Ethylene Is One of the Key Elements for Cell Death and Defense Response Control in the Arabidopsis Lesion Mimic Mutant *vad1*[1][W]

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Although ethylene is involved in the complex cross talk of signaling pathways regulating plant defense responses to microbial attack, its functions remain to be elucidated. The lesion mimic mutant *vad1-1* (for vascular associated death), which exhibits the light-conditional appearance of propagative hypersensitive response-like lesions along the vascular system, is a good model for studying the role of ethylene in programmed cell death and defense. Here, we demonstrate that expression of genes associated with ethylene synthesis and signaling is enhanced in *vad1-1* under lesion-promoting conditions and after plant-pathogen interaction. Analyses of the progeny from crosses between *vad1-1* plants and either *35S::ERF1* transgenic plants or *ein2-1*, *ein3-1*, *ein4-1*, *ctr1-1*, or *eto2-1* mutants revealed that the *vad1-1* cell death and defense phenotypes are dependent on ethylene biosynthesis and signaling. In contrast, whereas *vad1-1*-dependent increased resistance was abolished by *ein2*, *ein3*, and *ein4* mutations, positive regulation of ethylene biosynthesis (*eto2-1*) or ethylene responses (*35S::ERF1*) did not exacerbate this phenotype. In addition, *VAD1* expression in response to a hypersensitive response-inducing bacterial pathogen is dependent on ethylene perception and signaling. These results, together with previous data, suggest that VAD1 could act as an integrative node in hormonal signaling, with ethylene acting in concert with salicylic acid as a positive regulator of cell death propagation.

Plants regulate the different processes of their biology by producing a diverse set of hormones that act by modulating gene expression. Ethylene is one of them and affects myriad developmental processes, such as seedling emergence, leaf and flower senescence, formation of the vascular system, ripening, organ abscission, and also responses to biotic and abiotic stresses (Abeles et al., 1992; Bleecker and Kende, 2000; van Doorn, 2005). Interestingly, this hormone has been shown in certain cases to modulate programmed cell death (PCD) pathways, such as ethylene-induced leaf senescence, which depends on age-related changes (Jing et al., 2002; Lim et al., 2007) or fruit ripening (Adams-Phillips et al., 2004). PCD in plants is necessary for growth, but also for survival in response to environmental stresses, and occurs on a local or large scale (Pennell and Lamb, 1997). So far, the hypersensitive response (HR), a local and rapid cell death occurring in response to most pathogens and limiting growth of the microorganism, is the best-studied example of PCD. Plant defense (and HR) is regulated through a complex network of transduction pathways involving a number of signaling molecules: reactive oxygen species (ROS), nitric oxide, salicylic acid (SA), jasmonic acid (JA), and ethylene (Kunkel and Brooks, 2002). However, our current knowledge of the role of ethylene in plant defense is still limited.

It is well known that a large burst of ethylene is produced after the early steps of HR initiation and can induce defense-related genes (Boller, 1991). Treatment of plants with ethylene has long been known to increase either susceptibility or resistance, depending on the plant-pathogen interaction, and on the conditions of the interaction (Brown et al., 1993; Van Loon and Pennings, 1993; Diaz et al., 2002). More recently, the availability of plant mutants affected in ethylene production or signaling has enabled the study of the role of this phytohormone in a more functional way (Broekaert et al., 2006). However, conflicting results were obtained, showing in some cases that ethylene can act as a virulence factor of bacterial and fungal pathogens and, in other cases, indicating its involvement as a signaling compound in disease resistance (Broekaert et al., 2006; van Loon et al., 2006). These contrasting effects might be due to the fact that during plant-pathogen interactions, ethylene regulates PCD, which is observed both during the HR and disease development (Greenberg, 1997). The effects of ethylene in cells at different stages of infection and at

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different distances from the inoculation sites of the pathogens might also be quite different. Finally, as mentioned before, ethylene acts in concert with signaling molecules, such as the antagonistic interactions described between SA and JA/ethylene (Dong, 1998; van Loon et al., 2006) or the synergistic action of SA and ethylene (Schenk et al., 2000; Glazebrook, 2005).

Lesion mimic mutants (LMMs), which display spontaneous PCD and constitutively express defense responses, have been used as models for deciphering cell death signaling pathways (Lorrain et al., 2003). They are classified in two groups based upon the assumption that two mechanisms are involved in controlling cell death: initiation and propagation. Propagation mutants, unable to control the rate and extent of the lesions, are thought to be affected in genes controlling the suppression/limitation of PCD, whereas the other LMMs, the so-called initiation mutants, would be altered in the initiation of the process (Walbot et al., 1983). Many of these mutants have more than one permanently activated signaling pathway. However, the SA pathway predominates in these studies because the HR often triggers activation of SA-dependent signaling (Lorrain et al., 2003). However, plant defense responses are also controlled by mechanisms depending on ethylene and/or JA and, indeed, several LMMs (*cpr5*, *cpr22*, *ssi1*, and *hrl1*) show *PDF1.2* expression, a marker of activation of ethylene and JA pathways (Penninckx et al., 1998). Appearance of the lesions in the *acd5*, *hrl1*, and *dll1* mutants is correlated with increased ethylene production (Greenberg et al., 2000; Devadas et al., 2002; Pilloff et al., 2002). In addition, when treated with ethylene, the *acd1* mutant presents accelerated cell death with extension of necrosis in leaves (Greenberg and Ausubel, 1993). Crosses of a few LMMs (*acd5*, *cpr5*, *hrl1*, *cpr22*, *cet*, *dnd1*, *dnd2*) with mutants impaired in JA and/or ethylene pathways (*jar1*, *ein2*) showed that the appearance of lesions in initiation mutants is either unaffected (*dnd1*, *dnd2*; Genger et al., 2006) or delayed with more or less severity, suggesting in this latter case that ethylene plays a role in the proper timing and amplification of cell death. In propagation mutants, with the exception of *dll1* for which the role of ethylene remains unclear (Pilloff et al., 2002; Brodersen et al., 2005), no data are available. In terms of disease resistance analysis, these double mutants show various degrees of responses, consistent with the cell death phenotypes observed, suggesting that ethylene differentially affects resistance against pathogens with different lifestyles (necrotrophs, biotrophs) and plays an important role in mediating induced resistance (van Loon et al., 2006).

LMMs have also been powerful tools for investigating the role of defense-signaling pathways such as SA and its cross talks with other pathways (Clarke et al., 2000; Rusterucci et al., 2001). The role of ethylene is still an enigma during plant-pathogen interactions and has been poorly investigated using LMMs (Brodersen et al., 2002; Devadas et al., 2002; Pilloff et al., 2002). Because this hormone might play a key role in cell death control rather than resistance/susceptibility per se, analysis of the regulation of these processes by ethylene in the context of a well-characterized LMM should help to clarify the complex role of this hormone in disease resistance modulation in plants. The *vascular associated death1* (*vad1*) Arabidopsis (*Arabidopsis thaliana*) mutant displays necrotic HR-like lesions propagating along the vascular system and enhanced expression of defense genes, accumulation of high levels of SA, and increased resistance to virulent and avirulent strains of *Pseudomonas syringae* pv *tomato* (*Pst*). Analysis of the progeny from crosses between *vad1-1* plants and either *nahG* transgenic plants, *sid1*, *sid2*, *npr1*, e*ds1*, or *ndr1* mutants revealed the *vad1-1* cell death phenotype to be dependent on SA biosynthesis, but NPR1 independent. The mutant *vad1-1* not only exhibits increased expression of *PR1* (a marker of the SA-signaling pathway), but also of *PDF1-2* and *PR3* (markers of ethylene/JA-signaling pathways), suggesting that several defense pathways are activated (Lorrain et al., 2004). These data associated with strong and conditional regulation of cell death and resistance in the *vad1-1* mutant make *vad1-1* an interesting model for evaluating the role of ethylene in cell death and defense pathways as well as its possible cross talk with SA.

Here we exploit different approaches to elucidate the role of ethylene in (1) cell death and resistance phenotypes of *vad1-1*; and (2) transcriptional regulation of this negative regulator of cell death by ethylene and/or the ethylene-signaling pathway. By using the *vad1-1* mutant in combination with *ein2-1*, *ein3-1*, *ein4-1*, *ctr1-1*, and *eto2-1* mutants and an *ERF1* overexpressor, our results show that ethylene plays a major role in the cell death program controlled by VAD1. Transcriptional activation of *VAD1*, which was previously shown to occur late during plant-pathogen interactions, appears to be dependent on ethylene production and signaling.

RESULTS

Expression of Genes Associated with Ethylene Synthesis and Signaling Is Enhanced in *vad1-1* under Lesion-Promoting Conditions and during Plant-Pathogen Interaction

vad1-1 cell death and resistance phenotypes are dependent on SA biosynthesis (Lorrain et al., 2004). High levels of SA are accumulated and *PR1* gene expression is increased in the mutant under lesion-promoting conditions. Under the same conditions, *PDF1-2* and *PR3*, whose expression depends on the ethylene/JA-signaling pathways, are also expressed. We therefore investigated whether the *vad1-1* mutation could affect the different components of the ethylene pathway. For this purpose, we monitored by quantitative reverse transcription (RT)-PCR the expression of genes involved in ethylene biosynthesis and ethylene-signaling pathways in *vad1-1* under lesion-promoting

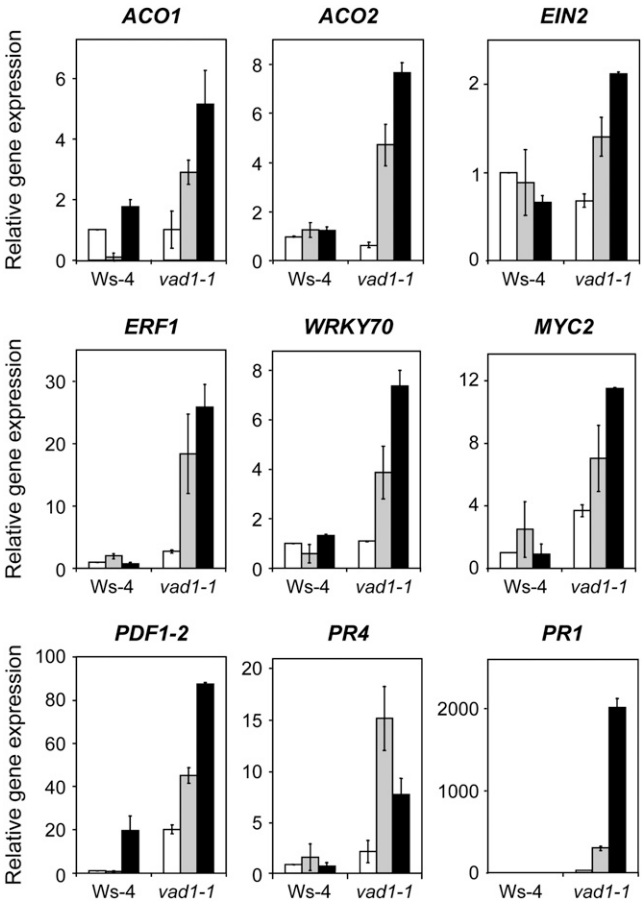

Figure 1. Ethylene-associated gene expression in wild-type and *vad1-1* plants. Quantitative RT-PCR analysis of gene expression in leaves of *ACO1*, *ACO2*, *EIN2*, *ERF1*, *WRKY70*, *MYC2*, *PDF1-2*, *PR4*, and *PR1* in wild-type (Ws-4) and *vad1-1* plants 17 (white bars), 21 (lesion formation in *vad1-1*; gray bars), and 31 d (black bars) after transplanting under lesion-promoting conditions. Each measurement is an average of two or three replicates, and the experiment was repeated three times with similar results. See "Materials and Methods" for details.

conditions as compared to the wild type (Wassilewskija-4 [Ws-4]). Figure 1 shows that, as compared to the wild type, marker gene expression for ethylene biosynthesis *ACC OXIDASE1* (*ACO1*) and *ACO2* is significantly increased in mutant in lesion-promoting conditions (*ACO1*, 2- to 3-fold higher in *vad1-1* than in the wild type; *ACO2*, 4- to 8-fold). In contrast, expression of another key gene of ethylene biosynthesis, *ACC SYNTHASE5* (*ACS5*), was found to be unaffected (data not shown). Genes involved in the ethylene-signaling pathway were then analyzed. *EIN2*, a central component of the ethylene-signaling pathway, is slightly induced (2-fold) after lesion appearance as compared to the wild type. Expression of transcription factors *ERF1*, *WRKY70*, *MYC2*, known to participate to a node of convergence for ethylene-SA-JA-mediated signals in plant defense responses (Thomas, 2002; Boter et al., 2004; Li et al., 2004), was increased in *vad1-1* under lesion-promoting conditions as compared to the wild type, from 4-fold when the lesions start (21 d after transplanting) to 25-fold when the lesions are fully expanded (31 d after transplanting; Fig. 1). Expression of *PDF1-2*, *PR4*, and *PR1*, defense-related genes associated with ethylene, ethylene/JA, and SA pathways, respectively, is induced in *vad1-1* as compared to the wild type at lesion appearance (45-, 15-, and 300-fold, respectively) and after lesion appearance (80-, 7-, and 2,000-fold, respectively). In agreement with these findings, *vad1-1* was shown to produce more ethylene (2.19 ± 0.23 nL h^{-1} g^{-1} fresh weight) than the wild type (0.8 ± 0.04 nL h^{-1} g^{-1} fresh weight) after lesion appearance (3 d; 24 d after transplanting). The values are significantly different from ethylene production in *vad1-1* mutant compared to the wild type according to Student's *t* test ($P \leq 0.005$). Thus, *VAD1* expression may be critical for the negative regulation of ethylene biosynthesis and defense-signaling pathways.

To gain insight into how the ethylene biosynthesis and signaling pathways are regulated in *vad1-1* during an interaction with a pathogen, gene expression was analyzed by quantitative RT-PCR in Ws-4 and *vad1-1* plants inoculated with *Pst DC3000* harboring the avirulence gene *avrRpm1* (Fig. 2). In the wild type, expression of *ACO1* was induced from 12 to 50 h postinoculation, whereas in *vad1-1*, *ACO1* transcript accumulation reached higher levels than the wild type, with a maximum at 72 h postinoculation (9-fold; Fig. 2). Similar data were obtained for *EIN2*, except that its expression in *vad1-1* during the first hours of the interaction was similar or slightly reduced as compared to the wild type. A similar high level of expression 24 h postinoculation was detected for *ERF1* and *WRKY70* in *vad1-1* and wild-type plants (Fig. 2). As expected, *PDF1-2* was induced in the wild type during the interaction with a maximum at 24 h (4-fold), whereas this maximal induction was observed 30 h postinoculation (8-fold) in *vad1-1*. It must be noted that, besides a high level of expression before inoculation (already reported), *PDF1-2* appears to be repressed between 12 and 24 h after inoculation. For comparison, *PR1* exhibits a similar profile of expression, although the repression level in *vad1-1* during the first hours following inoculation and the overexpression level 30 h postinoculation are less important than for *PDF1-2*, as compared to that of the wild type.

Based on these findings, it can be concluded that, during an incompatible interaction, ethylene-associated genes are more induced at later time points in the mutant *vad1-1* as compared to the wild type. This is in good agreement with the fact that VAD1 has been shown to act late during the HR and at the lesion borders, and with a possible role in cell death limitation. This is also in accordance with the observation that other defense marker genes, such as *PR1*, are constitutively expressed in *vad1-1* and overexpressed in response to pathogen attack, and that *vad1-1* shows enhanced resistance to *Pst* (Lorrain et al., 2004).

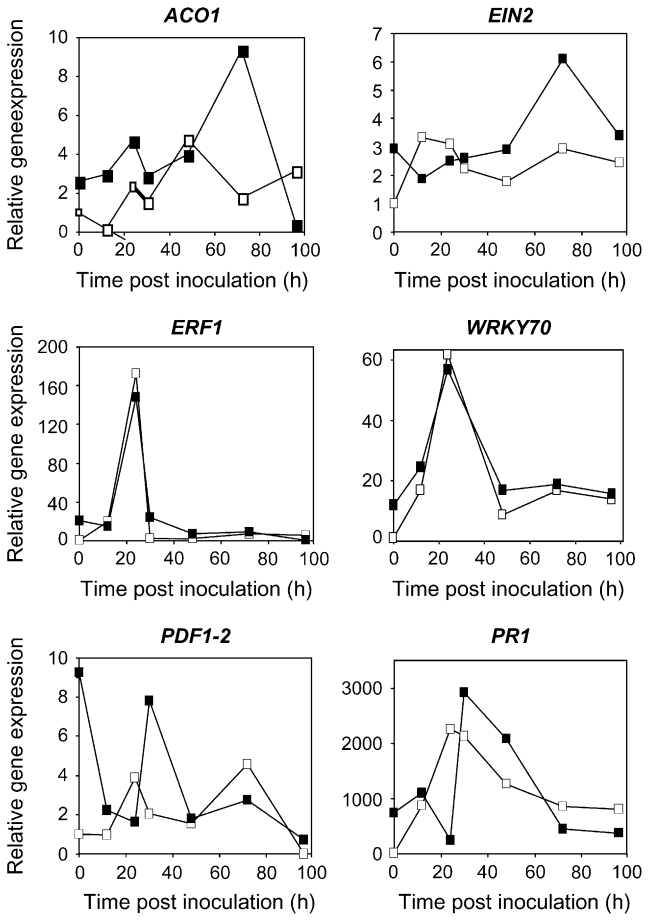

Figure 2. Ethylene-associated gene expression in wild-type (Ws-4) and *vad1-1* plants (26 d posttransplanting plants) after inoculation with the avirulent bacteria *Pst DC3000/avrRpm1*. Quantitative RT-PCR analysis of gene expression of *ACO1, EIN2, ERF1, WRKY70, PDF1-2,* and *PR1* in wild type (white squares) and *vad1-1* (black squares). Each measurement has been performed on 16 to 20 leaves collected from four independent plants, and the experiment was repeated two times with similar results. See "Materials and Methods" for details.

Cell Death and Defense Phenotypes of *vad1-1* Require the Positive Ethylene-Signaling Components EIN2, EIN3, and EIN4

To investigate whether EIN2, EIN3, and EIN4, known as positive regulators of the ethylene-signaling pathway (Guzman and Ecker, 1990; Roman et al., 1995), play a role in *vad1-1* phenotypes, we generated and analyzed double mutants (homozygous F3 lines) between *vad1-1* and the corresponding ethylene-insensitive mutants (*ein2-1*, *ein3-1*, and *ein4-1*). Because these mutations are in a Columbia-0 (Col-0) background, a backcross of *vad1-1* (background Ws-4) to Col-0 was performed as a control, showing that the *vad1-1*-conferred cell death phenotype segregated as in a parental backcross and appeared with the same kinetics and intensity under the same conditions (data not shown).

Under conditions where *vad1-1* would normally form lesions, the single mutants *ein2-1*, *ein3-1*, and *ein4-1* never exhibited lesions and presented a phenotype similar to the wild type, even though *ein4-1* plants show some differences in leaf morphology (Fig. 3A). Double mutants presented a similar leaf morphology to that of the single ethylene mutants during the different developmental stages, indicating that *vad1-1* leaf morphology is dependent on the ethylene-signaling pathway (Supplemental Fig. S1; Fig. 3A). For lesion formation and under the same conditions, the double mutant *vad1-1/ein3-1* never exhibited lesions, at least until 31 d after transplanting in *vad1-1* (Fig. 3A). In *vad1-1/ein2-1* and *vad1-1/ein4-1* double mutants, a similar phenotype was observed, although some lesions of faint intensity could be observed in some plants (Fig. 3A). These observations were confirmed by kinetic and quantitative evaluation of leaves presenting lesions (50 plants for each line) in double mutants in comparison with *vad1-1* (Table I). UV microscopy was used to observe leaves of double mutants and revealed that, whereas *vad1-1* developed lesions, no cell death symptoms could be observed, not even microscopic HRs (data not shown). Thus, the cell death phenotype of *vad1-1* is dependent on the signaling and perception components EIN2, EIN3, and EIN4.

To assess the defense phenotype of these mutants, expression of *PDF1-2* and *PR1* defense genes was analyzed in two independent lines for all the double mutants generated, in comparison with *vad1-1. PDF1-2* and *PR1* expression was abolished in the *vad1-1/ein2-1* double mutant, as well as in the *ein2-1* mutant, and drastically reduced in the *vad1-1/ein3-1* and *ein3-1* mutants. In good agreement with the *PR1* expression pattern in *vad1-1/ein2-1* and *vad1-1/ein3-1*, *ISOCHORISMATE SYNTHASE1* (*ICS1*) expression was found to be abolished in double mutants (Fig. 3B). *AtrbohD*, as a marker of ROS production, was also assessed and exhibited a similar expression profile (Fig. 3B). In the double mutant *vad1-1/ein4-1*, a delay in the accumulation of *PDF1-2* and *PR1* transcripts was observed, *PDF1-2* and *PR1* transcripts accumulating only after lesion appearance when *ICS1* and *AtrbohD* expression is strongly reduced, which could indicate partial involvement of the EIN4 ethylene receptor (Fig. 3B). These results are consistent with the data previously obtained for defense genes related to other signaling pathways (Lorrain et al., 2004) and indicate an intricate SA/ethylene/ROS network. Thus, ethylene seems also to be required for the *vad1-1*-conferred defense phenotype.

We then tested whether the mutations *ein2-1*, *ein3-1*, and *ein4-1* might also affect the observed enhanced resistance of *vad1-1* to different strains of *Pst* (Lorrain et al., 2004). For this purpose, resistance phenotypes of the different mutant lines were evaluated following inoculation with virulent (*DC3000*) and avirulent (*DC3000/avrRpm1*) strains of *Pst* by measuring in planta bacterial growth (Fig. 3C; data not shown). Importantly, the enhanced resistance observed in *vad1-1* to both *Pst* strains was abrogated when the *ein3-1* mutation was present (Fig. 3C). The resistance of *vad1-1/ein3-1*

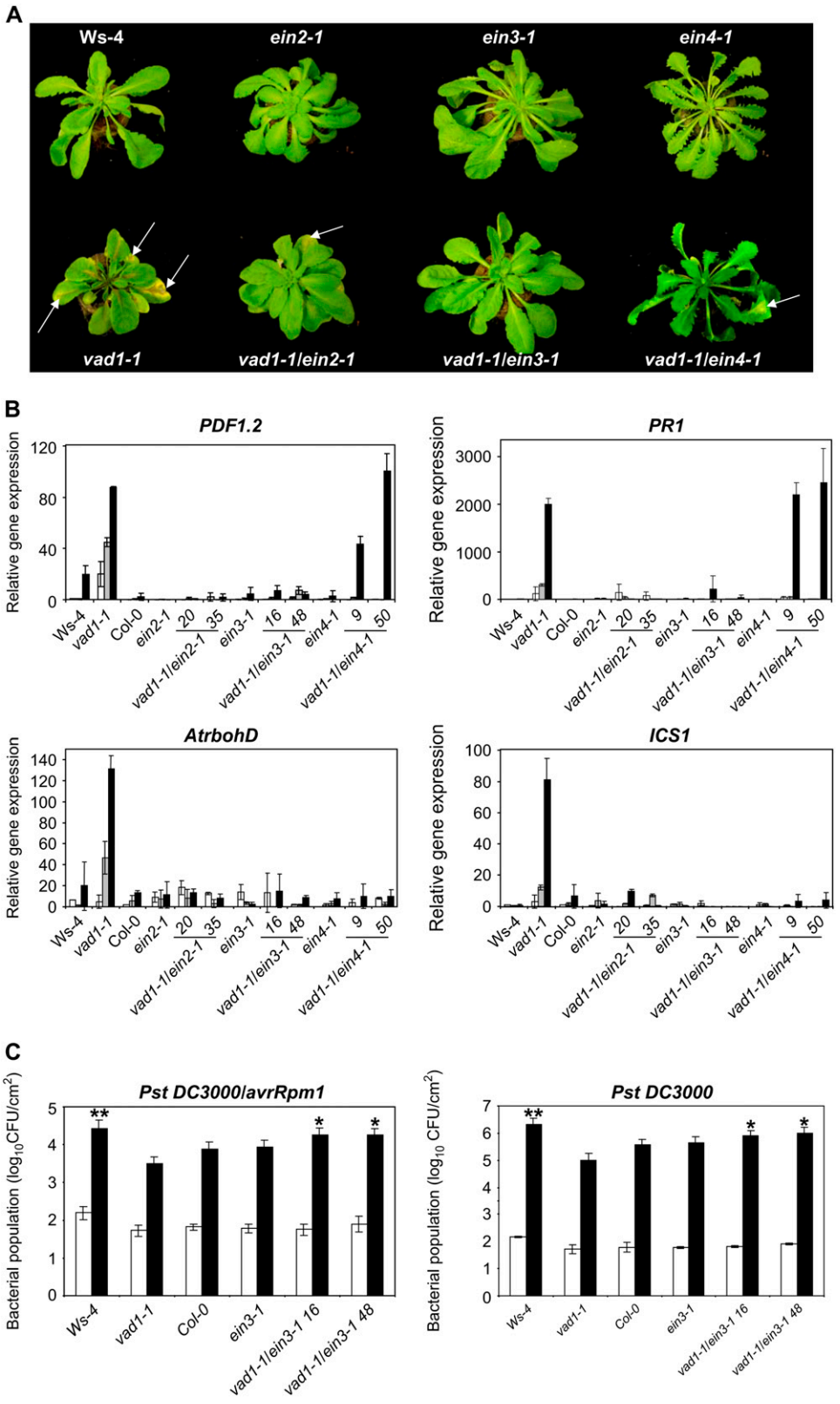

Figure 3. Effect of *ein2-1*, *ein3-1*, and *ein4-1* mutations on the cell death and defense phenotypes of *vad1-1* plants. A, Six-week-old single- and double-mutant plants 31 d after transplanting (10 d after lesion formation in *vad1-1*). Arrows indicate lesion formation on plants. B, Transcript levels of the defense-related genes *PDF1.2* and *PR1* in wild-type, single-, and double-mutant plants evaluated by quantitative RT-PCR. Plants were grown under lesion-promoting conditions and harvested 17 (white bars), 21 (lesion formation in *vad1-1*; gray bars), and 31 d (black bars) after transplanting. Each measurement is an average of two replicates and experiments were repeated two or three times and similar results were obtained. C, Bacterial populations in wild-type, single-, and double-mutant plants. Inoculations with *Pst* strain *DC3000* and strain *DC3000* expressing *avrRpm1* were performed on leaves without lesion with a bacterial suspension at 2×10^5 and 5×10^5 cfu mL^{-1}, respectively. Bacterial populations were measured at 0 (white bars) and 3 d (black bars) postinoculation. Mean bacterial densities are shown (three to five replicates with corresponding SDs) for one representative experiment from two or three independent experiments. Asterisks denote significantly different values from bacterial number in *vad1-1* according to Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.005$). See "Materials and Methods" for details.

Table I. *Percentage of leaves with lesions in vad1-1, vad1-1/ein2-1, vad1-1/ein3-1, vad1-1/ein4-1, vad1-1/eto2-1, vad1-1/ctr1-1, and vad1-1/35S::ERF1 double mutants*

Results indicated are mean values of two independent experiments and represent a percentage of leaves with lesions, which was similar for all the plants. Fifty plants of each mutant or double mutant have been used in this experiment.

Mutant Lines	Percentage of Leaves with Lesions			
	17	21	24	31
	d posttransplanting			
vad1-1	0	10	50	60
vad1-1/ein2-1	0	0	5	15
vad1-1/ein3-1	0	0	0	5
vad1-1/ein4-1	0	0	8	20
vad1-1/eto2-1	0	33	77	85
vad1-1/ctr1-1	10	40	65	80
vad1-1/35S::ERF1	10	25	70	80

mutant plants was compromised upon inoculation with the avirulent strain, with bacterial growth similar to *ein3-1* 3 times higher than in wild-type Col-0 and 5 times higher than in the *vad1-1* mutant. Similar differences were observed with the virulent strain *Pst DC3000* (Fig. 3C) and for the *vad1-1/ein2-1* double mutant (data not shown). Resistance for the *vad1-1/ein4* double mutant was not statistically different from *vad1-1*, certainly due to the late expression of defense-related genes observed in double mutants. This suggests that EIN4 is certainly not the only ethylene receptor associated with *vad1-1*-increased resistance (data not shown). The phenotypes of leaves infected under these conditions indicated that weak chlorosis was observed 4 d postinoculation, the symptom intensity correlating with bacterial population measurement.

Taken together, these results indicate that the cell death, defense, and resistance phenotypes of *vad1-1* are dependent on positive regulation of the ethylene pathway by EIN2 and EIN3.

Overexpression of the Positive Regulator *ERF1*, Depletion of the Negative Regulator CTR1, and Constitutive Activation of ACS5 in the *eto2-1* Mutant Lead to Amplification of the Cell Death and Defense Phenotypes of *vad1-1*

We demonstrated that crosses with mutants impaired in positive components of the ethylene-signaling pathway abolish the *vad1-1*-associated phenotypes. To gain more insight into the role of the ethylene-signaling pathway in *vad1-1* phenotypes, we tested three other signaling components, ETO2, CTR1, and ERF1. The dominant *eto2-1* mutation affects the C-terminal domain of ACC SYNTHASE5 (ACS5) and, as a result, stabilizes the protein (Chae et al., 2003). *CTR1* encodes a Ser-Thr protein kinase, which acts as a negative regulator of the ethylene-signaling pathway (Kieber and Ecker, 1993). ERF1, a convergence point of ethylene/JA-signaling pathways, encodes a transcription factor that regulates the expression of pathogen response genes (Lorenzo et al., 2003). The mutants *ctr1-1* and *eto2-1* and the transgenic line harboring the *35S::ERF1* transgene present a constitutive response to ethylene (Kieber et al., 1993; Berrocal-Lobo et al., 2002). Furthermore, the *eto2-1* mutant and the *35S::ERF1* line exhibit a delay in development: They are generally smaller than the wild type. The *ctr1-1* mutant is also affected in development because it presents a very small rosette as compared to the wild type. The presence of these mutations or the transgene in *vad1-1* accelerated lesion formation (Fig. 4A). In addition, plant development was also affected, the double-mutant plants showing a smaller rosette than the single ethylene mutants and presenting *vad1-1* leaf morphology in relation to lesion appearance (Supplemental Fig. S1; Fig. 4A). At later time points, leaf lesion intensity and number were also increased in the double mutants (Table I), and lesion propagation was more effective (Fig. 4A). Interestingly, treatment of *vad1-1* with ethylene led to acceleration and strong intensification of lesion development (Table II), whereas 1-methylcyclopropene (1-MCP), an inhibitor of ethylene perception (Sisler and Serek, 1997), abolished cell death at least until 5 d of treatment.

The effect of *eto2-1*, *ctr1-1*, and *35S::ERF1* was then studied on the expression of defense-related genes in *vad1-1* (Fig. 4B). The *ctr1-1* and *eto2-1* mutations and the *35S::ERF1* transgene accelerated *PDF1-2* transcript accumulation as compared to the mutant *vad1-1*: 17 d after transplanting, double mutants showed *PDF1-2* expression, whereas the *vad1-1* mutant did not exhibit detectable *PDF1-2* expression. In addition, *ctr1-1* and *eto2-1* mutations did not only affect the timing of *PDF1-2* expression, but also its level, which appeared to be significantly increased in double mutants compared to *vad1-1*. Thus, we showed that *vad1-1*-associated expression of *PDF1-2* was accelerated and increased when the ethylene-signaling pathway is constitutively activated (*vad1-1/ctr1-1* and *vad1-1/eto2-1*), whereas its expression was only accelerated in *vad1-1/35S::ERF1* lines. *PR1* expression was also accelerated in double mutants as compared to *vad1-1*, but was not increased (Fig. 4B). This is consistent with the results obtained with ethylene-insensitive mutants and strengthens the hypothesis that *vad1-1*-associated defense gene expression is dependent on ethylene synthesis and signaling pathways.

These data were extended by in planta bacterial growth experiments performed on all the double mutants except *vad1-1/ctr1-1*, because of the reduced size of the leaves of the double mutant. In response to avirulent (*DC3000/avrRpm1*) and virulent (*DC3000*) strains of *Pst*, *vad1-1/eto2-1* double mutants (shown as an example) exhibited similar levels of resistance as *vad1-1*, clearly more resistant than the wild-type and the mutant *eto2-1* (5-fold; Fig. 4C). Similar results were obtained for double mutants *vad1-1/35S::ERF1* (data not shown). Leaf phenotypes, in good agreement with observations performed on crosses between *vad1-1*

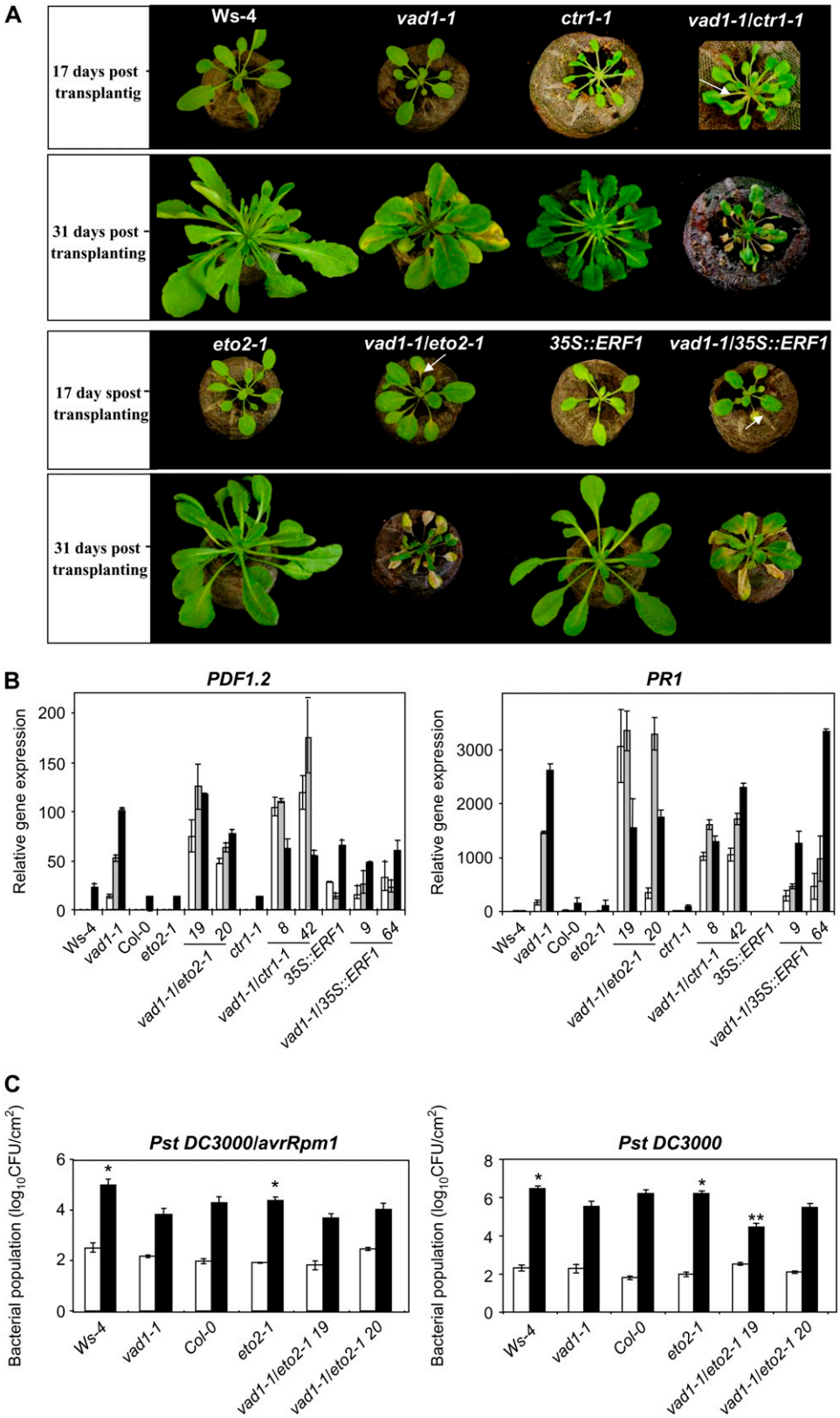

Figure 4. Effect of *eto2-1* and *ctr1-1* mutations and *ERF1* transgene on the cell death and defense phenotypes of *vad1-1* plants. A, Single- and double-mutant plants, 17 and 31 d after transplanting. A closeup (3×) of the double mutant *vad1-1*/*ctr1-1* is presented. Arrows indicate lesion formation on plants. B, Transcript levels of defense-related genes *PDF1.2* and *PR1* in wild-type, single-, and double-mutant plants evaluated by quantitative RT-PCR. Plants were grown under lesion-promoting conditions and harvested 17 (white bars), 21 (lesion formation in *vad1-1*; gray bars), and 31 d (black bars) after transplanting. Each measurement is an average of two replicates and experiments were repeated two times with similar results. C, Bacterial populations in wild-type, single-, and double-mutant plants. Inoculations with *Pst* strain *DC3000* and strain *DC3000* expressing *avrRpm1* were performed on leaves without lesion with a bacterial suspension at 2×10^5 and 5×10^5 cfu mL^{-1}, respectively. Bacterial populations were measured at 0 (white bars) and 3 d (black bars) postinoculation. Mean bacterial densities are shown (three to five replicates with corresponding SDs) for one representative experiment from two or three independent experiments. Asterisks denote significantly different values from bacterial number in *vad1-1* according to Student's *t* test (*, $P \leq 0.05$; **, $P \leq 0.005$). See "Materials and Methods" for details.

Table II. *Cell death phenotype of vad1-1 after ethylene and 1-MCP treatment*

Results indicated are mean values of two experiments and are expressed as percentage of leaves presenting lesions. Observations were made 5 d after treatment on *vad1-1* mutant (23 d after transplanting). At time 0 of the experiment, no lesion was visible on *vad1-1* plants.

Treatment	Percentage of Leaves with Lesions (5 d after Treatment)
Air	17
C_2H_4 5 μL L^{-1}	52
C_2H_4 20 μL L^{-1}	66
C_2H_4 80 μL L^{-1}	72
MCP 1 μL L^{-1}	0

and ethylene-insensitive mutants, retained the *vad1-1* leaf morphology once lesions appear.

Thus, we conclude that the cell death and defense phenotypes, but not the increase in resistance of *vad1-1*, are amplified by overexpression of ERF1, a positive regulator of ethylene responses, by depletion of CTR1, a negative regulator of ethylene signaling, and by activation of ACS5, a protein involved in ethylene biosynthesis.

VAD1 Expression Is Dependent on Ethylene Biosynthesis and Signaling Pathways

We have shown that *vad1-1*-associated phenotypes are dependent on ethylene biosynthesis and signaling pathways. To get a better understanding of how ethylene can regulate cell death via *VAD1*, we analyzed *VAD1* expression in ethylene mutants or transgenic *35S::ERF1* lines, previously described, during plant-pathogen interactions. *VAD1* expression was monitored by quantitative RT-PCR after inoculation with an avirulent strain of *Pst* (*DC3000/avrRpm1*; Fig. 5, A–D). Whereas *VAD1* expression was maximal 48 h after inoculation in the wild type (7-fold) after transient and early induction between 3 and 6 h postinoculation (3-fold), it was abolished in ethylene-insensitive mutants (*ein2-1*, *ein3-1*, and *ein4-1*; Fig. 5A). Surprisingly, *VAD1* expression in mutants presenting a constitutive ethylene response (*eto2-1*, *ctr1-1*) and in the *35S::ERF1* line presented the same profile as in ethylene-insensitive mutants (Fig. 5B). As a control, *PDF1-2* expression was analyzed in the different lines and showed expression patterns consistent with previous data (Berrocal-Lobo et al., 2002; Fig. 5, C and D). In addition, treatment with 1-aminocyclopropane-1-carboxylic acid (ACC; ethylene precursor) at a physiological dose (100 μM) transiently induced *VAD1* expression (4- to 5-fold over the control between 6 and 24 h after treatment; Fig. 5E). *ACO1* expression, used as a control of ACC treatment, is constantly activated.

In agreement with these results, ethylene is also able to induce *VAD1* expression in a dose-dependent manner (Fig. 5G), *VAD1* expression being maximal at 72 h with 5 μL L^{-1} of ethylene treatment. 1-MCP treatment (1 μL L^{-1}) abolished *VAD1* expression. *EIN2* expression, used as a positive control, was constantly activated by ethylene treatment and was inhibited by 1-MCP.

These data indicate that *VAD1* expression is dependent on ethylene production, perception, and signaling. In addition, the same expression profiles were observed in response to pathogen inoculation and to ACC treatment, indicating that ethylene could contribute to *VAD1* expression regulation during an interaction with avirulent bacteria.

DISCUSSION

LMMs showing aberrant regulation of cell death constitute powerful tools not only for the identification of genes involved in the regulation and/or execution of PCD, but also to decipher cell death and defense pathways in plants (Lorrain et al., 2003). In this article, we provide genetic and molecular evidence that (1) VAD is involved in a signaling cascade that modulates (directly or indirectly) gene expression associated with ethylene synthesis and signaling; (2) VAD acts through the ethylene pathway; and (3) VAD expression is regulated by ethylene.

Ethylene and Cell Death in *vad1*

As previously demonstrated for SA, ethylene is a requisite modulator of cell death pathways activated in *vad1-1.* Our results clearly show that the cell death phenotype of *vad1-1* is dependent on ethylene perception, through EIN4, and on positive regulators of the signaling pathway of ethylene, such as EIN2 and EIN3. Conversely, the extent of cell death in the mutant *vad1-1* is increased and its time of appearance accelerated in lines overexpressing ERF1, a positive regulator of ethylene responses, and in the *ctr1* mutant, depleted of CTR1, a negative regulator of ethylene signaling. These effects are quantitatively important (Table I) and demonstrate without ambiguity that the ethylene-signaling pathway is required for cell death initiation and propagation in *vad1-1.* In addition, no HR-like or microscopic HR could be observed in the double mutants at the time of lesion appearance in *vad1-1.* Ethylene biosynthesis also positively influences the *vad1-1* cell death phenotype: Although to a lesser extent than the ethylene-signaling mutations, the mutation *eto2-1*, which leads to activation of ACS5, a protein involved in ethylene biosynthesis, significantly accelerates cell death appearance in *vad1-1* and increases its propagation. In addition, ethylene biosynthesis (except ACS5, known to be regulated at the translational level) and signaling-associated genes are all activated in the mutant as soon as lesions appear, indicating a role for ethylene in lesion development.

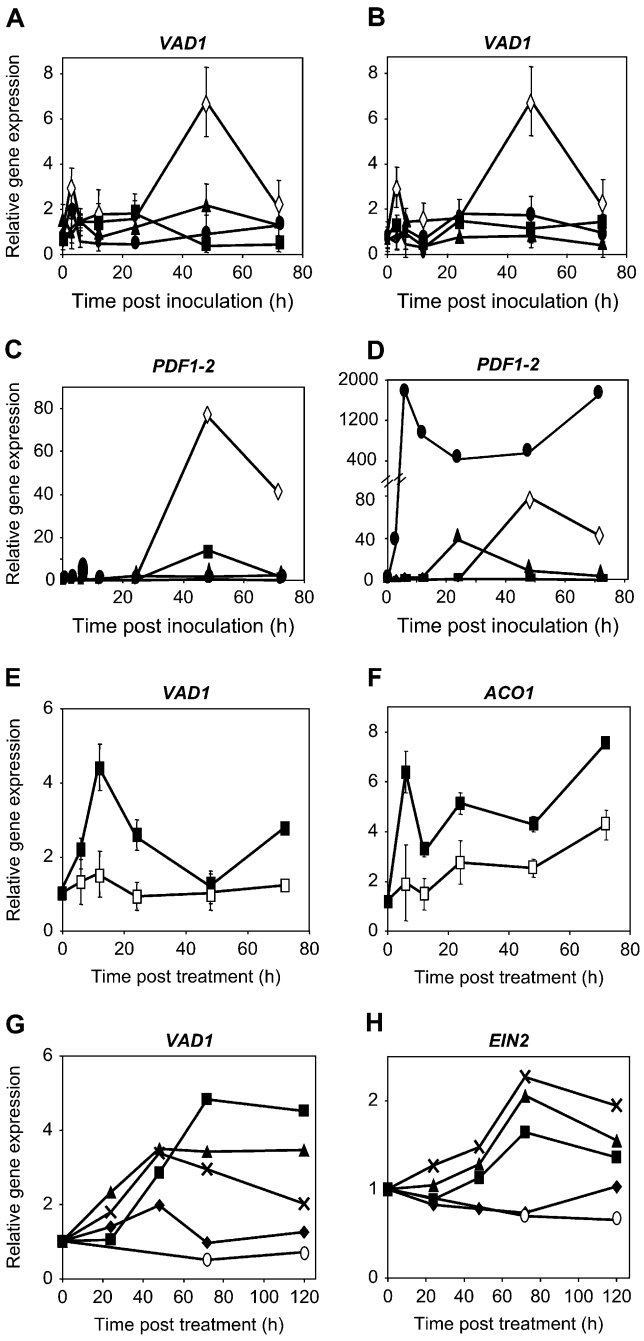

Figure 5. VAD1 expression is dependent on ethylene biosynthesis and signaling pathways. Transcript levels were quantified by quantitative RT-PCR. A, *VAD1* transcript levels in Col-0 (diamonds), *ein2-1* (squares), *ein3-1* (triangles), and *ein4-1* (circles) plants in response to inoculation with the avirulent pathogen *Pst DC3000/avrRpm1*. B, *VAD1* transcript levels in Col-0 (diamonds), *eto2-1* (squares), *ctr1-1* (triangles), *35S::ERF1* (circles) plants, in response to inoculation with the avirulent pathogen *Pst DC3000/avrRpm1*. C, *PDF1.2* transcript levels in Col-0 (diamonds), *ein2-1* (squares), *ein3-1* (triangles), and *ein4-1* (circles) plants in response to inoculation with the avirulent pathogen *Pst DC3000/avrRpm1*. D, *PDF1.2* transcript levels in Col-0 (diamonds), *eto2-1* (squares), *ctr1-1* (triangles), *35S::ERF1* (circles) plants, in response to inoculation with the avirulent pathogen *Pst DC3000/avrRpm1*. Each measurement is an average of two replicates and experiments were repeated two times with similar results. E and F, *VAD1* expression in response to ACC treatment. Ten-day-old wild-type (Col-0) plants were treated with water (white squares) or with ACC (black squares) at a final concentration of 100 μM. Mean values with corresponding SDs are shown for three independent experiments. G and H, *VAD1* and *EIN2* transcript levels after treatment of Ws-4 plants (23 d after transplanting) with air (black diamonds), 5 μL L^{-1} ethylene (black squares), 20 μL L^{-1} ethylene (black triangles), 80 μL L^{-1} ethylene (black crosses), and 1 μL L^{-1} 1-MCP (white circles). Each measurement is an average of two replicates and experiments were repeated two times with similar results. See "Materials and Methods" for details.

However, it should be noted at this point that all these effects on cell death symptoms are partial; a complete suppression of the phenotype, or the complete collapse of the plants was not observed. This suggests that ethylene-dependent cell death in *vad1-1* comprises both ethylene-dependent and ethylene-independent components, such as SA, previously demonstrated to play an essential role. Concerning the other propagation mutants already characterized, ethylene has been very rarely studied as a regulator of the control of cell death, except for *acd1* (Greenberg and Ausubel, 1993), which exhibits expended leaf necrosis when treated with ethylene. The same observations have been performed for *vad1-1*, reinforced by the fact that 1-MCP treatment of *vad1-1* leads to complete inhibition of cell death propagation (Table II). Ethylene has been shown to act as a promoting factor for cell death propagation in the ozone-sensitive Arabidopsis mutant *rcd1* (Overmyer et al., 2000). In this case, ethylene was suggested to be responsible for amplification of superoxide accumulation, which, in turn, promotes the execution of spreading cell death. RCD1, which has been identified as a WWE protein-protein interaction domain protein (Ahlfors et al., 2004), is thought to be an integrative node in hormonal signaling and regulation of several stress responses. As discussed later, a similar function can be envisaged for VAD1.

Ethylene and Defense/Resistance in *vad1*

Ethylene has a complex and still not completely elucidated function in plant defense because its role is probably different according to the plant-pathogen interaction and according to the invasion strategy of the pathogen and also because in-depth cell biology approaches and/or simplified tools are necessary to address the question of spatial and temporal action of ethylene (Glazebrook, 2005; van Loon et al., 2006). It is generally admitted that ethylene, in concert with JA, contributes to resistance to necrotrophic, but not biotrophic, pathogens (Thomma et al., 2001; van Loon et al., 2006). For pathogens with mixed lifestyles, such as *Hyaloperonospora parasitica* or *P. syringae*, all three signaling pathways (SA, JA, and ethylene) are involved, with exceptions and complexities related to the nature of the pathosystem and the way the inoculation tests are performed (Devadas and Raina, 2002;

O'Donnell et al., 2003; Glazebrook, 2005). In *vad1-1*, defense activation and resistance to the bacterial pathogen *Pst* are dependent on the ethylene components EIN2, EIN3, and EIN4. Interestingly, whereas defense gene activation is also dependent on ACS5 activation and *ERF1* expression, increased resistance is not affected by these ethylene components under our experimental conditions. This might be explained by the high level of increased resistance already reached in *vad1-1*, a level that cannot be further affected by ethylene overproduction and/or defense overactivation. These results suggest either (1) an important role of ethylene, together with SA, in limiting *Pst* growth; or, more likely, (2) participation of the ethylene pathways to the increased resistance phenotype of *vad1-1*, as the result of its constitutive activation, in concert with the SA pathway, by pathogen attack in the absence of VAD1. In favor of this latter hypothesis, most of the ethylene mutants tested in response to pathogens, such as *Pst* or *H. parasitica*, do not show significant phenotypic variations as compared to the wild type (Thomma et al., 2001; Devadas and Raina, 2002). In some cases, *ein2* or *35S::ERF1* lines showed slightly modified phenotypes in response to *Pst*: enhanced or decreased tolerance, respectively (Bent et al., 1992; Berrocal-Lobo et al., 2002), that we did not reproduce, probably because of the rather low inoculum used in our experiments. In conclusion, ethylene and SA pathways seem to act synergistically in defense regulation in the mutant *vad1-1*, probably as a consequence of the propagative cell death occurring in the mutant.

Cross Talk between Ethylene, SA, JA, and ROS for Cell Death/Defense Regulation in *vad1*

Besides ethylene, *VAD1* expression and *vad1-1*-conferred phenotypes have been shown to be dependent on SA (Lorrain et al., 2004). JA signaling also has been shown to be involved in cell death limitation (Overmyer et al., 2000). These three stress-related signaling pathways can either cooperate or act as antagonists as function of the different stresses applied and they play an important role in defense-signaling pathways. Cross talk between SA-, ethylene-, and JA is thought to be essential in fine tuning complex defense responses established by the type of interaction (Reymond and Farmer, 1998; Lorenzo and Solano, 2005). The fact that the phenotypes of *vad1-1* are abolished/decreased in the double mutants *vad1-1/nahG*, similarly to the observations made with the ethylene double mutants, implies that VAD1 operates upstream of SA and ethylene, and that basal resistance is intimately related to cell death in *vad1-1.* In favor of synergistic action of the two signals, blocking the SA pathway in *vad1-1* either by *sid1* or *nahG* resulted in a dramatic decrease not only in *PR1* expression, but also in *PDF1-2* and *PR5* genes. In parallel, positive or negative regulation of the ethylene pathways in *vad1-1* by the different ethylene-related mutations resulted not only in changes in *PDF1-2* expression, but also in *PR1* and *ICS1* expression, suggesting that SA production is affected in these double mutants (Figs. 3 and 4). Thus, ethylene and SA act together in the regulation of cell death and defense phenotypes of *vad1-1*.

To address the question of the role of JA in the regulation of cell death in *vad1-1*, we crossed *vad1-1* with *jar1-1*, a JA-insensitive mutant (Staswick et al., 1992). Whereas the *jar1-1* mutant did not exhibit lesions under our growth conditions, the double mutant *jar1-1/vad1-1* showed accelerated lesion formation and increased severity of the lesions, as compared to *vad1-1* (Supplemental Fig. S2A). In terms of defense expression, *PDF1-2* expression was clearly accelerated and increased (Supplemental Fig. S2B). A similar pattern of expression was observed for *PR1* expression. Our results demonstrate clearly that JA negatively regulates propagation of cell death in *vad1-1*, and that, as demonstrated for the ozone-sensitive mutant *rcd1* (Tuominen et al., 2004), there is mutual antagonism between the two hormones for the regulation of cell death propagation in *vad1-1*. Genetic and pharmacological experiments aimed at deciphering more precisely the cooperative, synergistic, or antagonistic interactions between the three hormones should allow a better assessment of the cell death phenotype conferred by *vad1-1*.

In this context, the accumulation of ROS, which has been implicated in the HR cell death (Torres et al., 2002) and that is potentiated by SA and ethylene in combination with avirulent pathogens (Kotchoni and Gachomo, 2006), might be the common regulator of these pathways in *vad1-1*. As previously demonstrated, the *vad1-1* mutant accumulates elevated levels of ROS, evaluated through *AtrbohD* expression, and microscopic observation of hydrogen peroxide production at the lesion sites (Lorrain et al., 2004). Crosses of the mutant *vad1-1* with the ethylene-insensitive mutants (*ein2*, *ein3*, *ein4*) drastically reduced expression of the *AtrbohD* gene to the background level, suggesting that ethylene, as well as SA (or SA-regulated signals), may both, in concert, potentiate the accumulation of ROS. As described by Kankasjarvi and collaborators, in the case of ozone stress, ROS production might be the central component of a self-amplifying loop termed the oxidative cell death cycle (Kangasjarvi et al., 2005) in which the three hormones play a critical regulatory role.

All together, these findings support a model in which VAD1 exerts a negative control on cell death-dependent ROS accumulation, promoted by SA and ethylene, and limited by JA (Fig. 6). More precisely, pathogen perception is accompanied by an oxidative burst resulting in rapid production of ROS, which is dependent on ethylene and which drives the SA-dependent HR cell death. After cell death initiation, for which VAD1 does not seem to play a major role, cell death propagates until VAD1 and JA-dependent pathways negatively control the process and lead to

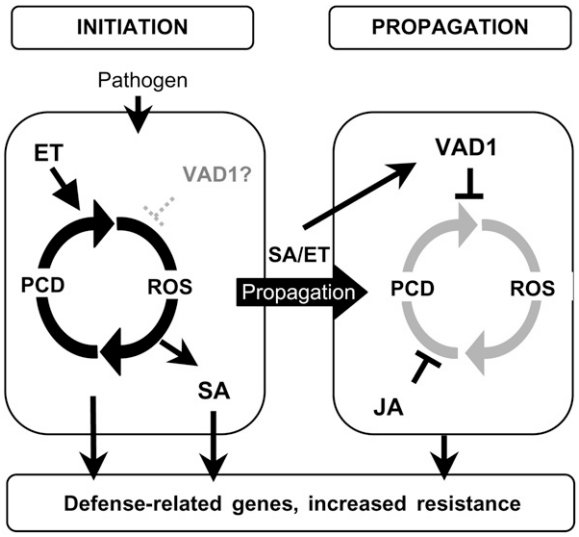

Figure 6. Schematic representation of cell death regulation by VAD1 during plant-pathogen interaction. Black arrows indicate positive regulation, whereas gray arrows indicate the absence of positive regulation. Black end-blocked lines indicate negative regulation, whereas gray end-blocked lines indicate the absence of negative regulation. ET, Ethylene.

lesion containment. This model is in good agreement with the kinetic and spatial expression of *VAD1* during an incompatible interaction (i.e. a major peak of expression late during the interaction [48 h postinoculation], localized at the periphery of the HR lesions [Lorrain et al., 2004]).

Signaling Messengers and *VAD1* Function

VAD1 encodes a plant membrane protein containing two domains, one with an unknown function and a GRAM domain that is found in a variety of proteins associated with membrane-coupled processes and signal transduction (Doerks et al., 2000). In humans, the crystal structure of the myotubularins revealed that the myotubularin GRAM domain is part of a larger motif that encompasses a Pleckstrin homology (PH) domain (Choudhury et al., 2006) and the PH/G (PH/GRAM) domain is able to bind to phosphoinositidine lipids (Lorenzo et al., 2005). Taking into account these structural motifs and their putative functions (signaling, binding with pro-cell death lipid messengers), it can be speculated that VAD1 might regulate (1) cell death signaling, which in turn could modulate cell death regulators such as ROS, ethylene, and SA; or (2) ROS production systems, known to be at least in part constituted by membrane proteins such as AtRBOHD (Laloi et al., 2004) and AtGPX1 (Iqbal et al., 2006).

Further approaches, including VAD1 protein subcellular localization, VAD1 overexpression in the cell, and a search for interactors, would undoubtedly shed some light on the biochemical functions of this cell death regulator and its implication in the complex network of hormones that regulates cell death in plants.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) plants, accessions Col and Ws, were used in these experiments. Seeds of the mutant plants *ein2-1* (Guzman and Ecker, 1990), *ein3-1* (Chao et al., 1997), *ein4-1* (Roman et al., 1995), *eto2-1* (Vogel et al., 1998), *ctr1-1* (Kieber et al., 1993), and *jar1-1* (Staswick et al., 1992) were obtained from the Nottingham Arabidopsis Stock Centre. The *35S::ERF1* overexpressor line was kindly provided by R. Solano (CNB Campus Universidad Autonoma).

For all experiments, mutant and wild-type seeds were sterilized and sown on Murashige and Skoog plates as previously described (Balagué et al., 2003) under a light period of 16 h (71 μmol m^{-2} s^{-1}) at 21°C. Seedlings were transplanted 7 d after sowing to Jiffy pots and grown in a growth chamber under a light period of 9 h (192 μmol m^{-2} s^{-1}) at 21°C and 40% to 70% humidity. Most experiments were performed with 4- to 6-week-old plants. In these growth conditions, lesions appear on the *vad1-1* mutant 21 d after transplanting.

Generation of Double Mutants

Generation of double mutants between different signaling mutants and *vad1-1* (pollen donor) was confirmed for the presence of the *vad1-1* mutation by selection on kanamycin. For all double mutants, *vad1-1* and other mutant plants were crossed, the F1 plants selfed, and the segregating F2 plants genotyped for the mutations of interest. Homozygous *vad1-1* plants were then selected by PCR as described previously (Lorrain et al., 2004). *ein2-1* and *ein3-1* mutations were selected using the already described cleaved amplified polymorphic sequence, using, respectively, the primer pairs 5′-GCTGGTGGTTTGAGATGGAA-3′ and 5′-TTTACATCAGAGTCTTCCTTCAGACT-3′ (Nandi et al., 2003), and 5′-AGGCAGTCTCAAGAGCAAGC-3′ and 5′-CATTCATCAGAAGCGAGCAA-3′ (Wubben et al., 2004). *vad1-1/ein4-1* double mutants were selected on Murashige and Skoog agar containing 50 μM ACC and kanamycin 50 μg mL^{-1}. Plants showing no triple response were selected. *ctr1-1* and *eto2-1* mutations and *35S::ERF1* lines were selected on the constitutive triple response conferred by the mutations (Kieber et al., 1993; Solano et al., 1998). Experiments were performed with F3- or F4-derived plants for each double mutant. Three to eight lines per double mutant were phenotyped, then at least three were further used in our experiments and two were presented in the results.

Plant Treatments

Seeds were sterilized, sown on Murashige and Skoog agar, and grown under a light period of 16 h (71 μmol m^{-2} s^{-1}) at 21°C. Ten days after sowing, 100 μM ACC (Sigma-Aldrich) or water were added on Murashige and Skoog plates. Ten plantlets were randomly collected at indicated time points.

Ethylene treatment was performed in 3-L tins containing 10 plants and ethylene treatments at different concentrations (5, 20, 80 μL L^{-1}) were injected through a septum. The same experiment was performed with 1-MCP treatment (1 μL L^{-1}). The experiment was done in duplicate.

Ethylene accumulation from adult plants was measured after 8-h incubation in air-tight rubber-cap containers (250 mL). Plants were taken 3 d after lesion appearance in the *vad1-1* mutant. Mean values were obtained from four independent experiments and corresponding SDS are mentioned. Accumulation of ethylene was determined by gas chromatography.

RNA Extraction and Quantitative RT-PCR

Total RNA extraction was performed from leaves with the Nucleospin RNA kit following the manufacturer's instructions (Macherey-Nagel). Total RNA (1 μg) was subjected to cDNA synthesis in a 20-μL reaction, using 0.5 μL of SuperScript II reverse transcriptase (Invitrogen), 1 μg of oligo(dT), and 10 nmol of dNTP. cDNA (diluted 1:10) was used as a template in the quantitative RT-PCR analysis. Quantitative RT-PCR was performed using gene-specific

primers (Supplemental Table S1), LightCycler reagents, and apparatus (Roche Diagnostics). Quantitative RT-PCR was performed using the SYBR GREEN I protocol using 5 pmol of each primer and 1 μL of a 10-fold dilution of RT reaction product in a 10-μL final reaction volume. The PCR conditions are 9 min at 95°C, followed by 45 cycles of 5 s at 95°C, 10 s at 65°C, and 20 s at 72°C. Quantitative PCR reactions were performed with three independent biological assays. Desmine was used as an internal control for RT. *β-tubulin4* and a gene (At2g28390) whose deduced protein belong to the SAND family protein whose expression has been shown to be extremely stable under different physiological conditions (Czechowski et al., 2005) were used as biological controls. Data are expressed as fold induction of each point as compared to the wild type.

Bacterial Strains and Inoculation Procedure

The virulent and avirulent *Pst* strains were grown at 28°C on King's B medium supplemented with the appropriate antibiotics: rifampicin 50 μg mL^{-1} (*DC3000*), rifampicin 50 μg mL^{-1}, and tetracyclin 10 μg mL^{-1} (*DC3000/avrRpm1*).

Four- or 5-week-old plants were used for bacterial inoculation. For this objective, they were kept at high humidity 12 h before experiments and then grown under light-promoting lesions under the following conditions: 9-h light/15-h dark and 90% humidity. Plants were infiltrated with a bacterial suspension of 2×10^5 colony forming units (cfu) mL^{-1} (*DC3000*) or 5×10^5 cfu mL^{-1} (*DC3000/avrRpm1*) for determination of in planta bacterial growth, and 5×10^7 cfu mL^{-1} for gene expression analysis. Determination of in planta bacterial growth was performed as previously described by Lorrain et al. (2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Developmental phenotypes of double mutants.

Supplemental Figure S2. Effect of the *jar1-1* mutation on cell death and defense phenotypes of *vad1-1* plants.

Supplemental Table S1. Primers used for real-time RT-PCR experiments.

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