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ORIGINAL PAPER

Regulation of glutamine synthetase isoforms in two differentially drought-tolerant rice (*Oryza sativa* L.) cultivars under water deficit conditions

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Abstract
Key message **The regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. OsGS1;1 and OsGS2 were differentially regulated in IR-64 (drought-sensitive) and Khitish (drought-tolerant) cultivars of rice.**
Abstract Water deficit (WD) has adverse effect on rice (*Oryza sativa* L.) and acclimation requires essential reactions of primary metabolism to continue. Rice plants utilize ammonium as major nitrogen source, which is assimilated into glutamine by the reaction of Glutamine synthetase (GS, EC 6.3.1.2). Rice plants possess one gene (OsGS2) for chloroplastic GS2 and three genes (OsGS1;1, OsGS1;2 and OsGS1;3) for cytosolic GS1. Here, we report the effect of WD on regulation of GS isoforms in drought-sensitive (cv. IR-64) and drought-tolerant (cv. Khitish) rice cultivars. Under WD, total GS activity in root and leaf decreased significantly in IR-64 seedlings in comparison to Khitish seedlings. The reduced GS activity in IR-64 leaf was mainly due to decrease in GS2 activity, which correlated with decrease in corresponding transcript and polypeptide contents. GS1 transcript and polypeptide accumulated in leaf during WD, however, GS1 activity was maintained at a constant level. Total GS activity in stem of both the varieties was insensitive to WD. Among GS1 genes, OsGS1;1 expression was differently regulated by WD in the two rice varieties. Its transcript accumulated more abundantly in IR-64 leaf than in Khitish leaf. Following WD, OsGS1;1 mRNA level in stem and root tissues declined in IR-64 and enhanced in Khitish. A steady OsGS1;2 expression patterns were noted in leaf, stem and root of both the cultivars. Results suggest that OsGS2 and OsGS1;1 expression may contribute to drought tolerance of Khitish cultivar under WD conditions.

Keywords Rice · Water deficit · Glutamine synthetase · Drought tolerance

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Introduction

Nitrogen (N) is an essential nutrient for plant growth and productivity. In higher plants, the main pathway for assimilation of inorganic-N to organic form is the Glutamine synthetase (GS, EC 6.3.1.2)/Glutamate synthase (GOGAT, E.C.1.4.7.1) cycle. GS catalyzes the ATP-dependent assimilation of ammonium (NH_4^+) into glutamate to yield glutamine, which is then used for the biosynthesis of essentially all nitrogenous compounds. GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). GS2 is encoded by 1 gene and GS1 is encoded by 3–5 genes depending on the species (Forde and Cullimore 1989; Miflin and Habash 2002). During the vegetative stage, GS2 is the predominant isoform in the leaf, where it assimilates ammonia originating from nitrate reduction and photorespiration (Bernard et al. 2008; Wallsgrove et al. 1987). Immunocytochemical studies have demonstrated that GS1 protein is localized in the vascular tissue suggesting its metabolic role in assimilation of NH_4^+ to glutamine for transport and distribution throughout the plant (Bernard and Habash 2009; Pereira et al. 1996). In root, GS1 facilitates the assimilation of NH_4^+ taken from the soil or from symbiotic N-fixation (Hirel et al. 1987). GS

isoforms are differentially regulated in response to plant N-status as well as environmental signals (Oliveira and Coruzzi 1999). Although, transcriptional control of GS is important in determining polypeptide abundance and cellular localization, the enzyme is also regulated by post-transcriptional and post-translational mechanism or by protein turnover (Lima et al. 2006).

Drought, heat and salinity are the most important abiotic stresses that have adverse effect on plant growth and productivity. All these major stresses result in water deficit (WD) stress. During prolonged period of WD, decrease in water availability for transport-associated process leads to limited uptake of N and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by the disturbances in carbon (C) and N metabolism (Foyer et al. 1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue.

Rice (*Oryza sativa* L.) is adversely affected by WD stress. The effect of water deprivation varies with variety, degree and duration of stress (Blum 1982). NH_4^+ is the main form of N available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-N transported through vascular tissues (Ishiyama et al. 2004a). Rice plants possess one gene (OsGS*2*) for GS2. The GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. These isoforms show organ and cell specific patterns of expression and are developmentally regulated. They are also differentially regulated at the level of gene expression by light, inorganic-N, soluble carbohydrate and amino acids (Kamachi et al. 1991; Kusano et al. 2011; Tabuchi et al. 2007). However, regulation of these isoforms during WD has not been investigated in detail. The role of GS isoforms in controlling N metabolism during WD can be understood by studying their regulation in differentially drought-tolerant rice varieties. The work presented here focuses on differential effect of WD on activity and expression of GS isoforms in leaf, stem and root of two differentially drought-tolerant rice cultivars.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* var. *indica*) seeds (cv. Khitish, Triguna, Satabdi, PNR-519, IR-8 and IR-64) were obtained from Rice Research Station, Chinsurah, West Bengal. Seeds were germinated in moist cotton bed at 30 °C for 2–3 days in dark. About 50 germinated seedlings were transplanted to each pot containing a mixture of soil: soilrite (3:1 v/v). Seedlings were grown under 250 μmol m^{-2} s^{-1} photon flux density (16 h/8 h day/night regime) at 27 ± 2 °C and 70–80 % relative humidity, in a Plant Growth Chamber (Conviron, Canada). After 3 weeks of sowing, WD was imposed by withholding water. Seedlings were harvested from individual pots at indicated days of stress treatment till day 12, frozen in liquid N_2, and stored at −80 °C for further analysis. Fresh plant tissue immediately after harvest was used for the determination of relative water content (RWC), electrolyte leakage, proline and protein contents. For determination of dry weight (DW), 1.0 g fresh leaf, stem and root tissues were dried in an oven at 80 °C for 48 h.

Determination of RWC

Leaf tissue was weighed to determine fresh weight (FW) and then rehydrated in water at 4 °C for 24 h and turgid weight (TW) was recorded. Finally, the sample was dried in an oven at 80 °C for 48 h and dry weight (DW) was recorded. The RWC was calculated by the following formula: RWC (%) = (FW − DW/TW − DW) × 100. The experiment was carried out in triplicates.

Proline estimation

Free proline was quantified according to Bates et al. (1973). The leaf tissue was homogenized in 3 % aqueous sulfosalicylic acid, and the homogenate was centrifuged at 10,000 rpm for 20 min. 2 ml of supernatant was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid and boiled at 100 °C for 1 h. After termination of the reaction on ice, the reaction mixture was extracted with 4 ml of toluene, and the absorbance of the aqueous phase was recorded at 520 nm. The assay was done in triplicates. Amount of proline was expressed as μmol proline g^{-1} dry wt.

Electrolyte leakage assay

Electrolyte leakage was assayed by estimating the ions leaching from the leaf into Milli-Q water. Leaf tissue was placed in 20 ml of Milli-Q water in two sets. The first set was kept at room temperature for 4 h, and its conductivity (C1) was recorded using a conductivity meter. The second set was autoclaved and its conductivity was also recorded (C2). Electrolyte leakage (1 − C1/C2) × 100 was calculated. The experiment was carried out in triplicates.

Protein estimation

Quantitative estimation of protein was carried out by following the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Amount of protein was expressed as mg protein g^{-1} dry wt.

GS extraction and assay

Frozen rice tissue was homogenized in GS extraction buffer (5 ml g^{-1} fresh wt) containing 0.05 M Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was desalted on pre-equilibrated Biogel P-2 column. GS activity was determined by semisynthetase reaction (Washitani and Sato 1977). A 1 ml reaction mixture contained 25 mM Tris–HCl (pH 7.5), 200 mM glutamate, 10 mM ATP, 5 mM hydroxylamine hydrochloride, 20 mM $MgCl_2$, and 100 µl enzyme preparation. The reaction mixture was incubated at 37 °C for 30 min and terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20 % (w/v) tri-chloroacetic acid). After 20 min, the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

Separation of GS isoforms

All the steps of GS isoforms separation were performed at 4 °C. 1 g of frozen rice tissue was homogenized in 5 ml GS extraction buffer. The homogenate was centrifuged at 10,000 rpm for 15 min. 1 ml of desalted supernatant was loaded onto a diethylaminoethyl (DEAE)-sephacel (Sigma, USA) column (5 × 1 cm) pre-equilibrated with the GS extraction buffer. The column was washed with the same buffer until no protein was detectable in the eluate. Elution of the adsorbed proteins was carried out by the buffer containing, first 0.15 M and then 0.30 M KCl. The flow rate was maintained at 20 ml h^{-1}. 2 ml fractions were collected and assayed for GS activity. The activities of chloroplastic and cytosolic isoforms were estimated from the area of the corresponding elution profile after fractionation. About 80–90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

RNA extraction and RT-PCR analysis

Total RNA was isolated from rice tissues using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction and quantified spectrophotometrically. To ensure comparability of the resulting band intensity, spectrophotometric quantitation of RNA was confirmed by applying equal amount of total RNA to an agarose gel. First-strand cDNA was synthesised using 1 µg of total RNA, oligo (dT) primer and AMV reverse transcriptase (Promega) in 25 µl of reaction. Semiquantitative RT-PCR was performed using 5 µl of cDNA as template in 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′-AGT ATGGCTTCTCTCACCGATCTCGTC-3′) and OsGS1;1-R (5′-GTACCTCGAG GGGCT TCCAGATGATGGTGGTC T-3′) for OsGS1;1, OsGS1;2-F (5′-GACTCATATGGC CAACCTCACC GACCTCGTT-3′) and OsGS1;2-R (5′-T AGCGGCCGCGTTCTGCTTCCACAGCAGCGTG-3′) for OsGS1;2, OsGS2-F (5′-AGAACTTGGACGATGAATC GG-3′) and OsGS2-R (5′- CATTTTATTTCGAGGGAA GG-3′) for OsGS2 and OsActin-F (5′-GTCAGAATGGG ATGATATGG-3′) and OsActin-R (5′-TCTCCTTGCTCA TCCTGTCAG-3′) for actin. PCR was performed for 27–29 cycles within a linear range of amplification of these genes. Expression of actin gene was used as a control to equalize cDNA quantity in different reactions. 7 µl of the PCR product were loaded and separated on 1 % agarose Tris–acetate EDTA gel. Gels were scanned using a gel documentation system (Spectronics, USA) and quantified by ImageAide version 3.06.04, to calculate changes in gene expression. Results were repeated three times and representative one time gel pictures are shown.

Protein gel blot analysis of GS protein

Proteins were separated by 12.5 % SDS-PAGE (Laemmli 1970) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the anti-GS antibody raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptide was visualized with a goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers.

Results

Screening of Rice varieties for WD tolerance

Preliminary experiments were carried out to evaluate the drought tolerance characteristics of rice cultivars. 3 weeks old rice seedlings of different varieties were subjected to WD and relative water content (RWC), proline and protein contents and electrolyte leakage of leaf tissues were monitored at various stages of WD. IR-64 and Khitish cultivars were finally selected for further studies due to their relative drought-sensitive and tolerant properties, respectively. The results of drought tolerance characteristics of these two cultivars are shown in Figs. 1 and 2. WD treatment resulted in rapid decrease in the water content of IR-64 seedlings in comparison to Khitish seedlings. Consequently, a greater increase in dry wt g^{-1} of leaf and root tissue was noted in

IR-64 seedlings. At 12 days of WD, soluble protein content in leaf and root was decreased by 58 % and 40 % in IR-64 and by 17 and 10 % in Khitish, respectively (Fig. 1). The RWC measured at 3 h photoperiod was almost constant in well-watered control plants. WD treatment for 12 days reduced RWC to 26 and 69 % in IR-64 and Khitish varieties, respectively (Fig. 2a). Although, proline content was increased during WD in both the cultivars, its accumulation was greater in Khitish from the beginning of stress treatment (Fig. 2b). In IR-64 leaf, proline content was maximum at day 12 of WD, followed by a severe decline on further dehydration. On the other hand, proline accumulation kept on increasing in Khitish leaf with intensification of WD. The effect of WD on membrane integrity was evaluated by measuring electrolyte leakage from leaf. The electrolyte leakage was maintained at an almost constant level in Khitish until day 6 and increased marginally during later stages of WD. In contrast, a sharp rise in electrolyte leakage was noted in IR-64 cultivar (Fig. 2c).

GS isoforms in leaf, stem and root of rice seedlings

The mRNA and protein contents of GS isoform in rice seedlings were determined by quantitative RT-PCR and Western blot analysis, respectively. The activity of GS1 and GS2 was determined by fractionating them from the total cell free extract by anion exchange chromatography followed by estimating the activity from the area of corresponding elution profile after fractionation. GS1 activity was present in all the organs. GS2 activity was present in leaf and stem but was not detectable in root. The contribution of GS2 to total GS activity was about 80 and 30 % in leaf and stem, respectively (Table 1). Among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs. OsGS1;1 transcript was more abundant in leaf, whereas OsGS1;2 was the predominant form in stem and root. Moreover, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish. In the present study, OsGS1;3 gene expression was not studied as the previous reports indicate its absence in vegetative stage of growth of rice seedlings (Ishiyama et al. 2004a; Tabuchi et al. 2007). Western blot analysis indicated the presence of two proteins of about 39 and 43 kDa in leaf and stem that correspond to the molecular size of GS1 and GS2, respectively. Only GS1 was found to express in root. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in the immunoblot.

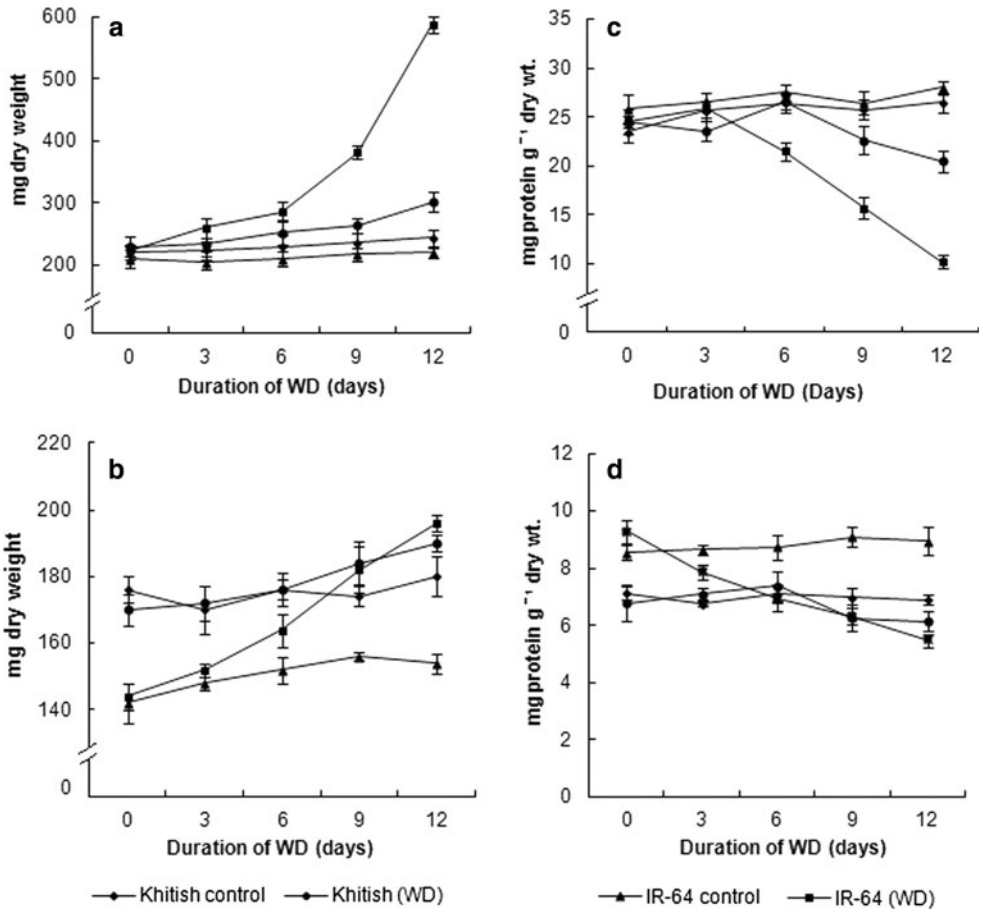

Fig. 1 Effect of WD on dry weight and protein content. Comparative analysis of dry weight of (**a**) leaf, (**b**) root and protein content of (**c**) leaf, (**d**) root between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. For determination of dry weight, 1 g of fresh tissue was dried at 80 °C for 48 h. All experiments were done in triplicates ($n = 3$), and average mean values were plotted against duration of WD

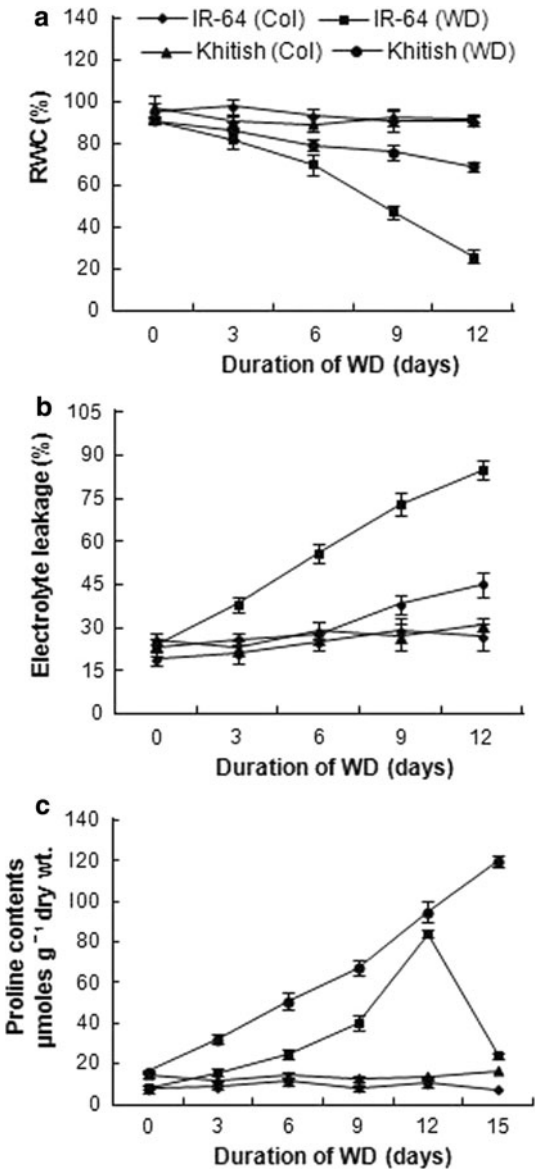

Fig. 2 Effect of WD on leaf RWC, proline content and electrolyte leakage. Comparative analysis of (**a**) RWC, (**b**) electrolyte leakage and (**c**) proline accumulation between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. All experiments were done in triplicates ($n = 3$), and average mean values were plotted against duration of dehydration

Effect of WD on expression of GS isoforms in leaf

3 weeks old, IR-64 and Khitish seedlings were subjected to WD for 12 days and activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs, at various stages. The results in Fig. 3 show the effect of WD on activity and expression of leaf GS isoforms. Total GS activity was almost similar in leaf of both the varieties at the beginning of stress treatment. However, it decreased by 52 and 10 % in IR-64 and Khitish leaf, respectively, at 12 days of WD (Table 1; Fig. 3a). The rapid reduction in activity in IR-64 was mainly due to disappearance of GS2 activity. The differential rate of decline in GS2 activity decreased the GS2 to GS1 ratio from 4.18 to 2.28 in IR-64 and from 4.00 to 3.75 in Khitish (Fig. 3b). The OsGS1;1 mRNA accumulated differentially in the two varieties. Its level was increased by about 5 and 2.5-fold in IR-64 and Khitish cultivars, respectively. On the other hand, OsGS1;2 mRNA content enhanced almost equally in both the varieties. It is also noticeable that OsGS2 mRNA content was reduced to about half in IR-64 leaf by the end of WD treatment, but no such change was noticed in Khitish leaf (Fig. 3c, d). The results of immunoblot analysis indicate that the GS1 polypeptide content was increased in both the varieties. GS2 polypeptide level was decreased in IR-64 leaf and remained almost unaltered in Khitish leaf (Fig. 3e, f).

Effect of WD on expression of GS isoforms in stem

GS isoforms in stem responded differently to WD. Total GS activity did not alter significantly in both the cultivars. In IR-64 stem, GS1 activity was reduced with WD. The reduction in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity (Table 1; Fig. 4a). These changes in GS1and GS2 activities raised the ratio of GS2 to GS1 from 0.46 to 1.20. The activity of GS isoforms did not alter significantly in Khitish stem (Fig. 4b). A varietal variation was also noted in OsGS1;1 mRNA expression as its level declined to less than half in IR-64 and enhanced about fourfold in Khitish, by the end of stress treatment. The OsGS1;2 mRNA was almost equally increased in both the varieties and was reflected in the time course of GS1 polypeptide abundance (Fig. 4 c–f). During WD treatment, OsGS2 transcript level was enhanced about twofold in IR-64 and was almost maintained in Khitish (Fig. 4c, d). The increased level of OsGS2 mRNA in IR-64 stem corresponded with the abundance of GS2 polypeptide (Fig. 4e, f).

Effect of WD on expression of GS isoforms in root

The cytosolic GS1 was the only GS isoform in rice root. WD treatment reduced the total GS activity of root by almost 50 % in IR-64, but hadn't much effect on Khitish cultivar (Table 1; Fig. 5a). OsGS1;1 mRNA expression also differed in root of the two cultivars. The initial higher level of OsGS1;1 transcript declined with WD in IR-64. In contrast, its expression increased almost twice in Khitish. OsGS1;2 transcript content declined faster in IR-64 in comparison to Khitish. As a result, its level fell to a minimum at day 4 and day 8 of WD in IR-64 and Khitish, respectively and then increased on further WD treatment

Table 1 Total GS, GS1 and GS2 activities in leaf, stem and root of rice (cv. IR-64 & Khitish) seedlings at various stages of WD

Rice varieties	Tissue	Days of WD	GS activity*			Ratio GS2; GS1
			Total GS	GS1	GS2	
Khitish	Leaf	0	28.50 ± 1.51	5.28 ± 0.10	21.12 ± 2.42	4.00
		4	30.00 ± 2.12	5.45 ± 0.25	21.52 ± 1.84	3.95
		8	27.20 ± 2.40	5.25 ± 0.15	19.68 ± 2.80	3.75
		12	25.80 ± 3.21	5.11 ± 0.30	19.16 ± 3.41	3.75
	Stem	0	12.10 ± 1.10	7.89 ± 0.25	4.10 ± 0.02	0.52
		4	10.92 ± 1.25	6.77 ± 0.31	3.84 ± 0.03	0.56
		8	11.50 ± 2.50	7.10 ± 0.15	3.82 ± 0.10	0.53
		12	12.20 ± 1.80	7.00 ± 0.10	4.05 ± 0.02	0.57
	Root	0	17.00 ± 2.10	Total activity was present as GS1		
		4	16.70 ± 2.41			
		8	16.68 ± 2.15			
		12	15.69 ± 2.80			
IR-64	Leaf	0	32.06 ± 3.21	5.45 ± 0.02	22.78 ± 3.10	4.18
		4	34.00 ± 3.80	5.86 ± 0.14	22.45 ± 2.50	4.00
		8	23.00 ± 4.25	5.30 ± 0.04	15.88 ± 3.12	3.00
		12	17.22 ± 5.50	5.13 ± 0.10	11.69 ± 3.12	2.28
	Stem	0	12.40 ± 2.10	8.21 ± 2.11	3.80 ± 1.45	0.46
		4	11.95 ± 3.20	6.95 ± 1.61	4.32 ± 3.12	0.62
		8	11.38 ± 1.26	5.42 ± 2.23	5.35 ± 2.54	0.98
		12	11.60 ± 3.24	4.95 ± 3.41	6.00 ± 3.61	1.21
	Root	0	20.00 ± 3.22	Total activity was present as GS1		
		4	16.00 ± 2.65			
		8	10.00 ± 2.65			
		12	08.40 ± 1.82			

GS1 and GS2 from various tissues were isolated by anion exchange chromatography. The enzyme activity in different fractions was assayed by semisynthetase method as described in "GS extraction and assay"

*One unit of GS activity represents 1.0 μmol of γ-glutamylhydroxamate produced 30 min^{-1}

(Fig. 5b, c). The time course of GS1protein detected on Western blot corresponded with the OsGS1;2 transcript level (Fig. 5d, e).

Discussion

Drought induces complex change in N metabolism due to decreased water availability for transport, leading to limited uptake of N-nutrients. GS is a key enzyme of N metabolism in rice crop and has pivotal role in survival of plant during WD. Present study describes the effect of WD on expression of GS isoforms in leaf, stem and root of two rice cultivars differently tolerant to drought conditions.

Dehydration tolerance in plants is attained by the maintenance of metabolic and physiological functions at low water status, which serve as the driving force for plant productivity. A few characteristics such as osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops. These are expressed in terms of RWC of the plant, accumulation of compatible solutes like proline, and membrane permeability of ions and electrolytes (Blum 2005). In the present study, rice varieties were initially screened for drought tolerance characteristics. During WD, Khitish variety maintained high RWC and showed maximum accumulation of proline and marginal increase in electrolyte leakage. Under similar conditions, IR-64 cultivar showed the least RWC and proline accumulation and sharp rise in electrolyte leakage. As a consequence, dry wt g^{-1} of leaf and root tissues of IR-64 seedling was increased significantly. The results thus indicated more susceptibility of IR-64 to WD as compared to Khitish and hence were designated as drought-sensitive and -tolerant cultivar, respectively.

The activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs of the two cultivars, at various stages of WD. The WD mediated alteration in total GS activity in leaf and root was directly related to dehydration tolerance characteristics of rice varieties, as it declined

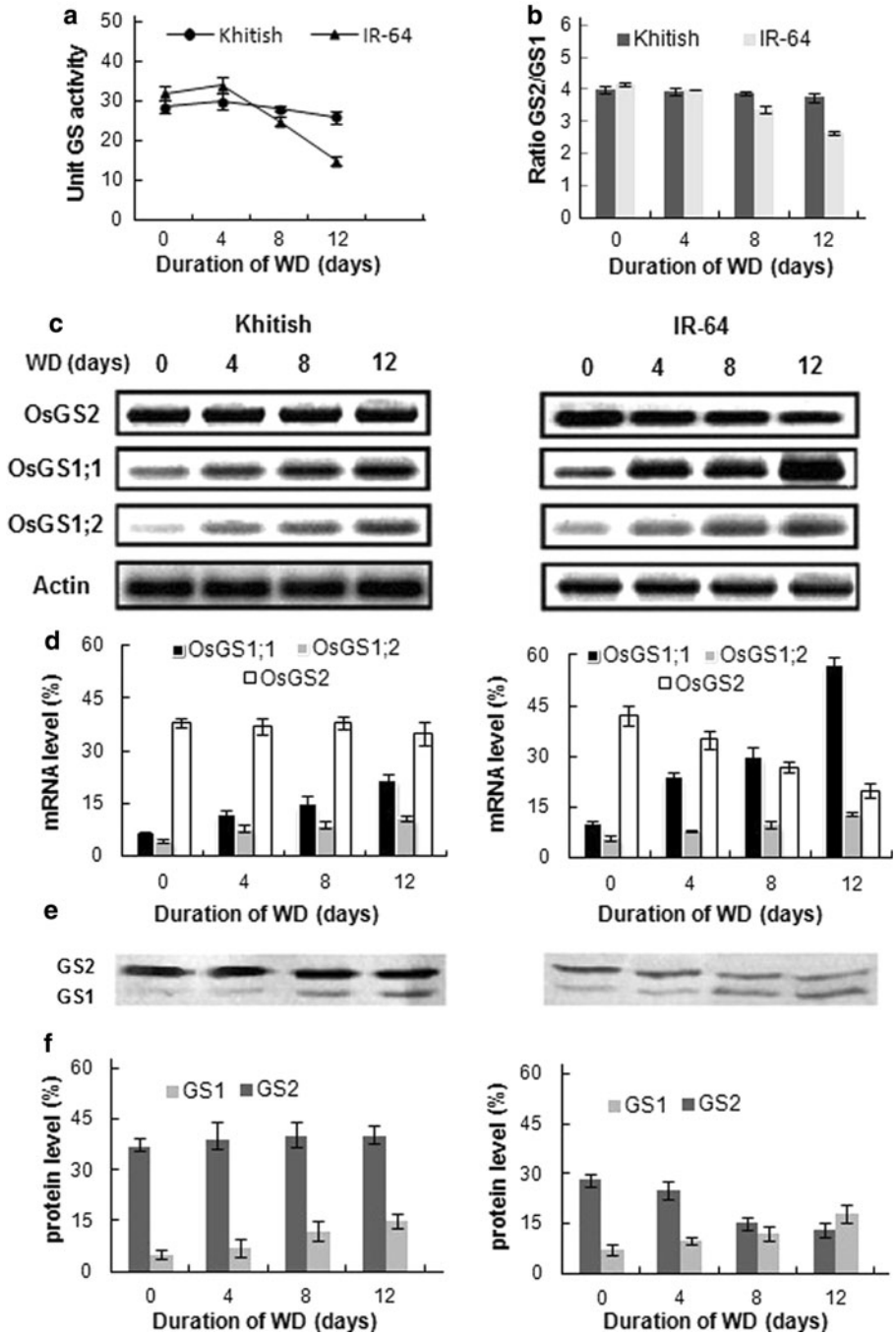

Fig. 3 GS activity and expression in leaves of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD. (**a**) Relative change in total GS activity of IR-64 (*black triangle*) and Khitish (*black circle*), (**b**) change in ratio of GS2/GS1 activity in IR-64 (*grey square*) and Khitish (*black square*) seedlings, (**c**) analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR, (**d**) *bar diagram* of GS mRNA level, (e) Western blot analysis of GS1 and GS2 polypeptides and (f) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 µg of total soluble protein extracted from leaf. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}

significantly in IR-64 and did not change markedly in Khitish cultivar. The decreased GS activity in IR-64 leaf was due to preferential reduction of GS2 activity and was correlated with decreased level of GS2 mRNA and protein. Under similar conditions, an almost constant GS2 mRNA and corresponding polypeptide maintained a steady GS2 activity in Khitish leaf. The results suggest that WD mediated GS2 regulation resides mainly at the transcriptional and/or mRNA stability levels. An important physiological function of GS2 is reassimilation of NH_4^+ produced during photorespiration, a rate limiting step of the pathway (Hausler et al. 1994; Hoshida et al. 2000). The physiological importance of GS2 has been demonstrated in transgenic plants overexpressing GS2 in the leaves. The transgenic plant line accumulating 1.5 fold more GS2 than the control plant had an increased photorespiration capacity. They also retained more than 90 % photosystem II activity when grown under osmotic stress treatment for 2 weeks (Hoshida et al. 2000). Hence in the present study, a relatively unaltered GS2 expression in Khitish leaf could

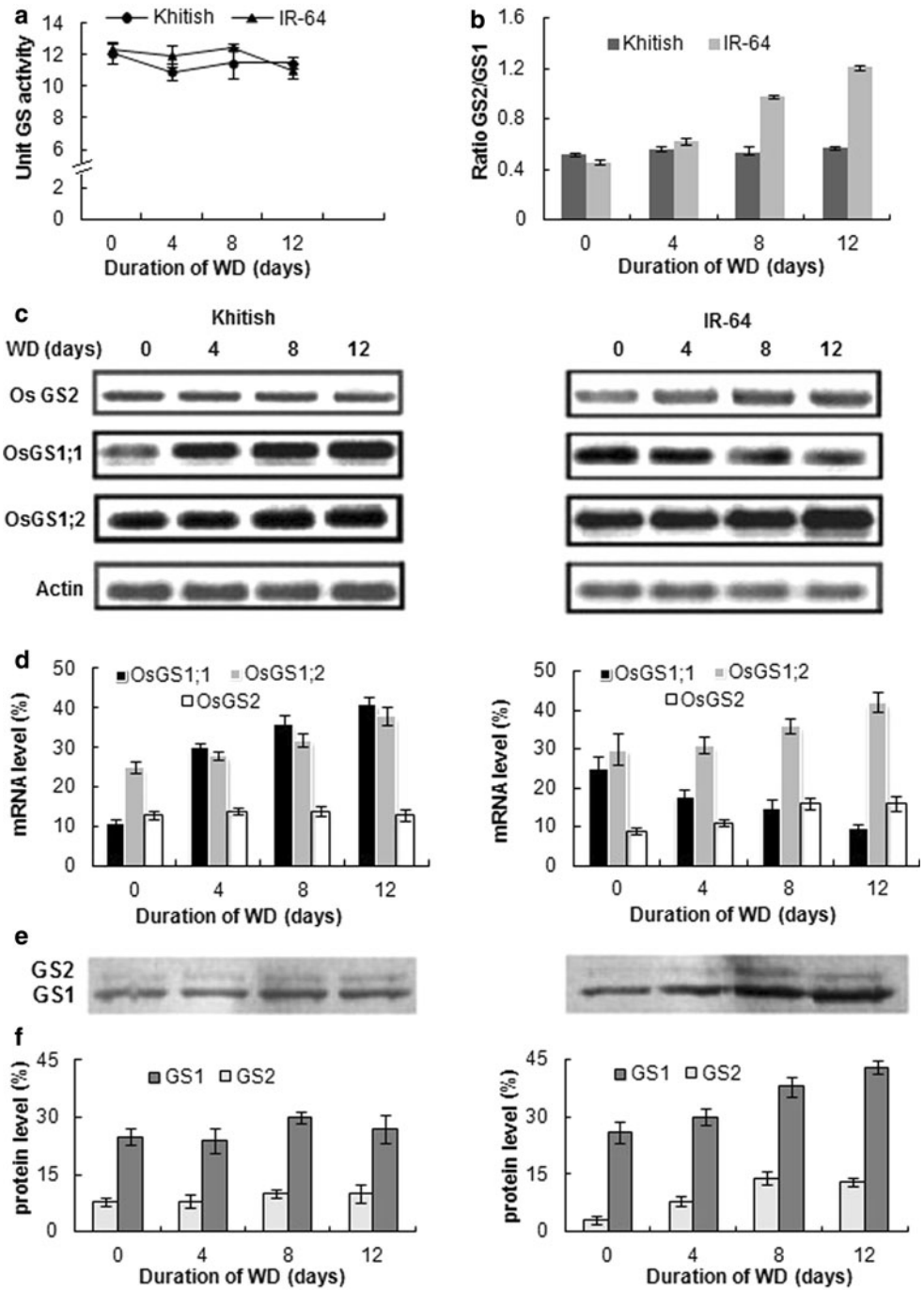

Fig. 4 GS activity and expression in stem of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (**a**) Relative change in total GS activity of IR-64 (*black triangle*) and Khitish (*black circle*), (**b**) changes ratio of GS2/GS1 activity in IR-64 (*grey square*) and Khitish (*black square*), (**c**) analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR, (**d**) *bar diagram* of GS gene expression, (**e**) Western blot analysis of GS1 and GS2 polypeptides and (**f**) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 µg of total soluble protein extracted from stem. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}

maintain the photorespiratory capacity at limited water availability that improves tolerance of the cultivar to WD.

The WD treatment increased the expression of GS1 transcripts and polypeptide in leaf of both IR-64 and Khitish. Although the total GS1 transcript level has already been reported to increase in leaf during natural senescence and in response to biotic and abiotic stress, the response of individual cytosolic GS gene to abiotic stress has not been studied. The observed increase in GS1 expression in rice leaf corresponded with an almost unchanged GS1 activity during WD. As in the present study, the nitrogen nutrition-mediated increase in GS1 transcript and polypeptide in *Arabidopsis* root was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004b). Moreover, a significantly greater expression of OsGS1;1 mRNA in IR-64 leaf could be due to its higher rate of protein degradation (Fig. 1c), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown as suggested earlier by Tabuchi et al. (2007).

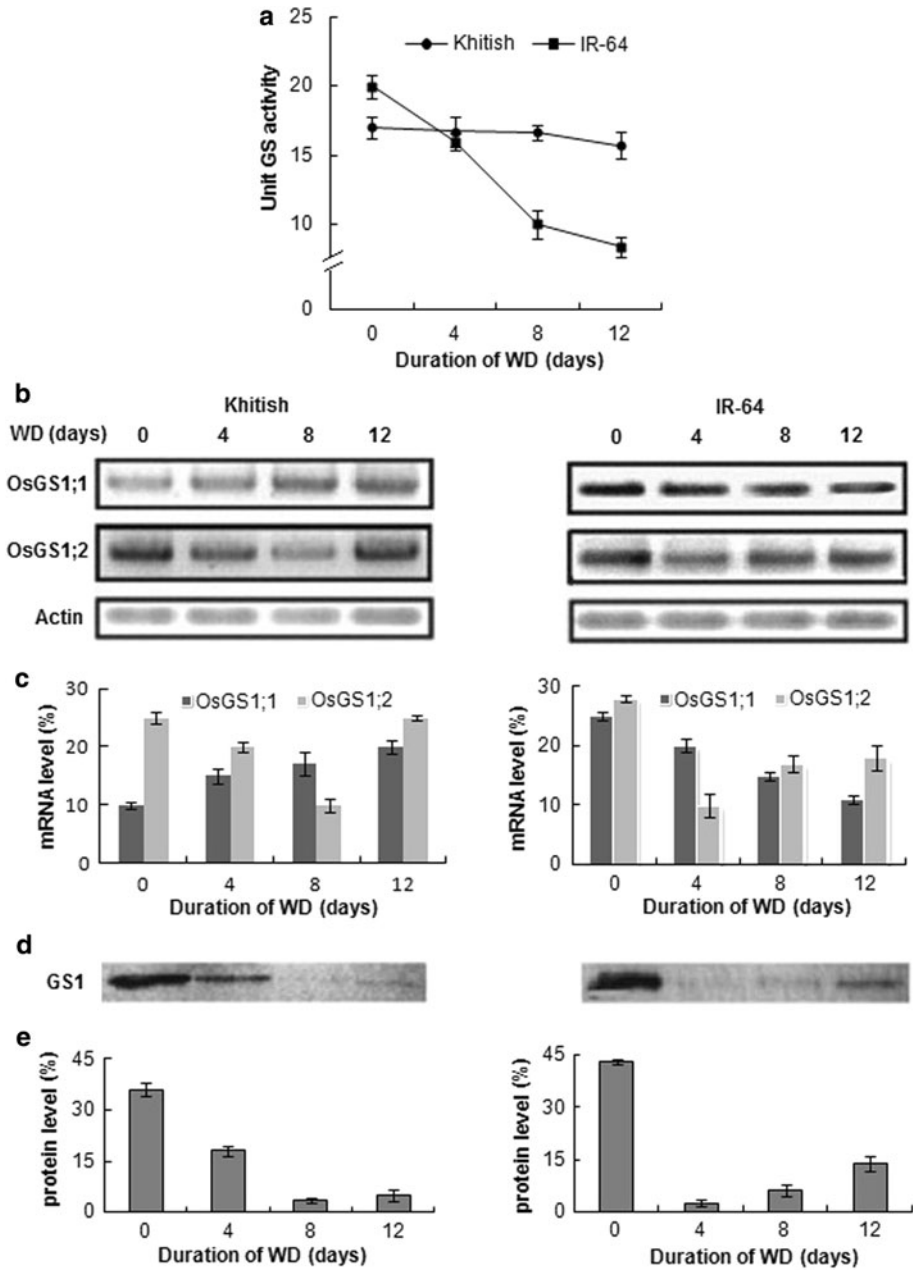

Fig. 5 GS activity and expression in roots of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (**a**) Relative change in total GS activity of IR-64 (*black square*) and Khitish (*black circle*), (**b**) analysis of OsGS1;1 and OsGS1;2 transcripts by RT-PCR, (**c**) *bar diagram* of GS gene expression, (**d**) Western blot analysis of GS1 polypeptide and (**e**) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 μg of total soluble protein extracted from root. One unit of GS activity represents 1.0 μmol of γ-glutamylhydroxamate produced 30 min^{-1}

In contrast to leaf, OsGS1;1 transcript level was found to decrease in IR-64 stem and root in response to WD. Nitrogen remobilization from protein breakdown constitutes the major source of nitrogen in vascular tissue and glutamine is the most abundant free amino acid for transport in rice plant (Tabuchi et al. 2007). The repression in OsGS1;1 might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in leaf. The transcriptional down-regulation of OsGS1;1 has already been documented in presence of NH_4^+ in roots of *Arabidopsis* and rice seedlings (Ishiyama et al. 2004a, b; Kusano et al. 2011). Several other rice genes associated with N-uptake and metabolism, such as, OsGS1;2, OsNADH-GOGAT1, OsAMT1;1 and OsAMT1;2, are also regulated by exogenous NH_4^+ ions (Ishiyama et al. 2004a; Sonoda et al. 2003; Tabuchi et al. 2007). However, pharmacological studies have suggested glutamine rather than NH_4^+ ions, being the real signaling molecule in regulation of expression of these genes (Kamachi et al. 1991; Oliveira and Coruzzi 1999; Tabuchi et al. 2007). The study by Ishiyama et al. (2004b) also reported glutamine as negative feedback regulator of GS1 in *Arabidopsis*.

WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. The GS1;1 gene has been

previously reported to be involved in remobilization and reutilization of nitrogen in rice plant during senescence (Tabuchi et al. 2007) and thus seems to play significant role in performance of plant under stress condition. The significance of OsGS1;1 in ammonium assimilation has also been studied earlier by Ishiyama and co-workers (2004a). A comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root indicated former having more substrate affinity for ammonium and approximately twofold higher V_{max} value. The result supported the importance of OsGS1;1 in promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions (Ishiyama et al. 2004a). The implication of OsGS1;1 in NH_4^+ assimilation has been further indicated by the over accumulation of free ammonium in the leaf sheath and roots of the rice mutant lacking OsGS1;1(Kusano et al. 2011). The decline in GS1 activity in IR-64 stem was compensated by increment in GS2 protein and activity, maintaining almost unchanged total GS activity. The increased GS2 protein can be due to observed increase in total protein content of stem during WD (data not shown). As in the present study, a tissue-specific response of WD stress was noted in *Lupinus albus* with strikingly increase in concentration of N and S in stem with intensification of water stress (Pinheiro et al. 2001). Thus, a different metabolic status may contribute to the maintenance of GS protein and activity in stem during WD.

In the present study, the two rice varieties did not differ much in expression pattern of OsGS1;2 during WD, despite difference in their drought tolerance properties. Its transcript level was found to enhance in leaf and stem. However, in root the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. Although, the time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance, it was not correlated with total GS activity in all the three tissues. The result is supported by study of Ishiyama et al. (2004b) on effect of ammonium nutrition in Arabidopsis root GS isoforms. Arabidopsis root has four different isoforms of GS1, among them amount of GS1;1, GS1;3, and GS1;4 mRNA decreased and GS1;2 mRNA was increased by ammonium nutrition. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation was due to lower affinity of GS1;2 for ammonium as compared to other isoform.

In conclusion, the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought-sensitive and -tolerant rice cultivars. GS2 is the major GS isoform in leaf and its over-expression in leaf has been found to be associated with the maintenance of photosynthetic and photorespiratory capacity of the plant. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen. A higher substrate affinity of the enzyme for ammonium signifies its promotion of the rapid conversion of ammonium to glutamine even under low ammonium conditions. Hence from the results, it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 may contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

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