Rice *oxalate oxidase* gene driven by green tissue-specific promoter increases tolerance to sheath blight pathogen (*Rhizoctonia solani*) in transgenic rice

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SUMMARY

Rice sheath blight, caused by the necrotrophic fungus *Rhizoctonia solani*, is one of the most devastating and intractable diseases of rice, leading to a significant reduction in rice productivity worldwide. In this article, in order to examine sheath blight resistance, we report the generation of transgenic rice lines overexpressing the rice *oxalate oxidase 4* (*Osoxo4*) gene in a green tissue-specific manner which breaks down oxalic acid (OA), the pathogenesis factor secreted by *R. solani*. Transgenic plants showed higher enzyme activity of oxalate oxidase (OxO) than nontransgenic control plants, which was visualized by histochemical assays and sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Transgenic rice leaves were more tolerant than control rice leaves to exogenous OA. Transgenic plants showed a higher level of expression of other defence-related genes in response to pathogen infection. More importantly, transgenic plants exhibited significantly enhanced durable resistance to *R. solani*. The overexpression of *Osoxo4* in rice did not show any detrimental phenotypic or agronomic effect. Our findings indicate that rice OxO can be utilized effectively in plant genetic manipulation for sheath blight resistance, and possibly for resistance to other diseases caused by necrotrophic fungi, especially those that secrete OA. This is the first report of the expression of defence genes in rice in a green tissue-specific manner for sheath blight resistance.

INTRODUCTION

Sheath blight of rice, the second most devastating disease after rice blast, leading to a reduction in grain yield and quality, is caused by the basidiomycetous necrotrophic fungus *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk]. The pathogen is mainly soil water borne, has a wide host range and can infect plants belonging to more than 32 plant

families (Gangopadhyay and Chakraborty, 1982). In addition to the host range and variability of the pathogen, the major constraint in sheath blight management is the inability to identify genetic sources, from either cultivated or wild varieties of rice, which provide adequate levels of durable and inheritable sheath blight resistance (Bonman *et al.*, 1992). Consequently, the generation of the sheath blight resistance trait of rice by traditional breeding processes is not yet possible. Hence, the deployment of transgenic cultivars by the incorporation of defence-related genes may be the most effective strategy to reduce yield losses caused by sheath blight.

The different genetic strategies which have been undertaken to date to generate transgenic rice plants conferring enhanced resistance to sheath blight have involved the constitutive expression of pathogenesis-related (PR) proteins, such as PR-3 (chitinases) (Baisakh *et al.*, 2001; Datta *et al.*, 2000, 2001; Kumar *et al.*, 2003; Lin *et al.*, 1995; Ramana Rao *et al.*, 2011; Sridevi *et al.*, 2003; Sriprya *et al.*, 2008) and PR-5 (thaumatin-like proteins) (Datta *et al.*, 1999) or plant defensin genes, such as *Dm-AMP1* (*Dahlia merckii*-antimicrobial protein 1) (Jha *et al.*, 2009) and *npr1* (*non-expressor of PR-1*) (Cao *et al.*, 1998), *puroindoline* (Krishnamurthy *et al.*, 2001) and nonspecific lipid transfer proteins (ns-LTPs) (Patkar and Chattoo, 2006) under the control of the cauliflower mosaic virus (*CaMV*) 35S or *ubiquitin* promoter. The simultaneous co-expression of some PR proteins, such as rice chitinase (*chi11*) and β -1,3-glucanase (Sridevi *et al.*, 2008), *chi11* and thaumatin-like protein (Kalpana *et al.*, 2006), and the maize ribosome-inactivating protein gene *MOD1* and a rice basic chitinase gene *RCH10* (Kim *et al.*, 2003), under different constitutive promoters, such as *CaMV* 35S, *ubiquitin*, *rbcS* and *Act1* (*actin 1*), showed enhanced resistance against sheath blight infection. In order to minimize the metabolic load caused by the constitutive expression of the transgene and to reduce the effectiveness of sheath blight infection, the expression of target genes at the site of infection, such as the sheath for *R. solani*, is highly desirable. The present study aimed to develop transgenic rice plants expressing the necessary target gene for sheath blight resistance in a tissue-specific manner.

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Oxalic acid (OA) is a nonhost-specific toxin, secreted early in the pathogen–plant interaction and accumulated during the developmental process of infection (Dutton and Evans, 1996). The rice sheath blight pathogen *R. solani* also produces OA in advance of mycelial growth. Virulent isolates of *R. solani* produce more OA than do less virulent isolates (Nagarajkumar *et al.*, 2005). During pathogenesis, OA can cause the acidification of host tissues, sequestration of calcium from host cell walls and formation of calcium oxalate crystals, which weaken cell walls and thereby allow polygalacturonase to cause degradation more rapidly in a synergistic response (Dutton and Evans, 1996).

OA can be degraded by germin-like oxalate oxidase (OxO) enzymes. OxO enzymes degrade OA and generate CO₂ and hydrogen peroxide (H₂O₂) (Lane, 1994). H₂O₂ is a reactive oxygen species, involved in the plant defence response mechanism. In relation to disease resistance, OxO-generated H₂O₂ may function as a secondary messenger in the activation of phytoalexin biosynthetic pathways, hypersensitive response (HR), systemic acquired resistance (SAR) and *PR* gene expression in plants (Carter *et al.*, 1998). H₂O₂ also plays an important role in senescence and wound response in plants. H₂O₂ generation was detected at wound sites within 1 h after wounding in tomato leaves. The wound-generated H₂O₂ in the veins may also play a defensive role against bacteria, fungi or viruses, as they invade leaves wounded by herbivores (Orozco-Cardenas and Ryan, 1999). It has been shown that wounding promotes H₂O₂ production for the activation of expression of defence genes and wound healing (Orozco-Cardenas *et al.*, 2001). The induction of senescence is accompanied by an increase in endogenous H₂O₂ content in rice leaves and in rye grass leaves (Hung and Kao, 2004; Le Deunff *et al.*, 2004). It has been reported that 14 senescence-associated genes (SAGs) are induced by H₂O₂ treatment in *Arabidopsis thaliana* (Balazadeh *et al.*, 2011). The accumulation of H₂O₂ during ageing coincides with increases in both oxalate level and OxO activity (Davoine *et al.*, 2001). Germin-like OxO enzymes belong to the cupin superfamily and have been effectively used in the engineering of plant disease resistance (Dunwell *et al.*, 2000). The transgenic expression of wheat OxO in American chestnut callus tissue prevents the reduction in lignin content in oxalate-containing medium (Welch *et al.*, 2007). Soybean plants expressing an active oligomeric OxO from the wheat germin gene have been shown to be resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum* (Donaldson *et al.*, 2001). Barley OxO expression has also been reported to confer fungal resistance in transgenic peanut (Livingstone *et al.*, 2005). Genome analyses of rice showed four tandemly duplicated *oxo* genes in chromosome 3, *Osoxo1–Osoxo4*, with *Osoxo4* playing a role in disease resistance (Carrillo *et al.*, 2009). In cereals, particularly in rice, flag leaf and sheath tissues play a key role in providing nutrition to plant growth and grain filling. Sheath blight infection takes place in sheath tissues and moves

vertically, causing the disruption of metabolic functions and, eventually, yield loss.

In this study, we have generated transgenic rice plants showing resistance against *R. solani* by overexpressing the rice oxalate oxidase gene (*Osoxo4*) under the control of the green tissue-specific promoter *P_{D540}*. Molecular [real-time polymerase chain reaction (PCR)] and biochemical (OxO activity assay, OA tolerance assay) analyses showed higher expression of *Osoxo4* in transgenic than in nontransgenic plants, correlating with 50% resistance to *R. solani*, as demonstrated by bioassay. Transgenic plants showed higher activity of OxO visualized by histochemical and in-gel assay. Hence, this article, which reports the design of transgenic rice plants to stop the vertical movement of sheath blight disease by the introduction of the green tissue-specific promoter-driven *Osoxo4* gene, represents a significant contribution to the management of sheath blight disease.

RESULTS

Generation, screening and integration of transgenic *Osoxo4*-overexpressing line

Initially, the cassette pBI-*P_{D540}*-*gus* was introduced into rice by biolistic method to check the green tissue specificity of the promoter [Fig. 1, part (i)]. β -Glucuronidase (GUS) histochemical assay of the PCR-positive (*gus* gene-specific) plants clearly showed that the promoter drives gene expression only in green tissues [Fig. 1, part (iii)]. After the confirmation of promoter function and specificity, 5000 mature embryo explants were bombarded with a mixture of the transformation cassette with *Osoxo4* driven by *P_{D540}* [Fig. 1, part (ii)] and the selectable marker gene cassette with the *hygromycin phosphotransferase* (*hpt*) gene. Thirty-five putative T₀ transgenic plants were obtained from several transformation experiments. Primary screening of positive transformants was performed by *Osoxo4* gene-specific PCR using the junction primer as stated in experimental procedures. PCR-positive plants were selected for Southern blot analysis. The result of Southern blot hybridization showed stable integration of the 1.5-kb *Osoxo4* transformation cassette into transgenic plants (data not shown).

Expression analysis of *Osoxo4* in transgenic plants

Overexpression of the *Osoxo4* gene in transgenic plants was analysed by real-time PCR using gene-specific primers. *Osoxo4* transcripts were substantially elevated in different *Osoxo4* overexpressing plants (PON-1, PON-4, PON-5 and PON-16), whereas a very low level of *Osoxo4* transcript was detected in the nontransgenic control plants (Fig. 2A). All transgenic plants showed variable fold induction of the mRNA level over that of control plants. The plants PON-1 and PON-5 showed 7.78- and 6.72-fold higher expression, respectively, of the *Osoxo4* gene than

Fig. 1 (i) The P_{D540} promoter:*gus* construct for tissue specificity checking. (ii) The rice *oxalate oxidase 4* (*Osoxo4*) gene construct (1.5 kb) for the generation of the *Osoxo4*-overexpressing line. (iii) Histochemical β -glucuronidase (GUS) staining of different parts of rice plants transformed with the P_{D540} :*gus* construct, showing blue stain only in green parts, indicative of the green tissue specificity of the P_{D540} promoter. (A) Mature panicle. (B) Root. (C) Young panicle. (D) Transverse section (TS) of sheath. (E) TS of leaf. (F) Ligule and leaf. (G) Mature seed. (H) Longitudinal section (LS) of leaf. Each bar represents 2 mm.

that of the nontransgenic controls, whereas a slightly lower level of fold induction (2.87- and 3.09-fold) was observed in PON-4 and PON-16. The result indicates that the *Osoxo4* gene shows significantly higher expression in transgenic plants PON-1, PON-4, PON-5 and PON-16 than in nontransgenic control plants ($P < 0.0001$).

Elevated expression of *phenylalanine ammonia lyase* (*PAL*) and *PR* genes in *Osoxo4*-overexpressing plants

Quantitative real-time PCR was used to measure the transcript level of *PAL* and *PR* gene *RC24* in *Osoxo4*-overexpressing plants

and control plants after inoculation with *R. solani*. The transcript of *PAL* was shown to be significantly induced on pathogen infection in all four transgenic plants in comparison with the nontransgenic control plant, ranging from 10- to 96-fold ($P < 0.0001$) (Fig. 2B). The *PR* gene-like *RC24* transcript was also measured, and showed an enhanced level (27–58-fold) of transcript accumulation in over-expressing plants when compared with control plants (Fig. 2C).

Histochemical staining

OxO activity was histochemically detected by a simple staining procedure with the substrate 4-chloro-1-naphthol. Dark blue

Fig. 2 (A) Relative quantity of rice *oxalate oxidase 4* (*Oxoxo4*) mRNA in leaves of overexpressing T_0 lines (PON-1, PON-4, PON-5 and PON-16) and wild-type (WT) lines as determined by real-time polymerase chain reaction (PCR). (B) Enhanced expression of *phenylalanine ammonia lyase* (PAL) gene in *Oxoxo4*-overexpressing lines. (C) Relative expression of *RC24* gene in overexpressing lines. mRNA was isolated from leaves 2 days after inoculation with *Rhizoctonia solani*. All the results are the mean $\pm$ standard error (SE) of three independent experiments.

precipitation was observed in the microcentrifuge tube containing staining solution and transgenic leaves incubated overnight. Control nontransgenic leaves in staining solution did not show any blue precipitation (Fig. 3). No blue precipitation was observed in

Fig. 3 Oxalate oxidase (OxO) activity staining in transgenic and wild-type (WT) leaves. (A) Blue precipitation of 4-chloro-1-naphthol shows overexpression in transgenic leaves. (B) Light microscopy of histochemical detection of OxO activity. Violet blue coloration in the vein region indicates the presence of OxO activity in transgenic leaves. Magnification bars represent 2 mm length.

staining solution containing roots of transgenic plants because of the green tissue-specific nature of expression of the gene driven by the P_{DS40} promoter. No blue staining occurred in the solution without OA, which clearly demonstrates the substrate specificity of the enzyme produced by the introduced gene. Blue stain was mainly found in the vein region of the leaf section, which shows that rice OxO is secretory in nature.

OxO activity assay

In-gel assay of OxO activity was performed by running sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with protein extracts from T_1 transgenic and nontransgenic plant leaves, followed by incubation of the gel in developer solution overnight. A clear dark purple–blue band was detected in the lanes corresponding to transgenic plants, whereas a very faint band was detected in the lane corresponding to the nontransgenic control (Fig. 4A). The enzyme OxO retains its activity in SDS-PAGE. From this, it was established that rice OxO is SDS tolerant. When the protein extracts were heated prior to loading in SDS-PAGE and the assay was performed, no bands were developed in-gel (data not shown).

Detection of OxO enzyme activity in solution was carried out by the incubation of protein extracts from transgenic and nontransgenic control plant leaves with OxO solution developer, followed

Fig. 4 Oxalate oxidase (OxO) activity assay in transgenic and wild-type (WT) plants. (A) In-gel (sodium dodecylsulphate-polyacrylamide gel electrophoresis, SDS-PAGE) detection of rice OxO activity in protein samples from the leaves of WT plants and rice *oxalate oxidase 4* (*Osoxo4*)-overexpressing transgenic plants. (B) In-solution detection of OxO activity in transgenic and WT plant lines. The activity was measured using a spectrophotometric assay at an optical density at 555 nm (OD₅₅₅). Values are represented by the mean $\pm$ standard error (SE) of three independent experiments.

by measurement using a spectrophotometric assay and expression as the optical density at 555 nm (OD₅₅₅). A dark purple colour was developed in the case of the protein sample from transgenic plants, whereas the protein from the nontransgenic plant showed a faint purple colour because of endogenous enzyme activity. All transgenic plant lines exhibited significantly ($P < 0.05$) higher OxO activities than the nontransgenic control line (Fig. 4B). Lines 1, 4 and 5 showed OD values of 2.91-, 2.54- and 2.19-fold greater, respectively, than the nontransgenic line. The OD₅₅₅ value for line 16 was almost four times higher than that of the nontransgenic line.

Resistance to OA

OA can cause yellowing and death of plant tissue on exposure. Two different concentrations (50 and 100 mM) of OA were applied to excised leaves to determine whether the *Osoxo4* transgenic plants showed increased resistance to the damaging effects of OA. Transgenic rice plants showed increased tolerance to exogenous OA at both concentrations in comparison with nontransgenic plants (Fig. 5). *Osoxo4* transgenic plants showed a larger green

Fig. 5 Tolerance to exogenous oxalic acid (OA). Percentage of green area remaining after treatment with 50 and 100 mM OA in transgenic and wild-type (WT) plant leaves. Bars represent the means with standard error for five replicates.

area in the leaf section after 30 min and 1 h of treatment with 50 and 100 mM OA than did nontransgenic plants. There was a significant difference ($P < 0.0001$) between transgenic and nontransgenic plants when the measured green area was compared after treatment. However, there was no significant difference between the nontransgenic and transgenic plants treated with HCl solution at the same pH as OA (50 and 100 mM).

Osoxo4-overexpressing lines with increased resistance to *R. solani*

In the detached leaf bioassay, the numbers of infection cushions in transgenic and nontransgenic plants were counted under a stereomicroscope, 72 h after inoculation (hai). The number of infection cushions developed in transgenic lines was significantly lower ($P < 0.0001$) than that in the leaves of control transgenic plants (Fig. 6A). Furthermore, the leaves of transgenic plants showed a reduction in the size of the lesions, with characteristic browning around the lesions, demonstrating effective restriction of pathogen invasion (Fig. 6C).

The transgenic *Osoxo4*-overexpressing rice lines and nontransgenic control plants were subjected to bioassay with a virulent Hyderabad isolate of *R. solani* AG1-1A. The transgenic lines showed an increased level of sheath blight resistance, as demonstrated by a low sheath blight score in comparison with nontransgenic control plants (Fig. 7). The mean disease scores of transgenic lines were significantly lower ($P < 0.05/P < 0.01/P < 0.001$) than that of the nontransgenic control line at 14 and 21 days post-inoculation (dpi). The result was not significant for 7 dpi in lines 4, 5 and 16, whereas line 1 showed a significantly lower ($P < 0.05$) disease score than the nontransgenic control at 7 dpi.

Osoxo4-overexpressing lines do not show any phenotypic cost or agronomic imbalance

In order to investigate the effect of overexpression of *Osoxo4* in rice on its phenotype and agronomic performance, five different

Fig. 6 (A) Detached leaf bioassay shows reduced infection cushion formation in transgenic lines (PON-1, PON-4, PON-5 and PON-16) than in the wild-type (WT). The values are shown as the mean of 10 plants from each line $\pm$ standard error (SE). Experiments were replicated three times. (B) A stereomicroscopic enlarged view of a portion of infected leaf, showing infection cushions (indicated by black arrows). Magnification bar represents 2 mm. (C) Representative images of reduced lesion formation in transgenic leaves (PON-1, PON-4, PON-5, PON-16) relative to WT in the detached leaf bioassay.

important phenotypic and agronomic parameters were analysed for the overexpressing line and control nontransgenic counterpart (data not shown). Plant heights were taken from the soil surface to the tip of the tallest panicle. There were no significant difference in plant height ($P = 0.8665$) between the overexpressing lines and nontransgenic control line. The panicle number per plant, seeds per panicle and weight of 100 seeds were measured and showed no significant difference between the transgenic overexpressing line and nontransgenic control ($P = 0.0694$ – 0.7856). There was no

significant difference in germination percentage between the transgenic and nontransgenic seeds, and no change in agronomic performance.

DISCUSSION

The introduction of high-yielding varieties (HYVs) and increased use of nitrogen fertilizer have caused the rice plant to become more vulnerable to sheath blight disease (Savary *et al.*, 1995). The

Fig. 7 Overexpression of rice *oxalate oxidase 4* (*Osoxo4*) enhances resistance against the sheath blight pathogen *Rhizoctonia solani*. (A) Disease severity was measured as the relative lesion height and expressed as the percentage disease index (PDI) in transgenic line and wild-type (WT) plants. The values are shown as the mean $\pm$ standard error (SE) ($n = 10$). (B) Disease symptoms in transgenic (T) and WT plants. (C) Zoomed view of a portion of tillers showing symptoms from mock (not inoculated) (M), WT and transgenic (PON-1-7, PON-4-8, PON-5-1, PON-16-3) plants.

wide host range of the pathogen, its ability to survive in soil for a long time with the formation of sclerotium and its high genetic variability make resistance breeding difficult. Consequently, the generation of a sheath blight resistance trait of rice by traditional breeding processes is not yet possible. There is also a lack of effective fungicides to control or eradicate sclerotium, which is otherwise able to infect healthy rice plants in the next crop season. An alternative strategy could be the activation of the

plant's own defence system at an early stage of infection by the expression of plant defence response genes in a green tissue-specific manner.

Fungal pathogens are able to produce toxic compounds that influence plant metabolism and play an important role in fungal pathogenicity. OA is a nonhost-specific toxin secreted by several fungal species (Bateman and Beer, 1965). OA released by necrotrophic fungi, including *R. solani*, helps fungi to colonize sites of

infection and impedes subsequent downstream defence response signalling. However, H_2O_2 , produced as the breakdown product of OA, aids in plant defence via cell wall modification through oxidative cross-linking of lignin and proteins, activation of gene expression for transcription-dependent defence and direct action as an antimicrobial agent to inhibit pathogen development (Lamb and Dixon, 1997). As OA is released at the very initial stage of infection by *R. solani*, degradation of OA and the subsequent induction of the plant's own defence response would be very effective in the prevention of sheath blight infection.

Earlier reports have shown tissue-specific promoter expression in rice, e.g. green leaf tissue-specific promoter- or pith tissue-specific promoter-driven *Bt* gene expression (Datta *et al.*, 1998). The *cis*-acting 544-bp DNA element, immediately upstream of the D540 translational start site, can drive the expression of the *gus* reporter gene only in green tissues, such as sheath, leaf and young panicle, and has been designated as *P_{D540-544}* (Cai *et al.*, 2007). Therefore, the expression of the rice *oxo4* gene under the control of the *P_{D540-544}* promoter region would help to degrade the OA released by *R. solani* at the site of infection. As such degradation would happen at the onset of the infection process, it would not only decrease the effectiveness of *R. solani* infection by impeding the colonization of the fungus, but also induce an HR, followed by the triggering of SAR and the subsequent induction of *PR* genes.

In this study, we initially investigated the expression of the *P_{D540-544}* *cis*-acting promoter element cloned from indica rice cultivar IRBB21 available from the Directorate of Rice Research, Hyderabad, India, in green tissues of the sheath blight pathogen-susceptible cultivar *Oryza sativa* L. ssp. *indica* var. Pusa Sugandhi II (PSII) [Fig. 1, part (iii)] through histochemical staining of the *gus* gene cloned under the control of *P_{D540-544}*. The images clearly indicate that *P_{D540-544}* is strongly expressed in all green tissues, such as the leaf, sheath, ligule and lemma-palea of the young panicle, but not in nongreen tissues, such as the lemma-palea of the mature panicle, endosperm, embryo and root. We developed rice lines expressing the *Osoxo4* gene under the control of the *P_{D540-544}* promoter element. When the *Osoxo4*-overexpressing rice lines were analysed by quantitative real-time PCR, they all showed variable levels of increased mRNA expression (three- to eight-fold) compared with the wild-type (Fig. 2A). All four *Osoxo4*-overexpressing rice lines showed that the *Osoxo4* gene is functional, as evident from histochemical OxO staining analysis (Fig. 3), in-gel assay (Fig. 4A) and in-solution OxO activity assay (Fig. 4B). The transgenic leaves showed the production of a dark blue stain as a result of the production of H_2O_2 from oxalate and its subsequent interaction with 4-chloro-1-naphthol in the presence of horseradish peroxidase. The blue stain was mainly found in the cut end and vein region (vascular region) of the leaves, which indicates the localization of OxO in this apoplastic region. A similar result was obtained in the case of rye grass OxO1 and wheat OxO, where most of the OxO activity was recovered in the cell wall and

in the apoplastic fluid (Davoine *et al.*, 2001; Walz *et al.*, 2008). The stain in the cut end is relatively darker in appearance. One reason for such a result may simply be the greater accessibility to the staining solution. Another reason may be the involvement of OxO in wound (cutting)-induced production of endogenous H_2O_2 (Le Deunff *et al.*, 2004). The result of in-gel and in-solution assays also showed the production of H_2O_2 from oxalate (no band was observed in SDS-PAGE with incubation in developer solution devoid of oxalate). The results of these experiments are similar to the findings in transgenic poplar, transgenic tomato expressing wheat OxO (Liang *et al.*, 2001; Walz *et al.*, 2008) and transgenic peanut expressing barley OxO (Livingstone *et al.*, 2005). From these experiments, it is evident that rice OxO is virtually indistinguishable from other OxOs discovered to date (Dumas *et al.*, 1993; Lane *et al.*, 1993). In our study, only one OxO band was observed in the in-gel activity assay. However, transgenic tobacco and transgenic poplar plants expressing the wheat *oxo* gene showed two bands of two OxO isoforms (Berna and Bernier, 1997; Liang *et al.*, 2001). OA is corrosive and can cause extensive damage to plant tissues (Bateman and Beer, 1965). Exogenous OA-treated rice leaves showed yellowing and bleaching of leaf tissue, which progressed inwardly from the cut end. However, the leaves from *Osoxo4*-overexpressing lines were found to be more tolerant to OA than the nontransgenic control (Fig. 5), indicating that the *Osoxo4*-overexpressing lines may have the ability to detoxify exogenously applied OA. Moreover, a lower concentration (5, 10, 20 or 30 mM) of exogenous OA for a longer exposure time (24 h) was tested in tomato leaves expressing wheat OxO, and greater resistance was exhibited by transgenic than nontransgenic plants (Walz *et al.*, 2008). Therefore, transgenic rice plants may also be able to detoxify OA secreted by the pathogen *R. solani* and subsequently produce H_2O_2 . The H_2O_2 produced in this pathway may play a key role in activating defence responses, such as phytoalexin biosynthesis, the activation of different defence-related genes and cell wall lignification. However, H_2O_2 -mediated cell wall modification depends on another enzyme, peroxidase (Bradley *et al.*, 1992). Proteomic and genetic studies have indicated a complex response of rice to challenge by *R. solani* which involves the simultaneous induction of proteins from multiple defence pathways (Lee *et al.*, 2006). PAL mediates an important regulatory step in the shikimic acid pathway for the synthesis of many phenolic compounds, including lignin, which play a role in the defence mechanism. A rice phytoalexin sakuranetin (flavanones) is also synthesized from this pathway. The mRNA expression levels of the *PAL* gene, a marker gene for SAR induction in plants, and of the *RC24* gene, the early *PR*-marker gene, were also increased relative to the wild-type after *R. solani* infection (Fig. 2), indicating that the degradation of OA released by *R. solani* at the early infection stage induces an HR, followed by the triggering of SAR and subsequent induction of *PR* genes. The transcript levels of a different set of genes, such as *PR5-1*, *defensin* and *SCO* (*Sun-*

flower Carbohydrate Oxidase), were analysed and found to be significantly induced in the leaves of transgenic sunflower plants expressing a wheat OxO relative to the untransformed leaves (Hu *et al.*, 2003). Liang *et al.* (2001) also proposed that PR proteins are involved in the interaction between transgenic poplar overexpressing OxO and *Septoria musiva*.

Transgenic expression of wheat OxO in soybean, sunflower, tomato and oilseed rape led to increased resistance to *S. sclerotiorum* in both laboratory bioassays and field tests (Donaldson *et al.*, 2001; Dong *et al.*, 2008; Hu *et al.*, 2003; Walz *et al.*, 2008). The expression of barley OxO in transgenic peanut led to enhanced resistance to *Sclerotinia minor* (Livingstone *et al.*, 2005). However, no reports have been published of the overexpression of rice OxO in transgenic plants and its effect on the sheath blight pathogen. For the first time, we have studied the resistance level of rice OxO-overexpressing transgenic plants challenged with *R. solani*. *Osoxo4*-overexpressing plants exhibit enhanced resistance to the sheath blight pathogen *R. solani*, as evident from both the detached leaf bioassay (Fig. 6) and whole-plant bioassay (Fig. 7). The detached leaf bioassay (Fig. 6) indicated more than a 2.5-fold reduction in the number of infection cushions for all the four overexpressing rice lines. Infection cushions are aggregates of convoluted hyphae developed from the runner hyphae of *R. solani* (Fig. 6B). The cushions play an important role in the infection by *R. solani* by enzymatic degradation and physical penetration of the leaf surface (Anderson, 1982). On average, a 50% reduction in the percentage disease index (PDI) in *Osoxo4*-overexpressing plants relative to nontransgenic plants was observed. The sheath blight symptoms caused tiller lodging and eventual plant collapse in all nontransgenic control plants 1 month after inoculation, whereas the PON transgenic lines showed a restriction in vertical growth of the disease symptoms. The result of a previous report has shown that sheath blight disease severity is, on average, 25% lower in transgenic rice plants co-expressing ribosome-inactivating protein and a rice chitinase relative to control plants (Kim *et al.*, 2003). A 45% reduction in disease symptoms has been observed in transgenic rice plants expressing a *Rs-AFP2* defensin gene (Jha and Chattoo, 2009).

Constitutive expression can be problematic for several reasons. It may have several adverse effects, such as metabolic load, unexpected consequences on plant growth and development. It may result in enhanced susceptibility to other pathogens (Berrocal-Lobo *et al.*, 2002; Stuijver and Custers, 2001). If a specific transgene is overexpressed at the incorrect time in development, in tissues in which it is not normally expressed, or at very high levels, it can have unexpected consequences on plant growth and development (Potenza *et al.*, 2004). Constitutive expression of rice OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants (Zhang *et al.*, 2008). Therefore, in our study, the use of a green tissue-specific promoter helps OxO to be expressed at the site of infection (mainly sheath),

which is more important, rather than its expression in a constitutive manner. It is also evident from the present study of different phenotypic and agronomic data of transgenic and nontransgenic plants that the tissue-specific overexpression of the gene *Osoxo4* does not adversely affect the growth and yield of transgenic plants. As the yield of transgenic plants did not show any impairment with growth, it is presumed that the nutritional quality will remain consistent, which, in turn, will help in breeding.

Very recently, the draft genome sequence of *R. solani* AG1-1A has been published and 25 pathogen effectors have been identified (Zheng *et al.*, 2013). These newly available resources could provide a better understanding of plant–pathogen interaction, the mechanism of pathogenesis of *R. solani* and the consequences of oxalate degradation in host resistance.

In summary, we have developed sheath blight-resistant rice lines expressing the *Osoxo4* gene under the control of a green tissue-specific promoter *P_{DS40-544}*. As the *oxo4* gene is expressed at the sheath (the site of *R. solani* infection) and at an early stage during the onset of the *R. solani* infection process, the developed lines show enhanced resistance to the fungal disease rice sheath blight. In addition, these lines do not have any negative impact on agronomically important traits. The rice *oxo4* gene may be used to engineer other important crop plants to develop fungal resistance, especially against oxalate-secreting fungi. This transgenic strategy with the *oxo4* gene may also be used for the management of the OA-secreting rice blast fungus, *Magnaporthe oryzae*.

EXPERIMENTAL PROCEDURES

Plant material

Oryza sativa L. ssp. *indica* var. Pusa Sugandhi II (PSII) was used as plant material for this study. It is a susceptible cultivar to the sheath blight pathogen *R. solani*.

Fungal material and culture conditions

The rice sheath blight pathogen *R. solani* AG1-1A (Chinsurah, Hyderabad and Pantanagar isolates) was used in this study. The fungal pathogen was maintained in potato dextrose agar (PDA) medium at 28 °C by subculturing every 14 days. Inocula cultured in PDA medium were transferred into a bottle containing a rice hull–rice grain mixture and incubated at room temperature (28–30 °C) for 7–10 days. The inocula with the rice hull–rice grain mixture were used for bioassay.

Cloning of green tissue-specific promoter

A fragment from +41 to –544 of the green tissue-specific promoter *P_{DS40}* was isolated from the indica rice cultivar IRBB21 by genomic DNA PCR using HotStar HiFidelity DNA Polymerase (Qiagen, Hilden, Germany) and cloned into TOPO vector (Invitrogen, Carlsbad, CA, USA). The fragment was

sequenced for verification. Subsequently, it was subcloned upstream of the *gus* reporter gene in pBI121 at the *HindIII/BamHI* site.

Determination of tissue-specific activity of promoter by histochemical GUS staining

In order to determine the green tissue-specific expression of the P_{D540} promoter, the construct pBI- P_{D540} -*gus* was integrated into rice plants by biolistic transformation. Transgenic plants, confirmed by positive PCR results with *gus* gene-specific primers, were subjected to qualitative GUS assay (by histochemical staining) by immersing different parts, namely sheath, lamina, young panicle, ligule, root, mature panicle and seeds, in GUS staining solution (Jefferson, 1987).

Isolation and cloning of *Osoxo4* gene

Total RNA was isolated from the leaves of young rice seedlings (IRBB21) using the RNeasy Plant Mini Kit (Qiagen). cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). PCR amplification was performed with gene-specific primers using the prepared cDNA as template employing HotStar HiFidelity DNA Polymerase (Qiagen). *BamHI* and *KpnI* restriction sites were used in the forward and reverse primer sequences, respectively. The 690-bp PCR product was cloned into TOPO vector (Invitrogen) and sequenced for verification.

Vector construction

A cassette was constructed in the multiple cloning site (MCS) of the pUC19 vector. The promoter P_{D540} was cloned at the *HindIII/BamHI* site. Downstream of the promoter, the 690-bp *Osoxo4* gene was cloned at the *BamHI/KpnI* site. The *nopaline synthase* (*nos*) terminator sequence was cloned at *KpnI/EcoRI* for the generation of *pUC-P_{D540}-oxo4-nos* (pUC-PON). The constructed vector was used in the transformation. Another vector pGL2, containing the *hpt* marker gene cassette, was used in the present study (Datta *et al.*, 1990).

Rice transformation and tissue culture

The two vector DNAs, pUC-PON and pGL2, were delivered together into the scutella of freshly isolated mature embryos of rice cultivar PSII by a particle delivery system (PDC-1000:He system; BioRad, Hercules, CA, USA) following the protocol described previously (Datta *et al.*, 2001). DNA coating by gold particles was performed according to the manufacturer's instructions. After bombardment, the embryos were transferred to selection medium [Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 8 g/L agar and 50 mg/L hygromycin B solution] as described previously (Datta *et al.*, 1998). Developing calli were subcultured every 2 weeks in fresh selection medium for three to four cycles. The calli were transferred to regeneration medium [MS supplemented with 2 mg/L kinetin and 1 mg/L 1-naphthaleneacetic acid (NAA)] and kept in the light at 28 °C for 2–3 weeks. Regenerated plantlets were transferred to rooting medium for root development. The rooted plantlets were transferred to a glasshouse for further development.

PCR-based screening

DNA was extracted from rice leaves as described previously (Huang *et al.*, 1997). In order to avoid the problem related to the endogenous presence of the *oxo4* gene in rice, we used junction primers (forward primer from the junction of promoter and gene, reverse primer from the junction of gene and terminator) to screen transformants.

Southern blot analysis

The PCR-positive plants were used in Southern blot analysis. Total DNA was isolated from leaves according to a modified Dellaporta method (Dellaporta *et al.*, 1983). The DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher, Franklin, MA, USA). Ten micrograms of DNA were digested with *HindIII/EcoRI* restriction endonucleases (Fermentas, Ontario, Canada). The digested DNA samples were separated by 1% agarose (w/v) gel electrophoresis. DNA transfer to membrane, hybridization and washing were performed according to standard protocols (Sambrook and Russell, 2001). A 690-bp PCR product of *Osoxo4* was used as a probe and was labelled with (α -³²P)dCTP using a DecaLabel™ DNA Labelling Kit (Fermentas). The membrane was exposed for autoradiography.

RNA extraction and cDNA synthesis

Rice leaf tissues were snap frozen in liquid N₂ and total RNA was extracted from 100 mg of frozen leaf samples using Trizol reagent (Invitrogen). RNA pellets were washed with 70% ethanol, dried and dissolved in RNase-free water. RNA samples were treated with recombinant DNase I (Roche Applied Science, Penzberg, Germany) to remove all traces of genomic DNA from the samples. Five micrograms of RNA were used for cDNA synthesis employing a Maxima First Strand cDNA Synthesis Kit (Fermentas).

Quantitative real-time PCR analysis

All quantitative real-time PCRs were performed using a CFX 96 real-time system (BioRad) with Maxima qPCR Master Mix containing SYBR green (Fermentas) according to the manufacturer's instructions. PCR cycling conditions were as follows: initial DNA denaturing steps of 3 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The primers were synthesized to quantify the transcript level of *Osoxo4* (F, 5'-CGACACCGGGAACAGGTACT-3'; R, 5'-GTCTTGCCAACGTTGAACT-3'; accession no. NM_001057501), *pal/zb8* (F, 5'-GTGTTCTGCGAGGTGATGAA-3'; R, 5'-TTCCTCCAAGATGTGCTTC-3'; accession no. X87946.1) and *rc24* (F, 5'-TCTGCTGCAGCCAGTACG-3'; R, 5'-GGAGAAGCATCTGGTCAAG-3'; accession no. X87109.1). Rice *tubulin* gene (F, 5'-GGAGTCACATGCTGCCTAAGGTT-3'; R, 5'-TCACTGC CAGCTTACGGAGG-3'; accession no. X78143) was used as reference to normalize all data. Quantitative variation among different samples was determined using the $\Delta\Delta$ CT method. All the data were analysed using Bio-Rad CFX manager software (BioRad). The mean values for the expression levels of the genes were calculated from three independent experiments.

OxO assay

In-gel detection of OxO activity was performed as described previously (Zhang *et al.*, 1996). Total protein was extracted from 1 g of transgenic

and nontransgenic rice leaves by grinding in liquid N₂ and homogenizing in 2 mL of water, followed by centrifugation at 10 000 *g* for 30 min. The supernatant was treated at 80 °C for 3 min and centrifuged for 10 min at 10 000 *g*. The protein was precipitated with ammonium sulphate at 70% saturation and collected by centrifugation at 10 000 *g* for 10 min. The pellet was dissolved in 200 µL of water and the protein concentration was measured by the method described previously (Bradford, 1976). SDS loading buffer without reducing agent was added to 60 µg of protein sample and loaded directly onto an SDS-PAGE gel without heat treatment. After electrophoresis, the gel was washed at room temperature overnight in OxO gel developer [40 mM succinic acid/NaOH, pH 3.8, 60% (v/v) ethanol, 2 mM OA, 3 units/mL horseradish peroxidase, 50 mg/100 mL 4-chloro-1 naphthol]. In-solution detection of OxO activity was also performed by adding 80 µg of protein sample to 1 mL of OxO solution developer [40 mM succinic acid/NaOH, pH 3.8, 60% (v/v) ethanol, 2 mM OA, 5 units/mL horseradish peroxidase, 20 µL/100 mL *N,N*-dimethylaniline, 8 mg/100 mL 4-antipyrine], incubating for 2 h at 37 °C and measuring OD at 555 nm. The reaction was terminated by the addition of 20 µL of 1 M NaOH before measuring OD. The spectrophotometer was blanked by protein plus control developer without OA.

Histochemical staining of OxO activity

Tissue localization of OxO activity was assayed by a histochemical assay according to a method described previously (Dumas *et al.*, 1995). Sliced leaves from transformed and untransformed rice plants were incubated with OA (2.5 mM) in succinate buffer (25 mM succinic acid, 3.5 mM ethylenediaminetetraacetic acid, pH 4.0) containing 4-chloro-1-naphthol (0.6 mg/mL) as staining reagent at room temperature in the dark overnight. As controls, leaves from transformed and untransformed plants were also incubated in staining solution without OA.

In vitro OA tolerance assay of transgenic leaves

Excised leaves of the same size from five transgenic plants at the maximum tillering stage were treated with 50 and 100 mM exogenously applied OA solution and incubated at room temperature for 1 h. Nontransgenic PSII plant leaves of the same age were used as controls. HCl solution of the same pH (1.7) as the 100 mM OA solution was used as a control. Five replicates were used for each of the transgenic plants. The total leaf area and remaining green area were measured according to a method described previously (Partridge-Telenko *et al.*, 2011). After the time period, the leaves were photographed. The total and remaining green areas of the digital images were measured using the measuring tools of Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA). The ability of transgenic plants to tolerate exogenous OA was calculated as the percentage of green area after 30 min and 1 h.

Bioassay

Bioassay using detached leaves was performed as described previously (Kumar *et al.*, 2003). Sterilized Petriplates were lined with sterile cotton pad and sprayed with sterile distilled water. Sterile glass slides, with their ends inserted into slits cut 6 cm apart on a supporting Whatman 3MM

filter paper (Whatman plc, Kent, UK), were placed inside each Petri plate. A fresh piece of young leaf (6 cm) was detached from each plant. The leaf piece was surface sterilized and two cut ends were inserted into the slits and placed on the slide, keeping the abaxial side up. A 5-mm-diameter mycelial disc from the peripheral region of a 5-day-old PDA culture of *R. solani* was placed in the middle of the leaf surface. The lid was sealed with parafilm and kept at room temperature for 3 days.

Bioassay of transgenic rice plants was performed as described previously (Datta *et al.*, 1999). Five grams of rice hull–rice grain inocula mixture were placed in the middle of the tillers of each plant at the maximum tillering stage, and all the tillers were tied with a rubber band. Sterile water was sprayed every morning to keep the environment highly humid. The symptoms caused by fungal infection were scored after 7, 14 and 21 days using a scale ranging from 0 to 9, which is based on the relative lesion height on the whole plant, according to the Standard Evaluation System for Rice (IRRI, 2002).

Agronomic evaluation of transgenic plants

Ten plants from each of the *Osox4*-overexpressing lines and the nontransgenic control line were evaluated for agronomic performance. Plant height, panicle number per plant, seeds per panicle, weight of 100 seeds and germination percentage were measured for each individual plant. All the data were scored according to the Standard Evaluation System for Rice (IRRI, 2002).

Statistical analysis

The data were analysed using Graphpad prism 5 software (GraphPad Software, La Jolla, CA, USA). One-way and two-way analyses of variance (ANOVAs) were used to compare the differences between the nontransgenic control and the transgenic plants. *P* < 0.05 was considered to be statistically significant.

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REFERENCES

- Anderson, N.A. (1982) The genetics and pathology of *Rhizoctonia solani*. *Annu. Rev. Phytopathol.* **20**, 329–347.
- Baisakh, N., Datta, K., Oliva, N., Ona, I., Rao, G., Mew, T. and Datta, S.K. (2001) Rapid development of homozygous transgenic rice using anther culture harboring rice chitinase gene for enhanced sheath blight resistance. *Plant Biotechnol.* **18**, 101–108.
- Balazadeh, S., Kwasniewski, M., Caldana, C., Mehrnia, M., Zanor, M.I., Xue, G.P. and Mueller-Roeber, B. (2011) ORS1, an H₂O₂-responsive NAC transcription factor, controls senescence in *Arabidopsis thaliana*. *Mol. Plant*, **4**, 346–360.
- Bateman, D.F. and Beer, S.V. (1965) Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. *Phytopathology*, **55**, 204–211.
- Berna, A. and Bernier, F. (1997) Regulated expression of a wheat germin gene in tobacco: oxalate oxidase activity and apoplastic localization of the heterologous protein. *Plant Mol. Biol.* **33**, 417–429.

- Berrocal-Lobo, M., Segura, A., Moreno, M., López, G., Garci'a-Olmedo, F. and Molina, A. (2002) Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiol.* **128**, 951–961.
- Bonman, J., Khush, G. and Nelson, R. (1992) Breeding rice for resistance to pests. *Annu. Rev. Phytopathol.* **30**, 507–528.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.
- Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell*, **70**, 21–30.
- Cai, M., Wei, J., Li, X., Xu, C. and Wang, S. (2007) A rice promoter containing both novel positive and negative cis-elements for regulation of green tissue-specific gene expression in transgenic plants. *Plant Biotechnol. J.* **5**, 664–674.
- Cao, H., Li, X. and Dong, X. (1998) Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. *Proc. Natl. Acad. Sci. USA*, **95**, 6531–6536.
- Carrillo, M.G.C., Goodwin, P.H., Leach, J.E., Leung, H. and Vera Cruz, C.M. (2009) Phylogenomic relationships of rice oxalate oxidases to the cupin superfamily and their association with disease resistance QTL. *Rice*, **2**, 67–79.
- Carter, C., Graham, R.A. and Thornburg, R.W. (1998) *Arabidopsis thaliana* contains a large family of germin-like proteins: characterization of cDNA and genomic sequences encoding 12 unique family members. *Plant Mol. Biol.* **38**, 929–943.
- Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M.F., Oliva, N., Abrigo, E., Khush, G. and Datta, S.K. (1998) Constitutive and tissue-specific differential expression of the cryIA(b) gene in transgenic rice plants conferring resistance to rice insect pest. *Theor. Appl. Genet.* **97**, 20–30.
- Datta, K., Velazhahan, R., Oliva, N., Ona, I., Mew, T., Khush, G., Muthukrishnan, S. and Datta, S.K. (1999) Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. *Theor. Appl. Genet.* **98**, 1138–1145.
- Datta, K., Koukolikova-Nicola, Z., Baisakh, N., Oliva, N. and Datta, S. (2000) *Agrobacterium*-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems. *Theor. Appl. Genet.* **100**, 832–839.
- Datta, K., Tu, J., Oliva, N., Ona, I., Velazhahan, R., Mew, T.W., Muthukrishnan, S. and Datta, S.K. (2001) Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Sci.* **160**, 405–414.
- Datta, S.K., Peterhans, A., Datta, K. and Potrykus, I. (1990) Genetically engineered fertile indica-rice recovered from protoplasts. *Nat. Biotechnol.* **8**, 736–740.
- Davoine, C., Le Deunff, E., Ledger, N., Avise, J.C., Billard, J.P., Dumas, B. and Huault, C. (2001) Specific and constitutive expression of oxalate oxidase during the ageing of leaf sheaths of ryegrass stubble. *Plant Cell Environ.* **24**, 1033–1043.
- Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA miniprep: version II. *Plant Mol. Biol. Rep.* **1**, 19–21.
- Donaldson, P.A., Anderson, T., Lane, B.G., Davidson, A.L. and Simmonds, D.H. (2001) Soybean plants expressing an active oligomeric oxalate oxidase from the wheat gf-2.8 (germin) gene are resistant to the oxalate-secreting pathogen *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* **59**, 297–307.
- Dong, X., Ji, R., Guo, X., Foster, S.J., Chen, H., Dong, C., Liu, Y., Hu, Q. and Liu, S. (2008) Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*). *Planta*, **228**, 331–340.
- Dumas, B., Sailland, A., Cheviet, J.P., Freyssinet, G. and Pallett, K. (1993) Identification of barley oxalate oxidase as a germin-like protein. *C. R. Acad. Sci. III*, **316**, 793–798.
- Dumas, B., Freyssinet, G. and Pallett, K.E. (1995) Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. *Plant Physiol.* **107**, 1091–1096.
- Dunwell, J.M., Khuri, S. and Gane, P.J. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiol. Mol. Biol. Rev.* **64**, 153–179.
- Dutton, M.V. and Evans, C.S. (1996) Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* **42**, 881–895.
- Gangopadhyay, S. and Chakraborty, N. (1982) Sheath blight of rice. *Rev. Plant Pathol.* **61**, 451–455.
- Hu, X., Bidney, D.L., Yalpani, N., Duveck, J.P., Crasta, O., Folkerts, O. and Lu, G. (2003) Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol.* **133**, 170–181.
- Huang, N., Angeles, E., Domingo, J., Magpantay, G., Singh, S., Zhang, G., Kuma-ravadivel, N., Bennett, J. and Khush, G. (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. *Theor. Appl. Genet.* **95**, 313–320.
- Hung, K.T. and Kao, C.H. (2004) Hydrogen peroxide is necessary for abscisic acid-induced senescence of rice leaves. *J. Plant Physiol.* **161**, 1347–1357.
- IRRI (2002) Standard Evaluation System for Rice (SES). Manila, Philippines: International Rice Research Institute.
- Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.
- Jha, S. and Chattoo, B.B. (2009) Expression of a plant defensin in rice confers resistance to fungal phytopathogens. *Transgenic Res.* **19**, 373–384.
- Jha, S., Tank, H.G., Prasad, B.D. and Chattoo, B.B. (2009) Expression of Dm-AMP1 in rice confers resistance to *Magnaporthe oryzae* and *Rhizoctonia solani*. *Transgenic Res.* **18**, 59–69.
- Kalpana, K., Maruthasalam, S., Rajesh, T., Poovannan, K., Kumar, K.K., Kokiladevi, E., Raja, J.A.J., Sudhakar, D., Velazhahan, R. and Samiyappan, R. (2006) Engineering sheath blight resistance in elite indica rice cultivars using genes encoding defense proteins. *Plant Sci.* **170**, 203–215.
- Kim, J.K., Jang, I.C., Wu, R., Zuo, W.N., Boston, R.S., Lee, Y.H., Ahn, I.P. and Nahm, B.H. (2003) Co-expression of a modified maize ribosome-inactivating protein and a rice basic chitinase gene in transgenic rice plants confers enhanced resistance to sheath blight. *Transgenic Res.* **12**, 475–484.
- Krishnamurthy, K., Balconi, C., Sherwood, J.E. and Giroux, M.J. (2001) Wheat puroindolines enhance fungal disease resistance in transgenic rice. *Mol. Plant–Microbe Interact.* **14**, 1255–1260.
- Kumar, K.K., Poovannan, K., Nandakumar, R., Thamilarasi, K., Geetha, C., Jayashree, N., Kokiladevi, E., Raja, J.A.J., Samiyappan, R., Sudhakar, D. and Balasubramanian, P. (2003) A high throughput functional expression assay system for a defence gene conferring transgenic resistance on rice against the sheath blight pathogen, *Rhizoctonia solani*. *Plant Sci.* **165**, 969–976.
- Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Biol.* **48**, 251–275.
- Lane, B. (1994) Oxalate, germin, and the extracellular matrix of higher plants. *FASEB J.* **8**, 294–301.
- Lane, B., Dunwell, J., Ray, J., Schmitt, M. and Cuming, A. (1993) Germin, a protein marker of early plant development, is an oxalate oxidase. *J. Biol. Chem.* **268**, 12 239–12 242.
- Le Deunff, E., Davoine, C., Le Dantec, C., Billard, J.P. and Huault, C. (2004) Oxidative burst and expression of germin/oxo genes during wounding of ryegrass leaf blades: comparison with senescence of leaf sheaths. *Plant J.* **38**, 421–431.
- Lee, J., Bricker, T.M., Lefevre, M., Pinson, S.R.M. and Oard, J.H. (2006) Proteomic and genetic approaches to identifying defence-related proteins in rice challenged with the fungal pathogen *Rhizoctonia solani*. *Mol. Plant Pathol.* **7**, 405–416.
- Liang, H., Maynard, C.A., Allen, R.D. and Powell, W.A. (2001) Increased *Septoria musiva* resistance in transgenic hybrid poplar leaves expressing a wheat oxalate oxidase gene. *Plant Mol. Biol.* **45**, 619–629.
- Lin, W., Anuratha, C., Datta, K., Potrykus, I., Muthukrishnan, S. and Datta, S.K. (1995) Genetic engineering of rice for resistance to sheath blight. *Nat. Biotechnol.* **13**, 686–691.
- Livingstone, D.M., Hampton, J.L., Phipps, P.M. and Grabau, E.A. (2005) Enhancing resistance to *Sclerotinia minor* in peanut by expressing a barley oxalate oxidase gene. *Plant Physiol.* **137**, 1354–1362.
- Nagarajkumar, M., Jayaraj, J., Muthukrishnan, S., Bhaskaran, R. and Velazhahan, R. (2005) Detoxification of oxalic acid by *Pseudomonas fluorescens* strain PfMDU2: implications for the biological control of rice sheath blight caused by *Rhizoctonia solani*. *Microbiol. Res.* **160**, 291–298.
- Orozco-Cardenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl. Acad. Sci. USA*, **96**, 6553–6557.
- Orozco-Cardenas, M.L., Narvaez-Vasquez, J. and Ryan, C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell*, **13**, 179–191.
- Partridge-Telenko, D.E., Hu, J., Livingstone, D.M., Shew, B.B., Phipps, P.M. and Grabau, E.A. (2011) Sclerotinia blight resistance in Virginia-type peanut transformed with a barley oxalate oxidase gene. *Phytopathology*, **101**, 786–793.
- Patkar, R.N. and Chattoo, B.B. (2006) Transgenic indica rice expressing ns-LTP-like protein shows enhanced resistance to both fungal and bacterial pathogens. *Mol. Breed.* **17**, 159–171.

- Potenza, C., Aleman, L. and Sengupta-Gopalan, C. (2004) Targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. *In Vitro Cell. Dev. Biol. Plant*, **40**, 1–22.
- Ramana Rao, M.V., Parameswari, C., Sriprya, R. and Veluthambi, K. (2011) Transgene stacking and marker elimination in transgenic rice by sequential *Agrobacterium*-mediated co-transformation with the same selectable marker gene. *Plant Cell Rep.* **30**, 1241–1252.
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Savary, S., Castilla, N.P., Elazegui, F.A., McLaren, C.G., Ynalvez, M.A. and Teng, P.S. (1995) Direct and indirect effects of nitrogen supply and disease source structure on rice sheath blight spread. *Phytopathology*, **85**, 959–965.
- Sridevi, G., Sabapathi, N., Meena, P., Veluthambi, K., Nandakumar, R., Samiyappan, R. and Muthukrishnan, S. (2003) Transgenic indica rice variety Pusa Basmati 1 constitutively expressing a rice chitinase gene exhibits enhanced resistance to *Rhizoctonia solani*. *J. Plant Biochem. Biotechnol.* **12**, 93–101.
- Sridevi, G., Parameswari, C., Sabapathi, N., Raghupathy, V. and Veluthambi, K. (2008) Combined expression of chitinase and β -1,3-glucanase genes in indica rice (*Oryza sativa* L.) enhances resistance against *Rhizoctonia solani*. *Plant Sci.* **175**, 283–290.
- Sriprya, R., Raghupathy, V. and Veluthambi, K. (2008) Generation of selectable marker-free sheath blight resistant transgenic rice plants by efficient co-transformation of a cointegrate vector T-DNA and a binary vector T-DNA in one *Agrobacterium tumefaciens* strain. *Plant Cell Rep.* **27**, 1635–1644.
- Stuiver, M.H. and Custers, J.H.H.V. (2001) Engineering disease resistance in plants. *Nature*, **411**, 865–868.
- Walz, A., Zingen-Sell, I., Loeffler, M. and Sauer, M. (2008) Expression of an oxalate oxidase gene in tomato and severity of disease caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Plant Pathol.* **57**, 453–458.
- Welch, A.J., Stipanovic, A.J., Maynard, C.A. and Powell, W.A. (2007) The effects of oxalic acid on transgenic *Castanea dentata* callus tissue expressing oxalate oxidase. *Plant Sci.* **172**, 488–496.
- Zhang, J., Peng, Y. and Guo, Z. (2008) Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. *Cell Res.* **18**, 508–521.
- Zhang, Z., Yang, J., Collinge, D.B. and Thordal-Christensen, H. (1996) Ethanol increases sensitivity of oxalate oxidase assays and facilitates direct activity staining in SDS gels. *Plant Mol. Biol. Rep.* **14**, 266–272.
- Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., Ding, L., Wang, Y., Chen, Y., Liu, Y., Sun, Z., Feng, H., Liang, X., Fu, R., Tang, C., Li, Q., Zhang, J., Xie, Z., Deng, Q., Li, S., Wang, S., Zhu, J., Wang, L., Liu, H. and Li, P. (2013) The evolution and pathogenic mechanisms of the rice sheath blight pathogen. *Nat. Commun.* **4**, 1424. doi:10.1038/ncomms2427.