Nitric oxide mediates elicitor-induced saponin synthesis in cell cultures of *Panax ginseng*

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Abstract. The elicitor oligogalacturonic acid (OGA) stimulated nitric oxide (NO) accumulation in the growth medium of ginseng suspension cultures and induced increased nitric oxide synthase (NOS) activity in ginseng cells. OGA also stimulated accumulation of saponin, transcription of genes encoding squalene synthase (sqs) and squalene epoxidase (sqe), two early enzymes of saponin synthesis, and the accumulation of β-amyrin synthase (β-AS). Saponin accumulation, sqs and sqe gene expression, and increases in β-AS content were also induced by exposure to NO via the NO donor sodium nitroprusside (SNP). Inhibitors of mammalian nitric oxide synthase reduced both OGA-induced NO accumulation and NOS activity, suggesting that OGA-induced NO production occurs via a NOS-like enzyme. OGA-induced accumulation of β-AS and saponin, and transcription of sqs and sqe, were suppressed by treatments that removed NO or inhibited its production, indicating a role for NO in mediating OGA effects on these defence responses. NO accumulation and increased NOS activity were inhibited by calcium channel inhibitors and a protein kinase inhibitor, but not by a protein phosphatase inhibitor, indicating the requirement for calcium and protein phosphorylation during OGA-induced NO production. Saponin production and transcription, and accumulation of saponin biosynthetic genes and enzymes were also suppressed by these treatments, as well as by the protein phosphatase inhibitor okadaic acid.

Keywords: elicitor, ginseng, NO, OGA, saponin.

Introduction

The chemically simple, gaseous free radical nitric oxide is now well established as a signalling molecule in animals and plants (Furchgott 1995; Neill et al. 2002, 2003). In animals, it regulates a variety of biological processes including blood pressure homeostasis, platelet aggregation and immune functions (Furchgott 1995). In plants, substantial data indicate a role for NO in a growing number of processes including plant defence, programmed cell death and stomatal movements (Durner and Klessig 1999; Neill et al. 2003). Some studies have indicated that NO can be synthesised from arginine by a nitric-oxide-synthase-like enzyme (Cueto et al. 1996; Durner et al. 1998; Durner and Klessig 1999; Modolo et al. 2002; Neill et al. 2003), whereas other work demonstrates that nitrite can also act as a cellular source of NO, in a reaction catalysed by the enzyme nitrate reductase (Yamasaki and Sakihama 2000; Desikan et al. 2002; Neill et al. 2003). Elicitors derived from fungal or plant cell walls induce defence responses in plants by means of complex signalling mechanisms that include ion fluxes across the plasma membrane, reversible protein phosphorylation and the generation of reactive oxygen and nitrogen species (Lamb and Dixon 1997; Durner and Klessig 1999). NO can induce phytoalexin synthesis in potato (Noritake et al. 1996) and, in soybean, induction of phytoalexin biosynthesis by a fungal elicitor from *Diaporthe phaseolorum* f.sp. *meridionalis* was dependent on NOS activity (Modolo et al. 2002).

Triterpenoid saponins are secondary metabolites synthesised by many different plant species during normal growth and development. Examples of these species include those exploited as sources of drugs, such as liquorice and ginseng, as well as crop plants such as legumes and oats (Osborn et al. 1997; Suzuki et al. 2002). Interest in these molecules

Abbreviations used: β-AS, β-amyrin synthase; CPTIO, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DHEA, diethyldithiocarbamic acid; DMU, dimethylthiourea; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; fw, fresh weight; Hb, haemoglobin; JA, jasmonic acid; L-NAA, Nω-nitro-L-arginine; NOS, nitric oxide synthase; OA, okadaic acid; OGA, oligogalacturonic acid; PBITU, S,S′-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea; RR, ruthenium red; SNP, sodium nitroprusside; sqe, squalene epoxidase; sqs, squalene synthase.
relates to their medicinal properties, anti-microbial activity and potential role as determinants of plant disease resistance (Bouarab et al. 2002). The anti-fungal activity of triterpenoid saponins has been documented (Favel et al. 1994). In general, little is known of the enzymes and biochemical pathways involved in saponin biosynthesis. However, triterpenoid saponins are synthesised by the isoprenoid pathway by cyclisation of 2,3-oxidosqualene to give primarily oleanane (β-amyrin) or dammarane triterpenoid skeletons. The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent mono-oxygenases, glycosyltransferases and other enzymes (Haralampidis et al. 2002). Squalene synthase and squalene epoxidase are responsible for the synthesis of 2,3-oxidosqualene (Osborn et al. 1997). β-amyrin synthase, catalysing the cyclisation of oxidosqualene into β-amyrin, generates five rings and eight asymmetric centres in a single transformation (Kushiro et al. 1998).

Ginseng (Panax ginseng CA Meyer) a member of the Araliaceae family, is one of the most valuable oriental herbs. It has been used as a healing drug and health tonic in countries such as China, Japan and Korea since ancient times. Ginseng has many beneficial bioactive effects on human health, such as anti-tumour, anti-stress and anti-ageing effects, as well as improved immune function. It has been increasingly used as a health tonic formulated into a variety of commercial health products including ginseng capsules, soups, drinks and cosmetics, used widely in the Orient as well as many other countries around the world (Wu and Zhong 1999). The triterpenoid saponins are widely believed to be the major bioactive ingredients of ginseng. It has been reported that saponin accumulation in cultured ginseng cells can be stimulated by various elicitors such as chitosan (Hu et al. 2002), an oligogalacturonic acid elicitor (Hu et al. 2003a), yeast cell wall fractions and saponin precursors (Wu and Zhong 1999; Lu et al. 2001).

In the present study, the potential role of NO in mediating OGA-induced saponin accumulation and transcription of genes encoding squalene synthase and squalene epoxidase, two enzymes of squalene synthesis, in ginseng cells was determined. Treatment with OGA induced the release of NO from challenged cells. To determine the role of NO in mediating saponin synthesis in elicited cells, NOS activities of cells exposed to different treatment were assayed and the effects of NOS inhibitors and NO scavengers on saponin synthesis determined. The data reported herein indicate that NO acts as an endogenous signal mediating the responses of ginseng cells to OGA.

Materials and methods

Chemicals

Sodium nitroprusside (SNP), Nω-nitro-l-arginine (l-NNA) and S,S′-1,3-phenylene-bis (1,2-ethanediyl)-bis-isothiourea (PBITU) were obtained from Sigma (St Louis, MO, USA). l-[3H]arginine was obtained from Amersham Pharmacia Biotech Ltd (Quarry Bay, Hong Kong). The Dig-DNA labelling and detection kit was obtained from Roche Diagnostics Ltd (Mannheim, Germany). All other chemicals were obtained from Shanghai Chemical Co. (Shanghai, China).

Cell cultures

The ginseng cell line used in this work was originally obtained from stems of Panax ginseng CA Meyer. Suspension cultures were maintained on 67-V medium (Velicky and Martín 1970), supplemented with 1.5 mg L–1 dichlorophenoxyacetic acid, 1 mg L–1 indole-3-acetic acid, 0.1 mg L–1 naphthalene acetic acid and 0.25 mg L–1 kinetin, pH 5.8. Stock cultures were grown in 150-mL flasks with 50 mL in each, on a rotary shaker shaking at 150 rpm at 24°C in the dark, and subcultured every 30 d. To study elicitor-induced responses, 5 mL cells collected from the stock culture were inoculated into 50 mL fresh medium [0.1 g fresh weight (fw) mL–1] and incubated under the same conditions as for the stock cultures for 3 d prior to elicitor addition. Inhibitors and scavengers were added to the cultures 30 min before the addition of elicitor. Preliminary tests indicated negligible effects on cell growth or the assays described below.

Oligogalacturonic acid

Oligogalacturonic acid was purified from acid hydrolysate of citrus pectin (Sigma), as described by Nothnagel et al. (1983) and Legendre et al. (1993). The OGA preparation was subsequently dialysed against distilled water using dialysis tubing with a cut-off of approximately 500 Da. The prepared OGA was adjusted to a final concentration of 1 mg mL–1 of galacturonic acid equivalents (gal. equiv.) with distilled water, as determined by the method of Blumenkrantz and Asboe-Hansen (1973).

NO assay

Nitric oxide production was assayed by monitoring the conversion of oxyhaemoglobin (HbO2) to methaemoglobin (metHb) (Delledonne et al. 1998). In brief, 5 mL ginseng suspension culture were collected and incubated with 50 U catalase and 50 U superoxide dismutase (to remove any reactive oxygen species) before adding 5 µM oxyhaemoglobin. After 2 min, the change in absorbance of the medium at 421 nm and 401 nm was determined and the concentration of NO calculated by using an extinction coefficient of 77 mM–1 cm–1 [A401 (metHb) – A421 (HbO2)].

NOS assay

The assays for total NOS activity were performed for 20 min at 37°C in a reaction medium containing 40 mM HEPES buffer, pH 7.2, 0.2 mM 3-[[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate (CHAPS), 10 µg mL–1 calmodulin, 1.25 mM CaCl2, 1 mM NADPH, 10 µM flavin dinucleotide (FAD), 10 µM flavin mononucleotide (FMN), 10 µM tetrahydrobiopterin, variable amounts of protein samples, and a variable concentration of l-arginine supplemented with l-[3H]arginine (37 MBq mL–1) in a total volume of 220 µL (Delledonne et al. 1998). The reaction was terminated by addition of 1.5 mL of a cation exchange resin (Dowex 50W, 8% cross-linkage, 200–400 mesh, Na+ form; Fluka AG, Buchs, Switzerland) at 4°C. Water (5 mL) of was added and the resin allowed to settle for 10 min An aliquot (3.5 mL) of the supernatant was then removed for liquid scintillation counting. The level of l-[3H]citrulline was computed after subtracting the blank value, which represented the non-specific radioactivity in the absence of enzyme. In addition, the values obtained in the experiments performed in the absence of NADPH, which indicate the amount of l-[3H]citrulline formation independent of a specific NOS activity, were also subtracted from the total level of l-citrulline. NOS activity was expressed as picomol of l-[3H]citrulline min–1 mg–1 protein.
**Saponin assay**

The cell suspension was treated with OGA for 21 d with or without different inhibitors and then collected by centrifugation (1200 g, 5 min). The cell pellet was washed with a large volume of distilled water and centrifuged again before drying at 50°C for several days to a constant weight. Saponin (total) in the dried ginseng cells was extracted with water-saturated n-butanol, isolated by thin-layer chromatography and quantified by measuring the absorbance after reacting with vanillin in acetic acid and perchloric acid, based on the methods described by Zhong and Wang (1998). The inhibitors used were: L-NAA and PBITU, inhibitors of NOS, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), scavengers of NO, LaCl3, and ruthenium red (RR), inhibitors of calcium channel, K252a, an inhibitor of protein kinase, okadaic acid (OA), an inhibitor of phosphatase, diethyldithiocarbamic acid (DIECA), an inhibitor of jasmonic synthesis pathway, and dimethylthiourea (DMTU), a scavenger of H2O2. The concentrations of inhibitors (or scavengers) used were: L-NAA (100 µM), PBITU (50 µM), CPTIO (50 µM), LaCl3 (10 µM), RR (10 µM), K252a (10 µM), OA (10 µM), DIECA (500 µM) and DMTU (10 µM). Inhibitors or scavengers were added into ginseng cell culture 30 min prior to OGA treatment.

**RT–PCR and northern blotting**

Total RNA was isolated from ginseng cells as described in Maniatis et al. (1982) and first-strand CDNA synthesised using SuperScript (Life Technologies, Rockville, MD, USA) in a reaction at 42°C with 1 µg RNA as the template, according to the manufacturer’s instructions. The primers for sqs amplification were 5’-AGGTTGAATTG-GCTCGGCC-3’ and 5’-CGGGCTTACGAAGGGTGCTG-3’, and those for sqc amplification were 5’-GCTCTGTGTTACTCTA-3’ and 5’-TTATGTCCTCCACCTCC-3’. PCR amplification was performed in a Hybrid PCR Sprint thermocycler (Midwest Scientific, MO, USA) with an initial 5-min step at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 56°C, and 60 s at 72°C, with a final extension time of 10 min at 72°C. The amplified products were ethanol-precipitated and dissolved in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). PCR fragments were labelled using digoxigenin–dUTP (Dig-system, Roche Diagnostics GmbH, Mannheim, Germany). RNA (30 µg) was electrophoresed on a 1.2% (v/v) agarose gel with 0.67% formaldehyde, and RNA transferred in 10x SSC (1.5 mM NaCl, 150 mM trisodium citrate) onto Hybond-N membrane (Amersham Pharmacia Biotech, Amersham, Buckinghamshire, UK). The membrane was then incubated at 62°C for 2 h in pre-hybridisation solution [10x Denhardt’s solution (for 50x Denhardt’s, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin fraction V, 1% (w/v) ficoll 400), 3x SSC, 10% (w/v) dextran sulphate, 7% (w/v) SDS, 167 µg mL−1 of single-stranded salmon sperm DNA]. The Dig-labelled probe was added to the pre-hybridisation solution and incubated for 18–36 h at 60°C. The hybridised membrane was then processed using the Dig DNA labelling and detection kit. Uniformity of mRNA loading was verified by hybridisation with a conserved *Arabidopsis thaliana* 18S RNA probe (ABRC, Columbus, OH, USA).

**SDS–PAGE and western blotting**

Ginseng cells (2 g fw) were ground in a mortar and pestle before adding 2 mL of extraction buffer [125 mM Tris–Cl, 7.75% (w/v) SDS, 10% (v/v) mercaptoethanol, pH 7.0] and grinding further. The mixture was transferred to a centrifuge tube, raised to room temperature, and centrifuged in a swing-out rotor at 3500 g for 10 min. The supernatant was mixed with 6x (v/v) sample buffer [0.1 M Tris–Cl, 12% (w/v) SDS, 9% (v/v) glycerol, 60% (v/v) mercaptoethanol, pH 6.8] and 60 µL separated by SDS–PAGE (Laemmli 1970). After electrophoresis, the separated proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech, Sydney, NSW, Australia) using a Multiphor II semi-dry blotting apparatus (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Hybridisation was detected using the BM Chemiluminescence Blotting Substrate POD system (Roche, Mannheim, Germany). The anti-AS antibodies were used at a dilution of 1:1000. Antibodies were raised against the peptide LEKKNFQRTI, corresponding to amino acid residues 71–80 in the deduced amino acid sequence of the β-amyrin synthase (Genbank No. AB009030; Kusihoro et al. 1998). The peptides were conjugated to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysuccinimide ester and injected into rabbits. Serum was collected and used for antibody probing.

**Results**

**OGA induces NO production and NOS activity in ginseng cells**

To determine the effect of OGA on the production of NO and the activity of NOS in cultured ginseng cells, 3-d-old cultures were treated with OGA at different concentrations, and the NO content of the medium was determined after 12 h. The effects of OGA on NO production were dose-dependent. NO production was detectable at 10 µg gal. equiv. mL−1 and saturated at ca 50 µg gal. equiv. mL−1 (Fig. 1A). OGA-induced NO production commenced after 5 h and concentrations in the cell culture peaked after 12 h treatment, remaining at this level for another 12 h (Fig. 1B). OGA-induced increases in the culture medium NO content were inhibited by the NO scavenger CPTIO and the NOS inhibitors 1-NAA and PBITU. In addition, OGA-induced NO production was also inhibited by treatment with LaCl3 or RR, which are blockers of plasma membrane and internal membrane calcium channels respectively. The general protein kinase inhibitor K252a also inhibited NO production, but OA, an inhibitor of protein phosphatase 1/2A, had no effect (Fig. 1C). Neither DMTU, an H2O2 scavenger, nor DIECA, an inhibitor of jasmonic acid (JA) biosynthesis, compounds that both inhibit OGA-induced accumulation of JA and saponin in ginseng cultures (Hu et al. 2003a) had a significant effect on NO production (Fig. 1C). OGA treatment rapidly increased NOs activity in ginseng cells, such increased activity continuing until 8 h after challenge with OGA (Fig. 2A). OGA-increased NOs activity was suppressed by 1-NAA and PBITU, LaCl3, RR and K252a pre-treatment, but not by OA, similar to the effects of these compounds on NO production (Fig. 2B).

**OGA and NO induce saponin accumulation in ginseng cells**

Oligogalacturonic acid induces saponin accumulation in a dose-dependent manner, such that at 60 µg gal. equiv. mL−1 OGA, the saponin content is 2- to 3-fold that of the control cells (Hu et al. 2003a). The NO scavenger CPTIO and the NOS inhibitors 1-NAA and PBITU all reduced OGA-induced saponin production (Fig. 3). NO itself, provided by the NO donor SNP, induced increase of saponin...
OGA-induced saponin accumulation was inhibited to varying degrees by LaCl₃, RR, K252a and OA (Fig. 3).

** OGAs induces the transcription of sqs and sqe in ginseng cells**

Our previous study showed that sqs and sqe gene expression is induced in ginseng cells by 50 µg gal. equiv. mL⁻¹ OGA treatment. Increased transcription of both these genes could be observed within 6 h and was strongly induced after 24 h (Hu et al. 2003a). NO treatment (via SNP) also induced transcription of these two genes (Fig. 4A). Removal of NO by CPTIO or inhibition of NO production by L-NAA and

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**Fig. 1.** OGA induces NO accumulation in the culture medium of cultured ginseng cells. (A) Dose-response (12 h). Closed circles, + OGA; open circles, control. (B) Time course (50 µg gal. equiv. mL⁻¹). (C) Modulation of OGA-induced NO accumulation by various treatments. Compounds were added 30 min prior to addition of OGA (50 µg gal. equiv. mL⁻¹) and medium NO content assayed after 12 h. l-NAA (100 µM), PBITU (50 µM), CPTIO (50 µM), LaCl₃ (10 µM), RR (10 µM), K252a (10 µM), OA (10 µM), DIECA (500 µM) and DMTU (10 mM) were used. Bars = standard error (s.e.; n = 5).

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**Fig. 2.** OGA induces NOS activity in ginseng cells. (A) Kinetics of increased NOS activity following treatment with 50 µg gal. equiv. mL⁻¹ OGA. Closed circles, + OGA; open circles, control. (B) Inhibition of OGA-induced NOS activity by various treatments. Inhibitors were added 30 min prior to addition of OGA (50 µg gal. equiv. mL⁻¹) and medium NO content assayed after 12 h. l-NAA (100 µM), PBITU (50 µM), CPTIO (50 µM), LaCl₃ (10 µM), RR (10 µM), K252a (10 µM) and OA (10 µM) were used. Bars = s.e. (n = 5).
PBITU inhibited induction of \( sqs \) and \( sqe \) gene expression, as did treatment with \( \text{LaCl}_3 \), RR and K252a. Interestingly, OA pre-treatment also inhibited the transcription of these genes (Fig. 4B).

**OGA and NO induce the accumulation of \( \beta \)-AS**

At 50 \( \mu \)g gal. equiv. mL\(^{-1} \), OGA treatment induced a gradual accumulation of \( \beta \)-AS in ginseng cells (Fig. 5A). A similar response to NO was also observed (Fig. 5B). L-\( \text{NAA}, \) PBITU, CPTIO, \( \text{LaCl}_3 \), RR, K252a and OA pre-treatment all resulted in an inhibition of OGA-induced accumulation of \( \beta \)-AS (Fig. 5C).

**Discussion**

Oligogalacturonic acid derived from plant cell walls induces multiple plant defence responses. These include a rapid oxidative burst (Apostol *et al.* 1989; Lee *et al.* 1999), releasing reactive oxygen species via a pathway that involves receptor binding (Horn *et al.* 1989), G-protein activation (Legendre *et al.* 1992), influx of Ca\(^{2+} \) ions (Chandra and Low 1997), stimulation of phospholipase C (Legendre *et al.* 1993), and activation of a number of kinases (Chandra and Low 1995). In the present study we have demonstrated that OGA induces NO accumulation in ginseng suspension cultures. Increases in the NO content of the culture medium were dependent on the OGA dose; the NO content of the medium increased over the range 10–50 gal equiv mL\(^{-1} \) but appeared to saturate at higher OGA concentrations. A similar saturation effect was reported for elicitor-induced \( \text{H}_2\text{O}_2 \) accumulation in ginseng (Hu *et al.* 2003a) and the brown alga *Laminaria digitata* following treatment with oligoguluronates (Küpper *et al.* 2001). These observations suggest the presence of a receptor by which the elicitor is recognised and with which it interacts, such interaction being required for the generation of intracellular signalling and subsequent defence responses, including NO production.

The biosynthetic origins of NO in plants have not been fully resolved and there is more than one possible route (Neill *et al.* 2003). In the present study we found that OGA-treated ginseng cells can use arginine in a NOS assay that converts arginine to citrulline, similar to mammalian cells. In addition, inhibitors of mammalian NOS reduced both NO accumulation and OGA-induced NOS activity. These data suggest that ginseng cells possess a NOS activity with some biochemical similarity to mammalian NOS. Increases in cytosolic calcium and protein phosphorylation are central features of intracellular signalling in plant cells. It is not surprising then, that \( \text{LaCl}_3 \) and RR, both calcium channel inhibitors, suppressed NO accumulation and OGA-induced NOS activity. Protein phosphorylation also appears to be required for OGA-induced NO production, as the general
protein kinase inhibitor K252a inhibited both NO accumulation and NOS activity. The protein phosphatase inhibitor OA had no effect.


