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Transgenic rice lines expressing maize *C1* and *R-S* regulatory genes produce various flavonoids in the endosperm

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Summary

Flavonoids, compounds that possess diverse health-promoting benefits, are lacking in the endosperm of rice. Therefore, to develop transgenic lines that produce flavonoids, we transformed a white rice cultivar, *Oryza sativa japonica* cv. Hwa-Young, with maize *C1* and *R-S* regulatory genes. Expression of these transgenes was restricted to the endosperm using the promoter of a rice prolamin gene. The pericarp of the *C1*/*R-S* homozygous lines became dark brown in accordance with their maternal genotype, whereas the endosperm turned chalky, similar to the opaque kernel phenotype. Analysis via high-performance liquid chromatography (HPLC) revealed that numerous kinds of flavonoids were produced in these transgenic kernels. To identify individual flavonoids, the number of HPLC peaks was reduced through moderate acid hydrolysis, followed by ethyl acetate partitioning. Amongst the major flavonoids, dihydroquercetin (taxifolin), dihydroisorhamnetin (3′-*O*-methyl taxifolin) and 3′-*O*-methyl quercetin were identified through liquid chromatography/mass spectrometry/mass spectrometry and nuclear magnetic resonance analyses. Fluorescence labelling with diphenylboric acid showed that the flavonoids were highly concentrated in the cells of four to five outer endosperm layers. More importantly, a high fluorescence signal was present in the cytosol of the inner endosperm layers. However, the overall signal in the inner layers was significantly lower because starch granules and protein bodies occupied most of the cytosolic space. Our estimate of the total flavonoid content in the transgenic kernels suggests that *C1*/*R-S* rice has the potential to be developed further as a novel variety that can produce various flavonoids in its endosperm.

Keywords: antioxidant, dihydroflavonols, endosperm, flavonoids, genetic engineering, rice.

Introduction

Over 2000 plant flavonoids are involved in the pigmentation of flowers and fruits, protection against ultraviolet (UV) radiation and pathogens, signalling to symbiotic microorganisms and male fertility (reviewed in Shirley, 1996; Winkel-Shirley, 2001a,b, 2002). All flavonoids possess the basic skeleton phenylbenzopyrone, which is synthesized through sequential condensation reactions of a *p*-coumaroyl-coenzyme A (*p*-coumaroyl-CoA) and three molecules of malonyl-CoA. In addition to subtle modifications in the phenylbenzopyrone itself, flavonoids are diversified by the addition of various moieties, such as hydroxyl, methyl or sugar groups, to the basic structure.

Flavonoids have been studied extensively with regard to their biological activities, including antioxidant, antiviral,

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anticancer, antiaging and hepatoprotective effects, as well as for their ability to enhance the immune system and to improve serum lipid quality, which leads to a decrease in the risk of cardiovascular disease (Dixon and Steele, 1999; Yousef *et al*., 2004). Although most health-promoting roles are primarily associated with their antioxidant properties in functioning as reducing agents, hydrogen donors and free radical quenchers, a number of non-antioxidant activities have also been reported with regard to their inhibition of carcinogenesis (Jankun *et al*., 1997; reviewed in Ren *et al*., 2003) and modulation of receptors and intracellular signalling enzymes (reviewed in Williams *et al*., 2004). Pinent *et al*. (2004) have found that procyanidins (i.e. oligomeric flavonoids) have antidiabetic properties, possibly through their interaction with signalling components, such as phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase. Likewise, Enomoto *et al*. (2004) have demonstrated the inhibitory effect of 3-methoxyquercetin on human aldose reductase, platelet aggregation and blood coagulation.

Amongst the major secondary metabolic pathways in plants, flavonoid biosynthesis has been best characterized in molecular genetics studies of genes and in the biochemical mechanisms of enzymes involved in the pathway (reviewed in Holton and Cornish, 1995; Winkel-Shirley, 2001a,b). Members of the *C1* and *R* regulatory gene families that encode Myb-type transcription factors and basic helix–loop–helix-type transcription factors, respectively, either individually or mutually activate different sets of structural genes in the flavonoid biosynthetic pathway (Quattrocchio *et al*., 1993, 1998). Genes in both families have been isolated from a number of monocot and dicot species; members are highly conserved within the corresponding families. For this reason, some *C1* and *R* family members are functionally interchangeable amongst different plant species (reviewed in Schijlen *et al*., 2004). For example, ectopic expression of both maize *C1* and *R* is sufficient to produce anthocyanins in root, petal and stamen tissues that normally lack these pigments in *Arabidopsis* and tobacco (Lloyd *et al*., 1992). Similarly, Bovy *et al*. (2002) have reported that tomato plants transformed with both maize *C1* and *LC* (a member of the *R* gene family) produce anthocyanins and additional flavonoids in the leaves and fruit flesh, respectively. In addition to regulatory genes, the enhanced production of certain flavonoids in specific tissues of tomato fruit has been demonstrated by the elevated expression of biosynthetic enzyme genes for chalcone synthase, chalcone isomerase and flavonol synthase (Verhoeyen *et al*., 2002).

The levels and types of flavonoids vary significantly, depending not only on the plant species but also on the tissue type. For example, cereal endosperms lack flavonoids (Sharp, 1991), whereas the leaves of green tea (*Camellia sinensis*) contain high levels, accounting for 10%–30% of their dry weight (Graham, 1992). Varieties of black rice produce a few types of anthocyanin (pigmented flavonoids), exclusively in the pericarp, which is readily removed on polishing. Therefore, it must be consumed without polishing, which adversely affects the shelf life and palatability. It is unknown whether the tissue-specific production of flavonoids is solely attributed to the expression of biosynthetic genes or requires additional factors, such as substrate specificity of enzymes and subcellular organization. Thus, it is of great interest and challenge to develop cereal varieties that accumulate flavonoids in the endosperm. In this study, we analysed the endosperm-specific expression of maize *C1* and *R-S* genes that caused the accumulation of flavonoids in transgenic rice kernels.

Results

We developed transgenic rice lines in which flavonoids were produced throughout the endosperm. Because most of the genes involved in this biosynthetic pathway normally are not expressed in this cereal endosperm, our strategy was to transform regular white rice with the maize *C1* and *R-S* (a seed-specific member of the *R* gene family) genes in order to activate various structural genes in the pathway. To avoid possible side-effects resulting from transgene expression in the vegetative tissues of transformed plants, we used an endosperm-specific promoter to regulate the expression of *C1* and *R-S*.

Production of transgenic rice lines expressing maize *C1* and *R-S* transgenes

The maize *C1* and *R-S* cDNA clones were individually placed between the rice prolamin *NPR33* promoter (Wu *et al*., 1998) and the *NOS* terminator (Tnos) in the pUC plasmid. These fusion molecules were subcloned into the binary vector pCambia3301 that contains the phosphinotricin-resistant *BAR* gene (Figure 1A). *Agrobacterium tumefaciens* LBA4404 harbouring the construct was used to infect calli induced from germinating embryos of *Oryza sativa japonica* cv. Hwa-Young. Through selection and regeneration of the infected calli, we produced 27 independent primary transgenic plants (T_0) and grew them to maturity (Figure 1B–E). The presence of the transgenes in the T_0 plants was conveniently determined via genomic DNA polymerase chain reaction (PCR) for the *BAR* gene (Figure 2A). The germination of T_1 kernels on agar plates containing 4 mg/L of phosphinotricin indicated that most *C1*/*R-S* lines possessed a single locus of the transgenes, except for a few lines possibly having two loci. Our

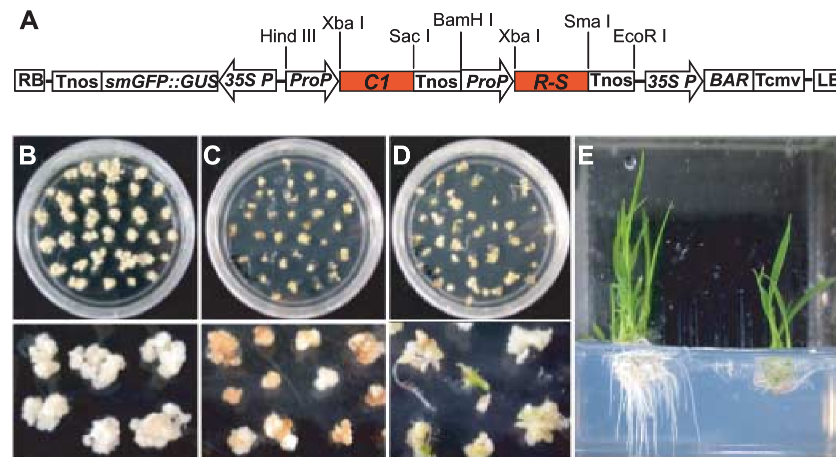

Figure 1 Production of transgenic rice lines transformed with maize *C1* and *R-S* transgenes. (A) Construct map consists of RB (T-DNA right border), Tnos (nopaline synthase gene terminator), *smGFP::GUS* (soluble modified green fluorescent protein gene fused with β-glucuronidase coding sequence), *35S P* [cauliflower mosaic virus (CaMV) 35S promoter], *ProP* (a rice 13-kDa prolamin *NPR33* promoter), *C1* (maize MYB-type transcription factor, accession number AF320614), *R-S* (maize bHLH-type transcription factor, accession number X15806), *BAR* (phosphinotricin-resistant gene), Tcmv (CaMV 35S terminator) and LB (T-DNA left border). (B) Calli induced from germinating embryos of *Oryza sativa japonica* cv. Hwa-Young. (C) Calli infected with *Agrobacterium tumefaciens* LBA4404 harbouring transgenes on phosphinotricin-containing selection medium. (D) Calli with regenerating plantlets. (E) Regenerated seedlings transferred to Magenta box containing Murashige–Skoog medium.

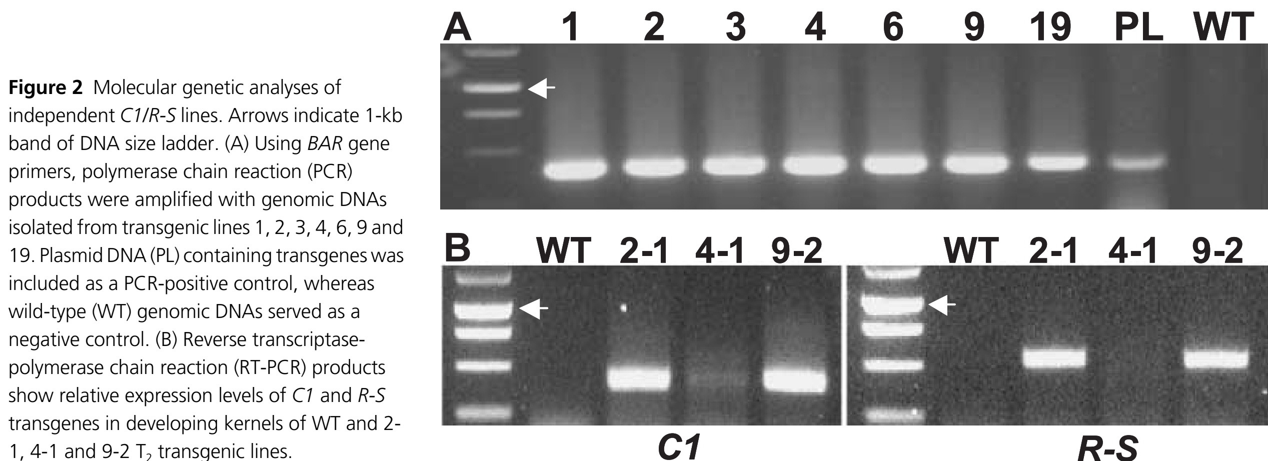

Figure 2 Molecular genetic analyses of independent *C1/R-S* lines. Arrows indicate 1-kb band of DNA size ladder. (A) Using *BAR* gene primers, polymerase chain reaction (PCR) products were amplified with genomic DNAs isolated from transgenic lines 1, 2, 3, 4, 6, 9 and 19. Plasmid DNA (PL) containing transgenes was included as a PCR-positive control, whereas wild-type (WT) genomic DNAs served as a negative control. (B) Reverse transcriptase-polymerase chain reaction (RT-PCR) products show relative expression levels of *C1* and *R-S* transgenes in developing kernels of WT and 2-1, 4-1 and 9-2 T_2 transgenic lines.

genomic DNA Southern blot analysis with a ^{32}P-labelled *BAR* gene probe also demonstrated that most of the transgenic lines contained a single locus for the transgenes (data not shown).

Expression of the transgenes was estimated by reverse transcriptase-polymerase chain reaction (RT-PCR), using RNAs isolated from the developing kernels of homozygous lines plus primer sets specific for the maize *C1* and *R-S* genes. Representative images are shown in Figure 2B. The amounts of RT-PCR products relative to transcript levels ranged from hardly detectable, as in line 4-1, to high levels, in lines 2-1 and 9-2. However, the expression of *C1* and *R-S* transgenes was quite similar within individual lines.

Numerous types of flavonoids are produced in kernels of *C1/R-S* rice

T_2 kernels harvested from the herbicide-resistant T_1 plants did not differ from the wild-type (WT) in their germination and growth rates. However, the progression of seed development, particularly dehydration, in the T_2 kernels was notably delayed, with kernels remaining fresh and soft on the panicles up to twice as long as the WT kernels. Growing the T_2 plants in a glasshouse during the winter rather than the summer resulted in the production of thin and light kernels, indicating that grain filling was more adversely affected by poor growing conditions in the transgenic lines than in the WT.

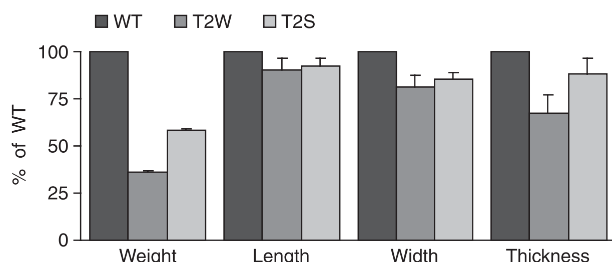

Figure 3 Measurements of T_2 and wild-type (WT) kernels. WT, kernels harvested from WT plants grown in a glasshouse during the winter season; T2W, *C1/R-S* homozygous T_2 kernels in the winter; T2S, T_2 kernels in the summer. For weight, five replicates of 100 kernels were weighed and compared with those of the WT. For length, width and thickness, 50 kernels of each type were measured and compared with those of the WT.

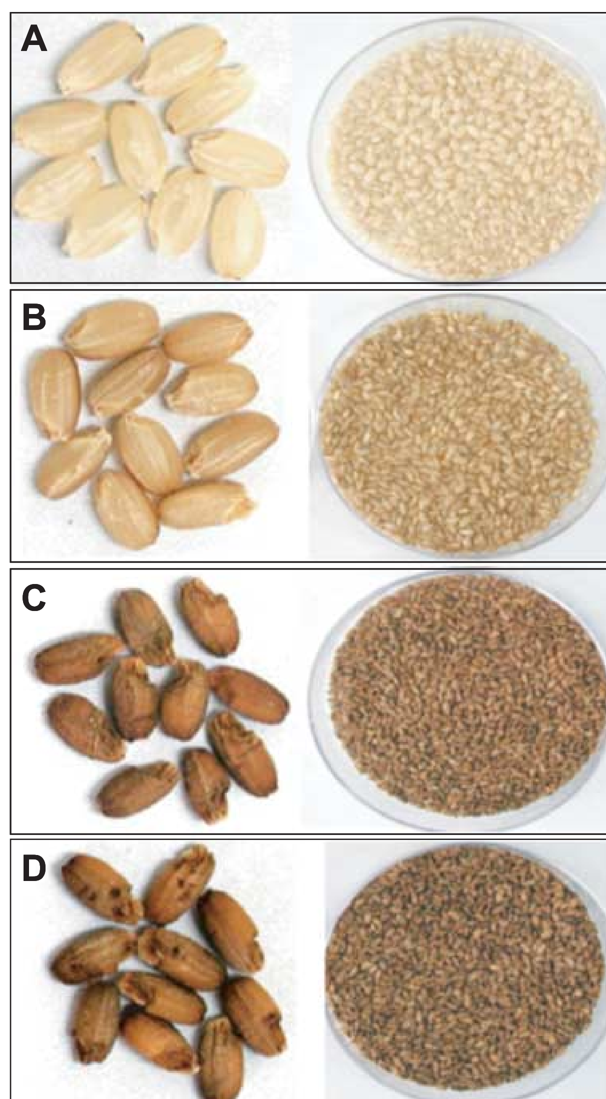

Figure 4 Comparison of untransformed, T_1, T_2 and T_3 *C1/R-S* rice kernels. Untransformed (A) and T_1 hemizygous transgenic (B) rice kernels are larger and lighter in pigmentation than T_2 (C) and T_3 (D) homozygous transgenic kernels.

The kernel weight and thickness of the winter-grown T_2 homozygous lines were substantially reduced to 36% and 67% of the WT, respectively. However, the values of the same T_2 lines grown in the following summer recovered to 58% and 88% of the WT (Figure 3). From the homolines in which all T_2 kernels exhibited herbicide resistance, we selected two sublines, 2-1 and 9-2, to further analyse their T_3 kernels. Both T_2 and T_3 kernels from the homozygous transgenic lines were darker and smaller than the T_1 kernels, which, in turn, were slightly darker than the untransformed WT kernels (Figure 4).

Flavonoids were extracted from the kernels of the 'Hwa-Young' WT, the black rice cultivar 'Mil-Yang 188' and a homozygous *C1/R-S* 2-1 rice line. Their high-performance liquid chromatography (HPLC) profiles showed that the *C1/R-S* 2-1 kernels contained various levels of several flavonoids (Figures 5C,D and 6A). In contrast, flavonoids were hardly detectable in the extracts from WT kernels (Figure 5A); only a few flavonoids, presumably anthocyanins, were found in black rice (Figure 5B). Because of the numerous types of flavonoids produced in *C1/R-S* rice, it was not feasible to separate their individual peaks and identify them by matching the retention times with those of reference compounds. To circumvent such technical problems, we performed acid hydrolysis, followed by ethyl acetate partitioning, which resulted in a substantial decrease in the total number of peaks, as well as an increase in the levels of some major peaks (Figure 5D). On the basis of our liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) and nuclear magnetic resonance (NMR) analyses, we finally identified peaks I, II and III as dihydroquercetin (taxifolin), dihydroisorhamnetin (3′-*O*-methyl taxifolin) and 3′-*O*-methyl quercetin, respectively (Figure 6). Using authentic taxifolin as a reference compound and the quantification function of Waters Millennium 32 software (Milford, MA, USA), we estimated the amounts of nine major compounds (Table 1), and found that 3′-*O*-methyl taxifolin (peak 6; 330.3 μg/g) was most abundant. We also measured the total flavonoid contents in the WT, an existing black rice cultivar, the T_3 *C1/R-S* 2-1 rice and green tea leaves, comparing their A_{280} values with those of known taxifolin concentrations (Table 2). On the basis of this rough estimation, the total flavonoid contents in *C1/R-S* rice were at least 30 times and six times higher than in the WT and existing black rice, respectively. However, the *C1/R-S* rice contained only about one-tenth of the total flavonoids detected in green tea leaves. The total flavonoid content of 15% in the dried tea leaves suggests that our quantification system is reasonable, because a value in the range 10%–30% has commonly been reported by other research groups (Graham,

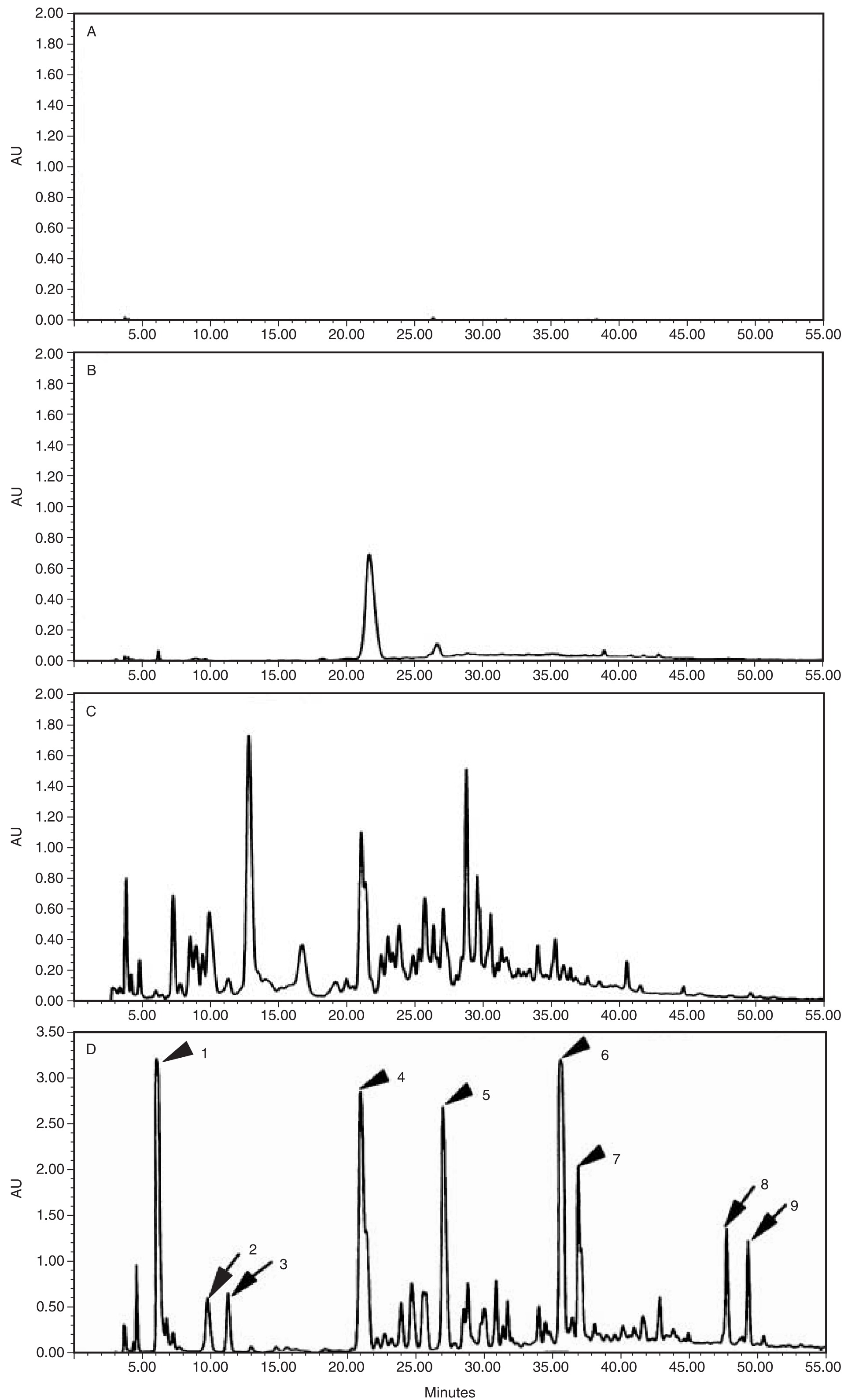

Figure 5 High-performance liquid chromatography (HPLC) profiles of flavonoids extracted from kernels of Hwa-Young wild-type (A), existing black rice 'Mil-Yang 188 Ho' (B) and T_3 *C1/R-S* 2-1 rice without acid hydrolysis and ethyl acetate partitioning (C). (D) HPLC profile of flavonoids extracted from T_3 *C1/R-S* 2-1 rice kernels subsequently acid hydrolysed and partitioned with ethyl acetate. Retention times given in minutes; AU, absorption units.

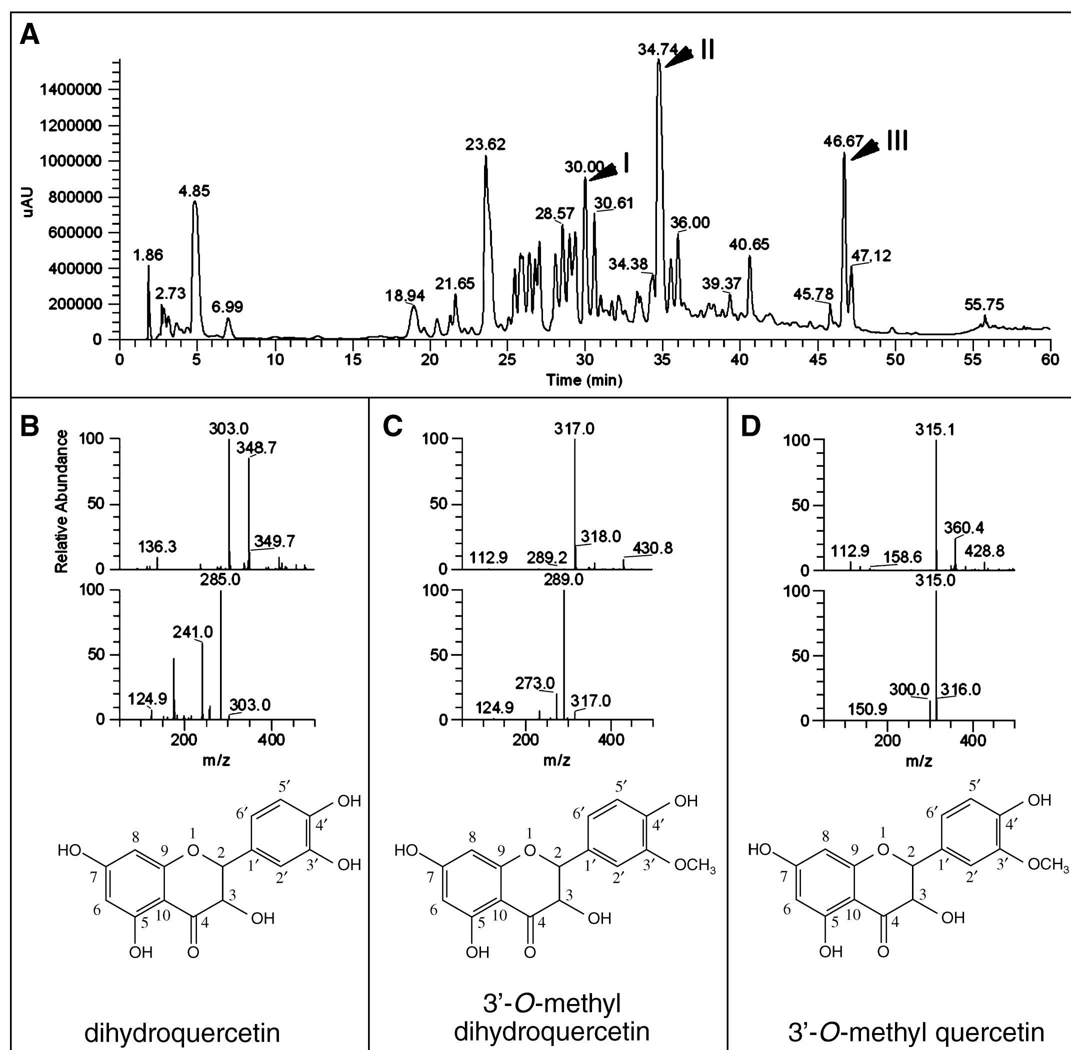

Figure 6 Liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analyses of flavonoid extract from T_3 *C1/R-S* rice kernels, and chemical formulae identified by nuclear magnetic resonance (NMR) analyses. (A) Liquid chromatogram of extract that was acid hydrolysed and partitioned with ethyl acetate. (B–D) MS/MS spectra for compounds I (B), II (C) and III (D) in (A). The chemical formulae identified by NMR analyses are shown under the spectra.

Table 1 Estimated concentrations of major peaks in high-performance liquid chromatography (HPLC) profile. The peak number, retention time (RT) and absorption units (AUs) correspond to the HPLC profile in Figure 5D. Peaks 5, 6 and 8 correspond to peaks I, II and III in Figure 6A, respectively, as identified by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) and nuclear magnetic resonance (NMR) analyses. The concentration (μg/g) represents the microgram of flavonoids per gram of dry T_3 *C1/R-S* rice kernels

Peak	RT (min)	AUs	μg/g
1	6.33	3.20	268.9
2	9.85	0.35	29.4
3	11.20	0.42	35.3
4	20.97	2.10	176.5
5	26.91	1.85	155.5
6	35.66	3.93	330.3
7	36.86	1.19	100.0
8	47.67	0.66	55.5
9	49.22	0.64	53.8

Table 2 Concentrations of total flavonoids estimated using taxifolin at 280 nm wavelength as reference. SD, standard deviation calculated from five measurements per sample

Sample	Flavonoid (mg/g)	SD
Hwa-Young WT	0.414*	0.0144
Black rice	1.900	0.0036
C1/R-S T_3	12.880	0.0467
Green tea	151.660	0.0846

*The low level of flavonoids in wild-type (WT) kernels probably arose from their embryos.

1992). The sum of the major compounds (Table 1) was approximately 1.2 mg/g, accounting for about 10% of the estimated total flavonoids in the *C1/R-S* rice kernels. This indicates that a larger portion of the total flavonoids is partitioned into the aqueous phase rather than the ethyl acetate phase.

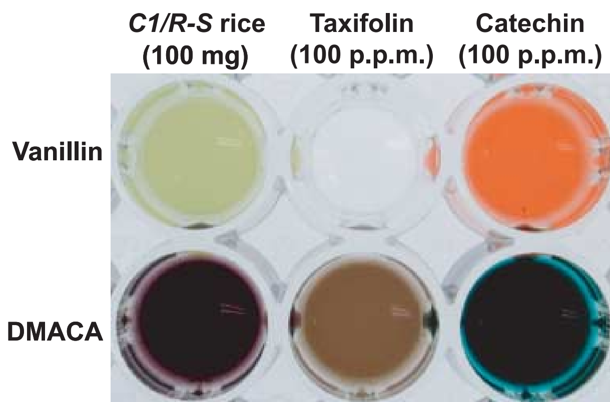

Figure 7 Reaction assays of flavonoids. Colours of vanillin- and dimethylaminocinnamaldehyde (DMACA)-stained extracts of 100 mg of dry kernels of T_3 *C1/R-S* rice were compared with those of 0.1 mg/mL taxifolin and 0.1 mg/mL catechin. Vanillin, 1% vanillin in 5 M HCl; DMACA, 0.5% DMACA and 5% H_2SO_4 in methanol.

To test whether the flavonoid extracts of *C1/R-S* rice contained catechins and catechin derivatives, we used a catechin-specific dye, vanillin, for the colour reaction (Figure 7). Although a concentration of 0.1 mg/mL catechin reacts with vanillin to produce a bright red colour, our *C1/R-S* rice extract showed no response with vanillin, suggesting a lack of catechin-type flavonoids. In contrast, we confirmed the presence of taxifolin-type flavonoids in *C1/R-S* rice via the dimethylaminocinnamaldehyde (DMACA) colour assay (Figure 7).

Flavonoids are produced throughout the endosperm of *C1/R-S* rice

For general labelling of flavonoids, we incubated thin sections of *C1/R-S* 2-1 and WT kernels with saturated diphenylboric acid (DPBA). Epifluorescence microscopy with an fluorescein isothiocyanate (FITC) filter set revealed that the DPBA-derived fluorescence level was much higher in the transgenic rice (Figure 8). Because DPBA fluoresces only on binding to flavonoids, the strong fluorescence signal in *C1/R-S* rice kernels indicated the presence of high levels of flavonoids. In the labelled section of the *C1/R-S* rice kernels, the concentrated fluorescence signal of cells in the outer endosperm layers appeared as a thick, green band (Figure 8A). At higher magnification, we determined that this highly fluorescent band comprised four to five cell layers, including the aleurone layer (Figure 8D). The fluorescence signal was abruptly attenuated in the inner starchy endosperm layers, mainly because starch granules and protein bodies limited the free cytosolic space and simultaneously blocked the fluorescence signal of the DPBA-labelled flavonoids.

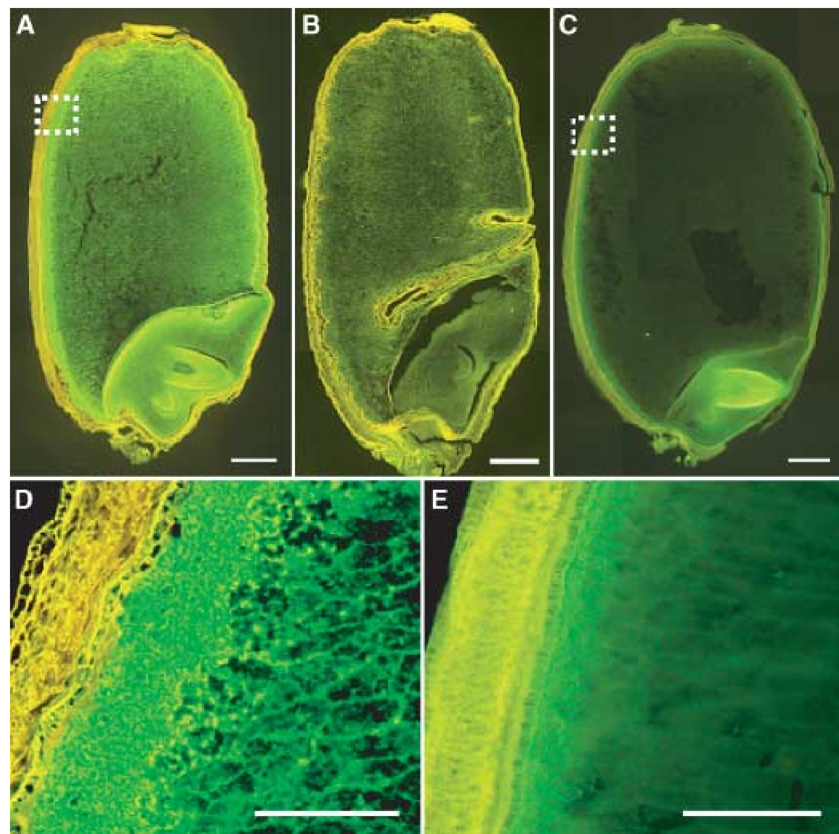

Figure 8 Thin sections of *C1/R-S* 2-1 rice (A, D) and wild-type (WT) (C, E) kernels labelled with diphenylboric acid (DPBA). Labelled flavonoids are depicted as a green fluorescent signal. (B) Section of T_3 *C1/R-S* 2-1 rice kernel without DPBA labelling as a negative control. Signal in embryo axis of WT kernel (C) serves as a positive control. (D) and (E) are insets of (A) and (C), respectively, at higher magnifications. Scale bars: (A–C) 0.5 mm; (D, E) 0.2 mm.

Flavonoid biosynthetic pathway genes activated by *C1*/*R-S* transgenes

Genes activated by the *C1* and *R-S* transgenes were identified by RT-PCR using RNAs extracted from the developing kernels of the *C1*/*R-S* 2-1 rice line and primer sets for various genes involved in the flavonoid biosynthetic pathway. These genes included those for phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavone synthase (*FNS*), 2-hydroxyisoflavone synthase (*IFS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3′-hydroxylase (*F3′H*), flavonol synthase (*FLS*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin reductase (*LAR*), anthocyanidin synthase (*ANS*) and glutathione-*S*-transferase (*GST*). Of these, the expression of *PAL, CHS*, *F3H, F3′H* and *DFR* was substantially increased in the transgenic lines, but hardly detectable or at relatively lower levels in the WT (Figure 9). Expression of *CHI*, *LAR* and *ANS* was not detectable in the developing transgenic kernels. Likewise, *GST* transcript levels were significant in the WT, but remained unaffected in the *C1*/*R-S* transgenic lines. These results suggest that the biosynthetic pathway is activated up to the steps for dihydroflavonols, leucoanthocyanidins and flavin-4-ols (Figure 10). Although two of the identified major flavonoids, taxifolin and 3′-*O*-methyl taxifolin, belong to the dihydroflavonol group, we did not determine whether high activation of the *DFR* gene resulted in the production of leucoanthocyanidins and flavin-4-ols, because no members of these two flavonoid groups were identified from the extracts of *C1*/*R-S* kernels.

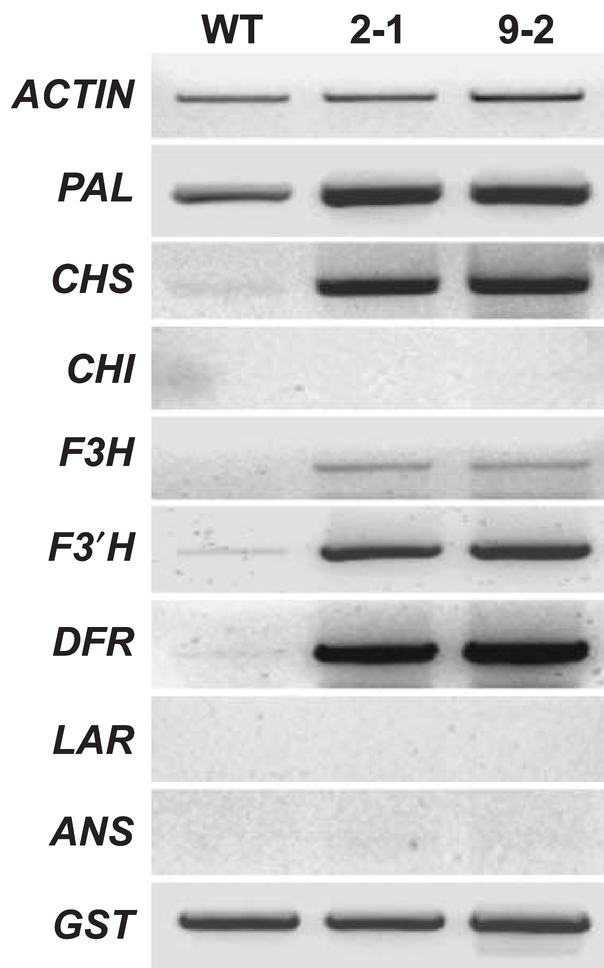

Figure 9 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of flavonoid biosynthetic pathway genes using RNAs extracted from developing kernels of wild-type (WT) and T_3 *C1*/*R-S* rice homozygous lines (2-1 and 9-2). *ACTIN*, used as reference for PCR template quantity; *PAL*, phenylalanine ammonia lyase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *F3H*, flavanone 3-hydroxylase; *F3′H*, flavonoid 3′-hydroxylase; *DFR*, dihydroflavonol 4-reductase; *LAR*, leucoanthocyanidin reductase; *ANS*, anthocyanidin synthase; *GST*, glutathione-*S*-transferase.

Discussion

Well-elucidated biosynthetic pathways and numerous lines of evidence for the health-promoting effects of flavonoids have made them an attractive target for the genetic engineering of crop plants (Dixon and Steele, 1999). Despite a number of attempts to ectopically express various genes involved in the pathways of diverse systems (reviewed in Schijlen *et al.*, 2004), our study is the first to report the development of transgenic rice lines that produce high levels of various flavonoids in the endosperm. Because, normally, the endosperms of cereals (including rice) completely lack the expression of most flavonoid biosynthetic genes, we used two maize regulatory genes (*C1* and *R-S*) for ectopic expression in the endosperm of *C1*/*R-S* rice to activate structural genes in the pathway. Our rationale for selecting *C1* and *R-S*, rather than the corresponding members in rice, was based on previous studies showing that the maize *C1* and *R* family members together are functionally conserved amongst diverse plant species to induce biosynthesis even in plant tissues that naturally do not produce flavonoids (Lloyd *et al.*, 1992; Quattrocchio *et al.*, 1993, 1998; Hu *et al.*, 2000).

Analysis of the *C1*/*R-S* rice revealed that *PAL, CHS, F3H, F3′H* and *DFR* were highly activated by the transgenes, whereas the expression of *CHI, ANS* and *LAR* was not detected. For *CHI*, it has been reported that, without its detectable expression, the pathway proceeds to produce anthocyanins in *C1*- and *R*-over-expressing maize suspension cells (Bruce *et al.*, 2000). Considering that the majority of the flavonoids produced in our study seemed to be dihydroflavonols and their derivatives, the flavonoid biosynthetic pathway in *C1*/*R-S* rice appears to be activated up to the step prior to the

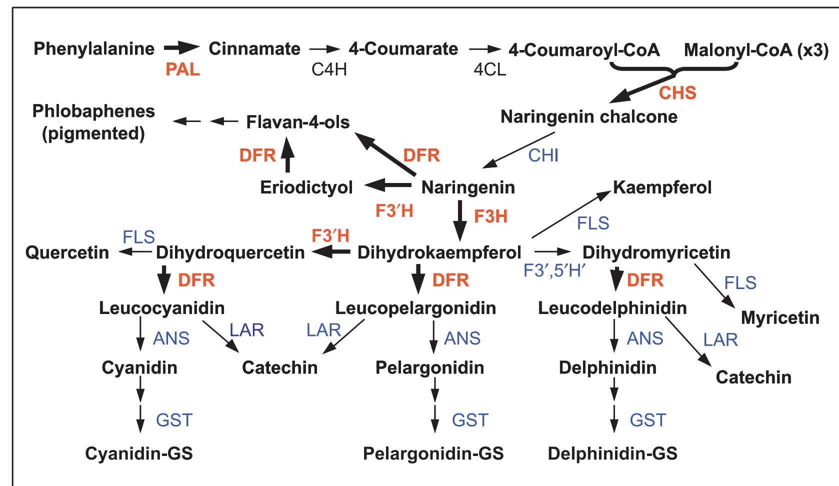

Figure 10 Flavonoid biosynthetic pathway and structural genes activated by *C1* and *R-S* transgenes. Enzymes in red are from genes whose expression increased in transgenic plants. Blue- and black-coloured enzymes are from genes whose expression was unchanged and untested, respectively. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; FNS, flavone synthase; IFS, 2-hydroxyisoflavone synthase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; GST, glutathione-*S*-transferase.

production of anthocyanins and catechins. However, the activated steps in the pathway cannot be determined until all the flavonoids, other than the three named here, have been identified in the extracts of *C1*/*R-S* rice kernels.

Our results are comparable with those from other studies, in which subsets of flavonoid biosynthetic genes have been activated by introduced *C1* and *R* regulatory genes. For example, Grotewold *et al*. (1998) and Bruce *et al*. (2000) have reported that anthocyanin production is induced in the cell suspension lines of inbred maize 'Black Mexican Sweet' that ectopically expresses *C1* and *R*, as a result of the activation of *ANS*, *PAL*, *CHS*, *F3H* and *DFR*. In contrast, Bovy *et al*. (2002) developed transgenic tomato lines expressing maize *C1* and *LC* genes that were controlled under either *35S* or a fruit-specific promoter. For any combination of transgenes and promoters, activation of the pathway genes, including *PAL, CHS, F3H, FLS, DFR* and *ANS*, was insufficient for anthocyanin production in the fruit flesh, whereas *C1* alone was able to induce anthocyanin production in the leaves. It was suggested that no expression of *F3′H* and flavanone-3′,5′-hydrozylase (*F3′,5′H*) genes and the substrate specificity of FLS and DFR enzymes could be responsible for the accumulation of kaempferol and the absence of anthocyanins in *C1*/*LC* tomato flesh. In contrast, the expression of *F3′H* was activated in our *C1*/*R-S* rice, whereas the expression of *FLS*, *F3′,5′H* and *ANS* was not detected (Figure 9). These gene expression data were in agreement with the accumulation of dihydroquercetin and its derivatives, other than dihydrokaempferol, in *C1*/*R-S* rice (Figures 7 and 10). Although the role of *ANS* in anthocyanin production was not discussed in Bovy *et al*. (2002), no expression of the *ANS* gene may be the primary reason for the absence of anthocyanin production in *C1*/*R-S* rice. This explanation can be further confirmed by the transformation of *C1*/*R-S* rice with *ANS*. Nevertheless, despite the acknowledgement of the tissue specificity of *C1*/*R* transgenes for the activation of flavonoid biosynthetic genes, the factors inducing anthocyanin production in rice endosperm and tomato fruit flesh remain unknown.

Although we noted no difference in growth habit between *C1*/*R-S* rice and the WT, kernel maturation was greatly delayed in the former, resulting in a substantial decrease in kernel weight and, in turn, productivity. This may be explained by the possible role of flavonoids in the actions of plant hormones, such as auxin, ethylene, abscisic acid, gibberellins and cytokinin. For example, Buer and Muday (2004) reported that the interaction of flavonoids with auxin inhibited its transport. Therefore, it is probable that such inhibited auxin transport in the *C1*/*R-S* rice endosperm also results in the altered homeostasis of other hormones, e.g. abscisic acid, gibberellins and ethylene, which are directly responsible for the maturation processes of dehydration and senescence. The reduced grain filling of *C1*/*R-S* rice, as indicated by smaller kernels and intrusions of the pericarp into the inner endosperm, may also be partially associated with the interaction of flavonoids with plant hormones in the endosperm. Further work is needed to determine the mechanisms by which flavonoid production is related to phenotypes such as delayed maturation and smaller kernel sizes in *C1*/*R-S* rice.

In summary, the research discussed here represents the first report of flavonoids being produced in the rice endosperm, making this a significant product of plant biotechnology. The total flavonoid content in our transgenic kernels was about 10-fold greater than that measured in existing black rice kernels, but was still only 10% of the level found in the dried leaves of a green tea cultivar. Nevertheless, because most flavonoids have a two- to 25-fold higher antioxidant capacity

than ascorbic acid, or vitamin C (Latte and Kolodziej, 2004), our rough estimate of about 12 mg/g dry weight from *C1/R-S* rice kernels shows great potential in developing a novel rice variety that can promote human health either through daily consumption or as a pharmaceutical product. Furthermore, this *C1/R-S* rice accumulates flavonoids throughout the endosperm, so that the compounds remain in the transgenic grains even after extensive polishing, in contrast with their substantial loss from existing black rice during post-harvest processing.

Experimental procedures

Construction of *C1/R-S* transgene

The prolamin *NPR33* promoter (a 5′ untransformed region between –652 and –13 from the ATG start site of a rice 13-kDa prolamin gene; Wu *et al*., 1998) in the pUC18 plasmid was subcloned into the *Hin*dIII/*Xba*I sites of a modified pGEM7Zf(+) plasmid (Promega Co., Madison, WI, USA) that had the *NOS* terminator (Tnos) at the *Sac*I/*Eco*RI sites. It was designated as pYMPP. *C1* cDNA (822 bp) was amplified by PCR using the p35SC1 plasmid, which contained *C1* cDNA as template, a forward primer with an *Xba*I restriction site (5′ATTCTAGACGAGCTTGATCGACGAGAGAGCGAG3′) and a reverse primer with an *Sac*I site (5′CGAGCTCGACGTGTACTTGTTGTCTACGCAAG3′). The PCR product was digested with *Xba*I and *Sac*I, and then ligated into the same restriction sites of the pYMPP plasmid, to be designated as pYMPPC1. Up to this step, *R-S* cDNA (1839 bp) was similarly subcloned into the pYMPP plasmid, but using a different primer set (5′GCTCTAGACGTTCAGCAGGCGCGTGATG3′ and 5′CCCCCGGGGGCTGCCCCTTCACCGCTTCCCT3′) corresponding to the *R-S* sequence. It had an *Xba*I site at the end of the forward primer and an *Sma*I site at the end of the reverse primer, and was redesignated as pYMPPR-S. Using the pYMPPC1 plasmid DNAs as template, a forward primer for the *NPR33* promoter with an *Hin*dIII site (5′AAGCTTGGTGTAGCAACACGACTT3′) and a reverse primer for Tnos with a *Bam*HI site (5′CGGGATCCCGGATCTAGTAACATAGATGACAC3′), we digested the PCR products with *Hin*dIII and *Bam*HI, followed by ligation into the same restriction sites of a modified pCambia3301 binary vector that contained *smGFP::GUS* and a phosphinotricin-resistant *BAR* gene, each of which was under the control of the 35S cauliflower mosaic virus (CaMV) promoter within the T-DNA borders. Subsequently, we PCR-amplified the *R-S* coding sequence, using the pYMPPR-S plasmid DNAs as template, a forward primer for the *NPR33* promoter with a *Bam*HI site (5′GGATCCGGTGTAGCAACACGAGTT3′) and a reverse primer for Tnos with the *Eco*RI site (5′GGAATTCCGATCTAGTAACATAGATGACAC3′). The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the same restriction sites of a modified pCambia3301 (Cambia, Black Mountain, Australia) that contained *C1*. The modified pCambia3301 possessed coding regions for the *C1* and *R-S* genes, each having a prolamin *NPR33* promoter and Tnos, and being fused at a common restriction site, *Bam*HI. This new plasmid, pYMPPC1/R-S, was then transformed into *Agrobacterium tumefaciens* LBA4404 by the freeze–thaw method (An *et al*., 1988).

Rice transformation

Calli were produced from the germinating embryos of *Oryza sativa japonica* cv. Hwa-Young. We used a callus-inducing medium (N6CI, pH 5.8) that contained N6 basal salts and vitamins, 1 g/L casamino acid, 30 g/L sucrose and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and which was solidified by 2 g/L gelite. Within 1 or 2 weeks of subculturing on fresh medium, the calli were infected with *Agrobacterium tumefaciens* LBA4404 harbouring the binary vector pYMprolaminProC1/R-S, and then co-cultured for 3 days in the dark at 25 °C with *Agrobacterium* on an N6CI medium supplemented with 100 μM acetosyringone. Transformed callus cells were selected by culturing for about 3 weeks on a selection medium consisting of N6 basal salts and vitamins, 1 g/L casamino acid, 30 g/L sucrose, 2 mg/L 2,4-D, 500 mg/L carbenicillin and 6 mg/L phosphinotricin. Subsequently, the calli were transferred to a pre-regeneration medium containing N6 basal salts and vitamins, 2 g/L casamino acid, 30 g/L sucrose, 30 g/L sorbitol, 1 mg/L 2,4-D, 0.5 mg/L 6-benzyladenine, 0.25 g/L cefotaxime and 6 mg/L phosphinotricin. Following 10 days of culturing on this medium, the calli were transferred to a regeneration medium (Murashige–Skoog basal salts and vitamins, 2 g/L casamino acid, 30 g/L sucrose, 20 g/L sorbitol, 1 mg/L naphthaleneacetic acid, 5 mg/L kinetin, 0.125 mg/L cefotaxime and 6 mg/L phosphinotricin). After about 4 weeks, the calli showed greening and regeneration of plantlets. These plantlets were moved to a growth medium (Murashige–Skoog basal salts and vitamins, 30 g/L sucrose and 3 g/L gelite; pH 5.8) and grown to about 10 cm in height before being transferred to soil.

Genomic DNA isolation, genomic DNA PCR and Southern blot analysis to verify transgene integration

About 2 g of leaf tissue of WT and several independent *C1/R-S* T_1 plants was ground in liquid nitrogen to a fine powder and homogenized in 9 mL of an extraction solution consisting

of 1% cetyltrimethylammonium bromide (w/v), 1% β-mercaptoethanol (v/v), 700 mM NaCl and 10 mM ethylenediaminetetraacetic acid (EDTA) in 100 mM Tris-HCl buffer (pH 7.5). The homogenate was incubated at 65 °C for 1–1.5 h, with shaking every 15 min. After cooling, 5 mL of chloroform/isoamyl alcohol (24 : 1) was added and gently shaken for 5 min. The mixture was centrifuged at 1900 ***g*** for 10 min, and the supernatant was transferred to a new 15-mL Falcone tube. The extract was treated with RNase A at a final concentration of 40–50 μg/mL at 37 °C for 30 min. Genomic DNAs were precipitated by adding 6 mL of cold isopropanol and centrifuging at 3380 ***g*** for 5 min. The DNA pellet was transferred to a 1.5-mL microfuge tube and washed twice in 0.5 mL of 70% ethanol. It was then briefly air-dried and resuspended in 1 mL of nuclease-free water. PCR products were amplified using about 100 ng of genomic DNA as template and a primer set of the *BAR* gene, 5′TACATCGAGACAAGCACGGTCAACTT3′ and 5′TGCCAGAAACCCACGTCATGCCAGTT3′.

For Southern blot analysis, 10 μg of genomic DNA was digested with either *Eco*RI alone or *Eco*RI and *Bam*HI together. The blots were hybridized with a ^{32}P-labelled *BAR* gene probe, and exposed to X-ray film for about 20 h. Other details of this analysis essentially followed the protocol of Church and Gilbert (1984).

RNA isolation and semiquantitative RT-PCR to determine the expression of transgenes and genes involved in the flavonoid biosynthetic pathway

Total RNAs were isolated and purified from kernels of the T_3 2-1 homozygous line at 10–15 days after pollination, based on the acid phenol–guanidinium thiocyanate–chloroform extraction protocol (Sambrook and Russell, 2001). The first-strand cDNAs of purified total RNAs were synthesized at 42 °C for 60 min in a total volume of 25 μL that contained 3 μg of total RNAs, 1.5 μg oligo dT and 200 units of M-MLV reverse transcriptase. The relative concentrations of RNAs in different samples were estimated by second PCRs using a primer set of rice actin, 5′AACTGGGATGATATGGAGAA3′ and 5′CCTCCAATCCAGACACTGTA3′. The relative expression levels of the *C1* and *R-S* transgenes, as well as most of the genes in the flavonoid biosynthetic pathway, were determined by second PCRs using the same first-strand cDNA batches.

Extraction of flavonoids from rice kernels and HPLC analysis

Flavonoids were extracted from 1 g of fine powder from T_3 *C1*/*R-S* 2-1 rice kernels in 10 mL of 70% methanol. This process involved 2 min of vigorous vortexing, 30 min of sonication and incubation with shaking either for 3 h at room temperature or overnight at 4 °C. After the mixture had been centrifuged for 10 min at 1900 ***g***, the supernatant was taken and filtered through a 0.45-μm syringe filter. After partitioning with 20 mL of n-hexane to remove lipids, 0.5-mL aliquots of the extract were vacuum dried for 2 h at room temperature. The dried aliquot was then resuspended in 100 μL of 10% methanol, and 20 μL was injected on to a Waters 600 Series HPLC system equipped with a Waters Symmetry C18 guard column, Waters XTerra Phenyl column [250 mm × 4.6 mm internal diameter (i.d.)] and a Waters 996 photodiode array detector. The binary mobile phase consisted of 6% acetic acid in 2 mM sodium acetate (v/v; final pH 2.5; solvent A) and 25% of solvent A in acetonitrile (v/v; solvent B). The HPLC flow rate was 1 mL/min, with a gradient running programme of 100% solvent A for 10 min, 0%–50% solvent B for 40 min and 50%–100% solvent B for 5 min. Data were processed with Waters Millennium 32 software. All flavonoid extraction and HPLC conditions primarily followed the guidelines described by Tsao and Yang (2003).

For moderate acid hydrolysis and ethyl acetate partitioning, each dried aliquot of the 0.5-mL extract was dissolved in 100 μL of 1 M HCl containing 0.1% ascorbic acid (w/v), and treated for 10 min at 90 °C. To purify the aglycones of the flavonoids, three volumes of ethyl acetate were added to this acid-hydrolysed mixture and briefly vortexed. After the ethyl acetate phase (upper phase) had been vacuum dried, two aliquots were dissolved in 100 μL of 10% methanol for injection into the HPLC system.

LC coupled with electrospray ionization/MS

We used the Finnigan Surveyor Modular HPLC System (Thermo Electron Co., Waltham, MA) with a Waters XTerra MS C18 (5 μm, 2.1 mm × 150 mm) for individual flavonoid separation, plus the Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co.), equipped with a Finnigan electrospray source, for electrospray ionization/MS. These systems were operated by Xcalibur software (version 1.3 SP2, Thermo Electron Co.). The HPLC mobile phase consisted of water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid. HPLC was conducted at a flow rate of 0.2 mL/min, with the following gradient programme: 0–3 min, 5% solvent B; 3–50 min, 5%–50% solvent B; 50–55 min, 50%–100% solvent B; and 55–75 min, 100% solvent B. MS was performed with an electrospray ionization source, under the following conditions: spray needle voltage, 5 kV; ion transfer capillary temperature, 200 °C; nitrogen

sheath gas flow rate, 60 arbitrary units; auxiliary gas flow rate, 5 arbitrary units. Full-scan mass spectra were obtained in the range of 100–1000 *m/z*, with three microscans and 200 ms of maximum ion-injection time. Data were acquired from both positive and negative modes of the LCQ mass spectrometers, and by using software provided with the system.

NMR determination of flavonoids isolated from T_3 kernels of *C1/R-S* transgenic rice

Samples (1–3 mg) of individual flavonoids isolated via HPLC were dissolved in 0.15 mL of CD_3OD. Each sample was transferred to a 3-mm-diameter NMR tube, and its ^1H and ^{13}C NMR spectra were measured with a Varian Unity Plus 500 instrument (Walnut Creek, CA, USA) at 500 MHz and 125 MHz, respectively. The NMR gHSQC, gCOSY and gHMBC pulse sequences were used to determine the chemical structures of these isolated flavonoids.

Fluorescent labelling of flavonoids in rice kernels

Kernels of WT and T_3 *C1/R-S* 2-1 transgenic rice at 25 days after pollination were fixed in 3.7% paraformaldehyde and 0.2% picric acid in a 50 mM potassium/5 mM EGTA buffer (pH 6.8). After washing away the fixative in the same buffer, the samples were dehydrated and infiltrated with paraffin. The embedded tissue blocks were then sliced to a 30-μm thickness; these sections were dewaxed, rehydrated and labelled with saturated (0.25%, w/v) DPBA for 5 min. The DPBA-labelled sections were examined with an epifluorescence microscope (Olympus BX 51), and fluorescent images were acquired with an FITC filter set and a digital camera on the microscope.

Colour reaction assays of *C1/R-S* rice kernel extracts with vanillin and DMACA

A working solution of 1% vanillin was prepared by dissolving 0.1 g of vanillin in 10 mL of 5 M HCl; 5% DMACA was prepared in methanol containing 5% H_2SO_4. The lyophilized extract from 100 mg of dry kernels was then dissolved in either 0.5 mL of vanillin or DMACA. Taxifolin and catechin (0.1 mg/mL each) were used as reference compounds for the colour reactions. Vanillin is catechin-specific and produces a bright red hue, but no colour develops when it reacts with taxifolin. DMACA reacting with catechin produces a dark greenish-blue colour; with taxifolin, the coloration is purple.

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