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# Harpin-induced expression and transgenic overexpression of the phloem protein gene *AtPP2-A1* in *Arabidopsis* repress phloem feeding of the green peach aphid *Myzus persicae*

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## Abstract

**Background:** Treatment of plants with HrpN<sub>Ea</sub>, a protein of harpin group produced by Gram-negative plant pathogenic bacteria, induces plant resistance to insect herbivores, including the green peach aphid *Myzus persicae*, a generalist phloem-feeding insect. Under attacks by phloem-feeding insects, plants defend themselves using the phloem-based defense mechanism, which is supposed to involve the phloem protein 2 (PP2), one of the most abundant proteins in the phloem sap. The purpose of this study was to obtain genetic evidence for the function of the *Arabidopsis thaliana* (*Arabidopsis*) PP2-encoding gene *AtPP2-A1* in resistance to *M. persicae* when the plant was treated with HrpN<sub>Ea</sub> and after the plant was transformed with *AtPP2-A1*.

**Results:** The electrical penetration graph technique was used to visualize the phloem-feeding activities of apterous agamic *M. persicae* females on leaves of *Arabidopsis* plants treated with HrpN<sub>Ea</sub> and an inactive protein control, respectively. A repression of phloem feeding was induced by HrpN<sub>Ea</sub> in wild-type (WT) *Arabidopsis* but not in *atpp2-a1/E/142*, the plant mutant that had a defect in the *AtPP2-A1* gene, the most HrpN<sub>Ea</sub>-responsive of 30 *AtPP2* genes. In WT rather than *atpp2-a1/E/142*, the deterrent effect of HrpN<sub>Ea</sub> treatment on the phloem-feeding activity accompanied an enhancement of *AtPP2-A1* expression. In PP2OETAt (*AtPP2-A1*-overexpression transgenic *Arabidopsis thaliana*) plants, abundant amounts of the *AtPP2-A1* gene transcript were detected in different organs, including leaves, stems, calyces, and petals. All these organs had a deterrent effect on the phloem-feeding activity compared with the same organs of the transgenic control plant. When a large-scale aphid population was monitored for 24 hours, there was a significant decrease in the number of aphids that colonized leaves of HrpN<sub>Ea</sub>-treated WT and PP2OETAt plants, respectively, compared with control plants.

**Conclusions:** The repression in phloem-feeding activities of *M. persicae* as a result of *AtPP2-A1* overexpression, and as a deterrent effect of HrpN<sub>Ea</sub> treatment in WT *Arabidopsis* rather than the *atpp2-a1/E/142* mutant suggest that *AtPP2-A1* plays a role in plant resistance to the insect, particularly at the phloem-feeding stage. The accompanied change of aphid population in leaf colonies suggests that the function of *AtPP2-A1* is related to colonization of the plant.

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## Background

Harpins are multifunctional proteins produced by Gram-negative plant pathogenic bacteria [1,2]. The first-characterized [1] and well-studied harpin [2-7], HrpN<sub>Ea</sub>, is secreted by *Erwinia amylovora*, the bacterial pathogen that causes fire blight disease in rosaceous plants [1]. Multiple functions of harpin proteins, especially in eliciting plant defense responses, were also elucidated initially by studies using HrpN<sub>Ea</sub> as a paradigm [1-3]. Early studies demonstrated that the external application of HrpN<sub>Ea</sub> was able to induce resistance in a variety of plant species [3-7], and that the induced resistance effectively protected plants from attacks by insect herbivores [2,7-9]. HrpN<sub>Ea</sub>-induced resistance to insects first was suggested based on observations of field-grown peppers. Plants that had been treated with HrpN<sub>Ea</sub> incurred fewer injuries from the European corn borer than comparable untreated plants [2]. A deterrent effect on striped cucumber beetles was observed in HrpN<sub>Ea</sub>-treated cucumber; striped cucumber beetles preferred to colonize untreated control plants rather than HrpN<sub>Ea</sub>-treated plants [8]. HrpN<sub>Ea</sub>-induced resistance was also effective in impeding infestations of aphids, an important type of phloem-feeding herbivores [9,10]. In cucumbers grown under environmentally controlled conditions, HrpN<sub>Ea</sub> treatment had a deterrent effect on colonization by the muskmelon aphid *Aphis gossypii* (Glover), which preferred to colonize control plants rather than HrpN<sub>Ea</sub>-treated plants [9]. In *Arabidopsis thaliana* (Arabidopsis), moreover, HrpN<sub>Ea</sub>-induced resistance was shown to repress infestation of the green peach aphid *Myzus persicae* (Sulzer), a generalist phloem-feeding insect [10].

Phloem-feeding insects are highly specialized in their mode of feeding [11] and present a unique stress on plant fitness [12-15]. These insects use their slender stylets to feed from a single-cell type, the phloem sieve element [8,16]. The feeding process can be monitored by the electrical penetration graph (EPG) technique [16]. Pivotaly, a stylet puncturing of the host plant cell, shown as a probe in the EPG, may lead to uptake of the phloem sap. In order to prevent protein clogging inside the sieve element, ejection of watery saliva is essential in feeding from the phloem [13,16]. This ejection is detected in the EPG as E1 salivation and always precedes phloem sap ingestion [16]. During ingestion from the sieve element, the watery E2 salivation occurs, and this E2 saliva is added to the ingested sap, thought to prevent phloem proteins from clogging inside the capillary food canal [16]. Therefore, salivation is a crucial event during the phloem-feeding process for insects to overcome a number of phloem-related plant properties and reactions [13-19].

In response to the phloem-feeding stress, plants defend themselves specifically using the phloem-based

defense (PBD) mechanism [14-16], which can be also activated by other cues, such as wounding [20-22], besides insect attacks [14,20-22]. Proposed components of PBD include the phloem protein 1 (PP1) and phloem protein 2 (PP2), which represent a type of the most abundant proteins in the phloem sap [23]. PP2 is a phloem lectin conserved in plants [23,24] and is believed to play a role in the establishment of PBD induced by insect attacks [21,25,26] and other stresses, such as wounding [16,21,22,26] and oxidative conditions [25]. In pumpkin, PP1 monomers and PP2 dimers are covalently cross-linked via disulphide bonds, forming high molecular weight polymers that close the sieve pores [21,25,26]. This response is induced by oxidative stress [25] but normally accompanies the synthesis of the  $\beta$ -1,3-glucan callose by callose synthase [20] that accumulates on sieve plates after different stress treatments [21]. Phloem protein plugging and callose closure of sieve pores, and callose coagulation on sieve plates as well, is hypothesized to serve as a physical barrier to prevent the insect from phloem-feeding activity [26]. Nevertheless, evidence for the function of phloem proteins in insect defense has been in paucity.

In the completely sequenced Arabidopsis genome, *PP2* (previously *PP2-like*) genes were identified as a large multigene family constituted of 30 members [23,27], *AtPP2-A1* to *AtPP2A-15* and *AtPP2-B1* to *AtPP2-B15* [23]. To our knowledge, however, little has been known about bioprocesses affected by these genes and properties of the encoded proteins. Although Arabidopsis mutants that represent multiple mutation alleles of *AtPP2* have been generated [27,28], subsequent biological effects have not been studied, and especially, effects of *AtPP2* mutations on the plant resistance to insects are unclear. For example, different types of Arabidopsis mutants were generated by T-DNA insertion at distinct locations in the *AtPP2-A1* DNA sequence; *atpp2-a1/P/-210* resulted from the insertion at nucleotide residue -210 in the promoter region. When grown on an artificial medium, the *atpp2-a1/P/-210* mutant performs as the wild-type (WT) plant in response to infestations of *M. persicae* adults and newborn nymphs in 24 hours after colonization by the adults [29]. There is as yet no evidence to show if *atpp2-a1/P/-210* impacts longer behaviors and feeding activities of the insect and if other mutation alleles of *AtPP2-A1* have biological effects [27,28].

The purpose of this study was to obtain genetic evidence that could elucidate a function of *AtPP2-A1* in Arabidopsis resistance to *M. persicae*. We began with determining the effect of *AtPP2-A1* on phloem feeding of aphids that colonized the plants treated with HrpN<sub>Ea</sub> according to previous evidence that the HrpN<sub>Ea</sub> treatment and *M. persicae* infestation had some degrees of

overlapping effects on the induction of plant responses. For example, formation of the PP2-PP1 complex needs reactive oxygen burst in cucurbit [25] while reactive oxygen burst is a conserved response in Arabidopsis treated with any harpins [30,31]. *M. persicae* infestation induces an elevation of the ethylene level [32] and triggers modest induction of ethylene-dependent responses [32,33], whereas, HrpN<sub>Ea</sub> induces resistance to *M. persicae* by activating the ethylene-signaling pathway [4,34]. Therefore, we devised to determine the possibility that HrpN<sub>Ea</sub>-induced resistance involves the PBD mechanism to encounter with *M. persicae* infestation. In order to further test this hypothesis, we generated *AtPP2-A1*-overexpression plants and investigated them to elucidate the supposed function of *AtPP2-A1*. In this article, we report evidence that harpin-induced expression and transgenic overexpression of *AtPP2-A1* induce a repression in the phloem-feeding activity of *M. persicae*.

## Results

### HrpN<sub>Ea</sub> treatment in Arabidopsis induces a repression in phloem feeding and colonization by *M. persicae*

The HrpN<sub>Ea</sub> protein used in this study was produced by prokaryotic expression with a vector that carried a *hrpN<sub>Ea</sub>* gene insert; the *hrpN<sub>Ea</sub>*-absent Empty Vector Preparation (EVP) that contained inactive proteins but not HrpN<sub>Ea</sub> was used as a control [6]. We investigated activities of *M. persicae* feeding from Arabidopsis (ecotype Col-0) WT plants following treatment with EVP and HrpN<sub>Ea</sub>, respectively. Because a period of five days is usually required for the induction of plant defense responses [3-8], plants at the fifth day posttreatment (dpt) were artificially colonized with uniform ten-day-old apterous (wingless) agamic *M. persicae* females transferred from an Arabidopsis nursery. Aphid feeding activities were studied by the EPG technique applied to 20 aphids that colonized leaves of Arabidopsis plants treated with EVP and HrpN<sub>Ea</sub>, respectively. Feeding activities were depicted as different waveform patterns recognized according to the standard previously established [35] and widely used [13,16,17,36]. Based on the EPG patterns, all the 20 aphids tested in five repetitions of the experiments for each treatment accomplished major steps of the feeding process, but aphid activities varied greatly depending on feeding stages (Table 1).

Figure 1a shows a four-hour EPG record of aphid feeding from the WT plant. The nonpuncturing phase (Figure 1a, np) indicated the stylet staying outside the cuticle. Cell puncturing (Figure 1a, probe) led to the pathway phase (Figure 1a, path) in which the stylet penetrated between cells en route to the vascular tissue [35]. In the four-hour EPG record, total number and duration of the nonpuncturing phase, time to the first cell puncturing or the first pathway phase, and total number and

duration of the pathway phase were all similar in HrpN<sub>Ea</sub>-treated plants as in control plants (Table 1). The pathway phase represents insect's efforts in navigating the phloem and preparing to ingest sap from sieve elements [16,17]. Subsequently, aphids may proceed to the phloem phase (Figure 1a, PP) in which ingestion of the phloem sap may occur [16]. The pathway phase may be also connected with the xylem phase, indicating stylet penetration of the xylem in the vascular tissue [16], but xylem phase was not found in this study. Analyses of the four-hour EPG record as a whole suggested that the plant treatment with HrpN<sub>Ea</sub> did not evidently change aphid activities outside vascular tissues when evaluated in a four-hour course of surveys (Table 1). However, analyses by hour offered additional information. In the first hour, especially, the nonpuncturing phase was more frequent with longer duration while the pathway phase was more but shorter under the HrpN<sub>Ea</sub> treatment condition compared with control. This result suggested that the HrpN<sub>Ea</sub> treatment impeded aphids in early feeding activities, both puncturing of the plant cell and navigating of the phloem. Subsequently, however, the phloem phase was always shorter, in HrpN<sub>Ea</sub>-treated plants than in control plants, no matter if the EPG patterns were analyzed by hour or based on the four-hour record as a whole (Table 1).

Based on the four-hour EPG record, the proportions of times within the pathway phase and time to the first phloem phase were much longer, suggesting the impediment to aphids in locating the ingestion site within the vascular tissue, in HrpN<sub>Ea</sub>-treated plants compared with control plants (Figure 1a; Table 1). On HrpN<sub>Ea</sub>-treated plants, moreover, aphids took fewer actions to puncture cells (Table 1, Number of cell puncturing) and to enter the phloem phase (Table 1, Number of phloem phase) after the first entry of phloem phase. These results suggested that phloem properties of HrpN<sub>Ea</sub>-treated plants were changed as unfavourable to aphid feeding. In consistence with this notion, total duration of the phloem phase was markedly shorter in HrpN<sub>Ea</sub>-treated plants than in control plants (Table 1). Noticeably, duration of the phloem phase in the second hour of the EPG monitoring, being 30 in HrpN<sub>Ea</sub>-treated plants and 14 min in control plants, on average, strongly suggested the deterrent effect of the HrpN<sub>Ea</sub> treatment on the phloem-feeding activity of *M. persicae*.

In the phloem phase, E1 and E2 salivations were recognized by dissecting the EPG waveform patterns (Figure 1b). Compounds of E1 and E2 saliva produced by aphids after stylet entry of the phloem are believed to function in preventing protein clogging inside the sieve element and preventing phloem proteins from clogging inside the capillary food canal, respectively [16]. Thus, E1 and E2 salivations play an important role in ingestion of the phloem sap by the insects [13,16]. As shown in Table 1, durations of both

**Table 1 Four-hour electrical penetration graph (EPG) analyses of the green peach aphid *Myzus persicae* feeding from wild-type (WT) Arabidopsis plants**

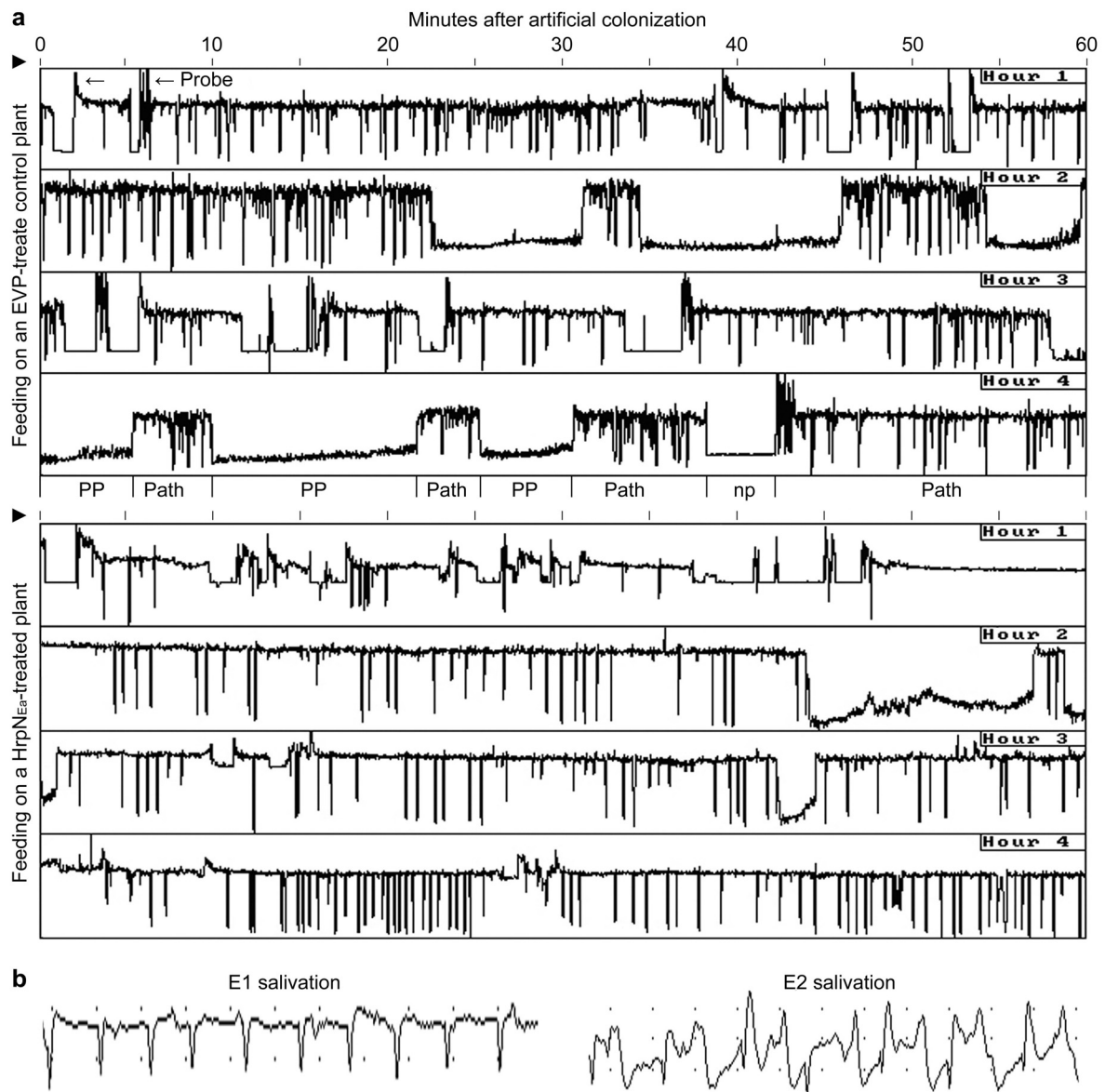
Activity examined		Control group mean (SD*)	HrpN <sub>Ea</sub> treatment group mean (SD*)	Student's t-test (n = 20)
Number of nonpuncturing phase	total	13.5 (2.2)	16.0 (3.5)	**
	1st h	6.0 (1.0)	13 (2.5)	$p < 0.01$
	2nd h	0	0	
	3rd h	6.5 (0.8)	2 (0.5)	$p < 0.01$
	4th h	1.0 (0.3)	1.0 (0.3)	**
Duration of nonpuncturing, min	total	19.8 (5.2)	16.8 (4.6)	**
	1st h	4.9 (0.3)	15.0 (3.9)	$p < 0.01$
	2nd h	0	0	
	3rd h	11.1 (3.6)	1.3 (0.4)	$p < 0.01$
	4th h	3.8 (1.2)	0.5 (0.2)	$p < 0.01$
Time to 1st cell puncturing, min		2.1 (0.6)	2.1 (0.3)	**
Time to 1st pathway, min		3.3 (0.5)	3.0 (0.4)	**
Number of pathway phase	total	19.5 (2.0)	16.5 (1.5)	**
	1st h	5.2 (0.5)	11.5 (1.0)	$p < 0.01$
	2nd h	3.0 (0.3)	2.0 (0.1)	**
	3rd h	7.3 (1.0)	2.0 (0)	$p < 0.01$
	4th h	4.0 (0.5)	2.0 (0.2)	$p < 0.01$
Duration of pathway phase, min	total	175.7 (48.9)	205.0 (62.5)	$p < 0.01$
	1st h	55.1 (6.7)	45.0 (7.5)	$p < 0.01$
	2nd h	37.2 (3.5)	43.9 (7.2)	$p < 0.05$
	3rd h	47.4 (5.6)	56.6 (8.0)	$p < 0.01$
	4th h	36/0 (3.2)	59.5 (10.5)	$p < 0.01$
Time to 1st phloem phase, min		85.6 (10.7)	104.3 (12.0)	$p < 0.01$
Number of cell puncturing after 1st phloem phase		20.5 (2.0)	11 (1.6)	$p < 0.01$
Number of phloem phase	total	7 (1.0)	3.0 (0.2)	$p < 0.01$
	1st h	0	0	
	2nd h	3.0 (0.5)	1.5 (0.5)	$p < 0.01$
	3rd h	1.0 (0)	1.5 (0.5)	**
	4th h	3.0 (0.5)	0	$p < 0.01$
Duration of phloem phase, min	total	44.5 (8.5)	18.2 (3.6)	$p < 0.01$
	1st h	0	0	
	2nd h	22.8 (5.0)	16.1 (3.5)	$p < 0.01$
	3rd h	1.5 (0.5)	2.1 (0.6)	$p < 0.01$
	4th h	20.2 (3.5)	0	$p < 0.01$
Duration of phloem feeding, min	total	44.5 (8.5)	18.2 (3.6)	$p < 0.01$
	E1	12.6 (2.8)	5.0 (1.4)	$p < 0.01$
	E2	31.9 (3.5)	13.2 (3.1)	$p < 0.01$

\*SD, standard deviation. \*\*Insignificant difference at  $p < 0.05$ .

E1 and E2 salivations were much shorter in HrpN<sub>Ea</sub>-treated plants than control plants, confirming the deterrent effect of the HrpN<sub>Ea</sub> treatment on the phloem-feeding activity of *M. persicae*.

To correlate repression in the phloem-feeding activity with colonization of Arabidopsis by *M. persicae*, we monitored a large-scale population of the insect and surveyed a

24-hour fluctuation in leaf colonies. A total of 1,200 uniform individuals of apterous and agamic *M. persicae* females were monitored in four repetitions of the experiments for plants treated with EVP and HrpN<sub>Ea</sub>, respectively. The number of aphids that stayed in their colonies on leaves was counted and the number of aphids that run away from the leaf colonies was calculated at intervals in

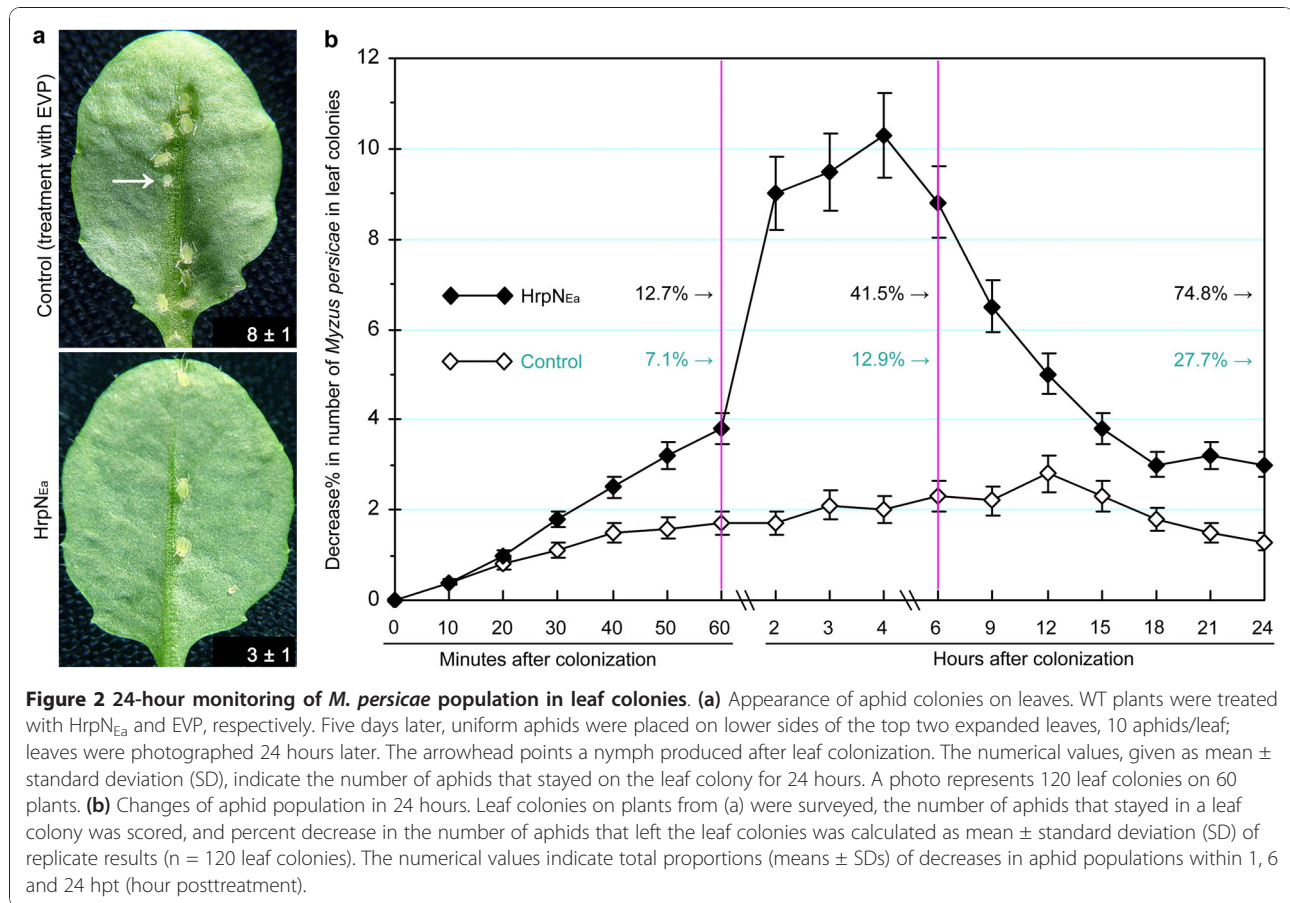


**Figure 1** PG patterns and waveforms of the green peach aphid *Myzus persicae* on wild-type (WT) *Arabidopsis*. **(a)** Four-hour EPG record. Plants were treated with the bacterial harpin protein HrpN<sub>Ea</sub> and specific control protein preparation EVP, respectively. Five days later, uniform ten-day-old apterous aphid females were placed on upper sides of the top first expanded leaves. Feeding activities were detected immediately with a four-channel Giga-4 direct current amplifier, which enabled simultaneous recording from four individual aphids. The EPG record represents 20 aphids feeding on 20 plants treated differently and monitored in five repetitions of experiments. Reiteratively appeared EPG waveforms are indicated once at proper spaces. PP, phloem phase; Path, pathway phase; np, no probing. **(b)** Two important waveforms in the phloem phase dissected every five second using the EPG analysis software STYLET 2.5.

24 hours (Figure 2). At each time point, the number of aphid individuals run away from their colonies on leaves of HrpN<sub>Ea</sub>-treated plants was greater than the number of the insect run away from colonies on leaves of control plants (Student's *t*-test,  $P < 0.01$ ). Proportions of aphids escaped from leaf colonies in control plants were close at the different intervals, but much higher proportions of aphid escapes from leaf colonies in HrpN<sub>Ea</sub>-treated plants were

observed in the short period of two to four hours. And this period was critical to the effect of HrpN<sub>Ea</sub> treatment on colonization of the plant, consistent with the effect on the phloem-feeding activity (Figure 1a). In 24 hours, a total of 74.8% aphids on average run away from their colonies on leaves of HrpN<sub>Ea</sub>-treated plants, in contrast to totally 17.7% aphids escaped from leaf colonies in control plants (Figure 2; Student's *t*-test,  $P < 0.01$ ). In subsequent





days, aphids that had run away from the original leaf colonies were found in a drifting status, died, and appeared as white carcasses on other different parts of the plants. These observations indicate that the HrpN<sub>Ea</sub> treatment impairs the stability of Arabidopsis colonization by *M. persicae*.

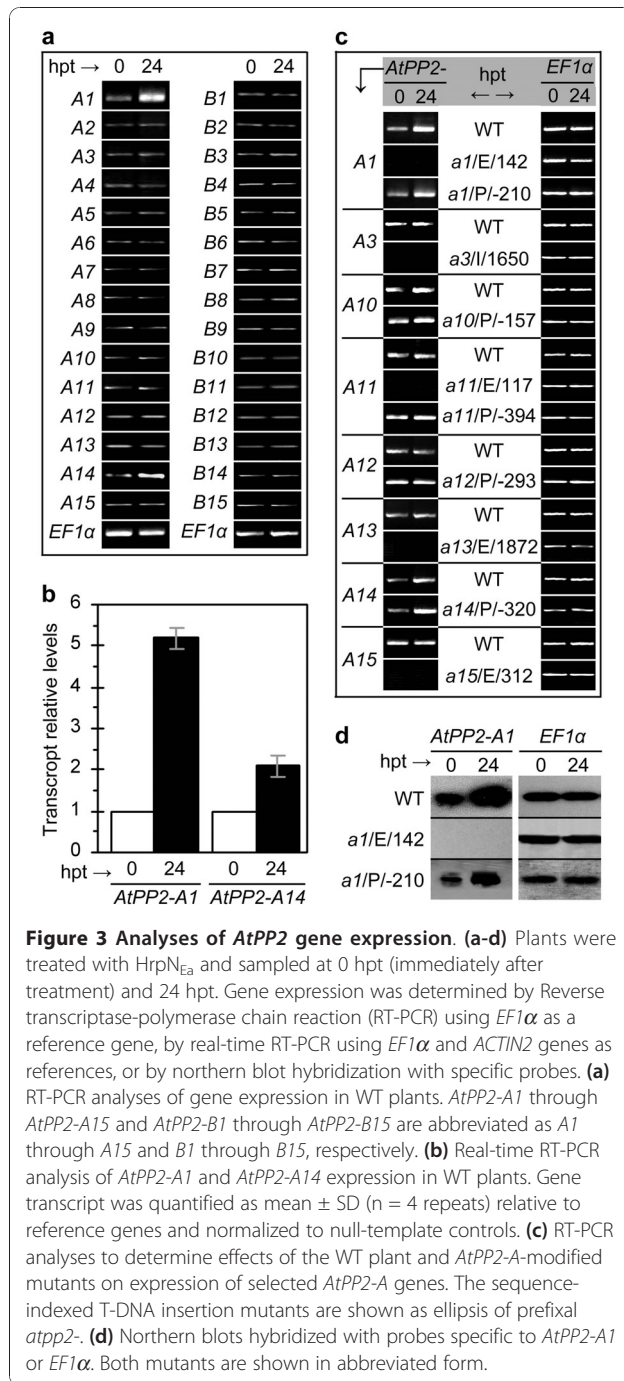
#### Arabidopsis *atpp2-a1/E/142* mutant pampers *M. persicae* in phloem feeding

To gain information about relationships between previously identified 30 *AtPP2* genes [23] and HrpN<sub>Ea</sub>-induced repression in the phloem-feeding activity of *M. persicae*, we studied expression of these genes in HrpN<sub>Ea</sub>-treated WT Arabidopsis plants. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the *EF1α* gene as a reference [6,37] to detect the expression of 15 *AtPP2-A* genes and 15 *AtPP2-B* genes [23]. As shown in Figure 3a, transcript levels of the genes, except *AtPP2-A1* and *AtPP2-A14*, in HrpN<sub>Ea</sub>-treated plants were similar when tested at the 24th hour posttreatment (hpt) as tested at 0 hpt (immediately after the plant treatment). However, both *AtPP2-A1* and *AtPP2-A14* were expressed at enhanced extents in HrpN<sub>Ea</sub>-treated plants. Subsequent real-time RT-PCR analyses using the *EF1α* and *Actin2* genes as references [37,38] revealed a greater expression level of

*AtPP2-A1* than *AtPP2-A14*. Relatively, *AtPP2-A1* and *AtPP2-A14* transcripts accumulated in 24 hours were 5 and 2 times more, respectively, in HrpN<sub>Ea</sub>-treated plants than in control plants (Figure 3b).

To correlate the role of HrpN<sub>Ea</sub> in enhancing gene expression with the role in repressing phloem feeding of *M. persicae*, we investigated Arabidopsis mutants previously generated by T-DNA insertion at *AtPP2-A* sequences. Two *AtPP2-A1* sequence-indexed lines were chosen for the test because the *AtPP2-A1* protein had been shown to affect weight gain in *M. persicae* nymphs [24], and the other eight *AtPP2-A*-modified mutants were considered for comparison because the *AtPP2-A* genes differed from *AtPP2-A1* in response to HrpN<sub>Ea</sub> (Figure 3a). The ten mutants were confirmed for the presence of T-DNA insert according to available information (Table 2); they were named conventionally after lowercase gene symbols, suffixed with the insert locations, including gene DNA components (P, promoter; E, exon; I, intron) and nucleotide residue sites at the gene DNA sequences. Mutants were compared with WT in expression of the corresponding genes and aphid behaviors on leaf colonies.

Parallel RT-PCR analyses of RNA samples isolated at 0 and 24 hpt revealed that the *AtPP2-A* genes performed



differently in corresponding mutants compared with the WT plant (Figure 3c). Both the basal expression (0 hpt) and HrpN<sub>Ea</sub>-induced expression (24 hpt) of *AtPP2-A1* was detected in the *atpp2-a1/P/-210* mutant as in WT but not in the *atpp2-a1/E/142* mutant (Figure 3c). This result was confirmed by northern blot hybridization (Figure 3d). And this result conformed to the PLACE Web Signal Scan [39], which revealed 37 types of *cis*-acting regulatory DNA elements present in the predicted

344-bp promoter of *AtPP2-A1*. Eighteen elements exist as a single copy and 19 elements have multiple copies, located at distant 83 sites in the promoter sequence. However, none of the elements was disrupted by T-DNA insertion and this might account for *AtPP2-A1* expression in *atpp2-a1/P/-210*. Similarly, none of 35 types of *cis*-acting regulatory DNA elements scanned in the upstream -370 region of the *AtPP2-A14* DNA sequence was disrupted in *atpp2-a14/P/-320*. This mutant performed as WT in both the basal expression and HrpN<sub>Ea</sub>-induced expression of *AtPP2-A14* (Figure 3c). The other eight mutants behaved differently in expression of the corresponding *AtPP2-A* genes. *AtPP2-A3*, *-A11*, *-A13*, and *-A15* were not expressed in their corresponding mutants *atpp2-a3/I/1650*, *-a11/E/177*, *-a13/E/1872*, and *-a15/E/312*. In contrast, *atpp2-a10/P/-157*, *a11/P/-394*, *a12/P/-293*, *a14/P/-320* performed as WT in the expression of the corresponding *AtPP2-A* genes. In *atpp2-a12/P/-293* and *atpp2-a12/P/-293*, T-DNA insert did not disrupt any DNA regulatory motifs present in *AtPP2-A11* and *AtPP2-A12* promoters. In *atpp2-a10/P/-157*, T-DNA insert disrupted the pollen-specific transcription activator element AGAAA (#S000245) [40,41] located between -159 and -155 in the *AtPP2-A10* sequence. In *atpp2-a11/P/-394*, the MYB recognition site TGGTTT (#S000408) [42] located between -398 and -393 in the *AtPP2-A11* sequence was disjoined by T-DNA insertion. However, both mutations did not affect basal expression of the genes (Figure 3c). In the ten mutants, therefore, only *atpp2-a1/E/142* represents an effective mutation allele, which may be responsible for a transcriptional stop of *AtPP2-A1* in the plant and result in experimental compromises in both the basal expression and HrpN<sub>Ea</sub>-induced expression of the gene.

The ten *AtPP2-A*-modified mutants were compared with the WT plant in terms of colonization and feeding by aphids. Based on monitoring of large-scale populations of apterous and agamic *M. persicae* females (1,200 aphids/treatment/plant genotype), the insect colonies on leaves of *atpp2-a1/E/142* were stable, shown as a smaller rate of the population decrease in 24 hours, than those on WT and the other nine mutants (Figure 4a; ANOVA test, *p* < 0.01). In *atpp2-a1/E/142*, the deterrent effect of HrpN<sub>Ea</sub> on colonization by the insect was little, but the effect was evident in the other mutants as in WT (Figure 4a). Based on the four-hour EPG record, total durations of nonpuncturing and pathway phases had little and insignificant differences between WT and *atpp2-a1/E/142* under the same condition, HrpN<sub>Ea</sub> treatment or control (Table 3). Then, the four-hour EPG record of aphid feeding from leaves was analyzed to particularly calculate total duration of the phloem phase (Figure 4b), which well reflected HrpN<sub>Ea</sub>-induced repression in aphid feeding from the WT phloem (Table 1). Apparently, aphids preferred to feed from *atpp2-a1/*

**Table 2 Information on *AtPP2-A*-defected *Arabidopsis* mutants investigated in this study**

Gene name	Locus no.	Mutant name	T-DNA insertion site	Mutant seed stock no. <sup>a</sup>	TAIR <sup>b</sup> annotations
<i>AtPP2-A1</i>	AT4G19840	<i>atpp2-a1/E/142</i>	Exon, 142	CS837256	T-DNA insertion lines; a modified approach of thermal asymmetric interlaced-PCR was used to amplify DNA fragments flanking the T-DNA left border from the transformed lines; no phenotype information available at this time.
<i>AtPP2-A11</i>	AT1G63090	<i>atpp2-a1/P/-394</i>	Promoter, -394	CS842726	
<i>AtPP2-A1</i>	AT4G19840	<i>atpp2-a1/P/-210</i>	Promoter, -210	SALK_080914C	Sequence-indexed T-DNA insertion lines; presence of the insertion was analyzed by PCR; kanamycin resistance gene may be silenced; PCR- or hybridization-based segregation analysis is required to confirm presence and homozygosity of insertion; may be segregating for phenotypes that are not linked to the insertion; may have additional insertions potentially segregating; no phenotype information available at this time.
<i>AtPP2-A10</i>	AT1G10155	<i>atpp2-a10/P/-157</i>	Promoter, -157	SALK_107807C	
<i>AtPP2-A3</i>	AT2G26820	<i>atpp2-a3/I/1650</i>	Intron, 1650	SALK_005443C	
<i>AtPP2-A11</i>	AT1G63090	<i>atpp2-a11/E/117</i>	Exon, 117	SALK_080546	
<i>AtPP2-A12</i>	AT1G12710	<i>atpp2-a12/P/-293</i>	Promoter, -293	SALK_015774	
<i>AtPP2-A13</i>	AT3G61060	<i>atpp2-a13/E/1872</i>	Exon, 1872	SALK_046907	
<i>AtPP2-A14</i>	AT5G52120	<i>atpp2-a14/P/-320</i>	Promoter, -320	SALK_066553	
<i>AtPP2-A15</i>	AT3G53000	<i>atpp2-a1/E/312</i>	Exon, 312	SALK_022649	

<sup>a</sup>Distribution seeds of *atpp2-a1/P/-210*, *atpp2-a10/P/-157* and *atpp2-a3/I/1650* are from confirmed lines and T2 or T3 generation for the other mutants.

<sup>b</sup>TAIR, The Arabidopsis Information Resource <http://www.arabidopsis.org> databases.

E/142 (Figure 4c). In the mutant, total duration of the phloem phase in 4 hours was much longer than that in the other mutants and WT as well (Figure 4b; Table 3). Both the second and fourth hour of the EPG record indicated significant deterrent effect of the HrpN<sub>Ea</sub> treatment on aphid feeding from the WT phloem (Table 1), but the deterrent effect was lost in *atpp2-a1/E/142* (Figure 4c; Table 3). Duration of the phloem phase in the second-hour EPG was much shorter in WT plants treated with HrpN<sub>Ea</sub> vs. EVP, but the duration was close in *atpp2-a1/E/142* in despite of treatments (Figure 4c; Table 3). These results suggest that *atpp2-a1/E/142* pampers *M. persicae* in phloem feeding and that *AtPP2-A1* plays a role in HrpN<sub>Ea</sub>-induced repression of the phloem-feeding activity.

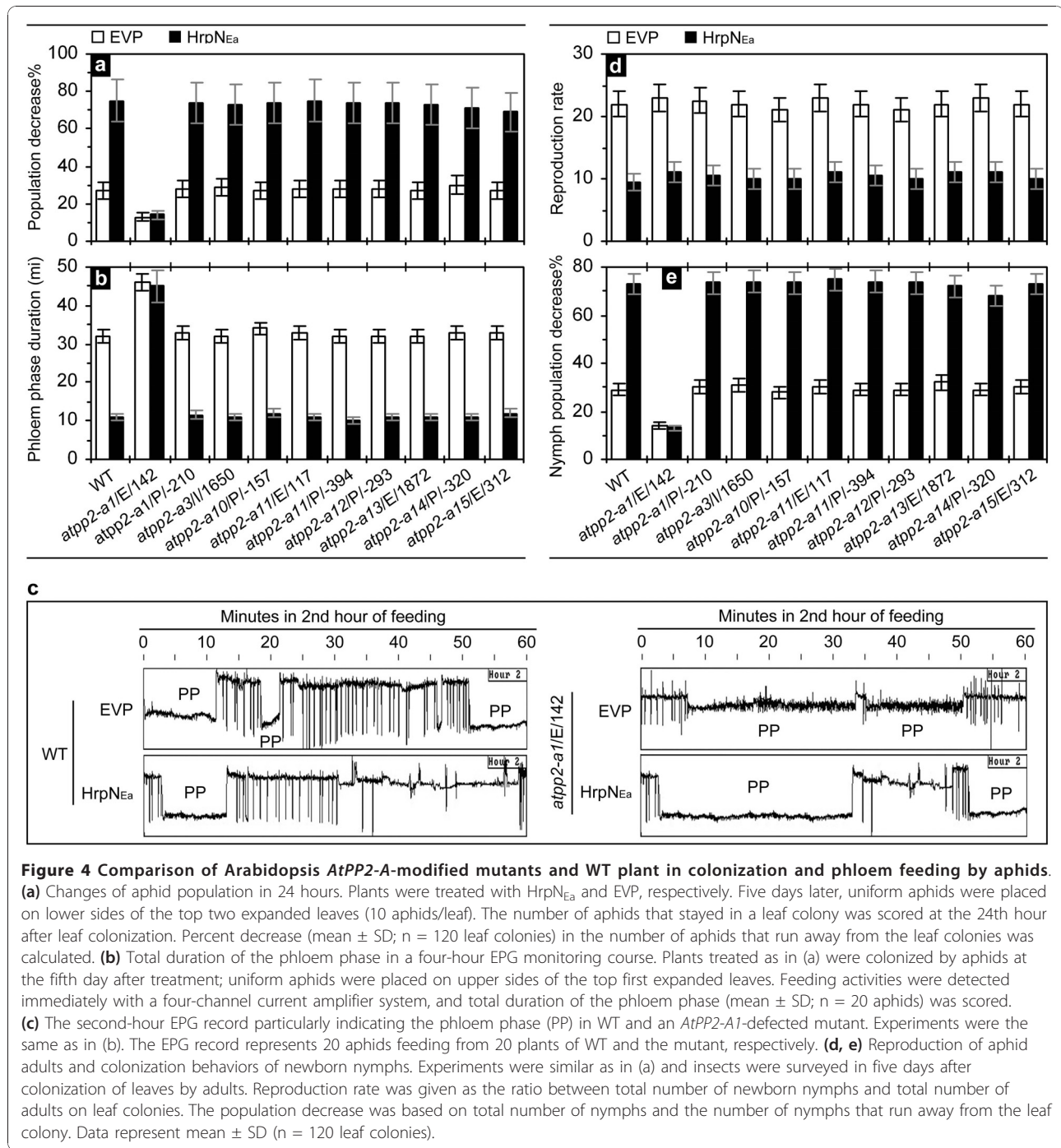
To gain information about the general function of *AtPP2-A1* in Arabidopsis resistance to *M. persicae*, we compared *atpp2-a1/E/142* with the other nine mutants and with WT as well in the effects on multiplication of the insect and subsequent nymph activities. The reproduction rate was scored as the ratio between total numbers of newborn nymphs and total numbers of aphid adults that stayed on leaves in five days after colonization. As shown in Figure 4d, reproduction rates were much smaller under the condition of HrpN<sub>Ea</sub> treatment vs.

control (Student's *t*-test,  $p < 0.01$ ) irrespective of the plant genotypes, suggesting that HrpN<sub>Ea</sub>-induced repression of *M. persicae* multiplication [4] was not related to the *AtPP2-A1* gene. The gene, however, showed a repressive effect on plant colonization by newborn nymphs. Nymph colonies were more stable on *atpp2-a1/E/142* with a smaller proportion of the population decrease than the other mutants or WT (Figure 4e; ANOVA test,  $p < 0.01$ ). In *atpp2-a1/E/142*, the deterrent effect of HrpN<sub>Ea</sub> on colonization by nymphs was little, but the effect was evident in the other mutants as in the WT plant (Figure 4e). Evidently, *AtPP2-A1* does not affect aphid reproduction, but instead, the gene plays a role in repressing plant colonization by nymphs as by adults.

#### ***AtPP2-A1*-overexpression confers repressed phloem feeding of *M. persicae***

The *AtPP2-A1* gene was cloned into the binary vector pBI121 under control by the cauliflower mosaic virus 35S promoter (35S), creating *pBI121::35S::AtPP2-A1* (Figure 5a). Transformation of WT Arabidopsis with the recombinant unit generated PP2OETat (*AtPP2-A1*-overexpression transgenic *A. thaliana*) plants. Ten PP2OETat lines were selected and designated as PP2OETat1 through PP2OETat10 according to *AtPP2-A1* expression levels





(Figure 5b). Transformation of the WT plant with the empty pBI121 vector, containing neither *uidA* nor *AtPP2-A1*, generated the transgenic control plant, which behaved as WT in all the tests (Figure 5b-5d). Also, WT, transgenic control and PP2OETat plants did not have evident differences in morphology. Homozygous T3 progenies of the PP2OETat lines were compared the WT and transgenic

control plants in *AtPP2-A1* expression and in colonization and feeding by apterous *M. persicae* females.

Real-time RT-PCR was conducted with RNA samples from leaves and primers specific to *AtPP2-A1*. As shown in Figure 5b, levels of the *AtPP2-A1* transcript varied with the different PP2OETat lines, and levels of the transcript were greater in all the PP2OETat lines than

**Table 3 Four-hour EPG analysis of aphid feeding from WT Arabidopsis and the *atpp2-a1/E/142* mutant**

Activity examined	WT group			<i>atpp2-a1/E/142</i> group		
	EVP treatment mean (SD)	HrpN <sub>Ea</sub> treatment mean (SD)	Student's <i>t</i> -test (n = 20)	EVP treatment mean (SD)	HrpN <sub>Ea</sub> treatment (SD)	Student's <i>t</i> -test (n = 20)
Total duration of nonpuncturing, min	21.1 (4.8)	18.9 (3.5)	$p > 0.05$	31.4 (8.3)	28.5 (6.4)	*
Duration of pathway phase, min	175.0 (50.5)	201.5 (58.6)	$p < 0.05^*$	160.0 (42.0)	162.5 (45.5)	*
Total duration of phloem phase, min	43.9 (6.3)	19.6 (3.9)	$p < 0.005^*$	48.6 (9.2)	49.0 (11.5)	*

\*Insignificant difference at  $p < 0.05$ .

the transgenic control plant. Compared with the transgenic control plant, PP2OETAt lines seemed more resistant to colonization and feeding by aphids. Smaller populations of aphids were able to stay for 24 hours on leaf colonies of PP2OETAt than the transgenic control plant (Figure 5c). Consistently, aphids preferred to feed from the transgenic control plant rather than PP2OETAt (Figure 5d). Total duration of the phloem phase in the four-hour EPG record was much shorter in PP2OETAt than in the control plant (Figure 5d).

Based on statistical analyses (ANOVA test,  $p < 0.01$ ), the ten PP2OETAt lines differed significantly from the transgenic control plant in levels of *AtPP2-A1* expression (Figure 5b), the number of aphids that were able to stay for 24 hours on leaf colonies (Figure 5c), and durations of the phloem phase (Figure 5d). In the ten PP2OETAt lines, the number of aphids that were able to stay for 24 hours on leaf colonies was increased (Figure 5c), but durations of the phloem phase was decreased (Figure 5d), with increases in levels of *AtPP2-A1* expression (Figure 5b). The PP2OETAt1 line showed as the greatest expresser of *AtPP2-A1* and the greatest repressor of colonization and feeding by *M. persicae*. In addition, a greater repression of phloem feeding by aphids was observed in the presence than the absence of HrpN<sub>Ea</sub> treatment (not shown), suggesting that original and introduced versions of the *AtPP2-A1* gene might be able coordinate their functions and might function simultaneously, in PP2OXTA1.

#### ***AtPP2-A1* expression in different organs of PP2OETAt1 is consistent with repression of phloem feeding by *M. persicae***

PP2OETAt1 was further investigated in the genomic integration of the introduced *AtPP2-A1* gene, organ specificity of the gene expression, and the effect of *M. persicae* feeding from the phloem. The Southern blot of specifically ingested genomic DNA hybridized with the *AtPP2-A1*-specific probe revealed that the introduced *AtPP2-A1* gene had been integrated into the genome and existed as a double copy in PP2OETAt1 (Figure 6a). Overexpression of the gene was confirmed by northern

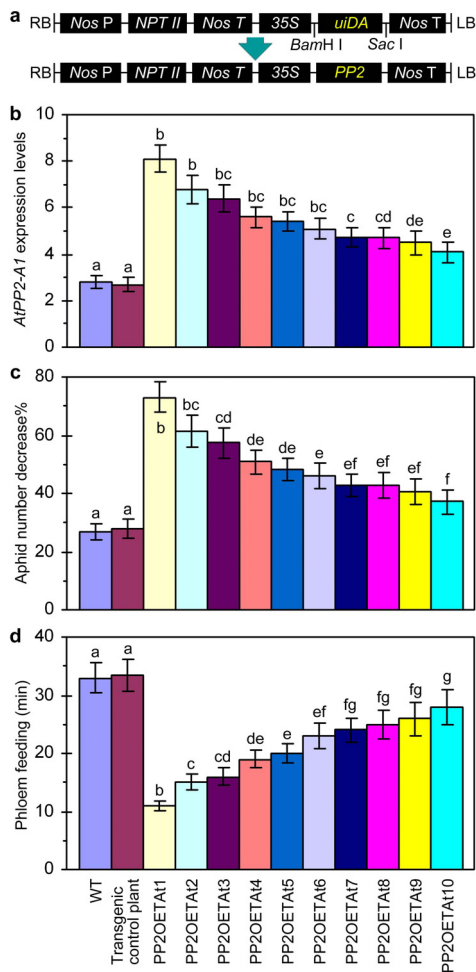
blot of leaf RNA samples hybridized with the probe specific to *AtPP2-A1* (Figure 6b).

Real-time RT-PCR analyses revealed that *AtPP2-A1* expression varied greatly in different organs of PP2OETAt1. The expression of *AtPP2-A1* was conspicuous in leaves, stems, calyces, and petals but little transcript was detected from flower stalks (Figure 6c). Amounts of the *AtPP2-A1* transcript were much greater in leaves, stems, calyces, and petals of PP2OETAt1 than the transgenic control plant. However, close amounts of the transcript were detected from flower stalks of both plants. This result suggested the overexpression of *AtPP2-A1* in all the organs except flower stalks of PP2OETAt1. Levels of the gene overexpression were higher in leaves, calyces, and petals compared with stems (Figure 6c; ANOVA test,  $p < 0.01$ ).

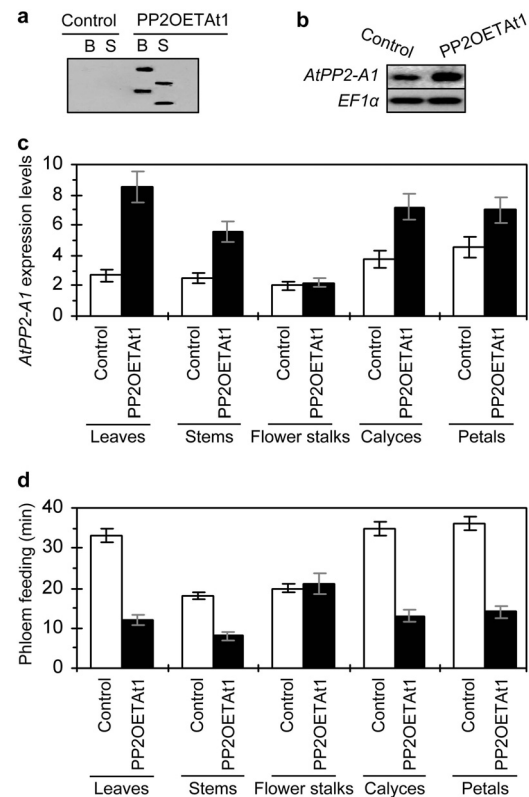
The organ-differential levels of *AtPP2-A1* overexpression were negatively correlated with the extents by which apterous agamic *M. persicae* females fed from the different organs. Based on total duration of the phloem phase in the four-hour EPG record (Figure 6d), aphids preferred to feed from leaves, calyces, and petals, but aphids were also able to feed from stems and flower stalks. However, durations of the phloem phase were much shorter when aphids were feeding from leaves, stems, calyces, and petals of PP2OETAt1 compared with the transgenic control plant (Student's *t*-test,  $p < 0.01$ ), suggesting that the phloem-feeding activity was repressed in the different organs of PP2OETAt1. Inversely, the phloem phase of aphid feeding from the PP2OETAt1 flower stalk lasted as longer as feeding from the same organ of the transgenic control plant (Figure 6d), suggesting that aphids did not have a preference between both plants in feeding from flower stalks.

#### **Expression of *AtPP2-A1* promoter-GUS is organ-unspecific**

Because the introduced copies of *AtPP2-A1* (Figure 6a) are under direction by 35S (Figure 5a), the organ-differential expression in PP2OETAt1 (Figure 6c) does not offer significant information about organ specificity of the gene expression. Lack of the organ specificity was



**Figure 5 Genetic construction used in generation of PP2OETAt (*AtPP2-A1*-overexpression transgenic *Arabidopsis thaliana*) and comparison of PP2OETAt and control plants in *AtPP2-A1* expression and aphid activities on leaves. (a) The construct. The *AtPP2-A1* (*PP2*) gene was inserted into the binary vector pBI121 at the *Bam*H I and *Sac* I restriction sites to replace *uidA*, a reporter gene encoding  $\beta$ -D-glucuronidase. *Nos P*, promoter from the nopaline synthase-encoding gene (*Nos*); *NPT II*, kanamycin resistance gene; *Nos T*, *Nos* transcription terminator; *35S*, the cauliflower mosaic virus 35S promoter. (b-e) Experiments were done with 35-day-old plants. Different letter labels in histograms indicate significant differences (ANOVA test,  $p < 0.01$ ). (b) Real-time RT-PCR analysis of *AtPP2-A1* expression in leaves. The gene transcript was quantified as mean  $\pm$  SD ( $n = 3$  repeats) relative to reference genes (*EF1 $\alpha$*  and *ACTIN2*) and normalized to the null-template control. (c) Changes of aphid population in 24 hours. Uniform aphids were placed on lower sides of the top two expanded leaves (10 aphids/leaf). Leaf colonies were surveyed, the number of aphids that stayed in a leaf colony was scored, and percent decrease (mean  $\pm$  SD;  $n = 120$  leaf colonies) in the number of aphids that run away from the leaf colonies was calculated. (d) Total duration of the phloem phase in a four-hour EPG monitoring course. Uniform aphids were placed on upper sides of the top first expanded leaves. Feeding activities were detected immediately with a four-channel current amplifier system, and total duration of the phloem phase (mean  $\pm$  SD;  $n = 20$  aphids) was scored.**



**Figure 6 Comparison of PP2OETAt1 and transgenic control plants in organ-unspecific *AtPP2-A1* expression and effects on colonization and phloem feeding by aphids. (a, b)** In the experiments, PP2OETAt1 was compared with the transgenic control plant (Control); 35-day-old plants grown in long day were investigated. (a) Southern blot hybridized with the *AtPP2-A1*-specific probe. Prior to blotting, the genomic DNA had been digested with the restriction enzymes *Bam*H I (B) and *Sac* I (S). (b) Northern blots hybridized with probes specific to *AtPP2-A1* and the reference gene *EF1 $\alpha$* . (c) Real-time RT-PCR analysis of *AtPP2-A1* expression in the different organs of the plants. The gene transcript was quantified as mean  $\pm$  SD ( $n = 3$  repeats) relative to reference genes and normalized to null-template controls. (d) Total duration of the phloem phase in a four-hour EPG monitoring course. Uniform aphids were placed on the indicated organs. Feeding activities were detected immediately with an EPG monitoring system, and total duration of the phloem phase (mean  $\pm$  SD;  $n = 20$  aphids) was scored.

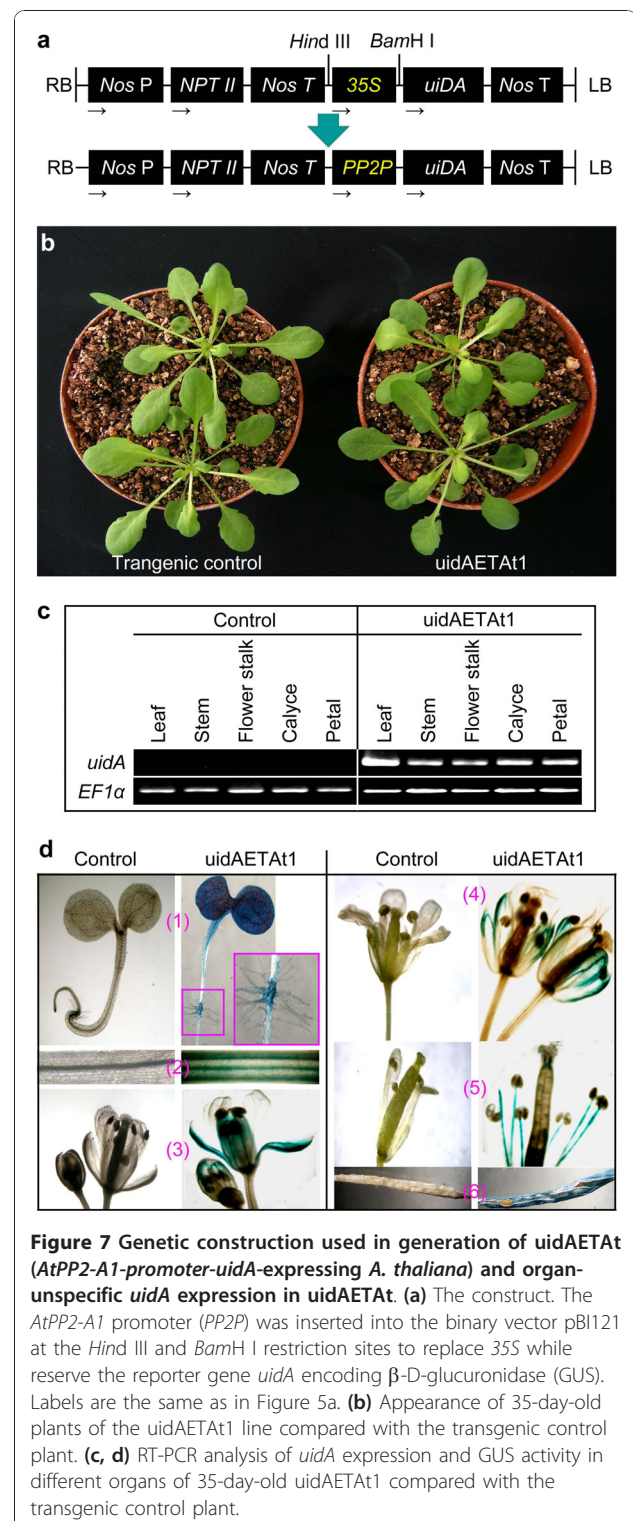
indicated by the transcript detected from different organs of the transgenic control plant (Figure 6c). In an experimental design to test whether the organ-unspecific *AtPP2-A1* expression was related with activity of the *AtPP2-A1* promoter, the promoter placed in front of the *uidA* reporter gene (Figure 7a) was able to drive the gene expression in the *uidA*ETAt (*uidA*-expressing transgenic *A. thaliana*) plant (Figure 7b). Six *uidA*ETAt lines were observed. They seemed to resemble each other closely and were also similar to the transgenic control plant (Figure 7b; *uidA*ETAt1 as a representative

line). In the uidAETAt1 line, *uidA* was expressed markedly in the stem, flower stalk, calyx, and petal, whereas, stronger expression was found in the leaf (Figure 7c). The *uidA* gene encodes  $\beta$ -glucuronidase (GUS) enzyme [43]. GUS activity was detected in the root, stem, calyx, and petal of PP2OETA1 (Figure 7d). GUS activity was not found in the flower stalk (Figure 7d), possibly due to no *uidA* expression or little GUS beyond detectable level. In addition, uidAETAt1 showed as tolerant as the transgenic control plant to phloem feeding by *M. persicae* (Table 4). This result indirectly suggests that *AtPP2-A1* plays a role, only when expressed itself, in repressing the phloem-feeding activity.

### Discussion

Although harpin proteins and their functions as proteinaceous elicitors in eliciting plant defense responses have been found for decades [1-3], many aspects of the mechanisms that underlie harpin-induced defenses remain unclear. Important questions include, for example, how a harpin protein as an exogenous signal is perceived by plants and how the signal perception is connected to a transducer to trigger a cellular pathway. Great attentions have been paid to plant signal transduction in harpin-induced resistance to pathogens [3,6,30,31,37,44] and insect herbivores [2,4,8,34]. We have used HrpN<sub>Ea</sub>, the first-characterized [1] and well-studied harpin [2-7], as a model of proteinaceous elicitors to characterize induced resistance against insect herbivores [2,4,8,34], particularly the green peach aphid *M. persicae*, a generalist phloem-feeding insect [10]. It has been shown that the HrpN<sub>Ea</sub> treatment and *M. persicae* infestation have some overlapping effects on the induction of plant responses [4,25,30-34], especially the PBD mechanism that is suggested to involve the lectin-type phloem protein PP2 [23,24] as a component [14-16,25,26]. Although 30 members of the PP2 multigene family have been identified in Arabidopsis [23] and *AtPP2*-modified Arabidopsis mutants generated [27], little is known about biological effects, especially on resistance to insects, of the genes and mutants <http://www.arabidopsis.org>. The purpose of this study is to elucidate the function of *AtPP2-A1* in resistance to *M. persicae* in Arabidopsis plants when treated with HrpN<sub>Ea</sub> and under the condition of *AtPP2-A1* overexpression.

We show that the treatment of Arabidopsis with HrpN<sub>Ea</sub> induces a repression in *M. persicae* feeding from the plant phloem (Figure 1; Table 1) and colonization of plants by the insect (Figure 2). Based on the EPG patterns, applying HrpN<sub>Ea</sub> to WT Arabidopsis impedes aphids in stylet puncturing of the plant cell, en route to the vascular tissue while looking for the phloem, and, especially, in the phloem-feeding activity. So the HrpN<sub>Ea</sub> treatment is likely to induce changes in cell wall properties unfavorable to



aphid feeding, but this notion remains to be examined. HrpN<sub>Ea</sub>-induced deterrent effect on the phloem-feeding activity has been found in the EPG data analyzed either by hour or based on the four-hour record as a whole (Table 1). The phloem-feeding activity could be reflected



**Table 4 Analysis of major activities of aphid feeding from uidAETAt (*uidA*-expressing transgenic *Arabidopsis thaliana*) and transgenic control plants**

Activity examined	Control plant (SD)	uidATEAt1 (SD)	Student's t-test (n = 20)
Total duration of nonpuncturing, min	23.5 (5.2)	21.9 (4.5)	*
Duration of pathway phase, min	181.8 (56.0)	182.6 (61.6)	*
Total duration of phloem phase, min	34.7 (5.5)	35.5 (5.6)	*

\*Insignificant difference at  $p < 0.05$ .

in the EPG by duration of the phloem phase composed of E1 and E2 salivations (Figure 1), which are essential for ingestion of the phloem sap [13,16,17,35]. Shortened duration of the phloem phase, in both E1 and E2 salivations (Table 1), suggests that the insect's effort in ingestion of the phloem sap is repressed under the HrpN<sub>Ea</sub> treatment condition compared with control. It is also pertinent to propose that the HrpN<sub>Ea</sub> treatment impacts the insect-plant interaction. In terms of the insect, E1 and E2 saliva are believed to prevent protein clogging inside the sieve element and prevent phloem proteins from clogging inside the capillary food canal [13,16], respectively. In the plant side, phloem protein plugging of the sieve element presumably serves as a physical barrier to aphid feeding from the phloem [26]. The lectin-type phloem protein PP2 [23,24] is supposed to play a role in plant response to the feeding stress [21,25,26].

Molecular and genetic evidence supports a role of *Arabidopsis PP2* gene *AtPP2-A1* in HrpN<sub>Ea</sub>-induced repression of *M. persicae* feeding from the plant phloem. RT-PCR analyses (Figure 3) suggest that *AtPP2-A1* is the most HrpN<sub>Ea</sub>-responsive gene of 30 members of the *PP2* multigene family [23]. PLACE Web Signal Scan [39] provides a clue to molecular basis of HrpN<sub>Ea</sub> response and the *AtPP2-A1* induction as well. For example, the gene promoter contains three copies of the consensus GT-1 binding box GA/GA/TAAA/T (#S000508) [45]. This element is involved in the regulation of salicylic acid signaling [45,46], which otherwise can be activated by HrpN<sub>Ea</sub> treatment in *Arabidopsis* [3]. Moreover, previous studies have shown that HrpN<sub>Ea</sub>-induced resistance to *M. persicae* is regulated by the ethylene signaling pathway [4], which essentially involves perception of the ethylene signal by the receptor ETR1, the signal transduction to the integral membrane protein EIN2 [4], and the regulation of ethylene responsive factors (ERFs) [47,48]. The W-box TGACC/T (#S000457) present in the *AtPP2-A1* promoter has been shown as required for wounding-induced activation of the *ERF3* gene [47]. The *ERF3* protein is a regulator of ethylene signaling [48], which otherwise is activated to regulate induced resistance to *M. persicae* in *Arabidopsis* plants responding to HrpN<sub>Ea</sub> treatment [4]. Thus, *AtPP2-A1* is pertinently thought a part of the signaling pathway that is required for HrpN<sub>Ea</sub> response, at least during induction of the plant resistance to *M.*

*persicae* [4]. A role of *AtPP2-A1* in the induced resistance has been elucidated by evidence obtained from investigating ten mutants (Table 2) in comparison with the WT plant. The investigation demonstrates that deterrent effect of the HrpN<sub>Ea</sub> treatment on aphid feeding from the phloem requires a functional *AtPP2-A1* gene in the plant (Figure 4). This notion is especially supported by the absence of HrpN<sub>Ea</sub>-induced repression of the phloem-feeding activity in the *atpp2-a1/E/142* mutant (Figure 4; Table 3). This result offers a novel angle to further understanding on the PBD mechanism. Previously, this defensive mechanism was known as a result of plant responses to attacks by phloem-feeding insects [14,15,20-22] and other stresses, such as wounding [16,21,22,26]. Now, the PBD mechanism is known to occur as a result of plant response to HrpN<sub>Ea</sub>, a proteinaceous elicitor of plant defenses [1-7,49]. This notion, however, remains to be examined in regard to how *AtPP2-A1* contributes to PBD in response to the HrpN<sub>Ea</sub> treatment.

The function of *AtPP2-A1* in conferring repression of the phloem-feeding activity is further supported by evidence obtained from investigating PP2OETAt (*AtPP2-A1*-overexpression transgenic *A. thaliana*) plants (Figure 5). Levels of *AtPP2-A1* expression are significantly greater in the 10 tested PP2OETAt lines than in the transgenic control plant, conforming to the experimental design for the gene overexpression. In the different PP2OETAt lines, durations of the phloem phase are decreased with increases in levels of *AtPP2-A1* expression, suggesting that *AtPP2-A1* overexpression confers a repression in the phloem-feeding activity of *M. persicae*. These observations also identify the PP2OETAt1 line as the greatest expresser of *AtPP2-A1* and the greatest repressor of colonization and feeding by the insect. Reminiscently of cell-to-cell *PP2* RNA movement in cucurbits [50] and distant phloem transport flowering signals [51], little amount of the *AtPP2-A1* transcript in flower stalks (Figure 6) may result from organ-to-organ trafficking to fulfill the requirement for flower development. In the other organs, however, *AtPP2-A1* expression is consistent with the repression of aphid feeding from the organs (Figure 6). The organ-unspecific feature of *AtPP2-A1* expression and function is also suggested indirectly by investigating the transgenic plant uidAETAt1. In the

plant, *uidA* expression under direction of the *AtPP2-A1* promoter is found in various organs (Figure 7), but these organs do not have a repressive effect on aphid feeding (Table 4). This result indirectly supported that *AtPP2-A1* plays a role, only when expressed itself, in repression of the phloem-feeding activity. Consistent to our observations on *uidAETAt1*, a previous study detected GUS activity in different organs of transgenic plants that expressed *uidA* under direction by the *AtPP2-A* promoter [23]. In the present study, both *uidA* transcript and GUS activity were detected in different organs of *uidAETAt1* (Figure 7). Due to our failure in obtaining transgenic plants that had been designed to express *AtPP2-A1-uidA* under control by the *AtPP2-A1* promoter, now we can not provide more convincing evidence for coincident organ localization in *AtPP2-A1* expression and aphid feeding repression. However, repression of the phloem-feeding activity seems a consistent attribute of the different PP2OETAt lines (Figure 5) and a consistent attribute of the different organs of PP2OETAt1 (Figure 6) as well, owing to *AtPP2-A1* overexpression in both cases. In the case of PP2OETAt1, whenever the level of *AtPP2-A1* expression is greater in an organ than in the others, aphid feeding from the organ incurs a stronger repression (Figure 6). These observations offer a convincing support for the function of *AtPP2-A1* in conferring the plant resistance shown as a repression in phloem-feeding activity of the insect. The results also indicate a defensive significance of ubiquitous organ-unspecific expression of *PP2* genes in plants demonstrated previously [23] and observed in this study (Figure 7).

The contribution of lectin-type phloem proteins, such as PP2, to the PBD mechanism is believed owing to their functions as a physical barrier that prevents insects from phloem feeding [26]. A preceding event is the formation of the PP1-PP2 complex, which, however, has been demonstrated only in cucurbits, whereas, other plant families do not have any PP1-like protein [52]. The role of PP1-PP2 aggregation in the clogging of sieve plates has been the matter of long standing debates that have not yet been solved, and still remains a hypothesis that is beyond elucidating scopes of the present study. Lectin-type phloem proteins take only a small proportion of phloem sap proteins that have potential of defensive significance in plants under attacks by phloem-feeding insects [21]. Thus, lectin-type phloem proteins are only one of different PBD components and are not likely to play an entire role in plant resistance against attacks by the insects [14-21]. Subtle differences in aphid population, the insect escape from leaf colonies, for example, between HrpN<sub>Ea</sub>-treated plants and control plants (Figure 2), between *atpp2-a1/E/142* and WT (Figure 4), and between PP2OETAt and transgenic control plants

(Figure 5), also imply components alternative to *AtPP2-A1* in impacting aphid behaviors while colonizing the plants. Alternative defense components are further indicated by HrpN<sub>Ea</sub>-induced impediments to aphid feeding activities observed in the first-hour EPG monitoring (Table 1). However, we do not have evidence yet to show a proportion of *AtPP2-A1*'s contribution to resistance against *M. persicae* in Arabidopsis plants either when treated with HrpN<sub>Ea</sub> or under the condition of *AtPP2-A1* overexpression.

Moreover, *AtPP2-A1* is a member of the *PP2* multi-gene family [23,27] and *atpp2-a1/E/142* is one of *AtPP2* mutation alleles in Arabidopsis [24]. The other *AtPP2* genes and *AtPP2*-modified mutants seem not involved in HrpN<sub>Ea</sub>-induced repression of aphid feeding from the phloem (Figure 4). This result suggests that different members of the *PP2* multigene family may have different functions in the plant. So far, *AtPP2-A1* is the only phloem protein demonstrated as a lectin with the ability to bind N-acetylglucosamine oligomers, and recombinant *AtPP2-A1* has been shown to affect weight gain in *M. persicae* nymphs in an artificial diet [24]. The induction of *AtPP2-A1* may be an indirect effect of the HrpN<sub>Ea</sub> treatment, which is multifunctional, inducing plant growth enhancement [4], resistance to pathogens [3], insects [4] and drought stress [5], and resistance-associated cell death [1,6]. These multiple effects have been determined separately; and so whether they are simultaneous is unclear. It is also unclear if other *PP2* genes affect plant defenses rather than resistance. In several species of angiosperms, including Arabidopsis, different *PP2* genes are expressed in various organs during plant growth and development [23]. The ubiquitous organ-unspecific *PP2* expression suggests that different *PP2* genes may fulfill distinct functions at a special stage of plant growth and development. It is possible that a particular *PP2* gene may have different functions depending on plant growth and development processes or depending on an immediate requirement for encountering with distinct challenges, such as attacks by insects and infection by pathogens. Studies to test this hypothesis represent an interesting avenue for further research.

## Conclusions

The HrpN<sub>Ea</sub> treatment has a deterrent effect on the phloem-feeding activity of *M. persicae* and the deterrent effect occurs in WT Arabidopsis rather than the *atpp2-a1/E/142* mutant. The phloem-feeding activity can be also repressed as a result of *AtPP2-A1* overexpression. Both sets of evidence support the conclusion that *AtPP2-A1* plays a role in Arabidopsis resistance to the insect, particularly at the phloem-feeding stage. The accompanied change of aphid population in leaf colonies suggests that the function of *AtPP2-A1* is related to

colonization of the plant and may have a broader importance for the plant-insect interaction.

## Methods

### Plant growth and treatment

*Arabidopsis* genotypes used in this study included the ecotype Col-0, transgenic plants created in this study, and *AtPP2-A* sequence-indexed T-DNA insertion lines generated previously (Table 2). Both types of modified plants were created under the background of Col-0 and characterized as homozygous at the transgene and T-DNA insertion loci, respectively, before use in the experiments. Plants were grown in 9-cm pots, 1 plant/pot for the EPG monitoring and 5 plants/pot for other experiments, under 22°C and 250  $\mu\text{E}/\text{m}^2/\text{s}$  illumination [53]. A long day (16-h light/8-h dark) photoperiod was applied to plants for transformation and *AtPP2-A1* expression in different organs, and short day (with 12-h light/12-h dark) was used in other experiments. Plants grown in short day were used at different stages of growth and development depending on experimental purposes. Transgenic plants were used in different experiments since the 35th day after planting. Thirty-day-old plants of the WT and mutants were treated with EVP and HrpN<sub>Ea</sub>, respectively. EVP and HrpN<sub>Ea</sub> were prepared [1,6] as 10  $\mu\text{g}/\text{ml}$  aqueous solutions and were applied in the presence of surfactant Silwet-77 (0.02%) by spraying plant tops with a low-pressure atomizer. Treated plants were used at 5 dpt in monitoring of aphid behaviors, and were used at 0 and 24 hpt in determination of gene expression.

### Aphid culture

A single isolate of *M. persicae* was collected from the field-grown radish (*Raphanus sativus* L.) near Nanjing in China. A clone of apterous agamic females was obtained by acclimatization in WT *Arabidopsis* grown in the chamber (22°C; 250  $\mu\text{E}/\text{m}^2/\text{s}$ ; short day). The colony was maintained in nursery WT *Arabidopsis* seedlings and was transferred to fresh plants every two weeks. Uniform ten-day-old aphids were used in this study and were transferred to experimental plants with a fine paintbrush.

### Aphid feeding behavior

Aphid feeding activities were observed by the EPG technique using the Giga Amplifier system (Laboratory of Entomology, Wageningen Agricultural University, Wageningen, The Netherlands; <http://www.epgsystems.eu/systems.htm>). Uniform ten-day-old aphids were placed on upper side of the top first expanded leaves of plants. For each genotype of the plant or each combination of a genotype and treatment (with EVP or HrpN<sub>Ea</sub>), 20 aphids placed on 20 plants were monitored in five repetitions of

experiments. Immediately after aphids were placed on leaves, a 20-mm diameter gold wire was attached to the dorsal surface of each aphid's abdomen using silver conductive paint. The other end of the wire was connected to a four-channel Giga-4 direct current amplifier with four channels and  $10^9\text{-}\Omega$  input resistance in an electrical circuit that is also connected to the plant via an electrode placed in the soil. The behavior of individual aphids was monitored for 4 hours. Voltage waveforms were digitized at 100 Hz with an A/D converter USB device. Waveform patterns were identified according to previously described categories [35]. Waveform recordings were dissected each 5 second with the EPG analysis software STYLET 2.5 installed in a computer connected to Giga-4 direct current amplifier.

### Plant colonization

Uniform ten-day-old aphids were placed on the lower sides of the top two expanded leaves of plants; 10 aphids per leaf. A total of 1,200 aphids were monitored in four repetitions of the experiments for each single recombination of a treatment and a plant genotype. In each experimental repetition, 300 aphids were placed on 30 leaves of 15 plants treated specifically. Aphid movement from leaf colonies was monitored for five days, and the number of aphids in a leaf colony was scored at intervals in 24 hours. Aphid reproduction was surveyed twice a day, and in each survey, newborn nymphs were counted. Reproduction rate was quantified as the ratio between total numbers of nymphs produced in five days and total numbers of aphid adults that stayed in leaf colonies during the same period. Nymphs produced in five days were also monitored; the number of nymphs that run away from leaf colonies was accounted.

### Determination of gene expression in plants

Total RNA was isolated from leaves of EVP-treated plants and HrpN<sub>Ea</sub>-treated WT plants, and was isolated from leaves, stems, flower stalks, calyces, and petals of transgenic plants. Gene expression was determined by northern blot hybridization [31] and RT-PCR or real-time RT-PCR [54] as described previously. Northern blots of leaf RNA samples were hybridized to a digoxigenin-labeled *AtPP2-A1* probe prepared using the DIG Nucleic Acid Detection Kit [Roche Diagnostics (Shanghai) Trading Co., LTD]. An established quantitative method [55] was adopted in real-time RT-PCR using *ACTIN2* and *EF1 $\alpha$*  as reference genes [54,56]. Genes were amplified <26 cycles with a range of template concentration increases by 0.5 ng and from 0 to 3.0 ng in 25  $\mu\text{l}$  reaction solutions to select desired doses. Reaction treatments, RT-PCR protocols, product cloning and sequencing verification were performed as described [5,6]. The 25  $\mu\text{l}$  reaction mixture was composed of 1  $\mu\text{l}$

first-strand cDNA diluted 1:10, 2.5  $\mu$ M primer and 1 $\times$ SYBR Premix Ex Taq (TaKaRa Biotech. Co., Ltd, Dalian, China). All reactions were performed in triplicate with null-template controls in which cDNA was absent. PCR cycling was: 95°C for 3 min, followed by 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Average expression levels of the genes were normalized to the null-template controls. Average level of the *AtPP2-A1* transcript was quantified relative to *EF1 $\alpha$*  and *ACTIN2*. The expression of *uidA* in different organs

of the *uidA*TEAt plant was determined by RT-PCR using the superscript II RNase H<sup>-</sup> Reverse Transcriptase (Invitrogen Biotech. Shanghai Trading Co., LTD). Primers and related information are provided in Table 5.

#### Mutant screening

Information on sequence-indexed T-DNA insertion Arabidopsis mutants tested in this study (Table 2) was from The Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org>) seed stock database. Mutant seeds

**Table 5 Information on genes analyzed by reverse transcriptase-polymerase chain reaction in this study**

Gene	Locus no.	Primers	Product size (bp)
<i>ACTIN2</i>	AT3G18780	5'-CCCCTGAGGAGACCCAGTTCTA-3'; 5'-CATACCCCTCGTAGATTGGCACAG-3'	219
<i>AtPP2-A1</i>	AT4G19840	5'-GCCTAACGGTAAGGAGAA-3'; 5'-TTACTGTTTGGGACGAAT-3'	205
<i>AtPP2-A2</i>	AT4G19850	5'-TCAATTACATGGGCAGAGTCTCAA-3'; 5'-TCTCCACCCACTTGTTCCTTCTA-3'	401
<i>AtPP2-A3</i>	AT2G26820	5'-TGTGGTGGACGGAAGGTGCT-3'; 5'-CCTCTGGCCTACTGTTGATGTA AAA-3'	716
<i>AtPP2-A4</i>	AT1G33920	5'-GATCTACGCAAGGGATCTTAGCATT-3'; 5'-CTCCAGCATTATCTGGTGATGCACGAACT-3'	371
<i>AtPP2-A5</i>	AT1G65390	5'-GTAAAGTCAATCGTCAAGGCTGTAA-3'; 5'-TTCTCCCAAGTATTCGGCAAGTC-3'	524
<i>AtPP2-A6</i>	AT5G45080	5'-ATGGCTTCTTCTCCTCGGTTGTG-3'; 5'-GAGTTTGGTGCCTCGTTGATGGT-3'	797
<i>AtPP2-A7</i>	AT5G45090	5'-TAATGAATCCGCCGATGAAGC-3'; 5'-CAACACCTTTGACCACGAGCC-3'	638
<i>AtPP2-A8</i>	AT5G45070	5'-AATGCGATTCCCATCTTCTACAAAC-3'; 5'-CACTCATAACCACCTTCAGCGTCA-3'	565
<i>AtPP2-A9</i>	AT1G31200	5'-GTTGCGATCATAAGGCAGACTCCA-3'; 5'-TTCTTGAACAAAGGCTTCGTGGA-3'	521
<i>AtPP2-A10</i>	AT1G10150	5'-AATCCCTAACAGCTTGAAGCAGATC-3'; 5'-TGCAATAGCCTCAGTCCACCC-3'	694
<i>AtPP2-A11</i>	AT1G63090	5'-CGCTTCTGGGCTGATTTTCG-3'; 5'-GACTCCAGTTTCTGCTTCGGTTA-3'	533
<i>AtPP2-A12</i>	AT1G12710	5'-TTGTCTTCTCATCTTGTTTTGGGG-3'; 5'-CCGCTTCAACTGGTCTTACACGAG-3'	837
<i>AtPP2-A13</i>	AT3G61060	5'-CAGATTGGTGGATTTACCTGAGAATT-3'; 5'-TTGTTGGTTGTCCGAAGTGGC-3'	598
<i>AtPP2-A14</i>	AT5G52120	5'-AGACAAACTTATTTACCGC-3'; 5'-AACTGCTTCTAACCACCAT-3'	244
<i>AtPP2-A15</i>	AT3G53000	5'-TTTCGTGGTGCGGCTTCTTC-3'; 5'-TGCGTGCACTCAATCTGTTTCAT-3'	659
<i>AtPP2-B1</i>	AT2G02230	5'-CGAGTCTCTGGGACGCTTGT-3'; 5'-CCACGGACGCCTCATCTAAA-3'	620
<i>AtPP2-B2</i>	AT2G02250	5'-CCGGTTCTTCGTCGATGGTG-3'; 5'-AAGCCGAGTAACGGGTTCCAG-3'	537
<i>AtPP2-B3</i>	AT2G02270	5'-TTTTGCTGCTTCGGTTTCG-3'; 5'-CCCATGAGATCACCATTCCCT-3'	792
<i>AtPP2-B4</i>	AT2G02280	5'-ATGAATACTCAAATCCTATC-3'; 5'-TTATGGGCTTTTCGTAGGGCGGATA-3'	435
<i>AtPP2-B5</i>	AT2G02300	5'-GTTCTTGCTGCTTTGGTTTCG-3'; 5'-CCATCCACCCATCTTGCTCT-3'	536



**Table 5 Information on genes analyzed by reverse transcriptase-polymerase chain reaction in this study (Continued)**

<i>AtPP2-B6</i>	AT2G02310	5'-TGGAACTATCGGTGGAGGCG-3', 5'-CAACTTGATAGGCAAATCTCGTAAGC-3'	570
<i>AtPP2-B7</i>	AT2G02320	5'-AGCCGTTGCTTTGGGTGATT-3', 5'-ACGTTTCGTATTGCGCTGAGTAG-3'	755
<i>AtPP2-B8</i>	AT2G02340	5'-TTCACAAGCCCTCAAGATGCG-3', 5'-CACCACCTCAACTACAACCTTCTACGG-3'	498
<i>AtPP2-B9</i>	AT2G02350	5'-TGCAACTGCGATGAATCTATCAAG-3', 5'-CTGCTGGGCGTATTTACCCCTCT-3'	448
<i>AtPP2-B10</i>	AT2G02360	5'-GCGTCGCTGCTACGGTTTCG-3', 5'-GCTCAATCTCCATCCACCCATCTT-3'	579
<i>AtPP2-B11</i>	AT1G80110	5'-TGCGGCACCTGCTGGTCTTC-3', 5'-CCCTTTGTCTCCTTGAGGCTCATCTC-3'	558
<i>AtPP2-B12</i>	AT5G24560	5'-GCGGCGGATTCCAATACCA-3', 5'-AAGTTCAATCTCCAACCACCCATC-3'	525
<i>AtPP2-B13</i>	AT1G56240	5'-CCAACATCCTTGCCTTCACATC-3', 5'-TCTCCAACCACCCGCTCT-3'	690
<i>AtPP2-B14</i>	AT1G56250	5'-ATAGCCAACATCCTTGCCTTCA-3', 5'-TCAATCTCCATCCATCCGTCAT-3'	698
<i>AtPP2-B15</i>	AT1G09155	5'-ATCTCTGTCGGCGGCTGTCTC-3', 5'-CTATCTCCATCCACCCATCGTCTC-3'	649
<i>EF1<math>\alpha</math></i>	AT1G07930	5'-CCCCTTCGCTCCCACTTCAGGATGCTA-3', 5'-GTTGTACCTGGAAGTGCCTCAAGAAG-3'	189
<i>Kan<sup>r</sup></i>	HM047294	5'-GGCTATGACTGGGCACAACAGACAA-3', 5'-GCGGCGATACCGTAAAGCACGAGGA-3'	683
<i>uidA</i>	U00096	5'-GGGGTGGCAGTGAAGGGCGAACAGT-3', 5'-TGGGAGAACATTAGGTAGACGCAGGTGA-3'	533

were provided as either homologous (*atpp2-a1/P/-210*, *atpp2-a10/P/-157*, and *atpp2-a3/I/1650*) or heterozygous (the other seven mutants) at the insertion loci (Table 2). Homozygous progenies of heterozygous mutants were obtained by a PCR-based screening protocol according to information shown in Table 2. Provided mutant seeds were used to grow progeny plant lines and new seeds were harvested separately from five lines of each mutant. In the next generation, five lines of a mutant were grown for use to analyze the T-DNA insert and identify homozygous plants. Genomic DNA was isolated separately from ten plant individuals of each line and subjected to PCR analyses with *Kan<sup>r</sup>*-specific primers (Table 5). Seeds from the line that had *Kan<sup>r</sup>* in all the ten plant individuals were regarded as homozygous at the insertion locus.

#### Promoter analyses

Promoter sequences of the *AtPP2-A* genes (Figure 3b) were predicted with the AtcisDB program <http://arabidopsis.med.ohio-state.edu/>. Presence and locations of plant *cis*-acting regulatory DNA elements in the promoter sequences were determined by analyses with the PLACE Web Signal Scan program <http://www.dna.affrc.go.jp/PLACE/signalup.html> [39]. The *cis*-acting regulatory DNA elements were correlated with genes and processes by browsing linked web information and publications.

#### Generation and characterization of transgenic plants

The binary vector pBI121 (EMD Bioscience Inc., Gibbstown, NJ, USA), which contains the *NPT II* gene encoding kanamycin resistance, *35S* and *uidA*, was used to construct transformation units. Full length cDNA of the *AtPP2* gene used in construction of pBI121::*35S::AtPP2-A1* was obtained by RT-PCR conducted with RNA isolate from leaves of HrpN<sub>Ea</sub>-treated plants and *AtPP2-A1*-specific primers (5'-CGGGATCCATGAGCAAGAAACATTGCTCAG-3' and 5'-CGAGCTCTTACTGTTTGGGACGAATTGCAACAC-3'; underline indicates protection bases; italics indicate *Bam*H I and *Sac* I restriction bases). The gene was inserted into the pBI121 vector at the *Bam*H I and *Sac* I restriction sites to replace *uidA* (Figure 5a). The *AtPP2-A1* promoter was obtained by PCR using the genomic DNA from WT plant and the specific primers (5'-CCCAAGCTTGATAATTTTTCAAGACCC-3' and 5'-CGGGATCCAAACCAGTATGATGTATT-3'; underline indicates protection bases; italics indicate *Hind* III and *Bam*H I restriction bases). The promoter sequence was inserted pBI121 at the *Hind* III and *Bam*H I restriction sites to replace *35S* (Figure 7a), creating pBI121::*PP2P::uidA*.

Recombinant vector was transferred into cells of *Agrobacterium tumefaciens* strain EHA105. A suspension of EHA105 cells containing the empty pBI121 vector (without *AtPP2-A1* and *uidA* inserts) or the recombinant

vector were used to transform WT *Arabidopsis* by blossom infiltration. Transformation with pBI121::GUS::*AtPP2-A1* and pBI121::PP2P::uidA generated PP2OETAt and uidAETAt plants, respectively. Both types of transgenic plants were screened, multiplied and characterized by a previously described protocol [31]. The phenotype of kanamycin resistance was used in screening of PP2OETAt candidates and the transgenic control plant candidates, respectively; the phenotype of both kanamycin resistance and GUS activity was used in screening of uidAETAt candidates. GUS activity was determined using the histochemical techniques described previously [57]. Screened transgenic plants were allowed to self-pollinate and selected to the T3 generation [58]. T3 homozygous progenies were used in this study. The genomic integration of the transgene in PP2OETAt was detected by Southern blot analysis [54]. For Southern blots, *Arabidopsis* genomic DNA was digested with *Bam*H I and *Sac* I, and transferred to a nylon membrane, followed by hybridization to a digoxigenin-labeled *AtPP2-A1* probe prepared using the DIG Nucleic Acid Detection Kit [Roche Diagnostics (Shanghai) Trading Co., LTD].

#### Data treatment

Experiments were done three or four times with similar results. The student's *t*-test was used to compare data obtained from HrpN<sub>Ea</sub>-treated plants with those obtained from EVP-treated plants, and to compare data obtained from the transgenic control plant with those obtained from each line of PP2OETAt. Quantitative data were also analyzed by the ANOVA test to compare differences among the transgenic control plant and different lines of PP2OETAt, and among different organs of transgenic plants.

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#### Authors' contributions

CZ, XW and SZ carried out EPG studies, investigated insect population, and performed the statistical analysis. CZ and LC also participated in the design of the study and drafted the manuscript. HS and BL did bioinformatics analyses and determined gene expression. HS also investigated aphid nymphs. XW, LY and RL generated and characterized transgenic plants. BL,

JQ, WS and ZY participated in the insect monitoring experiments. HD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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#### References

1. Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV: Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 1992, **257**:85-88.
2. Kim JF, Beer SV: *hrp* genes and harpins of *Erwinia amylovora*: a decade of discovery. In *Fire blight and its causative agent, Erwinia amylovora*. Edited by: Vanneste JL. C.A.B. International, Wallingford, U.K; 2000:141-162.
3. Dong H, Delaney TP, Bauer DW, Beer SV: Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J* 1999, **20**:207-215.
4. Dong HP, Peng J, Bao Z, Meng X, Bonasera JM, Chen G, Beer SV, Dong H: Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense. *Plant Physiol* 2004, **136**:3628-3638.
5. Dong HP, Yu H, Bao Z, Guo X, Peng J, Yao Z, Chen G, Dong H: The *ABI2*-dependent abscisic acid signalling controls HrpN-induced drought tolerance in *Arabidopsis*. *Planta* 2005, **221**:313-327.
6. Peng J, Dong H, Dong HP, Delaney TP, Bonasera BM, Beer SV: Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes. *Physiol Mol Plant Pathol* 2003, **62**:317-326.
7. Ren X, Liu F, Bao Z, Zhang C, Wu X, Chen L, Liu R, Dong H: Root growth of *Arabidopsis thaliana* is regulated by ethylene and abscisic acid signaling interaction in response to HrpN<sub>Ea</sub>, a bacterial protein of harpin group. *Plant Mol Biol Rep* 2008, **26**:225-240.
8. Zitter TA, Beer SV: Harpin for insect control. *Phytopathology* 1998, **88**: S104-S105.
9. Gou Z, Zhang S, Dong H: Effects of HrpN<sub>Ea</sub> on inducing aphid repellency on *Cucumis melo*. *Acta Agric Boreali-Sinica* 2009, **24**:188-192.
10. Pollard DG: Plant penetration by feeding aphids (Hemiptera, Aphidoidea): A review. *Bul Entomol Res* 1972, **62**:631-714.
11. Douglas AE: Phloem-sap feeding by animals: problems and solutions. *J Exp Bot* 2006, **57**:747-754.
12. Kempema LA, Cui X, Holzer FM, Walling LL: *Arabidopsis* transcriptome changes in response to phloem-feeding silver leaf whitefly nymphs. Similarities and distinctions in responses to aphids. *Plant Physiol* 2007, **143**:849-865.
13. Mutti NS, Louis J, Pappan LK, Pappan K, Begum K, Chen M, Park Y, Dittmer N, Marshall J, Reese JC, Reeck GR: A protein from the salivary glands of the pea aphid, *Acyrtosiphon pisum*, is essential in feeding on a host plant. *Proc Nat Acad Sci USA* 2008, **105**:9965-9969.
14. De Vos M, Jander G: *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. *Plant Cell Environ* 2009, **32**:1548-1560.
15. Will T, van Bel AJ: Induction as well as suppression: How aphid saliva may exert opposite effects on plant defense. *Plant Signal Behav* 2008, **3**:427-430.
16. Tjallingii WF: Salivary secretions by aphids interacting with proteins of phloem wound responses. *J Exp Bot* 2006, **57**:739-745.
17. Klingler J, Creasy R, Gao L, Nair RM, Calix AS, Jacob HS, Edwards OR, Singh KB: Aphid resistance in *Medicago truncatula* involves antixenosis and phloem-specific, inducible antibiosis, and maps to a single locus flanked by NBS-LRR resistance gene analogs. *Plant Physiol* 2005, **137**:1445-1455.
18. Pegadaraju V, Louis J, Singh V, Reese JC, Bautor J, Feys BJ, Cook G, Parker JE, Shah J: Phloem-based resistance to green peach aphid is controlled by *Arabidopsis PHYTOALEXIN DEFICIENT4* without its signaling partner *ENHANCED DISEASE SUSCEPTIBILITY1*. *Plant J* 2007, **52**:332-341.
19. Louis J, Leung Q, Pegadaraju V, Reese J, Shah J: *PAD4*-dependent antibiosis contributes to the *ssi2*-conferred hyper-resistance to the green peach aphid. *Mol Plant-Microbe Interact* 2010, **23**:618-627.
20. Stone BA, Clarke AE: Chemistry and physiology of higher plant 1,3- $\beta$ -glucans (callose). In *Chemistry and biology of 1,3- $\beta$ -glucans*. Edited by: Stone BA, Clarke AE. Bundoora Australia: La Trobe University Press; 1992:365-429.

21. Kehr J: Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. *J Exp Bot* 2006, **57**:767-774.
22. Willats WGT, Knox JP: Molecules in context: probes for cell wall analysis. In *The Plant Cell Wall*. Edited by: Rose JKC. Oxford UK: CRC Press; 2003:92-110.
23. Dinant S, Clark AM, Zhu Y, Vilaine F, Palauqui JC, Kusiak C, Thompson GA: Diversity of the superfamily of phloem lectins (phloem protein 2) in angiosperms. *Plant Physiol* 2003, **131**:114-128.
24. Beneteau J, Renard D, Marche L, Douville E, Lavenant L, Rahbe Y, Dupont D, Vilaine F, Dinant S: Binding properties of the N-acetylglucosamine and high-mannose N-glycan PP2-A1 phloem lectin in *Arabidopsis*. *Plant Physiol* 2010, **153**:1345-1361.
25. Read SM, Northcote DH: Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (pumpkin). *Eur J Biochem* 1983, **134**:561-569.
26. Will T, van Bel AJE: Physical and chemical interactions between aphids and plants. *J Exp Bot* 2006, **57**:729-737.
27. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, *et al*: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 2003, **301**:653-657.
28. McElver J, Tzafirir I, Aux G, Rogers R, Ashby C, Smith K, Thomas C, Schetter A, Zhou Q, Cushman MA, *et al*: Insertional mutagenesis of genes required for seed development in *Arabidopsis thaliana*. *Genetics* 2001, **159**:1751-1763.
29. Divol F, Vilaine F, Thibivilliers S, Kusiak C, Helene Sauge M, Dinant S: Involvement of the xyloglucan endotransglycosylase/hydrolases encoded by celery XTH1 and *Arabidopsis* XTH33 in the phloem response to aphids. *Plant Cell and Environ* 2007, **30**:187-201.
30. Liu F, Liu H, Jia Q, Wu X, Guo X, Zhang S, Song F, Dong H: The internal glycine-rich motif and cysteine suppress several effects of the HpaG<sub>Xooc</sub> protein in plants. *Phytopathology* 2006, **96**:1052-1059.
31. Peng J, Bao Z, Dong H, Ren H, Wang J: Expression of harpin<sub>Xooc</sub> in transgenic tobacco induces pathogen defense in the absence of hypersensitive cell death. *Phytopathology* 2004, **94**:1048-1055.
32. De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC, Dicke M, Pieterse CMJ: Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol Plant-Microbe Interact* 2005, **18**:923-927.
33. Thompson GA, Goggin FL: Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *J Exp Bot* 2006, **57**:755-766.
34. Liu R, Lü B, Wang X, Zhang C, Zhang S, Qian J, Chen L, Shi H, Dong H: Thirty-seven transcription factor genes differentially respond to a harpin protein and affect resistance to the green peach aphid in *Arabidopsis*. *J Biosci* 2010, **35**:435-450.
35. Tjallingii WF, Esch TH: Fine-structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiol Entomol* 1993, **18**:317-328.
36. Tjallingii WF: Electrical recording of stylet penetration activities. In *Aphids: Their Biology, Natural Enemies and Control. Volume 2B*. Edited by: Minks AK, Harrewijn P. Elsevier Amsterdam; 1987:95-108.
37. Chen L, Qian J, Qu S, Long J, Yin Q, Zhang L, Wu X, Sun F, Wu T, Hayes M, Beer SV, Dong H: Identification of specific fragments of HpaG<sub>Xooc</sub>, a harpin from *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhance growth in plants. *Phytopathology* 2008, **98**:781-791.
38. Sun L, Ren H, Liu R, Li B, Wu T, Sun F, Liu H, Wang X, Dong H: An h-type thioredoxin functions in tobacco defense responses to two species of viruses and an abiotic oxidative stress. *Mol Plant-Microbe Interact* 2010, **23**:1470-1485.
39. Higo K, Ugawa Y, Iwamoto M, Korenaga T: Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res* 1999, **27**:297-300.
40. Bate N, Twell D: Functional architecture of a late pollen promoter: pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. *Plant Mol Biol* 1998, **37**:859-869.
41. Filichkin SA, Leonard JM, Monteros A, Liu PP, Nonogaki H: A novel endo- $\beta$ -mannanase gene in tomato LeMAN5 is associated with anther and pollen development. *Plant Physiol* 2004, **134**:1080-1087.
42. Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K: Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 2003, **15**:63-78.
43. Jefferson RA, Burgess SM, Hirsh D:  $\beta$ -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc Natl Acad Sci USA* 1986, **83**:8447-8451.
44. Miao WG, Wang XB, Li M, Song CF, Wang Y, Hu DW, Wang JS: Genetic transformation of cotton with a harpin-encoding gene *hpa<sub>Xooc</sub>* confers an enhanced defense response against different pathogens through a priming mechanism. *BMC Plant Biol* 2010, **10**:67.
45. Buchel AS, Brederode FT, Bol JF, Linthorst HJM: Mutation of GT-1 binding sites in the *Pr-1A* promoter influences the level of inducible gene expression *in vivo*. *Plant Mol Biol* 1999, **40**:387-396.
46. Yu D, Chen C, Chen Z: Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell* 2001, **13**:1527-1540.
47. Nishiuchi T, Shinshi H, Suzuki K: Rapid and transient activation of transcription of the *ERF3* gene by wounding in tobacco leaves: Possible involvement of NtWRKYs and autorepression. *J Biol Chem* 2004, **279**:55355-55361.
48. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M: Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 2000, **12**:393-404.
49. Zhang C, Bao Z, Liang Y, Yang X, Wu X, Hong X, Dong H: Abscisic acid mediates *Arabidopsis* drought tolerance induced by HrpN<sub>Ea</sub> in the absence of ethylene signaling. *Plant Mol Biol Rep* 2007, **25**:98-114.
50. Balachandran S, Xiang Y, Schobert C, Thompson GA, Lucas WJ: Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell to cell through plasmodesmata. *Proc Natl Acad Sci USA* 1997, **94**:14150-14155.
51. Giakountis A, Coupland G: Phloem transport of flowering signals. *Curr Opin Plant Biol* 2008, **11**:687-694.
52. Clark AM, Jacobsen KR, Bostwick DE, Dannenhoffer JM, Skaggs MI, Thompson GA: Molecular characterization of a phloem-specific gene encoding the filament protein, phloem protein 1 (PP1), from *Cucurbita maxima*. *Plant J* 1997, **12**:49-61.
53. Zhang S, Yang X, Sun M, Sun F, Deng S, Dong H: Riboflavin-induced priming for pathogen defense in *Arabidopsis thaliana*. *J Integr Plant Biol* 2009, **51**:167-174.
54. Wu T, Guo A, Zhao Y, Wang X, Wang Y, Zhao D, Li X, Ren H, Dong H: Ectopic expression of the rice lumazine synthase gene contributes to defense responses in transgenic tobacco. *Phytopathology* 2010, **100**:573-581.
55. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>- $\Delta$ ACT</sup> method. *Methods* 2001, **25**:402-408.
56. Volkov RA, Panchuk II, Schöffl F: Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. *J Exp Bot* 2003, **54**:2343-2349.
57. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 1987, **6**:3901-3907.
58. Weigle D, Glazebrook J: *Arabidopsis: a Laboratory Manual*. Cold Spring New York: Cold Harbor Laboratory Press; 2002.

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