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RESEARCH PAPER

Evidence for long-distance xylem transport of signal peptide activity from tomato roots

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Abstract

Several types of small, endogenous signal peptides are now known to induce a wide range of local and systemic responses in plants, but how such signal peptide activity is transported over long distances remains unclear. In particular, the possible occurrence and root-to-shoot transport of signal peptide activity in the xylem does not appear to have been previously investigated. Suspension-cultured cells of wild tomato *Lycoporsicon peruvanium* L. were used in an established bioassay for detecting nanomolar concentrations of signal peptides via the induction of alkalinizing activity. Xylem sap naturally exuded from the cut and washed stem-surfaces of de-topped tomato plants *(Lycopersicon esculentum* L. cv. Castlemart) was collected, partially purified, concentrated, and shown by the bioassay consistently to contain significant alkalinizing activity. Plant salinity treatment induced further small increases in activity. Subsidiary experiments indicated that the alkalinizing activity found in the xylem-sap had properties similar to those of known plant signal peptides and was root derived. Thus, it was (i) detectable within minutes, (ii) eluted similarly during HPLC chromatography, (iii) destroyed by incubation with proteases and stable in the presence of protease inhibitor cocktail, and (iv) not found in bioassays of simulated xylem sap placed on the cut stem-surfaces of non-exuding roots in order to detect any significant release of wound peptides from the stem. Further investigations of the signal peptide activity in root xylem sap could provide new insights into its identity, genes, receptors, origins, and possible hormonal roles in regulating shoot growth and development.

Key words: Bioactive peptides, bioassay, hormone, long-distance signal, *Lycopersicon,* xylem-sap.

Introduction

Long-distance signalling between roots and shoots and the induction of appropriate responses is essential for the optimal functioning of higher plants in their continually changing soil and aerial environments. Chemical, hydraulic, or electrical signalling may be involved (Malone, 1992; Chazen and Neumann, 1994). Most of the available evidence concerns chemical signalling via the traditional plant hormones. For example, leaf epinastic responses to root flooding have been related to increased root-to-shoot xylem transport of the ethylene precursor ACC (Bradford and Yang, 1980; Else *et al.*, 1995). Similarly, leaf stomatal closure has been related to the induction by soil-water deficits of increased root-to-shoot xylem transport of abscisic acid (Davies and Zhang, 1991; Sobeih *et al.*, 2004). This article raises the possibility that bioactive peptides, which are now considered major components of signalling for defence, leaf shape, self incompatibility, nodule development, organ abscission, cell division, cell differentiation, and root growth (see reviews by Ryan *et al.*, 2002; Matsubayashi and Sakagami, 2006), may also have a role in long-distance root-to-shoot signalling via the xylem.

Some evidence supports the occurrence of long-distance signalling via phloem transport of small RNA and signal peptides such as systemins (Pearce *et al.*, 1991; Chen and Kim, 2006; Lough and Lucas, 2006). Systemins are 18 amino acid peptides produced in plants responding to the stress of localized wounding. They induce a systemic activation of plant defence proteins such as the serine protease inhibitors which may upset protein digestion in

predator insects. However, the possibility that systemin itself (or other peptides) undergo long-distance phloem transport throughout the plant remains controversial (Stratmann, 2003). Any involvement of the other long-distance transport system, i.e. the apoplastic xylem network, in transporting endogenous peptide signal activity from roots to shoots does not appear to have been investigated.

The xylem sap of monocot and dicot species is known readily to transport water, mineral ions, amino acids, carboxylic acids, protons, and traditional non-peptide hormones from the roots to the shoots (Gollan *et ai,* 1992; Goodger *et al.,* 2005). It also transports a range of large enzyme proteins including serine, aspartyl and cysteine proteases (Biles and Abeles, 1991; Satoh *et al.,* 1992; Rep *et al,* 2002; Bhutz *et al.,* 2004; Kehr *et al.,* 2005; Alvarez *et al.,* 2006). Although xylem transport of small root-produced signal peptides has not been reported, exogenous signal peptides artificially supplied to transpiring shoot explants via the cut xylem have been shown to induce appropriate metabolic responses in distant target cells in the leaves (Pearce *et al.,* 1991, 2001; Scheer *et al.,* 2005; Huffaker *et al.,* 2006). These reports suggest that any signal peptides naturally secreted into the root xylem would be able to move into leaf cells after root-to-shoot transport.

Bioactive signal peptides of plant and micro-organism origin have been shown to block a proton pumping ATPase, thereby reducing the outward flux of protons to the apoplast and causing an alkalinization of the medium of suspension-cultured cells (Felix and Boiler, 1995; Schaller and Decking, 1999). The regulation of root and shoot growth has also been partially associated with altered transport of protons into (expanding) cell walls (Rayle and Cleland, 1992; Bogoslavsky and Neumann, 1998; Peters and Felle, 1999; Winch and Pritchard, 1999; Fan and Neumann, 2004; Fan *et al,* 2006). Moreover, the RALF signal peptide has been shown to inhibit root growth in seedlings (Pearce *et al,* 2001). An investigation of possible root-to-shoot transport of peptide signal activity was therefore of interest in the context of conceivable involvement in shoot growth regulation, for example, for ongoing maintenance of root:shoot ratios or in response to root stresses.

A sensitive bioassay based on induced alkalinization of the medium of suspension-cultured wild tomato cells has been developed and used conveniently to detect nanomolar concentrations of several different bioactive signal peptides in purified extracts from plant shoots (Pearce *et al,* 2001; Pearce and Ryan, 2003; Scheer *et al,* 2005; Huffaker *et al,* 2006). This report concerns the use of this cell-suspension alkalinizing assay to search for the presence of signal peptide activity in xylem sap naturally exuded from the roots of detopped greenhouse-grown tomato plants under well-watered or saline conditions.

Materials and methods

Plant growth

Tomato plants were grown in a greenhouse. Batches of 6-10-week-old plants were used for exudate collection. Some plants in each batch were salinized by irrigating with nutrient solution containing 100 mM NaCl for 2 d. At 30 min before sap collection the salinized soil was thoroughly flushed with nutrient solution containing 20 mM NaCl in order to decrease osmotic restraints to root pressure development.

Sap *collection and purification*

The shoots of control or salinized tomato plants were excised with a fresh razor blade 2-3 cm above the soil level and discarded. The cut stem surfaces protruding from the soil were repeatedly rinsed with streams of water and blotted before collection of the exuding xylem sap droplets by use of a pipette. Sap was stored on ice in graduated plastic tubes and a pooled volume of 1.5 ml or more was collected after about 2 h from batches of 5-20 plants per treatment. Protein levels in the sap of control plants ranged between 70 jig ml^{-1} and 100 ug ml^{-1}. Collected sap was desalted and partially purified by passage through Cig Sep-Pak cartridges (Waters, Milford, MA) and elution of the adsorbed activity with 1 ml aliquots of 25%, 50%, and 75% acetonitrile in 0.1% trifluoroacetic acid (TFA). The pooled eluates were freeze-dried and suspended in 0.1% TFA prior to assay of alkalinizing activity using *L. peruvianwn* cell suspensions.

Alkalinizing assay

L. peruvianum cell-suspension cultures were maintained at 24 °C under weak fluorescent light in 125 ml flasks on orbital shakers in Murashige and Skoog media, excluding buffer, adjusted to pH 5.6 with KOH, and renewed weekly as in Scheer and Ryan (1999). After 4-7 d, aliquots of 1 ml of cell suspension were transferred to wells of 24 well cell-culture cluster plates (Costar, Coming Inc.) and allowed to equilibrate for 1 h under laboratory conditions on an orbital shaker at 160 rpm prior to addition of 1-10 ul of sap preparations dissolved in 0.1% trifluoroacetic acid. Addition of trifluoroacetic acid alone caused minor decreases in the pH of cell suspensions. Any pH increases (alkalinization of the cell suspension medium) induced by signal peptide activity in sap preparations were detected up to 30 min after adding the sap, with an Orion semi-micro pH electrode attached to an Orion EA940 pH meter (Thermo Orion, Beverly, MA). The pH of control cell suspensions without sap addition were monitored in parallel.

Statistics

Paired two-sample *t* tests of means, using the analysis tool box in Excel, were performed to compare signal activity in exudates of tomato plants from the same batches of circa 30 plants which had been divided into salt-treated or control treatments.

Results

Alkalinizing activity in root exudates

Preliminary experiments indicated that alkalinizing activity in the xylem sap was best assayed after desalting and partial purification by selective adsorption of the alkalinizing activity in Cig Sep-Pak cartridges. The removal from the sap constituents of polar solutes with osmotic and pH

buffering capacity appeared to reduce adverse effects on the wild tomato cell bioassay. The less polar material subsequently eluted from the cartridges by increasing concentrations of acetonitrile in 0.1% TFA was shell frozen in liquid nitrogen, freeze-dried, and resuspended in of 0.1% trifluoroacetic acid (TFA) to give 100-fold concentration of the original sap volume. Microlitre aliquots of the partly purified, concentrated, and acidified sap were assayed for alkalinizing activity 30 min after adding to

1 ml aliquots of cell suspension. Control cell suspensions without sap additions were assayed at the same time. Additions of up to 10 ul aliquots of 0.1% TFA alone to 1 ml batches of cell suspension slightly reduced pH. However, similar additions of the part-purified and

concentrated xylem sap preparations suspended in 0.1% TFA, consistently induced pH increases, even at a 40 times dilution of the original sap volume (not shown).

Table 1 summarizes the results from five independent experiments. In each, the alkalinizing activity induced by root xylem sap preparations from matched batches of well-watered and salinized plants, were bioassayed. The additions of sap clearly induced pH increases in comparison with control cell suspensions to which no sap was added, A paired *t* test of the means indicated that these increases were statistically significant *(P* >99%). Interestingly, the pH values in the saline treatments were consistently more alkaline than those in the well-watered treatments and although the differences were not large they were statistically significant *(P* >95%). Thus, signal peptide activity was consistently present in root-derived xylem sap from well-watered and salinity-treated plants and the activity was somewhat increased by the salinity treatments. Additional subsidiary experiments were carried out to support or possibly refute the findings.

Elution profile and UV absorbtion of alkalinizing activity

Previous work has used C_{18} column elution profiles and UV absorption further to identify signal peptides in extracts from plant leaves. A root xylem sap preparation was therefore analysed using a narrow bore HPLC Cj_g column (2.1 mm inner diameter, Vydac 218TP52, Hesperia, CA) equilibrated with 0.1% TFA in water at a flow rate of 0.25 ml min"[1] and a gradient to 50% acetonitrile in 0.1% TFA. Fractions eluting at 1 min intervals from the Cig column between 20 min and 70 min exhibited various UV-absorbing components when measured at 214 nm (data not shown). Several peaks exhibited alkalinizing activity, with a large, broad activity peak in fractions 48 to 60 and a smaller peak associated with fractions 60 to 70 (Fig. 1).

Proteinase susceptibility of alkalinizing activity

Signal peptides are expected to be deactivated by the protease trypsin at neutral pH. In order to test the protease susceptibility of the signal activity in xylem sap, active fractions from the C_{18} column were pooled, vacuum centrifuged to dryness, and resuspended in 50 mM ammonium bicarbonate buffer. Aliquots were then incubated overnight at 36 °C with or without additions of trypsin (1 ul of 1 mg ml"[1] trypsin per 20 ul sample). The samples were then freeze-dried for 24 h to remove ammonium bicarbonate buffer, resuspended in 0.1% TFA and bioassayed for alkalinizing activity. Surprisingly, the alkalinizing activity in the sap was lost in both cases (not shown). Thus, both trypsin and xylem sap alone appeared spontaneously to destroy alkalinizing activity when incubated overnight at neutral pH and 36 °C.

The spontaneous loss of alkalinizing activity in xylem exudates was confirmed in an independent experiment in which partly purified and freeze-dried xylem sap was also resuspended in ammonium carbonate buffer and incubated at 36 °C for only 6,h, with or without the addition of aliquots of a solution of proteinase inhibitor cocktail (Roche Applied Sciences-complete mini protease inhibitor).

Table *1.* *Induction by xylem sap preparations from well-watered and salinized plants of increased alkalinizing activity in wild tomato cell-suspension bioassays*

Data summarize five separate experiments each involving xylem sap from matched batches of 5-20 well-watered or salinized plants. Microlitre aliquots of partially purified and concentrated xylem sap preparations in 0.1% TFA (trifluoroacetic acid) were added to 1 ml aliquots of stirred wild tomato cell suspensions at room temperature and pH recorded after 30 min. The first column gives pH of the appropriate cell suspension without sap additions.

Experiment	Cell suspension pH		
	Without sap	+Well-watered sap	+Salinized sap
1	4.35	5.09	5.41
2	4.12	4.55	4.58
3	4.11	4.99	5.04
4	4.22	4.56	4.84
5	4.42	4.84	5.02

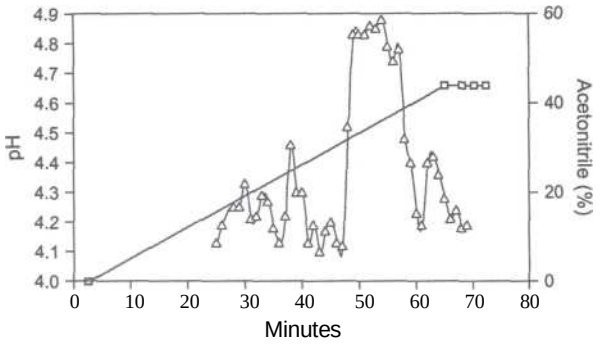

Fig. 1. Alkalinizing activity in HPLC fractions of xylem sap. Concentrated xylem sap was applied to a narrow bore Cig HPLC column and fractions of 0.25 ml were collected. Aliquots of 10 ul from each fraction were added to 1 ml of stirred wild tomato *(L. peruvianum)* cell suspension for assay of pH (triangular symbols) after a 30 min incubation. Acetonitrile gradient, square symbols.

These samples were bioassayed for alkalinizing activity as above. The pH of 1 ml batches of cell suspension media after additions of 2, 5, or 10 *il* aliquots of sap treated without the addition of proteinase inhibitor cocktail, averaged pH 4.43 (Table 2). This was similar to the average of pH 4.42 (n=3) for cell suspensions without sap addition. Thus, sap preparations incubated at neutral pH and high temperature for 6 h without proteinase inhibitor lost their capacity to alkalinize cell suspension media. By contrast, the cell suspension to which the similarly treated sap plus proteinase inhibitor was added, reached above background pHs (up to pH 5.21). These findings suggest that the proteinase inhibitor cocktail inhibited the spontaneous degradation of bioactive peptides by the endogenous proteases known to be present in xylem sap.

Table 2. *Proteinase inhibitor cocktail prevents the spontaneous loss of alkalinizing activity in xylem sap preparations*

Xylem sap preparations plus proteinase inhibitor cocktail dissolved in 50 mM ammonium bicarbonate buffer (+PI) or ammonium bicarbonate buffer alone (-PI) were each incubated for 6 h at 36 °C to allow any endogenous protease activity to occur. Ammonium bicarbonate was then removed by freeze-drying, the sap residues were suspended in 0.1% TFA, and any signal peptide activity bioassayed by measuring the alkalinizing effects of indicated aliquots of sap preparations on 1 ml batches of wild tomato cell suspensions. The mean pH of cell suspension without additions of xylem sap was pH 4.42 (n=3).

Sap aliquot	Cell suspension pH	
	-PI	+PI
2ul 5	4.42	4.67
Hi 10	4.45	4.97
pi	4.43	5.21

Kinetics of the alkalinizing response

Xylem sap preparations from both control and previously salinized plants initiated progressive alkalinizing responses in tomato cell suspension media within min. For example, the average pH increase induced in 10 min by sap preparations from separate batches of plants was 0.56±0.16 pH units (means ±SE, *n-3).* The speed of this alkalinizing response to root-derived xylem sap was within the range previously shown by known plant signal peptides extracted from tomato leaves.

Contamination from the cut stem

To minimize any potential accumulation in the sap of alkalinizing activity due to the possible release of wound peptides at the site of collection, the cut stems were washed and blotted three or more times over several min before collecting root exudates from their surfaces. A measure of the relative effects of potential contaminants was obtained by assaying the alkalinizing activity collected after repeatedly placing fresh 50 ul drops of simulated sap, consisting of a dilute solution with salt composition similar to that of natural xylem sap, directly on the cut stem surfaces of plants in which root pressure and natural xylem sap exudation had been osmotically prevented. This was achieved by irrigating plants to run off with 100 mM NaCl for 2 d and then with 250 mM NaCl at 30 min before excising and discarding the shoots. The salt treatment completely prevented appearance of root exudate at the cut stem surfaces. A total of 3 ml of simulated sap was then collected from the cut stems over 1.5 h, part-purified in the usual way, and suspended in 0.1% TFA before bioassay (Table 3). The mean pH of cell suspensions at 30 min after additions of aliquots of the part-purified artificial xylem sap was 4.43 (n=3), as compared with 4.42 *(n=3)* for the same cell suspensions without sap addition. By contrast, an equal volume of

Table 3. *Comparative absence of peptide signal activity in artificial xylem solution contacted with and collected from non-exuding cut stem surfaces*

Droplets of simulated xylem solution (50 ul containing 0.1 mM $CaCl_2$ and KC1) were applied to and removed from the cut and washed stem surfaces of de-topped plants the roots of which had been osmotically stressed with 250 mM NaCl to prevent root exudation of natural xylem sap. Natural sap was then collected from the same plants after flushing out soil salinity by repeated irrigation with dilute nutrient solution. After partial purification and concentration, any alkalinizing effects were assayed 30 min after the addition of indicated aliquots of artificial or natural xylem sap preparations to 1 ml aliquots of stirred wild tomato cell suspension. The mean pH of cell suspensions without additions of xylem sap was pH 4.42 (n=3).

Sap aliquot	Cell suspension pH	
	Artificial sap	Natural sap
2 ul 5ul 10 pi	4.47	5.00
	4.47	5.02
	4.39	4.74

natural root-derived xylem sap which was collected from the same cut stems after repeatedly flushing the roots with non-salinized nutrient solution to remove the osmotic restraint and then processed in the same way, induced strong alkalinizing activity. This indicated that the alkalinizing activity in the root sap was derived from xylem exudate rather than wounded tissues at the cut surface of the stem collection site.

Though not on a scale which allows detailed statistical analyses, the subsidiary experiments, both individually and when taken together, provide further support for the presence of peptide signal activity in root-derived xylem sap.

Discussion

Experiments were conducted to explore the possibility that root-derived xylem sap collected from de-topped tomato

plants might contain a peptide or peptides with signal activity. Several signal peptide molecules that activate both defensive and developmental plant genes have been previously shown to induce alkalinization in wild tomato *(L. peruvianum)* cell suspension cultures when added in nanomolar concentrations (Felix and Boiler, 1995; Schaller and (Decking, 1999; Pearce *et al,* 2001; Pearce and Ryan, 2003; Scheer *et al.,* 2005). The main finding in this report is that signal peptide activity can be consistently detected by the cell suspension bioassay in microlitre aliquots of partly purified xylem sap from well-watered and salt-treated plants. The probable peptide nature and root origin of this signal activity is further supported by subsidiary findings.

(i) The alkalinizing activity in a xylem sap preparation was found to elute during a Qg column chromatography run at concentrations of non-polar solvent previously shown to elute bioactive plant peptides and the activity was associated with UV absorption at 214 nm (indicative of peptide carbonyl linkages).

(ii) The alkalinizing activity in pH neutralized sap decreased spontaneously during incubation at 36 °C. The activity was possibly degraded by one or more of the protease types reported to be present in the xylem sap of numerous plant species (Bhutz *et al.,* 2004; Alvarez *et al.,* 2006). This spontaneous loss of xylem sap alkalinizing activity could be prevented by a cocktail of protease inhibitors.

(iii) Alkalinizing activity was induced in the tomato cell suspension bioassay within minutes of adding xylem sap preparations and showed kinetics similar to those induced by signal peptides extracted from the shoots of plants. Classical plant hormones such as abscisic acid and methyl jasmonate which may be transported in the xylem did not induce similar alkalinizing responses (Felix and Boiler, 1995; Pearce *et al,* 2001).

(iv) The alkalinizing activity in the xylem sap appeared to be root-derived rather than an artefact due to the release of wound peptides from the cut stem surfaces from which the sap was collected. Thus, the activity produced by simulated xylem sap droplets applied to and removed from the cut stem surface of non-exuding plants was negligible compared with the activity levels produced by natural xylem exudates similarly collected from the same cut stems after removal of the high root salinity which provided an osmotic restraint to exudation. Several previous investigations have used other approaches to conclude similarly that the enzyme proteins also found in root xylem sap of various plant species were not contaminants derived from the cut stem surfaces where the sap was collected (Bhutz *et al.,* 2004; Kehr *et al.,* 2004; Alvarez *et al.,* 2006).

It has been suggested that the enzyme proteins detected in xylem sap might result from either passive release accompanying the maturation into vessels of young xylem cells at the growing root tips and/or from secretion by xylem-adjacent cells (Biles and Abeles, 1991; Satoh *et al.,* 1992; Rep *et al.,* 2002; Bhutz *et al.,* 2004; Kehr *et al.,* 2004; Alvarez *et al.,* 2006). How large protein molecules may be secreted by xylem parenchyma cells into the xylem sap is unknown. Smaller signal peptide molecules should be more mobile and it is noteworthy that exogenous signal peptides supplied to the cut stems of explants can be transported through the xylem to induce metabolic responses in target cells in the leaves (Pearce *et al.,* 1991, 2001; Scheer *et al.,* 2005; Huffaker *et al.,* 2006).

Salinity is reported to induce some plant metabolic responses similar to those induced by bioactive peptides (Chao *et al.,* 1999; Dombrowski, 2003). Salinity treatment was shown here consistently to cause small increases in the alkalinizing activity of root xylem sap. It is not known whether these increases were related to increased secretion of bioactivity into the xylem of salt-treated roots or to the concentrating effect of a salinity-induced lowering of water flux through the roots (Neumann and Stein, 1984; Evlagon *et al.,* 1990). In the latter case, the peptide activity in the xylem may be more related to developmental processes than to salt-stress responses.

In conclusion, several lines of evidence indicate that signal peptides are transported from tomato roots to shoots via the xylem. The most important evidence is that partially purified root-derived xylem sap from both well-watered and salinized plants repeatedly showed significant levels of alkalinizing activity in a bioassay which has been shown specifically to detect the presence of signal peptides. Subsidiary support comes from the findings that (i) the kinetics of alkalinization induced by xylem sap preparations were similar to those induced by known signal peptides, (ii) a sap preparation showed UV absorbance and C_{J8} column elution traits similar to those shown by known signal peptides, (iii) as expected for peptides, the alkalinizing activity in xylem sap appeared to be protease sensitive and its spontaneous loss was prevented by a protease inhibitor cocktail, and (iv) any alkalinizing activity generated by the potential release of wound peptides from the cut stem surfaces was found to be negligible compared with the activity levels in the natural xylem exudates subsequently collected from the same plants.

This report appears to provide the first evidence for transport in xylem sap of endogenous signal peptide activity from the roots. Further investigations of xylem-borne signal peptides could provide new insights into their identity, genes, receptors, origins, and possible hormonal roles in regulating shoot growth and development.

Note added in proof

Maryani *etal. [JXB,* Vol. 54, 1533-1564 (2003)] detected an immunoreactive natriuretic-like peptide with ion and

water transport activity in the xylem exudate of a perennial forest sage *(P. ciliatus).*

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