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Authors

Veronese, P
Narasimhan, M L
Stevenson, R A
et al.

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Identification of a locus controlling *Verticillium* disease symptom response in *Arabidopsis thaliana*

Paola Veronese¹, Meena L. Narasimhan¹, Rebecca A. Stevenson², Jian-K. Zhu², Stephen C. Weller¹, Krishna V. Subbarao³ and Ray A. Bressan^{1,*}

¹Center for Plant Environmental Stress Physiology, 625 Agriculture Mall Drive, Purdue University, West Lafayette, IN 47907-2010, USA,

²Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA, and

³Department of Plant Pathology, University of California, Davis c/o U.S. Agricultural Research Station, 1636 E. Alisal Street, Salinas, CA 93905, USA

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*For correspondence (fax +1 765 494 0391; e-mail bressan@hort.purdue.edu).

Summary

Verticillium dahliae Klebahn is a soil-borne fungal pathogen causing vascular diseases. The pathogen penetrates the host through the roots, spreads through the xylem, and systemically colonizes both resistant and susceptible genotypes. To elucidate the genetic and molecular bases of plant–*Verticillium* interactions, we have developed a pathosystem utilizing *Arabidopsis thaliana* and an isolate of *V. dahliae* pathogenic to both cruciferous and non-cruciferous crops. Relative tolerance (based on symptom severity) but no immunity was found in a survey of *Arabidopsis* ecotypes. Anthocyanin accumulation, stunting, and chlorosis were common symptoms. Specific responses of the more susceptible ecotype Columbia were induction of early flowering and dying. The more tolerant ecotype C-24 was characterized by pathogen-induced delay of transition to flowering and mild chlorosis symptoms. Genetic analysis indicated that a single dominant locus, *Verticillium dahliae*-tolerance (*VET1*), likely functioning also as a negative regulator of the transition to flowering, was able to convey increased tolerance. *VET1* was mapped on chromosome IV. The differential symptom responses observed between ecotypes were not correlated with different rates of fungal tissue colonization or with differential transcript accumulation of *PR-1* and *PDF1.2* defense genes whose activation was not detected during the *Arabidopsis*–*V. dahliae* interaction. Impairment in salicylic acid (SA)- or jasmonic acid (JA)-dependent signaling did not cause hypersensitivity to the fungal infection, whereas ethylene insensitivity led to reduced chlorosis and ABA deficiency to reduced anthocyanin accumulation. The results of this study clearly indicated that the ability of *V. dahliae* to induce disease symptoms is also connected to the genetic control of development and life span in *Arabidopsis*.

Keywords: *Arabidopsis*, *Verticillium*, plant–microbe interaction, disease tolerance, ABA, ethylene.

Introduction

Verticillium species are soil-borne fungi with worldwide distribution, causing vascular disease that results in severe yield and quality losses in fruit and nut crops, legumes, vegetables, forest trees, and woody and herbaceous ornamentals (Bhat and Subbarao, 1999; Goth and Webb, 1981; Subbarao *et al.*, 1995; Wilhelm, 1981). Most crop diseases are caused by the two species *Verticillium dahliae* Klebahn and *V. albo-atrum* Reinke & Berthier, which differ in morphology, host range, and growth characteristics (Schnathorst, 1981). The *Verticillium* species persist in infested soils as spores, melanized hyphae, or resting

structures called microsclerotia (Schnathorst, 1981). The germination of these propagules is stimulated by root exudates, and the germ tube penetrates the host through the roots, proceeds to grow both inter- and intracellularly in the cortex, and spreads into the xylem. Systemic invasion occurs when successive generations of conidia are produced and then transported through the xylem transpiration stream to the aerial parts of the plant. In many hosts, the infection results in gradual wilting, senescence, eventual defoliation, and death. Stunting or, in some cases, a sudden overall wilt and death can occur (Goth and Webb,

1981; Wilhelm, 1981). Multiple factors contribute to the *Verticillium* disease symptoms. In cotton, potato, cauliflower, sunflower, and pepper, *Verticillium* disease has been associated with several physiological and biochemical changes in the host, including reductions in leaf water potential, stomatal conductance, and rate of carbon assimilation and accumulation of proline, soluble sugars and ABA (Bowden *et al.*, 1990; Goicoechea *et al.*, 2000; Sadras *et al.*, 2000; Tzeng and DeVay, 1985; Tzeng *et al.*, 1985; Xiao and Subbarao, 1998). *Verticillium* spp. colonization generally causes water stress by decreasing the hydraulic conductance of the xylem (Hall and MacHardy, 1981). Pathogenicity factors have been identified as protein-lipopolysaccharide complexes and small peptides released by the fungus that are able to evoke host leaf chlorosis and necrosis and root growth inhibition (Meyer *et al.*, 1994; Nachmias *et al.*, 1987, 1990).

The control of *Verticillium* spp. is especially difficult because they can survive in the soil as resting structures for several years (Schnathorst, 1981). Furthermore, as they lack a high degree of host specificity, they can multiply and survive by colonizing several different plant species (Krikun and Bernier, 1987; Subbarao *et al.*, 1995). *Verticillium* spp.–host interactions are characterized by the absence of a direct correlation between degree of colonization of the fungus and disease severity. In cotton, mint, potato, and tomato, both susceptible and resistant genotypes are systemically invaded by the fungus, usually to the same extent (Brandt *et al.*, 1984; Corsini *et al.*, 1988; Gold *et al.*, 1996; Heinz *et al.*, 1998; Lynch *et al.*, 1997; Schnathorst, 1981). Although resistance can be associated with a lower rate of pathogen proliferation, resistance is more closely associated with only the absence of disease symptoms (Bell and Mace, 1981). The genetic basis and the molecular mechanisms underlying plant resistance are thus poorly understood, and it appears that pathogen-mediated alterations in the growth, development and especially senescence of host plants may play important roles in disease etiology (Goicoechea *et al.*, 2001; Pegg, 1981; Sadras *et al.*, 2000; Tzeng and DeVay, 1985).

Genetic control of resistance is polygenic in strawberry, potato and alfalfa and linked to a single dominant gene in cotton, sunflower, and tomato (Lynch *et al.*, 1997). The only known resistance gene is the tomato *Ve* gene that has been recently identified as a cell surface-like receptor. It specifically confers resistance to race 1, but not to race 2, of *V. albo-atrum* when transferred to susceptible tomato and potato (Kawchuk *et al.*, 2001).

Little information is available describing the utilization of active defense responses against *Verticillium* spp. colonization. In tomato and cotton, defense response of resistant cultivars was associated with a more rapid suberin coating in the xylem and to an increased activity of phenylalanine ammonia-lyase, the enzyme that catalyzes the first step of

the phenylpropanoid pathway providing precursors for phytoalexin, lignin, and salicylic acid biosynthesis (Lee *et al.*, 1992; Smit and Dubery, 1997). In cotton, a higher amount of methylated sesquiterpenoid phytoalexins was found in the infected vascular tissues of resistant genotypes (Garas and Waiss, 1986). Such defense responses may have the same effect on the lateral movement of the fungus in roots and stems. However, they are seen as generally ineffective in arresting the upward distribution of the pathogen in the stems.

In this study, we present results describing host phenotypic changes associated with the interaction of *A. thaliana* with *V. dahliae* Kleb. We have identified the occurrence of a single dominant locus in the ecotype C-24 that controls the severity of specific aspects of symptom development in response to *V. dahliae* infection, in particular, induction of early flowering and senescence-like symptoms. However, this allele did not result in reduced pathogen growth or influence the ability of the pathogen to induce expression of defense genes controlled by either the systemic acquired resistance (SAR) or ethylene/jasmonic acid (ET/JA) response pathways.

Results

Identification of A. thaliana ecotypes that differ in tolerance to V. dahliae infection

Several *Arabidopsis* ecotypes originating from different habitats were evaluated for susceptibility to the isolate VdBob.70 (90–02) of *V. dahliae* Kleb. The isolate is pathogenic to both cruciferous and non-cruciferous crops (Bhat and Subbarao, 1999; Subbarao *et al.*, 1995). A root-dip inoculation technique was first applied because it has been routinely used in cross-pathogenicity and germplasm resistance studies involving *Verticillium* spp. and major crops (Atibalentja and Eastburn, 1997; Subbarao *et al.*, 1995). Disease symptoms developed slowly, becoming evident after 3 weeks. One month after the inoculation of 4-week-old plants, asymptomatic plants were not found. Symptoms observed in all ecotypes included stunted growth (measured as reduction in FW) and increased level of leaf chlorosis (Table 1). FW reduction resulted from reduced leaf size in all ecotypes and also from reduced inflorescence height in those ecotypes that completed the flowering process during the course of the experiment (Figure 1a; Table 1). Reduction in FW and height as a result of fungus-induced stunting ranged from 20 to 56% and 39 to 60%, respectively, in the ecotypes tested. Chlorosis could be observed in mock-inoculated control plants after flowering, as typically observed in *Arabidopsis*. Chlorosis always developed more rapidly in the inoculated plants. Forty per cent of the inoculated plants of the late-flowering

Table 1 Ecotype dependence of disease symptoms induced by *V. dahliae* inoculation in soil-grown *A. thaliana* plants

Accession	FW (g)			Inflorescence height (cm)			Chlorosis ^d	
	Control ^a	Inoculated	Loss ^b , %	Control	Inoculated	Loss ^b , %	Control	Inoculated
C-24	2.2 ± 0.1	1.4 ± 0.1	36	30.1 ± 0.4	17.2 ± 0.6	43	2	3
Columbia	2.3 ± 0.1	1.0 ± 0.1	56	28.6 ± 0.4	11.5 ± 1.0	60	2	4
Aa-0	2.9 ± 0.1	1.5 ± 0.1	48	33.5 ± 0.4	18.5 ± 1.3	48	2	3
Bl-1	2.0 ± 0.1	1.6 ± 0.1	20	–	–	– ^c	1	2
Bla-6	2.2 ± 0.1	1.1 ± 0.1	50	–	–	– ^c	1	2
Cal-0	2.4 ± 0.2	1.8 ± 0.1	25	–	–	– ^c	1	2
Co-1	2.0 ± 0.1	1.1 ± 0.1	45	29.5 ± 0.6	15.5 ± 0.8	47	2	3
Co-2	2.7 ± 0.2	1.3 ± 0.1	52	26.9 ± 1.9	14.5 ± 1.5	46	2	3
Ler-0	2.0 ± 0.2	1.1 ± 0.1	45	26.3 ± 1.0	13.0 ± 0.8	50	2	5
LI-0	2.5 ± 0.1	1.6 ± 0.1	36	26.0 ± 1.1	15.8 ± 1.4	39	2	3

Four-week-old plants were inoculated by root-dip procedure (5×10^7 conidia ml⁻¹) and scored for disease symptoms after 4 weeks. Data represent the mean ± SE ($n = 16$); similar results were obtained from two independent experiments.

^aMock-inoculated plants.

^bFW and height loss were calculated comparing data from mock- and fungal-inoculated plants.

^cLate-flowering ecotype not flowering or just bolting at the time of disease symptom evaluation.

^dExtent of leaf chlorosis was visually rated on a scale of 0–5 in which 0 = no symptom, 1 = up to 20% chlorotic leaves, 2 = up to 40% chlorotic leaves, 3 = up to 60% chlorotic leaves, 4 = up to 80% chlorotic leaves, and 5 = up to 100%.

ecotypes Bl-1 were bolting at the time of symptom evaluation, whereas no transition to flowering was observed in mock-inoculated plants, indicating that, in this ecotype, fungal infection accelerated the flowering process (data not shown). Hyperbranching was a response to *V. dahliae* infection observed only in the ecotypes C-24 and Co-1. The increase in the number of stems developing from the rosette, in comparison to mock-inoculated plants, was 37% for C-24 (Figure 1a) and 29% for Co-1 (data not shown). The roots and the crowns of all infected plants showed extensive browning and softening of the tissues. Microsclerotia (resting structures made of melanized spores) formation was observed on dead leaves (data not shown). From this screening of *A. thaliana* ecotypes, it was concluded that *V. dahliae* infection induces symptoms in *Arabidopsis* that are typical of *Verticillium* disease such as stunting (consisting of reduction in height and leaf area), chlorosis, and alteration of the flowering

process (Goicoechea *et al.*, 2001; Goth and Webb, 1981; Schnathorst, 1981). Based on the degree of development of these symptoms, the ecotype C-24 was observed to be more tolerant compared to the ecotype Columbia, which was found to be more susceptible.

Development of an in vitro technique to study A. thaliana–V. dahliae interaction

To facilitate further studies of the *A. thaliana–V. dahliae* interaction, an *in vitro* inoculation method was developed that allows the analysis of hundreds of seedlings in a relatively small and fully controlled environment. Two-week-old *A. thaliana* seedlings, grown on the surface of a cellophane membrane placed on solidified plant growth medium, were inoculated by placing a drop of fungal conidial suspension directly on the roots. At 3 days post-inoculation (dpi), trypan blue staining of roots revealed

Figure 1. *Verticillium dahliae* disease symptoms of C-24 and Columbia *A. thaliana* plants.

(a) Disease symptoms of soil-grown plants at 30 dpi. Four-week-old plants were inoculated following a root-dip procedure with 5×10^7 conidia ml⁻¹. The photographs show mock-inoculated control (C) and *V. dahliae*-inoculated (Vd) plants. *V. dahliae*-induced stunting and chlorosis of both ecotypes, greater severity of these symptoms in the Columbia ecotype, and increase in number of stems specific to the C-24 ecotype are evident.

(b–d) Disease symptoms of *in vitro* grown plantlets. Two-week-old seedlings were inoculated with *V. dahliae* (c. 10 conidia per seedling). Shown are data for uninoculated control (left, green bars, C) and inoculated (right, yellow bars, Vd) plantlets of the indicated ecotypes. *V. dahliae*-induced stunting, anthocyanin accumulation, and chlorosis of both ecotypes are shown at 42 dpi in (b). The greater magnitude of these symptoms as well as early flowering induced in the Columbia ecotype is evident. *V. dahliae*-induced inhibition of flowering in C-24 and induction of early flowering in Columbia were examined quantitatively. The number of uninoculated C-24 ($n = 82$), uninoculated Columbia ($n = 79$), and inoculated Columbia ($n = 73$) plantlets that flowered on the indicated dpi is shown in (c). In inoculated C-24 ($n = 77$) plantlets, the transition from vegetative rosette leaf to reproductive inflorescence development was not observed, and the number of flowering plantlets was recorded as zero. Inset are photographs taken at 60 dpi showing the inhibition of the transition to flowering occurring in *Verticillium*-inoculated C-24 compared to uninoculated plantlets (left), and at 15 dpi showing the induction of early flowering occurring in *Verticillium*-inoculated Columbia compared to uninoculated plantlets (right). *V. dahliae*-induced decline of photosynthetic parameters, examined at 49 dpi, are shown in (d). The chlorophyll content per unit of FW in uninoculated and inoculated C-24 and Columbia plantlets was measured in extracts of plant tissues above the crowns. The data are mean ± SE of six measurements. The relative amount of the large subunit of Rubisco in these samples is also shown. Rubisco was detected in total soluble proteins (30 µg) after SDS-PAGE, by staining the gel with Coomassie Brilliant Blue.

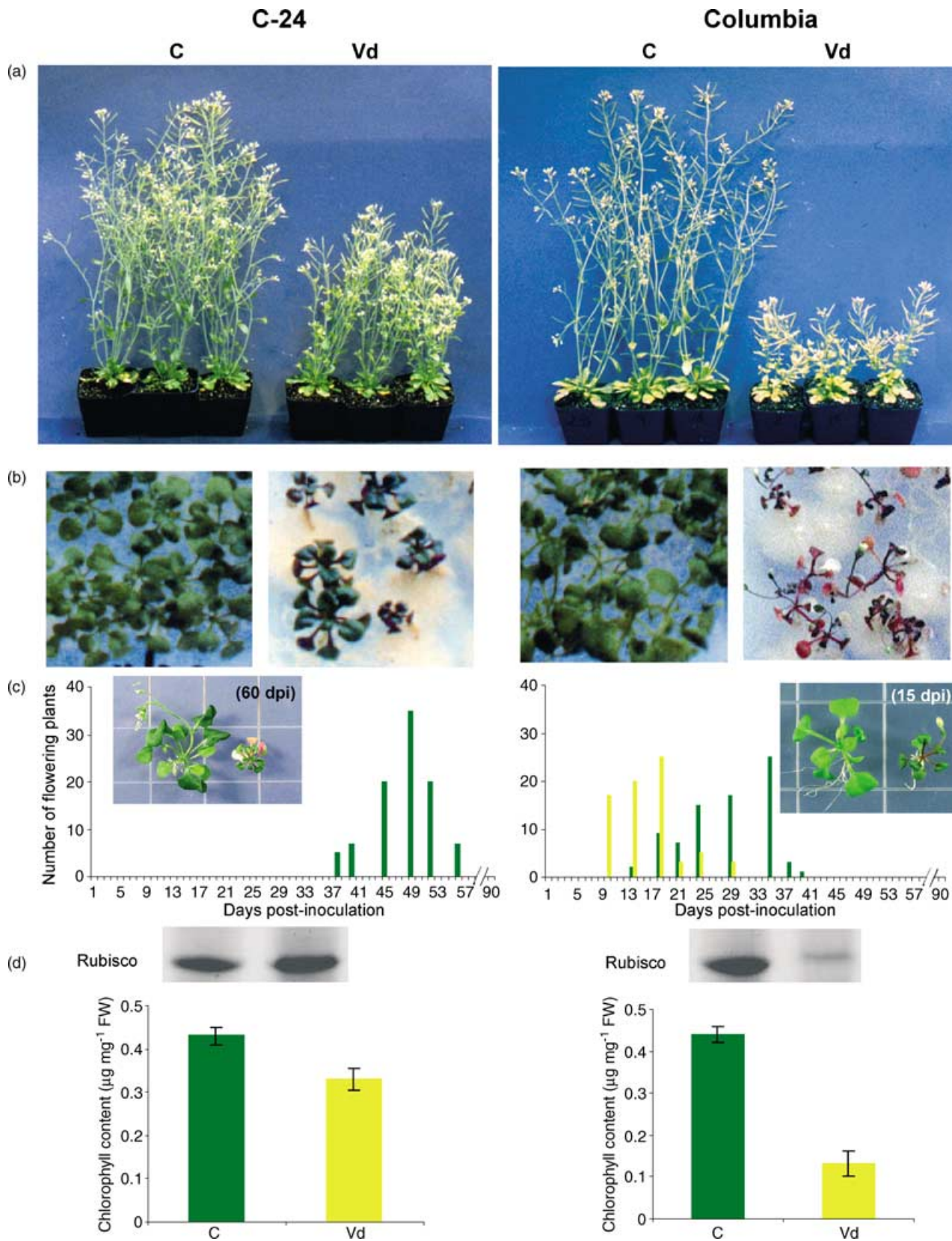


Table 2 Symptom development in plantlets of *A. thaliana* ecotypes *in vitro* inoculated with *V. dahliae*

Accession	14 dpi			28 dpi			
	Anthocyanin ^a	Flowering plants (%) ^b		Stunting ^c	Chlorosis ^d	Flowering plants (%)	
		Control	Inoculated			Control	Inoculated
C-24	1	0	0	2	1	0	0
Columbia	3	3	36	3	3	37	89
Aa-0	2	0	0	2	2	6	0
An-1	2	5	43	2	3	60	91
Ang-0	1	0	0	2	2	6	0
Bl-1	2	0	0	2	2	0	0
Bla-1	1	0	0	2	2	0	0
Bla-2	1	0	0	2	2	0	0
Bla-6	1	0	0	2	1	0	0
Cal-0	1	0	0	2	1	0	0
Co-1	1	0	0	2	1	0	0
Co-2	3	0	0	3	1	0	0
Col-7	3	0	35	3	2	23	61
Ct-1	1	0	0	2	2	27	30
Cvi-0	1	0	0	3	2	0	0
Enkheim	1	0	0	2	2	8	1
Kas-1	3	0	0	2	2	0	0
Ler-0	2	8	40	3	3	59	96
Petergof	2	0	0	2	2	0	0
Uk-4	2	0	0	2	2	0	1
Wa-1	3	0	0	2	2	10	2

Two-week-old plantlets were inoculated and scored for symptom development after 2 and 4 weeks using a binocular microscope. Results shown were obtained from two independent experiments with at least 80 uninoculated and 80 inoculated plantlets for each ecotype.

^aAnthocyanin accumulation intensity was rated on a scale of 0–3 in which 0 = no accumulation, 1 = mild accumulation along stem and petioles, 2 = intense accumulation along stem and petioles, and 3 = intense accumulation along stem, petioles, and leaves.

^bPercentage calculated comparing the number of flowering plantlets with total number of plantlets.

^cExtent of stunting was rated on a scale of 0–3 in which 0 = no stunting, 1 = moderate reduction of leaf area, 2 = strong reduction of leaf area, and 3 = strong reduction of leaf area, leaf number, and length of stem.

^dExtent of leaf chlorosis was rated on a scale of 0–3 in which 0 = no symptom, 1 = up to 25% chlorotic leaves, 2 = up to 50% chlorotic leaves, 3 = up to 75% chlorotic leaves, and 4 = up to 100% chlorotic leaves.

extensive inter- and intracellular fungal colonization that extended towards the vascular system, without any obvious symptoms on the plantlets (data not shown). Disease symptoms were visually scored at 14 and 28 dpi (Table 2). At 14 dpi, the most common symptom was anthocyanin accumulation along stems and petioles that was never observed in uninoculated control plantlets. Anthocyanin accumulation was the first visible response of *A. thaliana* to *V. dahliae* infection. In some ecotypes (Columbia, Col-7, Kas-1, and Wa-1), it occurred as early as 7 dpi (data not shown), subsequently becoming more intense and extending to the leaves (Table 2). Acceleration of the flowering process by fungal infection was observed only in the ecotypes Columbia, An-1, Col-7, and Ler-0. Thirty-five to 43% of the inoculated plantlets of these ecotypes were flowering at 14 dpi compared to 0–8% of the control plantlets (Table 2). At 28 dpi, the stunting and chlorosis became evident on the *in vitro* cultured plantlets of all tested ecotypes (Table 2). Stunting was generally evident as a reduction in leaf area resulting in smaller rosettes (Figure 1b,c). In the ecotypes

Columbia, An-1, Col-7 and Ler-0 that had an accelerated flowering response to fungal infection, stunting was also evident as reduction in leaf number and length of the flowering stems. Chlorosis was a major symptom that differentiated more and less susceptible ecotypes, and was initiated at the cotyledons but then extended to the true leaves in later stages of infection and involved 50–75% of the leaves in the severely affected ecotypes Columbia, An-1, and Ler-0. Chlorosis was never observed in uninoculated control plantlets. Fungal infection-induced flowering was still evident at 28 dpi in the ecotypes Columbia, An-1, Col-7, and Ler-0, with 61–96% of the inoculated plantlets flowering compared to 23–60% of the uninoculated control plantlets. The ecotypes Aa-0, Enkheim, and Wa-1 exhibited a slightly delayed flowering response after the infection. *V. dahliae* infection also caused root branching at the crown and microsclerotia formation on dead tissues in all ecotypes (data not shown). Thus, in the *in vitro* inoculated *A. thaliana* plantlets, symptoms of *Verticillium* disease (stunting, chlorosis, alteration of the flowering process)

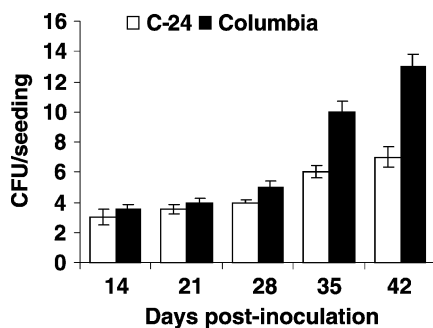


Figure 2. Estimation of the extent of *V. dahliae* colonization in C-24 and Columbia plantlets.

The number of CFU was determined by maceration of plant tissues above the crown in sterile water followed by plating aliquots on potato dextrose agar medium (PDA). After 12 days, *V. dahliae* colonies were counted. Results are mean \pm SE from two independent experiments.

qualitatively resembled those observed in soil-grown inoculated plants, indicating that this system could be used for genetic analyses of disease susceptibility or resistance.

More susceptibility of Columbia is based on accelerated transition to flowering and early dying

In the *in vitro* inoculations, the ecotypes C-24 and Columbia exhibited maximum differences in all four symptoms evaluated, namely anthocyanin accumulation, stunting, development of leaf chlorosis, and acceleration of the flowering process (Figure 1b; Table 2). The differences in stunting (measured as reduction in FW and height of inflorescence stems) and leaf chlorosis after infection were also significant in soil-grown plants (Figure 1a; Table 1). Therefore, the interaction of *V. dahliae* with these two ecotypes was investigated in greater detail. Daily quantitative analysis of the flowering process in the *Arabidopsis* ecotypes C-24 and Columbia was performed for 3 months after the *in vitro* *Verticillium* infection, and the number of plants that flowered on the indicated dpi is shown in Figure 1(c). The two ecotypes exhibited a differential flowering response to the *V. dahliae* infection. In the absence of the fungus, C-24 plantlets completed the flowering process within 56 days from the date of the treatment, whereas inoculated plants did not start bolting during this time period (Figure 1c). Inoculated C-24 plantlets continued to produce small round leaves forming a compact rosette and maintained high chlorophyll content (Figure 1c), suggesting that the infection resulted in the extension of the vegetative growth phase and inhibition of the transition to flowering. Uninoculated Columbia plants started to flower at the seven-to-eight-true-leaf stage and completed the flowering process within 40 dpi (Figure 1c). Infection induced early flowering in Columbia plantlets. Inoculated plantlets started to flower at the four-to-five-true-leaf stage and completed flowering within 30 dpi (Figure 1c). Thus, in

C-24, *V. dahliae* infection inhibited flowering whereas in Columbia the flowering process was accelerated. In Columbia, interaction with the pathogen resulted in a shortened life cycle characterized by earlier flowering followed by very severe chlorosis and premature death (Figure 1b), all responses that have been associated with *Verticillium* susceptibility symptoms (Goicoechea *et al.*, 2001; Goth and Webb, 1981; Schnathorst, 1981). No visible signs of leaf chlorosis were observed in uninoculated C-24 and Columbia control plantlets (Figure 1b). To evaluate the nature of fungus-induced chlorosis at this time, the chlorophyll content and the relative amount of the large subunit of ribulose biphosphate carboxylase oxygenase (Rubisco) complex were compared in uninoculated and inoculated plantlets at 49 dpi (Figure 1d). In C-24, *V. dahliae* infection caused 22% reduction in chlorophyll content and no significant difference in the relative amount of Rubisco. In Columbia, *V. dahliae* infection had a much greater effect on both photosynthetic parameters. Chlorophyll content was reduced by 71%, and Rubisco was barely detectable by SDS-PAGE. As flowering was accelerated and photosynthetic parameters (chlorophyll content and Rubisco) were subsequently reduced upon fungal infection in plantlets of the Columbia ecotype, it is proposed that more susceptibility of Columbia is mainly based on an accelerated life cycle, development, and senescence-like syndrome induced by the fungus. More tolerance of the C-24 ecotype, on the other hand, was associated with a blockage of the transition to flowering by the fungus, thereby delaying the appearance of senescence-like symptoms. The extent of fungal penetration into shoots at different intervals was estimated by maceration of tissues above the crown, plating the extracts on fungal growth medium and counting the number of colonies formed (Figure 2). No significant differences in the colony-forming units (CFU) were observed in C-24 and Columbia up to 28 dpi when symptoms were fully expressed in Columbia. At later dpi, susceptible Columbia supported more CFU presumably because extensive tissue death supported saprophytic fungal growth. Thus, disease symptom development in the more susceptible Columbia ecotype was not the result of differences in the extent of tissue colonization by *V. dahliae*.

The C-24 ecotype contains the dominant allele of a single locus that controls increased tolerance to V. dahliae

To investigate the genetic basis of C-24 responses to *V. dahliae* (inhibition of the transition to flowering and reduced chlorosis), reciprocal crosses with Columbia were performed and the *Verticillium* susceptibility phenotypes of the progenies were studied 49 days after inoculation. The inoculated plantlets of the F₁ progeny did not flower during the course of the experiment and retained a chlorophyll content comparable to that of inoculated C-24 (Figure 3b),

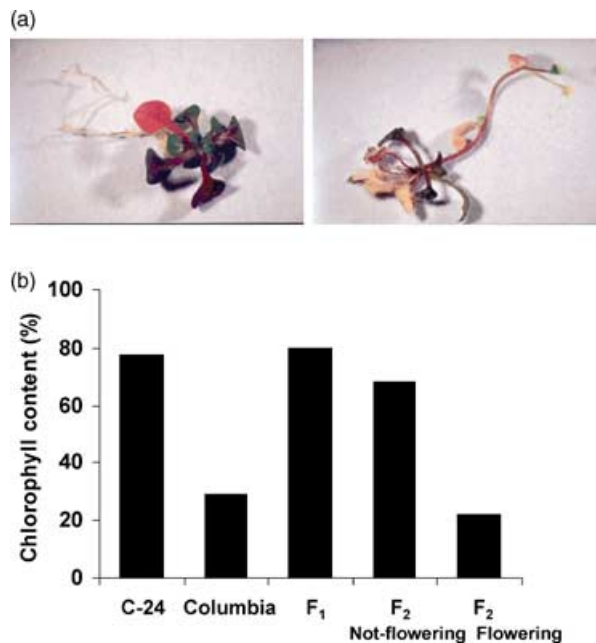


Figure 3. Phenotype of C-24 that is more tolerant to *V. dahliae* is inherited as a single dominant trait.

Two-week-old seedlings of F₁ and F₂ progenies of the cross of C-24 and Columbia were *in vitro* inoculated, and *V. dahliae* disease phenotypes at 49 dpi are shown.

(a) The C-24-like tolerant response (inhibition of flowering and extension of vegetative growth) exhibited after inoculation by the entire F₁ population and the three-quarters of the F₂ population is shown on the left. On the right is shown the Columbia-like susceptible response (early flowering and dying) of one-quarter of the inoculated F₂ population. Flowering was completed within 30 dpi.

(b) Chlorophyll was extracted in triplicates from pools of 15 plantlets. Average chlorophyll content, relative to uninoculated plantlets, in C-24, Columbia, and their F₁ and F₂ progenies is shown.

indicating that increased fungal susceptibility of the Columbia ecotype is a recessive trait. The disease response of a total of 812 F₂ plantlets was analyzed. In this F₂ population, 600 plantlets did not flower after the infection and showed a C-24-like response (Figure 3a) with only a 20% reduction in chlorophyll content compared with that of uninoculated plantlets (Figure 3b). One hundred and ninety-three inoculated plantlets flowered within 28 days and expressed the Columbia-like accelerated senescence-like syndrome

(Figure 3a) characterized by 80% or more chlorophyll reduction (Figure 3b). From these results, summarized in Table 3, it was concluded that *Verticillium*-increased disease tolerance is conveyed by a single dominant gene that is also likely involved in the control of the flowering process. To obtain a chromosomal map position for this locus, designated *VET1*, for *Verticillium dahliae* tolerance, genomic DNA was extracted from 90 individual F₂ homozygous, more susceptible plants and analyzed for linkage of more susceptibility to microsatellite markers (Bell and Ecker, 1994). The *VET1* locus was linked to the SSLP marker *nga8*, which is located on the top of chromosome IV (Table 4). Genes that are relevant to *Arabidopsis* development or disease resistance and map to that chromosome region include *FRI*, *FCA*, and *VRN2* of the vernalization/autonomous pathways controlling flowering time (Mouradov *et al.*, 2002) and the *RPP5* supergene family controlling resistance to the fungal pathogen *Pero­nospora parasitica* (Parker *et al.*, 1997).

V. dahliae infection does not appear to trigger systemic induction of PR-1 and PDF1.2 defense gene expression in *A. thaliana*

Northern blot analysis of C-24 and Columbia plantlets was performed at various times after the *in vitro* inoculation with *V. dahliae* to investigate the inducibility of the pathogenesis-related genes *PR-1* and *PDF1.2*, markers of salicylic acid (SA)- and ET/JA-dependent defense response pathways, respectively (Glazebrook, 2001). No detectable pathogen-induced systemic activation of either genes was observed in both ecotypes as early as 2 dpi (data not shown) and up to 9 dpi (Figure 4) when extensive fungal colonization had occurred (data not shown) and disease symptoms such as anthocyanin accumulation were visible (data not shown). Gene expression was detected, as expected, in plantlets treated with SA or methyl jasmonate (MeJA), respectively (Figure 4), indicating that the *in vitro* grown *Arabidopsis* plantlets were fully responsive to increased levels of both phytohormones. These results show that the *in vitro* *A. thaliana*-*V. dahliae* interaction is not characterized by a clear activation of either the SA- or

Table 3 Co-segregation of *V. dahliae*-induced early flowering and more severe chlorosis phenotypes in progenies from a cross of more tolerant C-24 and more susceptible Columbia

C-24 × Col cross progeny	Inoculated plants	Flowering		Not flowering		χ^2
		Less chlorosis	More chlorosis ^a	Less chlorosis	More chlorosis ^a	
F ₁	21	0	0	21	0	0.53
F ₂	812	0	193	600	19	

Two-week-old seedlings were *in vitro* inoculated with *V. dahliae* and disease symptoms were scored at 49 dpi.

χ^2 was calculated for the segregation ratio 3 : 1, $0.5 > P > 0.1$.

^aMore chlorosis was defined as having 30% (or lower) of the chlorophyll content of uninoculated plants.

Table 4 Genetic mapping of *VET1*

Markers	Chromosome	<i>n</i> ^a	Recombination frequency ^b (% ± SE)
<i>nga248</i>	I	23	46 ± 5.4
<i>nga1126</i>	II	28	46 ± 5.6
<i>nga168</i>	II	23	39 ± 6.0
<i>AthGAPab</i>	III	29	48 ± 4.2
<i>nga8</i>	IV	90	17 ± 4.7
<i>nga158</i>	V	29	52 ± 6.4

^aNumber of samples analyzed.

^bCalculated by the Kosambi function (Koornneef and Stam, 1992).

ET/JA-dependent defense signal transduction pathways, as detected by analyses of *PR-1* and *PDF1.2* gene expression. Defense gene induction was more responsive to exogenous SA and MeJA in the more tolerant C-24 ecotype. In the absence of any detectable difference in fungal growth in the two ecotypes, the importance of this, if any, may be related only to symptom development.

ABA and ET control A. thaliana symptom responses to V. dahliae

Several plant hormones are well established as mediators of plant disease responses, especially SA, ET, and JA

(Glazebrook, 2001). Plant hormones also control many plant developmental processes including cell necrosis, anthocyanin accumulation, senescence, and flowering (Bleecker and Patterson, 1997; Dangl *et al.*, 1996; Kao *et al.*, 1996; Nam, 1997), all of which have been clearly shown to be part of host-pathogen interactions (Goicoechea *et al.*, 2001; Hipskind *et al.*, 1996; O'Donnell *et al.*, 2001; Sadras *et al.*, 2000). *Verticillium* disease symptom development was examined in *A. thaliana* mutants that are deficient or insensitive to SA, ET, JA, and abscisic acid (ABA). Significantly altered anthocyanin levels in response to *V. dahliae* were observed in the ABA-deficient mutant *aba 2-1*, which accumulated 68% less anthocyanin than Columbia wild type (Figure 5a). The ET-insensitive mutant *etr 1-1* showed less severe senescence-like symptoms, as revealed by a 58% higher chlorophyll content, compared to the wild type (Figure 5b). These data clearly indicate that the phytohormones ABA and ET act as regulators of *A. thaliana* responses to *V. dahliae* infection, whereas SAR impairment or JA insensitivity do not appear to influence plant symptom development. It is also interesting that each hormone response mutant affected specific aspects of *V. dahliae* symptoms (anthocyanin versus chlorosis). This indicates that *V. dahliae* infection can affect symptoms by separate signal pathways mediated by different hormones, and may act early in plant signal perception. We also did not observe any effect of the impairment of phytoalexin synthesis caused by the phytoalexin deficient (PAD) mutations (Figure 5a,b) even though phytoalexin accumulation has been correlated with a tolerant response to *V. dahliae* in other plant species (Joost *et al.*, 1995; Resende *et al.*, 1996).

Discussion

Verticillium dahliae is a major fungal pathogen affecting a wide range of crops (Bhat and Subbarao, 1999; Goth and Webb, 1981; Wilhelm, 1981). *Verticillium* wilt disease is usually managed by a combination of chemical and cultural practices. In some species such as tomato, genetic resistance has been available for many years (Goth and Webb, 1981). However, germplasm screens for most crops have yielded only tolerance, which can be polygenic or based on a single gene (Goth and Webb, 1981; Wilhelm, 1981). Even though *Verticillium* spp. are often described as non-host-specific or as having a broad host range, isolates from the two major pathogenic species *V. dahliae* and *V. albo-atrum* do not exhibit indiscriminate pathogenicity (Baergen *et al.*, 1993; Bhat and Subbarao, 1999), and the only gene that has been identified for *V. albo-atrum* resistance (*Ve* from tomato) conveys resistance to only race 1 of the tomato pathogen (Kawchuk *et al.*, 2001).

The etiology of *Verticillium* wilt disease is also well documented. The fungus enters the plant from the root, growing both inter- and intracellularly and reaching the

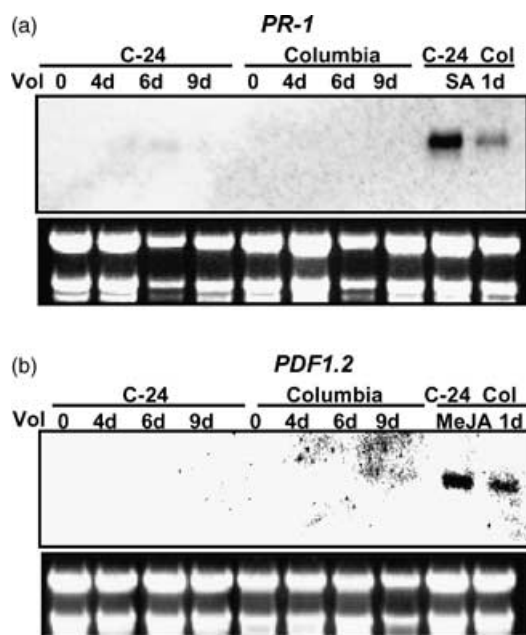


Figure 4. *Verticillium dahliae* colonization does not induce systemic accumulation of (a) *PR-1* and (b) *PDF1.2* transcripts in C-24 and Columbia *A. thaliana*.

Two-week-old seedlings were *in vitro* inoculated with *V. dahliae* or sprayed with 0.5 mM SA or 100 μM MeJA. Total RNA was isolated from plant tissues above the crown at 0, 4, 6, and 9 dpi, and 1 day after SA or MeJA treatment. Ethidium bromide-stained ribosomal bands were used as loading control.

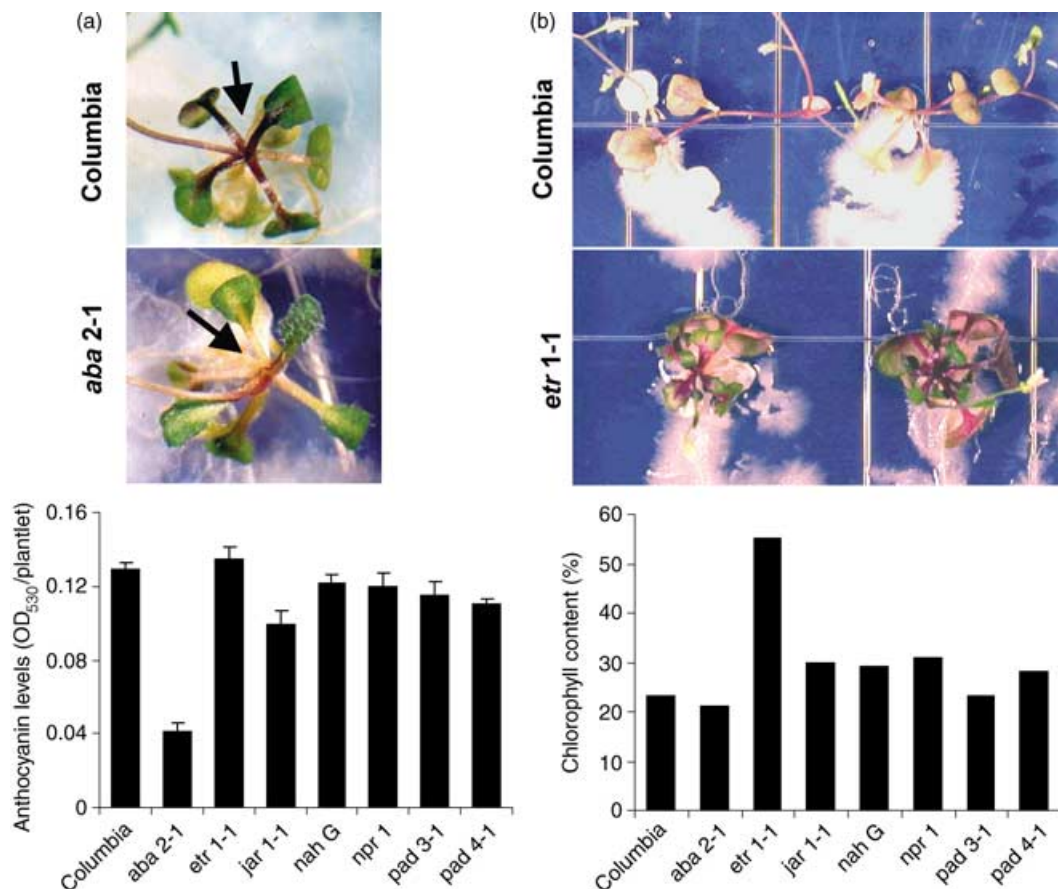


Figure 5. Plant hormone dependence of *V. dahliae* disease symptom expression in *A. thaliana*.

Two-week-old seedlings of Columbia ecotype and indicated mutants of Columbia background were *in vitro* inoculated with *V. dahliae*, and their anthocyanin and chlorophyll levels were measured at 14 and 49 dpi, respectively. Results shown were obtained from two independent experiments with at least 60 uninoculated and 60 inoculated plants for each genotype.

(a) ABA deficiency attenuates anthocyanin accumulation. Anthocyanin accumulation phenotypes of the wild type Columbia and the ABA-deficient mutant *aba 2-1* are shown. Bars indicate the average anthocyanin content (\pm SE) per *V. dahliae*-inoculated plantlet of Columbia and indicated mutants ($n = 6$).

(b) Ethylene insensitivity attenuates chlorosis. The early dying phenotype of Columbia and the reduced leaf chlorosis occurring in the ET-insensitive mutant *etr 1-1* are shown. Bars indicate the chlorophyll content, relative to uninoculated plantlets, of *V. dahliae*-inoculated Columbia and mutant plantlets.

vessel elements of the root vasculature. Within vessels, the mycelia quickly produce spores that are carried upward through the transpiration stream. Spread of the fungus through the vascular system is slowed by trapping of spores in pit cavities or vessel end walls where they must germinate allowing hyphae to penetrate into adjacent vessels. This secondary infection process, called determinative phase II, usually occurs in waves that may be related to peaks of defensive activities initiated by the host plant (Beckman, 1987; Gold and Robb, 1995; Heinz *et al.*, 1998). Often observed defensive responses of the host include xylem suberization, phytoalexin synthesis, tylosis formation, and callose deposition (Beckman, 1987; Gold and Robb, 1995). Although the ability of the pathogen to rapidly colonize the host, undoubtedly, can play a role in disease development and severity (Gold *et al.*, 1996), *Verticillium* wilt diseases are unusual in the sense that there is often little or no correlation between degree of fungal prolifera-

tion and symptom development (Brandt *et al.*, 1984; Corsini *et al.*, 1988; Gold *et al.*, 1996; Heinz *et al.*, 1998; Lynch *et al.*, 1997; Schnathorst, 1981). We also did not find a good correlation between pathogen growth and severity of symptoms (Figures 1 and 2).

Few studies on the molecular characterization of plant responses to *Verticillium* infection have been conducted, and very little evidence exists that links *V. dahliae* infection responses to any of the major signal transduction components that are known to control host resistance or tolerance to other pathogens. In fact, the only molecular genetic component linked to *Verticillium* tolerance response is the Ve protein that is related to the Leucine-Rich-Repeats (LRR) superclass of defense receptor genes (Kawchuk *et al.*, 2001). Ve is a cell-surface LRR glycoprotein with receptor-mediated endocytosis-like signals and leucine zipper or Pro-Glu-Ser-Thr (PEST)-like sequences. We have identified a single dominant locus called *VE1* that confers increased

tolerance to *V. dahliae*. *VET1* maps to chromosome IV in the vicinity of genes involved with vernalization or autonomous pathways controlling flowering time and genes of the *RPP5* superfamily controlling resistance to the fungal pathogen *P. parasitica* (Mouradov *et al.*, 2002; Parker *et al.*, 1997). Resistance to *Peronospora parasitica* (*RPP5*) family members are nucleotide-binding leucine-rich repeat proteins with Toll/interleukin-1 receptor domains (Parker *et al.*, 1997). Until we clone the *VET1* gene, the relationship between *VET1* and the genes in the same map vicinity will remain unknown. Although some of the downstream target genes of pathogen signal-response pathways, such as glucanases (*PR-2*), chitinases (*PR-3*) and *Ypr10*-like (*PR-10*), have been shown to be induced by *V. dahliae* infection (McFadden *et al.*, 2001), surprisingly little information is available showing that major defense gene expression responses occur after inoculation of plants with *V. dahliae*. We also failed to observe any major induction of *PR-1* or defensin *PDF1.2* genes in response to *V. dahliae* infection (Figure 4). These genes are controlled by the two major pathogen defense-response pathways that are induced by SA (*PR-1*) and ET/JA (*PDF1.2*) (Glazebrook, 2001).

The unusual disparity between pathogen proliferation and symptom severity and the lack of induction of genes that are commonly pathogen associated further emphasizes the unusual nature of the host–pathogen interaction between *V. dahliae* and its target plant. This interesting decoupling between pathogen proliferation and symptomatology has been noted for both bacterial (Bent *et al.*, 1992; Lund *et al.*, 1998; O'Donnell *et al.*, 2001) and viral (Cecchini *et al.*, 2002) plant–pathogen interactions and may in part be explained by the possibility that symptoms can result from pathogen-induced signal events that cause changes in normal plant growth and development. Changes such as accelerated flowering, senescence, and programmed cell death are then manifested as disease symptoms that are more dependent on the genotypes of pathogen and host and interacting environmental factors (e.g. short versus long days) than on the degree of pathogen growth (Cecchini *et al.*, 2002; Dietrich *et al.*, 1994; Lund *et al.*, 1998; O'Donnell *et al.*, 2001; Piloff *et al.*, 2002). Such host symptom responses may or may not be defensive because they may reflect host co-opted signal responses designed to minimize pathogen success, although they may also represent pathogen-directed signals designed to exploit the host. Tolerance of the host could therefore be based primarily on reduced susceptibility of the host development signal transduction system to intervention by the pathogen. It is important to note that pathogen-induced symptom development, even in susceptible responses, can be separated into stages of infection and symptom progression (Lund *et al.*, 1998). Also, the complexity and pervasiveness of these apparent signaling interactions between host and pathogen that affect symptoms is underscored by the

observation that many host mutations that affect disease severity are pleiotropic and also mediate changes in plant growth and development in the absence of the pathogen. Several constitutive defense response mutants such as *cpr1*, *ssi1*, *cpr5*, *cpr6*, *acd6*, *lsd6*, *mpk4*, *dnd1*, *agd2*, and *cev1* all exhibit some degree of dwarfism and altered leaf shape (Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998; Clough *et al.*, 2000; Ellis and Turner, 2001; Petersen *et al.*, 2000; Rate and Greenberg, 2001; Rate *et al.*, 1999; Shah *et al.*, 1999; Weymann *et al.*, 1995). *Cev1* mutant plants also have short roots with an excess of root hairs and increased anthocyanin accumulation in petioles (Ellis and Turner, 2001), whereas *crp5* plants present an altered trichome development and an early senescence phenotype (Bowling *et al.*, 1997; Yoshida *et al.*, 2002). This complexity is further evidenced by the observation that altering the levels of host signal molecules may mimic but not exactly duplicate pathogen-induced symptoms (Bent *et al.*, 1992; Chang *et al.*, 1997). The dependence of interactions of pathogen with host signaling on the host genetic status is emphasized by the distinct difference in flowering response by Columbia and C-24 whereby *V. dahliae* accelerates flowering in Columbia and delays flowering in C-24 (Figure 1c). This likely reflects different sets of genes that are active in the two ecotypes and are able to modify the flowering response to *V. dahliae* infection.

Our analysis of *A. thaliana* mutants that are impaired in hormone responsiveness revealed that *V. dahliae* susceptibility in *A. thaliana* is specifically affected by mutations that affect ET response and ABA biosynthesis, both phenomena that are connected to plant aging and senescence and may share a common signal pathway that controls hormone-mediated leaf senescence (Grbic and Bleeker, 1995; Oh *et al.*, 1997; Weaver *et al.*, 1998; Woo *et al.*, 2001) possibly through senescence mediators like F-box proteins that also control flowering response in *A. thaliana* (Woo *et al.*, 2001). The strong connection between susceptibility to *V. dahliae* and flowering suggests that other genes that mediate flowering response in *Arabidopsis* may affect *Verticillium* tolerance. Likewise, environmental factors that affect flowering such as photoperiod may also play a strong role in pathogen susceptibility, as was demonstrated for fungal and viral susceptibility (Cecchini *et al.*, 2002; Pegg, 1981).

The ability of *V. dahliae* to elicit morphological and developmental changes in *A. thaliana*, which are manifested as disease symptoms (stunting, accelerated flowering, chlorosis, increased branching, anthocyanin accumulation), could be mediated by *V. dahliae*-originated signal molecules. A good candidate for such a molecule is the glycoprotein toxin produced by *V. dahliae* that is able to produce host-specific symptoms in the absence of fungal cells (Meyer *et al.*, 1994; Nachmias *et al.*, 1987, 1990). Although the only identified gene capable of conveying resistance to

Verticillium encodes a class of cell-surface glycoproteins that share homology to other LRR-type plant resistance genes (Kawchuk *et al.*, 2001), any requirement of the *Ve* protein to recognize the *V. dahliae* glycoprotein is unknown. Yet, recognition of some *V. dahliae*-based ligand is most likely a part of the function of the *Ve* receptor of tomato. An ability to either induce a host plant defense response (inhibiting pathogen infection) or block pathogen-induced symptom development could be derived from ligand-*Ve* receptor recognition and result in the observed resistance phenotype.

The *in vitro* *A. thaliana*-*V. dahliae* interaction that we have described appears to display all the important features of a natural infection: (i) the pathogen specifically infects the root system; (ii) disease symptoms evolve slowly and progressively; (iii) typical *V. dahliae* disease symptoms are induced such as stunted growth and leaf chlorosis; (iv) microsclerotia form in senescent and dead host plant tissues at a very late stage of the interaction.

A genotype-dependent degree of susceptibility was observed in the ecotypes Columbia, An-1 and *Ler-0* and was especially characterized by induction of early flowering and extensive leaf chlorosis. The ecotypes C-24 and Columbia represent the most tolerant and most susceptible ecotypes, respectively, being consistent with the results obtained from infections of greenhouse-grown plants. Thus, a clear genetic basis for controlling increased resistance to *V. dahliae* in *A. thaliana* plants cultured in the *in vitro* system has been demonstrated here, and should allow efficient screening of mutants and subsequent identification of other genetic loci controlling altered susceptibility to *V. dahliae*.

Experimental procedures

Plant growth conditions, inoculations, and chemical applications

The Arabidopsis Biological Resources Center (ABRC, Ohio State University, Columbus, USA) provided the seeds of all the *A. thaliana* ecotypes and mutants used in this study except the transgenic line *nahG*, which was kindly provided by Dr X. Dong (Duke University, Durham, NC, USA). The Columbia ecotype utilized in all the experiments carries the *glabrous 1 (gl-1)* mutation. *Arabidopsis* seeds were stored for 4 days at 4°C before sowing. Plants were grown in environmentally controlled growth rooms at 22 ± 1°C, 60–70% relative humidity, and light intensity of 100 µmol E m⁻² sec⁻¹ on a 16-h light/8-h dark cycle. Plants used for *V. dahliae* root dip inoculations were grown in steam-sterilized soil (Metro-Mix 360; The Scotts Company, Marysville, OH, USA) for 4 weeks. They were then gently uprooted and rinsed in sterile water, and their roots were dipped for approximately 1 min in conidial suspensions (5 × 10⁷ conidia ml⁻¹). Fresh inoculum was used for every 32 plants. Root systems of mock-inoculated plants were dipped in sterile water. Plants were immediately transplanted into potting mix after treatment and kept in high-humidity environ-

ment for 2 days. For the *in vitro* inoculation technique, *Arabidopsis* seeds were surface-sterilized with 2% sodium hypochlorite and sown in Petri dishes on the surface of a cellophane membrane (cat. 1650963 Bio-Rad, Richmond, CA, USA) laid over the Murashige and Skoog medium (Sigma Chemical Co., St Louis, MO, USA) solidified with 0.8% agar and supplemented with 2% sucrose. The roots of 2-week-old seedlings were inoculated with 2-µl drop of conidial suspension (5 × 10³ conidia ml⁻¹), and symptoms were visually scored using a binocular microscope.

The cauliflower isolate VdBob.70 (90-02) of *V. dahliae* was used throughout this study. Cultures of the isolate were grown on potato dextrose agar (PDA, Difco, Detroit, MI, USA). Conidia were harvested from 4- to 5-day-old plates by flooding the surface of plates with sterile distilled water and filtering through two layers of sterile cheesecloth. Conidia were counted using a calibrated haemocytometer. Fungus was stored as a conidial suspension in 15% aqueous glycerol at -70°C.

For treatments with SA and MeJA, 2-week-old seedlings growing in Petri dishes were sprayed with 0.5 mM SA in water or 100 µM MeJA in 0.1% (v/v) ethanol, respectively.

Visualization and quantification of *V. dahliae* in infected plant material

Fungal hyphae in plantlet tissues were visualized by lactophenol-trypsin blue staining according to the method described by Keogh *et al.* (1980). The extent of colonization of plant tissues was also determined by a maceration and plating technique (Brandt *et al.*, 1984; Lynch *et al.*, 1997). Shoots of 20 seedlings were detached from roots just below the crown, rinsed with sterile distilled water, and homogenized in 4 ml of sterile distilled water using a high-speed blender (Sorvall Omnimixer, Newton, CT, USA). Two-hundred-microliter triplicate aliquots of the suspensions were plated on PDA, and colonies were counted after 12 days.

Measurement of photosynthetic parameters

Forty-nine days after *in vitro* inoculation with *V. dahliae*, chlorophyll and total soluble protein were extracted from pools of four uninoculated and 10–15 inoculated plantlets. Shoot tissues were ground into powder in liquid nitrogen and weighed. Chlorophyll was extracted in 80% acetone, and the concentration per FW was calculated as described by Lichtenthaler (1987). Total soluble protein was extracted, and protein concentration was determined as described by La Rosa *et al.* (1989). All measurements were performed in triplicate. The large subunit of Rubisco was detected by SDS-PAGE as described by Oh *et al.* (1997).

Anthocyanin assay

Fourteen days after *in vitro* inoculation with *V. dahliae*, anthocyanins were extracted and quantified as described by Gareth *et al.* (1997) from pooled shoot tissues of 10–15 plantlets. Results are expressed as OD₅₃₀ per seedling. Measurements were performed in triplicate.

Genetic analysis

For genetic mapping of the *VET1* locus, C-24 and Columbia (*gl-1*) plants were crossed and F₂ seeds were obtained from self-pollinated F₁ plants. Homozygous *V. dahliae*-sensitive plantlets

in the segregating F₂ population were selected 49 days after fungal inoculation. Mapping of *VE1* locus was carried out following the procedures of Bell and Ecker (1994).

RNA gel blot analysis

Total RNA was isolated from shoot tissues of *in vitro* treated plantlets using the RNeasy total RNA isolation kit (Qiagen Inc., Valencia, CA, USA). RNA aliquots (10 µg) were resolved electrophoretically in denaturing formaldehyde-agarose gels and transferred onto nylon membranes (Scheicher & Schell, Keene, New Hampshire, USA) as described by Ausubel *et al.* (1996). Hybridization with DIG-labeled DNA probes and washes were performed as described by Yokoi *et al.* (2002). The *PR-1* probe was amplified from genomic DNA using gene-specific primers (Rogers and Ausubel, 1997). *PDF1.2* probe was amplified from the expressed sequence tag (EST) with GenBank accession number T04323 obtained from ABRC.

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