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INORGANIC AND ORGANIC SOLUTES IN APOPLASTIC AND SYMPLASTIC SPACES CONTRIBUTE TO OSMOTIC ADJUSTMENT DURING LEAF ROLLING IN *CTENANTHE SETOSA*

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In *Ctenanthe setosa* we studied changes in apoplastic and symplastic sugar, proline, ions and organic acids under drought stress causing leaf rolling. Leaf extractions were made at visually judged leaf rolling stages (not rolled, slightly rolled, strongly rolled, completely rolled). Glucose and sucrose content increased in the symplast. Glucose declined during leaf rolling in the apoplast, and sucrose was not present. Symplastic and apoplastic proline content increased during leaf rolling; citrate increased in both compartments, and malate increased in the symplast but declined in the apoplast. Symplastic and apoplastic K^+ declined during rolling. Ca^{2+} increased at slightly rolled stage but then began to decrease in both compartments. Na^+ level increasing in the symplast but decreased in the apoplast. Cl^- decreased in both compartments during rolling. Glucose, proline, Na^+ and K^+ are preferred for osmotic adjustment during leaf rolling under drought.

Key words: Apoplast, ions, leaf rolling, organic acids, proline, sugars.

INTRODUCTION

A decrease in osmotic potential in response to water stress is a well-known mechanism by which many plants can cope with drought conditions. Most organisms increase the cellular concentration of osmotically active compounds, termed "compatible solutes," under desiccation by drought or lowering of osmotic potential (Bohnert and Shen, 1999). Because of this function they are often called osmolytes. Compatible solutes may have other functions as well, namely in protection of enzyme activity and membrane structure and in scavenging of radical oxygen species (Bohnert and Shen, 1999). All known osmolytes are amino acids and their derivatives, polyols, soluble sugar and ions (Yancey, 2001), whose responses can be stress-specific (Chen et al., 2005)

Leaf rolling is a dehydration avoidance mechanism in some plants under drought stress. Leaf rolling is not the only response to water deficit stress for plants; some biochemical changes in the leaves may also occur together with leaf rolling (Kadioglu and Terzi, 2007). One of these changes occurs in the levels of osmolytes such as proline and sugar. Proline and soluble sugar are important solutes for adaptation to low water potential with leaf rolling (Kadioglu and Turgut, 1999). Hsiao et al. (1984) found that tissue viability was maintained because osmotic adjustment delayed the beginning of leaf rolling and leaf drying.

The apoplast is the first plant compartment encountering environmental signals (Gao et al., 2004). It is involved not only in the response to stress but also in the perception and transduction of various environmental signals in cooperation with the plasma membrane (Hoson, 1998). The apoplast also contributes to plant development (Almeida and Huber, 1999). Apoplastic pH exerts a strong influence on turgor and wall loosening, possibly via control of hydrolytic reactions and intermolecular interactions between structural carbohydrates and proteins (Rayle and Cleland, 1992). In tomato, inorganic ions K^+, Na^+ and Ca^{2+} accounted for the osmolarity of the apoplastic fluid (Almeida and Huber, 1999).

Ctenanthe setosa Eichl. (Marantaceae) is a tropical herbaceous perennial plant cultivated as a greenhouse ornamental and houseplant for its attractive foliage. It prefers semishade and rolls its

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leaves in response to drought stress. *C. setosa* is a convenient model for leaf rolling studies because its leaves roll gradually, taking 30–40 days, making it easy to observe and follow leaf rolling (Kadioglu and Terzi, 2007).

While there are some studies on accumulation of osmotically active compounds during leaf rolling, as cited, there is no study of changes in inorganic and organic compounds in apoplastic and symplastic areas during leaf rolling. In this work we examined changes in compounds contributing to osmotic adjustment in apoplastic and symplastic spaces during leaf rolling under drought stress in *Ctenanthe setosa*.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Ctenanthe setosa (Rosc.) Eichler (Marantaceae) plants were vegetatively propagated from their rhizomes and grown for twelve months in plastic pots containing peat and sand (5:1) in a growth chamber with the following parameters: 16 h photoperiod, 25°C, relative humidity 70%, and photon flux density at leaf surface 300 μmol m^{-2} s^{-1}. Plants at the same age were selected. Old and wilted leaves were trimmed. Some plants were well-watered (tap water) throughout the experiment and remained unrolled (control). The other plants were subjected to drought stress for 35, 47 and 56 days in order to cause leaf rolling, judged visually and scored as follows: stage 1 – not rolled (⎯), stage 2 – slightly rolled (◡), stage 3 – strongly rolled (⊂), and stage 4 – completely rolled (○). O'Toole and Moya (1978) also judged leaf rolling stages visually in their work. The parameters described below were measured in apoplastic and symplastic spaces during leaf rolling.

ANALYSIS OF SUGARS

Apoplastic washing fluid (AWF) for determination of apoplastic and symplastic sugars were extracted by vacuum infiltration according to Hernández et al. (2001). Fresh leaves were cut to 1 cm lengths and rinsed in tap water six times to remove cell proteins from the cut ends. The leaves were vacuum-infiltrated for 20 min in 50 mM potassium-phosphate buffer (pH 6.5) containing 0.2 M KCl and 0.1 mM $CaCl_2$. Then the leaves were blotted dry with thin paper tissues and the apoplastic solution was collected in microcentrifuge vials by centrifuging the leaf pieces at 1450 g for 15 min at 4°C. Cytoplasmic contamination of the apoplast during the extraction procedure was below detection limits (1%), as assessed from measurements of the activity of the marker enzyme glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) (Kornberg and Horecker, 1955). Apoplastic washing fluid was lyophilized before use for high-performance liquid chromatographic (HPLC) analysis.

Residual leaf (0.5 g) was homogenized in 80% acetone. The homogenates were centrifuged for 15 min at 7000 rpm at 4°C. The supernatants were collected and lyophilized. Apoplastic and symplastic lyophilizates were dissolved in the same extraction solvent, centrifuged for 10 min at 10,000 rpm at 4°C, filtered (0.45 μm Millipore filter) and directly injected (10 μl) to the HPLC system.

The HPLC system we used consisted of an Agilent 1100 Series (Palo Alto, CA) equipped with a quaternary HPLC pump, micro vacuum degasser (MVD), thermostated column compartment (TTC), refractive (RID) detector, and standard micro and preparative autosampler. Sugar analyses were performed on an EC 250/4 NUCLESIL carbohydrate (250 mm × 4.0 mm i.d., 10 μm particle size, Düren, M&N, Germany) column (25 cm × 4.6 mm i.d., 10 μm particle size) containing acetonitrile/water (79:21, v/v) in mobile phase, operating at 22°C with flow rate 1 ml/min. The automatic injection system used was a 5–10 μl sample loop. Sugar content was identified by comparison of their retention times to those of authentic standards under the above analysis conditions. Standard solutions of sucrose and glucose at the proper concentration in mobile phase were injected into the column. Calibration curves for each standard sugar solution were made, later used for assessing the concentrations corresponding to the different peaks in the chromatograms. The areas of the peaks of compounds were quantified using HP ChemStations software. Means of three replicates of three different extractions of leaf samples and standards were used in HPLC analyses.

ANALYSIS OF PROLINE

Apoplastic washing fluid was extracted by vacuum infiltration according to Nielsen and Schjoerring (1998). Fresh leaves were cut into 1 cm lengths, washed with deionized water and infiltrated with 440 mM sorbitol. Then the leaves were blotted and centrifuged as above. The proline concentration in the apoplastic water space was determined by multiplying the proline concentration of the fluid extracted with sorbitol by the dilution factor.

Dried ground leaves (0.25 g) were used for proline extraction of residual leaf. Samples were homogenized in 5 ml 3% sulfosalicylic acid and extracts were centrifuged at 8000 g for 15 min. Proline in the apoplast and symplast was determined according to the method described by Bates et al. (1973) against a standard curve.

ANALYSIS OF ORGANIC ACIDS

Fresh leaves were vacuum-infiltrated for 20 min in 10 mM KCl (Takahama and Oniki, 1992). Apoplastic extract was collected from the bottom of the tubes after leaves were centrifuged at 1450 g for 15 min as above.

Two grams of residual leaves were homogenized in 5% m-phosphoric acid. The homogenates were centrifuged for 15 min at 7000 rpm at 4°C. The supernatants were collected and lyophilized. Apoplastic and symplastic lyophilizates were dissolved in the same extraction solvent, centrifuged for 10 min at 10,000 rpm at 4°C, filtered (0.45 μm Millipore filter) and directly injected (10 μl) to the HPLC consisting of a UV/VIS detector. Organic acid analyses were performed on an Ace 5 C18 (ACE, Scotland) column (25 cm × 4.6 mm i.d., 10 μm particle size) operating at 25°C with flow rate 2 ml/min. The mobile phase was an isocratic 0.02 M potassium phosphate solution (pH 2.04, adjusted *meta*-phosphoric acid). The automatic injection system used was a 10 μl sample loop. Detection was done with an HP 1100 series multivariable wavelength detector at 210 nm. Acid components were identified by comparison of their retention times with those of authentic standards under the above analysis conditions. All the samples were directly injected to the column after filtration through a 0.45 μm Millipore filter and injected to the HPLC. Standard solutions of malic and citric acids, each containing 25 mg/ml in mobile phase, were injected into the column. Calibration curves for each standard organic acids were made (malic acid, R^2 = 1.00000, y=94405.59501.x + 45.24800; citric acid, R^2=1.00000, y=9685.98240.x + 0.907071). Later they were used for quantification. Means of three replicates of three different extractions of samples and standards were used in HPLC analyses.

ANALYSIS OF INORGANIC IONS

Extraction of Apoplastic solution for measurement of ions was performed as in proline determination. Following collection of AWF, the residual leaf (0.5 g) was homogenized with liquid nitrogen in 5 ml deionized water. The symplastic homogenate was boiled in a water bath for 10 min. The precipitate was removed by centrifugation (Schroppel-Meier and Kaiser, 1988). Symplastic and apoplastic ion content (K^+, Ca^{2+}, Cl^- and Na^+) was measured with a pH/mV/Temp meter (JENCO 6230N).

ANALYSIS OF pH

Apoplastic pH was directly measured with the pH/mV/Temp meter. Samples (0.5 g FW 20 ml) were treated for 120 min in a basal solution containing 0.5 mM $CaSO_4$, 5 μM DCMU and 20 mM MES, adjusted to the required pH (routinely pH 6 unless otherwise indicated) with LiOH or H_2SO_4 depending on the presence of the weak bases. At the end of the treatments the leaves were washed for 3 min at 0°C with 0.5 mM $CaSO_4$ to clear the free space from the external medium, blotted on filter paper, transferred to plastic syringes and frozen at -30°C for at least 3 h. The pH was directly measured with the pH/mV/Temp meter in the cytoplasmic sap obtained by squeezing the leaves after freeze-thawing (Romani et al., 1998).

STATISTICAL ANALYSIS

All analyses were made three times with three independent extractions. Variance of mean values was checked with Duncan's multiple comparison test using SPSS software for Microsoft Windows ver. 10.0 (SPSS Inc., U.S.A.) at 5% ($P<0.05$) significance.

RESULTS

Our analyses showed the presence of sucrose and glucose in the symplast and glucose in the apoplast of *Ctenanthe setosa* leaves. Symplastic sucrose significantly increased until stage 3 but decreased at 4, the last stage; at stage 3 its concentration was 1.3 times the value at stage 1. Glucose increased in the symplast during leaf rolling but decreased in the apoplast except at stage 3, when the concentration was 1.1 times the level at stage 1 (Tab. 1).

Symplastic proline significantly increased up to stage 3 but at the last stage dropped below the level at stage 1; at stage 3 its concentration was 1.2 times the level at stage 1. In apoplastic spaces, proline increased from stage 1 to stage 2 and then slightly declined later; its concentration at stage 2 was 1.4 times the level at stage 1 (Tab. 1).

Malate increased during leaf rolling in the symplast but declined in the apoplast as leaf rolling proceeded. Citrate concentrations increased in both the apoplast and symplast (Fig. 1).

Symplastic K^+ declined until stage 3 and then increased at stage 4; its concentration at stage 1 was 1.5 times the level at stage 2 and 3.2 times the level at stage 3, but by stage 4 it was higher than the level at stage 1 by a factor of 1.7 (Fig. 2). K^+ declined continuously during leaf rolling in the apoplast; Ca^{2+} significantly increased at stage 2 in both compartments, then declined (Fig. 2). Through the leaf rolling process, Na^+ concentration showed opposite trends in the symplast and apoplast; when it increased in the symplast it decreased in the apoplast, and vice versa. The initial Na^+ concentration in the apoplast was higher than at stage 2 by

TABLE 1.Changes in sugar and proline levels during leaf rolling

Leaf rolling stage	Symplast			Apoplast		
	Glucose (mg g^{-1} fw)	Sucrose (mg g^{-1} fw)	Proline (µg g-1 dw)	Glucose (mg g^{-1} fw)	Sucrose (mg g^{-1} fw)	Proline (µM)
1	0.87±0.09*a	0.76±0.05 b	144.9±7.6 b	0.47±0.01 c	-**	87.1±0.2 a
2	1.01±0.06 b	1.11±0.10 d	165.1±2.4 c	0.32±0.06 b	-	125.8±2.8 d
3	1.19±0.10 c	1.01±0.04 c	176.7±4.3 d	0.53±0.03 d	-	115.4±3.1 c
4	1.59±0.06 d	0.65±0.11 a	115.5±2.5 a	0.29±0.01 a	-	105.2±1.0 b

* Standard deviation; ** Not detected. Values with different letters within columns differ significantly (P<0.05). Means of nine replicates.

a factor of 1.4, and higher than at stage 3 by a factor of 1.2; the change at stage 4 was not significant. Cl^- concentrations declined in both compartments as leaf rolling proceeded, but at the final stage it increased in the apoplast (Fig. 2).

Drought stress reduced the pH of apoplastic fluid from 5.49 to 3.87. Symplastic pH did not significantly change during leaf rolling; the pH was 6.11 at stage 1 and 6.10 at stage 4 (Fig. 3).

DISCUSSION

In this work we examined the changes in content of organic and inorganic solutes in leaf apoplastic and symplastic spaces during leaf rolling. Symplastic glucose continuously increased during leaf rolling, while apoplastic glucose decreased except at stage 3. We infer that glucose may be transported from apoplast to symplast during leaf rolling. Symplastic sucrose increased at stage 2 and then gradually decreased, and was not detected in apoplastic fluid. This result suggests hydrolysis of sucrose to glucose in the leaf apoplast of *C. setosa*. Indeed, Dunford (1998) reported that transported sugar such as sucrose can be metabolized in the apoplast. Sugars and especially glucose are the preferred organic solute for osmotic adjustment in cells, allowing the plant to continue water uptake under drought stress causing leaf rolling.

Symplastic proline concentrations increased from stage 1 to stage 3. Proline accumulates under a broad range of stress conditions such as water shortage, high salinity, extreme temperatures, high light intensity and air pollution (Aspinall and Paleg, 1981). It is known that proline makes an important contribution to osmotic adjustment and adaptation to stress (Gzik, 1996). In our study, however, symplastic proline concentration at the last stage declined versus stage 1 (control). We suggest that the plant protects itself against stress through the first three stages, but then the proline synthesis pathway is affected by drought stress as a result of water loss. In the apoplastic area the proline concentration increased at stage 2 versus stage 1 and then slightly declined from the level at stage 2. Extracellular proline increased with the increase of intracellular proline, providing considerable support for the role of proline in stress tolerance during leaf rolling.

Malate is considered a strong osmoticum in higher plants. Its level increases in response to drought conditions in some plant species (Irigoyen et al., 1992; Tschaplinski and Tuskan, 1993). Malate involved in turgor phenomena (Martinoia and Reutsch, 1994) plays a role as a mobile storage

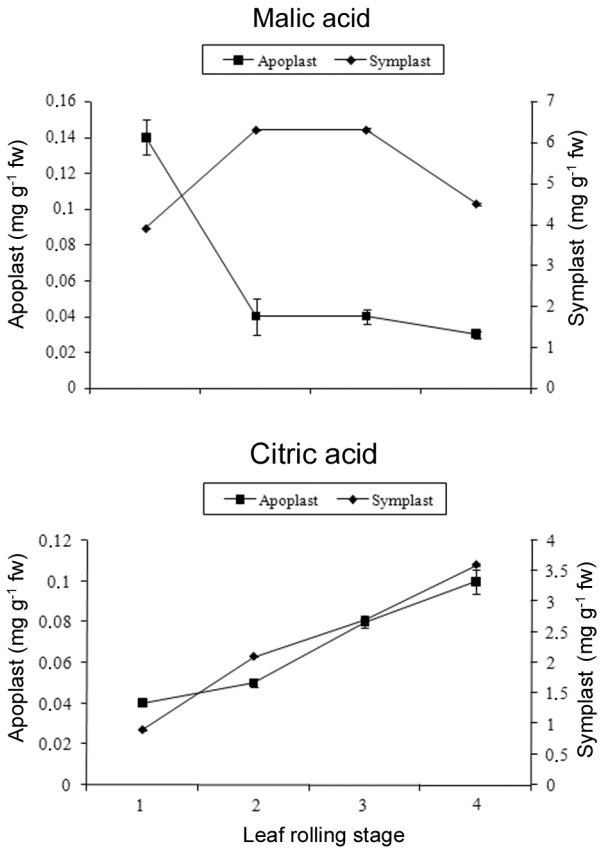

Fig. 1. Changes in organic acid concentration during leaf rolling.

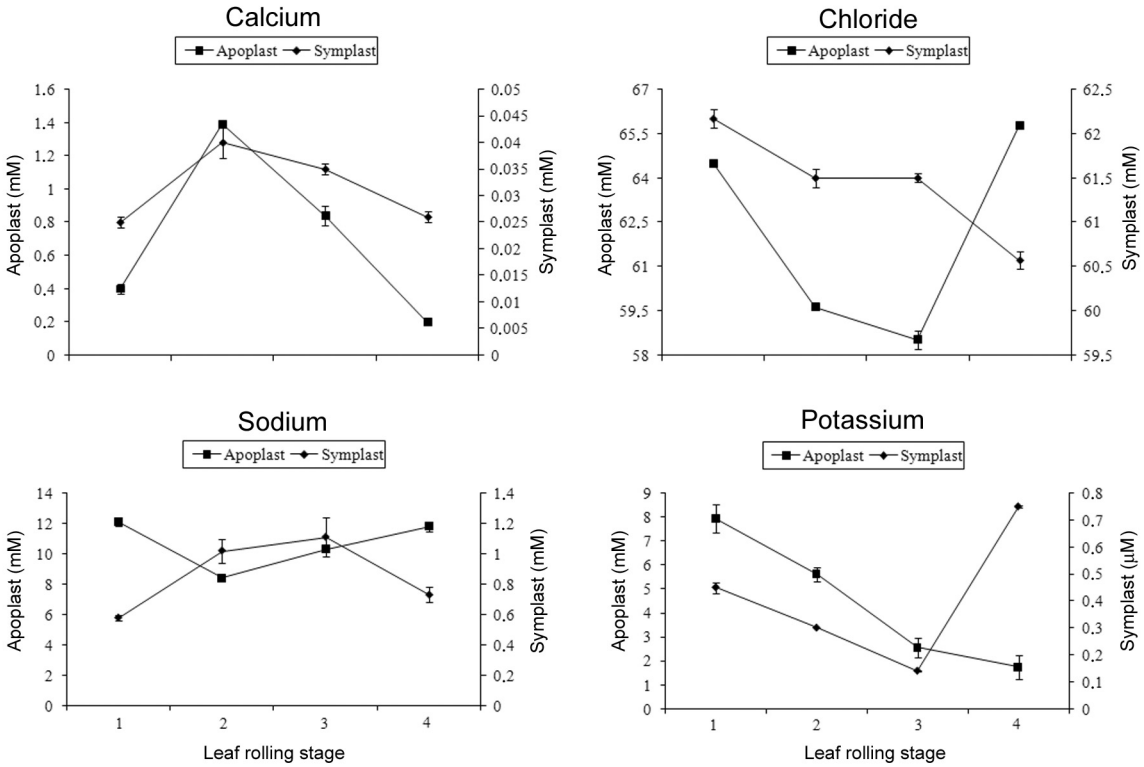

Fig. 2. Changes in apoplastic and symplastic ion concentrations during leaf rolling.

molecule for CO_2 and as a source of anions and protons for controlling intracellular pH and electrical balance. In the current study it is difficult to interpret the role of malate since the leaves contain quite appreciable amounts of Ca^{2+}. Malate level increased in the symplast but declined in the apoplast during leaf rolling. We can suggest that malate may serve as a chelator for Ca^{2+} ions. In addition, the malate accumulated in symplastic areas might be utilized in synthesis of citrate as a source of energy and carbon, because citrate concentrations increased in the symplast. Malate declined in the apoplastic fluid, however. Malate in the apoplast may have been transported to the symplast by a malate transporter located in the plasma membrane of the cell. Another reason for that decline could be high malate dehydrogenase activity associated with the cell plasma membrane, as shown in onion roots (Córdoba-Pedregosa et al., 1998). Citrate increased in both the apoplast and symplast in *C. setosa*. Increased citrate may help increase utilization of light (which decreases due to the shading effects of leaf rolling) for electron transport. In other work of ours on *C. setosa*, the electron transport rate was kept constant during rolling up to stage 3 in spite of severe drought (Nar et al., 2009).

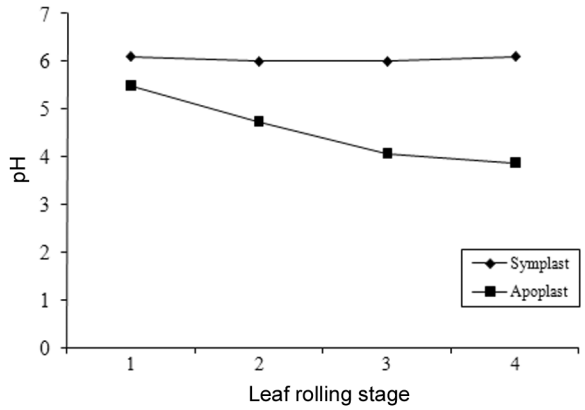

Fig. 3. Apoplastic and symplastic pH changes during leaf rolling.

In our study the Ca^{2+} concentration significantly increased in both compartments up to stage 2 and then declined. The increase may be related to the increase in H_2O_2 during drought. It is known that H_2O_2 regulates Ca^{2+} channels (Pei et al., 2000). In our previous study, H_2O_2 increased during leaf rolling under drought (Saruhan et al., 2010). Ca^{2+}

ions control water use efficiency by initiating stomatal closure (Atkinson, 1991). The increased Ca^{2+} concentrations in our stressed *C. setosa* plants may be responsible for reduction of stomatal conductance. In other work of ours we found that stomatal conductance sharply decreased at stage 2 and the decrease slowly continued at stages 3 and 4 (Nar et al., 2009). Ca^{2+} also plays a role in stress tolerance, stabilizing membranes and reducing oxidative damage by elevating antioxidants (Larkindale and Knight, 2002). An increase of cytosolic Ca^{2+} induced by drought and salt stress up-regulated proline biosynthesis (Knight et al., 1997). Rising Ca^{2+} concentrations may help protect *C. setosa* against stress by inducing proline accumulation or antioxidant enzymes in leaves. Similarly to our Ca^{2+} results, in other work on *C. setosa* we observed that some antioxidant enzymes such as ascorbate peroxidase, catalase and monodehydroascorbate reductase increased in the first two stages but slightly declined later.

Inorganic ions play important roles in controlling the osmotic potential of plants. K^+ is generally regarded as the most important cationic osmolyte, and various studies indicate that K^+ is accumulated up to an osmotically significant degree in leaf cells of a wide range of species in response to drought (Matoh et al., 1988; Liu and Luan, 1998). In this study, however, symplastic K^+ concentrations decreased up to stage 3 but increased at the last stage, whereas the apoplastic K^+ level declined continuously. At the last stage the decrease of K^+ in the apoplast indicates the influx of K^+ towards the symplast; the decline of K^+ in both the symplast and apoplast suggests that K^+ might be converted to different forms such as potassium salts in the symplastic area to maintain osmosis.

Sodium affects the water relations of plants and often enables crops to withstand drought conditions that would otherwise produce severely adverse effects (Durrant et al., 1978). The increase in symplastic sodium up to stage 3 is likely a consequence of inactivation of the plasma membrane (sodium/proton) antiporter or its homologues. The increase in symplastic sodium in *C. setosa* provides evidence that sodium is a preferred ion for osmotic adjustment. Apoplastic sodium declined at stage 2 but rose at later stages, in contrast to the trend for symplastic sodium. To maintain the cytosolic Na^+ concentration at a low level to prevent toxicity, *C. setosa* may increase Na^+ efflux from the cytosol into the apoplast.

It is known that chloride has a role in controlling stomatal movement. In the present study the Cl^- concentration decreased in both compartments as leaf rolling proceeded, but at the final stage it increased in the apoplast. The decrease may show that Cl^- is needed in regulation of the stoma during drought. The increase may indicate that Cl^- is transported from the symplast to the apoplast to contribute to osmotic adjustment. K^+, Na^+ and Ca^{2+} were higher in the apoplast than in the symplast. These results show the important role of the apoplast in adjusting osmolarity so that the plant can maintain water content during drought.

In this study the symplastic sap of leaf exhibited constant pH, while apoplastic pH gradually decreased during leaf rolling. It is known that increasing symplastic Ca^{2+} deactivates plasmalemma H^+/ATPase and also activates K^+/H^+ symport. The inflow of K^+ and H^+ depolarizes the membrane, so the apoplast becomes less acidic (Netting, 2000). However, symplastic Ca^{2+} decreased during leaf rolling. We can say that Ca^{2+} did not affect plasmalemma H^+/ATPase activity and did not activate K^+/H^+ symport, so the apoplast became more acidic than in other plant species. Apoplastic pH has been reported many times from different species, with the majority of values ranging between 5.3 (Kosegarten and Englisch, 1994) and 6.7 (Dannel et al., 1995).

We found that the concentrations of organic acids, ions, proline and sugar changed in apoplastic and symplastic spaces during leaf rolling. The changes in inorganic ions and organic solutes between apoplastic and symplastic spaces contributed to osmotic adjustment during leaf rolling. Among the organic compounds, glucose and proline were preferred for osmotic adjustment, and among the inorganic ions Na^+ and K^+ were preferred. On the other hand, Cl^- was the less preferred ion, but it may have roles in controlling regulation of stomata during rolling. Ca^{2+} may be important in controlling stomatal behavior and antioxidant synthesis. The changes in solutes were very pronounced, especially in the apoplastic space, indicating that the apoplast plays important roles in controlling leaf rolling under drought. Further studies on solute transport and sequestration mechanisms in the apoplast during leaf rolling should help elucidate those roles.

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