The role of Fusarium oxysporum effector protein Avr2 in resistance and pathogenicity

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CHAPTER 7

Phenotypic characterization of transgenic plants constitutively expressing *AVR2* from *Fusarium oxysporum*

Abstract

Fusarium oxysporum f.sp. *lycopersici* (*Fol*) causes vascular wilt disease on tomato (*Solanum lycopersicum*). Upon colonization of the host the fungus secretes many small proteins in the xylem sap. Eleven of these Six proteins have been identified, among them is Six3, which is a genuine virulence factor of *Fol*. Six3 is also referred to as Avr2 since it triggers host defence on *I-2*-carrying tomato plants. We previously showed that Avr2 activates I-2 intracellularly resulting in the induction a cell death response. To examine the *AVR2* expression *in planta,* we made stable transgenic Arabidopsis and tomato plants constitutively expressing the *AVR2* gene. Heterologous expression of *AVR2* in *Arabidopsis thaliana* caused enhanced susceptibility towards *Verticillium dahliae,* a xylem-colonizing fungal pathogen. In tomato, *AVR2* expression resulted in plants with elongated internodes and smaller leaf angles resulting in upward pointing leaves.

This chapter is prepared as:
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Introduction

Fusarium oxysporum is a soil-borne and highly destructive pathogen that causes vascular wilt disease on a wide range of plants. Wilt diseases lead to the severe losses in crops such as tomato, cotton, banana and melon (Dean *et al.*, 2012; Michielse and Rep, 2009). The difficulties to control *F. oxysporum* has urged plant pathologist to pursue the molecular mechanisms underlying susceptibility and resistance to *F. oxysporum*. In the past decade the interaction between tomato and *F. oxysporum* f.sp. *lycopersici* (*Fol*) has evolved as an excellent model to study these molecular mechanisms (Takken and Rep, 2010). The *Fol*-tomato interaction complies with the gene-for-gene model in which dominant and monogenic *R* genes confers race specific resistance by mediating recognition of distinct avirulence gene products. Currently, three *Fol* avirulence genes, including *AVR1*, *AVR2* and *AVR3,* have been cloned ((Takken and Rep, 2010) and chapter 3). The resistance (*R*) gene that mediates recognition of Avr2, called *I-2* for *Immunity 2*, has been cloned as well (Simons *et al.*, 1998). *I-2* encodes a classical NB-LRR protein carrying a central nucleotide-binding domain and a leucine rich repeat region.

Avr2 is a small protein without recognisable motifs or homology to known proteins. Upon colonization of the host *Fol* secretes Avr2 in the xylem sap where it is N-terminally processed into an 11-14 kDa form (Chapter 3 and 4). Although Avr2 is secreted into the xylem sap the protein appears to be recognized inside the host cell as co-expression of *I-2* and Δ*spAVR2* (a truncation lacking the sequence encoding the signal peptide) in leaves of *N. benthamiana* triggers a cell death response (Chapter 3). A knockout of *AVR2* reduces virulence of *Fol* on susceptible plants (Chapter 3) demonstrating that *AVR2* has a dual role: it can trigger *I-2*-mediated plant immunity and has an important role in pathogenicity. To gain more insights on the activity of Avr2 *in planta*, we constitutively expressed the gene in Arabidopsis and assessed its susceptibility towards *Verticillium dahliae*. In addition, the phenotype of tomato plant constitutively expressing *AVR2* is characterized.

Results and discussion

Generation of transgenic *Arabidopsis thaliana* plants stably expressing *AVR2*

To test whether Avr2 induces a phenotype in Arabidopsis, implying the presence of an effector target that is conserved between tomato and Arabidopsis, transgenic Arabidopsis lines in the Columbia-0 (Col-0) background were generated. Expression of the Δsp*AVR2* gene, without its secretion-signal coding sequence, was driven by the constitutive CaMV 35S promoter. The gene was translated in frame with a sequence encoding a C-terminal HA-SBP tag. After segregation analyses, six five independent homozygous single-integration lines (35S-Avr2: 3, 4, 6, 9, 13 and 25) identified that were retained for further analyses. To

select the homozygous lines with the highest levels of Avr2 accumulation, total protein extracts from the six independent lines was used for immunoblotting. Detection of the Avr2 protein was done using an HA-antibody recognizing the protein tag (Figure 1A). Two lines (35S-Avr2-6 and 35S-Avr2-25) that exhibit the highest levels of Avr2 accumulation were selected for further analysis. When grown under standard conditions the *AVR2*-expressing lines were indistinguishable from wild-type Col-0 and no macroscopically visible phenotypic anomalies were apparent (Figure 1B, left panel). However, we could not exclude that Avr2 induces a phenotype under other conditions or when challenged with pathogens.

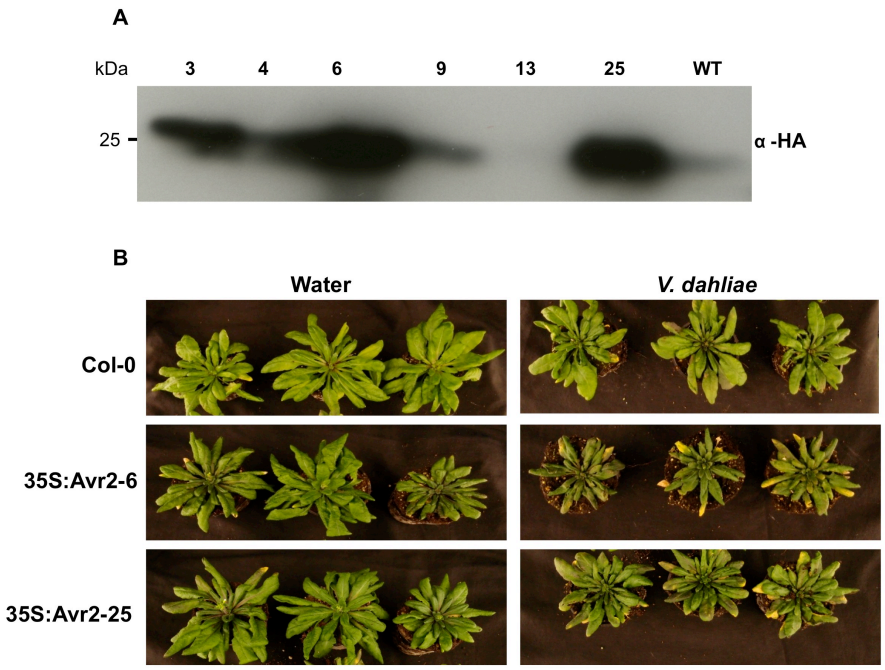

Figure 1. *AVR2*-expressing Arabidopsis plants are more susceptible to *V. dahliae*. A) Immunoblot of ΔspAvr2-HA-SBP fusion protein accumulating in homozygous *AVR2*-expressing Arabidopsis lines. Protein extracts from 21-day-old plants were subjected to immunoblot and subsequently probed with an anti-HA antibody (α-HA). WT represents wild-type Columbia-0 plants. Apparent protein sizes in kDa are indicated on the left. **B)** Typical disease symptoms are shown on *V. dahliae*-infected Columbia-0 (Col-0) and two *AVR2*-expressing plant lines (35S:Avr2-6 and 35S:Avr2-25). 21-day-old soil-grown plants were inoculated with a *V.dahliae* conidial suspension (10^6 conidia/mL) in water. As negative control, water-inoculated plants were included. *AVR2*-expressing lines show enhanced disease symptoms, reflected by their reduced plant sizes and more severe chlorosis, as compared to *V.dahliae*-infected Col-0 lines. Representative plants were photographed 23 days after inoculation.

Heterologous expression of *AVR2* in Arabidopsis enhances susceptibility toward *Verticillium*

To assess whether the *AVR2* Arabidopsis plants have altered defence responses against pathogens the 35S-Avr2-6 and 35S-Avr2-25 lines were challenged with the vascular

pathogen *Verticillium dahliae*. Wild-type Arabidopsis Col-0, which is susceptible to this pathogen, was included as positive control. As shown in Figure 1B, enhanced disease symptoms were observed in both *AVR2*-expressing lines as compared to the infected-Col-0 control. These symptoms are more severe chlorosis and enhanced wilting of the leaves and a reduced plant size when compared to the Col-0 control (Figure 1). The occurrence of a phenotype supports our previous findings that Avr2 functions inside plant cells as the produced Avr2 is cytosolic since it lacks the signal peptide for extracellular secretion (Houterman *et al.*, 2009). Although Arabidopsis is not a host for *Fol*, the enhanced disease symptoms indicate that Avr2 has a target that is conserved between Arabidopsis and tomato. Recently, an Arabidopsis-infecting *F. oxysporum* isolate Fo5176 was identified (Thatcher *et al.*, 2012), and it will be interesting to assess disease development of this strain on the *AVR2*-expressing Arabidopsis plants to test whether is also exhibits enhanced susceptibility. Inoculation with other types of pathogens, such as bacteria or oomycetes, could reveal whether the Avr2 target is specifically involved in resistance against fungal wilt diseases or whether it is involved in other resistances as well, thereby providing clues about the identity of the target. These plants also provide an excellent resource to directly identify an Avr-2 target. The produced Avr2 is equipped with an HA-SBP tag that allows affinity based pull-down assays that could co-precipitate the Avr2 plant target.

Phenotypic characterization of *AVR2*-expressing tomato plants

To explore whether Avr2 also induces a phenotype in the endogenous host of *Fol*, transgenic tomato plants were generated that constitutively produce Avr2. The same binary construct as used for generation of *AVR2*-expresssing Arabidopsis was used to transform tomato cultivar Moneymaker that does not carry resistance genes against *Fol*. To assess accumulation of Avr2 protein in T0 transgenic tomato plants, six independent 35S-Avr2 lines were subjected to immunoblotting analysis using an anti-HA antibody (35S-Avr2: 1, 2, 3, 4, 10 and 11). Expression and accumulation of ΔspAvr2-HA-SBP fusion protein was detected in all six independent lines (Figure 2A). Four lines 35S-Avr2-2, 35S-Avr2-3, 35S-Avr2-4 and 35S-Avr2-10 accumulated higher level of fusion proteins as compared to other two lines (Figure 2A), and those four were used for further analysis. Although *AVR2*-expressing Arabidopsis lines did not show phenotypic aberrations in the absence of a pathogen the 35S:Avr2 transgenic plants showed clear morphological aberrations in that they had erected petioles and elongated internodes (Figure 2B). This phenotype resembles the hyponastic response frequently observed in submerged plants as part of an escape mechanism towards flooding (Polko *et al.*, 2011; van Zanten *et al.*, 2010). In these plants it was found that elevated accumulation of ethylene in the submerged tissues is an important factor to trigger the

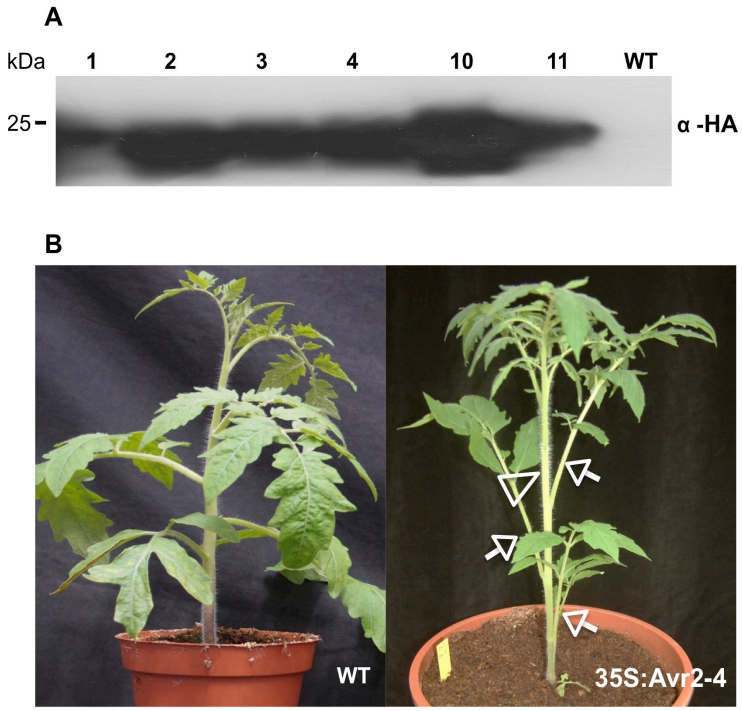

Figure 2. *AVR2*-expressing tomato plants exhibit a distinct phenotype. **A)** Immunoblot of ΔspAvr2-HA-SBP fusion proteins accumulating in T0 *AVR2*-expressing tomato plants (35S:Avr2). Protein extracts from six individual 35S:Avr2 plant lines were subjected to immnuoblot analysis. Blots were probed with an α-HA antibody. WT represents the non-transformed control. Position and size of a protein molecular mass marker is shown. **B)** 35S:Avr2 plants have erected petioles (arrowhead) and elongated internodes (arrow). A representative picture was taken three weeks after the primary transformant was transferred to soil.

hyponastic response (Peeters *et al.*, 2002; Cox *et al.*, 2004). In addition, exogenous application of ethylene to plants like Arabidopsis results in a hyponastic response (Millenaar *et al.*, 2005). Such a hyponastic response, however, is plant-species specific as for tomato flooding or ethylene treatment typically results in an epinastic, rather than a hyponastic response. An epinastic response is characterised by reduced plant stature and downward curling of the leaves and petioles (Bradford and Dilley, 1978). Since the Avr2-induced hyponastic phenotype is the opposite of that found after ethylene treatment, we speculated that Avr2 might interfere with ethylene production, sensing or signalling in tomato. To test whether ethylene responsiveness is affected in the 35S-Avr2 plants we applied exogenous ethylene to cuttings of the T0 35S-Avr2-4 line. Ethylene was applied by spraying ethephon that in aqueous solutions decomposes into ethylene, phosphate and chloride. As shown in Figure 3, ethephon-treated 35S-Avr2 plants showed reduced curling of their petioles and leaves as compared with the progenitor plants treated the same way, indicating that ethylene responsiveness is affected in the 35S-Avr2 plants.

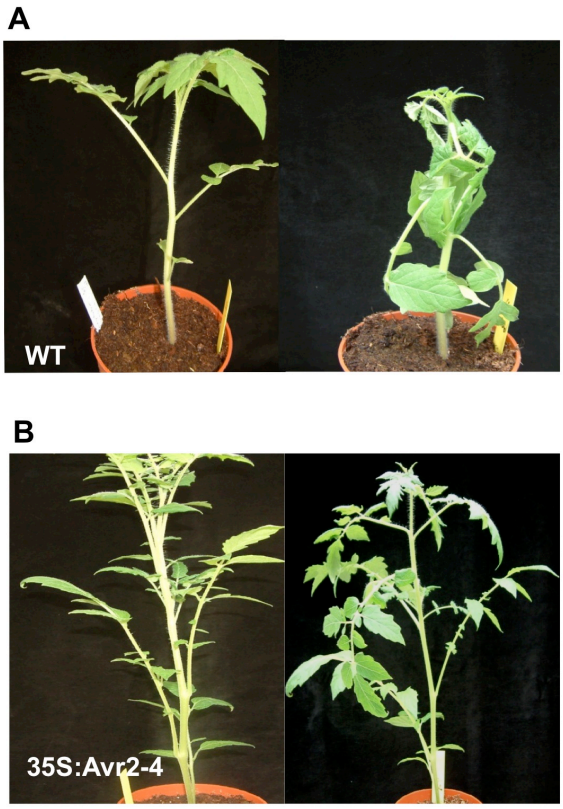

Figure 3. *AVR2*-expressing tomato plants exhibit a reduced epinastic response after ethephon treatment. **A)** Phenotypes of the ancestor (WT) before ethephon treatment (left) and after ethephon treatment (right). Ethephon-treated WT plants display strong epinastic responses reflected by extensive curling of the petioles (right). **B)** Phenotypes of the 35S:Avr2-4 before (left) and after ethephon treatment (right). The 35S:Avr2-4 plants show a strongly reduced curling of their petioles upon ethephon-treatment. A representative plant was photographed 16 hours after ethephon treatment.

Interestingly, flower development and fruit ripening of 35S-Avr2 plants was similar to that of the parental lines, although their seed yield was variable (data not shown). The latter phenotype might be a tissue-culture effect, which will become apparent when seed set of T1 plants will be analysed when they become available. As in tomato ethylene plays a critical role in determining fruit development and maturation, the development of normal fruits suggest that ethylene-production is not abolished and that perception or signalling might be affected in the *AVR2*-expressing plants (Cara and Giovannoni, 2008). Six ethylene receptors have been identified in tomato that display distinct expression patterns in the reproductive tissues (flowers and fruit), (Cara and Giovannoni, 2008). Hence, Avr2 might interfere either with specific receptors or distinct signalling pathways present in specific plant parts. Since ethylene has been reported to play an important role in disease resistance of tomato plants against many fungal pathogens such as *Botrytis cinerea*, *Verticillium dahliae*, and *Fusarium oxysporum* f. sp. *lycopersici* (Diaz *et al.*, 2002; Lund *et al.*, 1998; van Loon *et al.*, 2006), the tentative link between Av2 and ethylene might reflect its actual

activity during *Fol* infection. To confirm such a link an ethylene-insensitive *Never ripe* (*Nr*) mutant or an ETR4 (ethylene receptor)-silenced tomato plants should be infected with either a wild-type *Fol* or an *AVR2* knockout. If these plant lines exhibit the same susceptibility to both *Fol* strains this supports the hypothesis that Avr2 exhibits its virulence function via ethylene. These results have been obtained using either the primary transformants or segregating F1 lines. When homozygous lines become available, it will be interesting to assess their susceptibility to pathogens such as *V. dahliae,* to test whether Avr2 also in tomato results in enhanced susceptibility to this pathogen as found in the *AVR2*-expressing Arabidopsis plants.

Phenotypic characterization of F1 progeny derived from a cross between 35S-Avr2 and *I-2* plants

Disease resistance is often a developmentally regulated process, in that resistance is observed at the seedling stage while adult plants are susceptible or vice versa (Develey-Riviere and Galiana, 2007). To test whether *I-2*-mediated Avr2 perception and defense signalling are also developmentally regulated we crossed two independent Avr2 lines (35S-Avr2-4 and 35S-Avr2-10) with tomato plants carrying *I-2*. These crosses resulted in tomato fruits containing viable seeds, which show that the *AVR2/I-2* combination does not result in embryo lethality or seed abortion. The F1 seeds germinated normally and also growth of the seedlings, including the emergence of the hypocotyl, cotyledons and the first true leave was identical to that of *I-2* seedlings (data not shown). Around 16 days after sowing, when the seedlings were transplanted to new pots, seedlings from one cross (35-Avr2-4 × I-2) spontaneously developed necrotic sectors in the leaves (Figure 4A), whereas seeds derived from another cross (35S-Avr2-10 × I-2) did not show necrosis. Necrosis remained confined to small spots in the leaves and did not result in seedling death. Actually, the affected 35-Avr2-4 × I-2 plants continued to grow and around 25 days the F1 plants were slightly taller with elongated stem than the F1 plants that did not show necrosis, although their weight was reduced (Figure 4B). The leaves of a majority of F1 plants are tilted upward when compared to the *I-2* plants (data not shown). This elongation together with the small angle between leafs and stem resembles the hyponastic phenotype observed in the parental 35S-Avr2 lines. The plants continued to grow and even flowered but failed to set fruit. It must be noted that necrosis was only observed in crosses in which the 35S-Avr2-4 line was used as pollen donor. Seedlings derived from crosses using the 35S-Avr2-10 line did not show necrosis although in the parental line Avr2 was readily detectable. In the future the progeny plants should be analysed for expression of *AVR2* as the gene might have been silenced explaining

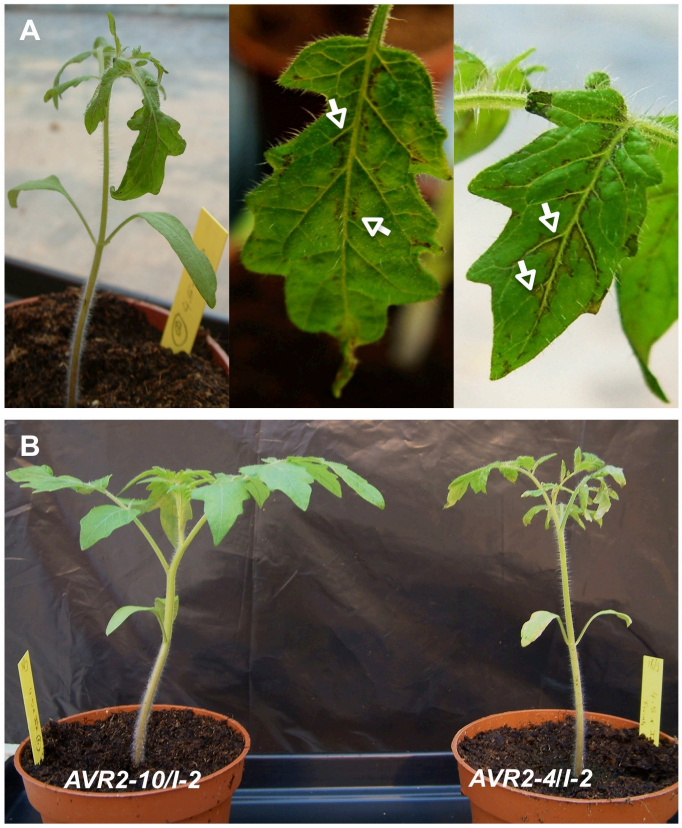

Figure 4. Phenotypic characterization of F1 plants carrying *AVR2/I-2*. A) Macroscopic symptoms of a 16-day-old F1 seedling. A whole seedling is shown in the left panel. Necrotic spots developed in young leaves (middle panel) and fully stretched older leaves (right panel). Arrows indicate the necrotic spots. **B)** A 25-day-old F1 plant with necrotic spots (right) from the cross Avr2-4 × I-2 has elongated stem and is slightly taller, but more fragile, than an F1 plant from the cross Avr2-10 × I-2 that did not show necrosis.

the lack of phenotype. Also additional crosses using independent homozygous *AVR2*-expressing lines are required to assess the reproducibility of the observed phenotype. Future studies, such as transcriptome and metabolome analysis, using these F1 plants could provide more insights on the downstream signalling events induced by the interaction between Avr2 and I-2 and the specific defence responses they trigger.

Materials and methods

Plant transformation

For *in planta* production of Avr2, the SLDB3104::Δsp*AVR2* vector was used (Chapter 4). *AVR2* was cloned behind the cauliflower mosaic virus 35S promoter for constitutive expression. The *AVR2* gene was cloned in frame with a C-terminal HA-streptavidin-binding peptide (HA-SBP) tag. The resulting vector was introduced by electroporation into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) or LBA4404 (Hoekema *et al.*, 1983) for Arabidopsis or tomato transformation, respectively. *A. tumefaciens* transformants were selected on Luria-Bertani mannitol (LBman) medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin.

Arabidopsis thaliana ecotype Columbia (Col) was transformed by the floral dip method (Clough and Bent, 1998). First-generation transformants were selected on half strength Murashige-Skoog salts medium containing kanamycin, timentin and nystain (40, 100 and 100 mg/L, respectively) and subsequently transferred to soil. Several independent homozygous single insertion lines were selected according to segregation analyses, and homozygous T3 lines were used for pathogen inoculation.

S. lycopersicum cv. Money-maker was transformed with the construct described above using *Agrobacterium*-mediated transformation as described before (Cortina and Culianez-Macia, 2004). Briefly, surface-sterilized seeds were sown on Murashige and Skoog (MS) agar supplemented with sucrose (15 g/L). The seeds were incubated in the dark in a growth chamber at 25 ˚C for 2 days, and subsequently exposed to light. After 10 days, the base and the tip of the cotyledons was removed and the cotyledons were placed upside up in Petri dishes containing co-cultivation medium (MS agar supplemented with 30 g/L sucrose, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES) [Duchefa] and 0.2 mM Acetosyringone, pH 5.75). The plates were incubated for 24 hours at 25 ˚C in dark. Transgenic *A. tumefaciens* carrying the construct of interest was grown in 30 mL LBman at 29˚C overnight (max 16 - 18 hours). After harvesting, the bacteria were resuspended in 30 mL LM2 medium (4.4 g/L Murashige and Skoog (MS), 30 g/L sucrose, 0.5 g/L MES [Duchefa] and 0.2 mM Acetosyringone, pH 5.75). Subsequently, the explants were incubated in the bacterial suspension for maximal 1 min, briefly dried on sterile filter paper and placed on co-cultivation plates. The plates were incubated in the dark for 48 hours at 25 ˚C after which the explants were transferred to selection plates (SIM) (MS agar supplemented with 30 g/L sucrose, 0.5 g/L MES, 0.5 mg/L zeatin riboside, 0.5 mg/L indole-3-acetic acid (IAA), 250 mg/L carbenicilline, 100 mg/L vancomycin, and 40 mg/L kanamycin, pH 5.75). Explants were transferred to fresh selection plate every two weeks. When callus appeared, it was transferred to new selection plates until shoots appeared. Upon shoot development, the shoots were harvested and transferred to root-inducing medium (MS agar supplemented with 15 g/L sucrose, 0.5 g/L MES, 4 g/L gelrite, 50 mg/L kanamycin, pH 5.75). Once roots developed, the plantlets were potted in soil and transferred to the greenhouse where they were grown under standard greenhouse with conditions of a 16 h photoperiod and 70% relative humidity at 25 ˚C.

Immunoblotting

To verify presence of Avr2 in transgenic Arabidopsis and tomato plants, leaves were harvested and snap-frozen in liquid nitrogen. After grinding the tissue with a mortar and a pestle, the powder was allowed to thaw in 2 ml protein extraction buffer per gram of tissue [25 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 5 mM DTT, 0.1% NP-40, 1× Roche complete protease inhibitor cocktail (http://www.roche.com) and 2% PVPP]. Extracts were centrifuged at 12 000 ***g,*** 4 °C for 10 min, and the supernatant was passed over four layers of Miracloth (http://www.calbiochem.com/miracloth) to obtain a “total” protein lysate. 40 µL samples were mixed with Laemmli sample buffer and were run on 13% SDS–PAGE gels and blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as a blocking agent. The membranes were subjected to immunoblotting using anti-HA peroxidase at a dilution of 1:3000 (clone 3F10; Roche). The secondary antibody goat-anti-rat (P31470, Pierce) was used at a 1:5000 dilution. The luminescent signal was visualized by ECL using BioMax MR film (Kodak, http://www.kodak.com).

Application of ethephon

10 mM ethephon (2-chloroethylphosphonic acid; Sigma, St. Louis, MO, USA) was applied using a hand atomizer. Plants were sprayed until the leaves where evenly wetted and then left for half hour to dry before the plants were returned to the greenhouse. Sixteen hours after spraying plants were monitored for ethylene responses and representative plants were photographed.

***Verticillium* infection assay**

Inoculation of Arabidopsis with *Verticillium dahliae* JR2 was performed as described (van Esse *et al.*, 2008; Yadeta *et al.*, 2011). Briefly, 21-day-old soil-grown plants were uprooted and inoculated by dipping the roots for 5 min in a conidial suspension (10^6 conidia/mL) in water. After replanting in soil, plants were incubated in a growth chamber with a 16 h photoperiod and 70% relative humidity at a constant 21 ˚C. Disease symptoms were monitored and pictures were taken 23 days after inoculation.

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