Altered Expression of a Chloroplast Protein Affects the Outcome of Virus and Nematode Infection

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The chloroplast-resident RNA helicase ISE2 (INCREASED SIZE EXCLUSION LIMIT2) can modulate the formation and distribution of plasmodesmata and intercellular trafficking. We have determined that ISE2 expression is induced by viral infection. Therefore, the responses of *Nicotiana benthamiana* plants with varying levels of ISE2 expression to infection by *Tobacco mosaic virus* and *Turnip mosaic virus* were examined. Surprisingly, increased or decreased ISE2 expression led to faster viral systemic spread and, in some cases, enhanced systemic necrosis. The contributions of RNA silencing and hormone-mediated immune responses to the increased viral susceptibility of these plants were assessed. In addition, *Arabidopsis thaliana* plants with increased ISE2 expression were found to be more susceptible to infection by the beet cyst nematode *Heterodera schachtii*. Our analyses provide intriguing insights into unexpected functional roles of a chloroplast protein in mediating plant-pathogen interactions. The possible roles of plasmodesmata in determining the outcomes of these interactions are also discussed.

There is abundant and increasing evidence that chloroplasts control multiple facets of plant defense (Bobik and Burch-Smith 2015; Zhao et al. 2016). Chloroplasts are the source of the defense hormones salicylic acid (SA), jasmonic acid (JA) and its derivatives, and abscisic acid (ABA). They are also important for generating reactive oxygen species (ROS) and other redox-related molecules that are critical mediators of signaling during plant-pathogen interactions (Rojas et al. 2014; Sewelam et al. 2014; Torres et al. 2006). During defense responses, chloroplasts also generate retrograde signals that can modify expression of nucleus-encoded genes (Chan et al. 2016; Nomura et al. 2012). Moreover, recent studies have shown that chloroplasts can physically interact with nuclei and other cellular components through tubular extensions called stromules. Indeed, they are likely part of the mechanism of N immune receptor-mediated defense against *Tobacco mosaic virus* (TMV) (Caplan et al. 2008, 2015), and are also likely involved in infection by DNA viruses (Krenz et al. 2012).

Cell-to-cell communication in plants is also an emerging focus in studies of the plant defense response. Much is known about the role of plasmodesmata (PD) in various aspects of plant growth and development (Burch-Smith et al. 2012; Lucas and Lee 2004; Yadav et al. 2014), and it is becoming clear that through their roles in micro- and macromolecular trafficking PD are important players in defense (Faulkner 2013; Lee and Lu 2011; Lee 2015; Lim et al. 2016; Wang et al. 2013). Among plant pathogens viruses are best known for their use of the PD-mediated symplasmic route for systemic spread during infection. The plant antiviral response includes the deposition of callose at the necks of PD to effectively restrict cell-to-cell spread (Ellinger et al. 2013; Lee 2015; Levy et al. 2007). Cyst nematodes (*Heterodera* and *Globodera* spp.) are another important group of plant pathogens in which PD play a key role in the etiology and structure of their feeding sites, the syncytia, formed in the root vascular tissues. The importance of PD to successful nematode parasitism was demonstrated by manipulating the level of callose deposited at syncytial PD; reduced callose degradation led to smaller syncytia and a higher female-to-male ratio (Hofmann et al. 2010). The role of PD in modulating viral and nematode infections has been extended to almost all other classes of plant pathogens by recent findings demonstrating the importance of PD-mediated intercellular trafficking in systemic acquired resistance (Carella et al. 2015; Lim et al. 2016).

Since PD function is essential to plant survival, few mutants with abnormal PD function have been identified. Interestingly, one of these is a defect in the chloroplast-resident DEAH-type RNA helicase named INCREASED SIZE EXCLUSION LIMIT2 (ISE2) for its loss-of-function phenotype of increased PD-mediated trafficking (Burch-Smith and Zambryski 2010; Kim et al. 2002; Kobayashi et al. 2007). *Arabidopsis ise2* mutants have severely defective development, and some alleles led to arrested embryonic development (Kobayashi et al. 2007). Mutant *ise2* embryos have increased numbers of twinned and branched PD, as compared with wild-type embryos at the same developmental stage (Burch-Smith and Zambryski 2010). *Nicotiana benthamiana* leaves in which the ISE2 homolog has been silenced similarly contain a higher frequency of twinned and branched PD, compared with nonsilenced plants, and increased PD-mediated intercellular trafficking of fluorescent probes (Burch-Smith and Zambryski 2010).

Given that ISE2 is a chloroplast protein that can influence the formation and function of PD, the role of ISE2 in plant-pathogen interactions was investigated. Viral accumulation, spread, and disease induction were examined through ISE2 knockdown or overexpression in *N. benthamiana* plants. In addition, the effects

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of elevated *ISE2* expression in *Arabidopsis* on beet cyst nematode infection were also determined. The results provide insight into the interplay between PD and chloroplasts in mediating plant defense responses to pathogens.

RESULTS

ISE2 expression is increased upon TMV infection.

The level of *NbISE2* expression was examined in wild-type *N. benthamiana* plants in response to TMV or Turnip mosaic virus (TuMV) infection. Leaves were agroinfiltrated with cultures expressing either TMV or TuMV and samples were analyzed for *ISE2* expression at 0, 6, 12, 18, and 24 h post-viral infection (hpi). *NbISE2* expression was dramatically increased at 18 hpi upon infection with either virus (Fig. 1A and B). *Rubisco activase* (*RCA*) and *AtpC* (ATP synthase- γ subunit gene) are two other nuclear genes encoding chloroplast proteins, and both genes are involved in TMV infection (Bhat et al. 2013). Expression of *RCA* and *AtpC* is repressed in response to TMV infection (Bhat et al. 2013), a finding confirmed in our system (Supplementary Fig. S1).

Silencing *ISE2* increases susceptibility to TMV.

Based on the phenotypes of increased intercellular trafficking and PD formation observed in plants depleted of *ISE2*, we hypothesized that decreased *ISE2* expression would lead to increased local intercellular spread of viral pathogens and result in increased systemic spread. Tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS) was used to knock down *ISE2* expression (Burch-Smith and Zambryski 2010). Fully expanded leaves of 5-week-old plants were then mechanically inoculated with plant sap containing 65 ± 5 infectious particles of TMV(30B)-GFP (TMV-based vector 30B expressing green fluorescent protein). Development of systemic infection in the upper leaves of the infected plants was monitored for 4 days by the detection of GFP signal with UV light. Representative plants with systemic infection are shown in Figure 2A. At that time point, TMV(30B)-GFP had spread systemically in fewer than 5% of TRV-infected, nonsilenced control plants, while approximately 40% of *ISE2*-silenced plants were systemically infected (Fig. 2B). Local intercellular movement of the virus in the TRV-infected, nonsilenced controls and *ISE2*-silenced plants was quantified by imaging fluorescent lesions in the inoculated leaves at 4 days postinfection (dpi). The foci in *ISE2*-silenced leaves were slightly larger than those on control leaves, although the difference was not statistically significant (Fig. 2C).

Increased *ISE2* expression enhances susceptibility to TMV.

We had previously generated stable transgenic *N. benthamiana* lines expressing *Arabidopsis ISE2* fused to GFP and expressed under the control of the constitutive *Cauliflower mosaic virus* 35S promoter (*35S:AtISE2-GFP*) (Burch-Smith et al. 2011). After assessing the level of transgene expression and *AtISE2*-GFP accumulation in 12 lines, two lines, OE1 and OE3, with consistently high levels of *AtISE2* expression and *ISE2*-GFP accumulation were selected for further analysis. These lines appeared indistinguishable from wild-type *N. benthamiana* plants, and chloroplast development and function were apparently unaffected by increased *ISE2* levels (Supplementary Fig. S2), except for increased starch accumulation in chloroplasts of overexpressing (OE) plants (Supplementary Fig. S3).

The effect of increased *ISE2* expression on viral infection was examined in those lines. It is important to mention that, in these and subsequent experiments, wild-type and OE plants were agroinfiltrated with a nonsilencing TRV-VIGS vector when used in experiments that included *TRV-ISE2*, so that all plants used contained TRV (Supplementary Fig. S4). Thus, any

effects could be attributed to the changes in *ISE2* expression and not TRV infection. Four days after inoculation with TMV (30B)-GFP, almost 40% of OE1 plants and 60% of OE3 plants were systemically infected, compared with fewer than 5% of TRV-infected, nonsilenced control plants (Fig. 2B). The increased TMV(30B)-GFP spread in OE plants was confirmed in plants not containing TRV. TMV(30B)-GFP infection foci on OE1 and OE3 leaves tended to have smaller diameters than those on TRV-*ISE2* and control infiltrated leaves (Fig. 2C). Interestingly, *ISE2*-GFP accumulated in the OE lines during TMV infection, despite transgene expression being driven by the 35S promoter (Supplementary Fig. S5).

TMV infection also led to the development of systemic necrosis in both OE lines (Fig. 2D), with more than 60% of those plants displaying wilting, browning, and drying of the upper, systemically infected leaves (Fig. 2E). Strikingly, no necrotic symptoms were visible in TRV-infected, nonsilenced control, or *ISE2*-silenced plants (Fig. 2D and E). Systemic necrosis is a consequence of the massive production of ROS and programmed cell death induced by SGT1 (SUPPRESSOR OF G-TWO ALLELE OF *SKP1*) and RAR1 (REQUIRED FOR *MLA12* RESISTANCE) in complex with HSP90 (HEAT SHOCK PROTEIN OF 90 KDA) (Aguilar et al. 2015; Komatsu et al. 2010). Compared with nonsilenced, TRV-infected control, and TRV-*ISE2* plants, *SGT1* and *RAR1* expression was more strongly induced in the *ISE2*-OE plants in response to TMV infection (Supplementary Fig. S6).

ISE2 modulates susceptibility to TuMV.

Next, we asked if *ISE2* also modulates plant responses to other viruses. Representative plants systemically infected with the evolutionarily distinct virus TuMV expressing GFP (TuMV-GFP), at 9 dpi, are shown in Figure 3A. Although the long-distance spread of TuMV was slower than that of TMV (9 versus 4 dpi), systemic invasion was significantly enhanced in one line overexpressing *ISE2* (OE3) and in TRV-*ISE2* plants compared with nonsilenced, TRV-infected control plants (Fig. 3B). The other line overexpressing *ISE2* (OE1) was also systemically infected more frequently. Another characteristic of the infection caused by TuMV-GFP was necrosis of the vascular tissues, particularly in the petioles of the bottom leaves (Fig. 3C). Necrotic streaks appeared between 10 and 14 dpi on leaf petioles and major stems, causing the entire plant to wilt. By 14

Fig. 1. Effect of Tobacco mosaic virus (TMV) and Turnip mosaic virus (TuMV) infection on *ISE2* expression in wild-type *Nicotiana benthamiana* plants. **A**, The relative accumulation of *NbISE2* mRNA in TMV(30B)-GFP-inoculated leaves over 24 h was measured by quantitative polymerase chain reaction (qPCR). **B**, The relative accumulation of *NbISE2* mRNA in TuMV-GFP-inoculated leaves over 24 h was measured by qPCR. Expression was normalized to mock-infected leaves. Results are representative of three biological replicates. Asterisks indicate *P* values compared with control plants at $P < 0.05$ (Student's *t* test). cDNA was prepared from three leaves collected from three different plants. Error bars represent standard deviation.

dpi, the majority of *ISE2*-silenced or *ISE2*-OE plants showed severe necrosis of the four oldest leaves compared with the control plants (Fig. 3D). TuMV has been shown to move systemically through xylem, which induces cell death of the phloem without affecting xylem integrity (Wan et al. 2015). Thus, altered *ISE2* expression led to increased susceptibility to two unrelated viruses, TMV and TuMV.

Arabidopsis plants overexpressing *ISE2* are more susceptible to cyst nematode infection.

ISE2 expression is higher in *Arabidopsis* shoots than in roots (Kobayashi et al. 2007). A previous transcriptome analysis of pure syncytial tissue isolated by microaspiration revealed that *ISE2* expression was strongly induced by *Heterodera schachtii* compared with noninfected root tissues (Szakasits et al. 2009). Therefore, we measured *ISE2* expression in *H. schachtii*-infected roots over the course of syncytium development. At 3 dpi, *ISE2* expression was about 3.7-fold higher in infected roots than in uninfected roots and this increased to approximately

sixfold higher expression by 7 dpi (Fig. 4A). However, by 10 dpi, *ISE2* expression in infected roots had returned to almost the same level as in uninfected control roots (Fig. 4A). The induction of *ISE2* expression during the establishment phase of the *H. schachtii* infection supports the idea that *ISE2* has an important role in this host-pathogen interaction and is consistent with the changes observed to *ISE2* expression during viral infections.

Several *Arabidopsis* lines rescued by expression of the *AtISE2-GFP* transgene and, therefore, with varying levels of *ISE2* expression (called RL lines) (Fig. 4B and C) (Kobayashi et al. 2007) were examined for their susceptibility to the beet cyst nematode *H. schachtii*. Ten-day-old plants were infected with juvenile stage 2 nematodes (J2) and the number of J4 females was scored 3 weeks later and used to determine nematode susceptibility levels. Three of the four lines examined showed statistically significant increases in nematode susceptibility compared with the wild-type Col-0 control plants (Fig. 4D). Moreover, the size and structure of the fully developed

Fig. 2. Tobacco mosaic virus vector 30B–green fluorescent protein [TMV(30B)-GFP] infection of *Nicotiana benthamiana* plants. **A**, Representative images of control, TRV-*ISE2*, OE1, and OE3 plants showing systemic infection at 4 days postinfection (dpi) as detected with UV light. Scale bar = 3 cm. The infected leaves are indicated with arrowheads. **B**, Quantitative representation of systemic infection in plants at 4 dpi. Asterisks indicate *P* values compared with control plants at *P* < 0.05 (Z test for two population proportions). Average number of systemically infected plants was calculated from at least six independent experiments; 10 plants of each plant line were used in each experiment. Error bars represent standard deviation. **C**, The distribution of the sizes of the fluorescent local lesions induced by TMV(30B)-GFP inoculated onto leaves and measured at 4 dpi. Bar markers indicate average foci size. *n* = 28. **D**, Systemic necrosis induced by TMV in *N. benthamiana* plants at 9 dpi. Asterisks (*) indicate *P* < 0.05 (Z test for two population proportions). Plants used for quantitation of systemic infection were monitored for systemic necrosis. Error bars represent standard deviation. **E**, Representative images of the plants imaged at 9 dpi. Stem necrosis and lodging of the apical regions indicated by white arrows.

H. schachtii-induced syncytia (feeding sites) were monitored. In *H. schachtii*-infected *Arabidopsis* roots, callose accumulated along the outer cells of the fully developed syncytia and was much more prominent in the roots of RL plants, as detected by aniline blue staining (Fig. 4E). At 3 weeks after infection, syncytia on the roots of *Arabidopsis* RL plants were slightly larger than those on the control plants (Fig. 4F). Similar experiments examining the effects of reduced *ISE2* expression were not possible, due to the unavailability of *Arabidopsis* tissue with the appropriate *ISE2* knockdown. Nonetheless, the results with the rescued lines demonstrate that *ISE2* is important for determining susceptibility to cyst nematode infection.

Given that both viral and nematode infections are known to depend on PD function and were both affected by changes in *ISE2* expression, the effect of *ISE2* overexpression on a pathogen that does not explicitly depend on PD trafficking was examined. For this, *N. benthamiana* TRV-*ISE2*-infected or OE plants were infected with the pathogenic bacteria *Pseudomonas syringae* DC3000. No differences in *P. syringae* DC3000 CFU or in expression of the *NbPR5* marker gene were observed between control, *ISE2*-silenced, and OE plants at 4 dpi (Supplementary Fig. S7). Thus, overexpression of *ISE2* appeared to dramatically influence susceptibility to two different types of pathogens whose successful invasion is PD-dependent, but there was no effect on pathogens whose invasion is PD-independent.

***ISE2* is a negative regulator of intercellular trafficking.**

In order to understand the altered pathogen susceptibility in *ISE2*-silenced and *ISE2*-OE plants, PD-mediated symplasmic trafficking in *N. benthamiana* plants with altered *ISE2* expression was measured, using the passive diffusion of a fluorescent reporter molecule consisting of two tandem copies of GFP (2×GFP; molecular mass of 54 kDa) (Brunkard et al. 2015). Examples of fluorescent foci of different sizes formed by movement of 2×GFP from the primary transformed cell are provided in Figure 5A. We confirmed that silencing *ISE2* increased intercellular movement of fluorescent marker proteins (Fig. 5) (Burch-Smith and Zambryski 2010). This increased symplasmic flux could explain the increased systemic movement of TMV and TuMV in *ISE2*-silenced plants.

In the nonsilenced TRV-infected control plants, the 2×GFP probe moved in about 38% of the foci (Fig. 5B) and almost no foci were extended to 3 or more cell layers (Fig. 5C), while in striking contrast 2×GFP movement was significantly restricted in OE plants, with no movement of the 2×GFP probe in about 54% of OE1 foci and 83% of OE3 foci (Fig. 5B), and no foci were larger than two cell layers in both lines (Fig. 5C). Further, silencing *ISE2* expression led to increased probe movement from the epidermal to mesophyll layers of leaves (Fig. 5D), as previously reported (Burch-Smith and Zambryski 2010). However, increased *ISE2* expression did not affect 2×GFP movement between leaf tissues. These data support *ISE2*'s role

Fig. 3. Turnip mosaic virus-green fluorescent protein (TuMV-GFP) infection of *Nicotiana benthamiana* plants. **A**, Representative images of control, TRV-*ISE2*, OE1, and OE3 plants showing systemic infection at 9 days postinfection (dpi) as detected with UV light. Infected leaves indicated by arrowheads. Scale bar = 3 cm. **B**, Quantitative representation of the appearance of systemic infection in plants at 9 dpi. Average number of systemically infected plants was calculated from at least three independent experiments; 10 plants of each plant line were used in each experiment. Asterisks indicate *P* values compared with control plants at *P* < 0.05 (Z test for two population proportions). Error bars represent standard deviation. **C**, Quantitation of plants with cell death at 14 dpi. Plants used for quantitation of systemic infection were monitored for systemic necrosis. Asterisks indicate *P* values compared with control plants at *P* < 0.05 (Z test for two population proportions). Error bars represent standard deviation. **D**, Representative images of TuMV-GFP-infected plants; necrotic regions on the older leaves are indicated with white arrows. Scale bar = 3 cm.

as a negative regulator of PD-mediated intercellular trafficking. However, they do not explain the increased pathogen susceptibility of those plants.

The regulated deposition and removal of the polysaccharide callose at the necks of PD is a major mechanism for modulating PD-mediated intercellular trafficking. Callose deposition decreases plasmodesmal flux (Levy et al. 2007). Therefore, we measured callose levels at PD by aniline blue staining. *ISE2*-silenced leaves contained fewer aniline blue foci than did nonsilenced TRV-infected control plants (Fig. 5E). Intriguingly, OE plants also had significantly fewer aniline blue foci than did TRV-infected control plants (Fig. 5E), despite having reduced intercellular trafficking. This is in contrast to the observed increase in callose deposition in *H. schachtii*-induced syncytia (Fig. 4D). Together, the data suggest that the mechanism used by *ISE2* to alter intercellular trafficking does not depend solely on callose.

Factors leading to increased susceptibility in plants with increased *ISE2* expression.

We hypothesized that the enhanced pathogen susceptibility of plants with increased *ISE2* expression could be attributed to changes in chloroplast-mediated defense responses instead of

to PD. The chloroplast hormones SA, JA, ABA, and auxin have been implicated in antiviral defense (Alazem and Lin 2015). The expression of marker genes that reflect the activity of pathways induced by these hormones was, therefore, monitored in leaves 48 hpi with TMV. The *PR5* marker for SA-activated pathways revealed no difference between TRV-infected, non-silenced control plants and plants with elevated *ISE2* expression (Fig. 6A). Similarly, ABA-induced *ABI1* expression was not differentially affected between control and OE plants. In contrast, expression of the *PDF1.2* marker of JA signaling was significantly induced in TMV-infected OE plants compared with similarly infected control plants (Fig. 6C). In addition, the concentrations of these hormones in 4-week-old plants in the presence or absence of TMV infection were measured. Uninfected OE3 plants overexpressing *ISE2* had smaller amounts of ABA, SA, and auxin (indole 3-acetic acid) than wild-type plants, while JA and its derivatives (JA-isoleucine [JA-Ile], 12-oxophytodienoic acid) were not detected in either set of plants. Meanwhile, consistent with the gene expression analysis OE3 plants infected with TMV(30B)-GFP had significantly more JA-Ile but lower levels of ABA than did wild-type plants. There was no difference in SA or auxin content between control and OE3 plants during infection (Supplementary Fig. S8).

Fig. 4. Nematode infection in *Arabidopsis* plants with increased *ISE2* expression. **A**, Expression of *ISE2* in *Heterodera schachtii*-infected roots relative to uninfected roots measured by quantitative polymerase chain reaction (qPCR). Expression levels were normalized against *ACTIN8* as an internal reference. Error bars represent standard deviation from three biological replicates and asterisks indicate statistically significant difference from 1.0 (no change; $P < 0.05$, Student's *t* test). **B**, Validation of *ISE2-GFP* (green fluorescent protein) expression by qPCR. Error bars represent standard deviation. **C**, Representative images of 3-week-old *ISE2-GFP* overexpressing rescued lines (RL). Scale bar = 10 mm. **D**, Transgenic *Arabidopsis* plants overexpressing *ISE2* revealed increased susceptibility to *H. schachtii*. RL seeds were planted on modified Knop's medium, and 10-day-old seedlings were inoculated with approximately 250 surface-sterilized J2 *H. schachtii* nematodes. Three weeks after inoculation, the number of J4 females per root system was determined. Data are presented as the mean $\pm$ standard error ($n = 20$). Asterisks indicate statistically significant differences (indicated by asterisks [*], $P < 0.05$, Student's *t* test, $n = 3$). **E**, Representative images of syncytia formed by *H. schachtii*. Arrows indicate callose deposition in the cell walls of syncytia, as determined by aniline blue staining. Scale bar = 130 μ m. **F**, Average size of syncytia formed by *H. schachtii* in the roots of control and RL plants. Error bars represent standard deviation, $n = 47$ each for control and RL.

A second hypothesis tested was that overexpression of *ISE2* could interfere with the host small interfering RNA (siRNA)-mediated antiviral defense response. To this end, accumulation of mRNA transcripts of a subset of genes that play crucial roles in the antiviral siRNA response, *RDR6* (RNA-DEPENDENT RNA POLYMERASE 6), *SGS3* (SUPPRESSOR OF GENE SILENCING 3), and *DCL4* (ENDORIBONUCLEASE DICER-LIKE 4) was measured 24 hpi with TMV. Strikingly, the two OE lines displayed different responses of the siRNA machinery to TMV infection for *SGS3* and *RDR6* expression. While the OE1 line showed enhanced expression of *SGS3* and *RDR6* during infection, expression of both genes was suppressed in the OE3 line (Fig. 6E and F). We interpret this to mean that *SGS3* and *RDR6* are likely not playing a role in the enhanced viral susceptibility observed in those lines. In contrast, expression of *DCL4* was less strongly induced in both OE lines compared with wild-type tissues (Fig. 6D). Perhaps the reduced expression of *DCL4* in those tissues could account for a compromised antiviral response in those lines, leading to enhanced susceptibility. Together, these data indicate that *ISE2* expression affects other aspects of the plant defense machinery, and these combined changes could be, at least partly, responsible for the increased pathogen susceptibility of plants with increased *ISE2* expression.

DISCUSSION

In this study, we have shown that increased or decreased *ISE2* expression enhanced susceptibility to pathogen infections. The chloroplast RNA helicase *ISE2* is important for the development and function of both chloroplasts and PD; loss of *ISE2*, required for the splicing of at least a subset of chloroplast group II introns (Carlotto et al. 2016), results in defective chloroplasts with reduced thylakoid formation, increased stromal volume, and, ultimately, leaf chlorosis (Burch-Smith et al. 2011), and *ISE2* knockdown leads to increased intercellular trafficking and defects in plasmodesmal biogenesis, notably increased formation of twinned and branched PD (Burch-Smith and Zambryski 2010; Kobayashi et al. 2007). Both chloroplasts and PD have important roles in mediating the outcome of plant-pathogen interactions. Interestingly, *ISE2* expression was induced by viral infection (Fig. 1), suggesting that it could have a role in plant-virus interactions. Similarly, nematode infection also induced *ISE2* expression (Fig. 5). We, therefore, investigated *ISE2*'s role in plant-pathogen interactions.

We hypothesized that plants with reduced *ISE2* expression would be more susceptible to infection by pathogens. Viruses use PD for their cell-to-cell spread at sites of infection, to enter the vasculature and, again, to exit the vasculature in sink tissues (Heinlein 2015; Hipper et al. 2013) and chloroplasts have important roles in viral replication and spread in their plant hosts, including defense hormone production (Murphy and Carr 2002; Zhao et al. 2016). First, we considered it likely that viruses would be able to spread cell-to-cell more easily in *ISE2*-silenced leaves than in leaves with normal *ISE2* levels, since there would be more PD available for intercellular spread. TMV moves between cells as a ribonucleoprotein complex (Heinlein et al. 1998), while TuMV moves via tubules derived from the viral coat protein (Wan et al. 2015). The ability of both viruses to move faster in *ISE2*-silenced plants suggests that there is a general defect in the control of PD trafficking in those plants. Second, the defective chloroplasts in *ISE2*-silenced leaves could have resulted in compromised or diminished responses to viral infection. It has long been known that the chlorotic and mosaic phenotypes associated with viral infection are due to disrupted chloroplast function (Manfre et al. 2011). Indeed, the susceptibility of *N. benthamiana* to TuMV was found to depend on the

amount of light available to infected plants and the function of the photosynthetic machinery (Manfre et al. 2011). It is also known that viruses recruit chloroplast proteins during infection. This is exemplified by the small subunit of Rubisco RbcS, which interacts with the *Tomato mosaic virus* (ToMV) movement protein and is required for local cell-to-cell spread during infection (Zhao et al. 2013). In addition, several chloroplast proteins have been implicated in defense against viral infection (Reinero and Beachy 1986, 1989; Schoelz and Zaitlin 1989). For example, AtpC and RCA are nucleus-encoded chloroplast proteins that physically interact with TMV replicases (Bhat et al. 2013) and reduced expression of either protein decreased viral accumulation and local spread, revealing that these proteins normally act to limit susceptibility to tobamoviruses. Another important function of chloroplasts during defense responses is the production of ROS (Bobik and Burch-Smith 2015). Chloroplasts in *ISE2*-silenced leaves are in a more reduced state compared with nonsilenced control plants (Stonebloom et al.

Fig. 5. Cell-to-cell movement of 2xGFP in *Nicotiana benthamiana* plants with various levels of *ISE2* expression. **A**, Examples of 2xGFP movement between cell layers. Images are three-dimensional projections of Z-stacks. Scale bar = 10 μm. **B**, Fraction of foci showing no movement of 2xGFP. *ISE2*-overexpressing plants show decreased 2xGFP movement compared with nonsilenced control plants. **C**, Number of foci with three or more layers of 2xGFP movement is increased in *ISE2*-silenced plants. **D**, Number of foci showing 2xGFP movement from epidermal to adjacent mesophyll cells is increased in plants with decreased *ISE2* expression. Average number of fluorescent foci was calculated from at least five independent experiments in which a minimum of 10 foci was scored. Error bars represent standard deviation. **E**, Number of aniline blue foci detected per 100 μm² of cell wall, as measured from single images of a Z-stack collected by confocal microscopy. Error bars represent standard deviation over three independent experiments. For B through E, asterisks indicate statistically significant differences compared with control plants, at $P < 0.05$ (Z test for two population proportions).

2012). Due to their reduced thylakoid content and photosynthetic capacity (Burch-Smith et al. 2011), it seems unlikely that chloroplasts in ISE2-silenced leaves would be able to generate significant amounts of ROS during viral infection. Conversely, perhaps the increased ISE2 levels in the ISE2-OE lines leads to increased ROS production. Indeed it has been shown that increased oxidation of chloroplasts leads to decreased intercellular trafficking (Benitez-Alfonso et al. 2009; Stonebloom et al. 2012). This could explain the systemic necrosis observed in the OE lines infected with TMV but not in the ISE2-silenced lines (Fig. 2). However, this does not explain the systemic necrosis observed in all lines in response to TuMV infections (Fig. 3). Nonetheless, our hypothesis was confirmed by the finding that reduced ISE2 expression led to increased viral systemic spread during infection (Figs. 2 and 3).

In contrast, we predicted that increased ISE2 levels would lead to decreased susceptibility to viruses and other pathogens. This prediction was based on ISE2's role as a negative regulator

of intercellular trafficking (Burch-Smith and Zambryski 2010; Kobayashi et al. 2007). Indeed, plant lines ectopically expressing *AtISE2* markedly decreased intercellular trafficking of GFP probes (Fig. 5). However, increasing ISE2 levels led to unexpected outcomes of plant-virus and plant-cyst nematode interactions, with increased susceptibility to both viruses (Figs. 2 and 3) and nematodes (Fig. 4). Studies of syncytia induced by *H. schachtii* in *Arabidopsis* roots have suggested that there is temporal regulation of PD formation and intercellular trafficking during syncytium formation (Hofmann and Grundle 2006; Hofmann et al. 2010; Hoth et al. 2005, 2008). However, the roles of PD and intercellular communication during nematode infection remain largely unexplored.

Transgenic *N. benthamiana* plants expressing both endogenous *NbISE2* and *AtISE2-GFP* (OE plants) were morphologically similar to nonsilenced control *N. benthamiana* plants. The levels of the photosynthetic pigments and the quantum efficiency of photosystem II in OE plants were similar to those of

Fig. 6. Quantitative polymerase chain reaction (qPCR) quantification of key marker genes associated with viral infection in plants with increased *ISE2* expression. **A**, Expression of *NbPR5* marker gene for SA-mediated defense signaling, **B**, *NbABI1* marker for ABA-mediated defense signaling, and **C**, *PDF1.2* marker for JA-mediated defense signaling was measured by qPCR. RNA was isolated from uninfected tissue or tissue collected 48 h after *Tobacco mosaic virus* (TMV) infection. **D** to **F**, Expression of genes encoding components of the RNA silencing machinery. Expression of *NbDCL4* (**D**), *NbSGS3* (**E**), and *NbRDR6* (**F**) was measured by qPCR. RNA was isolated from uninfected tissue or tissue collected 24 h after TMV infection. Expression was normalized to uninfected wild-type (WT) leaves. Error bars represent standard deviation. Results are representative of three biological replicates. Lower case letters denote the following: a, statistically significant difference between uninfected and infected tissues of a given line; b, statistically significant difference between uninfected WT and OE tissues; and c, statistically significant difference between TMV-infected WT and OE tissues. Statistical significance was $P < 0.05$ (Student's *t* test).

nonsilenced control plants. At the ultrastructural level, the chloroplasts of newly emerged OE leaves differed only in the presence of large starch granules, as compared with the chloroplasts of wild-type control leaves of the same age. Similarly, *Arabidopsis* RL expressing *AtISE2-GFP* were also identical in appearance to wild-type plants (Fig. 4). This suggests that the chloroplasts in OE plants were competent for photosynthesis, at least under our growth conditions. Nonetheless, our analysis revealed that *N. benthamiana* plants with increased *ISE2* expression displayed enhanced activation of JA-responsive gene expression in response to infection (Fig. 6). JA is a derivative of linoleic acid whose synthesis begins in the chloroplast but is completed in peroxisomes. JA has been reported to act through suppression of SA-mediated resistance to increase susceptibility of tobacco plants to TMV (Oka et al. 2013). Thus, while the chloroplasts in plants with increased *ISE2* expression appear normal, they likely have altered hormone metabolism and signaling. Understanding the effects of altered *ISE2* expression on chloroplast lipid metabolism could help illuminate the response of these plants to infection. An intriguing observation in OE plants is the reduced induction of expression of the DCL4 component of the RNA silencing machinery. Thus, *ISE2* could act indirectly to increase susceptibility to pathogens, through its role in modulating chloroplast signaling and hormone production.

Given that *ISE2* expression was increased in response to viral infection (Fig. 1) and that the *ISE2-GFP* protein accumulated in chloroplasts during infection, it is possible that *ISE2* could have a direct role as a host factor for viral replication and spread. As mentioned above, several chloroplast proteins are known to have positive roles in establishing viral infections. Although we have not observed its accumulation outside the chloroplast during infection, it cannot be ruled out that viruses recruit *ISE2* to their sites of replication or spread or both. Further experiments will specifically address *ISE2*'s interaction with viral proteins to address the possibility of this RNA helicase being co-opted into virus replication and possibly spread.

How might decreased intercellular trafficking lead to increased susceptibility to pathogens? Occlusion of PD to reduce intercellular trafficking has been shown to be a successful strategy to limit viral infection. Overexpression of *RGP2* led to its accumulation at PD and reduced TMV spread (Zavaliev et al. 2010). Similarly, overexpression of the membrane raft protein Remorin, which also localizes to PD, led to decreased *Potato virus X* spread in tomato (Raffaele et al. 2009). The PD-LOCALIZED PROTEIN5 (PDL5) belongs to a family of eight type I membrane receptors (Bayer et al. 2006; Thomas et al. 2008). *PDL5* overexpression in *N. benthamiana* delayed systemic spread of TMV but not *Cucumber mosaic virus* (CMV) (Lee et al. 2011). In stark contrast, the decreased intercellular trafficking caused by increased *ISE2* expression (Fig. 5) did not compromise viral local intercellular spread or systemic movement (Figs. 2 and 3). This finding suggests that limited PD trafficking capacity may, in some situations, be sufficient for virus intercellular and eventual systemic spread. It should be noted that, unlike the aforementioned proteins, *ISE2* localizes to chloroplasts and not to PD, indicating that there are different mechanisms to restrict PD flux. Also, our data suggest that *ISE2*'s role in regulating PD flux is not solely accomplished through the regulated deposition or removal of callose at the necks of PD (Fig. 5).

In addition to the controlled formation of new PD, the regulated deposition and removal of callose at PD is also crucial to determining the outcome of cyst nematode syncytia formation. Callose was detected at PD in the cell walls of the syncytial margins, and the amount of callose detected gradually decreased from day 4 to day 7 of infection (Hofmann et al. 2010).

An *Arabidopsis* mutant with a T-DNA insertion in the *AtBG_PAP* gene encoding a β -1,3-glucanase and, hence, reduced callose removal from PD was able to limit the size of syncytia and showed a smaller female/male ratio than wild-type plants (Hofmann et al. 2010). Similarly, overexpression of the RAP2.6 transcription factor led to increased callose deposition and smaller syncytia (Ali et al. 2013). Based on these previous findings, we had expected that overexpression of *ISE2* would confer enhanced resistance to *H. schachtii* infection. The observation that *Arabidopsis* RL plants with reduced intercellular trafficking were more susceptible to *H. schachtii* infection (Fig. 4) was, therefore, unexpected.

We propose two possibilities for how increased *ISE2* expression could lead to enhanced nematode infection. The first possibility is that either hormone production, distribution, or both are compromised in RL roots. The phytohormones auxin (Hewezi 2015, 2014), cytokinin (Shanks et al. 2016; Siddique et al. 2015), and ethylene (Wubben et al. 2001, 2004) have critical roles in successful nematode infection. Auxin moves symplasmically via PD (Coudert et al. 2015; Han et al. 2014), and mutants with altered callose synthesis had defective auxin distribution (Han et al. 2014). Long-distance movement of cytokinin has also been demonstrated, and this was disrupted by callose accumulation at PD (Bishopp et al. 2011). The decreased PD flux in RL roots could alter auxin and cytokinin flux in those roots, locally increasing their levels and leading to increased susceptibility to nematode infection. There is also evidence that other defense molecules move systemically from shoot to root (Kehr and Buhtz 2008; Lim et al. 2016) and that PD have a significant role in this process. The second possibility recalls the accumulated starch in chloroplasts of *ISE2*-RL sink leaves, likely due to altered carbohydrate metabolism or partitioning. Perhaps, similar to the young sink leaves in those plants, there are increased carbon levels in the *Arabidopsis* RL roots. At early times of infection, the J2 nematodes would, therefore, have access to elevated nutrient levels, allowing for its faster development. In this scenario, the growth of the nematode would simply outpace the plant's ability to launch an effective defense response. Perhaps the disrupted intercellular trafficking combined with defective defense-hormone production result in a compromised defense response against nematodes.

Conclusion.

Taken together, our results demonstrate that *ISE2* affects the structure and function of chloroplasts, which, in turn, affects cell-to-cell communication via PD, a phenomenon we previously termed ONPS (Burch-Smith et al. 2011). We have shown that the level of *ISE2* expression can modulate plant defense responses. Although low or high expression of *ISE2* affects symplastic transport in different ways, the outcome of the compromised defense response is unambiguous. These observations apply to pathogens whose life cycle is strictly dependent on symplastic transport. A practical outcome of this study is that chloroplast-PD crosstalk may result in severely compromised plant immunity. In total, the data presented here reveal a complex relationship between PD and chloroplasts, especially with respect to pathogen susceptibility and the outcomes of infection. They also suggest there are unknown connections between different defense mechanisms and that the cell integrates these signals to produce a final outcome that cannot be determined *prima facie*. As we move to engineer plants with altered carbon partitioning or chloroplasts in attempts to optimize plant productivity to better fit human needs, care must be taken to ensure that these changes do not inadvertently compromise plant disease resistance.

MATERIALS AND METHODS

Plant materials, VIGS, virus strains, and virus inoculations.

Nicotiana benthamiana plants were grown on light carts at $28 \pm 1^\circ\text{C}$ with 16 h of light (approximately $172 \mu\text{mol m}^{-2} \text{s}^{-1}$) and $22 \pm 1^\circ\text{C}$ in 8 h of dark. *Arabidopsis thaliana* plants were grown on plates in a growth chamber, as described in the infection assay.

The construct used to silence *ISE2* was described previously (Burch-Smith and Zambryski 2010). VIGS was done according to published protocols (Liu et al. 2004). Briefly, two leaves of 3-week-old *N. benthamiana* plants were infiltrated with *Agrobacterium tumefaciens* cultures carrying relevant TRV constructs. Plants were kept on light carts for 2 weeks, until assays were performed or samples collected. Photographs of plants were taken with a Nikon D60 camera (Nikon, Inc.). For assays involving time courses, infected leaves were detached from the plants after 16, 22, 28, 34, and 40 h. Allowing 16 h for *Agrobacterium*-mediated T-DNA transfer, this corresponded to 0, 6, 12, 18, and 24 h of viral infection.

To check replication and systemic movement of TMV(30B)-GFP, TRV-*ISE2*, OE1, and OE3 *N. benthamiana* plants were inoculated with sap from TMV(30B)-GFP agroinfiltrated leaves of the same species. Plant sap containing viruses were obtained by grinding infected leaf tissue with a mortar and pestle and were diluted by the addition of approximately 2 volumes of sterile deionized water. Viral extracts were inoculated directly to plants with a sterile sponge, as described previously (Dinesh-Kumar et al. 2000). For expression of TMV, *agrobacteria* containing the U1 strain of TMV were infiltrated into plants at an optical density at 600 nm (OD_{600}) = 1.0, after resuspending in inoculation medium (10 mM MES, 10 mM MgCl_2 , 200 μM acetosyringone). Plants were evaluated for symptoms for 9 days. GFP expression was visualized by illumination with a hand-held long-wave UV lamp, as previously described (Liu et al. 2004). TuMV-GFP (Lellis et al. 2002) was agroinfiltrated at OD_{600} = 1.0 to infect plants. Plants infected with TuMV were monitored for the development of systemic infection and necrosis for 14 days.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

Total RNA from leaf tissues was extracted using Trizol Reagent (Thermo Fisher Scientific) and was treated with DNase I (Ambion). First-strand cDNA was synthesized using 21-base oligo (dT) primer (Invitrogen) or random Primer mix (New England Biolabs). Fivefold diluted cDNA (2.5 μl), 0.5 μl of each gene-specific primer (Supplementary Text; Supplementary Table S1), and 5 μl of the Power SYBR green master mix (Applied Biosystems) were used in 10- μl reactions for qRT-PCR analyses of mRNA levels of with an iQTM5 multicolor RT-PCR detection system (Bio-Rad). To normalize the mRNA levels of target genes between samples, relative actin mRNA levels were determined, using actin-specific primers and a relative quantification method (Pfaffl 2001). The efficiency of the primers used in qPCR was no lower than 92%.

H. schachtii infection assay.

Seeds of four independent homozygous transgenic lines overexpressing *ISE2* (T3 generation) along with the wild-type *Arabidopsis* ecotype Col-0 were surface-sterilized and were planted on modified Knop's medium (Sijmons et al. 1991) in 12-well tissue culture plates (BD Biosciences). Each line was replicated 20 times in a randomized complete block design. Plants were grown in a growth chamber at constant temperature of 24°C under 16 h of light and 8 h of darkness. Ten-day-old

plants were inoculated with approximately 250 J2 *H. schachtii* nematodes per root system. Inoculated plants were kept under the same conditions for an additional 3 weeks, and then, the numbers of female nematodes per plant were scored, using a dissecting microscope. The numbers of female nematodes developed on each transgenic line were used to calculate nematode susceptibility levels relative to wild-type control plants, using unadjusted paired *t* tests on SAS with *P* value < 0.05 for significance.

2×GFP movement assay, callose detection, and confocal microscopy.

Upper leaves displaying the silencing phenotype or similar leaves in nonsilenced (control) or OE plants were infiltrated with *agrobacterium*-containing constructs encoding 2×GFP at OD_{600} = 0.0001, 2 weeks after VIGS induction. GFP fluorescence in plant tissues was viewed at approximately 43 hpi. Confocal analysis was performed on a Leica SP8 confocal microscope, using a 488-nm excitation laser for GFP with emission maximum at 509 nm. At least 10 2×GFP foci in each of three independent experiments were imaged for each plant line. Whole leaves from control, TRV-*ISE2*, OE1, or OE3 plants were cut at the petiole, were placed immediately in a 0.1-mg/ml solution of aniline blue in 67 mM K_3PO_4 , pH 9.5. Tissues were stained in the dark for 10 min and were then observed under a confocal microscope. Aniline blue fluorescence was detected using a $\times 25$ objective and 405-nm laser excitation with a 420- to 480-nm band-pass emission filter.

Syncytium measurements and callose detection.

The sizes of single-female, fully-developed syncytia were determined as previously described (Hewezi et al. 2008). Briefly, 3 weeks postinfection with *H. schachtii*, 10 syncytia were randomly selected from *ISE2* overexpression plants and wild-type Col-0 plants and were imaged using differential interference contrast (DIC) microscopy. For DIC microscopy, infected *Arabidopsis* roots were fixed overnight in ethanol/acetic acid (9:1) and were cleared in a chloral hydrate solution (chloral hydrate/water/glycerol, 8:1:1) for approximately 24 h. Structure of syncytia was observed using a Nikon Eclipse 80i microscope with DIC optics, and pictures were obtained with a 12-megapixel cooled color DXM-1200c (Nikon) camera. The size of syncytia was measured using NSI-Elements BR 2.30. Callose deposition at the cell wall of the syncytia was visualized in chloral hydrate-cleared tissues neutralized in 1 M Tris-HCl buffer (pH 7.5) and was stained with aniline blue as described above.

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