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Iron availability alters ascorbate-induced stress metabolism in *Glehnia littoralis* root cultures

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ABSTRACT

Our previous study indicated that formation of furanocoumarin phytoalexins could be induced in *Glehnia littoralis* root cultures by treatment with 10–40 mM ascorbic acid (AsA). This furanocoumarin production is much less evident when *G. littoralis* roots are treated with AsA under iron-deficient conditions. Instead, two large unknown peaks appeared in the HPLC chromatogram, whose chemical structures were elucidated by spectroscopic methods as being 6, β-dihydroxyphenethyl ferulate (DF) and 6-hydroxyphenethyl ferulate (HF), respectively. Their maximal level of induction was observed at 20 mM AsA, and the production of DF always exceeded that of HF. This is the first report of these compounds in *G. littoralis* and of the modulation of the phytoalexin biosynthetic pathway in *G. littoralis* by iron deficiency.

1. Introduction

Ascorbic acid (AsA) is ubiquitous in the plant cell and is present within the cytosol, the chloroplasts, the mitochondria and the apoplast (Liso et al., 2004; Horemans et al., 2000; Smirnoff, 1996), although the content varies depending on species, tissue, organelle and age. AsA is a powerful reducing agent and is itself oxidized to dehydroascorbic acid (DHA) via the free radical, monodehydroascorbic acid (MDA), or to further irreversible hydrolysis products such as 2,3-diketo-L-gulonic acid, L-threonic acid, L-erythrulose and oxalic acid (Atanassova and Tzatchev, 2008; Barros et al., 2010; Linster and Van Schaftingen, 2007; Washko et al., 1992). Plant cells have a glutathione-dependent system for recycling DHA to AsA that is important in regulating the AsA concentration (Arrigoni and De Tullio, 2002; Pignocchi and Foyer, 2003; Smirnoff, 2000). AsA is well known for its function as an antioxidant and radical scavenger to protect plant cells from the oxidative hazards associated with processes such as photosynthesis and stress responses (Horemans et al., 2000; Smirnoff, 1996). In addition, AsA has important roles not only in cell growth, including the cell cycle, cell elongation and cell differentiation (Córdoba-Pedregosa et al., 2005; de Pinto and De Gara, 2004; Potters et al., 2000), but also as a coenzyme in various biochemical reactions such as AsA-dependent dioxygenases (Arrigoni and De Tullio, 2002).

AsA (vitamin C) is essential in the human diet and in its pure form is also widely consumed; its prophylactic administration for the prevention of influenza pandemics has even been proposed (Banerjee and Kaul, 2010). AsA and its derivatives are also commonly used as antioxidant components in foods and beverages and cosmetics (Balaguer et al., 2008; Elmore, 2005). Against this background, its potential toxicity to human cells has been recognized on account of the generation of reactive oxygen species (Ros) that can occur in the presence of iron/copper, and on this basis it is administered in cancer therapy (Chen et al., 2005, 2007; Hadi et al., 2010). In principle – and in contrast to its antioxidant and radical-scavenging roles – AsA might also exhibit toxicity in plants, although to date this has received little attention. Previously it was found, for the first time, that AsA acted as an elicitor if it was exogenously supplied at 10–40 mM to root cultures of *Glehnia littoralis*, and that furanocoumarin phytoalexin biosynthesis was strongly induced (Ishikawa et al., 2008).

Glehnia littoralis (Apiaceae) is a perennial herb indigenous to the seashores of East Asia. Fresh sprouts are edible and have a role as a commercially important vegetable, and underground parts are used in traditional medicine in Japan. One of the candidate biologically active compounds in this plant is furanocoumarin (Masuda

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et al., 1998). In an investigation of the mechanism of AsA-dependent furanocoumarin induction, attention was paid to the redox reactions in which AsA is involved (Apel and Hirt, 2004; Arrigoni and De Tullio, 2002; Smirnoff, 2000). Furthermore, there was a focus on the role of iron, because it participates in electron transport reactions and iron and AsA together are involved in many biochemical reactions as enzyme cofactors (Arrigoni and De Tullio, 2002). In this context, it was noted that *G. littoralis* plants are generally found in alkaline soils (pH 9.2–9.7) with consequently very restricted iron availability.

In the present study, the effect of iron on AsA-dependent elicitation was determined using *G. littoralis* root cultures. Surprisingly, furanocoumarin induction was almost undetectable when the roots were treated with AsA under iron deficiency, and instead, two new and unfamiliar compounds were detected. In this report, this unique phenomenon and the elucidation of the chemical structures of these two compounds is proved.

2. Results and discussion

It was earlier discovered that production of the phytoalexin, furanocoumarin, is induced by ascorbic acid (AsA) treatment in *G. littoralis* root cultures (Ishikawa et al., 2008). These root cultures were usually maintained in Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium, supplemented with 0.1 mM ferrous iron together with EDTA as a chelating agent. As in earlier work, *G. littoralis* cultured roots were pre-propagated in MS medium for 10 days. The culture media was then replaced with either ferrous iron-deficient modified MS medium or with normal MS medium and AsA was added to a final concentration of 20 mM. As a control, water was added. After 24 h, the respective culture media were extracted with EtOAc and the extracts were analyzed for furanocoumarin production by HPLC. The HPLC profile obtained following AsA treatment without iron was extremely different from that seen following AsA treatment with iron: only traces of the furanocoumarins, psoralen, xanthotoxin and bergapten, were now present and instead two large, unfamiliar peaks, accompanied by some minor peaks, were observed (Fig. 1). The compounds (**1** and **2**) corresponding to the two large peaks were designated RT20 (**1**) and RT35 (**2**), according to their retention times. In media from cultures untreated with AsA, no unusual peaks were found, regardless of iron availability.

HPLC and TLC analysis showed that RT20 (**1**) was more polar than RT35 (**2**), although the compounds exhibited very similar UV absorption spectra. It was thus suspected that the two compounds might be furanocoumarin intermediates that accumulated as a result of an effect of iron starvation upon the later stages of the furanocoumarin biosynthetic pathway. Various candidate compounds, including umbelliferone, marmesin, demethylsuberosin, bergaptol and xanthotoxol (Bourgaud et al., 2006; Ishikawa et al., 2009; Stanjek et al., 1999), were examined both by HPLC and TLC, but none corresponded to either of the unknown compounds. It was therefore decided to purify the two compounds for structure elucidation. About 50 g of roots was treated with 20 mM AsA for 24 h under iron-deficient conditions, and 1.2 l of harvested culture medium was obtained for isolation of the target compounds. The EtOAc extract from the medium was subjected to HPLC separation, resulting in the isolation of compound **1** (RT20) 12 mg and compound **2** (RT35) 3 mg, respectively.

The molecular weight of compound **1** (RT20) was established as 330 from FAB-MS ($[M+Na]^+$, *m/z* 353; $[M+H-H_2O]^+$, *m/z* 313; $[M-H]^-$, *m/z* 329), consistent with a molecular formula of $C_{18}H_{18}O_6$. Similarly, the molecular weight and formula of compound **2** (RT35), deduced from FAB-MS, were respectively 314 and $C_{18}H_{18}O_5$ ($[M+H]^+$, *m/z* 315; $[M-H]^-$, *m/z* 313). The two compounds appeared to be similar, their structural formulae differing only by an oxygen atom. The IR spectra of compounds **1** and **2** showed expected carbonyl absorptions at 1683 and 1684 cm^{-1}, hydroxyl absorptions at 3377 and 3383 cm^{-1}, olefin absorptions at 1630 and 1631 cm^{-1}, and aromatic absorptions at 3010/1593/1513 and 3010/1594/1516 cm^{-1}, respectively. According to these observations and ^1H–^1H COSY and HMBC data (Table 1), the structures of compounds **1** and **2** were therefore confirmed as 6, β-dihydroxyphenethyl ferulate (DF) and 6-hydroxyphenethyl ferulate (HF), respectively (Fig. 2).

The response of DF/HF induction to AsA concentration, from 0 to 80 mM, was investigated; this indicated that over the range 10–80 mM AsA, though not at 5 mM AsA, DF and HF were produced abundantly. Maximal production was observed at 20 mM AsA and at all AsA concentrations, the production of DF always exceeded that of HF (Fig. 3). A time-course analysis (up to 120 h) of these products in the root cultures was undertaken and showed that the medium became yellowish in color after 12 h, deep yellow after 24–48 h, and finally colorless, with white turbidity, after 72 h. Concurrently, DF and HF began to appear in the medium after 9 h of AsA treatment, became maximal at around 24–36 h and then almost disappeared after 72 h (Fig. 4), suggesting that the yellow color was associated with the elicitation of DF and HF, the compounds being detectable only in the medium, not in the root tissues.

It was therefore, demonstrated for the first time to our knowledge, that ferrous iron is closely involved in furanocoumarin induction by AsA and that iron deficiency causes a drastic change in its production in *G. littoralis* root cultures. The production of the new compounds **1** and **2** was both very rapid and very sensitive to iron status. Both furanocoumarins and these compounds established here are phenolics biosynthesized via the phenylpropanoid pathway. Compounds **1** and **2** are non-coumarin, *p*-hydroxycinnamic acid derivatives, i.e. esters of ferulic acid with derivatives of phenethyl alcohols, such as tyrosol. The isolation of both ferulic acid and tyrosol has been reported from the cell walls of elicitor-treated parsley cells upon alkaline hydrolysis (Kauss et al., 1993), suggesting that precursors of cell-wall phenolics could be conjugated with each other, or that the conjugates could be direct precursors of cell-wall phenolics. In fact, it was found in *G. littoralis* roots that a moderate amount of free ferulic acid and a small amount of free *p*-hydroxycinnamic acid were detectable after yeast-extract treatment and that these exhibited a transient increase followed by a decrease, in good agreement with the increases observed in their cell-wall-bound forms (Ishikawa et al., 2007). Elicitation by AsA under iron-deficient conditions did not lead to furanocoumarin biosynthesis, but the production of cell-wall phenolics could nevertheless be increased. This is the first report of the occurrence of compounds **1** and **2** in *G. littoralis*, although they have been detected individually in other Apiaceae, *Angelica purpuraefolia* (Lee et al., 2007), *Peucedanum decursivum* (Kong and Yao, 2000) and *Heracleum lanatum* (Nakata et al., 1982).

Furanocoumarins such as psoralen, bergapten, and xanthotoxin are well-known phytoalexins induced by various elicitors (Masuda et al., 1998; Tietjen et al., 1983). Although compounds **1** and **2** have not been recognized as phytoalexins, it may be legitimate to consider them as a kind of phytoalexin because they are transiently elicited in the medium in response to AsA treatment (Tietjen et al., 1983). Since compounds **1** and **2** are ferulic acid derivatives possessing multiple hydroxyl groups in their structures (Fig. 2), biologic properties such as antioxidant activity can be expected, as with ferulic acid itself (Kanski et al., 2002; Kikuzaki et al., 2002).

The precise mechanism of elicitation by AsA in *G. littoralis* roots is unclear at this stage. It is may hypothesized that when, as a result of physical or biologic stresses, AsA is leached out from damaged plant cells to their apoplasts, apoplastic AsA might induce further damage, depending upon iron availability in the apoplasts. To explore this hypothesis and to clarify the biologic processes