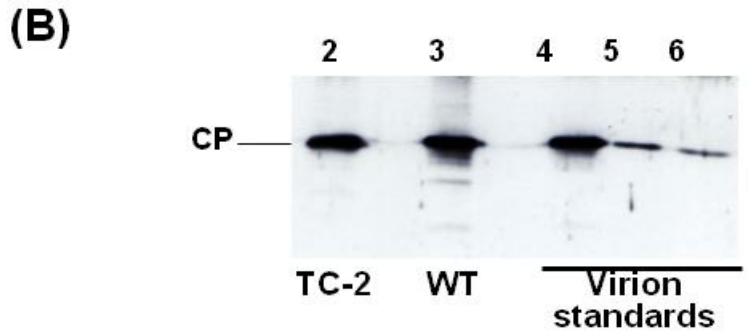
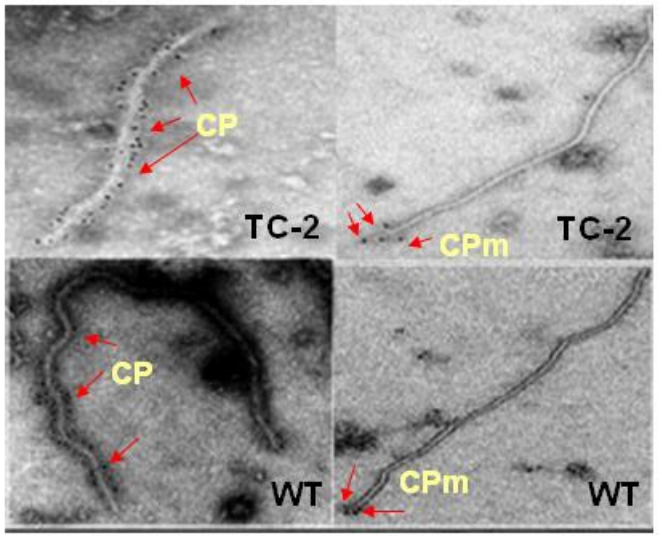

TC-2 (CP-C):
3' end of CP ORF
(nt #4943-4962)
TC-4 (CPm-C):
3' end of CPm ORF
(nt #6298-6317)
RNA 2
P59
P26
CPm
P5
HSP70 h
CP
3'
P9
TC-5 (CPm-M):
Middle of CPm ORF
(nt #5533-5552)
TC-1 (CP-N):
5' end of CP ORF
(nt #4223-4242)
TC-3 (CPm-N):
5' end of CPm ORF
(nt #4972-4991)

Figure B-2. Analysis of TC-2 virion-like particles purified from tobacco protoplasts. (A) SDS-PAGE in-gel fluorescence analysis was performed on FlAsH-labeled TC-2 virion-like particles (lane 2) along with FlAsH-labeled LIYV WT virion-like particle (lane 3) and LIYV standards (lane 4, 50ng; lane 5, 10ng; lane 6, 5ng). Low molecular weight prestained protein standards was also included as a reference in lane 1. Unincorporated/excess FlAsH is indicates by asterisks at bottom of lanes 2 and 3. (B) Immunoblot analysis was also performed on the same sample mentioned above with LIYV virion IgG. The position of a 25kDa protein standard and LIYV CP are indicated to the left of the gels. (C) Immunogold labeling TEM analysis of CP and CPm of TC-2 and LIYV virion-like particles.

Figure B-2



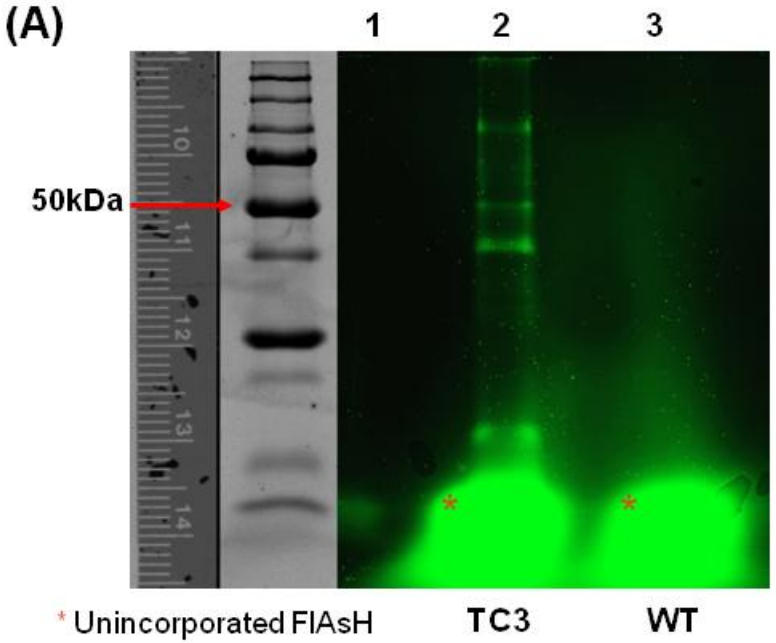


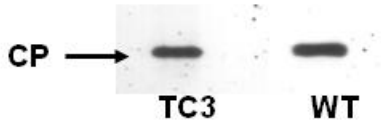


Courtesy of Tongyan Tian for Immunogold labeling

Figure B-3. Analysis of TC-3 virion-like particles purified from tobacco protoplasts (A) SDS-PAGE in-gel fluorescence analysis was performed on FlAsH-labeled TC-3 virion-like particles (lane 2) along with FlAsH-labeled LIYV WT virion-like particle (lane 3). Low molecular weight prestained protein standards was also included as a reference in lane 1. Unincorporated/excess FlAsH is indicates by asterisks at bottom of lanes 2 and 3. (B) Immunoblot analysis was also performed on the same sample mentioned above with LIYV virion IgG. The position of a 50kDa protein standard and LIYV CP are indicated to the left of the gels. (C) Immunogold labeling TEM analysis of CP and CPm of TC-3 and LIYV virion-like particles.

Figure B-3


(A)
1
2
3
50kDa
* Unincorporated FlAsH
TC3
WT


(B)
CP
TC3
WT

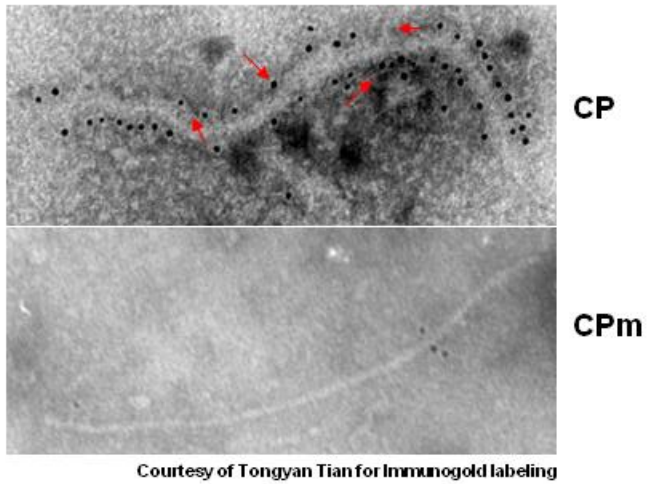
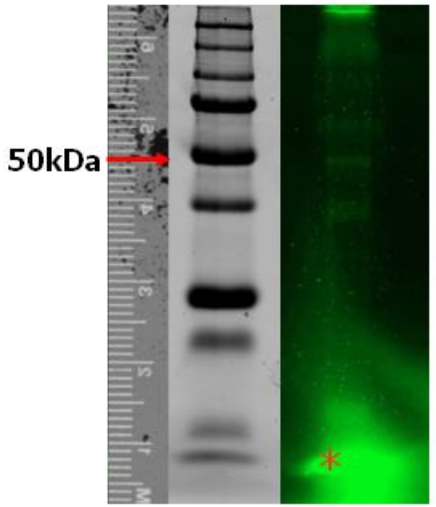

(C)
CP
CPm
Courtesy of Tongyan Tian for Immunogold labeling

Figure B-4. Analysis of TC-4 virion-like particles purified from tobacco protoplasts. (A) SDS-PAGE in-gel fluorescence analysis was performed on FlAsH-labeled TC-4 virion-like particles. Low molecular weight prestained protein standards was also included as a reference. Unincorporated/excess FlAsH is indicates by asterisks. (B) Immunoblot analysis was also performed on the same sample mentioned above with LIYV virion IgG. The position of a 50kDa protein standard and LIYV CP are indicated to the left of the gels. (C) Immunogold labeling TEM analysis of CP and CPm of TC-4 and LIYV virion-like particles.

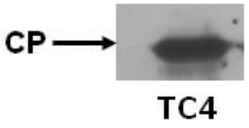
Figure B-4

(A)

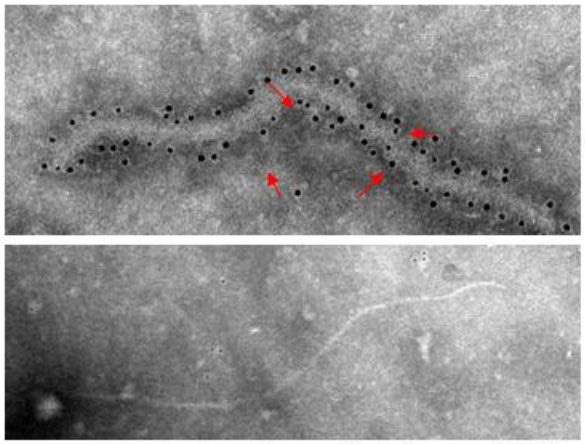


* Unincorporated FlAsH

(B)



(C)

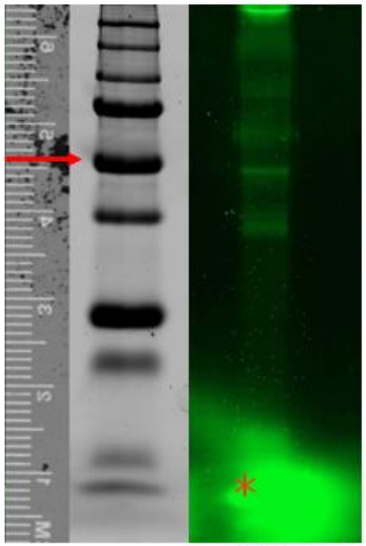


Courtesy of Tongyan Tian for Immunogold labeling

Figure B-5. Analysis of TC-5 virion-like particles purified from tobacco protoplasts. (A) SDS-PAGE in-gel fluorescence analysis was performed on FlAsH-labeled TC-5 virion-like particles. Low molecular weight prestained protein standards was also included as a reference. Unincorporated/excess FlAsH is indicates by asterisks. (B) Immunoblot analysis was also performed on the same sample mentioned above with LIYV virion IgG. The position of a 50kDa protein standard and LIYV CP are indicated to the left of the gels. (C) Immunogold labeling TEM analysis of CP and CPm of TC-5 and LIYV virion-like particles.

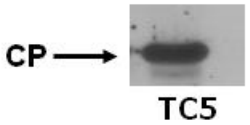
Figure B-5

(A)

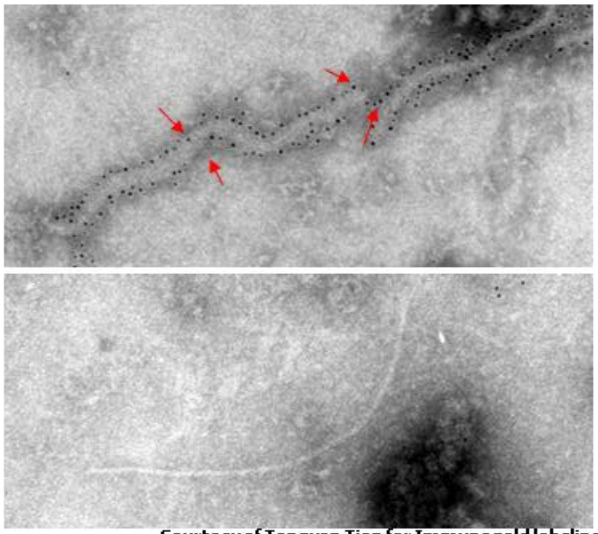


* Unincorporated FlAsH

(B)



(C)



Courtesy of Tongyan Tian for Immunogold labeling

Figure B-6. Immunoblot analysis of purified preparations from LIYV WT and TC-2 agro-inoculated plants with LIYV virion IgG. The position of 37kDa and 25kDa protein standard and LIYV CP are indicated to the right and left of the gels, respectively.

Figure B-6

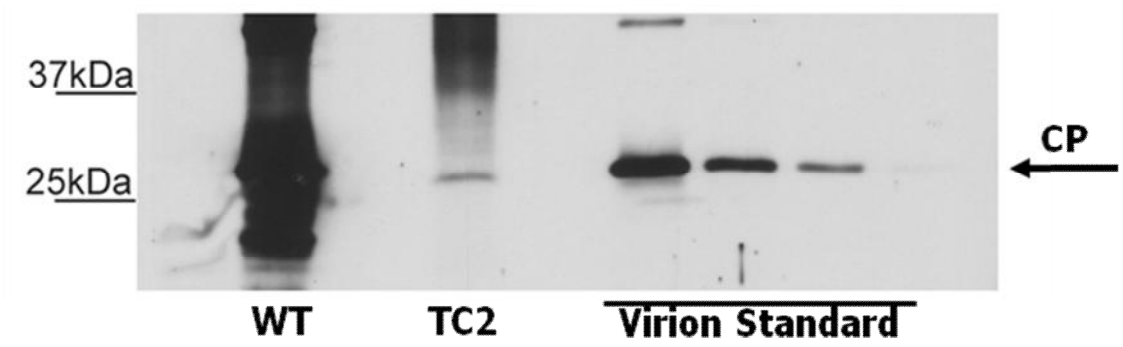
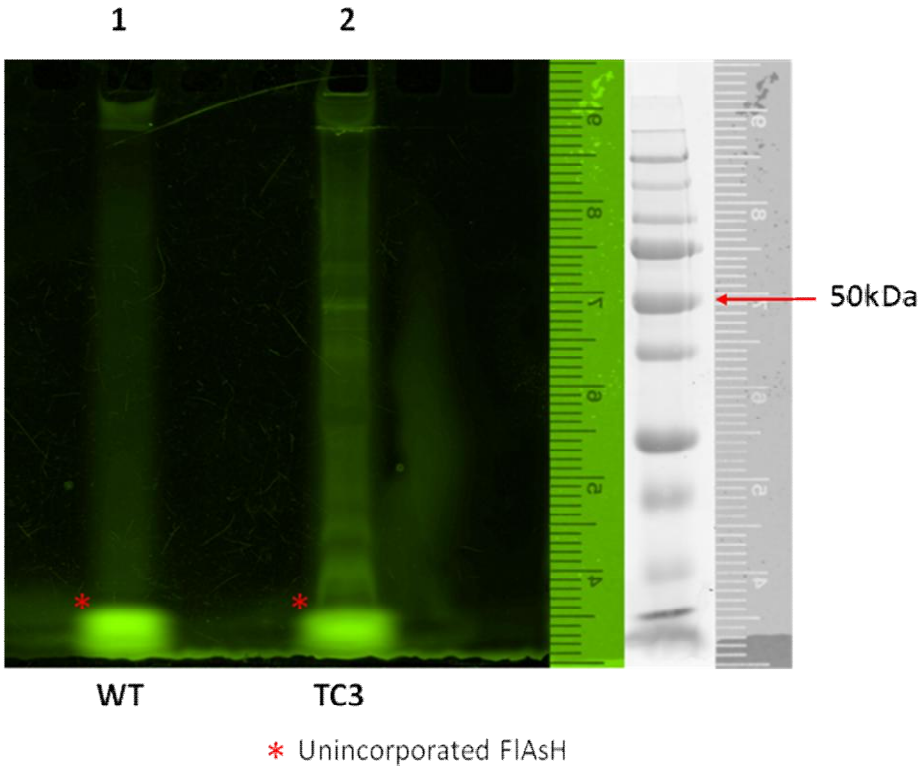


Figure B-7. Analysis of virion purified from TC-3 agro-infiltrated plants. (A) SDS-PAGE in-gel fluorescence analysis was performed on FlAsH-labeled TC-3 virion and FlAsH-labeled LIYV WT virion. Low molecular weight prestained protein standards was also included as a reference. Unincorporated/excess FlAsH is indicates by asterisks. The position of a 50kDa protein standard is indicated to the right of the gels. (B) The same sample mentioned above were also subjected to collodial blue-stained SDS-PAGE. The position of 25kDa and 37kDa protein standard and LIYV CP are indicated to the left and right of the gel, respectively.

Figure B-7

(A)


1
2
50kDa
WT
TC3
* Unincorporated FlAsH

(B)

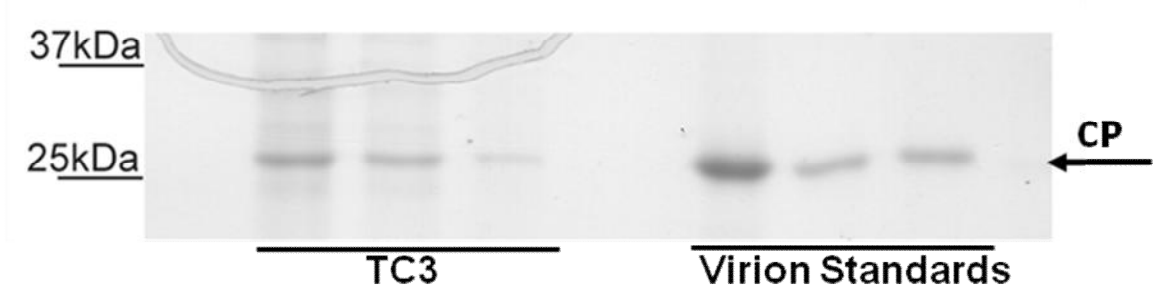

37kDa
25kDa
CP
TC3
Virion Standards

Table B-1. Infection rate of TC-2 agro-infiltrated *N. benthamiana* plants and LIYV WT agro-infiltrated *N. benthamiana* plants.

Experiment	#1	#2	#3	#4
TC-2 mutant	0/32	0/32	0/32	0/32
LIYV WT	20/32	16/32	16/32	16/32

(# of infected plants/ # of agro-infiltrated plant)

Table B-2. Infection rate of TC-2 agro-infiltrated heterologous CP expressed transgenic *N. benthamiana* plants and LIYV 5b and 5bm-1 agro-infiltrated *N. benthamiana* plants.

Experiment	#1	#2
TC-2 mutant	0/32	0/32
5b mutant*	14/32	13/32
5b-m1 mutant*	13/32	14/32

(# of infected plants/ # of agro-infiltrated plant)

Table B-3. Infection rate of TC-3 agro-infiltrated *N. benthamiana* plants and LIYV WT agro-infiltrated *N. benthamiana* plants.

Experiment	#1	#2	#3
TC-3 mutant	16/32	14/32	15/32
LIYV WT	13/32	13/32	10/32

(# of infected plants/ # of agro-infiltrated plant)

Table B-4. Infection rate of TC-5 agro-infiltrated *N. benthamiana* plants and LIYV WT agro-infiltrated *N. benthamiana* plants.

Experiment	#1	#2
TC-5 mutant	3/32	6/32
LIYV WT	6/32	10/32

(# of infected plants/ # of agro-infiltrated plant)

Appendix C. Investigation of the relationship between the stem-loop (SL) in 3' NCR of LCV RNA 2 and LCV RNA-dependent RNA polymerase

Summary:

The result from chapter 3 has demonstrated the importance of the 3' NCR LCV RNA 2 SL in regulating minus-strand RNA synthesis as any disruption of the overall structure of SL resulted in abolishment of minus-strand RNA 2 synthesis. However, the question remains as to how this particular SL is specifically involved in the process of synthesizing minus-strand. One hypothesis is that the predicted SL promotes minus-strand RNA synthesis by directly interacting with the viral RdRp. To examine the relationship between LCV RNA 2 SL and the viral RdRp, the recombinant LCV RdRp was produced from an *E. coli* expression system and subjected to *in vitro* binding assay and electrophoretic mobility shift assay (EMSA) with biotin-labeled transcripts of LCV RNA 2 SL.

First, LCV ORF1b sequence, which encodes RdRp, was cloned into a pRSET A expression vector through the use of PCR and restriction digestion. The resulting expression plasmid was transformed into *E. coli* BL21(DE3)pLysS and pilot expression was performed to determine the optimal expression condition (final concentration of 0.5mM IPTG and expressing for 3 hours at 37^0C after IPTG induction). The expressed protein containing an N-terminal polyhistidine tag was purified using Ni-NTA resin packed column and eluted with a standard elution buffer (250 mM immidazole pH 8, 50 mM NaH_2PO_4, and 300 mM NaCl). The buffer of eluted protein was changed from standard elution buffer to buffer E (20 mM HEPES-KOH, pH 7.5, 60 mM KCl, 12.5 mM

$MgCl_2$, 0.1 mM EDTA, 17% (v/v) glycerol, and 2 mM DTT) as the purified protein needed to be in suitable buffer for *in vitro* binding reaction. The protein was next be concentrated into a smaller volume using a concentrator spin column and the concentration of concentrated protein was quantified by comparing to known amount of BSA standards in SDS-PAGE gels (Fig. C-1).

The template for synthesizing *in vitro* transcripts of 3' NCR LCV RNA 2 SL (39 nucleotides) was constructed by annealing two oligonucleotide primers and then TA-cloning into pGEM-T Easy vector. In addition, a template for synthesizing transcript of GFP with a size of 39-nucleotides, which will serve as negative control, was also constructed. The resulting transcripts from both templates were labeled with biotinylated cytidine (bis)phosphate at the 3'-termini by T4 RNA ligase according to to Pierce RNA 3' End Biotinylation kit (Catalog #20160)**.**

The biotin-labeled transcript of LCV RNA 2 SL was incubated with the recombinant LCV RdRp according to Shikanai and Okuda (2011). In addition, the biotin-labeled transcripts of GFP and IRE control RNA (from Thermo Scientific LightShift Chemiluminescent RNA EMSA kit) were also subjected to the same treatment with the recombinant LCV RdRp as the negative control reaction. Furthermore, BSA was also used as the protein negative control when incubating with both the LCV RNA 2 SL and GFP transcripts. The reactions were then separated on non-denaturing polyacrylamide gel and were transferred onto nylon membrane. The shifting of transcript migration resulting from RNA-protein interaction was detected from biotin using the Thermo Scientific Chemiluminescent Nucleic Acid Detection Module Kit.

Electrophoretic mobility shift assay of binding reactions of containing recombinant LCV RdRp at various concentrations (0, 0.1, 0.15, and 0.25μg) and biotinylated transcripts of LCV RNA 2 SL, GFP, and IRE control RNA (1nM, 1nM, 0.1nM, respectively) showed a clear retardation of GFP and IRE control RNA transcripts, but no retardation of LCV RNA 2 SL transcripts, suggesting that rLCV RdRp binds to both GFP and IRE control RNA transcripts, but did not bind to RNA 2 SL transcripts (Fig. C-2 and C-3). Additional EMSA of binding reaction with an increased amount of rLCV RdRp (0.5μg) and biotin-labeled transcripts of LCV RNA 2 SL and GFP showed a smeared shift of transcript of LCV RNA 2 SL, but also a clear shift for GFP transcripts (Fig. C-4). In addition, the BSA of same amount (500ng) did not cause any retardation of LCV RNA 2 SL and GFP transcripts (Fig. C-4), suggesting that a higher amount of rLCV RdRp (possibly included other protein purified along rLCV RdRp) was the reason for the smearing of LCV RNA 2 SL transcripts. In conclusion, the result from EMSA indicated that LCV RNA 2 SL did not directly interacted with the viral LCV RdRp

Procedure:

Construction of cDNA clones of LCV SL and GFP: See Notebook #16 pg. 1257

Due to the short length of 39 nucleotides of LCV RNA 2 SL sequences, instead of PCR amplification of 60 nucleotides product, two single-stranded complimentary oligonucleotide primers, RNA2SL+ and RNA2SL-, were annealed together into dsDNA with 3'-terminal adenine overhang and directly TA-cloned into the pGEMT-Easy vector. The primers contain a bacteriophage T3 promoter immediately upstream of LCV RNA 2 SL sequences (nucleotide positions 8495-8533) followed by NcoI restriction digestion site (Table C-1). For the negative control RNA transcript, another set of oligonucleotide primers, GFPSL+ and GFPSL-, was designed and constructed in a same manner as those of LCV RNA 2 SL except the LCV RNA 2 SL sequences are replaced by 39 nucleotides of GFP sequences from pM5gfp.1.

1.) 1µl of 10µM or 1mM of each complementary oligonucleotides primers (RNA2SL+ with RNA2SL-; GFPSL+ with GFPSL-) was added into PCR tube with 8µl dH_2O (total volume of 10µl with final concentration of 100µM or 1µM)

2.) Heat the reaction to 95^0C for 3 min and let the reaction cool to room temperature for 60 min

3.) Add 4µl of the reaction to pGEMT-Easy ligation reaction:

Annealing reaction	4µl
2x ligation buffer	5µl
pGEMT-Easy	1µl

(Save 1µl for pre-ligation before adding T4 ligase)

T4 ligase 1µl

4.) Incubate ligation reaction at 4^0C overnight

5.) Use 4µl ligation reaction to transform plasmid into DH5α

6.) Miniprep and sequence the plasmid

Result:

R2SL 1mM #1	6/4/12	pg. 1265
GFP 1mM #3, 4; 10µM #7, 8, 9, 10	6/4/12	pg. 1265

In vitro transcription of LCV RNA 2 SL and GFP:

Because the LCV RNA 2 SL and GFP templates are very short inserts (39 nucleotides) in a much larger pGEMT-Easy vector (3.0kb), no transcript was produced after a standard *in vitro* transcription reaction when templates were simply linearized with NcoI restriction enzyme. In order to produce transcripts using a smaller DNA fragment, PCR was performed using R2SL as a template with primers LCV-164-CM and LCV-223-CM. The product was recovered by Qiagen PCR purification kit, digested with NcoI restriction enzyme, and gel-purified. 2pmol of gel-purified product (368ng of 60bp DNA) was used in *in vitro* transcription except the reaction was incubated at 37^0C for 6 hours instead of 2 hours. Transcript was produced, but the recovered transcript ran with two different sizes when the product was subjected to denatured 7M Urea/ 15% PAGE gel.

In order to obtain RNA product with single band in denatured PAGE gel, PCR products with exact 3'-termini was used to bypass the restriction enzyme digestion step. PCR was performed using primers LCV-223-CM and R2SL+ with RNA2SL templates, and primers pGEMT and GFP+ with GFP templates, and then were recovered by gel purification. 2pmol of recovered products (364ng of 300bp DNA) were used in *in vitro* transcription reaction except the reaction was incubated at 37^0C for 6 hours, treated with DNaseI, extracted with phenol: chloroform, and recovered by ethanol precipitation (instead of LiCl precipitation as LiCl precipitation does not efficiently precipitate RNA smaller than 300 nucleotides according to Ambion mMESSAGEe mMACHINE T3 manual) . The product was analyzed by denatured 7M Urea/ 15% PAGE gel.

Result:

In vitro transcripts of R2SL and GFP 7/5/12 pg.1281

A. PCR: Notebook #17 pg. 1281

dH_2O	33µl		
5x Herculase Buffer	10µl		
10mM dNTP	1µl		
10µM Forward primer	2µl	RNA2SL: R2SL+; GFP: GFP+	
10µM Reverse primer	2µl	RNA2SL: LCV-223-CM; GFP: pGEMT	
Herculase II Polymerase	1µl		
Plasmid (10ng)	1µl	RNA2SL: R2SL 1mM #1	6/4/12
		GFP: GFP 10mM #3	6/4/12

94^0C	2min	

30x cycles		
94^0C	45sec	
T_m-5^0C	45sec	RNA2SL: 53.8^0C; GFP: 45.9^0C
72^0C	30sec	

72^0C	10min	
4^0C	∞	

B. **Gel-purification**: Elute with 13µl Buffer EB

C. **In vitro transcription and ethanol precipitation:** According to Ambion mMESSAGEe mMACHINE T3 manual (also see Notebook #17 pg. 1281)

1.) Set up the reaction:

Linearized plasmid	2pmol (368ng of 60bp DNA)
2x NTP/CAP mix	10µl
10x reaction mix	2µl
T3 enzyme	2µl
Nuclease-free dH_2O	top up to 20µl

2.) Incubate at 37^0C for 6 hours

3.) Add 1µl DNaseI and incubate at 37^0C for 15 min

4.) Add 115µl dH_2O, 15µl Ammonium acetate solution, and 150µl Phenol: chloroform

5.) Vortex 30 sec and centrifuge 13krpm at 4^0C for 3 min

6.) Collect 145µl upper phase and add 145µl chloroform

7.) Vortex 30 sec and centrifuge 13krpm at 4^0C for 3 min

8.) Collect 140µl upper phase

9.) Add 280µl absolute EtOH (2x) and 14µl 3M NaOAc (pH 5.2) (1/10x)

10.) Incubate at -20^0C overnight

11.) Centrifuge 13krpm at 4^0C for 20 min

12.) Remove supernatant

13.) Add 700µl 70% EtOH and centrifuge 13krpm at 4^0C for 10 min

14.) Remove wash, air-dry and resuspend with 15µl nuclease-free dH_2O

D. Analyze transcripts on denaturing 7M/15% PAGE gel: According to Kenji's small RNA PAGE protocol

1.) Prepare 7M Urea/ 15% acrylamide solution: for 2x 1mm thick gels

Urea	5.04g
30% acrylamide, 0.8% bis	6ml
5x TBE	1.2ml
dH_2O	1.2ml (top up to 12ml)

Dissolving urea by heating to 60^0C

10% Ammonium persulfate	72µl
TEMED	5.6µl

2.) Transfer the solution into the gel cassettes, insert combs, and wait for 30min

3.) Remove the comb and wash the wells with 0.5x TBE 2-3 times

4.) Pre-run the gel in 500ml of 0.5x TBE at 150V for 30min

- Only fill the center reservoir with 0.5x TBE; not the whole tank

5.) Prepare the RNA sample:

- Xµl of RNA + 4µl formamide (40% final) + top up with DEPC- dH_2O to 10µl
- Incubate the sample at 65^0C for 5 min and immediately put on ice for 1+min
- Add 1µl 10x RNA loading dye

6.) Wash the wells against with 0.5x TBE before loading RNA sample

7.) Run at 150V for 1hr 20min

- Xylene cyanol and bromophenol blue will migrate corresponding to 60 and 15bp DNAs, respectively.

8.) Stain the gel in Ethidium Bromide solution for 30min and visualize

Biotinylation of LCV RNA 2 SL and GFP transcripts:

In order to detect the shift of the LCV RNA 2 SL transcript when subjecting to binding assay with LCV RdRp protein, the LCV RNA 2 SL and GFP control transcripts were labeled with biotin at 3' end, and then the biotin labeling efficiency were determined according to Pierce RNA 3' End Biotinylation kit (Catalog #20160).

Result:

Biotinylated transcripts of R2SL and GFP 7/10/12 pg.1286

A. Biotin labeling reaction: According to Pierce RNA 3' End Biotinylation kit (Catalog #20160)

1.) Thaw all components on ice except thaw DMSO at room temperature and warm 30% PEG at 37^0C for 5-10 min until the solution is fluid

2.) Heat 50pmol of RNA (600ng for 39bp RNA) in 5 µl volume with 25% DMSO at 85^0C for 5 min and put immediately on ice

- Heating in presence of 25% DMSO might increase efficiency for RNA with significant secondary structure.

3.) Prepare biotin labeling reaction:

Nuclease-free dH_2O	3µl
10X RNA ligase reaction buffer	3µl
RNase inhibitor	1µl
50 pmol RNA	5µl

Biotinylated Cytidine (Bis) Phosphate	1µl
T4 RNA Ligase	2µl
30% PEG	15µl

- Add components in order and 30% PEG should be carefully added last using a new pipette tip to mix the ligation reaction after the PEG addition

4.) Incubate 16 ^{0}C for 2 hrs

5.) Add 70µl nuclease-free dH_2O and then add 100µl chloroform

6.) Vortex 30 sec and centrifuge 13krpm at 4^{0}C for 3 min

7.) Collect top aqueous phase (approx. 90-95µl) and transfer into new tube

8.) Add 10µl of 5M NaCl, 1µl of glycogen, and 300µl ice-cold 100% ethanol

9.) Incubate -20 ^{0}C overnight

10.) Centrifuge 13krpm at 4^{0}C for 20 min

11.) Remove the supernatant and add 300µl of ice cold 70% ethanol

12.) Centrifuge 13krpm at 4^{0}C for 10 min

13.) Remove the wash and air dry the pellet

14.) Resuspend with 20µl nuclease-free dH_2O

15.) Aliquot into individual tube and store -80 ^{0}C

B. Dot blotting for determining labeling efficiency: According to Pierce RNA 3' End Biotinylation kit (Catalog #20160)

1.) Dilute the biotinylated IRE RNA control 25-fold to make 5 nM stock solution

- 2µl of IRE RNA control + 48µl dH_2O

2.) Prepare the standard from the stock according to table:

Biotin %	100	75	50	25	0
Final concentration (Stupina et al.)	1	0.75	0.5	0.25	0
Biotinylated IRE RNA control (µl)	12	9	6	3	0
dH_2O (µl)	48	51	54	57	60

3.) Prepare biotinylated RNA sample:

- Prepare stock: 1µl sample RNA + 249 µl water = 250µl of A (10 nM)
- Prepare a series of 2x-fold dilution (4x times)
    i. 25µl from A + 25µl water = 50µl of B (5 nM)
    ii. 25µl from B + 25µl water = 50µl of C (2.5 nM)
    iii. 25µl from C + 25µl water = 50µl of D (1.25 nM)
    iv. 25µl from D + 25µl water = 50µl of E (0.625 nM)

4.) Spot 2µl of each dilution of standard and RNA sample onto nylon membrane

5.) UV crosslink with optimal setting at Rao's lab

6.) Processing the blot according to LightShift Chemiluminescent RNA EMSA kit

Construction of LCV ORF1b expression vector:

For the construction of LCV ORF1a/1b in expression vector, PCR was performed using pCM1 as a template with oligonucleotide primers LCV-243-CM and LCV-244-CM. The resulting PCR product, containing a XhoI restriction site upstream of the full length sequences of ORF1a/1b without the first ATG codon and immediately followed by NcoI restriction site, was gel-purified, TA cloned into pGEM-T Easy vector, and transformed into *E. coli* DH5α. After scale up the plasmid and sequence confirmation, the LCV ORF1a/1b sequence was cloned into pRSET A expression vector by digestion with XhoI and NcoI restriction enzymes. The resulting plasmid, pRSET + ORF1a/1b, was transformed into both *E. coli* DH5α for storage and *E. coli* BL21(DE3)pLysS for expression. The pilot expression was performed to determine optimal expression condition. However, the expressed recombinant ORF1a/1b protein with N-terminal poly Histidine contains P-Pro, which cleaves itself off expressed recombinant ORF1a/1b protein. This cleavage by P-Pro also removed the N-terminal poly Histidine, which is needed for protein purification step. So another two vectors for expressing recombinant LCV ORF1a without P-Pro (pORF1a without P-Pro + pRSET) and LCV ORF1b proteins (pORF1b + pRSET) were constructed in the same manner as those of pORF1a/1b + pRSET except the oligonucleotide primers LCV-249-CM with LCV-244-CM and LCV-275-CM with LCV-276-CM were used for pORF1b + pRSET and pORF1a without P-Pro + pRSET, respectively.

Result:

pRSET + ORF1a/1b #7	1/8/12	pg. 1169
pORF1b + pRSET #6	3/15/12	pg. 1222
pORF1a without P-Pro + pRSET #4	1/30/13	pg. 1389

Expression of LCV ORF1b (RdRp):

The pRSETA + ORF1b plasmid was transformed into *E. coli* BL21(DE3)pLysS competent cells according to Invitrogen pRSET A, B, C manual. The liquid culture of the transformant was confirmed by miniprep and was made into glycerol stock for long-term storage. In order to determine the optimal expression condition for LCV ORF1b, the pilot expression of LCV ORF1b were performed according to Invitrogen pRSET A, B, C manual. The bacteria collected from pilot expression were analzyed by SDS-PAGE and Western Blot. For the optimal expression for LCV ORF1b, the culture (OD600 = 0.4-0.6) should be induce with IPTG to a final concentration of 0.5mM and grow at 37^0C for 3 hours after IPTG induction.

Result:

pRSET + ORF1a/1b #1 in BL21(DEe)pLysS	1/19/12	pg. 1180
pORF1b + pRSET #1 in BL21(DE3)pLysS	3/19/12	pg.1224
pORF1a without P-Pro + pRSET in solu BL21(DE)	2/25/13	pg. 1408

A. Transformation of pRSETA + ORF1b into *E. coli* BL21(DE3)pLysS: According to Invitrogen pRSET A, B, C manual

1.) Add 10ng of plasmid into 100µl of BL21(DE3)pLysS competent cell

2.) Incubate ice for 30 min

3.) Heat shock at 42^0C for 45 sec

4.) Incubate ice for 2 min

5.) Add 1ml SOC media and shake 200+rpm for 45 min at 37^0C

6.) Centrifuge 13krpm 1min, remove the media, and resuspend with 100µl SOC media

7.) Plate 10µl and 90µl onto SOB plates with 50µg/ml ampicillin (for pRSET) and 35µg/ml chloramphenicol (for *E. coli* BL21(DE3)pLysS)

8.) Pick colony for making glycerol stock and perform minilysate to confirm the transformant contains the correct plasmid

B. Pilot expression of LCV ORF1b: According to Invitrogen pRSET A, B, C manual

1.) Inoculate 5ml of SOB containing 50µg/ml ampicillin and 35µg/ml chloramphenicol and grow overnight at 37^0C

2.) Inoculate overnight culture into 4x 25ml of SOB to OD600 of 0.1

3.) Shake the culture at 37^0C until OD600 = 0.4-0.6

4.) Collect 1ml of culture before IPTG induction, centrifuge, remove supernatant and store cell pellet at -80^0C (pre-induction)

5.) Add IPTG to appropriate final concentration and grow the culture at appropriate temperature

- #1 = 37^0C with 0.5mM IPTG final
- #2 = 37^0C with 1mM IPTG final
- #3 = Room temperature with 0.5mM IPTG final
- #4 = Room temperature with 1mM IPTG final

6.) Collect 1ml of culture every 1 hr for 5 hr

7.) Analyze the protein by SDS-PAGE and Western Blot

- Resuspend the cell pellet with 100µl 1x sample buffer
- Vortex, spin down, and can be stored at -20^0C
- For SDS-PAGE, use 10µl resuspended solution
- For Western Blot, use 5µl resuspended solution
    - Primary antibody: 1/5000x His-tag antibodies
    - Secondary antibody: 1/3000x Goat Anti-mouse HRP IgG

C. Expression of LCV ORF1b: According to Invitrogen pRSET A, B, C manual

1.) Streak pRSETA + ORF1b #1 in *E. coli* BL21(DE3)pLysS 3/19/12

2.) Pick colony into 25ml SOB and shake 250rpm at 37 ^{0}C overnight

3.) Transfer 10ml of culture into 500ml SOB

4.) Shake 250rpm at 37 ^{0}C until OD600 = 0.4-0.6

5.) Add 5ml 100 mM IPTG for final concentration = 0.5 mM

6.) Grow 250rpm at 37^{0}C for 3 hours

7.) Collect the bacteria by spin down in BSA bottle and remove the media

8.) Measure the weight of the pellet and store at -80 ^{0}C

Purification of LCV ORF1b:

After expressing the LCV ORF1b with optimal expression condition, the recombinant RdRp containing the 6x His tag was purified using Ni-NTA agarose according QIAexpressionist manual and was eluted from the column with a standard elution buffer (250 mM immidazole pH 8, 50 mM NaH_2PO_4, and 300 mM NaCl). As the purified protein was need to be in a suitable buffer for RNA binding reactions, after elution of protein from the Ni-NTA column, the elution buffer was changed from standard elution buffer to buffer E (20 mM HEPES-KOH, pH 7.5, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% (v/v) glycerol, and 2 mM DTT) using GE Healthcare PD-10 Desalting Columns. The protein was next concentrated into a smaller volume using a Amicon Ultra-4 Centrifugal Filter Devices 10k spin column. The concentrated protein was then analyzed by Western blot and quantified by densitometry using known amount of co-electrophoresed BSA standards in SDS-PAGE.

A. Purification of LCV ORF1b: According to QIAexpressionist manual

1.) Thaw cell pellet on ice for 15min

2.) Prepare the resuspension solution in blue cap tube:

- 3ml of lysis buffer per gram weight of cell pellet
- 12.5µl protease inhibitor (Sigma) per 1ml lysis buffer used

3.) Resuspend cell pellet by swirling (no vortex)

- If the cell pellet didn't resuspend well, use glass rod (clean with 70% EtOH) to help resuspend the cell pellet

4.) Transfer the resuspended into blue cap tube and measure the total volume

5.) Add 10mg/ml lysozyme solution for final concentration of 1mg/ml

- Make 10mg/ml lysozyme solution by dissolving lyzozyme in lysis buffer

6.) Incubate on ice 30 min

7.) Sonicate the resuspened pellet: Boyce 2294 (Roper's lab)

- Clean probe with 70% EtOH and dry with Kim Wipe
- Transfer the resuspension into GSA bottle or smaller bottle
- Insert the probe into the solution with the solution on ice
- Turn on machine and set the strength knob at "4"
- Press "Program" twice and then Press "Start"
- The machine will sonicate for 5 sec and pause for 5 sec with total sonication time of 5 min.
- After sonication, solution should not be viscous.
- Clean probe with 70% EtOH and dry with Kim Wipe

8.) Centrifuge JA-20 11.2krpm (10000x*g*) for 30 min at 4^0C

9.) Collect supernatant and transfer into another JA-20 tube

10.) Repeat centrifuge JA-20 11.2krpm for 30 min at 4^0C

11.) While waiting:

- Set up column in 3ml syringe by inserting glass wool with forceps (clean with 70% EtOH) and pushing them to the bottom using syringe plunger
- Attach 3-way valve at the tip and wet the wool with 1ml of lysis buffer
- Add Ni-NTA agarose (0.5ml for 1L cell pellet)
- Equilibrate the agarose with 6x volumes of lysis buffer (3 ml)
- Close the valve and add additional lysis buffer until protein sample are ready to be pass through the column (Don't let the agarose dry)

12.) Collect supernatant from centrifugation and store on ice

- Save 15µl from supernatant total lysate
- Resuspend the pellet with 2ml lysis buffer (insoluble)

13.) Move the column and the protein supernatant to the cold room (Batchelor 4143)

14.) Pass the supernatant through column and collect the flow-through

15.) Pass the collected flow-through through column again

- Save the second flow-through

16.) Wash the column with 20x volume of Washing buffer (10 ml)

- Save the first 1 ml wash, 8 ml wash in blue cap tube, and last 1 ml wash

17.) Elute 6x times with 1ml Elution buffer (collect in 6x separate 1ml tubes)

18.) Keep all solutions on ice in refrigerator overnight or proceed to desalting step

B. Desalting/ Buffer Exchange of LCV ORF1b: According to GE Healthcare PD-10 Desalting Columns manual

1.) Set up the desalting column by empty the storage buffer and equilibrate with 25ml Buffer E

2.) After determining which elution contains the most amount of protein, combine those elution up to 2.5ml (save 10µl of each elution used)

- For LCV ORF1b: 1ml Elution #1 + 1ml Elution #2 + 0.5ml Elution #3

3.) Add 2.5 ml eluted protein into column and save flow-through

4.) Elute the protein with 3.5ml Buffer E and save 10µl of desalted protein

C. Concentrating LCV ORF1b: According to Amicon Ultra-4 Centrifugal Filter Devices 10k manual

1.) Add 3.5ml desalted proteins into filter

2.) Insert the filter tube into the adapter for JA-14 rotor

3.) Centrifuge JA-14 8.7krpm for 30 min at 4 ^{0}C

4.) Collect concentrated protein from filter

- Approximately 35µl can be recovered from the filter after centrifuge fro 30 min

8.) Analyze the protein by SDS-PAGE and Western Blot

- For SDS-PAGE, use 5µl resuspended solution
    - BSA should also be included for calculating concentration
- For Western Blot, use25µl resuspended solution
    - Primary antibody: 1/5000x His-tag antibodies
    - Secondary antibody: 1/3000x Goat Anti-mouse HRP IgG

Electrophoretic mobility shift assay of LCV RNA 2 SL transcript and LCV ORF1b protein:

The biotin-labeled transcript of LCV RNA 2 SL was incubated with the recombinant LCV ORF1b (RdRp). The biotin-labeled transcript of GFP was also subjected to the same treatment with the recombinant LCV ORF1b as the negative control. In addition, BSA was also used as protein negative control in the binding reaction with biotinylated RNA 2 SL and GFP transcripts. The reaction was then separated on non-denaturing polyacrylamide gel and transferred onto nylon membrane. The shift of transcript migration resulting from RNA-protein interaction will be observed by detecting biotin using the Thermo Scientific Chemiluminescent Nucleic Acid Detection Module Kit.

A. Preparation of non-denaturing PAGE gel: According to Kenji's small RNA PAGE protocol

1.) Prepare 6% acrylamide solution: for 2x 1mm thick plate gels

30% acrylamide, 0.8% bis	2.4ml
5x TBE	1.2ml
dH_2O	8.4ml (top up to 12ml)
10% Ammonium persulfate	72µl
TEMED	5.6µl

1.) Transfer the solution into the gel cassettes, insert combs, and wait for 30min

2.) Remove the comb and wash the wells with 0.5x TBE 2-3 times

3.) Pre-run the gel in 500ml of 0.5x TBE at 100V for 30min

- Only fill the center reservoir with 0.5x TBE; not the whole tank

B. Preparation of RNA transcripts:

1.) Incubate biotinylated RNA at 85 ^{0}C for 5 min.

2.) Gradually cool transcripts down to room temperature so the transcripts can form secondary structure

C. *In vitro* RNA-protein binding reaction: According to *In vitro* RNA-Binding Assay for Studying Trans-Factors for RNA Editing in Chloroplasts by Shikani and Okuda, 2011

1.) Set up the following reaction:

- Pμl of protein (1 fmol to 1 pmol or fixed amount) in Buffer E
  - 1pmol of LCV RdRp (62kDa) = 62ng
  - or fixed amount of proteins (i.e. 100-500ng)
- Top up to 5μl with Buffer E
- 1μl of 20x buffer IVT (1x final)
- Top up with nuclease-free dH_2O so the reaction will have final volume of 20μl after RNA is added

2.) Incubate room temperature for 10min

3.) Add prepared RNA transcripts and incubate at 25^{0}C for 15min

4.) Add 2μl of 80% glycerol

D. Electrophorese Binding Rections: According to Thermo Scientific LightShift Chemiluminescent RNA EMSA kit (Catalog #20158)

1.) Wash the well with 0.5x TBE and apply the sample to the gel

2.) Add 1x RNA loading dye in one empty lane as a electrophorese marker

3.) Run at 100V for 30min (Bromophenol blue dye should be in middle of gel.)

E. Electrophoretic Transfer from PAGE gel to Nylon memberane: According to Thermo Scientific LightShift Chemiluminescent RNA EMSA kit (Catalog #20158)

1.) Pre-chill the 1000ml of 0.5x TBE before using for transferring

2.) For one gel, prepare 9.5cm x 7cm 1x Hybond N+ membrane and 6x filter papers (larger than gel size of 8.3 cm x 6 cm)

- A gel tends to become larger after transfer according to Kenji

3.) Fill a glass tray with 0.5x TBE and assemble the transfer sandwich

- Black side > sponge > 3x filter papers > PAGE gel > membrane > 3x filter papers > sponge > White Side

4.) Put the sandwich and ice-filled small white container into the electrophoresis tank

5.) Fill the tank with 0.5x TBE to the top (not just the middle reservoir)

6.) Place the tank into Styrofoam box and fill it with ice

- Transferring process will generate a lot of heat in buffer

7.) Transfer at constant 0.4A (400mA) for 30 min.

8.) UV Cross-link using auto optimal setting at Rao's lab

F. Detect Biotin-labeled RNA by Chemiluminescence: According to Thermo Scientific LightShift Chemiluminescent RNA EMSA kit (Catalog #20158)

1.) Gently warm the Nucleic Acid Detection Blocking Buffer and 4x Wash Buffer at 37-50^{0}C until all precipitate is dissolved.

2.) Add 20 ml Blocking Buffer to membrane and gently shake for 15 min

3.) Prepare conjugate/blocking buffer solution by adding 66.7µl Streptavidin-HRP conjugate with 20ml Blocking buffer

4.) Decant blocking buffer and add conjugate/blocking buffer solution

5.) Gently shake for 15 min

6.) Prepare 1x wash solution by adding 40ml of 4x Wash Buffer to 120ml water

7.) Transfer membrane to new container and rinse with 20ml 1x wash solution

8.) Wash 4x times for 5 min each with 20ml 1x wash solution with gently shaking

9.) Transfer membrane to new container with 30ml Substrate Equilibration Buffer

10.) Gently shaking for 5 min

11.) Prepare substrate working solution by adding 6 ml Luminol/Enhancer Solution to 6 ml Stable Peroxide Solution (store the solution in the dark at 4^{0}C)

12.) Transfer membrane into plastic wrap

13.) Add substrate working solution and incubate for 5 min

14.) Expose to X-ray film

Buffers used in Appendix C:

Lysis buffer: According to QIAexpressionist manual

Final concentration: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole

Total volume: 1 L

NaH_2PO_4	6.90 g
NaCl	17.54 g
Imidazole	0.68 g

Adjust pH to 8.0 with NaOH, filter-sterilized, and store at 4^0C

Washing buffer: According to QIAexpressionist manual

Final concentration: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole

Total volume: 1 L

NaH_2PO_4	6.90 g
NaCl	17.54 g
imidazole	1.36 g

Adjust pH to 8.0 with NaOH, filter-sterilized, and store at 4^0C

Elution buffer: According to QIAexpressionist manual

Final concentration: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole

Total volume: 1 L

NaH_2PO_4	6.90 g
NaCl	17.54 g
imidazole	17 g

Adjust pH to 8.0 with NaOH, filter-sterilized, and store at 4^0C

Buffer E: According to *In vitro* RNA-Binding Assay for Studying Trans-Factors for RNA Editing in Chloroplasts by Shikani and Okuda, 2011

Final concentration: 20 mM HEPES-KOH, pH 7.5 (modified from pH 7.9), 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% (v/v) glycerol, 2 mM DTT

Total Volume: 1 L

HEPES	4.766 g
KCl	4.473 g
$MgCl_2$	2.541 g
0.5M EDTA, pH 8.0	0.2 ml
Glycerol	17 ml
DTT	0.308 g

Adjust pH to 7.5 with KOH, filter-sterilized, and store at 4^0C

20X Buffer IVT: According to *In vitro* RNA-Binding Assay for Studying Trans-Factors for RNA Editing in Chloroplasts by Shikani and Okuda, 2011

Final concentration: 75 mM $MgCl_2$, 40 mM DTT, 2700 mM KCl

Total Volume: 1 L

$MgCl_2$	15.246 g
DTT	6.16 g
KCl	201.285 g

Filter-sterilized and store at 4^0C

5X TBE stock solution:

Total Volume: 1L

Tris-Base	54 g
Boric acid	27.5 g
0.5M EDTA (pH8.0)	20 ml

Top up with dH_2O to 1L and autoclave

All solutions and buffers used for biotin labeling reaction and detection of biotin-labeled RNA came with the Pierce RNA 3' End Biotinylation kit (Catalog #20160) and Thermo Scientific LightShift Chemiluminescent RNA EMSA kit (Catalog #20158), respectively.

Figure C-1. Detection of E. coli expressed recombinant LCV RdRp. Purified proteins were separated on 12% SDS-polyacrylamide gels. The gel was detected and quantified by comparing to known amount of BSA standards by Collodial blue staining (A) and another gel was detected by anti-polyhistidine antibody after transferred onto nitrocellulose membrane (B). The position of the recombinant LCV RdRp (62kDa) is indicated by an arrow. Additional (37, 50 and 75kDa) size markers are included as references.

Figure C-1

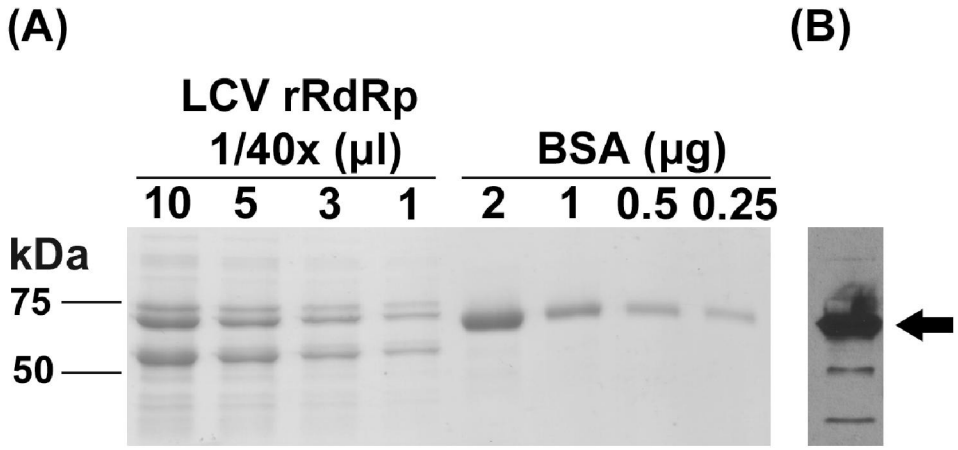


Figure C-2. Electrophoretic mobility shift assay of binding reactions containing recombinant LCV RdRp at various concentrations (0.1, 0.15, 0.25, and 0µg) and biotinylated transcripts of LCV RNA 2 SL and GFP (1nM)

Figure C-2

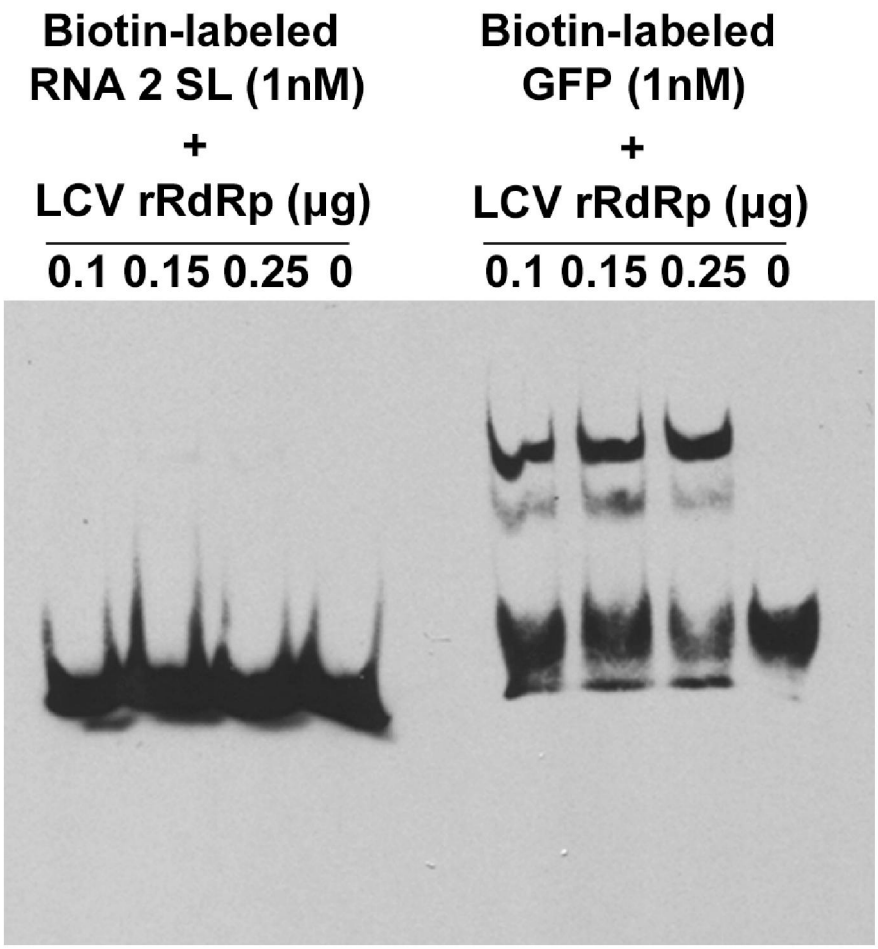

Biotin-labeled
RNA 2 SL (1nM)
+
LCV rRdRp (µg)
0.1 0.15 0.25 0
Biotin-labeled
GFP (1nM)
+
LCV rRdRp (µg)
0.1 0.15 0.25 0

Figure C-3. Electrophoretic mobility shift assay of binding reactions containing recombinant LCV RdRp at various concentrations (0.1, 0.15, 0.25, and 0µg) and biotinylated transcripts of LCV RNA 2 SL and IRE control RNA (1nM and 0.1nM, respectively)

Figure C-3

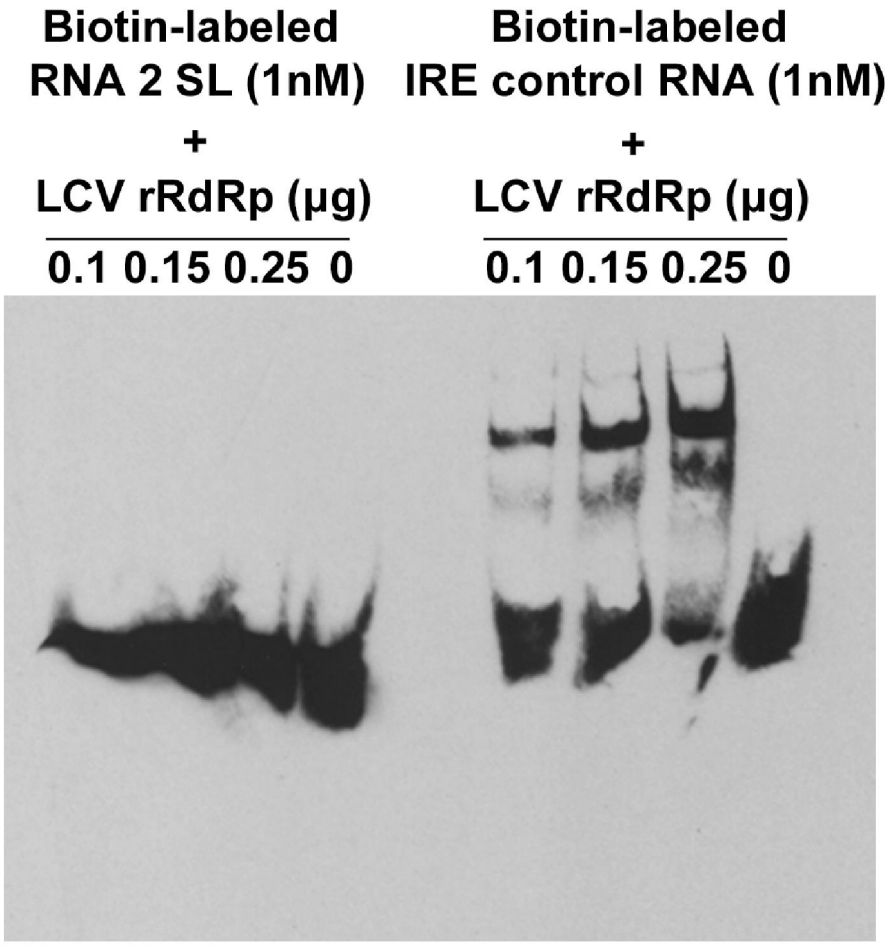

Biotin-labeled
RNA 2 SL (1nM)
+
LCV rRdRp (µg)
0.1 0.15 0.25 0
Biotin-labeled
IRE control RNA (1nM)
+
LCV rRdRp (µg)
0.1 0.15 0.25 0

Figure C-4. Electrophoretic mobility shift assay of binding reactions containing recombinant LCV RdRp and BSA at various concentrations (0.5, 0.25, and 0µg) and biotinylated transcripts of LCV RNA 2 SL and IRE control RNA (1nM)

Figure C-4

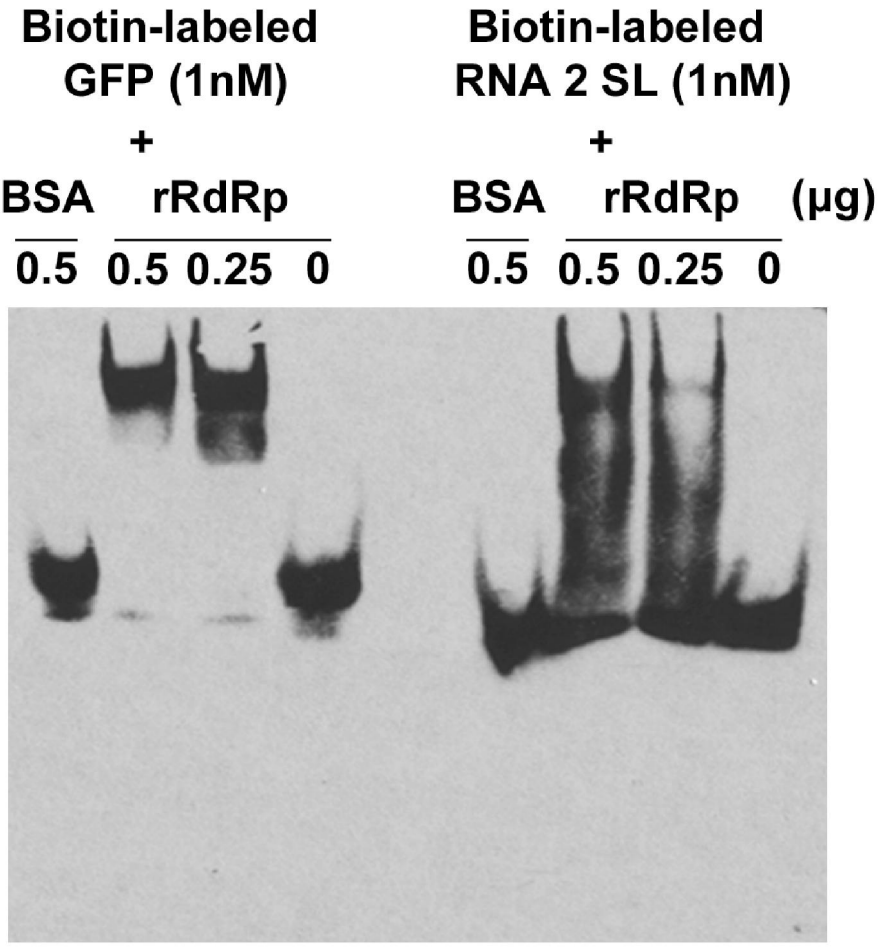

Biotin-labeled
GFP (1nM)
+
BSA rRdRp
0.5 0.5 0.25 0
Biotin-labeled
RNA 2 SL (1nM)
+
BSA rRdRp (µg)
0.5 0.5 0.25 0

Table C-1. List of Oligonucleotide primers used in appendix C

Primer	Sequence (5' - 3')	Description
RNA2SL+	**AATTAACCCTCACTAAG**TCGTTCAAAAGAATCCACTAGGGATCGCCTTGAACGAC *CATGG* A	Bacteriophage T3 promoter (bold), nucleotides corresponding to LCV RNA 2 position 8459-8533 (underlined), NcoI restriction site (italic), and overhanging A
RNA2SL-	*CCATG G* TCGTTCAAGGCGATCCCTAGTGGATTCTTTTGAACGA**CTTTAGTGAGGGTTAATT**A	A NcoI restriction site (italic), nucleotides complementary to LCV RNA 2 position 8533-8459 (underlined) and bacteriophage T3 promoter (bold), and overhanging A
GFPSL+	**AATTAACCCTCACTAAAG**AAAACTTACCCTTAAATTTATTTGCACTACTGGAAAAC *CATGG* A	Bacteriophage T3 promoter (bold), nucleotides corresponding to pM5GFP position 1174-1211 (underlined), NcoI restriction site (italic), and overhanging A
GFPSL-	*CCATG G* TTTTCCAGTAGTGCAAATAAATTTAAGGGTAAGTTTTCTTT**AGTGAGGGTTAATT**A	A NcoI restriction site (italic), nucleotides complementary to pM5GFP position 1211-1174 (underlined) and bacteriophage T3 promoter (bold), and overhanging A
LCV-164-CM	GGGTTGAGTGTTGTTCCAGTTTG	Nucleotides complementary to position 492-514 of the pGEM-T Easy backbone of pCM2
LCV-223-CM	GCAGTGAGCGCAACGCAATTA	Nucleotides complementary to position 289-309 of the pGEM-T Easy backbone of pCM1
pGEMT	GCGCTTAATGCGCCGCTACA	Nucleotides corresponding to position 2808-2827 of pGEM-T Easy vector
LCV-243-CM	**CTCGAG**GCCGTGATGTCGCACTCTC	A XhoI restriction site (bold) and nucleotides correspoding to LCV RNA 1 position 76-94
LCV-244-CM	**TCCATGG**TTACACATTCACACTGAATCTTTC	A NcoI restriction site (bold) and nucleotides complementary to LCV RNA 1 position 7561-7538
LCV-249-CM	**CTCGAG**AGCATATATAATCTCCGCATTG	A XhoI restriction site (bold) and nucleotides correspoding to LCV RNA 1 position 6044-6065
LCV-275-CM	**CTCGAG**GTGAAACTTGAGAATGAGGCTGAC	A XhoI restriction site (bold) and nucleotides correspoding to LCV RNA 1 position 1495-1518
LCV-276-CM	**TCCATGG**CTAAACACGCTGTTTAAACATGT	A NcoI restriction site (bold) and nucleotides complementary to LCV RNA 1 position 6045-6023

Appendix D. Modified 5' Rapid Amplification of cDNA Ends (5' RACE)

1st strand DNA synthesis (RT):

Gene specific primer #1	2.5µl of 1µM
Total RNA	5µg
RNase-free dH_2O	top up to 15.5µl
	70^0C 10min
	Ice 1min
10x PCR buffer	2.5µl
25mM $MgCl_2$	2.5µl
10mM dNTP	1µl
0.1M DTT	2.5µl
	42^0C 1min
	Add 1µl Superscript RT
	42^0C 50min
	70^0C 15min (deactivation)

All buffers can be found in Invitrogen 5'RACE Kit or with Invitrogen Superscript RT.

Superscript RT can be bought separately from the 5' RACE kit.

(Save 3-5µl for confirmation in case)

Purification of 1st strand DNA:

Proceed to QIAGEN PCR Purification Kit

Elute 30µl Buffer EB

Tailing ssDNA with TdT:

Purified 1st strand DNA	10µl
dH_2O	6.5µl
5x tailing buffer	5µl
2mM dCTP (or any other dNTP)	2.5µl

TdT and 5x tailing buffer can be found in Invitrogen 5'RACE kit.

TdT also can be bought separately from many suppliers including Invitrogen.

94^0C 3min

Ice 1min

Add 1µl TdT

37^0C 10min

65^0C 10min

PCR 1st reaction:

dH_2O	29µl
5x Herculase Buffer	10µl
10mM dNTP	1µl
10µM Gene specific primer #2	2µl
Abridged anchor primer	2µl
(Use primer complementary to dNTP used in TdT step)	
Herculase II Polymerase	1µl
dN-tailed cDNA	5µl

94^0C 2min

30x cycles

94^0C 45sec

T_m-5^0C 45sec

72^0C 1min per 1kb

72^0C 7min

4^0C ∞

Herculase II Polymerase and Buffer can be bought from Agilent Technologies.

Abridged anchor primer (complementing dC-tailed DNA), can be found in 5' RACE kit and bought individually from Invitrogen.

Alternatively, abridged anchor primer can be designed and ordered from any primer supplier (complementing to other dNTP beside dCTP).

(Check Nida's Primer Stock Box and Chawin's Primer Stock Box for abridged anchor primer of other

PCR 2nd reaction:

dH_2O	31µl
5x Herculase Buffer	10µl
10mM dNTP	1µl
10µM Gene specific primer #3	1µl
10µM AUAP	1µl
Herculase II Polymerase	1µl

AUAP (abridged universal amplification primer) can be bought from Invitrogen or designed and ordered from any primer supplier.

(Check Nida's Primer Stock Box for AUAP)

Diluted PCR product 1st reaction 5µl

(Dilution: 5µl + 495µl = 500µl)

94^0C	2min

30x cycles	
94^0C	45sec
T_m-5^0C	45sec
72^0C	1min per 1kb

72^0C	7min
4^0C	∞

Additional Information:
In case, PCR using Herculase polymerase did not work.

Taq polymerase is also work for the PCR or DMSO can be added to Herculase PCR reaction according to Herculase's manual.

Additional information for 5' RACE of minus-strand LCV RNAs:

LCV RNA 1:

LCV-27 (+)	for RT step	nucleotide position 7923-7947
LCV-28 (+)	for PCR 1st reaction	nucleotide position 8006-8025
LCV-29 (+)	for PCR 2nd reaction	nucleotide position 8076-8100

Final size of PCR product from 2nd reaction = approximately 515nt

LCV RNA 2:

LCV-161 (+)	for RT step	nucleotide position 7312-7337
LCV-103 (+)	for PCR 1st reaction	nucleotide position 7894-7923
LCV-180 (+)	for PCR 2nd reaction	nucleotide position 8290-8314

Final size of PCR product from 2nd reaction = approximately 266nt

Troubleshooting for RNA 2:

LCV-70 (+) [7592-7612] and LCV-99 (-) [7926-7950] can be use to amplify internal region of the fragment to check whether you obtained product for each step.

Appendix E. Modified 3' Rapid Amplification of cDNA Ends (3' RACE)

RNase A treatment (digesting ssRNA): taken from Nida's dsRNA isolation protocol

Total RNA	10µg
20x SSC	1.7µl
0.1M $MgCl_2$	1µl
RNase A (100ng/µl)	1.5µl
RNase-free dH_2O	top up to 15µl

Dilution of RNase A:

RNase A from Fermantas concentration = 10mg/ml = 10µg/µl

Dilute: 1µl RNase A (10µg/µl) + 999µl nuclease-free dH_2O= 1000µl (10ng/µl)

Dilute again: 2µl RNase A (10ng/µl) + 18µl nuclease-free dH_2O = 20µl (1ng/µl)

37^0C 1hr

Isolation of dsRNA:

Proceed to QIAGEN PCR Purification Kit

Elute with 15µl Buffer EB

Poly-A tailing:

Isolated dsRNA from PCR column	13 µl
10x Poly (A) buffer	2µl
10mM ATP	2µl
Poly (A) Polymerase	1µl (5U)
RNase-free dH_2O	2µl

E. coli Poly (A) Polymerase can be bought from NEB.

37^0C 10min

65^0C 20min (deactivation)

RT: Add the following component directly into the Poly-A tailing reaction:

10x PCR buffer	2.5µl
25mM $MgCl_2$	---µl
(10x Poly (A) buffer already contains enough $MgCl_2$)	
10mM dNTP	1µl
0.1M DTT	2.5µl
1µM Abridged anchor primer	2.5µl
Superscript RT	1µl
RNase-free dH_2O	2µl

All buffers can be found in Invitrogen 5' RACE Kit or with Invitrogen Superscript RT.

Superscript RT can be bought separately from the 5' RACE kit.

Abridged anchor primer can be found in Nida's primer box. (Primer with multiple T).

42^0C 50min

70^0C 15min

PCR 1st reaction:

dH_2O	24µl
5x Herculase Buffer	10µl
10mM dNTP	1µl
10µM Gene specific primer #2	2µl
10µM Abridged anchor primer	2µl
Herculase II Polymerase	1µl
RT product	10µl

Herculase II Polymerase and Buffer can be bought from Agilent Technologies.

Abridged anchor primer (complementing dA-tailed DNA) can be designed and order from DTT oligo.

(Check Nida's Primer Stock Box for the primer)

95^0C	2min

30x cycles	
95^0C	45sec
T_m-5^0C	45sec
72^0C	1min per 1kb

72^0C	7min
4^0C	∞

PCR 2nd reaction:

dH_2O	31µl
5x Herculase Buffer	10µl
10mM dNTP	1µl
10µM Gene specific primer #3	1µl
10µM AUAP	1µl
Herculase II Polymerase	1µl
Diluted PCR product 1st reaction	5µl

(Dilution: 5µl + 495µl = 500µl)

95^0C	2min

30x cycles	
95^0C	45sec
T_m-5^0C	45sec
72^0C	1min per 1kb

72^0C	7min
4^0C	∞

AUAP (abridged universal amplification primer) can be bought from Invitrogen or designed and ordered from Fisher Oligo.

(Check Nida's Primer Stock Box for AUAP)

Additional Information:
In case, PCR using Herculase polymerase did not work.

Taq polymerase is also work for the PCR or DMSO can be added to Herculase PCR reaction according to Herculase's manual.

Additional information for 3' RACE of plus-strand LCV RNAs:

LCV RNA 1:

LCV-28 (+)	for PCR 1st reaction	nucleotide position 8006-8025
LCV-29 (+)	for PCR 2nd reaction	nucleotide position 8076-8100

Final size of PCR product from 2nd reaction = approximately 515nt

LCV RNA 2:

LCV-103 (+)	for PCR 1st reaction	nucleotide position 7894-7923
LCV-180 (+)	for PCR 2nd reaction	nucleotide position 8290-8314

Final size of PCR product from 2nd reaction = approximately 266nt

Appendix F. Modified Prehybridization and Hybridization of Northern Blot

The protocol is adopted from Rao lab.

1.) Prior to prehybridization step, the prehybridization/hybridization buffer is needed to be warm up until all precipitates completely dissolved into the solution.

2.) After processing the blot according to II. Transfer using VacuGene XL Vacuum blotting system (Amersham Bioscience) from Ng lab's protocol, transfer the membrane into the hybridization tube.

3.) Add 15ml prehybridization/hybridization buffer (maximum of 20ml per blot)

4.) Incubate the blot at 65^0C by rotating in the incubator overnight (>14 hours)

5.) Add probe (1.5µg: final contration = 100ng/ml) into 50µl RNase-free dH_2O, boil for 5min, cool on ice for 1+min, and add into prehybridization buffer

6.) Incubate the blot at 65^0C by rotating in the incubator overnight

7.) Prior to the washing step, pre-warm the wash buffer at 65^0C

8.) Discard hybridization buffer, add 50ml of first wash, rotate blot at 65^0C for 15min

9.) Discard the wash, add another 50ml of first wash, rotate blot at 65^0C for 15min

10.) Discard the wash, add 50ml of second wash, rotate blot at 65^0C for 30min

11.) Discard the wash, add another 50ml of second wash, rotate blot at 65^0C for 30min

12.) Proceed to Detection of DIG-labeled probe Ng lab's protocol

Prehybridization/hybridization Buffer:

	Total: 100ml	Final concentration:
Formamide	50ml	50%
20X SSC	25ml	5X
20% SDS	5ml	1%
0.2M phosphate buffer (pH 7.0)	10ml	0.02M
50X Denhardt's buffer	10ml	5X
10mg/ml sheared salmon sperm DNA	1.5ml	0.15mg/ml
50mg/ml yeast RNA	1ml	0.5mg/ml

When making buffer, the buffer may turn cloudy or precipitate at room temperature so the buffer can be warmed up to 65^0C to dissolve the precipitate.

Store buffer at -20^0C and warm up to 65^0C prior to use for prehybridization step

Recipe of components of Prehybridization/hybridization Buffer:

Formamide:

Can be taken directly from the stock solution (non-deionized)

20X SSC:

Final concentration: 0.3M Sodium Citrate and 3M NaCl (pH7.0)

Dissolve 88.2g of Sodium citrate and 175.3g of NaCl into 800ml of dH_2O

Adjust pH to 7.0 with concentrated HCl, top up with dH_2O to 1L, and autoclave

20% SDS:

Dissolve 100g in 400ml of dH_2O and heat up to 68^0C

Stir until all SDS dissolve, top up with dH_2O to 500ml and filter sterilize

0.2M phosphate buffer, pH 7.0: According to Molecular Cloning Volume 3 page A1.5

Add 23.08ml of 1M Na_2HPO_4 and 16.92ml of 1M NaH_2PO_4

Add 160ml dH_2O and autoclave

50X Denhardt's buffer: According to Molecular Cloning Volume 3 page A1.15

	Total volume: 100ml	Final concentration:
Ficoll 400	1g	1% (w/v)
Polyvinylpyrrolidone	1g	1% (w/v)
BSA (Sigma, Fraction V)	1g	1% (w/v)

Fliter sterilize into 50ml blue cap tube and stored at -20^{0}C.

Warmed up to 65^{0}C before making prehybridization/hybridization buffer

50mg/ml yeast RNA: According to Soon from Rao's lab

Dissolve 50mg yeast RNA into 800µl autoclaved dH_2O, top up to 1ml, and directly use for making prehybridization/hybridization buffer.

10mg/ml sheared salmon sperm DNA: According to Venky from Rao's lab

1.) Measure 50ml of autoclaved dH_2O, transfer into autoclaved beaker, mark the position of final volume, and decant water to below the marked position
2.) Microwave the water for 5 seconds, and add 500mg of salmon sperm DNA
3.) Add autoclaved stir bar, top up with autoclave dH_2O to the mark position, and stir for 1+ hr with gentle heat (setting 2-2.5)
    - Solution will be viscous
4.) Put the beaker on ice and bring to Ding's lab (Genomic Building 3239)
5.) Clean the probe of Biolock model 300 VT Ultrasonic Homogenizer with dH_2O and dry the probe with Kim wipe
6.) Sonicate the solution for several minutes until the solution become watery
    - pulse: 90%; timer: off; power: 20
7.) Test the solution using syringe and 16G 1/2 needle
    - Solution should be easily pass through the needle
    - If the solution is still viscous, continue sonication until the solution can easily pass though the needle
8.) Clean the probe with dH_2O and dry it with Kim Wipe
9.) Take 1µl to measure concentration, aliquot the solution into 2ml microcentrifuge tube and store at -20^0C

Wash Buffer:

First wash buffer:

Final concentration: 2X SSC and 0.2% SDS

	Total volume:	50ml	100ml
5X SSC		20ml	40ml
10% SDS		1ml	2ml
Autoclaved dH_2O		29ml	58ml

Second wash buffer:

Final Concentration: 0.2X SSC and 0.2% SDS

	Total volume:	50ml	100ml
5X SSC		2ml	4ml
10% SDS		1ml	2ml
Autoclaved dH_2O		47ml	94ml

Appendix G. Modified Protoplast Isolation and Inoculation Protocol

Combining and redistribution of inoculated protoplasts (3x plates method):

The procedure is developed in order to normalize the variability of protoplast infection as each inoculation of transcript/virion RNA can have different rates of infection.

After removing the PEG solution and resuspending the inoculated protoplasts with 10ml culture medium in snap-cap tube, instead of directly transfer the inoculated protoplasts into individual small 60x20mm plate (Nunc Lab-Tek Extra-Depth Polystyrene Dishes; 60 x 20mm):

1.) Combine the 3 separate inoculation of the same sample/treatment (3x 10ml inoculated protoplasts + culture medium) into a single 100x20mm plate
2.) Incubate at 26^0C overnight
3.) Redistribute 10ml of the inoculated protoplasts into individual 60x20mm plate
4.) Incubate at 26^0C until harvesting time

Harvesting inoculated protoplasts:

2x 5ml of inoculated protoplasts will be harvested into a single 2ml microcentrifuge tubes from inoculated protoplasts of one sample/treatment (10ml):

1.) Transfer 2ml of inoculated protoplasts into 2ml microcentrifuge tube
2.) Centrifuge 10krpm for 5 min at 4^0C
3.) Remove the supernatant
4.) Transfer additional 2ml of inoculated protoplasts
5.) Repeat centrifugation and removal of supernatant
6.) Transfer last 1ml of inoculated protplasts
7.) Repeat centrifugation and removal of supernatant
8.) Store at -80^0C

- Use a single tube of protoplast pellet (5ml) for total RNA extraction

Appendix H. SHAPE analysis protocol

Construction of cassette plasmid:

In order to accurately read the extreme 5'- and 3'-terimini nucleotides of RNA fragment, the template for *in vitro* transcription should be constructed in the structure cassette plasmid (Fig. H-1). The structure cassette contains bacteriophage T3 promoter, two linker regions, which fold into stable stem-loop structures at 5' and 3' termini of transcript, and restriction digestion site immediately after 3'-linker sequence (XbaI restriction site in Fig. H-1). The 3'-linker is designed for binding of primer and reverse transcriptase enzyme, while the 5'-linker is designed to eliminate full length cDNA products (resulting from primer extension) from obscuring reactivity information at 5' end of the RNA (Merino et al., 2004; Vachon and Conn, 2012). Additional restriction sites can also be designed in the stem region of both 5'- and 3'-linkers for future cloning with another cDNA of target RNA (BamHI and XhoI restriction sites in Fig. H-1).

The structure cassette plasmid with the region of interest is constructed by two PCRs amplification. Each PCR is performed with 40-50 oligonucleotide long primers that designed with 20-30 nucleotides annealing to template and 20-30 nucleotides of linker and/or bacteriophage T3 promoter. The amplified product is gel-purified, adenylated and cloned into the pGEM-T Easy vector.

***In vitro* transcription:** According to Ambion mMESSAGEe mMACHINE T3 manual and Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution by Wilkinson et al., 2006

For making transcript of small template (less than 300 nt such as 3' NCR of LCV RNA 2 + 5'- and 3'- linker), the plasmid linearizes by restriction enzyme (XbaI for 3' NCR of LCV RNA 2 + 5'- and 3'-linker), is then extracted by phenol:chloroform and recovered by ethanol precipitation. The recovered linearized plasmid can be directly used for *in vitro* transcription according to Ambion mMESSAGEe mMACHINE T3 manual except the reaction should be incubated at 37^0C for 6 hours instead of 2 hours. The reaction is then treated with DNaseI, extracted with phenol: chloroform, and recovered by ethanol precipitation (instead of LiCl precipitation) according to Ambion mMESSAGEe mMACHINE T3 manual. The product should be resuspended with 0.5x TE (pH8.0) instead of nuclease-free water and 1µl of transcript should be analyzed by both denatured HEPES gel according Ng's lab Northern blot protocol and denatured 7M Urea/ 10% PAGE gel according to according to Kenji's small RNA PAGE protocol. (See appendix C *in vitro* transcription section for detailed protocol) (Fig. H-2).

Transcript from large template (more than 300 nt such as LCV dRNA 2 P4 + 3'-linker) can made according Ng's lab *in vitro* transcription protocol except the transcript should be resuspended with 0.5x TE (pH8.0) instead of nuclease-free water.

RNA folding: According to Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution by Wilkinson et al., 2006

1.) Add 2 pmol RNA transcript in PCR tube

- M.W. of ssRNA = (#nt x 320.5) + 159.0 (according to Invitrogen's website)

2.) Top up the volume to 12µl with 0.5X TE, pH 8.0

3.) Heat at 95°C for 2 min, then immediately put on ice for 2 min

4.) Add 6µl Folding mix and mix by gently pipetting up and down (total: 18µl)

5.) Distribute 9µl to two 1.5ml microcentrifuge tubes [for NMIA (+) and NMIA (-)]

6.) Incubate at 37°C for 20 min

RNA modification with NMIA: According to Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution by Wilkinson et al., 2006

While waiting: make NMIA solution to appropriate concentration (130mM for 3' NCR of LCV RNA 2 + 5'- and 3'-linker, 130, 70, and 10mM for LCV dRNA 2 P4 + 3'-linker) The solution should be made fresh before use for the reaction and color of solution will be brown.

For 130mM: dissolve 4.6mg in 200µl DMSO

For 70mM: dilute 10µl of 130mM stock with 8.5µl DMSO

For 10mM: dilute 10µl of 130mM stock with 120µl DMSO

A. RNA modification by NMIA:

1.) Add 1µl NMIA to the NMIA (+) tube

- The solution will be cloudy after adding NMIA solution

2.) Add 1µl DMSO to the NMIA (-) tube

3.) Incubate tubes at 37°C for 50 min (approximately 5 half-lives for NMIA)

B. Recovery of modified RNA by ethanol precipitation:

1.) Add the following components into each tube:

dH_2O	90µl
5M NaCl	4µl
20mg/ml glycogen	1µl
100mM EDTA, pH 8.0	2µl
Absolute Ethanol	350µl

2.) Incubate at -80°C for 30 min

3.) Centrifuge 13krpm at 4°C for 30 min

4.) Remove supernatant, air dry and resuspend with 10µl 0.5xTE, pH 8.0

- After centrifugation, there will be white RNA pellet

Primer extension: According to High-Throughput SHAPE and Hydroxyl Radical Analysis of RNA Structure and Ribonucleoprotein Assembly by McGinnis et al., 2009

A. Preparation for primer extension

1.) Make following mixes:

SHAPE enzyme mix:

Superscript RT III first strand buffer	40µl	(4x part)
0.1M DTT	10µl	(1x part)
10mM dNTP	10µl	(1x part)

Acid stop mix:

4M NaOAc, pH 5.2	50µl
100mM EDTA, pH 8.0	50µl

B. Primer extension:

1.) Set up the following reaction in PCR tubes:

#1: NMIA (+) primer extension

10µl from NMIA (+) + 3µl of 0.3 µM VIC primer

#2: NMIA (-) primer extension

10µl from NMIA (+) + 3µl of 0.3 µM NED primer

#3: NMIA (+) sequencing reaction

2pmol of transcript + top up to 8 µl with 0.5xTE, pH 8.0 + 3µl of 0.3 µM NED primer

#4: NMIA (-) sequencing reaction

2pmol of transcript + top up to 8 µl with 0.5xTE, pH 8.0 + 3µl of 0.3 µM VIC primer

2.) Incubate at 95°C for 5min, 37°C for 1min, and then ice for 1min

3.) Add 6µl SHAPE enzyme mix to each tube

4.) For the sequencing reaction (#3 and 4), add 1µl of 5mM ddTTP

5.) Add 1µl Superscript III RT (Catalog #18080-093)

6.) Incubate at 37°C for 5min, 52°C for 20min, and then 60°C for 5min

7.) Add 4µl Acid stop mix and incubate ice

8.) Combine the primer extension with the sequencing reaction (#1 + #3; #2 + #4)

C. Recovery of cDNA by ethanol precipitation:

1.) Add 240µl absolute Ethanol

2.) Incubate at -80°C for 15min

3.) Centrifuge 13krpm at 4°C for 15min

4.) Remove supernatant and wash with 800µl 70% Ethanol

5.) Centrifuge 13krpm at 4°C for 15min

6.) Remove the wash and wash with 800µl 70% Ethanol

7.) Centrifuge 13krpm at 4°C for 15min

8.) Remove the wash

9.) Air dry and resuspend with 10µl deionized formamide

D. RNA sequencing

1.) Transfer the product into 0.2ml PCR tube

2.) Place the tube on the pipette tip rack and wrap the rack with aluminum foil

3.) Send to Genomic center by choosing "Tube – DNA Seq. Only (Investigator does Big Dye seq. reactions and cleanup)"

E. Data analysis

1.) The raw data will come back in fsa format that can be directly used in Qushape software.

2.) The software and instruction for QuShape can be found at: http://www.chem.unc.edu/rna/qushape/

Buffers used in Appendix H:

1x TE buffer, pH8.0:

Final concentratin: 10mM Tris, 1mM EDTA

Dissolve 0.12g of Tris Base in 90ml dH_2O

Add 0.2ml of 0.5M EDTA pH8.0

Adjust the pH to 8.0 with concentrated HCl

Filter sterilize

3.3x RNA Folding Buffer: According to Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution by Wilkinson et al., 2006

Final concentration: 333mM HEPES, pH 8.0, 20mM $MgCl_2$, 333mM NaCl

	Total Volume: 1L	500ml
HEPES	79.52g	39.72g
$MgCl_2$	4.06g	2.03g
NaCl	19.45g	9.72g

Adjust the pH to 8.0 with NaOH and autoclave

5M NaCl:

Dissolve 29.21g in 90ml dH_2O, top up to 100ml with dH_2O, and filter sterilize

4M NaOAc, pH 8.0:

Dissolve 32.81g in 50ml dH_2O, adjust pH to 8.0 with glacial acetic acid, top up to 100ml with dH_2O and autoclave

20mg/ml glycogen:

Can be purchase from Invitrogen Catalog # 10814-010

0.1M DTT**:**

Come with Superscript III Reverse Transcriptase Catalog # 18080-093

Figure H-1. Organization of the structure cassette plasmid with RNA of interest for in vitro transcription and secondary RNA structure of transcript from the structure cassette plasmid containing RNA of interest. The structure cassette plasmid contains a bacteriophage T3 promoter for *in vitro* transcription, 5'-linker, RNA of interest, 3'-linker and RT primer binding site along with several restriction sites for cloning and linearization for *in vitro* transcription.

Figure H-1

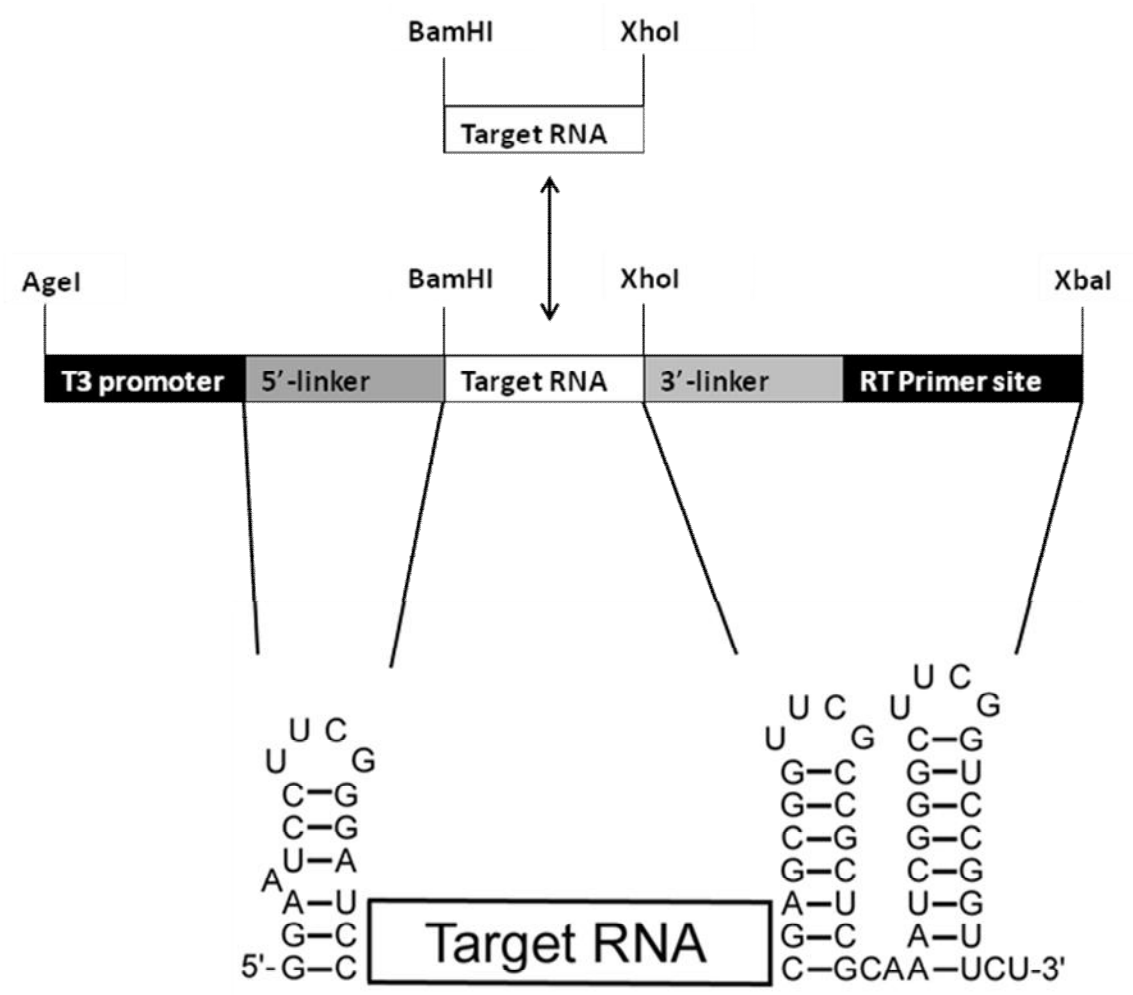
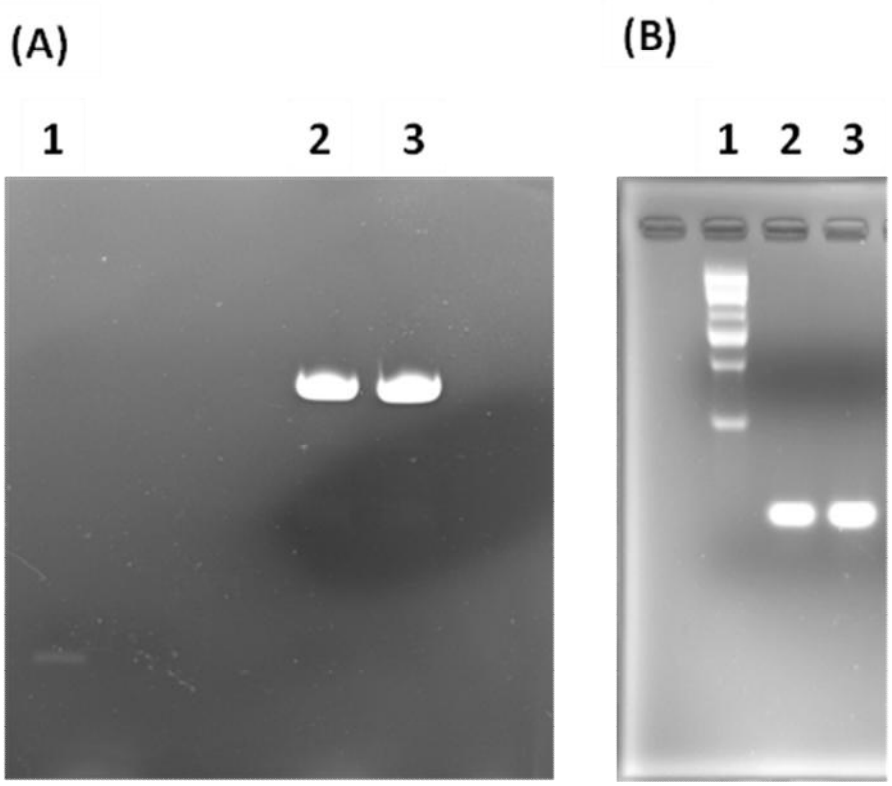

BamHI
XhoI
Target RNA
AgeI
BamHI
XhoI
XbaI
T3 promoter
5′-linker
Target RNA
3′-linker
RT Primer site
Target RNA
5'-
-3'

Figure H-2. Ethidium bromide-stained 7M Urea/ 10% PAGE gel (A) and ethidium bromide-stained HEPES gel (B) of in vitro transcripts from structure cassette plasmid containing 3' NCR of LCV RNA 2 (lanes 2 and 3). DNA oligo with size of 60 nucleotides and RNA marker are also included as reference (lane 1).

Figure H-2



Appendix I. Discovery of Defective RNA associated with LCV RNA 2 and Construction of Infectious cDNA clone of Defective RNA 2 #6

In a previous attempt to synthesize full-length cDNA of LCV RNA 2, the first strand cDNA of LCV RNA2 was generated using purified LCV virion RNAs, the oligonucleotide primer LCV-57, and ThermoScript^{TM} Reverse Transcriptase according to the manufacturer's instructions. The second-strand cDNA synthesis and PCR amplification were performed using the Expand Long Template PCR system (systems 2 and 3; Roche) with the oligonucleotide primers LCV-56 and LCV-57, corresponding and complementary to the extreme 5' and 3' termini, respectively. The full length cDNA was gel-purified and an attempt had been made to ligate into pGEM-T Easy vector. However, no recombinant plasmid contains the full length cDNA of LCV RNA 2. However, six randomly chosen recombinant plasmids with shorter inserts were sequenced. The steps described above were done by Carlo.

Interestingly, the result showed that these products exhibited genome organization of defective RNA: the 5' terminal nucleotides from LCV RNA 2 position 1 to between positions 965 and 997, a non-contiguous region of nucleotides from positions between 5036 and 5322 to positions between 5352 to 6217, and followed immediately by 3' terminal nucleotides from between positions 7321 and 8132 to position 8556 (the last nucleotide) (Fig. I-1). Two of the defective RNAs 2 (#1 and 2) contains additional (non-viral) nucleotides in between the two nucleotide junctions, and one of the defective RNA 2 contains an entire CP ORF in the middle junction of defective RNA (Fig. I-1).

In order to construct the infectious cDNA clone of DRNA2 #6 (P4), pDR2-6, a bacteriophage T3 promoter and an NgOMIV restriction site were engineered immediately upstream of the 5'-terminus and downstream of the 3'-terminus of the defective LCV RNA 2 sequence by PCR and oligonucleotide primers LCV-101-CM and LCV-172-AC. The PCR product was gel-purified, adenylated, and cloned into pGEM-T Easy vector. To test the biological activity of the defective RNA2, *in vitro* transcript of pdR2-1 was synthesized and inoculated into tobacco protoplasts in the same manner as mentioned above. The analysis of defective RNA 2 sequence was performed by Dr. Angel Chen.

Figure I-1. Genomic organization of defective RNA derived from LCV RNA 2.

Figure I-1

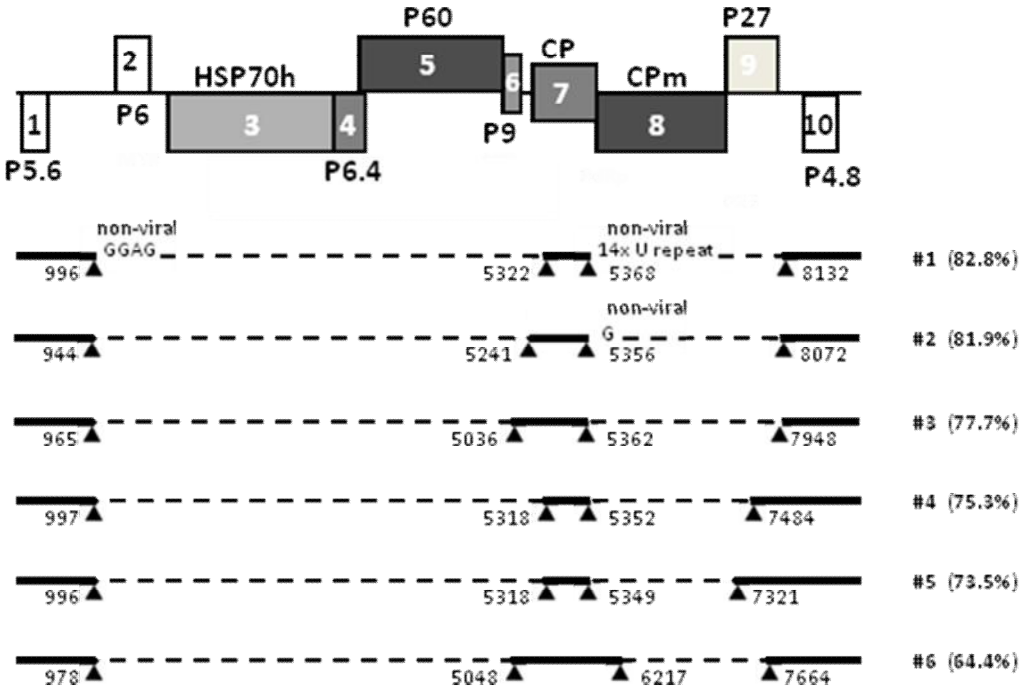

P60
P27
CP
2
HSP70h
5
6
7
CPm
9
1
P6
3
4
P9
8
10
P5.6
P6.4
P4.8
non-viral
GGAG
non-viral
14x U repeat
996
5322
5368
8132
#1 (82.8%)
non-viral
G
944
5241
5356
8072
#2 (81.9%)
965
5036
5362
7948
#3 (77.7%)
997
5318
5352
7484
#4 (75.3%)
996
5318
5349
7321
#5 (73.5%)
978
5048
6217
7664
#6 (64.4%)

Table I-1. List of Oligonucleotide primers used in appendix I

Primer	Sequence (5' - 3')	Description
LCV-56	CAAAGCGTC**GCATGC**GAAATTTCCACGGTTTCCCCGAG	SphI restriction site (bold) and nucleotides corresponding to LCV RNA 2 position 1-23
LCV-57	ATCTGATT**GCGGCCGC**CGGCCTAGCTATACTACTAACTAGTCGTTCAAG	NotI restriction site (bold) and nucleotides complementary to LCV RNA 2 position 8556-8524
LCV-101-CM	*ccc**ggg**GCATGC* **AATTAACCCTCACTAAAG**GAAATTTCCACGGTTTCCCCGAG	XmaI (italic lower case) and SphI (italic upper case) restriction sites; a T3 promoter (bold) and nucleotides corresponding to LCV RNA 2 position 1-23 (underlined)
LCV-172-AC	**GGGCCC**GCCGGC *GGCCTAGCTTACTACTAAC*	ApaI (bold) and NgoMIV (underlined) restriction sites, and nucleotides complementary to defective LCV RNA 2 position 3041-3022 (italic)

Appendix J. Construction of cDNA clone of Defective RNA 1 with GFP

In order to assess whether defective (D) RNA derived from LCV RNA 1can be replicated by LCV RNA 1, we constructed infectious cDNA clone of DRNA1 protoplast #1 (Fig. 1-7) [with a bacteriphage T3 promoter for *in vitro* transcription, an NgOMIV restriction site for linearization of the plasmid] with an addition of GFP sequence in frame with open reading frame presented in DRNA as a marker for distinguishing by the input DRNA 1 from the *de novo* DRNA 1 generated by the original helper virus LCV RNA 1. The bacteriophage T3 promoter was engineered immediately upstream of the 5'-terminus of the DRNA1 by PCR using DRNA1 protoplast #1 as template, and oligonucleotide primers LCV-89-CN and LCV-196-CM. To engineer GFP sequence into the DRNA1, a second PCR was performed with pM5GFP (Yeh et al., 2001) as template and oligonucleotide primers LCV-197-CM and LCV-198-CM. The amplified products from first two PCRs were gel-purified and were used to perform overlapping PCR using oligonucleotide primers LCV-89-CN and LCV-198-CM, resulting in intermediate PCR fragment, pD101, containing a bacteriophage T3 promoter, DRNA1nucleotide position 1-72, GFP sequence from pM5GFP, and followed immediately by DRNA1 nucleotide position 950- 969. To introduce the 3'-terminal of DRNA and an NgoMIV restriction site, third PCR was performed using DRNA1 protoplast #1 as template with oligonucleotide primers LCV-199-CM and LCV-92-CN. The amplified product was gel-purified along with pD101 and both were subjected to overlapping PCR using oligonucleotide LCV-89-CN and LCV-92-CN. The final amplified product was gel-purified, adenylated and

cloned into the pGEM-T Easy vector, resulting in pDRNA1-GFP, an infectious clone of DRNA1 protoplast #1 with GFP sequence.

An *in vitro* transcript of pDRNA1-GFP was inoculated into protoplast. The total RNAs were extracted from the inoculated protoplasts and were subjected to Northern blot analysis with minus-strand specific GFP riboprobe. The result showed that the DRNA1 was detected from 0hpi to the final time point 96hpi and did not increase in accumulation level.

Table J-1. List of Oligonucleotide primers used in appendix J

Primer	Sequence (5' - 3')	Description
LCV-89-CN	GGC**AATTAACCCTCACTAAA**GAAATCAAACTTTCCTTCGTACGAAGAG	A T3 promoter sequence (bold) and nucleotides corresponding to those of LCV RNA1 position 1-28 (underlined)
LCV-92-CN	CTGAAT**GCCGGC**CGGCCTAGTTATTCTATTAACTAGTCTC	A NgoMIV restriction site (bold) and nucleotides complementary to those of LCV RNA 1 position 8591-8564 (underlined)
LCV-196-CM	CTTTACTGAAGTTATTATTATTATTGTTATTTGGATTTTTGG	Nucleotides complementary to LCV RNA 1 position 222-191 and pM5gfp position 1056-1065 (underlined)
LCV-197-CM	TAATAATAACTTCAGTAAAGGAGAAGAACTTTTCAC	Nucleotides corresponding to LCV RNA 1 position 213-222 and pM5gfp position 1056-1081 (underlined)
LCV-198-CM	AGCCTTATTCTTAAAGCTCATCATGTTTGTAT	Nucleotides complementary to LCV RNA 1 position 8410-8401 and pM5gfp position 1884-1862 (underlined)
LCV-199-CM	TGAGCTTTAAGAATAAGGCTGTTTCGGTAATAG	Nucleotides corresponding to LCV RNA 1 position 8401-8423 and GFP position 1853-1862 (underlined)

Appendix K. Quick and Dirty Purification of LIYV virion from infected C. Murales

1.) Collect 8g of infected tissues

2.) Grind in liquid nitrogen

3.) Dissolve in 40ml extraction buffer (5x weight of tissue)

4.) Add triton 100x (final: 2%) drop wise while the buffer is stirring

5.) Stir 2hr on ice

6.) Centrifuge JA-20 8300rpm for 10min at 4^0C

7.) Collect supernatant and transfer 9ml into Ti70.1 polycarbonate tube

8.) Add 1ml 20% sucrose cushion with TE buffer

9.) Centrifuge Ti70.1 35000rpm for 2hrs at 4^0C

10.) Remove supernatant

11.) Rinse the wall of the tube and dry the tube

12.) Resuspend with 25ul 1x TE buffer (pH 7.4)

13.) Set on ice 4^0C overnight

14.) Flick the tube to dislodge the pellet into the solution

15.) Transfer the supernatant and the pellet into 1.5ml microcentrifuge tube

16.) Centrifuge 10000rpm for 1.5min at 4^0C

17.) Collect the supernatant into a new 1.5ml microcentrifuge tube

Appendix L. Colloidal Coomassie Blue Staining of SDS-PAGE gel

Colloidal Coomassie Blue Staining Solution:

	Total Volume: 500ml	Final concentration:
Distilled Water	325ml	
Ammonium Sulfate	50g	10%
5% Coomasie Blue Solution	10ml	0.1%
Ortho-phosphoric acid	15ml	3%
95% Ethanol	105ml	20%

1.) Dissolve 0.5g of Coomasie G-250 into 10ml of dH_2O to make 5% Coomasie solution

2.) Add each component in the order listed into beaker

- Before adding 5% Coomasie Blue solution, make sure that all Ammonium Sulfate is dissolved

3.) After adding every components, bring the total volume 500ml with dH_2O

Staining SDS-PAGE gel:

1.) After the electrophoresis, rinse the gel twice with 100 ml dH_20 for 3 minutes

2.) Add Colloidal Coomasie Blue solution and stain for at least 2 hours

- Staining can be as long as overnight

3.) De-stain the gel several times with dH_2O and dry on the apparatus

Appendix M. Construction of pCM2 mutants

Mutant constructs were engineered by PCR-mediated mutagenesis using the WT LCV cDNA clone, pCM2 (RNA 2), as templates (Mongkolsiriwattana et al., 2011), and Herculase II fusion DNA polymerase with high fidelity proofreading capability (Agilent Technologies). All oligonucleotide primers used for the construction of pCM2 mutants are indicated in Table M1.

Mutants p3'LS, p3'ΔUS1, p3'ΔUS2, p3'ΔIL, p3'ILS1, p3'ILS2, p3'ILS3, p3'ILS4, p3'ILS5, and p3'ΔLUI: the pCM2 mutants with specific mutations engineered in the stem loop located within the 98-nt (-24 to -62 nts upstream of the final nt at the 3' end) of RNA 2, were constructed in the same manner as pR1-3'R2 (see Materials and Methods in Chapter 2) except for p3'ILS4 and p3'ILS5, where p3'ΔUS1 was used as template instead of pCM2. The first PCR product was generated using the forward oligonucleotide primer LCV-161-CM and following reverse primers: LCV 252-CM (p3'LS), LCV-254-CM (p3'ΔUS1), LCV-256-CM (p3'ΔUS2), LCV-258-CM (p3'ΔIL), LCV-262-CM (p3'ILS1), LCV-264-CM (p3'ILS2), LCV-266-CM (p3'ILS3), LCV-268-CM (p3'ILS4), LCV-270-CM (p3'ILS5), and LCV-281-CM (p3'ΔLUI). The second PCR product was generated using reverse primer LCV-164-CM and following forward primers: LCV 253-CM (p3'LS), LCV-255-CM (p3'ΔUS1), LCV-257-CM (p3'ΔUS2), LCV-259-CM (p3'ΔIL), LCV-263-CM (p3'ILS1), LCV-265-CM (p3'ILS2), LCV-267-CM (p3'ILS3), LCV-269-CM (p3'ILS4), LCV-271-CM (p3'ILS5), and LCV-282-CM (p3'ΔLUI). Overlapping extension PCR of 50ng of each of the gel-purified form of the above PCR products was performed using oligonucleotide primers LCV-161-CM and LCV-164-CM. The

resulting PCR amplified products were gel-purified, adenylated, and cloned into pGEM-T Easy vector. Further PCRs were required to introduce the desired mutations for making p3'SLR and p3'ΔIL. The first PCR was performed using oligonucleotide primer LCV-161-CM and the following primers: LCV-191-CM (for constructing p3'SLR) and LCV-260-CM (p3'ΔIL); the second PCR was performed using LCV-164-CM and following primers: LCV-192-CM (for constructing p3'SLR) and LCV-261-CM (p3'ΔIL). Overlapping extension PCR (using LCV-161-CM and LCV-164-CM) and cloning of the gel purified products into pGEM-T Easy vector were as described above.

The recombinant pGEM-T Easy vectors containing the PCR products for constructing p3'LS, p3'ΔUS1, p3'ΔUS2, p3'ΔIL, p3'ILS1, p3'ILS2, p3'ILS3, p3'ILS4, p3'ILS5, and p3'ΔLUI, were digested with AatII and ApaI , and the released DNA fragments were subcloned into similarly digested pCM2, resulting in the respective final products.

Table M-1. List of Oligonucleotide primers used in appendix M

Primer	Sequence (5' - 3') and polarity (+ or -)	Description	Use for
LCV-161-CM	TGATAAGTTGAGAGTGTCCGATCAGG	Nucleotides corresponding to LCV RNA 2 position 7313-7339	Construction of p3'LS, p3'ΔUS1, p3'ΔUS2, p3'ΔIL, p3'ILS1, p3'ILS2, p3'ILS3, p3'ILS4, p3'ILS5, and
LCV-164-CM	GGGTTGAGTGTTGTTCCAGTTTG	Nucleotides complementary to position 492-514 of the pGEM-T Easy backbone of pCM2	Construction of p3'LS, p3'ΔUS1, p3'ΔUS2, p3'ΔIL, p3'ILS1, p3'ILS2, p3'ILS3, p3'ILS4, p3'ILS5, and
LCV-252-CM	*GATCCC* **ATCA**GGATTCTTTTGAACGAC	Nucleotides complementary to LCV RNA 2 position 8521-8516 (italic), 8511-8495 (underlined), and nucleotide substitution position 8515-8512 with non-viral nucleotide (bold)	Construction of p3'LS
LCV-253-CM	GAATCC**TGAT***GGGATCGCCTTGAACG*	Nucleotides corresponding to LCV RNA 2 position 8506-8511(underlined), 8516-8531 (italic) and nucleotides substitution position 8512-8515 with non-viral nucleotides (bold)	Construction of p3'LS
LCV-254-CM	**CGTTCAAGGC**TTTTGAACGACGACTTTATT	Nucleotides complementary to LCV RNA 2 position 8531-8522 (bold) and 8505-8486	Construction of p3'ΔUS1
LCV-255-CM	TCGTTCAAAA**GCCTTGAACGACTAGTTAGTAG**	Nucleotides corresponding to LCV RNA 2 position 8496-8505 and 8522-8543 (bold)	Construction of p3'ΔUS1
LCV-256-CM	**GTTCAAGGCG**CTTTTGAACGACGACTTTAT	Nucleotides complementary to LCV RNA 2 position 8530-8521 (bold) and 8506-8487	Construction of p3'ΔUS2
LCV-257-CM	CGTTCAAAAG**CGCCTTGAACGACTAGTTAG**	Nucleotides corresponding to LCV RNA 2 position 8497-8506 and 8521-8540 (bold)	Construction of p3'ΔUS2
LCV-258-CM	**TAGTGGATTC**TTGAACGACGACTTTATTTAATG	Nucleotides complementary to LCV RNA 2 position 8515-8506 (bold) and 8503-8481	Construction of p3'ΔIL
LCV-259-CM	CGTCGTTCAA**GAATCCACTAGGGATCGCC**	Nucleotides corresponding to LCV RNA 2 position 8494-8503 and 8506-8524 (bold)	Construction of p3'ΔIL
LCV-260-CM	**AGTCGTTCAAG**ATCCCTAGTGGATTC*TTGA*	Nucleotides complementary to LCV RNA 2 position 8534-8525 (bold), 8521-8506, 8503-8500 (italic)	Construction of p3'ΔIL
LCV-261-CM	ACTAGGGATC**TTGAACGACTAGTTAGTAGT**	Nucleotides corresponding to LCV RNA 2 position 8512-8521 and 8525-8544 (bold)	Construction of p3'ΔIL
LCV-262-CM	*GTGGATTCT* **G**TTGAACGACGACTTTATTTA	Nucleotides complementary to LCV RNA 2 position 8513-8505 (italic), 8503-8484 (underlined), and nucleotide substitution position 8504 with non-viral nucleotide (bold)	Construction of p3'ILS1
LCV-263-CM	GTCGTTCAA*CAGAATCCACTAGGGATCGCC*	Nucleotides corresponding to LCV RNA 2 position 8495-8503 (underlined), 8505-8524 (italic) and nucleotides substitution position 8504 with non-viral nucleotides (bold)	Construction of p3'ILS1
LCV-264-CM	*TC GTTCAAG* **T**CGATCCCTAGTGGATTCTTT	Nucleotides complementary to LCV RNA 2 position 8532-8524 (italic), 8522-8503 (underlined), and nucleotide substitution position 8523 with non-viral nucleotide (bold)	Construction of p3'ILS2
LCV-265-CM	TAGGGATCG**A***CTTGAACGACTAGTTAGTAG*	Nucleotides corresponding to LCV RNA 2 position 8514-8522 (underlined), 8524-8543 (italic) and nucleotides substitution position 8523 with non-viral nucleotides (bold)	Construction of p3'ILS2
LCV-266-CM	*TCGTTCAA* **T**GCGATCCCTAGTGGATTCTTT	Nucleotides complementary to LCV RNA 2 position 8532-8525 (italic), 8523-8503 (underlined), and nucleotide substitution position 8524 with non-viral nucleotide (bold)	Construction of p3'ILS3
LCV-267-CM	AGGGATCGC**A***TTGAACGACTAGTTAGTAGT*	Nucleotides corresponding to LCV RNA 2 position 8515-8523 (underlined), 8525-8544 (italic) and nucleotides substitution position 8524 with non-viral nucleotides (bold)	Construction of p3'ILS3
LCV-268-CM	*CGTTCAAGGC* **G**TTTGAACGACGACTTTATT	Nucleotides complementary to LCV RNA 2 position 8544-8525 (italic), 8504-8486 (underlined), and nucleotide substitution position 8505 with non-viral nucleotide (bold)	Construction of p3'ILS4
LCV-269-CM	TCGTTCAAA*CGCCTTGAACGACTAGTTAGT*	Nucleotides corresponding to LCV RNA 2 position 8496-8504 (underlined), 8522-8541 (italic) and nucleotides substitution position 8505 with non-viral nucleotides (bold)	Construction of p3'ILS4
LCV-270-CM	*CGTTCAAGG* **A**TTTTGAACGACGACTTTATT	Nucleotides complementary to LCV RNA 2 position 8531-8523 (italic), 8505-8486 (underlined), and nucleotide substitution position 8522 with non-viral nucleotide (bold)	Construction of p3'ILS5
LCV-271-CM	TCGTTCAAAA*TCCTTGAACGACTAGTTAGT*	Nucleotides corresponding to LCV RNA 2 position 8496-8505 (underlined), 8523-8541 (italic) and nucleotides substitution position 8522 with non-viral nucleotides (bold)	Construction of p3'ILS5
LCV-281-CM	**AGTCGTTCAAT**TGAACGACGACTTTATTTA	Nucleotides complementary to LCV RNA 2 position 8534-8525 (bold) and 8503-8484	Construction of p3'ΔLUI
LCV-282-CM	CGTCGTTCAA**TTGAACGACTAGTTAGTAGT**	Nucleotides corresponding to LCV RNA 2 position 8494-8503 and 8525-8544 (bold)	Construction of p3'ΔLUI

Appendix N. Inventory

-80^{0}C freezer

Level #1 Tower #3:

Box#:	**Box Name:**	**Content:**
#1	Chawin's protoplast #1	Total RNA and collected inoculated protoplasts: pCM1 and pCM2 (1:3) (2ug:6ug) (0-96hpi) pCM1 and pCM2 (1:1) (2ug:2ug) (0-96hpi) pCM2 only (96hpi) Total protein lysate from protoplasts inoculated with pCM1 and pCM2. Total RNA extracted from LCV-infected and healthy plants (C. murales and lettuce) using TRIZOL and LiCl method
#2	Chawin's protoplast #2	Various riboprobes for Northern blot
#3	Chawin's protoplast #3	Total RNA and collected inoculated protoplasts: Clone #1-5 (24 and 96hpi) Clone #6-9 (24 and 96hpi)
#4	Chawin's protoplast #4	Clone #6-7 (24 and 96hpi) Clone #8-9 (24 and 96hpi) Clone #5-9 (96hpi)
#5	Chawin's protoplast #5	Clone #5-9 (24 and 96hpi) Clone #1-5 (24 and 96hpi)
#6	Chawin's protoplast #6	pCM1 and pCM2 (0-96hpi) pCM1 only (0-96hpi) pCM1 and dRNA1-GFP (0-96hpi) pCM1, pCM2 and dRNA1-GFP (0-96hpi)
#7	Chawin's protoplast #7	Clone #12-13 (24 and 96hpi) Clone #10-11 (24 and 96hpi) Clone #5 and #13 (24, 48, and 96hpi) Clone #6 and #7 (24, 48, and 96hpi)
#8	Chawin's protoplast #8	pR1-3'R2 and pR2-3'R1 (24 and 96hpi)

		pR1-3'R2 and pR2-3'R1 (24, 48, and 96hpi)
#9	Chawin's protoplast #9	pR1-3'R2 and pR2-3'R1 (24, 48, and 96hpi) Clone #14 (24, 48, and 96hpi)
#10	Chawin's protoplast #10	Clone #15-22 (24, 48, and 96hpi)
#11	Chawin's protoplast #11	Clone #17-23 (24, 48, and 96hpi)
#12	Chawin's transcript #6	Transcripts of pCM1, pCM2, 3' NCR LCV RNA 2 Clone #6, 7, 10, 11, 24, 25 and dRNA2 P4 + linker

Level #1 Tower #4:

Box#:	**Box Name:**	**Content:**
#1	5b and 5bm1 plant tissue	Plant tissue from agro-infiltrated with 5b and 5bm1
#2	Chawin's temporary total RNA	Plant tissue and total RNA from: LCV infected agro-infiltrated pJW168, TC2, TC3, and 5b
#3	Chawin's vRNA #1	Virion RNA of BYV extracted with proteinase K and without proteinase K
#4	Chawin's vRNA #2	Virion RNA of CMV and BYV extracted with proteinase K
#5	Chawin's glycerol stock #1	Glycerol Stocks of various constructs
#6	Chawin's glycerol stock #2	Glycerol stocks of agro-construct transformed into agrobacterium GV3101 and C58C1
#7	Chawin's glycerol stock #3	Glycerol stocks of expression vector ORF1a/1b and ORF1b in *E.Coli* strain BL21(DE) pLysS
#8	Chawin's transcript #5	Transcripts of pCM1, pCM2, 3' NCR LCV RNA 2 Clone #17-22, and SHAPE cassette + 3' LCV RNA2 XbaI/Ngo
#9	Chawin's transcript #1	Transcripts of CMV Fny 109, 209, 309, TMV30BGFP, pCM1 and pCM2

#10	Chawin's transcript #2	Transcript of 3' NCR LCV RNA 2 Clone #1-13 and defective RNA 1 with GFP (dRNA1-GFP)
#11	Chawin's transcript #3	Transcripts of pCM1, pCM2, pR1-3'R2, pR2-3'R1, and 3' NCR LCV RNA 2 Clone #14
#12	Chawin's transcript #4	Transcripts of 3' NCR LCV RNA 2 Clone #15-16, M5GFP, LCV RNA2 SL, GFP, Biotinylated LCV RNA2 SL, GFP, and RNA control from kit

Level 2 Tower #3:

Box#:	**Box Name:**	**Content:**
#9	Chawin's protoplast #12	Clone #10, 11, 23, 25 (24, 48, and 96 hpi) dRNA2 P4 (24, 48, and 96 hpi)
#10	Chawin's protoplast #13	Clone #10-11, 20-22, 24, 25 (24, 48, and 96hpi)

-20^{0}C freezer

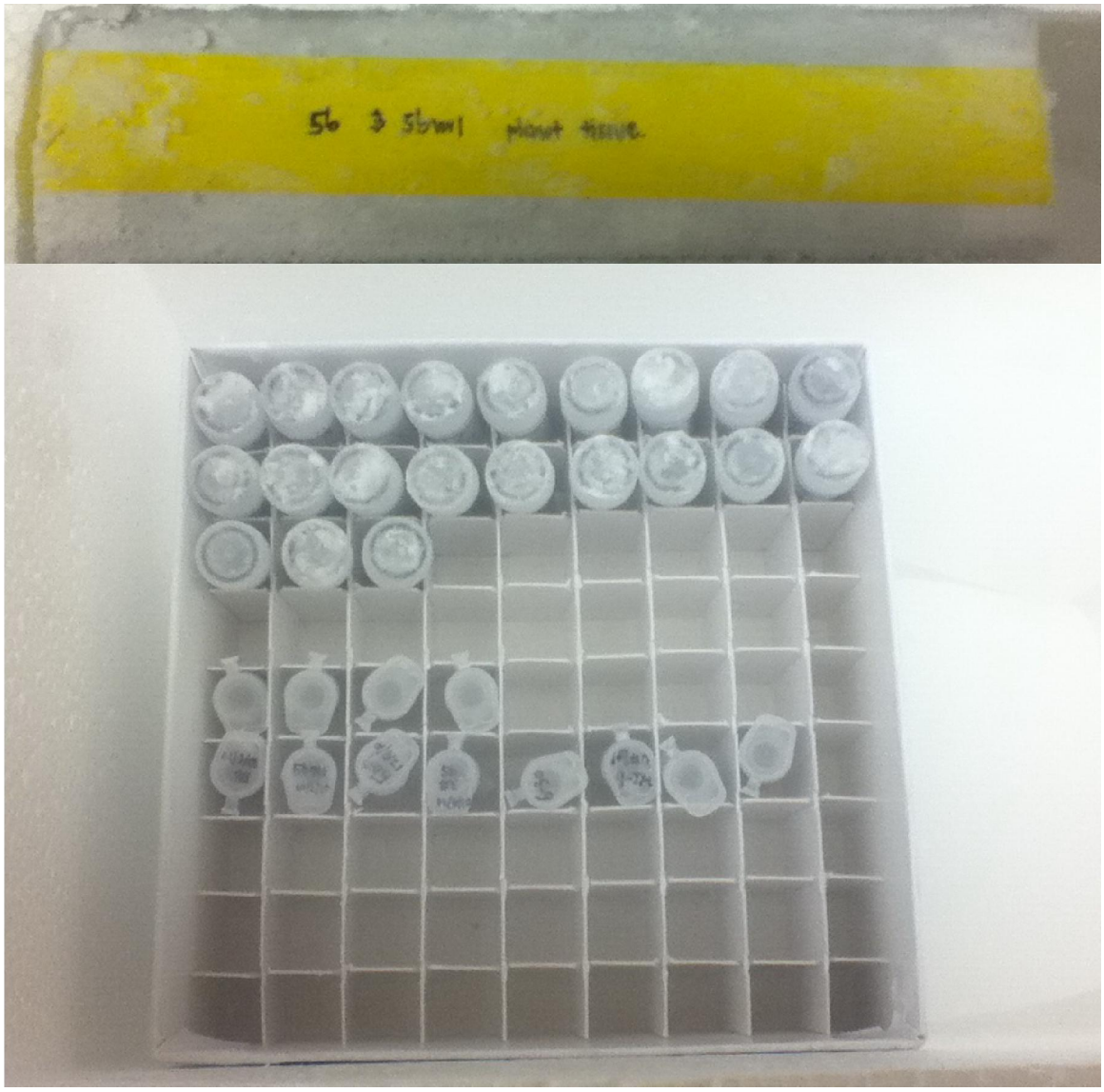
Box Name:	**Content:**
Chawin's Box #1	various final clones and constructs
Chawin's Box #2	various final clones and constructs
Chawin's Box #3-6	various final 5'RACE products
Chawin's Work box #1-12	various intermediate products and clones
Chawin's Primer Stock #1-2	various primer stock used for
Chawin's Virion #1	various purified LIYV and LCV virions

Box Schematic:

	1	2	3	4	5	6	7	8	9
A	A1	A2	A3	A4	A5	A6	A7	A8	A9
B	B1	B2	B3	B4	B5	B6	B7	B8	B9
C	C1	C2	C3	C4	C5	C6	C7	C8	C9
D	D1	D2	D3	D4	D5	D6	D7	D8	D9
E	E1	E2	E3	E4	E5	E6	E7	E8	E9
F	F1	F2	F3	F4	F5	F6	F7	F8	F9
G	G1	G2	G3	G4	G5	G6	G7	G8	G9
H	H1	H2	H3	H4	H5	H6	H7	H8	H9
I	I1	I2	I3	I4	I5	I6	I7	I8	I9

5b & 5bm1 plant tissue: -80^{0}C Level #1 Tower #4 Box #1

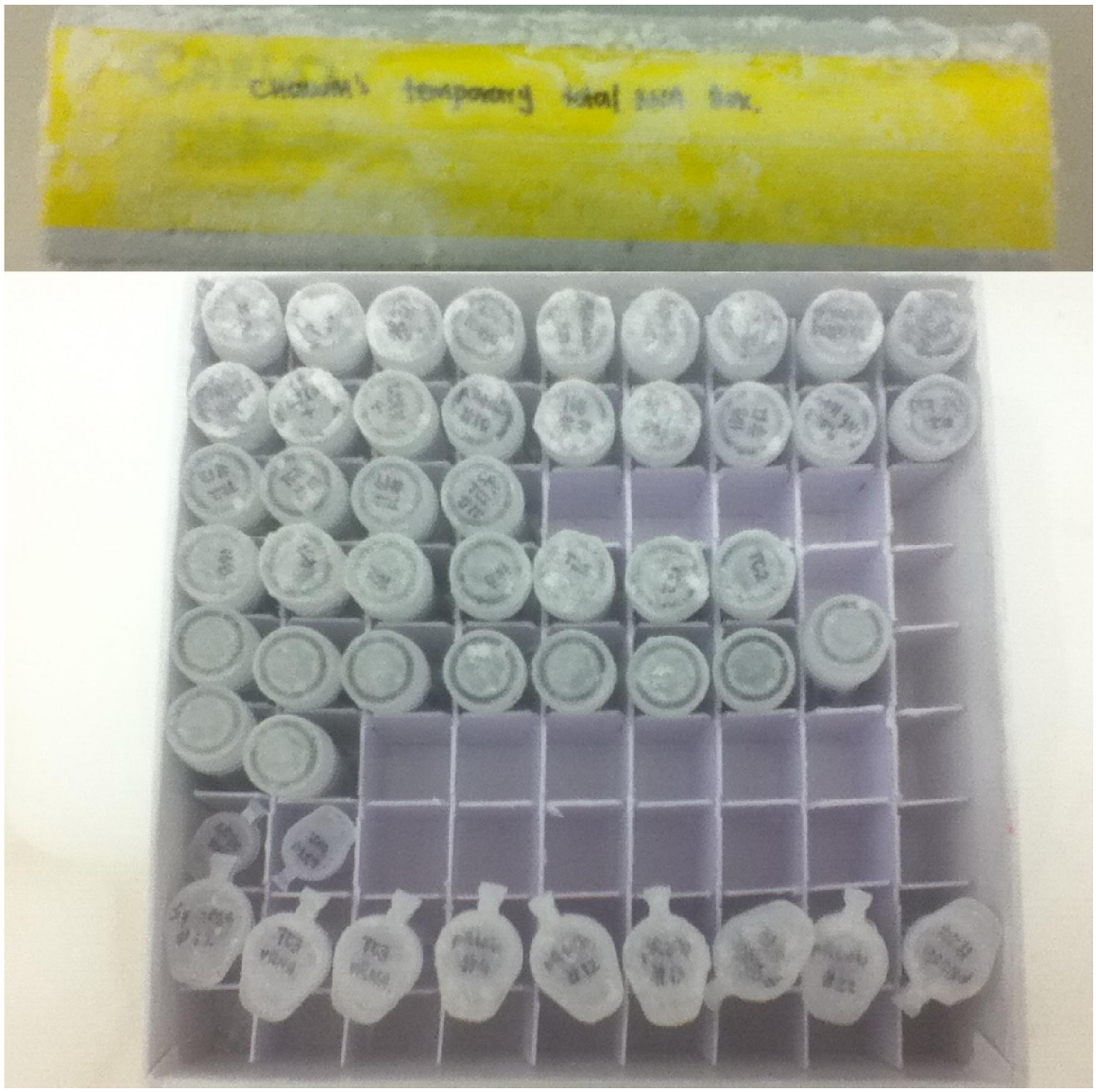
	Description:	Date:	NB pg:
A1	5b	10/6/10 collect 6/21/11	1050
A2	5b	10/6/10 collect 6/21/11	1050
A3	5bm1	10/6/10 collect 6/21/11	1050
A4	5bm1	10/6/10 collect 6/21/11	1050
A5	5b #1	12/8/10 collect 6/21/11	1050
A6	5b #1	12/8/10 collect 6/21/11	1050
A7	5b #2	12/8/10 collect 6/21/11	1050
A8	5b #3	12/8/10 collect 6/21/11	1050
A9	5bm1 #1	12/8/10 collect 6/21/11	1050
B1	5bm1 #1	12/8/10 collect 6/21/11	1050
B2	5bm1 #2	12/8/10 collect 6/21/11	1050
B3	5bm1 #2	12/8/10 collect 6/21/11	1050
B4	5bm1 #3	12/8/10 collect 6/21/11	1050
B5	5bm1 #3	12/8/10 collect 6/21/11	1050
B6	5b	2/16/11 collect 6/21/11	1050
B7	5b	2/16/11 collect 6/21/11	1050
B8	5b	3/24/11 collect 6/21/11	1050
B9	5b	3/24/11 collect 6/21/11	1050
C1	5bm1	3/24/11 collect 6/21/11	1050
C2	5bm1	3/24/11 collect 6/21/11	1050
C3	5bm1	3/24/11 collect 6/21/11	1050
C4			
C5			
C6			
C7			
C8			
C9			
E1	Total RNA 5bm1 #3 by Steve	12/8/10 collect 6/21/11 ext. 8/29/11	
E2	Total RNA 5bm1 #2 by Steve	12/8/10 collect 6/21/11 ext. 8/29/11	
E3	Total RNA 5bm1 #1 by Steve	12/8/10 collect 6/21/11 ext. 8/29/11	
E4	Total RNA 5b #3 by Steve	12/8/10 collect 6/21/11 ext. 8/29/11	
E5			
E6			
E7			
E8			
E9			
F1	Total RNA 5b by CM	10/6/10 collect 6/21/11 ext. 6/21/11	1050
F2	Total RNA 5bm1 by CM	10/6/10 collect 6/21/11 ext. 6/21/11	1050
F3	Total RNA 5b #1 by Steve	12/18/10 collect 6/21/11 ext. 6/22/11	
F4	Total RNA 5b #2 by Steve	6/30/10 collect 7/18/11 ext. 7/18/11	
F5	Total RNA 168 #8 by Steve	4/27/11 collect 7/18/11 ext. 7/18/11	
F6	Total RNA 5b by Steve	3/24/11 collect 6/21/11 ext. 9/7/11	
F7	Total RNA 5b by Steve	2/16/11 collect 6/21/11 ext. 9/7/11	
F8			
F9			


56 3 5bml plant tissue

Chawin's temporary total RNA box: -80^{0}C Level #1 Tower #4 Box #2

	Description:	Date:	NB pg:	
A1	LCV infected Murale	11/10/2009		
A2	LCV infected Murale	11/10/2009		
A3	LCV infected Lettuce	12/8/2009		
A4	LCV infected Lettuce	12/8/2009		
A5	LCV infected Murale	12/8/2009		
A6	Healthy Murale	12/8/2009		
A7	Healthy Murale	12/8/2009		
A8	Healthy Lettuce	12/8/2009		
A9	Healthy Lettuce	12/8/2009		
B1	Healthy Lettuce	12/8/2009		
B2	LIYV #5 Lettuce	7/24/2009		
B3	LIYV #5 Lettuce	7/24/2009		
B4	pJW168 #10	9/24/2009		622
B5	pJW168 #2	11/12/2009		667
B6	pJW168 #21	11/12/2009		667
B7	pJW168 #24	11/12/2009		667
B8	TC2 Infiltrated Leaves	8/18/10 collect 9/7/10		848
B9	TC2 Infiltrated Leaves	8/18/10 collect 9/7/10		848
C1	TC2 #13	11/18/2010		891
C2	TC2 #18	11/18/2010		891
C3	TC2 #19	11/18/2010		891
C4	TC2 #15 NB CP+	12/8/2010		909
C5				
C6				
C7				
C8				
C9				
D1	pJW168 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D2	pJW168 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D3	pJW168 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D4	pJW168 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D5	TC2 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D6	TC2 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D7	TC2 Infiltrated Leaves	3/2/11 collect 3/8/11	965	
D8				
D9				
E1	TC3 #1 Lettuce	7/20/11 collect 8/18/11	1074	
E2	TC3 #2 Lettuce	7/20/11 collect 8/18/11	1074	
E3	TC3 #3 Lettuce	7/20/11 collect 8/18/11	1074	
E4	TC3 #4 Lettuce	7/20/11 collect 8/18/11	1074	
E5	TC3 #5 Lettuce	7/20/11 collect 8/18/11	1074	
E6	TC3 #6 Lettuce	7/20/11 collect 8/18/11	1074	
E7	TC3 #7 Lettuce	7/20/11 collect 8/18/11	1074	
E8	TC3 #8 Lettuce	7/20/11 collect 8/18/11	1074	
E9				

F1	LIYV #9 Lettuce	7/20/11 collect 8/18/11	1074
F2	LIYV #10 Lettuce	7/20/11 collect 8/18/11	1074
F3			
F4			
F5			
F6			
F7			
F8			
F9			
G1	LIYV WT	ext. 1/13/11	920
G2	LIYV WT	ext. 1/12/11	920
G3			
G4			
G5			
G6			
G7			
G8			
G9			
H1	S. purse #12 BFNB2-9/16/02-5		
H2	TC3 vRNA	8/16/2010	844
H3	TC3 vRNA	9/20/2010	878
H4	pR65b #24	2/24/10 ext. 3/3/10	749
H5	pR65b #22	2/24/10 ext. 3/3/10	749
H6	pR65b #18	2/24/10 ext. 3/3/10	749
H7	pR65b #13	2/24/10 ext. 3/3/10	749
H8	pR65b #12	2/24/10 ext. 3/3/10	749
H9	pR65b #4	2/24/10 ext. 3/3/10	749
I1			
I2			
I3			
I4			
I5			
I6			
I7			
I8			
I9			



Chawin's vRNA box #1: -80^{0}C Level #1 Tower #4 Box #3

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A3	BYV vRNA ext. with proteinase K	5/2/2008	110
A4	BYV vRNA ext. with proteinase K	5/2/2008	110
A5	BYV vRNA ext. with proteinase K	5/2/2008	110
A6	BYV vRNA ext. with proteinase K	5/2/2008	110
A7	BYV vRNA ext. with proteinase K	5/2/2008	110
A8	BYV vRNA ext. with proteinase K	5/2/2008	110
A9	BYV vRNA ext. with proteinase K	5/2/2008	110
B1	BYV vRNA ext. with proteinase K	5/2/2008	110
B2	BYV vRNA ext. with proteinase K	5/2/2008	110
B3	BYV vRNA ext. with proteinase K	5/2/2008	110
B4	BYV vRNA ext. with proteinase K	5/2/2008	110
B5	BYV vRNA ext. with proteinase K	5/2/2008	110
B6	BYV vRNA ext. with proteinase K	5/2/2008	110
B7	BYV vRNA ext. with proteinase K	5/2/2008	110
B8	BYV vRNA ext. with proteinase K	5/2/2008	110
B9	BYV vRNA ext. with proteinase K	5/2/2008	110
C1	BYV vRNA ext. with proteinase K	5/2/2008	110
C2	BYV vRNA ext. with proteinase K	5/2/2008	110
C3	BYV vRNA ext. with proteinase K	5/2/2008	110
C4	BYV vRNA ext. with proteinase K	5/2/2008	110
C5	BYV vRNA ext. with proteinase K	5/2/2008	110
C6	BYV vRNA ext. with proteinase K	5/2/2008	110
C7	BYV vRNA ext. with proteinase K	5/2/2008	110
C8	BYV vRNA ext. with proteinase K	5/2/2008	110
C9	BYV vRNA ext. with proteinase K	5/2/2008	110
D1	BYV vRNA ext. with proteinase K	5/2/2008	110
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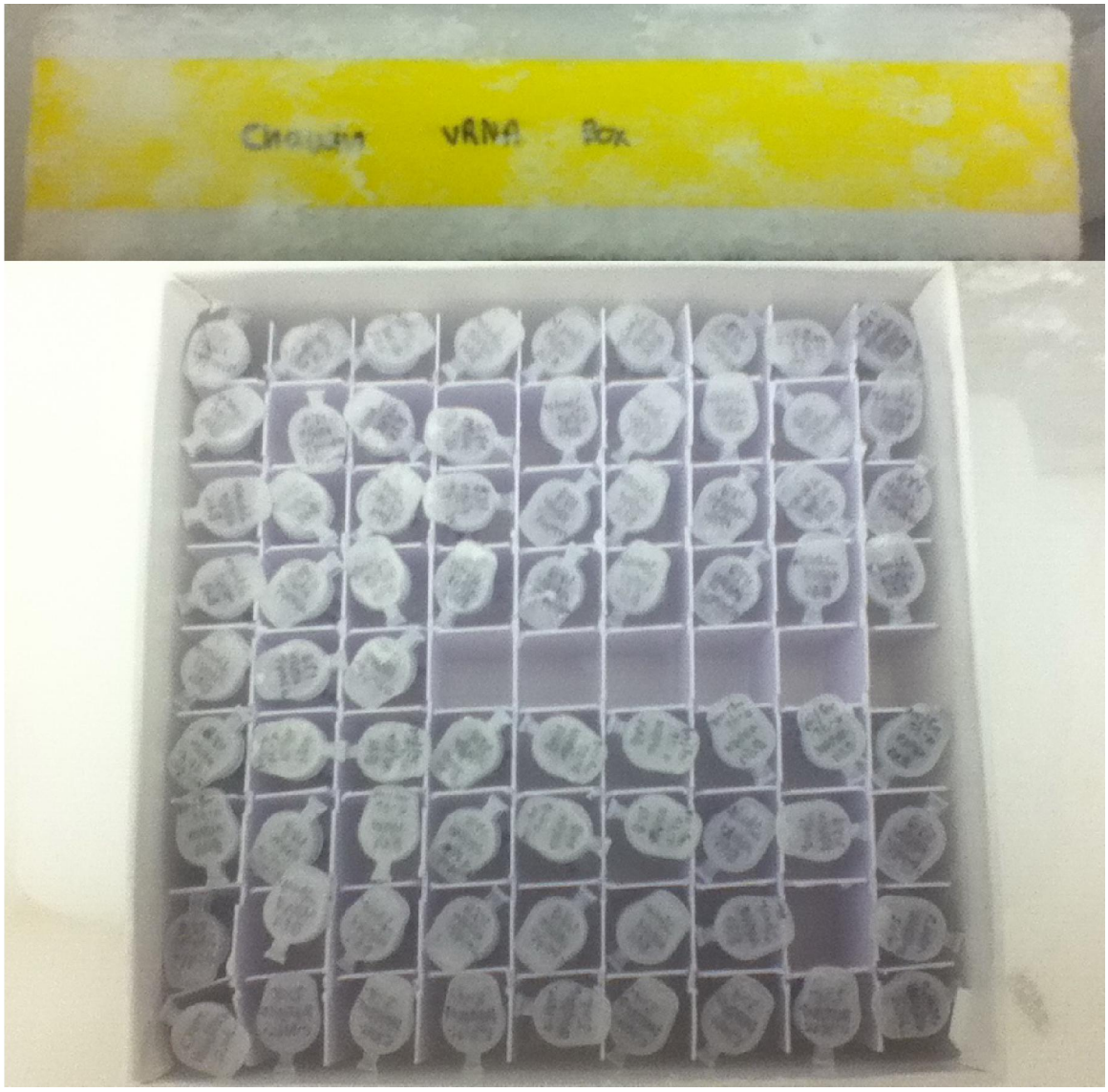
E2	BYV vRNA ext. with proteinase K	5/2/2008	110
E3	BYV vRNA ext. with proteinase K	5/2/2008	110
E4			
E5			
E6			
E7			
E8			
E9			

F1	BYV vRNA	5/2/2008	110
F2	BYV vRNA	5/2/2008	110
F3	BYV vRNA	5/2/2008	110
F4	BYV vRNA	5/2/2008	110
F5	BYV vRNA	5/2/2008	110
F6	BYV vRNA	5/2/2008	110
F7	BYV vRNA	5/2/2008	110
F8	BYV vRNA	5/2/2008	110
F9	BYV vRNA	5/2/2008	110
G1	BYV vRNA	5/2/2008	110
G2	BYV vRNA	5/2/2008	110
G3	BYV vRNA	5/2/2008	110
G4	BYV vRNA	5/2/2008	110
G5	BYV vRNA	5/2/2008	110
G6	BYV vRNA	5/2/2008	110
G7	BYV vRNA ext. with proteinase K	7/10/2008	147-148
G8	BYV vRNA ext. with proteinase K	7/10/2008	147-148
G9	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H1	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H2	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H3	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H4	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H5	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H6	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H7	BYV vRNA ext. with proteinase K	7/10/2008	147-148
H8			
H9	C. Murale Healthy	10/3/2008	232-233
I1	C. Murale Healthy	10/3/2008	232-233
I2	C. Murale Healthy	10/3/2008	232-233
I3	C. Murale Healthy	10/3/2008	232-233
I4	C. Murale Healthy	10/3/2008	232-233
I5	C. Murale Healthy	10/3/2008	232-233
I6	C. Murale Healthy	10/3/2008	232-233
I7	C. Murale Healthy	10/3/2008	232-233
I8	C. Murale Healthy	10/3/2008	232-233
I9	C. Murale Healthy	10/3/2008	232-233


VRNA Box

Chawin's vRNA box #2: -80^{0}C Level #1 Tower #4 Box #4

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A1	CMV vRNA	4/30/2009	429
A2	CMV vRNA	4/30/2009	429
A3	CMV vRNA	4/30/2009	429
A4	CMV vRNA	4/30/2009	429
A5	CMV vRNA	4/30/2009	429
A6	CMV vRNA	4/30/2009	429
A7	CMV vRNA	4/30/2009	429
A8	CMV vRNA	4/30/2009	429
A9	CMV vRNA	4/30/2009	429
B1	CMV vRNA	4/30/2009	429
B2	CMV vRNA	4/30/2009	429
B3	CMV vRNA	4/30/2009	429
B4	CMV vRNA	4/30/2009	429
B5	CMV vRNA	4/30/2009	429
B6	CMV vRNA	4/30/2009	429
B7	CMV vRNA	4/30/2009	429
B8	CMV vRNA	4/30/2009	429
B9	CMV vRNA	4/30/2009	429
C1	CMV vRNA	4/30/2009	429
C2	CMV vRNA	4/30/2009	429
C3	CMV vRNA	4/30/2009	429
C4	CMV vRNA	4/30/2009	429
C5	CMV vRNA	4/30/2009	429
C6	CMV vRNA	4/30/2009	429
C7	CMV vRNA	4/30/2009	429
C8	CMV vRNA	4/30/2009	429
C9	CMV vRNA	4/30/2009	429
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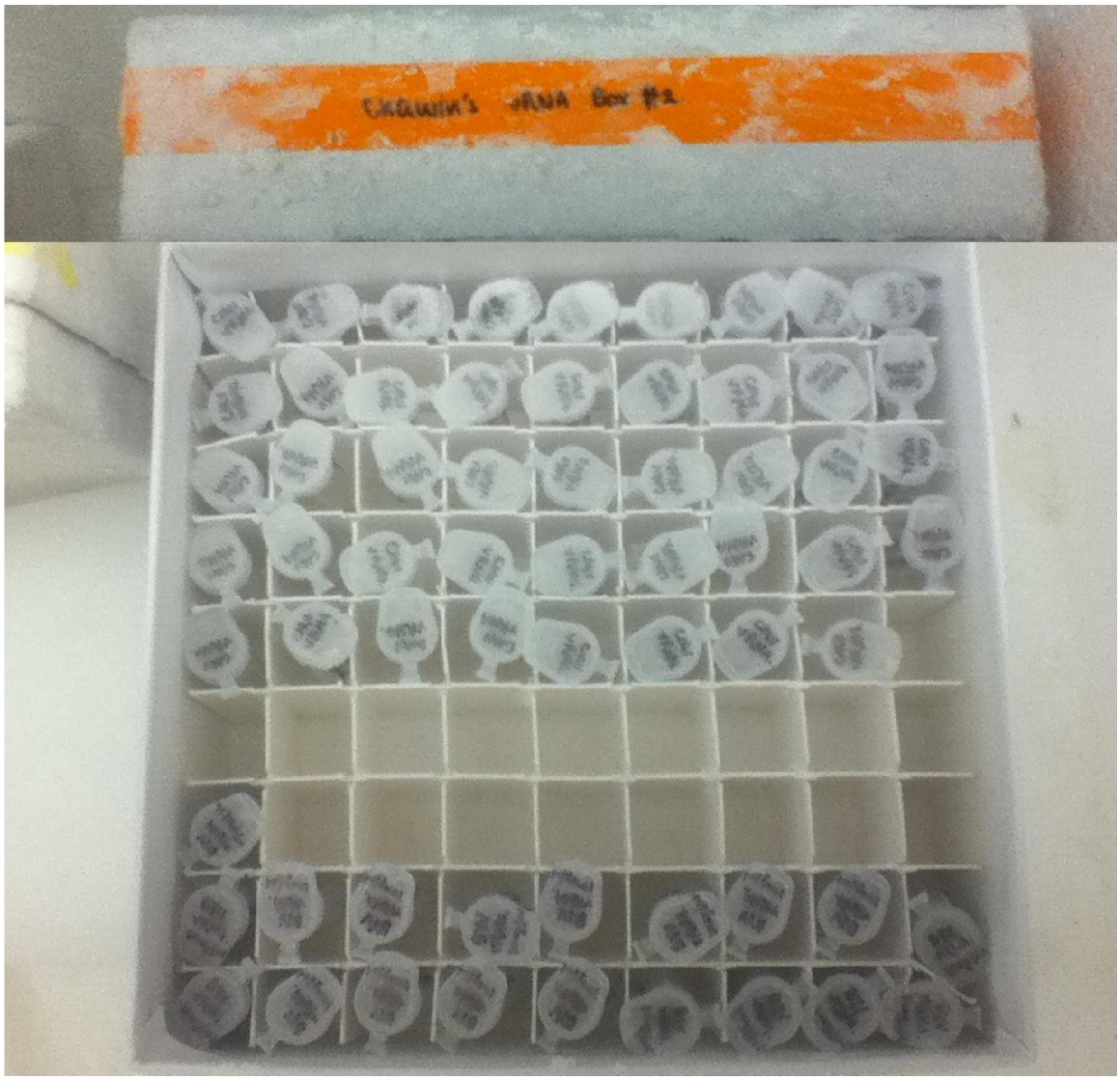
D2	CMV vRNA	4/30/2009	429
D3	CMV vRNA	4/30/2009	429
D4	CMV vRNA	4/30/2009	429
D5	CMV vRNA	4/30/2009	429
D6	CMV vRNA	4/30/2009	429
D7	CMV vRNA	4/30/2009	429
D8	CMV vRNA	4/30/2009	429
D9	CMV vRNA	4/30/2009	429
E1	CMV vRNA	4/30/2009	429
E2	CMV vRNA	4/30/2009	429
E3	CMV vRNA	4/30/2009	429
E4	CMV vRNA	4/30/2009	429
E5	CMV vRNA	4/30/2009	429
E6	CMV vRNA	4/30/2009	429
E7	CMV vRNA	4/30/2009	429
E8	CMV vRNA	4/30/2009	429
E9			

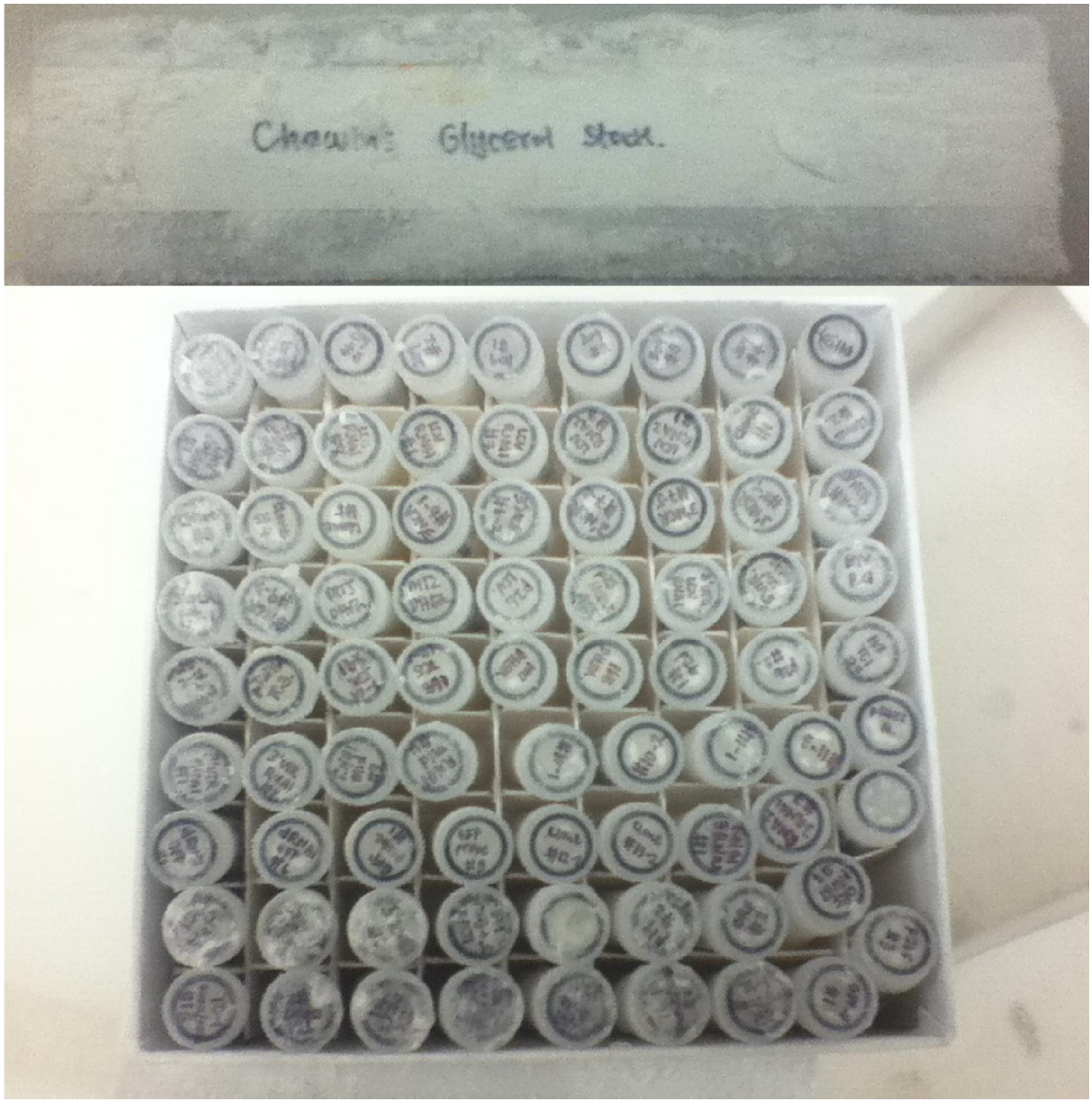
F1			
F2			
F3			
F4			
F5			
F6			
F7			
F8			
F9			
G1	BYV vRNA ext. with proteinase K	5/2/2008	110
G2			
G3			
G4			
G5			
G6			
G7			
G8			
G9			
H1	BYV vRNA ext. with proteinase K	5/2/2008	110
H2	BYV vRNA ext. with proteinase K	5/2/2008	110
H3	BYV vRNA ext. with proteinase K	5/2/2008	110
H4	BYV vRNA ext. with proteinase K	5/2/2008	110
H5	BYV vRNA ext. with proteinase K	5/2/2008	110
H6	BYV vRNA ext. with proteinase K	5/2/2008	110
H7	BYV vRNA ext. with proteinase K	5/2/2008	110
H8	BYV vRNA ext. with proteinase K	5/2/2008	110
H9	BYV vRNA ext. with proteinase K	5/2/2008	110
I1	BYV vRNA ext. with proteinase K	5/2/2008	110
I2	BYV vRNA ext. with proteinase K	5/2/2008	110
I3	BYV vRNA ext. with proteinase K	5/2/2008	110
I4	BYV vRNA ext. with proteinase K	5/2/2008	110
I5	BYV vRNA ext. with proteinase K	5/2/2008	110
I6	BYV vRNA ext. with proteinase K	5/2/2008	110
I7	BYV vRNA ext. with proteinase K	5/2/2008	110
I8	BYV vRNA ext. with proteinase K	5/2/2008	110
I9	BYV vRNA ext. with proteinase K	5/2/2008	110



Chawin's glycerol stock #1: -80^{0}C Level #1 Tower #4 Box #5

	Description:	Date:	NB pg:
A1	CMV p109 #6	11/26/2008	289
A2	CMV p109 #5	11/26/2008	289
A3	CMV p209 #3	11/26/2008	289
A4	CMV p209 #2	11/26/2008	289
A5	CMV p309 #2	1/6/2009	293
A6	CMV p309 #3	1/6/2009	293
A7	CMV p309 #3	1/13/2009	303
A8	CMV p309 #4	1/13/2009	303
A9	E. coli DH5a for making competent cell		
B1	TMV30BGFP #1	1/9/2009	299
B2	TMV30BGFP #2	1/9/2009	299
B3	LCV RNA1 #8 (pCM1)	3/31/2009	400
B4	LCV RNA1 #1 (pCM1) midiprep final	4/30/2009	430
B5	LCV RNA1 #7 (pCM1) midiprep final	4/30/2009	430
B6	LCV RNA2 #7 (pCM2)	8/13/2009	585
B7	LCV RNA2 #1 (pCM2)	8/13/2009	585
B8	3'NCR RNA2 Clone #1	11/19/2009	675
B9	3'NCR RNA2 Clone #2-1	11/12/2009	675
C1	3'NCR RNA2 Clone #3-1	11/19/2009	675
C2	3'NCR RNA2 Clone #4-1	11/12/2009	675
C3	3'NCR RNA2 Clone #5	11/12/2009	675
C4	3'NCR RNA2 Clone #6-1	2/8/2010	728
C5	3'NCR RNA2 Clone #6-2	2/8/2010	728
C6	3'NCR RNA2 Clone #7-2	2/8/2010	728
C7	3'NCR RNA2 Clone #7-5	2/8/2010	728
C8	3'NCR RNA2 Clone #8-1	2/8/2010	728
C9	3'NCR RNA2 Clone #8-2	2/8/2010	728
D1	3'NCR RNA2 Clone #9-1	2/8/2010	728
D2	3'NCR RNA2 Clone #9-3	2/8/2010	728
D3	MT5#3 p1-5b1 (agro) maxi DH5a	12/17/2009	695
D4	MT2#1 p1-5bm1 (agro) maxi DH5a	12/17/2009	695
D5	LCV p26 pGEMT for probe	4/7/2010	769
D6	LCV p23 (A23 from AC) for probe	5/15/2009	450
D7	5'UTR LCV RNA1 for probe	8/30/2010	864
D8	5'UTR LCV RNA2 for probe	8/30/2010	864
D9	BYV p21 (from AC) for probe	8/24/2010	850
E1	pJW168+TC2 #19 maxiprep DH5a	4/9/2009	411
E2	pJW168+TC3 #4 DH5a	5/7/2009	451
E3	pJW168+TC5 #10-1 DH5a	7/13/2009	490
E4	pJW168+TC5 #10-2 DH5a	7/13/2009	490
E5	pJW100 DH5a	10/19/2010	871
E6	pJW168 DH5a	10/19/2010	871
E7	LCV p26-1 pGEMT	11/8/2010	887
E8	LCV p26-2 pGEMT	11/8/2010	887
E9	pJW168+TC1 #7 maxiprep DH5a	5/18/2011	1030

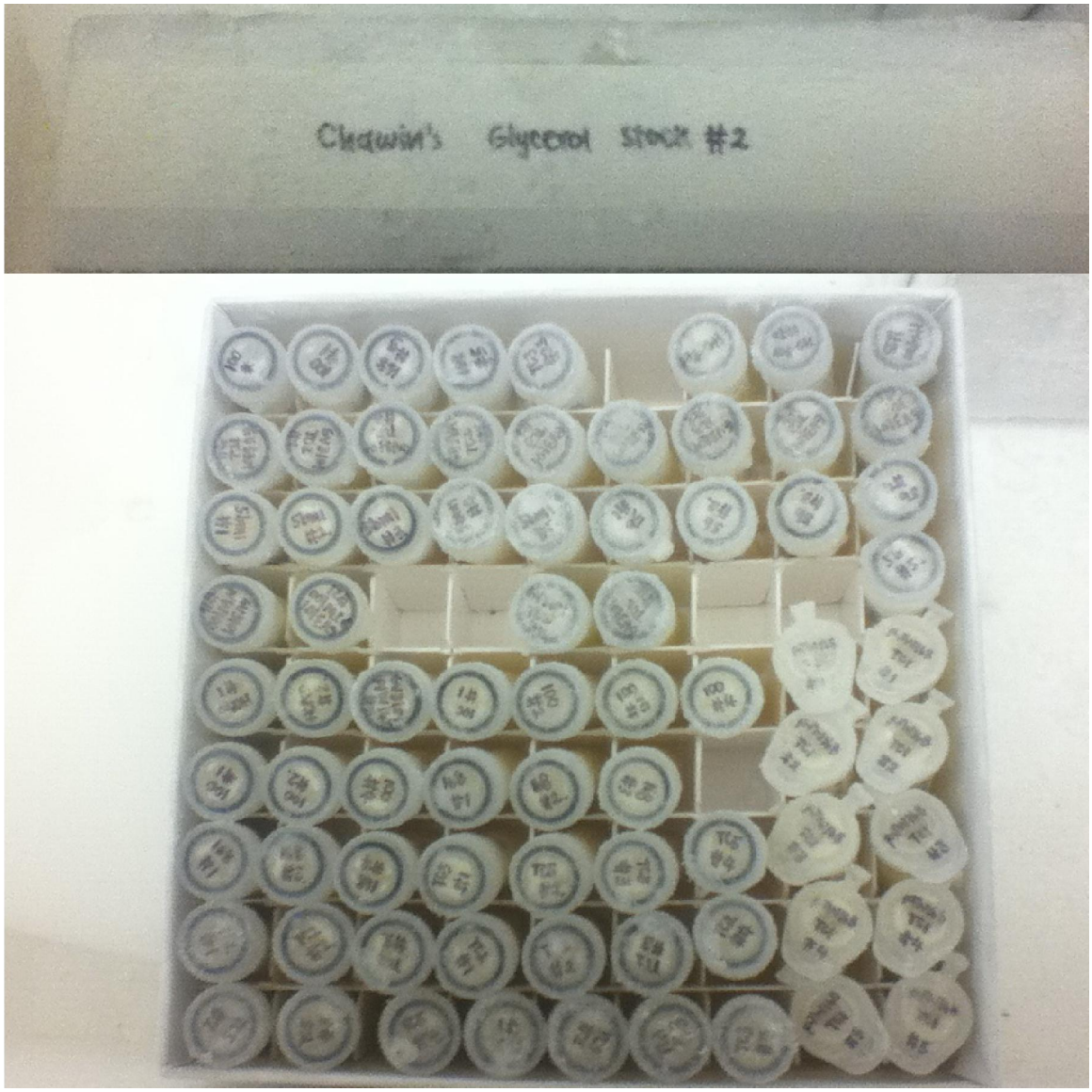
F1	3'UTR LCV RNA1 #1 for probe	12/11/2010	912
F2	3'UTR LCV RNA1 #2 for probe	12/11/2010	912
F3	LCV RNA1 mid probe #3	1/20/2011	923
F4	LCV RNA1 mid probe #4	1/20/2011	923
F5	3'NCR RNA2 Clone #10-1	3/10/2011	974
F6	3'NCR RNA2 Clone #10-2	3/10/2011	974
F7	3'NCR RNA2 Clone #11-1	3/10/2011	974
F8	3'NCR RNA2 Clone #11-3	3/10/2011	974
F9	pRSET (A10 from AC) DH5a	12/23/2011	1163
G1	LCV RNA1 dRNA-GFP #1 PCR #5	5/22/2011	1027
G2	LCV RNA1 dRNA-GFP #6 PCR #5	5/22/2011	1027
G3	LCV RNA1 dRNA-GFP #1 PCR #2 for GFP probe	5/22/2011	1027
G4	LCV RNA1 dRNA-GFP #3 PCR #2 for GFP probe	5/22/2011	1027
G5	3'NCR RNA2 Clone #12-2	6/30/2011	1064
G6	3'NCR RNA2 Clone #13-2	6/30/2011	1064
G7	LCV RNA1-3'NCR RNA2 #1	9/16/2011	1107
G8	LCV RNA2-3'NCR RNA1 #7	9/16/2011	1107
G9	3'NCR RNA2 Clone #14-1	3/1/2012	1207-1208
H1	LCV RNA2 5'end + frag#1 - #6	7/29/2009	509
H2	LCV RNA2 3'end #8	7/28/2009	508
H3	LCV RNA2 5'end #1 mini	6/10/2009	478
H4	LCV RNA2 5'end + frag#1+2 - #3	8/7/2009	515
H5	LCV RNA2 frag#1 Clone#3-#1	7/8/2009	486
H6	p19 #2 (agro) in C58C1	5/14/2009	446
H7	ORF1a/1b in pGEMT #2	12/15/2011	1157
H8	ORF1a/1b in pGEMT #7	12/15/2011	1157
H9			
I1	TC1 overlap #1 colony #1	10/24/2008	263
I2	TC1 overlap #1 colony #2	10/24/2008	263
I3	TC1 overlap #2 - #2	1/6/2009	292
I4	TC1 overlap #2 - #1	1/6/2009	292
I5	TC1 #1 SwaI/NdeI	2/17/2009	343
I6	TC1 #3 SwaI/NdeI	2/17/2009	343
I7	TC1 #1 SwaI/NdeI	3/17/2009	382
I8	GFP-F#1 M5GFP probe	1/7/2012	1168-1169
I9	p26F#3 M5GFP probe	1/7/2012	1168-1169


Glycerol Stock.

Chawin's glycerol stock #2: -80^{0}C Level #1 Tower #4 Box #6

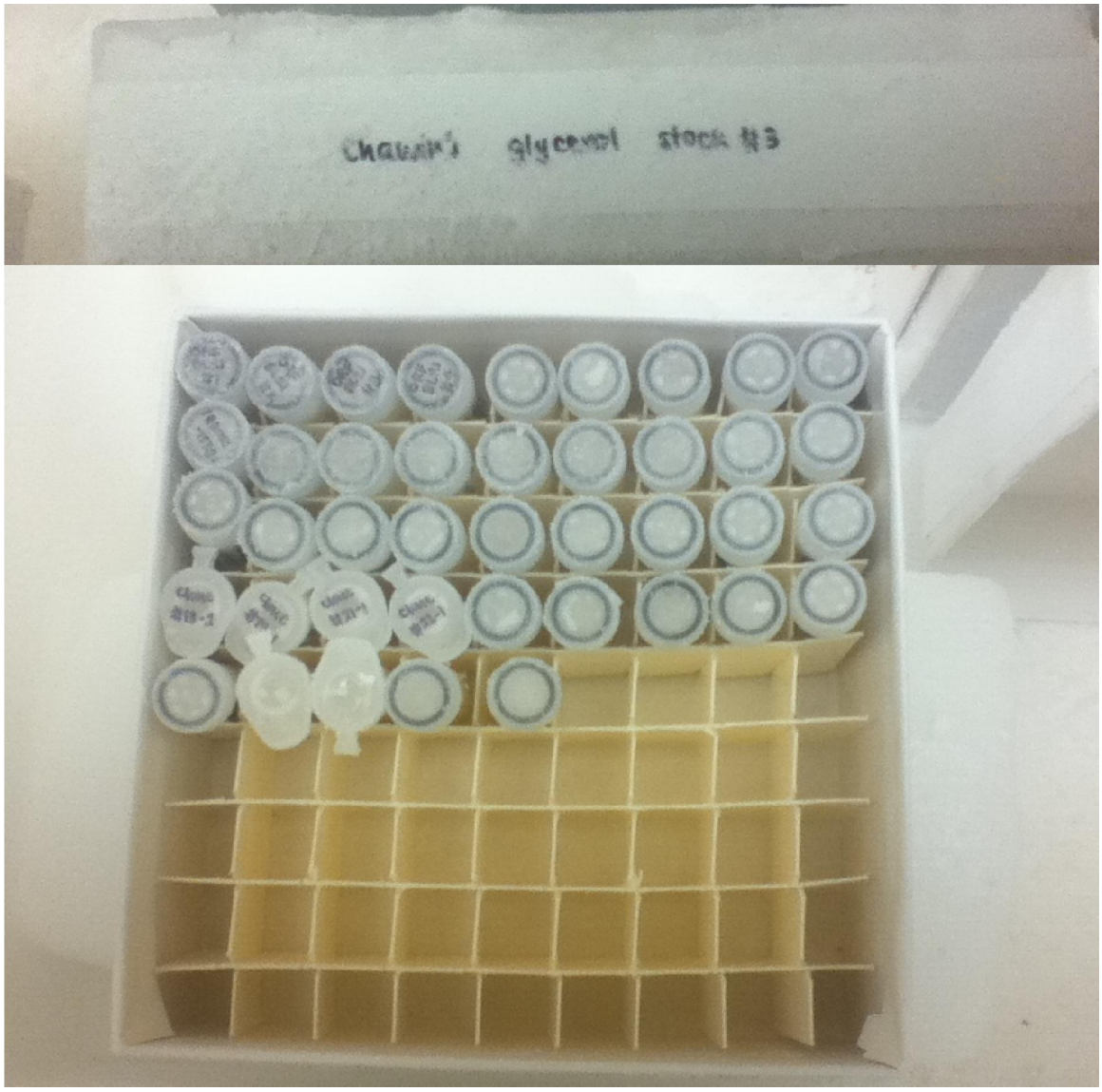
	Description:	Date:	NB pg:
A1	pJW100 * C58C1	11/12/2009	667
A2	pJW100 #1 C58C1	11/12/2009	667
A3	pJW168 #3 C58C1	11/12/2009	667
A4	pJW168 #5 C58C1	11/12/2009	667
A5	TC3 #25 C58C1	3/24/2010	
A6			
A7	HCPro	10/18/2009	636
A8	HCPro #1S	12/7/2009	688
A9	HCPro #2S	12/7/2009	688
B1	TC2 #1S GV3101	5/2/2010	783
B2	TC2 #3S GV3101	5/2/2010	783
B3	TC2 #4M GV3101	5/2/2010	783
B4	TC2 #5M GV3101	5/2/2010	783
B5	TC3 #1S GV3101	5/2/2010	783
B6	TC3 #2S GV3101	5/2/2010	783
B7	TC3 #3S GV3101	5/2/2010	783
B8	TC3 #4M GV3101	5/2/2010	783
B9	TC2 #3M GV3101	5/2/2010	783
C1	5bm1 #1 GV3101	7/11/2010	830
C2	5bm1 #2 GV3101	7/11/2010	830
C3	5bm1 #3 GV3101	7/11/2010	830
C4	5bm1 #4 GV3101	7/11/2010	830
C5	5bm1 #5 GV3101	7/11/2010	830
C6	5b #1 GV3101	7/11/2010	830
C7	5b #2 GV3101	7/11/2010	830
C8	5b #3 GV3101	7/11/2010	830
C9	5b #4 GV3101	7/11/2010	830
D1	pJW100 #1S GV3101	5/12/2010	783
D2	pJW100 #2S GV3101	5/12/2010	783
D3			
D4			
D5	TC2 #1S GV3101	5/12/2010	783
D6	TC2 #2S GV3101	5/12/2010	783
D7			
D8			
D9	5b #5 GV3101	7/11/2010	830
E1	HCPro #1 AC C181	11/9/2010	890
E2	HCPro #2 AC C181	11/9/2010	890
E3	pJW168 #1S GV3101	5/12/2010	783
E4	pJW100 #1 GV3101	4/3/2011	992
E5	pJW100 #2 GV3101	4/3/2011	992
E6	pJW100 #3 GV3101	4/3/2011	992
E7	pJW100 #4 GV3101	4/3/2011	992
E8	TC1 GV3101	6/6/2011	1042
E9	TC1 GV3101	6/6/2011	1042

F1	pJW100 #1 GV3101	1/28/2011	936
F2	pJW100 #2 GV3101	1/28/2011	936
F3	pJW100 #4 GV3101	1/28/2011	936
F4	pJW168 #1 GV3101	4/3/2011	992
F5	pJW168 #2 GV3101	4/3/2011	992
F6	pJW168 #3 GV3101	4/3/2011	992
F7			
F8	TC1 #2 GV3101	6/6/2011	1042
F9	TC1 #2 GV3101	6/6/2011	1042
G1	pJW168 #1 GV3101	1/28/2011	936
G2	pJW168 #3 GV3101	1/28/2011	936
G3	pJW168 #4 GV3101	1/28/2011	936
G4	TC5 #1 GV3101	4/3/2011	992
G5	TC5 #2 GV3101	4/3/2011	992
G6	TC5 #3 GV3101	4/3/2011	992
G7	TC5 #4 GV3101	4/3/2011	992
G8	TC1 #3 GV3101	6/6/2011	1042
G9	TC1 #3 GV3101	6/6/2011	1042
H1	TC2 #1 GV3101	1/28/2011	936
H2	TC2 #2 GV3101	1/28/2011	936
H3	TC2 #3 GV3101	1/28/2011	936
H4	TC2 #1 GV3101	4/17/2011	1001
H5	TC2 #2 GV3101	4/17/2011	1001
H6	TC2 #3 GV3101	4/17/2011	1001
H7	TC2 #4 GV3101	4/17/2011	1001
H8	TC1 #4 GV3101	6/6/2011	1042
H9	TC1 #4 GV3101	6/6/2011	1042
I1	TC5 #2 GV3101	1/28/2011	936
I2	TC5 #3 GV3101	1/28/2011	936
I3	TC5 #4 GV3101	1/28/2011	936
I4	TC3 #1 GV3101	4/17/2011	1001
I5	TC3 #2 GV3101	4/17/2011	1001
I6	TC3 #3 GV3101	4/17/2011	1001
I7	TC3 #4 GV3101	4/17/2011	1001
I8	TC1 #5 GV3101	6/6/2011	1042
I9	TC1 #5 GV3101	6/6/2011	1042


Chewin's Glycerol Stock #2

Chawin's glycerol stock #3: -80^{0}C Level #1 Tower #4 Box #7

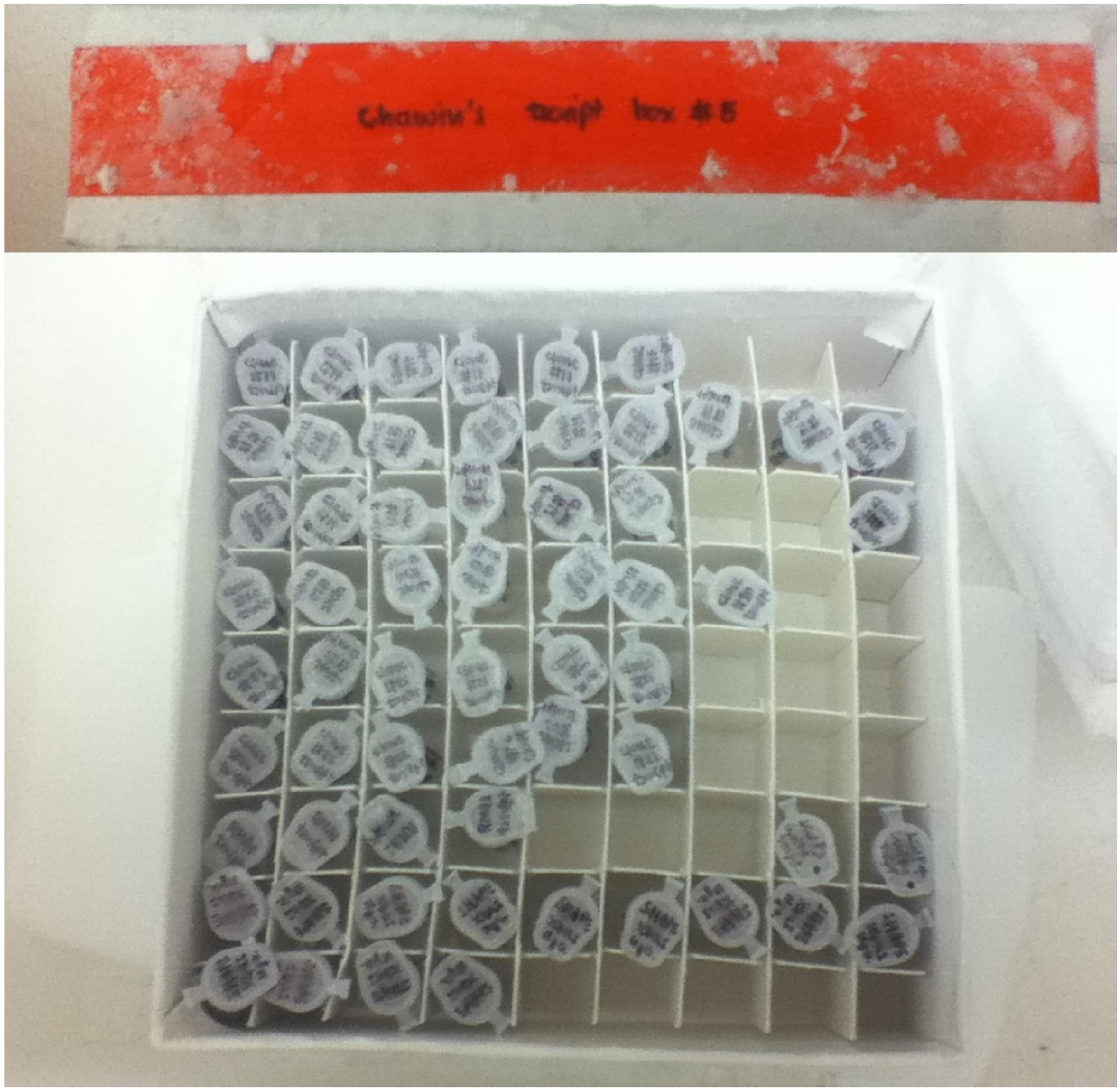
	Description:	Date:	NB pg:
A1	pRSET A + ORF1a/1b #1 BL21(DE)pLysS	1/19/2012	1180
A2	pRSET A + ORF1a/1b #2 BL21(DE)pLysS	1/19/2012	1180
A3	pRSET A + ORF1a/1b #3 BL21(DE)pLysS	1/19/2012	1180
A4	pRSET A + ORF1a/1b #4 BL21(DE)pLysS	1/19/2012	1180
A5	pRSET A + ORF1b #1 BL21(DE)pLysS	3/19/2012	1224
A6	pRSET A + ORF1b #2 BL21(DE)pLysS	3/19/2012	1224
A7	pRSET A + ORF1b #3 BL21(DE)pLysS	3/19/2012	1224
A8	pRSET A + ORF1b #4 BL21(DE)pLysS	3/19/2012	1224
A9	pRSET A + ORF1b #5 BL21(DE)pLysS	3/19/2012	1224
B1	RNA2SL 1mM #1	6/4/2012	1265
B2	RNA2SL 10uM #3	5/22/2012	1258
B3	RNA2SL 10uM #4	5/22/2012	1258
B4	GFP 10uM #3	5/22/2012	1258
B5	GFP 10uM #4	5/22/2012	1258
B6	GFP 10uM #5	5/22/2012	1258
B7	GFP 10uM #6	5/22/2012	1258
B8	GFP 1mM #3	6/4/2012	1265
B9	GFP 1mM #4	6/4/2012	1265
C1	Clone #17-1 1/10/13	1/17/2013	1383
C2	Clone #17-2 1/10/13	1/17/2013	1383
C3	Clone #18-2 9/28/12	1/17/2013	1383
C4	Clone #19-1 9/28/12	1/17/2013	1383
C5	Clone #20-1 9/28/12	1/17/2013	1383
C6	Clone #21-1 10/22/12	1/17/2013	1383
C7	Clone #22-1 10/22/12	1/17/2013	1383
C8	SHAPE cassette + 3' NCR RNA 2 #1	1/15/2013	1382
C9	SHAPE cassette + 3' NCR RNA 2 #2	1/15/2013	1382
D1	Clone #18-2	9/27/2012	1329
D2	Clone #19-1	9/27/2012	1329
D3	Clone #21-1	10/18/2012	1343
D4	Clone #22-1	10/18/2012	1343
D5	ORF1a + pRSET A #4 DH5a	1/29/2013	1389
D6	ORF1a + pRSET A #8 DH5a	1/29/2013	1389
D7	ORF1a + pRSET A #8 solu BL21	2/25/2013	1408
D8	LCV dRNA2 P4 #1 T3 from AC (B161)	5/8/2013	1483
D9	LCV CP probe from AC (B101)	5/8/2013	1483
E1	LCV dRNA2 P4 + 3' linker #1	5/20/2013	1492
E2	Clone #24-5	6/27/2013	1516
E3	Clone #24-6	6/27/2013	1516
E4	Clone #25-2	8/5/2013	1547
E5	Clone #25-3	8/5/2013	1547
E6			
E7			
E8			
E9			


glycerol

Chawin's transcript #5: -80^{0}C Level #1 Tower #4 Box #8

	Description:	Date:	NB pg:
A1	Transcript of RNA 2 Clone #17	1/24/2013	1387
A2	Transcript of RNA 2 Clone #17	1/24/2013	1387
A3	Transcript of RNA 2 Clone #17	1/24/2013	1387
A4	Transcript of RNA 2 Clone #17	1/24/2013	1387
A5	Transcript of RNA 2 Clone #17	2/6/2013	1395
A6	Transcript of RNA 2 Clone #17	2/6/2013	1395
A7			
A8			
A9			
B1	Transcript of RNA 2 Clone #18	1/12/2013	1378
B2	Transcript of RNA 2 Clone #18	1/12/2013	1378
B3	Transcript of RNA 2 Clone #18	1/12/2013	1378
B4	Transcript of RNA 2 Clone #18	1/24/2013	1387
B5	Transcript of RNA 2 Clone #18	1/24/2013	1387
B6	Transcript of RNA 2 Clone #18	1/24/2013	1387
B7	Transcript of RNA 2 Clone #18	1/24/2013	1387
B8	Transcript of RNA 2 Clone #18	1/24/2013	1387
B9	Transcript of RNA 2 Clone #18	1/24/2013	1387
C1	Transcript of RNA 2 Clone #19	1/17/2013	1381
C2	Transcript of RNA 2 Clone #19	1/17/2013	1381
C3	Transcript of RNA 2 Clone #19	1/17/2013	1381
C4	Transcript of RNA 2 Clone #19	1/17/2013	1381
C5	Transcript of RNA 2 Clone #19	1/17/2013	1381
C6			
C7			
C8			
C9	Transcript of RNA 2 Clone #18	1/24/2013	1387
D1	Transcript of RNA 2 Clone #20	10/4/2012	1334
D2	Transcript of RNA 2 Clone #20	10/4/2012	1334
D3	Transcript of RNA 2 Clone #20	10/4/2012	1334
D4	Transcript of RNA 2 Clone #20	10/4/2012	1334
D5	Transcript of RNA 2 Clone #20	10/4/2012	1334
D6	Transcript of RNA 2 Clone #20	10/4/2012	1334
D7	Transcript of RNA 2 Clone #20	10/4/2012	1334
D8			
D9			
E1	Transcript of RNA 2 Clone #21	1/17/2013	1381
E2	Transcript of RNA 2 Clone #21	1/17/2013	1381
E3	Transcript of RNA 2 Clone #21	1/17/2013	1381
E4	Transcript of RNA 2 Clone #21	1/17/2013	1381
E5	Transcript of RNA 2 Clone #21	1/17/2013	1381
E6	Transcript of RNA 2 Clone #21	1/17/2013	1381
E7			
E8			
E9			

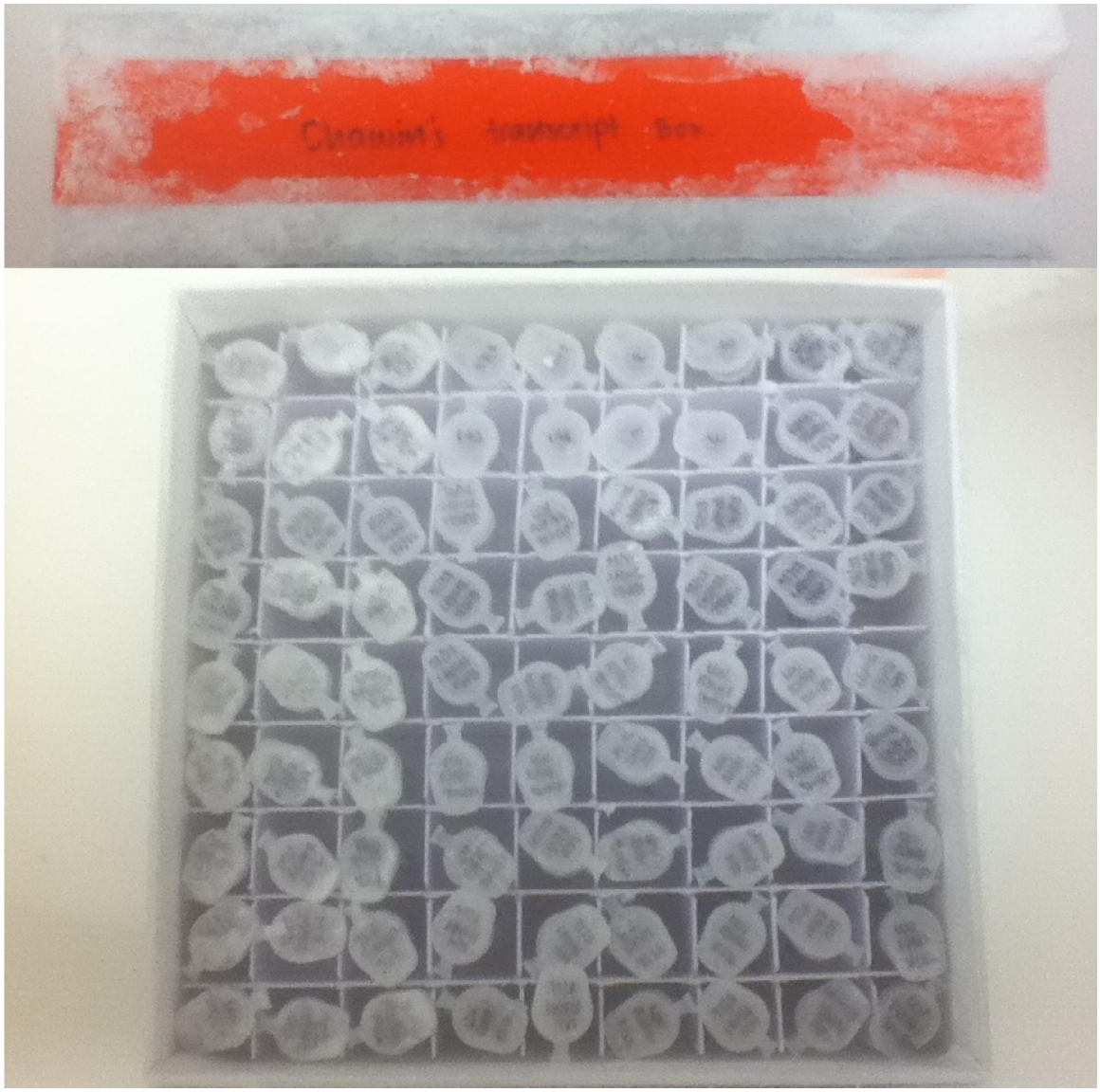
F1	Transcript of RNA 2 Clone #22	1/17/2013	1381
F2	Transcript of RNA 2 Clone #22	1/17/2013	1381
F3	Transcript of RNA 2 Clone #22	1/17/2013	1381
F4	Transcript of RNA 2 Clone #22	1/17/2013	1381
F5	Transcript of RNA 2 Clone #22	1/17/2013	1381
F6	Transcript of RNA 2 Clone #22	1/17/2013	1381
F7			
F8			
F9			
G1	Transcript of LCV RNA1 (pCM1)	1/24/2013	1387
G2	Transcript of LCV RNA1 (pCM1)	1/24/2013	1387
G3	Transcript of LCV RNA2 (pCM2)	1/24/2013	1387
G4	Transcript of LCV RNA2 (pCM2)	1/24/2013	1387
G5			
G6			
G7			
G8	Transcript of LCV dRNA2 P3 from AC	1/15/2010	
G9	Transcript of LCV dRNA2 P4 from AC	1/15/2010	
H1	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H2	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H3	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H4	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H5	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H6	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H7	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H8	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
H9	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
I1	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
I2	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
I3	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
I4	Transcript of SHAPE cassette + 3' RNA2 XbaI/Ngo	2/6/2013	1395-1396
I5			
I6			
I7			
I8			
I9			


Chawin's script box #5

Chawin's transcript #1: -80^{0}C Level #1 Tower #4 Box #9

	Description:	Date:	NB pg:
A1	Transcript of CMV 109 (RNA 1)	1/7/2009	294
A2	Transcript of CMV 209 (RNA 2)	1/7/2009	294
A3	Transcript of CMV 309 (RNA 3)	1/7/2009	294
A4	Transcript of CMV 109 + 209 (RNA 1+2)	9/19/07-1	20-21
A5	Transcript of CMV 109 + 209 (RNA 1+2)	9/19/07-1	20-21
A6	Transcript of CMV 309 (RNA 3)	9/19/07-1	20-21
A7	Transcript of CMV 309 (RNA 3)	9/19/07-1	20-21
A8	Transcript of LCV RNA 2 (pCM2)	1/5/2011	914
A9	Transcript of LCV RNA 2 (pCM2)	1/5/2011	914
B1	Transcript of CMV 109 (RNA 1)	1/7/2009	294
B2	Transcript of CMV 209 (RNA 2)	1/7/2009	294
B3	Transcript of CMV 309 (RNA 3)	1/7/2009	294
B4	Transcript of CMV 109 + 209 (RNA 1+2)	9/19/07-1	20-21
B5	Transcript of CMV 109 + 209 (RNA 1+2)	9/19/07-1	20-21
B6	Transcript of CMV 309 (RNA 3)	9/19/07-1	20-21
B7	Transcript of CMV 309 (RNA 3)	9/19/07-1	20-21
B8	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
B9	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
C1	Transcript of LCV RNA 1-3' RNA2	11/23/2011	1146
C2	Transcript of LCV RNA 1-3' RNA2	11/23/2011	1146
C3	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
C4	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
C5	Transcript of LCV RNA 2 (pCM2)	10/21/2010	873-874
C6	Transcript of LCV RNA 2 (pCM2)	1/5/2011	914
C7	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
C8	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
C9	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
D1	Transcript of TMV30BGFP	1/27/2009	322-323
D2	Transcript of TMV30BGFP	1/27/2009	322-323
D3	Transcript of TMV30BGFP	1/27/2009	322-323
D4	Transcript of LCV RNA1 (pCM1)	12/16/2011	1159
D5	Transcript of LCV RNA1 (pCM1)	10/21/2010	873-874
D6	Transcript of LCV RNA1 (pCM1)	1/15/2011	914
D7	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
D8	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
D9	Transcript of LCV RNA 2 (pCM2)	12/16/2011	1159
E1	Transcript of TMV30BGFP	1/27/2009	322-323
E2	Transcript of TMV30BGFP	1/27/2009	322-323
E3	Transcript of TMV30BGFP	1/27/2009	322-323
E4	Transcript of LCV RNA1 (pCM1)	12/16/2011	1159
E5	Transcript of LCV RNA1 (pCM1)	12/16/2011	1159
E6	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
E7	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
E8	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
E9	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094

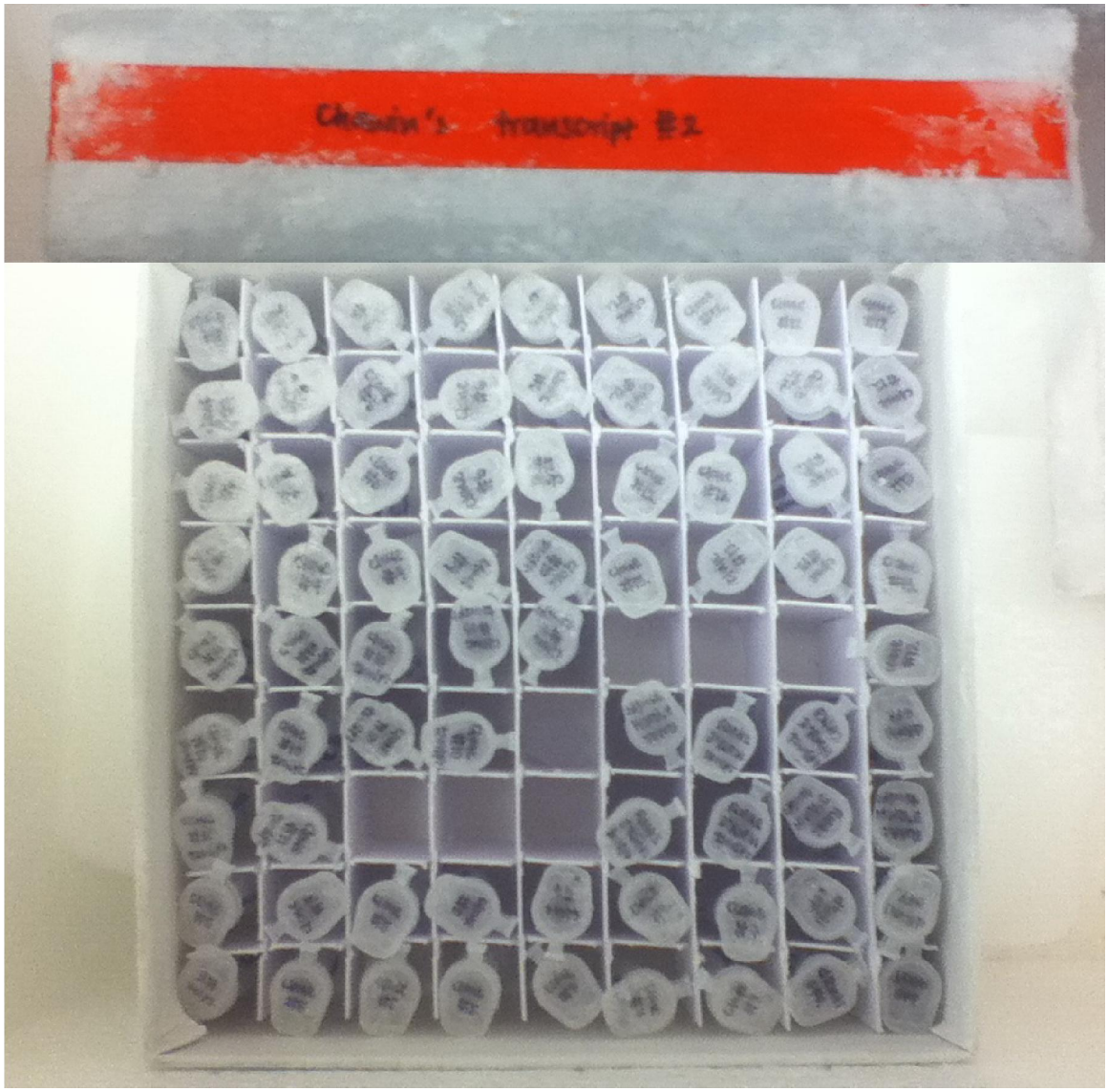
F1	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
F2	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
F3	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
F4	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
F5	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
F6	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
F7	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
F8	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
F9	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G1	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
G2	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
G3	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
G4	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G5	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G6	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G7	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G8	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
G9	Transcript of LCV RNA1 (pCM1)	8/31/2011	1094
H1	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
H2	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
H3	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
H4	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
H5	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
H6	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
H7	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
H8	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
H9	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
I1	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
I2	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
I3	Transcript of TMV30BGFP LiCl	3/6/2009	364-365
I4	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
I5	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
I6	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
I7	Transcript of LCV RNA1 (pCM1)	8/26/2011	1091
I8	Transcript of LCV RNA1 (pCM1)	7/2/2010	824-825
I9	Transcript of LCV RNA1 (pCM1)	7/2/2010	824-825



Chawin's transcript #2: -80^{0}C Level #1 Tower #4 Box #10

	Description:	Date:	NB pg:
A1	Transcript of RNA 2 Clone #1	11/19/2009	677-678
A2	Transcript of RNA 2 Clone #1	11/19/2009	677-678
A3	Transcript of RNA 2 Clone #2	11/19/2009	677-678
A4	Transcript of RNA 2 Clone #2	11/19/2009	677-678
A5	Transcript of RNA 2 Clone #3	11/19/2009	677-678
A6	Transcript of RNA 2 Clone #12	7/15/2011	1069
A7	Transcript of RNA 2 Clone #12	7/15/2011	1069
A8	Transcript of RNA 2 Clone #12	7/15/2011	1069
A9	Transcript of RNA 2 Clone #12	7/15/2011	1069
B1	Transcript of RNA 2 Clone #4	11/19/2009	677-678
B2	Transcript of RNA 2 Clone #4	11/19/2009	677-678
B3	Transcript of RNA 2 Clone #6	6/6/2010	804-805
B4	Transcript of RNA 2 Clone #6	6/6/2010	804-805
B5	Transcript of RNA 2 Clone #6	6/6/2010	804-805
B6	Transcript of RNA 2 Clone #12	7/15/2011	1069
B7	Transcript of RNA 2 Clone #12	7/15/2011	1069
B8	Transcript of RNA 2 Clone #12	7/15/2011	1069
B9	Transcript of RNA 2 Clone #12	7/15/2011	1069
C1	Transcript of RNA 2 Clone #6	6/6/2010	804-805
C2	Transcript of RNA 2 Clone #6	6/6/2010	804-805
C3	Transcript of RNA 2 Clone #6	6/6/2010	804-805
C4	Transcript of RNA 2 Clone #6	6/6/2010	804-805
C5	Transcript of RNA 2 Clone #6	6/6/2010	804-805
C6	Transcript of RNA 2 Clone #12	7/15/2011	1069
C7	Transcript of RNA 2 Clone #12	7/15/2011	1069
C8	Transcript of RNA 2 Clone #12	7/15/2011	1069
C9	Transcript of RNA 2 Clone #13	7/15/2011	1069
D1	Transcript of RNA 2 Clone #7	6/6/2010	804-805
D2	Transcript of RNA 2 Clone #9	6/6/2010	804-805
D3	Transcript of RNA 2 Clone #9	6/6/2010	804-805
D4	Transcript of RNA 2 Clone #9	6/6/2010	804-805
D5	Transcript of RNA 2 Clone #10	6/24/2011	1063
D6	Transcript of RNA 2 Clone #13	7/15/2011	1069
D7	Transcript of RNA 2 Clone #13	7/15/2011	1069
D8	Transcript of RNA 2 Clone #13	7/15/2011	1069
D9	Transcript of RNA 2 Clone #13	7/15/2011	1069
E1	Transcript of RNA 2 Clone #10	6/24/2011	1063
E2	Transcript of RNA 2 Clone #10	6/24/2011	1063
E3	Transcript of RNA 2 Clone #10	6/24/2011	1063
E4	Transcript of RNA 2 Clone #10	6/24/2011	1063
E5	Transcript of RNA 2 Clone #10	6/24/2011	1063
E6			
E7			
E8			
E9	Transcript of RNA 2 Clone #13	7/15/2011	1069

F1	Transcript of RNA 2 Clone #10	6/24/2011	1063
F2	Transcript of RNA 2 Clone #10	6/24/2011	1063
F3	Transcript of RNA 2 Clone #10	6/24/2011	1063
F4	Transcript of RNA 2 Clone #10	6/24/2011	1063
F5			
F6	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
F7	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
F8	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
F9	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
G1	Transcript of RNA 2 Clone #11	6/24/2011	1063
G2	Transcript of RNA 2 Clone #11	6/24/2011	1063
G3			
G4			
G5			
G6	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
G7	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
G8	Transcript of RNA 2-3' RNA 1	11/23/2011	1146
G9	Transcript of dRNA 1-GFP	5/26/2011	1032
H1	Transcript of RNA 2 Clone #5	8/31/2011	1094
H2	Transcript of RNA 2 Clone #5	8/31/2011	1094
H3	Transcript of RNA 2 Clone #5	8/31/2011	1094
H4	Transcript of RNA 2 Clone #5	8/31/2011	1094
H5	Transcript of RNA 2 Clone #7	8/31/2011	1094
H6	Transcript of RNA 2 Clone #7	8/31/2011	1094
H7	Transcript of RNA 2 Clone #7	8/31/2011	1094
H8	Transcript of dRNA 1-GFP	5/26/2011	1032
H9	Transcript of dRNA 1-GFP	5/26/2011	1032
I1	Transcript of RNA 2 Clone #5	8/31/2011	1094
I2	Transcript of RNA 2 Clone #5	8/31/2011	1094
I3	Transcript of RNA 2 Clone #5	8/31/2011	1094
I4	Transcript of RNA 2 Clone #5	8/31/2011	1094
I5	Transcript of RNA 2 Clone #7	8/31/2011	1094
I6	Transcript of RNA 2 Clone #7	8/31/2011	1094
I7	Transcript of RNA 2 Clone #7	8/31/2011	1094
I8	Transcript of dRNA 1-GFP	5/26/2011	1032
I9	Transcript of dRNA 1-GFP	5/26/2011	1032


transcript #2

Chawin's transcript #3: -80^{0}C Level #1 Tower #4 Box #11

	Description:	Date:	NB pg:
A1	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A2	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A3	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A4	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A5	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A6	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A7	Transcript of LCV RNA1 (pCM1)	1/20/2012	1181-1182
A8			
A9			
B1	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B2	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B3	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B4	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B5	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B6	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B7	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B8	Transcript of LCV RNA 2 (pCM2)	1/20/2012	1181-1182
B9			
C1	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C2	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C3	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C4	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C5	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C6	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C7	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C8	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
C9	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D1	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D2	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D3	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D4	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D5	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D6	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D7	Transcript of LCV RNA 1-3' RNA2	1/20/2012	1181-1182
D8			
D9			
E1	Transcript of RNA 2-3' RNA 1	1/20/2012	1181-1182
E2	Transcript of RNA 2-3' RNA 1	1/20/2012	1181-1182
E3	Transcript of RNA 2-3' RNA 1	1/20/2012	1181-1182
E4	Transcript of RNA 2-3' RNA 1	1/20/2012	1181-1182
E5	Transcript of RNA 2-3' RNA 1	1/20/2012	1181-1182
E6			
E7			
E8	Transcript of RNA 2 Clone #14	3/7/2012	1211
E9	Transcript of RNA 2 Clone #14	3/7/2012	1211

F1	Transcript of RNA 2 Clone #14	3/7/2012	1211
F2	Transcript of RNA 2 Clone #14	3/7/2012	1211
F3	Transcript of RNA 2 Clone #14	3/7/2012	1211
F4	Transcript of RNA 2 Clone #14	3/7/2012	1211
F5	Transcript of RNA 2 Clone #14	3/7/2012	1211
F6	Transcript of RNA 2 Clone #14	3/7/2012	1211
F7	Transcript of RNA 2 Clone #14	3/7/2012	1211
F8	Transcript of RNA 2 Clone #14	3/7/2012	1211
F9	Transcript of RNA 2 Clone #14	3/7/2012	1211
G1	Transcript of RNA 2 Clone #14	3/7/2012	1211
G2	Transcript of RNA 2 Clone #14	3/7/2012	1211
G3	Transcript of RNA 2 Clone #14	3/7/2012	1211
G4	Transcript of RNA 2 Clone #14	3/7/2012	1211
G5	Transcript of RNA 2 Clone #14	3/7/2012	1211
G6	Transcript of RNA 2 Clone #14	3/7/2012	1211
G7	Transcript of RNA 2 Clone #14	3/7/2012	1211
G8	Transcript of RNA 2 Clone #14	3/7/2012	1211
G9	Transcript of RNA 2 Clone #14	3/7/2012	1211
H1	Transcript of RNA 2 Clone #15	4/19/2012	1240
H2	Transcript of RNA 2 Clone #15	4/19/2012	1240
H3	Transcript of RNA 2 Clone #15	4/19/2012	1240
H4	Transcript of RNA 2 Clone #15	4/19/2012	1240
H5	Transcript of RNA 2 Clone #15	4/19/2012	1240
H6	Transcript of RNA 2 Clone #15	4/19/2012	1240
H7	Transcript of RNA 2 Clone #15	4/19/2012	1240
H8	Transcript of RNA 2 Clone #15	4/19/2012	1240
H9	Transcript of RNA 2 Clone #15	4/19/2012	1240
I1	Transcript of RNA 2 Clone #16	4/19/2012	1240
I2	Transcript of RNA 2 Clone #16	4/19/2012	1240
I3	Transcript of RNA 2 Clone #16	4/19/2012	1240
I4	Transcript of RNA 2 Clone #16	4/19/2012	1240
I5	Transcript of RNA 2 Clone #16	4/19/2012	1240
I6	Transcript of RNA 2 Clone #16	4/19/2012	1240
I7	Transcript of RNA 2 Clone #16	4/19/2012	1240
I8	Transcript of RNA 2 Clone #16	4/19/2012	1240
I9	Transcript of RNA 2 Clone #16	4/19/2012	1240

Chawin's transcript #4: -80^{0}C Level #1 Tower #4 Box #12

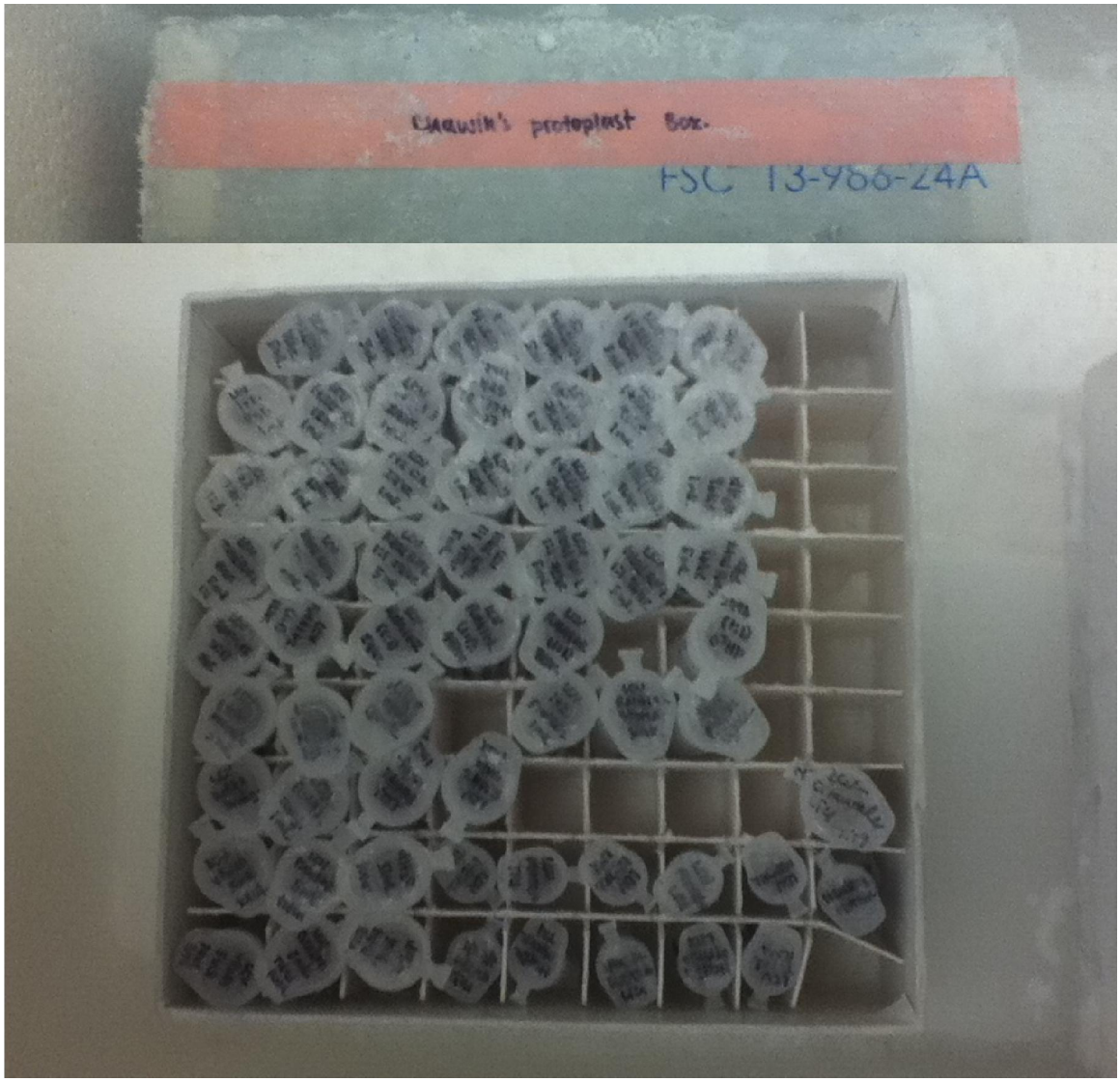
	Description:	Date:	NB pg:
A1	Transcript of M5GFP	4/19/2012	1240
A2	Transcript of M5GFP	4/19/2012	1240
A3	Transcript of M5GFP	4/19/2012	1240
A4	Transcript of M5GFP	4/19/2012	1240
A5	Transcript of M5GFP	4/19/2012	1240
A6	Transcript of M5GFP	4/19/2012	1240
A7	Transcript of M5GFP	4/19/2012	1240
A8	Transcript of M5GFP	4/19/2012	1240
A9	Transcript of M5GFP	4/19/2012	1240
B1	Transcript of R2SL 6/11	6/19/2012	1273
B2	Transcript of R2SL 6/11	6/19/2012	1273
B3	Transcript of R2SL 6/11	6/19/2012	1273
B4	Transcript of R2SL 6/11	6/19/2012	1273
B5	Transcript of R2SL 6/11	6/19/2012	1273
B6	Transcript of R2SL 6/11	6/19/2012	1273
B7	Transcript of R2SL 6/11	6/19/2012	1273
B8	Transcript of R2SL 6/11	6/19/2012	1273
B9	Transcript of R2SL 6/11	6/19/2012	1273
C1	Transcript of R2SL 6/14	6/19/2012	1273
C2	Transcript of R2SL 6/14	6/19/2012	1273
C3	Transcript of R2SL 6/14	6/19/2012	1273
C4	Transcript of R2SL 6/14	6/19/2012	1273
C5	Transcript of R2SL	6/25/2012	1276
C6	Transcript of R2SL	6/25/2012	1276
C7	Transcript of R2SL	6/25/2012	1276
C8	Transcript of R2SL	6/25/2012	1276
C9	Transcript of R2SL 6/11	6/19/2012	1273
D1	Transcript of R2SL	6/25/2012	1276
D2	Transcript of R2SL	6/25/2012	1276
D3	Transcript of R2SL	6/25/2012	1276
D4	Transcript of R2SL	6/25/2012	1276
D5	Transcript of R2SL	6/25/2012	1276
D6	Transcript of R2SL	6/25/2012	1276
D7	Transcript of R2SL	6/25/2012	1276
D8	Transcript of R2SL	6/25/2012	1276
D9	Transcript of R2SL	6/25/2012	1276
E1	Transcript of R2SL	7/5/2012	1281-1282
E2	Transcript of R2SL	7/5/2012	1281-1282
E3	Transcript of R2SL	7/5/2012	1281-1282
E4	Transcript of R2SL	7/5/2012	1281-1282
E5	Transcript of R2SL	7/5/2012	1281-1282
E6	Transcript of R2SL	7/5/2012	1281-1282
E7			
E8			
E9			

F1	Transcript of GFP	7/5/2012	1281-1282
F2	Transcript of GFP	7/5/2012	1281-1282
F3	Transcript of GFP	7/5/2012	1281-1282
F4	Transcript of GFP	7/5/2012	1281-1282
F5	Transcript of GFP	7/5/2012	1281-1282
F6	Transcript of GFP	7/5/2012	1281-1282
F7	Transcript of GFP	7/5/2012	1281-1282
F8	Transcript of GFP	7/5/2012	1281-1282
F9	Transcript of GFP	7/5/2012	1281-1282
G1	Biotin transcript of R2SL	7/10/2012	1286
G2	Biotin transcript of R2SL	7/10/2012	1286
G3	Biotin transcript of GFP	7/10/2012	1286
G4	Biotin transcript of GFP	7/10/2012	1286
G5	Biotin transcript of GFP	7/10/2012	1286
G6			
G7	Biotin transcript of RNA control	7/10/2012	1286
G8	Biotin transcript of RNA control	7/10/2012	1286
G9	Biotin transcript of RNA control	7/10/2012	1286
H1	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H2	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H3	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H4	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H5	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H6	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H7	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H8	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
H9	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
I1	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
I2	Transcript of SHAPE cassette + 3' RNA2 XbaI	2/6/2013	1395-1396
I3			
I4			
I5			
I6			
I7			
I8			
I9			

Chawin's protoplast #1: -80^{0}C Level #1 Tower #3 Box #1

	Description:	Date:	NB pg:
A1			
A2	Total RNA LCV 1+2 1:3 12hr 5ml	8/31/2009	603
A3	Total RNA LCV 1+2 1:3 24hr 5ml	8/31/2009	603
A4	Total RNA LCV 1+2 1:3 48hr 5ml	8/31/2009	603
A5	Total RNA LCV 1+2 1:3 72hr 5ml	8/31/2009	603
A6	Total RNA LCV 1+2 1:3 96hr 5ml	8/31/2009	603
A7	Total RNA dH2O 96hr 5ml	8/31/2009	603
A8			
A9			
B1	Protos LCV 1+2 1:3 0hr 1ml	8/24/2009	598-599
B2	Protos LCV 1+2 1:3 12hr 1ml	8/25/2009	598-599
B3	Protos LCV 1+2 1:3 24hr 1ml	8/25/2009	598-599
B4	Protos LCV 1+2 1:3 48hr 1ml	8/26/2009	598-599
B5	Protos LCV 1+2 1:3 72hr 1ml	8/27/2009	598-599
B6	Protos LCV 1+2 1:3 96hr 1ml	8/28/2009	598-599
B7	Protos dH2O 96hr 1ml	8/28/2009	598-599
B8			
B9			
C1	Protos LCV 1+2 1:3 0hr 1ml	8/24/2009	598-599
C2	Protos LCV 1+2 1:3 12hr 1ml	8/25/2009	598-599
C3	Protos LCV 1+2 1:3 24hr 1ml	8/25/2009	598-599
C4	Protos LCV 1+2 1:3 48hr 1ml	8/26/2009	598-599
C5	Protos LCV 1+2 1:3 72hr 1ml	8/27/2009	598-599
C6	Protos LCV 1+2 1:3 96hr 1ml	8/28/2009	598-599
C7	Protos dH2O 96hr 1ml	8/28/2009	598-599
C8			
C9			
D1	Total RNA LCV 1+2 1:1 0hr 5ml	9/8/2009	608
D2	Total RNA LCV 1+2 1:1 12hr 5ml	9/8/2009	608
D3	Total RNA LCV 1+2 1:1 24hr 5ml	9/8/2009	608
D4	Total RNA LCV 1+2 1:1 48hr 5ml	9/8/2009	608
D5	Total RNA LCV 1+2 1:1 72hr 5ml	9/8/2009	608
D6	Total RNA LCV 1+2 1:1 96hr 5ml	9/8/2009	608
D7	Total RNA dH2O 96hr 5ml	9/8/2009	608
D8			
D9			
E1	Total RNA LCV 1+2 1:1 0hr 2ml	9/30/2010	887
E2	Total RNA LCV 1+2 1:1 12hr 2ml	9/30/2010	887
E3	Total RNA LCV 1+2 1:1 24hr 2ml	9/30/2010	887
E4	Total RNA LCV 1+2 1:1 48hr 2ml	9/30/2010	887
E5	Total RNA LCV 1+2 1:1 72hr 2ml	9/30/2010	887
E6			
E7	Total RNA dH2O 96hr 5ml	9/30/2010	887
E8			
E9			

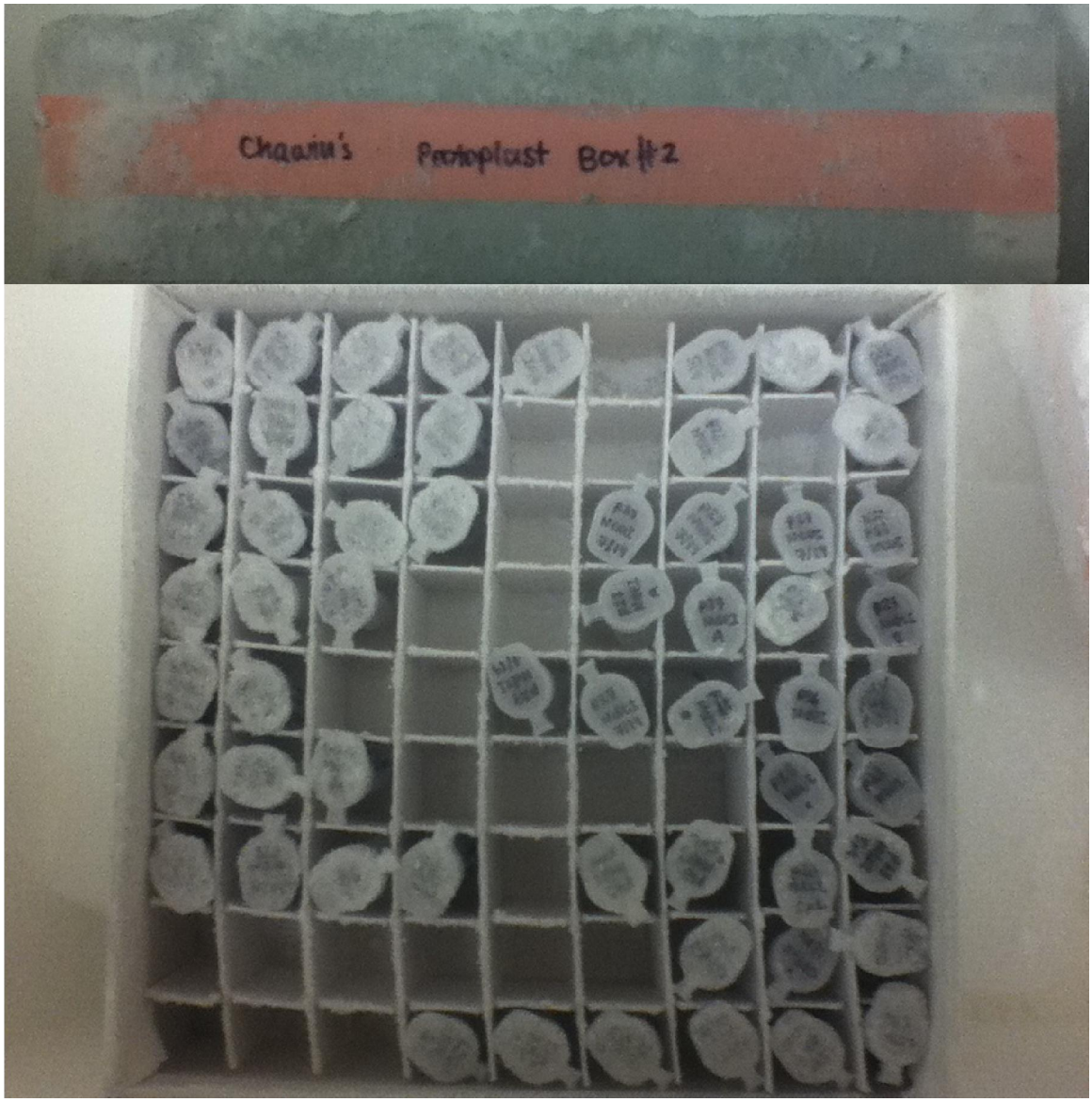
F1	Total protein LCV RNA1+2 5ml	9/14/2009	617
F2	Total protein LCV RNA1+2 1ml	10/23/2009	648-649
F3	Total protein dH2O 1ml	10/23/2009	648-649
F4			
F5	Total protein LCV RNA1+2 1ml	9/14/2009	617
F6	Total protein LCV RNA1+2 1ml	10/23/2009	648-649
F7	Total protein dH2O 1ml	10/23/2009	648-649
F8			
F9			
G1	Total RNA LCV 2 only	5/11/2010	789
G2	Protos LCV 2 only 1ml	1/25/2010	707-708
G3	Protos LCV 2 only 1ml	1/25/2010	707-708
G4	Protos LCV 2 only 1ml	1/25/2010	707-708
G5			
G6			
G7			
G8			
G9	Total RNA LCV C.Murale LiCl	1/5/2009	
H1	Total RNA LCV vRNA	11/9/2009	666
H2	Total RNA LCV 1+2	11/9/2009	666
H3	Total RNA dH2O	11/9/2009	666
H4	Total RNA LCV lettuce TRIZOL	12/9/2009	693
H5	Total RNA LCV C.Murale TRIZOL	12/9/2009	693
H6	Total RNA LCV lettuce LiCl	12/9/2009	693
H7	Total RNA LCV C.Murale LiCl	12/9/2009	693
H8	Total RNA LCV C.Murale TRIZOL	11/13/2009	669
H9	Total RNA Healthy C.Murale TRIZOL	11/13/2009	669
I1	Total RNA LCV vRNA	9/13/2010	870
I2	Total RNA LCV 1+2	9/13/2010	870
I3	Total RNA dH2O	9/13/2010	870
I4	Total RNA Healthy lettuce TRIZOL	12/9/2009	693
I5	Total RNA Healthy C. Murale TRIZOL	12/9/2009	693
I6	Total RNA Healthy lettuce LiCl	12/9/2009	693
I7	Total RNA Healthy C. Murale LiCl	12/9/2009	693
I8	Total RNA LCV lettuce	NB7-2/6/09-1	
I9			


protoplast Box.
FSC 13-966-24A

Chawin's protoplast #2: -80^{0}C Level #1 Tower #3 Box #2

	Description:	Date:	NB pg:
A1	5'UTR RNA1 NcoI/Sp6 detect +	10/1/2010	858
A2	5'UTR RNA1 NdeI/T7 detect -	9/8/2010	865-867
A3	5'UTR RNA1 NcoI/Sp6 detect + by AC	1/16/2009	
A4	3'UTR RNA1 NdeI/T7	2/1/2011	934-935
A5	3'UTR RNA1 NdeI/T7	2/1/2011	934-935
A6			
A7	p26 NcoI 5/3/10 CM detect -	5/4/2010	779
A8	p26 NcoI 5/3/10 CM detect -	5/4/2010	779
A9	p26 NcoI 5/3/10 CM detect -	5/4/2010	779
B1	RNA1 mid probe NcoI/Sp6 detect +	1/26/2011	930-931
B2	RNA1 mid probe NcoI/Sp6 detect +	1/26/2011	930-931
B3	RNA1 mid probe NdeI/T7 detect -	1/26/2011	930-931
B4	RNA1 mid probe NdeI/T7 detect -	1/26/2011	930-931
B5			
B6			
B7	p26 NcoI detect -	4/30/2010	779
B8	p26 NcoI detect -	4/30/2010	779
B9	p26 NcoI detect -	4/30/2010	779
C1	5'UTR RNA2 NcoI/Sp6 detect +	11/19/2010	893-894
C2	5'UTR RNA2 NcoI/Sp6 detect +	11/19/2010	893-894
C3	5'UTR RNA2 NcoI/Sp6 detect -	9/8/2010	865-867
C4	5'UTR RNA2 NcoI/Sp6 detect -	9/8/2010	865-867
C5			
C6	p26 NcoI detect -	7/19/2013	1534-1535
C7	p26 NcoI detect -	7/19/2013	1534-1535
C8	p26 NcoI detect -	7/19/2013	1534-1535
C9	p26 NcoI detect -	5/2/2013	1478-1479
D1	BYV p21	9/13/2010	874
D2	BYV p21	9/13/2010	874
D3	BYV p21 by AC	5/27/2008	
D4			
D5			
D6	p27 NdeI A detect +	5/31/2013	1501-1502
D7	p27 NdeI A detect +	5/31/2013	1501-1502
D8			
D9	p27 NdeI B detect +	5/31/2013	1501-1502
E1	PCR#2-1 dRNA1GFP ApaI/Sp6 detect +	6/18/2011	1047-1048
E2	PCR#2-1 dRNA1GFP ApaI/Sp6 detect +	6/18/2011	1047-1048
E3			
E4			
E5	p27 NdeI detect +	7/19/2013	1534-1535
E6	p27 NdeI detect +	7/19/2013	1534-1535
E7	p27 NdeI detect +	9/13/2010	874
E8	p27 NdeI detect +	9/13/2010	874
E9	p27 NdeI detect + by AC	9/18/2008	

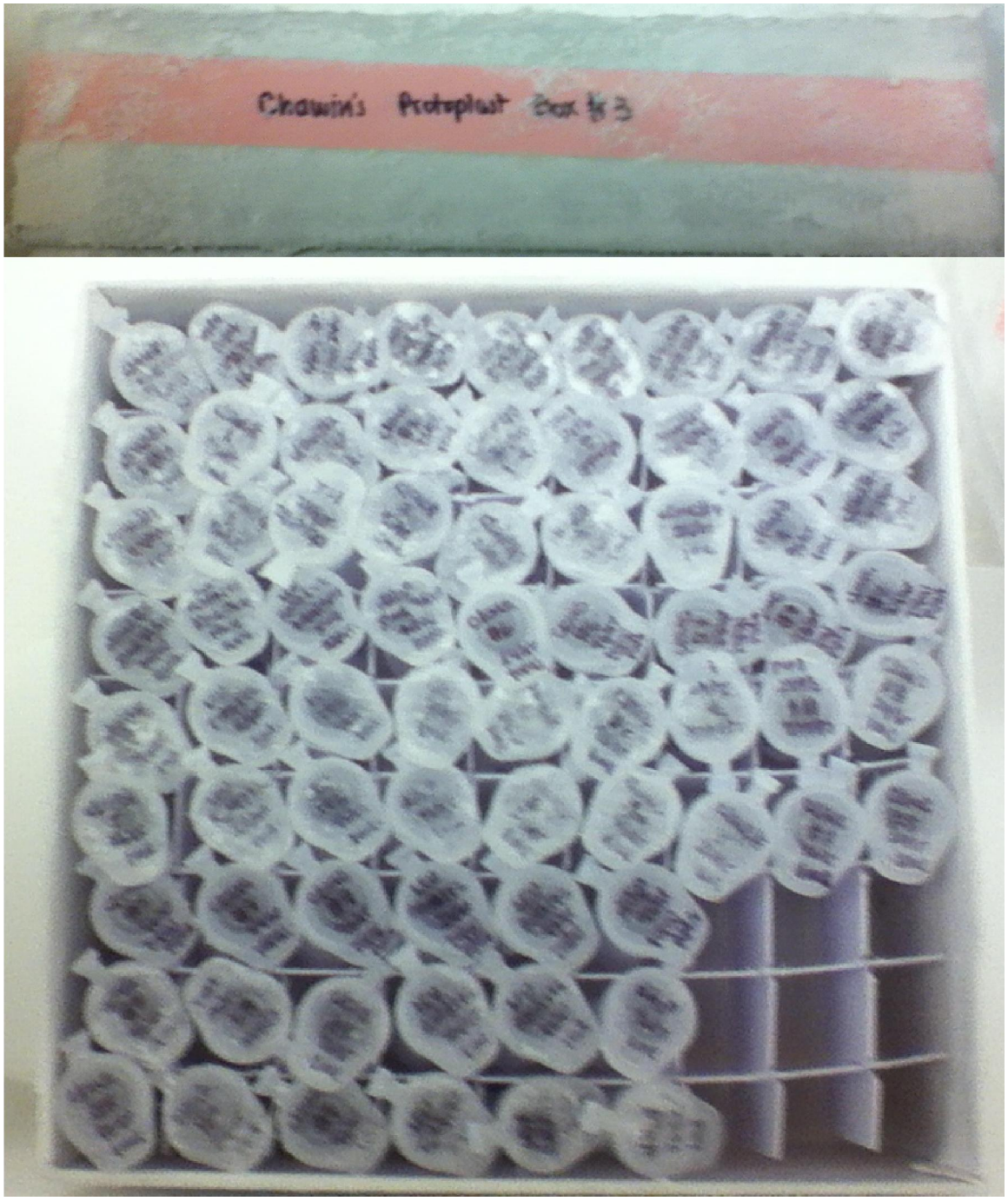
F1	PCR#2-1 dRNA1GFP Nsi/T7 detect -	6/18/2011	1047-1048
F2	PCR#2-1 dRNA1GFP Nsi/T7 detect -	6/18/2011	1047-1048
F3			
F4			
F5			
F6			
F7			
F8	p23 NcoI	10/28/2011	1140-1141
F9	p23 NcoI	10/28/2011	1140-1141
G1	LCV vRNA 0.55ug/ul	3/27/08-1	87
G2	LCV vRNA 1ug/ul	10/30/08-1	268
G3	LCV vRNA 10ng/ul	10/30/08-1	268
G4	LCV vRNA 150ng/ul	3/27/08-1	87
G5			
G6	p23 NdeI	6/8/2009	470
G7	p23 NdeI	6/8/2009	470
G8	p23 NdeI	6/8/2009	470
G9	p23 NdeI	6/8/2009	470
H1			
H2			
H3			
H4			
H5			
H6			
H7	GFP-F NcoI	1/20/2012	1188
H8	GFP-F NcoI	1/20/2012	1188
H9	GFP-F NcoI	2/28/2012	1206
I1			
I2			
I3			
I4	LIYV p26 NcoI	10/28/2011	1140-1141
I5	LIYV p26 NcoI	10/28/2011	1140-1141
I6	LIYV p26 NcoI	10/28/2011	1140-1141
I7	LIYV p26 NcoI	10/28/2011	1140-1141
I8	LIYV p26 NcoI	1/5/2011	915
I9	LIYV p26 NcoI	9/13/2011	1103


Chaaru's Petroplast Box #2

Chawin's protoplast #3: -80^{0}C Level #1 Tower #3 Box #3

	Description:	Date:	NB pg:
A1	Total RNA Clone #1 24hr 5ml	11/25/2009	683
A2	Total RNA Clone #1 96hr 5ml	11/25/2009	683
A3	Total RNA Clone #2 24hr 5ml	11/25/2009	683
A4	Total RNA Clone #2 96hr 5ml	11/25/2009	683
A5	Total RNA Clone #3 24hr 5ml	11/25/2009	683
A6	Total RNA Clone #3 96hr 5ml	11/25/2009	683
A7	Total RNA Clone #4 24hr 5ml	11/25/2009	683
A8	Total RNA Clone #4 96hr 5ml	11/25/2009	683
A9	Total RNA Clone #5 24hr 5ml	11/25/2009	683
B1	Protos Clone #1 24hr 1ml	11/25/2009	679-680
B2	Protos Clone #1 96hr 1ml	11/25/2009	679-680
B3	Protos Clone #2 24hr 1ml	11/25/2009	679-680
B4	Protos Clone #2 96hr 1ml	11/25/2009	679-680
B5	Protos Clone #3 24hr 1ml	11/25/2009	679-680
B6	Protos Clone #3 96hr 1ml	11/25/2009	679-680
B7	Protos Clone #4 24hr 1ml	11/25/2009	679-680
B8	Protos Clone #4 96hr 1ml	11/25/2009	679-680
B9	Protos Clone #5 24hr 1ml	11/25/2009	679-680
C1	Protos Clone #1 24hr 1ml	11/25/2009	679-680
C2	Protos Clone #1 96hr 1ml	11/25/2009	679-680
C3	Protos Clone #2 24hr 1ml	11/25/2009	679-680
C4	Protos Clone #2 96hr 1ml	11/25/2009	679-680
C5	Protos Clone #3 24hr 1ml	11/25/2009	679-680
C6	Protos Clone #3 96hr 1ml	11/25/2009	679-680
C7	Protos Clone #4 24hr 1ml	11/25/2009	679-680
C8	Protos Clone #4 96hr 1ml	11/25/2009	679-680
C9	Protos Clone #5 24hr 1ml	11/25/2009	679-680
D1	Total RNA Clone #5 96hr 5ml	11/25/2009	679-680
D2	Total RNA RNA1+2 24hr 5ml	11/25/2009	679-680
D3	Total RNA RNA1+2 96hr 5ml	11/25/2009	679-680
D4	Total RNA dH2O 96hr 5ml	11/25/2009	679-680
D5	Total RNA Clone #6 24hr 5ml	2/25/2010	745
D6	Total RNA Clone #6 96hr 5ml	2/25/2010	745
D7	Total RNA Clone #7 24hr 5ml	2/25/2010	745
D8	Total RNA Clone #7 96hr 5ml	2/25/2010	745
D9	Total RNA Clone #8 24hr 5ml	2/25/2010	745
E1	Protos Clone #5 96hr 1ml	11/25/2009	679-680
E2	Protos RNA1+2 24hr 1ml	11/25/2009	679-680
E3	Protos RNA1+2 96hr 1ml	11/25/2009	679-680
E4	Protos dH2O 96hr 1ml	11/25/2009	679-680
E5	Protos Clone #6 24hr 1ml	2/25/2010	737
E6	Protos Clone #6 96hr 1ml	2/25/2010	737
E7	Protos Clone #7 24hr 1ml	2/25/2010	737
E8	Protos Clone #7 96hr 1ml	2/25/2010	737
E9	Protos Clone #8 24hr 1ml	2/25/2010	737

F1	Protos Clone #5 96hr 1ml	11/25/2009	679-680
F2	Protos RNA1+2 24hr 1ml	11/25/2009	679-680
F3	Protos RNA1+2 96hr 1ml	11/25/2009	679-680
F4	Protos dH2O 96hr 1ml	11/25/2009	679-680
F5	Protos Clone #6 24hr 1ml	2/25/2010	737
F6	Protos Clone #6 96hr 1ml	2/25/2010	737
F7	Protos Clone #7 24hr 1ml	2/25/2010	737
F8	Protos Clone #7 96hr 1ml	2/25/2010	737
F9	Protos Clone #8 24hr 1ml	2/25/2010	737
G1	Total RNA Clone #8 96hr 5ml	2/25/2010	745
G2	Total RNA Clone #9 24hr 5ml	2/25/2010	745
G3	Total RNA Clone #9 96hr 5ml	2/25/2010	745
G4	Total RNA RNA1+2 24hr 5ml	2/25/2010	745
G5	Total RNA RNA1+2 96hr 5ml	2/25/2010	745
G6	Total RNA dH2O 96hr 5ml	2/25/2010	745
G7			
G8			
G9			
H1	Protos Clone #8 96hr 1ml	2/25/2010	737
H2	Protos Clone #9 24hr 1ml	2/25/2010	737
H3	Protos Clone #9 96hr 1ml	2/25/2010	737
H4	Protos RNA1+2 24hr 1ml	2/25/2010	737
H5	Protos RNA1+2 96hr 1ml	2/25/2010	737
H6	Protos dH2O 96hr 1ml	2/25/2010	737
H7			
H8			
H9			
I1	Protos Clone #8 96hr 1ml	2/25/2010	737
I2	Protos Clone #9 24hr 1ml	2/25/2010	737
I3	Protos Clone #9 96hr 1ml	2/25/2010	737
I4	Protos RNA1+2 24hr 1ml	2/25/2010	737
I5	Protos RNA1+2 96hr 1ml	2/25/2010	737
I6	Protos dH2O 96hr 1ml	2/25/2010	737
I7			
I8			
I9			


Chawin's Protoplast Box #3

Chawin's protoplast #4: -80^{0}C Level #1 Tower #3 Box #4

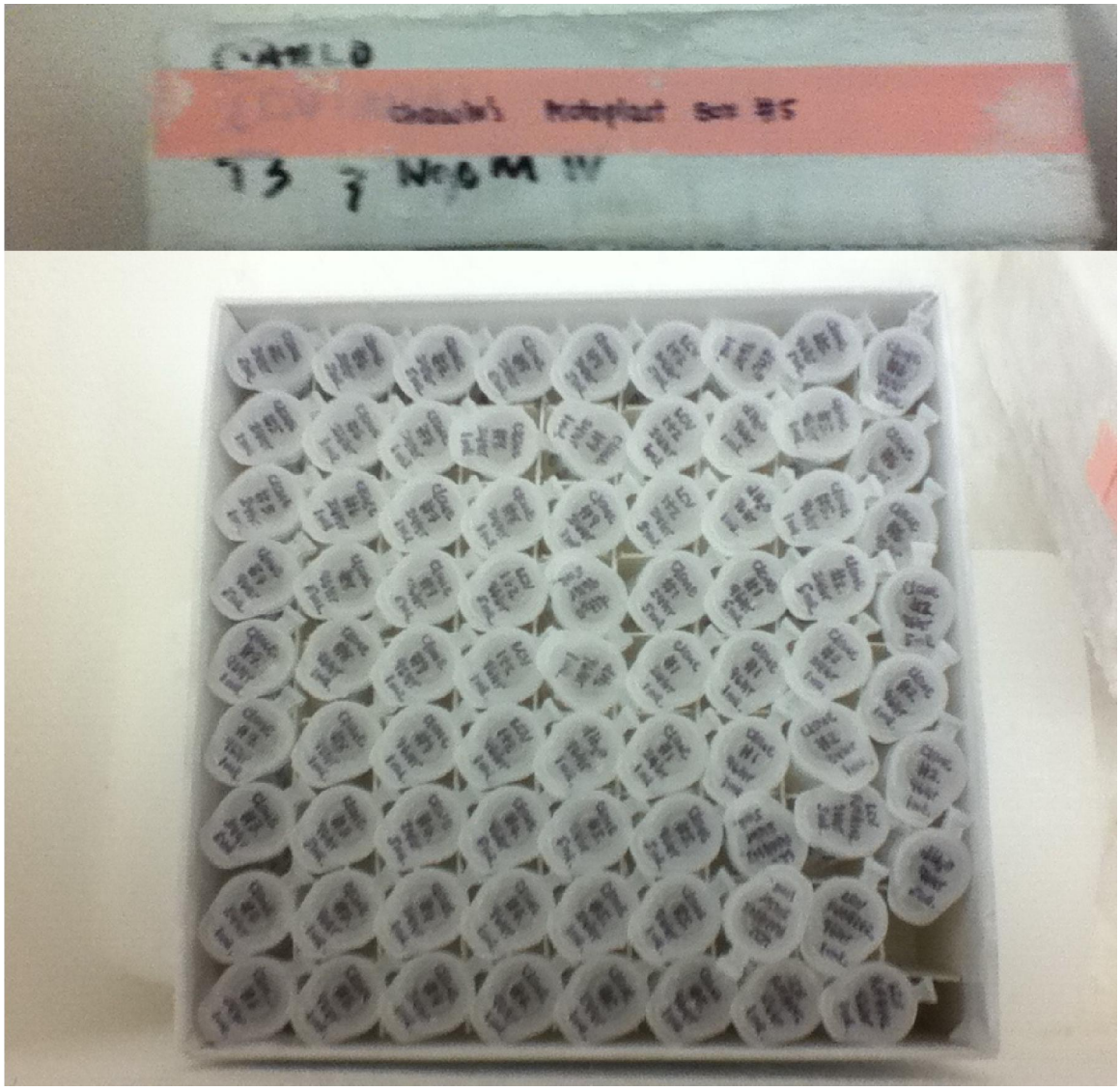
	Description:	Date:	NB pg:
A1	Total RNA Clone #6 24hr 5ml	5/11/2010	789
A2	Total RNA Clone #6 96hr 5ml	5/11/2010	789
A3	Total RNA Clone #7 24hr 5ml	5/11/2010	789
A4	Total RNA Clone #7 96hr 5ml	5/11/2010	789
A5	Total RNA RNA1+2 24hr 5ml 4/7/10	5/11/2010	789
A6	Total RNA RNA1+2 96hr 5ml 4/7/10	5/11/2010	789
A7	Total RNA RNA1+2 96hr 5ml 2/17/10	5/11/2010	789
A8	Total RNA dH2O 96hr 5ml	5/11/2010	789
A9			
B1	Protos Clone #6 24hr 1ml	5/3/2010	781-782
B2	Protos Clone #6 96hr 1ml	5/3/2010	781-782
B3	Protos Clone #7 24hr 1ml	5/3/2010	781-782
B4	Protos Clone #7 96hr 1ml	5/3/2010	781-782
B5	Protos RNA1+2 24hr 1ml 4/7/10	5/3/2010	781-782
B6	Protos RNA1+2 96hr 1ml 4/7/10	5/3/2010	781-782
B7	Protos RNA1+2 96hr 1ml 2/17/10	5/3/2010	781-782
B8	Protos dH2O 96hr 1ml	5/3/2010	781-782
B9			
C1	Protos Clone #6 24hr 1ml	5/3/2010	781-782
C2	Protos Clone #6 96hr 1ml	5/3/2010	781-782
C3	Protos Clone #7 24hr 1ml	5/3/2010	781-782
C4	Protos Clone #7 96hr 1ml	5/3/2010	781-782
C5	Protos RNA1+2 24hr 1ml 4/7/10	5/3/2010	781-782
C6	Protos RNA1+2 96hr 1ml 4/7/10	5/3/2010	781-782
C7	Protos RNA1+2 96hr 1ml 2/17/10	5/3/2010	781-782
C8	Protos dH2O 96hr 1ml	5/3/2010	781-782
C9			
D1	Total RNA Clone #8 24hr 5ml	5/11/2010	789
D2	Total RNA Clone #8 96hr 5ml	5/11/2010	789
D3	Total RNA Clone #9 24hr 5ml	5/11/2010	789
D4	Total RNA Clone #9 96hr 5ml	5/11/2010	789
D5	Total RNA RNA1+2 24hr 5ml 4/7/10	5/11/2010	789
D6	Total RNA RNA1+2 96hr 5ml 4/7/10	5/11/2010	789
D7	Total RNA RNA1+2 96hr 5ml 2/17/10	5/11/2010	789
D8	Total RNA dH2O 96hr 5ml	5/11/2010	789
D9			
E1	Protos Clone #8 24hr 1ml	5/6/2010	785-786
E2	Protos Clone #8 96hr 1ml	5/6/2010	785-786
E3	Protos Clone #9 24hr 1ml	5/6/2010	785-786
E4	Protos Clone #9 96hr 1ml	5/6/2010	785-786
E5	Protos RNA1+2 24hr 1ml 4/7/10	5/6/2010	785-786
E6	Protos RNA1+2 96hr 1ml 4/7/10	5/6/2010	785-786
E7	Protos RNA1+2 96hr 1ml 2/17/10	5/6/2010	785-786
E8	Protos dH2O 96hr 1ml	5/6/2010	785-786
E9			

F1	Protos Clone #8 24hr 1ml	5/6/2010	785-786
F2	Protos Clone #8 96hr 1ml	5/6/2010	785-786
F3	Protos Clone #9 24hr 1ml	5/6/2010	785-786
F4	Protos Clone #9 96hr 1ml	5/6/2010	785-786
F5	Protos RNA1+2 24hr 1ml 4/7/10	5/6/2010	785-786
F6	Protos RNA1+2 96hr 1ml 4/7/10	5/6/2010	785-786
F7	Protos RNA1+2 96hr 1ml 2/17/10	5/6/2010	785-786
F8	Protos dH2O 96hr 1ml	5/6/2010	785-786
F9			
G1	Total RNA Clone #5 96hr 5ml	6/14/2010	812
G2	Total RNA Clone #6 96hr 5ml	6/14/2010	812
G3	Total RNA Clone #7 96hr 5ml	6/14/2010	812
G4	Total RNA Clone #8 96hr 5ml	6/14/2010	812
G5	Total RNA Clone #9 96hr 5ml 2/17/10	6/14/2010	812
G6	Total RNA Clone #9 96hr 5ml 6/6/10	6/14/2010	812
G7	Total RNA RNA1+2 96hr 5ml 2/17/10	6/14/2010	812
G8	Total RNA RNA1+2 96hr 5ml 4/7/10	6/14/2010	812
G9	Total RNA dH2O 96hr 5ml	6/14/2010	812
H1	Protos Clone #5 96hr 1ml	6/7/2010	808-809
H2	Protos Clone #6 96hr 1ml	6/7/2010	808-809
H3	Protos Clone #7 96hr 1ml	6/7/2010	808-809
H4	Protos Clone #8 96hr 1ml	6/7/2010	808-809
H5	Protos Clone #9 96hr 1ml 2/17/10	6/7/2010	808-809
H6	Protos Clone #9 96hr 1ml 6/6/10	6/7/2010	808-809
H7	Protos RNA1+2 96hr 1ml 2/17/10	6/7/2010	808-809
H8	Protos RNA1+2 96hr 1ml 4/7/10	6/7/2010	808-809
H9	Protos dH2O 96hr 1ml	6/7/2010	808-809
I1	Protos Clone #5 96hr 1ml	6/7/2010	808-809
I2	Protos Clone #6 96hr 1ml	6/7/2010	808-809
I3	Protos Clone #7 96hr 1ml	6/7/2010	808-809
I4	Protos Clone #8 96hr 1ml	6/7/2010	808-809
I5	Protos Clone #9 96hr 1ml 2/17/10	6/7/2010	808-809
I6	Protos Clone #9 96hr 1ml 6/6/10	6/7/2010	808-809
I7	Protos RNA1+2 96hr 1ml 2/17/10	6/7/2010	808-809
I8	Protos RNA1+2 96hr 1ml 4/7/10	6/7/2010	808-809
I9	Protos dH2O 96hr 1ml	6/7/2010	808-809

Chawin's protoplast #5: -80^{0}C Level #1 Tower #3 Box #5

	Description:	Date:	NB pg:
A1	Total RNA Clone #5 24hr 5ml	6/28/2010	819
A2	Total RNA Clone #6 24hr 5ml	6/28/2010	819
A3	Total RNA Clone #7 24hr 5ml	6/28/2010	819
A4	Total RNA Clone #8 24hr 5ml	6/28/2010	819
A5	Total RNA Clone #9 24hr 5ml	6/28/2010	819
A6	Total RNA RNA1+2 24hr 5ml	6/28/2010	819
A7	Total RNA dH2O 24hr 5ml	6/28/2010	819
A8	Total RNA Clone #5 96hr 5ml	6/28/2010	819
A9	Total RNA Clone #6 96hr 5ml	6/28/2010	819
B1	Protos Clone #5 24hr 1ml	6/21/2010	814-815
B2	Protos Clone #6 24hr 1ml	6/21/2010	814-815
B3	Protos Clone #7 24hr 1ml	6/21/2010	814-815
B4	Protos Clone #8 24hr 1ml	6/21/2010	814-815
B5	Protos Clone #9 24hr 1ml	6/21/2010	814-815
B6	Protos RNA1+2 24hr 1ml	6/21/2010	814-815
B7	Protos dH2O 24hr 1ml	6/21/2010	814-815
B8	Protos Clone #5 96hr 1ml	6/21/2010	814-815
B9	Protos Clone #6 96hr 1ml	6/21/2010	814-815
C1	Protos Clone #5 24hr 1ml	6/21/2010	814-815
C2	Protos Clone #6 24hr 1ml	6/21/2010	814-815
C3	Protos Clone #7 24hr 1ml	6/21/2010	814-815
C4	Protos Clone #8 24hr 1ml	6/21/2010	814-815
C5	Protos Clone #9 24hr 1ml	6/21/2010	814-815
C6	Protos RNA1+2 24hr 1ml	6/21/2010	814-815
C7	Protos dH2O 24hr 1ml	6/21/2010	814-815
C8	Protos Clone #5 96hr 1ml	6/21/2010	814-815
C9	Protos Clone #6 96hr 1ml	6/21/2010	814-815
D1	Total RNA Clone #7 96hr 5ml	6/28/2010	819
D2	Total RNA Clone #8 96hr 5ml	6/28/2010	819
D3	Total RNA Clone #9 96hr 5ml	6/28/2010	819
D4	Total RNA RNA1+2 96hr 5ml	6/28/2010	819
D5	Total RNA dH2O 96hr 5ml	6/28/2010	819
D6	Total RNA Clone #1 24hr 5ml	7/26/2010	831
D7	Total RNA Clone #1 96hr 5ml	7/26/2010	831
D8	Total RNA Clone #2 24hr 5ml	7/26/2010	831
D9	Total RNA Clone #2 96hr 5ml	7/26/2010	831
E1	Protos Clone #7 96hr 1ml	6/21/2010	814-815
E2	Protos Clone #8 96hr 1ml	6/21/2010	814-815
E3	Protos Clone #9 96hr 1ml	6/21/2010	814-815
E4	Protos RNA1+2 96hr 1ml	6/21/2010	814-815
E5	Protos dH2O 96hr 1ml	6/21/2010	814-815
E6	Protos Clone #1 24hr 1ml	7/8/2010	827-828
E7	Protos Clone #1 96hr 1ml	7/8/2010	827-828
E8	Protos Clone #2 24hr 1ml	7/8/2010	827-828
E9	Protos Clone #2 96hr 1ml	7/8/2010	827-828

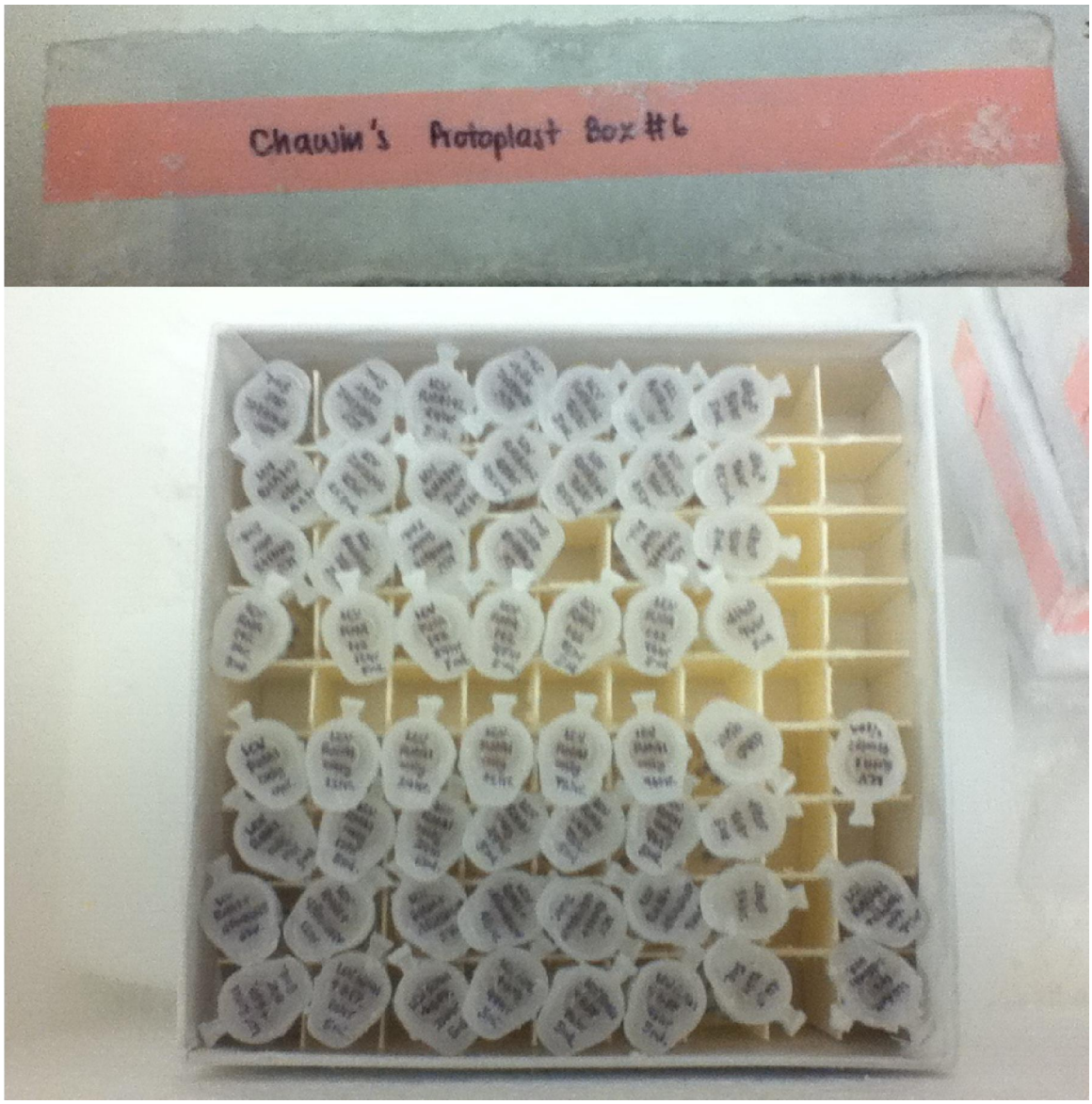
F1	Protos Clone #7 96hr 1ml	6/21/2010	814-815
F2	Protos Clone #8 96hr 1ml	6/21/2010	814-815
F3	Protos Clone #9 96hr 1ml	6/21/2010	814-815
F4	Protos RNA1+2 96hr 1ml	6/21/2010	814-815
F5	Protos dH2O 96hr 1ml	6/21/2010	814-815
F6	Protos Clone #1 24hr 1ml	7/8/2010	827-828
F7	Protos Clone #1 96hr 1ml	7/8/2010	827-828
F8	Protos Clone #2 24hr 1ml	7/8/2010	827-828
F9	Protos Clone #2 96hr 1ml	7/8/2010	827-828
G1	Total RNA Clone #3 24hr 5ml	7/26/2010	831
G2	Total RNA Clone #3 96hr 5ml	7/26/2010	831
G3	Total RNA Clone #4 24hr 5ml	7/26/2010	831
G4	Total RNA Clone #4 96hr 5ml	7/26/2010	831
G5	Total RNA Clone #5 24hr 5ml	7/26/2010	831
G6	Total RNA Clone #5 96hr 5ml	7/26/2010	831
G7	Total RNA RNA1+2 24hr 5ml	7/26/2010	831
G8	Total RNA RNA1+2 96hr 5ml	7/26/2010	831
G9	Total RNA dH2O 96hr 5ml	7/26/2010	831
H1	Protos Clone #3 24hr 1ml	7/8/2010	827-828
H2	Protos Clone #3 96hr 1ml	7/8/2010	827-828
H3	Protos Clone #4 24hr 1ml	7/8/2010	827-828
H4	Protos Clone #4 96hr 1ml	7/8/2010	827-828
H5	Protos Clone #5 24hr 1ml	7/8/2010	827-828
H6	Protos Clone #5 96hr 1ml	7/8/2010	827-828
H7	Protos RNA1+2 24hr 1ml	7/8/2010	827-828
H8	Protos RNA1+2 96hr 1ml	7/8/2010	827-828
H9			
I1	Protos Clone #3 24hr 1ml	7/8/2010	827-828
I2	Protos Clone #3 96hr 1ml	7/8/2010	827-828
I3	Protos Clone #4 24hr 1ml	7/8/2010	827-828
I4	Protos Clone #4 96hr 1ml	7/8/2010	827-828
I5	Protos Clone #5 24hr 1ml	7/8/2010	827-828
I6	Protos Clone #5 96hr 1ml	7/8/2010	827-828
I7	Protos RNA1+2 24hr 1ml	7/8/2010	827-828
I8	Protos RNA1+2 96hr 1ml	7/8/2010	827-828
I9			



Chawin's protoplast #6: -80^{0}C Level #1 Tower #3 Box #6

	Description:	Date:	NB pg:
A1	Total RNA LCV 1+2 0hr 5ml 10/25/10	11/1/2010	885
A2	Total RNA LCV 1+2 12hr 5ml 10/25/10	11/1/2010	885
A3	Total RNA LCV 1+2 24hr 5ml 10/25/10	11/1/2010	885
A4	Total RNA LCV 1+2 48hr 5ml 10/25/10	11/1/2010	885
A5	Total RNA LCV 1+2 72hr 5ml 10/25/10	11/1/2010	885
A6	Total RNA LCV 1+2 96hr 5ml 10/25/10	11/1/2010	885
A7	Total RNA dH2O 96hr 5ml 10/25/10	11/1/2010	885
A8			
A9			
B1	Total RNA LCV 1+2 0hr 4x1ml 10/25/10	12/7/2010	908
B2	Total RNA LCV 1+2 12hr 4x1ml 10/25/10	12/7/2010	908
B3	Total RNA LCV 1+2 24hr 4x1ml 10/25/10	12/7/2010	908
B4	Total RNA LCV 1+2 48hr 4x1ml 10/25/10	12/7/2010	908
B5	Total RNA LCV 1+2 72hr 4x1ml 10/25/10	12/7/2010	908
B6	Total RNA LCV 1+2 96hr 4x1ml 10/25/10	12/7/2010	908
B7	Total RNA dH2O 96hr 4x1ml 10/25/10	12/7/2010	908
B8			
B9			
C1	Total RNA LCV 1+2 0hr 5ml 1/10/11	1/18/2011	925
C2	Total RNA LCV 1+2 12hr 5ml 1/10/11	1/18/2011	925
C3	Total RNA LCV 1+2 24hr 5ml 1/10/11	1/18/2011	925
C4	Total RNA LCV 1+2 48hr 5ml 1/10/11	1/18/2011	925
C5			
C6	Total RNA LCV 1+2 96hr 5ml 1/10/11	1/18/2011	925
C7	Total RNA dH2O 96hr 5ml 1/10/11	1/18/2011	925
C8			
C9			
D1	Protos LCV 1+2 0hr 5ml	1/10/2011	918-919
D2	Protos LCV 1+2 12hr 5ml	1/10/2011	918-919
D3	Protos LCV 1+2 24hr 5ml	1/10/2011	918-919
D4	Protos LCV 1+2 48hr 5ml	1/10/2011	918-919
D5	Protos LCV 1+2 72hr 5ml	1/10/2011	918-919
D6	Protos LCV 1+2 96hr 5ml	1/10/2011	918-919
D7	Protos dH2O 96hr 5ml	1/10/2011	918-919
D8			
D9			
E1			
E2			
E3			
E4			
E5			
E6			
E7			
E8			
E9			

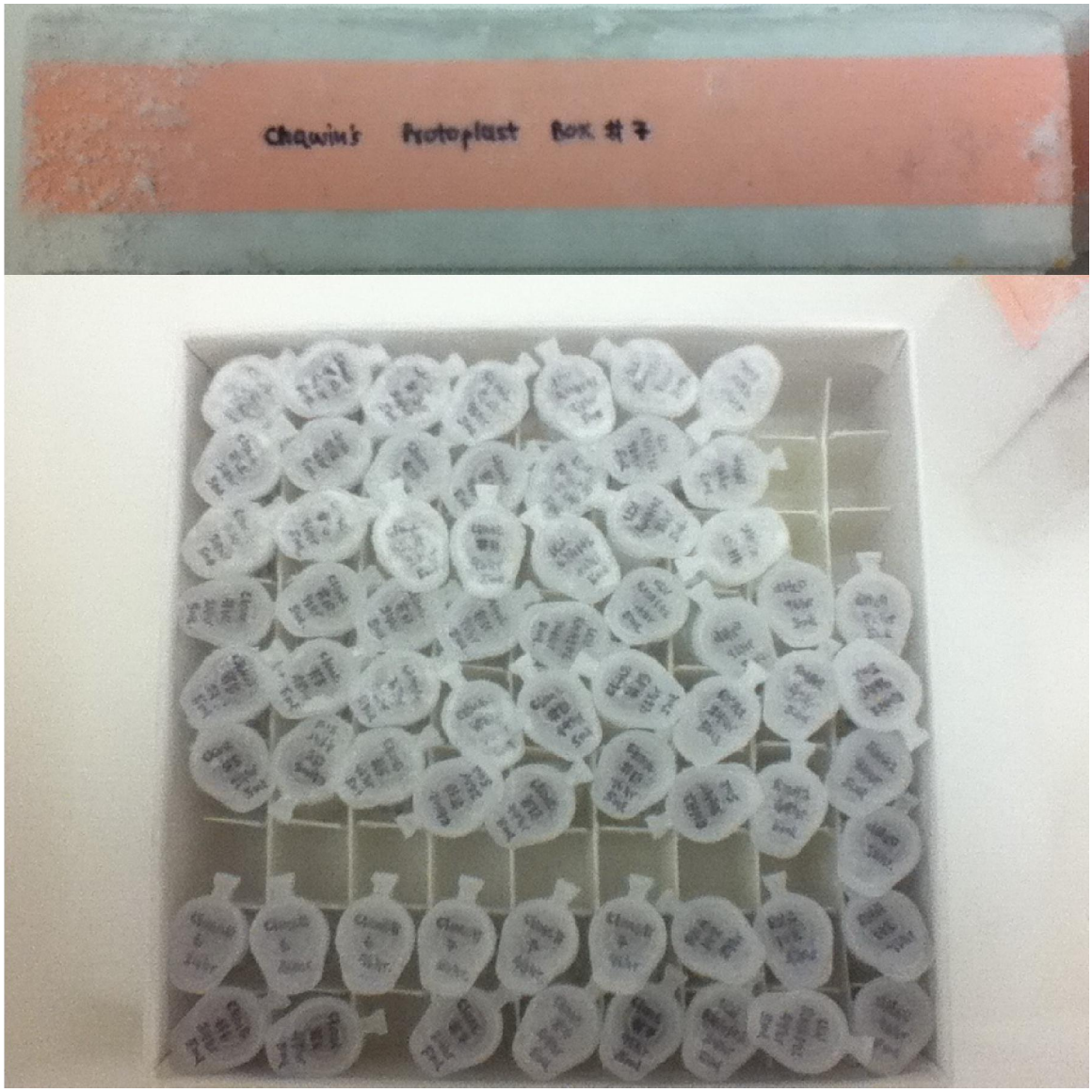
F1	Protos LCV 1 0hr 5ml	4/18/2011	997-998
F2	Protos LCV 1 12hr 5ml	4/18/2011	997-998
F3	Protos LCV 1 24hr 5ml	4/18/2011	997-998
F4	Protos LCV 1 48hr 5ml	4/18/2011	997-998
F5	Protos LCV 1 72hr 5ml	4/18/2011	997-998
F6	Protos LCV 1 96hr 5ml	4/18/2011	997-998
F7	Protos dH2O 96hr 5ml	4/18/2011	997-998
F8			
F9			
G1	Total RNA LCV 1 0hr 5ml	4/26/2011	1009
G2	Total RNA LCV 1 12hr 5ml	4/26/2011	1009
G3	Total RNA LCV 1 24hr 5ml	4/26/2011	1009
G4	Total RNA LCV 1 48hr 5ml	4/26/2011	1009
G5	Total RNA LCV 1 72hr 5ml	4/26/2011	1009
G6	Total RNA LCV 1 96hr 5ml	4/26/2011	1009
G7	Total RNA dH2O 96hr 5ml	4/26/2011	1009
G8			
G9	LCV RNA1 transcript 1/10x		925
H1	Total RNA LCV 1+ dRNA1-GFP 0hr 5ml 5/31/11	6/5/2011	1040
H2	Total RNA LCV 1+ dRNA1-GFP 12hr 5ml 5/31/11	6/5/2011	1040
H3	Total RNA LCV 1+ dRNA1-GFP 24hr 5ml 5/31/11	6/5/2011	1040
H4	Total RNA LCV 1+ dRNA1-GFP 48hr 5ml 5/31/11	6/5/2011	1040
H5	Total RNA LCV 1+ dRNA1-GFP 72hr 5ml 5/31/11	6/5/2011	1040
H6	Total RNA LCV 1+ dRNA1-GFP 96hr 5ml 5/31/11	6/5/2011	1040
H7	Total RNA dH2O 96hr 5ml 5/31/11	6/5/2011	1040
H8			
H9	Total RNA LCV 1+2+ dRNA1-GFP 72hr 5ml 6/2/11	6/5/2011	1040
I1	Protos LCV 1+ dRNA1-GFP 0hr 5ml 5/31/11	6/5/2011	1033-1034
I2	Protos LCV 1+ dRNA1-GFP 12hr 5ml 5/31/11	6/5/2011	1033-1034
I3	Protos LCV 1+ dRNA1-GFP 24hr 5ml 5/31/11	6/5/2011	1033-1034
I4	Protos LCV 1+ dRNA1-GFP 48hr 5ml 5/31/11	6/5/2011	1033-1034
I5	Protos LCV 1+ dRNA1-GFP 72hr 5ml 5/31/11	6/5/2011	1033-1034
I6	Protos LCV 1+ dRNA1-GFP 96hr 5ml 5/31/11	6/5/2011	1033-1034
I7	Protos dH2O 96hr 5ml 5/31/11	6/5/2011	1033-1034
I8			
I9	Protos LCV 1+2+ dRNA1-GFP 72hr 5ml 6/2/11	6/2/2011	1036-1037


Chawin's Protoplast Box #6

Chawin's protoplast #7: -80^{0}C Level #1 Tower #3 Box #7

	Description:	Date:	NB pg:
A1	Protos Clone #12 24hr 5ml	7/21/2011	1075-1076
A2	Protos Clone #12 96hr 5ml	7/21/2011	1075-1076
A3	Protos Clone #13 24hr 5ml	7/21/2011	1075-1076
A4	Protos Clone #13 96hr 5ml	7/21/2011	1075-1076
A5	Protos RNA1+2 24hr 5ml	7/21/2011	1075-1076
A6	Protos RNA1+2 96hr 5ml	7/21/2011	1075-1076
A7	Protos dH2O 96hr 5ml	7/21/2011	1075-1076
A8			
A9			
B1	Total RNA Clone #12 24hr 5ml	7/26/2011	1077
B2	Total RNA Clone #12 96hr 5ml	7/26/2011	1077
B3	Total RNA Clone #13 24hr 5ml	7/26/2011	1077
B4	Total RNA Clone #13 96hr 5ml	7/26/2011	1077
B5	Total RNA RNA1+2 24hr 5ml	7/26/2011	1077
B6	Total RNA RNA1+2 96hr 5ml	7/26/2011	1077
B7	Total RNA dH2O 96hr 5ml	7/26/2011	1077
B8			
B9			
C1	Protos Clone #10 24hr 5ml	7/28/2011	1080-1081
C2	Protos Clone #10 96hr 5ml	7/28/2011	1080-1081
C3	Protos Clone #11 24hr 5ml	7/28/2011	1080-1081
C4	Protos Clone #11 96hr 5ml	7/28/2011	1080-1081
C5	Protos RNA1+2 24hr 5ml	7/28/2011	1080-1081
C6	Protos RNA1+2 96hr 5ml	7/28/2011	1080-1081
C7	Protos dH2O 96hr 5ml	7/28/2011	1080-1081
C8			
C9			
	Description:	Date:	NB pg:
D1	Total RNA Clone #10 24hr 5ml	8/2/2011	1082
D2	Total RNA Clone #10 96hr 5ml	8/2/2011	1082
D3	Total RNA Clone #11 24hr 5ml	8/2/2011	1082
D4	Total RNA Clone #11 96hr 5ml	8/2/2011	1082
D5	Total RNA RNA1+2 24hr 5ml	8/2/2011	1082
D6	Total RNA RNA1+2 96hr 5ml	8/2/2011	1082
D7	Total RNA dH2O 96hr 5ml	8/2/2011	1082
D8	Total RNA dH2O 96hr 5ml	9/6/2011	1097
D9	Protos dH2O 96hr 5ml	8/30/2011	1092-1093
E1	Protos Clone #5 24hr 5ml	8/29/2011	1092-1093
E2	Protos Clone #5 48hr 5ml	8/30/2011	1092-1093
E3	Protos Clone #5 96hr 5ml	8/30/2011	1092-1093
E4	Protos Clone #13 24hr 5ml	8/30/2011	1092-1093
E5	Protos Clone #13 48hr 5ml	8/30/2011	1092-1093
E6	Protos Clone #13 96hr 5ml	8/30/2011	1092-1093
E7	Protos RNA1+2 24hr 5ml	8/30/2011	1092-1093
E8	Protos RNA1+2 48hr 5ml	8/30/2011	1092-1093
E9	Protos RNA1+2 96hr 5ml	8/30/2011	1092-1093

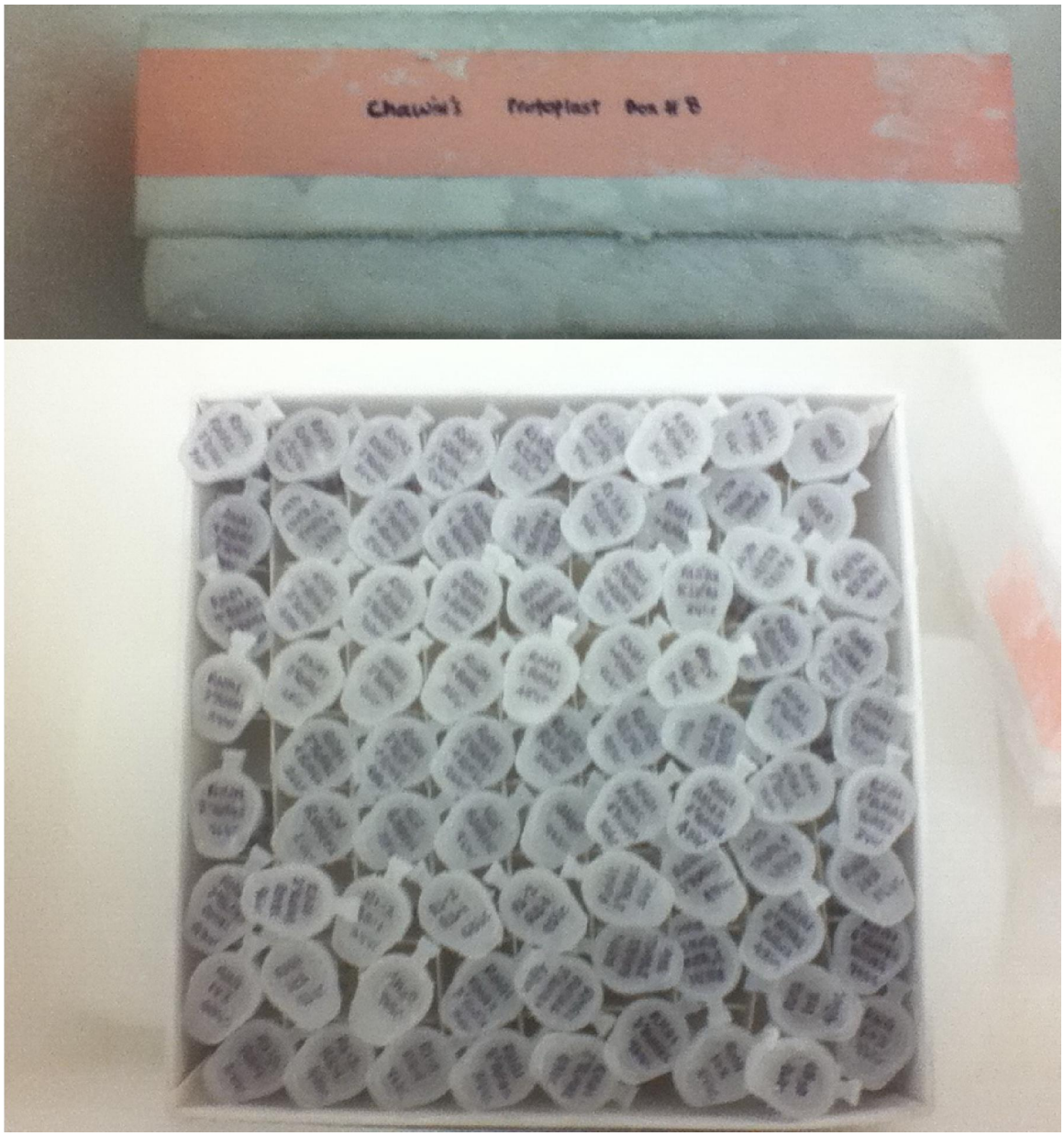
F1	Total RNA Clone #5 24hr 5ml	9/6/2011	1097
F2	Total RNA Clone #5 48hr 5ml	9/6/2011	1097
F3	Total RNA Clone #5 96hr 5ml	9/6/2011	1097
F4	Total RNA Clone #13 24hr 5ml	9/6/2011	1097
F5	Total RNA Clone #13 48hr 5ml	9/6/2011	1097
F6	Total RNA Clone #13 96hr 5ml	9/6/2011	1097
F7	Total RNA RNA1+2 24hr 5ml	9/6/2011	1097
F8	Total RNA RNA1+2 48hr 5ml	9/6/2011	1097
F9	Total RNA RNA1+2 96hr 5ml	9/6/2011	1097
	Description:	Date:	NB pg:
G1			
G2			
G3			
G4			
G5			
G6			
G7			
G8			
G9	Total RNA dH2O 96hr 5ml	3/19/2012	1225
H1	Total RNA Clone #6 24hr 5ml	3/19/2012	1225
H2	Total RNA Clone #6 48hr 5ml	3/19/2012	1225
H3	Total RNA Clone #6 96hr 5ml	3/19/2012	1225
H4	Total RNA Clone #7 24hr 5ml	3/19/2012	1225
H5	Total RNA Clone #7 48hr 5ml	3/19/2012	1225
H6	Total RNA Clone #7 96hr 5ml	3/19/2012	1225
H7	Total RNA RNA1+2 24hr 5ml	3/19/2012	1225
H8	Total RNA RNA1+2 48hr 5ml	3/19/2012	1225
H9	Total RNA RNA1+2 96hr 5ml	3/19/2012	1225
I1	Total RNA Clone #6 24hr 5ml	9/19/2011	1109
I2	Total RNA Clone #6 48hr 5ml	9/19/2011	1109
I3			
I4	Total RNA Clone #7 24hr 5ml	9/19/2011	1109
I5	Total RNA Clone #7 48hr 5ml	9/19/2011	1109
I6	Total RNA Clone #7 96hr 5ml	9/19/2011	1109
I7	Total RNA RNA1+2 24hr 5ml	9/19/2011	1109
I8	Total RNA RNA1+2 48hr 5ml	9/19/2011	1109
I9	Total RNA dH2O 96hr 5ml	9/19/2011	1109


Chawin's Protoplast Box #7

Chawin's protoplast #8: -80^{0}C Level #1 Tower #3 Box #8

	Description:	Date:	NB pg:
A1	Protos R1 + R2 3'R1 24hr 5ml	10/3/2011	1119-1120
A2	Protos R1 + R2 3'R1 96hr 5ml	10/3/2011	1119-1120
A3	Protos R1 3'R2 + R2 24hr 5ml	10/3/2011	1119-1120
A4	Protos R1 3'R2 + R2 96hr 5ml	10/3/2011	1119-1120
A5	Protos R1 3'R2 + R2 3'R1 24hr 5ml	10/3/2011	1119-1120
A6	Protos R1 3'R2 + R2 3'R1 96hr 5ml	10/3/2011	1119-1120
A7	Protos RNA1+2 24hr 5ml	10/3/2011	1119-1120
A8	Protos RNA1+2 96hr 5ml	10/3/2011	1119-1120
A9	Protos dH2O 96hr 5ml	10/3/2011	1119-1120
B1	Total RNA R1 + R2 3'R1 24hr 5ml	10/10/2011	1125
B2	Total RNA R1 + R2 3'R1 96hr 5ml	10/10/2011	1125
B3	Total RNA R1 3'R2 + R2 24hr 5ml	10/10/2011	1125
B4	Total RNA R1 3'R2 + R2 96hr 5ml	10/10/2011	1125
B5	Total RNA R1 3'R2 + R2 3'R1 24hr 5ml	10/10/2011	1125
B6	Total RNA R1 3'R2 + R2 3'R1 96hr 5ml	10/10/2011	1125
B7	Total RNA RNA1+2 24hr 5ml	10/10/2011	1125
B8	Total RNA RNA1+2 96hr 5ml	10/10/2011	1125
B9	Total RNA dH2O 96hr 5ml	10/10/2011	1125
C1	Protos R1 + R2 3'R1 24hr 5ml	10/17/2011	1128-1129
C2	Protos R1 + R2 3'R1 48hr 5ml	10/17/2011	1128-1129
C3	Protos R1 + R2 3'R1 96hr 5ml	10/17/2011	1128-1129
C4	Protos R1 3'R2 + R2 24hr 5ml	10/17/2011	1128-1129
C5	Protos R1 3'R2 + R2 48hr 5ml	10/17/2011	1128-1129
C6	Protos R1 3'R2 + R2 96hr 5ml	10/17/2011	1128-1129
C7	Protos R1 3'R2 + R2 3'R1 24hr 5ml	10/17/2011	1128-1129
C8	Protos R1 3'R2 + R2 3'R1 48hr 5ml	10/17/2011	1128-1129
C9	Protos R1 3'R2 + R2 3'R1 96hr 5ml	10/17/2011	1128-1129
D1	Protos R1 3'R2 24hr 5ml	10/17/2011	1128-1129
D2	Protos R1 3'R2 48hr 5ml	10/17/2011	1128-1129
D3	Protos R1 3'R2 96hr 5ml	10/17/2011	1128-1129
D4	Protos RNA1+2 24hr 5ml	10/17/2011	1128-1129
D5	Protos RNA1+2 48hr 5ml	10/17/2011	1128-1129
D6	Protos RNA1+2 96hr 5ml	10/17/2011	1128-1129
D7	Protos dH2O 96hr 5ml	10/17/2011	1128-1129
D8	Total RNA R1 + R2 3'R1 24hr 5ml	10/24/2011	1138
D9	Total RNA R1 + R2 3'R1 48hr 5ml	10/24/2011	1138
E1			
E2	Total RNA R1 3'R2 + R2 24hr 5ml	10/24/2011	1138
E3	Total RNA R1 3'R2 + R2 48hr 5ml	10/24/2011	1138
E4	Total RNA R1 3'R2 + R2 96hr 5ml	10/24/2011	1138
E5	Total RNA R1 3'R2 + R2 3'R1 24hr 5ml	10/24/2011	1138
E6	Total RNA R1 3'R2 + R2 3'R1 48hr 5ml	10/24/2011	1138
E7	Total RNA R1 3'R2 + R2 3'R1 96hr 5ml	10/24/2011	1138
E8	Total RNA R1 3'R2 24hr 5ml	10/24/2011	1138
E9	Total RNA R1 3'R2 48hr 5ml	10/24/2011	1138

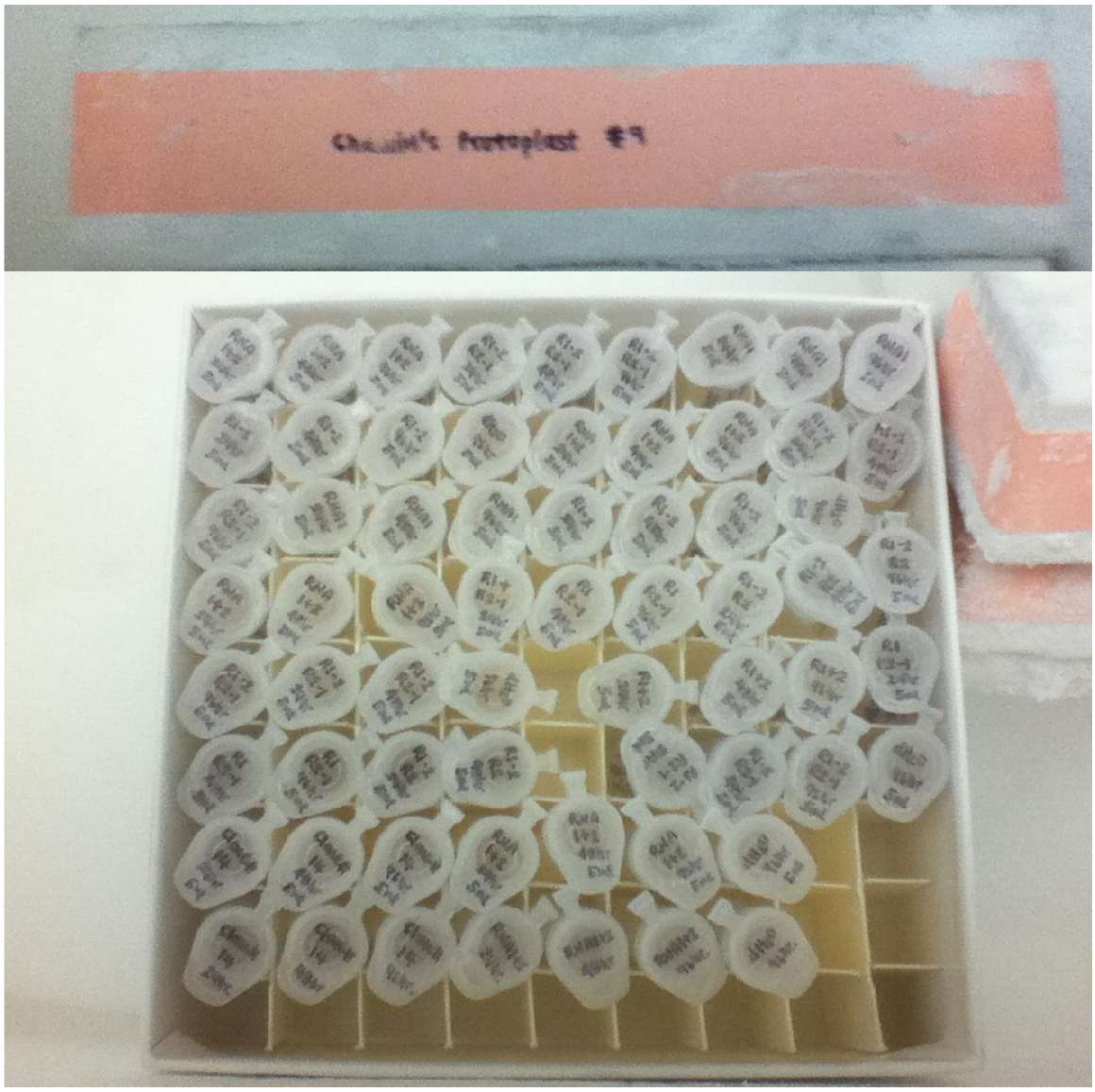
F1	Total RNA R1 3'R2 96hr 5ml	10/24/2011	1138
F2	Total RNA RNA1+2 24hr 5ml	10/24/2011	1138
F3	Total RNA RNA1+2 48hr 5ml	10/24/2011	1138
F4	Total RNA RNA1+2 96hr 5ml	10/24/2011	1138
F5	Total RNA dH2O 96hr 5ml	10/24/2011	1138
F6	Protos R1 + R2 3'R1 24hr 5ml	11/28/2011	1148-1149
F7	Protos R1 + R2 3'R1 48hr 5ml	11/28/2011	1148-1149
F8	Protos R1 + R2 3'R1 96hr 5ml	11/28/2011	1148-1149
F9	Protos R1 3'R2 + R2 24hr 5ml	11/28/2011	1148-1149
G1	Protos R1 3'R2 + R2 48hr 5ml	11/28/2011	1148-1149
G2	Protos R1 3'R2 + R2 96hr 5ml	11/28/2011	1148-1149
G3	Protos R1 3'R2 + R2 3'R1 24hr 5ml	11/28/2011	1148-1149
G4	Protos R1 3'R2 + R2 3'R1 48hr 5ml	11/28/2011	1148-1149
G5	Protos R1 3'R2 + R2 3'R1 96hr 5ml	11/28/2011	1148-1149
G6	Protos R1 3'R2 24hr 5ml	11/28/2011	1148-1149
G7	Protos R1 3'R2 48hr 5ml	11/28/2011	1148-1149
G8	Protos R1 3'R2 96hr 5ml	11/28/2011	1148-1149
G9	Protos RNA1+2 24hr 5ml	11/28/2011	1148-1149
H1	Protos RNA1+2 48hr 5ml	11/29/2011	1148-1149
H2	Protos RNA1+2 96hr 5ml	11/29/2011	1148-1149
H3	Protos dH2O 96hr 5ml	11/29/2011	1148-1149
H4	Total RNA R1 + R2 3'R1 24hr 5ml	12/5/2011	1150
H5	Total RNA R1 + R2 3'R1 48hr 5ml	12/5/2011	1150
H6	Total RNA R1 + R2 3'R1 96hr 5ml	12/5/2011	1150
H7	Total RNA R1 3'R2 + R2 24hr 5ml	12/5/2011	1150
H8	Total RNA R1 3'R2 + R2 48hr 5ml	12/5/2011	1150
H9	Total RNA R1 3'R2 + R2 96hr 5ml	12/5/2011	1150
I1	Total RNA R1 3'R2 + R2 3'R1 24hr 5ml	12/5/2011	1150
I2	Total RNA R1 3'R2 + R2 3'R1 48hr 5ml	12/5/2011	1150
I3	Total RNA R1 3'R2 + R2 3'R1 96hr 5ml	12/5/2011	1150
I4	Total RNA R1 3'R2 24hr 5ml	12/5/2011	1150
I5	Total RNA R1 3'R2 48hr 5ml	12/5/2011	1150
I6	Total RNA R1 3'R2 96hr 5ml	12/5/2011	1150
I7	Total RNA RNA1+2 24hr 5ml	12/5/2011	1150
I8	Total RNA RNA1+2 48hr 5ml	12/5/2011	1150
I9	Total RNA RNA1+2 96hr 5ml	12/5/2011	1150
	Total RNA dH2O 96hr 5ml	12/5/2011	1150


Chawin's Protoplast Box # 3

Chawin's protoplast #9: -80^{0}C Level #1 Tower #3 Box #9

	Description:	Date:	NB pg:
A1	Protos RNA1+2 24hr 5ml	12/20/2011	1160-1161
A2	Protos RNA1+2 48hr 5ml	12/20/2011	1160-1161
A3	Protos RNA1+2 96hr 5ml	12/20/2011	1160-1161
A4	Protos R1 3'R2 + R2 3'R1 24hr 5ml	12/20/2011	1160-1161
A5	Protos R1 3'R2 + R2 3'R1 48hr 5ml	12/20/2011	1160-1161
A6	Protos R1 3'R2 + R2 3'R1 96hr 5ml	12/20/2011	1160-1161
A7	Protos R1 24hr 5ml	12/20/2011	1160-1161
A8	Protos R1 48hr 5ml	12/20/2011	1160-1161
A9	Protos R1 96hr 5ml	12/20/2011	1160-1161
B1	Protos R1 3'R2 24hr 5ml	12/20/2011	1160-1161
B2	Protos R1 3'R2 48hr 5ml	12/20/2011	1160-1161
B3	Protos R1 3'R2 96hr 5ml	12/20/2011	1160-1161
B4	Protos dH2O 96hr 5ml	12/20/2011	1160-1161
B5	Total RNA RNA1+2 24hr 5ml	12/27/2011	1165
B6	Total RNA RNA1+2 48hr 5ml	12/27/2011	1165
B7	Total RNA RNA1+2 96hr 5ml	12/27/2011	1165
B8	Total RNA R1 3'R2 + R2 3'R1 24hr 5ml	12/27/2011	1165
B9	Total RNA R1 3'R2 + R2 3'R1 48hr 5ml	12/27/2011	1165
C1	Total RNA R1 3'R2 + R2 3'R1 96hr 5ml	12/27/2011	1165
C2	Total RNA R1 24hr 5ml	12/27/2011	1165
C3	Total RNA R1 48hr 5ml	12/27/2011	1165
C4	Total RNA R1 96hr 5ml	12/27/2011	1165
C5	Total RNA R1 3'R2 24hr 5ml	12/27/2011	1165
C6	Total RNA R1 3'R2 48hr 5ml	12/27/2011	1165
C7	Total RNA R1 3'R2 96hr 5ml	12/27/2011	1165
C8	Total RNA dH2O 96hr 5ml	12/27/2011	1165
C9			
D1	Protos RNA1+2 24hr 5ml	1/23/2012	1183-1184
D2	Protos RNA1+2 48hr 5ml	1/23/2012	1183-1184
D3	Protos RNA1+2 96hr 5ml	1/23/2012	1183-1184
D4	Protos R1 + R2 3'R1 24hr 5ml	1/23/2012	1183-1184
D5	Protos R1 + R2 3'R1 48hr 5ml	1/23/2012	1183-1184
D6	Protos R1 + R2 3'R1 96hr 5ml	1/23/2012	1183-1184
D7	Protos R1 3'R2 + R2 3'R1 24hr 5ml	1/23/2012	1183-1184
D8	Protos R1 3'R2 + R2 3'R1 48hr 5ml	1/23/2012	1183-1184
D9	Protos R1 3'R2 + R2 3'R1 96hr 5ml	1/23/2012	1183-1184
E1	Protos R1 3'R2 + R2 3'R1 24hr 5ml	1/23/2012	1183-1184
E2	Protos R1 3'R2 + R2 3'R1 48hr 5ml	1/23/2012	1183-1184
E3	Protos R1 3'R2 + R2 3'R1 96hr 5ml	1/23/2012	1183-1184
E4	Protos dH2O 96hr 5ml	1/23/2012	1183-1184
E5			
E6	Total RNA RNA1+2 24hr 5ml	1/30/2012	1190
E7	Total RNA RNA1+2 48hr 5ml	1/30/2012	1190
E8	Total RNA RNA1+2 96hr 5ml	1/30/2012	1190
E9	Total RNA R1 + R2 3'R1 24hr 5ml	1/30/2012	1190

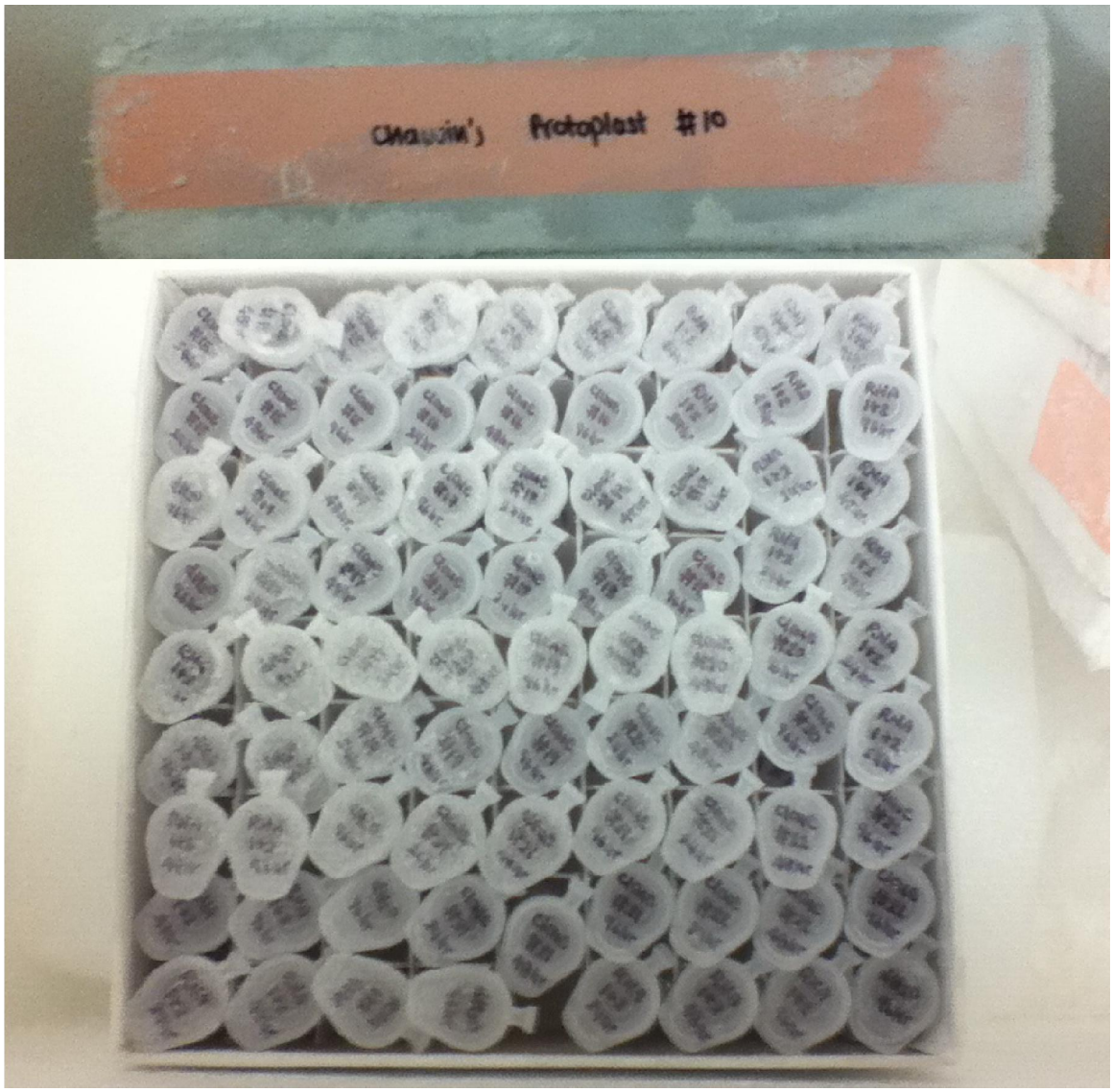
F1	Total RNA R1 + R2 3'R1 48hr 5ml	1/30/2012	1190
F2	Total RNA R1 + R2 3'R1 96hr 5ml	1/30/2012	1190
F3	Total RNA R1 3'R2 + R2 24hr 5ml	1/30/2012	1190
F4	Total RNA R1 3'R2 + R2 48hr 5ml	1/30/2012	1190
F5			
F6	Total RNA R1 3'R2 + R2 3'R1 24hr 5ml	1/30/2012	1190
F7	Total RNA R1 3'R2 + R2 3'R1 48hr 5ml	1/30/2012	1190
F8	Total RNA R1 3'R2 + R2 3'R1 96hr 5ml	1/30/2012	1190
F9	Total RNA dH2O 96hr 5ml	1/30/2012	1190
G1	Protos Clone #14 24hr 5ml	3/12/2012	1219-1220
G2	Protos Clone #14 48hr 5ml	3/12/2012	1219-1220
G3	Protos Clone #14 96hr 5ml	3/12/2012	1219-1220
G4	Protos RNA1+2 24hr 5ml	3/12/2012	1219-1220
G5	Protos RNA1+2 48hr 5ml	3/12/2012	1219-1220
G6	Protos RNA1+2 96hr 5ml	3/12/2012	1219-1220
G7	Protos dH2O 96hr 5ml	3/12/2012	1219-1220
G8			
G9			
H1	Total RNA Clone #14 24hr 5ml	3/19/2012	1225
H2	Total RNA Clone #14 48hr 5ml	3/19/2012	1225
H3	Total RNA Clone #14 96hr 5ml	3/19/2012	1225
H4	Total RNA RNA1+2 24hr 5ml	3/19/2012	1225
H5	Total RNA RNA1+2 48hr 5ml	3/19/2012	1225
H6	Total RNA RNA1+2 96hr 5ml	3/19/2012	1225
H7	Total RNA dH2O 96hr 5ml	3/19/2012	1225
H8			
H9			
I1			
I2			
I3			
I4			
I5			
I6			
I7			
I8			
I9			



Chawin's protoplast #10: -80^{0}C Level #1 Tower #3 Box #10

	Description:	Date:	NB pg:
A1	Protos Clone #15 24hr 5ml	4/23/2012	1244-1245
A2	Protos Clone #15 48hr 5ml	4/23/2012	1244-1245
A3	Protos Clone #15 96hr 5ml	4/23/2012	1244-1245
A4	Protos Clone #16 24hr 5ml	4/23/2012	1244-1245
A5	Protos Clone #16 48hr 5ml	4/23/2012	1244-1245
A6	Protos Clone #16 96hr 5ml	4/23/2012	1244-1245
A7	Protos RNA1+2 24hr 5ml	4/23/2012	1244-1245
A8	Protos RNA1+2 48hr 5ml	4/23/2012	1244-1245
A9	Protos RNA1+2 96hr 5ml	4/23/2012	1244-1245
B1	Total RNA Clone #15 24hr 5ml	4/30/2012	1248
B2	Total RNA Clone #15 48hr 5ml	4/30/2012	1248
B3	Total RNA Clone #15 96hr 5ml	4/30/2012	1248
B4	Total RNA Clone #16 24hr 5ml	4/30/2012	1248
B5	Total RNA Clone #16 48hr 5ml	4/30/2012	1248
B6	Total RNA Clone #16 96hr 5ml	4/30/2012	1248
B7	Total RNA RNA1+2 24hr 5ml	4/30/2012	1248
B8	Total RNA RNA1+2 48hr 5ml	4/30/2012	1248
B9	Total RNA RNA1+2 96hr 5ml	4/30/2012	1248
C1	Protos dH2O 96hr 5ml	4/23/2012	1244-1245
C2	Protos Clone #17 24hr 5ml	1/14/2013	1379-1380
C3	Protos Clone #17 48hr 5ml	1/14/2013	1379-1380
C4	Protos Clone #17 96hr 5ml	1/14/2013	1379-1380
C5	Protos Clone #18 24hr 5ml	1/14/2013	1379-1380
C6	Protos Clone #18 48hr 5ml	1/14/2013	1379-1380
C7	Protos Clone #18 96hr 5ml	1/14/2013	1379-1380
C8	Protos RNA1+2 24hr 5ml	1/14/2013	1379-1380
C9	Protos RNA1+2 48hr 5ml	1/14/2013	1379-1380
D1	Total RNA dH2O 96hr 5ml	4/30/2012	1248
D2	Total RNA Clone #17 24hr 5ml	1/22/2013	1385
D3	Total RNA Clone #17 48hr 5ml	1/22/2013	1385
D4	Total RNA Clone #17 96hr 5ml	1/22/2013	1385
D5	Total RNA Clone #18 24hr 5ml	1/22/2013	1385
D6	Total RNA Clone #18 48hr 5ml	1/22/2013	1385
D7	Total RNA Clone #18 96hr 5ml	1/22/2013	1385
D8	Total RNA RNA1+2 24hr 5ml	1/22/2013	1385
D9	Total RNA RNA1+2 48hr 5ml	1/22/2013	1385
E1	Protos RNA1+2 96hr 5ml	1/14/2013	1379-1380
E2	Protos dH2O 96hr 5ml	1/14/2013	1379-1380
E3	Protos Clone #19 24hr 5ml	1/28/2013	1391-1392
E4	Protos Clone #19 48hr 5ml	1/28/2013	1391-1392
E5	Protos Clone #19 96hr 5ml	1/28/2013	1391-1392
E6	Protos Clone #20 24hr 5ml	1/28/2013	1391-1392
E7	Protos Clone #20 48hr 5ml	1/28/2013	1391-1392
E8	Protos Clone #20 96hr 5ml	1/28/2013	1391-1392
E9	Protos RNA1+2 24hr 5ml	1/28/2013	1391-1392

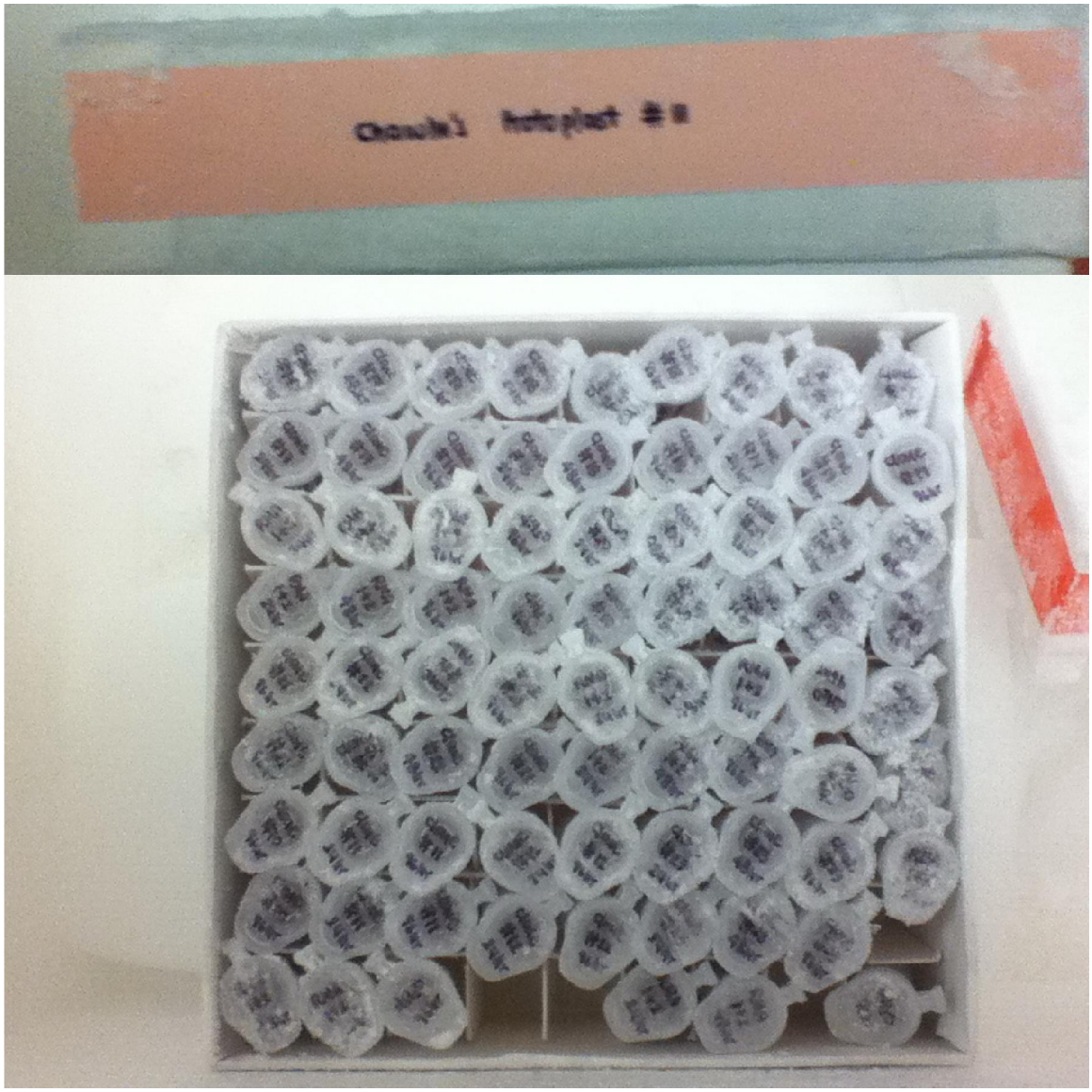
F1	Total RNA RNA1+2 96hr 5ml	1/22/2013	1385
F2	Total RNA dH2O 96hr 5ml	1/22/2013	1385
F3	Total RNA Clone #19 24hr 5ml	2/5/2013	1402
F4	Total RNA Clone #19 48hr 5ml	2/5/2013	1402
F5	Total RNA Clone #19 96hr 5ml	2/5/2013	1402
F6	Total RNA Clone #20 24hr 5ml	2/5/2013	1402
F7	Total RNA Clone #20 48hr 5ml	2/5/2013	1402
F8	Total RNA Clone #20 96hr 5ml	2/5/2013	1402
F9	Total RNA RNA1+2 24hr 5ml	2/5/2013	1402
G1	Protos RNA1+2 48hr 5ml	1/28/2013	1391-1392
G2	Protos RNA1+2 96hr 5ml	1/28/2013	1391-1392
G3	Protos dH2O 96hr 5ml	1/28/2013	1391-1392
G4	Protos Clone #21 24hr 5ml	2/4/2013	1393-1394
G5	Protos Clone #21 48hr 5ml	2/4/2013	1393-1394
G6	Protos Clone #21 96hr 5ml	2/4/2013	1393-1394
G7	Protos Clone #22 24hr 5ml	2/4/2013	1393-1394
G8	Protos Clone #22 48hr 5ml	2/4/2013	1393-1394
G9	Protos Clone #22 96hr 5ml	2/4/2013	1393-1394
H1	Total RNA RNA1+2 48hr 5ml	2/4/2013	1393-1394
H2	Total RNA RNA1+2 96hr 5ml	2/4/2013	1393-1394
H3	Total RNA dH2O 96hr 5ml	2/4/2013	1393-1394
H4	Total RNA Clone #21 24hr 5ml	2/13/2013	1404
H5	Total RNA Clone #21 48hr 5ml	2/13/2013	1404
H6	Total RNA Clone #21 96hr 5ml	2/13/2013	1404
H7	Total RNA Clone #22 24hr 5ml	2/13/2013	1404
H8	Total RNA Clone #22 48hr 5ml	2/13/2013	1404
H9	Total RNA Clone #22 96hr 5ml	2/13/2013	1404
I1	Protos RNA1+2 24hr 5ml	2/5/2013	1402
I2	Protos RNA1+2 48hr 5ml	2/5/2013	1402
I3	Protos RNA1+2 96hr 5ml	2/5/2013	1402
I4	Protos dH2O 96hr 5ml	2/5/2013	1402
I5			
I6	Total RNA RNA1+2 24hr 5ml	2/13/2013	1404
I7	Total RNA RNA1+2 48hr 5ml	2/13/2013	1404
I8	Total RNA RNA1+2 96hr 5ml	2/13/2013	1404
I9	Total RNA dH2O 96hr 5ml	2/13/2013	1404


Protoplast #10

Chawin's protoplast #11: -80^{0}C Level #1 Tower #3 Box #11

	Description:	Date:	NB pg:
A1	Protos Clone #17 24hr 5ml	3/4/2013	1423-1424
A2	Protos Clone #17 48hr 5ml	3/4/2013	1423-1424
A3	Protos Clone #17 96hr 5ml	3/4/2013	1423-1424
A4	Protos Clone #18 24hr 5ml	3/4/2013	1423-1424
A5	Protos Clone #18 48hr 5ml	3/4/2013	1423-1424
A6	Protos Clone #18 96hr 5ml	3/4/2013	1423-1424
A7	Protos Clone #19 24hr 5ml	3/4/2013	1423-1424
A8	Protos Clone #19 48hr 5ml	3/4/2013	1423-1424
A9	Protos Clone #19 96hr 5ml	3/4/2013	1423-1424
B1	Total RNA Clone #17 24hr 5ml	3/12/2013	1431
B2	Total RNA Clone #17 48hr 5ml	3/12/2013	1431
B3	Total RNA Clone #17 96hr 5ml	3/12/2013	1431
B4	Total RNA Clone #18 24hr 5ml	3/12/2013	1431
B5	Total RNA Clone #18 48hr 5ml	3/12/2013	1431
B6	Total RNA Clone #18 96hr 5ml	3/12/2013	1431
B7	Total RNA Clone #19 24hr 5ml	3/12/2013	1431
B8	Total RNA Clone #19 48hr 5ml	3/12/2013	1431
B9	Total RNA Clone #19 96hr 5ml	3/12/2013	1431
C1	Protos RNA1+2 24hr 5ml	3/4/2013	1423-1424
C2	Protos RNA1+2 48hr 5ml	3/4/2013	1423-1424
C3	Protos RNA1+2 96hr 5ml	3/4/2013	1423-1424
C4	Protos dH2O 96hr 5ml	3/4/2013	1423-1424
C5	Protos Clone #20 24hr 5ml	3/11/2013	1428-1429
C6	Protos Clone #20 48hr 5ml	3/11/2013	1428-1429
C7	Protos Clone #20 96hr 5ml	3/11/2013	1428-1429
C8	Protos Clone #21 24hr 5ml	3/11/2013	1428-1429
C9	Protos Clone #21 48hr 5ml	3/11/2013	1428-1429
D1	Total RNA RNA1+2 24hr 5ml	3/12/2013	1431
D2	Total RNA RNA1+2 48hr 5ml	3/12/2013	1431
D3	Total RNA RNA1+2 96hr 5ml	3/12/2013	1431
D4	Total RNA dH2O 96hr 5ml	3/12/2013	1431
D5	Total RNA Clone #20 24hr 5ml	3/19/2013	1437
D6	Total RNA Clone #20 48hr 5ml	3/19/2013	1437
D7	Total RNA Clone #20 96hr 5ml	3/19/2013	1437
D8	Total RNA Clone #21 24hr 5ml	3/19/2013	1437
D9	Total RNA Clone #21 48hr 5ml	3/19/2013	1437
E1	Protos Clone #21 96hr 5ml	3/11/2013	1428-1429
E2	Protos Clone #22 24hr 5ml	3/11/2013	1428-1429
E3	Protos Clone #22 48hr 5ml	3/11/2013	1428-1429
E4	Protos Clone #22 96hr 5ml	3/11/2013	1428-1429
E5	Protos RNA1+2 24hr 5ml	3/11/2013	1428-1429
E6	Protos RNA1+2 48hr 5ml	3/11/2013	1428-1429
E7	Protos RNA1+2 96hr 5ml	3/11/2013	1428-1429
E8	Protos dH2O 96hr 5ml	3/11/2013	1428-1429
E9	Protos Clone #10 24hr 5ml	3/18/2013	1435-1436

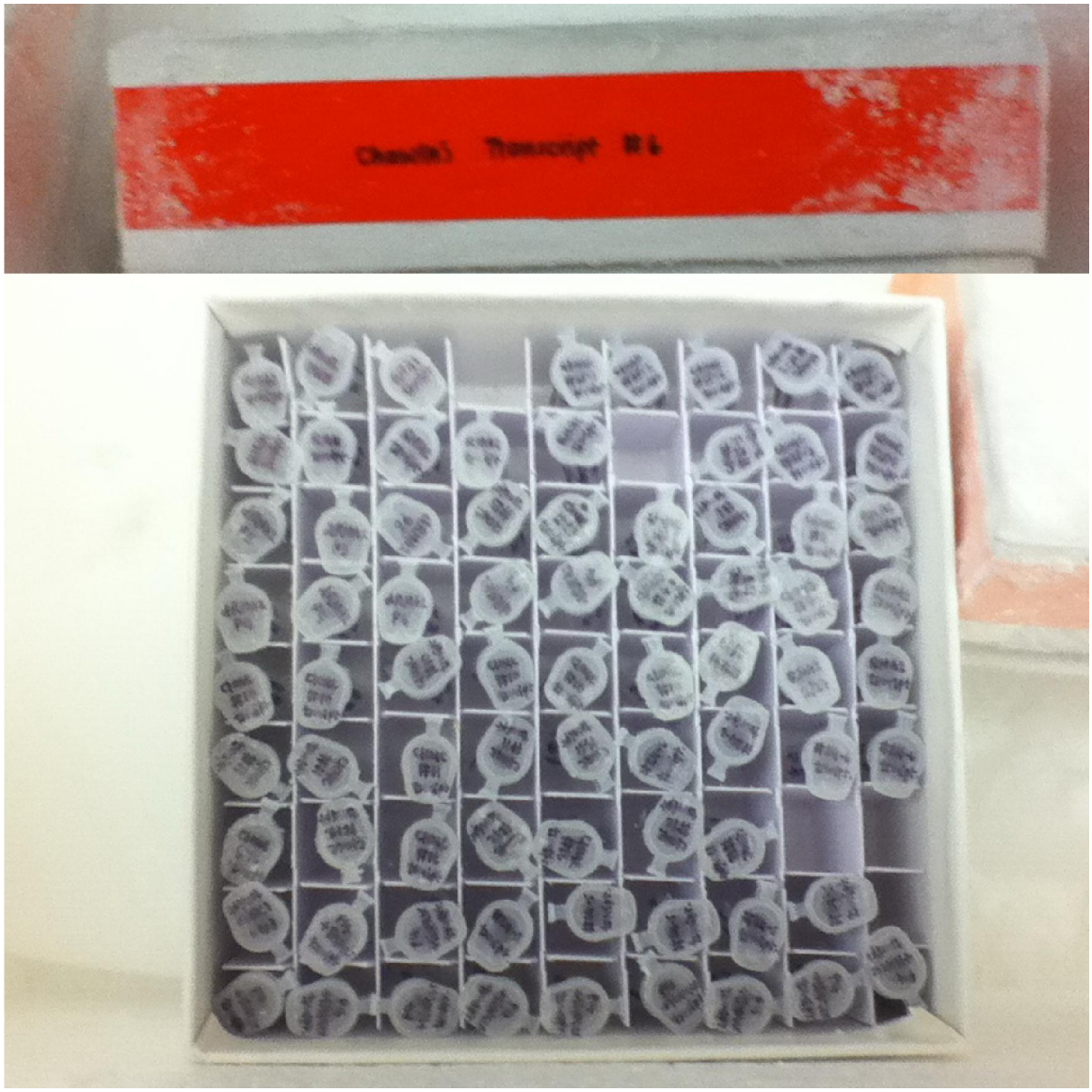
F1	Total RNA Clone #21 96hr 5ml	3/19/2013	1437
F2	Total RNA Clone #22 24hr 5ml	3/19/2013	1437
F3	Total RNA Clone #22 48hr 5ml	3/19/2013	1437
F4	Total RNA Clone #22 96hr 5ml	3/19/2013	1437
F5	Total RNA RNA1+2 24hr 5ml	3/19/2013	1437
F6	Total RNA RNA1+2 48hr 5ml	3/19/2013	1437
F7	Total RNA RNA1+2 96hr 5ml	3/19/2013	1437
F8	Total RNA dH2O 96hr 5ml	3/19/2013	1437
F9	Total RNA Clone #10 24hr 5ml	3/25/2013	1442
G1	Protos Clone #10 96hr 5ml	3/18/2013	1435-1436
G2	Protos Clone #11 24hr 5ml	3/18/2013	1435-1436
G3	Protos Clone #11 96hr 5ml	3/18/2013	1435-1436
G4	Protos Clone #12 24hr 5ml	3/18/2013	1435-1436
G5	Protos Clone #12 96hr 5ml	3/18/2013	1435-1436
G6	Protos Clone #23 24hr 5ml	3/18/2013	1435-1436
G7	Protos Clone #23 48hr 5ml	3/18/2013	1435-1436
G8	Protos Clone #23 96hr 5ml	3/18/2013	1435-1436
G9	Protos RNA1+2 24hr 5ml	3/18/2013	1435-1436
H1	Total RNA Clone #10 96hr 5ml	3/25/2013	1442
H2	Total RNA Clone #11 24hr 5ml	3/25/2013	1442
H3	Total RNA Clone #11 96hr 5ml	3/25/2013	1442
H4	Total RNA Clone #12 24hr 5ml	3/25/2013	1442
H5	Total RNA Clone #12 96hr 5ml	3/25/2013	1442
H6	Total RNA Clone #23 24hr 5ml	3/25/2013	1442
H7	Total RNA Clone #23 48hr 5ml	3/25/2013	1442
H8	Total RNA Clone #23 96hr 5ml	3/25/2013	1442
H9			
I1	Protos RNA1+2 48hr 5ml	3/18/2013	1435-1436
I2	Protos RNA1+2 96hr 5ml	3/18/2013	1435-1436
I3	Protos dH2O 96hr 5ml	3/18/2013	1435-1436
I4			
I5			
I6	Total RNA RNA1+2 24hr 5ml	3/25/2013	1442
I7	Total RNA RNA1+2 48hr 5ml	3/25/2013	1442
I8	Total RNA RNA1+2 96hr 5ml	3/25/2013	1442
I9	Total RNA dH2O 96hr 5ml	3/25/2013	1442



Chawin's transcript #6: -80^{0}C Level #1 Tower #3 Box #12

	Description:	Date:	NB pg:
A1	Transcript of LCV RNA1 (pCM1)	4/12/2013	1462-1463
A2	Transcript of LCV RNA1 (pCM1)	4/12/2013	1462-1463
A3	Transcript of LCV RNA1 (pCM1)	4/12/2013	1462-1463
A4			
A5	Transcript of RNA 2 Clone #25-2	8/7/2013	1549-1550
A6	Transcript of RNA 2 Clone #25-2	8/7/2013	1549-1550
A7	Transcript of RNA 2 Clone #25-2	8/7/2013	1549-1550
A8	Transcript of RNA 2 Clone #25-2	8/7/2013	1549-1550
A9	Transcript of RNA 2 Clone #25-2	8/7/2013	1549-1550
B1	Transcript of LCV RNA1 (pCM1)	4/12/2013	1462-1463
B2	Transcript of LCV RNA1 (pCM1)	4/12/2013	1462-1463
B3	Transcript of LCV RNA 2 (pCM2)	4/12/2013	1462-1463
B4	Transcript of LCV RNA 2 (pCM2)	4/12/2013	1462-1463
B5	Transcript of LCV RNA 2 (pCM2)	4/12/2013	1462-1463
B6			
B7	Transcript of RNA 2 Clone #25-3	8/7/2013	1549-1550
B8	Transcript of RNA 2 Clone #25-3	8/7/2013	1549-1550
B9	Transcript of RNA 2 Clone #25-3	8/7/2013	1549-1550
C1	Transcript of LCV dRNA2 P3	2/27/2013	1415-1416
C2	Transcript of LCV dRNA2 P3	2/27/2013	1415-1416
C3	Transcript of LCV dRNA2 P3	2/27/2013	1415-1416
C4	Transcript of RNA 2 Clone #23	3/15/2013	1434
C5	Transcript of RNA 2 Clone #23	3/15/2013	1434
C6	Transcript of RNA 2 Clone #6	4/12/2013	1462-1463
C7	Transcript of RNA 2 Clone #6	4/12/2013	1462-1463
C8	Transcript of RNA 2 Clone #6	4/12/2013	1462-1463
C9	Transcript of LCV RNA 2 (pCM2)	4/12/2013	1462-1463
D1	Transcript of LCV dRNA2 P4	2/27/2013	1415-1416
D2	Transcript of LCV dRNA2 P4	2/27/2013	1415-1416
D3	Transcript of LCV dRNA2 P4	2/27/2013	1415-1416
D4	Transcript of LCV dRNA2 P4	2/27/2013	1415-1416
D5	Transcript of LCV dRNA2 P4	2/27/2013	1415-1416
D6	Transcript of RNA 2 Clone #7	4/12/2013	1462-1463
D7	Transcript of RNA 2 Clone #7	4/12/2013	1462-1463
D8	Transcript of RNA 2 Clone #7	4/12/2013	1462-1463
D9	Transcript of LCV RNA 2 (pCM2)	4/12/2013	1462-1463
E1	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E2	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E3	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E4	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E5	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E6	Transcript of RNA 2 Clone #10	4/4/2013	1452-1453
E7	Transcript of LCV RNA1 (pCM1)	2/27/2013	1415-1416
E8	Transcript of LCV RNA 2 (pCM2)	2/27/2013	1415-1416
E9	Transcript of LCV RNA 2 (pCM2)	4/12/2011	

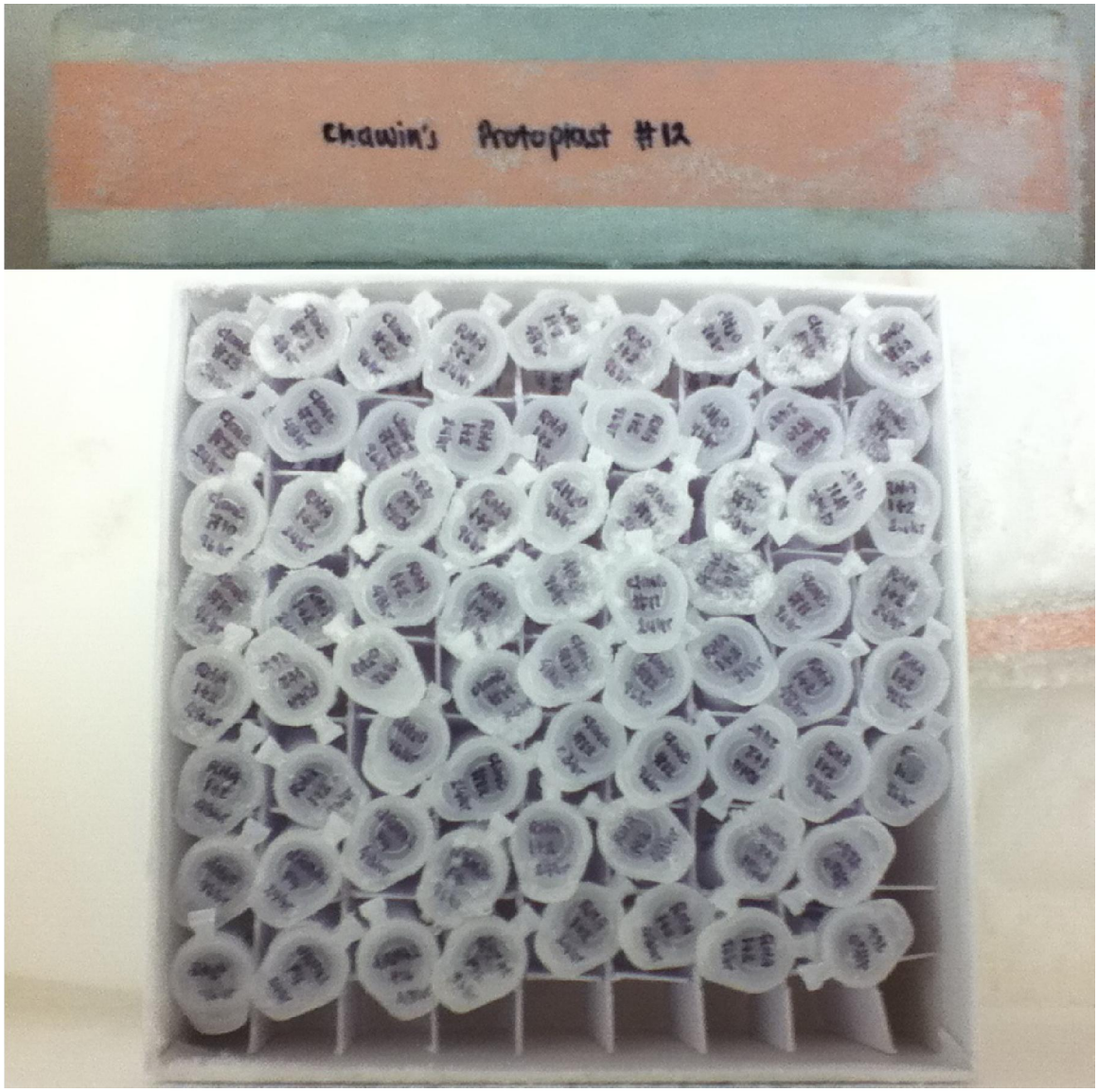
F1	Transcript of RNA 2 Clone #11	4/4/2013	1452-1453
F2	Transcript of RNA 2 Clone #11	4/4/2013	1452-1453
F3	Transcript of RNA 2 Clone #11	4/4/2013	1452-1453
F4	Transcript of RNA 2 Clone #11	4/4/2013	1452-1453
F5	Transcript of RNA 2 Clone #11	4/4/2013	1452-1453
F6	Transcript of RNA 2 Clone #24-6	6/30/2013	1518-1519
F7	Transcript of RNA 2 Clone #24-6	6/30/2013	1518-1519
F8	Transcript of RNA 2 Clone #24-6	6/30/2013	1518-1519
F9	Transcript of RNA 2 Clone #24-6	6/30/2013	1518-1519
G1	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G2	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G3	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G4	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G5	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G6	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G7	Transcript of RNA 2 Clone #12	4/4/2013	1452-1453
G8			
G9			
H1	Transcript of dRNA2 P4 + linker	5/29/2013	1496-1497
H2	Transcript of dRNA2 P4 + linker	5/29/2013	1496-1497
H3	Transcript of dRNA2 P4 + linker	5/29/2013	1518-1519
H4	Transcript of RNA 2 Clone #24-5	6/30/2013	1518-1519
H5	Transcript of RNA 2 Clone #24-5	6/30/2013	1518-1519
H6	Transcript of RNA 2 Clone #24-5	6/30/2013	1518-1519
H7	Transcript of RNA 2 Clone #24-5	6/30/2013	1518-1519
H8	Transcript of RNA 2 Clone #24-5	6/30/2013	1518-1519
H9			
I1	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I2	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I3	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I4	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I5	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I6	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I7	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I8	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519
I9	Transcript of dRNA2 P4 + linker	6/30/2013	1518-1519



Chawin's protoplast #12: -80^{0}C Level #2 Tower #3 Box #9

	Description:	Date:	NB pg:
A1	Protos Clone #23 24hr 5ml	4/1/2013	1449-1450
A2	Protos Clone #23 48hr 5ml	4/1/2013	1449-1450
A3	Protos Clone #23 96hr 5ml	4/1/2013	1449-1450
A4	Protos RNA1+2 24hr 5ml	4/1/2013	1449-1450
A5	Protos RNA1+2 48hr 5ml	4/1/2013	1449-1450
A6	Protos RNA1+2 96hr 5ml	4/1/2013	1449-1450
A7	Protos dH2O 96hr 5ml	4/1/2013	1449-1450
A8	Protos Clone #10 24hr 5ml	4/8/2013	1459-1460
A9	Protos Clone #10 48hr 5ml	4/8/2013	1459-1460
B1	Total RNA Clone #23 24hr 5ml	4/16/2013	1466
B2	Total RNA Clone #23 48hr 5ml	4/16/2013	1466
B3	Total RNA Clone #23 96hr 5ml	4/16/2013	1466
B4	Total RNA RNA1+2 24hr 5ml	4/16/2013	1466
B5	Total RNA RNA1+2 48hr 5ml	4/16/2013	1466
B6	Total RNA RNA1+2 96hr 5ml	4/16/2013	1466
B7	Total RNA dH2O 96hr 5ml	4/16/2013	1466
B8	Total RNA Clone #10 24hr 5ml	4/16/2013	1466
B9	Total RNA Clone #10 48hr 5ml	4/16/2013	1466
C1	Protos Clone #10 96hr 5ml	4/8/2013	1459-1460
C2	Protos RNA1+2 24hr 5ml	4/8/2013	1459-1460
C3	Protos RNA1+2 48hr 5ml	4/8/2013	1459-1460
C4	Protos RNA1+2 96hr 5ml	4/8/2013	1459-1460
C5	Protos dH2O 96hr 5ml	4/8/2013	1459-1460
C6	Protos Clone #11 24hr 5ml	4/11/2013	1461
C7	Protos Clone #11 48hr 5ml	4/11/2013	1461
C8	Protos Clone #11 96hr 5ml	4/11/2013	1461
C9	Protos RNA1+2 24hr 5ml	4/11/2013	1461
D1	Total RNA Clone #10 96hr 5ml	4/16/2013	1466
D2	Total RNA RNA1+2 24hr 5ml	4/16/2013	1466
D3	Total RNA RNA1+2 48hr 5ml	4/16/2013	1466
D4	Total RNA RNA1+2 96hr 5ml	4/16/2013	1466
D5	Total RNA dH2O 96hr 5ml	4/16/2013	1466
D6	Total RNA Clone #11 24hr 5ml	4/16/2013	1466
D7	Total RNA Clone #11 48hr 5ml	4/16/2013	1466
D8	Total RNA Clone #11 96hr 5ml	4/16/2013	1466
D9	Total RNA RNA1+2 24hr 5ml	4/16/2013	1466
E1	Protos RNA1+2 48hr 5ml	4/11/2013	1461
E2	Protos RNA1+2 96hr 5ml	4/11/2013	1461
E3	Protos dH2O 96hr 5ml	4/11/2013	1461
E4	Protos Clone #12 24hr 5ml	4/15/2013	1465
E5	Protos Clone #12 48hr 5ml	4/15/2013	1465
E6	Protos Clone #12 96hr 5ml	4/15/2013	1465
E7	Protos RNA1+2 24hr 5ml	4/15/2013	1465
E8	Protos RNA1+2 48hr 5ml	4/15/2013	1465
E9	Protos RNA1+2 96hr 5ml	4/15/2013	1465

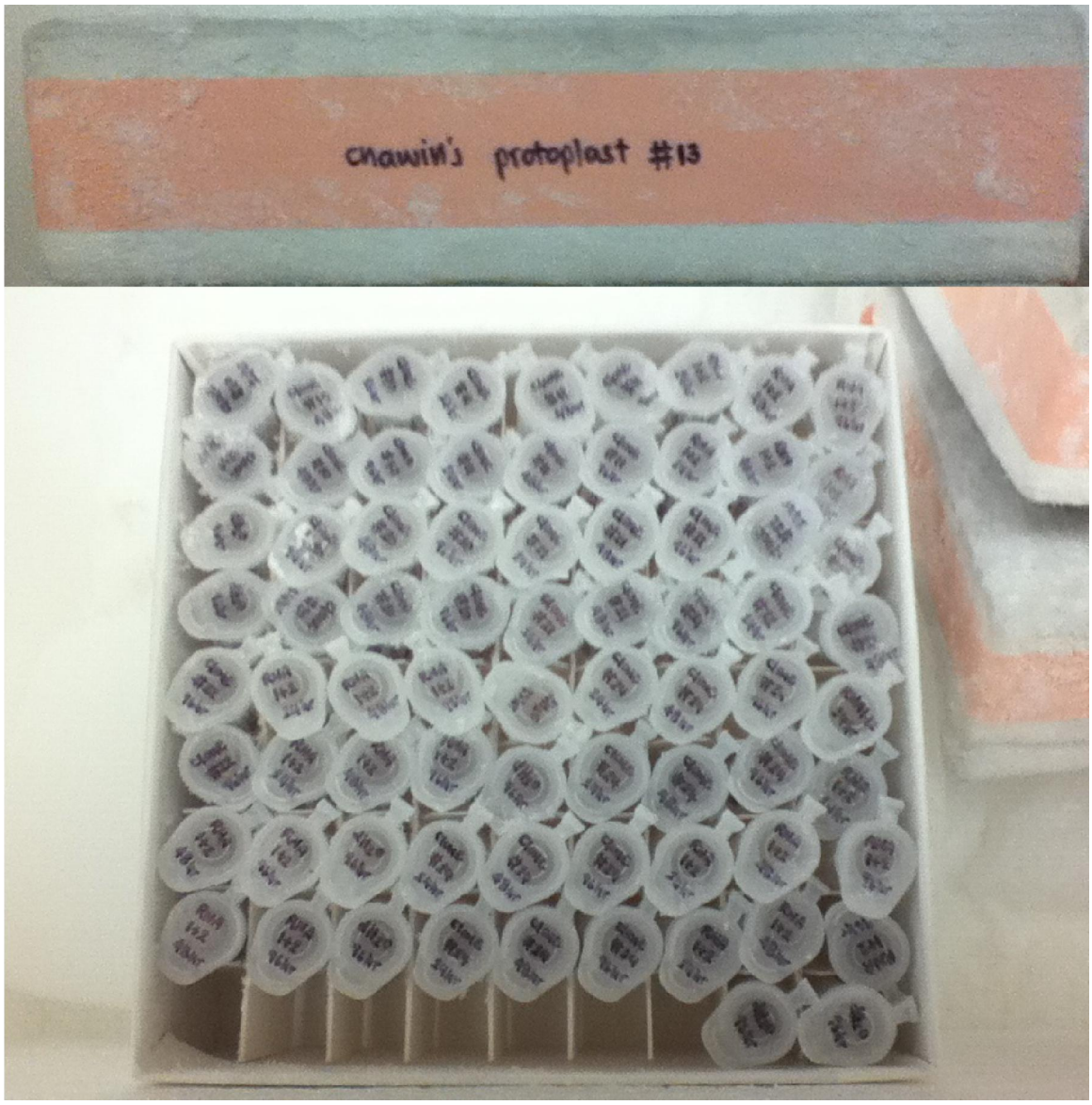
F1	Total RNA RNA1+2 48hr 5ml	4/16/2013	1466
F2	Total RNA RNA1+2 96hr 5ml	4/16/2013	1466
F3	Total RNA dH2O 96hr 5ml	4/16/2013	1466
F4	Total RNA Clone #12 24hr 5ml	5/29/2013	1500
F5	Total RNA Clone #12 48hr 5ml	5/29/2013	1500
F6	Total RNA Clone #12 96hr 5ml	5/29/2013	1500
F7	Total RNA RNA1+2 24hr 5ml	5/29/2013	1500
F8	Total RNA RNA1+2 48hr 5ml	5/29/2013	1500
F9	Total RNA RNA1+2 96hr 5ml	5/29/2013	1500
G1	Protos dH2O 96hr 5ml	4/15/2013	1465
G2	Protos dRNA2 P4 24hr 5ml	4/18/2013	1468
G3	Protos dRNA2 P4 48hr 5ml	4/18/2013	1468
G4	Protos dRNA2 P4 96hr 5ml	4/18/2013	1468
G5	Protos RNA1+2 24hr 5ml	4/18/2013	1468
G6	Protos RNA1+2 48hr 5ml	4/18/2013	1468
G7	Protos RNA1+2 96hr 5ml	4/18/2013	1468
G8	Protos dH2O 96hr 5ml	4/18/2013	1468
G9			
H1	Total RNA dH2O 96hr 5ml	5/29/2013	1500
H2	Total RNA dRNA2 P4 24hr 5ml	5/29/2013	1500
H3	Total RNA dRNA2 P4 48hr 5ml	5/29/2013	1500
H4	Total RNA dRNA2 P4 96hr 5ml	5/29/2013	1500
H5	Total RNA RNA1+2 24hr 5ml	5/29/2013	1500
H6	Total RNA RNA1+2 48hr 5ml	5/29/2013	1500
H7	Total RNA RNA1+2 96hr 5ml	5/29/2013	1500
H8	Total RNA dH2O 96hr 5ml	5/29/2013	1500
H9			
I1	Total RNA Clone #25 24hr 5ml	8/13/2013	1552
I2	Total RNA Clone #25 48hr 5ml	8/13/2013	1552
I3	Total RNA Clone #25 96hr 5ml	8/13/2013	1552
I4	Total RNA RNA1+2 24hr 5ml	8/13/2013	1552
I5	Total RNA RNA1+2 48hr 5ml	8/13/2013	1552
I6	Total RNA RNA1+2 96hr 5ml	8/13/2013	1552
I7	Total RNA dH2O 96hr 5ml	8/13/2013	1552
I8			
I9			


Chawin's Protoplast #12

Chawin's protoplast #13: -80^{0}C Level #2 Tower #3 Box #10

	Description:	Date:	NB pg:
A1	Protos Clone #10 24hr 5ml	4/29/2013	1476
A2	Protos Clone #10 48hr 5ml	4/29/2013	1476
A3	Protos Clone #10 96hr 5ml	4/29/2013	1476
A4	Protos Clone #11 24hr 5ml	4/29/2013	1476
A5	Protos Clone #11 48hr 5ml	4/29/2013	1476
A6	Protos Clone #11 96hr 5ml	4/29/2013	1476
A7	Protos RNA1+2 24hr 5ml	4/29/2013	1476
A8	Protos RNA1+2 48hr 5ml	4/29/2013	1476
A9	Protos RNA1+2 96hr 5ml	4/29/2013	1476
B1	Total RNA Clone #10 24hr 5ml	5/7/2013	1481
B2	Total RNA Clone #10 48hr 5ml	5/7/2013	1481
B3	Total RNA Clone #10 96hr 5ml	5/7/2013	1481
B4	Total RNA Clone #11 24hr 5ml	5/7/2013	1481
B5	Total RNA Clone #11 48hr 5ml	5/7/2013	1481
B6	Total RNA Clone #11 96hr 5ml	5/7/2013	1481
B7	Total RNA RNA1+2 24hr 5ml	5/7/2013	1481
B8	Total RNA RNA1+2 48hr 5ml	5/7/2013	1481
B9	Total RNA RNA1+2 96hr 5ml	5/7/2013	1481
C1	Protos dH2O 96hr 5ml	4/29/2013	1476
C2	Protos Clone #20 24hr 5ml	5/2/2013	1480
C3	Protos Clone #20 48hr 5ml	5/2/2013	1480
C4	Protos Clone #20 96hr 5ml	5/2/2013	1480
C5	Protos Clone #21 24hr 5ml	5/2/2013	1480
C6	Protos Clone #21 48hr 5ml	5/2/2013	1480
C7	Protos Clone #21 96hr 5ml	5/2/2013	1480
C8	Protos Clone #22 24hr 5ml	5/2/2013	1480
C9	Protos Clone #22 48hr 5ml	5/2/2013	1480
D1	Total RNA dH2O 96hr 5ml	5/7/2013	1481
D2	Total RNA Clone #20 24hr 5ml	5/7/2013	1481
D3	Total RNA Clone #20 48hr 5ml	5/7/2013	1481
D4	Total RNA Clone #20 96hr 5ml	5/7/2013	1481
D5	Total RNA Clone #21 24hr 5ml	5/7/2013	1481
D6	Total RNA Clone #21 48hr 5ml	5/7/2013	1481
D7	Total RNA Clone #21 96hr 5ml	5/7/2013	1481
D8	Total RNA Clone #22 24hr 5ml	5/7/2013	1481
D9	Total RNA Clone #22 48hr 5ml	5/7/2013	1481
E1	Protos Clone #22 96hr 5ml	5/2/2013	1480
E2	Protos RNA1+2 24hr 5ml	5/2/2013	1480
E3	Protos RNA1+2 48hr 5ml	5/2/2013	1480
E4	Protos RNA1+2 96hr 5ml	5/2/2013	1480
E5	Protos dH2O 96hr 5ml	5/2/2013	1480
E6	Protos Clone #24 24hr 5ml	7/1/2013	1526
E7	Protos Clone #24 48hr 5ml	7/1/2013	1526
E8	Protos Clone #24 96hr 5ml	7/1/2013	1526
E9	Protos RNA1+2 24hr 5ml	7/1/2013	1526

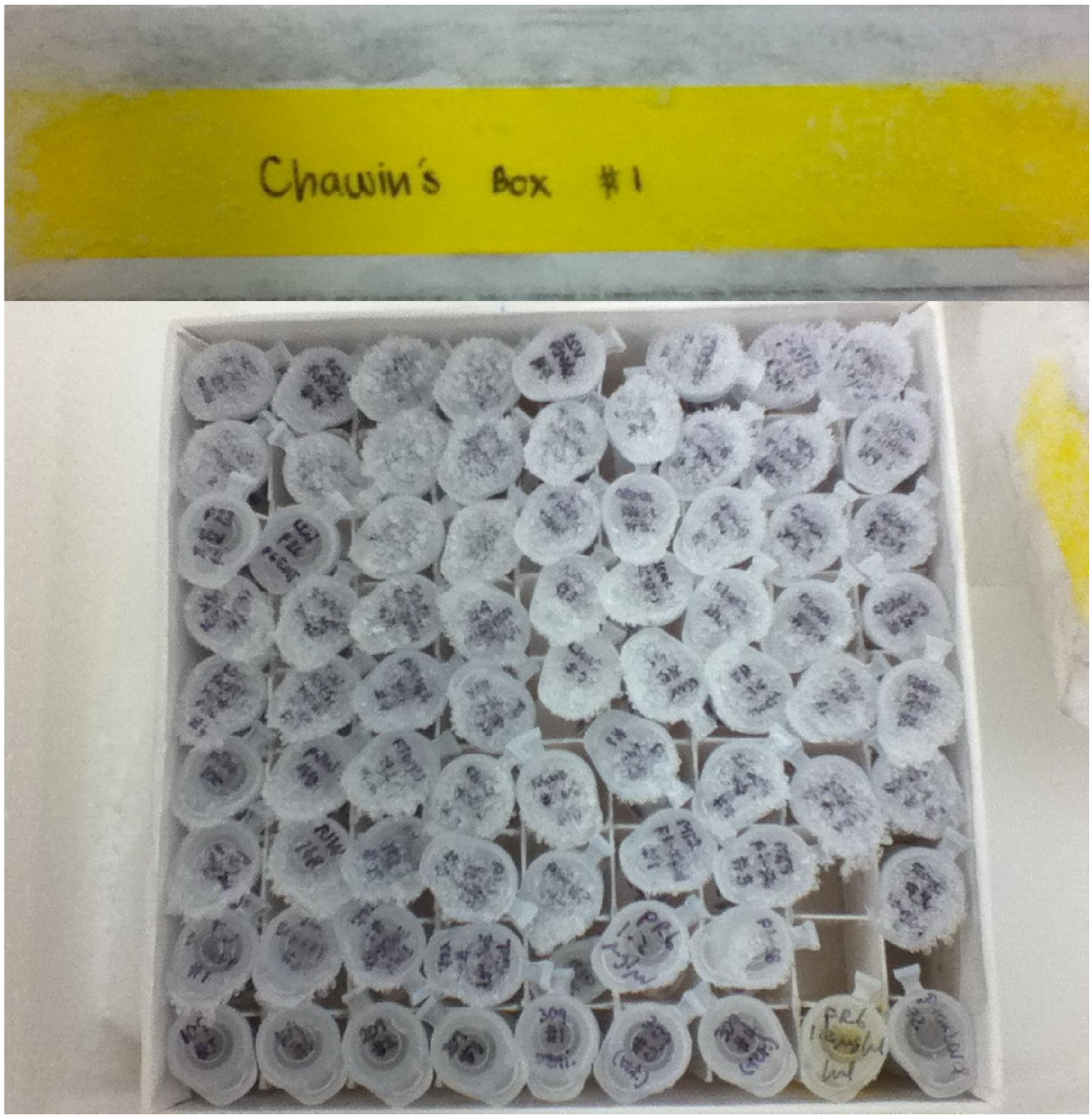
F1	Total RNA Clone #22 96hr 5ml	5/7/2013	1481
F2	Total RNA RNA1+2 24hr 5ml	5/7/2013	1481
F3	Total RNA RNA1+2 48hr 5ml	5/7/2013	1481
F4	Total RNA RNA1+2 96hr 5ml	5/7/2013	1481
F5	Total RNA dH2O 96hr 5ml	5/7/2013	1481
F6	Total RNA Clone #24 24hr 5ml	7/8/2013	1528
F7	Total RNA Clone #24 48hr 5ml	7/8/2013	1528
F8	Total RNA Clone #24 96hr 5ml	7/8/2013	1528
F9	Total RNA RNA1+2 24hr 5ml	7/8/2013	1528
	Desciption:	Date:	NB pg:
G1	Protos RNA1+2 48hr 5ml	7/1/2013	1526
G2	Protos RNA1+2 96hr 5ml	7/1/2013	1526
G3	Protos dH2O 96hr 5ml	7/1/2013	1526
G4	Protos Clone #24 24hr 5ml	7/15/2013	1530
G5	Protos Clone #24 48hr 5ml	7/15/2013	1530
G6	Protos Clone #24 96hr 5ml	7/15/2013	1530
G7	Protos RNA1+2 24hr 5ml	7/15/2013	1530
G8	Protos RNA1+2 48hr 5ml	7/15/2013	1530
G9	Protos RNA1+2 96hr 5ml	7/15/2013	1530
H1	Total RNA RNA1+2 48hr 5ml	7/8/2013	1528
H2	Total RNA RNA1+2 96hr 5ml	7/8/2013	1528
H3	Total RNA dH2O 96hr 5ml	7/8/2013	1528
H4	Total RNA Clone #24 24hr 5ml	7/24/2013	1538
H5	Total RNA Clone #24 48hr 5ml	7/24/2013	1538
H6	Total RNA Clone #24 96hr 5ml	7/24/2013	1538
H7	Total RNA RNA1+2 24hr 5ml	7/24/2013	1538
H8	Total RNA RNA1+2 48hr 5ml	7/24/2013	1538
H9	Total RNA RNA1+2 96hr 5ml	7/24/2013	1538
I1	Protos Clone #25 24hr 5ml	8/8/2013	1551
I2	Protos Clone #25 48hr 5ml	8/8/2013	1551
I3	Protos Clone #25 96hr 5ml	8/8/2013	1551
I4	Protos RNA1+2 24hr 5ml	8/8/2013	1551
I5	Protos RNA1+2 48hr 5ml	8/8/2013	1551
I6	Protos RNA1+2 96hr 5ml	8/8/2013	1551
I7	Protos dH2O 96hr 5ml	8/8/2013	1551
I8	Total RNA dH2O 96hr 5ml	7/24/2013	1538
I9	Protos dH2O 96hr 5ml	7/15/2013	1530


chawin's protoplast #13

Chawin's Box #1: -20^{0}C

	Description:	Date:	NB pg:
A1	LCV RNA1 #1 (pCM1) midiprep final	4/30/2009	430
A2	LCV RNA1 #7 (pCM1) midiprep final	8/20/2009	593
A3	LCV RNA2 #7-1 (pCM2) midiprep	8/12/2009	585
A4	LCV RNA2 #7-2 (pCM2) midiprep	8/12/2009	585
A5	LCV RNA2 #7 (pCM2) midiprep	8/20/2009	593
A6			
A7	pM5GFP BFNB2-9/16/02-1 (0.5ug/ul)		
A8	pT7/T3-GFP		
A9	pM5GFP from JN (1.4ug/ul)		
B1	3'NCR RNA2 Clone #1-1	11/17/2009	675
B2	3'NCR RNA2 Clone #2-1	11/17/2009	675
B3	3'NCR RNA2 Clone #3-1	11/17/2009	675
B4	3'NCR RNA2 Clone #4-1	11/17/2009	675
B5	3'NCR RNA2 Clone #5-1	11/17/2009	675
B6	3'NCR RNA2 Clone #6-1	2/10/2010	728
B7	3'NCR RNA2 Clone #7-5	2/10/2010	728
B8	3'NCR RNA2 Clone #8-2	2/10/2010	728
B9	3'NCR RNA2 Clone #9-1	2/10/2010	728
C1	LCV p23 (A23 from AC) for probe	6/5/2009	469
C2	LCV p26 pGEMT for probe	4/8/2010	769
C3	BYV p21 (from AC) for probe	8/24/2010	850
C4	LIYV p26 #1 for probe	1/6/2011	916
C5	LIYV p26 #2 for probe	11/8/2010	887
C6	3'NCR RNA2 Clone #6-1-2	5/27/2010	800
C7	3'NCR RNA2 Clone #7-5-2	5/27/2010	800
C8	3'NCR RNA2 Clone #8-2	2/22/2010	739
C9	3'NCR RNA2 Clone #9-1	2/22/2010	739
D1	3'UTR LCV RNA1 #1 for probe	12/11/2010	912
D2	3'UTR LCV RNA1 #2 for probe	12/11/2010	912
D3	3'UTR LCV RNA1 #3 for probe	12/11/2010	912
D4	3'UTR LCV RNA1 #4 for probe	12/11/2010	912
D5	3'NCR RNA2 Clone #5	8/23/2011	1090
D6	3'NCR RNA2 Clone #10-1	3/11/2011	974
D7	3'NCR RNA2 Clone #10-2	3/11/2011	974
D8	3'NCR RNA2 Clone #11-1	3/11/2011	974
D9	3'NCR RNA2 Clone #11-3	3/11/2011	974
E1	LCV RNA1 mid probe #3	1/20/2011	923
E2	LCV RNA1 mid probe #4	1/20/2011	923
E3	pJW168 TC1 #7	5/17/2011	1023
E4	pJW168 TC1 #12	5/17/2011	1023
E5	3'NCR RNA2 Clone #7	8/25/2011	1090
E6	3'NCR RNA2 Clone #12-1	5/17/2011	1021
E7	3'NCR RNA2 Clone #12-2	5/17/2011	1021
E8	3'NCR RNA2 Clone #13-1	5/17/2011	1021
E9	3'NCR RNA2 Clone #13-2	5/17/2011	1021

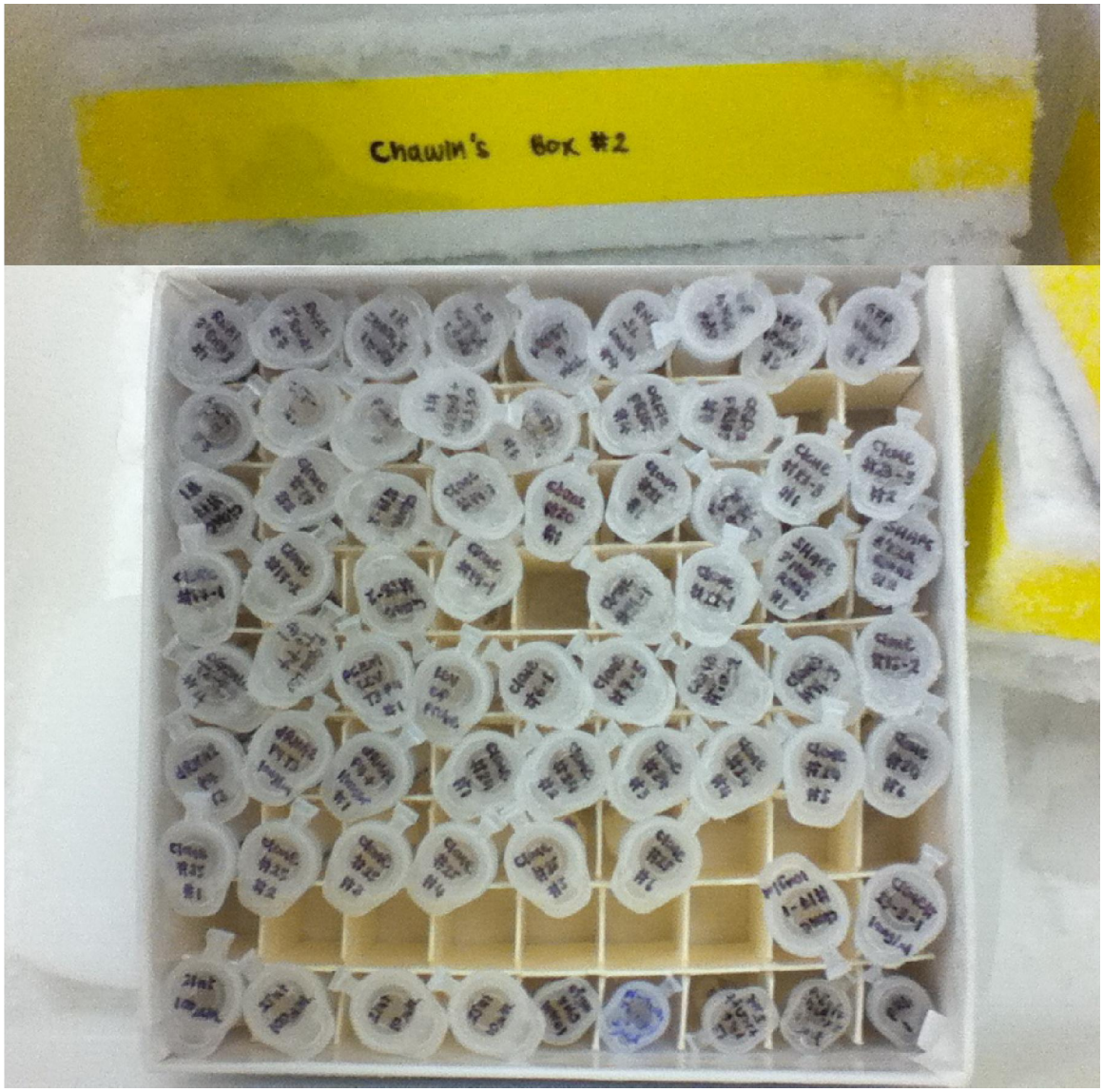
F1	pJW100	10/19/2010	871
F2	pJW168	10/19/2010	871
F3	pJW168 TC1 maxiprep	5/24/2011	1030
F4	3'NCR RNA2 Clone #12-2	7/8/2011	1064
F5	3'NCR RNA2 Clone #13-2	7/8/2011	1064
F6	3'NCR RNA2 Clone #14-1	3/2/2012	1208
F7	3'NCR RNA2 Clone #14-7	3/2/2012	1208
F8	3'NCR RNA2 Clone #15-1	4/16/2012	1238
F9	3'NCR RNA2 Clone #16-1	4/16/2012	1238
G1	pJW100 from Carlo (68ng/ul)	1/22/2009	
G2	pJW168 from Carlo (94ng/ul)	1/22/2009	
G3	pJW168 TC2 #19	4/9/2009	411
G4	pJW168 TC3 #4	5/15/2009	451
G5	pJW168 TC5	7/17/2009	496
G6	MT5#3 p1-5b1 (agro plasmid)	1/6/2010	700
G7	MT2#1 p1-5bm1 (agro plasmid)	1/6/2010	700
G8			
G9			
H1	TMV30BGFP #1	11/26/2008	288
H2	TMV30BGFP #2	11/26/2008	288
H3	TMV30BGFP #1 retransform	1/9/2009	299
H4	TMV30BGFP #2 retransform	1/9/2009	299
H5	TMV30BGFP C3	10/10/2003	
H6	pR6 from JN (1.3ug/ul)		
H7	p1-5b	11/28/2007	38
H8			
H9			
I1	CMV p109 #5	11/26/2008	289
I2	CMV p109 #6	11/26/2008	289
I3	CMV p209 #2	11/26/2008	289
I4	CMV p209 #3	11/26/2008	289
I5	CMV p309 #1	11/21/2008	281
I6	CMV p309 #3	1/13/2009	303
I7	CMV p309 #4	1/13/2009	303
I8	pR6 diluted (5ng/u)		
I9	pGEM LCV CP#2 with stop codon from AC	9/2/2008	


Chawin's Box #1

Chawin's Box #2: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	RNA1 3' RNA2 #1	9/29/2011	1116
A2	RNA2 3' RNA1 #7	9/29/2011	1116
A3	RNA1 3' RNA2 #1	11/19/2011	1145
A4	RNA2 3' RNA1 #7	11/19/2011	1145
A5	pRSET A	12/23/2011	1163
A6	R2SL 10mM #4	5/23/2012	1258
A7	GFP 10uM #4	5/23/2012	1258
A8	GFP 10uM #5	5/23/2012	1258
A9	GFP 10uM #6	5/23/2012	1258
B1	ORF1a/1b + pRSET #7	1/8/2012	1169
B2	GFP-F M5GFP probe #1	1/8/2012	1169
B3	p26-F M5GFP probe #3	1/8/2012	1169
B4	ORF1b + pRSET #1	3/15/2012	1222
B5	ORF1b + pRSET #6	3/15/2012	1222
B6	ORF1a + pRSET #4	1/30/2013	1389
B7	ORF1a + pRSET #8	1/30/2013	1389
B8			
B9			
C1	3'NCR RNA2 Clone #17-1	1/10/2013	1374
C2	3'NCR RNA2 Clone #17-2	1/10/2013	1374
C3	3'NCR RNA2 Clone #18-2	10/7/2012	1335
C4	3'NCR RNA2 Clone #19-1	10/7/2012	1335
C5	3'NCR RNA2 Clone #20-1	9/28/2012	1329
C6	3'NCR RNA2 Clone #21-1	10/22/2012	1343
C7	3'NCR RNA2 Clone #22-1	10/22/2012	1343
C8	3'NCR RNA2 Clone #23-3-1	3/12/2013	1426
C9	3'NCR RNA2 Clone #23-3-2	3/12/2013	1426
D1	3'NCR RNA2 Clone #17-1	1/17/2013	1383
D2	3'NCR RNA2 Clone #17-2	1/17/2013	1383
D3	3'NCR RNA2 Clone #18-2	1/17/2013	1383
D4	3'NCR RNA2 Clone #19-1	1/17/2013	1383
D5			
D6	3'NCR RNA2 Clone #21-1	1/17/2013	1383
D7	3'NCR RNA2 Clone #22-1	1/17/2013	1383
D8	SHAPE cassette + 3' NCR RNA2 #1	1/16/2013	1382
D9	SHAPE cassette + 3' NCR RNA2 #2	1/16/2013	1382
E1	pGEM T3 dRNA2 P3 #4 from AC	12/7/2009	
E2	pGEM T3 dRNA2 P4 #1 from AC	12/7/2009	
E3	pGEM T3 dRNA2 P3 #4	12/7/2009	
E4	3'NCR RNA2 Clone #6-1	4/2/2013	1451
E5	3'NCR RNA2 Clone #7-5	4/2/2013	1451
E6	3'NCR RNA2 Clone #10-2	4/2/2013	1451
E7	3'NCR RNA2 Clone #11-3	4/2/2013	1451
E8	3'NCR RNA2 Clone #12-2	4/2/2013	1451
E9			

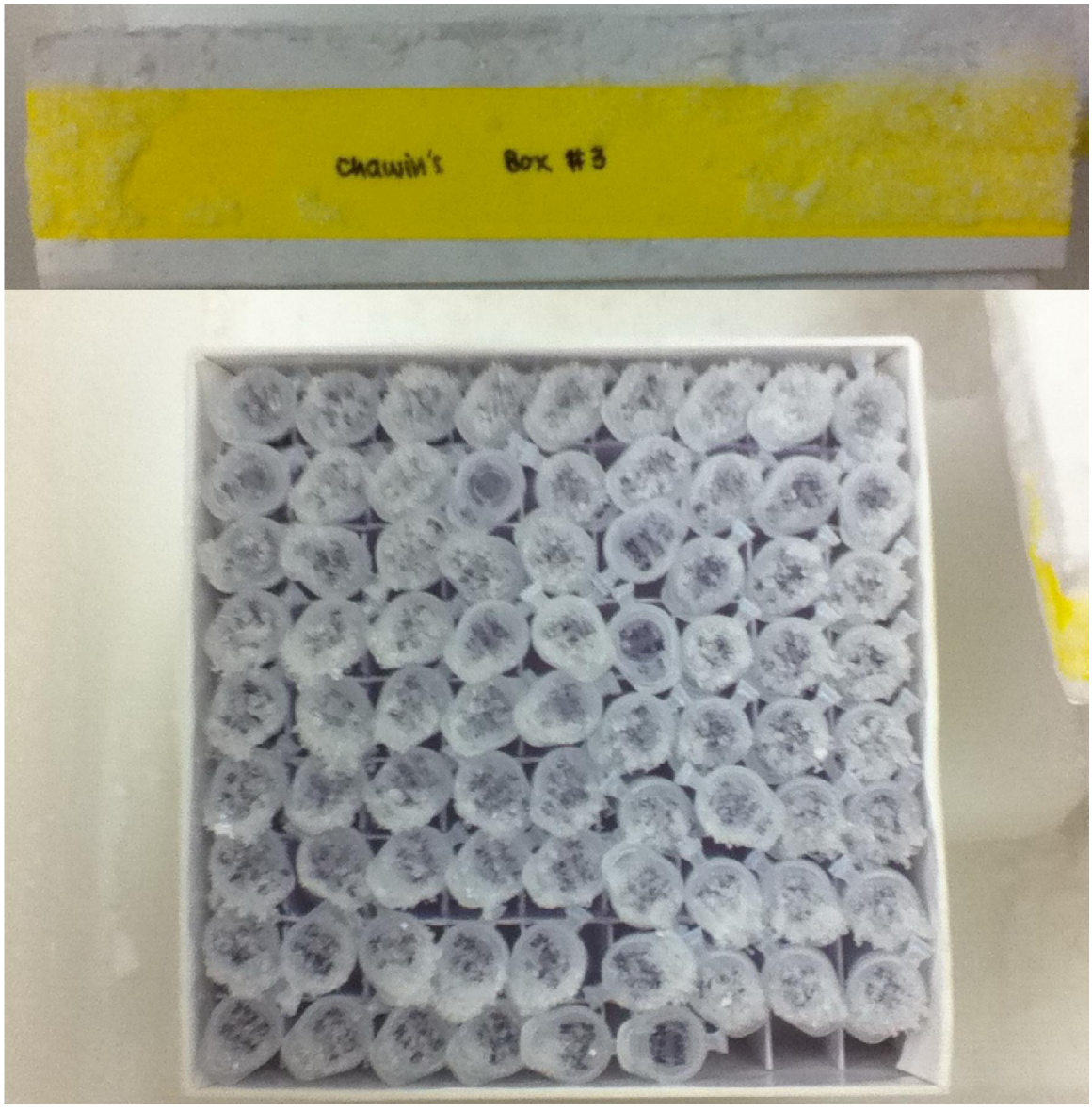
F1	dRNA2 P4 T3 #1	5/8/2013	1483
F2	dRNA2 P4 T3 #1 10ng/ul	5/8/2013	1483
F3	dRNA2 P4 T3 + linker #1	5/20/2013	1492
F4	3'NCR RNA2 Clone #24-1	6/27/2013	1516
F5	3'NCR RNA2 Clone #24-2	6/27/2013	1516
F6	3'NCR RNA2 Clone #24-3	6/27/2013	1516
F7	3'NCR RNA2 Clone #24-4	6/27/2013	1516
F8	3'NCR RNA2 Clone #24-5	6/27/2013	1516
F9	3'NCR RNA2 Clone #24-6	6/27/2013	1516
G1	3'NCR RNA2 Clone #25-1	8/5/2013	1547
G2	3'NCR RNA2 Clone #25-2	8/5/2013	1547
G3	3'NCR RNA2 Clone #25-3	8/5/2013	1547
G4	3'NCR RNA2 Clone #25-4	8/5/2013	1547
G5	3'NCR RNA2 Clone #25-5	8/5/2013	1547
G6	3'NCR RNA2 Clone #25-6	8/5/2013	1547
G7			
G8			
G9			
H1			
H2			
H3			
H4			
H5			
H6			
H7			
H8	3'NCR RNA2 Clone #17-1 10ng/ul	1/10/2013	
H9	3'NCR RNA2 Clone #23-3-1 10ng/ul	3/12/2013	
I1	21nt RNA oligo 100uM from Kenji		
I2	25nt RNA oligo 100uM from Kenji		
I3	21nt RNA oligo 10uM from Kenji		
I4	25nt RNA oligo 10uM from Kenji		
I5	60nt DNA oligo 10uM R2SL+		
I6	p1-5bm1 transcript NB2-11/8/04-14		
I7	LS pR6 transcript NB7-7/24/08-1		
I8	M5GFP transcript BFNB3-7/10/03-6		
I9	pR6 transcript NB2-4/16/02-1		


Chawin's Box #2

Chawin's Box #3: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	DRNA1 PCR + pGEMT 3C protos #1	3/9/2011	971
A2	DRNA1 PCR + pGEMT 3C protos #2	3/9/2011	971
A3	DRNA1 PCR + pGEMT 3C protos #3	3/9/2011	971
A4	DRNA1 PCR + pGEMT 3C protos #4	3/9/2011	971
A5	DRNA1 PCR + pGEMT 3C protos #5	3/9/2011	971
A6	DRNA1 PCR + pGEMT 3C protos #6	3/9/2011	971
A7	DRNA1 PCR + pGEMT 1C protos #1	3/9/2011	971
A8	DRNA1 PCR + pGEMT 1C protos #2	3/9/2011	971
A9	DRNA1 PCR + pGEMT 1C protos #3	3/9/2011	971
B1	DRNA1 PCR + pGEMT 1C protos #4	3/9/2011	971
B2	DRNA1 PCR + pGEMT 1C protos #5	3/9/2011	971
B3	DRNA1 PCR + pGEMT 1C protos #6	3/9/2011	971
B4	DRNA1 PCR + pGEMT RNA1 only #1	6/15/2011	1044
B5	DRNA1 PCR + pGEMT RNA1 only #2	6/15/2011	1044
B6	DRNA1 PCR + pGEMT RNA1 only #3	6/15/2011	1044
B7	DRNA1 PCR + pGEMT RNA1 only #4	6/15/2011	1044
B8	DRNA1 PCR + pGEMT RNA1 only #5	6/15/2011	1044
B9	PCR #2-1for GFP probe	5/23/2011	1027
C1	DRNA1 + GFP #1	5/23/2011	1027
C2	DRNA1 + GFP #6	5/23/2011	1027
C3	PCR #2-1 for GFP probe	5/23/2011	1027
C4	PCR #2-3 for GFP probe	5/23/2011	1027
C5	5'RACE #6-1	10/11/2011	1124
C6	5'RACE #6-2	10/11/2011	1124
C7	5'RACE #6-3	10/11/2011	1124
C8	5'RACE #6-6	10/11/2011	1124
C9	5'RACE #7-1	10/11/2011	1124
D1	5'RACE #7-2	10/11/2011	1124
D2	5'RACE #7-3	10/11/2011	1124
D3	5'RACE #7-4	10/11/2011	1124
D4	5'RACE #7-5	10/11/2011	1124
D5	5'RACE #13-1	10/11/2011	1124
D6	5'RACE #13-2	10/11/2011	1124
D7	5'RACE #13-3	10/11/2011	1124
D8	5'RACE #13-4	10/11/2011	1124
D9	5'RACE #13-5	10/11/2011	1124
E1	5'RACE vRNA #1-1	10/26/2011	1136
E2	5'RACE vRNA #1-2	10/26/2011	1136
E3	5'RACE vRNA #1-3	10/26/2011	1136
E4	5'RACE vRNA #1-4	10/26/2011	1136
E5	5'RACE vRNA #1-5	10/26/2011	1136
E6	5'RACE pCM1+2 #2-1	10/26/2011	1136
E7	5'RACE pCM1+2 #2-2	10/26/2011	1136
E8	5'RACE pCM1+2 #2-3	10/26/2011	1136
E9	5'RACE pCM1+2 #2-4	10/26/2011	1136

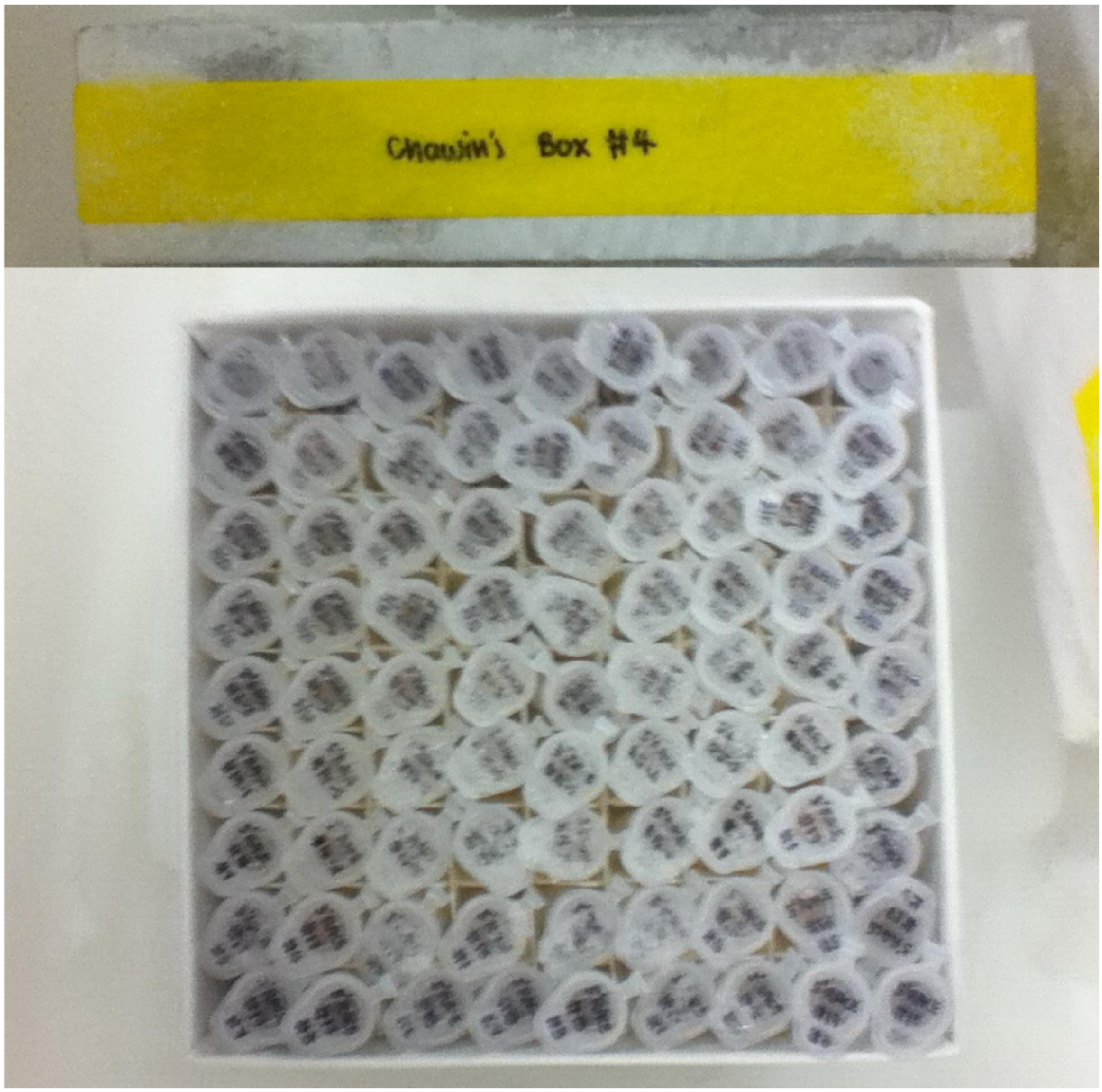
F1	5'RACE pCM1+2 #2-5	10/26/2011	1136
F2	5'RACE RNA2 of R1-2 + R2-1 #3-1	10/26/2011	1136
F3	5'RACE RNA2 of R1-2 + R2-1 #3-2	10/26/2011	1136
F4	5'RACE RNA2 of R1-2 + R2-1 #3-3	10/26/2011	1136
F5	5'RACE RNA2 of R1-2 + R2-1 #3-4	10/26/2011	1136
F6	5'RACE RNA2 of R1-2 + R2-1 #3-5	10/26/2011	1136
F7	5'RACE RNA1 of R1-2 + R2-1 #4-1	10/26/2011	1136
F8	5'RACE RNA1 of R1-2 + R2-1 #4-2	10/26/2011	1136
F9	5'RACE RNA1 of R1-2 + R2-1 #4-3	10/26/2011	1136
G1	5'RACE RNA1 of R1-2 + R2-1 #4-4	10/26/2011	1136
G2	5'RACE RNA1 of R1-2 + R2-1 #4-5	10/26/2011	1136
G3	3'RACE #1-1	1/17/2012	1176
G4	3'RACE #1-2	1/17/2012	1176
G5	3'RACE #1-5	1/17/2012	1176
G6	3'RACE #2-1	1/17/2012	1176
G7	3'RACE #2-2	1/17/2012	1176
G8	3'RACE #2-4	1/17/2012	1176
G9	3'RACE #3-1	1/17/2012	1176
H1	3'RACE #3-2	1/17/2012	1176
H2	3'RACE #3-3	1/17/2012	1176
H3	3'RACE #4-1	1/17/2012	1176
H4	3'RACE #4-2	1/17/2012	1176
H5	3'RACE #4-3	1/17/2012	1176
H6	3'RACE #2-5	2/6/2012	1193
H7	3'RACE #2-6	2/6/2012	1193
H8	3'RACE #2-7	2/6/2012	1193
H9	3'RACE #2-8	2/6/2012	1193
I1	3'RACE #2-9	2/6/2012	1193
I2	3'RACE #3-4	2/6/2012	1193
I3	3'RACE #3-5	2/6/2012	1193
I4	3'RACE #3-6	2/6/2012	1193
I5	3'RACE #3-7	2/6/2012	1193
I6	3'RACE #3-8	2/6/2012	1193
I7			
I8			
I9			


Chawin's Box #3

Chawin's Box #4: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	3'RACE #5-1	2/22/2012	1200
A2	3'RACE #5-2	2/22/2012	1200
A3	3'RACE #5-3	2/22/2012	1200
A4	3'RACE #5-4	2/22/2012	1200
A5	3'RACE #13-1	2/22/2012	1200
A6	3'RACE #13-2	2/22/2012	1200
A7	3'RACE #13-3	2/22/2012	1200
A8	3'RACE #13-4	2/22/2012	1200
A9	3'RACE #13-5	2/22/2012	1200
B1	3'RACE #13-7	2/22/2012	1200
B2	3'RACE R2-1 #3	2/22/2012	1200
B3	3'RACE R2-1 #4	2/22/2012	1200
B4	3'RACE R2-1 #6	2/22/2012	1200
B5	3'RACE R2-1 #7	2/22/2012	1200
B6	3'RACE R2-1 #8	2/22/2012	1200
B7	3'RACE Clone #5 #1-1	3/13/2012	1216
B8	3'RACE Clone #5 #1-2	3/13/2012	1216
B9	3'RACE Clone #5 #1-3	3/13/2012	1216
C1	3'RACE Clone #5 #1-4	3/13/2012	1216
C2	3'RACE Clone #5 #1-5	3/13/2012	1216
C3	3'RACE Clone #13 #2-1	3/13/2012	1216
C4	3'RACE Clone #13 #2-2	3/13/2012	1216
C5	3'RACE Clone #13 #2-3	3/13/2012	1216
C6	3'RACE Clone #13 #2-4	3/13/2012	1216
C7	3'RACE Clone #13 #2-5	3/13/2012	1216
C8	3'RACE R1 + R2-1 #3-1	3/13/2012	1216
C9	3'RACE R1 + R2-1 #3-2	3/13/2012	1216
D1	3'RACE R1 + R2-1 #3-3	3/13/2012	1216
D2	3'RACE R1 + R2-1 #3-4	3/13/2012	1216
D3	3'RACE R1 + R2-1 #3-5	3/13/2012	1216
D4	3'RACE R1-2 + R2 #4-1	3/13/2012	1216
D5	3'RACE R1-2 + R2 #4-2	3/13/2012	1216
D6	3'RACE R1-2 + R2 #4-3	3/13/2012	1216
D7	3'RACE R1-2 + R2 #4-4	3/13/2012	1216
D8	3'RACE R1-2 + R2 #4-6	3/13/2012	1216
D9	3'RACE R2 Agro #6-1	3/13/2012	1216
E1	3'RACE R2 Agro #6-2	3/13/2012	1216
E2	3'RACE R2 Agro #6-3	3/13/2012	1216
E3	3'RACE R2 Agro #6-5	3/13/2012	1216
E4	3'RACE R2 Agro #6-6	3/13/2012	1216
E5	5'RACE #14-1	4/16/2012	1238
E6	5'RACE #14-2	4/16/2012	1238
E7	5'RACE #14-3	4/16/2012	1238
E8	5'RACE #14-4	4/16/2012	1238
E9	5'RACE #14-5	4/16/2012	1238

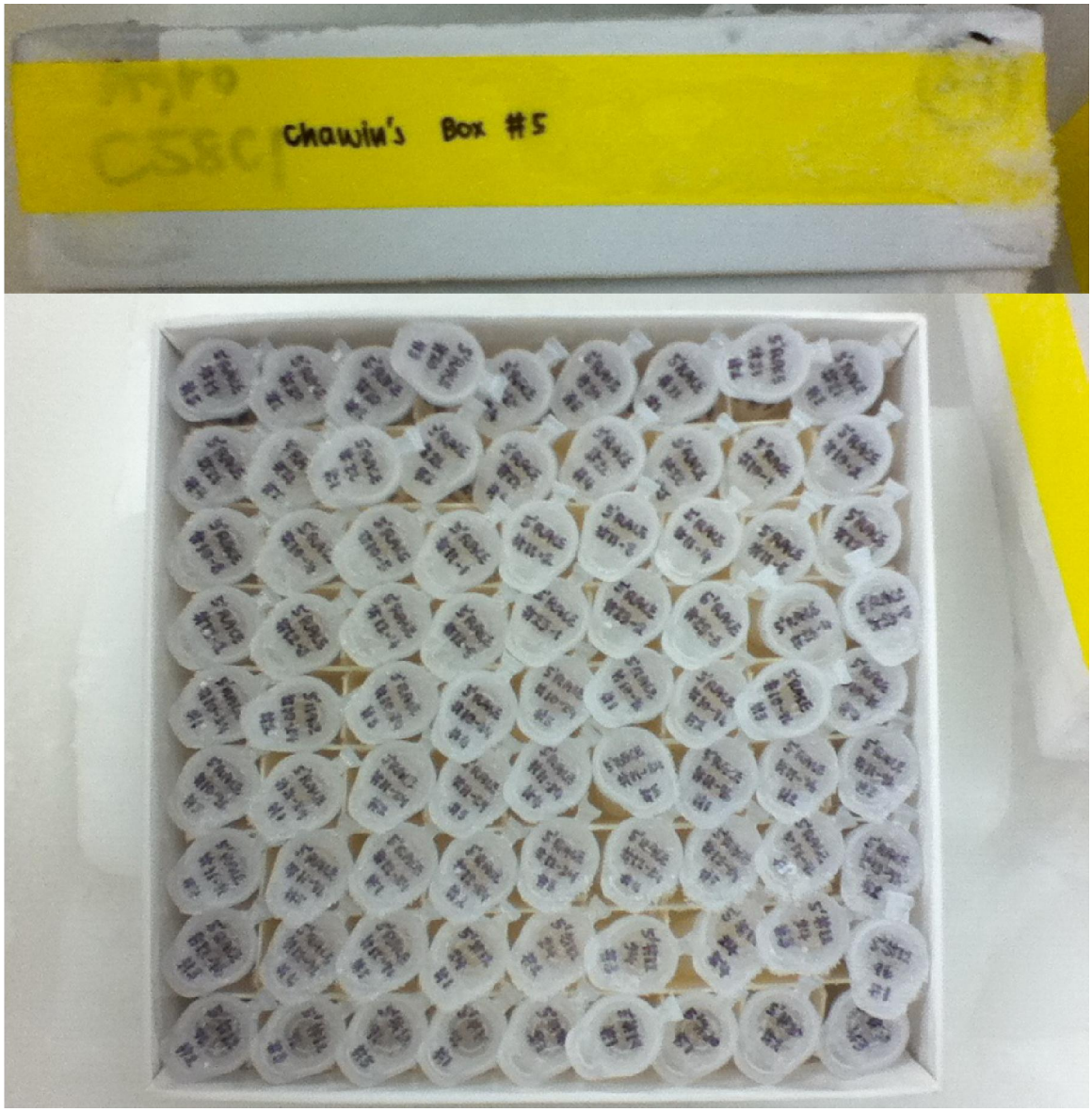
F1	5'RACE #14-1	5/16/2012	1254
F2	5'RACE #14-2	5/16/2012	1254
F3	5'RACE #14-3	5/16/2012	1254
F4	5'RACE #14-4	5/16/2012	1254
F5	5'RACE #15-1	5/16/2012	1254
F6	5'RACE #15-2	5/16/2012	1254
F7	5'RACE #15-3	5/16/2012	1254
F8	5'RACE #15-4	5/16/2012	1254
F9	5'RACE #15-5	5/16/2012	1254
G1	5'RACE #16-7	5/21/2012	1258
G2	5'RACE #16-2	5/16/2012	1254
G3	5'RACE #16-3	5/16/2012	1254
G4	5'RACE #16-4	5/16/2012	1254
G5	5'RACE #16-5	5/16/2012	1254
G6	5'RACE #16-6	5/16/2012	1254
G7	5'RACE #15-7	5/21/2012	1258
G8	5'RACE #17-1	3/4/2013	1421
G9	5'RACE #17-2	3/4/2013	1421
H1	5'RACE #17-3	3/4/2013	1421
H2	5'RACE #17-4	3/4/2013	1421
H3	5'RACE #17-5	3/4/2013	1421
H4	5'RACE #18-1	3/4/2013	1421
H5	5'RACE #18-2	3/4/2013	1421
H6	5'RACE #18-3	3/4/2013	1421
H7	5'RACE #18-4	3/4/2013	1421
H8	5'RACE #18-5	3/4/2013	1421
H9	5'RACE #17-6	3/21/2013	1440
I1	5'RACE #17-7	3/21/2013	1440
I2	5'RACE #17-8	3/21/2013	1440
I3	5'RACE #18-6	3/21/2013	1440
I4	5'RACE #18-7	3/21/2013	1440
I5	5'RACE #18-8	3/21/2013	1440
I6	5'RACE #19-1	3/21/2013	1440
I7	5'RACE #19-2	3/21/2013	1440
I8	5'RACE #19-3	3/21/2013	1440
I9	5'RACE #19-4	3/21/2013	1440


Chaussin's Box #4

Chawin's Box #5: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	5'RACE #19-5	3/21/2013	1440
A2	5'RACE #20-1	3/21/2013	1440
A3	5'RACE #20-2	3/21/2013	1440
A4	5'RACE #20-3	3/21/2013	1440
A5	5'RACE #20-4	3/21/2013	1440
A6	5'RACE #20-5	3/21/2013	1440
A7	5'RACE #21-1	3/21/2013	1440
A8	5'RACE #21-2	3/21/2013	1440
A9	5'RACE #21-3	3/21/2013	1440
B1	5'RACE #21-4	3/21/2013	1440
B2	5'RACE #21-5	3/21/2013	1440
B3	5'RACE #22-1	3/21/2013	1440
B4	5'RACE #22-2	3/21/2013	1440
B5	5'RACE #22-3	3/21/2013	1440
B6	5'RACE #22-4	3/21/2013	1440
B7	5'RACE #22-5	3/21/2013	1440
B8	5'RACE #10-1	4/9/2013	1457
B9	5'RACE #10-2	4/9/2013	1457
C1	5'RACE #10-3	4/9/2013	1457
C2	5'RACE #10-4	4/9/2013	1457
C3	5'RACE #10-5	4/9/2013	1457
C4	5'RACE #11-1	4/9/2013	1457
C5	5'RACE #11-2	4/9/2013	1457
C6	5'RACE #11-3	4/9/2013	1457
C7	5'RACE #11-4	4/9/2013	1457
C8	5'RACE #11-5	4/9/2013	1457
C9	5'RACE #12-1	4/9/2013	1457
D1	5'RACE #12-2	4/9/2013	1457
D2	5'RACE #12-3	4/9/2013	1457
D3	5'RACE #12-4	4/9/2013	1457
D4	5'RACE #12-5	4/9/2013	1457
D5	5'RACE #23-1	4/9/2013	1457
D6	5'RACE #23-2	4/9/2013	1457
D7	5'RACE #23-3	4/9/2013	1457
D8	5'RACE #23-4	4/9/2013	1457
D9	5'RACE #23-5	4/9/2013	1457
E1	5'RACE #10-24 #1	5/6/2013	1484
E2	5'RACE #10-24 #2	5/6/2013	1484
E3	5'RACE #10-24 #3	5/6/2013	1484
E4	5'RACE #10-24 #4	5/6/2013	1484
E5	5'RACE #10-24 #5	5/6/2013	1484
E6	5'RACE #10-96 #1	5/6/2013	1484
E7	5'RACE #10-96 #2	5/6/2013	1484
E8	5'RACE #10-96 #3	5/6/2013	1484
E9	5'RACE #10-96 #4	5/6/2013	1484

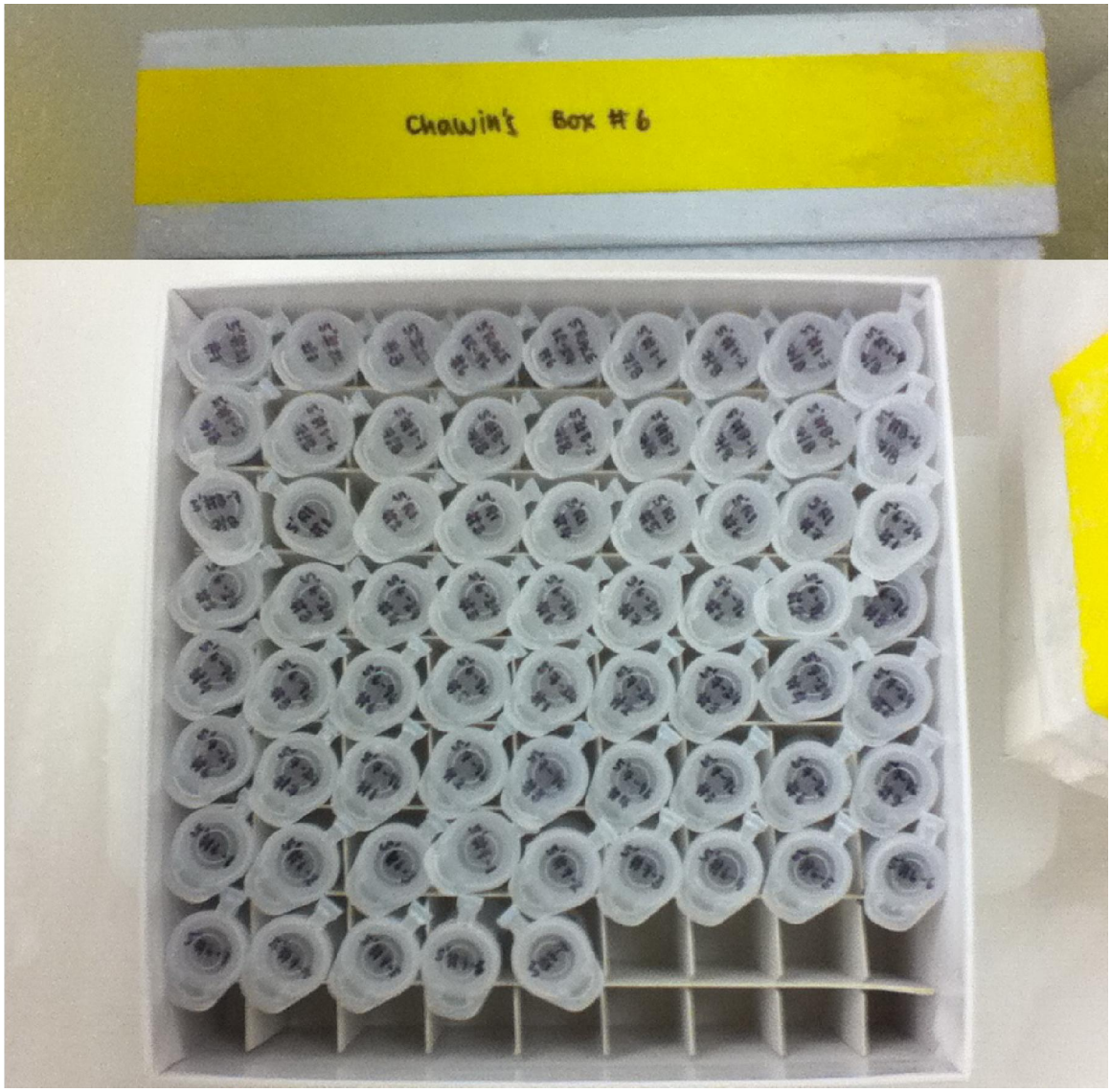
F1	5'RACE #10-96 #5	5/6/2013	1484
F2	5'RACE #11-24 #1	5/6/2013	1484
F3	5'RACE #11-24 #2	5/6/2013	1484
F4	5'RACE #11-24 #3	5/6/2013	1484
F5	5'RACE #11-24 #4	5/6/2013	1484
F6	5'RACE #11-24 #5	5/6/2013	1484
F7	5'RACE #11-96 #1	5/6/2013	1484
F8	5'RACE #11-96 #2	5/6/2013	1484
F9	5'RACE #11-96 #3	5/6/2013	1484
G1	5'RACE #11-96 #4	5/6/2013	1484
G2	5'RACE #11-96 #5	5/6/2013	1484
G3	5'RACE #12-24 #1	5/8/2013	1484
G4	5'RACE #12-24 #2	5/8/2013	1484
G5	5'RACE #12-24 #3	5/8/2013	1484
G6	5'RACE #12-24 #4	5/8/2013	1484
G7	5'RACE #12-24 #5	5/8/2013	1484
G8	5'RACE #12-96 #1	5/8/2013	1484
G9	5'RACE #12-96 #2	5/8/2013	1484
H1	5'RACE #12-96 #3	5/8/2013	1484
H2	5'RACE #12-96 #4	5/8/2013	1484
H3	5'RACE #12-96 #5	5/8/2013	1484
H4	5'RACE #12-24 #1	5/28/2013	1498
H5	5'RACE #12-24 #2	5/28/2013	1498
H6	5'RACE #12-24 #3	5/28/2013	1498
H7	5'RACE #12-24 #4	5/28/2013	1498
H8	5'RACE #12-24 #5	5/28/2013	1498
H9	5'RACE #12-96 #1	5/28/2013	1498
I1	5'RACE #12-96 #2	5/28/2013	1498
I2	5'RACE #12-96 #3	5/28/2013	1498
I3	5'RACE #12-96 #5	5/28/2013	1498
I4	5'RACE #10-96 #1	5/28/2013	1498
I5	5'RACE #18-1	5/28/2013	1498
I6	5'RACE #19-1	5/28/2013	1498
I7	5'RACE #20-1	5/28/2013	1498
I8	5'RACE #20-2	5/28/2013	1498
I9	5'RACE #20-3	5/28/2013	1498


Chawin's Box #5

Chawin's Box #6: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	5'RACE #21-1	5/28/2013	1498
A2	5'RACE #22-1	5/28/2013	1498
A3	5'RACE #22-3	5/28/2013	1498
A4	5'RACE #12-96 #6	5/28/2013	1498
A5	5'RACE #12-24 #6	5/28/2013	1498
A6	5'RACE #1-1	7/8/2013	1524
A7	5'RACE #1-2	7/8/2013	1524
A8	5'RACE #1-3	7/8/2013	1524
A9	5'RACE #1-4	7/8/2013	1524
B1	5'RACE #1-5	7/8/2013	1524
B2	5'RACE #1-6	7/8/2013	1524
B3	5'RACE #1-7	7/8/2013	1524
B4	5'RACE #8-1	7/8/2013	1524
B5	5'RACE #8-2	7/8/2013	1524
B6	5'RACE #8-3	7/8/2013	1524
B7	5'RACE #8-4	7/8/2013	1524
B8	5'RACE #8-5	7/8/2013	1524
B9	5'RACE #8-6	7/8/2013	1524
C1	5'RACE #8-7	7/8/2013	1524
C2	5'RACE R1 #1	7/10/2013	1522
C3	5'RACE R1 #2	7/10/2013	1522
C4	5'RACE R1 #3	7/10/2013	1522
C5	5'RACE R1 #4	7/10/2013	1522
C6	5'RACE R1 #5	7/10/2013	1522
C7	5'RACE R1 #6	7/10/2013	1522
C8	5'RACE R1 #7	7/10/2013	1522
C9	5'RACE #6-24 #1	7/10/2013	1522
D1	5'RACE #6-24 #2	7/10/2013	1522
D2	5'RACE #6-24 #3	7/10/2013	1522
D3	5'RACE #6-24 #4	7/10/2013	1522
D4	5'RACE #6-24 #5	7/10/2013	1522
D5	5'RACE #6-24 #6	7/10/2013	1522
D6	5'RACE #6-24 #7	7/10/2013	1522
D7	5'RACE #6-96 #1	7/10/2013	1522
D8	5'RACE #6-96 #2	7/10/2013	1522
D9	5'RACE #6-96 #3	7/10/2013	1522
E1	5'RACE #6-96 #4	7/10/2013	1522
E2	5'RACE #6-96 #5	7/10/2013	1522
E3	5'RACE #6-96 #6	7/10/2013	1522
E4	5'RACE #6-96 #7	7/10/2013	1522
E5	5'RACE #7-24 #1	7/10/2013	1522
E6	5'RACE #7-24 #2	7/10/2013	1522
E7	5'RACE #7-24 #3	7/10/2013	1522
E8	5'RACE #7-24 #4	7/10/2013	1522
E9	5'RACE #7-24 #5	7/10/2013	1522

F1	5'RACE #7-24 #6	7/10/2013	1522
F2	5'RACE #7-24 #7	7/10/2013	1522
F3	5'RACE #7-96 #1	7/10/2013	1522
F4	5'RACE #7-96 #2	7/10/2013	1522
F5	5'RACE #7-96 #3	7/10/2013	1522
F6	5'RACE #7-96 #4	7/10/2013	1522
F7	5'RACE #7-96 #5	7/10/2013	1522
F8	5'RACE #7-96 #6	7/10/2013	1522
F9	5'RACE #7-96 #7	7/10/2013	1522
G1	5'RACE #6-1	7/29/2013	1541
G2	5'RACE #6-2	7/29/2013	1541
G3	5'RACE #6-3	7/29/2013	1541
G4	5'RACE #7-1	7/29/2013	1541
G5	5'RACE #7-2	7/29/2013	1541
G6	5'RACE #7-3	7/29/2013	1541
G7	5'RACE #6-4	8/5/2013	
G8	5'RACE #6-5	8/5/2013	
G9	5'RACE #6-6	8/5/2013	
H1	5'RACE #6-7	8/5/2013	
H2	5'RACE #7-4	8/5/2013	
H3	5'RACE #7-5	8/5/2013	
H4	5'RACE #7-6	8/5/2013	
H5	5'RACE #7-7	8/5/2013	
H6			
H7			
H8			
H9			
I1			
I2			
I3			
I4			
I5			
I6			
I7			
I8			
I9			


Chawin's Box #6

Chawin's Primer stock #1: -20^{0}C

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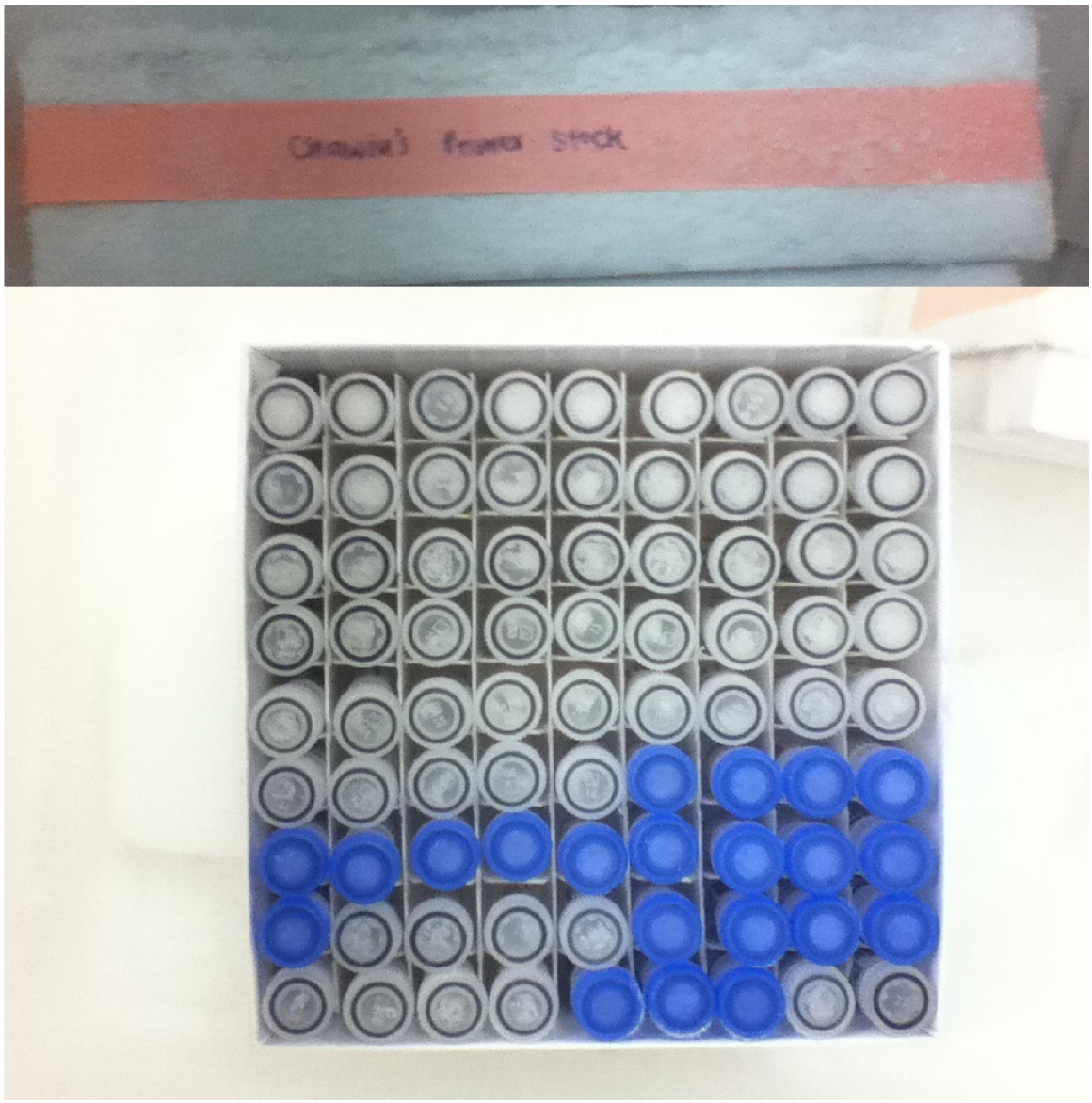
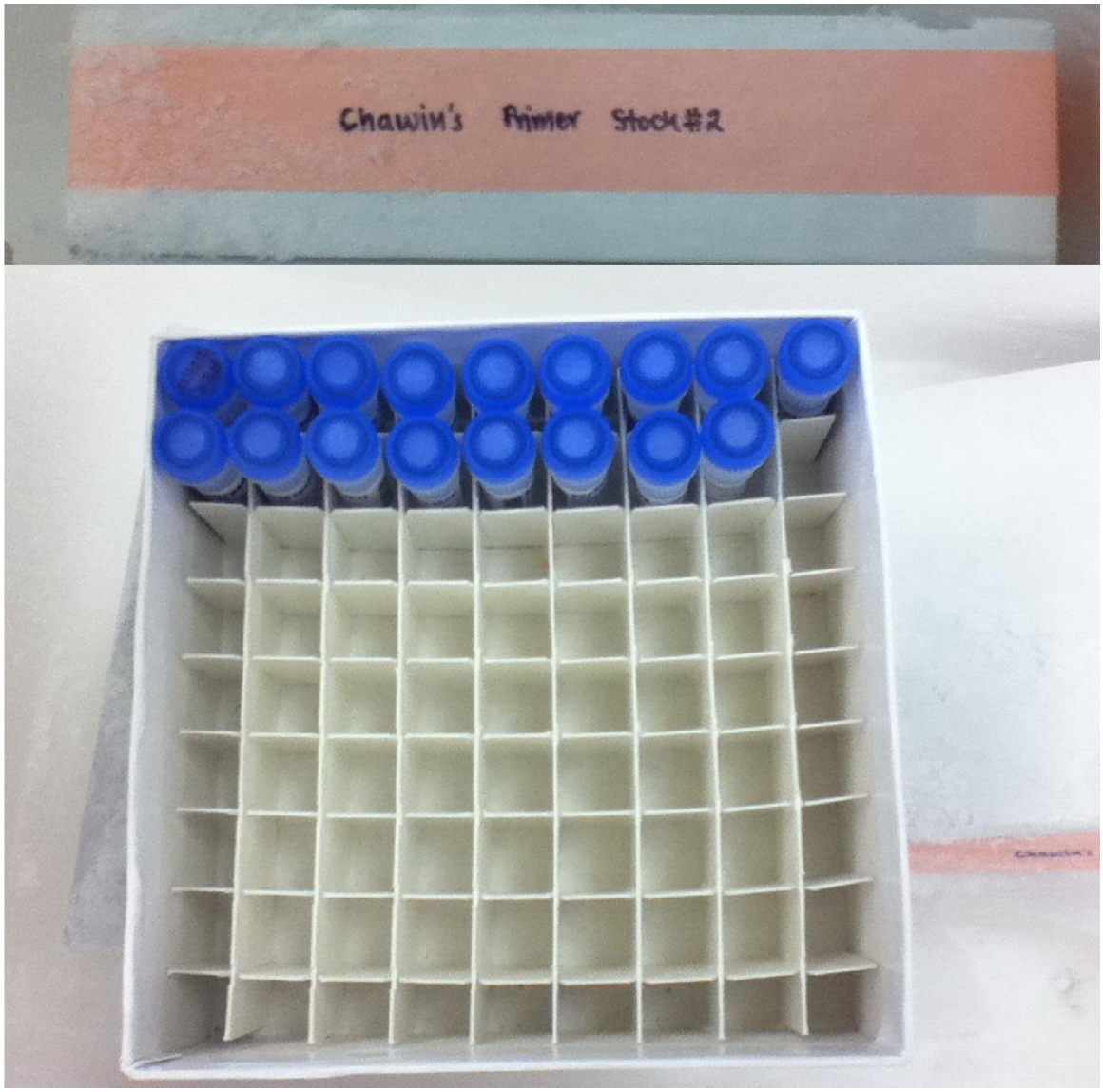
A1	LCV-101-CM		
A2	LCV-102-CM		
A3	LCV-103-CM		
A4	LCV-104-CM		
A5	LCV-124-CM		
A6	LCV-125-CM		
A7	LCV-161-CM		
A8	LCV-162-CM		
A9	LCV-163-CM		
B1	LCV-164-CM		
B2	LCV-165-CM		
B3	LCV-166-CM		
B4	LCV-167-CM		
B5	LCV-175-CM		
B6	LCV-176-CM		
B7	LCV-178-CM		
B8	LCV-179-CM		
B9	LCV-183-CM		
C1	LCV-184-CM		
C2	LCV-185-CM		
C3	LCV-186-CM		
C4	LCV-187-CM		
C5	LCV-188-CM		
C6	LCV-189-CM		
C7	LCV-190-CM		
C8	LCV-191-CM		
C9	LCV-192-CM		
D1	LCV-193-CM		
D2	LCV-194-CM		
D3	LCV-195-CM		
D4	LCV-196-CM		
D5	LCV-197-CM		
D6	LCV-198-CM		
D7	LCV-199-CM		
D8	LCV-219-CM		
D9	LCV-220-CM		
E1	LCV-221-CM		
E2	LCV-222-CM		
E3	LCV-223-CM		
E4	LCV-243-CM		
E5	LCV-244-CM		
E6	LCV-249-CM		
E7	LCV-250-CM		
E8	LCV-251-CM		
E9	LCV-252-CM		

F1	LCV-253-CM
F2	LCV-254-CM
F3	LCV-255-CM
F4	LCV-256-CM
F5	LCV-257-CM
F6	LCV-258-CM
F7	LCV-259-CM
F8	LCV-260-CM
F9	LCV-261-CM
G1	LCV-262-CM
G2	LCV-263-CM
G3	LCV-264-CM
G4	LCV-265-CM
G5	LCV-266-CM
G6	LCV-267-CM
G7	LCV-268-CM
G8	LCV-269-CM
G9	LCV-270-CM
H1	LCV-271-CM
H2	LIYV-67
H3	LIYV-68
H4	LIYV-69
H5	p26-r2
H6	TRSV-F1
H7	TRSV-R1
H8	TRSV-F2
H9	TRSV-R2
I1	RNA2SL + 5/14/12
I2	RNA2SL - 5/14/12
I3	GFPSL + 5/14/12
I4	GFPSL - 5/14/12
I5	RNA2SL + (short primer) 7/2/12
I6	GFP + 7/2/12
I7	pGEMT 6/21/12
I8	pGEMT-Easy + 5/11/11
I9	AAP(T) 1/11/11



Chawin's Primer stock #2: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	LCV-260-CM new		
A2	LCV-272-CM		
A3	LCV-273-CM		
A4	LCV-274-CM		
A5	LCV-275-CM		
A6	LCV-276-CM		
A7	LCV-277-CM		
A8	LCV-278-CM		
A9	LCV-279-CM		
B1	LCV-280-CM		
B2	LCV-281-CM		
B3	LCV-282-CM		
B4	LCV-283-CM		
B5	LCV-284-CM		
B6	LCV-285-CM		
B7	LCV-286-CM		
B8	LCV-287-CM		
B9			
C1			
C2			
C3			
C4			
C5			
C6			
C7			
C8			
C9			


Chawin's Primer Stock #2

Chawin's Virion #1: -20^{0}C

	Descriptions:	Date:	NB pg:
A1	5b agro dirty prep by JN 50% glycerol	5/13/2009	447
A2	5bm1 agro dirty prep by JN 50% glycerol	5/13/2009	447
A3	pR6-5b	2/9/2010	724
A4	pR6	4/6/2010	765
A5	pR6-5b	4/6/2010	765
A6	pR6-5b	4/28/2010	776
A7			
A8	LIYV virion from JN (1424.36ng/ul)		
A9	LIYV from Murale BFNB6-5/4/05-1 50% glycerol diluted to 1.33ug/ul	8/16/2010	
B1	LIYV p1-19 Murale BFNB2-2/14/02-5 (3.4ug/ul)		
B2	LIYV p1-2F Lettuce BFNB2-1/31/03-6 (34ng/ul)		
B3	LIYV pR6 Lettuce BFNB2-2/7/03-8 (232ng/ul)		
B4	LIYV pR6 Lettuce BFNB2-2/7/03-12 (6.1ug/ul)		
B5	LIYV WT Murale BFNB6-5/4/05-1 (6.6ug/ul)		
B6	LIYV Inflitrated leaves 50% glycerol	9/22/2010	880
B7			
B8			
B9			
C1	LIYV WT from inflitrated leaves 50% glycerol	5/22/2009	453
C2	TC3 virion 50% glycerol	8/12/2010	841
C3	TC2 dirty prep 50% glycerol	3/24/2011	986
C4	pJW168 dirty prep 50% glycerol	3/24/2011	986
C5	pJW168 dirty prep 50% glycerol	6/28/2011	1056
C6	TC1 dirty prep 50% glycerol	6/28/2011	1056
C7	TC3 virion 50% glycerol	7/20/2011	1072
C8			
C9			
E1	LCV from sugar beet supernatant 50% glycerol	2/6/2009	332
E2	LCV from protoplast 50% glycerol	9/14/2009	616
E3	LCV from protoplast 50% glycerol	6/7/2011	1038
E4	LCV from Murales	6/30/2011	1057
E5			
E6			
E7			
E8			
E9			
F1	NB total protein with Bradly Buffer	9/21/2010	881
F2	NB CP+ total protein with Bradly Buffer	9/21/2010	881
F3			
F4			
F5			
F6			
F7			
F8			
F9