Identification of a New Glucosinolate-Rich Cell Type in Arabidopsis Flower Stalk

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Distribution of K, Ca, Cl, S, and P in freeze-dried sections of Arabidopsis flower stalk was analyzed by energy dispersive x-ray imaging. Concentrations of these elements in different cell types were quantified by microanalysis of single-cell samples and phloem exudates. Results showed a differential pattern of distribution for all five elements. K concentration was found to be highest in the parenchymatous tissue around vascular bundles. Ca and Cl were present mainly in the central part of the flower stalk. P was largely located in the bundles and in the parenchyma surrounding them. S signal was extraordinary high in groups of cells (S-cells) situated between the phloem of every vascular bundle and the endodermis. Enzymatic hydrolysis by thioglucosidase of cell sap collected from S-cells using a glass microcapillary resulted in the release of glucose, indicating that these cells contain glucosinolates at high (> 100 mM) concentration, which is consistent with the concentration of S (> 200 mM) estimated by x-ray analysis of cell sap samples. Since their position outside of the phloem is ideally suited for protecting the long-distance transport system from feeding insects, the possible roles of these cells as components of a plant defense system are discussed.

Energy dispersive x-ray microanalysis (EDX) has been previously used for investigation of partitioning of various elements in tissues of the leaf and root (Pitman et al., 1981; Storey et al., 1983a, 1983b; Malone et al., 1991; Leigh and Storey, 1993; Williams et al., 1993). The probability of artificial ion and water shifts between intracellular compartments during the specimen preparation leading to the redistribution of mobile ions such as K can be minimized by fast freezing (Vonzglinicki, 1991). X-ray analysis of tissue sections allows the localizing of areas of accumulation of specific element, a first step in the future identification of cell-specific chemical compounds. The ratio of elements is often unique for individual cell types (Williams et al., 1993; Fricke et al., 1994) and therefore can be used as a marker for particular tissues.

All members of Brassicaceae to which Arabidopsis belongs take up sulfate and reduce it to a number of organic S-containing compounds such as amino acids and glucosinolates (Marschner, 1995; Halkier and Du, 1997; Schnug, 1997). Thus in plants grown at suboptimal sulfate supply most of the S is present in organic form. Sulfate accumulates only when its supply exceeds demands for optimal growth (Marschner, 1995).

Glucosinolates have been detected in all organs of the plant (Halkier and Du, 1997) where they accumulate in vacuoles. In addition to their role in insect defense, they function as a source of S during growth periods characterized by sulfate starvation (Bennett and Wallsgrove, 1994; Marschner, 1995; Halkier and Du, 1997). For young plants and developing seedlings of *Brassica napus* specific myrosin cells were identified by immunolocalization of their marker enzyme myrosinase (thioglucosidase, which hydrolyses glucosinolates) (Bones and Iversen, 1985; Bones et al., 1991). However, localization of glucosinolate-storing cells in mature plants has not yet been demonstrated. Much progress has recently been achieved in the field of glucosinolate biosynthesis (Halkier and Du, 1997; Bak et al., 1998). Heterogeneity of plant tissues, however, requires precise localization of cells where genes of interest are expressed and their regulation can be studied in response to their cellular and environmental context.

In the current study we determined the elemental composition of different cells of Arabidopsis flowering stalk as potential markers for the specific cell types. Glucosinolate content is highly correlated with the S content (Pinkerton et al., 1993; vanDalen, 1998) and, therefore, S, measured by x-ray analysis, represents a good marker for localization of glucosinolates. The S signal was used to identify glucosinolate-storing S-cells; samples of cell sap isolated from the S-cells were further analyzed by enzymatic microassay to characterize their organic solutes including glucosinolates.

RESULTS

Anatomy of the Young Flower Stalk

Transverse and longitudinal sections of Arabidopsis flower stalk are presented in Figure 1. A thick

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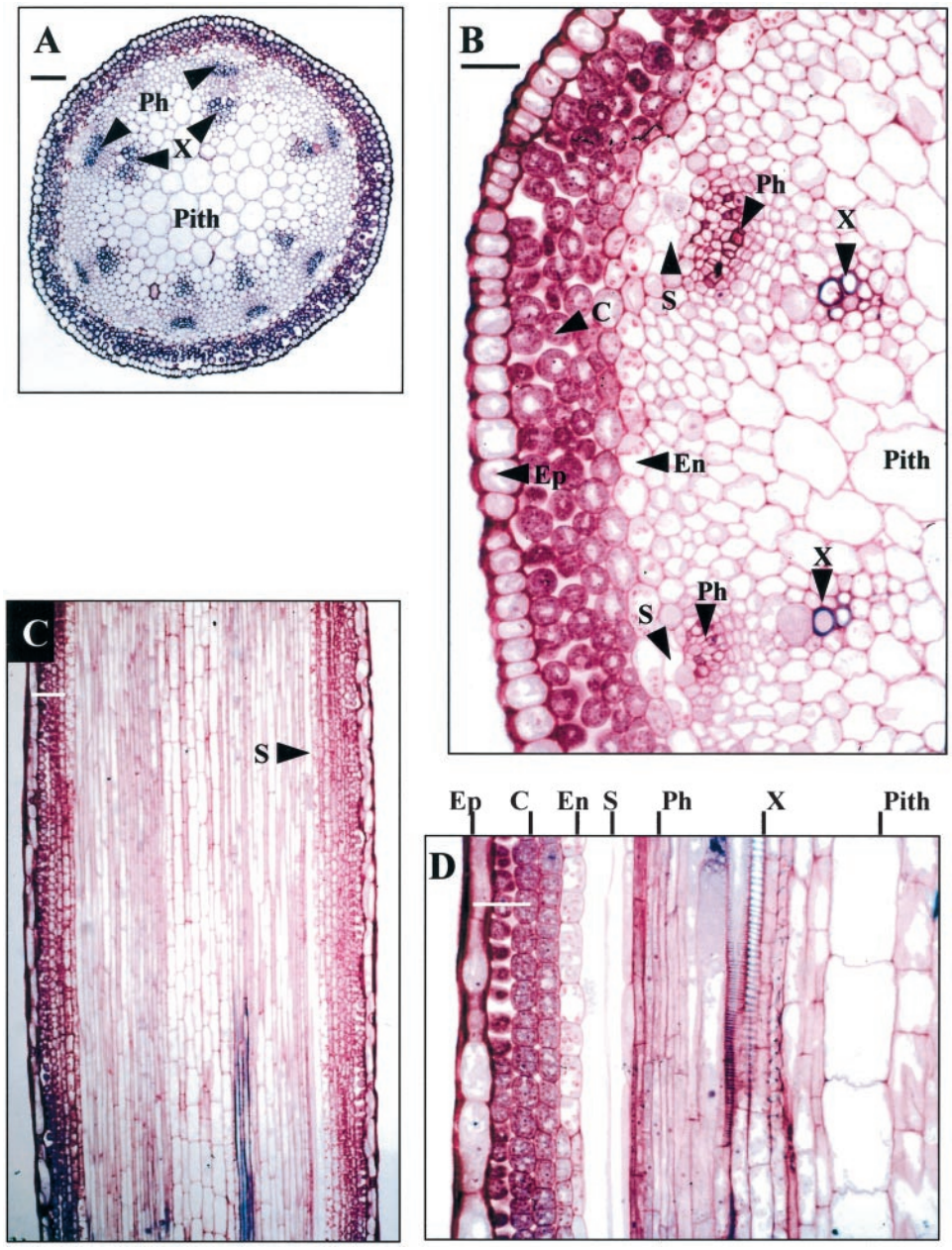

Figure 1. Histological sections of Arabidopsis flowering stalk stained with toluidine blue and periodic acid/Schiff reagent. Transverse (A and B) and longitudinal (C and D) sections. Ep, Epidermis; C, cortex chlorenchyma; En, endodermis; S, S-cells; Ph, phloem zone; X, xylem zone. Bars = 100 μm (A and C); = 25 μm (B and D).

layer of cuticular wax covers the epidermis. The cortical layer consists of chlorenchyma. Underneath is a single layer of endodermis (starch sheath) with cells larger than those of the cortex. They contain plastids packed with starch grains (Fig. 1, B and D). In between the endodermis and cells belonging to the vascular bundle usually one to six cells were found, which appear very long in the longitudinal sections. As described below, these cells are characterized by a very high-S content and therefore referred to as S-cells. The next layer of cells is a ring of six to nine vascular bundles separated by cells of inter-fascicular parenchyma. Each vascular bundle contains a phloem zone with phloem parenchyma and sieve element/companion cell complexes, a cambial zone, and zone of xylem parenchyma surrounding the xylem vessels. The center of the stem is composed of the pith with large, highly vacuolated cells.

Tissue-Specific Spectra of Elements and X-Ray Maps of the Flower Stalk

Small specified sections of freeze-dried flower stalk were used for EDX analysis at high magnification by

electron scanning microscopy to generate a spectrum of elements for individual cell types. The spectra in Figure 2 demonstrate typical ratios (derived from plants grown under high-light conditions) of elements for each cell type (data represent measurements on 12 sections from five plants). The data of Figure 2 cannot be used for quantitative analysis of concentrations in different tissue types because spectra could not be normalized due to the lack of an internal standard. To compare concentrations of elements in different tissues, maps of the distribution of single elements on whole sections of flower stalk were made (Fig. 3).

The highest concentration of K was observed in cells of the endodermis, phloem parenchyma, and interfascicular parenchyma (Figs. 2, C, E, and H, and 3). P was concentrated to the area of vascular bundles, and relatively high amounts were also present in epidermis and cortex (Figs. 2 and 3). A strikingly high amount of S was found in the region of S-cells (Figs. 2D and 3) and epidermis (Fig. 2A), although lower S peaks could be resolved in other tissues as well. The highest proportion of Ca was present in the cells of the pith (Figs. 2G and 3); other cells (except cortical) showed low-Ca signals. Note that the false impression of Ca presence in high-K samples is caused by the K-β peak, which with 10% of value of K-α peak overlies the Ca peak. Localization of Cl was very similar to that of Ca with more uniform distribution in the cells of the pith.

Maps of the distribution for single elements (Fig. 3) and the scanned image of the same flower stalk section showed a unique pattern of distribution for each element. Individual images were superimposed to produce false-color images of whole sections (Fig. 4A) or fragments of it at a higher magnification (Fig. 4B). The image on Figure 4 presents zones rich in individual elements. This information is complementary to the semiquantitative ratios between elements for each functional group of cells demonstrated in Figure 2.

A ring of high concentration just below the cortex is characteristic of the K distribution in a cross section (Fig. 3). The vascular bundles appeared to have less K, and the cortex and pith showed lowest concentrations of this cation. Groups of S-cells emitting a prominent S signal were identified between the phloem zone of each bundle and the endodermal cells (Fig. 3). A thin ring of cells, which had higher S contents than adjacent ones, connects the groups. The epidermal cells were quite high in S and P content, whereas the central part of the flower stalk, the pith, harbor the highest concentration of Ca and Cl (Fig. 3).

Line scans through the bundle at high magnification were run to ensure exact localization of the S peak near the phloem zone. Typical traces are shown in Figure 5. The background of other elements was very stable, and thus artifactual disturbances could be largely excluded. The lack of clefts between compartments due to large ice crystal formation or haloes around them can be taken as an appropriate criterion of absence of artificial shifts of ions and water over large distances (Vonzglinicki, 1991).

For the precise quantification of elemental distribution in several of the cell types analyzed before, the technique of single-cell sampling and analysis (SiCSA) was used (Tomos and Leigh, 1999). Thereby we used $RbNO_3$ as internal standard to determine the precise concentrations of individual elements from the sample spectra (Tomos et al., 1994; Hinde et al., 1998). EDX microanalysis of sap samples from pith cells again identified them as Cl and Ca rich (Table I).

Microanalysis of S-Cell Sap Samples

A typical spectrum from the sap for S-cells is shown on Figure 6 with the ratio between elements being very similar to those obtained from S-cells in the freeze-dried section (Fig. 2D). The composition of the cell sap from S-cells differed remarkably from the others (Fig. 7) with the concentration of S of 264 mM. Concentrations of S measured in phloem exudates obtained from aphids' stylets (27 mM) was lower than that in epidermal and cortical cells (108 mM) of Arabidopsis (ecotype Columbia) (Fig. 7). Analysis of S-cells in Arabidopsis (ecotype Wassiljewskija), grown under low-light conditions showed similar concentration of S as those of ecotype Columbia grown under the same conditions (Table II; Fig. 7). The concentration of K of 212 mM in S-cells (which accounted for one-half of the osmotic pressure) was as high as the S concentration. Average concentration of Glc was 10 mM, and Suc and Fru were below 1 mM each. When plants of ecotype Wassiljewskija were grown under high light, osmotic pressure increased due to the elevated accumulation of K, S, and free Glc (Table II).

Glucosinolates (146 mM) together with Glc (33 mM) accounted for 42% of the osmotic pressure of the S-cells from Arabidopsis (ecotype Wassiljewskija) grown at high-light conditions (Table II). Total osmotic pressure in these cells at high-light conditions (424 mosmol/kg) was only slightly higher than that at low-light conditions (407 mosmol/kg, Table II). The glucosinolate molecule is a monovalent anion containing two atoms of S and a Glc moiety. Quantitatively, the concentration of S in Table II equals the sum of the concentrations of glucosinolates and Glc, suggesting that this cell type stored additional 30 mM of S-containing compound other than glucosinolates.

DISCUSSION

The qualitative and quantitative analysis of the distribution of the major elements in the flower stalk of Arabidopsis presented here will provide the background for future analysis of Arabidopsis mutants altered in their capacity to transport and accumulate

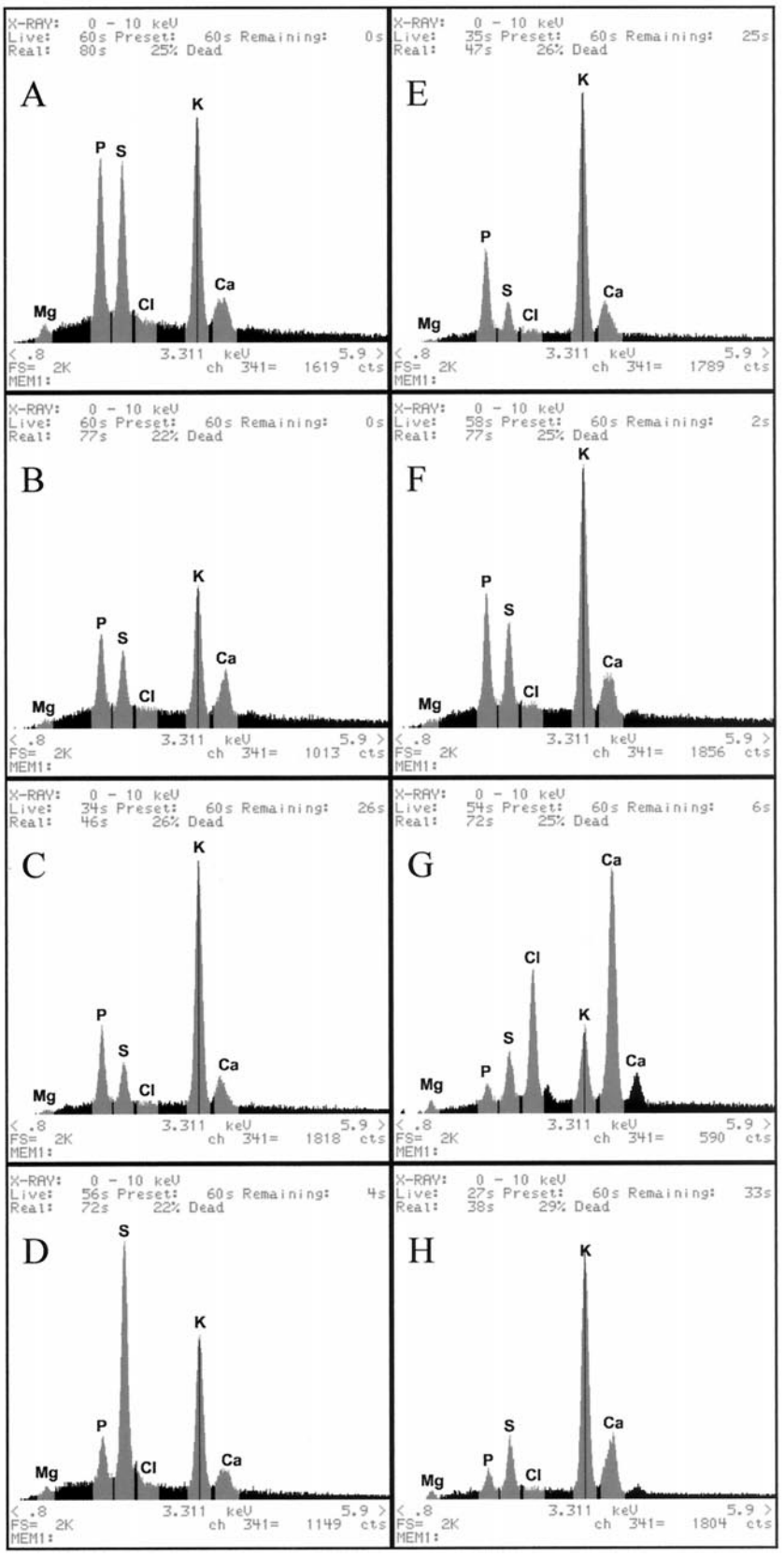

Figure 2. Spectra of elements for epidermis (A), cortex (B), endodermis (C), S-cells (D), phloem + cambium region (E), xylem region (F), pith (G), and interfascicular parenchyma (H). Plants grown at 350 μmol photons m^{-2} s^{-1}.

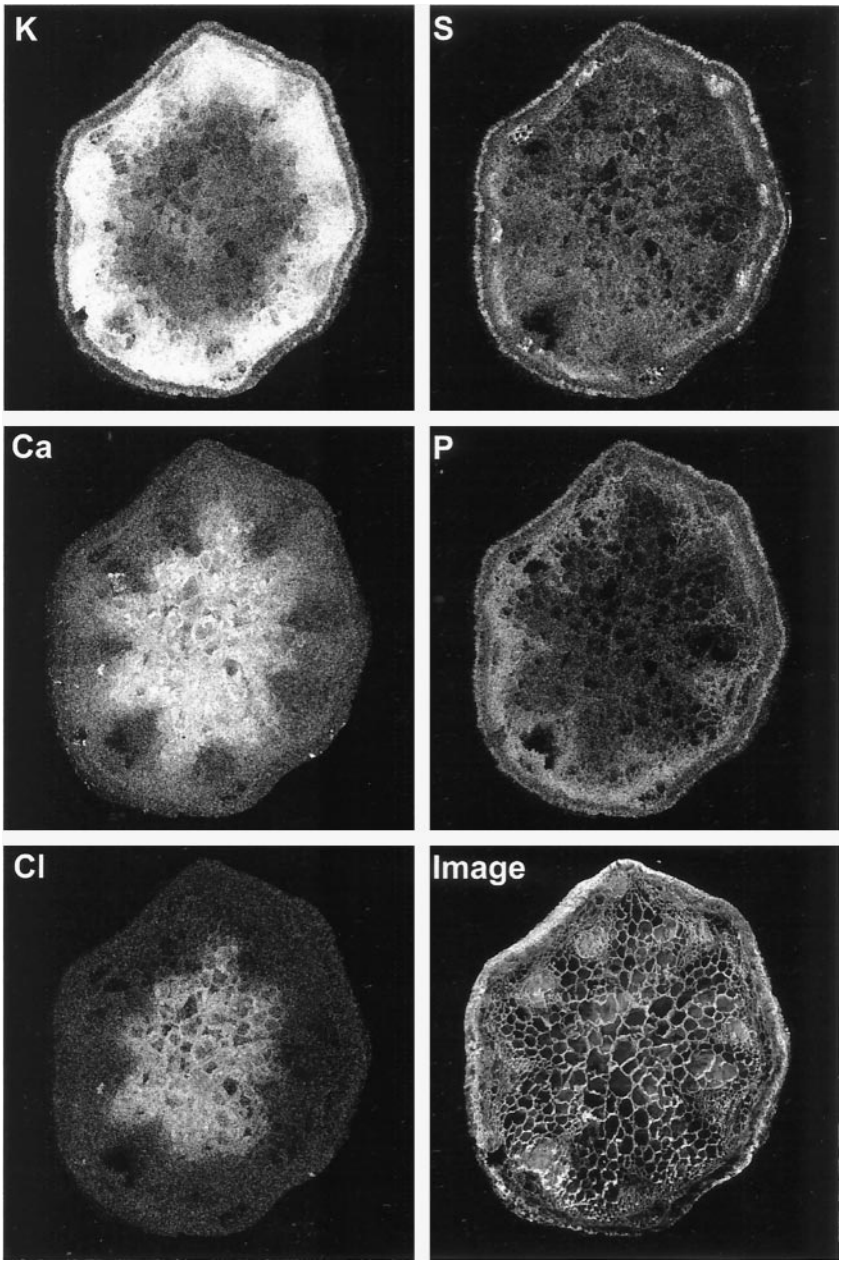

Figure 3. Image of a representative cross section and its x-ray maps of K, Ca, Cl, S, and P of a young flowering stalk. Plants grown at 350 μmol photons m^{-2} s^{-1}.

ions, sugars, and amino acids. Combining qualitative x-ray maps and quantitative SiCSA measurements we were able to overcome the shortcomings in each technique individually.

Distinct pattern of distribution was found for K, P, Ca, Cl, and S. K was most abundant in young, metabolically active tissues (with high cytosol to vacuole ratio) on the periphery of the flower stalk in the parenchymatous cells of the bundles and in the endodermal cells. The epidermal cells contained lower concentration of K. P was detected mainly in the bundles and in cortical cells. The cells in the bundle exhibit a high-metabolic activity related both to growth and phloem transport. Metabolic activity demands the presence of energy-rich intermediates such as ATP and pyrophosphates. In the cortex cells, which contain chloroplasts, inorganic phosphate is required for photosynthesis (Leigh and Tomos, 1993). The pith parenchyma cells have high concentrations of Cl and Ca. Relatively low concentrations of K, S, and P are present. The higher Ca to K ratios of the freeze-dried sections (Fig. 2F) compared with cell sap samples (Table I) suggest that some Ca is insolubly bound to the cell walls of these cells. Both the low cytosol to vacuole ratio and the composition of elements with a predominance of Ca and Cl is similar to those of epidermal cells of cereal leaves (Malone et al., 1991; Leigh and Tomos, 1993; Williams et al., 1993; Fricke et al., 1994). Accumulation of Ca and Cl in such structural cells may result from exclusion of

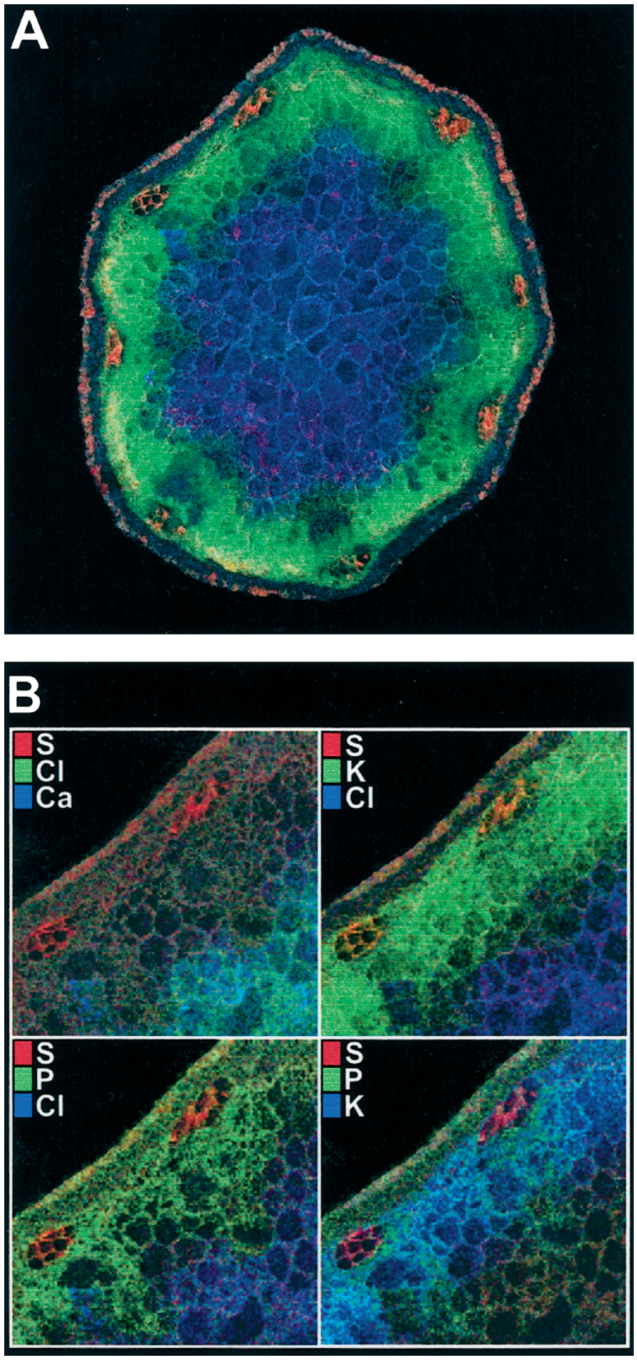

Figure 4. Image of cross section with superimposed x-ray maps of elements (of Fig. 3) in false colors. A, Whole section with maps of S (red), K (green), and Ca (blue). B, Fragment of cross section with four different combinations of elements, colors same as symbols of elements.

these ions from metabolically active tissues where they could inhibit metabolic processes (Leigh and Tomos, 1993). The accumulation of Ca in epidermal vacuoles during leaf aging was interpreted as an essential process to maintain high osmolality in the cells while exporting K (Fricke et al., 1994).

S-cells containing large amounts of S were localized on the periphery of the vascular bundles. To our knowledge, these cells have not been described before. S-cells are up to 1,000 μm long and up to 30 μm in diameter and surround the phloem zone of each vascular bundle. Large samples of cell sap (nanoliter range) can be obtained from them by SiCSA. A high-S signal was detected in S-cells of freeze-dried sections,

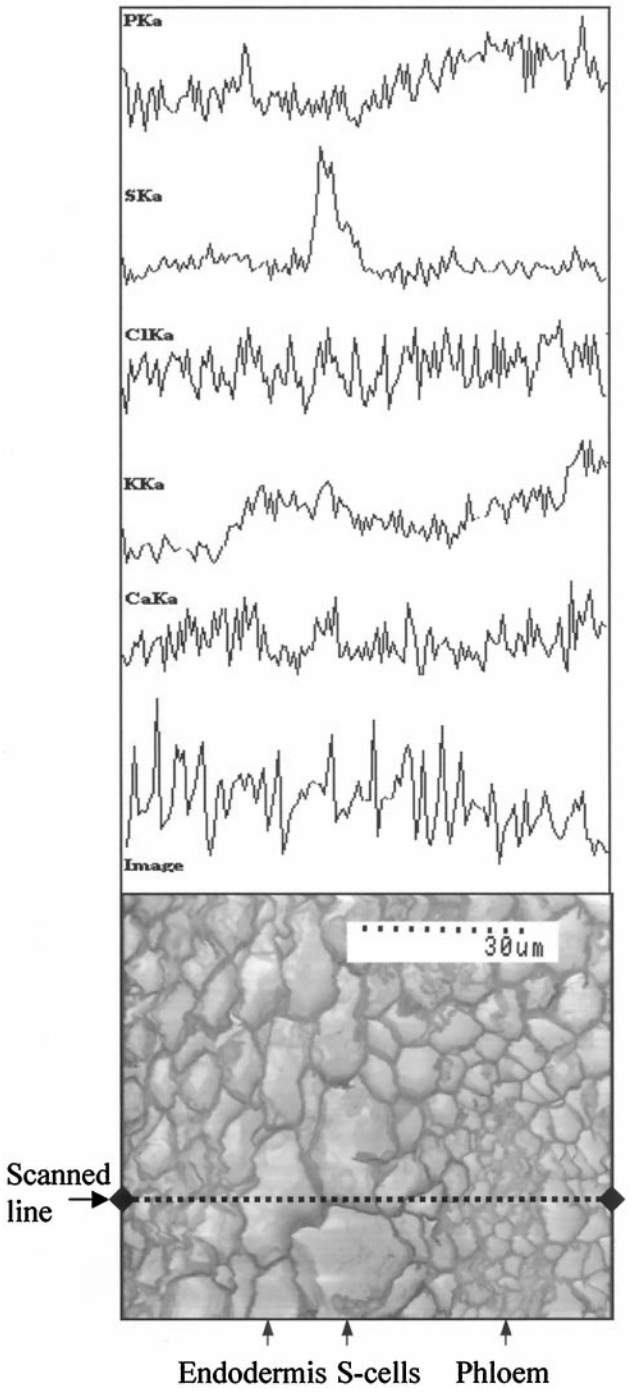

Figure 5. X-ray profiles of elements through the area of bundle shown below.

Table I. *Solute composition of pith cells in flower stalk Arabidopsis (ecotype Wassiljewskija) plants grown at 350 μmol photons m^{-2} s^{-1}*

Three samples from three plants were analyzed for each parameter.

Element	Concentration (Average ± SD)
	mM
K	192 ± 26
S	29 ± 7
Cl	93 ± 16
Ca	87 ± 15

and consequently a concentration of S of above 200 mM was measured in SiCSA samples from these cells. A weaker signal came from a ring of cells proximal to the endodermis and an outer ring in the epidermis. We have not yet fully identified all S-containing compounds in the S-cell sap, yet the estimation of concentration of glucosinolates (which usually contain two atoms of S per molecule) of 146 mM is consistent with the high-S concentration of 348 mM (Table II). Specific release of Glc after thioglucosidase addition suggests that glucosinolates harbor 84% of the S present (Table II).

The ability of standard methods such as chromatography and mass spectrometry to detect directly the presence of glucosinolates in picoliter-range samples from single cells has not yet been demonstrated. That the S-containing compounds are of low M_r is indicated by several observations. First, scanning of some sections for a second time after their storage under vacuum for 2 weeks showed a decreased signal, suggesting that the S was present in a relatively volatile compound. Second, the S (as well as P) signal was readily leached by ethanol dehydration from the S-cells of glutaraldehyde-fixed tissue (data not shown), whereas in phloem parenchyma and cortical cells, on the same sections, these elements were still present. Presumably in the latter cells the elements were conserved as the result of binding to or part of proteins that were immobilized when cross-linked by glutaraldehyde. High-sulfate concentration is unlikely because being divalent it would require higher levels of counter ions for a charge balance than we have identified. The concentration of the K was lower than S, and negligible Ca concentration was detected in these cells. Since the sum of solutes measured accounted for the cell osmotic pressure, a major contribution of additional cations could be neglected. The conditions of plant growth (low versus high light) appeared to have profound effect on accumulation of S in S-cells (Table II). In the same time, two different ecotypes of Arabidopsis (Wassiljewskija and Columbia) grown in low-light conditions had similar concentration of S in their S-cells (Table II; Fig. 7).

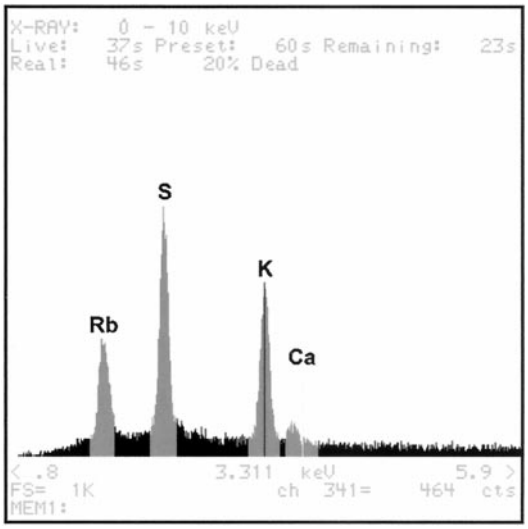

Figure 6. Typical spectrum for cell sap of S-cell with added standard solution of 200 mM $RbNO_3$.

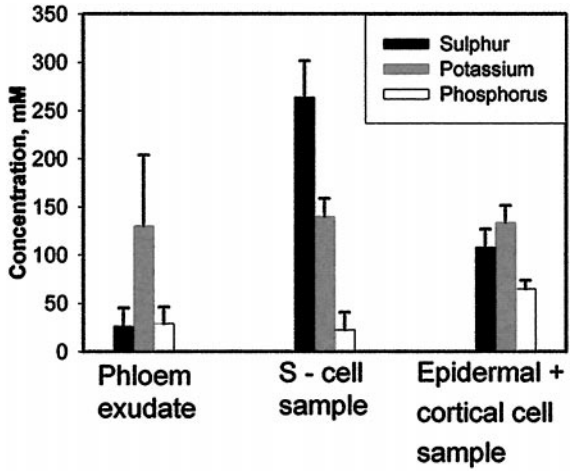

Figure 7. Single-cell analysis of phloem, S-cells, and pooled epidermal and cortical cells of Arabidopsis ecotype Columbia (plants grown at 120 μmol photons m^{-2} s^{-1}).

So far the biological role of S-cells is not known. The position of these cells outside of the phloem, however, is ideally suited as a storage site for repellents against phloem-feeding insects. Glucosinolates are stored in the vacuole and upon damage, infection, or pest attack they become exposed to the cytosolic hydrolases (myrosinases), and highly toxic products are released (Bennett and Wallsgrove, 1994; Bones and Rossiter, 1997). The myrosinase-glucosinolate system is known to affect the activities of a number of herbivorous insects (Peterson et al., 1998). Future studies will be directed toward the regulation of S-cell specific gene expression and response to phloem-feeding insects.

MATERIALS AND METHODS

Growth of Plants

Seeds of Arabidopsis (ecotypes Wassiljewskija and Columbia) were sown directly in soil and grown under low-

Table II. *Solute composition of S-cells in flower stalk Arabidopsis plants grown at 120 and 350 μmol photons m^{-2} s^{-1}*

Osmotic pressure values are given as mOsmol kg^{-1} and concentrations of solutes and elements as mM.

Parameter	Light Intensity	
	120 μmol Photons m^{-2} s^{-1}	350 μmol Photons m^{-2} s^{-1}
	average ± SD (n samples, n plants)	
Osmotic pressure	407 ± 51 (6, 3)	424 ± 67 (4, 3)
Suc	<1 (6, 3)	–
Glc	10 ± 12 (6, 3)	33 ± 19 (7, 4)
Fru	n.d.[a] (6, 3)	–
Glucosinolates	–	146 ± 58 (7, 4)
S	227 ± 47 (6, 3)	348 ± 86 (3, 3)
K	212 ± 45 (6, 3)	291 ± 52 (3, 3)
Ca	14 ± 12 (6, 3)	–
Cl	n.d. (6, 3)	–
P	23 ± 18 (6, 3)	–

[a] n.d., Not detected.

or high-light intensity (120 or 350 μmol photons m^{-2} s^{-1}, respectively) until flowering. Sections for x-ray analysis and single-cell samples were taken from the upper part of the main flower stalk.

Histological Sections

Upper parts of young flower stalks were cut into sections approximately 1 to 3 mm long and fixed immediately with fresh solution of 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) under vacuum to infiltrate tissue. The tissue was then dehydrated through an ethanol series followed by chloroform (10 min) and 100% (v/v) ethanol. After full dehydration, the tissue was infiltrated with 50% (v/v) Historesin infiltration solution 50% (v/v) ethanol and then with 100% (v/v) Historesin (Jung/Leica, Heidelberg). Finally tissue sections were embedded in Historesin. Using a microtome (Reichert, Jung, Germany) with a glass knife, 4-μm-thick sections of ribbon were cut from the Historesin block. The sections were stretched on a water surface before being placed on glass slides, dried, and stained with periodic acid/Shiff reagent and toluidine blue at room temperature.

Tissue Preparation for X-Ray Analysis

Small sections 4 to 6 mm long were cut from upper part of young flowering stalk, immediately frozen in nitrogen slush at −210°C, and then dried under vacuum at −45°C for 48 h. The freeze-dried stem sections were mounted using adhesive on a small piece of thick paper and then on to an aluminum stub. The distal ends of the stems were hand-sectioned using new double-edged razor blades to produce a flat even surface of a sample. The sections were carbon-coated and kept under vacuum until used for measurements of elemental distribution.

X-Ray Maps of Arabidopsis Flowering Stalk

X-ray analysis was performed using a scanning electron microscope (model S520, Hitachi, Tokyo) equipped with a Link Analytical LZ-4 detector coupled to a Link Analytical QX2000I micro-analytical system (Link Systems, High Wycombe, UK). The accelerating voltage of the electron beam was set at 12 kV with a condenser adjusted to prove an intensity of counting at approximately 2,000 counts s^{-1}. Scanning of the whole sample surface for 6 h (512 × 512 sampling points with beam dwell time of 60 ms per sampling point) provided x-ray maps with good signal to noise ratio for the selected elements. Smaller areas of whole cross section at higher magnification provided images after shorter scanning times (around 0.5 h). The information was collected and processed using Link Analytical QX 2000 software. Intensity of each x-ray map was adjusted to identify places of highest accumulation against the background concentration in the tissue.

SiCSA

Single-cell samples were isolated from flower stalks of intact plants under microscope, using a glass microcapillary filled with water-saturated paraffin oil to avoid evaporation of the cell sap (Tomos et al., 1994). The position of the S-cells outside the vascular bundles was extrapolated from the topography of the flower stalk surface. Cell samples characterized by large amounts (approximately 1 nL) of expelled cell sap entering the microcapillary (compared with 10–50 pL produced by surrounding cells of cortex) were assigned as coming from S-cells.

Aphid stylectomy technique (radio-frequency microcautery of aphid stylets) was used to obtain samples of phloem sap (Fisher and Frame, 1984). Phloem exudate from cut stylets covered by paraffin oil was collected with an oil-filled glass microcapillary.

Subsamples (approximately 10 pL) of cell saps or standard solutions were put on pioloform film-coated copper grids under a layer of paraffin oil using a constriction pipette (Malone et al., 1991; Tomos et al., 1994). Exactly the same volume of internal standard solution of 200 mM $RbNO_3$ was added by the same pipette to each droplet of sample or standard. Grids were then briefly washed in

hexane and isopentane (analytical grade) and dried. EDX was performed on a scanning electron microscope (see description above) at accelerating voltage of 14 kV. Calibration curves of standard solutions were used to calculate concentration of each element from element peak integral normalized to those of Rb (Tomos et al., 1994; Hinde et al., 1998).

Enzymatic Assay of Glucosinolate Concentration

Estimation of total concentration of glucosinolates in single-cell samples was based on their selective hydrolysis by the enzyme thioglucosidase (myrosinase), followed by measurement of the released Glc, an approach used in commercial food/crop analysis (Tholen et al., 1993; Stancik et al., 1995).

Micro-fluorometric assay was used to measure free sugars and the appearance of Glc after hydrolysis of glucosinolates in the single cell saps (Tomos et al., 1994; Koroleva et al., 1998). The assay involves enzymatic dehydrogenation of Glc-6-P derived from Glc, with corresponding reduction of NADP to NADPH. A Leitz MPV Compact 2 Fluorovert microscope photometer fitted with filter block A and Leitz software (Leitz, Wetzlar, Germany) was used to measure fluorescence of 4 to 5 nL droplets of reaction mixture, placed on a microscope slide inside a 4-mm-deep aluminum ring, under 3 mm of water-saturated paraffin oil. Solutions of sinigrin (Sigma, St. Louis) and Glc were used as standards for calibration of the resulting fluorescence. The reaction mixture contained 68 mM imidazole buffer (pH 7.5), 5.6 mM $MgCl_2$, 5.6 mM ATP, 0.1% (w/v) bovine serum albumin, and 23.8 mM NADP. The standards and samples (volume approximately 10 pL) were added to the droplets of reaction mixture previously placed on the slide using a constriction pipette. Initial fluorescence was recorded for all droplets, and Glc phosphorylation and dehydrogenation were started by adding of approximately 100 pL of hexokinase/Glc-6-P-dehydrogenase (HK/G6PDH) (Boehringer Mannheim, Germany) mixture (85/43 units/mL). After 10 to 15 min the reaction was complete (the fluorescence readings increased and then became stable) with the change in NADPH fluorescence being proportional to the amount of Glc in the droplet. The second step of the assay was started by adding approximately 100 pL of thioglucosidase (myrosinase, from *Sinapis alba* seed) (Sigma) (17 units/mL). The additional change in NADPH fluorescence after 10 to 15 min, corresponding to Glc released from glucosinolates, was recorded.

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