UNIVERSITY OF CALIFORNIA, SAN DIEGO

The *Arabidopsis* transcription factor ERF13 negatively regulates defense against *Pseudomonas syringae*

A thesis submitted in partial satisfaction of
the requirements for the degree Master of Science

in

Biology

by

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2013

The thesis of Kimberly F. Chia is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

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DEDICATION

In recognition of all the Briggs lab members I've had the privilege of interacting with during my stint here. It's been a pleasure doing research as part of this lab family! You have all collectively helped make my stay at the Briggs lab an enjoyable one. You've taught me that research is a community effort—that it's okay to ask for help and advice.

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Finally, in recognition of the God who created the universe and everything in it—including plant immune systems and the bacterial pathogens that attack them—and whose face all research ultimately reveals: "blessed be his glorious name for ever: and let the whole earth be filled [with] his glory; Amen, and Amen" (Psa 72:19).

EPIGRAPH

And God said, Let the waters under the heaven be gathered together unto one place, and let the dry [land] appear: and it was so. And God called the dry [land] Earth; and the gathering together of the waters called he Seas: and God saw that [it was] good. And God said, Let the earth bring forth grass, the herb yielding seed, [and] the fruit tree yielding fruit after his kind, whose seed [is] in itself, upon the earth: and it was so. And the earth brought forth grass, [and] herb yielding seed after his kind, and the tree yielding fruit, whose seed [was] in itself, after his kind: and God saw that [it was] good. . . .

And God said, Let us make man in our image, after our likeness: and let them have dominion over the fish of the sea, and over the fowl of the air, and over the cattle, and over all the earth, and over every creeping thing that creepeth upon the earth. So God created man in his [own] image, in the image of God created he him; male and female created he them. And God blessed them, and God said unto them, Be fruitful, and multiply, and replenish the earth, and subdue it: and have dominion over the fish of the sea, and over the fowl of the air, and over every living thing that moveth upon the earth.

And God said, Behold, I have given you every herb bearing seed, which [is] upon the face of all the earth, and every tree, in the which [is] the fruit of a tree yielding seed; to you it shall be for meat. And to every beast of the earth, and to every fowl of the air, and to every thing that creepeth upon the earth, wherein [there is] life, [I have given] every green herb for meat: and it was so.

And God saw every thing that he had made, and, behold, [it was] very good.

Genesis 1:9-12, 26-31a (KJV)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

1. ABA: abscisic acid
2. ABRE: abscisic acid response element
3. ACC: 1-aminocyclopropane-1-carboxylic acid, a precursor of ethylene
4. At: *Arabidopsis thaliana*
5. CE: coupling element
6. Cfu: colony-forming units
7. Chi-B: basic chitinase
8. Col-0: *Arabidopsis thaliana* ecotype Columbia-0
9. COR: coronatine, a bacterial phytotoxin
10. COR15A: cold-regulated 15A
11. Dex: dexamethasone
12. EMS: ethyl methanesulfonate
13. ERE: ethylene response element
14. ERF: ethylene response factor, or ethylene response element (ERE) binding factor
15. ET: ethylene
16. ETI: effector-triggered immunity
17. HA: hemagglutinin
18. iTRAQ: isobaric tag for relative and absolute quantitation
19. JA: jasmonic acid
20. MAMP: microbe/microorganism-associated molecular pattern
21. MAPK: mitogen-activated protein kinase

22. MeJA: methyl jasmonate

23. MRM: multiple reaction monitoring

24. NB-LRR: nucleotide-binding leucine rich repeat

25. OD: optical density

26. PAMP: pathogen-associated molecular pattern

27. PDF1.2a: plant defensin 1.2a

28. PR: pathogenesis-related

29. PRR: pathogen recognition receptor

30. *Pst: Pseudomonas syringae* pathovar (pv.) *tomato* (also *Pto)*

31. PTI: PAMP-triggered immunity

32. R protein: resistance protein

33. RLK: receptor-like kinase

34. RLP: receptor-like protein

35. RLU: relative light units

36. ROS: reactive oxygen species

37. RT-PCR: reverse transcription polymerase chain reaction

38. SA: salicylic acid

39. wt: wild-type

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

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This thesis also includes data generated in collaboration with Cheryl Philipsen in the course of her Master's thesis work in the Briggs lab on the project "Development and use of Mass Spectrometry techniques to study Pseudomonas induced PAMP-triggered and effector-triggered immunity in Arabidopis thaliana." Master's Thesis (Utrecht, The Netherlands: Utrecht University).

ABSTRACT OF THE THESIS

The *Arabidopsis* transcription factor ERF13
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by

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Professor Steven P. Briggs, Chair

Food security is a function of, among other factors, plant health. Because people subsist on a small number of key crops in many parts of the world, disease epidemics among such crops can have dire ramifications. It therefore behooves us to learn more about the plant immune system in order to better protect crops and safeguard the food supply. Many studies on plant immunity have focused in on the role of phytohormones, particularly salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), in negotiating defense strategies against pathogens with different lifestyles. Our work addresses the role of AtERF13, an *Arabidopsis* ethylene response factor previously found to enhance

abscisic acid (ABA)-mediated responses to abiotic stress. We found in proteome profiling of plant immune signaling that S168-phosphorylated ERF13 increases concurrent with early effector-triggered immunity mediated by the R protein RPM1. Using quantitative luminescence assays to measure the growth of bioluminescent bacteria *in planta*, we determined that overexpression of *ERF13* induces susceptibility to the bacterial pathogen *Pseudomonas syringae*. We also found that *ERF13* overexpression promotes chlorosis and inhibits plant growth. Semi-quantitative RT-PCR analysis furthermore demonstrated that ERF13 stimulates the expression of *PDF1.2a*, a JA/ET-inducible pathogenesis-related gene. We conclude that ERF13 lies at a junction in the signaling pathways of SA, JA, ET, and ABA, and that it promotes susceptibility to *P. syringae* by negotiating the crosstalk between these hormones which shapes and tailors the plant defense response.

INTRODUCTION

Plant immunity is relevant to food security

Plant disease is one of the primary contributing factors impacting the state of global food security. Plant pathogens run the gamut from viruses to nematodes, with fungi and oomycetes figuring prominently among these. Some of the diseases these phytopathogens cause can have devastating effects on crop yields. A few particularly infamous plant pathogens include *Phytophthora infestans*, the oomycete responsible for potato blight (Strange and Scott, 2005), *Fusarium oxysporum*, the fungus that causes Panama disease in bananas (Borges et al., 2003), and *Botrytis cinerea*, a necrotrophic fungus that afflicts hundreds of plant species (Rathi et al., 2012; Ferrari et al., 2003).

According to the Food and Agriculture Organization of the United Nations, pests and diseases account for 20-40% of global crop losses annually (FAO, 2012). It has been estimated that phytopathogens cause at least 10% of this loss, totaling US $220 billion in 2002 (Strange and Scott, 2005; Singh et al., 2012; Thompson and Tepfer, 2010). Crop losses are about more than money—in many parts of the world, especially where humans depend heavily on one or a few crops for food, plant disease can severely compromise food availability and affordability.

The plant immune system is therefore a topic of great practical and social relevance. How plants defend themselves from pathogen attack—and what makes a plant susceptible or resistant to a given pathogen—is directly applicable to how we can genetically engineer plants that are more robust. Considerable progress has already been made in this direction, producing such genetically modified (GM) crops as Bt corn and *Papaya ringspot virus* (PRSV)-resistant papaya. James (2010) estimates GM crops have

accrued an economic benefit of US$44 billion between 1996 and 2007, stemming from such effects as increased productivity and reduced pesticide use.

Success stories like Bt corn form the impetus driving such research such as that of Rathi et al. (2012), characterizing *Botrytis cinerea* virulence factors, and of Lu et al. (2011), to isolate banana resistance genes. These studies aim to, through a more complete understanding of the plant immune system, contribute to new strategies for crop protection against pathogens.

An overview of the plant immune system

Our current knowledge on the plant immune system is that it is solely innate, in that plants lack specialized immune cells (Spoel and Dong, 2012). The plant innate immune system is described as bipartite, with the first branch being basal or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI)—sometimes called microbe/microorganism-associated molecular pattern (MAMP)-triggered immunity—and the second branch being effector-triggered immunity (ETI) (**Figure 1A**). PTI is activated in response to recognition of such common and characteristic molecular motifs as LPS and flagellin by receptor-like kinases (RLKs) or receptor-like proteins (RLPs) known as pathogen recognition receptors (PRRs). It consists of responses such as callose deposition to thicken the cell wall and induction of pathogenesis-related (PR) genes via mitogen-activated protein kinase (MAPK) signaling cascades (Spoel and Dong, 2012).

ETI, meanwhile, is raised against effectors—molecules pathogens employ to bypass PTI responses and promote virulence. Plants have evolved resistance (R) proteins that either directly recognize effectors or otherwise respond to their modification of host

proteins. The latter scenario has been designated "the guard hypothesis," in which R proteins ("guards") monitor effector targets ("guardees"). When R proteins identify the presence of effectors, they become activated and trigger potent defense measures including the hypersensitive response (HR)—a kind of localized programmed cell death. Because of the specificity between effectors and R proteins, the ETI branch of plant immunity is described as "gene-for-gene resistance" (Chisholm et al., 2006). If the plant has the gene for the appropriate R protein to recognize an effector's host modification(s) or the effector itself, it mounts a defense response. If it does not, it will be susceptible to the pathogen. For this reason, effectors are considered "avirulence factors" (hence their naming conventions, e.g. AvrPto and AvrRpm1). Accordingly, pathogens are classified as either "virulent" or "avirulent" depending on whether they carry an avirulence gene that can be recognized by a given host.

One well-studied example of ETI is the AvrRpm1/RIN4/RPM1 system (**Figure 1B**). AvrRpm1, a *P. syringae* type III effector, causes the post-translational modification of RIN4, the guardee of RPM1 (an R protein) (Belkhadir et al., 2004). RPM1 recognizes AvrRpm1-induced phosphorylation of RIN4 and activates ETI (HR, resistance). Therefore, for plants armed with the RPM1 gene, *P. syringae* carrying AvrRpm1 gene are avirulent because they activate defense signaling. This is termed an incompatible interaction. By contrast, in the absence of RPM1 (e.g. in *rpm1-3* plants) no ETI is mounted in response to the pathogen, the interaction is termed compatible, and plants are susceptible (Katagiri et al., 2002).

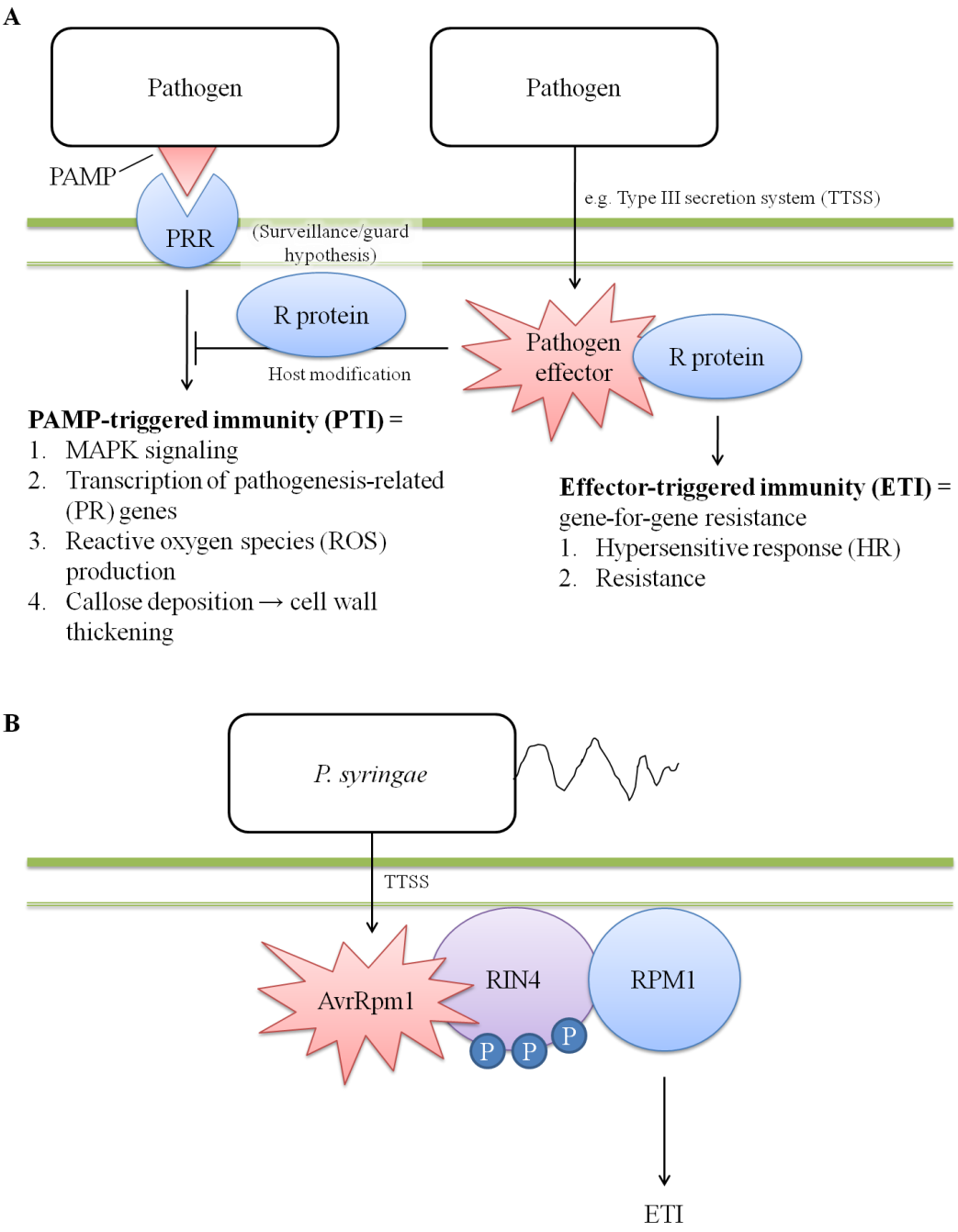

Figure 1. An overview of plant immunity.

(A) The plant immune system can be divided conceptually into basal or PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). PAMP, pathogen-associated molecular pattern. PRR, pathogen recognition receptor. **(B)** *P. syringae* carrying *AvrRpm1* can trigger ETI in *RPM1* plants. AvrRpm1 causes phosphorylation of RIN4, the guardee of RPM1. RPM1 recognizes RIN4 phosphorylation and initiates ETI signaling.

RIN4 has also been found to be targeted by at least two other effectors, AvrB and AvrRpt2. AvrB, like AvrRpm1, causes RIN4 phosphorylation that can be recognized by RPM1 (Belkhadir et al., 2004). AvrRpt2, meanwhile, cleaves RIN4 (Takemoto and Jones, 2005). This cleavage is recognized by RPS2, another R protein guarding RIN4 (Belkhadir et al., 2004).

Phytohormones play an important role in plant immunity

A key mediator of PTI and ETI is salicylic acid (SA), the accumulation of which is critical for expression of a subset of *PR* genes as well as for systemic acquired resistance (SAR), the stimulation of immunity in tissues beyond the area of local infection (Pieterse et al., 2012). SA is a plant hormone (phytohormone), a class of small signaling molecules that mediate growth, development, and stress responses (including disease). Phytohormones contribute an additional dimension to the picture of plant immunity at the systemic level.

SA, jasmonic acid (JA), and ethylene (ET) are three plant hormones that are important regulators of plant defense. These hormones can be classified roughly according to the type of pathogens they combat, which in turn are broadly categorized based on their lifestyle as biotrophic, necrotrophic, or hemibiotrophic. The dichotomy between defense against biotrophic and necrotrophic pathogens can be understood intuitively: biotrophic pathogens thrive off of living plant tissue, making HR a logical means of combating them, whereas this strategy could potentially facilitate the pathogenesis of necrotrophs, which feed on dead plant material. Generally, SA triggers pathways that primarily combat biotrophic pathogens such as *Peronospora parasitica* and

Erysiphe orontii, while JA and ET work mostly against necrotrophic pathogens such as *Alternaria brassicicola, Botrytis cinerea,* and *Fusarium oxysporum* (Glazebrook, 2005; McGrath et al., 2005; Oñate-Sánchez et al., 2007).

Also intuitive are the observed trends in hormone signaling interactions, as SA and ET/JA tend to oppose one another (Glazebrook et al., 2003). For example, SA dampens the pathogen-induced expression of some JA-inducible genes *(PDF1.2, VSP),* perhaps by interfering with JA synthesis itself (Spoel et al., 2003). Similarly, infection with virulent *Pseudomonas syringae* pv. *tomato (Pst)* DC3000 (a hemibiotrophic bacteria) results in compromised resistance to *Alternaria brassicicola* (a necrotrophic fungus) (Spoel et al., 2007). Conversely, MPK4 (a MAP kinase) promotes JA signaling while repressing SA responses (Koornneef and Pieterse, 2008).

Studies have also shown, however, that the aforementioned delineation is not so clean and easy. The evidences for extensive crosstalk amongst these defense hormones suggest that what goes on in a stressed plant is more a function of hormone balance than on/off switches. For example, AtERF14 (a putatively ET-responsive transcription factor induced by *Pst* DC3000 (AvrRpt2)), activates transcription of not just JA/ET-responsive genes but also PR1, a SA-induced gene (Oñate-Sánchez et al., 2007). It has also been reported that resistance to *Plectosphaerella cucumerina,* a necrotrophic fungus, requires SA accumulation (Berrocal-Lobo et al., 2002). Hemibiotrophic pathogens with both biotrophic and necrotrophic stages of infection further complicate the picture, as plants must defend against them using all three hormones.

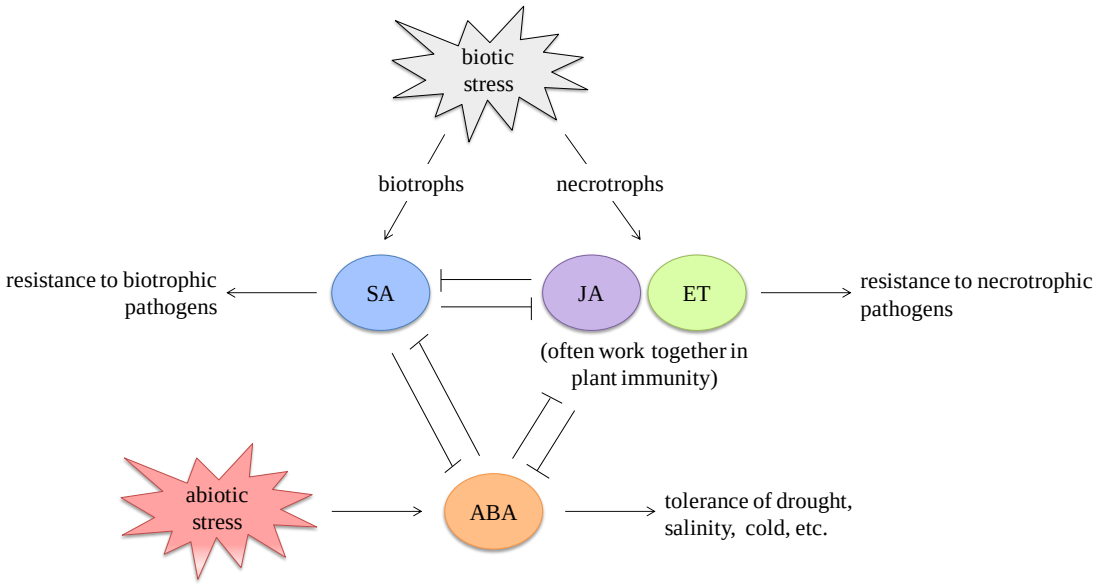

Figure 2. An overview of the crosstalk between the major plant hormones integrating biotic and abiotic stress responses.

In general, there is mutual antagonism between defense signaling against biotic stress caused by biotrophic pathogens (mediated by salicylic acid (SA)) and necrotrophic pathogens (mediated by jasmonic acid (JA) and ethylene (ET)). Likewise, responses against biotic stresses (mediated by SA, JA, and ET) and abiotic stresses (mediated by abscisic acid (ABA)) tend to be mutually antagonistic.

Plant immunity is also affected by abscisic acid (ABA), a key hormone for responses to abiotic stresses such as drought, salinity, and cold. Mutual antagonism between biotic and abiotic stress signaling has been described, suggesting that these two responses are largely incompatible (Mauch-Mani and Mauch, 2005). For instance, Kim et al. (2011) found that DFPM (5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione) inhibits ABA gene induction and responses while simultaneously inducing defense gene expression. Meanwhile, *Pst* DC3000 infection stimulates production of ABA to promote susceptibility by suppressing SA signaling (de Torres-Zabala et al., 2007 and 2009).

Figure 2 offers a summary of the general trends in the relationships between SA, JA, ET, and ABA signaling.

Ethylene response factors mediate stress responses downstream of various hormones

Loci such as the aforementioned AtERF14 form points of intersection and communication between hormone signaling pathways. In fact, AtERF14 belongs to a family of proteins that are comfortably embroiled in negotiating this crosstalk.

The *ERF* gene family encodes a group of plant transcription factors containing one copy of the AP2/ERF domain, which binds the GCC box (TAAGAGCCGCC) found in many ET- and JA-inducible *PR* genes (Gu et al., 2002; Nakano et al., 2006). *ERFs* can be induced upon biotic and abiotic stresses and in response to ET (Gu et al., 2002), and are collectively involved in effectuating ET, SA, JA, and ABA signaling (Nakano et al., 2006).

Pti4, a tomato ERF, directly links R protein activation and defense gene induction

The tomato *(Solanum lycopersicum)* protein Pti4 is one of the most well-studied ERFs due to its role in ETI initiated by the tomato R protein Pto kinase (Pto), which works with the LRR protein Prf to recognize the *Pst* effector AvrPto (Gu et al., 2000; Mucyn et al., 2006). Pto-mediated phosphorylation of Pti4 strengthens its binding to the GCC box and induction of a subset of *PR* genes (Gu et al., 2000). Thus, the AvrPto/Pto/Pti4 system draws a direct connection between effector recognition and initiation of ETI. An overview of the AvrPto-triggered immunity is shown in **Figure 3.**

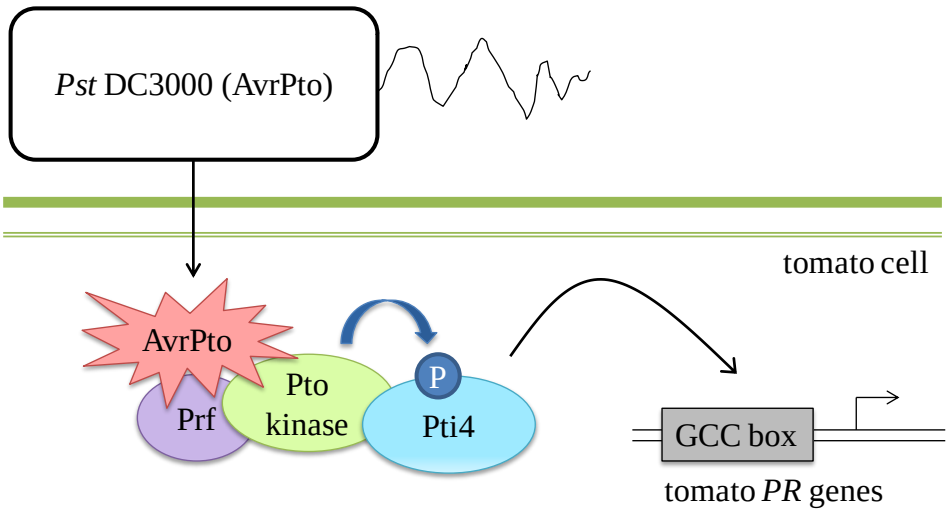

Figure 3. Pto-mediated immunity in tomato against *Pst* carrying the effector AvrPto.

Upon recognition of AvrPto by Pto and Prf, Pto phosphorylates Pti4. Pti4 phosphorylation increases its binding affinity to the GCC box and enhances its induction of various *PR* genes.

Arabidopsis ERFs mediate hormone crosstalk in biotic and abiotic stress responses

Arabidopsis thaliana is a model organism used in many plant studies because of features such as its small size, short generation time, and ease of genetic manipulation (Koornneef and Meinke, 2010). As a result, many heterologous studies have been conducted in *Arabidopsis* in the hopes of extrapolating these findings to crop plants such as tomato and corn. The function of tomato Pti4, for example, has been characterized in the model plant *Arabidopsis*, where its overexpression was found to result in enhanced resistance or tolerance to the biotrophic pathogens *Pseudomonas syringae* and *Erysiphe orontii*—presumably via its activation of various *PR* genes (Wu et al., 2002; Gu et al., 2002).

Demonstration of the activity of such ERFs in *Arabidopsis* opened the door for

further studies on native *Arabidopsis* ERFs (AtERFs), especially after bioinformatic efforts to classify the ERF family in *Arabidopsis* (Sakuma et al., 2002). Since then, many AtERFs have been researched extensively and found to be intimately involved in not just ET signaling but also that of SA, JA, and ABA.

Table 1 offers a summary of findings on several AtERFs, with emphasis on their roles in hormone signaling. Below are a few AtERF case studies of particular interest to our work.

ERF1 is induced by *B. cinerea,* against which it confers resistance (Berrocal-Lobo et al., 2002). Overexpression of ERF1 renders plants susceptible to *Pst* DC3000, which has led to speculation that it lies at an intersection between ET and SA pathways.

AtERF2 is induced by numerous pathogens *(A. brassicicola, P. syringae, F. oxysporum),* MeJA (methyl jasmonate, a biologically active derivative of JA), ET, and wounding. Overexpressing AtERF2 results in heightened resistance to *F. oxysporum,* potentially via its positive regulation of MeJA responses, such as *PDF1.2* and *Chi-B* (also known as *PR3)* expression (McGrath et al., 2005).

Not all ERFs are transcriptional activators; AtERF4 is a repressor-type ERF induced by *A. brassicicola* and *F. oxysporum* infection, as well as SA and MeJA treatment. It appears to negatively regulate MeJA responses, since overexpressing it causes susceptibility to *F. oxysporum* and decreases MeJA-mediated *PDF1.2* induction and inhibition of root elongation. Conversely, AtERF4 T-DNA lines display the opposite phenotypes: increased PDF1.2 levels in untreated plants, more inhibition of root elongation by MeJA, and increased resistance to *F. oxysporum* (McGrath et al., 2005).

AtERF4 has also been shown to mute expression of several ABA-responsive

genes (Yang et al., 2005). Another family member, AtERF7, is also a negative regulator of ABA signaling; when it is overexpressed plants are more susceptible to drought, and when it is knocked down plants are hypersensitive to ABA inhibition of seed germination and growth (Song et al., 2005).

Table 1. Summary of the known characteristics of several previously studied *Arabidopsis* ERFs.

Ethylene response factors (ERFs) in *Arabidopsis* are not exclusively involved in ET signaling pathways, but also contribute to SA, JA, and ABA responses. SA, salicylic acid; ET, ethylene; JA, jasmonic acid; MeJA, methyl jasmonate; ABA, abscisic acid. Only overexpression phenotypes are represented, as many ERFs do not have a non-wild-type knockout phenotype due to putative functional redundancy.

ERF (locus identifier)	**Hormone pathway**	**Induced by**	**Overexpression phenotype**	**Reference**
ERF1 (At3g23240)	SA ET	*B. cinerea*	Susceptibility to *Pst* DC3000	Berrocal-Lobo et al., 2002
AtERF2 (At5g47220)	JA ET	*A. brassicicola* *P. syringae* *F. oxysporum* MeJA ET wounding	Resistance to *F. oxysporum* Upregulation of *PDF1.2*, *Chi-B*	McGrath et al., 2005
AtERF4 (At3g15210)	SA JA ABA	*A. brassicicola* *F. oxysporum* SA MeJA	Suppression of MeJA-induced *PDF1.2* expression and inhibition of root elongation Susceptibility to *F. oxysporum* Suppression of ABA-responsive genes	McGrath et al., 2005 Yang et al., 2005
AtERF5 (At5g47230)	SA JA ET		Upregulation of JA/ET-responsive genes Resistance to *B. cinerea* Suppression of PR1 expression Susceptibility to *P. syringae*	Moffat et al., 2012

Table 1, continued. Summary of the known characteristics of several previously studied *Arabidopsis* ERFs.

Ethylene response factors (ERFs) in *Arabidopsis* are not exclusively involved in ET signaling pathways, but also contribute to SA, JA, and ABA responses. SA, salicylic acid; ET, ethylene; JA, jasmonic acid; MeJA, methyl jasmonate; ABA, abscisic acid.

ERF (locus identifier)	Hormone pathway	Induced by	Overexpression phenotype	Reference
AtERF6 (At4g17490)	SA JA ET	*B. cinerea* infection	Upregulation of JA/ET-responsive genes Resistance to *B. cinerea* Suppression of PR1 expression Susceptibility to *P. syringae*	Moffat et al., 2012 Meng et al., 2013
AtERF7 (At3g20310)	ABA		Suppression of ABA signaling	Song et al., 2005
AtERF13 (At2g44840)	ABA	MeJA High [salt] High [mannitol] Drought Wounding *P. syringae* infection *B. cinerea* infection *A. brassicicola* infection	ABA hypersensitivity Glucose hypersensitivity Upregulation of *COR15A*	Goda et al., 2008 Lee et al., 2010 Hasegawa et al., 2011 McGrath et al., 2005
AtERF14 (At1g04370)	SA JA ET	*Pst* DC3000 (AvrRpt2)	Induction of JA/ET-responsive genes Induction of *PR1*	Oñate-Sánchez et al., 2007
RAP2.2 (At3g14230)	ET	ET *B. cinerea* infection	Resistance to *B. cinerea*	Zhao et al., 2012

AtERF13 shares a high degree of homology with several other defense-related ERFs and is a potentiator of the ABA response.

AtERF13 (At2g44840, subsequently referred to as ERF13) is a member of the B-3 ERF family subgroup (reclassified to group IX in Nakano et al., 2006), which is mainly composed of transcriptional activators such as ERF1, AtERF2, and AtERF14 (Sakuma et

al., 2002; McGrath et al., 2005). It has been confirmed to bind and activate transcription at GCC box-containing promoters (Lee et al., 2010). High salt concentrations induce *ERF13* expression in roots, while high mannitol concentrations, drought, and wounding cause its systemic induction (Lee et al., 2010; Hasegawa et al., 2011). Infection by *P. syringae, B. cinerea,* and *A. brassicicola* also induce it somewhat (Winter et al., 2007; AtGenExpress; McGrath et al., 2005).

ERF13 binds a coupling element (CE1, TGCCACCGG) required for induction of many ABA-responsive genes, and when overexpressed it was found to cause hypersensitivity to both ABA and glucose (Lee et al., 2010). *ERF13*-overexpressing plants also had increased levels of *COR15A* transcripts, where *COR15A* is an ABA-inducible gene (Lee et al., 2010). These findings suggest that ERF13 is a positive regulator of the ABA response.

ERF13: a case study in proteomics validation and follow-up

All of the ERFs featured in the studies mentioned above were first identified through methods such as microarrays (Hasegawa et al., 2011), homology and phylogeny modeling (Solano et al., 1998; Meng et al., 2013), and yeast one- and two-hybrid (Lee et al., 2010; Song et al., 2005). The reliability of mRNA-based information, at least, has been questioned at least since 1999, as studies have indicated that there is only a "modest" correspondence between mRNA levels and protein abundance due to factors such as post-transcriptional and –translational regulation (Gygi et al., 1999; Nie et al., 2006; O'Brien et al., 2010; Walley et al. (manuscript in review)).

We report further characterization of ERF13 as a defense-related transcription

factor based on proteomics. Although ERF13 was previously studied in the context of the ABA response, it had not been implicated in plant defense against biotic stresses. Our disease proteome analysis suggests that phosphorylated ERF13 plays a role in ETI signaling.

In order to mimic and monitor the proteome changes that occur coincident with ETI and susceptibility, *AvrRpm1* expression was induced in wild-type or *rpm1-3* transgenic plants. Quantitative comparison of protein levels was achieved via isobaric tag for relative and absolute quantitation (iTRAQ) labeling, followed by mass spectrometric analysis. In addition to our analysis of the non-modified peptides in these samples, we sought to address the dimension of post-translational modifications by performing a phosphopeptide enrichment. Phosphorylated ERF13 peptides were detected in these phosphopeptide-enriched samples, indicating that ERF13 is involved in negotiating plant-pathogen interactions, and that its phosphorylated form may be important for this functionality.

The last step in any workflow, regardless of the screening technique, is validation and functional analysis. Since our phosphoproteome data suggested ERF13 may be phosphorylated upon initiation of ETI signaling through the R protein RPM1, we were interested to know what specific contribution (if any) ERF13 phosphorylation makes to the ETI branch of the plant defense response. Here we report our subsequent analysis of inducible ERF13 overexpression lines (using the GVG system (Aoyama and Chua, 1997)), which revealed that ERF13 suppresses defense against virulent and avirulent *P. syringae. ERF13* overexpression also causes stunting and chlorosis. Finally, ERF13 induces expression of *PDF1.2a,* suggesting that it may be involved in the crosstalk

between SA and JA/ET.

Acknowledgments

This thesis includes material from the project "Quantitative Phospho-proteomic and Proteomic Analysis of the Plant Innate Immune Response," which is being prepared for publication, and of which Chris van Schie is the principal author and Zhouxin Shen and Steven P. Briggs are co-authors. The thesis author's research is a continuation of this work.

This thesis also includes data generated in collaboration with Cheryl Philipsen in the course of her Master's thesis work in the Briggs lab on the project "Development and use of Mass Spectrometry techniques to study Pseudomonas induced PAMP-triggered and effector-triggered immunity in Arabidopis thaliana." Master's Thesis (Utrecht, The Netherlands: Utrecht University).

RESULTS

ERF13 is phosphorylated in early ETI signaling triggered by AvrRpm1

In order to assess changes in protein levels and post-translational modifications during ETI and virulence, we induced *AvrRpm1* expression in wild-type and *rpm1-3* plants, respectively, by treating with dexamethasone. Mass spectrometry-based profiling of non-modified and phosphorylated peptides was then conducted on samples collected 0, 2, and 4 hours after *AvrRpm1* induction. iTRAQ labeling was used to make quantitative comparisons.

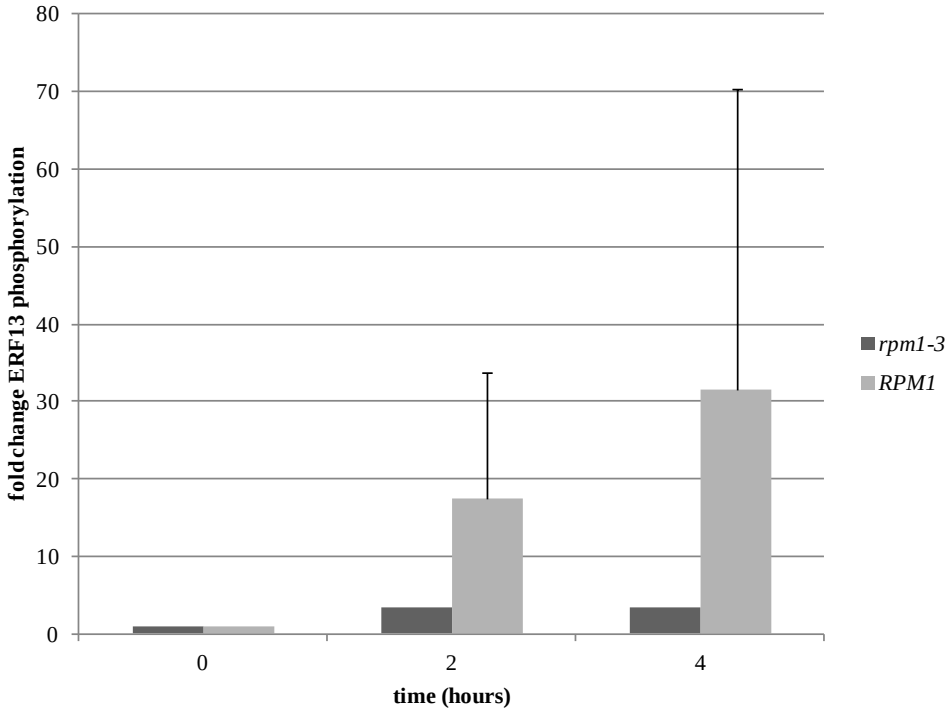

Figure 4. Early AvrRpm1-triggered ETI signaling causes an increase in ERF13 phosphorylation at S168.

Fold change iTRAQ label signal intensity 2 and 4 relative to 0 hours (set to 1), in 4-5-week-old *RPM1* and *rpm1-3* background plants carrying a dexamethasone (dex)-inducible *AvrRpm1* transgene. Values are normalized against mock treatment, 0.035% Silwet spray. Error bars represent standard deviation, n=2 for plants carrying *RPM1* at 2 and 4 hours. The absence of error bars indicates that phosphorylated ERF13 was detected in only one of the biological replicates.

We observed an increase in a phosphorylated ERF13 peptide in early ETI signaling initiated upon RPM1 recognition of AvrRpm1. This phosphopeptide, SPEPSVSDQLTSEQK (phosphorylated residue, S168, underlined), increased an average of 17.5- and 31.6-fold 2 and 4 hours, respectively, after induction of *AvrRpm1* expression (**Figure 4**). Meanwhile, in the absence of RPM1, AvrRpm1 triggered a more modest 3.4-fold increase at 2 hours, with no apparent change between 2 and 4 hours (**Figure 4**).

Because ERF13 was not detected in our profiling of non-modified peptides (samples not enriched for phosphorylated peptides), it is not possible to determine if the observed increases in phosphorylated ERF13 were due to an actual increase in ERF13 phosphorylation or an increase in the total protein levels. It should also be noted that the standard deviations for the fold changes of ERF13 in AvrRpm1-induced ETI were very high (16.3- and 38.3-fold at 2 and 4 hours, respectively), which adds uncertainty to the trends observed. In addition, ERF13 was not detected in several of the replicates.

Though the quantitative data is highly variable and suffers from a small sample size, the results nevertheless indicate at least that phosphorylated ERF13 was present in the conditions represented. As no phosphorylation of ERF13 has been previously reported, our finding of S168 phosphorylation is both a novel post-translational modification of ERF13 and a novel phosphorylation site.

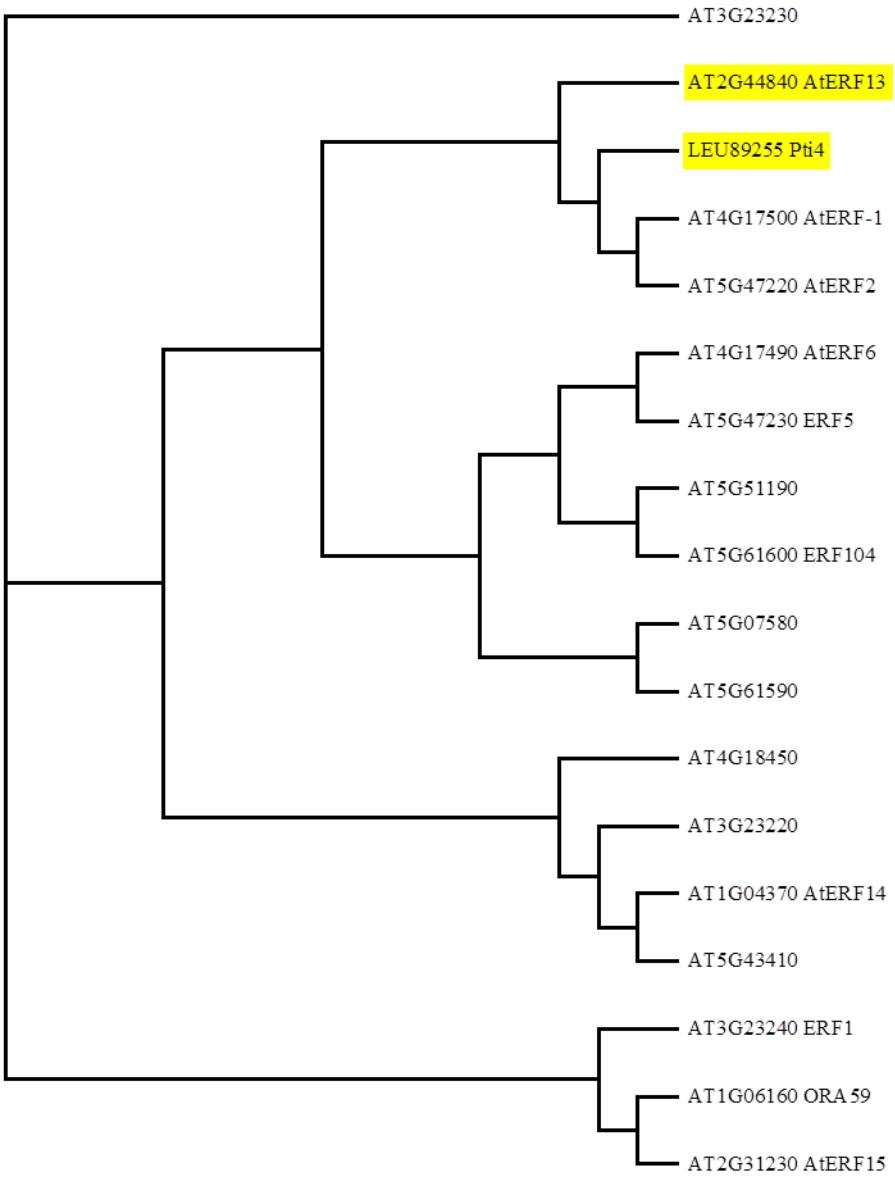

Figure 5. AtERF13 shares a high degree of homology with Pti4.

Rectangular cladogram depicting a ClustalX alignment of the amino acid sequences of ERF family group IX members (Nakano et al., 2006) and Pti4 amino acid sequences. All the unnamed genes are integrase-type DNA-binding superfamily proteins. LEU, *Lycopersicon esculentum* (also known as *Solanum lycopersicum).* ERF13 and Pti4 are highlighted. The figure was generated using TreeView.

ERF13's phosphorylation was intriguing due to the precedent of Pti4, the tomato

ERF phosphorylated by the R protein Pto kinase (Gu et al., 2000). In fact, ERF13 shares a high degree of homology with Pti4. A ClustalX multiple sequence alignment of Pti4, ERF13, and other AtERFs belonging to the ERF family group IX (as defined by Nakano et al. (2006)) indicated that ERF13 is the closest homolog of Pti4 in *Arabidopsis* (**Figure 5**). Pti4 is known to be a transcriptional activator of *PR* genes in Pto-mediated ETI. We therefore hypothesized that ERF13 lies downstream of and is required for proper RPM1-activated ETI.

ERF13 T-DNA knockouts do not have a defense phenotype distinct from wild-type plants

In order to assess any unique contribution ERF13 makes to AvrRpm1-induced, RPM-mediated immunity, we obtained two independent *ERF13* T-DNA insertion lines (GABI 121A12 and GABI 724B09, both exon insertions) and tested their defense phenotype against *Pst* DC3000 and *Pst* DC3000 (AvrRpm1) via bacterial growth assay. We found that neither line fostered significantly different levels of bacterial multiplication compared to wild-type Columbia-0 (Col-0) plants (**Figure 6**). Since extensive functional redundancy has been previously reported for members of the ERF family, we chose to generate and investigate the phenotype(s) of *ERF13* overexpression lines.

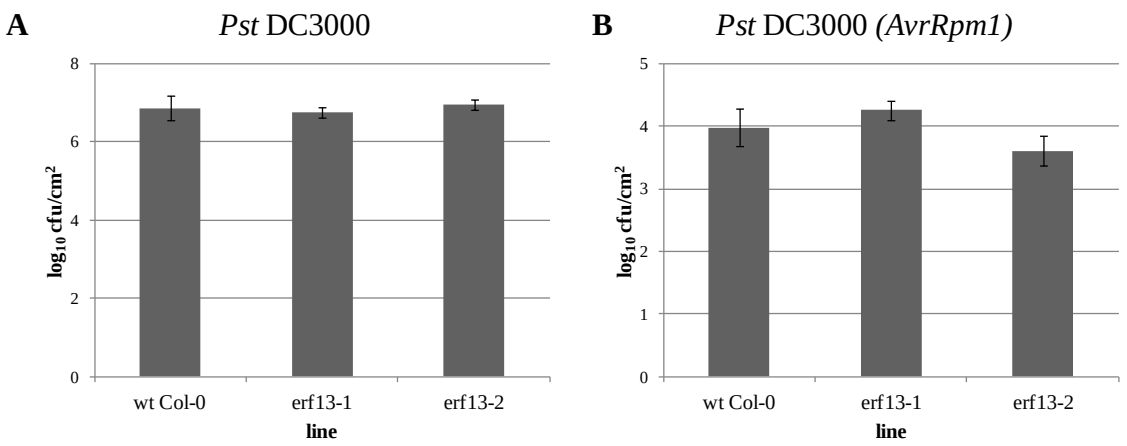

Figure 6. *erf13* and wild-type plants foster a similar amount of bacterial growth.

Wild-type and *erf13* plants were grown for 5 weeks and sprayed with an OD_{600} = 0.01 suspension of **(A)** *Pst* DC3000 and **(B)** *Pst* DC3000 *(AvrRpm1).* Bacterial growth was measured 3 days post-inoculation by colony counting. Results shown are the mean $\log_{10}$ colony-forming units (cfu) per cm^2 leaf tissue. Error bars represent SEM. Wt, wild-type. There was no significant difference in bacterial growth between wt Col-0 plants and plants from either *erf13* line.

Overexpression of *ERF13* causes stunting

We cloned *ERF13* into the pGreen vector behind the cauliflower mosaic virus *35S* promoter for constitutive expression (with N-terminal HA tag) and transformed the resulting construct via the *Agrobacterium tumefaciens*-mediated floral dip method into wild-type Col-0 plants. We also generated S168 phospho-mimetic (S168E) and phospho-dead (S168A) overexpression lines to investigate the role of ERF13 phosphorylation. However, *ERF13* expression could not be verified for these lines by Western blot, and T2-generation plants died off as seedlings when selected on soil with BASTA (glufosinate) spray, suggesting that overexpression of *ERF13* may be seedling-lethal.

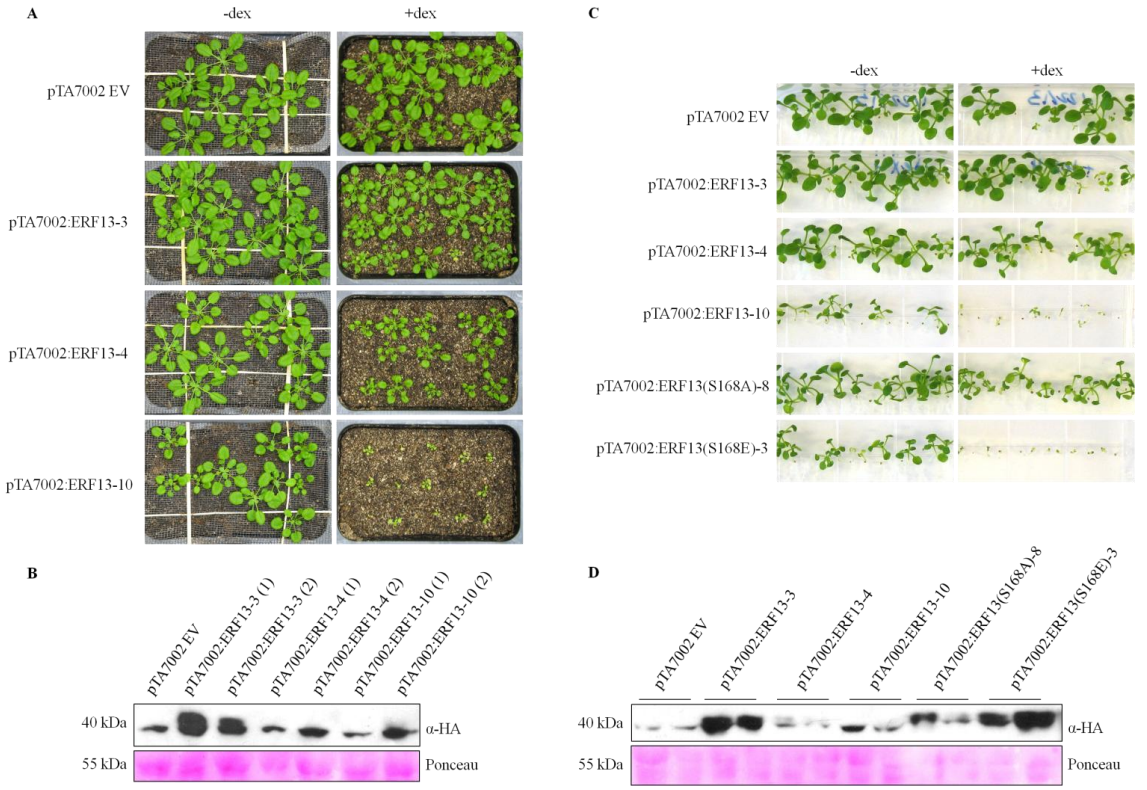

Figure 7. *ERF13* overexpression impairs plant growth.

(A) 1-week-old *ERF13*-overexpressing (pTA7002:ERF13) seedlings germinated with hygromycin selection (15 μg/mL) on plate were transplanted to soil and sprayed with dex every 48 hours for 3 weeks. pTA7002:ERF13 plants appeared stunted relative to the empty vector (pTA7002 EV) control. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). **(B)** Dex-treated plants were sampled after 3 weeks of treatment and ERF13-HA levels were measured by Western blot (probed with mouse anti-HA antibody). (1), protein extracted from smaller plants. (2), protein extracted from bigger plants. Equal loading was verified by Ponceau staining of the RuBisCO large subunit (~55 kDa). **(C)** 2-week-old pTA7002:ERF13 seedlings grown on hygromycin plates with or without 1 μM dex. All lines were from the T2 generation (segregating) except pTA7002:ERF13(S168E)-3 (T3, homozygous). **(D)** Hygromycin plates with 2-week-old pTA7002:ERF13 seedlings were flooded with dex solution to a final concentration of 1 μM dex and seedlings were harvested after 48 hours. ERF13-HA levels were quantified via Western blot using a mouse anti-HA mouse. Samples were collected and run in duplicate.

To overcome the possible seedling lethality of constitutive *ERF13* overexpression and ensure that we could detect ERF13 levels via Western blotting, we cloned

hemagglutinin (HA)-tagged *ERF13* (wild-type, S168A, and S168E) into the pTA7002 vector under the control of the dex-inducible GVG system with C-terminal HA tag (**Figure S2**). These constructs were then introduced into wild-type Col-0 plants, and expression was confirmed by Western blot.

To investigate the possibility that overexpression of *ERF13* is seedling-lethal, T2 seeds of dex-inducible *ERF13*-overexpressing plants (pTA7002:ERF13; see Aoyama and Chua, 1997) were germinated with hygromycin selection on plate and transferred to soil after a week. Seedlings were sprayed with dex every 48 hours. After two weeks of treatment, developmental differences between the pTA7002 empty vector control and pTA7002:ERF13 lines began emerging. After three weeks, three independent pTA7002:ERF13 were visibly stunted relative to the pTA7002 EV control (**Figure 7A**).

We observed that line 10 (pTA7002:ERF13-10) was far more severely affected than either of the other two independent lines tested, demonstrating significantly impaired growth, as well as some chlorosis. Several plants (roughly 6 out of 45, ~13%) died off during the course of the experiment. A few others (5 of 45, ~11%, not including the ones that eventually died) never grew more than two true leaves. This line does appear to have some growth impairment relative to the empty vector even when untreated, but there does not appear to be a consistent correlation between the degree of stunting and level of protein expression (**Figure 7B**).

Figure 8. *ERF13* overexpression causes long-term developmental phenotypes. 6-week-old T2 *ERF13*-overexpressing plants were treated with dex every 48 hours for 8 days. **(A)** Plants 19 days after mock and dex treatment. EV, empty vector. **(B)** Leaves detached on day 19 from mock- and dex-treated *ERF13*-overexpressing plants. Each leaf shown was taken from a different plant.

The developmental impairment of pTA7002:ERF13-3 and -4 was significantly attenuated when the seedlings were germinated on agar containing 1 μM dex (**Figure 7C**), suggesting that *ERF13* overexpression causes stunting via increased sensitivity to

environmental stresses. However, pTA7002:ERF13-10 and –(S168E)-3 both displayed severe growth impairment in the presence of dex. These phenotypes did not seem linked to protein levels (**Figure 7D**).

We furthermore observed that for many of the *ERF13*-overexpressing lines, developmental phenotypes were visible several weeks after dex treatment had been discontinued (**Figure 8**). These plants underwent a rapid onset of flowering, with multiplication of very small leaves (sometimes exhibiting epinasty), culminating in an inflorescence. Growth also seemed impaired for these plants, as their new leaves tended to be smaller. In addition, we also often observed the concomitant death of the older leaves.

ERF13 **overexpression causes susceptibility to** ***Pseudomonas syringae*** **pv.** ***tomato*** **DC3000** ***(lux, AvrRpm1)*** **and** ***Pseudomonas syringae*** **pv.** ***maculicola*** **ES4326** ***(lux)***

T2-generation (segregating) transgenic plants were subjected to bacterial growth assays with *Pst* DC3000 *(lux, AvrRpm1)* and *Psm* ES4326 *(lux), P. syringae* strains carrying the *Photorhabdus luminescens luxCDABE* operon *(lux)* to facilitate quantitation of bacterial growth (Fan et al., 2008). Dex-induced overexpression of ERF13 increased susceptibility to both the avirulent *Pst* DC3000 *(lux, AvrRpm1)* regardless of S168 phosphorylation state (**Figure 9A,B**). This phenotype was confirmed to be specifically dex-inducible (**Figure S4**).

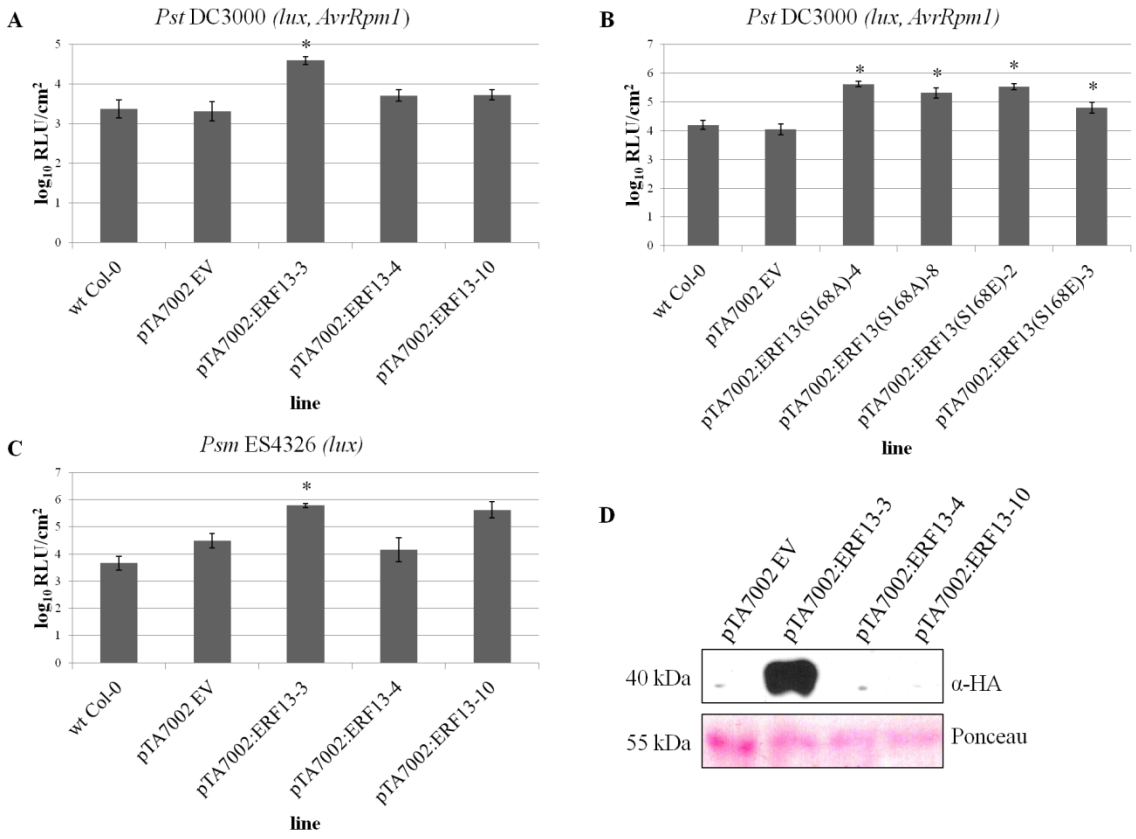

Figure 9. *ERF13* overexpression causes susceptibility to virulent and avirulent bacteria.

6-week-old dex-inducible *ERF13*-overexpressing (pTA7002:ERF13) plants were sprayed with 50 μM dex before and after vacuum inoculation with bacteria carrying the *Photorhabdus luminescens luxCDABE* operon. Bacterial growth was measured 3 days post-inoculation with a luminescence detector. Results shown are the mean $\log_{10}$ relative light units (RLU) per cm^2 leaf tissue. Error bars represent SEM, n=8 (n=7 for wt Col-0). Wt, wild-type. EV, empty vector. * indicates significant difference from pTA7002 EV by one-way ANOVA (Tukey method), $p<0.05$. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). **(A)** Plants were vacuum-inoculated with OD_{600} 0.0002 ($9.3x10^4$ cfu/mL) *Pst* DC3000 *(lux, AvrRpm1);* n=8. This experiment was conducted twice, with similar results. **(B)** Phospho-dead (S168A) and phospho-mimetic (S168E) *ERF13*-overexpressing plants inoculated with OD_{600} 0.0002 ($9.25x10^4$ cfu/mL) *Pst* DC3000 *(lux, AvrRpm1).* n=8 (n=7 for wt Col-0). The results shown were confirmed in one to three other independent experiments. **(C)** Plants were inoculated with OD_{600} 0.0001 ($5.9x10^4$ cfu/mL) *Psm* ES4326 *(lux).* n=8 (n=7 for wt Col-0). **(D)** (Top) Western blot of protein extracted from plants in (A), probed with mouse anti-HA antibody to measure ERF13-HA levels. (Bottom) Total protein was measured with Ponceau stain.

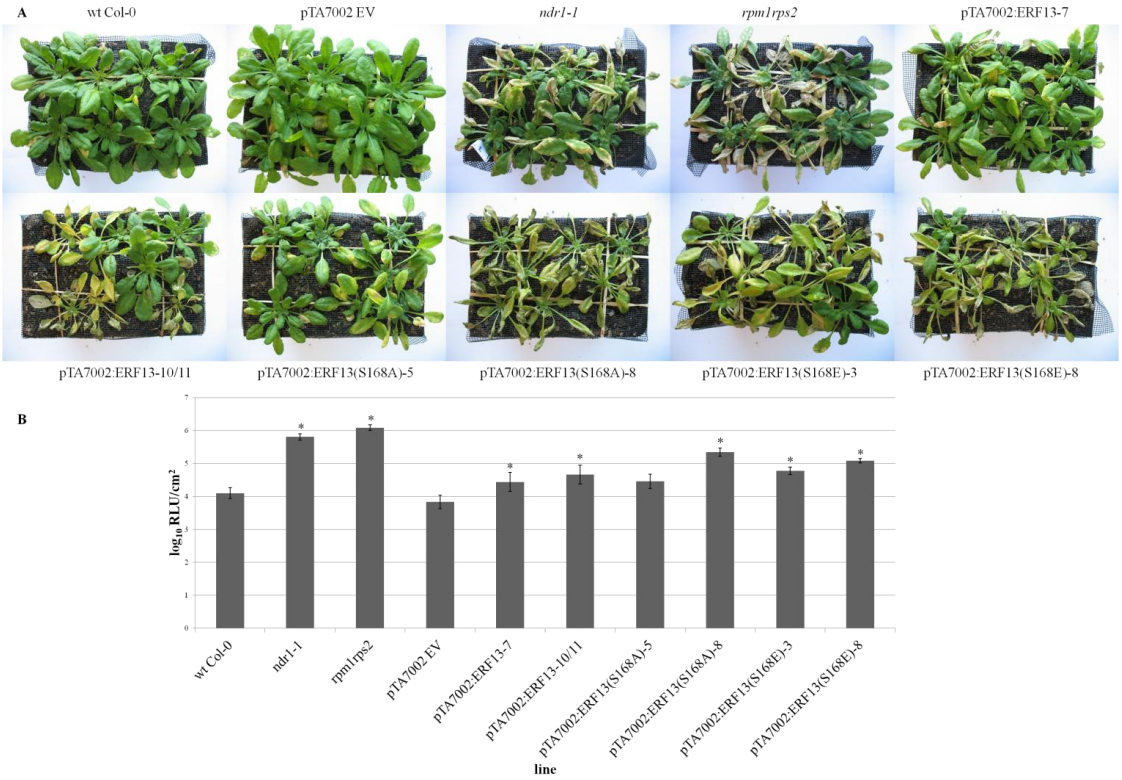

Figure 10. Overexpression of *ERF13* causes increased chlorosis and necrosis in response to infection with *Pst* DC3000 *(lux, AvrRpm1)*.

6-week-old T2 dex-inducible *ERF13*-overexpressing plants were vacuum-inoculated with *Pst* DC3000 *(lux, AvrRpm1)* at OD_{600} = 0.001 ($3.0x10^5$ cfu/ml) in 50 µM dex, 0.01% Silwet L-77, and 5 mM $MgSO_4$. *ndr1-1* and *rpm1rps2* are susceptible controls. Wt, wild-type. EV, empty vector. **(A)** Plants were photographed 7 days after inoculation. **(B)** Relative luminescence was measured 3 days after inoculation with a luminescence detector. Data presented are the mean log_{10} relative light units (RLU) per cm^2 leaf tissue. Error bars indicate the SEM; n=8. * indicates significant difference from pTA7002 EV by one-way ANOVA (Tukey method), $p<0.05$.

It should be noted that even when bacterial growth was not significantly different from wild-type Col-0 or the pTA7002 empty vector control, increased chlorosis and necrosis in the leaves of *ERF13*-overexpressing plants was consistently observed (**Figure 10**). We determined that there was a rudimentary correlation between *Pst* DC3000 *(lux, AvrRpm1)* susceptibility and ERF13 protein levels (**Figure 9A,D**).

Since susceptibility to biotrophic pathogens is sometimes associated with resistance to necrotrophic pathogens, the *ERF13*-overexpressing lines were also assayed for susceptibility to several different *B. cinerea* strains (B05.10, grape, and rose). However, the results of these experiments were inconclusive, as the pTA7002 empty vector control consistently developed larger lesions than wild-type Col-0 plants, suggesting that the GVG system and/or dex treatment may have confounded the data.

ERF13 promotes chlorosis in older plants

One of the symptoms of infection with *P. syringae* is chlorosis, yellowing of the leaves caused by insufficient chlorophyll production (or by the degradation of existing chlorophyll). Disease-related chlorosis has been linked to the bacterial phytotoxin coronatine (COR) (Santner and Estelle, 2007; Mecey et al., 2011). We observed that *ERF13*-overexpressing plants tended to develop enhanced chlorosis upon infection with *Pst* DC3000 *(lux, AvrRpm1)* (**Figure 10**), which sometimes culminated in the death of the plant after a few weeks (data not shown). In order to determine if *ERF13* overexpression was sufficient to induce chlorosis in plants, we treated 5-week-old pTA7002:ERF13 plants with dex and observed that chlorosis develops over the course of 6-8 days in an S168 phosphorylation-independent manner (**Figure 11A)**. Plants carrying the pTA7002 empty vector do also develop some chlorosis, suggesting that part of the chlorophyll loss is partly an effect of the GVG system (since wild-type Col-0 plants do not develop any chlorosis upon dex treatment (**Figure S5**). Mock-treated plants did not develop any chlorosis (**Figure S5**).

Dex-induced overexpression of *ERF13* also caused some other interesting effects:

for several lines, including pTA7002:ERF13-3, -4, and -10, some leaves that did not develop significant chlorosis shriveled up and became brittle (**Figure 11A**). We also observed some mottling of leaves, perhaps a precursor to full-on chlorosis. Leaves that had yellowed significantly also tended to be limp and wilted. It should be noted that the plants did not uniformly develop chlorosis (see **Figure S6A** for pictures of all the pots). In **Figure 11A,** if a plant had a chlorotic leaf, that leaf was sampled.

The amount of the ERF13-HA transgene product was measured via Western blot, with distinction between chlorotic and green leaf tissue (**Figure S6B**), but there was no obvious correlation between degree of chlorosis and level of ERF13 present.

Mecey et al. (2011) found that *SGR*, which encodes a protein involved in chlorophyll degradation, is induced upon infection with *Pst* DC3000. *SGR* can also be induced by COR treatment, except in *coi1* mutants, which are defective in JA signaling. Since *ERF13* overexpression appears to induce chlorosis and is itself induced by *Pst* DC3000 infection (Winter et al., 2007), we hypothesized that ERF13 acts through *SGR* to promote chlorosis. We therefore conducted semi-quantitative RT-PCR on dex-treated pTA7002:ERF13 plants and found that overexpression of *ERF13* does not impact *SGR* levels (**Figure S6. Overexpression of ERF13 causes chlorosis.Figure S6C**), suggesting that ERF13 may cause chlorosis via an alternative, non-SGR-regulated pathway.

Interestingly, the development of chlorosis upon *ERF13* overexpression appeared restricted to older plants (5+ weeks old), as chlorosis in seedlings was only observed for pTA7002:ERF13-10 (**Figure 7A,C**).

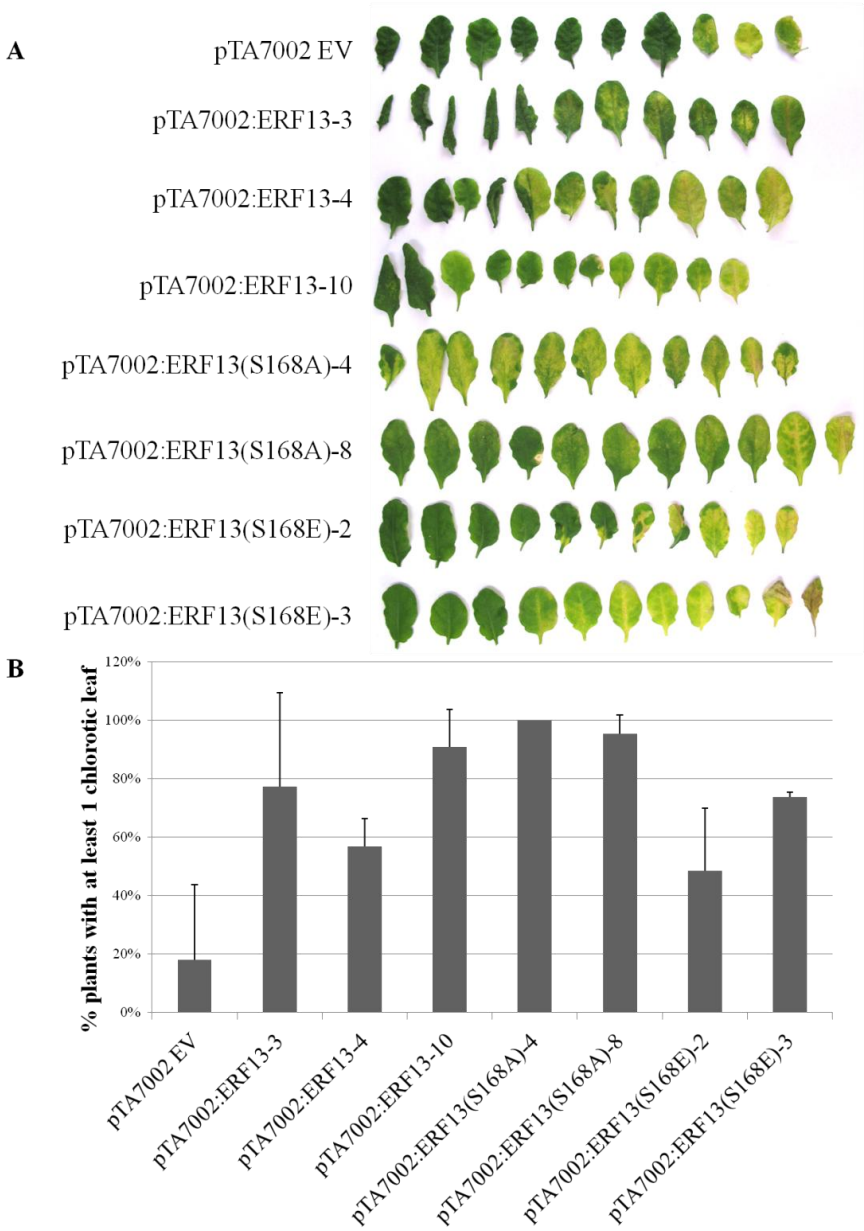

Figure 11. Overexpression of *ERF13* causes chlorosis.

5-week-old *ERF13*-overexpressing plants were sprayed with 50 μM dex + 0.02% Silwet every 48 hours for 6 days. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). EV, empty vector. **(A)** Leaves were detached and photographed on day 8. Each leaf represents a different plant (n=11). This experiment was repeated twice with similar results. **(B)** Percent plants with at least one chlorotic leaf, where chlorotic was defined as any lightening of the leaf relative to the pTA7002 EV. Error bars represent standard deviation, n=2 (results from two independent experiments). No error is shown for pTA7002:ERF13(S168A)-4 because in both experiments all the plants had at least one chlorotic leaf.

ERF13 induces *PDF1.2a* expression

ERF13 is a transcription factor putatively involved in ET signaling because of its AP2/ERF domain. Therefore, in order to elucidate why *ERF13*-overexpressing plants are susceptible to *P. syringae*, we conducted semi-quantitative RT-PCR of *PR1* (an SA marker gene), *PDF1.2a* and *Chi-B* (JA/ET-responsive genes), and *COR15A* (an ABA-induced gene) on total RNA extracted from dex-treated *ERF13*-overexpressing plants.

We found that ERF13 induces expression of *PDF1.2a* (**Figure 12**). We also observed upregulation of *PR1* and *Chi-B* (**Figure S7**), but these results were not reproducible. Additionally, although Lee et al. (2010) previously reported that overexpression of *ERF13* in *Arabidopsis* causes upregulation of *COR15A*, we did not observe the same result (**Figure S7**).

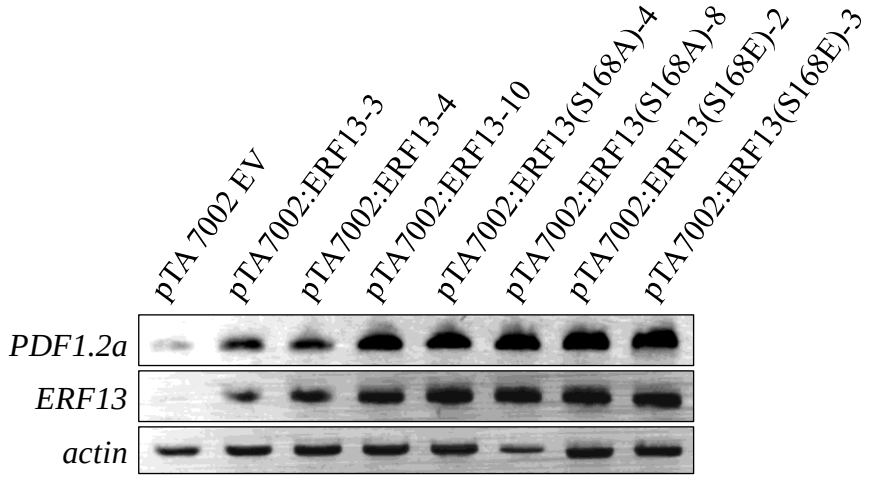

Figure 12. *ERF13* overexpression induces *PDF1.2a.*

6-week-old *ERF13*-overexpressing plants were treated with 50 μM dex and sampled after 48 hours. Transcript abundances were measured via semi-quantitative RT-PCR, using actin *(ACT2)* as a control. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). The experiment was conducted with 3 biological replicates; one representative replicate is shown here. EV, empty vector.

Acknowledgments

This thesis includes material from the project "Quantitative Phospho-proteomic and Proteomic Analysis of the Plant Innate Immune Response," which is being prepared for publication, and of which Chris van Schie is the principal author and Zhouxin Shen and Steven P. Briggs are co-authors. The thesis author's research is a continuation of this work.

This thesis also includes data generated in collaboration with Cheryl Philipsen in the course of her Master's thesis work in the Briggs lab on the project "Development and use of Mass Spectrometry techniques to study Pseudomonas induced PAMP-triggered and effector-triggered immunity in Arabidopis thaliana." Master's Thesis (Utrecht, The Netherlands: Utrecht University).

DISCUSSION

The goal of improving global food security by engineering resistant crops is a daunting one, and the road from gene discovery to functional studies to GM crops is long and arduous. Plant immunity is complicated and multifaceted, and there is still much room for growth and progress in our understanding of it before we can even begin to apply discoveries in practical ways.

Proteome profiling has tremendous potential as a method for large-scale analysis of the protein machinery underlying biological responses, and is therefore a valuable tool for deconvoluting the complexities of the plant defense response. However, even as instrument sensitivity and detection methods improve, validation remains a key part of the workflow, as changes in protein levels can at best only imply function. This work on ERF13, a protein identified via phosphoproteome profiling of *Arabidopsis* effector-triggered immunity, offers a case study in the extension of proteomics to functional studies.

We conclude that inducible overexpression is a useful way to address the challenge of functional redundancy in studying genes such as ERF family members, and that ERF13 is a suppressor of plant defense which acts as a mediator between various hormone signaling pathways.

ERF13 is functionally redundant

Lee et al. (2010) previously found that although *ERF13*-overexpressing plants showed an enhanced ABA response, RNAi-based ERF13 knockdown lines did not display ABA or glucose insensitivity. Similarly, our experiments indicated that *ERF13* T-

DNA lines did not permit a significantly different amount of bacterial growth compared to wild-type plants (**Figure 6**). These data lead us to conclude that ERF13 is either functionally redundant or not required for resistance against *Pst* DC3000 (±AvrRpm1).

ERF13 may interface between stress responses and growth programming in *Arabidopsis*

Our inability to obtain constitutive *ERF13* overexpression lines suggested a developmental impact of *ERF13* overexpression, which was confirmed in subsequent experiments on our dex-inducible overexpression lines. Although the degree of dex-inducible growth impairment varied between lines, it was consistently observed that the plants were smaller when dex-treated, compared to untreated plants from the same lines and plants carrying the empty vector (**Figure 7A**). Furthermore, *ERF13* overexpression appeared to have long-term effects, causing epinasty and accelerated flowering in the weeks after the dex treatment regimen was over. This suggests that a one-time spike in ERF13 levels is sufficient to alter the plant growth program, perhaps by triggering stress signaling that leads to rapid maturation in override of considerations such as size. Plants transition into emergency flowering mode when stressed (Wada et al., 2010), so if in fact ERF13 is a positive regulator of abiotic stress responses (Lee et al., 2010) this could offer an explanation for our results. ET is also known to promote flowering and senescence, so if ET signaling proceeds through ERF13 (as is suggested by ERF13's induction of *PDF1.2a* (**Figure 12**)) this could help account for the phenotypes we observed.

Our results are consistent with the work of Lee et al. (2010), who found that constitutive overexpression of ERF13 in *Arabidopsis* (ecotype Landsberg *erecta)* results

in mild stunting. In addition, overexpression of some other ERFs has also been shown to cause developmental phenotypes: overexpressing *AtERF14*, for example, results in stunting and sterility (Oñate-Sánchez et al., 2007). In fact, AtERF14 seems remarkably similar to ERF13. Besides belonging to the same ERF family sub-group (B-3), it is also induced by avirulent *Pst* DC3000 and activates transcription of *PDF1.2a* (among other *PR* genes) (Oñate-Sánchez et al., 2007). However, *AtERF14*-overexpressing and T-DNA insertion mutant plants were not tested for susceptibility to *Pst.* We might expect, though, that *AtERF14* overexpressers are susceptible to *Pst.* It is also worth note that overexpression of tomato *Pti4* in *Arabidopsis* was found to inhibit plant growth, suggesting that ERF13 and its putative tomato ortholog may have a conserved function in regulation of plant development (Gu et al., 2002; Wu et al., 2002).

The lack of germination of some seeds (**Figure 7C**) could be explained in terms of ERF13's function as a potentiator of the ABA response, since ABA inhibits seed germination and cotyledon expansion. Furthermore, given that ABA addresses abiotic stresses with such responses as stomatal closure to prevent water loss in drought, it is possible that this could help account for the stunting observed in ERF13 overexpressers. Plants that are chronically stressed are typically smaller due to the diversion of energy and resources in maintaining a constitutive stress response. For example, overexpression of the immune protein NDR1 also results in developmental impairment in *Arabidopsis* (Coppinger et al., 2004).

It should be noted that for some lines (namely pTA7002:ERF13-3, -4, and (S168A)-8), dex-inducible growth impairment was significantly attenuated on plate (**Figure 7C**). This observation suggests that the ERF13-induced stunting may be partially

a function of environmental stresses. Based on these results, we might hypothesize that overexpressing ERF13 causes increased sensitivity to stresses a plant grown on plate would not encounter, but which a plant grown on soil would—including, for example, the presence of soil microorganisms. If ERF13 is in fact a negative regulator of defense, this could also help explain the stunting of *ERF13* overexpressers on soil: they may be more susceptible to colonization by soil microorganisms to which plants would ordinarily be resistant, in a manner similar to how immunocompromised patients are vulnerable to opportunistic pathogens.

Another explanation for the line-to-line variations in degree of growth impairment could lie with the dex-inducible system, since a rough correlation between *GVG* expression level and severity of growth defects on dex-supplemented plates has been demonstrated (Kang et al., 1999). We did not check the levels of *GVG* mRNA or GVG protein, so it is possible that this is sufficient to account for the stunting observed.

Although the pTA7002:ERF13-10 line exhibited the most severe dex-inducible growth impairment, we were unable to reliably detect the presence of the transgene product in Western blots conducted on tissue sampled from these plants (**Figure *7***). Transcripts were, however, confirmed to be upregulated in pTA7002:ERF13-10 plants to a similar degree as plants from the other overexpression lines (**Figure 12**). It is possible that this line has such high transgene expression that it undergoes silencing, resulting in our failure to detect the ERF13-HA protein. In addition, it is possible that this line may have leaky expression, since we observed that even without dex treatment it appears to grow somewhat more slowly than wild-type Col-0 and pTA7002 EV plants. Western blots of untreated pTA7002:ERF13-10 plants did not seem to indicate the presence of

ERF13-HA, however (**Figure S3**). We also cannot rule out the possibility that the T-DNA insertion for this line may have disrupted a gene related to growth and development. (This was not further investigated.) We are currently conducting an experiment to construct a timecourse for ERF13-HA levels in pTA7002:ERF13-10 plants in the aftermath of dex treatment, as this may lend insight into any silencing occurring in this line.

ERF13 may be a negative regulator of PTI and ETI that is exploited by virulent and avirulent bacteria

We have found, based on bacterial growth assays, that ERF13 promotes susceptibility to virulent and avirulent *P. syringae* (**Figure 9**). *ERF13* was previously shown to be induced by both virulent and avirulent *Pst* (AtGenExpress; Winter et al., 2007). These data suggest that ERF13 is a negative regulator of basal resistance, and that it could be exploited by *P. syringae* to enhance its colonization of and multiplication in leaf tissues. AvrRpm1 is known to suppress PTI (Lim and Kunkel, 2004; Kim et al., 2005, 2009), so it is conceivable that it may induce *ERF13* expression to achieve this goal. In addition, *ERF13*-overexpressing plants' susceptibility to avirulent bacteria indicates that ERF13 could also oppose or otherwise suppress ETI, presumably via activation of JA/ET and/or ABA signaling (**Figure 9A, Figure *12***).

Our observations that ERF13 is a positive regulator of chlorosis support these data, as chlorosis is a disease symptom associated with infection by many pathogens, including *P. syringae.* We found that overexpression of *ERF13* enhanced chlorosis upon

P. syringae infection (**Figure 10**), that overexpression of *ERF13* alone was sufficient to cause chlorosis (**Figure 11**, **Figure S6**), and that this chlorosis was dex-inducible (**Figure S5**).

While the role of chlorosis in infection remains ambiguous, it is possible to decouple it from bacterial multiplication in leaves. For example, *noc1* mutants are defective in development of chlorosis but still support levels of bacterial growth comparable to wild-type plants (Mecey et al., 2011). A similar phenomenon was also reported for *dnd1npr1* plants, which showed elevated levels of bacterial growth that were comparable to *npr1* plants, but accompanied by reduced disease symptoms (including chlorosis) more comparable to *dnd1* plants (Genger et al., 2008).

Induction of chlorosis, at least in the case of *Pst* DC3000 infection, has been attributed to the phytotoxin COR (Mecey et al., 2011). COR is a JA-Ile lookalike, and is thought to hijack the JA signaling pathway to oppose SA signals raised in response to *Pst* infection, thereby promoting virulence (Zheng et al., 2012; Spoel and Dong, 2008). In fact, COI1, an F-box protein required for JA signaling, was named after the phenotype of its mutation: COR insensitivity (Pieterse et al., 2012).

Still, it remains to be determined if chlorosis is merely a side effect of COR toxicity, or if it actually plays some role in promoting pathogen growth. Mecey et al. (2011) found that bacterial growth 6 days after inoculation was higher for *noc1* plants than for wild-type, indicating that chlorosis may actually *restrict* bacterial multiplication. *noc1* is an ethyl methanesulfonate (EMS) point mutant of *SGR*, a COR-inducible gene encoding a protein that facilitates chlorophyll breakdown (Mur et al., 2010). Interestingly, it was found that SGR is also important for development of HR, as it

mediates chloroplastic ROS production (Mur et al., 2010). These results suggest that chlorosis may in fact be part of the plant's basal immune response, even though it has traditionally been considered a disease symptom—something like fever in humans. Alternatively, this could be part of *Pst*'s pathogenesis strategy, perhaps helping promote its transition to a necrotrophic lifestyle.

At a glance, *ERF13* seems strikingly similar to COR: it, too, feeds into the JA pathway, causes chlorosis, and promotes bacterial growth in infected leaf tissue. Since COR is known to induce *PDF1.2a* (Camañes et al., 2012), we might even hypothesize that ERF13 lies downstream of COR, or that they share some parts of their signaling pathways. However, we found that *ERF13* overexpression does not affect *SGR* transcript levels (**Figure S6C**), suggesting that ERF13-mediated chlorosis may generate chlorosis in a SGR-independent manner. This could be confirmed by investigating whether ERF13 can still effect chlorosis in *noc1* plants.

Many things other than disease can cause chlorosis—to name a few, iron deficiency and cold stress (Abadía et al., 2011; Yadav, 2010)—indicating that there are likely to be multiple pathways leading to its development. Nevertheless, it might be interesting to investigate *ERF13*'s responsiveness to COR treatment. **Figure 13** summarizes our hypotheses relating to ERF13's role in hormone-modulated immunity and *Pst*'s manipulation thereof.

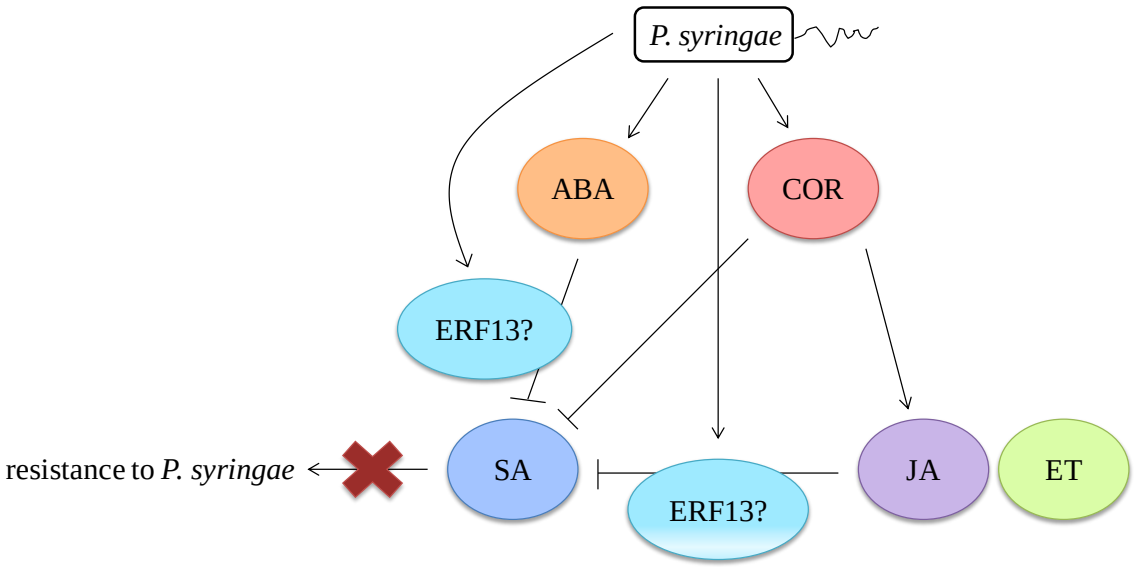

Figure 13. Hypothetical model of ERF13's role in the hormone dynamics triggered by *Pst* infection.

Coronatine (COR), a phytotoxin secreted by *P. syringae,* is a JA-Ile mimic that inhibits SA accumulation and triggers JA responses. *P. syringae* also stimulates ABA biosynthesis, which together with COR-induced JA signaling counter SA-mediated resistance and promote susceptibility against *P. syringae.* ERF13 may be involved in COR-, JA-, and/or ABA-mediated inhibition of SA signaling.

It is also due note that our observations of chlorosis were somewhat inconsistent: that is, not all plants from the same overexpression line developed chlorosis, and for those plants that did exhibit chlorosis, not all leaves were chlorotic (**Figure S5, Figure S6A**). These differences are potentially attributable to our application of dex via spray, as it is difficult with this method to control how much treatment each leaf or plant receives.

Another large factor that could have contributed to the observed variations is our use of T2-generation segregating lines, such that the genetic composition of each batch of plants was approximately 1/3 homozygous and 2/3 heterozygous. It is conceivable that the homozygous and heterozygous plants exhibited differential levels of *ERF13* overexpression, leading to differential phenotypes. However, we used one T3 generation

homozygous line (pTA7002:ERF13(S168E)-3) for several experiments and obtained similar varied results (**Figure 11**, **Figure S6A**). We were also unable to correlate protein levels and degree of chlorosis (**Figure S6B**), suggesting that genotype cannot fully account for the apparent inconsistencies we saw. We are currently in the process of obtaining homozygous T3 seed batches for the remainder of the lines used.

It is not clear why the development of chlorosis was restricted to older (5+ week old) plants for several of the *ERF13*-overexpressing lines. For pTA7002:ERF13-3 and -4, no significant chlorosis was observed when seedlings were sprayed with dex every two days (**Figure 7A**). However, 5-week-old plants from the same lines began showing chlorosis as early as 4 days after the start of dex treatment. The same was true for seedlings germinated on plates with dex (**Figure 7C**). In both cases, though, pTA7002:ERF13-10 displayed an extreme phenotype—including marked chlorosis—in response to application of dex. It is possible that, if in fact these plants undergo silencing but feature spurts of very high transgene expression, the difference can be explained solely in terms of ERF13 levels. Perhaps, for example, a certain threshold level of ERF13 is required in young plants to induce chlorosis because there are mechanisms in place to prevent chlorophyll breakdown in critical growth stages where such chlorosis would threaten the plant's survival.

Finally, we observed that in some of our experiments, dex-treated pTA7002 EV plants developed some chlorosis while dex-treated wild-type Col-0 plants never did (**Figure S6A**), indicating that the GVG system may contribute to the development of chlorosis. In fact, it has been previously found that this particular overexpression system can cause growth defects and defense activation in plants (Moore et al., 2006).

Furthermore, we used a somewhat high concentration of dex to induce expression (50 μM), so it is possible that dex in combination with the GVG fusion protein—or either of these on its own—caused toxicity leading to the chlorosis observed. We observed a similar result in our attempted *B. cinerea* susceptibility assays, where leaves detached from pTA7002 EV plants developed extensive chlorosis and unexpectedly large lesions compared to wild-type Col-0 plants. Nevertheless, we still conclude that *ERF13* overexpression causes chlorosis because in general the *ERF13*-overexpressing plants displayed more severe chlorosis than did pTA7002 EV plants.

All of these data are suggestive of an interesting function for the native ERF13 protein, leading us to ask what purpose ERF13 serves in the plant defense scheme apart from appropriation or hijacking by pathogens. We hypothesize that ERF13 may play a role in linking ETI to hormone signaling.

HR triggered by R proteins such as RPM1 is accompanied by a spike in SA, which in turn confers systemic acquired resistance (SAR)—the subsequent restriction of pathogen growth in tissues beyond the infected area (Glazebrook et al., 2005; Spoel and Dong, 2012; Pieterse et al., 2012). However, it is not known how or where the signaling of R proteins and defense-related hormones intersects. Given that it is induced by both MeJA and avirulent bacteria and negatively regulates resistance against avirulent bacteria (Goda et al., 2008; AtGenExpress; Winter et al., 2007; **Figure 9A,B**), *ERF13* could be one of the points at which these signals converge.

ERF13's appearance in our ETI signaling proteome data seems to corroborate this (**Figure 4**). RPM1 itself could conceivably induce *ERF13* as a kind of fine-tuning/negative feedback mechanism to limit the severity of the immune response (which

might be exaggerated into susceptibility when *ERF13* is overexpressed) (**Figure 14**). Immunity is costly to the plant, and maintaining constitutive defense can be considered a kind of stress itself, resulting in phenotypes such as stunting and delayed development (Coppinger et al., 2004). It is thus expected for plants to have mechanisms in place for modulating their own defense responses.

This hypothetical model could also help explain the unexpected differential effects of *ERF13* and tomato *Pti4* overexpression in *Arabidopsis*. It is conceivable that if Pti4 does not retain its normal function in *Arabidopsis*, it could interfere with the function of the native ERF13—thereby resulting in a dominant negative phenotype (including reduced chlorosis upon *Pst* infection).

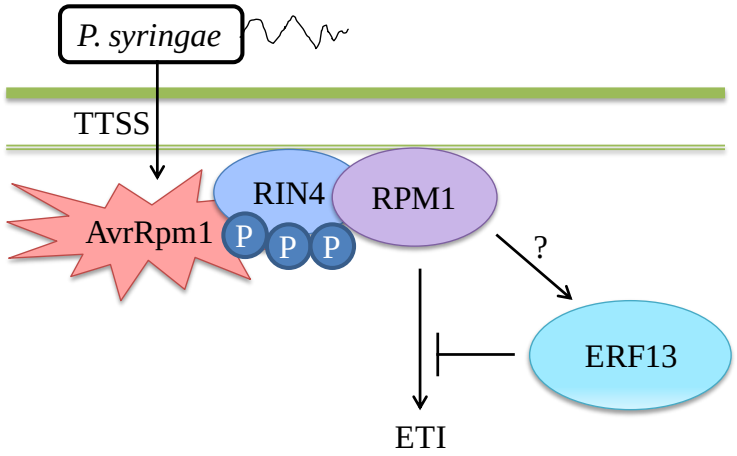

Figure 14. Hypothetical model for ERF13's involvement in RPM1-mediated ETI signaling.

ERF13 may be induced by RPM1 as an internal negative feedback mechanism to suppress AvrRpm1-triggered ETI.

We also observed enhanced cell death subsequent to *P. syringae* infection for *ERF13*-overexpressing plants (**Figure 10A**), suggesting that ERF13 may play a role in potentiating HR. Analyses of the mutants *ndr1-1* and *dnd1* have shown that resistance

(i.e., restriction of pathogen multiplication) and HR in ETI can be decoupled, since *ndr1-1* plants are susceptible to *Pst* DC3000 (AvrRpm1) but can still undergo HR in response to it, whereas the opposite is true for *dnd1* mutants (Century et al., 1995; Yu et al., 1998). If in fact ERF13 promotes HR and lies somewhere downstream of RPM1 activation, then it (like *ndr1-1* and *dnd1)* may be useful in parsing apart the distinction in signaling between resistance and HR, since if it lay upstream of this divergence one would expect it to either cause increased susceptibility and decreased HR or vice versa.

Associating ERF13 with RPM1 as a downstream signaling component could be verified by taking advantage of the fact that RPM1 is also known to recognize and initiate ETI in response to AvrB's phosphorylation of the RPM1 guardee RIN4. It may be interesting to see if induced expression of *AvrB* also leads to upregulation or phosphorylation of ERF13, and furthermore if *ERF13*-overexpressing plants are susceptible to *Pst* DC3000 (AvrB).

One important question which remains to be addressed is that of how ERF13 protein levels fluctuate upon pathogen infection and defense signaling. However, ERF13 could not be detected in our total proteome data and was not reliably detected in its phosphorylated form. The sensitivity and consistency issues with our mass spectrometry-based results can be circumvented by repeating the experiment with a larger number of replicates or by resorting to other protein-based assays such as Western blotting (of the native ERF13 or of tagged ERF13 under control of the native promoter) or multiple reaction monitoring (MRM).

Furthermore, while ERF13 seems definitively to positively regulate susceptibility in *Arabidopsis,* it is still unclear how *ERF13* expression is induced. It seems likely that

numerous stresses *(P. syringae* infection, *B. cinerea* infection, MeJA treatment) all converge at or upstream of ERF13, resulting in its expression. Unraveling the pathways these stress responses take to arrive at modulating ERF13 levels—and factoring in the possible impact of RPM1 signaling, which may also cause ERF13 induction—is of interest in better understanding the intricacies of hormone crosstalk and the defense response.

ERF13 works at a junction of the SA, JA/ET, and ABA pathways

The observed susceptibility of *ERF13*-overexpressing plants to *P. syringae* (**Figure 9**) is consistent with reports of ERF13's role as a positive regulator of ABA signaling (Lee et al., 2010). ABA and defense signaling have been found to oppose one another (Fujita et al., 2006; Mauch-Mani and Mauch, 2005), so if *ERF13* overexpression stimulates ABA responses, it is likely to antagonize SA and thereby render plants more susceptible to biotrophic pathogens such as *P. syringae.* Indeed, it has been shown that virulent and avirulent *P. syringae (Pst* DC3000 +/- AvrRpm1) infection stimulates *ERF13* expression (Winter et al., 2007), suggesting that the induction of *ERF13* may be a virulence strategy of this pathogen. A similar scenario has been observed with the *Pst* effector coronatine (COR), which mimics MeJA and thereby counteracts SA responses (Spoel and Dong, 2008). Since *ERF13* is MeJA-inducible, it could conceivably lie downstream of COR (**Figure 13**).

We were, however, unable to consistently identify changes in *PR1* transcript levels in *ERF13*-overexpressing plants. Although some of our data indicated that *ERF13*

overexpression resulted in *PR1* upregulation (**Figure S7**), our results appeared variable, as subsequent experiments did not confirm it. This is not necessarily surprising; *PR1* is considered a SA-responsive gene, but it is also subject to regulatory input by proteins such as AtERF14 (Oñate-Sánchez et al., 2007). Furthermore, while AtERF14 can impact *PR1* expression, it is not required for *PR1* induction in response to exogenous SA application (Oñate-Sánchez et al., 2007). These findings paint a complex picture of *PR1* regulation, in which numerous factors and conditions can impact its expression. Therefore, we speculate that ERF13 induces *PR1* under some circumstances. To tackle the task of deconvoluting the molecular basis behind *ERF13* overexpressers' susceptibility to *P. syringae*, it may be useful to investigate ERF13's impact on other SA-responsive *PR* genes (e.g. *PR2, PR5),* and/or to measure SA levels in *ERF13*-overexpressing plants.

Actually, contrary to earlier work on ERF13 (Lee et al., 2010), we did not observe any upregulation of the ABA-responsive gene *COR15A* in our system (**Figure 12**), suggesting that ERF13's activity may vary between ecotypes (Col-0 vs. L*er).* Alternatively, differences in sampling time and temperature may have affected our results, since *COR15A* is cold-responsive. As a future direction, we could attempt to verify the other ABA-related effects of *ERF13* overexpression. To confirm ERF13 as a positive regulator of ABA signaling, we would need to conduct further experiments, for example an ABA sensitivity assay on seedlings. We could also hypothesize that ERF13 represents an exception to the mutual antagonism between biotic and abiotic stress response pathways.

If ERF13 promotes ABA signaling, however, its induction of the JA/ET-

responsive *PR* gene *PDF1.2a* (and possibly *Chi-B)* (**Figure 12**, **Figure S7**) is surprising and even contradictory. We would expect a potentiator of ABA responses to exert an inhibitory effect on immune responses, culminating in the downregulation of markers such as the *PR* genes. Our results suggest that ERF13 may be an exception to the rule of ABA/immune opposition.

If ERF13 is indeed a positive regulator of the JA/ET response, as indicated by its induction upon *B. cinerea* infection (Winter et al., 2007, AtGenExpress) and stimulation of *PDF1.2a* expression (**Figure 12**), we would expect that *ERF13* overexpression should confer increased resistance to necrotrophic pathogens such as *B. cinerea.* We conducted several experiments to assay our *ERF13*-overexpressing lines for their ability to defend against various strains (B05.10, grape, rose) of this pathogen, but without conclusive results. Not all leaves developed lesions, and furthermore the pTA7002 EV control had an unexpectedly high average lesion diameter relative to wild-type Col-0 plants. It is possible that our dex-inducible overexpression system is not compatible with a *B. cinerea* susceptibility assay. There are methods for assessing resistance to other necrotrophic pathogens, such as *A. brassicicola* and *F. oxysporum,* so it may be worthwhile to investigate the effect of *ERF13* overexpression on immunity to these pathogens as well.

As a regulator of ET responses, ERF13 may be induced by EIN3, a transcription factor found to directly induce expression of *ERF1,* another group IX ERF family member (Zhu and Guo, 2008). This could be investigated via, for example, electrophoretic mobility shift assay (EMSA), or by determining if overexpressing *ERF13* in an *ein3* background will complement *ein3* ET insensitivity.

Finally, it should be noted that dex treatment at concentrations as low as 0.1 μM

has been shown to induce *PDF1.2a* expression in *Arabidopsis* plants carrying the GVG system transgene (Kang et al., 1999). Nevertheless, because we observe much stronger induction of *PDF1.2a* in *ERF13*-overexpressing plants relative to pTA7002 empty vector plants, we conclude that ERF13 does stimulate *PDF1.2a* expression. In addition, Meng et al. (2013) reported *PDF1.2a* induction in response to dex-induced expression of constitutively active *NtMEK2* in a *35S:ERF6* background, indicating that the dex-induced expression of *PDF1.2a* is negligible enough not to confound the results of such expression studies.

Exploring upstream and downstream of ERF13 will help explain the pleiotropic effects of *ERF13* overexpression

While our RT-PCR results indicate that ERF13 exerts transcriptional control over at least one JA/ET-inducible *PR* gene (**Figure 12**), it is not known whether this control is direct or indirect—i.e. if ERF13 is actually a transcription factor for these genes, or if it lies somewhere further upstream. This question could be addressed by conducting ChIP-PCR or -seq on *ERF13*-overexpressing lines. Results could then be verified by cloning putatively ERF13-regulated gene promoters in front of a reporter, e.g. luciferase, introducing these constructs into our *ERF13* overexpression lines, and looking for dex-induced reporter expression (e.g. luminescence).

Seminal members of the ERF family were described as transcription factors capable of binding to the GCC box found in JA/ET-inducible genes such as *PDF1.2a*, *Chi-B*, and *Thi2.1*. However, it has also been observed that ERFs can induce expression of genes whose promoters lack a GCC box (Oñate-Sánchez et al., 2007). Conversely, the

presence of a GCC box in a gene's promoter does not guarantee that ERFs will affect its transcription (McGrath et al., 2005). ERFs have also been found to bind other consensus sequences, e.g. the CE1 in ABA-responsive genes (Lee et al., 2010). We would expect ERF13 to regulate *PDF1.2a* expression via the GCC box (in addition to its regulation of *COR15A* via the CE1). This could be confirmed by mutating the GCC box in the *PDF1.2a* promoter and determining (e.g. via RT-PCR or reporter) if *ERF13* overexpression can still induce it.

For future directions in the vein of proteomics, it could be enlightening to investigate proteome-level changes concurrent with *ERF13* overexpression. Such analyses could help reveal what other proteins ERF13 partners with in order to achieve its various effects on defense, growth and development, and hormone signaling.

Also of interest is the matter of what transcription factors regulate *ERF13* expression. It might be useful to conduct a yeast one-hybrid screen of *Arabidopsis* transcription factors with the regulatory region of *ERF13* as bait. This could help us better understand how pathogen infection and plant defense responses feed into hormone pathways and ultimately translate into the phenotype of susceptibility or immunity.

The "promiscuity" of transcription factors like ERF13, which are involved in multiple hormone pathways, is intriguing and promises to be useful in untangling the crosstalk between SA, JA, ET, and ABA (among other hormones). Further studies of ERF13's regulatory portfolio—and of its own regulation profile—may do much to outline a pathway from hormone signal(s) to nuclear transcriptional reprogramming of *PR* genes.

The function of ERF13 S168 phosphorylation remains elusive

Our phosphoproteome data indicate that ERF13 is phosphorylated at Serine 168. No phosphorylation of ERF13 has been previously reported, so this is both a novel post-translational modification and phosphorylation site. Although our results suggest that ERF13 phosphorylation increases from 0 to 4 hours after initiation of ETI signaling through RPM, it is not possible to determine if the increase in S168-phosphorylated ERF13 was due to an increase in the total amount of the protein or to phosphorylation of the existing pool of the protein. Regardless of these uncertainties, our data are still useful because they show, for the first time, a phosphorylated ERF13 species in the context of immune signaling. Our results thus illustrate the utility of phospho-enrichment prior to mass spectrometry in discovering interesting proteins.

The total body of our experimental data on wild-type, phospho-dead, and phospho-mimetic ERF13 indicates that its S168 phosphorylation state is not important for mediating defense against *Pst*, enhancing chlorosis, inhibiting growth, or inducing *PDF1.2a* (**Figure 7C,** Figure ***9*B, Figure *11*, Figure *12***). These data suggest that S168 alone is not a vital phosphorylation site for ERF13's function in these capacities, or for the plant's control of these various processes and responses. In drawing these conclusions, we assume that the S168E mutation is a close enough structural approximation of S168-phosphorylated ERF13 to serve as a practical proxy in whatever role phosphorylation at this residue performs. Conversely, we also assume that S168A ERF13 in fact mimics the conformation and function of non-phosphorylated ERF13. Numerous studies have demonstrated the success of D/E and A substitutions as phospho-mimetic and phospho-dead, respectively (Fillebeen et al., 2003; Chung et al., 2011; Liu et al., 2011), lending confidence to the legitimacy of our results. However, there is still the

possibility that our results are false negatives raised because downstream components were unable to recognize S168E as phosphorylated ERF13.

An additional consideration is the amount of S168-phosphorylated ERF13 required to affect the aforementioned plant processes. If only a minimal threshold level of phosphorylated ERF13 is necessary, then it is conceivable that the effect of ERF13(S168A) overexpression could have been dampened by the phosphorylation of native ERF13, since we transformed the various pTA7002:ERF13 constructs into wild-type plants.

In terms of future directions, it remains to be determined if there are other phosphorylation sites on ERF13 which together have a synergistic effect on protein function, or if S168 phosphorylation is important for some other as-yet-unassessed processes in which ERF13 is involved. While multiple sequence alignments indicated that the tomato ERF Pti4 may have a motif corresponding to ERF13's S168 and flanking residues, previous work has indicated that Pti4 is phosphorylated by Pto kinase at several threonine residues, with no indication of phosphorylation at any serines (Gu et al., 2000).

Besides Pti4, other research has shown that phosphorylation is important for several other ERFs. AtERF6 (another member of the ERF family group IX) was found to be phosphorylated by the MAP kinases MPK3 and MPK6 in response to *B. cinerea* infection (Meng et al., 2013). This phosphorylation enhances both the protein's stability and resistance against *B. cinerea,* apparently by facilitating ERF6's induction of defense genes such as various *PDFs* (Meng et al., 2013). The MPK6-mediated phosphorylation of another group IX ERF, ERF104, was also found to contribute to protein stability (Bethke et al., 2009).

The work done on ERF6 and ERF104 phosphorylation leads us to speculate that unphosphorylated ERF13 may likewise be unstable, which might explain its poor detection in mass spectrometry-based assays (likely compounded by its predicted low abundance as a transcription factor). Protein instability could also account for its spotty detection in our Western blots (**Figure 7B,D;** Figure ***9*; Figure S3; Figure S6B**).

ERF6 first came to the attention of Meng et al. (2013) as a substrate of MPK3 and 6, two MAP kinases which appear to be positive regulators of defense. It could be of interest to investigate whether either of these MPKs or related kinases are in charge of ERF13 phosphorylation. Knowing what protein(s) is/are responsible for ERF13 phosphorylation could help shed light on the purpose of S168 phosphorylation.

Summary

We propose that ERF13 may serve as a negative regulator of SA-mediated immune responses in plants, in accordance with its affiliation with ABA and JA/ET as a positive regulator of signaling. We also propose that *P. syringae* exploits this built-in immunosuppression mechanism by inducing *ERF13* as a virulence strategy, thereby facilitating bacterial growth and the development of disease symptoms such as chlorosis. ERF13's promotion of susceptibility against *P. syringae* appears to occur independently of S168 phosphorylation, the role of which remains undefined. Further studies on the mechanism of *ERF13* induction will improve our understanding of ERF13's role in plant/pathogen interactions.

Acknowledgments

This thesis includes material from the project "Quantitative Phospho-proteomic and Proteomic Analysis of the Plant Innate Immune Response," which is being prepared for publication, and of which Chris van Schie is the principal author and Zhouxin Shen and Steven P. Briggs are co-authors. The thesis author's research is a continuation of this work.

This thesis also includes data generated in collaboration with Cheryl Philipsen in the course of her Master's thesis work in the Briggs lab on the project "Development and use of Mass Spectrometry techniques to study Pseudomonas induced PAMP-triggered and effector-triggered immunity in Arabidopis thaliana." Master's Thesis (Utrecht, The Netherlands: Utrecht University).

MATERIALS AND METHODS

Protein extraction and digestion, phosphopeptide enrichment, iTRAQ labeling, and mass spectrometry

4-5-week-old transgenic plants expressing *AvrRpm1* in a dex-inducible manner in an *rpm1-3* background with or without RPM1-myc (Mackey et al., 2002) were treated with 0.03% Silwet L-77 ± 50 μM dex and sampled at 0, 2, and 4 hrs. Tissue was processed as described in van Schie et al. (manuscript being prepared for publication). In brief: whole rosettes were flash-frozen and crushed in liquid nitrogen. Protein was precipitated in methanol + 0.2 mM vanadate and washed in acetone. Following dehydration and solubilization in 50 mM HEPES pH 7.3, samples were digested with trypsin and subjected to iTRAQ labeling approximately as described in O'Brien et al. (2010). Four differentially labeled samples from a given replicate and time point were pooled. Samples were then split, with the majority used for phosphopeptide enrichment, and LC-MS/MS analysis was conducted.

Figure S1 shows an overview of the workflow for this experiment.

Cloning of phospho-dead and –mimetic *ERF13*

Wild-type, phospho-dead (S168A), and phospho-mimetic (S168E) *ERF13* constructs were generated via PCR-based mutagenesis and cloned into pGreen vectors. These constructs were then subcloned from the pGreen vectors into pTA7002 in front of two copies of the strep tag (WSHPQFEK) and one copy of the human influenza hemagglutinin (HA) tag (YPYDVPDYA). All primers used for cloning are listed in **Table S1**. A map of the pTA7002:ERF13 vector is shown in **Figure S2**.

Plant materials and growth conditions

All *Arabidopsis thaliana* plants used in this study were in the Columbia-0 ecotype background. The T-DNA insertion lines (GABI 121A12 and 724B09) were obtained from the ABRC (stocks CS314316 and CS469429, respectively). Transgenic *ERF13*-overexpressing plants were generated via the floral dipping method: electrocompetent *Agrobacterium tumefaciens* (GV3101) were transformed with the vectors previously described and flowering wild-type plants were dipped using a standard protocol. Seeds were collected and plated on ½ Murashige & Skoog + Gamborg vitamins and 0.8-1.0% plant agar with 15 mg/L hygromycin, 50 mg/L carbenicillin, and 15 mg/L nystatin. Hygromycin-resistant (T1) seedlings were transplanted to soil and allowed to set seed. T2 plants were used for all further experiments on transgenic inducibly overexpressing *ERF13* plants.

For the assays conducted on *erf13* T-DNA lines, plants were germinated on soil. For the assays conducted on T2 *ERF13*-overexpressing lines, plants were germinated with hygromycin selection on plate as described by Harrison et al. (2006) and transplanted to soil after 1-2 weeks. Plants were kept in a growth chamber (Percival Scientific) under a 10-11-hour light cycle at ~22°C (temperature and humidity not controlled).

Dexamethasone treatment

Concentrated (1000x) dexamethasone stocks were created by dissolving dex in 1:1 DMSO/ethanol to a concentration of 50 mM. All dex treatments were carried out by

spraying plants with a solution of 50 μM dex and 0.02% Silwet L-77 in water. For the control/mock treatments, plants were sprayed with a solution of 1:1 DMSO/ethanol diluted 1000x in water and 0.02% Silwet. 50 μM was chosen as the concentration for all dex treatments on the basis of data indicating that dex-induced expression of *AvrRpm1* in the GVG system was maximized at 20 μM (Geng and Mackey, 2011). The concentration for the dex-supplemented MS plates was chosen based on the precedent of several other uses of 1 μM dex in seedling treatments (Kodaira et al., 2011; Meng et al., 2013).

For all assays to measure bacterial growth, chlorosis, or developmental phenotypes of soil-grown *ERF13*-overexpressing plants, we sprayed plants with dex every ~48 hrs. 48 hours was selected as the time point for re-treatment because of data showing that mRNA levels of a reporter gene in the GVG system decreased from peak levels between 48 and 72 hrs (Aoyama and Chua, 1997).

Bacterial growth assays

For the bacterial growth assays conducted on *erf13* T-DNA lines, we used two *Pseudomonas syringae* strains obtained from the Dangl lab: *Pst* DC3000 and *Pst* DC3000 *(AvrRpm1).* For all other bacterial growth assays, we used two *Pseudomonas syringae* strains described by and obtained from Fan et al. (2008), namely, *Pst* DC3000 *(lux, AvrRpm1)* and *Psm* ES4326 *(lux).*

Plants were grown for 5-6 weeks in pots covered with mesh. The night before the assay, plants were watered in excess and domed. For assays with the pTA7002:ERF13 lines, plants were sprayed with dex ~24 hrs before inoculation and again ~48 hrs later. Bacteria were grown overnight to 2 days at 28-30°C on King's B media plates (15 g/L

peptone, 10 mM K_2HPO_4, 10 mM $MgSO_4$, 1% glycerol, 1.5% bacteriological agar, pH 7.2) with selection (25 mg/L rifampicin, 50 mg/L kanamycin, 40 mg/L nystatin; 20-25 mg/L tetracycline added for *Pst* DC3000 *(lux, AvrRpm1)).* Bacteria were scraped off the plates into 5-10 mL sterile 5 mM $MgSO_4$.

For the assays on the T-DNA lines, plants were spray-inoculated with bacteria at OD 0.01 in 5 mM $MgSO_4$ and 0.035% Silwet. For the assays on pTA7002:ERF13 lines, plants were vacuum-inoculated with bacteria at OD 0.0002-0.001 *(Pst* DC3000 *(lux, AvrRpm1)* or 0.0001 *(Psm* ES4326 *(lux))* in 5 mM $MgSO_4$ and 0.01% Silwet. Vacuum inoculation was performed by inverting pots over pipet tip box lids filled with inoculum inside a vacuum chamber, applying the vacuum, and then releasing it rapidly. The inoculum was changed out between each pot.

For sampling, two well-inoculated leaves per plant were cut off from 8 plants and rinsed in 70% ethanol. Leaves were then washed in water and dried on a paper towel. One disk 6 mm in diameter was excised from each leaf using a handheld hole puncher. Two disks from the same plant were combined for each biological replicate. For the assays on T-DNA lines, the two disks were placed in one well of a 96-well deep-well plate with 500 μL 5 mM $MgSO_4$ and 1 stainless steel bead 5.6 mm in diameter. For the assays on pTA7002:ERF13 lines, the two disks were placed in one well of a 96-well PCR plate with 100 μL 5 mM $MgSO_4$ and 3 stainless steel beads 2.3 mm in diameter.

Bacteria were extracted by shaking the plate for 30 seconds (plate rotated after 15 seconds) at 30 Hz at room temperature with a Retsch Mixer Mill MM400. For the assays on T-DNA lines, 10-fold serial dilutions of the extract were made in 200 μL 5 mM $MgSO_4$ and 10 μL of select dilutions were streaked on King's B plates with the

appropriate selection. Plates were incubated at room temperature for 2 days, and colonies were counted thereafter. For the assays on pTA7002:ERF13 lines, 70 μL of the extract was removed into a 96-well flat-bottom white plate, which was read in a Berthold Mithras LB 940 luminescence detector (no filter, 10 seconds per well, 30-second delay before plate reading) (Schroeder lab). Mikrowin 2000 software was used to manage the luminescence detector and data collection.

Gene expression analysis

Total RNA was isolated using TRIzol (Ambion), starting with 100-200 mg flash-frozen and crushed leaf tissue. These samples were then cleaned up using a QIAGEN RNeasy kit. Since several of the genes assayed lack introns, all samples were subjected to DNase I treatment. Concentrations of the resulting RNA were measured using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo). cDNA was synthesized using a SuperScript III First-Strand Synthesis System kit (Invitrogen). PCR was conducted with Choice-Taq DNA polymerase (Denville Scientific). See

Table S2 for a complete list of RT-PCR primers and conditions.

Western blots were conducted by extracting protein from flash-frozen, crushed leaf tissue into a buffer containing 100 mM Tris pH 7.0, 10 mM $MgCl_2$, 100 mM KCl, 10-15% glycerol, 40 mM β-mercaptoethanol, 0.5% Triton X-100, and 1x protease inhibitor cocktail. Samples were incubated on ice or at room temperature for several minutes, then centrifuged for 5 min at 16,100 xg at 4°C. Protein concentration was determined via Bradford assay (Biorad). Equal loading was verified with either Ponceau or Memcode reversible protein stain (Pierce) following semi-dry transfer. Membranes were probed with primary anti-HA antibody generated by the Jamora lab.

Chlorophyll measurement

Chlorophyll was extracted by adding 1 mL 96% ethanol to ~100 mg (pre-weighed) flash-frozen crushed leaf tissue and incubating rocking in the dark at room temperature for 10 minutes. The samples were spun down for 15 min at 16,100 xg at 4°C. Spectrophotometry was conducted on the undiluted supernatant and a 1:10 dilution of the supernatant in order to obtain the mg chlorophyll a and b per mg tissue, as described in Lichtenthaler, 1987.

Phylogenetic analysis

Multiple sequence alignments of the IX group AtERFs and Pti4 were conducted using ClustalX 2.1. TreeView was used to generate a cladogram from the alignment.

Photography

All pictures of plants and leaves were taken with a Canon PowerShot A620.

Acknowledgments

This thesis includes material from the project "Quantitative Phospho-proteomic and Proteomic Analysis of the Plant Innate Immune Response," which is being prepared for publication, and of which Chris van Schie is the principal author and Zhouxin Shen and Steven P. Briggs are co-authors. The thesis author's research is a continuation of this work.

This thesis also includes data generated in collaboration with Cheryl Philipsen in the course of her Master's thesis work in the Briggs lab on the project "Development and use of Mass Spectrometry techniques to study Pseudomonas induced PAMP-triggered and effector-triggered immunity in Arabidopis thaliana." Master's Thesis (Utrecht, The Netherlands: Utrecht University).

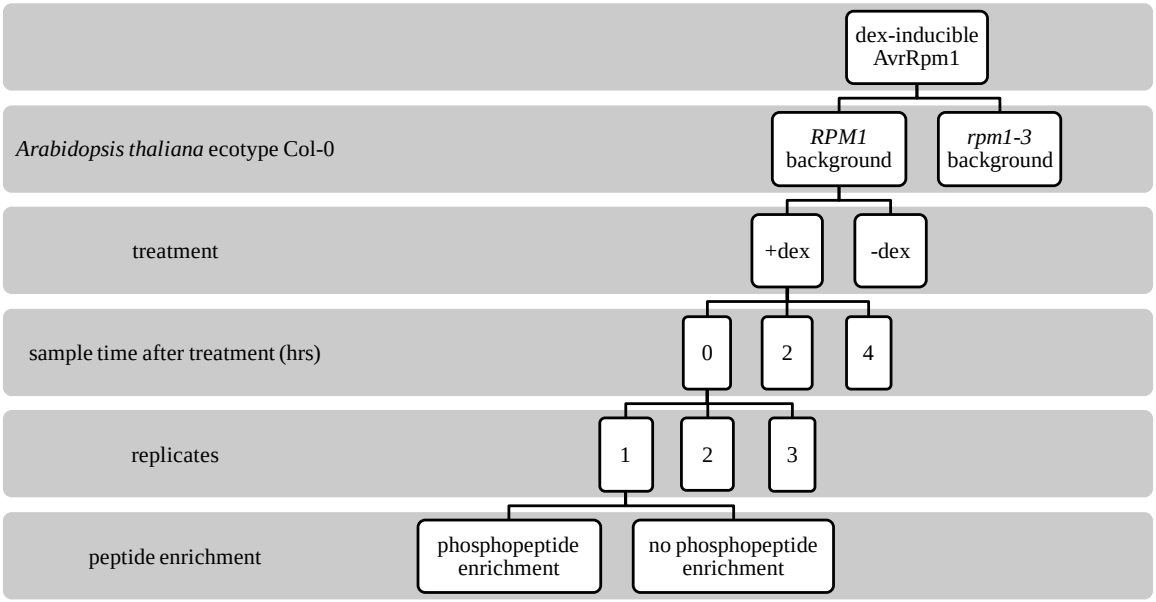

Figure S1. Experimental design for the quantitative mass spectrometry profiling of non-modified and phospho-enriched peptides.

Each branch is shown for only one of the lines/conditions in each horizontal module in the scheme, but applies to all lines/conditions listed in the module (e.g. *rpm1-3* plants were also subjected to ±dex treatment), for a total of 72 samples. Each final sample for mass spectrometry consisted of a pool of 4 differentially iTRAQ-labeled samples from a given time point (0, 2, or 4 hrs), replicate, and phosphopeptide enrichment status (yes/no): (1) dex-treated *RPM1*, (2) dex-treated *rpm1-3*, (3) mock-treated ("-dex," Silwet only) *RPM1*, and (4) mock-treated *rpm1-3*.

Table S1. Primers used for cloning.

List of primers employed in cloning phospho-dead and –mimetic *ERF13* and subcloning these *ERF13* constructs into the pTA7002 vector. All primer sequences are written 5' to 3'. Underlined sequences indicate the mutated codons (ERF13(S168A), ERF13(S168E)) or restriction sites (XhoI and SpeI for forward and reverse ERF13 subcloning primers, respectively).

Cloning	Forward primer	Reverse primer
ERF13(S168A)	CCGTCGCGCGCCGGAACCG TCAGTCTCC	GGTTCCGGCGCGCGACGG CGAGGCCTA
ERF13(S168E)	CCGTCGCGAGCCGGAACCG TCAGTCTCC	GGTTCCGGCTCGCGACGG CGAGGCCTA
Subcloning ERF13 from pGreen into pTA7002	ATCGCTCGAGATGAGCTCA TCTGATTCCGTTA	ATCGACTAGTTATCCGAT TATCAGAATAAGAAC

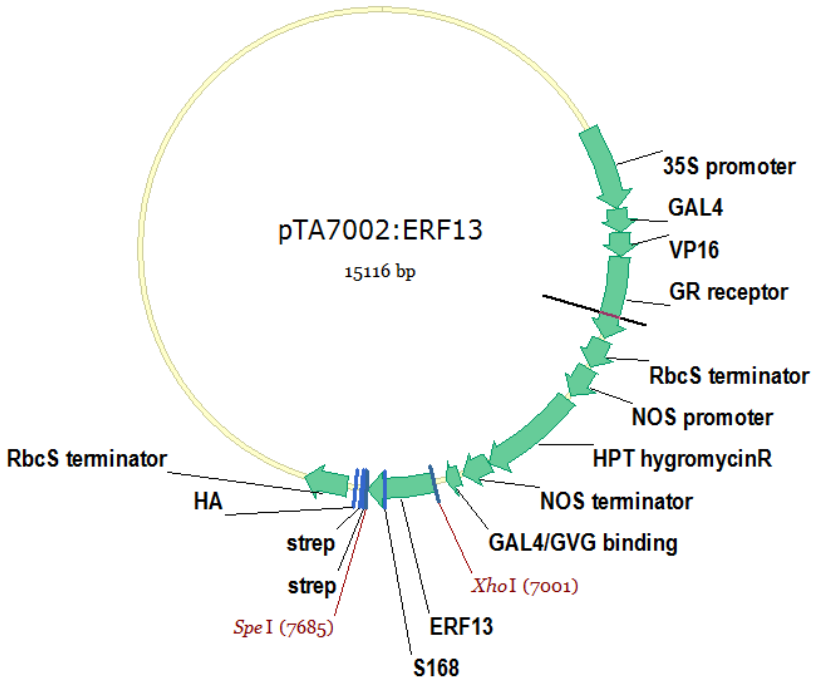

Figure S2. Diagram of the pTA7002:ERF13 vector.

ERF13 was cloned into the pTA7002 vector. 35S, Cauliflower mosaic virus promoter. GVG, GAL4/VP16/GR. GAL4, DNA binding domain of the yeast transcription factor. VP16, herpes viral protein. GR, rat glucocorticoid receptor. HPT hygromycinR, hygromycin resistance conferred by hygromycin phosphotransferase. HA, hemagglutinin tag. RbcS, RuBisCO small submit.

Table S2. RT-PCR primers and conditions.

List of all the primers and amplification conditions used for the RT-PCR-based gene expression analyses. All primers are written 5' to 3'.

Gene	**Forward primer**	**Reverse primer**	**T_a (°C)**	**Extension (seconds)**	**# cycles**	**Source**
PR1	AGGCAACTGCAGACTCATACAC	TCGCTAACCCACATGTTCAC	60	10	35	Tenai Eguen
SGR	ACTACCTGTGGTGTTGAAGG	CGACTTTGTTGAACTCATTGAC	55	10	34	Mecey et al., 2011
actin (ACT2)	GTCGTACAACCGGTATTGTGC	CACATCACACTTCATGATTGAG	56	30	27-30	Chris van Schie
ERF13	CGGAACCGTCAGTCTCCGAT	ACCGTGAAATCCAACTCCGGT	60	10	35	Chris van Schie
COR15A	CTCTCATGGCGATGTCTTTCTCAG	TTACCCTCCGCGAACTCTGCCG	55	35		Lee et al., 2010
PDF1.2a	TTTGCTGCTTTCGACGCAC	CGCAAACCCCTGACCATG	60	10	35	Walley et al., 2008
Chi-B	ATCAGCGCTGCAAAGTCCTTC	GTGCTGTAGCCCATCCACCTG	60	10	35	Oñate-Sánchez et al., 2007

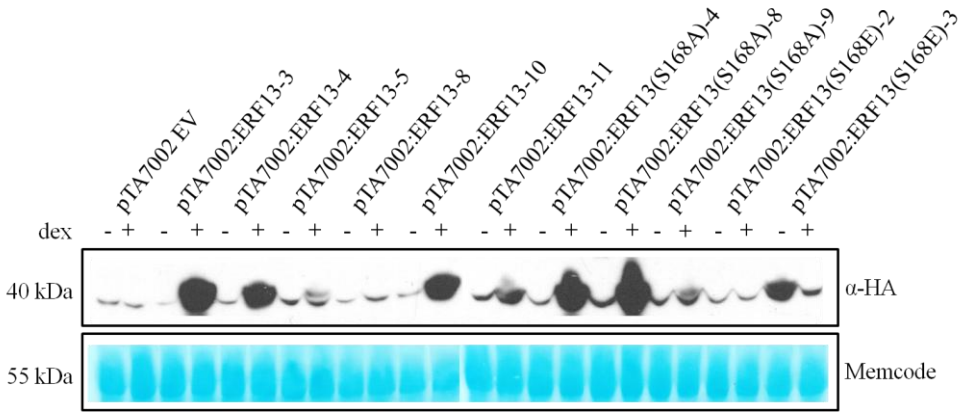

Figure S3. Dexamethasone-inducible expression of ERF13.

Dex-inducible *ERF13*-overexpressing plants were treated with 0.02% Silwet ± 50 μM dex and sampled after 48 hours. ERF13-HA levels were measured by Western blot (probed with mouse anti-HA antibody). Equal loading was verified by Memcode reversible protein stain (Pierce) of the RuBisCO large subunit (~55 kDa). All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). EV, empty vector.

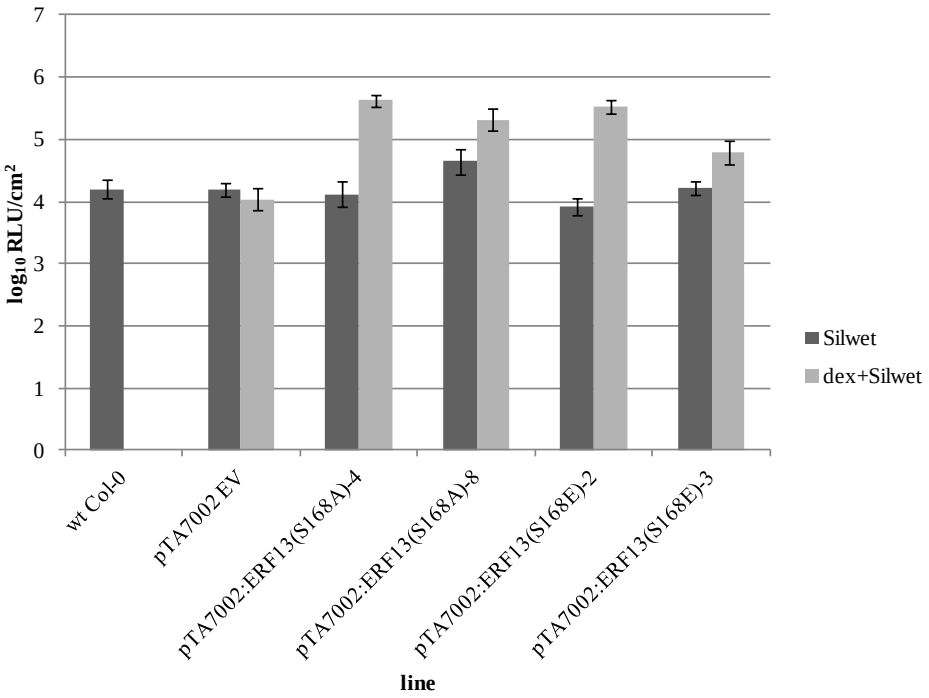

Figure S4. pTA7002:ERF13 phospho-dead and –mimetic lines display increased susceptibility to *Pst* DC3000 *(lux, AvrRpm1)* relative to wt Col-0 and pTA7002 empty vector plants only when treated with dexamethasone.

6-week-old T2 dex-inducible phospho-dead (S168A) and phospho-mimetic (S168E) *ERF13*-overexpressing plants were sprayed with 0.02% Silwet ± 50 μM dex and vacuum-inoculated with OD_{600} 0.0002 ($9.25x10^4$ cfu/mL) *Pst* DC3000 *(lux, AvrRpm1)* 24 hours later. Plants were sprayed again with Silwet ± dex 48 hours post-inoculation. Bacterial growth was measured by luminescence detector 3 days post-inoculation. Results shown are the mean log_{10} relative light units (RLU) per cm^2 leaf tissue; error bars represent SEM, n=8 (n=7 for wt Col-0 and Silwet-treated pTA7002:ERF13(S168A)-8). Wt, wild-type. EV, empty vector.

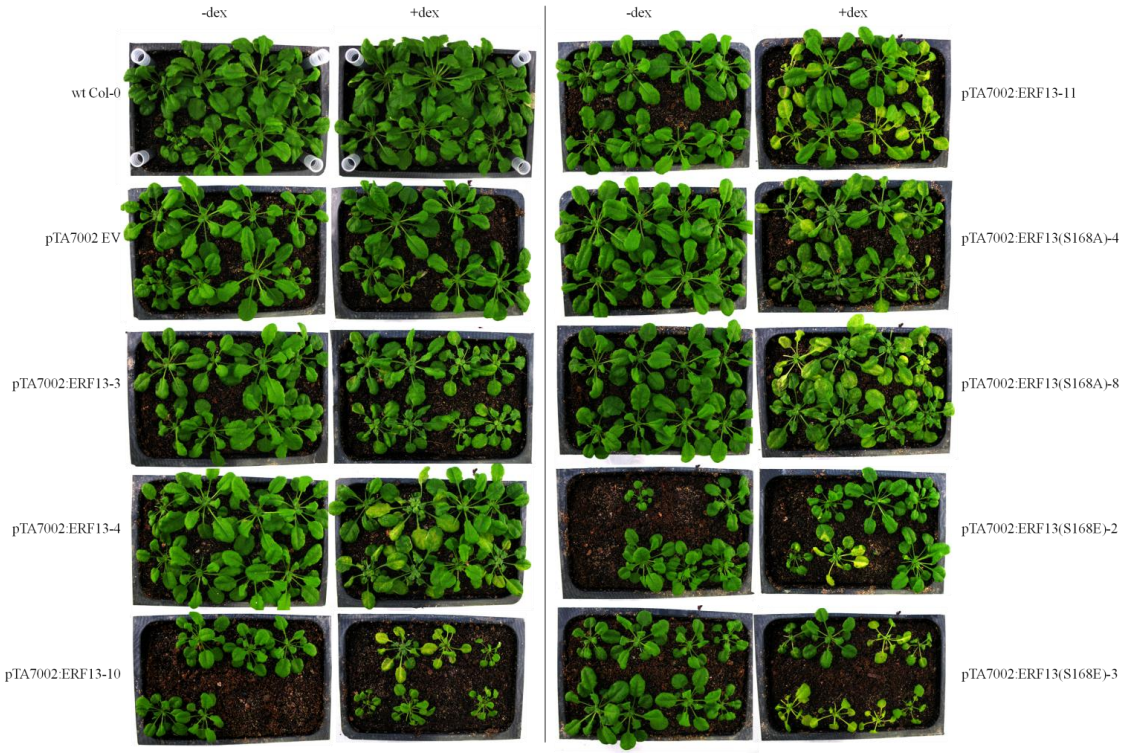

Figure S5. *ERF13* overexpression causes chlorosis in a dexamethasone-dependent manner.

5-week-old wild-type Columbia-0 and *ERF13*-overexpressing plants were sprayed with 0.02% Silwet ± 50 μM dex every 48 hours for 4 days. Plants were photographed on day 6. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). EV, empty vector.

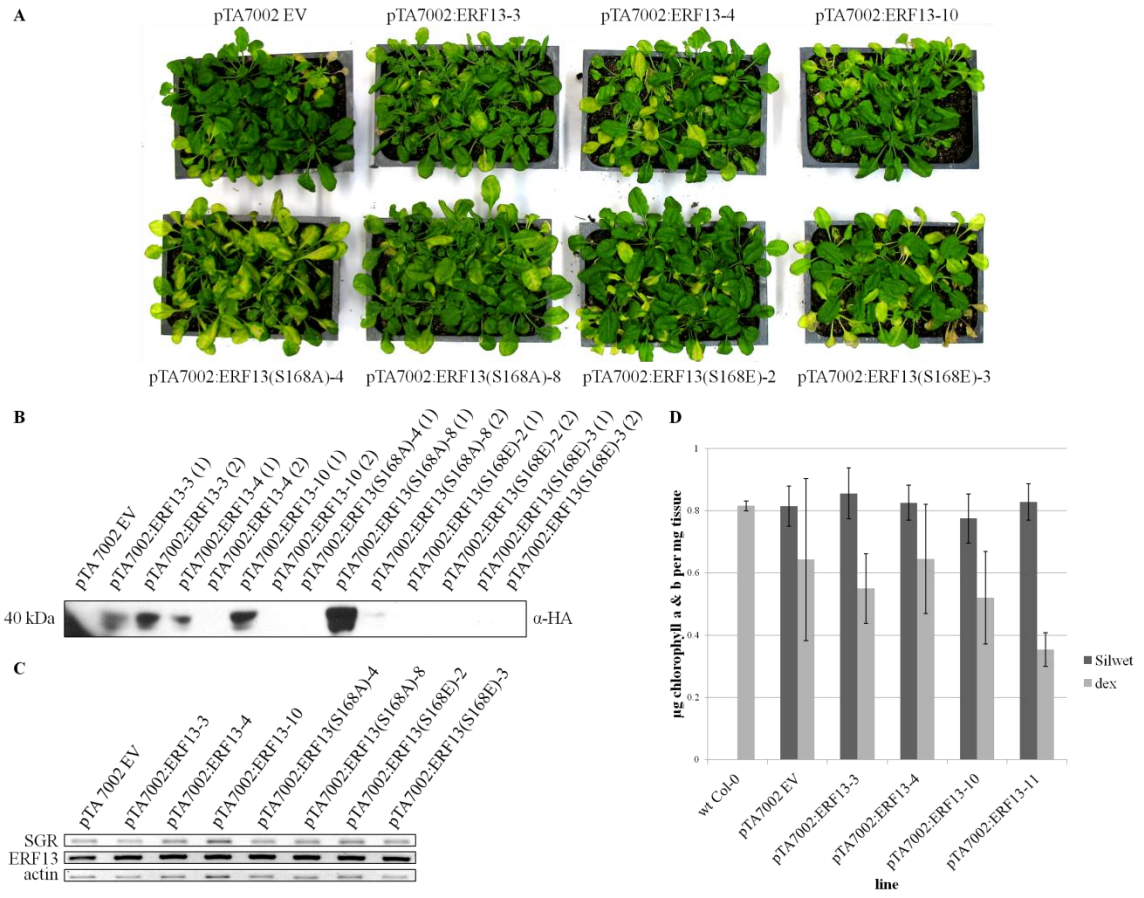

Figure S6. Overexpression of ERF13 causes chlorosis.

5-week-old *ERF13*-overexpressing plants were sprayed with 50 µM dex + 0.02% Silwet every 48 hours for 6 days. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). wt, wild-type. EV, empty vector. **(A)** Pots were photographed on day 8. Similar results were obtained in two other independent experiments. **(B)** Western blot of leaf tissue sampled on day 8, probed with mouse anti-HA antibody, to detect ERF13-HA levels. (1), protein sample extracted from chlorotic leaves. (2), sample from green leaves. **(C)** RT-PCR of *SGR* and *ERF13*, sampled 48 hours after dex treatment. Actin *(ACT2)* was amplified as a constitutive expression control.

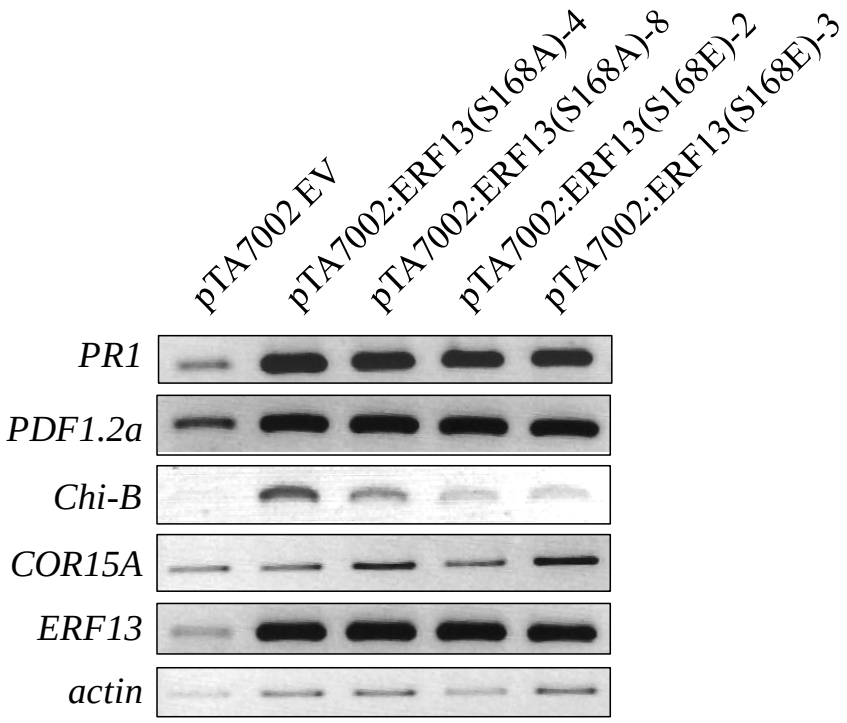

Figure S7. ERF13 may induce PR1, PDF1.2a, and Chi-B.

6-week-old *ERF13*-overexpressing plants were treated with 50 μM dex and sampled after 48 hours. Semi-quantitative RT-PCR was conducted, using actin *(ACT2)* as a control. All lines were from the T2 generation except pTA7002:ERF13(S168E)-3 (T3, homozygous). This experiment was conducted only with the phospho-dead and –mimetic lines. EV, empty vector.

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