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NRS1, a nitrogen story, and the fuTORe of the Amino Sensing Pathway

By

Nanticha A Lutt

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

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in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jacob Brunkard, Co-Chair
Professor Sarah Hake, Co-Chair
Professor Roberto Zoncu
Professor Jennifer Fletcher

Summer 2022

Abstract

NRS1, a nitrogen story, and the fuTORe of the Amino Sensing Pathway

By
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Doctor of Philosophy in Plant Biology

University of California, Berkeley

Professor Jacob Brunkard, Co-Chair

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This work addresses three pictures of nitrogen and amino acid sensing within a plant. Much of this work focuses on the role of the kinase TARGET OF RAPAMYCIN (TOR). TOR is the focus of many researchers interested in metabolic diseases and cancers since TOR dysregulation is a major cause of such diseases. The role of TOR in plants is relatively unexplored, however, and questions surrounding its upstream cues and downstream targets persist.

TOR is a deeply conserved Ser/Thr protein kinase that integrates an array of metabolic processes to coordinate growth and development with nutritional status in eukaryotes. TOR is often dysregulated in many human diseases including diabetes and cancer, and as such, has been relatively well studied in mammalian systems. The TOR protein and complex are well-conserved across the eukaryotic kingdom; however, their role in plant growth and development is not as well understood. Recent work in this emerging field has shown that TOR plays a similar role in plants, serving as a metabolic rheostat modulating developmental processes in response to signals like light, sugars, and phosphorus abundance. In response, TOR regulates key pathways like nucleotide biosynthesis, ribosome biogenesis, and leaf initiation.

In chapter one, as well as outlining the field of TOR in plants, I will also introduce the nuances and complexities of sensors, transducers, and transporters in the world of nitrogen sensing, detailing what it takes to be considered a “sensor” in plant cell biology. The section that follows will outline the developments in autonomous nitrogen pathways and the small body of knowledge of nitrogen sensing by TOR in plants, and explain important contextual background for nutrient dependent TOR regulation. Here, I introduce *NRS1* in it’s starring role in this thesis. The Brunkard lab discovered a null 1 allele in a forward genetic screen for defective embryonic cell-cell (plasmodesmatal) trafficking that repeatedly identified mutants defective in TOR signaling (Brunkard et al 2020, Kim et al 2002), which led us to speculate that *NRS1* could be required for TOR activity in plants. Jake Brunkard used RNA-Seq to define the *NRS1* transcriptome (>850 differentially expressed genes using stringent cut-off parameters), which exhibits several signatures of TOR inactivation, including significant repression of ribosomal

protein genes and induction of proteolytic and catabolism-related genes (Busche et al 2021, Brunkard et al 2020, Xiong et al 2013). *NRS1* mutants and *NRS1* knockdowns also show significantly less phosphorylation of S6K-T449 (orthologous to human S6K-T389), a residue that is uniquely phosphorylated by the TOR kinase in plants. In all, this makes *NRS1* an attractive candidate for study in the TOR field.

In chapter two, I tell a nitrogen story. I detail a series of experiments aimed at teasing out TOR's role in sensing and responding to different exogenous forms of nitrogen. The first picture of my thesis engages the hypothesis that application of exogenous nitrogen has an impact on the TOR signaling pathway. Here, I will describe how nitrogenous nutrients stimulate TOR activity, with the hypothesis that TOR activity is promoted by the exogenous addition of nitrogenous nutrients when plants are grown under nitrogen limiting conditions. I established N-limiting growth conditions to determine the impact of nitrogenous nutrients on TOR activity, and tested nitrogen and amino acid addition in N-deprived plants to identify conditions that promote TOR activity. I found that the addition of nitrogenous nutrients and glucose to a nitrogenous nutrient limiting environment increases the phosphorylation of SK1, with glucose having less effect than the other nitrogen-rich substrates. The addition of asparagine, a nitrogen rich amino acid, has more impact than glucose, however, the addition of nitrate and ammonia to the substrate caused the most significant S6K1 phosphorylation, indicative of higher TOR activity. These findings suggest there must be some sensor that exists that modulates external nitrogen suppliance with TOR activity.

In chapter three, I dive within plant metabolism, with the goal of elucidating the impact of genetically manipulating endogenous levels of specific amino acids on TOR activity. Unlike animals, plants synthesize all 20 of the amino acids, and have complex metabolic systems to produce and transport these amino acids throughout the plant. One of the limitations of only investigating the exogenous application of nitrogenous nutrient sources and the impact on TOR activity via western blot, is that it is not possible to know all the possible downstream effects of plant amino acid uptake and biosynthesis when adding exogenous amino acids and nitrogenous nutrients. The experiments in the previous chapter may guide our understanding of TOR activity as it responds to external output, but will not be sufficient to conclude that a particular metabolite is shifting TOR activity without looking at the plant's endogenous metabolism. I cloned the suite of *ASN* genes present in *A. thaliana* (*ASN1, ASN2, ASN3*) and the *L- ASPARAGINASES* (*Aspga1, Aspgb1)*. *Arabidopsis thaliana* plants were transformed to stably or transiently overexpress these genes. My hypothesis was that if asparagine is the primary amino acid involved in activating TOR in plants, then it is predicted that overexpressing L-asparaginase will decrease TOR activity, as measured by S6K1- Thr449 phosphorylation. In plants, these genes can either be stably overexpressed in transgenic plants or they can be transiently overexpressed in leaves by infiltration with *Agrobacterium*. However, the results showed that manipulating the individual asparaginases did not have a significant impact on TOR activity, turning my focus to *NRS1*.

In chapter four, I put a singular focus on *NRS1* and its paralogs. The known cytosolic NRS genes in Arabidopsis thaliana are *NRS1*, *NRS2*, and a recently evolved paralogue of *NRS1*, which is called *NRS3*. A few insertional mutants are available for *NRS*3, but none have been characterized or investigated for phenotypes, and the Brunkard Lab has shown that null *NRS2* alleles exhibit no obvious phenotypes under normal growing conditions. Significantly, however, *NRS1* mutants are lethal at the mid-torpedo stage of embryogenesis, which suggests that *NRS1* has some nonredundant function that is not complemented by *NRS2* or *NRS3*. Therefore, *NRS1* is unique among aminoacyl tRNA transferases in plants because it is not strictly essential for cell survival, but it is required for plant development, even though there are two other NRS genes in Arabidopsis thaliana. In combination with a previous experiment, I agroinfiltrated plants with the three aminoacyl tRNA synthetase constructs. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf samples were collected and TOR activity was assayed. As anticipated, overexpressing *NRS1* has a significant impact on S6K1 phosphorylation.

Finally, in chapter five, I embarked on a journey to tease out the molecular mechanisms of *NRS1* and TOR activity. This chapter proposes to build on the discovery that *NRS1* stimulates TOR activity by mechanistically defining how *NRS1* activates TOR at a molecular level. I disrupted key functions of *NRS1* as a tRNA synthetase and deleted the WHEP domain to establish the domains of *NRS1* necessary to activate TOR. I cloned truncated versions of *NRS1* to exclude the WHEP domain, i.e., *NRS1*ΔWHEP. This truncated *NRS1*ΔWHEP over-expression construct was compared against the Wt-*NRS1* construct in western blot assays. The WHEP domain of WARS, for example, acts like a molecular “bridge”, interacting with both the phosphatidylinositol kinase-like kinase (PIKK) DNA-PKcs and the poly[ADP-ribose] (PAR) polymerase 1 (PARP1) to facilitate PARylation of DNA- PKcs and subsequent activation of the tumor suppressor p53 (Guo et al, 2010). Thus, there exists a model in humans where the WHEP domain mediates interaction between a tRNA synthetase and a protein kinase.

Dedication

For my family, chosen and blood
and for the lengths in which I have traveled to find you



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Chapter One

Sensors and Transducers and Transporters

“I'm a flower, you're my bee,
It's much older than you and me.”
Father John Misty



Section 1.1

Amino acid signaling for TOR in eukaryotes: sensors, transducers, and a sustainable agricultural fuTORe

The following chapter is a modified version of an article published in *Biomolecules* (Lutt and Brunkard, 2022).

Abstract

Eukaryotic cells monitor and regulate metabolism through the atypical protein kinase TARGET OF RAPAMYCIN (TOR) regulatory hub. TOR is activated by amino acids in animals and fungi through molecular signaling pathways that have been extensively defined in the past ten years. Very recently, several studies have revealed that TOR is also acutely responsive to amino acid metabolism in plants, but the mechanisms of amino acid sensing are not yet established. Here, we will review these discoveries, emphasizing the diversity of amino acid sensors in human cells and highlighting pathways that are indirectly sensitive to amino acids, i.e., how TOR can monitor changes in amino acid availability without a bona fide amino acid sensor. We will then discuss the relevance of these model discoveries to plant biology. Since plants can synthesize all proteinogenic amino acids from inorganic precursors, we will focus especially on the possibility that TOR senses both organic metabolites and inorganic nutrients. We conclude that an evolutionary perspective on nutrient sensing by TOR will ultimately benefit both agricultural and biomedical sciences, contributing to ongoing efforts to generate crops for a sustainable agricultural future.

Introduction

TARGET OF RAPAMYCIN (TOR) is a serine/threonine kinase that senses environmental cues, especially nutrient availability, to coordinate eukaryotic cellular metabolism (Shi *et al.*, 2018; Valvezan and Manning, 2019; Liu and Sabatini, 2020; Brunkard, 2020; Burkart and Brandizzi, 2021). TOR supports growth by activating anabolic processes, such as mRNA translation (Thomas and Hall, 1997; Thoreen *et al.*, 2012; Meyuhas and Kahan, 2015; Scarpin *et al.*, 2020), nucleotide biosynthesis (Ben-Sahra *et al.*, 2013; Robitaille *et al.*, 2013; Valvezan *et al.*, 2017; Busche *et al.*, 2021), and lipid biosynthesis (Laplante and Sabatini, 2009; Düvel *et al.*, 2010; Thelen and Zoncu, 2017), while inhibiting catabolic processes, such as autophagy (Kamada *et al.*, 2000; Liu and Bassham, 2010; Kim *et al.*, 2011; Noda, 2017; Kazibwe *et al.*, 2020). Early investigations found that TOR is especially responsive to amino acid signals. Free amino acids stimulate TOR, which then increases the global rate of protein synthesis to metabolize those amino acids (Thomas and Hall, 1997; Masvidal *et al.*, 2017; Wolfson and Sabatini, 2017), thus maintaining metabolic homeostasis while promoting growth and development. Until recently, however, the stimulatory effect of amino acids on TOR had only been studied in heterotrophs, especially yeast, invertebrates, and mammals, that rely on dietary sources for the full suite of 20 proteinogenic amino acids. Unlike these model species, plants are autotrophs that can synthesize all proteinogenic amino acids from inorganic precursors (Binder, 2010; Maeda and Dudareva, 2012; Xu et al., 2012). Multiple studies have firmly established that TOR is also responsive to amino acid signals in autotrophic plants (Cao et al., 2019; Schaufelberger et al., 2019; O'Leary et al., 2020; Liu et al., 2021), but the precise amino acids sensed by TOR and the molecular mechanisms of amino acid sensing in plants remain unknown.

Here, we will summarize the current understanding of the molecular pathways of amino acid sensing by TOR in mammalian, invertebrate, and yeast models, discuss the recent literature on amino acid sensing by TOR in plants, address the possibility that TOR might respond to both inorganic and organic nitrogenous compounds in plant cells, and propose that a deeper understanding of nitrogen-TOR signaling is urgently needed to minimize reliance on chemical fertilizers for a sustainable agricultural future. We argue that a comparative, evolutionary perspective on nutrient sensing by TOR will ultimately benefit both agricultural and biomedical sciences, and we highlight how ongoing studies of TOR signaling in plants and algae can contribute to these fields.

Sensors and transducers in metabolic signaling

Currently, multiple amino acid sensors are proposed to regulate TOR activity in humans (Wolfson and Sabatini, 2017; Vellai, 2021). The relative significance of these sensors and their precise mechanisms of signal transduction are under debate, however. Before proceeding, therefore, we will begin by defining several terms in the field of metabolic signal transduction that will be useful for understanding the amino acid-TOR network. A "sensor" protein responds directly to an environmental cue, e.g., by binding to a metabolite that alters the protein's activity or by undergoing a structural change triggered by light or temperature. A classical example of a sensor from plant biology is

phytochrome, which reversibly changes conformations in response to red or far-red light (Ulijasz et al., 2010). Sensors then engage "transducer" proteins, whether indirectly or through direct protein-protein interactions, which may act on additional transducers to create a chain of signaling events in a pathway, or which may activate the response to a cue. Antibodies, ligand-binding proteins, and hormone receptors are all examples of sensors (A.R. Webb et al., 2017); mitogen-activated protein (MAP) kinases, G-proteins, and TOR itself are examples of transducers.

The distinction between "sensor" and "transducer" is crucial for understanding nitrogen- and amino acid-responsive signaling networks in eukaryotic cells. The debates over the function of GCN2 (General Control Non-derepressible 2), for example, revolve around whether GCN2 is a "sensor" or a "transducer". GCN2 is a serine/threonine kinase that is activated when cells experience extreme stress (Dever et al., 1992). Among other substrates, GCN2 phosphorylates the eukaryotic translation initiation factor eIF2α (at serine 51 in human eIF2α, which is orthologous to serine 56 in Arabidopsis eIF2α), which globally represses translation of most mRNAs while selectively upregulating translation reinitiation of transcripts that encode small, upstream open reading frames (uORFs) in addition to the primary ORF (Tzamarias and Thireos, 1988; Lageix et al., 2008; Zhang et al., 2008; Chikashige et al., 2020). Transcripts with uORFs encode various stress-inducible and starvation response transcription factors (Costa-Mattioli and Walter, 2020). Thus, when GCN2 is active, gene expression is regulated translationally and transcriptionally to promote stress responses.

GCN2 is often misleadingly called an "amino acid sensor", since GCN2 is rapidly activated in response to amino acid deprivation and mediates starvation responses in diverse eukaryotes (Li et al., 2013; Costa-Mattioli and Walter, 2020). Early studies confirmed that GCN2 does not directly sense amino acids, but did hypothesize that GCN2 senses uncharged tRNAs (i.e., tRNAs that have not been ligated with an amino acid by an aminoacyl-tRNA synthetase, presumably due to amino acid deprivation), and that physical interaction with uncharged tRNAs allosterically activates GCN2 (Wek et al., 1989, 1995; Dong et al., 2000; Li et al., 2013). Recent studies have upended this straightforward model, proposing instead that GCN2 senses translational stress through alternative mechanisms, such as by directly binding the exposed phosphoprotein (P)-stalk of stalled mRNA-bound ribosomes (Inglis et al., 2019; Harding et al., 2019) or by acting as a transducer downstream of other proteins that associate with stalled ribosomes (Wu et al., 2020; Pochopien et al., 2021). While the distinction among directly sensing uncharged tRNAs, directly sensing exposed P-stalks, or acting as a transducer for other sensors of translational stress may at first seem esoteric, these distinctions are critical for understanding human diseases (Mellado and Willis, 2021). In mouse, Drosophila, and human cell models of Charcot-Marie-Tooth (CMT) disease, a genetic disorder that causes peripheral neuropathies, tRNA synthetases carry partially dysfunctional mutations that limit synthesis of charged tRNAs, leading to translational stress and constitutive activation of GCN2 (Mellado and Willis, 2021; Spaulding et al., 2021; Zuko et al., 2021; Mendonsa et al., 2021). Strikingly, overexpressing tRNA genes rescues CMT symptoms and suppresses GCN2, apparently by increasing levels of both

charged tRNAs and uncharged tRNAs (Zuko et al., 2021; Mendonsa et al., 2021). This elegant experiment in a disease model directly demonstrates that accumulation of uncharged tRNAs is not sufficient to activate GCN2 in cells. Therefore, structural, biochemical, and in vivo experiments all indicate that GCN2 is not an amino acid sensor, nor an uncharged tRNA sensor, but most likely a sensor or transducer of translational stress per se in eukaryotic cells (Brunkard and Baker, 2018; Wu et al., 2020; Pochopien et al., 2021).

The growing chorus of amino acid sensors and transducers for mammalian TOR
From an evolutionary perspective, sensors could evolve through one of two general mechanisms: exaptation or adaptation (Brunkard, 2020). In the first case, a sensor could evolve from a protein that already interacted with the cue it senses (e.g., an enzyme and its substrate), later evolving to interact with a transducer that connects to a downstream signal transduction pathway. This situation is called “exaptation” (Gould and Vrba, 1982) because both the transduction pathway and the sensor already existed in cells, and they were coopted to create a new pathway that conferred some fitness benefit. When the sensor retains its ancestral function in addition to its new, exaptive role as a sensor, the sensor is often called a “moonlighting protein”. In the second case, an existing transducer or sensor could evolve the capacity to sense a new cue. This situation is called “adaptation” because the sensor did not already interact with a cue, but arose de novo through mutation of a previously non-sensor protein and conferred some fitness benefit. The distinction between exaptation and adaptation can be useful for understanding the evolutionary origins and diversity of amino acid sensors in eukaryotic cells.

With these definitions in mind, we will first present the major proposed amino acid sensors for TOR, and then discuss their proposed transducers that mediate the signaling pathway to TOR. In mammals, TOR is especially responsive to levels of the essential amino acid leucine (Leu) and the conditionally essential amino acid arginine (Arg) (Ban et al., 2004; Stipanuk, 2008). Leucyl tRNA synthetase (LARS) was the first proposed Leu sensor for TOR, identified through a protein-protein interaction screen for potential amino acid sensors in yeast (Bonfils et al., 2012) and through co-localization experiments in mammalian cells (Han et al., 2012). SLC38A9, an amino acid transporter localized to the membranes of lysosomes (lytic vacuoles), was the first proposed Arg sensor for TOR, identified through a protein-protein interaction screen for potential amino acid sensors in mammals (Wang et al., 2015). Both of these proposed sensors are examples of exaptation, since their ancestral functions already involved direct interactions with Leu and Arg, respectively, at a molecular level.

Protein-protein interaction screens later identified additional amino acid sensors for TOR in mammalian cells, including Leu sensors Sestrin1/2 (Saxton et al., 2016b; Wolfson et al., 2016) and Sar1b (Chen et al., 2021), Arg sensor Castor1 (Chantranupong et al., 2016; Saxton et al., 2016a), and a sensor of the methionine derivative S-adenosyl methionine (SAM) called SAMTOR (Gu et al., 2017). Sestrins have an ancestral role as transducers of stress signals to TOR (Lee et al., 2016;

Brunkard, 2020): Sestrin genes are transcriptionally activated by stress-responsive transcription factors, including p53 and the GCN2-stimulated ACTIVATING TRANSCRIPTION FACTOR 4 (ATF4). Very recently in the mammalian lineage, some Sestrins evolved a Leu-binding pocket that induces a conformational change to prevent their role in transducing stress signals to TOR. Even in humans, not all Sestrin paralogues interact with Leu (for example, Sestrin3 is completely Leu-insensitive), and Sestrin orthologues outside of mammals do not include a Leu-binding site, suggesting that the Leu sensor function of Sestrin1/2 is a recent adaptation and that Sestrin genes may be subfunctionalizing in the human lineage (Lee et al., 2016). Sar1b is a small GTPase in the Arf family that is conserved across eukaryotes and participates in endomembrane trafficking. Human and C. elegans Sar1b orthologues directly bind to Leu and signal Leu levels to TOR through interaction with transducers (Chen et al., 2021). Whether Sar1b orthologues in other lineages can bind to Leu is not established, but presumably the role of Sar1b as a Leu sensor is another example of adaptation. The evolutionary history of Castor1 and SAMTOR is less clear; Castor1 encodes two ACT domains, which are found in various metabolite-binding proteins and enzymes (Lang et al., 2014), and SAMTOR likely evolved from a SAM-dependent methyltransferase or may itself be a functional SAM-dependent methyltransferase enzyme (Gu et al., 2017). Neither gene has readily identifiable orthologues outside of vertebrates, suggesting that these sensors evolved very recently in the human lineage.

The transducers of amino acid signals to TOR were largely identified before the amino acid sensors, again primarily through screens for protein interactors of TOR and associated proteins, but also through genetic screens for regulators of TOR activity. The Rag family of small GTPases form a complex with regulatory proteins called the “Ragulator” that form a platform for the activation of TOR at the surface of lysosomes (Sancak et al., 2010). These Rag GTPases are regulated by the “GAP Activity TOwards the Rags 1” (GATOR1) complex, which stimulates hydrolysis of RagA/RagB-bound GTP to negatively regulate TOR (Bar-Peled et al., 2013; Panchaud et al., 2013; Shen et al., 2018). GATOR1, in turn, is negatively regulated by another multiprotein complex, GATOR2. GATOR2 transduces signals from Sestrins, Castor1, and Sar1b; GATOR1 transduces these signals plus signals from SAMTOR; and the Ragulator complex transduces all these signals plus signals from SLC38A9 and LARS (Figure 1.2).

Multiple groups have proposed that the diversity of amino acid sensors in mammalian cells reflects functional differences, rather than simple redundancy (Wolfson and Sabatini, 2017; Chen et al., 2021). The proposed sensors have distinct binding affinities, subcellular localizations, and expression profiles, and sensors can intersect with distinct transducers upstream of TOR, which conceivably allows the various sensors to coordinately act on TOR like “rheostats” that fine-tune TOR activity in response to dynamic metabolic conditions (Valvezan and Manning, 2019). To illustrate, Castor1 and SLC38A9 are localized to the cytosol and lysosome, respectively, responding to different subcellular pools of Arg (Rebsamen et al., 2015; Wang et al., 2015; Chantranupong et al., 2016; Saxton et al., 2016a). As another example, Sestrin1, Sestrin2, and Sar1b each bind specifically to Leu in the cytosol, but with high, moderate,

and low affinities (Wolfson et al., 2016; Chen et al., 2021). Indeed, in cells that express both Sestrins and Sar1b, Sestrins engage with GATOR2 and only partially suppress TOR activity when Leu levels are moderately low, but both Sar1b and Sestrins engage with GATOR2 and completely inactivate TOR when Leu is extremely scarce or absent (Chen et al., 2021).

Beyond leucine and arginine, many other amino acids can stimulate TOR, and the potency of TOR activation by specific amino acids varies across biological contexts (e.g., cell type, species, experimental conditions, etc.). Current evidence indicates that some of these amino acid sensitivities are mediated by the GATOR2-GATOR1-Ragulator transduction pathway, but others act independently. For example, glutamine and asparagine can activate TOR through Ragulator-independent mechanisms (Meng et al., 2020; Hesketh et al., 2020). The details of how glutamine and asparagine activate TOR are still under investigation, but several studies have pointed to a role for another small GTPase, Arf1, in transducing glutamine and asparagine signals to TOR (Jewell et al., 2015). Alternative (but not mutually exclusive) pathways have been proposed, including that glutamine drives synthesis of α-ketoglutarate (αKG, also known as 2-oxoglutarate or 2OG) via glutaminolysis, and that αKG promotes TOR activity in Ragulator-dependent mechanisms (Bodineau et al., 2021); or, that glutamine and asparagine elevate ATP/AMP ratios via asparagine synthase and the GABA shunt, which in turn inhibits the TOR-antagonizing AMP-activated kinase (AMPK) (Bodineau et al., 2021). Therefore, glutamine and asparagine might activate TOR indirectly, without any glutamine- or asparagine-specific sensor proteins in cells.

Even Leu may induce TOR indirectly, without a bona fide Leu sensor, under some circumstances. In cells that do not strongly express Sestrins, including HeLa cells, Leu instead stimulates TOR through a downstream metabolite, acetyl coenzyme A (AcCoA) (Son et al., 2019, 2020). AcCoA is synthesized in human cells from pyruvate, fatty acid, or branched chain amino acid (especially Leu) precursors (Mariño et al., 2014). Under Leu deprivation, AcCoA levels temporarily decrease, and can be restored by resupplying Leu or, eventually, by compensatory synthesis from the other precursors (Son et al., 2019). AcCoA is mobilized in the cytosol to acetylate the TOR-associated protein RAPTOR, which effectively increases TOR activity in cells; RAPTOR acetylation correlates tightly with cytosolic AcCoA concentrations, and thus acts as an AcCoA sensor (Son et al., 2020). Crucially, knocking down Sestrin1/2 or LARS does not prevent Leu from activating TOR in some cell types (e.g., HeLa cells), but knocking down the enzymes that metabolize Leu to produce AcCoA makes TOR unresponsive to Leu supply (Son et al., 2019, 2020). This discovery illustrates the complexity of amino acid signaling in cells and further highlights how TOR can integrate multiple dynamic cues as the hub of a signal transduction network.

Plant nutrient sensing: from inorganic precursors to organic metabolites

Most investigations relevant to plant amino acid signaling have focused on how upstream precursors are sensed by plant cells, including inorganic nutrients absorbed from the soil (e.g., nitrate, ammonium, and sulfate) and photosynthesis-related cues

(carbon dioxide, light, and sugars). To illustrate, the best-studied sensor of nitrogenous nutrients in plant cells is the plasma membrane-localized NITRATE TRANSPORTER 1.1 (NRT1.1, often called CHLORINA 1 or CHL1), a transceptor that alters nuclear gene expression when it detects environmental nitrate via a calcium-dependent protein kinase signaling cascade (Ho et al., 2009; Liu et al., 2017, 2020). Pioneering studies of plant TOR signaling focused on how TOR reacts to photosynthesized sugars rather than amino acids. In plant cells, sugars are directly sensed by proteins including hexokinase (HXK1) and the SNF1-related protein kinase (SnRK1, orthologous to human AMP-activated kinase, AMPK, but not sensitive to AMP or ATP) (Sheen, 2014). HXK1 has evolved an exaptive signaling role in addition to its critical metabolic function in glycolysis, signaling sugar availability in response to direct interaction with glucose (Frommer et al., 2003; Moore et al., 2003; Li et al., 2021). SnRK1 phosphorylates proteins to promote stress and starvation responses, but is repressed by a proposed direct interaction with trehalose-6-phosphate, an intermediate in sugar metabolism (Baena-González et al., 2007; Zhang et al., 2009; Zhai et al., 2018; Baena-González and Lunn, 2020). Although TOR activity is likely regulated by SnRK1 (Nukarinen et al., 2016), sugar activation of TOR also requires glycolysis and oxidative phosphorylation (Xiong et al., 2013; Riegler et al., 2021), suggesting that TOR responds primarily to ATP levels rather than sugars directly. This may be analogous to the mammalian model: TOR senses glycolytic intermediates (dihydroxyacetone phosphate), but only in cells that lack ATP sensing pathways (Orozco et al., 2020). Although no ATP sensors have been established in plant cells, a proposed ATP sensor for mammalian TOR, the co-chaperone R2TP ATPase complex, is conserved in plants and regulates TOR activity, hinting that R2TP may be an ATP sensor in plant cells (Brunkard et al., 2020; Chatterjee et al., 2021).

Recently, several forward genetic screens have refocused attention on how plants sense organic nitrogenous nutrients, especially amino acids and nucleotides. Two in-dependent genetic screens for Arabidopsis thaliana mutants that disrupt cellular patterning identified recessive alleles of ISOPROPYL MALATE SYNTHASE 1 (IPMS1), which en-codes an enzyme in the leucine biosynthetic pathway (Cao et al., 2019; Schaufelberger et al., 2019). ipms1 mutants display defects in cytoskeletal organization and leaf shape during early seedling development (Cao et al., 2019), and ipms1 also rescues the root hair abnormalities observed in leucine-rich receptor / extensin 1 (lrx1) mutants (Schaufelberger et al., 2019). Moreover, ipms1 mutants exhibit elevated TOR activity, can be partially phenotypically rescued by very low concentrations of TOR inhibitors, and are resistant to the growth-inhibiting effects of moderate concentrations of TOR inhibitors (Cao et al., 2019). Therefore, the effects of ipms1 on amino acid metabolism are somehow transduced to TOR, and this is likely the primary cause of ipms1 cellular/developmental phenotypes. Separately, a functional genetic screen for *Nicotiana benthamiana* genes that regulate TOR activity identified PHOSPHORIBOSYL PYROPHOSPHATE SYNTHETASE 4 (PRS4), which encodes an enzyme required for nucleotide biosynthesis in plant cells (Busche et al., 2021). Subsequent experiments using diverse approaches confirmed that plant TOR senses purine and pyrimidine availability, analogous to the recent discovery that mammalian TOR also monitors

nucleotide levels (Kazibwe et al., 2020; Busche et al., 2021). Thus, plant TOR reacts to disruptions in organic nutrient levels and biosynthetic path-ways, but the sensors and transducers involved remain enigmatic.

Beyond plants, the intersecting roles of nutrient sensing, amino acid metabolism, and TOR signaling in distantly-related microalgae, such as the chlorophyte Chlamydomonas reinhardtii, are currently under investigation by multiple groups. Several algae are excellent sources of triacylglycerols (TAGs) that could serve as biofuel feedstocks for a sustainable energy future (Takeuchi and Benning, 2019), and a series of studies demonstrated that inhibiting TOR is sufficient to significantly induce TAG biogenesis in candidate biofuel species, including C. reinhardtii, A. thaliana, and non-green algae, Cyanidioschyzon merolae and Phaeodactylum tricornutum (Caldana et al., 2013; Imamura et al., 2015, 2016; Mukaida et al., 2016; Prioretti et al., 2017). Alongside TAG accumulation, inhibiting TOR rapidly increases amino acid levels in C. reinhardtii cells (Jüppner et al., 2018). Detailed metabolomic investigations of the origin of these amino acids led to the unexpected discovery that amino acid accumulation upon TOR inhibition in C. reinhardtii is not primarily due to suppression of mRNA translation or autophagic recycling of proteins, but instead due to drastically elevated inorganic nitrogen uptake from the environment and subsequent de novo amino acid biosynthesis (Mubeen et al., 2018). Since TOR activity is stimulated by nitrogen and carbon sources in C. reinhardtii cells (Upadhyaya et al., 2020), a possible model is that TOR monitors intracellular nutrient status (whether inorganic nutrients or organic forms, like amino acids) to regulate ex-tracellular nutrient uptake and maintain carbon/nitrogen balance (Jüppner et al., 2018; Mubeen et al., 2018; Upadhyaya et al., 2020). A deeper understanding of TOR signaling and nutrient sensing in algae will help to illuminate how this signaling network evolved in eukaryotes, with likely practical benefits for the biofuel industry.

A major outstanding question in plant metabolic signaling is whether plant cells encode true amino acid sensors analogous to the mammalian amino acid sensors for TOR described above. Addressing this question, multiple groups have explored whether specific amino acids can activate TOR in plants. Amino acid profiles of ipms1 mutants, which show constitutively elevated TOR activity, revealed altered levels of almost every amino acid except for Lys and Met (Cao et al., 2019). The relative potency of TOR activation by amino acids was not clearly resolved by experiments using other mutants in the branched-chain amino acid biosynthetic pathway (Cao et al., 2019). In leaf discs floated on solutions containing either Ile or Gln at night, TOR activity is rapidly induced (O'Leary et al., 2020); no amino acids were conclusively shown to be incapable of activating TOR, but Gln did have a stronger effect on TOR activity than Ile (O'Leary et al., 2020). In *Arabidopsis* seedlings grown for 9 days without any source of nitrogen, TOR becomes in-active and growth arrests. Supplying most amino acids to these nitrogen-starved seedlings activated TOR within minutes, except for arginine, proline, and the aromatic amino acids phenylalanine, tryptophan, and tyrosine (Liu et al., 2021). Nitrate and ammonium could also activate TOR in this experimental system, even in the presence of tungstate (a nitrate reductase inhibitor) or methionine sulfoximine (a

glutamine synthetase inhibitor), which prevent the assimilation of nitrogen into amino acids (Liu et al., 2021). This could suggest that TOR specifically senses inorganic forms of nitrogen in plants and that amino acids supplied to N-deprived seedlings are catabolized to yield inorganic nitrogen forms. Alternatively, plant TOR could monitor multiple nitrogenous cues, including inorganic nitrogen and diverse amino acids, analogous to the diversity of amino acids and amino acid-related metabolites sensed by mammalian TOR. To summarize, although current data do not resolve whether plant cells directly sense amino acid cues, TOR does dynamically react to changes in amino acid metabolism.

The fuTORe of crops: deploying TOR for a sustainable agricultural future

Modern agriculture relies heavily on inorganic fertilizers to drive growth and increase yields (Tilman, 1998; Stewart et al., 2005). Fertilizers are primarily composed of ammonias to provide nitrogen (e.g., ammonium nitrate), phosphate rock to provide phosphorus, and potash to provide potassium. Crops use fertilizers inefficiently, however, resulting in significant fertilizer run-off that is environmentally disruptive (Veneklaas et al., 2012; Xu et al., 2012). Moreover, fertilizers are nonrenewable resources: potash and phosphate rock are both ores mined from limited underground sources. Estimates vary, but most studies agree that both potash and phosphate production will peak in the 21st century, reducing fertilizer availability even as agriculture faces other pressures from changing climates, dwindling arable land, and growing global populations (Cordell and White, 2011; Springmann et al., 2018). Therefore, a major goal of plant biology in the 21st century is to reduce reliance on external fertilizer sources and maximize nutrient use efficiency in diverse, resilient crop species.

We argue that a detailed mechanistic understanding of the TOR signaling network in plants will be critical for these efforts. By defining the nutrient sensors and signaling network transducers that act upstream of TOR, alongside the phosphoprotein effectors and downstream processes engaged by TOR, we expect to discover new targets for breeding and/or biotechnological interventions to improve plant nutrient use efficiency. In particular, whereas TOR signaling networks evolved to maintain homeostasis for plants that experience unpredictable, fluctuating environments in ecological competition with other plant species, domesticated crops do not face these same fitness costs and selective pressures. Ongoing investigations of TOR signaling in model systems, including Arabidopsis, will illuminate new targets for genetic modification, contributing to the larger project of establishing a sustainable global agricultural program.

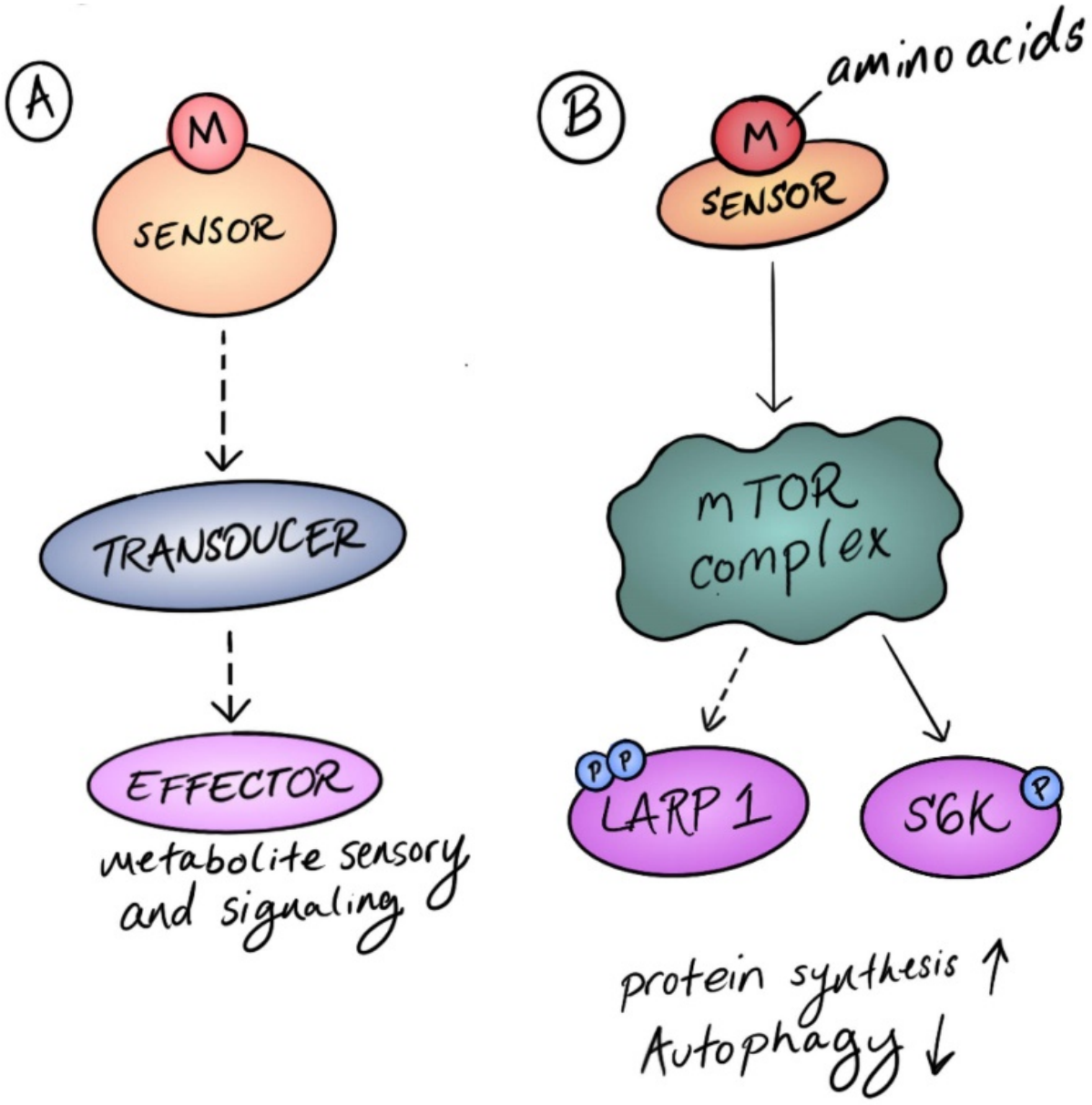


Figure 1.1 Sensors and transducers in metabolic signaling.
(A) Metabolic signaling is triggered by a metabolite sensor protein that directly binds to a metabolite. The sensor then engages transducers in a signaling pathway that eventually activate responses through an effector protein.
(B) In mammalian models, amino acids are sensed directly by diverse sensor proteins that activate the mechanistic TARGET OF RAPAMYCIN (mTOR), a central regulatory hub that transduces diverse upstream signals to coordinate metabolism. mTOR then phosphorylates additional transducers and effectors, such as LARP1 and S6K, to promote protein synthesis and repress autophagy.

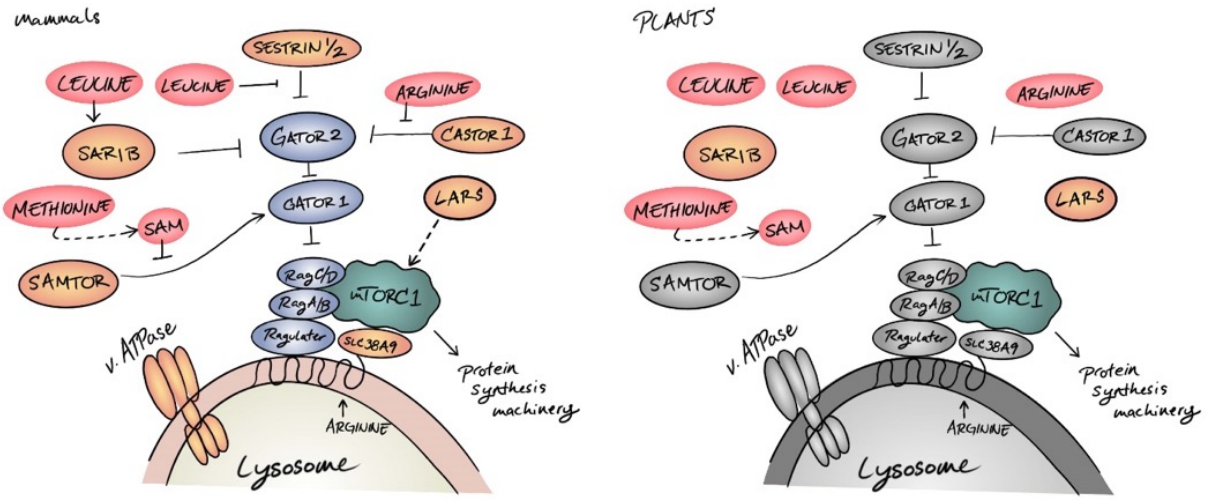


Figure 1.2 Amino acid signaling in eukaryotes.
In mammals (left panel), amino acids (red) are monitored by multiple sensors (orange) that engage transducers (blue) at multiple steps in in the GATOR2-GATOR1-Ragulator-Rag GTPase signaling cascade to coordinate TOR (teal) activity at the surface of the lysosome. Very few of these signaling components are conserved in plants (right panel; gray indicates not conserved to plants). Note that Sar1b and LARS, which have essential roles in membrane trafficking and tRNA synthesis, respectively, have been exapted as amino acid sensors in the animal lineage, and therefore may not act as amino acid sensors in plants.

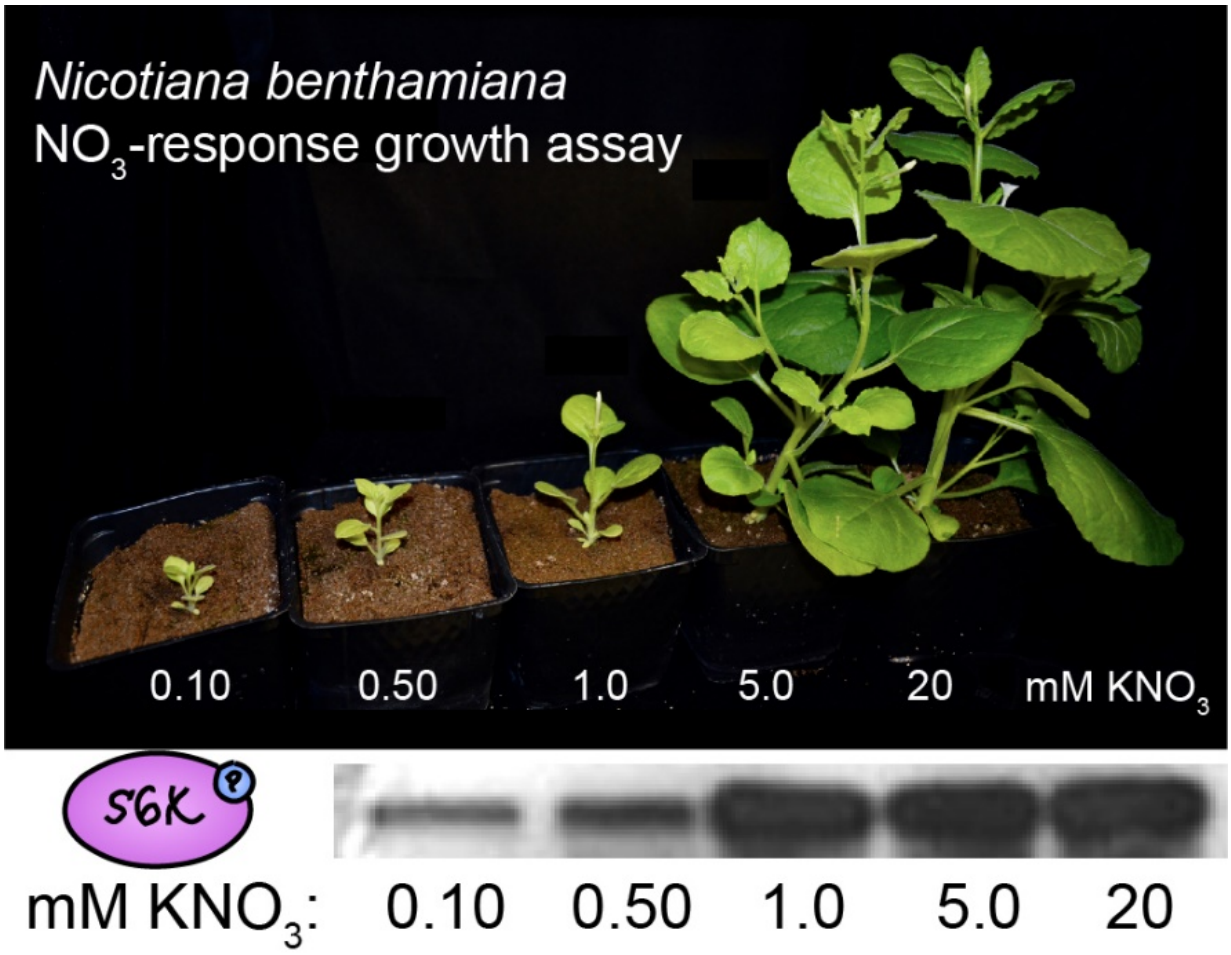


Figure 1.3 TOR monitors nitrogenous nutrient availability in plants. Plants can synthesize all 20 proteinogenic amino acids from inorganic precursors (nitrate or ammonium, carbon dioxide, and sulfate).
Here, *N. benthamiana* were grown on calcined clay and supplied with standard nutrients except for nitrogen, which was supplied as potassium nitrate at the indicated concentrations. Nitrate strongly stimulated plant growth and activated TOR, as measured by Western blots using phosphospecific antibodies against the canonical TOR substrate, S6K-pT449

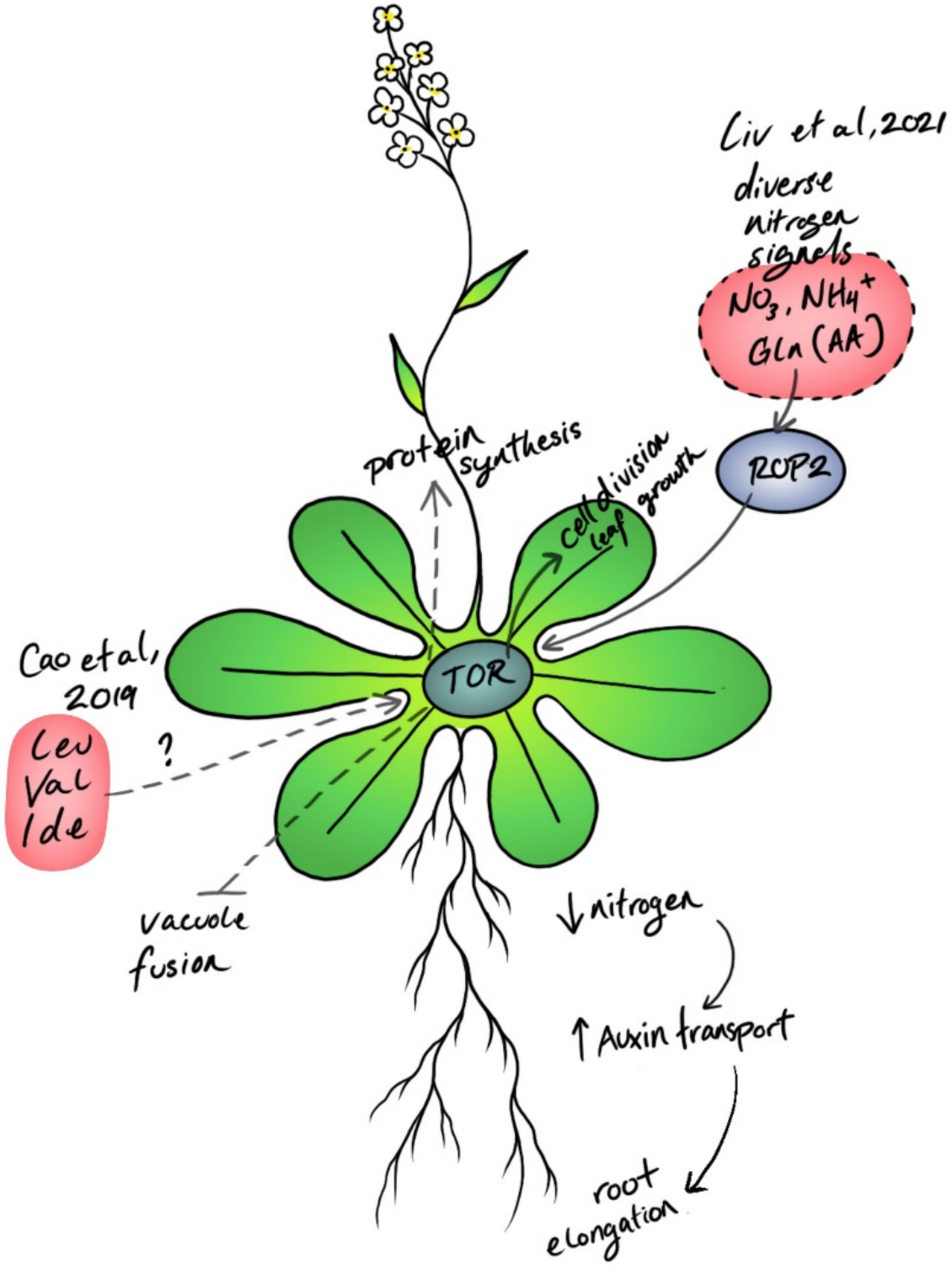


Figure 1.4 TOR responds to nitrogen and amino acid metabolism in plants to coordinate growth and development, protein synthesis, and cytoskeletal function

Section 1.2 What is the TOR Kinase and how does it (maybe) signal in plants

TOR kinase

All organisms must respond to changing conditions as they grow and develop. Most organisms utilize a sensing pathway to effectively respond to these changes. One mechanism by which eukaryotes interpret and integrate cues like nutrient availability, energy, hormones and stress to dynamically modulate growth and development is the TARGET OF RAPAMYCIN (TOR) protein kinase pathway. TOR is a phosphatidylinositol 3-kinase (PI3K) related kinase (PIKK) that integrates these signals. When TOR is active, promotes growth, anabolism, and cell proliferation, while inhibiting processes like autophagy and quiescence (Saxton and Sabatini, 2017).

The TOR protein is incredibly well conserved among eukaryotes, despite the evolutionary distance and dramatic differences in cellular physiology. the Arabidopsis thaliana and Homo sapiens TOR proteins share over 40% sequence identity with 75% sequence similarity in the protein kinase domain (Xiong and Sheen, 2015). While TOR is highly conserved across eukaryotes, its interacting partners have diversified in different lineages. In metazoans and yeast, TOR serves as the catalytic subunit of two structurally and functionally distinct protein complexes: the TOR complex 1 (TORC1) and the TOR complex 2 (TORC2). Phylogenetic analyses supported by in vivo protein interaction experiments have confirmed that the TORC1 components are somewhat conserved in plants. In the model species Arabidopsis thaliana, orthologs of REGULATORY ASSOCIATED PROTEIN OF TOR (RAPTOR), which helps recruit TOR substrates and is necessary for proper TORC1 subcellular localization, and LETHAL WITH SEC THIRTEEN 8 (LST8), which may aid in stabilization mTORC1 stability, have been identified. These three proteins dimerize to form a complex with two TOR subunits, two RAPTOR subunits, and two LST8 subunits (Figure 1.1). To date, a homologous TORC2 has not been identified in plant lineages (Nojima et al., 2003, Schalm et al., 2003, Yang et al., 2013, Mahfourz et al. 2006, Díaz-Troya et al., 2008). For this reason, only TORC1 will be discussed in this text and mentions of “TOR” refer to its function in TORC1.

As the name suggests, TORC1 is inhibited by the antibiotic rapamycin, a compound first isolated from the bacterium *Streptomyces hyprospopicus.* Rapamycin works by forming a inhibitory complex with FK506-binding protein 12 (FKBP12) leading to formation of noncovalent bonds between FKBP12 and the FKPB12-rapamycin binding (FRP) domain of the TOR protein. This prevents FKBP12 from disassociating from TORC1, potentially resulting in the disassociation of RAPTOR from TORC1 or preventing certain substrates from interacting with the complex. (Oshiro et al. 2004, Yip et al. 2010). Several ATP- competitive inhibitors exist as well that have proven to be more effective at inhibiting TOR than rapamycin. AZD-8055, Torin1, Torin2 specifically bind the TOR active site allowing for the targeted inhibition of TORC1 and TORC2 simultaneously (Chresta et al. 2010; Thoreen et al. 2009; Liu et al. 2013).

When TOR is active, there is a shift in cellular metabolism towards anabolic growth. This shift leads to the consumption of cellular resources. Thus, many of the upstream activators of TOR are the substrates that are consumed during anabolism, such as Glucose, energy (in the form of ATP), nucleotides, and amino acids have all been shown to promote TOR activity (Figure 1.2) (Emmanuel et al., 2017; Hoxhaj et al., 2017; Saxton and Sabatini, 2017). In plants, some of these molecules have been shown to promote TOR activity, but the specifics of how their abundance is sensed and how TOR is subsequently stimulates are still poorly understood in comparison to other organisms.

TOR responds to changes in upstream cues by phosphorylating several targets that affect fundamental processes including transcription, translation, metabolism, and autophagy to ultimately control cell growth and proliferation as well as organismal development (Xiong and Sheen, 2014, 2015; Dobrenel et al., 2016; Saxton and Sabatini, 2017; Schepetilnikov and Ryabova, 2018; Shi et al., 2018).

Ribosomal protein S6 kinase 1 (S6K1) is one such target that is phosphorylated by TORC1 directly when TORC1 is active, which leads to its own activation. S6K1, a serine/ threonine kinase itself, then phosphorylates the S6 ribosomal protein (eS6), promoting translation (Chung J et al. 1992). Work in my lab showed that TOR may also regulate translation through phosphorylation of the RNA-binding protein La-related protein 1 (LARP1) (Scarpin et al., 2020). Regina Scarpin demonstrated that LARP1 promotes TOR signaling in plants and is required to repress translation of certain mRNAs in response to TOR inhibition. These data point to a LARP1-mediated signaling pathway through which TOR regulates the translation of a specific subset of genes.

Another downstream member of the TOR signaling pathway in plants is the transcription factor E2Fa. Work performed in the Sheen Lab discovered that TOR phosphorylates E2Fa in response to activation by glucose, leading to the expression of S-phase genes in the root meristem and establishing a glucose-TOR signaling network in plants that regulates stem cell activation in response to nutrient availability (Xiong et al., 2013a).

TOR signaling in plants: a big black box!

Most of what is known about TOR's protein structure, function, and regulation come from studies performed in fungal and mammalian systems, despite the central role of TOR in many biological processes across the eukaryotic kingdom. The specific role that TOR plays in plant biology is a large "black box" that has been only explored relatively recently. Because of how widely TOR is conserved, the deep evolutionary conservation of the protein itself, and the extensiveness of tools developed in other systems, the existing knowledge base allows us to study TOR complexes in plants with informed intent. Other studies in other organisms have helped us understand TOR's part in the coordination of plant growth and development.

Plant metabolism, unlike other model species, are autotrophs that can synthesize all proteinogenic amino acids from inorganic precursors. Plant metabolism is unique in that way, since many of the biomolecules consumed in anabolism are produced by the plant

itself. For example, CO2 is fixed from the air to produce glucose, and inorganic nutrients like phosphate, nitrate, and sulfate are taken up from the environment and incorporated into amino acids, nucleotides, and energy-storing molecules like ATP. Plants can also synthesize all of their amino acids, instead of consuming them from other sources.

This means plants must balance the consumption of these products with their production. Emerging work in plant molecular biology, including some of the research discussed here, places TOR as the coordinator of both processes, not only initiating the consumption of nutrients but ensuring their synthesis as well.

TOR complex controls significant processes in plants. Activated TOR engages downstream plant growth and development characteristics; embryogenesis, meristem activation, root and leaf growth, orchestrating complex transcriptional networks integrate of growth and development (Xiong, 2013). One of the first analysis of a *tor* mutant in plants showed that disruption of TOR leads to the premature arrest of endosperm and embryo development (Menand et al, 2002). Growth arrest occurs in *tor* cells as the consequence of an incapacity to synthesize cell components during the transition between two modes of cell division. Just like in most other eukaryotes, TOR controls many of the same basic processes such as translation, ribosome biogenesis, and cell cycle progression. TOR thus impacts several developmental programs that are unique to plants, including seed germination, hypocotyl elongation, shoot and root expansion, flowering, meristem activation, and leaf initiation. *tor* knockouts in A. thaliana are embryo lethal, demonstrating that TOR is necessary for embryo development as well (Xiong and Sheen, 2012). TOR regulates these processes in response to a variety of upstream cues.

Upstream of TOR

TOR is sensitive to sugar

In plant metabolism, fixation of carbon into sugars through photosynthesis is the central axis of all plant metabolism. TOR has been conclusively shown to regulate cellular metabolism and proliferation in response to photosynthates. Sucrose and glucose activate the TOR complex, as measured by phosphorylation of S6K1 at the TOR-catalyzed phosphosite T449 by these metabolites independent of photosynthesis (Xiong et al., 2013a). A massive TOR-dependent transcriptional and physiological response occurs shortly after photosynthetically-limited seedlings are treated with exogenous glucose o sucrose. Shoots and roots begin to expand and over 2,000 genes are differentially expressed only 2 hours after treatment (Xiong and Sheen, 2012; Xiong et al., 2013a). Glucose stimulated TOR activation can impact the expression of genes in several metabolic pathways, not unlike what is seen in mammalian systems. . Glycolysis, the citric acid cycle, the mitochondrial electron transport chain, ribosome assembly, and the nucleotide and amino acid biosynthesis pathways are all upregulated in a TOR-dependent response to glucose treatment. These shows that the function of TOR as a metabolic switch from catabolic to anabolic growth is well conserved in plants and indicates that glucose is a key upstream activator of the TOR signaling pathway in

plants (Deprost et al., 2007; Moreau et al., 2012; Ren et al., 2012 ; Caldana etal., 2013; Xiong etal., 2013a/b; Dong etal., 2015).

TOR is sensitive to amino acids and other nutrients

As mentioned in the first chapter, although plants lack equivalents of these mammalian amino acid sensors and RAGs (Ragulator-Rag GTPases) it is known other nutrients such as nitrogen (N), phosphate (Pi) and sulfur (S) play crucial roles in promoting plant growth and development, and recent studies suggest that these may function via a TOR-based pathway. Sulfur availability coordinates glucose signaling to activate TOR (Dong et al., 2017), nitrate acts as a nutrient nutrient signal to promote system-wide shoot growth and root establishment in Arabidopsis (Liu et al., 2017), and Arabidopsis seedlings overexpressing TOR are hyposensitive to high nitrate inhibition of roots (Deprost et al., 2007). Based on enhanced S6K phosphorylation, amino acids also activate TOR signaling, and future research may thus identify plant-specific amino amino acid transporter-sensors involved in TOR regulation.

Figures

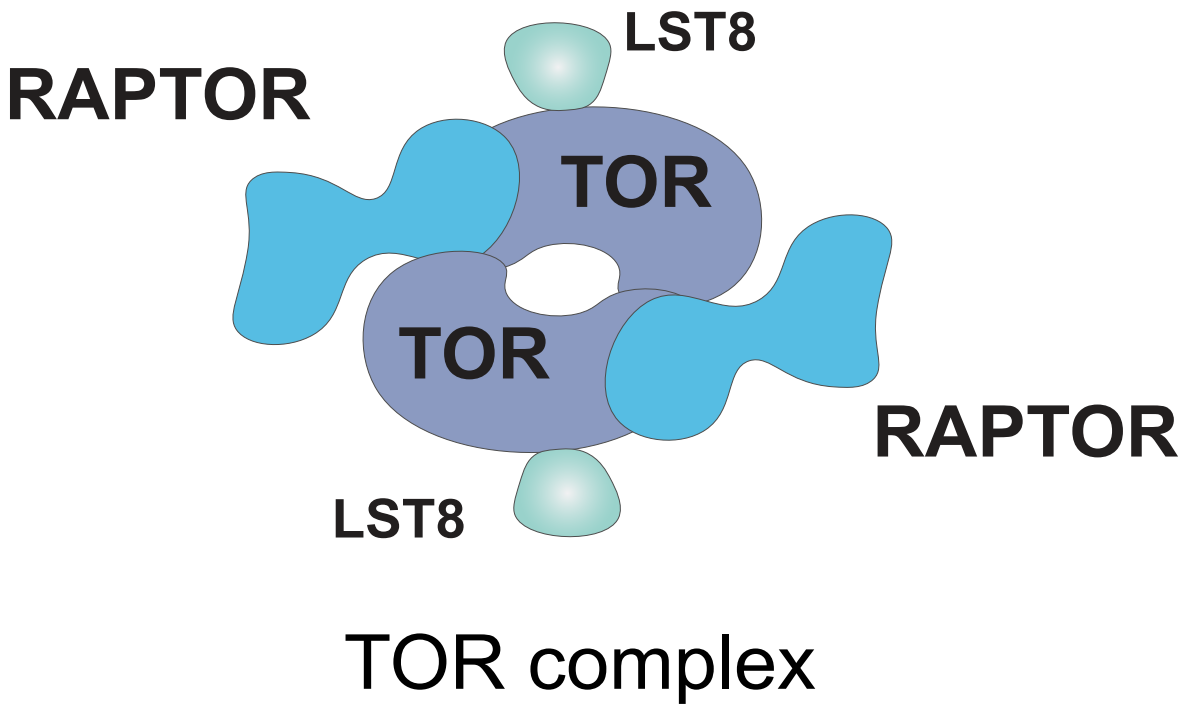


Figure 1.5 Cartoon of plant TORC1 components. REGULATORY ASSOCIATED PROTEIN OF TOR (RAPTOR) and LETHAL WITH SEC THIRTEEN 8 (LST8) associate with the TOR protein. These three proteins dimerize to form a complex with two TOR subunits, two RAPTOR subunits, and two LST8 subunits.

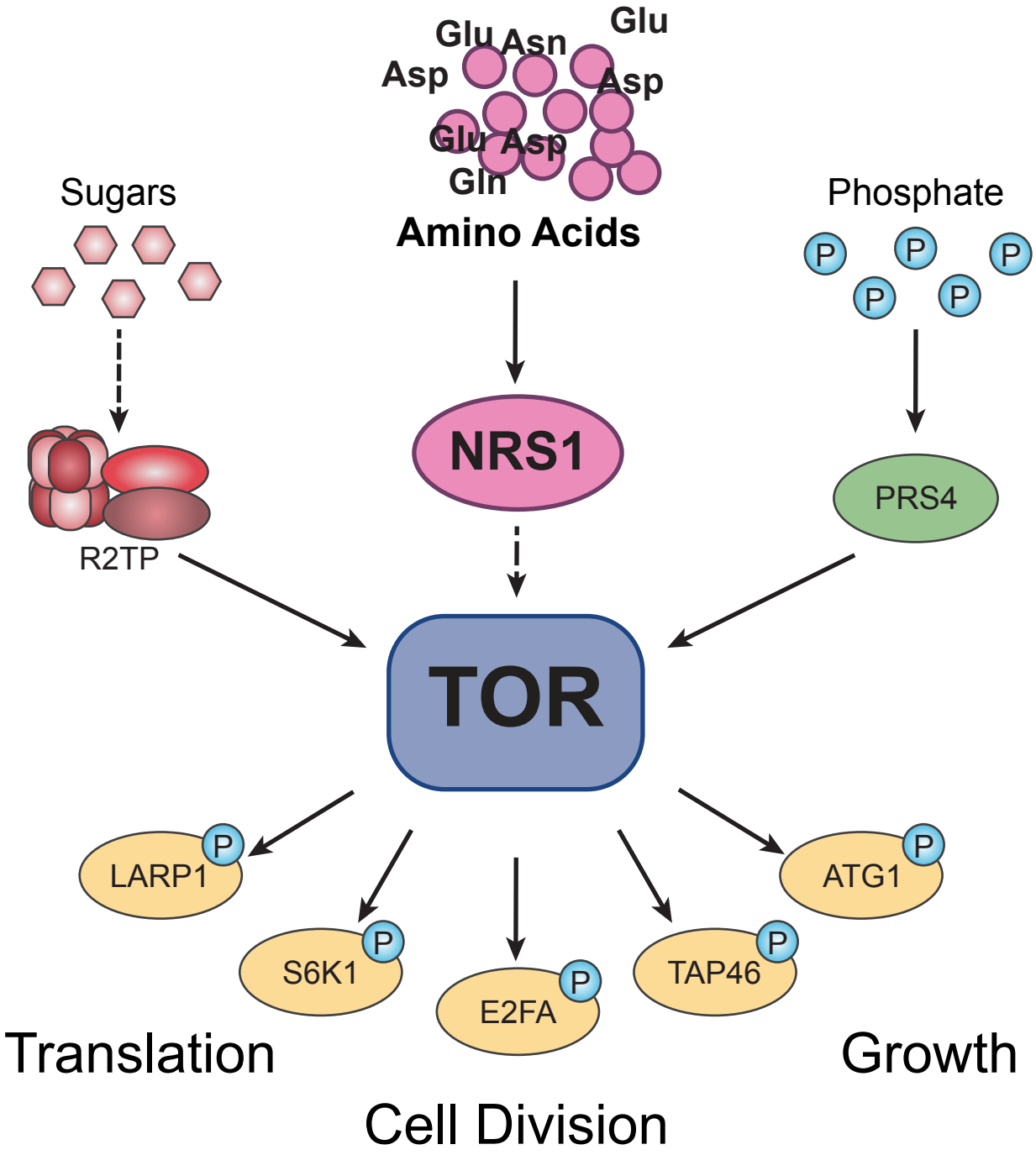


Figure 1.6 TOR regulates developmental processes in response to nutrient availability. TOR senses the abundance of sugar, amino acids, phosphate, and other metabolites and regulates processes including translation, cell division, and growth in response.

Section 1.3 Developments in Autonomous Nitrogen Pathways and Nitrogen Sensing by TOR in Plants

Nitrogen status sensing in plants

Plants require more nitrogen than any other macronutrients derived from the soil. Nitrogen is a major constituent of several of the most important plant substances, including amino acids, the building blocks of proteins (Swan *et al.*, 1971). Nitrogen-deficient plants show stunted growth and chlorosis and necrosis in older leaves (Roy *et al.,* 2006). The availability of nitrogen is a major limiting factor for plant growth, with availability corresponding positively with biomass accumulation (Marschner, 1995; Epstein and Bloom, 2005). Commercial fertilizers are responsible for over 60% of global crop yields, primarily due to nitrogen deficiencies in arable soil (Stewart 2005, and Tillman 2002), and in natural conditions, the availability of nitrogen constrains biomass production in ecological systems where climate and other nutrients are adequate to support plant growth. Levels of exogenous nitrogen application in our agricultural systems are currently at ecologically unsustainable levels, due to the negative impacts of nitrogen leaching into groundwater, fertilizer runoff creating algal blooms, and reducing the amount of organic carbon available in the soil (Springmann, 2018, Hoekstra *et al*, 2014).

Despite the fundamental importance of nitrogen for agriculture, the body of knowledge about how plants sense nitrogen availability or coordinate nitrogen allocation across the plant body is lacking, confounding efforts to intelligently breed plants that are resistant to low nitrogen soils (Krapp 2015). Plants can absorb and metabolize inorganic nitrogen (nitrate or ammonium) or organic nitrogen (primarily amino acids). Although amino acids represent the largest fraction of low molecular weight dissolved organic N in many soils (Jones *et al*, 2005), and are the primary transported form of nitrogen from the root to the shoot in many plant species (Inselsbacher, 2012), no *bona fide* amino acid sensor has yet been identified in plant cells. There have been significant efforts to elucidate early steps in sensing and response to external nitrate, but the mechanisms by which cells monitor internal N status remain unclear. The only clearly defined nitrogen sensor in plants is a low- and high-concentration (i.e., dual-affinity) nitrate transporter, CHL1, that triggers a phosphorylation cascade in response to nitrate availability (Ho *et al*, 2009).

Developments in Autonomous Nitrogen Pathways

Adverse environmental conditions are impossible for sessile organisms to escape, so in order to cope with the barrage of constant limiting challenges, plants must adjust their growth, physiology, and development. Plants have evolved to respond dynamically to shifting environmental conditions and must maintain an adequate nutrient supply under various stresses. Plants require nitrogen in the greatest amount compared to other minerals, and the availability of nitrogen is a major limiting factor in plant growth. (Marschner, 1995; Epstein and Bloom, 2005) Commercial fertilizers are responsible for over 60% of global crop yields, primarily due to nitrogen deficiencies in arable soil (Stewart 2005, and Tillman 2002), and in natural conditions, nitrogen is most often

highly sought after. Plants can acquire nitrogen via two mechanisms; autonomously, via internal uptake mechanisms, or through association with many and functionally diverse microorganisms that can also contribute to an adequate nitrogen supply. This minireview will specifically address the existing body of knowledge and recent advances in the autonomous nitrogen pathway, and review what nitrogen compounds are sensed and in which part of the plant, as well as the identity of the signaling pathways responsible for their detection.

How do plants physiologically respond to nitrogen in the environment?
In plants there is considerable developmental plasticity in response to variations in nitrogen concentration and distribution. This physiological background is necessary to provide the framework for recent advances and efforts to identity molecular and genetic components in nitrogen acquisition. One of the most notable and well-studied plant adaptations to ensure adequate nitrogen uptake is the modulation of the root system architecture in response to nitrogen supply. The evidence shows that primary root growth and lateral root elongations are modifications to plant architecture in response to the availability of the nitrogen supply (Zhang and Forde, 1998; Zhang *et al*., 1999; Vidal *et al*., 2010a). Plants that are grown in a local high concentration of nitrate or ammonium display a dramatic proliferation of lateral roots (Drew *et al* 1973, Drew 1975). This response is specific to the localized nitrate-rich zone, and the dramatic proliferation of lateral roots occurs in a variety of plant species and represents an established adaptation phenomenon (Hodge 2004, Robinson 1994). However, although the phenotypic descriptions of plant response to nitrogen supply in plants have largely been characterized, the full picture of nitrogen sensors and signaling pathways mediating their integrated effects are still not well understood.

How do plants uptake nitrogen from the soil?
Plants have evolved two categories of autonomous nitrogen uptake. The systems of inorganic and organic nitrogen uptake assist the plant in coping with the heterogeneous nitrogen availability in the soil. Nitrate and ammonium are the widely available forms of inorganic nitrogen for plants to take up in the environment. For the transport of nitrate and ammonium, the membrane transporter proteins involved have been identified at the in model species, and the related gene families have been shown to be conserved between species, allowing fast progress in research on crops.

The well-studied families of nitrate transporters are NPF (NRT1) nitrate transporter 1 family, and NRT2, nitrate transporter 2 family, identified originally in Arabidopsis (Tsay *et al*., 2007). Also, chloride channel family (CLC) and slow anion channel homologues (SLAC1/SLAH) families have been added to the list of nitrate transporters, making four gene families identified (Fan *et al*, 2017, Li *et al*, 2017). These families collectively represent more than 73 genes encoding nitrate transporters (Krapp, 2015). However, despite the complexity and number of genes involved, nearly all steps of nitrate transport have been characterized with the function of at least one member of these families (Fan *et al*. 2017). The AMT1 family of high affinity transporters is responsible for our molecular understanding of ammonium transport, localized at the root uptake (Li

et al., 2017). All transporters that contribute significantly to the uptake of nitrates and ammonium from the external medium have been identified in Arabidopsis as well; *NRT2.1, NRT2.2, NRT2.4, NRT2.5* for nitrate, and *AMT1.1, AMT1.2, AMT1.3 and AMT1.5* for ammonium. These transporters have largely been identified due to the phenotypes of multiple mutants in the associated genes, which are impaired in root nitrogen acquisition of either nitrogen source (Yuan *et al*, 2007, Lezheva *et al*, 2014). In Arabidopsis, out of the 73 genes identified, only 35 have been characterized in detail, and only 24 are known nitrate transporters. As detailed genomic information becomes more widely available, homologous gene families have been identified in other crops and trees (Li *et al*, 2017, Castro-Rodriguez *et al*, 2017). However, marked differences exist between different species. For example, there are nine *AMT2* genes in the *Populus trichocarpa* genome (Castro-Rodriguez *et al*, 2017), suggesting a need for further investigation of functional complexity in a diversity of organisms.

As robust as the research in inorganic nitrogen sources is, the existing body of knowledge of organic nitrogen uptake sensors is not complete. The molecular mechanisms are still yet to be elucidated, and generally at an early stage of investigation. Nitrogen transport between plant organs is functionally mediated by the movement of amino acids and is essential for nitrogen partitioning in the plant body as a whole (Tegeder, 2014.). During senescence, dying leaves can provide rich sources of nitrogen, and plants engage in N remobilization to complete nitrogen seed filling during the phase of reproductive development, but the functional mechanisms of amino acid or peptide transporters are still unclear (Have *et al* 2017).

How do plants sense their nitrogen status?

There have been significant efforts in elucidating the early steps in sensing and responding to external nitrate, but the mechanisms of internal monitoring of N status is rather unclear. The target of rapamycin (TOR) signaling pathway, the general control non-derepressible 2 (GCN2) pathway, and the PII-dependent pathway are all candidates that are considered for the role of nitrogen sensory systems (Gent, Forde 2017). A prevailing possibility is that the GLR family of Ca2+ channels, which are expressed throughout the whole plant and gated by several amino acids, could act upstream of a Ca2+ regulated signaling pathway as amino acid sensors. A candidate mechanism is the TOR pathway; to regulate the plant's response to changes in nitrogen status.

The TOR pathway has been implicated in regulating nitrogen metabolism (Caldana *et al*., 2013). Experiments that down regulate TOR itself, or downregulation associated proteins observe major shifts in metabolism that relate to amino acid accumulation. At a transcriptional level, this might mean that TOR is response for regulating the balance between primary nitrogen assimilation and nitrogen recycling internally through the ratio of amino acids (Dobrenel *et al*, 2016). While there is proof that sugar availability regulates intercellular TOR activity, there is no conclusive information or clarity on the mechanism of nitrogen sensing upstream of TOR or on which N metabolites are sensed.

However, the Glutamate-Like Receptor (GLR) family of glutamate receptor-like proteins may help shine some light on the intricacies of the nitrogen sensing pathway. At the molecular level, the evidence suggests that GLRs act as amino acid-gated Ca2+ channels (Weiland *et al*, 2016), with a wide range of interactive substrates. There's evidence to suggest that the GLR family has important roles throughout the plant in monitoring changes in amino acid distribution, which then can be symptomatic changes in the plant's nitrogen status (Yoshida *et al*, 2016; Li *et al*, 2013). The GLR family, with their role as a general and promiscuous amino acid sensor, could act upstream of the TOR-mediate amino acid signaling pathway (Gent and Forde 2017). This assumption draws upon older work done describing the phenotype of a GLR mutant line that demonstrates changes in the expression of carbon and nitrogen metabolism (Kang and Turano, 2003) that are very similar to the phenotypes described for lines defective in components of the TOR signaling pathway (Dobrenel *et al*, 2016). A large genetic redundancy exists among GLR family members that makes testing the link between GLR and TOR in amino acid signaling, but the monitoring of nitrogen status in plants is so essential to how the regulation of nitrogen acquisition, nitrogen metabolism and nitrogen storage that the molecular mechanisms of nitrogen sensing should be made part of future efforts based on these recent advances.

Some new developments have occurred over the past year, focusing on long-distance signaling of nitrate availability. Ohkubo *et al* 2017 demonstrates that a root-to-shoot-to-root relay system involving xylem mobile peptides and phloem-mobile glutaredoxin polypeptides is essential in the signaling of nitrate availability, and both Undurraga *et al* 2017 and Bellegarde at al 2017 demonstrates that there is a significant number of regulators that act putatively downstream from the signals of nitrogen status in Arabidopsis. These articles are referring to regulators that act on a genetic level, which is only one phase of the regulatory systems that are implicated in nitrogen response and signaling. Phosphorylation is now largely known to be of major importance for control of the transport and sensing functions of transporters in the NRT1 and AMT1 families in Arabidopsis (Jacquot *et al*, 2017), which harkens back to the previously mentioned need to investigate kinases of interest.

Figures

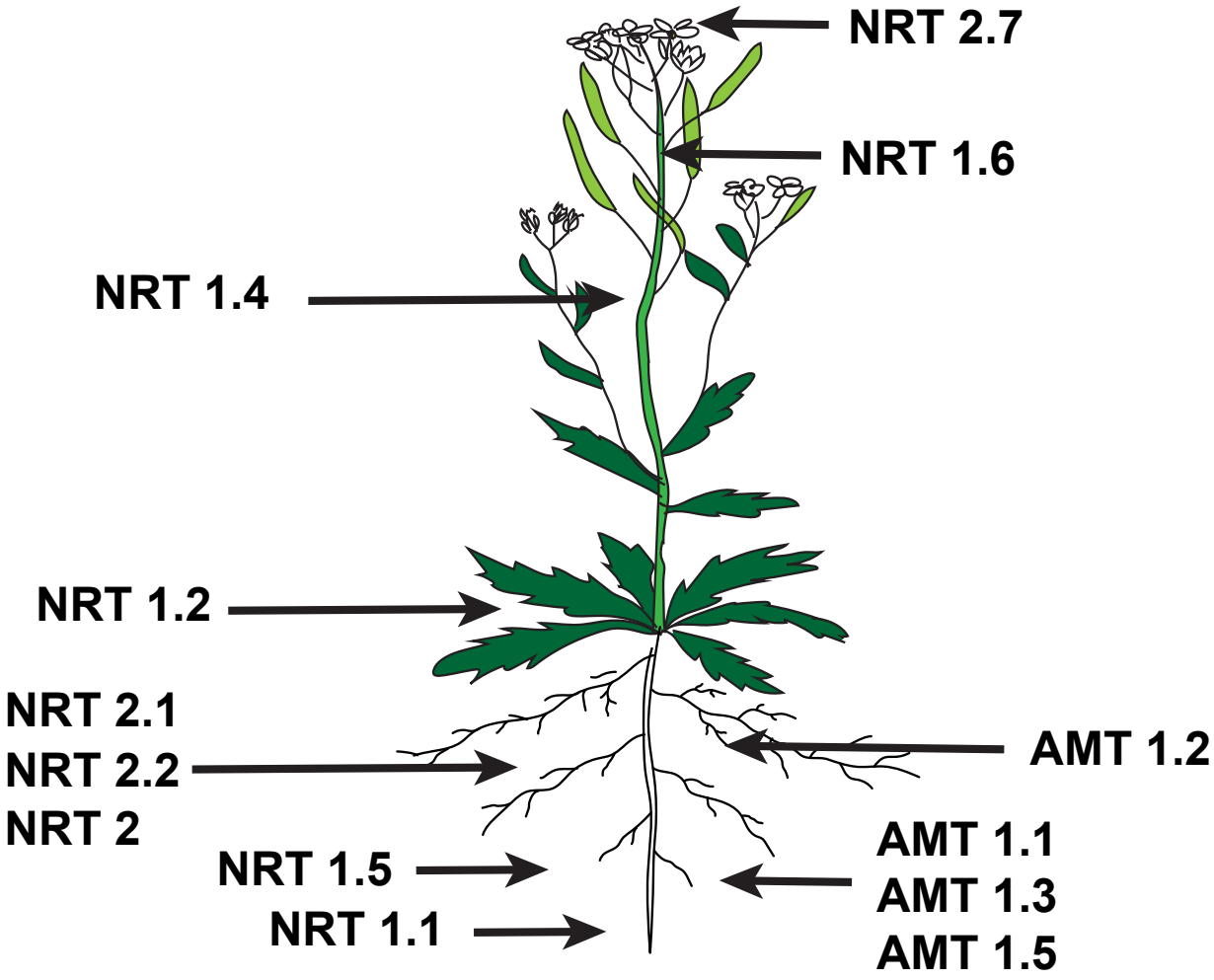


Figure 1.7 Schematic presentation of the known localization of the NRT1, NRT2 and AMT genes in Arabidopsis. These nitrate transport systems have been shown to coexists in plants and act coordinately to take up nitrate from the soil solution and distribute nitrate within the whole plant

(Image adapted from Masclaux-Daubresse et al 2009).

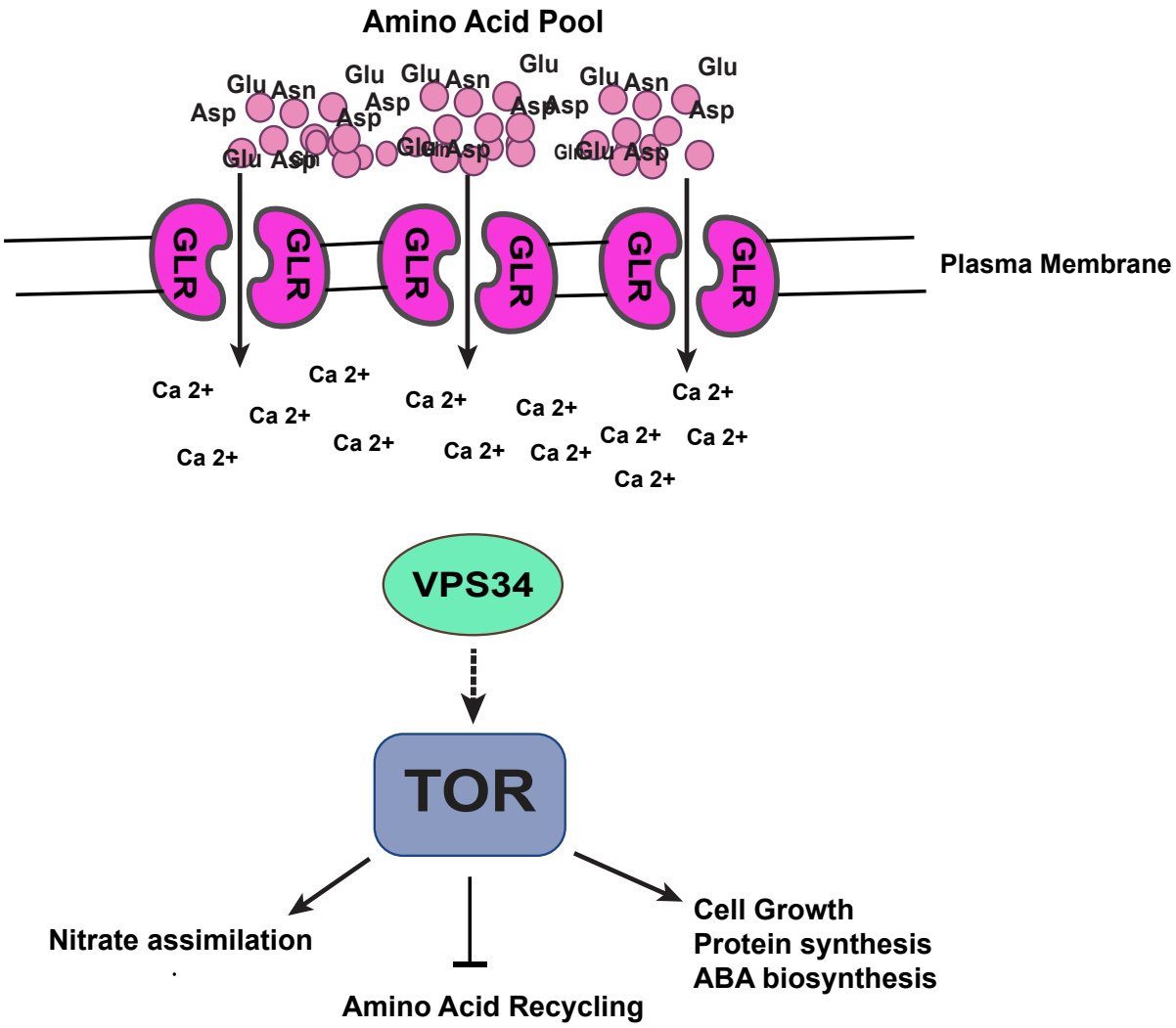


Figure 1.8 Hypothetical model for activation of the TOR complex by amino acid sensing through the family of GLR glutamate receptor-like Ca2+ channels present in plants. The TOR complex plays a central role in regulating plant growth and metabolism in response to changes in nutrient availability. The figure depicts a cell in which a diverse set of GLRs is expressed, each activated by a variety of different amino acid ligands.

Collectively, this panel of amino acid sensors would be able to respond to changes in the overall amino acid pool, therefore potentially providing an accurate reflection of the N status of the tissue. Ligand binding by the GLR is known to trigger Ca2+ influx, and it is proposed that this could be linked to activation of the TOR complex, perhaps through a pathway involving the Ca2+-dependent regulation of VPS34 and the downstream production of phosphatidic acid, which is an activator of TOR in animals. (Figure adapted from Gent and Forde 2016).

Section 1.4 Nutrient-dependent TOR Regulation

Introduction

Upon uptake by plant cells, inorganic nitrogen is first assimilated into amino acids before it is incorporated into downstream biological molecules. Therefore, amino acids are essential nutrients for cellular metabolism, not only as subunits for protein synthesis, but also as precursors for other metabolites, including nucleotides and phytohormones. In mammals and yeasts, amino acids are required for the activation of the TOR kinase. As stated previously, one of the recently identified amino acid sensors in animals is a leucyl tRNA synthetase (LRS) that is proposed to sense leucine to activate TOR. The LRS plays a critical role in amino acid-induced TOR activation by sensing intracellular leucine concentration and initiating molecular events that lead to the activation of the TOR complex. However, unlike the well-described mechanisms of TOR and nutrient sensing in mammals and yeasts, very little is known about the nutrient-sensing components of the TOR regulatory pathway in plants. The TOR pathway has been implicated in regulating nitrogen metabolism in plants, as well as coordinating N and C metabolism (Caldana *et al*., 2013). Experiments that repress TOR or components of the TOR complex observe major shifts in metabolism that relate to amino acid accumulation, resulting in massive increase in the levels of free amino acids. However, the mechanisms responsible for TOR involvement in these systems remain debated, and while there is evidence that sugar availability regulates TOR activity (Xiong *et al.*, 2013**), there is no conclusive information or clarity on the mechanism of nitrogen sensing upstream of TOR or on which N metabolites are sensed.** Amino acids in plants also play a key role in these complex metabolic networks of nutrient cycling and partitioning, which make them ideal candidates as signaling activators of the TOR pathway. I propose that an analogous mechanism to the animal LRS pathway exists in plants, through the asparyginyl tRNA synthase, *NRS1,* and attempt to elucidate the effects of nitrogen stimulus on TOR activity. In significance as well, tRNA synthetases, are the only putative amino acid- sensors proposed to be shared among plants, fungi, and animals. Figure 1.9 describes the overarching chapters of this thesis.

NRS1 has unexpected roles in growth and development

A null allele of *NRS1* was identified in a forward genetic screen for mutants that disrupt traffic through plasmodesmata, channels that connect neighboring plant cells and regulate nutrient distribution, in part by coordinating transport through the plant vascular system. In a screen to identify genes that regulate the sink-to-source transition, the Zambryski lab isolated six mutants that have strongly altered PD transport (five with increased PD transport, *ise1* through *ise4*, and one with decreased PD transport, dse1). We discovered that at least two of these mutants impact the TOR signaling pathway, *ise3* and *ise4.* Experimental treatments that promote TOR activity accelerate the sink-to-source transition, and treatments that decrease TOR activity slow the sink-to-source transition.

In experiments that utilize virus-induced gene silencing (VIGS) to knockdown expression of TOR or the direct TOR antagonist, SnRK1, silencing TOR slowed the sink-to-source transition while silencing SnRK1 accelerated the sink-to-source transition. Differential

gene expression between young sink leaves and mature sources leaves significantly overlaps with the set of genes regulated by TOR in response to glucose. Of the hundreds of overlapping genes between these datasets, over 85% are co-regulated, further confirming that TOR regulates the sink-to-source transition. TOR is a critical upstream regulator of leaves' physiological transition from sinks for nutritional resources to sources of carbohydrates for the whole plant (Brunkard et al. 2020).

TOR represses carbon distribution in plants by repressing PD transport in mature, photoautotrophic tissues, thereby promoting sugar export to other organs. One of these mutants identified in the screen, *NRS1,* is characterized here. *NRS1* has stunted growth and is defective in TOR signaling. In further analysis. we found that stably overexpressing *NRS1* causes phenotypes similar to TOR overexpression in Arabidopsis transgenics (e.g., increased organ and cell size, Figure 1.11.

Most of the mutants identified in this screen are disruptions in genes that encode core components of the TOR signaling network, hinting that *NRS1* may similarly disrupt TOR signaling. As shown in Figure 1.10, *nrs1* embryo is not only smaller, but has fewer cells, implying a defect in cell division processes. We analyzed the transcriptome of *NRS1*, which shows significant differences in expression of genes involved in late embryogenesis/seed maturation, cell wall synthesis, and amino acid metabolism (Figure 1.10), among other processes. Importantly, the *NRS1* transcriptome also shares qualities with a TOR deficient mutant: in both TOR-inhibited plants and *NRS1,* photosynthesis is down-regulated, ribosomal protein genes are down-regulated, autophagy is upregulated, and a number of development genes are misexpressed.

To test altered PD transport in *NRS1*, we performed a screen for altered trafficking of 10kDa FITC-dextran at the midtorpedo stage of embryogenesis of wild-type and two examples of *NRS1* mutants, with chlorophyll in red and the dye-loading assay in green (10 kDa FITC-Dextran). In Figure 1.13 the dye has moved rapidly through the *NRS1* embryos but not through wild-type. This demonstrates that *NRS1* clearly has increased PD transport. s. Beyond their roles in nutrient partitioning, PD also traffic a variety of signaling molecules, including transcription factors, cytosolic proteins, and hormones, and are the primary route for viral spread. Dynamic regulation of PD transport by TOR may therefore contribute to a variety of plant biological processes through *NRS1*.

What is *NRS1*?

In the literature, *NRS1* has been implicated in controlling plant development in ways that would not be readily predicted from its established enzymatic function as an asparaginyl tRNA synthetase. In an investigative screen for overexpressed cDNAs that impact agricultural traits in *Lotus japonica* (a model legume), *NRS1* was a standout (Yano *et al*, 2014). The overexpression of *NRS1* increased biomass and height while reducing branching, which are traits that are usually selected for during domestication and thus agronomically beneficial (Figure 1.12). These results suggest that *NRS1*, an asparaginyl tRNA synthetase that has not yet been linked to the TOR pathway, might play a role in

controlling some of the developmental and physiological processes that TOR activity is responsible for. Consideration of the family of asparaginyl tRNA synthetases also offers some insight on why the *NRS1* mutant is of interest. Flowering plants have evolved three major clades of asparaginyl tRNA synthetases: a cytosolic *NRS1* clade that is conserved throughout flowering plants, a cytosolic *NRS2* clade that has been lost in monocots, and a third clade that is targeted to the chloroplast and/or mitochondria. Significantly, however, *NRS1* mutants are lethal at the mid-torpedo stage of embryogenesis, which suggests that *NRS1* has some nonredundant function that is not complemented by NRS2 or NRS3. Therefore, *NRS1* is unique among aminoacyl tRNA transferases in plants because it is not strictly essential for cell survival, but it is required for plant development, even though there are two other *NRS* genes in *Arabidopsis thaliana*.

In combination, these results lead us to the hypothesis that *NRS1* acts in a pathway beyond its established enzymatic role in synthesizing Asn-tRNA. The chapters will test the hypothesis that *NRS1*, an asparginyl tRNA synthetase activates TOR, by defining TOR responses to nitrogen in plants, genetic manipulation of aminoacyl tRNA synthetases impacts on TOR activity, and establishing an *NRS1* and TOR interaction.

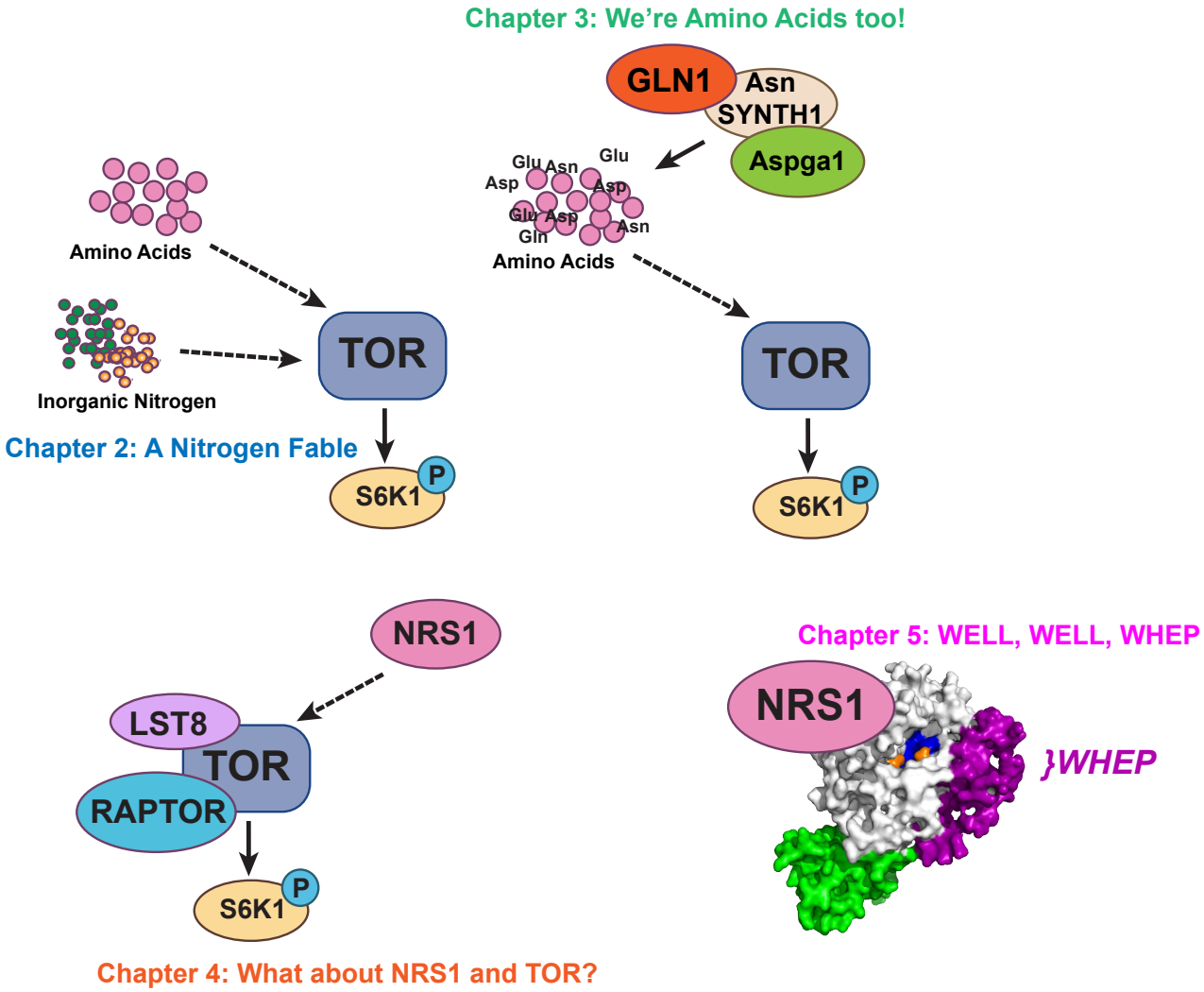


Figure 1.9 Schematic image of the chapters of this thesis

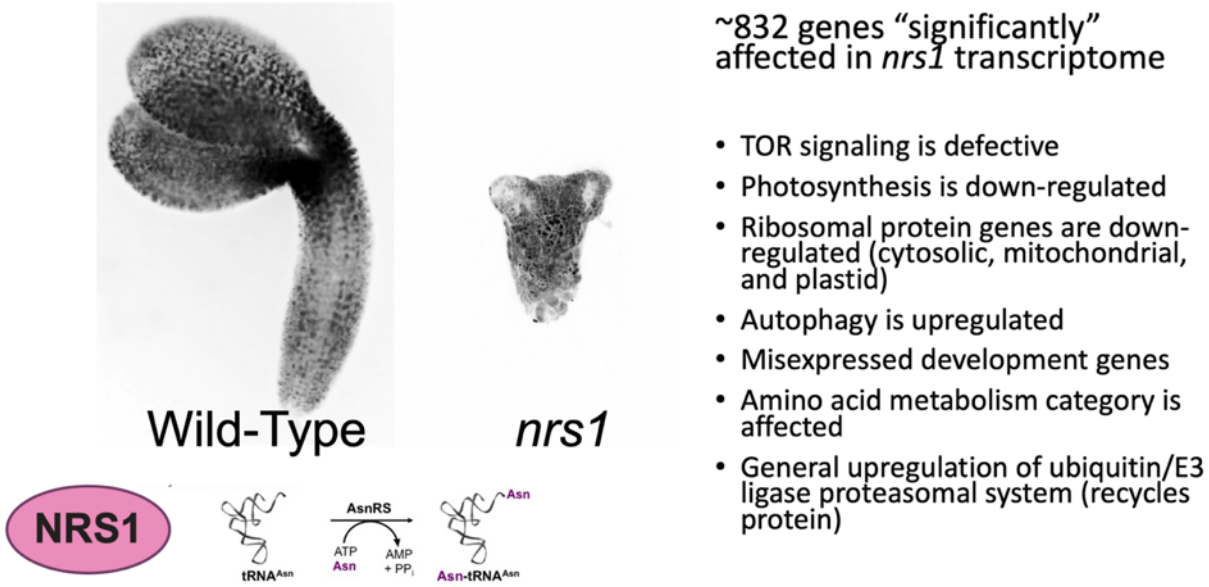


Figure 1.10 A wild type *Arabidopsis thaliana* seedling on the left, with an *NRS1* mutant on the right. Below, a schematic representation of *NRS1* tRNA activity. *NRS1* has stunted growth and is defective in TOR signaling. 832 genes are significantly affected, (p, q <0.05). Genes affected have overlap with TOR deficient mutants.

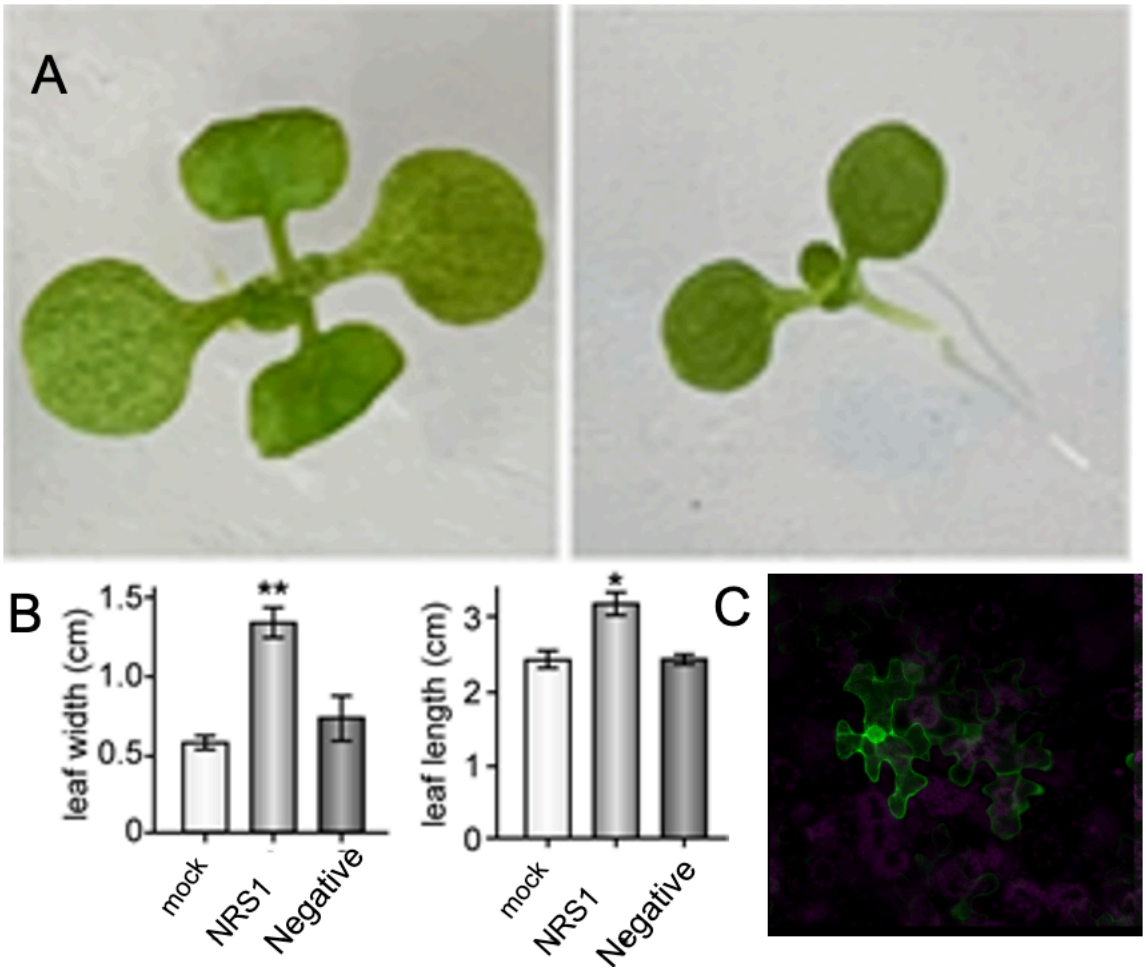


Figure 1.11 Overexpression of *NRS1* phenotype
(A) WT (left) and *NRS1* (right) *Arabidopsis* plants of similar ages.
(B) *NRS1*, negative control (related gene) and mock GFP stable transgenic lines were grown for 7 weeks on ½ MS agar plates. *NRS1* overexpression clearly stimulated vegetative growth. The length and width of leaf 6 was measured and compared by t tests (**p <10^-5, *p <10—2); n> 24)
(C) These images demonstrate the localization of each protein-GFP for *NRS1* overexpression lines (at 10X magnification under a confocal microscope.

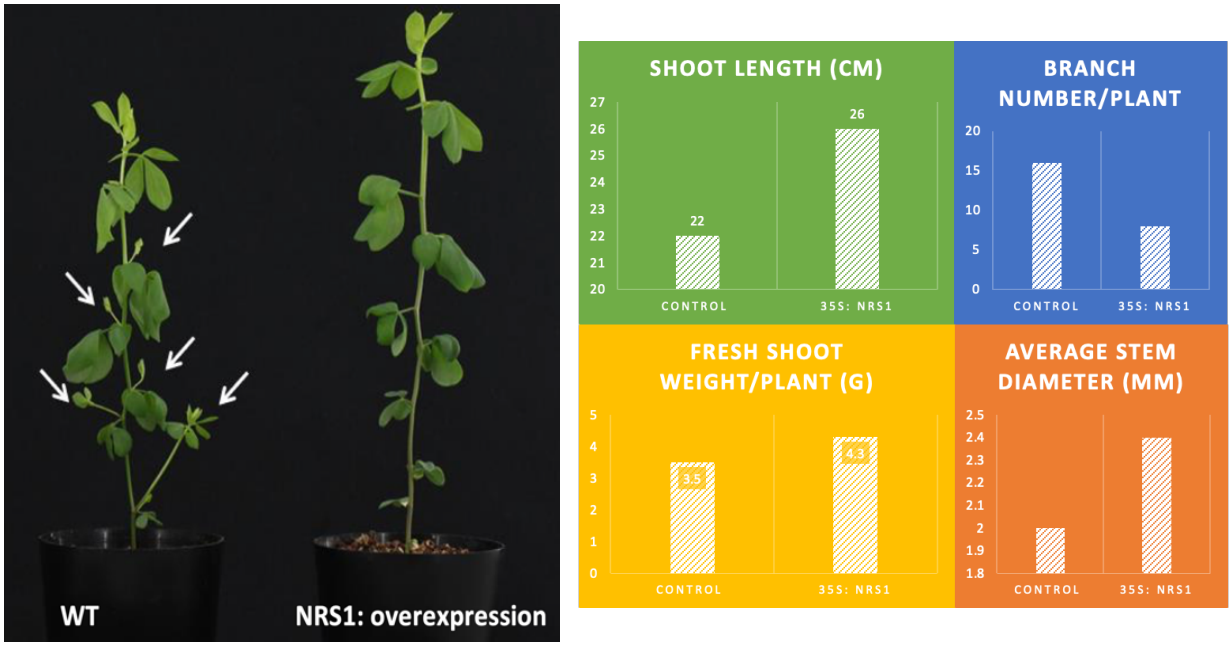


Figure 1.12 A wild type plant *Lotus japonica*, on the left, exhibiting normal branching patterns, and an overexpression line of *NRS1* on the right, showing increased shoot length and reduced branching. Shoot length, fresh shoot weight, and average stem diameter all increased in the transgenic plant, while branch number was reduced; traits that are usually selected for in domestication. Image and data modified from Yano *et al.*, 2013.

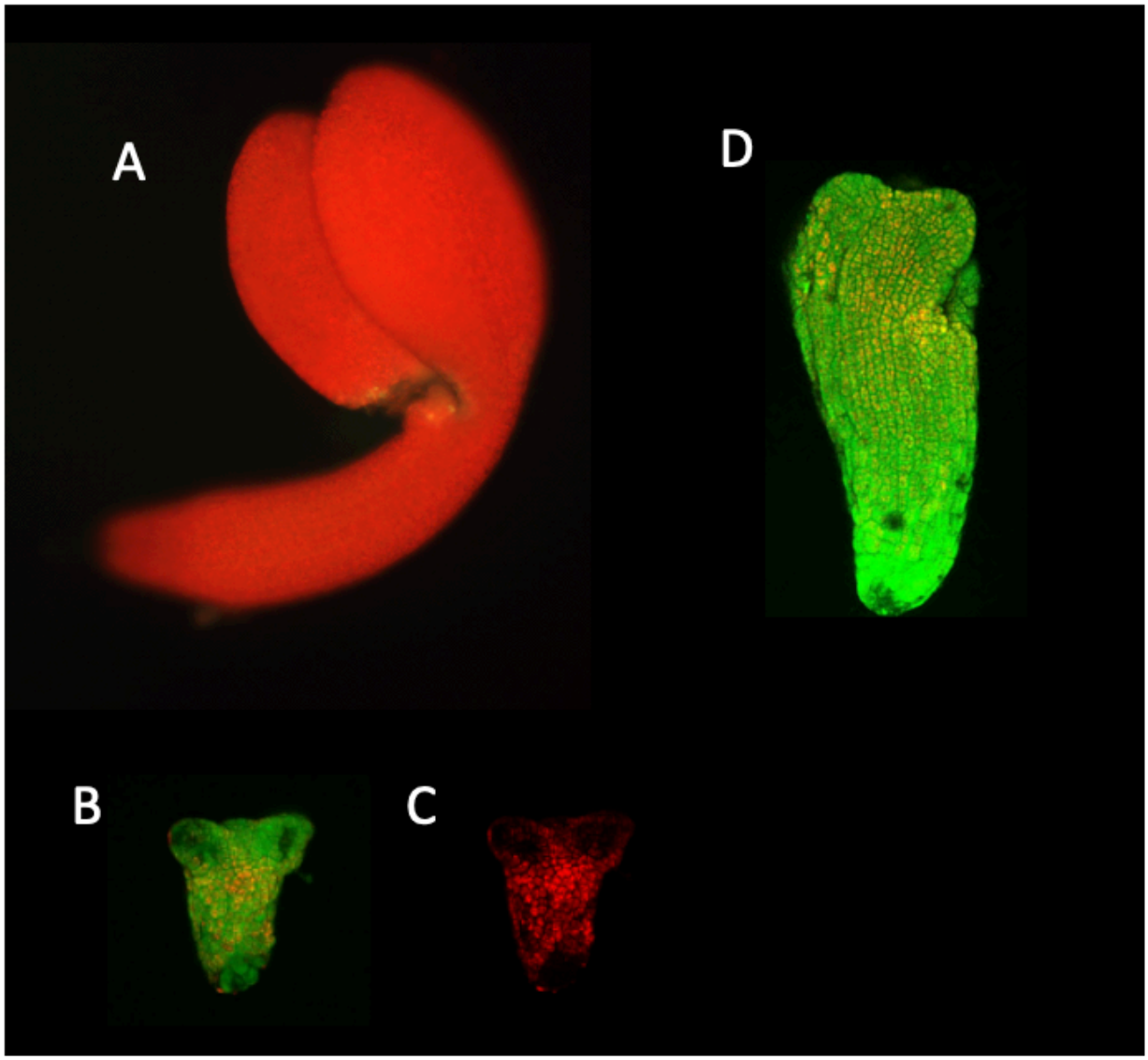


Figure 1.13 *NRS1* PD screen
(A) WT
(B) *NRS1* (embryo #1)
(C) *NRS1* (embryo #1, showing only chlorophyll)
(D) *NRS1* (embryo #2) The color red indicates chlorophyll autofluorescence while the color green represents 10 kDa FITC-Dex tran fluorescence (embryos are to scale)

Chapter Two
A Nitrogen Story

“People who deny the existence of dragons are often eaten by dragons.
From within.”
Ursula K. Le Guin



Section 2.1 Determine if nitrogenous nutrients stimulate TOR activity

The first picture of my thesis engages the hypothesis that application of exogenous nitrogen has an impact on the TOR signaling pathway. Here, I will describe how nitrogenous nutrients stimulate TOR activity, with the hypothesis that TOR activity is promoted by the exogenous addition of nitrogenous nutrients when plants are grown under nitrogen limiting conditions. To test the hypothesis that TOR is stimulated by nitrogenous nutrients in plants, I have employed two experimental approaches. Firstly, establishing N-limiting growth conditions to determine the impact of nitrogenous nutrients on TOR activity. Secondly, testing nitrogen and amino acid addition in N-deprived plants to identify conditions that promote TOR activity to isolate plant reactivity to exogenous treatments.

Establish N-limiting growth conditions to determine the impact of nitrogenous nutrients on TOR activity.

Different inorganic or organic forms of nitrogen might impact growth and development patterns. For these experiments, performing TOR assays under conditions that are nitrogen limiting are key. My hypothesis is that TOR activity is promoted by the exogenous addition of nitrogenous nutrients when plants are grown under nitrogen-limiting conditions. To test this hypothesis, I have developed a variety of functional nutrient-controlled systems to observe the morphological and developmental impact of limiting nitrogen availability in *Arabidopsis thaliana* and *Nicotiana benthamiana.* This hypothesis is illustrated in Figure 1. All plant experiments are grown in ½ Murashige and Skoog (MS) media or MS media without nitrogenous sources (½ MS-N). The ½ MS medium will serve as a positive control for plant growth under nitrogen-replete conditions, whereas the ½ MS-N medium is the negative control for plant growth, as a total lack of nitrogen severely hinders plant growth, and results in nitrogen-stress phenotypes (Epstein and Bloom, 1995). The nitrogenous compounds that will be applied to the nitrogen controlled environments outlined in this experiment are listed in Table 2.1. Figure 2.1 shows the phenotype of a seedling grown in nitrogen-deprived conditions.

Assaying TOR activity in plants with S6K1 phosphorylation: A reliable, direct, and powerful assay of TOR activity is to monitor the phosphorylation status of Thr449 of S6K1 (homologous with Thr389 in mammalian TOR), which is phosphorylated by active TOR and dephosphorylated by protein phosphatase 2A when TOR is inactive (Peterson *et al*, 1999). S6K1 is highly conserved in eukaryotes, and the Brunkard Lab has successfully developed consistent methods to test S6K1-Thr449 phosphorylation. We have reliably used commercially available antibodies raised against conserved S6K1 epitopes to detect overall levels of S6K1 and phosphospecific antibodies to detect the level of Thr449 phosphorylation in protein extracts from *N. benthamiana* and *A. thailana*. As shown in Figure 2.3, a demonstrative blot of total S6K phosphorylation and S6K-Thr449 phosphorylation are indicative of TOR activity, as measured by western blot. All of the methods described in Table 2.1 and following will be sampled and protein extracted at different time points and developmental stages to run Western blots that will measure total S6K1 and S6K1-pThr449 levels.

Test nitrogen and amino acid addition in N-deprived plants to identify conditions that promote TOR activity

In addition to exogenous nitrogen sources, I also tested the impact of amino acid metabolism on TOR activity, with the hypothesis that TOR activity positively correlates with endogenous asparagine levels and the activity of asparagine synthetases and asparaginases.

The experimental systems developed in the previous section have been used in parallel with assays of TOR activity to determine if various forms of nitrogen or amino acids stimulate TOR. The aim of 1b is to test nitrogen and amino acid addition in N-deprived plants to identify conditions that promote TOR activity. I will be testing ranges of amino acid treatment that have been estimated based on free amino acid levels measured in wild-type *A. thaliana,* ecotype Columbia-0 (Col-0), in experimental data collected by Snigdha Chatterjee in the Brunkard Lab and by Watanabe *et al*, 2013. Glutamate, glutamine, aspartate and asparagine are the primary products of nitrogen assimilation and function as the forms of transported nitrogen within the plant body, and therefore are the focus of this aim. The nitrogenous compounds that were applied to the nitrogen controlled environments outlined in previously are listed in Table 1. To this background solution of ½ MS or ½ MS-N media, different nutrient treatments have been applied.

As previously stated, the amino acids that I have tested first are those that are the early products of the nitrogen assimilation pathway. Namely, I have tested the impact of supplying asparagine, glutamine, aspartic acid, glutamic acid, or a mix of all the amino acids in my liquid seedling assay, hydroponic boxes, and protoplast experiments and assay TOR activation in response. This thesis has primarily focused on tsting asparagine for several reasons. First, asparagine found at very high concentrations in many plant species. Second, of proteinogenic amino acids, asapargine has the highest nitrogen to carbon ratio. Third, the Brunkard Lab has also found that addition of 5.0 mM asparagine to *N. benthamiana* leaves rapidly induces TOR activity, as measured by S6K1-Thr449 phosphorylation. Fourth, the *NRS1* mutant encodes an asparaginyl tRNA synthetase, and we postulate that *NRS1* is an amino acid sensor for TOR. Since leucine and arginine are amino acids that are known to activate TOR in mammals, they are also included for analysis.

Section 2.2 Materials and Methods

Seedling liquid assays: The Brunkard Lab has also optimized a sterile system for highly reproducible nutrient and chemical assays in the form of six-well liquid plate experiments, based on experimental approaches developed in Jen Sheen's lab (Xiong *et al.*, 2013). *A. thaliana* seedlings are added into six well plates containing a background medium of ½ MS or ½ MS-N on Day 0. On Day 3, when seedlings have completed germination, I can replace the nutrient media with a nitrogenous or amino acid treatment (outlined in Aim1b) and then observe growth phenotypes for up to 12 days in the liquid culture. *A. thaliana* seedlings deplete their maternal carbohydrate stores in approximately three days if grown under low light and in liquid media, conditions that limit photosynthesis (Xiong *et al*, 2013). Eventually, germinated seedlings require an external nitrogen source to continue growing, otherwise they enter a quiescent state. Seedlings will be grown in nitrogen-limiting conditions until they enter a quiescent state, and then protein will be extracted and S6K1 phosphorylation will be measured to identify nitrogen deprivation conditions that turn TOR off. This method is described in Figure 2.4.

Hydroponic boxes: Hydroponic boxes also provide a flexible system to assess plant responses to chemicals, amino acids, or macronutrients (Monte-Bello *et al*, 2018) under sterile and highly reproducible conditions. Using tip boxes, agar of ½ MS or ½ MS-N is filled in each tip hole, and left to solidify. The bottom of the tip box is filled with ½ MS or ½ MS-N. By treating the liquid base of the hydroponic box with varying forms of nitrogen, amino acids, and TOR inhibitors, I will monitor the action of these compounds and their response on plant growth and TOR activity. I have performed preliminary experiments that demonstrate that the hydroponics system works as a delivery system for nitrogen, with plant growth varying based on the levels of exogenous nitrogen added (Figure 2.5). These plants can grow comfortably in this system without needing transfer to soil for 5 weeks, sufficient time to observe how treatments impact plant morphology and physiological responses. Figure 2.5 demonstrates the phenotype of *Arabidopsis* seedlings grown in different liquid concentrations of exogenous nitrogen.

Soilless potting substrate (Turface): I have also developed a system to observe the limiting growth effects of nutrients in the greenhouse, using a Turface watering system. Turface is a soil conditioner, a calcine clay product that is devoid of nitrogen and many other macronutrients that plants need for growth, unlike many other soilless potting substrates, such as vermiculite, that are not nitrogen-free. With the background media of ½ MS or ½ MS-N, I can add a variety of exogenous nitrogen treatments directly to the turface when watering the plants. I have shown that *N. benthamiana* plants successfully grow and flower in this media given ½ MS media and varying levels of KNO_3. *N. benthamiana* is an ideal organism to grow in this system, since the plant is too large to fit in an hydroponics box. In addition to this, *N. benthamiana* is an organism where nitrogen stress conditions result in more developmentally plastic phenotypes. *A. thaliana* produces leaves in a rosette morphology, and does not undergo branching, while the model species in the *Solanaceae* family*, N. benthamiana,* has an upright

morphology with elongated internodes, and undergoes both primary and axillary branching, which N conditions have the potential to affect (Xu *et al,* 2015). Observing these treatment conditions in an additional species will allow us to see if growth or development phenotypes in response to nitrogen treatments are conserved in distantly-related eudicots.

Section 2.3 Results

TOR activity positively correlates with plant growth and nitrogen application

The first illuminating result from these experiments was from the Turface assay with *N. benthamiana* plants. I tested inorganic nitrogen, in the form of KNO_3 and $NH_4^+PO_4^-$, using agriculturally-relevant concentrations (Haboudane *et al*, 2002); KCl and KH_2PO_4 have been added to the solutions, respectively, to control for the impact of introducing additional potassium or phosphate into the plant growth media in the Turface media. In Figure 2.6, the results suggest that TOR activity positively correlates with plant growth and nitrogen application (Figure 2.6). I grew *N. benthamiana* plants in Turface at varying KNO_3 (N) concentrations watered with supplemental ½ Murashige and Skoog (MS) medium without any nitrogen, and collected plant tissue from the plants at 7 weeks. I performed a Western blot to assay S6K1-pThr449 levels, which are low in nitrogen-deprived plants but rescued by inorganic nitrogen supply. This evidence indicates that TOR activity is stimulated by nitrogen application to the plant, and motivated me into further study for establishing more specific delineations of amino acid or nitrogen conditions that activate TOR.

TOR activity positively correlates with exogenous nutrient application under nitrogen starved conditions

The limitations of a Turface assay are several, however, with the plants being grown in a greenhouse with various amounts of sunlight and other environmental variables that are difficult to control and ascertain. These results were tested in tandem with the liquid seedling assay, where young seedlings were used. *A. thaliana* seedlings deplete their maternal carbohydrate stores in approximately three days if grown under low light and in liquid media and thus provide a significantly nitrogen-starved environment to test exogenous treatment in. Figure 2.2 demonstrates the phenotypic response from the seedling (producing many root hairs in search of nutrient in the immediate media).

A.thailana were grown in 6-well plates supplied with standard nutrients except for treatment conditions that exempted nitrogen (1/2MS-N). Treatments of indicated nitrogenous nutrient was added on Day 3, and protein was collected after 2 hours of treatment. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. Figure 2.7 (A) and (B) demonstrate representative western blots of the results of this seedling assay. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these graphs, the control lane is either the 1/2MS lane or the 1/2MS-N lane). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane.

In (A), the total S6K1 bands remain consistent throughout the treatments. The S6K1-pThr449 bands on the western blot demonstrates that the addition of glucose in the +1/2MS media a small impact of S6K phosphorylation compared to the control band of +1/2MS media with no treatment. Similarly, adding NO3- or NH4 to the seedling assay under non-nitrogen limiting conditions have a small increase in impact on TOR activity compared to the control (1/2MS media). However, we can see that the band for S6K1-

pThr449 in the nitrogen starved seedlings (1/2MS-N with no treatment) is significantly less, indicating that nitrogen starvation reduced phosphorylation of S6K, a hallmark of reduced TOR activity. This reduction in TOR activity is rescued (and even increased) by addition of nitrogenous nutrients in the media, such as NO3-, NH4+, and asparagine, with glucose having little to no effect. Figure 2.7 (B) shows similar results.

S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these graphs, the control lane is either the 1/2MS lane or the 1/2MS-N lane). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. In Figure 2.7 (C) illustrates that the addition of nitrogenous nutrients and glucose to a non-nutrient limiting environment increases the phosphorylation of SK1. Graph (D) illustrates that the addition of nitrogenous nutrients and glucose to a nitrogenous nutrient limiting environment increases the phosphorylation of SK1, with glucose having less effect than the other nitrogen-rich substrates. Addition of asparagine, a nitrogen rich amino acid, has more impact than glucose, however, the addition of nitrate and ammonia to the substrate caused the most significant S6K1 phosphorylation, indicative of higher TOR activity. Figure 2.9 summarizes the results of the liquid seedling experiment into a box plot (B) with a representative western (A), illustrating that addition of nitrogenous nutrient sources positively correlate with TOR activity.

To test the impact of asparagine, since our gene of interest is an asparginyl tRNA synthetase (discussed in later chapters), and because asparagine is the most nutrient rich amino acid, I conducted hydroponic box experiments where I could apply asparagine in a nitrogen-starved environment. A.thailana were grown in hydroponic boxes supplied with standard nutrients without nitrogen. Treatments of asparagine (with concentrations indicated) was added on Day 5, and protein was collected after 2 hours of treatment. In Figure 2.8, a representative western blot is shown. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in this blot, the control is no asparagine added-mock lane). As illustrated, the addition of increasing amounts of asparagine to the hydroponic box positively correlates with TOR activity, indicating that S6K1 phosphorylation can be linked to addition of asparagine, nitrogenous amino acid, in nitrogen limiting environments.

Additionally, in Figure 2.8 (B) to test the impact of exogenous asparagine in non-nitrogen limiting conditions, N. benthamiana were grown for five weeks with minimal fertilizer application to support growth. Leaves were infiltrated with 5 mM Asn or mock controls via syringe. 2 h after treatment, leaves were flash frozen in liquid N2. TOR activity positively correlates with nitrogen levels and plant growth, as demonstrated by the larger plants and the phosphorylation of S6K-pT449.

In Figure 2.8 (C) a graph plots the S6K-pT449/Ponceau ratios are shown as a number normalized to a control lane (in this blot, the control is no asparagine added- mock lane)

of the hydroponic asparagine experiment. The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. The plot shows that asparagine exogenous treatments correlate well with increased phosphorylation of S6K, a hallmark of increased TOR activity.

Section 2.4 Discussion

TOR activity positively correlates with plant growth and exogenous nutrient application under both full nutrient conditions and nitrogen starved conditions
Repeated western blots show two key findings: TOR activity positively correlates with plant growth and nitrogen application, and TOR activity positively correlates with exogenous nutrient application under nitrogen starved conditions. The first conclusion confirms what a large body of previous research has already investigated about TOR and plant biology; that plant growth is impacted by TOR activity, and the levels of available nitrogen in the plant media have significant impacts on plant size and development. Low nitrogen conditions result in smaller plants, while higher nitrogen conditions result in growth, leading to TOR activity. The second finding allows us to deepen our understanding of TOR and nitrogen response, since the seedlings I used in this experiment were totally nitrogen starved in the liquid seedling assay. The various media applications also offered insight on how other biomolecules necessary for plant growth (such as differently charged forms of nitrogen, glucose, and amino acids) impact S6K phosphorylation.

However, the mechanism of nucleotide sensing by TOR in plants remains unresolved by these series of experiments. there is no conclusive information or clarity on the mechanism of nitrogen sensing upstream of TOR or on which N metabolites are sensed. Potentially, the nitrogen sensing pathway may act through the Glutamate-Like Receptor (GLR) family of glutamate receptor-like proteins may shine some light on the intricacies of the nitrogen sensing pathway. At the molecular level, the evidence suggests that GLRs act as amino acid-gated Ca2+ channels (Weiland *et al*, 2016), with a wide range of interactive substrates. There's evidence to suggest that the GLR family has an important role throughout the plant in monitoring changes in amino acid distribution, which then can be symptomatic changes in the plant's nitrogen status (Yoshida *et al*, 2016; Li *et al*, 2013). The GLR family, with their role as a general and promiscuous amino acid sensor, could act upstream of the TOR-mediate amino acid signaling pathway (Gent and Forde 2017).

Alternative hypothesis *CHL1* as a nitrate sensor
The only defined nitrogen sensor in plants is a low and high concentration nitrate transporter, *CHL1 (AtNRT1.1* nitrate transporter 1), that triggers a phosphorylation cascade in response to nitrate availability (Ho *et al*., 2009). Thus, an alterative hypothesis for nitrogen and TOR interaction is through the *CHL1* pathway, where *CHL1* could potentially function as a nitrate sensor, and TOR is stimulated by nitrate. It is known that phosphorylated *CHL1* functions as a high-affinity nitrate transporter, whereas dephosphorylated *CHL1* functions as a low-affinity transporter. This phosphorylation switch allows it to function as an ion sensor in soils (Bouguyon *et al*., 2015). To date, there have been no investigations to determine whether TOR coordinates nutrient signaling with the known nitrate transporters, even though TOR functions as a master regulator of nutrient signaling. In addition to endogenously

manipulating asparagine levels, a future experiment could investigate if manipulating the perception of nitrate through the CHL1 pathway affects TOR activity through nutrient sensing. If *CHL1* is the coordinator of nitrogenous nutrients to TOR activity, then it might engage with some of the downstream pathways of TOR activation, or bind to one of the TOR interacting partners to engage TOR activity.

TOR and Nitrogen sensing for sustainable agriculture

The TOR signaling network is a promising candidate for improving agricultural crops. Current agricultural systems are unsustainable, but biotechnological enhancement of crops can make significant contributions to sustainability efforts (Springmann et al., 2018). To date, most studies of nutrient sensing in plants have focused on pathways that detect inorganic nutrients, such as nitrate, ammonium, and phosphate (Chiou and Lin, 2011; Xu et al., 2012). In natural settings, however, plants consume both inorganic and organic nutrients, such as dissolved nitrogen and amino acids (Näsholm et al., 1998). Moreover, once nutrients are absorbed by plants, many species rapidly convert inorganic nitrogen and phosphate to organic forms for transport and metabolism. Therefore, understanding the mechanisms of organic nutrient sensing in plants, including the TOR signaling network, should accelerate efforts to develop sustainable agricultural crop varieties. As TOR positively correlates to nitrogen application, accordingly, genetically or chemically dampening TOR activity could reduce nitrogen demands. Alternatively, for plants grown with excess nitrogen supply, stimulating TOR activity could increase growth rates and crop yields by increasing nitrogen use efficiency. Therefore, rewiring the TOR signaling network in crop species could theoretically contribute to enhancing crop yields and reducing reliance on fertilizers.

Figures

Treatment	Nutrient Supplied	3-letter symbol	Properties	Charge Grouping	Assay Ranges	Testable mM Ranges	in Col-0 at 24C
Positive Control	1/2 MS	1/2 MS	N/A	N/A	N/A	N/A	N/A
Negative Control	1/2 MS	1/2 MS - N	N/A	N/A	N/A	N/A	N/A
Inorganic Nitrogen	Ammonium	NH_4P0_{4-}	N/A	N/A	0.1-5mM	1-20mM	N/A
Inorganic Nitrogen	Nitrate	KNO_3	N/A	N/A	0.1-5mM	1-20mM	N/A
Amino Acid	Asparagine	Asn	Amino acid with polar neutral side chains	Neutral	0.1-5mM	1-50mM	24.72
Amino Acid	Glutamine	Gln	Amino acid with polar neutral side chains	Neutral	0.1-2mM	1-20mM	89.39
Amino Acid	Aspartic acid	Asp	Amino acid with electrically charged side chains	Acidic	0.1-1mM	1-10mM	60.47
Amino Acid	Glutamic acid	Glu	Amino acid with electrically charged side chains	Acidic	0.1-5mM	1-50mM	228.3
Amino Acid	Arginine	Arg	Amino acid with electrically charged side chains	Basic	0.1-0.5mM	1-5mM	8.437

Table 2.1 Nutrient limiting conditions, with listed properties

Nitrogen Controlled Environments	**Nutrient treatment regime applied**	**TOR Outputs Analyzed**
Hydroponic Boxes	All treatments from Table 2.1	S6K1 phosphorylation
Soilless potting substrate	All treatments from Table 2.1	S6K1 phosphorylation
Seedling Liquid Assays	All treatments from Table 2.1	S6K1 phosphorylation

Table 2.2 List of nitrogen controlled environment experiments, with growth and development phenotype assays and TOR outputs analyzed.

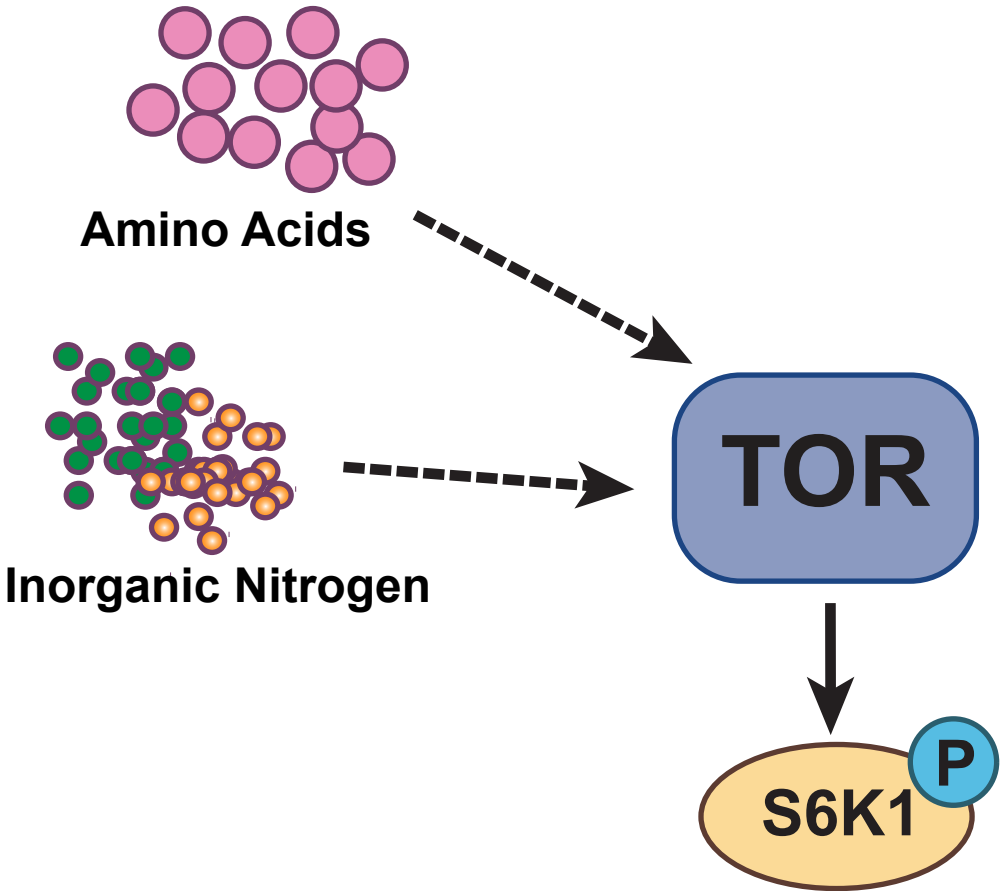


Figure 2.1 Hypothesis of nitrogenous nutrient supply impact on TOR activity

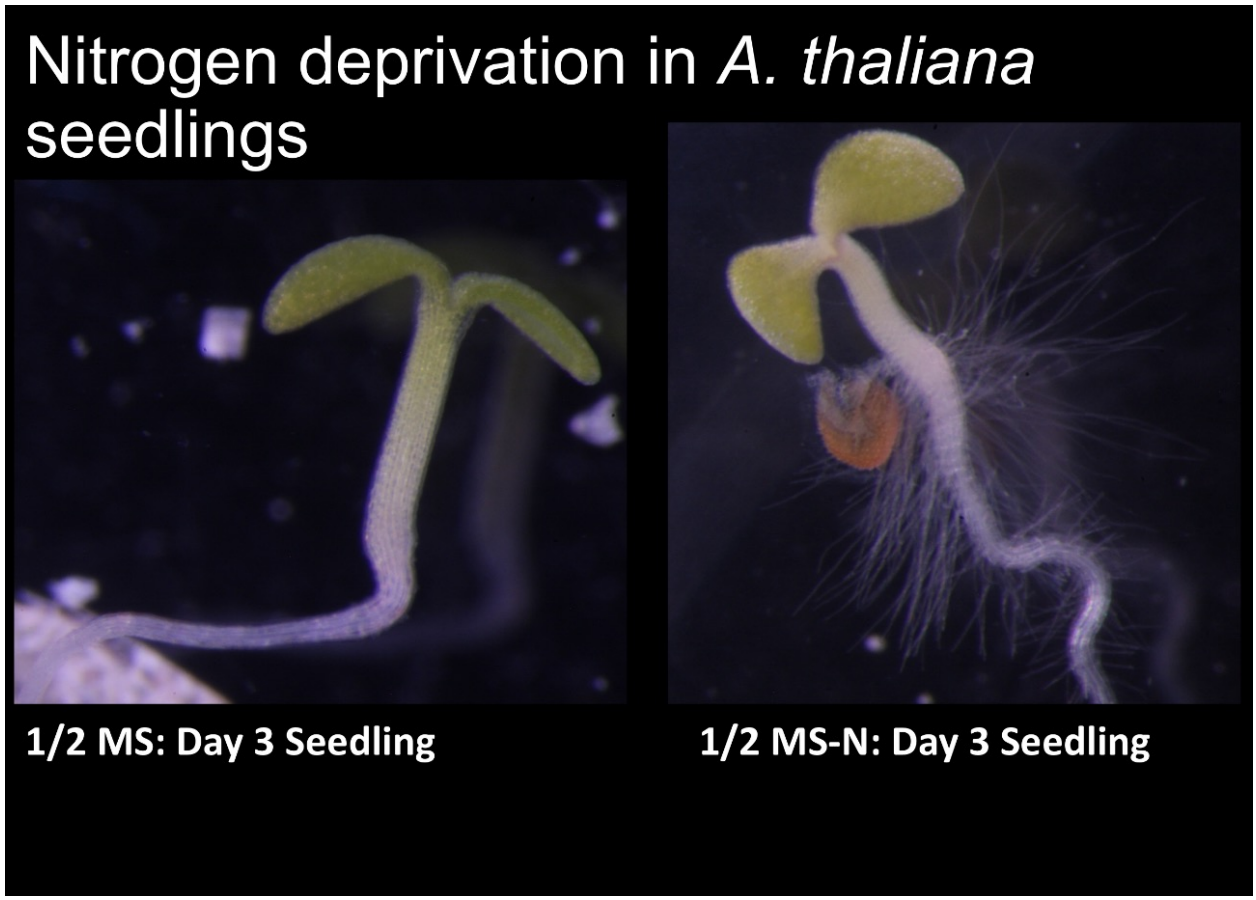


Figure 2.2 Arabidopsis thaliana seedlings grown in full media and in nitrogen deprived conditions

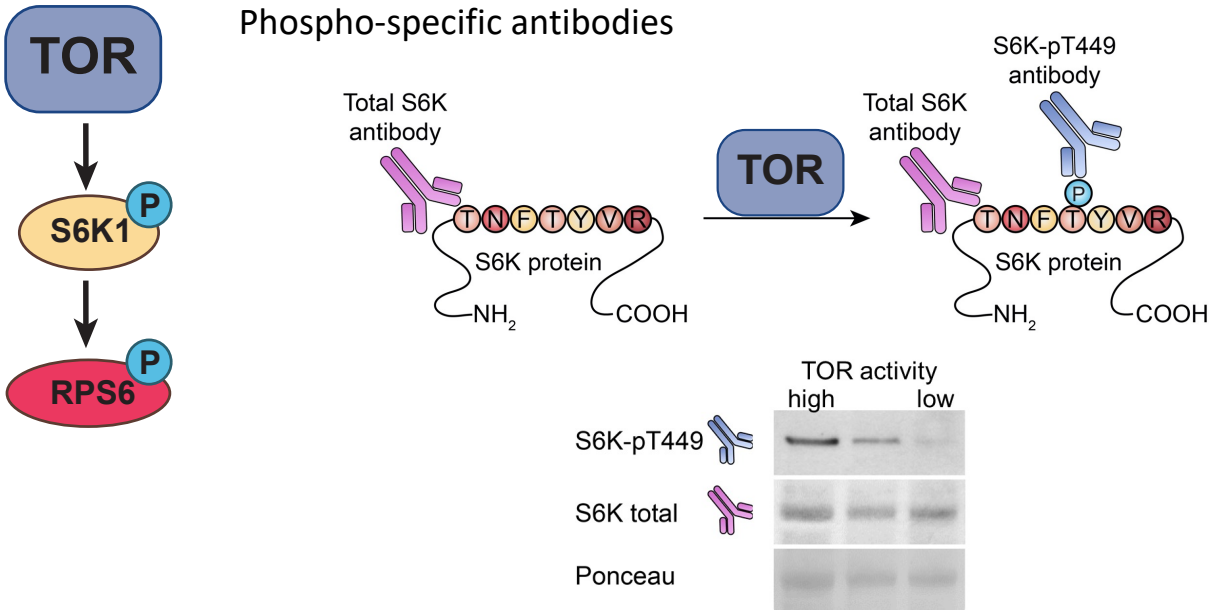


Figure 2.3 Schematic of the function of phosphor-specific antibodies to assay TOR activity

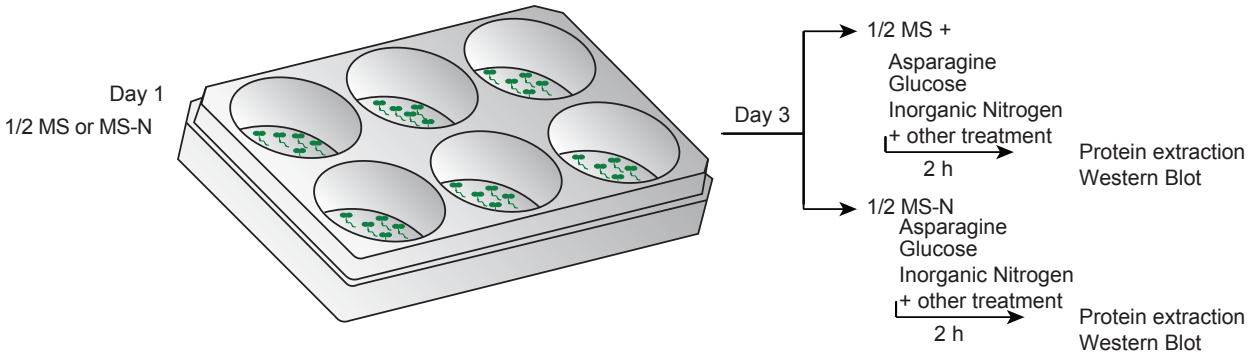


Figure 2.4 Visual representation of the liquid seedling assay with treatment types

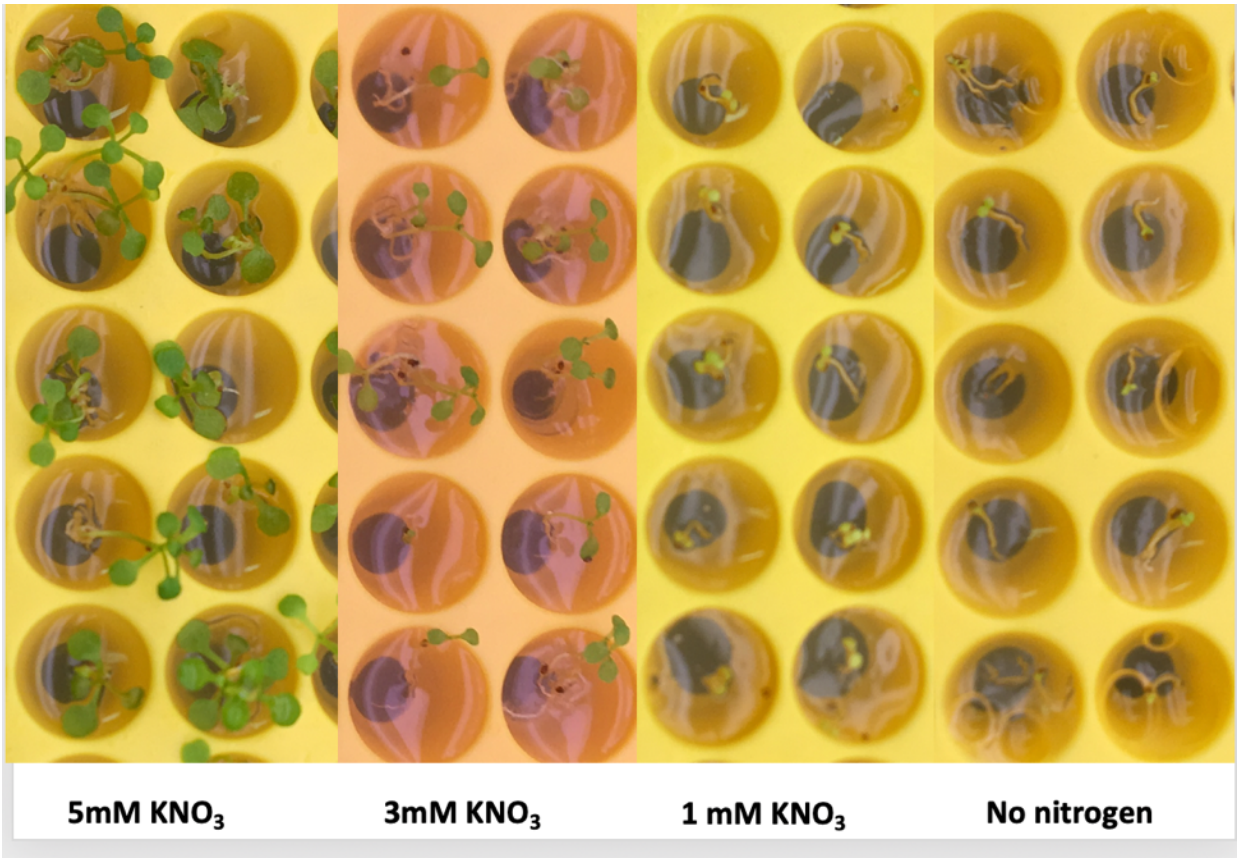


Figure 2.5 *Arabidopsis thaliana* seedlings grown in a flexible hydroponics system developed by Monte-Bello *et al.*, 2018. Seeds are planted in ½ MS-N agar medium, allowed to germinate, then treated once a week with inorganic nitrogen. Seedling growth corresponds positively to the amount of nitrogen added in the liquid medium.

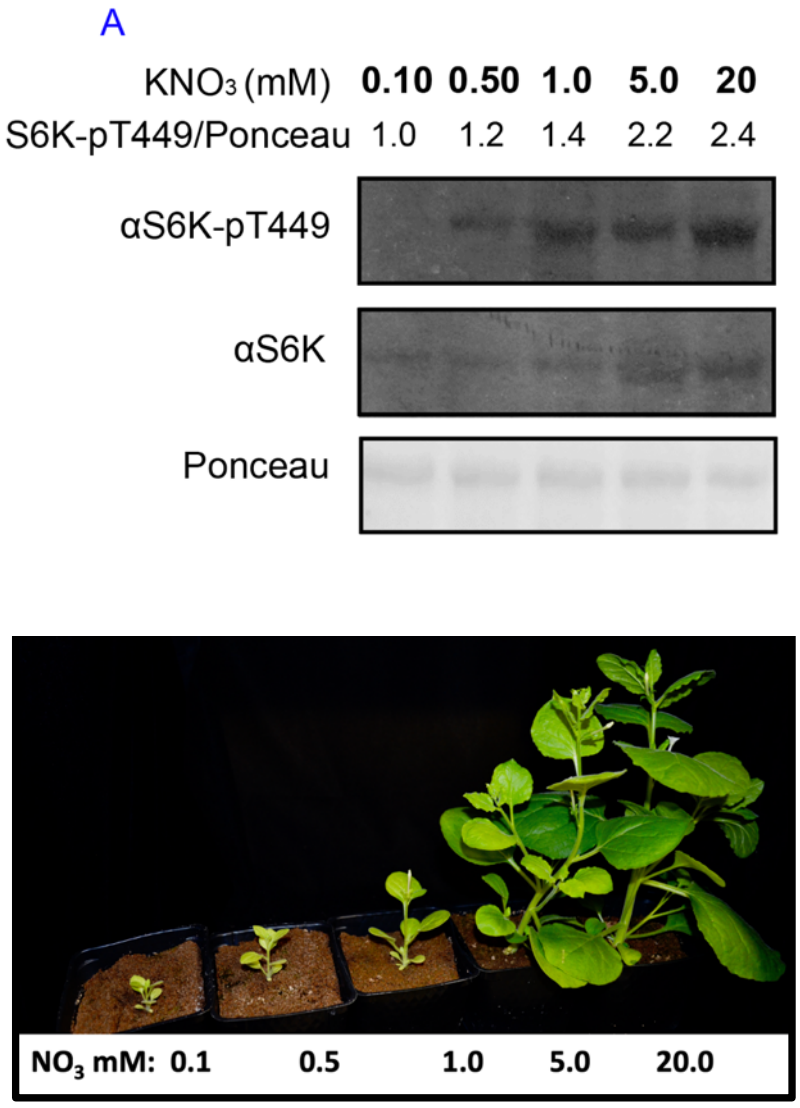
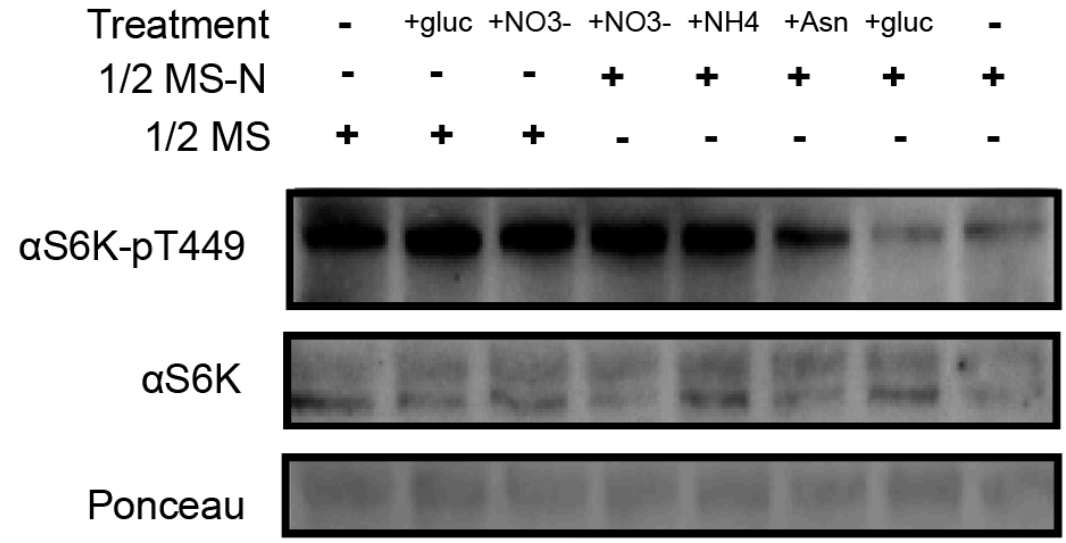


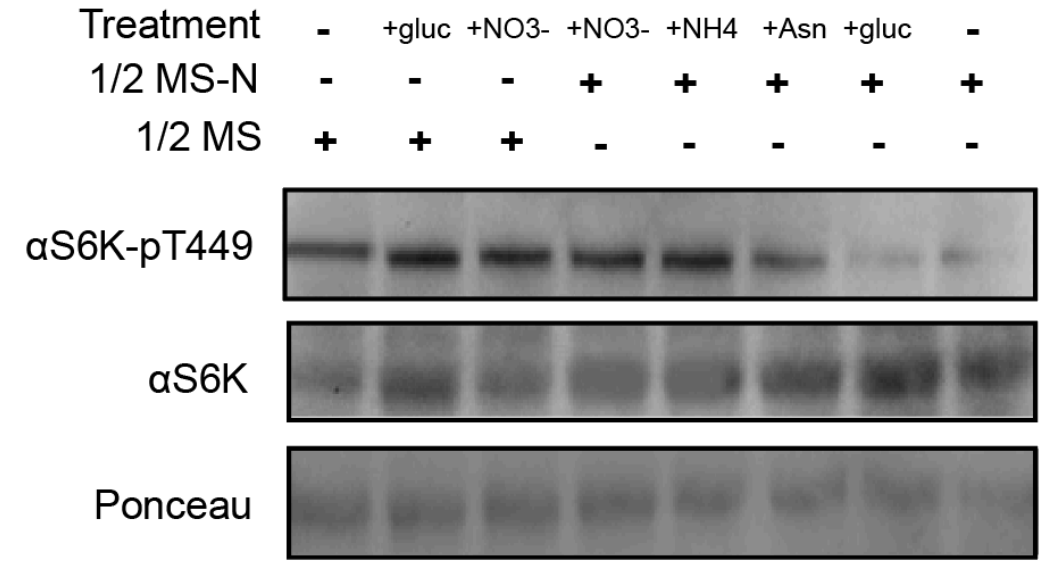
Figure 2.6 TOR positively correlates with plant growth and variable forms of nitrogen application.

(A) *N. benthamiana* was fertilized with varying concentrations of nitrogen once a week for seven weeks. At the seventh week, leaves were flash frozen 2 h after treatment, leaves were flash frozen in liquid N_2. Total protein was extracted in standard buffers, separated by SDS-PAGE, transferred to a membrane, and probed with anti-S6K1-pT449. TOR activity positively correlates with nitrogen levels and plant growth, as demonstrated by the larger plants and the phosphorylation of S6K-pT449.

A


Treatment - +gluc +NO3- +NO3- +NH4 +Asn +gluc -
1/2 MS-N - - - + + + + +
1/2 MS + + + - - - - -
αS6K-pT449
αS6K
Ponceau

B


Treatment - +gluc +NO3- +NO3- +NH4 +Asn +gluc -
1/2 MS-N - - - + + + + +
1/2 MS + + + - - - - -
αS6K-pT449
αS6K
Ponceau

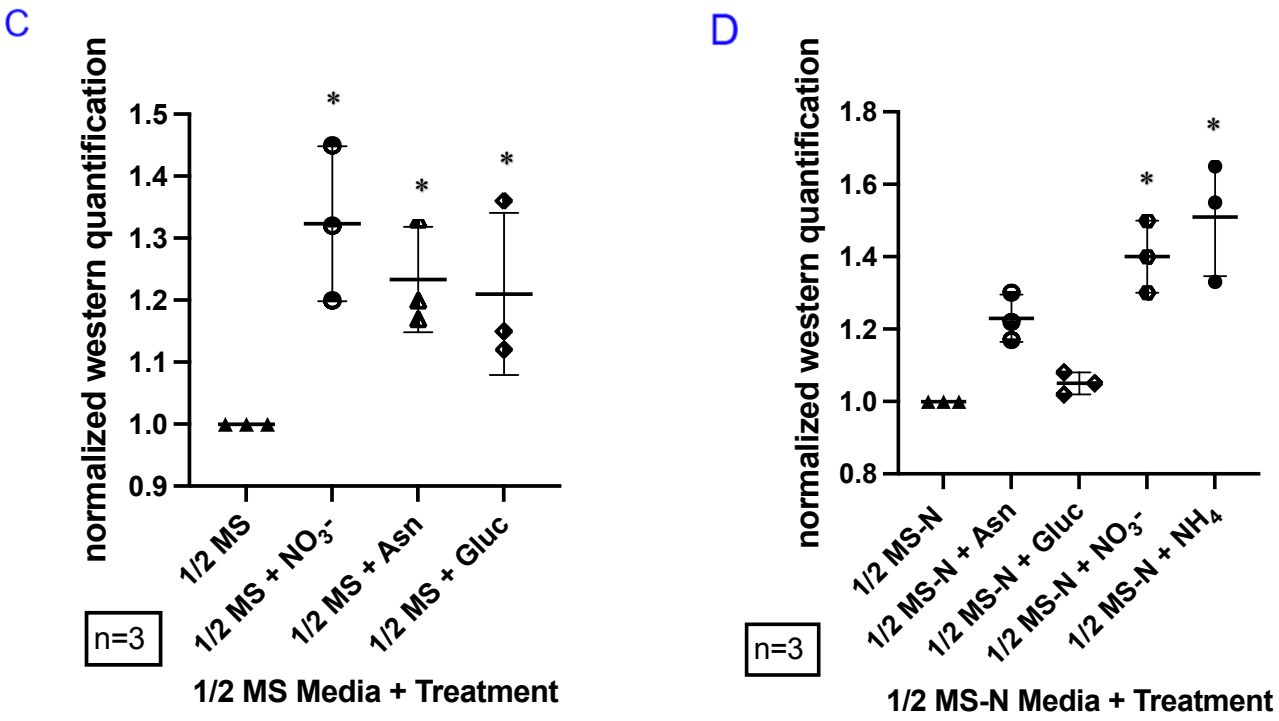


Figure 2.7 Exogenous nutrient western blots and graphs
(A-B*) A.thailana* were grown in 6-well plates supplied with standard nutrients except for treatment conditions that exempted nitrogen (1/2MS-N). Treatments of indicated nitrogenous nutrient was added on Day 3, and protein was collected after 2 hours of treatment. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control.
(C-D) S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these graphs, the control lane is either the 1/2MS lane or the 1/2MS-N lane). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated three times; representative results are shown here. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

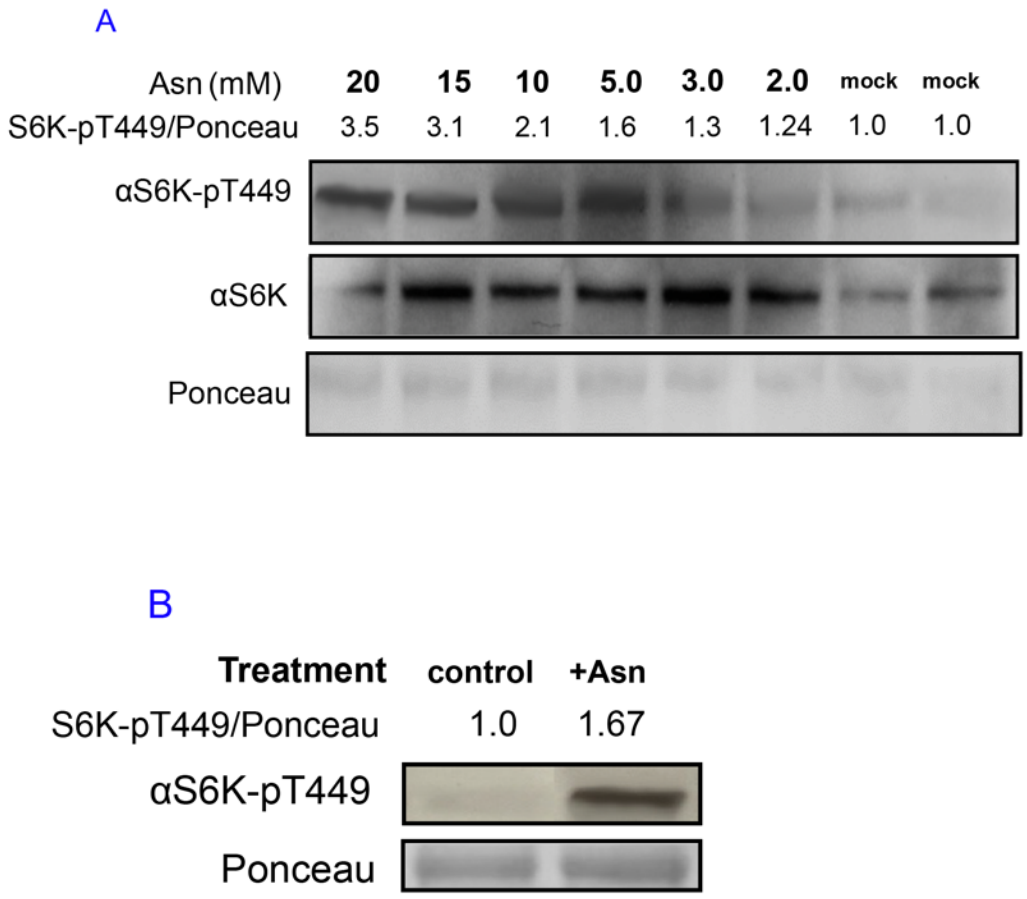


Figure 2.8 Asparagine Western Blots

(A) *A.thailana* were grown in hydroponic boxes supplied with standard nutrients without nitrogen. Treatments of asparagine (with concentrations indicated) was added on Day 5, and protein was collected after 2 hours of treatment. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in this blot, the control is no asparagine added- mock lane).

(B) N. benthamiana were grown for five weeks with minimal fertilizer application to support growth. Leaves were infiltrated with 5 mM Asn or mock controls via syringe. 2 h after treatment, leaves were flash frozen in liquid N2. Total protein was extracted in standard buffers, separated by SDS-PAGE, transferred to a membrane, and probed with anti-S6K1-pT449. TOR activity positively correlates with nitrogen levels and plant growth, as demonstrated by the larger plants and the phosphorylation of S6K-pT449.

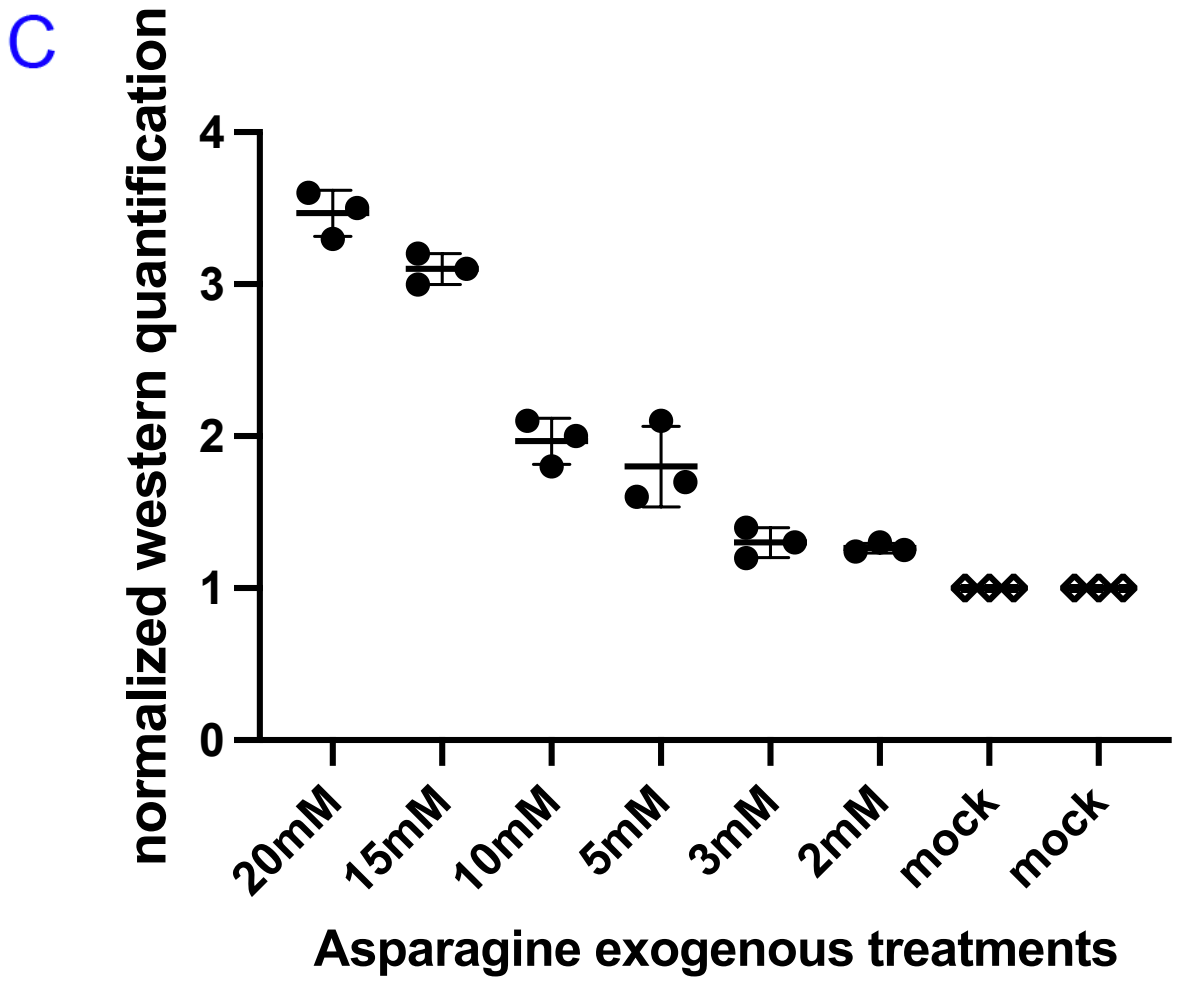


Figure 2.8 Asparagine Western Blots
(C) *A.thailana* were grown in hydroponic boxes supplied with standard nutrients. Treatments of asparagine (with concentrations indicated) was added on Day 5, and protein was collected after 2 hours of treatment. Graph plots the S6K-pT449/Ponceau ratios of the in the are shown as a number normalized to a control lane (in this blot, the control is no asparagine added- mock lane). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated three times.

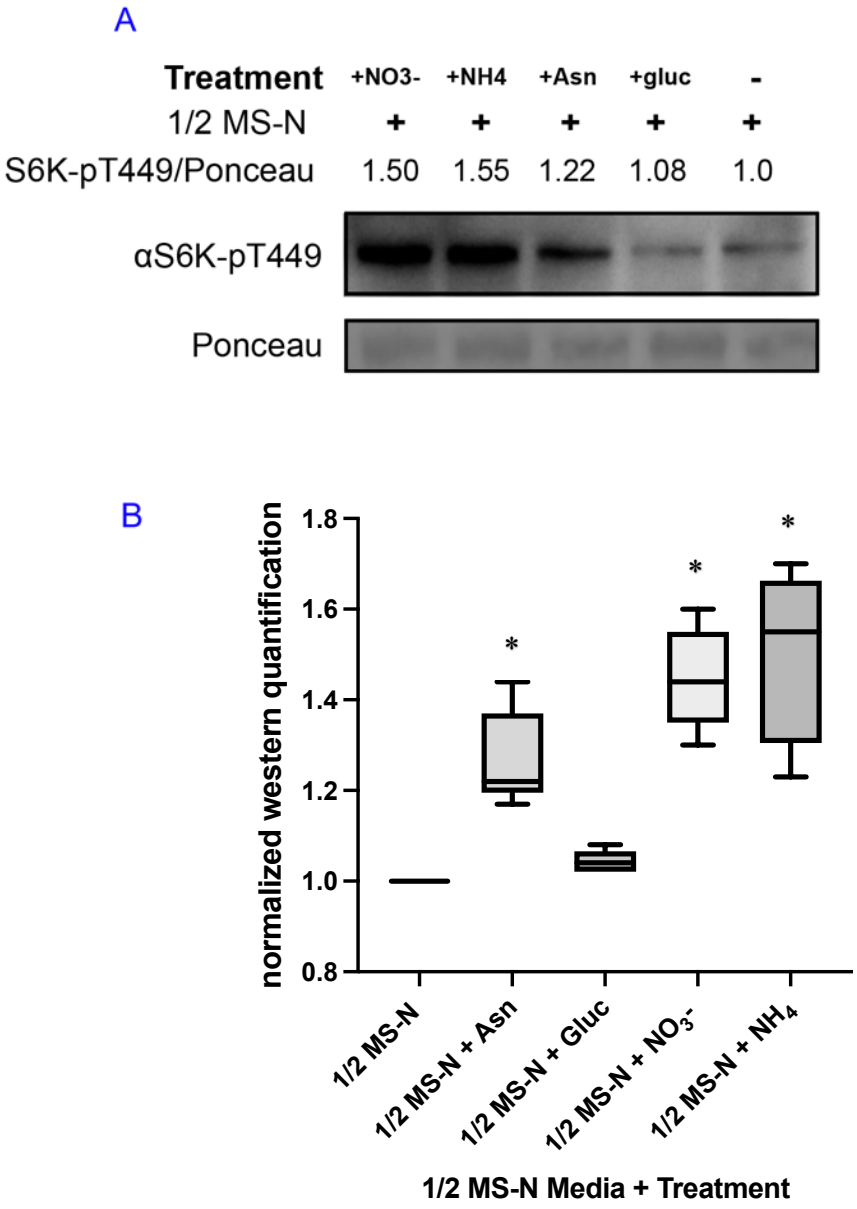


Figure 2.9 Nitrogen starved nitrogenous nutrient western blots
(A) *A.thailana* were grown in 6-well plates supplied with standard nutrients except for nitrogen (1/2MS-N). Treatments of indicated nitrogenous nutrient was added on Day 3, and protein was collected after 2 hours of treatment. TOR activity was assayed using Western blots against S6K-pT449 with Ponceau staining shown as a loading control.
(B) S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these graphs, the control lane is the 1/2MS-N lane with no treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated five times; representative results are shown here. Asterisks indicate t-tests performed.

Chapter Three
We're Amino Acids too!

"Fortunately, where reason failed, blind panic served well enough."
N.K. Jemisin



Section 3.1 Elucidate the impact of genetically manipulating endogenous levels of specific amino acids on TOR activity

Introduction

Unlike animals, plants synthesize all 20 of the amino acids, and have complex metabolic systems to produce and transport amino acids throughout the plant. One of the limitations of only investigating the exogenous application of nitrogenous nutrient sources and the impact on TOR activity via western blot is that it is not possible to know all the possible downstream effects of plant amino acid uptake and biosynthesis when adding exogenous amino acids and nitrogenous nutrients. The experiments in the previous chapter may guide our understanding of TOR activity as it responds to external output, but will not be sufficient to conclude that a particular metabolite is shifting TOR activity without looking at the plant's endogenous metabolism.

Manipulation of the endogenous metabolism of amino acids might have an effect on *TOR* activity. The results of the previous amino acid assays performed illuminate which synthetases to manipulate (Asn, Gln, Leu, etc) in the internal amino acid pathway to test their impact on *TOR.* Plants have deeply synchronized metabolomic systems, as demonstrated in Figure 3.1. Figure 3.1 provides a brief overview of the interactions of the nitrogen assimilation pathways in plants, and how their respective synthetases function to create endogenous pools of amino acids.

As stated previously, the Brunkard Lab has also found the external addition of 5.0 mM asparagine to *Arabidopsis* seedlings rapidly induces TOR activity, as measured by S6K1-Thr449 phosphorylation, in Figure 2.8. If the exogenous application of asparagine effects TOR activity, it logically follows that the manipulation of internal asparagine levels has an impact on TOR as well. In summary, the hypothesis is that by increasing the level of asparagine within the plant, TOR activity will also increase. In preliminary findings, TOR consistently and strongly represses expression of *ASN1,* the glutamine-dependent asparagine synthase. Upon glucose activation of TOR in quiescent seedlings, there is a 50-fold repression of *ASN1* transcript levels, compared to mock treated plants*,* and in plants treated with AZD-8055, a TOR inhibitor, there is a 13-fold decrease in *ASN1* transcript levels, compared to mock treated plants (Xiong *et al*, 2013). *ASN1, ASN2,* and *ASN3* are three genes that encode asparagine synthetases in A. *thaliana*. The asparagine synthetase is a chiefly cytoplasmic enzyme that generates asparagine from aspartate (Gaufichon *et al*., 2010). Ammonium is a form of inorganic nitrogen taken up from the soil or derived from several catabolic pathways, and is assimilated into glutamine and asparagine. The glutamine-dependent ASPARAGINE SYNTHETASE 1 (ASN1) catalyzes ammonium assimilation into asparagine through the hydrolysis of ATP and the use of glutamine as an amino donor (Larsen, 1999). In wild-type plants, *ASN1* expression during development correlates positively with free Asn levels in Arabidopsis, and in mutants that overexpress *ASN1,* Asn levels rise dramatically in all forms of plant tissue (Lam *et al.*, 2003). The *ASN* genes alone are not the full picture of asparagine metabolism in *A. thaliana; Aspga1* and *Aspgb1* are two genes that encode asparaginases, which catalyze the degradation of L-asparagine to L-aspartic acid and ammonia, and which have been

implicated in the catabolism of transported asparagine in the sink tissues of plants (Ivanov *et al*., 2012). I hypothesize that TOR activity positively correlates with endogenous asparagine levels, as modulated by ectopic expression of asparagine synthetases or asparaginases.

Section 3.2 Materials and Methods

Test the impact of manipulating asparagine metabolism through asparagine synthases and asparaginases on TOR activation and TOR-related outputs

Knockouts of *ASN* genes have not been isolated in *A. thaliana*, suggesting that each *ASN* gene is essential for plant development. *A. thaliana* mutants that lack the asparaginases (*Aspga1, Aspgb1)* develop normally, but exhibit enhanced root growth inhibition when supplied high concentrations of exogenous asparagine (Ivanov, 2011). To develop overexpression lines for the asparagine synthetases and asparaginases, I cloned several plasmids containing a 35S promoter, derived from cauliflower mosaic virus (CaMV) (Hull *et al,* 2000), to overexpress asparagine synthetase and L-asparaginases reporters of yellow fluorescent protein (YFP) and green fluorescent protein (GFP) (Table 3). These epitope tags are either an N-terminal YFP tag or a GFP C-terminal tag (Table 3). I used alternatively tagged vectors to control for possible negative effects of the N- or C-terminal tags to develop a variety of transgenic plants, listed in Table 3.1.

Transient expression with Agrobacterium: Agroinfiltrated *N. benthamiana* plants with constructs described in Table 3.1. Leaf samples are visualized using confocal fluorescence microscopy 48 hours after agroinfiltration, when transient gene expression is high. Additional samples are collected for analysis using the output methods described in the previous section (protein extraction) to assay TOR activity in these transient expression experiments.

Stable transgenics: I stably transformed these constructs (Table 3.1) in *Arabidopsis*. Utilizing the floral dip method in *A. thaliana*, it is possible to generate and screen several hundred independent transgenic lines with various levels of transgene expression.

Measurement of modulating asparagine metabolism on TOR activity: After generating these overexpression lines and establishing TOR-limiting conditions, TOR activity and other output assays described are performed on WT and mutant plants under TOR-activating and inactivation conditions to study transcriptional and translation effects of TOR and the asparagine metabolism genes. Collectively, the previous investigation into the nitrogenous nutrient story will establish limiting growth conditions where TOR is off, and then investigate nutrient stimulus that will activate TOR in wild type plants. After observing these growth limiting conditions, the data will help suggest how exogenous conditions can influence the TOR activity. Aim 2 seeks to recapitulate those results genetically. Again, these experiments specifically engaging asparagine metabolism because the Brunkard Lab's preliminary evidence suggests that asparagine is a potential activator of TOR.

Section 3.3 Results

I cloned the suite of *ASN* genes present in *A. thaliana* (*ASN1, ASN2, ASN3*) and the *L-ASPARAGINASES* (*Aspga1, Aspgb1)*. *Arabidopsis thaliana* plants were transformed to stably or transiently overexpress these genes. In plants, genes can either be stably overexpressed in transgenic plants or they can be transiently overexpressed in leaves by infiltration with *Agrobacterium*. Both techniques will be used to build strong genetic evidence that endogenous asparagine accumulation affects TOR activity. My hypothesis was that if asparagine is the primary amino acid involved in activating TOR in plants, then it is predicted that overexpressing L-asparaginase will decrease TOR activity, as measured by S6K1-Thr449 phosphorylation.

Constructs in Table 3.1 that were agroinfiltrated or stably transformed were confirmed by confocal microscopy. These representative images demonstrate the localization of each protein-GFP or protein-YFP fusion. pEG104 contains the YFP-protein, while pEG103 contains the GFP-protein. The green color indicates the localization of the GFP or YFP expression, while the magenta represents chlorophyll autofluorescence. They allowed me to visualize protein in the cytosol, organelles, and nucleus of the plant cell to confirm the successful transformation of the over expression constructs tagged with GFP or YFP.

To study the impact of overexpressing the suite of asparaginases in plants, agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag (Figure 3.5). Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane.

In Figure 3.5 (A) the western blot of asparagine synthetases compared against *NRS1* constructs (which will be discussed in a later chapter). Asparagine synthetases did not significantly impact SK6 phosphorylation. In Figure 3.5 (B) *NRS1* overexpression constructs compared against mock infiltrations, illustrating that asparagine synthetases have western blot bands more similar to mock infiltrations.
In (C), the normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane plotted in a box graph. Different asparagine synthetases seem to have very little impact on TOR activity in comparison to the control.

Next, I stably transformed *A. thaliana* with the constructs for agroinfiltration and western blotting to study any observable phenotypes. Figure 3.6 contains representative images of the A. thaliana stable transgenics phenotypes. (A-C) The phenotype of 35S:PRO: ASN1: pEG103, 35S:PRO: ASN3: pEG103 is very similar to a wild-type Col-0 plant. However, although some 35S:PRO: ASN2: pEG103 transgenics have a relatively normal phenotype, some transgenic plants became extremely dwarfed. Figure 3.8 demonstrates

the relative size comparison of two Col-0 wild type plant and the dwarf *A. thailana* 35S:PRO: ASN2: pEG103 transgenic plant.

Figure 3.7 contains representative images of the *A. thaliana* stable transgenics phenotypes. (A-C) The phenotype of 35S:PRO: ASN1: pEG103, 35S:PRO: ASN3: pEG103 is very similar to a wild-type Col-0 plant. Figure 3.6 contains representative images of the A. thaliana stable transgenics phenotypes. (A-C) The phenotype of 35S:PRO: Aspg1a: pEG103, 35S:PRO: Aspg1b: pEG103 is very similar to a wild-type Col-0 plant. However, although some 35S:PRO: Aspg1a: pEG103 transgenics have a relatively normal phenotype, some plants had leaves that were smaller than usual. Leaf width and length measurements were performed on all the transgenics at five weeks of plant growth. Figure 3.9 plots the results. However, the phenotypes of stably transformed plants of did not provide any obvious insight on the developmental and physiological impact of overexpressing asparaginases in *A. thailiana.* Further characterization experiments were not within the scope of this thesis.

In summary, my hypothesis was not correct; that if asparagine is the primary amino acid involved in activating TOR in plants, then it is predicted that overexpressing L-asparaginase will decrease TOR activity, as measured by S6K1-Thr449 phosphorylation. Instead, overexpressing the suite of asparaginases had little impact on TOR activity. In the interest of pursuing an alternative sensor, more in-depth phenotypic of the asparagine mutants were not performed in this dissertation and existing mutants did not offer any clear insights on how TOR activity was impacted in the stably transformed plants.

Section 3.4 Discussion

Manipulation of Asparagine Metabolism has no impact on S6K1 phosphorylation

The complexities of internal nitrogen sensing by TOR are outlined in Figure 3.1. All these various components of synthetases, amino acids, and inorganic nitrogen make up Alanine, aspartate, and glutamine metabolism, as well as arginine biosynthesis and asaparagine metabolism in a plant. To highlight ASN1, ASN2, and ASN3 are three genes that encode Asparagine synthetases in Arabidopsis thaliana. The asparagine synthetase is a chiefly cytoplasmic enzyme that generates asparagine from aspartate (Gaufichon et al 2010). Ammonium is a form of inorganic nitrogen derived from several metabolic pathways, and is assimilated into glutamine, glutamine, asparagine. The glutamine-dependent asparagine synthase (ASN1) catalyzes ammonium assimilation into asparagine through the hydrolysis of ATP the usage of glutamine or ammonium as an amino donor (Larsen, 1999). However, the hypothesis that TOR activity positively correlates with endogenous asparagine levels resulting from activity of asparagine synthetases and asparaginases was not proven to be true in this series of experiments, demonstrating instead that *NRS1* has the most significant impact on TOR activity and S6K1 phosphorylation.

The literature on how amino acid metabolism on TOR activation remains relatively vague. TOR activation through the phosphorylation of S6K1, which is phosphorylated by active TOR and dephosphorylated by protein phosphatase 2A when TOR is inactive (Peterson et al, 1999), is implicated in the pathway of amino acid signaling; experiments that repress TOR or components of the TOR complex observe major shifts in metabolism that relate to amino acid accumulation, resulting in massive increase in the levels of free amino acids (Xiong *et al.*, 2013), but no clear mechanism for this increase has been discovered. Chemical intervention targeting has also elucidated that homeostasis of branched-chain amino acids is critical for the activity of TOR signaling in *Arabidopsis* (Dinkeloo et al, 2018), and implies that further investigation of the uncharted downstream subcellular processes of TOR signaling might identify plant-specific amino acid transporter-sensors involved in TOR regulation.

Recent studies have confirmed that plant TOR activity is sensitive to disruptions in amino acid synthesis, but these studies have not determined which amino acid(s) TOR is sensing (Liu et al, 2021). For example, when Arabidopsis seedlings grown under extreme nitrogen starvation are supplied with nitrate, ammonium, and any amino acid except arginine, phenylalanine, proline, tryptophan, or tyrosine, TOR is activated—but since amino acids are rapidly interconverted and metabolized after uptake, this does not indicate that all fifteen amino acids are actually sensed by TOR. This indicates that there are yet-to-be discovered mechanisms through TOR may be activated by individual amino acids in plants.

In another new study that showcases the concert of inorganic and organic nitrogen sources' impact on TOR, researchers discovered that independent of nitrogen assimilation, nitrate and ammonium function as primary nitrogen signals to activate TOR in the Arabidopsis leaf primordium (Liu et al, 2021). 15 proteinogenic amino acids are also able to activate TOR, and the first AAs generated from plant specific nitrogen

assimilation (glutamine), sulfur assimilation (cysteine), and glycolate cycle (glycine), exhibit the highest potency. Interestingly, nitrate, ammonium, and glutamine all activate the small GTPase Rho-related protein from plants 2 (ROP2), and constitutively active ROP2 restores TOR activation under nitrogen-starvation conditions. These findings suggest that specific evolutionary adaptations of the nitrogen-TOR signaling pathway occurred in plant lineages, and ROP2 can integrate diverse nitrogen and hormone signals for plant TOR activation (Liu et al, 2021).

In *Chlamydomonas,* TOR inhibition triggers new amino acid synthesis by enhancing nitrogen assimilation (Umarah et al, 2018), which leads to new areas of inquiry around the conservation of TOR signaling pathways among land plants and their ancestors, as the TOR signaling pathway between organisms has demonstrated significant divergence between plants and animals.

Since the results from this section demonstrate that although external application of asparagine has an impact on TOR activity, the asparaginases that are responsible for the endogenous maintenance of metabolic levels within a plant have little impact on S6K1 phosphorylation. This indicates that the mechanisms by which plants regulate their internal amino acid levels probably operates independently from TOR signaling.

The next section will address the role of *NRS1* in the TOR-signaling pathway.

Figures

Gene Name	TAIR ID	Description	GFP (C-terminally tagged constructs)	YFP (N-terminally tagged) Constructs
ASN1	AT3G47340	glutamine-dependent asparagine synthase 1	*35S:$_{PRO}$: ASN1: pEG103*	*35S:$_{PRO:}$ ASN1: pEG104*
ASN2	AT5G65010	glutamine-dependent asparagine synthetase 2	*35S:$_{PRO}$: ASN2: pEG103*	*35S:$_{PRO:}$ ASN2: pEG104*
ASN3	AT5G10240	glutamine-dependent asparagine synthetase 2	*35S:$_{PRO}$: ASN3: pEG103*	*35S:$_{PRO:}$ ASN3: pEG104*
Aspga1	AT5G08100	asaparaginase 1 catalyzes the degradation of L-aspargine to L-aspartic acid	*35S:$_{PRO:}$ Asp1ga: pEG103*	*35S:$_{PRO:}$ Asp1ga: pEG104*
Aspgb1	AT3G16150	asaparaginase 2 catalyzes the degradation of L-aspargine to L-aspartic acid	*35S:$_{PRO:}$ Asp1gb: pEG103*	*35S:$_{PRO:}$ Asp1gb: pEG104*

Table 3.1 List of genes referenced

Plant	Observed Resistant Progeny (OR)	Expected Value (R)	Observed Suceptible Progeny (OS)	Expected Value (S)	(OR-R)^2/R	(OS-S)^2/S	(OR-R)^2/R + (OS-S)^2/S = X^2
ASN1 103 (2)	36	34.5	10	11.5	0.06521791	0.1956521 7	0.260869565
ASN1 103 (9)	39	35.25	8	11.75	0.39893617	1.1968085 1	1.595744681
ASN1 103 (1)	23	24	9	8	0.04166667	0.125	0.166666667
ASN1 103 (11)	32	30.75	9	10.25	0.05081308	0.1524390 2	0.203252033
ASN1 103 (12)	23	24	9	8	0.04166667	0.125	0.166666667
ASN2 103 (13)	37	33	7	11	0.48484885	1.4545454 5	1.939393939
ASN2 103 (11)	36	34.5	10	11.5	0.06521791	0.1956521 7	0.260869565
ASN2 103 (22)	39	35.25	8	11.75	0.39893617	1.1968085 1	1.595744681
ASN2 103 (33)	32	35.25	15	11.75	0.29964539	0.8989361 7	1.19858156
ASN2 103 (28)	32	28.5	6	9.5	0.42982456 1	1.2894736 8	1.719298246
ASN2 103 (12)	26	27	10	9	0.03703703 7	0.1111111 1	0.148148148
ASN2 103 (23)	45	38.25	6	12.75	1.19117647 1	3.5735294 1	4.764705882
ASN2 103 (29)	32	30.75	9	10.25	0.05081308	0.1524390 2	0.203252033
ASN2 103 (9)	23	24	9	8	0.04166667	0.125	0.166666667
ASN2 103 (26)	33	32.25	10	10.75	0.01744186	0.0523255 8	0.069767442
ASN2 103 (16)	26	23.25	5	7.75	0.32526881 7	0.9758064 5	1.301075269

ASN2 103 (21)	45	38.25	6	12.75	1.191176471	3.573529411	4.764705882
ASN2 103 (14)	32	30.75	9	10.25	0.050813008	0.152439024	0.203252033
ASN2 103 (17)	26	24	6	8	0.166666667	0.5	0.666666667
ASN2 103 (31)	33	30.75	8	10.25	0.164634146	0.493902439	0.658536585
ASN2 103 (18)	36	34.5	10	11.5	0.065217391	0.195652173	0.260869565
ASN2 103 (20)	39	35.25	8	11.75	0.398936171	1.196808511	1.595744681
ASN2 103 (15)	49	48	15	16	0.020833333	0.0625	0.083333333
ASN2 103 (2)	23	24	9	8	0.041666667	0.125	0.166666667
ASN2 103 (10)	32	31.5	10	10.5	0.007936508	0.023809524	0.031746032
ASN2 103 (37)	55	48.75	10	16.25	0.801282051	2.403846154	3.205128205
ASN2 103 (5)	23	27.75	14	9.25	0.813063063	2.439189189	3.252252252
ASN2 103 (7)	32	30.75	9	10.25	0.050813008	0.152439024	0.203252033
ASN2 103 (6)	29	27	7	9	0.148148148	0.444444444	0.592592593
ASN2 103 (4)	34	31.5	8	10.5	0.198412698	0.595238095	0.793650794
ASN3 104 (2)	23	24	9	8	0.041666667	0.125	0.166666667
Asplga 103 (9)	33	28.5	5	9.5	0.710526316	2.131578947	2.842105263
Asplga 103 (6)	29	27	7	9	0.148148148	0.444444444	0.592592593
Asplga 103 (2)	34	31.5	8	10.5	0.198412698	0.595238095	0.793650794
Asplga 103 (1)	39	35.25	8	11.75	0.398936171	1.196808511	1.595744681

Asplga 103 (4) (21 actually)	33	29.25	6	9.75	0.480769231	1.4423076 9	1.923076923
Asplga 103 (13)	29	27	7	9	0.148148148	0.4444444 4	0.592592593
Asplga 103 (29)	34	31.5	8	10.5	0.198412698	0.5952381	0.793650794
Asplga 103 (12)	23	24	9	8	0.041666667	0.125	0.166666667
Asplga 103 (2)	32	30.75	9	10.25	0.050813008	0.1524390 2	0.203252033
Asplga 103 (22)	33	29.25	6	9.75	0.480769231	1.4423076 9	1.923076923
Asplga 103 (7)	29	27	7	9	0.148148148	0.4444444 4	0.592592593
Asplga 103 (20)	34	31.5	8	10.5	0.198412698	0.5952381	0.793650794
Asplga 103 (30)	39	36.75	10	12.25	0.137755102	0.4132653 1	0.551020408
Asplga 103 (18)	32	35.25	15	11.75	0.299645439	0.8989361 7	1.19858156
Asplga 103 (16)	26	27	10	9	0.037037037	0.1111111 1	0.148148148
Asplga 103 (26)	45	38.25	6	12.75	1.191176471	3.5735294 1	4.764705882
Asplga 103 (25)	32	30.75	9	10.25	0.050813008	0.1524390 2	0.203252033
Asplga 103 (14)	26	24	6	8	0.166666667	0.5	0.666666667
Asplga 103 (3)	33	30.75	8	10.25	0.164634146	0.4939024 4	0.658536585
Asplga 103 (10)	39	35.25	8	11.75	0.398936171	1.1968085 1	1.595744681
Asplga 103 (5)	32	35.25	15	11.75	0.299645439	0.8989361 7	1.19858156
Asplga 103 (19)	23	24	9	8	0.041666667	0.125	0.166666667
Asplgb 103 (1)	26	25.5	8	8.5	0.009803922	0.0294117 6	0.039215686

Asplgb 103 (6)	36	34.5	10	11.5	0.065 2173 91	0.195 6521 7	0.260869565
Asplgb 103 (3)	23	24	9	8	0.041 6666 67	0.125	0.166666667
Asplgb 103 (7)	32	31.5	10	10.5	0.007 9365 08	0.023 8095 2	0.031746032
Asplgb 103 (16)	26	27	10	9	0.037 0370 37	0.111 1111 1	0.148148148
Asplgb 103 (6)	45	38.25	6	12.75	1.191 1764 71	3.573 5294 1	4.764705882
Asplgb 103 (2)	35	32.25	8	10.75	0.234 4961 24	0.703 4883 7	0.937984496
Asplgb 103 (13)	23	22.5	7	7.5	0.011 1111 11	0.033 3333 3	0.044444444
Asplgb 103 (20)	32	30.75	9	10.25	0.050 8130 08	0.152 4390 2	0.203252033
Asplgb 103 (10)	26	24	6	8	0.166 6666 67	0.5	0.666666667
Asplgb 103 (7)	48	42	8	14	0.857 1428 57	2.571 4285 7	3.428571429
Asplgb 103 (18)	34	31.5	8	10.5	0.198 4126 98	0.595 2381	0.793650794
Asplgb 103 (9)	23	24	9	8	0.041 6666 67	0.125	0.166666667
Asplga 103 (24)	36	34.5	10	11.5	0.065 2173 91	0.195 6521 7	0.260869565
Asplgb 103 (5)	39	35.25	8	11.75	0.398 9361 7	1.196 8085 1	1.595744681

Table 3.2 Transgenic segregation analysis of stably transformed asparagine synthetases in *A. thaliana*

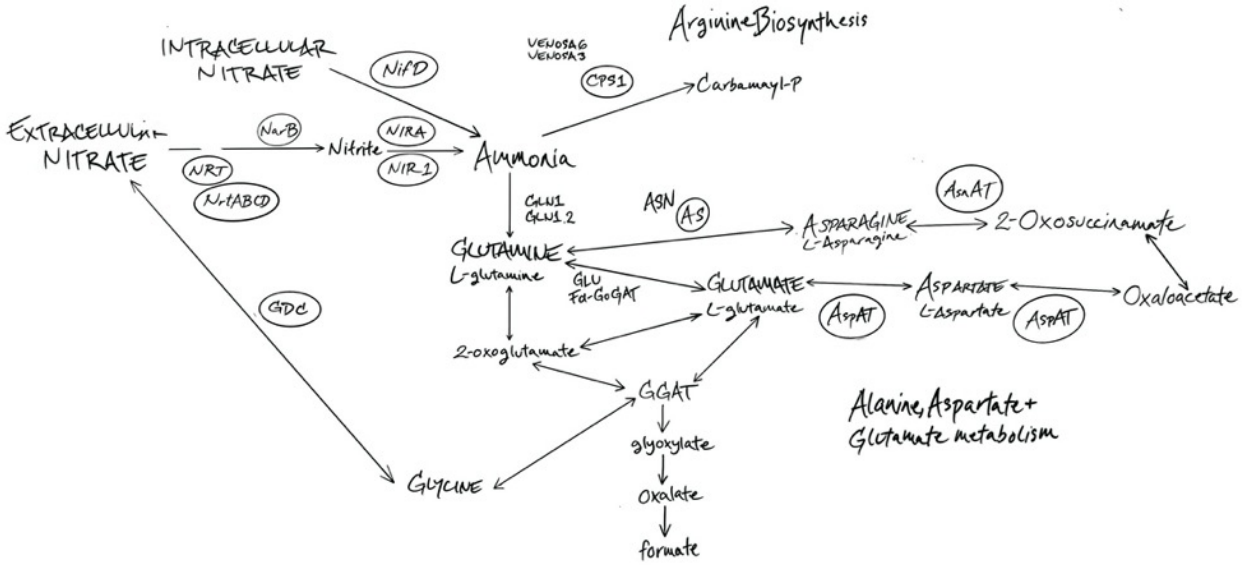


Figure 3.1 A snapshot of plant metabolism with focus on nitrogen metabolism

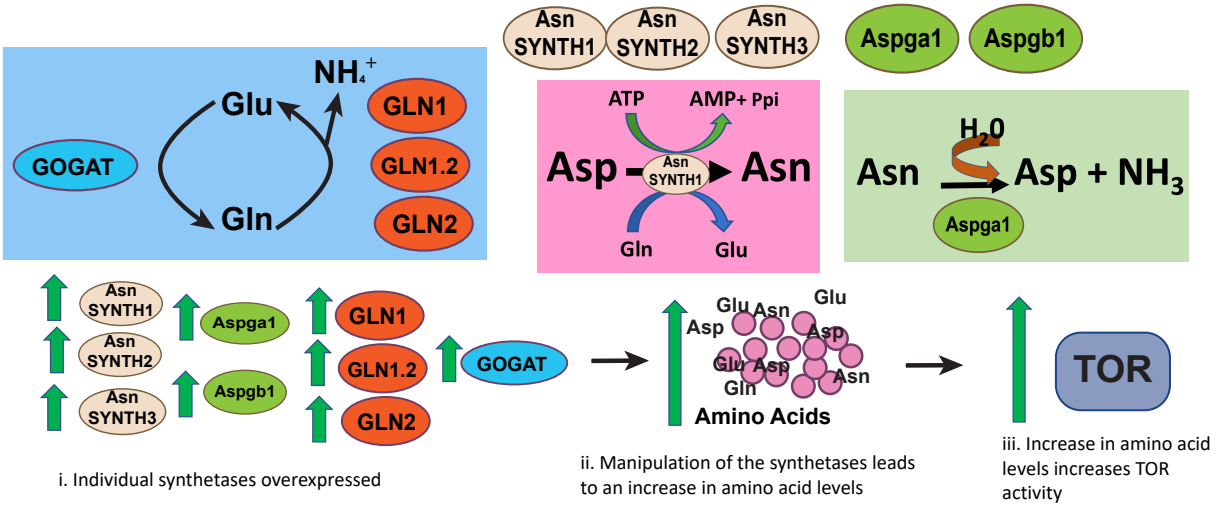


Figure 3.2 Visual representation of experimental protocol of overexpression constructs

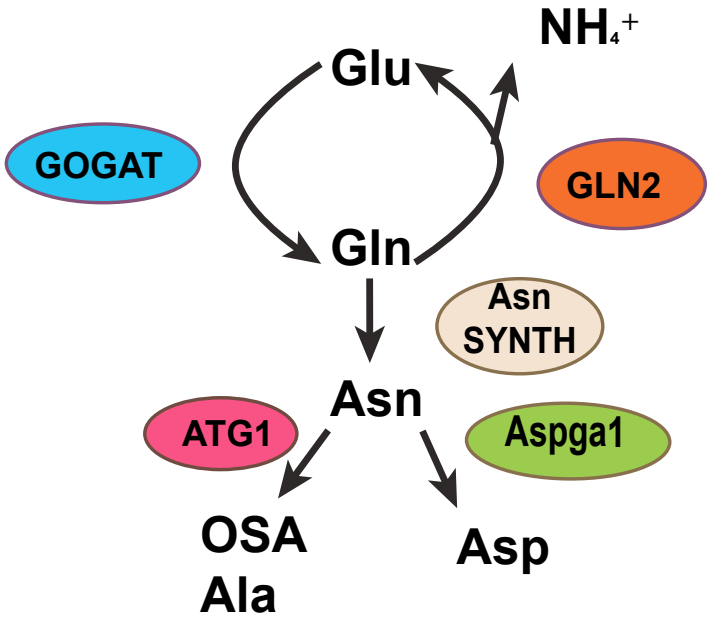


Figure 3.3 Plant metabolism with focus on nitrogen metabolism with the synthetases expressed

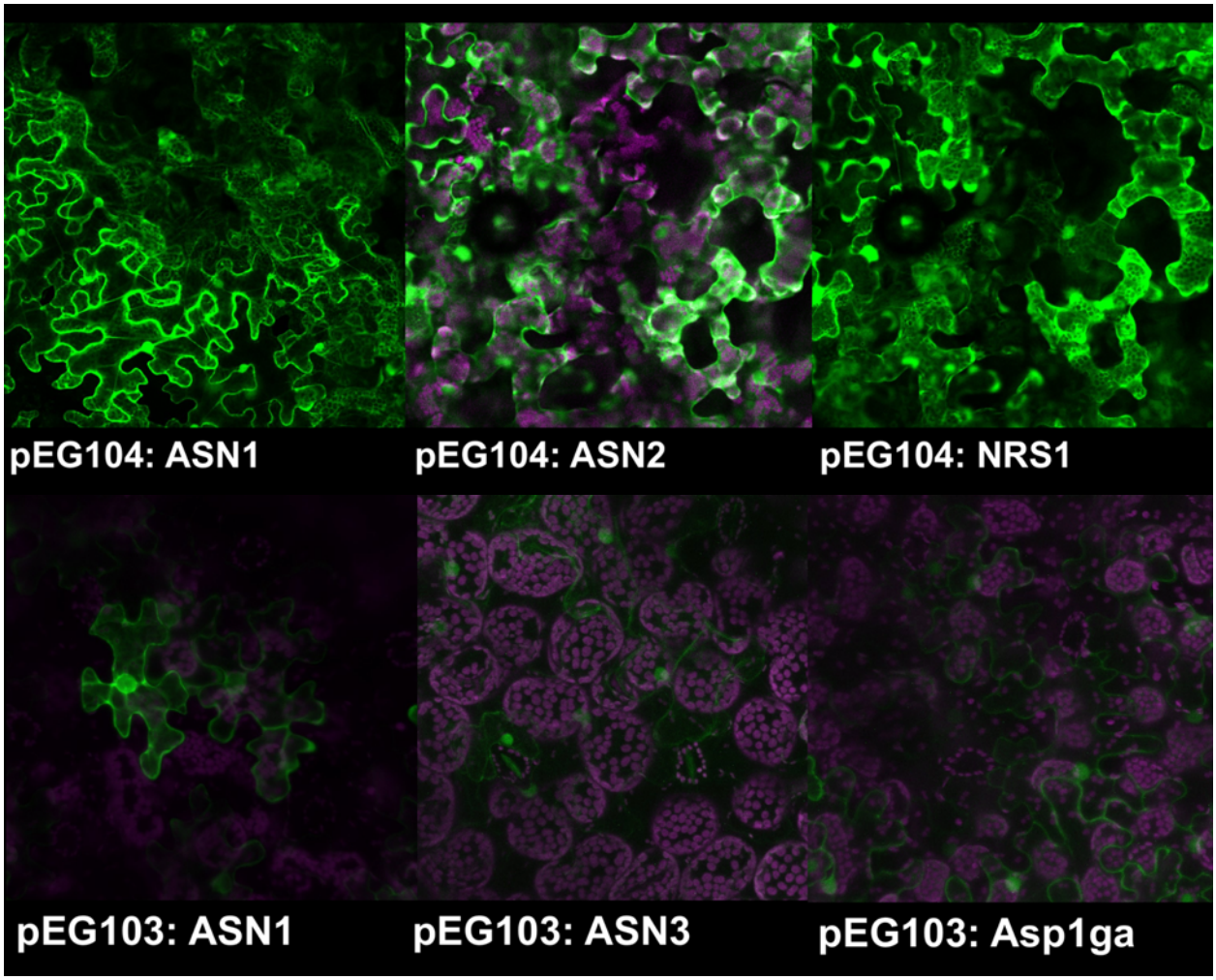


Figure 3.4 These images demonstrate the localization of each protein-GFP or protein-YFP fusion. pEG104 contains the YFP-protein, while pEG103 contains the GFP-protein. The green color indicates the localization of the GFP or YFP expression, while the magenta represents chlorophyll autofluorescence. They allow us to visualize protein in the cytosol, organelles, and nucleus of the plant cell.

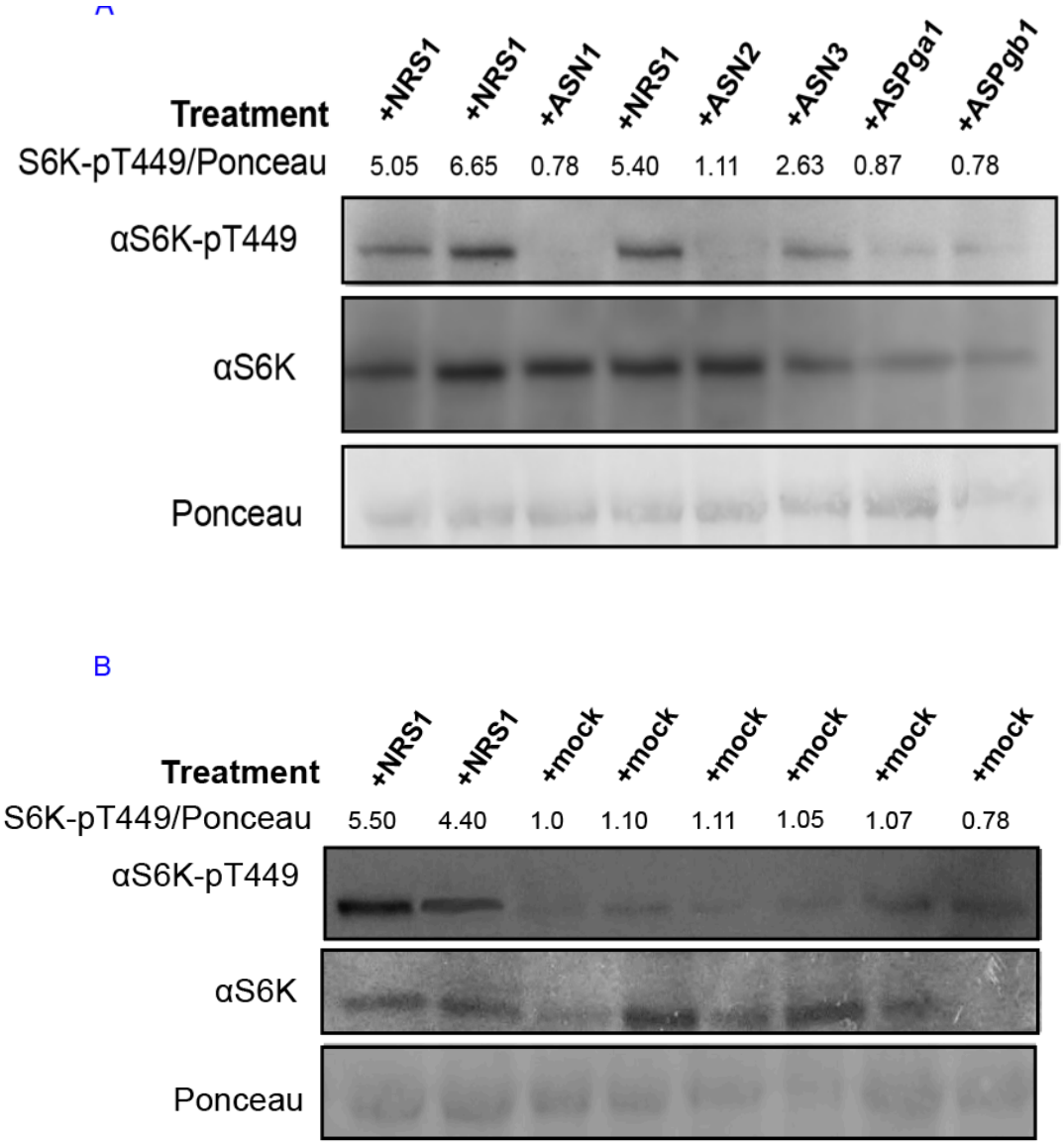


Figure 3.5 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated multiple times; representative results are shown here.
(A) Western blot of asparagine synthetases compared against *NRS1* overexpression constructs
(B) *NRS1* overexpression constructs compared against mock infiltrations

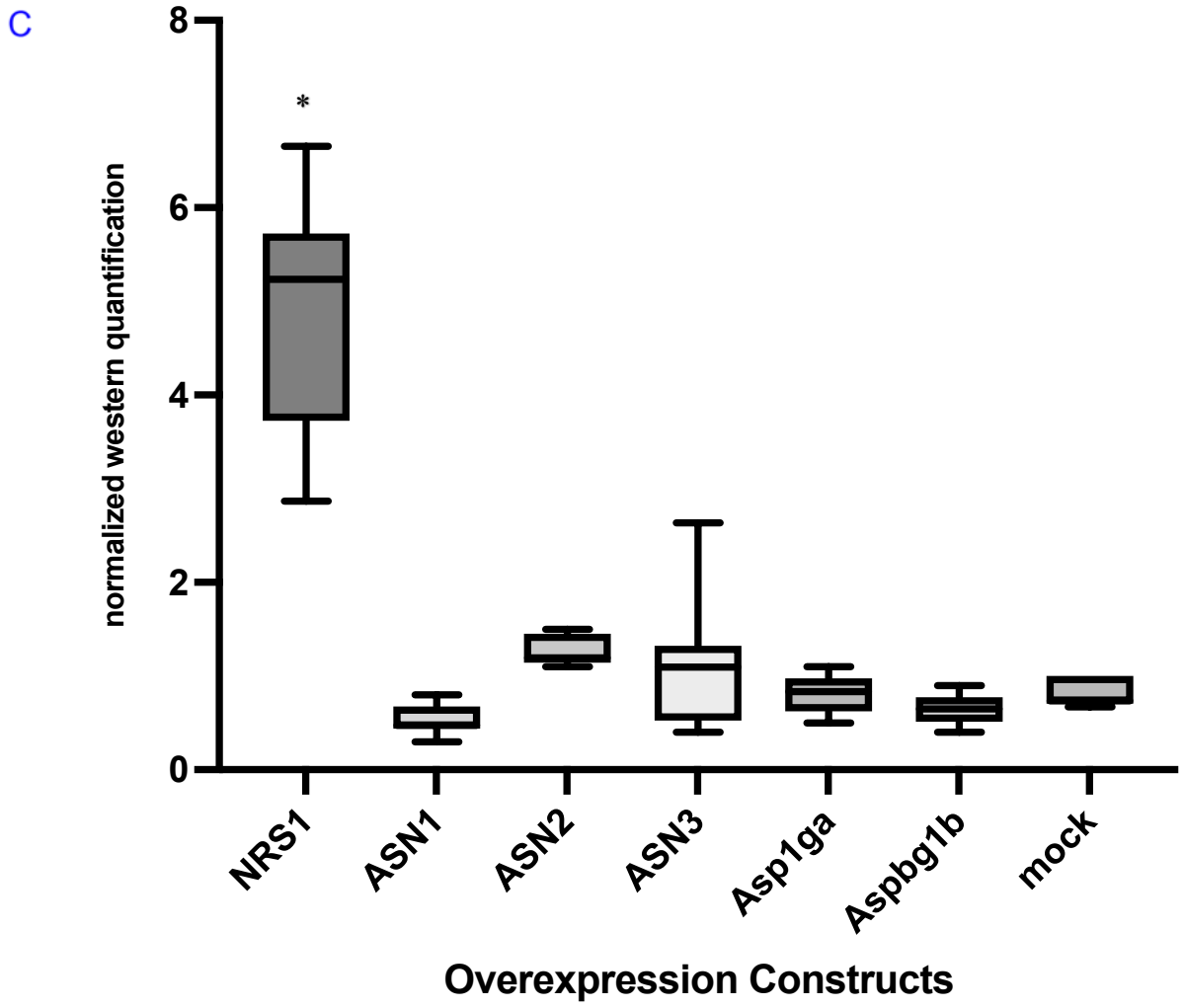
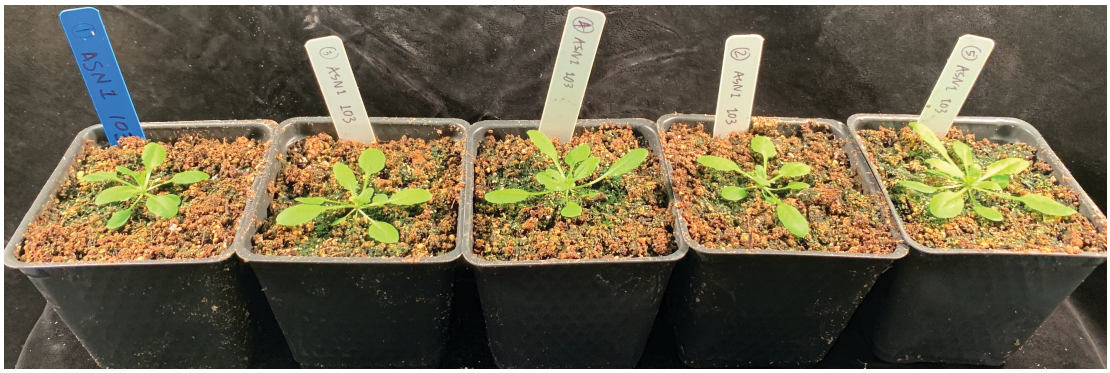


Figure 3.5 Plotted asparagine synthetases compared against *NRS1* overexpression constructs
(C) Graph is a plotted box plot of the normalized western quantification numbers of each treatment.
Asterisks show a t-test performed and statistical significance. * indicates P<0.05

A

35S:PRO: ASN1: pEG103



B

35S:PRO: ASN2: pEG103



C

35S:PRO: ASN3: pEG103

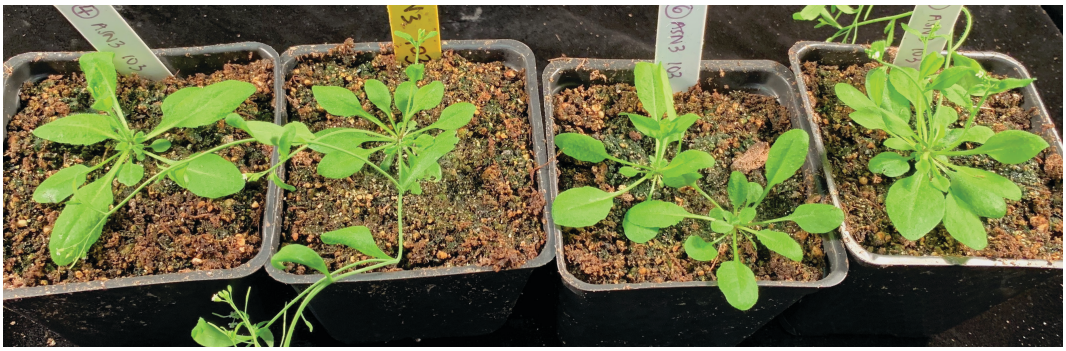
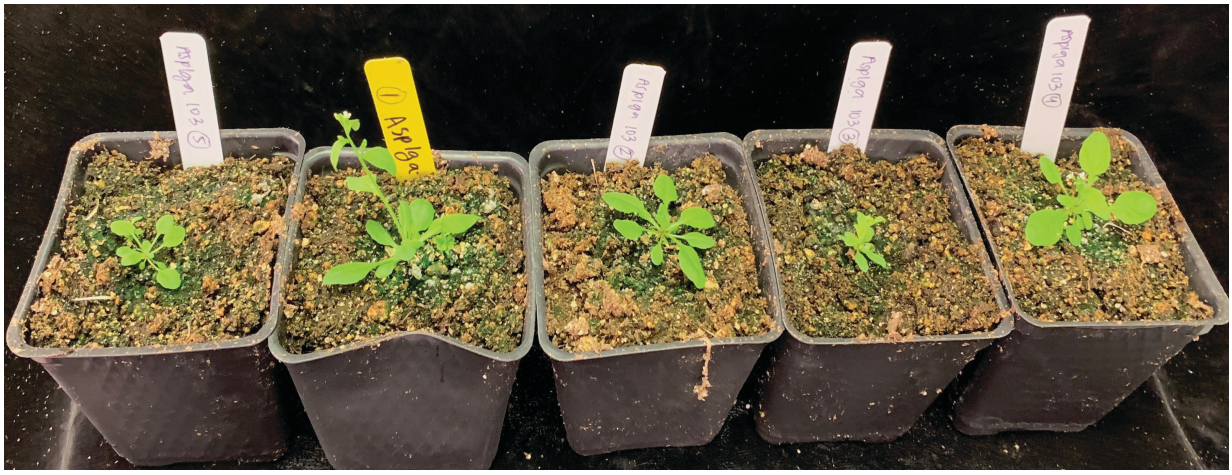


Figure 3.6 *A. thaliana* stable transgenics phenotypes
(A) *A. thaliana* stable transgenics with *35S:$_{PRO}$: ASN1: pEG103*
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: ASN2: pEG103*
(C) *A. thaliana* stable transgenics with *35S:$_{PRO}$: ASN3: pEG103*

A

35S:PRO: Aspg1a: pEG103



B

35S:PRO: Aspg1b: pEG103



Figure 3.7 *A. thaliana* stable transgenics phenotypes
(A) *A. thaliana* stable transgenics with *35S:$_{PRO}$: Aspg1a: pEG103*
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: Aspg1b: pEG103*

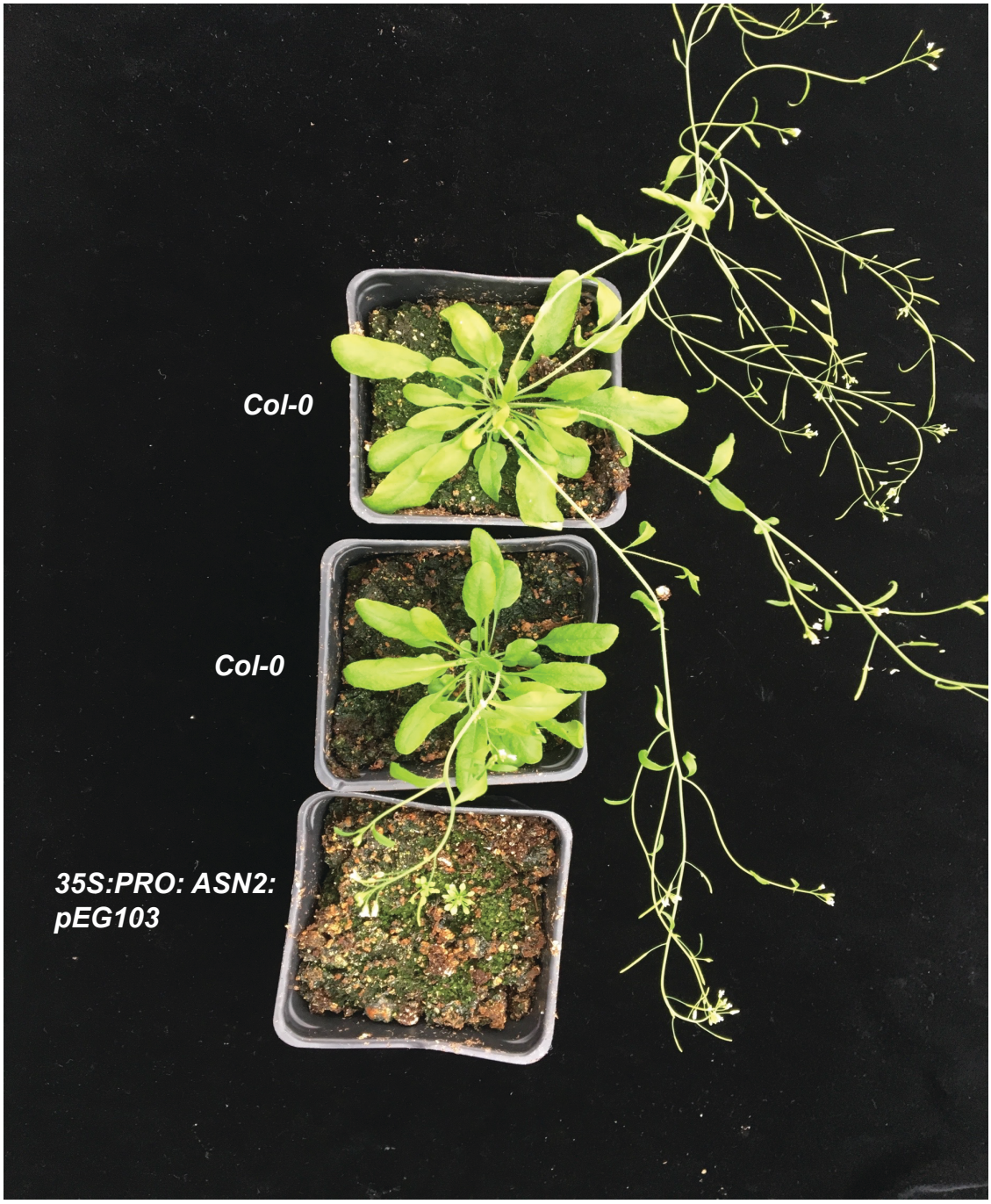


Figure 3.8 Col-0 wildtype plant and *A. thaliana* stable transgenics with *35S:$_{PRO}$: ASN2: pEG103*

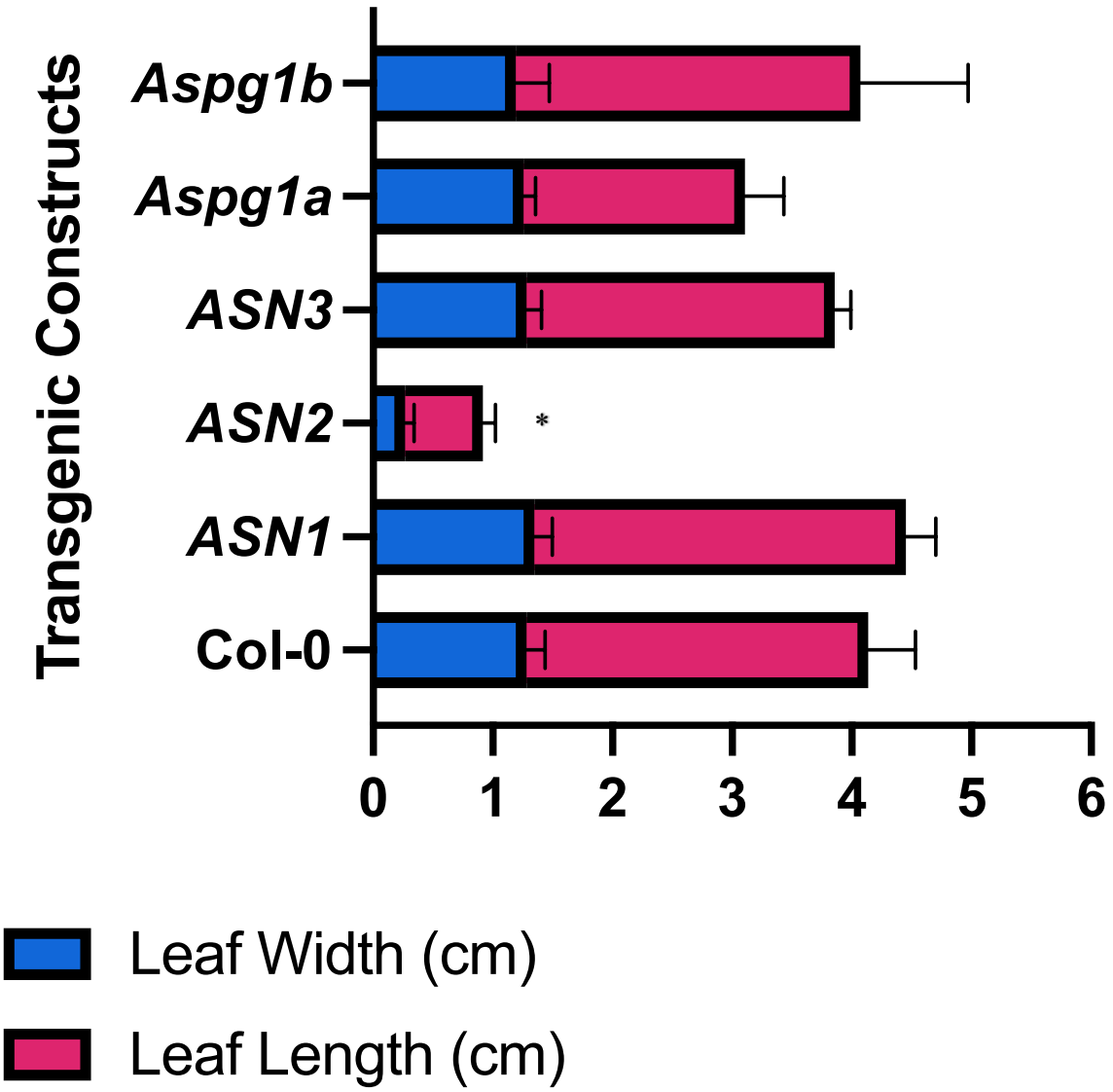


Figure 3.9 Leaf width and length measurements of stable asparagine synthetase transgenics compared against wild-type (Col-0). The length and width of leaf 6 was measured and compared by *t* tests (**$p < 10^{-5}$; *$p < 10^{-2}$; $n \geq 24$).

Chapter Four
What about *NRS1* and TOR?

"Chance is the first step you take, luck is what comes afterward."
Amy Tan



Section 4.1 A Sensor?

This next chapter will interrogate how a tRNA synthetase, *NRS1*, activates TOR in plant cells using biochemical and molecular methodologies. By using these orthogonal, rigorous approaches in a completely novel, unexplored biological, this project will sidestep the confusion and controversies surrounding human *LARS* and instead focus on fundamental questions about how cells sense and respond to amino acids through *NRS1*.

Overexpressing the asparagine synthetases and other t-RNA synthetases did not have the anticipated impact on TOR activity as hypothesized, so this next chapter will investigate how *NRS1* and TOR directly interacts with *NRS1,* and the potential mechanism of how this upstream sensor activates TOR*,* with the hypothesis that *NRS1* enzyme physically interacts with the TOR protein complex to stimulate TOR activity.

NRS1 has been implicated in controlling plant development in ways that would not be readily predicted from its established enzymatic function as an asparaginyl tRNA synthetase. *NRS1*, an asparaginyl tRNA synthetase that has not yet been linked to the TOR pathway, might play a role in controlling some of the developmental and physiological processes that TOR activity is responsible for. Consideration of the family of asparaginyl tRNA synthetases also offers some insight on why the *NRS1* mutant is of interest. Flowering plants have evolved three major clades of asparaginyl tRNA synthetases: a cytosolic *NRS1* clade that is conserved throughout flowering plants, a cytosolic *NRS2* clade that has been lost in monocots, and a third clade that is targeted to the chloroplast and/or mitochondria.

This chapter will focus on the known cytosolic *NRS* genes in *Arabidopsis thaliana* are *NRS1*, *NRS2*, and a recently evolved paralogue of *NRS1*, which is called *NRS3*. A few insertional mutants are available for *NRS3*, but none have been characterized or investigated for phenotypes, and the Brunkard Lab has shown that null *NRS2* alleles exhibit no obvious phenotypes under normal growing conditions. Significantly, however, *NRS1* mutants are lethal at the mid-torpedo stage of embryogenesis, which suggests that *NRS1* has some nonredundant function that is not complemented by NRS2 or NRS3. Therefore, *NRS1* is unique among aminoacyl tRNA transferases in plants because it is not strictly essential for cell survival, but it is required for plant development, even though there are two other *NRS* genes in *Arabidopsis thaliana*.

In combination, these results lead us to the hypothesis that *NRS1* acts in a pathway beyond its established enzymatic role in synthesizing Asn-tRNA.

Determine if *NRS1* activates *TOR*

I tested if *NRS1* specifically promotes TOR activity and if the *NRS1* enzyme physically interacts with the TOR protein complex to stimulate TOR activity in one key experimental ways; establishing whether genetically modulating expression of *NRS1* activates TOR (Figure 4.3).

Establish whether genetically modulating expression of *NRS1* activates TOR

Aminoacyl-tRNA synthetases are ubiquitous and essential components in translation needed to produce aminoacyl-tRNA. The genes encoding cytosolic aminoacyl tRNA synthetases present in Arabidopsis are named *NRS1*, *NRS2*, and *NRS3* (Table 4.1). These *NRS* genes have been suggested to have evolved from a repeated duplication of a gene transferred from an ancestral plastid (Peeters *et al*., 1999). As shown in Figure 4.1, *NRS1* and NRS3 share significant sequence and peptide similarity (84.53%), and are thus paralogues, whereas *NRS2* is more distantly related to the two other enzymes (43.5%). *NRS1* is lethal; as mentioned previously, in every other case, loss of cytosolic tRNA synthetases is either lethal at single cell reproductive stages (if only one copy of that tRNA synthetase is encoded by the genome) or causes no phenotype (if another tRNA synthetase encoded by the genome provides redundancy (Berg *et al*., 2005).

Therefore, *NRS1* is unique among aminoacyl tRNA transferases in plants because it is not strictly essential for cell survival but is required for plant development, since there are two other NRS genes in *Arabidopsis thaliana, NRS2* and *NRS3*. (Berg *et al*., 2005). As stated in the introduction, insertional mutants are available for *NRS3* but they have not been characterized or observed for any obvious phenotypes, and the Brunkard lab has shown that null *nrs2* alleles exhibit no obvious phenotypes under normal growing conditions. Since *NRS1* and *NRS3* share significant similarity, but *NRS3* has not yet been characterized, observing growth and development phenotypes for the two mutants generated from these genes will be of interest. I predict that *NRS1* encodes a tRNA synthetase that has properties beyond its enzymatic role in synthesizing Asn-tRNA to impact TOR activity.

To test that the impacts on TOR activity that *NRS1* has is specific to the asparginyl tRNA synthetases, I've also cloned GlnRS and LeuRS (the glutaminyl-tRNA synthetase and Leucyl-tRNA synthetase respectively) to test the impact of other tRNA synthetases on TOR activity.

Section 4.2 Materials and Methods

Transient expression with Agrobacterium: In combination with a previous experiment, I agroinfiltrated plants with the three aminoacyl tRNA synthetase constructs fused to an N-terminal YFP tag or a C-terminal GFP tag (Table 1). I utilized these alternatively tagged vectors to control for potential negative effects by the N- or C- terminal tag. Agroinfiltration allows for the visualization of the overexpression constructs via a confocal microscope. Confocal microscopy allows for the demonstration of the localization of each protein-GFP or protein-YFP fusion. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf samples can be collected for further analysis after visualization.

Stable transgenic lines: In addition to transiently expressing these constructs through agroinfiltration, the tRNA synthetases in expression vectors were used to generate stable transgenic lines in *A. thaliana* and *N. benthamiana.* I cloned *NRS1, NRS2* and *NRS3* into constructs (Table 4.1) and stably transformed these genes into *A. thaliana,* utilizing the floral dip method as described previously to generate a variety of expression lines. Using the constructs I cloned, *N. benthamiana* stable transgenics were transformed by Jeffery Tung of the Baker Lab, and were characterized by me.

Assaying TOR activity: After generating these overexpression lines I performed the TOR activity assays on WT and mutant plants under TOR-activating and inactivation conditions to study S6K phosphorylation and TOR activation in these lines.

Section 4.3 Results

Transgenics were generated in two species, *A. thaliana and N. benthamiana. A. thaliana* produces leaves in a rosette morphology, and does not typically produce branches during the vegetative growth phase. Studying the developmental morphology of *A. thaliana* would therefore be limited. A model species in the *Solanaceae* family*, N. benthamiana,* has an upright morphology with elongated internodes, and undergoes both primary and axillary branching. Nitrogen availability can promote shoot branching in plants (Xu, 2014), so modifying nutrient availability and observing phenotypes in both wild type and plants overexpressing of *NRS1* would be possible in *N. benthamina,* with its upright branching morphology.

Constructs in Table 4.1 that were agroinfiltrated or stably transformed were confirmed by confocal microscopy. Agroinfiltrated N. benthamiana plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. In Figure 4.4, TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane.

NRS1 correlates positively with TOR activity

In Figure 4.4 (A) *NRS1* overexpression constructs are compared against asparagine synthetases, with *NRS1* constructs inducing higher band density, indicative of higher S6K1 phosphorylation. In (B), *NRS1* overexpression constructs are blotted with a mock, with *NRS1* treatments inducing higher S6K1 phosphorylation. In (C-E), *NRS1*, NRS2, and *NRS3* on a blot together, aside a mock treatment, with varying S6K1 results. (F) Graph is a plotted box plot of the normalized western quantification numbers of each treatment.

NRS1 has the most significant impact on TOR activity in comparison to its paralogs

NRS1, NRS2 and NRS3 overexpression constructs are also assayed via western blot against the asparagine synthetases mentioned in the previous chapter. In Figure 4.5 Western blots of *NRS1* compared to asparagine synthetases; agroinfiltrated N. benthamiana plants with overexpression constructs and fused to a C-terminal GFP tag and then probed with S6K and S6Kp. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. *NRS1* and its paralogs has the most significant impact on TOR activity.

Other tRNA synthetases*; LeuRS* and *GlnRS* have some impact on on TOR activity
I tested other tRNA synthetases to see if they might also have an impact on TOR activity. I chose two synthetases, LeuRS and GlnRS for their significance in the TOR literature in mammals and their importance in the nitrogen metabolism of plants respectively. Figure 4.6 (A) illustrates a representative blot of LeuRS and GlnRS overexpression constructs compared against *NRS1* overexpression constructs. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. *NRS1* infiltrated constructs positively correlates with TOR activation as measured by S6k1 phosphorylation. Plotting at the normalized western blot ratios of S6K phosphorylation to ponceau bands, the graphical results demonstrate that *NRS1* has the most significant impact on TOR activity. However, LeuRS does cause S6K1 phosphorylation as compared to the mock infiltration band. Further investigation of this result is necessary, but since *NRS1* result is still the most significant, the investigation of this thesis primarily still focuses on the asparginyl tRNA synthetase.

Transgenics of *NRS1* show increased growth phenotypes
In addition to the agroinfiltration experiments in *N. benthamiana* and western blots to assay TOR activity, I also stably transformed *A. thaliana* with *NRS1, NRS2* and *NRS3* with the overexpression constructs to see if modifying expression of the tRNA synthetases could have any impact on the phenotype of *A. thaliana* plants. In Figure 4.7, on Day 4 *NRS2* is slowest to germinate of all the constructs, but catches up on Day 7 (Figure 4.8). During the first week of plant growth, the *Col-0* wild-type plants have little to no phenotype difference than the transgenics. However, as the plants mature, the over expression constructs of *NRS1, NRS2* and *NRS3* start to differ wildly. Figure 4.9, Figure 4.10 and Figure 4.12 show representative images of plants displaying two phenotypes of *35S:$_{PRO}$: NRS1: pEG103* at various ages and stages of development; one that is significantly dwarfed, and the other with a large leaf phenotype.

Figure 4.11 shows representative images of *35S:$_{PRO}$: NRS2: pEG103* and *35S:$_{PRO}$: NRS3: pEG103* at week 4 of development. The phenotype of these plants do not differ that significantly from wild-type plants. Figure 4.14 plots the results of leaf width and length measurements were performed on all the *A. thaliana* transgenics at five weeks of plant growth.

Section 4.4 Discussion

Expression of *NRS1* positively correlates with S6K1 phosphorylation
In this section, experiments were performed to investigate the impact of *NRS1* on TOR activity. The results strongly suggest that *NRS1* is deeply implicated in the TOR signaling pathway, due to how significantly S6k1 phosphorylation is impacted in western blot assays. In addition to that, *NRS1* over-expression transgenics show similar phenotypes to TOR over-expression constructs, illustrating that *NRS1* has some kind of impact on growth beyond it's canonical function as a tRNA synthetase, and likely functions in the TOR pathway.

There is evidence from the literature that tRNA synthetase, maybe such as *NRS1,* can interact with TOR or one of the TOR complexes or interacting partners and regulate its activity. There is a potential mechanism for how a tRNA synthease can modify TOR activity, as He *et al* reveals that tRNA synthetases sense sufficiency of amino acids and act as aminoacyl transferases to modify 3-amines of lysines in proteins in human cells, with leucylation of RagA/B regulating mTORC1 activity and glutaminylation of ASK1 inhibiting apoptosis. However, this mechanism would imply that *NRS1* would also engage the endogenous amino acid metabolism network, such as the synthetases, which were overexpressed in Chapter 3. The overexpression of the synthetases did not have an impact on TOR activity as anticipated, so *NRS1* must sense amino acid sufficiency or levels external of a direct metabolic feedback loop.

There are no straightforward conclusions that can be drawn from phenotypes of the *NRS1* overexpressing plants, however, I believe that the "dwarf" or smaller *NRS1* plants are co-suppressing from the transgene (i.e., they are *NRS1* knockdowns instead of overexpressing). The other potential explanation for this phenotype is that the expression level matters; too much of *NRS1* expression and other plant developmental processes end up being mis-expressed (like sink/source dynamics) and the result is a smaller, stressed plant. The more compelling evidence for *NRS1* impact on growth phenotypes is the by observing the early growth stages of the transgenics (Figure 4.2) where we see young seedings that are about twice the size than the wild-type.

However, in the interest of investigating the functional components of *NRS1* that are necessary for TOR activation, further characterization of the *NRS1*, *NRS2, and NRS3* mutants were not performed in this dissertation. In both transgenic plants *N. benthamiana* and *A. thaliana* further characterization is necessary for a more complete picture of TOR investigation, but beyond the scope of this thesis. However, since these lines have been stably made; RNA-Seq will be used to quantify changes in gene expression that are regulated by the nitrogen-TOR signaling network. Future experiments will focus on the coding transcriptome of phenotypically interesting plants, quantifying gene expression at the gene level with RNA-seq. Future experiments could also include test for segregation of these phenotypes, measure expression levels by RT-qPCR, Westerns, and small RNA blots to look for silencing signals to confirm how *NRS1* expression levels correlate with phenotype.

Figures

Gene Name	TAIR ID	Description	GFP (C-terminally tagged constructs)	YFP (N-terminally tagged) Constructs)
NRS1	AT5G56680	putative cytosolic asparaginyl-tRNA synthetase	35S:$_{PRO:}$ *NRS1*: pEG103	35S:PRO: *NRS1*: pEG104
NRS2	AT3G07420	putative cytosolic asparaginyl-tRNA synthetase	35S:$_{PRO:}$ NRS2: pEG103	35S:PRO: NRS2: pEG104
NRS3	AT1G70980	putative cytosolic asparaginyl-tRNA synthetase	35S:$_{PRO:}$ NRS3: pEG103	35S:PRO: NRS3: pEG104
LeuRS	AT1G09620	Leucyl-tRNA synthetase	35S:$_{PRO:}$ LeuRS: pEG103	35S:$_{PRO:}$ LeuRS: pEG104
GlnRS	AT1G25350	glutaminyl-tRNA synthetase	35S:$_{PRO:}$ GlnRS: pEG104	35S:$_{PRO:}$ GlnRS: pEG104

Table 4.1 List of genes referenced

Plant	Observed Resistant Progeny (OR)	Expected Value (R)	Observed Suceptible Progeny (OS)	Expected Value (S)	(OR-R)^2/R	(OS-S)^2/S
NRS1 103 (1)	44	39.75	9	13.25	0.454402516	1.36320755
NRS1 103 (5)	26	24	6	8	0.166666667	0.5
NRS3 103 (2)	33	30.75	8	10.25	0.164634146	0.493 90244
NRS3 103 (12)	33	28.5	5	9.5	0.710526316	2.13157895
NRS3 103 (10)	20	19.5	6	6.5	0.012820513	0.038 46154
NRS3 103 (9)	23	24	9	8	0.041666667	0.125
NRS3 103 (7)	36	34.5	10	11.5	0.065217391	0.195 65217
NRS3 103 (5)	32	35.25	15	11.75	0.29964539	0.898 93617
NRS3 103 (8)	23	24	9	8	0.041666667	0.125
NRS3 103 (6)	23	22.5	7	7.5	0.011111111	0.033 33333
NRS2 103 (21)	32	30.75	9	10.25	0.050813008	0.152 43902
NRS2 103 (2)	33	29.25	6	9.75	0.480769231	1.442 30769
NRS2 103 (18)	29	27	7	9	0.148148148	0.444 44444
NRS2 103 (14)	34	31.5	8	10.5	0.198412698	0.595 2381
NRS2 103 (11)	23	24	9	8	0.041666667	0.125
NRS2 103 (2)	26	27	10	9	0.037037037	0.111 11111
NRS2 103 (7)	20	21	8	7	0.047619048	0.142 85714
NRS2 103 (3)	23	22.5	7	7.5	0.011111111	0.033 33333
NRS2 103 (8)	32	30.75	9	10.25	0.050813008	0.152 43902
NRS2 103 (17)	34	31.5	8	10.5	0.198412698	0.595 2381
NRS2 103 (12)	23	24	9	8	0.041666667	0.125

NRS2 103 (20)	32	31.5	10	10.5	0.0079 36508	0.023 80952
NRS2 103 (19)	48	42	8	14	0.8571 42857	2.571 42857
NRS1 104 (8)	23	22.5	7	7.5	0.0111 11111	0.033 33333
NRS1 104 (9)	34	31.5	8	10.5	0.1984 12698	0.595 2381
NRS1 104 (6)	23	24	9	8	0.0416 66667	0.125
NRS1 104 (7)	20	20.25	7	6.75	0.0030 8642	0.009 25926
NRS1 104 (19)	40	36.75	9	12.25	0.2874 14966	0.862 2449

Table 4.2 Transgenic segregation analysis of stably transformed *NRS A. thaliana* plants

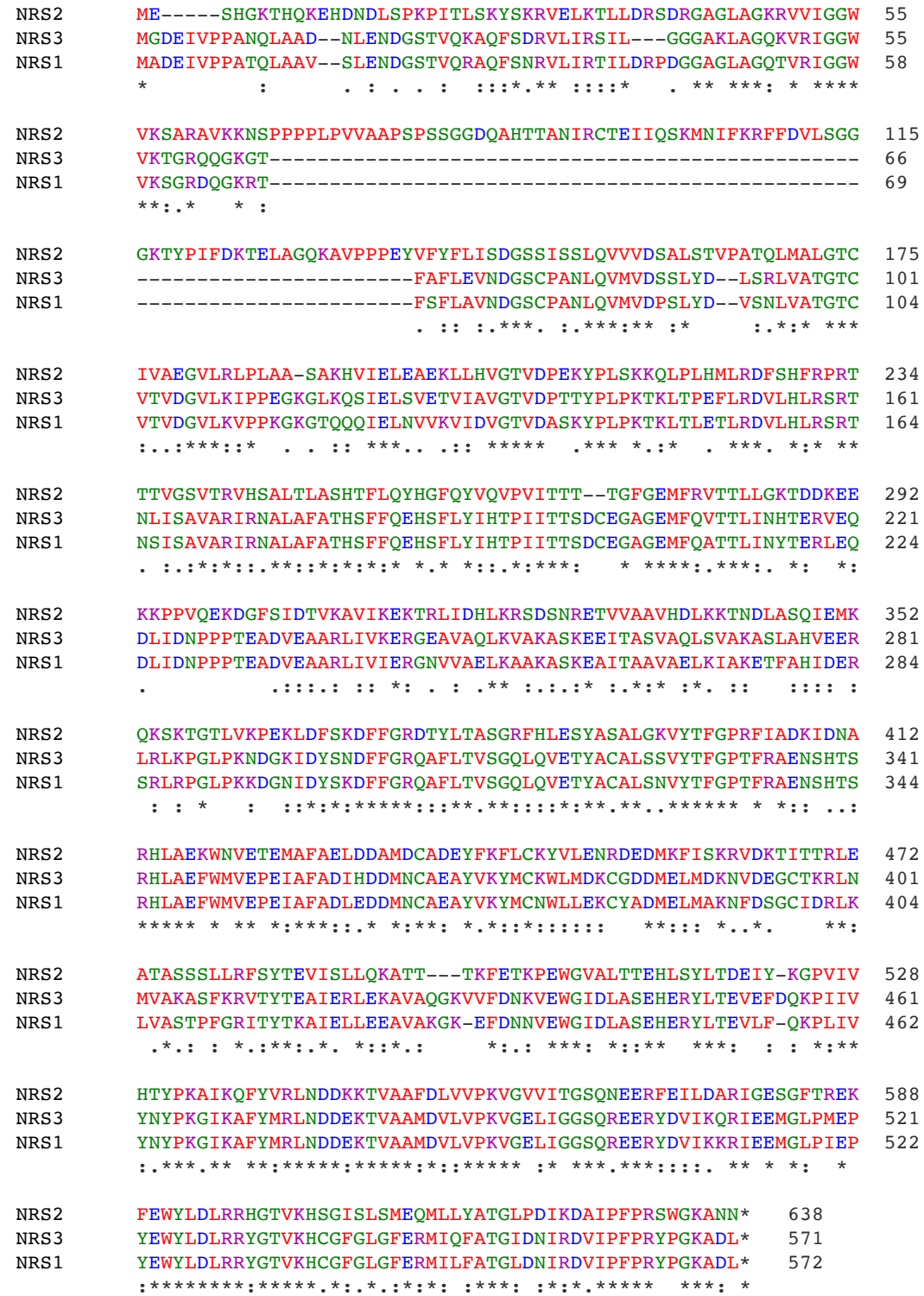


Figure 4.1 Peptide alignment of *NRS1,* NRS2, and NRS3 generated by CLUSTAL multiple sequence alignment.

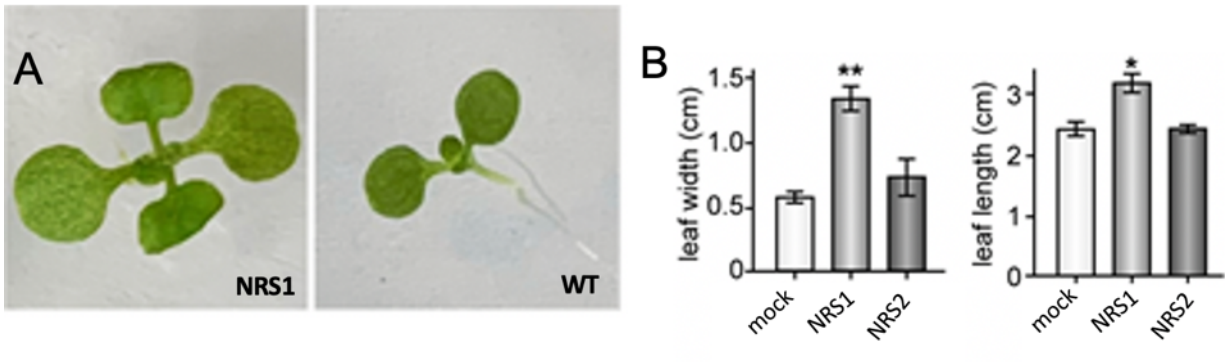


Fig 4.2 *NRS1*, *NRS2*, and mock (*GFP*) stable transgenic Arabidopsis lines were grown for 7 weeks. *NRS1* overexpression clearly stimulated vegetative growth. The length and width of leaf 6 was measured and compared by *t* tests (**$p < 10^{-5}$; *$p < 10^{-2}$; $n \geq 24$).

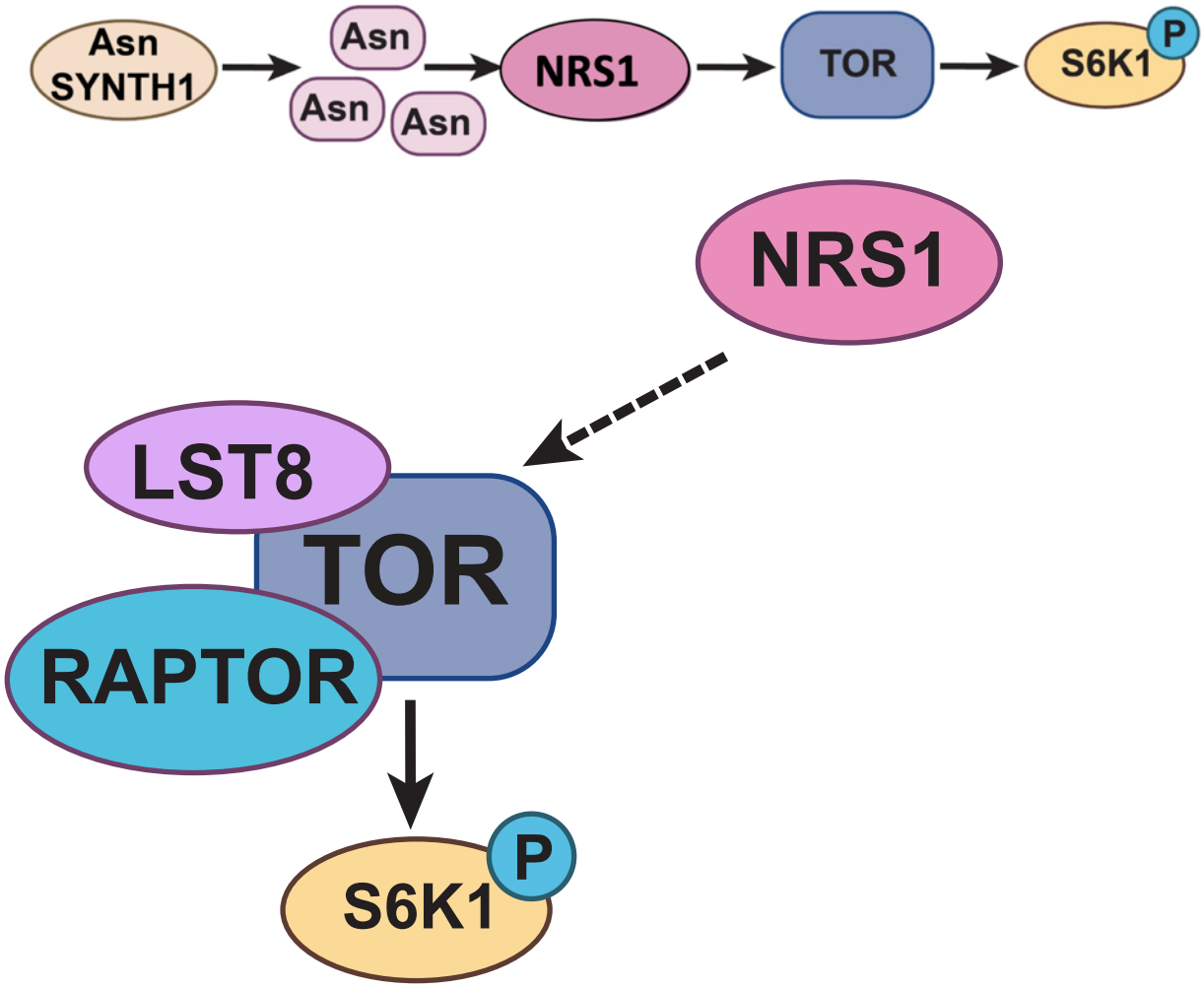


Figure 4.3 A potential model for ASN - *NRS1* - TOR interaction, where genes responsible for asparagine metabolism produce amino acids (specifically, asparagine), *NRS1* senses the level of the amino acids inside the plant body and then activates TOR.

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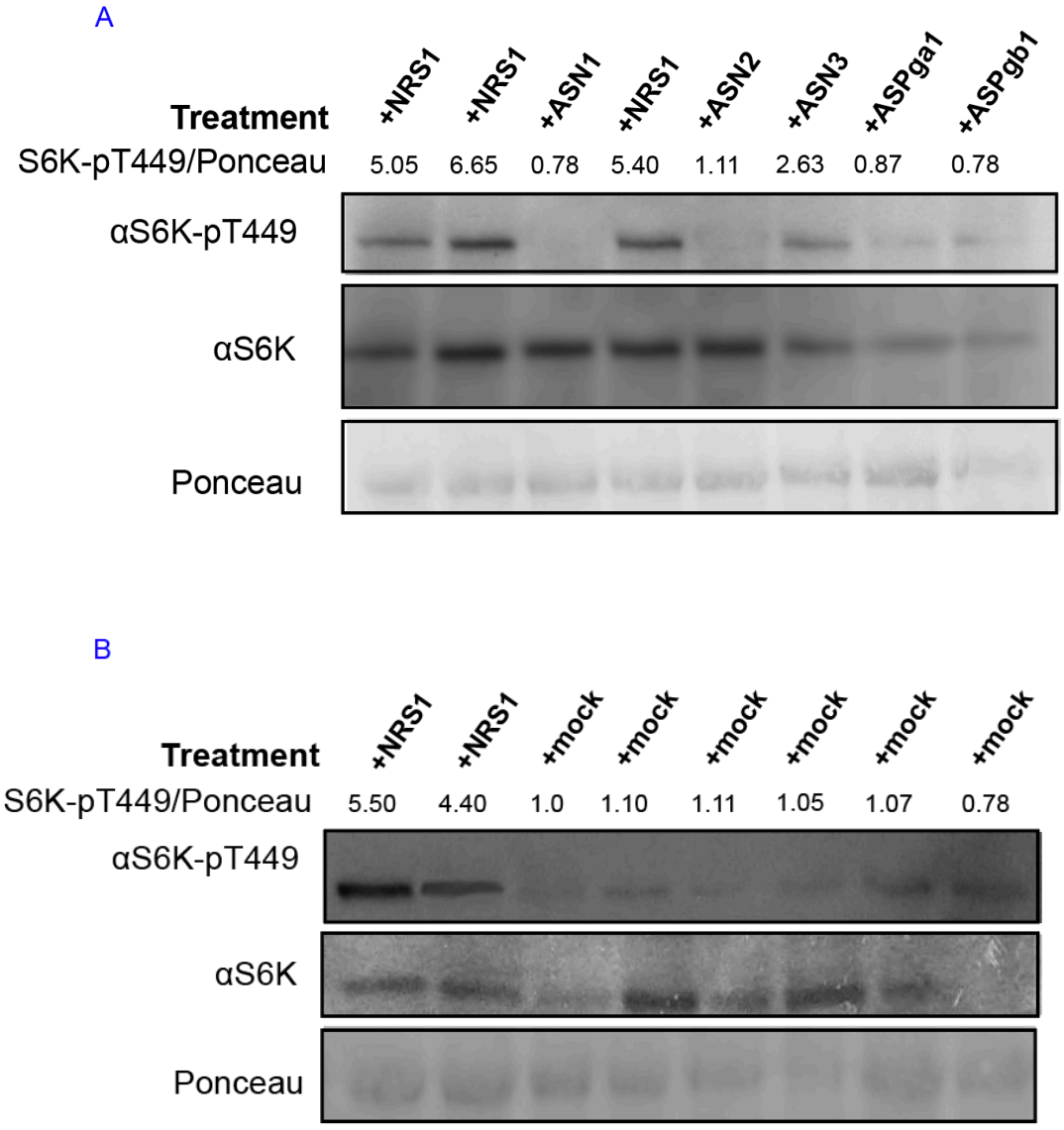


Figure 4.4 Overexpression construct western blots (full caption at the last figure section)
(A) *NRS1* western blots compared to asparagine synthetases and (B) mock infiltrations

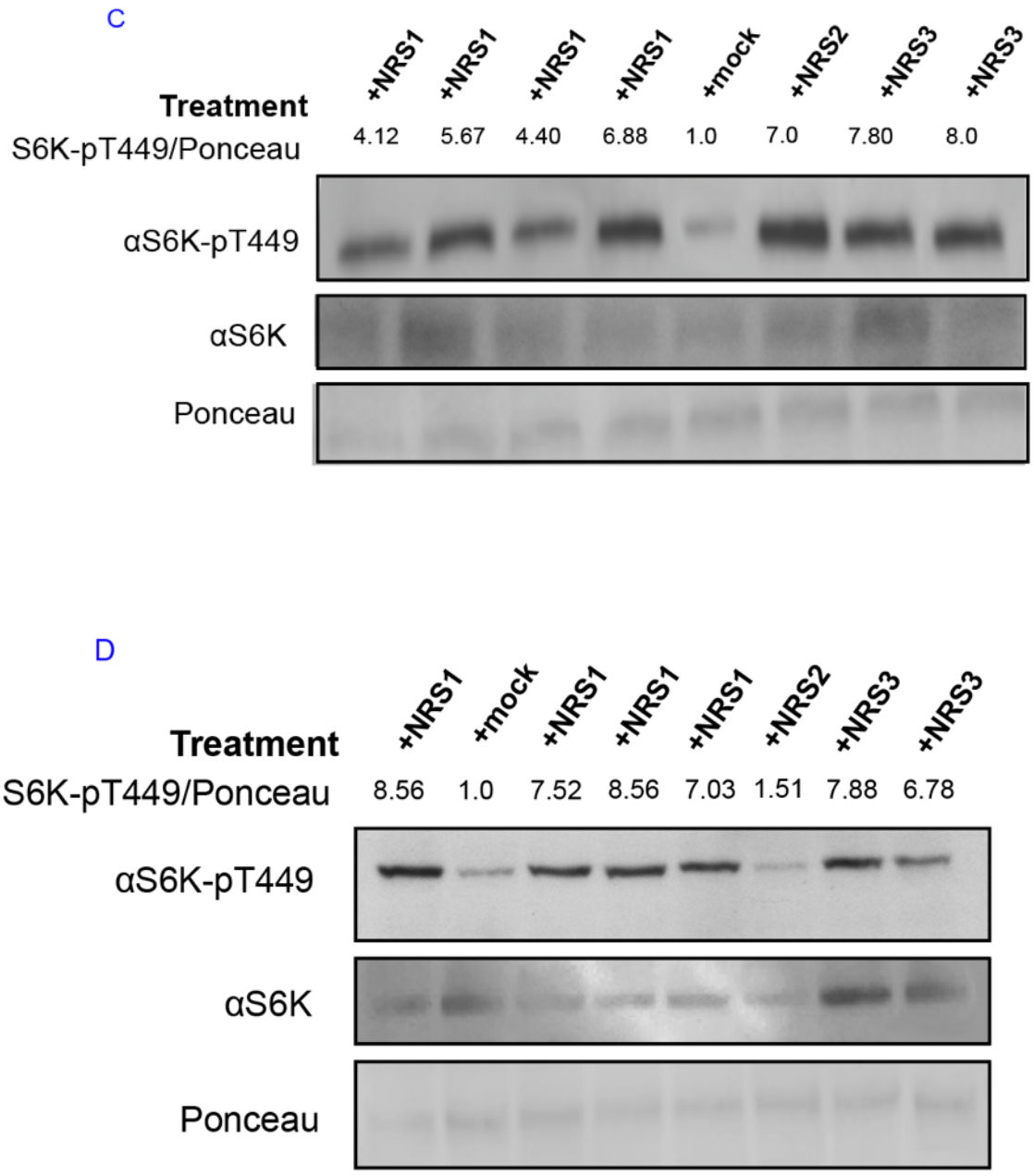


Figure 4.4 Overexpression construct western blots (full caption at the last figure section)
(A) *NRS1* western blots compared to NRS2 and NRS3 and mock infiltrations (B) repeat

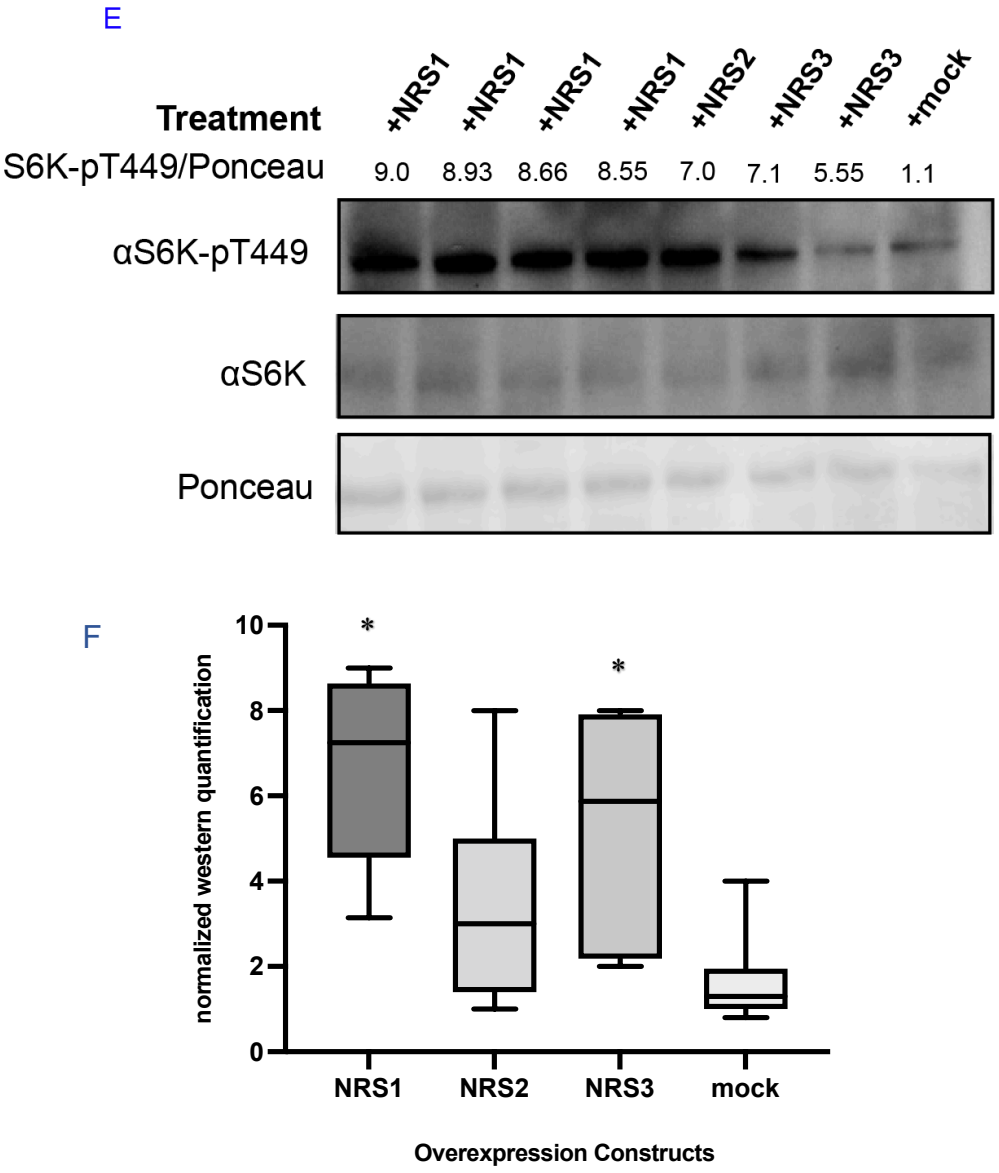


Figure 4.4 *NRS1* Western Blots and graph
Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

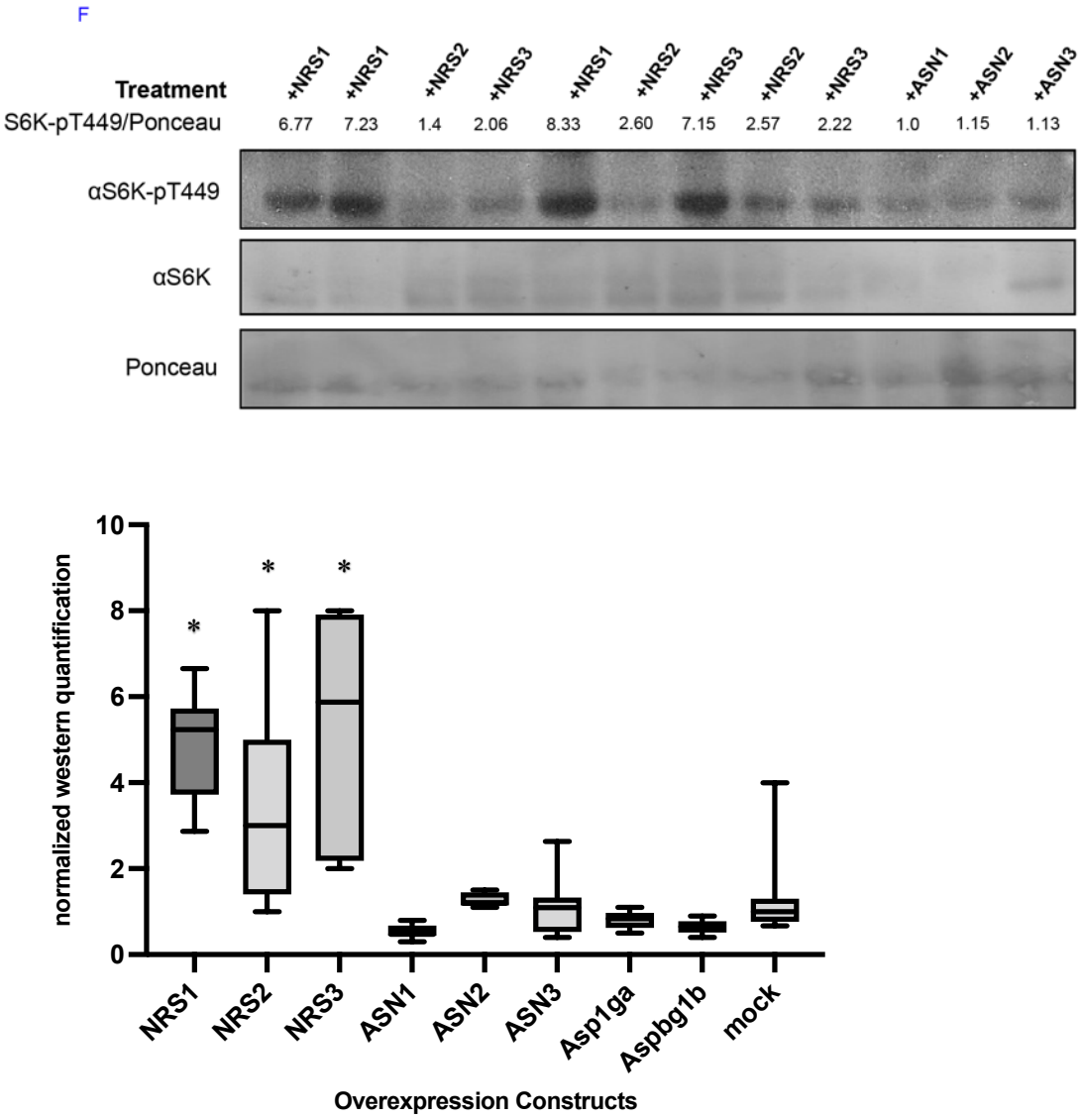


Figure 4.5 Western blots of *NRS1* compared to asparagine synthetases Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

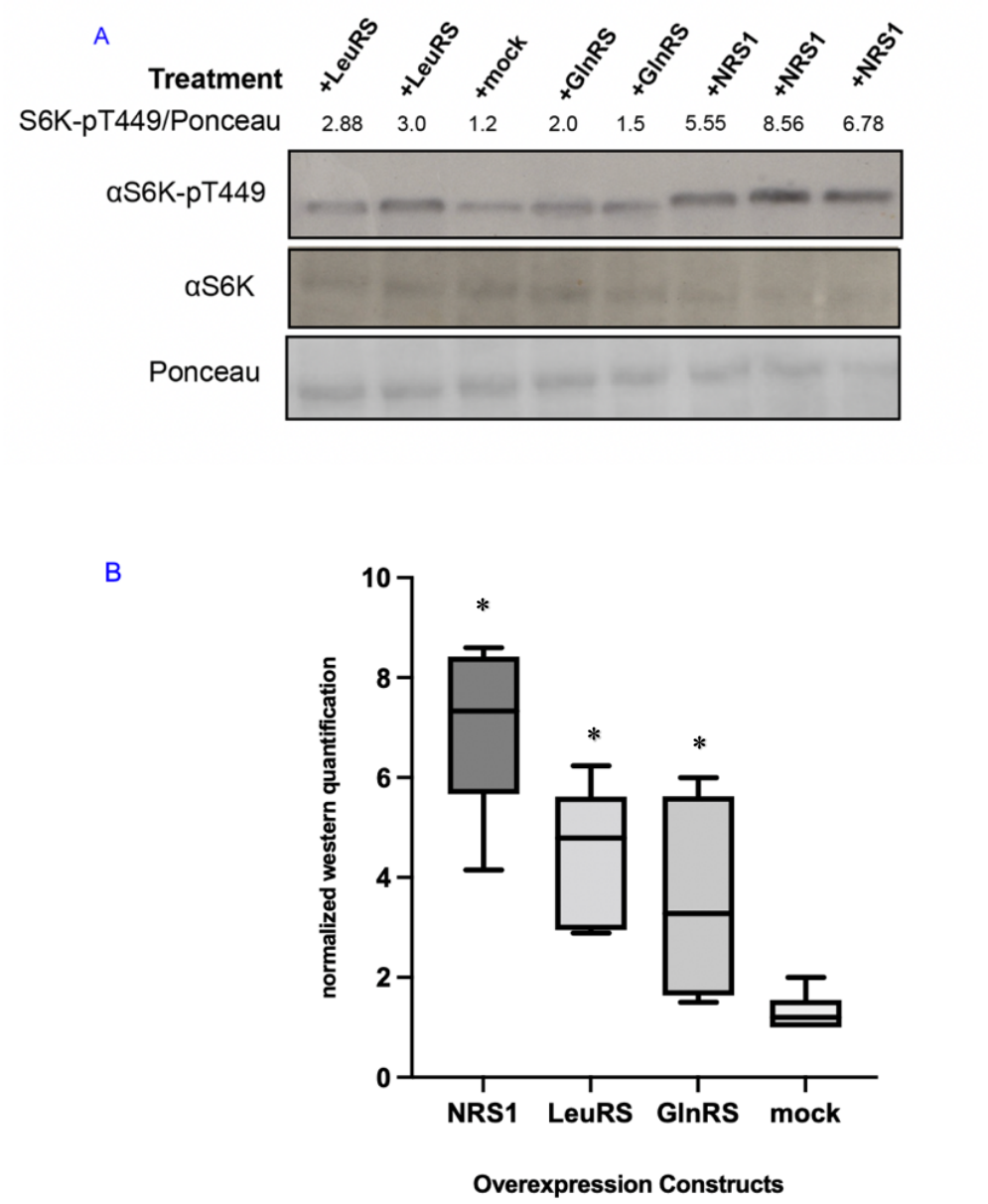


Figure 4.6 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. TOR activity was assayed using Western blots against S6K-pT449 and total S6K, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated multiple times; representative results are shown here. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$



Figure 4.7 *A. thaliana* stable transgenics phenotypes, seedlings on Day 4 images under 10X magnification
(A) *Col*-0 (wild-type)
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103*
(C) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS2: pEG103*

(D) *A. thaliana* stable transgenics with *35S:PRO: NRS3: pEG103*

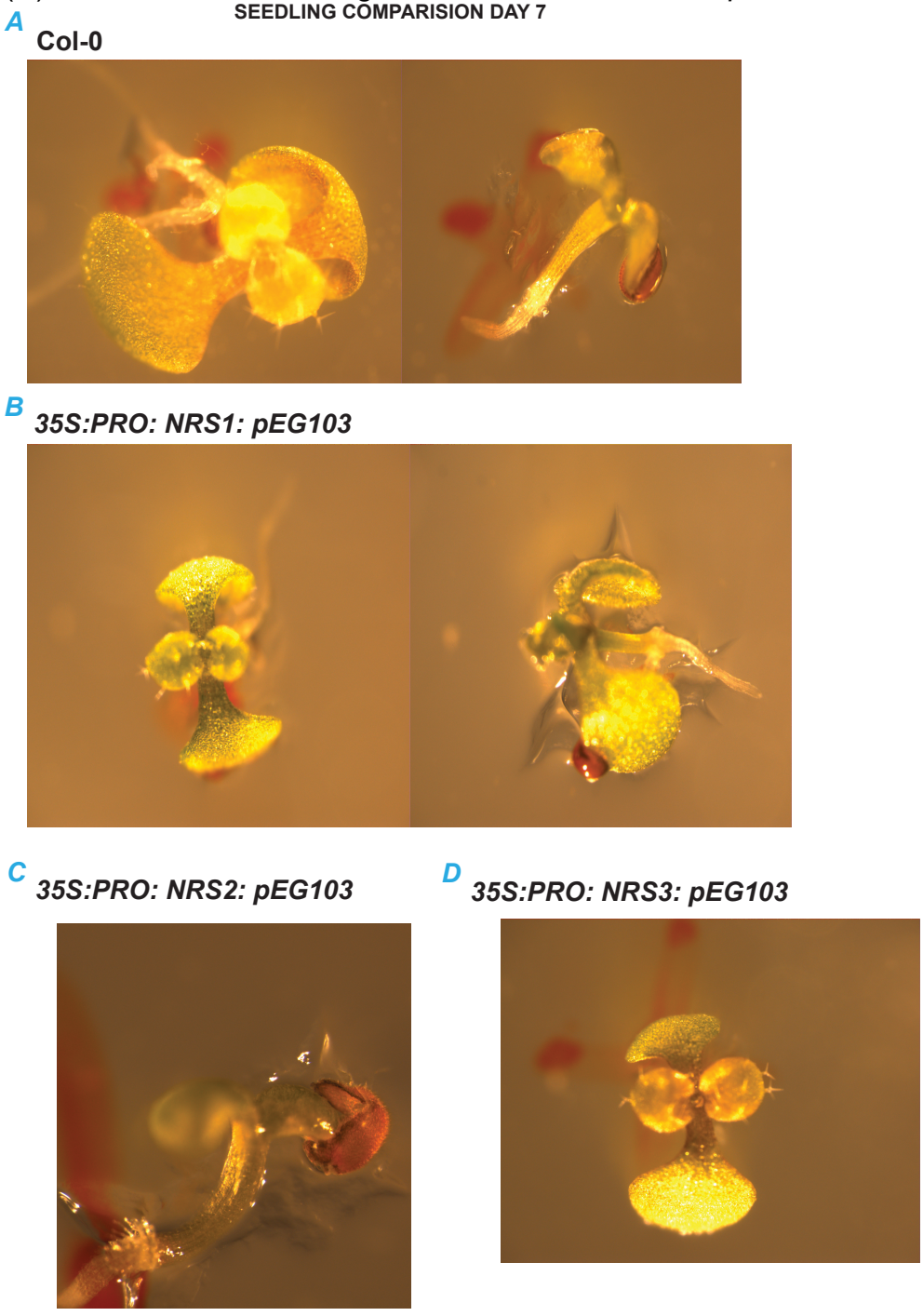


Figure 4.8 *A. thaliana* stable transgenics phenotypes, seedlings on Day 7 images under 10X magnification
(A) *Col*-0 (wild-type)
(B) *A. thaliana* stable transgenics with *35S:PRO: NRS1: pEG103*
(C) *A. thaliana* stable transgenics with *35S:PRO: NRS2: pEG103*

(D) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS3: pEG103*

35S:PRO: NRS1: pEG103



Figure 4.9 *A. thaliana* stable transgenics phenotypes, plants on week 5
(A) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a dwarf phenotype
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a large phenotype

35S:PRO: NRS1: pEG103

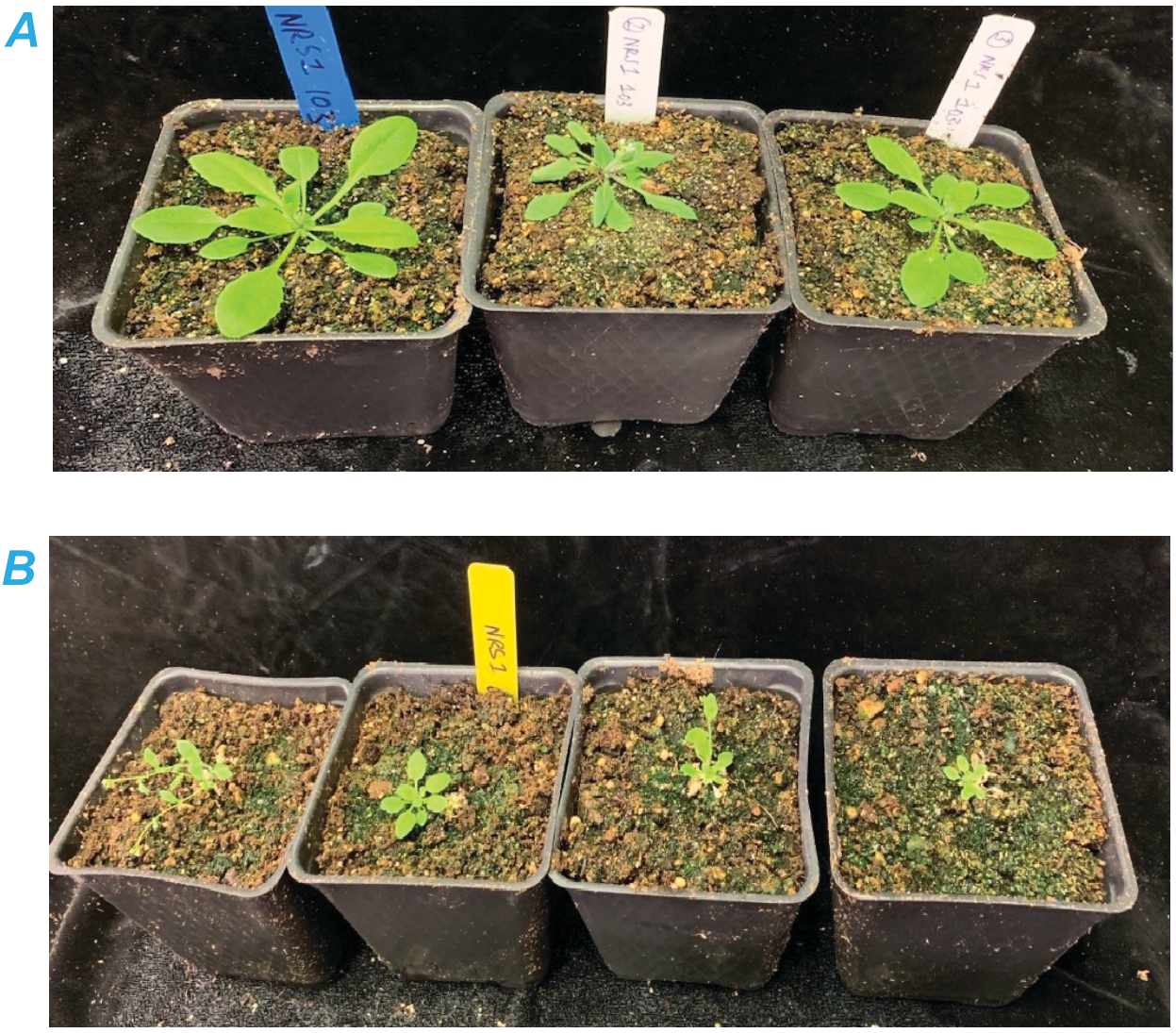
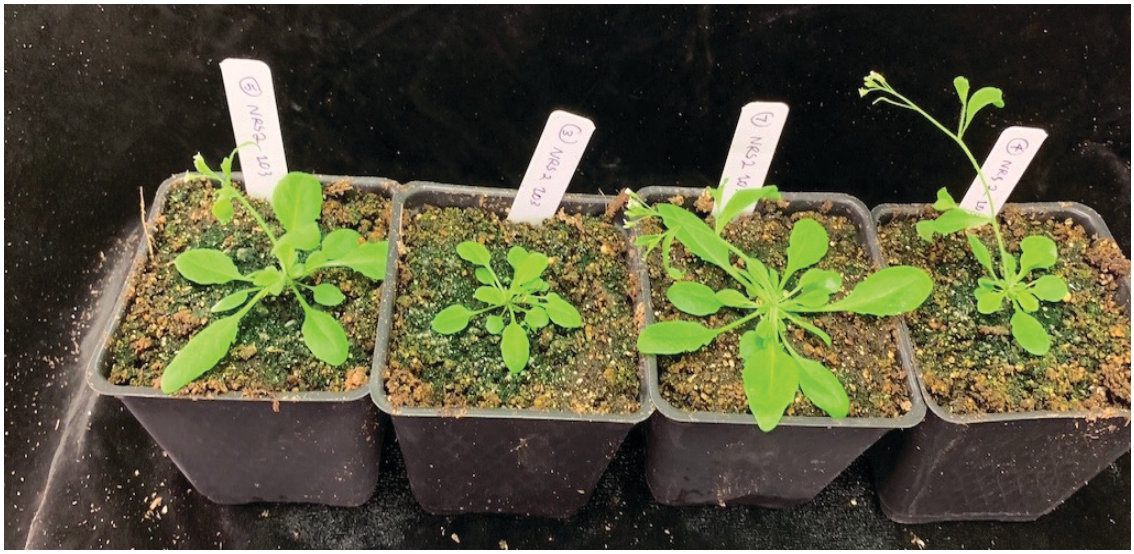


Figure 4.10 *A. thaliana* stable transgenics phenotypes, plants on week 4
(A) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a large phenotype
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a dwarf phenotype

A ***35S:PRO: NRS2: pEG103***



B ***35S:PRO: NRS3: pEG103***



Figure 4.11 *A. thaliana* stable transgenics phenotypes, plants on week 4
(A) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS2: pEG103*
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS3: pEG103*

A

Col-0



B

35S:PRO: NRS1: pEG103



C

35S:PRO: NRS1: pEG103



Figure 4.12 *A. thaliana* stable transgenics phenotypes, plants on week 4
(A) *Col-0* wild-type *A. thaliana* plants
(B) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a large phenotype
(C) *A. thaliana* stable transgenics with *35S:$_{PRO}$: NRS1: pEG103* with a dwarf phenotype

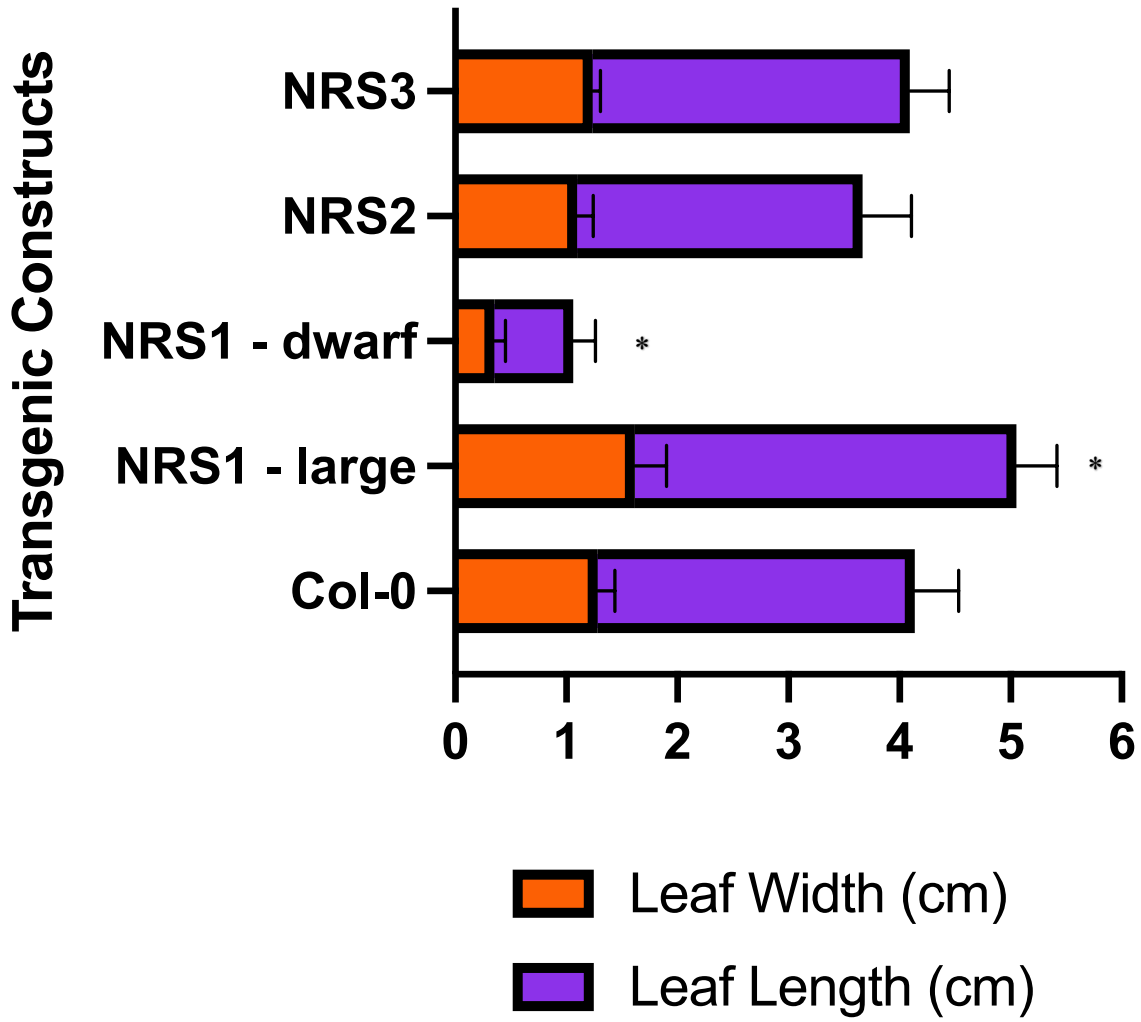


Figure 4.13 Leaf width and length measurements of stable *NRS1*, NRS2 and NRS3 transgenics compared against wild-type (Col-0). The length and width of leaf 6 was measured and compared by *t* tests (**$p < 10^{-5}$; *$p < 10^{-2}$; $n \geq 24$).

Chapter Five
WELL, WELL, WHEP

“The fruits of my immigrant father's lifelong efforts would be gobbled up and squandered by me, his lazy, disaffected daughter.”

Ling Ma



Section 5.1 WELL, WELL, WHEP

Introduction

This chapter proposes to build on the discovery that *NRS1* stimulates TOR activity by mechanistically defining how *NRS1* activates TOR at a molecular level. Beyond, TOR signaling, my experiments will also directly test the significance of the widely-observed but poorly-understood "WHEP" domains common to several tRNA synthetases but with no established enzymatic role.

As we have arrived at the final experimental chapter of this thesis, I find it relevant to summarize the story so far. The Brunkard lab discovered a null *1* allele in a forward genetic screen for defective embryonic cell-cell (plasmodesmatal) trafficking that repeatedly identified mutants defective in TOR signaling (Brunkard *et al* 2020, Kim *et al* 2002), which led us to speculate that *NRS1* could be required for TOR activity in plants. Jake Brunkard used RNA-Seq to define the *NRS1* transcriptome (>850 differentially expressed genes using stringent cut-off parameters), which exhibits several signatures of TOR inactivation, including significant repression of ribosomal protein genes and induction of proteolytic and catabolism-related genes (Busche et al 2021, Brunkard et al 2020, Xiong et al 2013). *NRS1* mutants and *NRS1* knockdowns also show significantly less phosphorylation of S6K-T449 (orthologous to human S6K-T389), a residue that is uniquely phosphorylated by the TOR kinase in plants. From all the previous experiments; we have understood that ***NRS1* expression is necessary to sustain TOR activity and sufficient to hyperactivate TOR** under standard growing conditions, supporting the overall hypothesis that *NRS1* acts as an upstream regulator of TOR. Moreover, we found that stably overexpressing *NRS1* causes phenotypes similar to TOR overexpression in Arabidopsis transgenics (e.g., increased organ and cell size).

The biological canonical function of Aminoacyl tRNA synthetases (aaRS), including *NRS1*, catalyze the ATP-dependent synthesis of "charged" tRNAs, that is, they covalently bind amino acids to their cognate tRNAs, which are then available to translate mRNA codons for protein synthesis. Beyond this canonical enzymatic role, however, tRNA synthetases have evolved various "moonlighting" roles in other biological processes, often through the addition of new domains (Paul and Schimmel, 2013, Luna et al, 2014, Chen et al 2018).

When human tRNA synthetases were first sequenced 30 years ago, independent groups discovered a helix-turn-helix motif shared by the tryptophanyl, histidyl, and glutamyl-prolyl tRNA synthetases (**W**ARS, **H**ARS, and **EP**ARS, respectively), now called the "**WHEP**" domain (Shiba 2002), that serves no biochemical role in catalyzing tRNA aminoacylation (Jia et al, 2008), but instead participates in protein-protein interactions (Ray et al 2014, Chang et al 2016, Halawani et al 2018, Guo et al, 2010). *NRS1* was the first example of a plant tRNA synthetase with a WHEP domain (Shiba 2002). The WHEP domain of WARS, for example, acts like a molecular "bridge", interacting with both the phosphatidylinositol kinase-like kinase (PIKK) DNA-PKcs and the poly[ADP-ribose] (PAR) polymerase 1 (PARP1) to facilitate PARylation of DNA-PKcs and subsequent activation of the tumor suppressor p53 (Guo et al, 2010). The

capacity for WARS-WHEP to activate DNA-PKcs and PARP1 is regulated at multiple levels. During aminoacylation, the binding of the WARS enzyme to the metabolic intermediate, Trp-AMP, prevents WHEP from interacting with DNA-PKcs and PARP1 (Sajish et al, 2012). Moreover, alternative splicing and/or proteolytic cleavage produce different isoforms of WARS (Otani et al 2002, Ahn et al 2016, Xu et al, 2017, Zhou et al, 2010), some of which lack the WHEP domain but are still fully functional tRNA synthetases. This example illustrates how tRNA synthetases can evolve new roles in regulating disease-related biological pathways.

Several studies have argued that leucyl tRNA synthetase (LARS) can stimulate TOR activity in humans and yeast, operating as an amino acid sensor for the essential nutrient leucine (Han et al 2012, Bonfils et al, 2012, Bar-Peled et al 2013, Yoon et al 2016, Lee et al 2018, Liu et al 2020, He et al 2018. Unlike WARS, however, LARS does not encode significant structures beyond the necessary domains for leucylating $tRNA_{Leu}$. A very recent study argued that tRNA synthetases, including LARS, can post-translationally aminoacylate lysine residues in proteins in a reaction analogous to the better-established post-translational modification, protein acetylation (Liu et al 2020). Human LARS can apparently leucylate Rag GTPase lysines, which promotes their GTPase activity and thus contributes to activating TOR (Liu et al 2020).

In this chapter, I will disrupt key functions of *NRS1* as a tRNA synthetase and delete the WHEP domain to establish the domains of *NRS1* necessary to activate TOR.

Section 5.2 Materials and Methods

Define the molecular features of *NRS1* required to stimulate TOR

NRS1 variants were transiently expressed using agrobacterium-mediated transformation of *N. benthamiana* leaves. Throughout, wild-type *NRS1* was used as a positive control for TOR induction and free GFP will was used a "mock" treatment negative control. TOR activity will then be screened from these samples using Western blots against S6K-pT449 and total S6K to determine how *NRS1* variants impact phosphorylation of this canonical TOR substrate. aaRS enzymes catalyze two reactions: amino acid adenylation (reaction **1**: AA + ATP◊AA-AMP + PPi) and subsequent esterification of tRNA with the aminoacyl group (reaction **2**: AA-AMP + tRNA ◊ AA-tRNA + AMP). aaRS structures are highly similar across all organisms, with highly conserved or invariant residues in the aaRS proteins predicted to be responsible for catalyzing tRNA aminoacylation and/or binding to amino acid-specific side chains, the amino acid backbone, ATP, co-enzymatic Mg^{++}, and the tRNA (Kaiser et al, 2020).

I mutated each of these conserved residues in *NRS1* and tested how these variants impact the stimulation of TOR by *NRS1* overexpression. The two reactions of ATP and Asn have key interaction residues that I have mutated listed in Table 5.2 and Table 5.3. I have also identified "random mutations" i.e. randomly selected amino acid sites in *NRS1* that are not highly conserved to serve as negative controls in these experiments (Table 5.1). By utilizing site-directed mutagenesis, I amplified the overexpression vector of *NRS1* and the pEG103 backbone by using primers that match the vector and have specific changes in the amino acid I had targeted. Most of the changes in the amino acid have changed the site to different amino acid than the wild type.

In addition to the key catalytic and control mutations, I mutated four sites on the amino acid backbone of *NRS1* (Figure 5.5, (B)) listed in Table 5.4 and the amino acid responsible for the dimerization interface of *NRS1* (Table 5.5). All 13 mutations I've introduced in each specific *NRS1* amino acid residue is listed in Table 5.6 along with the reaction or significance of the residue. I then amplified the two halves of the vector with each combination of the changed amino acid with the forward and the reverse, and QUICKSLIC-ed the reaction, moved it into ecoli and then transformed into agrobacterium.

Establish the minimal *NRS1* domains necessary to stimulate TOR

NRS1 is composed of three domains separated by flexible linkers: an N-terminal anticodon-binding domain, a C-terminal aminoacylation domain, and a WHEP domain within the C-terminal domain (Fig. 5.2). I have a cloned truncated versions of *NRS1* to exclude the WHEP domain, i.e., *NRS1*ΔWHEP. *In vivo*, *NRS1* forms a stable homodimer that is required for full enzymatic activity; I will also introduce a mutation, P196G, that removes an invariant proline residue required for tRNA synthetase dimerization (Table 5.5) (Eriani, 1993). As above, stability and subcellular localization of these proteins have been validated with fusions to GFP.

Section 5.3 Results

NRS1 could conceivably activate TOR through its enzymatic activity as an aaRS, through nonenzymatic protein-protein interactions, or through a combination of both. To distinguish among these possibilities, I will mutate key residues and/or domains of *NRS1* to define the minimal features of the *NRS1* protein required to activate TOR. I performed experiments to screen many *NRS1* variants and classify these variants by their effects on *NRS1* enzymatic function and *NRS1*-mediated activation of TOR.

NRS1 variant stability and subcellular localization are not expected to be disrupted by any of these mutations based on structural comparisons to orthologues in other systems, but to confirm this assumption, variant *NRS1*-GFP fusions have been expressed to test for protein stability by Western blot and subcellular localization by confocal fluorescence microscopy. If *NRS1* senses amino acids for TOR, the hypothesis here is that mutations disrupting reaction 2 (tRNA charging) may be dispensable for *NRS1* stimulation of TOR activity, whereas mutations disrupting reaction 1 (Asn aminoacylation) would abrogate *NRS1*-TOR signaling.

In Figure 5.6 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). In (A) Lys431 mutation compared against *NRS1* with mock infiltrations. In (B) Iso5 mutation compared against *NRS1*. In (C) this experiment was repeated multiple times; representative results are shown here. Graph is a plotted box plot of the normalized western quantification numbers of each treatment, with negative controls (random mutations) functioning well with Ly431 and Iso5, with very little different than the wild-type *NRS1* over expression construct.

Having established reliable negative controls, ATP catalysis and binding mutations were tested. Overexpression constructs of *NRS1* were compared against Arg337, Glu389 and Arg546. In Figure 5.7 (A) Arg337 and Glu389 mutants compared against *NRS1* and (B) Repeat representative blot of A, with (C) Arg546 mutant compared against *NRS1* and (D) Repeat representative blot of C. The normalized western quantification numbers of each treatment is plotted in a box plot in Figure 5.7 (E). It appears that mutations in the residues that are responsible for the ATP binding residues of the tRNA synthetase have some impact on *NRS1*'s ability to impact TOR, but the results are variable across different mutations; with the residue Arg546 having the most variability, Arg337 having moderate variability, and *NRS1* mutant residue Glu389 having decreased the tRNA synthetases' ability to phosphorylate S6K1 most significantly.

After testing the ability of mutants in the ATP catalysis and binding amino acid residues to impact the ability of *NRS1* to phosphorylate S6K1, subsequent esterification of tRNA

with the aminoacyl group (reaction 2: AA-AMP + tRNA à AA-tRNA + AMP) demonstrated that disrupting this pathway was not essential for *NRS1*-TOR signaling.

The other canonical reaction to interrupt in the aaRS enymze is the amino acid adenylation, the Asn binding reaction(reaction **1**: AA + ATPàAA-AMP + PPi). Overexpression constructs of *NRS1* were compared against agroinfiltrated constructs of mutated amino acid residues (A-B) Asp487, Arg502 and (C-D) Glu354. In (E) the normalized western blot quantification ratios are blotted in a box plot. The results indicate that mutating the amino acid adenylation function of *NRS1* has an impact on interrupting the *NRS1*-TOR signaling pathway, with some level of significance. The residue Glu5354 has the most variability, with Asp487 and Arg502 having more impact on reducing *NRS1*'s ability to phosphorylate S6K1.

The next four mutated amino acid resides I tested were the amino acid residues that make up conserved backbone interactions of the aaRS enzyme (Figure 5.5) Overexpression constructs of *NRS1* were compared against agroinfiltrated constructs of mutated amino acid residues in Figure 5.9 with (A) Gln316, (B) Ser314,(C) Tyr473 (D) Glu204. Representative blots of S6K1 phosphorylation shown, without ponceau staining. In Figure 5.9 (E) the normalized western blot quantification ratios of these experiments are blotted in a box plot. The results illustrate that the amino acid mutation in residues Tyr473 and Serin314 has no impact on *NRS1*'s ability to phosphorylate S6K1, whereas the mutations in the backbone amino acids Glu204 and Gln316 had some impact on the *NRS1*-TOR signaling pathway.

Next, I tested the mutant P196, that removes an invariant proline residue required for tRNA synthetase dimerization. Agroinfiltrated N. benthamiana plants with overexpression constructs and fused to a C-terminal GFP tag in Figure 5.10 (A) show representative western blot results. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane, and the results are plotted in a box plot (B), with results indicating that mutating the key residue in the dimerization interface has some diminished impact on *NRS1*-TOR signaling.

I have a cloned truncated versions of *NRS1* to exclude the WHEP domain, i.e., *NRS1*ΔWHEP. This truncated *NRS1*ΔWHEP over-expression construct was compared against the Wt-*NRS1* construct in western blot assays. To reiterate, the WHEP domain of WARS, for example, acts like a molecular "bridge", interacting with both the phosphatidylinositol kinase-like kinase (PIKK) DNA-PKcs and the poly[ADP-ribose] (PAR) polymerase 1 (PARP1) to facilitate PARylation of DNA- PKcs and subsequent activation of the tumor suppressor p53 (Guo et al, 2010). Thus, there exists a model in humans where the WHEP domain mediates interaction between a tRNA synthetase and a protein kinase. In Figure 5.11, agroinfiltrated N. benthamiana plants with overexpression constructs of *NRS1*ΔWHEP, wild-type *NRS1* and mock infiltrations (A-F). S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in

each lane. The results are plotted in the box blot graph (G) and demonstrate that *NRS1* can no longer activate S6K with deletion of the WHEP domain. Based on these results, it is possible that the WHEP domain is the interacting partner for the upstream TOR pathway.

Section 5.4 Discussion

These experiments test several hypotheses about the molecular mechanism of *NRS1* activation of TOR. In the human WARS-DNA-PKcs regulatory model, the WHEP domain of WARS is proposed to interact with either DNA-PKcs or PARP1; by dimerizing, WARS forms the quaternary structure that brings together these two proteins so that PARP1 can PARylate (and thus activate) DNA-PKcs. If *NRS1* activates TOR through an analogous model, then *NRS1* variants will **only stimulate TOR if they include the WHEP domain and can dimerize**. The N-terminal anticodon-binding domain may be dispensable in this situation.

This expands upon the known body of literature that encompasses TOR signaling, primarily within the realm of S6K activation, as a direct downstream target of TOR, and proves a novel mechanism by which S6K may be phosphorylated. As TORC complexes are hubs of huge signaling networks that govern key developmental regulation in plants, a key factor in unraveling the understanding of how the complex responds to external signals to coordinate multicellular growth appropriately (as plants are completely sessile).

Mounting evidence suggests that integrated signaling and metabolic networks play an instructive role in developmental programs and responses to environmental changes and stresses (Li and Sheen, 2016; Krejci and Tennessen, 2017). The results from this chapter show a potential mechanism for metabolic integration through the WHEP domain of *NRS1,* which is integral to amino acid charging and part of the complex nitrogen assimilation pathway of plants.

Building on the thorough mechanistic understanding of *NRS1*-TOR activation we will gain from this project, a major next step will be to resolve the protein structure of *NRS1* when bound versus not bound to its substrates. For example, in the case of human WARS-DNA-PKcs regulation, the WARS WHEP domain undergoes a conformational change when the enzyme's active site is bound to tryptophanyl- AMP; given the location of the *NRS1* WHEP domain flexibly linked near the Asn-binding pocket, a similar mechanism may reconfigure *NRS1*-WHEP's structure when the enzyme is bound to Asn.

At an organismal level, the precise mechanism(s) of *NRS1*-promoted growth in *NRS1* transgenic plants need to be further investigated using physiological and developmental approaches. A primary evolutionary question is how *NRS1* can broadly promote TOR in other plants or even non-plant eukaryotes, or if the 1-TOR signal transduction pathway only recently evolved in dicot flowering plants.

Figures

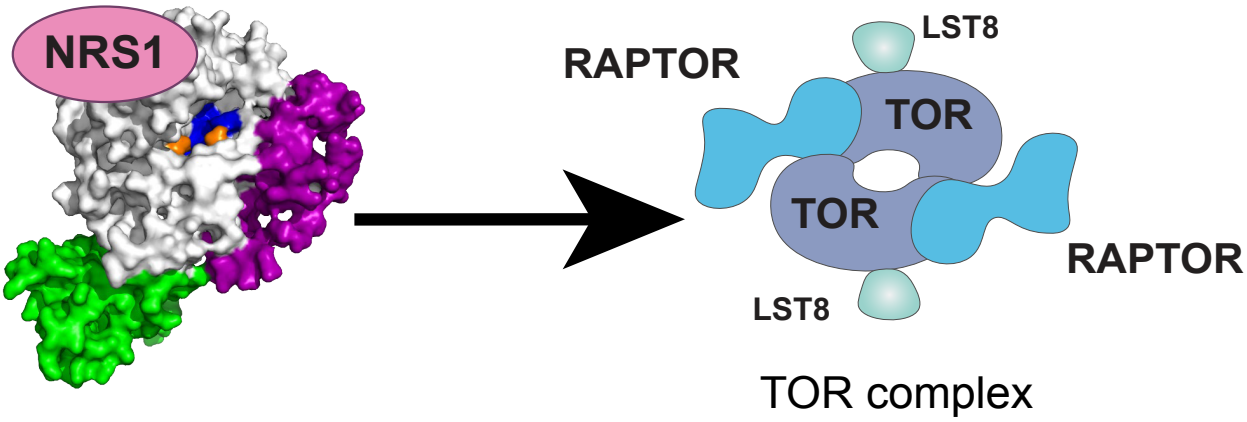


Figure 5.1 Summary of this chapter's goals; defining the molecular features of *NRS1* required to stimulate TOR

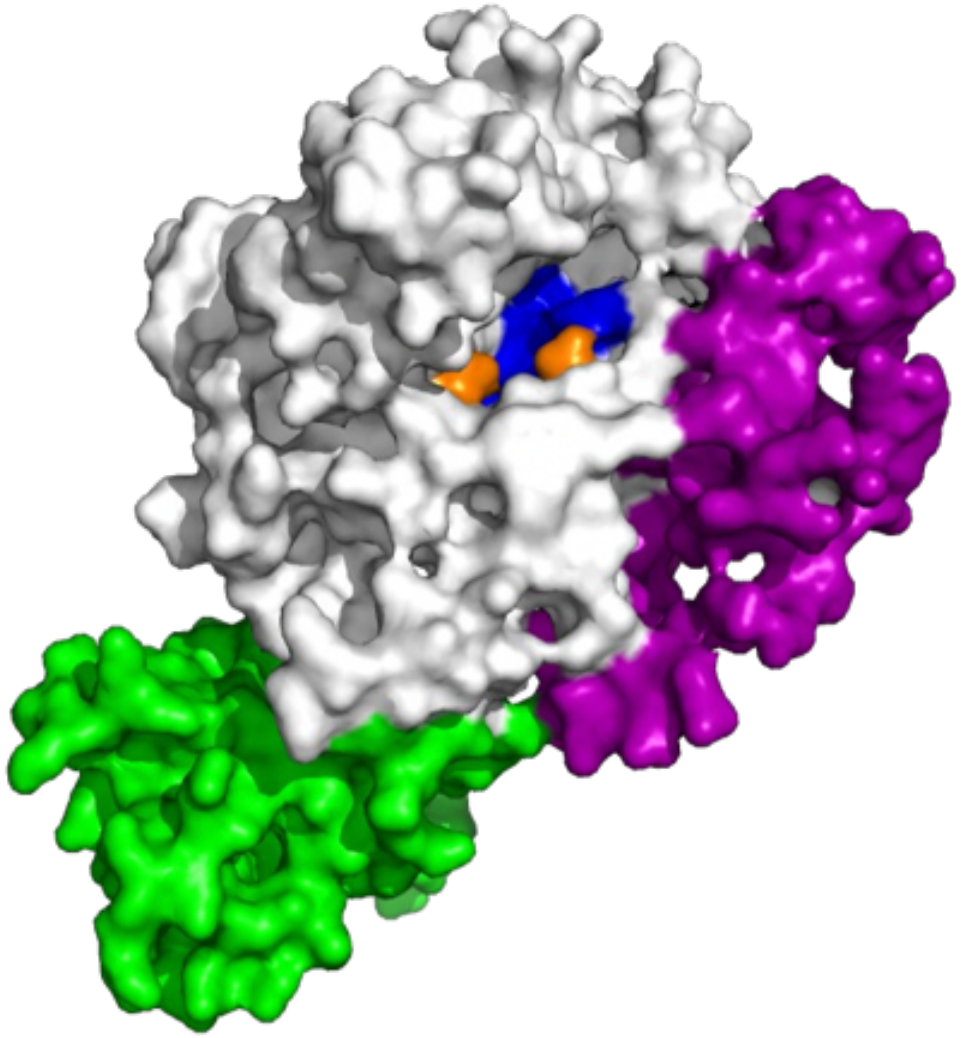


Figure 5.2 Model of *NRS1* structure, showing the N-terminal anticodon recognition domain (green), the C- terminal aminoacylation domain (white) with catalytic residues in a groove that bind to ATP (orange) or Asn (blue) indicated, and the WHEP domain (magenta), a helix- turn-helix motif that is connected by ~20aa long flexible linkers at either side to the aminoacylation domain.

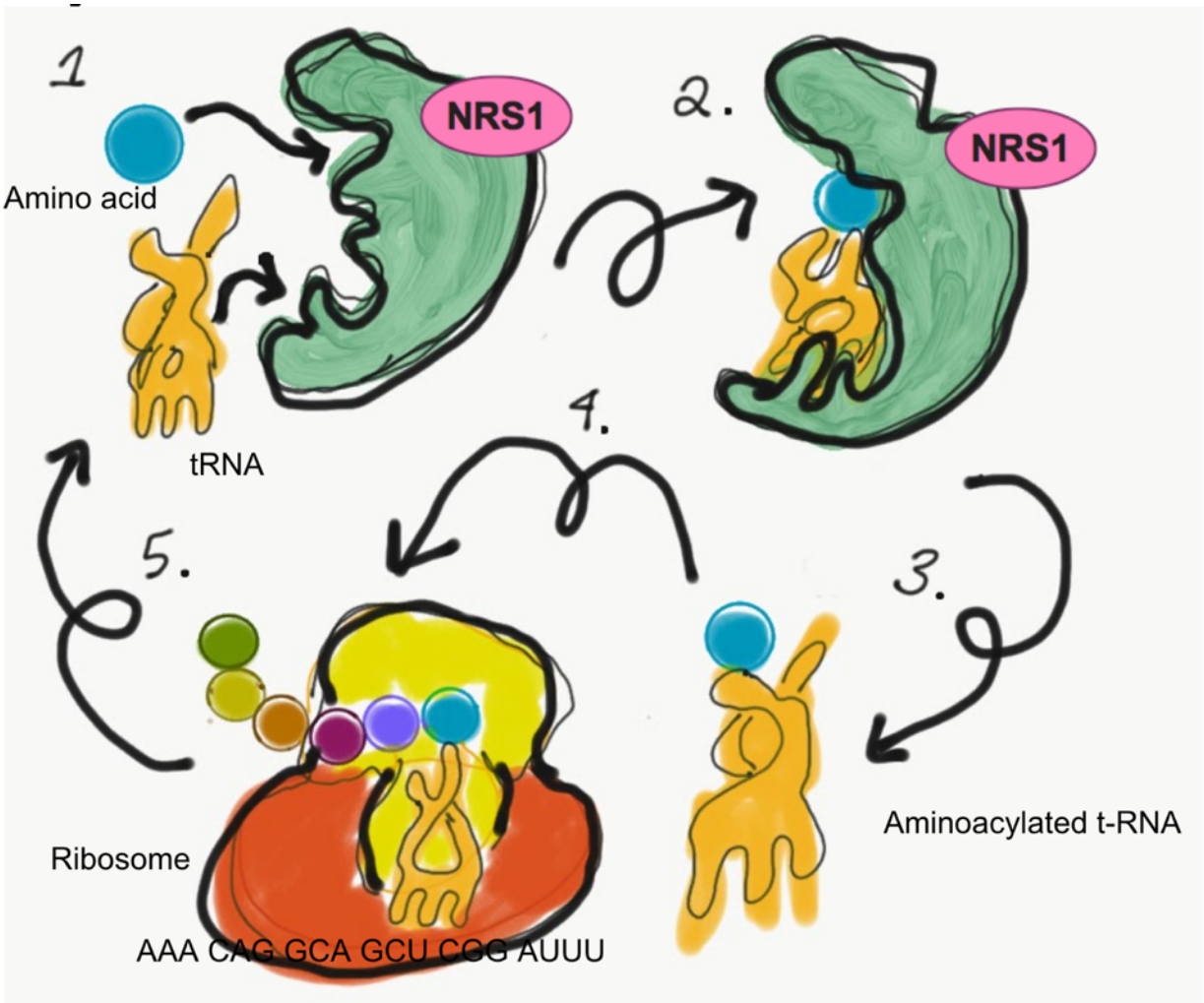


Figure 5.3 Aminoacyl-tRNA synthetase function illustrated

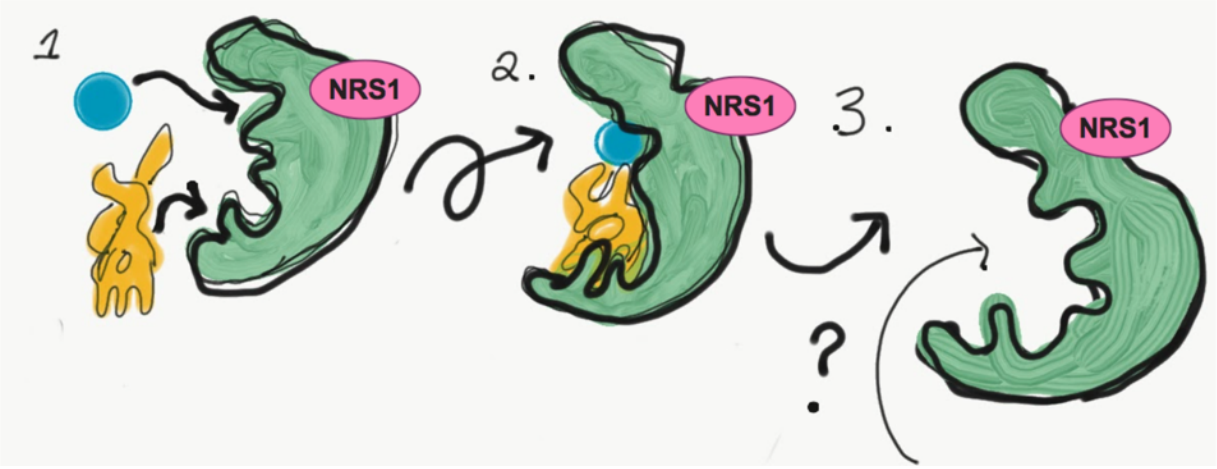


Figure 5.4 Experimental design illustrated; introducing mutations to *NRS1* that should prevent it from functioning as a tRNA synthetase

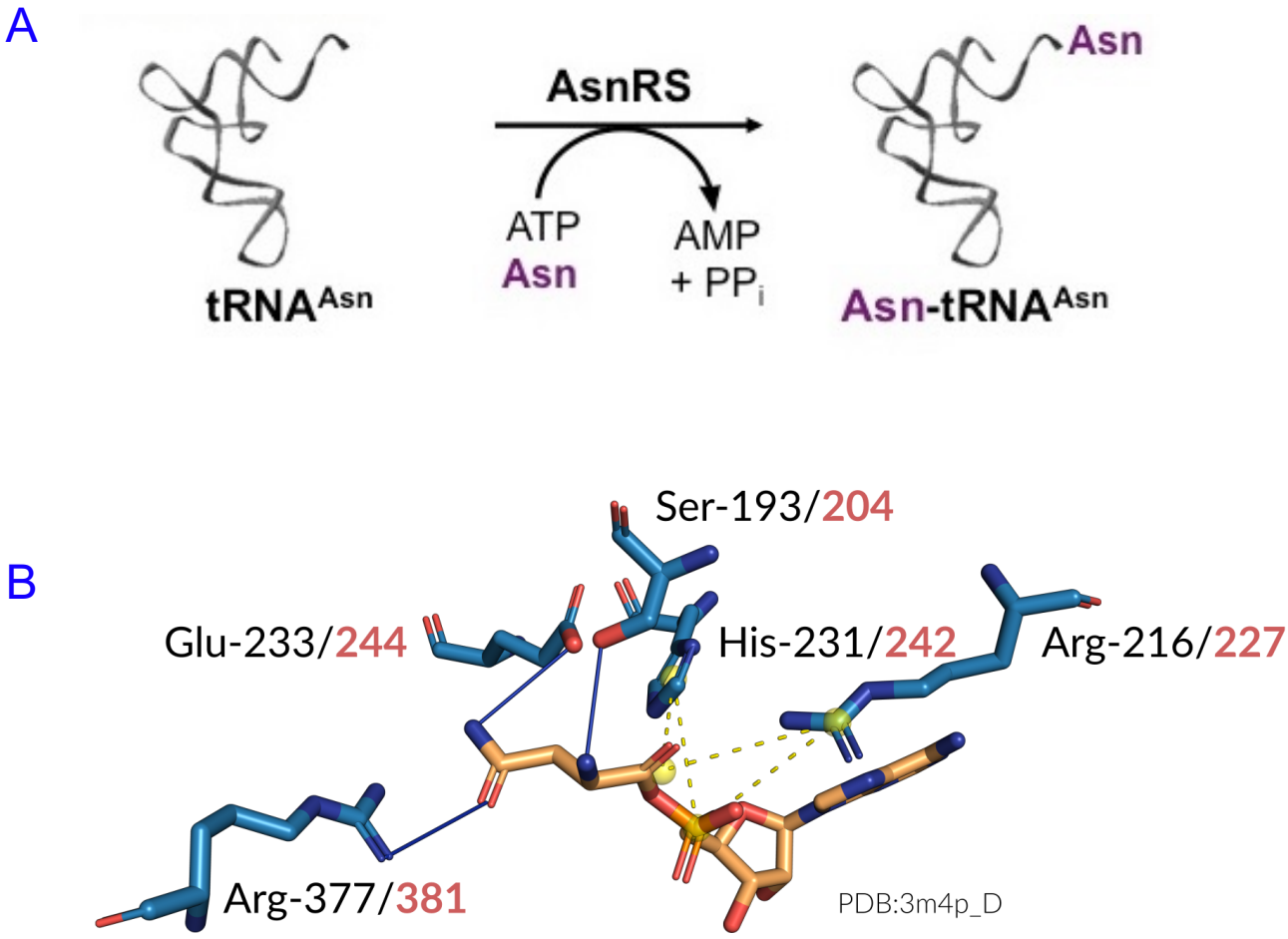


Figure 5.5 Asparginyl tRNA synthetase function
(A) The aminoacylation of the RNA involves three substrates: ATP, amino acids and tRNA
(B) The interaction patterns of asparyginyl -tRNA synthetase in *Entamoeba histolytica* (adapted from Kaiser et al, 2020)

AA Residue	Significance
Lys431	Random mutation
Iso5	Random mutation

Table 5.1

Mutation Site	Principal Interaction of Amino Acid Residue	Reaction Catalyzed
Glu389	Binds a structural and catalytic magnesium co-ion	Δ ATP
Arg337	Stabilizes the enzyme transition state	Δ ATP
Arg546	ATP binding	Δ ATP

Table 5.2

Mutation Site	Principal Interaction of Amino Acid Residue	Reaction Catalyzed
Asp487	Binds a structural and catalytic magnesium co-ion	Δ Asn
Arg502	Stabilizes the enzyme transition state	Δ Asn
Glu354	Asparagine specificity	Δ Asn

Table 5.3

AA Residue	Significance
Glu204	**amino acid backbone**
Serine314	**amino acid backbone**
Gln316	**amino acid backbone**
Tyr473	**amino acid backbone**

Table 5.4

AA Residue	Significance
Pro196	**Dimerization interface**

Table 5.5

AA residue	Reaction/ Significance
Arg337	ATP catalysis
Glu389	ATP catalysis
Arg546	ATP binding
Asp487	Asn binding
Arg502	Asn binding
Glu354	Asn binding
Glu204	amino acid backbone
Serine314	amino acid backbone
Gln316	amino acid backbone
Tyr473	amino acid backbone
Pro196	dimerization
Lys431	Random mutation
Iso5	Random mutation

Table 5.6

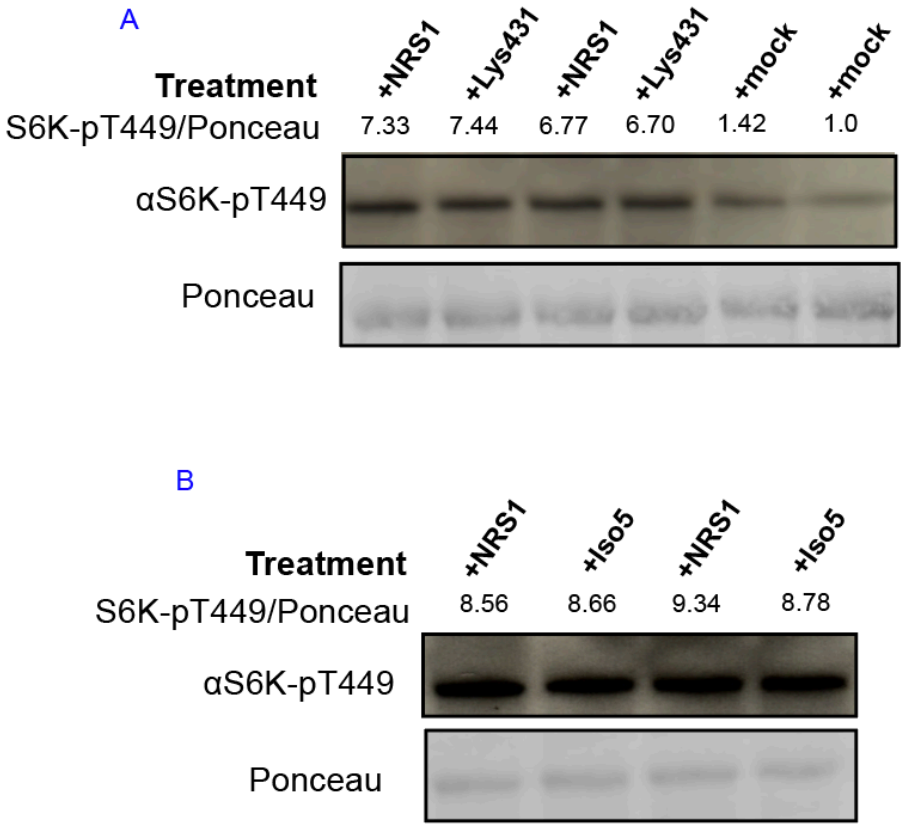


Figure 5.6 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment).
(A) Lys431 mutation compared against *NRS1*
(B) Iso5 mutation compared against *NRS1*

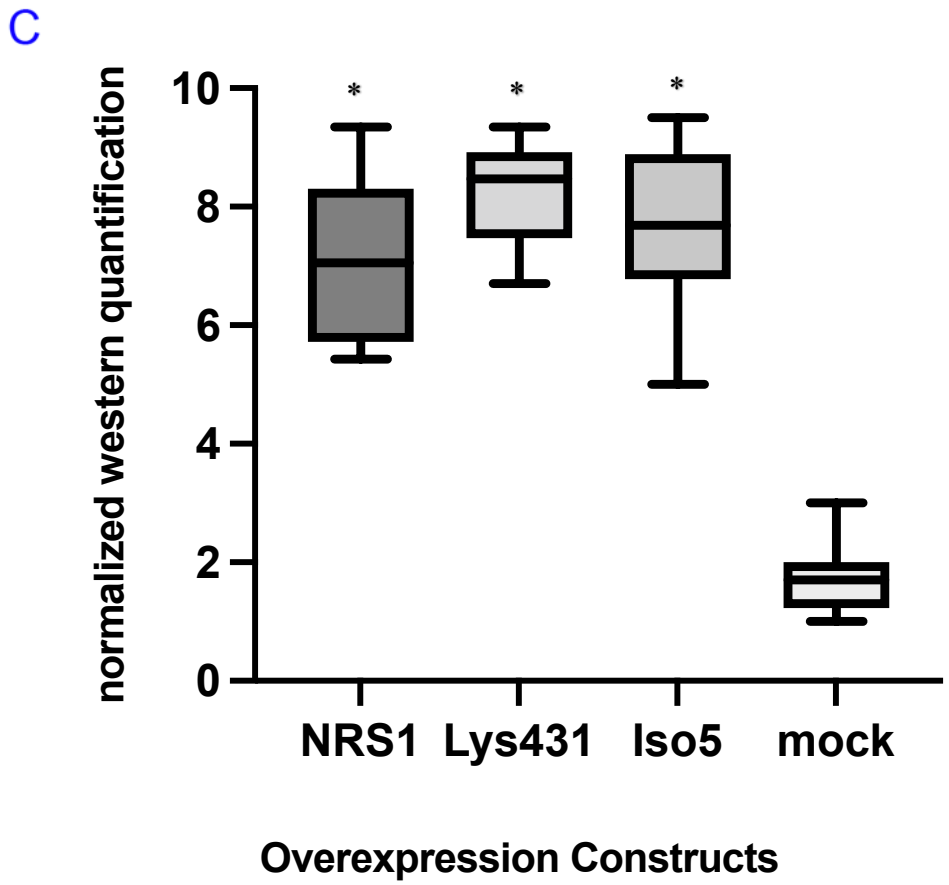


Figure 5.6 Graph comparing negative mutations to *NRS1*
(C) This experiment was repeated multiple times; representative results are shown here. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

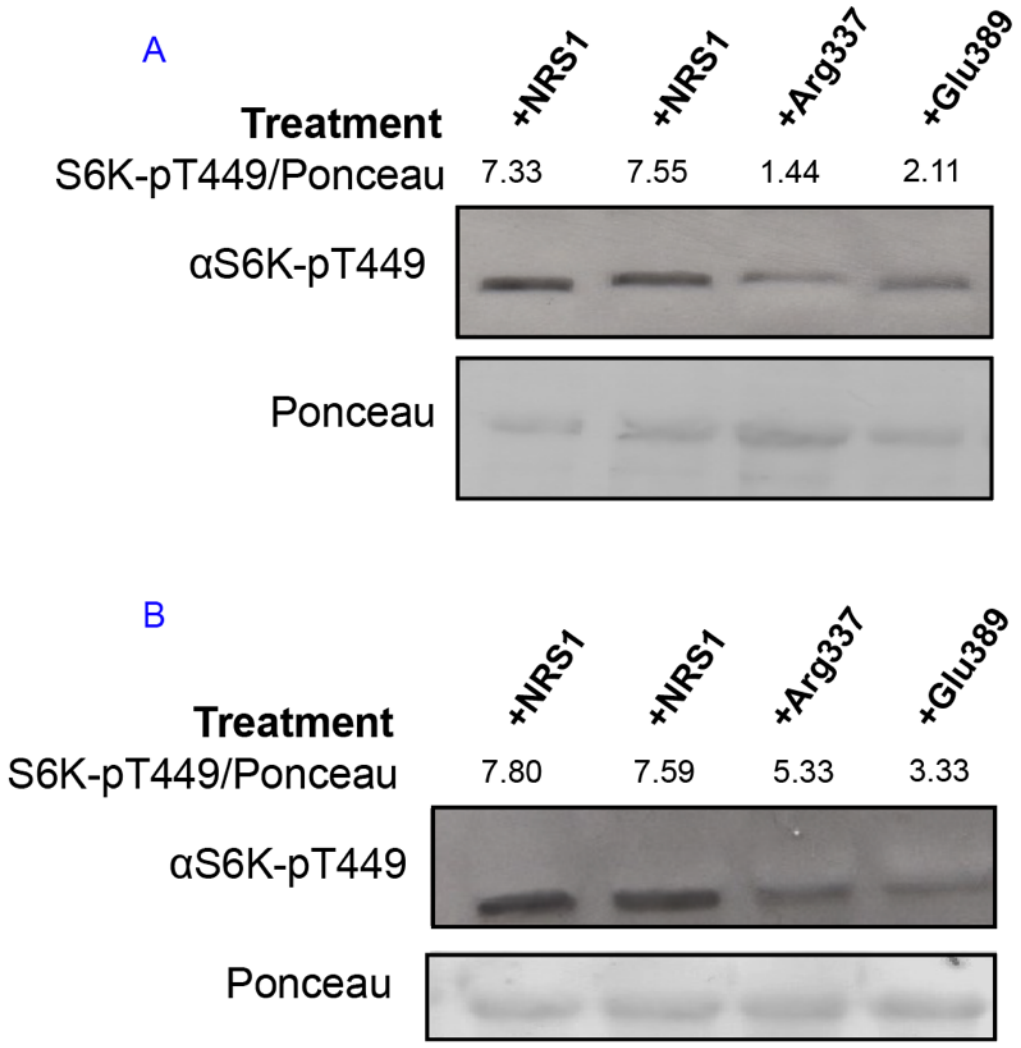


Figure 5.7 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment).
(A) Arg337 and Glu389 mutants compared against *NRS1*
(B) Repeat representative blot of A

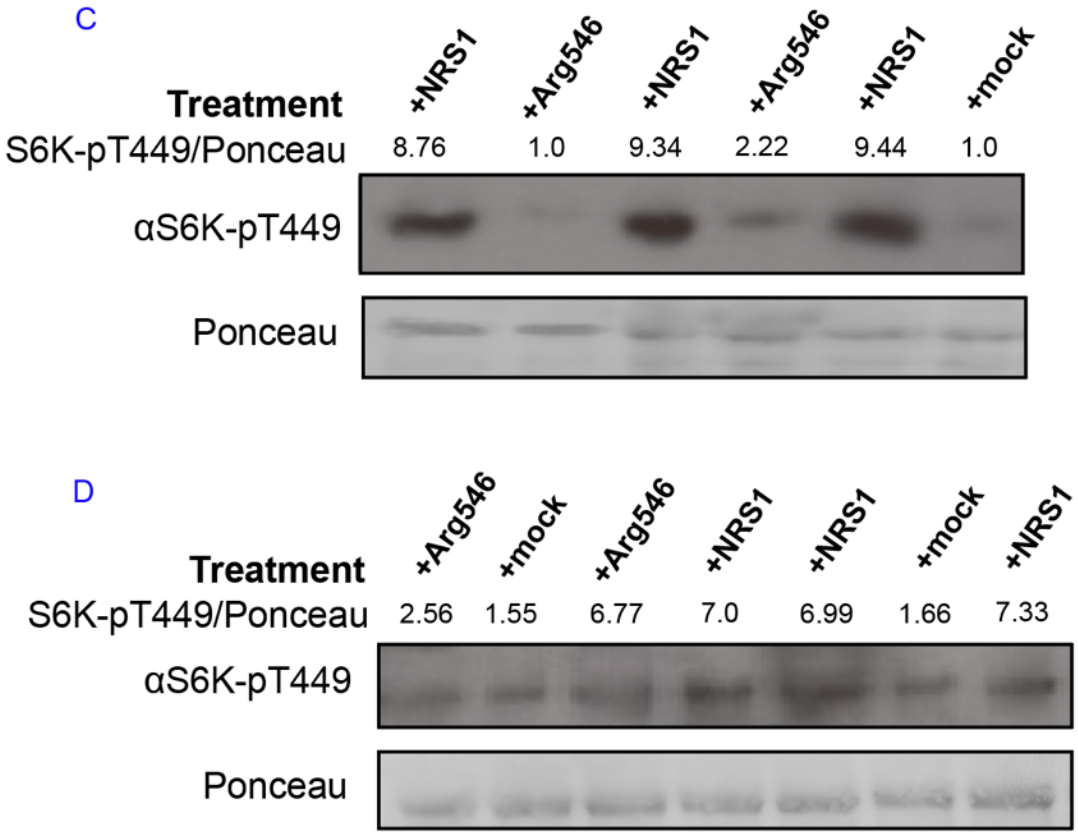


Figure 5.7 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment).
(C) Arg546 mutant compared against *NRS1*
(D) Repeat representative blot of C

E

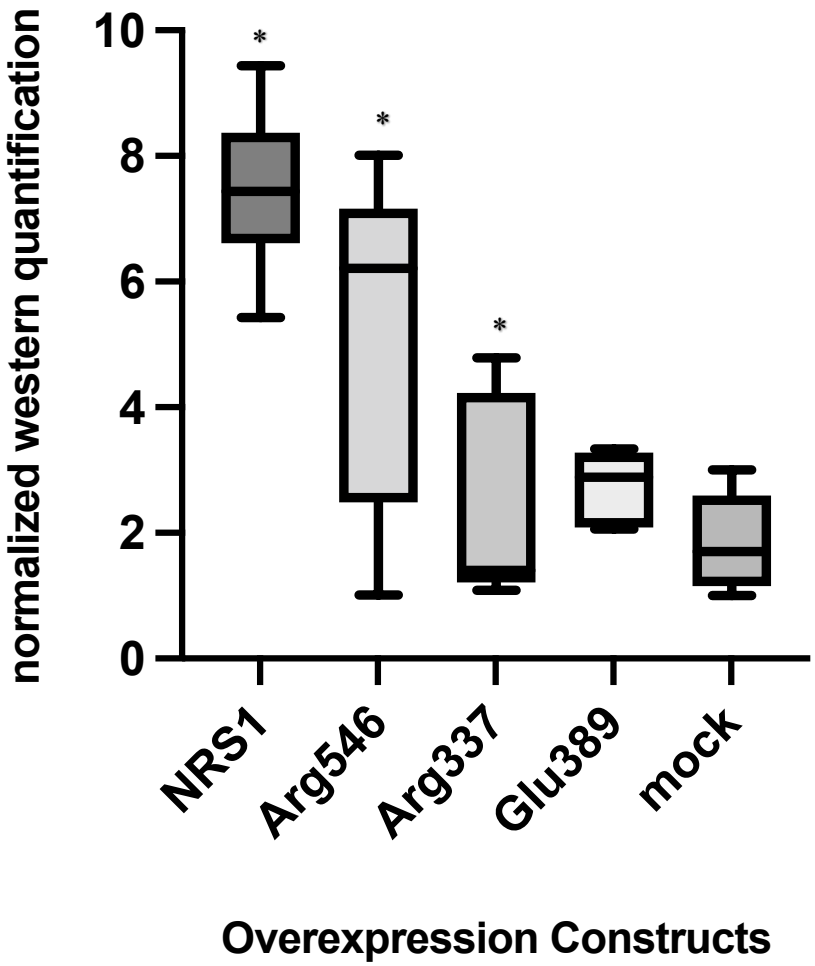


Figure 5.7 Graph of amino acids present in the catalytic residues that bind to ATP (E) This experiment was repeated multiple times; representative results are shown here. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

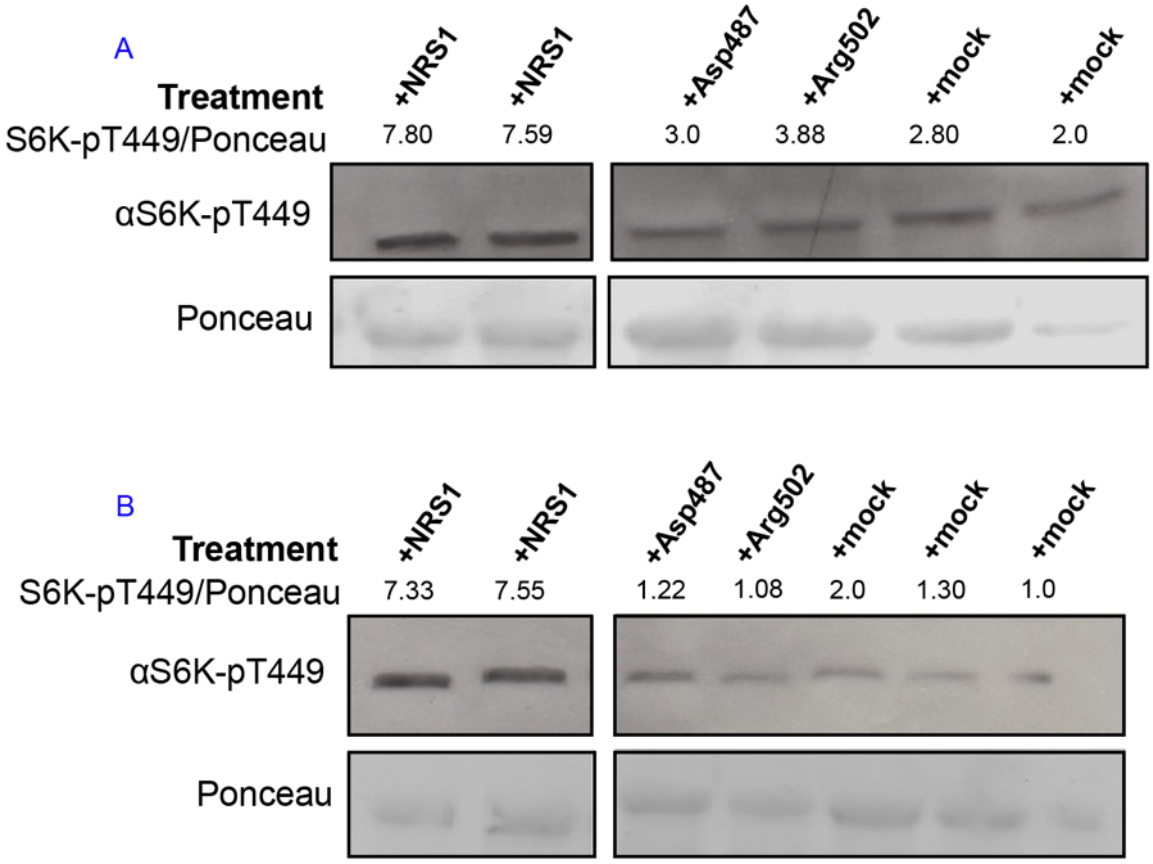


Figure 5.8 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment).
(A) Asp487 and Arg 502 mutants compared against *NRS1*
(B) Repeat representative blot of A

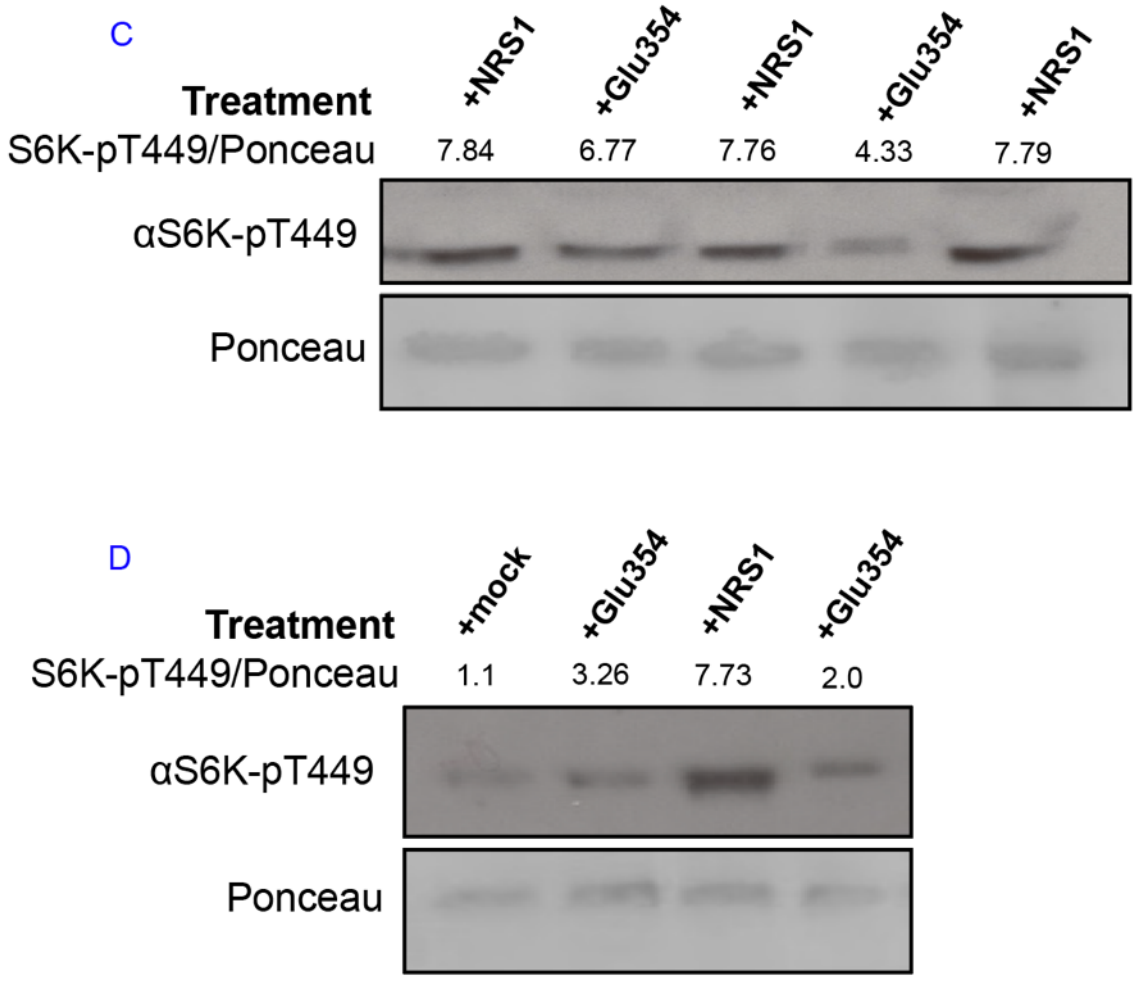


Figure 5.8 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449, with Ponceau staining shown as a loading control. S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment).
(C) Glu354 mutants compared against *NRS1*
(D) Repeat representative blot of C

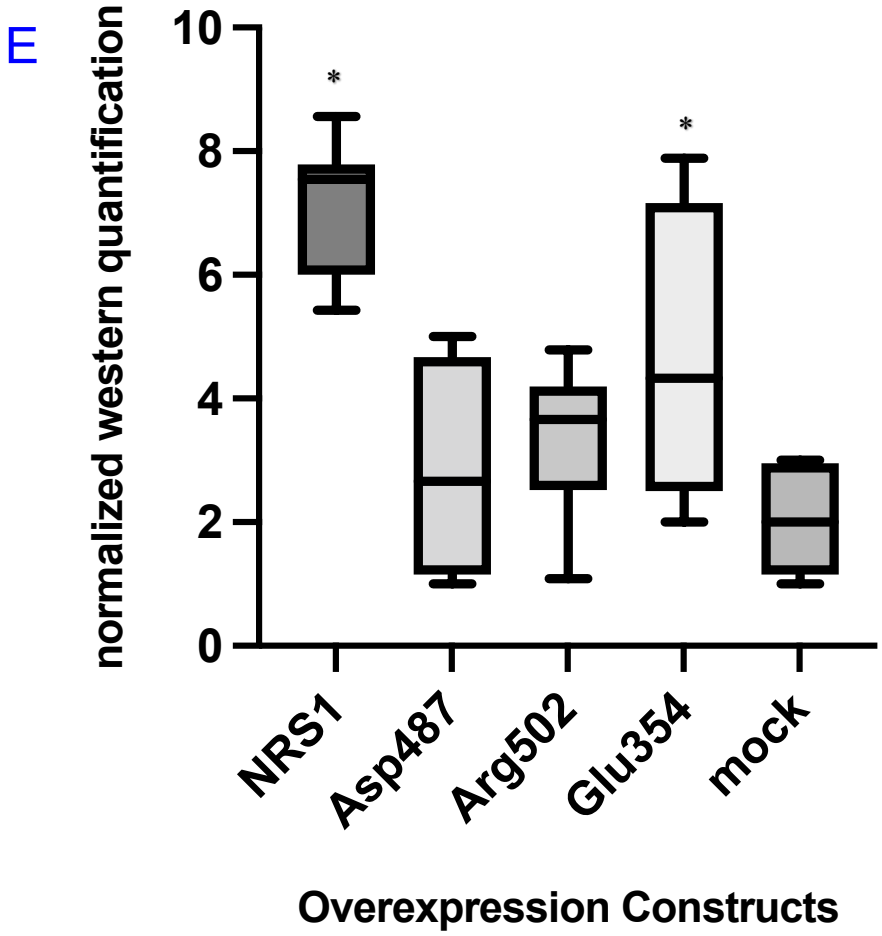


Figure 5.8 Graph of amino acids present in the catalytic residues that bind to ASN (E) This experiment was repeated multiple times; representative results are shown here. Graph is a plotted box plot of the normalized western quantification numbers of each treatment. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

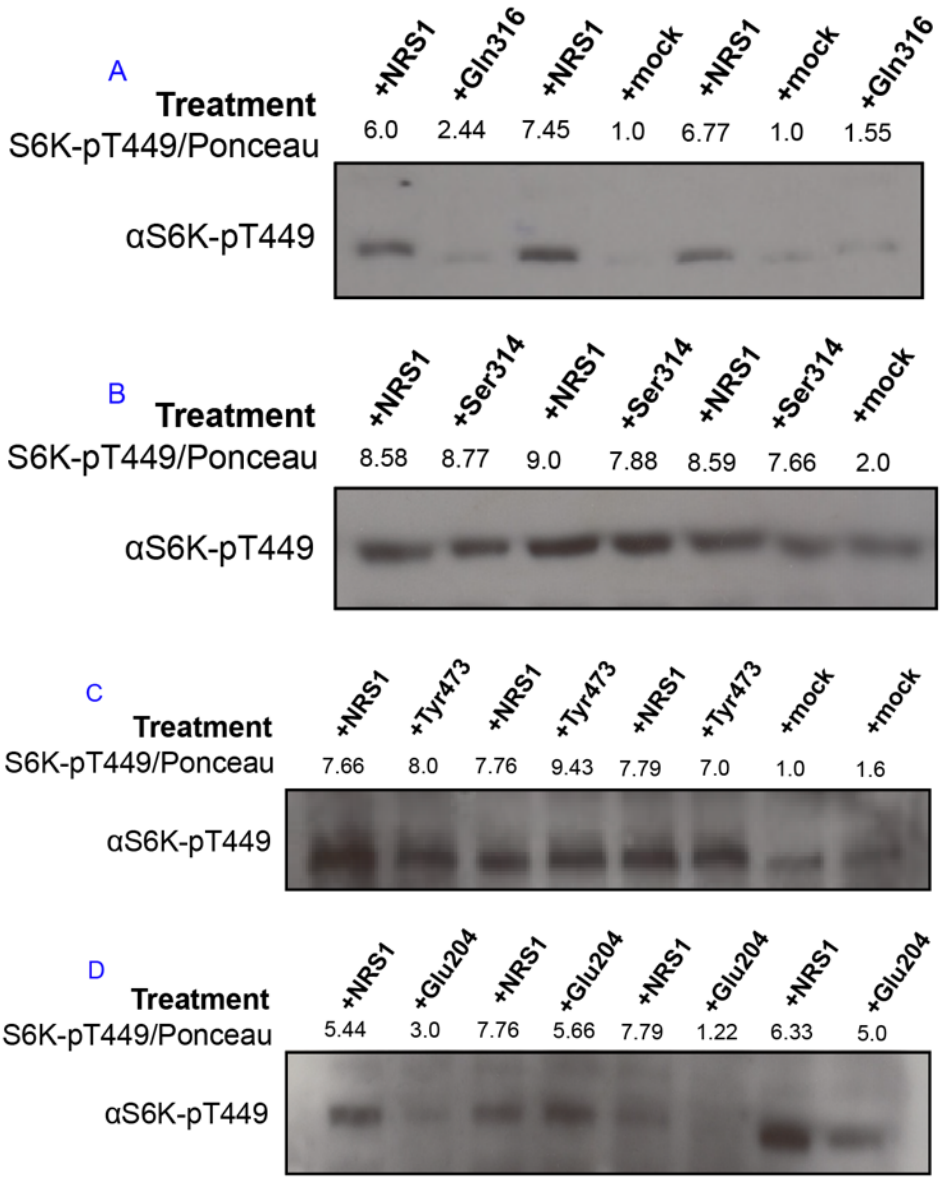


Figure 5.9 Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449. Ponceau staining not shown.
(A) Gln316 mutants compared against *NRS1*
(B) Ser314 mutants compared against *NRS1*
(C) Tyr473 mutants compared against *NRS1*
(D) Glu204 mutants compared against *NRS1*

E

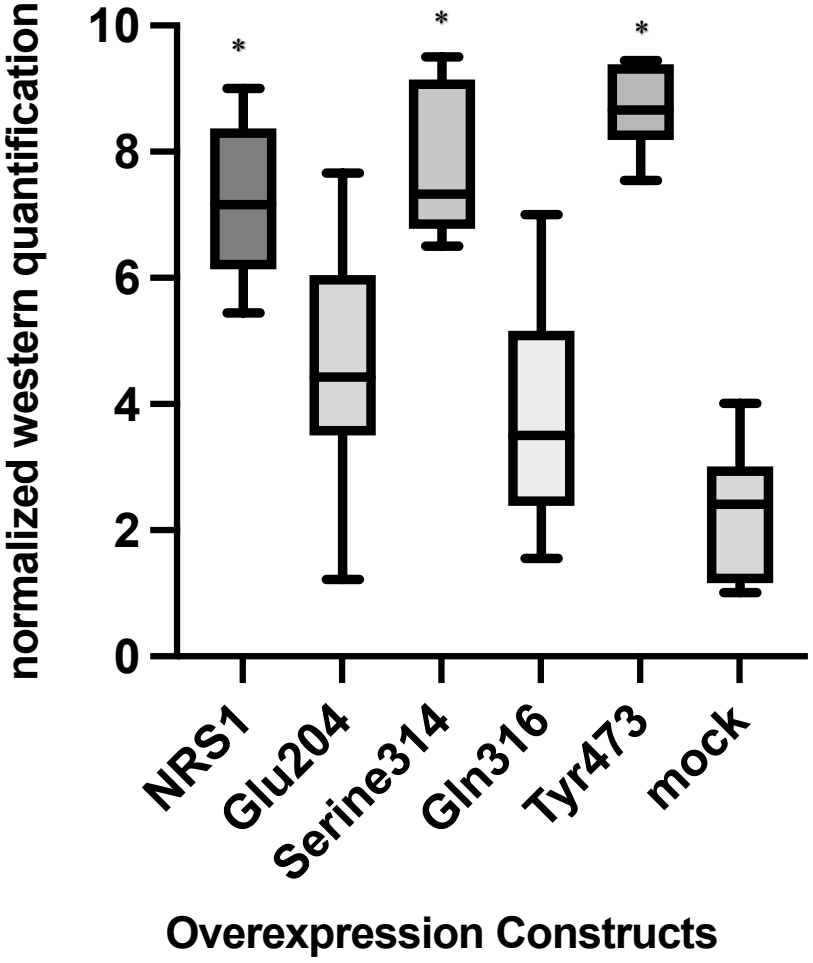


Figure 5.9 Graph of essential catalytic backbone amino acids plotted against *NRS1* (E) S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated multiple times; representative results are shown here. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

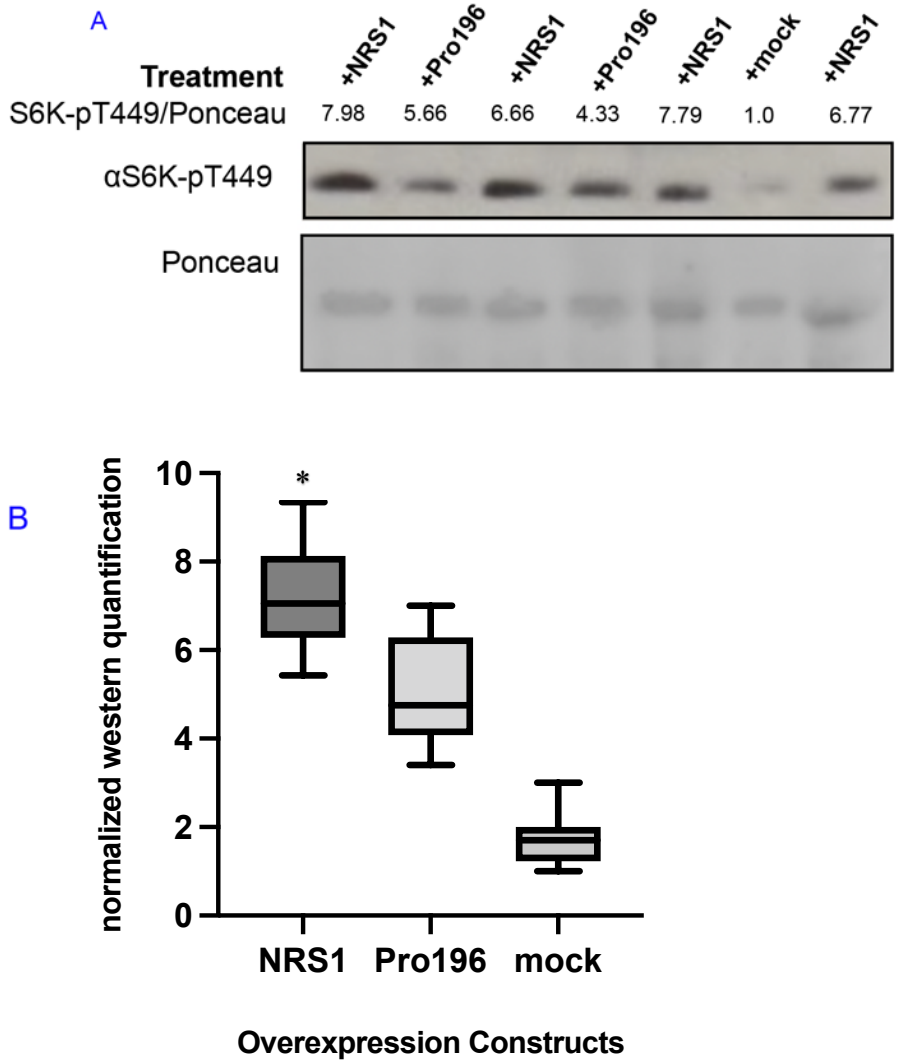


Figure 5.10 Dimerization mutants compared against *NRS1*
(A) Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449.
(B) S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated multiple times; representative results are shown here. * indicates $P<0.05$

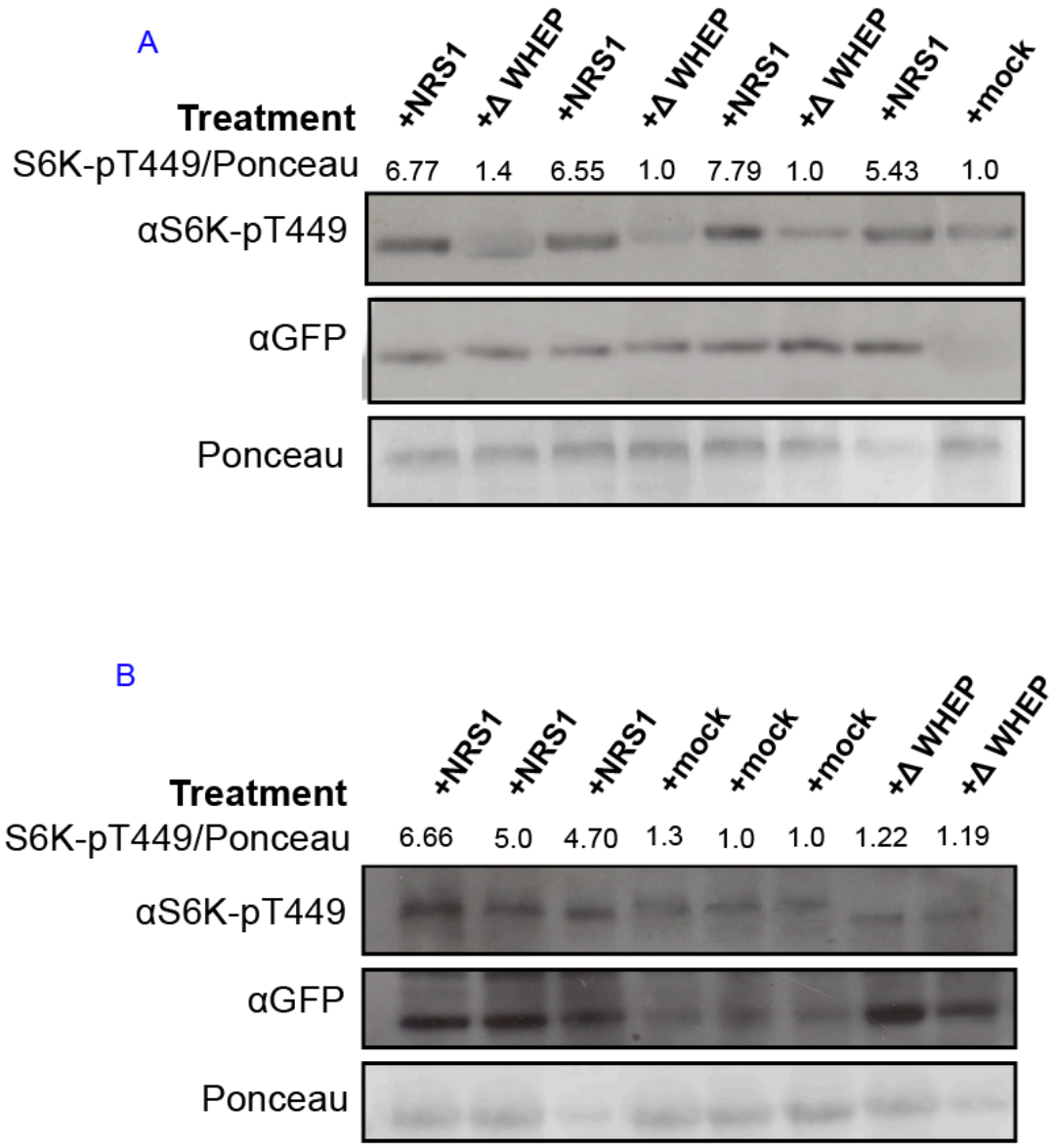
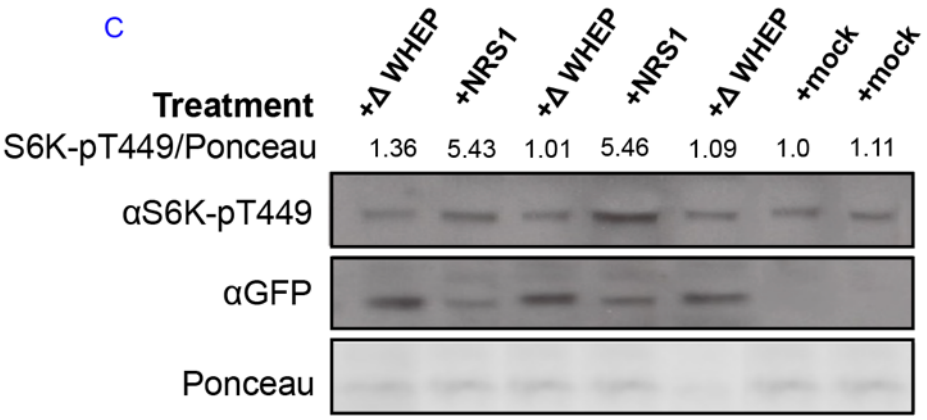
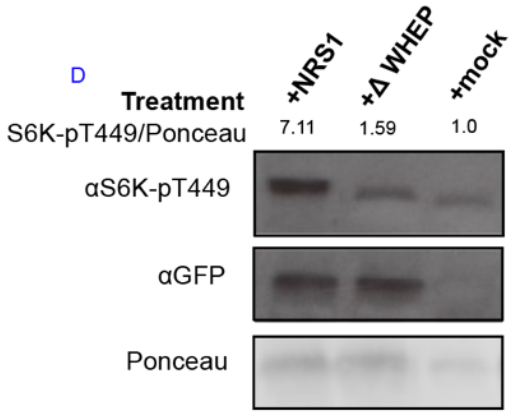


Figure 5.11 WHEP mutants and *NRS1* westerns
(A-B) Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449.

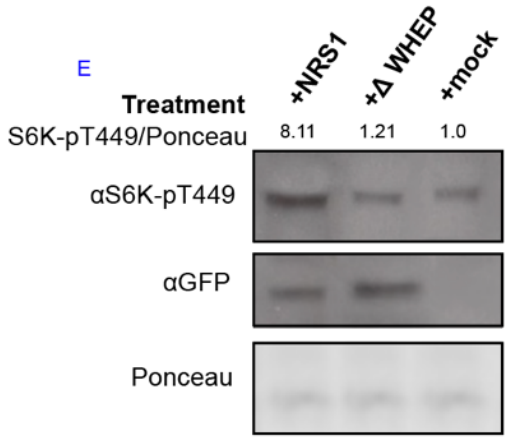
C


Treatment
+Δ WHEP
+NRS1
+Δ WHEP
+NRS1
+Δ WHEP
+mock
+mock
S6K-pT449/Ponceau
1.36
5.43
1.01
5.46
1.09
1.0
1.11
αS6K-pT449
αGFP
Ponceau

D


Treatment
+NRS1
+Δ WHEP
+mock
S6K-pT449/Ponceau
7.11
1.59
1.0
αS6K-pT449
αGFP
Ponceau

E


Treatment
+NRS1
+Δ WHEP
+mock
S6K-pT449/Ponceau
8.11
1.21
1.0
αS6K-pT449
αGFP
Ponceau

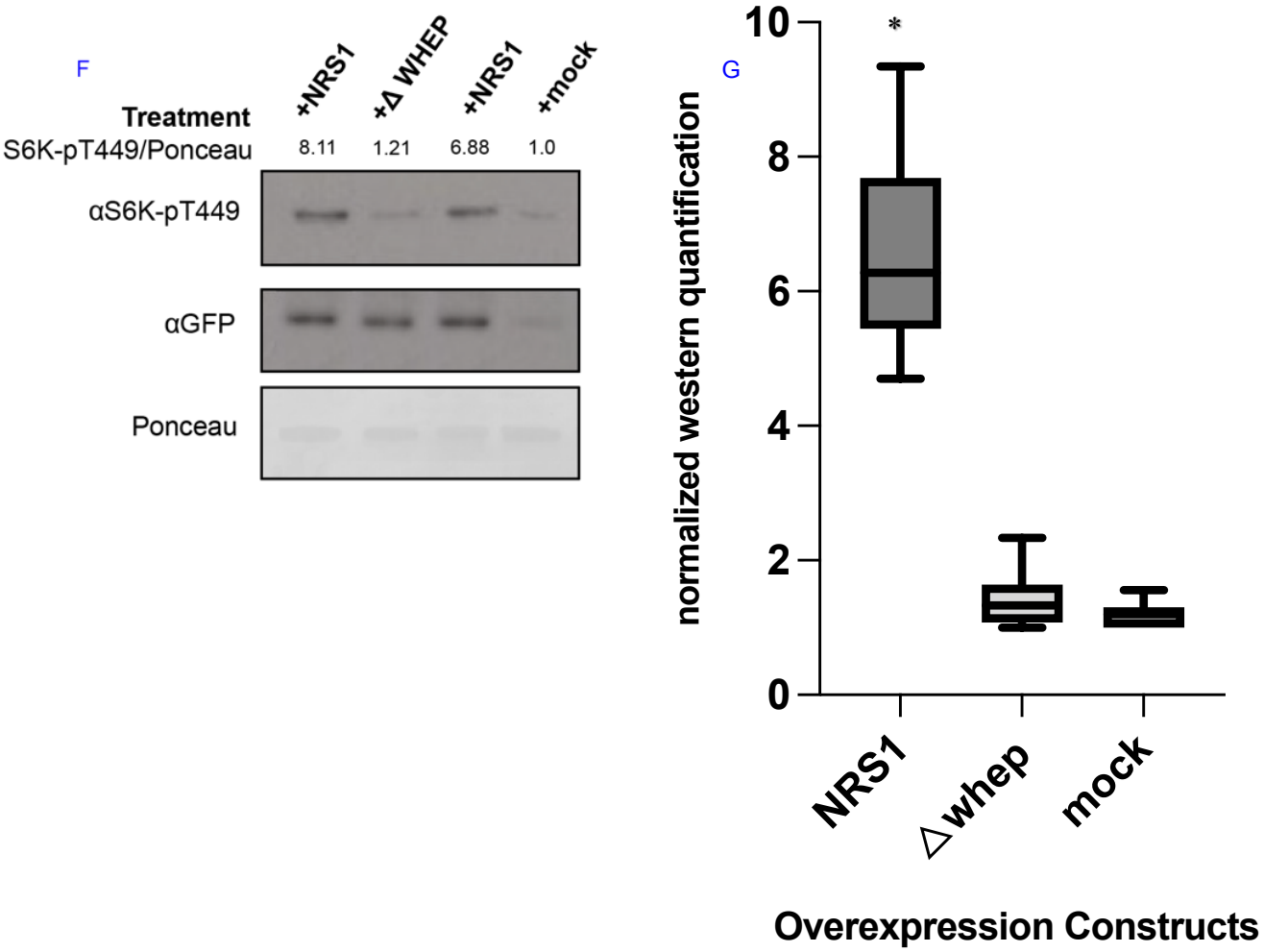


Figure 5.11 WHEP mutants and *NRS1* westerns and graph
(C-E) Agroinfiltrated *N. benthamiana* plants with overexpression constructs and fused to a C-terminal GFP tag. Genetic expression of the construct in the leaf occurs at 48 hours, and leaf protein samples were collected at this time for western blot analysis. TOR activity was assayed using Western blots against S6K-pT449.
(G) S6K-pT449/Ponceau ratios in the graphs are shown as a number normalized to a control lane (in these blots, the control lane is the mock treatment). The normalized ratio is the abundance of the target protein normalized to the total amount of protein in each lane. This experiment was repeated multiple times; representative results are shown here. Asterisks show a t-test performed and statistical significance. * indicates $P<0.05$

Chapter Six
Concluding Thoughts and Outlook

“She hopes for nothing except fine weather and a resolution. She wants to end properly, like a good sentence. ”
Zadie Smith



The End

This project focuses on one of the most promising targets, tRNA, which are the only putative amino acid- sensors proposed to be shared among plants, fungi, and animals. A fundamental, mechanistic understanding of how tRNA synthetases evolved amino acid-sensing roles will be foundational for ongoing efforts to develop clinically-relevant tRNA synthetase inhibitors and pivotal for understanding nitrogen sensing in agricultural contexts.

These results might help us to further elucidate the metabolic interactions of a plant through the TOR signaling network to external nitrogen sources. We know that nitrate, a major N source, behaves as a nutrient signal to promote system-wide shoot and root growth in Arabidopsis (Liu et al., 2017). Arabidopsis seedlings modified to overexpress *TOR* show hypersensitivity to high nitrate inhibition of roots (Deprost et al., 2007). By sensing the nutrient content in the cell, *TOR* kinase is able to initiate growth at a time when sufficient resources are available for healthy plant development. I propose that *NRS1* may act in a sensing role for nitrogen or amino acids in a similar fashion.

In chapter one, I reviewed the current understanding of TOR kinase in plant growth and development, highlighting the upstream signals it is sensitive to and some of the direct and indirect downstream targets that its activity affects. This introduction helps contextualize the data presented in chapters 2-5 and frames the research into a complete picture describing TOR kinase's role in plant development. In chapter two, I outline the a story of exogenous nitrogen, with findings that suggest there must be some sensor that exists that modulates external nitrogen suppliance with TOR activity. In chapter three, I attempt to untangle some of the asparagine metabolism and its relationship to TOR signaling; however, the results showed that manipulating the individual asparaginases did not have a significant impact on TOR activity, turning my focus to *NRS1*. In chapters four and five, I recount the deep dive of how *NRS1* and its mutant variant residues impact S6K1 phosphorylation, with the key finding that *NRS1* variants will **only stimulate TOR if they include the WHEP domain and can dimerize.** Together, the research in this dissertation forms a body of work that defines key functions of TOR signaling as it relates to nitrogen sensing and amino acids within plant biology.

Some of the results in this thesis were somewhat inconclusive, however, with the various phenotypes of overexpression of *NRS1* in plants. In other studies, overexpression of *TOR* results in larger seeds and plants (Deprost et al., 2007; Ahn et al., 2015), with bigger leaves due to larger epidermal cells and longer petioles. Other *TOR* overexpression lines possess twisted leaves and siliques (Deprost et al., 2007; Ahn et al., 2011), which might explain some of the dwarf plants I observed. Further segregation analysis of the *NRS1* overexpression mutants might connect the phenotypes more clearly to how *TOR* controls development through a sensor such as *NRS1.*

The TOR signaling pathway is vital to integrate information about the nutrient and energy status of cells and tissues to direct the appropriate developmental and physiological response (Dobrenel et al., 2016). This work contributes the identification of *NRS1* and the WHEP domain to to adapt growth and metabolism according to nutrient availability. As all eukaryotic organisms posses a functional TOR kinase, there is a strong body of research that demonstrates TOR kinase has a conserved regulatory role in photosynthetic organisms, to adapt growth and metabolism according to nutrient and hormone signals. Since plant immobility prevents their escape from hostile environments or nutrient scarcity, placing increased importance on their developmental plasticity in response to the environment. The study of potential plant “sensors”, such as *NRS1,* will allow further mechanistic study of the TOR signaling cascade, and give us a more complete understanding of this highly conserved complex.

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“There is no knowing for a fact. The only dependable things are humility and looking.”
— Richard Powers