



FR 1638

Final Report

of

Research Project No: RG/2007/BT/04

**Funded by the
National Science Foundation of Sri Lanka**

**Identification of Drought Resistant Varieties in
Rice (*Oryza sativa* L.) using Physiological and
Molecular Methods**

By

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(Principal Investigator)

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**Faculty of Agriculture
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December 2012

Section 1:**Information regarding Project/Project Personnel:**

i. Contract Number: RG/2007/BT/04

ii. Title of the Project:

Screening of candidate genes for drought tolerance in rice and quantification of gene expression

iii. Principal Investigator:

Prof. W.A.J.M. De Costa,
Department of Crop Science, Faculty of Agriculture, University of Peradeniya

iv. Co-Investigators:

Dr. Devika M. De Costa,
Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya

v. Institute(s) where research was being carried out:

Department of Crop Science, Faculty of Agriculture, University of Peradeniya

vi. Date of award: 01 April 2007

vii. Date of completion of project: December 2012

viii. Total allocation of funds: Although Rs. 2,892,392.00 was allocated only Rs. 1,630,860.66 was transferred to the University of Peradeniya

ix. Total spent: Rs. 1,621,634.18

x. Number of research students employed: Two (02).

From 01 November 2008 to 30 September 2010: Mr. H.R. Suranga

From 01 April 2011 to 31 May 2012: Ms. M.S.K. Amarakoon

xi. Postgraduate degrees completed with dates:

Mr. H.R. Suranga is writing his M.Phil. thesis. This has been delayed because he took up permanent employment as a Government School Teacher in October, 2011. However, he is in contact with the Principal Investigator and is working on the thesis.

Ms. M.S.K. Amarakoon joined the project in April, 2011. She was in the middle of the M.Phil. research work when the project was terminated in June 2012, despite a request by the Principal Investigator for an extension to the project to complete Ms. Amarakoon's research work, which had shown excellent progress as she had been successful in constructing a cDNA library from the drought tolerant rice variety Bg358. Despite the termination of the project by the NSF, Ms. Amarakoon is continuing her research work and her postgraduate degree on a part-time basis.

xii. Number of Technical Assistants and/or labourers employed and period of service:

None.

xiii. Publications/Communications arising from the project during the reporting period:

None. However, publications from the completed research work will be done in the future.

Section 2:

Executive Summary of the Project

Shortage of water (i.e. drought) is one of the principal constraints to increase rice production in Sri Lanka, especially in view of the increased frequency of drought due to long-term climate change. Therefore, identification of drought-resistant rice varieties from Sri Lankan rice germplasm and elucidation of candidate genes that are responsible for drought tolerance is extremely important. The present project consisted of two major activities: (1) Screening of a selected set of Sri Lankan rice varieties for drought tolerance during the vegetative and reproductive stages and identification of a drought tolerant variety to be used in molecular analysis of drought tolerance; (2) Identification of candidate genes that are responsible for drought tolerance of the selected drought tolerant Sri Lankan rice variety. Activity 1 was completed successfully. After overcoming considerable difficulties and delays initially, Activity 2 was progressing successfully when the project was terminated despite a request for extension by the investigators.

Screening of rice germplasm for drought tolerance in the vegetative and reproductive stages (i.e. Activity 1) was carried out in a rain-sheltered plant house using 30 selected rice varieties, which also included germplasm from International Rice Research Institute's Drought Screening Nurseries. Based on relative vegetative growth under induced drought at the vegetative stage (i.e. for 10 days before panicle initiation), varieties Bg300, DSN43, Bg358 and A14 were identified as drought tolerant at the vegetative stage. Based on relative yield under induced drought during the reproductive stage (i.e. for 10 days from panicle initiation), varieties Bg301, Bw302, A46, Bg358 and H10 were identified as drought tolerant at the reproductive stage. As Bg358 showed tolerance at both stages, it was selected for molecular analysis of drought tolerance to detect candidate genes.

For molecular analysis, shoot tissues of drought-stressed Bg358 were obtained from plants grown in the rain-shelter. In order to detect the expressed genes in response to drought stress, total RNA and mRNA were extracted. A cDNA library containing the expressed genes in Bg358 under drought stress was constructed from mRNA. At this point, the project had to be terminated on the directive of the NSF.

However, the investigators continued the project until November 2012 and carried out screening of the cDNA library.

During this period, the previously prepared cDNA library of the rice variety Bg 358, was further screened for drought responsive genes. The cDNA library was subjected to differential hybridization using cDNA probes prepared from drought stressed and unstressed rice leaves. Differential hybridization of 192 cDNA clones identified six up-regulated and 18 down-regulated genes, respectively due to drought stress. Out of the 24 cDNA clones identified by differential hybridization, six up-regulated and five down-regulated cDNA clones were subjected to DNA sequencing. Subsequent DNA/protein homology search identified ten putative gene products namely ubiquitin conjugating

enzyme E2, hypothetical protein, phosphoprotein phosphatase, stress-associated protein 8 and putative heat shock protein 82 as up regulated genes due to drought stress and serine/threonine protein kinase, putative transaldolase, MRG family protein and protein phosphatase 1 as down regulated genes due to drought stress in rice leaves of Bg 358.

These genes could be categorized in to the functional groups of growth and development (10% of the genes), protection and repair (10% of the genes), energy and metabolism (10% of the genes), protein synthesis, folding and stabilizing (60% of the genes) and hypothetical proteins (10% of the genes).

Section 3:

Report in detail

This part of the report has been prepared in two separate sections:

Section 3.I:

This section describes the achievements in Activity 1 of the project. This involved screening of a selected set of Sri Lankan rice varieties for drought tolerance during the vegetative and reproductive stages and identification of a drought tolerant variety to be used in molecular analysis of drought tolerance;

Introduction – Background, Scope and Objectives

Shortage of water (i.e. drought) is one of the principal constraints to increase rice production in Sri Lanka. Statistics during the last decade (1995-2004) show that the sown extent of rice in yala has been only 48-67% of that in maha. This is primarily because of water scarcity. A further analysis of sown extents shows that reductions in sown extent in yala occurs not only under rainfed conditions (where the reductions were greatest), but also in major and minor irrigation schemes. During the same period, the total rice production in yala was only 51-67% of that in maha. These statistics show that there is considerable scope for increasing rice production in Sri Lanka by increasing the sown extent in yala. This can be achieved by developing relatively drought-resistant rice varieties which are able to produce an economically-viable yield using a limited amount of water.

Most of the drought stress research in rice has been carried out on upland rice varieties (Mackill et al., 1996), which differ significantly from lowland varieties in terms of their growth, physiology and response to drought. Therefore, there is an urgent need to identify traits and mechanisms conferring drought resistance to lowland rice varieties, which are cultivated in Sri Lanka.

Mechanisms of drought resistance can be grouped in to three categories (De Costa, 2001): (a) *drought escape* involves completion of the crop's life cycle before drought begins to affect the physiological processes adversely; (b) *drought avoidance* involves maintaining a high internal water status even when there is a water shortage in the external environment; (c) *drought tolerance* involves the ability to maintain internal physiological processes at relatively higher levels (i.e. with minimum reductions) in the face of water deficits in the internal plant tissues.

Rice breeders in Sri Lanka have developed short-duration (i.e. 3-month and 2 ½ -month) rice varieties to be grown in drought-prone rainfed lowlands. This is an example of utilizing mechanisms of drought escape. However, the statistics presented earlier (Figs. 1 and 2) show that work has to be done to improve mechanisms of drought avoidance and tolerance as well in Sri Lankan rice varieties because drought escape alone will not

substantially improve our rice production any further. Very little, if any, work has been done in Sri Lanka on these aspects.

Therefore, the objectives of Activity 1 were:

1. To identify, from the Sri Lankan rice germplasm, rice varieties that are resistant to drought at the vegetative stage;
2. To identify, from the Sri Lankan rice germplasm, rice varieties that are resistant to drought at the reproductive stage

Materials and Methods

Location

The experiment conducted in a rain-sheltered, ventilated planthouse at the Agricultural Biotechnology Centre, University of Peradeniya, Peradeniya, Sri Lanka (Latitude 7° 15' 47N, Longitude 80° 36' 10E, Altitude 496 m above sea level).

Varieties used for screening

The following 30 selected rice varieties were screened for drought tolerance at the vegetative and reproductive stages:

Old Improved Varieties:

H 7, H 10,

New Improved Varieties:

At 16, At 303, At 353, At 354

Bg 305, Bg 352, Bg 358, Bg 94-1, Bg 250, Bg 357, Bg 305, Bg 750, Bg 300, Bg 350, Bg 300, Bg 301, Bg 360, Bg 34-6, Bg 276-5

BW 2726b, BW 302

LD 355

62-355

Breeding lines from International Rice Research Institute's Drought Screening Nurseries:

DSN 8, DSN 43

Aeron-51, Aeron 46, Aeron 14

Screening for drought tolerance at the vegetative stage was carried out in soil columns (90 cm high with a 6 cm diameter) (Plate 1). The soil columns were used in order to allow unrestricted root growth during the vegetative stage. Screening for drought tolerance at the reproductive stage was done in 10 L plastic pots.

Plants were established from seed and maintained with standard recommended fertilizer application and crop protection measures. All plants were maintained under well-watered conditions until subjected to drought stress.

Experimental treatments***Drought stress at the vegetative stage***

Drought stress was imposed at the vegetative stage by withholding water from 24 days after sowing onwards until the plants showed wilting symptoms.

Drought stress at the reproductive stage

Screening of the 30 varieties for drought tolerance at the reproductive stage was carried out in two rounds of 15 varieties each. Drought stress was imposed at the reproductive stage by withholding water at panicle initiation until the plants showed wilting symptoms.

Upon observation of wilting symptoms, drought stress at both stages was relieved by re-watering.

Experimental design

At both stages, sets of control plants were maintained under well-watered conditions. Each treatment was replicated four times in a randomized complete block design.

Measurements

At the end of the drought period at the vegetative stage, total dry weight of drought stressed and well-watered plants were measured by destructive sampling.

In the plants that were subjected to drought stress at the reproductive stage, grain yield was measured at crop maturity.



Plate 1: Varietal screening of rice for drought tolerance in the vegetative stage

Quantification of drought tolerance

The degree of drought tolerance of a given variety at the vegetative stage was quantified as the ratio between total plant dry weight under drought and that under well-watered conditions.

The degree of drought tolerance at the reproductive stage was quantified as the ratio between the final yield in the drought stressed treatment and that in the well-watered control.

Data analysis

Varietal variation in the degree of drought tolerance was determined by Analysis of Variance (ANOVA). Linear correlation analysis was used to determine the significance of the inter-relationships between measured variables.

Results and Discussion

Final grain yield of Round 1 of varietal screening for drought tolerance at reproductive stage

Final grain yield of Round 1 of varietal screening is given in Fig. 1.

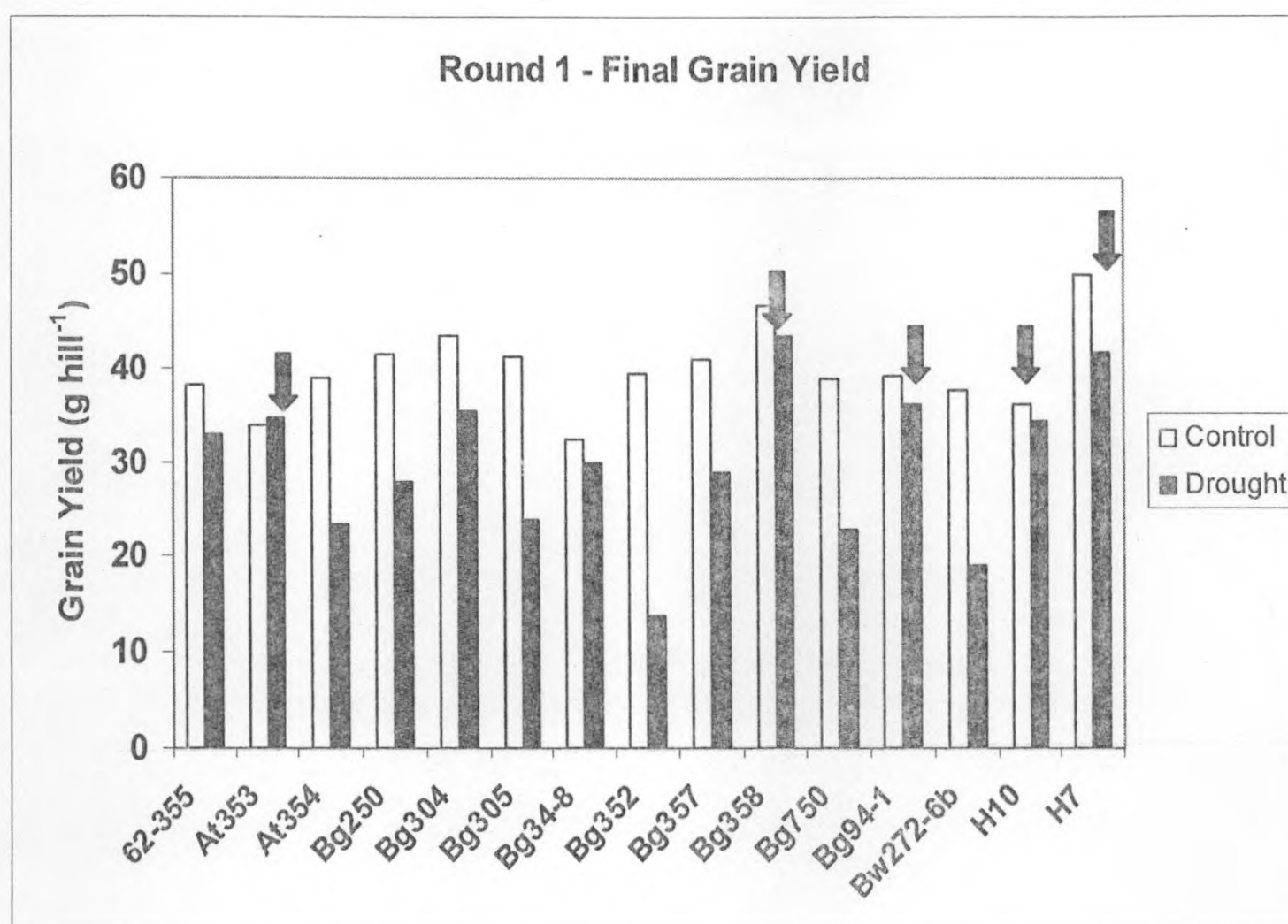


Figure 1: Final grain yield of Round 1 of varietal screening for drought tolerance at reproductive stage. Drought was imposed as three drying cycles during the reproductive stage. The control treatment was kept well-watered throughout. Identified drought tolerant varieties are shown by arrows.

Bg358 showed the highest grain yield under drought at the reproductive stage. It also showed a minimum yield reduction relative to the well-watered control. H7 showed the next highest yield under drought. Therefore, these two varieties are identified as drought tolerant at the reproductive stage, based on their higher absolute yields under drought. In addition, varieties At353, Bg94-1 and H10 are also identified as drought tolerant based on their combination of minimum yield reductions due to drought and comparatively higher absolute yields under drought.

Final grain yield of Round 2 of varietal screening for drought tolerance at reproductive stage

Final grain yield of Round 1 of varietal screening is given in Fig. 2.

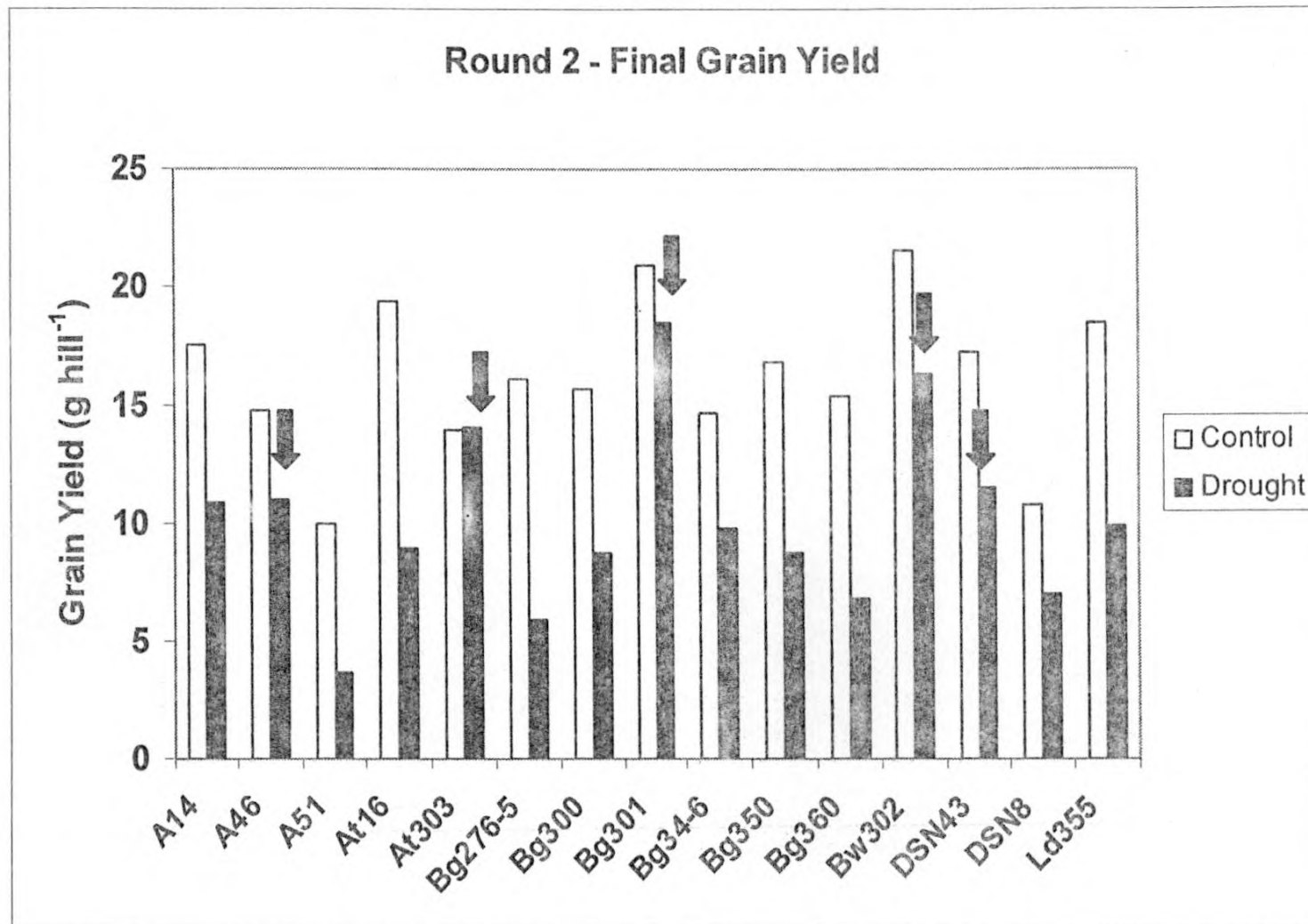


Figure 2: Final grain yield of Round 2 of varietal screening for drought tolerance at reproductive stage. Drought was imposed as three drying cycles during the reproductive stage. The control treatment was kept well-watered throughout. Identified drought tolerant varieties are shown by arrows.

Bg301 showed the highest grain yield under drought at the reproductive stage. Bw302 showed the next highest yield under drought. Therefore, these two varieties along with DSN43 and A46 are identified as drought tolerant at the reproductive stage, based on their comparatively higher absolute yields under drought. In addition, At303 is also identified as drought tolerant based on its combination of minimum yield reduction due to drought and comparatively higher absolute yield under drought.

Relative yield – Both rounds combined

Relative yield of a given variety was calculated as the ratio between final grain yield under drought and the corresponding yield in the well-watered control. This allows combination of yield data of the two rounds of varietal screening.

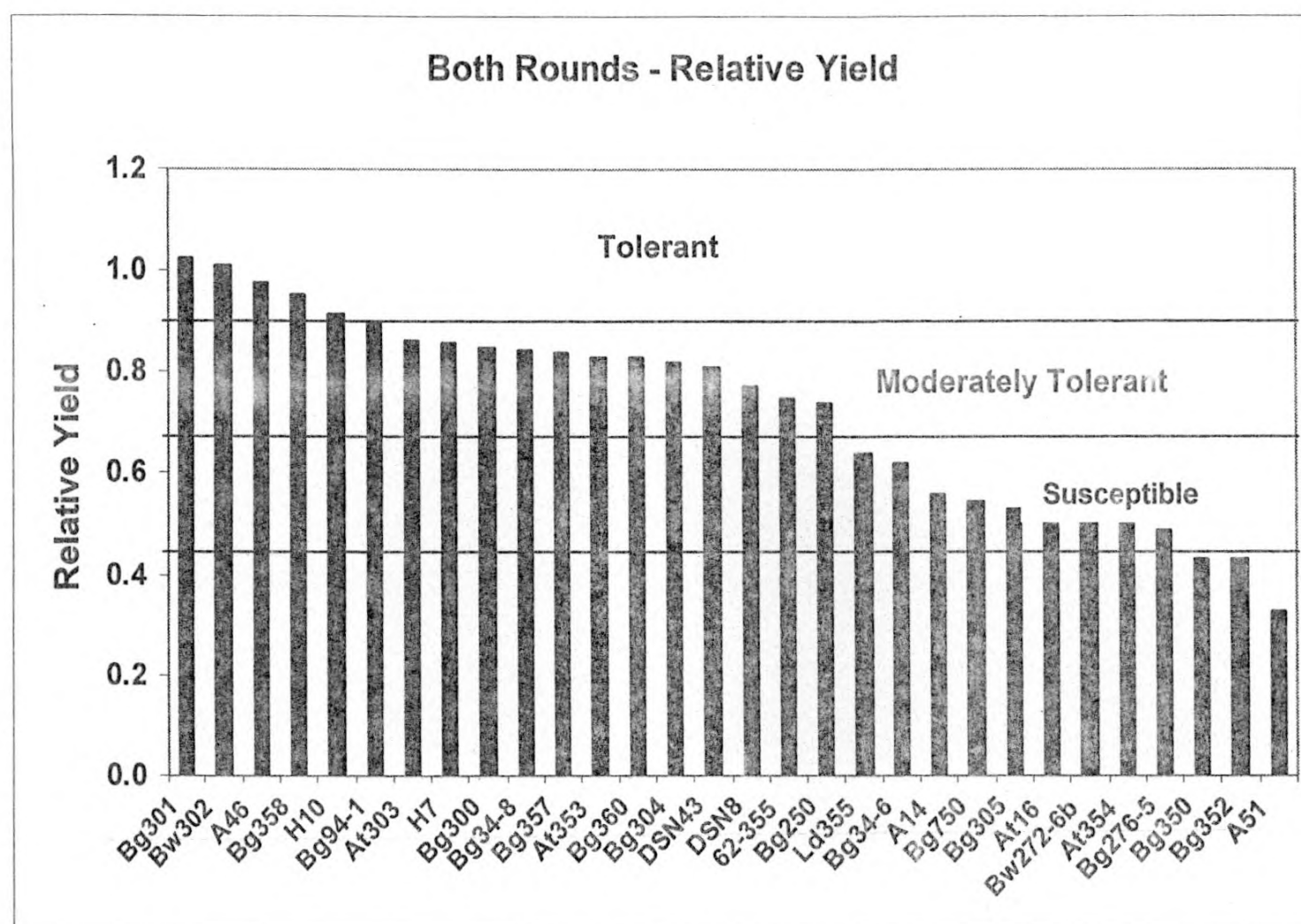


Figure 3: Variation of relative yield of the 30 rice varieties screened in two rounds for drought tolerance at the reproductive stage. The three varieties below the lowest line are categorized as 'highly susceptible'.

Based on relative yield, the 30 varieties were categorized into four groups (Fig. 3) based on their drought tolerance at the reproductive stage. There was a highly significant ($p < 0.0001$) positive correlation ($r = 0.871$) between grain yield under drought stress at the reproductive stage and grain yield under non-stressed conditions (Fig. 4).

Relative growth at the end of the vegetative stage

In order to identify drought tolerance at the vegetative stage, relative growth was calculated as the ratio between total dry weight under drought stress at the vegetative stage and the corresponding total dry weight in the well-watered control.

Based on relative total dry weight, the 30 varieties were categorized into four groups (Fig. 5) based on their drought tolerance at the vegetative stage.

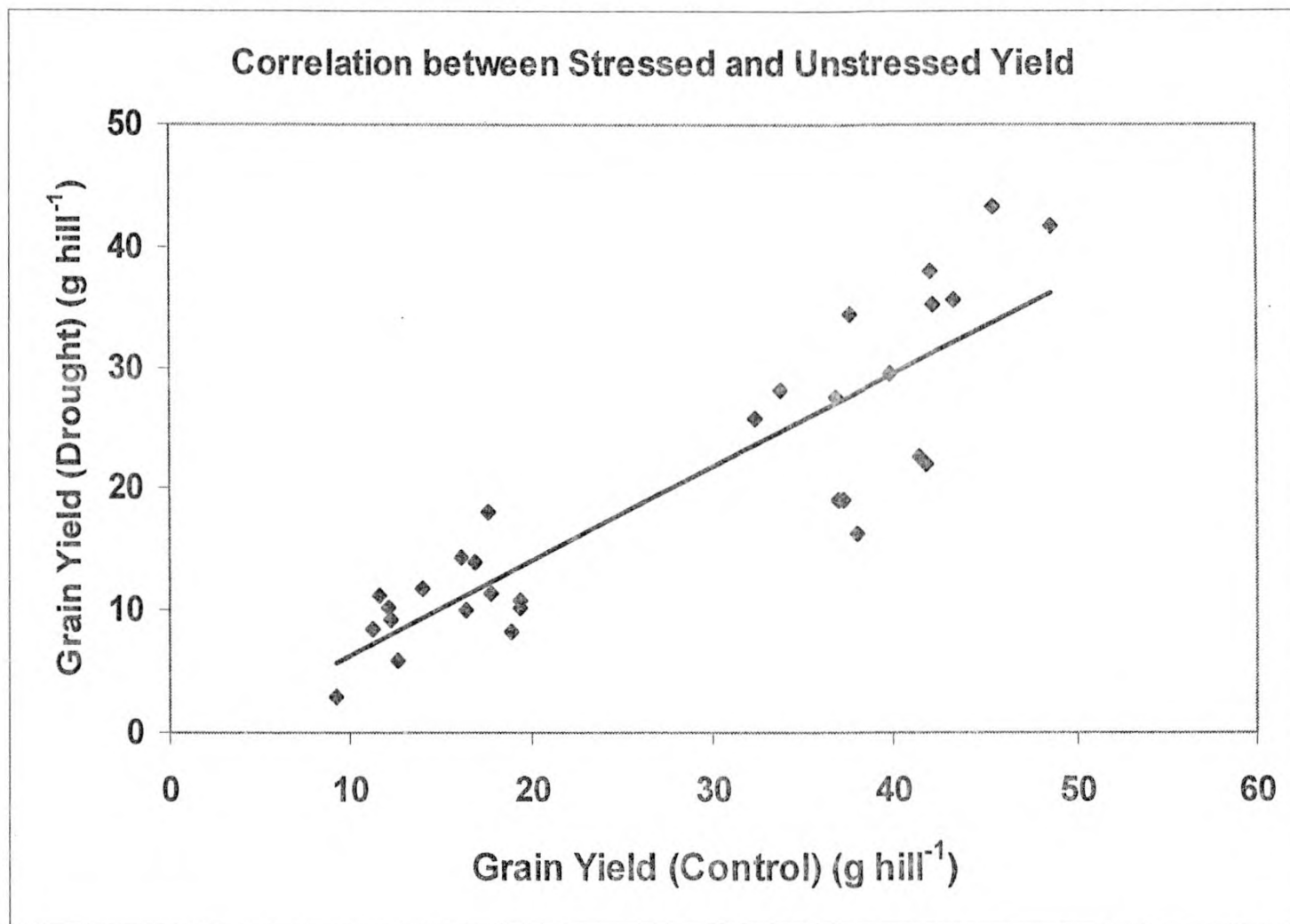


Figure 4: Correlation between grain yield under drought stress at the reproductive stage and yield in the well-watered control of the 30 rice varieties screened in two rounds.

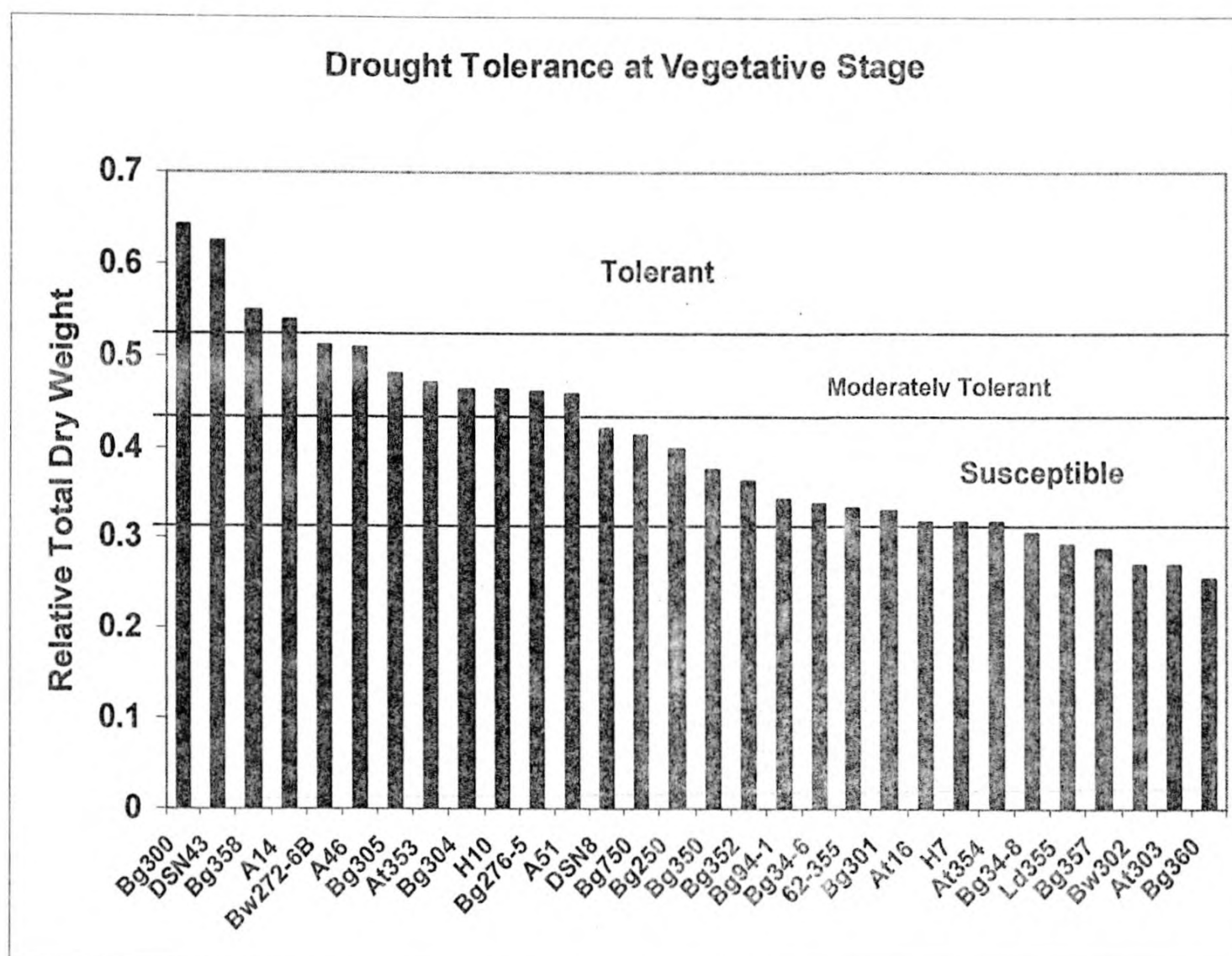


Figure 5: Variation of relative total dry weight of the 30 rice varieties screened in two rounds for drought tolerance at the vegetative stage. The six varieties below the lowest line are categorized as 'highly susceptible'.

Classification of varieties based on drought tolerance or susceptibility at both stages

Varieties identified as drought tolerant are given in Table 1.

Table 1: Varieties identified as drought tolerant at atleast one stage of their life cycle.

Variety	Tolerance to Reproductive Stage Stress	Tolerance to Vegetative Stage Stress
Bg358	Tolerant	Tolerant
A46	Tolerant	Moderately Tolerant
H10	Tolerant	Moderately Tolerant
Bg300	Moderately Tolerant	Tolerant
DSN43	Moderately Tolerant	Tolerant
Bg304	Moderately Tolerant	Moderately Tolerant
At353	Moderately Tolerant	Moderately Tolerant
Bg301	Tolerant	Susceptible
Bg94-1	Tolerant	Susceptible
Bw302	Tolerant	Highly Susceptible
A14	Susceptible	Tolerant

Out of the 30 varieties screened, there was only one variety (i.e. Bg358), which showed drought tolerance at both vegetative and reproductive stages. In addition, A46 and H10 showed a combination of tolerance at the reproductive stage and moderate tolerance at the vegetative stage. On the other hand, Bg300 and DSN43 showed moderate tolerance at the reproductive stage, but tolerance at the vegetative stage. There were four varieties (i.e. Bg301, Bg94-1, Bw302 and A14) which showed tolerance at one stage while showing susceptibility at the other stage.

Overall, there was no significant correlation between drought tolerance at the reproductive stage and that at the vegetative stage (Fig. 6).

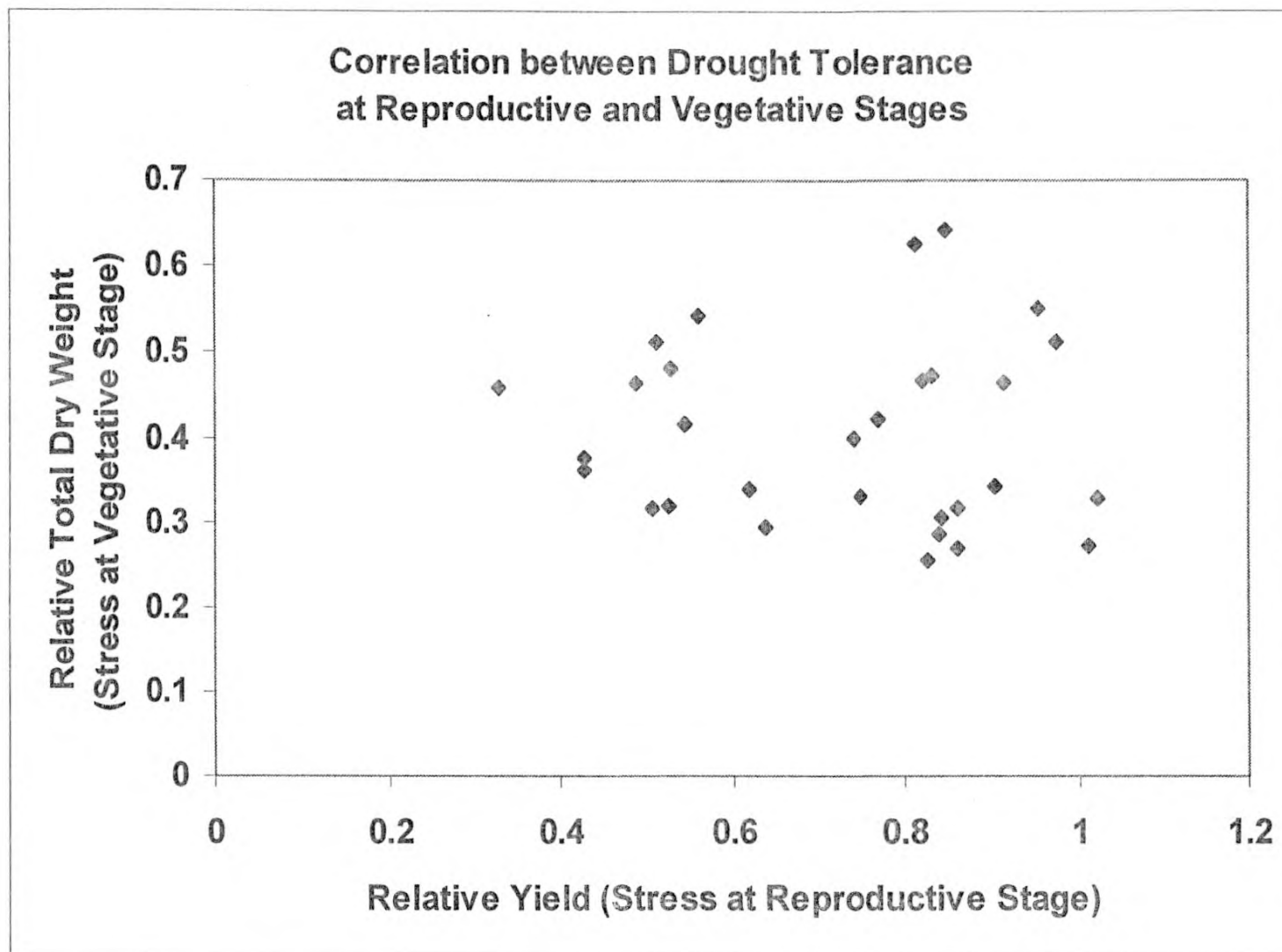


Figure 6: Comparative variation of relative yield at harvest under drought at the reproductive stage and relative total dry weight at the end of the vegetative stage under drought at the vegetative stage in the 30 varieties screened.

Section 3.II:

This section describes the achievements in Activity 2 of the project. This involved identification of candidate genes that are responsible for drought tolerance of the selected drought tolerant Sri Lankan rice variety.

Introduction – Background, Scope and Objectives

Drought *tolerance* (see above for definition) requires three principal functions at the cellular level: (a) protection of membranes and organelles from dehydration; (b) repair of damaged membranes and organelles; (c) detoxification of toxic compounds that are produced during desiccation. Several genes that encode proteins involved in the above functions have been identified (Ingram and Bartels, 1996; Datta, 2002; Yamaguchi-Shinozaki et al., 2002). The present research programme will focus on the following groups of genes.

Genes encoding enzymes of sugar metabolism

Sugars perform two important functions in dehydrated tissue. They function as compatible solutes under water stress by accumulating to high concentrations. This decreases cellular osmotic potential and enables the dehydrated cells to maintain turgor even at low total water potentials (i.e. *Osmotic adjustment*, OA). In addition to OA, sugars protect cellular membranes and organelles in dehydrated plant tissue via different mechanisms. For example, presence of certain sugars initiates the formation of a supersaturated liquid in the cytoplasm during drought. This is called ‘glass formation’ because the supersaturated liquid has properties of a solid. Therefore, during dehydration, it is able to fill the cellular space and thereby prevent cellular collapse (Koster, 1991). Disaccharide sugars such as sucrose, maltose and trehalose stabilize the activity of enzymes (e.g. phosphofructokinase) during dehydration. Garg et al. (2002) showed that increased production of trehalose in *indica* rice, by incorporating two trehalose-biosynthetic genes, increased its tolerance to drought, salinity and low-temperature stress.

Genes responsible for solutes that contribute to osmotic adjustment

In addition to sugars, several other solutes (osmolytes) also contribute to OA, which in turn, confers drought tolerance, in rice (Lilley and Ludlow, 1996; Nguyen et al., 1997). These osmolytes are molecules with low molecular mass and include quaternary amines, amino acids and sugar alcohols. These also can play a protective role by stabilizing membranes and other macromolecular structures (e.g. proteins) during water stress. Therefore, they are known as osmoprotectants. A summary of known candidate genes conferring drought tolerance by various mechanisms, including the synthesis of osmoprotectants, are given Table 2.

Table 2. Known candidate genes conferring drought tolerance by different mechanisms.

Gene	Gene action (Protein produced)	Functional expression (Effect)
<i>codA</i>	Glycine betaine synthesis (Choline oxidase)	Increased tolerance to drought and salinity
<i>P5cs</i>	Proline synthesis (Pyrroline carboxylate synthetase)	Increased biomass production under drought and salinity
<i>IMT1</i>	Myo-inositol synthesis (Myo-inositol O-methyl transferase)	Increased tolerance to drought and salinity
<i>otsA, otsB</i>	Trehalose synthesis (Trehalose-6-phosphate synthase)	Increased dry weight and photosynthetic activity under drought
<i>HVA1</i>	Synthesis of late embryogenesis abundant (LEA) proteins	Higher growth rates under stress, delayed stress-related damage, improved recovery from stress, increased water use efficiency
<i>Adc</i>	Synthesis of arginine decarboxylase	Reduced chlorophyll loss under drought
<i>OsCDPK7</i>	Transcription factor	Increased tolerance to drought and salinity
<i>DREB</i>	Transcription factor	Increased tolerance to drought and salinity
<i>Sod</i>	Super oxide dismutase synthesis	Detoxification of reactive oxygen species and reduction of cellular damage under drought

Sources: Datta (2002); Yamaguchi-Shinozaki et al. (2002); Ingram and Bartels (1996).

Therefore, the objective of this project activity was to detect and identify the genes that are responsible for conferring the drought tolerance of the identified Sri Lankan rice variety Bg358. The approach that was used was to examine the gene expression profiles in response to drought stress in Bg358.

Materials and Methods

Plant culture

A dapog nursery of paddy variety Bg 358 was maintained for two weeks and then the seedlings they were transplanted in 40 pots containing paddy soil at 5 plants per pot for a total of 200 plants. The plants were grown in a rain-sheltered, ventilated planthouse at the Agricultural Biotechnology Centre, University of Peradeniya, Peradeniya, Sri Lanka (Latitude 7° 15' 47N, Longitude 80° 36' 10E, Altitude 496 m above sea level).

Plant were maintained without any stress until three weeks after transplanting. Thereafter, half of the batch was kept without irrigation while maintaining the rest as control (Plate 2).

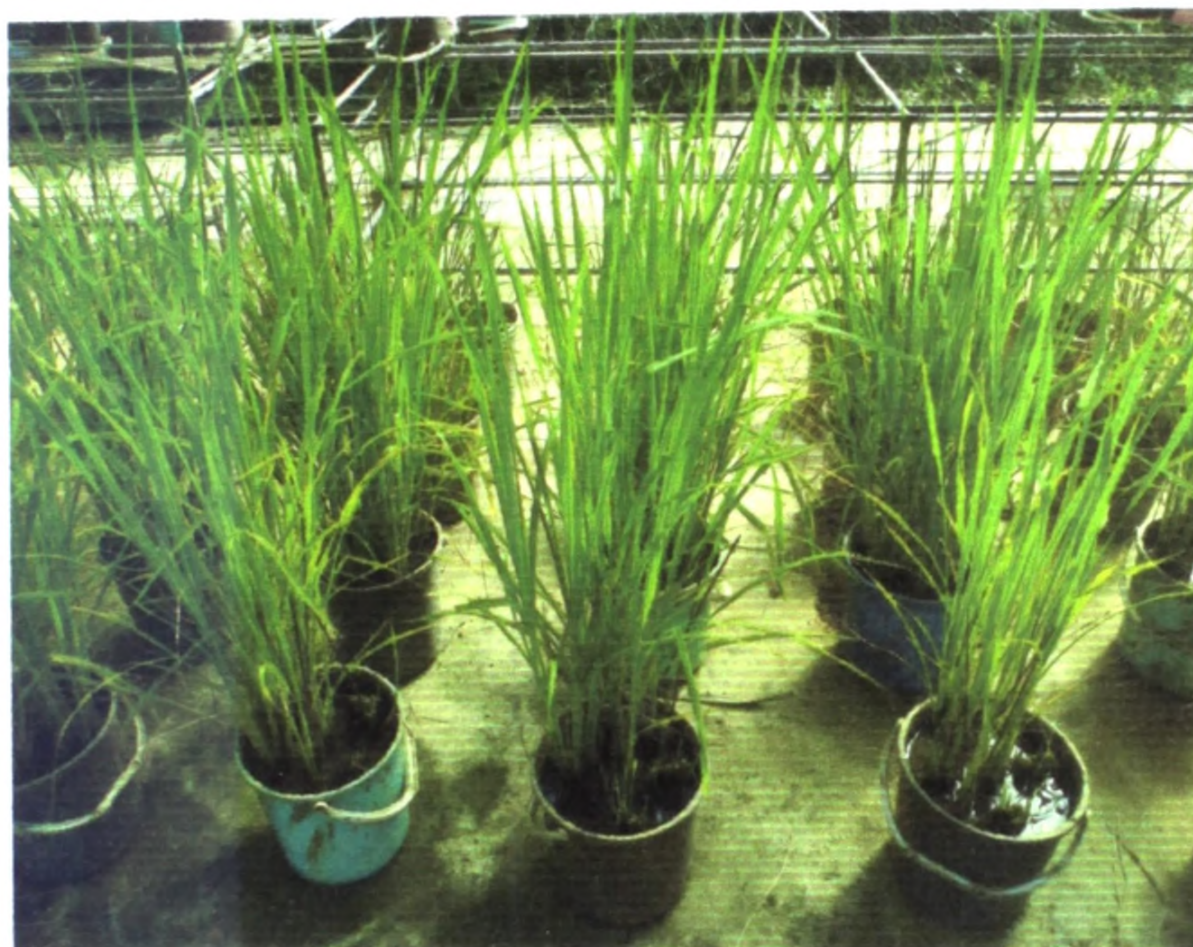


Plate 2: Arrangement of the pot experiment in the rainshelter

Then the sampling was started after one week from stopping the water supply (i.e. imposing the drought). Harvesting was done on every other day for a period of one week. Harvested samples were immediately frozen in liquid nitrogen (N_2). Tissues were ground with liquid N_2 and dispensed into 1.5 ml microcentrifuge tubes and kept at $-80^{\circ}C$ for further studies. At every harvesting, soil samples also were taken to measure the soil moisture content. During the whole period, the maximum minimum temperatures and the relative humidity of the plant house were monitored.

Preparation of chemicals for RNA extraction

All the required glassware were treated with DEPC prior to prepare the chemicals. Then the following chemicals were prepared. 1M Tris HCl (pH 8.0 and pH 9.0), 0.5 M EDTA (pH 8.0), 5 % SDS, 1 M TE Buffer (pH 8.0), 1M Sodium Citrate (pH 7.0), 3 M Sodium Acetate (pH 5.2), 1.4 M Sodium Chloride, 10 % Sarcocine water saturated phenol, 10 × MOPS Buffer and RNA Extraction buffer.

Total RNA extraction from harvested tissues

Total RNA from ground tissue was extracted according to following steps. Each ground plant samples in 1.5 ml micro-centrifuge tube was treated with 0.5 ml of RNA extraction

buffer, 0.5 ml of water saturated phenol and 50 μ l of 3 M Sodium acetate (pH 5.2) and vigorously shaken. Then the samples were kept in an ice bucket while shaking for 10 minutes. Then they were centrifuged at 12000 rpm for 10 minutes at 4^oC. After centrifugation supernatant of each tube was collected to a new eppendorf tube. For the supernatant 200 μ l of ice-cold chloroform was added and vigorously shaken for 15 seconds. Then the samples were centrifuged at 12000 rpm for 15 minutes at 4^oC after keeping at room temperature for 3 minutes. Then the supernatant was collected to a new eppendorf and treated with double the volume of ice- cold Isopropanol. Then they were kept at room temperature for 10 minutes and centrifuged at 12000 rpm for 10 minutes at 4^oC. After centrifugation supernatant was removed and the pellet was kept. After that the pellet was washed with 500 μ l 75 % Ethanol by centrifugation at 3800 rpm for 5 minutes at 4^oC. Then the pellet was dried and dissolved in 15 μ l of 1 \times TE buffer and kept at - 80^oC for further use.

Extracted RNA was run on a denaturing agarose gel to confirm the RNA extraction. RNA quantification was done with the use of Spectrophotometer. The ratio of the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) for pure RNA lies between 2 – 2.1.

Extraction of mRNA

Isolation of intact and biologically active mRNA is essential for cDNA library construction. In the present study, mRNA was extracted from the total RNA (isolated from the pooled shoot tissues subjected to moisture stress) by the paramagnetic particle technique using Dynal beads (Dynal Biotech ASA, Norway). Dynal beads and the paramagnetic particle technique was used to extract mRNA from total RNA samples isolated from rice shoot tissues collected after subjecting to moisture stress. The technique was used for extraction of mRNA according to the instruction of manufacturers (Dynal Biotech ASA, Norway). The method used was as follows:

Approximately 100 μ l (i.e.75 μ g) of total RNA was taken as starting material for mRNA extraction by the paramagnetic particle technique using Dynal beads. Sample was heated to 65^oC for 2 min to disrupt secondary structures of RNA molecules and placed on ice for quick chilling. In the meantime 200 μ l of re-suspended Dynabeads Oligo(dT)25 from the stock tube suspension was taken and dispensed in a 1.5 ml micro centrifuge tube and kept on the Dynal MPC at room temperature (i.e. 28^oC). Thirty seconds after keeping on the Dynal MPC, the supernatant collected in the microcentrifuge tube was pipetted off. This was done by removing the microcentrifuge tube from the magnet and resuspending Dynabeads Oligo(dT)25 in 100 μ l binding buffer and placing the tube back on the magnet. Supernatant was pipetted off in another 30 sec and the microcentrifuge tube was removed from the magnet. After the removal of the microcentrifuge tube from the MPT, an aliquot of 100 μ l of binding buffer was added to Dynabeads Oligo(dT)25. Then total RNA was added to Dynabeads Oligo(dT)25 in binding buffer and kept on a roller for 5 min at room temperature for continuous but gentle shaking. The tube was then placed on the magnet for 30 seconds until the solution gets cleared and then the supernatant was removed. The tube was removed from the MPT and the contents inside were washed twice with 200 μ l washing buffer B. All the supernatant was removed at each washing step and 20 μ l of Tris buffer was added. The sample was heated to 65^oC for 2 min and

placed immediately on the magnet and the mRNA was eluted to a new RNase free micro-centrifuge tube and the samples were stored at -80°C until cDNA libraries were prepared. cDNA library was constructed from mRNA of the rice shoot tissues subjected to moisture stress (collected at daily intervals after imposing the stress till one week). Construction of cDNA library was done using CloneMiner™ cDNA Library Construction kit (catalog no. 18249-029, Invitrogen, USA) based on Gateway^R Technology according to manufacturer's instructions as given below.

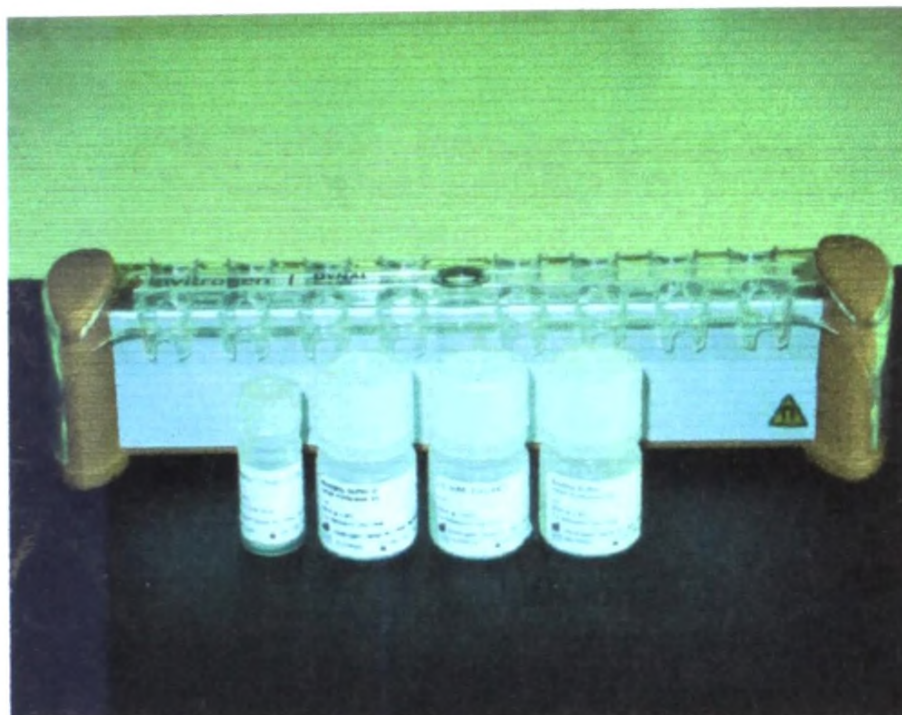


Plate 3: mRNA extraction kit

Synthesis of the first strand to construct the cDNA library

Synthesizing of the first strand consists of two reactions, namely priming reaction and first strand reaction. For the synthesis of the first strand of the cDNA libraries the priming reaction was conducted by adding the following ingredients in to an eppendorf tubes. To an eppendorf tube, 10 μl of mRNA, 1 μl of Biotin- *attB2*-Oligo (dT) primer (30 pmol/ μl) and 1 μl of dNTPs (10 mM) each were added.

Contents in the tube were mixed gently by pipetting and brief centrifugation was done for 2 seconds to spin down the sample. The sample was incubated at 65°C for 5 minutes and cooled to 45°C for 2 minutes. During these incubation steps First Strand Reaction was performed as below.

In to the above eppendorf tube the following ingredients were mixed to prepare the first strand reaction mixture. The ingredients used were 4 μl of 5x First Strand Buffer, 2 μl of 0.1M DTT and 1 μl of DEPC-treated double distilled water. Contents were mixed gently by pipetting and centrifuged for 2 seconds to spin down the contents . After cooling the priming reaction at 45°C for 2 min, it was mixed with the reaction mixture and the contents were incubated at 45°C for 2 min. Next, 1 μl of SuperScript II RT was added to the first strand reaction mixture and mixed gently by pipetting and incubated at 45°C for 60 min.

Synthesis of the second strand to construct the cDNA library

Upon the completion of first strand synthesis, the eppendorf tube containing the synthesized first strand DNA was subjected to synthesis of the second strand DNA. All the experimental steps of synthesizing of the second strand were performed quickly to prevent the rising of temperature above 16°C. The eppendorf tube containing first strand cDNA of the rice shoot tissues was placed on ice and following chemicals were added: 92 µl of DEPC-treated, double-distilled water, 30 µl of 5 x Second Strand Buffer, 3 µl of dNTPS each (10 mM), 1 µl of *E. coli* DNA ligase, 4 µl of *E. coli* DNA polymerase I (10 U/µl) and 1 µl of *E. coli* RNase H (2 U/µl).

Contents were mixed gently by pipetting and centrifuged for 2 seconds to spin down the contents and incubated at 16°C for 2 hours. Then 2 µl of T4 DNA Polymerase was added and the samples were incubated at 16°C for 5 minutes. DNA polymerase was added to the sample to create blunt-ended cDNA. Thereafter, the samples was mixed with 10 µl of 0.5 M EDTA (pH 8.0) to stop the reaction.

Confirmation of the synthesis of second strand DNA

To confirm the second strand synthesis, 10 µl of each cDNA sample was analyzed on 1% agarose gel. The remaining amount of the cDNA sample was subjected to phenol;chloroform extraction.

Phenol/Chloroform Extraction

For the extraction with phenol:chloroform, an aliquot of 160 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to cDNA sample and shaken thoroughly by inverting the eppendorf tube for approximately 30 seconds. Then sample was centrifuged at room temperature for 5 minutes at 14,000 rpm. The upper aqueous phase in the eppendorf tube was removed carefully to a new eppendorf tube and subjected to ethanol precipitation in order to precipitate the resulted in cDNA.

Ligating the attB1 Adapter

At the end of the ethanol precipitation, the tube should contain double-stranded, blunt-ended cDNA. As the next step of cDNA library construction, these cDNA fragments should be ligated with the *attB1* adapter before the construction of a recombinant molecule by ligating with the vector, pDONR.3222. Hence, the eppendorf tubes containing double stranded, bunt-ended cDNA were placed on ice and following reagents were added to each tube. The reagents introduced into the tube were 10 µl of 5x Adapter Buffer, 10 µl of *attB1* Adapter (1µg/µl), 7µl of 0.1 M DTT and 5 µl of T4 DNA Ligase. The contents were mixed gently by pipetting and incubated at 16°C for 16-24 hours. To stop the ligation reaction, the sample was incubated at 70°C for 10 minutes and placed on ice for quick chilling.

Size fractionation of cDNA by Column Chromatography

The cDNA fragments ligated with attB1 adapter was subjected to size fractionation by column chromatography. To this end, the columns were securely anchored in a support stand. A rack containing a series of 1.5 ml eppendorf tubes was placed below each column. The outlets of the columns were adjusted to be 1 to 2 cm above the 1.5 ml tubes so that rack could be moved freely under the columns.

cDNA size fractionation columns were packed in 20% ethanol. As ethanol should be removed completely before adding the cDNA sample, the following steps were taken to remove ethanol from the columns. The washing steps took approximately 1 hour and 30 min.. Initially, the top cap was removed and then bottom cap was removed. Ethanol was allowed to drain completely by gravity. Once the columns stopped dripping, 0.8 ml of TEN buffer was added to each column and allowed to drain completely. This washing step was repeated for another three more times with TEN buffer. Thereafter, the columns were drained and allowed to dry.

For collection of fractions of cDNA with attB1 adapter, twenty sterile 1.5 ml tubes were labeled from 1 to 20 and placed in a rack 1 to 2 cm below the bottom (outlet) of each column. Then, 100 μ l of TEN buffer was added to 50 μ l of heat-inactivated cDNA adapters prepared from rice shoots. Sample was mixed gently by pipetting and centrifuged for 2 seconds to collect the contents. Then, the entire sample was added to the column and let it drain into the resin bed and effluents were collected into tube 1. Next, tube 2 was moved under column outlet and 100 μ l of TEN buffer was added to the column. The effluents were collected into tube 2. Single-drop fractions of the next 100 μ l aliquot of TEN buffer, were collected into individual tubes starting from tube 3. Continue to add 100 μ l aliquots of TEN buffer until all 18 tubes (tubes 3-20) contain a single drop. Then, the contents in tube 1- 13 were pooled to get a volume of 600 μ l and rest of the tubes was discarded. Because pooling more than 600 μ l may contaminate cDNA library with attB1 adapters and would produce low average inserts size in the library.

The following reagents were added to the tube containing pooled cDNA: Glycogen (20 μ g/ μ l) -1 μ l; 7.5 M NH₄OAc 0.5 volume (*i.e.* 0.5 x volume of cDNA) and 100% ethanol 2.5 volumes [*i.e.* 2.5 x (volume of cDNA + NH₄OAc)].

The tube was placed at -20°C overnight and centrifuged at 4°C for 25 minutes at 14,000 rpm. Then the supernatant was removed carefully without disturbing the cDNA pellet. Next, 150 μ l of 70% ethanol was added to each sample. The samples were centrifuged at 4°C for 2 minutes at 14,000 rpm, supernatant was removed carefully and the 70% ethanol wash was repeated once. cDNA pellet was dried for at room temperature for 10 minutes. Pellet was re-suspended in 4 μ l of TE buffer by pipetting up and down 30-40 times. Samples were transferred into fresh tubes.

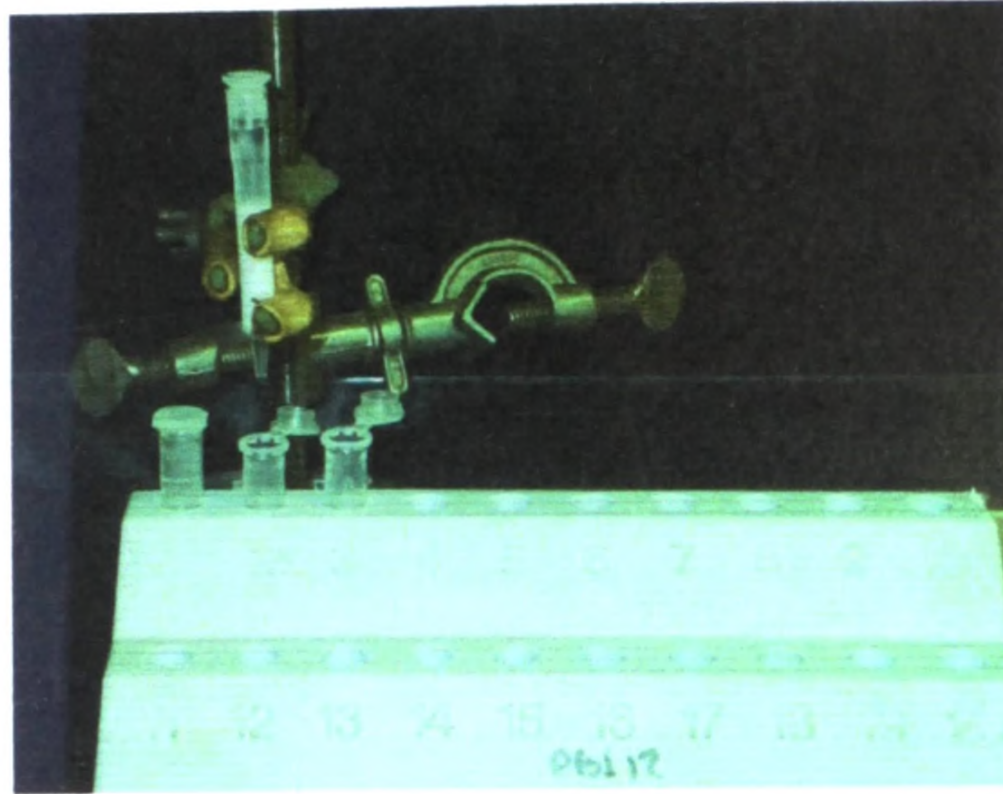


Plate 4: Size fractionation of cDNA by column chromatography

Performing the BP Recombination

After the ligation of *attB1* adapters, the following components were added at room temperature to a sterile 1.5 ml eppendorf tube to make recombinant cDNA molecules in the cloning vector pDONR.222. The reaction components added per eppendorf tube were 4 μ l of *attB1*-flanked cDNA, 1 μ l of pDONR.222 (250 ng/ μ l), 2 μ l of 5 x BP Clonase reaction buffer and 3 μ l of BP Clonase enzyme. The total volume of the reaction mixture in the eppendorf tube was 10 μ l and the mixture was incubated at 25 °C for 16-20 hours. Twenty hours after incubation, the BP recombination reaction was stopped by adding an aliquot of 2 μ l of Proteinase K to inactivate the BP Clonase enzyme. The mixture containing Proteinase K was incubated at 37°C for 15 minutes and then at 75°C for 10 minutes.

To the tubes containing pooled cDNAs, following reagents were added to precipitate cDNA: Sterile distilled water - 90 μ l; Glycogen (20 μ g/ μ l) -1 μ l; 7.5 M NH_4OAc – 50 μ l; 100% ethanol - 375 μ l.

The tubes were placed at -80°C for 10 minutes and centrifuged at 4°C for 25 minutes at 14,000 rpm. The supernatant was removed carefully without disturbing the cDNA pellets. 150 μ l of 70% ethanol was added to each sample and centrifuged at 4°C for 2 minutes at 14,000 rpm. The supernatant was removed carefully. The 70% ethanol wash was repeated and the pellets were dried at room temperature for about 10 minutes. Pellets were re-suspended in 9 μ l of TE buffer separately by pipetting up and down 30-40 times. Samples were transferred to fresh tubes.

Transformation

Three micro-liters of cDNA samples prepared from rice sheath tissues were transferred in to two separate ice cold eppendorf tubes. The cDNA in the eppendorf tubes were mixed separately with 50 μ l of thawed ElectroMAX DH10B, the competent cells used for transformation of the recombinant molecules. The contents were mixed by gentle pipetting without producing air bubbles. The entire contents were transferred to pre-cooled 0.1 cm cuvettes. The samples were electroporated with 1.8 kV voltage, at 200 Ω resistance and 25 μ F capacity. Soon after electroporation, 1 ml of SOC medium was added to the cuvette containing electroporated cells. Thereafter, the entire solution was transferred to 15 ml test tubes and shaken for 1 hour at 37°C at 225 rpm to allow expression of the Kanamycin resistance marker. After one hour of incubation at 37°C, an aliquot of 75 μ l of the culture was plated on LB medium supplemented with Kanamycin (50 μ g/ml) and incubated at 37°C for overnight. Plate 5 shows the development of transformed colonies on LB medium supplemented with Kanamycin.

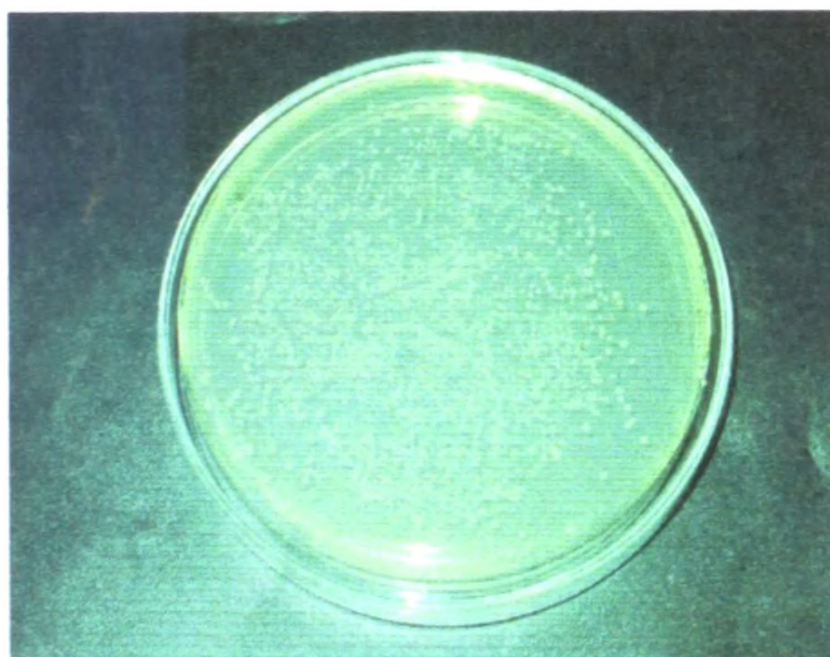


Plate 5: Single colonies of transformants on LB plate supplemented with Kanamycin

Storing the transformant colonies/cDNA libraries

Well separated transformed colonies appeared on LB medium containing kanamycin (50 μ g/ml) were transferred into microtiter plates with 96 wells having the LB broth medium supplemented with Kanamycin (50 μ g/ml) and grown overnight at 37°C. Each clone in a single well was designated with a code number. The colonies which were grown in each well of the microtiter plate were added with 30% sterilized glycerol, made in to 15% glycerol stocks and stored at -80°C.

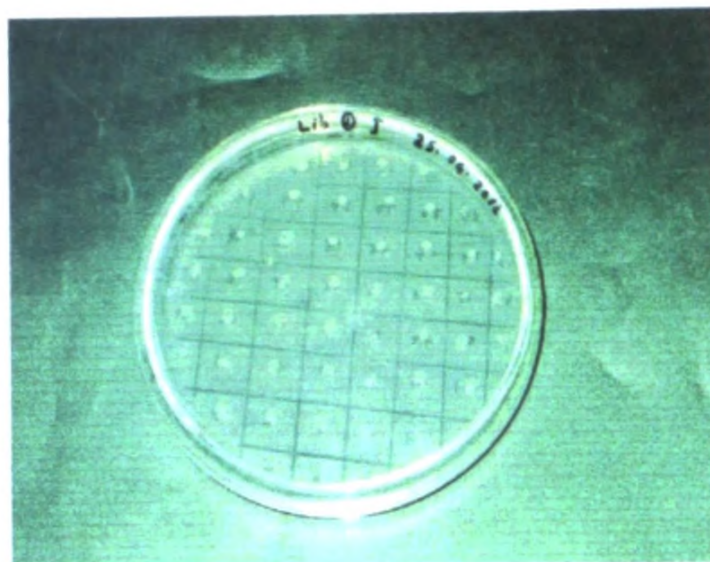


Plate 6: Single colonies of transformants prepared for storing

Work of the project had to be terminated at this point because of the termination of the research grant by the NSF despite a request by the PI for extension to complete the work.

Conclusions

1. Bg358 can be identified as a high yield rice variety which is relatively tolerant to drought at both vegetative and reproductive stages;
2. The cDNA library constructed from the shoot tissue of Bg358 subjected to drought stress at the vegetative stage contains genes that are expressed under drought stress. Among these genes are genes which are responsible for the drought tolerance of Bg358.

The following part was completed after the NSF officially terminated the project in May 2012. This work is still continuing.

Screening of the cDNA library

As explained earlier, rice variety Bg 358 was identified as a drought resistant local rice germplasm based on the selection index developed by measuring growth and physiological variables of rice grown under drought conditions. The physiological parameters considered for the selection index net photosynthesis rate, instantaneous transpiration efficiency, leaf temperature and chlorophyll fluorescence. The growth parameters used were relative growth, leaf area per unit rooting depth and visual scoring.

Identification of drought responsive genes of rice variety Bg 358

Determination of gene response to drought tolerance in rice varieties would be helpful in designing effective management measures against drought stress in paddy cultivation. Therefore, the objective of this work was to identification of drought responsive genes of

rice variety Bg 358, which is relatively tolerant to drought. A cDNA library was constructed from mRNA of rice plant tissues and collected at different time intervals after drought development, using pDONRTM222 vector and ElectroMAXTM DH10B competent host cells. From the cDNA library a set of 192 cDNA clones were arrayed on Hybond N+ nylon membranes and subjected to differential hybridization using Digoxigenin-labelled total cDNA probes (i.e. probes prepared from drought induced tissues and non drought induced tissues). Differential hybridization performed in the present study identified 6 cDNA clones carrying up regulated genes and 18 clones with down-regulated genes due to impose of drought (Plate 7). Out of the selected cDNA clones, which harbour up- and down- regulated genes, 11 were subjected to DNA sequencing and homology search was done with available DNA/protein databases. 10 putative genes/gene products were deduced for 6 up-regulated and 4 down- regulated genes (Table 3).

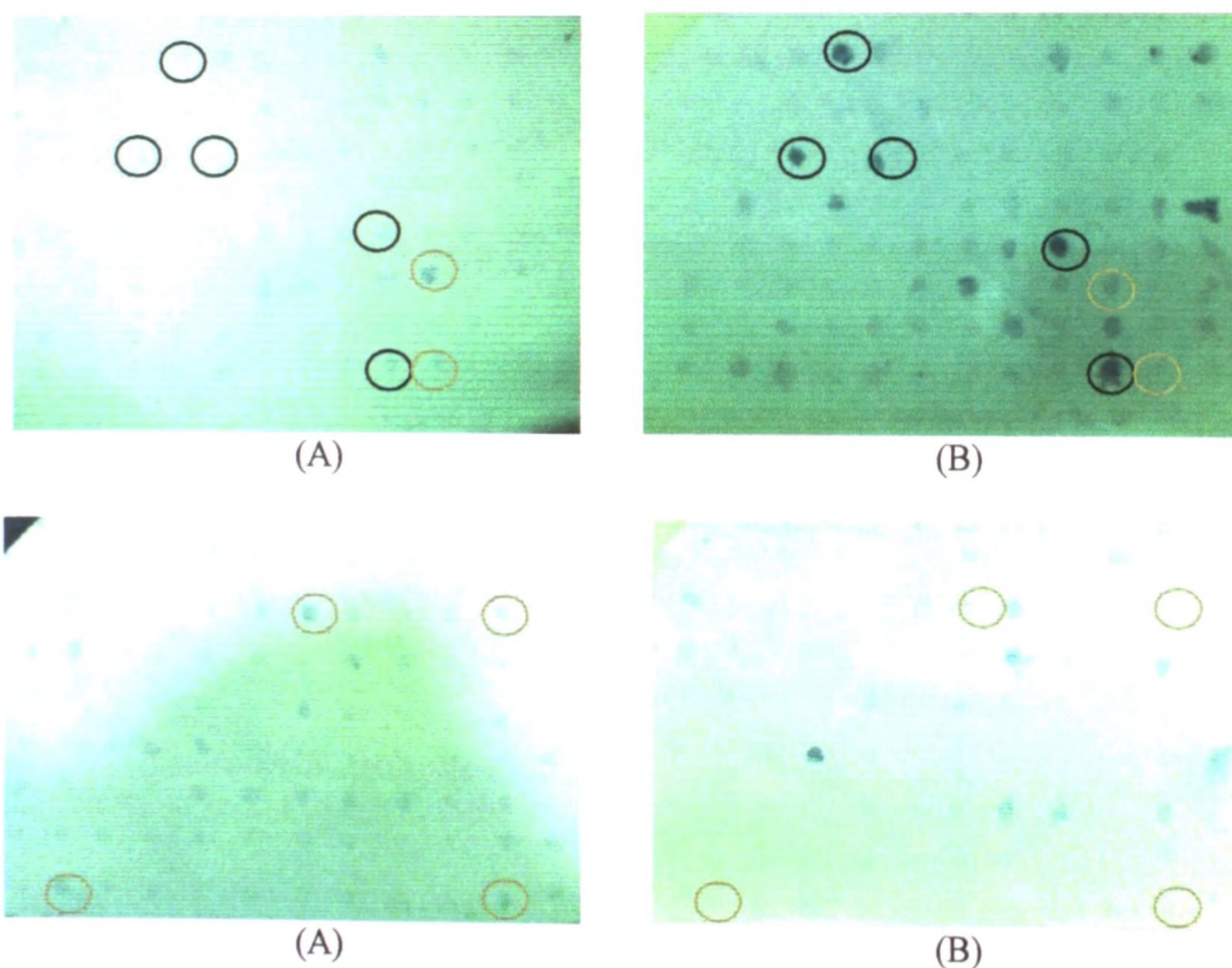


Plate 7: Signals developed by cDNA clones subjected to differential hybridization. Treatment probe (A) and Control probe (B). Red circles- Up regulated clones and Black circles- Down regulated clones

According to hybridization signals, 6 clones namely, 70, 93, 115, 119, 182 and 191 were identified as up regulated genes and 5 clones namely, 4, 27, 29, 57 and 94 were identified as down regulated genes. The selected 11 clones were subjected to plasmid extraction by Alkaline SDS method. Extracted plasmid DNA was diluted ten times and DNA concentration was measured by using 2 μ l.

Table 3. Spectrophotometric readings of plasmid DNA samples of the selected cDNA clones

Clone number	Nucleic acid concentration (ng/ μ l)	OD 260/280	OD 260/230
4	630.26	1.77	2.78
27	313.81	1.81	4.63
29	758.72	1.74	2.68
57	35.65	1.09	0.48
70	368.56	1.80	2.88
93	379.76	1.82	2.74
94	410.16	1.80	2.89
115	480.99	1.75	2.71
119	295.02	1.84	3.23
182	480.35	1.75	2.68
191	388.03	1.80	3.01

DNA samples can contain impurities that will affect the OD values at 260 nm. Therefore, it is recommended to measure wavelengths where impurities have an maximum absorbance. If contaminated by protein the highest absorbance will give at 280 nm wavelength and if contaminated with phenolate, thiocyanate the highest absorbance will give at 230 nm wavelength. A common method used to check the purity of a DNA sample is to calculate the ratio of OD values at 260nm and 280nm in order to look for protein contaminations. Pure DNA gives a ratio of 260nm/280nm between 1.8- 2.0 . Accordingly, except the plasmid DNA sample of clone 57, the other samples contained DNA with the acceptable purity.

To identify the relative insert sizes of cDNA fragments in the selected clones carrying up- and down-regulated genes, a restriction digestion was performed with *Hind*III. After the electrophoresis on a 1.8% agarose gel, diverse lengths of cDNA fragments could be observed (Plate 8). It indicated that different lengths of cDNA have been ligated with the vector pDONRTM222 during the cDNA library construction, which is a successful feature of the cDNA library.

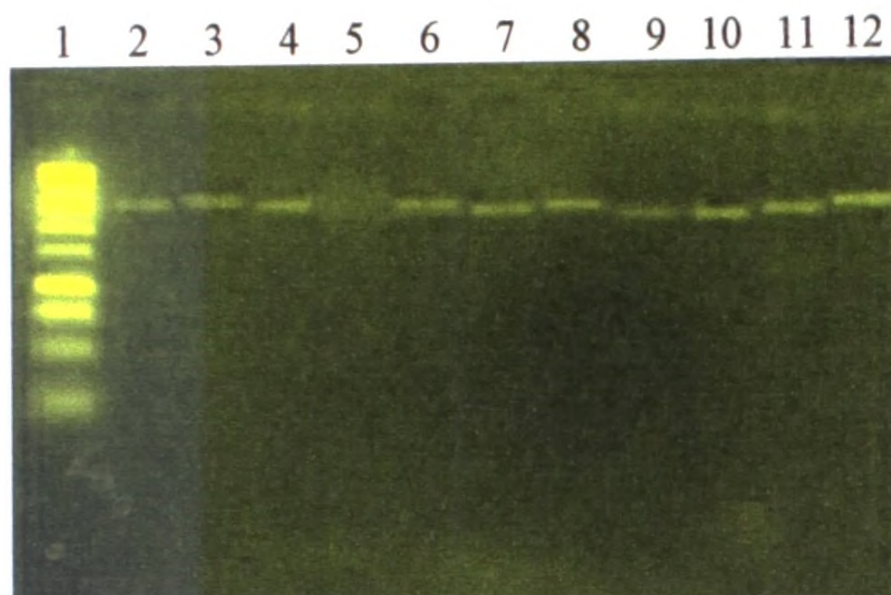


Plate 8: Gel electrophoresis of plasmid DNA digested with *Hind* III. Lane 1: 1 Kb ladder, Lane 2- 12: restriction digested plasmid DNA of the selected clones, namely clones 4, 27, 29, 57, 70, 93, 94, 115, 119, 182, 191.

Plasmid DNA extracted from the above, eleven selected cDNA clones were subjected to PCR amplification with the universal M 13 primers. Then PCR products were electrophoresed on 2% agarose gel. M13 amplified products gave various sizes and the sizes varied approximately from 1000bp to 500bp in length (Plate 9).

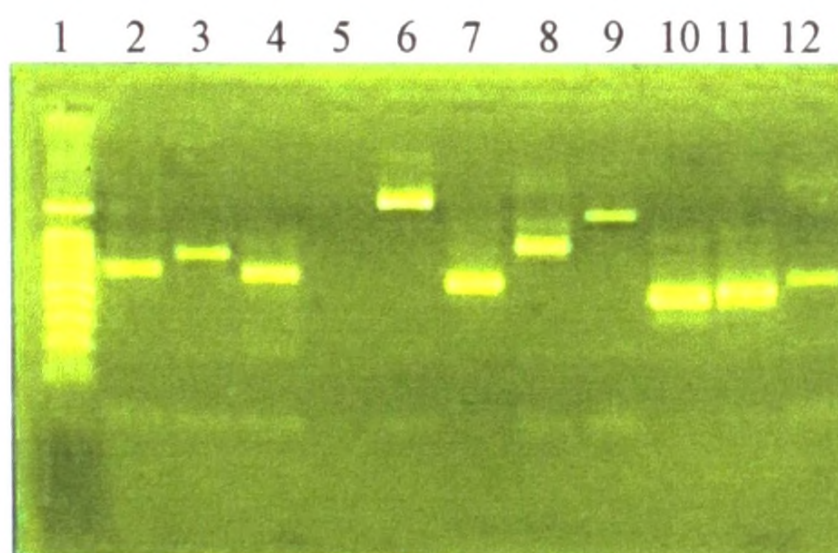


Plate 9: PCR amplified cDNA clone fragments separated on a 2% agarose gel. Lane 1: 100 bp ladder, Lane 2- 12: PCR products amplified with M 13 primer using cDNA clones namely, 4, 27, 29, 50, 70, 93, 94, 115, 119, 182, 191.

Based on the PCR products shown by Plate 9, it is clear that the selected cDNA clones are harbouring cDNA inserts of different sizes (all are above 500 bp) which is a desirable feature of a successful cDNA library.

DNA homology search results revealed that majority of the sequenced data are highly homologous with rice genome and it confirms the accuracy of the cDNA library prepared in this study (Table 4).

Table 4: Details of DNA homology search and the identified putative genes/protein produces of the query sequences

Clone	Differential Expression	Length sequenced	Accession Number (ID)	Homology	Identity	E-Value
Os DT 04	DR	440 bp	gb ABY60447.1 	serine/threonine protein kinase [<i>Adonis aestivalis</i> var. palaestina]	97	1e-131
Os DT 27	DR	926bp	dbj BAB89667.1 	putative transaldolase [<i>Oryza sativa</i> (japonica cultivar-group)]	81	2e-142
Os DT 29	DR	123bp	gb ABA94215.2 	MRG family protein, putative, expressed [<i>Oryza sativa</i> (japonica cultivar-group)]	67	5e-66
Os DT 70	UR	634bp	emb CAI29540.1 	ubiquitin conjugating enzyme E2 [<i>Oryza sativa</i> (indica cultivar-group)]	91	3e-65
Os DT 93	UR	516bp	ref NP_001067900.1 	hypothetical protein OsJ_032572 [<i>Oryza sativa</i> (japonica cultivar-group)]	93	10e-90
Os DT 94	DR	578bp	gb AAA74625.1 	protein phosphatase 1 [<i>Oryza sativa</i>]	81	1e-86

Os DT 115	UR	641bp	dbj BAE983 96.1 	phosphoprotein phosphatase [<i>Arabidopsis thaliana</i>]	96	5e-57
Os DT 119	UR	609bp	gb AAQ843 34.1 	stress-associated protein 8 [<i>Oryza sativa</i> (indica cultivar-group)]	66	2e-53
Os DT 182	UR	162bp	dbj BAD736 67.1 	putative heat shock protein 82 [<i>Oryza sativa</i> (japonica cultivar- group)]	86	4e-134
Os DT 191	UR	855bp	ref NP_1944 02.1 	phosphoprotein phosphatase (PPX-1) [<i>Arabidopsis thaliana</i>]	91	8e-107

These identified genes were grouped in to five functional categories namely, (1) Growth and development; (2) Protection and repair; (3) Hypothetical protein; (4) Energy and metabolism; (5) Protein synthesis, folding and stabilization (Figure 7; Table 5).

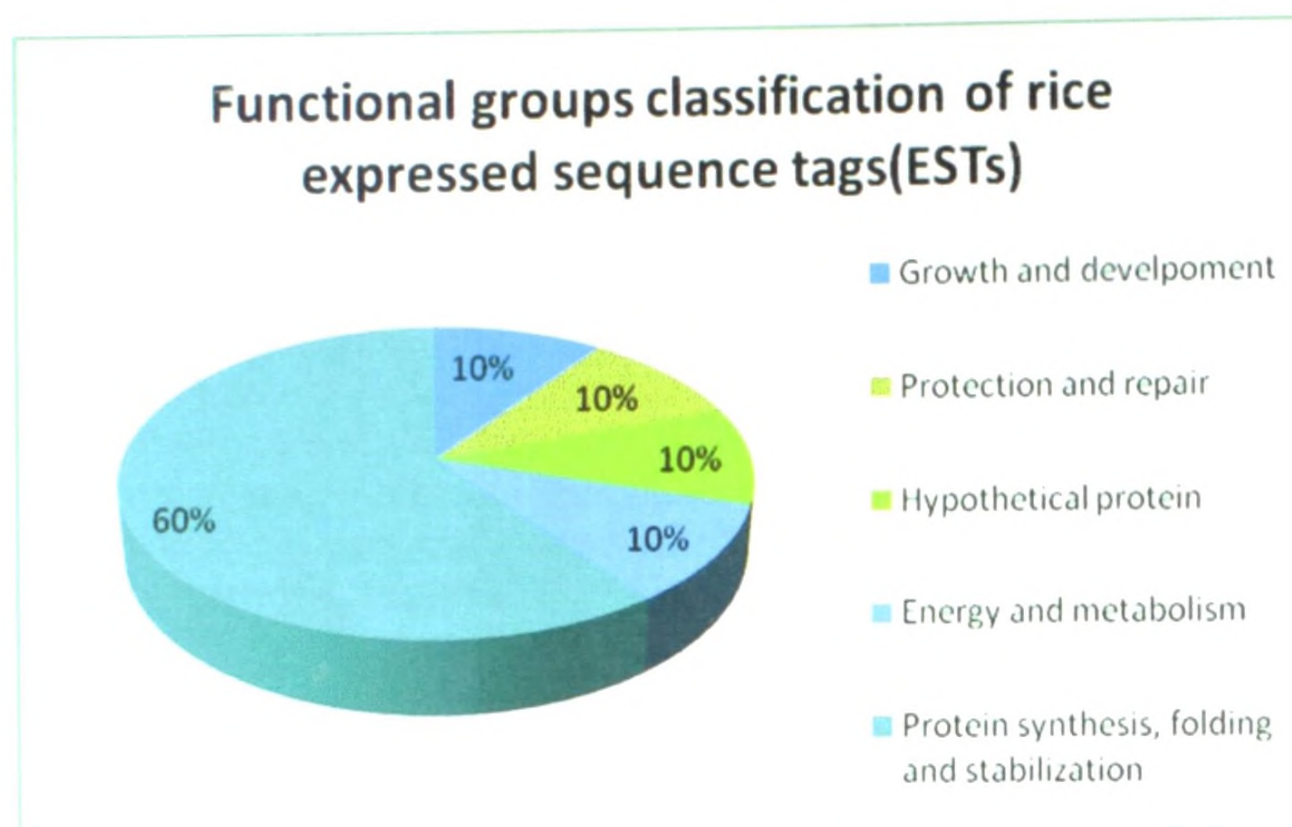


Figure 7: Functional groups of rice expressed sequence tags (ESTs) studied in the present study

Table 5: Summary of the functional classes and identified genes/protein products

Gene functional category	Genes
Growth and development	MRG family protein
Protection and repair	putative heat shock protein 82
Hypothetical protein	hypothetical protein
Energy and metabolism	putative transaldolase
Protein synthesis, folding and stabilization	Serine/threonine protein kinase, Ubiquitin conjugating enzyme E2 , Protein phosphatase 1 , Phosphoprotein phosphatase (PPX-1), Stress-associated protein 8

The largest sets of ESTs were assigned to the categories of Protein synthesis, folding and stabilization which accounted 60% of the total ESTs. The functional category of energy and metabolism contained 10% of the total ESTs and these included genes involved in photosynthesis and carbohydrate metabolism. The growth and development category accounted for 10% of the differentially expressed ESTs encoding MRG family protein and has shown important activities for cellular aging, proliferation, positive and negative transcriptional regulation and DNA damage repair.

The functional category of protection and repair comprised 10% of the ESTs including the genes involved in “protection and repair,” “drought,” “heat,” and “cold” within “ROS and Stress”. Finally the 10% of the total ESTs were categorized in to hypothetical group and the functions of this group was unknown.

Clone Os-DT-04 harboured a down- regulated transcript due to drought stress and the transcript was homologous with Serine/threonine protein kinase enzyme. Previous studies

have shown that Serine/threonine protein kinase plays important roles in plant abiotic stress responses. Drought stress triggers signal transduction-activating components of the drought response (Beck *et al.*, 2007). In cacao roots, TcRPK (putative receptor-like protein kinase), TcMKK4 (putative mitogen-activated protein), and TcSTK (putative serine/threonine protein kinase) were down regulated in response to drought stress.

According to the Hardie, (1999) the first plant protein kinase sequences were reported in 1989, but by mid-1998 there were more than 500, including 175 in *Arabidopsis thaliana* alone. Protein serine/threonine kinases from *A. thaliana* can be divided in to around a dozen major groups based on their sequence relationships. The network of Protein-serine/threonine kinases in plant cells appears to act as a “Central Processor Unit” (CPU), accepting input information from receptors that sense environmental conditions, phytohormones, and other external factors and converting it in to appropriate outputs such as changes in metabolism, gene expression and cell growth and division.

Clone Os-DT-29 has harboured a down regulated transcript due to drought stress and is homologous with MRG family protein, putatively, expressed in *Oryza sativa* (japonica cultivar-group) (Table 3). Previous studies have shown that MRG family protein plays important roles in plant abiotic stress responses. According to the Pena *et al.*, (2007), the MRG family of genes, of which MORF4 is a member, has shown important activities for cellular aging, proliferation, positive and negative transcriptional regulation and DNA damage repair. Cellular senescence is the dominant phenotype over immortality. According to the Chen *et al.*, (2010) they have conducted an experiment to identify senescence related genes. In this study they have cloned MORF4, which induced senescence in a subset of tumor cells. The MORF4 is a member of a family of seven genes, and MORF-related genes (Mrg) on chromosomes 15 (Mrg 15) and X (Mrg X) are also expressed. In contrast to MORF4, MRG 15 and MRGX are positive regulators of cell division. All 3 proteins interact with histone deacetylases and acetyltransferases suggesting that they function in regulation of chromatin dynamics.

Clone Os-DT-70 harboured a transcript up-regulated due to drought stress and homologous with Ubiquitin-conjugating enzyme-Previous studies have shown that ubiquitination plays important roles in plant abiotic stress responses. According to the Zhou *et al.*, (2010) the ubiquitin-conjugating enzyme gene *GmUBC2*, a homologue of yeast RAD6, was cloned from soybean and functionally characterized. *GmUBC2* was expressed in all tissues in soybean and was up-regulated by drought and salt stress. (*Arabidopsis* plants over expressing *GmUBC2* were more tolerant to salinity and drought stresses compared with the control plants. Through expression analyses of putative downstream genes in the transgenic plants, they found that the expression levels of two ion antiporter genes *AtNHX1* and *AtCLCa*, a key gene involved in the biosynthesis of proline, *AtP5CS*, and the copper chaperone for superoxide dismutase gene *AtCCS*, were all increased significantly in the transgenic plants. These results suggest that *GmUBC2* is involved in the regulation of ion homeostasis, osmolyte synthesis, and oxidative stress responses. Modulation of the ubiquitination pathway could be an effective means of improving salt and drought tolerance in plants through genetic engineering (Zhou *et al.*, 2010).

Clone Os-DT-93 had an up-regulated transcript under drought stress conditions which is homologous with hypothetical protein OsJ_032572. The functions of hypothetical protein was unknown and not described in literature.

Clone Os-DT-94 was identified as a down-regulated cDNA clone and the transcript of this cDNA clone was homologous with protein phosphatase 1. Previous studies have shown that protein phosphatase 1 plays important roles in plant abiotic stress responses. Protein phosphatase 1 involved in Abscisic acid signaling in guard cells as a central mediator.

Guard cells are located in the epidermis of plant leaves, and in pairs surround stomatal pores. These control both the influx of CO₂ as a raw material for photosynthesis and water loss from plants through transpiration to the atmosphere. Guard cells have become a highly developed system for dissecting early signal transduction mechanisms in plants. In response to drought, plants synthesize the hormone abscisic acid, which triggers closing of stomata, thus reducing water loss. Recently, central regulators of guard cell abscisic acid signalling have been discovered. The molecular understanding of the guard cell signal transduction network opens possibilities for engineering stomatal responses to control CO₂ intake and plant water loss. Guard cell signalling integrates water status, hormonal stimuli, light, CO₂ levels and other environmental conditions to regulate stomatal apertures. Guard cells are a well-developed model system for understanding how components interact within a signaling network in a single cell. They are well suited for dissecting the functions of genes and proteins in signalling cascades. A network of ion channels in the plasma membrane and the vacuolar membrane of guard cells, which controls stomatal movements, has been characterized. These ion channels are targets of early signalling branches and provide probes to identify and characterize upstream transducers. Abscisic acid (ABA)-induced stomatal closing is mediated by a reduction in the turgour pressure of guard cells, which requires an efflux of potassium and anions, sucrose removal and the conversion of malate to osmotically inactive starch. Box 1 shows an extension of early models for the proposed functions of ion channels during ABA-induced stomatal closing.

Phosphorylation events are central mediators of ABA signalling in guard cells. In this study some of the underlying protein kinase and phosphatase genes have been cloned. Both positively and negatively regulating protein kinases, and positively and negatively regulating PP2C- and PP1/2A-type protein phosphatases have been proposed to function in ABA signalling.

With one exception, dominant ABA-insensitive mutations in the known protein kinase and phosphatase genes ABI1, ABI2 and AAPK, have been analysed in guard-cell signalling. It will be important to isolate recessive loss-of-function mutations in these enzymes to test the proposed functions of the wild-type proteins. Scaffolding mechanisms may hold the key for target specificity of the many proposed protein kinases and phosphatases (Schroeder *et al.*, 2001).

Clone Os DT 115 and Os DT 191 which were up regulated and homologous with phosphoprotein phosphatase , putatively- expressed in *Arabidopsis thaliana* (Table 3). Previous studies have shown phosphoprotein phosphatase plays important roles in plant abiotic stress responses. Phosphoprotein phosphatases (PPP), these ancient and important regulatory enzymes are present in all eukaryotic organisms.

The Phosphoprotein phosphatase enzymes were involved in the regulation processes such as gene expression, DNA replication, morphogenesis, synaptic transmission, glycogen metabolism and apoptosis. Phosphoprotein phosphatase 1 is an enzyme of glycogen regulatory pathways that acts to reverse the effects of the Kinase cascade. Phosphoprotein phosphatase counters the effects of the Kinase cascade by removing phosphates from proteins. Thus it converts the phosphorylated glycogen phosphorylase a (more active) to the dephosphorylated phosphorylase b (less active) and also converts phosphorylated glycogen synthase D (less active) to the more active glycogen synthase 1 form (dephosphorylated). The activity of Phosphoprotein phosphatase 1 is controlled by Phosphoprotein phosphatase inhibitor. Inhibition of Phosphoprotein phosphatase (PP-1), whose activity would tend to restore activity of glycogen synthase. PP-1 and other Phosphoprotein phosphatases play converse roles in glycogenolysis, in which dephosphorylation of glycogen phosphorylase b kinase (SPK) causes its inactivation.

Clone Os DT 119 is homologous with stress-associated protein 8 and selected as an up-regulated cDNA clone due to drought stress. Previous studies have shown that stress-associated protein 8 plays important roles in plant abiotic stress responses. In the present study, the stress- associated protein 8 gene was homologous with *Oryza sativa* (indica cultivar- group). Abiotic stresses such as drought, salt, cold, heat and exposure to toxic metals adversely affect growth and productivity of crop plants and are serious threats to Agriculture. Members of Stress Associated Protein (SAP) family in rice have been shown to provide tolerance to multiple abiotic stresses. There are 18 and 14 reported members of SAP family in rice and *Arabidopsis*, respectively. These SAPs contain A20, AN1, or both A20/AN1 zinc finger domains at the N- or C-terminus. Some members of SAP family proteins also contain extra Cys2-His2 RING motifs on the C-terminus.

In here described the functional characterization of two novel SAP genes, *AtSAP10* and *AtSAP11*, from *Arabidopsis thaliana* ecotype Columbia. *AtSAP10* gene contains an A20 and AN1 zinc-finger domain at the N- and C-terminal, respectively. Over expression of *AtSAP11* in *Arabidopsis* conferred moderate tolerance to heavy metals As and Zn and slightly enhanced tolerance to drought stress. *AtSAP11* promoter-GUS fusion studies revealed a floral organ-specific and fruit specific expression of *AtSAP11*. *AtSAP11*-GFP fusion showed an ER like localization of the fusion protein. Thus these results showed that *AtSAP10* and *AtSAP11* are potentially useful candidate genes for engineering tolerance to heavy metals and to abiotic stress in cultivated plants (Dixit, 2011).

Clone Os DT 182 was up- regulated due to drought stress and homologous with putative heat shock protein 82- Previous studies have shown that putative heat shock protein 82 (HSP) plays important roles in plant abiotic stress responses. In the present study, the

putative heat shock protein 82 gene was homologue with *Oryza sativa* (japonica cultivar-group).

HSPs, which can be divided into five conserved families, have been shown to have particularly important stress-related chaperone functions in plants (Boston *et al.*, 1996; Waters *et al.*, 1996). These genes encoding the heat shock proteins (HSPs) and LEAs can be found in the subcategories of “protection and repair”, “drought”, “heat” and “cold” within “ROS and Stress. Cho and Hong 2006 described HSPs, which are induced by heat have been implicated in plant cell protection mechanisms under drought stress. Protein denaturation occurs under drought stress because decreased cellular volume increases the likelihood of degradation molecular interactions. HSPs maintain or repair companion protein structure an target incorrectly aggregated and non- native proteins for degradation and removal from cells.

In trees grown under mild stress, more genes within “heat” and “Protection and repair” had increased transcript levels compared with severe stress conditions. A more detailed analysis of some of the HSP and LEA genes identified as having altered transcript levels. The specific clones of HSP 70 and HSP 90 respond differently to mild and severe stress. For example, an HSP 70, DNA k-type homolog (clone NXSI_117_Co 8) and an HSP 90 homolog (NXSI_116_BO₄) show increased transcript levels during mild stress and no response during severe stress. Conversely, a mitochondrial HSP 70 (NXCI_022_Go1) and a different HSP 90 (NXNV_149_E10) show increased transcript levels during severe stress and no response during mild stress.

The expression of genes associated with protection and repair mechanisms have been correlated with responses to stress imposition (Lee and Vierling, 2000; Zhang *et al.*, 2000). The previously identified protection and repair genes as being positively regulated in response to drought and suggested that they aided in adaptation to drought stress by protecting processes within the cell (Heath *et al.*, 2002).

Lee and Vierling (2000) suggested that one protection afforded by HSPs, one group of protection and repair genes, is manifested through the interaction of small HSPs with denatured proteins, preventing aggregation and promoting refolding of denatured proteins (Watkinson *et al.*, 2003).

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Problems if any, encountered during the implementation of the project:

The project encountered the following problems during its implementation from the beginning until its end. All these difficulties were reported in the progress reports to the NSF:

- a. There was about a one-year delay in the commencement of the project activities owing to a delay in receiving project funds because of the financial constraints experienced by NSF in 2006/07.
- b. Construction of the rainshelter took a longer period than expected. Because of the delay in receiving project funds from the NSF, the cost that was estimated for the construction of the rainshelter (which was the estimated cost in 2006 at the submission of the research proposal) turned out to be a substantial

underestimation by 2008 on account of the increased prices of material such as iron pipes and polythene. Therefore, it was very difficult to find a contractor who could do the work within the budget given. However, this task was completed with great difficulty by December 2008.

- c. There were significant delays in paying the salary of the research assistant owing to the financial constraints faced by the NSF. For example, although the research assistant was recruited in November 2008, he had not been paid his monthly allowance even by February 2009. However, this DID NOT cause delays in the experimental work because the research assistant has continued his work without his salary.
- d. The research assistant (Mr. H.R. Suranga) left for permanent employment in October 2010. We could recruit a new research assistant (Ms. M.S.K. Amarakoon) only in April, 2011. This was because of the shortage of graduates skilled in molecular biological techniques and probably the unwillingness of those who are skilled to undertake fulltime postgraduate research in Sri Lanka for a monthly allowance as low as Rs. 25,000.00. Even this amount would be paid only if they register for a postgraduate degree. Otherwise, it was just Rs. 17,000.00, which was not attractive at all.
- e. Research work in the project was further delayed by three months from May to August, 2011 because of the trade union action by the academic staff of the Universities. During the three month period from May to August, 2011, we could not use the laboratories and plant houses of the university as they were not opened. Therefore, the new research assistant could only begin her research work in August, 2011.
- f. Perfecting and optimizing the RNA and mRNA extraction and cDNA library construction required several rounds of experimentation. This is not unusual for molecular biological studies. As has been shown in this report, we have been successful in constructing the cDNA library.

The above difficulties encountered throughout the project period were largely beyond control of the investigators. Progress of the project was significantly delayed because of them. However, in view of the significant progress made in the project despite the above difficulties, the investigators are extremely disappointed at the decision of the NSF to terminate the project before its successful conclusion. Furthermore, the decision to terminate the project also resulted in one of its research students, Ms. M.S.K. Amarakoon, having to temporarily abandon her M.Phil. research programme half-way before its conclusion. This is a tragedy in a situation where Sri Lanka is striving to increase its base of qualified research scientists, especially in the area of Biotechnology. However, to the credit of Ms. Amarakoon, she resumed her research work on a part-time basis after receiving employment through the graduate placement scheme in the government service.

This report should demonstrate that the investigators and the research students have done their maximum possible under difficult circumstances and achieved results, the usefulness of which can be evaluated by experts. Even at present, Ms. Amarakoon is continuing the screening of the cDNA library.

Major findings and follow-up activities

Despite its untimely termination, the project had two major findings as given below:

1. The relatively drought-tolerant, high-yielding Sri Lankan rice variety, Bg358, which can be used as a source of genes for drought tolerance
2. The cDNA library from drought-stressed shoot tissue of Bg358, which contains the full complement of genes expressed under drought

As a follow-up activity, we plan to apply for a fresh research grant from the NSF to continue the planned molecular biological work on the cDNA library.

Section 4:

Impact of Research results:

Relevance of results achieved to scientific advancement

The varietal screening yielded information about the differential response of rice germplasm to drought stress at different stages of its life cycle. It showed that except in Bg358, drought tolerance in rice is stage-specific.

If the molecular work had been allowed to continue, differential hybridization of the cDNA library would have yielded a wealth of information about the genes that are up-regulated or down-regulated in response to drought stress in Bg358. Among these would be genes conferring drought tolerance in rice. It is unfortunate that this important part of the project could not be continued up to a successful conclusion because of its untimely termination by the NSF.

Relevance of results achieved to national/socio-economic development

Drought is one of most critical environmental constraints for increasing rice production in Sri Lanka. This has been amply demonstrated by severe drought that has been experienced in the dry zone of Sri Lanka during the yala season of 2012. Therefore, development drought tolerant rice varieties is a top priority in ensuring food security in national/socio-economic development in Sri Lanka. Continuation of this project to its planned conclusion could have made a significant contribution towards national/socio-

economic development in Sri Lanka. It is unfortunate for the country that this could not be done because of the untimely termination of the project.

It must be understood that scientific research does not always progress smoothly along pre-defined time schedules. Research is fraught with unexpected difficulties and setbacks which may prevent the working progressing according to original schedules. Funding agencies such as the NSF has to be mature enough to understand these ground realities of scientific research that is taking place in the fields, plant houses and laboratories for the meagre investment in research to be given a chance to contribute to national development.

The principal investigator and co-investigator of this research project are academics who are not full-time researchers. As part of their duties, they are engaged in a heavy programme undergraduate and postgraduate lectures and practicals. Engaging in research and supervision of research students is done in addition to the above workload. Therefore, it is natural that certain deadlines for submission of progress reports to be missed. However, if Sri Lanka is to be given a chance reap the benefits of scientific research, research funding agencies such as the NSF needs to show a greater flexibility towards the relatively few scientists who have opted remain in Sri Lanka engage in research in this resource-poor environment.

Dissemination/application of research output

Results of varietal screening will be disseminated as research publications.

List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please attach copies)

Section 5:**Miscellaneous***List of major equipment acquired during the project period and their functionality*

Rainshelter. Functional at present.

List of publications/communications arising from the project and/or presentations made at seminars, workshops etc.


None so far.

Section 6:**Summary Statement of Expenditure**

Type of expenses	Funds received by the university (Rs.)	Total expenditure (Rs.)	Balance (Rs.)
Personal- Research student	5,99,066.66	731,296.14	(132,229.48)
Personal- Technical assistant	29,397.00	-	29,397.00
Consumables	425,000.00	387,183.04	37,816.96
Equipment	480,000.00	391,000.00	89,000.00
Miscellaneous	68,000.00	6,999.00	61,001.00
Other	29,397.00	105,156.00	(75,759.00)
Total	1,630,860.00	1,621,634.18	9,226.48

Section 7:

Grantees' signatures



Prof. W.A.J.M. De Costa
Principal Investigator



Dr. D.M. De Costa
Co-Investigator

Progress is satisfactory.



HEAD/DEPARTMENT OF CROP SCIENCE

Comments of the Head of the Department/signature:

Comments of the Dean/signature:

Recommended



03/1/13

Professor K Samarasinghe
Dean / Faculty of Agriculture
University of Peradeniya
Sri Lanka

Head of the Institution's signature



Vice-Chancellor
University of Peradeniya
Peradeniya
Sri Lanka

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