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## The chloroplast RNA helicase ISE2 is required for multiple chloroplast RNA processing steps in *Arabidopsis thaliana*

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**The chloroplast RNA helicase ISE2 is required for multiple chloroplast RNA processing steps in *Arabidopsis thaliana***

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**SUMMARY**

INCREASED SIZE EXCLUSION LIMIT2 (ISE2) is a chloroplast-localized RNA helicase that is indispensable for proper plant development. Chloroplasts in leaves with reduced *ISE2* expression have previously been shown to exhibit reduced thylakoid contents and increased stromal volume, indicative of defective development. It has recently been reported that ISE2 is required for the splicing of group II introns from chloroplast transcripts. The current study extends these findings, and presents evidence for ISE2's role in multiple aspects of chloroplast RNA processing beyond group II intron splicing. Loss of ISE2 from *Arabidopsis thaliana* leaves resulted in defects in C-to-U RNA editing, altered accumulation of chloroplast transcripts and chloroplast-encoded proteins, and defective processing of chloroplast ribosomal RNAs. Potential ISE2 substrates were identified by RNA immunoprecipitation followed by next-generation sequencing (RIP-seq), and the diversity of RNA species identified supports ISE2's involvement in multiple aspects of chloroplast RNA metabolism.

Comprehensive phylogenetic analyses revealed that ISE2 is a non-canonical Ski2-like RNA helicase that represents a separate sub-clade unique to green photosynthetic organisms, consistent with its function as an essential protein. Thus ISE2's evolutionary conservation may be explained by its numerous roles in regulating chloroplast gene expression.

## INTRODUCTION

An intriguing aspect of the plastid is its origin as a prokaryotic endosymbiont, necessitating the coordination of organelle and nuclear genomes for plant survival. The genomes of modern plastids typically encode around 100 proteins, considerably fewer than the estimated 3,000 proteins found in these organelles (Pesaresi *et al.* 2007). Numerous nucleus-encoded proteins are imported into the chloroplast in order to facilitate expression of the organelle genome; however, the molecular factors and mechanisms involved in the regulation of plastid gene expression have not been fully elucidated. RNA helicases are among the proteins imported into the chloroplasts to influence gene expression.

RNA helicases constitute a large group of highly conserved enzymes that use ATP to potentially engineer both RNA architecture and interactions with regulatory proteins (Jarnoskaite and Russell 2014). These enzymes have been implicated in all aspects of RNA metabolism (Linder and Owtrim 2009). On the basis of both their sequences and structure, most RNA helicases have been assigned to Superfamily (SF)1 and SF2, with the majority belonging to SF2 (Fairman-Williams *et al.*, 2010). The consensus sequence of conserved motifs is used to divide SF2 RNA helicases into three main families: (i) DEAD, (ii) DEAH/RHA and (iii) Ski2 (Superkiller 2; Jarnoskaite and Russell 2014). Members of the small family of Ski2-like RNA helicases are large proteins, and have molecular weights from 120 to 225 kDa in *Saccharomyces cerevisiae*. In *S. cerevisiae*, the Ski2 protein (ScSki2) is a component of the cytoplasm-localized SKI complex that modifies the RNA-degrading function of the exosome (Halbach *et al.* 2013). The Arabidopsis Ski2 homologue, encoded

by *At3g46960*, likely also functions in RNA degradation in the cytoplasm (Dorcey *et al.* 2012), and indeed it has been shown to prevent the formation of secondary siRNAs through its role in degrading 5'-cleavage fragments of the RISC complex (Branscheid *et al.* 2015). The single copy Arabidopsis *ISE2* gene encodes a chloroplast-localized RNA helicase with a predicted molecular weight of 132 kDa (Kobayashi *et al.* 2007; Burch-Smith *et al.* 2011a). *ISE2* was predicted to be a Ski2-like protein due to the presence of the consensus motifs typical of the Ski2 RNA helicase family, (Kobayashi *et al.* 2007; Linder and Owtrim 2009), although its C-terminus diverges from those of other Ski2 proteins.

The Arabidopsis nuclear genome encodes over 100 SF2 genes, of which 58 are predicted to be DEAD-box RNA helicases (Mingam *et al.* 2004; Umate *et al.* 2010). Eleven of these DEAD RNA helicases carry predicted N-terminal chloroplast-targeting sequences, and proteomic experiments suggest that six of these helicases localize to chloroplasts (Olinares *et al.* 2010), where they function to facilitate expression of the organellar genome (Tanner and Linder 2001; Goldschmidt-Clermont 1998 ; Stern *et al.* 2010). Three chloroplast DEAD-box helicases, RH3 (Asakura *et al.* 2012), RH22 (Chi *et al.* 2012) and RH39 (Nishimura *et al.* 2010), are required for ribosomal RNA (rRNA) processing in chloroplasts. In addition, RH3 is also involved in splicing of a subset of chloroplast group II introns (Asakura *et al.* 2012; Gu *et al.* 2014).

*ISE2* is the only chloroplast-localized, nucleus-encoded RNA helicase that does not belong to the DEAD family (Olinares *et al.* 2010). The importance of *ISE2* to plant development is illustrated by the defective embryogenesis of *ise2* mutants, including the *ise2-1* mutant that carries a single point mutation in the nucleotide-binding DEVH motif (Kobayashi *et al.* 2007). The involvement of *ISE2* in critical chloroplast processes was further supported by detailed TEM analysis that revealed severe changes to chloroplast ultrastructure in the absence of *ISE2* (Burch-Smith *et al.* 2011a). *ISE2*'s role in splicing group II introns from chloroplast transcripts has recently been reported (Carlotto *et al.* 2016). Unspliced transcripts from *rpl2*, *rps12*, *atpF* and *clpP1* accumulated in leaves of Arabidopsis

plants with reduced *ISE2* expression, demonstrating that *ISE2* is necessary for their proper splicing. Here we show that *ISE2*, in addition to group II intron splicing, is required for multiple other aspects of chloroplast RNA processing. Insight into *ISE2*'s function is provided by the identification of numerous *ISE2*-associated RNA species including introns and intergenic sequences as potential substrates for *ISE2* activity. Collectively, these results coupled to detailed phylogenetic analyses of *ISE2* orthologues, suggest that *ISE2* is a highly conserved plant-specific RNA helicase, and support the hypothesis that *ISE2* is a general chloroplast RNA processing factor.

## RESULTS

### ***ISE2* is required for site-specific chloroplast C-to-U RNA editing**

The involvement of RNA helicases in both mitochondrial and plastid cytidine-to-uridine (C-to-U) RNA editing was proposed to explain the dependence of the editing on ATP that could be fully substituted by any other dNTPs or NTPs, a feature characteristic for RNA helicase activity (Takenaka and Brennicke 2003; Nakajima and Mulligan 2005). However, the putative RNA helicase that may participate in RNA editing in either organelle has not yet been identified. *ISE2*'s potential involvement in plastid RNA editing was therefore investigated.

The embryonic-lethal phenotype of *Arabidopsis* *ise2* mutants is an obstacle to studying its role in mature plant tissues via knockouts. The *ise2* mutation had been complemented by the expression of a *35S:ISE2-GFP* transgene in the *ise2-2* homozygous background (Kobayashi *et al.* 2007). For several of the resulting lines, there occasionally appeared plants whose leaves developed chlorosis (Carlotto *et al.* 2016; Figure S1). Analysis of expression of the *35S:ISE2-GFP* transgene and accumulation of the *ISE2-GFP* protein in the green or chlorotic plants revealed that the chlorotic tissues showed reduced accumulation of the transgene transcript and no *ISE2-GFP* protein (Figure S1). Thus, the



transgene was silenced by cosuppression in the chlorotic tissues, and these tissues therefore contain very little, if any, ISE2 protein. Such cosuppression was observed in three of seven independent lines examined (Figure S1). We therefore used the rescued plants with ISE2-GFP (ISE2-OE) and plants cosuppressing the *ISE2-GFP* transgene (ISE2-SUP) plants from one line showing the most consistent appearance of cosuppression to further investigate ISE2 function in mature leaves (Figure S1). The loss of ISE2 in the cosuppressing ISE2-SUP leaves lead to severe chlorosis (Figure 1a-b) that was associated with reduced photosynthetic capacity ( $F_v/F_m$ ; Figure 1c-f) and decreased levels of photosynthetic pigments (Figure 1g).

To measure the degree of RNA editing, cDNA was generated from total RNA isolated from wild-type, ISE2-OE, or ISE2-SUP leaves, and fragments containing 34 known C-to-U chloroplast RNA editing sites were amplified and sequenced using published primers (Tseng *et al.* 2013). In leaves of ISE2-SUP plants 12 of the 34 sites examined exhibited statistically significantly altered degrees of RNA editing compared to the wild-type controls (Table S1 and Figure S2). Wild-type levels of RNA editing were restored in the ISE2-OE lines.

Interestingly, chlorosis has been associated with altered patterns of chloroplast RNA editing (Kakizaki *et al.* 2012; Tseng *et al.* 2013). To determine if the changes observed in RNA editing in tissues with decreased ISE2 levels (ISE2-SUP leaves) were due to the specific effects of ISE2 and not due to general chlorosis, RNA editing was also measured in chlorotic leaves resulting from disruption of other genes. We measured the relative editing efficiency at all 34 sites in tissues where the *PHYTOENE DESATURASE (PDS)* gene encoding an enzyme involved in carotenoid biosynthesis had been silenced by virus-induced gene silencing using the Tobacco rattle virus (TRV; Burch-Smith *et al.* 2006). From comparing the RNA editing defects in TRV-*PDS* leaves with those of TRV-alone control leaves and ISE2-SUP leaves we identified six sites whose editing appeared to be affected specifically by loss of ISE2 (Table S1). To confirm ISE2's role in RNA editing of those six sites, the *var2-2* variegation mutant defective in the Arabidopsis nuclear gene encoding

chloroplast-localized FtsH, a homolog of the bacterial FtsH protease, was used as another source of chlorotic tissue (Figure S2; Takechi *et al.* 2000). Total RNA was isolated from leaves of the same age as the ISE2-SUP leaves analyzed, and RNA editing at the six candidate ISE2-specific sites and several unaffected sites was measured (Table S1).

The *clpP1-559* site exhibited reduced RNA editing efficiency in both the ISE2-SUP and the *var2-2* leaves, but the extent of RNA editing in ISE2-SUP leaves (25%) was less than half of that in *var2-2* leaves (59%; Figure 2). Thus, depletion of ISE2 had a more drastic effect on the RNA editing efficiency at the *clpP1-559* site than depletion of FTSH2. Similarly, while the *var2-2* mutant displayed decreased RNA editing at *petL-5* and *rpoA-200*, the decrease at those sites was more drastic in ISE2-SUP leaves than in *var2-2* leaves (Figure 2). This suggests that although a general RNA editing machinery may control editing at these three sites, ISE2 may have an additional, specific role in their editing. The defects in RNA editing at the *clpP1-559*, *petL-5* and *rpoA-200* sites were confirmed in a second, independent line that cosuppressed the *ISE2-GFP* transgene (Figure S3). Interestingly, RNA editing at the sites *rpoB-338* and *-551*, and *rps14-149* were unaffected in the *var2-2* leaves (Figure 2 d-f), but in ISE2-SUP leaves RNA editing efficiency was reduced to 72% (*rpoB-338*), 76%, (*rpoB-551*) and 42% (*rps14-149*) of transcripts. These results support the specific role of ISE2 in RNA editing some sites. It also suggests that involvement of ISE2 in RNA editing is site specific, as different sites within the same transcript displayed distinct changes to RNA editing in the absence of ISE2. This is exemplified by the *rpoB* transcript where sites *rpoB-338* and *rpoB-551* appeared to depend on ISE2 for full RNA editing, while editing at the *rpoB-2432* site in this transcript was unaffected by the loss of ISE2 (Table S1 and Figure S2).

## Accumulation of chloroplast mRNAs is altered in the absence of ISE2

RNA helicases are involved in RNA transcription (Eisen and Lucchesi 1998). They also have important roles in modulating gene expression by regulating RNA stability (Owtrim 2006). We therefore measured the levels of all chloroplast transcripts by quantitative RT-PCR (qPCR) using primer pairs specific to each coding sequence within the Arabidopsis chloroplast genome (Chateigner-Boutin *et al.* 2008; de Longevialle *et al.* 2008). In both Arabidopsis mid-torpedo stage *ise2* embryos (within seeds) and chlorotic leaves of 3-week old ISE2-SUP plants, there were dramatic changes in the steady-state level of chloroplast transcripts (Figure 3a-b). In both sets of tissues, the lack of ISE2 resulted in decreased accumulation of transcripts encoding the photosynthetic machinery (PSII, Cyt  $b_6/f$ , PSI and ATPase). In striking contrast, chloroplast transcripts encoding proteins involved in chloroplast gene transcription and protein synthesis (RNA pol and Ribosomal) accumulated to higher levels in both *ise2* embryos and ISE2-SUP leaves as compared to wild type. The changes observed in leaves were more pronounced than those in embryos. Yet, even though the overall response profiles were similar in *ise2* embryos and 3-week old ISE2-SUP plants, transcripts of ten genes (*psbA*, *psbM*, *psaI*, *rps12A*, *rps14*, *rpl32*, *rpl33*, *accD*, *ccsA* and *ycf15*) were found to display opposite steady-state accumulation, suggesting development-related differences. The observed profile of transcription of chloroplast genes occurring in ISE2-SUP leaves is broadly reminiscent of transcriptional changes described previously in mutants lacking the plastid-encoded RNA polymerase (PEP) in which the expression of photosynthesis-related genes is repressed (Yagi and Shiina 2014). ISE2 may act at either the transcriptional or post-transcriptional level to modify chloroplast transcription or RNA stability.

## ISE2 is required for splicing of group II introns

Several chloroplast genes contain introns of prokaryotic origin called group II introns. Even though many group II introns undergo autocatalytic splicing *in vitro*, they require the assistance of proteins to accomplish splicing *in vivo*. The involvement of ISE2 in splicing of four chloroplast group II introns has been reported, and unspliced transcripts of the *atpF*, *rpl2*, *rps12* and *clpP1* accumulated in leaves of plants with reduced ISE2 levels (Carlotto *et al.* 2016). Using PCR with primers that could specifically detect the presence of the intron in a transcript, we confirmed that the ISE2 is required for splicing of the second intron from the *clpP1* transcript (Figure 4b). Using a similar approach, we found that ISE2 was also required for splicing of the first group II intron from the *ycf3* transcript, but not the second intron in the *ycf3* transcript (Figure 4c). The observation of altered intron processing was confirmed by Northern blotting with probes specific to either intron 2 from *clpP1* (Figure 4d) or intron 1 from *ycf3* (Figure 4e), where each probe revealed accumulation of intron-containing transcript in the ISE2-SUP leaves compared to WT and ISE2-OE leaves. These results confirm that ISE2 is involved in the splicing and processing of chloroplast group II introns and raise the possibility that more introns than those tested to date may depend on ISE2 for their removal.

## ISE2 is required for chloroplast rRNA processing

The involvement of nucleus-encoded RNA helicases in cytoplasmic, mitochondrial and chloroplast ribosome biogenesis is a known phenomenon (Kim *et al.* 2010; Chi *et al.* 2012; Asakura *et al.* 2012; Martin *et al.* 2013; Rodriguez-Galan *et al.* 2013). The chloroplast RNA helicases RH39 (Nishimura *et al.* 2010), RH22 (Chi *et al.* 2012), and RH3 (Asakura *et al.* 2012) have been shown to function in chloroplast rRNA maturation. Chloroplast 70S ribosomes consist of two subunits, the small 30S subunit and the large 50S subunit. The 30S subunit is composed of 16S rRNA and the 50S subunit is composed of 23S, 4.5S and

5S rRNAs. In addition to the RNA component, both subunits consist of ribosomal proteins encoded by either the nuclear or chloroplast genome. All chloroplast rRNAs are transcribed as a polycistronic transcript (Figure 5a) that is post-transcriptionally processed by the coordinated action of multiple enzymes including endonucleases, exonucleases and RNA helicases (Stern *et al.* 2008). Analysis of total RNA on a denaturing agarose gel revealed conspicuous changes in the rRNA profile of chlorotic ISE2-depleted leaves (Figure 5b). A striking difference observed in ISE2-depleted leaves as compared to WT leaves was the increased intensity of the two bands of 2.8 and 2.4 kb migrating between the cytoplasmic 25S and 18S rRNA bands (Figure 5b, top two asterisks). A similar phenomenon has been observed for the *rh39* (Nishimura *et al.* 2010), *clpR2-1* (Asakura *et al.* 2012) and *var2-5 svr7-1* (Liu *et al.* 2010) mutants. A third band migrating between 0.5 and 1 kb was also observed in ISE2-SUP derived RNA. Another obvious change is the decreased accumulation of 23S rRNAs of 1.3, 1.1 and 0.5 kb (Figure 5b).

Northern blot analyses using probes specific to individual rRNA species were used to examine changes occurring to individual rRNA species in ISE2-SUP leaves. Depletion of ISE2 in chlorotic ISE2-SUP leaves was associated with a changed profile of each rRNA including accumulation of high molecular weight bands of approximately 7.4 kb, presumably the full length polycistronic rRNA transcript (Figure 5c, d and f). Neither the ethidium bromide-stained gel nor Northern blots revealed any differences between the WT and ISE2-OE plants. The probe specific to the mature 23S rRNA detected increased accumulation of several intermediates in 23S rRNA processing at 3.2 kb (23S + 4.5S), 2.9 kb (full-length 23S before introduction of hidden breaks) and 2.4 kb (23S with the 0.5 kb fragment removed; Figure 5c). In addition, a band of unknown origin of about 0.8 kb was also detected. There were also changes in the case of the 16S rRNA, notably the accumulation of a band between 1.5 and 2 kb representing an intermediate in the processing to the mature 16S rRNA of 1.5 kb (Figure 5d). The 4.5S-specific probe detected a decrease in mature 4.5S rRNA at 0.1 kb and increased accumulation of 3.4-kb and 7.4-kb RNA molecules that

represent intermediates comprising the 4.5S and 23S rRNA and presumably the full rRNA transcript, respectively (Figure 5e). These defects were also detected with the 23S rRNA probe (Figure 5c). The 5S rRNA probe detected minimal changes in the processing of this rRNA species but overexposure of the blot revealed increased accumulation of the full rRNA transcript at 7.4 kb and a larger intermediate at 3.9 kb (Figure 5f). Together, these changes point to defective processing of rRNAs of both the 50S and 30S ribosomal subunits and ISE2's involvement in the processing of rRNAs of both the small and large ribosomal subunits.

### **ISE2 is required for accumulation of 30S and 50S ribosomal RNAs**

rRNAs only accumulate if they are incorporated into ribosomal subunits (Tiller and Bock 2014). Thus, the abundance of rRNA species has been interpreted as representative of the accumulation of ribosomal subunits, and it has been used to identify and characterize mutants with defects in ribosome biogenesis (Walter *et al.* 2010; Tiller *et al.* 2012; Fristedt *et al.* 2014). Using a microfluidics-based assay, the relative abundance of rRNA species on the cytosolic and chloroplast ribosomes was measured. The ratios of chloroplast to cytosolic ribosomal rRNAs and the ratios of small to large rRNAs of the plastid ribosome were then calculated (Figure 6a). ISE2-SUP leaves showed a drastic increase in the ratio of 16S to 23S H1 RNAs. This is best explained by a decrease in the levels of the 50S chloroplast ribosomal subunit RNAs in ISE2-SUP leaves, and is supported by the decreased 23S H1 to 18S ratios in those tissues. In addition, the 30S subunit RNA also showed decreased accumulation (evidenced by the decreased 16S to 18S ratio), although this was not as drastic a change as observed for the 50S subunit RNA. ISE2-OE plants accumulated wild-type levels of the RNAs of both the 30S and 50S chloroplast ribosomal subunits. The failures of both the 30S and 50S chloroplast subunit RNAs to accumulate to wild-type levels in the ISE2-SUP samples are consistent with the defects in rRNA processing observed for both the 16S and 23S rRNAs (Figure 5).

## **ISE2 is required for chloroplast protein accumulation**

Given that loss of ISE2 led to defects in chloroplast transcript accumulation, C-to-U editing, rRNA processing and splicing, the effects of reduced ISE2 levels on chloroplast protein accumulation were examined. Western blot analysis of total protein extracts with antibodies detecting chloroplast- or nucleus-encoded chloroplast proteins revealed changes to the levels of several chloroplast proteins in the absence of ISE2. Leaves from ISE2-SUP plants had greatly reduced amounts of chloroplast-encoded ClpP1, D1, PsaB and cyt b6 proteins compared to WT and ISE2-OE plants (Figure 6b). In contrast, the levels of the nucleus-encoded subunit A of the F-ATPase complex and Hsp70 showed only a mild decrease and no change, respectively (Figure 6b). Consistent with decreased accumulation of chloroplast-encoded proteins, ISE2-SUP total protein extracts contained reduced amounts of RbcL (Figure 6c). Notably, the level of the nucleus-encoded Lhcb proteins was dramatically diminished in ISE2-SUP leaves compared to the amounts in WT and ISE2-OE (Figure 6c and d). This reduction in Lhcb protein levels is consistent with the involvement of chloroplast-to-nucleus retrograde signaling in altering nuclear gene expression in response to disrupted chloroplast translation (reviewed in Bobik and Burch-Smith 2015). Thus, loss of ISE2 compromises chloroplast protein accumulation, and also affects expression of nuclear genes.

## **ISE2 associates with numerous chloroplast RNA species**

ISE2 carries a canonical RNA binding domain and previous work confirmed that ISE2 associates with RNA (Carlotto, *et al.* 2016). Given that ISE2 is likely involved in multiple aspects of chloroplast RNA metabolism we took an unbiased approach to identifying RNAs that are potential ISE2 substrates. For this, ISE2-GFP was immunoprecipitated from total protein extracts from leaves of ISE2-OE plants. Co-precipitated RNA was retrieved from the ISE2-GFP immunocomplexes and RNA-seq libraries were constructed from three biological

replicates for deep sequencing. As a negative control libraries were prepared similarly from plants expressing chloroplast-localized GFP (Stonebloom *et al.* 2012). The amount of RNA precipitated from ISE2-GFP samples was on average 20-fold more than that precipitated from GFP-alone samples; the three ISE2-GFP samples yielded on average 27.1  $\mu\text{g}$  RNA while the three GFP samples yielded an average of 1.36  $\mu\text{g}$  RNA. Since we found ISE2 was necessary for processing chloroplast rRNAs, rRNA was not depleted from our samples. These six samples were multiplexed and sequenced on a single lane of Illumina HiSeq1000 so the technical variation applied across all samples with no loss of sequencing depth. 100-bp paired end reads were generated and data was analyzed. Despite the use of total protein extract, roughly 45% of the approximately 167 million read pairs from across all three ISE2-GFP samples matched to the chloroplast genome, while fewer than 25% of those from the GFP-alone controls (92.7 million read pairs from all three samples) mapped to the chloroplast genome (Table 1 and Methods S1), indicating an enrichment of chloroplast RNAs in the ISE2-GFP samples compared to the negative controls.

Taking into consideration that ISE2 was likely required for different chloroplast RNA processing steps, we expected that ISE2 would bind numerous RNAs, including introns and intergenic regions. We therefore developed a custom pipeline for our data analysis (see Methods S1 for details). The distribution of reads over the entire chloroplast genome is shown in Figure S4, and indicates that transcripts from the entire chloroplast genome were represented in our dataset. After analysis through our pipeline 111 RNAs representing the introns, coding sequence, or downstream intergenic regions of 76 genes were identified as potential ISE2 substrates (Table 2 and Fig. 7a-b). Of the 133 genes in the Arabidopsis chloroplast genome, 76 were included in our set of potential ISE2 substrates, representing 57% of the chloroplast genome (Table 2). Most of the candidate substrates were



represented by their coding sequence although in some instances, 14 of the 76 candidate substrates, only RNA from the intron or intergenic region was identified (Fig 7b, Table 2).

Genes encoding proteins involved in all aspects of chloroplast function were represented in our dataset (Fig. 7c).

Seventy nucleus-encoded RNAs representing 67 loci were enriched in the ISE2-GFP samples (Table S2). This set of transcripts represents approximately 0.2% of the nuclear genome. The small number of nucleus-encoded transcripts identified indicates that the RNA immunoprecipitation procedure and subsequent analysis were largely successful at identifying transcripts that were likely *in vivo* targets of ISE2. Most of these genes are relatively highly transcribed, and their expression levels are comparable to that of *ACTIN2*. Such high expression could account for their non-specific association with ISE2-GFP.

#### *Chloroplast rRNAs are potential ISE2 substrates*

All chloroplast *rrn* transcripts were identified as potential ISE2 substrates (Table 1). Indeed, when ranked by p-values, *rrn16S* and *rrn23S* were the highest ranked ISE2 potential substrates (Table 2). Of the four *rrn* transcripts, the *rrn23S* transcript showed the highest enrichment (6.15-fold) in ISE2-GFP samples (Table 2). The other transcripts showed lower enrichment: both *rrn5S* and *rrn4.5S* were enriched approximately 4-fold in ISE2-GFP extracts compared to GFP alone controls, while *rrn16S* showed about 3-fold enrichment. In addition, several other regions of the *rrn* cistron are potential ISE2 substrates (Table 2), including the regions downstream of *rrn23S* (p-value 0.017), *rrn4.5S* (p-value 0.028), *rrn16S* (p-value 0.021) and *trnA* (p-value 0.014). ISE2's association with all chloroplast rRNAs and other regions of the *rrn* cistron is consistent with the observed defects in rRNAs in ISE2-SUP leaves detected by Northern blots (Figure 5c-f) and the decrease in both 50S and 30S ribosomal subunit RNAs (Figure 6a).

### *ISE2 associates with chloroplast introns*

Strikingly, all of the chloroplast group II introns except that of *rps16* were identified as potential ISE2 substrates (Figure 7, Table 2). Indeed, three introns were among those with the top 10 candidate substrates (ranked by p-values; Table 2). These findings support previous demonstrations of ISE2's involvement in chloroplast intron splicing (Carlotto *et al.* 2016; Figure 4). Interestingly, this finding suggests that ISE2 may associate with an intron but not directly affect its splicing, as seen for *clpP1* intron1 and *ycf3* intron 2 (Figure 4b and Table 2). Beside the group II introns, the *trnL* group I intron was also identified as a potential ISE2 substrate. The CRM-domain splicing factor CFM2 has been shown to be involved in splicing both group I and II introns (Asakura and Barkan 2007).

### *ISE2 associates with transcripts containing C-to-U editing sites*

We found that loss of ISE2 disrupted C-to-U editing (Figure 2). Consistent with this finding, 13 of the 18 transcripts (containing 34 sites) that were examined for C-to-U editing were also enriched in ISE2-GFP samples (Table 2). These 13 transcripts contain 27 editing sites. Indeed, all transcripts containing sites that showed statistically significant changes in editing on loss of ISE2 were identified as likely ISE2 substrates (Table 2), with the exception of *rpoB*. Interestingly, editing of the *rpoC1* transcript is affected in several chlorotic mutants including ISE2 but while the *rpoC1* intron was identified as a potential ISE2 substrate the *rpoC1* CDS containing the editing site was not. However, several transcripts that usually undergo editing but whose editing was unaffected by loss of ISE2, including *accD*, *atpF*, *ndhB*, *ndhD*, *ndhG*, and *rpl23*, were also identified as potentially associating with ISE2 *in vivo*. This suggests that ISE2's role in editing may be complex.

### *The PEP components are likely not ISE2 substrates*

The RNA transcripts associated with the plastid-encoded polymerase (PEP) show interesting association patterns with ISE2. The intron of *rpoC1* and the coding sequence of *rpoA* were the only PEP transcripts identified as potential ISE2 substrates (Table 2). This is

despite the changes in editing of the *rpoB* transcript (Figure 2) and the increases in the accumulation of *rpoA*, *rpoB*, *rpoC1* and *rpoC2* transcripts (Figure 3) observed in ISE2-SUP tissues. Perhaps ISE2 can also have indirect roles in RNA processing.

### **ISE2 is a member of a distinct phylogenetic clade of Ski2-like RNA helicases**

Arabidopsis contains two putative Ski2-like proteins, AtSKI2 and ISE2. AtSKI2, encoded by *At3g46960*, has been reported to be a *bona fide* homolog of ScSki2 and it likely also functions in regulating RNA degradation in the cytoplasm (Dorcey *et al.* 2012) and in small RNA biogenesis (Branscheid *et al.* 2015). While ISE2 contains all the Ski2-signature motifs in its N-terminal helicase domain, outside this domain it markedly diverges from other Ski2 proteins. Further, ISE2's function as an apparent general RNA processing factor in chloroplasts differs markedly from the specific function of AtSKI2 in the cytoplasm. A detailed phylogenetic analysis of full-length ISE2 and Ski2 proteins (Figure 8, or the more conserved helicase domains of those proteins (Figure S5) revealed that ISE2 clearly separates from Ski2 proteins. Moreover, the analysis supports that ISE2 is a plant-specific protein with primordial origins in cyanobacteria (Figure 8). This extends previous studies that placed ISE2 among the group of proteins that are specific to the plant lineage, the GreenCut2 inventory (Karpowicz *et al.* 2011). The alignment of multiple ISE2-like protein sequences exposed a highly conserved "AIRGENE" motif located within the DOB1/SKI2/helY-like DEAD box helicases C-terminal (DSHCT) domain near the C-termini of ISE2 homologs (Figure S6). This motif seems to coincide with photosynthetic/chloroplast-related functions of the host.

## DISCUSSION

ISE2 is a chloroplast-localized RNA helicase (Kobayashi *et al.* 2007; Burch-Smith *et al.* 2011a), for which involvement in splicing of some group II introns was recently demonstrated (Carlotto *et al.* 2016). Here we present further functional analysis that reveals ISE2's crucial role in multiple RNA processing steps in Arabidopsis chloroplasts. ISE2 is also required for site-specific C-to-U RNA editing, rRNA processing, transcript accumulation and protein accumulation in chloroplasts. Our identification of 111 chloroplast RNA features as possible ISE2 substrates supports the idea that this RNA helicase could indeed be involved in several aspects of chloroplast RNA metabolism. In the future, this dataset could be a valuable resource as the identified interactions are investigated and characterized by RNA-protein interaction assays.

C-to-U RNA editing of RNA residues is important for ensuring correct gene expression of organellar genomes (Takenaka *et al.* 2013). C-to-U RNA editing is perhaps best understood in the mitochondria of some protozoa. Here, a small guide RNA mediates the substitution of uracils for cytosines in transcripts. The DEAD-box RNA helicase REH1 and the Ski2-like REH2 helicase are involved in the RNA rearrangements that accompany RNA editing (Li *et al.* 2011; Hernandez *et al.* 2010; Li *et al.* 2011). In plant organelles, C-to-U RNA editing is accomplished by deamination of cytosines to uracils (Takenaka *et al.* 2013). RNA helicases have been predicted to be involved in plant mitochondrial editing based on the observation that the editing machinery could use any NTP or dNTP (Takenaka and Brennicke 2003; Nakajima and Mulligan 2005; Takenaka *et al.* 2013). RNA helicase are known to utilize various nucleotides (Bleichert and Baserga 2007). We found that C-to-U RNA editing at 12 of 34 sites tested was disrupted in chlorotic ISE2-SUP leaves, and ISE2 was specifically required for splicing of six of those sites (Figure 2). ISE2's role in RNA editing of these sites is supported by the identification of all of the eight transcripts containing the 12 significantly affected sites as potential ISE2 substrates (Table 2). To date, no other RNA helicase has been reported to affect chloroplast C-to-U editing.

Several DEAD-box RNA helicases are important for rRNA processing in chloroplasts. RH39 is required for the introduction of hidden breaks in the 23S rRNA during ribosome maturation, and RH39 bound directly to 23S rRNA *in vitro* (Nishimura *et al.* 2010). RH22 is involved in processing the rRNA of the 50S ribosome subunit (Chi *et al.* 2012). RH22 was found to bind the 5' fragment of the 23S rRNA and to interact with the L24 protein of the 50S subunit, and consistent with this, *rh22* mutants showed defective ribosome assembly. RH3 is a third DEAD-box RNA helicase required for chloroplast rRNA processing. RH3 apparently associates with assembling 50S ribosomal subunits, and Arabidopsis *rh3* mutants accumulated reduced amounts of 23S, 4.5S and 5S rRNA (Asakura *et al.* 2012), while 16S rRNA accumulation seemed to be affected in a development-dependent manner (Lee *et al.* 2013). We have shown that similar to these RNA helicases, ISE2 is also required for rRNA processing in chloroplasts (Figure 5). Indeed, the 23S, 16S, 5S and 4.5S rRNAs coimmunoprecipitated with ISE2 (Table 2). Thus, while multiple RNA helicases are required for the assembly of functional chloroplast ribosomes, apparently they have unique functions in the process, as mutations in individual RNA helicases result in phenotypes that cannot be mitigated by the other functional RNA helicases. The specialization of RNA helicases in the chloroplast is similar to the situation described for ribosome assembly in yeasts and mammals (Rodriguez-Galan *et al.* 2013). Four DEAD-box helicases are needed for *E. coli* ribosome assembly, while three DEAD-box, 15 DEAH/RHA and a Ski2-like RNA helicase are required in *S. cerevisiae*. The involvement of multiple RNA helicases with specific functions in rRNA processing is therefore not unprecedented.

RH3 and ISE2 are the only chloroplast RNA helicases reported to have functions beyond rRNA processing. RH3 was also found to be required for splicing of group II introns in Arabidopsis and maize (Asakura *et al.* 2012). Maize RH3 coimmunoprecipitated with RNAs from the *rps12*, *rpl2*, *trnA*, and *trnI* introns, and splicing of the *trnA*, *trnI* and *rpl2* introns was decreased in the weak *rh3-4* mutant. ISE2 was found to bind the *rpl2*, *atpF*, *clpP1* and *rps12* transcripts, and splicing of all these introns was reduced in plants lacking

ISE2 (Carlotto *et al.* 2016). Here we have confirmed those findings regarding ISE2, and we have extended them by showing that ISE2 associates with 20 of 21 introns encoded by the chloroplast genome (Table 2). Interestingly, association with ISE2 does not indicate that splicing of an intron depends on ISE2, as exemplified by the case of intron 1 of *clpP1* (Figure 4b).

In addition to changes in specific RNA processing steps, loss of ISE2 led to global changes in chloroplast transcript steady-state levels (Figure 3). The requirement of ISE2 for accumulation of numerous transcripts is consistent with our RIP-seq analysis that identified transcripts that map to almost half of the chloroplast genome as potential ISE2 substrates, in that they were enriched in ISE-GFP samples compared to GFP alone control samples (Table 2). The RNA transcript profile of ISE2-depleted tissues is generally reminiscent of that caused by the absence of functional plastid-encoded polymerase (PEP) with diminished amounts of photosynthesis-related transcripts and, with a few exceptions, elevated levels of transcripts associated with protein synthesis (Yagi and Shiina 2014). The changes in chloroplast transcripts are consistent with the reduced accumulation of chloroplast-encoded proteins in ISE2-depleted leaves (Figure 3).

It should be noted that *ise2*, *rh39*, *rh3*, and *rh22* null mutants are all embryonically lethal (Kobayashi *et al.* 2007; Nishimura *et al.* 2010; Chi *et al.* 2012; Asakura *et al.* 2012; Lee *et al.* 2013). It has been proposed that defects in rRNA processing and/or intron splicing was the cause of lethality in those mutants. In addition, several *ppr* mutants with defects in a single or just a few RNA editing sites also display early developmental defects. Examples of this include the Arabidopsis mutants *chloroplast biogenesis19* (Chateigner-Boutin *et al.* 2008), *vanilla cream1* (Tseng *et al.* 2010), and *seedling lethal1* (Pyo *et al.* 2013). Along with the RNA processing defects, loss of ISE2 results in defective plasmodesmata development and in increased intercellular trafficking (Kobayashi *et al.* 2007; Burch-Smith and Zambryski 2010). Numerous transcription factors and RNA molecules that regulate plant growth and development move from cell-to-cell via plasmodesmata (Lucas and Lee 2004; Burch-Smith

*et al.* 2011b; Han *et al.* 2014). Thus, since ISE2 affects numerous chloroplast RNA processing steps as well as intercellular trafficking, the primary cause of *ise2* embryonic lethality remains obscure. It is possible that the loss of chloroplast function, compromised intercellular communication, or some combination of the two may lead to arrested development and eventually death during embryogenesis.

Detailed phylogenetic analyses indicate that *ISE2* is evolutionarily conserved and is present in all photosynthetic organisms including cyanobacteria, but is absent in fungi and animals. It was previously proposed that ISE2 is a Ski2-type RNA helicase (Kobayashi *et al.* 2007). However, our analysis demonstrates a distinct separation of Arabidopsis *ISE2* and its orthologues from other Ski2-like proteins. The independent classification of those two groups is supported by the distinct subcellular localization of the helicases, with ISE2 proteins localized to chloroplasts and other Ski2-like proteins to the cytoplasm. Importantly, unlike ISE2, Ski2 is altogether absent from the genomes of some species (e.g. *Nostoc*). In addition, we identified a highly conserved “AIRGENE” signature within the C-terminal DSHCT domain of ISE2-related RNA helicases that is present only in photosynthetic organisms. A comprehensive phylogenetic analysis of proteins specific to the plant lineage identified ISE2 as a member of that set of proteins, the GreenCut2 (Karpowicz *et al.* 2011). ISE2 was also found to belong to the 124-member PlastidCut2 subset of GreenCut2 proteins, representing proteins conserved in the nuclear genomes of photosynthetic eukaryotes, regardless of whether or not they belonged to the plant lineage. The authors propose that these 124 proteins likely have critical roles in plastid metabolism (Karpowicz *et al.* 2011). Interestingly, while RH22 and RH39 also belong to the GreenCut2 group of proteins they did not belong to the PlastidCut2 set, and RH3 was not a member of the GreenCut2 set. Ski2 does not belong to the GreenCut2 group. Therefore, ISE2 is a plastid-specific RNA helicase whose role in RNA processing is essential for the expression of photosynthesis-related genes and genomes.



How may ISE2 fulfill so many roles in the chloroplast? SF2 RNA helicases function as RNA chaperones and also remodel ribonucleo-protein structures (Jarmoskaite and Russell 2014). Current models suggest that while DEAD-box RNA helicases act to unwind RNA internally with low processivity, DEAH/RHA and Ski2-like helicases unwind RNA by a mechanism that includes 3'-5' translocation along their substrates with a fair amount of processivity. Thus, helicases DEAH/RHA and Ski2-like could displace proteins from their substrates as they unwind their substrates. ISE2 could use such a displacement mechanism in a variety of contexts. RNA editing in the chloroplast genome requires PPR proteins and the MORFs (Takenaka *et al.* 2012). These proteins probably act together in a complex, the editosome. Perhaps ISE2 unwinds local RNA secondary structures to allow the editosome access to its substrates, or perhaps it acts to remove the complex on the completion of RNA editing and promote annealing of the edited RNA. We hypothesize that ISE2 could act as an RNA chaperone that non-sequence specifically recognizes structures and allows the actions of other processing factors. The RIP-seq data that has been generated in this study will be very useful in future studies that probe the mechanism(s) used by ISE2 to interact with its target substrates. ISE2's evolutionary conservation suggests that its generalist function is necessary for the correct expression of genomes encoding photosynthesis-related proteins.

## **Experimental Procedures**

### **Plant material and growth conditions**

*Arabidopsis thaliana* Columbia (Col-0) ecotype was used in all experiments. *Arabidopsis* seeds were kept at -80 °C for 48 h and then surface-sterilized by incubation with 95% ethanol for 5 min, followed by 5 min incubation with 30% bleach supplemented with 0.5% Triton-X 100. After extensive washing with autoclaved double-distilled water seeds were plated on quarter-strength Murashige and Skoog medium solidified with 3% phytagel and supplemented with 1% sucrose, pH 6.0. Seeds were then stratified at 4°C in the dark for 48



h. Seedlings were grown under long day conditions (16h/8h) with 60% relative air humidity at 22 °C for 12 days and then transferred to soil. The ISE2-GFP rescued lines were previously described (Kobayashi *et al.* 2007).

### **Chlorophyll a fluorescence measurements**

Chlorophyll a fluorescence emission was detected with either a portable OS30p hand held chlorophyll fluorometer device (Opti-Sciences) or a FluorCam 800MF station camera (Photon Systems Instruments). Plants were dark adapted for 20 min and then the maximum quantum efficiency of PSII was determined from the ratio of variable ( $F_v$ ) to maximum ( $F_m$ ) fluorescence ( $F_v/F_m$ ).

### **Virus-induced gene silencing (VIGS)**

VIGS of Arabidopsis *PDS* was performed on three week-old plants as previously described (Burch-Smith *et al.*, 2006). The non-silencing construct used as the control was also described previously (Burch-Smith and Zambryski 2010). TRV constructs were introduced into *Agrobacterium* strain GV3101 for plant infiltrations.

### **Total RNA isolation**

Seeds or leaves were collected and RNA prepared from three independent samples. The RNA was then used for cDNA synthesis, quantitative RT-PCR (qPCR) analysis (embryo and leaf tissue), and rRNA quantification, Northern blotting and measuring editing efficiency (leaf tissue). *Seeds*: RNA was isolated from approximately 125 *ise2* or 75 WT seeds at the mid-torpedo stage of development. Developing seeds were removed from siliques, introduced into 50  $\mu$ l RNA Later (Ambion) and then frozen in liquid nitrogen. Frozen seeds were ground and total RNA extracted with the RNeasy Kit (QIAGEN) according to the manufacturer's

protocol. *Leaves*: Rosette leaves of 3-4 week old Arabidopsis plants were harvested and ground in liquid nitrogen with a pestle to a fine, homogenous powder. 100 mg of obtained powder was transferred into a microcentrifuge tube and the RNA isolation was performed with TRIZOL Reagent (Invitrogen) according to manufacturer's instructions. Alternatively, total RNA was isolated from mature leaf tissue in WT, ISE2-OE and ISE2-SUP plants using a Plant RNeasy Mini Kit (QIAGEN). Isolated RNA was then treated with rDNase (DNA-free<sup>TM</sup>, Ambion) according to the manufacturer's protocol to remove any contaminating genomic DNA.

### **cDNA synthesis**

0.5 µg of random hexamers was added to 1 µg of total RNA and the volume brought to 15 µl with nuclease-free water. The RNA was then denatured by incubation at 70°C for 5 minutes and then transferred immediately to ice for another 5 minutes. A mixture of 0.5 mM deoxynucleotides, nuclease-free water, 25 U of M-MLV reverse transcriptase (Promega) and the corresponding 5x buffer were added to a final volume of 25 µl. cDNA synthesis was performed according to the manufacturer's instructions. At the end of the reaction the mixture including the cDNA was mixed with 25 µL of nuclease-free water and 1 µl was used as template for subsequent DNA amplification (PCR).

### **Efficiency of RNA editing**

Total RNA (1 µg) from mature leaves of the same age was reverse transcribed using Invitrogen SuperScript III Reverse Transcriptase (Life Technologies) and random hexamers. Primers for cDNA synthesis were designed to amplify chloroplast genes previously shown to undergo editing and that were previously published (Tseng *et al.* 2013). The PCR products were sequenced and the chromatograms generated were used to determine the degree of

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editing. The heights of the peaks corresponding to the editing site were measured and the following ratio calculated: Editing efficiency =  $T/(C+T)$ . RNA samples from at least three independent biological replicates of WT Col-0 and ISE2-SUP were analyzed in this way. One sample was analyzed for TRV alone controls, TRV-*PDS* and *var2-2*. Statistical significance was calculated using a Student's t-test after an analysis of variance.

### Measuring transcript abundance by qPCR

Total RNA (300 ng for embryo samples or 3,000 ng for leaf samples) was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamers. Quantitative PCR was performed using the LightCycler 480 SYBR Green I Master Mix and a LightCycler 480 Real Time PCR system (Roche) with the following thermal cycling program: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 sec, 60 °C for 10 sec and 72 °C for 20 sec. The primers used to assess transcript levels of 80 genes encoded in the chloroplast genome have been previously described (de Longevialle *et al.* 2008). The raw data generated was analyzed using the LightCycler 480 software (Roche) and then exported to Excel. Chloroplast transcript levels were normalized to the median of all values.

### rRNA quantification by microfluidics

rRNAs were quantified on a Bioanalyzer 2100 (Agilent Technologies) using the RNA 6000 nano kit and the Plant RNA Pico assay, according to manufacturer's instructions. RNAs were quantified as previously described (Walter *et al.* 2010; Tiller *et al.* 2012; Fristedt *et al.* 2014).

## Northern Blotting

2 µg of total RNA was separated on a 1.25% agarose denaturing gel and then transferred to positively charged nylon membranes (Roche) according to Church and Gilbert (Church and Gilbert 1984). The hybridization was performed at 57 °C, for 12 h, using PCR-generated DIG-labeled probes (PCR DIG Probe Synthesis Kit, Roche) using primers indicated in Table S3. Full-length probes were generated for 23S rRNA, 5S rRNA and 4.5S rRNA that correspond to 2811bp, 121bp and 103 bp, respectively. A probe corresponding to 770bp was generated to detect 16S rRNA. Probes for intron 2 of *clpP1* and intron 1 of *ycf3* were generated by PCR with primers indicated (Table S3). The detection was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Hybridization and detection procedures were according to the manufacturer's instructions.

## Total protein extraction and Western blots

Rosette leaves of 3-4 week old Arabidopsis plants were harvested and ground in liquid nitrogen with a pestle to a fine, homogenous powder. 100 mg of powder was transferred to fresh microcentrifuge tubes, 1 ml of 5% aqueous solution of trichloroacetic acid (TCA) was added and samples immediately mixed on a vortex. Samples were incubated on ice for 15 minutes and then centrifuged at maximum speed for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl of solubilization buffer (80 mM Tris, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 1% dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 µg ml<sup>-1</sup> each of leupeptin, aprotinin, antipain, pepstatin and chymostatin, pH 6.8 (HCl)) supplemented with 7 M urea. 15 µl of 1M Tris was added to restore the basic pH. Proteins were extracted by vigorous vortexing for 10 minutes at room temperature and the sample was then centrifuged for 10 minutes at maximum speed. The supernatant was carefully transferred into a new microcentrifuge tube and samples were kept at -80 °C. The RC/DC kit (Bio-Rad) with gamma-immunoglobulin protein

as a protein standard was used to estimate protein concentration. 20 µg of the total protein extract obtained using TCA-treatment was then loaded on an SDS-polyacrylamide gel.

Resolved proteins were then transferred to polyvinylidene difluoride membrane (Millipore) and probed with indicated antibodies at indicated dilutions in TBS buffer supplemented with 0.05% Tween 80, pH 7.6. Horseradish peroxidase-coupled secondary antibodies and ECL reagents were used to detect primary antibodies (Thermo Fisher). The primary polyclonal antibodies specific to the proteins indicated were used at the following dilutions: D1 at 1:20 000, ClpP1 at 1:2000, PsaB at 1:20 000, Cyt b6 at 1:20 000, Hsp60 at 1:20 000, Hsp70 at 1:20 000, LHCB1 at 1:2 500, and F-ATPase a at 1:2 500. Monoclonal antibodies specific to GFP were used at a dilution of 1:1000. Either anti-rabbit or anti-mouse secondary antibodies were used at a dilution of 1:20 000. Antibodies against D1 and ClpP1 proteins were kindly provided by Drs. Wataru Sakamoto and Zach Adam, respectively. Antibodies against Hsp60 and Hsp70 were kindly provided by Dr. Masato Nakai.

### **RIP-seq**

Total protein extracts from 3-4 week old ISE2-OE plants or Arabidopsis plants stably expressing chloroplast-targeted GFP (Stonebloom *et al.* 2012) were incubated with anti-GFP antibodies according to the protocol used for co-IP. After the last wash of beads with the dilution buffer, the supernatant was discarded and the beads were subjected to the Trizol-based RNA isolation protocol (Invitrogen) scaled-down to 100 µl. RNA concentration was determined with the NanoDrop 1000 spectrophotometer (NanoDrop) and RNA sizes were determined with the Agilent Bioanalyzer (Agilent Technologies). The University of Rochester Genomics Research Center prepared and sequenced the libraries for Illumina-based sequencing. Samples were not depleted of rRNA. Paired end reads of 100 nt were generated for each sample. Detailed methods of library preparation, sequencing and data analysis are presented in the Supplemental Materials.

## Phylogenetic analysis

The full-length of 109 RNA helicase protein sequences were aligned using the ClustalX program with Gonnet 250 as the protein-weight matrix. The default alignment parameters were applied. The alignment was then bootstrapped 1000 times using SEQBOOT from the Phylip 3.69 package. Protein distances were then calculated using PROTDIST from the Phylip 3.69 package using the Jones-Taylor-Thornton matrix with 100 data sets analyzed. The tree was calculated using NEIGHBOR from the Phylip 3.69 package and the neighbor-joining method. Consensus tree of the RNA helicase proteins was generated using the CONSENSE from the Phylip 3.69 software. The extensin from *Methanoculleus marisnigri* was used as the outgroup. The tree was plotted using TREEVIEW software and then manually edited in Illustrator (Adobe).

## ACCESSION NUMBER:

RIP-seq data are available from the NCBI Sequence Read Archive (SRA) under the BioProject accession number SRP069971.

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## SHORT SUPPORTING INFORMATION LEGENDS

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**Figure S1.** Cosuppression of *ISE2-GFP* transgene in chlorotic plants.

**Figure S2.** RNA editing efficiency across 34 sites.

**Figure S3.** RNA editing efficiency in independent ISE2-SUP line, L2.

**Figure S4.** Distribution of reads from RIP-seq analysis across the chloroplast genome

**Figure S5.** Phylogenetic analysis of N-terminal helicase domains of Ski2-like proteins.

**Figure S6.** Identification of the “AIRGENE” motif by sequence alignment.

**Table S1.** Average efficiency of chloroplast C-to-U RNA editing.

**Table S2.** List of nuclear RNA species identified as RNA substrates by RIP-seq.

**Table S3.** Oligonucleotides used in this study.

**Methods S1.** RIP-seq data analysis.

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**Table 1.** RNA-seq alignment results. Read counts and percentages of total read pairs are shown with read counts expressed in millions (M). Reads were mapped to the Arabidopsis genome (TAIR 10.0). “Concordant” means that both mates in a pair of reads align correctly to the same locus.

Sample	Total read pairs	Concordant alignments to full genome	Concordant alignments to chloroplast tiles	Concordant alignments to full genome tiles
ISE2 A	69.2M	67.3M (96.7%)	31.3M (45.2%)	56.9M (78.2%)
ISE2 B	49.5M	48.2M (96.7%)	21.9M (44.1%)	41.1M (78.8%)
ISE2 C	48.3M	46.9M (96.6%)	23.7M (49%)	39.4M (78.1%)
GFP A	31.4M	27.8M (88.0%)	6.7M (21.4%)	23.9M (71.4%)
GFP B	41.2M	39.0M (94.3%)	9.2M (22.3%)	33.8M (76.7%)
GFP C	20.1M	18.5M (90.5%)	5.7M (28.2%)	16.5M (78.3%)

**Table 2.** RNA species identified as associating with ISE2 by RIP-seq. CDS is coding sequence including all exons associated with a gene; I.R. is inverted region downstream of a gene. Fold is fold enrichment in ISE2-GFP samples compared to GFP alone controls.

Locus ID	Gene	CDS p-value	CDS Fold	Intron p-value	Intron Fold	I.R. p-value	I.R. Fold
ATCG00920	<i>rrn16S.1</i>	4.401e-05	2.874	-	-	0.02145	0.02145
ATCG00950	<i>rrn23S.1</i>	0.0004091	6.0152	-	-	0.01661	8.989
ATCG01000	<i>ycf1.1</i>	0.0006835	2.703	-	-	-	-
ATCG00040	<i>matK</i>	0.004438	6.248	-	-	-	-
ATCG01100	<i>ndhA</i>	0.006166	10.25	0.007169	8.24	-	-
ATCG00360	<i>ycf3-1</i>	0.006528	10.57	0.008046	4.453	-	-
	<i>ycf3-2</i>			0.03092	4.92		
ATCG00340	<i>psaB</i>	0.01044	3.821	-	-	-	-
ATCG01110	<i>ndhH</i>	0.01107	3.784	-	-	-	-
ATCG00130	<i>atpF</i>	0.01141	6.129	0.01096	5.35	0.02623	3.85
ATCG00380	<i>rps4</i>	0.01173	9.981	-	-	-	-
ATCG00840	<i>rpl23</i>	0.01342	8.083	-	-	-	-
ATCG00640	<i>rpl33</i>	0.01439	5.892	-	-	-	-
ATCG01050	<i>ndhD</i>	0.01485	4.974	-	-	-	-
ATCG00540	<i>petA</i>	0.01539	6.831	-	-	-	-



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<b>ATCG00740</b>	<i>rpoA</i>	0.01568	2.091	-	-	-	-
<b>ATCG00670</b>	<i>clpP1-1</i>	0.01568	6.038	0.01664	6.857	0.01568	5.972
	<i>clpP1-2</i>			0.025	9.73		
<b>ATCG00770</b>	<i>rps8</i>	0.01692	4.381	-	-	-	-
<b>ATCG00830</b>	<i>rpl2.1</i>	0.01739	4.269	0.01671	4.78	-	-
<b>ATCG01020</b>	<i>rpl32</i>	0.01741	14.61	-	-	-	-
<b>ATCG00960</b>	<i>rrn4.5S</i>	0.01776	3.968	-	-	0.02815	4.91
<b>ATCG00680</b>	<i>psbB</i>	0.01786	3.627	-	-	0.02115	11.45
<b>ATCG00480</b>	<i>atpB</i>	0.01786	5.333	-	-	0.01962	4.306
<b>ATCG00905</b>	<i>rps12.2</i>	0.01808	14.32	0.02348	3.662	0.02149	17.44
<b>ATCG00270</b>	<i>psbD</i>	0.01919	4.256	-	-	-	-
<b>ATCG00065</b>	<i>rps12</i>	0.01962	8.128	-	-	-	-
<b>ATCG00520</b>	<i>ycf4</i>	0.01966	3.219	-	-	0.0276	16.96
<b>ATCG00070</b>	<i>psbK</i>	0.0202	4.322	-	-	-	-
<b>ATCG00350</b>	<i>psaA</i>	0.02105	6.032	-	-	0.02932	8.991
<b>ATCG00780</b>	<i>rpl14</i>	0.02111	5.14	-	-	-	-
<b>ATCG00330</b>	<i>rps14</i>	0.02126	5.545	-	-	0.02006	7.32
<b>ATCG00280</b>	<i>psbC</i>	0.02258	5.054	-	-	0.04546	4.349
<b>ATCG00890</b>	<i>ndhB.1</i>	0.02321	5.302	0.02985	6.25	0.01388	4.744
<b>ATCG00120</b>	<i>atpA</i>	0.02374	5.794	-	-	0.02468	6.054
<b>ATCG00720</b>	<i>petB</i>	0.02439	6.716	0.02495	12.08	0.02636	8.912

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<b>ATCG01060</b>	<i>psaC</i>	0.02475	7.799	-	-	-	-
<b>ATCG00750</b>	<i>rps11</i>	0.02511	3.892	-	-	-	-
<b>ATCG00530</b>	<i>ycf10</i>	0.0255	10.45	-	-	0.01774	16.28
<b>ATCG00560</b>	<i>psbL</i>	0.02582	9.33	-	-	-	-
<b>ATCG00020</b>	<i>psbA</i>	0.02741	7.185	-	-	0.02498	5.959
<b>ATCG00810</b>	<i>Rpl22</i>	0.02708	3.55	-	-	-	-
<b>ATCG00150</b>	<i>atpI</i>	0.0271	3.429	-	-	-	-
<b>ATCG00900</b>	<i>rps7</i>	0.02728	9.117	-	-	-	-
<b>ATCG00500</b>	<i>accD</i>	0.0283	3.995	-	-	-	-
<b>ATCG00700</b>	<i>psbN</i>	0.0286	3.094	-	-	0.02416	10.82
<b>ATCG00430</b>	<i>psbG</i>	0.02943	5.95	-	-	-	-
<b>ATCG00790</b>	<i>rpl16.2</i>	0.02962	4.936	0.007707	4.92	-	-
<b>ATCG01070</b>	<i>ndhE</i>	0.03051	8.76	-	-	-	-
<b>ATCG00470</b>	<i>atpE</i>	0.03075	5.273	-	-	-	-
<b>ATCG00420</b>	<i>ndhJ</i>	0.03149	6.02	-	-	-	-
<b>ATCG00140</b>	<i>atpH</i>	0.0315	7.986	-	-	0.00937	4.368
<b>ATCG00570</b>	<i>psbF</i>	0.03175	12.83	-	-	-	-
<b>ATCG00490</b>	<i>rbcL</i>	0.03305	10.49	-	-	-	-
<b>ATCG00440</b>	<i>ndhC</i>	0.03306	4.916	-	-	-	-
<b>ATCG00080</b>	<i>psbI</i>	0.03384	5.305	-	-	-	-
<b>ATCG00580</b>	<i>psbE</i>	0.03407	13.29	-	-	-	-

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<b>ATCG01080</b>	<i>ndhG</i>	0.03566	4.348	-	-	-	-
<b>ATCG00550</b>	<i>psbJ</i>	0.03597	11.88	-	-	0.03178	22.1
<b>ATCG01090</b>	<i>ndhI</i>	0.03686	4.756	-	-	-	-
<b>ATCG00710</b>	<i>psbH</i>	0.03631	5.878	-	-	0.02365	4.908
<b>ATCG00300</b>	<i>ycf9</i>	0.04059	4.33	-	-	-	-
<b>ATCG00730</b>	<i>petD</i>	0.04479	2.994	0.002859	13.24	-	-
<b>ATCG00970</b>	<i>rrn5S</i>	0.049	4.458	-	-	-	-
<b>ATCG00180</b>	<i>rpoC1</i>	-	-	0.01523	3.165	-	-
<b>ATCG00360</b>	<i>trnV.1</i>	-	-	0.01617	3.165	-	-
<b>ATCG00030</b>	<i>trnK</i>	-	-	0.001641	9.032	-	-
<b>ATCG00930</b>	<i>trnI.2</i>	-	-	0.01866	7.543	-	-
<b>ATCG00100</b>	<i>trnG.1</i>	-	-	0.02067	6.733	-	-
<b>ATCG00400</b>	<i>trnL.1</i>	-	-	0.02489	9.813	0.003354	5.399
<b>ATCG00940</b>	<i>trnA.1</i>	-	-	0.02836	11.05	0.01413	3.48
<b>ATCG00660</b>	<i>Rpl20</i>	-	-	-	-	0.008622	3.137
<b>ATCG00460</b>	<i>trnM</i>	-	-	-	-	0.01776	6.482
<b>ATCG00610</b>	<i>trnW</i>	-	-	-	-	0.02028	37.69
<b>ATCG00160</b>	<i>Rps2</i>	-	-	-	-	0.02437	5.174
<b>ATCG00260</b>	<i>trnT.1</i>	-	-	-	-	0.03119	5.818
<b>ATCG00290</b>	<i>trnS.2</i>	-	-	-	-	0.04627	5.748
<b>ATCG00510</b>	<i>psaI</i>	-	-	-	-	0.01847	3.162

## FIGURE LEGENDS

**Figure 1.** Cosuppression of *ISE2* leads to decreased photosynthetic capacity.

(a, b) Wild-type Col-0 plants (a) were grown alongside transgenic plants stably expressing *ISE2*-GFP (*ISE2*-OE plants) (b). Some of the *ISE2*-OE plants developed yellow leaves (*ISE2*-SUP) and were smaller than the Col-0 or *ISE2*-OE plants with green leaves (b).

(c,d) Photosystem II efficiency was measured *in situ* for Arabidopsis WT Col-0 (c) or *ISE2*-GFP plants (d) after dark-adaptation. PS II fluorescence is presented as the ratio of variable to maximal fluorescence,  $F_v/F_m$ . Values are presented as a heat map.

(e)  $F_v/F_m$  for *ISE2*-OE plants was the same as in Col-0 control plants, while  $F_v/F_m$  for *ISE2*-SUP leaves was significantly reduced. Asterisks indicate  $p < 0.05$ .

(f) The initial fluorescence,  $F_o$ , was significantly increased in *ISE2*-SUP leaves compared to Col-0 controls and *ISE2*-OE plants. Asterisks indicate  $p < 0.05$ .

(g) The levels of chlorophylls and carotenoids were significantly reduced in *ISE2*-SUP leaves compared to Col-0 controls and *ISE2*-OE plants. Asterisks indicate  $p < 0.05$ .

**Figure 2.** Loss of *ISE2* compromises C-to-U RNA editing at specific sites.

Editing at sites *clpP1-559* (a), *petL-50* (b), *rpoA-200* (c), *rpoB-338* (d), *rpoB-551* (e) and *rps14-149* (f) was measured in WT Col-0, *ISE2*-SUP or *var2-2* leaves of the same age.

Editing efficiency is represented as a fraction, and calculated as  $T/(C+T)$ . Three independent biological replicates were used for WT Col-0 and *ISE2*-SUP, and one for *var2-2*. Error bars representation one standard deviation. Asterisk indicates statistically significant difference (Student's t-test) from WT Col-0. A representative chromatogram centered on the editing site is shown next to the corresponding bar for each graph. The yellow strip indicates the edited site.

**Figure 3.** Chloroplast transcripts show altered accumulation in the absence of ISE2.

(a) Levels of chloroplast transcripts in mid-torpedo stage embryos were measured by quantitative RT-PCR (qRT-PCR). Expression levels for Col-0 embryos are shown in black bars, levels for *ise2* embryos are in white bars. Chloroplast transcripts are grouped into functional categories. Error bars show standard deviation.

(b) Levels of chloroplast transcripts three-week old leaves were measured by quantitative RT-PCR (qRT-PCR). Expression levels for Col-0 leaves are shown in black bars, those for ISE2-OE leaves are in grey, and levels for ISE2-SUP leaves are in white bars. Chloroplast transcripts are grouped into functional categories and error bars show standard deviation.

**Figure 4.** ISE2 is required for splicing of a group II intron in *ycf3* transcripts.

(a) Schematic of the gene structures for *clpP1* and *ycf3*. Arrows indicate the relative annealing positions of primers spanning each intron.

(b) RT-PCR with primers for intron 1 (left panel) or intron 2 (right panel) of *clpP1* shows increased accumulation of intron 2 in ISE2-SUP leaves compared to Col-0 and ISE2- leaves.

(c) RT-PCR with primers for intron 1 (left panel) or intron 2 (right panel) of *ycf3* shows increased accumulation of intron 1 in ISE2-SUP leaves compared to Col-0 and ISE2- leaves.

(d-e) Northern blot using a probe corresponding to intron 2 of *clpP1* (d) or intron 1 of *ycf3* (e) (illustrated below the blot) shows a complex pattern of bands in ISE2-SUP tissues distinct from that observed in Col-0 or ISE2-OE leaves. Ethidium bromide stained 18S rRNA is shown as loading control.

**Figure 5.** Loss of ISE2 leads to defects in rRNA processing.

(a) A graphical representation of the chloroplast *rrn* operon. The full-length transcript is 7.4 kb. The mature rRNAs in their final processed forms and lengths are shown in the second row.

(b) Total RNA isolated from Arabidopsis WT Col-0, ISE2-OE and ISE2-SUP leaves. The gel was stained with ethidium bromide. Asterisks indicate positions of bright bands appearing in RNA from ISE2-SUP tissues but present at much lower levels in other tissues.

(c-f) RNA samples from Col-0, ISE2-OE and ISE2-SUP leaves were analyzed by Northern blot analyses of 23S, 16S, 4.5S and 5S rRNAs using specific DIG-labeled probes. Labels to the right of the gels indicate the origin of each band.

**Figure 6.** ISE2 is required for accumulation of chloroplast-encoded proteins

(a) Accumulation of rRNAs as a surrogate for ribosomal subunit abundance. 16S, the 16S rRNA of the small chloroplast subunit. 23S H1, the 1.1-kb fragment of the 23S rRNA after processing at the “hidden break” (H1) in the 50S subunit of the chloroplast ribosome. 18S, the 18S rRNA of the 40S subunit of the cytosolic ribosome. 25S, the 25S rRNA of the 60S subunit of the cytosolic ribosome. The measurements are based on at least three biological replicates, and the error bars represent one standard deviation. Asterisks indicate statistical significance,  $p < 0.05$ .

(b) Western blot analyses of total protein extracts isolated from WT, ISE2-OE or ISE2-SUP plants. Blots were probed with antibodies specific to indicated proteins.

(c) Colloidal Coomassie-stained gel is shown as loading control and to indicate changes to levels of RbcL (top arrow) and Lhcb (bottom arrow) proteins.

(d) The decrease in Lhcb proteins was confirmed by Western blot analysis with antibodies against Lhcb proteins.

**Figure 7.** Identification of RNAs that are potential ISE2 substrates.

(a) Proportions of gene features represented in RNAs enriched in ISE2-GFP samples compared to GFP alone control samples. IR: intergenic region downstream of a gene, CDS: coding sequence of a gene including all exons of a given gene.

(b) Proportional Venn diagram indicating how genes were represented in the set of potential ISE2 substrates. Diagram was generated using the BioVenn web application (Hulsen *et al.* 2008).

(c) Functional categories of proteins encoded by genes that are potential ISE2 substrates.

**Figure 8.** Phylogenetic analysis of ISE2 and Ski2 proteins. Asterisks indicate Arabidopsis proteins.

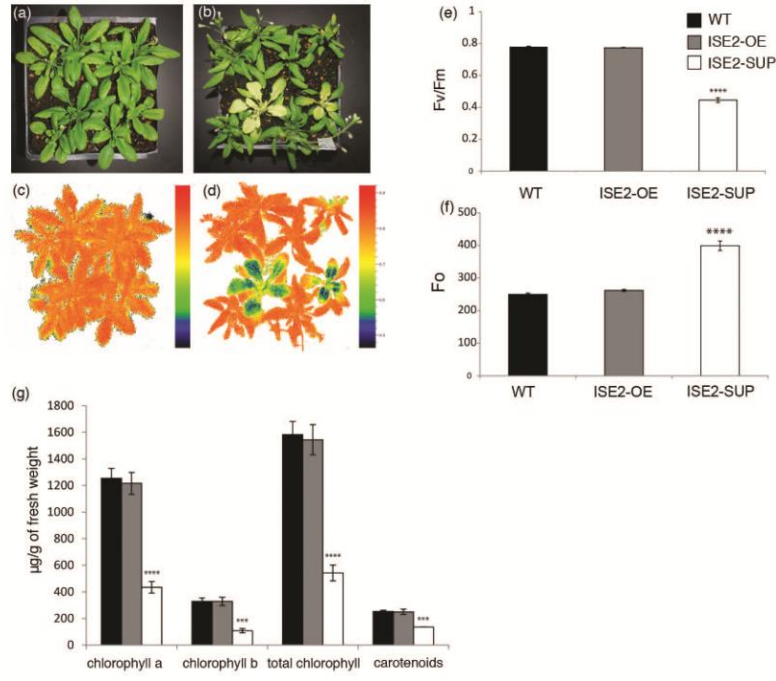


Figure 1



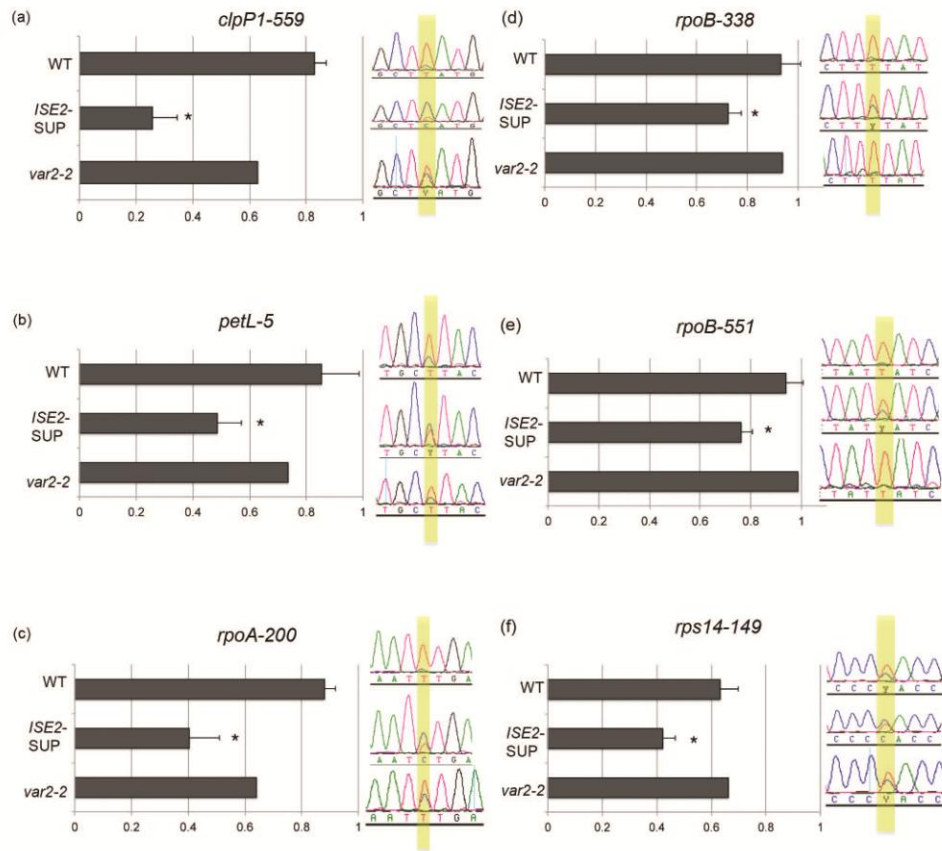
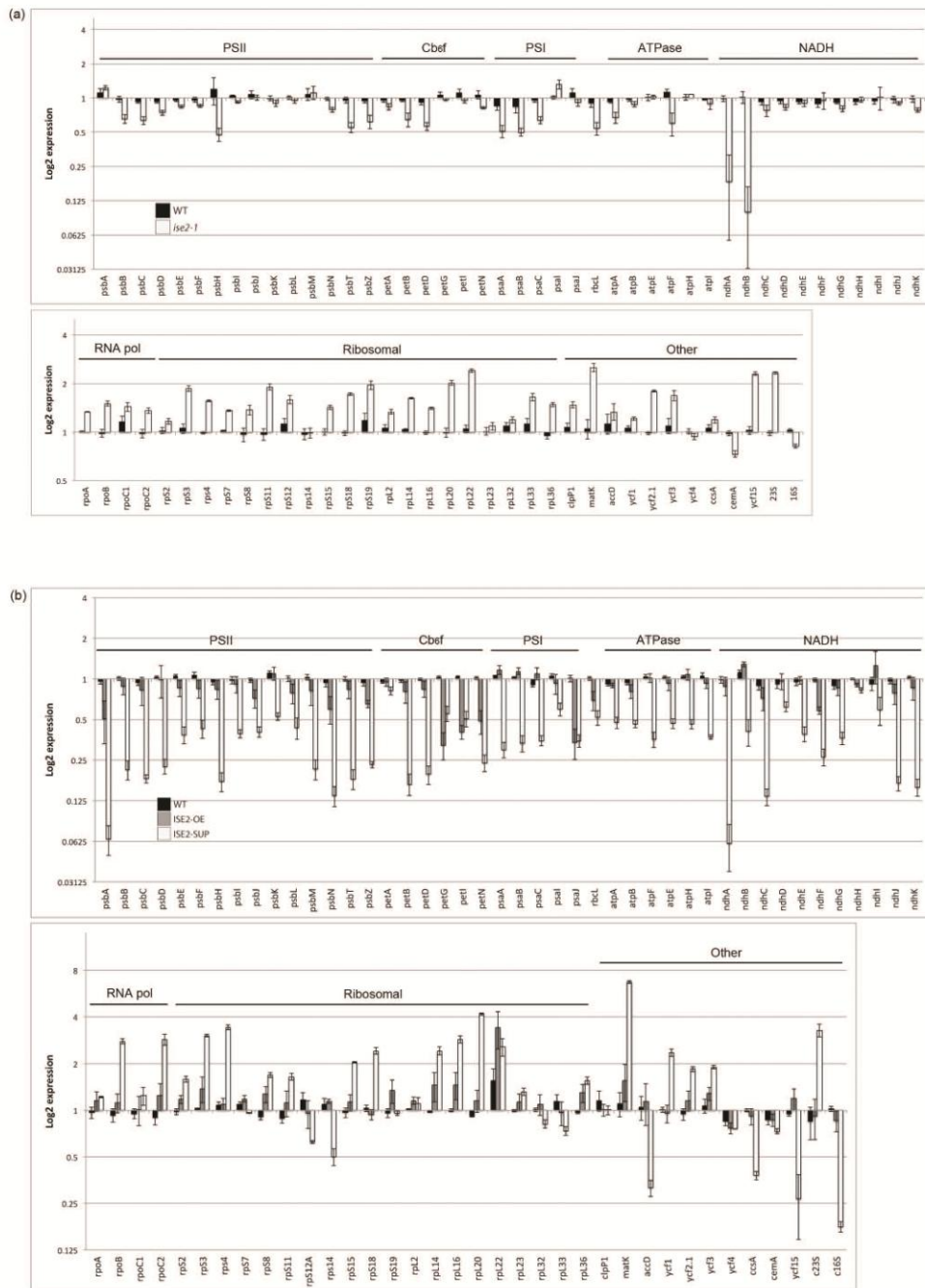


Figure 2



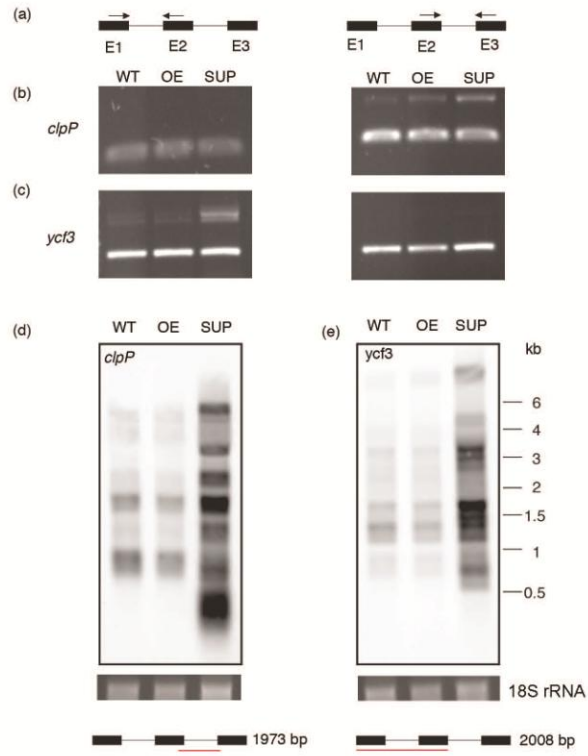


Figure 4

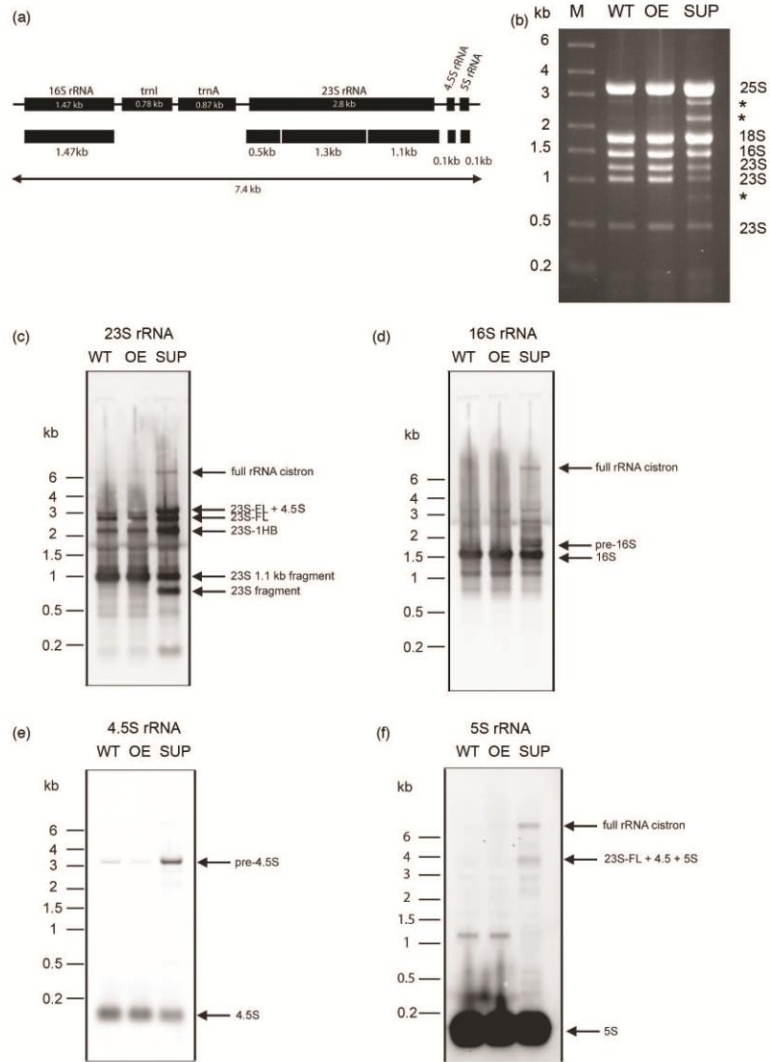


Figure 5

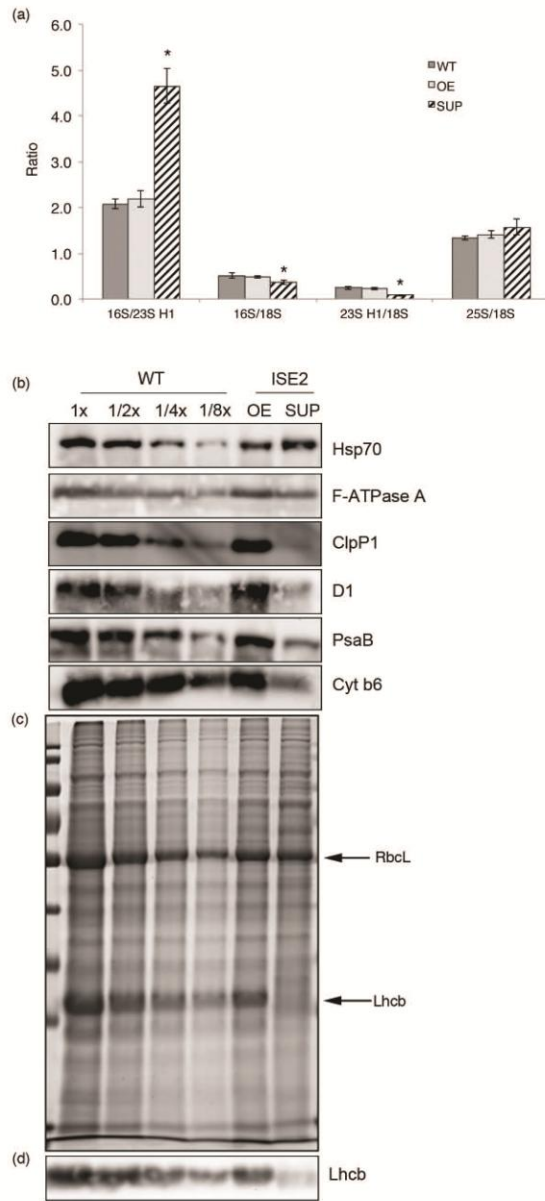


Figure 6

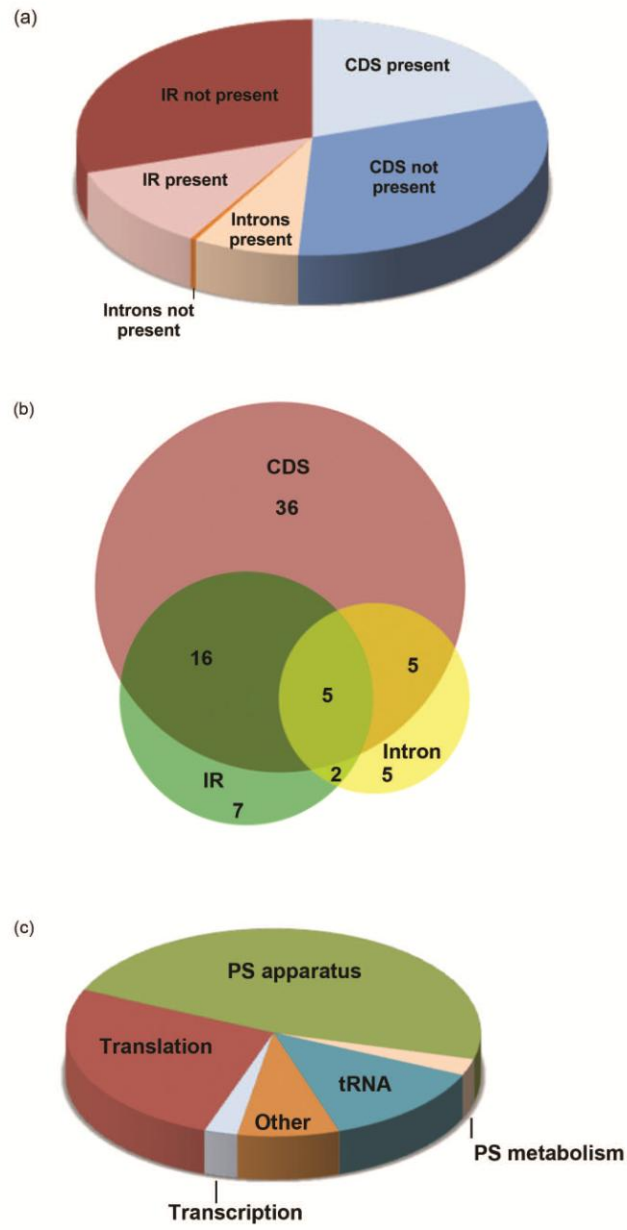


Figure 7

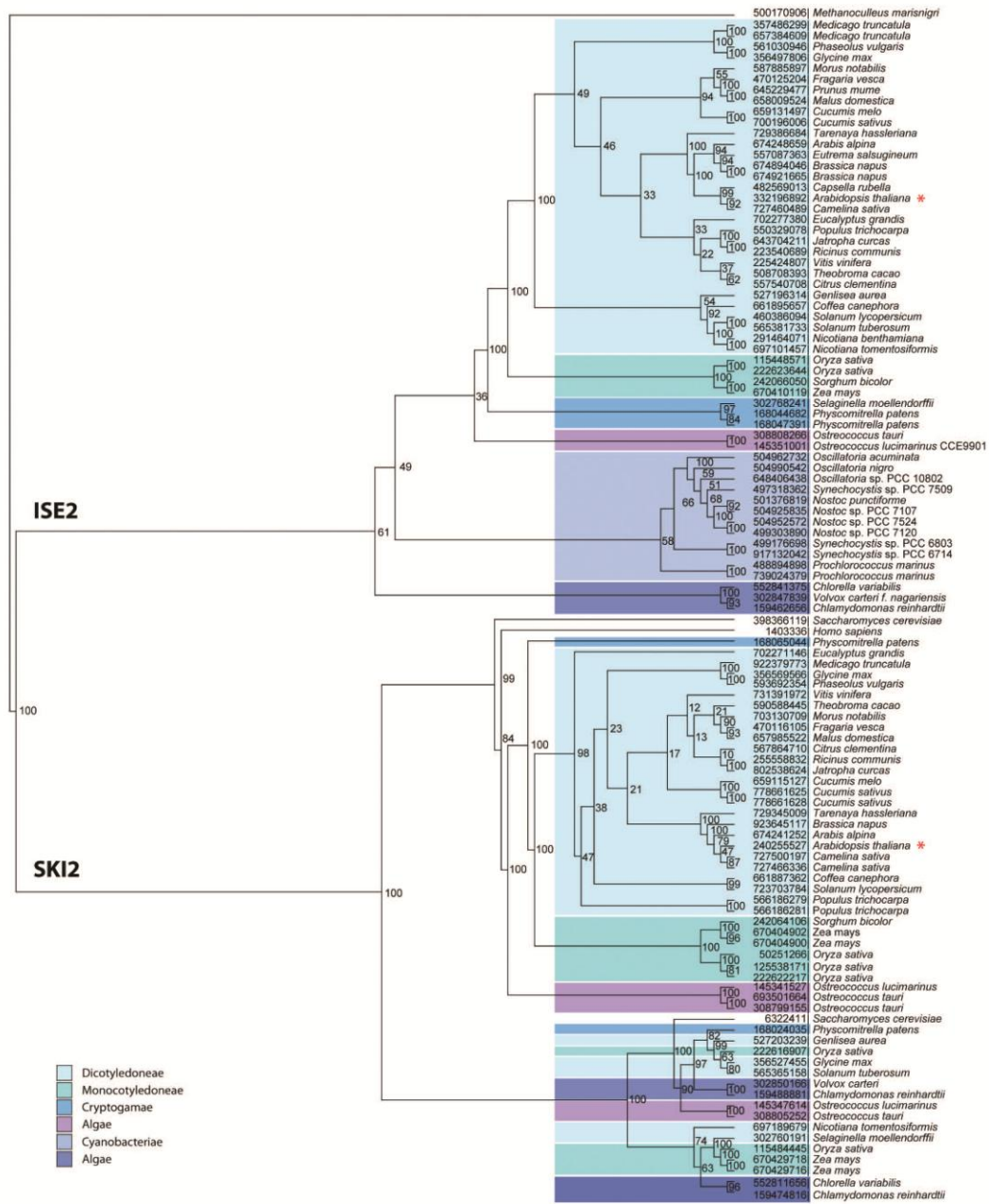


Figure 8