The biochemical function of the potyviral P3 protein is not known, although it is known to regulate virus replication, movement, and pathogenesis. We show that P3, the putative virulence determinant of soybean mosaic virus (SMV), targets a component of the translation elongation complex in soybean. Eukaryotic elongation factor 1A (eEF1A), a well-known host factor in viral pathogenesis, is essential for SMV virulence and the associated unfolded protein response (UPR). Silencing GmEF1A inhibits accumulation of SMV and another ER-associated virus in soybean. Conversely, endoplasmic reticulum (ER) stress-inducing chemicals promote SMV accumulation in wild-type, but not GmEF1A-knockdown, plants. Knockdown of genes encoding the eEF1B isoform, which is important for eEF1A function in translation elongation, has similar effects on UPR and SMV resistance, suggesting a link to translation elongation. P3 and GmEF1A promote each other’s nuclear localization, similar to the nuclear-cytoplasmic transport of eEF1A by the Human immunodeficiency virus 1 Nef protein. Our results suggest that P3 targets host elongation factors resulting in UPR, which in turn facilitates SMV replication and place eEF1A upstream of BIP in the ER stress response during pathogen infection.

Soybean mosaic virus (SMV) is a member of the largest and most successful genus of plant pathogenic viruses, *Potyvirus* (Adams et al., 2005). SMV is seed-borne and aphid-transmitted, and causes mosaic and severe necrosis in soybean. It affects both seed yield and quality and has caused up to ~35% reduction in yield. Based on the differential phenotypic reactions of specific soybean cultivars, seven distinct SMV strains have been described in North America (G1–G7, Cho and Goodman, 1979), and twenty-one strains in China (SC1–SC21; Wang et al., 2003; Li et al., 2010a). Resistance to the North American SMV strains is derived from the *Rsv* (Resistance to SMV) loci, which comprise dominant resistance (*R*) genes (Buss et al., 1999; Hayes et al., 2000; Gunduz et al., 2002; Liao et al., 2002; Gore et al., 2002; Zheng et al., 2005). Of these, resistance derived from *Rsv1* and *Rsv3* is effective against strains G1–G6 and G5–G7, respectively (Gunduz et al., 2002). *Rsv4* was initially thought to provide resistance against all North American strains of SMV but was later shown to exhibit a late susceptible phenotype to strains G1 and G2 (Chen et al., 1993; Ma et al., 1995; Ma et al., 2002; Gunduz et al., 2004). Although none of the *Rsv* genes have been cloned, some downstream signaling components that regulate *Rsv*-derived resistance have been identified (Fu et al., 2009; Zhang et al., 2012; Wang et al., 2014). The microRNA and small interfering RNA pathways are also known to regulate defense against SMV (Chen et al., 2015). Resistance to the strains from China is derived from the *Rsc* loci, and these have been mapped to chromosomes 2, 13, and 14 in the cultivars Keleng 1, Qihuang, and Dabaima, respectively (Wang et al., 2011; Ma et al., 2011; Zheng et al., 2014). The SMV genome is a single-stranded positive-sense RNA, which like other potyviruses, is processed as a single large polypeptide that is later cleaved by viral proteases.
encoded proteases to yield mature proteins. SMV encoded mature proteins include P1 (protease), helper component-proteinase (HC-Pro), P3, 6K1, cylindrical inclusion (CI), 6K2, viral protein genome-linked (VPg), nuclear inclusion a proteinase (Nia-Pro), nuclear inclusion b (Nib, RNA-dependent RNA polymerase), and coat protein (CP). Transcriptional slippage at a single nucleotide insertion site within the P3 cistron results in an additional polypeptide, P3N-PIPO. This polypeptide is only produced by a very small fraction of the viral RNA, which contains the single nucleotide insertion (Olspert et al., 2015). P3N-PIPO contributes to cell-to-cell movement of the virus in susceptible hosts (Chung et al., 2008; Wen and Hajimorad, 2010). HC-Pro, along with P3 and CI, regulates SMV virulence (Eggenberger et al., 2008; Hajimorad et al., 2008; Seo et al., 2009; Zhang et al., 2009; Hajimorad et al., 2011; Seo et al., 2011; Chowda-Reddy et al., 2011; Wen et al., 2011). The precise biochemical function of SMV P3, and indeed the potyviral P3 protein in general, is largely uncharacterized due to the lack of structural or functional motifs in its sequence. Roles in viral replication, movement, virulence activity, and in one case, avirulence, have been suggested (Rodriguez-Cerezo et al., 1993; Sænzer et al., 2000; Desbiez et al., 2003; Jenner et al., 2003; Choi et al., 2005; Hajimorad et al., 2005, 2006; Hjulsager et al., 2006; Eggenberger et al., 2008). Some potyviral P3 proteins were detected in the nucleoli of early-infected cells, whereas others were detected exclusively in the cylindrical inclusions of infected cells (Rodriguez-Cerezo et al., 1993; Langenberg and Zhang, 1997). Some potyviral P3 proteins interact with the Nia, HC-Pro, P1, and Nib of the respective virus, but these interactions have not yet provided major insights into the function of P3. In case of SMV, P3 from the G7H strain did not interact with any other viral protein, whereas the Pinellia ternata isolate showed weak interaction with Nia and Nib (Guo et al., 2001; Kang et al., 2004; Lin et al., 2009).

Here, we attempted to determine the role of the SMV P3 in soybean-SMV pathogenesis. We find that the ER-localized SMV P3 interacts with eukaryotic elongation factor 1 alpha (eEF1A), which is essential for viral virulence in the susceptible host. eEF1A is a cytoplasmic protein that delivers aminoacylated tRNAs to ribosomes during polypeptide elongation when bound to GTP. After tRNA transfer, eEF1A is recycled to its active GTP-bound form by the guanine nucleotide exchange factor, eEF1B. eEF1B interacts with the RNA dependent RNA polymerases (RdRp) of tobacco mosaic virus and turnip mosaic virus and is known to be associated with the replication complexes of potyviruses, tobamoviruses, and tombusviruses (Yama et al., 2006; Nishikiori et al., 2006; Thivierge et al., 2008; Li et al., 2010b). We also show that SMV replication is associated with the unfolded protein response (UPR) and that eEF1A contributes to this. UPR is associated with ER stress and involves transduction of the ER stress signal via the ER membrane receptor protein kinase-like ER kinase (PERK), the inositol-requiring transmembrane kinase and endonuclease 1α (IRE1), and the activation of transcription factor 6 (ATF6) in mammals (Walter and Ron, 2011). The plant UPR comprises two branches, one mediated by IRE1 and the other by ATF6-like receptors, which involves the transcription factors bZIP17 and 28 (Howell, 2013). Viral infection in plants is known to induce the IRE1 arm of UPR, which involves IRE1-mediated splicing of bZIP60 mRNA, resulting in the synthesis of a functional bZIP protein that can activate ER stress-inducible promoters in the nucleus (Ye et al., 2011, 2013; Verchot 2014). Examples include induction of bZIP60 and UPR chaperone gene expression by the ER associated movement protein of potato virus X (Ye and Verchot, 2011) and the turnip mosaic virus 6K2 protein-induced splicing of bZIP60 mRNA (Zhang et al., 2015). We show that plants lacking eEF1A or the associated eEF1B show reduced UPR in response to SMV infection and better resist viral accumulation.

RESULTS

SMV Infection Is Associated with the Induction of UPR-Associated Gene Expression in Soybean

In an effort to better understand the process of SMV infection in soybean, we carried out ultrastructural analysis of SMV-infected soybean leaves from resistant and susceptible cultivars at 10 and 20 d postinfection (dpi) with SMV. At these time points, the resistant cultivar did not exhibit visual symptoms of infection, whereas the susceptible cultivar showed clear mosaic symptoms (data not shown). In contrast to the resistant cultivar, the SMV-inoculated susceptible plants showed pinwheel-type inclusions in the cytoplasm, and there was a progressive increase in these inclusion structures at later time points of infection (Fig. 1A). No other major cellular changes were noticeable between the microscopic ultra sections prepared from SMV-inoculated resistant and susceptible cultivars. In wheat, the pinwheel structures associated with Wheat spindle streak virus infection are considered to be derived from the endoplasmic reticulum (ER; Langenberg and Schroder, 1973).

To test if there was any association between SMV and the ER membrane, we fractionated total extracts from SMV-infected soybean leaves on a Suc gradient and examined the presence of SMV coat protein (CP) in various subcellular fractions. Western blot analysis showed that SMV CP co-purified with fractions containing the ER-localized binding protein (BiP), chaperone protein that restores proper protein folding during ER stress, Fig. 1B). We next tested if ER membrane reorganization due to pinwheel structure formation in SMV infected plants was associated with UPR, involving the accumulation of unfolded proteins at the ER membrane. For this, we examined the mRNA levels of genes associated with UPR, including those encoding BiP, IRE1, the ER resident protein sensor; bZIP, the
downstream transcription factors that activate genes required for coping with UPR and the associated ER stress; and NRP, the cell death domain-containing Asn-rich protein that is associated with the ER stress-induced cell death response. qRT-PCR analysis showed that the soybean orthologs (Silva et al., 2015) of these genes BiP-D, NRP-A, bZIP68 (ortholog of AtbZIP60, downstream regulator of the IRE branch of UPR), and four isoforms of IRE1 were significantly induced in SMV (G5)-infected plants (cv Essex, Fig. 1C, left and middle panels). Interestingly, SMV-infected plants also showed induction of bZIP37 and bZIP38 (ortholog of AtbZIP17/28, Fig. 1C, right panel), which function in the ATF6-like receptor branch of UPR. Induction of UPR was further confirmed in western blot analyses of BiP protein, which was significantly higher in the SMV infected plants (Fig. 1D).

We next tested whether ER stress altered response to SMV. Soybean (Glycine max cv Essex) plants were treated with the ER stress-inducing agents, DTT and tunicamycin. DTT disrupts disulphide bonding, while tunicamycin inhibits N-linked glycosylation of secreted glycoproteins, both of which affect protein folding resulting in ER stress (Howell, 2013). Control plants were treated with water or DMSO, respectively. Interestingly, SMV was detected earlier and accumulated at much higher levels in plants treated with DTT or tunicamycin as compared to control plants treated with water or DMSO, respectively (Fig. 1, E and F). In contrast, DTT or tunicamycin did not alter the in planta levels of two other viruses that are not associated with the ER membrane (Fig. 1, G and H). Turnip crinkle virus (TCV) replicates on the outer mitochondrial membrane (Russo and Martelli, 1982; Blake et al., 2007), whereas cucumber mosaic virus (CMV) is thought to replicate in the cytoplasm (Zitter and Murphy, 2009). Accumulation of neither TCV nor CMV was significantly different in DTT- or tunicamycin-treated Arabidopsis (Arabidopsis thaliana) plants as compared to the respective control plants (Fig. 1, G and H). Together, these results suggested that SMV infection induces ER stress in soybean, which in turn promotes virus accumulation in the plant.
SMV P3 Interacts with GmEF1A

Based on the above results, we considered the possibility that SMV virulence proteins might target plant proteins to induce ER stress. Therefore, we conducted a yeast two-hybrid (Y2H) screen to identify soybean proteins that interacted with SMV P3 protein, because P3 is associated with virulence and localizes to the ER (see below). Of the several P3-interacting proteins identified, we proceeded to characterize the P3 interactor GmEF1A (Glyma17g186600) because it was identified multiple times in the Y2H screen. The predicted GmEF1A protein sequence contained conserved sequences of the eukaryotic eEF1A protein family, including the GTP/Mg^{2+} binding site (Andersen et al., 2001), the EF1B alpha binding site, the switch I and II regions that undergo conformational changes upon GTP binding (Sprang, 1997), and the G box motifs (GDP/GTP-binding motif elements) of the Ras superfamily GTPases (Colicelli, 2004; Wennerberg et al., 2005; Supplemental Fig. S1A).

The interaction of full length GmEF1A with SMV P3 was confirmed using bimolecular fluorescence complementation (BiFC) and communoprecipitation (IP) assays. For BiFC assays, proteins were tagged with reciprocal N- or C-terminal halves of enhanced yellow fluorescent protein (nEYFP and cEYFP) and transiently co-expressed in Nicotiana benthamiana. Coexpression of GmEF1A with SMV P3 cloned from G5 strain resulted in reconstitution of EYFP, indicating positive interaction (Fig. 2A). Likewise, GmEF1A also interacted with P3 from SMV strains G7 and SC15 (Supplemental Fig. S1B). In contrast, P3 did not interact with GST (glutathione-S-transferase), and GmEF1A did not interact with SMV HC-Pro (Fig. 2A), although all proteins

![Figure 2. eEF1A interacts with the P3 protein of SMV. A, Bimolecular fluorescence complementation (BiFC) assay showing in planta interactions. 40x magnification of micrographs at 48 h postinfiltration from plants co-expressing nYFP-GmEF1A with cYFP-P3, cYFP-HC-Pro, or cYFP-GST are shown. Transgenic N. benthamiana expressing CFP-H2B (nuclear localized histone 2B) were used for the BiFC assays. Images are representative of three separate infiltrations from two independent experiments for each interaction using both combinations of n/cYFP fused proteins. Scale bars = 10 μm. B, Communoprecipitation assay using proteins coexpressed in N. benthamiana. MYC-tagged SMV P3 was co-expressed with FLAG-tagged GmEF1A. Proteins were immunoprecipitated (IP-FLAG) from total extracts using α-FLAG antibodies and visualized using tag-specific antibodies. Results are representative of two independent repeats. C, C-terminal YFP/CFP-tagged proteins were transiently expressed individually (GmEF1A-CFP, P3-YFP) or co-expressed (P3-YFP, GmEF1A-CFP) in N. benthamiana via Agro-infiltration. Scale bars = 10 μm. GmEF1A-CFP was expressed in transgenic plants expressing RFP-H2B (nuclear localized). P3-YFP was expressed in transgenic plants expressing RFP localized to the ER via the N-terminal signal sequence of Arabidopsis basic chitinase and a carboxy-terminal HDEL ER-retention signal. Images in two different focal planes are shown for P3-YFP to enable ideal visualization of the ER as well as the nucleus (indicated by white arrowhead in the lowermost left panel). Bottom italicized letters indicate the fluorescent channel used for imaging. D, MYC-tagged SMV P3 or GmEF1A were expressed individually in N. benthamiana. Total (T) leaf extracts and membrane (M) or soluble (S) fractions were analyzed using MYC-specific antibodies. TIP (tonoplast intrinsic protein, membrane specific) and Hsc70 (cytosolic heat shock protein 70) were used to test purity of the fractions. Blots are representative for all experimental repeats.](http://www.plantphysiol.org/doi/abs/10.1104/pp.16.00400)
were adequately expressed in *N. benthamiana* (Supplemental Fig. S1C). For IP assays, proteins were transiently co-expressed in *N. benthamiana* as MYC- or FLAG-tagged derivatives. IP assays confirmed the interaction between P3 and GmEF1A (Fig. 2B). The GmEF1A-P3 interaction is unlikely to involve P3N-PIPO because this interaction was detected using heterologously expressed P3, whereas P3N-PIPO is specifically made from viral RNA variants generated by the potyviral replicase (Olspert et al., 2015, Rodamilans et al., 2015).

GmEF1A is predicted to be a cytoplasmic protein (PlantLoc probability: 1.0, http://cal.tongji.edu.cn/PlantLoc/index.jsp). Indeed, transiently expressed GmEF1A did localize primarily to the cell periphery (Fig. 2C, middle panel), and was detected in the soluble protein fraction, suggesting cytoplasmic localization (Fig. 2D). On the other hand, P3 co-localized primarily with the ER marker in planta (Fig. 2C, lower panel) and was detected in the membrane fractions of leaf extracts (Fig. 2D). However, the localization of GmEF1A and P3 proteins was inconsistent with the BiFC result that showed interaction in the cell periphery as well as in the nucleus (Fig. 2A). To address this, we assayed localization in leaves co-expressing both GmEF1A and P3 proteins. Interestingly, consistent with BiFC data, co-expression of GmEF1A and P3 resulted in increased nuclear localization of both GmEF1A and P3 (Fig. 2C, top panel). Together, these results suggested that P3 and GmEF1A promote each other’s relocation to the nucleus, which explains their interaction in the nuclear compartment.

**GmEF1A Proteins Negatively Regulate Soybean Resistance to SMV**

The *Glycine max* genome contains four other GmEF1A-like sequences. These include Glyma05g089000, Glyma10g212900, Glyma16g068000, and Glyma19g052400, the predicted protein sequences of which shared ~86% identity with GmEF1A (Supplemental Fig. S1A). Quantitative (q) RT-PCR analysis showed that transcript levels for GmEF1A (*Glyma17g186600*) was the most abundant in every tissue analyzed (cotyledon, leaf, stem, flower, and roots), and these were expressed to highest levels in leaf tissues (Supplemental Fig. S2A). Furthermore, SMV infection resulted in significant (*P < 0.0001*) induction of *Glyma17g186600* expression (Supplemental Fig. S2B). Although SMV infection also induced *Glyma05g089000* expression, its levels were much lower than those of *Glyma17g186600*.

We next tested if GmEF1A contributed to soybean resistance against SMV. For this, we knocked down the *GmEF1A* genes in soybean using the bean pod mottle virus (BPMV)-based VIGS (virus-induced gene silencing) vector (Zhang and Ghabrial, 2006; Kachroo and Ghabrial, 2012). We generated a vector derived from *Glyma17g186600* (Supplemental Fig. S3A), which was expected to knock down the expression of all isoforms simultaneously because the various isoforms share >90% nucleotide identity. Plants (cv Essex) infected with the vector were tested for *GmEF1A* transcript levels using qRT-PCR. Notably, mRNA levels of all but one (Glyma16g068000) *GmEF1A* isoforms were significantly reduced in the *GmEF1A*-knockdown plants (*GmEF1A*) as compared to that in plants infected with a BPMV control vector (contains a nonspecific sequence, V; Supplemental Fig. S3B). Consistent with the reduced transcript levels, GmEF1A protein levels were also significantly reduced in the *GmEF1A* plants as compared to V plants (Fig. 3A).

**Knockdown of GmEF1A Expression Renders Plants Insensitive to SMV- or Chemical-Induced UPR**

Our results that ER stress-inducing chemicals inhibited SMV replication, and that *GmEF1A* plants showed enhanced resistance to SMV, prompted us to test the possible link between eEF1A and UPR. We determined the response of the *GmEF1A* plants to chemically induced ER stress. The V and *GmEF1A* plants (cv Essex) were treated with the ER stress-inducing agent DTT, and control
Plants were treated with water. Plants were then assessed for expression of the BiP-D transcript using qRT-PCR analysis. BiP-D mRNA levels increased by ~1.6-fold in DTT-treated V plants as compared to the water-treated counterparts. In contrast, BiP-D mRNA levels showed minimal increase in response to DTT treatment in the SeF1A plants (Fig. 4A). This suggested that the SeF1A plants might be impaired in chemical-induced ER stress response. We next tested the transcript levels of other genes associated with UPR, including BiP-D, IRE1a-d, and ZIP37, 38, and 68, and NRP-A. SMV infection significantly (P < 0.0001) upregulated the mRNA levels of all of these markers in V plants (Fig. 4, B and C). In contrast, SMV-infected SeF1A plants showed induction of only bZIP68 and bZIP38 mRNA at levels comparable to V plants. The SMV-infected SeF1A plants also showed induction of IRE1b and IRE1c mRNA, though at significantly lower levels than V plants; IRE1b/c mRNA levels in SMV-infected SeF1A plants were comparable to their respective levels in uninfected V plants (Fig. 4C). This suggested that SMV-induced UPR was attenuated in the SeF1A plants. Consistent with their gene expression data, and unlike V plants, the SeF1A plants did not accumulate the BiP protein in response to SMV infection (Fig. 4D). We tested whether the ER stress inducers DTT or tunicamycin altered response to SMV in the SeF1A plants because these ER stress-inducing agents promoted SMV accumulation in wild-type plants. Both DTT and tunicamycin treatments resulted in increased SMV accumulation in V plants similar to wild-type plants (Fig. 1, E and F, and Fig. 4). In contrast, these chemicals did not significantly increase SMV accumulation in the SeF1A plants (Fig. 4E). Together, these results indicate that knockdown of GmEF1A expression alters the plants’ ability to induce ER stress, which in turn correlates with their enhanced resistance to SMV.

GmEF1A Is Required for Pathogen- and Chemical-Induced Cell Death

SMV G7 induces a typical systemic cell death phenotype termed lethal systemic hypersensitive response (LSHR) on plants containing the Rs01 locus (Hajimorad and Hill, 2001, Hajimorad et al., 2003). We noticed that SMV G7 did not induce visible or microscopic LSHR on SeF1A plants (Fig. 5, A and B). This in turn correlated with reduced induction of the HR marker gene Hsr203j in SMV G7-infected SeF1A plants (Fig. 5C). Together, these results indicate that knockdown of GmEF1A expression alters the plants’ ability to induce ER stress, which in turn correlates with their enhanced resistance to SMV.

**Figure 3.** Silencing GmEF1A alters soybean response to SMV. A, Western blot analysis showing GmEF1A protein levels in plants infected with the control BPMV vector (V) and the GmEF1A knockdown (SeF1A) plants. GmEF1A was detected using plant eEF1A specific antisera. Ponceau staining was used as control for protein loading. B, Visual symptoms of V and SeF1A plants (cv Essex) before (upper panel) and after (lower panel) infection with SMV-G5. C, Western blot detecting SMV coat protein (CP) in inoculated (I) and systemic leaves (S) of V and SeF1A plants at indicated days postinfection (dpi) with SMV. Proteins were detected using antibodies specific to SMV or BPMV CP. Total extracts of I and S leaves were used. Ponceau staining was used as control for protein loading. D, ELISA of SMV CP levels in V and SeF1A plants (cv Essex) at indicated dpi (4, 6 dpi from I, and 10 dpi from S) with SMV (G5 or G7). Error bars indicate SD (n = 3). Asterisks denote significant difference from V, t test, P < 0.0001. E and F, Quantitative RT-PCR analysis showing relative levels of SMV positive sense RNA (E) or PR1 mRNA (F) in V and SeF1A plants at indicated dpi with SMV. Error bars indicate SD (n = 3). Asterisks denote significant differences from V for each time point, t test, P < 0.0001. G, ELISA of Tobacco ring spot virus (TRSV) levels in V and SeF1A plants at indicated dpi. Results are representative of 2–3 independent repeats.
these results suggested a correlation between cell death and ER stress and that EF1A was required for SMV-induced cell death. The S<sub>EF1A</sub> plants also exhibited impaired cell death when treated with paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), which induces cell death by promoting the formation of reactive oxygen species (ROS; Farrington et al., 1973; Hiyama et al., 1993); paraquat induced cell death on V, but not on S<sub>EF1A</sub> plants (Fig. 5D). Expression analysis of genes encoding ROS-quenching enzymes showed elevated basal levels of ascorbate peroxidase (APX) and glutathione-S-transferase (GST) in S<sub>EF1A</sub> plants (Fig. 5E), which correlated with their increased tolerance to paraquat.

We next evaluated if silencing GmEF1A alters HR-associated cell death that occurs during the pathogen resistance response because ROS contributes to the cell-death response. We chose to assay HR response against the bacterial pathogen Pseudomonas syringae pv. glycinea (Psg) expressing avrB because Rsv1 conferred extreme resistance that is not associated with visible or microscopic cell death against SMV. To this end, we generated S<sub>EF1A</sub> plants in the Rpg1-b background (cv Merit) and infected them with Psg avrB. Rpg1-b is the R protein that specifies resistance to Psg avrB in soybean (Ashfield et al., 2004). As expected, Psg avrB induced HR-like microscopic cell death on V plants, but no cell death was observed in the Psg avrB-infected S<sub>EF1A</sub> plants (Fig. 5F). Consistent with their microscopic phenotype, S<sub>EF1A</sub> plants showed reduced ion leakage (Fig. 5G) and increased susceptibility to Psg avrB (Fig. 5H). This in turn correlated with induction of the Glyma17g23900 isoform of GmEF1A in response to infection by Psg avrB (Fig. 5I). Notably, Psg avrB infection induced transcript levels of the ER stress marker BiP-D in V plants but not in S<sub>EF1A</sub> plants (Fig. 5J), suggesting that Psg avrB-induced cell death is also associated with ER stress.

eEF1B Participates in ER Stress and SMV Resistance

Because eEF1A is a subunit of the eukaryotic translation elongation complex, it is possible that the role of eEF1A in ER stress and SMV resistance is linked to its role in translation elongation. eEF1A recruits codon-specific aminoacyl-tRNAs to the ribosome during translation elongation, and this involves conversion of GTP to GDP. The eEF1B subunit, a guanine nucleotide exchange factor, then restores the GTP moiety to reanimate eEF1A. Thus, lack of eEF1B should potentially...
Knockdown of GmEF1A expression inhibits the cell death response in soybean. A and D, Visual phenotypes on leaves of Essex-Rsv1 GmEF1A knockdown plants (S_{EF1A}) or those infected with the control BPMV vector (V) in response to infection by SMV-G7 (A) or spot treatment with paraquat (D). B and F, Trypan blue staining showing microscopic cell death in V and SEF1A leaves (cv Essex)–treatment with paraquat (D). B and F, Trypan blue staining showing control BPMV vector (V) in response to infection by SMV-G7 (A) or spot treatment with paraquat (D). B and F, Trypan blue staining showing microscopic cell death in V and SEF1A leaves (cv Essex)–treatment with paraquat (D). B and F, Trypan blue staining showing control BPMV vector (V) in response to infection by SMV-G7 (A) or spot treatment with paraquat (D).

C and E, Relative mRNA levels of the hypersensitive response marker gene Hsr203j (C), or the antioxidant genes ascorbate peroxidase (APX) and glutathione-S-transferase (GST) (E) as determined by quantitative RT-PCR. Error bars indicate SD (n = 5). Asterisks denote significant difference from 0 dpi (Student’s t test, P < 0.0001). G, Electrolyte leakage in V and SEF1A plants (cv Essex)–treatment with paraquat (D). B and F, Trypan blue staining showing microscopic cell death in V and SEF1A leaves (cv Essex)–treatment with paraquat (D).

Figure 5. Knockdown of GmEF1A expression inhibits the cell death response in soybean. A and D, Visual phenotypes on leaves of Essex-Rsv1 GmEF1A knockdown plants (S_{EF1A}) or those infected with the control BPMV vector (V) in response to infection by SMV-G7 (A) or spot treatment with paraquat (D). B and F, Trypan blue staining showing microscopic cell death in V and SEF1A leaves (cv Essex)–treatment with paraquat (D). B and F, Trypan blue staining showing control BPMV vector (V) in response to infection by SMV-G7 (A) or spot treatment with paraquat (D). B and F, Trypan blue staining showing microscopic cell death in V and SEF1A leaves (cv Essex)–treatment with paraquat (D).
infection did not induce expression of any of the UPR-associated genes in SEF1B plants (Supplemental Fig. S7). Furthermore, the ER stress inducers DTT or tunicamycin resulted in increased accumulation of SMV in V plants, but not in SEF1B plants (Fig. 6, F and E, data shown for tunicamycin). This suggested that, like SEF1A, the SEF1B plants were impaired in the chemical- and SMV-induced UPR. Together, these results indicate that GmEF1A and GmEF1B contribute to UPR, and thereby SMV resistance. Notably, unlike eEF1A, P3 did not interact with eEF1B in BiFC or Co-IP assays (Supplemental Fig. S8), suggesting that GmEF1B likely functions via GmEF1A in mediating UPR.

DISCUSSION

Eukaryotic elongation factor 1A (eEF1A) is an essential protein that is not only involved in protein translation but also performs a variety of cellular functions including nucleocytoplasmic trafficking, protein degradation, cytoskeletal regulation, and activation of phosphatidylinositol 4-kinase. Here, we show a new role for eEF1A as an important regulator of UPR. UPR involves the accumulation of unfolded proteins at the ER and often occurs during the ER stress response, an important coping mechanism for plants undergoing biotic or abiotic stresses (Liu and Howell, 2010). Depending on the severity and length of stress conditions, UPR can either induce autophagy as a mechanism of cell survival or result in cell death (Williams et al., 2014). We show that the potyvirus SMV induces UPR in soybean, and this in turn promotes virus accumulation in the plant. We further show that the eEF1A subunit of the translation elongation machinery is essential for this response and thereby SMV proliferation in soybean.

Considering its abundance (1%–3% of total protein) and multifunctional properties, eEF1A is an ideal pathogen target (reviewed by Li et al. [2013]). For instance, binding of eEF1A to aminoacylated viral RNA has been suggested to enhance viral protein translation while repressing viral minus-strand RNA synthesis (Matsuda et al., 2004). eEF1A has also been suggested to stabilize viral replicase complexes and facilitate viral replicase complex or particle assembly (Ott et al., 2000; Li et al., 2010b; Warren et al., 2012). This correlates with...
the fact that eEF1A binds RdRps of some viruses (Yamaji et al., 2006; Li et al., 2013). Indeed, in addition to SMV P3, GmEF1A also interacts with the SMV RdRP, Nlb (Supplemental Fig. S9). This is similar to the binding of eEF1A with multiple HIV-1 (human immunodeficiency virus) proteins (Cimarelli and Luban, 1999; Allouch and Cereseto, 2011; Abbas et al., 2014 Warren et al., 2012). In particular, the altered localization of GmEF1A in the presence of SMV P3 and the effect of GmEF1A on pathogen/paraquat-induced cell death is strikingly similar to the effect of the HIV-1 Nef protein on eEF1A and apoptosis. Nef associates with eEF1A and promotes its nuclear-cytoplasmic transport in monocyte-derived macrophages, which in turn inhibits ER stress-induced apoptosis (Abbas et al., 2014). Thus, P3 might affect GmEF1A function by promoting increased nuclear localization of the protein in infected cells. This, together with the comparable virulence-related roles of SMV P3 and HIV-1 Nef (enhances HIV-1 replication and survival in infected cells and facilitates in vivo spread of the virus) suggest possible parallel mechanistic functions for these proteins. For instance, Nef is known to bind tRNAs and promote annealing between primer tRNA and HIV-1 RNA (Ecchari et al., 1997). This in turn could be linked to the ability of eEF1A to bind aminoacylated tRNAs. Testing the ability of P3 to bind tRNAs and GmEF1A to bind SMV RNA might help determine the role of the P3-GmEF1A interaction in SMV pathogenesis. Clearly, the interaction between P3 and eEF1A does not appear to be important for R protein-derived signaling because P3 from several SMV strains is able to interact with eEF1A, and silencing GmEF1A did not inhibit resistance derived from the Rsu1 locus.

It is also possible that the interaction with P3 affects posttranslational modifications and thereby the function of GmEF1A. For example, the addition of a phosphatidylethanolamine (PE) moiety can affect the activity of eEF1A (Hiatt et al., 1982; Whiteheart et al., 1989; Rosenberry et al., 1989; Venema et al., 1991; Ransom et al., 1998; Zobel-Thropp et al., 2000). Indeed, all the five GmEF1A isoforms contain two PE binding sites that are highly conserved in plant eEF1A proteins but have not been reported in the yeast EF1A. Interestingly, in yeast, GmEF1A localizes to the nucleus in the absence of SMV P3 (Supplemental Fig. S10). This raises the possibility that PE binding could be important for the extra-nuclear localization of eEF1A, which may be impaired in the presence of P3 thereby facilitating the retention of eEF1A within the nucleus in planta. The biological significance of the nuclear localization of eEF1A or P3 proteins remains unknown at present.

SMV-induced UPR correlates with the copurification of SMV CP with the ER membrane fraction and is consistent with a previous report suggesting the ER as the initial site of replication of the potyvirus Turnip mosaic virus (TuMV; Wei et al., 2010). Notably, only the IRE branch of UPR, but not the bZIP17/28 branch, was shown to regulate TuMV pathogenesis (Zhang et al., 2015). In contrast, SMV infection resulted in the induction of IRE1 and its downstream regulator bZIP68, as well as that of bZIP37/38, orthologs of AtbZIP17/28 that function in the other branch of UPR (Silva et al., 2015). Functional analysis of bZIP37 and bZIP38 in SMV-infected plants would help resolve their true involvement in SMV pathogenesis. The mechanism underlying ER stress activation by plant viruses or the mechanism of viral perception by ER stress sensors remain unknown. Our results suggest that binding of plant eEF1A with the viral P3 protein could at least be part of such a stress response leading to ER stress. Consistent with this notion, knockdown of GmEF1A expression inhibited the plants ability to induce UPR in response to chemical ER stress inducers or pathogen infection. Thus, UPR promotes SMV accumulation in wild-type plants and knock down of GmEF1A results in enhanced resistance. The role of GmEF1A in ER stress and SMV resistance is likely linked to its function in translation elongation because silencing genes encoding the GmEF1B subunit of the translation elongation complex also enhanced resistance to SMV and inhibited ER stress. Testing the ER stress response in plants lacking other members of the translation elongation complex, and analyzing response to SMV in plants expressing the GTPase-defective derivative of GmEF1A, could further elucidate the precise relationship between translation elongation and the ER stress response. Notably, the GmEF1A/B knockdown plants did not exhibit enhanced resistance to another potyvirus, BYMV. The replication site for BYMV is not known, but it is possible that the involvement of eEF1A/B in ER stress affects plant response to only those viruses that recruit the ER membrane for proliferation.

Reduced BiP-d transcript in the GmEF1A and GmEF1B knockout plants suggests that eEF1A and eEF1B regulate the mRNA levels of BiP, which in turn is thought to negatively regulate UPR (Leborgne-Castel et al., 1999; Alvim et al., 2001; Carvalho et al., 2014). Notably, overexpression of BiP in soybean and tobacco amplified the HR response (Carvalho et al., 2014) and conversely BiP-silenced tobacco plants showed attenuated HR. Furthermore, 35S::BiP plants showed higher SA levels and increased expression of SA-responsive marker gene PR-1 (Carvalho et al., 2014). Together, these results suggest that BiP levels are critical for UPR as well as plant defense. Consistent with results reported for BiP-silenced tobacco plants (Carvalho et al., 2014), GmEF1A knockdown plants showed reduced expression of BiP and thereby attenuated HR thus placing eEF1A upstream of BiP. Interestingly, like SMV, Psg infection also induced ER stress in soybean. Reduced expression of BiP in GmEF1A knockdown plants likely enhanced susceptibility to Psg because BiP negatively regulates ER stress. It is possible that Psg- induced ER stress is dependent on pathogen-encoded effectors that are directed to the ER (Wang and Fobert, 2013). Indeed, at least one P. syringae effector (HopD1) is known to localize to the ER (Block et al., 2014). In fact, the conserved bacterial elongation factor Tu is itself a
well-characterized PAMP in Arabidopsis and other plants of the Brassicaceae (Zipfel et al., 2004). Together, these further highlight the importance of elongation factors in regulating host-pathogen interactions.

MATERIALS AND METHODS

Plant Growth Conditions

Soybean [Glycine max (L.) Merr.] cultivars Merit (Rg1-rh), Essex (rsl), Essex-Res1 (Rsl1), Nanning 1138-2 (rsc), and Qihuang 1 (Rsc3) were grown in the greenhouse with day and night temperatures of 25°C and 20°C, respectively. Arabidopsis plants (ecotype Col-0) were grown in MTS 144 Conviron (Winnipeg, MB, Canada) walk-in-chambers at 22°C, 65% relative humidity, and 14 h photoperiod.

Construction of Viral Vectors, in Vitro Transcription, and Plant Inoculation

For silencing experiments, construction of silencing vectors, in vitro transcription, and RNA-labeling of recombinant BMOV vectors on soybean leaves, and confirmation of silencing was carried out as described previously (Kachroo et al., 2008; Kachroo and Ghabrial, 2012). A 180 bp fragment (L63-G122) of Glyma17g186600 was used to generate vectors targeting GmEF1A. A 225 bp fragment (L134-P208) of Glyma13g073200 was used to generate vectors targeting GmEF1B.

Sequence Accessions and Phylogenetic Analysis

Database accessions for sequences used here are PR1 (AI930866), β-tubulin (M21297), BtP0 (Glyma5g219600), NAP-1 (Glyma2g061600), IRE1a (Glyma01g137800), IRE1b (Glyma16g111800), IRE1c (Glyma13g073200), IRE1d (Glyma19g126800), Hor203 (Glyma06g310100), APX (L10292), GST (AF243364), the GmEF1A family (Glyma17g186600, Glyma05g089000, Glyma10g212900, Glyma16g088000, Glyma19g052400), and the GmEF1B family (Glyma13g073200, Glyma02g26600, Glyma04g195100, and Glyma14g059100). Sequence alignment and phylogenetic analysis were carried out using the Megalign program in the DNASTAR package (Swofford, 2000).

Pathogen Infection Assays and Chemical Treatments

For SMV or BYMV inoculations, infected plant tissue was homogenized in 0.01 M phosphate buffer mixed with a small amount of carborundum and used for rice-inoculation of seeds. For ELISA assays of SMV levels, 0.3 g leaf tissue from inoculated plants was collected. Total extracts were prepared by grinding in PBS buffer containing 0.14 M NaCl, 3 mM KCl, 4.5 mM NaHPO4, 2 mM KH2PO4, 1% Tween 20, and centrifuged at 12,000 g. Extract corresponding to 200 ng of protein was used for ELISA assays with purified IgG against SMV coat protein. Bacterial strains and plant inoculations were carried as described previously (Sabeti et al., 2013). For ER stress treatments, plants (V2 stage) were vacuum-infiltrated with 4 mM DTT or 10 mM Tunicamycin. Control plants were infiltrated with water or 0.1 µM DMSO, respectively. Leaf tissue was sampled at 6 h, 12 h, and 24 h post infiltration for gene expression analysis. SMV was inoculated 24 h post infiltration for pathogenicity assays.

Cell Death Assays

For ion leakage, soybean leaves (V2 stage) were infiltrated with 1 x 10^3 CFU/mL of Pog anRB (cv Merit), rice-inoculated with SMV G7 (cv Essex-Res1), or spotted with 50 µM paraquat (6, 20 µl droplets each/leaf). 6 leaf discs per treatment (d = 0.7 cm) were collected at 24 h postinfection of Pog, 7 d postinfection with SMV, or 24 h posttreatment with paraquat, washed in distilled water for 30 min, and transferred to tubes containing 10 mL of distilled water. Conductivity of the solution was measured every 2 h for 24 h with an NIST traceable digital Conductivity Meter (Fisher Scientific, Waltham, MA). δσ was calculated from three replicate measurements per treatment per experiment. Results are representative of three independent experiments.

RNA Extraction and Quantitative RT-PCR Analysis

RNA from leaf tissues of soybean plants at V2/V3 growth stage was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA), per manufacturer’s instructions. Reverse transcription (RT) and first-strand cDNA synthesis were carried out using Superscript II (Invitrogen, Carlsbad, CA). Two to three independent RNA preparations were analyzed by quantitative RT-PCR to evaluate relative differences in transcript levels. Primers were designed to amplify gene-specific PCR products of <200 bp in size. Actin was employed as an internal control to normalize the cDNA. qRT-PCR was carried out in 96 well plate using SYBR Green Mix, with cycling conditions described previously (Wang et al., 2014). Gene expression was quantified using the relative quantification (ΔΔCt) method as described before (Livak and Schmittgen, 2001). Each sample or treatment was tested in at least three biological repeats and the same experiment was performed twice.

Protein Extraction and Immunoprecipitation Assays

Proteins were expressed as N-terminal epitope-tagged fusions in N. benthamiana using the pSITE vectors (Martin et al., 2009). Total extracts of plant proteins were prepared by grinding 1 g leaf tissue in buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 5 mM DTT, and 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Total protein extract was centrifuged at 10,000 g for 10 min, followed by a second centrifugation at 125,000 g. For immunoblot analysis, proteins (10–50 µg) were fractionated on 7–10% SDS-PAGE gels and subjected to immunoblot analysis using α-MYC, α-FLAG (Sigma-Aldrich, St. Louis, MO) or antibodies specific to coat proteins of respective viruses. Immuno-blots were developed using ECL detection (Roche) or alkaline-phosphate-based color detection. For Poncearsain-staining, PVDF membranes were incubated in Poncearsain solution (40% methanol [v/v], 15% acetic acid [v/v], 0.25%, Poncearsain). Then membranes were washed in deionized water. IP assays were performed by incubating total protein extracts with 2 ML-MAX-affinity beads (unless noted otherwise), followed by extensive washing with extraction buffer lacking PVPP. Proteins were separated on 10% SDS-PAGE at 100V for 1–2 h and detected by western blot analysis using protein-tag-specific antibodies. Expected Mr of proteins: GmEF1A ~ 50 kD, FLAG-GmEF1A ~ 50 kD, MYC-GmEF1A ~ 64 kD, MYC-P3 ~ 44 kD, FLAG-P3 ~ 39 kD, MYC-NB ~ 40 kD, SMV-CP ~ 24 kD, BVM-CP ~ 40 kD, CMV-CP ~ 24 kD, TCV-CP ~ 38 kD, BYMV-CP ~ 30 kD, BP ~ 74 kD.

Membrane Protein Isolation

Total microsomal membranes were obtained by homogenization of N. benthamiana leaves transiently expressing 3×-FLAG-GmEF1A/B or SMV P3 (via Agrobacterium-mediated expression). Leaf tissues were homogenized in buffer containing 250 mM Tris-HCl (pH 10.5), 200 mM Suc, 25 mM EDTA, 10 mM DTT, 1× Protease inhibitor cocktail (Roche), 1 mM PMSF and 2% PVPP. Crude lysate was filtered through four layers of cheese-cloth and centrifuged at 10,000 g for 10 min at 4°C. The clear supernatant constitutes the total protein (T) fraction. Total protein fractions were centrifuged at 100,000 g for 1 h at 4°C to obtain supernatant comprising the soluble (S) fraction and pellet comprising the microsomal (M) fraction. The microsomal membrane pellet was washed with homogenization buffer (lacking PVPP) to remove soluble protein contaminations. The total (T), soluble (S), and membrane (M) protein fractions were analyzed by standard SDS-PAGE (10% gel) followed by immunoblottedting with anti-FLAG M2 antibody (GmEF1A, or P3), anti-TIP (tonoplast intrinsic protein, membrane protein control) and anti-Hsc70 (soluble protein control, Agriser). The expected Mr of control proteins were: TIP ~ 23 kD, and Hsc70 ~ 70 kD.

ER membrane fractionation was carried out as described before (Edwards and Lloyd, 1977). Briefly, total proteins were extracted in homogenization buffer (0.5 M Suc, 10 mM KCl, 1 mM EDTA, 1 mM MgCl2, 2 mM DTT, 0.1 mM phenylmethyl-sulfonyl fluoride, 150 mM Tricine/KOH pH 7.5). 5 mL of the protein extract was layered on a preformed Suc gradient (8 mL step of 80% on 20% weight/volume of Suc). Fractions were collected from each layer and used for western blot analysis using antibodies specific to SMV-CP or BP (tumoral binding protein, Hsp70 family ER chaperone).

Yeast Two-Hybrid Assay

A soybean cDNA library (~0.68 x 10^7 clones) from SMV-SC15 infected soybean (cv Nanning1138-2) was cloned into the modified vector pPR3-N using Gateway technology. The SMV SC15 P3 protein cloned in pBT3 was used as bait.
to screen the library (32 clones) by cotransformation in yeast (NMY51). Yeast transformants expressing P3-interacting proteins were selected on synthetic dropout medium lacking tryptophane, Leu, His, and adenine. Yeast strains expressing P3 interactors were further assessed for β-galactosidase activity. Positive interactors including GmEF1A were identified based on sequence identity.

**Nuclear Localization Assay**

Full-length P3, eEF1A, and eEF1B were cloned into the pJNIA-Ca vector (Zaltsman et al., 2002), which produced in-frame fusion of the proteins to the C terminus of the mLexA-Gal4AD chimera. mLexA-Gal4AD induces reporter gene expression only if the test protein is able to enter the nucleus. The constructs were transformed into yeast strain L40 and selected for growth on synthetic dropout medium lacking Trp, Leu, His, and adenine at 29°C for 2–4 d.

**Bimolecular Fluorescence Complementation Assays**

BiFC assays were carried out as described before (Selote and Kachroo, 2010). Briefly, the various proteins were fused to the N/C-terminal halves of EYFP using the pSITE-n/cEYFP vectors (Martin et al., 2009) and introduced in Agrobacterium tumefaciens using the pSITE-n/cEYFP vectors (Martin et al., 2009) and introduced in Agrobacterium tumefaciens strain LBA4404. Agrobacterium strains expressing proteins fused to reciprocal halves of EYFP were co-infiltrated into CFP-H2B-tagged *N. benthamiana* plants (transgenic plants expressing nuclear localized CFP). 48 h later, water-mounted sections of leaf tissue were examined by confocal microscopy using a water-immersion PLAPO60XWLSM (NA 1.0) objective. Positive interactions were detected as yellow fluorescence upon reconstitution of the complete EYFP. CFP and YFP overlay images (40x magnification) are shown. All interactions were confirmed using both combinations of reciprocal nEYFP/eEYFP fusion proteins in two separate experiments (three replicates per experiment). Protein localization assays were done using agroinfiltration of C-terminal YFP/CFP tagged proteins in transgenic *N. benthamiana* plants expressing RFP-H2B or RFP tagged with the amino-terminal signal sequence of Arabidopsis thaliana basic chitinase and a carboxy-terminal HDEL ER-retention signal (Goodin et al., 2007).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Amino acid sequence alignment of GmEF1A isoforms.

**Supplemental Figure S2.** qRT-PCR analysis of eEF1A mRNA levels in soybean.

**Supplemental Figure S3.** Knockdown of GmEF1A in soybean.

**Supplemental Figure S4.** Effect of GmEF1A-knockdown on soybean response to SMV.

**Supplemental Figure S5.** Effect of GmEF1A-knockdown on soybean response to BYMV.

**Supplemental Figure S6.** Amino acid sequence alignment of GmEF1B isoforms.

**Supplemental Figure S7.** Effect of GmEF1B-knockdown on UPR-related gene expression.

**Supplemental Figure S8.** eEF1B does not interact with SMV P3.

**Supplemental Figure S9.** GmEF1A interacts with SMV Nib.

**Supplemental Figure S10.** Nuclear import assay of GmEF1A and SMV P3.

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**LITERATURE CITED**


Desbiez C, Gal-On A, Girard M, Wipf-Scheibl C, Legoc H (2003) Increase in Zucchini yellow mosaic virus symptom severity in tolerant zucchini cultivars is related to a point mutation in P3 protein and is associated with a loss of relative fitness on susceptible plants. Phytopathology 93: 1478–1484


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Fu DQ, Gabrial S, Kachroo A (2009) GmRAR1 and GmSCt1 are required for basal, R-gene-mediated and systemic acquired resistance in soybean. Mol Plant Microbe Interact 22:86–95


Verchot J (2014) The ER quality control and ER associated degradation machineries are vital for viral pathogenesis. Front Plant Sci 5: 66


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