Involvement of Reactive Oxygen Species in the Induction of (*S*)-*N*-*p*-Coumaroyloctopamine Accumulation by β-1,3-Glucooligosaccharide Elicitors in Potato Tuber Tissues

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β-1,3-Glucooligosaccharide, (*S*)-*N*-*p*-coumaroyloctopamine, *Solanum tuberosum*

Treatment of potato tuber tissues with β-1,3-glucooligosaccharide induces accumulation of (*S*)-*N*-*p*-coumaroyloctopamine (*p*-CO). We examined the role of reactive oxygen species (ROS) and nitric oxide (NO) in the signal transduction leading to *p*-CO accumulation. Induction was suppressed by an NADPH-oxidase inhibitor, diphenyleneiodonium chloride, and oxygen radical scavengers. H_2O_2 was generated in the tuber tissue within a few minutes of treatment with β-1,3-glucooligosaccharide. On the other hand, treatment with NO specific scavenger, nitric oxide synthase inhibitor, and serine protease inhibitor did not inhibit *p*-CO induction. Our findings suggest that ROS generated by the action of NADPH-oxidase play an important role in this system, while NO and serine protease are unlikely to be involved in this process.

Introduction

It has been reported that certain oligosaccharides exhibit an elicitor activity that can induce various defense responses in plants (John *et al.*, 1997). However, the signal transduction pathway activated in this process remains unclear.

In potato, treatment of tuber tissue with the hyphal wall component prepared from *Phytophthora infestans* (HWC elicitor) causes marked accumulation of soluble (*S*)-*N*-*p*-coumaroyloctopamine (*p*-CO) (Matsuda *et al.*, 2000; Miyagawa *et al.*, 1998). Accumulation of *p*-CO can also be induced by laminarin (Miyagawa *et al.*, 1998), a β-1,3-glucooligosaccharide derived from seaweed *Laminaria digitata* (Percival *et al.*, 1951). It has been reported that, in addition to the elicitor treatment, *p*-CO and its derivatives, hydroxycinnamic-acid amides of catecholamines such as *N*-feruloyltyramine, can be induced by wounding and pathogen-infection in potato (Keller *et al.*, 1996; Negrel *et al.*, 1993; Schmidt *et al.*, 1998), tobacco (Negrel *et al.*, 1987; Villegas *et al.*, 1990), tomato (Pearce *et al.*, 1998), carnation (Niemann *et al.*, 1991), and poppy (Facchini, 1998) plants. Previous studies have suggested that these accumulated phenolic amide compounds are then integrated into cell walls to provide a physical barrier to protect against wound and/or pathogen intrusions (Clarke, 1982; Matsuda *et al.*, 2000). The biosynthesis pathway of phenolic amide compounds has already been determined, in which hydroxycinnamic-acid and catecholamine moieties are synthesized from L-phenylalanine and L-tyrosine, respectively. Treatment by saccharide elicitors induces up-regulation of biosynthesis of related enzymes such as phenylalanine ammonia-lyase (PAL), tyrosine decarboxylase (TyrDC), and hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT) in a few hours (Matsuda *et al.*, 2000; Schmidt *et al.*, 1998).

Although various signal transduction mechanisms are known to be activated in the elicitor-treated plant tissue (Hammond-Kosack *et al.*,

Abbreviations: *p*-CO, (*S*)-*N*-*p*-coumaroyloctopamine; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide; DPI, diphenyleneiodonium chloride; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; *n*-PG, *n*-propylgallate; PAL, phenylalanine ammonia lyase; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; THT, hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt; Tris, Tris(hydroxymethyl)aminomethane; TyrDC, tyrosine decarboxylase.

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1996), the generation of reactive oxygen species (ROS) is thought to be one of the most important signaling events responsible for the induction of plant defense responses (Camp *et al.*, 1998). A rapid generation of high levels of ROS, also known as oxidative burst, has been observed in pathogen-infected or elicitor-treated plant tissue (see review by Wojtaszek, 1997). The generation process includes conversion of molecular oxygen to superoxide anion (O_2^-) by the activity of NADPH-oxidase located at the plasma membrane, and subsequent dismutation of the generated superoxide anion to hydrogen peroxide (H_2O_2) by superoxide dismutase. Several lines of evidences have indicate that these ROS act as the signals to induce the hypersensitive reaction (HR) (Jabs *et al.*, 1996) and accumulation of antifungal substances (Apostol *et al.*, 1989).

In addition, recent studies have demonstrated that another reactive oxygen-derived species, nitric oxide (NO), is also involved in the induction of the defense responses in pathogen-infected plant tissue (Delledonne *et al.*, 1998; Tada *et al.*, 2000). The signaling role of NO, which is synthesized from L-arginine by the activity of nitric oxide synthase (NOS), is well characterized in mammalian cells.

On the other hand, another ROS-independent signaling pathway has also been identified, which can lead to the establishment of HR (Yano *et al.*, 1999). This pathway is inhibited by treatment with PMSF, a serine protease inhibitor.

In the present study, we examined the roles of ROS, NO, and serine protease in β-1,3-glucooligosaccharide-induced accumulation of (*S*)-*N*-*p*-coumaroylocopamine in potato tuber tissue in order to understand the signal transduction pathways activated by the oligosaccharide elicitor.

Materials and methods

Plant materials

Potato tubers (*Solanum tuberosum* cv. Eniwa) stored at 4 °C for a minimum of 6 months following harvesting were used in the present studies.

Chemicals

Laminarin from *Laminaria digitata* (Sigma Chemical Co., St. Louis, MO), laminariheptaose (Seikagaku Co., Japan), Tiron, *n*-propylgallate (Nakalai Tesque Co., Japan), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (CPTIO) (Tocris Co., Ballwin, USA), diphenyleneiodonium chloride (DPI) (Alexis Co., San Diego, CA), N^G-monomethyl-L-arginine (L-NMMA), and 3-aminophthaloylhydrazine (luminol) (Wako Pure Chemical Industries, Osaka, Japan) were used.

Elicitor treatment

The internal part of the tuber was cut into disks (8 mm diameter, 2 mm thick), which were washed with water for 30 min and incubated at 18 °C in the dark under wet condition for 24 h. The disks were then treated with 10 µl of an aqueous test solution containing 0.5 mg/ml of the elicitor together with a specified concentration of test chemicals. The disks were incubated under the same condition.

Determination of p-CO levels

Twenty-four hours after elicitor treatment, three disks were combined, weighed, and extracted with 2.5 ml of 2% acetic acid (aq.) at 100 °C for 10 min. These extracts were centrifuged at 3,500×*g* for 10 min, followed by HPLC analysis of the supernatant (column, Cosmosil 5C18AR-II, 4.6 × 150 mm; solvent, 40% methanol in water containing 0.1% H_3PO_4; flow rate, 0.8 ml / min; detection, UV 310 nm).

Enzyme assay

Sixteen hours after elicitor treatment, three disks (about 0.5 g) were homogenized with sea sand in five volumes of 0.1 M sodium phosphate buffer (pH 7.5) containing 14.4 mM 2-mercaptoethanol. The homogenate was centrifuged at 15,000×*g* for 10 min, and the supernatant was desalted on a PD-10 column. The protein fraction was used as a crude enzyme extract. The activities of PAL, THT and TyrDC were determined by the methods described previously (Matsuda *et al.*, 2000).

Luminol assay

Potato tuber sticks (1.5 mm diameter, 15 mm length) were prepared in a manner similar to that

used for tuber disks described above. The sticks were soaked for 5 sec in an aqueous solution containing 5.0 mM of tris[tris(hydroxymethyl)aminomethane]HCl (pH 7.5), 1 mg/ml of laminarin, and 100 mg/ml of luminol. Following incubation for specified times under darkness, the sticks were placed into glass test tubes (3.5 mm diameter), and fluorescence from the tuber sticks was determined by a luminometer (Pico Lite®, Packard Co.). The counting time was 30 seconds.

Results

Effects of ROS scavengers on p-CO accumulation in potato tuber tissues treated with laminarin

In order to investigate the involvement of reactive oxygen species in the signaling pathway from elicitor recognition to the *p*-CO accumulation, we investigated the effects of ROS scavengers on accumulation of *p*-CO in laminarin-treated potato tuber tissues. The amount of *p*-CO was determined at 24 h after treatment with an aqueous laminarin solution containing a ROS scavenger (Fig. 1a, b). Tiron and *n*-propylgallate (*n*-PG) inhibited *p*-CO induction in a dose-dependent manner up to 10 and 4 mM, respectively. However, the maximum levels of inhibition by Tiron and *n*-PG were about 70% and 60%, respectively. No further inhibition was observed for both compounds, even were treated at higher concentrations. Tiron and *n*-PG inhibited *p*-CO induction in laminariheptaose treated disks (Fig. 2). Furthermore, diphenyleneiodonium chloride (DPI), a potent inhibitor of NADPH oxidase, also inhibited the laminarin-induced accumulation of *p*-CO (Fig. 1c). The inhibitory effect was about 50% at 1.0 nM. No further inhibition, however, was observed.

Effects of ROS scavengers on p-CO biosynthesis related enzyme activities

It has been demonstrated that laminarin treatment induced a transient increase in the activities of *p*-CO biosynthesis-related enzymes such as PAL, THT, and TyrDC in potato tuber tissues (Matsuda *et al.*, 2000). Tiron and *n*-PG suppressed the induction of THT, which catalyzes the key step of *p*-CO biosynthesis, in laminarin-treated potato tuber disks (Fig. 3). Furthermore, the induction of

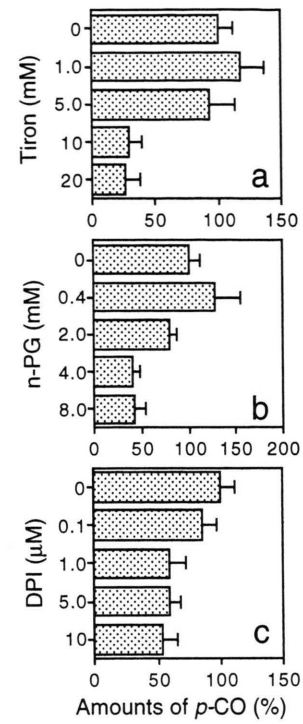

Fig. 1. Effects of Tiron (a), *n*-propylgallate (*n*-PG) (b), and diphenyleneiodonium chloride (DPI) (c) on the induction of (*S*)-*N*-*p*-coumaroyloctopamine (*p*-CO) accumulation upon laminarin treatment. The amount of *p*-CO was determined at 24 h after treatment of solutions containing various concentrations of inhibitors in the presence of 1.0 mg/ml laminarin. In tissue treated with 10 μl of water, the amount of *p*-CO was less than 3% of control. Data represent the mean ± SD of triplicate experiments.

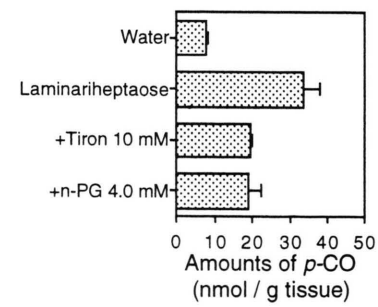

Fig. 2. Effects of Tiron and *n*-propylgallate (*n*-PG) on the induction of (*S*)-*N*-*p*-coumaroyloctopamine (*p*-CO) accumulation upon laminariheptaose treatment. The amount of *p*-CO was determined at 24 h after treatment of test solutions. Data represent the mean ± SD of triplicate experiments.

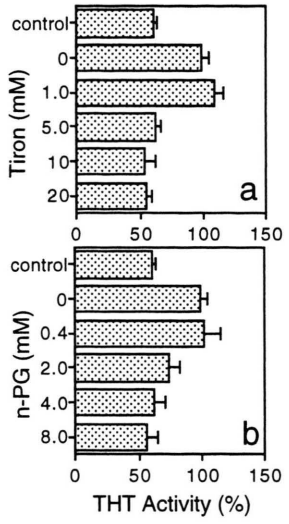

Fig. 3. Effects of Tiron (a), *n*-propylgallate (*n*-PG) (b) on the induction of hydroxycinnamoyl-CoA:tyramine *N*-(hydroxycinnamoyl)transferase (THT) activity upon laminarin treatment. The activity of THT was determined at 16 h after treatment of solutions containing various concentrations of inhibitors in the presence of 1.0 mg/ml laminarin. Data represent the mean ± SD of triplicate experiments.

Table I. The effects of Tiron (10 mM) on the induction of *p*-CO biosynthesis related enzymes upon laminarin treatment.

Additions	Enzyme activities (pkatal/mg protein) PAL	TyrDC
Control	51.5 ± 2.5	7.1 ± 1.2
Laminarin (1.0 mg/ml)	105.1 ± 1.7	14.7 ± 2.3
+ Tiron (10 mM)	73.9 ± 13.3	9.4 ± 0.9

PAL, phenylalanine ammonia lyase: TyrDC, tyrosine decarboxylase. Data represent the mean ± SD of triplicate experiments.

PAL and TyrDC was also inhibited by treatment of Tiron at a concentration of 10 mM (Table I).

Generation of H_2O_2 in laminarin-treated potato tuber tissues

The amount of H_2O_2 generated in the potato tuber tissue was determined by the luminol assay. The amount of H_2O_2 increased immediately after laminarin treatment and reached maximum levels in 10 minutes (Fig. 4), but gradually decreased to

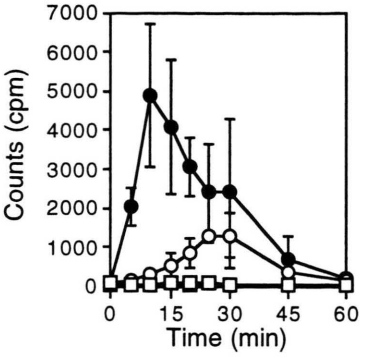

Fig. 4. Time-course of H_2O_2 generation in laminarin-treated potato tuber tissues. Following soaking in an aqueous test solution containing 5 mM of Tris-HCl (pH 7.5), 1 mg/ml of laminarin, and 100 mg/ml of luminol for 5 seconds, fluorescence from the tuber sticks was determined by a luminometer. The detection time was 30 seconds. Open squares: water control, solid squares: 1.0 mg/ml laminarin, open circles: 100 mg/ml luminol, solid circles: mixture of laminarin and luminol.

control levels at 60 minute after laminarin treatment. Accumulation of H_2O_2 was inhibited by Tiron and DPI to 0.2% and 36% of control, respectively (Table II).

Effects of NO scavenger, NOS inhibitors, and serine protease inhibitor on laminarin-induced accumulation of p-CO

We also investigated the effects of CPTIO and L-NMMA, an NO specific scavenger and a potent inhibitor of nitric oxide synthase, respectively, on the induction of *p*-CO in lamainarin-treated potato tuber tissues. As shown in figure 5a, b, both agents failed to influence laminarin-induced accumulation of *p*-CO, when used at concentrations ranging from 0.1 to 10 mM. Treatment with PMSF,

Table II. Effects of Tiron (10 mM) and diphenyleneiodonium chloride (DPI, 10 μM) on the generation of H_2O_2 in laminarin-treated potato tuber tissue.

Additions	Counts (cpm)
Laminarin (1.0 mg/ml)	32462 ± 13592
+ Tiron (10 mM)	76 ± 5
+ DPI (10 μM)	11740 ± 2824

Counts were determined at 10 min after treatment. Data represent the mean ± SD of 10 independent experiments.

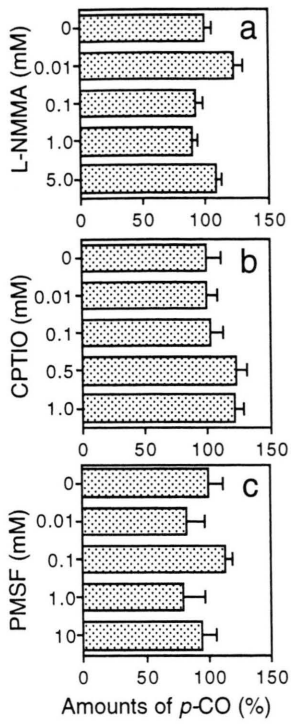

Fig. 5. Effects of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (CPTIO) (a), N^G-monomethyl-L-arginine (L-NMMA) (b) and phenylmethylsulfonyl fluoride (PMSF) (c) on the induction of (*S*)-*N*-*p*-coumaroyloctopamine (*p*-CO) accumulation upon laminarin treatment. The amount of *p*-CO was determined at 24 h after treatment of solutions containing various concentrations of inhibitors in the presence of 1.0 mg/ml laminarin. In tissues treated with 10 μl of water, the amount of *p*-CO was <3% of control. Data represent the mean ± SD of triplicate experiments.

a serine protease inhibitor, also failed to inhibit accumulation of *p*-CO (Fig. 5c).

Discussion

Our results showed that treatment of potato tuber tissue with ROS scavengers inhibited both the accumulation of *p*-CO (Fig. 1a, b) and activation of *p*-CO-biosynthesis related enzymes (Fig. 3, Table I) which are induced by β-1,3-oligosaccharide elicitor, or laminarin. A rapid and marked production of H_2O_2 was observed in potato tuber tissue after laminarin treatment (Fig. 4), in which the amount of H_2O_2 reached maximum 10 minutes after laminarin treatment. These findings suggest that ROS, including H_2O_2, play an important role in the signal transduction pathway from stimulation by laminarin to the induction of *p*-CO accumulation. Inhibition of both laminarin-induced *p*-CO biosynthesis and H_2O_2 generation by DPI (Fig. 1c, Table II) suggests that NADPH oxidase is likely to be involved in this generation of ROS. In potato; it has already been demonstrated that large amounts of ROS are generated shortly after inoculation of incompatible pathogen or elicitor treatment (Doke, 1983). The produced ROS are thought to function as the signal for the induction of HR (Doke, 1985) and accumulation of sesquiterpenoid phytoalexin including rishitin (Ellis *et al.*, 1993). The present study indicates that *p*-CO biosynthesis is controlled by the same mechanism involved in the defense responses in potato, although it follows a totally distinct pathway from that of rishitin. Thus, it is likely that ROS can regulate the activities of a broad-range of enzymes in the secondary metabolism of potato. While ROS generation has also been described in various plant/pathogen or plant/elicitor interactions (Wojtaszek, 1997), their signaling function with respect to the induction of phytoalexin accumulation has been demonstrated in parsley (Jabs *et al.*, 1997) and soybean (Apostol *et al.*, 1989) suspension cultures.

Recent studies have also demonstrated that NO is involved in the induction of rishitin in potato (Noritake *et al.*, 1996). However, the NO specific scavenger (CPTIO) and NOS inhibitor (L-NMMA) were inactive in the present study with respect to laminarin-induced *p*-CO accumulation (Fig. 5). These results suggest that the signal transduction mechanisms leading to the induction of rishitin and *p*-CO are probably different.

While the ROS scavengers completely inhibited the generation of H_2O_2 (Table II) and activation of THT in the elicitor-treated potato tuber tissues (Fig. 3), inhibition of *p*-CO accumulation was partial even in the presence of a high concentration of scavengers (Fig. 1a, b). Such incomplete inhibition suggests that ROS is probably not the sole signal for the induction of *p*-CO accumulation in potato tuber tissue and that there might be another unknown signal transduction pathway that is activated independent of ROS. In this regard, *p*-CO accumulation could not be induced by treatment of tuber disks with ROS generated by the xanthine/xanthine oxidase system (data not shown).

This finding suggests that a critical signaling step precedent to the generation of ROS is present, and ROS act as an amplifier of this critical signal, like the relationship between initiator and promotor in chemical carcinogenesis. The critical step might be among other signaling system(s) which have been demonstrated to operate in plants, involving such agents as calcium (Ishihara *et al.*, 1996), G-proteins (Rajasekhar *et al.*, 1999), kinase cascades (Seo *et al.*, 1995), cAMP (Kurosaki *et al.*, 1987), PLC (Senda *et al.*, 1998), and serine protease (Yano *et al.*, 1999) in plant defense responses. However, no involvement of serine protease was inferred in the present study, since PMSF was ineffective (Figure 5c). The involvement of other factors in the induction of *p*-CO in potato tuber disks is currently being examined in our laboratories.

As demonstrated for laminarin in potato tuber tissue (Fig. 4), previous studies have shown that other oligosaccharide elicitors such as oligogalacturonide and *N*-acetylchitopentaose, also cause a rapid and intense generation of ROS in some plant species (Legendre *et al.*, 1993; Ruth *et al.*, 1999). On the other hand, proteinous elicitors, including cryptogein from *Phytophthora cryptogea* can also elicit ROS production in some Solanaceae plants including potato (Allan *et al.*, 1998). While the proteinous elicitors are characteristically able to cause cell death, one of the typical phenomenon of HR (Pernollet *et al.*, 1993), laminarin appeared to cause no cell death (data not shown). This suggests that there are more than one signaling pathways, the activation of which depends on the elicitors.

Although it has been repeatedly shown that a definite class of saccharides act as elicitors in plants (John *et al.*, 1997), their perception mechanism(s) has not been well characterized. Chemical reactions underlying the macromolecular interactions between elicitor-active saccharides and their putative receptor are of great interest, since typical elicitors have considerably large molecular weights, and are unlikely to be recognized by a single proteinous molecule. It is anticipated that a closer examination of the action of laminarin in potato should unravel one of the actual events occurring at the interface of the plant-pathogen interaction. This should enhance our understanding of the labyrinth of plant defense responses.

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