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Engineering pathogen resistance in crop plants

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Abstract

As the world population continues to increase, food supplies must also grow to meet nutritional requirements. One means of ensuring the stability and plentitude of the food supply is to mitigate crop loss caused by plant pathogens. Strategies for combating disease include traditional technologies such as plant breeding and chemical applications; current technologies such as generating transgenic plants that express components of known defense signaling pathways; and the adaptation of newer technologies such as RNA silencing of pathogen and plant transcripts. Breeding has been used to pyramid resistance (R) genes into many different plants including rice. Chemical strategies include application of salicylic acid (SA) analogs to stimulate systemic acquired resistance (SAR) responses. Genetic screens in Arabidopsis have identified genes controlling SAR and these genes have been manipulated and used to engineer crop plants. The diseases caused by plant viruses are being thwarted through the initiation of endogenous RNA silencing mechanisms. Many of these strategies show great promise, some limitations, and exciting opportunities to develop many new tools for combating plant pests.

Introduction

Engineering resistance for agricultural improvement presently incorporates diverse strategies to mitigate the crop losses imposed by pathogens. For instance, traditional breeding has been strengthened and streamlined with the advent of new molecular markers for rapid selection of desired traits. Transgenic technology provides a complement to some of the weaknesses inherent in traditional breeding. This includes altering the expression of endogenous components from specific and broad-based pathogen resistance signaling pathways as well as utilizing genes from other species. Further analyses are providing insight into other means of inducing inherent plant defense responses through refined chemical and hormone treatments. The control of viruses and a bacterial pathogen using transgene expression *in planta* to initiate RNA silencing has also shown great promise. This review will address the wide number of avenues being explored by combining traditional genetics and breeding with newer transgenic approaches to generate enhanced resistance in plants.

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Qualitative resistance

Shortly after the re-discovery of Mendel's laws of heritability, qualitative traits were identified in wheat that conferred resistance to the rust pathogen *Puccinia striiformis* (Biffen, 1905). Subsequently, numerous qualitative loci have been identified in diverse plant species that confer resistance (Crute & Pink, 1996). Most of these loci do not confer broad-spectrum resistance; rather, the resistance is limited to subgroups within pathogen species. Likewise, diverse isolates of a particular pathogen species have been shown to contain loci that prevent the pathogen from successfully causing disease on the host (Flor, 1956). Flor developed a model based upon classical genetics using flax, *Linum ultissimom*, and the fungal pathogen *Melamspora lini*. His 'gene-for-gene' theory states that the plant resistance locus [R] and the pathogen avirulence determinant [avr] must both be present to

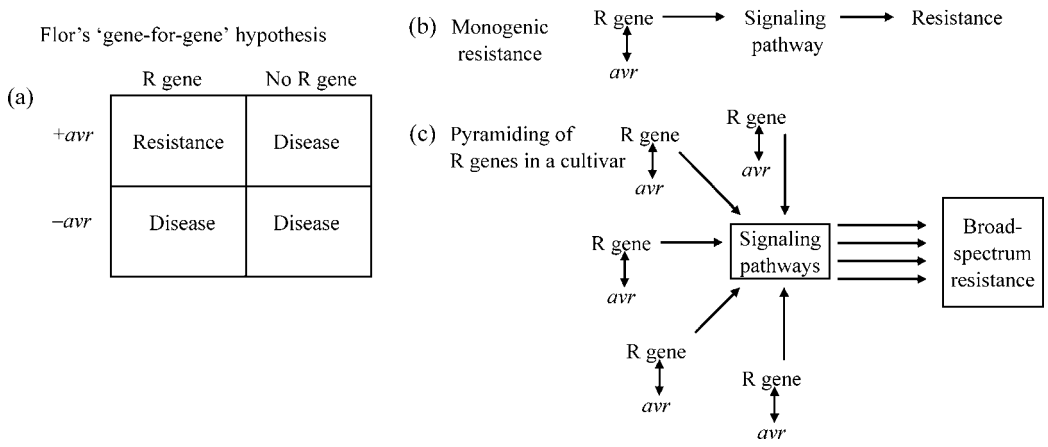

Figure 1. R gene-mediated resistance. (a) A schematic of Flor's gene-for-gene hypothesis. (b) Monogenic resistance involves the use of a single R gene. This R gene can be 'defeated' when the gene product of the avirulence locus is mutated. The dynamic nature of the pathogen genome to alter these avirulence loci has been observed as a loss of resistance in the field. (c) Pyramiding of resistance genes, however, has been shown to confer broad-spectrum resistance to pathogens. The incorporation of multiple R genes simultaneously requires a pathogen to eliminate all avirulence loci corresponding to the R gene combination to effect disease.

observe phenotypic resistance in the host (Flor, 1971). For any given plant and potential pathogen pair, an incompatible interaction (i.e., resistance) is the product of these two loci. If the plant lacks the R locus or the pathogen lacks the avr determinant, then a compatible interaction (i.e., disease on the host) is the outcome (see Figure 1(a)). The R gene products are hypothesized to act as receptors for the products of the avirulence locus. Thus Flor's findings demonstrate that for many host–pathogen relationships, resistance [R] and avirulence [avr] loci dictate the outcome of varying combinations of host and pathogen genotypes.

R gene-mediated resistance is an economical method to control losses in the field and breeders have mobilized R genes into virtually all improved lines. Often this genetic mechanism of resistance lacks long-lasting durability in the field. In terms of Flor's 'gene-for-gene' theory, the pathogen responds to selection pressure by altering or eliminating the avirulence determinant. When the pathogen eliminates the cognate avr gene product, the R gene (receptor) can no longer perceive the product (ligand) of the avirulence locus. Plants have responded to these dynamic genetic changes of the pathogen by generating their own clusters of diverse resistance loci (Michelmore et al., 2000; Richter & Ronald, 2000). Over the past decade, a number of R loci and avr loci have been cloned from diverse species of plants and pathogens. The specific structural details have been thoroughly described elsewhere (Hulbert et al., 2001; Bonas & Lahaye, 2002).

Pyramiding of resistance loci

As noted above, monogenic resistance frequently is not durable in the field when exposed to high levels of pathogen pressure. One strategy to control pathogen outbreaks in the field is to cultivate plants with diverse genetic backgrounds within a single field. This agricultural practice has been shown to dramatically reduce yield losses caused by the rice fungal pathogen *Magnaporthe grisea* in repeated field studies (Zhu et al., 2000). For many crops, utilization of this approach may provide a mechanism for conferring long-term, durable resistance.

For some crops, planting and harvesting a field planted with diverse germplasm may not always be practical. Thus it would be useful to develop disease control strategies that would benefit farming practices that still rely on monculture. The use of gene pyramiding offers an attractive mechanism by which multiple resistance loci, each recognizing a unique range of isolates of a pathogen species, may be incorporated into a single cultivar (Singh et al., 2001). The combined presence of these R loci ensures that only pathogens devoid of all cognate avirulence loci perceived by the R loci combination can cause disease (Figure 1(b) and (c)). With the advent of molecular markers tightly linked to resistance loci, simultaneous selection of multiple resistance loci has been facilitated. This strategy of marker assisted selection also allows for the selection against undesirable chromosomal segments known to carry unfavorable agronomic traits as well

as the successful incorporation of recessive resistance genes.

The interaction of cultivated rice, *Oryza sativa*, and a bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) offers a clear example of the benefits of gene pyramiding. One set of gene pyramiding experiments was performed using two completely dominant resistance genes, *Xa21* and *Xa4*, and two recessive genes, *xa5* and *xa13* (Li et al., 2001). Each of these resistance genes confers resistance to distinct isolates of *Xoo* and individually these R genes have been overcome by known field isolates (Ona et al., 1998; Adhikari et al., 1999; Lee et al., 1999; Shanti et al., 2001). The efficacy of the disease resistance conferred by combinations of these four different resistance loci was evaluated in controlled environments as well as in field studies. The incorporation of four genes simultaneously conferred complete resistance to all isolates of *Xoo* tested (Li et al., 2001). These results demonstrate the utility and efficacy of gene pyramiding for generating broad-spectrum resistance to *Xoo*, although this finding does not suggest that combinations of four genes will lead to durable resistance *per se*. One caveat in gene pyramiding is that any given resistance locus may have variable penetrance. It should not be assumed that each R gene introgressed into any genetic background will display full phenotypic resistance. Therefore each set of R genes incorporated into a cultivar must be evaluated systematically requiring a significant investment of time. However, it is clear that gene pyramiding offers an attractive mechanism for combining the individual specificities of R genes as well as taking advantage of their synergistic effects to generate broad-spectrum resistance.

Generating durable resistance by targeting a conserved avirulence locus

Occasionally, the loss or mutation of an avirulence locus is associated with reduced pathogen fitness. In pepper, bacterial spot disease has been controlled effectively using the *Bs2* resistance locus in breeding programs. The durability of *Bs2* is due to the widespread conservation of the *avrBs2* locus in diverse pathogen isolates of *Xanthomonas campestris* pv. *vesicatoria*. Mutation of *avrBs2* prevents wild-type levels of bacterial growth on susceptible pepper cultivars lacking *Bs2* (Tai et al., 1999). Another example is found in rice where a functional *avrXa7* avirulence locus is required for full virulence of *Xoo* (Bai et al., 2000). Isolates carrying an *avrXa7* mutation can cause disease in the presence of the *Xa7* R gene although greenhouse tests demonstrated that the severity of disease was reduced (Bai et al., 2000). Tests have further demonstrated that spontaneous mutants of *avrXa7* could be recovered from *Xa7* plants in the field; however these virulent isolates did not persist. Field tests over six years demonstrated that the presence of *Xa7* was sufficient to prevent any *Xoo* epidemics even when *avrXa7* mutants had appeared (Vera Cruz et al., 2001). In contrast, these field tests demonstrated that epidemics were common in fields planted with rice carrying the *Xa10* R gene. *Xoo* with mutations in *avrXa10* displayed no loss of fitness in controlled studies and disease in the field was common on *Xa10* plants over the same 6-year analysis (Bai et al., 2000; Vera Cruz et al., 2001). These results indicate that some R genes can confer durable resistance if loss of their cognate avirulence locus confers some fitness penalty for the pathogen. Using such R/avr combinations may be a rational strategy for generating durable resistance.

Introduction of resistance by transgenic technology

An alternate strategy to breeding is to directly introduce a cloned resistance gene into a plant via transgenic technology. Introduction of a gene by transgenic means can overcome the limitations of traditional breeding, namely interspecies sterility. Additionally, transgenic technologies allows multiple genes to be inserted simultaneously. However, validation of the function of the transgene and its stability and heritability after transformation requires a significant investment of time and resources. Further, the transgenic lines must also undergo subsequent analysis for agronomic traits before release. While the creation of transgenic plants may be relatively straightforward for a number of species, the strategy has its own substantial time requirements.

The greatest advantage of transgenic technology is its ability to overcome fertility barriers for the dissemination of genes originating from a different species; two examples from the *Solanaceae* family highlight this advance. *Bs2*, as mentioned above, was identified originally in pepper and its resistance has been durable in the field against isolates of *X. campestris* (Tai et al., 1999). Due to the fitness requirement associated with *avrBs2* locus, the incorporation of the resistance locus *Bs2* via transgenic technology may offer durable resistance in a number of plant systems

affected by *X. campetris*. To assess this hypothesis, tomato was transformed with the *Bs2* gene from pepper. Inoculations of *X. c.* pv *vesicatoria* isolates onto *Bs2*-containing transgenic tomato plants failed to cause disease therefore *Bs2* function was conserved in tomato (Tai et al., 1999). Tomato and pepper when crossed cannot form a fertile hybrid and this resistance could not have been utilized with standard breeding protocols. In another example the *N* gene from tobacco, conferring resistance to the tobacco mosaic virus (TMV), was transferred into tomato (Whitham et al., 1994, 1996). The resulting transgenic tomato plants, expressing the *N* resistance gene, were inoculated with TMV and complete resistance was observed. While TMV is not as devastating economically to tomato as is *X. campestris*, the conceptual notion that resistance loci can be transferred among species while retaining their function points illustrates a key advance for engineering resistance using transgenic technology. These examples demonstrate conservation in disease signaling pathways that can be exploited for cultivar improvement.

Strategies for engineering broad-spectrum disease resistance

Disease resistance research has largely focused on understanding the specific pathogen–host interactions mediated by R and avr loci. Recently, studies have revealed signaling components that function downstream of R genes or other pathogen sensors. Studies on broad-spectrum resistance pathways, such as the rhizobacteria-mediated, induced systemic resistance (ISR) pathway and the insect-responsive pathway involving jasmonic acid (JA) are rapidly gaining momentum (Pieterse et al., 1998; Turner et al., 2002). However, research on the pathway transducing a broad-spectrum defense response termed the systemic acquired resistance (SAR) response has progressed most rapidly. Chemical and abiotic inducers of SAR, along with inherent signaling components of this pathway identified by basic research in model plant systems, are among the initial targets being used to engineer multi-pathogen disease resistance in important crop plants.

The SAR defense response is manifested when a plant host is inoculated with a pathogen that results in a localized infection. This primary infection (often associated with plant necrosis) subsequently primes the host to resist secondary infections by viral, oomycete and bacterial pathogens (Ryals et al., 1996). In the model plant Arabidopsis, SAR is associated with a rise of internal levels of the plant hormone salicylic acid (SA), and is correlated with the increased expression of a set of genes termed pathogenesis-related (PR) genes (Ward et al., 1991; Ryals et al., 1996; Van Loon, 1999; Maleck et al., 2000; Muthukrishnan et al., 2001). Several PR genes encode proteins with antimicrobial activity and thus contribute to an overall defense response directly (Ward et al., 1991). Research aimed at modulating this pathway and generating broad-spectrum resistance has largely targeted three parts of this response for further study: (1) the ability of SA to trigger the response, (2) the increased expression of PR genes and (3) the identification and modulation of other signaling components.

Chemical and biotic induction of SAR in plants

SA is both necessary and sufficient to induce SAR in Arabidopsis and SA added exogenously initiates the SAR response (Delaney et al., 1994). Conversely, transgenic plants constitutively expressing the bacterial *nahG* gene, which encodes a salicylate hydroxylase, do not accumulate SA and do not mount an SAR response (Gaffney et al., 1993; Delaney et al., 1994; Vernooij et al., 1994). Other potent chemical inducers of SAR for higher plants are the synthetic chemicals benzo-(1,2,3)-thiadiazole-7-carbothioc acid (BTH) and isonicotinic acid (INA), both functional analogs of SA (Metraux et al., 1991; Ward et al., 1991; Uknes et al., 1992; Gorlach et al., 1996; Lawton et al., 1996). BTH (in the form of the commercial chemical BION®) in particular, is associated with low phytotoxicity and has demonstrated efficacy against multiple pathogens when applied in field trials. Notably, resistance is increased against the wheat powdery mildew fungus and the rice blast fungus, as well as against the late blight pathogen of tomatoes (Oostendorp et al., 2001).

An increase of internal SA levels after pathogen infection is a key feature of SAR in Arabidopsis and tobacco. These plants have low SA levels when not induced by pathogen attack. In contrast, high levels of endogenous SA are constitutively detected in some crop plants such as potatoes and rice (Silverman et al., 1995; Chen et al., 1997; Yu et al., 1997). Therefore, the role of SA in rice and potato SAR-like responses is not clear and these examples may indicate points of divergence in the composition of defense pathways in different plants. It will be important to understand the

individual differences before applying similar engineering strategies for all plant types. For example, BTH treatment of wheat enhances resistance to the pathogens *Erysiphe graminus* and *Puccinia recondita*, and induces the expression of five novel *WCI* (wheat chemically induced) genes (Gorlach et al., 1996). BTH does not induce the full subset of wheat PR genes (Gorlach et al., 1996) and the expression of the *WCI* genes after induction by BTH is not sufficient to provide resistance to disease caused by another pathogen, the wheat head blight fungal pathogen *Fusarium graminarium* (Yu et al., 2001). Infection of wheat by this fungus will induce the expression of wheat PR-like genes but not the WCI genes (Yu et al., 2001). Thus, at least in wheat, biotic and chemical inducers promote the expression of different genes sets suggesting roles for multiple defense response pathways. Studies in rice and barley yield similar results (Schweizer et al., 1999; Besser et al., 2000).

Evidence for the conservation of a SAR-like response in cereals and other economically important crop plants arises from studies indicating that defenses against multiple pathogens can be induced after treatment with the SAR-inducing chemicals SA and BTH. Biotic induction of systemic resistance has also been described. Systemic resistance to the rice blast pathogen, *Magnaporthe grisea*, is established after an initial infection with the non-host pathogen *Pseudomonas fluorescens* (Smith & Metraux, 1991). In wheat and barley, an initial infection by *E. graminus* heightens a plant's ability to respond to a subsequent infection by the same pathogen (Schweizer et al., 1989; Hwang & Heitefuss, 1992). Hence, several studies indicate that in multiple plants, including cereals, SA and similar chemicals can induce defense responses and that pathogens can prime secondary resistance, two key SAR features. It is then reasonable to postulate that at least some of these responses may be mediated via a SAR signaling pathway. Therefore, identifying and characterizing the components of such a pathway will likely provide targets that can be used to engineer resistance in plants.

Screening for SAR components: potential targets for engineering resistance

Genetic screens were initiated in model plant systems such as in Arabidopsis to identify genes involved in SAR. Many genes encoding components of the SAR signaling pathway have been identified through these screens (for recent reviews see Dong, 2001; Glazebrook, 2001). Below, a few examples which may provide targets for engineering resistance are discussed in detail.

Mutants identified by their enhanced disease resistance, and thus likely to function as negative regulators of the SAR pathway include the *edr1* (enhanced disease resistance) and *mpk4* (mitogen-activated protein kinase) mutants (Frye et al., 1998; Petersen et al., 2000). Plants carrying an *edr1* mutation have enhanced resistance to the fungal pathogen *Erisphye cicoracearum*. In addition, the phenotypic effects of this mutation can be suppressed by removal of endogenous SA from the mutant plants (Frye et al., 1998). The *EDR1* locus has been cloned and was shown to encode a protein with high sequence similarity to a MAPKK kinase (Frye et al., 2001). The *mpk4* mutant plants show strong expression of the PR genes 1, 2 and 5. When *mpk4* mutants carry the *nahG* transgene the resistance phenotype is lost and these plants no longer constitutively express *PR-1* indicating that the *mpk4*-mediated negative regulation of SAR requires SA accumulation (Petersen et al., 2000). Other mutants showing constitutive PR gene expression and enhanced pathogen resistance are the *cpr* mutants (Bowling et al., 1994, 1997; Clarke et al., 1998). The SA levels in these mutants are also increased. Several members of the *cpr* mutants and the *mpk4* mutant have growth defects, indicating metabolic problems with constitutively expressing defense responses (Bowling, 1994; Petersen, 2000).

A contrasting class of mutants contains members that can no longer express PR genes and fail to mount a defense response (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). The *NPR1* (non-expressor of PR genes) gene (also called *NIM1*) was isolated from this second class of mutants. *NPR1* was cloned and shown to encode a protein with limited overall homology to the mammalian immune response signaling protein IkB (Cao et al., 1997; Ryals et al., 1997). Subsequently, studies of *NPR1* have indicated that it is a key positive regulator of the SAR response in Arabidopsis that functions downstream of the SA signal. NPR1 may help induce PR gene expression through interaction with a class of transcription factors of the basic leucine-zipper type (see Figure 2) (Cao et al., 1997, 1998; Zhang et al, 1999; Kinkema et al., 2001; Fan & Dong, 2002). Recently, genomic-analysis technologies have identified novel defense-associated genes that may be additional targets for genetic engineering. In two examples, the expression of Arabidopsis genes under several

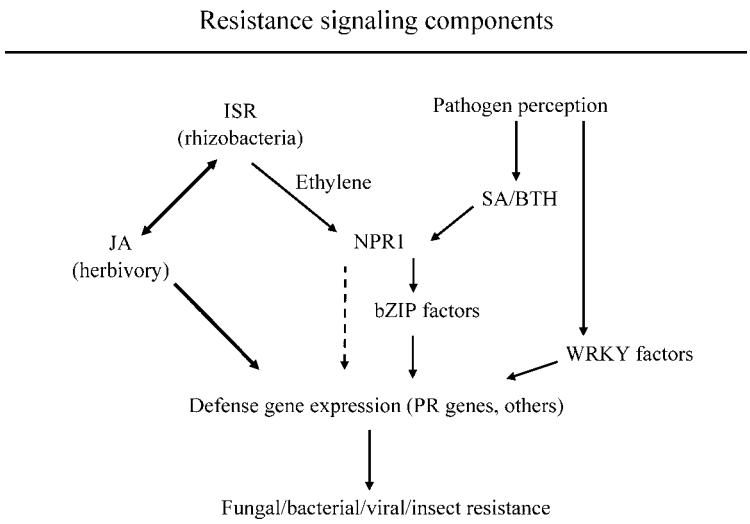

Figure 2. A simplified schematic of induced broad-spectrum resistance signaling pathways. SA and BTH initiate *NPR1*-mediated expression of defense genes that lead to SAR. JA can accumulate after herbivory by insects initiating a complex interaction with ISR to yield resistance. Studies suggest that there is significant cross-talk between the JA and SA signaling pathways. The ISR signaling pathway also requires some SA pathway components, such as *NPR1*, for resistance. The elevated expression of different sets of defense-related genes, including the SAR pathway PR genes, is a common feature of multiple induced resistance pathways. Gene expression may be regulated by transcription factors, possibly of the WRKY or bZIP types. Induction of any of these three broadly defined pathways results in enhanced resistance to a broad-spectrum of plant pests.

SAR-inducing conditions was analyzed using microarray technology (Maleck et al., 2000; Chen et al., 2002). Along with the transcripts for the well-characterized PR genes (PR-1, 2 and 5) the mRNAs upregulated by defense stressors include those that encode for proteins such as a zinc-copper, superoxide dismutase, and a WRKY transcription factor, *AtWRKY7* (Maleck et al., 2000). WRKY transcription factors are novel, plant transcription factors that can bind to promoters containing a 'W' box; these motifs can be found in high abundance in the promoters of defense-related genes (Chen et al., 2002), including *NPR1* (Yu et al., 2001). While these technologies for global gene analysis are starting to reveal additional components of defense pathways, work using these components in crop plants has so far focused on the transgenic expression of the PR genes and *NPR1*.

Engineering crop plants for enhanced resistance by manipulating PR gene expression

Attempts to alter the expression of individual PR genes and their encoded proteins were among the first examples of experiments manipulating SAR components to engineer crop plants to display broad-spectrum pathogen resistance. PR proteins were originally classified as plant host proteins that were induced only in pathological or related situations (Antoniw, 1980; Muthukrishnan et al., 2001). The function of PR-1 is not clear but PR-2 (a B-1-3 glucanase) and PR-3 (a chitinase) have anti-fungal properties *in vitro*. This suggests they may play a direct role in defense by attacking and degrading pathogen cell wall components (Kauffman et al., 1987; Legrand et al., 1987; Muthukrishnan et al., 2001).

Many PR and PR-like proteins have been identified in crop species including, rice, wheat, barley, sorghum and maize (for reviews see Ward et al., 1991; Van Loon, 1999; Muthukrishnan et al., 2001). In crop plants each PR gene class has multiple family members. For example, there are at least 17 unique members of the *PR-3* gene family, and seven *PR-2* family members in barley (Muthukrishnan et al., 2001). Increasing the expression of individual and multiple PR proteins in various crops has demonstrated some success in enhancing disease resistance to particular pathogens. Examples of enhanced protection conferred by altering PR gene expression are mainly derived from studies manipulating levels of the *PR-2* and *PR-3* gene family members. For example, over-expression of a rice *PR-3* gene, *Chi11*, slightly reduces the extent of infection by the rice sheath blight pathogen, *R. solani*, in rice and the extent of the disease reduction correlates with the expression level of the transgene (Lin et al., 1995; Muthukrishnan et al., 2001). Additionally, over-expression of another rice

chitinase, *Cht-2*, reduces the severity of the disease conferred by the rice fungal pathogen *M. grisea*, although the extent of this resistance was decreased in subsequent generations (Nishizawa et al., 1999).

This protective ability is not limited to rice; over-expression of a barley chitinase in wheat confers resistance, in isolated leaf assays, to the powdery mildew pathogen *E. graminus* (Bliffeld et al., 1999). Furthermore, in sorghum, over-expression of the rice chitinase, *Chi11*, enhances resistance to the fungal pathogen *Fusarium thapsinum* (Krishnaveni et al., 2001). The effects of altering the expression of the *PR-5* (thaumatin-like) gene family members have also been studied in wheat and maize (Reimann & Dudler, 1993). Constitutive over-expression of a rice *PR-5* gene in wheat delays the onset of the symptoms caused by the wheat scab pathogen, *Fusarium graminearusin*, in a stable and heritable manner (Chen et al., 1999). Hence, at least for a subset of PR genes, manipulation of their expression levels may offer some enhanced protection to crop plants, even if the protection is not the broad-spectrum resistance originally desired. It appears that over-expression of any single PR gene, similar to the results seen employing single R genes, will not be sufficient to generate broad, durable resistance. An alternate approach to engineering resistance using PR genes is to over-express regulatory genes, such as *NPR1*, that are found upstream of defense genes in signaling pathways.

Modulating NPR1 expression to enhance disease resistance

NPR1 plays an important role in stimulating many downstream components of the SAR pathway; it is therefore a natural target with which to engineer disease resistance in crop plants. Over-expression of *NPR1* in Arabidopsis leads to enhanced disease resistance to both bacterial and fungal pathogens in a dose-dependent manner (Cao et al., 1998; Friedrich et al., 2001). Plants containing the *NPR1* transgene display no obvious pleiotropic effects. Once induced by pathogen attack or by chemical induction, NPR1 localizes to the cell nucleus where it can interact with a class of basic-leucine zipper transcription factors (TGAs) that are predicted to modulate PR gene expression, thus mediating the SAR response (Zhang et al., 1999; Kinkema et al., 2001; Fan & Dong, 2002). In separate studies *NPR1* over-expression and enhanced resistance are correlated either with elevated or earlier expression of *PR* gene transcripts, thus supporting this theory (Cao et al., 1998; Friedrich et al., 2001).

Along with stimulating PR gene expression and priming plants to respond to infection, high *NPR1* expression levels enhance the sensitivity of plants to chemicals and fungicides including BTH, fosetyl, and $Cu(OH)_2$ (Friedrich et al., 2001). *NPR1* is also required for a BTH-induced defense priming indicating a great potential for coupling both chemical and transgenic disease strategies through plants expressing *NPR1* (Conrath et al., 2001; Friedrich et al., 2001; Kohler et al., 2002). For instance, plants can be engineered to over-express *NPR1* so that a lower chemical dose is required to confer efficient disease resistance. Genes with high sequence similarity to *NPR1* can be found in Arabidopsis, tobacco, tomato, rice and maize (Zhang et al., 1999; Chern et al., 2001; Yu and Muehlbauer, 2001) suggesting that this regulator will be conserved among many plant species. Over-expression of *NPR1* in rice has been shown to enhance resistance to the rice bacterial blight pathogen *Xoo* (Chern et al., 2001). Studies with a putative rice homolog of *NPR1* indicate that over-expression of the endogenous rice gene can also provide protection against *Xoo* (Chern & Ronald, unpublished). However, unlike in Arabidopsis, rice over-expressing *NPR1* grown under suboptimal conditions display a detrimental growth phenotype. These types of observations may predict an overall phenotype that will need to be further investigated when strongly over-enhancing SAR pathway components in plants (Fitzgerald & Ronald, unpublished). Together these data suggest that, in general, crop plants contain defense signaling components similar to those found in Arabidopsis. Potentially, over-expression of other endogenous signaling components other than *NPR1* may also be able to provide enhanced plant protection.

Biotic induction of resistance

Biotic infections that stimulate localized host cell death can stimulate SAR in a wide variety of plants, as indicated above. Similarly, root colonization by non-pathogenic *Rhizobacteria*, can stimulate induced systemic resistance (ISR) (Pierterse et al., 1998). This resistance is distinct from SAR, but interestingly shares one of the same components, *NPR1* (Pierterse et al., 1998) and can work additively with SAR to mount a heightened defense response (Van Wees et al., 2000). Induction of the ISR response requires that plants are able to properly respond to signals triggered

by JA and by the plant hormone ethylene. ISR is not functional in Arabidopsis mutants that are non-responsive to ethylene, although the SAR response remains intact (Knoester et al., 1999). Presently, most work utilizing ISR with field grown crops focuses on biocontrol. For example, when tomato plants or seeds are treated with dried *Rhizobacteria* spores, the severity of infection by the tomato mottle virus is reduced (Murphy et al., 2000). It is notable that the ISR pathway shares components with other defense pathways. Thus, altering the amount of a single component involved in multiple pathways, such as *NPR1*, may have unintended pleiotropic effects, both favorable and unfavorable that will need to be addressed before application in the field.

Plants induce defense responses not only against bacterial, fungal and viral pathogens but also against pests that can cause wounding. Thus, other biotic inducers of resistance include herbivorous insects. The chemical JA is important for triggering resistance to these pests (McConn et al., 1997). The defense pathways controlling insect defense and other induced responses are partially antagonistic. Treatment of plants with SA and BTH can inhibit the induction of JA-induced genes and conversely, application of JA reduces the defenses triggered by ISR inducers (Thaler et al., 1997, 1999; Stout, 1998; Heil, 2001). However, it appears that the SA and JA pathways can also, in some situations, act in concert to promote defenses against at least a subset of pathogens. In Arabidopsis, in studies where both SA and JA are applied to a plant these chemicals can work additively to protect plants against *Pseudomonas syringae* pv. *tomato* (van Wees et al., 2000). Potentially, upregulation of one induced resistance pathway may impart costs to a number of other pathways. With such complexity inherent in defense responses, it becomes clear that thorough field tests performed under multiple environmental, developmental and pathogen stressors will be essential for any plants engineered for enhanced resistance.

Costs and limitations to engineering resistance using inducers of broad-spectrum resistance

Basic research is providing an ever-expanding arsenal of genes with which to engineer disease resistance. Several of these genes have already proven useful and more will undoubtedly be discovered. However, the limitations and costs to using this technology are just starting to be explored. A thorough understanding of these areas will be increasingly important as the tools identified by basic research in plant defense mechanisms are applied more frequently to commercial crops. Previously, only a few studies have attempted to look at the costs, for example, in fitness to plants induced for one of these resistance responses and even fewer still of these studies have been with the economically important cereal crops (Heil et al., 1999; Hatcher & Paul, 2000; Heil, 2001).

Growth costs associated with chemical inducers of SAR under suboptimal growing conditions

Most of the genes involved in broad-spectrum resistance have yet to be inserted as transgenes into crops. Therefore, investigations into the costs of induced resistance have started by assaying the effects of using chemical inducers. Heil et al. (2000) have studied the fitness of wheat plants treated with BTH in the absence of pathogens. When plants were grown either hydroponically or in the field, water-treated control plants were able to achieve greater biomass than their BTH-treated cohorts. In field experiments, however, significant growth differences were not seen until approximately 6 weeks after treatment. The authors suggest that many of the potential fitness costs associated with induced resistance responses may be masked in laboratory experiments where growth conditions are kept optimal, and support this hypothesis with experiments performed growing plants under differing nitrogen concentrations. In addition, when the age of the plants induced for SAR was considered it was found that the growth-costs of BTH treatment could be reduced if the BTH was applied after the lateral shoot formation was complete (Heil et al., 2000). These data also underscore the importance of factoring plant developmental programs into any efficient strategy to enhance plant resistance by chemical treatment or genetic engineering.

Cell death – a possible byproduct of manipulating resistance pathways

Another unwanted effect that may arise from transgenic manipulation of genes involved in defense signaling pathways is spontaneous cell death. Spontaneous cell death has been uncovered in many genetic screens for enhanced disease resistance and recently, has been seen in transgenic plants. These mutants and transgenic plants are often collectively referred to as lesion-mimic (LM) mutants since they display

lesions similar to those observed in a defense response even in the absence of pathogens. This form of cell death in plants is sometimes influenced by alterations in environmental conditions such as light, temperature and humidity (Kiyosawa, 1970; Dietrich et al., 1994; Arase et al., 2000; Jambunathan et al., 2001). Therefore, both in basic research and in applied experiments, it will be important to understand the parameters controlling cell death. This research is critical not only for optimizing the situations where transgenes and chemicals will be most useful to generate disease resistance, but also to minimize negative effects on important agronomic factors such as development, fertility and yield.

Several dicot lesion-mimic mutants that lead to enhanced cell death have been well characterized including the Arabidopsis *acd* (accelerated cell death) and *lsd* (lesions simulating disease) mutants (Greenberg & Ausubel, 1993; Dietrich et al., 1994). A recessive mutation in the *LSD1* gene (encoding a zinc-finger transcription factor) leads to a lesion-mimic phenotype that is triggered under long-day light conditions and by treatment with SA and INA (Dietrich et al., 1994, 1997). The *lsd1* mutation appears to confer hypersensitivity to these compounds (Jabs et al., 1996). It is hypothesized that reactive oxygen species (ROS) accumulate in leaf tissues preceding formation of lesions and that *LSD1* normally functions to define the extent of lesion spread by suppressing cell death (Kliebenstein et al., 1999). ROS accumulation is observed in the initial stages of plant defense responses including during the hypersensitive response (HR). In wild-type plants, the HR precedes the formation of microlesions that are correlated with induced resistance and is associated with subsequent resistance to pathogen infection (Heath, 2000). Altered accumulation patterns of ROS in plants with heightened cell death or an HR suggest that ROS play a central role in regulating plant programmed cell death (PCD) (Jabs, 1999).

Lesion-mimic mutants have also been identified in cereals including rice, maize and barley (*sl*, *Les*, *lls*, *Mlo*) (Obanni et al., 1994; Hueckelhoven et al., 2000; Yin et al., 2000). In rice, one well-characterized class of lesion-mimics contains the *sl* (Sekiguchi lesion also known as *spl*) mutants (Kiyosawa, 1970; Yin et al., 2000). Many of the *sl* mutants display heightened resistance to *M. grisea* and increased expression of *PR-1* and peroxidase genes (Yin et al., 2000). Other rice lesion mimic mutants displaying enhanced resistance are the *cdr* (cell death and resistance) mutants (Takahashi et al., 1999). The *cdr* mutants also have elevated PR gene expression and cell cultures of the *cdr* mutants under certain conditions can accumulate H_2O_2. Thus, a subset of rice lesion mimics may have misregulated levels of ROS (Takahashi et al., 1999). Misregulation of ROS accumulation also occurs in transgenic rice engineered to express the *OsRac1* (a GTPase) gene. Overexpression of *OsRac1* in a wild-type stimulated H_2O_2 accumulation in leaf tissue and over-expression in an *sl* background stimulated cell death (Kawasaki et al., 1999).

While mutant rice genes leading to lesion-mimic phenotypes have only been hypothesized to play a role in ROS regulation, one lesion-mimic-inducing gene from maize, *Les22*, has been cloned. *Les22* encodes a uroporphyrinogen decarboxylase (UROD), an enzyme required for chlorophyll and heme biosynthesis (Hu et al., 1998). Mutations in the homologous human enzyme lead to the light-induced skin toxicity condition of porphyria. People with mutations in the UROD are predicted to accumulate high levels of uroporphyrin III that upon light excitation can become highly reactive resulting in toxic levels of ROS. While the *Les22* mutant phenotype does not appear to be associated with enhanced resistance to pathogens, a recessive *Les* mutant, *les9*, displays enhanced resistance to the pathogen, *Bipolaris maydis* (Nadimpalli et al., 2000). Another maize lesion-mimic mutant, *lls1* (lethal leaf spot1), a recessive mimic mutation is associated with enhanced resistance to the maize rust fungus, *Puccinia sorghi* (Simmons, 1998). The *LLS1* gene was cloned and found to encode a novel protein containing two binding motifs resembling aromatic ring-hydroxylating dioxygenase regions suggesting that this gene may also be involved in detoxification, perhaps of a phenolic compound important in mediating cell death (Gray et al., 1997). Finally, pathogen resistance is associated with lesion-mimic phenotypes in not only rice and maize, but also barley. The barley *mlo* allele has conferred durable resistance to all *E. graminus* isolates for decades (Wolter, 1993). The *mlo* mutation confers a spontaneous cell death phenotype upon pathogen challenge and noticeable formation of structural appositions under epidermal cells. Thus, the mutant phenotype confers a rapid death phenotype to the cells, halting fungal ingress at the point of challenge and preventing a compatible interaction. The wild-type allele *MLO* prevents cell death when challenged by *E. graminus* (Buschges et al., 1997).

Many mutants showing lesion-mimic or enhanced cell death phenotypes are associated with enhanced disease resistance. This does not necessarily suggest

that cell death is a requirement for defense, or that defense always de-represses cell death pathways. Simply, many defense components will likely have multiple roles in basic metabolism and stress responses throughout the plant that need to be characterized before utilizing these genes for resistance engineering.

Overcoming limitations of engineering broad-spectrum disease resistance

Many of the examples listed above, may appear as substantial challenges to engineering disease resistance, however, these challenges provide opportunities to create plants that are even more resistant than plants engineered based on our current knowledge. For instance if the already identified components of a signaling pathway are not the best candidates for durable resistance in the field, technologies such as microarrays will help to pinpoint novel targets of interest (Chen et al., 2002). When mutations involved in disease resistance have already been identified, but are recessive in nature such as the *mlo*, *edr1* and *mpk4* mutants, classical breeding strategies can be employed. These mutants cannot be placed into heterologous systems using transgenic technology but, as with gene-pyramiding, they are still useful in breeding. Or, as technology continues to improve, gene knockouts and silencing of homologs may be employed to generate mutants in diverse species. If research continues to suggest crosstalk between ISR, SAR and insect defense signaling pathways, there may be great potential for additive defense effects by manipulating overlapping components. So, while limitations and cost of engineering broad-spectrum defenses warrant much attention, it is useful to look at such challenges as means for streamlining and improving upon current engineering strategies.

Exploitation of post-transcriptional gene silencing (PTGS) to enhance viral and bacterial resistance

Another promising strategy for enhancing resistance in plants is the use of RNA homology-dependent silencing to combat viral and bacterial disease (see Wassenegger, this issue). The nature of this silencing has been evaluated in a number of systems where similar phenomena are called by different names; RNAi in animals and quelling in fungi (Mourrain et al., 2000). One conserved step leading to RNA homology-dependent silencing is the formation of a double-stranded RNA intermediate. This dsRNA intermediate is recognized by an enzymatic complex which targets degradation of all corresponding homologous RNA transcripts (Beclin et al., 2002). Several cases detailed below illustrate the possibilities for generating disease resistant plants by taking advantage of this inherent biological process.

RNA silencing for viral resistance

Viral resistance using RNA homology-dependent silencing has been successfully engineered into many plant systems. Single or multiple viral-derived transgenes can be expressed in plants leading to RNA homology-dependent silencing and subsequent viral resistance. The use of this transgenic technology may be particularly effective in thwarting viral diseases where little or no genetic resistance has been identified. Resistance to rice yellow mottle virus (RYMV) is one example where traditional breeding cannot be used for improvement due to fertility barriers and genetic resistance being a poorly defined polygenic trait (Pinto et al., 1999). The RYMV open reading frame 2 (ORF2) was highly expressed in transgenic rice. The resultant RYMV resistant lines carried very low or non-detectable amounts of the ORF2 RNA transcript.

Conversely, transgenic lines that were susceptible had abundant amounts of the ORF2 transcript. Therefore the resistance phenotype was correlated with the loss of the viral transgene expression. This indicates that the mechanism of resistance was due to silencing of the ORF2 present as the transgene and in the RYMV RNA genome. The ORF2 sequence variation among different RYMV field isolates was found to be less than 10% at the nucleotide level suggesting that an RNA homology-dependent silencing approach may be effective in the field (Pinto et al., 1999).

Viral resistance utilizing endogenous silencing mechanisms is not restricted to using a single open reading frame from one virus. Two ORF fragments from different viruses can be fused into a chimeric expression cassette to confer resistance to both viruses. One clear example was generated from using tomato spotted wilt virus (TSWV) and turnip mosaic virus (TuMV) (Jan et al., 2000). The open reading frame for the *N* gene encoding the nucleocapsid from TSWV was fused to the coat protein (CP) of TuMV and the resulting chimeric construct was used to transform tobacco. As with the example using RYMV,

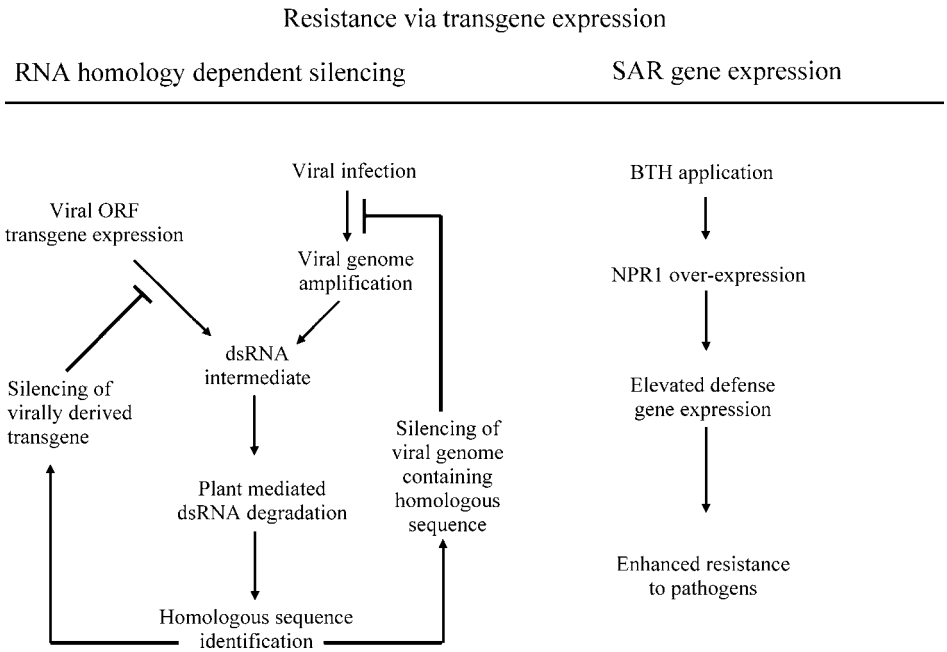

Figure 3. Transgene-mediated enhanced resistance. Two generalized examples of transgene expression are highlighted. RNA homology-dependent silencing requires the transgenic expression of a viral-derived sequence that forms a dsRNA intermediate. This intermediate is perceived by the plant and all sequences (plant or viral in origin) with significant homology to the transgene are silenced. *NPR1* over-expression alone enhances resistance to pathogens. When coupled with BTH application, *NPR1* over-expression may be a model for initiating resistance to diverse pathogens in the field.

resistance of the transgenic plants to both viruses corresponded with the loss of transcript accumulation from both viruses as detected by northern analysis. Transgenic plants susceptible to both viruses showed accumulation of the gene fragment transcript for both viruses. These two examples have been evaluated in greenhouse experiments; however, a well-described example of RNA homology-dependent silencing for viral resistance is presently being utilized successfully in the field.

Field successes for PTGS and crop protection

One clear commercial success of generating enhanced resistance by stable expression of a viral gene is against the papaya ringspot virus (PRSV). Papaya is grown throughout the tropics and subtropics and no natural resistance has been described for PRSV. A PRSV control strategy for the Hawaiian islands was developed using RNA homology-dependent silencing by expressing a mutated open reading frame for the coat protein (CP) from PRSV (Yeh et al., 1984; Fitch et al., 1992). Resistant transgenic plants were generated and were found to be devoid of the CP RNA indicating the RNA homology-dependent silencing of the plant-derived transgene and PRSV gene (Chiang et al., 2001). All PRSV strains present in Hawaii have been effectively controlled using silencing constructs derived from this mutant CP ORF. Sequence analysis demonstrated that these Hawaiian isolates had 97% or greater sequence homology to the mutant CP transgene. However, isolates of PRSV from outside of Hawaii can cause disease on the transgenic papaya lines. These geographically distinct isolates were found to have a lower sequence homology (89–94%) to the CP than the isolates from Hawaii. Thus, silencing of PRSV was contingent upon levels of sequence homology above 97% (Chiang et al., 2001). Interestingly, PRSV and RYMV require different levels of homology between transgene and the endogenous gene to induce silencing. The silencing in RYMV was successful for all variations tested (up to 10% divergence in nucleotide sequence) as compared with less than 3% divergence allowed for successful silencing in PRSV. Silencing is not only dependent upon the degree of homology but also the target sequence that is chosen. Much like the transgenic approach with R genes, each silencing construct must be carefully validated. Overall, RNA homology-dependant silencing has proven its utility in both the greenhouse and

the field, and appears to be among the most versatile mechanisms currently available to engineer resistance to viruses.

PTGS-mediated resistance to crown-gall disease

Crown-gall is a perennial problem in nurseries of fruit trees, nut trees and some bushy ornamental plants. Prevention of gall formation is a target for engineering resistance in these trees since breeding programs for resistance are not practical due to temporal considerations (e.g., decades). When replanted, the trunks suffer cuts that are an entry point for the bacterium *Agrobacterium tumefaciens*, the causal agent of the disease, and infection becomes apparent with the formation of galls. The bacterium causes disease by transforming the host cell with sets of oncogenes leading to uncontrolled cell division. These oncogenes encode biosynthetic genes for the production of plant hormones auxin and cytokinin. The endogenous plant genes and transferred oncogenes share no sequence homology making the bacterial genes an ideal target for RNA homology-dependent silencing (Zhao et al., 2001). Arabidopsis and tomato plants were transformed with constructs containing direct inverted repeats of the auxin and cytokinin oncogenes (Escobar et al., 2001). *In planta*, these tandem inverted repeats generate dsRNA molecules that in turn induce RNA homology-dependent silencing of the transformed *Agrobacterium* oncogenes. In the resulting transgenic tomato and Arabidopsis, *Agrobacterium*-mediated transformation was not prevented but the formation of galls (the result of uncontrolled cell division) by oncogene expression was abrogated completely. This was confirmed by the lack of detectable RNA from the bacterial oncogenes. The transgenic plants did not show any developmental phenotypic variation indicating that endogenous hormone production was not altered by the presence of the silencing construct (Figure 3).

Conclusions

For R gene-mediated resistance, gene pyramiding with marker assisted selection offers an improved method to confer resistance using endogenous signaling pathways. The manipulation of the components of these pathways to generate resistance also holds promise although alterations in the fine balance of expression may lead to undesired pleiotropic effects, such as cell death. Viral containment strategies using the endogenous RNA homology-dependent silencing machinery is highly adaptable and has proven successful in the field. These studies indicate that the combination of transgenic technologies with traditional breeding approaches can provide an excellent method for mitigating yield losses due to pathogens. The challenge clearly lies in the appropriate selection and incorporation of these strategies.

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