Differential Costs of Two Distinct Resistance Mechanisms Induced by Different Herbivore Species in Arabidopsis[1]

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Plants respond to herbivory with the induction of resistance, mediated by distinct phytohormonal signaling pathways and their interactions. Phloem feeders are known to induce plant resistance via the salicylic acid pathway, whereas biting-chewing herbivores induce plant resistance mainly via the jasmonate pathway. Here, we show that a specialist caterpillar (biting-chewing herbivore) and a specialist aphid (phloem feeder) differentially induce resistance against *Pieris brassicae* caterpillars in Arabidopsis (*Arabidopsis thaliana*) plants. Caterpillar feeding induces resistance through the jasmonate signaling pathway that is associated with the induction of kaempferol 3,7-dirhamnoside, whereas aphid feeding induces resistance via a novel mechanism involving sinapoyl malate. The role of sinapoyl malate is confirmed through the use of a mutant compromised in the biosynthesis of this compound. Caterpillar-induced resistance is associated with a lower cost in terms of plant growth reduction than aphid-induced resistance. A strong constitutive resistance against *P. brassicae* caterpillars in combination with a strong growth attenuation in plants of a transfer DNA (T-DNA) insertion mutant of WRKY70 (*wrky70*) suggest that the WRKY70 transcription factor, a regulator of downstream responses mediated by jasmonate-salicylic acid signaling cross talk, is involved in the negative regulation of caterpillar resistance and in the tradeoff between growth and defense. In conclusion, different mechanisms of herbivore-induced resistance come with different costs, and a functional WRKY70 transcription factor is required for the induction of low-cost resistance.

Herbivory is a common biotic stress that terrestrial plants frequently encounter during their life cycle. In their natural habitat, plants are continuously challenged by the same or different herbivore species. Hence, the ability to mount rapid and effective responses against subsequent herbivores is essential for plant fitness (Karban and Baldwin, 1997; Kessler and Baldwin, 2004; Bruce et al., 2007; Frost et al., 2008; Gális et al., 2009; Vos et al., 2013; Stam et al., 2014). To tailor their responses against subsequent herbivore feeding, plants can benefit from mechanisms that utilize the information from previous herbivore attack to modify their defense responses (Baldwin and Schmelz, 1996; De Vos et al., 2006; Bruce et al., 2007; Vos et al., 2013). Based on current understanding, plants mount responses to caterpillar feeding via intricate signaling events that integrate and synchronize various stress signals to orchestrate the elicitation of cost-effective defense responses (Heil and Silva Bueno, 2007; Frost et al., 2008). Among the various signaling pathways, phytohormones, especially jasmonic acid (JA), salicylic acid (SA), and their cross talk, are known to mediate plant responses to herbivory (Gols et al., 2003; Frost et al., 2008; Gális et al., 2009; Pieterse et al., 2012; Thaler et al., 2012; Vos et al., 2013; Menzel et al., 2014).

Jasmonates, a group of plant hormones including JA and its derivatives such as methyl jasmonate and jasmonic acid-isoleucine (JA-Ile), are important regulators of plant defenses against tissue-chewing herbivores and necrotrophic pathogens (Wasternack and Hause, 2013). To activate defense against herbivory, JA-Ile binds to the CORONATINE INSENSITIVE1 (COI1) receptor protein, which results in ubiquitination and subsequent degradation of JAZ proteins that act as repressors of JA signaling by binding to transcription factors (TFs) such as MYC2 that regulate JA-responsive genes (Chini et al., 2007; Thines et al., 2007; Wasternack, 2007; Browse and Howe, 2008; Chung et al., 2008; Yan et al., 2009). To be able to mount earlier and stronger defense responses against subsequent caterpillar feeding, an ability to enhance JA biosynthesis is required. Indeed, several studies have reported an earlier increased expression of

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JA biosynthetic genes after exogenous application of JA, wounding, or oral secretions to mimic caterpillar feeding in different model plants, including Arabidopsis (*Arabidopsis thaliana*) and *Nicotiana attenuata*, suggesting the existence of a regulatory network that augments JA biosynthesis in response to continuous or recurring biotic stresses (Reymond et al., 2000; Wasternack, 2007; Chung et al., 2008; Stork et al., 2009).

The major plant hormone that regulates and activates plant defenses in response to phloem-feeding herbivores and biotrophic pathogens is SA (Walling, 2008; Vlot et al., 2009). SA mediates transcriptional reprogramming through the NONEXPRESSOR OF PATHOGENESIS-RELATED GENE1 (NPR1) protein, which is a master regulator that synchronizes the activation of SA-responsive TFs and downstream upregulation of defense-related genes (Després et al., 2003; Vlot et al., 2009; Wu et al., 2012; Seyfferth and Tsuda, 2014). In addition to the widely proposed role as an activator of plant resistance against phloem-feeding herbivores such as aphids, a role of SA as a negative regulator of plant defense against tissue-chewing herbivores has also been reported. For instance, Cui et al. (2002) showed that the SA-deficient mutant (*sid2-1*) and the SA-insensitive mutant (*npr1*) of Arabidopsis were highly resistant to a generalist tissue-chewing caterpillar (*Trichoplusia ni*). Moreover, tomato (*Solanum lycopersicum*) and Brussels sprouts (*Brassica oleracea* var *gemmifera*) plants that had been exposed to a 7-d aphid infestation, resulting in a significant increase in SA, were more susceptible to subsequent caterpillar feeding (Rodriguez-Saona et al., 2005; Soler et al., 2012). The results from these studies indicate that antagonistic interactions between SA and JA signaling may interfere with JA-induced responses against caterpillar feeding in Arabidopsis.

The underlying molecular mechanisms of SA-JA antagonistic interactions or cross talk have been well studied in Arabidopsis. For instance, Spoel et al. (2003) demonstrated that a functional NPR1 protein is indispensable for the regulation of downstream transcript reprogramming mediated by SA-JA cross talk. Furthermore, Li et al. (2004) identified the WRKY70 TF, which acts downstream of NPR1 and COI1, as a transcriptional regulator that positively regulates SA-dependent genes but negatively regulates a subset of JA-inducible genes in response to pathogen infection. Recently, Shim et al. (2013) reported that the MYB44 TF regulates *WRKY70* in a regulatory network parallel to NPR1, thus facilitating SA-JA cross talk in plant-pathogen interactions. Based on the available information, it is now clear that the WRKY70 TF is a transcriptional regulator acting downstream of NPR1 and COI1 in mediating plant responses against necrotrophic pathogen infection (Li et al., 2004; Cui et al., 2005; De Vos et al., 2006; Mur et al., 2006; Van der Does et al., 2013; Caarls et al., 2015; Vos et al., 2015). However, the role of WRKY70 in plant defenses against biting-chewing herbivores such as caterpillars, particularly when they arrive as a second herbivore, remains to be elucidated. Interestingly, Kroes et al. (2015) reported a negative correlation between *WRKY70* and *MYC2* transcription in Arabidopsis plants that were simultaneously infested with specialist aphids (*Brevicoryne brassicae*) at a high density and specialist caterpillars (*Plutella xylostella*). Moreover, Li et al. (2015) demonstrated the involvement of *OsWRKY70* in rice (*Oryza sativa*) resistance against a chewing herbivore and in the tradeoff between growth and defense. These studies strongly indicate a role of *WRKY70* in plant responses to herbivore attack in both dicot and monocot plants.

In this study, we investigated the role of the WRKY70 TF in plant resistance against secondary *Pieris brassicae* caterpillar feeding in Arabidopsis. We found that a short (24-h) exposure to feeding by *P. brassicae* caterpillars or *B. brassicae* aphids rendered plants more resistant to subsequent *P. brassicae* caterpillars. However, enhanced resistance after previous exposure to herbivore feeding was only minor in a transfer DNA (T-DNA) insertion mutant of *WRKY70* (*wrky70*) that already had a strong constitutive resistance to *P. brassicae* caterpillars. Analysis of phytohormone and defense metabolite levels revealed that fundamentally different mechanisms, which incurred different costs in terms of plant growth, were responsible for an enhanced resistance in plants with prior exposure to caterpillar or aphid feeding. A strong growth reduction after subsequent caterpillar feeding in all treatments of *wrky70* plants suggests that the WRKY70 TF functions as a negative regulator of induced resistance against *P. brassicae* caterpillars and is also involved in optimizing plant fitness in response to herbivory in Arabidopsis. This study signifies the underlying mechanisms of the very different induced resistance mechanisms that are effective against the same specialist herbivore (i.e. *P. brassicae* caterpillars) but come with very different fitness costs.

RESULTS

Prior Feeding by Caterpillars or Aphids Enhances Plant Resistance against Subsequent *P. brassicae* Caterpillars in Arabidopsis

A previous exposure of plants to either caterpillar or aphid feeding, resulting in an alteration of JA or SA levels, respectively, may have a positive or negative effect on the resistance against subsequent caterpillar attack (Cui et al., 2002; Kessler and Baldwin, 2004; Rodriguez-Saona et al., 2005; De Vos et al., 2006; Soler et al., 2012; Rasmann et al., 2015). Here, we observed that short feeding (24 h) by *P. brassicae* caterpillars or *B. brassicae* aphids on Arabidopsis wild-type, ecotype Columbia-0 plants significantly induced an accumulation of JA or SA, respectively (Supplemental Fig. S1, A and D; Student's *t* test, $P_{[JA]} = 0.01$ and $P_{[SA]} = 0.05$). To investigate the effect of this short feeding by caterpillars or aphids on plant resistance against subsequent feeding by specialist caterpillars (i.e. *P. brassicae*), we

assessed the body mass of *P. brassicae* caterpillars that had fed on wild-type plants that had been exposed previously to either *P. brassicae* caterpillars or *B. brassicae* aphids for 24 h. Plants without prior herbivore exposure were used as a control (Fig. 1A). After 9 d of feeding, *P. brassicae* caterpillars had gained significantly less body mass when feeding on caterpillar-exposed (ANOVA, $P = 0.01$) or aphid-exposed (ANOVA, $P \leq 0.01$) plants than caterpillars that had fed on plants without prior herbivore exposure (Fig. 1B). Intriguingly, *P. brassicae* caterpillar body mass did not differ between caterpillars that had fed on caterpillar-exposed or aphid-exposed wild-type plants (Fig. 1B; ANOVA, $P = 0.84$). As we recorded a significant increase in SA level after 24 h of aphid feeding (Supplemental Fig. S1D; Student's *t* test, $P = 0.05$), enhanced resistance against subsequent *P. brassicae* caterpillars on aphid-exposed wild-type plants was unexpected, because SA signaling is known to suppress JA-induced responses to caterpillar feeding (Cui et al., 2002; Soler et al., 2012). It has been demonstrated that SA-JA cross talk affects plant resistance against pathogens through the WRKY70 TF that functions downstream of NPR1 and COI1 (Van der Does et al., 2013; Caarls et al., 2015). Moreover, the effect of WRKY70 on plant resistance against pathogens is independent of subsequent changes in SA and JA levels (Li et al., 2004). Therefore, we investigated whether the herbivore-enhanced plant resistance phenotypes recorded here are mediated by SA-JA cross talk and WRKY70 regulation. To this end, we measured the body mass of *P. brassicae* caterpillars that had fed on unexposed, caterpillar-exposed, or aphid-exposed plants of a T-DNA insertion mutant of the *WRKY70* gene (*wrky70* plants). We observed enhanced resistance to *P. brassicae* caterpillars but increased susceptibility to *B. brassicae* aphids in *wrky70* plants, which indicates a role of *WRKY70* in plant resistance against specialist caterpillars and aphids (Supplemental Fig. S2, A and B; Student's *t* test, $P_{[caterpillar]} = 0.04$ and $P_{[aphid]} = 0.01$). Unlike the situation in wild-type plants, caterpillar body mass after 9 d of feeding did not differ among caterpillars that had fed on unexposed, caterpillar-exposed, or aphid-exposed *wrky70* plants (Fig. 1B; ANOVA, $P = 0.74$). Although a significantly lower body mass was recorded for caterpillars that had fed on unexposed *wrky70* plants than for caterpillars that had fed on unexposed wild-type plants (Fig. 1B; Student's *t* test, $P = 0.04$), there was no significant difference in body mass between caterpillars that had fed on caterpillar-exposed wild-type or *wrky70* plants (Fig. 1B; Student's *t* test, $P = 0.51$) or between caterpillars that had fed on aphid-exposed wild-type or *wrky70* plants (Fig. 1B; Student's *t* test, $P = 0.63$). Moreover, similar trends of herbivore-induced plant resistance against subsequent *P. brassicae* caterpillars to that shown in *wkry70* plants were observed in SA-deficient mutant (*sid2-1*) plants that are impaired in SA biosynthesis (Supplemental Fig. S3). This indicates that an induced biosynthesis of SA was not required for the herbivore-enhanced resistance phenotypes observed in our study (Supplemental Fig. S3). Collectively, these results suggest that enhanced resistance

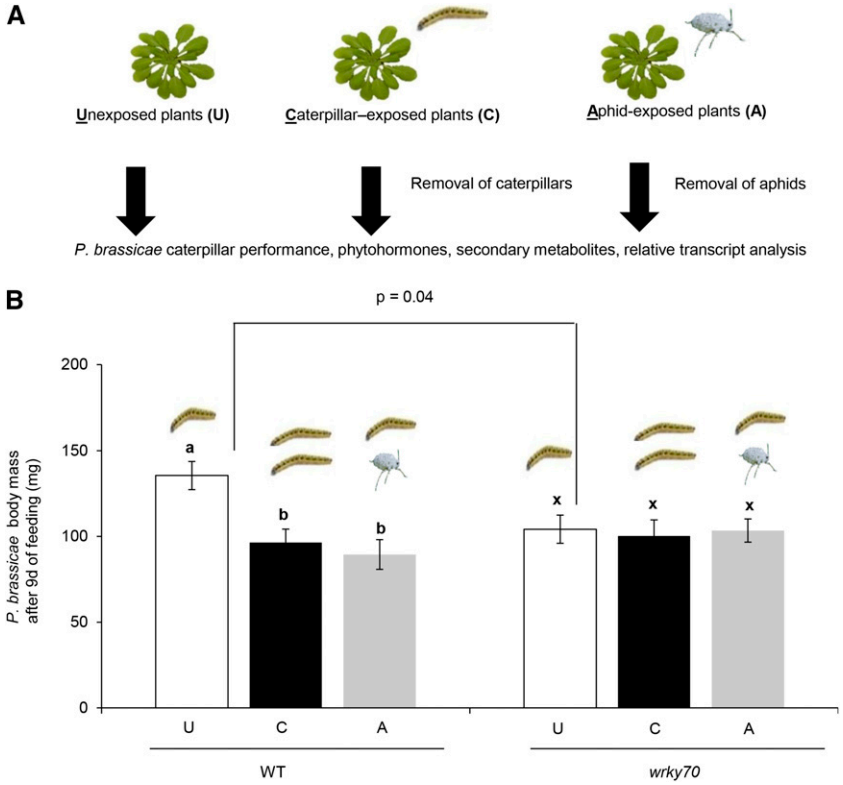

Figure 1. Plant resistance to *P. brassicae* caterpillars and effect of previous plant exposure to feeding by caterpillars or aphids. A, Wild-type and *wrky70* mutant plants were infested with five first instar *P. brassicae* caterpillars or five adult *B. brassicae* aphids for 24 h to create caterpillar-exposed (C) or aphid-exposed (A) plants. B, Effect of a prior exposure to caterpillar or aphid feeding on plant resistance against subsequent *P. brassicae* caterpillar feeding is determined by measuring caterpillar body mass (means ± SE) of 20 individual caterpillars feeding on caterpillar-exposed and aphid-exposed wild-type (WT) or *wrky70* mutant plants for 9 d. Caterpillar body mass from the group fed on plants without prior herbivore experience or unexposed (U) plants was used as a control to assess the positive or negative effect of previous herbivore exposure on plant resistance. Caterpillar body mass after 9 d of feeding on unexposed, caterpillar-exposed, and aphid-exposed wild-type or *wrky70* mutant plants was compared within the same genotype by one-way ANOVA followed by Tukey's honestly significant difference (HSD) posthoc test. Different letters indicate significant differences within a plant genotype among treatments ($P \leq 0.05$).

against *P. brassicae* caterpillars in plants with prior exposure to feeding by *P. brassicae* caterpillars or *B. brassicae* aphids is not regulated through SA-JA signaling cross talk and *WRKY70* TF regulation (Fig. 1B). To address this hypothesis, the transcript expression of biosynthetic genes and phytohormone levels were analyzed in wild-type or *wrky70* plants upon caterpillar feeding.

Impact of Herbivory History on JA and SA Signaling Pathways in Wild-Type and *wrky70* Plants

To assess the effect of caterpillar feeding on JA and SA signaling in plants that had been previously exposed to caterpillars or aphids, we analyzed the relative transcript levels of selected biosynthetic and responsive genes in the JA and SA signaling pathways as well as the phytohormone levels for unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. In addition, the transcript levels of *WRKY70* upon subsequent caterpillar attack were also analyzed. The relative transcript levels of *WRKY70* were undetectable before and after subsequent caterpillar feeding in *wrky70* plants of all three treatments (Supplemental Fig. S4D), which indicates that *WRKY70* was fully nonfunctional in the *wrky70* mutant plants. Interestingly, while subsequent caterpillar feeding hardly increased the expression of *WRKY70* in previously unexposed wild-type plants, *WRKY70* expression was distinctively increased in wild-type plants previously exposed to caterpillars or aphids after subsequent caterpillar feeding (Supplemental Fig. S4A).

The relative transcript levels of a JA biosynthetic gene, *ALLENE OXIDE SYNTHASE* (*AOS*), were transiently induced after 24 h of caterpillar feeding in unexposed and aphid-exposed wild-type and *wrky70* plants (Supplemental Fig. S4, B and E). In contrast, in wild-type and *wrky70* plants that had been previously exposed to caterpillar feeding, *AOS* expression was already significantly induced after 6 h of subsequent feeding by caterpillars (Fig. 2, A and C; ANOVA, $P_{[\text{wild type}]} = 0.01$ and $P_{[wrky70]} = 0.01$). Consistent with induced *AOS* expression, JA levels were significantly increased after 6 h of subsequent caterpillar feeding on caterpillar-exposed wild-type and *wrky70* plants (Fig. 2, B and D; ANOVA, $P_{[\text{wild type}]} = 0.01$ and $P_{[wrky70]} = 0.01$). Furthermore, JA-Ile levels in caterpillar-exposed wild-type and *wrky70* plants were also significantly induced after 6 h of subsequent caterpillar feeding (Fig. 3; ANOVA, $P_{[\text{wild type-JA-Ile}]} = 0.01$ and $P_{[wrky70\text{-JA-Ile}]} = 0.01$), whereas JA-Ile levels showed similar patterns for unexposed and aphid-exposed wild-type and *wrky70* plants (Fig. 3). It is noteworthy that the relative expression of *JAR1*, a JA-Ile biosynthetic gene, was higher in caterpillar-exposed wild-type and *wrky70* plants after 6 h of subsequent feeding; however, these expression levels were not significantly different from the levels in unexposed or aphid-exposed wild-type or *wrky70* plants (Fig. 3, A and C; ANOVA, $P_{[\text{wild type-}JAR1]} = 0.20$ and $P_{[wrky70\text{-}JAR1]} = 0.15$). It is interesting that the expression of *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), a well-established marker gene for JA-regulated defense responses, was higher in *wrky70* plants than in wild-type plants in unexposed, caterpillar-exposed, and aphid-exposed plants after 48 or 72 h of subsequent caterpillar feeding (Supplemental Fig. S5, A–C). These results suggest that the WRKY70 TF is a negative regulator of JA-induced responses.

The transcript levels of the SA biosynthetic gene *ISOCHORISMATE SYNTHASE1* (*ICS1*) and SA levels after subsequent caterpillar feeding in aphid-exposed plants were not significantly different from those in unexposed and caterpillar-exposed wild-type plants (Fig. 4; ANOVA, $P_{[0\,h]} = 0.85$, $P_{[6\,h]} = 0.13$, $P_{[24\,h]} = 053$, $P_{[48\,h]} = 0.16$, and $P_{[72\,h]} = 0.36$). In contrast, in *wrky70* plants, levels of *ICS1* expression and SA were significantly higher than in wild-type plants, regardless of the type of prior herbivory (Fig. 4). Furthermore, the expression of *PATHOGENESIS-RELATED GENE1* (*PR1*), an SA-responsive marker gene, was consistent with an increased expression of *ICS1* and SA levels in *wrky70* plants (Supplemental Fig. S5, D–F). The negative correlation between *WRKY70* expression on the one hand and *ICS1* expression and SA level on the other suggests that the WRKY70 TF is a negative regulator of the SA biosynthetic pathway, which supports data by Wang et al. (2006).

Thus, in comparison with unexposed plants, prior exposure to caterpillar feeding results in an earlier (6-h) induction of JA and JA-Ile in wild-type and *wrky70* plants upon subsequent caterpillar feeding (Figs. 2 and 3). However, because JA and JA-Ile levels in unexposed, caterpillar-exposed, and aphid-exposed *wrky70* plants are similar to the levels in wild-type plants, this suggests that *WRKY70* does not exert control on JA and JA-Ile biosynthesis (Figs. 2 and 3). In contrast, prior exposure of wild-type plants to aphid feeding hardly affected JA, JA-Ile, and SA levels during subsequent caterpillar feeding in comparison with unexposed wild-type plants (Figs. 2–4) and, importantly, did not correlate with enhanced resistance to subsequent caterpillar feeding in aphid-exposed wild-type plants (Fig. 1B). These results indicate that an earlier induction of JA and JA-Ile in caterpillar-exposed plants that is correlated with an enhanced resistance against subsequently feeding caterpillars is not affected by a nonfunctional WRKY70. Thus, aphid-enhanced plant resistance against subsequent caterpillar feeding seems to be mediated through a novel mechanism that does not depend on SA signaling, SA-JA cross talk, and *WRKY70* regulation.

Enhanced Resistance in Caterpillar-Exposed Plants Is Associated with a Significant Increase in Kaempferol 3,7-Dirhamnoside

In order to examine the effects of an earlier induction (6 h) of JA and JA-Ile in caterpillar-exposed plants on defense metabolite accumulations that consequently may enhance plant resistance against subsequent caterpillar feeding, we quantified levels of kaempferol 3,7-dirhamnoside (KRR), a defense metabolite against specialist *P. brassicae* caterpillars (Onkokesung et al., 2014), in previously unexposed, caterpillar-exposed,

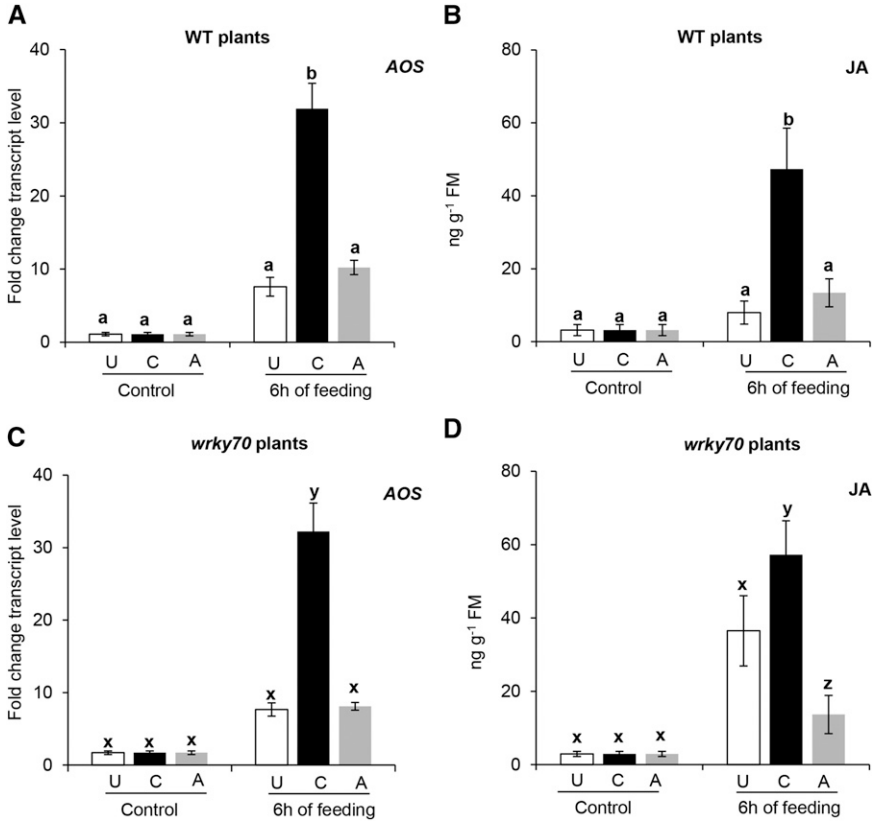

Figure 2. Transcript levels of *AOS*, a JA biosynthetic gene, and JA levels in previously unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type (WT) or *wrky70* plants after 6 h of subsequent *P. brassicae* caterpillar feeding. A and C, *AOS* transcript levels in unexposed, caterpillar-exposed, and aphid-exposed plants of the wild type and the *wrky70* mutant after subsequent feeding by five first instar *P. brassicae* caterpillars. Leaves were harvested just before caterpillar introduction and after 6 h of caterpillar feeding. Relative transcript levels were analyzed by quantitative real-time PCR (RT-qPCR). Values for each time point represent means ± SE ($n = 5$) of *AOS* transcript levels relative to those of the *ELONGATION FACTOR-1α* (*EF1α*) gene. B and D, JA levels in unexposed, caterpillar-exposed, and aphid-exposed plants of the wild type and the *wrky70* mutant after subsequent caterpillar feeding. The same leaf samples used for *AOS* transcript analysis were extracted, and JA levels were quantified by liquid chromatography quadrupole tandem mass spectrometry (LC-MS) using deuterium-labeled JA as an internal standard. Each graph shows means ± SE ($n = 5$) of the JA level in wild-type and *wrky70* plants. JA levels in wild-type and *wrky70* plants from the same treatment were compared by Student's *t* test. AOS expression and JA levels were compared among unexposed, caterpillar-exposed, and aphid-exposed treatments within the same genotype (wild-type or *wrky70* plants) using one-way ANOVA followed by Tukey's HSD posthoc test; different letters indicate significant differences within a plant genotype ($P \leq 0.05$). FM, Fresh mass.

and aphid-exposed wild-type and *wrky70* plants. In addition, levels of glucosinolates (GLSs), common defense metabolites effective against generalist herbivores (Mewis et al., 2005; Halkier and Gershenzon, 2006), were also analyzed. After 4 d of caterpillar feeding, total GLS and KRR levels were significantly higher in previously caterpillar-exposed than in previously unexposed and aphid-exposed wild-type or *wrky70* plants (Fig. 5, A–C; ANOVA, $P_{[\text{GLS-wild type}]} \leq 0.01$, $P_{[\text{KRR-wild type}]} \leq 0.01$, $P_{[\text{GLS-}wrky70]} \leq 0.01$, and $P_{[\text{KRR-}wrky70]} \leq 0.01$). Moreover, GLS and KRR levels did not differ between unexposed and aphid-exposed plants from both genotypes (Fig. 5, A–C). In addition, the accumulation patterns of other flavonol glycosides showed similar trends to those observed for KRR accumulation (Supplemental Fig. S6). Importantly, a significantly increased KRR level in caterpillar-exposed plants corresponds with enhanced resistance against subsequent caterpillar feeding in caterpillar-exposed wild-type and *wkry70* plants. From these results, we infer that caterpillar-enhanced plant resistance is associated with an early transient induction of JA and JA-Ile at 6 h of caterpillar feeding that contributes to an increase in KRR levels upon subsequent caterpillar feeding.

Aphid-Enhanced Plant Resistance against Subsequent Caterpillar Feeding Is Associated with Alteration of Sinapoyl Ester Levels

Besides GLS and KRR, sinapate esters, a group of flavonoid metabolites, play a role in plant resistance to caterpillars and necrotrophic pathogens (Leiss et al., 2011; Demkura and Ballaré, 2012). As we did not observe a correlation between KRR levels and aphid-enhanced plant

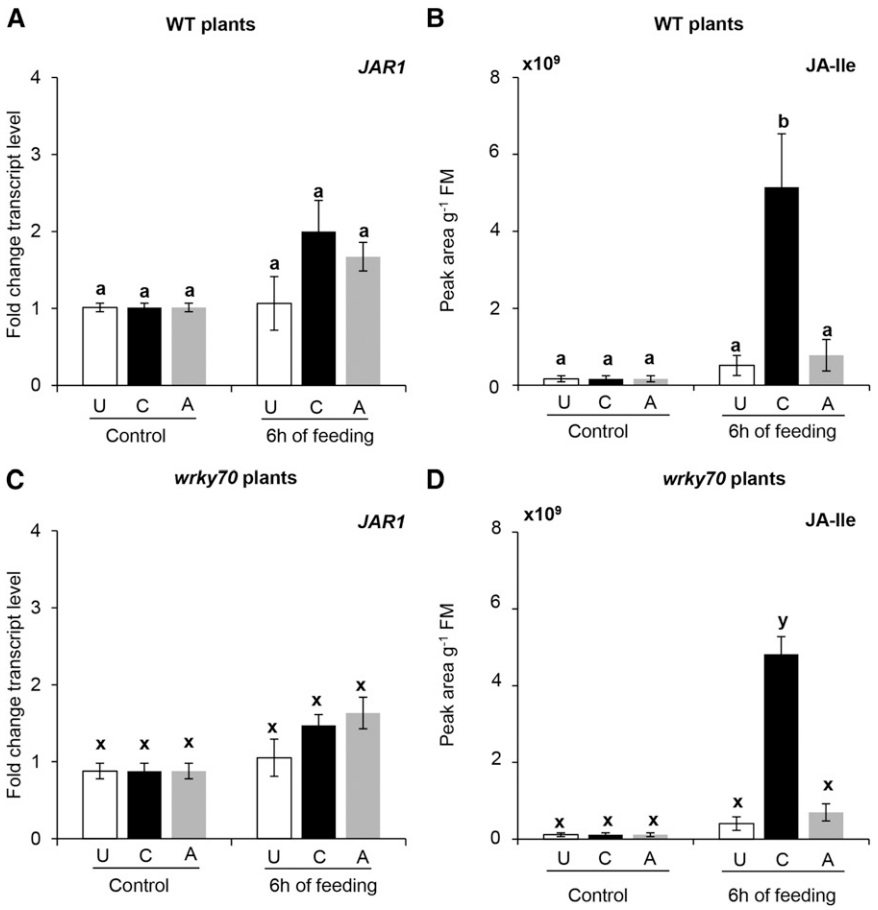

Figure 3. Transcript levels of *JASMONIC ACID AMIDO SYNTHETASE1* (*JAR1*), a JA-Ile biosynthetic gene, and JA-Ile levels in response to subsequent caterpillar feeding in unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type (WT) and *wrky70* plants. A and C, Means ± SE ($n = 5$) of *JAR1* relative transcript levels in unexposed, caterpillar-exposed, and aphid-exposed wild type and *wrky70* mutant plants before and after 6 h of subsequent feeding of five first instar larvae of *P. brassicae*. Relative transcript levels of *JAR1* were determined by RT-qPCR. The value at each time point represents the expression of *JAR1* relative to the *EF1α* gene in wild-type and *wrky70* plants. B and D, Means ± SE ($n = 5$) of JA-Ile levels in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* mutant plants before and after 6 h of subsequent feeding by five first instar larvae of *P. brassicae*. JA-Ile levels were determined by LC-MS followed by quantifying peak areas. Mean values of *JAR1* relative transcripts and JA-Ile levels from unexposed, caterpillar-exposed, and aphid-exposed treatment were compared among treatments within the same plant background (wild-type and *wrky70* plants) by one-way ANOVA by Tukey's HSD posthoc test; different letters indicate significant differences within a plant genotype ($P \leq 0.05$). FM, Fresh mass.

resistance against subsequent caterpillar feeding in wild-type and *wrky70* plants (Figs. 1B and 5D), we investigated whether sinapate esters play a role in an enhanced resistance against specialist caterpillars in our system. The levels of two sinapate esters that are abundant in Arabidopsis, namely sinapoyl glucose (SG) and sinapoyl malate (SM), were analyzed in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. SG levels in wild-type and *wrky70* plants were reduced after 4 d of subsequent caterpillar feeding on previously unexposed, caterpillar-exposed, and aphid-exposed plants (Supplemental Fig. S7B). While SM levels in unexposed and caterpillar-exposed wild-type plants hardly changed from the basal levels, a significant increase in SM level was observed in aphid-exposed wild-type plants (Fig. 5D; ANOVA, $P \leq 0.01$). In contrast, in the *wrky70* mutant, SM levels increased from the basal level after subsequent caterpillar feeding in previously unexposed, caterpillar-exposed, and aphid-exposed plants (Fig. 5D; ANOVA, $P \leq 0.01$). Consistent with an increase in SM levels, the relative transcript level of *SINAPOYLGLUCOSE:MALATE SINAPOYLTRANSFERASE* (*SMT*), a gene coding for an SM biosynthetic enzyme, was significantly higher in aphid-exposed than in unexposed and caterpillar-exposed wild-type or *wrky70* plants (Supplemental Fig. S8, B and E). In contrast, the transcript levels of *SINAPATE-1-GLUCOSYL TRANSFERASE* (*SGT*), an SG biosynthetic gene, did not correlate with SG levels in previously unexposed, caterpillar-exposed, and aphid-exposed of wild-type or *wkry70* plants (Supplemental Fig. S8, C and F). Importantly, the significant increase in SM corresponded with an enhanced resistance against subsequent caterpillar feeding in aphid-exposed wild-type plants, and in *wrky70* plants this was the case regardless of prior herbivory history (Fig. 1B). It is noteworthy that a significant increase in SM levels did not correlate with phytohormone profiles in aphid-exposed wild-type plants and in *wrky70* plants of all three treatments (Figs. 2–5). Taken together, our results suggest that SM is a defense metabolite effective against *P. brassicae* caterpillars and that SM biosynthesis is likely to be regulated through an independent mechanism rather than the JA or SA signaling cascade. We further investigated the role of SM in plant resistance against subsequent caterpillar feeding in an SM-deficient mutant plant.

SM Plays a Role in Enhanced Resistance to *P. brassicae* Caterpillars Feeding on Arabidopsis

A mutant plant silenced in *ferulic acid 5-hydroxylase* (*fah1*), a major enzyme in sinapate ester biosynthesis, has been reported to lack sinapate ester accumulation, including SM (Ruegger et al., 1999; Hagemeier et al., 2001). To examine the role of SM in enhanced

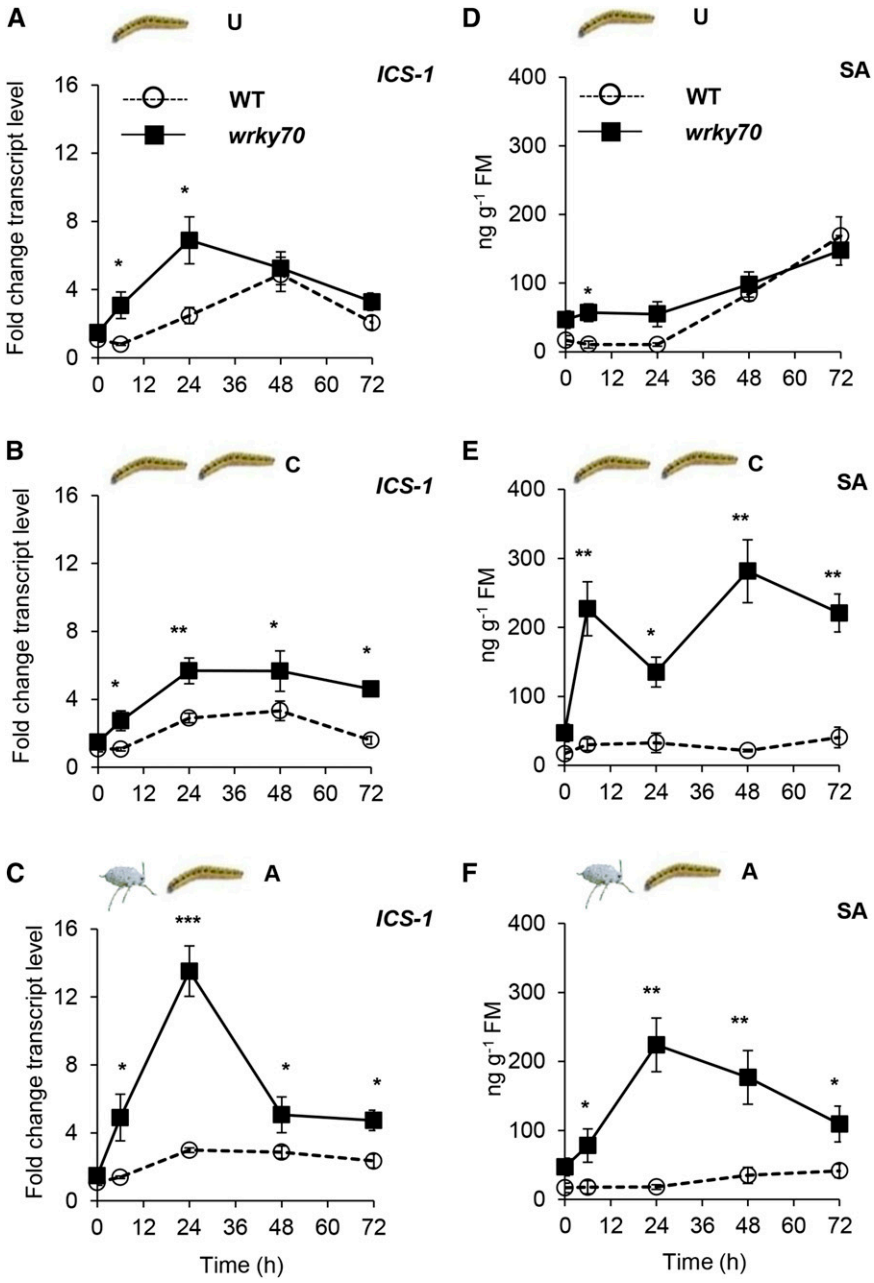

Figure 4. Expression profile of an SA biosynthetic gene, *ICS1*, and SA levels in unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type (WT) and *wrky70* plants after subsequent caterpillar feeding. A to C, Kinetics of *ICS-1* expression relative to the *EF1α* gene from unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants after subsequent feeding by five first instar *P. brassicae* caterpillars; graphs represent means ± SE (n = 5). D to F, Free SA levels at the designated time points after subsequent *P. brassicae* caterpillar feeding on unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. Free SA levels were determined by LC-MS using deuterium-labeled SA as an internal standard. Mean values of *ICS1* relative transcripts and SA levels at each time point from unexposed, caterpillar-exposed, and aphid-exposed treatment were compared between wild-type and *wrky70* plants from the same treatment by Student's *t* test; asterisks indicate significant differences: *, $P \leq 0.05$, **, $P \leq 0.01$ and ***, $P \leq 0.001$. FM, Fresh mass.

resistance against subsequent caterpillar feeding in aphid-exposed wild-type plants, we compared *P. brassicae* caterpillar performance on *fah1*-silenced (*fah1-7*) wild-type and *wrky70* plants. On unexposed *fah1-7* plants, *P. brassicae* caterpillars gained significantly higher body mass than on unexposed wild-type or *wrky70* plants (Fig. 6A; ANOVA, $P = 0.01$). As expected, enhanced resistance against subsequently feeding caterpillars, as observed in aphid-exposed wild-type plants, was not observed in *fah1-7* plants (Fig. 6A; ANOVA, $P_{[fah1\text{-}7]} = 0.40$). It is noteworthy that while constitutive and induced KRR levels in *fah1-7* plants were significantly lower compared with the levels in wild-type and *wrky70* plants after subsequent caterpillar feeding, enhanced resistance against subsequent *P. brassicae* caterpillars in caterpillar-exposed plants was still observed in *fah1-7* mutant plants (Fig. 6A; Supplemental Fig. S9D). These results suggest an involvement of other defense metabolites in mediating the caterpillar-enhanced resistance phenotype in *fah1-7* plants. Nevertheless, our results indicate that SM is a metabolite that provides resistance against *P. brassicae* caterpillars in Arabidopsis plants that had been previously exposed to aphid feeding.

Different Costs of Enhanced Resistance to Subsequent *P. brassicae* Caterpillar Feeding

An increased accumulation of distinct defense metabolites upon caterpillar feeding on either caterpillar-exposed

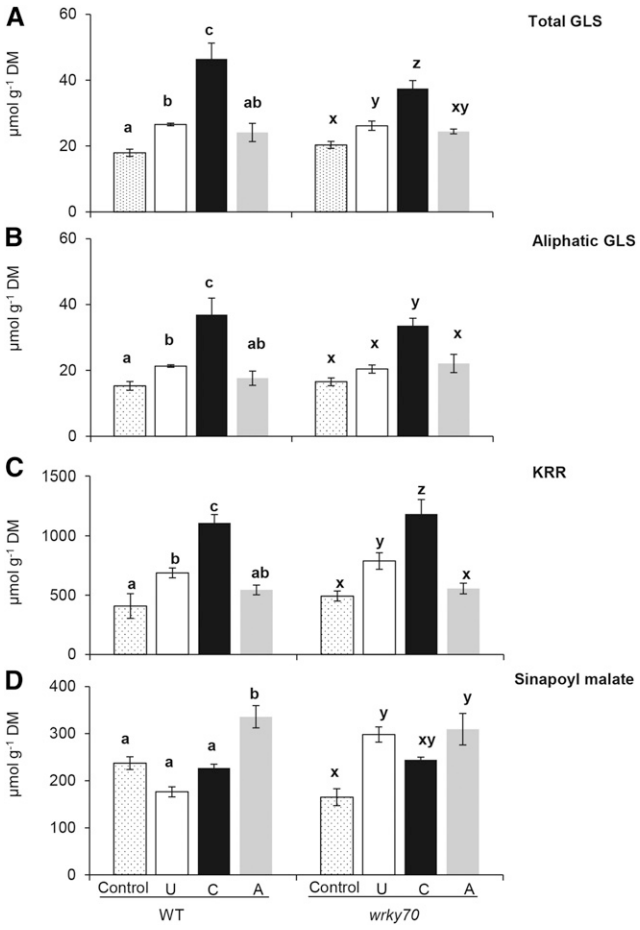

Figure 5. GLS, KRR, and sinapoyl malate (SM) production after 4 d of *P. brassicae* caterpillar feeding on previously unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type (WT) and *wrky70* plants. A and B, Means ± SE ($n = 5$) levels of total and aliphatic GLSs in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. GLS was determined by HPLC using the peak areas at 229 nm relative to the peak area of the internal standard. C and D, Means ± SE ($n = 5$) of KRR (C) and SM (D) in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* mutant plants after 4 d of subsequent feeding by five first instar *P. brassicae* caterpillars. KRR levels were quantified by HPLC based on an external standard curve of an authentic standard of KRR. SM levels were determined by HPLC based on an external standard curve of an authentic standard of sinapic acid. GLS, KRR, and SM levels in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* mutant plants were compared by one-way ANOVA followed by Tukey's HSD posthoc test; different letters indicate significant differences ($P \leq 0.05$) among treatments within the same genotype. DM, Dry mass.

plants (KRR) or aphid-exposed plants (SM) suggests that plants employ different defense mechanisms depending on previous herbivory history to enhance their defense against subsequent feeding by *P. brassicae* caterpillars (Fig. 5). As defense mechanisms are costly for plant growth and development (Gulmon and Mooney, 1986; van Hulten et al., 2006; Zavala and Baldwin, 2006; Onkokesung et al., 2010), we investigated the effect of the different enhanced-resistance mechanisms on plant growth in caterpillar-exposed and aphid-exposed wild-type and *wrky70* plants upon subsequent exposure to caterpillars. To determine plant growth, we measured stalk length and rosette area at 14 d after removing caterpillars for previously unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants and compared this with the respective control (undamaged) plants.

A slight, nonsignificant reduction in stalk length and rosette area was observed for previously unexposed and previously caterpillar-exposed wild-type plants; however, a large reduction of stalk length (approximately 50%) and a small but significant reduction in rosette area (approximately 6%) were observed in wild-type plants previously exposed to aphids compared with control plants (Fig. 7; ANOVA, $P_{[stalk]} = 0.02$ and $P_{[rosette]} = 0.01$). In contrast, a strong reduction of stalk length was observed in previously unexposed (41%), caterpillar-exposed (56%), and aphid-exposed (57%) *wrky70* plants (Fig. 7A; ANOVA, $P = 0.01$). Consistent with the stalk length reduction, rosette area was significantly reduced in *wrky70* plants regardless of prior herbivory conditions (Fig. 7B; ANOVA, $P = 0.02$). It is interesting that there was no significant difference in stalk length between undamaged control wild-type and *wrky70* plants (Fig. 7A; Student's *t* test, $P = 0.99$); however, the rosette area of undamaged *wrky70* plants was significantly smaller than the rosette area of undamaged wild-type plants (Fig. 7B; Student's *t* test, $P = 0.01$). In addition, although leaf area damaged after 4 d of caterpillar feeding showed no significant difference among previously unexposed, caterpillar-exposed, and aphid-exposed wild-type plants or among *wrky70* plants of these three treatments, the caterpillar-damaged leaf areas were significantly smaller in *wrky70* plants than in wild-type plants for unexposed and caterpillar-exposed plants (Supplemental Fig. S10; Student's *t* test, $P_{[unexposed]} = 0.007$ and $P_{[caterpillar]} = 0.04$). Taken together, our results indicate that enhanced resistance to caterpillars in wild-type plants that had previously been exposed to aphid feeding is more costly than for wild-type plants that had previously been exposed to caterpillars. A significant attenuation of plant growth after subsequent caterpillar feeding in *wrky70* plants, which have a high constitutive resistance against *P. brassicae* caterpillars, indicates an involvement of the WRKY70 TF in suppressing herbivore-induced resistance to maintain an acceptable plant growth during herbivory conditions.

DISCUSSION

A prior exposure to herbivore attack has been proposed to influence plant responses to subsequent attack from a similar or different herbivore species (Karban and Baldwin, 1997; Kessler and Baldwin, 2004; Rodriguez-Saona et al., 2005; De Vos et al., 2006; Poelman et al., 2008, 2010; Zhang et al., 2013a, 2013b; Rasmann et al., 2015). Here, we show that an enhanced

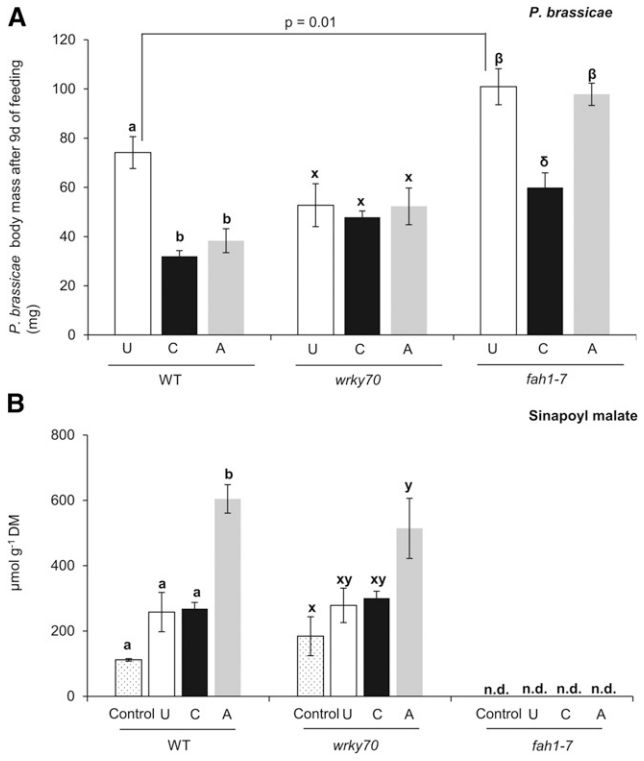

Figure 6. Biomass of *P. brassicae* caterpillars and SM production in previously unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type, *wrky70*, and *fah1-7* plants. A, *P. brassicae* caterpillar body mass (means ± SE; $n = 20$) after 9 d of feeding on unexposed, caterpillar-exposed, and aphid-exposed plants of the wild type and the *wrky70* and *fah1-7* mutants. Caterpillar body mass was compared among treatments within the same genotype by one-way ANOVA followed by Tukey's HSD posthoc test; different letters indicate significant differences within a plant genotype ($P \leq 0.05$). In addition, caterpillar body mass from the same treatment was compared between wild-type and *wrky70* plants or between wild-type and *fah1-7* plants by Student's *t* test. B, SM levels (means ± SE; $n = 5$) after 4 d of feeding by five first instar *P. brassicae* caterpillars on previously unexposed, caterpillar-exposed, and aphid-exposed wild-type, *wrky70*, and *fah1-7* plants. SM levels were quantified by HPLC using an external standard curve of an authentic sinapic acid standard. Mean values of SM levels were compared among unexposed, caterpillar-exposed, and aphid-exposed treatments within the respective genotypes (wild-type, *wrky70*, or *fah1-7* plants) by one-way ANOVA followed by Tukey's HSD posthoc test; different letters indicate significant differences within the same genotype ($P \leq 0.05$). DM, Dry mass; n.d., not detected.

resistance to *P. brassicae* caterpillars, which involves an early induction of JA signaling in wild-type plants previously exposed to caterpillar feeding, is more cost effective than an enhanced resistance via an alternative mechanism (SM) in wild-type plants that had been previously exposed to aphid feeding. A combination of enhanced JA responses and an increase in SM after *P. brassicae* feeding on *wrky70* plants, which occurs independent of herbivory history and which results in a caterpillar-resistant phenotype, indicates that the WRKY70 TF plays an important role in this. Subsequently, the fact that caterpillar feeding on *wrky70* plants results in growth reduction, compared with the effect of caterpillar feeding on wild-type plants, indicates that this TF, which is a negative regulator of JA-induced resistance, also mediates a tradeoff between plant growth and defense.

WRKY70 TF Regulation Is Involved in Suppressing Plant Resistance against *P. brassicae* Caterpillars

Jasmonates are major signaling molecules mediating the defense against caterpillars in various plant species, including Arabidopsis (Wasternack and Hause, 2013). A significant increase in susceptibility to generalist caterpillars (*Spodoptera exigua*) in an Arabidopsis genotype with an overexpressor of a negative regulator of JA (JAZ1Δ3A) indicated an important role for this negative regulator in the induced resistance against caterpillars (Chung et al., 2008). Because the WRKY70 TF has been identified as a negative regulator of JA-induced responses in Arabidopsis (Li et al., 2004), we hypothesized that *wrky70* plants would become resistant to *P. brassicae* caterpillars due to a lack of this negative regulator. Indeed, our data show that *wrky70* plants are more resistant to *P. brassicae* caterpillars than wild-type plants (Fig. 1B; Supplemental Fig. S2A). Although similar levels of JA and JA-Ile were observed in wild-type and *wrky70* plants upon caterpillar feeding, the transcript level of the JA-responsive marker gene *VSP2* was significantly higher in *wrky70* plants than in wild-type plants after caterpillar feeding (Figs. 2 and 3; Supplemental Fig. S4, A–C). Our results are consistent with previous studies on a T-DNA insertion mutant of WRKY70 that reported an increased expression of JA-responsive genes and increased JA sensitivity in *wrky70* mutant plants, while the transcript expression of JA biosynthetic genes and JA levels in *wrky70* plants remained at the same levels as in wild-type plants in response to necrotrophic pathogens (Li et al., 2004, 2006). Altogether, our data indicate that the WRKY70 TF is a negative regulator of JA-induced responses that lead to plant resistance against *P. brassicae* caterpillars feeding on Arabidopsis. It is interesting that in the monocot species rice, OsWRKY70 mediated plant resistance against chewing herbivores via positive regulation of JA biosynthesis rather than suppressing JA-induced responses (Li et al., 2015). Together with our results, this suggests a different evolution of WRKY70 functions in plant resistance against chewing herbivores in dicot and monocot plants.

Caterpillar-Induced Plant Resistance

An enhanced resistance to caterpillar feeding on plants previously exposed to the same caterpillar species has been reported in the Solanaceae and Brassicaceae families (Rodriguez-Saona et al., 2005; De Vos et al., 2006; Soler et al., 2012; Rasmann et al., 2015). Here, we report that a short-term (24-h) exposure to feeding by *P. brassicae* caterpillars was sufficient to significantly enhance plant resistance against subsequent

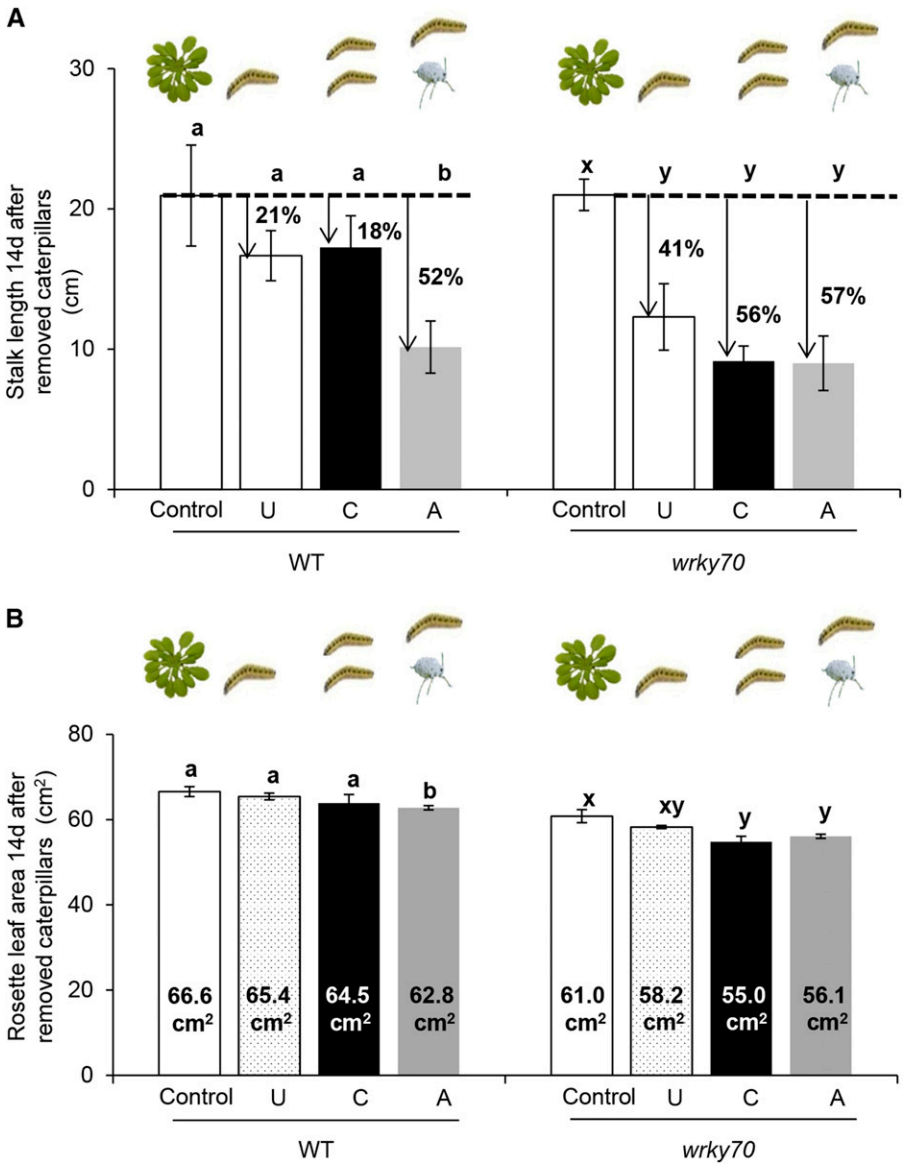

Figure 7. Plant growth in unexposed (U), caterpillar-exposed (C), and aphid-exposed (A) wild-type (WT) and *wrky70* plants. A, Stalk length (means ± SE; $n = 20$) of unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants at 14 d after removal of *P. brassicae* caterpillars. Five first instar *P. brassicae* caterpillars were removed from unexposed, caterpillar-exposed, and aphid-exposed plants of the wild type and the *wrky70* mutant after 4 d of feeding. The percentage of stalk length reduction in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants was calculated based on the stalk length of control (undamaged) wild-type plants. B, Rosette area (means ± SE; $n = 20$) at 14 d after *P. brassicae* caterpillars had been removed from unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. Five first instar *P. brassicae* caterpillars were allowed to feed freely for 4 d before removal from unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants. The entire rosette area of each plant from undamaged control, unexposed, caterpillar-exposed, and aphid-exposed treatments was photographed, and total rosette area was analyzed using ImageJ software. Mean values of stalk length and total rosette area were compared among control (undamaged), unexposed, caterpillar-exposed, and aphid-exposed plants within the respective plant type (wild type or *wrky70*) by one-way ANOVA followed by Tukey's HSD post-hoc test; different letters indicate significant differences within a plant genotype ($P \leq 0.05$).

P. brassicae caterpillar feeding in wild-type Arabidopsis plants (Fig. 1B). The induction of JA, caused by prior caterpillar attack, or an exogenous application of JA or methyl jasmonate can facilitate earlier and stronger responses to subsequent herbivory (Gols et al., 2003; Cui et al., 2005; Rasmann et al., 2012; Menzel et al., 2014). Indeed, we observed an early increase in the transcript expression of *AOS* and *JAR1* and the accumulation of JA and JA-Ile (at 6 h of subsequent feeding) in wild-type plants that had previously been exposed to caterpillars or that had been previously unexposed (Figs. 2 and 3). Moreover, GLS and KRR levels after 4 d of caterpillar feeding were significantly higher in wild-type plants that had previously been exposed to caterpillar feeding (approximately 2-fold) than in previously unexposed wild-type plants (Fig. 5, A and C). Many studies on Arabidopsis showed that specialist herbivores such as *P. brassicae* caterpillars are highly adapted to GLS (Hopkins et al., 2009; Müller et al., 2010; Winde and Wittstock 2011). Therefore, plant resistance against specialist caterpillars is unlikely to depend on an individual group of defense metabolites such as GLS (Rasmann et al., 2015). We have previously demonstrated that KRR is an effective defense metabolite against *P. brassicae* caterpillars (Onkokesung et al., 2014). The data presented here show that a short-term exposure of Arabidopsis to caterpillar feeding is sufficient to induce an early accumulation of JA and JA-Ile that is associated with an increase in the levels of KRR and an enhanced resistance against subsequent caterpillar feeding. Although unexposed *wrky70* plants were more resistant to *P. brassicae* caterpillars than unexposed wild-type plants, a comparable level of resistance against subsequent caterpillar feeding was observed in caterpillar-exposed wild-type and *wrky70* plants (Fig. 1B). Furthermore, the accumulation of KRR was

similar in caterpillar-exposed wild-type and *wrky70* plants (Fig. 5C). These results indicate that the lack of a negative regulator of JA (i.e. the WRKY70 TF) is unlikely to further enhance plant resistance against subsequent caterpillar feeding beyond the level represented in unexposed *wrky70* plants. Recently, it has been shown for Arabidopsis natural accessions that an enhanced resistance against subsequent caterpillar feeding was minor in the accessions with high constitutive resistance against caterpillars (Rasmann et al., 2015). Together, our results suggest a constraint of JA-induced plant resistance against caterpillars that restricts an induction of defense mechanisms to the level that allows enough resources to be allocated to other processes, particularly growth. The effect of enhanced resistance in herbivore-exposed wild-type plants and a highly resistant phenotype in *wrky70* plants on plant growth will be discussed below.

Aphid-Induced Plant Resistance

Aphid infestation is commonly known to increase the accumulation of SA, a major phytohormone in plant responses to phloem feeders (Zhu-Salzman et al., 2004; Walling, 2008). Previous studies have suggested that defense against caterpillars was suppressed after long-term (7-d) exposure to aphid feeding due to antagonistic interactions between SA and JA signaling (Rodriguez-Saona et al., 2005; Soler et al., 2012). Although a short-term (24-h) exposure to *B. brassicae* aphid feeding in wild-type plants was sufficient to significantly increase SA levels (Supplemental Fig. S1D), an unexpected enhanced resistance against subsequent caterpillar feeding was observed in aphid-exposed wild-type plants (Fig. 1B). Moreover, a similarly low body mass was recorded for *P. brassicae* caterpillars after 9 d of subsequent feeding on aphid-exposed wild-type, *wrky70*, or *sid2-1* plants (Fig. 1B; Supplemental Fig. S3). These results indicate that neither a functional WRKY70 TF nor an increase in SA level is required for the regulation of aphid-enhanced plant resistance to *P. brassicae* caterpillars. Furthermore, the levels of JA, JA-Ile, and KRR were similar for previously unexposed and previously aphid-exposed wild-type and *wrky70* plants, which suggests that a novel mechanism, independent of SA, JA, or their cross talk, regulates aphid-enhanced plant resistance against subsequent feeding by a specialist caterpillar (Figs. 2, 3, and 5).

Besides an increase in SA accumulation, aphid infestation is also known to induce oxidative stress in plant cells (Moran et al., 2002; Kempema et al., 2007). Demkura and Ballaré (2012) reported that a prior mild UV light treatment, which induced oxidative stress, made Arabidopsis plants highly resistant to (necrotrophic) pathogens that are sensitive to JA-dependent defenses through an induction of sinapate esters. Interestingly, we found a correlation between high SM accumulation and enhanced resistance to *P. brassicae* caterpillars in aphid-exposed wild-type plants as well as in *wrky70* plants regardless of herbivory history (Figs. 1B and 5D). An increased susceptibility to *P. brassicae* caterpillars and a lack of enhanced resistance against subsequent caterpillar feeding in aphid-exposed SM-deficient plants (*fah1-7*) further support the function of SM in plant defense against *P. brassicae* caterpillars in Arabidopsis. It is interesting that the accumulation of KRR, a metabolite that provides resistance against *P. brassicae* caterpillars (Onkokesung et al., 2014), was much lower (approximately 50%) in *fah1-7* plants than in wild-type and *wrky70* plants. Furthermore, caterpillar feeding failed to induce KRR levels in *fah1-7* mutant plants (Supplemental Fig. S6D). These results indicate that the combination of a lack of SM accumulation and a low KRR level contribute to the susceptibility to *P. brassicae* caterpillars in previously unexposed *fah1-7* plants (Fig. 6; Supplemental Fig. S9D). Further investigation of *P. brassicae* caterpillar resistance in Arabidopsis mutant plants silenced in *SMT* should provide additional information on the function of SM in plant resistance against specialist caterpillars.

SM induction after subsequent caterpillar feeding is a unique phenomenon in aphid-exposed wild-type plants. It is interesting that feeding by either *P. brassicae* caterpillars or *B. brassicae* aphids did not induce SM in wild-type plants, suggesting that the mechanism that regulated SM accumulation might act independently from JA- or SA-signaling pathways (Supplemental Fig. S11). Although we observed a tentatively negative correlation between *SMT* and *WRKY70* expression in unexposed, caterpillar-exposed, and aphid-exposed wild-type plants (Supplemental Fig. S8, A and B), the lack of an increased transcript level of *SMT* and SM accumulation at the basal level in *wrky70* mutant plants prevent us from drawing the conclusion that the WRKY70 TF is a negative regulator of SM biosynthesis. Moreover, Kim et al. (2015) reported that the level of indole-3-acetaldoxime, a precursor in indole GLS, auxin, and camalexin biosynthesis, negatively affected SM level in Arabidopsis. Based on available information on the SM biosynthetic pathway (Clauss et al., 2011) and our results, we speculate that SM biosynthesis is regulated through a novel molecular mechanism that may involve reactive oxygen species. Future studies using an integrative analysis of transcriptomics and metabolomics are required to identify the regulatory network of SM biosynthesis from signaling to metabolite biosynthesis.

It is noteworthy that KRR and SM both are flavonoid metabolites that are active defense metabolites against specialist caterpillars. Therefore, it is likely that other flavonoid metabolites might also be an active defense metabolite against specialist herbivores of Arabidopsis. Studying plant resistance against specialist herbivores in Arabidopsis mutant plants defective in transcriptional regulators of flavonoid biosynthesis pathways may result in the identification of novel defense metabolites against specialist herbivores.

Differential Costs of JA-Dependent and JA-Independent Herbivore-Induced Plant Resistance

The concept that plants utilize information of a previous encounter with biotic or abiotic stress in phytohormonal networks and enhance resistance against subsequent stress conditions in a cost-effective manner has been proposed previously (van Hulten et al., 2006; Bruce et al., 2007; Conrath, 2011; Vos et al., 2013). We found that an enhanced resistance against subsequent *P. brassicae* caterpillar feeding through a JA-independent mechanism involving SM biosynthesis in aphid-exposed wild-type plants strongly reduced plant growth. In contrast, enhanced resistance through JA-dependent mechanisms via the induction of GLS and KRR in caterpillar-exposed wild-type plants did not have a significant growth reduction compared with previously unexposed plants or control uninfested wild-type plants (Fig. 7). Although the induction of defense metabolites that is mediated by JA signaling after caterpillar feeding has been shown to incur costs in terms of plant growth reduction in Arabidopsis (Kliebenstein et al., 2001; Paul-Victor et al., 2010; Züst et al., 2011; Bekaert et al., 2012), this mechanism appears to be more cost effective than the induction of aphid-induced defense involving the metabolite SM. Nevertheless, we cannot rule out that a direct negative impact of aphid feeding on plant growth (Züst et al., 2011) leads to a stronger growth reduction upon caterpillar feeding in aphid-exposed wild-type plants.

Although the induction of JA signaling and defense metabolites in caterpillar-exposed or aphid-exposed *wrky70* plants were similar to those in wild-type plants, a strong growth reduction that followed subsequent caterpillar feeding was observed in *wrky70* plants (Fig. 7). Wang et al. (2006) reported a dual function of the WRKY70 TF as a negative and a positive regulator of SA biosynthesis and perception, respectively. It has been reported that high SA accumulation can suppress plant growth in Arabidopsis (Rivas-San Vicente and Plasencia, 2011). Indeed, *wrky70* plants had a significantly higher level of SA at the constitutive and induced levels than wild-type plants (Fig. 4). It is interesting that, although stalk length was comparable between undamaged control wild-type and *wrky70* plants, undamaged control *wrky70* plants had a smaller rosette than undamaged control wild-type plants (Fig. 7). These results suggest a possible negative effect of high SA level on plant growth in *wrky70* mutant plants. However, in comparison with undamaged control *wrky70* plants, a further and significant reduction of stalk length and rosette area was observed after subsequent caterpillar feeding on *wrky70* plants (Fig. 7). Taken together, we infer from our results that the growth attenuation in *wrky70* plants is a consequence of a high SA level and induced defense against caterpillars. Furthermore, a highly caterpillar-resistant phenotype that might derive from a strong induction of JA-dependent responses coincides with a strong growth reduction after caterpillar feeding on *wrky70* plants regardless of a prior herbivory history (Figs. 1B and 7). These results suggest that the WKRY70 TF is involved in a negative regulatory network that represses JA-inducible resistance against caterpillars to maintain an acceptable plant growth during herbivory stress conditions. More studies, for instance on the interaction between WRKY70 TF and other negative regulators of JA, particularly JAZ repressor proteins, are required to understand the role of the WRKY70 TF in a JA negative regulatory network that might also be involved in the regulation of a tradeoff between growth and defense during herbivory stress.

CONCLUSION

Our study shows that, although a prior exposure to short-term aphid or caterpillar feeding enhances plant resistance to subsequent caterpillar feeding, fundamentally different underlying defense mechanisms are involved. An enhanced resistance through the JA signaling network in caterpillar-exposed plants is more cost effective than an enhanced resistance through an alternative mechanism involving an increase in SM level, as observed in aphid-exposed plants. The constitutive caterpillar-resistant phenotype in *wrky70* plants coincides with a strong growth reduction, which indicates that plants utilize negative regulators of JA-induced responses, such as the WRKY70 TF, to restrict JA-induced resistance against caterpillar feeding while maintaining plant growth during subsequent herbivory.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used as the wild type. Seeds of a T-DNA insertion mutant of *WRKY70* (*wrky70*; SALK_025198; Li et al., 2006), a sinapate ester mutant (*fah1-7*; N8604; Ruegger et al., 1999), and an SA induction-deficient mutant (*sid2-1*; N16438; Wildermuth et al., 2001) in the Columbia-0 background were obtained from the European Arabidopsis Stock Centre. Surface-sterilized seeds as described previously (Onkokesung et al., 2014) were germinated on one-half-strength Murashige and Skoog medium

Table I. *Specific primers used for* RT-qPCR

Gene	Sequence (5′→3′)
WRKY70-Forward	ACCCGTTAAGGGTAAAAGAGGA
WRKY70-Reverse	CTTGGGTTCGAGCTCAACCT
AOS-Forward	TCCACCCAAAAACCGTACGA
AOS-Reverse	TGAAGAACTCTTCAGCTCCTTG
JAR1-Forward	GAAGCTGCTCACACCTAACC
JAR1-Reverse	CAATCCATCCTTCCGAGCTAC
ICS1-Forward	CACTAGATTCTCCCGCAAGAAG
ICS1-Reverse	TGGTCAATTGGAACCTGTAACC
VSP2-Forward	TCAGTGACCGTTGGAAGTTGTG
VSP2-Reverse	GTTCGAACCATTAGGCTTCAATATG
SMT-Forward	GAATGTTGGGCTAACGACGA
SMT-Reverse	CTTATCCAGGCTTGAGTTGCA
EF-Forward	TGAGCACGCTCTTCTTGCTTTCA
EF-Reverse	GGTGGTGGCATCCATCTTGTTACA

containing 3% (w/v) Suc. Plates were kept in the dark at 4°C for 2 d before transfer to a growth chamber at 21°C ± 1°C, 60% ± 5% relative humidity (RH), 120 μmol m^{-2} s^{-1} light intensity, and an 8/16-h (light/dark) photoperiod. Fourteen-day-old seedlings were subsequently transplanted into round plastic pots (diameter, 4.5 cm) containing sterilized substrate mix (Horticoop) and kept under environmental conditions as described above. Four- to 5-week-old plants were used in all experiments.

Insects

Caterpillars (*Pieris brassicae*) and aphids (*Brevicoryne brassicae*) from their respective stock colonies (Laboratory of Entomology, Wageningen University) were reared on Brussels sprouts plants (*Brassica oleracea* var *gemmifera* 'Cyrus') at 22°C ± 1°C, 60% ± 5% RH, and a 16/8-h (light/dark) photoperiod.

Herbivore Treatments

Four- to 5-week-old Arabidopsis plants were transferred from short (8/16-h light/dark) to long (16/8-h light/dark) photoperiod conditions, at 21°C ± 1°C and 60% ± 5% RH, 24 h before herbivore treatments.

For caterpillar-exposed plants, five first instar *P. brassicae* caterpillars were placed on each Arabidopsis plant and allowed to feed freely for 24 h before being removed from the plants.

For aphid-exposed plants, five adult *B. brassicae* aphids were placed on individual Arabidopsis plants, and the plants were kept in cylindrical plastic containers (diameter 8 cm × height 14 cm) covered with fine-mesh gauze under the same growth conditions as for the caterpillar-exposed treatment. After 24 h of feeding, the aphids were removed from the plants.

Plants without prior herbivore exposure (unexposed plants) were kept under the same growth conditions as caterpillar- and aphid-exposed plants for 24 h. After 24 h of exposure to caterpillars or aphids, unexposed, caterpillar-exposed, and aphid-exposed plants were subjected to *P. brassicae* caterpillar feeding.

P. brassicae Caterpillar Performance

To determine the effects of prior herbivory on the performance of *P. brassicae* caterpillars, a freshly hatched neonate caterpillar was placed on unexposed, caterpillar-exposed, or aphid-exposed plants (one caterpillar per plant). The larvae were allowed to continuously feed on the plants for 9 d, after which their body mass was determined. The fresh weights of 20 individual caterpillars fed on unexposed, caterpillar-exposed, and aphid-exposed plants were assessed.

Phytohormone Analysis

Five first instar larvae of *P. brassicae* were placed on each unexposed, caterpillar-exposed, and aphid-exposed plant. After removal of the caterpillars, caterpillar-damaged leaves were harvested after 6, 24, 48, or 72 h of feeding and pooled from two individual plants to obtain one biological replicate. Leaf tissue from undamaged plants was used as a control. Five biological replicates for each time point were harvested for unexposed, caterpillar-exposed, and aphid-exposed plants, flash frozen in liquid nitrogen, and kept at −80°C until analysis.

Approximately 0.1 g of finely pulverized leaf tissue from control, unexposed, caterpillar-exposed, and aphid-exposed plants was extracted and analyzed for JA, JA-Ile, and SA levels by LC-MS (Varian) as described in detail by Onkokesung et al. (2014). JA, JA-Ile, and SA were detected in the electrospray ionization (ESI) negative mode. JA was quantified based on the deuterium-labeled JA internal standard (C/D/N Isotope). SA and JA-Ile levels were quantified based on peak areas.

GLS Analysis

Each of 10 unexposed, caterpillar-exposed, and aphid-exposed plants were damaged by five first instar *P. brassicae* larvae for 4 d. Control (undamaged) plants were also kept under similar environmental conditions (16/8-h light/dark photoperiod, 21°C ± 1°C, and 60% ± 5% RH) to caterpillar-damaged plants. After removal of the caterpillars, damaged leaf tissues were harvested and pooled from two individual plants to obtain one biological replicate. Leaf tissue from undamaged plants was used as a control. Five biological replicates of unexposed, caterpillar-exposed, and aphid-exposed plants were harvested, flash frozen in liquid nitrogen, and kept at −80°C until analysis.

Approximately 20 mg of lyophilized tissue was used for GLS extraction and analyzed by HPLC and UV light detection; GLSs from the 80% (v/v) methanol extracts were bound to DEAE-Sephadex and converted to desulfoglucosinolates by the use of *Helix pomatia* sulfatase (Burow et al., 2006). An HPLC instrument (Agilent 1100 series), equipped with a C-18 reverse-phase column (Nucleodur Sphinx RP; 250 × 4.6 mm, 5-μm particle size; Macherey-Nagel), was used as described by Burow et al. (2006). Desulfoglucosinolates were identified based on comparison of retention times and UV light absorption spectra with those of known standards. GLS levels (μmol g^{-1} dry weight) were calculated from the peak areas at 229 nm relative to the peak area of the internal standard *para*-hydroxybenzyl GLS using the relative response factors 2 for aliphatic and 0.5 for indolic GLSs (Burow et al., 2006; Onkokesung et al., 2014).

Flavonol and Sinapate Ester Analysis

The same tissue samples from unexposed, caterpillar-exposed, and aphid-exposed plants that were used for GLS analysis were also used for flavonol and sinapate ester analysis. Approximately 20 mg of lyophilized tissue was extracted and analyzed by HPLC (Agilent HP1100 series) in conjunction with a C-18 reverse-phase column (Nucleodur Sphinx RP; 250 × 4.6 mm, 5-μm particle size; Macherey-Nagel) as described by Onkokesung et al., (2014). KRR (High-Purity Compound Standard), quercetin-3-glucoside (Sigma-Aldrich), and an authentic standard of sinapic acid (Fluka) were used as external standards for the quantification of kaempferol glycoside, quercetin glycoside, SG, and SM (Onkokesung et al., 2014).

RT-qPCR

Approximately 100 mg of finely ground frozen leaf tissue was used for total RNA isolation using the NucleoSpin RNA Plant Kit (Macherey-Nagel). Total RNA samples were treated with RQ1 DNase (Promega) followed by ethanol precipitation. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using an iScript cDNA synthesis kit for RT-qPCR (Bio-Rad) in a 20-μL reaction volume. RT-qPCR was performed in a CFX96 Touch Real Time PCR Detection System (Bio-Rad) in a total volume of 20 μL containing 1.5 μL of cDNA from 1 μg of RNA, 10 μL of iQ SYBR Green supermix (Bio-Rad), and 1.2 μL of 5 μM forward and reverse gene-specific primers. The primer sequences are listed in Table I. The reactions were run in a three-step program including melting curve analysis: preincubation at 95°C for 10 min, amplification for 40 cycles (95°C for 15 s, 59°C for 30 s, and 72°C for 45s), and melting analysis from 72°C to 95°C. For normalization, specific primers of EF1α from Arabidopsis (accession no. NM_001125992) were used. All reactions were performed with five biological replicates. Relative gene expression (fold change) was calculated based on an efficiency-corrected model (Pfaffl, 2001).

Plant Growth Analysis

The effect of enhanced resistance to *P. brassicae* caterpillars on plant growth was determined by measuring stalk length and rosette leaf area. Five first instar *P. brassicae* larvae were allowed to continuously feed on each unexposed, caterpillar-exposed, and aphid-exposed plant for 4 d. Caterpillars were removed from the plants after 4 d of feeding, and unexposed, caterpillar-exposed, and aphid-exposed plants were kept under 16/8-h light/dark photoperiod, 21°C ± 1°C, and 60% ± 5% RH conditions. Fourteen days after removal of caterpillars, stalk lengths were measured and rosette leaf area was determined from digital image analysis using ImageJ software (http://imagej.nih.gov/ij/index.html; Schneider et al., 2012). Undamaged plants were used as controls. In addition, the total damaged leaf area after 4 d of caterpillar feeding on unexposed, caterpillar-exposed, and aphid-exposed plants was quantified from digital image analysis by ImageJ software.

Statistical Analysis

The data were analyzed by Student's *t* test or ANOVA followed by Tukey's HSD posthoc test using SPSS 22 (IBM).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT3G56400 (WRKY70), AT5G42650 (AOS), AT2G46370 (JAR1), AT1G74710 (ICS1), AT5G24770 (VSP2), AT2G22990 (SMT), AT3G21560 (SGT1), AT4G36220 (FAH1), AT2G14610 (PR1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phytohormone levels in wild-type and *wrky70* mutant plants after feeding by *P. brassicae* caterpillars or *B. brassicae* aphids.

Supplemental Figure S2. Effect of functional WRKY70 TF on plant resistance against specialist caterpillars or specialist aphids in Arabidopsis.

Supplemental Figure S3. Effect of previous exposure to feeding by caterpillars or aphids on wild-type and SA-deficient mutant plants.

Supplemental Figure S4. Kinetics of transcript expression of *WRKY70*, *AOS*, and *ICS1* in unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* mutant plants after subsequent *P. brassicae* caterpillar feeding.

Supplemental Figure S5. *VSP2* and *PR1* relative transcript levels in previously unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants after subsequent *P. brassicae* caterpillar feeding.

Supplemental Figure S6. Flavonol glycoside levels after 4 d of feeding by *P. brassicae* caterpillars on previously unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants.

Supplemental Figure S7. Indole GLS and SG levels after feeding by *P. brassicae* caterpillars on previously unexposed, caterpillar-exposed, and aphid-exposed wild-type or *wrky70* plants.

Supplemental Figure S8. Relative transcript levels of *WRKY70*, *SMT*, and *SGT* after feeding by *P. brassicae* caterpillars on previously unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants.

Supplemental Figure S9. GLS and KRR levels upon *P. brassicae* caterpillar feeding on previously unexposed, caterpillar-exposed, and aphid-exposed wild-type, *wrky70*, and *fah1-7* plants.

Supplemental Figure S10. Percentage of total damaged leaf area after 4 d of feeding by *P. brassicae* caterpillars on unexposed, caterpillar-exposed, and aphid-exposed wild-type and *wrky70* plants.

Supplemental Figure S11. SM levels after 4 d of *P. brassicae* caterpillar feeding or after 7 d of *B. brassicae* aphid feeding on wild-type Arabidopsis.

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LITERATURE CITED

Baldwin IT, Schmelz EA (1996) Immunological "memory" in the induced accumulation of nicotine in wild tobacco. Ecology **77:** 236–246

Bekaert M, Edger PP, Hudson CM, Pires JC, Conant GC (2012) Metabolic and evolutionary costs of herbivory defense: systems biology of glucosinolate synthesis. New Phytol **196:** 596–605

Browse J, Howe GA (2008) New weapons and a rapid response against insect attack. Plant Physiol **146:** 832–838

Bruce TJA, Matthes MC, Napier JA, Pickett JA (2007) Stressful "memories" of plants: evidence and possible mechanisms. Plant Sci **173:** 603–608

Burow M, Müller R, Gershenzon J, Wittstock U (2006) Altered glucosinolate hydrolysis in genetically engineered *Arabidopsis thaliana* and its influence on the larval development of *Spodoptera littoralis*. J Chem Ecol **32:** 2333–2349

Caarls L, Pieterse CMJ, Van Wees SCM (2015) How salicylic acid takes transcriptional control over jasmonic acid signaling. Front Plant Sci **6:** 170

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666–671

Chung HS, Koo AJK, Gao X, Jayanty S, Thines B, Jones AD, Howe GA (2008) Regulation and function of Arabidopsis JASMONATE ZIM-domain genes in response to wounding and herbivory. Plant Physiol **146:** 952–964

Clauss K, von Roepenack-Lahaye E, Böttcher C, Roth MR, Welti R, Erban A, Kopka J, Scheel D, Milkowski C, Strack D (2011) Overexpression of sinapine esterase BnSCE3 in oilseed rape seeds triggers global changes in seed metabolism. Plant Physiol **155:** 1127–1145

Conrath U (2011) Molecular aspects of defence priming. Trends Plant Sci **16:** 524–531

Cui J, Bahrami AK, Pringle EG, Hernandez-Guzman G, Bender CL, Pierce NE, Ausubel FM (2005) *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proc Natl Acad Sci USA **102:** 1791–1796

Cui J, Jander G, Racki LR, Kim PD, Pierce NE, Ausubel FM (2002) Signals involved in Arabidopsis resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. Plant Physiol **129:** 551–564

Demkura PV, Ballaré CL (2012) UVR8 mediates UV-B-induced Arabidopsis defense responses against *Botrytis cinerea* by controlling sinapate accumulation. Mol Plant **5:** 642–652

Després C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D, Fobert PR (2003) The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. Plant Cell **15:** 2181–2191

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC, Pieterse CMJ (2006) Herbivore-induced resistance against microbial pathogens in Arabidopsis. Plant Physiol **142:** 352–363

Frost CJ, Mescher MC, Carlson JE, De Moraes CM (2008) Plant defense priming against herbivores: getting ready for a different battle. Plant Physiol **146:** 818–824

Gális I, Gaquerel E, Pandey SP, Baldwin IT (2009) Molecular mechanisms underlying plant memory in JA-mediated defence responses. Plant Cell Environ **32:** 617–627

Gols R, Roosjen M, Dijkman H, Dicke M (2003) Induction of direct and indirect plant responses by jasmonic acid, low spider mite densities, or a combination of jasmonic acid treatment and spider mite infestation. J Chem Ecol **29:** 2651–2666

Gulmon SL, Mooney HA (1986). Costs of defense and their effect on plant productivity. *In* TJ Givnish, ed, On the Economy of Plant Form and Function. Cambridge University Press, Cambridge, UK, pp 681–699

Hagemeier J, Schneider B, Oldham NJ, Hahlbrock K (2001) Accumulation of soluble and wall-bound indolic metabolites in Arabidopsis thaliana leaves infected with virulent or avirulent Pseudomonas syringae pathovar tomato strains. Proc Natl Acad Sci USA **98:** 753–758

Halkier BA, Gershenzon J (2006) Biology and biochemistry of glucosinolates. Annu Rev Plant Biol **57:** 303–333

Heil M, Silva Bueno JC (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc Natl Acad Sci USA **104:** 5467–5472

Hopkins RJ, van Dam NM, van Loon JJA (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annu Rev Entomol **54:** 57–83

Karban R, Baldwin IT (1997) Induced Responses to Herbivory. University of Chicago Press, Chicago

Kempema LA, Cui X, Holzer FM, Walling LL (2007) Arabidopsis transcriptome changes in response to phloem-feeding silverleaf whitefly nymphs: similarities and distinctions in responses to aphids. Plant Physiol **143:** 849–865

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. Plant J **38:** 639–649

Kim JI, Doland WL, Anderson NA, Chapple C (2015) Indole glucosinolate biosynthesis limits phenylproponoid accumulation in *Arabidopsis thaliana*. Plant Cell **27:** 1529–1546

Kliebenstein DJ, Lambrix VM, Reichelt M, Gershenzon J, Mitchell-Olds T (2001) Gene duplication in the diversification of secondary metabolism: tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in *Arabidopsis*. Plant Cell **13:** 681–693

Kroes A, van Loon JJA, Dicke M (2015) Density-dependent interference of aphids with caterpillar-induced defenses in Arabidopsis: involvement of phytohormones and transcription factors. Plant Cell Physiol **56:** 98–106

Leiss KA, Choi YH, Verpoorte R, Klinkhamer PGL (2011) An overview of NMR-based metabolomics to identify secondary plant compounds involved in host plant resistance. Phytochem Rev **10:** 205–216

Li J, Brader G, Kariola T, Palva ET (2006) WRKY70 modulates the selection of signaling pathways in plant defense. Plant J **46:** 477–491

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16:** 319–331

Li R, Zhang J, Li J, Zhou G, Wang Q, Bian W, Erb M, Lou Y (2015) Prioritizing plant defence over growth through WRKY regulation facilitates infestation by non-target herbivores. eLife **4:** e04805

Menzel TR, Weldegergis BT, David A, Boland W, Gols R, van Loon JJA, Dicke M (2014) Synergism in the effect of prior jasmonic acid application on herbivore-induced volatile emission by lima bean plants: transcription of a monoterpene synthase gene and volatile emission. J Exp Bot **65:** 4821–4831

Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiol **138:** 1149–1162

Moran PJ, Cheng Y, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Arch Insect Biochem Physiol **51:** 182–203

Müller R, de Vos M, Sun JY, Sønderby IE, Halkier BA, Wittstock U, Jander G (2010) Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. J Chem Ecol **36:** 905–913

Mur LAJ, Kenton P, Atzorn R, Miersch O, Wasternack C (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant Physiol **140:** 249–262

Onkokesung N, Gális I, von Dahl CC, Matsuoka K, Saluz HP, Baldwin IT (2010) Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* leaves. Plant Physiol **153:** 785–798

Onkokesung N, Reichelt M, van Doorn A, Schuurink RC, van Loon JJA, Dicke M (2014) Modulation of flavonoid metabolites in *Arabidopsis thaliana* through overexpression of the *MYB75* transcription factor: role of kaempferol-3,7-dirhamnoside in resistance to the specialist insect herbivore *Pieris brassicae*. J Exp Bot **65:** 2203–2217

Paul-Victor C, Züst T, Rees M, Kliebenstein DJ, Turnbull LA (2010) A new method for measuring relative growth rate can uncover the costs of defensive compounds in Arabidopsis thaliana. New Phytol **187:** 1102–1111

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:** e45

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annu Rev Cell Dev Biol **28:** 489–521

Poelman EH, Broekgaarden C, Van Loon JJA, Dicke M (2008) Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. Mol Ecol **17:** 3352–3365

Poelman EH, van Loon JJA, van Dam NM, Vet LEM, Dicke M (2010) Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. Ecol Entomol **35:** 240–247

Rasmann S, Chassin E, Bilat J, Glauser G, Reymond P (2015) Trade-off between constitutive and inducible resistance against herbivores is only partially explained by gene expression and glucosinolate production. J Exp Bot **66:** 2527–2534

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW, Jander G (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol **158:** 854–863

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell **12:** 707–720

Rivas-San Vicente M, Plasencia J (2011) Salicylic acid beyond defence: its role in plant growth and development. J Exp Bot **62:** 3321–3338

Rodriguez-Saona C, Chalmers JA, Raj S, Thaler JS (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. Oecologia **143:** 566–577

Ruegger M, Meyer K, Cusumano JC, Chapple C (1999) Regulation of *ferulate-5-hydroxylase* expression in Arabidopsis in the context of sinapate ester biosynthesis. Plant Physiol **119:** 101–110

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods **9:** 671–675

Seyfferth C, Tsuda K (2014) Salicylic acid signal transduction: the initiation of biosynthesis, perception and transcriptional reprogramming. Front Plant Sci **5:** 697

Shim JS, Jung C, Lee S, Min K, Lee YW, Choi Y, Lee JS, Song JT, Kim JK, Choi YD (2013) At*MYB44* regulates *WRKY70* expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. Plant J **73:** 483–495

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Funct Ecol **26:** 156–166

Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, Buchala AJ, Métraux JP, Brown R, Kazan K, et al (2003) NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15:** 760–770

Stam JM, Kroes A, Li Y, Gols R, van Loon JJA, Poelman EH, Dicke M (2014) Plant Interactions with Multiple Insect Herbivores: From Community to Genes. Annu Rev Plant Biol **65:** 689–713

Stork W, Diezel C, Halitschke R, Gális I, Baldwin IT (2009) An ecological analysis of the herbivory-elicited JA burst and its metabolism: plant memory processes and predictions of the moving target model. PLoS ONE **4:** e4697

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends Plant Sci **17:** 260–270

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF (COI1) complex during jasmonate signalling. Nature **448:** 661–665

Van der Does D, Leon-Reyes A, Koornneef A, Van Verk MC, Rodenburg N, Pauwels L, Goossens A, Körbes AP, Memelink J, Ritsema T, et al (2013) Salicylic acid suppresses jasmonic acid signaling downstream of SCFCOI1-JAZ by targeting GCC promoter motifs via transcription factor ORA59. Plant Cell **25:** 744–761

van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J (2006) Costs and benefits of priming for defense in Arabidopsis. Proc Natl Acad Sci USA **103:** 5602–5607

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol **47:** 177–206

Vos IA, Moritz L, Pieterse CMJ, Van Wees SCM (2015) Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions. Front Plant Sci **6:** 639

Vos IA, Pieterse CMJ, van Wees SCM (2013) Costs and benefits of hormone-regulated plant defences. Plant Pathol **62:** 43–55

Walling LL (2008) Avoiding effective defenses: strategies employed by phloem-feeding insects. Plant Physiol **146:** 859–866

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog **2:** e123

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot (Lond) **100:** 681–697

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot (Lond) **111:** 1021–1058

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565

Winde I, Wittstock U (2011) Insect herbivore counteradaptations to the plant glucosinolate–myrosinase system. Phytochemistry **72:** 1566–1575

Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, De Luca V, Després C (2012) The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell Reports **1:** 639–647

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, et al (2009) The *Arabidopsis* CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell **21:** 2220–2236

Zavala JA, Baldwin IT (2006) Jasmonic acid signalling and herbivore resistance traits constrain regrowth after herbivore attack in *Nicotiana attenuata*. Plant Cell Environ **29:** 1751–1760

Zhang PJ, Broekgaarden C, Zheng SJ, Snoeren TAL, van Loon JJA, Gols R, Dicke M (2013a) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in Arabidopsis thaliana. New Phytol **197:** 1291–1299

Zhang PJ, Li WD, Huang F, Zhang JM, Xu FC, Lu YB (2013b) Feeding by whiteflies suppresses downstream jasmonic acid signaling by eliciting salicylic acid signaling. J Chem Ecol **39:** 612–619

Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H (2004) Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. Plant Physiol **134:** 420–431

Züst T, Joseph B, Shimizu KK, Kliebenstein DJ, Turnbull LA (2011) Using knockout mutants to reveal the growth costs of defensive traits. Proc Biol Sci **278:** 2598–2603