A Single Effector Protein, AvrRpt2_{EA}, from *Erwinia amylovora* Can Cause Fire Blight Disease Symptoms and Induces a Salicylic Acid–Dependent Defense Response

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The AvrRpt2_{EA} effector protein of *Erwinia amylovora* is important for pathogen recognition in the fire blight-resistant crabapple *Malus × robusta* 5; however, little is known about its role in susceptible apples. To study its function in planta, we expressed a plant-optimized version of AvrRpt2_{EA} driven by a heat shock-inducible promoter in transgenic plants of the fire blight-susceptible cultivar Pinova. After induced expression of AvrRpt2_{EA}, transgenic lines showed shoot necrosis and browning of older leaves, with symptoms similar to natural fire blight infections. Transgenic expression of this effector protein resulted in an increase in the expression of the salicylic acid (SA)-responsive *PR-1* gene but, also, in the levels of SA and its derivatives, with diverse kinetics in leaves of different ages. In contrast, no increase of expression levels of *VSP2* paralogs, used as marker genes for the activation of the jasmonic acid (JA)-dependent defense pathway, could be detected, which is in agreement with metabolic profiling of JA and its derivatives. Our work demonstrates that AvrRpt2_{EA} acts as a virulence factor and induces the formation of SA and SA-dependent systemic acquired resistance.

Fire blight, the economically most important bacterial disease of apple, is caused by the necrogenic, gram-negative enterobacterium *Erwinia amylovora* (Burrill), Winslow et al. (Malnoy et al. 2012). The main symptoms of this disease are associated with progressive necrosis on actively growing aerial

parts and bacterial ooze formation (Paulin 1996; Slack et al. 2017). Since its first description in North America in 1780, fire blight was spread worldwide and is now present in more than 40 countries (Peil et al. 2009). The pathogenicity and plant colonization ability of *E. amylovora* is driven by different factors, including the exopolysaccharide amylovoran, levan production, biofilm formation, motility on the host, and the type III secretion system (T3SS) (Peil et al. 2009; Piqué et al. 2015; Vrancken et al. 2013). The T3SS is a conserved pilus-like protein structure found in many gram-negative plant- and animal-pathogenic bacteria, which allows the injection of bacterial type III effector proteins (T3Es) into the cytosol of host cells (Gaytán et al. 2016). In susceptible plant hosts, T3Es act as virulence factors manipulating host defense responses such as pathogen-associated molecular pattern (PAMP)-triggered immunity (Chisholm et al. 2006; Jones and Dangl 2006). Resistant plants are able to recognize T3Es by corresponding resistance (R) proteins, which can then lead to elicitation of effector-triggered immunity (ETI).

E. amylovora is known to secrete different T3Es such as DspA/E, Eop1, HopPtoC_{EA}, HrpN, HrpW, as well as AvrRpt2_{EA} (Khan et al. 2012; Malnoy et al. 2012; McNally et al. 2012). Within the genus *Erwinia*, the presence of AvrRpt2_{EA} is a unique feature of *E. amylovora*, and it was not found in closely related species (Zhao et al. 2006). AvrRpt2_{EA} is homologous to the avirulence protein AvrRpt2 of *Pseudomonas syringae* and shares high amino-acid similarity in the functional effector domain, whereas secretion domains are different (Zhao et al. 2006). After secretion into *Arabidopsis thaliana*, the enzymatic activity of the cysteine protease AvrRpt2 is activated by the endogenous cyclophilin AtROC1 (Coaker et al. 2006). The recognition of this effector fits the ‘guard’ model (Dangl and Jones 2001). AvrRpt2 was shown to cleave the guarded plant defense protein AtRIN4, which leads to an activation of the RIN4-interacting R protein AtRPS2 and RPS2-mediated resistance (Axtell and Staskiewicz 2003; Day et al. 2005; Mackey et al. 2003). Similarly to AvrRpt2, avirulence functions of AvrRpt2_{EA} were also described, as the deletion of AvrRpt2_{EA} leads to a breakdown of the fire blight resistance of the wild apple *Malus × robusta* 5 (Mr5) (Vogt et al. 2013). Moreover, AvrRpt2_{EA} is able to cleave RIN4 of *A. thaliana* (Vogt et al. 2013) and is genetically recognized by AtRPS2 when expressed in *P. syringae* pv. *tomato* DC3000 (Zhao et al. 2006),

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suggesting conservation of the avirulence functions of these related effectors. Interestingly, two forms of *AvrRpt2_{EA}* were identified, in different *E. amylovora* strains, that differ only in one amino acid (Ser/Cys) but change the ability of the pathogen to overcome the fire blight resistance of Mr5 (Vogt et al. 2013).

Beside their functions in mediating resistance of their host, *AvrRpt2* and *AvrRpt2_{EA}* are also required for virulence (Belkadir et al. 2004; Chen et al. 2000, 2004; Zhao et al. 2006). The classification of *AvrRpt2_{EA}* as a virulence factor was demonstrated by a reduced ability of an *E. amylovora AvrRpt2_{EA}* deletion mutant to cause infections on its native host and showing an enhanced growth of *P. syringae* pv. *tomato* DC3000 expressing *AvrRpt2_{EA}* on *A. thaliana rps2* mutants (Zhao et al. 2006). However, the mechanisms of *AvrRpt2* causing virulence are not completely understood. It is likely that the effector targets other host factors than *AtRIN4* to suppress host defenses, because *AvrRpt2* promotes virulence independently of *AtRIN4* (Belkadir et al. 2004; Lim and Kunkel 2004) and can cleave other targets carrying cleavage sites (Chisholm et al. 2005). Some studies on *AvrRpt2* point to a function of this effector in manipulation of hormone signaling (Chen et al. 2004, 2007; Cui et al. 2013; Geng et al. 2016; Guan et al. 2015; Lim and Kunkel 2005).

The phytohormones salicylic acid (SA) and jasmonic acid (JA) are major players in the regulation of the plant defense against pathogens. The SA-dependent response was shown to be mainly involved in the defense against biotrophic and hemibiotrophic pathogens, whereas the JA-mediated response is important for the control of necrotrophic pathogens and herbivorous insects (Glazebrook 2005). It seems to be a widespread principle that the SA- and JA-dependent pathways are, at times, capable of negatively regulating each other, which is often described as SA-JA antagonism (Robert-Seilantantz et al. 2011; Spoel and Dong 2008). This implies that an elevated resistance against biotrophs, driven by the SA-dependent response, correlates with an increased susceptibility against necrotrophic pathogens and vice versa. Many pathogens have evolved mechanisms to manipulate hormonal signaling networks as a virulence strategy to disturb host defenses (Spoel and Dong 2008). Analyses of the hormonal balance of different apple cultivars demonstrated an accumulation of SA after infection with *E. amylovora* (Dugé De Bernonville et al. 2012; Milčevićová et al. 2010). Furthermore, Dugé De Bernonville et al. (2012) demonstrated a T3SS-dependent downregulation of the JA pathway in a susceptible apple genotype and an increased resistance induced by the application of methyl-jasmonate (MeJA). Therefore, the suppression of the JA pathway seems to play a critical step in the apple-*E. amylovora* host-pathogen interaction (Dugé De Bernonville et al. 2012; Vrancken et al. 2013).

In this work, the effect of *AvrRpt2_{EA}* on a susceptible apple cultivar was investigated, using a heat shock (HS)-inducible system for transgenic expression of the effector in planta, which resulted in fire blight disease-like symptoms caused solely by expression of the effector *AvrRpt2_{EA}*. Furthermore, a potential role of the effector in deregulation of the hormonal SA or JA signaling was examined and revealed that *AvrRpt2_{EA}* induces increased SA levels and a SA-dependent defense response in a susceptible cultivar.

RESULTS

Generation of transgenic *AvrRpt2_{EA}* apple lines.

Four transformation experiments (Table 1) were performed and 800 wounded leaf disks of fire blight-susceptible cultivar Pinova (hereafter called Pin) were inoculated with the strain EHA105::3.p9N-HSP-avrRptII. From 1,650 explants, 29 putative transgenic shoots were selected, out of which three transgenic plant lines (M904, M905, M906) could be obtained, which were verified by polymerase chain reaction (PCR) and Southern hybridization. The presence of *AvrRpt2_{EA}* and *nptII* could be shown for each line, using the PCR primer pairs p9NAvr-F/R (Fig. 1B) and nptII-f/-r (Fig. 1C), respectively. To exclude the possibility of contaminations with *Agrobacterium tumefaciens* cells, the primer pair *virG*-f/-r was used to detect the bacteria-specific gene *virG* (Fig. 1D). A *virG*-specific amplicon could not be detected in any of the plant lines.

Integration of the T-DNA into the plant genome was confirmed by Southern hybridization using *AvrRpt2_{EA}*- (Fig. 1E) or *nptII*-specific (Fig. 1F) probes. Positive signals could be observed in samples of M904, M905, and M906 but not in Pin. Different signals could be detected for line M906, indicating multiple T-DNA integrations. Lines M904 and M905 exhibited only one signal with a comparable size in each blot, indicating a single T-DNA integration event. Because both lines originated from the same transformation experiment, it is assumed that these lines are identical to each other. However, we decided to handle them independently for all experiments and used line M904 as internal control.

HS-induced expression of *AvrRpt2_{EA}*.

To verify the HS-induced expression of *AvrRpt2_{EA}* in transgenic lines, micrografted plants (Fig. 2A) were grown in a climate chamber and were periodically treated with HS. Sampling was performed before, 1, and 4 days post-first HS treatment (dpHS). The *AvrRpt2_{EA}* expression level was determined in differently aged leaves of each plant (Fig. 2B). The wild-type Pin never shows *AvrRpt2_{EA}* expression. In contrast, *AvrRpt2_{EA}* expression was detectable in the transgenic lines for all tested leaves. Without HS treatment, transgenic plants already exhibited a weak expression in leaf 2, leaf 5, and the oldest leaf. At 1 dpHS, all transgenic lines showed a strong induction of *AvrRpt2_{EA}* expression. In leaf 2, the strongest induction was observed in comparison with the non-HS-treated transgenic control of the same line (397- to 674-fold) (Supplementary Fig. S1). The mean induction factor in these samples was also high in leaf 6 (61- to 256-fold) and in the oldest leaves (39- to 124-fold). At 4 dpHS, *AvrRpt2_{EA}* expression levels were elevated in comparison with the untreated control but differed between leaves of different ages (Fig. 2B). In the second leaf, the expression was still high (101- to 560-fold), whereas in the other leaves only an intermediate expression could be observed (seven- to 13-fold in leaf 6 and three- to 20-fold in the oldest leaf). In summary, HS treatment successfully induced expression of *AvrRpt2_{EA}* and provided an effective methodology to investigate the function of this bacterial effector in its native host apple.

Table 1. Generation of transgenic *AvrRpt2_{EA}* apple lines by *Agrobacterium tumefaciens*-mediated transformation^a

Transformation experiment	No. of explants	No. of regenerated shoots	No. of lines	Line name
1	660	0	0	
2	300	2	0	
3	405	21	2	M904, M905
4	285	6	1	M906
Total	1,650	29	3	

^a Four different transformation experiments were performed, using the *Agrobacterium tumefaciens* strain EHA105::3.p9N-HSP-avrRptII for the inoculation of 200 leaf disks each. The number of explants, regenerated shoots, and resulting apple lines is specified for each experiment. The *AvrRpt2_{EA}* lines M904 and M905 both originate from the third experiment and line M906 results from experiment four.

Reduced growth of transgenic *AvrRpt2EA* lines without HS treatment.

Because the growth of the *AvrRpt2EA* lines seemed to be reduced in comparison with Pin, the length of actively growing shoots of plants cultivated under glasshouse conditions without HS treatment was analyzed. Shoots of each plant were pruned at the same time to initiate and synchronize the growth of a new shoot. The length of the newly growing shoots of micrografted transgenic plants was significantly ($P < 0.05$ or less [Fig. 3B]) less after pruning compared with Pin, when repeated on three different plant sets (Fig. 3A; Supplementary Fig. S2).

To exclude the effect of ‘Golden Delicious’ (GD) seedlings used as rootstocks, the experiment was repeated with plants grafted onto M9 rootstock and, also, with own-rooted plants.

The shoot length of transgenic lines was always significantly reduced in comparison with Pin. Because weak *AvrRpt2EA* expression occurred prior to HS treatment (Fig. 2), inhibition of shoot growth in the transgenic lines was likely caused by a negative effect of even low-level expression of *AvrRpt2EA* on plant growth.

Induced expression of *AvrRpt2EA* leads to shoot necrosis and browning of older leaves.

Without HS treatment, the tissue of transgenic shoots exhibited no visible defect and the shape was comparable to Pin (Fig. 4). In contrast, at 4 dpHS induction, actively growing transgenic shoots showed necrosis, bending, and wilting in the region of the shoot tip (Fig. 4). Necrotic areas were first visible

Fig. 1. Genotypic characterization of the transgenic *AvrRpt2EA* apple lines. **A**, The T-DNA region of the binary plasmid 3.p9N-HSP-avrRptII, which was used for the generation of the transgenic *AvrRpt2EA* apple lines, flanked by left and right border sequences (LB and RB) and consists of a *nptII* resistance gene under the control of the promoter from the nopaline synthase gene (NOS-P) and the 35S terminator from *Cauliflower mosaic virus* (35S-T). The heat shock (HS)-inducible Gmhsp17.5-E promoter (Czarnecka et al. 1989) of *Glycine max* (HSP) regulates the expression of the effector gene *AvrRpt2EA* from *Erwinia amylovora*. Downstream of the *AvrRpt2EA* gene, the terminator from the octopine synthase gene (OCS-T) is located. Cutting sites of restriction enzymes (line) and primer binding positions (arrow) are marked. **B** and **C**, Standard polymerase chain reaction (PCR) analyses using specific primer pairs detected the transgenic elements *AvrRpt2EA* (**B**) and *nptII* (**C**) in each *AvrRpt2EA* apple line (Pin + *AvrRpt2EA*: M904, M905, M906) but not in the apple cultivar Pin used for the transformation. For a PCR positive control, plasmid 3.p9N-HSP-avrRptII was used as template. **D**, Using a bacteria-specific primer combination detecting *virG* from *Agrobacterium tumefaciens* EHA105::3.p9N-HSP-avrRptII in the control reaction, no specific PCR amplicon is produced in the reactions containing plant DNA from Pin and the *AvrRpt2EA* lines. The PCR negative control (non-template control) is negative for each primer pair. **E** and **F**, For Southern hybridization analyses, genomic DNA of the apple lines and the plasmid DNA (3.p9N-HSP-avrRptII) was restricted with *EcoRI* or *HindIII*, respectively, and was separated by fragment size. *AvrRpt2EA*- (**E**) or *nptII*-specific (**F**), ECF-labeled probes were used for the detection of T-DNA containing fragments. In each case, no signal was observed in the negative control Pin and a strong signal was seen in the positive control reaction containing the restricted plasmid. The *AvrRpt2EA* lines M904 and M905 displayed a single signal in each blot, with comparable fragment sizes to each other. Line M906 showed several signals with different fragment sizes in each blot.

at the youngest leaves and spread basipetally over time, affecting both leaves and stem (Fig. 4B). The extent of shoot necrosis was quantified at 4 dpHS as length of the necrotic region relative to the total length of the new shoot (Table 2). The experiment was done with several actively growing plant sets micrografted in different years as well as with plants grafted on M9. Pin never showed any necrotic tissue at the shoot tip after HS treatment. However, all transgenic plants exhibited shoot necrosis 4 dpHS. The extent of the necrosis in transgenic lines varied between 6 and 13% and was significantly ($P \leq 0.001$) greater than Pin (Table 2). In contrast, transgenic plants that had already terminated shoot growth exhibited less severe symptoms, with only some transgenic plants developing necrosis after HS treatment, but these differences were not statistically significant compared with Pin (Table 2).

Besides necrotic alterations on shoot tips, actively growing transgenic plants showed browning of older leaves (Fig. 5; Supplementary Figs. S3 and S4). These leaves looked fully intact and green before HS treatment but developed brownish discoloration 4 dpHS. The circular charts in Figure 5C reflect

the percentage of plants exhibiting brownish discolored leaves. All transgenic plants micrografted in 2014 were affected, whereas Pin did not exhibit any brownish discolored leaves (Fig. 5C). The browning effect was also found on plants micrografted and grafted on M9 in 2016. When analyzing plants micrografted in 2014 in the first HS experiment, it was striking that only leaves in the lower portion of the shoot were affected by browning (Fig. 5A and B). When the distribution of brownish discolored leaves on shoots was investigated in more detail in 2016, mainly leaves of the primary shoot were affected by browning (Supplementary Fig. S4). In summary, these results indicate that a single effector, *AvrRpt2_{EA}*, can cause a severe phenotype in a fire blight-susceptible apple cultivar that is characterized by necrosis of shoot tips and browning of leaves. Furthermore, this effect is dependent on the growth status of the plant and the age of the plant tissue as well as the degree of *AvrRpt2_{EA}* expression.

***AvrRpt2_{EA}* induces expression of the SA-responsive *PR-1* gene.**

To study the effect of *AvrRpt2_{EA}* on phytohormone-regulated defense pathways, the expression of SA- and JA-responsive genes was monitored in leaves of different ages (Fig. 6).

The expression of the *PR-1* gene can be used as a molecular marker for the upregulation of the SA-dependent pathway (van Loon et al. 2006) and was already established for apple (Milčevićová et al. 2010). Before HS treatment, the basal transcript level of *PR-1* differed between leaves of different ages, with the highest levels in the oldest leaves (Fig. 6A). An increase of *PR-1* expression could be observed in transgenic lines after HS treatment but not in Pin. At 1 dpHS, the *PR-1* expression level was dependent on the leaf age as compared with the non-HS-treated control, with a strong, significant elevation in leaf 2, a slight elevation in leaf 6, and no significant elevation in the oldest leaves (Supplementary Table S3). At 4 dpHS, a strong increase of *PR-1* expression could be detected in each of the tested leaves that was significantly ($P = 0.05$) greater than expression in Pin.

Expression of *AvrRpt2_{EA}* leads to increased levels of SA and its derivatives.

To confirm the induction of the SA-dependent defense pathway by *AvrRpt2_{EA}*, leaf material of the plants used for transcript analyses (Fig. 6) was also analyzed by ultra-high pressure liquid chromatography electrospray ionization quadrupole-time-of-flight mass spectrometry (UHPLC/ESI Q-TOF MS) for relative quantities of SA and its inactivated derivatives SA 2-*O*-glucoside (SAG), 1-*O*-salicyloyl-glucose (SGE), 2,5-dihydroxybenzoic acid 5-*O*-glucoside (2,5-DHBA5G), and 2,3-DHBA3G (Fig. 7; Supplementary Fig. S8). Before HS treatment, low levels of these SA-associated compounds were detected in Pin and the *AvrRpt2_{EA}* lines in all tested tissues (except for 2,5-DHBA5G, 2,3-DHBA3G, and SAG, which were elevated in the oldest leaves). In general, the quantity of the tested SA-associated compounds was not modulated by HS treatment in Pin. In contrast, a clear increase of SA and 2,5-DHBA5G was found in the shoot tip of *AvrRpt2_{EA}* lines by 1 dpHS, whereas the maximum level of these derivatives in young and old leaves was observed at 4 dpHS. A delayed elevation of the SAG- and SGE levels in the shoot tips of the transgenic lines was detected at 4 dpHS. In young and old leaves, the quantity of SA-inactivation products was clearly increased in the *AvrRpt2_{EA}* lines at 4 dpHS. Taken together, elevated levels of SA and increased expression of the SA-responsive gene *PR-1* as a consequence of an induced *AvrRpt2_{EA}* expression are evidence for the activation (directly or indirectly) of the SA-dependent pathway by *AvrRpt2_{EA}*.

Transcript levels of phenylalanine ammonia lyase (PAL)- (Fig. 6C) and isochorismate synthase 1 and 2 (ICS1 and ICS2)-encoding

Fig. 2. *AvrRpt2_{EA}* gene expression analysis. **A**, Schematic of micrografted plants, cultivated in a climate chamber and treated periodically with heat shock (HS) proteins at 42°C for 1 h each day. The sampling of defined plant material (leaves 2, 6, and old) for gene expression analyses was done before HS treatment, 1 and 4 days post-HS treatment (dpHS), by pooling the respective leaves from three individual plants. The experiment was performed three times. **B**, Bar chart showing the mean of the normalized relative *AvrRpt2_{EA}* gene expression determined by reverse transcription-quantitative polymerase chain reaction, shown with a logarithmic scale. Error bars show standard deviation. No *AvrRpt2_{EA}* expression was detectable in the non-transgenic control line Pin, either in any tissue or at any timepoint. The *AvrRpt2_{EA}* lines M904, M905, and M906 showed *AvrRpt2_{EA}* expression in each of the tested leaves and the expression level was induced by HS treatment.

genes (Fig. 6D; Supplementary Fig. S5), which are involved in parallel pathways for the biosynthesis of SA (Chen et al. 2009; Milčevićová et al. 2010), were also analyzed. Before HS treatment, the expression level of *PAL* was comparable in all plants but differed between leaf stages (Fig. 6C). No significant ($P > 0.05$) difference of *PAL* expression was observed after HS treatments in Pin, whereas a tissue-specific effect was found in transgenic lines. In old leaves, a significantly ($P = 0.05$) increased expression level was detected after HS treatment. In leaf 6, *PAL* expression was slightly elevated 1 and 4 dpHS, whereas a significant difference between transgenic lines and Pin was not always found. For *ICS1* (Fig. 6D) and *ICS2* no increase of transcript levels could be detected in transgenic plants after HS application. In contrast, the expression of *ICS1* decreased significantly, as compared with Pin, at 4 dpHS in leaf 6 and the old leaf (Fig. 6D). A similar expression pattern was found for *ICS2*.

Establishment of a JA-responsive transcriptional marker.

AtPDF1.2 (At5g44420) and *AtVSP2* (At5g24770) are used in *A. thaliana* as transcriptional markers for the JA-dependent defense pathway (Spoel et al. 2003; Van der Does et al. 2013). To find putative homologs in apple, BLAST search approaches were performed using predicted protein sequences. In both cases, numerous hits with good conservation of amino acid and nucleotide sequence were obtained within the apple genome sequence of GD (v.3). For the best hits, primer pairs were designed and established for reverse transcription-quantitative (RT-q)PCR. The establishment of primer pairs to distinguish potential apple *PDF1.2* paralogs turned out to be difficult. Therefore, one primer pair (*PDF1.2*_paralogs_FW/REV) (Supplementary Fig. S6A) was established that detects several potential *PDF1.2* paralogs (MDP0000157904, MDP0000136074, MDP0000188377, MDP0000164450, MDP0000368984, MDP0000573460, MDP0000520536). For potential *VSP2* candidates, one gene-specific primer set was established for MDP0000155698 (*VSP2_155698_FW/REV*) as well as a second primer pair (*VSP2_two_FW/REV*), which includes two genes MDP0000155698 and MDP0000274344 (Table 3).

As JA formation is known to be induced by mechanical damaging of plant tissue, leaves were wounded and harvested after

2, 5, and 90 min and 24 h. Using the *VSP2* two-primer pair, a strong induction of gene expression (about 150-fold) was observed 24 h after wounding. With the other *VSP2*-specific primer combination, an induction of gene expression was also detected 90 min and 24 h after wounding but to a lesser extent. Using the primer pair detecting potential *PDF1.2* paralogs, only a slight elevation of transcript level could be measured 90 min after wounding.

To elucidate the metabolism of JA in apple leaves, the same plant material was subjected to metabolite profiling by UHPLC/ESI Q-TOF MS. Using the JA metabolic pathway of *A. thaliana* as a reference, several JA-derived catabolites were identified and their accumulation kinetics were determined. Two minutes after wounding, the peak accumulation level of the JA precursor 12-oxo-phytodienoic acid was already reached. JA and its hormonally active amino-acid conjugate jasmonoyl-isoleucine (JA-Ile) became detectable 2 and 90 min after wounding, respectively, and showed increasing accumulation levels within the investigated time period. Several oxidized derivatives of JA (hydroxy-JA, hydroxy-JA hexose conjugate, hydroxy-JA sulfate conjugate, hydroxy-dihydro-JA sulfate conjugate, carboxy-JA) and JA-Ile (hydroxy-JA-Ile, carboxy-JA-Ile) were identified and showed maximal accumulation levels 24 h after wounding. The accumulation kinetics of JA derivatives correlates with the observed induction in gene expression of the potential *VSP2* paralogs. Further, after exogenous application of MeJA on apple plants, the gene expression measured by the *VSP2* two-primer pair was increased about 50-fold compared with the untreated control (0 h) (Supplementary Fig. S7). Together, because of the strong induction of gene expression on wounding and exogenous treatment with MeJA, the primer pair *VSP2_two_FW/REV* was used subsequently to monitor activation of the JA-dependent pathway.

The JA-dependent pathway is not activated by *AvrRpt2_{EA}*.

The relative quantities of JA-associated compounds were quantified in the fire blight-susceptible Pin and the *AvrRpt2_{EA}* lines. There was no increase of JA, JA-Ile, and their modified products hydroxy-JA and hydroxyl-JA-Ile after HS treatment in young or old leaves of each line. In shoot tips, the *AvrRpt2_{EA}* lines showed a transient increase in the level of JA and

Fig. 3. Reduced shoot length of *AvrRpt2_{EA}* lines without heat shock (HS) treatment. Shoots of apple plants, micrografted on ‘Golden Delicious’ (GD) seedling rootstocks, scion-grafted on M9 rootstocks, or rooted in 2016, were pruned and cultivated under glasshouse conditions. **A**, Two individual micrografted plants of each line five weeks after pruning. Transgenic lines M904, M905, and M906 were smaller than the control cultivar Pin. **B**, Lengths of the new shoot of the plants were measured 5 weeks (micro- and scion-grafted plant) or 20 days (rooted) after pruning. The mean values, standard deviations (error bar), sample size (N), and *P* values of a Student’s *t* test (one asterisk [*] ≤ 0.05 , two [**] ≤ 0.01 , three [***] ≤ 0.001) are given in the bar charts. In each plant set, the shoot length of *AvrRpt2_{EA}* lines M904, M905, and M906 was reduced in comparison with cultivar Pin.

hydroxyl-JA at 1 dpHS but not at 4 dpHS in comparison with Pin. A more sustained increase of JA-Ile in shoot tips of *AvrRpt2EA* lines could be detected at 1 and 4 dpHS compared with Pin, but the extent of the increase was clearly less compared with JA-Ile levels detected in the wounding experiment (e.g., 600 areas in the same amounts of fresh weight 24 h after wounding). The basal expression level of *VSP2* paralogs trended down with age between the tested leaves, with a high expression in leaf 2, an intermediate expression in leaf 6, and a weak expression in the old leaf (Fig. 6B). However, no significant difference was observed between the transgenic plants and Pin at 1 and 4 dpHS. Taken together, the findings suggest that the expression of *AvrRpt2EA* does not lead to a consistent increase in all hormonally active JA derivatives.

DISCUSSION

The effect of the T3E *AvrRpt2EA* of *E. amylovora* on a fire blight-susceptible apple cultivar was investigated using transgenic plants harboring a HS-inducible *AvrRpt2EA* expression construct. After induction of *AvrRpt2EA* expression, plants developed acute phenotypes, such as necrosis on actively growing shoots, bending of the shoot tip, commonly referred to as shepherd's crook, and browning of leaves (Fig. 4), which are

comparable to symptoms of a natural infection caused by *E. amylovora* (Khan et al. 2012; Paulin 1996; Peil et al. 2009). These results clearly demonstrate that *AvrRpt2EA* is able to act by itself as a virulence factor on its native host. Virulence activity of *AvrRpt2EA* was also suggested by Zhao et al. (2006), showing that the loss of *AvrRpt2EA* reduces the ability of *E. amylovora* to cause infection on immature pear fruits. Interestingly, *AvrRpt2EA* is not required for the ability of *E. amylovora* to cause necrotic symptoms on susceptible apple cultivars, as an *avrRpt2EA* deletion mutant is able to cause similar symptoms as its *E. amylovora* wild-type strain (Vogt et al. 2013). It is possible that redundant factors can overtake the virulence function of *AvrRpt2EA* in the *avrRpt2EA* deletion mutant to cause necrosis. Besides *AvrRpt2EA*, *E. amylovora* encodes at least four more effectors, namely, *Eop1* (OrfB or *EopB*), *Eop3* (*HopX1EA*), *DspA/E*, and *HopPtoCEA* (McNally et al. 2012, 2015). *DspA/E* seems to be the most important effector, because the deletion of *DspA/E* leads to a full loss of pathogenicity in immature pear and apple shoots (Barny et al. 1990; Bogdanove et al. 1998). Until now, virulence functions are not described for *Eop1*, *Eop3*, and *HopPtoCEA* (McNally et al. 2015). However, even though *AvrRpt2EA* itself is able to induce fire blight disease symptoms, the defined role it plays during a natural infection is still unknown.

The visible *AvrRpt2EA*-specific effects are dependent on the age of the plant tissue and the growth vigor of the shoot. On one hand, necrotic alterations of tissue were first seen at the shoot tip (Fig. 4) and were dependent on active shoot growth (Table 2); on the other hand, older leaves were affected specifically by browning whereas young, subapical leaves exhibited no visible effects (Fig. 5). These findings suggest that the biological network of the host cell itself is at least partly responsible for the development of fire blight-like symptoms caused by *AvrRpt2EA*.

Fig. 4. *AvrRpt2EA*-specific shoot necrosis. **A**, Shoots from representative, micrografted plants (Pin plant #11, M906 plant #28) before and 4 days post-heat shock (HS) treatment. Before HS treatment, both shoots were completely normal, whereas, 4 days after the first HS treatment, the shoot from the *AvrRpt2EA* line M906 is severely affected by necrosis, bending, and drying out. **B**, The *AvrRpt2EA*-specific phenotype of the shoot tip is shown in more detail. Leaves as well as the stem of the shoot tip are affected by necrosis. Arrows indicate the positions for measurement of the length of shoot necrosis.

Table 2. Quantification of shoot necrosis^a

Growth phase	Plant set	Statistical parameter	Pin	M904	M905	M906
Actively shooting	Micrografted, 2016	Mean	0.0	6.2	6.8	9.4
		Std	0.0	2.2	2.5	1.9
		N	11	11	11	11
		Significance	–	***	***	***
	Micrografted, 2015	Mean	0.0	6.9	7.4	10.7
		Std	0.0	3.1	3.5	3.1
		N	9	9	9	9
		Significance	–	***	***	***
	Micrografted, 2014	Mean	0.0	–	5.7	5.5
		Std	0.0	–	1.8	1.6
		N	9	–	9	8
		Significance	–	–	***	***
Shooting terminated	Scion-grafted, 2016	Mean	0.0	–	9.8	13.2
		Std	0.0	–	6.0	3.5
		N	12	–	12	12
		Significance	–	–	***	***
	Micrografted, 2016	Mean	0.0	0.0	1.0	4.6
		Std	0.0	0.0	1.9	6.2
		N	8	5	6	7
		Significance	–	n.s.	n.s.	n.s.

^a Plants were periodically treated with heat shock (HS) at 42°C for 1 h each day. The relative shoot necrosis length of shoot necrosis relative to the length of the total shoot was determined 4 days after the first HS treatment. Std = standard deviation. N = sample size. A significant difference, tested by Student's *t* test, relative to the control Pin is marked by three asterisks (***) (*P* < 0.001); n.s. = no significant difference (*P* > 0.05). The experiment was performed with several sets of plants, micrografted in 2016, 2015, or 2014 and scion-grafted in 2016. The untransformed Pin did not exhibit shoot necrosis in any of the experiments. Actively growing plants of the transgenic *AvrRpt2EA* lines M904, M905, and M906 exhibited significantly greater shoot necrosis than Pin. In the plant set with terminated growth, no significant difference between the *AvrRpt2EA* lines and Pin was found.

and that these may differ in different host tissues. This tissue-specific host reaction could be explained in several ways, including differences in the presence of unknown AvrRpt2_{EA} targets, altered activity of AvrRpt2_{EA}-affected pathways, and a different preexisting balance of combinations of hormonal derivatives or different responses to AvrRpt2_{EA}-driven signals.

Furthermore, the phenotype caused by AvrRpt2_{EA} seems to be dosage-dependent, as plants showing low AvrRpt2_{EA} expression without HS-induced expression exhibit reduced growth, whereas plants with strong AvrRpt2_{EA} expression (i.e., after HS) showed a severe necrotic phenotype (Figs. 2, 3, and 4). A low AvrRpt2_{EA} expression without HS treatment could be explained by the leakiness of the HS promoter (Würidig et al. 2013). It might be that the presence of small amounts of AvrRpt2_{EA} causes cell death of only a few cells in differentiating tissue at the shoot tip, which would lead to the mild growth symptoms observed.

Another important result of this study is that plants expressing AvrRpt2_{EA} exhibit enhanced levels of SA and its derivatives (Fig. 7). Besides its prominent role in local defense and systemic acquired resistance (SAR) against microbial pathogens (Vlot et al. 2009), SA is also known to be involved in the regulation of various other processes, such as seed germination, vegetative growth, photosynthesis, respiration, thermogenesis, flower formation, seed production, senescence, and a type of cell death that is not associated with the hypersensitive response (Rivas-San Vicente and Plasencia 2011). Furthermore, the induced expression of AvrRpt2_{EA} leads to an increased *PR-1* expression (Fig. 6A), which is known as a marker for SA-dependent activation of SAR (van Loon et al. 2006). The observed accumulation of SA and the activation of SA-dependent responses by AvrRpt2_{EA} are consistent with the findings that susceptible apple plants accumulate SA and show induced *PR-1* expression after infection with *E. amylovora* (Dugé De Bernonville et al. 2012; Milčevićová et al. 2010). SA-dependent defense responses are usually associated with hypersensitive response and cell death (Alvarez, 2000; Dangl et al. 1996; Greenberg, 1997). The up-regulation of these SA-dependent pathways may explain how the effector AvrRpt2_{EA} is able to cause fire blight disease symptoms such as necrosis that may be a consequence of induced host cell death. The biological relevance of this AvrRpt2_{EA}-mediated hyperactivation of SA-dependent responses causing disease-associated necrosis might be beneficial for the colonization of infected host plants by the necrotrophic *E. amylovora* (Kraepiel and Barny, 2016). However, if the strategy of *E. amylovora* includes the hyperactivation of the SA-dependent pathway, as suggested by the results in this study, the effectiveness of disease management strategies such as plant treatments with SA analogs (e.g., acibenzolar-S-methyl) to stimulate SAR (Aćimović et al. 2015) should be reconsidered critically.

For the biosynthesis of SA, two distinct pathways are proposed in plants that depend on either the enzyme PAL or ICS (Chen et al. 2009; Vlot et al. 2009). Interestingly, only an increased *PAL* expression was found, whereas *ICS1/2* expression was not altered or even reduced after AvrRpt2_{EA} induction. Whether the increased SA levels may be explained by an up-regulation of the PAL-dependent biosynthesis pathway or by the expression of unidentified *ICS1/2* paralogs is still unknown. However, although the *ICS1/2*-dependent pathway appears to be responsible for the bulk of pathogen-induced SA in plants (Garcion et al. 2008; Wildermuth et al. 2001), several reports describe the importance of high *PAL* activity for pathogen-induced SA formation (Coquoz et al. 1998; Elkind et al. 1990; Mauch-Mani and Slusarenko 1996; Meuwly et al. 1995; Pallas et al. 1996).

Generally, the antagonism between SA and JA signaling in plants is well-established (Gimenez-Ibanez and Solano 2013)

and its manipulation has been described as virulence strategy of necrotrophic pathogens (Rahman et al. 2012). One could speculate that the demonstrated upregulation of SA-dependent responses caused by AvrRpt2_{EA} might indirectly suppress JA-mediated reactions, which are important for the defense against necrotrophic pathogens (Glazebrook 2005). This, in consequence, may facilitate the colonization of the plant by the bacterium. Furthermore, the downregulation of the JA-dependent pathway in the host was described as an important factor for successful infections by *E. amylovora* (Dugé De Bernonville et al. 2012; Vrancken et al. 2013). To investigate a potential role of AvrRpt2_{EA} on the JA pathway, levels of JA and its derivatives were determined. After induction of AvrRpt2_{EA} expression, only small amounts of JA and its derivatives were noticeable in young and old leaves. In comparison with JA levels that were detectable after induction of the JA pathway by wounding of leaves, the measured levels of JA and its derivatives after induction of AvrRpt2_{EA} expression in young and old leaves could be considered as low. In contrast, enhanced levels of JA and its degradation derivative hydroxyl-JA were measured in shoot tips 1 dpHS, but the level of the hormonal active form JA-Ile is low. Possibly, high levels of JA are initially generated in shoot tips after induction of AvrRpt2_{EA} expression, which are directly converted into the inactive forms such as hydroxyl-JA. To get a more detailed view

Fig. 5. AvrRpt2_{EA}-dependent browning of older leaves 4 days after heat shock (HS) treatment (dpHS), shown in detail. **A**, A shoot from line M906 and **B**, representative plants, micrografted in 2014. The transgenic AvrRpt2_{EA} lines exhibited intense brown areas specifically on older leaves that developed after the application of periodical HS treatments. Younger leaves were not affected by browning 4 dpHS. **C**, Circular charts reflect the percentage of plants with or without brownish discolored leaves in different plant sets, micrografted in 2014 and 2016 and scion-grafted in 2016. The sample size (N) and the total numbers of plants with and without discolored leaves are given.

about a potential manipulation of the JA pathway by AvrRpt2_{EA}, a transcriptional marker for the activation of the JA pathway in apple was established. The expression of potential homologous apple genes of *AtVSP2*, which is highly sensitive to suppression by SA (van Wees et al. 1999; Spoel et al. 2003) and is used as a marker for JA-dependent defense response in *A. thaliana* (Spoel et al. 2003; Van der Does et al. 2013), was strongly induced by damaging of leaves, generally known to stimulate JA formation (Koo and Howe 2009). Further, the expression of *VSP2* paralogs correlated with increased levels of JA and its derivatives detected in the same plant material. Additionally, an induction of gene expression of the *VSP2* paralogs could also be provoked by exogenous treatment of apple plants with MeJA. Using this newly established marker gene for activation of JA-dependent pathways, no significant difference was detected in *VSP2* paralog expression in each tested tissue, including the shoot tip, after induction of *AvrRpt2*_{EA} expression. Together, a suppression of the JA-dependent defense response by *AvrRpt2*_{EA} could not be shown by experimental data from this study. But it is not clear whether the assumption of an existing antagonism of SA and JA pathways is true for apple or the established *VSP2* marker in apple is, indeed, suppressive by an upregulation of SA levels like it is in the model organism *A. thaliana*. Further work is needed to finally define the potential role of *AvrRpt2*_{EA} in manipulating JA-dependent responses.

As the functional effector domains of *AvrRpt2*_{EA} from *E. amylovora* and *AvrRpt2* from *P. syringae* share high similarity on amino-acid sequence level (Zhao et al. 2006), a comparison of their virulence function is of interest. Investigations of the virulence activity of *AvrRpt2* were done using *A. thaliana* mutant lines that are defective in one or both resistance genes *AtRPS2* and *AtRPM1* or the guard gene *AtRIN4* to avoid effects driven by ETI (Chen et al. 2000, 2004, 2007; Geng et al. 2016; Kim et al. 2009; Lim and Kunkel 2004). Because of the lack of knowledge concerning *AvrRpt2*_{EA}-responsive resistance genes, the Pin cultivar was used in this study. This cultivar is known to be susceptible to fire blight and, therefore, the results are not confounded by the presence of background resistance reactions. The induced expression of *AvrRpt2* in *A. thaliana* mutants (lacking one or both *AtRPS2* and *AtRPM1*) causes symptoms similar to those elicited during *P. syringae* infection and leads to an activation of SA and JA signaling, shown by an induced expression of the respective marker genes *AtPR-1* and *AtPDF1.2* (Geng et al. 2016; Kim et al. 2009). It was proposed, by Geng et al. (2016), that the antagonism between SA and JA signaling in *A. thaliana* partly explains *AvrRpt2* virulence activity. However, it is important to point out this was in the context of a different set of virulence factors (like coronatine) not found in *E. amylovora*. Another group, performing experiments with transgenic lines constitutively expressing the effector *AvrRpt2*, reported virulence function independent or downstream of SA, showing a

Fig. 6. Gene expression of *PR-1*, *VSP2*, *PAL*, and *ICS1* in *AvrRpt2*_{EA} lines. Quantitative reverse transcription-polymerase chain reaction was performed on the same material used for analysis of *AvrRpt2*_{EA} expression. The mean of the normalized, relative gene expression of **A**, *PR-1*, **B**, *VSP2*, **C**, *PAL*, and **D**, *ICS1*, shown with a logarithmic scale. Error bars show standard deviation (for samples marked by a +, only the upper error bar is shown).

suppression of SA-responsive pathogenesis-related genes by AvrRpt2 (Chen et al. 2004). Apart from differences in the experimental designs of the studies, a direct comparison of AvrRpt2 and AvrRpt2_{EA} virulence functions may be difficult, because the function of the effectors evolved in different specific systems, dependent on the host, different suites of effectors, as well as the different lifestyle and routes of preferred entry of the pathogens. Unlike necrotrophic *E. amylovora*, *P. syringae* is considered biotrophic or hemibiotrophic (Kraepiel and Barny 2016), which likely induces other defense response. It would be interesting to investigate if the functional divergence of these two homologous effectors could be attributable to their sequence difference. Therefore, an ectopic expression of AvrRpt2_{EA} in the *P. syringae* host *A. thaliana* would be intriguing in future experiments and could give new insights in the evolution of effectors in different host-pathogen systems.

Like AvrRpt2 from *P. syringae*, the counterpart AvrRpt2_{EA} from *E. amylovora* also combines virulence activity (Zhao et al. 2006; this work) and functions by triggering resistance of the host (Vogt et al. 2013). Various studies were focused on

elucidating the resistance function of these T3Es, which is associated with the proteolytic cleavage of the guard protein RIN4 by the T3E. However, the mechanism of virulence activity is not well understood. Because AvrRpt2 can cause virulence independently of *AtRIN4* (Belkhadir et al. 2004; Lim and Kunkel 2004), it is possible that additional molecular targets may be present, whose cleavage leads to virulence. Consistent with this hypothesis, the cysteine protease AvrRpt2 is able to cleave several host proteins, including AtRIN4 (Axtell and Staskawicz, 2003; Mackey et al. 2003). Because results of this work point to a manipulation of the hormone balance by AvrRpt2_{EA}, it may be that the function of the potential virulence target plays a role in hormone pathways, e.g., enzymes involved in biosynthesis or a feedback-loop of the hormones.

The major advantage to using an inducible, transgenic expression system to investigate the function of AvrRpt2_{EA} is that it isolates its effects on the host from other influences caused by a bacterial infection. However, it could obscure how the function of AvrRpt2_{EA} might be modulated in a natural infection, as the amount of the secreted protein, the timing, the affected

Fig. 7. Relative quantification of salicylic acid (SA) and its derivatives SA 2-*O*-glucoside (SAG), and 1-*O*-salicyloyl-glucose (SGE) in AvrRpt2EA lines. Defined material (shoot tip including the first completely unfolded leaf, young leaf [leaf 3], and old leaf) of micrografted plants was harvested before heat shock (HS) and at 1 and 4 days post HS treatment (dpHS) from five individual plants. A to C, The relative quantities of SA (A) and its derivative metabolites SAG (B) and SGE (C) were quantified by ultra-high pressure liquid chromatography electrospray ionization quadrupole-time-of-flight mass spectrometry analysis, and the average of area per fresh weight from two technical replicates is depicted in the bar charts. In comparison with Pin, the AvrRpt2EA lines exhibited an increase of SA, SAG, and SGE at both 1 and 4 dpHS.

tissue, and the interplay with other virulence factors (e.g., other T3Es) will likely have some influence. In contrast to bacterial infections, transgenic plants express AvrRpt2_{EA} in every cell after HS treatment, even in those cells that would not naturally be in contact with the bacterium. Further work is required to analyze the function of AvrRpt2_{EA} after delivery by *E. amylovora*.

The use of inducible transgenic expression of bacterial effectors provides a new dimension to the study of host-pathogen interactions during fire blight infection. This work used a HS-inducible system to demonstrate that AvrRpt2_{EA} acts as virulence factor and induces the formation of SA and SA-dependent SAR.

MATERIALS AND METHODS

Description of the binary vector.

The T-DNA of the binary plasmid 3.p9N-HSP-avrRptII (DNA Cloning Service, Hamburg, Germany) used for transformation is shown in Figure 1A. The sequence of the *Erwinia amylovora* effector gene AvrRpt2_{EA} was optimized for the codon usage in plants. A single nucleotide polymorphism (C instead G) at position 654 of AvrRpt2_{EA} is present in comparison with the Ea1189 wild-type allele leading to a F218L amino acid substitution near the end of the protein and not affecting any of the active sites.

Generation of transgenic apple plants.

Cells of *Agrobacterium tumefaciens* EHA105 (Hood et al. 1993) containing the binary plasmid 3.p9N-HSP-avrRptII were used for transformation of 3-week-old leaves of a proliferating axillary shoot culture of the apple *Malus domestica* Borkh. cultivar Pinova, which is susceptible to fire blight. Transformation, selection, regeneration, and propagation of putative transgenic

shoots were done according to Flachowsky et al. (2007). Pin (wild type) and AvrRpt2_{EA}-transgenic shoots were propagated in vitro on M8 media and were used for micrografting onto GD seedlings. Later, scion material of these plants was harvested and was grafted onto M9 rootstocks. Alternatively, rooting of in-vitro shoots and acclimatization were performed as described by Flachowsky et al. (2007).

PCR analyses.

Genomic DNA from in-vitro leaves was extracted using the DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The PCR reaction was performed in a total volume of 25 µl containing 25 ng of genomic DNA, 1× Dream Taq buffer, 0.2 mM dNTPs, 0.5 µM of each primer (Table 3), and 0.5 U of Dream Taq polymerase (Fermentas GmbH, St. Leon-Rot, Germany). With an initial denaturation at 94°C for 3 min, the PCR starts, followed by 35 amplification cycles of 30 s denaturation at 94°C, 60 s annealing at the specific annealing temperatures (T_a), and an elongation at 72°C of 30 s/kb. The final elongation at 72°C was done for 1 to 7 min.

Southern hybridization analysis.

Southern hybridization was done as described by Flachowsky et al. (2008). Genomic DNA (6 µg), isolated by the CTAB protocol (Saghai-Marooof et al. 1984), were restricted overnight at 37°C, using 80 U of *Eco*RI or *Hind*III (both Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.), respectively. For hybridization, a PCR-amplified, digoxigenin-labeled probe from the coding regions of AvrRpt2_{EA} (primer pair p9NAvr-F/-R) and *nptII* (primer pair nptII-f/-r) were used. The ECF random-prime labeling and detection kit (Amersham Biosciences, Freiburg, Germany) was used for hybridization and detection.

Table 3. Primer oligos and sequences used for standard polymerase chain reaction (PCR), Southern hybridization, and reverse transcription-quantitative PCR^a

Gene	Primer	Application	Sequence 5' to 3'	T _a	Bp ^b
<i>β-Actin</i>	MdActin-qFor	RT-qPCR	CTA TGT TCC CTG GTA TTG CAG ACC	63.9	82
	MdActin-qRev		GCC ACA ACC TTG TTT TTC ATG C		
<i>AvrRpt2_{EA}</i>	pN9Avr-F	PCR, S	GGT CCA ATC CCG AAA CTT CT	60.0	930
	pN9Avr-R		AGG CGT CTC GCA TAT CTC AT		
	Avr-RT-FW2	qRT-PCR	CGG AGG ATG CTT CGG AAA GA	63.9	79
	Avr-RT-REV2		TCC AGC GTT GCT TCT GCT TC		
<i>EF1α</i>	EF1-for	PCR	ATT GTG GTC ATT GGY CAY GT	56.0	707*
	EF1-rev		CCA ATC TTG TAV ACA TCC TG		905**
	EF1a_TM1	RT-qPCR	TCA AGC GTG GGT ACG TTG CTT C	66.0	77
	EF1a_TM2		GAT GAC CTG AGC GAT AAA GTT GGC		
<i>ICS1</i>	ICS1-FW	RT-qPCR	GCA TTA GTC AAA AAG GTA AAC CA	63.9	114
	ICS1-REV		CGA CCG TAG AAT CCA GAA GG		
<i>ICS2</i>	ICS2-FW	RT-qPCR	CGT AAA TTC CCT CGA GTC CA	65.0	120
	ICS2-REV		TGG AAA TCC ACA AAC TGC TG		
<i>NptII</i>	nptII-f	PCR, S	ACA AGA TGG ATT GCA CGC AGG	58.0	779
	nptII-r		AAC TCG TCA AGA AGG CGA TAG		
<i>PAL</i>	PAL-FW	RT-qPCR	TGA CAA ATG GCG AGA GTG AG	65.0	79
	PAL-REV		CTC TTC CTC GAA AGC TCC AA		
<i>PDF1.2</i>	PDF1.2_paralogs_FW	RT-qPCR	CAA GGC AGT TGA GGG GAA GA	61.2	87
	PDF1.2_paralogs_REV		CAG GTG TGT TTA CAG TTG TTA GA		
<i>PR-1</i>	PR1-FW	RT-qPCR	CTT GAC GTG GGA TGA CAA TG	63.9	118
	PR1-REV		AGT GCT CAT GGC AAG GTT TT		
<i>VirG</i>	virG-r	PCR	CGC ACG CGC AAG GCA ACC	65.0	~700
	virG-f		GCC GGG GCG AGA CCA TAG G		
<i>VSP2^c</i>	VSP2_two_FW	RT-qPCR	CTC CTC AGG CCG AAA TCC G	64.7	108
	VSP2_two-REV		GAA CAG TCT TCC AGT TGA TAA TG		
<i>VSP2^d</i>	VSP2_155698-FW	RT-qPCR	TTG TGA CGG AGA ATG AGT GGT	65.6	150
	VSP2_155698-REV		GAG AGA TTT TTC AGT ACA CAC G		

^a The primer pairs were used for different applications (standard PCR [PCR], Southern hybridization [S], RT-qPCR) with specific annealing temperatures (T_a). The size of the amplification product is indicated. T_a = annealing temperature in degrees centigrade.

^b Product size in base pairs. One asterisk (*), cDNA and two (**), genomic DNA.

^c Primer pair detecting transcripts of MDP0000155698 and MDP0000274344.

^d Primer pair detecting transcripts of MDP0000155698.

Plant growth conditions and HS induction of *AvrRpt2_{EA}* expression.

In-vitro cultures were grown in a culture chamber (day: 16 h of light at 21°C, night: 8 h of dark at 16°C). Grafted apple plants were cultivated in the glasshouse under a natural photoperiod, with extension of day time in spring at temperatures of 20 to 22°C and 18 to 20°C at night. For the HS induction, grafted plants were placed in a climate chamber and were grown under controlled conditions (18 h of light at 22°C, 6 h of darkness at 16°C, air humidity 55%). To induce the expression of *AvrRpt2_{EA}*, plants were periodically treated with a single HS at 42°C for 1 h every day.

Gene expression analysis.

Leaves of a specific age from three individual plants were pooled, were frozen in liquid nitrogen, and were homogenized. Total RNA was extracted from 100 mg of the plant powder, using the InviTrap Spin plant RNA mini kit (Stratag Molecular GmbH, Berlin). Genomic DNA contaminations were removed with the DNA-free kit (Thermo Fisher Scientific Inc.). cDNA synthesis was performed with the oligo(dT)₁₈ primer using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc.). In general, 1 µg of RNA was used for RT and the cDNA was diluted 1:10 for PCR. From old leaves, 0.2 µg of RNA were reverse transcribed (because of the low RNA yield) and the cDNA was diluted 1:2 for PCR. To verify the success of cDNA synthesis and to exclude genomic DNA contaminations, 1 µl of the diluted cDNA as well as 1 µl of the RNA preparation was used as template in a standard PCR with the primer pair EF1-for/-rev (Table 3) (Boudichevskaia et al. (2009)). PCR products resulting from genomic DNA and cDNA differ in fragment size, whereas no product should be generated using RNA.

For RT-qPCR, 2 µl of the cDNA were used for amplification on a CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The expression of *AvrRpt2_{EA}* and the endogenous apple genes (*β-Actin* [Joshi et al. 2011], *EF1α*, *ICS1*, *ICS2*, *PAL*, and *PR-1* [Milčevićová et al. 2010], *VSP2*) were measured in each sample, using gene-specific primer pairs (Table 3). The expression of two reference genes, *β-Actin* and *EF1α*, was used as internal control for normalization. The reaction was performed in a total volume of 20 µl, containing 10 µl of 2× iQ SYBR green supermix (Bio-Rad Laboratories, Inc.), 1 µl of each primer (10 pmol/µl), and 6 µl of double distilled H₂O. After an initial denaturation at 95°C for 10 s, annealing at *T_a* for 30 s, elongation at 72°C for 30 s, including signal measurement) followed. A melt curve was generated to validate the product specificity. The PCR efficiency was determined in each run by a standard curve (10-fold dilution), which was also used for inter-run calibration. The evaluation of RT-qPCR was done using the Bio-Rad CFX Manager 3.0 (Bio-Rad Laboratories, Inc.). Quantification cycle values were further analyzed by qbase+ version 2.6.1 (Biogazelle, Zwijnaarde, Belgium), which is based on geNorm and qBase technology (Hellemans et al. 2007; Vandesompele et al. 2002), using target and run-specific amplification efficiencies, and scaling was set to minimum. The gene-expression analyses were performed with three biological replicates for each treatment and three technical replicates for each sample.

Analysis of phytohormones and their derivatives.

Sample preparation. Plant material was sampled from five individual plants per genotype and treatment, was immediately shock-frozen in liquid nitrogen, and was pooled. The resulting plant material was homogenized under liquid nitrogen, using a precooled mortar and pestle. Two aliquots (100 ± 5 mg) were weighed into precooled 2-ml polypropylene tubes and were tempered at -80°C in a cooling rack (CoolRack, BioCision,

Larkspur, CA, U.S.A.). Afterward, 400 µl methanol/water, 4:1 (vol/vol), precooled at -80°C were added. The mixture was allowed to reach room temperature within 30 min, while vortexing occasionally. After addition of 100 µl of 4 µM biochanin A in methanol/water, 2:1 (vol/vol), as quality-control standard, the mixture was sonicated for 10 min at 22°C and was centrifuged for 10 min at 13,000 × *g* and 22°C. The supernatant was collected and the remaining pellet was subjected to a second round of extraction, using 400 µl methanol/water, 8:2 (vol/vol), as described above. The combined extracts were evaporated to dryness in a vacuum centrifuge at 40°C, were thoroughly reconstituted in 400 µl of methanol/water, 1:1 (vol/vol), were sonicated for 10 min at 22°C, and were centrifuged for 10 min at 13,000 × *g* and 22°C.

LC/MS analysis. One microliter of the supernatant was separated, using an Agilent Infinity 1290 UHPLC system (G4220A binary pump, G4226A autosampler with 20-µl loop, G1330B autosampler thermostat, G1316C thermostatted column compartment) (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a Zorbax RRHD Eclipses Plus C18 column (100 × 2.1 mm, 1.8 µm particle size, Agilent). The following binary gradient, at a flow rate of 400 µl min⁻¹, using 0.1% (vol/vol) formic acid in water as eluent A and 0.1% (vol/vol) formic acid in acetonitrile as eluent B, was applied: 0 to 20 min, linear gradient from 5 to 95% B, 20 to 23 min, isocratic, 95% B, 23 to 25 min, isocratic 5% B. The column and autosampler temperature were maintained at 40°C and 8°C, respectively. Eluting compounds were detected from *m/z* 70 to 1,200, using an Agilent 6550 iFunnel Q-TOF LC MS system equipped with a dual Agilent jet stream electrospray ion source in negative ion mode. The following instrument settings were applied: nebulizer gas, nitrogen, 35 pounds-force per square inch gauge; dry gas, nitrogen, 200°C, 18 liters min⁻¹; sheath gas, nitrogen, 300°C, 12 liters min⁻¹; capillary voltage, 3,000 V; nozzle voltage, 0 V; fragmentor voltage, 300 V; high pressure funnel, voltage drop 150 V, RF voltage 100 V; low pressure funnel, voltage drop 100 V, RF voltage 60 V; funnel exit DC 40 V; octopole RF voltage, 750 V; collision gas, nitrogen; collision energy, 0 V; acquisition rate 3 Hz. Mass spectra were acquired in centroid mode. The mass spectrometer was operated in extended dynamic range (2 GHz) mode and the slicer was set to high sensitivity. Using these settings, the mass resolution (full width at half maximum) at *m/z* 1,334 was approximately 27,000. The instrument was auto-tuned and calibrated on a daily basis, according to manufacturer's recommendations, using ESI-L tuning mix (Agilent). MassHunter Data Acquisition software (version B.06.01) was used for controlling the instrument and data acquisition.

Relative quantification of phytohormones and derivatives. For data evaluation MassHunter Qualitative and Quantitative Analysis software (version B.07.00) was used. Target compounds were relatively quantified by integration of extracted ion chromatograms (*m/z*-width 0.02) using quantifier ions and retention times from Supplementary Table S1. Resulting peak areas were normalized by sample fresh weight. Afterward, weight-normalized peak areas of technical replicates were averaged. Method validation data for SA and JA is presented in Supplementary Table S2.

Annotation of phytohormone derivatives. Accurate mass collision-induced dissociation mass spectra were acquired in targeted MS/MS mode. Precursor ions were isolated using the quadrupole (isolation width of *m/z* 1.3) and were fragmented inside the collision cell by applying collision energies in the range of 5 to 30 V and nitrogen as collision gas. For reference mass correction, a solution of purine (5 µM) and hexakis-(2,2,3,3-tetrafluoropropoxy)phosphazene (5 µM) in acetonitrile/water, 95:5 (vol/vol), was continuously introduced through the second sprayer of the dual ion source at a flow rate of 15 µl min⁻¹,

using an external HPLC pump equipped with a 1:100 splitting device.

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