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Activation of defense pathways: synergism between reactive oxygen species and salicylic acid and consideration of field applicability

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Abstract

Treatment of tobacco with a mixture containing reactive oxygen species (ROS) and salicylic acid (SA) provided greater protection of tobacco against infection by *Pseudomonas syringae* pv. *tabaci* than either treatment alone. Synergism in expression from the promoter of the defense gene *PR-1a* was also observed. Although the ROS hydrogen peroxide and peracetic acid were poor inducers alone, they enhanced the level of β-glucuronidase (GUS) activity expressed from the *PR-1a* promoter when applied with SA to a transgenic plant bearing a *PR-1a::GUS* fusion. *PR-1a* expression was not correlated with increased cell death as determined by Evans blue staining. There was no effect on the timing at which expression was increased by the mixture compared with the separate treatments. The mixture of hydrogen peroxide and SA partially mimicked the effect of a commercial product Oxycom™ that has field efficacy in improving plant performance. Repetitive applications of Oxycom™ enhanced expression from the *PR-1a* promoter and the production of the PR-1 protein. Enhanced activity occurred systemically both from aerial applications to single leaves and from root drenches. Root application strongly promoted veinal expression for the *PR-1a* promoter compared with confluent production in leaves of sprayed seedlings. Application methods and timing may aid in the success of activators of systemic acquired resistance in field conditions.

Abbreviations: AA – acetic acid; GUS – β-glucuronidase; H_2O_2 – hydrogen peroxide; *Pst* – *Pseudomonas syringae* pv. *tabaci*; ROS – reactive oxygen species; SA – salicylic acid; UV-C – ultraviolet-C.

Introduction

Stimulation of defense pathways in plants involves a complex of interactive pathways. Induction of systemic resistance occurs by at least two pathways, one salicylic acid (SA) dependent and the second involving methyl jasmonate/ethylene as key intermediates (Glazebrook, 2001; Orozco-Cardenas et al., 2001; Ryals et al., 1995; Sasaki et al., 2001; Ton et al., 2002). Antagonism between the pathways involving these signal molecules has been reported (Felton et al., 1999; Gupta et al., 2000). Salicylic acid reduces the activation of the defense genes associated with the jasmonate pathway. However, simultaneous activation of the methyl jasmonate pathway by saprophytic root colonizing pseudomonads and the SA pathway was fully compatible in *Arabidopsis* (Van Wees et al., 2000).

The role of interactions between SA and reactive oxygen species (ROS) in induction of plant defense genes is not resolved (Bi et al., 1995; Chamnongpol et al., 1996, 1998; Neuenschwander et al., 1995). Reactive oxygen species are proposed to be involved in the programmed cell death occurring in the hypersensitive response (Levine et al., 1994) and the resultant increase in SA is cited as being important in the subsequent induction of SAR (Ryals et al., 1996). However, studies by Chamnongpol et al. (1998) demonstrated enhanced *PR-1* expression without cell necrosis. The

induction of *PR-1* was generated by exposure to light of tobacco transformed to be deficient in catalase, a process that was accompanied by increased hydrogen peroxide formation.

The research in this paper was initiated in order to understand more fully how a commercial product, Oxycom™, helped to maintain yield and quality in crops grown under pathogen pressure (Kim et al., 2001). Treatment with Oxycom™ involves spray or drench with two components, termed A and B that are mixed at the time of application. Component A, peracetic acid, which for 5% peracetic acid contains 10–12% acetic acid and 20–22% hydrogen peroxide, and component B, contains fertilizers and includes SA (1 mM when applied at 3450 ppm). Genes activated after sprays of bean with Oxycom™ include those encoding enzymes involved in phenolic metabolism (phenylalanine ammonia lyase and chalcone synthase), phenolic oxidation (specific members of the peroxidase-gene family in bean) and cell wall structure (hydroxyproline rich glycoproteins) (Kim et al., 2001).

Treatment of tobacco with Oxycom™ induced a greater protection of leaves against subsequent infection by the wildfire pathogen, *Pseudomonas syringae* pv. *tabaci* (*Pst*), than treatment with SA alone (Yang et al., 2002). These studies were aimed to determine whether the combination of ROS with SA present in Oxycom™ was beneficial in stimulating plant resistance. Plant cell death was assessed to determine whether it was elicited by treatments with the ROS plus SA mixture. The effect of single and combined ROS and SA treatments on the extent and location of *PR-1a* expression were studied. Timing of response was investigated to see if the mixture of ROS with SA induced a more rapid induction of the genes. Because in the field several applications of the Oxycom™ products are applied to the same crop during the growing season, the effect of repeat applications was studied. An antibody to the PR-1 protein was used to determine whether the changes being measured at the level of GUS activity were extended to the protein level. We also studied application procedures to determine whether defense gene expression was induced systemically.

Materials and methods

Plant growth and defense-inducing treatments

Wild-type tobacco (*Nicotiana tabacum* cv. Xanthi nc (NN)) was grown at 29 °C for eight to ten weeks under a 14 h light cycle (photosynthetic photon flux density 144 μmol s^{-1} m^{-2} at container surface). Plants were grown in seed-starting soil (Garden Grow Co, Independence, OR) and were watered once every 48 h with fertilization from Osmocote granules. *Pseudomonas syringae* pv. *tabaci*, the pathogen to examine for induction of resistance, was grown in liquid King's medium B, containing 50 μg ml^{-1} rifampin, to stationary-phase. Cells were centrifuged and suspended in 10 mM $MgCl_2$. Infiltration at known doses was performed with a 3 ml syringe into the three upper, fully expanded leaves. Disease symptoms were recorded by photography. The experiment was repeated at least five times.

The *PR-1a::GUS* tobacco line carrying the tobacco pathogenesis-related protein *PR-1a* promoter fusion (Bi et al., 1995) was from Dr. J. Draper, United Kingdom. Tobacco seeds were surface-disinfested by immersion in 70% ethanol for 2 min followed by a sterile water rinse before putting the seeds into a 0.6% w/v hypochlorite solution for 10 min at room temperature. The surface-sterilized seeds were rinsed with five separate washes of sterile water. Seeds were placed singly into wells of 12 well microtiter plates containing 3 ml $well^{-1}$ of Murashige and Skoog (1962) basal medium (Sigma-Aldrich #M5519) prepared at 1×, pH 6, with 0.8% w/v Bacto-agar (Difco Laboratories #0140-01) and sterilized. Plants were grown for 4–6 weeks.

Chemical treatments included sterilized distilled- and deionized-water, 10 mM acetic acid, 30 mM hydrogen peroxide, 10 mM acetic acid plus 30 mM hydrogen peroxide, 1 mM SA in 0.1% v/v ethanol (Sigma-Aldrich #S3007), 1 mM SA plus 10 mM acetic acid, 1 mM SA plus 30 mM hydrogen peroxide, 5000 ppm Oxycom™ A (containing 4.1 mM peracetic acid, 30 mM hydrogen peroxide and 10 mM acetic acid) (Redox Chemicals, Inc., Burley, ID, USA), 3450 ppm Oxycom™ B (containing 1 mM SA) (Redox Chemicals, Inc., Burley, ID, USA), 5000 ppm Oxycom™A plus 3450 ppm Oxycom™ B. Spray treatment involved misting until run off. Root drenches were till run off for the mature plants or by injection of 500 μl of solution into the well plate medium. Spot treatment used single 20 μl droplets. After spray application plants were air dried for 1 h before being returned to the greenhouse or being covered by the well plate covers. At harvest, 4–10 plants from a single well plate treatment were removed gently from the growth medium. Roots were excised and leaves blotted dry and pooled together to create a single sample. The

material was frozen immediately in liquid nitrogen and ground to a fine powder using liquid nitrogen before storage at −80 °C. All experiments were repeated three times. In other studies, 10-week-old *PR-1a::GUS* tobacco plants were treated on single leaves with 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B mixture or with water. After 48 h, these leaves were frozen immediately in liquid nitrogen along with leaves at the third node above and the third node below the treated leaves. These samples were ground to a fine powder using liquid nitrogen and stored at −80 °C.

Assessment of plant cell death

Evans blue staining was used to assess whether treatments caused increased plant cell death (Baker and Mock, 1994). Seedlings raised for 4–6 weeks under sterile conditions were sprayed to run off with water as a control, or 30 mM hydrogen peroxide, 10 mM acetic acid, 1 mM SA, 1 mM SA plus 30 mM hydrogen peroxide, 5000 ppm Oxycom™A, 3450 ppm Oxycom™ B, 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B and leaves were harvested after 24 h. The tissue was immersed into 0.25% Evans blue aqueous solution for 20 min. The tissues were washed in water and placed into ethanol to remove the chlorophyll. The leaves were examined under ×10–40 microscopy. Studies were repeated with mature leaves from 10-week-old plants treated with water or with 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B. To provide a positive control, the intact plants were exposed for 24 h to a bacteriocidal UV-C lamp prior to Evans blue staining.

Glucuronidase assays

Powdered plant material was thawed with the addition of ice-cold, extraction buffer (50 mM MOPS pH 7.0, 10 mM Na_2EDTA, 0.1% v/v Triton X-100, 0.1% sodium lauryl sarcosine) using 3 ml per 1 g of tissue with light agitation on a vortex before a 30 min extraction period on ice. The homogenates were centrifuged at 10000× g at 8 °C for 30 min before collection of the supernatants that were assayed immediately for β-glucuronidase (GUS) activity and total protein. Total protein was determined using the BCA protein assay reagent (Pierce Chemical Company). β-glucuronidase activity was determined using the fluorometric assay with 4-methylumbelliferyl β-D-glucuronide (Sigma-Aldrich #M9130) as substrate (Jefferson, 1987).

Histochemical assays on intact plant material were performed by immersion into a reaction mixture: 100 mM sodium phosphate pH 7.0, 0.1% v/v Triton X-100, 0.1% v/v β-mercaptoethanol, 1 mM 5-bromo-4-chloro-3-indolyl phosphate, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$ and 20% methanol. After incubation at 37 °C for 14 h, leaves were fixed in 5% formaldehyde, 5% acetic acid, 20% ethanol for 2 h before clearing in 95% ethanol at 40 °C before photography.

Assessment of PR-1 protein levels

Leaves of treated plants were ground to a fine powder using a mortar and pestle and suspended in extraction buffer described by Zhang and Klessig (1997). The homogenate was centrifuged at 10000× g for 40 min, and the supernatants were stored at −80 °C until use. Protein was determined using the Bio-Rad protein assay kit (Hercules, CA) with bovine serum albumin as the standard. To detect the PR-1 protein, extracted proteins (15 µg) were separated on 15% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (Osmonics, Westborough, MA) by semi-dry electroblotting. After blocking for 1 h in TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% nonfat dried milk at room temperature, the blot was probed with PR-1 antibody (Carr et al., 1987) overnight at 4 °C. The blot was washed in TBS buffer three times, incubated with horseradish peroxidase-conjugated secondary antibody (Sigma #2304, 1 : 10000 dilution), and then the complexes were visualized using chemiluminescence according to standard protocol (Roche, Indianapolis, IN).

Results

Synergism in pathogen protection and activation of GUS activity by combined applications of ROS and SA

Symptoms in leaves of mature tobacco inoculated with *Pst* (1×10^5 to 10^6 cfu ml^{-1}) were reduced when the Oxycom™ A plus Oxycom™ B mixture was applied compared with treatment with either Oxycom™ A or Oxycom™ B alone (Figure 1).

In studies with younger plants grown in sterile growth medium, GUS activity in tobacco transformed

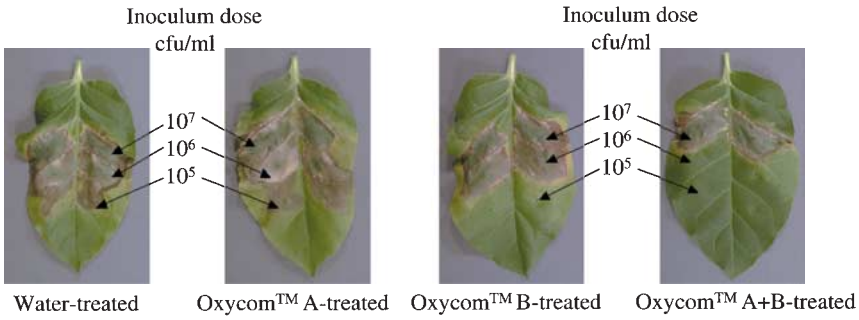

Figure 1. Protection of tobacco from *Pst* is enhanced by the mixture of ROS and SA components in the Oxycom™ product. Plants were sprayed to run off with water, 5000 ppm Oxycom™ A, 3450 ppm Oxycom™ B or the mixture of Oxycom™ A plus Oxycom™ B. After 48 h the leaves were challenged with *Pst* at three inoculum doses as indicated. Symptoms illustrated are after 8 days of incubation and are typical of five studies each with replicates of two plants per treatment.

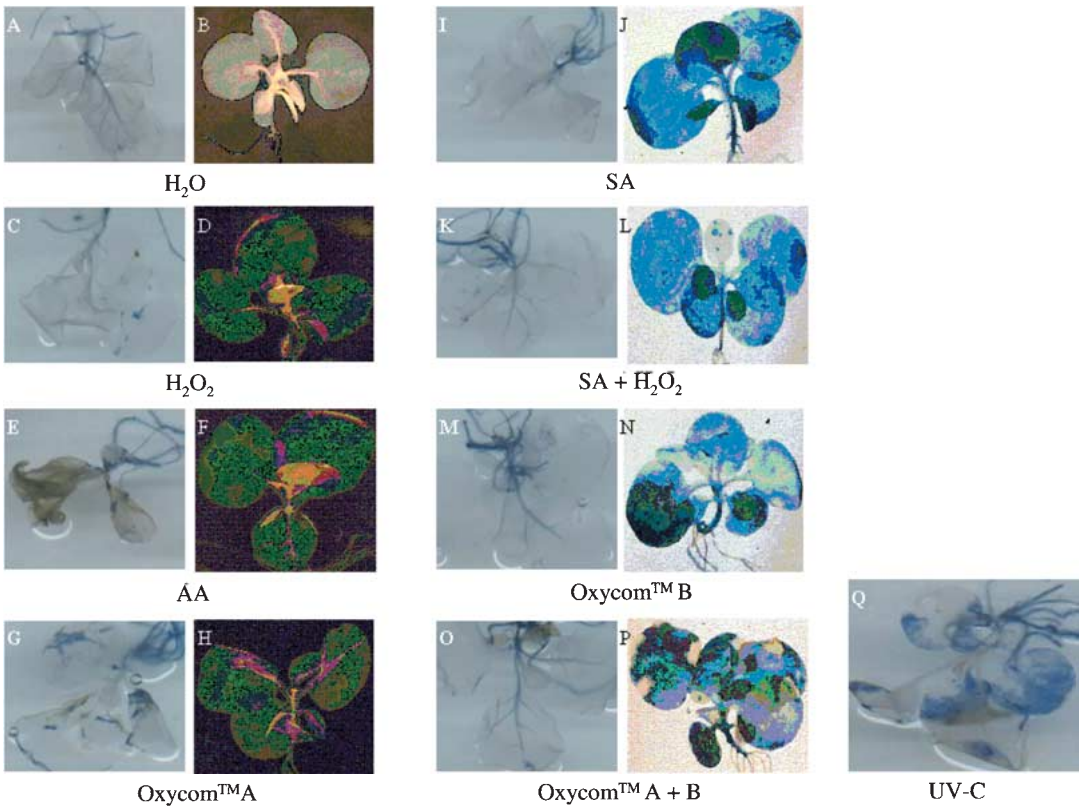

Figure 3. Assessment of plant cell death and GUS activity in leaves exposed to ROS, SA, Oxycom™ and UV-C. Gnotobiotic 6-week-old plants were sprayed to run off with H_2O, or 30 mM H_2O_2, 10 mM AA, 1 mM SA, 1 mM SA plus 30 mM H_2O_2, 5000 ppm Oxycom™ A, 3450 ppm Oxycom™ B, or the mixture of Oxycom™ A plus Oxycom™ B. Other plants were exposed to UV-C as a positive control. Leaves were harvested after 24 h and stained with Evans blue (A, C, E, G, I, K, M, O, Q) as described in the section 'Materials and methods'. Images are representative of three leaves examined from three plants for each of four studies. Histochemical staining (B, D, F, H, J, L, N, P) to detect GUS was performed 48 h after treatments as described in the section 'Material and methods'. Data are representative of 10 plants per treatment, in two replicated studies.

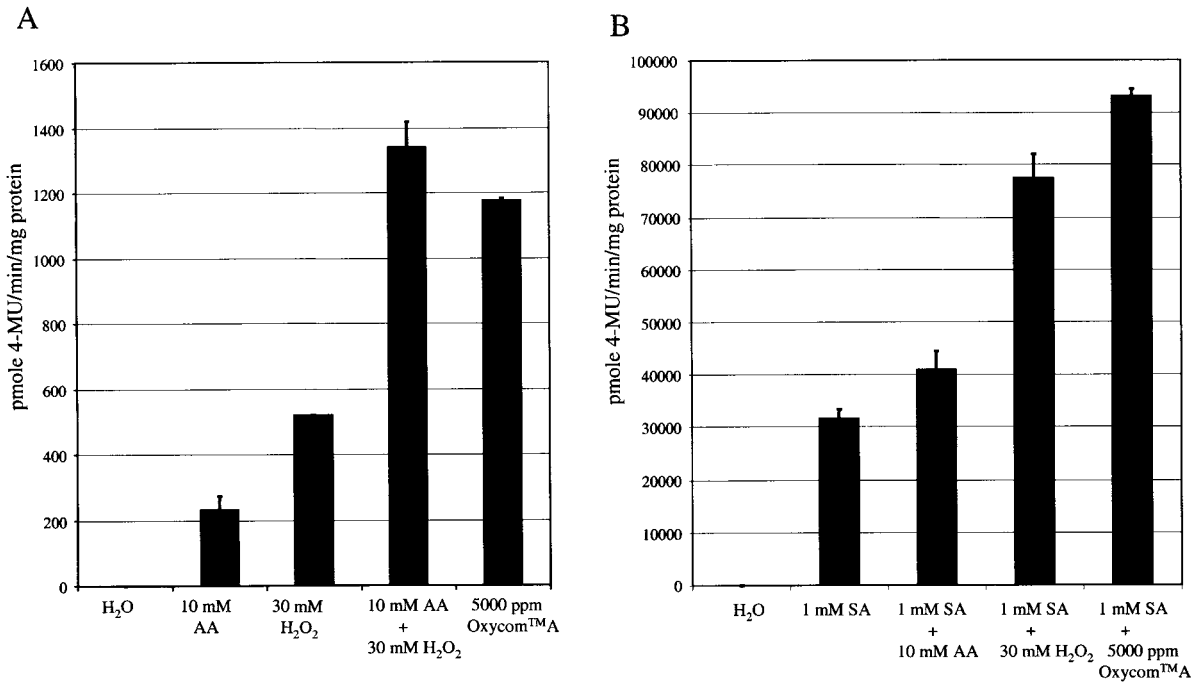

Figure 2. β-Glucuronidase activity in extracts from a transgenic tobacco line expressing GUS driven by the tobacco *PR-1a* promoter 48 h after treatments with ROS and SA. Plants were sprayed to run off with AA, H_2O_2, AA plus H_2O_2, or Oxycom™ A (A) and SA, SA plus AA, SA plus H_2O_2 or SA plus Oxycom™ A (B). Values are given as picomoles 4-methylumbelliferone formed per minute per milligram protein at 37 °C and are from extracts pooled from four to 10 seedlings for each treatment. Data presented are representative of four different studies.

with the *PR-1a::GUS* fusion was increased slightly after treatment with hydrogen peroxide and acetic acid at levels corresponding to those present in 5000 ppm peracetic acid in Oxycom™ A (Figure 2A). The peracetic acid in Oxycom™ A was a stronger inducer, 2–5 fold, than hydrogen peroxide or acetic acid alone (Figure 2A). Synergism in the level of GUS activity was also noted when the combination of acetic acid with hydrogen peroxide was applied (Figure 2A). Because the components were mixed just prior to spray, there was no time for the reaction to produce peracetic acid to occur so that the level of the peracetic acid should be negligible. These applications had slight pH differences (10 mM acetic acid: pH 2.91; 5000 ppm Oxycom™ A: pH 3.45; 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B: pH 4.75). The application of SA had a stronger inductive affect, with a sixty-fold increase over that generated by hydrogen peroxide treatments (Figure 2A and B). About three-fold increase in activation of the *PR-1a* promoter was observed when the SA was amended with hydrogen peroxide or peracetic acid (Figure 2B).

Effect of applications on tobacco cell death and PR-1a expression

Because of reported connections between plant cell death and SA, we assessed cell death in leaf tissue after ROS and SA applications. The most damaging treatment was with acetic acid. The leaves of 6-week-old plants had clear spots after treatments with acetic acid, and to a lesser degree the peracetic acid, Oxycom™ A and the Oxycom™ A plus Oxycom™ B mix. We correlated these sites to regions where the spray collected and pooled. Generally, leaves dried completely within 30 min of spraying. Within a week, leaves treated with acetic acid turned white and shriveled and the plants grew no further. In comparison, plants treated with hydrogen peroxide, SA, hydrogen peroxide plus SA, Oxycom™ A, Oxycom™ B or Oxycom™ A plus Oxycom™ B continued to grow and produced new leaves with no discoloration.

The results of Evans blue staining 24 h after treatment are shown in Figure 3(A,C,E,G,I,K,M,O,Q). Unexpectedly, cells surrounding the visibly-clear spots

after acetic acid (Figure 3E), Oxycom™ A (Figure 3G) and Oxycom™ A plus Oxycom™ B treatments (Figure 3O) displayed no blue staining characteristic of dead cells. The leaves treated with acetic acid were twisted and collapsed and retained a green-brown tint. Leaves exposed to UV-C irradiation as a control to check for staining of dead cells showed intense regions of blue-staining cells (Figure 3Q). None of the other treatments (hydrogen peroxide, SA, hydrogen peroxide plus SA, Oxycom™ B, Oxycom™ A plus Oxycom™ B) consistently stained more intensely with Evans blue than was observed in water-treated control plants (Figure 3). Leaves from the mature plants showed no increase in Evans blue staining after Oxycom™ A plus Oxycom™ B treatment than the control water-treated leaves (data not shown).

Histochemical staining for GUS activity was used to reveal the sites of activation of the *PR-1a* promoter (Figures 3). Application of hydrogen peroxide, acetic acid or the peracetic acid in Oxycom™ A (Figure 3D,F,H, respectively) resulted in little GUS activity although cell clearing was apparent. A confluent pattern of GUS activity on the leaves was seen with sprays of SA (Figure 3J), SA and hydrogen peroxide (Figure 3L), Oxycom™ B (Figure 3N) or Oxycom™ A plus Oxycom™ B (Figure 3P). The extent of staining was similar for the SA and the combined treatments of Oxycom™ A plus Oxycom™ B (Figure 3J and P).

Pattern of PR-1 expression is dependent on method of application

Application of Oxycom™ A plus Oxycom™ B mix as a single droplet to one leaf resulted in the detection of GUS activity in adjacent leaves (Figure 4A). No enhanced GUS activity was observed around the damaged area surrounding the application spot. When Oxycom™ was applied to the roots, GUS staining was observed in the leaves, being especially strong in veinal tissues (Figure 4B).

To study systemic effects further in mature plants, a selected leaf of 10-week-old plants was spray-treated with Oxycom™. Leaves three nodes up or down from the treated leaf (leaf #7) showed elevated GUS activity 48 h after the treatment (Table 1).

Synergism does not involve altered timing of gene expression or protein accumulation for the PR-1 gene

To investigate the mechanisms underlying the synergistic effect of the simultaneous application of ROS and SA, we studied the time at which GUS activity increased after treatment. Increased activity was observed at similar times whether treatments were single or combined (Figure 5A and B). Slight increases in GUS activity were observed by 9 h after a SA or the

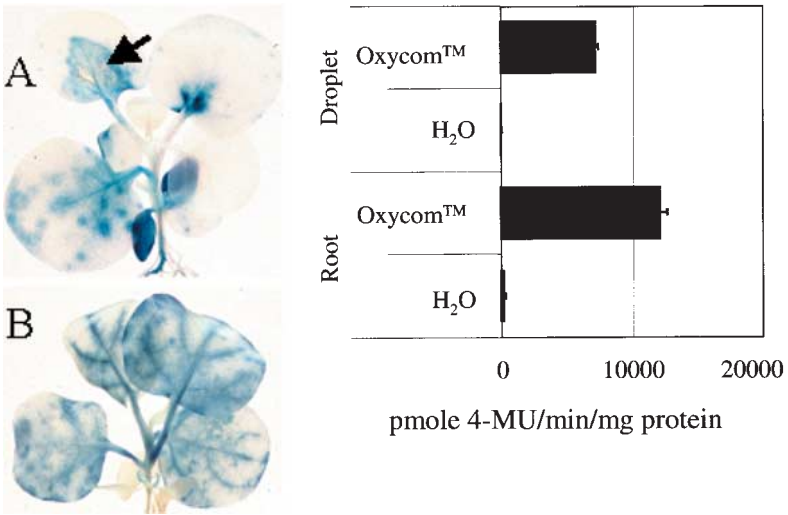

Figure 3. Comparison of the location of induced GUS activity and specific GUS activity resulting from two application methods for 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B. Activities were determined in extracts and *in planta* after 48 h of treatment by application of a droplet to a single leaf (A, indicated by arrow) and by root drench (B). The GUS specific activity was determined for pooled samples of 10 plants per treatment. The plants shown are typical of 10 plants each from two separate studies.

SA plus hydrogen peroxide treatment and this activity continued to increase for the 24 and 48 h sampling times (Figure 5A). Similar times of activation were observed when the A and B components of Oxycom™ were used (Figure 5B).

Repeat applications boost PR-1 activation

Because in the field applications of Oxycom™ are repeated during a growing season, the effects of repeat treatment were investigated. Plants were treated with Oxycom™ A plus Oxycom™ B mixture once, or twice, by a second application, applied 48 h after the first, or three times, when the third application was applied 48 h after the second treatment. Increased GUS activities were observed for the plants that received the second and third treatments over the single treatment (Figure 6A). Samples assayed at the same time for the level of PR-1 protein also showed accumulations above the single treatments in the double- and triple-treated plants (Figure 6B).

Table 1. Systemic induction of GUS reporter activity in tobacco after treatment of a single leaf

Treatment	GUS activity[1]		
	Leaf at third node above treated leaf	Leaf receiving treatment	Leaf at third node below treated leaf
H_2O	174 (11)[2]	85 (49)	0 (0)
Oxycom™ A + B	515 (9)	372 (127)	235 (29)

[1]Mean activity values are picomoles 4-methylumbelliferone formed per minute per milligram protein at 37 °C.
[2]Standard errors are shown in parentheses.

Discussion

Simultaneous application of ROS with SA in the Oxycom™ product enhanced protection in tobacco against *Pst* over the low level observed with ROS, or the heightened expression from SA alone. Synergistic increase in expression of the SA-regulated promoter of the *PR-1a* defense gene and the production of the PR-1 protein also was observed with the Oxycom™ product. Enhanced expression from the *PR-1a* promoter was mimicked by treatment with SA and hydrogen peroxide. This similar effect of the pure chemicals

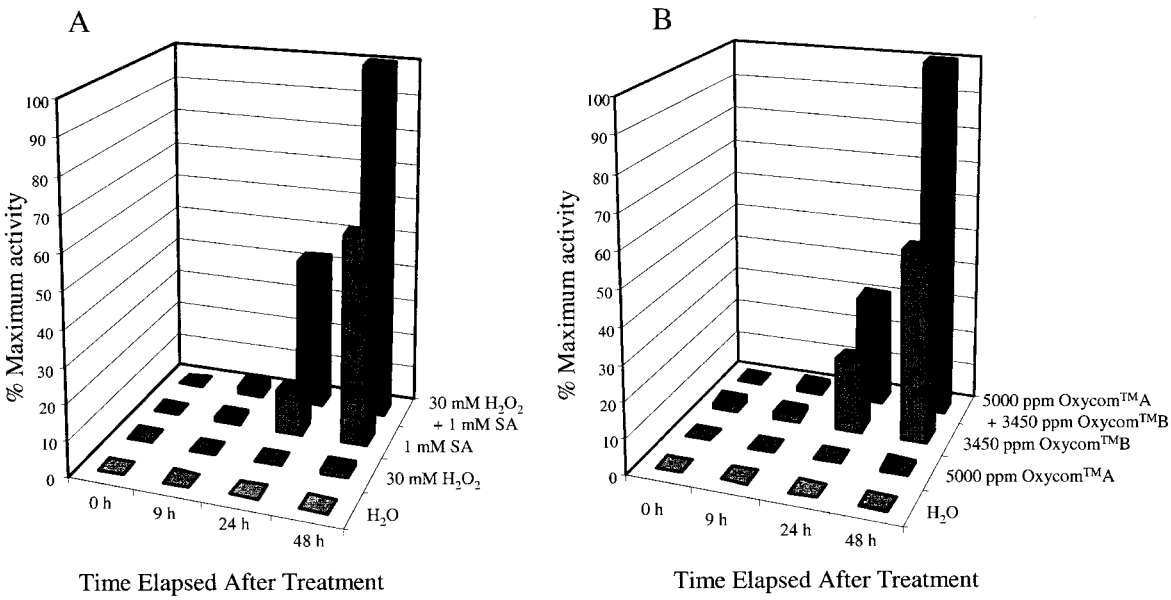

Figure 4. Time course for GUS activity from transgenic tobacco carrying *PR-1a::GUS* after treatments involving: (A) H_2O_2 and SA; (B) Oxycom™ A and Oxycom™ B. Whole plants were sprayed, harvested and assayed as described in the section 'Material and methods'. Values are given as the percentage of maximal GUS induction and represent data from four to 10 plants. Data are representative of three separate studies.

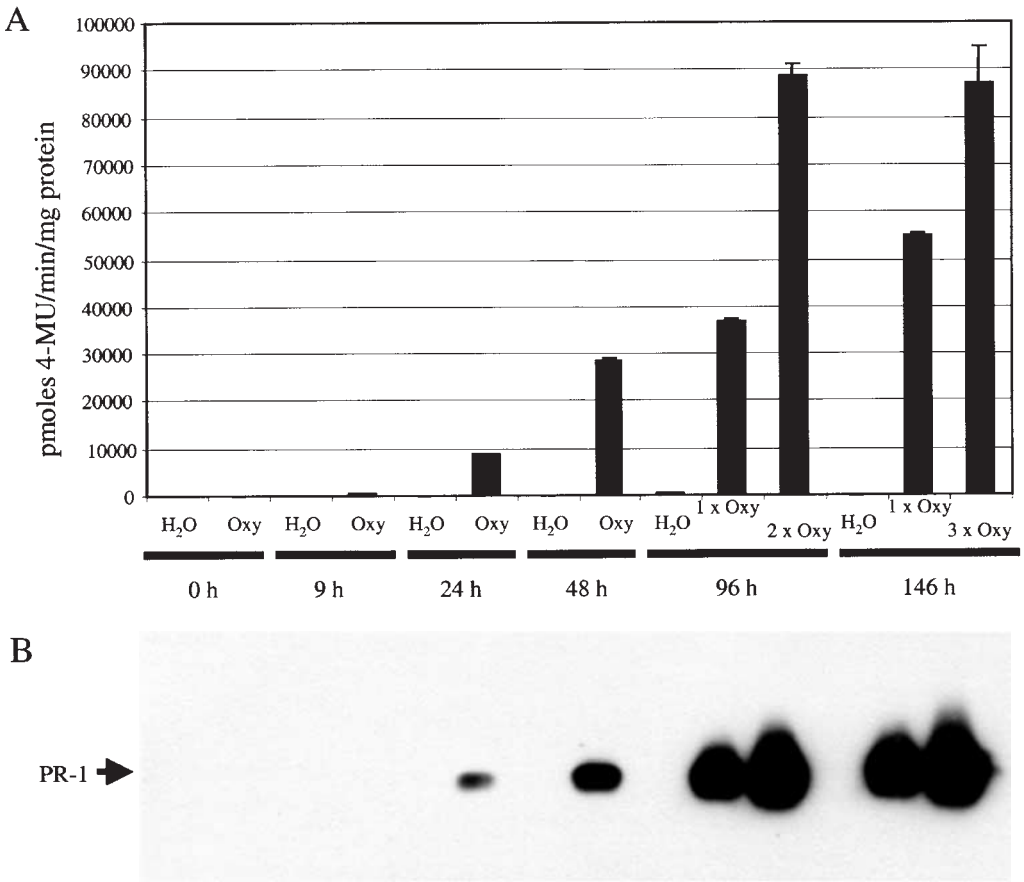

Figure 5. Effects of multiple 5000 ppm Oxycom™ A plus 3450 ppm Oxycom™ B treatments on GUS activity and PR-1 protein accumulation over time. (A) GUS activity from extracts of tobacco carrying the *PR-1a* reporter was determined at respective time points after a single spray treatment of either H_2O or the mixture of Oxycom™ A plus Oxycom™ B (Oxy). At time zero, two additional sets of plants were also treated with Oxycom™ A plus Oxycom™ B and kept to 48 h at which time they were sprayed again with Oxycom™ A plus Oxycom™ B. At 96 h one of these plant sets was harvested for assays (Oxy 2× treated) whereas the second set was sprayed with Oxycom™ A plus Oxycom™ B a third time before harvesting at 146 h (Oxy 3× treated). Plants were harvested by pooling material from four to 10 plants and GUS activity and protein assayed as described in the section 'Material and methods'. Values given are picomoles 4-methylumbelliferone formed per minute per milligram protein at 37 °C. Data are representative of two studies. (B) The combined extracts from the same plants were subjected to electrophoresis and blotted before detection of native PR-1 by monoclonal antibodies according to procedures described in the section 'Materials and methods'.

and the Oxycom™ product showed that the complex of materials in the Oxycom™ formulation did not conflict with plant defense activation by the SA-dependent pathway.

Previous workers have shown that ROS is a weak inducer of the *PR-1a* promoter except when there is enhanced cell death (Bi et al., 1995; Neuenschwander et al., 1995). The relationship between GUS expression and cell death is not clear in our studies. The visible damaged cell area resulting from acetic acid and in some studies peracetic acid treatments lacked staining with Evans blue, which is documented to detect cells undergoing cell death in a hypersensitive response (Baker and Mock, 1994). These acetic acid and ROS treated cells also lacked increased GUS staining, illustrating that not all plant cell death events are similar in triggering the SA-defense pathway. Perhaps the acid-induced death occurred too quickly for the SA signaling pathway to occur. Also, in our other treatments with ROS and SA although *PR-1a* expression was enhanced we could not document by Evans blue staining increases in plant cell death. Our findings instead agree with those from Chamnongpol et al. (1998) who present evidence to suggest synergism for

PR-1 expression between ROS and SA in transgenic plant deficient in catalase could occur in the absence of cell death.

How ROS and SA interact to enhance *PR-1a* expression remains unclear. Lawton et al. (1994) suggest that ethylene enhancement of the SA pathway for *PR-1a* expression is related to a lowering of sensitivity of the plant to the effect of SA. Low levels of SA are cited to potentiate defense responses upon an additional challenge causing an oxidative burst (Shirasu et al., 1997). Ryals et al. (1995) found no evidence for SA inducing hydrogen peroxide, although others report such findings (Shirasu et al., 1997). SA is found to generate superoxide anion in stomatal cells (Mori et al., 2001). Another possibility is that hydrogen peroxide is involved in the methyl jasmonate pathway of defense gene activation (Orozco-Cardenas et al., 2001), which may affect the SA pathway (Sasaki et al., 2001). Additionally ethylene production might be affected. Chamnongpol et al. (1998) showed that ethylene is enhanced in levels in transformed plants deficient in catalase when illuminated, conditions under which hydrogen peroxide is proposed to accumulate. In additional studies using gene chip microarray analyses of *Arabidopsis* seedlings treated with Oxycom™, we have observed induced expression of genes associated with the ethylene- and jasmonic acid-pathways as well as the anticipated genes regulated by SA (unpubl. data). Synergistic effects were observed for the accumulation of transcripts for the transcription activating factor, ATAF2, at 1 and 24 h a peroxidase, PrxCb, and the ethylene-responsive element binding factor, AtERF-1, when the combination of peracetic acid and SA was applied compared with either peracetic acid or SA alone (unpubl. data).

The simultaneous application of the ROS and SA did not alter the timing of activation of the *PR-1a* promoter from that with SA alone in the young tobacco seedlings that we examined for this paper. Priming effects of activators such as β-aminobutyric acid and acibenzolar-S-methyl has been reported in other plants to decrease the time required for resistance expression in tissues challenged by a pathogen (Latunde-Dada and Lucas, 2001; Zimmerli et al., 2000).

The ROS and SA mixture induced a systemic activation of *PR-1a* promoter activation in tobacco in these studies and in the previous studies in bean where other defense genes were monitored (Kim et al., 2001). These findings support the work of Chamnongpol et al. (1996) who found systemic expression for *PR-1* with SA only under conditions where hydrogen peroxide was thought to accumulate, otherwise the effects of SA were localized. Of interest is the pronounced veinal pattern of GUS expression in leaves when Oxycom™ was applied as a root drench rather than as a spray. A veinal pattern of PR-1 protein accumulation was reported previously where antibodies to PR-1 were used for detection (Carr et al., 1987).

These studies of dose and repeat applications indicated that promoter activity of *PR-1a* was responsive to dose, whether in a single application or administered as a repeat system. These findings have relevance to field applications where timing and placement of the treatment on leaves or roots may be varied with the anticipated disease pressure.

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