

Original Research

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Expression profiling of flavonoid biosynthesis genes in association with accumulation of flavonoid compounds in rice under water deficit stress

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Abstract

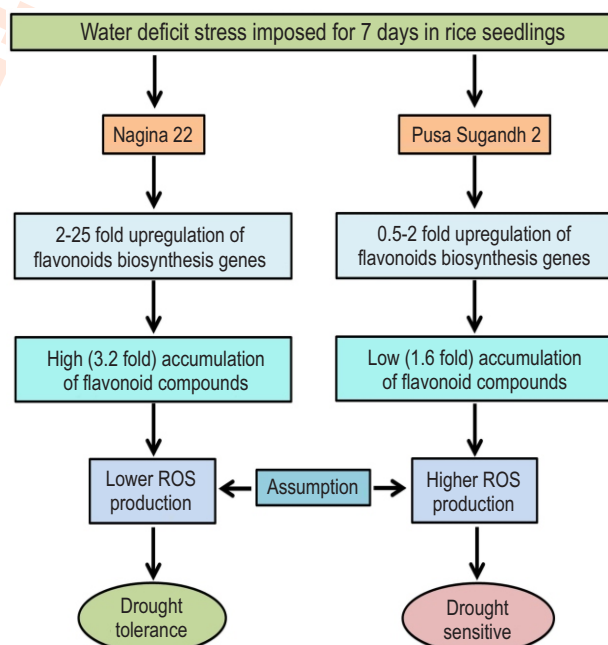
Aim: To investigate whether the phenolic and flavonoid contents have any direct relation with the water deficit stress tolerance in rice. If so, then, which genes in the flavonoid biosynthesis pathway are involved in the tolerance mechanism was explored by comparing a well-known water deficit tolerant cv. Nagina 22 (N22) as experiment material along with a sensitive cv. Pusa Sugandh 2 (PS2).

Methodology: Water deficit stress was imposed on N22 (drought tolerant) and PS2 (drought sensitive) cultivars at the seedling stage by withholding water for 7 days. Further, the expression level of flavonoid biosynthesis genes (*OsCHS*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsDFR*, and *OsANS*) and the accumulation of total contents of phenolics and flavonoids in rice leaves of N22 and PS2 under water deficit stress were also investigated.

Results: Quantitative RT-PCR analysis revealed enhanced expression of six major genes of the flavonoid biosynthesis pathway, namely *OsCHS*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsDFR*, and *OsANS* in the drought tolerant cv. N22 compared to the drought sensitive cv. PS2 under water deficit stress. Accumulation of total phenolics and flavonoids compounds increased during water deficit stress in both the genotypes, but, it was significantly higher in N22 as compared to PS2.

Interpretation: Our findings conclude that enhanced drought tolerance is positively correlated with an enhanced transcription of flavonoid biosynthesis genes and higher flavonoid content, suggesting that there could be differences in the genetic control of flavonoid metabolism in rice for drought stress response that, the enhanced expression and accumulation of flavonoids is promising strategy for rice improvement.

Key words: Antioxidant capability, Flavonoids, Nagina22, Rice, Water deficit tolerance



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Introduction

Crop growth, development, and productivity are adversely affected by several environmental stresses like drought, soil salinity, extreme temperatures, UV irradiation etc., and are serious issues threatening sustainable agriculture and the food security of the rapidly growing population worldwide (Bray *et al.*, 2000; Venuprasad *et al.*, 2008). Drought is a primary abiotic stress factor constraining rice production leading to severe economic and livelihood challenges. Exploiting the genetic variation available for drought stress tolerance in rice germplasm and understanding it at molecular level is a promising approach to enhance innate resilience of crop varieties. Accumulation of polyphenols in response to environmental stresses helps the plant to enhance stress tolerance as well as acclimatize them to survive under harsh environments (Lattanzio *et al.*, 2013; Pereira *et al.*, 2016). Accumulation of phenolics compounds stabilizes the negative impacts of abiotic stress in plants (Naikoo *et al.*, 2019). Hence, stimulation of plant secondary metabolism is a proven approach for better abiotic stress tolerance (Sharma *et al.*, 2012; Shahzad *et al.*, 2018 a; b). Phenolic compounds such as, phenolic acids and flavonoids are large families of plant secondary metabolites synthesized from the common shikimate-phenylpropanoid pathway (Shih *et al.*, 2008). These molecules plays an essential role in numerous biological functions, such as, plant growth and development, pollen development, male fertility, molecular signaling, cell cycle regulation, auxin transport regulation, besides resistance against biotic and abiotic stresses (Mol *et al.*, 1998; Winkel-Shirley, 2001; Winkel-Shirley, 2002).

Polyphenols have antioxidant properties, which protect plant cells from adverse effects of environmental stress by preventing the formation of reactive oxygen species (ROS) and scavenging of the formed ROS (Melidou *et al.*, 2005; Agati and Tattini, 2010; Li *et al.*, 2021). Many studies have focused on the protective role of flavonoids in response to drought stress. Under stress conditions, plants accumulate a high level of secondary metabolites like phenolic acids and flavonoids than under optimal condition (Selmar *et al.*, 2008). Sanchez-Rodriguez *et al.* (2011) found a high level of flavonoids accumulation (kaempferol and quercetin) in tomato under water stress and improved tolerance. Markham *et al.* (1998) reported that UV-B radiation or drought treatment in rice enhanced the C-glycosyl flavones content in tolerant cultivars. Ma *et al.* (2014) reported that upregulation of flavonoids biosynthesis genes led to increased accumulation of metabolite compounds (total contents of phenolics, flavonoids, and anthocyanins and shaftosides) in response to drought stress in wheat leaves.

The study of Ithal and Reddy *et al.* (2004) reported significant upregulation of rice flavonoid biosynthesis genes *OsDFR* and *OsANS* in N22 rice cultivar under drought, high salt, and ABA stresses. It has been reported that upregulation of structural genes of flavonoids biosynthesis pathway in potato plays a significant role in the enhancement of drought stress tolerance (Andre *et al.*, 2009). Liu *et al.* (2013) reported

upregulation of F3H (Flavanone 3-hydroxylase) transcript level in *Reaumuria soongorica* under UV-B irradiation and drought stress. More evidence for the role of flavonoids in imparting drought stress tolerance has come from functional genomic tools. For instance, overexpression of F3H gene from *Lycium chinense* in the transgenic tobacco plants showed a higher degree of flavonoid accumulation and enhanced tolerance to drought stress (Song *et al.*, 2016). Transcriptomic and metabolomic analyses revealed that higher level accumulation of flavonoids was closely associated with enhanced drought tolerance in *Arabidopsis* under drought stress (Nakabayashi *et al.*, 2014). Further, many studies have shown that the use of exogenous phenolic compounds enhanced drought tolerance in plants.

Fayez *et al.* (2014) reported that 50µM salicylic acid treated barely plants showed improved drought and salinity tolerance in response to salt and water deficit conditions. Different concentrations of elicitors like methyl jasmonate, salicylic acid and paclobutrazol treated seeds of N22 have shown increased total phenolics content and antioxidant activity than elicitors treated seeds of PS5 at the seedling stage under drought stress (Samota *et al.*, 2017). Thus, the relation between drought tolerance and flavonoid accumulation is well-known in literature (Selmar *et al.*, 2008; Sharma *et al.*, 2019). However, only few studies have examined the synthesis of flavonoids, and most of these studies have explored the expression pattern of a single gene or a few key genes in the flavonoid biosynthesis pathway for the assessment of their effect on abiotic stresses (Liu *et al.*, 2014; Song *et al.*, 2016; Thiyagarajan *et al.*, 2016; Wang *et al.*, 2016). In present study a well-known water deficit stress tolerant rice cv. N22 and a sensitive cv. PS2 were selected as test plants, and not only the flavonoid and phenolic content were estimated but also all the genes involved in the flavonoids biosynthesis pathway (CHS, CHI, F3H, F3'H, DFR, and ANS) were studied for their expression, to know whether the flavonoids and phenolics content have any inherent molecular basis in the contrasting rice genotypes. This is the first study in rice to analyze the expression pattern of flavonoids biosynthesis pathway genes between two genotypes contrasting in their response to water deficit stress.

Materials and Methods

Plant materials and water deficit stress treatment: Two *indica* rice genotypes showing extremely contrasting response to drought stress, N22 (drought tolerant) (Prakash *et al.*, 2016) and PS2 (drought sensitive) (Samota *et al.*, 2017; Latha *et al.*, 2019), were selected in the present study. Seeds available with the Rice Genomics and Molecular markers unit of the host institute, ICAR-National Institute for Plant Biotechnology, New Delhi were used in the experiment. Seeds were surface sterilized by 70% ethanol for 1min, treated with 0.1% HgCl₂ for 15min, rinsed with sterile distilled water thrice, and placed for germination on a petri dish at 28°C for 2 days under dark condition. Four days after sowing, uniformly germinated seeds were transplanted into soilrite. Pots with seedlings were maintained at 28°C under a light/dark cycle of 16/8 hrs till four to five leaf stages were attained. A relative

humidity of 60-70% was maintained and seedlings were watered with appropriate amount of water daily. Three biological and three technical replicates were maintained for the entire study. The experiments were conducted at the phenotyping facility of ICAR-National Institute for Plant Biotechnology, Pusa Campus, New Delhi, India during the months of August-September (*Kharif* season) in the year 2019. Once the seedlings attained four to five leaf stages, drought stress was imposed by withholding water for 7 days. Concurrently, a set of well watered seedlings that served as control were maintained in three replications. Top three leaves of control and water deficit stress treated plants were harvested during morning time (between 10 a.m.-12 p.m.) and immediately frozen in liquid nitrogen and stored at -80°C for further analysis. Biochemical parameters were assessed at two stages: before imposing stress and one week after the imposition of water deficit stress. All the parameters were estimated in three biological and three technical replications.

Determination of total phenolic and flavonoid content: Total phenolic content was estimated by the Folin-Ciocalteu colorimetric method (Singleton *et al.*, 1999). Gallic acid was used as a phenol standard. Total flavonoid content was quantified by the aluminum chloride colorimetric method (Bao *et al.*, 2005), using Rutin as a standard. In both biochemical experiments, the absorbance of the solution was read at 495nm and 725nm wavelength using Epoch2 Microplate Spectrophotometer (BioTek Instruments Inc, USA).

RNA isolation and quantitative RT-PCR: Total RNA was extracted from leaf samples of N22, and PS2 rice genotypes of control and water deficit stressed rice seedlings using a pure link RNA mini kit (Ambion, USA) according to the manufacturer's instructions. RNA samples were reverse transcribed with Superscript III first-strand cDNA synthesis kit (Invitrogen, USA).

The cDNA solution was used as a template for quantitative RT-PCR (Agilent Technologies Pvt Ltd, USA), which was performed using SYBR green kit (Applied Biosystems, Warrington, UK), as per manufacturer's instructions. Sequence information on the genes of flavonoids biosynthesis pathway, namely *OsCHS1*, *OsCHS2*, *OsCHI*, *OsF3H*, *OsF3'H*, *OsDFR*, and *OsANS* in rice, were retrieved from MSU Rice Genome Annotation Project Database (Table 1). Gene-specific primers were designed from 3'-UTR regions of different rice flavonoids biosynthesis genes for quantitative RT-PCR. Relative expression values were calculated for fold change following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and *OsActin* was used as an internal control to normalize the reaction. The primers used in the present study are listed in Table 1.

Statistical analyses: In this study, all experiments and/or estimations were conducted with three biological and three technical replications. The data were analyzed statistically by analysis of variance (ANOVA) for each parameter separately using the MSTAT-C software under completely randomized design. Significant variations between N22 and PS2 genotypes under control and water deficit stressed conditions were determined using Duncan's Multiple Range Test at $p \leq 0.05$.

Results and Discussion

In the present study, seedlings of both rice genotypes remained healthy before water deficit treatment (Fig. 1A). After 7 days of water deficit stress, drought tolerant genotype N22 seedlings exhibited lesser morphological changes with respect to leaf rolling, wilting, and chlorosis as compared to PS2. In the present study, N22 leaves were green till the end of water deficit stress (Fig. 1B). Similar morphological observation was found in the study of Samota *et al.* (2017), which revealed that the drought sensitive PS5 exhibited severe leaf rolling and wilting than the

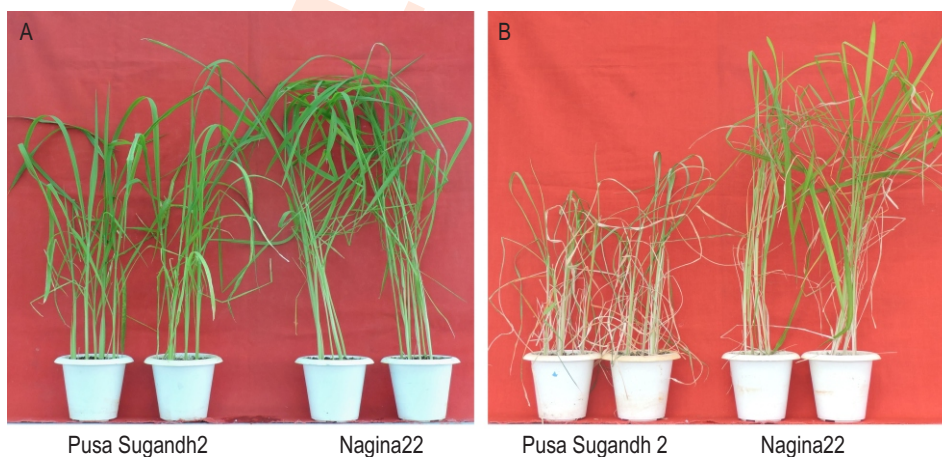


Fig. 1: Phenotypical performance of contrasting rice (N22 and PS2) cultivar seedlings in response to water deficit stress: (A) Growth performance of 4-5th leaf stage of contrast (N22 and PS2) rice seedlings, before water stress treatment and (B) Phenotype appearance of N22 and PS2 rice cultivar seedlings were subjected to water deficit stress by withholding water for a week.

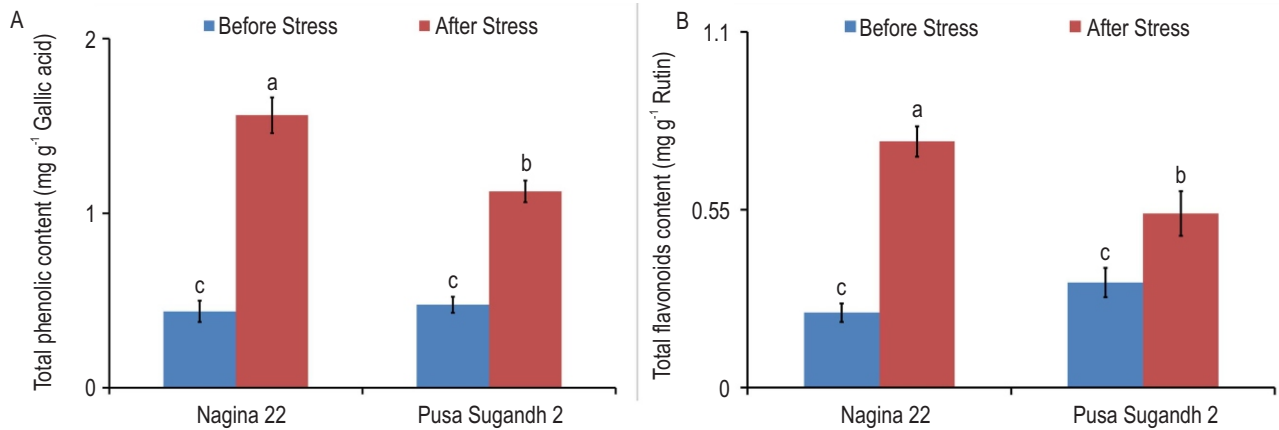


Fig. 2: Accumulation of total contents of phenolics and flavonoids in contrasting rice genotypes (N22 and PS2) under water deficit stress: (A) Total phenolics content and (B) total flavonoids content of contrasting rice seedlings (N22 and PS2) subjected to water deficit stress by withholding water for a week. Each value is mean of three biological and three technical replicates and the vertical bars give standard error (SE) of mean. Alphabets above bars indicate significant differences (p -value ≤ 0.05) between N22 and PS2 genotypes.

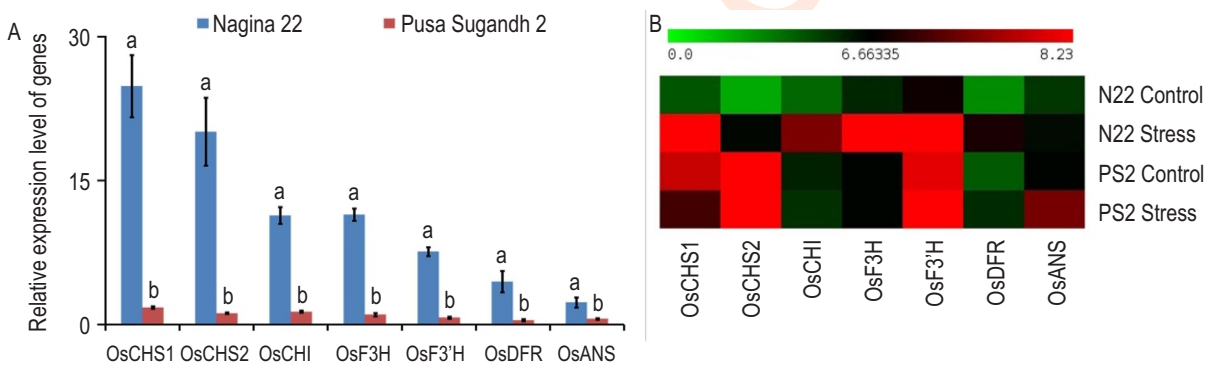


Fig. 3: Expression profiling of genes involved in flavonoid biosynthesis pathway: (A) Relative expression analysis of flavonoids biosynthesis genes (CHS1, CHS2, CHI, F3H, F3'H, DFR, and ANS) in contrasting rice genotypes of N22 and PS2 under water deficit stress. The results are represented as mean of fold change in relative expression of three biological and three technical replicates, normalized with respect to *OsActin* as housekeeping gene. Alphabets above bars indicate significant differences (p -value ≤ 0.05) between N22 and PS2 genotypes. (B) A heat map showing transcript levels of flavonoids biosynthesis genes (CHS1, CHS2, CHI, F3H, F3'H, DFR, and ANS) in N22 and PS2 rice genotypes under control and water deficit stress. Red color in this figure indicates a high level of expression while green denotes low level of expression.

drought tolerant N22 under drought stress. Phenolic compounds are considered as biochemical indicators of biotic and abiotic stress tolerance in plants (Akula *et al.*, 2016). Several studies have reported that flavonoids serve as an antioxidant in response to biotic and abiotic stress in various plant species, such as wheat (Ma *et al.*, 2014), *Arabidopsis* (Zhang *et al.*, 2018), maize (Li *et al.*, 2021), tobacco (Wang *et al.*, 2021).

In this study also the total phenolic and flavonoid accumulation increased significantly in both the genotypes under water deficit treatment, while the total content of phenolics and flavonoids in both the genotypes were at par under normal growth condition; however, the increase was

almost 1.5 times (for both TPC and TFC) more in N22 as compared to PS2 (Fig. 2A, B). Similarly, Samota *et al.* (2017) observed that the seed priming with elicitors induced a higher accumulation of total phenolic content in N22 when compared to PS5 under drought stress. Our results are also in line with the study of Quan *et al.* (2016) who reported that the accumulation of total phenols, flavonoid compounds, and accelerated antioxidant activities under water deficit condition in rice. It was observed that secondary metabolite content and antioxidant activity were closely associated with the degree of drought stress tolerance in rice. Accordingly, higher accumulation of secondary metabolites in N22 rice cv. is one of the reasons for its drought tolerance.

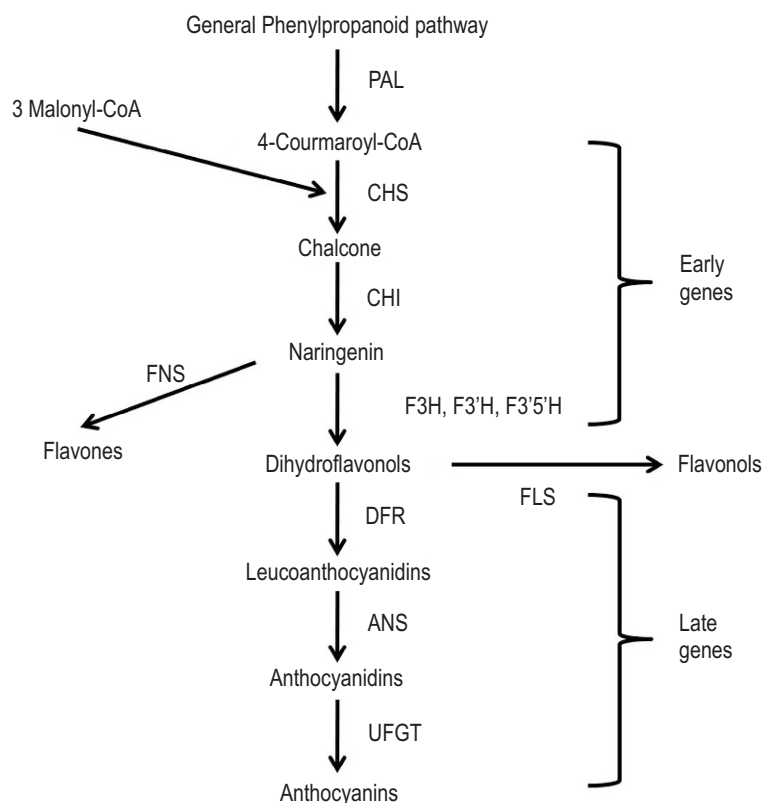


Fig. 4: Biosynthetic pathway of flavonoids: (Adopted from Shih *et al.*, 2008; Ma *et al.*, 2014). CHS -Chalcone synthase, CHI - Chalcone isomerase, F3H - Flavanone 3- β hydroxylase F3'H - Flavanone 3'- β hydroxylase FLS - Flavonol synthase, FNS -Flavone synthase, DFR - Dihydroflavonol 4-reductase, ANS -Anthocyanidin synthase and UFGT - UDP-glucose flavonoid glucosyltransferase.

In plants, ROS act as critical signaling molecules in responses to biotic and abiotic stresses (Huang *et al.*, 2019), which causes corrosion of membrane biomolecules (proteins, lipids, carbohydrates, and nucleic acids), eventually leading to cell death (Xie *et al.*, 2019). In maize seedlings, a higher level accumulation of flavonoids improved tolerance to drought stress by modulating ROS homeostasis (Li *et al.*, 2021). Previous studies have highlighted that higher level accumulation of flavonoid compounds enhanced abiotic stress tolerance in plants by regulating the homeostasis of ROS. A similar finding was made in rice for ROS modulation under drought stress, and the ability of N22 to withstand drought has been ascribed to the differences in the peroxidase, catalase, and superoxide dismutase encoding genes (Prakash *et al.*, 2016). Thus, the accumulation of flavonoid compounds which serve as antioxidants reduces the intracellular generation of ROS as well as increases the ROS scavenging activity under abiotic stress (Prakash *et al.*, 2016).

The expression level of *OsCHS1*, *OsCHS2*, and *OsCHI* genes were nearly 20 fold higher in N22 under water deficit stress when compared to PS2, and also the highest among all the genes

studied (Fig. 3A). Fig. 3B shows heat map representing the transcript levels of flavonoids biosynthesis genes (CHS, CHI, F3H, F3'H, FLS, DFR, and ANS) in N22 and PS2 rice genotypes in response to control and water deficit stress. Previous studies have reported increased expression levels of CHS and CHI genes under abiotic stresses conditions in many Plant Species conditions such as *Ginkgo biloba* (Cheng *et al.*, 2011), tobacco (Lijuan *et al.*, 2015; Chen *et al.*, 2017), Arabidopsis (Wang *et al.*, 2018) and rice (Jayaraman *et al.*, 2021). Vasquez-Robinet *et al.* (2008) found higher gene expression level of CHS in 'Sullu' potato variety (drought tolerant) than 'Negra Ojosa'(drought sensitive) during severe drought stress. Similar results were also reported in wheat which showed upregulation of *TaCHS* and *TaCHI* genes in two wheat cultivars under drought stress, with higher expression in Aikang 58 (AK) cultivar than Chinese Spring (CS) as compared to their respective control (Ma *et al.*, 2014). Thus, the flux in the flavonoid content observed in this study was due to the difference in the expression of early genes of the flavonoids pathway between the two genotypes.

The expression level of *OsF3H* and *OsF3'H* genes increased more than 10 fold in N22 as compared to PS2 under

Table 1: Primer sequences used for quantitative RT-PCR analysis of genes involved in flavonoid biosynthesis pathway.

Gene Name	Locus ID	Primer Name	Primer's Sequence (5'-3')
CHS	LOC_Os11g32650	OsCHS1_F	ATCTCGGACTGGAAGCTC
		OsCHS1_R	GACATGTTGCCGTACTC
	LOC_Os07g11440	OsCHS2_F	TCCATCTTCTGGGTGGT
		OsCHS2_R	TACTCGGAGAGGACGTT
CHI	LOC_Os06g10210	OsCHI_F	GAAGCTGGGACCTAAGTAT
		OsCHI_R	TAAGGCCGTTGTAGTGAA
F3H	LOC_Os04g56700	OsF3H_F	CGCCAAGCTCAAGAAGAA
		OsF3H_R	GAGACTTGGGCTTCTGTG
F3'H	LOC_Os10g17260	OsF3'H_F	CAGCCTTCTCGGTTTCT
		OsF3'H_R	TCATCAGTGTGACCATCC
DFR	LOC_Os01g44260	OsDFR_F	GACATGTTCCCGGAGTA
		OsDFR_R	TGTACCTGAACCTGAACC
ANS	LOC_Os01g27490	OsANS_F	TGGAGATCCTCACCAAC
		OsANS_R	CGTGCTGCTTGAATGTG
Actin	LOC_Os03g50885	OsActin_F	GATCTGGCATCACACCTTCTAC
		OsActin_R	CTGGGTCATCTTCTCAGATTG

water deficit stress (Fig. 3A). Several studies have reported that overexpression of F3H in transgenic tobacco plants upregulated the transcript level and induced flavonoids accumulation, which was often accompanied by increased antioxidant ability and abiotic stress tolerance (Mahajan and Yadav, 2014; Meng *et al.*, 2015; Song *et al.*, 2016). Likewise, Watkinson *et al.* (2006) and Liu *et al.* (2013) reported increased F3H expression, when potato and *Reaumuria soongorica* plants were exposed to UV-B irradiation and drought stress, respectively. Previous studies have reported that the expression level of downstream genes of the flavonoid biosynthesis pathway were also enhanced by abiotic stress in grape berries (Castellarin *et al.*, 2007) and *Populus euramericana* (Kim *et al.*, 2012). Hua *et al.* (2013) in this study reported that *Ginkgo biloba* DFR (*GbDFR*) transcripts are induced when plants were exposed to wounding stress. Correspondingly, in this study, the higher transcript levels of *OsDFR* and *OsANS* genes were observed in N22, as compared to PS2 (Fig. 3A). The results corroborates with the study of Ithal and Reddy *et al.* (2004), who showed upregulated expression levels of rice flavonoid biosynthesis genes *OsDFR* and *OsANS* in N22 rice cultivar after stress treatment. Ma *et al.* (2014) found that increased expression levels of *TaDFR* and *TaANS* genes in two wheat cultivars under drought stress, which suggested that increased gene expression, accumulation of flavonoids, and anthocyanin content might be closely related to drought tolerance. However, as shown in Fig. 3A, the expression level of upstream or early genes (CHS, CHI, F3H, F3'H) of flavonoids biosynthesis pathway was higher than downstream genes (DFR and ANS) in drought tolerant N22 genotype, and overall, the expression pattern of flavonoids biosynthesis pathway genes progressively decreased from *OsCHS* to *OsANS* (Fig. 4).

Thus, the results of this study clearly showed that the expression level of flavonoids biosynthesis was not only significantly higher in N22 under water deficit stress when compared to PS2, but also the fold change was perfectly

correlated with the progress of the biosynthetic pathway of flavonoids. In plants, the expression of early genes involved in flavonoids biosynthesis pathway encoding CHS, CHI, and F3H and the downstream or late genes encoding DFR, ANS, and UFGT are regulated separately (Quattrocchio *et al.*, 2006). It is known that phenolic and flavonoids are essential for stress tolerance, however, which genes in the pathway are more responsible for the water deficit stress tolerance in case of N22 is not known. Our study clearly suggests that it is worthwhile to work with early genes in the pathway. In fact, recent work from our laboratory found that stress-inducible expression of a single gene (*Chalcone Isomerase2*) could give multiple abiotic stress tolerance in the transgenic rice plants of susceptible genotype PS2 (Jayaraman *et al.*, 2021).

The present study clearly established that one of the mechanisms of the drought stress tolerance of N22 rice variety is due to the increased expression of flavonoids biosynthesis pathway genes coupled with the antioxidative capability through the accumulation of flavonoid compounds. These findings open up an avenue for improving drought tolerance in rice through modern breeding technologies.

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Add-on Information

Authors' contribution: K. Jayaraman: Performed experiments, data analysis, and drafted the manuscript; A.M. Sevanthi:

Helped in water deficit stress treatment, data analysis and manuscript editing; **V.K. Raman**: Helped in quantitative RT-PCR analysis and manuscript drafting; **P.K. Mandal**: Designed the experiments, revised the manuscript, and supervised the entire work.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Experimental Biology*.

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