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Compartmentation and Dynamics of Flavone Metabolism in Dry and Germinated Rice Seeds

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Among secondary metabolites, flavonoids are particularly important for the plant life cycle and could be beneficial for human health. The study of *Arabidopsis thaliana transparent testa* mutants showed that seed flavonoids are important for environmental adaptation, reactive oxygen species homeostasis, dormancy and longevity. Compared with *Arabidopsis* and maize (*Zea mays* L.), far less research has been conducted on rice (*Oryza sativa* L.) particularly for cultivars with non-pigmented seeds. In this study, we describe the localization, nature and relative abundance of flavonoids in mature and germinated non-pigmented Nipponbare seeds using a combination of confocal microscopy, mass spectrometry and gene expression analysis. The mature seed exclusively accumulates flavones mostly in the embryo and to a lesser extent in the pericarp/testa. Due to the variety of flavone conjugation patterns, 21 different flavones were identified, including sulfated flavones never mentioned before in cereals. Schaftoside (apigenin-6-C-glucoside-8-C-arabinoside) and its two isomers represent nearly 50% of all rice seed flavones and are the only flavonoids accumulated in the pericarp/testa seed compartment. These 21 conjugated flavones showed a very stable profile during rice seed germination *sensu stricto*, while expression of key flavone synthesis genes strongly increases before the completion of germination. We discuss the potential roles of these rice seed flavones in a seed biology context.

Keywords: Compartmentation • Embryo • Flavonoid • Germination • Rice • Seed.

Abbreviations: ANS, anthocyanidin synthase; bHLH, basic helix–loop–helix; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; DPBA, diphenylboric acid 2-aminoethyl ester; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FNS, flavone synthase; HAI, hours after imbibition; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; MALD-IMS, matrix-assisted laser desorption/ionization imaging mass spectrometry.

Introduction

Flavonoids are a large family of plant polyphenolic compounds that play key roles in plant reproduction, resistance to pathogens or survival in the dry state (Rajjou and Debeaujon 2008, Buer et al. 2010, Petroni and Tonelli 2011, Falcone Ferreyra et al. 2012, Agati et al. 2013, Liu et al. 2013). In addition, these plant secondary metabolites are of major interest for human health and cosmetic uses (Arct and Pytkowska 2008, Kumar and Pandey 2013, Mamta et al. 2013). Flavonoids can be categorized into several classes (e.g. chalcones, flavones, flavonols, dihydroflavonols, anthocyanins and proanthocyanidins) based on the degree of heterocyclic C-ring oxidation, the position of hydroxyl groups and the degree of polymerization (Fig. 1). They act as UV filters, serve as a line of defense against pathogens, color flower petals and participate in fertility (Falcone Ferreyra et al. 2012). In particular, flavonoids accumulate to high levels in the seed testa, embryo and sometimes in the aleurone layer of cereal seeds (Lepiniec et al. 2006, Petroni and Tonelli 2011, Falcone Ferreyra et al. 2012). The structural diversity of seed flavonoids has a significant impact on seed color depending on the accumulated flavonoids. In turn, this visible phenotype served to isolate genes involved directly in flavonoid biosynthesis or indirectly as regulatory elements in plants (Lepiniec et al. 2006, Petroni and Tonelli 2011, Falcone Ferreyra et al. 2012). Thus, mostly from studies on *Arabidopsis* (*Arabidopsis thaliana*) and maize (*Zea mays*), a very complete flavonoid biosynthesis pathway has emerged (Fig. 1).

In South-Eastern Asia, rice (*Oryza sativa* L.) is by far the main cereal in terms of food supply (FAO 2009) and exhibits a considerable genetic diversity exemplified by the existence of black, brown, red or colorless (white) rice seeds (Pereira-Caro et al. 2013a). Black rice seeds show the greatest polyphenolic content compared with red and white rice seeds (Finocchiaro et al. 2010). The black pigmentation of rice seeds is due to accumulation of the cyanidin-3-glucoside and peonidin 3-glucoside anthocyanins (Abdel-Aal et al. 2006, Yoshimura et al. 2012, Pereira-Caro et al. 2013a). In turn, the pigmentation of red rice is mainly due to the presence of proanthocyanidins and/

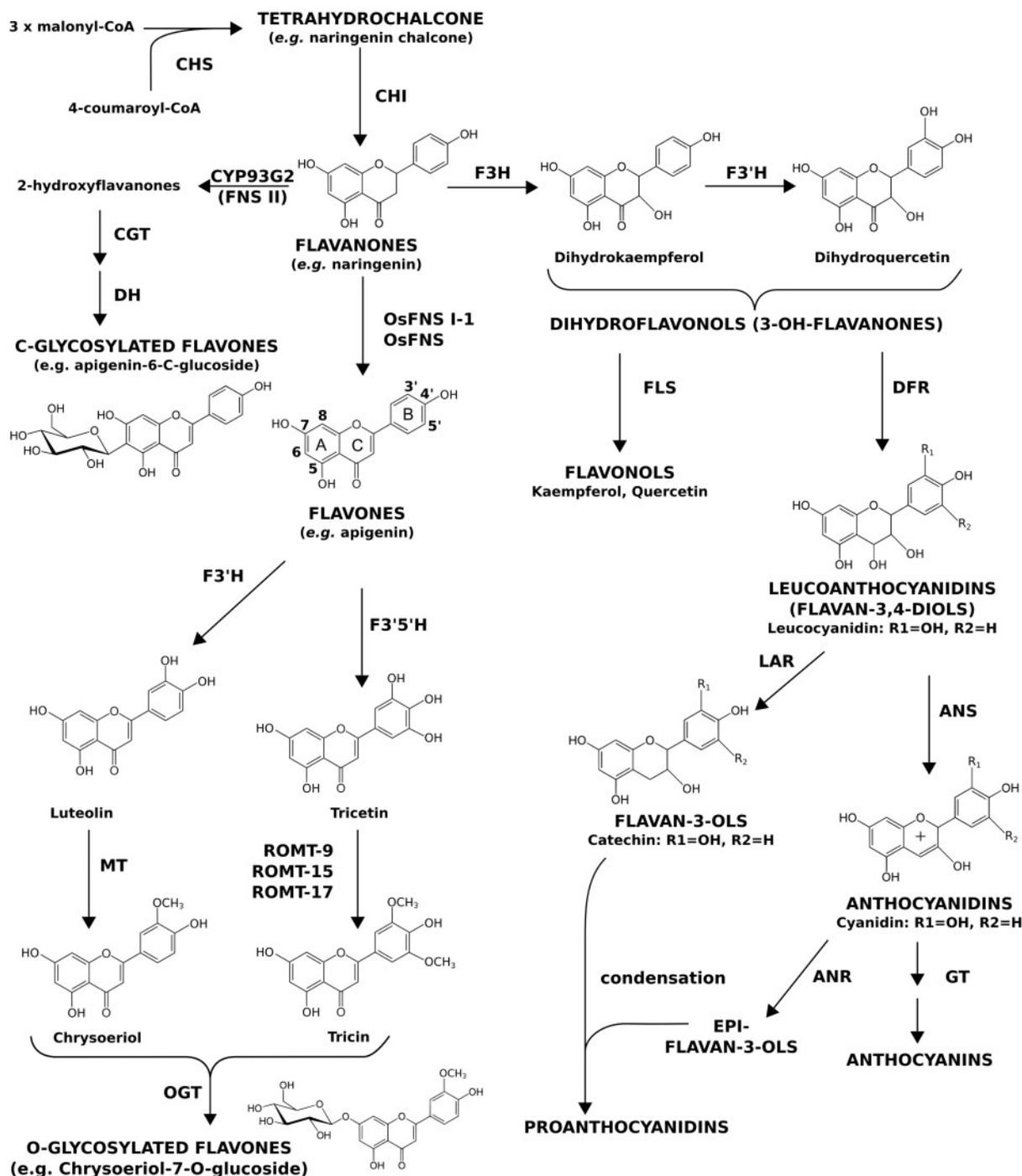


Fig. 1 Biosynthesis of rice flavonoids with an emphasis on flavones. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CGT, C-glycosyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; CYP93G2, P450 flavanone 2-hydroxylase; DFR, dihydroflavonol 4-reductase; DH, dehydratase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavone 3',5'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; GT, glucosyltransferase; LAR, leucoanthocyanidin reductase; MT, O-methyltransferase; OGT, O-glycosyltransferase; ROMT, rice O-methyltransferase.

or anthocyanins within the seed coat (Finocchiaro et al. 2010, Pereira-Caro et al. 2013a). Classical and molecular cloning studies showed that the red rice pericarp trait is determined by the complementary effect of the *Rc* and *Rd* loci (Furukawa et al. 2007). On the one hand, the *Rc* locus codes for a basic helix–loop–helix (bHLH) transcription factor that positively

regulates not only flavonoid accumulation but also ABA biosynthesis, thereby contributing to seed dormancy (Sweeney et al. 2006, Furukawa et al. 2007, X.Y. Gu et al. 2011). On the other hand, the *Rd* locus encodes a dihydroflavonol-4-reductase (DFR) that is involved in proanthocyanidin and anthocyanin synthesis (Furukawa et al. 2007). The presence of

anthocyanins, proanthocyanidins, flavones and flavonols may not be exclusive since black rice seeds exhibit different classes of flavonoids simultaneously (Finocchiaro et al. 2010, Kim et al. 2010, Pereira-Caro et al. 2013a, Pereira-Caro et al. 2013b).

In rice, the sequential events from chalcone synthesis to proanthocyanidin and anthocyanin synthesis have been deciphered by genetic approaches. It has been demonstrated that rice chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), DFR and anthocyanidin synthase (ANS) were able to complement their ortholog defect in *Arabidopsis* *tt4*, *tt5*, *tt6*, *tt7*, *tt3* and *tt18* mutants, respectively (Shih et al. 2008).

Most of the work on rice seed flavonoids has been done on pigmented *O. sativa* landraces with either red or black seeds. However, the majority of cultivated rice varieties have colorless (white) seeds since the seed coat pigmentation trait was lost during rice domestication (Sweeney et al. 2006, Gross et al. 2010, X.Y. Gu et al. 2011). Indeed, modern plant breeding has been associated with the loss of several ancestral traits (Kovach et al. 2007, Martinez-Andujar et al. 2012). One of them is the loss of pigmented pericarps of cereal seeds and, consequently, wild *Oryza* species exhibit pigmentation while most of the cultivated rice cultivars (*O. sativa*) exhibit colorless seeds (Sweeney et al. 2006, Furukawa et al. 2007, Kovach et al. 2007, Gross et al. 2010, X.Y. Gu et al. 2011). The change from red to white rice pericarp is the result of the removal of 14 bp on the *Rc* locus, leading to a truncated protein (Kovach et al. 2007).

In terms of chemical diversity, flavonoids present in colorless rice seeds seem to be restricted to flavones and flavonols (Kim et al. 2010, Matsuda et al. 2012). However, even if devoid of proanthocyanidins and anthocyanins, non-pigmented rice cultivars show a comparable amount of flavones with pigmented rice cultivars (Kim et al. 2010). Flavones are often *O*- or *C*-conjugated by two distinct pathways (Brazier-Hicks et al. 2009). *O*-Glycosylated flavones are formed from flavanones by the combined action of a flavone synthase (FNS) and *O*-glucosyltransferases (OGT) (Fig. 1). Two types of FNS, FNSI and FNSII, were classified as Fe²⁺/2-oxoglutarate-dependent dioxygenase and Cyt P450-dependent monooxygenase, respectively (Heller and Forkmann 1993, Martens and Mithofer 2005). In rice, the *OsFNSI-1* gene (LOC_Os10g39140) was found to be homologous to *Medicago truncatula* FNSI and capable of converting naringenin to apigenin, at least in vitro (Lee et al. 2008b). Moreover, very recently, two studies have confirmed the role in vitro and in vivo of the rice FNSII CYP93G1 (LOC_Os04g01140) in the production of flavone *O*-glycosides (Brazier-Hicks and Edwards 2013, Lam et al. 2014). Regarding *C*-glycosylflavone biosynthesis, the flavanone 2-hydroxylase (F2H) CYP93G2 (LOC_Os06g01250) can produce 2-hydroxyflavanones which are then *C*-glycosylated by a *C*-glucosyltransferase (*OsCGT*, e.g. LOC_Os06g18010) (Du et al. 2010, Brazier-Hicks and Edwards 2013). Finally, 2-hydroxyflavanone *C*-glucosides are dehydrated by an as yet unknown enzyme to produce the *C*-glycosylated flavones (Brazier-Hicks et al. 2009). Other enzymes responsible for flavone synthesis, flavonoid *O*-methylation or *O*-glycosylation were also reported and characterized by in vitro approaches (Kim et al. 2006, Kim et al. 2008, Lee et al.

2008a, Lee et al. 2008b, Luang et al. 2013). Thus, a nearly complete flavonoid biosynthesis pathway in pigmented and non-pigmented rice genotypes is now available (Fig. 1).

Regarding non-pigmented rice flavonoids, at least three important issues remain to be elucidated. The first issue tackles the precise localization of flavonoids within the mature seed tissues. Indeed, a single study has addressed flavonoid distribution in a black rice seed cultivar (Yoshimura et al. 2012), whereas no comparable work exists on colorless rice seeds. The second issue deals with the chemical diversity of flavonoids in non-pigmented rice seeds in terms of both the nature and quantity on a per seed basis. Finally, the third issue addresses the dynamics of flavonoids in the rice embryo during germination *sensu stricto*, a topic largely covered concerning leguminous plants but surprisingly unaddressed concerning other plant families (Lin and Lai 2006, Wu et al. 2012). Here, rice seed germination is characterized as the transition from a dry mature seed to a fully imbibed germinated seed where the coleoptile surrounding the first leaves is the first organ to emerge. The present work demonstrates that flavonoids are highly compartmented within the rice seed. The investigation of the chemical nature and relative abundance of flavonoids from the dry mature seed and from germinated rice embryos revealed the presence of 21 different flavone derivatives together with an absence of flavonols. Based on the nature and localization of rice seed flavones, their stability during germination and the expression of flavonoid biosynthesis genes, we suggest that rice seed flavones may serve as the first line of defense to cope with environmental stresses during seedling establishment.

Results

Histochemical localization of flavonoids in rice dry and germinated seeds

Recently, eight different flavones were identified in rice grains from two *japonica* and *indica* varieties (Matsuda et al. 2012). However, with the exception of one study on black-colored rice seeds (Yoshimura et al. 2012), the precise flavonoid distribution in the different tissues of non-pigmented mature rice seeds (Fig. 2A) remains to be established. It is known that germination can significantly influence flavonoid content in leguminous species (Wu et al. 2012) and that some flavonoids can modify the ABA hormonal content in *Arabidopsis* seeds (Jia et al. 2012). However, to date, there was no report of flavonoid localization in rice seed tissues during germination.

To fill this gap, a detailed cytological analysis of flavonoid localization was performed using both confocal and epifluorescence microscopy. To do so, we embedded whole mature rice seeds and isolated embryos in resin and obtained semi-thin sections. Then, the diphenylboric acid 2-amino ethyl ester (DPBA) that exhibits secondary fluorescence upon binding with flavonoids (Hutzler et al. 1998) was used to detect flavonoids in both whole mature seeds and germinated embryos (Fig. 3).

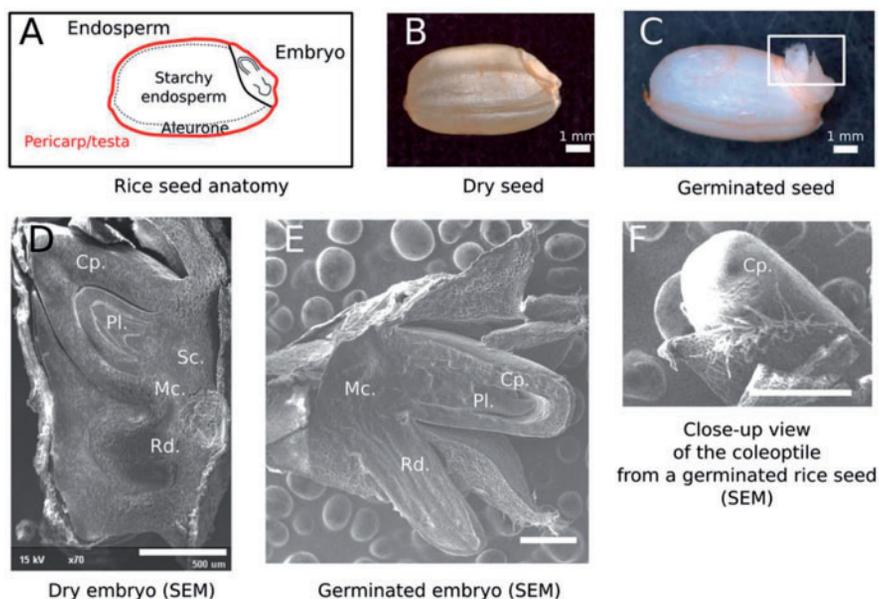


Fig. 2 Rice seed anatomy during germination *sensu stricto*. (A) Scheme of rice seed structure with the maternal tissues (pericarp and testa) and the zygotic tissues (embryo, starchy endosperm and aleurone layer). (B, C) Representative pictures of dry (B) or germinated rice seeds with an emerged coleoptile (C). (D–F) Scanning electronic microscopy (SEM) of dry (D) and germinated (E, F) embryos. (F) Close-up view of the coleoptile (inset in C) from a germinated embryo. Scale bars represent 500 μm .

In the rice mature seed, flavonoids were easily detected in the embryo axis and pericarp/testa (Fig. 3A–C). In the embryo axis, the coleoptile was strongly stained in comparison with the radicle and surrounding scutellum (Fig. 3A, B). Flavonoids could also be revealed in the seed pericarp/testa but not in the aleurone layer or in the starchy endosperm (Fig. 3C). A vanillin assay was also performed that confirmed the absence of proanthocyanidins (data not shown). To compare the distribution of flavonoids in whole dry seeds and germinated embryos, we took advantage of an epifluorescence microscope equipped with an ApoTome device (Fig. 3D–G). This analysis confirmed that flavonoids were mostly present in the coleoptile (Fig. 3D–G).

Identification and quantification of flavonoids present in mature dry rice seed

These cytological observations prompted us to characterize the chemical nature of the flavonoids detected in the different dry mature rice seed tissues and in germinating embryos. A relative quantification of the flavonoids on a seed equivalent basis was established in parallel using an internal standard. First, we manually dissected the embryos from mature seeds. Then, a laboratory bench polishing machine was used to obtain a pericarp/testa-enriched fraction from the embryoless endosperms (see the Materials and Methods and Supplementary Fig. S1 for details). Flavonoids were extracted with methanol and the extracts were subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis. This work revealed that flavones were the only flavonoids detected in Nipponbare grains (Table 1). A full-scan MS analysis using high and low in-source fragmentation alternatively was used to measure the mass of the detected compounds but also

to screen aglycones and *O*-conjugated flavonoids. *C*-Glycosylated flavones previously found in cereals were sought on the basis of the expected m/z value of the $[M+H]^+$ ions. A second analysis by targeted MS/MS product ion mode allowed specific fragments of $[M+H]^+$ ions to be obtained as complementary clues of flavonoid identity: whereas the glycosidic bond in *O*-glycosylated flavonoids is cleaved upon MS/MS, water losses and cross-ring cleavages of the sugar rings are prominent for fragmentation of *C*-glycosylated flavonoids, with characteristic ions for each sugar [further details regarding product ions are explained in Domon and Costello (1988) and Cavaliere et al. (2005)]. Similar cleavages have been found in MS high in-source fragmentation. A third analysis by pseudo MS3 (MS/MS on in-source produced aglycone) was used in order to confirm the similarity and identity of aglycones of *O*-conjugated flavones (Kerhoas et al. 2006).

In summary, 21 different flavones were annotated. These compounds derive from four flavone aglycone moieties, namely apigenin, luteolin and the methylated flavonoids chrysoeriol and tricetin (Table 1; Fig. 1). Tricetin and chrysoeriol aglycones were also detected, a feature rarely seen in non-hydrolyzed native extracts (Table 1). All other flavones are conjugated with sulfate (tricetin sulfate and tricetin-*O*-hexoside-*O*-sulfate; Supplementary Fig. S2), sugars (glucose, rhamnose or arabinose) and sugars acylated with hydroxycinnamic (ferulic and sinapic) acids (Table 1).

Relative quantification of the flavonoids present in the dry mature rice seed and in the germinated embryo

The 21 flavones were quantified relatively using the flavonol rhamnetin (7-methylquercetin) as an internal standard.

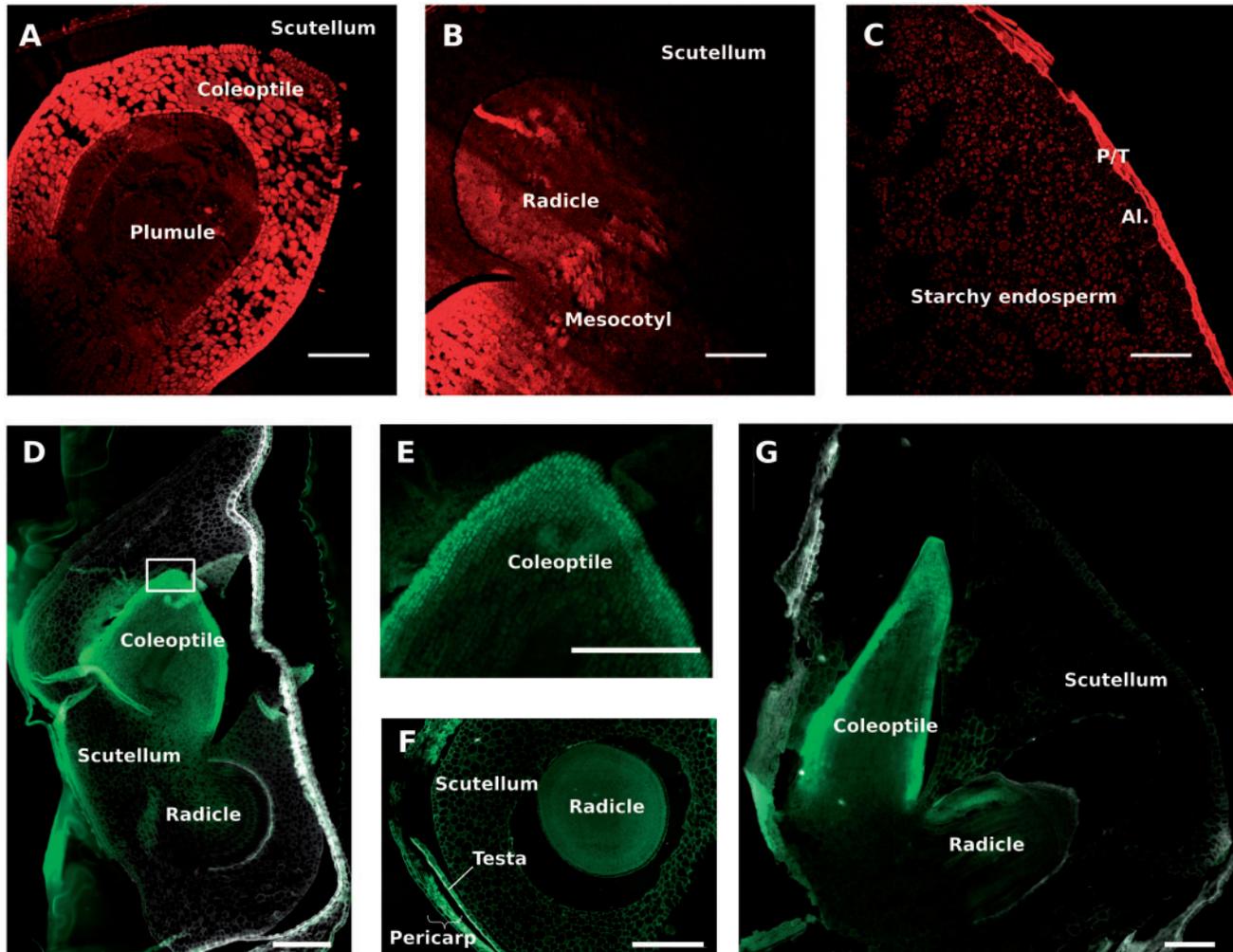


Fig. 3 Flavonoid distribution in the rice seed. (A–C) Flavonoid distribution in dry embryonic rice seed tissues was visualized with a confocal microscope using a 1% (w/v) diphenylboric acid stain solution on 5 μm semi-thin sections of resin-embedded seeds. (A, B). Flavonoids are mainly present in the embryo coleoptile (A) and to a smaller extent in the radicle (B), but not in the scutellum. (C) In the endosperm (pericarp/testa, aleurone layer and starchy endosperm), flavonoids are detected only in the pericarp/testa (C) but not in the starchy endosperm or in the aleurone layer. The scale bar represents 100 μm . (D–G) Flavonoid distribution in whole embryos was visualized with an Axioimager equipped with an ApoTome device using a 1% (w/v) diphenylboric acid stain solution on 5 μm semi-thin sections of resin-embedded dry and germinated embryos. Localization of flavonoids in dry (D–F) and germinated (G) whole embryos by epifluorescence. The scale bar represents 200 μm .

Schaftoside and its two isomers were the major flavones present in the embryo, accounting for 54% of the flavonoid signal distribution (Fig. 4; Supplementary Table S1). Tricin and its conjugated derivatives accounted for 24% of the flavonoid signal distribution, making them the second largest contributor to the embryo total flavone content (Fig. 4; Supplementary Table S1). In contrast, the pericarp/testa fraction accumulated exclusively schaftoside and two schaftoside isomers (Fig. 4; Supplementary Table S1). Therefore the embryo has both O- and C-glycosylated conjugates while the pericarp/testa fraction accumulates only C-glycosylated flavones (Fig. 4; Supplementary Table S1). In addition, the pericarp/testa C-glycosylflavones represent a very small proportion of the seed C-glycosylflavones (Fig. 4). We measured the dry weight of the embryo and starchy endosperm (without seed pericarp, testa and aleurone layer, Supplementary Fig. S1). While the embryo represents only 2% of the whole dry seed weight, the starchy

endosperm makes up 88% of the total dry seed weight. Therefore, the seed pericarp/testa fraction accounts for 10% of the total dry seed weight. These results indicate that the embryo flavone content is very high compared with that of the pericarp/testa. Thus, in colorless Nipponbare rice seeds, the embryo is by far the most important source of flavones. This chemical analysis completes our confocal observations on rice seed sections stained with the flavonoid-specific dye DPBA (Fig. 3).

No major change in the flavone distribution within the rice embryo tissues was recorded during germination *sensu stricto* (Fig. 3D–G). To address the question of whether quantitative flavonoid changes would occur, embryonic flavones were extracted from germinating embryos from a vigorous rice seed batch that germinated almost completely in 24 h ($T_{50} = 16$ h, $G_{\text{max}} = 98\%$). Similarly to dry seeds, the same 21 flavones were detected in germinating seeds (Fig. 5; Supplementary Table S2). Furthermore, on a rhamnetin and seed equivalent basis, only a

Table 1 List of the flavones identified in the rice embryo and pericarp/testa

ID ^a	RT ^b	m/z ^c	Product ions ^d	Composition ^e	Tissue ^f	References ^g
1	30	301	286, 258, 153	Chrysoeriol	EM	–
2	29.32	331		Tricin	EM	Jung et al. (2013), Goufo and Trindade (2014)
3	25.65	411	331	Tricin sulfate ^h	EM	–
4	18.5	449	287	Luteolin 4'-O-glucoside	EM	Goufo and Trindade (2014)
5	21.3	463	399, 388, 301	Chrysoeriol-O-hexoside	EM	Cavaliere et al. (2005), Gong et al. (2013)
6	21.28	493	331	Tricin-O-hexoside	EM	Cavaliere et al.(2005), Gong et al. (2013)
7	9.1	565	547, 529, 511, 499, 493, 481, 469, 457, 451, 445, 427, 415, 409, 397, 379	Schaftoside isomer 1 (apigenin-C-hexoside-C-pentoside)	EM and P	Cavaliere et al. (2005) Matsuda et al. (2012), Pereira-Caro et al. (2013a)
8	14.49	565	547, 529, 511, 499, 493, 481, 469, 457, 451, 445, 427, 415, 409, 397, 379	Schaftoside (apigenin-6C-glucoside-8C-arabinoside)	EM and P	Cavaliere et al. (2005)
9	15.88	565	547, 529, 511, 499, 493, 481, 469, 457, 451, 445, 427, 415, 409, 397, 379	Schaftoside isomer 2 (apigenin-C-hexoside-C-pentoside)	EM and P	Pereira-Caro (2013), Matsuda et al. (2012) (<i>Oryza sativa</i>); Cavaliere et al. (2009) (<i>Triticum durum</i>)
10	18.03	573	411, 331	Tricin-O-hexoside-O-sulfate ^h	EM	–
11	19.72	579	433, 271	Rhoifolin (apigenin-7-O-neohesperidoside)	EM	Matsuda et al. (2012)
12	11.88	595	577, 559, 541, 529, 523, 511, 499, 487, 481, 475, 457, 445, 439, 427, 409	Chrysoeriol-C-hexoside-C-pentoside isomer 1	EM	Cavaliere et al. (2005), Matsuda et al. (2012)
13	16.1	595	577, 559, 541, 529, 523, 511, 499, 487, 481, 475, 457, 445, 439, 427, 409	Chrysoeriol-C-hexoside-C-pentoside isomer 2	EM	Cavaliere et al. (2005), Matsuda et al. (2012)
14	17.77	595	449, 287	Luteolin-O-deoxyhexoside-O-hexoside	EM	–
15	20.16	609	463, 301	Chrysoeriol-7-O-rutinoside	EM	Cavaliere et al. (2005), Gong et al. (2013)
16	20.4	639	493, 331	Tricin-O-deoxyhexoside-O-hexoside	EM	Cavaliere et al. (2005), Matsuda et al. (2012), Gong et al. (2013)
17	22.43	771	433, 321, 271, 207 ^j	Apigenin-O-hexoside-O-sinapoylpentoside	EM	–
18	23.38	771	463, 301, 177 ⁱ	Chrysoeriol-O-feruloylpentoside-O-hexoside	EM	–
19	22.65	801	463, 301, 207 ^j	Chrysoeriol-O-sinapoylpentoside-O-hexoside	EM	–
20	23.16	801	493, 331, 177 ⁱ	Tricin-O-feruloylpentoside-O-hexoside	EM	–
21	22.42	831	493, 331, 207 ^j	Tricin-O-sinapoylpentoside-O-hexoside	EM	–

Flavone methanolic extracts were prepared from 400 rice embryos ($n = 3$) or from the pericarp of 500 embryoless seeds ($n = 3$). These extracts were then subjected to HPLC-ESI-MS/MS. C- and O-conjugated flavones were identified relative to known standards (shown in bold) or by MS/MS fragmentation of selected ions.

^a ID: number assigned to the compound.

^b RT: retention time in minutes.

^c m/z: [M-H]⁺ molecular ion weight as given by ESI-MS in positive mode. For each compound, the negative ESI-MS mode confirmed the expected compound mass.

^d Product ions: fragment ions obtained by MS/MS with a collision energy of 20–60 eV.

^e Composition: proposed structure of the compound based on RT and m/z matching with a chemical standard (names in bold) or deduced from MS and MS/MS spectra.

^f Tissue: compound detected in embryo (EM, dry and germinated) and/or pericarp/testa fraction (P).

^g References: references of previous studies where the compound was identified (rice, wheat).

^h Presence of a sulfate group could be confirmed by isotopic profiles for triclin-sulfate but not for triclin-O-hexoside-sulfate due to low abundance.

ⁱ Sinapoyl⁺ ion identified at m/z 207.

^j Feruloyl⁺ ion identified at m/z 177.

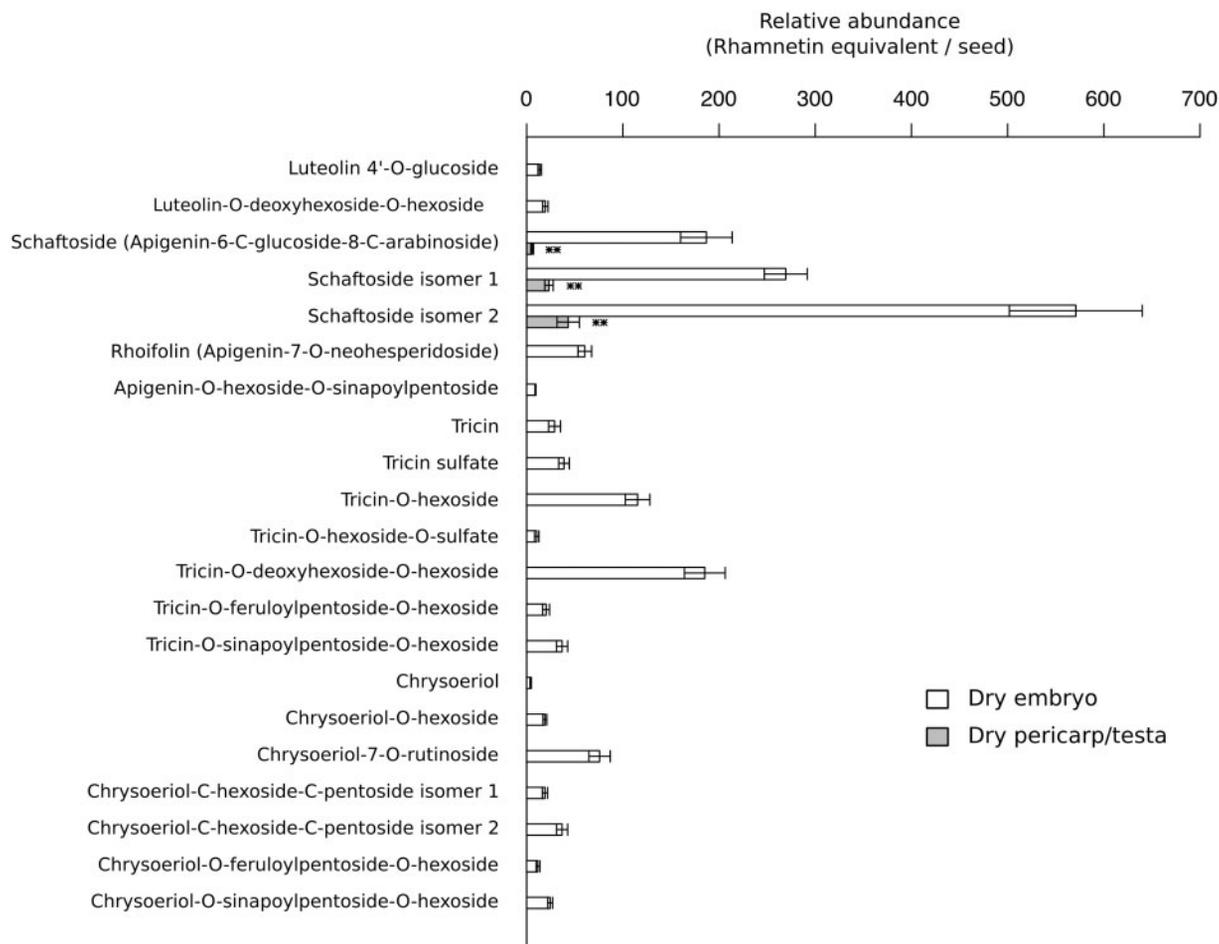


Fig. 4 Relative quantification of flavone abundance on a per seed basis in mature rice seed tissues. Relative quantification (seed and rhamnetin equivalent) for each flavone was obtained from methanolic extracts from isolated embryos and pericarp/testa-enriched fractions. Each bar represents the mean \pm SD ($n = 3$) relative abundance for each flavone. Asterisks indicate significant differences at $P < 0.05$ (**) according to Student's *t*-test.

few quantitative differences were observed between dry and germinating embryos as evidenced by a principal component analysis (Supplementary Fig. S3). However, three flavones decreased in abundance (chrysoeriol, tricin and luteolin-4'-O-glucoside, $P < 0.05$; Fig. 5) while seven flavones, in particular chrysoeriol-7-O-rutinoside, showed a significant but slight increase during germination *sensu stricto* (Fig. 5). These results might therefore indicate that the flavone content is very stable during germination *sensu stricto*.

Expression of flavone-related genes increases during rice embryo germination

From a literature survey, we collected 75 genes potentially or experimentally involved in flavonoid metabolism (Supplementary Table S3). These genes were either predicted from the rice genome sequence, experimentally confirmed or annotated from a metabolic quantitative trait loci analysis (Supplementary Table S3; Gong *et al.* 2013). In parallel, we generated Affymetrix microarray expression data on rice embryos during a germination time series ranging from 0 to 24 hours after imbibition (HAI; 0, 4, 6, 12, 18 and 24 HAI). Finally,

the expression of these 75 selected flavonoid genes was studied during rice seed germination (Fig. 6). After microarray normalization (Supplementary Fig. S4A), two groups of genes could be distinguished easily depending on their expression (Supplementary Fig. S4B). While a reliable expression could be observed for 25 flavonoid biosynthesis genes (cluster 1; Supplementary Fig. S4B), a large proportion, i.e. 50 genes, had a very weak expression close to background noise and were omitted from our study (cluster 2; Supplementary Fig. S4B). Further clustering helped to distinguish three subclusters in cluster 1 (named 1a, 1b and 1c; Fig. 6). While clusters 1a and 1c are characterized by a stable expression (with gene expression levels stronger in cluster 1c than in cluster 1a), cluster 1b comprises several key flavone biosynthesis enzymes (Figs. 1, 6) involved in flavone precursor synthesis (OsPAL2, LOC_Os02g41680; Os4CL2, LOC_Os02g46970; OsCHS1, LOC_Os11g32650), synthesis of C-glycosylflavones (CYP93G2, LOC_Os06g01250) and O-methylated flavones (FNS, LOC_Os03g03034; ROMT-9, LOC_Os08g06100). Altogether, based on these expression data, it seems that genes involved in C- and O-glycosylated flavones could be induced after several hours of imbibition.

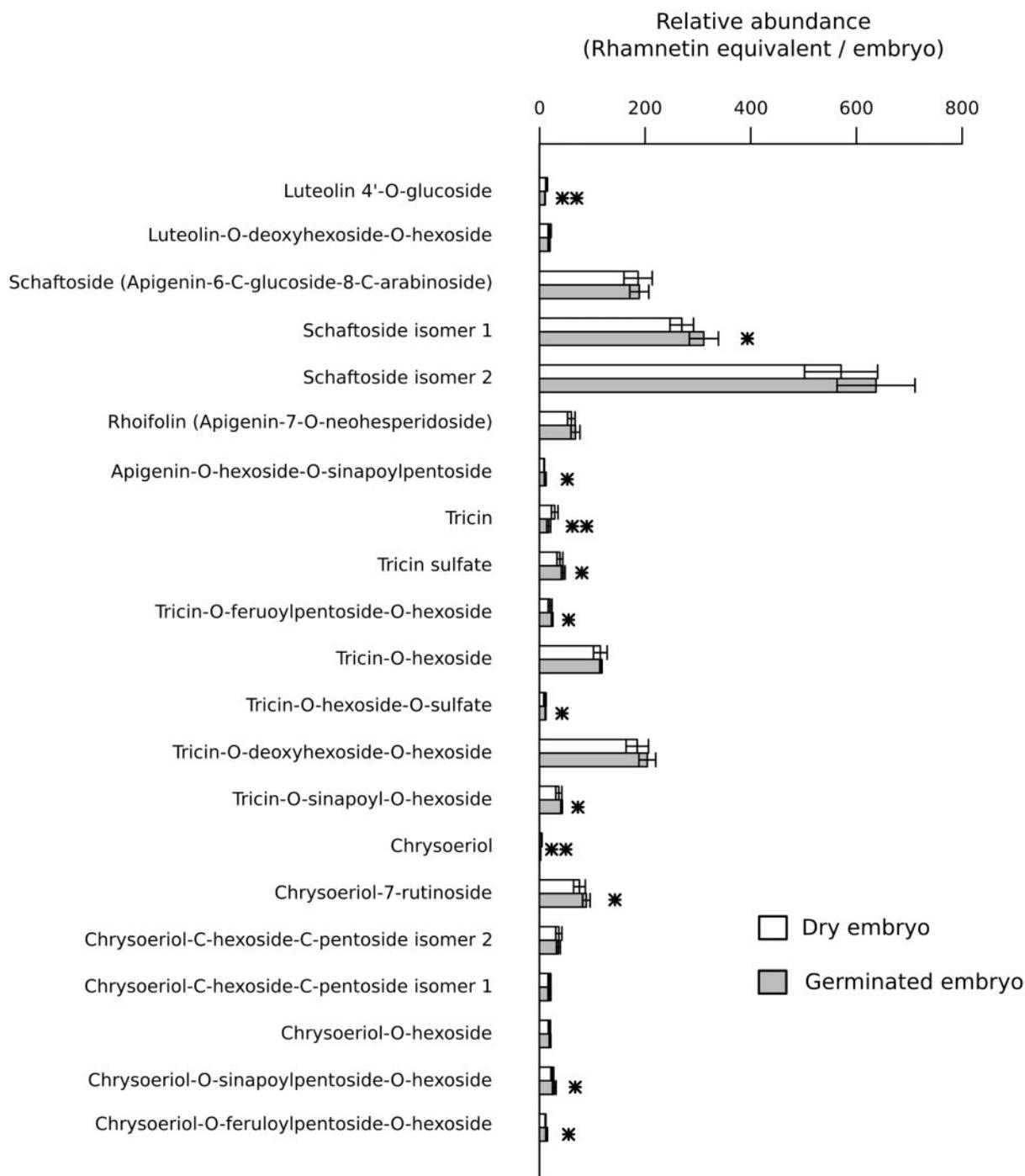


Fig. 5 Relative quantification of flavone abundance in the embryos of dry and germinated rice seeds. A total of 400 embryos were hand-dissected from dry and germinated seeds (24 HAI). Then, methanolic extracts were prepared and flavones were extracted in the presence of a rhamnetin internal standard to obtain relative measurements for each flavone on a per seed basis. Asterisks indicate significant differences at $P < 0.1$ (*) or $P < 0.05$ (**) according to Student's *t*-test.

Discussion

Nipponbare non-pigmented rice seeds exclusively accumulate flavones

The rice genotype Nipponbare produces white rice seeds (Furukawa et al. 2007, Finocchiaro et al. 2010). The non-pigmented seed phenotype in rice is related to the absence of

expression of F3H and DFR gene expression together with the 14 bp deletion of the *Rc* locus that leads to a non-functional bHLH domain (Sweeney et al. 2006, Shih et al. 2008). Indeed, in our microarray data, the probe signals of *DFR* and *F3H* are very weak (**Supplementary Table S4**). In addition, in developing seeds of the Nipponbare genotype, the alternative translation of the *DFR* mRNA produces only two DFR proteins (33 and

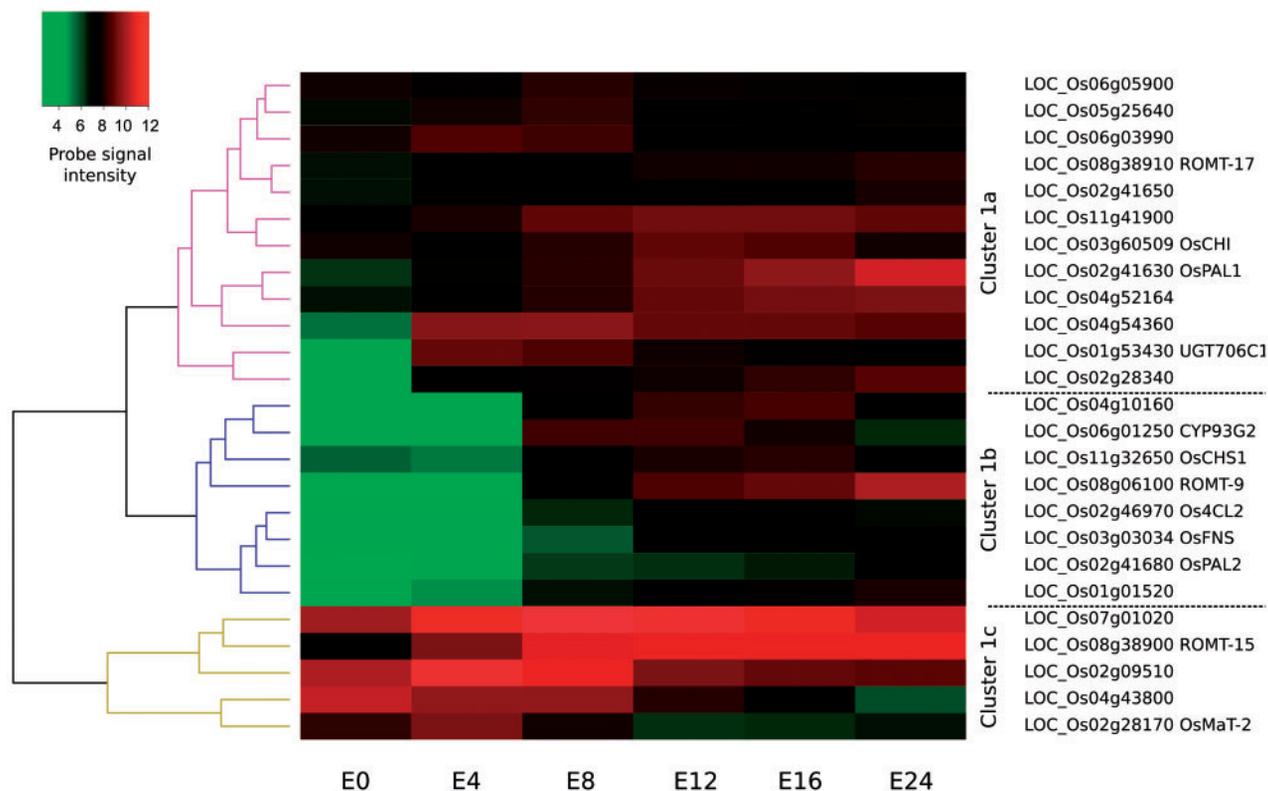


Fig. 6 Gene expression profiles of flavonoid biosynthesis genes during rice seed germination. The heat map of the 25 putative and confirmed flavonoid biosynthesis genes with a reliable expression during rice embryo germination is presented. When several probes corresponded to the same gene, their signal intensity was averaged (only locus-specific probes were kept in the analysis). Gene expression profiles were clustered (Euclidean distance, complete linkage) based on their corresponding GC-RMA-normalized probe intensity (log₂ scale). Gene names are indicated when available.

22 kDa) instead of the three DFR proteins of 46, 33 and 22 kDa normally seen in colored rice cultivars (Furukawa *et al.* 2007). Since the DFR enzymatic activity is responsible for the conversion of dihydroflavonols to leucoanthocyanidins (Lepiniec *et al.* 2006), there are neither anthocyanins nor proanthocyanidins. Altogether, the absence of flavonols, proanthocyanidins and anthocyanins in non-pigmented Nipponbare seeds is consistent with the findings of previous studies (Shih *et al.* 2008, Pereira-Caro *et al.* 2013a). As a consequence, the whole flavonoid pathway is channeled towards flavone biosynthesis (Table 1). This situation is similar to that previously observed in other rice plant organs (Besson *et al.* 1985, Du *et al.* 2010, Jung *et al.* 2013).

Flavonoids are strictly compartmented qualitatively and quantitatively within the rice dry mature seed

The distribution of flavonoids within the rice seed has been previously studied in black-pigmented rice seeds (Yoshimura *et al.* 2012). Using matrix-assisted laser desorption ionization coupled to imaging mass spectrometry (MALDI-IMS), these authors detected anthocyanins exclusively in the pericarp of black rice seeds (Yoshimura *et al.* 2012). Yet, to date, there was no similar study on colorless rice seeds. Therefore, the present work is the first describing flavonoid distribution within the different tissues of a colorless rice seed and during

germination *sensu stricto*, a key developmental transition from a quiescent to a very active metabolism (Figs. 2, 3). Strikingly, the pericarp/testa fraction accumulates only C-glycosylated flavones, suggesting that the synthesis of O-conjugated flavones is restricted to the embryo (Table 1). It indicates that flavone synthase enzymes (FNS, e.g. OsFNS, LOC_Os03g03034) are exclusively present in the embryo, while Cyt P450 enzymes such as the CytP450 enzyme CYP93G2 (LOC_Os06g01250) would be active in both embryo and seed pericarp/testa.

Based on a rhamnetin internal standard, we could also compare the relative abundance of each flavone in the embryo and in the pericarp/testa on a per seed basis (Fig. 4; Supplementary Table S1). These results clearly highlighted that schaftoside (apigenin-6C-glucoside-8C-arabinoside) and its two isomers are the major flavones in rice seed. These two isomers could be neoschaftoside and isoschaftoside, two schaftoside isomers previously detected in rice leaves (Besson *et al.* 1985).

A variety of flavone conjugation: impact on flavonoid localization and putative roles

Recently, several studies have identified flavones in rice leaves (Kim *et al.* 2008), dry seeds (Matsuda *et al.* 2012) or in 72 h germinating seedlings (Gong *et al.* 2013). The glycosylation, acylation or sulfation of flavone aglycones is likely to modify their solubility and/or biological activity. Consequently, within

rice seed, the different conjugation patterns of flavones might be related to precise tissue and subcellular distributions.

At the tissue level, flavonoids have been found in almost every plant tissue and organ (Zhao and Dixon 2010). In the maize leaf, flavonoids are localized immediately underneath the epidermis and in the parenchyme, especially upon UV radiation (Tossi et al. 2012). Flavonoids were also widely found in roots since they might participate in gravitropism even though in planta demonstration of their inhibitory action on auxin transport is still lacking (Buer et al. 2013). In pigmented rice pericarp/testa, cyanidin-*O*-hexoside is predominant in the posterior dorsal region, and cyanidin-*O*-pentoside is broadly disseminated within the whole rice seed (Yoshimura et al. 2012). The tissue-specific accumulation of *O*-glycosylated flavones in the embryo could be explained by a tissue-specific gene expression of transcription factor-activating genes that encode flavonoid glucosyltransferases, sulfotransferases and acyltransferases. Transcription factors of the MYB and bHLH types involved in flavonoid biosynthesis at the level of the rice seed have been identified (X.Y. Gu et al. 2011, Kim et al. 2011, Xue et al. 2012). Their targets at the level of the flavone pathway remain to be determined.

At the subcellular level, glycosylated flavonoids are mostly accumulating in the vacuole but can also be found in the nucleus, the external side of the endoplasmic reticulum, the chloroplast and the cell wall (Zhao and Dixon 2010). In *lisianthus* (*Eustoma grandiflorum*) petals, acylated hydroxycinnamoylated flavonoids are exclusively found in the vacuole while in *Coniferae* they have been retrieved in the cell wall (Strack et al. 1988, Markham et al. 2000). The primary cell wall of cereals is rich in ferulic acid (1–5% of dry weight) (Vogel 2008). In a preliminary experiment, we could easily detect sinapic acid by LC-ESI-MS/MS in the whole rice seed soluble fraction (data not shown). Whether our five acylated hydroxycinnamoylated flavones are present at the cell wall level and/or in the vacuole requires further investigations, possibly by MALDI-IMS (Yoshimura et al. 2012).

Finally, the present results describe sulfated flavones in rice and particularly in seeds for the first time (**Table 1; Supplementary Fig. S2**). Although sulfated flavones have been found in the *Poaceae* family, this is the first time that they have been found in a cultivated cereal species especially in the seed organ. The rice genome contains 32 predicted sulfotransferases that could theoretically transfer a sulfate preferentially to position 3, 7 or 4' of flavonoids. (Ouyang et al. 2007). According to the Predicted Rice Interactome Network (H. Gu et al. 2011), we did not find any interaction between these 32 sulfotransferases and any flavonoid-related genes. However, one or several of these sulfotransferases might be involved in resistance to pathogens and even regulation of seed germination. Indeed, in *Arabidopsis*, the SULFOTRANSFERASE 12 (AtSOT12, At2g03760) can sulfonate salicylic acid which in turn influences the plant resistance to *Pseudomonas syringae* (Lacomme and Roby 1996). Moreover, the *Arabidopsis atsot12* knock-out mutant is more sensitive to ABA or NaCl during germination, thereby establishing a link between seed physiology and flavonoid conjugation.

Putative roles of flavonoids in the biology and germination of rice seed

The mature rice 'seed' is a caryopsis in which the true botanical seed (diploid embryo, triploid endosperm and diploid testa) is covered by the pericarp (Krishnan and Dayanandan 2003). After fertilization and early during rice seed development (7 d after pollination), the maternal pericarp, integuments and nucellus degenerate to become a thin protective layer around the zygotic embryo and endosperm (Krishnan and Dayanandan 2003).

Our study showed that rice Nipponbare unpigmented seed exhibits flavonoids of the flavone type in the pericarp/testa and embryo compartments and that 1 d imbibition does not significantly change this flavonoid profile. Nevertheless, the transcription of several flavonoid biosynthetic genes is boosted during this period. The major change in flavonoid composition is therefore expected to occur later in the germination process, particularly during plantlet development. In leguminous seeds such as soybean, the peak of isoflavone biosynthesis is recorded after 3 d of germination, the total content being 4.68 mg g⁻¹ dry matter (Huang et al. 2014). Similar studies in rice addressed phenolic acid composition rather than flavonoids. For instance, it has been shown that after 21 HAI, the ferulic acid content of brown rice increased significantly from 0.32 to 0.48 mg g⁻¹ flour (Tian et al. 2004).

In *Arabidopsis*, *transparent testa* mutants impaired in flavonoid metabolism exhibit a reduced seed longevity (Debeaujon et al. 2000) and a decreased tolerance of plants to oxidative stress due in particular to UV-B radiation (Li et al. 1993) and drought (Nakabayashi et al. 2014). We expect that flavones accumulated in rice seeds during their development and that remain abundant during germination *sensu stricto* may exert a similar protective action. We propose that flavones present in the rice embryo coleoptile would probably be involved mostly in seed UV resistance. Several lines of evidence support this hypothesis. First, the accumulation of C-glycosylflavones is strongly linked to UV stress resistance as exemplified in maize (Casati and Walbot 2005, Tossi et al. 2012, Park et al. 2013). Secondly, leaf epidermal flavone glycosides rather than flavones present in the mesophyll cells underneath are strongly linked to the UV-A and UV-B absorbance capacity (Burchard et al. 2000). Finally, the embryo flavone content is relatively stable during germination *sensu stricto* which could indicate post-germinative roles (**Fig. 4**). Since the coleoptile is the first tissue to emerge and due to the absence of light-absorbing Chl in this colorless tissue, it is tempting to speculate that coleoptile flavones would prevent UV damage to biomolecules such as DNA.

We observed an exclusive accumulation of C-glycosylflavones in the rice seed pericarp/testa (**Table 1**). Several studies have pinpointed a role for C-glycosylflavones in plant-microbe interactions. Rice root and shoot secondary metabolites, which include flavones, are strongly altered by the colonization of beneficial soil bacteria of the genus *Azospirillum* (Chamam et al. 2013). Thus, a possibility exists that flavones in the

pericarp/testa would influence beneficial seed-borne bacteria. In cereal seeds, the pericarp/testa barrier is also the first line of defense against microorganisms (Bily et al. 2003, Tasleem-Tahir et al. 2011). C-Glycosylflavones such as maysin are critical for defense against plant-eating insects (Elliger et al. 1980, McMullen et al. 1998). In cucumber (*Cucumis sativus* L.) leaves, C-glycosylflavones are synthesized at the site of fungal penetration (McNally et al. 2003). Taken together, the specific accumulation of schaftoside and two schaftoside isomers in the rice pericarp during seed development could be related to a role in defense against pathogens and pests.

During late seed development, seeds can enter a dormancy state, defined as the absence of germination, even when exposed to favorable environmental conditions (Graeber et al. 2012). As previously stated, the red pigmentation trait related to the presence of proanthocyanidins in the pericarp is closely linked to the seed dormancy trait. Indeed, the bHLH transcription factor encoded by the *Rc* locus regulates both flavonoid accumulation in the pericarp and the ABA hormone level (X.Y. Gu et al. 2011). In *Arabidopsis*, flavonoids present in the seed testa are major determinants of seed dormancy (Debeaujon et al. 2000). Yet, most cultivated *japonica* rice varieties including Nipponbare have poor seed dormancy (Sugimoto et al. 2010). Therefore, flavones present in our Nipponbare seeds are unlikely to participate to seed dormancy (Elliger et al. 1980).

In summary, our work has provided new insights into flavonoid metabolism of a non-pigmented rice variety by characterizing their flavone composition together with their tissue localization. Apart from contributing to the understanding of potential physiological roles of flavonoids in rice seeds, this study paves the way for future work aiming at improving the health-beneficial properties of this important crop. Indeed, rice seed flavonoids could be used as a functional food, in particular by setting appropriate germination conditions and by breeding genotypes with elevated flavonoid contents.

Materials and Methods

Rice biological material

Dry mature rice seeds (*Oryza sativa* ssp *japonica* cv Nipponbare) were harvested in September 2010 at the 'Centre Français du Riz' (Mas du Sonnailler, Arles, France). At the lab, seeds were dehulled (JLGJ-45; Zhengzhou Chinatown Grain Machinery Co. Ltd.) and dissected into one embryo and one endosperm/testa/pericarp fraction with a sharp scalpel. The pericarp/testa fraction was obtained by using a laboratory bench rice milling machine (JMNJ-3; Zhengzhou Chinatown Grain Machinery Co. Ltd.) which removed the pericarp/testa plus aleurone layer of the rice seeds (Supplementary Fig. S1). Dry weight was determined on 10 bulks comprising 10 rice seeds. The corresponding embryo, endosperm and starchy endosperm were placed in a dry oven at 115°C for 48 h and weighed on a precision laboratory balance (XP204; Mettler-Toledo).

Rice seed germination was performed during 24 h in 450 ml of sterile distilled water in the dark at 30°C and under constant air bubbling (6 l min⁻¹, air compressor N86 KN.18; KNF Neuberger). In these conditions, 50% and 91% of the seeds had germinated at 16 and 24 HAI, respectively, as evidenced by coleoptile emergence, while the final germination percentage (G_{max}) was equal to 98%.

Photonic microscopy

All products except for a few indicated exceptions were obtained from Sigma-Aldrich (France) and inclusion steps were performed at 4°C in the dark. For fixation and histoiresin embedding, mature or germinated rice embryos as well as dry mature embryoless endosperms were fixed under vacuum infiltration in 5 ml of fixation buffer that contained 2% paraformaldehyde (w/v), 1% glutaraldehyde (v/v), 1% caffeine (w/v) and 0.05% Triton X-100 (v/v) buffered with sodium phosphate at pH 7.2. Then, samples were left overnight on a rotary shaker (100 r.p.m.). For dehydration, samples were placed in successive baths of ethanol: 50% for 30 min, 70% for 1 h, 95% for 30 min and finally 100% ethanol for 1 h. Then, samples were placed in a 1:1 ethanol/butanol solution (v/v) for 1 h before being placed in pure butanol (butan-1-ol) for 24 h. To embed the samples in Technovit[®] 7100 histoiresin (Kulzer), samples were placed in increasing concentrations of histoiresin: 3:1 (v/v) butanol/histoiresin for 24 h, 2:2 (v/v) butanol/histoiresin for 24 h, 1:3 (v/v) butanol/histoiresin for 24 h and finally in pure histoiresin for 7 d. The histoiresin was then polymerized by placing the samples in histoiresin with a hardener agent (resin/hardener, 15:1, v/v) and leaving them at 37°C for 1 week. Finally, samples embedded in histoiresin were cut with a microtome at 5 µm.

Confocal laser scanning fluorescence and epifluorescence microscopy

In dry seeds, flavonoids were imaged by confocal scanning fluorescence microscopy (Leica TCS SP2; Leica Microsystems SAS) using DPBA (also called Naturstoffreagenz A) (Hutzler et al. 1998, Buer et al. 2007). Semi-thin 5 µm sections of histoiresin-embedded embryos and embryoless endosperms were stained in 100 µl of a DPBA solution that contained 0.25% (w/v) DPBA and 0.005% Triton X-100 (v/v). For DPBA imaging, we followed settings from Hutzler et al. (1998) and set the excitation wavelength at 488 nm since secondary flavonoid fluorescence occurred only at 488 nm when conjugated with DPBA. The emission signal was collected between 515 and 635 nm.

To obtain complete embryo images of flavonoid localization during germination, we localized flavonoids in whole dry and germinated embryos in semi-thin 5 µm sections of histoiresin-embedded embryos colored with a solution of DPBA (see above). Observations were performed using an epifluorescence microscope (Zeiss Axio Imager 2 equipped with an ApoTome system; Carl Zeiss SAS) under illumination with a short-arc metal halide lamp (Illuminator HXP 120V; Zeiss) with emission filtered between 507 and 547 nm (Sp.Green filter F36-720; AHF analysentechnik AG).

Flavonoid analysis by LC-MS/MS

The method for flavonoid extraction was adapted from the literature (Kim et al. 2008). Embryo flavonoids were extracted from three replicates of 400 embryos that were ground in liquid nitrogen using a mortar and pestle. Pericarp/testa flavonoids were extracted from three replicates of 500 embryoless endosperms milled with a laboratory bench milling machine (JMNJ-3; Zhengzhou Chinatown Grain Machinery Co. Ltd.) for 15 min. Then, 1 ml of 80% (v/v) methanol was added to the embryo or pericarp/testa samples and membranes were further dissociated by placing the samples in an ultrasonic bath for 20 min at 25 kHz at 4°C (TI-H-5; Elma Hans Schmidbauer GmbH & Co.) and then on a rotating wheel (MiniLabroller; Dominique DUTSCHER SAS) for 1 h. After centrifugation for 10 min at 20,000 × g at 4°C, the pellet was extracted further with 1 ml of 80% (v/v) methanol overnight at 4°C on a rotating wheel. The two resulting supernatants were pooled, filtered at 0.45 µm (0.45 µm Millipore PTFE membrane; EMD Millipore Corporation) and dried on a SpeedVac (Savant SPD131DDA; Thermo Fisher Scientific) for approximately 8 h. The dry pellet was dissolved in methanol/water/acetone/trifluoroacetic acid (40:32:28:0.05, v/v) and we added 20 ng of rhamnetin (Extrasynthese) as the internal standard. Finally, the extract was filtered again (Paper filter grade GF/A; Whatman International Ltd.).

Embryo and pericarp/testa extracts were then analyzed by HPLC-ESI-MS/MS. The compounds were introduced in the ESI source using a Waters 2695 separation module (Alliance; WatersA) equipped with a Waters 2487 dual UV detector. Separation was achieved on a reverse-phase column (Uptisphere C18 ODB, 150 × 2.1 mm; Interchim) using a flow rate of 0.20 ml min⁻¹ and a binary gradient: (A) acetic acid 0.1% (v/v) and (B) acetic acid 0.5% (v/v) in acetonitrile.

The solvent gradient was programmed as follows: 0–5 min 86% A, 5–31 min 45% A, 31–41 min 100% B, 41–48 min 86% A (Kerhoas et al. 2006). Analyses were performed on a Waters Quattro LC triple quadrupole mass spectrometer (Waters) operating in MS full scan for the semi-quantification and different scanning modes for the identification of the compounds (ESI positive and negative modes). Relevant instrumental parameters were set as follows: capillary 2.70 kV, extractor 3 V, source block and desolvation gas temperatures 120°C and 350°C, respectively. Nitrogen gas was used to assist nebulization and desolvation (78 and 272 l h⁻¹, respectively). For the MS/MS mode (for confirmation of the identification: fragmentation ‘Daughter mode’ and molecular ions ‘Parent mode’), argon gas was used as the collision gas at 2.83 μbar.

RNA isolation and microarray analyses

Three independent biological replicates (100 embryos per replicate) of dry (0 HAI) or germinating embryos (4, 8, 12, 16 and 24 HAI) were obtained. Total RNA was isolated according to the protocol described by Li and Trick (2005). Then, total RNA was purified following the RNA cleanup protocol from the QIAGEN RNeasy Plant kit with the DNase I digestion step. RNA quantity and quality were evaluated by spectrophotometry (NanoDrop ND-1000; ThermoScientific) using the A₂₆₀/A₂₈₀ ratio and by the Agilent RNA 6000 kit on a Bioanalyzer 2100 (Agilent Technologies France SAS). A 250 ng aliquot of total RNA was used to synthesize biotin-labeled cRNAs with the GeneChip® 3’ IVT Express Kit according to the manufacturer’s recommendation (Affymetrix). The quantity of the cRNA labeled with Quant-It RiboGreen® RNA Quantification Reagent (Promega France) was determined after cleanup by RNA Binding Beads (Affymetrix). Fragmentation of 15 μg of labeled cRNA was carried out for 35 min at 94°C. For all samples, hybridization on the Affymetrix GeneChip® Rice Genome Array was performed for 16 h at 45°C. After hybridization, the arrays were washed with two different buffers (stringent, 6 × SSPE, 0.01% Tween-20; and non-stringent, 100 mM MES, 0.1 M [Na+], 0.01% Tween-20) and stained with a complex solution including streptavidin R–phycoerythrin conjugate (Life Technologies SAS) and anti-streptavidin biotinylated antibody (Vectors Laboratories). The washing and staining steps were performed in a GeneChip® Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip® Rice Genome Arrays were finally scanned with the GeneChip® Scanner 3000 7G piloted by the Command Console Launcher Tool.

For the flavonoid gene expression profile analysis in the germinating embryo, probes not specific for a single rice locus were removed, which gave us a final number of 15,339 probes. The CEL files were normalized with the RMA algorithm available in the Bioconductor package from R (Gentleman et al. 2004, R Development Core Team 2012). All raw CEL files are available from the Gene Expression Omnibus under the accession No. GSE43780.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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