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RESEARCH PAPER

A mutation in amino acid permease *AAP6* reduces the amino acid content of the *Arabidopsis* sieve elements but leaves aphid herbivores unaffected

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Abstract

The aim of this study was to investigate the role of the amino acid permease gene *AAP6* in regulating phloem amino acid composition and then to determine the effects of this altered diet on aphid performance. A genotype of *Arabidopsis thaliana* (L.) was produced in which the function of the amino acid permease gene *AAP6* (At5g49630) was abolished. Plants homozygous for the insertionally inactivated *AAP6* gene had a significantly larger mean rosette width than the wild type and a greater number of cauline leaves. Seeds from the *aap6* mutant were also significantly larger than those from the wild-type plants. Sieve element (SE) sap was collected by aphid stylectomy and the amino acids derivatized, separated, and quantified using Capillary Electrophoresis with Laser Induced Fluorescence (CE-LIF). In spite of the large variation across samples, the total amino acid concentration of SE sap of the *aap6* mutant plants was significantly lower than that of the wild-type plants. The concentrations of lysine, phenylalanine, leucine, and aspartic acid were all significantly lower in concentration in the *aap6* mutant plants compared with wild-type plants. This is the first direct demonstration of a physiological role for an amino acid transporter in regulating SE composition *in vivo*. The amino acid availability in sieve element sap is thought to be the major limiting factor for aphid growth and reproduction. Despite the changes in their diet, the aphid *Myzus persicae* (Sulzer) displayed only small changes in feeding behaviour on mutant plants when measured using the Electronic Penetration Graph (EPG) technique. Salivation by the aphid into the SE (E1 phase) was increased on mutant plants but there was no significant effect on other feeding EPG behaviours, or in the rate of honeydew production. Consistent with the small effect on aphid feeding behaviour, there was only a small effect of reduced sieve element amino acid concentration on aphid reproduction. The data are discussed in relation to the regulation of phloem composition and the role of phloem amino acids in regulating aphid performance.

Key words: *AAP6*, amino acid, aphid, *Arabidopsis thaliana*, capillary electrophoresis, EPG, herbivore, *Myzus persicae*, phloem, sieve element.

Introduction

The translocation of organic nutrients round the plant largely occurs in the phloem. Tissues exporting nutrients ('sources', such as photosynthesizing leaves) load them into the elongated sieve tubes that link different parts of the plant. Water follows by osmosis generating a hydraulically driven bulk flow of sap through files of sieve elements until

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they are unloaded in tissues that are net importers of nutrients ('sinks', such as the root system and the growing shoot tips). The pressures exerted in the translocation system are obvious since sap exudes from the severed stylets of phloem-feeding aphids (Pritchard, 1996). The translocation system is of vital importance to plants since the delivery of sugars, amino acids, and other nutrients provides the solutes required to drive cell elongation and the metabolites required both for respiration and for the synthesis of the macromolecules that form new structures. Regulation of the delivery of solutes to different parts of the plant is critical for a fundamental understanding of growth and development and informs attempts to manipulate resource allocation (e.g. during grain filling) in crop plants.

While there is information on the individual solute transporters in some plant species, there is no comprehensive information on the transport processes that regulate concentrations of a wide range of solutes at sources and sinks (Pritchard, 2007). The solutes present at the highest concentrations in the phloem are sucrose, amino acids, and K^+ ions. There is an increasing amount of information about the phloem-located SUC2/SUT1 transporter that loads sucrose into sieve tubes (Stadler and Sauer, 1996; Doering Saad *et al.*, 2002; Slewinski *et al.*, 2009). Potassium ions are present at concentrations of between 50–150 mM and play a crucial role in the phloem transport of photoassimilates (Gould *et al.*, 2004). For example, *Arabidopsis thaliana* mutant plants lacking K^+ phloem channels (AKT2/3) were found to contain only half the sucrose content of the wild type. It is believed that they regulate the phloem osmotic potential which, in turn, controls the activity of sucrose transporters (Deeken *et al.*, 2002). Other work has indentified mRNAs for aquaporins, nitrate transporters, ATPases, CNG channels, ABC, and sucrose transporters in the sieve element sap of barley and *Ricinus* (Doering Saad *et al.*, 2002, 2006; Gaupels *et al.*, 2008).

Amino acids form the second most abundant class of organic compounds found in the phloem sap after sucrose (Rentsch *et al.*, 1998). Two main super-families of amino acid transporters have been identified so far: the amino acid, polyamine, and choline (APC) transporter super-family and the amino acid transporter (ATF) super-family (Fischer *et al.*, 1995). The plant APCs are subdivided into two subgroups: the cationic amino acid transporters (CATs) and proteins most homologous to the yeast γ-aminobutyric acid (GABA) permease-related family (Fischer *et al.*, 1998). The only transporter of the APC superfamily to have been characterized in any detail is AtCAT1 (Frommer *et al.*, 1995). Recently, a bi-directional amino acid transporter, BAT1 has been identified in *Arabidopsis* and its location in vascular tissue has led to the suggestion that it may be involved in phloem unloading (Dündar and Bush, 2009).

Much more is known about members of the ATF family and, to date, five subclasses have been defined. These include the amino acid permeases (AAPs) of which eight members have been identified so far (Fischer *et al.*, 1995, 1998; Okumoto *et al.*, 2002), the lysine, histidine transporters (LHTs) such as LHT1 (Chen and Bush, 1997), the proline transporters (ProTs) such as ProT1 and ProT2 (Rentsch *et al.*, 1996), the putative auxin transporters (AUXs) with AUX1 identified as an auxin carrier (Bennett *et al.*, 1996), and the aromatic and neutral amino acid transporter (ANT1) which has been identified as a transporter of aromatic and neutral amino acids as well as arginine and auxin (Chen *et al.*, 2001).

The AAPs (amino acid permeases) have been the most heavily studied group in the model species *Arabidopsis thaliana*. AAP1 was the first of the group to be characterized (Frommer *et al.*, 1993; Hsu *et al.*, 1993) and, subsequently, seven related permeases (AAP2–AAP8) have been reported in this species (Fischer *et al.*, 1995; Kwart *et al.*, 1993; Okumoto *et al.*, 2002). Some have been associated with SE-specific transport of amino acids (Frommer *et al.*, 1993; Kwart *et al.*, 1993; Fischer *et al.*, 1995, 2002; Hirner *et al.*, 1998; Okumoto *et al.*, 2002; Koch *et al.*, 2003; Slewinski *et al.*, 2009; Tedeger *et al.*, 2007; Williams and Miller 2001).

AAP6 has been characterized as a high affinity transporter, efficiently transporting neutral amino acids and other acidic amino acids (Rentsch *et al.*, 1996). *AAP6* was expressed mainly in sink tissues such as roots, sink leaves, and cauline leaves, which depend on the supply of nitrogen from other organs. GUS expression studies revealed that *AAP6* was also expressed in the xylem parenchyma cells of *Arabidopsis* (Okumoto *et al.*, 2002). The xylem contains concentrations of amino acids that are typically orders of magnitude below that of phloem sap (Bi *et al.*, 2007), thus movement of amino acids into the SE necessitates a high affinity transporter (Rentsch *et al.*, 1996) leading to the hypothesis that a function of AAP6 is the transfer of amino acids from the xylem to the phloem (Okumoto *et al.*, 2002).

As well as its central importance in plant growth and development, the phloem is also a key site for the interaction between plants and some sap-feeding insects. Aphids cause direct damage by withdrawing nutrients from the SEs; they also transmit viruses and the physical lesions and honeydew produced can encourage secondary infections. Aphid growth and reproduction are thought to be limited by the availability of nitrogen in the form of amino acids; a subset of nine amino acids are thought to be essential for aphid growth since they cannot be synthesized by the herbivore (Douglas, 2003; Wilkinson and Douglas, 2003).

In some studies, the nutritional quality of the sieve elements correlates positively with the performance and behaviour of the aphid herbivore. In potato, aphid performance increased at higher phloem amino acid levels (Karley *et al.*, 2002). Similarly, aphids feeding on barley growing with additional nitrogen had a higher reproductive rate than those feeding on nutritionally deprived plants (Ponder *et al.*, 2000). However, other studies have failed to demonstrate a positive correlation between SE amino acid concentrations and aphid performance. In a study on a range of native British grasses, SE amino acids increased following drought but aphid reproductive performance was reduced (Hale *et al.*, 2003).

In this study, experiments utilizing a mutation at the *AAP6* locus in *A. thaliana* are described. Having demonstrated that a T-DNA insertion results in the inactivation of the gene, the effects of this loss of function have been examined first at a general phenotypic level and then on the concentrations of amino acids within sieve elements. The latter experiments make use of the collection of sap from individual sieve elements followed by the quantification of individual amino acids in these small samples using a capillary electrophoresis-laser induced florescence (CE-LIF) protocol (Zhu *et al.*, 2005). The effects of changes in sieve element amino acid levels have then been followed in the aphid *Myzus persicae* with regard to its detailed feeding behaviour, rate of ingestion of sieve element sap, and reproduction. The aims of this study are 2-fold: to determine the role of AAP6 in regulating SE amino acid composition and to test the relationship between the amino acid composition of aphid diet and performance *in vivo*.

Materials and methods

Plant material

An *Arabidopsis thaliana* genotype putatively carrying a copy of T-DNA within gene At5g49630 (the *AAP6* gene) was identified in the Syngenta *Arabidopsis* Insertion Library (SAIL) collection (Garlic_1232_C08.b.1a.Lb3Fa) and obtained from the Nottingham *Arabidopsis* Stock Centre. Plants were grown in compost consisting of two parts John Innes loam-based compost, two parts peat-based compost, one part Silvaperl, and one part Osmocote (a slow release fertilizer) in a growth room maintained at 20–22 °C with an 18/6 h light/dark regime.

Genetic analysis of the aap6 *mutant*

The occurrence of a T-DNA insertion within an *AAP6* allele was confirmed using *AAP6*-specific primers (AAP6F; CGTTGAACAGAGCTTCCCGGAGC, AAP6R; GGAGCCTGGAAAGGCTTGAAATCC) and a T-DNA-specific primer (SAIL LB3; CTGAATTTCATAACCAATCTCGATACAC). The exact T-DNA insertion site was determined by sequencing appropriate amplification products. Individuals homozygous for (i) the *AAP6* allele carrying the T-DNA insertion, and (ii) the wild-type gene ('negative segregants') were initially identified by screening using PCR; tests were carried out on their progeny (following selfing) to confirm their homozygous status.

RT-PCR analysis

mRNA populations were extracted from the rosette leaves of wild-type and *aap6* mutant *Arabidopsis* plants and used to produce single-stranded cDNA using the SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. These cDNA populations were used as template DNA in PCRs using (i) primers specific to a positive control transcript (actin2: ACT2For: 5′-TGCTGACCGTATGAGCAAAG; ACT2Rev: 5′-CAGCATCATCACAAGCATCC) to give a product of 400 bp, or (ii) to the *AAP6* transcript (AAP6For: 5′-TCCCGGAGCATGAAATTGGC; AAP6Rev: 5′-GGCTTGAAATCCTTGAGACTTTG) to yield a product of 1400 bp. In both cases, the primers were designed to span at least one intron present in the gene so that artefactual amplification from the genomic DNA template would be detected. All PCR products were analysed using gel electrophoresis.

Southern blotting to test for T-DNA insertion number

DNA was extracted from wild-type and homozygous *aap6* mutant rosette leaves and digested using *Hin*dIII before separation by electrophoresis in a 0.8% agarose gel and blotting onto a nitrocellulose filter. A probe was designed against a ~350 bp region of the *bar* (BASTA) resistance gene, which is present in the T-DNA of SAIL lines but not elsewhere in the plant genome. This was amplified using primers BastaF (5′-CATGAGCCCAGAACGACGCC) and BastaR (5′-TCTTGAAGCCCTGTGCCTCC), ligated into pGEM-T, transformed into *E. coli* strain DH5α and its sequence verified. The *bar* fragment was excised from the pGEM-T plasmid and labelled with α-^{32}P cytosine using the hexanucleotide labelling method (Feinberg and Vogelstein, 1983) and was used to hybridize to complementary DNA on the filter before subjection to autoradiography using Kodak X-O-Mat film (Amersham).

Developmental screening of the aap6 *mutant*

Wild-type and homozygous *aap6* mutant plants were grown as described above in a random block design with a row of 'guard' plants around them. The stages of plant development were catalogued using the 'principal growth stages' of development indices developed by Boyes *et al.* (2001).

Measurements of seed volume were carried out as an indication of genotype fitness. Plants were bagged at 3–4-weeks-old (when flower buds became visible; growth stage 5.10) and not watered after 6–7 weeks (when the first siliques started to burst; growth stage 8.00). When drying was complete (growth stage 9.70), seed was collected, 100 seeds randomly selected from each individual plant, and the length and width of each seed measured under a microscope. The equation for the volume of an ellipse ($4/3 \times \text{width} \times \text{height} \times \text{length}$) was used to calculate the volume of the seeds.

Aphid material

Experimental aphids were taken from an anholocyclic *Myzus persicae* culture derived from a single individual and maintained at the University of Birmingham on well-watered *A. thaliana* (Columbia-0; Col-0) plants, grown in compost in a controlled environment growth room maintained at 20–22 °C with a 18/6 h L/D cycle. Only apterous aphids were used in the experiments. Prior to each experiment, first instar offspring from an individual aphid were taken from the culture and reared for two complete generations in sponge-sealed cylindrical clip cages on either homozygous *aap6* mutant or wild-type *A. thaliana* plants in order to eliminate maternal effects on subsequent analyses.

Sieve element (SE) sap collection and analysis

Plants were grown until approximately 4 weeks old and bolting had occurred (principal growth stage 5.1–6.0). Cylindrical specimen cages (Agar Scientific Ltd) were secured to the stems and left for 2–3 d. Between 5 and 10 *M. persicae* adults were then placed in each cage and left overnight. Stylectomy was performed on feeding aphids using high-frequency microcautery (Downing and Unwin, 1977), as outlined in Hale *et al.* (2003). Stylectomy was always performed in daylight h and at 18–20 °C. Two sets of between 8 and 12 samples of exuding SE sap were collected from the severed stylet bundles of aphids feeding on individual homozygous *aap6* mutant and wild-type plants: one set for amino acid analysis and the other for osmotic pressure measurements.

For amino acid analyses, SE sample droplets were expelled from microcapillaries into water-saturated paraffin oil (grade BP). Diameters of the suspended sample droplets were measured using a microscope. Sample volume was calculated using the formula for the volume of a sphere; $V=(4/3)\ \pi r^3$. Oil-free samples were then collected in further microcapillaries and then expelled into sterile 0.5 ml microcentrifuge tubes. Samples were stored at –20 °C prior

to analysis. Samples were air-dried, amino acids derivatized, and then separated and quantified using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) at the University of Nottingham as described previously (Zhu *et al.*, 2005). Picolitre osmometry was used to measure osmotic pressure by recording the depression of melting temperature of samples compared to pure water (Tomos *et al.*, 1994).

Aphid reproductive performance

Replicate *aap6* mutant and wild-type plants were set up in a randomized block design in a controlled growth room (conditions as described above). Single, first instar aphid nymphs, less than 24 h old, were placed in clip cages on the stems of these plants at growth stage 6.0–6.5. The number of days until the onset of aphid reproduction (d) was recorded and the total number of offspring counted (Md) and removed daily for 'd' days. Performance was assessed by calculation of the intrinsic rate of increase (r_m) (Wyatt and White, 1977) according to the formula $r_m=0.738\times(\ln Md)/d$.

Aphid feeding behaviour and growth analysis

To study the detailed feeding behaviour of *M. persicae*, electrical penetration graphs (EPGs) were obtained using an 8-channel Direct Current (DC) system (Tjallingii and Hogen Esch, 1993). Adult, apterous *M. persicae* aphids were starved in a Petri dish for 1 h prior to experimentation. A 25 μm diameter gold wire was attached to the dorsum of each aphid using silver conductive paint (Ponder *et al.*, 2001; Hale *et al.*, 2003). Each aphid was placed onto the stem of a plant (*aap6* mutant or wild type, as above). Stylet 2.5® software (WF Tjallingii, Wageningen, The Netherlands) was used for data acquisition. The following aspects of aphid feeding behaviour were recorded: (i) total duration of E2 periods (ingestion from SE); (ii) mean frequency of G periods (xylem feeding); (iii) duration of G periods; (iv) mean duration of individual E1 periods (salivation into SE followed by E2); (v) length of time to reach E2; (vi) duration of non-probing activities; and (vii) proportion of probes containing E2 periods.

Honeydew production was measured by clamping plants horizontally suspended over rotating Petri dishes for collection (Hale *et al.*, 2003). Honeydew droplets were collected between 20.00 h and 08.00 h, at a temperature of 20–22 °C. The number of honeydew droplets in each dish was counted using a microscope. The mean honeydew droplet volumes for aphids feeding on several individuals of each of the two experimental plant types were calculated by measuring the diameter of 20–25 honeydew droplets expelled directly into silicone oil (200/50 cS, Dow Corning Corporation, USA). The rate of droplet production was combined with mean droplet volumes to obtain the mean hly volume of honeydew produced by aphids. Honey dew amino acid concentration was measured in the same manner as SE sap.

Adult aphid growth rates were assessed by caging preweighed groups of 10 adult aphids on *Arabidopsis* plants and reweighing them after 12 h. Nine independent replicates were used for aphids feeding on each of the wild-type and *aap6* mutant plants. To assess weight loss due to respiration and water loss, nine groups of 10 adult aphids were taken off plants and left for 30 min to expel any residual honeydew. They were then weighed, left for 4 h in a Petri dish, and reweighed after 4 h.

Aphid haemolymph collection and analysis

Aphids were placed under paraffin oil and a leg excised using the stylectomy needle. Exuding haemolymph was collected in glass capillaries and stored and analysed in the same manner as SE sap.

Statistical analyses

The Student *t* test was used to detect significant differences between *aap6* mutant and wild-type plants, or aphids feeding on them, with respect to data relating to whole plant development, silique production, seed volume, osmotic pressures of SE sap and honeydew, aphid reproductive performance, aphid feeding rate, and the concentrations of total, essential, non-essential, and individual amino acids in SE sap, honeydew, and haemolymph. Where necessary, these data were first logarithmically transformed to achieve normalization (assessed by the Ryan–Joiner test for normality).

Pair-wise comparisons of the different feeding parameters measured by EPG were made between aphids on *aap6* mutant and wild-type plants using the non-parametric Mann-Whitney U-test ($\alpha=0.05$) since the data sets were not normally distributed and transforming the data did not make the data sufficiently normal.

Results

Arabidopsis *genotype analysis*

Leaf DNA was extracted from individuals of *Arabidopsis thaliana* that putatively contained a mutant allele (with a T-DNA insertion) and from Col-0 (the background genotype of the putative mutant). This was used as the template in PCRs using a combination of primers specific to either the *AAP6* locus or to the T-DNA used for mutagenesis. It was confirmed that individuals of the SAIL line used contain a T-DNA insertion (within intron 2). To determine the effect of the T-DNA insertion in the *aap6* locus, the abundance of its transcript was assessed using RT-PCR. Although the predicted 1400 bp amplification product of the *AAP6* transcript was readily detectable in extracts from the wild-type rosette leaves, no such product was amplified from *aap6* mutant samples (Fig. 1). Amplification products representing a part of a positive control transcript (an actin mRNA) were detected in both wild-type and *aap6* mutant samples.

Southern blot analysis was carried out using genomic DNA extracts from mutant *aap6* leaves in order to determine the number of T-DNA inserts within the genome of this line. The *Hin*dIII-digested DNA samples were probed

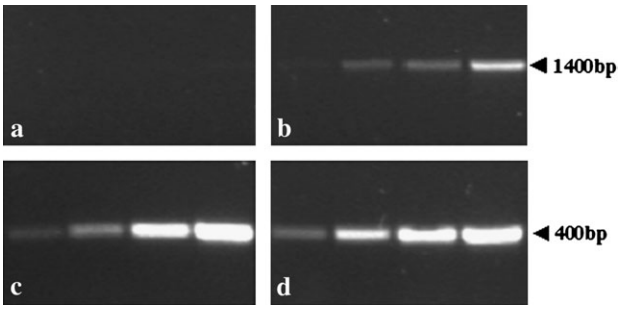

Fig. 1. RT-PCR analysis on cDNA obtained from *aap6* mutant and wild-type rosette leaves using: (a) *AAP6* gene-specific primers with *aap6* cDNA; (b) *AAP6* gene-specific primers with WT cDNA; (c) actin2-specific primers with *aap6* cDNA; (d) actin2-specific primers with WT cDNA. The four lanes show PCR amplification after 20, 22, 24, and 26 cycles.

with a portion of the *bar* gene, which forms part of the T-DNA construct used to produce the SAIL insertion library. The results (Fig. 2) are consistent with the occurrence of a single T-DNA insertion into the genome and the size of the hybridizing restriction fragment is the same as that predicted by the allele model presented (Fig. 2d) where 4.7 kbp separate *Hin*dIII restriction sites within the T-DNA and the *AAP6* gene.

Effects of abolition of the function of AAP6 *on plant phenotype*

Whole plant: There were no obvious developmental differences between wild-type and homozygous *aap6* mutant plants. However, measurement of quantitative characters revealed some small but significant changes (Table 1). For example, at the time of flowering, the mean rosette width of the *aap6* mutant was significantly larger than the wild type and it had a greater number of cauline leaves. Seeds from the *aap6* mutant were significantly larger (0.061 mm^3) than those from the wild-type plants (0.056 mm^3) (P=0.01).

Sieve element: The total amino acid concentration of SE sap of *aap6* plants was significantly lower than that of the wild-type plants (56.04±9.30 mM and 184.71±60.45 mM, respectively; P=0.032, Fig. 3). Similarly, the mean concentrations of essential and non-essential amino acid concentrations in SE sap of *aap6* mutant plants were significantly lower than those of the wild types.

Sixteen amino acids were individually identified and quantified; histidine and valine co-eluted and are reported together (Fig. 3). Lysine, phenylalanine, leucine, and aspartic acid were all significantly lower in concentration in

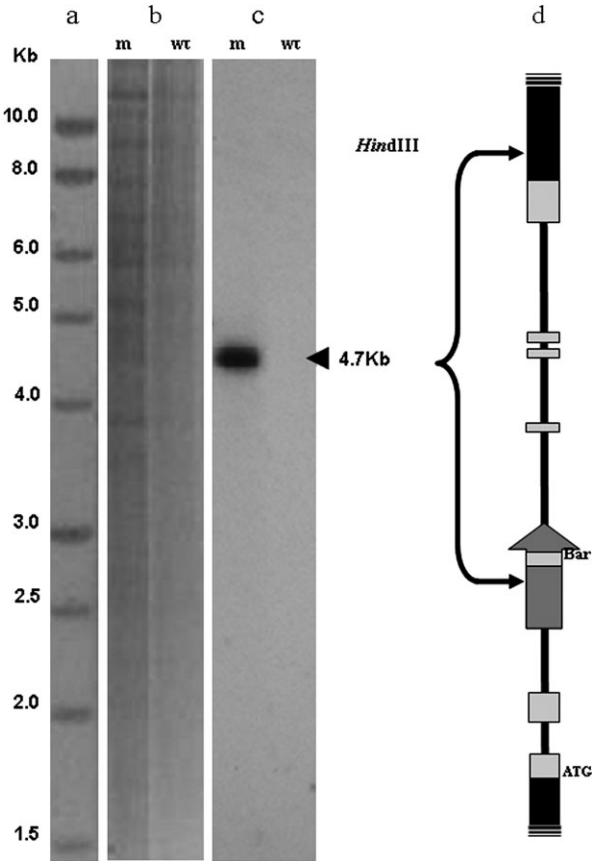

Fig. 2. Southern blot analysis of *Arabidopsis* genomic DNA using a probe to the BASTA (*Bar*) gene on the T-DNA insertion: (a) 1 kb ladder. (b) Image of ethidium bromide-stained agarose gel showing genomic DNA digested with *Hin*dIII. (c) Autoradiograph revealing hybridization to a single 4.7 kb fragment in the mutant (m) but not the wild type (wt) line. (d) A model of the mutant *aap6* allele showing exons (light grey boxes), introns (solid black lines), the position and orientation of the T-DNA insertion including the *bar* gene, and the positions of two *Hin*dIII sites which result in the production of a 4.7 kb DNA fragment to which the *bar* probe hybridizes.

Table 1. Randomised plot trial of quantitative traits of homozygous *aap6* mutant (n=18) and wild type (n=18) plants. Mean values (± standard errors) are given for each genotype; *p* values show the results of t-tests and are marked '*' if significant.

	***aap6* loss of function mutant**	**Wild type (Col-0)**	***P*-value**
Germination in days	4.7 (0.2)	4.3 (0.1)	0.20
Rosette leaf number on day 20	8.8±0.3	9.1±0.2	0.46
Rosette width on day 20 (mm)	31.0±2.2	29.3±1.6	0.54
Height on day 30 (mm)	206.5±19	219.7±10	0.55
Height on day 35 (mm)	339.4±15	336.9±7.3	0.88
Days to flowering	21.4±0.4	21.1±0.4	0.50
Height at flowering (mm)	57.7±3.3	52.7±2.5	0.24
Rosette leaf number at flowering	11.39±0.29	10.89±0.23	0.19
Number of cauline leaves at flowering	2.94±0.10	2.44±0.15	0.008*
Rosette width at flowering (mm)	66.1±1.6	60.6±0.	0.005*
Number of siliques on day 35	54.7±8.7	57.1±7.5	0.84
Seed Volume (mm^3)	0.061±0.0012	0.056±0.0011	0.01*

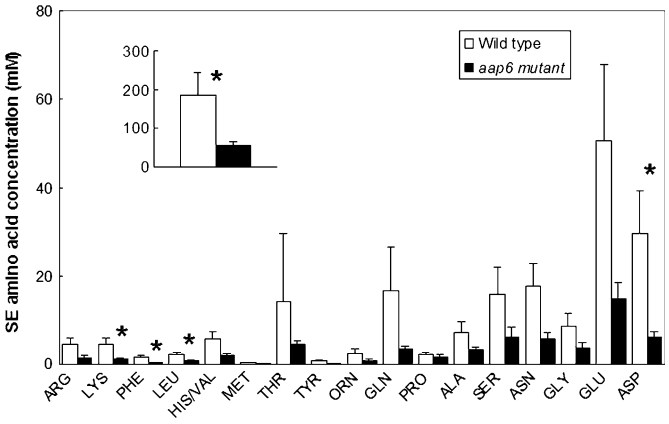

Fig. 3. Mean concentrations (mM ±SE) of amino acids in sieve element sap of wild-type and *aap6* mutant plants collected by aphid stylectomy and measured using CE-LIF. Insert indicates the total concentration of amino acids. Asterisks denote significant differences at P <0.05 in the concentration (mM) of amino acids between wild-type and *aap6* mutant plants.

aap6 mutant plants compared with wild-type plants, being reduced to 27%, 29%, 32%, and 20% of the wild-type values respectively. The large variances resulted in non-significance of the differences between the mean values of the remaining amino acids.

The mean osmotic pressures of SE sap of the homozygous *aap6* mutant and wild-type plants were not significantly different (0.80±0.04 MPa and 0.73±0.04 MPa, respectively).

Effects of the plant mutation on aphid phenotype

Aphid reproductive performance: Despite the large reduction in SE amino acids, aphid reproductive performance, assessed as r_m, was only slightly reduced when feeding on *aap6* mutant plants (0.268±0.008 compared with 0.243±0.008, respectively; P=0.039).

Aphid feeding behaviour: The results from the EPG experiments analysing aphid feeding behaviour on wild-type and *aap6* mutant plants are summarized in Table 2. The mean duration of individual 'successful' E1 periods (the salivation period which precedes SE sap ingestion by the aphid) was significantly greater for aphids feeding on *aap6* mutant plants than on wild-type plants (P=0.01). There were no other differences in any of the parameters measured for aphids feeding on the two genotypes (P >0.05).

Table 2. Summary of EPG parameters measured

The mean duration of individual 'successful' E1 periods (the salivation period which precedes SE sap ingestion by the aphid) was significantly greater for aphids feeding on *aap6* mutant plants than on wild-type plants (P=0.01).

	Wild type (n=15)	*aap6* mutant (n=13)
Mean frequency of		
Potential drops (pds)	76.2±10.7	74.5±12.6
Non-probing behaviour	4.7±1.2	4.4±1.0
E1 (total)	1.9±0.4	2.0±0.5
E1 (followed by E2 >10 min)	1.5±0.3	1.3±0.3
E2 periods	1.7±0.3	1.5±0.4
E2 periods (>10 min)	1.5±0.3	1.3±0.3
Xylem feeding (G)	0.4±0.3	0.5±0.1
Mean total duration (s) of		
Non-probing behaviour	1905.9±742.0	832.2±265.3
Pathway activities	4727.6±849.8	5390.2±900.9
E1 (total)	226.0±139.9	571.7±283.2
E1 (followed by E2)	57.5±11.5	224.8±114.7
E2 periods	14361.6±1675.5	12485.0±1941.3
E2 periods (>10 min)	15381.6±1373.6	14693.9±1328.1
Xylem feeding (G)	386.6±302.7	2372.5±1201.1
Mean duration (s) of individual		
E1 (followed by E2)	34.7±3.5	171.9±79.2
Mean time (s) from start of experiment to		
First E1	5328.1±1342.8	5642.2±1729.7
First E2 (>10 min)	6458.9±1681.8	7949.2±1991.9
Proportion of probes containing		
E2 periods (>10 min)	0.49±0.12	0.37±0.09

Honeydew production: The absence of large differences in the EPG feeding behaviour between aphids feeding on wild type and *aap6* mutants was paralleled by parity in honeydew production. Aphids feeding on wild-type plants produced 2.5±0.2 droplets h^{-1}, compared with 2.5±0.3 droplets h^{-1} for aphids feeding on *aap6* mutant plants. Similarly, there was no difference in the mean volumes of individual honeydew droplets produced by aphids feeding on wild-type plants (10.3±2.7 nl) and *aap6* mutants (11.5±3.1 nl). The rates of droplet production and volumes were combined to compare the rate of honeydew volume production (Fig. 4). Overall, there was no difference between the volume of honeydew produced h^{-1} for aphids feeding on wild-type and aap6 mutant plants.

Honeydew amino acids: The mean total concentration of amino acids in the honeydew from aphids feeding on *aap6* mutant plants was 17.1±10.5 mM compared to 2.2±0.8 mM for those feeding on wild-type plants (Fig. 5); these differences were not significant (P=0.157). There were also no differences in the mean concentrations of essential, non-essential or individual amino acids in the honeydew produced by aphids feeding on each plant type (P >0.05). The mean osmotic pressures of honeydew droplets produced by aphids feeding on wild-type and *aap6* plants were similar at 0.84±0.03 MPa and 0.78±0.03 MPa, respectively. In addition, there were no differences in the concentrations of total, essential and non-essential amino acids in the haemolymph of aphids feeding on the two plant genotypes (data not shown, P >0.05) nor in the concentrations of individual amino acids.

Discussion

This study demonstrated a role of the AAP6 transporter in the regulation of sieve element amino acid composition.

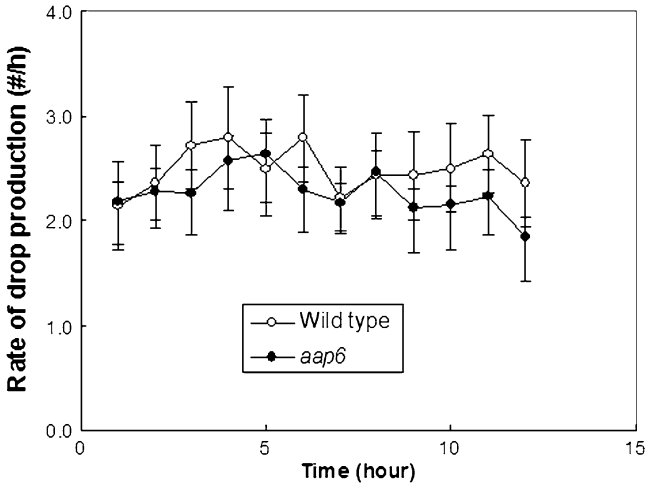

Fig. 4. Rate of honeydew production by aphids feeding on wild type or *aap6* mutants over a 12 h period commencing at 08.00 h. Each point is the mean of 14 determinations of droplet production rate ±SE. ANOVA indicated no significant difference in honeydew production over time or between genotypes.

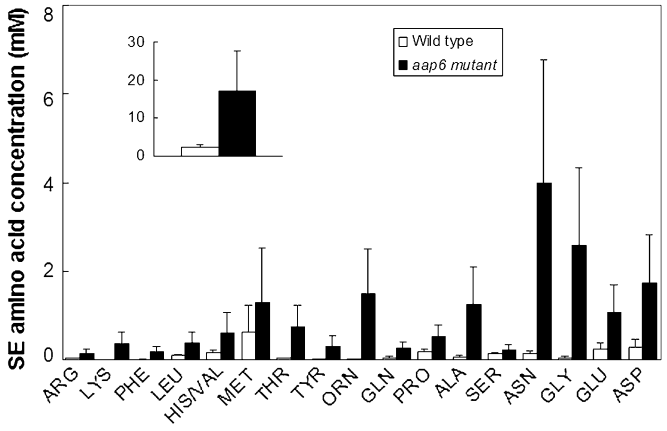

Fig. 5. Mean concentrations (mM ±SE) of amino acids in honeydew collected from aphids feeding on wild-type (n=11) and *aap6* (n=10) plants. Insert indicates the total concentration of amino acids. No differences were significant at P <0.05.

Despite the change in their diet, there was no large change in the metabolism, behaviour or reproduction of phloem-feeding aphids. The direct measurement of the concentrations of amino acids in sieve element sap has, until recently, been difficult. Aphid stylectomy provides pure sap from single sieve elements, but only provides nanolitre sample volumes. However, the development of the CE-LIF protocol for amino acid quantification has provided a method for measuring concentrations within such small samples (Zhu *et al.*, 2005). The technique involves derivatization of the amino acids, separation by capillary electrophoresis, and detection of the fluorescent analytes by laser induced fluorescence. Using this technique with wheat, it has recently been shown that there is great variation in the concentrations of amino acids in individual sieve elements of the same plant (Gattolin *et al.*, 2008) and this necessitates the collection of large numbers of samples and careful statistical analysis when two genotypes are to be compared.

In the present study, the abolition of *AAP6* function reduced the mean total amino acid concentration of SE sap to around 30% of the concentration of the wild type. Despite care taken to sample SE from the same site on the bolting stem from plants of the same age there was large variation in SE amino acid concentration. The previous work in wheat had indicated that such variation had a biological, not an experimental, origin and it was hypothesized that the basis of this variation lies at the regulation of the individual SE-CCC or domain (Gattolin *et al.*, 2008). However, in the present study, despite similar variation in *Arabidopsis* SE amino acid concentrations, significant reductions were detected in the levels of lysine, phenylalanine, leucine, and aspartic acid. The reduction in the total amino acid concentration of SE sap is consistent with the proposal that the AAP6 transporter moves amino acids from the xylem to the SE (Okumoto *et al.*, 2002). Okumoto *et al.* (2002) located AAP6 to xylem parenchyma of both roots and leaves of plants prior to bolting. In the present study, sap collection was performed on bolting stems. The two studies are consistent since reduced loading of amino acids in rosette leaf xylem parenchyma in the AAP6 knockout will result in a decrease in amino acids in the SE of the stem supplying the developing seed.

In *Arabidopsis*, as with other plants, there are multiple AAP transporters with overlapping specificities, so that it is not surprising that not all neutral and acidic amino acids were affected. It has been reported that the *AAP6* gene encodes a transporter of aspartic acid as well as other neutral and acidic amino acids (Rentsch *et al.*, 1996; Fischer *et al.*, 2002; Okumoto *et al.*, 2002). In the present study, SE aspartic acid concentration was decreased by 80% in the *aap6* mutant. In addition, the neutral amino acids phenylalanine and leucine were also significantly reduced. The significant decrease in lysine implies that the AAP6 transporter is also able to transport some basic amino acids.

In spite of the clear reduction in total amino acid concentration within the *aap6* mutants, the morphological phenotype and general development of the plants was little changed when grown under 'ideal' conditions. Phloem translocation is achieved by the hydraulically driven flow of sap through sieve tubes (Pritchard, 2007). Since SE osmotic pressure and whole plant phenotype was largely unchanged despite the large reduction in SE amino acid concentration, it is likely that the levels of other SE solutes (e.g. sucrose or cations) were elevated to maintain SE osmotic pressure and therefore translocation efficiency. However, there were some small but significant differences between the two genotypes. The mutant had a larger mean rosette width and a greater number of cauline leaves than the wild type. While seed number was unchanged in mutant plants (data not shown), seed volume was significantly greater in the *aap6* mutant plants. This is consistent with a role for *AAP6* gene function in the allocation of resources to seeds; however, there was no significant effect on the number of siliques produced (data not shown). A seed-related phenotype is not restricted to *aap6* plants; for example, a loss of function mutant in AAP8 significantly reduced seed number in *Arabidopsis* (Schmidt *et al.*, 2007). It is not obvious how loss of function in the AAP6 transporter would increase allocation to seeds, especially as SE amino acids were reduced. Increased seed volume may represent a secondary effect, due to, for example, a larger rosette diameter increasing capture and allocation of resources.

Sieve element sap is the major component of aphid diet and its composition can affect aphid growth. Generally, SE sap represents a non-ideal diet for aphids, being hyperosmotic and low in amino acids (Douglas, 2006). In addition to such direct effects, SE composition may influence aphid performance by affecting location and acceptance by a probing aphid. Despite the large variation in SE concentration, aphids on the different genotypes would experience altered amino acid concentration in their diet since over the period of the r_m measurement they would sample many individual SEs. The significant reduction in the average SE amino acid concentration between the wild type and the mutant was also determined from an average of a number of SEs. Therefore, despite the variation in SE amino acids, aphids on the *aap6* knockout mutant would

indeed receive a reduced amino acid concentration in their diet. The alteration in diet did not greatly affect aphids feeding on the *aap6* mutant; r_m was slightly but significantly reduced from 0.268 to 0.243. The aphid *Rhopalosiphum padi* feeding on barley grown under low nitrogen conditions had its r_m reduced from 0.46 to 0.41 (Ponder *et al.*, 2000). By contrast, *Arabidopsis* with a mutation in the amino acid carrier ANT1 had an altered SE amino acid composition but no significant change in r_m (Hunt *et al.*, 2006).

Despite the absence of large effects on reproduction, there were some small changes in aphid feeding behaviour. While there was no difference in the time taken ingesting sap from the SE (E2 phase), *Myzus persicae* spent an increased length of time salivating into the SEs of *aap6* mutant plants (E1 phase), suggesting the reduced acceptance of SE sap on the mutant plants. During E1, aphids inject a watery saliva into the SE which is proposed to condition the sap at the feeding site (Tjallingii and Hogen Esch, 1993). Aphids feeding from plants on which they perform well, salivate into the sieve element for a shorter period of time before initiating sustained ingestion (Van Helden and Tjallingii, 1993; Wilkinson and Douglas, 1998). Aphid saliva has recently been demonstrated to overcome SE blockage by forisomes (Will *et al.*, 2007). In addition to overcoming direct defence, some aphids are able to increase the nutritional quality of their diet, sometimes by altering SE amino acid composition (Prado and Tjallingii, 1997), with a role hypothesized for E1 salivation. Ponder *et al.* (2000) reported an increase in the time spent salivating (E1) prior to the onset of ingestion (E2) by aphids feeding on nitrogen-deficient barley.

Whilst the electrical penetration graph method system determines the duration of phloem ingestion (E2) feeding, it does not directly measure the rate of sap ingestion; this was achieved by measuring honeydew production. Since the amino acid content of the SE was reduced in the *aap6* mutant, a compensatory change in the rate of SE sap ingestion might have been expected. However, this was not observed, since *M. persicae* feeding on wild-type and *aap6* mutant plants produced similar amounts of honeydew. Amino acid analyses of the honeydew confirmed that the efficiency with which aphids are able to remove these compounds from their diet was similar between the two genotypes since there was no significant difference in the amino acid content of honeydew or haemolymph.

Previous studies have shown that nitrogen can positively influence aphid reproductive performance (Van Emden, 1966; Harrewijn, 1970; Ponder *et al.*, 2000, 2001; Sandström, 2000; Karley *et al.*, 2002). It is generally assumed and often empirically demonstrated that increased dietary amino acids correlated with increased performance. It was therefore predicted that aphids feeding on the *aap6* knockout would have reduced performance. However, there was no large difference in reproductive performance despite the significant change in SE amino acid composition. Other studies have reported a lack of a clear positive correlation between SE amino acid composition and reproductive rate. In droughted grasses, an increase in SE amino acids was accompanied by a decrease in aphid performance (Hale *et al.*, 2003) while an increase in the proportions of certain SE amino acids in the *Arabidopsis* mutant ANT1 did not result in any change in aphid fitness (Hunt *et al.*, 2006). It is becoming clear that prediction of aphid performance is more complex that a simple correlation with the nitrogen content of the diet, with many other factors potentially contributing to output. For example, a study using artificial diets concluded that the presence of secondary plant compounds in the diet was more important in determining aphid fitness than amino acid concentration (Tosh *et al.*, 2003). The cost of osmoregulation is receiving more attention (Shakesby *et al.*, 2009), but this is unlikely to be a factor in the present study as there was no significant difference in the osmotic pressures of the sieve element sap between the wild type and the mutant.

It may be that the knockout of *AAP6* did not reduce SE amino acids below the critical threshold for aphid performance; this may be possible in plants containing knockouts of two or more amino acid transporters in order to alter dietary nutritional quality to a greater magnitude, but this may also grossly impair plant function. The contribution of essential amino acids to the aphid by the bacterial symbiont *Buchnera* is critically important in determining the relationship between aphid performance and diet (Douglas, 2003); the response of aphids without these symbionts to dietary changes may reveal more direct relationships than can be observed in their presence (Douglas *et al.*, 2001).

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