Harpin-induced expression and transgenic overexpression of the phloem protein gene\textit{AtPP2-A1} in \textit{Arabidopsis} repress phloem feeding of the green peach aphid \textit{Myzus persicae}

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Abstract

\textbf{Background:} Treatment of plants with Hrp\textsubscript{N\textsubscript{Ea}}, a protein of harpin group produced by Gram-negative plant pathogenic bacteria, induces plant resistance to insect herbivores, including the green peach aphid \textit{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 \textit{Arabidopsis thaliana} (Arabidopsis) PP2-encoding gene \textit{AtPP2-A1} in resistance to \textit{M. persicae} when the plant was treated with Hrp\textsubscript{N\textsubscript{Ea}} and after the plant was transformed with \textit{AtPP2-A1}.

\textbf{Results:} The electrical penetration graph technique was used to visualize the phloem-feeding activities of apterous agamic \textit{M. persicae} females on leaves of \textit{Arabidopsis} plants treated with Hrp\textsubscript{N\textsubscript{Ea}} and an inactive protein control, respectively. A repression of phloem feeding was induced by Hrp\textsubscript{N\textsubscript{Ea}} in wild-type (WT) \textit{Arabidopsis} but not in \textit{atpp2-a1/E/142}, the plant mutant that had a defect in the \textit{AtPP2-A1} gene, the most Hrp\textsubscript{N\textsubscript{Ea}}-responsive of 30 \textit{AtPP2} genes. In WT rather than \textit{atpp2-a1/E/142}, the deterrent effect of Hrp\textsubscript{N\textsubscript{Ea}} treatment on the phloem-feeding activity accompanied an enhancement of \textit{AtPP2-A1} expression. In PP2OET\textit{At} (\textit{AtPP2-A1}-overexpression transgenic \textit{Arabidopsis thaliana}) plants, abundant amounts of the \textit{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 Hrp\textsubscript{N\textsubscript{Ea}} treated WT and PP2OET\textit{At} plants, respectively, compared with control plants.

\textbf{Conclusions:} The repression in phloem-feeding activities of \textit{M. persicae} as a result of \textit{AtPP2-A1} overexpression, and as a deterrent effect of Hrp\textsubscript{N\textsubscript{Ea}} treatment in WT \textit{Arabidopsis} rather than the \textit{atpp2-a1/E/142} mutant suggest that \textit{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 \textit{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], HrpNEa, 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 HrpNEa as a paradigm [1-3]. Early studies demonstrated that the external application of HrpNEa 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]. HrpNEa-induced resistance to insects first was suggested based on observations of field-grown peppers. Plants that had been treated with HrpNEa incurred fewer injuries from the European corn borer than comparable untreated plants [2]. A deterrent effect on striped cucumber beetles was observed in HrpNEa-treated cucumber; striped cucumber beetles preferred to colonize untreated control plants rather than HrpNEa-treated plants [8]. HrpNEa-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, HrpNEa treatment had a deterrent effect on colonization by the muskmelon aphid Aphis gossypii (Glover), which preferred to colonize control plants rather than HrpNEa-treated plants [9]. In Arabidopsis thaliana (Arabidopsis), moreover, HrpNEa-induced resistance was shown to repressinfestation 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]. Pivotal, 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 β-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 AtPP2B-1 to AtPP2B-15 [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 HrpNEa according to previous evidence that the HrpNEa 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, HrpNea induces resistance to *M. persicae* by activating the ethylene-signaling pathway [4,34]. Therefore, we devised to determine the possibility that HrpNea-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 resister plants and 14 min in control plants, on average, duration of the pathway phase were all similar in HrpNea-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 also be connected with the xylem phase, indicating styllet 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 HrpNea 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 HrpNea treatment condition compared with control. This result suggested that the HrpNea 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 HrpNea-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 HrpNea-treated plants compared with control plants (Figure 1a; Table 1). On HrpNea-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 HrpNea-treated plants were changed as unfavourable to aphid feeding. In consistence with this notion, total duration of the phloem phase was markedly shorter in HrpNea-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 HrpNea-treated plants and 14 min in control plants, on average, strongly suggested the deterrent effect of the HrpNea treatment on the phloem-feeding activity of *M. persicae*.

In the phloem phase, E1 and E2 salivations were recognized by dissecting the EPG waveform pattern (Figure 1b). Compounds of E1 and E2 saliva produced by aphids after styllet 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

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**Results**

**HrpNea treatment in Arabidopsis induces a repression in phloem feeding and colonization by *M. persicae***

The HrpNea protein used in this study was produced by prokaryotic expression with a vector that carried a hrpNea gene insert; the hrpNea-absent Empty Vector Preparation (EVP) that contained inactive proteins but not HrpNea 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 HrpNea, 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 HrpNea, 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 styllet staying outside the cuticle. Cell puncturing (Figure 1a, probe) led to the pathway phase (Figure 1a, path) in which the styllet 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 time to the first cell puncturing or the first pathway phase, and total number and time to the first cell puncturing or the first pathway phase, and total number and
E1 and E2 salivations were much shorter in HrpNea-treated plants than control plants, confirming the deterrent effect of the HrpNea 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 HrpNea, 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

### 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*) | HrpNea 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 (3.2)                  | 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)                   | 20 (0.1)                 | **                               |
| 3rd h 7.3 (1.0)                   | 20 (0)                   | *p < 0.01                        |
| 4th h 4.0 (0.5)                   | 20 (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 (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 (1.6)                      | *p < 0.01                |
| Number of phloem phase            |                          |                                  |                          |
| total 7 (1)                       | 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)                  | **                               |
| 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 15 (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 126 (2.8)                      | 50 (1.4)                 | *p < 0.01                        |
| E2 319 (3.5)                      | 13.2 (3.1)               | *p < 0.01                        |

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

Zhang et al. BMC Plant Biology 2011, 11:11
http://www.biomedcentral.com/1471-2229/11/11
Page 4 of 19
24 hours (Figure 2). At each time point, the number of aphid individuals run away from their colonies on leaves of HrpN$_{Ea}$-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$_{Ea}$-treated plants were observed in the short period of two to four hours. And this period was critical to the effect of HrpN$_{Ea}$ 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$_{Ea}$-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 carcases on other different parts of the plants. These observations indicate that the HrpNEa treatment impairs the stability of Arabidopsis colonization by *M. persicae*.

**Arabidopsis atpp2-a1/E142 mutant pampers *M. persicae* in phloem feeding**

To gain information about relationships between previously identified 30 *AtPP2* genes [23] and HrpNEa-induced repression in the phloem-feeding activity of *M. persicae*, we studied expression of these genes in HrpNEa-treated WT Arabidopsis plants. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the *EF1a* 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 HrpNEa-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 HrpNEa-treated plants. Subsequent real-time RT-PCR analyses using the *EF1a* 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 HrpNEa-treated plants than in control plants (Figure 3b).

To correlate the role of HrpNEa 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 HrpNEa (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_{Ea}-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_{Ea}-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-a3/1/1650, -a11/E/177, -a13/E/1872, and -a15/E/312. In contrast, atpp2-a10/P/-157, a11/E/-394, a12/P/-329, 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_{Ea}-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_{Ea} 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_{Ea} 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_{Ea}-induced repression in aphid feeding from the WT phloem (Table 1). Apparently, aphids preferred to feed from atpp2-a1/E/142.
Table 2 Information on AtPP2-A-defected Arabidopsis mutants investigated in this study

| Gene name | Locus no. | Mutant name | T-DNA insertion site | T-DNA stock no. | TAIR a annotations |
|-----------|-----------|-------------|---------------------|-----------------|------------------|
| AtPP2-A1  | AT4G19840 | atpp2-a1/E/142 | 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. |
| AtPP2-A11 | AT1G63090 | atpp2-a1/P-394 | Promoter, -394 | CS842726 | 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. |
| AtPP2-A1  | AT4G19840 | atpp2-a1/P-210 | Promoter, -210 | SALK_080914C | |
| AtPP2-A10 | AT1G10155 | atpp2-a10/P-157 | Promoter, -157 | SALK_107807C | |
| AtPP2-A3  | AT2G26820 | atpp2-a3/1650 | Intron, 1650 | SALK_005443C | |
| AtPP2-A11 | AT1G63090 | atpp2-a11/E/117 | Exon, 117 | SALK_080546 | |
| AtPP2-A12 | AT1G12710 | atpp2-a12/P-293 | Promoter, -293 | SALK_015774 | |
| AtPP2-A13 | AT3G61060 | atpp2-a13/E/1872 | Exon, 1872 | SALK_046907 | |
| AtPP2-A14 | AT5G52120 | atpp2-a14/P-320 | Promoter, -320 | SALK_066553 | |
| AtPP2-A15 | AT3G35000 | atpp2-a1/E/312 | Exon, 312 | SALK_022649 | |

*aDistribution seeds of atpp2-a1/E/210, atpp2-a10/P-157 and atpp2-a3/I/1650 are from confirmed lines and T2 or T3 generation for the other mutants.*  
*bTAIR, The Arabidopsis Information Resource [http://www.arabidopsis.org](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_Ea 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_Ea 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_Ea-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_Ea treatment vs. control (Student’s t-test, p < 0.01) irrespective of the plant genotypes, suggesting that HrpN_Ea-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_Ea 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.
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). Also, WT, transgenic control and PP2OETAt plants did not have evident differences in morphology. Homozygous T3 progenies of the PP2OETAt lines were compared with 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 control plants in *AtPP2-A1* expression and in colonization and feeding by apterous *M. persicae* females.

### Figure 4 Comparison of Arabidopsis *AtPP2-A* modified mutants and WT plant in colonization and phloem feeding by aphids

(a) Changes of aphid population in 24 hours. Plants were treated with HrpNea and EVP, respectively. Five days later, uniform aphids were placed on lower sides of the top two expanded leaves (10 aphids/leaf). The number of aphids that stayed in a leaf colony was scored at the 24th hour after leaf colonization. Percent decrease (mean ± SD; n = 120 leaf colonies) in the number of aphids that run away from the leaf colonies was calculated. (b) Total duration of the phloem phase in a four-hour EPG monitoring course. Plants treated as in (a) were colonized by aphids at the fifth day after treatment; 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 ± SD; n = 20 aphids) was scored. (c) The second-hour EPG record particularly indicating the phloem phase (PP) in WT and an *AtPP2-A1* defected mutant. Experiments were the same as in (b). The EPG record represents 20 aphids feeding from 20 plants of WT and the mutant, respectively. (d, e) Reproduction of aphid adults and colonization behaviors of newborn nymphs. Experiments were similar as in (a) and insects were surveyed in five days after colonization of leaves by adults. Reproduction rate was given as the ratio between total number of newborn nymphs and total number of adults on leaf colonies. The population decrease was based on total number of nymphs and the number of nymphs that run away from the leaf colony. Data represent mean ± SD (n = 120 leaf colonies).
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 HrpNea 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

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**Table 3 Four-hour EPG analysis of aphid feeding from WT Arabidopsis and the atpp2-a1/E142 mutant**

| Activity examined                      | WT group                          | atpp2-a1/E142 group                       |
|----------------------------------------|-----------------------------------|-------------------------------------------|
|                                        | EVP treatment mean (SD)           | HrpNea treatment mean (SD)                | Student’s t-test (n = 20) | EVP treatment mean (SD) | HrpNea treatment (SD) | Student’s t-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.
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 uidAETAt (uidA-expressing transgenic A. thaliana) plant (Figure 7b). Six uidAETAt lines were observed. They seemed to resemble each other closely and were also similar to the transgenic control plant (Figure 7b; uidAETAt1 as a representative

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 BamHI and SacI restriction sites to replace uidA, a reporter gene encoding β-D-glucuronidase. NosP, promoter from the nopaline synthase-encoding gene (Nos); NPT II, kanamycin resistance gene; Nos T, Nos transcription terminator; 3SS, the cauliflower mosaic virus 3SS 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 ± SD (n = 3 repeats) relative to reference genes (EF1α 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 ± 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 the indicated organs. Feeding activities were detected immediately with an EPG monitoring system, and total duration of the phloem phase (mean ± 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 BamHI (B) and SacI (S). (b) Northern blots hybridized with probes specific to AtPP2-A1 and the reference gene EF1α. (c) Real-time RT-PCR analysis of AtPP2-A1 expression in the different organs of the plants. The gene transcript was quantified as mean ± 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 ± SD; n = 20 aphids) was scored.
line). In the uidAETAt1 line, uidA was expressed markedly in the stem, flower stalk, calyce, and petal, whereas, stronger expression was found in the leaf (Figure 7c). The uidA gene encodes β-D-glucuronidase (GUS) enzyme [43]. GUS activity was detected in the root, stem, calyce, and petal of PP2OETAt1 (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 HrpNE, 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 HrpNE 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 HrpNE and under the condition of AtPP2-A1 overexpression.

We show that the treatment of Arabidopsis with HrpNE 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 HrpNE to WT Arabidopsis impedes aphis 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 HrpNE treatment is likely to induce changes in cell wall properties unfavorable to aphid feeding, but this notion remains to be examined. HrpNE-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...
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 HrpNea treatment condition compared with control. It is also pertinent to propose that the HrpNea 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 HrpNea-induced repression of M. persicae feeding from the plant phloem. RT-PCR analyses (Figure 3) suggest that AtPP2-A1 is the most HrpNea-responsive gene of 30 members of the PP2 multigene family [23]. PLACE Web Signal Scan [39] provides a clue to molecular basis of HrpNea 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 HrpNea treatment in Arabidopsis [3]. Moreover, previous studies have shown that HrpNea-induced repression of 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 to require wound-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 HrpNea treatment [4]. Thus, AtPP2-A1 is pertinently thought a part of the signaling pathway that is required for HrpNea 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 HrpNea 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 HrpNea-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 HrpNea, 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 HrpNea 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 PP2OETAt 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 convening 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\textsubscript{Ea}-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\textsubscript{Ea}-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\textsubscript{Ea} or under the condition of *AtPP2-A1* overexpression.

Moreover, *AtPP2-A1* is a member of the *PP2* multigene 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\textsubscript{Ea}-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\textsubscript{Ea} 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\textsubscript{Ea} 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 μE/m²/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_{Ea}, respectively. EVP and HrpN_{Ea} were prepared [1,6] as 10 μg/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, China. A clone of apterous agamic females was obtained by acclimatization in WT Arabidopsis grown in the chamber (22°C; 250 μE/m²/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_{Ea}), 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^{5}-Ω 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_{Ea}-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 EF1α 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 μl reaction solutions to select desired doses. Reaction treatments, RT-PCR protocols, product cloning and sequencing verification were performed as described [5,6]. The 25 μl reaction mixture was composed of 1 μl...
first-strand cDNA diluted 1:10, 2.5 μM primer and 1×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 \textit{AtPP2-A1} transcript was quantified relative to \textit{EF1a} and \textit{ACTIN2}. The expression of \textit{uidA} in different organs of the uidATEAt plant was determined by RT-PCR using the superscript II RNase H\textsuperscript{-} 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) |
|--------|-----------|----------------------------------|------------------|
| ACTIN2 | AT3G18780 | 5'CCCCTGAGGAGCACCCAGTCTTA-3', 5'CATAACCCTCCTGAGATGGCGCAG-3' | 219               |
| \textit{AtPP2-A1} | AT4G19840 | 5'GCCCTACCGTGAAAGCGAA-3', 5'TTACGTGTGTTGAGGAAT-3' | 205               |
| \textit{AtPP2-A2} | AT4G19850 | 5'TCATTATGTCGCAAGTCGCTCA-3', 5'CTGCTGGCGCTGTGATGCTAA-3' | 401               |
| \textit{AtPP2-A3} | AT2G26820 | 5'TGTGGTGCAGGGAGGATGTTG-3', 5'CCTCAGCAATTCGCTGAGGAG-3' | 716               |
| \textit{AtPP2-A4} | AT1G3920 | 5'CATCTACGCAAGGGATCTTAGCATT-3', 5'CTGCTGGCGCTGTGATGCTAA-3' | 371               |
| \textit{AtPP2-A5} | AT5G45080 | 5'ATGGCTTCTTCTTCCTCGGTTG-3', 5'GAGTTTGGTGCCTCGTTGATGGT-3' | 524               |
| \textit{AtPP2-A6} | AT5G45090 | 5'TAATGAATCCGCCGATGAAGC-3', 5'CAACACCTTTGACCACGAGCC-3' | 638               |
| \textit{AtPP2-A7} | AT1G65390 | 5'GTAAAGTCAATCGTCAAGGCTGTTA-3', 5'TTCTCCCAAGTATTCGGCAAGTC-3' | 533               |
| \textit{AtPP2-A8} | AT1G31200 | 5'GTTCGCATCA TAAGGCAGACTCCA-3', 5'TTCTTGAACAAAGGCTTCGTGGA-3' | 521               |
| \textit{AtPP2-A9} | AT1G10150 | 5'AATCCCTAACAGCTTGAAGCAGATC-3', 5'TGCAATAGCCTCAGTCCACCC-3' | 694               |
| \textit{AtPP2-A10} | AT1G12710 | 5'TTGTCTTCTTCATCTTGTTTTGGGG-3', 5'CCGCTTCAACTGGTCTTTACACGAG-3' | 837               |
| \textit{AtPP2-A11} | AT3G61060 | 5'CAGATTTGCTGAGATTACCTGGA-3', 5'CCTTCAGCTCTTCTTACAGGAG-3' | 598               |
| \textit{AtPP2-A12} | AT3G53000 | 5'TTTCTCCTTTCTTATCTGGG-3', 5'CCGCTTCAACTGGTCTTTACAGGAG-3' | 244               |
| \textit{AtPP2-A13} | AT2G02230 | 5'GGCTGCTTCTATTCTCTGCGG-3', 5'CCTACGACTCGGCGGTGGTG-3' | 659               |
| \textit{AtPP2-A14} | AT2G02250 | 5'CCGCTTCTTCTCGCTGGG-3', 5'CCGCTTCAACTGGTCTTTACAGGAG-3' | 537               |
| \textit{AtPP2-A15} | AT2G02270 | 5'GGCTGCTTCTTCTCGCTGGG-3', 5'CCGCTTCAACTGGTCTTTACAGGAG-3' | 792               |
| \textit{AtPP2-A16} | AT2G02280 | 5'GGCTGCTTCTTCTCGCTGGG-3', 5'CCGCTTCAACTGGTCTTTACAGGAG-3' | 435               |
| \textit{AtPP2-A17} | AT2G02300 | 5'GGCTTCTTCTCGCTGGG-3', 5'CCGCTTCAACTGGTCTTTACAGGAG-3' | 536               |
were provided as either homologous (attp2-a1/P/-210, attp2-a10/P/-157, and attp2-a31/l/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 (the other seven mutants) at the insertion loci (Table 2).

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.afrc.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.

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

| AtPP2-B6 | AT2G02310 | 5'-TGGGAGCTCTAGTATGAGAGAAGCC-3', 5'-CAACTTGTATAGGACGAAATCCTGAAAGC-3' | 570 |
| AtPP2-B7 | AT2G02320 | 5'-ACGCGGTGGTTGCTTATGAGG-3', 5'-ACACCGTCGCTGGGCTATTTATGACG-3' | 755 |
| AtPP2-B8 | AT2G02340 | 5'-TTACCAAGCCCTCAAGATGCG-3', 5'-CCCACTCTCAACTAACACTTCTGAGC-3' | 498 |
| AtPP2-B9 | AT2G02350 | 5'-TGCAACTGCGTAGATCAATGAA-3', 5'-CTGTGGGGGCTATTTATGACG-3' | 448 |
| AtPP2-B10 | AT2G02360 | 5'-GGTGCGCTCTAGGCTTTTG-3', 5'-GCTCAATCCATGACCGGTATCT-3' | 579 |
| AtPP2-B11 | AT1G80110 | 5'-TGGGGGACACCTGTCGATTC-3', 5'-CCCCTTGTTTCTTGTACGCT-3' | 558 |
| AtPP2-B12 | AT5G24560 | 5'-GGGGGGCATATAGCCTCAACACAC-3', 5'-AAGTTCATCTCTCCACCAACCATC-3' | 525 |
| AtPP2-B13 | AT1G56240 | 5'-CCCAACTCATGCGCTTACATC-3', 5'-TCCTCAACCATGCGGTGTCT-3' | 690 |
| AtPP2-B14 | AT1G56250 | 5'-ATAGCCAAACATCTTGTACCTCAAC-3', 5'-TCAATCTCATCCATCACCGGTCT-3' | 698 |
| AtPP2-B15 | AT1G90155 | 5'-ATCTCGCTGCGGGCTGTCTC-3', 5'-CTATCCTCATCCACCGGTCT-3' | 649 |
| EF1α | AT1G07930 | 5'-CCCCCTCCCTCCACCTACCTGAGATGCT-3', 5'-GTTGACCTGCGAAAGTCGCTCAAGA-3' | 189 |
| Kanr | HM047294 | 5'-GGGTTATGACGGCCACAGACACAGAC-3', 5'-GGGGCAATACGGCAGGACCGGA-3' | 683 |
| uidA | U00096 | 5'-GGGGTGGGCAAGCTGAGACCCATG-3', 5'-TGCGGAAGACATTAGGTA -157, and 5'-GGGGTGCGGTGGTTGCTTATGAGG-3', 5'-ACACCGTCGCTGGGCTATTTATGACG-3' | 533 |

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 was used in construction of pBI121::35S::AtPP2-A1 was obtained by RT-PCR conducted with RNA isolate from leaves of HrpNea-treated plants and AtPP2-A1-specific primers (5'-CGGGATCCATGACGGCAAAACCCATTTGCTCAG-3' and 5'-CCGGATCCATGACGGCAAAACCCATTTGCTCAG-3'; underline indicates protection bases; italics indicate BamHI and SacI restriction bases). The gene was inserted into the pBI121 vector at the BamHI and SacI 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'-CCCAAGCTTTGAAATTTTATGACG-3' and 5'-CCGGATCCATGACGGCAAAACCCATTTGCTCAG-3'; underline indicates protection bases; italics indicate Hind III and BamHI restriction bases). The promoter sequence was inserted pBI121 at the Hind III and BamHI 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 pBl121::GUS::AtPP2-AI and pBl121::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 BamH I and Sac I, and transferred to a nylon membrane, followed by hybridization to a digoxigenin-labeled AtPP2-AI 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 t-test was used to compare data obtained from HrpN_3-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, JW 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. JW, 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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Zhang et al. BMC Plant Biology 2011, 11:11
Page 18 of 19

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BMC Plant Biology 2011, 11:11

Page 18 of 19
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