Unique Roles for ISE2 in Chloroplast RNA Metabolism and Regulation of Plasmodesmata-mediated Intercellular Trafficking

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ABSTRACT

INCREASED SIZE EXCLUSION LIMIT 2 (ISE2) is a nuclear gene encoding a chloroplast-localized RNA helicase that is essential for *Arabidopsis thaliana* embryogenesis, chloroplast RNA metabolic events and the regulation of plasmodesmal permeability. Here I report that ISE2 is essential for the editing of several chloroplast transcripts.

Emb175/PPR103 is a nuclear gene encoding a pentatricopeptide repeat (PPR) protein that was previously reported to be required for embryogenesis in *Arabidopsis thaliana* and for seedling survival in *Zea mays*. EMB175/PPR103 was previously identified in our lab in a yeast-two-hybrid interaction screen with ISE2 and subsequently named ISE2 PROTEIN INTERACTOR (IPI)1. Confocal fluorescence microscopy illustrates that IPI1-YFP, similar to ISE2-YFP, localizes to chloroplasts, consistent with its predicted chloroplast N-terminal targeting sequence. In *Nicotiana benthamiana*, silencing of *emb175/PPR103/IPI1* in mature leaf tissue produces a chlorotic phenotype coupled to defective chloroplast structural integrity. Interestingly, virus induced gene silencing (VIGS) of *N. benthamiana emb175/PPR103/IPI1* or *N. benthamiana ISE2* revealed defects in the RNA editing of *N. benthamiana* chloroplast transcripts. However, *ISE2*-silenced plants displayed increased plasmodesmata-mediated intercellular trafficking, whereas no intercellular trafficking defect was observed in *N. benthamiana* plants silenced for *emb175/PPR103/IPI1*. These results indicate that ISE2 performs unique functions in the regulation of PD permeability.

Collectively, our results identify IPI1 as an ISE2 interacting protein that localizes to the chloroplast and that participates in the proper RNA editing of select *N. benthamiana* chloroplast transcripts. These observations add to the rapidly growing knowledge base of RNA helicase and PPR protein function in plants.

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CHAPTER ONE INTRODUCTION

Plasmodesmata (PD) are essential to plant survival as they are necessary for plant developmental coordination and environmental responses. PD achieve this role by serving as pores to transfer essential molecular information between cells. Informational molecules that traffic though PD include endogenous transcription factors, small RNA molecules, water and solutes (Sevilem, Miyashima et al. 2013). In addition to mediating cell-to-cell communication, PD aid in the long-distance transport of molecules by functioning in the loading and unloading of the phloem (Sevilem, Miyashima et al. 2013).

A current objective for plant researchers is to identify how PD are regulated. While several regulatory factors have been reported to localize to and/or affect PD structure and function, such factors generally participate in multiple developmental and environmental signaling responses. Thus, it is challenging to identify the unique functionalities of these factors that specifically affect PD function.

Phloem unloading of chloroplast-generated sugars is necessary to deliver sugars from actively-photosynthesizing source leaves to developing sink leaves (Lalonde, Weise et al. 2003). PD have been found to play a role in the cell-to-cell movement of photosynthetically assimilated sucrose and in the phloem unloading of sucrose in sink leaves (Sevilem, Miyashima et al. 2013). Additionally, during the sink to source transition, whereby sink leaves switch from a role as a net importer of sucrose to a net exporter of sucrose, PD structure and function changes (Oparka, Roberts et al. 1999). More specifically, during the sink to source transition, PD structure changes from simple (allowing the trafficking of non-specific proteins) to branched (restricting the trafficking of nonspecific proteins) (Oparka, Roberts et al. 1999). These observations link sugar transport with PD structure and function. As chloroplasts are the source of sugar

production, a logical assumption may be that chloroplasts and PD function as crucial components of a plant signaling network that coordinates the transport of available sugar in the plant (Burch-Smith, Brunkard et al. 2011).

Furthermore, chloroplasts are well-known targets of pathogens (Padmanabhan and Dinesh-Kumar 2010), (de Torres Zabala, Littlejohn et al. 2015); thus it is proposed that chloroplasts may generate signals, which inform the PD that the plant cell is under attack. Such chloroplast to plasmodesmata communication may lead to a restriction in PD-mediated trafficking in order to prevent the spread of harmful pathogen-derived effectors through the PD. Indeed, the chloroplast-derived phytohormone, salicylic acid, is produced upon pathogen attack and inhibits PD-mediated intercellular trafficking (Wang, Sager et al. 2013). For these reasons, it seems likely that chloroplasts generate signaling intermediates that regulate PD function (Burch-Smith, Brunkard et al. 2011). Despite a proposed role for chloroplasts in the regulation of PD-mediated permeability, a detailed mechanism by which chloroplasts may regulate PD permeability has not yet been demonstrated.

Understanding in detail how PD are regulated requires separating the contributing signaling pathways by at the minimum (i) identifying the associated regulatory and informational factors and subsequently (ii) dissecting apart the functions of these regulatory factors that are specific to the regulation of PD function.

Previously, a chloroplast-localized DEVH RNA helicase was found to disrupt chloroplast RNA processing events and PD permeability (Burch-Smith and Zambryski 2010). DEVH RNA helicases are characterized by the presence of a central motif containing the amino acids aspartic acid, glutamic acid, valine, and histidine. We used this RNA helicase as a proxy to understand how chloroplast RNA processing events may generate signals that affect PD function. There are two possibilities for how chloroplasts RNA processing events may affect PD

function (i) general defects in chloroplast RNA metabolism may affect PD function or (ii) specific signals are generated from particular chloroplast RNA metabolic events that affect PD function. My dissertation work illustrates that not all mutants that disrupt chloroplast RNA metabolic events affect PD permeability. More likely, PD permeability is affected by a 'particular defect pattern' or a 'unique signature' produced by disruption of particular chloroplast RNA metabolic processes. Excitingly, my dissertation work has identified novel roles in chloroplast RNA metabolism for two chloroplast-localized proteins. While these two proteins function in the same chloroplast metabolic events, defects in each produce a unique defective RNA metabolic signature. More specifically, defects in each cause particular defects in chloroplast rRNA processing and RNA editing. However, a knock-down in gene expression encoding only one these proteins drastically affects PD permeability. Therefore, following the notion of the 'unique RNA signature', I infer that each mutation results in a distinct spectrum of molecular defects that in turn defines the principles that may regulate PD permeability.

Intercellular Trafficking in Higher Plants

In multi-cellular organisms intercellular communication, or the transfer of information between cells, is necessary for the coordination of cellular function and thus for the survival of the organism. In plants, this informational transfer is impeded by the presence of cellulosic cell walls. Plant cells overcome this barrier through the presence of structures called plasmodesmata. Plasmodesmata are membrane- lined cytoplasmic pores that connect adjacent plant cells and that regulate the intercellular movement of molecules. Molecules that traffic through PD include water, nutrients, signaling molecules, viral components and developmentally important macro molecules such as transcription factors and RNA (Burch-Smith, Stonebloom et al. 2011), (Sager and Lee). The main route of trafficking for these molecules through PD involves cytoplasmic continuity of two

adjacent cells and is referred to as symplastic trafficking. For these reasons, PD are essential to plant survival, growth and development; a characteristic buttressed by the observation that several PD mutants are either embryonically lethal or exhibit severe developmental abnormalities (Kim and Zambryski 2005); (Benitez-Alfonso, Cilia et al. 2009).

PD can form during cell division, originating when strands of the endoplasmic reticulum (ER) become trapped in the cell plate prior to Golgi-mediated cell wall deposition (primary PD). Alternatively, PD may form after cell division through the pre-existing cell wall (secondary PD), an action that likely involves the breakdown of the existing cell wall, and turgor pressure to insert the ER through the plasma membranes that connect adjacent cells (Burch-Smith, Stonebloom et al. 2011). Primary PD typically present as a single channel, while secondary PD (that form in the absence of cell-division) can exist in a variety of complex structures (Fig. 1.1). Branched forms resemble Y, X and H in addition to more complex shapes, and twinned PD are comprised of two simple parallel tunnels within 100 nm of each other (Burch-Smith, Stonebloom et al. 2011). Simple PD are mostly found in young immature tissue and branched PD are predominately found in mature tissues (Lucas, Ding et al.), (Oparka, Roberts et al. 1999), (Xu, Cho et al. 2012).

At early embryonic stages, PD connect all cells in the embryo allowing the embryo to exist as one symplastic unit. However, as development proceeds starting at the torpedo stage, intercellular trafficking through PD becomes restricted at defined symplastic boundaries within the embryo (Fig. 1.2a; (Kim, Hempel et al. 2002). Such a restriction allows the developmental coordination of individual organs to occur (Fig. 1.2b; (Kim and Zambryski 2005)). For example, subdomains that are restricted at the mid-torpedo embryonic stage later develop into different organs of the plant such as the shoot apex, cotyledons, hypocotyl and root (Fig. 1.2b).

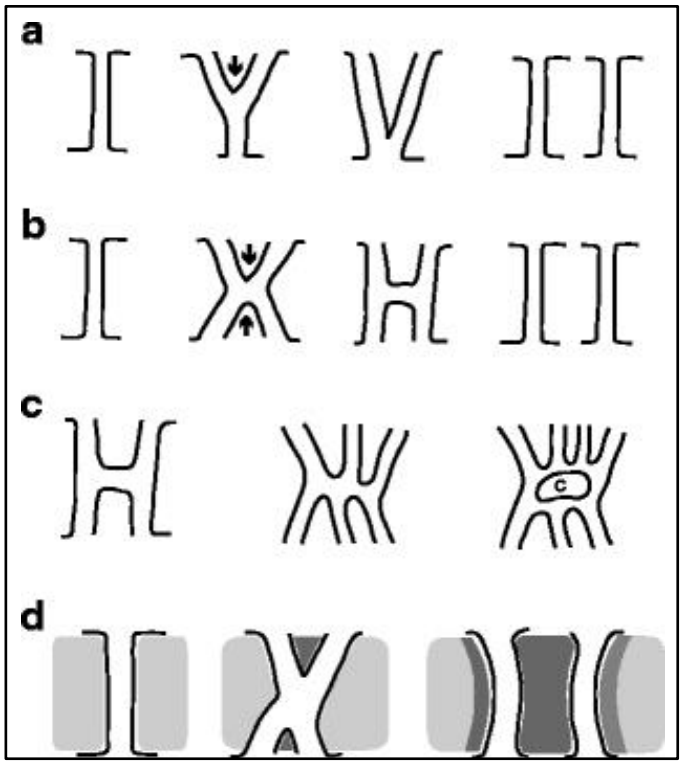


Figure 1.1 Representative PD structures and models for their formation

PD adopt several conformational shapes (a,b) two models by which twined PD may form due to the fission of simple PD originating from (a) one end or (b) both ends of a simple PD (c) complex PD structures may arise from branched PD, generating a central cavity (d) a model for how twinned, branched or complex PD as shown in (a-c) may arise from deposited cell wall material (dark grey). A representation for the formation of twinned PD is illustrated. Desmotubule is not shown for simplification. Figure adopted from (Burch-Smith, Stonebloom et al. 2011). Permissions obtained from Dr. Patricia Zambryski.

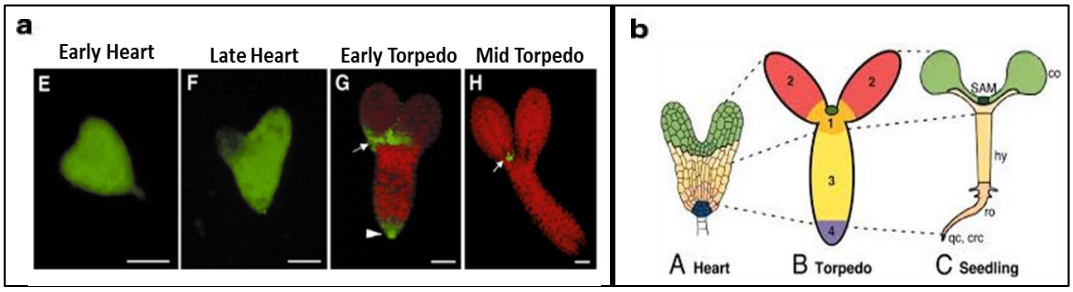


Figure 1.2 PD permeability during embryogenesis

PD-mediated intercellular trafficking becomes restricted at the torpedo stage in wild-type embryos (a) 10 kDa F-dextran (green) freely moves within early heart and late heart-stage embryos, but becomes restricted in torpedo-stage embryos. Chlorophyll fluorescence (red) represents the cytoplasm. Arrows indicate the radical tip and the regions that the cotyledons join as regions where the movement of uptook dextran is restricted (b) Subdomains that are restricted at the mid-torpedo stage later develop into (1) shoot apex (2) cotyledons (3) hypocotyl and (4) root, respectively. Figure 1.2a is adopted from (Kim, Hempel et al. 2002) and Figure 1.2b is adopted from (Kim and Zambryski 2005). Adapted with permission from Development Journal and Current Opinion Plant Journal.

The size exclusion limit is defined as the molecule size above which movement is restricted through PD (Burch-Smith, Stonebloom et al. 2011). The PD size exclusion limit in tobacco cells of mature leaves has been reported to be dynamic and dependent on leaf developmental stage (Burch-Smith, Stonebloom et al. 2011)

The size exclusion limit of PD is regulated in response to the initiation of cellular differentiation and to prevent the invasion of harmful, pathogen-derived signaling molecules from spreading cell to cell (Sager and Lee 2014). Despite the fundamental importance of PD to plant survival, growth and development, the identification of regulatory mechanisms that govern PD size exclusion limit and thus PD function, remain elusive.

Regulation of Intercellular Trafficking

A thorough understanding of PD regulation requires the identification of factors and cellular processes that affect PD permeability. An example of a well-known

mechanism of PD regulation involves callose deposition at PD neck regions. However, it has been postulated that callose deposition may regulate PD

aperture in response to dormancy as a long-term response but is not likely to be a transient regulator of PD during development (Burch-Smith, Stonebloom et al. 2011). Here I summarize molecular factors and potential cellular processes that may regulate PD aperture also referred to as the size exclusion limit.

Molecular Factors that Regulate Intercellular Trafficking

Callose, or β-1,3-glucan, is a homo polymer of glucose that is deposited or removed at PD neck regions in order to regulate PD permeability. Callose deposition and removal at PD are regulatory events that affect the size exclusion limit of plasmodesmata; increases in callose deposition can lead to a decrease in PD permeability while decreases in callose deposition can lead to an increase in PD permeability. The two main enzymes that regulate the synthesis and degradation of callose are callose synthases (CalSs) and β-1,3-glucanases (BGS) (De Storme and Geelen 2014).

Enzymes that Affect Callose Deposition at PD

Typically, genes encoding CalSs are required for the accumulation of callose at PD (De Storme and Geelen 2014). There are around 12 CalSs in *Arabidopsis thaliana*, and these are referred to as GLUCAN SYNTHASE-LIKE (AtGSL1 to AtGSL12) proteins (De Storme and Geelen 2014). GSLs typically possess several transmembrane domains and are typically located in the plasma membrane. Three of the twelve CalSs in *Arabidopsis* are reported to play a direct role in callose deposition at PD: CalS10/GSL8, CalS7/GSL7, and CalS3/GSL12 (De Storme and Geelen 2014).

In a mutant background of *ERECTA LIKE (ERL1 and ERL2)* receptor-like kinases, which inhibit differentiation of meristemoids into guard cells, loss of function in the gene *GSL8* (or *CHORUS)* leads to a reduction in callose

deposition at PD and also to an increase in the intercellular trafficking of macro-molecules. One such macromolecule is SPEECHLESS (SPCH), a transcription

factor involved in stomata cell differentiation (Guseman, Lee et al. 2010). The increased intercellular trafficking of SPCH is evidenced by a stomata cluster phenotype observed in *chor* mutants; this defect was not caused by a general cytokinesis defect (Guseman, Lee et al. 2010). The *CHOR* gene encodes a predicted callose synthase, GLUCAN SYNTHASE-LIKE 8 (GSL8). GSL8 is required for callose deposition at the cell plate, cell wall and plasmodesmata regions (Guseman, Lee et al. 2010). Interestingly, *CHORUS* expression is differentially regulated in mutants of the RNA helicase, *ISE2* that also displays an increased intercellular trafficking mutant phenotype.

PD-callose-binding protein 1 (PDCB1) localizes to plasmodesma and binds the callose polysaccharide 1,3- beta glucan. Mutations in *PDCB1* do not display a PD intercellular trafficking defect but do lead to reduced intercellular trafficking of GFP when over-expressed (Simpson, Thomas et al. 2009). Another group of PD-localized enzymes that may potentially regulate callose deposition are reversibly glycosylated polypeptides (RGPs). The silencing of RGPs resulted in increased intercellular trafficking, while the overexpression of RGPs resulted in decreased intercellular trafficking (Burch-Smith, Cui et al. 2012). These reported observations collectively suggest that PDCB1 and RGP may facilitate the deposition of callose at PD.

Enzymes that Affect Callose Removal at PD

There are many beta-1,3-glucanses in plant cells, yet only a few localize to PD. The PD-localized BGLs regulate callose deposition by degrading callose; mutations in such BGLs lead to increased callose deposition at plasmodesmata neck regions and reduced virus movement in tobacco (Sager and Lee 2014).

KOBITO (KOB) 1 is a predicted glycosyltransferase that plays roles in carbohydrate metabolism, the biosynthesis of cellulose during cell elongation,

and that also affects plasmodesmata closure (Kong, Karve et al. 2012). Mutations in KOB1 phenotypically resemble *chorus* mutant phenotypes (Guseman, Lee et al. 2010). In the *erl1* and *erl2* mutant backgrounds, mutations in *KOB1* lead to an increased intercellular trafficking phenotype as evidenced by increased and clustered stomata. Although KOB1 functions in the biosynthesis of cellulose, a disruption in cellulose-biosynthesis alone was not found to affect PD permeability (Kong, Karve et al. 2012). It has been postulated that the increased stomata mutant phenotype of *erl1 erl2 kob1* is due to increased PD permeability, allowing bHLH transcription factors (e.g SPEECHLESS, MUTE, FAMA, ICE1/SCREAM and SCREAM2) to traffic between cells and thus promote differentiation leading to stomata clustering (Kong, Karve et al. 2012). Thus, KOB1 was postulated to play a role in regulating the plasmodesmata size exclusion limit (Kong, Karve et al. 2012). Interestingly, *kob1-3* is allelic to both *ELONGATION DEFECTIVE 1* (*eld1-1)* and *ABSCISIC ACID INSENSITIVE 8 (abi8)*; *eld1* and *abi8* mutant alleles have been reported to lead to the misregulation of cell proliferation and ABA/sugar responses, respectively (Cheng, Su et al. 2000), (Brocard-Gifford, Lynch et al. 2004), (Huang, Yu et al. 2015). Although increased intercellular trafficking (such as that seen in *kob1-3* mutants) is sometimes associated with decreased callose deposition at PD neck regions (Sager and Lee 2014), a slight increase in callose accumulation actually occurs in the *kob1-1* mutant, suggesting that KOB1 may affect callose removal (Kong, Karve et al. 2012).

ATP-binding Proteins Affect PD Permeability

ATP-dependent proteins may also function in the regulation of molecular trafficking through PD. Cytoskeleton-associated proteins, including actin and myosin have been reported to localize to PD (Sager and Lee 2014). The inhibition of actin with the drug latrunculin led to an increase in PD aperture between leaf mesophyll cells coupled to a decrease in cellular ATP levels in all tissue types examined (reviewed in (Burch-Smith, Stonebloom et al. 2011) and in

(Burch-Smith and Zambryski 2012)). ATP-dependent RNA helicases are also involved in the regulation of PD aperture size and in intercellular trafficking. RNA helicases function in a diverse array of metabolic processes. In addition to unwinding secondary structures in an ATP-dependent manner, RNA helicases function as adapters to bring together multi-subunit RNA-protein complexes (Linder and Jankowsky 2011). RNA helicases have been implicated as having roles in developmental and abiotic stress responses likely due to their reported roles in RNA maturation, ribosome biogenesis, RNA splicing, transport, turnover, transcription, translation, RNAi and RNA editing (Owttrim 2006), (Linder and Jankowsky 2011).

Chloroplast-localized Proteins Affect PD Permeability

The chloroplast-localized RNA helicase INCREASED SIZE EXCLUSION LIMIT 2 (ISE2) and the mitochondria-localized RNA helicase INCREASED SIZE EXCLUSION LIMIT 1 (ISE1) were identified in a forward genetic screen designed to identify critical regulators of PD-mediated permeability in *Arabidopsis thaliana* embryos (Kobayashi, Otegui et al. 2007), (Kim, Hempel et al. 2002). Interestingly, the majority of misregulated genes in *ise1* or *ise2* mutants are predicted to function in chloroplast processes (Burch-Smith, Brunkard et al. 2011). *ISE2* is a nuclear gene encoding a DEVH RNA helicase (Fig. 1.3). ISE2 is essential for *Arabidopsis thaliana* embryogenesis, and mutations in *ISE2* led to developmental arrest at the mid-torpedo embryonic stage (Kim, Hempel et al. 2002). At this stage of embryogenesis, *ise2* mutant embryos displayed increased symplastic movement of a 10-kD fluorescent dextran (Fig. 1.4a,b; (Kobayashi, Otegui et al. 2007) and (Kim, Hempel et al. 2002)). The increased intercellular trafficking defect is uniquely seen in *ise2* mutants and not in other mutants that arrest at the midtorpedo embryonic stage. Furthermore, *ISE2*-silenced mature leaves displayed increased intercellular trafficking of a GFP dimer (2X GFP), demonstrating that ISE2 is additionally essential for the regulation of PD

permeability in mature plant leaves (Fig. 1.4c,d; (Burch-Smith and Zambryski 2010)).

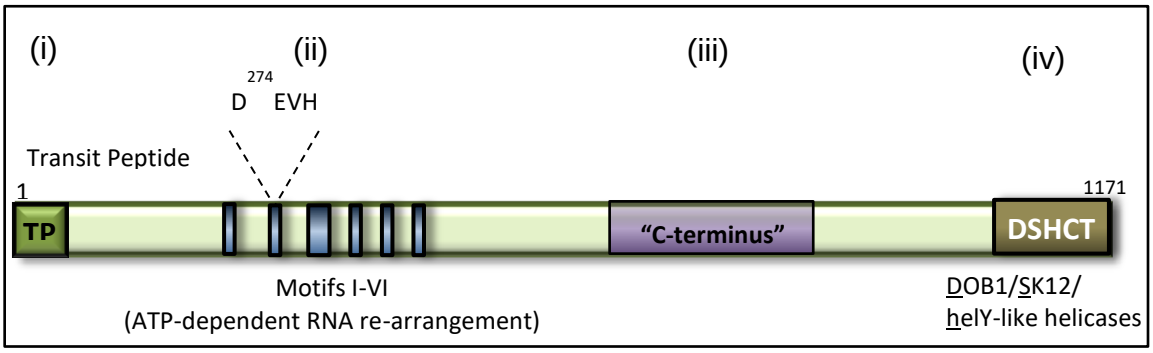


Figure 1.3 Domain architecture of the DEVH box RNA helicase ISE2

ISE2 is a nuclear encoded gene that encodes for a chloroplast targeted ski-type RNA helicase. The ISE2 protein product belongs to the DEVH box RNA helicase family and is characterized by: (i) an N-terminal transit peptide (ii) motifs I-VI that function in ATP-dependent RNA rearrangement (motif II contains the amino acids D, E, V and H that are shared in other DEVH RNA helicases), (iii) a "C-terminus region" that contains homology to C-terminal regions of other RNA helicases within the DEVH family and (iv) a far C-terminal conserved DSHCT domain.

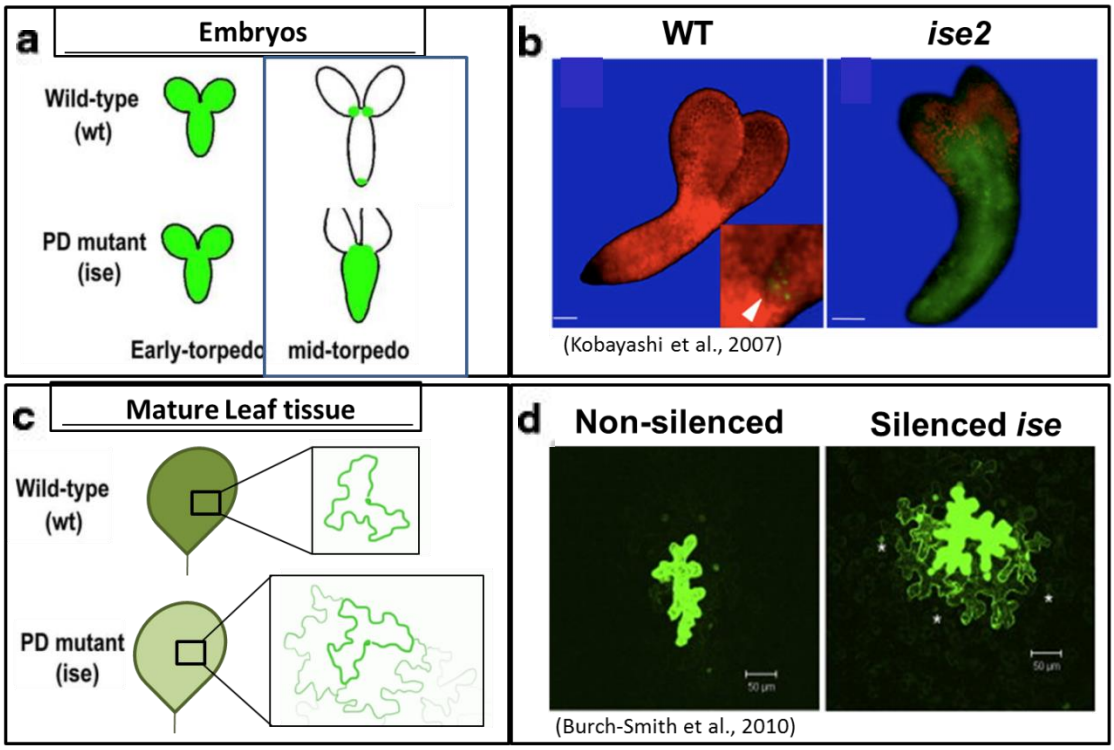


Figure 1.4 Intercellular trafficking is misregulated in *ISE2* mutants

Leaves silenced for *ISE2* lead to increased plasmodemal permeability. (a) Increased movement of F-Dextran occurs in *ise2* mid-torpedo stage embryos. A schematic illustrating the differences in movement of a tracer dye in wild-type (WT) and in *ise* mutants are early-torpedo and mid-torpedo embryonic stages is shown (b) Microscope image depicting movement differences of the tracer dye in WT as compared to *ise2* mutant embryos (c) Increased movement of 2XGFP occurs in mature leaf tissue silenced for *ISE.* A schematic illustrating the differences in movement of 2XGFP in wild-type and in *ise* mutant mature leaf tissue is shown (d) Confocal image of movement differences of 2XGFP in WT as compared to leaf tissue silenced for *ISE.*

In addition to the intercellular trafficking defect seen in mature *ISE2*-silenced leaves, the leaves were chlorotic. Transmission electron microscopy (TEM) of chloroplast ultrastructure revealed fewer grana and starch grains and a greater stromal area in *ISE2*-silenced leaves as compared to non-silenced control leaves (Burch-Smith and Zambryski 2010). Further characterization of ISE2 function revealed that it is essential for the processing of chloroplast rRNA (generates 23S rRNA species), splicing of chloroplast group II introns (including *ycf3*) and for the accumulation of chloroplast transcripts (Bobik, McCray et al. 2016). Developmentally arrested *Arabidopsis* embryos harboring mutations in *ISE2* also displayed defects in the expression of genes transcribed in the chloroplast and in nucleus-encoded genes that function in photosynthesis or PD-mediated trafficking (Burch-Smith, Brunkard et al. 2011).

Additionally, several mutants that exhibit defects in chloroplast function also exhibit defects in trafficking and in the expression of cell wall genes (Bobik and Burch-Smith 2015). Besides defects in *ISE2* function, other examples of gene mutations that affect PD function and chloroplast function include: (i) sucrose export defective1 (sxd1) and (ii) gfp arrested trafficking (gat)1 ((Bobik and Burch-Smith 2015), see sugar metabolism section).

Mutations in the chloroplast-localized thioredoxin, GFP Arrested Trafficking (GAT-1) exhibit decreased intercellular trafficking coupled to increased callose production and elevated ROS production in the roots (Benitez-Alfonso, Jackson et al. 2011). It has been postulated that GAT-1 functions in modulating chloroplast redox homeostasis and that when this process is perturbed, PD permeability is affected (reviewed in (Burch-Smith and Zambryski 2012)). These results further support the existence of a relationship between chloroplast signaling and PD function (Burch-Smith, Brunkard et al. 2011).

Retrograde signaling is a process of signaling that involves chloroplast-to-nucleus communication. Chloroplasts generate signals reflecting their metabolic

status that are then perceived by the nucleus. In response to chloroplast-derived signals, the expression of nearly 750 nuclear genes are modulated to in turn regulate chloroplast function (Chan, Phua et al. 2016). For example, a reduced plastoquinone pool was found to affect the expression of nuclear-encoded *cab* and *plastocyanin* genes (Escoubas, Lomas et al. 1995), (Pfannschmidt, Schutze et al. 2001), (Piippo, Allahverdiyeva et al. 2006). Treatment of plant cells with norflurazon (NF), a photo bleaching herbicide that inhibits the carotenoid synthesis enzyme phytoene desaturase (PDS) or lincomycin (Lin), an inhibitor of chloroplast translation, results in the reduction of at least 1,000 encoded mRNAs (Terry and Smith 2013). These observations indicate that chloroplasts generate signals that reflect their metabolic status and that affect nuclear gene expression (Terry and Smith 2013). Additionally, mutations in genes affecting chloroplast transcription, chloroplast RNA editing, chloroplast protein synthesis, or chloroplast protein import also repress nuclear gene expression (Terry and Smith 2013). In addition to its roles in the regulation of chloroplast RNA metabolism and in the regulation of PD permeability, ISE2 has been proposed to regulate retrograde signaling; this signaling may eventually be transmitted to the PD via a process called Organelle Nucleus Plasmodesmata Signaling (ONPS) (Burch-Smith, Brunkard et al. 2011). Alternatively, ISE2 may generate signals-arising from the ISE2-mediated processing of chloroplast RNAs-that directly regulate PD permeability.

Chloroplast-derived Signals May Regulate Intercellular Trafficking

Organelle metabolism has been proposed to control plasmodesmal permeability (Burch-Smith, Brunkard et al. 2011). A signaling defense response of chloroplasts to plasmodesmata includes a direct line of communication between chloroplast and plasmodesmata involving stromules (Bobik and Burch-Smith 2015). Stromules are extensions that form from the chloroplasts under stress (Caplan, Kumar et al. 2015). In addition to a direct line of communication between chloroplast and PD via stromules, chloroplasts may produce signals that

eventually converge on the regulation of PD function (Bobik and Burch-Smith 2015). A primary goal of our lab is to understand how signals from plant organelles such as the chloroplast may regulate plasmodesmata-mediated intercellular trafficking. Chloroplast to plasmodesmata signaling may involve chloroplast-to-nucleus retrograde signaling mechanisms that include the production of chloroplast-derived signals such as (i) ROS (ii) hormones (iii) tetrapyrroles (iv) sugars or (v) other signaling intermediates (Barajas-Lopez Jde, Blanco et al. 2013), (Chan, Phua et al. 2016).

ROS

Levels of reaction oxygen species (ROS), well-known secondary messengers involved in plant developmental and stress responses, have been postulated to regulate PD aperture (reviewed in (Benitez-Alfonso, Jackson et al. 2011)). Support for the involvement of ROS in the regulation of PD permeability come from histological studies that demonstrate that H_20_2 and peroxidases are detected at PD in response to stress (Shapiguzov, Vainonen et al. 2012). Additionally, studies indicate that in response to stress such as exposure to pathogens or heavy metals, the simultaneous induction of both callose and ROS is correlated with regulation of PD-mediated permeability (Scharte, SchÖN et al. 2005), (Jones and Dangl 2006). In wheat roots, an increase in PD permeability occurs when roots are exposured to anaerobic conditions, as compared to un-treated roots (Cleland, Fujiwara et al. 1994). Further, ROS stress was reported to restrict the intercellular movement of pSUC2-GFP and phloem unloading in roots (Sager and Lee 2014). However, ROS production by itself does not lead to a particular effect on PD transport. For example, although mitochondrial RNA helicase *ise1* mutants and chloroplast thioredoxin *gat1* mutants both have increased ROS production, they have opposite effects on PD-mediated intercellular trafficking: *ise1* mutants cause an increase in PD permeability while *gat1* mutants cause a decrease in PD permeability (Benitez-Alfonso, Cilia et al.

2009), (Stonebloom, Burch-Smith et al. 2009), (Sager and Lee 2014). This may indicate that unique redox-responsive signaling factors- affected in each particular mutant- are involved in the regulation of plasmodesmal permeability. Additionally, there may be a correlation between the amount of ROS produced and the particular effect on PD trafficking, with low levels of hydrogen peroxide leading to an increase in PD-mediated intercellular movement, whereas 10-fold higher concentrations decrease it (Rutschow, Baskin et al. 2011). It has been also been postulated that in addition to the amount of ROS produced, the site of ROS production (i.e the redox status of the chloroplasts versus the mitochondria) is an important factor in affecting PD permeability (Stonebloom, Brunkard et al. 2012). Increased ROS production in mitochondria led to increased PD permeability while increased ROS production in chloroplasts led to reduced PD permeability (Stonebloom, Brunkard et al. 2012). These results indicate that the redox state of the chloroplast and mitochondria differentially regulate PD-mediated intercellular trafficking (Burch-Smith and Zambryski).

Hormone Metabolism

Several studies have indicated that elevated levels of chloroplast-derived hormones such as salicylic acid (SA) lead to increased intercellular trafficking. Secondary metabolites such as SA, abscisic acid (ABA) and jasmonic acid (JA) are produced in the chloroplast. The chloroplast-derived phytohormones SA, JA, ABA, and non chloroplast-derived ethylene are the primary hormones that regulate plant defense responses (Alazem and Lin 2015). Thus, these phytohormones are likely to influence the regulation of PD permeability in response to stress induced by pathogen or viral attack. Indeed, SA induces PD closure in response to pathogen attack (Wang, Sager et al. 2013) and ABA was found to affect the expression of *PATHOGENESIS-RELATED PROTEIN 2 (PR2)* leading to increased callose deposition (Oide, Bejai et al. 2013). Additionally, ABA was found to regulate PD permeability in moss (Kitagawa and Fujita 2011).

Jasmonic acid accumulates at locations near wounds (Farmer, Gasperini et al. 2014). And both jasmonic acid and salicylic acid were found to increase callose deposition in response to the bacterial flagellin (flg22), a primary inducer of innate immunity (Yi, Shirasu et al. 2014). The phytohormone auxin was also found to affect PD permeability (Han, Hyun et al. 2014).

Sugar Metabolism

As previously mentioned, mutations in the maize gene, *sucrose export defective1 (sxd1)* led to defects in chloroplast and PD function (Russin, Evert et al. 1996), (Botha, Cross et al. 2000), (Bobik and Burch-Smith 2015). *sdx1* mutants exhibited increased callose deposition at PD coupled to decreased intercellular trafficking and were unable to export photosynthates from the site of their production in source leaves. This defect led to an accumulation of sugars in source leaves and co-occurred with defects in PD permeability (Botha, Cross et al. 2000).

In pathogen infected root nodules-which develop many secondary plasmodesmata as a response to infection- symplastic transport processes to partition carbon predominantly involve the expression of sugar transporters and the activity of sucrose-degrading enzymes (Schmidt, Kuhbacher et al. 2011). The results from this study indicate that sugar metabolism and the formation of *de novo* plasmodesmata co-occur and may be inter-linked processes that occur in response to pathogen attack of root nodules.

Hormone metabolism and sugar metabolism are also closely linked. For example, many ABA insensitive mutants have known functions in sugar metabolism. For example, *KOB1* is a gene that is known to affect plasmodesmal permeability and is also allelic to *ABI8*.

Organelle Metabolism

The observation that the *ISE* genes encode organelle-targeted proteins that regulate RNA metabolism suggests that organelle RNA processing may be connected to plasmodesmata function. RNA processing events disrupted in ISE2 mutants include the post-transcriptional processing of chloroplast 23S ribosomal RNA, splicing of group II introns (i.e *ycf3*) and changes in the steady state level of transcription and translation, which is consistent with general functions of RNA helicases (Bobik, McCray et al. 2016). ISE2 was found to physically interact with POLYNUCLEOTIDE PHOSPHORYLASE (PNPase), an exoribonuclease enzyme (unpublished), implicating roles for ISE2 in the degradation of chloroplast RNAs (Burch-Smith, Brunkard et al. 2011). ISE2 was also found to interact with RH3, a chloroplast-localized DEAD box RNA helicase that functions in RNA processing and RPL15, a chloroplast ribosomal protein (K. Bobik. pers. comm.). Microarray analysis of transcripts that are affected in *ise2* mutant mid-torpedo stage *Arabidopsis* embryos reveal the misregulation of many chloroplast –encoded genes and nuclear genes that encode chloroplast-targeted proteins (Burch-Smith, Brunkard et al. 2011). Microarray analysis additionally revealed that *ise2* mutants resulted in defects in the expression of nuclear-encoded plasmodesmata-related genes. For example, genes that encode callose-binding proteins, which are localized to plasmodesmata neck regions, and cell wall genes are misexpressed in *ise2* mutants. These include: *CHORUS/GLUCAN SYNTHASE-LIKE 8* (callose synthesis), *ATBG_PPAP* (callose degradation), *RGP3, PDL5,6,8* (encode glycosylation enzymes). Misregulated cell wall genes include those encoding for: cellulose glycosyltransferases, XyG endotransglucosylase/hydrolases (XTH) and expansins (Burch-Smith, Brunkard et al. 2011). The results from this study may provide a starting point to investigate the link between disrupted nuclear gene expression and PD function seen in *ise2* mutants; however the overall expression pattern of the misregulated genes (induced/repressed) do not fully explain the increased intercellular

trafficking phenotype observed in *ise2* mutants (Burch-Smith, Brunkard et al. 2011). For example, genes that are proposed to result in a decreased size exclusion limit of plasmodesmata were actually increased in *ise2* mutants. These genes include: *REVERSIBLY GLYCOSYLATED POLYPEPTIDE (RGP2), PDLP1 and PDLP*5 (Burch-Smith, Cui et al. 2012). Additionally, *ATBG_PPAP* encodes a callose degradation enzyme that is expected to increase PD SEL by removing callose, but it was downregulated in *ise2* mutants (Burch-Smith, Brunkard et al. 2011). Therefore gene expression changes alone cannot explain the intercellular trafficking defect seen in *ise2* mutants. Despite their different organelle localizations, loss of function in either ISE1 or ISE2 resulted in increased intercellular trafficking and interestingly the majority of genes misregulated in both mutants are implicated in chloroplast function. Comparison of the misregulated nuclear and chloroplast-encoded genes in both *ise1* and *ise2* mutants is shown in Fig.1.5 (Figure modified from (Burch-Smith, Brunkard et al. 2011).

Regulation of Organelle RNA Metabolism

The observations that (i) genes encoding proteins with proposed roles in chloroplast function are predominantly affected in *ise2* or *ise1* mutants (ii) chloroplasts are known to signal their status to modulate nuclear gene expression and that (iii) both chloroplast RNA processing defects and PD permeability are affected in *ise2* mutant suggest that an ISE2-mediated RNA processing event may generate a signal that affects PD permeability. Thus, a careful examination of the chloroplast-localized factors that regulate chloroplast metabolism is necessary to understand the mechanism behind which chloroplasts may generate signals to regulate PD permeability. Subcellular fractionation experiments of the chloroplast reveal that the chloroplast proteome contains over 3000 proteins; both nucleus and chloroplast-encoded (Rolland et al. 2012). Nucleus-encoded factors that participate in chloroplast function include proteins encoding components of photosystem II (PSII), PSI

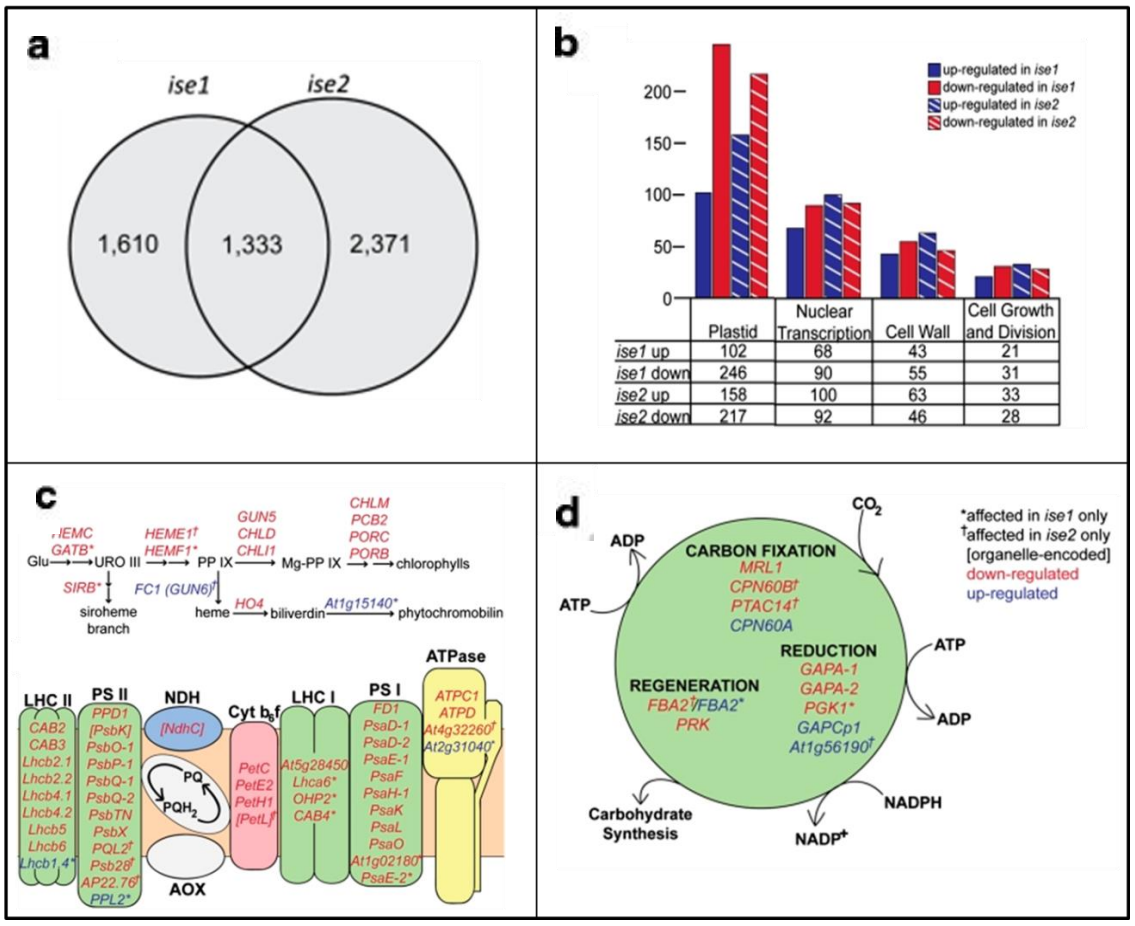


Figure 1.5 Gene expression changes in *ise1* or *ise2* mutants

Misregulated genes in *ise1* or *ise2* mutants compared with WT midtorpedo embryos. (a) Misregulated genes that are common in *ise1* and *ise2* mutants or that are unique to *ise1* or *ise2* mutants (*b*) Function and quantification of genes exhibiting altered expression in *ise1* or *ise2*. As indicated in Burch Smith, 2011, most affected genes in both *ise1* and *ise2* mutants belong to genes involved in chloroplast function (c,d) Misregulated genes in *ise1* or *ise2* that encode proteins involved in chloroplast function include those involved in tetrapyrrole synthesis (top) and in the photosynthetic electric transport chain (bottom). As indicated in Burch Smith, 2011, most affected genes are repressed in mutants and genes in brackets are encoded by the chloroplast genome; all other genes are nuclear (d) Misregulated genes in *ise1* or *ise2* mutants encoding chloroplast proteins of the Calvin Cycle. Figure adapted from Burch Smith, 2011.

photosynthetic and Calvin cycle redox reactions, tetrapyrrole/chlorophyll metabolism, fatty acid biosynthesis, and chloroplast RNA processing complexes (Alberts, Johnson et al. 2002), (Stern, Goldschmidt-Clermont et al. 2010). Several organelle-targeted RNA helicases were reported to have a role in the processing of chloroplast transcripts, likely in part due to their ability to modify secondary/tertiary RNA structures. RH3, RH22 and RH39 are chloroplast DEAD-box proteins that are required for rRNA processing in chloroplasts. RH3, a late assembly protein of the chloroplast 40S subunit, has been identified as an interacting partner of ISE2 and *RH3*-silenced plants exhibit increased intercellular trafficking of 2xGFP (K. Bobik. Pers. Comm., E. Ganusova. Pers. Comm.). Another ISE2 interacting protein that has a role in chloroplast RNA processing is PNPase. In addition to roles in RNA degradation, PNPase functions in the editing of *Arabidopsis* chloroplast transcripts (Ruwe, Castandet et al. 2013).

In addition to RNA helicases, a group of RNA-binding proteins found to heavily influence organelle RNA metabolic events are the pentatricopeptide repeat (PPR) proteins (see chapter 2.2 for characterization of the ISE2 Interacting PPR protein, IPI1). The PPR protein family is greatly expanded in plants with over 450 PPR proteins encoded by the *Arabidopsis* and rice genome. Most are predominantly targeted to chloroplasts or mitochondria (Nakamura, Yagi et al. 2012).

The focus of this dissertation will be on two key regulators of chloroplast RNA metabolism: the RNA helicase, ISE2 and the PPR protein, IPI1. Their role in chloroplast RNA metabolic processes with a focus on chloroplast RNA editing will be examined.

To further elucidate ISE2's role in unknown chloroplast RNA metabolic processes, we set out to further examine an extended role in RNA editing, a major metabolic function in plant organelles. RNA editing is a post-transcriptional

mechanism to change the transcript sequence information prior to organelle translation. In angiosperms, RNA editing is the site-specific conversion of a cytosine (C) to uracil (U) within chloroplast and mitochondrial transcripts (Fig.1. 6) (Bock 2000).

RNA editing in plant organelles is thought to proceed via a deamination reaction, involving deamination of a cytosine in order to produce a uracil. In most plants, only a subset of plastid and mitochondrial RNA transcripts undergo RNA editing. Generally, residues modified by C-to-U RNA editing function to restore conserved amino acids, change the physiochemical properties of a protein and to change a codon to a start codon (i.e *ndhD*). Additionally, the editing and splicing of chloroplast transcripts is necessary for organelle function and editing may be a prerequisite for splicing in a subset of plant organelle transcripts (Tillich and Krause 2010), (Gray and Covello 1993), (Castandet, Choury et al. 2010), (Farre, Aknin et al. 2012). RNA editing is also essential for gene expression (Grennan 2011), (Hirose, Kusumegi et al. 1999), translation (Stevenson and McCarthy 2008) and plant immunity (Garcı´a-Andrade, Ramirez et al. 2013). As unedited mature RNA was found in both polysomal and non-polysomal fractions, there appears to be no difference in translation efficiency between edited and unedited transcripts (Chasan, 1991). The translation of unedited transcripts suggests that unedited transcripts may also play important roles under certain conditions.

There are *cis*-elements required for the editing of chloroplast and mitochondria transcripts and they normally occur upstream within 30 nucleotides (nt) 5' and downstream within 10 nt 3' of the C editing target (Robbins, Heller et al. 2009). In tobacco (*Nicotiana tabacum* cv. Petit Havana), about 30 of the known 34 sites are clustered into 2-5 types based on sequence similarity 5' of the edited C in chloroplasts (Chateigner-Boutin, Hanson et al.2002). The action of a site-specific *trans*-factor in RNA editing was suggested due to results showing reduced editing of other transcript members when one transcript member (belonging to a

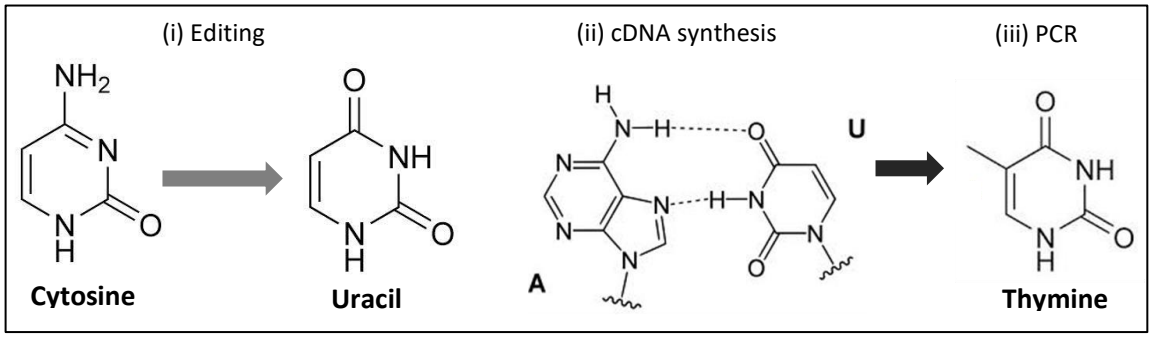


Figure 1.6 RNA editing is the conversion of a C to U

RNA editing in plant organelles is thought to proceed via a deamination reaction (i) cytosine is deaminated in order to produce a uracil (gray arrow) (ii) subsequent cDNA synthesis and (iii) PCR amplification produces a thymine (black arrow).

group with the same consensus sequence) was over-expressed (Chateigner-Boutin, Hanson et al.2002). Further support of a *trans*-factor involved in editing comes from results where exogenous expression of the *rpoB* transcript resulted in reduced editing efficiency of the endogenous *rpoB* transcript (Stern, Goldschmidt-Clermont et al. 2010). Additionally, in maize, Arabidopsis and tobacco, some sites are edited and others are not within the same transcript; therefore, specific *trans*-factors for each editing site have been postulated (Peeters and Hanson 2002), (Tseng, Sung et al. 2010). While some *trans*-factors may be specific to one site, other *trans*-factors may edit several sites (Robbins, Heller et al. 2009). Compared to tobacco leaves, most of the NADH dehydrogenase subunits show drastically reduced editing in roots, illustrating that the presence and/or the activity of *trans*-factors that are required for RNA editing depend on the tissue type (Chateigner-Boutin, Hanson et al.2002).

Not surprisingly, RNA editing does not require chloroplast translation of *rpoB*, a subunit of the plastid-encoded polymerase, supporting the idea that the factors that participate in the RNA editing of chloroplast transcripts are nuclear-encoded (Zeltz, Hess et al. 1993).

Chloroplast Editing Factors

In plants, the nuclear-encoded pentatricopeptide repeat (PPR) protein family is known to contain proteins that function in RNA editing (Manna 2015). PPR protein appearance and the appearance of RNA editing originated about the same time during the water to land transitioning of plants (Tillich and Krause 2010).

The PPR protein family consists of a well-conserved group of RNA-binding proteins that are critical for diverse roles in plant signaling. Loss of a single PPR protein can result in embryonic arrest or severe developmental defects, demonstrating their fundamental importance to plant survival and development.

PPR proteins have been classified as site-specific *trans*-factors involved in group II intron splicing, intergenic processing, stabilization and RNA editing of organelle transcripts (Nakamura and Sugita 2008), (Mach 2009).

Plant PPR proteins are typically targeted to either the mitochondria or chloroplast, where they act by binding to one or several single-stranded RNA molecules via 2-30 35 amino acid tandem helical repeat motifs (Shikanai 2006), (Barkan, Rojas et al. 2012), (Okuda, Myouga et al. 2007), (Tavares-Carreon, Camacho-Villasana et al. 2008). Within plant mitochondria and chloroplasts, most characterized PPR proteins mediate specific events in post-transcriptional processing and the maturation of RNA (Schallenberg-Rudinger, Kindgren et al. 2013) by influencing RNA splicing (Khrouchtchova, Monde et al. 2012), (Ban, Ke et al. 2013), (Ke, Chen et al. 2013), (Tan, Tan et al. 2014), (de Longevialle, Hendrickson et al. 2008), (Hattori, Miyake et al. 2007), (Hattori and Sugita 2009), (Meierhoff, Felder et al. 2003), (de Longevialle, Meyer et al. 2007), RNA cleavage (Hattori, Miyake et al. 2007), (Meierhoff, Felder et al. 2003), (Fisk, Walker et al. 1999), (Schmitz-Linneweber, Kushnir et al. 2005), (Hashimoto, Endo et al. 2003), RNA stability/turn-over (Liu, He et al. 2010), (Beick, Schmitz-Linneweber et al. 2008), (Yamazaki, Tasaka et al. 2004), (Johnson, Wostrikoff et al. 2010), (Loiselay, Gumpel et al. 2008), (Williams-Carrier, Kroeger et al. 2008), (Pfalz, Liere et al. 2006), translation (Schmitz-Linneweber, Williams-Carrier et al. 2005), (Zoschke, Kroeger et al. 2012), (Wang, Zou et al. 2006), (Williams and Barkan 2003), (Uyttewaal, Mireau et al. 2008) and the site-specific sequence alteration of RNA transcripts through RNA editing (Barkan and Small 2014). Defects in genes encoding PPR proteins can also affect nuclear gene expression (Liu, He et al. 2010), (Koussevitzky, Nott et al. 2007), (Ding, Liu et al. 2006). Additionally, several *ppr* mutants display chlorotic or albino phenotypes and exhibited defective chloroplast structures coupled to defects in RNA metabolism (Tseng, Sung et al. 2010), (Hammani, Takenaka et al. 2016).

PPR proteins are classified into the P subfamily or the plant-specific PLS subfamily. The PLS subfamily is further classified according to the following domains present in the C terminal region: (i) E groups are classified as those containing a E domain (ii) E+ groups are classified as those containing an E and an E+ and (iii) DYW groups are classified as those containing an E, E+ and a DYW domain, the DYW domain is traditionally named after the C-terminal amino acids (asp, tyr,trp) (Fig. 1.7; (Shikanai 2006)). Typically, PPR proteins that belong to the DYW subclass are involved in the RNA editing of organelle transcripts. The first discovered editing defects were found to be due to mutations in genes encoding PPR proteins (Kotera, Tasaka et al. 2005). Since then, several studies have documented editing defects that were not only due to mutations in genes encoding for PPR proteins but also genes encoding for other factors involved in general RNA metabolism. For example, mutations in the exoribonuclease PNPase, which functions in 3' end maturation and tRNA precursor degradation, (Ruwe, Castandet et al. 2013) was found to cause editing defects. Interestingly, PNPase was identified as an ISE2 interacting partner and *pnpase* mutants display a very similar defective editing profile as *ISE2*-silenced mutants in *Arabidopsis* (Ruwe, Castandet et al. 2013). Additionally cp31, a common chloroplast RNA-binding protein was found to be required for editing several sites (Hirose and Sugiura 2001). It should also be mentioned that stress or defects in chloroplast function and members of the tetrapyrrole biosynthetic pathway were found to also cause RNA editing defects (Castandet, Hotto et al. 2013), (Kakizaki, Yazu et al. 2012), (Tseng, Sung et al. 2010), (Zhang, Tang et al. 2014), although the particular pattern of editing defects are unique.

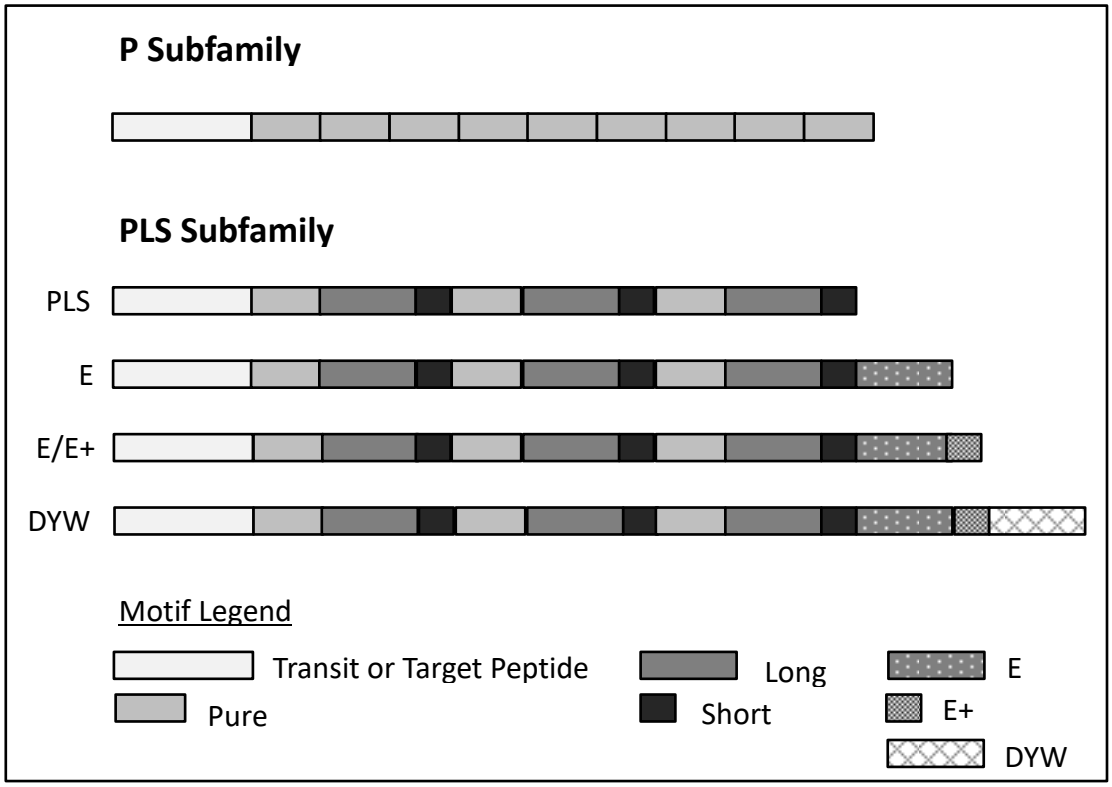


Figure 1.7 Domain architecture of the PPR protein family in plants

PPR proteins are divided into the P subfamily or the plant-specific PLS subfamily. The PLS subfamily is further classified according to the following domains present in the C terminal region: (i)E groups are classified as those containing a E domain (ii) E+ groups are classified as those containing an E and an E+ and (iii) DYW groups are classified as those containing an E, E+ and DYW domain

Although the DYW domain is commonly found in PPR proteins that are necessary for the editing of certain sites within transcripts, it is not required. For example, while the DYW PPR protein RARE 1 has required functions in RNA editing and contains a DYW domain, other proteins with reported roles in RNA editing such as CRR4, CRR21, and CLB19 lack a DYW domain (Robbins, Heller et al. 2009). Interactions among PPR proteins have also been reported and non-DYW containing proteins may gain access to DYW domains through physical protein-protein interaction. For example, both DYW1, a DYW domain-containing protein and CRR4, a non DYW-domain containing PPR protein, physically interact and are necessary for editing the chloroplast *ndhD-1* editing site (Boussardon, Salone et al. 2012). Several PPR proteins (i.e CRR4 and CRR21) were found to specifically bind to the transcripts that they are necessary for editing (i.e *ndhD*) (Okuda, Nakamura et al. 2006).

Other non-DYW domain-containing proteins that have been found to affect editing include members of the *Arabidopsis* RNA editing-Interacting Protein (RIP) family. RIP1 has been identified as an essential component of the plant RNA editing machinery that is involved in the editing extent of 75% of mitochondrial sites and around 20% of plastid sites (Bentolila, Oh et al. 2013). RIP3 and RIP8, together with RIP1 are also involved in the editing of over 85% of mitochondrial sites (Bentolila, Oh et al. 2013).

What role might the RNA helicase, ISE2, play in the chloroplast RNA editing process? RNA helicases in other organisms have been implicated as playing a functional role in RNA editing. For example, RNA helicases have been proposed to be involved in the editing of pre-mRNAs in *Trypanosomes* and in *Drosophila* (Simpson, Sbicego et al. 2003), (Kruse, Voigt et al. 2013), (Bass, 2001), (Seeburg, 2000). RNA helicases are known to hydrolyze NTP for energy in order to rearrange RNA (Schwer, Meszaros, 2000). Plastid RNA editing requires an energy source, such as hydrolysable NTP which may indicate that an RNA helicase may be necessary for plastid editing (Schmitz-Linneweber and Barkan

2007). Despite the proposed involvement of an RNA helicase in RNA editing, an RNA helicase that is necessary for RNA editing has not yet been identified in plants.

Ph.D Dissertation Aims

Based on the information stated in the introduction, the specific aims of this Ph.D dissertation are to:

(i) Examine additional roles for ISE2 in chloroplast RNA metabolism by investigating a potential role for ISE2 in the editing of chloroplast transcripts

(ii) Characterize an interacting partner of ISE2 (the PPR protein IPI1) to examine if IPI1 shares functions with ISE2 in the regulation of chloroplast RNA metabolism and in the regulation of PD permeability and

(iii) Examine the global impact of IPI1 on chloroplast RNA metabolism.

The observations made from the investigation of these aims indicate that, in general, the disruption of chloroplast RNA metabolism is not sufficient to disrupt PD permeability. Thus, not all regulators of chloroplast RNA metabolism affect intercellular trafficking. More likely, particular defects in chloroplast RNA processing events may produce a ‘unique signature’ that signals to and affects PD permeability. Such a scenario indicates that specific regulators of chloroplast RNA metabolism exert specific effects on PD permeability.

CHAPTER TWO RESULTS

This chapter describes a potential role for ISE2 in the editing of chloroplast transcripts. Besides long-held speculation of the involvement of an RNA helicase in RNA editing, support for the idea that ISE2 is involved in RNA editing comes from the observation that ISE2 physically interacts with a PPR protein. Due to ISE2's involvement in RNA post-transcriptional processing events and its interaction with a PPR protein, we asked whether ISE2 is necessary for efficient editing of chloroplast transcripts. If so, then RNA editing of chloroplast transcripts should be affected by loss of ISE2 function.

Generation of *Arabidopsis* Plants That Were Used to Measure Editing

In *Arabidopsis thaliana*, complementation lines with *ISE2* expressed under the control of the constitutive 35S promoter were generated to rescue the embryo developmental defect seen in *ise2* homozygous mutants (Kobayashi, Otegui et al. 2007). All subsequent generations derived from this independent line display a chlorotic phenotype in approximately one in eight plants (Fig. 2.1a). The chlorotic phenotype results from silencing the *ISE2* transgene expression by cosuppression. This is mediated by the plant's endogenous RNA silencing machinery, and it is a response to the presence of the transgene (Jorgensen 2003), (Ketting, Plasterk 2000). Confirmation of *ISE2* cosuppression is presented in (Bobik, McCray et al. 2016). We thus used cosuppressed leaf tissue to test a role for ISE2 in chloroplast RNA editing. A role for ISE2 in chloroplast editing has not been previously explored.

Generation of Amplicons from Arabidopsis Chloroplast Transcripts That Were Used to Measure Editing

There are 34 well-known editing sites in plastids of seed plants (Tseng, Lee et al. 2013), affecting 24 transcripts. RNA was isolated from wild type, *ISE2*-cosuppressed (*ISE2*-silenced) and rescued plants, and complementary DNA (cDNA) was generated by reverse transcription. The same cDNA reaction without reverse transcriptase was performed in parallel on all RNA samples to ensure that no genomic DNA contamination was present. Polymerase chain reaction (PCR), with primer pairs specific to editing site locations, was then used to amplify transcripts that contain known editing sites from the cDNA samples (Fig. 2.1b). For each primer specific pair, a PCR reaction was also performed in parallel on samples that were not treated with reverse transcriptase (-RT). The transcripts were then sequenced and the sequences analyzed for editing defects (Appendix for all chromatograms). The data indicates that ISE2 is required for RNA editing of several chloroplast transcripts, including transcripts that function in photosynthetic electron transport, protein degradation and chloroplast transcription.

ISE2 is Necessary for the Full Editing of a Subset of Transcripts in Arabidopsis Mature Leaf Tissue

Editing defects in *ISE2*-silenced leaves are seen in 7 out of the 34 edited sites within chloroplast transcripts (Fig. 2.2). The results from the RNA editing experiments were highly reproducible (Fig. 2.3), illustrating that the Sanger sequencing method is as reliable as the poisoned primer extension (PPE) method (Asakura and Barkan, 2006). Additionally, the editing profile for the wild-type *Arabidopsis* plants is consistent with previous reports (Tseng, Lee et al. 2013). The functional classifications of transcripts with reduced editing

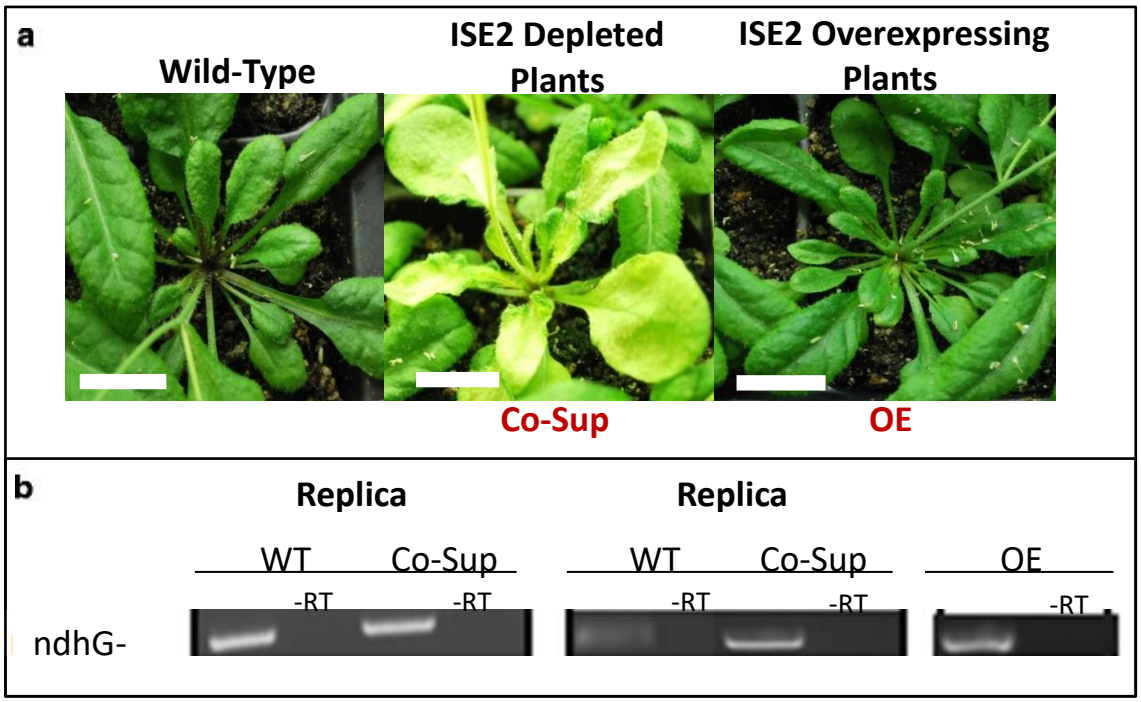


Figure 2.1 ISE2 co-suppressed tissue displays a chlorotic phenotype and RT-PCR results indicate the absence of genomic DNA contamination from isolated RNA

(a) *ISE2*-silenced plants from the Nth generation of the original homozygous independent complementation line display chlorosis, a phenotypic defect that is suppressed in D3G plants. Col-0 is WT, co-Sup is a plant with reduced ISE2 levels, and OE are plants over-expressing ISE2 in the homozygous *ise2* mutant background and therefore rescuing the *ise2* embryo-defective phenotype. Scale bar is approx.1.7 centimeters (b) Representative RT-PCR results indicate that there is no genomic DNA contamination in the –RT control (same RT-PCR reaction without reverse transcriptase).

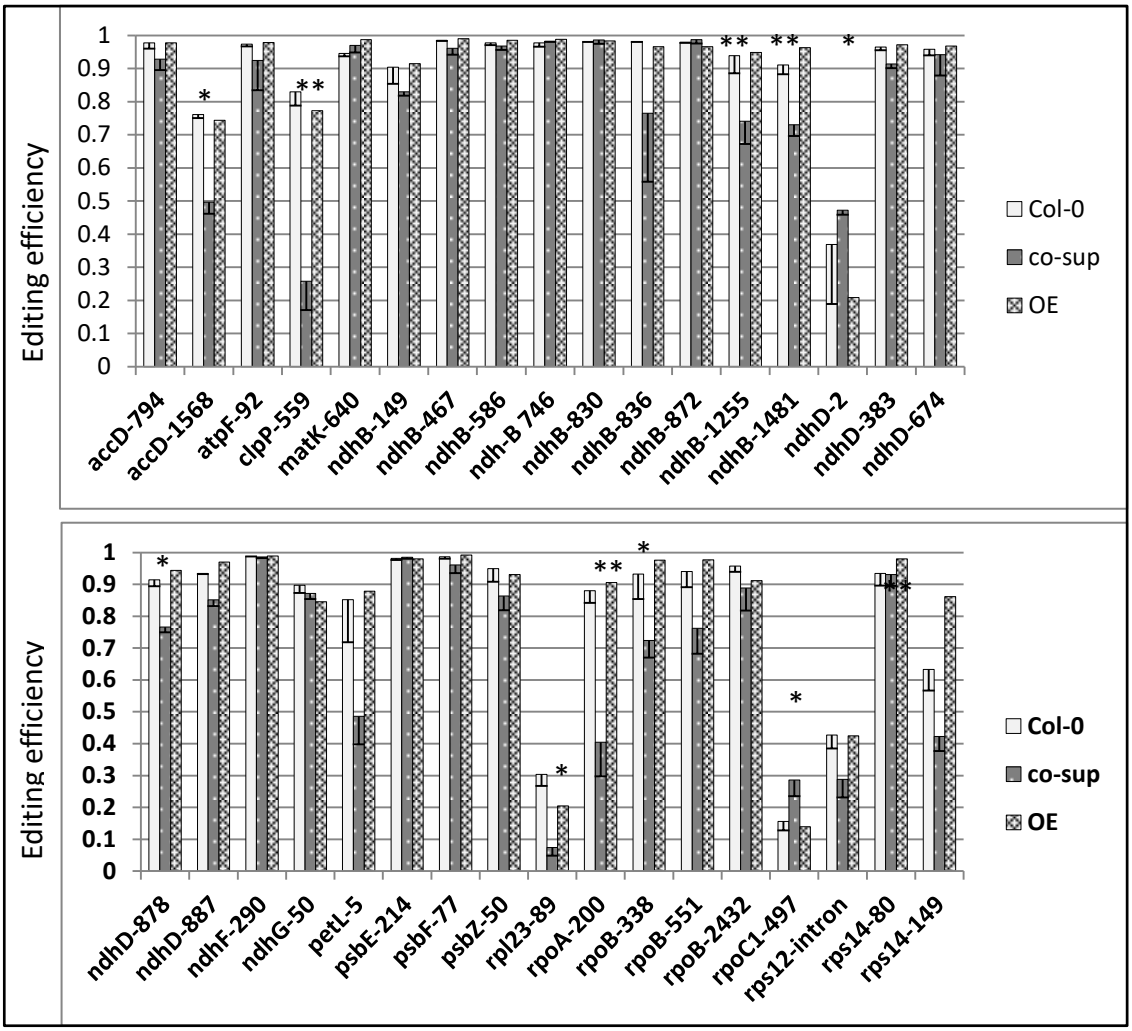
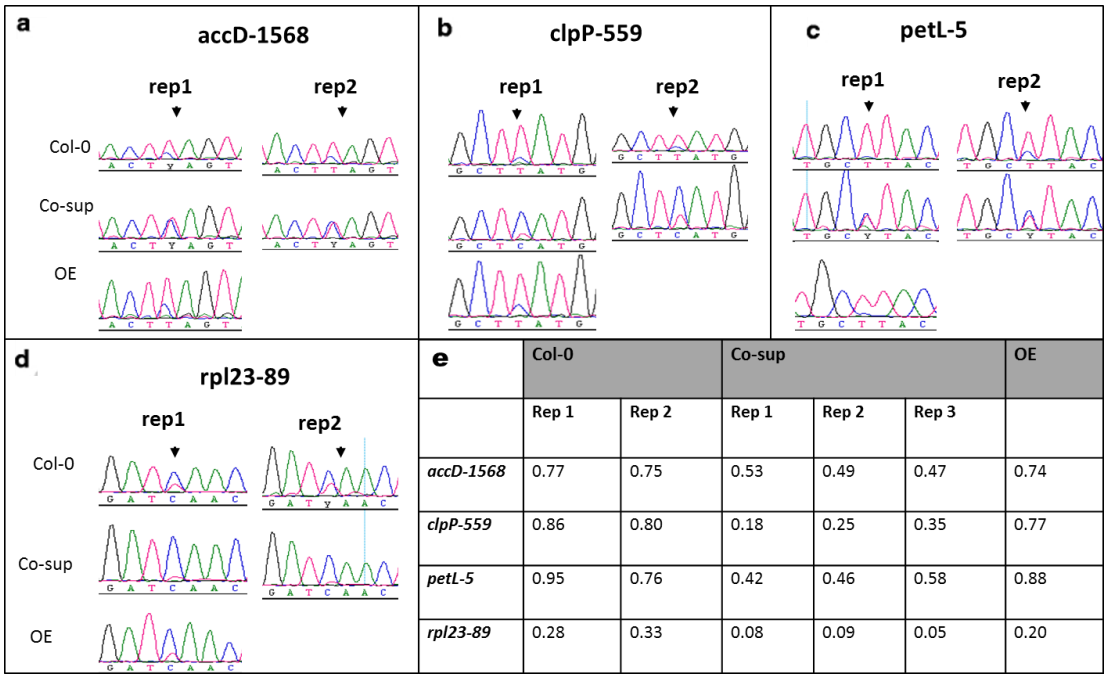


Figure 2.2 Defects in cosuppressed plants affect individual editing sites within chloroplast transcripts

Editing efficiencies at several chloroplast transcript edited sites are affected in *ISE2* co-suppressed leaf tissue (co sup) as compared to wildtype leaf tissue (Col-0) and this defect is rescued in the non-cosuppressed complementation lines that over express ISE2 (OE). *ClpP* and *PetL* are the most severely affected by loss of ISE2. *Rpl23* transcripts are edited to a lesser extent than WT in *ISE2*-silenced mutants, while *rpoC1* transcripts are edited to a higher extent in cosuppressed plants than in WT. ISE2 is needed for full editing at site 1568 but not at site 794 within the *AccD* transcript. Editing extent in Col-0, Co-sup, and OE plants is calculated as the peak intensity of fluorescence for T divided by the total fluorescence (T/(T+C)). The error bars represent standard deviations between biological samples. The number of biological samples (n) is two or three for the majority of Col-0 and co-sup leaf tissue. Data in figure a-c are representative results of sequencing reactions from the same Col-0, co-suppressed (co-sup) or complemented leaf tissue (OE). One asterisk represents statistical significant at p-value <0.05 and two asterisks represent statistical significant at p-value <0.01.



e

	Col-0		Co-sup			OE
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 3	
accD-1568	0.77	0.75	0.53	0.49	0.47	0.74
clpP-559	0.86	0.80	0.18	0.25	0.35	0.77
petL-5	0.95	0.76	0.42	0.46	0.58	0.88
rpl23-89	0.28	0.33	0.08	0.09	0.05	0.20

Figure 2.3 Reproducibility is observed between biological replicates

a.) ISE2 is needed for full editing at site 1568 but not at site 794 within the *accD* transcript. b-c.) *clpP* and *petL* are the most severely affected by loss of ISE2 d.) *rpl23* transcripts are edited to a lesser extent than WT in *ISE2*-silenced mutants. e.) Editing extent in Col-0, cosuppressed, and complemented plants is calculated as the peak intensity of fluorescence for the edited base (T) divided by the total fluorescence (T/(T+C)). The error bars represent standard deviations between biological samples. The number of biological samples (n) is two or three for the majority of Col-0 and co-sup leaf tissue. Data in figure a-d are representative results of sequencing reactions from Col-0, co-suppressed (co-sup) or complemented leaf tissue (OE).

Table 2.1. Functions of chloroplast transcripts whose editing extents are reduced in cosuppressed plants. ISE2 is necessary for the full extent of editing for the transcripts listed to the left. **Transcripts that require ISE2 for editing at some edited sites but not others

Gene	Functions of proteins whose transcripts are required ISE2 for Editing
accD **	Encodes the carboxytransferase beta subunit of the Acetyl-CoA carboxylase (ACCase) complex in chloroplasts (fatty acid biosynthesis).
clpP	Encodes the only ClpP (caseinolytic protease) encoded within the chloroplast genome. Part of the 350 kDa chloroplast Clp complex (protein degradation).
*ndhB***	NADH dehydrogenase ND2 (ATP synthesis coupled electron transport, NADH dehydrogenase (ubiquinone) activity, chloroplast, oxidation-reduction process).
ndhD	Represents a chloroplast-encoded subunit of a NAD(P)H dehydrogenase complex (ATP synthesis coupled electron transport).
petL	Cytochrome b6f subunit (photosynthesis electron transport).
rpl	One of two chloroplast genes that encode chloroplast ribosomal protein L23, a constituent of the large subunit of the ribosome (RNA binding/translation).
rpoA	RNA polymerase beta subunit-1 (transcription).
rpoB	Chloroplast DNA-dependent RNA polymerase B subunit (transcription).
rps12-intron	chloroplast gene encoding ribosomal protein S12 (translation).
rps14	30S chloroplast ribosomal protein S14(translation).
psbZ	Subunit of photosystem II (photosynthesis).

efficiency are listed in Table 2.1. c*lpP* and *petL* are the most severely affected showing approximately 60% and 40% reduced editing efficiency in *ISE2*-silenced plants as compared to wild type, respectively (Fig. 2.2). However, the editing extent of transcripts whose editing efficiency is affected in the absence of ISE2 varies. For example, *rpl23* transcripts are edited to a lesser extent than WT in *ISE2*-silenced plants, while *rpoC1* transcripts are edited to a non-statistically significant higher extent in *ISE2*-silenced plants than in WT plants (Fig. 2.2). Additionally, ISE2 is necessary for editing some sites but not others within the same chloroplast transcript. For example, ISE2 is needed for full editing at site 1568 but not at site 794 within the *accD* transcript (Fig. 2.2). These defects are likely due to reduced ISE2 function as the editing defects are suppressed in complementation lines that overexpress *ISE2* (Fig. 2.2 and Fig. 2.3).

Editing Sites are Conserved in *Nicotiana benthamiana* 4 Week Old Leaf Tissue

Conserved edited sites have been reported for Arabidopsis*, Zea mays* (maize), *Oryza sativa* (rice) and *Nicotiana tabacum* (tobacco) (Tseng, Lee et al. 2013), (Hirose, Kusumegi et al. 1999). To confirm that these editing events extend to *Nicotiana benthamiana*, we examined conserved editing extents at several leaf developmental stages. Editing was detected in *N. benthamiana* for the majority of transcripts, and interestingly there appears to be a developmental dependence of editing for at least some of the transcripts with overall reduced editing extent in older leaf tissue (Fig. 2.4).

Editing sites within the *rpoA*, *ndhB*, and *ndhD* transcripts are well-known to be conserved between the dicots *Arabidopsis thaliana* and *Nicotiana tabacum* (Tseng, Lee et al. 2013). We detected editing at the *rpoA* editing site at about

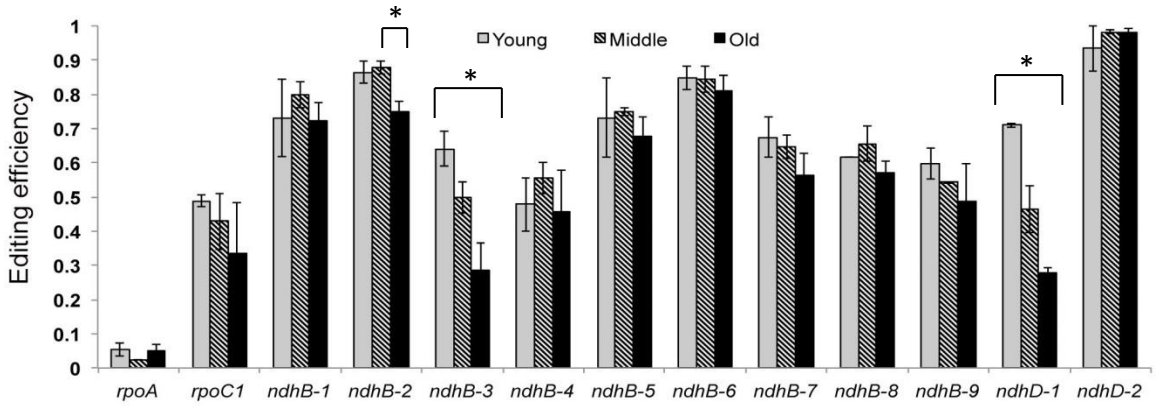


Figure 2.4 Editing occurs in 4 week old *N. benthamiana* leaves

Editing extent was examined in leaves of different ages. The y-axis represents the editing efficiency fraction (e.g 0.1 is equivalent to 10% editing efficiency). The x-axis represents the conventional name of the edited transcript. Note that *ndhB* and *ndhD* transcripts contain multiple edited sites and these are numerically distinguished according to the order in which they occur along the length of the transcript in the 5'->3' orientation. Error bars represent standard deviation from 2 independent biological samples. A test was performed to assess statistical significant. Asterisks represent p-values <0.05 between *ndhB*-2 (middle and old) leaves, *ndhB*-3 (young and old), and *ndhD*-1 (young and old) leaves.

90% efficiency in 5 week old wild-type *Arabidopsis thaliana* plants and about 5% efficiency in 4 week old wild type or 6 week old control *N. benthamiana* leaves (compare Fig 2.2, Fig 2.4, and Fig 1.4). Previous published work has reported the editing of *rpoA* transcripts in tobacco in four week old wild-type leaves (Hirose, Kusumegi et al. 1999). The reason for limited editing at the *rpoA* editing site in the four-week-old *N. benthamiana* wild-type leaves is unclear.

Editing efficiencies at *ndhB* editing sites is at least 90% efficient in wild-type *Arabidopsis* leaves but varies widely in *N. benthamiana* wild type leaves, ranging from approx. 50% to 80% in young leaf tissue (compare Fig 2.2, Fig 2.4). These results suggest that there are editing efficiency differences at conserved editing sites between different species. Thus, although editing at conserved sites may be a common occurrence amongst species, editing efficiencies at these sites may differ.

In Arabidopsis, editing at sites *ndhD*-2 and *ndhD*-383 (conventionally named after the transcript followed by the edited nucleotide position from the initial coding nucleotide) is conserved with editing at sites *ndhD*-1 and *ndhD*-2 (conventionally named according to the order in which the edited sites occur in the 5'->3' orientation of the transcript) in *N. benthamiana.* Editing at this site changes the first *ndhD* codon from an A<u>C</u>G to the start codon, A<u>U</u>G. Editing at the *Arabidopsis ndhD*-2 or the conserved *N. benthamiana ndhD*-1 site is around 50% on average in wild-type plants of both species but is also highly variable in both species (compare Fig. 2.2 and Fig. 2.4). Editing at the next conserved edited site along the *ndhD* transcript (*Arabidopsis ndhD*-383 or *N. benthamiana ndhD*-2) is around 100% (compare Fig. 2.2 and Fig 2.4). Thus, the pattern of editing efficiencies at these two *ndhD* sites is consistent between *Arabidopsis thaliana* and *N. benthamiana* editing sites.

In *Arabidopsis,* editing at the *rpoC1* editing site changes the encoded amino acid from a serine to a conserved leucine (Tseng, Lee et al. 2013). We observed a low editing efficiency of about 15% at the Arabidopsis *rpoC1*-497 editing cite (Fig.2.2). In tobacco, the *rpoC1* transcript already encodes the conserved L amino acid, and thus editing does not occur at this site (Tseng, Lee et al. 2013). Interestingly, however, in *N. benthamiana* young leaf tissue, we observed about a 50% editing efficiency at this site. The reason for the difference between the documented *rpoC1* editing occurrence in *N. tabacum* and *N. benthamiana* is not clear, but it has been previously documented that editing efficiencies at the same editing site can also widely vary even within the same *Nicotiana* genus (Okuda, Habata et al. 2008).

Collectively, these results indicate that editing events and editing efficiencies at the same site can vary not only between plants belonging to different genera but can also vary between plants belonging to the same genera.

Editing Defects are Detected in *Nicotiana benthamiana ISE2*-silenced Plants

N. benthamiana plants silenced for *ISE2* produce a chlorotic phenotype 14 days after Tobacco rattle virus (TRV)-mediated virus induced gene silencing (VIGS) (Fig 2.5). We used same-aged plants that were infiltrated with agrobacterium containing the silencing vector but no target sequence for silencing as the control. A minor non-statistically significant increase in the editing extent of *rpoA* was observed in plants silenced for *ISE2* in *N. benthamiana* relative to the non-silenced control (Fig. 2.14). Editing at site *ndhB3* is also increased while editing at sites *ndhB*-4 and *ndhD*-1 are reduced in *ISE2*-silenced plants (Fig. 2.14). Additionally, the editing efficiency is reduced at editing sites *atpF* and *ndhA*-1 (Fig. 2.6).These results indicate that edited sites are uniquely affected in *ISE2*-silenced plants.

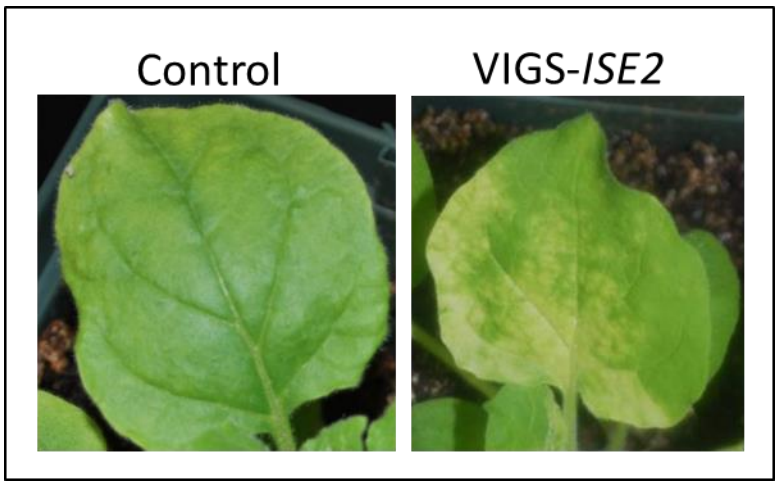


Figure 2.5 Phenotype of *ISE2*-silenced *N. benthamiana* leaves

N. benthamiana plants silenced for *ISE2* produce a chlorotic phenotype approx.14 days after tobacco rattle virus (TRV)-mediated virus induced gene silencing (VIGS).

In *Arabidopsis thaliana,* there is reduced editing for the majority of edited sites in *ISE2*-silenced leaves (Fig. 2.2). In *N. benthamiana,* however, there is increased editing efficiency in *ISE2*-silenced plants relative to the control (Fig. 2.14 and Fig. 2.6). These results indicate that ISE2 may affect editing efficiency in a different manner in Arabidopsis as compared to *N. benthamiana* leaves. It appears that ISE2 performs a promotive function in the *Arabidopsis* editing reactions and an inhibitory function in the *N. benthamiana* editing reactions. The reason for the different effects of ISE2 on the *Arabidopsis* and *N. benthamiana* editing reactions is unclear but may be due to species-specific differences in the presence and/or amounts of other regulatory editing machinery components.

Characterization of ISE2 Protein Interactor (IPI1)

In an effort to understand the connection between ISE2's effect on chloroplast RNA metabolism and plasmodesmata function, the Burch-Smith lab set out to identify protein interactors of ISE2 and thus used ISE2 as bait in a yeast two hybrid screen of a commercially available, normalized Arabidopsis cDNA library representing 11 different plant tissues. One clone identified in this screen represented a portion of the *AT5G03800* locus. The protein encoded by this locus was subsequently named ISE2 PROTEIN INTERACTOR (IPI). IPI1 belongs to the DYW class of PPR proteins, typically known for their role in RNA metabolic events such as translation, stability/turn-over, rRNA processing, splicing or RNA editing in chloroplasts and mitochondria (Barkan and Small 2014). Recently, the maize orthologue of IPI1, PPR103 was reported to function in rRNA processing and stabilization (Hammani, Takenaka et al. 2016). More specifically, mutations in *PPR103* resulted in a drastic reduction of chloroplast rRNAs (Hammani, Takenaka et al. 2016). We thus set out to examine a role for IPI1 in the RNA processing of chloroplast transcripts in *N. benthamiana* using a combination of cell biology and biochemical methods.

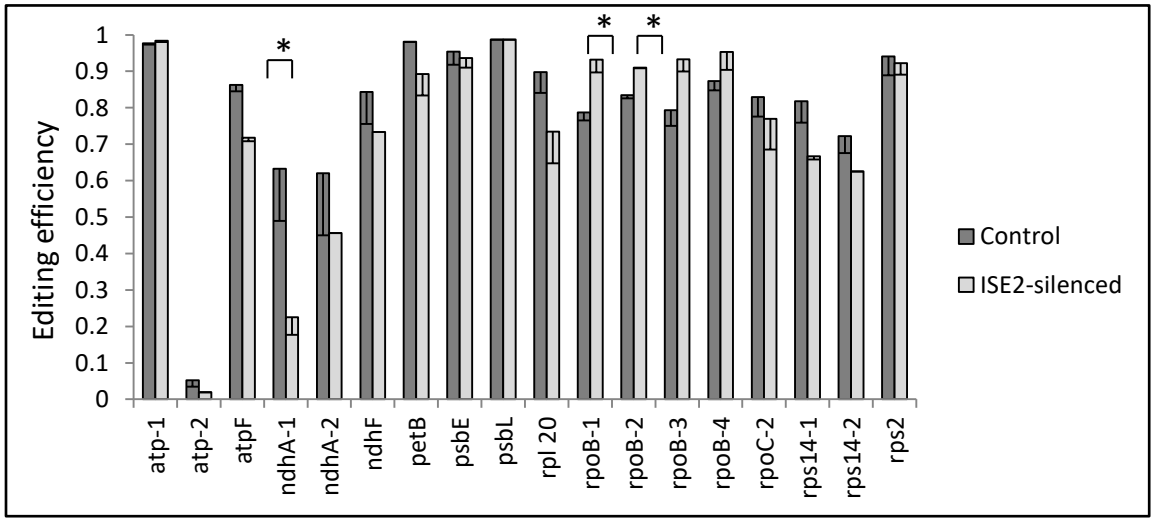


Figure 2.6 Editing defects are seen in *ISE2*-silenced leaves

A statistically significant slight editing efficiency increase in TRV-*ISE2* leaves occurs at sites *rpoB*-1 and *rpoB*-2. A statistically significant decrease in editing efficiency in TRV-*ISE2* leaves occurs at site *ndhA*-1.Control plants represent plants that were infiltrated with the same buffer and an empty vector control used for virus induced gene silencing. Two control biological replicates were and three replicates of *ISE2*-silenced leaves *(TRV-ISE2)* from individual plants were tested.

As previously mentioned, ISE2 regulates PD permeability (see Fig. 1.4). Although IPI1 was identified in an interaction screen with ISE2, a potential role for IPI1 in the regulation of PD permeability has not been examined. Thus, we additionally set out to examine a role for IPI1 in the regulation of PD permeability in *N. benthamiana.*

IPI1 physically Interacts With ISE2 via the C-terminal Region

As previously mentioned, ISE2 is a chloroplast RNA helicase that has multiple functions in chloroplast RNA processing (Bobik, McCray et al. 2016). ISE2 has been identified in high molecular weight chloroplast protein complexes (Olinares, Ponnala et al. 2010); (Majeran, Friso et al. 2012). In an effort to identify proteins that could interact with ISE2 in these complexes, a yeast-two-hybrid screen of a normalized *Arabidopsis thaliana* cDNA library with full-length ISE2 as bait was performed. After verifying that ISE2 did not autoactivate expression of the reporters, more than $2x10^7$ colonies were screened. In this way, 27 putative ISE2 partners were identified (Table 2.2). Thirty-three and 67% of identified proteins were predicted to localize to chloroplasts and other compartments, respectively. One of these proteins, encoded by *At5g03800*, was predicted to encode a PPR protein. Two mutant alleles of this gene had previously been reported to cause embryonic lethality (Cushing, Forsthoefel et al.), *emb175-1* and *-2*, although no molecular function had been ascribed to the gene product. IPI1 contains a predicted chloroplast targeting peptide (cTP) at its N-terminus, followed by 13 pure long short (PLS) PPR motifs and finally a C-terminal DYW motif (Fig. 2.7).

The original clone identified in the yeast two-hybrid screen encoded only the C-terminal half of the protein. The full-length gene was therefore cloned and the

Table 2.2 Proteins identified as interacting with ISE2 in yeast two-hybrid screen

Locus	Description	No. of clones
AT3G21295	[R]Uncharacterized protein Tudor/PWWP/MBT superfamily protein	8
AT1G31817	[R]Ribosomal L.8/L5e family protein (NUCLEAR FUSION DEFECTIVE 3 [NFD3])	6
AT3G25920	[R]Putative 50S ribosomal protein L15	6
AT1G59660	Nucleoporin autopeptidase	3
AT2G43130	RAS-related protein ARA-4	2
AT5G03800	[R]PPR protein, emb175/emb166	2
AT5G42780	Homeobox protein 27	2
AT4G11450	Hypothetical protein, DUF3527	2
AT1G21580	Zinc-finger CCH domain-containing protein	2
AT2G33800	[R]30S ribosomal protein S5	2
AT3G16060	Kinesin family protein	2
AT4G36980	Unknown	2
AT2G39460	[R]60S ribosomal protein L23A1	1
AT1G51745	[R]Tudor/PWWP/MBT superfamily protein	1
AT1G02780	[R]Ribosomal protein 60S L193 family, emb2386	1
AT5G55300	DNA topoisomerase I	1
AT1G35210	Hypothetical protein, DUF740 domain	1
AT5G22830	Magnesium transporter MRS2-11	1
AT4G03292	[R]RNaseH domain-containing protein	1
AT4G03300	Similar to Ulp1 protease family protein (transposable element gene)	1
AT4G04220	Receptor-like protein 46, RLP46	1
AT1G51510	[R]RNA binding protein 8A	1
AT5G59430	Telomere repeat binding protein 1 (TRP1)	1
AT2G31280	Conserved peptide uORF 7 (CPUORF7)	1
AT3G16840	[R]DEAD-box RNA helicase 13	1
AT4G39960	Molecular chaperone Hsp40/DnaJ family protein: Chloroplast thylakoid	1

[R] Denotes proteins with known or predicted role in RNA processing

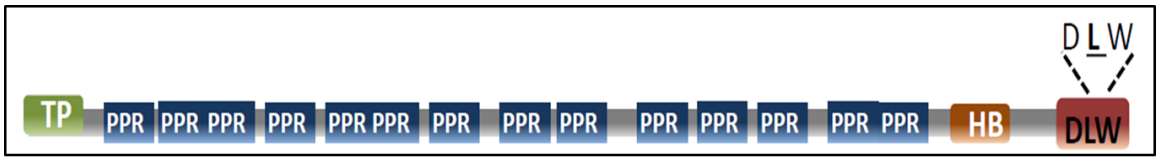


Figure 2.7 Domain architecture of IPI1

AtIPI1 contains an N-terminal transit peptide (TP), 13 PLS PPR motifs, a predicted zinc and heme binding motif (HB) and a C-terminal DYW domain. Notably, IPI1 contains a C-terminal DLW amino acid variation.

gene product tested for interaction with full-length ISE2. The interaction between the full-length IPI1 protein and ISE2 was confirmed (Fig. 2.8), and the protein was named ISE2 PROTEIN INTERACTOR1 (IPI1). In order to refineunderstanding of how EMB75/IPI1 and ISE2 interacted, truncations of IPI1 and ISE2 full-length protein were constructed and tested for interaction using a yeast two-hybrid assay. A weak interaction through the C-terminal domains of both ISE2 and IPI1 was detected (Fig. 2.8). The C-terminal domain of ISE2 remains uncharacterized. IPI1's C-terminal region contains high sequence similarity to cytidine deaminases and to other DYW PPR proteins that also high similarity to cytidine demainases within their C-terminal region and that have been found to both function in RNA editing and to bind zinc (Boussardon, Avon et al. 2014).

IPI1 is a Conserved PPR Protein in Plants

IPI1 belongs to the DYW subclass within the plant-specific PLS subfamily of PPR proteins in higher land plants (see Fig. 1.7). A homology search using BLAST and subsequent alignment of homologous proteins using the Basic Local Alignment Search Tool (BLAST) and the aligner MUltiple Sequence Comparison by Log- Expectation (MUSCLE) revealed that NbIPI1 has orthologues in maize, rice, and Arabidopsis ((Hammani, Takenaka et al. 2016), Fig. 2.9).The protein sequence alignment illustrates the presence of a highly conserved C-terminal region containing a predicted CXXCH heme binding motif (Nakamura and Sugita 2008), (Cushing, Forsthoefel et al.). However, no study has yet demonstrated the ability of any PPR protein to bind heme. Interestingly, this predicted heme binding motif is also predicted to be a zinc binding motif and the C and H residues within this motif have been previously shown to bind zinc and to contribute to the RNA editing of chloroplasts transcripts (Boussardon, Avon et al.). The terminal DxW amino acids are found in *N*. *benthamiana* and the *Arabidopsis* IPI1 orthologue but not in rice or maize IPI1 orthologues (Fig 2.9).

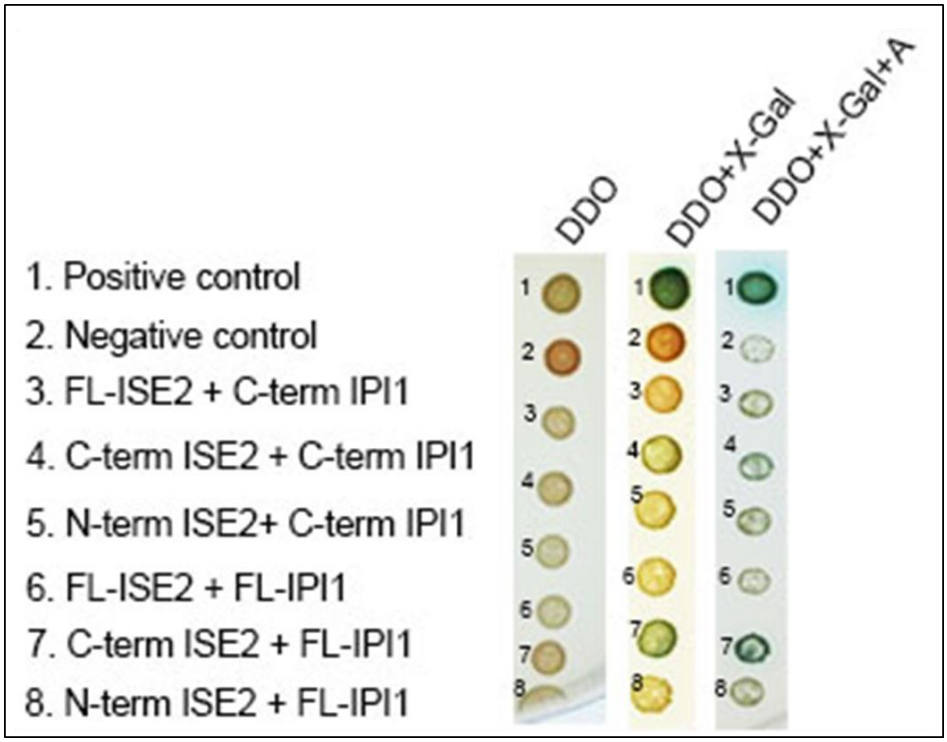


Figure 2.8 IPI1 physically interacts with ISE2 via its C-terminal region

A yeast two hybrid mating assay was conducted to confirm the interaction between ISE2 and IPI1 as identified in the original yeast two hybrid screen. Results were visualized after four days of mated yeast cell growth on plates. The interaction between p53 and SV40 large T-antigen represents the positive control and the interaction between Lamin and the SV40 large T-antigen represent the negative control in this figure. Full_ISE2 and Full_IPI1 represent full length ISE2 without its transit peptide and full length IPI1 without its transit peptide, respectively. C-term_ISE2 and C-term_IPI1 represent C-terminal truncations of ISE2 and IPI1 proteins, respectively. N-term_ISE2 and N-term_IPI1 represent N-terminal truncations of ISE2 and IPI1 proteins, respectively. Undilluted yeast grown from diploid cells after mating are shown. Growth media details are indicated at the top of the figure (A indicates the antibiotic Aureobasidin A. DDO: double drop out Media (-Leu-Trp), TDO: triple drop out media (-Leu-Trp-His)). Descriptions to the left of the figure are highlighted in red to indicate a positive (albeit weak) interaction.

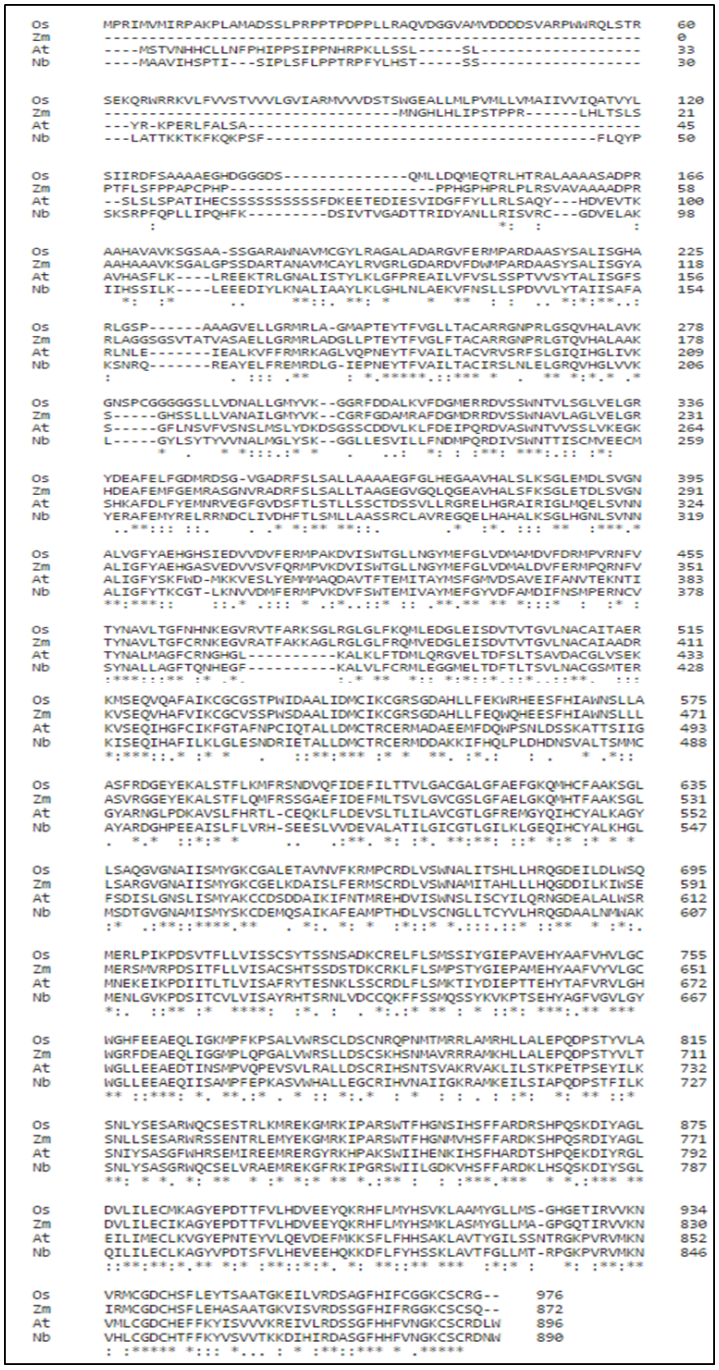


Figure 2.9 Sequence alignment the full-length IPI1 orthologues

At represents *Arabidopsis thaliana*, Nb represents *Nicotiana benthamiana*, and Os represents *Oryza sativa, Zm* represents *Zea mays*. Full length IPI1 from the respective plant species were aligned using CLUSTAL 0 (1.2.3) multiple sequence alignment. Asterisks represent conserved residues and dots represent similar residues.

IPI1 is Localized to Chloroplasts

The putative chloroplast transit peptide (cTP) of IPI1 suggested that the protein localized to chloroplasts. Further, AtIPI1 was identified as one of the 8071 proteins found in the plastid proteome database (ppdb.tc.cornell.edu). To confirm the subcellular localization of IPI1, the full length Arabidopsis *IPI1* coding sequence was cloned upstream of a yellow fluorescent protein (YFP) tag. The resulting fusion protein (IPI1-YFP) was transiently expressed in *N. benthamiana* leaves and visualized by confocal laser scanning fluorescence microscopy. Fluorescence from the IPI-YFP fusion colocalized with chloroplast autofluorescence, indicating that IPI1-YFP localized to chloroplasts (Fig. 2.10 A-D). A western blot conducted on the same leaf tissue used for confocal analysis confirmed that the full-length IPI1-YFP fusion was expressed in plants infiltrated with full-length IPI1-YFP under the 35S promoter but not full-length IPI1-Myc (Fig 2.10). The ability of the predicted N-terminal chloroplast targeting peptide (cTP) to direct import into chloroplasts was also examined. To test IPI1 cTP function, the putative cTP, plus 20 amino acids downstream of the cTP of NbIPI1 or ATIPI1, was fused N-terminal to YFP and the fusions (NbIPI1-cTP-YFP and AtIPI1-cTP-YFP) were transiently expressed in *N. benthamiana*. Fluorescence from both NbIPI1-cTP-YFP and AtIPI1-cTP-YFP co-localized with chloroplast auto fluorescence, indicating that the fusions were imported into the chloroplast. As further confirmation, time lapse imaging revealed that the cTP-YFP fusions remained confined within the chloroplast over time (movie found in McCray, 2017 in prep.). Transient expression of YFP alone under the control of the 35S promoter did not localize to chloroplasts (Fig. 2.10 M-P). Additionally, we could observe chlorophyll autoflorescence but not YFP signal in all un-infiltrated WT plants (data not shown). Thus the IPI1 cTP is functional and IPI is a chloroplast protein.

Phenotypic Characterization of Plants with Reduced NbIPI1 Expression

Loss of *AT5G03800* gene product in *Arabidopsis* results in arrested embryogenesis (*emb175-1* and *-2* mutant alleles*)* (Cushing, Forsthoefel et al. 2005), precluding further analysis of gene function in Arabidopsis. We therefore knocked down *NbIPI1* expression in *N. benthamiana* by Tobacco rattle virus (TRV)-mediated virus induced gene silencing (VIGS). The efficacy of VIGS was confirmed by qPCR in three independent biological samples (data not shown). Silencing *NbIPI1* expression produced severely bleached leaves, resembling a phenotype reported for the knock down of the maize orthologue of IPI1, PPR103 (Hammani, Takenaka et al. 2016) (Fig. 2.11a). A BLAST search of the *N. benthamiana* genome using the sequence of the primers used to silence *NbIPI1* returned only IPI1, suggesting that the phenotypic defects are due to the specific knock down of *NbIPI1*. Consistent with the severe chlorosis, the levels of chlorophyll (data not shown) and the quantum efficiency of photosystem II in silenced leaves were severely reduced (Fig. 2.11b). The chloroplast ultrastructure in *NbIPI1*-silenced leaves was examined by transmission electron microscopy (TEM) (Fig. 2.11c). This revealed defective chloroplast ultra-structure with reduced thylakoids, as has been observed for other PPR mutants and chloroplast RNA processing factors (Tseng, Sung et al. 2010).

IPI1 is Necessary for the Accumulation of Chloroplast rRNA Species

Disruption of the maize IPI1 orthologue, PPR103 produced an albino phenotype coupled to drastically reduced chloroplast ribosomal RNA (rRNA) levels in leaf tissue (Hammani, Takenaka et al. 2016). To test whether NbIPI1 may have a similar role in chloroplast rRNA biogenesis, we performed a northern blot of chloroplast rRNAs in *NbIPI1*-silenced *N. benthamiana* leaves using sequence specific probes. The northern blots revealed that silencing *NbIPI1* led to major

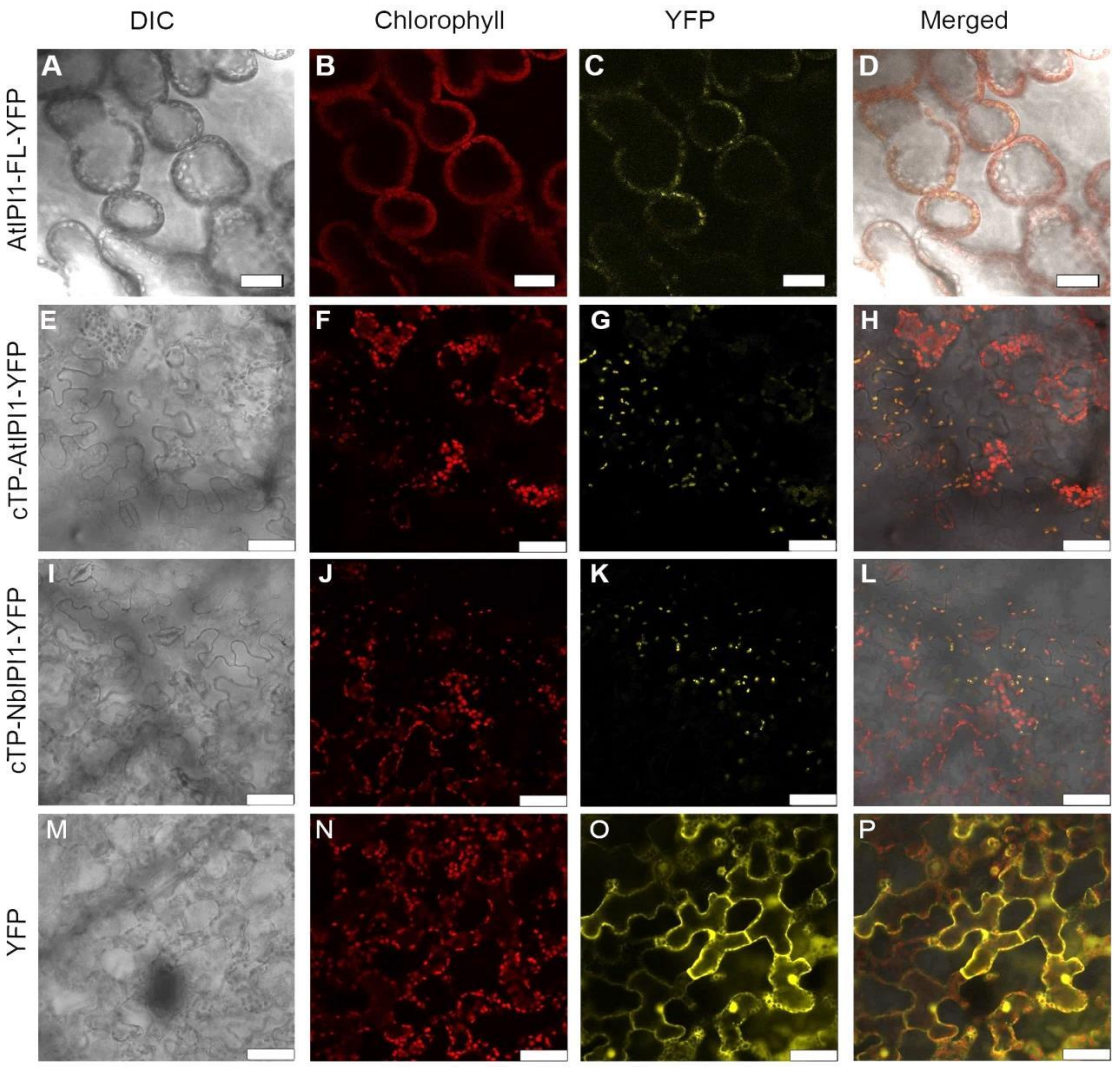


Figure 2.10 Confocal microscopy images of full-length and cTP IPI1 fragments

Top panel indicates Differential Interference Control (DIC), YFP excitation wavelength channel, red light excitation wavelength channel and merged, from left to right. All constructs were transiently expressed under the control of the 35S promoter and the *YFP* coding sequence was fused to the C-terminal end of *IPI1* cDNA coding sequences. The left panel indicates full-length *Arabidopsis* IPI1 (AtIPI1)(A-D), *Arabidopsis* transit peptide (AtTP)(E-H), *Nicotiana benthamiana* transit peptide (NbTP)(I-L) and YFP alone (M-P).

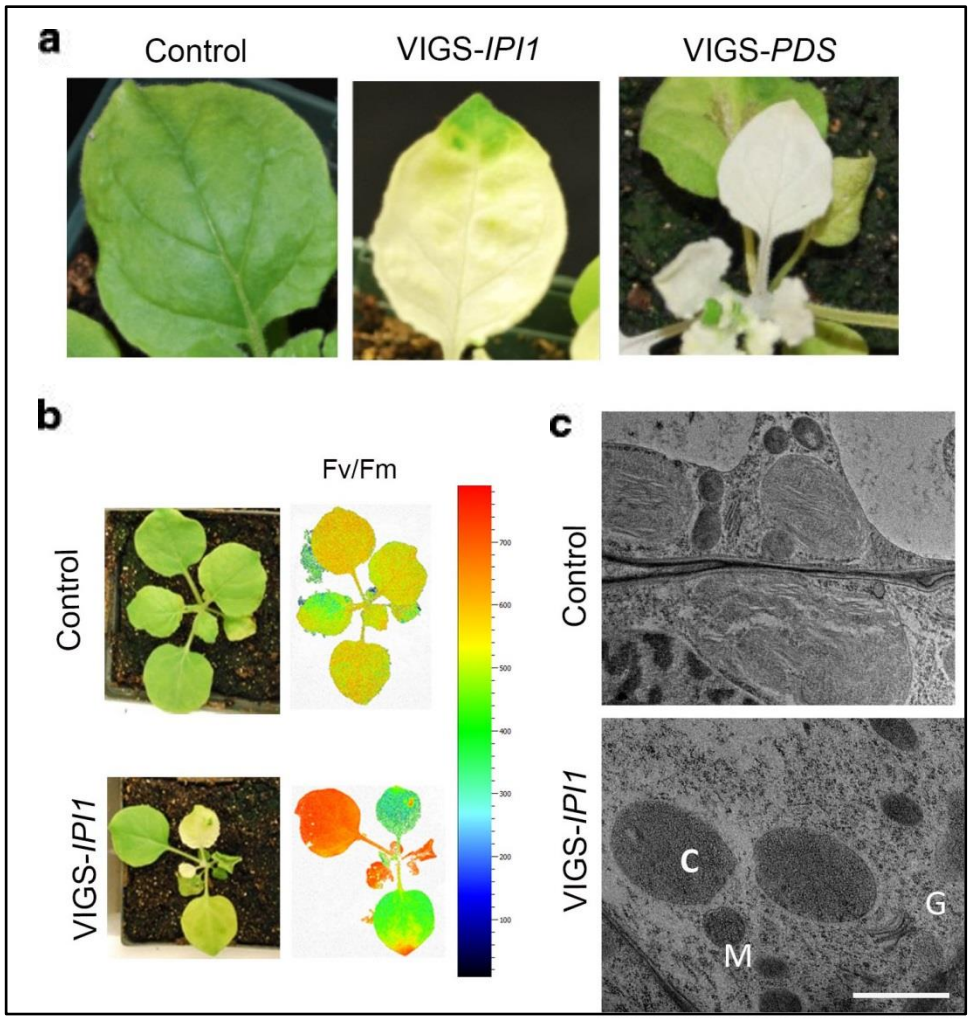


Figure 2.11 Phenotypic characterization of *IPI1*-silenced plants

IPI1-silenced leaves show defects in chloroplast function (a) A severe chlorotic phenotype is produced after *IPI* is silenced by VIGS in *Nicotiana benthamiana* leaf tissue (b) The PSII quantum yield is reduced to levels of zero in *IPI1*-silenced leaves, presumably due to the absence of chlorophyll. Fo: fluorescence at the ground state (prior to excitation), Fm represents maximal fluorescence, Fv/Fm represents variable fluorescence divided by the maximal fluorescence as an indication of the ability of PSII to photochemically quench excited electrons (c) TEM analysis reveals defective chloroplasts in *IPI1*-silenced leaves. C,M, and G represent chloroplast, mitochondria, and golgi, respectively.

defects in chloroplast rRNA processing, with drastic reductions in the 4.5S, 16S, 5S and 23S rRNAs (Fig. 2.12c). rRNA processing defects were seen for 16S and 23S rRNA in *IPI1*-silenced leaves as compared to the rRNA processing pattern observed in the non-silenced control. Drastic reductions were observed for 4.5S and 5S rRNA. These reductions may be due to transcriptional defects in *IPI1*-silenced leaves.

Overall, the reductions in total rRNA were severe enough to be observed on an agarose gel stained with ethidium bromide (Fig. 2.12a). These results indicate that IPI1's role in rRNA processing is conserved between *maize* and *Nicotiana.*

Leaves silenced for *ISE2* produce a less severe chlorotic defect than leaves silenced for *IPI1* or for the transcript encoding the carotenoid synthesis enzyme, phytoene desaturase *(PDS)* (Fig. 2.5). Previous reports in maize demonstrated that severe chlorotic phenotypes were associated with greater defects in rRNA processing, as compared to mild chlorotic phenotypes (Hammani, Takenaka et al. 2016). To compare the rRNA processing defects seen in *IPI1*-silenced leaves to rRNA processing defects seen in leaves that produce a less severe (e.g TRV-*ISE2*) or more severe (eg TRV-*PDS*) chlorotic phenotype, northern blots were also conducted on RNA isolated from *N. benthamiana* leaves silenced for *ISE2* or for *PDS,* for comparison. Previous data indicates that *Arabidopsis* co suppressed tissue exhibits defects in the processing of 23SrRNA (Bobik, McCray et al. 2016). The northern blot of total RNA from *N. benthamiana ISE2*-silenced leaves confirmed this defect. Additionally, the northern blot of *ISE2*-silenced-leaves revealed less severe defects in 23S rRNA processing than seen in leaves silenced for *IPI1* (Fig. 2.13). However, leaves silenced for *PDS* produced a drastic reduction in rRNA species, similar to that seen in leaves silenced for *IPI1* (Fig. 2.13). These data indicate that, in *N. benthamiana,* the degree of rRNA processing defects may correlate with the severity of chlorosis in particular mutants, as was previously reported in maize (Hammani, Takenaka et al. 2016)

IPI1 is Necessary for Editing Chloroplast Transcripts

Although the degree of rRNA processing defects may correlate with the severity of the chlorotic phenotype that is seen in leaf tissue, several similarly chlorotic mutants displayed unique patterns of RNA editing defects (Chateigner-Boutin, Ramos-Vega et al. 2008). For example, mutations in the PPR gene *vanilla cream 1 (vac1)* produce a severe chlorotic phenotype coupled to drastic defects in chloroplast ultrastructure; however only a subset of transcripts exhibit editing defects (Tseng, Sung et al. 2010). Examples of transcripts that exhibit editing defects in *vac1* mutants include *ndhF* and *accD* (Tseng, Sung et al. 2010). Similarly, mutations in the PPR gene, *chloroplast biogenesis 19,* display a severe chlorotic phenotype, but only editing within the *rpoA* and *clpP* transcripts are affected (Chateigner-Boutin, Ramos-Vega et al. 2008)). Further, mutations in the gene encoding the maize orthologue of IPI1, PPR103, exhibited a severe chlorotic phenotype coupled to drastic reductions in rRNA processing but no significant editing defects were reported (Hammani, Takenaka et al. 2016). Interestingly however, the maize orthologue does not contain the C-terminal amino acid triplet, DYW, that is found in other PPR proteins known to function in editing (Zehrmann, Verbitskiy et al. 2009), (Brehme, Császár et al. 2015), (Wagoner, Sun et al. 2015), (Boussardon, Avon et al. 2004) (Fig. 2.9).

N. benthamaina, IPI1 contains the terminal DxW (Asp, variable,Trp) amino acids (Fig. 2.7) and we found editing defects for a subset of edited sites in *NbIPI1*-silenced *N. benthamaina* leaves (Fig. 2.14). We have demonstrated a role for the interacting protein of IPI1, ISE2 in the editing of several chloroplast transcripts in mature *Arabidopsis* and *N.benthamiana* leaf tissue (Fig. 2.2 and Fig. 2.14). Interestingly, the editing defects for editing sites *rpoA*, *ndhB4* and *ndhD1* observed in *IPI1*-silenced *N. benthamiana* leaves appear to resemble the editing defects observed in *ISE2*-silenced *N. benthamiana* leaves (Fig. 2.14).

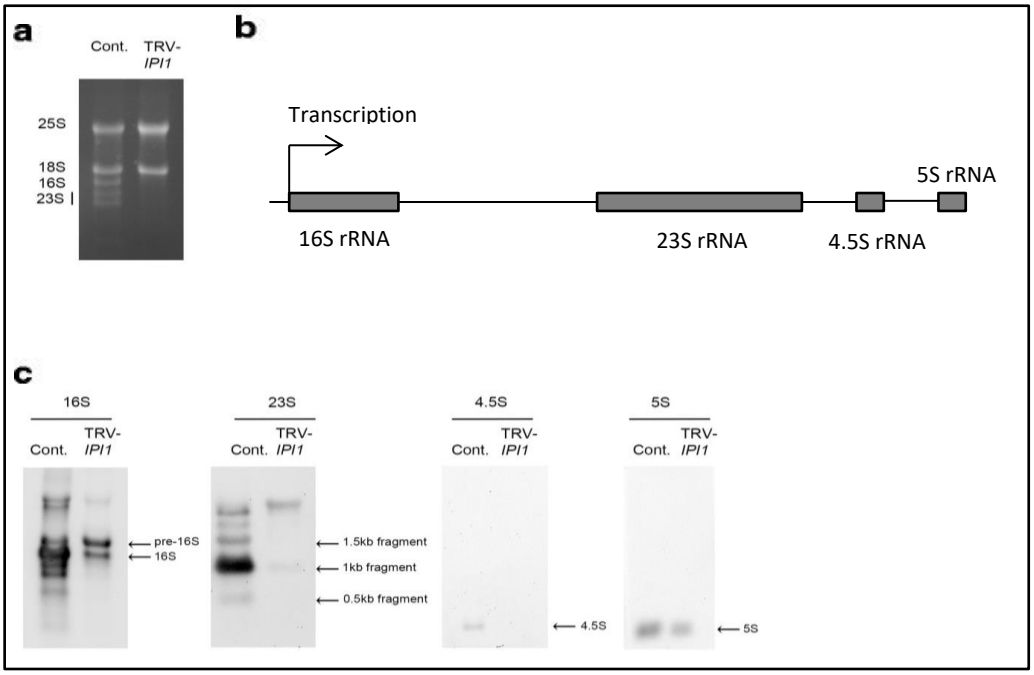


Figure 2.12 rRNA processing defects are seen in *IPI1*-silenced leaves

Analysis of rRNA processing defects in the control vs *IPI1*-silenced leaves (TRV-*IPI1*) revealed that a) defects are readily apparent on a denaturing gel b) schematic of rRNA locations c) probes against 16S, 4.5S,5S and 23S reveal drastic reductions in the respective rRNA species in *IPI1*-silenced plants as compared to the control non-silenced plants. Results are representative images from two biological replicates in which similar results were obtained.

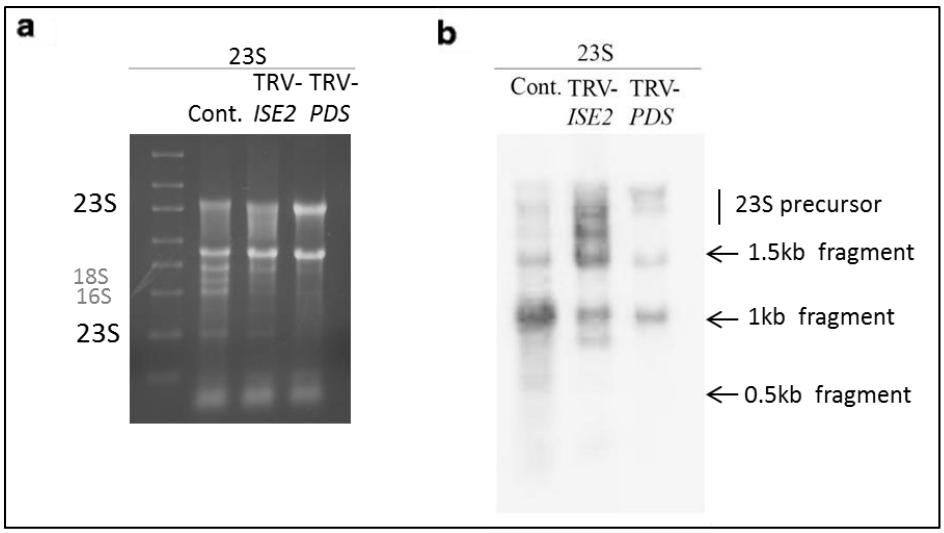


Figure 2.13 rRNA processing defects are seen in *ISE2* and *PDS*-silenced leaves

Analysis of rRNA processing defects in the control vs *ISE2*-silenced (TRV-*ISE2*) or *PDS*-silenced leaves revealed that a) defects are readily apparent on a denaturing gel and b) probes against 23S reveal processing defects in the respective 23S rRNA species in *ISE2*-silenced leaves as compared to control leaves. These are represented as an accumulation of unprocessed precursor rRNA species and is reminiscent of rRNA processing defects in *Arabidopsis ISE2*-silenced leaves (cosuppressed). TRV-*PDS* exhibit severe reductions in 23S rRNA. Results are representative images from two biological replicates in which similar results were obtained.

To examine whether these editing events are due to a secondary or a general stress response to chlorosis, we examined editing in mature *N. benthamiana* leaf tissue in which the carotenoid biosynthesis gene, *PYTOENE DESATURASE (PDS*) was silenced. *PDS*-silenced *N. benthamiana* leaves also exhibited editing defects at several editing sites (Fig. 2.14). As plants silenced for *ISE2, IPI1* or *PDS* produced similar defective editing patterns at sites *rpoA, ndhB4*, and *ndhD1,* editing at these sites are likely due to a secondary defect in chloroplast function. However, the examination of editing defects at other edited sites revealed that the editing "signatures" of the mutants are distinct (Fig. 2.14). For example, edited site *ndhB3* is uniquely affected in *ISE2*-silenced plants and *ndhB2, ndhB3, ndhB5-ndhB8* sites are uniquely affected in *IPI1*-silenced plants.

This result indicates that while some editing defects may be due to a general stress response, each factor may differentially affect the downstream editing reaction at specific sites within particular transcripts. Future analysis includes ascertaining whether IPI1 has a direct role in editing *ndhB2* (an editing site that is not affected in *ISE2* or *PDS*-silenced leaves) by assessing whether IPI1 binds to RNA within the vicinity of this RNA editing site.

No Intercellular Trafficking Defects are Seen in IPI1 Mutants

Because the IPI1 interacting partner, ISE2 displays defects in PD-mediated intercellular trafficking as well as defects in rRNA processing and RNA editing, we checked whether intercellular trafficking defects are observed in *IPI1*-silenced plants. To this end, *IPI1*-silenced plants and non-silenced control plants were infiltrated with 2xGFP and the extent of the intercellular movement of 2xGFP was compared 48 hours later. Unlike plants that are silenced for *ISE2*, where an increase in the movement of 2xGFP is observed relative to control plants, no major intercellular trafficking defects were observed in plants that were silenced for *IPI1* (Fig. 2.15). These results suggest that ISE2 and IPI1 may physically

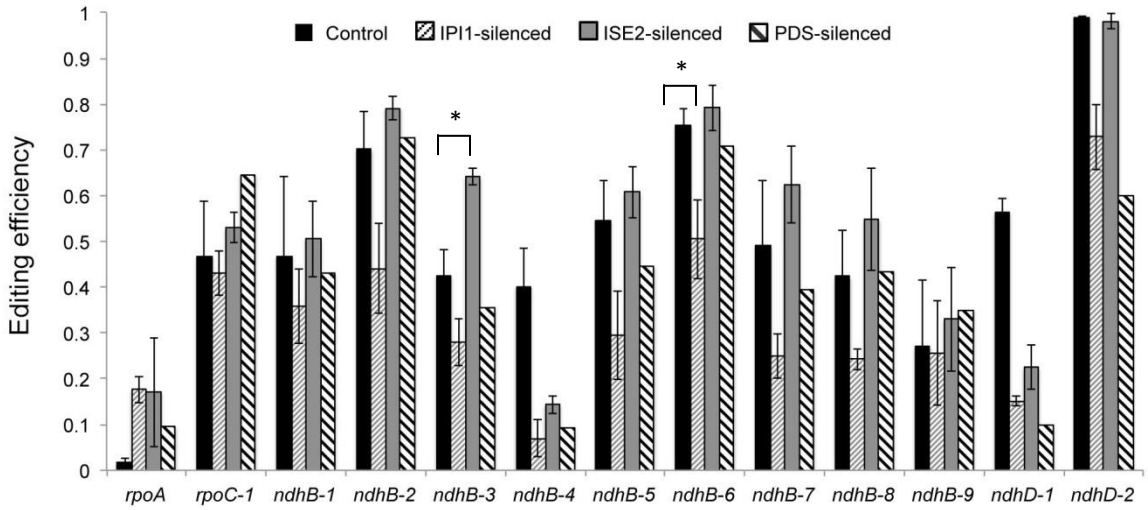


Figure 2.14 Comparison of RNA editing in control, *ISE2*-silenced, *IPI1*-silenced and *PDS*-silenced leaves

RNA editing defects are compared in plants silenced for *ISE2* and *IPI1*. TRV-*PDS*-silenced plants are shown for comparison. Editing experiments were conducted on the same leaf number (leaf #11) from control, *IPI1, ISE2, or PDS*-silenced plants grown at the same time and under the same conditions. Results represent two control, threeTRV-*ISE2,* and three *TRV-IPI1* biological replicates. An ISE2-specific statistically significant difference is observed between control and *ISE2*-silenced leaves at site *ndhB*-3. Sites *ndhB*-4 and *ndhD*-1 are also affected in ISE2-silenced plants; however these sites are also affected in *PDS*-silenced plants. An IPI1-specific statistically difference is observed between control and *IPI1*-silenced leaves at site ndhB-6. Asterisks denote p-value <0.5 as determined using a one-tailed ttest assuming unequal variance.

interact for a role that is related to a specific RNA processing event; this RNA processing event may not relate to ISE2's role in the regulation of PD-mediated trafficking.

Transcriptome Wide Analysis

A comprehensive understanding of chloroplast RNA metabolism requires the characterization of chloroplast-localized proteins that function in chloroplast RNA metabolism. PPR proteins are chloroplast-localized proteins that represent a greatly expanded protein family in plants. Although several PPR genes have been characterized over the last decade, their global effect on chloroplast (cp) RNA metabolism remains poorly understood. Insight into how PPR proteins may affect the abundance and architecture of the chloroplast transcriptional landscape can provide insight into chloroplast gene regulation; however comprehensive transcriptional studies in *ppr* mutants are limited. I demonstrate the use of a high-throughput Illumina sequencing approach on isolated chloroplasts to comprehensively and quantitatively assess the RNA metabolic response of a mutant lacking the chloroplast-localized pentatricopeptide repeat protein, IPI1 (characterized in chapter 2.2). Our data provide a widespread view of changes to the chloroplast transcriptome in *N. benthamiana* leaves that are silenced for *IPI1*. In this section of chapter 2, we (i) report that known editing sites are conserved in *N. benthamiana,* (ii) identify new editing site in *N. benthamiana,* (iii) and demonstrate that several editing sites within transcripts are specifically affected by the silencing of *IPI1*. The data additionally implicate new stabilization functions for this PPR gene. Overall, our results provide a glimpse into the global regulatory function of IPI1 within chloroplasts.

In *N. benthamiana*, many aspects of global chloroplast RNA metabolic changes, caused by mutations in *PPR* genes, remain poorly understood. Although such

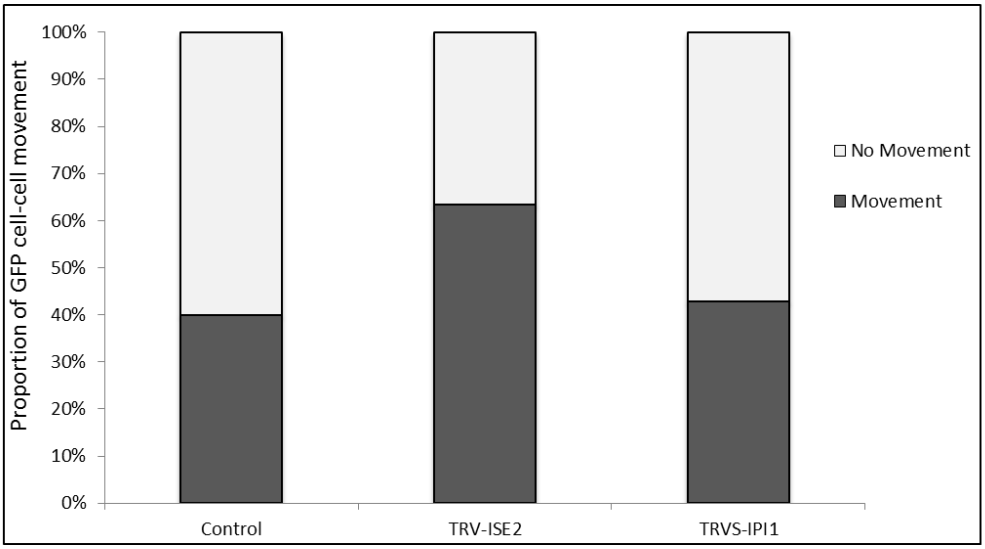


Figure 2.15 Analysis 2xGFP movement in control, *ISE2*-silenced or *IPI1*-silenced leaves

Control, *ISE2*-silenced or *IPI1*-silenced plants were bombarded with 2xGFP and examined using confocal microscopy for 2xGFP movement 48 hours later. The plants descriptions are indicated on the x-axis and the percentage of 2xGFP movement in each plant background is indicated on the Y-axis. Light grey indicates that 2XGFP remained in the primary infected cells. Dark grey indicates that 2xGFP moved at least one cell layer away from the primary infected cell into adjacent cells. n=3 for all plants. 18 loci were counted in the control, 30 loci were counted in *ISE2*-silenced plants and 28 loci were counted in *IPI1*-silenced plants. The p-values obtained from a chi-squared test indicated that the changes in movement between the control and *ISE2*-silenced plants were significant (p-value =9.6 e-5), while the changes in movement between the control and *IPI1*-silenced plants were insignificant (p-value > .05).

information is crucial to understanding the regulatory function of PPR proteins and although pleiotropic effects are often observed in *ppr* mutants, to my knowledge, there is only one report that comprehensively examines the global defects of a *ppr* mutant (Zoschke, Watkins et al. 2016). Additionally, to our knowledge, very few organelle RNA-sequencing experiments using purified organelles as starting material have been conducted.

We have examined the transcriptome-wide response of isolated chloroplasts from *IPI1*-silenced leaves in *N. benthamiana*. The Illumina high-throughput sequencing platform offers a powerful tool to investigate the global regulation of organelle RNA metabolism and to discover how such processes are affected in *ppr* mutations. To this end, total RNA was isolated from chloroplasts of intact leaf tissue from control plants or *IPI1*-silenced plants (Fig. 2.16). After an RNA quality check, cDNA synthesis, and library prep using the Next Tera Illumina kit, reads were amplified and subjected to sequencing reactions on a Miseq Illumina instrument.

Overall, the sequenced and mapped bases yielded a transcriptome profile and identified a number of edited sites that are differentially affected between the non-silenced tissue control and *IPI1*-silenced tissue (TRV-*IPI1*). IPI1 is a protein that contains a domain that may contribute to RNA editing reactions. We observed editing defects (as well as differential expression defects) in the *IPI1*-silenced leaf tissue relative to the control. Our analysis additionally led to the discovery of new previously unidentified editing sites in the model plant *Nicotiana benthamiana*. This study highlights transcriptome sequencing as a key tool for understanding the extent of organelle RNA-based regulation by PPR proteins.

General Pipeline

N.benthamiana plants silenced for *IPI1* produce a severe chlorotic phenotype at about 5 weeks of age. After the bleached phenotype was apparent (Fig. 2.5), chloroplasts were isolated from control green leaf tissue and *IPI1*-silenced

bleached tissue (Fig. 2.16). Visualization of intact chloroplasts using an inverted microscope illustrated that phenotypically normal chloroplasts were isolated from non-silenced control plants whereas almost all chloroplasts isolated from *ppr* mutant tissue were distinctively defective with no thylakoid structures or starch granules apparent (Fig. 2.16). Total RNA was subsequently isolated from chloroplasts, treated with DNase and subjected to quality inspection using microfluidics. To ensure that enough chloroplasts were available for RNA isolation and subsequent cDNA library preparation, leaf tissue was pooled from all leaves that exhibited a bleached phenotype or from leaves of the same age and developmental stage in the control plant. We did not enrich for mRNA by depleting the 16S and 23S ribosomal RNAs from our samples in order to detect processing rRNA defects in the *IPI1*-silenced leaf tissue. Double-stranded cDNA was prepared using random-hexamer priming on total RNA and then subjected to Next Tera library preparation with paired end adapter-ligation. The prepared libraries were again subjected to quality check using the microfluidics instrument to ensure that high quality cDNA libraries were used to sequence on the MiSeq Illumina platform. Resulting adapter-ligated cDNA fragments were sequenced using the Miseq illumina platform. Three control libraries (non-silenced control) and four mutant libraries (TRV-*IPI1*) were used for the analysis. The 250 base pair sequenced reads revealed a high quality mapping score before (e.g Fig. 2.17), and after the adapters were trimmed ensuring that good quality contigs are subsequently mapped to the *N. benthamiana* chloroplast reference genome. The total assembled reads for control or TRV-*IPI1* is represented in Table 2.3.

Analysis was conducted on a similar number of sequenced reads from each library. After trimming adapters, the short sequenced reads were mapped to the *N. benthamiana* genome using DNA Array Star software (SeqMan NGen). A small percentage (roughly 25%) of the reads uniquely mapped to the chloroplast genome. These results may be due to the fact that the *N. benthamiana* chloroplast genome is not complete or due to contamination from non-chloroplast

sequenced contigs. The 250 nt reads were mapped to the *N. benthamiana* chloroplast genome with no mismatch penalty to allow the detection of multiple editing events –recognized as SNPs- within the same transcript. The number of assembled reads to the *N. benthamiana* genome is represented in Table 2.3.

Detection of RNA Editing Defects in IPI1-silenced Leaves

Studies over the past several years have defined editing sites in *Nicotiana tabacum* (Hirose, Kusumegi et al.), (Tseng, Lee et al. 2013), and we used those data to identify a set of 34 expected editing sites in the closely related *Nicotiana benthamiana* species. These sites are listed in Table 2.5 and they all exhibit some degree of editing. Comparison of the mapped RNA-seq results from the non-silenced control and *IPI1*-silenced plants revealed editing efficiency differences between the two plant backgrounds for known edited sites (Fig. 2.18, Table 2.4). Sites that are affected by *IPI1*-silencing include *atpA-2, atpF, ndhA-1, ndhB-3, ndhB-4, ndhB-5, ndhB-6, ndhB-7, ndhB-8, ndhD1,2,* and *petB*. Notably, *atpA-1, atpA-2, atpF, ndhB-6, ndhD-1, ndhD-2, petB, psbE,psbL, rpoC2, rps2-* sites that were not significantly affected by loss of *IPI1*-exhibited very little variation among either the 3 PYC1 control or the 4 *IPI1*-silenced plants (Fig 2.18). This observation suggests that editing at these sites is robust and may be unaffected by biological variation or slight environmental changes experienced amongst the different plants. Edited sites that exhibited large variation (represented by large error bars) may reflect biological variation of individual plants for editing these particular sites and for the requirement of *IPI1* for the full extent of editing at these sites. The majority of edited sites were detected by the DNA ARRAY STAR Seq Man Pro SNP algorithm; however four of the editing sites (*rpoA, petB, ndhA-1*, *ndhB4*) were not detected with the SNP algorithm and had to be manually detected. An ideal criterion for analysis would be that each edited site contains at least 10 sequence reads that were mapped to that site. However, the *rpoA* edited site, that was previously identified and characterized in

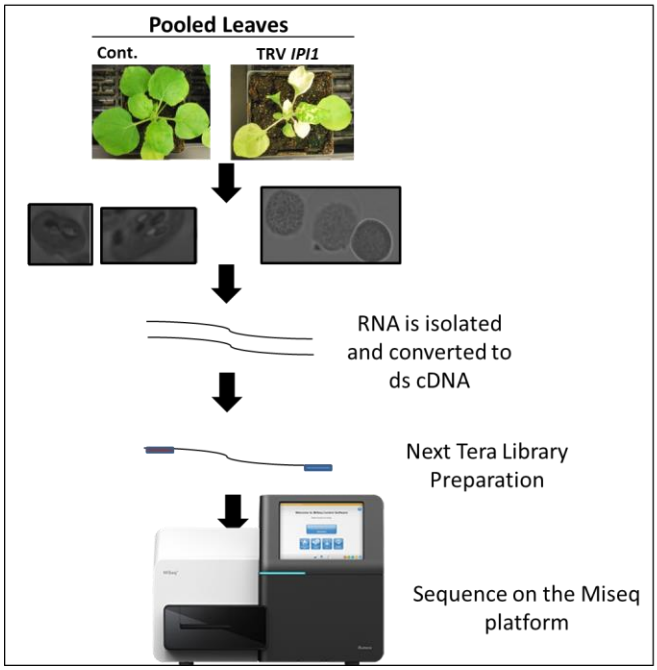


Figure 2.16 Isolation of chloroplasts and RNA for RNA-seq schematic

Chloroplasts were isolated from intact *Nicotiana benthamiana* leaves and used for total chloroplast RNA extraction using the Trizol method A) Representative plants and respective chloroplasts are shown. Chloroplasts were isolated from all *IPI1*-silenced leaves that showed the chlorotic phenotype in individual plants and from the corresponding leaf numbers in the VIGS-PYC1 control plant .Three control plants and four *IPI1*-silenced plants were used to construct three control and four mutant individual libraries for sequencing. Chloroplasts were also isolated from WT leave for comparison B) A schematic of the chloroplast isolation procedure (left) representative results achieved prior to the visualization, further purification and freeze fracture of the chloroplast membranes C) Bioanalyzer results from the initial RNA that was isolated from chloroplasts. Two methods were used: RNeasy kit (left) and Trizol (right). RNA from the RNeasy kit was used for double strand cDNA synthesis and library preparation.

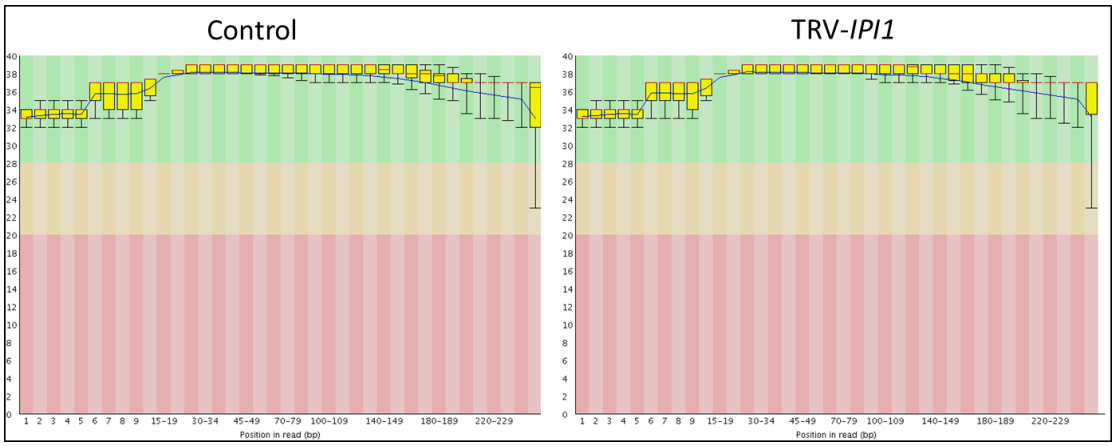


Figure 2.17 Representative map quality scores from control and TRV-*IPI1* sequenced reads

Quality scores across all bases for 1 representative read from control or TRV-IPI1 libraries using the Sanger/Illumina sequencing platform. Representative quality scores before trimming illustrate that high quality reads were obtained from the Miseq instrument. X axis represents the base pair position along the length of the read and y-axis represents the quality score. Most reads reflect phread quality scores above 30 for the majority of the read length.

Table 2.3 Mapped reads using SeqMan NGen software

	Total Reads Assem.			
		Single Seq Cnt	**Split Seq Cnt**	**Seqs score =<100%**
Control	645570	9573	2319	647889
***IPI1*-silenced**	559697	127037	2045	561742
	Unassem. Seq.			
		Unaligned Cnt	**ExcessiveCov. Seq Cnt**	**Split Fragments**
Control	3373	3373	1336118	558
***IPI1*-silenced**	2399638	10095	560214	473

N. tabacum (Hirose, Kusumegi et al.), only contained about 4 reads on average that were mapped, potentiallyindicating its low expression. Nonetheless, the level of mapped reads were not used as a filtering criteria in downstream analysis of SNP detection.

Identification of Novel Chloroplast RNA Editing Sites in Nicotiana benthamiana Leaves

Excitingly, novel sites were identified that have not been reported in any *Nicotiana* species (Fig. 2.19, Table 2.5). These sites were generally edited in all 7 libraries (3 PYC1 control and 4 *IPI1*-silenced). Many of these newly identified editing sites occur within the *ndhD* transcript which is involved in electron transport. There are currently only 5 reported editing sites in the *Nicotiana tabacum ndhD* transcript (Tseng, Lee et al. 2013), (Hirose, Kusumegi et al. 1999). These are as follows (i) *ndhD*-1 changes the first codon to a translational start site (ii) one changes the amino acid from an S to an L at position 128 and (iii) one changes the amino acid from an S to an L at position 225 (Tseng, Lee et al. 2013). The amino acid changes for the new edited sites are indicated in table 2.5. Interestingly, these newly identified amino acid changes reveal a non-synonymous change from a serine (S) to a leucine (L) amino acid, the most common amino acid change that occurs for known edited sites (Table 2.5).

I identified novel S to L editing at sites 293, 433, and 437 within the *N. benthamiana ndhD* transcript (Table 2.5). Interestingly, editing at position 293 is not reported in *Nicotiana tabacum* as the codon for this position encodes the conserved amino acid in the chloroplast genome and for this reason, does not need to undergo editing (Fig. 2.20). However the coding sequence for editing sites *ndhD*-433 and *ndhD*-437 in both *N. tabacum* and *N. benthamiana* encodes an S that is normally edited to an L in other plant species (Tseng, Sung et al. 2010).

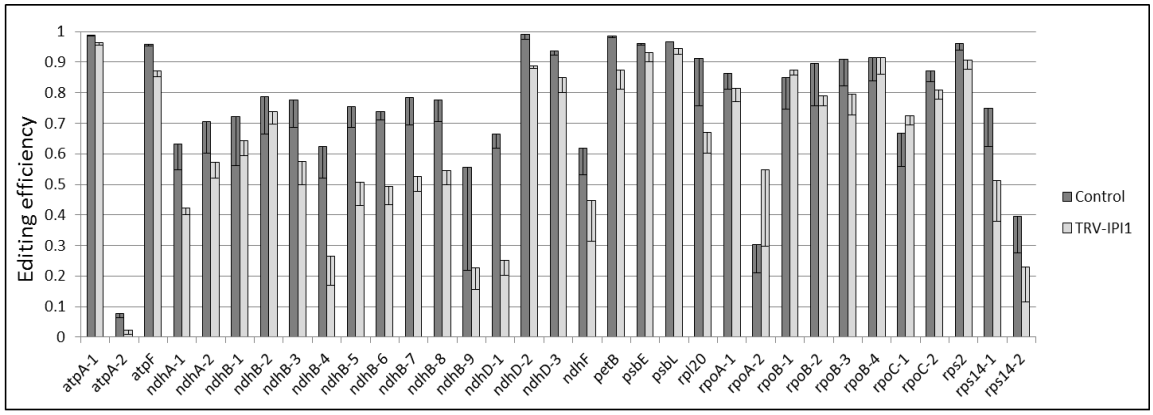


Figure 2.18 RNA editing efficiencies from RNA-seq data analysis

RNA-Seq analysis confirms the pattern seen for editing defects in plants silenced for *IPI1* (examined in chapter 2.2) and extends examination to all known editing sites. Standard error bars represent variation obtained from individual plant (as individual sequenced plant library) editing values at the respective sites. Statistical significance is represented in Table 2.4.

Table 2.4 Statistical significance between RNA-seq control and TRV-*IPI1* RNA-seq libraries

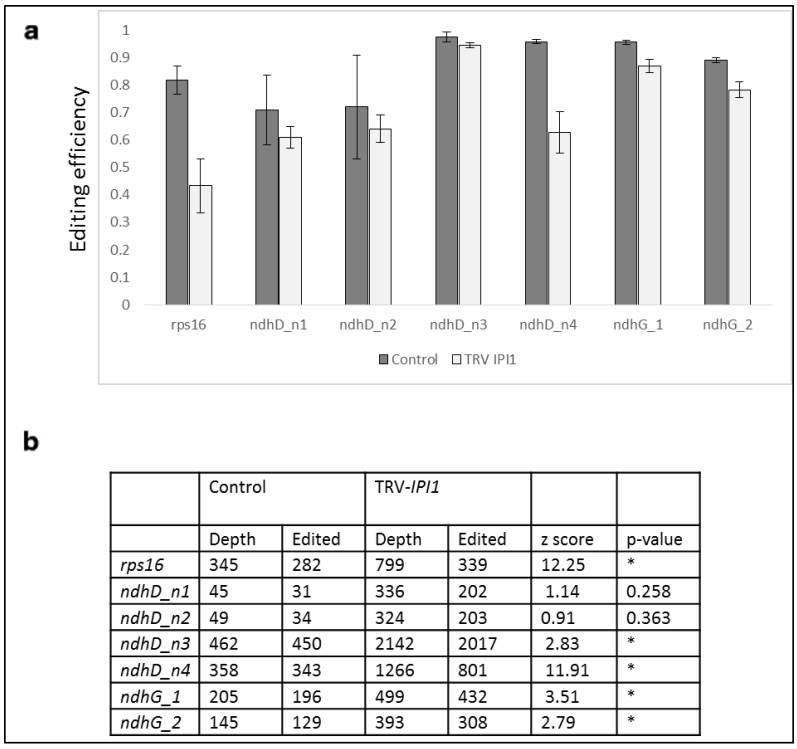
	Control		**TRV-*IPI1***			
	Depth	**Edited**	**Depth**	**Edited**	**Z Score**	**pValue**
atpA-1	1122	1109	2742	2645	4.04	*
atpA-2	1143	87	2386	52	7.76	*
atpF	2432	2330	3013	2625	11.13	*
ndhA-1	327	208	1416	594	7.08	*
ndhA-2	333	236	1370	764	5.02	*
ndhB-1	143	107	1033	688	1.97	4.9 e -2
ndhB-2	243	195	1107	806	2.4	1.6 e -2
ndhB-3	209	161	1039	596	5.31	*
ndhB-4	199	124	1040	261	10.39	*
ndhB-5	131	100	766	371	5.91	*
ndhB-6	123	91	743	358	5.3	*
ndhB-7	107	84	571	306	4.78	*
ndhB-8	112	87	583	318	4.55	*
ndhB-9	25	15	251	64	3.64	2.8 e -4
ndhD-1	367	246	1525	325	17.13	*
ndhD-2	415	412	2141	1907	6.56	*
ndhF	52	31	76	37	1.22	0.22
pet B	2379	2341	1617	1422	13.85	*
psbE	2951	2833	1933	1806	4.03	*
psbL	1870	1808	908	856	3.01	2.6 e -3
rpl20	42	38	250	170	2.98	2.8 e -3
rpoA-1	565	490	1977	1590	3.43	6.2 e -4
rpoA-2	34	9	118	53	-1.93	5.3 e -2
rpoB-1	91	76	1250	1089	-0.99	0.33
rpoB-2	62	55	795	627	1.85	6.4 e -2
rpoB-3	60	54	689	539	2.15	3.2 e -5
rpoB-4	98	88	868	797	-0.68	0.49
rpoC-1	93	61	1120	800	-1.19	0.23
rpoC-2	176	153	1692	1365	2.02	0.04338
rps14-1	127	97	275	145	4.5	*
rps14-2	39	15	144	31	2.16	0.03078
rps2	247	237	1855	1673	2.95	*

Single askerisk denontes statistical significance at p <0.01

Expectedly, we detected editing at these sites for *N. benthamiana,* but it is unclear as to why these sites have never been previously reported to be edited in *N. tabacum.* Nevertheless, we report here the newly identified edited sites in the model plant, *N. benthamiana.* It appears that IPI1 does not affect every edited site, indicating that there may be at least some level of selectivity (Fig. 2.18). The *ndhB* and *ndhD* transcript edited sites are the most affected in plants that are silenced for *IPI1*. NAD(P)H-quinone oxidoreductase (NDH) is composed of 16 subunits, 5 that are nucleus-encoded and 11 that are chloroplast-encoded (Peng, Yamamoto et al. 2011). The chloroplast-encoded *ndhB* and *ndhD* subunits comprise components of the fully assembled NDH complex located in the chloroplast thylakoid membrane. NDH is involved in cyclic electron transport around photosystem I (PSI) (Peng, Yamamoto et al. 2011). The NDH function is to shuttle 2 electrons from NAD(P)H:plastoquinone to ubiquinone (forming plastoquinols) in the chloroplast photosynthetic electron transport chain via route through the FMN and iron-sulfur (Fe-S) centers. This reaction creates a proton gradient and allows proton translocation for eventual ATP synthesis. Because the reaction involves the acceptance of electrons, this reaction also alleviates oxidative stress generated from the NDH complex, a major source of reactive oxygen species generation due to electron transport from FMNH (Fisher 2000). The ndhB2 (subunit 2A of the NDH dehydrogenase) is a multi-pass membrane protein (Peng, Yamamoto et al. 2011). It is not clear where the quinone binding site is, but if the NDH complex cannot bind quinone, oxidative stress may occur (Fisher 2000). Editing within transcripts encoding subunits of the plastid-encoded polymerase appear to be unaffected by *IPI1*-silencing; however IPI1 may regulate the stability of these transcripts as a separate role. It is additionally possible that IPI1 may exert an effect on the transcription rate of the *rpo* plastid-encoded polymerase transcripts. However as PPR proteins are well-known to bind transcripts in order to aid in degradation, stabilization, editing, or splicing, it is more likely that wild-type IPI1 plays a role in the degradation of particular transcripts.

Table 2.5 RNA-seq analysis identifies known edited sites and identifies unreported edited sites. Nomenclature as reported in Hirose, 1999 for known sites.

Known Edited Sites		**Novel Edited Sites**	
Transcript Name	**Amino Acid Change**	**Transcript Name**	**Amino Acid Change**
atpA-1	P264L	*rps16*	intron
atpA-2	P265S	*ndhD_n1*	S437L
atpF	P31L	*ndhD_n2*	S433L
ndhA-2	S114L	*ndhD_n3*	S293L
ndhA-5	S358F	*ndhD_n4*	S200L
ndhB-1	S50L	*ndhG_1*	S116L
ndhB-2	P156L	*ndhG_2*	S17L
ndhB-3	H196Y		
ndhB-4	S204L		
ndhB-5	P246L		
ndhB-6	S249F		
ndhB-7	S277L		
ndhB-8	S279L		
ndhB-9	P494L		
ndhD-1	T1M(Start)		
ndhD-2	S128L		
ndhD-3	S225L		
ndhF-2	S97L		
petB	P204L		
psbE	P72S		
psbL	T1M (Start)		
rpl20	S103L		
rpoA-1	S67F		
rpoA-2	S277L		
rpoB-1	S113F		
rpoB-2	S158L		
rpoB-3	S184L		
rpoB-4	S667F		
rpoC1	S21L		
rpoC-2	S1248L		
rps2	S83L		
rps14-1	S27L		
rps14-2	P50L		



	Control		TRV-*IPI1*			
	Depth	Edited	Depth	Edited	z score	p-value
rps16	345	282	799	339	12.25	*
ndhD_n1	45	31	336	202	1.14	0.258
ndhD_n2	49	34	324	203	0.91	0.363
ndhD_n3	462	450	2142	2017	2.83	*
ndhD_n4	358	343	1266	801	11.91	*
ndhG_1	205	196	499	432	3.51	*
ndhG_2	145	129	393	308	2.79	*

Figure 2.19 RNA editing efficiencies of novel sites

a) The editing efficiencies were compared for the newly identified sites. Dark grey represents editing efficiency in control plants and light grey represents editing efficiency average in light grey plants. b) a z score statistical test was used to assess the statistical significance between 3 control and 4 TRV-*IPI1* library populations. Single asterisk represents significance between control and TRV-*IPI1* at $p<0.01$.

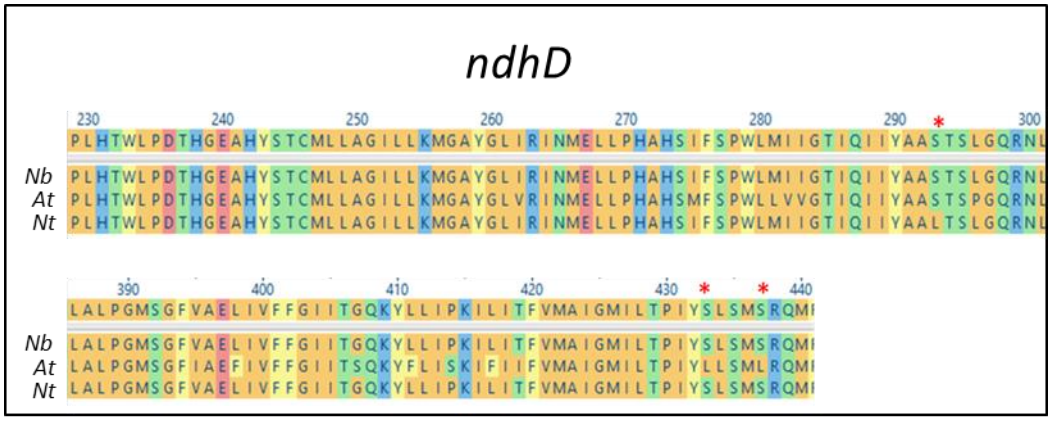


Figure 2.20 Alignment of *Nicotiana benthamiana, Arabidopsis thaliana* and *Nicotiana tabacum* chloroplast ndhD protein sequences surrounding the location of the S->L amino acid change caused by editing events within the *ndhD* transcript

Fasta file protein sequences were downloaded from NCBI and aligned using MUSCLE. Abbreviations: Nb *(Nicotiana benthamiana),* At *(Arabidopsis thaliana)* and Nt *(Nicotiana tabacum).* Amino acids are colored according to similarity. The numerical positon of amino acids is indicated above the figure. Red asterisks represent newly identified editing sites from our RNA-seq analysis in *Nicotiana benthamiana* that were not reported in *Nicotiana tabacum.*

It will be interesting to observe how the expression of all edited transcripts correlates with their editing efficiency in control and *IPI1*-silenced plants and whether IPI1 directly binds to some of the transcripts.

Conclusion

In this results section of chapter 2, we offer an approach to examining the global consequences of defective PPR gene function on chloroplast RNA editing metabolism. Very few experiments have reported the use of high-throughput sequencing technology to examine the organelle RNA metabolic profile of samples of interest by using isolated RNA from purified organelles. And very few publications have reported the use of this approach to characterize the global

organelle RNA metabolic defects in any *ppr* mutant. Here we report the use of the Mi-seq platform to sequence libraries prepared from total RNA that was isolated from purified chloroplast organelles. Our results confirm that conserved editing events in other species (including other *Nicotiana* species) are conserved in *Nicotiana benthamiana.* Additionally, our findings extend our knowledge of editing in *Nicotiana benthamiana* through the identification of novel editing sites. Finally, our results offer insight into the global role of IPI1 on chloroplast RNA metabolism. This technique can be applied to the examination of other plants that are defective in *ppr* genes.

Care was taken to isolate plastids from bleached tissue (tissue that showed the *IPI1*-silenced phenotype). It has been previously documented that white leaves or white parts of leaves (in mutants) do not contain ribosomes and lack all plastid-encoded proteins (Zhelyazkova, Sharma et al. 2012). Interestingly, however, transcription still occurs, albeit by the nuclear encoded polymerase (NEP) instead of by the plastid encoded polymerase (PEP), indicating that plastid protein import is not completely abolished. In fact, NEP activity is reported to be higher in white leaves than in green leaves, while the PEP is usually

downregulated in chlorotic tissue (Zhelyazkova, Sharma et al. 2012), (Chateigner-Boutin, Ramos-Vega et al. 2008), (Tseng, Sung et al. 2010), (Tseng, Lee et al. 2013). Thus, the editing defects are likely not due to defects in protein import.

Overall, our pipeline has allowed us to confirm RNA editing defects, identify new edited sites and examine IPI1's global role on chloroplast RNA metabolism in *N. benthamiana.*

CHAPTER THREE DISCUSSION

Biological Implications of the Role of ISE2 in RNA Editing

Functions of Arabidopsis ISE2-affected Edited Transcripts

Our data indicate that ISE2 is required for the RNA editing of the following *Arabidopsis thaliana* chloroplast transcripts at the following sites: *accD-1568, clpP-559, petL-5, rpoA-200, rpoC1-497, rpl23-89, rps12*-intron and *ndhD-2* (Fig. 2.2). The *accD* gene encodes a carboxyltransferase beta subunit of the acetyl CoA carboxylase (ACCase), which catalyzes the carboxylation of acetyl CoA to malonyl CoA, the initial fatty acid biosynthesis step in chloroplasts. The C-to-U editing of the accD transcript at the site 794 was found to be dependent on a PPR-DYW protein called RARE1 in Arabidopsis chloroplasts (Robbins, Heller et al. 2009); however, the particular editing defect at the *accD-794* site did not affect plant survival or growth under standard growth conditions (Robbins, Heller et al. 2009). AtISE2-silenced plants produce a chlorotic phenotype coupled to an editing defect at site 1568 (not site 794) within the *accD* transcript. The editing site 1568 corresponds to the 3'UTR within the *accD* transcript. Defective editing at sites 794 and 1568 of the *accD* transcript are seen in mutations of the *Arabidopsis* DYW-containing protein, VAC1 (Tseng, Sung et al. 2010). Interestingly *vac1* mutants produce an albino phenotype coupled to defective retrograde signaling (Tseng, Sung et al. 2010). Editing at *accD* -1568 may be a general defect due to stress as editing at this site exhibited the same pattern in Arabidopsis plants defective for VAC1, VARIGATED 2 (var2) and PDS (see chapter 2). In several plant species, the editing of *accD* is required for both functional acetyl-CoA carboxylate and development in tobacco and has been suggested to be a general housing keeping gene (Sasaki, Kozaki et al. 2001), (Kode, Mudd et al. 2005), (Lee, Jeong et al. 2004).

The subunit pet L plays a role in the assembly, stability and dimerization of the cytochrome b6f complex in tobacco (Schwenkert, Legen et al. 2007), (Schottler, Flugel et al. 2007). The chloroplast *clpP* transcript encodes the catalytic subunit of the plastid ClpP RS protease complex (Chateigner-Boutin, Ramos-Vega et al. 2008). Interestingly, reduced accumulation of regulatory subunit (clpR2) led to reduced accumulation of the clpPRS protease complex and a yellow phenotype (Chateigner-Boutin, Ramos-Vega et al. 2008). Moreover, an E/E' subclass PPR mutant, *chloroplast biogenesis* (*clb19)*, shows defective editing at *rpoA-200* and *clpP-559* (Chateigner-Boutin, Ramos-Vega et al. 2008), two sites that are defective in *ISE2*-silenced plants. Both clpP and accD are essential for tobacco development, illustrating their importance outside of the chloroplast (Kode, Mudd et al. 2005). Additionally, clpP has been implicated as paying a role in retrograde signaling (Ramundo, Casero et al. 2014). Although the editing sites within the *rpoA* and *clpP* transcripts are affected by the same PPR protein, they do not contain apparent conserved *cis* elements (Chateigner-Boutin, Ramos-Vega et al. 2008). This may indicate that unique multi-subunit ribonucleic acid complexes function in the editing of particular transcripts. Collectively, our results indicate that ISE2 may additionally function in distinct protein complexes with at least one PPR protein to target particular editing sites.

Importantly, editing at site *ndhD-2,* in which the first codon ACG is converted to an AUG, was recently found to be abolished in the *cia5-2* (impaired in chloroplast protein import), *ispF*, and *ispG (ispF and ispG* mutants are defective in plastid isoprenoid biosynthesis) albino mutants and in norflurazon-treated or lincomycin-treated seedlings (Tseng, Lee et al. 2013). Editing at sites *ndhB-148* and *ndhB-1255*, two sites unaffected by loss of ISE2, were also unaffected in these albino mutants. As *ndhD* encodes a subunit of the NDH complex, the observation that editing is abolished in the *ISE2*-silenced plants and in these albino mutants suggests that the NDH complex may not be functional in these particular mutants, and thus plants may be more susceptible to photooxidative damage

(Tseng, Lee et al. 2013). Photooxidative damage may be a secondary effect that occurs due to elevated ROS in bleached or chlorotic leaves (Tseng, Lee et al. 2013).

The observation that similar editing sites are affected by loss of ISE2 and in several other PPR mutants –some of which cause albino phenotypes-and that ISE2 physically interacts with a PPR protein collectively suggest that ISE2 is functionally linked to at least some PPR-mediated RNA editing events in *Arabidopsis*.

Defects in *Arabidopsis* plants with reduced ISE2 function affect about 50% of transcripts to some extent but about 10% are severely affected (statistically significant). Therefore, ISE2 may specifically interact with PPR proteins in unique protein complexes that are required for editing the respective transcripts. Alternatively, ISE2 may unwind some complex RNA structures to facilitate access of editing factors to some RNA editing sites. If an RNA helicase is directly involved in un-winding, it may associate with nucleic acid sequences within the RNA transcript. AtISE2 has been found to associate with several transcripts in Arabidopsis, a subset of which are edited (Bobik, McCray, 2016).

Differences in the Role of ISE2 in Editing Arabidopsis thaliana vs. Nicotiana benthamiana Transcripts

A comparison of the edited transcripts between *Arabidopsis thaliana* and *Nicotiana benthamiana* revealed that 21 of the 34 known edited sites are conserved *(atpF, ndhB1-7, ndhB9, ndhD1,2,3, ndhF, ndhG, psbE, rpl23, rpoA, rpoB1,2, and rps14 1,2)* (Tseng, Lee et al. 2013). In *Arabidopsis*, defects in ISE2 affect editing sites within transcripts *ndhB, ndhD, rpl23, rpoA and rpoB.* Although there is a non-statistically significant decrease in editing efficiency within these transcripts in *Arabidopsis* plants silenced for *ISE2*, there is a slight non-

statistically significant increase in editing efficiency in *Nicotiana* plants silenced for *ISE2* (compare Fig. 2.2 and Fig. 2.14). Additionally, the *N. benthamiana* transgenic plants that contain the Arabidopsis *ISE2* coding sequence driven by the 35S promoter, exhibit a reduction in editing efficiency relative to WT (data now shown). The reason for opposite effects of ISE2 on the chloroplast editing reactions in *Arabidopsis* and *N. benthamiana* is unclear but may be due to differences in the editing machinery of both plants. In *Arabidopsis*, *rpoA* editing is drastically decreased in *ISE2*-silenced leaves and this is the opposite trend observed in *N. benthamiana,* where a slight increase in *rpoA* editing efficiency is apparent in *ISE2*-silenced leaves. Additionally, there is a (non-statistically significant) increase in *ndhD* editing efficiency in *Arabidopsis ISE2-silenced* leaves but a decrease in *N. benthamiana ISE2* –silenced leaves. Therefore, although most ISE2-affected editing sites are conserved between *Arabidopsis* and *Nicotiana,* the mechanistic contribution of ISE2 is different at the conserved edited sites between the two species (the same is true for the ISE2-interacting PPR protein partner that did not appear to display any editing defects in *Arabidopsis* as it does in *N. benthamiana* for a subset of conserved sites (data not shown).

Overall, the observation of editing defects *Arabidopsis thaliana ISE2*-silenced leaves but not *Nicotiana benthamiana* leaves may indicate that the species differ in editing machinery and/or their requirement for ISE2-mediated editing may depend on other molecular factors.

The Role of ISE2 in Editing May be Exclusive to its Role in PD Regulation

Currently, it is still unclear whether or not ISE2's role in RNA editing may be related to its role in the regulation of PD structure and function. Although retrograde signaling has been postulated as one mechanism to regulate PD function and defects in retrograde signaling have been correlated with defects in

editing, the published defects in RNA editing have not been found to directly cause defects in retrograde signaling. Defects in chloroplast RNA metabolic processes such as RNA editing may be secondary defects of altered chloroplast function, supported by the observation that RNA editing defects co-occur with general stress responses in the plant (Zhu, Dugardeyn et al. 2013). However, mutations in chloroplast-localized PPR proteins were found to result in defective chloroplast function but not necessarily editing defects (Hammani, Takenaka et al. 2016).

We show here that ISE2 may function in an RNA metabolic role that results in RNA editing defects. However, **i**t seems likely that ISE2s role in RNA editing may be exclusive to its role in the regulation of PD function. However, we cannot exclude the possibility that the unique combinatorial RNA editing signature produced in *ISE*-silenced plants may lead to the alteration of PD function or that ISE2 may affect other chloroplast RNA metabolic events that somehow result in the alteration of PD function.

Interestingly, a mammalian DEAD box RNA helicase was found to coordinate transcription and ribosomal RNA processing by sensing the transcriptional status of RNA polymerase (Pol) I and II in human cells (Calo, Flynn et al. 2015). Whether or not ISE2 performs a similar function in the chloroplast by sensing the transcriptional activity of the plastid encoded polymerase and/or the nuclear encoded polymerase and coordinating transcription with other RNA processing events is unknown. Such a role may result in the initiation of a particular retrograde signal or ‘signature’ that reflects a particular metabolic state of the chloroplasts. How such a signal may affect PD regulation is currently unknown, but merits further investigation.

Our results put ISE2 upstream of chloroplast RNA editing events in *Nicotiana* and *Arabidopsis*. In *ISE2*-silenced leaves, defective editing is likely due to the inability

of a PPR protein to access particular unwound transcripts. Experiments have been conducted to identify the association sites of ISE2 within the transcripts that it is necessary to fully edit (Bobik, McCray et al. 2016) and such results are likely to differentiate between the transcripts that are direct targets of ISE2 and the ones that are defective in *ISE2*-silenced leaves as a result of secondary defects. The identification of ISE2 interacting partners that may perform a similar function in RNA editing or other RNA metabolic processes but that may not affect PD permeability may allow us to tease apart or separate specific RNA metabolic functions for ISE2 that may relate to its role in the regulation of PD function.

Biological Implications of RNA Editing Defects in *IPI1*-silenced Leaves

Function of Edited Transcripts affected in VIGS-IPI1 plants

We report here chloroplast RNA metabolic defects found in *N. benthamiana IPI1*-silenced leaves confirming its role in rRNA processing that was previously reported for its maize orthologue PPR103 and extending its role to the RNA editing of chloroplast transcripts. Interestingly, the terminal amino acid residues (DxW) are not found at the C-terminal end of the maize IPI1 orthologue, PPR103 (Fig. 2.9), where almost no editing events were disrupted in the mutant (Hammani, Takenaka et al. 2016). This observation may suggest that the specific DYW or a variation of the DYW amino acid residues may contribute to the editing reaction at several IPI1-targeted chloroplast editing sites in *Nicotiana bethamiana*.

As mentioned previously, between *Arabidopsis thaliana* and *Nicotiana benthamiana*, 21 of the 34 known edited sites are conserved (*atpF, ndhB1-7, ndhB9, ndhD1,2,3, ndhF, ndhG, psbE, rpl23,rpoA,rpoB1,2*, and *rps14 1,2*) (Tseng, Lee et al. 2013). Out of these sites, *atpF, ndhB1, ndhB4,ndhB6,ndhD2,3,*

ndhF, ndhG, psbE,rpl23,rpoA, rpoB1 and *rps14-2* are not conserved in the monocots: maize or rice because the genomic position of the corresponding edited site is already encoded and does not require editing. The only exception is *ndhB6* that is edited in rice but not maize (Tseng, Lee et al. 2013). It may be interestingly to compare the terminal amino acid motifs for all DYW-containing PPR proteins in monocots as compared to dicots. Perhaps the occurrence of less IPI1-mediated editing events in monocots may be correlated with its presence of a DYW domain in dicots, as compared to monocots.

In *Nicotiana benthamiana*, editing efficiency at sites *rpoA, ndhB-2, ndhB-3, ndhB-4, ndhB-5, ndhB-6, ndhB-7, ndhB-8, ndhD-1* and *ndhD-2* are affected in *IPI1*-silenced leaves vs. control leaves as revealed by Sanger sequencing. Editing efficiency differences between *IPI1*-silenced and control leaves at these sites are additionally confirmed using the RNA-seq method. For example, editing efficiency at the *rpoA*- editing site is increased in *IPI1*-silenced leaves as compared to the control using both the chromatogram and the RNA-seq methods (compare Fig.2.14 and Fig. 2.18). Additionally, editing efficiencies at *ndhB-2*, *ndhB-4* and *ndhD-1* sites is decreased using both the chromatogram and the RNA-seq methods (compare Fig.2.14 and Fig. 2.18). The RNA-seq method additionally revealed editing efficiency differences at sites *atpF*, *ndhA-1* and *ndhA-2* in *IPI1*-silenced leaves relative to the control.

The chromatogram method indicates that editing efficiency at the *rpoA, ndhB-3, ndhB-4, ndhD-1, atpF, ndhA-1, rpoB-1, rpoB-2* and *rpoB-3* sites are affected in *ISE2*-silenced mutants (see Fig. 2.6 and Fig. 2.14). Interestingly, editing efficiencies at the *rpoA, ndhB-4* and *ndhD-1* sites are all decreased in either *ISE2* or *IPI1*-silenced leaves. However, silencing of *PHYTOENE DESATURASE (PDS)* produces a similar result indicating that the editing defect seen at the *rpoA, ndhB-4* and *ndhD-1* sites are likely due to non-specific retrograde signaling defects (Fig. 2.14). These sites were originally chosen because they are

Table 3.1 RNA editing changes in *ISE2*-or *IPI1*-silenced plants

Site examined	VIGS-ISE2	VIGS-IPI1 (RNA-seq)	VIGS-IPI1
rpoA	**++++(++)**		**++++(++)**
rpoC1	**++++(+)**	**++++(+)**	**++++**
ndhB-1	**++++(+)**	**++++**	**++++**
ndhB-2	**++++(+)**	**++++**	**+++**
ndhB-3	**++++(++)**	**+++**	**+++**
ndhB-4	**++**	**++**	**+**
ndhB-5	**++++**	**+++**	**+++**
ndhB-6	**++++**	**+++**	**+++**
ndhB-7	**++++(++)**	**+++**	**++**
ndhB-8	**++++(++)**	**+++**	**+++**
ndhB-9	**++++(+)**	**+++**	**++++**
ndhD-1	**++**	**++**	**++**
ndhD-2	**++++**	**++++**	**+++**
atpA-1	**++++**	**++++**	
atpA-2	**++**	**++**	
atpF	**++++**	**++++**	
ndhA-1	**++**	**+++**	
ndhA-2	**+++**	**++++**	

Table 3.1 cont.

Site examined	*VIGS-ISE2*	*VIGS-IPI1 (RNA-seq)*	*VIGS-IPI1*
ndhF	**++++**	**+++**	
petB	**++++**		
psbE	**++++**	**++++**	
psbL	**++++**	**++++**	
rpl20	**++++**	**+++**	
rpoB-1	**++++(+)**	**++++**	
rpoB-2	**++++(+)**	**++++**	
rpoB-3	**++++(+)**	**++++**	
rpoB-4	**++++(+)**	**++++**	
rpoC2	**++++**	**+++**	
rps14-1	**++++**	**+++**	
rps14-2	**++++**	**+++**	
rps2	**++++**	**++++**	

Table Legend:
Symbols represent the following degrees of editing efficiency relative to control samples: + 0.25 or less; ++ 0.26 – 0.5; +++ 0.5 to 0.75; ++++ 0. 76 – 1.05; ++++(+) 1.06 – 1.25

examples of editing sites that are conserved between Arabidopsis and *N. tabacum* (Tseng, Lee et al. 2013). Indeed defects in tetrapyrrole metabolism or other key biosynthetic pathways were found to result in defects in editing, likely involving retrograde signaling (Zhang, Tang et al. 2014), (Tseng, Sung et al. 2010), (Dong, Deng et al. 2007). Nonetheless, there are unique signatures of editing defects observed in *IPI1*- as compared to *ISE2*-silenced leaves that are likely not due to general stress. For example, *ndhB2* and *ndhB8* are uniquely affected in *IPI1*-silenced leaves and *ndhB3* is uniquely affected in *ISE2*-silenced leaves. The sites that are affected by loss of IPI1 exhibit a reduction in editing efficiency, while these same sites exhibit a slight (statistically insignificant) increase in editing efficiency in *ISE2*-silenced leaves. In fact, a general trend for editing defects is an increase observed in *ISE2*-silenced leaves and a decrease observed in *IPI1*-silenced-leaves, suggesting that ISE2 and IPI1 perform antagonistic roles with respect to the editing of *N. benthamiana* chloroplast transcripts (Table 2.6). This observation is interesting as ISE2 and IPI1 interact via their C-terminal regions and the C-terminal region of IPI1 contains the predicted sequence that is likely to be necessary for the editing reaction. These results indicate that ISE2 may normally function to inhibit IPI1-mediated editing events at certain transcripts.

IPI1 Does Not Affect Plasmodesmata Function

ISE2 and IPI1 likely play antagonistic roles with respect to RNA editing. However, unlike ISE2, IPI1 does not significantly affect PD permeability. These results suggest that ISE2s role in RNA editing may not affect its role in PD regulation.

Although identified in our lab as an interacting partner of ISE2, no defect in intercellular trafficking of 2xGFP was observed in *IPI1*-silenced plants. None the less, our study sheds light on the unique roles of chloroplast-localized proteins that are involved in either (i) both RNA processing and intercellular trafficking or

(ii) solely RNA processing. Future studies include the further dissection of these functionalities to identify shared roles that are unique to those groups that affect intercellular trafficking. Such examinations will eventually clarify the roles that specific RNA processing events may have on the regulation of intercellular trafficking.

Although the results presented here demonstrate that general defects in RNA editing are not sufficient to affect PD function, we cannot rule out that 'unique signatures' of editing defects seen in particular mutants may somehow affect PD function. To address this question, mutants that produce the same 'editing signature' that ISE2 produces would have to be examined for defects in intercellular trafficking. Another chloroplast-derived signaling event that leads to defects in intercellular trafficking is increased ROS production and the inability to edit the NDH subunits may be a source of ROS generation. It would be interesting to measure the levels of ROS in PPR silenced tissue. If there are elevated levels of ROS, this may indicated that elevated ROS production alone is not sufficient to regulate PD permeability. In such a scenario, we still cannot rule out that a certain structural type of and level of ROS potentially in combination with other produced 'unique signatures' together form a complex signaling event that eventually is perceived by an event/molecular factor that affects PD permeability. RNA editing of *ndhB* is also modulated in wild type Arabidopsis plants that are challenged with pathogens and this RNA editing event results in enhanced pathogen resistance (García-Andrade, Ramírez et al. 2013). As NDH subunits are involved in cyclic electron flow around PSI, perhaps the editing of NDH subunits in response to pathogens enhances the ability of plants to produce ATP but not sugar. This defect precedes callose deposition (García-Andrade, Ramírez et al. 2013). Thus, a unique pattern of editing defects within the *ndhB* transcript may correlate with PD-mediated callose deposition in response to pathogen attack. Interestingly, the predominate editing change within the *ndhB* and *ndhD* transcript changes the coding amino acid from an S to an L. Might this

editing event be a way to change the phosphorylation status or activity of the ndhB and ndhD proteins in response to abiotic stress? The answer to this question awaits further investigation.

Biological Significance of ISE2 and IPI1 Protein Interaction

IPI1 and ISE2 are found to interact through their C-terminal regions. While the exact function of the C-terminal region of ISE2 is unknown, the C-terminal region of IPI1 displays sequence similarity to cytidine deaminases which are known to function in RNA editing and to other known editing factors in plants that have been found to bind zinc and contribute to the editing reaction (Boussardon, Avon et al. 2014). The interaction of ISE2 with the C-terminal region of IPI1 indicates that ISE2 may regulate the function of IPI1 with respect to RNA editing, suggesting antagonistic functions. Indeed, comparison of the complete ISE2 editing signature in *ISE2*-silenced plants and the editing signature in *IPI1*-silenced plants reveal an opposite effect on editing; ISE2 seems to perform a slight inhibitory role while IPI1 perform a promotive role at certain edited sites (Table 3.1, Fig. 2.14). Interestingly, the sites that are similarly affected in *ISE2*-silenced or in *IPI1*-silenced leaves (exhibiting a reduction in both respective mutants) are also affected in same-aged leaves that are silenced for *PDS* (Fig. 2.14). These results indicate that defective editing at these sites may be due to a general stress response of the plant. Additionally, IPI1 and ISE2 do not appear to share a role in the regulation of intercellular trafficking (see chapter 2). Perhaps ISE2 and IPI1 interact in an antagonist manner in a chloroplast complex that is involved in RNA editing; this interaction may not affect PD permeability. Or alternatively, the unique pattern of defective editing sites-or ‘signatures’-produced in both mutants may differently signal to downstream events. Consistent with this idea, as mentioned previously, reports have suggested that specific editing events with the *ndhB* transcript (*ndhB-2, ndhB-3,ndhB-4,* and

ndhB-6) are edited rapidly in response to pathogen attack and prior to callose deposition and are thought to modulate cyclic electron flow around PSI in response to pathogen attack (García-Andrade, Ramirez et al. 2013). Thus, we cannot rule out the possibility that the unique defective pattern of RNA editing events seen in *ISE2*-silenced leaves may contribute to its role in the regulation of PD permeability. Further research may address this hypothesis.

Transcriptome Wide Profiling of Chloroplast Localized Factors

Our assumption is that ISE2-mediated RNA processing events are connected to the ISE2-mediated regulation of PD function. In order to comprehensively identify RNA processing events that are likely to/not to contribute to the regulation of PD permeability, the global examination of RNA processing events need to be compared to the RNA metabolic processes that are affected in *ise2* mutants. We present an approach to examine the global consequences of defective PPR gene function on chloroplast RNA metabolism.

Overall, our pipeline has allowed us to confirm RNA editing defects, identify new edited sites and examine IPI1's global role on chloroplast RNA metabolism in *N.benthamiana*. Similar examinations coupled to examination of intercellular trafficking in other mutants of ISE2-interacting partners will allow us to clarify roles for specific 'signatures' that are produced from RNA metabolic events that are also correlated with PD meditate intercellular trafficking.

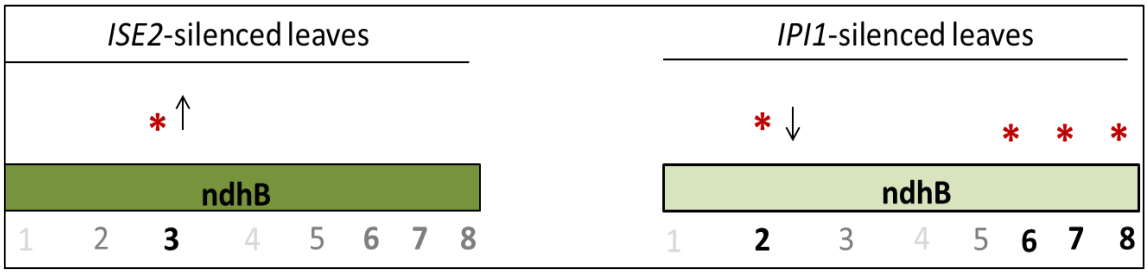


Figure 3.1. Summary of *ndhB* edited sites that are affected in *ISE2* or *IPI1*-silenced leaves.

Red astericks and bold numbering indicate editing sites that are uniquely affected in each mutant (i.e not affected in the other mutant and not affects in TRV-*PDS* leaves). Arrows indicated whether editing is increased (pointing up) or decreased (pointing down) in the respective mutant. ndhB-1 and ndhB-4 (light grey) are sites that are affected in ISE2, IPI1, and PDS-silenced plants and therefore are likely involved in a general stress signaling pathway.

CHAPTER FOUR MATERIALS AND METHODS

Materials

Plant Materials and Molecular Constructs

The Columbia (Col-0) accession, D3Y and D3G plants are published in (Bobik, McCray 2016). Virus Induced Gene Silencing (VIGS) constructs for PYC1 and ISE2 are described in (Burch-Smith, 2010). The VIGS-IPI1 construct was made by cloning a unique C-terminal fragment of IPI1 into a silencing vector. The plant species and lines together with their functions are indicated in Table 4.1.

Agrobacteria and Yeast Strains

Agrobacterium strains

The *Agrobacterium tumefaciens* GV3101 (pMP90RK) strain was used to house VIGS constructs that were used for gene silencing and transient expression in *N. benthamiana*. This strain has the C58 chromosomal background with the rifampicin-resistance marker gene and the pMP90RK (pTiC58DT-DNA) Ti plasmid with the gentamicin and kanamaycin-resistance marker genes. The associated opine is nopaline. Strains were obtained from Dr. Patricia Zambryski at UC Berkeley.

Saccharomyces cerevisiae strains

pGBKT7 and pGADT7 (plasmids containing the GAL4-DNA binding and transcriptional activation domain, respectively) PPR constructs were transformed

Table 4.1 Plants used in this dissertation

Plant Lines	Function
Arabidopsis thaliana Col-0	Wild-type control to compare editing defects.
Arabidopsis thaliana D3G Stable transgenic lines	ISE2 complementation line where *ISE2* is overexpressed in an *ise2* homozygous mutant background. These plants were used to ensure that editing efficiencies are restored in complemented plants to levels that are seen in wild-type.
Arabidopsis thaliana D3Y	Plants exhibiting reduced *ISE2* expression due to cosuppression of *ISE2* in D3G plants. These plants were used to test for editing defects that are seen in plants with reduced levels of *ISE2* expression.
Nicotiana benthamiana VIGS-PYC1	Virus Induced Gene Silencing (VIGS) of *N. benthamiana* plants was conducted with the empty vector control, pYC1.
Nicotiana benthamiana VIGS-*ISE2*	VIGS of *N. benthamiana* plants was conducted with a specific fragment against *ISE2.*
Nicotiana benthamiana VIGS-*IPI1*	VIGS of *N. benthamiana* plants was conducted with a specific C-terminal fragment against *IPI1.*
Nicotiana benthamiana 35S::*AtIPI1-Myc* Transient expression	Wild-type *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* containing the full-length *Arabidopsis IPI1* sequence with a C-terminal *Myc* tag under the control of the Cauliflower mosaic virus (CaMV) 35S (overexpressing) promoter. These plants were used to verify IPI1 protein expression in Nb leaves.
Nicotiana benthamiana 35S::AtIPI1-YFP Transient expression	Wild-type *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* containing the full length *Arabidopsis IPI1* sequence with a C-terminal *YFP* under the control of the CaMV 35S (constitutive) promoter. These plants were used for localization and western blot experiments.
Nicotiana benthamiana 35S::*AtIPI1_TP-YFP* Transient expression	Wild-type *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* containing the Arabidopsis IPI1 transit peptide, coding sequence for ~20 amino acids after the transit peptide, with a C-terminal tag under the control of the CaMV 35S promoter.
Nicotiana benthamiana 35S::*NbIPI1_TP-YFP* Transient expression	Wild-type *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* containing the Nicotiana IPI1 transit peptide coding sequence followed by a coding sequence for ~20 amino acids after the transit peptide, with a C-terminal tag under the control of the CaMV 35S promoter.

into yeast strains Y2HGold (pGBKT7) and Y187 (pGADT7). Yeast strains are described in Table 4.2 (adopted from the Match Maker Gold Yeast Two Hybrid System Manual (Clontech, U.S.A)), Aholt, unpublished, James et al., 1996, Nguyen, unpublished). Y2H strains housing ISE2 Y2H constructs are published in Bobik, McCray 2016).

Table 4.2 Yeast host strain genotypes

Strain	Genotype	Reporters	Transformation Marker
Y2HGold	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$–$Gal1_{TATA}$–His3, $GAL2_{UAS}$–$Gal2_{TATA}$–Ade2 URA3 : : $MEL1_{UAS}$–$Mel1_{TATA}$ AUR1-C MEL1	HIS3, ADE2, MEL1	trp1, leu2
Y187	MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3 : : $GAL1_{UAS}$–$Gal1_{TATA}$–LacZ, MEL1	MEL1, LacZ	trp1, leu2

Vectors

The following vectors listed in Table 4.3 were previously generated or generated in this study.

Table 4.3 Vectors used in this study

Vector	Purpose
pDONR 221	Gateway™ Entry cloning vector to clone PCR products of the C-terminal *IPI1* region for VIGS, or full length and truncations of *IPI1* for transient expression. The cloning is achieved via a recombination mechanism (Invitrogen). pDONR221 has a Zeomycin^r and Kanamycin^r (50 µg/ml) in *E coli.*
VIGS	
pRMW102	Contains a cDNA fragment used to silence the C-terminal portion of *IPI1* in the PYC1 backbone plasmid for VIGs.
	Transient Expression
pGWB417	Gateway™ Destination cloning vector used to generate the expression cassette 35S::*IPI1*-4xMyc. Spectinomycin^r (50µg/ml) in *E coli.* Used to detect IPI1 protein expression via western blot. The input gateway cassette is P35S-attR1-Cmr-ccdB-attR2-4xMyc-TNOS (accession number: AB294441).
pGWB441	Gateway™ Destination cloning vector used to generate the expression cassette 35S::IPI1-EYFP. Spectinomycin^r (50µg/ml) in *E coli.* Used to detect IPI1 (full-length and transit peptide) localization via confocal microscopy and IPI1 protein expression via western blot in the same leaf tissue that was used for confocal microscopy. The input gateway cassette is P35S-attR1-Cmr-ccdB-attR2-EYFP-TNOS (accession number: AB294457).
pAN569	Binary expression vector from Dr. Andreas Nebenführ. 35S::YFP. Kanamycin (25 µg/ml)
	Yeast 2 Hybrid
pGADT7	Vector used for Yeast Two-Hybrid analysis. Full-length and C-terminal fragment of *IPI1* were cloned into pGADT7 vectors to assay for an interaction with ISE2. pGADT7 contains the GAL4-activation domain (Clontech™). Plasmid confers resistance to ampicillin (100 µg/ml) in E. coli. Plasmid contains the gene encoding leucine biosynthesis used for selection in *S.cerevisiae*.
pGBKT7	Vector used for Yeast Two-Hybrid analysis. Full-length and C-terminal fragment of ISE2 were cloned into pGADT7 vectors (as described in Bobik et al., 2016). pGADT7 contains the GAL4-binding domain (Clontech™). Plasmid confers resistance to kanamycin (50 µg/ml) in E. coli. Plasmid contains the gene encoding for Tryptophan used for selection in *S. cerevisiae*.
pGBKT7-53	Binding Domain control plasmid. pGBKT7-53 is a positive control plasmid that encodes a fusion of the murine p53 protein (a.a. 72–390) and the GAL4 DNA-BD (a.a. 1–147) (Clontech™).
pGADT7-T	Activating Domain control plasmid. pGADT7-T is a positive control plasmid that encodes a fusion of the SV40 large T antigen (a.a. 87–708) and the GAL4 AD (a.a. 768–881) (Clontech™).

Oligonucleotides

The following primers listed in Table 4.4 were ordered from Invitrogen (Carlsbad, CA) for RNA editing, qPCR and Northern blot experiments for *Nicotiana benthamina* transcripts used in this study. The primers arrived lyophilized and therefore were resuspended in ddH2O to a 100µM final concentration. Working solutions were used at 10 µM. Primers for *Arabidopsis* RNA editing experiments were already published in (Tseng et al., 2013).

Table 4.4 Oligonucleotides used in this study

Edited Site	**Oligo Sequence (5' -> 3')**	**Purpose**
atpA 1,2	TGGCCCAGGTCGTAACTACT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *atpA* 1,2 editing sites
	CGTGAGAGGAGCTGATTGGG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *atpA* 1,2 editing sites
atpF	ATCGCGAAATGCTATGGTTCTT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *atpF* editing sites
	TTCGTTTCTTTGGGCCACTG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *atpF* editing sites

Table 4.4 cont.

Edited Site	Oligo Sequence (5' -> 3')	Purpose
ndhA1	TGGACTTTACCGAGGCTGAG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhA* 1 editing sites
	CTGGCGGCTCGTATTGTTTG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhA* 1 editing sites
ndhA2	GAAAATTCGCCCACCAGGTT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhA* 2 editing sites
	CTGCCCGTAGACCACCTAAA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhA* 2 editing sites
ndhB1	TCTCCCCCGGATGAACCATA	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*1 editing site
	TAGTGGATGCTGCCAAAGGG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*1 editing site
ndhB 2-6	GATTCGTCGTTCCTGACCCT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*2-6 editing sites
	AGGGGGAATGTTTTTATGCGG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*2-6 editing sites

Table 4.4 cont.

Edited Site	Oligo Sequence (5' -> 3')	Purpose
ndhB 7-9	TTCCCATTTTGGGCGGAACA	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*7-9 editing sites
	CACTTAGGAGCCGTGTGAGA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhB*7-9 editing sites
ndhD 1,2	ACAGACGTTTCTTTCCTCCCC	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhD* 1,2 editing sites
	AGATGTGAATCCGCCTGTCC	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhD* 1,2 editing sites
ndhF	TTCGCCGTATGTGGGCTTTT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *ndhF* editing site
	CGCAACAGGTCGTGTAAACC	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *ndhF* editing site
petB	GGTGTCCCTGACGCTATTCC	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *petB* editing site
	TGTATAGGGCTTACACGGCG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *petB* editing sites

Table 4.4 cont.

Edited Site	Oligo Sequence (5' -> 3')	Purpose
psbE	AAAGACGCCCTCGGTACAAT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *psbE* editing site
	TAGGTACAGCTAGGCCGTGAA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *psbE* editing sites
psbL	ACAGTACGATGGTTGGCTGT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *psbL* editing site
	TTACCCCACTTCCCTCCAGA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *psbL* editing site
rpl20	CGTCGTTTGTGGATCACTCG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpl20* editing site
	ACCTTCCCGGAGTTCGTTCT	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpl20* editing site
rpoA	CCTTTGGTTGGGCATTGGTG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpoA* editing site
	CCTGTTCGAAACGCGAATCA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpoA* editing site

Table 4.4 cont.

Edited Site	Oligo Sequence (5' -> 3')	Purpose
rpoB-1,2,3	CGGGGATGGAAATGAGGGAA	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpoB*1-3 editing site
	CGGATCGCCACCTACACAAG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpoB*1-3 editing sites
rpoB-4	TTGGTGGCGAACTTGCTTTG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpoB*4 editing site
	ACTTTTTCAGGGCCTTGGCT	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpoB*4 editing site
rpoC1	TCCACAAGCACAAATTCCGC	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpoC1* editing site
	AGAAGGGTTTGGGGTTGCTC	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpoC1* editing site
rpoC2	AAATGGACCGCCCCTCAAAT	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rpoC2* editing site
	AACCAATCGATACGACCCCG	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rpoC2* editing site

Table 4.4 cont.

Edited Site	Oligo Sequence (5' -> 3')	Purpose
rps2	TTTGACGCAGCAAGTAGGGG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rps2* editing site
	TTCGGGAGACGGTTGAGTCT	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rps2* editing site
rps14-1,2	TTTCTTTCGACGGAGAGGGG	Forward primer to amplify *N. benthamiana* chloroplast transcript containing *rps14* 1,2 editing sites
	AACGTCGATGAAGGCGTGTA	Reverse primer to amplify *N. benthamiana* chloroplast transcript containing *rps14* 1,2 editing sites
AtIPI1 TP Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTatgtccaccgttaatcatcactg	attB Forward primer to amplify the AtIPI1 transit peptide plus codons for ~ 60 nucleotides of the *IPI1* coding sequence to be used for localization experiments
	GGGGACCACTTTGTACAAGAAAGCTGGGTG CCCATCAATAACAGATTCAATGTCTTCCG	attB Reverse primer to amplify the AtIPI1 transit peptide plus codons for ~ 60 nucleotides of the *IPI1* coding sequence to be used for localization experiments

Table 4.4 cont.

Edited Site	**Oligo Sequence (5' -> 3')**	**Purpose**
Nb IPI1 TP Gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGCCGTTATCCACAGCCCC	attB Forward primer to amplify the *N. benthamiana* transit peptide plus codons for ~ 60 nucleotides of the *IPI1* coding sequence to be used for localization experiments
	GGGGACCACTTTGTACAAGAAAGCTGGGTG ATCACCACACCGAACAGAAATACG	attB Reverse primer to amplify the *N. benthamiana* transit peptide plus codons for ~ 60 nucleotides of the *IPI1* coding sequence to be used for localization experiments
qPCR Nb *IPI1*	TGATCGTATTGAGACGGCAT	qPCR *NbIPI1* Forward Primer
	CAAGTGGCAGCTGATGAAAT	qPCR *NbIPI1*Reverse Primer
Northern 4.5S	GAAGGTCACGGCGAGACGAGC	5' *rrn4.5S* Based on chloroplast sequence NC_000932
	GTTCAAGTCTACCGGTCTGTTAGGATG	3' *rrn4.5S* Based on chloroplast sequence NC_000932
Northern 5S	TATTCTGGTGTCCTAGGCGTAGAGG	forward for *rrn5S* Based on chloroplast sequence NC_000932
	ATCCTGGCGTCGAGCTATTTTT	reverse for *rrn5S* Based on chloroplast sequence NC_000932

Table 4.4 cont.

Edited Site	Oligo Sequence (5’ -> 3’)	Purpose
Northern 23S	GAAACTAAGTGGAGGTCCGAACCGAC	5' for *rrn23S* IRB23F from Heinz, works with *Arabidopsis* and tobacco.
	CGCTACCTTAGGACCGTTATAGTTAC	5' for *rrn23S* IRB23R from Heinz, works with Arabidopsis and tobacco.
Northern 16S	ACG GGT GAG TAA CGC GTA AG	5' for *rrn16S* 16S-F from Heinz
	TGAGTTTCATTCTTGCGAACGTACTC	3' for *rrn16S* IRB18R from Heinz

Methods

General Methods

Plant Material and Growth Conditions

Nicotiana benthamiana seedlings were grown on a light cart at 25^{o}C under fluorescent white light in a 16:8 hr light/dark cycle. Around 1 week old seedlings were transplanted to individual pots and typically silenced around 2 weeks of age.

Arabidopsis thaliana seeds were surface-sterilized and imbibed at 4 degrees in the dark for at least two days. Vernalized seeds were sown to 0.25x MS plates (1.075g MS, 10g sucrose, pH 6.0 with KOH, 3.4g phytagel up to 1 L) and put in a 16hr light/8hr dark growth chamber (long day) for about 14 days. Around 2 week old seedlings were transferred to individual soil containers and subsequently transferred to long day plant growth chambers.

Molecular Biology Methods

Virus Induced Gene Silencing

Cloning for VIGS of *IPI1* was achieved by targeting a unique C-terminal region within the *IPI1* transcript. VIGS of *IPI1*, *ISE2*, *GUS* intron (negative control) and *PDS* (positive control) were performed according to Liu et al., 2008. Approximately 3 week old *N. benthamiana* plants were infiltrated with the respective constructs and then grown under standard growth conditions.

RNA Editing

RNA Editing in Arabidopsis thaliana

Total RNA was isolated from mature leaf tissue in Col-0 and *ise2* cosuppressed plants using a Plant RNeasy Mini Kit (QIAGEN) and reverse transcribed using

Invitrogen SuperScript III Reverse Transcriptase (Life Technologies) and random hexamers. Primers used for cDNA synthesis designed to amplify chloroplast genes, which have previously been shown to undergo editing, were previously published (Tseng et al., 2013).

RNA Editing in Nicotiana benthamiana

For editing in the non-silenced control, *ISE2*-, *IPI1*- or *PDS*-silenced leaves, RNA was isolated from leaf number 11 of approximately six-week-old plants using Trizol (Invitrogen™). RNA concentration and quality values were measured with a NanopDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE). The RNA was treated with DNase at least once with the DNA-free kit™ according to manufacturer's protocol (Ambion ®). RT-PCR was conducted according to manufacturer's instructions in the Promega M-MLV RT protocol manual using random primer hexamers. A typical reaction included 1 µg RNA, 1.2 µl random hexamer (60 uM)and 0.8 µl reverse transcriptase (200 units/µl). The same reaction without the reverse transcriptase was performed in parallel with the experimental cDNA synthesis reaction to ensure the absence of genomic DNA contamination. Second-strand PCR synthesis was performed according to standard Taq polymerase protocol using primers in Table 4.4. PCR was conducted with an annealing temperature of 53 °C for almost all primer pairs. PCR products were gel purified using the Gel Extraction Wizard Promega kit. Purified amplicons were sequenced by the UTK sequencing center.

For examination of editing in 4 week old WT *N. benthamiana* plants, plants were grown under standard growth conditions. Older, middle-aged and younger leaves (leaf number 5, 7 and 9) were snap frozen in liquid nitrogen at 4 weeks of age. RNA was isolated using Trizol and RT-PCR was conducted using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega) according to manufacturer's instructions. PCR was conducted on cDNA to amplify specific transcripts containing editing sites of interest. PCR products were gel purified

using Promega Wizard gel purification kit, diluted to about 5ng per 100 bp and sent to sequencing. Three individual biological replicates were analyzed. The same cDNA used for editing experiments was also used to assay *NbIPI1* expression using qPCR.

Biochemistry Methods

Western Blot

Approximately 6 week old Nb plants were infiltrated with agrobacterium containing either 35S:IPI1-Myc or 35S:IPI1-YFP (the same tissue used for confocal visualization of full length IPI1-YFP). About 65 hours after infiltration, infiltrated leaf tissue was snap-frozen in liquid nitrogen and stored at -80°C until protein was isolated from the tissue. Total protein was isolated from the snap-frozen tissue, run on a 10% SDS-PAGE, and the gel was then transferred to a Nylon membrane for 2 hours at 8V. The membrane was subsequently blocked with 5% powered milk for 1 hour. Western blot was performed about using the primary antibody anti-GFP or anti-myc (mouse) at a 1:1000 concentration. The secondary antibody mouse IgG (rabbit host) was used at 1:10,000. The blot was developed using Chemiluminescent Western Blot Detection kit Supersignal with the west data extended duration substrate (Thermo fisher Scientific).

Northern Blot

About 1 microgram of total RNA was run on a denaturing formaldehyde gel, transferred to positively charged nylon membranes (Roche), and hybridized with DIG-labeled 23S rRNA, 16S rRNA, 5S rRNA and 4.5S rRNA probes (PCR DIG Probe Synthesis Kit, Roche) according to manufacturer's instructions. Bands corresponding to ribosomal RNA species were detected using the DIG High

Prime DNA Labeling and Detection Starter Kit II (Roche). The same RNA that was used for the RNA editing experiments was used for the Northern Blot.

Chloroplast Isolation for RNA-seq

Chloroplasts were extracted according to “Extraction of Chloroplast Proteins from Transiently Transformed *Nicotiana benthamiana* Leaves” bio protocol (http://www.bio-protocol.org/e1238). Briefly, fresh leaf tissue was ground, filtered and centrifuged through a Percoll gradient, and chloroplasts were visualized on an inverted microscope. Chloroplasts were then shock-frozen and total RNA was isolated from purified chloroplasts using the Trizol method or the Qiagen RNeasy method as per manufacturer’s instructions. For each plant, approximately 100 mg of tissue was ground from each leaf to isolate chloroplast RNA. Leaves from individual plants were pooled. Removal of cp DNA was done by treating the samples with DNase (Ambion). Because rRNA typically constitutes over 75% of total RNA and its depletion can results in very low yields of RNA for cDNA preparation, a rRNA depletion was not performed. The RNA integrity of the isolated RNA was examined on a Bioanalyzer machine and quantitated on a NanoDrop 1000 spectrophotometer prior to library preparation. For cDNA synthesis, about one microgram of non rRNA-depleted RNA was used to make double strand cDNA (ds-cDNA) and dsDNA was produced using the Invitrogen SuperScript II Double Stranded cDNA Synthesis kit with random hexamers instead of oligo (dT) primers for first-strand synthesis. The cleaned ds-cDNA was then used to construct a library using the Illumina Next Tera Library prep kit with no adaptations (Illumina, Inc). After examination of the library quality using the bioanalyzer, multiplexed libraries were sequenced using the Illumina Miseq sequencing platform at the UTK Genomics Core Facility (Knoxville, TN) according to standard Miseq run parameters (Illumina protocol manuals).

Cellular Biology Methods

Confocal Microscopy

Confocal fluorescence microscopy was performed using a Leica SP2 confocal laser scanning microscope (Leica Microsystems Heidelberg GmbH) equipped with a high-resolution camera, a beam slitter and differential interference contrast (Nomarski) optics. A 40x or 63x HCX PL APO objective was used for image acquisition. The samples were excited with an excitation line of 458/514 nm for YFP and 488/543 for GFP. The numerical aperture of the objective was 1.32.

Transmission Electron Microscopy (TEM)

TEM was performed as referenced in (Burch-Smith, 2010, Bobik et al., 2014). Briefly young leaf control and *IPI1*-silenced samples were fixed by high-pressure freezing (HPF) and quick freeze substitution (QFS). Subsequently, samples were embedded in epoxy resin, sliced into thin sections, and visualized on a Libra 200M TEM/STEM (Zeiss) at 200 kilo Volts.

Chlorophyll a Fluorescence

Chlorophyll a fluorescence, F, was measured with an Os-30p hand held device (Opti-Sciences, Inc.) under kinetics mode with a 5 second flash and with Flurocam (Photo Systems Instruments) on approx. 5 week old plants. The readings were used to calculate Fv/Fm, the ratio of variable to maximal fluorescence.

Yeast Two Hybrid

A series of fragments of the ISE2 coding sequence or the IPI1 coding sequence were cloned into both PGKBT7 (AD; Gal 4 activating domain) and pGADT7 (BD; Gal4 binding domain) vectors. Full length and C-terminal constructs were transformed into yeast strain Y187 using LiCl transformation and subsequently

plated on Sc –Leu plates. Yeast Two Hybrid mating assay was performed according to manufactures instructions in the Match Maker Y2H protocol (Clontech). Briefly, the bait and prey plasmids were incubated for 20-24 hrs at 30 degrees until yeast culture Optical Density (O.D) reached around 0.85.

One to ten (1:10), 1:100, and 1:1000 dilutions were plated on single Trp and Leu drop out plates, Trp/Leu double drop out (DDO) plates, Trp/Leu double drop out (DDO) plates supplemented with x-alpha gal and the antibiotic Aureobasidin, Trp/Leu/His triple drop out plates (TDO), and Trp/Leu/His/Ade quadruple knock out plates supplemented with x-alpha gal.

Computational Methods

Mapping and Data Statistical Analysis

Sequence data generated from the Miseq platform were examined for sequence quality, trimmed using trimmomatic software or the base space graphical user app (Base Space, Illumina, Inc) and mapped to the *Nicotiana benthamiana* genome using DNA Array Star Next Gen Seq software (version 11) permitting more than 8 mismatches in order to detect multiple editing events (SNPs) within the same transcript. Mapped contigs were visualized in Seq Man Pro software or the Integrative Genomics Viewer (IGV) software.

Sequence Alignments

IPI1 orthologue sequences for *Arabidopsis thaliana* (UniProtKB Q9ffN1, GenPept Accession 79506598, sequence ID NP 196000.2), *Zea mays* (Accession 670380462), *Nicotiana benthamiana* (XP 008670964) and *Oryza sativa* (XP 015645871) were obtained from NCBI blastp using default parameters. Resulting fasta sequences were aligned in Meg align pro using Muscle alignment.

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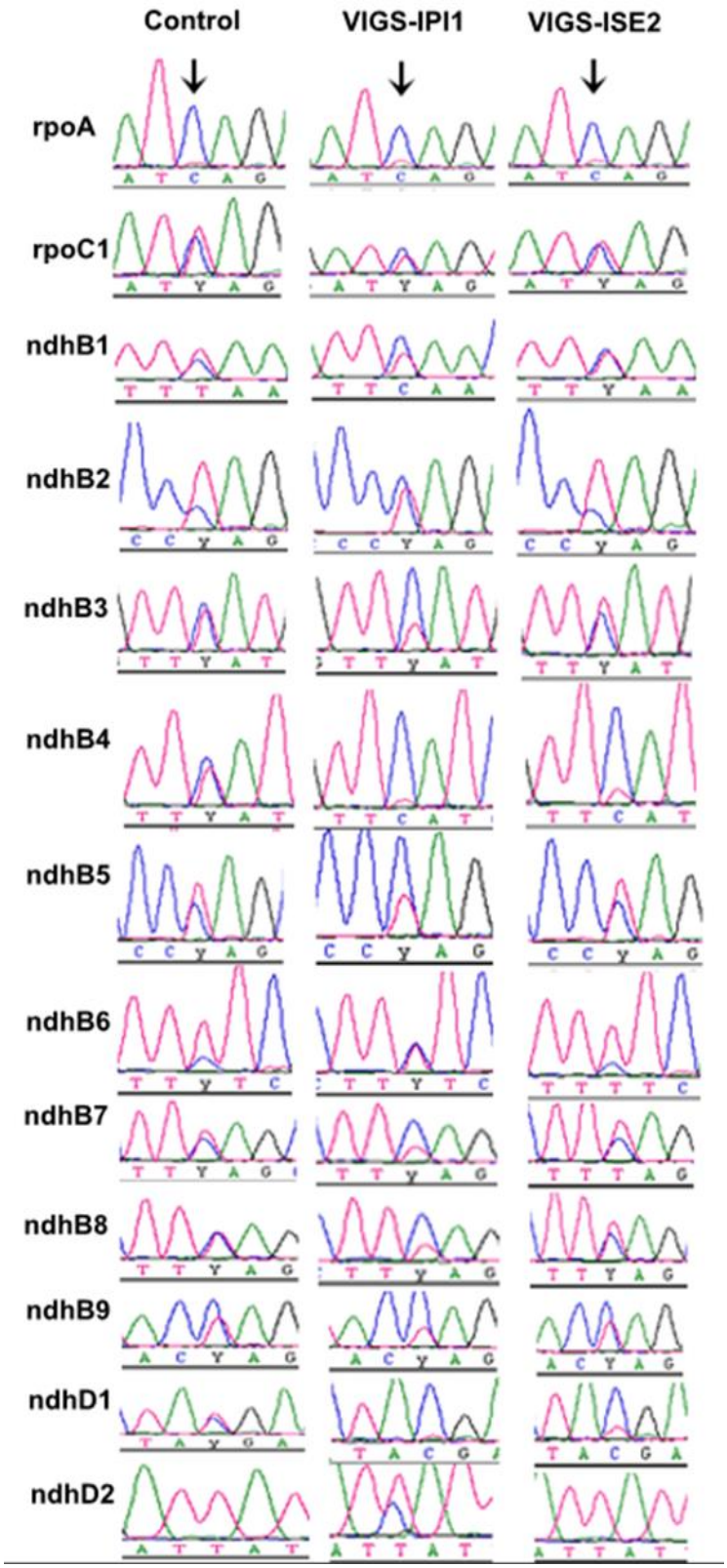
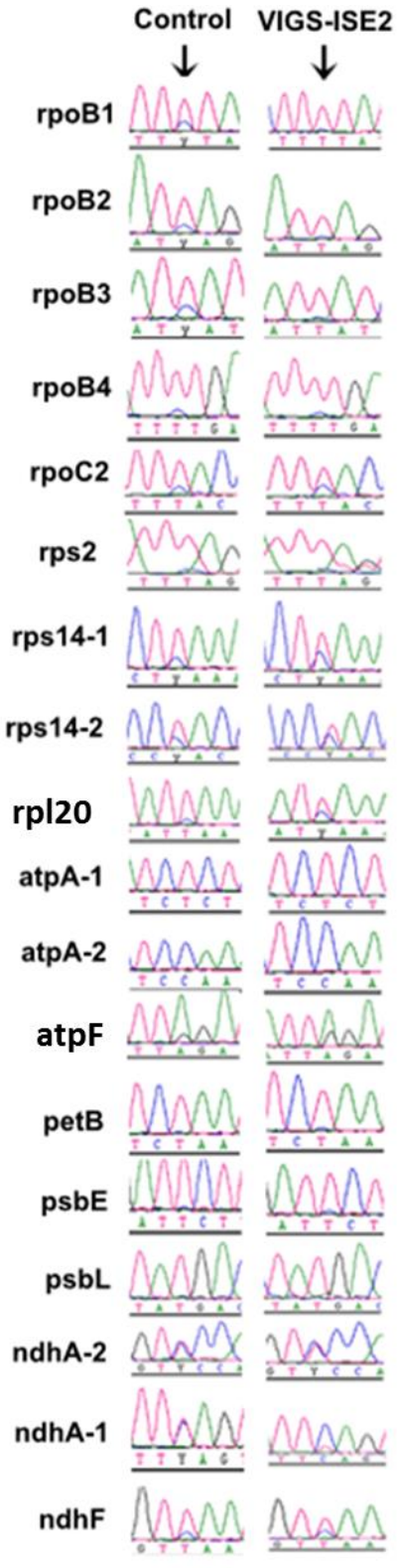
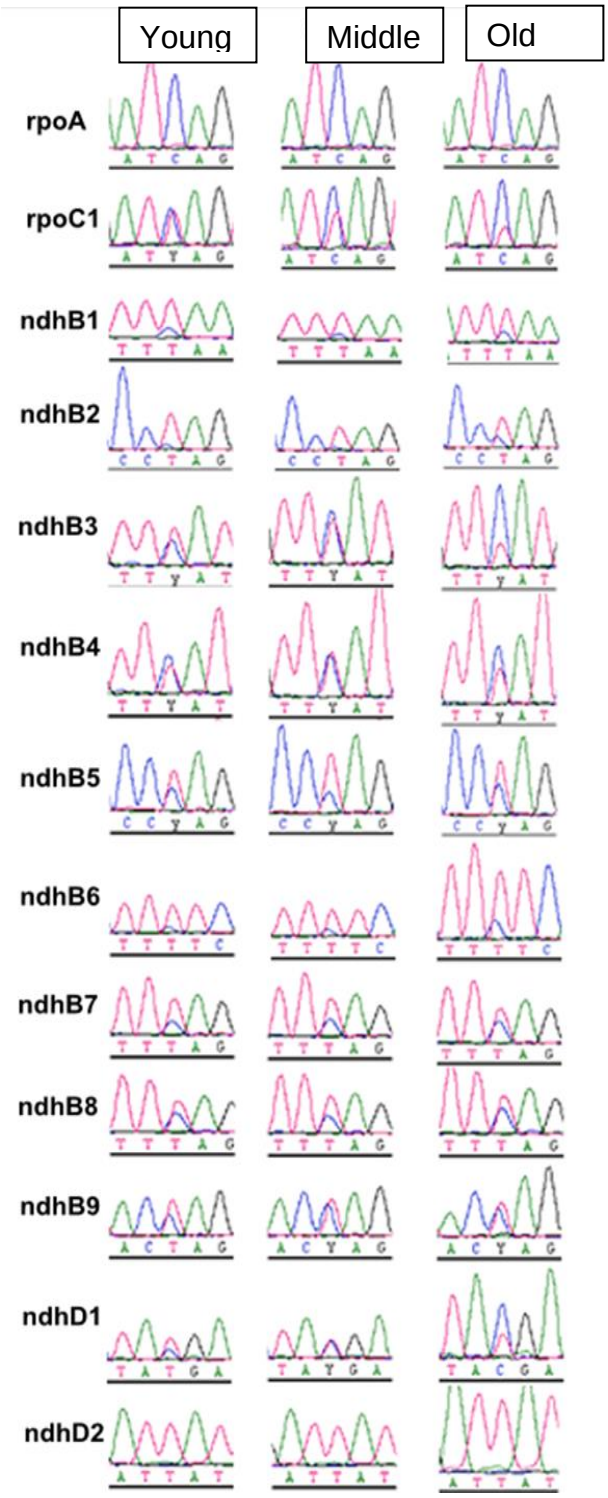
APPENDIX



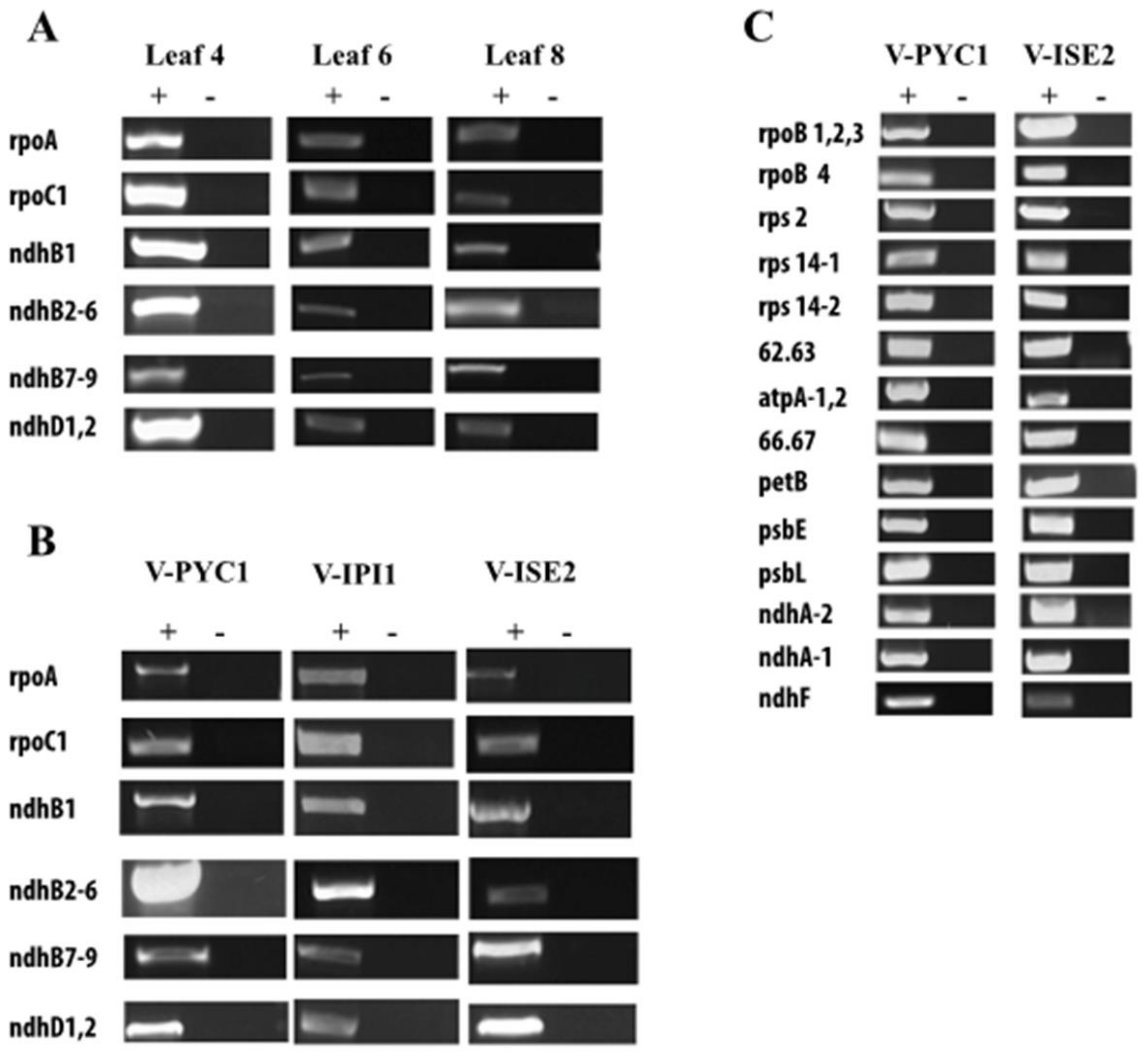
Representative Chromatograms from *ISE1* and *IPI1* –silenced plants



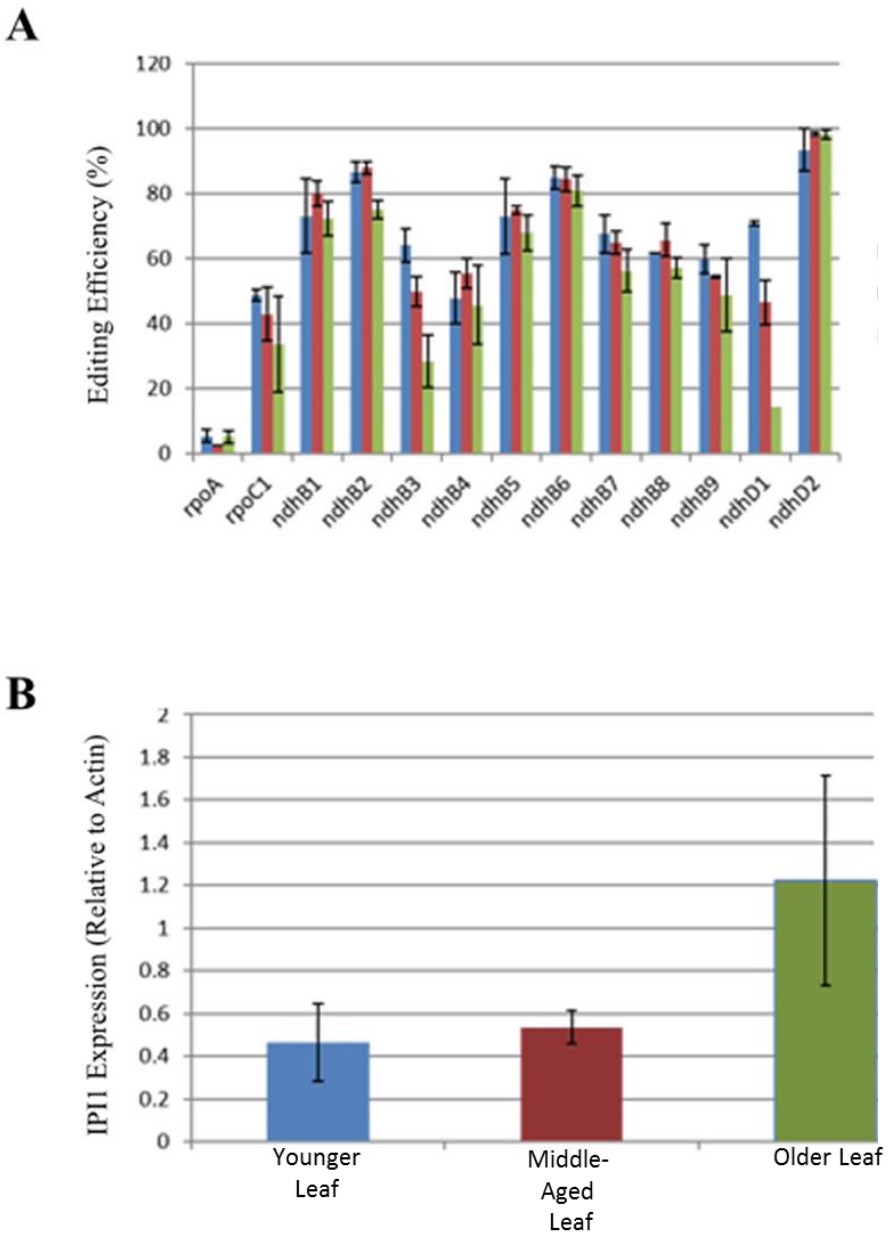
Chromatograms of Editing Sites from *ISE2*-silenced plants con't



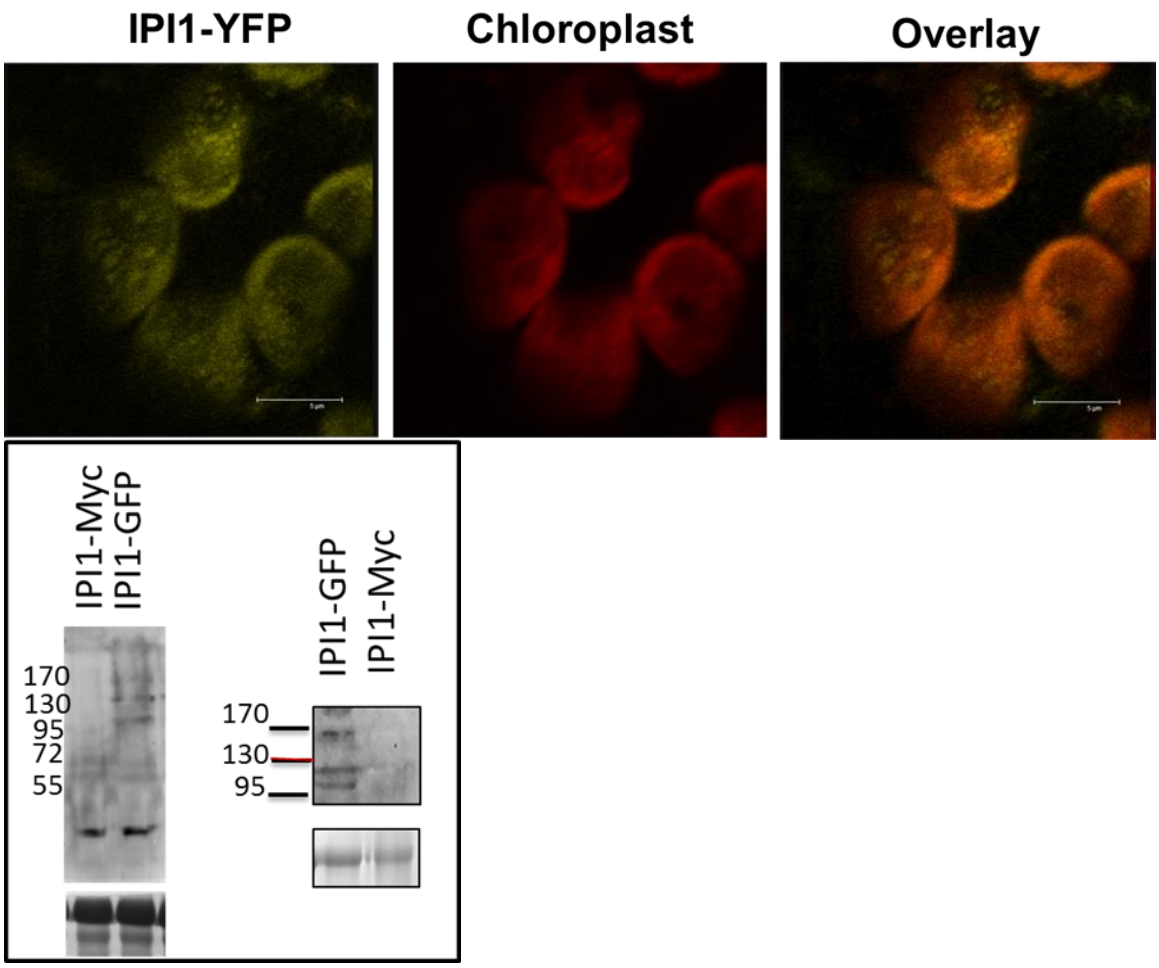
Representative Chromatograms from Different Aged *Nicotiana benthamiana* Leaves



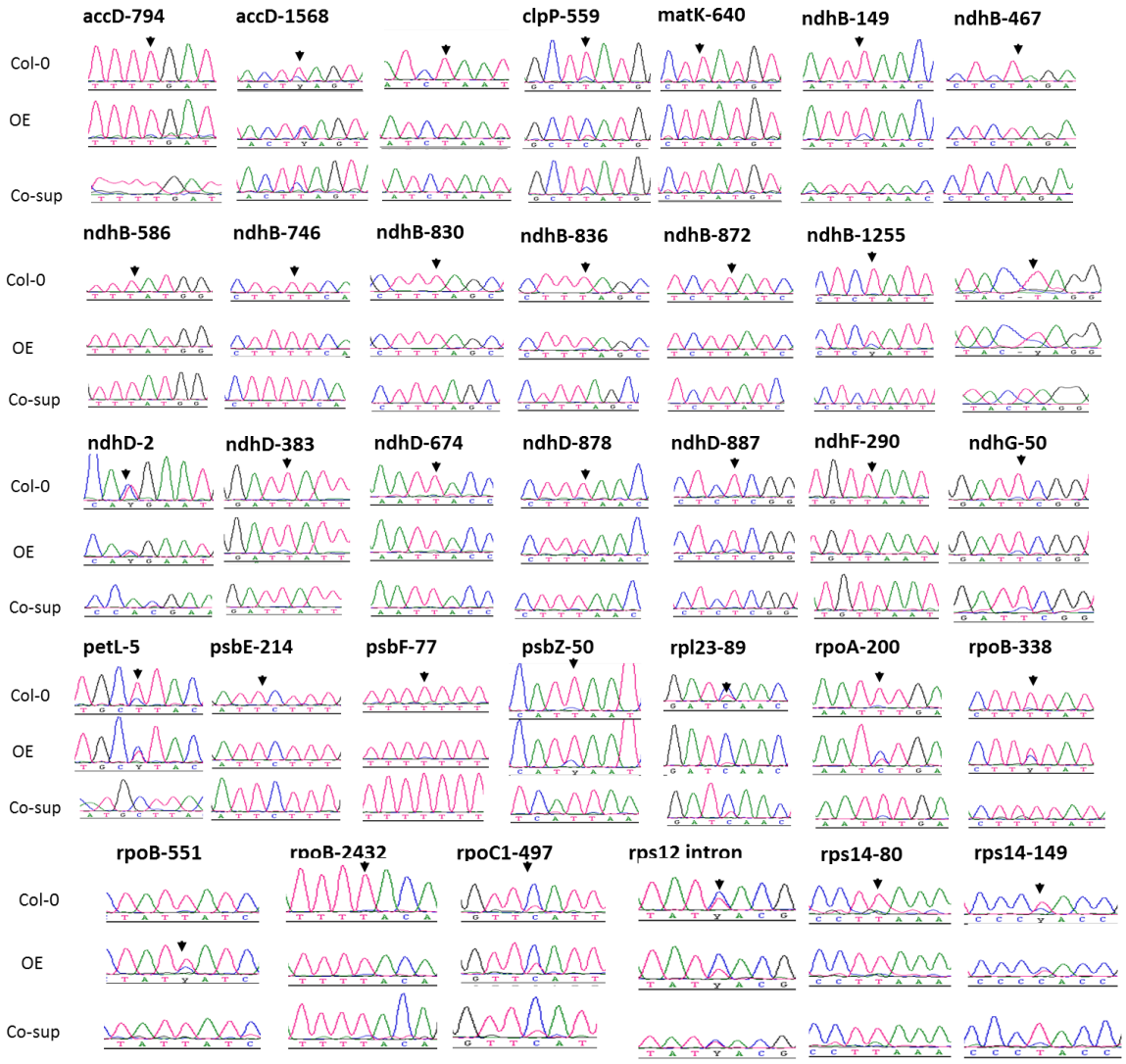
Representative Gels Used For Editing Experiments. + indicated that Reverse transcriptase (RT) was added to the RT-PCR reaction and – indicates cDNA that was used for PCR where RT was not added to the RT-PCR reaction.



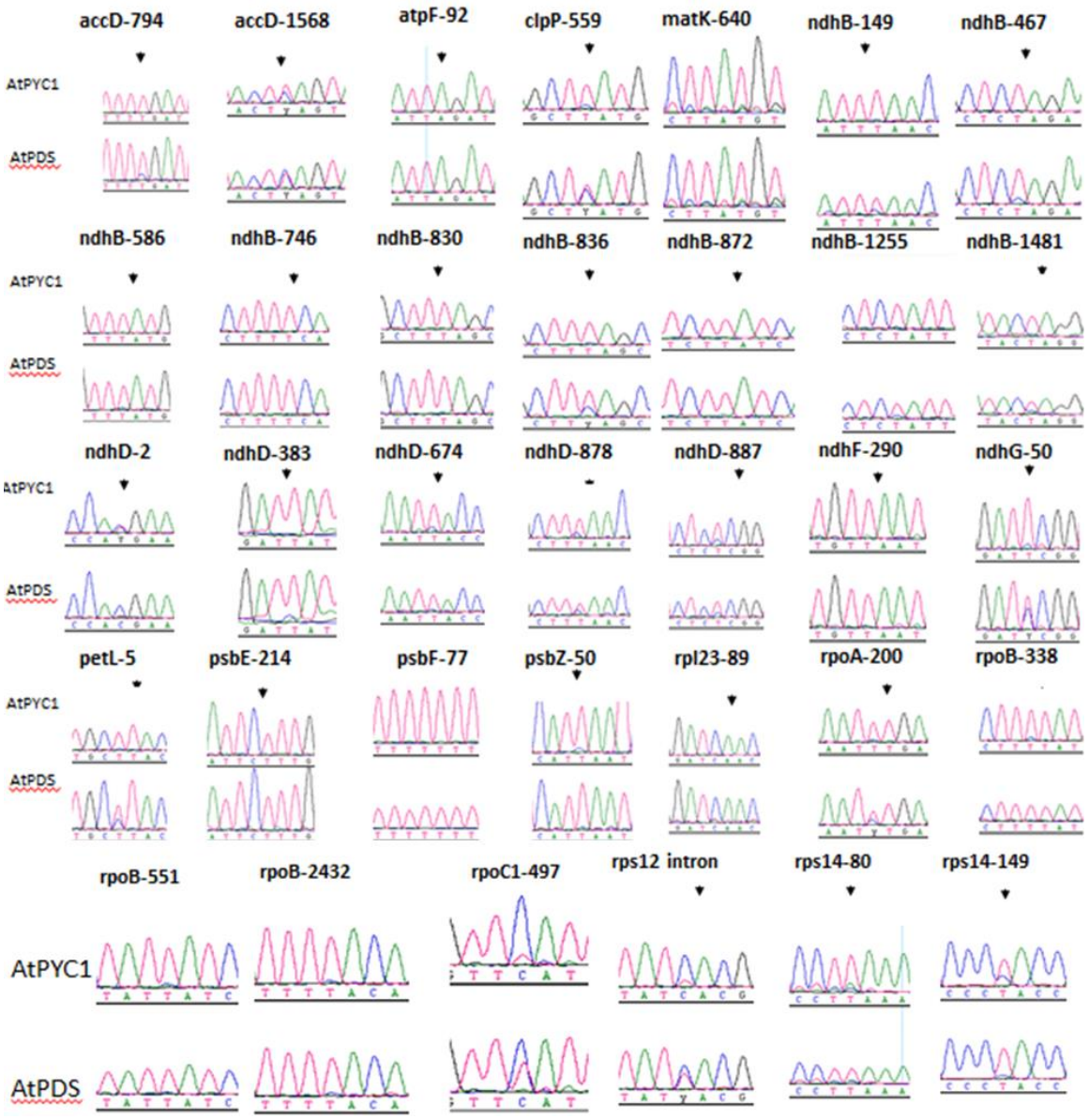
qPCR Expression indicates that IPI1 is expressed in WT leaves that were assayed for developmental-dependence of Editing. Figure A is replicated from chapter 2.1.



A Western blot (bottom) was done on the same tissue that was used to visualize full-length IPI1-YFP localization using confocal microscopy (top).



Chromatogram sequencing results of WT, cosuppressed and rescued plants from Replica 1.
Arabidopsis chloroplast transcript name and editing position are listed above each chromatogram. Col-0 is WT, cosuppressed are plants with reduced ISE2 levels, and OE are plants over-expressing ISE2.



Chromatogram sequencing results of control (PYC1) and TRV-PDS in Arabidopsis. Arabidopsis chloroplast transcript name and editing position are listed above each chromatogram.

VITA

Tyra McCray received her Bachelor of Science from U.C Davis. She then went on to obtain two Masters of Science degrees in Molecular Biology from San Francisco State and Yale University. She hopes to obtain her PhD degree from the University of Tennessee in Genome in Science and develop a career in creative scientific writing and computational science.