CHAPTER 2

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

2.1. INTRODUCTION

Water deficit (WD) or dehydration is the most crucial environmental factor that limits crop productivity and the geographic distribution of several important crops such as rice, wheat and maize. The specific plant responses to WD are dependent on the rate and amount of water loss, the duration of the stress and the stage of plant development. Adaptation to WD at biochemical and molecular levels involves the activation/increased expression or induction of genes, transient increase in ABA level, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. During prolonged period of WD, decrease in water availability for transport associated process also leads to limited uptake of nitrogen and reduced availability of CO₂ for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. GS transcript and protein are shown to be regulated in response to both the plant status and environmental cues and hence, the enzyme constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production (Swarbreck et al. 2011).
WD is one of the major constraint depressing rice (*Oryza sativa* L.) production. The effect of WD varies with the variety, degree, growth stage and duration of stress. \( \text{NH}_4^+ \) is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS2) for GS2. The GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. OsGS1;1 and OsGS1;2 are expressed in all organs with higher expression in leaf blades. They are present as minor form as compared to GS2. OsGS1;3 is expressed mainly in roots and spikelets, respectively (Tabuchi et al. 2005). These isoforms have been shown to be regulated by a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et al. 1991; Kusano et al. 2011; Tabuchi et al. 2007). The role of GS isoforms in controlling N-metabolism during WD can be understood by studying their regulation in differentially drought tolerant rice varieties. Present study describes the regulation of GS isoforms in various organs of drought sensitive and tolerant cultivars of rice in response to WD.

### 2.2. MATERIALS AND METHODS

#### 2.2.1. Chemicals and reagents

All the chemicals used in this investigation were purchased from Sigma-Aldrich, USA; E. Merck, Germany; Sisco Research Laboratory, India and HiMedia Laboratory, India. DEAE-Sephacel was purchased from Sigma-Aldrich, USA. Biogel P-2 and P-100 were from Bio-Rad, USA. Molecular biology kits were from Promega, USA; QIAGEN, Germany and Invitrogen, USA. GS-antibody was purchased from Agrisera, Sweden.

#### 2.2.2. Plant materials and growth conditions

Rice (*Oryza sativa* L.) seeds (cv. Khitish, Pokkali, Triguna, Satabdi, IR-64, IR-8, PNR-519) were obtained from Rice Research Station, Chinsurah, West Bengal. Seeds were germinated in moist cotton bed at 30°C for 2-3 days under dark conditions. About 50 germinated seedlings were transferred 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 day12, frozen in liquid nitrogen, and stored at -80 °C for further analysis. Fresh leaf tissue immediately after harvest was used for determination of RWC, electrolyte leakage, proline and protein contents.

2.2.3. Determination of RWC

The relative water content (RWC) of leaves was measured according to Barrs and Weatherley (1962). 1 g of leaf tissue was weighed immediately after sampling to determine fresh weight (FW) and then rehydrated in water at 4 °C for 24 h and blotted dry 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 leaf relative content was calculated using the following formula: RWC = [(FW – DW)/ (TW – DW)] x 100. The experiment was carried out in triplicates.

2.2.4. Electrolyte leakage assay

Electrolyte leakage was assayed by (Bhusan et al. 2007) 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) x 100 was calculated. The experiment was carried out in triplicates.

2.2.5. Estimation of proline

Free proline content was estimated following the method of Bates et al (1973). The leaf tissue (100 mg) was powdered with the help of liquid nitrogen and extracted in 3% aqueous sulphosalicylic acid. The homogenate was centrifuged at 10,000 rpm for 10 min. and the supernatant was collected in a fresh vial. 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 ml of acetic acid at boiling temperature to which 20 ml of ortho-phosphoric acid was added) and 2 ml of glacial acetic acid and boiled at 100 °C for 1 h. After termination of reaction on ice, the reaction mixture was extracted with 4 ml of toluene. The chromophore containing aqueous phase was aspirated, warmed to room temperature (RT) and the absorbance was recorded.
at 520 nm. Proline concentration was calculated from a standard curve using 0-100 µg L-proline (Figure 2.1). The assay was done in triplicates. Amount of proline was expressed as µmole proline g⁻¹ dry wt.

![Figure 2.1. Standard curve for proline.](image)

### 2.2.6. Protein estimation

One gram frozen tissue of rice (leaves, stems and roots) was homogenized in 5 ml of extraction buffer containing 50 mM Tris HCl (pH 8.0), 1 mM MgCl₂, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was filtered through four layers of muslin. The filtered homogenate was centrifuged at 10,000 rpm for 15 min at 4 ºC. Quantitative estimation of protein was carried out by following the method of Bradford (1976) using bovine serum albumin (BSA) as standard (Figure 2.2).

![Figure 2.2. Standard curve for bovine serum albumin (BSA).](image)
To 100 μl of protein sample, 3.0 ml of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol; to the solution 100 ml 85% (w/v) phosphoric acid was added and diluted to 1 litre] was added. The reaction mixture was incubated at RT for 10 min. followed by determination of absorbance at 595 nm. Amount of protein was expressed as mg protein g⁻¹ dry wt.

2.2.7. GS extraction and assay

Frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in GS Extraction Buffer (5 ml g⁻¹ fresh wt.) containing 50 mM Tris HCl (pH 8.0), 1 mM MgCl₂, 2 mM cysteine hydrochloride and 15% glycerol. After filtering through four layers of muslin the extract 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 in the supernatant was determined by either transferase or semisynthetase reaction (Washitani and Sato 1977). For semisynthetase reaction, 1 ml reaction mixture contained 25 μmol Tris HCl (pH 7.5), 200 μmol glutamate, 10 μmol ATP, 5 mM hydroxylamine hydrochloride, 20 μmol MgCl₂, and 100 μl enzyme preparation. For transferase activity, the reaction mixture in a final volume of 1 ml contained 100 μmol Tris HCl (pH 7.5), 100 μmol glutamine, 60 μmol hydroxylamine hydrochloride, 20μmol sodium hydrogen arsenate, 1 μmol MnCl₂, and 10 μl of the enzyme preparation.

![Figure 2.3. Standard curve for γ-glutamylhydroxamate.](image)
The reaction was started by adding enzyme preparation and both the assays were carried out at 37 °C for 30 min. The reaction was terminated by adding 2 ml of FeCl₃ reagent (0.67 M FeCl₃, 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. γ-glutamylhydroxamate concentration was determined from a reference curve prepared with 0 - 2 μmole γ-glutamylhydroxamate (Figure 2.3). In blank, FeCl₃ reagent was added prior to the addition of enzyme preparation. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min⁻¹.

2.2.8. Separation of GS isoforms from leaf, stem and root of rice seedlings

GS isoforms in rice leaf, stem and root were separated by anion-exchange chromatography in a diethylaminoethyl (DEAE)-Sephacel column.

2.2.8.1. Preparation of DEAE-Sephacel

The pre-swollen DEAE-Sephacel slurry (Sigma-Aldrich, USA) was treated with 0.50 N HCl with slow stirring. The acid treated slurry was washed with distilled water till attaining pH 4. After discarding the supernatant, 0.50 N NaOH was added to the slurry followed by washing with distilled water till pH 6 to 7. Finally, the ion-exchange material was equilibrated with the GS Extraction Buffer, pH 8.0. About 10 ml of equilibrated slurry was packed into the column (10 x 2 cm) for separation of GS isoforms.

2.2.8.2. Separation of GS isoforms

All the steps of GS isoforms separation were performed at 4 °C. One gram of frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in 5 ml of GS extraction buffer. After filtering through four layers of muslin the extract was centrifuged at 10,000 rpm for 15 min at 4 °C. The homogenate was centrifuged at 10,000 rpm for 15 min. One ml of desalted supernatant was loaded onto a DEAE-Sephacel column (5x2 cm) pre-equilibrated with the GS extraction buffer. The column was washed with the same buffer until no protein was detectable in the eluate. Gradient elution of the adsorbed proteins was carried out by buffer containing, 0 - 0.50 M KCl. The flow rate was
maintained at 20 ml h\(^{-1}\). Two 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.

2.2.9. **RT-PCR analysis of GS genes**

2.2.9.1. *Isolation of total RNA from leaf, stem and root of rice seedlings*

Total RNA was isolated from rice tissues by using TRIZOL reagent (Invitrogen, USA) following the manufacturer’s instruction. 100 mg of plant tissue was homogenized in liquid nitrogen to fine powder and suspended in 1 ml of TRIZOL reagent. The homogenized sample was incubated at RT for 5 min to permit complete dissociation of the nucleoprotein complex. To the sample 0.20 ml of chloroform was added followed by vigorous shaking and incubation at RT for 2-3 min. The sample was centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was taken in a fresh tube and 0.50 ml of 100% isopropanol was added. The tube was incubated at RT for 10 min followed by centrifugation at 10,000 rpm for 10 min at 4°C. The RNA pellet obtained after centrifugation was washed with 75% ethanol, air dried and dissolved in 25 µl of RNase free water.

2.2.9.2. **Quantification of RNA**

The purity and concentration of RNA were determined spectrophotometrically by measuring absorbance at 260 and 280 nm using a UV-spectrophotometer (Thermo, USA). The RNA concentration was calculated using the following formula:

\[
\text{Concentration of RNA (µg/ml)} = A_{260} \times \text{dilution factor} \times 40
\]

The ratio of \(A_{260}\) and \(A_{280}\) determined the purity of the RNA preparation.

2.2.9.3. **Agarose gel electrophoresis of RNA**

1% agarose in 1X TAE (Tris-acetate EDTA) buffer was melted in a microwave and then cooled to 50-60°C. It was then supplemented with 5µg ml\(^{-1}\) ethidium bromide. The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. RNA sample was mixed with RNA loading dye (1X) prior to loading in the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer. RNA bands were visualized on a UV-transilluminator (Genei, India) (Figure 2.4).
2.2.9.4. **PCR Cloning of OsGS1;1, OsGS1;2 and OsGS2 ORF and sequence analysis**

GS isoform genes were isolated by RT-PCR. The ImProm-II™ Reverse Transcription System (Promega, USA) was used for synthesis of first-strand cDNA in preparation for PCR amplification. The experimental RNA (1μg) and Oligo (dT) primer (0.50 μg) were combined in nuclease free water to a final volume of 5 μl. The tube was closed tightly and placed into a preheated 70 °C heat block for 5 min followed by immediate chilling in ice water for 5 min. To the tube other components of reverse transcriptase reaction were added. The reaction mixture in a final volume of 15 μl contained ImProm-II™ 5X Reaction Buffer, 4 μl; 25 mM MgCl2, 1.20 μl; 10 mM dNTP mix, 1μl; Recombinant RNasin® Ribonuclease inhibitor, 20 units and 1μl ImProm-II™ reverse transcriptase. The reaction mix was incubated at 37 °C for 60 min. The reaction was terminated by incubation of reaction mix at 90 °C for 10 min. The cDNA was used as a template for PCR amplification in a 25 μl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5’-AGTATGGCT TCTCTCACCAGATCTCGTC3’) and OsGS1;1-R (5’-GTACCTCGAGGGCGTTTCCA GATGATGTTGTC T-3’) for OsGS1;1; OsGS1;2-F (5’-GACTCATATGCGCAAC CTCACCGACCTCGTC-3’) and OsGS1;2-R (5’-TAGCGGCGCTGTCTCCTCCTCA CA GCAGCGTG-3’) for OsGS1;2 PCR was performed for 35 cycles. The PCR products were loaded and separated on 1% agarose TAE gel. The PCR products were then cloned.
into pGEMT-Easy vector. For cloning the PCR product was extracted from the agarose gel by using Gel Extraction Kit (QIAGEN, Germany). The ligation reaction mixture in a total volume of 10 µl contained: 2X rapid ligation buffer, 5µl; pGEM-T-Easy vector, 50 ng (1µl); PCR product, 2µl; T4 DNA ligase (3weiss unit/µl), 1µl and 1µl H2O. The reaction mixture incubated over night at 4 ºC was transformed into competent E. coli JM109 cells by heat shock at 42 ºC for 90 sec. The transformed cells were revived for 1.5 hours at 37 ºC in a shaking incubator. The transformants were selected in Ampicillin (50 µg ml−1) agar plate supplemented with 20% IPTG and 2% (w/v) X-gal by incubating over night at 37 ºC. Plasmid was isolated from the recombinant colonies by Alkaline lysis method (Birnboirm and Dolly, 1979). The recombinant plasmids were analysed for the presence of DNA inserts by restriction digestion. The cloned PCR products were subjected to sequence analysis.

2.2.9.5. Quantitative RT-PCR analysis of GS gene expression under WD

Semiquantitative RT-PCR was performed by 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′-AGTATGGCTTCTCTCAACCCATCTCGT-3’) and OsGS1;1-R (5′-GTACCTGAGGCTTCCACAGATGATGGTGTTCT-3’) for OsGS1;1, OsGS1;2-F (5′-GACTCATATGGCCACCTCACCACAGCTCTCGTT-3’) and OsGS1;2-R (5′-TAGCGGCCCCTGTTCTGCTCCACACAGCGCGTG-3’) for OsGS1;2, OsGS2-F (5′-AGAACCTTGGACATGAATCGG-3’) and OsGS2-R (5′-CATTTTATTTTCGAGGAGG-3’) for OsGS2 and OsActin-F (5′-GTCAGATGGGATGATATGG-3’) and OsActin-R (5′-TCTCCTTGGCTCCTCCTGTCAG-3’) for actin. GS specific primers were designed according to the sequences of BAC clones represented in Table 2.1. PCR was performed for 27 to 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. Seven microliters of the PCR products were loaded and separated on 1% agarose TAE gels. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.
Table 2.1. Detail of the BAC cloned genes of glutamine synthetase isoforms.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Product</th>
<th>BAC clone accession numbers</th>
<th>Chromosome location</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsGS1;1</td>
<td>GS1</td>
<td>AP004880 (P0487D09)</td>
<td>chr02</td>
</tr>
<tr>
<td>OsGS1;2</td>
<td>GS1</td>
<td>AC105364 (OJ1743A09)</td>
<td>chr03</td>
</tr>
<tr>
<td>OsGS2</td>
<td>GS2</td>
<td>AL662953(OSJNBA0011F23)</td>
<td>chr04</td>
</tr>
</tbody>
</table>

2.2.10. Immuno blot analysis of GS isoforms

2.2.10.1. Extraction of total soluble protein and SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out following the discontinuous method described by Laemmli (1970). Total soluble protein was resolved by 12.50 % SDS-PAGE (Figure 2.5). Resolving gel [30% acrylamide, 4 ml; 1.50 M Tris-Cl (pH 8.8), 2.50 ml; 10% SDS, 0.10 ml; 10% APS, 0.10 ml; TEMED 0.004 ml and 3.30 ml water] of 5 cm length was poured between two glass plates which were clamped together but held apart by plastic spacers and was allowed to set.

Figure 2.5. SDS-PAGE of total protein isolated from (a) protein marker, (b) leaf, (c) root, and (d) stem of rice seedlings.

The stacking gel (0.80 cm) [1M Tris HCl (pH 6.8), 0.38 ml; 10% SDS, 0.03 ml; 10% APS, 0.03 ml; TEMED, 0.003 ml; 2.10 ml water] was poured on the top of resolving gel and a plastic comb was placed on the stacking gel. After polymerization the comb was removed to provide loading wells. Glass plates with gel were placed in vertical electrophoresis system with running buffer tank containing running buffer [25 mM...
TrisHCl (pH 8.0), 250 mM glycine, 0.10 % (w/v) SDS]. The protein sample was mixed with SDS gel loading buffer [50 mM TrisHCl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.10 % bromophenol blue, 10 % glycerol] and heated in a boiling water bath for 10 min. Protein samples and prestained protein molecular weight marker were loaded in the wells and electric field was applied. When dye reached at the bottom of the tank, power was turned off. Gel was removed carefully from the glass plates and subjected to immunoblot analysis of GS isoforms.

2.2.10.2. Immunoblot analysis of GS isoforms

Proteins separated by 12.50 % SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry method using Electroblotting apparatus (Atto, Japan). The membrane was incubated with the anti-GS antibody (Agrisera, Sweden) raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptide was visualised with a secondary antibody, 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. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.
2.3. RESULTS

2.3.1. Screening of rice cultivars for tolerance to WD stress

Seven different varieties of rice (cv. IR-64, Khtish, Triguna, IR-8, Pokkali, PNR-519 and Satabadi) were screened for their drought tolerance characteristics. For this rice seedlings were grown for 3 weeks under controlled conditions and WD was imposed thereafter by withholding watering. Drought tolerance characteristic of rice cultivars was evaluated by monitoring changes in leaf relative water content (RWC), electrolyte leakage, proline and protein contents at indicated days of WD treatment.

2.3.1.1. Effect of WD on RWC in leaves of rice cultivars

The results of the effect of WD on RWC in leaves of different cultivars of rice are shown in Figure 2.6. The RWC measured at 3 h photoperiod was almost constant in well watered control plants.

![Figure 2.6](image-url)
A differential effect of WD on rice cultivars was noted from day 8 of WD and became more significant on further treatment. Consequently, at 12 days of WD the decline was maximum in IR-64 (82 %); moderate in Pokkali (48 %), Satabadi (52 %), Triguna (56 %), PNR-519 (63 %), IR-8 (67 %); and least in Khitish (31 %).

**2.3.1.2. Effect of WD on proline content in leaves of rice cultivars**

The WD condition resulted in an increase in proline content in leaves of all the rice varieties. As can be seen from result in *Figure 2.7* that leaves of Khitish, Satabadi, Pokkali and Triguna cultivars had greater level of proline from the beginning of stress treatment. In these seedlings the accumulation of proline kept on increasing continuously throughout the duration of WD. However, in rice cultivars IR-8, PNR-519, and IR-64, proline content was maximum at day 12 of WD, followed by a severe decline on further dehydration.

![Figure 2.7. Proline content in leaves of different rice (*O. sativa*) cultivars at 0, 3, 6, 9, 12 and 15 days of WD. All experiments were done in triplicates (*n* = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated](image)

At 15 days of WD, proline content increased to about 120, 90, 80 and 72 μmole g⁻¹ dry wt. in Khitish, Pokkali, Satabadi and Triguna cultivars, respectively. Under similar condition
proline accumulation of about 52, 40 and 26 μmole g\(^{-1}\) dry wt. was noted in PNR-519, IR-8 and IR-64, respectively.

### 2.3.1.3. Effect of WD on protein content in leaves of rice cultivars

Influence of WD conditions on protein level in leaves of various rice cultivars is shown in Figure 2.8. As can be seen that total soluble protein increased throughout the experimental period in leaves of well watered control seedlings. WD treatment resulted in significant decline in protein level of all the varieties, however, the effect was variable. A comparison of protein content at day 12 of WD indicates that its decline was minimum in Khitish (15 %), moderate in Pokkali (33 %), Satabadi (35 %), Triguna (43 %), IR-8 (48 %), PNR-519 (48 %) and was highest in IR-64 (58 %).

![Protein content in leaves of different rice (O. sativa) cultivars at 0, 3, 6, 9, and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated](image)

### 2.3.1.4. Effect of water-deficit on electrolyte leakage

The effect of WD on cell membrane integrity was evaluated by measuring electrolyte leakage from leaf. It is evident from the results in Figure 2.9 that the electrolyte leakage was almost constant in well-watered control rice plants. Although, WD
treatment caused increase in electrolyte leakage from leaves, the response was variable in
different rice cultivars. The effect of WD on leakage of electrolyte was more pronounced
in IR-64, PNR-8 and IR-8 varieties. At 12 days of WD electrolyte leakage was enhanced
by 3.6 and 2.6 folds in IR-64 and IR-8 cultivars, respectively. Electrolyte leakage was
least in case of Khitish cultivar. Results of above studies indicated that *Oryza sativa* cv.
IR-64 was the least tolerant, whereas *Oryza sativa* cv. Khitish was most tolerant to WD.
Hence, IR-64 and Khitish cultivars were designated as drought-sensitive and drought-
tolerant rice cultivar, respectively, and were selected for further studies.

![Figure 2.9](image)

**Figure 2.9.** Electrolyte leakage from leaves of different rice (*O. sativa*) cultivars during 0, 3, 6, 9
and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were
plotted against duration of WD. *C = Control, **S = WD treated.

Ready comparisons of the effect of WD on morphological and biochemical changes in
seedlings of these two varieties are shown in Figure 2.10, 2.11 and 2.12. IR-64 and
Khitish seedlings were grown under similar conditions with same level of irrigation for
three weeks and then subjected to WD for 12 days. WD treatment caused rapid decrease in
the water content of IR-64 as compared to Khitish. The susceptibility of IR-64 to WD is
clearly visible in *Figure 10a*. At 12 days of WD, morphology of IR-64 seedlings altered significantly with drying of shoot and rolling of leaves, however, such features were not observable in Khitish seedlings throughout the experimental duration.

**Figure 2.10.** Effect of WD on IR-64 and Khitish cultivars of rice. Comparison of morphological characteristics of (a) shoot and (b) root of IR-64 and Khitish seedlings at 0, 4, 8 and 12 days of WD.
Drought tolerant and sensitive nature of Khitish and IR-64 are also evident from their root architectures. The root system of Khitish seedlings were more branched, longer and thicker as compared to that of IR-64 (Figure 2.10 b). The dry weight of leaves and roots of these seedlings were measured various days of WD treatment. The dry weights increased more significantly in IR-64 in comparison to Khitish. For example WD treatment for 12 days raised the dry wt. g$^{-1}$ of leaves from 112 mg to 294 mg in IR-64 and from 115 mg to 151 mg in Khitish cultivar (Figure 2.11a). Under same condition, the dry wt. g$^{-1}$ of root enhanced from 140 mg to 190 mg in IR-64, whereas it increased only marginally in Khitish cultivar (Figure 2.11 b). As a result RWC in leaf declined at a faster rate in IR-64 as compared to Khitish.

**Figure 2.11.** Effect of WD on dry weight and relative water content. Comparative analysis of dry weights of (a) leaf, and (b) root and (c) RWC of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit and dry weight and RWC were determined at 0, 3, 6, 9 and 12 days of treatment. For determination of dry weight 0.50 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.
WD treatment for 12 days reduced leaf RWC to 26 and 69 % in IR-64 and Khitish varieties, respectively (Figure 2.11 c). The protein contents in leaves and roots declined more rapidly in IR-64 seedlings. At the beginning of WD treatment protein content in leaves of both the cultivars was about 25 mg g⁻¹ dry wt., which declined to 15 and 22 mg in IR-64 and Khitish, respectively, at day 9 of WD. At the end of WD treatment the soluble protein content in leaf and root was decreased by 58 % and 40 % in IR-64 and by 17 and 10 % in Khitish, respectively (Figure 2.12 a, b).

![Figure 2.12](image)

**Figure 2.12.** Effect of WD on protein content, proline content and electrolyte leakage. Comparative analysis of protein contents: (a) leaf, and (b) root; (c) proline content and (d) electrolyte leakage of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit. All experiments were done in triplicates (*n* = 3), and average mean values were plotted against duration of WD.

Although, the level of proline was increased during WD in leaves of both the varieties, its accumulation was greater in Khitish from the beginning of stress treatment (Figure 2.12)**
c). In IR-64 seedlings, proline content was maximum at day 12 of WD, followed by its severe decline on further dehydration. On the other hand, proline accumulation kept on increasing with WD in Khitish 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 (Figure 2.12 d).

2.3.2. Optimization of GS extraction and assay conditions

GS has been studied in many higher plants. However, the optimal conditions for its extraction vary with tissue as well as the plant species (Lea et al. 1990). Hence, the optimal conditions for extraction of the enzyme with respect to pH of the buffer and concentration of protective/stabilizing agents were established in order to ensure maximal extraction and recovery of the enzyme. Maximum recovery of the enzyme was obtained when 50 mM Tris HCl (pH 8.0) supplemented with 1 mM MgCl₂, 2 mM cysteine hydrochloride and 15% glycerol was used as extraction buffer. The optimum pH of assay media for GS activity of was 7.5. The rate of semisynthetase activity rose proportionately upto 300 µl of enzyme extract. The amount of γ-glutamylhydroxamate produced increased proportionately with the reaction period upto 30 min when 300 µl of enzyme extract was used for assay. Hence in all the subsequent experiments GS activity was routinely determined over a period of 30 min with 300 µl of enzyme preparation.

2.3.3. GS isoforms in leaf, stem and root of rice varieties

GS was extracted from root, stem and leaf of three weeks old rice seedlings raised in 3:1mixture of soil and soilrite, under 250 µmol m⁻² s⁻¹ photon flux density (16h/8h day/night regime) at 27 ± 2 °C and 70-80 % relative humidity in a plant growth chamber.

The activity of GS in crude extract was determined by semisynthetase assay based on rate of formation of γ-glutamylhydroxamate. Anion-exchange chromatography was used to separate the isoforms of GS in leaf, stem and root extracts. As can be seen from result in Figure 2.13a, on subjecting the leaf extract to chromatography in DEAE-Sephacel column, the enzyme was resolved in two distinct peaks which were recovered with gradient elution buffer containing 0 - 0.50 M KCl. These two enzyme forms were
designated as GS1 and GS2, respectively. In case of the stem extract (Figure 2.13 b) also the enzyme activity was resolved into two peaks at the same salt concentration as GS1 and GS2 in the leaf extract. However, ion-exchange chromatography of root extract eluted almost the entire activity as a single peak at the same salt concentration as GS1 in leaf and stem extracts and no peak corresponding to GS2 was detectable (Figure 2.13 c). From the results in Table-2.2 it is apparent that rice leaves contained 25 and 75 % of activity as GS1 and GS2, respectively. In stem most of the GS activity (70 %) was present as GS1 and rest 30 % activity represented as GS2.

2.3.4 Detection and quantization of GS isoforms mRNA and protein
2.3.4.1 Standardization of RT-PCR amplification of GS isoforms

Before performing the experiments on quantitative GS gene expression analysis, the reaction conditions for RT-PCR of full length GS isogenes were established. Total RNA was isolated from leaf, stem and root tissues of three week old rice seedlings. 1 µg of total RNA was used for synthesis of first strand cDNA by reverse transcriptase (RT) followed by PCR using gene specific forward and reverse primers. The number of cycles and annealing temperature were optimized for each gene specific primer pairs. To ascertain the PCR products as OsGS1;1, OsGS1;2 and OsGS2, the amplified PCR products (Figure 2.14) were cloned in pGEMT vector and sequenced. The sequence showed homology with OsGS sequences in the database.

2.3.4.2 Quantification of GS isoforms transcripts in rice seedlings

The transcript levels of GS isoform in various organs of rice plant were determined by RT-PCR. The PCR amplified full length OsGS1;1, OsGS1;2 and OsGS2 ORFs were resolved by agarose gel electrophoresis and quantified by ImageAide version 3.06.04, to calculate the level of gene expression. From the results in Figure 2.15 it is apparent that among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs, whereas OsGS2 was expressed in leaf and stem but not in root. In leaf tissue the OsGS2 mRNA level was considerably higher than that of cytosolic isoforms and its accumulation was found to be about 4.20 folds greater than OsGS1;1. OsGS2 was present as minor form in stem.
Figure 2.13. Elution profile during anion-exchange chromatography of GS isoforms (a) leaf, (b) stem and (c) root tissues of 3 weeks old rice seedlings. The enzyme activity in different fractions was assayed by the semisynthetase assay. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min⁻¹.
Table 2.2. Relative proportion of GS1 and GS2 in leaf, stem and root of rice seedlings.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total GS</th>
<th>GS1</th>
<th>GS2</th>
<th>Ratio GS2/GS1</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf</td>
<td>31.25</td>
<td>6.10</td>
<td>22.70</td>
<td>3.72</td>
<td>92.16</td>
</tr>
<tr>
<td>Stem</td>
<td>13.20</td>
<td>8.21</td>
<td>3.80</td>
<td>0.46</td>
<td>90.90</td>
</tr>
<tr>
<td>Root</td>
<td>20.00</td>
<td>18.5</td>
<td>N.D.</td>
<td>0</td>
<td>92.50</td>
</tr>
</tbody>
</table>

N.D.: Not detectable

GS1 and GS2 were isolated by anion-exchange chromatography (Section 2.2.8.2) from leaf, stem and root tissues of 3 weeks old rice (*Oryza sativa* cv. IR-64) seedlings raised in plant growth chamber. The enzyme activity was assayed by the semisynthetase assay. *One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min⁻¹.

Figure 2.14. RT-PCR amplification of GS isoforms and agarose gel electrophoresis analysis of PCR products. Lane1- 500bp ladder, Lane2- OsGS1;2 (1171 bp), Lane3- OsGS1;1 (1039 bp) and Lane 4: OsGS2 (1255bp)

Among the GS1 isoforms OsGS1;1 was more abundant than OsGS1;2 in leaf. OsGS1;2 was the predominant form in stem and root of both IR-64 and Khitish cultivars. However, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish. OsGS1;3 gene expression was not studied, as previous reports indicate its absence in vegetative stage of growth of rice seedlings (Ishiyama et al. 2004b; Tabuchi et al. 2007).
2.3.4.3. Quantification of GS isoforms polypeptides in rice seedlings

The polypeptide levels of GS1 and GS2 isoforms in leaves, stems and roots of rice seedlings were determined by immunoblotting. Total soluble proteins (10 µg) of leaf, stem and root tissues were resolved by 12.50 % SDS-PAGE and transferred to PVDF membrane followed by probing the membrane with the anti-GS antibody raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptides were visualised with secondary antibody-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP).
The result in Figure 2.16 indicates the presence of two protein bands of approximately 39 and 43 kDa in leaf and stem that correspond to the molecular size of GS1 and GS2, respectively. The immunoblot analysis of root protein highlighted only one protein band corresponding to GS isoform. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in immunoblot.

2.3.5. Effect of WD on activity and expression of GS isoforms in leaf, stem and root of IR-64 and Khitish seedlings

Rice seedlings of IR-64 and Khitish cultivars grown for 3 weeks under controlled conditions were subjected to WD by withholding watering and activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously determined in leaf, stem and root tissue at 0, 4, 8 and 12 days of the treatment.

2.3.5.1. Effect of WD on total GS, GS1 and GS2 activities in leaf, stem and root of IR-64 and Khitish seedlings

Results of the influence of WD on total GS, GS1 and GS2 activities in leaves, stem and roots of IR-64 and Khitish cultivars are shown in Table 2.3. At the beginning of WD treatment the total GS activity was almost similar in both the cultivars. WD treatment caused rapid decline of GS activity in leaves and roots of IR-64 seedlings as compare to that of Khitish. In IR-64 leaf total GS activity decreased by 28 and 52 % at 8 and 12 days of WD, whereas in Khitish leaf a significant decrease of only 10 % was noted at day 12 of treatment. The rapid reduction in GS activity of IR-64 leaf was mainly due to disappearance of GS2 activity. For example, WD condition for 12 days reduced the GS2 activity from 23 to 11 in IR-64 and from 21 to 19 in Khitish cultivar. The differential rate of decline in GS2 activity in the two varieties decreased the ratio of GS2 to GS1 from 4.18 to 2.28 in IR-64 and from 4.00 to 3.75 in Khitish.

Total GS activity in stem of IR-64 and Khitish seedlings was almost unaffected by WD. However, alteration in both GS1 and GS2 activities was noted in stem of IR-64 seedling. In this seedling GS1 activity was reduced by 16, 36 and 41% on 4, 8 and 12 days of WD. On the other hand, the activity of GS2 was enhanced by 40 % at 12 days of stress.
Thus the decline in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity. Alterations in GS1 and GS2 activities raised the ratio of GS2 to GS1 from 0.46 to 1.20 in IR-64 stem. Such change in ratio was not noticeable in Khitish stem as both GS1 and GS2 activities were almost constant throughout the WD treatment. The cytosolic GS1 was the only GS isoform in rice root. WD treatment had not much effect on GS activity in Khitish root. In contrast, the GS activity in roots of IR-64 cultivar was quite sensitive to WD. A rapid reduction in activity of about 50 and 60% was noted at 8 and 12 days of WD treatment, respectively.

Table 2.3. Effect of WD on total GS, GS1 and GS2 activities in leaves, stem and root of *O. sativa* cv. IR-64 and Khitish.

<table>
<thead>
<tr>
<th>Rice Varieties</th>
<th>Tissues</th>
<th>Days of WD</th>
<th>Total GS</th>
<th>GS activity*</th>
<th>GS2</th>
<th>Ratio GS2:GS1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Khitish</strong></td>
<td>Leaf</td>
<td>0</td>
<td>28.50 ± 1.51</td>
<td>5.28 ± 0.10</td>
<td>21.12 ± 2.42</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>30.00 ± 2.12</td>
<td>5.45 ± 0.25</td>
<td>21.52 ± 1.84</td>
<td>3.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>27.20 ± 2.40</td>
<td>5.25 ± 0.15</td>
<td>19.68 ± 2.80</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>25.80 ± 3.21</td>
<td>5.11 ± 0.30</td>
<td>19.16 ± 3.41</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0</td>
<td>12.10 ± 1.10</td>
<td>7.89 ± 0.25</td>
<td>4.10 ± 0.02</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>10.92 ± 1.25</td>
<td>6.77 ± 0.31</td>
<td>3.84 ± 0.03</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>11.50 ± 2.50</td>
<td>7.10 ± 0.15</td>
<td>3.82 ± 0.10</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>12.20 ± 1.80</td>
<td>7.00 ± 0.10</td>
<td>4.05 ± 0.02</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>0</td>
<td>17.00 ± 2.10</td>
<td>Total activity was present as GS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>16.70 ± 2.41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>16.68 ± 2.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>15.69 ± 2.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IR-64</strong></td>
<td>Leaf</td>
<td>0</td>
<td>32.06 ± 3.21</td>
<td>5.45 ± 0.02</td>
<td>22.78 ± 3.10</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>34.00 ± 3.80</td>
<td>5.86 ± 0.14</td>
<td>22.45 ± 2.50</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>34.00 ± 4.25</td>
<td>5.86 ± 0.14</td>
<td>15.88 ± 3.12</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>17.22 ± 5.50</td>
<td>5.86 ± 0.14</td>
<td>11.69 ± 3.12</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>0</td>
<td>12.40 ± 2.10</td>
<td>8.21 ± 2.11</td>
<td>3.80 ± 1.45</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>11.95 ± 3.20</td>
<td>6.95 ± 1.61</td>
<td>4.32 ± 3.12</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>11.38 ± 1.26</td>
<td>5.42 ± 2.23</td>
<td>5.35 ± 2.54</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>11.60 ± 3.24</td>
<td>4.95 ± 3.41</td>
<td>6.00 ± 3.61</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>0</td>
<td>20.00 ± 3.22</td>
<td>Total activity was present as GS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>16.00 ± 2.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>10.00 ± 2.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>08.40 ± 1.82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Total GS activity was resolved into GS1 and GS2 by anion-exchange chromatography. Enzyme activity in different fractions was assayed by semisynthetase reaction. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min⁻¹.
2.3.5.2. Effect of WD on expression of GS1 and GS2 mRNA in leaf, stem and root of IR-64 and Khitish seedlings

Total RNA was isolated from various tissues of rice seedlings at indicated days of WD and mRNA levels of OsGS1;1, OsGS1;2 and OsGS2 were determined by semi-quantitative RT-PCR.

The results of WD mediated alteration in GS transcripts expression in leaf tissue are shown in Figure 2.17. Though, OsGS1;1 and OsGS1;2 mRNA levels increased in both IR-64 and Khitish cultivars in response to WD, expression level of OsGS1;1 mRNA was considerably higher in IR-64 leaf. OsGS1;1 mRNA level was increased by about 2.5 and 5 folds in IR-64 leaf and by 2 and 2.5 folds in Khitish leaf at 8 and 12 days of WD, respectively. On the other hand, OsGS1;2 mRNA content enhanced almost equally in both the varieties. It is also noticeable that OsGS2 mRNA level was significantly affected by WD in IR-64 cultivar. Its level declined by about 40% at 8 days of WD. A further...
reduction of upto 50% was observed at 12 days of treatment. Under similar conditions no such change in OsGS2 expression was noticed in Khitish leaf.

![Graph showing the effect of WD on OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR.](image)

**Figure 2.18.** Effect of WD on expression of GS mRNA in stem of *O. sativa* cv. IR-64 and Khitish. (a) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The influence of WD on expression of GS mRNAs in stem of was significantly different from that of leaf. The results in *Figure 2.18* indicate a varietal variation in expression of OsGS1;1 in stem of IR-64 and Khitish seedling in response of WD. In stem, OsGS1;1 transcript content declined with the intensification of stress. Its level decreased to about 50% at 8 days of WD followed by a further decline of upto 60% at 12 days of treatment. In contrast, OsGS1;1 mRNA was found to increase in Khitish stem with about 3 and 4 folds enhancement at 4 and 12 days of WD, respectively. The OsGS1;2 transcript responded almost equally in both the varieties, with almost 2 fold increase in its abundance at 12 days of stress treatment.
Figure 2.19. Effect of WD on expression of GS mRNA in root of *O. sativa* cv. IR-64 and Khitish (a) Analysis of OsGS1;1 and OsGS1;2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The chloroplastic GS2 mRNA was found to express in stem, but its mRNA level was significantly lower than that of cytosolic GS isoforms. A differential response of WD on GS2 mRNA expression was also noted. WD treatment for 12 days resulted in enhancement of OsGS2 transcript content by about 2 fold in IR-64 stem. However, such alteration in OsGS2 mRNA quantity was undetectable in Khitish stem, which maintained an almost constant level of the transcript throughout the treatment period.

Among GS transcripts only GS1 isoforms were expressed in root. As can be seen from the results in *Figure 2.19*, the expression of OsGS1;1 mRNA differed in root of the two cultivars. Its level was initially higher in IR-64 that declined with WD. At 8 days of WD the OsGS1;1 mRNA content was reduced to less than half in IR-64 root and increased to almost twice in Khitish root. Although, WD treatment resulted in initial decline of OsGS1;2 transcript content in roots of both the cultivar, the rate of decline was faster in IR-64, in comparison to Khitish. As a result, OsGS1;2 level fell to a minimum at day 4
and day 8 of WD in IR-64 and Khitish, respectively and then increased on further treatment.

2.3.5.3. Effect of WD on expression of GS1 and GS2 polypeptide in leaf, stem and root of IR-64 and Khitish seedlings

To ascertain whether the WD induced alterations in GS mRNA expression was reflected in corresponding polypeptides, immunoblot analysis of GS isoforms was carried out. The result of the effect of WD on expression of GS polypeptides in leaf tissue is shown in Figure 2.20. As can be seen that GS1 isoforms i.e. OsGS1;1 and OsGS1;2, couldn’t be distinguished in immunoblot and is represented as single band. This is because of the development of GS antibody against conserved region of the GS polypeptide. As shown in Figure 2.20a, GS1 polypeptide accumulated in leaf of both IR-64 and Khitish seedlings with progress of WD.

![Figure 2.20](image)

**Figure 2.20.** Effect of WD on expression of GS polypeptides in leaf of *O. sativa* cv. IR-64 and Khitish (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

Its level increased by more than 2 fold by the end of the treatment. The GS2 polypeptide content of IR-64 leaf was reduced by about 50% at day 12 of WD, however, such change was not noticeable in Khitish leaf (**Figure 2.20b**).
The results in *Figure 2.21* show the effect of WD on expression of GS polypeptides in the stem of rice seedlings. WD significantly affected GS2 polypeptide expression in stem of IR-64 cultivar than that of Khitish cultivar. At 12 days of WD, GS2 polypeptide level rose by about 2 fold in IR-64 stem but remained almost unaltered in Khitish stem.

**Figure 2.21.** Effect of WD on expression of GS polypeptides in stem of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

**Figure 2.22.** Effect of WD on expression of GS polypeptide in root of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptide (b) Bar diagram of GS polypeptide levels.
Similarly the abundance of GS1 polypeptide was enhanced by 1.5 fold in IR-64 and didn’t change significantly in Khitish. The immunoblot analysis of root proteins highlighted a single protein band of molecular size 39 kDa, which corresponds to the cytosolic GS1 isoform. In both IR-64 and Khitish cultivars, initial level of GS1 polypeptide was significantly higher. WD treatment resulted in drastic decline GS1 protein content. The rate of decline was faster in drought sensitive IR-64 as compared to Khitish. As a consequence its level was reached to a minimum on day 4 in IR-64 and on day 8 in Khitish. These declines began to reverse on further WD treatment in both the seedlings (Figure 2.22).
2.4. DISCUSSION

Environmental factors that impose water deficit (WD) stress, such as drought, salinity and temperature extremes, place major limits on plant productivity (Cushman and Bohnert 2000). The specific plant responses to WD are dependent on the amount and rate of water loss, duration of the stress and stages of plant development. Adaptation to WD at biochemical and molecular levels involves the activation or increased expression of several genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways (Xiong et al. 2002; Waseem et al. 2011). During prolonged periods of WD, the decrease in water availability for transport-associated processes leads to changes in the concentrations of many metabolites, followed by disturbances in amino acid and carbohydrate metabolism. Acclimation to WD requires responses that allow essential reactions of primary metabolism to continue and enable the plant to tolerate WD (Foyer et al. 1998). Recent studies have indicated that nitrogen assimilation to be critical to plant acclimation to fluctuating environmental conditions (Swarbrek et al. 2011). However, the effect of WD on nitrogen metabolism remains relatively unexplored.

Rice is an important crop worldwide. It is also considered to be a model plant for monocots because of its relatively small genome size. WD is one of the major constraints depressing rice production (Jonaliza et al. 2004). Rice plants in paddy fields prefer to utilize ammonium as a major nitrogen source. GS serves for assimilation of ammonia to glutamine, which is the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b; Tabuchi et al. 2007). Present research work describes the effect of WD conditions on regulation of activity and expression of GS isoforms in leaf, stem and root of seedlings of two rice cultivars differently tolerant to WD 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 maintenance of RWC, osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops (Bhushan et al. 2007). RWC is considered to be the best integrated measure of plant water status, which represents variations in water potential (WP), turgor potential
(TP), and osmotic potential (OP). The choice of RWC as the best representation of plant water status in terms of genetic variation is also supported by genetic association between RWC and plant production under dehydration (Bhusan et al. 2007). Plants accumulate metabolites under stress conditions, which have been proposed as one of the mechanisms of stress tolerance. Plant cells accumulate some kind of compatible solutes, such as proline, betaine, polyols, polyamines and ions (i.e. potassium) when subjected to abiotic and biotic stress. They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor et al. 1995; Bajji et al. 2000). It is shown to be involved in tolerance mechanisms against oxidative stress, which is the main strategy of plants to avoid detrimental effects of water stress (Vendruscolo et al. 2007). The WD stress on plant also results in inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids in a variety of monocots and dicots (Gilbert et al. 1998). Moreover, the capacity to avoid or repair membrane damage during dehydration processes is also pivotal for the maintenance of membrane integrity, especially for those membranes in which functional proteins are embedded.

In the present study, rice varieties were initially screened for their dehydration tolerance characteristics by measuring RWC, accumulation of compatible solute like proline, and membrane permeability of ions and electrolytes. During WD, the dry wt. g⁻¹ fresh wt. of root and leaf tissue of IR-64 seedling was increased significantly; however, no such change in dry weight was noted in Khitish. The RWC, protein and proline level declined markedly and the electrolyte leakage was increased sharply in IR-64 cultivar in response to WD treatment. Under similar condition, Khitish variety maintained relatively higher RWC, protein and proline level. Proline level kept on increasing continuously throughout the treatment period. Moreover, electrolyte leakage from Khitish leaf increased only marginally during WD. The results thus indicated more susceptibility of IR-64 to WD in comparison to Khitish and hence, were designated as drought- sensitive and tolerant- cultivar, respectively. These two cultivars were used for studying the effect of WD on regulation of GS isoforms.
GS isoforms in rice seedlings was determined by Anion exchange chromatography. GS activity in leaves and stem were resolved into two distinct peaks eluting at 0.15 M and 0.30 M and were designated as GS1 and GS2, respectively. GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their localization in cytosol and chloroplast, respectively. In rice root GS activity was localized only in cytosol. Anion exchange chromatography has commonly been used for separation of isoforms of GS from various plant tissues. This technique has been successfully employed for resolution of isoforms of GS from leaves of rice (Hirel and Gadal 1980), sorghum (Hirel and Gadal 1982), wheat (Tobin et al. 1985), maize (Becker et al. 1993), sunflower (Cabello et al. 1994, Larios et al. 2004), *Pennisetum glaucum* (Ghosh 2004), tobacco (Pageau et al. 2005) and potato (Teixeira et al. 2005); roots of rice, bean, maize (Suzuki et al. 1981), pea and alfalfa (Vezina et al. 1987) and nodules of *Phaseolus vulgaris* (Robert and Wong 1986). As in the present study, leaves of several C3 plants were found to contain most of the GS activity as GS2 (Mc Nally et al. 1983). Majority of studies conducted on roots indicate the presence only cytosolic isoform of GS in the tissue (Suzuki et al. 1981; Mack 1995; Ishiyama et al. 2004; Bernard et al. 2008). In few studies, substantial activity of GS has also been shown to be associated with plastid fraction of the root cells, such as in roots of pea and alfalfa (Vezina et al. 1987). Brugiere et al (1999) reported the occurrence of both GS1 and GS2 in phloem. They showed the major role of GS2 in stem in controlling proline production. In leaves of sorghum (Hirel and Gadal 1982) and soybean (Kang and Hymowitz 1988) GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their cytosolic and chloroplastic localization, respectively. The non-overlapping localization of GS1 and GS2 was further confirmed by subcellular and immunocytochemical studies. Immunolocalization studies in tobacco (Brugiere et al. 1999), pine (Canovas et al. 2007), potato (Pereira et al. 1995), rice (Tabuchi 2005) have shown predominant vascular location of GS1 in different plant organs. A detailed immunolocalization study of mature flag leaf of wheat using anti-GS antibody showed the presence of GS2 label in the plastid of mesophyll parenchyma and in the plastid of parenchyma cells in the perifascicular sheath surrounding the vascular bundles (Bernard et al. 2008).
As in present study, a single chloroplastic GS2 is form has been reported in many higher plants. However, in soybean and alfalfa several GS2 isoforms have been identified (Zozaya-Garza and Sengupta Gopalan 1999). Two cytosolic GS isoforms, OsGS1;1 and OsGS1;2 were present in root, stem and leaf of IR-64 and Khitish seedling. Majority of studies indicated the presence of multiple homologous but distinct genes for cytosolic GS1 (Tingey and Corruzi 1987; Ireland and Lea 1999; Yamaya and Oak 2004; Canovas et al, 2007; Bernard et al. 2008). Earlier it was believed that GSr (OsGS1;2) is the only GS1 isoform expressed in rice root (Sakamoto et al. 1989). However, present investigation indicated the presence of both OsGS1;1 and OsGS1;2 in root of rice seedlings, as reported by Ishiyama et al (2004b). OsGS1;2 was the major cytosolic GS in root and stem, whereas OsGS1;1 was the major isoform of GS1 in leaf. Previous study (Ishiyama et al. 2004a,b) also showed approximately 2.5-fold greater abundance of OsGS1;2 as compared to OsGS1;1 in root. A varietal variation in expression of OsGS1;1 was noted in all the organs tested. At the beginning of WD treatment OsGS1;1 transcript level was significantly higher in IR-64 in comparison to Khitish. Genetic linkage studies using segregating mapping populations have implicated cytosolic GS genes with grain production in maize (Hirel et al. 2007; Fontaine et al. 2009), rice (Obara et al. 2004) and wheat (Habash et al. 2007). In rice crop significant correlations were obtained between grain number/size and the locus for OsGS1;1 protein content (Obara et al. 2004). A higher level of OsGS1;1 in all the organs of IR-64 indicates that the cultivar can perform better under proper growth conditions.

To evaluate the effect of WD on GS isoform, 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. The detailed view of regulation of GS isoforms in leaf of IR-64 and Khitish seedlings in response to WD has been depicted in Figure 2.23. Total GS activity declined significantly in IR-64 and didn’t 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. As in the IR-64 seedlings, total GS activity declined to less than a quarter of its initial level during the natural senescence of rice leaves and this decline was mainly caused by a decrease in the GS2 level (Kamachi et al. 1991). Similarly, other studies have also shown the susceptibility of chloroplastic GS2 to other abiotic and biotic stresses as well as dark (Larios et al. 2004; Ghosh 2004; Santos et al. 2004; Pageau et al. 2005).

An important physiological function of GS2 is reassimilation of NH$_4^+$ produced during photorespiration (Wallsgrove et al. 1979). Photorespiration is a metabolic pathway in which CO$_2$ is released by light and is linked to Calvin-Benson cycle through the oxygenase activity of ribulose- bisphosphate carboxylase (Rubisco). Although photorespiration includes many metabolic steps which are performed across chloroplast, mitochondria and peroxisomes, several studies suggest that rate limiting step is the GS2 catalyzed reassimilation of ammonia (Hauser et al. 1994; Hosida et al. 2000). The photorespiration activity has been reported to be induced by abiotic stress and plays a protective role (Hosida et al. 2000). The overexpression of GS2 in leaf of transgenic rice increased their photorespiration capacity and improved their salt tolerance. The transgenic rice 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 two weeks indicating the physiological importance of GS2 in abiotic stress tolerance (Hosida et al. 2000). Hence, in the present study a relatively unaltered GS2 expression in Khitish leaf could maintain the photorespiratory capacity of the plant at limited water availability that improves tolerance of the cultivar to WD.

WD treatment increased the expression of both OsGS1;1 and OsGS1;2 transcripts in leaf of IR-64 and Khitish cultivars. The time course of increase in GS1 transcripts corresponded with the accumulation of GS1 protein detected on Western blot. Although, the response of individual cytosolic GS genes to abiotic stress has not been studied earlier, the total GS1 transcript and polypeptide level have already been shown to accumulate in leaf during natural senescence and in response to biotic and abiotic stress (Kamachi et al. 2004).
Figure 2.23. GS activity and expression in leaves of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (a) Relative change in total GS activity of IR-64 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings, (c) Analysis of OsGS1:1, OsGS1:2 and OsGS2 transcripts by RT-PCR, (d) Bar diagram for GS mRNA level, (e) Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamyldihydroxamate produced 30 min⁻¹.
In natural senescing potato plant GS1 transcript accumulation was coupled to increased synthesis of corresponding polypeptide (Teixeira et al. 2005). However, during natural senescence of rice leaves translatable GS1 mRNA level increased by four folds without affecting the corresponding polypeptide content (Kamachi et al. 1991). Similarly, the GS1 protein quantity has also been shown to increase in *Nicotiana tabacum* leaf during *Potyvirus* infection (Pageau et al. 2005). In the present study, WD mediated increase in GS1 transcript and protein expression in rice leaf didn’t correspond with GS1 activity. A similar type of response to GS1 mRNA overexpression was observed in *Arabidopsis* root. In this tissue the nitrogen nutrition mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004a). A lack of correlation between constitutive overexpression of GS1 mRNA and abundance of corresponding polypeptide and activity has been reported earlier in leaf of Alfalfa (Ortega et al. 2001). WD treatment caused significantly greater OsGS1;1 accumulation in leaf of IR-64 as compared to that of Khitish. The increased OsGS1;1 in IR-64 leaf could be due to its higher rate of protein degradation (*Figure 2.12*), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown. The contention is supported by previous studies showing the localization of GS1 protein in companion cells and vascular parenchyma cells in senescing leaf blade of rice (Kamachi et al. 1992) and wheat (Kichey et al. 2005) plants. The research work by Tabuchi et al (2005) with OsGS1;1 knockout mutant also showed the importance of OsGS1;1 in remobilization and reutilization of nitrogen in rice plant. Knockout mutants created by the insertion of Tos17 into the exon of OsGS1;1 were screened and characterized. Homozygously inserted mutants exhibited a severe retardation in growth rate and grain filling when grown under normal nitrogen fertilizer concentrations. Reintroduction of OsGS1;1 cDNA under the control of its own promoter into the mutant successfully complemented the slow growth phenotype.

In contrast to leaf, WD treatment reduced GS1 activity and OsGS1;1 transcript level in stem and root of IR-64 seedlings (*Figure 2.24 and 2.25*). 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. 2005).
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 IR-64 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; Ishiyama et al. 2004b; 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. 2004; 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 (Oliveira and Coruzzi 1999; Tabuchi et al. 2007). Oliveira and Coruzzi (1999) reported the association of metabolic regulation of GS1 with relative abundance of carbon skeleton verses amino acids accumulated in the root tissue. Their results suggested a negative feedback regulation of GS1 by glutamine or the downstream nitrogen metabolites.

A comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root was carried out by Ishiyama et al. (2004b). The Vmax values were approximately 2-fold higher with OsGS1;1 than with OsGS1;2. In addition, OsGS1;1 exhibited extremely high substrate affinity for ammonium, as indicated from its Km value: the Km for ammonium was 2.7-fold lower in OsGS1;1 than in OsGS1;2. 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. 2004b). Similarly, in Arabidopsis root, GLN1;1 exhibited an extremely high affinity for ammonium (Km < 10 µM) as compared to GLN1;2 (Km = 2450 µM) (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). In the present investigations WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. Hence, OsGS1;1 seems to play significant role in performance of plant under stress condition.
Figure 2.24. 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 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings, (c) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR, (d) Bar diagram for GS mRNA level, (e) Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min."
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. 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. At 13 days of WD, the ratio of stem protein concentration of WD to control plant increased from 0.8 to 1.3 (Pinheiro et al. 2001). Translation of some proteins has also been found to increase in stem in other type of stress conditions as well. Recently, Moller et al (2011) in their proteomic based study indicated an increase in synthesis of some proteins of chloroplastic transcription and translation machinery under N-starvation condition. The condition is quite similar to that encountered by rice plant under WD leading to limited uptake of nutrient from soil. Thus, a different metabolic status may contribute to the maintenance of GS protein and activity in stem during WD.

WD treatment had no differential effect on expression of OsGS1;2. The transcript level of OsGS1;2 was found to enhance progressively and almost equally in leaf and stem of IR-64 and Khitish seedlings. However, in roots of both the cultivars the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. The rate of decline was faster in IR-64 root in comparison to Khitish root. The time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance in all the three tissues. The result is supported by study of Ishiyama and co-worker (2004a) on effect of ammonium nutrition in *Arabidopsis* root GS isoforms. Arabidopsis root contained 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. Furthermore, GS1;2 has been reported to overexpress in leaves and roots by ammonium supply. GS1;2 knockout mutants of *Arabidopsis* displayed lower GS activity, higher ammonium concentration, and reduced rosette biomass compared with the wild type (WT) under ample nitrogen supply only.
Figure 2.25. GS activity and expression in root of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment; (a) Relative change in total GS activity of IR-64 and Khitish, (b) Analysis of OsGS1;1 and OsGS1;2 OsGS2 transcripts by RT-PCR, (c) Bar diagram for GS mRNA level, (d) Western blot analysis of GS1 and GS2 polypeptide and (e) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min⁻¹.*
However, it showed no significant difference from the wild type under nitrogen limiting conditions (Lothier et al. 2010). A similar nitrogen limiting condition prevailed during WD. IR-64 seedlings being more susceptible to WD showed faster decline in OsGS1;2 mRNA and protein in root with minimum expression levels at day 4 of treatment due to limited uptake of nitrogen from soil. The enhancement in OsGS1;2 expression on further intensification of WD might be related to increased accumulation of ammonium ion due to observed increased protein degradation.

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 IR-64 and drought tolerant Khitish cultivars of rice. GS2 was the major GS isoform in leaf. GS2 expression in leaves decreased in IR-64 seedlings and remained almost unaltered in Khitish seedlings in response to WD. The maintenance of GS2 expression in leaf might be associated with the maintenance of photosynthetic and phorespiratory capacity of the Khitish and hence, with WD tolerance characteristics of the cultivar. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen during senescence and other stress conditions characterized by high rate of protein degradation. A higher substrate affinity of the enzyme for ammonium signifies its promoting 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 in might contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.
2.5. BIBLIOGRAPHY


Birnboim HC and Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7:1513-1523


McNally SF, Hirel B, Gadal P, Mann AF and Stewart GR (1983) Evidence for a specific isoform content related to their possible physiological role and their compartmentation within the leaf. Plant physiology 72: 22-25


Sakamoto A, Qgawa M, Masumura T, Shibata D, Takeba G, Tanaka K and Fuji S
Three cDNA sequences coding for glutamine synthetase polypeptides in *Oryza sativa* L. Plant Molecular Biology 13:611-614


