Constitutive overexpression of the pollen specific gene SKS13 in leaves reduces aphid performance on Arabidopsis thaliana

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Abstract

Background: Plants have developed a variety of mechanisms to counteract aphid attacks. They activate their defences by changing the expression of specific genes. Previously we identified an activation tag mutant of Arabidopsis thaliana on which Myzus persicae population development was reduced. Activation tag mutants are gain-of-function in which the expression of a gene is increased by the insertion of the Cauliflower mosaic virus 35S enhancer that acts on the natural promoter. By further characterizing this previously identified mutant we identified a gene that reduces performance of M. persicae and also provided clues about the mechanism involved.

Results: We show that SKU5 SIMILAR 13 (SKS13), a gene whose expression in wild type plants is restricted to pollen and non-responsive to M. persicae attack, is overexpressed in the A. thaliana mutant showing reduced performance of M. persicae. Monitoring M. persicae feeding behaviour on SKS13 overexpressing plants indicated that M. persicae have difficulties feeding from the phloem. The constitutive expression of SKS13 results in accumulation of reactive oxygen species, which is possibly regulated through the jasmonic acid pathway. The enhanced resistance is not aphid species specific as also the population development of Brevicoryne brassicae was affected.

Conclusions: We demonstrate that constitutive expression in leaves of the pollen-specific gene SKS13 can enhance plant defence, resulting in a reduction of M. persicae population development and also decreases the transmission of persistent viruses. Overexpression of SKS13 in A. thaliana also affects B. brassicae and possibly other phloem feeding insects as well. Identifying genes that can enhance plant defence against insects will be important to open up new avenues for the development of insect resistant crop plants.

Keywords: Activation tag mutant, Brevicoryne brassicae, Electrical penetration graph, Jasmonic acid, Myzus persicae, Phloem-feeding insect, Reactive oxygen species

Background

Aphids have a sophisticated feeding strategy in which they use their stylets to penetrate plant tissue and puncture cells along the intercellular pathway towards the phloem [1]. To facilitate the probing and feeding processes, aphids secrete saliva into the plant tissue to degrade cell walls and to overcome occlusion of the feeding site [2,3]. Once an aphid establishes a feeding site it can feed from the phloem of a susceptible plant for hours or even days [1]. Aphid infestation limits plant productivity due to the depletion of photo-assimilates and the deposition of excess sugars as honeydew that encourages growth of mold. In addition, aphids are important vectors of numerous plant viruses that can be transmitted during probing and feeding, resulting in additional damage to plants [4].

Plants have evolved a series of defense traits to directly affect the aphid’s feeding behavior. These defenses include physical and chemical traits that can be constitutively present or induced upon aphid attack [5]. Physical traits, such as hairs and glandular trichomes, hinder aphid settling on a plant [6]. Chemical traits include the production of secondary metabolites and proteins that are repellent or toxic to aphids thereby affecting their performance [7]. For example, the brassicaceous-specific secondary metabolites glucosinolates have been shown to negatively affect the performance of the generalist...
aphid *Myzus persicae* [8]. Contrary to constitutive traits, inducible defenses require recognition of the attacking aphid and subsequent transcriptional reprogramming. This also includes the activation of general wound responses. An increasing body of evidence suggests that reactive oxygen species (ROS), which were always thought to be induced as a general wound response, can play a role in plant defense towards aphids as well [9,10]. For example, an early accumulation of ROS upon Russian wheat aphid infestation was suggested to be a defense response in aphid resistant wheat [11]. In contrast, an increasing concentration of ascorbic acid, a compound that is capable of reducing ROS, leads to an enhanced aphid fecundity [10], further underpinning the role of ROS in plant defense towards aphids. Moreover, ROS can act as signaling molecules, along with JA, to confer aphid resistance [12]. The activation of plant hormone pathways, especially jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), plays an important role in plant defense against aphids [13,14]. These pathways interact in a network, regulating the expression of specific groups of defense-related genes [15]. Although all pathways can be involved in defense, the JA pathway is thought to be the most effective against aphids [16,17]. Constitutive activation of the JA pathway in an *Arabidopsis thaliana* mutant leads to enhanced aphid resistance, whereas blocking the JA pathway results in aphid susceptibility [14]. It has been shown that certain genes, for instance *IQ-Domain1* and *MPL1* (*Myzus persicae* -induced lipase 1) can confer plant resistance to insects when their level of expression is increased or the location of expression is changed [18-20]. Such genes may be identified by screening activation tag mutant collections for insect resistance [18,21]. In these mutants, tagged genes are overexpressed by a tetramer *Cauliflower mosaic virus* (CaMV) 35S enhancer adjacent to the natural promoter, resulting in a dominant gain-of-function phenotype [22]. By screening such a mutant collection of *A. thaliana*, we have identified several mutants with enhanced resistance against *M. persicae* [23]. In the present paper we characterize one of these mutants, leading to the identification of *SKUS SIMILAR 13 (SKS13)* as a gene responsible for enhanced resistance to *M. persicae*. We analyzed the feeding behavior of *M. persicae* on the mutant using the electrical penetration graph (EPG) technique [24] to get information about the location of resistance factors. Based on the putative involvement of SKS13 in oxidation/reduction reactions we visualized the accumulation of ROS in leaves. Finally, we monitored the expression of several JA-, SA, and ethylene-pathway marker genes to study the possible interaction of SKS13 with these hormone pathways that may explain the aphid resistance conferred by SKS13 overexpression.

**Results**

**Phenotypic characterization of mutant 3790**

Mutant 3790 was previously identified as an *Arabidopsis thaliana* activation tag mutant on which *Myzus persicae* shows a longer pre-reproductive period and produces smaller numbers of offspring than on its corresponding wild type *Wassilewskija* (Ws) [23]. Compared to Ws, mutant 3790 has smaller and darker green colored leaves (Figure 1), shows a delayed flowering, a reduced height of the main stem and an increased number of lateral branches.

**Identification of SKS13 as a gene conferring enhanced resistance to *M. persicae***

Using inverse PCR we could determine that mutant 3790 contains a T-DNA including a 35S enhancer that is located on chromosome 3 at position 4,350,852 (according to the TAIR website; http://www.arabidopsis.org) in the 3’-UTR region of the *Brassinosteroid Receptor Like* gene (*BRL3*, At3g13380; Figure 2a). Additionally, two other genes, *SKU5 Similar 11 (SKS11, At3g13390)* and *SKU5 Similar 13 (SKS13, At3g13400)* are located within a distance of approximately 8 kb of the enhancer (Figure 2a), a distance over which the enhancer can effectively activate the expression of genes [25]. To determine whether the transcript levels of these three genes were affected by the enhancer, we first performed quantitative RT-PCR (qPCR). The transcript level of *BRL3* was two-fold higher in mutant 3790 than in Ws (Figure 2b). No transcripts of *SKS11* and *SKS13* were detectable in Ws but they could clearly be detected in mutant 3790 (Figure 2b).

As *A. thaliana* knockout mutants for many genes are publically available, we determined whether impaired expression of *BRL3* affects the performance of *M. persicae*. To this purpose, we performed no-choice aphid assays and

![Figure 1 Phenotype of *A. thaliana* mutant 3790. Rosette leaf phenotype of six-week old Wassilewskija (Ws) and mutant 3790.](http://www.biomedcentral.com/1471-2229/14/217)
compared *M. persicae* population development on *BRL3* knockout mutants *brl3-2* and *brl3-3* [26] with that on wild type Columbia-0 (Col-0). The numbers of *M. persicae* on these mutants (18.5 ± 5.6 on *brl3-2* and 16.2 ± 4.1 on *brl3-3*) did not differ from that on Col-0 (19.5 ± 7.0; Kruskal–Wallis followed by Mann–Whitney U test, *P* > 0.05, *n* = 15). Because *SKS11* and *SKS13* are not expressed in control leaves of Ws plants (Figure 2b), we performed a qPCR experiment to reveal whether these genes are induced upon infestation by *M. persicae*. Induced expression of *Lipoxygenase 2* (*LOX2*; data not shown) indicated an efficient infestation of *M. persicae* [27], but the expression of *SKS11* and *SKS13* remained undetectable in Ws leaves six and 24 hours after infestation of *M. persicae*. Therefore, we did not evaluate *M. persicae* performance on *SKS11* or *SKS13* knockout mutants.

Due to the orientation regarding the position of the transposon (Figure 2a) and strongest overexpression impact (Figure 2b), we decided to focus on *SKS13* for the continuation of this study. To confirm that overexpression of *SKS13* enhances resistance to *M. persicae*, we generated transgenic Col-0 lines (G101, G102 and G103) in which *SKS13* is overexpressed by the CaMV 35S promoter. Compared to Col-0, these lines showed significantly higher expression levels of *SKS13* (Figure 3a) and lower numbers of *M. persicae* (Figure 3b). Similar to mutant 3790, plants of these transgenic lines had smaller, rounder rosette leaves than their corresponding wild type (Figure 3c), and delayed flowering. The height of the main stem and the numbers of lateral branches of plants from these transgenic lines did not differ from Col-0.

**Feeding behavior of *M. persicae* on mutant 3790**

To reveal whether aphid feeding behavior was affected by overexpression of *SKS13* we compared electrical penetration graph (EPG) [1] recordings of *M. persicae* on mutant 3790 and Ws plants. The EPG parameters relevant for our study are summarized in Table 1. No differences were observed for EPG parameters related to epidermal or xylem tissue. Also the total time of the pathway phase was similar on Ws and mutant 3790 (Table 1). *Myzus persicae* showed a significantly longer duration of the non-probing phase on mutant 3790 than on Ws (Table 1). Significant differences were also observed for pre-phloem and phloem phase-related activities. Compared to Ws, *M. persicae* on mutant 3790 needed double the amount of time to the first phloem
Figure 3 Gene expression analysis, *Myzus persicae* aphid performance and phenotype of three independent SKS13 overexpressing transgenic lines. (a) Quantitative RT-PCR expression analysis of SKS13 in rosette leaves of Columbia-0 (Col-0) and the three transgenic lines G101, G102 and G103. Values are the means ± SD (n = 3). (b) Performance of *M. persicae* on plants of Col-0 and the three transgenic lines G101, G102 and G103. Values are the means ± SD (n = 15). Bars marked with different letters are significantly different from each other (Kruskal–Wallis followed by Mann–Whitney U tests, *P* < 0.05). (c) Rosette leaf phenotype of six-week-old Col-0 and transgenic lines G101, G102 and G103.

Table 1 Electrical Penetration Graph (EPG) parameters considered and their relation to *Myzus persicae* feeding activity on *Arabidopsis thaliana* Ws and mutant 3790

| Related tissue | EPG parameter                                | Wild type (Ws) n¹ = 18 | Mutant 3790 n = 15 | *P* value² |
|----------------|----------------------------------------------|-------------------------|--------------------|------------|
| Epidermal      | Time to first probe (min)                    | 2.5 ± 0.7               | 2 ± 0.4            | 0.870      |
|                | Prephloem                                    |                         |                    |            |
|                | Number of probes before first phloem contact | 10.5 ± 2.6              | 13.2 ± 4.6         | 0.969      |
|                | Time from first probe to first phloem contact (min) | 61.6 ± 12.4             | 113.6 ± 15.5       | 0.024      |
| Phloem         | Total time of phloem salivation (min)        | 10.8 ± 1.5              | 8.1 ± 1.6          | 0.025      |
|                | Number of phloem salivation events           | 14.3 ± 1.8              | 10.1 ± 1.8         | 0.240      |
|                | Average duration of phloem salivation (min)  | 0.8 ± 0.1               | 0.7 ± 0.1          | 0.462      |
|                | Total time of phloem ingestion (min)         | 97.5 ± 10.4             | 33.3 ± 8.2         | 0.001      |
|                | Number of phloem ingestion events            | 13.3 ± 1.6              | 8.7 ± 1.7          | 0.110      |
|                | Average duration of phloem ingestion (min)   | 7.8 ± 0.8               | 4.3 ± 1.0          | 0.003      |
|                | Total time of sustained (>10 min) phloem ingestion | 64.2 ± 9.4             | 20.4 ± 2.5         | 0.002      |
|                | Number of sustained (>10 min) phloem ingestion | 3.9 ± 0.6              | 1.2 ± 0.3          | 0.001      |
|                | Average duration of sustained (>10 min) phloem ingestion | 17.4 ± 2.5             | 12.7 ± 1.7         | 0.032      |
| All tissues    | Total time of non-probing (min)              | 106.4 ± 16.9            | 148.3 ± 11.3       | 0.023      |
|                | Total time of pathway phase (min)            | 247.5 ± 11.5            | 261.4 ± 13.3       | 0.278      |
|                | Number of aphids with sustained (>10 min) phloem ingestion | 18.0 (100%) | 11.0 (73%) | 0.030 |
| Xylem          | Total time of G                              | 15.2 ± 5.8              | 16.2 ± 4.7         | 0.912      |
|                | Number of G                                  | 0.7 ± 0.3               | 0.3 ± 0.2          | 0.195      |

Values are means ± SE of EPG parameters during 8 h monitoring. ¹EPG replicates; ²Mann Whitney U (duration) or Fisher exact (number) test *P* values.
phase, but spend only about one third of the total time in this phase (Table 1). Additionally, fewer *M. persicae* showed sustained phloem sap ingestion on mutant 3790 than on WS and the ones that did show this activity on mutant 3790 did this a smaller number of times (Table 1). Furthermore, aphids on Ws spent significantly more time salivating into the phloem and ingesting phloem sap than aphids on mutant 3790 (Table 1).

**Accumulation of reactive oxygen species in mutant line 3790**

*SKS13* has a putative function in oxidation/reduction reactions [28,29] and its co-expressed genes function in the generation of reactive oxygen species (ROS) [30,31]. Therefore, we hypothesized that overexpression of *SKS13* may lead to an accumulation of ROS in leaves. To visualize ROS we used 3-3’-diaminobenzidine (DAB) staining on the leaves of Ws, mutant 3790, Col-0 and transgenic line G101 (Figure 4). Each leaf was injured by forceps to serve as a positive control for the DAB staining [32]. In comparison to Ws and Col-0 leaves, darker browning was observed in leaves of mutant 3790 and transgenic line G010, respectively (Figure 4).

**Brevicoryne brassicae performance on mutant 3790**

It has been suggested that ROS accumulation plays a general role in plant defense against aphids [11,12]. Therefore, we hypothesized that *SKS13* overexpressing plants would not only affect the generalist *M. persicae* but also other aphid species. This hypothesis was tested by infesting mutant 3790 and Ws with the specialist *B. brassicae*. At 14 days after infestation, an average of four *B. brassicae* was found on mutant 3790 and 18 *B. brassicae* on WS plants (Mann–Whitney *U* test *P* < 0.001, *n* = 15).

**Effect of SKS13 overexpression on transcription of known JA-, SA- and ET-defense genes**

To determine whether overexpression of *SKS13* affects the plant hormone pathways known to be involved in plant defense against herbivorous insects, we monitored the expression levels of JA-, SA- and ET-marker genes in mutant 3790, Ws, *SKS13* overexpressing transgenic lines and Col-0 without aphid infestation. In leaves of mutant 3790 the expression levels of the JA-marker genes LOX2 (*Lipoxygenase 2*), VSP2 (*Vegetative Storage Protein 2*) and PDF1.2 (*Putative plant defensin 1.2*) as well as SA-marker genes PAD4 (*Phytoalexin Deficient4*) and PR1 (*Pathogenesis-related 1*) were similar as in leaves of Ws (data not shown). However, the expression level of the ET-marker gene ERF1 (*Ethylene response factor 1*) was significantly higher in mutant 3790 than in WS (Figure 5). Conversely to mutant 3790 (in Ws background), the *SKS13* overexpressing transgenic lines showed significant higher expression levels of the JA-marker genes compared to their corresponding wild type Col-0 (Figure 5). The SA- and ET-marker genes were not affected in these lines (data not shown).

**Discussion**

**Overexpression of SKS13 in leaves enhances resistance to M. persicae in a. thaliana**

Mutant 3790 was previously identified as an *A. thaliana* mutant on which the population development of *M. persicae* was reduced [23] and in the present paper we show that this is, at least partly, due to the constitutive overexpression of *SKS13*. The negative effect of *SKS13* on aphid population development was confirmed in transgenic plants that embraced the *SKS13* under the control of CaMV 35S promoter. An analysis of expression profiles in publicly available microarray data sets revealed that *SKS13* is exclusively expressed in pollen (https://www.genevestigator.com/) [30]. This is in agreement with our observation that *SKS13* was not expressed in leaves of Ws or Col-0. We also demonstrated that the expression of *SKS13* was not induced upon infestation of *M. persicae*. This is consistent with previous microarray studies in which no induction of *SKS13* expression in *A. thaliana* after *M. persicae* infestation was found [33-35].

**Overexpression of SKS13 affects feeding behavior of M. persicae probably due to ROS accumulation**

Analysis of *M. persicae* feeding behavior by the EPG technique can provide insight into the plant resistance

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**Figure 4 Accumulation of reactive oxygen species (ROS) in SKS13 overexpressing plants.** 3-3’-diaminobenzidine (DAB) staining of detached leaves from Ws, mutant 3790, Col-0 and *SKS13* overexpressing transgenic line G101. The arrows indicate the part of each leaf that was injured by forceps to serve as a positive control for the DAB staining.
mechanisms [36]. The EPG results suggest that plant resistance conferred by overexpression of SKS13 was phloem based. This was supported by the fact that the phloem phase of *M. persicae* on SKS13 overexpressing plants was delayed in time and reduced in length, while the length of the pathway phase was not significantly different from the control. The phloem based resistance was further indicated by the reduced number of sustained phloem sap ingestions. As sustained phloem sap ingestion is required for the transmission of persistently transmitted viruses [37], the phloem based resistance explains the decreased transmission of such a virus, i.e. *Turnip yellows virus*, as previously observed in mutant 3790 [23].

To uncover the role of SKS13 in the phloem based plant resistance to *M. persicae*, we explored the possible biological function of this gene. As structurally related to multiple-copper oxidases, ascorbate oxidases and laccases, SKS13 has been suggested to function in oxidation/reduction reactions [28,29]. Furthermore, SKS13 is co-expressed with genes involved in ROS generation (https://www.genevestigator.com) [30,31]. Therefore we hypothesized that constitutive overexpression of SKS13 results in an accumulation of ROS in leaves and confirmed this by DAB staining the leaves of SKS13 overexpressing plants. The effect of ROS accumulation on aphid feeding behavior has also been shown for a triticate cultivar with a high concentration of ROS on which cereal aphids displayed a reduced time in the phloem phase and a prolonged time in the non-probe phase [38]. This is similar to our observations of *M. persicae* feeding behavior on SKS13 overexpressing plants. The accumulation of ROS was suggested to play a role in plant resistance to several aphid species [11,38]. This is also in line with our results, as aphid resistance on SKS13 overexpressing plants not only affected *M. persicae* but also *B. brassicae* performance.

Besides enhancing aphid resistance, excessive ROS can damage proteins, lipids and nucleic acids and can eventual be harmful to plant growth [39], thereby explaining the reduced size of SKS13 overexpressing plants.

**Overexpression of SKS13 affects plant hormone pathways in A. thaliana**

Several studies suggest that ROS accumulation is linked with the JA, SA and ET plant hormone pathways to play a role in plant defense against aphids [11,12,40,41]. For instance, the *A. thaliana* *RbohD* mutant, in which JA-induced ROS accumulation does not occur, promotes a four times larger aphid population development than its wild type Col-0 [12,42], suggesting that aphid resistance conferred by activation of the JA pathway is probably mediated by ROS accumulation. In our study, we observed a similar activation of the JA pathway in SKS13 overexpressing Col-0 plants, as indicated by the significantly higher expression levels of three JA marker genes.

In mutant 3790, SKS13 is overexpressed in *A. thaliana* accession Ws and the ET pathway is activated instead of the JA pathway, which may be due to the genetic differences between Col-0 and Ws in response to ROS accumulation [43]. Furthermore, ROS may indirectly affect plant growth through altered signaling pathways. Kerchev et al. [44] concluded that a reduced Arabidopsis plant growth results from a low ascorbate, a compound that buffers the production of ROS, that triggers ABA- and JA dependent signaling. As ascorbate buffers the production of ROS, low levels of this compound would result in enhanced ROS accumulation. This is consistent with the observed higher ROS accumulation and reduced plant growth for both mutant 3790 and SKS13 overexpressing transgenic lines in our study. Our observation that signaling pathways were differently affected suggests that other factors may influence plant growth as well. In addition to SKS13,
the higher expression of BRL3 and/or SKS11 may contribute to this difference. Alternatively the additional differences may be attributed to unknown interactions among BR, ET and ROS. Studying the interaction between SKS13 and JA-/ET-mediated defense responses may lead to a better understanding of activation of JA and ET responses and their contribution to aphid resistance.

**Conclusions**

Overexpression of SKS13 in *A. thaliana* leads to a reduced phloem feeding of *M. persicae*, which probably is due to accumulation of ROS in leaves. The reduced phloem feeding results in the suppression of the population development of *M. persicae* and also decreases the transmission of persistent viruses. Overexpression of SKS13 in *A. thaliana* also affects *B. brassicae* and possibly other phloem feeding insects as well. The enhanced resistance towards *M. persicae* and *B. brassicae* in SKS13 overexpressing *A. thaliana* plants reduces plant development.

**Methods**

**Plants**

Mutant 3790, was previously identified from an *Arabidopsis thaliana* accession Wassilewskija (Ws) activation tag library as a mutant on which *M. persicae* showed a reduced population development [23]. Seeds of this mutant and its corresponding background accession Ws were obtained from the library present at Wageningen UR Plant Breeding [22]. Seeds of *brl3-2* and *brl3-3* mutants and their corresponding background accession Columbia-0 (Col-0) were kindly provided by Prof. S.C. de Vries, Laboratory of Biochemistry Wageningen University [26]. To induce germination, seeds were placed at 4°C in the dark for 3 days under high humidity. Subsequently, seeds were transferred to potting compost (Lentse Potgrond®) and plants were cultivated in a climate chamber (20 ± 2°C, RH 60-70%, 6 h: 18 h (light: dark). Plants were watered every other day and no pest control was applied. In all experiments we used plants in their vegetative stage, i.e. before they start flowering.

**Insects**

*Myzus persicae* (green peach aphid) was reared in cages on Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* cv. Granaat). *Brevicoryne brassicae* (cabbage aphid) was reared on Brussels sprouts (*Brassica oleracea* L. var. *gymnifera* cv. Cyprus) at the Laboratory of Entomology, Wageningen University. Both rearings were maintained in an acclimatized room at 20 ± 2°C, RH 60-70%, 18 h: 6 h (light: dark). Plants were watered every other day and no pest control was applied. In all experiments we used plants in their vegetative stage, i.e. before they start flowering.

**Inverse PCR**

Genomic DNA was extracted from leaves of mutant 3790 using the DNeasy Plant Mini kit (Qiagen), digested with restriction enzyme EcoRI (Thermo, product # ER0275) or BamHI (Thermo, product # ER0051) and subsequently ligated with T4 DNA ligase (Fermentas, product # EL0011). Inverse PCR was performed according to the method described previously [21]. PCR products were sequenced and then blasted against the *A. thaliana* genome (http://www.arabidopsis.org/) [45].

**Time course experiment of *M. persicae* infestation**

To determine if the SKS11 and SKS13 gene are induced upon *M. persicae* infestation, we performed a time course experiment. Four-week-old plants were infested with 15 wingless aphids of assorted life stages. Leaf material was collected after zero, six and 24 hours of aphid infestation. Aphids were gently brushed away from the leaf tissue and uninfested plants were also brushed. Leaf material was immediately flash frozen in liquid nitrogen and stored at −80°C until use.

**Quantitative RT-PCR**

Samples were designed in three biological replicates, with 17 individual plants pooled per replicate. Total RNA isolation, cDNA synthesis and quantitative RT-PCR (qPCR) were performed according to the methods described previously [21]. Gene specific primers were designed with Primer-3-Plus software [46] and are listed in Table 2. Threshold cycle (Ct) values obtained with qPCR were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *ACTIN8* (At1g49240) [47] from the Ct value of the gene of interest. Normalized gene expression was then calculated as $2^{-\Delta\Delta Ct}$ and Log-transformed prior to analysis. Independent-samples *t*-test or ANOVA followed by Tukey tests were used to determine the significance between genotypes/treatments ($P < 0.05$).

**Generation of transgenic plants**

To generate transgenic lines overexpressing SKS13, the coding region fragment of SKS13 was amplified from

Table 2 Sequences of gene specific primers used for quantitative RT-PCR analyses

| Gene name | Forward primer (5'→3') | Reverse primer (5'→3') |
|-----------|------------------------|------------------------|
| BRL3      | GGACATACCCGGGGAGTACCT  | CCGGTGCTCAGATTGGT    |
| SKS11     | CAACGTGGAATGGGACAGCG   | GGTGACAAAGCACCTTGA   |
| SKS13     | CATCAGCAAGGAGAAGCACCG  | CACTGCCGTTAAGGTCCAT  |
| LOX2      | AGATCCAAAGGAAAGCTCA    | ACAAACACAGCTCTGACTCT |
| VSP2      | TACGAAACGAAACCGAAGTTCT | GGCCAGCTGTAGCAGTCT   |
| PDF1.2    | CACCTCATATCTCCTGCTTC   | GCACAAATTCTCTGTCCCA  |
| PAD4      | GTTCTTTCCTGGCGCTATCG   | CCGTATACCCAGCTGTTTT |
| PRI       | GGCCCTAGGGGAAAAACTTA   | CTGCCTAACCACATGTCCA  |
| ERF1      | CTGCCGAGATTGCAATGCCG   | TCTGACCCGGGAAAGATCC  |
| ACTIN8    | GATGGAGAATCTCGGACACCTA| AAAAGGACTTGCTGGCAGGTC|
Col-0 cDNA using primers AttB1_SKS13_F (GGGGACAAATTTGTAACAAGAAAAAACGAGGCTAGTAGGAGGA GAGATTCAAAT) and AttB2_SKS13_R (GGGGACACATTGTAACAAGAAAAAGACTGAGTGTTCTCTCT CTTGATTGAAAATGA) in a PCR reaction containing the Phusion™ enzyme (Finnzymes, Product codes: F-5305, 100U). The following PCR program was used: 30 seconds at 98°C followed by 35 cycles of 98°C for 10 sec, 64°C for 10 sec, and 72°C for 3 min with a final extension at 72°C for 10 min. The resulting PCR product was extracted from a 1% agarose gel using the Qiaquick Gel Extraction Kit (Qiagen) and sequenced for verification. The verified coding region fragment of SKS13 was transferred into donor vector pDONR207 using the Gateway™ BP Clonase™ II enzyme mix (Invitrogen) to generate entry vector pDONR207::SKS13. The entry vector was subsequently cloned into Gateway Donor vector pFATT-R02 [48] using the Gateway LR™ Clonase™ II enzyme mix (Invitrogen) to generate the expression construct pFAST-R02-SKS13 in which SKS13 is under the control of the CaMV 35S promoter. The construct was transformed into E. coli and transformants were checked by colony PCR using primers AttB1_F (GGGGACAAATTTGTAACAAGAAAAAACGAGGCTAGTAGGAGGA GAGATTCAAAT) and AttB2_R (ACCACTTGTTTGGACGAGATGTTCTCTCTC TGGATTGAAAATGA). After verifying the accuracy of the coding region fragment of SKS13, the construct was transformed into Agrobacterium tumefaciens strain GV3101 [49] by electroporation. Agrobacterium mediated transformation [50] was used to introduce the pFAST-R02-SKS13 plasmid into Col-0 flowers. Seeds containing the construct were selected using fluorescence microscopy (Zeiss, StEREO Discovery.V8) [48].

No-choice aphid assays

Nymph producing adult aphids (both M. persicae and B. brassicae) were collected from rearing plants and placed on detached cabbage leaves in a petri dish overnight. Newly born one-day-old nymphs were placed in the centre of three-week-old A. thaliana plants using a fine brush. Each plant received one nymph and the total number of aphids was counted 14 days after infestation. The plants were randomly organized with 15 biological replicates per genotype. Plants were separated by a water barrier to prevent aphids crossing over from one plant to the other. The Mann–Whitney U test or Kruskal–Wallis test followed by Mann–Whitney U test were used to determine if differences between genotypes were significant (P < 0.05).

Electrical penetration graph

The electrical penetration graph (EPG) technique [24] was employed to monitor the feeding behavior of Myzus persicae. A gold wire (diameter 20 μm) was attached onto the dorsum of young adult aphids using conductive water-based silver glue. The wired aphid was placed on a mature leaf of a five-week-old plant that was connected to a recording system via a copper electrode in the soil. All tested aphids stayed at the underside of the leaf. The EPGs were recorded at 22°C with constant light for 8 hours. The EPG data were analyzed using the PROBE 3.0 software (Wageningen University, the Netherlands) to distinguish the various waveforms. Waveform C represents the pathway phase, when the aphid stylet is penetrating through the leaf tissue; waveform E2 represents phloem sap ingestion; Waveform F is associated with penetration difficulties; and waveform G indicates active uptake of water from the xylem. Both sequential and non-sequential parameters were analyzed [51] to characterize probing behavior of individual M. persicae. At least 15 recordings of individual aphids (one aphid per plant) were obtained for each genotype. The Mann–Whitney U and Fisher exact test were used to determine the significance difference between genotypes (P < 0.05).

Determination of reactive oxygen species (ROS) accumulation

To visualize reactive oxygen species (ROS), leaves were cut from four-week-old plants and submerged overnight in an HCl solution containing 1 mg mL⁻¹ 3-3′-diaminobenzidine (DAB), pH 3.7 [52]. Chlorophyll was extracted with 96% ethanol overnight at room temperature. Leaves were subsequently photographed in 80% glycerol.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

XC carried out the molecular and insect behavior studies, performed the DAB staining study, analyzed the data and drafted the manuscript. ZZ generated the transgenic overexpressing lines. BV and CB conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Cindy ten Broeke and Freddy Tjallingii for assistance with the EPG recordings; Gerrit Stunnenberg and Taede Stoker for taking care of the plants; Geet Steenhuis-Broers and Leon Westerd for looking after the aphid rearings; Roeland Vooip for suggestions on statistics; Wei Liu for assistance on molecular analysis; Weicong Qi for valuable discussions; three anonymous reviewers for their valuable comments and suggestions.

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Received: 28 March 2014 Accepted: 4 August 2014
Published: 14 August 2014

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doi:10.1186/s12870-014-0217-3

Cite this article as: Chen et al.: Constitutive overexpression of the pollen specific gene SKS13 in leaves reduces aphid performance on Arabidopsis thaliana. BMC Plant Biology 2014 14:217.