Induction of Plant gp91 phox Homolog by Fungal Cell Wall, Arachidonic Acid, and Salicylic Acid in Potato

Hirofumi Yoshioka, Kenichi Sugie, Hae-Jun Park, Hirotaka Maeda, Naoki Tsuda, Kazuhito Kawakita, and Noriyuki Doke

Plant Pathology Laboratory, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan
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The oxidative burst has been suggested to be a primary event responsible for triggering the cascade of defense responses in various plant species against infection with avirulent pathogens or pathogen-derived elicitors. The molecular mechanisms of rapid production of active oxygen species (AOS), however, are not well known. We isolated homologs of gp91 phox, a plasma membrane protein of the neutrophil NADPH oxidase, from a potato cDNA library. Molecular cloning of the cDNA showed that there are two isogenes, designated StrbohA and StrbohB, respectively. The RNA gel blot analyses showed that StrbohA was constitutively expressed at a low level, whereas StrbohB was induced by hyphal wall components (HWC elicitor) from Phytophthora infestans in potato tubers. Treatment of potato tubers with HWC elicitor caused a rapid but weak transient accumulation of \( \text{H}_2\text{O}_2 \) (phase I), followed by a massive oxidative burst 6 to 9 h after treatment (phase II). Diphenylene iodonium (DPI), an inhibitor of the neutrophil NADPH oxidase, blocked both bursts, whereas pretreatment of the protein synthesis inhibitor cycloheximide with the tuber abolished only the second burst. These results suggest that the expression of StrbohA and StrbohB contributes to phase I and II bursts, respectively. The same is true for arachidonic acid, whose role in triggering the oxidative burst, we examined in a causal relationship between the oxidative burst and expression of defense genes induced by the HWC elicitor. The transcript accumulation of genes related to sesquiterpenoid phytoalexin synthesis (lubimin and rishitin) and phenylpropanoid pathway was inhibited slightly by the DPI treatment, suggesting that the oxidative burst is not essential to activate these genes. Interestingly, the concomitant presence of DPI with the elicitor resulted in an increase in lubimin accumulation and a decrease in rishitin accumulation. Because it is known that lubimin is metabolized into rishitin via oxylubimin, we propose that AOS mediates the synthesis of rishitin from lubimin.

The production of active oxygen species (AOS) at the cell surface, called the “oxidative burst,” is one of the earliest events detected during incompatible interactions between plants and pathogens (Doke 1983a; Hammond-Kosack and Jones 1996; Lamb and Dixon 1997). Elegant experimental evidence with plant mutants showed that \( \text{O}_2^- \) is an indispensable factor for the hypersensitive cell death (Dietrich et al. 1996; Jabs et al. 1996). The rapid production of AOS could inhibit pathogen growth through direct microbiocidal action (Peng and Kuc 1992) by restricting pathogen penetration via cross-linking cell wall glycoproteins (Bradley et al. 1992; Brisson et al. 1994), induction of phytoalexin accumulation (Apostol et al. 1989), and activating salicylic acid (SA) biosynthesis (Léon et al. 1995), which leads to the induction of defense-related genes (Chen et al. 1993; Yang et al. 1997).

Exposure of potato tuber tissues to Phytophthora infestans, the late-blight pathogen, causes multiple defense responses, including the oxidative burst, accumulation of phenylpropanoid compounds, and sesquiterpenoid phytoalexins (lubimin and rishitin), which are accompanied by de novo synthesis of the enzymes involved in their production (Choi et al. 1992; Doke 1983a; Hahlbrock and Scheel 1989). All of these reactions also are induced in potato tubers by treatment with a crude elicitor preparation (hyphal wall components [HWC]) from the mycelium of the fungus (Doke et al. 1996). Several lines of evidence demonstrated that the oxidative burst in plants reflects activation of an NADPH oxidase, closely resembling that which operates in activated neutrophils. An

Corresponding author: H. Yoshioka;
E-mail: hyoshiok@agr.nagoya-u.ac.jp

The nucleotide and amino acid sequences reported here have been submitted to the DDBJ, EMBL, and GenBank databases, under accession nos. AB050660 for StrbohA mRNA and AB050661 for StrbohB mRNA.
NADPH-dependent \( \text{O}_2^- \) -generating system is present in microsomal fractions from potato tubers (Doke 1983b), incubation of tuber slices with an incompatible race of *P. infestans*, or HWC-elicitor stimulates of NADPH-dependent \( \text{O}_2^- \) -generating activity, which was located predominantly in the plasma membrane fraction (Doke 1985; Doke and Miura 1995). Diphenylene iodonium (DPI), an inhibitor of the neutrophil NADPH oxidase, blocks the oxidative burst in plant cells (Auh and Murphy 1995; Dwyer et al. 1996; Levine et al. 1994). Recently, homologs of gp91 \( \text{phox} \) (respiratory burst oxidase homolog [rboh]), which is a plasma membrane protein of the neutrophil NADPH oxidase, was isolated from rice (Groom et al. 1996), Arabidopsis (Keller et al. 1998; Torres et al. 1998), and tomato (Amicucci et al. 1999).

Signal transduction leading to defense responses in the plant cell is illustrated by the role of the oxidative burst in signal generation. Earlier works provided evidence that signaling events preceding the burst include the involvement of a homolog of human Rac (Kawasaki et al. 1999), calcium influx into the cytoplasm (Chandra and Low 1997; Piedras et al. 1998), and changes in protein phosphorylation (Chandra and Low 1995; Kauss and Jeblick 1995). Unequivocal evidence for a causal relationship between the upstream signal transduction pathways preceding the elicitor-stimulated oxidative burst and regulation of the plant gp91 \( \text{phox} \) homolog, however, is still lacking. There are also contradictory reports that suggest the oxidative burst is independent of phytoalexin accumulation or other defense responses (Dorey et al. 1999; Rustérucci et al. 1996; Sasabe et al. 2000). Accumulating evidence suggests that there are marked differences in the responses of plant cell cultures and intact plant tissue (Dixon and Harrison 1994). Most data on the signal transduction leading to the plant defense response have been obtained from cell cultures. Here, we isolated plant gp91 \( \text{phox} \) homologs from a potato cDNA library and report that calcium-independent protein phosphorylation is involved in the regulation of gene activation in potato tuber tissues. We also address whether the elicitor-stimulated oxidative burst participates in defense gene activation related to the synthesis of sesquiterpenoid phytoalexins and phenylpropanoid derivatives.

RESULTS

Two homologs of human gp91 \( \text{phox} \) in potato.

By screening a potato cDNA library with a 552-bp reverse-transcription-polymerase chain reaction (RT-PCR) product of potato rboh (see below), seven positive clones were isolated under low-stringency conditions. We identified the potato homologs of human gp91 \( \text{phox} \), designated StrbohA and StrbohB. The longest StrbohA cDNA was 3,733 bp, and it had an open reading frame (ORF) for 963 amino acids with a predicted molecular mass of 99 kDa (accession no. AB050661). Although the topology and functions of plant rboh proteins have not been tested experimentally, several features of the amino acid sequence and comparisons with human gp91 \( \text{phox} \) allow for a prediction (Yu et al. 1998). StrbohA and StrbohB proteins showed the six transmembrane-spanning domains (TMD-1 to TMD-6), which correspond to those identified in human gp91 \( \text{phox} \). TMD-3 and TMD-5 contain pairs of histidine residues, which are important for heme binding (Finegold et al. 1996). The C-terminal regions of each homolog contain conserved flavin adenine dinucleotide, NADPH–ribose, and NADPH–adename binding sites and are therefore likely to be located in the cytoplasm. Moreover, human gp91 \( \text{phox} \) amino acid residues Pro-415 and Asp-500, which are indispensable for the catalytic activity (Segal et al. 1992), are also conserved in StrbohA and StrbohB. The N-terminal regions of the proteins that are hydrophilic and seen only in plant rboh contain two \( \text{Ca}^{2+} \)–binding EF hands. Calcium-binding activity of these domains was found experimentally by Keller et al. (1998) for Arabidopsis AtrbohA.

Comparison of the Strboh proteins and genes.

Figure 2A shows a phylogenetic tree for polypeptides of the human gp91 \( \text{phox} \) and the 10 characterized plant rbhos. Arabidopsis AtrbohA (accession no. AF015301), referred to by Keller et al. (1998), corresponds to AtrbohF (accession no. AB008111), referred to by Torres et al. (1998). The plant rbhos, except for AtrbohE, were divided into two groups. The deduced amino acid sequence of StrbohA had a maximum homology to tomato Lerboh1 (92% identity). Amicucci et al. (1999) reported that the transcripts were relatively abundant in cold-stressed tomato seedlings. StrbohB, however, showed a high homology (63% identity) to Arabidopsis AtrbohB, which was expressed at a low level in all tissues tested (Torres et al. 1998). It was reported that Arabidopsis thaliana contains at least six Atrboh genes (Torres et al. 1998). Screening of the potato cDNA library under low-stringency conditions showed that potato also contains at least two isoforms. Gel blot hybridizations were performed with potato genomic DNA samples under high-stringency conditions when an entire sequence of StrbohA cDNA and a truncated sequence of StrbohB cDNA were used as probes (Fig. 2B). Despite the high homology between StrbohA and StrbohB (51% identity), hybridization patterns of the blots were different, indicating that these probes detect each Strboh sequence. We cannot rule out the possibility that more Strboh genes exist. The analysis of cDNAs obtained under low-stringency conditions from 6 \( \times \) 10\(^5\) plaques of the cDNA library prepared from tuber tissues treated with HWC elicitor suggests that, if there are more gp91 \( \text{phox} \) homologs in potato, they are unlikely to be expressed in elicitor-stimulated tuber tissues.

Induction of the StrbohB gene by pathogen signals.

We estimated changes with time in the transcript levels of StrbohA and StrbohB in total RNA isolated from potato tubers that were treated with the HWC elicitor, archidonic acid (AA), or SA as potent fungal signals of *P. infestans* after an aging period of 24 h (Fig. 3). The StrbohB probe showed that treatment with 1 mg of HWC elicitor per ml gave a drastic and transient increase in the transcript level, peaking at 3 to
6 h and then declining. Treatment with AA, a fatty acid elicitor present in the lipids of *P. infestans* (Bostock et al. 1981) that induces defense responses on potato tubers at a concentration of 0.1 mM (data not shown), also induced the transient increase 3 h after treatment. Moreover, exogenous SA stimulated only weak accumulation of StrbohB transcripts at a concentration of 0.5 mM, which is sufficient for the induction of the oxidative burst in plant cell suspensions (Kauss and Jeblick 1995; Shirasu et al. 1997). In contrast, StrbohA was constitutively expressed at a low level during each treatment. In many cases, more than 7 days exposure of X-ray film is necessary to detect StrbohA transcripts by RNA gel blot

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**Fig. 1.** Predicted amino acid sequence alignment of StrbohA, StrbohB, and human gp91 phox. Amino acids conserved within each group are in black boxes. Motifs homologous with other oxidoreductases are inside large squares (Segal et al. 1992). Six potential transmembrane-spanning domains (TMD1-6) are indicated by a bar. Conserved histidine residues, demonstrated to bind heme in yeast FRE1 (Finegold et al. 1996), are indicated. EF-hand motifs in the C-terminal domain of Strboh are overlined. Beneath these motifs are the sequence of a canonical EF-hand (Kretzinger 1996). N is usually hydrophobic residues. Dashes indicate variable amino acid residues. X, Y, Z, and −X, Y, Z contain oxygen within their side chain. Carbonyl oxygen of # serves as ligand. −Z usually is glutamic acid. Dashes indicate gaps in sequence to allow for maximal alignment. Multiple alignment of the plant respiratory burst oxidase homolog was made with the CLUSTAL W program (Thompson et al. 1994). Dots under the sequences indicate amino acid residues involved in N-glycosylation.
analysis, whereas 1 day of exposure is sufficient for StrbohB detection. Thus, the StrbohB gene is stimulated by various pathogen signals.

**Oxidative burst by pathogen signals and the effects of inhibitors.**

The oxidative burst was measured as an increase in chemiluminescence caused by H$_2$O$_2$ (Fig. 4A). Treatment of HWC elicitor with potato tubers caused an immediate increase in the phase I oxidative burst, peaking at 1 h and then returning to near-control level (Fig. 4B). A massive phase II oxidative burst occurred 6 to 9 h after HWC treatment. The similar two-phase kinetics were observed in potato leaves (Chai and Doke 1987b), tobacco cells (Baker et al. 1993; Dorey et al. 1999), soybean cells (Levine et al. 1994), and parsley cells (Jabs et al. 1997). The concomitant presence of DPI with the elicitor, however, dramatically abolished the phase I and II bursts, suggesting that the enzyme operating both bursts in potato tuber is similar to NADPH oxidase in mammalian cells, as reported with other plants. In many cases, 10 µM DPI is sufficient for nearly complete inhibition of elicitor-induced oxidative burst in plant suspension cells (Dorey et al. 1999; Jabs et al. 1997; Rustérucci et al. 1996). Here, we used 50 µM DPI for complete inhibition of the burst because our previous experiments with other inhibitors indicated that responses in potato discs generally require a higher concentration of an inhibitor to exert an effect than the concentration level reported to inhibit responses in cell suspensions (Miura et al. 1995). Treatment of tuber discs with AA also stimulated biphasic oxidative burst. The second burst, however, was modest, which is in agreement with StrbohB expression (Fig. 3). Administration of SA induced only a weak second burst. H$_2$O$_2$ concentration began to increase 6 h after treatment, reaching a maximum at 9 h. The SA-induced burst also was blocked by treatment with 50 µM DPI (data not shown). We examined whether de novo protein synthesis is required for the elicitation of phase I and II bursts with the use of a protein synthesis inhibitor cycloheximide. The treatment of aged tuber discs with cycloheximide at a concentration of 100 µM for 1 h was shown to inhibit when following $^3$H-leucin incorporation into tuber disc protein for 18 h by 94% relative to corresponding control discs (Yoshioka and Doke 1994). The cycloheximide...

![Fig. 2. Strboh gene organization](image)

A. Phylogenetic tree of various plant respiratory burst oxidase homologs. Sequences origins [accession nos.]: Arabidopsis respiratory burst oxidase homolog (AtrbohA–F) [AF055353, AF055354, AF055355, AF055357, AF055356, and AB008111, respectively]; rice rboh (Osrboh) [AF015301]; tomato rboh (Lerboh1) [AF088276]; potato rboh (StrbohA) [AB050660]; (StrbohB) [AB050661]; and human gp91 phox (human gp91) [S70773]. Arabidopsis AtrbohA (accession no. AF015301), referred to by Keller et al. (1998), corresponds to the AtrbohF (accession no. AB008111), referred to by Torres et al. (1998). An unrooted tree was constructed by the neighbor-joining method (Saitou and Nei 1987). The length of the horizontal lines connecting the sequences is proportional to the estimated genetic distance between these sequences. The number above or below each horizontal line is the frequency with which a given branch appeared in 1,000 bootstrap replications. B. Southern blot hybridization of genome DNA. Genome DNA was digested with HindIII (H), EcoRI (E), or SpeI (S) and hybridized with $^{32}$P-labeled StrbohA or StrbohB cDNAs. DNA molecular size markers are indicated.

![Fig. 3. Accumulation of Strboh transcripts in potato tuber discs after treatment with elicitors](image)

Potato tuber discs were aged for 24 h prior to treatment with 1 mg of hyphal wall component (HWC) elicitor per ml, 0.1 mM arachidonic acid (AA), 0.5 mM salicylic acid (SA), or water as a control. Total RNA was hybridized with $^{32}$P-labeled cDNAs for StrbohA and StrbohB and for 18S rRNA as an RNA loading control.
treatment only abolished the phase II burst; the phase I burst occurred as early as 30 min after HWC treatment (Fig. 4A), indicating that the second burst requires new protein synthesis.

In plants, two kinds of enzymes are believed to contribute to an \( \text{O}_2^{-} \)-generation reaction in response to pathogen-derived elicitors, i.e., NADPH oxidase in the plasma membrane and peroxidase in the cell wall (Kawano et al. 1998; Kiba et al. 1997). In some plant systems, DPI appears to mimic the effects of certain antioxidants that use peroxidases to scavenge \( \text{H}_2\text{O}_2 \) and NAD(P)H as a reductant (Baker et al. 1998). In fact, other recent experiments that employed pharmacological agents suggest that at least two distinct sources, including NADPH oxidase and peroxidase-type enzyme, contribute to the generation of redox cues (Allan and Fluhr 1997; Grant et al. 2000). These reports indicate that the dominant source of AOS generation depends upon the experimental system. We provide evidence for a dominant role of NADPH oxidase in the generation of AOS in potato tuber.

**Involvement of calcium-independent protein kinase in the expression of the StrbohB gene.**

As reported in previous studies in potato tubers (Miura et al. 1995) and tobacco cells (Atkinson et al. 1990; Baker et al. 1993), a sustained calcium influx and protein phosphorylation are required for the elicitation of phase I and II bursts. Little is known, however, about the signal pathway leading to the expression of plant gp91 phox. To gain insight into whether expression of the StrbohB gene by the HWC elicitor might have similar requirements, we tested the effects of two serine-threonine kinase inhibitors, with broad specificity, and two calcium antagonists. Stauroporine and K252a, at 10 \( \mu \text{M} \), were treated for 1 h prior to treatment with a mixture of these inhibitors and HWC elicitor because it was reported that the continuous presence of inhibitor at 10 \( \mu \text{M} \) is required for the complete inhibition of the oxidative burst in cultured soybean cells (Chandra and Low 1995). An extracellular calcium chelator ethylene glycol-bis(\( \beta \)-aminoethyl ether)-\( \text{N},\text{N},\text{N}'\text{,N}' \)-tetraacetic acid (EGTA) at 1 \( \text{mM} \) or 100 \( \mu \text{M} \) calcium channel blocker verapamil were treated for 1 h prior to treatment with HWC elicitor because it was reported that the continuous presence of inhibitor at 10 \( \mu \text{M} \) is required for the complete inhibition of the oxidative burst in cultured soybean cells (Chandra and Low 1995). An extracellular calcium chelator ethylene glycol-bis(\( \beta \)-aminoethyl ether)-\( \text{N},\text{N},\text{N}'\text{,N}' \)-tetraacetic acid (EGTA) at 1 \( \text{mM} \) or 100 \( \mu \text{M} \) calcium channel blocker verapamil were treated for 1 h prior to treatment with HWC elicitor because it was reported, the oxidative burst and the activation of the phenylalanine ammonia-lyase (PAL) gene induced by HWC elicitor in potato tubers were suppressed dramatically by EGTA and verapamil at these concentrations (Miura et al. 1995; Miura et al. 1999). Both protein kinase inhibitors completely blocked the accumulation of the StrbohB transcript, whereas the extracellular Ca\(^{2+}\)-chelator EGTA partially inhibited gene activation (Fig. 5). The verapamil, however, did not prevent StrbohB expression by HWC elicitor. We conclude from these results that StrbohB gene expression results from certain calcium-independent protein kinase activity.

**Fig. 4.** Oxidative burst in potato tuber discs. A, Oxidative burst as images of luminol-mediated chemiluminescence on the potato tuber discs. Photons from the chemiluminescence from potato discs aged for 24 h were incorporated integrally for 5 min at 1 h after treatment with 1 mg of hyphal wall component (HWC) elicitor per ml. HWC elicitor plus 50 \( \mu \text{M} \) diphenylene iodonium (DPI) (HWC + DPI), or water (control) in the presence of 2.5 mM luminol in 10 mM Tris-\( \text{HCl} \) buffer (\( \text{pH} \) 7.4). Signal intensities become stronger toward red. B, Time-course observation of the oxidative burst on the potato tuber discs. Chemiluminescence was counted at various times after the addition of water, 1 mg of HWC elicitor per ml, 0.1 mM arachidonic acid (AA), 0.5 mM salicylic acid (SA), HWC elicitor + 50 \( \mu \text{M} \) DPI, or HWC elicitor + 100 \( \mu \text{M} \) cycloheximide (CHX; see text). Values are the average at designated times in three experiments.
Role of oxidative burst in expression of defense genes and phytoalexin synthesis.

To address whether the elicitor-stimulated oxidative burst participates in defense gene activation related to the sesquiterpenoid phytoalexins, lubimin and rishitin (Fig. 6), and lignin synthesis, we examined the DPI effect on the levels of several defense genes such as 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; HMG1, HMG2, and HMG3), sesquiterpene cyclase (SC), PAL, and caffeoyl CoA o-methyltransferase (CCoAOMT). Nitric oxide (NO) has been implicated in redox-active signaling leading to hypersensitive cell death, an increase in PAL transcript levels and phytoalexin accumulation (Delledonne et al. 1998; Noritake et al. 1996). Durner et al. (1998) found that DPI also has a weak inhibitory effect on NO synthase at 250 µM in tobacco leaves. We believe that the 50 µM DPI administrated here, however, has no effect on activity. Treatment with HWC elicitor gave transient increases in transcript levels of HMG2, HMG3, and SC that are responsible for phytoalexin biosynthesis (Fig. 7) (Choi et al. 1992; Yoshioka et al. 1999). In contrast, the wound-induced HMG1 and squalene synthase (SS) transcripts, which participate in sterol and steroid glycoalkaloid biosynthesis (Choi et al. 1994; Schaller et al. 1995; Yoshioka et al. 1999), were suppressed during elicitation. Similar results were observed by Zook and Kuc (1991), who showed that treatment of potato tubers with an elicitor results in the induction of SC activity and suppression of SS activity. These results support the concept of coordinated regulation of HMGR, SC, and SS genes in potato during elicitation of sesquiterpenoid phytoalexin synthesis. Because these enzymes are positioned at a key point in the isoprenoid pathway, the induction of one enzyme and the suppression of the other could be an important mechanism controlling carbon flow, as proposed by Chappell (1995). The transcript accumulation of HMG2, HMG3, SC, and CCoAOMT was inhibited partially by DPI treatment, especially at 6 h, whereas the amounts of HMG1, SS, and PAL transcripts were not affected, even in the presence of DPI, suggesting that the oxidative burst is not essential to activate these genes. To test whether the oxidative burst has a role in sesquiterpenoid phytoalexin synthesis, we also examined the effect of DPI on the accumulation of lubimin and rishitin. Interestingly, HWC-induced rishitin accumulation was decreased and, simultaneously, lubimin accumulation was increased by the concomitant presence of DPI with the elicitor (Fig. 8).

DISCUSSION

Role of Ca2+ in the activation of plant NADPH oxidase.

Cloning gp91 phox homologs in potato plants provides valuable material in which to study the involvement of these genes in the production of AOS and their role in disease resistance. Potato tubers expressed at least two homologs, designated StrbohA and StrbohB. The Strboh gene products show pronounced similarity to the 69-kDa apoprotein of the gp91...
phox subunit of the neutrophil NADPH oxidase and carry an extra N-terminal extension containing regions that show conserved amino acids and two putative Ca\(^{2+}\) binding motifs (Fig. 1). Keller et al. (1998) provided evidence for \(^{45}\)Ca\(^{2+}\) binding to the two EF-hand motifs of AtrbohA in Arabidopsis. Sequence matching of AtrbohA EF hands and the presence of conserved residues in this region indicate that Strboh proteins probably carry two EF hands. The presence of the EF hands in plant gp91 phox homologs suggests a role for Ca\(^{2+}\) in the regulation of NADPH oxidase activity (Amicucci et al. 1999; Keller et al. 1998; Torres et al. 1998). Our previous pharmacological studies indicate that calcium influx and protein phosphorylation are involved in the activation process of the elicitor-stimulated oxidative burst in potato tubers (Miura et al. 1995) and in the isolated plasma membrane fraction (Doke and Miura 1995). On the basis of the requirement of Ca\(^{2+}\) for the oxidative burst, it is conceivable that some of the kinases involved are calcium-dependent protein kinases (CDPK). In the Cf9–Avr9 gene-for-gene interaction, a 68- to 70-kDa CDPK is activated in the plasma membrane fraction of tobacco cell cultures. Inhibitor studies are consistent with the CDPK being located upstream in the signal pathway that leads to the induction of AOS generation (Romeis et al. 2000). Furthermore, AA, a lipid elicitor of P. infestans, induces activation of 78-kDa protein kinase C-like enzyme in potato tubers in a calcium-dependent manner (Subramaniam et al. 1997). These lines of evidence suggest that plant NADPH oxidase is activated by direct binding of Ca\(^{2+}\) to the EF hands and/or certain CDPK activity.

Induction of StrbohB gene by pathogen signals.

Previous work in this laboratory has established that HWC elicitor stimulates the oxidative burst in potato tubers (Doke 1983b), yet here it is demonstrated that treatment with the elicitor not only activates the oxidase but also leads to the induction of StrbohB gene expression (Fig. 3). RNA gel blot analyses indicated that StrbohA was expressed constitutively at a low level (7 days exposure), whereas StrbohB was induced by HWC elicitor. It is possible that, in plants, elicitor induction of the gp91 phox homolog is one of the mechanisms controlling the oxidative burst machinery and, perhaps, leading to the long-term generation of AOS. Recently, it was reported that the Arabidopsis AtrbohD gene was induced by the bacterial elicitor harpin and exogenous H\(_2\)O\(_2\) at 10 mM (Desikan et al. 1998). This finding led us to believe that a rapid phase I burst by the HWC elicitor stimulates StrbohB gene expression, followed by a massive phase II burst. Treatment of potato tubers with 10 mM H\(_2\)O\(_2\) or xanthine–xanthine oxidase, however, did not induce StrbohB expression (data not shown). In addition, although SA did not stimulate the first burst, StrbohB gene was induced, suggesting that the early production of AOS is not required to initiate the StrbohB gene. Protein phosphorylation–dephosphorylation is the most widely used mechanisms for signal transduction in eukaryotic cells (Hubbard and Cohen 1993). In plants, it also is involved in most cases of signal pathways, leading to defense reactions initiated by external and internal stimuli (Felix et al. 1991; Sheen 1996). Recently, it was suggested that mitogen-activated protein (MAP) kinases participate in the plant defense response. For instance, some members of the MAP kinase family are stimulated by HWC elicitor, SA, or the Cf9–Avr9 interaction in tobacco cells. The activities of these kinases were determined with the use of myelin basic protein (MBP) as substrate (Romeis et al. 1999; Suzuki and Shinshi, 1995; Zhang and Klessig 1997; Zhang et al. 1998). We demonstrated previously that HWC elicitor, AA, and SA activate a 51-kDa MBP kinase in potato tubers (Katou et al. 1999). Here, these pathogen signals also stimulated the phase II burst and StrbohB gene (Figs. 3 and 4).

![Fig. 7. Effect of diphenylene iodonium (DPI) on the expression of several defense genes induced by the HWC elicitor. Potato discs were aged for 24 h at 20°C prior to treatments with 1 mg of HWC elicitor per ml, HWC elicitor + 50 µM DPI, or water. Ten micrograms of RNA was separated by electrophoresis on a 1.2% agarose–formaldehyde gel. The RNA gel blot was probed with potato HMG1-, HMG2-, HMG3-specific cDNAs; potato sesquiterpene cyclase (SC) cDNA; potato squalene synthase (SS) cDNA; potato phenylalanine ammonia–lyase (PAL) cDNA; potato caffeoyl CoA o-methyltransferase (CCoAOMT) cDNA; or 18S cDNA as a RNA loading control. One blot was reprobed successively in the present experiments.](image-url)
Regulation of the StrbohB gene by calcium-independent protein kinases.

To characterize the signaling pathway leading to expression of the StrbohB gene by the HWC elicitor, we tested the effects of two serine-threonine kinase inhibitors with broad specificity and two inhibitors of extracellular Ca\(^{2+}\) movement (Fig. 5). Interestingly, K252a and staurosporine completely blocked the accumulation of the StrbohB transcript, whereas the extracellular Ca\(^{2+}\)-chelator EGTA partially inhibited gene activation. The Ca\(^{2+}\) channel blocker verapamil, however, did not prevent StrbohB expression by HWC elicitor, suggesting that certain calcium-independent protein kinases have an important role in StrbohB gene expression (Fig. 9). Ádám et al. (1997) reported that the Ca\(^{2+}\) channel blocker La\(^{3+}\) did not affect activation of a 49-kDa harpin\(_{eucalyptus}\)-activated protein kinase (HAPK) in tobacco leaves. Additionally, EGTA partially inhibited HAPK activation. Likewise, the HWC-induced, 51-kDa MBP kinase in potato tubers was not influenced by La\(^{3+}\) (Katou et al. 1999), indicating that calcium-independent protein kinase mediates HWC-stimulated defense responses. The protein kinase responsible for StrbohB expression remains to be identified.

Is potentiation of the oxidative burst a major factor for defense gene expression?

The rapid production of AOS is one of the earliest events in defense responses (Lamb and Dixon 1997). The oxidative burst may contribute directly to plant defense and also play an important role in signaling, thus leading to the induction of defense genes (Jabs et al. 1997; Yang et al. 1997). Studies with other systems concentrated mainly on genes with roles in phenylpropanoid phytoalexin production or cellular protectants such as glutathione S-transferase and glutathione peroxidase. In soybean cells, exogenous H\(_2\)O\(_2\) induces expression of the gene required for cellular protection, but not those that encode PAL and chalcone synthase, which are involved in phytoalexin synthesis (Levine et al. 1994). In contrast, Jabs et al. (1997) reported that antibiotics that induce H\(_2\)O\(_2\) accumulation elicit the expression of a set of furanocoumarin phytoalexin biosynthetic genes, including PAL in parsley cell cultures. To address whether the elicitor-stimulated oxidative burst is involved in defense gene activation related to the sesquiterpenoid phytoalexins lubimin and rishitin (Fig. 6) as well as to phenylpropanoid biosynthesis, we examined the effect of DPI on the levels of those genes. We found that all defense genes tested here were not blocked by DPI and so are not dependent on AOS (Fig. 7). Yin et al. (1997) made a similar observation in elicitor-treated tobacco leaf tissues. Exogenous H\(_2\)O\(_2\) had little effect on the induction of endogenous sesquiterpene cyclase activity and GUS activity driven by the promoter of the cyclase gene in transgenic plants. Antioxidant treatments fail to inhibit sesquiterpenoid phytoalexin accumulation in tobacco cell cultures treated with an elicin peptide from Phytophthora cryptogea (Rustérucci et al. 1996). Moreover, in cell suspension cultures derived from Cf-9 tobacco plants, 273 cDNA fragments were elicited rapidly when challenged with the Avr9 elicitor. Of these fragments, 263 were induced by Avr9, even in the presence of DPI, indicating that rapid elicitor-responsive genes are independent on AOS (Durrant et al. 2000). Rishitin production was induced by HWC elicitor, whereas the concomitant presence of DPI with the elicitor results in an increase in lubimin accumulation and a decrease in rishitin accumulation (Fig. 8). Murai et al. (1982) reported that lubimin is metabolized into rishitin via oxylubimin; thus, we think that there is a possibility that AOS participates in the lubimin-to-rishitin biosynthetic process (Fig. 6).

Role of the oxidative burst in the defense reaction in plant-pathogen interactions.

Exogenous H\(_2\)O\(_2\) induces PR-1 gene expression in tobacco leaves (Chen et al. 1993). Likewise, transgenic potato plants expressing glucose oxidase, which constitutively elevate the level of H\(_2\)O\(_2\), accumulate the transcripts of the anionic peroxidase, acidic chitinase, and conjugated SA (Wu et al. 1997). Therefore, the role of AOS in defense responses appears to be plant and gene specific and situation dependent. It was reported that inoculation with an avirulent pathogen rapidly induces secondary oxidative bursts in distant tissues and uninoculated leaves, leading to systemic acquired resistance (SAR) (Alvarez et al. 1998; Chai and Doke 1987a). Park et al. (1998) provided similar evidence for the resistance-mediating function of the oxidative burst in the distant area of potato tubers without phytoalexin accumulation. The oxidative burst induced by fungal signals may play a key role in systemic immunity by activation of defense responses throughout the plant. Pathogen-inducible SA has been implicated as an internal signal in SAR ( Ryals et al. 1996). We demonstrate here that exogenous SA induced StrbohB expression and phase II burst. Furthermore, SA potentiates a gain control for amplification of avirulence signals upstream of the oxidative burst and induces the PAL gene, the first enzyme in SA biosynthesis (Shirasu et al. 1997). We propose that SA serves as a mobile signal to potentiate AOS amplification circuits in the induction of secondary oxidative bursts in distant areas and/or tissues.
MATERIALS AND METHODS

Plant materials, HWC elicitor, chemicals, and treatment protocols.

Tubers of the potato cultivar Rishiri carrying the R1 gene were stored at 4°C until use. The tuber discs, 2.0 cm in diameter and 2 mm thick, were prepared in the dark. HWC elicitor was prepared from the mycelium of *P. infestans* (Doke and Tomiyama 1980). SA and AA were purchased from Sigma (St. Louis, MO, U.S.A.). The volume of all solutions applied to the tuber surface was 100 µl. It has been suggested that the aging process, after slicing the potato tubers, is essential for the rapid expression of host resistance (Furuichi et al. 1979). Thus, tuber discs were aged for 24 h prior to treatment with various pathogen signals. Cycloheximide, EGTA, and verapamil (all from Sigma) were pretreated for 1 h. The solutions were then wiped off before elicitor treatment. In all experiments, the treated discs were incubated at 20°C in the dark.

Isolation of gp91 phox homologs from potato.

To generate a probe in order to screen a potato cDNA library, PCR primers forward 5′-CAATAATGTGGTGAGTGAATG-3′ and reverse 5′-TTCTATICCTTTTGAAACCA-3′ were designed on the basis of expressed sequence tags from rice (accession no. X93301) and Arabidopsis (accession no. AB008111). Total RNA (1 µg) from potato tubers was used as a template for RT-PCR reactions. RT-PCR was conducted with a commercial kit (rTth DNA polymerase RT-PCR high-Plus; Toyobo, Osaka, Japan). PCR was followed by the cDNA synthesis reaction, which was performed with denaturing and annealing plus extension temperatures of 94°C for 1 min and 47°C for 1.5 min, respectively, for a total of 40 cycles. A 552-bp RT-PCR product was cloned into the TA cloning site of pGEM-T easy (Promega, Madison, WI, U.S.A.) and sequenced. This cDNA fragment was used as a probe to screen an oligo(dt)-primed Uni-ZAP XR library (Stratagene, La Jolla, CA, U.S.A.), which was constructed from poly(A)+ RNAs of potato tubers treated with HWC elicitor for 4 h. From 6 × 10⁵ plaques of the library, seven positive clones were isolated under low-stringency conditions. Positive phage plaques were excised directly in vivo into pBluescript SK(−) phagemid, according to the manufacturer’s instructions (Stratagene). Nucleotide sequences were determined for both strands with the Prism Dye Termination Cycle Sequencing Ready Reaction Kit and the Model 373A DNA sequencing system (Applied Biosystems, Foster, CA, U.S.A.).

RACE.

RACE was performed to obtain 5′ cDNA of StrbohB with the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA, U.S.A.). Gene-specific primer (5′-ACCGCGGGAGATGTTTGATGCA-3′) and the 5′ adapter primer (5′-CCATCCTAATACGACTCACTATAGGGC-3′) were used for PCR reaction. PCR was performed with denaturing and annealing plus extension temperatures of 94°C for 5 s and 68°C for 4 min, respectively, for a total of 25 cycles. The PCR products were cloned into the TA cloning site of pGEM-T easy for sequencing.

Gel blot hybridization.

Total RNA from potato tuber discs was prepared as described by Yoshioka et al. (1996). Total potato genomic DNA was extracted from leaves, according to the method described by Murray and Thompson (1980). Ten micrograms of DNA

Fig. 9. Schematic representation of a Strboh protein and elicitor-induced signal transduction in potato cells. Indicated are the orientation of the protein in the membrane with six transmembrane domains and the putative position of the two hemes, the FAD and NAD/P (r, ribose; a, adenine) binding sites and the EF hands. CDPK, calcium-dependent protein kinase; CIPK, calcium-independent protein kinase.
was digested with HindIII, EcoRI, or SpeI and then separated by electrophoresis on a 0.8% agarose gel. Ten micrograms of RNA was fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel. The separated DNA and RNA were transferred from gels to Hybond N+ nylon membranes (Amersham, Arlington Heights, IL, U.S.A.) and hybridized, according to the manufacturer’s recommendations. The cDNA probes were labeled with 32P-dCTP with a random-primer DNA labeling kit (Takara, Kusatsu, Japan).

cDNA probes.

The probes used in this study were a 3.7-kbp, nearly full-length cDNA for StrbOhA and a 2.3-kbp StrbOhB cDNA fragment containing partial sequence (this study). HMG1 cDNA contains a 5′ untranslated region of hmg1. HMG2 and HMG3 cDNAs contain 3′ untranslated regions of hmg2 and hmg3, respectively. These represent specific mRNAs for three classes of HMG from potato tuber (Choi et al. 1992). Additional probes were a 2.0-kbp potato SC cDNA fragment (Yoshioka et al. 1999), a 1.7-kbp potato SS cDNA fragment (Yoshioka et al. 1999), and a 1.2-kbp potato PAL cDNA fragment (Joos and Hahlbrock 1992). A 1.0-kbp potato CCoAOMT cDNA was isolated from a potato tuber cDNA library made in the Uni-Zap XR vector by differential hybridization. The isolated clone was sequenced to confirm identity. An 0.6-kbp potato 18S cDNA fragment was used for RNA loading control.

AOS monitoring.

The relative intensity of H2O2 generation was principally determined by counting photons from luminol-mediated chemiluminescence on the luminol-applied tissue surface. The chemiluminescence was monitored continuously by a photon image processor equipped with a sensitive CCD camera in a dark chamber at 20ºC (Park et al. 1998). Photons from the chemiluminescence from potato discs aged for 24 h were integrated for 5 min after treatment with each solution in the presence of 2.5 mM luminol in 10 mM Tris-HCl buffer (pH 7.4). The different samples were used at each time point because continuous existence of luminol on the tuber surface interferes with AOS generation.

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LITERATURE CITED


Doke, N. 1983a. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible races of *Phytophthora infestans* and to the hypopall wall components. Physiol. Plant Pathol. 23:345-357.


