A Pharmacological Approach to Test the Diffusible Signal Activity of Reactive Oxygen Intermediates in Elicitor-Treated Tobacco Leaves

Laurent Costet 1, Stephan Dorey 2, Bernard Fritig and Serge Kauffmann 3

Institut de Biologie Moléculaire des Plantes du C.N.R.S., Université Louis Pasteur, 12 rue du Général Zimmer, F-67084 Strasbourg, France

1 Present address: CIRAD-CA, Station de la Bretagne, Laboratoire de Phytopathologie, BP 20, F-97408 St Denis, France.
2 Present address: The Sainsbury Laboratory, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, U.K.
3 Corresponding author: E-mail, serge.kauffmann@ibmp-ulp.u-strasbg.fr; Fax, +33-388-61-4442.

The capacity of H₂O₂, the most stable of the reactive oxygen species (ROI), to diffuse freely across biological membranes and to signal gene expression suggests that H₂O₂ could function as a short-lived second messenger diffusing from cell to cell. We tested this hypothesis in tobacco plants treated with a glycoprotein elicitor. Applied at 50 nM, it induces H₂O₂ accumulation and the hypersensitive response restricted to the infiltrated zone 1 tissue. Stimulation of a set of defense responses also occurs in the surrounding zone 2 tissue without diffusion of the elicitor. ROI levels in zone 1 were modulated using N-acetyl-l-cysteine (NAC) as a ROI scavenger and Rose Bengal (RB) as a ROI generator. We found that ROI appeared to act as a signalling intermediate in pathways leading to salicylic acid accumulation, to PR1, PR5 and 3-hydroxy-3-methylglutarylCoA reductase expression in glycoprotein-treated zone 1 tissues. Compared to the treatment with the elicitor alone, coinfiltration of the glycoprotein and NAC increased the surface of zone 2 showing PR1 and O-methyltransferase expression. Application of RB had the opposite effect. The data suggest that, in our system, ROI did not act as a cell-to-cell diffusible signal to activate PR protein and O-methyltransferase expression in zone 2.

Key words: Cell-to-cell signalling — Hydrogen peroxide — Nicotiana tabacum — Tobacco.

Abbreviations: DPI, diphenylene iodonium; HR, hypersensitive response; NAC, N-acetyl-l-cysteine; RB, Rose Bengal; ROI, reactive oxygen intermediates; SA, salicylic acid.

Introduction

Reactive oxygen intermediates (ROI) are thought to display a pleiotropic activity in plants developing a hypersensitive response (HR) to pathogen infection. They can act as direct antimicrobial components. For instance, hydrogen peroxide (H₂O₂) inhibits spore germination of number of fungal pathogens (Peng and Kuc 1992). They function in the rapid cross-linking of cell wall structural proteins (Bradley et al. 1992), making the cell wall more refractory to digestion by microbial enzymes.

ROI have been involved as key mediators of defense gene activation. Superoxide anions (O₂⁻) were shown to drive mRNA accumulation of PR1, a typical plant defense protein with antifungal activity (Fritig et al. 1998), in an Arabidopsis cell death (lsd1) mutant (Jabs et al. 1996) and in parsley cells (Jabs et al. 1997). In tobacco plants, H₂O₂ has been implicated in PR1 gene expression (Chamnongpol et al. 1998, Chen et al. 1993, Takahashi et al. 1997) and salicylic acid (SA) biosynthesis (Leon et al. 1995). Implication of ROI in the induction of the HR cell death was reported in Arabidopsis (Jabs et al. 1996), soybean (Levine et al. 1994), and tobacco (Chamnongpol et al. 1998, Mittler et al. 1999, Mittler et al. 1996, Schraudner et al. 1998, Takahashi et al. 1997).

Production of ROI through an oxidative burst is a hallmark of incompatible plant-pathogen interactions (Baker and Orlandi 1995, Lamb and Dixon 1997). It was first reported by Doke (Doke 1983) who showed a generation of O₂⁻ at the onset of the HR of potato to Phytophthora infestans. Since then, the generation of O₂⁻ and/or H₂O₂ was shown to occur in different plant-pathogen systems, as well as in plants and plant cell cultures treated with pathogen-derived components (Baker and Orlandi 1995, Lamb and Dixon 1997). The oxidative burst is thought to result from a combination of ROI overproduction and suppression of ROI scavenging systems (Dorey et al. 1998, Mittler et al. 1998, Mittler et al. 1999). In plant cell suspensions, the oxidative burst occurs within the first min after pathogen or elicitor treatments. Diphenylene iodonium (DPI) was shown to block this oxidative burst in different cell culture systems indicating that most of the ROI production seems to implicate a membrane bound NADP(H) oxidase (Desikan et al. 1996, Keller et al. 1998, Levine et al. 1994, Xing et al. 1997). The NADP(H) oxidase generates O₂⁻, which is readily dismutated into H₂O₂ either spontaneously or by superoxide dismutase (Sutherland 1991). Other sources may also account for ROI production. They involve peroxidases (Bestwick et al. 1997) and amine oxidase-type enzyme(s) (Allan and Fluhr 1997). The oxidative burst was described as a two-phase process in tobacco (Baker and Orlandi 1995, Dorey et al. 1999, Schraudner et al. 1998), soybean (Levine et al. 1994) and parsley (Jabs et al. 1997) cell cultures inoculated with avirulent pathogens or treated with elicitors.

The speed of the oxidative burst associated with the capacity of H₂O₂ to diffuse freely and rapidly across biological...
membranes and to signal gene expression suggest that \( H_2O_2 \) could function as a short-lived second messenger diffusing from cell to cell. This question is poorly documented, however. A first clue came from the work by Levine et al. (1994). They used two populations of cultured soybean cells separated by a pair of dialysis membranes. One population was inoculated with avirulent bacteria to induce \( H_2O_2 \) production. They observed that cells of the second population accumulated transcripts encoding glutathione-S-transferase and glutathione peroxidase, which are antioxidant enzymes, and that gene induction was suppressed when catalase enzyme was included in the medium between the dialysis membrane. Whether \( H_2O_2 \) could act as a cell-to-cell diffusible signal in planta awaits further investigations, however.

Here, we tested this hypothesis in planta using a pharmacological approach. We have previously shown that infiltration into tobacco leaves of a fungal glycoprotein (50 nM) triggers the HR (Baillieul et al. 1995) and typical defense responses in tissues located in the vicinity of the HR lesion (Dorey et al. 1997). The elicitor-infiltrated tissue is called zone 1 and underlies tissues located in the vicinity of the HR (Baillieul et al. 1995) and typical defense responses in the HR (Enyedi 1999, Dorey et al. 1998). Because a strong rise in \( H_2O_2 \) occurs in the elicitor-treated zone 1 tissues (Dorey et al. 1997), when we showed that the elicitor remained strictly localized to the infiltrated zone 1 tissue (Dorey et al. 1997). Thus, a plant signal(s), and not the elicitor, is released from the cells undergoing the HR and diffuses to induce the defense responses.

The nature of this diffusible signal(s) is currently unknown. In previous reports, we have shown that at least another molecule, different from SA, diffuses from the HR zone 1 to zone 2 to induce various defense responses (Costet et al. 1999, Dorey et al. 1997). Because a strong rise in \( H_2O_2 \) levels occurred in the elicitor-treated zone 1 tissues (Dorey et al. 1998), \( H_2O_2 \) could be the (or one of the) diffusible signal(s) inducing the (or part of) defense responses in zone 2 tissues, thus functioning as a cell-to-cell diffusible signal. We have previously shown that the co-infiltration of the elicitor with DPI did not prevent catalase gene induction in zone 2 strongly supporting that \( H_2O_2 \) is not the diffusible signal inducing catalase expression in zone 2 (Dorey et al. 1998). In this report, we analysed PR gene expression in zone 2 upon treatments aimed to modulate the oxidative burst in zone 1. The rationale was that a treatment designed to decrease the levels of ROI produced by HR cells was expected to prevent PR gene expression in zone 2. Conversely, a treatment leading to ROI overproduction would enhance PR gene expression in zone 2.

### Results

In order to modulate the levels of ROI in our in planta system we used two molecules, \( N \)-acetyl-L-cysteine (NAC) and Rose Bengal (RB). NAC has proven to be a powerful antioxidant able to scavenger in vitro different ROI (Aruoma et al. 1989). It is widely used in animal (Mayer and Noble 1994, Schreck et al. 1991) and plants systems (Conrath et al. 1995, Green and Fluhr 1995) to study ROI signalling function. RB is a water-soluble xanthene dye (4,5,6,7-tetracloro-2',4',5',7'-tetraiodofluorescein) that forms singlet oxygen (\( ^1O_2 \)) upon irradiation with light (Knox and Dodge 1984). In plants, \( ^1O_2 \) is rapidly and spontaneously converted into other ROI (Foyer et al. 1994). In tobacco, UVB-induced PR1 expression was suppressed by NAC (Green and Fluhr 1995) and PR1 accumulation was induced by RB (Enyedi 1999, Green and Fluhr 1995). As a prerequisite of the study, we investigated whether ROI, as reported in other systems, were acting as signalling intermediates in the expression of different defense responses in our in planta system. We have shown previously that the

![Fig. 1 Modulation of PR1-GUS expression in tobacco leaves treated with the glycoprotein, or the glycoprotein and NAC, or RB. The glycoprotein (GP) was infiltrated into attached Xanthi nc PR1-GUS transgenic tobacco leaves at the indicated concentration, or in the presence of 10 mM NAC (+NAC). RB was infiltrated at 10 μM or 0.5 μM (as indicated). As a control, 10 μM light-inactivated RB (Rbi) was also infiltrated. The upper row shows the leaf symptoms observed 48 h after treatments. The two rows beneath show leaf disks punched out from the treated leaves and stained for GUS activity. Infiltration of 10 mM NAC alone did not induce either necrotic symptoms or GUS expression (not shown).](image-url)
and SA accumulated after stimulation of HMGR gene and HMGR is an early induced gene, PR5 is a late-induced gene, that, in zone 2 of leaves treated with a HR dose of elicitor, and PR5 mRNA and accumulation of SA. It should be noted with 0.25 nM glycoprotein induced accumulation of HMGR injected to the same treatments (Fig. 2). As expected, treatments expression and SA accumulation in Samsun NN plants sub-
resulted in reduced GUS staining compared to the treatment with the elicitor alone. Both treatments, with or without NAC, were performed on the same leaf and repeated several times with similar results. We also analysed HMGR and PR5 gene expression and SA accumulation in Samsun NN plants subjected to the same treatments (Fig. 2). As expected, treatments with 0.25 nM glycoprotein induced accumulation of HMGR and PR5 mRNA and accumulation of SA. It should be noted that, in zone 2 of leaves treated with a HR dose of elicitor, HMGR is an early induced gene, PR5 is a late-induced gene, and SA accumulated after stimulation of HMGR gene and before induction of PR genes (Dorey et al. 1997). The non-HR treatment, thus, maintained the differential kinetics of induc-
Effect of NAC on HMGR and PR5 gene expression and on salicylic acid accumulation in tobacco leaves infiltrated with a non-HR dose of elicitor. Samsun NN tobacco leaves were infiltrated with 0.25 nM glycoprotein or 10 mM NAC or both. (A) RNA analysis. The blot was hybridized with HMGR or PR5 or 25S rRNA probes. Amounts of radioactivity were quantified and normalized. Black bars: RNA from tissue infiltrated with the glycoprotein. White bars: RNA from tissue co-infiltrated with the glycoprotein and NAC. (B) Salicylic acid analysis. Salicylic acid was analysed from tissue infiltrated with the glycoprotein (closed squares), from tissue co-infiltrated with the glycoprotein and NAC (closed circles), and from tissue infiltrated with NAC alone (open diamonds).

Fig. 2  Effect of NAC on HMGR and PR5 gene expression and on salicylic acid accumulation in tobacco leaves infiltrated with a non-HR dose of elicitor. (A) RNA blot analysis. The blot was hybridized with HMGR or PR5 or 25S rRNA probes. Amounts of radioactivity were quantified and normalized. Black bars: RNA from tissue infiltrated with the glycoprotein. White bars: RNA from tissue co-infiltrated with the glycoprotein and NAC. (B) Salicylic acid analysis. Salicylic acid was analysed from tissue infiltrated with the glycoprotein (closed squares), from tissue co-infiltrated with the glycoprotein and NAC (closed circles), and from tissue infiltrated with NAC alone (open diamonds).
have been repeated at least four times, on different batches of plants at different periods of the year, with similar results. We observed variations in the intensity of the biological phenomenon we describe here. However, we repeatedly observed the stronger PR1 and OMT expression in zone 2 when the elicitor was co-infiltrated with NAC or ascorbate.

NAC’s activity in our system led us to investigate NAC’s target. NAC is often used as an inhibitor of apoptosis in animal cells (Mayer and Noble 1994). In our system, co-infiltration of NAC and a HR-dose of the glycoprotein did not suppress the HR. Using a leaf disk assay, we found that the NAC treatment delayed the elicitor-induced cell death (Fig. 4A). The difference in the kinetics was significant and reproduced over several experiments. NAC’s ability to scavenge ROI was often put forward to explain its anti-cell death activity. Indeed, NAC was shown, in vitro, to scavenge efficiently the hydroxyl radical, to react with $\text{H}_2\text{O}_2$ but not with $\text{O}_2^–•$ (Aruoma et al. 1989). Therefore, we examined whether NAC was able to decrease or suppress the $\text{H}_2\text{O}_2$ burst known to occur in zone 1 of leaves treated with a HR dose of elicitor (Dorey et al. 1998). The experiment was repeated several times, and we never measured a significant decrease in $\text{H}_2\text{O}_2$ levels in the HR-zone 1. The results of a typical experiment are shown in Fig. 4B. In this experiment, $\text{H}_2\text{O}_2$ levels in tissue infiltrated with elicitor+NAC were slightly higher than those measured in tissues treated with the elicitor alone. In other experiments, there was no such difference.

In animal cells, NAC is easily deacetylated to form cysteine which efficiently supports glutathione biosynthesis (Meister and Andersson 1983). Accordingly, there are reports indicating that the intracellular glutathione levels increased after treatment with NAC (Bush et al. 1999). As shown in Fig. 4C (closed circles and open diamonds), infiltration of NAC alone or together with a HR dose of elicitor resulted in a rapid increase in glutathione levels which correlated well with the sharp drop in non-protein thiol levels which included the infiltrated NAC (Fig. 4D, closed circles and open diamonds). Comparatively, infiltration of the elicitor alone had only a slight effect (Fig. 4D, closed squares). There was no increase in non-protein thiols in zone 2 tissues surrounding those infiltrated with NAC alone or together with the elicitor (data not shown),
indicating that NAC remained strictly localized to its application site. In plants, glutathione has been described as a major antioxidant system, scavenging free radicals (Alsher 1989). Thus, in our system, NAC's effect was to increase rapidly the cellular antioxidant capacity either directly or indirectly via glutathione.

If the decrease in ROI levels due to NAC activity was the cause of the increased PR1-GUS expression in zone 2, one may expect that an excess of ROI would have the opposite effect. Similarly to the 50 nM glycoprotein treatment, infiltration of 10 μM RB into PR1-GUS transgenic tobacco leaves induced HR symptoms which were strictly limited to the infiltration site shown by the black pen line in Fig. 1, lane RB 10 μM. GUS staining in zone 2 surrounding the RB-treated tissue was reduced in terms of surface of tissue involved in the staining, being almost undetectable, compared to the glycoprotein treatment (Fig. 1, compare lane GP, 50 nM to lane RB, 10 μM). This result was reproduced over several experiments. Infiltration of light-inactivated RB caused neither HR symptoms nor GUS expression (Fig. 1, lane Rbi), indicating that RB effects were through ROI generation.

We also applied 10 μM RB and 10 μM RB+10 mM NAC to Samsun NN tobacco plants, collected different zone 2 tissues as shown in Fig. 3A, and measured PR1 and OMT expression (Fig. 3B, C). PR1 and OMT expression were slightly higher in zone 2a after RB+NAC treatment compared to treatment with RB alone. In distal tissues, as zones 2b, 2c, and 2d, we could not detect PR1 nor measure significant increase in OMT activity. Furthermore, we observed that both PR1 and OMT expression was lower in zone 2a after RB+NAC treatment compared to the elicitor+NAC treatment. The results obtained with RB+/−NAC are thus in good agreement with those obtained with elicitor+/−NAC and are in favor that ROI did not act as a diffusible signal stimulating PR1 and OMT expression in zone 2.

Discussion

In previous reports we have shown that inhibiting SA synthesis in the elicitor-induced HR-zone 1 did not prevent neither SA (Dorey et al. 1997) nor PR protein (Cordeliers and Kauffmann, manuscript in preparation) accumulation in zone 2. It strongly suggested that SA was not diffusing from the HR zone to the LAR zone. Furthermore, NahG tobacco plants, which constitutively degrade SA, still express a subset of biochemical defense responses in zone 2 (Costet et al. 1999), indicating that at least another molecule different from SA was acting as a short distance diffusing signal. Thus, in this report we have investigated whether ROI could act as such a signal in our system.

ROI are thought to display a pleiotropic activity in plants reacting actively to pathogen infection. They have been involved as direct antimicrobial agents, as co-substrate for the oxidative crosslinking of proteins into the cell wall, as mediators of the HR cell death activation and of PR gene expression,
used a non-HR dose of elicitor or of RB to check this important prerequisite because expression of defense responses in tissues treated with a HR dose was rapidly compromised due to cell death (Dorey et al. 1997). The results with SA are in good agreement with a previous report (Dorey et al. 1999) in which we have shown that DPI treatment suppressed SA accumulation in elicitor-treated tobacco cell suspensions. Second, treatment with 0.5 μM RB triggered PR1-GUS expression limited to the infiltration site. These results are, thus, in good agreement with previous evidence that ROI are mediators of PR protein expression (Chamnongpol et al. 1998, Chen et al. 1993) and SA accumulation in tobacco plants (Chamnongpol et al. 1998, Leon et al. 1995) and cell suspensions (Dorey et al. 1999).

Our results lead to different arguments that are not in favor of ROI acting as a cell-to-cell diffusible signal upregulating PR gene and OMT expression in zone 2 tissues surrounding a HR lesion. The first argument implies that ROI issuing from either an early or late oxidative burst are not acting as a signal diffusing in zone 2 to stimulate the PR1 and OMT expression. Concerning the late elicitor-induced oxidative burst, we have shown that the increase in H$_2$O$_2$ levels in our system was not affected by the NAC treatment. The increased surface of tissue showing the GUS staining upon co-infiltration of the elicitor and NAC suggested that higher levels of signal(s) were produced/released and/or the signal(s) remained active over a longer period of time. A consequence of both processes would be a higher rate of diffusion of the signal. Earlier work has shown that no H$_2$O$_2$ could be measured in zone 2 tissues, indicating a lack of diffusion of measurable levels of H$_2$O$_2$ (Dorey et al. 1998). The NAC treatments and the measure of H$_2$O$_2$ levels suggest that this specific H$_2$O$_2$ was not involved in PR and OMT expression in zone 2 tissues. The kinetics of liberation of the diffusible signal(s) out of the HR cells also argues against a direct involvement of this specific H$_2$O$_2$ in enhanced PR and OMT expression. Analyzing PR1 expression and OMT activity, we observed that the signal(s) was liberated between 3 and 6 h after elicitor application (unpublished results) which is well before the measured augmentation of H$_2$O$_2$ levels. In our system, the increase in H$_2$O$_2$ occurred rather late, several hours after elicitor application (Fig. 3 and Costet et al. 1999), questioning the occurrence of an early oxidative burst which is known to occur within min in elicitor-treated cell suspensions (Dorey et al. 1999, Levine et al. 1994, Pugin et al. 1997). Unfortunately, assays developed to quantify H$_2$O$_2$ in tissues are much less easy to run than those used for cell suspensions. The test used to measure H$_2$O$_2$ in tissues involved an extraction procedure prior to the quantification. An extraction procedure is not necessary when cell suspensions are the biological material. This extraction procedure is thought to decrease the sensitivity of the test. Although we did not measure an early increase in H$_2$O$_2$ levels, such an oxidative burst occurred most probably in our system since an intracellular source of ROI was postulated in elicitin-treated tobacco plants (Allan and Fluhr 1997). This intracellular oxidative burst implied H$_2$O$_2$ and was induced within min after elicitin application. Interestingly, it was completely abolished by NAC. Our glycoprotein shares common epitopes with the elicitins (Baillieul et al. 1996) and its amino acid sequence displays an elicitin domain with the six conserved cysteine residues. Similarly to the glycoprotein, elicitins induce the HR and defense responses in zone 2 surrounding the HR lesion when infiltrated into tobacco leaves (Baillieul et al. 1996). Our current interpretation of the data thus involves a two-phase oxidative burst in the glycoprotein-treated leaf tissues and NAC would exert its antioxidant activity on the first phase. It is not clear whether this two-phase kinetics corresponds to that reported in cell suspensions (Lamb and Dixon 1997). The first phase would result from the specific perception of the glycoprotein by the cells, as specific high-affinity binding sites for elicitins were characterized in tobacco leaf plasma membranes (Wendehenne et al. 1995). The metabolic origin of the second and massive H$_2$O$_2$ burst is not certain. We observed that light is an important factor for the HR to develop in glycoprotein-treated tissue. Plants submitted to different stresses show increased ROI levels due to photo-oxidative processes, and photorespiration was shown to be required for the development of leaf necrosis in catalase-deficient plants submitted to a high light regime (Willemsen et al. 1997). We suggest that the massive H$_2$O$_2$ burst in our system is the result of two processes: a stress-induced photorespiration and a suppression of ROI scavenging systems (Dorey et al. 1998, Mittler et al. 1998, Mittler et al. 1999). Concerning the latter process, we have shown that the strong H$_2$O$_2$ increase in our system closely paralleled the drop in catalase activity (Dorey et al. 1998). This H$_2$O$_2$ found in millimolar concentrations in elicitor-treated tissues could function as a direct antimicrobial mechanism.

The gain-of-function experiments with RB supported the conclusion drawn from the loss-of-function experiments using NAC, in that ROI were not acting as a diffusible signal upregulating PR gene expression in zone 2 tissues. The lesion caused by the treatment with 10 μM RB was reminiscent to the glycoprotein-induced HR lesion. One major difference was the clear decrease in the surface of zone 2 tissue showing PR1-GUS expression compared to that observed after the glycoprotein treatment. PR1 and OMT expression was also reduced in zone 2a tissue compared to the treatments with elicitor or elicitor+NAC. GUS staining and PR and OMT expression were analysed on two different tobacco cultivars, a transgenic cultivar Nicotiana tabacum cv. Xanthi nc (the plants used for the GUS staining) and a non-transgenic cultivar N. tabacum cv. Samsun NN (the plants used for PR1 and OMT analysis). Thus the biological phenomenon described here can be observed on different cultivars using different techniques. The results with NAC and RB are rather in favor of an inverse relationship between ROI present in tissue undergoing the HR and expression of defense genes in surrounding cells: the higher ROI levels in zone 1, the reduced surface of zone 2 tissue exhibiting...
PR1-GUS expression and the reduced expression of PR1 and OMT in zone 2. Conversely the lower ROI levels in zone 1 the augmented surface of zone 2 tissue exhibiting PR1-GUS expression and the higher PR1 and OMT expression.

Another factor could also explain the increased PR1 and OMT expression in zone 2 surrounding tissues co-infiltreated with NAC and the elicitor. Yet this factor is closely related to the levels of ROI present in the elicitor-infiltreated tissue undergoing the HR. NAC treatment delayed the elicitor-induced HR cell death (Fig. 4A). ROI scavenging by NAC would explain this phenotype since ROI were shown to activate HR cell death (Draper 1997, Mittler et al. 1999), bean (Levine et al. 1994) and Arabidopsis (Jabs et al. 1996). A possible scenario supposes that the plant cells perceiving the elicitor would have more time to produce and/or release the diffusible signal(s) resulting in elevated levels of signal, which would be able to diffuse to a longer distance explaining the enhanced surface of zone 2 tissue showing PR1 and OMT expression.

In conclusion, (i) ROI appear as key mediators in the activation of elicitor-induced PR, HMG gene expression and SA accumulation in zone 1, (ii) the data are not in favor of ROI acting as a diffusible signal released from the HR cells to induced PR1 and OMT expression in the surrounding zone 2 cells. The inverse relationship between the levels of ROI and the levels of PR gene activation in zone 2 suggests that ROI could affect the activity of a diffusible signal(s) resulting in a negative feed back control.

Materials and Methods

Plant material and treatments

Leaves of 3- to 4-month-old plants, N. tabacum cv. Samsun NN and PR1-GUS transgenic N. tabacum cv. Xanthi nc kindly provided by Novartis, were infiltrated with the different solutions to cover areas of (1995). The test probes were a cDNA encoding RNA analysis and quantification was performed as in Baillieul et al. In situ detection of GUS activity, RNA analysis and cell death assay stock solution (100 mM) in the light for 1 week. Tissue samples were prepared immediately before use as a 100 mM stock solution. Megasperma (1995)Plant material and treatments

In situ detection of GUS activity, RNA analysis and cell death assay

GUS staining was performed as described in Costet et al. (1999). RNA analysis and quantification was performed as in Baillieul et al. (1995). The test probes were a cDNA encoding N. tabacum PR5 and a cDNA encoding N. sylvestris HMG. Cell death was monitored by means of a leaf disk assay as described earlier (Dorey et al. 1997).

PR1 immunodetection and OMT activity analysis

Protein extraction was performed from 150 mg FW tissue. Tissues were ground in 750 l of 20 mM Na-phosphate buffer, pH 7.5, containing 15 mM l-mercaptoethanol, and charcoal. The crude extract was centrifuged at 10,000×g for 30 min and the supernatant was used to perform OMT activity and PR protein analysis. OMT activity was assayed as described elsewhere (Pellegrini et al. 1993). PR1 was analysed by Western blotting as described in Baillieul et al. (1995). Protein extracts corresponding to 4 mg FW were loaded onto the gels. Rabbit antisera to probe PR1 has been raised against the tobacco acidic PR1b. The polyclonal antibodies crossreact with the two other acidic PR1 proteins, PR1a and PR1e. Immunodetection was performed with the immun-star chemiluminescent kit of Bio-Rad.

Salicylic acid, H2O2, non-protein thiol and glutathione analysis

SA and H2O2 were analysed as previously described (Dorey et al. 1998). Total glutathione (reduced and oxidized form, GSH+GSSG) was assayed following a modified method (Griffith 1980). Leaf tissues were sampled as for the cell death assay and ground in 5% sulfosalicylic acid (v/v). After 1 h at 4°C, extract was centrifuged at 10,000×g for 10 min. The supernatant was neutralized with 1.5 volumes of 0.5 M K-phosphate buffer. Total glutathione was quantified by taking 40 l of the neutralized extract to which was added 100 l of 10 mM 5,5′-dithiobis-(2-nitrobenzenzoic) acid and 1.7 l of 0.1 M Na-phosphate buffer pH 7.4. The change in absorbance at 412 nm was followed for 5 min at 25°C. Non-protein thiols (NPSH) were assayed by taking 0.8 l of the neutralized extract to which was added 20 l of 10 mM 5,5′-dithiobis-(2-nitrobenzenzoic acid) and 2.18 l of 0.1 M Na-phosphate buffer pH 7.4. Absorbance was measured at 412 nm. Amounts of NPSH were calculated from a standard made with GSH.

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References


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