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The extensin multigene family responds differentially to superoxide or hydrogen peroxide in tomato cell cultures

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Abstract Changes in extensin gene expression were examined in cultured tomato cells following treatments leading to the production of activated oxygen species. Digitonin, a steroid glycoalkaloid compound, has been shown to trigger a rapid and transient production of superoxide anion, $O_2^{-\bullet}$. 6 h after application of 50 or 100 μM of digitonin, the accumulation of four extensin transcripts (1.5, 2.6, 4.0 and 6.1 kb) was observed. Superoxide dismutase strongly inhibited the digitonin-mediated response, suggesting a key role of $O_2^{-\bullet}$ in the signalling cascade. Furthermore, cells treated with enzymatically produced $O_2^{-\bullet}$ generated by xanthine oxidase (0.015 U/ml) gave a similar extensin response and again, SOD exerted a strong inhibitory effect on the response. On the other hand, H_2O_2 (2 mM) or the enzymatic H_2O_2 generator, glucose oxidase (0.34 U/ml), elicited the accumulation of only three of the four transcripts (1.5, 2.6 and 4.0 kb), indicating that the corresponding genes could be regulated either by H_2O_2 or $O_2^{-\bullet}$ but that the gene encoding the 6.1 kb transcript was exclusively expressed in response to $O_2^{-\bullet}$. Finally, it was shown that lipid peroxidation, which was only induced when cells were exposed to H_2O_2, did not participate in the AOS-mediated gene expression for extensin. It can be concluded from these results that tomato cells are able to discriminate H_2O_2 from $O_2^{-\bullet}$ and they probably sense the latter by the specific oxidation of an extracellular component.

Key words: Active oxygen species; Extensin; Hydrogen peroxide; Lipid peroxidation; Superoxide anion; Tomato cell

1. Introduction

The oxidative burst is an ubiquitous and early defence mechanism of eukaryotes and has been widely reported in plants challenged with microbes, bacterial or fungal elicitors, plant cell wall fragments as well as through abiotic stress generated by wounding or chemical compounds. This phenomenon is characterized by the rapid and transient production of active oxygen species (AOS) such as $O_2^{\bullet-}$ and H_2O_2 [1–5]. The AOS may serve (i) to destroy the pathogen by oxidation, (ii) to provoke a local host cell death and (iii) to control the expression of various defense-related genes [6]. In connection with the defense-related genes, data indicated that genes encoding antioxidative enzymes such as glutathione S-transferase and glutathione peroxidase [7] or pathogenesis-related proteins (PR proteins) could be triggered by H_2O_2 [8]. Furthermore, it has recently been demonstrated that parsley cells responded to the superoxide anion by producing furanocoumarin phytoalexins [9]. This was the first evidence indicating that plants, like bacteria [10], are able to sense $O_2^{-\bullet}$ and respond to it by activating complex physiological processes.

Hydroxyproline-rich glycoproteins or extensins are encoded by defense-related genes belonging to a multigene family. These structural proteins are thought to play a central role in the primary cell wall organisation [11] but the expression of genes encoding these proteins can be stimulated in plants under several stress situations such as interactions with pathogens, wounding [12] and ozone treatments [13]. Considering that these conditions were shown to lead to oxygen activation or to modification of the cell redox balance [14,15], we assumed that the regulation of extensin genes might be AOS-dependent. Because evidence shows that elicited plant cells are faced with both $O_2^{-\bullet}$ and H_2O_2 during the course of the oxidative burst [5], we tried to establish whether the extensin gene activity could be differentially regulated by either of the two AOS.

In this study, we analyse the AOS produced in cultured cells of tomato, challenged with the steroid glycoalkaloid compound, digitonin, previously reported to trigger the AOS production in various plant species [16]. In addition, we provide evidence for a causal relationship between the digitonin-induced oxidative burst and extensin mRNA accumulation. The participation of AOS in the signalling cascade which led to the extensin response was further demonstrated in cells treated with the AOS producing enzymes, xanthine oxidase (XO) for $O_2^{-\bullet}$ and glucose oxidase (GO) for H_2O_2. The involvement of lipid peroxidation in the AOS-mediated signalling cascade was excluded because of the lack of correlation between the intensities of the oxidative process and the extensin response. Our results clearly show that extensin genes differentially respond to $O_2^{-\bullet}$ and H_2O_2 and that the transduction of the $O_2^{-\bullet}$ signal would require the presence of a $O_2^{-\bullet}$-specific sensor at the plant cell surface.

2. Materials and methods

2.1. Chemicals

Cytochrome *c* (CytC, bovine heart), superoxide dismutase (SOD, EC 1.15.1.1, horseradish), catalase (CAT, EC 1.11.1.6, bovine liver), XO (EC 1.1.3.22, butter milk), GO (EC 1.1.3.4, type II, *Aspergillus niger*) were obtained from Sigma. Digitonin was purchased from Boehringer Mannheim. All other chemicals were from Calbiochem. The oligogalacturonide elicitor preparation (PGA) was kindly provided by Prof. P.S. Low (Purdue University, West Lafayette, IN, USA).

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Abbreviations: AOS, active oxygen species; CytC, cytochrome *c*; GO, glucose oxidase; PGA, polygalacturonic acid; PR protein, pathogenesis-related protein; XO, xanthine oxidase

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2.2. Cell cultures and treatments

Tomato cell suspension cultures (Lycopersicon esculentum Mill., cv. sweet 100, Vilmorin, France) were grown in MS medium as described earlier [17]. Cells were subcultured every 2 weeks. To investigate extensin gene induction, 7 days old cultures were used. Digitonin was prepared in ethanol and added to the cell suspension culture at the appropriate concentration. PGA was used at a final concentration of 5 μg/ml of galacturonic acid equivalent [18]. Exogenous H_2O_2 was either generated by the addition of GO and glucose, or added directly to the culture medium whereas exogenous $O_2^{\bullet-}$ was produced by the addition of XO and xanthine. In order to properly investigate the eliciting efficiencies of $O_2^{-\bullet}$ and H_2O_2, we determined the activity of the enzymatic AOS generators XO and GO at a pH value of 5.1 and a temperature of 25°C that corresponded to the cell culture medium conditions and differed from the condition mentioned by the supplier. Indeed, the XO unit was set at pH 7.5 and 25°C whereas the GO unit was at pH 5.1 and 35°C. Consequently, we re-evaluated both enzyme activities according to the procedures described by Bergmeyer et al. [19] and our results gave the corresponding equivalences: 1 U of XO at pH 7.5 and 25°C was equivalent to 0.44 U at pH 5.1 and 25°C, 1 U of GO at 35°C and pH 5.1 corresponded to 0.67 U at 25°C and pH 5.1. In the following work, enzyme concentrations were given as the number of corrected units per ml of cell suspension and tomato cultured cells were exposed to XO (0.015 U/ml) plus xanthine (0.1 mM) or to GO (0.04–0.34 U/ml) plus glucose (6 mM). Controls were performed by treating cells with xanthine or glucose in the absence of the corresponding enzymes. After treatment, tomato cells were harvested by filtering through a coarse glass filter, frozen in liquid N_2 and stored at −80°C until used.

2.3. Measurement of the superoxide anion production

The $O_2^{-\bullet}$ generating activity was assayed on 40 mg of tomato cells by measuring spectrophotometrically the reduction of exogenously supplied CytC according to Martinez et al. [20]. Each experiment was performed in triplicate and data represented the mean ± S.D.

2.4. RNA isolation and RNA gel blot hybridisation analysis

Total RNA was isolated from samples (3 g) of frozen cells as described by Haffner et al. [21]. RNA (10 μg) was then denaturated and separated by electrophoresis on agarose gel according to Maniatis et al. [22]. The gel was then blotted onto a nylon membrane (Hybond N, Amersham) and the membranes were pre-hybridized for 2 h in 50% formamide, 0.5% SDS, 1×Denhardt's solution, 2×SSC and 50 μg/ml denatured salmon sperm (carrier) DNA. The DNA clone encoding carrot extensin (pDC5A1) [23] was isolated from its plasmid vector and labelled by nick-translation with [α-^{32}P]dCTP. Hybridisations were carried out overnight under the same conditions with approximately 15 ng of DNA probe per ml of hybridisation solution. Filters were then washed twice with 2×SSC, 0.5% SDS at 58°C and once with 1×SSC, 0.5% SDS at 58°C before autoradiography.

2.5. Lipid peroxidation measurements

Cultured tomato cells were challenged as described above, harvested at various times and pentane was measured by gas chromatography as previously described [24]. Data represented the mean ± S.D. of two experiments of five replicates each.

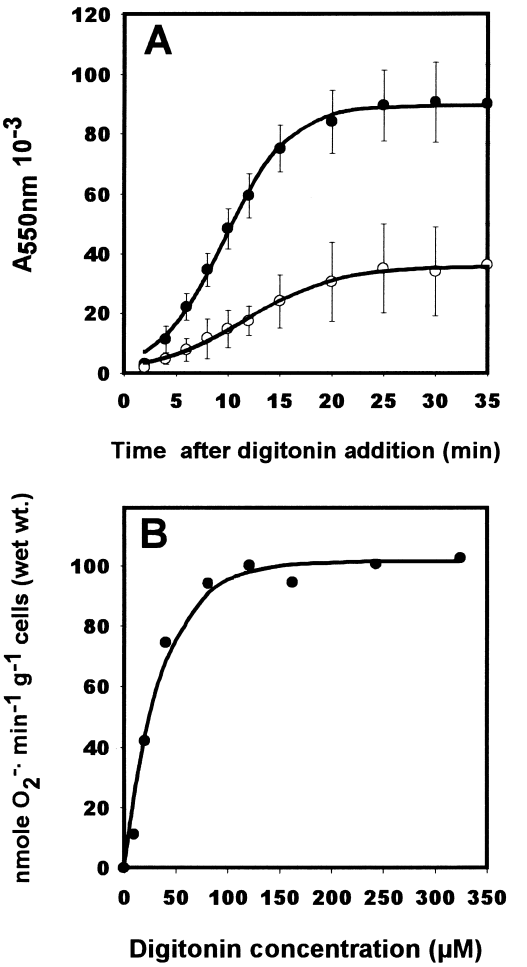

Fig. 1. Digitonin-induced oxidative burst on tomato cells. (A) The CytC reducing activity of cultured cells after a treatment with: ●, 80 μM digitonin; ○, 80 μM digitonin with SOD (100 U/ml). Results are expressed as the difference of absorbance at 550 nm between a sample of challenged cells and a sample of untreated cells. Each experiment was performed in triplicate and data represented the mean ± S.D. (B) Dose response for the production of $O_2^{-\bullet}$ by digitonin. The $O_2^{-\bullet}$ production rates were determined by measuring the initial slopes of the curves (increase in $A_{550\,nm}$ during the first 8–10 min) taking into account the inhibition scores with SOD and the absorbance constant of ferricytochrome *c* (0.89×10^4/M/cm) and ferrocytochrome *c* (2.99×10^4/M/cm).

3. Results

3.1. Extracellular superoxide production by digitonin-treated tomato cells

As previously described by Doke [25] on potato leaves or protoplasts and by Martinez et al. [20] on cotton cotyledons, we showed here that digitonin rapidly triggered an extracellular $O_2^{-\bullet}$ production on tomato cells (Fig. 1). Indeed, about 2 min after treatment of the cells with 80 μM of digitonin, a CytC-reducing activity was observed and lasted for a further 15 min. Superoxide anion was the main CytC-reducing component since addition of an optimum concentration of SOD (100 U/ml) gave 75% inhibition of this process (Fig. 1A). The intensity of the $O_2^{-\bullet}$ production was dose-dependent, the optimum burst (100 nmol/min/g of cells) being reached for a digitonin concentration of about 100 μM.

3.2. Effects of the superoxide anion and hydrogen peroxide on the expression of tomato extensin genes and lipid peroxidation

As shown in Fig. 2A, four transcripts (6.1, 4.0, 2.6 and 1.5 kb) accumulated when cells were treated with digitonin at 50 or 100 μM. A similar profile was observed when cells were elicited with 5 μg/ml of the biotic elicitor PGA, known to activate the AOS production on soybean cells [18]. Similarly, treatment of cells with the enzymatic $O_2^{-\bullet}$ generator, XO (0.015 U/ml), resulted in the accumulation of the four extensin transcripts (Fig. 2B). In these conditions, the 4.0 and 1.5 kb transcripts were the most abundant, representing about 80%

of the whole extensin response. Both digitonin and XO responses were strongly inhibited when SOD (220 U/ml) was added to the culture medium (Fig. 2B). On the other hand, when tomato cells were treated either with 2 mM of H_2O_2 or with GO (0.34 U/ml), three of the four extensin transcripts accumulated, the 6.1 kb mRNA being never detected (Fig. 2C). The peroxide-mediated response mainly corresponded to the elicitation of the 1.5 kb transcript which represented about 80% of the whole extensin response. Treatment with lower GO concentrations (0.04 or 0.08 U/ml) gave less intense responses where at 0.04 U/ml only the 1.5 kb transcript accumulated and at 0.08 U/ml two of them (1.5 and 2.6 kb) were detected. When catalase (240 U/ml) was added to the GO-treated cells (0.34 U/ml), the accumulation of the three transcripts was about 30% inhibited (Fig. 2C).

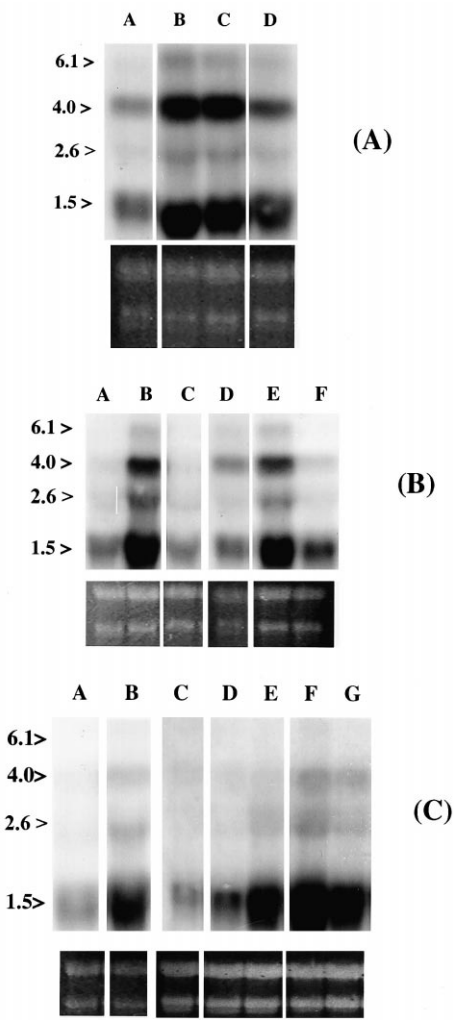

Fig. 2. (A) The effect of digitonin and PGA elicitor on extensin mRNA accumulation. Lane A, control; lane B, 50 μM digitonin; lane C, 100 μM digitonin; lane D, PGA (5 μg/ml). (B) Northern blotting analysis of extensin mRNA in tomato cells challenged with digitonin and the enzymatic $O_2^{-\bullet}$ producer XO. Lane A, xanthine (0.1 mM); lane B, XO (0.015 U/ml)+xanthine (0.1 mM); lane C, XO (0.015 U/ml)+xanthine (0.1 mM)+SOD (220 U/ml); lane D, control; lane E, 50 μM digitonin; lane F, 50 μM digitonin+SOD (220 U/ml). (C) Northern blotting analysis of extensin mRNA in tomato cells challenged with hydrogen peroxide and the enzymatic H_2O_2 producer glucose oxidase. Lane A, control; lane B, 2 mM H_2O_2; lane C, glucose (6 mM); lane D, GO (0.04 U/ml)+glucose (6 mM); lane E, GO (0.08 U/ml)+glucose (6 mM); lane F, GO (0.34 U/ml)+glucose (6 mM); lane G, GO (0.34 U/ml)+glucose (6 mM)+CAT (240 U/ml). Total RNA from 1 week old cultures was isolated after 6 h of treatment and a 10 μg sample was subjected to a RNA blot analysis. The specific mRNAs were hybridized with pDC5A1, a carrot extensin probe labelled with [α-^{32}P]dCTP by nick-translation. The bottom panels with ribosomal RNAs show the equalisation of RNA samples used for analysis. Markers indicate the size of the extensin transcripts.

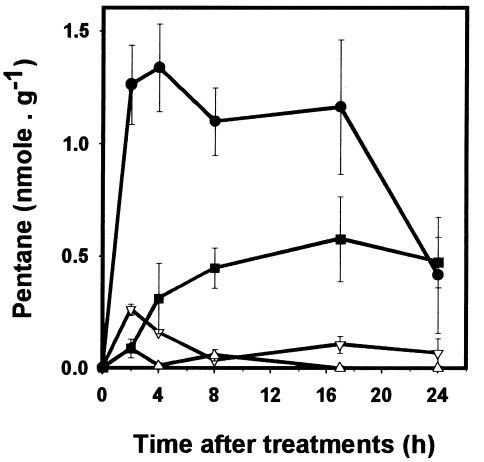

Fig. 3. Kinetics of lipid peroxidation measured as the pentane production by cultured tomato cells challenged with: (●), 2 mM H_2O_2; (■), GO (0.34 U/ml)+glucose (6 mM); (△), XO (0.015 U/ml)+xanthine (0.1 mM) or (▽), 50 μM digitonin. Values are means ± S.D. for two experiments with five replicates each.

The time course of lipid peroxidation was estimated in AOS-treated tomato cells by quantification of the pentane production. As shown in Fig. 3, the action of $O_2^{-\bullet}$ (induced by 50 μM of digitonin or by 0.015 U/ml of XO, respectively) resulted in a very low or insignificant lipid peroxidation, whereas 2 mM of H_2O_2 or 0.34 U/ml of GO both induced it. However, strong differences in the intensity and kinetics were noticed between the last two treatments. Indeed, whereas the direct application of H_2O_2 rapidly resulted in the development of an intense peroxidation process (1.25 nmol of pentane/g of cells) which lasted about 15 h, treatment of the cells with GO induced a slower response which reached a maximum between 4 and 8 h, was two-fold less intense and also lasted more than 10 h.

4. Discussion

We report in the present work that digitonin is able to trigger an extracellular $O_2^{-\bullet}$ production in tomato cells. The production was shown to be dose-dependent, beginning a few minutes after application of the compound and lasting for about 15–20 min. Nevertheless, the $O_2^{-\bullet}$ burst intensity determined in the standard conditions (i.e. in a phosphate buffer, pH 7.8, which did not contain any trace of metals) did not allow us to easily predict the $O_2^{-\bullet}$ production when digitonin was directly added to the culture medium. Indeed, the pH value (pH 5.1) of the culture medium and the presence of iron (Fe^{3+}) might lead to either a proton-catalysed dismutation of the superoxide anion into H_2O_2 or to its re-oxidation

into oxygen by ferric ions. In addition, the pH might modulate the activity of the enzymes involved in the $O_2^{-\bullet}$ production. Similarly, when XO+xanthine was used to artificially generate $O_2^{-\bullet}$ in the culture medium, it was difficult to estimate parameters such as the anion steady state concentrations and the duration of enzymatic activity. However, we could overcome these difficulties by considering the effect of a SOD application on the both treatments where $O_2^{-\bullet}$ was supposed to be active.

Our present results indicate that digitonin- or XO-treated tomato cells were able to trigger the accumulation of a set of four extensin transcripts. A similar response was induced by the biotic elicitor PGA. Since SOD application resulted in a strong inhibition of the extensin response, induced by either digitonin or XO, we concluded that $O_2^{-\bullet}$ was involved as a signal. Furthermore, because $O_2^{-\bullet}$ (which is not cell permeable) was produced beyond the plasma membrane in both treatments and SOD was only supposed to exert its inhibitory activity in the extracellular space, we can infer for the presence of an extracellular $O_2^{-\bullet}$-specific sensor in tomato cells. Even though such an element has not been identified in plants so far, redox-sensitive transcription factors are present in bacteria, particularly the Fe-SoxR which contains a redox active iron-sulfur cluster susceptible to oxidation by $O_2^{-\bullet}$ [10]. Such a protein located in the apoplasm or at the external surface of the plasma membrane of tomato cells could transduce the $O_2^{-\bullet}$ signal. Hydrogen peroxide was also shown to induce the accumulation of extensin transcripts and catalase partially inhibited the effect of GO. However, neither direct application of H_2O_2 nor exposure of cells to GO resulted in the accumulation of the 6.1 kb transcript, even when higher H_2O_2 concentrations, up to 10 mM, were used (data not shown). This suggested that the corresponding gene was only expressed in response to $O_2^{-\bullet}$. On the other hand, genes encoding the three other transcripts could be regulated by either $O_2^{-\bullet}$ or H_2O_2. However, our results indicated that $O_2^{-\bullet}$ was more efficient than H_2O_2 in triggering the response of extensin 4.0 kb. Indeed, in order to induce a strong extensin transcript accumulation it was necessary to generate $O_2^{-\bullet}$ at a rate of 1.2 μmol/min, equivalent to a total amount of 1.2 U of XO added to 80 ml of culture medium containing 7 g of cells. Similarly, the digitonin-induced extensin response was optimal around 50 μM that corresponded to a $O_2^{-\bullet}$ production rate of 0.5 μmol/min, assuming that the pH value of the medium (5.1) did not markedly alter the activities of enzymes involved in this production. On the other hand, 3.2 U of GO (corresponding to a H_2O_2 production rate of 3.2 μmol/min) resulted in a significant but very low accumulation of only the 1.5 kb transcript. The three transcripts (1.5, 2.6 and 4.0 kb) were only detectable when at least 27 U of GO were used (i.e from a H_2O_2 rate production of 27 μmol/min) or when 2 mM H_2O_2 was directly added (i.e. a peroxide total amount of 160 μmol). According to this, the SOD inhibition of XO or digitonin effects could be explained by assuming that the resulting H_2O_2 production was too low to trigger any extensin response. Finally, our results show that H_2O_2 is more efficient for induction of lipid peroxidation in tomato cells. Particularly, a massive addition of 2 mM H_2O_2 triggered an intense lipid oxidation whereas GO (0.34 U/ml) resulted in a slower increase in the cell fatty acid hydroperoxide levels that did not exceed half of those observed when cells were directly treated with H_2O_2. On the other hand, although 50 μM digitonin triggered a very weak lipid peroxidation visible around 2–4 h, XO (0.015 U/ml) was not able to induce the oxidative process. Because the extensin response intensity was not correlated with the lipid peroxidation intensity, we concluded that the latter phenomenon could not be considered as a cellular signal leading to the extensin gene activation.

In conclusion, we demonstrate that some genes, notably those encoding extensins, are elicited in cells exposed to low levels of $O_2^{-\bullet}$. This response observed in the absence of lipid peroxidation suggests that cell death, often associated with this oxidative process, is not necessary for the activation of extensin genes. Although we still do not know whether $O_2^{-\bullet}$ is produced at sufficient levels during the oxidative burst that takes place in incompatible plant microbe interactions, we have observed that the extensin response triggered on tomato cells by the biotic elicitor PGA was similar to the one observed with digitonin or XO. The signalling elements required for these responses are now under investigation and we will focus particularly on the characterisation of a putative extracellular component that could be specifically oxidized by $O_2^{-\bullet}$ and would serve to transduce the signal emanating from this AOS.

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