Multiple faces of Fusarium oxysporum effector protein Avr2

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Publication date
2017
Document Version
Other version
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Chapter 3

Involvement of SA, ET and JA signaling pathways in susceptibility of tomato for *Fusarium oxysporum*

This chapter has been provisionally accepted:
Di,X., Gomila, J., Takken, F. L. W. (2017) Involvement of salicylic acid, ethylene and jasmonate acid signaling pathways in susceptibility of tomato for *Fusarium oxysporum.* Molecular Plant Pathology

Abstract

Phytohormones such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) play key roles in plant defense following pathogen attack. Involvement of these hormones in plant susceptibility following *Fusarium oxysporum* (*Fo*) infection has mostly been studied in *Arabidopsis thaliana*. However, *Fo* causes vascular wilt disease in a broad range of crops, including tomato (*Solanum lycopersicum*). Surprisingly little is known about the involvement of these phytohormones in tomato toward *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) susceptibility. Here we investigate their involvement by analyzing the expression of ET and SA marker genes, following *Fol* infection and using bioassays on tomato mutants affected in either hormone production or -perception. *Fol* inoculation triggered expression of SA and ET marker genes showing activation of these pathways. *NahG* tomato in which SA is degraded, was hyper-susceptible to *Fol* infection and exerted stronger disease symptoms than wild-type. In contrast *ACD* and *Never ripe* (*Nr*) mutants in which respectively ET biosynthesis or -perception is impaired, showed decreased disease symptom development and a reduced fungal colonization upon infection. Susceptibility of the *def1* tomato mutant, which is deficient in JA biosynthesis, was unaltered, while a prosystemin over-expressing line, showed slightly more disease symptoms concomitantly with increased fungal colonization. The latter is surprising as prosystemin indirectly regulates JA biosynthesis. Our results show that SA is a negative and ET a positive regulator of susceptibility. The SA and ET signaling pathways appear to act synergistically as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*.

Key words: *Fusarium oxysporum*, SA, ET, JA, tomato, susceptibility

Introduction

The root-infecting fungal pathogen *Fusarium oxysporum* (*Fo*) causes vascular wilt disease in over 100 different plant species, including banana, cotton, palm, Arabidopsis and tomato (Michielse and Rep, 2009). *Fo* represents a species complex comprising many individual pathogenic strains, each capable to infect one or a few host species only. Based on host specifity, strains have been grouped into *formae speciales*. Infection by *Fo* starts upon its attachment to the plant root surface. Fungal hyphae enter roots through wounds or cracks at the root tip or at sites of lateral root formation. Ultimately the fungus reaches the xylem vessels and proliferates there. Subsequently, disease ensues (Rep et al., 2002, di Pietro et al., 2003, Berrocal-Lobo and Molina, 2008). In attempting to arrest the pathogen when it spreads through the vascular tissues, the plant blocks its infected vessels thereby compromising their ability to transport water and nutrients. Vascular browning, stunting, progressive wilting and eventually plant death are typical disease symptoms in infected plants (di Pietro et al., 2003, Agrios, 2005).

In general, plant defense responses against pathogens are controlled by complex signalling routes often involving the classical defense-phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Robert-Seilaniantz et al., 2011). Usually, SA signaling triggers resistance against biotrophic and hemibiotrophic pathogens, whereas a combination of JA and ET signaling activates resistance against necrotrophs (Glazebrook, 2005).

Due to the extensive availability of genetic and genomic resources most studies on phytohormone involvement in defense against *Fo* have been performed in Arabidopsis. (Edgar et al., 2006). Arabidopsis is susceptible to *Fo forma specialis* (f.sp.) *conglutinans* (*Focn*). Arabidopsis lines that express the salicylate hydroxylase transgene (*NahG*), or that carry the SA *induction-deficient 2* (*sid2*) mutant, are impaired in SA accumulation. Both lines exert increased susceptibility to *Fo* showing involvement of SA in disease susceptibility (Berrocal-Lobo and Molina, 2004, Diener and Ausubel, 2005).

Pre-treatment of Arabidopsis seedlings with either Methyl JA (MeJA) or the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) led to enhanced disease symptom development upon *Fo* inoculation, indicating that both ET and JA are involved in disease susceptibility (Trusov et al., 2009). The ethylene-insensitive Arabidopsis mutants *ein2* and *etr1-1* showed a reduction of disease symptoms compared to Col-0 plants when inoculated with *Focn* (Trusov et al., 2009, Pantelides et al., 2013). In contrast, various JA biosynthesis mutants such as *jasmonate resistant 1* (*jar1-1*) and *allene oxide synthase* (*aos*) did not exhibit increased susceptibility to *Fo* (Thatcher et al., 2009, Trusov et

al., 2009). Surprisingly, a point mutation in *CORONATINE INSENSITIVE* 1 (*COI1*), an essential component of JA perception, strongly reduced disease symptom development following *Fo* infection (Thatcher et al., 2009, Trusov et al., 2009). Additionally, disruption of *MYC2*, *PFT1* and *LBD20*, transcriptional regulators of JA signaling, also resulted in an increased resistance to *Fo* (Anderson et al., 2004, Kidd et al., 2009, Thatcher et al., 2012). Taken together, ET and JA are positive regulator of susceptibility in Arabidopsis.

The role of phytohormones in determining host colonization and disease symptom development is known to vary for different *formae speciales* of *Fo* and their respective hosts (Di et al., 2016). To obtain a better insight in these processes it is therefore crucial to investigate the role of phytohormones in defense responses to *Fo* in plant species other than Arabidopsis. Tomato (*Solanum lycopersicum*), a major and important vegetable crop (Panthee and Chen, 2010), is susceptible to *Fo* f.sp. *lycopersici* (*Fol*) resulting in significant yield losses each year (McGovern, 2015). The interaction between tomato and *Fol* is well studied (Takken and Rep, 2010). Like other *formae speciales* of *Fo Fol* colonizes the vasculature and infected plants exhibit vascular browning, leaf epinasty, stunting, progressive wilting and eventually death (di Pietro et al., 2003). During colonization the fungus secretes virulence factors called effector proteins (Houterman et al., 2007). Deletion of specific effectors, such as Avr2, compromises virulence of the fungus making it less pathogenic than wild-type *Fol* (Houterman et al., 2007, Houterman et al., 2009).

For tomato a great collection of lines is available that are compromised either in hormone perception, -metabolism or -signaling. In our study, mutants affected in the biosynthesis and signaling pathway of specific defense-related hormones were analyzed for their susceptibility to *Fol*. The lines used included; transgenic *NahG* plants that express the bacterial enzyme salicylate hydroxylase, which converts SA into biologically inactive catechol, making the plants deficient in SA accumulation (Brading et al., 2000). *Never ripe*, a dominant ET-insensitive mutant, carrying a single base substitution in the region encoding the N-terminus of ETR3, which is a homolog of the Arabidopsis ETR1 receptor (Wilkinson et al., 1995). The transgenic tomato line *ACD*, which expresses the *ACCD* (1-amino-cyclopropane-1-carboxylic acid deaminase) gene; encoding the ACCd enzyme that catalyzes the degradation of ACC. JA-deficient mutant *defenseless-1* (*def1*), which has a defect in the jasmonate pathway between 13-HPOTE and 12-oxo-PDA. This mutant fails to form JA and to systemically accumulate proteinase inhibitors in response to systemin and oligosaccharide elicitors (chitosan and polygalacturonide) (Li et al., 2002). In addition, a *35S::prosystemin* transgenic line overexpresses prosystemin was included. Prosystemin is a positive regulator of JA signaling, and hence the plants constitutively accumulate high levels of proteinase inhibitor proteins (Howe and Ryan, 1999).

Here, we report on our inoculation assays of the various tomato lines affected in SA, ET and JA signaling with wild-type *Fol* and the *FolΔAvr2* mutant. In contrast to JA signaling, which appears to be involved mainly in the ability of the fungus to colonize the plant, SA and ET have major and opposing roles in disease susceptibility and development. The SA and ET signaling pathways appear to act synergistically as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*. A model for the role of SA, ET and JA signaling in tomato toward susceptibility against *Fol* is proposed and compared to that in Arabidopsis.

Results

NahG tomato plants show enhanced disease symptom development upon *Fol* infection

To assess a potential role of SA in modulating susceptibility against *Fol*, three-week-old wild-type tomato plants (cultivar Moneymaker) and transgenic *NahG* plants impaired in SA accumulation, were inoculated with either water (mock) or wild-type *Fol*, notably race 2 isolate *Fol007*. Additionally, to allow assessment of hyper-susceptibility, a *Fol007 Avr2* knockout strain (*FolΔAvr2*) was included. This mutant is compromised in virulence and causes less disease symptoms on susceptible plants (Houterman et al., 2009). As shown in Figure 1A, *NahG* plants inoculated with *Fol007* exhibited more severe disease symptoms than wild-type plants. These symptoms include extensive wilting and a more severe stunting three weeks after inoculation. Consistent with this, the fresh weight of *Fol007*-infected *NahG* tomato plants was significantly lower than that of corresponding wild-type plants. Moreover, all vascular bundles of infected *NahG* plants had turned brown, and plants were either dead or very small and wilted. On a scale from 0 to 4 (Gawehns et al., 2014) the infected *NahG* plants scored the maximal disease index (Figure 1B and C). As expected, *FolΔAvr2*-inoculated plants developed less severe disease symptoms (Figure 1A). Similar to *Fol007*, *FolΔAvr2* infected *NahG* plants showed a significant reduction in fresh weigh and a higher disease index as compared to wild-type plants (Figure 1B and C).

To investigate whether the augmented disease symptom development in *NahG* plants correlated with increased host colonization, a fungal recovery assay was performed. Thereto, sections were taken from *Fol*-inoculated wild-type and *NahG* plants at different heights of the stem, notably at the position of 1) the cotyledon, 2) the second node and 3) the fourth node, and upon sterilization placed on CDA plates and incubated for 5 days at 25°C. As shown in Figure 1D, in 30% of the cases the fungus was observed in stem sections collected at the fourth node of *Fol007*-inoculated wild-type plants, whereas in 70% of the cases of that in *Fol007*-inoculated *NahG* plants. Typically, colonization of

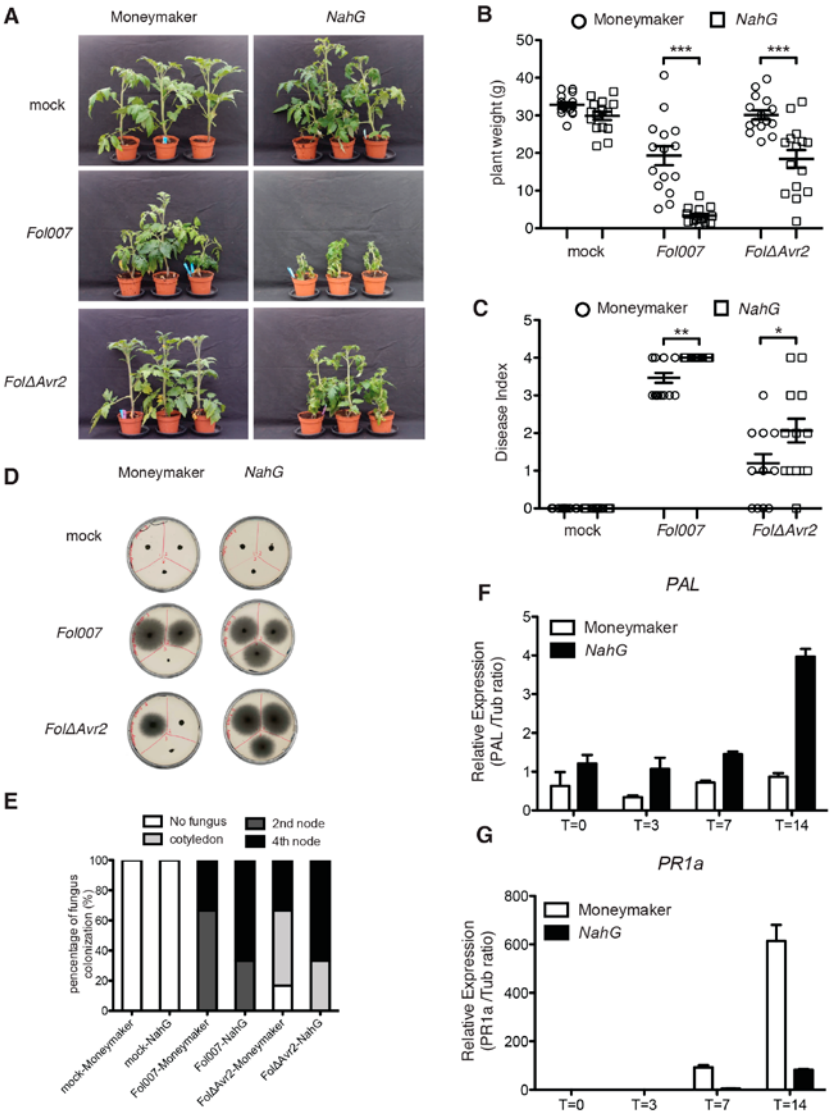

Figure 1. Impaired SA signaling enhances *Fol* disease symptom development in tomato. (A). Three-week-old wild-type Moneymaker and *NahG* tomato plants inoculated with either water (mock), *Fol007* or *FolΔAvr2* at 21 days post-infection (dpi). (B) Disease development was scored by measuring fresh plant weight and (C) determining the disease index (ranging from 0-4) of 20 plants/genotype. Circles and squares indicate respectively Moneymaker and *NahG* plants. Plant weight and disease index were subjected to a pairwise comparison with a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual *Fol*-inoculated plants (n=6) after incubation for five days on CDA plates. (E). Percentage of infected slices showing fungal outgrowth. Fungal progression in the stem was expressed as infected percentage of all stem pieces. (F) Transcription patterns of *PAL* and (G) *PR1a* in *Fol007*-inoculated wild-type Moneymaker and *NahG* at 0, 3, 7 and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

wild-type plants was much reduced from stem sections of *FolΔAvr2*-inoculated wild-type plants, while *FolΔAvr2* was found to efficiently colonize *NahG* plants as fungal outgrowth was often observed up to the fourth node (Figure 1E). Together these data suggest that *NahG* plants are hyper-susceptible to *Fusarium* infection and that the increased symptoms correlate with increased fungal colonization of the transgenic plants.

SA is biosynthesized in the phenylpropanoid pathway through the activity of phenylalanine ammonia-lyase (PAL) (Lee et al., 1995). To assess expression of the *PAL* gene during infection, an RT-qPCR analysis was carried out on hypocotyls. Samples were taken at 0, 3, 7 and 14 days after inoculation of wild-type and *NahG* plants, and expression levels were measured and normalized to tubulin. We found that at 14dpi transcript levels of *PAL* were up-regulated in *NahG* plants compared to wild-type plants (Figure 1F).

Very often *PR1a* expression is used as a reporter for SA-dependent defense signaling (Kunkel and Brooks, 2002). To assess whether *PR1a* expression is altered during *Fol* infection, its expression at 0, 3, 7 and 14 days after inoculation was measured. Transcript levels of *PR1a* were strongly induced in wild-type plants upon infection, suggesting that SA signaling is activated during *Fol* infection (Figure 1G). Compared to wild-type plants the expression of *PR1a* in infected *NahG* plants was very much reduced. This correlates with the loss of SA accumulation in *NahG* plants, confirming the proposed phenotype of the transgenic line. Overall, these data suggest that impaired SA signaling enhances *Fol* susceptibility and disease symptom development in tomato.

ET enhances susceptibility to *Fol* in tomato plants

Pre-treatment of Arabidopsis seedlings with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) leads to enhanced disease symptom development upon *Focn* inoculation, indicating that ET is involved in disease susceptibility (Trusov et al., 2009). To investigate the role of ET in disease symptom development in tomato, transgenic plants impaired in ET-biosynthesis were analyzed for their susceptibility to *Fol* infection. In the transgenic line *ACD*, constitutively expressing a bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase gene, ET production is reduced 90% compared to wild–type (Klee et al., 1991). Since the transgene is present in cultivar UC82B, this cultivar was used as wild-type control. Whereas UC82B showed severe wilting and stunting following *Fol* infection, most *ACD* plants showed only mild disease symptoms (Figure 2A). Plant weight of infected *ACD* plants was significantly higher than that of infected wild-type plants (Figure 2B). In addition, the disease index in *ACD* plants was also significantly attenuated as compared to the wild-type plants (Figure 2C). A similar reduction in

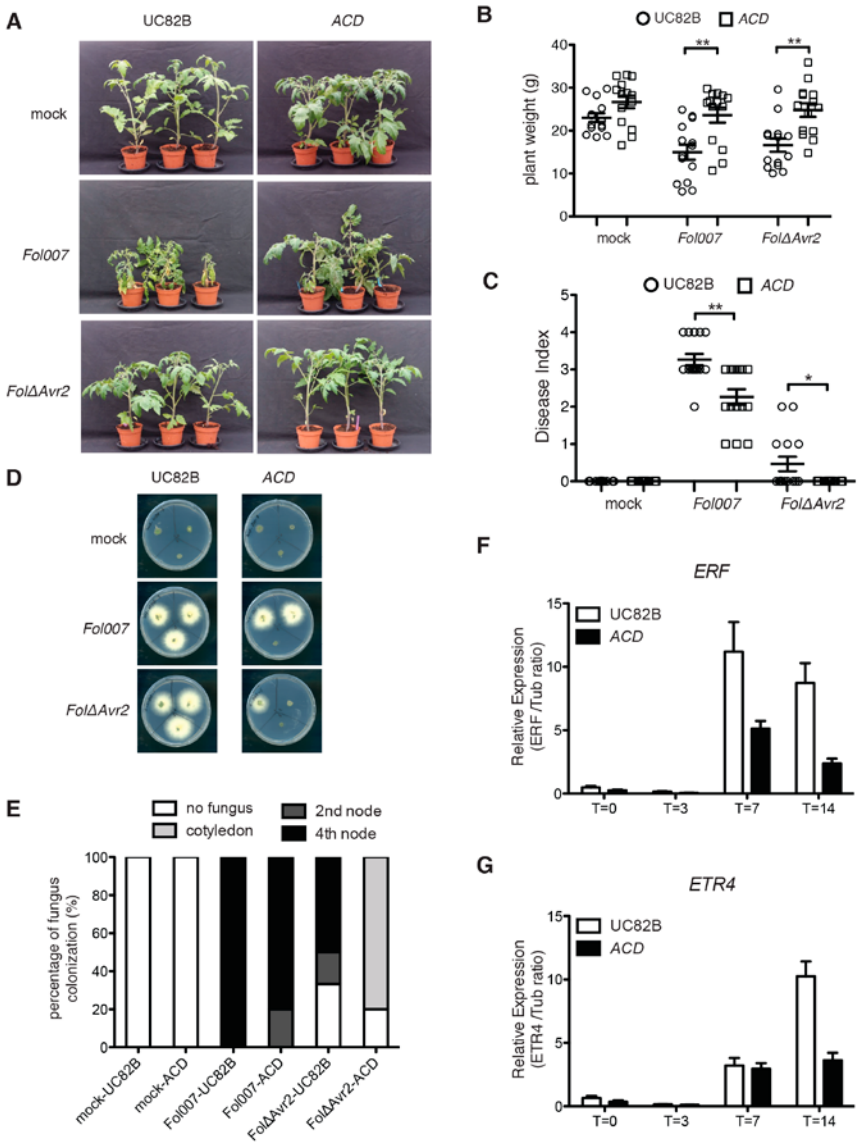

Figure 2. Impaired ET biosynthesis and production in tomato reduces disease susceptibility to *Fol*. (A). Three-week-old wild-type UC82B and *ACD* tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease development was scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circles and squares indicate UC82B and *ACD* plants (n = 20), respectively. Plant weight and disease index were subjected to a pairwise comparison using a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6). (F). Transcription patterns of ET-regulated marker genes *ERF* and *ETR4* in *Fol007*-inoculated UC82B and *ACD* at 0, 3, 7 and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

symptom development was also observed in *ACD* lines inoculated with *FolΔAvr2*. To monitor fungal colonization stem sections were taken (see above) and incubated on CDA plates. The fungal recovery assay showed that *Fol007* grew out from most stem sections of both wild-type and the *ACD* line, whereas much less fungal growth was observed in *FolΔAvr2*-inoculated *ACD* plants. A typical example of a plate assay is shown in Figure 2D and the data from fungal recovery assay is summarized in Figure 2E. *FolΔAvr2* was found to efficiently colonize wild-type plants as fungal outgrowth was often observed up to the fourth node. Colonization of *ACD* plants was much reduced: in 80% of the cases the fungus was only observed in stem sections collected at the cotyledons. These data indicate that the *ACD* line exerts a reduced susceptibility towards Fol infection concomitantly with a reduction in symptom development.

It has been reported before that the ET receptor gene *ETR* and ET responsive factor *ERF* are induced by *Fo* infection (Berrocal-Lobo and Molina, 2008, Pantelides et al., 2013). As shown in Figure 2F and 2G, transcription of *ETR4* and *ERF* was highly induced in wild-type plants following *Fol* inoculation. Compared to wild-type, *Fol* infected *ACD* plants showed reduced expression of the tested genes. Apparently, the lack of ET accumulation in the *ACD* line results in a reduction of *ETR4* and *ERF* expression suggesting that the ET mediated signaling pathway is activated during *Fol* infection.

To distinguish whether the ET synthesis contributes to increased susceptibility by affecting the virulence of the fungus or the responses of the plant, the involvement of ET perception by the host was investigated. To study whether ET perception is required for disease symptom development following *Fol* infection, bioassays were performed with wild-type tomato cultivar Pearson and the ET insensitive Pearson mutant *Never-ripe* (*Nr*). Upon inoculation of wild-type Pearson with *Fol007*, the older leaves of infected plants became chlorotic and the plants showed mild wilting symptoms (Figure 3A). Upon inoculation with *FolΔAvr2*, wild-type plants showed hardly any symptom (Figure 3A). Notably, no obvious disease symptoms were observed in inoculated *Nr* plants. Although the fresh weight of *Nr* plants was identical to that of Pearson plants after *Fol* infection (Figure 3B), the *Nr* plants exhibited a significantly lower disease index than Pearson, as fewer brown vessels were observed in the stems (Figure 3C). Fungal recovery assays revealed that *FolΔAvr2* either completely failed to colonize *Nr* plants, or in the rare cases it could, it only reached the basal part of the stem that forms the hypocotyl (Figure 3D and E). Taken together the data suggest that the inability to synthesize ethylene as well as the perception of the hormone are essential for both disease development and the ability of the fungus to colonize the plant.

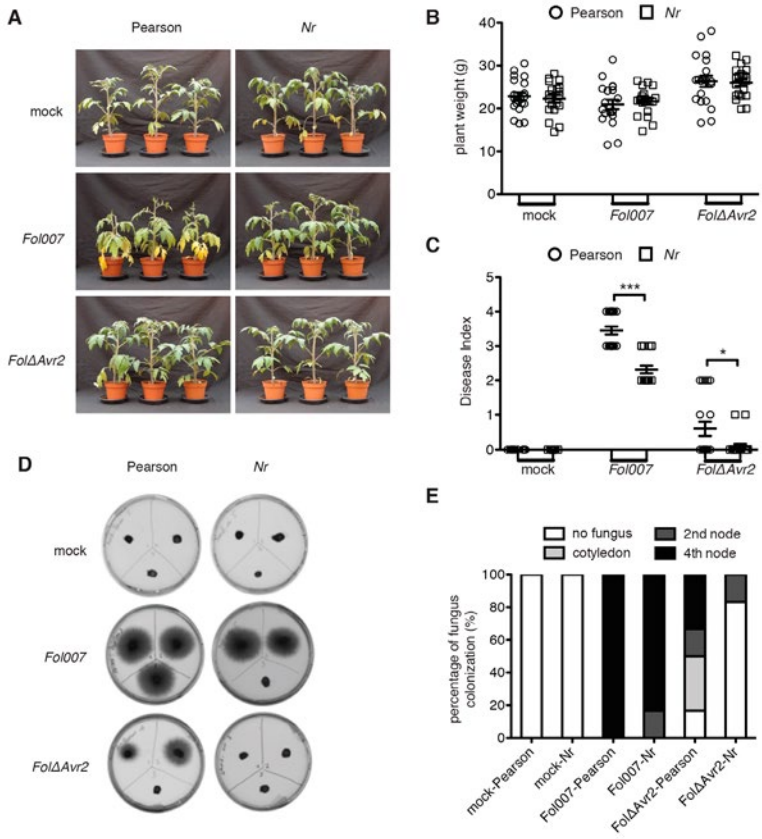

Figure 3. ET perception is required for *Fol* disease symptom development in tomato. (A). Three-week-old wild-type and *Nr* Pearson tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease symptoms were scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circle and square indicate Pearson plant and *Nr* plant (n = 20), respectively. Plant weight and disease index were subjected to a pairwise comparison using a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6).

JA plays a minor role in the ability of the fungus to colonize tomato

In Arabidopsis, various JA biosynthesis mutants such as *jasmonate resistant 1* (*jar1-1*) and *allene oxide synthase* (*aos*) do not exhibit increased susceptibility to *Focn* (Thatcher et al., 2009, Trusov et al., 2009). To investigate the role of JA biosynthesis in susceptibility of tomato to *Fol*, we selected the *def1* mutant. This line has a defect in its octadecanoid biosynthesis pathway, which provides the precursors for JA synthesis, making the plant hyper-susceptible to herbivores due to its impaired accumulation of proteinase inhibitors (PIs) I and II in response to wounding (Lightner et al., 1993). Besides *def1* also *35S::prosystemin* plants were analyzed. In these plants the prosystemin gene is

constitutively overexpressed by the 35S cauliflower mosaic virus promoter. Prosystemin is the precursor of systemin, which initiates a signaling pathway that leads to synthesis of JA from linolenic acid (Ryan, 2000). The constitutive induction of the JA pathway in *35S::prosystemin* plants results in the systemic accumulation of high levels of PIs in these plants (McGurl et al., 1994).

As shown in Figure 4A, *Fol007* infected *def1* and *35S::prosystemin* lines both became more chlorotic than the wild-type parental cultivar Castlemart. Inoculation with the less pathogenic *FolΔAvr2* mutants did not result in obvious disease symptoms in any of the lines. Although *def1* and *35S::prosystemin* plants exhibited a slightly higher disease

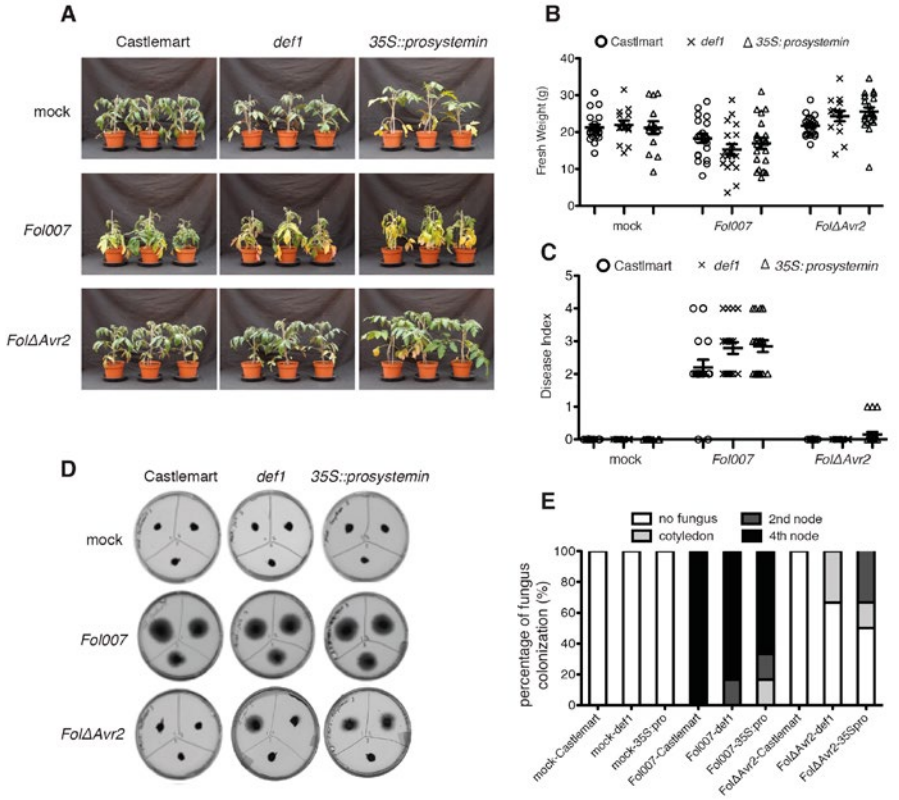

Figure 4. JA signaling is affects the ability of the fungus to colonize tomato. (A). Three-week-old wild-type Castlemart, *def1* and *35S:prosystemin* tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease symptoms were scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circle, × and Δ indicate Castlemart, *def1* and *35S:prosystemin* (n = 20), respectively. Plant weight and disease index were subjected to one-way ANOVA with Dunnet's *post-hoc* test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6).

index and a reduction in fresh weight as compared to wild-type plants, this difference was not significant for neither *Fol007* nor *FolΔAvr2* infection (Figure 4B and C). Fungal recovery assay showed that in wild-type Castlemart *Fol007* was present in all probed stem sections, indicating that the fungus has colonized the entire stem until the forth node (Figure 4D and E). In 20-30% of the inoculated *def1* and *35S::prosystemin* plants *Fol007* did not colonize the stem until the 4th node. Interestingly, in contrast to *Fol007*, *FolΔAvr2* completely failed to colonize wild-type Castlemart plants, which is consistent with the lack of disease symptoms in these plants. However, from the symptomless *35S::prosystemin* plants the fungus could be observed to grow out from stem sections collected either at the position of the cotyledon or of the second node in 40% of the cases (Figure 4E). Also from the *def1* mutant the fungus could be cultured from the lowest node in 30% of the plants. These data suggest that altered JA homeostasis enhances the ability of the fungus to colonize the plant, but does not significantly affects disease symptom development.

SA and ET signaling pathways act synergistically in tomato susceptibility against *Fol* infection

Since low levels of SA enhance susceptibility to *Fol*, while impaired ET signaling reduces susceptibility, we wanted to test whether SA interacts with ET in the response ofv tomato to *Fol*, and to identify which of the two pathways is dominant. To this end, the expression of *PR1a* was monitored over time (0-14 dpi) in the *ACD* ethylene synthesis mutant and wild-type upon *Fol007* infection. As shown in Figure 5A, SA marker gene *PR1a* was greatly induced in wild-type plants, but much less in *ACD* lines, clearly indicating that induction of *PR1a* following *Fol* infection requires an intact ET pathway. Subsequently, the expression of *ERF* and *ETR4* were assessed in the *NahG* line and in its parental wild-type line Moneymaker following *Fol007* inoculation. As shown in Figure 5B and C, the ET signaling pathway was strongly induced in wild-type plants, but repressed in *NahG* plants inoculated with *Fol*. The above results suggest that SA and ET signaling pathways act synergistically in tomato following *Fol* infection: impairment of one pathway compromises the activation of the other.

Discussion

Here the role of SA, ET and JA in modulating susceptibility of tomato plants to *Fol* was investigated. *NahG* plants that fail to accumulate SA were hyper-susceptible to *Fol* infection and showed severe disease symptoms and extensive fungal colonization of their xylem vessels. Together with the strong induction of the SA marker gene *PR1a*, which was not induced in the *NahG* plants, these data show that SA plays a positive role in reducing disease susceptibility. This conclusion is in agreement with chemical

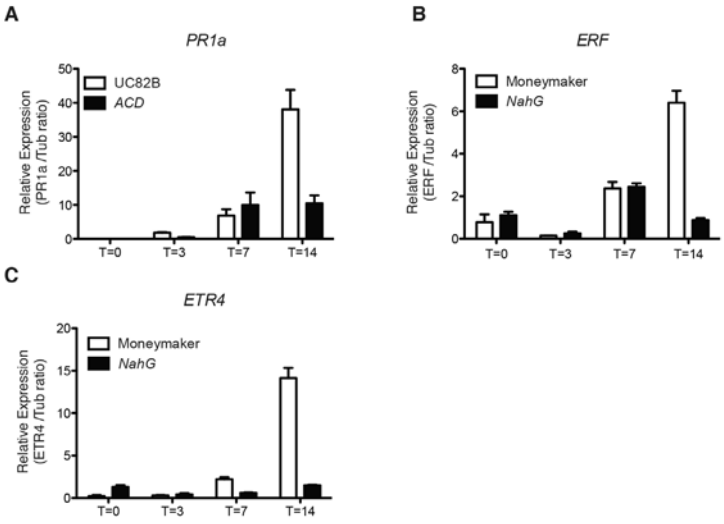

Figure 5. Time course of transcription patterns of SA and ET marker genes upon *Fol* inoculation. (A) Expression of the SA marker gene *PR1a* in wild-type UC82B and the transgenic *ACD* line at 0, 3, 7, and 14 dpi. (B-C) Transcription patterns of the ET-marker genes *ERF* and *ETR4* in *NahG* and the Moneymaker progenitor at 0, 3, 7, and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

studies, in which exogenous application of SA to tomato through root feeding or foliar sprays reduced vascular browning, leaf yellowing and wilting following *Fol* inoculation (Mandal et al., 2009). The positive role of SA in *Fo* resistance in tomato is consistent with studies with *NahG* Arabidopsis showing an increased susceptibility to *Fo* (Berrocal-Lobo and Molina, 2004, Diener and Ausubel, 2005, Thatcher et al., 2009, Trusov et al., 2009). Therefore, the role of SA in *Fo* susceptibility seems conserved in both plant species. Interestingly, in several studies, elevated SA is reported to enhance susceptibility to necrotrophic pathogens, but to promote resistance to hemibiotrophs (Bari and Jones, 2009, El Oirdi et al., 2011). Our finding in which SA reduces the susceptibility of tomato plants to *Fol* is in line with the hemibiotroph lifestyle of latter.

The *ACD* tomato line, in which ET biosynthesis is compromised, showed a reduced susceptibility to *Fol* infection. Upon *Fol* infection the transgenic line showed less disease symptoms and a reduced fungal colonization as compared to the wild-type UC82B cultivar (Figure 2). The ET marker genes *ETR4* and *ERF* were highly induced in wild-type plants, but not in the *ACD* line, indicating that ET signaling is induced in response to *Fol* infection and is important for disease development. The ET-insensitive tomato line *Nr* as well was found to be less susceptible than wild-type to infection with *Fol007* (Figure 3). These data are consistent with a study by Lund and coworkers in which a reduction of disease symptoms in the *Nr* mutant was found as well upon infection with a different *Fol* isolate (Lund et al., 1998). The fact that both ET synthesis and perception were found

to be important for disease development as well as for fungal colonization suggests that *Fol* has hijacked the host's ET signaling pathway to cause disease.

The role of ET is multifaceted in Arabidopsis (Di et al., 2016). Similar to the tomato *ethylene insensitive2* (*ein2-1*) and *etr1-1* mutants grown in soil, a reduction of disease symptoms compared to wild-type Col-0 plants is seen when inoculated with *Focn* (Trusov et al., 2009, Pantelides et al., 2013). Fo inoculation of plants carrying the *ein2-5* allele revealed a markedly enhanced susceptibility to *Fo* in plate assays (Berrocal-Lobo and Molina, 2004). These differences might be explained by the different mutations or by the different inoculation methods. The *ein2-1* mutation harbors a stop codon after the sequence encoding transmembrane domain, which might result in a partial functional EIN2, whereas the *ein2-5* mutant contains a frame-shift in the transmembrane-spanning α-helix and hence is likely a complete loss-of-function mutant (Alonso et al., 1999). As inoculation of soil-grown plants best mimics the natural infection process, which resembles that of our tomato assays, it seems that in both plant species the role of ET is conserved in that its absence reduces disease symptom development (Trusov et al., 2009, Pantelides et al., 2013).

No significant difference in disease index and fresh weight between wild-type and the JA deficient *def1* line was found after *Fol007* or *FolΔAvr2* inoculation (Figure 4). These findings contrast those of Thaler and coworkers who showed that the weight of *def1*, but not wild-type plants, was reduced upon inoculation with a race 1 *Fol* isolate (Thaler et al., 2004). One reason for the discrepancy could be the different *Fusarium* race used, or differences in assay conditions. The observation that the colonization of the vasculature of the *35S::prosystemin* plant by *Fol007* was reduced compared to wild-type plants whereas it was increased for the *FolΔAvr2* variant, indicates that Avr2 might manipulate the JA signaling pathway to promote infection. Involvement of *Fo* effectors in manipulating hormone signaling of the host has been suggested before (Di et al., 2016). Future experiments could reveal whether *Fol* effectors are indeed manipulating the JA pathway by performing bioassays on the JA mutants using different *Fol* races and/or the effector knockout strains previously described (Gawehns et al., 2015).

Upon *Fol007* infection the *35S::prosystemin* plants became slightly more chlorotic with a higher, but non-significant increase in disease index together with a minor reduction of fresh weight as compared to wild-type tomato. Fungal recovery assays revealed a more extensive fungal colonization in *35S::prosystemin* plants compared to wild-type. Thus, under our assays conditions JA appears not to play a major role in development of disease symptoms, but it facilitates fungal colonization.

In different host-*Fo* pathosystems JA can promote either resistance or susceptibility (Di

et al., 2016). A point mutation in *CORONATINE INSENSITIVE 1* (*COI1*) in Arabidopsis, an essential component for JA perception, strongly reduced susceptibility to *Fo* (Thatcher et al., 2009, Trusov et al., 2009). In contrast, *jar1* mutants that are defective in synthesis of the bioactive JA-Isoleucine conjugate showed wild-type-like symptoms or only a slight increase in susceptibility (Thatcher et al., 2009, Trusov et al., 2009). Additionally, Cole reported that infection by *Fo* f.sp. *conglutinans* and *Fo* f.sp. *matthioli*, which produce isoleucine- and leucine-conjugated jasmonate (JA-Ile/Leu), respectively is suppressed in *coi1*. In contrast, *Fo* f.sp. *raphani*, which produces no detectable JA-Ile/Leu, has no effect on infection in *coi1*. Furthermore, *Fol*, which produces no detectable jasmonates, has no effect on wilt disease in *jasmonic acid-insensitive* (*jai1*) tomato plants (Cole et al., 2014). Therefore different *formae speciales* may adopt different strategies to infect their host and to cause disease symptoms.

The SA, ET and JA signaling pathways are entangled in a complex network in which the different pathways influence each other through positive and negative regulatory interactions (Grant and Jones, 2009). We observed that compared to wild-type plants the expression of *PR1a* was reduced in the ET biosynthesis mutant *ACD* (Figure 5). Similarly, following *Fol* inoculation the induction of ET signaling, as monitored by reduced *ETR4* and *ERF* expression, was strongly decreased in *NahG* lines as compared to wild-type plants. Collectively, these results indicate that in tomato SA and ET signaling act synergistically during *Fol* infection as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*. Also for *X. campestris* pv. *vesicatoria* infection of tomato accumulation of SA was found to require ET synthesis, suggesting that ET positively regulates SA-induced defenses (O'Donnell et al., 2003).

All data together allow us to propose a model for involvement of SA, ET and JA signaling in tomato toward susceptibility against *Fol* (Figure 6A). SA and ET signaling interact and have opposite roles in disease susceptibility. Infection of tomato plants by *Fol* activates both the ET and SA pathways. The ET response enhances susceptibility to *Fol* infection and disease development whereas SA responses restrict colonization. The role of JA in infection is less clear and seems mostly confined to the ability of the fungus to colonize the plants. A comparison with the reported roles of these phytohormones in Arabidopsis to *Fo* infection reveals shared and unique effects between Arabidopsis and tomato. As shown in Figure 6B, SA signaling also negatively regulates susceptibility to *Fo*, whereas ET signaling likewise positively enhances susceptibility. Notably, JA can be hijacked by the fungus to enhance pathogenicity in Arabidopsis, but only enhances the ability of the fungus to colonize tomato plants.

The role of the ET, SA and JA in susceptibility to *Fo* seems broadly conserved between *A. thaliana* and the crop tomato. This insight is relevant to allow translation of molecular

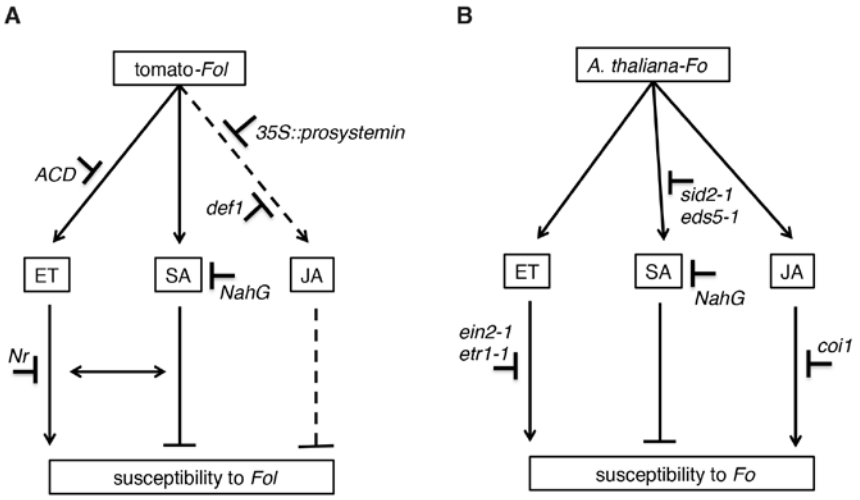

Figure 6. Proposed models for the involvement of the JA, SA, and ET signaling in tomato (A) and Arabidopsis (B) upon *Fol* infection are shown. Compromised ET biosynthesis and -perception reduce disease susceptibility, while compromised SA signaling promotes hyper-susceptibility to *Fol* infection. The SA and ET pathways act synergistically, in that induction of one pathway requires intactness of the other. JA affects the ability of the fungus to colonize tomato, but does not significantly affect disease symptom development (dashed line). By convention, the arrowhead implies positive regulation (stimulation) and the T-bar implies negative regulation.

insights obtained in this Arabidopsis model into actual crops when aiming for a reduced susceptibility to wilt disease. The molecular mechanisms underlying susceptibility, however, are currently unknown and their elucidation is a challenge for future studies.

Materials and Methods

Plant materials

Ten different tomato (*Solanum lycopersicum*) genotypes were used in these studies including the four wild-type cultivars from which these mutants are derived: Moneymaker, UC82B, Pearson and Castlemart. The transgenic *NahG* line, which is compromised in SA accumulation, is in a Moneymaker background (Brading et al., 2000). The ET-impaired mutant *ACD* (Klee et al., 1991) and the *Never ripe* mutation (Lanahan et al., 1994) are in a UC82B background and Pearson cultivar, respectively. The JA-impaired mutant *def1* (Howe et al., 1996) and prosystemin overexpressing line *35S::prosystemin* are in the Castlemart background (McGurl et al., 1994). Tomato seedlings were grown in a conditioned green house with day–night temperatures of 23–18°C and a 16h light/8h dark regime.

Fusarium inoculation assay

Wild-type *Fusarium* strain *Fol007* (race 2) and the derived *FolΔAvr2* mutant have been descripted before (Houterman et al., 2009). *Fol* strains were grown on Czapek Dox Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) at 25°C for 7-10 days, Subsequently, a piece of agar carrying the fungus was transferred to 100ml minimal medium (100 mM KNO3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids or ammonia). Conidial spores were harvested after 3-5 days of cultivation at 25 °C with shaking. After washing with sterilized water the spores were diluted to 10^6 spores/ml. For bioassay, 3-week-old tomato seedlings were uprooted from the soil. The seedlings were placed for 5 min in the Fol spore suspension (10^6 spores/ml) and subsequently potted. Disease progression was evaluated after 3 weeks. Plant weight and disease index (Gawehns et al., 2014) were scored for 20 plants/treatment.

Fungal recovery assay

Fungal colonization in tomato plants was assessed at 21 days after inoculation. Stem sections at cotyledon, 2nd node and 4th node were collected separately. The stem pieces were surface sterilized in 70% ethanol, rinsed in sterile distilled water after which the ends of the stem were removed with a sterile scalpel. Stem sections of about 5 mm thick were cut and placed on potato dextrose agar (PDA) supplemented with 200mg/l streptomycin and 100mg/l penicillin at 25°C, allowing the fungus to grow out of the stem sections. Pictures were taken after 5 of incubation at 25°C. Data were expressed as a percentage of slices showing fungal outgrowth.

Analysis of gene expression by RT-qPCR

RNA isolation and cDNA synthesis was done as described before (Gawehns et al., 2014). Briefly, total RNA from tomato stem beneath cotyledon was extracted using Trizol-Reagent (Invitrogen, Life Technologies, Grand Island, NY, U.S.A.) according to the manufacturer's instructions. The RNA was subsequently purified with RNeasy Mini kit (Qiagen, Düsseldorf, Germany) and DNA was removed by on-column treatment with RNase-free DNase(Qiagen). cDNA was synthesized using the M-MulV reverse-transcriptase RNase H minus kit (Fermentas, Thermo Scientific, Pittsburgh PA, U.S.A.). Stem tissue was collected from tomato plants 0, 3, 7, 14 days upon pathogen infection. The conditions of RT-qPCR (reverse transcription-quantitative polymerase chain reaction) experiments and the relative quantification of specific mRNA levels was performed according to Lopez-Raez et al. (2010) (Lopez-Raez et al., 2010) and using the gene-specific primers described in Table 1. PCRs were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, http://www.appliedbiosystems.com), using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The 20ul PCR reactions contained 0.25 uM of each primer, 0.1 ul ROX reference dye and 1 ul of cDNA. The cycling program was set to 5 min at 50°C, 5 min at 95°C, 40 cycles of 15 sec at 95°C and

1 min at 60°C, followed by a melting curve analysis. The expression levels of selected genes were normalized to tomato α-Tubulin (Solyc04g077020.2) expression. Relative gene expression was calculated using the $2^{-\Delta CT}$ methods. Three biological replicates for each of the selected genes were performed.

Statistical analyses

The statistical significance of the results was determined by performing PRISM 5.0 (GraphPad, http://www.graphpad.com). The data on plant weight and disease index in SA and ET related tomato plants were subjected to a pairwise comparison with the Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). For data on JA related tomato plants, a one-way ANOVA with Dunnet's *post-hoc* test (*P<0.05; **P<0.01; ***P<0.001) were made for each genotype. Pairwise comparisons with Student's *t*-test were also made for expression analysis for each gene in the different genotypes. All the experiments were performed twice, with similar results.

Acknowledgement

We thank Jonathan D.G. Jones (Sainsbury Laboratory) for providing the transgenic *NahG* line. *ACD* and *Never ripe* mutants were obtained from M. Mudgett, Stanford University (provided by H. Klee, University of Florida). *def1* is obtained from M. Mudgett, Stanford University (provided by G.Howe, Michigan State University). We also thank M. Kant (University of Amsterdam) for providing *35S::prosystemin*. The authors wish to thank Ben Cornelissen for critically reading and commenting on the manuscript.

Table1. Primer sequences used in the gene expression analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
α-tubulin	TCGTGGCCACTATACCATTG	AGTGACCCAAGACCTGAACC
PR1a	TGGTGGTTCATTTCTTGCAACTAC	ATCAATCCGATCCACTTATCATTTTA
ERF	TCGTCGGGAAACGGTTCCAT	GACATCCAACTTGCATGACACTTG
ETR4	GGTAATCCCAAATCCAGAAGGTTT	CAATTGATGGCCGCAGTTG
PAL	CGTTATGCTCTCCGAACATC	GAAGTTGCCACCATGTAAGG

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