$HrpN_{Ea}$-induced deterrent effect on phloem feeding of the green peach aphid *Myzus persicae* requires *AtGSL5* and *AtMYB44* genes in *Arabidopsis thaliana*

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In *Arabidopsis thaliana* (*Arabidopsis*) treated with the harpin protein $HrpN_{Ea}$, resistance to the green peach aphid *Myzus persicae*, a generalist phloem-feeding insect, develops with induced expression of the *AtMYB44* gene. Special *GLUCAN SYNTHESIS-LIKE* (*GSL*) genes and β-1,3-glucan callose play an important role in plant defence responses to attacks by phloem-feeding insects. Here we report that *AtGLS5* and *AtMYB44* are both required for $HrpN_{Ea}$-induced repression of *M. persicae* feeding from the phloem of *Arabidopsis* leaves. In 24 h successive surveys on large-scale aphid populations, the proportion of feeding aphids was much smaller in $HrpN_{Ea}$-treated plants than in control plants, and aphids preferred to feed from the 37 tested *atgsl* mutants rather than the wild-type plant. The *atgsl* mutants were generated previously by mutagenesis in 12 identified *AtGSL* genes (*AtGSL1* through *AtGSL12*); in the 24 h survey, both *atgsl5* and *atgsl6* tolerated aphid feeding, and *atgsl5* was the most tolerant. Consistently, *atgsl5* was also most inhibitive to the deterrent effect of $HrpN_{Ea}$ on the phloem-feeding activity of aphids as monitored by the electrical penetration graph technique. These results suggested an important role of the *AtGSL5* gene in the effect of $HrpN_{Ea}$. In response to $HrpN_{Ea}$, *AtGSL5* expression and callose deposition were induced in the wild-type plant but not in *atgsl5*. In response to $HrpN_{Ea}$, moreover, the *AtMYB44* gene known to be required for repression of aphid reproduction on the plant was also required for repression of the phloem-feeding activity. Small amounts of the *AtGSL5* transcript and callose deposition were detected in the *atmyb44* mutant, as in *atgsl5*. Both mutants performed similarly in tolerating the phloem-feeding activity and impairing the deterrent effect of $HrpN_{Ea}$, suggesting that *AtGSL5* and *AtMYB44* both contributed to the effect.

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1. Introduction

Harpins are multifunctional proteins produced by Gram-negative plant pathogenic bacteria (Kim and Beer 2000; Peng *et al.* 2004; Liu *et al.* 2006; Chen *et al.* 2008). $HrpN_{Ea}$ is the first-characterized (Wei *et al.* 1992), well-studied harpin (Dong et al. 1999, 2004, 2005a; Kim and Beer 2000; Peng *et al.* 2003; Zhang *et al.* 2007; Ren *et al.* 2008; Liu *et al.* 2010a,b; Sun *et al.* 2010) secreted by *Erwinia amylovora*, the bacterial pathogen that causes fire blight disease in rosaceous plants (van der Zwet and Beer 1999). Harpins' multiple functions, especially in eliciting plant defence responses, were also elucidated initially by studies using $HrpN_{Ea}$ as a paradigm (Wei *et al.* 1992; Dong *et al.* 1999; Kim and Beer 2000). Early studies demonstrated that the external application of $HrpN_{Ea}$ was able to induce resistance in a variety of plant species

Keywords. *Arabidopsis*; *AtGSL5*; *AtMYB44*; callose; green peach aphid

Abbreviations used: EPG, electrical penetration graph; *GSL*, *GLUCAN SYNTHESIS-LIKE*; HRB, hexyl rhodamine B; RT-PCR, reverse transcriptase–polymerase chain reaction; WT, wild type

(Dong *et al.* 1999, 2004, 2005a; Peng *et al.* 2003; Ren *et al.* 2008), and that the induced resistance effectively protected plants from attacks by herbivorous insects (Zitter and Beer 1998; Dong *et al.* 2005a; Gou *et al.* 2009; Liu *et al.* 2010a). $HrpN_{Ea}$-induced resistance to insects was first suggested on the basis of observations of field-grown peppers. Plants that had been treated with $HrpN_{Ea}$ incurred fewer injuries from the European corn borer *Ostrinia nubilalis* Hubner than comparable untreated plants (Kim and Beer 2000). A deterrent effect on the striped cucumber beetle *Acalymma vittatum* Fabricius was observed in $HrpN_{Ea}$-treated cucumber; striped cucumber beetles preferred to colonize untreated control plants rather than $HrpN_{Ea}$-treated plants (Zitter and Beer 1998). $HrpN_{Ea}$-induced resistance was also effective in impeding infestations of aphids, which represent typical phloem-feeding herbivores (Dong *et al.* 2005a; Gou *et al.* 2009; Liu *et al.* 2010a). In cucumbers grown in environmentally controlled conditions, the plant treatment with $HrpN_{Ea}$ had a deterrent effect on colonization by the muskmelon aphid *Aphis gossypii* Glover (Gou *et al.* 2009). In *Arabidopsis*, $HrpN_{Ea}$-induced resistance was shown to repress multiplication of the green peach aphid *Myzus persicae* Sulzer (Dong *et al.* 2004), a generalist phloem-feeding insect (Pollard 1972).

Phloem-feeding insects are highly specialized in their mode of feeding and present a unique stress on plant fitness (Douglas 2006). They use their slender stylets to feed from a single-cell type, the phloem sieve element; the feeding process can be monitored by the electrical penetration graph (EPG) technique (Tjallingii 1987, 2006). Of pivotal importance, 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. This ejection is detected in the EPG as E1 salivation and always precedes phloem sap ingestion. During ingestion from the sieve element, the watery E2 salivation occurs, and this E2 saliva is added to the ingested sap, and is thought to prevent phloem proteins from clogging inside the capillary food canal. Therefore, salivation is a crucial event during the phloem-feeding process for insects to overcome a number of phloem-related plant properties and reactions (Klingler *et al.* 2005; Pegadaraju *et al.* 2007; Mutti *et al.* 2008; Will and van Bel 2008; De Vos and Jander 2009; Louis *et al.* 2010).

In response to the phloem-feeding stress, plants defend themselves by using the phloem-based defense mechanism (Kehr 2006; Will and van Bel 2006, 2008). This mechanism involves the biosynthesis of β-1,3-glucan callose and subsequent closure of sieve pores and coagulation on sieve plates (Stone and Clarke 1992; Willats and Knox 2003; Kuśnierczyk *et al.* 2008; Saheed *et al.* 2009; Villada *et al.* 2009). Biochemical evidence and molecular studies in several plant species indicate that callose is synthesized by a class of enzymes, termed callose synthases or glucan synthases (Hong *et al.* 2001; Verma and Hong 2001). Twelve genes encoding putative glucan synthase have been identified and designated as *AtGSL1* through *AtGSL12* in *Arabidopsis* (for review, *see* Chen and Kim 2009). Based on analyses of mutations made by knocking out the individual *GSL* genes, it is found that the genes exhibit partially redundant roles in plant growth and development; a single *GSL* gene can also have diverse functions (Chen and Kim 2009). For example, GSL5 is responsible for the synthesis of wound-inducible and pathogen-inducible callose in leaf tissue (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Wawrzynska *et al.* 2010); it also plays an important role in exine formation and pollen wall patterning (Dong *et al.* 2005b; Enns *et al.* 2005). However, genetic evidence is incomplete regarding functions of the different *GSL* genes; so far, evidence is available only for *AtGSL1*, *2*, *5*, *6*, *8* and *10* (Kaliff *et al.* 2007; Dong *et al.* 2008; Huang *et al.* 2008; Töller *et al.* 2008; Chen *et al.* 2009a; Consonni *et al.* 2009; Xie *et al.* 2009).

One of the main purposes of this study was to elucidate if *AtGSL* and callose play a role in $HrpN_{Ea}$-induced resistance to *M. persicae* in *Arabidopsis*. We present evidence that *AtGSL5* is required for $HrpN_{Ea}$-induced repression in aphid feeding activities. Recently we showed that *AtMYB44* is the most $HrpN_{Ea}$-inducible of the 37 transcription factor genes tested and that *AtMYB44* is required for the induction of resistance to *M. persicae* in *Arabidopsis* (Liu *et al.* 2010a). In this study, we sought to verify if *AtMYB44* also affects the repression of aphid feeding activities.

2. Materials and methods

2.1 *Plant growth and treatment*

Arabidopsis genotypes used in this study included the wild type (WT) of the ecotype Col-0 and both *atmyb44* and *atgsl* mutants previously generated under the background of Col-0 (table 1). Plants were grown in 9 cm pots (5 plants/pot) and incubated in an environmentally controlled chamber (22°C; 14 h illumination at 250 $\mu E/m^2/s$) for 40 days before use. $HrpN_{Ea}$ was prepared and purified as described (Dong *et al.* 1999). The protein was used in a 10 μg/ml aqueous solution and applied in the presence of surfactant Silwet-77 (0.02%, v/v) by spraying plant tops with a low-pressure atomizer. Similar treatment with pure water in the presence of Silwet-77 (0.02%, v/v) was used as a control.

2.2 *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 (wingless) agamic females was obtained

Table 1. Information on *Arabidopsis* genes and mutants tested in this study

Gene[a]	Synonyms	AGI[b] code	Mutant	T-DNA insertion site[c]	Mutant seed stock number
AtGSL1	*GSL01, T32N4.8*, *T32N4_8*	AT4G04970	*atgsl1-1*	Promoter −120	SALK_021172
			atgsl1-2	Intron 5788	SALK_040711
			atgsl1-3	Exon 5457	SALK_045624
AtGSL2	*ATGSL02, Callose Synthesis-like 5 (CalS5)*, *CASL5*	AT2G13680	*atgsl2-1*	Exon 3850	SALK_009227
			atgsl2-2	Intron 4278	SALK_009234
			atgsl2-3	Intron 617	SALK_072226
			atgsl2-4	Exon 3772	SALK_026354
AtGSL3	*F22D22.29*, *F22D22_29*	AT2G31960	*atgsl3-1*	Exon 438	CS331075
			atgsl3-2	Intron 8186	SALK_131153
			atgsl3-3	Intron 216	SALK_011560
AtGSL4	*ATGSL4*, *ATGSL04*	AT3G14570	*atgsl4-1*	Exon 7135	SALK_015030
			atgsl4-2	Intron 157	CS358972
			atgsl4-3	Exon 6991	SALK_000507
AtGSL5	*POWDERY MILDEW RESISTANT 4* (*PMR4*), *GSL05*, *T5L23.4*	AT4G03550	*atgsl5-1*	Exon 5433	SALK_090017
			atgsl5-2	Promoter −663	SALK_021415
			atgsl5-3	Exon 1833	CS319625
AtGSL6	*CalS1*	AT1G05570	*atgsl6-1*	Intron 5713	SALK_071591
			atgsl6-2	Intron 5378	SALK_071595
			atgsl6-3	Exon 5671	SALK_007820
			atgsl6-4	Exon 5733	SALK_071100
AtGSL7	*atgsl7*, *F12K11.17*, *F12K11_17*, *gsl07*	AT1G06490	*atgsl7-1*	Intron 9756	SALK_040051
			atgsl7-2	Intron 9735	SALK_040339
			atgsl7-3	Exon 2145	SALK_048921
AtGSL8	*ATGSL08*, *T1J8.3*, *T1J8_3*	AT2G36850	*atgsl8-1*	Intron 1009	SALK_111094
			atgsl8-2	Promoter −122	SALK_001434
			atgsl8-3	Intron 4765	SALK_057120
AtGSL9	*F5H8.14*, *F5H8_14*, *gsl09*	AT5G36870	*atgsl9-1*	Intron 8311	SALK_007717
			atgsl9-2	Exon 9592	SALK_026245
			atgsl9-3	Intron 5917	SALK_147529
AtGSL10	*ATGSL10*, *CALS9*, *T1B9.18*	AT3G07160	*atgsl10-1*	Exon 2208	SALK_060111
			atgsl10-2	Intron 4763	SALK_031800
			atgsl10-3	Promoter −547	SALK_023245
AtGSL11	*F17J16.150*	AT3G59100	*atgsl11-1*	Exon 3179	SALK_019539
			atgsl11-2	Intron 5577	SALK_054207
			atgsl11-3	Promoter −402	SALK_073636
AtGSL12	*gsl12*, *T24H18.170*, *T24H18_170*	AT5G13000	*atgsl12-1*	Promoter −896	SALK_018046
			atgsl12-2	Promoter −986	SALK_003469
AtMYB44	*MYBR1*, *ATMYBR1*, *K8K14.2*, *K8K14_2*	AT5G67300	*atmyb44*	Promoter −96	SALK_008606

[a] *GLS*, *GLUCAN SYNTHASE-LIKE.*

[b] AGI, Arabidopsis Genome Initiative.

[c] Mutagenesis map and characterization of each mutant can be found in The Arabidopsis Information Resource database (*http://www.arabidopsis.org*). The numbers refer to nuclear acid sites of T-DNA insertion at the gene DNA sequences.

by acclimatization in WT *Arabidopsis* grown in the chamber (22°C; 14 h illumination at 250 μE/m^2/s). The colony was maintained in nursery WT *Arabidopsis* seedlings and was transferred to fresh plants every 2 weeks. Uniform 10-day-old apterous agamic females were used in this study and were transferred to experimental plants with a fine Chinese writing brush.

2.3 *Plant colonization*

Plants were treated with pure water and purified $HrpN_{Ea}$ separately. Water-treated and $HrpN_{Ea}$-treated plants were distributed alternately in 45×30 cm plastic trays. Four days after treatment, plants were fed by root irrigation with a 2 μM aqueous solution of hexyl rhodamine B (HRB), a rose fluorescence dye with the chemical character of water solubility and plant-permissible diffusion (Boevink *et al.* 1996; Zheng *et al.* 2005). Twenty-four hours later, HRB-fed plants were colonized with uniform 10-day-old aphids by placing the aphids on lower sides of the top two expanded leaves of the plants (10 aphids per leaf). A total of 1200 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. The leaves and aphids were observed at intervals in 24 h using a fluorescence microscope of low magnification. Aphid feeding activities were judged on the basis of HRB fluorescence from their esophagus globes. The number of feeding aphids in a leaf colony was scored.

2.4 *Aphid feeding behaviour*

Activities of aphids placed on leaves of HRB-fed plants were observed with the aid of HRB fluorescence, either from wounds caused by the stylet puncturing of leaves or from esophagus globes of feeding aphids. HRB fluorescence spots on leaves were regarded as a result of spillage of the plant sap. The term 'feeding aphids' was used to indicate aphid individuals that had accomplished a feeding process and had ingested the plant sap, seen as strong signals of HRB fluorescence from esophagus globes of the insects. The effect of $HrpN_{Ea}$ on feeding activities was quantified as a percentage decrease in the number of feeding aphids using the formula:

$$\text{Effect of } HrpN_{Ea} \text{ treatment } (\%) = 100 \times (x - y) \div x.$$

Here, x and y refer to proportions of feeding aphids in plants treated with water and $HrpN_{Ea}$, respectively.

Aphid feeding activities were also 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 10-day-old aphids were placed on the upper sides 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 the experiments. Immediately after the aphids were placed on the leaves, a 20-mm-diameter gold wire was attached to the dorsal surface of each aphid's abdomen using the silver conductive paint. The other end of the wire was connected to a four-channel Giga-4 direct-current amplifier with 10^9 Ω input resistance in an electrical circuit that was also connected to the plant via an electrode placed in the soil. The behaviour of individual aphids was monitored for 4 h. Voltage waveforms were digitized at 100 Hz with an A/D converter USB device. Waveform patterns were identified according to previously described categories (Tjallingii and Esch 1993). Waveform recordings were dissected every 5 s with the EPG analysis software STYLET 2.5 installed in a computer connected to Giga-4 direct-current amplifier.

2.5 *AtGSL5 expression and callose deposition*

The gene expression in $HrpN_{Ea}$-treated plants and water-treated control plants was determined immediately and 3 days after treatment. Total RNA isolated from plant tops and the top two leaves was subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) performed using superscript II RNAse H^- Reverse Transcriptase (Invitrogen) and using the *EF1α* gene as a reference (Peng *et al.* 2003). Specific primers were synthesized as per reported sequences of *AtGSL5* and *EF1α* (*Arabidopsis* Genome Initiative Numbers AT4G03550 and AT1G07930). Primers specific to *AtGSL5* were 5′-CCGCCACCGCACCGTCCCGCCG CAA-3′ and 5′-CGTCGTAGTTCCTCCACTTACAATG-3′, yielding a 752 bp product. Primers specific to *EF1α* were 5′-CCCCTTCGTCTCCCACTTCAGGATGTCTA-3′ and 5′-GTTGTCACCTGGAAGTGCCTCAAGAAG-3′, yielding a 189 bp product. RT-PCR products were confirmed by sequencing and sequence comparison. They were resolved by electrophoresis and visualized by staining with ethidium bromide.

Callose deposition in $HrpN_{Ea}$-treated plants and water-treated control plants was determined 3 days after treatment. Callose deposition in leaves was visualized as a violet colour by staining with aniline blue (Reuber *et al.* 1998; Zhang *et al.* 2009). The top two leaves were infiltrated with 5 ml of a solution made of phenol, glycerol, lactic acid, water and 95% ethanol (1:1:1:1:2, v/v). Leaves in solution were incubated in a 65°C bath until they were judged clear and then stained with aniline blue. The staining reaction was held in the dark for 4 h. Samples were observed by microscopy under ultraviolet field. Leaf photos were resolved by Adobe Photoshop to determine relative levels

of callose in leaves. The size of a leaf and the size of callose-deposited areas were determined using the Magic Wand and Edit Tools of Adobe Photoshop as described (Kwack *et al.* 2005; Sun *et al.* 2010). The relative level of callose in a leaf was qualified as the ratio of violet callose-deposited area vs. leaf size.

2.6 *Data treatment*

The experiments were repeated three to five times with similar results. The Student's *t*-test was used to compare data obtained from $HrpN_{Ea}$-treated plants with those obtained from water-treated control plants, and to compare data obtained from the WT and mutant plants. Quantitative data were also analysed using the ANOVA test to compare differences among plants of the WT and different mutants.

3. Results

3.1 *Plant treatment with $HrpN_{Ea}$ reduces proportion of feeding aphids*

We devised a luminescence assay using HRB, a fluorescence dye with the chemical character of water solubility and plant-permissible diffusion (Boevink *et al.* 1996), to monitor *M. persicae* behaviours after colonization of *Arabidopsis* leaves. After the plants were fed by root irrigation with an aqueous HRB solution, leaves were stained rose, whereas feeding sites appeared as orange spots when uniform apterous females of the insect were placed on the leaves (10 aphids/leaf) of HRB-fed plants (figure 1A, section a). The stylet puncturing of leaves occurred in 1 h (figure 1A, section b); puncturing sites appearing as orange fluorescence spots was thought to indicate spillages of the plant sap from wounds caused by the stylet puncturing of leaves (figure 1A, sections b–c). Subsequently, the number of puncturing sites and the number of plant sap spillages increased with time during the course of 24 h monitoring experiments (figure 1A, sections b–c). At the 24th hour, strong signals of HRB fluorescence were found in esophagus globes of aphids on leaves of water-treated control plants but not $HrpN_{Ea}$-treated plants (figure 1B), suggesting that feeding activities were repressed by the plant treatment with $HrpN_{Ea}$. The luminescence assay method was used to investigate 24 h feeding activities of a large-scale aphid population of 1200 aphids placed on $HrpN_{Ea}$-treated plants and water-treated control plants, respectively. As shown in figure 1C, $HrpN_{Ea}$-treated plants had much smaller number of feeding aphids than water-treated control plants (Student's *t*-test, $P<0.01$) at each time point from 1 h after colonization. The largest degree of difference in the number of feeding aphids between $HrpN_{Ea}$-treated and control plants was found at the 4th hour. Similar degrees of difference were observed subsequently. The proportion of feeding aphids remained smaller in $HrpN_{Ea}$-treated plants than in control plants (figure 1C), suggesting that the $HrpN_{Ea}$ treatment induced a deterrent effect on *M. persicae* feeding on the plant.

3.2 *Tests of 37 atgsl alleles identify atgsl5 and atgsl6 as compromised in $HrpN_{Ea}$-induced repression of aphid feeding activities*

To determine if any of the 12 *AtGSL* genes already identified in *Arabidopsis* (Hong *et al.* 2001; Verma and Hong 2001; Chen and Kim 2009) plays a role in $HrpN_{Ea}$-induced deterrent effect on *M. persicae* feeding activities, we investigated *Arabidopsis* mutants generated previously by T-DNA insertion into the individual *AtGSL* genes (Alonso *et al.* 2003; Jacobs *et al.* 2003; Nishimura *et al.* 2003; Enns *et al.* 2005; Huang *et al.* 2008; Töller *et al.* 2008; http://www.arabidopsis.org). A total of 37 mutants were selected as representative of mutagenesis alleles of the individual *AtGSL* genes, including 3 and 4 mutation alleles of *AtGSL5* and *AtGSL6*, respectively (table 1). The 37 mutants were compared with the WT plant in terms of response to $HrpN_{Ea}$ and the effects on *M. persicae* feeding. Feeding activities were visualized by the HRB fluorescence assay applied to 1200 aphids in each of the plant genotypes treated with $HrpN_{Ea}$ or water. As shown in figure 2, different genotypes of the plant varied greatly in regard to aphid feeding activities and the effect of $HrpN_{Ea}$. The proportion of feeding aphids was decreased by $HrpN_{Ea}$ treatment compared with water treatment control (Student's *t*-test, $P<0.01$) in the WT plant, and also in 30 *atgsl* mutants rather than *atgsl5* and *atgsl6*. In *atgsl5* and *atgsl6* mutants, especially *atgsl5-1* and *atgsl6-2*, $HrpN_{Ea}$ treatment did not reduce the proportion of feeding aphids to a significant extent. These observations suggested that *atgsl5* and *atgsl6* were both inhibitive to the deterrent effect of $HrpN_{Ea}$ on aphid feeding. Based on multiple comparisons (ANOVA test, $P<0.01$), the three *atgsl5* alleles were found to be more inhibitive than the four *atgsl6* alleles, and *atgsl5-1* was the strongest inhibitor of the $HrpN_{Ea}$-induced deterrent effect on *M. persicae* feeding activities. So, both *AtGSL5* and *AtGSL6* genes were important for the effect of $HrpN_{Ea}$ with *AtGSL5* being more critical than *AtGSL6*.

3.3 *Deterrent effect of $HrpN_{Ea}$ on aphid feeding occurs mainly in phloem phase*

To further elucidate the effects of $HrpN_{Ea}$ and *AtGSL* genes on *M. persicae* feeding from *Arabidopsis*, feeding activities and the phloem-feeding behaviour in particular were studied

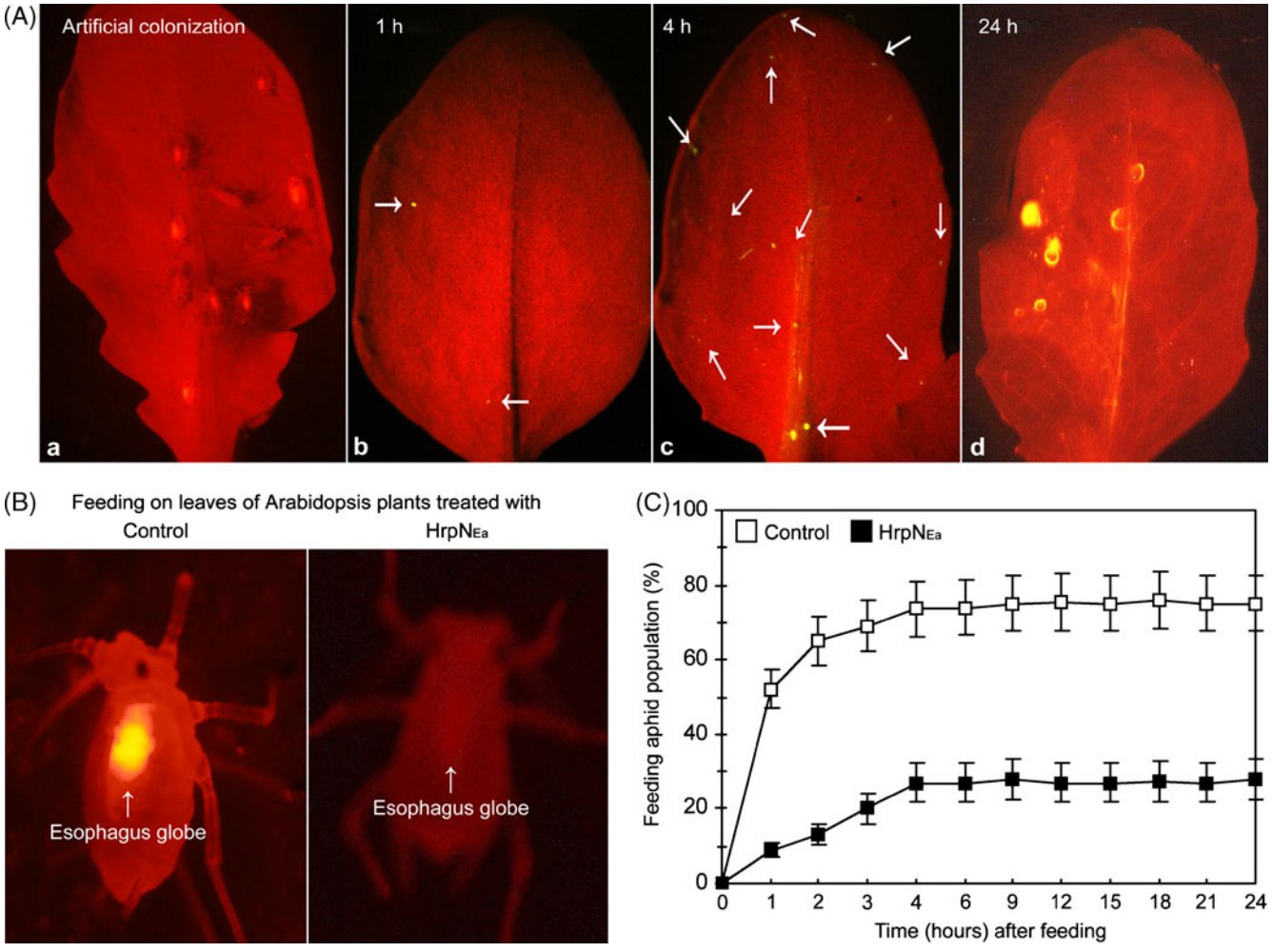

Figure 1. Hexyl rhodamine B (HRB) fluorescence assays to observe the green peach aphid *Myzus persicae* feeding on leaves of wild-type (WT) *Arabidopsis* plants. (**A**–**C**) Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Four days later, treated plants were fed by root irrigation with an aqueous HRB solution. Twenty-four hours later, the top two expanded leaves of HRB-fed plants were colonized by uniform aphids with 10 adults placed on the lower side of the top two expanded leaves. Leaves and aphids were observed with a fluorescence microscope. (**A**) Leaves of $HrpN_{Ea}$-treated plants photographed immediately (section **a**) and at the indicated time points (sections **b–c**) after colonization. (**B**) Aphids on leaves photographed at the 24th hour after colonization. (**C**) A 24 h survey on proportions of feeding aphids monitored at a large-scale population (1200 aphids/treatment). The curves represent mean±standard deviation (SD) of the results obtained from four repetitions of experiments (15 plants/repetition; 20 aphids/plant).

by the EPG technique. The technique was used to first characterize the feeding behaviour and the effect of $HrpN_{Ea}$ treatment. As shown in figure 3A, aphid feeding activities were detected in the EPG as different waveform patters recognized according to the standard previously established (Tjallingii and Esch 1993) and widely used (Tjallingii 1987, 2006; Klingler *et al.* 2005; Mutti *et al.* 2008). Based on a 4 h EPG record of aphid feeding activities, it was observed that all the 20 aphids tested in five repetitions of the experiments for $HrpN_{Ea}$-treated plants and water-treated control plants, respectively, accomplished major steps of the feeding process, but aphid activities varied greatly depending on the feeding process (figure 4; table 2) and varied greatly with different genotypes of the plant (figures 3 and 4).

In the WT plant, the proportion of time that aphids spent outside the cuticle (nonpenetration; figure 3A, np) until the first stylet puncturing of leaf cells (figure 3A, probe) under the condition of $HrpN_{Ea}$ treatment was similar to that under the condition of water treatment control (table 2). Under both conditions, equivalent proportion of time was used to penetrate between cells en route to the vascular tissue, referred to as the pathway phase in the EPG (figure 3A, path; table 2). The pathway phase represents insect's efforts in navigating the phloem

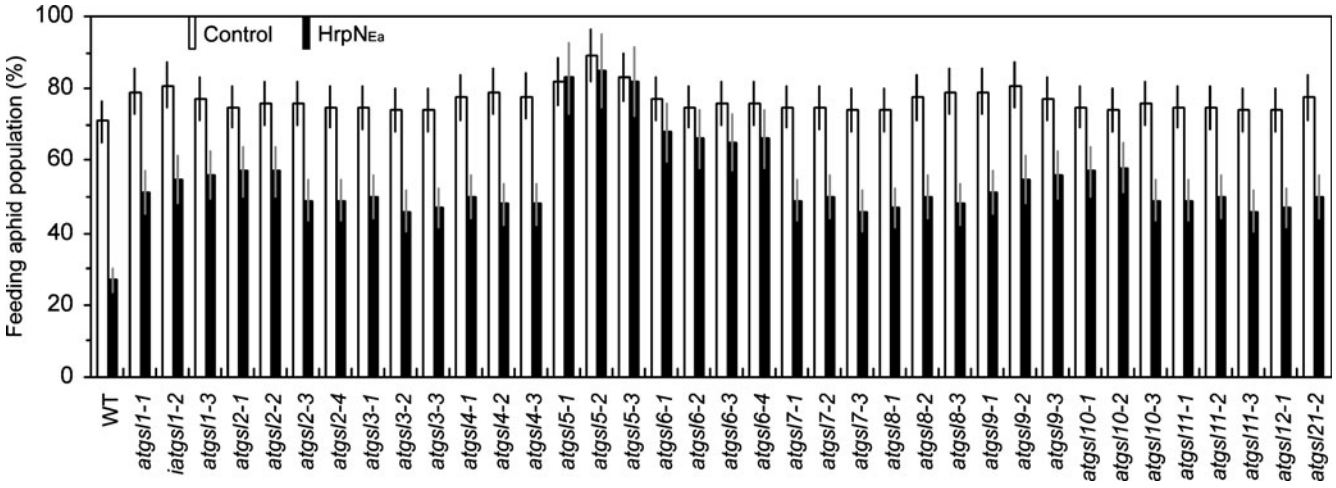

Figure 2. Quantification of proportions of aphids feeding on wild-type (WT) *Arabidopsis* and the *atgsl* mutants that represent different mutation alleles of 12 *AtGSL* genes. Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Four days later, treated plants were fed by root irrigation with an aqueous hexyl rhodamine B (HRB) solution. Twenty-four hours later, the top two expanded leaves of HRB-fed plants were colonized by uniform aphids with 10 adults placed on the lower side of the top two expanded leaves. Leaves and aphids were observed with a fluorescence microscope. The number of feeding aphids that had the HRB fluorescence in their esophagus globes was scored. Histograms represent mean±SD of the results obtained from four repetitions of experiments (15 plants/repetition; 20 aphids/plant).

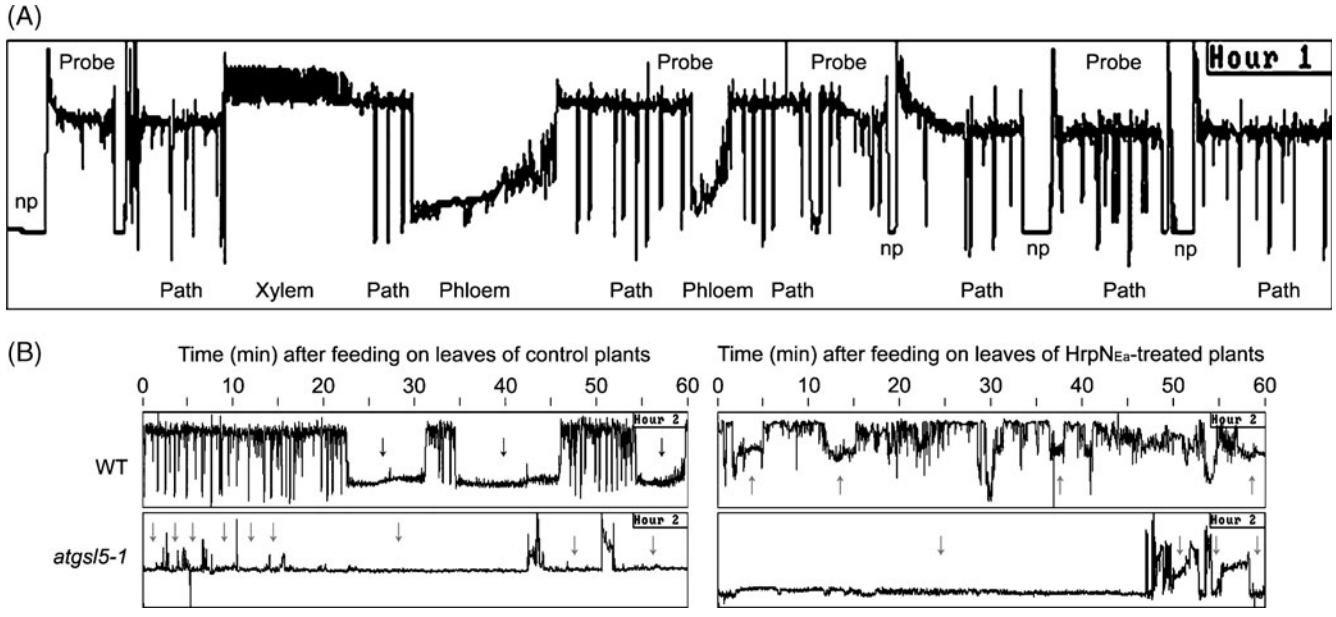

Figure 3. Electrical penetration graph (EPG) analyses of aphid feeding on *Arabidopsis* leaves. (**A**) The EPG waveforms obtained from untreated wild-type (WT) plants [Probe, the stylet puncturing of leaf; np, no probe (nonpuncturing); Path, pathway; Xylem, the xylem phase; Phloem, the phloem phase]. (**B**) The 2nd hour EPG patterns of aphid feeding from the WT plant and *AtGSL5*-deficient mutant *atgsl5-1*. Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Five days later, four uniform aphids were placed on the top first-expanded leaves of four plants; feeding behaviours were monitored by a four-channel Giga Amplifier system (Laboratory of Entomology, Wageningen Agricultural University, Wageningen, The Netherlands). Each EPG picture represents 20 aphids tested in five repetitions of the experiments.

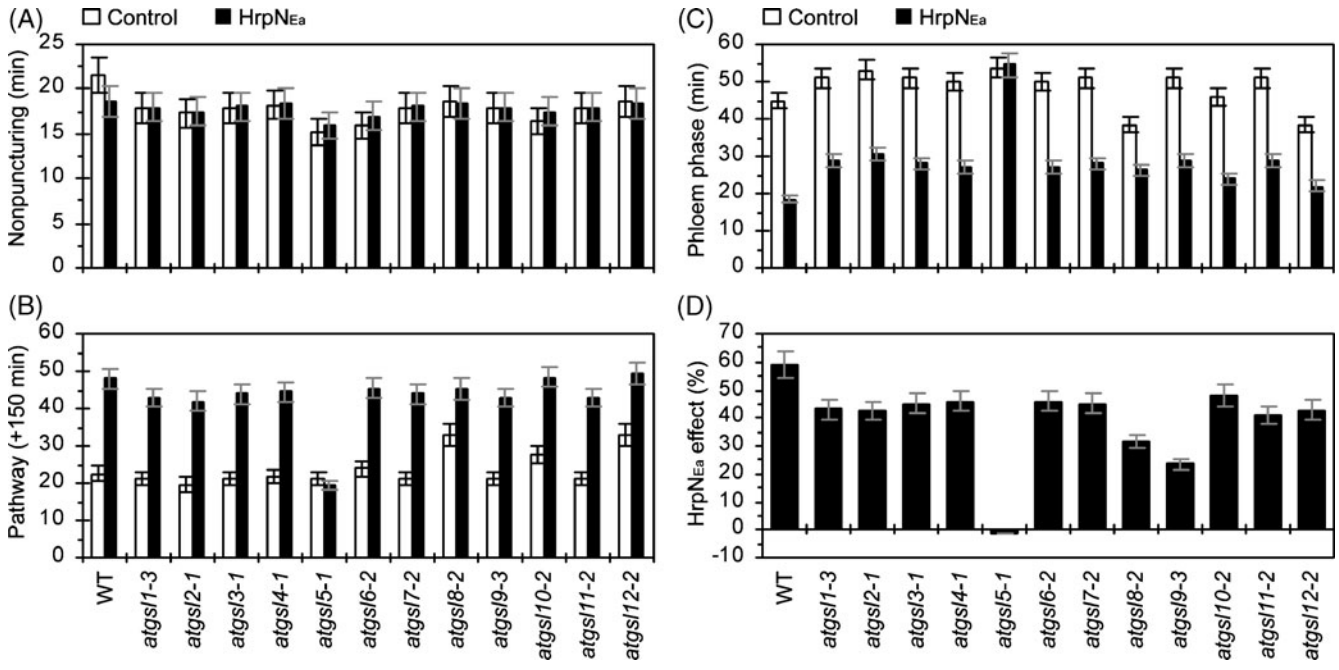

Figure 4. Key parameters of the EPG analyses of aphid feeding from the wild-type (WT) plant and representative *atgsl* mutants. (**A**–**C**) Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Five days later, four uniform aphids were placed on the top first-expanded leaves of four plants; feeding behaviours were monitored by the Giga Amplifier system (Laboratory of Entomology, Wageningen Agricultural University, Wageningen, The Netherlands). The total durations of the three phases were scored from 4 h EPG records. (**D**) Relative effect of $HrpN_{Ea}$ on the phloem-feeding activity. The effect in each genotype of the plant was quantified as a percentage reduction in total duration of the phloem phase under the condition of $HrpN_{Ea}$ treatment in contrast to water treatment control. Each histogram in (**A**–**D**) represents 20 aphids monitored in five repetitions of the EPG study.

and preparing to ingest sap from sieve elements (Klingler *et al.* 2005; Tjallingii 2006). Subsequently, aphid stylets may proceed to the phloem phase (figure 3A, phloem) in which ingestion of the phloem sap may occur (Tjallingii 2006). The pathway phase may be also connected with the xylem phase (figure 3A, Xylem), indicating stylet penetration of the xylem in the vascular tissue (Tjallingii 2006). The EPG records indicated that aphid activities did not

Table 2. Four-hour electrical penetration graph analyses of the green peach aphid *Myzus persicae* feeding from WT plant and an *atgsl5* mutant of *Arabidopsis*

Activity examined	WT		*atgsl5-1*	
	Control mean (SD)	$HrpN_{Ea}$ treatment mean (SD)	Control mean (SD)	$HrpN_{Ea}$ treatment mean (SD)
Total duration of nonpuncturing (min)	21.5 (5.0)^a	18.6 (4.8)^a	15.2 (3.5)^b	16.0 (4.0)^b
Time to first cell puncturing (min)	2.5 (0.6)^a	2.5 (0.3)^a	1.8 (0.4)^b	1.9 (0.4)^b
Time to first pathway (min)	3.8 (0.5)^a	3.3 (0.4)^a	2.3 (0.3)^b	2.4 (0.3)^b
Total duration of pathway phase (min)	172.5^a (45.0)	198.0 (58.5)^b	171.1^a (46.0)	169.5 (45.5)^a
Time to first phloem phase (min)	87.0 (11.5)^a	106.5 (13.2)^b	65.0 (9.5)^c	66.5 (9.6)^c
Number of phloem phase	7.0 (1.0)^a	3.2 (0.3)^b	6.5 (1.0)^a	7.0 (1.1)^a
Total duration of phloem phase (min)	45.0 (6.8)^a	18.5 (3.7)^b	53.7 (8.5)^c	54.5 (7.8)^c
Number of xylem phase	1^a	1^a	0^b	0^b
Total duration of xylem phase (min)	1 (0.2)^a	4.9 (1.3)^b	0^c	0^c

SD, standard deviation.

Different superscript letters indicate significant difference (ANOVA test, $P<0.01$).

change evidently in $HrpN_{Ea}$-treated plants compared with control plants until the stylet entry of vascular tissues (table 2). Proportions of time in the pathway phase and in the first phloem phase were much longer in $HrpN_{Ea}$-treated plants than in control plants, suggesting a $HrpN_{Ea}$-induced impediment to aphids in locating the ingestion site within the vascular tissue. Moreover, on $HrpN_{Ea}$-treated plants, fewer penetrations of the phloem were observed after the first stylet entry of phloem phase (table 2). These results suggest that phloem properties of $HrpN_{Ea}$-treated plants were unfavourable for feeding. Consistently, the total duration of the phloem phase was much shorter in $HrpN_{Ea}$-treated plants than in control plants (Student's *t*-test, $P<0.01$) during the course of the 4 h EPG monitoring (table 2). In particular, durations of the phloem phase in the 2nd hour of EPG monitoring were 32 and 15 min on average in control plants and $HrpN_{Ea}$-treated plants (Student's *t*-test, $P<0.01$), respectively, further supporting the deterrent effect of $HrpN_{Ea}$ treatment on the phloem-feeding activity of *M. persicae* (figure 4B).

3.4 *atgsl5-1 impairs deterrent effect of $HrpN_{Ea}$ on phloem feeding of aphids*

The effects of *AtGSL* genes on the phloem-feeding behaviour of *M. persicae* were elucidated by investigating 12 *atgsl* mutants using the EPG technique. The 12 mutants were selected because they represented highly effective alleles of *atgsl1* through *atgsl12* in suppressing the $HrpN_{Ea}$-induced deterrent effect on aphid feeding activities (figure 2). In the EPG experiments, the total duration of the non-puncturing phase in all the plants was close under conditions of water treatment control and $HrpN_{Ea}$ treatment, suggesting that the *atgsl* alleles had little effects on the stylet puncturing of leaf cells (figure 4A). However, feeding behaviours subsequent to the stylet entry of leaf tissues changed greatly due to the $HrpN_{Ea}$ treatment in WT and 11 *atgsl* mutants but not in *atgsl5-1*. In WT and the 11 mutants, the total duration of the pathway phase was longer in $HrpN_{Ea}$ treatment than in control (Student's *t*-test, $P<0.01$), suggesting that aphids incurred frustrations in navigating the phloem owing to the effect of $HrpN_{Ea}$ (figure 4B). In *atgsl5-1*, however, the total duration of the pathway phase in $HrpN_{Ea}$ treatment was as long as in the control (figure 4B). Moreover, in *atgsl5-1*, the total duration of the phloem phase were also close, suggesting that aphids fed from the phloem equally well, under $HrpN_{Ea}$ treatment and control conditions (figures 3B and 4C). On the contrary, the other 11 mutants performed as did WT in supporting the deterrent effect of $HrpN_{Ea}$ on the phloem-feeding activity. In these plants, total durations of the phloem phase were decreased significantly by the $HrpN_{Ea}$ treatment compared with the control (figure 5C; Student's *t*-test, $P<0.01$).

The relative degree of the $HrpN_{Ea}$-induced deterrent effect on the phloem-feeding activity in each genotype of the plant was quantified as the rate of decrease in total duration of the phloem phase under the $HrpN_{Ea}$ treatment condition in contrast to the control (figure 4D). Based on this criterion, only 3 of the 12 mutants were found to be inhibitive to the effect of $HrpN_{Ea}$. The three mutants were *atgsl5-1*, *atgsl8-2*, and *atgsl9-3* – all inhibited the effect of $HrpN_{Ea}$ to significant extents (ANOVA test, $P<0.01$). Comparatively, *atgsl5-1* proved to be most inhibitive to the $HrpN_{Ea}$-induced deterrent effect on phloem feeding by *M. persicae*, confirming the importance of *AtGSL5*.

3.5 *atgsl5-1 does not accumulate AtGSL5 transcript and callose*

The role of *ATGSL5* in repression of aphid feeding activities was correlated with induced expression of the gene in *Arabidopsis*. Three days after treatment with $HrpN_{Ea}$, the expression of *AtGSL5* was induced conspicuously in WT but not in *atgsl5-1* (figure 5A). Moreover, in response to $HrpN_{Ea}$, an abundant amount of callose was induced in WT rather than *atgsl5-1* (figure 5B). In WT, callose had a greater level when induced by the $HrpN_{Ea}$ treatment compared with control; in *atgsl5-1*, however, the relative level of callose was lower and did not change evidently in response to $HrpN_{Ea}$ (figure 5C).

3.6 *AtGSL5 expression and callose deposition are arrested in atmyb44*

Recently we showed that *AtMYB44* was the most $HrpN_{Ea}$-responsive of the 37 transcription factor genes tested and that *AtMYB44* was required for the induction of resistance to *M. persicae* in *Arabidopsis* (Liu *et al.* 2010a). In this study, we found that *AtMYB44* was required for the induction of *AtGSL5* expression and callose deposition. Both events were induced in WT but not *atmyb44*, an *Arabidopsis* mutant generated previously through T-DNA insertion at the promoter region of *AtMYB44* (Liu *et al.* 2010b; *http://www.arabidopsis.org*). The expression of *AtGSL5* was induced in WT but not in *atmyb44* following treatment with $HrpN_{Ea}$ (figure 5A). In contrast to the absence of *AtGSL5* expression in *atmyb44* and in water-treated control plants of WT, the gene was highly expressed in $HrpN_{Ea}$-treated WT plants tested 3 days after treatment. Moreover, in response to $HrpN_{Ea}$, an abundant amount of callose was induced in WT rather than *atmyb44* (figure 5B). In WT, callose had a greater level as induced by the $HrpN_{Ea}$ treatment compared with control; in *atmyb44*, however, the level of callose was lower and did not change evidently in response to $HrpN_{Ea}$ (figure 5C).

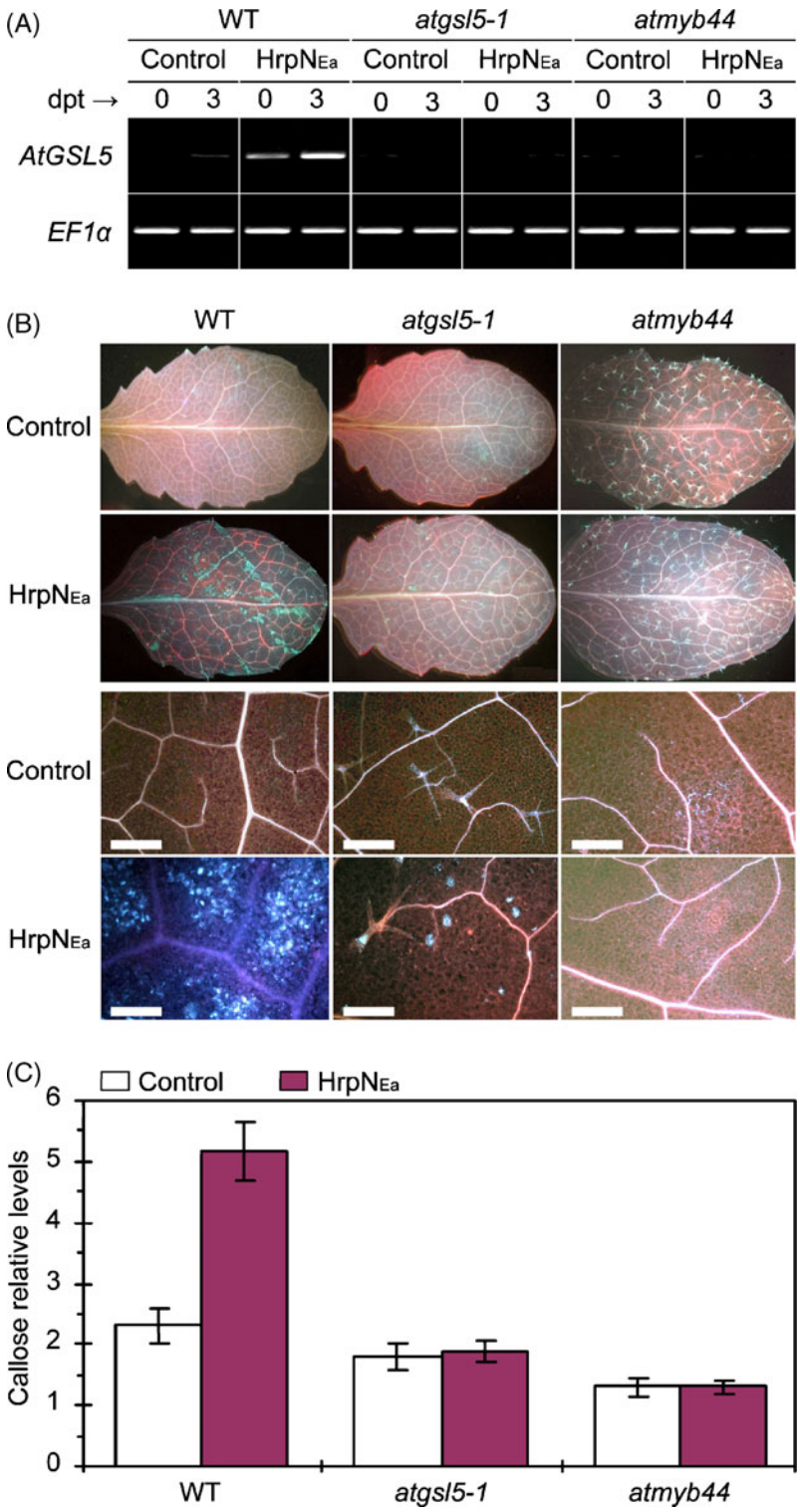

Figure 5. Determinations of *AtGSL5* expression and callose deposition in WT *Arabidopsis* and the *atgsl5-1* and *atmyb44* mutants that have defects in *AtGSL5* and *AtMYB44* genes. (**A**) Reverse transcriptase–polymerase chain reaction analysis of *AtGSL5* expression in plants treated with pure water (control) and purified $HrpN_{Ea}$, respectively [dpt, day(s) post-treatment; *EF1α*, a reference gene]. (**B**) Leaf callose deposition visualized as a violet colour by staining the leaves with aniline blue at 3 hpt (hours post-treatment) (scale bar, 1 mm). (**C**) Relative content of callose in leaves. Relative levels of callose were given as the violet callose-deposited area/leaf size ratio. Data are presented as mean±SD of the results obtained from three repetitions of experiments (15 plants/repetition).

3.7 *atmyb44 is more susceptible than atgsl5-1 to phloem feeding by aphids*

Comparison of data shown in figure 5 revealed that the amount of callose was smaller in *atmyb44* than in *atgsl5-1*. This difference was found to be consistent with the extents by which *atmyb44* and *atgsl5-1* compromised the $HrpN_{Ea}$-induced deterrent effect on aphid feeding activities. In parallel experiments, WT, *atmyb44* and *atgsl5-1* treated with water or $HrpN_{Ea}$ were colonized each with 1200 and 20 aphids, respectively, for monitoring by the HRB fluorescence and EPG assays. In comparison with WT, both *atmyb44* and *atgsl5-1* allowed greater proportions of feeding aphids (figure 6A) and longer durations of the phloem phase (figure 6B). However, *atmyb44* differed from *atgsl5-1* in both assays. More aphids failed to feed from *atgsl5-1* than from *atmyb44* in cases of both control and $HrpN_{Ea}$ treatment (figure 6A). In *atgsl5-1*, a smaller proportion of feeding aphids was scored in the case of $HrpN_{Ea}$ treatment compared with control (Student's *t*-test, $P<0.01$). In *atmyb44*, however, the proportion of feeding aphids in the case of $HrpN_{Ea}$ was similar to that in control. Based on 4 h EPG records, the total duration of the phloem phase was longer in *atmyb44* than in *atgsl5-1* and was reduced by $HrpN_{Ea}$ treatment to a greater extent in *atgsl5-1* than in *atmyb44* (figure 6B). These analyses suggested that *atmyb44* was more susceptible than *atgsl5-1* while both mutants were more susceptible than WT to phloem feeding by aphids. Moreover, *atmyb44* was more inhibitive than *atgsl5-1* to the deterrent effect of $HrpN_{Ea}$ on aphid feeding activities, especially the phloem-feeding behaviour (figure 6C).

4. Discussion

One purpose of this study was to screen and identify *GLS* genes involved in the induction of resistance to *M. persicae* in *Arabidopsis* following treatment with $HrpN_{Ea}$, a bacterial harpin protein (Wei *et al.* 1992). Previously, the induced resistance was shown to inhibit colonization of *Arabidopsis* by *M. persicae* (Liu *et al.* 2010a) and to inhibit multiplication of the insect on the plant (Dong *et al.* 2004). HRB fluorescence assays suggest that the $HrpN_{Ea}$ treatment causes a decrease in feeding population of aphids that colonizes leaves of treated *Arabidopsis* (figure 1). Use of the technique to investigate previously generated 37 *atgsl* alleles has identified *atgstl5* and *atgsl6* as mostly compromised in $HrpN_{Ea}$-induced repression of aphid feeding activities (figure 2). The 37 mutants represent different mutagenesis alleles of the 12 *AtGSL* genes (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Enns *et al.* 2005; Huang *et al.* 2008; Töller *et al.* 2008; *http://www.arabidopsis.org*) previously identified in *Arabidopsis* (Hong *et al.* 2001; Verma and Hong 2001; Chen and Kim 2009). The different mutation alleles of individual *AtGSL* genes perform consistently in response to $HrpN_{Ea}$ and in the effect on aphid feeding activities (figure 2), suggesting that the response and effect are a stable attribute of the genes under the conditions in this study. The *atgsl* alleles mostly inhibitive to the effect of $HrpN_{Ea}$ include *atgsl5-1* and *atgsl6-2*. Subsequent studies by the EPG technique suggest that the deterrent effect of $HrpN_{Ea}$ on aphid feeding activities occurs mainly in the phloem phase (figures 3 and 4; table 2). It is *atgsl5-1*, rather than *atgsl6-2*, that markedly compromises, but does not eliminate, the deterrent effect (figure 4), suggesting that *AtGSL5* is required for a significant proportion of the effect.

Apart from *atgsl5-1*, the other 11 *atgsl* alleles seem somewhat inhibitive to the effect of $HrpN_{Ea}$ (figure 4), indicating that the *AtGSL* genes other than *AtGSL5* play some roles in repressing the phloem-feeding activity. This result indicates possible redundancy in functions of *GSL* genes. Different *GSL* genes exhibit partially redundant roles in plant growth, development and defences (Chen and Kim 2009). It has been shown that the GSL5 enzyme is responsible for the synthesis of wound- and pathogen-inducible callose in leaf tissue (Jacobs *et al.* 2003; Nishimura *et al.* 2003; Wawrzynska *et al.* 2010). GSL5 also plays an important role in exine formation and pollen wall patterning (Dong *et al.* 2005b; Enns *et al.* 2005). When the *AtGSL5* gene plays a dominant role in the $HrpN_{Ea}$-induced deterrent effect on the phloem-feeding activity of aphids, other *AtGSL* genes may be partially redundant in function with *AtGSL5* and play additive roles in the effect. The representative *atgsl1* though *atgsl12* alleles were investigated (figure 2; table 1) because genetic evidence regarding functions of different *GSL* genes was available for several but not all of the genes (Kaliff *et al.* 2007; Dong *et al.* 2008; Huang *et al.* 2008; Töller *et al.* 2008; Chen *et al.* 2009b; Consonni *et al.* 2009; Xie *et al.* 2009). However, the present study does not provide information about contributions of the different individual *AtGSL* genes to the repression of aphid feeding activities except evidence of the major role of *AtGSL5* in the $HrpN_{Ea}$-induced deterrent effect on the phloem-feeding behaviour.

A related issue is that evidence is lacking in terms of regulation of *GSL*s' defensive function. In *Arabidopsis*, $HrpN_{Ea}$-induced resistance to *M. persicae* develops via the ethylene signalling pathway (Dong *et al.* 2004). The pathway uses different regulators to fulfill distinct functions (Guo and Ecker 2004; Chen *et al.* 2009a; Camehl *et al.* 2010). EIN2 is an integral membrane protein and an essential regulator of ethylene signalling, and therefore is indispensable for multiple processes, including defense responses (Alonso *et al.* 1999; Wang *et al.* 2002; Dong *et al.* 2004; Camehl *et al.* 2010). In $HrpN_{Ea}$-treated plants, induced *EIN2* expression is critical for the induction of resistance to *M. persicae* (Dong *et al.* 2004). Induced *EIN2* expression is regulated by AtMYB44 (Liu *et al.* 2010b), a

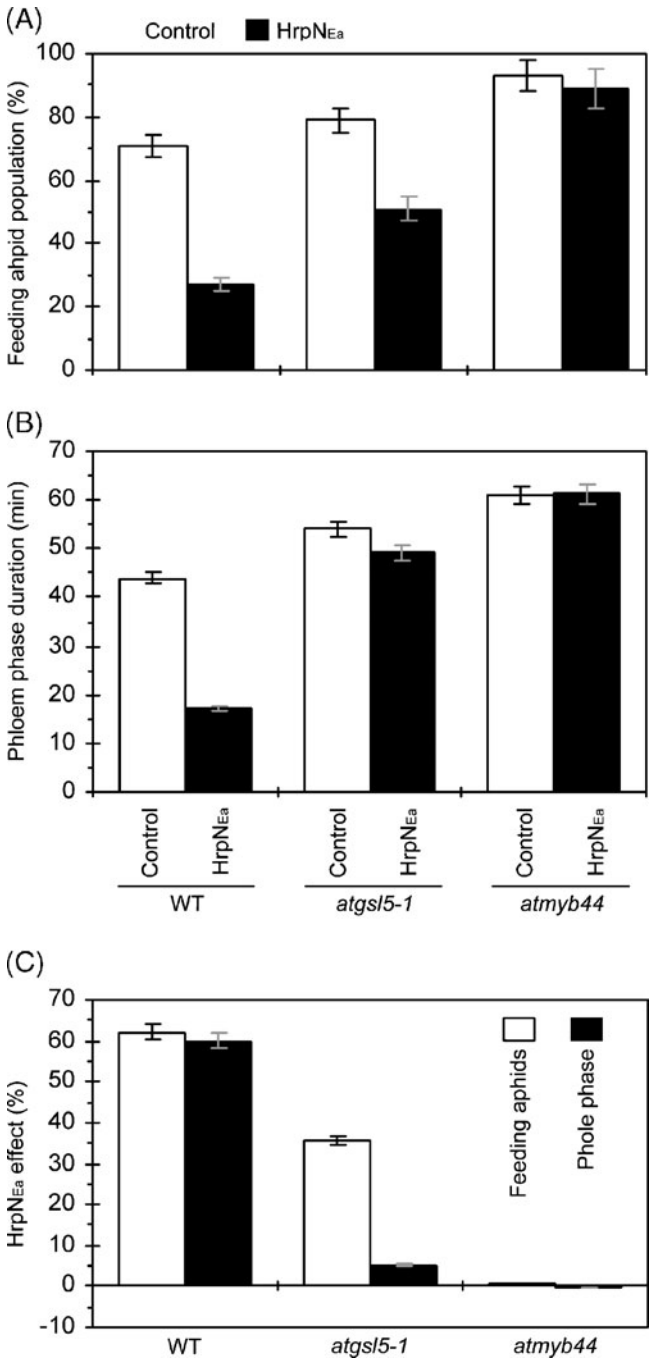

Figure 6. Comparison of WT, *atgsl5-1* and *atmyb44* in terms of effects on aphid feeding activities. (**A**) Proportions of feeding aphids. Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Four days later, treated plants were fed by root irrigation with an aqueous hexyl rhodamine B (HRB) solution. Twenty-four hours later, the top two expanded leaves of HRB-fed plants were colonized by uniform aphids with 10 adults placed on the lower side of the top two expanded leaves. Leaves and aphids were observed with a fluorescence microscope. Proportions of feeding aphids were scored (mean±SD) with a large-scale population – 1200 aphids per treatment-plant genotype combination, in four repetitions of the experiments. (**B**) Plants were treated with pure water (control) and purified $HrpN_{Ea}$ separately. Five days later, four uniform aphids were placed on the top first-expanded leaves of four plants; feeding behaviours were monitored by the Giga Amplifier system (Laboratory of Entomology, Wageningen Agricultural University, Wageningen, The Netherlands). The total duration of the phloem phase was scored from 4 h EPG records. (**C**) The effect of $HrpN_{Ea}$ on aphid feeding activities. Relative levels of the effect was quantified as a percentage reduction in the number of feeding aphids, or quantified as a percentage reduction in total duration of the phloem phase, under the condition of $HrpN_{Ea}$ treatment in contrast to water treatment control. Each histogram in (**B**) and (**C**) represents 20 aphids.

multifunctional transcription factor (Kirik et al. 1998; Kranz *et al.* 1998; Jung *et al.* 2008, 2010; Pitzschke *et al.* 2009). Downstream events were unclear.

Our evidence suggests that *AtMYB44* is required for $HrpN_{Ea}$-induced expression of *AtGLS5* and the concomitant deposition of callose in *Arabidopsis* (figure 5). *AtMYB44* is also required for the $HrpN_{Ea}$-induced deterrent effect on the phloem-feeding activity of *M. persicae* (figure 6). On comparison of the plant mutants *atmyb44* and *atgsl5-1*, *atmyb44* is found to be more susceptible to aphids and more inhibitive to the $HrpN_{Ea}$-induced deterrent effect on the phloem-feeding activity. These results indicate other components, besides AtGSL5 and callose, are involved in $HrpN_{Ea}$-induced resistance to *M. persicae* in the plant (Zhang *et al.* 2011), and especially support the function of AtMYB44 as a regulator of the induced resistance (Dong *et al.* 2004; Liu *et al.* 2010a,b). It would be great of interest to study in the future the physiological connection between AtMYB44 and AtGSL5 during induction of the defense.

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