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Ethylene perception via *ETR1* is required in *Arabidopsis* infection by *Verticillium dahliae*

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SUMMARY

Vascular wilts caused by *Verticillium* spp. are very difficult to control and, as a result, are the cause of severe yield losses in a wide range of economically important crops. The responses of *Arabidopsis thaliana* mutant plants impaired in known pathogen response pathways were used to explore the components in defence against *Verticillium dahliae*. Analysis of the mutant responses revealed enhanced resistance in *etr1-1* [ethylene (ET) receptor mutant] plants, but not in salicylic acid-, jasmonic acid- or other ET-deficient mutants, indicating a crucial role of *ETR1* in defence against this pathogen. Quantitative polymerase chain reaction analysis revealed that the decrease in symptom severity shown in *etr1-1* plants was associated with significant reductions in the growth of the pathogen in the vascular tissues of the plants, suggesting that impaired perception of ET via *ETR1* results in increased disease resistance. Furthermore, the activation and increased accumulation of the *PR-1*, *PR-2*, *PR-5*, *GSTF12*, *GSTU16*, *CHI-1*, *CHI-2* and *Myb75* genes, observed in *etr1-1* plants after *V. dahliae* inoculation, indicate that the outcome of the induced defence response of *etr1-1* plants seems to be dependent on a set of defence genes activated on pathogen attack.

INTRODUCTION

Verticillium dahliae Kleb. is a soil-borne fungal pathogen with a worldwide distribution, causing vascular disease in a wide range of plants, including vegetables (artichoke, aubergine, pepper, potato and tomato), fruits (grapevine, olive and strawberry), flowers (chrysanthemum), oilseed crops (sunflower), fibre crops (cotton, flax) and woody perennials (Pegg and Brady, 2002; Schnathorst, 1981). The control of *V. dahliae* is especially difficult because the fungus survives in the soil as resting structures, microsclerotia, for several years (Schnathorst, 1981), and there are no effective chemical treatments to combat the disease. Furthermore, as it lacks a high degree of host specificity, it can multiply and survive by colonizing several different plant species (Klimes *et al*., 2006; Krikun and Bernier, 1987; Subbarao *et al*., 1995).

On pathogen recognition by plants, several signal transduction pathways are activated. The role of the signalling pathways mediated by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) in the *Arabidopsis* innate immune response is well established (Glazebrook, 2005). However, not much is known about induced plant defence responses to soil-borne pathogens. In some recent studies (Johansson *et al*., 2006; Tjamos *et al*., 2005; Veronese *et al*., 2003), efforts have been made to explore the signal transduction network controlling *Arabidopsis* resistance to *Verticillium* spp. by analysing the pathogen susceptibility, at different developmental stages, of mutant plants defective in ET, JA and SA pathways.

In some case studies, enhanced resistance or tolerance to *V. dahliae* infection was not observed in *Arabidopsis* genotypes affected in different steps of SA signalling, when compared with wild-type (WT) plants (Veronese *et al*., 2003), whereas, in other studies involving *V. longisporum*, the SA mutants *sid2-1* and *pad4-1* were found to be less affected than WT plants (Johansson *et al*., 2006). The results obtained on the activity of the biocontrol agent *Paenibacillus alvei* K165 strain show, however, that the biocontrol-induced response against *V. dahliae* in *Arabidopsis* is dependent on SA via *NPR-1* (Tjamos *et al*., 2005), which further suggests that SA has a positive influence on resistance. Moreover, JA insensitivity does not appear to influence *Verticillium* symptom development in *Arabidopsis* plants (Veronese *et al*., 2003). In addition, none of the *jar1-1*, *coi1-16* or *eds8-1 A. thaliana* mutants impaired in JA signalling show any enhancement of susceptibility when compared with WT in response to *V. longisporum*, which suggests an independence of JA signalling (Johansson *et al*., 2006). Of the *A. thaliana* ET-insensitive mutants tested, some showed enhanced susceptibility, others had similar responses to WT plants and only *etr1-1* was more tolerant than WT on *Verticillium* inoculation (Johansson *et al*., 2006; Tjamos *et al*., 2005; Veronese *et al*., 2003). However, these studies were either performed *in vitro* (Johansson *et al*., 2006; Veronese *et al*., 2003), or the direct aim of the

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study was not the elucidation of the *ETR1*—*Verticillium* interaction (Tjamos *et al*., 2005). In addition, a correlation between *Verticillium* spp. endophytic levels and disease severity in tolerant and susceptible *Verticillium* spp.–host interactions has never been reported, as it was found that *Verticillium* spp. colonization reached the same extent in both types of interaction (Brandt *et al*., 1984; Corsini *et al*., 1988; Gold *et al*., 1996; Heinz *et al*., 1998; Lynch *et al*., 1997; Schnathorst, 1981; Veronese *et al*., 2003).

The ability of plants to induce the expression of defence genes in response to *V. dahliae* via the SA or ET/JA response pathway remains unclear. In a study of the *Arabidopsis–V. dahliae* interaction, no transcripts of *PR-1* or *PDF1.2*, two common marker genes for SA, JA and ET signalling pathways, were observed (Veronese *et al*., 2003); however, in more recent studies, overexpression of the SA, JA and ET marker genes, *PR-1*, *PR-2*, *PR-4*, *PR-5* and *PDF1.2* (Johansson *et al*., 2006; Tjamos *et al*., 2005), was observed on *Verticillium* spp. infection.

The published data on the *A. thaliana*—*V. dahliae* interaction indicate that the molecular mechanisms that control resistance to this pathogen remain poorly understood. Therefore, there is much interest in determining the molecular basis of plant innate immunity against this major pathogen for a broad spectrum of important crops.

In view of the above, the objectives of this study were as follows: (i) to clarify *in planta* the role of SA, JA and ET signalling mutants in defence against *V. dahliae*; (ii) to determine the correlation between disease severity and degree of fungal proliferation in the vascular tissues by real-time quantitative polymerase chain reaction (qPCR); and (iii) to monitor the expression levels of several defence genes involved in the SA, ET/JA and abscisic acid (ABA) response pathways in tolerant and susceptible *V. dahliae*—*A. thaliana* mutant interactions.

RESULTS

Impaired perception of ET via *ETR1* reduces *V. dahliae* wilt symptoms

The role of SA, JA and ET signalling in defence against *V. dahliae* was assessed in *A. thaliana* mutant plants impaired in these pathways. The plant responses to this pathogen were evaluated by the recording of wilt symptoms.

The first *V. dahliae* symptoms appeared in the form of wilting, especially on older leaves at 5 days post-inoculation (dpi), and were recorded until 30 dpi. Disease severity progressed rapidly in all genotypes, except for *etr1-1*, which showed less prominent symptoms and slower disease development (Figs 1A and 2). At 30 dpi, the disease incidence (percentage of plants with wilt symptoms) in *etr1-1* plants was 93% (data not shown) and the disease severity was 22%, whereas, in the other genotypes, the disease incidence ranged from 82% to 100% (data not shown) and the disease severity from 50% to 95% (Fig. 1A). The relative area under the disease progression curve (AUDPC) in *etr1-1* plants was 5.5%, whereas, in the other genotypes, it ranged from 21% to 43.5% (Fig. 1B).

qPCR fungal quantification in ET-insensitive mutants

The data obtained from the pathogenicity tests revealed that only the *etr1-1* mutant plants were less affected by the pathogen of all the SA, JA and ET mutant plants tested and the WT plants. On the basis of these results, we focused our efforts on the investigation of the interaction of *V. dahliae*—ET-insensitive mutants. To determine whether the reduced wilt symptoms of *etr1-1* mutants and the impaired perception of ET via *ETR1* have an impact on fungal growth and colonization in vascular tissues, Col-0 WT plants and the ET-insensitive mutants *etr1-1*, *ein2-1*, *ein3-1*, *ein4* and *ein5-1* were inoculated with *V. dahliae*, and the level of fungal colonization was assessed in each genotype by real-time qPCR. In these experiments, we included all ET-insensitive mutants in an effort to obtain information on each component of the ET signalling pathway and to investigate whether the disease symptoms are associated with the amount of the pathogen in the vascular tissues of the plants.

qPCR analysis showed that, by 5 dpi, *V. dahliae* was present in the vascular tissues of all genotypes (Fig. 3). The presence of *V. dahliae* decreased at 10 dpi, except for Col-0 and *ein2-1*, and then increased steadily until 25 dpi (Fig. 3). The same colonization pattern was observed in all genotypes; however, in the ET receptor mutant plants *etr1-1*, the levels of *V. dahliae* were significantly lower than in the WT plants and the rest of the other ET mutants at all sampling time points, except 5 dpi (Fig. 3). At 10 dpi, the amount of *V. dahliae* in *etr1-1* plants was at least 4.9 times less than that in the other genotypes, and at 15, 20 and 25 dpi the difference ranged from 4.4 to 6.6 times. Interestingly, the amount of *V. dahliae* in *ein4* plants, the other ET receptor mutant, was significantly greater than that in *etr1-1* plants at each sampling time point, ranging from 5.2 times at 5 dpi to a maximum of 11.3 times at 20 dpi.

Transcriptional changes in response to *V. dahliae* infection

Our data from the pathogenicity tests and fungal quantification indicated that *etr1-1* plants showed increased resistance to *V. dahliae*. We reasoned that the identification of genes up-regulated in *etr1-1* plants compared with WT plants would elucidate potential components of basal resistance operating in this mutant. We used Affymetrix Arabidopsis ATH1 GeneChips representing approximately 24 000 genes to examine the transcriptional profiles of WT and *etr1-1* plants. By comparing the

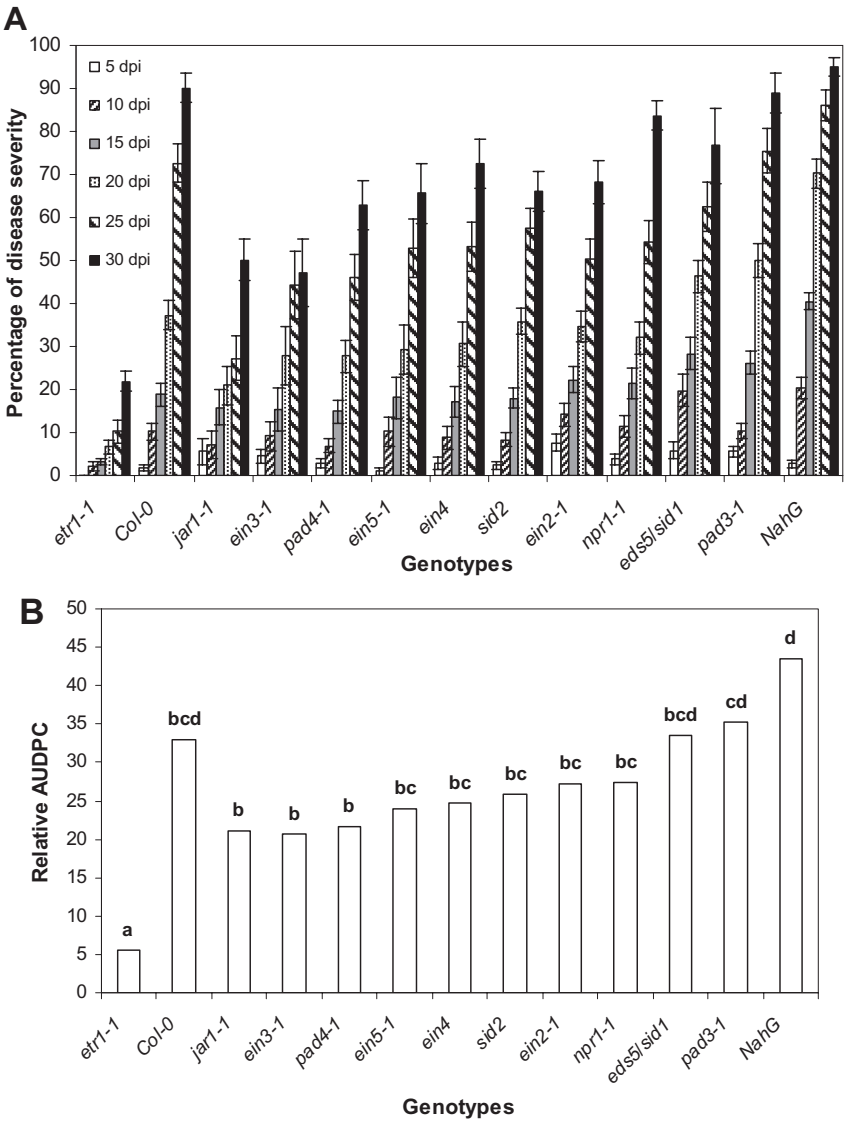

Fig. 1 (A) Percentage disease severity of *Arabidopsis thaliana* plants after inoculation by *Verticillium dahliae*. Disease severity at each observation was calculated from the number of leaves that showed wilting as a percentage of the total number of leaves of each plant. Each genotype consisted of 15 plants and the experiment was repeated three times with similar results. The columns represent the means of 15 plants and the vertical bars indicate the standard errors. (B) Disease ratings were plotted over time to generate disease progress curves; subsequently, the area under the disease progress curve (AUDPC) was calculated by the trapezoidal integration method (Campbell and Madden, 1990), and disease was expressed as a percentage of the maximum possible area for the whole period of the experiment, which is referred to as the relative AUDPC (Korolev *et al*., 2001). Columns with different letters are statistically significantly different according to Tukey's multiple range test at $P \leq 0.05$.

transcriptional profiles of *etr1-1* with WT plants, we identified 95 genes up-regulated by more than two-fold in *etr1-1* plants (Table S1, see Supporting Information). The Affymetrix microarray experiment was not repeated; therefore, we considered these genes as candidates for verification. From the candidate genes, we selected, for qPCR analysis over time, a group of nine genes (Table S1) that were reported in the literature to be involved in the resistance of plants against pathogens. The qPCR expression analyses were performed at different time points after *V. dahliae* inoculation. In these experiments, we also included 23 marker genes of the SA, ET, JA and ABA defence response pathways (Table 1) in an effort to obtain more information about the defence responses of plants at different sampling days. The experimental set-up included the ET receptor mutants *etr1-1* and *ein4* and Col-0 WT plants. We included *ein4* plants in our analysis in an initial attempt to dissect the role of ET perception between *ETR1* and *EIN4* in the defence response of plants against *V. dahliae*, as both *ETR1* and *EIN4* encode ET receptors. ETR1 is a

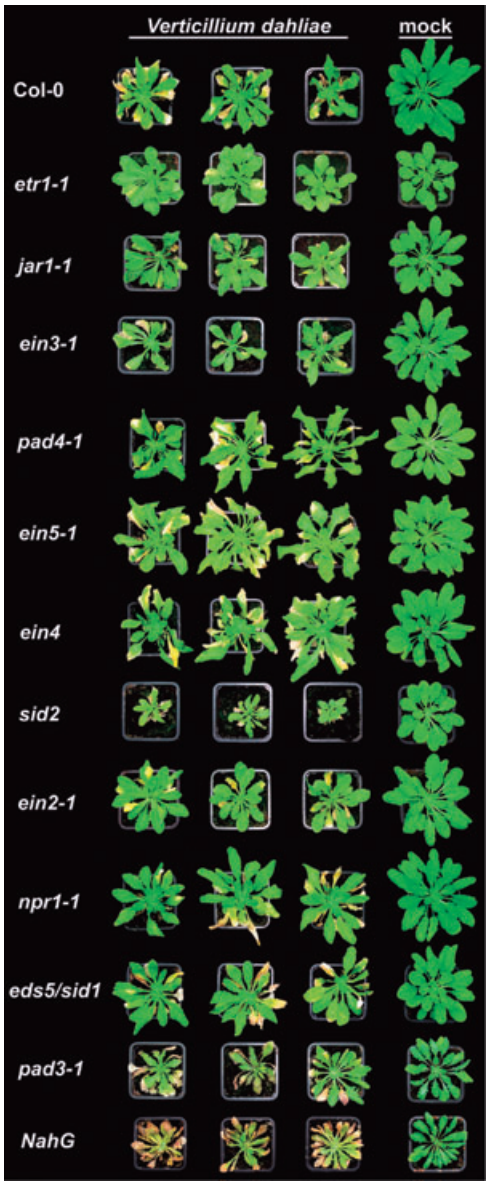

Fig. 2 Typical symptoms caused by *Verticillium dahliae* on *Arabidopsis* salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) mutants. The mutants *etr1-1*, *ein2-1*, *ein3-1*, *ein4*, *ein5-1*, *jar1-1*, *pad3-1*, *pad4-1*, *sid2*, *npr1-1*, *NahG*, *eds5/sid1* and the corresponding wild-type Col-0 were inoculated with *V. dahliae* or mock inoculated. *V. dahliae*-inoculated *etr1-1* mutants show reduced symptom development, including less severe stunting, wilting and tissue necrosis, when compared with Col-0 and the other mutant plants. Photographs were taken at 20 days post-inoculation.

member of the type I and EIN4 is a member of the type II subfamily of ET receptors (Guo and Ecker, 2004), but, as shown in this study, they exhibited opposite defence responses against *V. dahliae*. qPCR analysis confirmed constitutively higher expression of the candidate genes *GSTF12*, *GSTU16* (glutathione-*S*-transferases, GSTs), *CHI-1*, *CHI-2* (chitinases), *PR-5* (thaumatin-like), *PR-1*, *PR-2* (β-1,2-glucanase) and the *Myb75* transcription factor in *etr1-1* plants compared with WT and *ein4* plants over time (Fig. 4). The mRNA level of this set of genes was induced at 2 dpi in all genotypes (Fig. 4). The expression level of *GSTF12* and *PR-5* was greater in *etr1-1* mutants than in *ein4* and WT plants, whereas there was no difference between treatments in the expression levels of the other genes at 2 dpi (Fig. 4A,G). Similarly, but to a greater extent, the expression levels of all genes at 6, 10 and 14 dpi were higher in the *V. dahliae*—*etr1-1* interaction compared with the other treatments. The genes *GSTU16* and *PR-2* exhibited the strongest up-regulation in *etr1-1* plants at 10 dpi, whereas *GSTF12* and *PR-1* reached their maximum expression value at 14 dpi (Fig. 4A,B,E,F). *CHI-2* and *PR-5* exhibited similar expression patterns in all treatments, and showed the highest expression values at 10 and 14 dpi in *etr1-1* plants (Fig. 4D,G); the transcript levels of *CHI-1* and *Myb75* were strongly expressed from 6 to 14 dpi (Fig. 4C,H). Interestingly, overexpression of the transcript levels of all examined genes was observed in all genotypes in response to *V. dahliae*, indicating the activation of plant defence mechanisms to fungal attack. It is worth mentioning that the expression of these genes in mock-inoculated *etr1-1* plants was at least at the same level as that of mock-inoculated WT and *ein4* plants (data not shown).

DISCUSSION

Verticillium dahliae is a soil-borne plant pathogen with a worldwide distribution which causes vascular diseases resulting in severe yield losses in a wide range of economically important crops. Vascular wilts caused by *V. dahliae* constitute serious threats for their hosts, as there are no effective chemical control treatments. Although numerous studies have examined the interaction of *Verticillium* spp. with many hosts, the genetic basis and molecular mechanisms that control resistance to this pathogen remain poorly understood.

SA, JA and ET are plant hormones that act as signalling molecules in basal defence responses of plants, as well as in gene-for-gene-mediated defence (Thomma *et al*., 2001). The role of these signalling pathways in the reaction of plants to *Verticillium* spp. has been documented in a number of recent studies (Johansson *et al*., 2006; Tjamos *et al*., 2005; Veronese *et al*., 2003), but their influence remains unclear.

Veronese *et al*. (2003) demonstrated *in vitro* that *V. dahliae* infection in *A. thaliana* SA mutants did not result in enhanced resistance or tolerance to the pathogen; however, in a more recent *in vitro* study implicating *V. longisporum*, the SA mutants *sid2-1* and *pad4-1* were less affected compared with WT plants, suggesting a role of SA in resistance to *V. longisporum* (Johansson *et al*., 2006). In addition, the role of SA has been proposed in the protection of cotton callus cells from *V. dahliae* toxins (Zhen and Li, 2004). In the present study, it was shown *in planta* that all

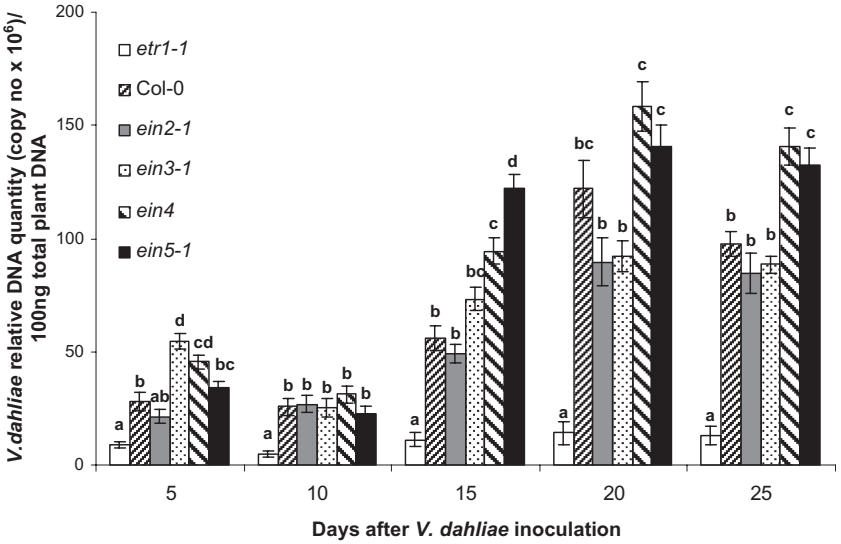

Fig. 3 Quantification of the *Verticillium dahliae* DNA levels in the ethylene (ET) mutants *etr1-1*, *ein2-1*, *ein3-1*, *ein4*, *ein5-1* and Col-0 plants. The fungal DNA levels were estimated by quantitative polymerase chain reaction (qPCR) using total plant DNA isolated from the above-ground parts of 10 plants per genotype and sampling at 5, 10, 15, 20 and 25 days post-inoculation (dpi). The experiment was repeated three times. The columns represent the means of 30 plants and the vertical bars indicate the standard errors. At each sampling day, columns with different letters differ significantly according to Tukey's multiple range test at $P \leq 0.05$.

the SA screened mutants had similar responses to Col-0 after *V. dahliae* inoculation (Figs 1 and 2), suggesting that SA is not involved in the resistance of plants against this vascular wilt pathogen. Our results are in agreement with the *in vitro* studies of Veronese *et al*. (2003), in which a *V. dahliae* isolate was used.

Mutants impaired in JA signalling (*jar1-1*, *coi1-16* and *eds8-1*) did not appear to influence disease severity caused by *V. dahliae* or *V. longisporum* in *in vitro* experiments (Johansson *et al*., 2006; Veronese *et al*., 2003). In our study, *jar1-1* plants showed similar levels of disease severity to WT plants after *V. dahliae* inoculation (Figs 1 and 2), indicating that JA does not influence the disease outcome incited by this pathogen.

Furthermore, the role of ET is even more complicated in disease resistance mechanisms and it appears to be involved in only particular classes of pathogens (Hoffman *et al*., 1999; Knoester *et al*., 1998; Thomma *et al*., 1999). In recent *in vitro* studies (Johansson *et al*., 2006; Veronese *et al*., 2003), it was shown that, on *Verticillium* spp. inoculation, the *etr1-1* mutant was more tolerant than WT plants, whereas the *ein3-1* and *eto1-1* mutants showed similar responses, and the *ein2-1*, *ein4-1* and *ein6-1* mutants exhibited enhanced susceptibility, compared with WT plants (Johansson *et al*., 2006). Of the ET-insensitive mutants tested *in planta* in the present study, we observed that the *etr1-1* plants were the only resistant mutants compared with WT against *V. dahliae* (Figs 1 and 2). ETR1 is a member of the type I and EIN4 is a member of the type II subfamily of ET receptors (Guo and Ecker, 2004), and this might explain the different responses of *etr1-1* and *ein4* mutant plants to *V. dahliae*. The observed difference in disease severity between *etr1-1* and *ein2-1*, *ein3-1* and *ein5-1* may originate from the fact that *EIN2*, *EIN3* and *EIN5* genes are positive regulators of ET responses, acting downstream of *ETR1*, which acts as a negative regulator of ET (Guo and Ecker, 2004).

A lack of a positive correlation between pathogen growth within plant tissues and symptom severity has been noted in several fungal (Brandt *et al*., 1984; Corsini *et al*., 1988; Gold *et al*., 1996; Heinz *et al*., 1998; Lynch *et al*., 1997; Schnathorst, 1981; Veronese *et al*., 2003), bacterial (Bent *et al*., 1992; Lund *et al*., 1998; O'Donnell *et al*., 2001) and viral (Cecchini *et al*., 2002) plant–pathogen interactions. A possible explanation of this interesting decoupling could be that symptoms can result from pathogen-induced signalling events that cause changes in normal plant growth and development, such as accelerated flowering, senescence and programmed cell death (Cecchini *et al*., 2002; Dietrich *et al*., 1994; Lund *et al*., 1998; O'Donnell *et al*., 2001; Piloff *et al*., 2002). However, in this study, the extent of *V. dahliae* growth within the vascular tissues of plants was determined to be positively correlated ($r^2 = 0.744$; d.f. = 28; $P < 0.01$) with disease severity. qPCR analysis revealed that the level of *V. dahliae* DNA in the ET mutant *etr1-1*, which exhibited the most resistant phenotype against *V. dahliae*, was significantly lower than that in WT plants and the other ET mutant plants at each sampling day, except for 5 dpi (Fig. 3).

In the *V. dahliae*—*Arabidopsis* bioassays, it was observed that the pathogen DNA level reached a maximum at 20 dpi and then declined (Fig. 3), whereas the maximum disease severity was observed at 30 dpi (Fig. 1A). This failure to increase after 20 dpi probably reflects reduced sporulation in the stem and coincides with the cyclical periods of fungal elimination that characterize the lifestyle of *V. dahliae* in the vascular system of plants, such as tomato and oilseed rape (Chen *et al*., 2004; Eynck *et al*., 2007; Heinz *et al*., 1998). Overall, qPCR analysis revealed that the pathogen levels in the vascular tissues of resistant *etr1-1* plants remained substantially lower than in WT and the other ET mutant plants at all sampling days. It is evident that the *etr1-1* resistance response is based on the activation of defence mecha-

Table 1 List of examined genes and their primer pairs used in the quantitative polymerase chain reaction (qPCR) experiments.

Gene	AGI code	Primer pairs
PR-1	AT2G14610	5′-TCACAACCAGGCACGAGGAG-3′
		5′-CACCGCTACCCCAGGCTAAG-3′
PR-2	AT3G57260	5′-GCTCTCCGTGGCTCTGACATC-3′
		5′-TACCGGAATCTGACACCATCTCTG-3′
PR-3	AT3G12500	5′-TTATCACCGCTGCAAAGTCCT-3′
		5′-TGGCGCTCGGTTCACAGTA-3′
PR-4	AT3G04720	5′-ATAATCCGGCGCAGAATAAT-3′
		5′-GCGGTCCAGCCATACTTG-3′
PR-5	AT1G75040	5′-GACTCCAGGTGCTTCCCGACAG-3′
		5′-ACTCCGCCGCCGTTACATCTT-3′
PDF1.2	AT5G44420	5′-CTGTTACGTCCCATGTTAAATCTACC-3′
		5′-CAACGGGAAAATAAACATTAAAACAG-3′
CHI-1	AT2G43620	5′-CGGCTGCCCAATCGTTCG-3′
		5′-ACCGCGGCCGTAGTAGTCTTTTC-3′
CHI-2	AT2G43570	5′-GCTTCGGTGCTTCCATCTCC-3′
		5′-GCCATAGTAGCCCTTTCCTTGTG-3′
WRKY18	AT4G31800	5′-AGCGCAAGTGAGTTACGAG-3′
		5′-ATCCGGGTCTTGTTTTCTTTTCT-3′
WRKY22	AT4G01250	5′-CCCGTAAACAAGTGGAGCGAAATA-3′
		5′-CACCGGAGACGATGAATAAGTAGC-3′
WRKY30	AT2G38470	5′-AGTACGGACAAAAACAGGTGAAA-3′
		5′-TCGTTGGACAATTAGGGAAAGT-3′
WRKY40	AT1G80840	5′-GCTTAAACCGCCACATCTCT-3′
		5′-GTAGTCACCGGCACAGTCAAG-3′
WRKY53	AT4G23810	5′-CAGACGGGGATGCTACGGTTTTC-3′
		5′-CGGCGAGGCTAATGGTGGTGT-3′
WRKY60	AT2G25000	5′-TAATCTTATGGAGGAGTTGC-3′
		5′-ACCGTCGCTTTATCGTT-3′
ERF	AT5G50080	5′-GTTTGGCTCGGGACGTTTGATAC-3′
		5′-AGAGGAGGGGGAGGAGGAAGAAT-3′
ERF2	AT3G16770	5′-CGGGGAAACGGAGGAAGAGG-3′
		5′-GCGATGACGGCGGAGGAGTAT-3′
Myb32	AT4G34990	5′-TCATCAAACTACATAGCCTTCTCG-3′
		5′-CCCTTTTCTTAATAGCTTCCTCTT-3′
Myb75	AT1G56650	5′-GATCTTCTTCTTCGCCTTCA-3′
		5′-CACGGTTCATGTTTCTTACTCA-3′
VSP1	AT5G24780	5′-TTTTACGCCAAAGGACTTGC-3′
		5′-AATCCCGAGTTCCAAGAGGT-3′
VSP2	AT5G24770	5′-TCAGTGACCGTTGGAAGTTGTG-3′
		5′-GTTCGAACCATTAGGCTTCAATATG-3′
ATGSTF8	AT2G47730	5′-CTCGAAGGTAAGCTCCAGAAAG-3′
		5′-TCACCAGCCAAGAACTCAGATT-3′
ATGSTF12	AT5G17220	5′-GGTCAAGTTCCAGCCATAGA-3′
		5′-TTGCCCAAAAGGTTCGT-3′
ATGSTU16	AT1G59700	5′-TCGCTCTTCGTCTCAAATCAGTGG-3′
		5′-AATCGGTTTGTTGTTGTGGAGGAG-3′
ATGSL05	AT4G03550	5′-GAATGCATTATGGCCACCCTGAT-3′
		5′-TTAAACCCGGCAAAGATGTCCTC-3′
RAB	AT3G02480	5′-AGGATGCTGCTGCTTCA-3′
		5′-TTAGTGGCTTTTGTTCAT-3′
RAB18	AT5G66400	5′-AGTGGTGGTGGCTTGGGAGGAAT-3′
		5′-ACCACCGTAGCCACCAGCATCAT-3′
UGT73B1	AT4G34138	5′-CATATCGGTCCGCTTTCCTTAG-3′
		5′-CTTGCCTTTTTGCCTCTTTCTG-3′
SDR1	AT1G52340	5′-AACTCGCTTTGGCTCATTTG-3′
		5′-GTCAGTTCCACCCCTTTTAGATTC-3′
AtMYC2	AT1G32640	5′-TCATACGACGGTTGCCAGAA-3′
		5′-AGCAACGTTTACAAGCTTTGATTG-3′
RD22	AT5G25610	5′-CTGTTTCCACTGAGGTGGCTAAG-3′
		5′-TGGCAGTAGAACACCGCGA-3′
ABI1	AT4G26080	5′-CGGCAAAACTGCACTTCCAT-3′
		5′-CACGAGCTCCATTCCACTGAA-3′
KIN1	AT5G15960	5′-GCTGGCAAAGCTGAGGAGAA-3′
		5′-TTCCCGCCTGTTGTGCTC-3′

AGI, Arabidopsis Genome Initiative.

nisms. These mechanisms have been partially investigated in plant—*V. dahliae* interactions, but never in *etr1-1* mutant plants.

To effectively intercept the attack by pathogens, plants have evolved complex defence strategies and responses (Dicke and Hilker, 2003; Jones and Dangl, 2006; Pieterse and Van Loon,, 2004) regulated by SA, JA and ET signal transduction pathways (Glazebrook, 2001; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998; Thomma *et al*., 2001). SA, JA and ET accumulate in response to pathogen infection, leading to the activation of distinct sets of defence-related genes (Glazebrook *et al*., 2003; Schenk *et al*., 2000). Earlier studies on the induction of SA, JA and ET marker genes in the *Arabidopsis–Verticillium* interaction exhibited conflicting results. In one case, no transcripts of *PR-1* and *PDF1.2* were detected (Veronese *et al*., 2003), whereas, in other studies, the expression levels of *PR-1*, *PR-2*, *PR-4*, *PR-5* and *PDF1.2* genes were induced after *Verticillium* inoculation (Johansson *et al*., 2006; Tjamos *et al*., 2005). However, no data are available on the expression of marker genes of the different signalling pathways in SA-, JA- and ET-deficient mutant plants showing either a susceptible or tolerant response after *V. dahliae* inoculation.

Preliminary results of microarray genome analysis (DNA Vision S.A., Charleroi, Belgium) on RNA isolated from WT and *etr1-1* plants indicated an overexpression of a set of genes in *etr1-1* plants 6 days after *V. dahliae* inoculation (Table S1). The expression level of a group of 32 defence-related genes (Table 1) was determined by qPCR analysis at different time points after inoculation with *V. dahliae*. The results revealed that eight genes (*GSTF12*, *GSTU16*, *CHI-1*, *CHI-2*, *PR-1*, *PR-2*, *PR-5*, *Myb75*) were overexpressed in *etr1-1* plants compared with WT and *ein4* plants (Fig. 4), suggesting that this set of genes might be possible determinants for the resistant phenotype against the pathogen. In a previous study based on microarrays, transcription profiles were determined for stem tissue of Craigella tomato plants infected with two different *V. dahliae* isolates, Vd1 and E6, resulting in a compatible and a tolerant interaction, respectively (Robb *et al*., 2007). In this study, as with our findings, up-regulation of PR1 (P4, P6), PR2 (β-1,3-glucanase), endo-β-1-3-glucanase (SGN-U144863), acidic 25-kDa endochitinase (SGN-U144297) and class IV chitinase (SGN-U145299) was observed in both types of interaction, whereas only endochitinase 3 and a putative GST (SGN-U143283) were elevated in the tolerant interaction (Robb *et al*., 2007). In a more recent study, tomato transcriptional responses to *V. dahliae* race1 were monitored after inoculation on MoneyMaker (susceptible genotype) and Motelle (resistant genotype against race 1 *Verticillium* strains) tomato plants using microarrays (van Esse *et al*., 2009). van Esse *et al*. (2009) noted the induction of *PR5* and chitinase genes in both the roots and foliage of the incompatible interaction, which is in agreement with our results.

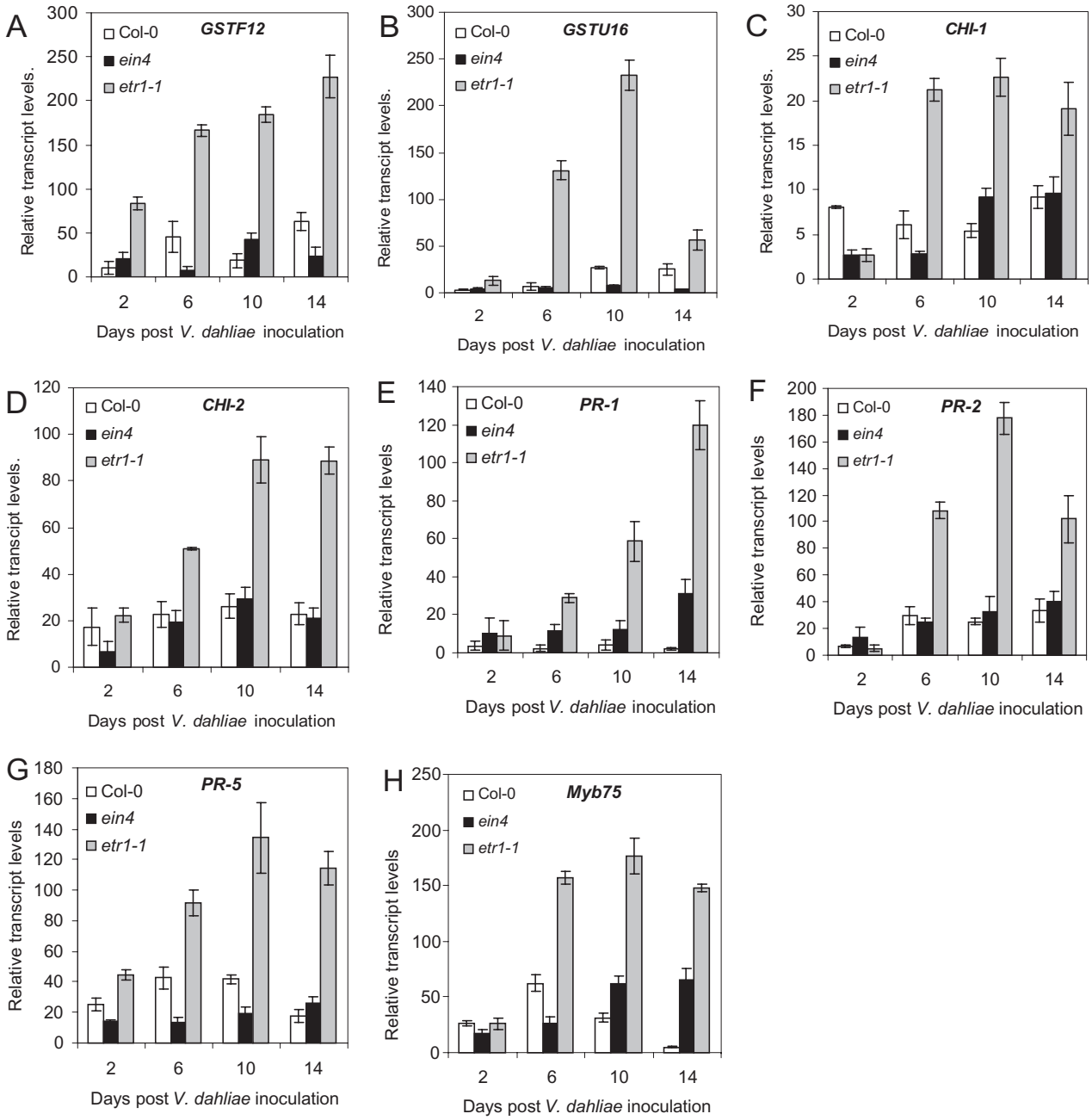

Fig. 4 Expression of *GSTF12* (A), *GSTU16* (B), *CHI-1* (C), *CHI-2* (D), *PR-1* (E), *PR-2* (F), *PR-5* (G) and *Myb75* (H) in *Arabidopsis thaliana* Col-0, *ein4* and *etr1-1* plants in response to infection with *Verticillium dahliae*. RNA was isolated from the above-ground parts of 10 plants per genotype with sampling at 2, 6, 10 and 14 days post-inoculation (dpi). Poly(A)$^+$-RNA was reverse transcribed to cDNA, and subjected to quantitative polymerase chain reaction (qPCR). Transcript levels in the different samples were normalized to those of the constitutive gene α-tubulin. The relative mRNA level was calculated with respect to the level of the corresponding transcript in uninfected plants. Each genotype consisted of 80 plants (40 *V. dahliae*- and 40 mock-inoculated) and the experiment was repeated twice. The columns represent the means of 20 plants and the vertical bars indicate the standard errors.

In the present study, the SA marker genes *PR-1*, *PR-2* and *PR-5* were clearly up-regulated in the *etr1-1*–*V. dahliae* resistance response, even though SA mutant plants did not show a tolerant or a more susceptible phenotype than WT plants (Fig. 1), indicating the occurrence of cross-talk between the different signalling pathways (De Vos *et al*., 2005; Koornneef and Pieterse, 2008; Koornneef *et al*., 2008). Previous studies have shown that PR-1, PR-2 and PR-5 proteins have a role in resistance to fungal

infection (Abad *et al*., 1996; Alexander *et al*., 1993; Li *et al*., 2003; Menu-Bouaouiche *et al*., 2003; Niderman *et al*., 1995; Pressey, 1997; Wessels and Sietsma, 1981). PR-1 represents a dominant group of PRs induced by pathogens or SA, and is commonly used as a marker for systemic acquired resistance. There is increasing evidence that PR-1 proteins may play a role in the resistance to fungal infection, but their mode of action and relationship to other proteins are unknown (van Loon and van Strien, 1999). The PR-2 family consists of β-1,3-glucanases that hydrolyse β-1,3-glucans, which are major fungal cell wall components (Wessels and Sietsma, 1981), therefore weakening the fungal cell wall and preventing hyphal colonization. However, reaction products of their enzymatic activity may act as elicitors of host defence responses (Menu-Bouaouiche *et al*., 2003). Recent work has demonstrated the different timing and level of β-1,3-glucanase activity between a *V. dahliae*-susceptible and *V. dahliae*-tolerant cotton cultivar in response to treatment with a *V. dahliae* toxin, with the activity of β-1,3-glucanase increasing to a higher level at an earlier time point in the resistant cultivar (Li *et al*., 2003). The PR-5 family includes proteins that are homologous to the protein thaumatin with *in vitro*-demonstrated antifungal activity against *V. dahliae* (Abad *et al*., 1996; Pressey, 1997).

Another class of proteins that has been studied extensively in plant defence is the family of chitinases, because of their potential in defence reactions against various pathogens. Chitinases of all families were found to have antifungal activity *in vitro* (Melchers *et al*., 1994; Ponstein *et al*., 1994) against fungi that contain chitin in their cell walls. However, only a few fungi are sensitive to chitinases alone. Most of the fungi are sensitive to the synergistic action of chitinases and β-1,3-glucanases (Mauch *et al*., 1988; Sela-Buurlage *et al*., 1993). *In vivo*, the constitutive expression of a chitinase in transgenic wheat resulted in enhanced resistance against *Fusarium graminearum* (Shin *et al*., 2008). In the present study, two putative chitinases have been found to be highly expressed in *etr1-1* plants after *V. dahliae* inoculation. qPCR analysis revealed an early transcript accumulation of the two putative chitinases at 2 dpi in all genotypes. The high expression levels of *PR-2*, *CHI-1* and *CHI-2* in *etr1-1* plants may indicate a synergistic action of the enzymatic products of these genes against *V. dahliae*, without excluding the participation of PR-1 and PR-5 (Mauch *et al*., 1988; Sela-Buurlage *et al*., 1993).

In addition to the aforementioned pathogenesis-related proteins, several studies have indicated that plants respond to infection by pathogens with an increased expression of signalling or transcription factors, such as GST and Myb (Hahn and Strittmatter, 1994; Mauch and Dudler, 1993; Wagner *et al*., 2002). In a recent work, *GST* gene expression was induced more rapidly and more strongly in a *V. dahliae*-resistant cotton cultivar than in a susceptible cultivar (Jia *et al*., 2007). In the present study, two *GST* genes, *GSTF12* and *GSTU16*, were up-regulated in all genotypes after *V. dahliae* inoculation. *GSTF12* was found to be expressed more strongly in *etr1-1* plants compared with WT and *ein4* plants at each sampling day. Interestingly, the expression level of the *GSTU16* gene was not substantially up-regulated in *V. dahliae*-inoculated *ein4* plants compared with mock-inoculated *ein4* plants. It is evident that, in addition to the observed differences in pathogenesis-related genes (*PR1*, *PR2*, *PR5* and chitinases), differences in the signalling cascade may also be responsible for the observed *V. dahliae* tolerance of *etr1-1* plants, as revealed by the overexpression of *GSTF12* and *GSTU16* genes in *etr1-1* plants.

Recent genetic analyses have shown that several genes encoding Myb transcription factors have important roles in plant immune responses, with some Myb factors binding to promoters of defence-associated genes (Rushton and Somssich, 1998). A T-DNA insertion in a Myb-encoding gene of *Arabidopsis* resulted in enhanced disease symptoms after infection with several biotrophic and necrotrophic pathogens (Mengiste *et al*., 2003). In the present study, the transcription factor-encoding gene *Myb75* was induced at 2 dpi after *V. dahliae* inoculation in all genotypes. It was highly expressed in *etr1-1* plants at 6, 10 and 14 dpi.

A molecular basis for the control of increased resistance to *V. dahliae* in *A. thaliana* plants has been shown in the present study, indicating for the first time in the literature that impaired ET perception via *ETR1* induces altered expression of a subset of defence-related genes. These transcriptional changes led to reduced fungal growth in vascular tissues and symptom development in *etr1-1* plants. It is an open question whether *V. dahliae* employs ET perception via *ETR1* to escape the activation of the plant defence mechanisms, as it is known that *V. dahliae* produces ET *in vitro* (Tzeng and DeVay, 1984). *Verticillium dahliae* constitutes a serious threat for a number of crops, as there is no chemical treatment; in the present study, we have attempted to elucidate and show the key role of the *ETR1* gene in a plant defence mechanism against this vascular wilt pathogen as a step towards the understanding and uncoupling of a plant–pathogen interaction that threats and reduces the agricultural capita in a world that demands additional safer plant products.

EXPERIMENTAL PROCEDURES

Origin of seeds

Seeds of *A. thaliana* ecotype Columbia (Col-0) and the transgenic line NahG (Delaney *et al*., 1994) were provided by Syngenta (Basle, Switzerland); Col-0 accession mutants *etr1-1* (Bleecker *et al*., 1988), *ein2-1*, *ein3-1*, *ein4*, *ein5-1* (Roman *et al*., 1995), *jar1-1* (Staswick *et al*., 1992), *npr1-1* (Cao *et al*., 1997),

pad3-1 (Glazebrook and Ausubel, 1994) and *pad4-1* (Glazebrook *et al*., 1996) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK), and *eds5/sid1* and *sid2* (Nawrath and Métraux, 1999) were provided by J. P. Métraux (University of Fribourg, Switzerland). All seeds were stored at 4 °C. Seeds were sown directly into 6-cm-diameter pots, each containing approximately 200 cm^3 of soil (Potground; Klasmann, Deilmann, Germany). The pots were placed at 25 °C with a 14 h photoperiod at 65–70% relative humidity in a controlled-environment growth chamber. The plants were watered and fed with a nutrient solution (XL 60, Hortifeeds, Lincoln, UK) as needed.

Fungal strains and inoculum preparation

The *V. dahliae* (Tjamos *et al*., 2005) isolate with known pathogenicity against *A. thaliana* plants was used in the experiments. The fungal strain was cryopreserved by freezing a suspension of 4×10^7 conidia/mL in 25% aqueous glycerol at −80 °C (Maniatis *et al*., 1982). Before being used, fungus was transferred to potato dextrose agar (Merck, Darmstadt, Germany) at 25 °C for 5 days. For the bioassays, conidia of the fungus were prepared in sucrose sodium nitrate (Sinha and Wood, 1968) in Erlenmeyer flasks of 250 mL capacity containing 100 mL of medium. The *V. dahliae* isolate was incubated in an orbital incubator at 120 rpm and 22 °C for 5 days. Suspensions were centrifuged at 10 000 ***g*** at 12 °C for 10 min, and resuspended by vortexing in sterile distilled water before treatment of the plants.

Verticillium dahliae—*Arabidopsis* bioassays

Three-week-old plants were inoculated with *V. dahliae* by root drenching 10 mL of 1×10^7 conidia/mL sterile distilled water (Tjamos *et al*., 2005). Control plants were mock inoculated with 10 mL of sterile distilled water. Disease severity at each observation was calculated from the number of leaves that showed wilting as a percentage of the total number of leaves of each plant, and was periodically recorded for 30 days after inoculation. Disease ratings were plotted over time to generate disease progression curves. AUDPC was calculated by the trapezoidal integration method (Campbell and Madden, 1990). Disease was expressed as a percentage of the maximum possible area for the whole period of the experiment, which is referred to as the relative AUDPC. The experiment was repeated three times with 15 replicates per experiment.

DNA extraction and qPCR fungal quantification

Ten plants from each treatment were harvested for real-time qPCR analysis at 5-day intervals from 5 dpi to 25 dpi. For each sampled plant, the above-ground parts were cut at soil level, rinsed with sterile distilled water and ground to a fine powder using an autoclaved mortar and pestle in the presence of liquid nitrogen. Roots were sampled as well, but they are not included in the analysis as there was a statistical fluctuation of fungal quantification. The fluctuation probably reflected the impossibility of discriminating fungal biomass within the roots from fungus partly attached to the root surface through inoculation (Eynck *et al*., 2007). Total DNA was isolated according to Dellaporta *et al*. (1983), and was quantified by spectrophotometry and agarose gel electrophoresis. qPCR assays for the quantification of *V. dahliae* were conducted using the primer pair Vd-F (5′-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3′) and Vd-R (5′-CGCCTGCGGGACTCCGATGCGAGCTGTAAC-3′) designed on the ITS1 and ITS2 regions of the 5.8S ribosomal RNA gene (Z29511) of *V. dahliae*. qPCR was performed in a Stratagene Mx3005P™ thermocycler and, for the amplification reactions, QuantiFast™ SYBR® Green PCR (Qiagen, Valencia, CA, USA) master mix was used. The results were analysed with MxPro qPCR software. The levels of the *Arabidopsis* α2-tubulin gene (M84696), detected using the primer pair TUBa-F (5′-TCCGCGAAACGAAAATG-3′) and TUBa-R (5′-TGGCTCAAGATCAACAAAGAC-3′), were used as internal standards to normalize the differences in DNA template amounts. PCR cycling started with an initial step of denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The primer specificity and formation of primer dimers were monitored by dissociation curve analysis. All qPCRs were performed in duplicate. To quantify the DNA levels of *V. dahliae*, a PCR product of the primer pair Vd-F/Vd-R was cloned into the commercial vector pGEM (Promega, Madison, WI, USA) and seven 10-fold dilutions of the plasmids, ranging from 3×10^9 copies to 3×10^3 copies, were used to generate a standard curve ($r^2 \geq 0.99$). The experiments were repeated three times.

RNA isolation and qPCR determination of transcript levels

For *Verticillium*-caused wilt diseases, the resistance or susceptibility of the host plant is generally considered to be determined mainly by the cellular interactions of plant against fungus, occurring in the stem (Beckman, 1987; Beckman and Talboys, 1981; Heinz *et al*., 1998). In the present study, above-ground parts from the pathogen- and mock-inoculated plants were collected for RNA analysis. Samples were collected from 10 randomly selected plants per treatment per experiment and were immediately frozen in liquid nitrogen and stored at −80 °C. For each sample, total RNA was extracted from 100 mg of tissue ground with liquid nitrogen using TRIzol® Reagent (Invitrogen, Paisley, Renfrewshire, UK), according to the manufacturer's instructions. The RNA samples were treated with DNase I (Invitrogen) to eliminate traces of contaminating genomic DNA. The RNA concentration was measured on a Nanodrop ND-1000 spectropho-

tometer (Saveen Werner, Malmö, Sweden). First-strand cDNA was synthesized using SuperScript II (Invitrogen) following the manufacturer's procedure. Gene-specific primers for the analysed genes were designed (Table 1). PCR efficiency for each amplicon was calculated by employing the linear regression method on log(fluorescence) per cycle number data, using LinRegPCR software (Remakers *et al*., 2003). The qPCRs were performed in duplicate, as described previously. The absence of nonspecific products and primer dimers was confirmed by the analysis of melting curves. The *A. thaliana* α2-tubulin gene (M84696) was used as an internal standard to normalize small differences in cDNA template amounts. For data analysis, average threshold cycle (C_t) values were calculated for each gene of interest (Pfaffl, 2001) on the basis of two independent biological samples.

Microarray sample preparation and data analyses

Total RNA from the same biological samples as used for qPCR at 6 dpi was employed for microarray analysis. Material harvested from mock-inoculated WT and *etr1-1* plants at 6 dpi was used as the reference sample with which *V. dahliae*-inoculated WT and *etr1-1* samples, respectively, were compared. RNA extraction was performed as described previously. Total RNA was sent to DNA Vision S.A. for further processing. RNA was hybridized onto the Affymetrix Arabidopsis ATH1 GeneChip Genome Array (Affymetrix, Inc. US, Santa Clara, CA, USA), which contains more than 22 500 probe sets representing approximately 24 000 gene sequences. Probe preparations, GeneChip hybridizations, washes and chip reading were conducted at DNA Vision S.A. following standard Affymetrix procedures.

To isolate candidate genes from the microarray expression data, the FiRe macro was used (Garcion *et al*., 2006). The selection of candidate genes was based on their fold-change ratios after *V. dahliae* inoculation compared with mock-inoculated plants. Genes showing at least twofold mRNA up-regulation in *etr1-1* plants and not in WT plants were selected for further analysis by qPCR.

Statistics

Data on relative AUDPC and *V. dahliae* DNA quantification were transformed with the $\sqrt{x}+1$ transformation before analysis of variance (ANOVA) was applied. When a significant ($P \leq 0.05$) *F*-test was obtained for treatments, data were subjected to means separation by Tukey's multiple range test.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Genes showing a twofold or greater difference in normalized expression in *etr1-1* plants inoculated with *Verticillium dahliae* relative to mock-inoculated *etr1-1* plants from the Affymetrix ATH1 GeneChip experiment.

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