A Diurnal Component to the Variation in Sieve Tube Amino Acid Content in Wheat[1][OA]

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We have used high-sensitivity capillary electrophoresis coupled to a laser-induced fluorescence detection method to quantify 16 amino acids in wheat (*Triticum aestivum*) sieve tube (ST) samples as small as 2 nL collected by severing the stylets of feeding aphids. The sensitivity of the method was sufficient to determine a quantitative amino acid profile of individual STs without the need to bulk samples to produce larger volumes for analysis. This allowed the observation of the full range of variation that exists in individual STs. Some of the total concentrations of amino acids recorded are higher than those reported previously. The results obtained show variation in the concentrations of phenylalanine (Phe), histidine/valine (His/Val), leucine/isoleucine (Leu/Ile), arginine, asparagine, glutamine, tyrosine (Tyr), and lysine (Lys) across the ST samples. These could not be explained by plant-to-plant variation. Statistical analyses revealed five analytes (Tyr, Lys, Phe, His/Val, and Leu/Ile) that showed striking covariation in their concentrations across ST samples. A regression analysis revealed a significant relationship between the concentrations of Tyr, Lys, Phe, Leu/Ile, His/Val, asparagine, arginine, and proline and the time of collection of ST samples, with these amino acids increasing in concentration during the afternoon. This increase was confirmed to occur in individual STs by analyzing samples obtained from stylet bundles exuding for many hours. Finally, an apparent relationship between the exudation rate of ST sap and its total amino acid concentration was observed: samples containing higher total amino acid concentrations were observed to exude from the severed stylet bundles more slowly.

The phloem system provides a means of transport for reduced nitrogen, as amino acids, within the plant. It is generally thought that sugars, potassium, and amino acids are the principal osmotic components of phloem sap (Patrick et al., 2001). The mature sieve element (SE) consists of an elongated cell closely connected to a companion cell by plasmodesmata. Phloem loading occurs in the companion cell mainly in the minor vein networks of source leaves and within storage organs (van Bel, 1996). Specific solute transporters mediate SE composition (for review, see Lalonde et al., 2003; Sauer, 2007), and their specificities and patterns of expression ensure a qualitative control on the phloem sap composition of a particular sieve tube (ST). Sap exchange between adjacent STs in a vascular bundle, however, is possible, as lateral sieve areas have been observed in angiosperms (Esau and Cheadle, 1959; Evert et al., 1966; Walsh and Melaragno, 1976), and osmolytes can also be exchanged between SEs by unloading and subsequent reloading of the SE (Thorpe and Minchin, 1987). The composition of phloem sap is not constant and can vary between day and night, for example, in barley (*Hordeum vulgare*; Winter et al., 1992), grapevine (*Vitis vinifera*; Gholami et al., 2004), and *Ricinus* (Smith and Milburn, 1980). In lupin (*Lupinus* spp.), the amino acid concentration of phloem exudate varied over the diurnal cycle in concert with the amount of photosynthate (Sharkey and Pate, 1976). Plant developmental stage was also shown to affect the composition of phloem sap in rice (*Oryza sativa*; Fukumorita and Chino, 1982; Weibull, 1987; Hayashi and Chino, 1990).

Alongside studies elucidating Suc loading into the ST (for review, see Kühn, 2003), phloem loading of amino acids has become a subject of great interest, as more than 50 amino acid transporters have been described in Arabidopsis (*Arabidopsis thaliana*; for review, see Rentsch et al., 2007). The loss of function of ANT1, a member of the aromatic-neutral transporter family, was recently associated with a change in ST amino acid composition in Arabidopsis (Hunt et al., 2006). Furthermore, members of the AAP transporter subfamily have shown a specific pattern of expression linked with the vascular tissue (Fischer et al., 1995; Hirner et al., 1998; Okumoto et al., 2002, 2004) and also represent good candidates for the regulation of amino acid concentration and composition in the phloem.

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Members of the AAP subfamily were suggested to have overlapping spectra of transported substrates but distinct expression patterns in various plant tissues, indicating that they may play a role in determining qualitative and quantitative import or export of amino acids between plant organs.

A simple technique to sample relatively pure phloem sap samples involves the collection of exudates in an EDTA solution (King and Zeevaart, 1974), and this method has been used to compare amino acid profiles in different plant species (Wilkinson and Douglas, 2003). This technique is limited, since the volume of sap collected cannot be determined, and its use is restricted to assessment of the relative proportions of ST components. Sampling of phloem sap from individual STs can also be achieved using aphid stylectomy (Fisher and Frame, 1984). Aphids feed exclusively from the phloem (for review, see Will and van Bel, 2006), and a large literature shows that their stylet bundles penetrate and extract phloem sap from an individual ST (Kimmins and Tjallingii, 1985; Tjallingii and Esch, 1993). It is possible to sever the stylets of feeding aphids and collect samples of phloem sap (Unwin, 1978; Pritchard, 1996; Zhu et al., 2005), and studies have confirmed that the samples obtained represent pure ST sap (Tjallingii and Esch, 1993). This technique has been used in previous studies to measure ST composition, and in particular amino acids, using HPLC for analysis. Limitations in the sensitivity of the HPLC technique, however, meant that analysis could only be performed where enough phloem sap was obtained. In the literature, volumes between 15 and 500 nL were used to determine directly the ST solute concentrations in various species using HPLC (Weibull et al., 1990; Lohaus et al., 1994; Girousse et al., 1996; Bernays and Klein, 2002; Hale et al., 2003), while lower collected volumes could not be analyzed directly. Since severed stylets exude at a range of rates, limitations of analytic techniques have previously restricted study to those stylets with a high exudation rate, thus producing larger sample volumes and leading to potentially unrepresentative data in the literature.

We recently reported (Hunt et al., 2006) refinements of analytical techniques to measure amino acids (Zhu et al., 2005) in sap from individual exuding stylets using capillary electrophoresis coupled to laser-induced fluorescence detection (CE-LIF). CE-LIF is a powerful tool in bioanalysis, especially where sample volume is limited and high sensitivity is required (Tseng et al., 2007). This approach provides significant advantages in terms of sensitivity compared with HPLC methods and opens up the possibility of detailed investigations of the amino acid composition of individual STs when coupled with aphid stylectomy sampling.

Here, we have examined in detail the relationship between the sampled volume of phloem sap and its amino acid concentration, and this has allowed us to confidently examine sample volumes lower than has been possible previously. We used young wheat (*Triticum aestivum*) plants to analyze the amino acid concentrations in phloem sap obtained from single STs, avoiding the need for bulking sap samples from multiple plants. This approach allowed us to use statistical tools to examine the range of amino acid concentrations in individual STs and to address the question of how much variation exists in ST amino acid composition.

RESULTS

Correcting for Potential Evaporative Volume Loss

To assess absolute concentrations of amino acids in ST sap, it is essential that the volumes of the samples collected from severed stylets be measured accurately. In previous work (Hunt et al., 2006), exuding ST sap was collected in paraffin oil to prevent evaporation of the sample. However, it has become apparent that the oil can potentially interfere with the subsequent analysis of amino acid contents by CE-LIF (data not shown). In this study, therefore, sap was collected from exuding stylets into microcapillary tubes filled with a small volume of water (20 nL), to prevent sap gelling and clogging. To estimate sap volume, the rate of sap exudation was measured by briefly (5–20 s) removing the capillary tip from the exuding droplet and measuring the rate of increase in diameter of the sphere of sap in air. Total sap volume collected was

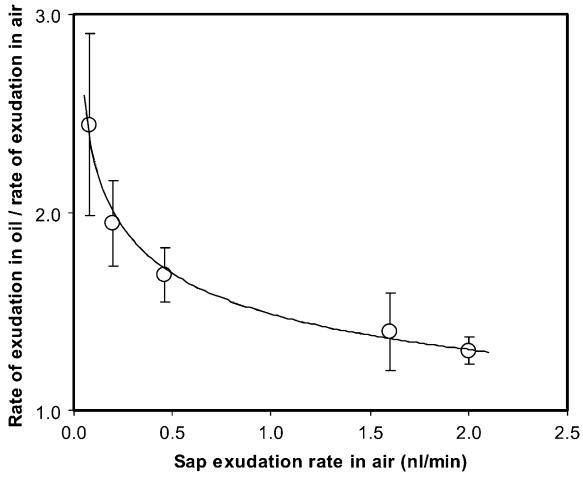

Figure 1. Ratio of exudation rate of ST sap collected in oil to that measured in air over a range of exudation rates measured in air. Each point represents the average of four to 15 comparisons ± SE. For individual exuding stylet bundles, sap exudation was estimated with minimal evaporation (collecting into oil-filled capillaries) and directly into air. Stylet bundles with an exudation rate in air above 0.5 nL min^{-1} had a significantly lower ratio of exudation in oil to that in air (Mann-Whitney test; $P = 0.015$) than those with an exudation rate below 0.5 nL min^{-1}. The mean data for each exudation rate were fitted with a power function ($y = 1.49x^{-0.19}$; $r^2 = 0.99$), which was used for all subsequent sap sample volumes measured in air to correct for evaporation.

calculated from the elapsed time. During the necessary measurement of the volume of exuding sap in air, any significant evaporation would reduce the observed diameter of the exuding sap droplet, resulting in an overestimation of in vivo concentrations, a problem previously recognized by Fisher and Cash-Clarke (2000). Evaporation would be greater for more slowly exuding samples, as these require a longer period for volume measurement and have a greater average surface area-to-volume ratio. To correct for evaporation, ST sap exuding from severed stylet bundles was collected over measured time periods into oil-filled capillaries to minimize evaporation. The capillary was then removed from the stylet bundle, and the rate of sap exudation of the same stylet in air was measured. The sap collected in the oil-filled capillary was then expelled into paraffin oil, and the diameter of the resulting sphere of sap was measured and used to calculate the volume, and so the rate of exudation, into oil. The ratio of exudation in oil (low evaporation) to exudation in air (high evaporation) was calculated for each individual stylet bundle. The oil-water exudation ratio was above unity for all stylet bundles (Fig. 1), indicating an underestimation of sap volume during collection in air. In addition, there was an increase in the oil-air exudation ratio at slower exudation rates: stylet bundles with an exudation rate above 0.5 nL min^{-1} had a lower oil-air volume ratio (1.35 ± 0.10; $n = 8$) compared with stylet bundles with an exudation rate below 0.5 nL min^{-1}, which had an oil-air volume ratio of 1.90 ± 0.13 ($n = 33$). The Mann-Whitney nonparametric test showed that the difference in the ratio of oil to air volume at high and low exudation rates was significant ($P = 0.015$). The mean data for each exudation rate were fitted with a power function ($y = 1.49x^{-0.19}$; $r^2 = 0.99$). In all of the subsequent work reported here, the volumes of samples were corrected using the measured ratios of evaporative loss represented by the fitted line shown in Figure 1.

Collection of ST Sap for CE-LIF Amino Acid Analysis: Effect of Small Sample Volume

A further potential source of error is the less reliable estimation of amino acid concentrations by CE-LIF in very small biological samples. On some occasions, the volumes of ST sap obtained by collection from severed stylet bundles were low because the bundles did not

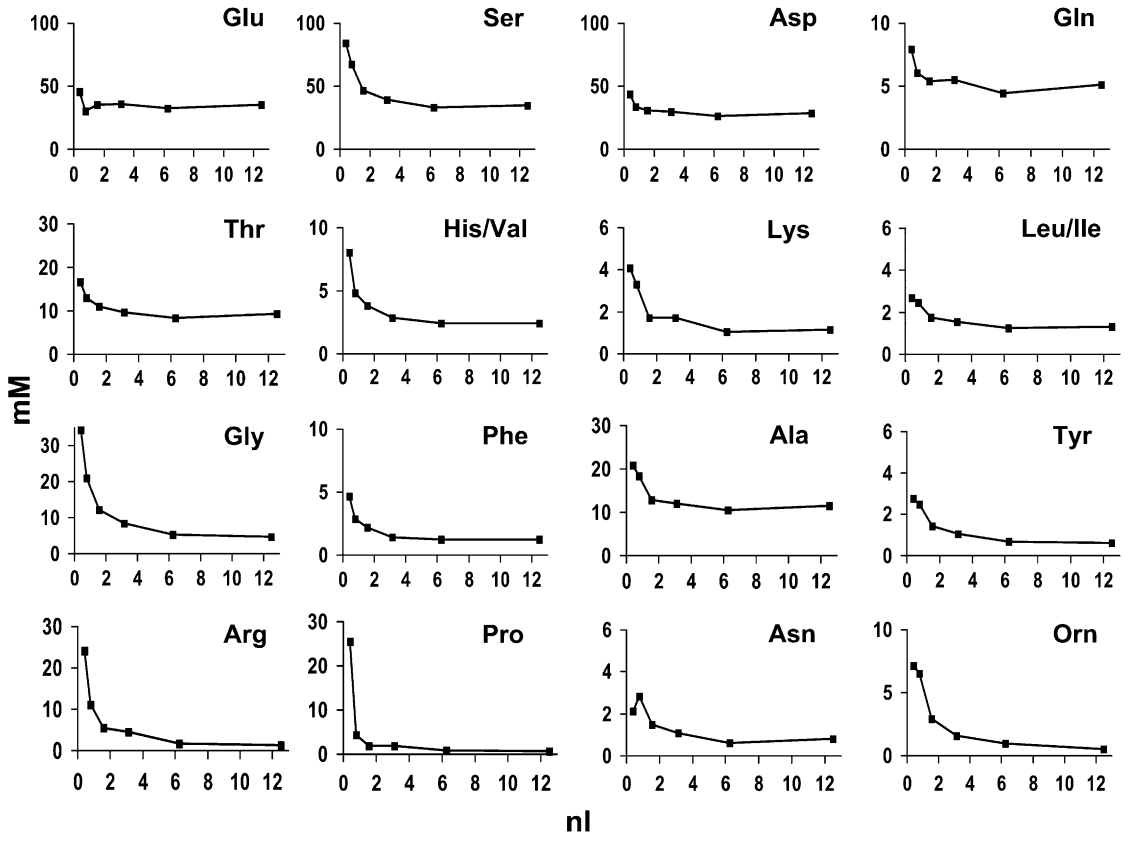

Figure 2. Determination of the lowest effective sample volume measured by CE-LIF on the calculated amino acid concentration of an undiluted ST sap sample. A single large sample of ST sap was serially diluted to produce a series of aliquots containing the equivalent of between 0.4 and 12.5 nL of the original, undiluted sap sample. These were each analyzed (in triplicate) using the CE-LIF system, and the calculated amino acid concentrations in the original sap sample (*y* axis) were plotted against the actual volume of original sap sample in the analyzed aliquot (*x* axis).

exude for long and/or exudation was very slow. After air drying and resuspending these samples, the concentrations of amino acids were low. In order to test the robustness of quantification of amino acids for these samples by CE-LIF, a single high-volume ST sap sample was subjected to serial dilution and the amino acid concentrations were measured by CE-LIF at each dilution (Fig. 2).

The dilutions produced aliquots, each injected into the CE-LIF system, containing the equivalents of between 0.40 and 12.5 nL of undiluted ST sap. The concentrations of amino acids at most dilutions were consistent with the dilutions employed. However, at higher dilutions, although the technique proved sufficiently robust to accurately assay Glu, Asp, Gln, Leu/Ile, and Pro in volumes equivalent to only 0.8 nL of undiluted ST sap, we observed an overestimation of the concentration of some other amino acids. Since the higher dilutions contained concentrations of amino acids equivalent to those yielded by less than 1 nL of undiluted sap, any ST sample volumes below 2.0 nL were rejected in this study. The volumes of ST sap samples used in further analyses ranged from 2.1 to 26.0 nL, with a mean of 9.2 nL.

Variation of Amino Acid Concentration in Phloem Sap Samples

Having eliminated evaporation and low sample volumes as potential sources of error, further experimentation was carried out to establish the amount of variation in amino acid concentrations across ST samples collected by stylectomy. First, ST sap was collected from three exuding stylet bundles on each of four different wheat plants. Amino acid concentrations were assessed using the CE-LIF method, and the total levels are shown in Table I. A one-way ANOVA was carried out, and this revealed that between-plant differences were not significant for any amino acid concentration. This analysis, however, provided a genuine estimate of random error variance for each amino acid that could be used elsewhere. Single ST sap samples were then collected from a larger sample of 22 wheat plants, and the variance of their amino acid contents was compared with the respective random error variance (see above) as a variance ratio test. This test showed that the variation in concentrations of six individual amino acids (Fig. 3) was significant (Table II), and Lys and Tyr also showed high variance ratios compared with the total variance. The total amino acid concentrations ranged from 134 to 1,047 mM (Table I).

Pair-wise correlations were then calculated to determine whether the concentrations of any of the amino acids were associated across the 22 samples. This analysis revealed that the concentrations of Tyr, Lys, Phe, Leu/Ile, and His/Val were very strongly associated (Table III). Asn and Arg also showed a significant association with these five amino acids and with each other, while the concentration of Gln was not critically associated with that of any other amino acid.

Variation in Amino Acid Concentrations with Time of Day

The striking associations between the concentrations of certain amino acids could not be explained in terms of variation between plants, since we have been unable to detect significant plant-to-plant variation in the concentrations of individual amino acids. In order to test whether there was an effect of the time of day at which ST sap had been collected (expressed as minutes after 8 AM) on the amino acid concentrations (millimolar), these were compared using regression analysis. There was a significant relationship between the concentrations of Leu/Ile, His/Val, Lys, Phe, Tyr, Asn, Arg, and Pro and the time of collection, with these amino acids increasing in concentration during the afternoon (Table IV).

Occasionally, but not predictably, a severed aphid stylet bundle would exude sap for extended periods of time. This occurred twice during our study and has allowed the comparison of the concentrations of individual amino acids over time within the same ST. For one ''long-exuding'' stylet bundle, the total amino acid concentration increased from 139 to 164 mM over a 5-h collection period between 12:30 PM and 5:30 PM (Fig. 4). The second stylet bundle with extended exudation (on a different plant) had an initial total amino acid concentration of 306 mM at 12:30 PM, which had increased to 525 mM after 5 h. Analyzing the two long-exuding samples together using the Kruskal-Wallis test revealed that the amino acids that showed a significant relationship with time of collection (Arg, Tyr, Lys, Phe, His/Val, Leu/Ile, Pro, and Asn) also increased significantly in proportion over the three collection periods of this experiment ($P = 0.000$),

Table I. *Mean total amino acid concentrations ± SD in ST sap samples from each of 22 wheat plants and from four plants each sampled three times*

Measure	Individual Collections	Plant 1	Plant 2	Plant 3	Plant 4
Mean concentration (mM)	459.94 ± 263.83	537.00 ± 168.95	658.02 ± 347.03	448.49 ± 293.26	486.13 ± 24.57
Lowest concentration (mM)	134.29	393.72	265.29	240.76	457.76
Highest concentration (mM)	1,046.61	723.30	1,010.02	783.95	500.39
n	22	3	3	3	3

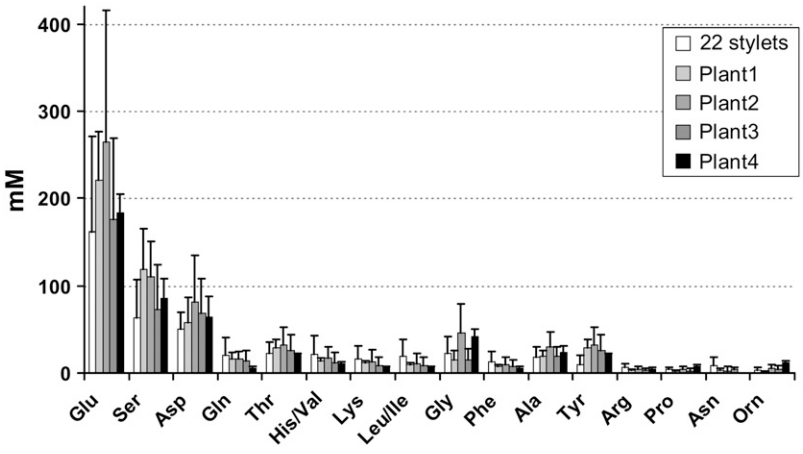

Figure 3. Mean concentration ± SD of each amino acid in ST sap from 22 individual plants and from three collections on each of four individual plants.

whereas the group showing no significant covariance (Ala, Asp, Gln, Glu, Gly, Ser, Thr, and Orn) did not change significantly over the same time periods ($P = 0.312$).

Total Amino Acid Concentration of ST Sap Is Linked to Exudation Rate

During collection, it was observed that ST sap exuded at different rates from individual severed stylet bundles. A regression analysis of exudation flux rate against the time of collection revealed no significant relationship (data not shown). However, regression analysis of flux rate against the total amino acid concentration revealed that samples with slower exudation rates possessed significantly higher ($P = 0.032$) total amino acid concentrations (Fig. 5A). This effect was not due to volume underestimation due to evaporation for slower exuding samples or to overestimation at low sample volumes, as these have already been taken into account (see above). The variation in total amino acid concentration with flux could not be explained by differences in the levels of any individual amino acid. A plot of the concentrations of each individual amino acid in a set of slow-exuding samples (0.1–0.3 nL min^{-1}) against their concentrations in a set of fast-exuding samples (1.0–3.8 nL min^{-1}) had a linear relationship, with an r^2 value of 0.92 (Fig. 5B).

DISCUSSION

This study has identified a high level of variation in the concentrations of amino acids in individual STs in wheat plants. This has been achieved by exploiting technical advances that have enabled analysis of smaller volumes of sap than was previously possible. We have also shown that this variation of ST amino acid concentration may be due, in part, to the coordinated changes in levels of a subset of ST amino acids during the day. In addition, this study has revealed a range of exudation rates of ST sap through cut aphid stylet bundles, with slower exudation rates being associated with higher amino acid concentrations.

Most previous studies examining ST amino acid composition have used HPLC for analysis. This has required the use of large volumes of ST sap, usually requiring the pooling of samples from different STs. For example, 15- to 60-nL samples were used for sugar beet (*Beta vulgaris*) analysis (Lohaus et al., 1994), 30- to 100-nL samples were collected by stylectomy for the Mexican sunflower (*Tithonia diversifolia*; Bernays and Klein, 2002), while only samples above 50 nL were used for a study of alfalfa (*Medicago sativa*; Girousse et al., 1996). A mean volume of 34 nL was reported in studies of oat (*Avena sativa*) and barley (Weibull et al., 1990), and 250 to 500 nL was used in a study of British grasses (Hale et al., 2003). Work on Arabidopsis employed sap volumes between 10 and 50 nL (Hunt et al., 2006). Lower volumes of approximately 5 nL

Table II. *Variance of amino acid concentrations of 22 plants, error variance, and the variance ratio test*

df, Degrees of freedom.

Amino Acid	Variance (21 df)	Error Variance (8 df)	Variance Ratio
Glu	1,2085.10	8,772.00	1.38[a]
Ser	1,912.07	1,719.00	1.11[a]
Asp	351.54	1,435.00	0.24[a]
Gln	419.91	69.90	6.01[b]
Thr	147.99	207.00	0.71[a]
His/Val	437.42	79.40	5.51[b]
Lys	231.26	77.60	2.98[a]
Leu/Ile	374.87	61.70	6.08[b]
Gly	356.89	348.00	1.03[a]
Phe	156.48	35.00	4.47[c]
Ala	128.59	128.00	1.00[a]
Tyr	117.49	38.60	3.04[a]
Arg	23.82	5.65	4.22[c]
Pro	6.71	5.93	1.13[a]
Asn	102.99	8.30	12.41[b]
Orn	11.52	10.50	1.10[a]
Total	69,609.1	63,762	1.09[a]

[a] $P > 0.05$ (not significant). [b] $P < 0.01$. [c] $P \leq 0.05$.

Table III. *Pearson's correlations among the concentrations of eight amino acids in STs*

Amino Acid	Lys	Phe	Leu/Ile	His/Val	Asn	Arg	Gln
Tyr	0.96	0.94	0.94	0.97	0.51	0.52	0.26[a]
Lys		0.91	0.91	0.96	0.60	0.57	0.28[a]
Phe			1.00	0.98	0.55	0.62	0.27[a]
Leu/Ile				0.98	0.59	0.65	0.23[a]
His/Val					0.57	0.54	0.28[a]
Asn						0.78	0.50[a]
Arg							0.38[a]

[a]NS, Not significant.

were analyzed in spinach (*Spinacea oleracea*), but in that study amino acid concentrations were not measured directly and were reported as estimates (Riens et al., 1991). In contrast, in the work reported here, we used a CE-LIF technique that allowed the simultaneous and quantitative profile of amino acids in sap volumes as low as 2 nL, so that comparisons could be made across sap samples obtained from numerous individual STs.

In this study, the average ST sap total amino acid concentration was 460 mM, with a range of 134 to 1,047 mM. These values tend to be higher than previously published figures, which ranged from just below 200 mM to over 400 mM in alfalfa (Girousse et al., 1996) and were 180 mM in barley seedlings (Ponder et al., 2000), 69 mM in *Dactylis glomerata* (Hale et al., 2003), 262 mM in wheat, and 125 mM in rice (Hayashi and Chino, 1986, 1990). However, high values of up to 1,232 mM have been reported in maize (*Zea mays*; Faria et al., 2007).

The proportions of different amino acids in ST sap reported here are similar to those of a previous study on wheat in which the profile was dominated by Glu and Asp (Hayashi and Chino, 1986). However, our data indicate higher Ser and Gly and lower amounts of Leu/Ile and Asn than were reported previously. The amino acid profile we obtained is also similar to those reported previously for barley (Winter et al., 1992), oat (Weibull et al., 1990), rice (Hayashi and Chino, 1990), and maize (Faria et al., 2007). Moreover, despite the lower total ST amino acid concentration in Arabidopsis (Hunt et al., 2006), the amino acid profile was similar to our results for wheat, with the exception that Arabidopsis had higher Asn and lower Ser concentrations.

While previous studies have been constrained by the need for higher sample volumes, potentially masking "within-plant" ST variation in individual STs, other authors have noted variation in ST amino acid concentration within a species: for example, in Arabidopsis (Hunt et al., 2006) and in alfalfa, in which ST amino acid concentration varied by a factor of three (Girousse et al., 1996). Our study demonstrated a significant variation in absolute levels of specific amino acids in individual STs. ST osmotic pressure was not systematically analyzed in this study; however, melting point depression of picoliter samples of ST sap (Pritchard, 1996) indicated a high and variable osmotic pressure (4.6 ± 0.8 MPa; $n = 18$), showing similar high variation to that of ST amino acid concentrations. Overall, average total amino acid concentration accounted for 19.8% of the average ST osmotic pressure.

There is no clear correlation between the amino acids that varied in concentration during the day in this study and those observed to change during the dark period in barley, although Leu/Iso, Lys, and Tyr all increased in the barley study (Winter et al., 1992). Sap composition is known to be affected by the diurnal cycle; for example, in *Ricinus communis*, there was a diurnal reciprocity between phloem potassium and Suc concentrations (Smith and Milburn, 1980).

Amino acids are loaded and unloaded into the ST through transporters, some localized to the vascular tissue (Rentsch et al., 2007). The striking covariation of Tyr, Lys, Phe, Leu/Ile, and His/Val in ST sap observed in this study might be explained by a tight regulation by the diurnal cycle of one or more of these transporters responsible for their loading or retrieval. A loss-of-function mutation in the amino acid transporter ANT1 led to a change in ST concentration of nine amino acids in Arabidopsis (Hunt et al., 2006); however, only three of those amino acids showed variation in our study, which is inconsistent with the involvement of an ANT1 ortholog in the changes observed in the wheat STs. Amino acid permeases are relatively nonspecific and transport a range of amino acids (Rentsch et al., 2007), but one, or a combination, of them could have higher affinity for a particular subset of amino acids, and their expression or activity could be regulated by a circadian rhythm.

A consequence of the ability to analyze very low volumes of ST sap in this study has been that it has been possible to acquire amino acid concentration data from slow-exuding stylet bundles; the technical requirement for larger volumes of samples has previously precluded measurements from this subset of STs. Many stylet bundles exude at a slow rate (between

Table IV. *Linear regression analysis obtained using the amino acid concentrations and the time of collection (in minutes after 8 AM) across 34 independent ST samples*

A significant relationship was found for the amino acids shown.

Amino Acid	Regression Coefficient
Leu/Ile	0.0725 ± 0.0225[a]
His/Val	0.0627 ± 0.0248[a]
Lys	0.0500 ± 0.0186[b]
Phe	0.0411 ± 0.0149[a]
Tyr	0.0331 ± 0.0131[b]
Asn	0.0277 ± 0.0127[b]
Arg	0.0173 ± 0.0059[a]
Pro	0.0106 ± 0.0038[a]

[a]$P < 0.01$. [b]$P \leq 0.05$.

A mM

	1st Collection	2nd Collection	3rd Collection
Glu	35.34	36.07	41.27
Ser	34.70	26.70	28.71
Asp	28.46	33.02	28.00
Gln	5.13	6.30	3.53
Thr	9.25	11.80	11.31
His/Val	2.41	6.15	8.27
Lys	1.15	4.51	6.51
Leu/Ile	1.30	5.09	7.13
Gly	4.75	5.99	5.28
Phe	1.24	3.40	4.55
Ala	11.42	7.97	8.68
Tyr	0.60	1.86	2.82
Arg	1.26	2.51	3.79
Pro	0.65	0.75	0.64
Asn	0.80	2.27	3.11
Orn	0.48	0.35	0.33
Σ	138.95	154.73	163.93

	1st Collection	2nd Collection	3rd Collection
Glu	73.21	115.02	120.04
Ser	52.74	76.47	88.68
Asp	47.77	52.66	48.24
Gln	28.97	37.57	36.67
Thr	17.36	26.47	36.80
His/Val	13.74	23.11	38.98
Lys	12.85	21.60	30.50
Leu/Ile	12.02	22.41	37.40
Gly	16.02	13.41	14.33
Phe	9.76	16.76	25.74
Ala	9.70	17.80	19.98
Tyr	4.46	8.43	13.92
Arg	4.75	6.95	10.25
Pro	1.72	2.02	3.02
Asn	0.67	0.00	0.00
Orn	0.93	0.42	0.53
Σ	306.67	441.09	525.07

B

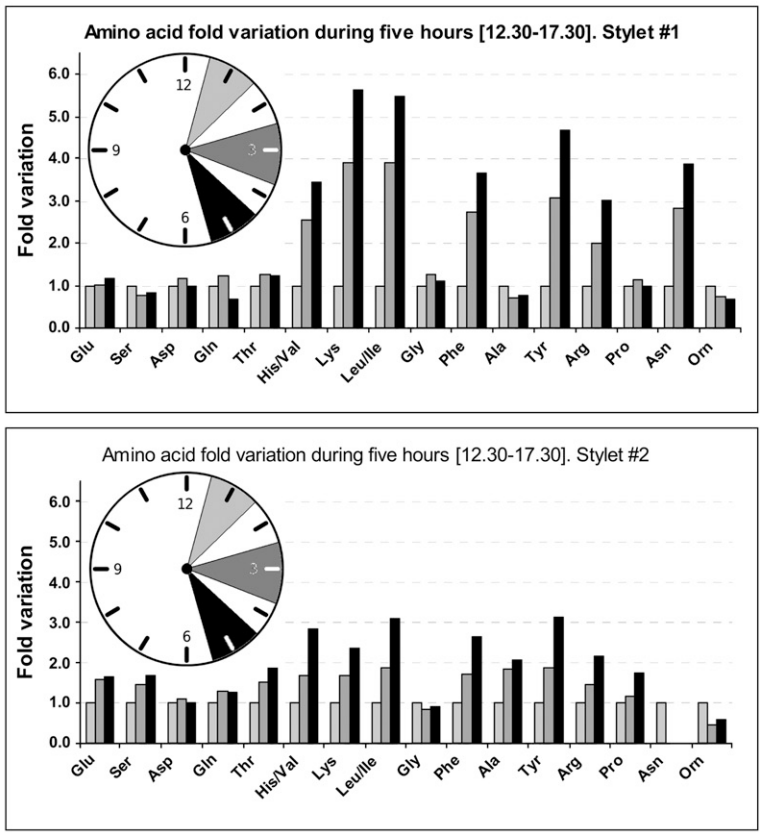

Figure 4. A, Increase in amino acid concentration during 5 h, from 12:30 PM to 5:30 PM, in sap collected from two independent severed stylets. B, Fold increase for each amino acid. The clock shows the periods of the day during which collections were made. Each measurement is the mean of three technical replicates of 12.5 and 10.5 nL of sap analyzed for each severed stylet bundle.

10 and 30 nL h^{-1}), but the basis for the different exudation rates is not clear. Recent research has identified forisome proteins as sites of defense against aphids in the Fabaceae (Will and van Bel, 2006), and it is possible that these proteins act to affect exudation rates. These proteins do not exist in monocotyledonous plants, but a qualitatively similar mechanism has been proposed for these (Will and van Bel, 2006). Sap exuded from cut stylet bundles 2.8 times more slowly on resistant compared with susceptible alfalfa varieties, consistent with such partial occlusion by a ST defense system (Girousse and Bourneville, 1994).

The aphids used in this study were all adults of the same size and age, so it is unlikely that differences in the diameter of the stylet food canal affected the rate of exudation. If resistance to flow is similar between stylet bundles, differences in exudation rate could be attributed to differences in ST turgor. Changes in ST pressure correlated positively with the rate of exudation from cut stylets in willow (*Salix* spp.; Peel and Weatherly, 1963). However, in our study, slower exudation rates were accompanied by elevated amino acid concentrations, which would be expected to generate higher pressures and hence higher exudation. Despite the marked difference in concentration, fast- and slow-exuding stylets had the same proportions of different amino acids. If high ST amino acid concentrations are accompanied by lower ST turgor (and hence the lower exudation), an uncoupling between ST water potential and water influx is implied. Experimentally induced changes in exudation from cut stylet bundles were not accompanied by changes in ST Suc concentration (Peel and Weatherly, 1963), implying that solute import was rapidly regulated to maintain ST solute concentration and/or turgor.

It is not clear whether the variations of exudation rate and amino acid concentration are of biological significance or are as yet unidentified artifacts of stylectomy-based sampling. If individual STs have similar variations in amino acid concentration in vivo, then it will be interesting to examine the underlying molecular mechanisms that regulate ST composition between species and within individual plants. If such variation is widespread, this would suggest that aphids do not use total ST amino acid concentration to select a ST on which to feed.

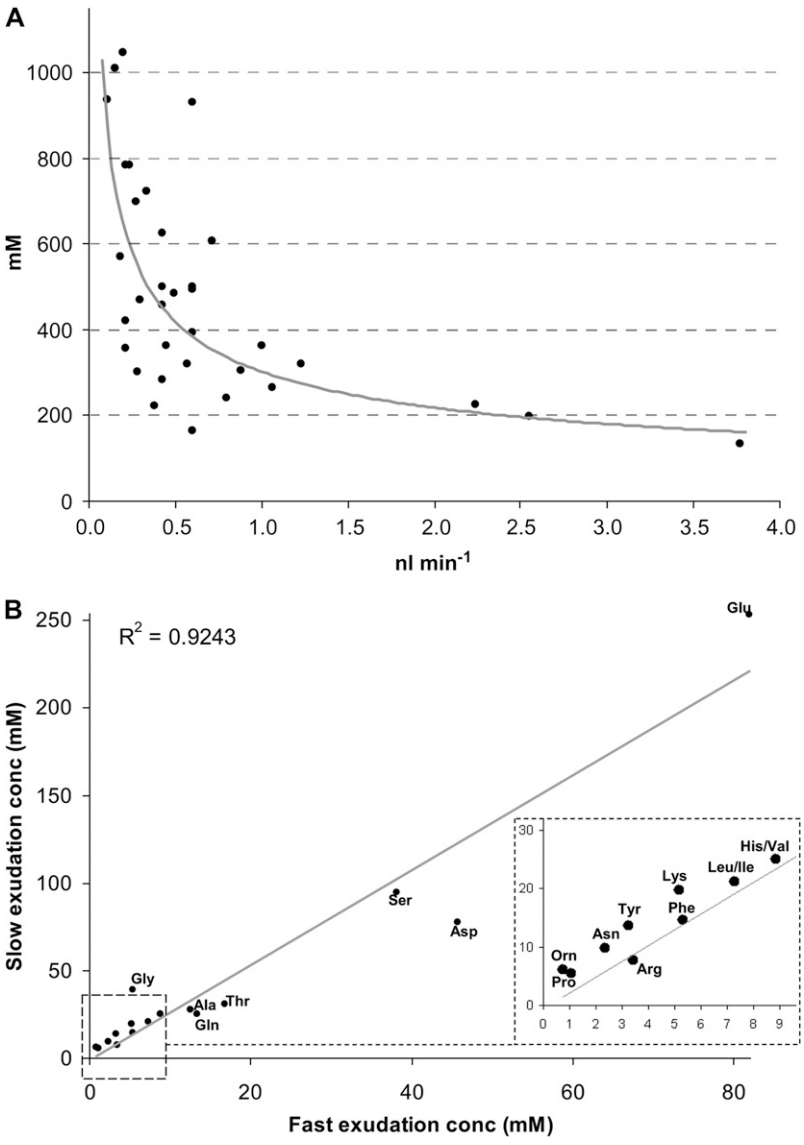

Figure 5. A, Correlation between the rate of exudation of ST sap from cut stylet bundles on wheat leaf sheaths and the total amino acid concentration of that sample. Each point represents an individual determination. B, Correlation of mean individual amino concentrations for fast-exuding (1.0–3.8 nL min^{-1}; $n = 6$) and slow-exuding (0.1–0. 3 nL min^{-1}; $n = 11$) stylets. These data are a subset of the data in A. The solid line represents the linear regression.

MATERIALS AND METHODS

Biological Material

Wheat (*Triticum aestivum* 'Paragon') seedlings were grown in 55-mm-diameter pots in compost containing six parts loam-based compost, six parts John Innes compost, and 1.5 part Silvaperl in a growth room maintained at 20°C to 22°C with a 16-h/8-h light/dark regime at 100 μmol m^{-2} s^{-1}. Aphids were taken from an anholocyclic *Rhopalosiphum padi* culture derived from a single individual and maintained at the University of Birmingham on well-watered wheat plants grown as above. Only apterous aphids were used in the experiments.

ST Sap Collection and Analysis

Eight to 12 adult aphids were placed on the leaf sheath region of 3-week-old wheat plants in sponge-sealed cylindrical clip cages (25 mm diameter) and left to establish overnight. Stylectomy was performed on feeding aphids using a high-frequency microcautery device (Unwin, 1978) as outlined by Ponder et al. (2000) and Hale et al. (2003). Stylectomy was always performed between 12 PM and 5.30 PM at 18°C to 20°C. A volume of sterile distilled water of approximately 20 nL was aspirated into the end of a microcapillary tube to facilitate the collection of nanoliter volumes of sap samples. The diameters of spheres of ST sap exuding in air from severed stylet bundles were measured using microscopy with a calibrated eyepiece graticule, and volumes were calculated using the formula $V = (4/3)\ \pi r^3$. Flux rates were estimated by measuring the time an exuding sap droplet took to reach a specified diameter. For all samples, the volume increase of the droplet was measured for 5 to 20 s during each minute of collection. If a decrease in flux was observed, or if the flux ceased altogether, collection was abandoned and the sample was discarded. Hence, the flux associated with samples can be considered as constant over the collection periods.

In order to assess the rate of reduction of sample volumes (and apparent flux rates) because of evaporation during collection in air, ST sap collections from the same severed stylet bundles were immediately made into microcapillary tubes back-filled with water-saturated paraffin oil (grade BP) and then expelled into water-saturated paraffin oil. Diameters of the suspended sample droplets were measured in the same way. Samples were stored at −20°C prior to amino acid analysis.

CE-LIF Methodology

Air-dried samples were analyzed for amino acid content by CE-LIF based on the method of Zhu et al. (2005). Briefly, the samples were derivatized with

the fluorogen 4-fluor-7-nitrobenzo-2,1,3-oxadiazol, separated by micellar electrokinetic chromatography, and detected by argon ion (488 nm) laser-induced fluorescence. Quantification used an internal standard method and individual amino acid calibration standards. The use of the ST sap collection described above, along with the novel derivatization and CE-LIF separation and detection systems, meant that it was possible to analyze individual amino acid concentrations at sample volumes approximately one-tenth of those that we (Hunt et al., 2006) and other authors had been compelled to use in previous analyses of ST amino acid levels.

Statistical Analyses

One-way ANOVA was used to calculate the random error variance for the concentration of each amino acid in ST sap using samples from three exuding stylet bundles on each of four different wheat plants. These random error variances were then used to determine whether the observed variation in amino acid concentrations was significant in a variance ratio test; this was carried out using the concentration of each amino acid in single samples from each of 22 plants. Having demonstrated that, for several amino acids, this variation was significant, Pearsons's pair-wise correlations were calculated to determine whether the concentrations of any of these amino acids were associated across 22 ST sap samples. To test whether there was an effect of the time of day at which ST sap had been collected (expressed as minutes after 8 AM) on the amino acid concentrations (millimolar), these were compared across 34 samples using linear regression analysis. Further analyses were next carried out using two ST sap samples that it had been possible to collect over many hours. For sets of amino acids that previously had, or had not, been shown to vary significantly in concentration with collection time, the Kruskal-Wallis test was employed to determine whether there was also a relationship between collection time and amino acid concentration for these two independent samples. Linear regression analysis was again employed in order to determine whether there was a significant relationship between the exudation flux rate and (1) the time of collection and (2) the total amino acid concentration across 34 samples.

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