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

Yukino Yakabe[1)], Masahiro Terato[2)], Ataru Higa[1)], Kouji Yamada[2)], Yoshie Kitamura[1, 3)*],

1) Graduate School of Science and Technology, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

2) Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

3) Faculty of Environmental Studies, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

* Corresponding author

Faculty of Environmental Studies, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Tel: +81 95 819 2759; Fax: +81 95 819 2759

E-mail address: k-yoshie@nagasaki-u.ac.jp (Y. Kitamura).

Abstract

Our previous study indicated that furanocoumarin phytoalexins could be induced in *Glehnia littoralis* root cultures by treatment with 10-40 mM of ascorbic acid (AsA). This furanocoumarin production is much less apparent when *G. littoralis* roots are treated with AsA under iron-deficient conditions. Instead, two large unfamiliar peaks appear in the HPLC chromatogram. The chemical structures of these compounds have been elucidated by spectroscopic methods as 6, β-dihydroxyphenethyl ferulate (DF) and 6-hydroxyphenethyl ferulate (HF). Their maximal 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.

Keywords: Glehnia littoralis; Apiaceae; root culture; iron availability; elicitation; ascorbic acid; furanocoumarin; hydroxyphenethyl ferulate.

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 the pure compound 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 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 role - AsA might also exhibit toxicity in plants, although to date this has received little attention. Previously we 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 seashore 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 et al., 1998). In our investigation of the mechanism of AsA-dependent furanocoumarin induction, we have paid attention to the redox reactions in which AsA is involved (Apel and Hirt, 2004; Arrigoni and De Tullio, 2002; Smirnoff, 2000); and we have focused on the role of iron, because iron 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, we have 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, we determined the effect of iron on AsA-dependent elicitation 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. We therefore present this unique phenomenon and the elucidation of the chemical structures of these two compounds.

2. Results and Discussion

We earlier discovered that the production of the phytoalexin, furanocoumarin, is induced by ascorbic acid (AsA) treatment in *Glehnia littoralis* root cultures (Ishikawa et al., 2008). These root cultures were usually maintained in Murashige and Skoog (MS, 1962) basal medium, supplemented with 0.1 mM ferrous iron together with EDTA as a chelating agent. As in our earlier work, *G. littoralis* cultured roots were pre-propagated in MS medium for 10 days. The culture media were 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 corresponding to the two large peaks were designated RT20 and RT35, 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 was more polar than RT35, although the compounds exhibited very similar UV absorption spectra. We suspected that the two compounds might be furanocoumarin intermediates 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 RT20 (12 mg) and RT35 (3 mg), respectively.

The molecular weight of 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 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 RT20 and RT35 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 RT20 and RT35 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 revealed 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.

We therefore demonstrate, 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 DF and HF is both very rapid and very sensitive to iron status. Both furanocoumarins and these compounds revealed here are phenolics biosynthesized via the phenylpropanoid pathway. DF and HF 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, we 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 DF and HF in *G. littoralis*, although DF and HF 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 DF and HF have not been recognised 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 DF and HF are ferulic acid derivatives possessing multiple hydroxyl groups in their structures (Fig. 2), biological 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. We may hypothesize that when, as a result of physical or biological 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 biological processes from the initiation of signal to the induction of stress metabolism, further careful investigations are clearly needed.

3. Conclusion

Ascorbic acid (AsA) is ubiquitous and plays various important roles in the plant cell. Nevertheless, stress metabolism was induced in *G. littoralis* root cultures when AsA was exogenously provided at concentrations $\geqq$10 mM and this induction was altered by iron availability. Whereas furanocoumarins were produced in the presence of Fe, in the absence of Fe hydroxyphenethyl ferulates were produced instead. These findings indicate that Fe plays a key role in AsA dependent-phytoalexin induction. Further biochemical and physiological studies are needed to clarify the mechanism of this phenomenon and the biological activity of hydroxyphenethyl ferulates.

4. Experimental

4.1. Chemicals

Marmesin and demethylsuberosin were gifts kindly provided by Dr. Jocelyn G. Millar (California University, USA).

Sodium ascorbate was obtained from Wako Chemicals. Umbelliferone (Nacalai Tesque, Japan), 4-methylumbelliferone (Wako Chemicals, Japan), bergapten (Extrasynthese), xantotoxin (Extrasynthese), bergaptol (Extrasynthese) and xanthotoxol (Extrasynthese) were obtained commercially. Other chemicals used for culture and analysis were of the highest quality commercially available.

4.2. Plant materials, culture methods and stress treatment

Root cultures of *G. littoralis* Fr. Schmidt ex Miquel (Apiaceae) were established and maintained in liquid Murashige and Skoog (MS) (1962) basal medium, supplemented with 5 mgl^{-1} of IBA, by subculturing at 2-month intervals in the dark at 80 rpm at 25 ˚C, as reported previously（Ishikawa et al, 2007）. For stress treatment, roots (100 mg) were inoculated in 100 ml flasks containing 25 ml of MS medium with 5 mgl^{-1} of IBA and cultured for 10 days prior to stress treatment.

For stress treatment, the culture medium was completely removed from 10-day-old root cultures using glass pipettes and then, after washing with 5 ml of distilled water, the roots were placed in new medium (25 ml /flask) with/without iron. Iron-deficient MS medium was

prepared by the removal of ferrous iron-EDTA from MS basal medium. Aqueous solutions of ascorbic acid (AsA) sodium salt were prepared, adjusted to pH 5.3 with HCl and then filter-sterilized just before addition.

Usually AsA was added to achieve a final concentration of 20 mM, but final concentrations of 0, 5, 10, 20, 40, and 80 mM were used in preliminary studies to investigate the concentration-dependence of elicitation by AsA. Water was used as a control. Incubation was usually carried out for 24 h, or for up to 120 h for a time-course experiment. To analyze metabolites, root cultures were harvested by vacuum filtration, separated into root tissue and culture media and stored at -20˚C until analysis.

4.3. Extraction and HPLC analysis of furanocoumarin and new products

Furanocoumarin extraction from root tissues and culture media was carried out as previously reported, except for the internal standard used (Ishikawa et al., 2005). As an internal standard, 4-methylumbelliferone (50 μL, 5mM MeOH solution) was added to the culture medium before extraction. The sample was applied to an HPLC system (LC-10, Shimadzu, Japan) equipped with a photodiode array detector (SPD-20A, Shimadzu, Japan). HPLC analysis was carried out with a Finepak SIL C 18T column (5 μm, 4.6 x 150 mm) (Jasco, Japan) at 40˚C, using 1 ml min^{-1} flow rate and stepwise gradient elution. Gradient conditions: solvent A, water; solvent B, acetonitrile; at 0 min, B = 20 %; at 20, 30, 40 and 50 min, B = 30, 35, 40 and 20 %, respectively. The eluent was monitored at 220, 254, and 320 nm. Peak identification was based on retention times and absorption spectra, by comparison with authentic standards.

4.4. Isolation and determination of new compounds

New compounds produced in response to AsA treatment under iron-deficient conditions were extracted and detected as described in section *4.3*. For structure elucidation, culture medium (1.2 l, from a culture of 52 g of roots) was taken for purification. The medium was extracted twice with an equivalent volume of EtOAc, the combined EtOAc extracts were evaporated under vacuum, and the residue (218 mg) was finally dissolved in 1 ml of MeOH for separation by HPLC as mentioned above. From replicate separations, the fractions corresponding to peaks with retention times (RT) of 20 and 35 min, respectively, were collected and pooled. After the removal of acetonitrile by vacuum evaporation, the aqueous residue was extracted with EtOAc three times and the combined EtOAc solution was evaporated to dryness, leading to the isolation of metabolite RT20 (12 mg) and RT35 (3 mg).

IR spectra of RT20 and RT35 were recorded in KBr discs using a JASCO FT/IR-410K spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HMQC, and HMBC, were acquired in CD_3OD at 400 MHz (^{1}H) and 125 MHz (^{13}C) with a JEOL JNM-AL400 spectrometer, using TMS as an internal standard. FAB-MS spectra were taken on a JEOL JMX-DX 303 mass spectrometer.

RT 20 : *6, β-dihydroxyphenethyl ferulate (DF)*

Amorphous powder; FAB-MS (positive and negative modes) *m/z*: 353 $[M+Na]^+$, 313 $[M+H-H_2O]^+$, 329 $[M-H]^-$ (calcd for $C_{18}H_{18}O_6$). IR ν_{max} (film) cm^{-1}: 3377 (OH), 1683 (α, β-unsaturated carbonyl), 1630 (olefin), 3010, 1593, 1513 (aromatic ring). ^{1}H-NMR, ^{13}C-NMR

and ^{1}H-^{1}H COSY: Table 1.

RT 35: 6 -hydroxyphenethyl ferulate (HF)

Amorphous powder; FAB-MS (positive and negative modes) *m/z*: 315 $[M+H]^{+}$, 313 $[M-H]^{-}$ (calcd for $C_{18}H_{18}O_5$). IR ν_{max} (film) cm^{-1}: 3383 (OH), 1684 (α, β-unsaturated carbonyl), 1631 (olefin), 3010, 1594, 1516 (aromatic ring). ^{1}H-NMR, ^{13}C-NMR and ^{1}H-^{1}H COSY: Table 1.

Acknowledgements

We are very grateful to Dr. Jocelyn G. Millar (California University, USA) for kind gifts of authentic demethylsuberosin and marmesin, and Dr Nicholas Walton (UK) for editing the English text. We also thank Dr. Aya Ishikawa and Ms. Natsuko Yamada for their technical assistances. This work was supported in part by a Grant-in-Aid (C, 18580255) from the Japan Society for the Promotion of Science.

References

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.

Arrigoni, O., De Tullio, M.C., 2002. Ascorbic acid: more than just an antioxidant. Biochim. Biophys. Acta 1569, 1-9.

Atanassova, B.D., Tzatchev, K.N., 2008. Ascorbic acid - important for iron metabolism. Folia Medica 50, 11-16.

Balaguer, A., Chisvert, A., Salvador, A., 2008. Environmentally friendly LC for the simultaneous determination of ascorbic acid and its derivatives in skin-whitening cosmetics. J. Sep. Sci. 31, 229-236.

Banerjee, D., Kaul, D., 2010. Combined inhalational and oral supplementation of ascorbic acid may prevent influenza pandemic emergency: A hypothesis. Nutrition 26, 128-132.

Barros, A.I.R.N.A., Silva, A.P., Gonçalves, B., Nunes, F.M., 2010. A fast, simple, and reliable hydrophilic interation liquid chromatography method for the determination of ascorbic acid and isoascorbic acid. Anal. Bioanal. Chem. 396, 1863-1875.

Bourgaud, F., Hehn, A., Larbat, R., Doerper, S., Gontier, E., Kellner, S., Matern, U., 2006. Biosynthesis of coumarins in plants: a major pathway still to be unraveled for cytochrome P450 enzymes. Phytochem. Rev. 5, 293-308.

Chen, Q., Espey, M.G., Krishna, M.C., Mitchell, J.B., Corpe, C.P., Shacter, E., Choyke, P.L., Buettner, G.R., Shacter, E., Levine, M., 2005. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. Proc. Natl. Acad. Sci. U. S. A. 102:13604-13609.

Chen, Q., Espey, M.G., Sun, A.Y., Lee, J.-H., Krishna, M.C., Shacter, E., Choyke, P.L., Pooput, C., Kirk, K.L., Buettner, G.R., Levine, M., 2007. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid *in vivo*. Proc. Natl. Acad. Sci. U. S. A. 104, 8749-8754.

Córdoba-Pedregosa, M.C., Villalba, J.M., Córdoba, F., González-Reyes, J.A., 2005. Changes in intracellular and apoplastic peroxidase activity, ascorbate redox status, and root elongation induced by enhanced ascorbate content in *Allium cepa* L. J. Exp. Bot. 56, 685-694.

de Pinto, M.C., De Gara, L., 2004. Changes in the ascorbate metabolism of apoplastic and symplastic spaces are associated with cell differentiation. J. Exp. Bot. 55, 2559-2569.

Elmore, A.R., 2005. Final report of the safety assessment of L-ascorbic acid, calcium ascorbate, magnesium ascorbate, magnesium ascorbyl phosphate, sodium ascorbate, and sodium ascorbyl phosphate as used in cosmetics. Int. J. Toxicol. 24 suppl 2, 51-111.

Hadi, S.M., Ulah, M.F., Shamim, U., Bhatt, S.H., Azmi, A.S., 2010. Catalytic therapy of cancer by ascorbic acid involves redox cycling of exogenous/endogenous copper ions and generation of reactive oxygen species. Chemotherapy 56, 280-284.

Horemans, N., Foyer, C.H., Potters, G., Asard, H., 2000. Ascorbate function and associated transport systems in plants. Plant Physiol. Biochem. 38, 531-540.

Ishikawa, A., Kitamura, Y., Ozeki, Y., Itoh, Y., Yamada, A., Watanabe, M., 2005. Post-stress

metabolism involves umbelliferone production in anthocyanin-producing and non-producing cells of *Glehnia littoralis* suspension cultures. J. Plant Physiol. 162, 703-710.

Ishikawa, A., Kitamura, Y., Ozeki, Y., Kobayashi, N., 2007. Different responses of shoot and root cultures of *Glehnia littoralis* to yeast extract. J. Nat. Med. 61, 30-37.

Ishikawa, A., Kobayashi, N., Kitamura, Y., 2008. Ascorbic acid induces furanocoumarin production in organ cultures of *Glehnia littoralis*. Planta Med. 74, 1517-1519.

Ishikawa, A., Kuma, T., Sasaki, H., Sasaki, N., Ozeki, Y., Kobayashi, N., Kitamura, Y., 2009. Constitutive expression of bergaptol *O*-methyltransferase in *Glehnia littoralis* cell cultures. Plant Cell Rep. 28, 257-265.

Kanski, J., Aksenova, M., Stoyanova, A., Butterfield, D.A., 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*: structure-activity studies. J. Nutr. Biochem. 13, 273-281.

Kauss, H., Franke, R., Krause, K., Conrath, U., Jeblick, W., Grimmig, B., Matern, U., 1993. Conditioning of parsley (*Petroselinum crispum* L.) suspension cells increases elicitor-induced incorporation of cell wall phenolics. Plant Physiol. 102, 459–466.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., Taniguchi, H., 2002. Antioxidant properties of ferulic acid and its related compounds. J. Agric. Food Chem. 50, 2161–2168.

Kong, L.Y., Yao, N.H., 2000. Coumarin-glycoside and ferulate from *Peucedanum decursivum*. Chinese Chem. Lett. 11, 315-318.

Lee, H.-K., Oh, A.-R., Kwon, O.-K., Ahn, K.-S., Lec, J., Kim, J.-C., Min, B.-S., Joung, H., 2007. Isolation of coumarins and ferulate from the roots of *Angelica purpuraefolia* and the antitumor activity of khellactone. Phytother. Res. 21, 406-409.

Linster C.L., Van Schaftingen, E., 2007. Vitamin C: Biosynthesis, recycling and degradation in mammals. FEBS Journal 274, 1-22.

Liso, R., De Tullio, M.C, Ciraci, S., Balestrini, R., La Rocca, N., Bruno L, Chiappetta, A., Bitonti, M.B., Bonfante, P., Arrigon, O., 2004. Localization of ascorbic acid, ascorbic acid oxidase, and glutathione in roots of *Cucurbita maxima* L. J. Exp. Bot. 55, 2589-2597.

Masuda, T., Takasugi, M., Anetai, A., 1998. Psoralen and other linear furanocoumarins as phytoalexins in *Glehnia littoralis*. Phytochemistry 47, 13-16.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Nakata, H., Sashida, Y., Shimomura, H., 1982. A new phenolic compound from *Heracleum lanatum* Michx. var. nippinicum Hara. II. Chem. Pharm. Bull. 30, 4554-6.

Pignocchi, C., Foyer, C.H., 2003. Apoplastic ascorbate metabolism and its role in the regulation of cell signaling. Curr. Opin. Plant Biol. 6, 379-389.

Potters, G., Horemans, N., Caubergs, R.J., Asard, H., 2000. Ascorbate and dehydroascorbate influence cell cycle progression in a tobacco cell suspension. Plant Physiol. 124, 17-20.

Smirnoff, N., 1996. The function and metabolism of ascorbic acid. Ann. Bot. 78, 661-669.

Smirnoff, N., 2000. Ascorbic acid: metabolism and functions of a multi-facetted molecule. Curr. Opin. 3, 229-235.

Stanjek, V., Piel, J., Boland W., 1999. Biosynthesis of furanocoumarins: mevalonate-independent prenylation of umbelliferone in *Apium graveolens* (Apiaceae). Phytochemistry 50, 1141-1145.

Tietjen, K.G., Hunkler, D., Matern, U., 1983. Differential responses of cultured parsley cells to

elicitors from two non-pathogenic strains of fungi. Eur. J. Biochem. 131, 401-407.

Washko, P.W., Welch, R.D., Dhariwal, K.R., Wang, Y., Levine, M., 1992. Ascorbic acid and dehydroascorbic acid analyses in biological samples. Anal. Biochem. 204, 1-14.

Figure captions

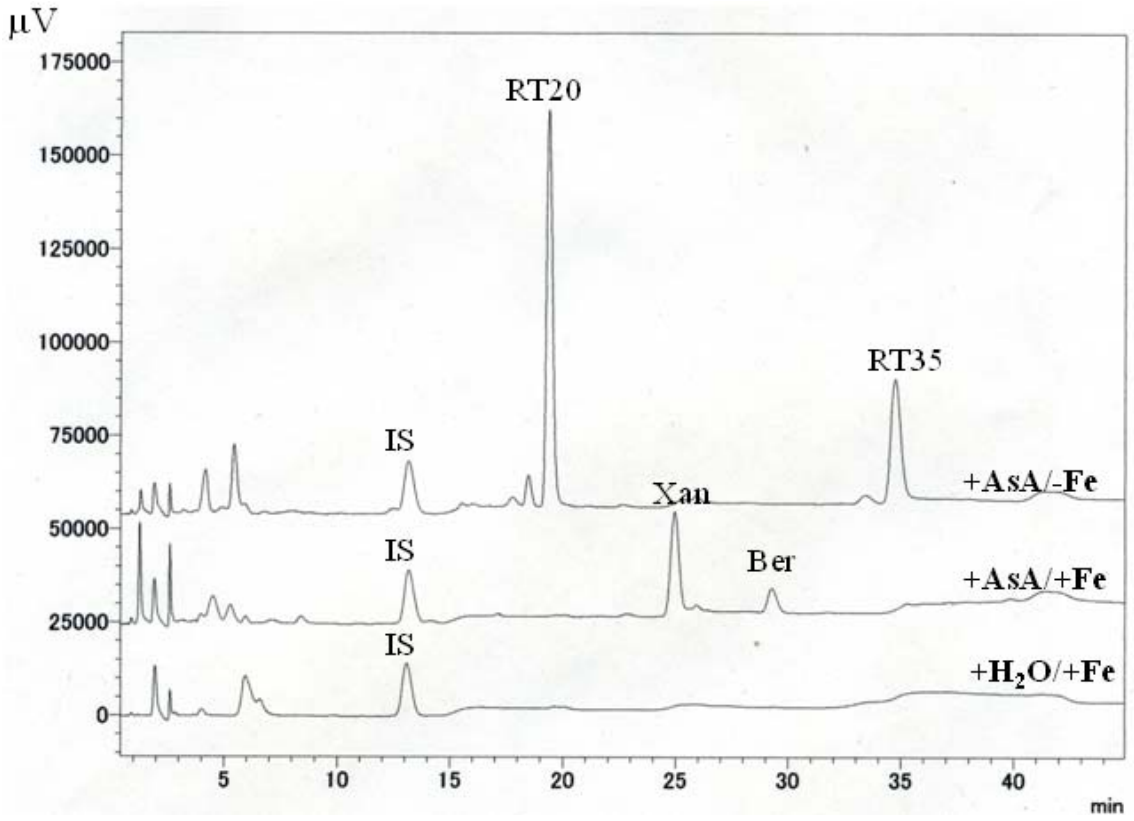

Figure 1. HPLC chromatograms

G. littoralis cultured roots were pre-propagated in MS medium for 10 days. The culture media were 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 incubation, culture media were extracted with ethyl acetate and the extracts, dissolved finally in MeOH, were analyzed by HPLC. As an internal standard (IS), 4-methylumbelliferone was added before extraction.

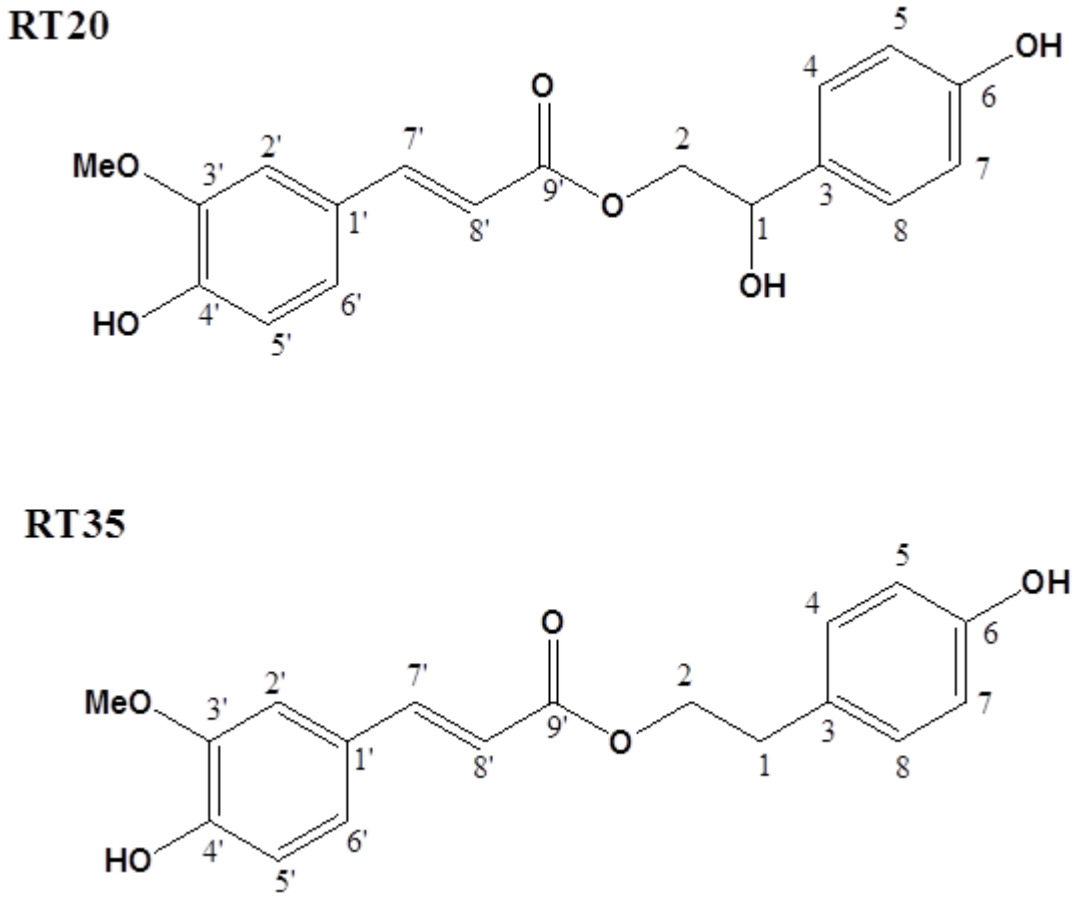

Figure 2. Structures of new compounds found in *G. littoralis* root cultures.

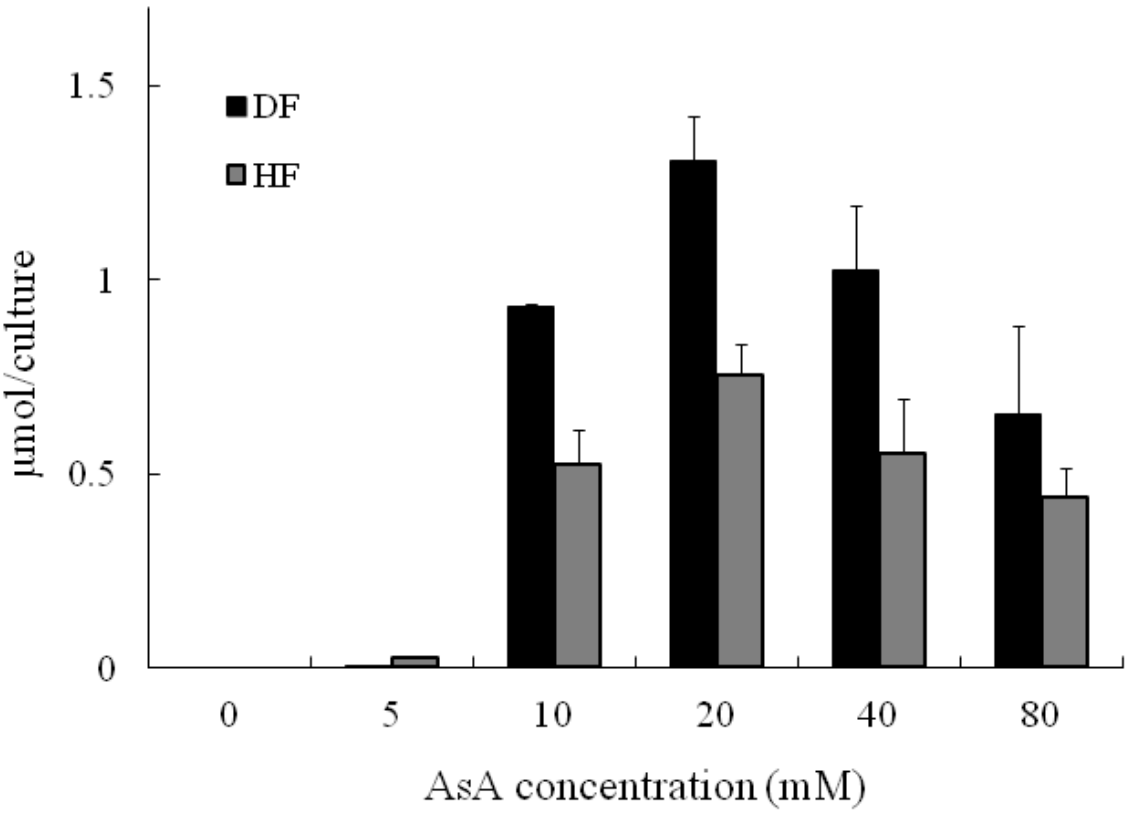

Figure 3. AsA concentration-dependent production of DF and HF

Legend as for Figure 2, except for medium used and AsA concentration added. Ferrous-iron-deficient modified MS medium was used.

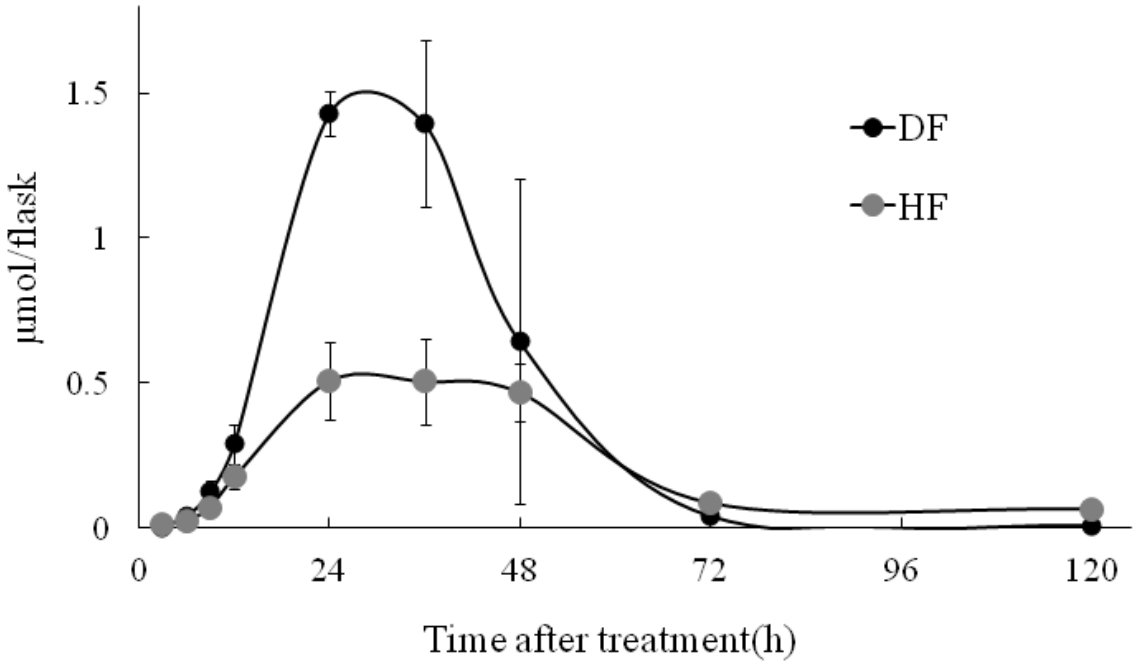

Figure 4. Time-course analysis of DF and HF

Legend as for Figure 2, except for medium used and incubation time. Ferrous iron-deficient modified MS medium supplemented with 20 mM AsA was used.

Table 1 ^{1}H- and ^{13}C-NMR, and ^{1}H -^{1}H COSY data of compounds RT20 and RT35.

	RT20			RT35		
Position	δ_H	δ_c	^{1}H-^{1}H COSY	δ_H	δ_c	^{1}H-^{1}H COSY
1	3.34(1H, brs)	72.7		2.88(2H, d, *J*=7.1 Hz)	35.4	H1
2	4.18(1H, dd, *J*=3.7, 11.0 Hz)	70.1	H2b	4.03(2H, t, *J*=7.1 Hz)	66.5	H2
	4.24(1H, dd, *J*=4.4, 11.0 Hz)		H2a			
3	-	133.2		-	130.1	
4	7.25(1H, d, *J*=8.8 Hz)	128.7	H5	7.07(1H, d, *J*=8.3 Hz)	130.9	H5
5	6.77(1H, d, *J*=8.8 Hz)	116.2	H4	6.72(1H, d, *J*=8.3 Hz)	116.3	H4
6	-	158.3		-	157.1	
7	6.77(1H, d, *J*=8.8 Hz)	116.2	H8	6.72(1H, d, *J*=8.3 Hz)	116.3	H8
8	7.25(1H, d, *J*=8.8 Hz)	128.7	H7'	7.07(1H, d, *J*=8.3 Hz)	130.9	H7'
1'	-	127.7		-	127.7	
2'	7.17(1H, d, *J*=2.0 Hz)	111.8	H6'	7.03(1H, dd, *J*=2.0, 7.0 Hz)	115.5	H6'
3'	-	149.4		-	149.4	
4'	-	150.6		-	150.6	
5'	6.81(1H, d, *J*=8.8 Hz)	116.4	H6'	6.89(1H, d, *J*=7.0 Hz)	124.1	H6'
6'	7.07(1H, dd, *J*=2.0, 8.9 Hz)	124.1	H2', H5'	7.03(1H, dd, *J*=2.0, 7.0 Hz)	115.5	H2', H5'
7'	7.63(1H, d, *J*=16.1 Hz)	147	H8'	7.57(1H, d, *J*=15.8 Hz)	147.8	H8'
8'	6.36(1H, d, *J*=16.1 Hz)	115.4	H7'	6.33(1H, d, *J*=15.8 Hz)	111.7	H7'
9'	-	169.1		-	169.2	
OMe	3.88(1H, s)	56.4		3.88(1H, s)	56.5	