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# Induction of $H_2O_2$ in transgenic rice leads to cell death and enhanced resistance to both bacterial and fungal pathogens

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*Key words:* transgenic rice, enhanced resistance, hydrogen peroxide, glucose oxidase, cell death

**Abstract**

Oxidative burst, mediated by hydrogen peroxide ($H_2O_2$), has been recognized as a key component of plant defense response during an incompatible interaction. To determine if elevated levels of $H_2O_2$ lead to cell death, activation of defense genes and enhanced resistance to diverse pathogens, transgenic rice plants expressing a fungal glucose oxidase gene (*GOX*) were generated using both constitutive and inducible expression systems. Constitutive or wound/pathogen-induced expression of GOX also allowed us to determine the effectiveness of these systems in conferring long lasting resistance to various pathogens. Both constitutive and wound/pathogen-induced expression of GOX lead to increases in the endogenous levels of $H_2O_2$, which in turn caused cell death. Elevated levels of $H_2O_2$ also activated the expression of several defense genes and these transgenic plants showed enhanced resistance to both bacterial and fungal pathogens. In comparison to inducible expression, constitutive expression of GOX resulted in 3–10-fold higher levels of the *GOX* transcript and the corresponding enzymatic activity. Such increased levels of GOX, which would result in elevated levels of $H_2O_2$, caused improper seed set and decreased seed viability in transgenic plants constitutively expressing *GOX*. Our results suggest that pathogen inducible expression of heterologous genes may be a practical and robust way of generating broad spectrum disease resistance.

## Introduction

Plants and pathogens have evolved an array of interactions described as compatible (host susceptible, pathogen virulent) or incompatible (host resistant, pathogen avirulent). An incompatible interaction activates a battery of host defense mechanisms, including hypersensitive response (HR) or programmed cell death (PCD) at the site of infection (Greenberg et al., 1994; Dangl et al., 1996; Greenberg, 1996; Jabs et al., 1996; Mittler et al., 1997; del Pozo & Lam, 1998), cell wall reinforcement (Dixon & Lamb, 1990) and induction of pathogenesis-related (*PR*) genes (Bowles, 1990; Ward et al., 1991; Meier et al., 1993; Shirasu et al., 1996).

Rapid generation of hydrogen peroxide ($H_2O_2$) and superoxide ($O_2{}^-$) during the oxidative burst has been recognized as a central component of plant defense responses to pathogen challenge (Lamb & Dixon, 1997; Tiedemann, 1997; Wojtaszek, 1997; Cazale et al., 1998). Accumulation of $H_2O_2$ during the oxidative burst not only directly inhibits microbes (Kiraly et al., 1993; Tzeng & De Vay, 1993), but also plays a key role in the oxidative cross-linking of

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cell wall proteins (Bradley et al., 1992; Brisson et al., 1994), lignification (Olson & Varner, 1993), and in the orchestration of HR (Levine et al., 1994; Tenhaken et al., 1995). In addition, $H_2O_2$ has been shown to be a diffusible signal during systemic acquired resistance (SAR) (Alvarez & Lamb, 1997; Lamb & Dixon, 1997). Elevated $H_2O_2$ levels have also been suggested to trigger downstream components of the defense pathway and induce expression of defense-related genes associated with SAR (Chen et al., 1993). Both $O_2^-$ and $H_2O_2$ have been shown to mediate a reiterative signal network underlying systemic as well as local resistance responses (Alvarez et al., 1998).

Increasing the endogenous levels of $H_2O_2$ either by overexpressing *GOX* in potato (Wu et al., 1995) or GTP-binding protein OsRac1 in rice (Kawasaki et al., 1999) are suggestive of a link between elevated levels of $H_2O_2$, cell death and disease resistance. Transgenic tobacco expressing a chimeric glucose oxidase gene showed massive cell death and PR-1a gene induction in the presence of exogenous glucose (Kazan et al., 1998). The cell death phenotype could further be enhanced by exogenous application of salicylic acid. Constitutive expression of the *GOX* gene in potato conferred resistance to potato soft rot caused by *Erwinia carotovora* subsp. *carotovora* and late blight caused by *Phytophthora infestans*. In addition, the elevated levels of $H_2O_2$ in the potato plants induced the accumulation of total SA and *PR* gene expression

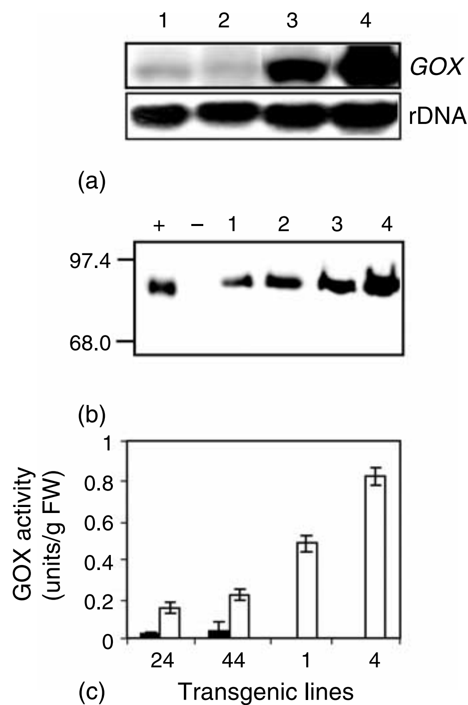


*Figure 1.* Northern and western blot analysis and, enzymatic assay of GOX activity in *UB::GOX* and *PAL::GOX* transgenic plants. (a) 20 μg of total RNA from wound-induced *PAL::GOX* 24 (lane 1) and *PAL::GOX* 44 (lane 2) lines or the *UB::GOX* lines 1 and 4 (lanes 3 and 4, respectively) were hybridized to the full length *GOX* fragment. The blot was reprobed with rDNA, which served as internal loading control. (b) Protein blots were prepared by running approximately 10 μg of total protein from wound-induced *PAL::GOX* lines 24 and 44 (lanes 1 and 2, respectively) or the *UB::GOX* lines 1 and 4 (lanes 3 and 4, respectively) and GOX protein was detected using anti-GOX antibodies. Purified commercial GOX protein (+) was used as a positive control while protein from untransformed plant served as a negative control (−). (c) Glucose oxidase activity was estimated using a colorimetric assay with glucose and *o*-dianisidine as substrates. Protein from *PAL::GOX* lines 24 and 44 was extracted at 0 (closed bars) and 12 h (open bars) post-induction. *UB::GOX* lines 1 and 4 did not require any 0 h controls as they express *GOX* constitutively. The data are presented as the mean and standard error from two independent experiments.

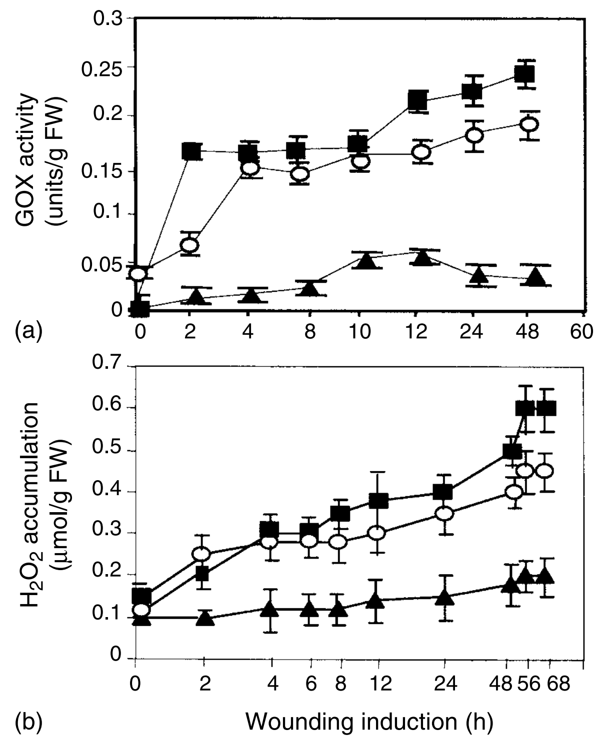


*Figure 2.* GOX activity and $H_2O_2$ accumulation in wounded leaf tissues of transgenic rice. Leaf tissue from wounded leaves was assayed for GOX enzymatic activity (a) 0–48 h, post-treatment and $H_2O_2$ accumulation (b) 0–68 h, after wounding. Lines shown are untransformed Taipei 309 (closed triangles), and transgenic lines *PAL::GOX* 24 (open circles) and *PAL::GOX* 44 (closed squares). GOX activity and $H_2O_2$ levels are shown as means per gram fresh weight (FW) with standard deviations ($n = 3$).

(Wu et al., 1997). These results suggest that manipulating $H_2O_2$-generation could be a practical approach for engineering broad-spectrum plant disease resistance. However studies conducted thus far have used constitutive expression of various transgenes to up-regulate $H_2O_2$ levels, which is rather undesirable and likely to have deleterious effects on plants. This is further supported by a recent study conducted by Lee et al. (2002), which shows that elevated levels of $H_2O_2$ can lead to partial to pronounced sterility in transgenic tobacco plants. In the present study we have compared constitutive and pathogen inducible expression of the *Aspergillus niger GOX* gene in transgenic rice plants. As expected, constitutive expression of *GOX* leads to higher levels of GOX enzymatic activity and a concomitant increase in $H_2O_2$, but these plants show various developmental abnormalities. By contrast, pathogen inducible expression of GOX results in timely accumulation of $H_2O_2$, which is sufficient to confer enhanced resistance to various pathogens. Furthermore, pathogen inducible expression does not cause any developmental abnormalities suggesting that this may be a better approach for engineering plant disease resistance.

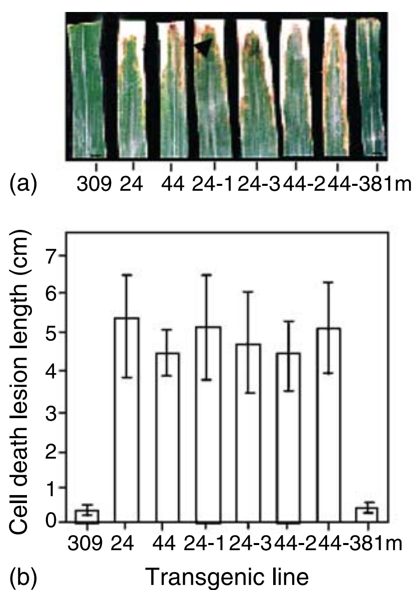


*Figure 3.* Cell death lesions induced by wounding in *PAL::GOX* plants. (a) Expression of *GOX* in *PAL::GOX* transgenic plants was induced by wounding (cutting off leaf tips) and lesion development was photographed 10 days after wounding. Lines shown from left to right are untransformed Taipei 309, GOX transgenic lines *PAL::GOX* 24 ($T_0$), *PAL::GOX* 44 ($T_0$), *PAL::GOX* 24-1 ($T_1$), *PAL::GOX* 24-3 ($T_1$), *PAL::GOX* 44-2 ($T_1$), *PAL::GOX* 44-3 ($T_1$) and vector-transformed line 381m. Cell death lesions characterized by yellowish regions on green leaves are indicated by arrows. (b) Lesion size was measured in untransformed control or transformed lines (as in Figure 3(a)). Data represent the mean from 15 different wounding experiments.

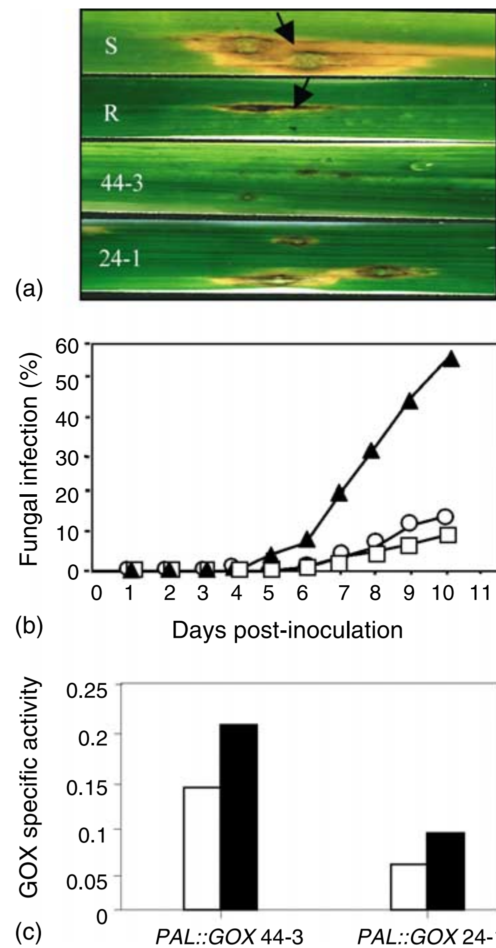


*Figure 6.* Enhanced disease resistance of *PAL::GOX* transgenic rice plants to rice blast. (a) Disease symptoms on blast susceptible wt Taipei 309 (S), a blast resistant cultivar (R) and the *PAL::GOX* transgenic lines 24-1 and 44-3 were evaluated at 10 dpi. Large spreading lesions on susceptible plants as opposed to small hypersensitive lesions on resistant plants are indicated by arrows. (b) The blast disease developed very rapidly in wt Taipei 309 (closed triangles) plants as compared to the GOX transgenic lines, *PAL::GOX* 24-1 (open circles) and *PAL::GOX* 44-3 (open squares). Disease development was recorded as the percentage of infected leaf area, 10 dpi ($n = 15$). (c) GOX activity was measured in *PAL::GOX* transgenic lines 24-1 and 44-3 before (open bars) and after (closed bars) inoculation with *M. grisea*. Data shown is a mean of two independent estimations of GOX activity.

## Results

### *Induction of GOX activity leads to generation of $H_2O_2$ and cell death in transgenic rice*

Transgenic rice lines expressing the glucose oxidase gene from *A. niger* were generated by placing *GOX* under the control of a constitutive maize ubiquitin (*UB*) promoter or a wound/pathogen inducible phenylalanine ammonia-lyase (*PAL*) promoter from rice. Molecular analysis of these transgenic plants revealed that constitutive levels of GOX transcript and the corresponding protein in *UB::GOX* plants were much higher as compared to levels obtained after wound induced stimulation of *PAL::GOX* plants (Figure 1(a) and (b)). The increased amount of GOX protein in *UB::GOX* plants also corresponds to a 3–10-fold increased GOX enzymatic activity (Figure 1(c)). However, the number of *UB::GOX* transgenic plants obtained was much lower than the *PAL::GOX* transgenics and moreover, *UB::GOX* transgenics yielded very few seeds that showed reduced viability and poor germination. By contrast, *PAL::GOX* plants were phenotypically similar to the wild-type (wt) and vector-transformed control plants. Due to these problems with the *UB::GOX* plants all further analysis was conducted on two of the *PAL::GOX* lines designated 24 and 44.

Since GOX enzymatic activity leads to production of $H_2O_2$, we next determined levels of $H_2O_2$ after wound treatment of leaves from *PAL::GOX* and wt or vector-transformed control plants. GOX activity was induced after 2 h of wounding and the peak activity was seen 48 h post-wounding (Figure 2(a)). A 2–10-fold increase in the GOX activity upon wounding of *PAL::GOX* leaves is consistent with the activation profile of the *PAL* promoter upon wounding (Zhu et al., 1995). By contrast, little or no GOX activity was detected in the vector transformed or wt plants. Consequently, the levels of $H_2O_2$ were elevated in *PAL::GOX* plants upon wounding and correlated with the increase in GOX activity. A 2–3-fold increase in the $H_2O_2$ level was observed in *PAL::GOX* plants as compared to control plants (Figure 2(b)).

Upon wounding, *PAL::GOX* leaves developed large disease-like lesions (Figure 3(a) and 3(b)). The extent of cell death induced by wounding was the same among $T_0$ and $T_1$ transgenic plants carrying the *GOX* transgene. These results indicate that induction of *GOX* expression leads to cell death by increasing the $H_2O_2$ levels, which is known to cause oxidative damage (Levine et al., 1994; Tenhaken et al., 1995).

### GOX *gene expression leads to activation of* PR *genes*

*GOX* mRNA accumulation was determined by reverse transcriptase polymerase chain reaction (RT-PCR) using the rice ubiquitin gene as an internal control (Figure 4(a)). *GOX* transcript started accumulating within 2 h of wounding and peak amplification levels were seen around 8–12 h after wounding. As expected, uninduced and wt plants did not show any amplification of *GOX* cDNA. The expression levels of *GOX* gene upon induction also correlated with the GOX enzymatic activity and $H_2O_2$ accumulation (Figure 2).

Defense-related or *PR* gene induction has been recognized as a down-stream event activated by the oxidative burst (Chen et al., 1993; Ryals et al., 1996; Shirasu et al., 1997). To investigate the effect of $H_2O_2$ generated by wounding, on defense-related gene expression, we analyzed the expression of the chitinase *RCH10* (Zhu et al., 1991), *PIR2* (thaumatin-like), and *PIR3* (peroxidase) (Schweizer et al., 1998) genes in the *PAL::GOX* plants (Figure 4(b)). Upon wounding of *PAL::GOX* plants both *PIR2* and *RCH10* were induced as early as 2 and 4 h, respectively, and accumulated to higher levels as compared to control plants. Interestingly, levels of peroxidase *PIR3* transcript that has been suggested to function in the plant defense response (Esnault & Chibber, 1997), was inhibited in *PAL::GOX* plants. By contrast, *PIR3* was induced 4 h post-wounding in the wt control and showed maximum levels at 12 h after wounding. This result suggests that either the GOX protein, or *GOX*-generated $H_2O_2$ causes repression of *PIR3* expression.

### *GOX expression leads to enhanced resistance to bacterial and fungal pathogens*

Both $H_2O_2$ and GOX are known to inhibit growth of bacterial and fungal pathogens under *in vitro* conditions (Frederick et al., 1990; Wu et al., 1995; Murray et al., 1997). We tested the effect of $H_2O_2$ and GOX on the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and the rice blast pathogen *Magnaporthe grisea*. Bacterial growth and fungal spore germination, were completely inhibited at a concentration of 150 μM $H_2O_2$, and 0.1 and 0.5 units GOX/ml, respectively (Table 1).

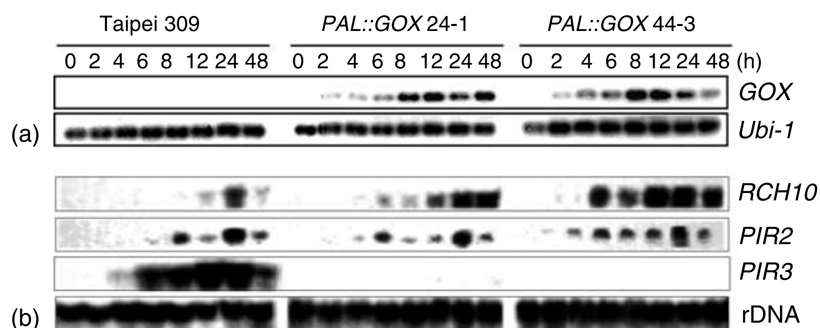


*Figure 4.* *GOX* gene induction and defense gene activation in *PAL::GOX* plants. (a) Total RNA was extracted from wounded leaf tissues of transgenic and wt plants 0–48 h post-wounding. RT-PCR was carried out using *GOX* primers described earlier and amplification of rice ubiquitin (*Ubi-1*) cDNA served as an internal control. Lines used were wt Taipei 309 and *PAL::GOX* transgenic lines 24-1 and 44-3. (b) Northern blot analysis showing differential activation of *PR* genes *RCH10* (chitinase), *PIR2* (thaumatin-like) and *PIR3* peroxidase in wt Taipei 309 and *GOX*-expressing lines 24-1 and 44-3. The blot was sequentially probed with the indicated genes and rDNA was used as an internal control for loading.

*Table 1.* *In vitro* inhibition of *Xoo* growth and *M. grisea* spore germination by $H_2O_2$ and GOX

| Pathogen | $H_2O_2$ (μM) | | | | | | Glucose oxidase (units/ml) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 50 | 100 | 150 | 200 | 0 | 0.001 | 0.01 | 0.1 | 0.5 | 1.0 |
| *Xoo*[a] | 100 | 89 | 43 | 18 | 0 | 0 | 100 | 100 | 51 | 0 | 0 | 0 |
| *M. grisea*[b] | 100 | 96 | 64 | 29 | 0 | 0 | 100 | 100 | 72 | 14 | 0 | 0 |

[a] $10^4$ cells/ml of the *Xoo* race 6 strain PXO99A were incubated in liquid medium with 10 g/l glucose and various amounts of $H_2O_2$ or *A. niger* GOX at 28°C for 48 h. Bacterial growth (%) in $H_2O_2$ or GOX containing medium calculated as compared to 100% growth in control medium without GOX/$H_2O_2$ was determined by measuring absorbance at 600 nm.

[b] $10^5$ spores/ml of the *M. grisea* isolate CAL-1 were germinated in 50 mM sodium acetate, pH 7.0, with 10 g/l glucose and various amounts of $H_2O_2$ or GOX at 25°C for 24 h. Percentage spore germination in $H_2O_2$ or GOX buffer was as compared to 100% germination in medium with buffer control.

To determine the resistance status of *GOX* expressing transgenic plants to a bacterial pathogen, 6-week-old *PAL::GOX* and control plants were inoculated with the *Xoo* race 6 strain PXO99A. Severity of infection was determined by measuring the lesion size and bacterial growth 12 days post-inoculation (dpi). Since wounded leaves of *PAL::GOX* plants also develop disease-like lesions, we included another set of plants that were wounded but not exposed to the pathogen (Figure 5(a)). Lesion size in the *PAL::GOX* transgenic plants inoculated with pathogen or post-wounding was comparable suggesting that both are equally effective in inducing expression of the transgene. However, in comparison to the transgenic plants, control plants inoculated with pathogen showed bigger spreading type lesions indicative of their susceptible nature. This was further confirmed by quantifying bacterial growth in control and transgenic plants at 0–12 dpi. In comparison to the control plants, a 100-fold reduction in bacterial count was observed in *PAL::GOX* transgenic plants (Figure 5(b)).

To determine whether *PAL::GOX* plants could confer broad spectrum disease resistance to diverse pathogens, we next tested their resistance to a fungal pathogen that causes blast disease in rice. Fungal infections were carried out by inoculating spores of *M. grisea* isolate CAL-1 and, IC-9 on 20-day-old seedlings. Rice blast infection of wild-type susceptible plants exhibited typical blast lesions (type 4-5) at 4 dpi. By contrast, the appearance of lesions in the GOX-expressing transgenic plants was not only delayed (visible at 5–6 dpi), but also reduced to a type 1-2 lesion size, which is typical of a resistant response (Valent et al., 1991) (Figure 6(a)). Furthermore, subsequent disease manifestation was more severe in the susceptible control plants with the lesions spreading rapidly (Figure 6(b)). In comparison, the lesions seen in *PAL::GOX* plants remained localized and also did not sporulate. Consequently, the mean area of disease development in the transgenic lines was reduced to 16–35% of that observed in the susceptible control plants. Subsequent to the

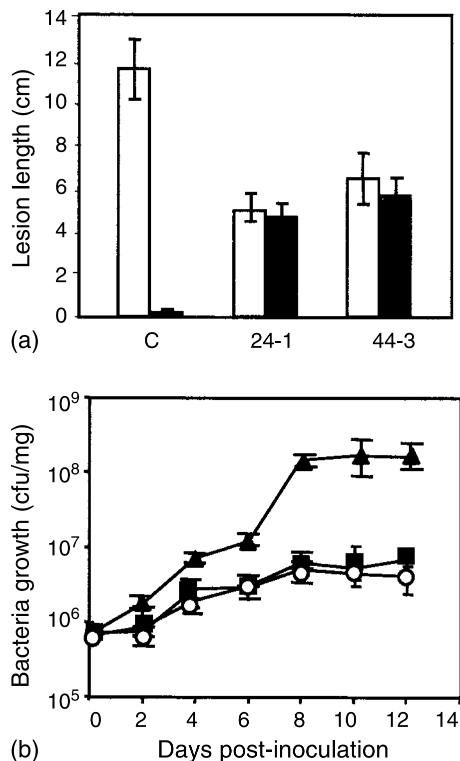


*Figure 5.* Enhanced resistance of *PAL::GOX* transgenic lines to bacterial blight disease. (a) Leaves of wt Taipei 309 (c) or *PAL::GOX* transgenic lines 24-1 and 44-3 were either inoculated with *X. oryzae* (open bar) or wounded (closed bar) and evaluated for lesion size, 12 days after treatment. Data shown is a mean from 24 independent experiments. (b) Density as well as rate of growth of *Xanthomonas*, in wt Taipei 309 (closed triangles) is much higher than that in transgenic lines *PAL::GOX* 24-1 (open circles) and *PAL::GOX* 44-3 (closed squares) expressing GOX. Bacterial density was measured as colony forming units (cfu) per mg of leaf tissue, 0–12 dpi ($n = 3$).

blast infection, *PAL::GOX* transgenic lines showed an increase in GOX activity which correlated with the enhanced resistance observed in these plants (Figure 6(c)).

## Discussion

Engineering broad-spectrum disease resistance in crops has significant agricultural potential (Lamb, 1998). Here, we show that pathogen inducible expression of a fungal gene encoding glucose oxidase, which generates $H_2O_2$ upon oxidation of glucose, confers enhanced resistance towards both bacterial and fungal pathogens of rice. Attempts to express the *GOX* gene under a constitutive promoter resulted in transgenic plants that showed poor germination and did not produce viable $T_1$ seeds. Analysis of transgenic plants expressing *GOX* constitutively was therefore limited to $T_0$ generation and showed that these plants expressed 3–10-fold higher levels of GOX compared to transgenic plants containing the pathogen inducible expression system. Since expression of *GOX* results in a concomitant increase in $H_2O_2$ levels, it is quite likely that such high levels of $H_2O_2$ may have caused metabolic disturbances interfering with normal growth and development of transgenic plants constitutively expressing *GOX*.

A basal level of GOX activity was observed in transgenic plants expressing *GOX* under the control of the *PAL* promoter. Both wounding and pathogen infection led to up-regulation of GOX activity and a corresponding increase in the levels of $H_2O_2$, which resulted in cell death. Since a pathogen/wound inducible promoter was used to control the expression of *GOX* gene, enhanced resistance in these transgenic plants could be triggered by either of these responses and was equally effective. Wounding of transgenic plants prior to challenge with a pathogen did not enhance the resistance status of such plants further, suggesting that pathogen infection alone was sufficient to confer enhanced resistance in these plants.

It has been shown that plants produce $O_2^-$ and $H_2O_2$ through a mechanism similar to the mammalian neutrophil NADPH oxidase system (Auh & Murphy, 1995; Pugin et al., 1997; Kawasaki et al., 1999). Redox enzymes in the plant membrane, transfer electrons from cytosolic NAD(P)H to molecular oxygen to produce $O_2^-$. A plant homolog of the NADPH oxidase gene has been cloned and characterized (Keller et al., 1998). This may account for $H_2O_2$ accumulation in the wild-type plants during wound induction (Figure 2(b)). Rapid generation of $H_2O_2$ during the oxidative burst is a key component of the plant defense response to pathogen challenge (Levine et al., 1994; Alvarez et al., 1998). Elevation of $H_2O_2$ levels can affect plant defense in several ways, presumably by stimulating cross-linking of proline-rich proteins of the cell wall (Bradley et al., 1992; Brisson et al., 1994), and inducing several plant genes involved in cellular protection and defense (Chen et al., 1993; Mehdy, 1994; Lamb & Dixon, 1997). $H_2O_2$ is also required for initiating programmed cell death which leads to SAR (Dangle et al., 1996; Alvarez et al., 1998). We observed that programmed-like cell death was initiated by wounding and pathogen infection (Figures 3(b), 5(b) and 6(a)), and defense-related genes were induced stronger (Figure 4(b)) in

*PAL::GOX* rice plants. These results suggest that elevated $H_2O_2$ levels in transgenic rice, confers disease resistance through an oxidative burst-mediated-like defense response.

It is quite surprising that the peroxidase gene *PIR3* was found to be completely suppressed at the RNA level in *GOX*-expressing plants (Figure 4(b)). Plants contain a multigene family of peroxidases, suggesting that different peroxidases function differentially in diverse plants. For example, viral induced PCD in tobacco was found to be accompanied by the suppression of cytosolic ascorbate peroxidase (*cAPX*) expression at the post-transcriptional level (Mittler et al., 1998), while the *GOX*-expressing potato, induced accumulation of anionic peroxidase at both mRNA and protein level (Wu et al., 1997). Peroxidase genes were also found to be induced at the transcript level in the legume *Stylosanthes humilis* (Curtis et al., 1997), and in rice infected by *Xoo* (Chittoor et al., 1997). Our results suggest that peroxidase might play an important role in $H_2O_2$-mediated defense response. The suppression of peroxidase is likely to reduce the capability of cells to scavenge $H_2O_2$, which in turn stimulates the accumulation of $H_2O_2$ and acceleration of PCD (Mittler et al., 1998). It will be worth studying, whether severely reduced peroxidase levels will result in enhanced disease resistance in peroxidase antisense transgenic plants like in the case of reduced catalase levels (Takahashi et al., 1997; Chamnongpol et al., 1998).

In the present study, we have shown that pathogen inducible $H_2O_2$-manipulation, leads to programmed-like cell death and PR gene activation in transgenic rice. Furthermore, we also show that pathogen inducible expression of heterologous genes is an effective way to confer broad spectrum disease resistance in crop plants and is less likely to cause any developmental abnormalities or metabolic errors.

## Experimental procedures

### *Plasmid construction and rice transformation*

The 1.9 kb region encoding glucose oxidase (*GOX*) was isolated from genomic DNA of *A. niger* by the polymerase chain reaction (PCR) using the primers GO-1 (5′-TTCCCTCATCTGCCCATCAT-3′) and GO-2 (5′-ATACCACTCACTGCATGGAA-3′) designed according to the published sequence (Frederick et al., 1990). The gene was sequenced and cloned into the vector pUC18 under the control of the rice phenylalanine-ammonia lyase promoter (Zhu et al., 1995), yielding the plasmid pPAL-GOX. The plasmid along with pMON410 carrying a hygromycin resistance marker (a gift from Monsanto Company) were co-transformed into rice (*Oryza sativa* L. ssp. *Japonica* cv. Taipei 309) using particle bombardment as described (Chen et al., 1998). Independent *PAL::GOX* transgenic $T_0$ and $T_1$ lines were screened by Southern blot analysis using a 0.8 kb *Pst*I/*Sal*I *GOX* fragment from the plasmid pPAL-GOX or by PCR using the primers GOX-1 (5′-CGGCCGCACGGTCGACTACATCAT-3′) and GOX-2 (5′-GGCATCGGAGCGCACTTGGTCTT-3′) to amplify a 650 bp GOX fragment. Two independent transgenic lines *PAL::GOX* 24-1 and *PAL::GOX* 44-3 were used for molecular analysis and disease resistance assays while wild-type (wt) and vector transformed transgenic plants (381m) were used as the controls.

### *Western hybridizations*

For GOX protein immunodetection, total protein from wound induced (12 h) transgenic, vector control or wt plants, were electrophoresed on 8% SDS-polyacrylamide gels, followed by electrotransfer to Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ). The immunoblots were developed with antibody raised against *A. niger* glucose oxidase (kindly provided by Dr John Markwell, University of Nebraska, USA) using the enhanced chemiluminescence immunodetection procedure (Amersham, Piscataway, NJ).

### *GOX activity, $H_2O_2$ levels and cell death*

GOX activity was assayed using the *o*-dianisidine colorimetric method (Sigma assay procedure for product G-7016, Sigma, St. Louis, MO). Hydrogen peroxide was measured using the titanium tetrachloride precipitation method as described by Brennan and Frenkel (1977). Briefly, wounded leaf tissue was homogenized in cold acetone and filtered to remove cell debris. Two milliliters of titanium reagent (20% titanic tetrachloride in conc. HCl) was added to 20 ml of the tissue extract, followed by 4 ml of conc. ammonium hydroxide to precipitate the titanium-peroxide complex. The precipitate was solubilized in 15 ml 2 N sulfuric acid, washed several times with acetone and, brought to a final volume of 20 ml with water to measure absorbance

at 415 nm. Cell death was detected using trypan blue staining (Koch & Slusarenko, 1990).

*Northern and RT-PCR analysis*

Total RNA was isolated from leaf tissues using the TRIZOL reagent and following manufactures instructions (GIBCO BRL Life Technologies, Gaithersburg, MD). Approximately 20 μg of total RNA was electrophoresed, blotted onto Hybond-N+ membranes (Amersham, Piscataway, NJ) and probed either with 0.8 kb *Pst*I/*Sal*I *GOX* fragment from the plasmid pPAL-GOX, a 1.2 kb *Nsi*I/*Hin*dIII fragment of *RCH10* (Zhu & Lamb, 1991), a 800 bp *Eco*RI/*Xho*I fragment of *PIR2* cDNA (thaumatin-like) or a 1.5 kb *Eco*RI/*Xho*I fragment of *PIR3* cDNA (peroxidase) (Schweizer et al., 1998). These probes were labeled by random priming method (Sambrook et al., 1989). Hybridization was done for 16–24 h in Church buffer (Church & Gilbert, 1984) and the membranes were washed twice for 20 min at room temperature in 2× SSC and 0.1% SDS followed by two washes for 15 min at 65°C in 1× SSC and 0.1% SDS.

RT-PCR analysis was carried out as described before (Kinoshita et al., 1992). Amplifications were carried out using *GOX* primers GOX-1 and GOX-2 or ubiquitin primers Rubi-1 (5′-GACGGACGCACCCTG GCGAACTAC-3′) and Rubi-2 (5′-TGCTGCCAATTA CCATATACCACGAC-3′) (Nishi et al., 1993).

*Bacterial blight assays*

*In vitro* growth inhibition of *Xoo* by GOX or $H_2O_2$ was assayed in liquid medium containing 10 g/l peptone, 10 g/l glucose, 5 g/l sucrose and 1 g/l sodium glutamate, pH 7.0. Various amounts of *A. niger* GOX or $H_2O_2$ (Sigma, St. Louis, MO) were added and $10^4$ cells/ml of the *Xoo* race 6 strain PXO99A were shake-incubated at 28°C for 48 h. The bacterial growth was determined by measuring absorbance at 600 nm. Bacterial inoculations were done by leaf-clip inoculation of 12–15 plants, each of *PAL::GOX* 24-1 *PAL::GOX* 44-3, wt and vector transformed control, with $10^9$ cells/ml *Xoo* race 6 stain PXO99A. Bacterial colony-forming units (cfu) were estimated as described (Song et al., 1995).

*Rice blast assays*

Infection assays with *M. grisea* were carried out as described by Valent et al. (1991). Briefly, 20-day-old $T_1$ and wt plants were inoculated with $3 \times 10^5$ spores/ml of *M. grisea* and the plants were incubated for 12–14 h in a dew chamber (Percival, USA) maintained at 26°C and 90% relative humidity. Subsequently plants were shifted to a growth chamber maintained at 28°C and 90% relative humidity. Blast symptoms and disease development were monitored over a period of 14 dpi and the lesion types were scored as described (Valent et al., 1991). At least 15 plants were scored for each line.

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