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Development of transgenic rice plants expressing maize anthocyanin genes and increased blast resistance

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Abstract

The functional association of flavonoids with plant stress responses, though widely reported in the literature, remains to be documented in rice. Towards this end we chose a transgenic approach with well characterized regulatory and structural genes from maize involved in flavonoid biosynthesis. Activation of anthocyanin pathway in rice was investigated with the maize genes. Production of purple anthocyanin pigments were observed in transformed Tp309 (a *japonica* rice variety) calluses upon the introduction of the maize regulatory genes *C1* (coloured-1), *R* (red) and the structural gene *C2* (coloured-2, encoding chalcone synthase). In addition, stable transgenic plants carrying the maize *C2* gene under the control of the maize *Ubiquitin* promoter were generated. A localized appearance of purple/red pigment in the leaf blade and leaf sheath of R_0 *C2* transgenic seedlings was observed. Such a patchy pattern of the transgene expression appears to be conditioned by the genetic background of Tp309, which is homozygous for dominant color inhibitor gene(s) whose presence was unravelled by appropriate genetic crosses. Southern blot analysis of the transgenic plants demonstrated that *c2* cDNA was integrated into the genome. Western blot analysis of these primary transgenics revealed the CHS protein while it was not detected in the control untransformed Tp3O9, suggesting that Tp309 might have a mutation at the corresponding *C2* locus or that the expression of this gene is suppressed in Tp309. Further analysis of *C2* transgenics revealed CHS protein only in three out of sixteen plants that were western-positive in the R_0 generation, suggesting gene silencing. Preliminary screening of these R_1 plants against the rice blast fungus *Magnaporthe grisea* revealed an increase in resistance.

Introduction

Flavonoids, the ubiquitous secondary metabolites accumulating in fruits, flowers and foliage are implicated in a wide range of functions in higher plants. These include diverse biological processes such as signaling molecules in different transduction pathways, modulation of hormone responses, pollination, male fertility, plant microbe interactions, plant's defense, UV-B response, biotic and abiotic stress responsive processes. Of the above mentioned functions, the role of flavonoids in biotic stress response as anti-microbial and antiviral compounds is well documented (Jambunathan et al. 1990; Snyder and Nicholson 1990; Bloor 1995; Geibel 1995; Lima et al. 1996; Lo et al. 1996; Malhotra et al. 1996). Not only flavonoids, but also the related proanthocyanidins (polymeric anthocyanidins) have been reported to act as defence compounds in plants (Kodama et al. 1991). The light induced *Sorghum* seedlings show an enhanced accumulation of anthocyanins along with 3-deoxyanthocyanidins during the

infection with *Cochliobolus heterostrophus* (Lo and Nicholson 1998). Similarly, maize silks have been shown to accumulate maysin (C-glycosylflavone) during the infection with corn earworm, *Helicoverpa zea*, and show a direct relationship between increased maysin concentration and plant resistance (Byrne et al. 1996). The levels of sakuranetin (methyl ether of naringenin) under blast infection have been found to increase significantly in a resistant cultivar (Grayer et al. 1995) compared to the susceptible cultivar (Dillon et al. 1997). A transient increase in flavonoid levels *in vivo* thus seems to act as a prophylactic measure against pathogens.

Transgenic approaches uncovered a strong relationship between phenylpropanoid/flavonoid gene expression and a plant's defence response levels. For instance, transfer of bean *Pal* (encoding phenylalanine ammonia-lyase, catalysing the first step of the phenylpropanoid pathway which supplies the precursors for flavonoid synthesis) into tobacco resulted in the sense suppression of *Pal* activity leading to an increased susceptibility to disease caused by *Cercospora nicotianae* (Maher et al. 1994).

In view of the potential of the flavonoid pathway in plant defense response, attempts are being made in several laboratories to genetically engineer plants for increased flavonoid levels by over-expressing the structural and/or the regulatory genes of the flavonoid pathway. The maize regulatory genes *C1* and *R* are extensively being used to trans-activate the anthocyanin pathway in homologous (Ludwig et al. 1990) as well as in heterologous plants such as *Arabidopsis*, tobacco, pea and white clover (Lloyd et al. 1992a; Majnik et al. 1998). The maize *Lc*, a duplicate allele of the *R*, activates anthocyanin pathway in transgenic *Arabidopsis*, tomato and *Petunia* (Quattrocchio et al. 1993; Goldsbrough et al. 1996). The *del* allele of *Antirrhinum* (showing an extensive sequence homology with *Lc* and other members of the maize *R* family) activates anthocyanin production in transgenic tobacco and tomato (Mooney et al. 1995). Similarly, the structural genes such as *A1* (encoding dihydroflavonol reductase) was introduced into *Petunia* line *RL101* which produced novel brick red flowers (Meyer et al. 1987). Chalcone synthase, under the control of a constitutive promoter, CaMV 35S, has been used to genetically transform *Chrysanthemum, Cyclamen, Pelargonium, Petunia, Gerbera* and *Lisianthus* to produce a range of floral colour patterns (Elomma et al. 1993; Courtney-Gutterson et al. 1994; Davies et al. 1997).

Rice, with a relatively small genome, high-density molecular maps, availability of entire genomic sequence data and reproducible transformation protocols, is very amenable for genetic and molecular analysis of flavanoid pathway and its role in defence responses. Recent studies on the genetics and molecular biology of the anthocyanin pathway in rice lead to the clear identification of genotypes and definition of the anthocyanin pathway in *indica* rice (Reddy et al. 1998). In addition, several cDNAs corresponding to the key structural genes such as the chalcone synthase, anthocyanidin synthase, dihydroflavonol reductase and the regulatory genes *C1* and *R*, showing extensive homology to *myb* and *myc* genes (encode transcription factors which bind to the promoters of the structural genes of the pathway) have been cloned and characterized from rice (Scheffler et al. 1995; Hu et al. 1996; Reddy et al. 1996a, b 1998).

The aim of the study is to test the role of the flavonoid pathway in plant defence response. Working towards this goal a set of plasmid constructs carrying the maize structural and regulatory genes under the control of the maize *Ubiquitin* promoter were generated, and a few of them were tested in rice transformation studies (Madhuri et al. 1998; Madhuri and Reddy 1999). This report deals with the genetic transformation of rice with well-characterized maize genes and their expression. We demonstrate here the activation of anthocyanin pathway and production of purple pigment in Tp309 embryogenic calli co–bombarded with the maize regulatory genes *C1, R* and the structural gene *C2* encoding chalcone synthase. We also demonstrate that the maize *C2* transgene activates the anthocyanin pathway leading to the accumulation of purple anthocyanins in transgenic rice. Further, our preliminary screening of these transgenics against rice blast show that the *C2* transgenic seedlings exhibit an increased resistance against rice blast infection.

Materials and methods

Construction of plant expression vectors

The pUOH series of plasmids were used as the source of the cDNAs of the anthocyanin biosynthetic pathway (Reddy 1996). These plasmids carry the respective cDNAs of the anthocyanin pathway under the control of actin promoter. pAHC 17 is the source of the ubiquitin promoter (Christensen and Quail 1996); pMON 999 is the source of E35S promoter (kindly provided

by Monsanto). p35H containing the *hph* gene under the control of CaMv 35S promoter was used as a marker plasmid for the selection of the transgenic calluses. The 1.4 kb maize *C2* cDNA (Wienand et al. 1986) fragment was excised from pUOH *C2* (Reddy 1996) with *Bam*HI and cloned into the *Bam*HI site of pAHC17 (Christensen and Quail 1996) to form pMAC2. The plasmid pMAC1 was constructed by excising a 2.8 kb fragment from pUOH *Myb C1* containing the maize *C1* cDNA (Paz-Ares et al. 1986) and the *Nos* terminator with *Hin*dIII-*Bgl*II and cloned into *Hin*dIII-*Bam*HI sites of pMON999 (660 bp *Hin*dIII-*Bam*HI fragment from pMON999 was excised) to form the intermediate plasmid p99C1S. The *Hin*dIII-*Cla*I fragment was excised from p99C1S and ligated into *Hin*dIII-*Cla*I site of pKS17 to form pMAC1. Plasmid pMAR was constructed by excising a 2.5 kb *Eco*RI fragment of the maize *R* cDNA from pUOHR and cloned under the control of the E35S promoter in pMON999. All plasmids were verified by restriction analysis and sequencing of the junctions.

Expression of purple colour in calluses

Scutellum-derived embryogenic calluses from mature seeds of Tp309 were used for transformation. The plasmids used for anthocyanin expression in calli carry individually the maize regulatory sequences *C1* and *R*, a structural gene *C2* and the selection marker gene *hph*. The gene constructs (pMAC1: pMAR: pMAC2: p35H) were mixed in the ratio of 3:3:4:1 w/w on 1 μm (O.D) gold particles (5 μg of plasmid DNA per 2.5 mg particles) and bombarded onto the calluses with the PDS 1000 He biolistics system (BioRad, Hercules, CA). Control experiments include the bombardment of pMAC1:pMAR and p35H. Callus initiation, subculture, particle coating, bombardment conditions, and selection were performed according to protocols described (Chen et al. 1998). The calluses were bombarded on NBO medium (NB medium with 0.256 M of mannitol and sorbitol) overnight on that medium, transferred onto NH50 medium (NB medium supplemented with 50 mg/1 hygromycin) for 3 weeks, PRNH50 (NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D) but with 2 mg/l of BAP, 1 mg/l NAA, 5 mg/l ABA and 50 mg/l of ABA) for 1 week and finally on RNH50 (NB medium with 2 mg/l 2,4-D, 3 mg/l BAP, 0.5 mg/l NAA, 50 mg/l hygB). The purple calluses observed on RNH50 experienced a photoperiod of 16 h at 110–130 μmol/m^2 PAR.

Generation of transgenic rice

Genetic transformation and regeneration of Tp309 calluses were according to the described protocol (Chen et al. 1998). The plasmids containing the gene of interest (pMAC2) and the selection marker (p35H) were co-bombarded in the ratio of 7:1 w/w.

Crosses

Reciprocal crosses of the rice lines Tp309 (*japonica*) and Purpleputtu (*indica*) were made in the greenhouse. Emasculation was done during the sunset time around 17:00–18:00 and pollination was done around 09:00–10:00 with the standard bagging method. The mature seeds were harvested three to four weeks after pollination and the F_1 seeds were embryo-rescued by culturing on the 1/2 MS medium. The cultured embryos were incubated in dark and subsequently transferred to light. The F_1 plant phenotype was scored.

Western blot analysis

Protein extracts were made from transgenic plants according to a slightly modified procedure (de Kochko et al. 1994) and concentration was estimated with the BioRad reagent. Western analysis of the plants was done with maize anti-CHS antibodies (Courtesy Dr Loverine Taylor, WSU, Pullman, WA). Total proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes with a Bio-Rad electrotransfer apparatus. Membranes were incubated with the maize primary antibody (anti-CHS antiserum) and then with mouse anti-rabbit alkaline phosphate-conjugated IgG as a secondary antibody. Protein bands were visualized in alkaline phosphatase substrate buffer (Promega, Madison, WI).

Southern blot analysis

Genomic DNA was isolated from young leaves as described (Dellaporta et al. 1983). Genomic DNA (3–5 μg) from transgenic plants were digested with *Hin*dIII to estimate the copy number, *Bam*HI to excise the 1.4 kb *C2* cDNA, and *Hin*dIII + *Bam*HI double digest to excise the 2 kb segment corresponding to *Ubi* promoter and 1.4 kb *C2* cDNA. Untransformed Tp309 was used as a control. The digested DNA was electrophoresed on 0.8% agarose and the digested DNA was transferred onto Hybond N+ membrane (Amersham). The blots were then probed with the radiolabelled 2 kb *Ubi* promoter and 1.4 kb *C2* cDNA

(labelling was done with the Prime-it II kit, Stratagene, San Diego, CA, or by the Random Primer Labeling kit, BARC, India). Pre-hybridization, hybridization and washing were performed at 65 °C with standard procedures and the autoradiographs were developed on Kodak films.

Screening transgenics for blast resistance

Screening of the transgenic plants for rice blast was conducted in seedling beds at the Directorate of Rice Research, Hyderabad, India. R_1 seeds along with seeds from Tp309, HR12 (susceptible) and IR64 (resistant) controls were germinated in small pots containing clay soil. Thirty-day old seedlings were subjected to *Magnaporthe grisea* strain IC9 (international Race C, Group 9, raised from a single-spore culture; origin Directorate of Rice Research, Hyderabad) (Ling and Ou 1969) infection either by spraying the spore suspension or infecting it with fully infected HR12 rice leaves. The day temperature during the infection season was around 28 °C at 85% relative humidity and the night temperature was around 25 °C, 90% relative humidity. In addition, artificial sprinklers maintained the high relative humidity and during the night the beds were covered with polythene paper pre-wetted with water. Response of the infected plants was scored within 10 days after infection with the SES scale (Standard Evaluation Scale) which is based on the length and type of lesion, percentage of disease spread, and overall performance of the seedlings (Ling and Ou 1969). The disease response is classified into the following types: resistant plants show no lesions or, if present, lesions are restricted and non-spreading with a pin head to a small round size and receive a score of 0–2; moderately resistant plants where the number of small round lesions are localized to the upper leaves receive a score of 3; plants with score 4 begin to show typical blast lesions with less than 4% of the infected area; and plants with score 5 have 4–10% of the infected leaf area; moderately susceptible plants which have around 25–50% of leaf area infected exhibit a score of 6–7; and sensitive plants show severe necrotic lesions with 51–71% of leaf infection with a score of 8–10.

Results

Production of purple colour in Tp309 calluses

Embryogenic calluses were co-bombarded with four different plasmid constructs containing the maize anthocyanin genes, *C1*, *C2* and *R*, and the selectable marker *hph*. A few of these calluses showed a bright pink colour resulting from the localized production of anthocyanins (Figure 1b) within two to three days after placing on RNH50 medium supplemented with 2 mg/ml IAA under light (16 h at 110–130 μmol/m^2 PAR). However, the frequency of the purple calluses was very low (three calluses out of the five plates bombarded) The pink colour was stable for a week after which it faded off or was masked by rapidly growing green areas. Control untransformed calluses did not show a purple colour (Figure 1a). In order to decipher the contribution of the *C1*, *R* and *C2* genes in production of purple colour in calluses, a set of experiments were performed with the co-bombardment of *C1* and *hph*; *R* and *hph*; C1, *R* and *hph*; and a control experiment with gold particles alone. None of the calluses obtained with these bombardments developed purple colour.

Generation of C2 *transgenics*

The genetic transformation of Tp3O9 was carried out by co-bombardment of pMAC2 containing the maize *C2* gene under the maize *Ubiquitin* promoter and r35H containing *hph* under the 35S promoter mixed in the ratio of 7:1 w:w. A total of 50 regenerants were obtained on the selection medium from three independent bombardment experiments. Of these, 11 individual R_1 seedlings exhibited purple pigmentation with the intensity ranging from pale to intense pink/purple (Figure 1d–h). The untransformed Tp3O9 (Figure 1c) plants and the control regenerated plants bombarded with gold particles alone did not show any purple colour when grown under the same conditions. Transformed plants showed red/purple colour within a week after placing on the 1/2MSH5O medium (supplemented with 50 mg/l hygromycin). Two primary transformants (C2-5, C2-7) showed a bright red pigmentation in leaf blade and leaf sheath, while nine others showed a lighter pigmentation. Some of the transgenic plants show patchy distribution of colour in seedlings (Figure 1e and 1g) while some others show an intense purple colour in the leaf sheath (Figure 1d, f and h). In a few others, the pigmentation was barely detectable. The colour persisted for a month

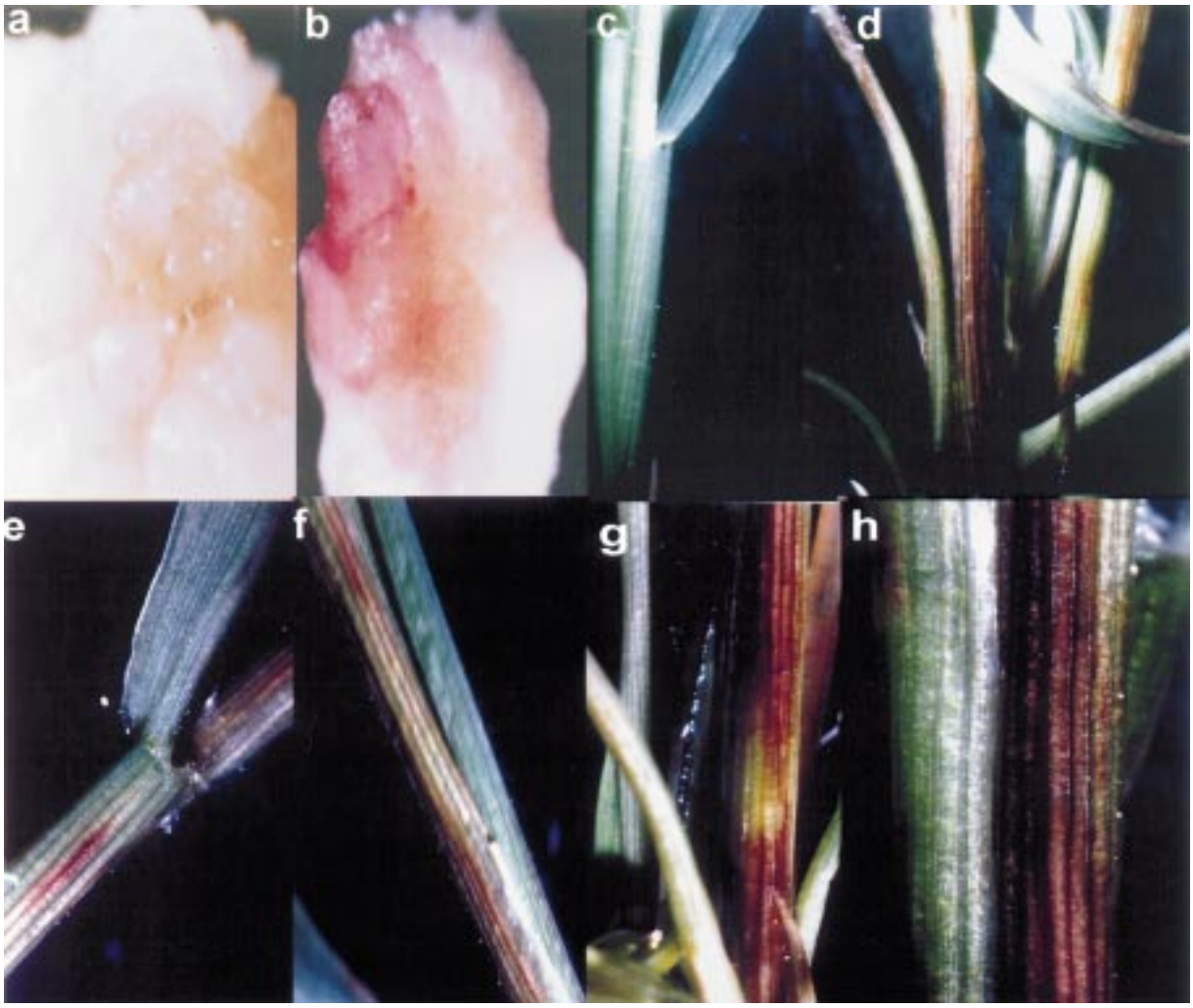

Figure 1. Anthocyanin pigmentation in transformed calluse and plants. a. Control untransformed calluses. b. Tp309 calluses co-bombarded with pMA*C1*, pMA*C2* and pMA*R*. c. Untransformed *Tp309* seedlings. d–g. Transgenic *Tp309* showing a patchy pigmentation in leaf sheath and leaf blade.

after which purple regions were overgrown by green areas upon transfer to the growth chamber. The growth and fertility of all but one (which died) transgenic plants were found to be normal. The transformants were checked for the presence of the marker gene *hph* by PCR and for the *C2* transgene by Southern blot analysis (data not shown). One-month old primary *C2* transgenic seedlings were tested for the accumulation of CHS protein using the antisera raised against the maize chalcone synthase protein. A representative western blot of the transgenic plants is shown in Figure 2. The western blot revealed the presence of CHS protein in 16 out of the 50 R_0 plants analyted. The CHS protein was not detectable in the leaf extracts of untransformed Tp3O9. Of the 16 transgenics that showed the presence of CHS protein, 6 were purple. The apparent molecular mass of the CHS protein in these transgenic plants was ca. 44 kDa, identical to that observed in Purpleputtu, a highly pigmented *indica* rice variety (Reddy et al. 1996a). Based on the phenotype and western results, the R_0 transgenics were classified into four groups. Group I plants exhibited purple pigment and also showed the presence of CHS, Group II plant did not show purple colour but hade detectable levels of CHS protein, and Group III plants neither exhibited colour nor accumulated CHS. Group IV plants were intensely coloured but did not survive in the greenhouse.

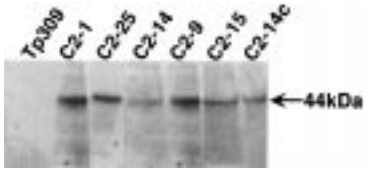

Figure 2. Western blot of R_0 plants showing the immuno-detectable CHS protein. Transgenic plant number is indicated on the top. Equal amounts (30 μg) of total soluble protein was loaded into each slot. *Anti*-CHS antibody from maize was used.

Analysis of R_1 transgenic plants

The R_1 plants were analysed for the presence of the transgene, expression of purple colour, accumulation of CHS protein and, finally, their performance against leaf blast caused by *M. grisea*. Among the R_1 plants tested only C2-1 exhibited a pale pink colour in young leaf sheath which subsequently faded off. Surprisingly, none of the other R_1 transgenic plants showed purple colour. Western blot analysis of the R_1 plants revealed CHS protein only in C2-1, C2-6 and C2-25 (Table 1). The R_1 plants were phenotypically similar to the untransformed Tp309 with respect to the morphology, vegetative growth and seed setting.

The loss of purple colour in R_1 plants could be due to the presence of dominant colour suppressor gene(s) in Tp309. To test this hypothesis, Tp309 was crossed with the fully coloured rice cultivar Purpleputtu. All the F_1 plants from the two reciprocal crosses were found to be colourless indicating that Tp309 carries at least one dominant suppressor (data not shown).

The Southern blot revealed the 3.5 kb band in *Hin*dIII digest, hybridizing to *C2* as well as *Ubi* probes indicating the presence of the intact cassette (Figure 3a and 3b). The *Bam*HI digest revealed the expected 1.4 kb *C2* fragment when probed with *C2*. The presence of 2 kb *Ubi* fragment was detected with *Bam*HI-*Hin*dIII double digests. The southern data confirmed the presence of intact *Ubi-C2* cassettes in *C2* transgenic plants.

The performance of the representative R_1 transgenics that were found to be western-positive was evaluated against the blast fungus *M. grisea*, and the disease responsiveness was recorded on the basis of SES scoring. The score values represent the overall mean of the two replicates of the three independent challenge experiments performed in the nursery beds. The response of the ten individual plants from each experiment to blast infection was measured as the number of infected lesions per leaf (represented as mean number of lesions per leaf), percentage of the

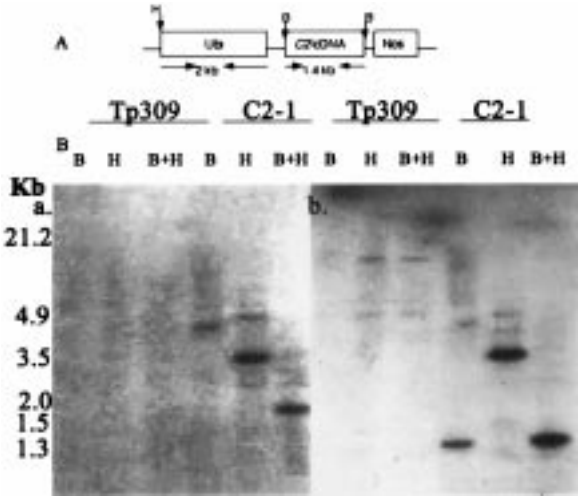

Figure 3. Southern blot analysis of transgenic plants. Restriction map of the pMAC2 cassette is shown on the top. 3 μg of digested DNA was loaded in each lane and the blot was hybridized with (a) the 2.0 kb *Ubi* fragment and (b) the 1.4 kb maize *C2* gene. Lanes: B, *Bam*HI; H, *Hin*dIII; B+H, *Bam*HI and *Hin*dIII double digests.

affected leaf area (represented as % disease in Table 2), and the size of the disease lesion. The crop damage is assessed on the SES scoring for the leaf blast. The nature and frequency of lesions on leaves of representative plants are shown in Figure 4. It is clear that HR12, the susceptible control, shows large necrotic lesion, having more than 75% of leaf infected (Figure 4a and Table 2) while untransformed Tp309 shows lesions spreading over about 10–20% of the area (Figure 4b). The *C2* transgenics exhibit lesions occupying less than 10% of the infected leaf area and the lesions are fewer in number (Figure 4c and d and Table 2). Though the data suggest that transgenic *C2* plants show relatively reduced symptoms upon infection, it requires more direct quantitative analysis under rigorously controlled conditions to confirm the conclusion. The overall performance of these transgenics based on SES score is given in Table 2. Most of these *C2* transgenics exhibited a SES score of 4–5, except C2-14 which showed 6 equal to the Tp309 (5–6). The corresponding scores of the resistant control IR 64 and the susceptible HR12 were 1–2 and 9–10 respectively. We observed that the extent of infection varied somewhat between experiments depending on age, density of the inoculum and relative humidity during the season. The presence of the transgene in these plants was further confirmed by Southern analysis in the R_2 generation (Table 1).

Table 1. Molecular characterization of representative *C2* transgenics.

Plant	Phenotype R0/R1		CHS protein[a] R0/R1		*C2* transgene[b]			Group*
					R_0	R_1	R_2	
C2-6	P	−	+	+	+	+	+	I
C2-9	P	−	+	−	+	+	+	I
C2-25	P	−	+	+	+	+	+	I
C2-1	−	P	+	+	+	+	+	II
C2-17	−	−	−	−	NA	+R	+R	III
C2-24	−	−	−	−	NA	+R	+R	III

P, Purple/Red phenotype of the seedling tissue.
[a]Presence (+) or absence (−) of CHS protein as detected by maize anti-CHS antibodies;
[b]Detection (+) of the intact *C2* transgene or a rearranged version (+R) by Southern blot.
NA, data not available.
*as described in the text.

Discussion

We have observed the production of purple colour in Tp309 calluses by co-transformation with the *C1*, *R* and *C2* constructs. The untransformed calluses did not show colour. The calluses bombarded with *C1* and *R* constructs along with the marker *hph* or co-transformed with *C1* and *hph*, R and *hph* also did not show any colour. We conclude that the appearance of anthocyanin coloration in calli is a result of over expression of the *C2* transgene. One cause of failure to produce colour with *C1* and *R* constructs (without *C2*) in Tp309 calluses is presumably that Tp309 is a mutant at the chalcone synthase locus. For instance, a mutation in regulatory *cis* elements of the promoter of the endogenous *Chs* gene would lead to chalcone synthase deficiency and thus the absence of colour even when the *C1* and *R* transcription factors are over-expressed. It is also possible that Tp309 is homozygous for a mutation in the coding region of the *chs* gene thus making it *chs*-deficient. In addition, it appears that *C1* and *R* may also play a role in the production of purple colour in calluses co-bombarded with *C1*, *R* and *C2*. The maize *C1* and *R* loci encode *myb* and *myc* transcriptional activator proteins, respectively, that control the tissue-specific expression of anthocyanin biosynthetic pathway genes in maize (Neuffer et al. 1997).

The production of purple colour in *C2* transformants (R_0) demonstrates that the overexpression of *C2* transgene is required to activate the anthocyanin pathway in transgenic Tp309 seedlings. This observation, in conjunction with the appearance of purple colour in calluses and in *C2* transgenic R_0 plants (showing immuno-detectable CHS protein) further strengthens the argument that chalcone synthase is required for the production of purple colour in transgenic Tp309. Absence of the CHS protein in the leaf extracts of Tp309 suggests either the total lack of CHS protein or the accumulation is so low that it cannot be detected by the immuno-based procedure used. In any event such low levels of CHS are insufficient to produce purple colour, as the non-transgenic Tp309 seedlings are absolutely devoid of anthocyanins. Western analysis of *C2* transgenics of the R_1 generation revealed the accumulation of the CHS protein in leaf extracts of three lines (C2-1, C2-25 and C2-6) (Table 1) in contrast to the sixteen plants showing CHS protein in the R_0 generation.

Among the western-positive R_1 plants, only those from the C2-1 line express the colour phenotype. The absence of purple phenotype in R_1 plants is due to the action of colour inhibitor allele(s) on the expression of anthocyanin gene(s) whose presence was detected in the appropriate genetic crosses. The loss of protein accumulation in a majority of the transgenic progeny (3 positives out of 16 in R_0) could be attributed to gene silencing.

The *C2* transgenic plants generated in this study were classified into four groups based on phenotypic and western data. The plants belonging to group I exhibited the purple phenotype due to the functional CHS protein, while the plants belonging to group III could not produce colour due to the lack of such a CHS protein, as they are western-negative. Group II plants are atypical with no simple explanation as they

Table 2. Screening of transgenic plants against the rice blasst, *Magneporthe grisea*.

Transgenic plant	Mean lesions per leaf[a]	% Disease spread[b]	Mean size of lesion (mm)	SES[c]
C2-1	10 ± 2	7 ± 1.4	4 ± 0.7	4–5
C2-6	13 ± 2	7 ± 1.3	6 ± 0.9	4–5
C2-9	12 ± 3	8 ± 1.8	4 ± 0.9	4–5
C2-14	15 ± 3	9 ± 1.7	5 ± 0.8	5
C2-25	14 ± 4	7 ± 1.6	4 ± 0.7	6
Tp309	18 ± 3	20 ± 1.1	7 ± 0.5	5–6
IR64	6 ± 3	1 ± 0	1 ± 0.2	1
HR12	$20 \pm$[4]	75 ± 0.5	10 ± 0.3	9–10

[a] Average number of infected lesions on a single leaf blade.
[b] % Disease spread: extent of disease spread.
[c] Standard evaluation score for leaf blast. A SES score of 1–3 is resistant; 8–10 is susceptible; 5–7 is moderately susceptible; 3–5 is moderately resistant. Data represent the mean values of three independent experiments done in two seasons in infection beds at the Directorate of Rice Research, Hyderabad.

do show immuno-detectable CHS protein but lack the purple colour. The absence of colour in these plants could be due to the non-functional transgene-encoded CHS protein or these plants were not able to overcome the effect of colour inhibitor allele(s) identified in Tp309 genome. The observation that the transgenic line C2-1, belonging to Group II, showed colour in the R_1 generation, but not in R_0, suggests leaky expression of *C2* overcoming the effect of the colour suppressor genes. The apparent lethality exhibited by group IV seedlings with intense purple colour is an interesting phenomenon that needs further analysis. Transgenic sugarcane seedlings over-expressing anthocyanins and intense purple colour were reported to die in early stages after regeneration (Bower et al. 1996). Such seedling lethality could be due to low threshold levels of pigmentation in young transgenic seedlings or to the deleterious effects of the over-expressed gene. It is possible, however, that dramatically high accumulation of anthocyanins in cytosol due to a defective transportation into vacuole, the natural location of anthocyanin pigments in a vast majority of plants, might become toxic and affect cellular functions. In tobacco transgenics the alteration of the phenylpropanoid biosynthesis resulted in abnormal plant development (Elkind et al. 1990).

The appearance of colour in R_0 plants is rather patchy and significantly different from that of the fully coloured purple seedlings such as Purpleputtu. The most likely reason for such a phenomenon is the activity of a class of genes that suppress purple colour production in rice. Such inhibitor alleles and their effect on tissue-specific distribution have been well documented in rice (Dhulappanaver 1973; Kinoshita and Takahashi 1991; Reddy et al. 1995). Trough, the genetic complement of Tp309 with respect to anthocyanin pathway is not yet defined; the F_1 progeny of the reciprocal cross Tp309 (green) $\times$ Purpleputtu (fully purple) having no colour suggest that the Tp309 carries suppressor alleles responsible for the elimination of the purple colour. Such dominant suppressor alleles, such as *C1-I* (suppressor of Colored-1) of maize, eliminates colour in aleurone even in heterozygous conditions. However, the triploid maize aleurone cells that are genetically *C1-I C1 C1* show specks of colour but *C1-I C1-I C1-I* do not normally show any colour (Coe 1962; Neuffer et al.1997). The patchy distribution of colour in transgenic seedlings is somewhat similar to the behavior of *C1-I* heterozygotes of maize. In *C2* transgenics, the constitutive *Ubi* promoter is not responsive to such a suppressor but these plants will not produce colour because the remaining structural genes of the pathway are responsive. However, further elaboration of Tp309 genotype is required for a clear interpretation of this observation.

The R_1 transgenic *C2* seedlings exhibited an increased resistance against blast infection under the test conditions. In addition, the R_1 plants exhibiting the improved blast resistance also are found to have the immuno-detectable CHS protein (Table 1) thus sug-

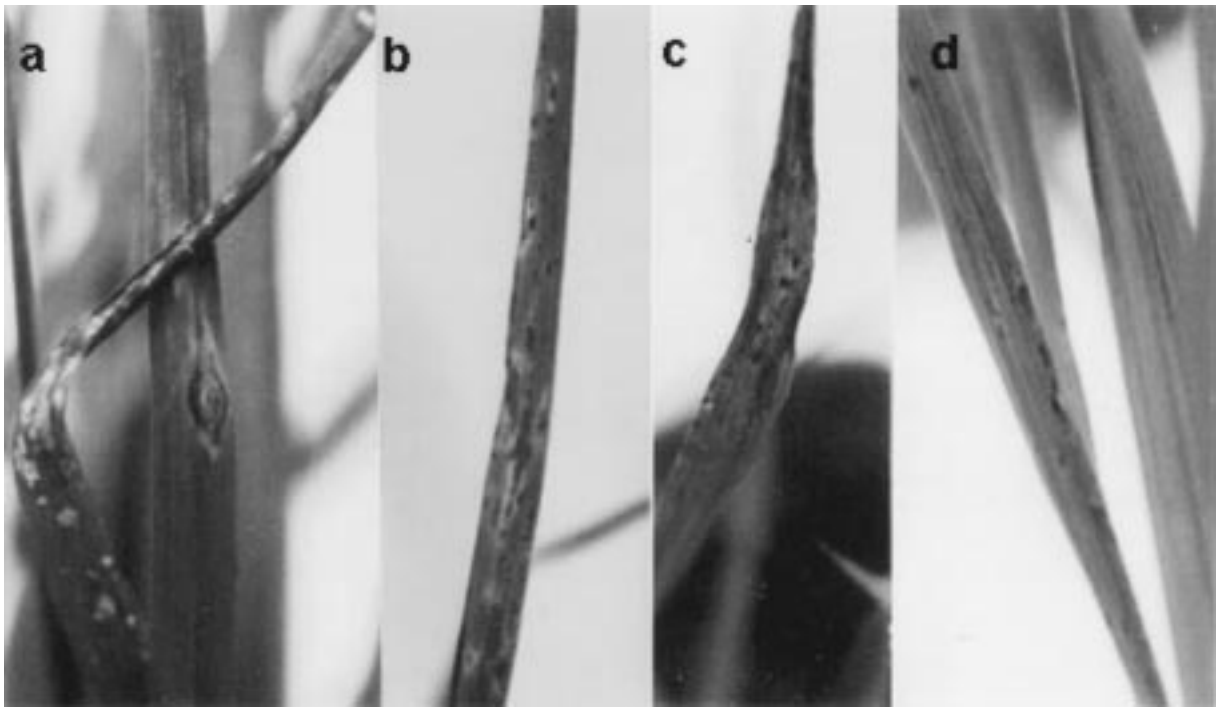

Figure 4. Lesions on the leaves of transgenic plants under infection by *Magnaporthe grisea*. a. Susceptible control HR12. b. Untransformed Tp3O9. c and d. Tp309 transgenics.

gesting that the functional CHS protein product is essential for improved performance against pathogen. Future studies will be aimed at the cosegregation of the CHS protein and the blast resistance phenotype for transgenic evaluation. In addition, these transgenics will be screened for resistance against other pathogens including leaf blight and sheath blight.

The increase in resistance in these transgenic plants against rice blast has to be confirmed by further evaluation under field and controlled conditions with different races of the pathogen. In fact, the screening conditions used in our trial certainly impose a more severe stress at the seedling stage compared to the mature plants. The increase in resistance in *C2* transgenics is not very surprising because flavonoids are known to contribute to plants' defence mostly in the form of restricting the pathogen proliferation and slowing down the disease spread by forming a flavonoid-anthocyanin barrier as a response to pathogen attack. The observation that lesions are comparatively small in transgenics agrees well with the general observation that the onset of red colour, consisting of anthocyanins and flavonoids, upon infection is comparatively faster in resistant plants than in susceptible ones, thus providing a quick barrier for the spread of the disease. The flavonoid pathway is essentially a component of the disease response mechanisms and therefore only a marginal but not dramatic improvement in resistance is expected in *C2* transgenics. Earlier studies have suggested the involvement of chalcone synthase in defence response processes in plants. For instance, a rapid and transient increase of the *Chs* transcript was observed during elicitor treatment and infection in bean and soybean cells (Lawton and Lamb 1987; Ryder et al. 1987; Wingeder et al. 1989). In addition, the reaction product of *C2* -naringenin (5,7,4′-trihydroxyflavonone) and its methyl derivative, sakuranetin (7-*O*-methyl ether) were shown to inhibit *in vitro* spore germination of *Pyricularia* (Grayer et al. 1995). Further, an *in vivo* increase of naringenin was observed in rice leaves during UV-B stress and blast infection (Grayer et al. 1995).

Our results highlight the possibility of using chalcone synthase as a reporter system in Tp309 transformation with further refinement. The *C2* transgenics can be used effectively to test the concept that the flavonoids contribute to disease resistance in different plant systems against different pathogens. More exhaustive production of transgenics with both structural and regulatory genes of the pathway and their

rigorous testing under controlled laboratory conditions and field are necessary in elucidating the role of this pathway in conferring disease resistance in rice.

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