The Combined Action of ENHANCED DISEASE SUSCEPTIBILITY1, PHYTOALEXIN DEFICIENT4, and SENESCENCE-ASSOCIATED101 Promotes Salicylic Acid-Mediated Defenses to Limit *Fusarium graminearum* Infection in *Arabidopsis thaliana*

Ragiba Makandar,^{1,2} Vamsi J. Nalam,^{1,3} Zulkarnain Chowdhury,¹ Sujon Sarowar,¹ Guy Klossner,¹ Hyeonju Lee,⁴ Dehlia Burdan,⁴ Harold N. Trick,⁴ Enrico Gobbato,⁵ Jane E. Parker,⁵ and Jyoti Shah¹

¹Department of Biological Sciences, University of North Texas, Denton, TX 76203, U.S.A.; ²Department of Plant Sciences, University of Hyderabad, Gachibowli, Hyderabad, India; ³Department of Biology, Indiana University-Purdue University, Fort Wayne, IN 46805, U.S.A.; ⁴Department of Plant Pathology, Kansas State University, Manhattan, KS 66506, U.S.A.; ⁵Max-Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, Carl von Linné Weg 10, 50829 Cologne, Germany

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Fusarium graminearum causes *Fusarium* head blight (FHB) disease in wheat and other cereals. *F. graminearum* also causes disease in *Arabidopsis thaliana*. In both *Arabidopsis* and wheat, *F. graminearum* infection is limited by salicylic acid (SA) signaling. Here, we show that, in *Arabidopsis*, the defense regulator EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) and its interacting partners, PAD4 (PHYTOALEXIN-DEFICIENT4) and SAG101 (SENESCENCE-ASSOCIATED GENE101), promote SA accumulation to curtail *F. graminearum* infection. Characterization of plants expressing the PAD4 noninteracting *eds1*^{L262P} indicated that interaction between EDS1 and PAD4 is critical for limiting *F. graminearum* infection. A conserved serine in the predicted acyl hydrolase catalytic triad of PAD4, which is not required for defense against bacterial and oomycete pathogens, is necessary for limiting *F. graminearum* infection. These results suggest a molecular configuration of PAD4 in *Arabidopsis* defense against *F. graminearum* that is different from its defense contribution against other pathogens. We further show that constitutive expression of *Arabidopsis* PAD4 can enhance FHB resistance in *Arabidopsis* and wheat. Taken together with previous studies of wheat and *Arabidopsis* expressing salicylate hydroxylase or the SA-response regulator NPR1 (NON-EXPRESSION OF PR GENES1), our results show that exploring fundamental processes in a model plant provides

important leads to manipulating crops for improved disease resistance.

The genus *Fusarium* includes several important fungal pathogens of plants. *Fusarium graminearum* is a devastating pathogen with a hemibiotrophic lifestyle that causes seedling blight, root rot, and *Fusarium* head blight (FHB) or scab disease in small grains such as wheat (*Triticum aestivum*) (Bai and Shaner 2004; Parry et al. 1995; Walter et al. 2010; Xu and Nicholson 2009). Infection initiates in the floral tissues at anthesis, with stomates providing a potential entry point. Hydrolytic enzymes secreted by the fungus also likely facilitate direct penetration of the host surface by the fungus (Walter et al. 2010). During the early stages of infection, *F. graminearum* exhibits a biotrophic phase in which fungal growth is observed in the extracellular spaces (Goswami and Kistler 2004). Subsequently, it enters an aggressive necrotrophic stage that is accompanied by increased fungal colonization. The fungus moves from the infected spikelet to other spikelets within the spike. Disease symptoms include bleaching and necrosis. Grain yield losses result from floret sterility and shriveled and lightweight kernels. In addition, mycotoxins that accumulate in the infected grain further reduce acceptability of the grain for human consumption or in animal feed. Annual losses due to FHB in the United States alone have approached \$3 billion (Johnson et al. 2003; Nganje et al. 2004; Windels 2000). In the absence of a known source of monogenic resistance against FHB, current control measures include deployment of partially resistant varieties, along with crop rotation and fungicide applications. Despite the importance of this pathogen, current understanding of the one or more signaling mechanisms engaged by the plant to naturally restrict *F. graminearum* infection is extremely limited.

In the laboratory, *F. graminearum* and the related species *F. culmorum* can colonize inflorescences of *Arabidopsis thaliana* (Cuzick et al. 2008; Makandar et al. 2010; Urban et al. 2002; Van Hemelrijck et al. 2006). In addition, *F. graminearum* can also colonize *Arabidopsis* leaves (Chen et al. 2006, 2009; Makandar et al. 2006, 2010; Savitch et al. 2007; Skadsen and

R. Makandar and V. J. Nalam contributed equally to this work.

Arabidopsis sequence data is available under the following accession numbers: *ACT8* (At1g49240), *EDS1* (At3g48090), *EF1α* (At5g60390), *PAD4* (At3g52430), *PR1* (At2g14610), *RAR1* (At5g51700), *SAG101* (At5g14930), *SID2* (At1g74710).

Corresponding Author: J. Shah; E-mail: Shah@unt.edu; Telephone: +1.940.565.3535; Fax: +1.940.565.4136.

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Hohn 2004; Van Hemelrijck et al. 2006). Efficiency of leaf colonization was enhanced by infiltrating the fungus into *Arabidopsis* leaves (Makandar et al. 2006, 2010; Savitch et al. 2007), thereby presumably by-passing leaf surface barriers. A compatible interaction between *Arabidopsis* and *F. graminearum* has been utilized to identify host genes and signaling mechanisms contributing to defense or, alternatively, promoting infection (Chen et al. 2009; Makandar et al. 2006, 2010; Urban et al. 2002). Salicylic acid (SA) signaling was found to be critical for controlling *F. graminearum* infection in leaves and floral tissues of *Arabidopsis* (Makandar et al. 2010). Resistance to *F. graminearum* was associated with increased SA content and expression of the SA-inducible *PR1* (*PATHOGENESIS-RELATED 1*) gene. Furthermore, *F. graminearum* disease severity was higher in the *sid2* (*salicylic acid induction deficient2*) mutant lacking an isochorismate synthase (ICS1) activity required for SA biosynthesis and in transgenic plants expressing the *Pseudomonas putida NahG* gene-encoded salicylate hydroxylase, which blocks SA accumulation by catalyzing its conversion to catechol (Makandar et al. 2010; Savitch et al. 2007). *F. graminearum* disease severity was also higher in the *npr1* (*nonexpresser of PR genes1*) mutant (Makandar et al. 2010), which lacks an active SA-response regulator (Fu and Dong 2013). SA promotes the translocation of NPR1 from the cytosol into the nucleus and also facilitates NPR1 turnover, which is critical for its activity as a transcription coactivator in SA signaling (Fu et al. 2012; Spoel et al. 2009). In agreement with a role for *NPR1* in curtailing disease severity, overexpression of *NPR1* conferred enhanced resistance against *F. graminearum* in *Arabidopsis* (Makandar et al. 2006, 2010).

SA also contributes to the response of wheat spikelets to *F. graminearum* infection. Expression of SA-responsive genes was upregulated in fungus-inoculated spikelets (Ding et al. 2011; Li and Yen 2008; Makandar et al. 2006, 2012). Also, a soil-drench application of SA enhanced FHB resistance in wheat (Makandar et al. 2012). Moreover, overexpression of the *Arabidopsis NPR1* gene in transgenic NPR1-OE (overexpressing) wheat plants enhanced FHB resistance by sensitizing wheat to SA (Makandar et al. 2006, 2012). Compared with nontransgenic wheat, SA-induced expression of the *PR1* gene was faster in NPR1-OE wheat plants. *PR1* expression was also more rapid and stronger in fungus-infected NPR1-OE than in nontransgenic plants (Makandar et al. 2006, 2012). *NahG* expression compromised basal resistance as well as the *NPR1* overexpression-conferred resistance to FHB (Makandar et al. 2012). Notably, FHB resistance in winter wheat correlated with allelic variants of *NPR1*-like genes (Diethelm et al. 2014), underscoring the importance of SA-NPR1 signaling in wheat defense against FHB.

The fact that *NPR1* overexpression sensitizes wheat plants to respond faster and stronger to SA, thereby reducing FHB disease severity, suggests that the timely activation of SA accumulation and signaling is a crucial factor determining the ability of wheat to limit *F. graminearum* infection. Indeed, it was shown previously that, compared with the wheat cv. Bobwhite, SA accumulation was higher and upregulation of *PR1* expression faster and stronger in *F. graminearum*-inoculated spikelets of the more FHB-resistant wheat cv. Sumai-3 (Makandar et al. 2012). Similarly, Ding et al. (2011) observed that SA levels peaked more rapidly and with higher amounts in the FHB-resistant wheat variety Wangshuibai compared with the relatively FHB-susceptible mutant Meh0106. Therefore, we postulated that, in addition to improving sensitivity of wheat to SA, FHB resistance might be enhanced by improving the ability of wheat to accumulate SA. In order to effectively target the SA defense pathway for enhancing plant resistance to *F. graminearum*, it is important to understand the one or more signaling mechanisms underlying the promotion of SA accumulation in response to *F. graminearum* infection.

In *Arabidopsis* basal resistance against virulent strains of *Pseudomonas syringae* bacteria or the oomycete pathogen *Hyaloperonospora arabidopsidis*, and SA accumulation is potentiated by the lipase-like defense regulator PHYTOALEXIN DEFICIENT4 (PAD4) (Feys et al. 2001). PAD4 is stabilized by its interaction with a key immune regulator, EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1) (Feys et al. 2001, 2005; Rietz et al. 2011; Wagner et al. 2013) and an EDS1-PAD4 complex is required for PAD4 to feedback positively on its own expression and to potentiate SA accumulation in pathogen-infected tissues (Rietz et al. 2011). Also, EDS1 and PAD4 are important regulators of effector-triggered immunity mediated by resistance (*R*) genes belonging to a large class of intracellular toll interleukin-1 receptor-nucleotide binding-leucine-rich repeat (TNL) proteins (Feys et al. 2001, 2005; Rietz et al. 2011). Notably, EDS1 was found to reside in complexes with several TNL proteins, suggesting that EDS1 molecularly connects TNL effector recognition to downstream defense signaling (Bhattacharjee et al. 2011; Heidrich et al. 2011; Kim et al. 2012). *Arabidopsis* EDS1 forms complexes with another lipase-like protein, SAG101 (SENESCENCE-ASSOCIATED GENE101), but in basal and TNL-conferred resistance against *P. syringae* and *H. arabidopsidis*, loss of *SAG101* was largely compensated for by *PAD4* (Feys et al. 2005; Rietz et al. 2011). A contribution of *SAG101* to basal and TNL immunity was only revealed in a *pad4 sag101* double mutant in which resistance was completely abolished (Feys et al. 2005).

The coiled-coil nucleotide binding-leucine-rich repeat (CNL) proteins are another large class of *R* proteins involved in effector-triggered immunity against pathogens. Although in general, *EDS1* is not required for immunity conferred by the CNL proteins, there are a few exceptions. For example, *EDS1* was required for the *Turnip crinkle virus* (TCV) coat protein-elicited resistance conferred by *HRT*, which encodes a CNL protein (Chandra-Shekara et al. 2004). However, *EDS1* and SA were found to have some redundant functions in *HRT*-conferred resistance against TCV (Venugopal et al. 2009). Similarly, *EDS1* was required for resistance against powdery mildew conferred by RPW8, an atypical CNL protein (Xiao et al. 2005), and for disease resistance conferred by ADR1, a noncanonical CNL protein (Roberts et al. 2013).

Here, we show that *Arabidopsis EDS1*, *PAD4*, and *SAG101* promote SA accumulation in response to *F. graminearum* infection and are required for controlling severity of *F. graminearum* infection. Our results show that EDS1-PAD4 interaction as well as a conserved lipase-site catalytic serine at position 118 in *PAD4* are necessary for defense against *F. graminearum*. Also, constitutive expression of *PAD4* in *Arabidopsis* and wheat enhanced SA accumulation, which was accompanied by stronger resistance to *F. graminearum*. Therefore, improving the kinetics of SA accumulation in wheat is a potential strategy for enhancing FHB resistance.

RESULTS

Both *EDS1* and *PAD4* are necessary for controlling *Fusarium graminearum* infection in *Arabidopsis*.

To determine if one or both *EDS1*- or *PAD4*-dependent mechanisms have a role in *Arabidopsis* response to *F. graminearum*, we first monitored *EDS1* and *PAD4* expression in *Arabidopsis* leaves inoculated with *F. graminearum*. *EDS1* and *PAD4* transcript levels were elevated in *F. graminearum*-inoculated leaves compared with mock-inoculated leaves of wild-type (WT) plants (Fig. 1). To establish if *EDS1* and *PAD4* have a role in limiting *F. graminearum* infection, we monitored *F. graminearum* disease severity in leaves of the *eds1-1* and *pad4-5* null mutants, which are in the accession Wassilewskija (Ws) and the *pad4-1* mutant, which is in the accession Columbia (Col). Disease severity was

also monitored in the *eds1-1 pad4-5* double mutant and was significantly higher on leaves of the *eds1* and *pad4* single and double mutant plants compared with the corresponding WT (Fig. 2A). *EDS1* and *PAD4* were also required for controlling *F. graminearum* disease severity in floral tissues. As shown in Tables 1 and 2, the *Fusarium-Arabidopsis* disease (FAD) score was higher on *eds1-1*, *pad4-1*, and *pad4-5* mutants and the *eds1-1 pad4-5* double mutant compared with the respective WT plants. By contrast, in comparison with the WT accession Ws, disease severity in leaves and inflorescence tissues was lower in the 35S:*PAD4* transgenic line in which *PAD4* was constitutively expressed from the *Caulliflower mosaic virus* 35S promoter (Fig. 2B; Table 1). Taken together, these results suggest that an *EDS1* and *PAD4*-dependent mechanism is critical for controlling *F. graminearum* infection in *Arabidopsis*.

As mentioned earlier, *EDS1* and *PAD4* are involved in R gene-mediated effector-triggered immunity. Defense signaling mediated by various R proteins is dependent on a chaperone complex involving RAR1 (REQUIRED FOR Mla12 RESISTANCE) that is involved in stabilizing and maintaining R proteins in a signaling-competent state (Kadota et al. 2010; Seo et al. 2008). Like *EDS1* and *PAD4*, *RAR1* expression is upregulated in response to *F. graminearum* infection (Supplementary Fig. 1). However, compared with the higher level of disease in the *eds1* and *pad4* mutants, disease severity was not significantly different between the *rar1-1* mutant and WT accession Ws plants (Fig. 2C; Table 1), indicating that *RAR1* is genetically dispensable for this *EDS1*/*PAD4*-dependent defense mechanism against *F. graminearum* infection of *Arabidopsis*.

***EDS1* and *PAD4* promote SA accumulation in response to *Fusarium graminearum* infection of *Arabidopsis*.**

An *EDS1*-*PAD4* complex potentiates SA accumulation during infection by bacterial and oomycete pathogens (Rietz et al. 2011). To determine if *EDS1* and *PAD4* are similarly involved in potentiating SA accumulation in response to *F. graminearum* infection of *Arabidopsis*, we monitored the accumulation of SA in *F. graminearum*-inoculated and as control, in mock-inoculated leaves of the Ws *eds1-1* and *pad4-5* null mutant plants and the *eds1-1 pad4-5* double mutant. SA content was comparable between the mock-inoculated WT and mutant plants (Fig. 3). As previously reported (Makandar et al. 2010), in WT plants *F. graminearum* inoculation resulted in significant increase in SA content compared with mock-inoculated plants (Fig. 3). By comparison, SA content in *F. graminearum*-inoculated leaves of the *eds1-1*, *pad4-5* and *eds1-1 pad4-5* mutant plants, which are in the accession Ws, and the *pad4-1* mutant in accession Columbia (Col), were significantly lower than that in *F. graminearum*-inoculated WT accession Ws and Col plants, respectively (Fig. 3), thus underscoring a requirement for both *EDS1* and *PAD4* in promoting SA accumulation in response to *F. graminearum* infection. *PR1* transcript accumulation was also weaker in *F. graminearum*-inoculated leaves of the *eds1-1*, *pad4-1*, *pad4-5*, and *eds1-1 pad4-5* mutants compared with the respective WT plants (Fig. 4A), thus indicating weaker activation of SA-mediated defense response in *F. graminearum*-inoculated mutants. In contrast, constitutive expression of *PAD4* resulted in higher basal as well as *F. graminearum* infection-induced accumulation of SA and *PR1* transcript in the 35S:*PAD4* compared with the WT plant (Figs. 3 and 4B), thus further highlighting the importance of a *PAD4*-dependent mechanism in promoting SA accumulation and signaling in response to *F. graminearum* infection.

In the *Arabidopsis*-*F. graminearum* pathosystem, a requirement of *EDS1* for promoting SA accumulation (Fig. 3) points to a function of *EDS1* upstream of SA. However, previously genetic redundancy between *SID2*-dependent SA and *EDS1* was

observed in the *Arabidopsis* *HRT*-mediated resistance to TCV (Venugopal et al. 2009). Therefore, the relationship between *EDS1* and SA was further tested by comparing *F. graminearum* disease severity between the *eds1-1*, *sid2-1*, and the *eds1-1 sid2-1* double mutant plants. Disease severity in *Arabidopsis* leaves was not significantly different between *sid2-1* and the *eds1-1 sid2-1* double mutant plants but was higher in *eds1-1 sid2-1* compared with *eds1-1* (Fig. 5). By comparison, in the floral tissues, disease severity was 10 to 20% higher in the *eds1-1 sid2-1* double mutant compared with the *eds1-1* and *sid2-1* single mutants (Table 1). Since, *eds1-1* and *sid2-1* are in two different accessions, we also tested disease severity between the *eds1-22*, *sid2-1*, and *eds1-22 sid2-1* plants, which are all in the accession Col. Disease severity was comparably high in the leaves of *eds1-22*, *sid2-1*, and *eds1-22 sid2-1* plants (Fig. 5) and 15% higher in floral tissues of the *eds1-22 sid2-1*, compared with the single mutant plants (Table 1). These results suggest that although *EDS1* is required for promoting SA accumulation in response to *F. graminearum* infection, some redundancy is prevalent between *EDS1* and *SID2* functions in *Arabidopsis* defense against *F. graminearum*.

***EDS1* interaction with *PAD4* is required for controlling *F. graminearum* infection.**

Although constitutive expression of *PAD4* from the 35S promoter resulted in enhanced *F. graminearum* resistance in leaves and floral tissues of *Arabidopsis pad4-5 35S:PAD4* plants, we found that 35S:*PAD4* was unable to confer enhanced resistance to *F. graminearum* infection in leaves and floral tissues of the *eds1-1 pad4-5 35S:PAD4* plants (Fig. 2B; Table 1). This shows that *EDS1* is required for the constitutive *PAD4* expression-conferred enhanced resistance to *F. graminearum*. *PAD4* and *EDS1* physically interact in yeast and in planta and this enhances *PAD4* steady state accumulation (Feys et al. 2001, 2005;

Fig. 1. *EDS1* and *PAD4* expression is upregulated in *Arabidopsis* leaves inoculated with *Fusarium graminearum*. Quantitative real-time polymerase chain reaction was performed on RNA extracted from leaves of mock- (white bars) and *F. graminearum*-inoculated (black bars) leaves of wild-type accession Ws plants harvested at 0, 6, and 24 h postinoculation (hpi Fg). Gene expression was quantified relative to *ACT8*. Values are means $\pm$ SE ($n = 3$). Asterisks indicate values that are significantly different from the other treatments ($P < 0.05$).

Rietz et al. 2011). Moreover, interaction between PAD4 and EDS1 is necessary for basal resistance against virulent strains of *P. syringae* pv. *tomato* DC3000 and *H. arabidopsidis* (Rietz et al. 2011). Preventing PAD4-EDS1 association by a missense mutation (*eds1*^{L262P}) that results in a leucine to proline exchange at amino acid 262 in the corresponding EDS1 protein, strongly attenuated basal immunity against these pathogens (Rietz et al. 2011). *P. syringae* pv. *tomato* DC3000-induced PAD4 expression and SA accumulation were also lower in *eds1-1 eds1*^{L262P} transgenic lines in which *eds1*^{L262P} protein is expressed from the native *EDS1* promoter in the *eds1-1* mutant background (Rietz et al. 2011). To test whether interaction

between PAD4 and EDS1 is critical for limiting *F. graminearum* infection, we measured *F. graminearum* disease severity in the *eds1-1 eds1*^{L262P} transgenic lines. Disease was higher in leaves and inflorescences of the *eds1-1* null mutant and two independent transgenic lines expressing *eds1*^{L262P} (Rietz et al. 2011, lines 1 and 2) compared with WT (Fig. 6; Table 1). Taken together with the inability of constitutively expressed 35S:PAD4 to enhance resistance against *F. graminearum* in the absence of *EDS1*, these results show that EDS1 molecular interaction with PAD4 in a complex is necessary for controlling *F. graminearum* disease severity.

PAD4 catalytic residue S118 is important for restriction of *F. graminearum* infection.

The N-terminal domains of PAD4 and EDS1 have the structural topology of eukaryotic α/β -fold acyl hydrolase enzymes with the catalytic triad residues—a serine, an aspartate and a histidine (Brady et al. 1990; Wagner et al. 2013). Although the catalytic residues are evolutionarily conserved in PAD4 and EDS1 orthologs across the angiosperm plant

Fig. 2. *EDS1*, *PAD4*, and *SAG101* are required for controlling *Fusarium graminearum* disease in leaves of *Arabidopsis*. *F. graminearum* disease severity in *Arabidopsis* leaves. Disease severity was recorded at 4 days post-inoculation in leaves of the indicated genotypes. The percentage of inoculated leaves exhibiting chlorosis covering <25%, 25 to 50%, 50 to 75%, and >75% of the leaf area was determined for each genotype. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other ($n = 60$). **A**, *EDS1*, *PAD4*, and *SAG101* are required for limiting *F. graminearum* infection. Top panel, disease severity in wild-type (WT) accession Ws, *eds1-1*, *pad4-5*, and *eds1-1 pad4-5* mutants. Lower panel, disease severity in leaves of WT accession Col, *pad4-1*, *sag101-1*, and *sag101-1 pad4-1* mutants. **B**, *EDS1* is required for the enhanced resistance against *F. graminearum* conferred by constitutive expression of *PAD4*. Disease severity in WT (Ws), *pad4-5*, *eds1-1*, *eds1-1 pad4-5*, *pad4-5* 35S:PAD4, and *eds1-1 pad4-5* 35S:PAD4 plants. **C**, *RAR1* is not required for limiting *F. graminearum* infection. Disease severity in WT (Ws) and the *rar1-1* mutant. Different letters next to the bars indicate disease ratings that are significantly different ($P < 0.05$) from each other ($n = 60$).

Table 1. *EDS1* and *PAD4* are required for limiting *Fusarium graminearum* disease severity on *Arabidopsis* inflorescence

Genotype ^a	FAD ^b
Wild type (WT) (Ws)	5.0 ± 0.3
<i>pad4-5</i>	10.3 ± 0.7
<i>eds1-1</i>	10.2 ± 0.8
<i>eds1-1 pad4-5</i>	12.2 ± 0.1
<i>pad4-5</i> (35S:PAD4)	1.7 ± 0.1
<i>eds1-1 pad4-5</i> (35S:PAD4)	10.4 ± 0.4
<i>eds1-1</i> (<i>eds1</i> ^{L262P})	9.6 ± 0.5
<i>pad4-5</i> (PAD4)	10.0 ± 0.1
<i>pad4-5</i> (<i>pad4</i> ^{S118A})	5.4 ± 0.1
	5.8 ± 0.5
	10.0 ± 0.2
	10.2 ± 0.3
<i>rar1</i>	5.1 ± 0.1
<i>sid2-1</i>	9.8 ± 0.7
<i>eds1-1 sid2-1</i>	11.8 ± 0.2
<i>eds1-22</i>	9.9 ± 0.2
<i>eds1-22 sid2-1</i>	11.3 ± 0.1
WT (Col)	4.7 ± 0.3

^a *sid2-1* and *eds1-22* are in the Col background. All other single mutants and transgenics are in the Ws background.

^b *Fusarium-Arabidopsis* disease (FAD) value, which combines the disease severity on flowers and siliques, was evaluated at 7 days postinoculation (Urban et al. 2002). Genotypes with FAD values ≤3 are considered highly resistant to *Fusarium graminearum* and those with FAD values ≥9 are considered highly susceptible to *Fusarium graminearum*.

lineages, they were found to be dispensable for basal disease resistance to *P. syringae* or *H. arabidopsidis* in *Arabidopsis* (Louis et al. 2012; Wagner et al. 2013). However, triad residue serine 118 in PAD4 was required for some of the PAD4-dependent defense activities against the green peach aphid (*Myzus persicae* Sülzer), a phloem sap-consuming insect (Louis et al. 2012). EDS1 and SA were not required for the PAD4-dependent resistance against the green peach aphid (Louis et al. 2012). We tested whether PAD4 serine 118 is needed for limiting *F. graminearum* infection by inoculating plants of two independent *pPAD4:pad4*^{S118A} lines (Louis et al. 2012) that express a mutant version of PAD4 in which serine 118 is exchanged by alanine. *F. graminearum* disease severity was higher on plants expressing *pad4*^{S118A} compared with control transgenic lines expressing the WT PAD4 or the original WT accession Ws (Fig. 7; Table 1). Since *pad4*^{S118A} in these lines is expressed at a similar level as WT PAD4 (Louis et al. 2012), we reasoned that the defect is unlikely to be due to lower *pad4*^{S118A} accumulation. Rather, the PAD4 molecular arrangement with EDS1 in heterocomplexes required for controlling *F. graminearum* infection appears to be different

from that mediating basal resistance against *P. syringae* or *H. arabidopsidis*.

SAG101 contributes to SA accumulation and limiting *F. graminearum* infection in *Arabidopsis*.

EDS1 can also form heterodimers with SAG101, which might molecularly compete with PAD4, since their heterodimer interfaces and main contact sites are similar (Feys et al. 2005; Wagner et al. 2013; Xing and Chen 2006). Notably, while loss-of-function mutations in *SAG101* are largely compensated for by *PAD4* in resistance to *P. syringae* or *H. arabidopsidis* (Feys et al. 2005; Wagner et al. 2013), a clear nonredundant *SAG101* action was shown to be required for the full extent of HRT-mediated SA accumulation in TCV-inoculated *Arabidopsis* plants (Zhu et al. 2011). We therefore tested whether *SAG101* is also necessary for limiting *Arabidopsis F. graminearum* infection. *F. graminearum* disease severity was as high in leaves and floral tissues of the *sag101-1* single mutant as *pad4-1* or *sag101-1 pad4-1* mutants (Fig. 2A; Table 2). As in the *eds1* and *pad4* mutant plants, fungal infection-induced accumulation of SA and *PR1* expression was also attenuated in *sag101-1* and *sag101-1 pad4-1* mutants compared with WT (Figs. 3 and 4A). These data show that *SAG101* contributes in a nonredundant

Table 2. *PAD4* and *SAG101* are required for limiting *Fusarium graminearum* disease severity on *Arabidopsis* inflorescence

Genotype ^a	FAD ^b
Wild type (Col)	5.7 ± 0.7
<i>pad4-1</i>	12.4 ± 0.9
<i>sag101-1</i>	15.3 ± 1.1
<i>pad4-1 sag101-1</i>	16.8 ± 2.7

^a The *pad4-1* and *sag101-1* mutant alleles are in the *Arabidopsis* accession Columbia (Col).

^b FAD = *Fusarium-Arabidopsis* disease.

Fig. 3. *EDS1*, *PAD4*, and *SAG101* promote salicylic acid (SA) accumulation in response to *Fusarium graminearum* infection. SA content in *Arabidopsis* leaves. Free SA content (micrograms per gram of fresh weight [FW] ± standard error; *n* = 3) was monitored in mock- and *F. graminearum* (*Fg*)-inoculated leaves of *Arabidopsis* genotypes harvested at 24 h post-inoculation. Top panel, free SA content in wild-type (WT) accession Ws, *eds1-1*, *pad4-5*, and *eds1-1 pad4-5* mutants and a transgenic line *35S:PAD4* in which *PAD4* is expressed from the *35S* promoter. Lower panel, free SA content in WT accession Col and the *pad4-1*, *sag101-1*, and *sag101-1 pad4-1* mutants. Different letters above bars indicate values that are significantly different from each other (*P* < 0.05).

Fig. 4. *EDS1*, *PAD4*, and *SAG101* promote salicylic acid (SA) signaling in response to *Fusarium graminearum* infection. **A**, Northern blot analysis of *PR1* expression in plants deficient in *EDS1*, *PAD4*, and *SAG101* function. RNA was isolated at 24 h postinoculation (hpi) from mock (M)- and *F. graminearum* (F)-inoculated leaves of the indicated genotypes. A ³²P-labeled *PR1* gene-specific probe was used to monitor *PR1* transcript accumulation, and ethidium bromide staining ensured quality and equal gel loading of RNA. **B**, Quantitative reverse transcription-polymerase chain reaction analysis of *PR1* expression in plants constitutively expressing *PAD4*. RNA was isolated at 24 hpi from mock- and *F. graminearum* (*Fg*)-inoculated leaves of wild-type and transgenic plants that express *PAD4* from the *35S* promoter. *PR1* expression was quantified relative to *EF1α*. Values are means ± standard error (*n* = 3). Different letters above bars indicate values that are significantly different from each other (*P* < 0.05).

manner with *PAD4* to *F. graminearum*-induced SA accumulation and to limiting *F. graminearum* infection in *Arabidopsis*.

Wheat plants expressing *Arabidopsis PAD4* display stronger SA accumulation and enhanced FHB resistance.

In spikes of the moderately *F. graminearum*-resistant wheat cv. Sumai-3, *F. graminearum* infection resulted in higher accumulation of SA than the *F. graminearum*-susceptible cv.

Fig. 5. *EDS1* and *SID2* function together to control *Fusarium graminearum* infection. Top panel, *F. graminearum* disease severity in wild-type (WT) accessions Ws and Col, *eds1-1*, *sid2-1*, and *eds1-1 sid2-1* mutants. Lower panel, *F. graminearum* disease severity in WT accession Col, *eds1-22*, *sid2-1*, and *eds1-22 sid2-1* mutants. Different letters next to the bars indicate disease ratings that are significantly different ($P < 0.05$) from each other ($n = 60$).

Fig. 6. Interaction between *PAD4* and *EDS1* is required for limiting *Fusarium graminearum* infection. *F. graminearum* disease severity in wild-type accession Ws, *eds1-1*, and *eds1-1* plants expressing the *eds1^{L262P}* mutant allele. Different letters next to the bars indicate disease ratings that are significantly different ($P < 0.05$) from each other ($n = 60$).

Bobwhite (Makandar et al. 2012), suggesting that the lack of robust SA accumulation may be one of the factors that limit induction of defenses controlling FHB in wheat. Indeed, SA treatment enhanced FHB resistance in cv. Bobwhite (Makandar et al. 2012). To determine whether increased ability to accumulate SA can enhance resistance to FHB, we generated transgenic 'Bobwhite' plants overexpressing the *Arabidopsis PAD4* coding sequence. The maize *Ubiquitin (Ubi)* gene promoter (Christensen and Quail 1996) was used to drive expression of *PAD4*, and two independent transgenic lines (610C and 882D) that constitutively express *PAD4* (Fig. 8A) were taken for further analysis. Constitutive expression of *PAD4* resulted in significantly higher basal and *F. graminearum* infection-induced accumulation of SA in the spikes of transgenic wheat lines 610C and 882D than in nontransgenic 'Bobwhite' plants (Fig. 8B). These results indicate that, like in *Arabidopsis*, constitutive *PAD4* expression can improve SA accumulation in wheat.

The higher accumulation of SA in the transgenic lines 610C and 882D was accompanied by enhanced resistance against *F. graminearum* (Fig. 8C). Compared with the nontransgenic 'Bobwhite' plants, FHB disease severity was significantly lower on spikes of 610C and 882D. These results reinforce the notion that improving the ability of wheat spikes to accumulate SA is a potentially attractive strategy for enhancing FHB resistance in wheat.

DISCUSSION

Here we demonstrate that *EDS1*, *PAD4*, and *SAG101* are necessary for promoting SA accumulation and signaling and that this promotes *Arabidopsis* defense against *F. graminearum*. Compared with WT plants, fungal infection-induced accumulation of SA and *PR1* expression was attenuated and *F. graminearum* disease was more severe in the *Arabidopsis eds1*, *pad4*, and *sag101* mutants (Figs. 2A and B, 3, and 4A). Conversely, constitutive expression of *PAD4* resulted in more robust SA accumulation and was accompanied by enhanced resistance against *F. graminearum* infection that was dependent also on *EDS1* (Figs. 2A and 3). The heightened disease severity in plants expressing *eds1^{L262P}* (Fig. 6), a stable *EDS1* protein variant that does not interact with *PAD4* (Rietz et al. 2011),

Fig. 7. Serine 118 is required for involvement of *PAD4* in defense against *Fusarium graminearum*. *F. graminearum* disease severity in wild-type (WT) Ws, *pad4-5*, and two independently derived transgenic lines each, for *Arabidopsis* expressing WT and *pad4^{S118A}* mutant versions of *PAD4* from the native *PAD4* promoter in the *pad4-5* genetic background. Different letters next to the bars indicate disease ratings that are significantly different ($P < 0.05$) from each other ($n = 60$).

suggests that formation of EDS1 and PAD4 heterocomplexes is critical for controlling *F. graminearum* infection. In addition, the catalytic Ser118 in PAD4 was required for defense against *F. graminearum* (Fig. 7). Association between EDS1 and PAD4 was previously shown to be necessary for SA accumulation and basal immunity to *P. syringae* and *H. arabidopsidis* but, surprisingly, not for TNL-mediated effector-triggered TNL immunity (Rietz et al. 2011). Similarly, PAD4 but not EDS1 was shown to be required for controlling green peach aphid infestation (Louis et al. 2012; Pegadaraju et al. 2007). As discussed below, increasing evidence points to the engagement of different molecular configurations of EDS1 and PAD4 in defense against different types of pests, including *F. graminearum*.

EDS1 also forms stable heterodimers with SAG101 (Rietz et al. 2011; Wagner et al. 2013) and *EDS1*, *PAD4*, and *SAG101* were all found to be required for the *HRT*-mediated induction of SA accumulation and immunity to the TCV (Chandra-Shekara et al. 2004; Venugopal et al. 2009; Zhu et al. 2011). It is likely that EDS1 with both PAD4 and SAG101 functions similarly as a resistance signaling node against *F. graminearum* infection. The absence of additive effects on *F. graminearum* disease severity in the *eds1-1 pad4-5* and *sag101-1 pad4-1* double mutants compared with the respective single mutants (Figs. 2A and 3; Tables 1 and 2), supports the notion that EDS1, PAD4, and SAG101 function in a nonredundant manner to promote SA synthesis via the isochlorismate pathway involving the *SID2*-encoded isochlorismate synthase (Wildermuth et al. 2001). It is possible that EDS1 forms a ternary complex containing both PAD4 and SAG101 as a single signaling unit, as suggested by Zhu et al. (2011), based on transient expression assays in *Nicotiana benthamiana*. Functional analysis of the crystal structure of an EDS1-SAG101 heterodimer and a derived EDS1-PAD4 structural model, indicate that a ternary arrangement with a 1:1:1 stoichiometry is highly improbable (Wagner et al. 2013). However, an oligomeric complex containing multiple EDS1 molecules with PAD4 and SAG101 cannot be excluded, since EDS1 is also able to self-associate (Wagner et al. 2013). Whatever the molecular arrangements of EDS1 with PAD4 and SAG101 in resistance to *F. graminearum*, it is clear that PAD4 and SAG101 have functions, together with EDS1, that are independent of each other.

Although the α/β hydrolase catalytic serine 118 (Ollis et al. 1992) is not required for PAD4 conditioned defense against *P. syringae* and *H. arabidopsidis* (Louis et al. 2012; Wagner et al. 2013), expression of a *pad4*^{S118A} variant in which serine at position 118 was replaced by alanine, was unable to restore resistance against *F. graminearum* in the *pad4-5* null mutant background (Fig. 7), indicating that serine 118 in PAD4 is necessary for limiting *F. graminearum* infection. While this shows that a catalytic mechanism might be needed for certain PAD4 actions, there is no evidence that PAD4 or EDS1 possesses lipase or acyl hydrolase enzymatic activity. Several plant hormone receptors utilize the α/β hydrolase topology to bind bioactive hormone molecules, which causes a conformational change in the protein that is essential for signal relay rather than catalytic activity (Guo et al. 2013; Janssen and Snowden 2012). A good example is the rice gibberellin receptor *GID1*, in which gibberellin binds to a modified catalytic triad, resulting in a conformational change in the protein, allowing it to connect with the downstream signaling machinery (Mura et al. 2008; Shimada et al. 2008). It is conceivable that PAD4 serine 118 also facilitates a conformational change in PAD4 that impacts defense activity in cells and tissues responding to *F. graminearum* infection. Serine 118 is also required for some of the PAD4 functions in *Arabidopsis* defense against the green peach aphid (Louis et al. 2012). However, PAD4 operates independently of EDS1 and SAG101 in *Arabidopsis* defense

against the green peach aphid (Louis et al. 2012; Pegadaraju et al. 2007). Thus, PAD4 has the capacity to adopt one or more varying states or activities in plant responses to different stresses. However, considering that PAD4 interaction with EDS1 is required for limiting *F. graminearum* infection (Fig. 6), it is highly improbable that, in the *Arabidopsis*-*F. graminearum* pathosystem, the requirement of serine 118 is associated with a PAD4 function that is independent of EDS1.

Fig. 8. Expression of *Arabidopsis* *PAD4* in wheat promotes higher salicylic acid (SA) accumulation in response to *Fusarium graminearum* infection and enhanced *Fusarium* head blight (FHB) resistance. **A**, Top panel: Polymerase chain reaction (PCR) analysis to confirm presence of the *Ubi:PAD4* transgene in transgenic wheat. PCR primers specific for *PAD4* were used on genomic DNA from two plants each of two independent transgenic lines, 610C and 882D. DNA extracted from the nontransgenic spring wheat cv. Bobwhite (Bw) provided the negative control for PCR. Lower panel, reverse transcription (RT)-PCR analysis of *PAD4* expression in leaves of the nontransgenic 'Bobwhite' and the *Ubi:PAD4* transgenic wheat (610C and 882D) plants. The wheat *Tubulin* (*TUB*) gene provided a positive control for RT-PCR signals. Cloned *PAD4* cDNA provided a positive control (C) for the PCR step. **B**, Free SA content (micrograms per gram of fresh weight [FW] $\pm$ standard error; $n = 3$) was monitored in mock (M)- and *F. graminearum* (F)-inoculated spikelets of 'Bobwhite' and the *Ubi:PAD4* transgenic lines 610C and 882D, harvested at 24 h postinoculation. Different letters above bars indicate values that are significantly different from each other ($P < 0.05$). **C**, FHB severity in wheat. FHB disease rating was monitored in nontransgenic wheat cv. Bobwhite (Bw) and the *Ubi:PAD4* transgenic wheat lines 610C and 882D. Disease incidence was recorded as the percentage of infected spikelets in each spike at 3, 9, 15, and 21 days postinoculation (dpi). A minimum of 20 plants per genotype were tested in each treatment. Different letters above bars indicate disease ratings at 21 dpi that are significantly different ($P < 0.05$) from each other.

Monocot plants contain orthologs of *EDS1* and *PAD4* (Wagner et al. 2013), suggesting that the EDS1-PAD4 basal immunity function is conserved across angiosperm lineages. Indeed, our results demonstrate that *PAD4* from *Arabidopsis*, a dicot, when constitutively expressed in wheat, a monocot, promotes SA accumulation and enhances disease resistance (Fig. 8B and C). However, monocots lack a *SAG101* homolog (Wagner et al. 2013). Therefore, we suggest that this *PAD4* effect in wheat operates independently of *SAG101*. Whether the resistance-promoting actions of *Arabidopsis PAD4* against *F. graminearum* infection require one or more wheat *EDS1* orthologs is not known. Besides its key function in basal resistance, EDS1-driven immune responses are also essential for effector-triggered immunity conferred by the TNL family of receptor proteins, which have expanded in eudicot plants (Yue et al. 2012). Our finding that *PAD4* operates with *EDS1* in promoting the SA pathway and limiting *F. graminearum* infection of *Arabidopsis* supports engagement of their basal resistance activities rather than TNL-mediated effector-triggered immunity. This prospect is recapitulated in wheat plants, which lack *TNL* genes (Yue et al. 2012). Monocots, however, contain genes encoding the CNL immune receptors (Jacob et al. 2013; Yue et al. 2012). Moreover, recognition of effectors by certain CNL receptors triggers immunity via EDS1 and SA-dependent mechanisms. For example, HRT-triggered immunity to TCV requires EDS1 and SA (Chandra-Shekara et al. 2004). EDS1 and SA are suggested to have redundant functions in the HRT-conferred resistance to TCV (Venugopal et al. 2009). EDS1 is also required for promoting SA accumulation and resistance conferred by ADR1, a noncanonical CNL (Roberts et al. 2013), as well as for resistance against powdery mildew conferred by the RPW8, an atypical CNL (Xiao et al. 2005). However, to date, an *R* gene-mediated effector-triggered immune mechanism that targets *F. graminearum* has not been demonstrated. Furthermore, RAR1, which is a component of a chaperone complex that has important functions in maintaining R proteins in a signaling-competent state (Kadota et al. 2010; Seo et al. 2008), was not required for limiting *F. graminearum* infection (Fig. 3C). Thus, *EDS1*, *PAD4*, and *SAG101* in *Arabidopsis* likely contribute to basal resistance against *F. graminearum*.

Constitutive expression of *PAD4* in *Arabidopsis* and wheat resulted in higher basal and fungal infection-induced accumulation of SA that was accompanied by enhanced resistance to *F. graminearum* (Figs. 2C, 3, and 8B and C). This increase in resistance in transgenic plants compared with nontransgenic plants could result from the higher accumulation of SA in response to *F. graminearum*-infection compared with the nontransgenic plants. It is equally plausible that the heightened resistance in the *PAD4*-expressing plants is due to the higher basal content of SA. In comparison with the transgenic *Arabidopsis*, in which *PAD4* was constitutively expressed, the enhancement of SA accumulation as well as disease resistance was weaker in the *PAD4* expressing wheat (Figs. 3 and 8B). These differences could be due to a variety of factors, including the fact that the strong 35S promoter compared with the maize *Ubi* promoter were used to drive *PAD4* expression in *Arabidopsis* and wheat, respectively. Differences in codon usage efficiency between *Arabidopsis* and wheat as well as potential differences in the strength of interaction between the *Arabidopsis PAD4* and EDS1 from *Arabidopsis* and wheat could also contribute to this difference in protection conferred by constitutive *PAD4* expression between *Arabidopsis* and wheat. Also, as noted above wheat lacks a *SAG101* homolog. Furthermore, whether wheat EDS1 is engaged in the *PAD4* expression-conferred resistance in wheat is not known.

In conclusion, we show that a resistance signaling mechanism requiring EDS1, *PAD4*, and *SAG101* promotes SA

accumulation in response to *F. graminearum* infection in *Arabidopsis*. Our observation that constitutive expression of *Arabidopsis PAD4* results in stronger SA accumulation in *Arabidopsis* and wheat, which is accompanied by heightened resistance against *F. graminearum* infection, suggests conservation of this mechanism between the eudicot and monocot plant lineages. Previously, we had shown that FHB resistance in wheat could be enhanced by increasing the sensitivity of wheat plants to SA through overexpression of the SA response regulator NPR1 (Makandar et al. 2006, 2012). It will be interesting to explore whether a combined strategy of improving SA accumulation and increasing sensitivity to SA in wheat can further improve resistance to FHB.

MATERIALS AND METHODS

Plant growth conditions.

Arabidopsis and wheat plants were cultivated in autoclaved peat-based planting mixture (Fafard). *Arabidopsis* was cultivated at 22°C in a growth chamber programmed for a 14-h-light (100 $\mu\text{E m}^{-2}\text{s}^{-1}$) and 10-h-dark cycle. Wheat plants were cultivated three seeds per gallon-capacity pot in a greenhouse set at 21 and 18°C during the day and night, respectively.

Arabidopsis mutant and transgenic plants.

Arabidopsis eds1-1, pad4-5, eds1-1 pad4-5 (Feys et al. 2001, 2005), and *rar1-1* (Azevedo et al. 2006) mutants are in the accession Ws. The *pad4-1* (Glazebrook et al. 1997), *sag101-1*, *sag101-1 pad4-1* (Feys et al. 2005), *sid2-1* (Nawrath and Métraux 1999), and *eds1-22* (Yang and Hua 2004) mutants are in accession Columbia (Col). Two tandem repeats of *EDS1* (At3g48090 and At3g48080) are present in the *Arabidopsis* accession Col (Yang and Hua 2004). Only the At3g48090 locus is disrupted in the *eds1-22* mutant (Yang and Hua 2004). The *eds1-1 sid2-1* line has been previously described (Venugopal et al. 2009). The 35S:*PAD4* (Pegadaraju et al. 2007, line 1) expresses a C-terminal StrepII epitope-tagged *PAD4* from the *Cauliflower mosaic virus* 35S promoter in the *pad4-5* mutant background. This *pad4-5* 35S:*PAD4* line was crossed with *eds1-1* to generate the *eds1-1 pad4-5* 35S:*PAD4* line. The *pad4-5 pad4*^{S118A} line in which N-terminal cMyc epitope-tagged *pad4*^{S118A} is expressed from the native *PAD4* promoter and the corresponding nonmutated cMyc-PAD4 expressing lines have been described previously (Louis et al. 2012). The *eds1-1 eds1*^{L262P} transgenic plants (Rietz et al. 2011, lines 1 and 2) expresses an N-terminal 3xHA-tagged *eds1*^{L262P} from the native *EDS1* promoter.

Fungal cultivation and plant infections.

F. graminearum isolate Z-3639 was cultured for 7 days on half-strength potato dextrose agar (Difco Laboratories) at 22°C. Fungal hyphae was washed off the plate using a brush in 10 ml of sterile distilled water and was filtered through four layers of sterile cheese cloth. The fungal suspension was infiltrated into the abaxial surface of *Arabidopsis* leaves with a needleless syringe. Four leaves per plant were inoculated and the plants kept covered with a clear plastic dome. Disease severity was scored 4 days postinoculation (dpi) by determining the fraction of inoculated leaves that exhibited chlorotic symptoms over <25% (category I), 25 to 50% (category II), 50 to 75% (category III), and >75% (category IV) of the leaf area (Makandar et al. 2010). A minimum of 60 leaves were analyzed for each genotype. Disease severity among different genotypes or treatments was compared by single factor analysis of variance (ANOVA) ($P < 0.05$) (SAS v5.1).

For bioassays on *Arabidopsis* inflorescence, approximately 40-day-old *Arabidopsis* plants that had open flowers on the

terminal inflorescence and few developing siliques were used. A fungal spore suspension (30,000 spores per milliliter) was sprayed on the inflorescence until run-off. Plants sprayed with water provided the control. For each genotype, 15 to 20 plants were treated. The inoculated plants were watered and covered individually with plastic separators to maintain high relative humidity. Disease severity was recorded at 7 dpi as previously described (Urban et al. 2002). Genotypes with FAD values ≤ 3 were considered highly resistant to *F. graminearum* and those with FAD values ≥ 9 were considered highly susceptible to *F. graminearum*.

Inoculation of wheat spikes was conducted as previously described (Makandar et al. 2012). At the anthesis stage, two central spikelets were inoculated with 10 μ l of a *F. graminearum* spore suspension containing 300 fungal spores. High humidity was maintained for 3 days by covering the inoculated spikes with a moistened zip-lock bag. Disease severity was monitored at periodic intervals by determining the percentage of spikelets in each spike that exhibited disease symptoms. ANOVA as calculated by the general linear models procedure available in Minitab (version 15; Minitab Inc.) was used to determine significant differences in disease severity between genotypes.

Plasmids and wheat transformation.

The *Ubi:PAD4* chimeric gene was generated by cloning the coding sequence for the *Arabidopsis PAD4* (At3g52430) gene in the pJS406 backbone (Makandar et al. 2006), such that the *PAD4* coding sequence is flanked on the 5' end by the maize *Ubi* gene promoter plus intron (Christensen and Quail 1996) and on the 3' end by the *Agrobacterium tumefaciens nos* (*nopaline synthase*) gene terminator. For cloning into pJS406, *PAD4* cDNA was amplified with the AtPAD4-Clone-F (5'-TCGGATCCATGGAACAAAACTTAT'), which contains a *Bam*HI restriction site and a c-Myc Epitope, and the AtPAD4-Clone-R (5'-GTTTTTCGTACGAGAATATATAGTA') primer, which contains a *Sph*I restriction site. The resulting 1.7-kb fragment was cloned into pJS406 that was partially digested with *Bam*HI and *Sph*I. To generate transgenic wheat, the *Ubi:PAD4* DNA was cobombarded into immature embryos from the spring wheat cv. Bobwhite with a plasmid containing the *Ubi:bar* construct (pAHC20) (Christensen and Quail 1996). Regeneration of wheat embryos was as described by Anand et al. (2003). The *BAR* gene, which confers resistance to glufosinate (Liberty; Bayer Crop Sciences), was used as a selectable marker for transgenic wheat. No detrimental growth or developmental phenotypes were observed in the independently-derived transgenic wheat *Ubi:PAD4* lines 610C and 882D.

Molecular analysis of transgenic *Ubi:PAD4* wheat.

DNA for polymerase chain reaction (PCR) was extracted from wheat leaves as previously described (Makandar et al. 2006). Presence of *Ubi:PAD4* was monitored by PCR using the *PAD4*-RT primer set (Supplementary Table 1). Expression of the *Ubi:PAD4* construct in wheat was monitored by reverse transcription (RT)-PCR using the *PAD4*-RT primer set. Expression of the wheat *Tubulin* gene was monitored as an internal control for RNA quality by RT-PCR using the Tub-F (5'-GACATCAACATTGAGAGACCATC-3') and Tub-R (5'-ATCTGTGCCTTGACCGTATCAGG-3') primers for the PCR.

Gene expression analysis.

All *Arabidopsis* and wheat tissues were frozen in liquid nitrogen, prior to RNA extraction using a guanidinium thiocyanate-phenol-chloroform solution (Chomczynski and Sacchi 1987). For RNA blot analysis, 10 μ g of RNA was denatured and resolved on formaldehyde-agarose gels. After transfer to Hybrid-N+ nylon membrane (Amersham Biosciences), the

blots were probed with a 32 P-labeled probe corresponding to the *Arabidopsis PRL* gene (At2g14610). The conditions for all RT-PCR reactions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min. Real-time PCR was performed with Sybr Green PCR master mix (Applied Biosystems) on an Eco qPCR system (Illumina), using the following amplification protocol: 10 min polymerase activation and denaturation at 95°C, 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. This was followed by a product melt to confirm presence of a single PCR product. The level of gene expression was normalized to that of *EF1 α* or *ACT8*. The *t* test or analysis of means using the general linear model was used to separate the means ($P < 0.05$).

SA measurements.

Free SA content in *Arabidopsis* leaves was quantified by a gas chromatography-mass spectrometry method (Schmelz et al. 2004), with modifications as described by Makandar et al. (2010). Each sample consisted of 100 to 200 mg of fresh weight of leaf tissue. A modified biosensor strain of an *Acinetobacter* sp., ADP1, was used for quantification of free SA in the wheat plants. Spike tissue was extracted in fresh Luria Bertani medium and was sonicated on ice for 5 min and centrifuged. The supernatant was used for free SA estimation using the biosensor strain as described previously (Huang et al. 2005, 2006).

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