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Ascorbic acid conjugates isolated from the phloem of *Cucurbitaceae*

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ABSTRACT

Analysis of phloem exudates from the fruit of *Cucurbitaceae* revealed the presence of several compounds with UV–visible absorption spectra identical to that of L-ascorbic acid. In *Cucurbita pepo* L. (zucchini), the compounds could be isolated from phloem exudates collected from aerial parts of the plant but were not detected in whole tissue homogenates. The compounds isolated from the phloem exudates of *C. pepo* fruit were eluted from strong anion exchange resin in the same fraction as L-ascorbic acid and were oxidised by ascorbate oxidase (E.C. 1.10.3.3). The major compound purified from *C. pepo* fruit exudates demonstrated similar redox properties to L-ascorbic acid and synthetic 6-*O*-glucosyl-L-ascorbic acid (6-GlcAsA) but differed from those of 2-*O*-glucosyl-L-ascorbic acid (2-GlcAsA) isolated from the fruit of *Lycium barbarum* L. Parent and fragment ion masses of the compound were consistent with hexosyl-ascorbate in which the hexose moiety was attached to C5 or C6 of AsA. Acid hydrolysis of the major *C. pepo* compound resulted in the formation of L-ascorbic acid and glucose. The purified compound yielded a proton NMR spectrum that was almost identical to that of synthetic 6-GlcAsA. A series of L-ascorbic acid conjugates have, therefore, been identified in the phloem of *Cucurbitaceae* and the most abundant conjugate has been identified as 6-GlcAsA. The potential role of such conjugates in the long-distance transport of L-ascorbic acid is discussed.

1. Introduction

Over the last decade significant advances have been made in the understanding of the physiology and metabolism of L-ascorbic acid (AsA) in plants. The *de novo* biosynthetic pathway was identified in 1998 (Wheeler et al., 1998) and all of the pathway enzymes have now been identified and the corresponding genes cloned (Østergaard et al., 1997; Keller et al., 1999; Wolucka et al., 2001; Gatzek et al., 2002; Laing et al., 2004; Dowdle et al., 2007). Advances have been made in understanding the pathways of ascorbate breakdown (Green and Fry, 2005) and the role of AsA as a precursor for organic acids (DeBolt et al., 2007). A number of advances have also been made in understanding the role of ascorbate in plant physiology aided by the availability of the AsA deficient vtc mutants of *Arabidopsis thaliana* that have revealed roles for AsA in developmental processes such as flowering, senescence and morphogenesis (Pavet et al., 2005; Attolico and De Tullio, 2006; Barth et al., 2006; Olmos et al., 2006). The vtc mutants have also aided the analysis of the role of AsA in the modulation of plant responses to both biotic (Pavet et al., 2005) and abiotic (Pignocchi and Foyer, 2003) stresses.

In many plant tissues the concentration and redox status of the AsA pool is tightly controlled. For example, with the exception of the apoplast, the aerial parts of plants have a highly reduced AsA pool with dehydroascorbic acid (DHA) representing less than 10% of the total (Noctor, 2006) while in root tissues the AsA pool is more oxidised with up to 30% DHA (Córdoba-Pedregosa et al., 2003). The mechanisms controlling AsA concentration and redox status are still largely mysterious with large areas of uncertainty concerning the genetic and biochemical controls of pathway flux (Hancock and Viola, 2005). Further uncertainty exists regarding the control of distribution of AsA at the whole plant level. Several studies have indicated that long-distance AsA transport via the phloem can occur (Franceschi and Tarlyn, 2002; Tedone et al., 2004) however, in the only systematic study undertaken to date, AsA accumulation in blackcurrant (*Ribes nigrum*) fruit was correlated with *in situ* synthesis and the contribution of AsA transport from source tissues was negligible (Hancock et al., 2007). It remains unclear to what extent AsA transport contributes to sink accumulation in other species and organs. Although the phloem is capable of synthesising AsA from sugars (Hancock et al., 2003), radiolabelling evidence also suggests active uptake of AsA in source leaf phloem (Franceschi and Tarlyn, 2002; Tedone et al., 2004) although the transporters are yet to be identified. These experiments were undertaken in apoplastic phloem loaders and currently no information is available regarding the uptake of AsA by the phloem of symplastic loaders.

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During research to gain greater insight into the roles of phloem AsA, a number of compounds with physicochemical properties similar to those of authentic AsA were isolated. In the present work, the presence of such compounds in several *Cucurbitaceae* species are documented and detailed physicochemical analysis of the most abundant AsA analogue from *Cucurbita pepo* was undertaken.

2. Results

2.1. ***Cucurbitaceae*** *phloem exudates contain multiple AsA analogues*

Analysis of deproteinated phloem exudates from several species of *Cucurbitaceae* by cation interaction HPLC, revealed the presence of several peaks with identical absorption spectra to AsA but different retention times. Retention times and relative peak areas of compounds with maximum absorbance 245 ± 3 nm from several *Cucurbitaceae* fruit are presented in Table 1. Only AsA was detected in *C. maxima* however, in all other species analysed at least one additional peak was detected and in several species a number of additional peaks were observed. For the majority of species sampled, AsA was the most abundant peak however, in *C. maxima* × *C. moschata* the peak at 9.0 min was almost four times as abundant as AsA and in *Cucumis sativus* (cucumber) the peak eluting at 11.7 min was slightly more abundant than AsA.

Due to the relatively large volumes of exudate that could be collected from *C. pepo* (zucchini) fruit, the presence of at least three of the four unidentified peaks and the ready availability of fruit from local markets throughout the entire year, further characterisation was undertaken on exudates from this species. To enrich for putative AsA analogues, crude exudates were collected into an equal volume of 10% $HClO_4$ containing 10 mM TCEP. After neutralisation with 5 M K_2CO_3, sample supernatants were applied to strong anion exchange cartridges and AsA and putative analogues were eluted with 300 mM formic acid. Following purification and concentration an additional peak was observed eluting after 13.5 min (Fig. 1).

Confirmation that the peaks eluting in the 300 mM formic acid fraction were related to AsA was achieved following incubation with ascorbate oxidase (E.C. 1.10.3.3) which resulted in the removal of all peaks (Fig. 2). Ascorbate oxidase specifically oxidises AsA, its 5-carbon analogue D-erythroascorbate (Hancock et al., 2000) and a few closely related glycosyl conjugates (Loewus, 1999).

The distribution of AsA related compounds within plant tissues was quantified in zucchini plants grown under glass. The most abundant analogue (Fig. 1, peak 2) was detected in trace amounts in flowers but could not be detected in any other whole tissue extract (Table 2). On the contrary, AsA was found in all tissues examined at concentrations similar to those reported in previous studies (Ranieri et al., 1993; Logan et al., 1998). Both AsA and its most abundant analogue were found in phloem exudates collected from fruit, leaf petioles and stems. In comparison to our previous findings in locally purchased zucchini (Hancock et al., 2003), phloem AsA concentrations were approximately 10-fold lower. This may be related to the growing conditions of the fruit which failed to exude as strongly as those obtained commercially. The position of phloem sampling had little effect on the AsA concentration determined with similar values recorded whether sampled from the stem, the leaf petiole or from fruit. In contrast, the concentration of the major analogue was dependent on the part of the plant from which phloem was sampled with low concentrations in phloem exudates from the stem, intermediate concentrations in fruit exudates and high concentrations in leaf petiole exudates.

2.2. The major zucchini analogue is a glucoside

Acid hydrolysis resulted in the progressive conversion of the primary analogue isolated from zucchini phloem to AsA as determined by cation interaction HPLC with diode array detection (Fig. 3, panel A). The other reaction product was glucose as determined by anion exchange HPLC with pulsed amperometric detection (Fig. 3, panel B). These data indicate that the major AsA analogue present in the phloem of zucchini is an L-ascorbic acid glucoside.

2.3. The most abundant zucchini conjugate has physicochemical properties similar to AsA and 6-GlcAsA but different from 2-GlcAsA

To further characterise the major AsA glucoside isolated from zucchini fruit exudate (Fig. 1, peak 2), its absorption spectrum was compared with that of AsA, synthetic 6-*O*-β-D-glucosyl AsA (6-GlcAsA) and 2-*O*-β-D-glucosyl AsA (2-GlcAsA) isolated from *Lycium barbarum* L. fruit (Toyoda-Ono et al., 2004). AsA, 6-GlcAsA and the glucoside isolated from zucchini fruit exudates all showed similar absorbance spectra at both pH 2.0 and pH 7.6 with λ_{max} close to 243 nm and 265 nm, respectively. On the contrary, 2-GlcAsA had lower absorbance maxima of 235 and 259 nm at acid and alkaline pH (Table 3).

AsA, 6-GlcAsA and the unknown zucchini glucoside demonstrated a strong capacity to reduce dichlorophenolindophenol (DCPIP). On the contrary, 2-GlcAsA demonstrated a very weak capacity to reduce DCPIP (Table 4). The mechanism of DCPIP reduction by AsA involves both the C2 and C3 hydroxyl groups (Rao et al., 1987) suggesting that these groups were unmodified in the zucchini analogue.

AsA was readily oxidised by both H_2O_2 and ascorbate oxidase while synthetic 6-GlcAsA and the zucchini analogue showed an intermediate oxidation rate approximately 40% of that of AsA. 2-GlcAsA was completely resistant to oxidation by either H_2O_2 or

Table 1
Relative concentration of compounds with similar optical properties to AsA isolated from *Cucurbitaceae* phloem

Species	Peak retention (min)				
	7.7	9.0	10.0	11.7	12.7
C. maxima × *C. moschata*	0.25 ± 0.01	3.69 ± 0.04	0.14 ± 0.02	ND	1.00 ± 0.03
Cucurbita moschata	ND	0.38 ± 0.05	ND	ND	1.00 ± 0.05
C. maxima	ND	ND	ND	ND	1.00
C. pepo (zucchini)	0.03 ± 0.01	0.50 ± 0.01	0.03 ± 0.01	ND	1.00 ± 0.01
C. pepo (squash)	ND	0.73 ± 0.01	0.01 ± 0.0	ND	1.00 ± 0.01
Cucumis sativus	ND	ND	ND	1.29 ± 0.05	1.00 ± 0.05

Phloem exudates from a number of *Cucurbitaceae* species were harvested from cut fruits and stabilised by dilution into an equal volume of 10% metaphosphoric acid containing 10 mM TCEP. Protein precipitates were removed by centrifugation and the supernatant filtered through a 0.22 μm filter and injected onto a Coregel 64H cation exchange column. UV traces were collected at 245 nm and absorbance spectra of individual peaks monitored between 200 and 600 nm using a UVD 340U Diode array detector. Under the conditions described authentic AsA eluted at 12.7 min and had a maximum absorbance at 245 nm. Compounds with similar optical properties were defined as those with a λ_{max} 245 ± 3 nm. Values are presented relative to AsA ± SE (n = 3). ND = peak not detected.