Layered pattern receptor signaling via ethylene and endogenous elicitor peptides during *Arabidopsis* immunity to bacterial infection

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Recognition of molecular patterns characteristic of microbes or altered-self leads to immune activation in multicellular eukaryotes. In *Arabidopsis thaliana*, the leucine-rich-repeat receptor kinases *FLAGELLIN-SENSING2* (*FLS2*) and *EF-TU RECEPTOR* (*EFR*) recognize bacterial flagellin and elongation factor EF-Tu (and their elicitor-active epitopes flg22 and elf18), respectively. Likewise, *PEP1 RECEPTOR1* (*PEPR1*) and PEPR2 recognize the elicitor-active Pep epitopes conserved in *Arabidopsis ELICITOR PEPTIDE PRECURSOR*s (*PROPEP*s). Here we reveal that loss of *ETHYLENE-INSENSITIVE2* (*EIN2*), a master signaling regulator of the phytohormone ethylene (ET), lowers sensitivity to both elf18 and flg22 in different defense-related outputs. Remarkably, in contrast to a large decrease in *FLS2* expression, *EFR* expression and receptor accumulation remain unaffected in *ein2* plants. Genome-wide transcriptome profiling has uncovered an inventory of EIN2-dependent and EFR-regulated genes. This dataset highlights important aspects of how ET modulates EFR-triggered immunity: the potentiation of salicylate-based immunity and the repression of a jasmonate-related branch. EFR requires ET signaling components for *PROPEP2* activation but not for *PROPEP3* activation, pointing to both ET-dependent and -independent engagement of the PEPR pathway during EFR-triggered immunity. Moreover, PEPR activation compensates the *ein2* defects for a subset of EFR-regulated genes. Accordingly, *ein2 pepr1 pepr2* plants exhibit additive defects in EFR-triggered antibacterial immunity, compared with *ein2* or *pepr1 pepr2* plants. Our findings suggest that the PEPR pathway not only mediates ET signaling but also compensates for its absence in enhancing plant immunity.

microbe-associated molecular pattern | danger-associated molecular pattern | disease resistance | plant hormone | defense-related genes

Like animals, plants have evolved a repertoire of pattern recognition receptors (PRRs) that recognize molecular signatures typical of a class of microbes (microbe-associated molecular patterns; MAMPs). MAMPs include bacterial flagellin, EF-Tu, lipopolysaccharides, peptidoglycans, and fungal cell wall-derived chitin fragments (1, 2). Perception of different MAMPs by cognate PRRs triggers immune responses that restrict the propagation of microbes, designated MAMP-triggered immunity (MTI). Loss of single PRRs allows enhanced growth of adapted and nonadapted bacterial pathogens, providing evidence for the significance of MAMP sensing and signaling in plant immunity (3–6).

Upon MAMP perception, PRRs trigger a stereotypic set of defense-associated outputs, including changes in ion fluxes, reactive oxygen species (ROS) spiking, MAPK activation, ethylene (ET) production, *GLUCAN SYNTHASE-LIKE5*-dependent callose deposition, extensive transcriptional reprogramming, and metabolic changes (1, 2). Our earlier molecular genetic work points to a critical role for sustained transcriptional reprogramming in EFR-triggered immunity (7). Close association of this late-phase MTI output with immune activation has been also described for the FLS2 pathway (8, 9). However, the mechanisms that couple MAMP recognition with sustained transcriptional reprogramming remain elusive.

In addition to MAMPs, perception of danger-associated molecular patterns (DAMPs) that represent altered-self also leads to immune activation (1, 10). The so-called Pep epitopes derived from PROPEPs are perceived by the two closely related transmembrane receptors PEPR1 and PEPR2, thereby triggering an immune response that resembles MTI (11, 12). However, the lack of an N-terminal signal peptide for targeting PROPEPs to the canonical secretory pathway leads to a model in which loss of membrane integrity upon cellular stresses facilitates the release of Pep ligands from the cytoplasm to the extracellular spaces or endomembrane compartments. Of six *Arabidopsis PROPEP* members, *PROPEP2* and *PROPEP3* are massively induced during defense activation (13), implying their selective contribution to immunity. However, compelling evidence is missing for the significance of this putative DAMP pathway in MTI. Moreover, possible cross-talk between different PRR pathways has been underexplored.

Different defense-related hormones become engaged and play a pivotal role for optimizing transcriptional reprogramming in MTI. Salicylate (SA) is a major plant hormone that is central to inducible defenses against biotrophic and hemibiotrophic pathogens, whereas jasmonate (JA) and ET primarily contribute to defenses against necrotrophic pathogens (14, 15). Genetic evidence demonstrates a role for SA in MTI (8). ET modulates SA-based immunity both positively and negatively (15): ET has synergistic effects on SA-induced expression of *PR1* (16), encoding a defense-related secreted protein (17), whereas the ET-responsive transcription factors EIN3 and *EIN3-LIKE1* (*EIL1*) attenuate SA biosynthesis by direct binding and repression of *SALICYLIC ACID INDUCTION DEFICIENT2* (*SID2*), encoding an SA biosynthesis enzyme (18). As such, defense-related hormone pathways constitute a complex signaling network that provides a basis for flexible control of immune response. Although antagonism is typically seen between SA- and JA-mediated immunity (14, 15), extensive genetic studies involving either separate or simultaneous disruption of the SA, JA, and ET pathways have revealed their synergistic interactions in promoting MTI (8).

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In *Arabidopsis*, *FLS2* transcription is under the direct control of EIN3 and EIL1 and requires intact ET signaling (19, 20). Hence, the marked decrease in steady-state FLS2 levels seen in ET dysfunctional mutants obscures the possible contribution of this hormone to later stages of MTI, downstream of the receptor. Therefore, it is unclear whether the previously described impairment of flg22-triggered outputs in these mutants can be attributed to the lowered FLS2 expression or signaling defects (21–24).

Here we reveal that *ein2* plants are less sensitive to both elf18 and flg22, pointing to a role for EIN2 in both PRR pathways. Remarkably, in contrast to FLS2, EFR expression is retained in the absence of ET signaling components. This allows us to dissect the contributions of ET to MTI other than PRR generation. Our genetic and genomic evidence suggests that ET-dependent and ET-independent functions of the PEPR pathway respectively mediate ET signaling or provide a backup for ET signaling defects in MTI. Consistent with this, simultaneous disruption of EIN2 and PEPRs severely reduces EFR-triggered immunity to bacterial infection. Together, our results suggest that ET and PEPR signaling provide critical steps between MAMP recognition and effective MTI activation.

Results

***Arabidopsis priority in sweet life6* Mutant, Carrying a Dysfunctional *ein2* Allele, Is Less Sensitive to both flg22 and elf18.** In *Arabidopsis* seedlings, sucrose-induced flavonoid accumulation is repressed upon exposure to different MAMPs (6, 9). Using this readout, we screened ~60,000 ethyl methanesulfonate-mutagenized M2 seedlings for individuals that were unresponsive to elf18. Among >50 lines of *priority in sweet life* (*psl*) mutants, the majority were less sensitive to elf18 but retained WT-like responsiveness to flg22, including *efr* alleles and nonreceptor *psl* alleles described earlier (6, 7). Notably, *psl6* mutant seedlings substantially accumulated anthocyanin in the presence of not only elf18 but also flg22 (Fig. 1*A* and *B*). This became apparent at low MAMP doses, although WT-like anthocyanin repression occurred in *psl6* plants with elf18 at 500 nM (Fig. 1*B*). We also found that *psl6* mutants showed a reduction in ROS spiking and callose deposition in response to both elf18 and flg22 (Fig. 1 *C* and *D*). Moreover, both elf18- and flg22-triggered induction of *PATHOGENESIS-RELATED GENE1* (*PR1*), an indicator for SA-based immunity and MTI (7, 17), was also much reduced in *psl6* plants (Fig. 1*E*). Together, these data indicate that PSL6 contributes to both EFR and FLS2 function. Consistent with these defects in the two PRR pathways, *psl6* plants showed strong supersusceptibility to the virulent bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Fig. 1*F*).

We identified *PSL6* by positional cloning and subsequent characterization of another allele. *PSL6* encodes the transmembrane protein EIN2 (*SI Materials and Methods* and Fig. S1), which is essential for the vast majority of ET responses (25). Our data suggest that residual EIN2 activity is greater in the *psl6* allele compared with the *ein2-1* allele (Fig. 1*B* and Fig. S1*D*), which correlated with the predicted difference in preservation of the cytoplasmic domain (Fig. S1*B*). We then found that elf18-triggered ROS spiking and callose deposition were also reduced in *ein2* mutants (Fig. S1 *E* and *F*). However, both *psl6* and *ein2-1* plants were largely indistinguishable from WT plants in MAPK activation and the ET levels upon elf18 treatment (Fig. S1 *G* and *H*). These results indicate that EIN2 is rate-limiting for ROS spiking and callose deposition but dispensable for MAPK activation and ET production in the EFR pathway.

EIN2 Is Dispensable for *EFR* Expression but Is Required for *FLS2* Expression. Next we determined EFR expression levels in *ein2* plants. Immunoblot analysis of total protein extracts derived from nonelicited seedlings revealed that the steady-state EFR accumulation was largely unaffected in both *ein2* alleles (Fig. 2*A*). Quantitative RT-PCR (qPCR) analysis verified that *ein2* plants show WT-like *EFR* mRNA accumulation, either with or without elf18 application (Fig. 2 *B* and *C*). By contrast, *FLS2* transcripts and receptor protein accumulation were greatly reduced in both

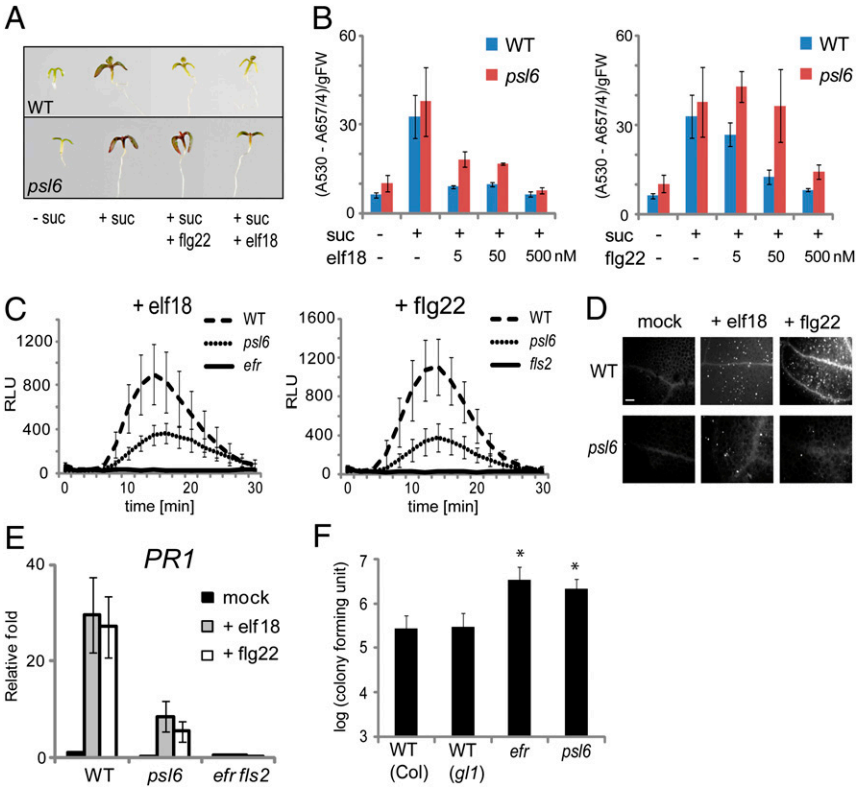

Fig. 1. *psl6* mutant plants exhibit reduced elf18- and flg22-triggered responses. (*A*) Seedlings grown in the absence (–suc) or presence of 100 mM sucrose (+suc) without or with 50 nM flg22 or 50 nM elf18. (*B*) Anthocyanin content of seedlings grown in the absence (–suc) or presence of 100 mM sucrose (+suc) without or with the indicated concentrations of elf18 (+elf18) or flg22 (+flg22). Mean ± SD ($n \geq 12$) is shown. (*C*) ROS spiking triggered in leaf disks treated with 100 nM elf18 or flg22. Mean ± SD ($n = 16$). (*D*) Callose deposition in seedlings treated with water (mock), 1 μM elf18 or flg22 for 16 h. (*E*) qPCR analysis for *PR1* expression in seedlings treated with water (mock) or 1 μM elf18 for 24 h. The relative induction (in fold) is shown, with the gene/At4g26410 (reference gene) value in mock-treated WT plants = 1. A representative dataset with mean ± SD of three experimental replicates is shown. The same conclusion was obtained in three independent experiments. (*F*) Growth of *Pst*. DC3000 in 4-wk-old plant leaves 4 d after spray inoculation with bacteria at 5×10^8 cfu/mL. *Statistically significant differences compared with WT at $P < 0.05$. WT used in *A–D* is *gl1*.

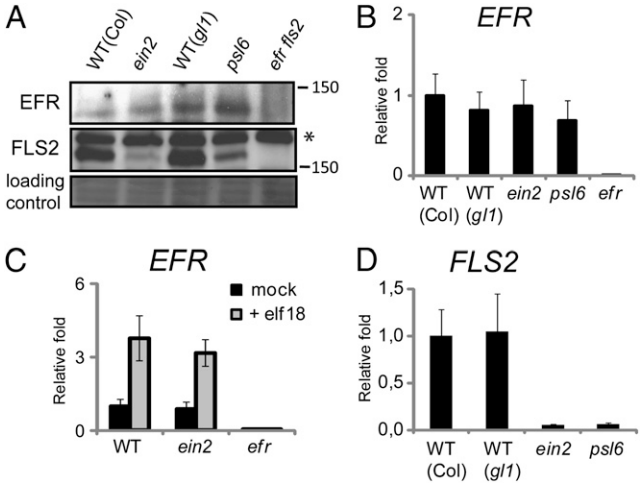

Fig. 2. Steady-state accumulation of EFR but not of FLS2 is retained in *ein2* plants. (*A*) Imunoblot analysis of total protein extracts from nonelicited WT, *ein2-1*, *psl6*, and *efr fls2* seedlings with anti-EFR and anti-FLS2 antibodies. A Coomassie blue-stained blot is presented as loading control. Positions of molecular weight markers (kDa) are indicated on the right. The asterisk indicates a cross-reacting band to the antibodies used. (*B–D*) qPCR analysis for *EFR* and *FLS2* expression in 12-d-old seedlings. Nonelicited seedlings (*B* and *D*) or seedlings treated with water (mock) or 1 μM elf18 for 24 h (*C*) were subjected to qPCR analysis as described in Fig. 1*E*.

ein2 alleles (Fig. 2 *A* and *D*), as described earlier (19, 20). Accordingly, there is no discernible EIN3/EIL1 binding site in the *EFR* promoter within 2,000 bp upstream of the transcriptional initiation site, whereas EIN3 binding was demonstrated in the *FLS2* promoter, which is predicted to harbor nine potential binding sites (19). Taken together, the essentially normal receptor accumulation, MAPK activation, and ET production in *ein2* plants (Fig. 2*A* and Fig. S1 *G* and *H*) indicate that EIN2 is dispensable for the generation of functional EFR. Rather, our results suggest the existence of an ET-dependent step(s) located downstream of receptor accumulation required for ROS spiking, callose deposition, anthocyanin repression, and transcriptional reprogramming.

Genome-Wide Identification of EFR-Regulated Genes Influenced by EIN2. To have an overview of the role of ET in EFR-triggered immunity, we obtained transcriptome profiles (Affimetrix ATH1 microarray) from seedlings exposed to elf18. This identified a total of 15,796 genes expressed above background levels. Plotting the relative expression fold change in elf18-treated plants vs. nontreated controls revealed a large overlap in the overall transcriptome profiles between WT and *ein2* plants. However, the divergence between the profiles was more pronounced at 10 h compared with 2 h after elf18 treatment (Fig. 3 *A* and *B*).

In elf18-treated WT plants, 1,218 and 967 genes showed ≥twofold up- or down-regulation ($q < 0.05$) at 2 h, whereas 1,932 and 2,195 genes were up- or down-regulated at 10 h, respectively, compared with control plants (Dataset S1). Of these EFR-regulated genes, we scored the genes whose up- or down-regulation was EIN2 dependent (using a cutoff of ≥twofold difference in transcript levels between WT and *ein2* plants). On this basis, 153 up- and 111 down-regulated genes at 2 h, and 234 up- and 232 down-regulated genes at 10 h, respectively, were defined as EIN2-dependent EFR-regulated genes (EFR-EIN2 regulons; Dataset S2). Of these, 121 and 110 genes showed a ≥twofold decrease or increase, respectively, in the basal transcript levels in nonelicited *ein2* plants compared with WT plants (Dataset S3).

To gain insight into the mechanisms by which ET influences EFR-triggered immunity, we performed hierarchical clustering analysis for 466 EFR-EIN2 regulons scored at 10 h after elf18 treatment. The majority (93%) of these genes could be classified into six clusters according to their distinct requirements for ET (Fig. 3*C*, Fig. S2, and Dataset S4): in the promotion or attenuation of EFR-mediated induction (clusters 1 and 2, respectively); in the promotion of both EFR-dependent induction and EFR-independent basal expression (cluster 3); in alleviating EFR-mediated repression (cluster 4); in the avoidance of induction, with or without elf18 (cluster 5); and in EFR-mediated repression (cluster 6).

For each cluster, we separately cross-referenced the constituent genes to a previously published transcriptome database for their

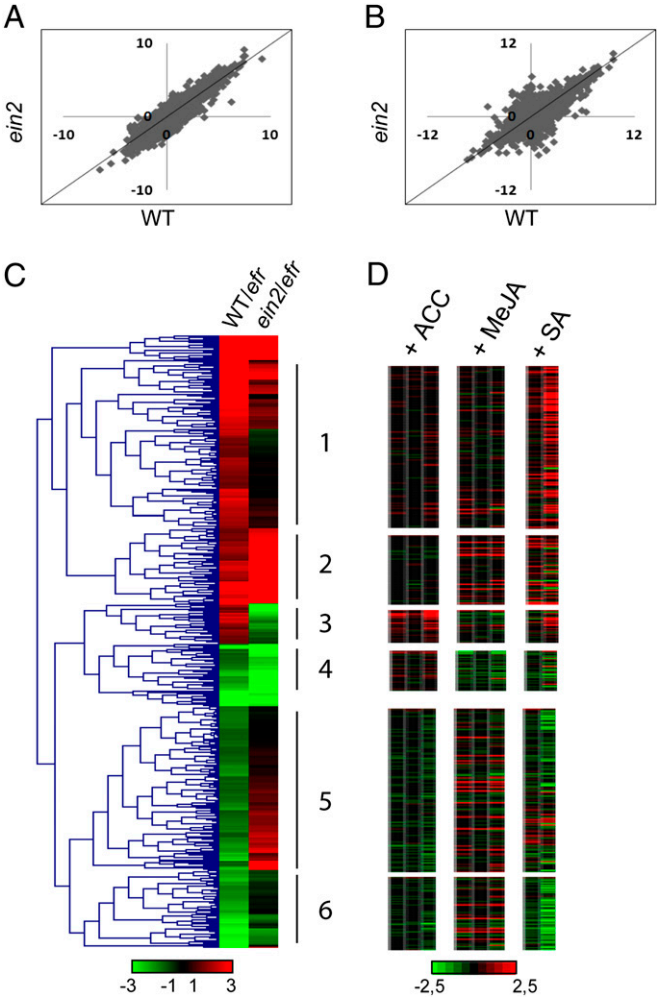

Fig. 3. Genome-wide profiling of EFR-triggered transcriptional reprogramming. (*A* and *B*) Analysis of global gene expression in 12-d-old WT and *ein2-1* seedlings upon elf18 application for 2 h (*A*) and for 10 h (*B*). The *x* axis shows the log2 ratios of transcript levels in elf18-treated WT seedlings vs. untreated WT seedlings, and the *y* axis shows the log2 ratios of transcript levels in elf18-treated *ein2* seedlings vs. untreated *ein2* seedlings (*A*). The *x* axis shows the log2 ratios of transcript levels in elf18-treated WT seedlings vs. elf18-treated *efr* seedlings, and the *y* axis shows log2 ratios of transcript levels in elf18-treated *ein2* seedlings vs. elf18-treated *efr* seedlings (*B*). (*C*) Hierarchical clustering analysis of EIN2-dependent elf18-responsive genes. Each column and row represents the mean values of three biological replicates and a gene probe, respectively. Six clusters are recognized in 466 genes that show EFR-triggered changes of transcript levels in WT seedlings (at a cutoff of 1 [$\log_2$], $q < 0.05$), which are altered in *ein2* seedlings (at a cutoff of 1 [$\log_2$]), upon elf18 application for 10 h. (*D*) The genes of six identified clusters are cross-referenced to a public database for their expression responses to ACC, MeJA, or SA (Genevestigator v3). Each column represents the values of biologically independent different datasets. The relative expression (in $\log_2$ ratios) is colored red for induction and green for repression as illustrated in the fold change color bars (*C* and *D*).

expression responses to ET [1-aminocyclopropane-1-carboxylic acid (ACC)], methyl-JA (MeJA), and SA (Fig. 3*D*). This revealed that SA-inducible and SA-repressible genes are respectively overrepresented in elf18-inducible and elf18-repressible genes (Table S1), pointing to a close correlation between EFR- and SA-mediated immunity. By contrast, EFR- and MeJA-regulons do not show such a correlation (Table S1).

Notably, elf18-induced genes, whether promoted or attenuated by ET (clusters 1 and 2, respectively), included an overrepresentation of SA-inducible genes. ET alone seems to be insufficient to induce the vast majority of cluster 1 genes (Fig. 3*D*), including *PR1*. These data are in accordance with the previously described dual role of ET in positive and negative modulation of SA-mediated responses (15). By contrast, cluster 3 genes are typically induced upon ACC treatment (Fig. 3*D*), including *FLS2*. The majority of cluster 3 genes require *EIN2* for basal expression (Fig. S2*A*), implying a direct role of ET in their expression.

JA-repressible and/or ET-inducible genes were overrepresented in cluster 4 (Table S1). Conversely, JA-inducible and/or ET-repressible genes were overrepresented in clusters 5 and 6, as well as cluster 2 (Table S1). These results imply that ET serves to antagonize these JA-responsive genes in the EFR pathway.

Ethylene Signaling Contributes to EFR-Triggered Transcriptional Reprogramming. To further investigate the EFR-EIN2 regulons, we traced the expression of different defense-related genes in *ein2* plants. ET-inducible *ETHYLENE RESPONSIVE FACTOR1* (*ERF1*) and *MYB DOMAIN PROTEIN51* (*MYB51*) encode transcription factors that promote ET responses and glucosinolate biosynthesis, respectively (26, 27). Both transcript levels were elevated at 2 h of elf18 application in WT plants but were much lower in *ein2* plants (Fig. 4*A*). Together with the EIN2 dependence of late-responsive *PR1* induction (Fig. 4*A*), our results indicate that ET influences EFR-mediated transcriptional reprogramming in the early and late phases. According to their basal expression in nonelicited *ein2* plants (Fig. 4*A*), *ERF1*/*PR1* and *MYB51* represent the characteristic features of gene clusters 1 and 3, respectively. We also verified that EFR represses, in particular, the transcription factor MYC2-regulated JA branch (28) in an EIN2-dependent manner (Table S2). A marker for this MYC2 branch, *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), is derepressed in nonelicited and elf18-treated *ein2* plants (Fig. 4*A*), which well represents the features of cluster 5 genes.

We confirmed that the loss of both EIN3 and EIL1 reduces elf18-induced activation of *ERF1* and *PR1* (Fig. S3). Notably, rapid ET elevation is retained in *ein2* plants in response to elf18 (Fig. S1*H*). We thus conclude that dysfunctional ET signaling causes improper transcriptional reprogramming during EFR-triggered immunity.

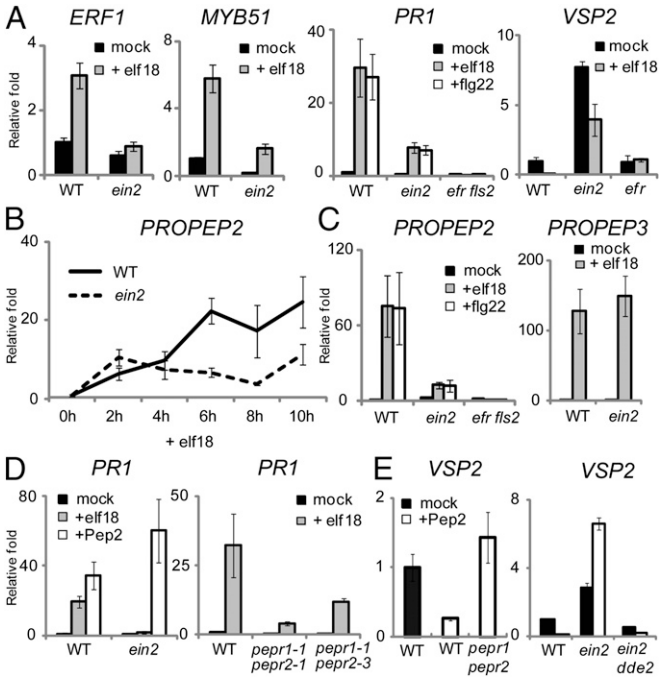

Fig. 4. Functional link between ET- and PEPR-signaling in EFR-triggered transcriptional reprogramming. (*A*) Gene expression in 12-d-old seedlings treated with water (mock) or 1 μM elf18 for 2 h (for *ERF1* and *MYB51*) or 24 h (for *PR1* and *VSP2*), as well as with 1 μM flg22 for 24 h for *PR1*. (*B*) *PROPEP2* expression in seedlings upon 1 μM elf18 for the indicated times. (*C*) *PROPEP2* and *PROPEP3* expression in seedlings treated with water (mock) or 1 μM elf18 for 24 h. (*D*) *PR1* expression in seedlings treated with water (mock), 1 μM elf18, or 1 μM Pep2 for 10 h. (*E*) *VSP2* expression in seedlings treated with water (mock) or 1 μM Pep2 for 24 h. *ein2-1* and *pepr1-1 pepr2-3* were used in *A*–*E*.

Functional Link Between ET and PEPR Signaling in the EFR Pathway. It has been proposed that the PEPR pathway serves to amplify defense signaling in MTI (10). We found a substantial decrease of elf18-induced *PROPEP2* activation in *ein2* and *ein3 eil1* plants (Fig. 4*B*, Fig. S3, and Dataset S2). By contrast, elf18-induced *PROPEP3* activation is largely unaffected in *ein2* plants (Fig. 4*C*). These results suggest that PEPR ligands are generated in both ET-dependent and -independent manners, thereby allowing the engagement of the PEPR pathway in EFR-triggered immunity, irrespective of ET availability.

We reasoned that if the PEPR pathway mediates ET signaling in the EFR pathway, its enforced activation would rescue the observed defects of *ein2* mutants in EFR-triggered outputs. In line with this model, *PR1* is activated in *ein2* plants upon Pep2 application, as in elf18-treated WT plants (Fig. 4*D*). The above model would also predict the impairment of elf18-triggered outputs in *pepr1 pepr2* mutants that are insensitive to Pep peptides (Fig. S4 *A* and *B*) (11, 12). Indeed, elf18-induced *PR1* activation is much reduced in *pepr1 pepr2* plants (Fig. 4*D*), while in contrast the coactivation of MAPKs, ROS spiking, and ET production remains essentially intact (Fig. S4 *C*–*E*). This points to a selective requirement of PEPRs for EFR-triggered transcriptional reprogramming among the tested outputs. The EFR-triggered signal might be sequentially relayed via ET signaling and then the PEPR pathway leading to *PR1* activation. Our data indicate the presence of similarly regulated genes (i.e., those requiring EIN2 for elf18 induction but not for Pep2 induction) (Fig. S5 and Dataset S5). This suggests that PEPR activation keeps this branch on and thus compensates in part for the MTI defects caused by dysfunctional ET signaling. Accompanying studies also support this notion (29).

We also found that Pep2 as well as elf18 down-regulated *VSP2* expression in WT plants (Fig. 4 *A* and *E*). However, *VSP2* transcript levels were constitutively elevated in *ein2* plants even in the presence of Pep2 (Fig. 4*E*), indicating that PEPRs require ET to repress this gene. The *VSP2* elevation in *ein2* plants is dependent on JA, because it is abolished in the (*delayed dehiscence2*) *dde2* mutant background that is defective in JA biosynthesis (30) (Fig. 4*E*). Thus, our results imply that the PEPR pathway contributes to EFR-mediated repression of the MYC2-JA branch.

EIN2-independent activation of subsets of defense genes upon Pep2 treatment (Fig. 4*D* and Fig. S5) prompted us to test whether PEPR function is tolerant to ET signaling perturbations. *PEPR1* expression was retained or even enhanced in *ein2* plants with or without elf18 treatment (Fig. S6*A*). Pep2-induced *PROPEP2* activation and root growth retardation remained unaffected in the mutant (Fig. S6 *B* and *C*). Moreover, we found that PEPR subfunctions are retained in the absence of the *N*-glycosylation-dependent endoplasmic reticulum (ER) protein-folding pathway (Fig. S6 *D* and *E*), in which EFR biogenesis and function are collapsed (6, 7). Our data indicate that PEPR signaling is robust

against all these perturbations that impair FLS2 and/or EFR function, providing a possible advantage for the engagement of this pathway in MTI.

Ethylene and PEPRs both Contribute to EFR-Triggered Immunity. To test the significance of ET and PEPRs in MTI, we first spray-inoculated plants with *Pst* DC3000. We observed enhanced bacterial growth in *ein2* and *pepr1 pepr2* plants (Fig. 5*A*). Consistent with the aforementioned functional link between ET and PEPRs, *ein2 pepr1 pepr2* plants showed strong supersusceptibility to the bacteria upon their inoculation onto leaf surfaces (Fig. 5*A*). However, it is unclear whether the enhanced bacterial growth is related to the EFR signaling defects. To unambiguously assess EFR-triggered immunity in these mutant plants, we next measured elf18-induced resistance, which we defined as the decrease of bacterial growth in elf18-pretreated leaves compared with mock controls (4). The bacteria were pressure infiltrated into the apoplast so that stomatal defense (31) can be largely ignored in this assay. We verified that elf18 pretreatment greatly reduces the growth of *Pst* DC3000 and its mutant strain lacking the phytotoxin coronatine (COR) (32) in an *EFR*-dependent manner (Fig. 5 *B* and *C*). In mock-treated *ein2* plants, the bacterial growth was slightly lower compared with WT plants for the two tested *Pst* strains (Fig. 5 *B* and *C*). However, importantly, the elf18-induced resistance against the *COR* (-) strain was reduced similarly in both *ein2* and *pepr1 pepr2* plants and was more severely impaired in *ein2 pepr1 pepr2* plants (Fig. 5*B*). Elf18-induced resistance against *Pst* DC3000 was retained in *ein2* or *pepr1 pepr2* plants but was weakened in the triple mutants (Fig. 5*C*). Our results suggest that ET and PEPRs substantially and independently contribute to EFR-triggered immunity to bacterial infection. Given the aforementioned EIN2-independent PEPR function (Fig. 4 *C* and *D* and Dataset S5), the enhanced immune defects shown by the triple mutants might reflect an important contribution of the PEPR pathway to EFR-triggered immunity when ET signaling is disrupted. Moreover, unexpectedly, the mutational effects of *EIN2* or *PEPRs* were less apparent upon challenge with the *COR* (+) bacteria under our assay conditions (Fig. 5*C*).

Discussion

The identification of the *Arabidopsis psl6* mutant has revealed a role for EIN2 in subsets of EFR- and FLS2-triggered outputs, including transcriptional reprogramming. Of particular note, *PR1* and *PROPEP2* activation is similarly impaired in *ein2* plants in response to flg22 or elf18 (Fig. 4 *A* and *C*), despite marked differences in the expression levels of the cognate receptors. The virtually normal accumulation of EFR in *ein2* plants argues for a critical role of ET at stages of MTI subsequent to receptor accumulation.

Our transcriptome profiling has uncovered important mechanisms by which ET influences EFR-triggered immunity. A striking feature of the EFR-EIN2 regulons is the high correlation between elf18- and SA-responsiveness. This points to a central role of SA in EFR-triggered immunity, as described earlier (8), and its modulation by ET. ET exerted either synergistic or antagonistic effects on distinct subsets of SA-related genes for their elf18 responsiveness (Fig. 3*C*). Numerous SA-related genes were also scored as EIN2-independent EFR-regulated genes (Table S1). Thus, our results suggest that ET modulates SA signaling rather than SA levels during EFR-triggered immunity. A second characteristic feature of the EFR-EIN2 regulons is the negative correlation between ET- and JA-responsiveness. This suggests JA-responsive genes that are antagonized, rather than synergized, by ET are targeted by ET-mediated EFR signaling, as exemplified by the ET-dependent repression of *VSP2* by EFR. Overall, our studies support a model in which EFR relies on ET signaling to augment SA-based immunity and to antagonize subsets of JA signaling branches during MTI. The two effects are not mutually exclusive, because both features are common to numerous genes in the EFR-EIN2 regulons (Fig. 3*D*). Notably, we obtained these data from seedlings exposed to elf18 under sterile conditions. However, SA overaccumulation has been described in soil-grown *ein3 eil1* plants (17, 18). This might account for a slight decrease of bacterial growth after pressure infiltration into *ein2* leaves without elf18 pretreatment. Nevertheless, in the absence of EIN2 and/or PEPRs, elf18-induced resistance to the *Pst COR* (-) strain is much reduced (Fig. 5*B*). This presents direct evidence for the existence of EIN2- and/or PEPR-dependent steps in EFR-triggered immunity to bacterial infection and therefore verifies the significance of ET- and PEPR-signaling in MTI.

A proper balance between defense-related hormones, in particular between SA, JA, and ET, is crucial in plant immunity. However, MTI is greatly reduced upon perturbations of even single hormone pathways (8), pointing to its vulnerability to hormone imbalance. Indeed, *Pst* exploits COR, a mimic of the bioactive JA-Isoleucine conjugate, to subvert SA-dependent and -independent defenses in MTI (15, 22, 32). The ERF1- and MYC2-mediated JA branches (represented by the *PDF1.2* and *VSP2* markers) are promoted or antagonized by ET, respectively (15), of which the latter is required for COR activities (33). Our transcriptome data suggest that PEPR activation also contributes to ET-mediated repression of this MYC2-JA branch during EFR-triggered immunity. Thus, it is conceivable that ET and PEPR signaling might influence the interplay between host MTI and bacterial COR. In *ein2 pepr1 pepr2* plants, elf18-induced resistance is also significantly reduced to the *Pst* COR (+) strain (Fig. 5*C*), further supporting the contribution of EIN2 and PEPRs to EFR-triggered immunity.

Our results suggest the occurrence of separate pathways emanating from EFR that differentially engage ET and PEPR signaling. First, the PEPR pathway works in concert with ET to effectively mount SA-based immunity and to repress the MYC2-JA branch. Second, ET-independent PEPR function might compensate for ET signaling defects that disable EFR and FLS2 function during MTI, as shown by the additive defects of EFR-triggered immunity in *ein2 pepr1 pepr2* plants. This seems to be achieved in part by the activation of *PROPEP2* and *PROPEP3* in an ET-dependent or -independent manner, respectively.

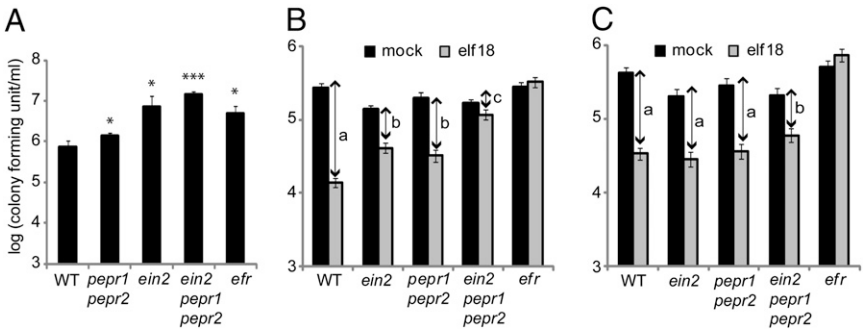

Fig. 5. EIN2 and PEPRs are both required for EFR-triggered immunity to bacterial infection. (*A*) Growth of *Pst*. DC3000 in leaves of 4-wk-old plants 4 d after spray inoculation with bacteria at 5×10^8 cfu/mL. Asterisks indicate statistically significant differences compared with WT at $P < 0.05$ (*) and $P < 0.01$ (***). The means with SDs are shown. (*B* and *C*) Elf18-induced resistance to *Pst*. DC3000 (COR-) (*B*) and *Pst* DC3000 (*C*). Bacterial growth 3 d after infiltration with bacteria at 10^5 cfu/mL on 4-wk-old plant leaves pretreated with water (mock) or 1 μM elf18 for 24 h. The means with SE of three independent experiments are shown. Different letters denote significant differences at $P < 0.01$. *ein2-1* and *pepr1-1 pepr2-3* were used.

Consistent with this model, Liu et al. (29) have reported that PEPR signaling mediates and can partially mimic ET signaling in growth inhibition and plant immunity. Third, PEPR-independent ET function also contributes to EFR-triggered immunity, an aspect of which is seen in elf18-induced ROS spiking. Moreover, our results suggest that elf18-induced resistance against the *COR* (+) bacteria remains largely unaffected upon their infiltration into leaves of *ein2* or *pepr1 pepr2* plants, and is substantially retained even in *ein2 pepr1 pepr2* plants. This might be explained by a contribution of COR to the compensation for the lack of EIN2 and PEPRs in EFR-mediated postinvasive defenses. However, this possibility remains to be tested.

The presence of MAMPs in a wide range of nonpathogenic microbes leads to the notion that the simultaneous detection of MAMPs and DAMPs underlies immune recognition of pathogens, thereby leading to robust defense activation (34). Although the molecular basis is currently unknown, the robustness of PEPR function seems to meet one of the predicted demands that DAMP signaling must work when pathogens interfere with MAMP receptor signaling. Future studies will be required to elucidate whether, and if so, how the PEPR pathway acts in DAMP sensing and signaling.

Materials and Methods

Plant Materials and Growth Conditions. The *Arabidopsis* M2 population used for *psl* mutant screening is in the Columbia *glabrous1* (*gl1*) background (Lehle Seeds). The WT control was Col-0 unless otherwise stated. The *efr* (4), *fls2* (3), *efr fls2* (5), *ein2-1*, and *ein2-5* (25), *ein3 eil1* (35), *ein2-1 dde2* (8), and *pepr1-1 pepr2-1* (11) mutants used were described earlier. Plant growth conditions were described earlier (7, 9), and more details are provided in *SI Materials and Methods*.

Bioassays for MAMP-Induced Responses. MAMP response assays were performed as described earlier (7). In essence, anthocyanin content of whole seedlings was determined as previously described (9), using three sets of at least 10 seedlings per treatment. For MAPK assays, the whole seedlings were treated with elf18 or flg22 at 1 μM for the indicated times. For ROS assays, leaf discs (5-mm diameter) were kept overnight on water, and ROS production was induced by application of 100 nM elf18 or flg22. To induce callose deposition, whole seedlings were treated with 1 μM elf18 or flg22 for 16 h. Callose deposits were stained with Aniline blue and visualized under UV light. For gene expression analysis whole seedlings were treated with 1 μM elf18, flg22, or Pep2 for the indicated times.

Pathogen Inoculation and Growth Assays. Bacterial inoculation assays were performed as described previously (7) with the following modifications. Four-week-old plants were spray inoculated with *Pst*. DC3000 at 5×10^8 cfu/mL. Infected plants were kept in a covered container for 2 d, and representative leaves were harvested 4 d after inoculation. For MAMP-induced resistance assays, plants were syringe infiltrated with 1 μM elf18 or water (mock) 24 h before bacterial inoculation. *Pst*. DC3000 and DB29 (DC3000 COR-) were syringe infiltrated at 10^5 cfu/mL into two to three leaves per plant. These experiments have been repeated at least three times with the same conclusion.

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