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Generation of Active Oxygen in *Arabidopsis thaliana*

By

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Key words: Active oxygen, *Arabidopsis thaliana*, hydrogen peroxide, NADPH oxidase.

S u m m a r y

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Suspension cultures of *Arabidopsis thaliana* generated active oxygen species (AOS) on challenge with the bacterial protein elicitor harpin, the protein kinase activator, phorbol 12-myristate 13-acetate (PMA), or the calcium ionophore, ionomycin. The release of AOS was inhibited by the protein kinase inhibitor, K-252a and by a NADPH oxidase inhibitor diphenylene iodonium (DPI). A substantial reduction in measured AOS was also observed following the addition of a superoxide dismutase inhibitor, diethyldithiocarbamate (DDC). Immunoblotting analysis revealed the presence of proteins immunologically related to those of the mammalian phagocytic NADPH oxidase, and reconstitution experiments demonstrated that a combination of human neutrophil cytosol and *Arabidopsis* membranes could generate superoxide. Current work is using RT-PCR technology to identify clones in an *Arabidopsis* suspension culture cDNA library with potential homology to NADPH oxidase components.

I n t r o d u c t i o n

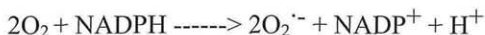
The attempted infection of plant tissues by pathogenic organisms initiates a series of complex biochemical and molecular interactions that culminates in the visual symptoms associated with disease or disease resistance. One of the earliest events related to disease resistance is the hypersensitive response (HR), characterised by a rapid collapse of cells around the site of infection. Associated with the HR is a rapid release of active oxygen species (AOS), often referred to as an oxidative burst. The released AOS include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) (SUTHERLAND 1991, MEHDY 1994). The formation of H_2O_2 during HR has been shown to be involved in induction of

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cellular protection genes (LEVINE & al. 1994), cross linking of the plant cell wall (BRISSON & al. 1994, LEVINE & al. 1994) and induction of phytoalexin synthesis.

The release of AOS is also used by mammalian phagocytes in their role as defensive cells. Here, AOS are generated by an enzyme complex referred to as NADPH oxidase, which catalyses the reaction:



This enzyme resides in the plasma membrane of mammalian white blood cells as a complex of several polypeptides (HENDERSON & CHAPPELL 1996). The addition of an electron to molecular oxygen is catalysed by a heterodimeric flavocytochrome, consisting of subunits of 22kDa and between 76 and 91kDa, these polypeptides being referred to as p22-phox and gp91-phox respectively. For full activity, at least three cytosolic polypeptides are also translocated to the membrane and in some way facilitate the catalytic turnover of the enzyme complex. These are polypeptides of 40kDa, 47kDa and 67kDa, referred to as p40-phox, p47-phox and p67-phox. Although the exact functions and interactions of these polypeptides with the cytochrome are unknown at present, they contain SH3 domains used in their interactions, and their presence in the complex appears to facilitate electron flow through the redox centres (CROSS & CURNUTTE 1995).

Recent work has suggested that plants may be generating AOS by a mechanism analogous to that used by mammalian cells. DOKE & MIURA 1995 have suggested the presence of a plasma membrane-bound NADPH oxidase in potato and immunological evidence has been reported showing the presence of polypeptides in plant tissues which are immunologically related to those of the mammalian system (TENHAKEN & al. 1995, DWYER & al. 1996). Work in our own laboratory has shown that cell suspension cultures of *Arabidopsis thaliana* also contain polypeptides which are recognised by antibodies raised to the components of mammalian NADPH oxidase (DESIKAN & al. 1996). Here we describe further characterisation of the AOS release by *Arabidopsis thaliana* and report preliminary studies on the cloning of the polypeptides which may be responsible for this activity.

Materials and Methods

Suspension cultures of *Arabidopsis thaliana*, originally obtained from DR M. MAY Oxford, UK, were subcultured every 7 days with AT3 medium (MURASHIGE & SKOOG medium supplemented with 3% (w/v) sucrose, 0.5mg/l naphthalene acetic acid, 0.05mg/l kinetin, pH5.5). Cell cultures were incubated on a rotatory shaker (110 rpm) in the dark at 24°C.

For elicitor treatment, cells were washed three times in sterile water, resuspended in AT3 medium and finally left to equilibrate on the shaker for 4 hours. Harpin, (obtained from DR S.Y. HE, University of Kentucky, USA), phorbol 12- myristate 13-acetate (PMA: in DMSO, Sigma) and ionomycin (in DMSO, Calbiochem) were used at concentrations indicated in the figure legend. Diethyldithiocarbamate (DDC: sodium salt, Sigma), diphenylene iodonium (DPI: from PROF. O. JONES, University of Bristol, UK) or K-252a (in DMSO, Calbiochem) were added to the cell

suspensions at the appropriate concentrations as indicated ten minutes before the addition of elicitors.

The release of active oxygen species was estimated by chemiluminescence (CL). Elicitor or control cell supernatant (500µl) was added to 3.35ml potassium phosphate buffer (50mM, pH 7.9), 150µl horse radish peroxidase (1mg/ml stock) and 1ml luminol (5-amino-2,3-dihydro-1,4-phthalazinedione: 3.5 mg/ml in DMSO) and the luminescence measured in a LKB scintillation counter in out-of-coincidence mode, and counts reported every 15 seconds for 1 minute, with the last two values averaged (AUH & MURPHY 1995).

Cell free reconstitution assays of superoxide generation were carried out as described by JONES & al. 1994.

Results and Discussion

Cultured cells of *Arabidopsis thaliana* produced AOS constitutively at a low level. On the addition of harpin, a heat stable protein isolated from gram negative plant pathogenic bacteria (HE & al. 1993), the release of AOS increased dramatically (Fig. 1). This release of AOS was dose dependent and continued for at least two hours, although it declined after a maximum at 1 hour. In the presence of the SOD inhibitor DDC, the rate was reduced to approximately 15% of that seen with harpin alone, suggesting that the primary product is superoxide, which is dismuted to hydrogen peroxide. Hydrogen peroxide is probably the most significant AOS produced as far as a cell signalling molecule is concerned due to the short half lives and small diffusion distances predicted for $O_2^{\cdot-}$ and $OH^{\cdot}$.

On the addition of a NADPH oxidase inhibitor, DPI, a flavin analog (CROSS & JONES 1986), the rate of AOS release was reduced by 55% (Fig.1), suggesting that the enzyme responsible for this activity may be a flavin containing protein and show similarities to the mammalian NADPH oxidase as suggested by previous work (DESIKAN & al. 1996) and by others (TENHAKEN & al. 1995, DWYER & al. 1996). Addition of the protein kinase inhibitor, K-252a reduced the rate of harpin-elicited AOS release by 90% suggesting the involvement of a protein kinase in the activation of the system, while AOS release could be stimulated by the addition of the protein kinase activator, PMA. PMA-elicited AOS could also be inhibited by the addition of DDC, DPI and K-252a. In animal cells, PMA has been shown to activate protein kinase C, and although this enzyme has not been found in plant cells, these data do suggest that plants contain a protein kinase which is sensitive to phorbol esters. Addition of ionomycin, a calcium ionophore, also stimulated the release of AOS by *Arabidopsis* cells. Interestingly, this response was also inhibited by DDC, DPI and K-252a (Fig.1). Our data suggest therefore, that generation of AOS requires calcium-induced phosphorylation of one or more proteins (Fig. 2).

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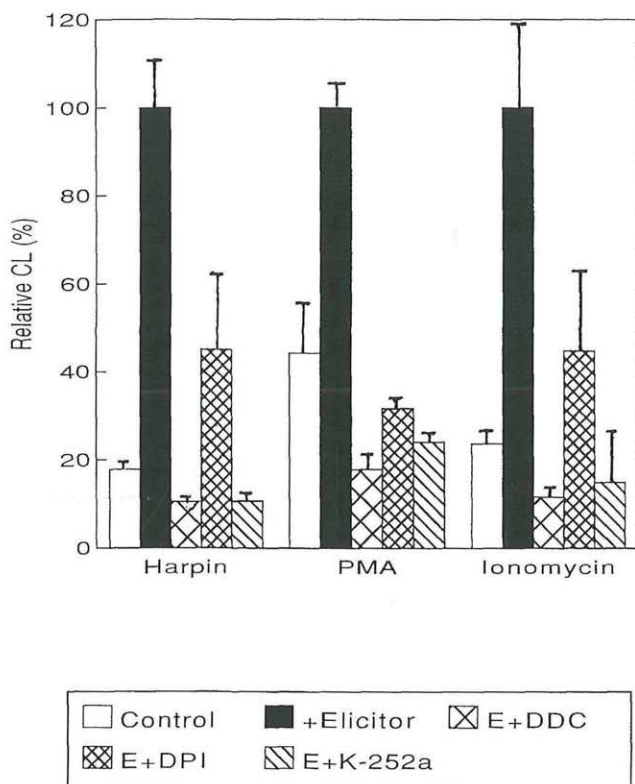


Fig.1. Effect of elicitors and inhibitors on AOS generation in *Arabidopsis*. Hydrogen peroxide release from cultured *Arabidopsis* cells was measured by chemiluminescence as described in the methods section. Final concentrations of elicitors and inhibitors used was as follows: harpin, 100ng/ml; PMA, 1 μ g/ml; ionomycin, 2 μ M; DDC, 1mM; DPI, 10 μ M; K-252a, 2 μ M. Values are represented as relative chemiluminescence (CL) with 100% taken as the maximal CL measured after the addition of elicitor. Harpin and ionomycin-induced CL was measured after 1hour, and PMA-induced CL after 30min. Values are mean $\pm$ S.E. of 3 individual experiments.

Antisera raised to the components of the mammalian NADPH oxidase were used to probe *Arabidopsis* proteins. Using both anti-peptide antisera and anti-recombinant protein antisera for the p47-phox component, bands were seen at approximately 47kDa and 95kDa, while anti-peptide p67-phox antisera showed a band at approximately 67kDa, amongst others, as published elsewhere (DESIKAN & al. 1996). Further to this, mixing of a membrane fraction derived from *Arabidopsis* cells and a soluble cytosol fraction derived from human neutrophils could reconstitute low levels of SOD inhibitable activity (0.04 μ mol/min/mg membrane

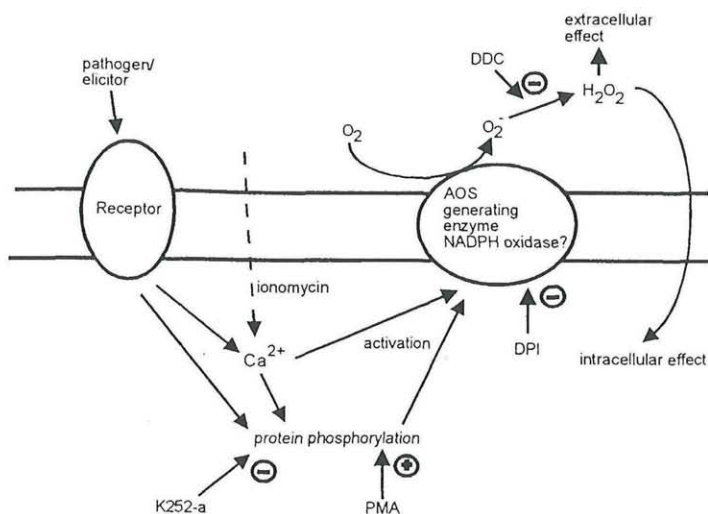


Fig. 2. A schematic representation of the possible activation pathway leading to the release of AOS by *Arabidopsis thaliana*.

protein: DESIKAN & al. 1996). Taken together, these data suggest that plant cells do indeed contain an AOS generating system which is related in some way to that seen in mammalian phagocytes. With this in mind, we have used PCR primers designed against the human NADPH oxidase sequences, to probe cDNA derived from cultured *Arabidopsis* cells. Bands were seen using primer pairs for p22-phox and p47-phox components. These products have been subsequently used to pick out clones from an *Arabidopsis* suspension culture cDNA library. These clones have been re-screened with PCR products generated from human neutrophil derived cDNA, and positive clones obtained, suggesting that *Arabidopsis* may contain an enzyme which has regions of sequence which are related to human NADPH oxidase, supporting the cross reactivity of the antibodies seen previously (TENHAKEN & al. 1995, DESIKAN & al. 1996, DWYER & al. 1996). These clones are now being sequenced.

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