Identification and characterization of traditional rice genotypes for drought tolerance through proteomic approach

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Identification and Characterisation of traditional rice genotypes for drought tolerance through proteomic approach"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me any degree, diploma, fellowship or other similar title of any other University or Society.

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ABBREVIATIONS

ABA	Abscisic acid
ATP	Adenosine triphosphate
^0C	Degree Celsius
cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DIC	Distributed Information Centre
DNA	Deoxyribo Nucleic Acid
ds	Double stranded
DSVS	Drying Score at Vegetative Stage
EC	Electrical Conductivity
g	Gram
HSP	Heat shock protein
H_2O_2	Hydrogen peroxide
IRGA	Infra red gas analyser
IRRI	International Rice Research Institute
KAU	Kerala Agricultural University

Kb	Kilo base
kDa	kilo Dalton
LEA	Late Embryogenesis Abundant
M	Molar
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation- Time of Flight
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrometry
MSI	Membrane Stability Index
µg	Microgram
µl	Microlitre
µM	Micromole
OD	Optical Density
PCR	Polymerase Chain Reaction

pH	Hydrogen ion concentrartion
%	Per cent
RNA	Ribo Nucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl sulphate
Sec	Second
UV	Ultra violet
V	Volts

Introduction

1. INTRODUCTION

Rice is the world's most important staple food crop, which not only provided food but also influenced traditions, religions, culture and life style since Vedic period. 'Rice is life' for human beings especially in Asian subcontinent, where 90 per cent of world's rice is grown and consumed by 60 per cent of population and where, about two-thirds of world's poor live (Khush and Virk, 2000). As an important staple, rice has a central position in global food security and is directly related to alleviating hunger and poverty in Asia and Africa. It is the agricultural commodity with the third-highest worldwide production, after sugarcane and maize, according to data of FAOSTAT 2012. Only 4-5 per cent of world rice production enters the global market. Hence, any shortfall in rice production in the major rice growing countries could be disaster for food security. Moreover, it is the most important grain with regard to human nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by the humans.

Rice is a unique crop as it has ability to grow in a wide range of hydrologic environment. This crop can be cultivated in several ecosystems like upland, rainfed lowland, flood prone and irrigated. It routinely faces abiotic stresses in all these ecosystems except under irrigated ecosystem. Worldwide there are 54 million ha of rainfed lowlands, which contribute 19% of the world's total rice production and 14 million ha of rainfed uplands, which contribute 4% of the world's total rice production (Maclean *et al.,* 2002). Rainfed low lands are characterized by uncertain and erratic rain fall, which destabilizes the yield in yearly fluctuations. Therefore, drought is considered to be one of the major constrait for rice production in the World (Herdt, 1991)

Drought is the largest constraint to rice production, affecting 19 million ha of upland rice and 14 million ha of rainfed lowland rice (Pandey *et al*., 2007). Rice

yields in drought prone rainfed systems remain low at 1.0 to 2.5 t ha^{-1} and tend to be unstable because of erratic and unpredictable rainfall (O'Toole, 2004). The drought vulnerability scenarios are likely to worsen in future with predicted climate change scenarios (Wassmann *et al*., 2009). In the context of current and predicted water scarcity, increasing irrigation is generally not a viable option for alleviating drought problems in rainfed rice growing systems.

Rice productivity is affected by adverse environmental conditions (Jagadish *et al*., 2012), and the reproductive stage water stress is particularly debilitating (O'Toole, 1982). Extensive genetic variation for drought resistance exists in rice germplasm. Increasing crop tolerance to drought stress would be the most economical approach to improve productivity and to reduce agricultural use of fresh water resource. To survive against the stress, plants have evolved a number of morphological, physiological, biochemical and metabolic responses. Many changes in plant due to drought induced gene expression have been revealed and a large number of genes have been identified. Proteins encoded by some of these identified genes have been confirmed to tolerate drought stress and protect cellular structure or involve in the signal transduction pathway.

Among the several factors contributing to enhanced drought resistance root characters are believed to be vital components in the mechanisms of dehydration postponement since they contribute to regulation of plant growth, extraction of water and nutrients from deeper unexplored soil layers (Price *et al.,* 1997; Toorchi *et al.,* 2006). Significant genetic variation exists among different rice cultivars for root morphological traits (O'Toole, 1982) such as root diameter (Armento-Soto *et al.,* 1983), root depth (Kato *et al.,* 2007), root pulling force (Ekanayake *et al.,* 1985; O'Toole 1982), deep root to shoot ratio (Yoshida and Hasegawa, 1982), root number (Armento-Soto *et al.,* 1983), root growth plasticity (O'Toole, 1982; Ingram *et al.,* 1994; Price *et al.,* 2002) and root penetration ability (Ali *et al.,* 2000; Babu *et al.,* 2001; Clark *et al.,* 2008).

Wilkins *et al*. (1996) coined the term "proteome" to refer to the total set of proteins encoded by the genome of an organism. "Proteomics" is the global study of the proteins comprising the proteome including the changes in structure and abundance in response to developmental and environmental cues. In recent years the term proteomics has also been applied to all the proteins expressed in a particular organelle or in response to a particular stress.

The analysis of stress responsiveness in plants is an important route to the discovery of genes conferring stress tolerance and their use in breeding programmes. Proteomic analysis provides a broad view of plant responses to stress at the level of proteins. In recent years this approach has increased in sensitivity and power as a result of improvements in two-dimensional polyacrylamide gel electrophoresis (2DE), protein detection, quantification, finger printing and partial sequencing of proteins by mass spectrometry (MS), MS MALDI-TOF, bioinformatics and methods for gene isolation. 2DE provides information on changes in abundance and electrophoretic mobility of proteins.

With this background the present study was undertaken to screen 15 upland rice genotypes for drought tolerance, and to characterize proteins responsible for drought tolerance in the selected genotype. The identified lines can be utilized for developing drought tolerant lines in rice through conventional as well as molecular breeding methods.

Review of Literature

2. Review of Literature

2.1. Drought:

As plants are sessile they experience many types of abiotic stress on a regular basis. These stresses include extremes in temperature, salinity, water logging, high light, strong winds and drought. Abiotic stresses are the biggest limiting factor in crop yields in many parts of the world, causing an average loss of more than 50 per cent of potential yield (Boyer, 1982). Drought is one of the most important abiotic stresses to which plants are exposed and which affect plant growth and yield.

In meteorological term drought may be defined as lack of precipitation over a prolonged time period. In agronomical term drought refers a situation where any area receives annual rainfall less than its average rainfall. In physiological terms drought a situation where transpiration rate exceeds absorption rate, so plants experiences stress.

According to statistics, the percentage of drought affected land areas more than doubled from 1970s to early 2000s in the world (Isendahl and Schmidt, 2006). Drought is a world spread problem seriously influencing grain production and quality with the increasing population and global climate change making this situation more serious (Hongbo *et al*., 2005).

As world's population grows to an estimated 8.9 billion in 2030, agriculture must respond to the increasing demand for food and compete for scarce water with other users. Many of the over 800 million people in the world who still go hungry live in water scarce regions (FAO report, 2001). Of the 1,500 million hectares of global cropland, only 250 million hectares (17 %) are irrigated. Breeding for drought tolerance is a slow and insufficient process. This situation arises from three problems related to nature of drought stress. First,

screening for drought tolerant landraces is highly sensitive to environmental conditions such as weather, soil chemistry and texture. Second, for each time of one set of drought, all plant tissues are affected which resulted in multiplex responses and complex genetic control of stress. Third, the distribution of rainfall is uneven during the plant growth cycle and plants may face drought during vegetative or reproductive stages which are essentially different challenges. Therefore traits conferring drought adaption in specific environments may differ and the genetic basis of plant responses to drought may involve many gene functions regulated by water availability (Reynolds and Tuberosa, 2008)

Rice genotypes cultivated in Kerala and their characteristics reported by Rosamma *et.al.* (2003) which were used for the present study

Ptb 1 (Aryan)

It is a traditional tall indica variety developed through pure line selection pure line selection from Aryan, a first crop (*Virippu*) variety popular in northern Kerala especially in the districts of Palakkad and Malappuram. Local strain used for crop improvement was collected from Valluvanad- Ponnanithaluks. Pureline selection in the local strain started during 1927 and the improved variety exhibited 20 percent improvement over the local strain used. Ptb1, released in 1934, is the first paddy strain of the then Agricultural Research Station and present RARS, Pattambi. The variety matures in 145 days and the kernel colour is red. The variety is best suited for the first crop season in wet lands, here there is no scarcity for water. It performs well in temporarily flooded areas as well. Average yield is 3000 kg ha^{-1} of grain with a rice recovery is 76.7 percent and the quality is well accepted. The variety possesses tolerance to blast and moderate resistance to white tip nematode.

Ptb 7 (Parambuvattan)

This is a relatively short duration (120 days) first crop variety selected from a drought tolerant local strain *viz.,* Parambuvattan, grown extensively in single cropped pailiyal lands of Ponnani, Valluvanad and Palakkad taluks. The variety is characterised by awned spikelets, which turns black on ripening. Ptb7 was released in 1935. This variety has red kernel colour and has low rice recovery of 58.3 per cent. It adapts to moisture stress during growing periods and can withstand salinity to a certain extent. Its performance is the best in high-level palliyals and yields more than 15 per cent over the parent. The variety is tolerant to gall fly, foot rot and drought. Rice is of high quality and is preferred for certain special preparations

Ptb8 (Thavalakkannan)

This variety is a selection from the local strain Thavalakkannan, which was popular in Malabar region. Distinguishing morphological character of the variety is its purple pigmentation in the apiculusof the spikelet, which helps in easy identification. Grains are short bold resembling eyes of the frog and hence the name 'Thavalakkannan'. Due to the red kernel colour, the strain is popularly known as "Chuvannari Thavalakkannan". Purple colour on leaves and stem enables weeding and removal of wild rice especially in the early growing stages. The variety matures in 130 days and was released during 1936. Average grain yield recorded by Ptb 8 is 2500 kg ha^{-1} and milling recovery is 78.2 percent. The variety can adapt to adverse soil and other conditions. Ptb 8 has got a strong resistance to Green Leaf Hopper (GLH), the vector for Tungro virus disease of rice. A recessive gene (glh 4) conferring resistance to Green Leaf Hopper has been identified in this variety.

Ptb 10 (Thekkancheera)

This is one of the most popular short duration tall indica varieties of South Malabar, suited to all the three cropping seasons. It was selected from the

cosmopolitan popular short duration local strain 'Thekkancheera' through pure line selection and was released during 1936. The duration varies from 90-100 days. Though the strain performs well in all the three seasons, its performance is the best during the third crop (*puncha*) season and yields over 2500kg ha^{-1}. The rice quality is very good and the milling recovery is high.

This variety has been extensively utilized as a parent in many hybridization programmes, especially for the evolution of short duration high yielding photo insensitive varieties in Kerala. As an early duration photo insensitive variety inheriting quite a number of desirable attributes and good combining ability, the variety has won name and fame in other states of the country as well as in other nations. The variety is reported to have genes for better photosynthetic efficiency, translocation efficiency, and better utilization of solar energy. Besides, Ptb 10 exhibits a strong resistance to gall fly. It has moderate resistance to Brown Plant Hopper and stem borer. The extremely short growth duration, good combining ability, resistance to pests and wider adaptability accounts for the wider utilization of this material in the evolution of modern varieties, throughout the country. In Kerala, 17 high yielding varieties of rice have been evolved utilizing this variety as a direct or indirect parent.

Ptb 15 (Kavunginpoothala)

Ptb 15 is a pure line selection from the popular local strain viz; Kavunginpoothala. This is a unique variety, grown as an intermediate crop between regular first crop and second crop seasons, in highly water logged and shallow flooded areas. The special type of cultivation is locally known as '*karimkora*' and Ptb15, since it can withstand water logged condition is well adapted to this type of cultivation. It is a photosensitive *mundakan* variety with white kernel colour. Panicle shape of the variety resembles the inflorescence of arecanut palm and hence the name 'Kavunginpoothala'. Ptb 15 recorded 20 per cent yield improvement over the local

strain and was released during 1941. Duration of the variety varies according to time of sowing and normally it takes 160 to 165 days from seeding to maturity. Plants are vigorous with high level of tillering and grow very tall with robust stem. Grains are long and narrow with a milling recovery of 75.9 per cent. Ptb15 has tolerance to Brown Plant Hopper and Yellow Stem Borer.

Ptb 20 (Vadakkanchitteni)

This popular photosensitive, second crop, tall *indica* variety was isolated from a well known local cultivar of North Malabar, viz., Vadakkanchitteni, also popular as Chuvannachitteni. It matures in 125 days and is having red kernel colour. Milling percentage is 79.3 and is with preferable grain qualities. In die initial evaluation trials, an average increase of 44.7 percent over the parent was recorded by the strain. The average yield is 2500 kg ha^{-1}. Good cooking and grain qualities make the variety more acceptable to the farmers. The highly versatile nature of the strain makes it suitable for cultivation under a wide range of rice growing eco-systems. It is being cultivated in normal wetlands and in special systems of cultivation such as as Karinkora (early *mundakan*). Cultivation of this variety is also popular in acid sulphate soils of kuttanad and sandy soils of Onattukara. Moderate level of resistance to major rice pests like BPH, Gall fly and disease like Sheath blight is an added attribute to this variety.

Ptb26 (Chenkayama)

Ptb26 was developed through pure line selection from the local cultivar "Chenkayama" which is characterized by purple pigmentation of the plant especially on leaf sheath, blade and apiculus. This is an important tall indica variety popular in Palakkad district. Cultivation of this variety facilitates identification of wild rice and similar weeds which 'mimic' rice, thereby making hand weeding more effective. Compared to Thavalakkannan, this is less lodging and shorter in duration, maturing in 120 days. It recorded 25 per cent increase in grain yield over the parent cultivar and

the average yield is 2500 kg ha^{-1}. The rice kernel is red in colour, quality good and milling recovery is 78.1 per cent. This variety is also recommended for *Koottumundakan* system of cultivation. Ptb 26 was released during 1948.

Ptb 28 (Kattamodan)

Ptb 28 is a drought tolerant variety developed through mass selection from Kattamodan, a popular upland race. This is often raised as a rainfed crop and recommended for *modan* cultivation which is a special system of rice cultivation in upland fields of Kerala, in which paddy crop is wholly depending on monsoon showers. Short duration varieties are preferred for this type of cultivation. Performance of Ptb 28 is satisfactory in uplands and also in wetlands. The variety matures in 120 days. Panicles are long with golden grains. Rice quality is good and milling recovery is 77.5 per cent. Ptb 28 was released during 1951. Average yield of the variety is 2700 kg ha^{-1}. Tolerance to drought and high initial seedling vigour makes the variety more adapted to upland cultivation. Tolerance to blast is another special character of Ptb 28.

Ptb29 (Karuthamodan)

Ptb29 was developed through mass selection from a local upland strain viz. Karuthamodan. This is another drought tolerant *kharif season* variety suited to the uplands. The varietv is having black coloured glumes and hence the name Karuthamodan. Being selection from a traditional upland variety, it can tolerate drought and is usually recommended for *modan* cultivation. Ptb29 has duration of 110 days and is suitable for broad casting as well as transplanting. Kernel colour is red and rice recovery is 78.6 per cent. The variety was released during 1951.

Ptb30 (Chuvanna Modan)

This variety was developed through mass selection from Chuvanna Modan, a local *kharif season* upland strain, and was released during 1951. On maturity the

grain develops a brownish red colour which gives an attractive appearance to the panicle. Duration of the variety is 105 days and it has a milling out-turn of 76.5 per cent. Its performance is satisfactory both under broadcasting and transplanting. Considering its drought tolerance character it is particularly recommended for 'modan cultivation'. The kernel colour is red and average yield recorded is 2200 kg ha^{-1}.

Ptb39 (Jyothi)

This is the most widely accepted and popular high yielding rice variety released from Pattambi as well as from the state. It is a short duration high yielding variety with wide adaptability and is grown extensively in a wide range of field conditions in the state in all the three seasons. The variety has good coverage in other states too. This is short in duration (110 to 120 days), red kernelled and photo insensitive. The cross between the famous short duration improved local strain viz., Ptb 10 and the internationally famous high yielding genotype viz., IR8 led to the evolution of this variety. This was the same cross from which Ptb 36 (Rohini), a short duration variety having white rice was released during 1972. Jyothi was released during 1974. The rice recovery is 72.9% and the quality is very good. The variety can be cultivated by transplanting or sowing, including dry sowing. The average yield of the variety is 6 tons per hectare. Jyothi is moderately resistant to BPH and blast; but susceptible to sheath blight. Wider adaptability to different systems of cultivation, soil types and environmental conditions is a unique feature of this variety.

2.2. Crop losses due to drought:

Drought is one of the inherent abiotic constraints that affect agricultural productivity worldwide. It is estimated that drought stress can potentially reduce nearly twenty per cent of crop yield around the world (Scheiermeier, 2008). Water is needed at every phase of plant growth from seed germination to plant maturation (Athar and Ashraf, 2005) and any degree of imbalance in the uptake would pose a

serious threat to agriculture by adversely affecting the growth and grain yield (Wang *et al*., 2001).

Current rice production systems rely on ample supply of water and it is estimated that on an average rice requires 1900 liters of water to produce one Kg of grain. Even a short period of water deficit is highly sensitive to rice farming and rice productivity (O'Toole, 2004). Different developmental stages of rice such as tillering phase, panicle initiation and heading known to respond differently to drought stress (Botwright *et al*., 2008). However, factors such as timing, intensity and duration of stress have detrimental effect on plant growth. Drought is one of the major constraints on rice production. At least twenty three million ha of rice are drought prone. Rice yield is more sensitive to drought during the flowering and grain formation stage.

Luz *et al.* (1997) reported that water stress reduced cotton yield with greatest effect at the flowering and fruiting stages.

Upreti *et al*. (2000) studied the response of pea cultivars to water stress. The results revealed that pea cultivars differed widely in vigour under the conditions of moisture stress. There was significant reduction in plant height in all the cultivars and the effect of moisture stress was more pronounced at vegetative stage than at flowering stage.

The average reduction of 45 per cent in biomass and 67-70 per cent in grain yield has been reported to be due to drought stress in rice (Babu *et al*., 2003). Pantuwan *et al*. (2001) also reported reduction of rice grain yield up to 80 per cent under drought.

Naidu *et al*. (2001) screened the green gram genotypes for drought tolerance under receding soil moisture and they reported about 60 per cent reduction in seed yield under drought stress and attributed to corresponding reduction in yield parameters.

In Thailand, the drought of 2004 alone is estimated to have affected 2 million ha of cropped area and over 8 million people (Asia Times, 2005).

Liu *et al*. (2006) reported that reproductive stage especially during flowering is more vulnerable to stress and cause spikelet sterility. The loss in rice yield during drought years was estimated to be in the range of 25-40 per cent in Jharkhand and Orissa but was almost 100 per cent in Chhattisgarh there was almost complete crop failure during the 2002 drought. The period of flowering delay is partly related to extent of stress the rice genotypes experienced and those with longer delay will tend to produce less grain (Kumar and Kujar, 2003).

In India, major droughts in 1918, 1957-58 and 1965 resulted in famines in the 20^{th} century (FAO, 2001). The 1987 drought affected almost 60 per cent of the total cropped area and 285 million people across India (Sinha, 1999). Similarly, the average annual drought-affected area in China over the period 1978-2003 is estimated at 14 million ha and the direct economic cost of drought is estimated at 0.5-3.3 per cent of agriculture-sector GDP.

Severe droughts can result in starvation and death of the affected population. However, different types of economic costs arise before such severe consequences occur. As a result of market failures, farmers attempt to 'self-insure' by making costly adjustments in their production practices and adopting conservative measures to reduce the negative impact in drought years.

2.3. Different ways to induce drought conditions

Blum (1988) reported that the major criteria to evaluate the performance of genotypes against drought under field conditions are drought score, grain yield and spikelet fertility. Delayed leaf rolling under water stress for dehydration avoidance is also an important selection criterion as the genotypes that have the capacity to maintain high leaf water potential show less leaf rolling.

Screening with aqueous solutions of poly ethylene glycol-6000 and mannitol (Costa *et al.,* 2004; Fanti and Perez, 2004) aided the identification of cultivars having higher levels of tolerance to drought in rice (Pirdashti *et al.,* 2003). According to (Turkan *et al.,* 2005; Landjeva *et al.,* 2008) Screening under stimulated water stress conditions induced by osmotic substances having high molecular weight like polyethylene glycol (PEG) for identification of tolerant genotypes against drought is one of the popular approaches.

Polyethylene glycol is a non-penetrating inert osmoticum that can lower the water potential of nutrient solutions without being taken up or being phytotoxic (Lawlor, 1970). It has been reported that an increase in drought stress by PEG was accompanied by a sudden decline in moisture content of tissues (El-Tayeb and Hassanein, 2000) as PEG mimics in a way similar to soil drying. This approach has been used to simulate drought stress in plants and selection of tolerant genotypes in different crops (Nepomuceno *et al.,* 1998; Cherian and Reddy 2003; Badiane *et al.,* 2004) and it was reported to be an effective strategy for selection at the early growth stages of rice (Jing and Chang, 2003).

2.3.1. Field conditions

Evaluation of genotypes under field conditions in the dry season was found to be ideal for identification of drought tolerant genotypes that are able to retain a large proportion of green living tissues under soil water deficit both at vegetative and reproductive stages (Chang *et al.,* 1974; De Datta *et al.,*1998)

2.3.2. Assessment of drought resistance among wild rice accessions based on single-tiller propagation and PVC-tube cultivation

To assess the variation in drought resistance among wild rice accessions, a protocol was developed based on single-tiller propagation and PVC-tube cultivation. Severe water stress was applied at late vegetative growth stage. The responses of

eight accessions of *Oryza rufipogon* and one accession of *O. officinalis* were evaluated by measuring the morphological and physiological traits, including leaf rolling score, leaf water potential, free proline content, chlorophyll content, above-ground biomass, maximum root depth, maximum root length, root weight, deep root percentage and root/shoot ratio.

2.4. Drought tolerance in rice

Rice being adapted to a semi aquatic environment is extremely sensitive to water shortage. When the soil water content drops below saturation, growth and yield formation are affected, predominantly because of reduced leaf surface area, photosynthesis rate, and sink size (Yoshida, 1982; Bouman and Tuong, 2001).

Rice plants show a clear sensitivity to water stress at early growth stage, during the formation of panicles and flowering and results in reduced yields (Bhattacharjee *et al.,* 1973; De Datta *et al.,* 1973; O'Toole, 1982; Garrity and O'Toole, 1994; Fukai *et al.,* 2001 and Kamoshita *et al.,* 2008). Several researchers have concluded that moisture stress at late vegetative and reproductive stages resulted in reduction of number of panicles per plant, percentage of filled grain and 1000 grain weight. (Krupp *et al.,* 1971; De Datta *et al.,*1973; Cruz and O'Toole 1984; Namuco and O'Toole 1986; Ekanayake *et al.,* 1989; Garrity and O'Toole 1994; Nour *et al*., 1994 and Fabre *et al.,* 2005).

Drought tolerance can greatly enhance the productivity of rainfed rice areas. However, improvement in this direction through selection for yield per se has not been rewarding due to low heritability under stress. It has been suggested that combining selection based on yield with secondary traits into selection indices can improve selective response, if the physiological processes contributing to grain yield in the target environment are well understood and if the secondary traits can be repeatedly and inexpensively measured (Araus *et al.,* 2002; Bernier *et al.,* 2008).

Several traits have been considered important in adaptation to stress. Drought related traits have been categorized as those for drought escape, drought avoidance and drought tolerance. Drought escape is through shorter growth duration to escape terminal drought. Drought avoidance is by either maintaining a favourable water balance or by protecting the cellular functions from dehydration. Such traits include small plant size, deep root system and thick cuticle. Drought tolerance is through mechanisms such as osmotic adjustment, dehydration tolerance (Blum, 1988; Levitt 1980).

Drought avoidance mechanisms can be expressed even in the absence of stress and are then considered constitutive while drought tolerance mechanisms are the result of a response triggered by drought stress itself and are therefore considered adaptive (Bernier *et al.,* 2008). A number of physiological, morphological and phenological traits putatively associated with drought tolerance have been reported and reviewed by many scientists (Ludlow and Muchow, 1990; Fukai and Cooper, 1995; Nguyen *et al.,* 1997; Price and Courtois, 1999; Kamoshita *et al.,* 2008 and Gowda *et al.,* 2011).

One hundred and twenty eight genotypes were evaluated by Pantuwan *et al.* (2004) under non stress and four different types of drought stress conditions. They found that drought stress developed prior to flowering delayed the time of flowering of genotypes, and the delay in flowering is negatively associated with grain yield, fertile panicle percentage and filled grain percentage. Genotypes with a longer delay in flowering time had extracted more water during the early drought period and had high water deficit and were consistently associated with a larger yield reduction under drought.

Kamoshita *et al.,* (2004) evaluated six diverse rice genotypes selected from rainfed lowland germplasm to examine the development of a deep root system and osmotic adjustment, and their relationship with biomass production during drought

and after rewatering, under two different drought durations (shorter and prolonged). Two genotypes NSG19 and KDML105 showed superior drought recovery even after a prolonged drought period in which they suffered a greater reduction in transpiration, water use efficiency, and biomass production which was attributed to with the larger plant size by the end of the drought period rather than with plant water status during drought, such as osmotic adjustment or leaf water potential.

Liu *et al.* (2006) observed a lesser reduction in spikelet fertility in Moroberekan (16%) than in IR64 (80%) after 6 days of with holding water, starting three days before heading. This was attributed to better anther dehiscence and higher stigma pollen density in Moroberekan.

Zou *et al. (*2007) evaluated 187 genotypes under well-watered and drought stress conditions, imposed at panicle initiation stage. The relationship of genotypic variation in yield under drought conditions to potential yield, heading date and flowering delay, reduction in plant height, and to a drought response index (DRI) was detected. The results indicate that genotypes with drought resistance can be identified by measuring yield potential, delay in flowering, reduction in plant height, or DRI under test environments of well-watered and drought stress.

Kato *et al.* (2007) evaluated six *japonica* cultivars varying in drought tolerance by using a system that restricted root growth to no more than 25 cm below the soil surface by means of a water-permeable sheet and showed that genotypes with deeper root development and with larger plant size could maintain higher leaf water potential when there was no root restriction, but suffered from a greater reduction in leaf water potential land increased leaf death score with the presence of root restriction.

Kamoshita *et al.* (2008) divided drought-resistance traits into primary traits, secondary traits, integrative traits, phenology, and plant type traits. Primary traits were further divided into constitutive traits (e.g., rooting depth, root thickness,

branching angle, and root distribution pattern) and induced traits (e.g., hardpan penetration and osmotic adjustment). They also reviewed the association between these traits and yield under drought stress. Secondary traits include maintenance of plant water status, canopy temperature, leaf rolling score, and leaf death score, which are influenced by primary traits. These secondary traits in turn influence integrative traits like spikelet fertility and yield components. The plant-type traits such as tiller number and plant height alter the expression of secondary and integrative traits by affecting transpiration demand.

Genotypes with greater plant height are often larger in overall plant size, intercept more light and use water faster by transpiration, leading to lower plant water status (Pantuwan *et al.,* 2002; Kato *et al.,* 2007).

Farooq *et al.,* (2010) evaluated the *indica* rice cultivar IR64 and four of its near-isogenic lines unique for leaf size traits, for changes in leaf growth and its water status under two soil water regimes, well watered and progressive soil drying. The IR64-derived lines with broader leaves were found to perform better than those with narrow and short leaves under drought.

Chromosome segment substitution lines (CSSLs) derived from Nipponbare and Kasalath crosses were evaluated by Kano *et al.,* (2011) under soil moisture gradients with line source sprinkler system up to around heading. CSSL50 was found consistently to show significantly higher shoot dry matter production than its parent Nipponbare. It showed phenotypic plasticity through greater total root length through promoted lateral root branching and elongation than Nipponbare, which was found to be key trait that effectively contributed to plant dry matter production through increased total root length and thus water uptake.

Several other putative drought tolerance traits have been reported and in some cases their role has been well demonstrated. Genotypic variation in the amounts of

epicuticular wax has been reported. Production of epicuticular wax is observed to increase under water stress

Maintaining ion balance under tissue water deficit is important in drought resistance. Genotypic variation in cell membrane stability (Tripathy *et al.,* 2000; Babu *et al*., 2004) and osmotic adjustment has been demonstrated in rice (Lilley *et al.,* 1996; Babu *et al.,* 2001).

Carbon isotope discrimination often associated with transpiration efficiency and water use efficiency is also reported to vary with rice genotypes (Price *et al.,* 2002).

Haider *et al.,* (2012) studied morphological and yield related traits of twenty (20) genotypes to ascertain the genetic and phenotypic correlation among some drought related and morphological traits and contribution of these traits to the yield under drought stress directly and indirectly in rice.

2.5. Response of plants to drought conditions:

Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields. Understanding the biochemical and molecular responses to drought is essential for a holistic perception of plant resistance mechanisms to water-limited conditions. Drought impacts include growth, yield, membrane integrity, pigment content, osmotic adjustment water relations and photosynthetic activity (Benjamin and Nielsen, 2006; Praba *et al*., 2009). Drought stress is affected by climatic, edaphic and agronomic factors. The susceptibility of plants to drought stress varies independence of stress degree, different accompanying stress factors, plant species and their developmental stages (Demirevska *et al*., 2009). Acclimation of plants to water deficit is the result of different events, which lead to adaptive changes in plant growth and physio-biochemical processes, such as changes in plant structure, growth rate, tissue osmotic

potential and antioxidant defenses (Duan *et al*., 2007). It has become imperative to elucidate the responses and adaptation of crops to water deficit and take actions to improve the drought resistance ability of crop plants and to ensure higher crop yields against unfavorable environmental stresses.

2.5.1. Morphological responses:

Environmental stresses trigger a wide variety of plant responses, ranging from altered gene expression and cellular metabolism to changes in growth and productivity.

2.5.1.1. Growth:

To ensure that food supplies keep pace with population growth, a complete understanding of the processes involved in crop growth and development is required to inform agronomic practices. The optimization of plant performance and crop sustainability under variable environmental stress conditions will be dependent on the degree to which plant vegetative and reproductive growth patterns can be regulated.

Plant growth is a function of complex interplay between sources and sink limitations of the two main organs of a plant, the root system and the shoot, establishing functional equilibrium. The permanent or temporary water deficit severely hampers the plant growth and development more than any other environmental factor.

The first and foremost effect of drought is impaired germination and poor stand establishment (Harris *et al*., 2002). Cell growth is considered one of the most drought sensitive physiological processes due to the reduction in turgor pressure. Growth is the result of daughter-cell production by meristematic cell divisions and subsequent massive expansion of the young cells. Under severe water deficiency, cell elongation of higher plants can be inhibited by interruption of water flow from the xylem to the surrounding elongating cells (Nonami, 1998). Drought caused impaired

mitosis; cell elongation and expansion resulted in reduced growth and yield traits (Hussain *et al*., 2008).

Water deficits reduce the number of leaves per plant and individual leaf size, leaf longevity by decreasing the soil's water potential. Leaf area expansion depends on leaf turgor, temperature and assimilating supply for growth. Drought-induced reduction in leaf area is ascribed to suppression of leaf expansion through reduction in photosynthesis (Rucker *et al*., 1995).

A common adverse effect of water stress on crop plants is the reduction in fresh and dry biomass production (Zhao *et al*., 2006). Khan *et al*. (2001) conducted a study comprising of six treatments, namely, control (six irrigations), five, four, three, two and one irrigation in maize. It was concluded that plant height, stem diameter, leaf area decreased noticeably with increasing water stress. The reduction in plant height could be attributed to decline in the cell enlargement and more leaf senescence in the plant under water stress (Manivannan *et al*., 2007a).

Drought led to substantial impairment of growth related traits of maize in terms of plant height, leaf area, number of leaves/plant, cob length, shoot fresh and dry weight/plant. Furthermore, Kamara *et al*. (2003) revealed that water deficit imposed at various developmental stages of maize reduced total biomass accumulation at silking by 37 per cent, at grain-filling period by 34 per cent and at maturity by 21 per cent.

2.5.1.2. The root

O'Toole (1982) opined that for relatively large soil water reservoir (deep soil), increase in rooting depth, conductance and root to shoot ratio (by weight) results in increased soil water uptake capacity. Passioura (1982) reported that in deep wet soils, large root density at depth is necessary to extract water from deeper layers. Mumbani and Lal (1983) reported similar results in studies conducted on response of upland

rice varieties to drought stress. O'Toole and De Datta (1986) opined that increased rooting depth and density would increase the plant's capacity to extract water in rice. Deep roots may also reduce the production of chemical signals from roots under drought conditions, which may otherwise reduce leaf growth and expansion and stomatal conductance.

Yoshida and Hasegawa (1982) analysed the tillering and rooting pattern of rice plant. A tiller and its roots come simultaneously from the same node that is, when the leaf emerges, a tiller and its roots start emerging from 3rd node and also reported the significant differences in the root length density between upland and lowland rice. Also demonstrated that under upland conditions genotype which has thicker and deeper roots will extract large amount of water from deeper layers and maintain high plant water status.

O'Toole and Bland (1987) reviewed the genotypic variations in root systems and reported that plant root systems have the capability of coping with changes in environment factors such as water and temperature. Rice has a high rooting density in the surface soil compared to other crops, which is attributed to tillering habit of rice.

Ekanayake *et al.* (1985) found predominantly additive gene effects for root volume along with root thickness. However, Price *et al.* (1997) failed to detect any significant additive or dominance gene effects for this trait.

Haque *et al.* (1989) checked the consistency in the production of roots. They used four varieties of the Aus type and four of the hill type along with a drought resistant and a susceptible check. These were grown in aeroponic and hydroponic culture. Root number of the genotypes differed in these two cultures revealing its inconsistent nature.

Root studies are arduous under actual field conditions. For convenience some scientists have conducted root studies in aeroponic or hydroponics. Gomathinayagam

et al. (1988) studied seminal roots of rice seedlings in solution culture and suggested association of long seminal roots with drought tolerance. Similar association between total root length and drought tolerance was observed in aeroponic study by (Gomathinayagam *et al.*, 1992). Several studies have been conducted to determine the root length at different stages of growth. During vegetative stage, the root growth is rapid and declines towards the reproductive stage. Maximum root length was observed at panicle initiation by (Beyrouty *et al.*, 1988). Though root growth was rapid during vegetative stage, Gomathinayagam *et al.* (1988) could not discriminate genotypes with respect to root length at 45 days after sowing.

Zuno-Altoveros *et al.* (1990) conducted an experiment to determine the root volume of some selected upland and lowland varieties. They found that Rikuto Norin12, a Japanese upland variety had very high root volume and a lowland variety, IR20 had low root volume.

Sorte *et al.* (1992) reported 74 per cent reduction in root weight when soil moisture was reduced by 44 per cent. Survival during stress reflects on capacity of roots to function. The drought tolerant genotypes should have greater root weight as compared to upland and drought susceptible cultivars (Vijayalakshmi and Nagarajan, 1994).

Root traits being constitutive traits, provide the ability of the plant to meet the evapotranspirational demand during the drought stress by extracting water from deeper layers of soil (Lilley and Fukai, 1994).

Thick roots persist longer and produce more and larger branch roots, thereby increasing root length density and water uptake capacity (Ingram *et al.,* 1994). Such large diameter roots also have large xylem vessels with higher axial conductance (Fukai and Cooper, 1995). The plant with small number of well-developed tillers has fewer long roots and this result in high root length density at depth.

Sorte *et al.* (1992) quantified reduction in root length under water stress. They imposed water stress for five days at 30 days after sowing and observed 19% reduction in root length when the reduction in moisture content was 44%. Rao *et al.* (1994) studied the root systems under stress. They supplied the roots with water at deeper zones to relieve the stress but the stress was not relieved. This led them to give a divergent conclusion that, water deficit occurring up to a depth of 30-40 cm was critical in determining the drought tolerance and not the length of the root system.

Ingram *et al.* (1994) showed that among the root traits studied, total root length is strongly related to drought tolerance under rainfed upland conditions. Root traits found to confer drought tolerance under rainfed lowland conditions are root length density in the 10- 30 cm soil layer and dynamic shedding of roots and production of root length in response to changing moisture conditions. Toorchi (2001) observed that in the sampling at maturity, significant increases in mean values of root length under low-moisture stress conditions. Grain yield showed maximum reduction (28%) under LMS condition. Lalitha (2001) studied the root and shoot-related traits under WW and LMS conditions. She observed higher expression of RIL's for shoot characters under well-watered (WW) condition than that under LMS (LMS) condition.

Ray *et al.* (1996) observed that root penetrating ability is an important factor for rice drought resistance in areas with soils subjected to both compaction and periodic water deficits. Breeding for root penetration ability is inhibited by difficulties with measuring root traits. Courtosis *et al.* (1996) reported that cultivars of upland origin are more deeply rooted and have larger diameter of main axes compared to cultivars of lowland origin.

Yadav *et al.* (1997) demonstrated a deep thick root system have a positive effect on yield of upland rice under water stress conditions. A number of physiological and morphological traits have been reported to improve the

performance of crops affected by drought. The ability of root systems to provide for evapotranspirational demand from deep soil moisture and capacity for osmotic adjustment forces to consider roots as major drought tolerance traits in rice (Passioura, 1982: Nguyen *et al.,* 1997).

Deep and thick root traits contribute to better growth and higher yield under drought stress and the strong association between root length density and the amount of water extracted has been reported and well demonstrated by several authors (Lafitte and Courtois, 2002 and Chandra Babu *et al.,* 2003).

Kato *et al.* (2007) evaluated six *japonica* cultivars varying in drought tolerance by using a system that restricted root growth to no more than 25 cm below the soil surface by means of a water-permeable sheet and showed that genotypes with deeper root development and with larger plant size could maintain higher leaf water potential when there was no root restriction, but suffered from a greater reduction in leaf water potential and increased leaf death score with the presence of root restriction.

Yoichiro *et al.* (2007) study, were constructed screening facilities to evaluate the performance of rice cultivars under drought conditions and to assess the roles of deep roots. Two experiments were conducted with six rice cultivars, including drought-tolerant and drought-susceptible cultivars, grown in two root environments: In the root restricted treatment, in which root growth was restricted to the surface 25-cm layer, leaf water potential decreased faster in cultivars with a large canopy during drought stress and there was little difference in panicle weight among cultivars. With a normal (unrestricted) root environment, the deepest rooting cultivar (IRAT109) maintained higher leaf water potential during drought, although panicle weight under drought stress was affected by yield potential as well as by deep rooting. Under the intermittent drought stress in the raised bed, deep rooting cultivars accumulated more nitrogen and produced more biomass and the difference in panicle weight between

deep-rooting drought-tolerant and shallow-rooting drought-susceptible cultivars was magnified by the raised bed compared with the yield differences under drought in a normal root environment.

Anbumalarmathi *et al.* (2008) study was undertaken to evaluate 13 parents and their 40 hybrids for drought resistance in order to use them in drought resistant breeding programmes. The parents and their hybrids were studied under PVC pipe condition observations were recorded on seven important root traits *viz.*, root length, root volume, root length density, total number of roots, root thickness, root dry weight and root:shoot ratio. Six genotypes showed significantly superior mean values than grand mean for most of the root traits

Hanamarathi *et al.* (2008) reported that majority of the traditional varieties in rainfed upland tolerate moisture stress and possess strong root system under field condition. Land races Dodiga and Navalisali in early and medium maturity groups respectively, were found significantly superior for yield and productivity traits under varied moisture stress situation over three years.

Drought avoidance mechanisms can be expressed even in the absence of stress and are then considered constitutive while drought tolerance mechanisms are the result of a response triggered by drought stress itself and are therefore considered adaptive (Bernier *et al.,* 2008).

Root traits for drought tolerance in rice (*Oryza sativa*) under controlled (PVC pipes) condition was studied by Ganapathy *et al.* (2010) and were reported significant mean values for seven important root traits *viz.*, root length, root volume, root length density, total number of roots, root thickness, root dry weight and root to shoot ratio.

Chromosome segment substitution lines (CSSLs) derived from Nipponbare and Kasalath crosses were evaluated under soil moisture gradients with line source sprinkler system up to around heading. CSSL50 was found consistently to show

significantly higher shoot dry matter production than its parent Nipponbare. It showed phenotypic plasticity through greater total root length through promoted lateral root branching and elongation than Nipponbare, which was found to be key trait that effectively contributed to plant dry matter production through increased total root length and thus water uptake (Kano *et al.,* 2011).

Pandey *et al.* (2012) in a study involving thirteen genotypes of rice were evaluated for root characters namely, maximum root length, root volume, dry root weight, fresh root weight and root to shoot ratio. The study revealed greater relative variability for all the root characters under irrigated and water stress (rainfed) regimes. The genotypes, Dula, Aditya, IR36 and Browngora were found to be superior to other genotypes with respect to ideal root architecture. They concluded that, these genotypes could be best used as parental lines in introgression breeding programme to derive drought tolerant lines.

2.5.1.3. Leaf rolling scores

At IRRI, it was reported that 100 crosses made among rice genotypes revealed that leaf rolling was increased when one of the parents involved in the cross was tall upland rice (IRRI, 1976). Singh and Mackil (1989) in their studies of genetics of leaf rolling in rice under vegetative stage drought stress reported a major gene for leaf rolling.

For many soils, it takes at least 2 rainless weeks to cause marked differences in drought sensitivity during the vegetative stage and at least 7 rainless days during the reproductive stage to cause severe drought injury.

The ability of the plant to recover after drought was described to be more important than drought tolerance (Maji, 1994). Chang *et al.* (1974) and De Datta *et al.* (1975) also considered drought recovery as the determinant of grain yield under

stress condition. Also Malabuyoc *et al.,* (1984) stated from their studies that, poor recovery from stress could be a major cause of reduced grain yield.

Table 1. Leaf rolling scores description

Scale	Description
0	Leaves healthy
1	Leaves starts to fold
3	Leaves folding (deep V- shaped)
5	Leaves fully cupped (U- shaped)
7	Leaves margins touching (O-shaped)
9	Leaves tightly rolled

Table 2. Drought score at vegetative stage

Scale	Description	Rate
0	No symptom	Highly resistant
1	Slight tip drying	Resistant
3	Tip drying extended to ¼ length in most leaves	Moderately resistant
5	¼ to ½ of the leaves fully dried	Moderately susceptible
7	More than 2/3rd of leaves fully dried	Susceptible
9	All plants apparently dead	Highly susceptible

2.5.2. Physiological responses:

2.5.2.1. Root signaling under drought stress:

An extensive root system is advantageous to support plant growth during the early crop growth stage and extract water from shallow soil layers that is otherwise easily lost by evaporation. There are controversial evidences on effect of drought

stress on root growth. An increased root growth due to water stress was reported in *Catharanthus roseus* (Jaleel *et al*., 2008). However, the root growth was not substantially inhibited under water stress in maize (Sacks *et al*., 1997).

Generally, when water availability is limited, the root: shoot ratio of plants increases because roots are less sensitive than shoots to growth inhibition by low water potentials (Wu and Cosgrove, 2000). Under drought stress conditions roots induce a signal cascade to the shoots *via* xylem causing physiological changes eventually determining the level of adaptation to the stress.

Abscisic acid (ABA), cytokinins, ethylene, malate and other unidentified factors have been implicated in the root–shoot signaling. This drought induced root-to-leaf signalling through the transpiration stream results in stomatal closure, which is an important adaptation to limited water supply in the field.

ABA promotes the efflux of K+ ions from the guard cells, which results in the loss of turgor pressure leading to stomata closure. Dehydration of plants has been shown to cause ABA level increase up to 50-fold due to loss of cell turgor or cell membrane perturbation (Guerrero and Mullet, 1986). In addition, the dominant role of ABA as a root to shoot signal has been challenged by experiments showing that the ABA concentrations of xylem sap from drought stressed plants were much lower than the concentrations of exogenous ABA required to close stomata in detached leaves (Munns and King, 1988). Overall, ABA is a dominate signal in controlling growth and transpiration, but other factors could also be important.

Cytokinins could also be an important signal traveling from roots to the shoots. Root-produced cytokinins are clearly involved in responses to nutrient deprivation and as they are produced mainly in roots, could be important in drought responses (Schachtman and Shin, 2007). Although recent data show decreased cytokinin concentrations in the xylem under drought stress, it is still not clear that all

plant species respond in the same way to cytokinin at the concentrations found in the leaf and guard cells (Dodd, 2003).

2.5.2.2. Proline:

Proline accumulation is a common metabolic response of higher plants to water deficits, salinity stress and has been the subject of numerous reviews over the last 20 years (Rhodes *et al*., 1999). Proline protects membranes and proteins against the adverse effects of high concentrations of inorganic ions and temperature extremes (Santoro *et al*., 1992). Proline may also function as a protein-compatible hydrotrope Srinivas and Balasubramanian (1995) and as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).

This highly water soluble imino acid is accumulated by leaves of many halophytic higher plant species grown in saline environments Briens and Larher (1982), in leaf tissues and shoot apical meristems of plants experiencing water stress Jones *et al*. (1980), in desiccating pollen Lansac *et al*.(1996), in root apical regions growing at low water potentials Sharp *et al.* (1994) and in suspension cultured plant cells adapted to water stress (Rhodes *et al*., 1986).

In winter wheat the hydroxyproline-resistant lines are significantly more frost tolerant than wild-type (Dorffling *et al*., 1993). Salt tolerant and polyethylene glycol resistant mutants of *Nicotiana plumbaginifolia* have been derived from protoplast culture and appear to have enhanced proline accumulation in comparison to wild-type (Sumaryati *et al*., 1992).

Proline accumulation in maize root apical meristems in response to water deficits involves increased proline deposition to the growing region and appears to require abscisicacid (ABA) (Sharp *et al*., 1994).

2.5.2.3. Stomatal conductance

Another mechanism of drought avoidance in the rice shoot is quick stomatal closure which acts to reduce water loss (O'Toole and Cruz, 1980). Genotypic differences in the sensitivity of stomatal conductance to leaf water status have been reported (Dingkuhn *et al*., 1989; Dingkuhn *et al*., 1991; Price *et al*., 1997; Hoque and Kobata, 1998). The stomata of rice plants close noticeably in response to a reduction in leaf water potential causing marked reduction in photosynthetic rate (Hirasawa *et al*., 1999). Yeo *et al*. (1997) observed that plants had a stomatal conductance greater than expected for their carbon assimilation rate. They concluded that improvement in water acquisition is important than decreasing water loss. The contribution of stomatal conductance to drought performance in the field is yet unknown. However, a plant with sensitive stomata would only be adapted to a situation of relatively severe drought (Price and Courtois, 1999). Price *et al*. (1997) reported varietal differences for stomatal response and its contribution to drought tolerance in Bala rice variety which could be because of better osmotic adjustment.

2.5.2.4. Transpiration

Transpiration is a vital process in the life cycle of plants, which gives cooling effect besides promoting water and nutrient absorption (O'Toole and De Datta, 1986). The rate of water intake is determined largely by the rate of water loss by transpiration (Kramer, 1937) and is most sensitive to water stress (Hsiao, 1973). Transpiration rate reduced markedly by water stress (Dingkuhn *et al*., 1989; Kobata *et al*., 1996; Cabuslay *et al*.,1999; Wade *et al*., 2000; Ravindrakumar *et al*., 2003). Cultivar differences were reported by many authors (Kobata *et al*., 1996; Cabuslay *et al*., 1999; Wade *et al*., 2000). In general cultivars with high relative transpiration were rated tolerant on the basis of leaf rolling and leaf drying, which further supports

the role of transpiration in water uptake and cell enlargement. A high transpiration rate under conditions of water deficit also implies high stomatal conductance, which is associated with continued water extraction (Cabuslay *et al*., 1999 and Kamoshita *et al*., 2000). The results on genotypic variation in relative transpiration by Cabuslay *et al*. (1999) suggested that, drought tolerant genotypes maintained fairly open stomatas under stress.

Relative transpiration during water deficit was highly and positively correlated with relative leaf area (Cabuslay *et al*., 1999), which is expected because the leaf is the organ of transpiration. Leaf expansion is much more sensitive to water stress and maintenance of leaf area is necessary under rainfed environments. At the onset of drought and especially when solar radiation is high, having an initially large leaf area may be disadvantageous to plants because of high transpirational demand that is not met due to limited water supply. Initial leaf area was negatively correlated with relative transpiration and positively correlated with visual drought score. This indicates that small leaf area initially gives the advantage of having less leaf surface exposed to intense solar radiation so that, at the onset of drought, photorespiration and water loss from leaf tissues are minimized.

2.6. Molecular basis of drought tolerance:

Genes induced during water-stress conditions are thought to function in protecting cells from water deficit by production of important metabolic proteins and regulation of genes for signal transduction in water-tress response. Recently, a number of droughts - responsive genes were cloned and characterized from different plant species (Nepomuceno *et al*., 2000). Transcription of many of these genes is unregulated by drought stress. Initial attempts to develop transgenics (mainly tobacco) for abiotic stress tolerance involved ‘‘single action genes’’ i.e., genes responsible for modification of a single metabolite that would confer increased tolerance to salt or drought stress. Stress-induced proteins with known functions such

as water channel proteins, key enzymes for osmolyte (proline, betaine, sugars such as trehalose, and polyamines) biosynthesis, detoxification enzymes, and transport proteins were the initial targets of plant transformation.

Various genes respond to drought stress in several species, and functions of their gene products have been predicted from sequence homology with known proteins. Such genes that are induced during drought stress conditions provides tolerance to plant by functioning directly in protecting cells from water deficit by the production of important metabolic proteins and indirectly by regulating other genes for signal transduction (Shinozaki and Yamaguchi-Shinozaki 1996).

These gene products can be classified into three major groups:

1. Those that encode products and directly protect plant cells against stresses.
E.g. Heat stress proteins or chaperones, LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes and free radicals scavengers (Bray *et al*., 2000).

2. Those that are involved in signaling cascades and in transcriptional control.
E.g. MAPKs, CDPKs (Ludwig *et al*., 2004) and kinase (Zhu, 2001), phosopholipases and transcriptional factors (Shinozaki and Yamaguchi-Shinozaki, 2000).

3. Those that are involved in water and ion uptake and transport such as aquaporins and ion transporters .

Drought associated genes and proteins

2.6.1.1. Heat shock proteins and chaperones

Heat shock proteins (Hsps) and molecular chaperons, as well as late embryogenesis abundant (LEA) protein families, are reported to be involved in plant drought stress tolerance (Wang *et al*., 2004). High temperature and drought stress can cause denaturation and dysfunction of many proteins.

Hsps and LEA proteins help to protect against stresses by controlling the proper folding and conformation of both structural (*i.e.* cell membrane) and functional (*i.e.* enzymes) proteins (Almoguera and Jordano, 1992). Small hsps are also found to be associated with plant desiccation tolerance. These act as molecular chaperones during seed dehydration and first few days of rehydration (Hoekstra *et al.*, 2001). It has been shown that two of hsps, hsp 70 in maize and hsp 27 in soybean can also be induced by water stress (Sachs and David, 1986).

Over expression of LEA proteins was correlated in several cases with desiccation tolerance, although the actual function of these proteins is still unknown (Villalobos *et al.,* 2004). Over expression of HVA1, a group 3 LEA protein isolated from barley (*Hordeum vulgare*) conferring dehydration tolerance to transgenic plants was reported (Chandra Babu *et al.,* 2001).

Wade *et. al.* (2002) reported three-week old plants of rice (*Oryza sativa* L. cv CT9993 and cv IR62266) developed gradual water stress over 23 days of transpiration without watering, during which period the mid-day leaf water potential declined to ~2.4 MPa, compared with ~1.0 MPa in well-watered controls. More than 1000 protein spots that were detected in leaf extracts by proteomic analysis showed reproducible abundance within replications. Of these proteins, 42 spots showed a significant change in abundance under stress, with 27 of them exhibiting a different response pattern in the two cultivars. However, only one protein (chloroplast Cu-Zn superoxide dismutase) changed significantly in opposite directions in the two cultivars in response to drought. The most common difference was for proteins to be up-regulated by drought in CT9993 and unaffected in IR62266; or down-regulated by drought in IR62266 and unaffected in CT9993. By 10 days after rewatering, all proteins had returned completely or largely to the abundance of the well-watered control. Mass spectrometry helped to identify 16 of the drought-responsive proteins, including an actin depolymerizing factor, which was one of three proteins detectable under stress in both cultivars but undetectable in well watered plants or in plants 10

days after rewatering. The most abundant protein upregulated by drought in CT9993 and IR62266 was identified only after cloning of the corresponding cDNA. It was found to be an S-like RNase homologue but it lacked the two active site histidines required for RNase activity. Four novel drought-responsive mechanisms were revealed by this work: up-regulation of S-like RNase homologue, actin depolymerizing factor and rubiscoactivase, and down-regulation of isoflavonereductase-like protein.

Huaqin *et al.* (2009) reported, the differential expression of proteins and phosphoproteins induced by drought in rice using proteomic approaches. Three drought-responsive proteins were identified. Late embryogenesis abundant (LEA)-like protein and chloroplast Cu–Zn superoxide dismutase (SOD) were up-regulated by drought whereas Rieske Fe–S precursor protein was down-regulated. Ten drought-responsive phosphoproteins were identified: NAD-malate dehydrogenase, OSJNBa0084K20.14 protein, abscisic acid- and stress-inducible protein, ribosomal protein, drought-induced S-like ribonuclease, ethylene-inducible protein, guanine nucleotide binding protein beta subunit-like protein, r40c1 protein, OSJNBb0039L24.13 protein and germin-like protein 1. Seven of these phosphoproteins have not previously been reported to be involved in rice drought stress. These results provide new insight into the regulatory mechanism of drought-induced proteins and implicate several previously unrecognized proteins in response to drought stress.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Identification and characterization of traditional rice genotypes for drought tolerance through proteomic approach" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period of 2013-2015. The materials used and methodologies adopted are discussed in this chapter.

3.1. Laboratory chemicals, equipment and machinery

The chemicals utilized in this study were procured from Bio-Rad, Merck India Ltd., HIMEDIA and Bangalore Genei Ltd. All the plastic wares were obtained from Tarson India Ltd., and borosilicate glass wares were used.

For sterilization of glasss wares and supplies hot air oven and autoclave were used. High precision electronic balance (Shimadzu), pH meter (EuTech Instruments PC 510), micropipettes (Eppendorf), Icematic (F100 compact) and high speed refrigerated centrifuge (KUBOTA 6500) were used for protein extraction. Extracted protein samples were stored at -80^{o}C and reagents were stored at -20^{o}C (SANYO medical freezer). Some chemicals were stored at 4^{o}C in refrigerator (Samsung). First dimensional isoelectric focusing was carried out in 2D (Protean^{R}i12^{TM} IEF cell- BioRad), second dimensional SDS-PAGE separation was carried out in large and small units (Protean^{R} II xi cell- BioRad) circulatory cooler (Analab) was connected to the SDS unit to prevent preheating of the samples. Staining was aided by Rocker 25 (Labnet). The gels were visualized and converted into digital image data using the GS-900™ calibrated densitometer (BioRad). Softwares used for the analysis of the digital image data are Quantity one and PD Quest.

3.2. Experimental material

The material for the study comprised of fifteen traditional genotypes of rice (*Oryza sativa* L.) collected from 1) Regional Agricultural Research Station (RARS), KAU, Pattambi, Palakkad 2) Regional Agricultural Research Station, KAU, Ambalavayal. The list of genotypes included in the study is given in Table 3

Table 3.Plant material selected for the study

S. No	Genotype	Source and details of genotype
1	Karuthamodan	RARS, Pattambi. Traditional rice variety recommended for uplands
2	Chuvanna Modan	RARS, Pattambi. Drought tolerant variety
3	Karanavara	RARS, Ambalavayal Traditional rice variety recommended for uplands
4	Thekkancheera	RARS, Pattambi. Traditional rice variety with short duration.
5	Vadakanchitteni	RARS, Pattambi. Photosensitive and being cultivated in wet lands.
6	Navara Black	RARS, Ambalavayal
7	Thavalakkannan	RARS, Pattambi. Traditional rice variety less prone to lodging.
8	Chenkayama	RARS, Pattambi. Released during 1948, less lodging and short duration
9	Parambuvattan	RARS, Pattambi. Traditional rice variety recommended for uplands
10	Kalladiyaran	RARS, Pattambi. Traditional rice variety recommended for uplands
11	Kavunginpoothala	RARS, Pattambi. Traditional rice variety recommended for flooded condition.

12	Kattamodan	RARS, Pattambi. Drought tolerant variety
13	Jyothi	RARS, Pattambi. High yielding variety released in 1974 (PTB 10 x IR-8)
14	Kanali	RARS, Ambalavayal
15	Aryan	RARS, Pattambi. Traditional rice variety recommended for wet lands

3.3. Cultural Operations in raising seedlings

Seeds of different genotypes were treated with *Pseudomonas fluorescens* solution for overnight, the seeds were then covered in a cloth and incubated for 24-48 hrs for sprouting, and the sprouted seeds were sown in pots. After 25 days of sowing, seedlings were transplanted in poly bags of 100 cm length and 60cm diameter filled with potting mixture in three replications and also transplanted on poly bags of 100 cm length and 30 cm diameter with single plant per bag in two seasons. The transplanted seedlings were maintained in field capacity without stagnation of water and at reproductive stage (visual panicle initiation) water stress was imposed to rice seedlings (Plate2).

Leaf drying score at vegetative stage stress (DSVS): Leaf drying score was recorded based on visual observations as per the standard evaluation system (IRRI, 1996) under stress.

3.4. Phenotyping of genotypes for drought tolerance

The physiological observations were recorded by using IRGA, later the intact roots pulled out from the polybags were evaluated for water mining traits.

3.4.1. Physiological traits

The observations on physiological traits were recorded during peak stress condition at reproductive stage. Similar observations were recorded under control condition also.

3.4.1.1. Photosynthesis related traits:

Photosynthetic rate and other gas exchange parameters were measured on the second fully expanded leaf of three representative plants per genotype with a portable photosynthesis system (LI-6400 LICOR, Nebraska, Lincoln, USA) as shown in plate 4. The measurements were taken between 9.00 AM to 11.00 AM on all sampling dates. The measured parameters were as follows:

Parameters		Units
Transpiration rate (TR)	:	m mol of H_2O m^{-2} s^{-1}
Stomatal conductance (SC)	:	µ mol of CO_2 m^{-2} s^{-1}
Electrical Conductivity (EC)	:	μsm^{-1} or dsm^{-1}
Membarane Stability Index	:	%

Based on EC value Membrane stability Index (MSI) % was calculated by using a formula = (1-(EC2/EC1)*100)

3.4.2. Evaluation of root phenology of selected genotypes for water mining traits

Observations were recorded for maximum shoot and root lengths, total root volume, total shoot and root dry weight, and root to shoot ratio for all the fifteen genotypes for identifying the drought tolerant and susceptible genotypes.

The poly bags were cut open and loosened the soil by watering. The roots were washed thoroughly. Then the following observations were recorded

3.4.2.1. Maximum root length (cm)

Root length was measured from crown of the root to tip of the root and expressed in centimeter as shown in plate 3.

3.4.2.2. Total root volume (ml)

The total root volume of each genotype was taken by water displacement method as shown in plate 3.

3.4.2.3. Total shoot and root dry weight (g)

The plant samples with intact shoot ant roots together were dried in hot air oven for 2 days at 70 degree Celsius. The dry weights of three replications have been recorded by using electronic balance and the mean value is taken, expressed in grams.

3.4.2.4. Root to Shoot ratio

After weighing the root and shoot dry weights the root to shoot ratio was measured for each sample.

3.5. Statistical analysis

The statistical analysis of the data on individual characters of selected genotypes was performed using OP stat for completely randomized design with two factor analysis by fisher method of analysis of variance (Gomez and Gomez 1976).

Genotypes selected for the study

Liquid formulation of *Pseudomonas*

Seed treatment with *Pseudomonas*

Incubation of seeds for germination

Plates 1. Pre sowing operations for seed germination

Plate 2. Evaluation of rice genotypes for response to moisture stress in polybags

1. Karuthamodan
2. Chenkayama
3. Karanavara
4. Thekkancheera
5. VadakanChitteni
6. Navara Black
7. Thavalakkannan
8. ChuvannaModan
9. Parambuvattan
10. Kalladiyaran
11. Kavunginpoothala
12. Kattamodan
13. Jyothi
14. Kanali
15. Aryan

Measurement of root lengths of different genotypes

Measurement of root volume of different genotypes

Plates 3. Evaluation of water mining traits

Plate 4. Monitoring of stomatal conductance (µ mol of CO_2 m^{-2} s^{-1}) and transpiration rate using IRGA (m mol of H_2O m^{-2} s^{-1})

Plate 5. Measurement of Electrical Conductivity using EUTECH CON-510 (µsm^{-1} or dsm^{-1})

3.6. Analysis of the Proteome for drought tolerance

Based on phenotypic screening the most tolerant and susceptible genotypes for drought was identified and they were used for proteome analysis.

3.6.1. Collection of leaf Sample

Rice leaf samples from treated and control plants of highly tolerant and susceptible genotypes Chuvanna Modan and Parambuvattan respectively were collected in liquid Nitrogen. The samples were packed in aluminium foil and stored at -80^0 C for further analysis.

3.7. Proteome Analysis

3.7.1. Protein extraction from rice leaf

The extraction procedure was based on the reports of (Damerval *et al.,* 1986 and Kamo *et al.*, 1995) with some modifications. One gram of rice leaf was ground into fine powder using autoclaved and prechilled mortar and pestle in liquid nitrogen. This fine powder was then added to an Oakridge tube containing 1 ml TCA extraction buffer (A) (pre-cooled at -20ºC). These tubes were then kept in freezer at -20 for one hour for incubation. These were then centrifuged at 12000 rpm for 15 minutes in a precooled rotor (precooled up to 4ºC). Supernatant was discarded and 10ml wash buffer was added and kept for one hour incubation at 20ºC. Tubes were again centrifuged at 12000rpm for 15 min (4ºC). Supernatant was discarded and washing was carried out for 2 more times. The pellet was lyophilized and stored at -80ºC.

3.7.2. Protein solubilisation

Fifteen microgram of lyophilized sample protein was suspended in 250µl of lysis buffer and incubated for one hour at 37^{o}C with intermittent vortexing at 10 minutes interval. These were then centrifuged at 12000rpm for 15 minutes at room temperature. The clear supernatant was transferred to eppendorf tubes and stored at -80^{o}C.

3.7.3. Protein quantification

Protein quantification was carried out with the help of protein estimation kit (Lowry's method). For estimation of total protein using Lowry's method BSA standards were prepared. One millilitre of distilled water was added to 5g of BSA, from which 0.1ml was taken and mixed with 0.9ml distilled water to make the concentration to 0.5mg/ml.

Complex forming reagent was prepared by mixing solution I and solution II in the ratio 1:100. BSA standards were prepared with following concentrations: 0.05µg/µl,0.1µg/µl, 0.2µg/µl, 0.3µg/µl and 0.4µg/µl. Two millilitre of complex forming reagent was added to 200µl of BSA standards and protein samples mixed well and kept at room temperature for 10 minutes. 0.2ml of solution III was then added to each of the tubes and was kept for 30 minutes incubation. The optical density was measured in spectro-photometer at 660nm.

3.8. Protein profiling

The isolated protein was confirmed by SDS-PAGE before analysis by 2D-Page.

3.8.1. Sample treatment

Twenty microlitre of protein sample having 50 µg concentrations from four samples were mixed well with 5 µl of sample loading buffer in a microfuge tube

boiled for 3 min at 95^0 C and quickly snap cooled on ice. Similarly, 20 µl of ready to use markers were boiled for 1 min and quickly snap cooled on ice.

3.8.2. Standardization of SDS-PAGE protocol

SDS PAGE (Sodium dodecyl Sulphate- Polyacrylamide Gel Electrophoresis) analysis was done by the standardized protocol (Laemmili, 1970) with minimum modifications by adjusting the APS and TEMED concentrations.

3.8.3. SDS-PAGE analysis:

The plates were washed well with tap water and wiped with distilled water followed by ethanol. The plates were then assembled in a gel casting apparatus. Separating gel mixture of 12 per cent was prepared (volume according to thickness of spacer and plate size used) and poured into the plates. A layer of water about one cm was poured over the gel mix and allowed to solidify for 45 min with the presence of light and absence of air. After solidification, the layer of water was removed by tilting, washed and dried with filter paper. Stacking gel mixture of 4 per cent (volume according to the thickness of spacer and plate size used) was then prepared and poured above. Appropriate comb was placed at the top immediately and allowed to solidify for 30 min.

After complete polymerization, the plates were separated from the casting apparatus and fixed in the gel running gasket vertically. The inert plate was fixed on the other side and the set up was tightened before placing inside the buffer tank containing 1X tank buffer. The combs were then removed carefully and the wells were washed thoroughly to remove the unbound acrylamide (Annexure I)

Treated samples containing equal volume and concentration of proteins derived from different treatments were loaded into the wells. The medium range molecular weight marker was then loaded and electrophoresis was carried out at constant voltage of 80 V until the samples travelled through the stacking gel. Then the voltage was adjusted to 120 V until the samples reached the bottom of the separating gel.

To prevent the sample from degradation due to the heating of the buffer while running, polar packs were kept on both sides of the buffer tank for the small unit. The large unit was connected to a cooling water circulator (Analab).

When the tracking dye reached the bottom of the plates, the power was switched off and the chords were removed. The gel running gasket containing the plates was taken out and the lock was relaxed. The inert plate was taken first followed by the glass plate assembly. The two plates were then separated carefully using the tool provided with the set up and the fragile gel sticking to one of the plates was removed patiently with the help of water squeeze without breaking the gel.

3.9. Staining

3.9.1. Silver staining

The gel box was taken out of the tank and gel was removed from the glass plates and placed on a tray containing the fixer solution. After an incubation of 10 minutes the fixer was drained out and the gel was washed with distilled water twice. Gel was then incubated for 10 minutes with silver stain. The stain was removed and the gel was washed again with distilled water. Later developer was added to the gel,

and was drained out as soon as the spots appeared on the gel and the fixer was poured over the gel. Fixer was then decanted after 3-4 minutes. (Annexure II)

3.9.2. Coomassie brilliant blue staining

The gel was transferred to staining solution and kept overnight with uniform shaking on rocker shaker. The next day the gel was immersed into destaining solution with uniform shaking for one and half hours and the process was repeated at least twice until the background of the gel became colorless. (Annexure III)

3.10. Documentation

The protein profile was viewed in white light trans illuminator and documented by placing over a conversion screen in gel documentation unit connected to the computer having Quantity 1 software (Biorad) using epi-white option.

3.11. Peptide mass fingerprinting by MALDI-TOF/MS and *In-silico* analysis

The differentially expressed spots were identified, cut from the gel and sent for peptide sequencing by Matrix Assisted Lazer Desorption/ Ionization – Time of Flight Mass Spectrometry (MALDI-ToF/MS). The peaks obtained were analysed with the online bioinformatics tool, MASCOT/MS peptide search engine for the characterization of proteins.

3.12. 2-Dimensional Gel Electrophoresis using Protean^{R} i12^{TM} IEF cell- BioRad unit

3.12.1. IPG strip rehydration

The solubilized protein was mixed with appropriate amount of rehydration buffer to make up the concentration suitable for focusing. Appropriate volume of this mixture is then pipetted as a line along the back edge of channel of the rehydration tray. Care was taken not to insert any bubbles. Coversheet of the IPG strips of p^{H} 3-10 were peeled off and the strips were placed gel side down onto the sample in the rehydration tray precaution was taken not to trap any air bubbles beneath the strips. 2 to 3 ml of mineral oil was added on top of the strips to prevent evaporation during rehydration step. The rehydration tray was covered using plastic lid and the tray was left on a level bench overnight (11 to 16 hours). (Annexure V)

3.12.2. Isoelectric focusing

Clean and dry PROTEAN IEF focusing tray the same size as that of the IPG strips were placed on the lab bench. Paper wicks were dipped in distilled water and placed on both ends of the channels covering the wire electrodes using forceps. The lid was removed from the rehydration tray, the strips were taken out and the mineral oil was drained out from the strips using tissue paper. The IPG strips were then transferred to focussing tray (gel side down configuration). Mineral oil was added over the strips as before and focussing was done 600V for 1 hr, 1000V for 1hr and 35000 Vhr for and the tray was placed in the PROTEAN IEF cell. A three step protocol was programmed in the PROTEAN IEF cell, default temperature was setup to be 20^{0}C and maximum current of 50µA/strip. The electrophoresis run was initiated by pressing the START button as depicted in Plate 6. (Annexure V)

3.12.3. Second dimensional focusing

After completion of first dimensional focusing the strips were taken out and the oil was drained out. Equilibration buffer I was added to the channels of fresh rehydration tray and strips were placed on these channels. This setup was then kept in shaker for 10 minutes. At the end of 10 minutes the setup taken from the shaker and the buffer was decanted. Equilibration buffer II was added to each of the strips and once again the setup was placed on the shaker for 10 minutes. During the incubation of 10 minutes overlay agarose was melt with a help of microwave oven. After the incubation the buffer was decanted. SDS-PAGE gel was prepared and poured onto the plates. The strips were taken out of the tray and dipped in 1X TGS (tris-glycine-SDS) buffer. The strip was then inserted into plates containing the solidified gel in such a way that the strip should touch the gel using forceps. Care was taken not to introduce any bubbles. Over the strip melted agarose was poured using a pipette. The plate having the gel was then mounted over the gel box. The tank was then filled with 1X TGS buffer and electrophoresis was initiated. Electrophoresis was carried out at 20mA for 6hrs and at 20 ^{0}C until the dye front was approximately 1 mm from the bottom of the gel. All gels were stained with colloidal Coomassie Brilliant blue G-250 or with Silver staining.

3.13. Staining

3.13.1. Silver staining

The gel box was taken out of the tank and gel was removed from the glass plates and placed on a tray containing the fixer solution. After an incubation of 10 minutes the fixer was drained out and the gel was washed with distilled water twice. Gel was then incubated for 10 minutes with silver stain. The stain was removed and the gel was washed again with distilled water. Later developer was added to the gel,

and was drained out as soon as the spots appeared on the gel and the fixer was poured over the gel. Fixer was then decanted after 3-4 minutes. (Annexure II)

3.13.2 Coomassie brilliant blue staining

The gel was transferred to staining solution and kept overnight with uniform shaking on rocker shaker. The next day the gel was immersed into destaining solution with uniform shaking for one and half hours and the process was repeated at least twice until the background of the gel became colorless. (Annexure III)

3.14. Documentation

The protein profile was viewed in white light transilluminator and documented by placing over a conversion screen in gel documentation unit connected to the computer having Quantity 1 software (Biorad) using epi-white option.

3.15. Peptide mass fingerprinting by MALDI-TOF/MS and *In-silico* analysis

The differentially expressed spots were identified, cut from the gel and send for peptide sequencing (Sandor Proteomics, Hyderabad) by Matrix Assisted Lazer Desorption/ Ionization – Time of Flight Mass Spectrometry (MALDI-ToF/MS). The peaks obtained were analysed with the online bioinformatics tool, MASCOT/MS peptide search engine for the characterization of proteins.

Plate 6. Overview of 2D gel electrophoresis

Results

4. RESULTS

The traditional genotypes of rice were screened using morpho-physiological parameters at the reproductive stage i.e., visual panicle initiation stage for water stress. The phenotypically drought tolerant and susceptible genotypes were further characterized on molecular basis. The results of different experiments carried out to identify and characterize the traditional rice genotypes and further to characterize the proteome related to drought tolerance. The characterization of genotypes for water stress was carried out by analyzing the proteome using SDS-page (1D) and 2D gel electrophoresis.

4.1. Identification of drought tolerant genotypes by phenotypng traditional rice genotype according to IRRI scores

After visual panicle initiation water was with held continuously in rice grown in polybags and the plants which showed wilting symptoms were screened according to IRRI leaf score 7.0. The plant Chuvanna Modan took 25 days to show wilting symptoms at the IRRI leaf rolling score of 7.0 and was identified as drought tolerant genotype and the genotype Parambuvattan took only 11days to wilt was identified as susceptible genotype. These genotypes were selected for proteomic analysis. The genotypes were showing significant variation in number of days taken for wilting. (Table 4). The effect of water stress on plants can be seen in Plate no 7

4.2 Morpho-physiological observations

When 90% of plants have reached IRRI score 7.0 morpho-physiological observations were recorded.

4.2.1 Morphological observations

The morphological observations analyzed were water mining traits like- root length, root volume, root dry weight, root to shoot ratio

4.2.1.1 Measurements on root length

The mean of rice root length was given in (Table 5). The root length of each genotype significantly varied under water stress condition. Under water stress the genotypes Parambuvattan, Karuthamodan and Chuvanna Modan had the highest root lengths of 93.33 cm, 93.00 cm and 92.67 cm respectively and the genotype Kanali had lowest root length of 58.00 cm when compared with control plants.

4.2.1.2 Measurement on total root volume

The effect of water stress on root volume also varied among the genotypes. The highest root volume was recorded in Karanavara followed by Karuthamodan and Chuvanna Modan as (163.33 ml, 123.33 ml and 120 ml) respectively and lowest in Thavalakkannan followed by Navara Black and Chenkayama (13.33 ml, 20.00 ml and 20.00ml) respectively when compared to control plants. There was significant difference between the genotypes (Table 6)

4.2.1.3 Measurement on total root dry weight

Root dry mass varied significantly among the genotypes and in many genotypes root dry mass in water stress condition was on par with the control plants. The highest root dry weight of 10.78g was recorded in Chuvanna Modan followed by 10.07 g in Karuthamodan and lowest in Thekkancheera (1.40 g) followed by Thavalakkannan (1.99 g) in water stress condition. Chuvanna Modan recorded (9.47g) with highest root dry wt. followed by Karnavara and (8.19 g) and lowest in Thekkancheera (0.99 g) followed by 1.50 g in Thavalakkannan (Table 7).

4.2.1.4 Measuremnt on root to shoot ratio

These traditional rice genotypes have shown significant variation in root to shoot ratio. The root to shoot ratio of many genotypes was on par under stress and control condition. Under stress Chuvanna Modan recorded highest root to shoot ratio

of 0.72 followed by Parambuvattan (0.48). Vadakkanchitteni was on par with Thekkancheera, Navara Black, Kanali and Thavalakkannan with lowest root to shoot ratio values of 0.10, 0.11, 0.14, 0.15 and 0.16 respectively (Table 8)

Plate7. Genotypes selected for proteomic analysis

Chuvanna modan (Tolerant genotype) - (18 days after imposition of stress)

Parambuvattan- Susceptible genotype (11 days after imposition of stress)

Table 4. No. of days taken by genotypes for reaching IRRI leaf rolling score 7.0

S.NO	Genotypes	No. of days taken
1	Karuthamodan	14^{f}
2	Chenkayama	19^{b}
3	Karanavara	17^{cd}
4	Thekkancheera	17^{cde}
5	Vadakkan Chitteni	17^{cd}
6	Navara Black	11^{gh}
7	Thavalakkannan	18^{c}
8	Chuvanna Modan	25^{a}
9	Parambuvattan	11^{h}
10	Kalladiyaran	15^{def}
11	Kavunginpoothala	$15e^{f}$
12	Kattamodan	17^{cde}
13	Jyothi	11^{h}
14	Kanali	12^{h}
15	Aryan	13^{fg}

Table 5. Root length of the genotypes in different treatments

		Root length (cm)	
S.NO	Genotypes	Stress treatment	Control
1	Karuthamodan	93.00^{a}	65.67^{ab}
2	Chenkayama	61.67^{defgh}	52.67^{bcd}
3	Karanavara	69.00^{bcdef}	46.67^{e}
4	Thekkancheera	59.33^{fgh}	59.00^{abc}
5	Vadakkan Chitteni	63.67^{cdef}	55.33^{bcde}
6	Navara Black	70.67^{bcd}	48.67^{de}
7	Thavalakkannan	62.00^{defgh}	58.67^{abc}
8	Chuvanna Modan	92.67^{a}	59.33^{abcde}
9	Parambuvattan	93.33^{a}	53.33^{cde}
10	Kalladiyaran	71.00^{bc}	54.00^{cde}
11	Kavunginpoothala	69.33^{bcde}	68.33^{a}
12	Kattamodan	70.67^{bcd}	63.00^{abc}
13	Jyothi	78.67^{b}	54.67^{cde}
14	Kanali	58.00^{g}	54.67^{abc}
15	Aryan	61.00^{defgh}	59.33^{abc}

Table 6. The root volume of genotypes in different treatments

		Root vol. (ml)	
S.NO	Genotypes	Stress	Control
1	Karuthamodan	123.33^{a}	48.33^{a}
2	Chenkayama	20.00^{fg}	16.67^{b}
3	Karanavara	103.33^{b}	48.33^{a}
4	Thekkancheera	33.33^{efg}	8.33^{b}
5	Vadakkan Chitteni	70.00^{cd}	46.67^{a}
6	Navara Black	20.00^{fg}	15.00^{b}
7	Thavalakkannan	13.33^{g}	11.67^{b}
8	Chuvanna Modan	120.00^{a}	21.67^{b}
9	Parambuvattan	78.33^{c}	25.00^{b}
10	Kalladiyaran	43.33^{def}	31.67^{a}
11	Kavunginpoothala	75.00^{c}	41.67^{a}
12	Kattamodan	60.00^{cde}	25.00^{b}
13	Jyothi	76.67^{c}	21.67^{b}
14	Kanali	23.33^{fg}	8.33^{b}
15	Aryan	53.33^{cde}	28.33^{a}

Table 7. Root dry wt. of genotypes in different treatments

		root dry wt. (g)	
S.NO	Genotypes	Stress	Control
1	Karuthamodan	10.07^{b}	8.19^{b}
2	Chenkayama	4.92^{def}	3.55^{e}
3	Karanavara	4.83^{ef}	3.52^{e}
4	Thekkancheera	1.40^{j}	0.99^{h}
5	Vadakkan Chitteni	3.80^{gh}	2.55^{f}
6	Navara Black	3.52^{i}	2.55^{f}
7	Thavalakkannan	1.99^{ij}	1.50^{gh}
8	Chuvanna Modan	10.78^{a}	9.47^{a}
9	Parambuvattan	5.35^{c}	5.51^{c}
10	Kalladiyaran	4.34^{f}	3.88^{e}
11	Kavunginpoothala	5.51^{d}	4.51^{d}
12	Kattamodan	4.55^{f}	3.79^{e}
13	Jyothi	7.54^{c}	6.04^{c}
14	Kanali	2.55^{h}	1.74^{g}
15	Aryan	5.30^{de}	4.55^{d}

Table 8. Root to shoot ratio of genotypes in different treatments

		Root to shoot ratio	
S.NO	Treatments	Stress	Control
1	Karuthamodan	0.41^{c}	0.27^{b}
2	Chenkayama	0.25^{de}	0.15^{d}
3	Karanavara	0.27^{de}	0.18^{bc}
4	Thekkancheera	0.11^{e}	0.06^{d}
5	Vadakkan Chitteni	0.10^{e}	0.06^{d}
6	Navara Black	0.14^{e}	0.08^{d}
7	Thavalakkannan	0.16^{e}	0.10^{d}
8	Chuvanna Modan	0.72^{a}	0.49^{a}
9	Parambuvattan	0.48^{c}	0.28^{b}
10	Kalladiyaran	0.23^{de}	0.16^{cd}
11	Kavunginpoothala	0.22^{de}	0.14^{d}
12	Kattamodan	0.35^{cd}	0.20^{bc}
13	Jyothi	0.33^{cd}	0.18^{bc}
14	Kanali	0.15^{e}	0.07^{d}
15	Aryan	0.18^{de}	0.11^{d}

4.2.2 Physiological Observations

The physiological observations analyzed were stomatal conductance, transpiration rate and Electrical conductivity.

4.2.2.1 Stomatal conductance

Chuvanna Modan recorded highest stomatal conductance of 1.11 μ mol of CO_2 m^{-2} s^{-1} under water stress condition and the response of other genotypes were almost similar for stomatal conductance and Parambuvattan was showing lowest stomatal conductance of 0.02 μ mol of CO_2 m^{-2} s^{-1}. However, in controlled condition Chuvanna Modan was showing highest stomatal conductance of 1.18 μ mol of CO_2 m^{-2} s^{-1} and Parambuvattan showed stomatal conductance of 0.30 μ mol of CO_2 m^{-2} s^{-1}. This showed the controlled plants has more stomatal conductance than treated plants.

In stress condition there was less significant variation between Chuvanna Modan and all other genotypes. The stomatal conductance in all genotypes except Chuvanna Modan was on par. In normal condition all the genotypes have significance variation and also on par relationship (Table 9)

4.2.2.2 Transpiration rate

It can be observed from the (table 10) that both in control and water stress condition Chuvanna Modan recorded more transpiration rate of 13.37 m mol of H_2O m^{-2} s^{-1} and 10.06 m mol of H_2O m^{-2} s^{-1} than all other genotypes. The transpiration rate showed significant variations among the genotypes in water stress and under control condition.

Lowest transpiration rate was recorded in Karanavara under water stress which was on par with Jyothi, Kanali and Kavunginpoothala (0.51 m mol of H_2O m^{-2} s^{-1}, 0.71 m mol of H_2O m^{-2} s^{-1} and 0.98 m mol of H_2O m^{-2} s^{-1}) respectively where as

in control condition Jyothi and Aryan were showing less transpiration rate of 2.87 m mol of H_2O m^{-2} s^{-1} and 3.30 m mol of H_2O m^{-2} s^{-1} .

4.2.2.3 Electrical Conductivity

The effect of water stress on electrical conductivity varied significantly among genotypes. Under water stress condition, Jyothi showed highest EC value of 157.00 d Sm^{-1} followed by Navara Black and Kavunginpoothala (153.97 d $Sm^{-1}$and 145.73 d Sm^{-1}), lowest EC value of 54.30 d Sm^{-1} was recorded in Chuvanna Modan, followed by 56.40 d Sm^{-1} and 57.37 d Sm^{-1} in Karanavara and Vadakanchitteni. In controlled condition Jyothi showed highest EC 111.37 d Sm^{-1} and Chuvanna Modan showed lowest EC 30.83 d Sm^{-1} values (Table 11)

Based on EC values Membrane stability has calculated and it showed significant variation among genotypes. Chuvanna Modan has shown highest MSI value of 43 whereas Parambuvattan has showed value of 18.

Table 9. Stomatal conductance of rice genotypes in different treatments

		Stomatal conductance (μ mol of CO_2 m^{-2} s^{-1})	
S.NO	Genotypes	Stress	Control
1	Karuthamodan	0.08^{b}	0.22^{cd}
2	Chenkayama	0.08^{b}	0.22^{cd}
3	Karanavara	0.11^{b}	0.89^{b}
4	Thekkancheera	0.06^{b}	0.17^{cd}
5	Vadakkan Chitteni	0.06^{b}	0.26^{cd}
6	Navara Black	0.03^{b}	0.18^{cd}
7	Thavalakkannan	0.05^{b}	0.26^{cd}
8	Chuvanna Modan	1.11^{a}	1.18^{a}
9	Parambuvattan	0.02^{b}	0.30^{cd}
10	Kalladiyaran	0.08^{b}	0.28^{cd}
11	Kavunginpoothala	0.02^{b}	0.21^{cd}
12	Kattamodan	0.03^{b}	0.29^{cd}
13	Jyothi	0.03^{b}	0.21^{cd}
14	Kanali	0.03^{b}	0.12^{d}
15	Aryan	0.06^{b}	0.41^{c}

Table 10. Transpiration rate of rice genotypes in different treatments

		Transpiration rate (m mol of H2O m^{-2} s^{-1})	
S.NO	Genotypes	Stress	Control
1	Karuthamodan	1.81^{e}	4.61^{de}
2	Chenkayama	2.82^{bc}	5.82^{bcd}
3	Karanavara	0.65^{b}	4.98^{cd}
4	Thekkancheera	2.41^{bcd}	6.54^{b}
5	Vadakkan Chitteni	2.75^{bc}	6.67^{b}
6	Navara Black	1.46^{def}	3.55^{ef}
7	Thavalakkannan	2.06^{cde}	6.54^{b}
8	Chuvanna Modan	10.06^{a}	13.37^{a}
9	Parambuvattan	0.65^{f}	6.39^{b}
10	Kalladiyaryan	3.31^{b}	5.74^{bcd}
11	Kavunginpoothala	0.98^{f}	5.82^{bcd}
12	Kattamodan	1.10^{f}	5.99^{bc}
13	Jyothi	0.71^{f}	2.87^{f}
14	Kanali	0.71^{f}	4.60^{de}
15	Aryan	1.39^{e}	3.30^{f}

Table 11. Electrical Conductivity of rice genotypes in different treatments

		Electrical conductivity (d Sm^{-1})	
S.No	Genotypes	Stress	Control
1	Karuthamodan	73.70^{fg}	45.30^{def}
2	Chenkayama	72.70^{fg}	55.53^{de}
3	Karanavara	56.40^{gh}	44.13^{efg}
4	Thekkancheera	75.73^{fgh}	48.90^{def}
5	Vadakkan Chitteni	57.37^{gh}	35.70^{ef}
6	Navara Black	153.97^{a}	102.17^{ab}
7	Thavalakkannan	88.63^{ef}	54.43^{def}
8	Chuvanna Modan	54.30^{h}	30.83^{f}
9	Parambuvattan	108.04^{cd}	88.47^{bc}
10	Kalladiyaran	91.83^{de}	59.87^{de}
11	Kavunginpoothala	145.73^{ab}	88.70^{bc}
12	Kattamodan	69.00^{fgh}	47.57^{de}f
13	Jyothi	157.00^{a}	111.37^{a}
14	Kanali	129.23^{b}	84.03^{cd}
15	Aryan	82.77^{efg}	60.80^{cde}

Table 12. Membrane stability Index of rice genotypes in different treatments

		EC		
S.NO	Genotypes	Stress (EC1)	Control(EC2)	MSI (1-(EC2/EC1)*100)
1	Karuthamodan	73.70^{fg}	45.30^{def}	39
2	Chenkayama	72.70^{fg}	55.53^{de}	24
3	Karanavara	56.40^{gh}	44.13^{efg}	22
4	Thekkancheera	75.73^{fgh}	48.90^{def}	35
5	Vadakkan Chitteni	57.37^{gh}	35.70^{ef}	38
6	Navara Black	159.97^{a}	102.17^{ab}	36
7	Thavalakkannan	88.63^{ef}	54.43^{def}	39
8	Chuvanna Modan	54.30^{h}	30.83^{f}	43
9	Parambuvattan	108.04^{cd}	88.47^{bc}	18
10	Kalladiyaran	91.83^{de}	59.87^{de}	35
11	Kavunginpoothala	145.73^{ab}	88.70^{bc}	39
12	Kattamodan	69.00^{fgh}	47.57^{def}	31
13	Jyothi	157.00^{a}	111.37^{a}	29
14	Kanali	129.23^{b}	84.03^{cd}	35
15	Aryan	82.77^{efg}	60.80^{cde}	27

4.4. Protein profiling

4.4.1 Extraction and quantification of total proteins

A high concentration of proteins ranging between 16 to 32 mg/g of leaf sample was extracted using TCA-Acetone precipitation method from the control and the treated samples of Chuvanna Modan and Parambuvattan genotypes.

4.4.2. Standardization of SDS-PAGE protocol

Laemmlli (1970) protocol for the SDS-PAGE analysis was further appropriated to the laboratory conditions by increasing the APS and TEMED concentration to promote the polymerization of the SDS-PAGE gel.

4.4.3. Protein profiling in relation to water stress in traditional rice genotypes

The SDS-PAGE analysis of the protein isolated has shown distinct expression of the proteins in resistant and susceptible rice genotypes. The morpho-physiological screening of traditional rice genotypes identified Chuvanna Modan as the most tolerant and Parambuvattan as the most suceptible among the genotypes.

Many proteins were observed in each lane in all the four samples and there was difference in the expression of the proteins in tolerant, susceptible genotypes under water stressed and normal condition. The similar proteins were found in tolerant and susceptible genotypes which may be constitutive proteins for housekeeping and chaperons. The molecular weights of the bands were elucidated by comparing with a protein maker (6.4- 201.2 kD). Among them 7 up regulated and differentially expressed bands were observed in the size range of 20 kD to 44 kD between control and treatments of tolerant and susceptible genotypes (Plate 8)

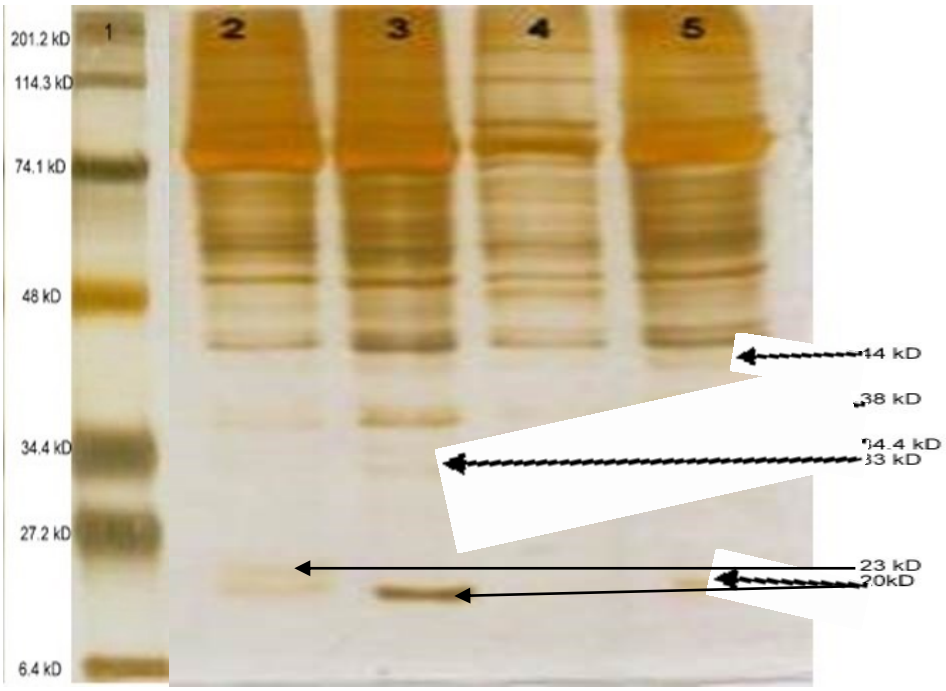


Plate 8. Protein profile of rice genotypes (control and water stress condition).

1. Protein Marker 2. Parambuvattan Control 3. Parambuvattan Treatment 4. Chuvanna Modan Control 5. Chuvanna Modan Treatment

4.4.4. SDS-PAGE profile of proteins in tolerant and susceptible genotype

In Chuvanna Modan the tolerant genotype showed differential regulation of four proteins of size 20 kD, 34.4 kD, 38 kD and 44 kD (plate 8) upon imposition of water stress

In Parambuvattan the susceptible genotype showed differential regulation of three proteins of size 20 kD, 23 kD and 33kD (Plate 8) upon imposition of water stress. In tolerant as well as in susceptible genotype a similar sized band of 20 kD was expressed under water stress condition which may be a chaperon protein constitutively expressed by house keeping genes under water stress condition.

4.5. Peptide mass fingerprinting by MALDI-TOF/MS

The two differentially expressed protein bands from Chuvanna Modan and Parambuvattan of size 44kD and 33 kD (Plate 8) respectively were cut from the gel and sent for peptide mass fingerprinting by Matrix Assisted Laser Desorption and Ionization- Time of Flight Mass spectrometry (MALDI-TOF/MS) (Sandor Proteomiocs, Hyderabad) for the identification of the differentially expressed protein bands. The protein bands were eluted from the gel, digested with trypsin and analysed by MALDI-TOF Mass spectrometry. Mass spectrometric analysis of the intact digest mixture thus provided a set of peptide molecular masses with the corresponding peaks separately for the 44kD and 33kD proteins as shown in fig 1 and 4 respectively

4.6. *In-silico* analysis

The mass spectrometry data having the peak values of peptide mass finger print of the 44kDand 33kD analyzed with Mascot Sever software from Matrix science which is basically utilized for identification, characterization and quantification of

proteins using mass spectrometry data. By comparing the peak values of different peptides with all available protein databases, the 44 kD proteins were identified to have shown higher similarity with glycosyl transferase At5g03795 isoform X2, leucine rich protein 12 and extended PHD finger ATX3, 4, 5, and similar proteins whereas 33 kD protein have shown high similarity with kinesin like protein NACK-1 like isoform X1, TPR and ankyrin-repeat containing protein 1 isoform and proteosome activator sub unit 4 as shown in Fig 2, 3 and 5, 6.

4.7. 2D gel electrophoresis

On image analysis, many protein spots were detected were matched between tolerant and susceptible gels, which are proteins constitutively expressed by house keeping genes. In order to compare protein expression between treatments, a difference map was generated between control and water stress treated groups (Plate 9, 10). A total of 2 upregulated and 2 differential protein spots were detected in Chuvanna Modan under stress depicted as 1, 2, 3, 4 and 1 differential spot was present in control depicted as A whereas 1 differential protein spot was present in Parambuvattan treatment indicated as 1 and four protein spots were present in control indicated as A, B, C, D.

4.8. Peptide mass fingerprinting by MALDI-ToF/MS

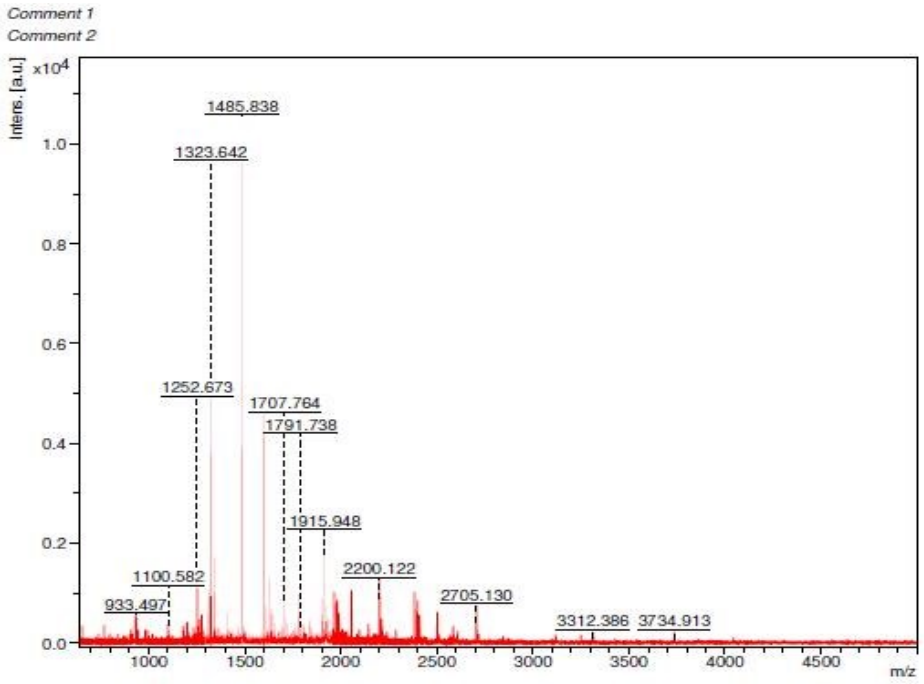
After comparing the 2D gel pictures of drought tolerant Chuvanna Modan four differential and up-regulated spots were identified and cut from the gel and sent for peptide mass fingerprinting by matrix Assisted Laser Desorption and Ionization-Time of Flight Mass spectrometry (MALDI-ToF/MS) from Sandor Proteomcs, Hyderabad). The protein from the spots were eluted from the gel, digested with trypsin and analysed by MALDI-ToF mass spectrometry. Mass spectrometric analysis of intact digest mixture from a spot thus provided a set of peptides with

varying molecular masses for each spot as shown in Fig.7, 10, 13, and 16 respectively.

4.9 *In-silico* analysis

The mass spectrometry data having the peak values of peptide mass fingerprint of the 4 spots were analysed with Mascot Server software from Matrix Science which is basically utilized for the identification, characterization and quantification of proteins using mass spectrometry data. The protein showing maximum similarity was used as query for Blastp and Smart BLAST to get the closely related proteins. By comparing the peak values of different peptides with all available protein databases, spot 1 and 2 proteins showed higher similarity with ribulose bisphosphate carboxylase small chain c, Rubisco Complexed with 2- Carboxyarabinitol-1,5-bisp and ribulose-bisphosphate carboxylase. The spot 3 has shown similarity with Protein tyrosine (pTyr) phosphorylation, serine/threonine-protein kinase and TPA: nicotianamine amino transferase whereas spot 4 has shown similarity with Photosystem II assembly stability factor HCF 136, protein LOC 102664781 isoform X1 and putative ferredoxin-NADP(H) oxidoreductase. The screen shots of *In-silico* analysis are depicted in the Fig. 8, 9, 11, 12, 14, 15, 17 and 18 respectively.

Fig 1: Peptide mass finger print of the 44 kD protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.303	6.3	569	5243	331.53	67
740.345	2.5	80	3711	133.53	44
768.501	6.6	1972	6138	346.52	76
842.459	2.3	60	3267	120.93	54
870.516	2.0	235	6372	108.72	27
908.469	4.8	152	7112	257.06	63
933.497	10.6	6248	7141	578.48	145
938.454	8.4	4168	5392	455.41	147
945.502	4.4	357	5601	238.04	75
983.493	4.2	651	5443	240.81	83
997.508	3.0	142	4246	172.18	77
1021.526	2.7	91	5138	154.15	59
1100.582	5.6	1527	6025	341.05	129
1179.610	4.8	662	7086	306.46	109
1198.709	5.4	285	7731	346.47	115
1201.564	4.1	329	3431	261.16	197
1234.650	4.4	377	6184	291.24	131
1252.673	17.4	18929	8032	1147.13	411
1264.658	5.9	930	5708	393.99	196
1276.653	8.1	2515	6860	545.51	231
1282.638	3.1	497	8635	208.99	70
1308.659	8.5	899	7081	577.90	249
1311.643	15.2	5845	7179	1038.96	440
1320.598	14.6	160	7418	1003.05	418
1323.642	75.0	4098	8689	5115.97	1877
1341.652	27.6	5454	9118	1936.04	700
1351.662	2.8	170	5110	191.72	120
1363.709	2.5	131	4799	174.07	117
1365.658	2.1	106	4977	142.99	93
1406.705	6.8	1317	6045	474.62	267
1419.727	2.8	216	4836	199.73	144
1436.708	2.4	99	5541	168.53	106
1465.750	4.2	687	9799	312.44	123
1485.838	142.5	32552	9102	10536.68	4558
1493.726	2.9	192	5676	215.29	145
1601.799	64.8	59623	9221	4839.33	2367
1614.820	2.6	77	4369	194.87	197
1631.809	17.0	13925	9543	1295.29	628

Fig 2 : *In-silico* analysis of the 44 kD protein using Mascot Server software

MATRIX SCIENCE **Mascot Search Results**

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150815 (71457443 sequences; 25669551608 residues)
Taxonomy        : Viridiplantae (Green Plants) (3133767 sequences)
Timestamp       : 24 Aug 2015 at 10:04:48 GMT
Top Score       : 98 for gi|697129658, PREDICTED: probable glycosyltransferase At5g03795 isoform X2 [Nicotiana tomentosiformis]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 77 are significant (p<0.05).

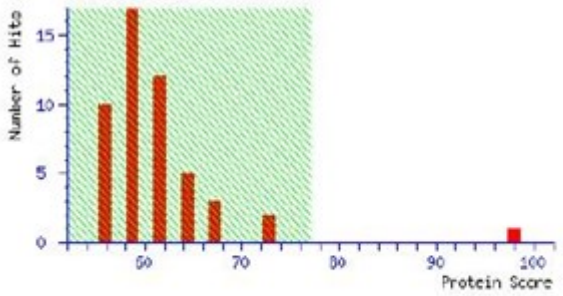


Fig 3: *In-silico* analysis of the 44 kD protein using Mascot Server software

Index

	Accession	Mass	Score	Description
1.	gi|697129658	64809	98	PREDICTED: probable glycosyltransferase At5g03795 isoform X2 [Nicotiana tomentosiformis]
2.	gi|255089322	48879	74	predicted protein [Micromonas sp. RCC299]
3.	gi|697129656	75244	72	PREDICTED: probable glycosyltransferase At5g03795 isoform X1 [Nicotiana tomentosiformis]
4.	gi|674242599	114086	68	hypothetical protein AALP_AA5G275500 [Arabis alpina]
5.	gi|674242598	113958	68	hypothetical protein AALP_AA5G275500 [Arabis alpina]
6.	gi|255577566	144229	66	Structural maintenance of chromosome, putative [Ricinus communis]
7.	gi|595811256	169802	65	hypothetical protein PRUPE_ppa000194mg [Prunus persica]
8.	gi|302836746	28548	65	hypothetical protein VOLCADRAFT_90254 [Volvox carteri f. nagariensis]
9.	gi|573947286	67807	64	PREDICTED: microtubule-associated protein 70-3-like isoform X2 [Oryza brachyantha]
10.	gi|670371515	59263	64	PREDICTED: uncharacterized protein LOC100191495 isoform X1 [Zea mays]
11.	gi|566188208	139871	63	TITAN7 family protein [Populus trichocarpa]
12.	gi|566196267	40135	62	hypothetical protein POPTR_0012s01620g [Populus trichocarpa]
13.	gi|302811229	68464	62	hypothetical protein SELMODRAFT_235261 [Selaginella moellendorffii]
14.	gi|593701193	24239	62	hypothetical protein PHAVU_004G011500g [Phaseolus vulgaris]
15.	gi|145344149	80415	62	predicted protein [Ostreococcus lucimarinus CCE9901]
16.	gi|605263764	23371	62	PREDICTED: 40S ribosomal protein S19, mitochondrial-like [Brassica rapa]
17.	gi|32489462	109551	61	OSJNBa0028M15.8 [Oryza sativa Japonica Group]
18.	gi|224117148	85061	61	dynamin family protein [Populus trichocarpa]
19.	gi|743792234	139829	61	PREDICTED: structural maintenance of chromosomes protein 3 isoform X1 [Populus euphratica]
20.	gi|552841082	55781	61	hypothetical protein CHLNCDRAFT_30221, partial [Chlorella variabilis]

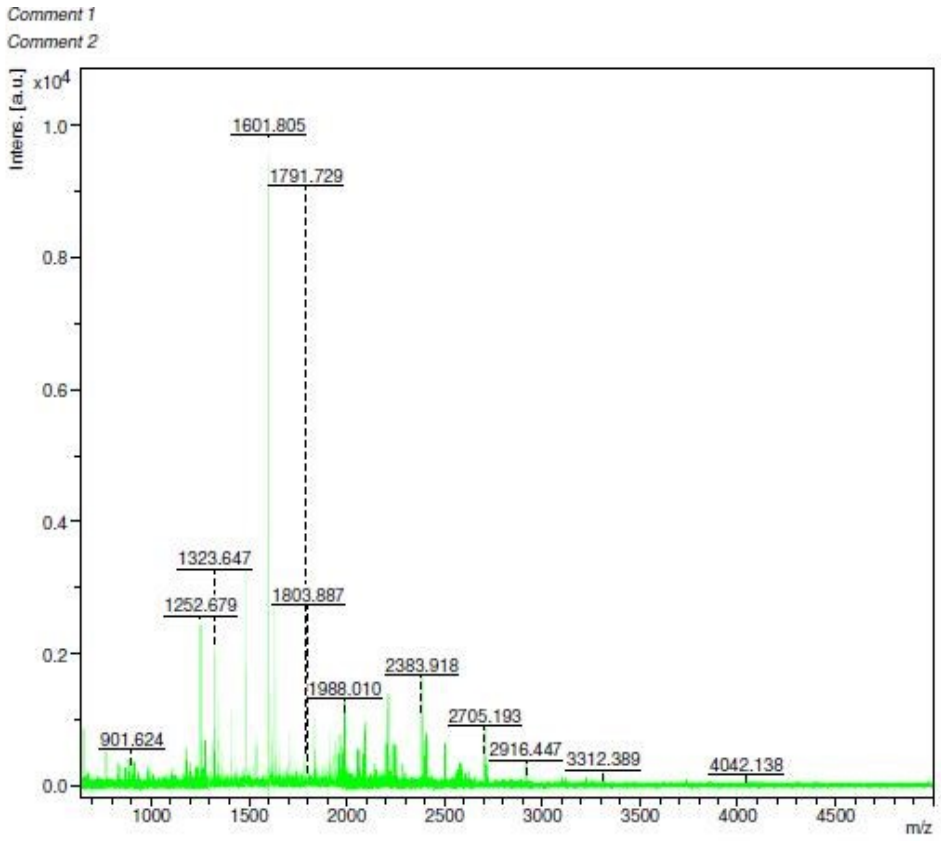
Results List

1. gi|697129658 **Mass:** 64809 **Score:** 98 **Expect:** 0.0005 **Matches:** 25
PREDICTED: probable glycosyltransferase At5g03795 isoform X2 [Nicotiana tomentosiformis]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
643.3114	642.3042	642.3561	-80.92	404 - 408	0		K.RPSQR.E
740.3335	739.3262	739.3905	-86.96	563 - 568	0		R.VFQIAY.-
842.4513	841.4440	841.3575	103	457 - 462	0		R.YCICAR.G
945.5068	944.4995	944.4563	45.8	164 - 171	0		K.EATDRPEK.G
1108.5601	1107.5529	1107.5309	19.8	71 - 79	0		R.QFNDNLTTR.V
1179.6165	1178.6092	1178.5754	28.7	445 - 453	1		R.KTDYIQHMK.S + Oxidation (M)
1234.6570	1233.6497	1233.6023	38.4	152 - 163	0		R.NGSTMTNSPVVK.E
1282.6407	1281.6334	1281.6387	-4.10	238 - 247	1		R.SYELMERNLK.V
1310.6261	1309.6188	1309.7506	-100.64	524 - 533	2		R.RYLKLYNNVK.K
1323.6425	1322.6352	1322.6077	20.8	429 - 438	1		K.YWQNKDPNMK.I
1365.6192	1364.6120	1364.6507	-28.35	446 - 456	1		K.TDYIQHMKSSR.Y
1382.6492	1381.6420	1381.7717	-93.92	245 - 255	2		R.NLKVYIYREGK.R
1493.7130	1492.7057	1492.7456	-26.76	445 - 456	2		R.KTDYIQHMKSSR.Y
1657.7783	1656.7710	1656.8334	-37.66	266 - 279	1		K.GIYASEGMFMKQLK.A
1673.8316	1672.8243	1672.8283	-2.38	266 - 279	1		K.GIYASEGMFMKQLK.A + Oxidation (M)
1707.7638	1706.7565	1706.9930	-138.54	509 - 523	1		K.DIPNLKSILESIPLR.R
1762.9270	1761.9197	1761.9315	-6.68	534 - 547	1		K.KVQQHFLMHSEPVK.Y
1928.9926	1927.9853	1928.0578	-37.62	11 - 29	2		R.SGVELLKSNGTLAPDTAKK.V
1940.9031	1939.8958	1940.0057	-56.65	325 - 339	2		K.NYVDLIKGRYPFWNR.T
2286.0804	2285.0731	2285.1110	-16.60	186 - 206	0		R.SHPSSLLTKPMMSSASDEGLR.S
2398.9818	2397.9745	2398.0868	-46.82	360 - 379	2		R.HEMANCIKSFCNADLKEGFK.L
2408.1136	2407.1064	2407.1590	-21.87	30 - 50	1		K.VGYIFNGNNENVAPMLEDQRK.D
2508.2796	2507.2723	2507.2546	7.08	164 - 185	2		K.EATDRPEKGVVSISEMTKMMLR.S
3117.4430	3116.4357	3116.6519	-69.37	210 - 237	2		K.SQIENAANIIVDPGLHAPVYHNVSKFKR.S
3312.3576	3311.3504	3311.6972	-104.74	51 - 79	1		K.DDIVPLANNVASLPMISPLRQFNDNLTTR.V + Oxidation (M)

No match to: 639.3150, 653.3032, 768.5010, 795.3983, 797.3192, 870.5161, 886.1372, 901.5849, 909.4680, 916.4704, 917.2357, 933.4974, 938.4542, 983.4931, 997.5042, 1021.5373, 1100.5807, 1198.7128, 1201.5398, 1229.6209, 1246.6044, 1252.6735, 1264.6578, 1276.6526, 1308.6588, 1311.6429, 1320.5977, 1324.6121, 1341.6515, 1343.6202, 1351.6430, 1363.6897, 1406.7053, 1419.7104, 1426.6901, 1436.6950, 1465.7511, 1475.7139, 1485.8379, 1497.7860, 1506.7428, 1524.7400, 1588.7082, 1601.7986, 1614.8004, 1621.7632, 1631.8093, 1638.8311, 1642.7650, 1687.9051, 1690.7705, 1721.8987, 1755.8895, 1776.7990, 1791.7060, 1803.8762, 1806.7722, 1838.9036, 1845.8849, 1877.5562, 1901.0091, 1902.8954, 1915.9485, 1949.9149, 1963.9338, 1979.9355, 1987.9918, 2001.9740, 2006.9406, 2020.9424, 2054.9323, 2096.9321, 2143.0361, 2171.0602, 2200.1219, 2211.0439, 2215.0290, 2239.0598, 2383.9213, 2409.1306, 2411.1408, 2501.2055, 2566.2231, 2584.1637, 2607.3486, 2705.1299, 2717.0358, 2844.3171, 3248.5655, 3734.8861, 4042.0531

Fig 4: Peptide mass finger print of the 33 kD protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
639.317	4.4	247	3430	255.34	77
653.303	15.3	2290	5473	899.43	176
768.495	8.2	2442	4982	473.92	123
829.210	4.3	158	3195	247.87	112
842.486	4.7	964	4956	272.86	83
864.462	3.6	127	4699	214.29	70
868.536	4.2	518	5961	247.80	65
870.512	3.8	319	6200	227.29	59
886.148	4.7	159	3151	276.38	139
901.624	5.3	649	3888	314.89	133
917.285	4.3	492	3607	253.76	118
933.502	2.9	313	5342	170.96	55
983.503	2.7	76	4448	163.62	68
997.570	2.6	108	3672	158.12	82
1165.600	2.4	164	3690	152.31	102
1179.617	7.9	3077	6167	506.40	208
1198.711	3.8	189	4964	248.63	129
1229.793	4.2	546	6307	279.92	123
1234.659	3.3	148	4040	222.79	149
1237.827	2.0	147	2074	136.19	178
1252.679	37.6	30051	7861	2533.32	932
1265.651	2.5	74	4365	168.90	109
1277.683	8.9	4440	6868	601.83	252
1294.597	2.1	125	9027	151.82	78
1308.656	5.8	354	5993	399.04	200
1311.656	10.0	2520	6725	684.76	313
1320.595	11.5	566	6822	795.75	358
1323.647	30.9	5352	6919	2143.12	960
1325.648	4.6	224	4932	319.07	196
1341.662	17.3	4449	6906	1203.04	551
1343.653	3.1	169	5991	219.05	115
1363.712	2.6	70	4399	180.87	132
1365.647	5.6	1175	6251	394.51	205
1406.724	15.1	16567	8222	1084.27	463
1427.743	2.8	113	3893	199.04	179
1434.756	2.6	230	4696	185.68	141
1465.749	2.6	155	6670	194.56	107
1475.769	3.5	167	10529	257.87	94
1485.834	47.4	13835	7975	3525.90	1708

Fig 5: *In-silico* analysis of the 33 kD protein using Mascot Server software

MATRIX SCIENCE

Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150815 (71457443 sequences; 25669551608 residues)
Taxonomy        : Viridiplantae (Green Plants) (3133767 sequences)
Timestamp       : 24 Aug 2015 at 10:14:07 GMT
Top Score       : 85 for gi|356531188, PREDICTED: kinesin-like protein NACK1-like isoform X1 [Glycine max]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 77 are significant (p<0.05).

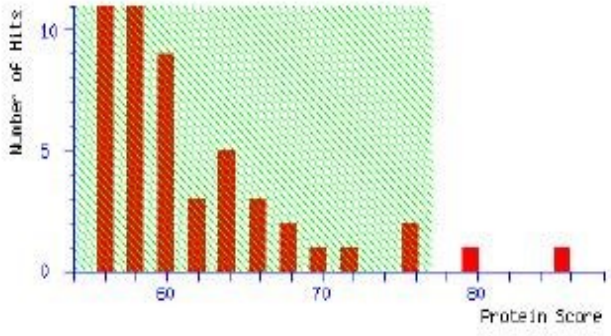


Fig 6: *In-silico* analysis of the 33 kD protein using Mascot Server software

Index

	Accession	Mass	Score	Description
1.	gi|356531188	109540	85	PREDICTED: kinesin-like protein NACK1-like isoform X1 [Glycine max]
2.	gi|848872098	180551	80	PREDICTED: uncharacterized protein LOC105957228 [Erythranthe guttatus]
3.	gi|734382910	109610	76	Kinesin-related protein 4 [Glycine soja]
4.	gi|720089625	205283	76	PREDICTED: proteasome activator subunit 4 [Nelumbo nucifera]
5.	gi|565385468	18949	71	PREDICTED: SNF2 domain-containing protein CLASSY 4-like [Solanum tuberosum]
6.	gi|4558555	136127	69	hypothetical protein [Arabidopsis thaliana]
7.	gi|238481454	111066	69	TIR-NBS-LRR class disease resistance protein [Arabidopsis thaliana]
8.	gi|698569407	66526	67	PREDICTED: ruBisCO large subunit-binding protein subunit beta, chloroplastic isoform X1 [Nicoti
9.	gi|242074314	209758	66	hypothetical protein SORBIDRAFT_06g028450 [Sorghum bicolor]
10.	gi|125537864	60708	65	hypothetical protein OsI_05639 [Oryza sativa Indica Group]
11.	gi|727580534	115492	65	PREDICTED: formin-like protein 14 [Camelina sativa]
12.	gi|22328224	146881	64	WD40 domain-containing protein [Arabidopsis thaliana]
13.	gi|604336241	93916	64	hypothetical protein MIMGU_mgv1a001333mg [Erythranthe guttata]
14.	gi|693499768	82262	64	DNA polymerase A [Ostreococcus tauri]
15.	gi|255085080	34329	63	predicted protein [Micromonas sp. RCC299]
16.	gi|674959497	59405	63	BnaA03g28450D [Brassica napus]
17.	gi|685287611	59405	63	PREDICTED: T-complex protein 1 subunit theta-like [Brassica rapa]
18.	gi|115443881	67850	62	Os02g0121700 [Oryza sativa Japonica Group]
19.	gi|110741935	111004	61	disease resistance like protein [Arabidopsis thaliana]
20.	gi|848267671	76402	60	phytochrome, partial [Cosmarium granatum]

Results List

1. gi|356531188 **Mass:** 109540 **Score:** 85 **Expect:** 0.009 **Matches:** 23

PREDICTED: kinesin-like protein NACK1-like isoform X1 [Glycine max]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
768.4951	767.4879	767.4766	14.7	37 -	42	0	R.LRPLNR.R
997.5447	996.5374	996.4811	56.5	140 -	147	0	K.HIMNTPER.D
1165.5839	1164.5766	1164.6363	-51.28	312 -	322	0	R.ILQHSLGGNAR.T
1179.6170	1178.6097	1178.6659	-47.69	720 -	729	1	R.SIRAYVTELK.E
1266.6948	1265.6876	1265.6550	25.7	922 -	933	1	R.LVGFRTGGNMSK.E
1276.6656	1275.6583	1275.6493	7.08	544 -	555	0	K.NVEVGSMVSINK.S
1308.6559	1307.6486	1307.5928	42.7	709 -	719	0	K.MFQNAAEENVR.S
1325.6341	1324.6268	1324.7350	-81.69	378 -	389	1	R.LEAVLRTPDPSK.E
1390.6786	1389.6714	1389.6484	16.5	213 -	225	0	R.QVGETALNDNSSR.S
1406.7238	1405.7165	1405.7201	-2.56	153 -	164	0	K.ISGLEIYNENVR.D
1601.8054	1600.7981	1600.8031	-3.14	140 -	152	1	K.HIMNTPERDFTIK.I
1615.8278	1614.8205	1614.8213	-0.50	351 -	365	0	K.EVTNNAQVNVVVSDK.Q
1635.7935	1634.7862	1634.7610	15.4	809 -	822	0	K.GDPADQIYMEVELR.R
1791.7294	1790.7221	1790.8621	-78.16	809 -	823	1	K.GDPADQIYMEVELRR.L
1900.9563	1899.9491	1900.0278	-41.47	620 -	636	1	K.LLPLSSSNAANRQNFLR.S
1909.8601	1908.8528	1908.9833	-68.35	82 -	99	1	K.VFGPASVTEAVYEEGVKK.V
1940.9203	1939.9131	1940.0327	-61.65	515 -	532	1	R.LGNQDAAETIAKLQAEIR.G
1957.9906	1956.9833	1957.0125	-14.90	275 -	291	0	K.EGCHINLSLMTLTTVIR.K
1988.0102	1987.0029	1986.9270	38.2	657 -	674	0	R.VPENDDIVSTDTLPESEK.E
2082.9856	2081.9783	2082.0204	-20.23	592 -	610	0	K.LVMSLPNNFQHSPSEASPK.N
2152.0605	2151.0532	2151.0201	15.4	469 -	486	1	R.QSMRQSSTAPFTLMHEIR.K + 2 Oxidation (M)
2215.0247	2214.0174	2214.1864	-76.33	273 -	291	1	R.LKEGCHINLSLMTLTTVIR.K + Oxidation (M)
2916.4314	2915.4241	2915.5042	-27.47	902 -	926	1	K.LWTDPHDQIHVQESAEIVARLVGFR.T

No match to: 639.3215, 653.3032, 829.1938, 842.4875, 864.4635, 868.5337, 870.5134, 886.1417, 901.6253, 917.2743, 933.5079, 938.4415, 983.5009, 1106.5620, 1125.5318, 1198.7124, 1229.7970, 1234.6558, 1237.8072, 1252.6789, 1264.6542, 1277.6828, 1311.6559, 1320.5946, 1323.6475, 1341.6620, 1343.6102, 1363.7090, 1365.6470, 1380.6938, 1427.7389, 1434.7434, 1465.7488, 1475.7748, 1485.8342, 1487.7214, 1493.6972, 1503.7347, 1525.7805, 1534.8111, 1544.8310, 1618.8371, 1631.8209, 1638.8496, 1649.7819, 1657.7978, 1673.8303, 1687.9129, 1694.7755, 1707.7601, 1722.7978, 1742.8918, 1762.8742, 1776.7978, 1803.8769, 1831.9479, 1838.8974, 1845.8829, 1851.9082, 1875.9256, 1880.8456, 1891.8952, 1915.9526, 1929.9615, 1963.9438, 1969.8931, 1979.9297, 2001.9906, 2054.9590, 2060.0614, 2064.9046, 2094.0524, 2142.0535, 2144.0195, 2184.0420, 2200.1048, 2211.0735, 2225.0643, 2239.0940, 2251.0511, 2286.0717, 2383.9180, 2398.9944, 2408.1421, 2501.2046, 2560.1072, 2566.2132, 2576.1307, 2584.1835, 2588.2529, 2607.3613, 2621.2939, 2705.1927, 2717.0629, 2836.2465, 3312.3688, 4043.1324

2. gi|848872098 **Mass:** 180551 **Score:** 80 **Expect:** 0.032 **Matches:** 25

PREDICTED: uncharacterized protein LOC105957228 [Erythranthe guttatus]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
933.5079	932.5006	932.4239	82.2	656 -	663	0	R.ASSPEEWK.S
938.4415	937.4342	937.5094	-80.14	1116 -	1124	0	K.GNVATLHAR.H
1106.5620	1105.5547	1105.5437	9.92	344 -	353	1	R.LKNGDLMGDK.M + Oxidation (M)
1179.6170	1178.6097	1178.5098	84.7	417 -	426	1	K.SRNCDEGSVR.R

Plate 9a. Protein profile generated from rice genotype Chuvanna Modan under water stress

Plate 9b. Protein profile generated from rice genotype Chuvanna Modan under control

Plate 10a. Protein profile generated from rice genotype parambuvattan under control condition

Plate 10b. Protein profile generated from rice genotype parambuvattan under water stress

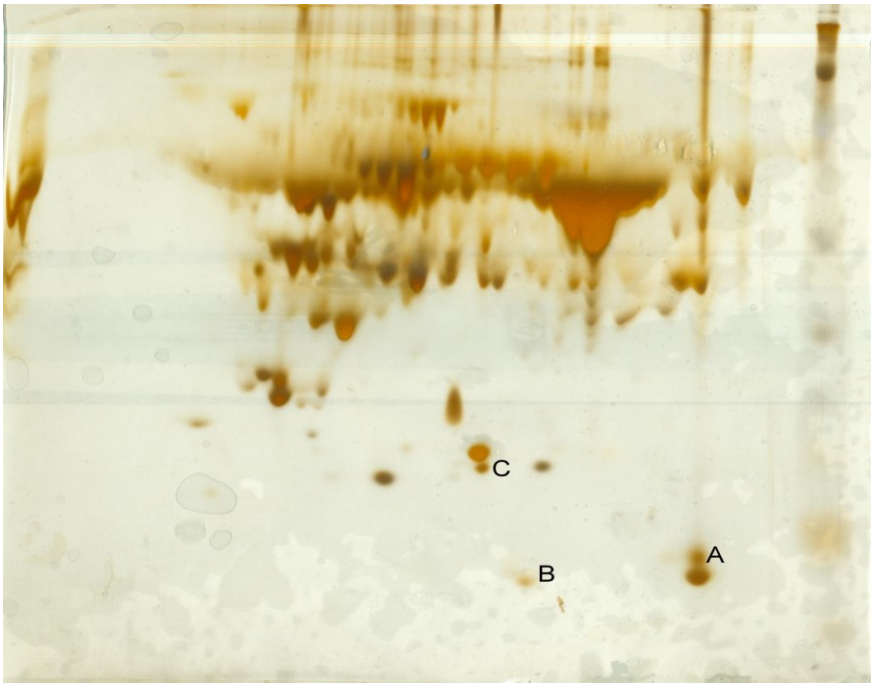
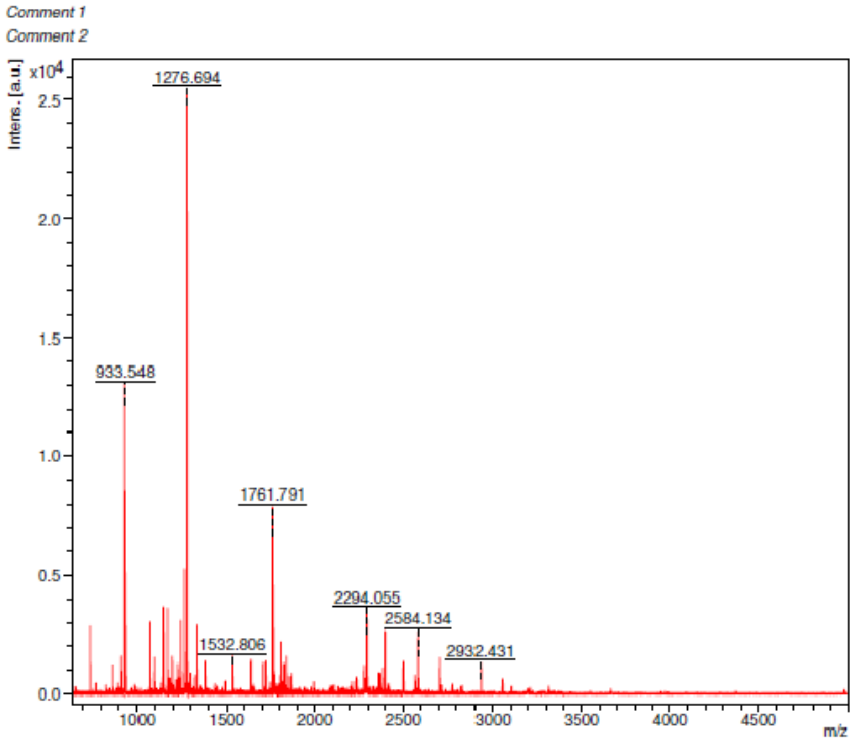


Fig 7: Peptide mass finger print of the spot 1 protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
740.416	31.4	5347	4061	2694.21	827
866.444	11.1	704	3057	992.71	499
914.441	16.4	1295	3806	1485.70	664
933.548	133.3	19414	3991	12147.68	5348
934.536	29.1	733	7154	2657.48	638
938.507	49.2	4564	3748	4477.47	2082
1066.602	29.9	9270	4685	2846.44	1311
1092.247	11.5	914	1054	1067.49	2300
1143.718	31.7	1911	1783	3037.73	4098
1166.696	33.3	7296	4051	3174.15	1949
1193.689	10.0	588	1617	956.85	1506
1223.750	8.4	209	1852	798.88	1138
1225.753	6.6	756	2549	633.96	666
1240.282	24.8	3504	2880	2360.49	2251
1261.832	47.3	9608	3055	4477.19	4167
1276.694	263.3	24223	3990	24761.34	18149
1294.781	6.7	245	3596	633.87	520
1320.630	6.6	506	3739	625.80	513
1333.699	28.6	6650	5066	2690.94	1667
1380.725	11.1	280	3050	1053.53	1141
1532.806	13.5	1373	3902	1270.46	1302
1638.847	14.9	7914	5714	1340.79	1091
1707.760	13.4	6937	5735	1181.81	1041
1723.485	11.8	2233	3589	1037.22	1482
1761.791	75.9	12071	4603	6669.48	7735
1765.888	20.6	5010	4098	1808.77	2351
1770.327	6.4	1117	2257	548.67	1322
1803.918	6.1	551	3980	535.59	751
1809.780	22.6	14112	5779	1982.84	1961
1825.776	9.0	617	3888	787.63	1150
1838.883	11.7	1279	4549	1022.66	1298
1866.803	7.7	836	5351	667.30	747
2236.084	6.5	520	3908	430.44	818
2278.074	12.2	1070	4203	776.21	1422
2294.055	40.0	20386	5634	2531.81	3565
2360.141	8.0	1006	4444	472.37	860
2368.990	7.8	503	3715	457.21	987

Fig 8: *In-silico* analysis of the spot 1 protein using Mascot Server software

MATRIX SCIENCE **Mascot Search Results**

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150912 (71310511 sequences; 25955008131 residues)
Taxonomy        : Oryza sativa (rice) (135928 sequences)
Timestamp       : 23 Sep 2015 at 10:10:40 GMT
Top Score       : 135 for gi|149392567, ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).

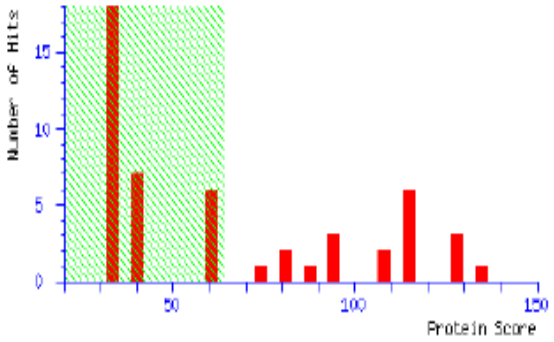


Fig 9: *In-silico* analysis of the spot 1 protein using Mascot Server software

Index

	Accession	Mass	Score	Description
1.	gi|149392567	12196	135	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
2.	gi|56966763	15091	131	Chain S, Crystal Structure Of Activated Rice Rubisco Complexed With 2- Carboxyarabinitol-1,5-bis
3.	gi|671740	15111	128	ribulose-bisphosphate carboxylase [synthetic construct]
4.	gi|383875291	15222	128	Chain S, Structure Of Rice Rubisco In Complex With 6pg
5.	gi|115488238	19714	118	Os12g0291400 [Oryza sativa Japonica Group]
6.	gi|125536346	19936	114	hypothetical protein OsI_38046 [Oryza sativa Indica Group]
7.	gi|115488234	19846	114	Os12g0291100 [Oryza sativa Japonica Group]
8.	gi|115488240	19876	114	Os12g0292400 [Oryza sativa Japonica Group]
9.	gi|115488144	19862	112	Os12g0274700 [Oryza sativa Japonica Group]
10.	gi|149392260	18112	112	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
11.	gi|149392158	9927	109	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
12.	gi|383875287	15269	109	Chain S, Structure Of Rice Rubisco In Complex With Nadp(h)
13.	gi|125551406	19834	97	hypothetical protein OsI_19041 [Oryza sativa Indica Group]
14.	gi|347451	19292	97	ribulose 1,5-bisphosphate carboxylase (chloroplast) [Oryza sativa]
15.	gi|2407281	19733	96	ribulose 1,5-bisphosphate carboxylase small subunit [Oryza sativa Indica Group]
16.	gi|218210	19847	84	small subunit of ribulose-1,5-bisphosphate carboxylase [Oryza sativa Japonica Group]
17.	gi|3063524	19813	83	ribulose 1,5-bisphosphate carboxylase small subunit [Oryza sativa Japonica Group]
18.	gi|149392361	8463	81	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
19.	gi|149392308	15152	75	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
20.	gi|149392711	14758	60	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]

Results List

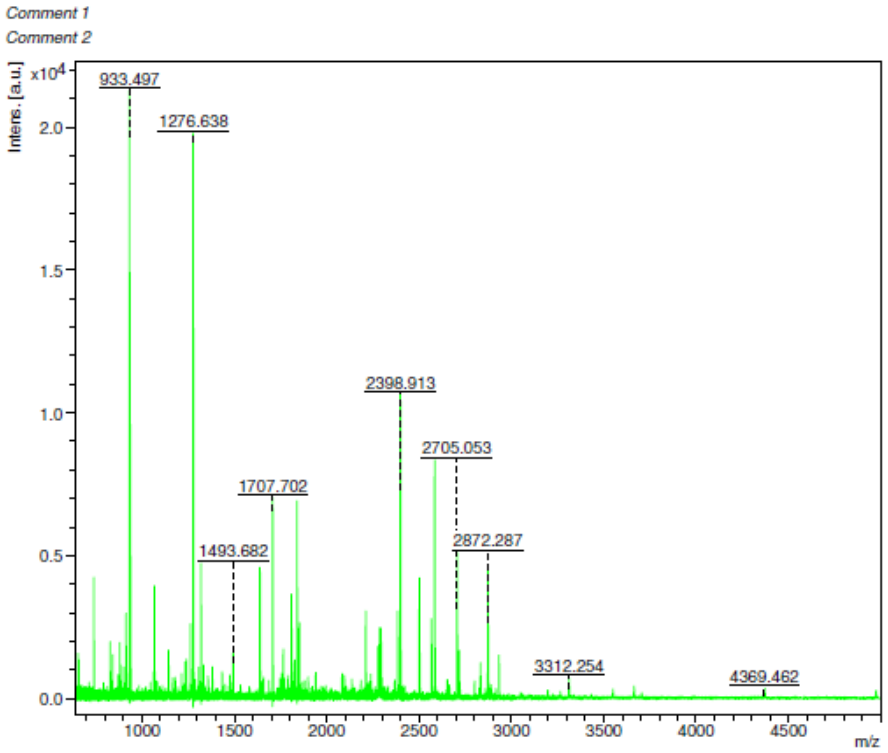
1. gi|149392567 **Mass:** 12196 **Score:** 135 **Expect:** 4.3e-09 **Matches:** 11

ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
740.4157	739.4084	739.4017	9.02	22 -	27	0	K.VGFVYR.E
914.4409	913.4336	913.4156	19.7	40 -	45	0	R.YWIMWK.L
933.5481	932.5409	932.5080	35.3	75 -	82	0	R.IIGFDNVR.Q
934.5358	933.5285	933.5283	0.21	4 -	10	0	K.QIEYLLR.S
938.5065	937.4992	937.4658	35.7	67 -	74	0	K.AYPDAFVR.I
1066.6017	1065.5945	1065.5607	31.7	66 -	74	1	K.KAYPDAFVR.I
1276.6943	1275.6870	1275.6472	31.2	22 -	31	1	K.VGFVYRENHR.S
1380.7249	1379.7176	1379.6907	19.4	11 -	21	1	R.SKWVPCLEFSK.V
1809.7801	1808.7729	1808.7981	-13.93	32 -	45	1	R.SPGYYDGRYWIMWK.L
1825.7757	1824.7685	1824.7930	-13.44	32 -	45	1	R.SPGYYDGRYWIMWK.L + Oxidation (M)
2294.0552	2293.0479	2293.1049	-24.85	83 -	103	0	R.QVQLISFIAYKPPGCEESGGN.-

No match to: 866.4444, 1092.2468, 1143.7180, 1166.6960, 1193.6892, 1223.7502, 1225.7527, 1240.2821, 1261.8323, 1294.7813, 1320.6304, 1333.6988, 1532.8059, 1638.8475, 1707.7599, 1723.4849, 1761.7907, 1765.8883, 1770.3271, 1803.9176, 1838.8829, 1866.8027, 2236.0835, 2278.0741, 2360.1412, 2368.9895, 2383.9009, 2398.9571, 2501.1768, 2566.2031, 2584.1337, 2705.0964, 2932.4305, 3052.5449

Fig 10: Peptide mass finger print of the spot 2 protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.347	10.3	544	3952	1452.17	389
658.417	7.2	483	2823	999.45	368
740.373	28.0	3225	3607	3829.36	1308
829.238	10.4	379	2561	1405.36	792
842.485	10.1	1213	4020	1363.92	504
881.268	10.6	380	2798	1409.18	796
914.409	19.6	1278	3705	2614.72	1188
917.444	7.6	375	1895	1004.79	882
933.497	146.9	10199	4365	19635.56	7841
934.525	32.2	487	4574	4303.69	1600
938.457	67.0	12495	4488	8956.39	3527
1066.552	28.7	10344	4974	3693.06	1605
1143.673	9.6	717	1702	1228.94	1712
1214.633	6.1	2616	6063	768.09	337
1234.634	8.8	1299	5304	1095.19	562
1240.252	7.3	426	2635	911.44	945
1261.796	16.5	749	2856	2051.07	2025
1276.638	156.4	13744	5207	19453.38	10941
1307.643	7.6	955	5823	947.48	490
1320.552	33.4	7273	5515	4149.31	2321
1333.652	7.9	728	4847	982.21	631
1380.672	6.7	223	4156	837.38	666
1493.682	10.0	1640	6134	1243.29	780
1638.808	35.6	16954	6345	4417.42	3234
1707.702	53.3	21460	6573	6537.36	5009
1765.828	9.9	718	4894	1211.51	1321
1809.748	27.0	6762	5752	3312.93	3252
1825.743	7.2	672	4315	878.34	1160
1838.835	50.5	19132	6340	6154.80	5664
1851.840	18.1	5411	5628	2207.64	2312
2210.980	21.5	5573	6135	2069.60	2467
2276.993	13.4	522	4301	1208.34	2132
2283.031	8.4	105	5248	757.41	1096
2285.026	14.5	350	4926	1298.03	2012
2293.990	18.0	1147	5294	1613.02	2346
2383.840	25.0	6812	5874	2070.40	2913
2398.913	89.1	31905	6552	7308.05	9291
2501.133	36.7	22622	6634	2744.67	3655
2566.165	24.4	3981	6137	1719.49	2555

Fig 11: *In-silico* analysis of the spot 2 protein using Mascot Server software

MATRIX SCIENCE **Mascot Search Results**

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150912 (71310511 sequences; 25955008131 residues)
Taxonomy        : Oryza sativa (rice) (135928 sequences)
Timestamp       : 23 Sep 2015 at 10:12:27 GMT
Top Score       : 121 for gi|149392567, ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).

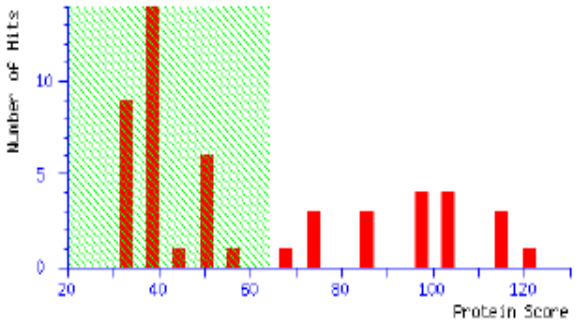


Fig 12: *In-silico* analysis of the spot 2 protein using Mascot Server software

1.	gi|149392567	12196	121	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
2.	gi|56966763	15091	117	Chain S, Crystal Structure Of Activated Rice Rubisco Complexed With 2- Carboxyarabinitol-1,5-bis
3.	gi|671740	15111	115	ribulose-bisphosphate carboxylase [synthetic construct]
4.	gi|383875291	15222	114	Chain S, Structure Of Rice Rubisco In Complex With 6pg
5.	gi|115488238	19714	104	Os12g0291400 [Oryza sativa Japonica Group]
6.	gi|125536346	19936	101	hypothetical protein OsI_38046 [Oryza sativa Indica Group]
7.	gi|115488234	19846	101	Os12g0291100 [Oryza sativa Japonica Group]
8.	gi|115488240	19876	101	Os12g0292400 [Oryza sativa Japonica Group]
9.	gi|115488144	19862	99	Os12g0274700 [Oryza sativa Japonica Group]
10.	gi|149392260	18112	99	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
11.	gi|149392158	9927	98	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
12.	gi|383875287	15269	97	Chain S, Structure Of Rice Rubisco In Complex With Nadp(h)
13.	gi|347451	19292	86	ribulose 1,5-bisphosphate carboxylase (chloroplast) [Oryza sativa]
14.	gi|125551406	19834	86	hypothetical protein OsI_19041 [Oryza sativa Indica Group]
15.	gi|2407281	19733	84	ribulose 1,5-bisphosphate carboxylase small subunit [Oryza sativa Indica Group]
16.	gi|218210	19847	74	small subunit of ribulose-1,5-bisphosphate carboxylase [Oryza sativa Japonica Group]
17.	gi|149392361	8463	73	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
18.	gi|3063524	19813	73	ribulose 1,5-bisphosphate carboxylase small subunit [Oryza sativa Japonica Group]
19.	gi|149392308	15152	66	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]
20.	gi|149392565	4583	54	ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]

Results List

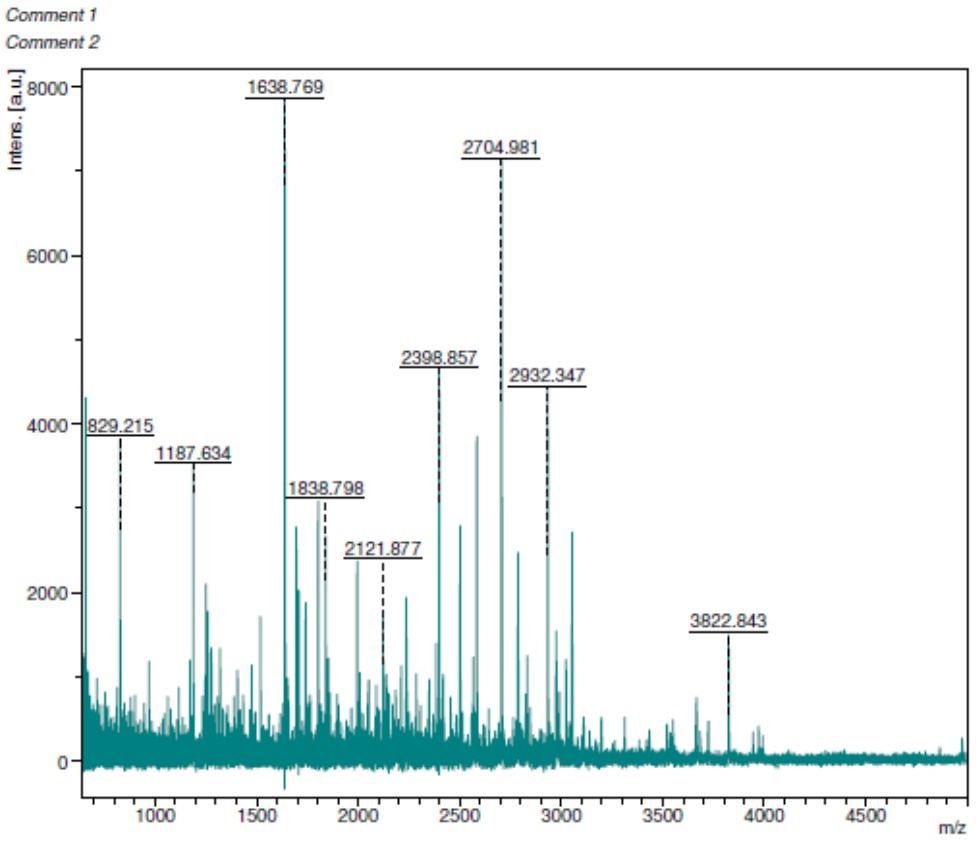
1. gi|149392567 **Mass:** 12196 **Score:** 121 **Expect:** 1.1e-07 **Matches:** 11

ribulose bisphosphate carboxylase small chain c, partial [Oryza sativa Indica Group]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
740.3729	739.3656	739.4017	-48.84	22	27	0	K.VGFVYR.E
914.4087	913.4014	913.3930	9.25	32	39	0	R.SPGYYDGR.Y
933.4972	932.4899	932.5080	-19.33	75	82	0	R.IIGFDNVR.Q
934.5247	933.5174	933.5283	-11.72	4	10	0	K.QIEYLLR.S
938.4573	937.4500	937.4658	-16.79	67	74	0	K.AYPDAFVR.I
1066.5521	1065.5449	1065.5607	-14.87	66	74	1	K.KAYPDAFVR.I
1276.6382	1275.6309	1275.6472	-12.80	22	31	1	K.VGFVYRENHR.S
1380.6718	1379.6646	1379.6907	-18.99	11	21	1	R.SKWVPCLEFSK.V
1809.7485	1808.7412	1808.7981	-31.44	32	45	1	R.SPGYYDGRYWTMWK.L
1825.7433	1824.7360	1824.7930	-31.23	32	45	1	R.SPGYYDGRYWTMWK.L + Oxidation (M)
2293.9903	2292.9830	2293.1049	-53.13	83	103	0	R.QVQLISFIAYKPPGCEESGGN.-

No match to: 653.3471, 658.4167, 829.2377, 842.4849, 881.2684, 917.4439, 1143.6732, 1214.6328, 1234.6337, 1240.2520, 1261.7962, 1307.6434, 1320.5521, 1333.6522, 1493.6824, 1638.8075, 1707.7020, 1765.8278, 1838.8353, 1851.8400, 2210.9803, 2276.9932, 2283.0315, 2285.0259, 2383.8397, 2398.9130, 2501.1325, 2566.1654, 2584.1017, 2651.2700, 2705.0527, 2716.9687, 2831.2797, 2872.2865, 2932.4315, 3312.2541, 3662.7832, 4369.4624

Fig 13: Peptide mass finger print of the spot 3 protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
644.359	7.7	251	2909	1363.07	490
658.411	21.7	752	3344	3846.51	1204
829.215	16.6	548	2960	2755.14	1343
1170.607	6.2	556	4327	927.90	531
1187.634	21.1	1539	5018	3180.06	1625
1249.585	10.3	1220	4076	1531.08	1052
1259.643	10.2	330	4328	1525.03	1002
1277.661	7.8	1450	5457	1170.03	624
1320.544	6.8	637	4699	1021.57	670
1518.676	9.7	1838	5823	1496.51	1023
1638.769	43.9	6638	6415	6845.59	4944
1695.782	14.2	2138	5652	2211.74	1914
1707.679	10.2	1323	5168	1593.15	1543
1741.790	10.2	3074	5774	1587.95	1436
1803.814	16.0	4392	5509	2506.71	2530
1838.798	13.6	2924	6372	2140.99	1945
1993.852	12.7	6534	6327	1873.70	1913
2121.877	8.4	901	4866	1142.28	1642
2236.035	9.6	733	4443	1228.58	2063
2383.811	6.4	729	5208	733.76	1163
2398.857	26.6	13289	6453	3070.08	3937
2501.064	15.1	5531	6065	1644.68	2376
2565.128	6.0	333	4203	624.05	1320
2584.035	23.9	12402	6548	2439.34	3436
2704.981	46.2	23676	6937	4281.37	6166
2786.204	15.9	7589	6552	1386.77	2214
2831.055	7.3	1358	5460	616.85	1213
2932.347	31.2	16564	7079	2454.67	3951
2974.312	9.3	792	4799	704.93	1671
3022.301	8.7	3162	6812	636.99	1135
3052.455	18.6	9361	7116	1339.01	2280
3663.717	8.2	392	6156	355.52	895
3822.843	14.4	3094	6918	539.77	1316

Fig 14: *In-silico* analysis of the spot 3 protein using Mascot Server software

MATRIX SCIENCE Mascot Search Results

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150912 (71310511 sequences; 25955008131 residues)
Taxonomy        : Oryza sativa (rice) (135928 sequences)
Timestamp       : 23 Sep 2015 at 10:29:06 GMT
Top Score       : 72 for gi|297599968, Os02g0771400 [Oryza sativa Japonica Group]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).

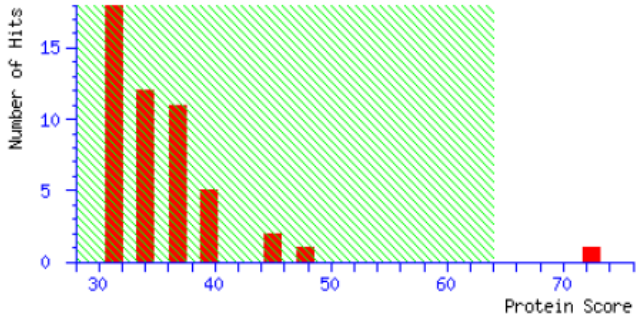


Fig 15: *In-silico* analysis of the spot 3 protein using Mascot Server software

Index

	Accession	Mass	Score	Description
1.	gi|297599968	23499	72	Os02g0771400 [Oryza sativa Japonica Group]
2.	gi|218200767	68288	47	hypothetical protein OsI_28446 [Oryza sativa Indica Group]
3.	gi|115451935	23396	45	Os03g0250900 [Oryza sativa Japonica Group]
4.	gi|115450215	16605	44	Os03g0109500 [Oryza sativa Japonica Group]
5.	gi|353351804	48383	40	TPA: nicotianamine aminotransferase homolog [Oryza sativa Japonica Group]
6.	gi|34394973	9047	40	hypothetical protein [Oryza sativa Japonica Group]
7.	gi|218187606	83167	39	hypothetical protein OsI_00611 [Oryza sativa Indica Group]
8.	gi|297725159	46880	39	Os06g0664400 [Oryza sativa Japonica Group]
9.	gi|13786456	18120	38	hypothetical protein [Oryza sativa Japonica Group]
10.	gi|215741526	28068	37	unnamed protein product [Oryza sativa Japonica Group]
11.	gi|222641845	58519	37	hypothetical protein OsJ_29872 [Oryza sativa Japonica Group]
12.	gi|218190758	7134	37	hypothetical protein OsI_07236 [Oryza sativa Indica Group]
13.	gi|62733827	137816	37	retrotransposon protein, putative, Ty3-gypsy sub-class [Oryza sativa Japonica Group]
14.	gi|222616640	48236	37	hypothetical protein OsJ_35224 [Oryza sativa Japonica Group]
15.	gi|222616665	51996	37	hypothetical protein OsJ_35278 [Oryza sativa Japonica Group]
16.	gi|125575762	32280	37	hypothetical protein OsJ_32537 [Oryza sativa Japonica Group]
17.	gi|56783729	10258	36	hypothetical protein [Oryza sativa Japonica Group]
18.	gi|222635444	91987	36	hypothetical protein OsJ_21077 [Oryza sativa Japonica Group]
19.	gi|125547747	12634	36	hypothetical protein OsI_15355 [Oryza sativa Indica Group]
20.	gi|125585623	16458	36	hypothetical protein OsJ_10156 [Oryza sativa Japonica Group]

Results List

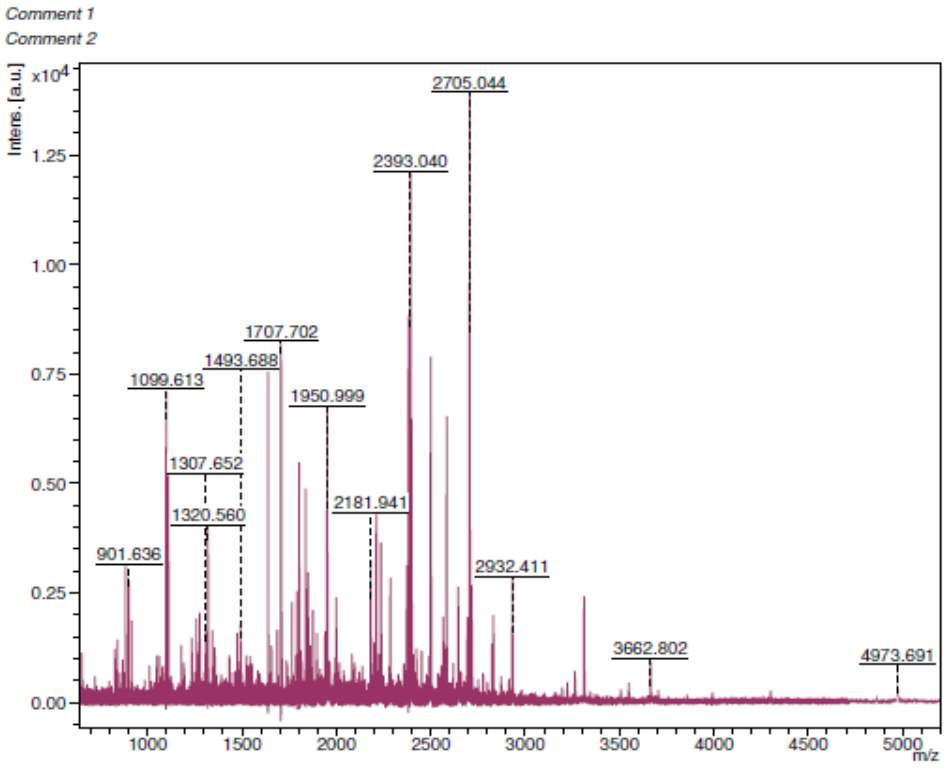
1. gi|297599968 **Mass:** 23499 **Score:** 72 **Expect:** 0.0078 **Matches:** 8

Os02g0771400 [Oryza sativa Japonica Group]

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
653.3391	652.3318	652.3881	-86.24	145 -	149	2	K.RGKHR.T
714.4499	713.4426	713.4436	-1.38	130 -	135	0	K.VILDVR.N
842.4752	841.4679	841.4657	2.61	2 -	8	0	M.QLEISPR.Q
1518.6755	1517.6682	1517.8170	-98.05	150 -	162	2	R.TGCVVGCLRKLQK.W
1803.8135	1802.8062	1803.0189	-117.95	130 -	144	1	K.VILDVRNQPVLIHCK.R
2236.0351	2235.0279	2235.0994	-31.99	78 -	95	1	K.LRSIVYLCPEPYPEENTR.F
2704.9815	2703.9742	2704.1931	-80.97	182 -	204	1	R.STDQRFMELFDTSSLMHLTASQC.-
2932.3474	2931.3401	2931.3313	3.00	180 -	204	2	K.ARSTDQRFMELFDTSSLMHLTASQC.-

No match to: 639.3523, 642.3247, 644.3594, 647.3407, 651.3375, 655.9999, 658.4108, 671.9727, 672.4318, 685.8822, 686.3947, 687.9437, 695.4073, 723.4308, 739.4192, 753.4161, 755.4610, 767.4672, 811.5194, 813.5133, 829.2147, 832.4474, 850.3972, 881.2237, 885.5277, 886.1450, 901.5692, 929.5221, 972.4101, 985.5332, 1061.5230, 1106.5251, 1116.5427, 1136.4792, 1148.5052, 1170.6072, 1179.5351, 1187.6344, 1234.6094, 1245.6203, 1247.5676, 1249.5851, 1259.6433, 1261.5502, 1277.6612, 1285.6082, 1293.6232, 1307.6158, 1316.6270, 1320.5441, 1334.6446, 1351.6096, 1357.6400, 1377.5517, 1387.6325,

Fig 16: Peptide mass finger print of the spot 4 protein obtained by MS-MALDI TOF



m/z	S/N	Quality Fac.	Res.	Intens.	Area
653.331	7.5	346	3803	991.37	271
829.234	6.5	231	3414	876.93	371
842.489	10.6	1246	6009	1448.20	370
886.191	18.4	1870	3407	2525.17	1178
901.636	19.2	4298	5224	2647.47	848
917.297	11.2	690	4455	1544.33	580
1096.570	36.4	3632	5315	5312.02	2247
1099.613	44.2	8612	6013	6455.17	2433
1110.598	33.1	11076	5836	4880.35	1937
1179.591	7.3	767	5783	1107.43	486
1234.646	7.4	558	5270	1163.87	601
1259.656	10.1	1382	6118	1600.03	741
1266.633	6.1	321	5625	973.63	492
1275.676	6.8	509	6535	1077.35	477
1277.673	9.4	1164	6170	1507.64	712
1307.652	8.5	183	6747	1378.39	622
1320.560	22.8	7098	6600	3703.88	1748
1343.611	7.9	677	5735	1307.76	720
1357.687	6.7	376	6441	1114.01	557
1475.709	8.0	2430	7226	1392.38	731
1493.688	8.2	2387	6735	1444.13	824
1638.795	42.2	23866	7956	7610.97	4453
1687.887	8.5	1228	8144	1540.13	935
1707.702	43.7	27237	7560	7956.00	5327
1711.726	6.1	639	4969	1116.70	1126
1765.873	10.9	3069	7012	1997.44	1499
1791.652	10.1	1036	6036	1852.29	1679
1803.846	24.2	4077	5846	4426.94	4190
1838.837	23.1	14371	7642	4231.58	3240
1851.844	11.8	1387	5637	2166.04	2262
1875.859	8.5	1178	6994	1561.46	1328
1940.861	6.6	968	6380	1180.45	1150
1950.999	25.0	1382	5574	4420.76	4942
2000.025	9.1	791	5739	1560.11	1752
2181.941	15.6	3605	6664	2370.61	2567
2210.978	20.9	12450	7572	3111.43	3023
2236.061	15.4	1033	4842	2232.46	3406
2285.993	13.8	5963	7043	1914.16	2099
2374.010	15.4	1835	6742	1966.10	2359

Fig 17: *In-silico* analysis of the spot 4 protein using Mascot Server software

MATRIX SCIENCE **Mascot Search Results**

```
User            : joy
Email           : joyprashant88@gmail.com
Search title    :
Database        : NCBInr 20150912 (71310511 sequences; 25955008131 residues)
Taxonomy        : Oryza sativa (rice) (135928 sequences)
Timestamp       : 23 Sep 2015 at 11:31:17 GMT
Top Score       : 71 for gi|75252730, RecName: Full=Photosystem II stability/assembly factor HCF136, chloroplastic; Flags: Precu
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).

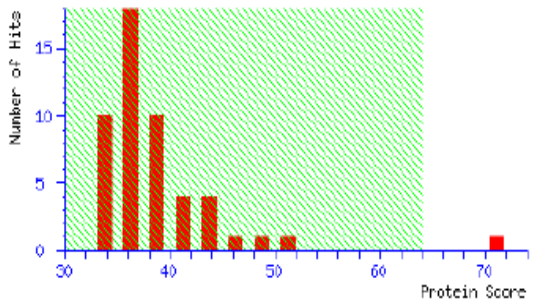


Fig 18: *In-silico* analysis of the spot 4 protein using Mascot Server software

Index

	Accession	Mass	Score	Description
1.	gi|75252730	45498	71	RecName: Full=Photosystem II stability/assembly factor HCF136, chloroplastic; Flags: Precursor
2.	gi|297607748	8710	52	Os07g0658100 [Oryza sativa Japonica Group]
3.	gi|41052915	41095	48	putative ferredoxin-NADP(H) oxidoreductase [Oryza sativa Japonica Group]
4.	gi|55297163	17613	47	hypothetical protein [Oryza sativa Japonica Group]
5.	gi|149390933	25650	44	ferredoxin--nadp reductase, leaf isozyme, partial [Oryza sativa Indica Group]
6.	gi|115451935	23396	44	Os03g0250900 [Oryza sativa Japonica Group]
7.	gi|218191731	32550	43	hypothetical protein OsI_09249 [Oryza sativa Indica Group]
8.	gi|115456081	24302	43	Os03g0807200 [Oryza sativa Japonica Group]
9.	gi|125546129	35775	42	hypothetical protein OsI_13990 [Oryza sativa Indica Group]
10.	gi|32489066	9150	41	OSJNBb0089B03.10 [Oryza sativa Japonica Group]
11.	gi|115445943	13174	41	Os02g0446100, partial [Oryza sativa Japonica Group]
12.	gi|125537696	41123	40	hypothetical protein OsI_05475 [Oryza sativa Indica Group]
13.	gi|115443657	39180	40	Os02g0103800 [Oryza sativa Japonica Group]
14.	gi|62734234	20564	40	hypothetical protein LOC_Os11g08350 [Oryza sativa Japonica Group]
15.	gi|125551712	40467	40	hypothetical protein OsI_19351 [Oryza sativa Indica Group]
16.	gi|218191241	38842	39	hypothetical protein OsI_08215 [Oryza sativa Indica Group]
17.	gi|47497217	43942	39	putative carboxypeptidase D [Oryza sativa Japonica Group]
18.	gi|125553198	34523	39	hypothetical protein OsI_20862 [Oryza sativa Indica Group]
19.	gi|77554332	18667	39	retrotransposon protein, putative, Ty3-gypsy subclass [Oryza sativa Japonica Group]
20.	gi|38344592	113332	38	OSJNBa0079M09.12 [Oryza sativa Japonica Group]

Results List

1. gi|75252730 **Mass:** 45498 **Score:** 71 **Expect:** 0.011 **Matches:** 15

RecName: Full=Photosystem II stability/assembly factor HCF136, chloroplastic; Flags: Precursor

Observed	Mr(expt)	Mr(calc)	ppm	Start	End	Miss	Peptide
832.4613	831.4541	831.4702	-19.36	109	115	0	R.QTILETK.N
1096.5699	1095.5626	1095.5713	-7.93	345	354	0	R.GFGILDVGYR.S
1099.6127	1098.6055	1098.6186	-11.94	289	298	0	R.ADGGLWLLVR.G
1275.6759	1274.6687	1274.6983	-23.24	402	413	0	K.GYVLGNDGVLLR.Y
1359.6812	1358.6740	1358.7154	-30.49	212	224	0	K.AAVQETVSATLNR.T
1542.6408	1541.6335	1541.6634	-19.37	125	137	0	R.SIPSAEDEDFNYR.F
1588.7332	1587.7259	1587.8257	-62.79	355	370	1	R.SKDEAWAAGGSGVLLK.T
1867.8380	1866.8307	1866.9417	-59.48	299	315	1	R.GGGLFLSKGSGFQFFYR.G
1949.9618	1948.9545	1949.0371	-42.37	396	413	1	K.FLGDNKGYVLGNDGVLLR.Y
2181.9408	2180.9336	2181.0338	-45.98	225	245	0	R.TVSSGISGASYYTGTFNTVNR.S
2328.9786	2327.9713	2328.0540	-35.48	185	206	0	K.ATGEQSAEMVTDEGAIYVTSNR.G
2374.0097	2373.0024	2373.1079	-44.47	258	276	0	R.GNFYLTWEPGQPFWQPHNR.A
2540.0040	2538.9967	2539.1325	-53.50	125	146	1	R.SIPSAEDEDFNYRFNSVSFMGK.E
2551.1450	2550.1377	2550.2867	-58.39	147	169	0	K.EGWIIGKPAILLHTSDAGDSWER.I
2694.1213	2693.1140	2693.2682	-57.24	225	250	1	R.TVSSGISGASYYTGTFNTVNRSPDGR.Y

Discussion

5. DISCUSSION

This study was taken up to screen different traditional rice genotypes at reproductive stage for water stress and to identify the tolerant genotype based on the morpho-physiological analysis and further to characterize the proteins in tolerant genotype by proteome analysis. The results presented in the previous chapter are discussed here under.

5.1 Phenotypical observations of each genotype according to IRRI scores

After panicle initiation, water stress was induced continuously in rice grown in polybags. When 90% of plants showed wilting symptoms according to IRRI leaf scoring system, the plants were screened for tolerance and susceptibility. Parambuvattan and Jyothi have taken only 11 and 12 days to reach 7.0 IRRI score and were identified as most susceptible genotypes under water stress condition. Chuvanna Modan which was showing no wilting symptom upto 18 days was identified as most tolerant genotype followed by Chenkayama which was showing slight tip drying with IRRI score of 1.0. One hundred and nine rice varieties were screened for drought to select promising varieties for hybridization programs programs and identified Danboto at a score of 4 was selected for further crop improvement programme (Gana, 2011). In the present study also tolerant genotype was selected at a score of 4.

5.2 Morpho-physiological observations

Drought stress could be applied at vegetative or reproductive stage of rice plants. It was found that the results of evaluation at two stages could be largely different, suggesting possible differences in drought tolerance mechanisms (Lilley and Fukai, 1993; Boonjung and Fukai, 1996). The ability of rice plant to overcome the drought stress during the initiation of panicles appeared to be more important for

the final grain yield than the drought during early stages. But the drought resistance at early growth stage is a precondition of final grain yield by keeping the seedlings survive or in well growth situation under drought at this period.

When 90% of plants have reached IRRI score 7.0 morpho-physiological observations were recorded to identify which trait was responsible for the drought tolerance in Chuvanna Modan.

5.2.1. Maximum root length

Many of the genotypes were showing positive effect on rice root length when stress was initiated compared with control. Under water stress the genotypes Parambuvattan, Karuthamodan and Chuvanna Modan gave the highest root lengths of 93.33 cm, 93.00 cm and 92.67cm and the same genotypes were showing less significant effect on control with Parambuvattan, Karuthamodan and Chuvanna Modan of 53.33 cm, 65.67 cm and 56.67cm which may be the reason for Chuvanna Modan's drought tolerance character (Table 5). This is in confirmation with the findings of Jaleel *et al*. (2008) in which he reported that an extensive root system is advantageous to support plant growth during the early crop growth stage and extract water from shallow soil layers that is otherwise easily lost by evaporation. An increased root growth due to water stress was reported in *Catharanthus roseus*.

5.2.2. Total Root volume

Maximum root length showed positive effect on total root volume (Table 6). Under stress conditions same genotypes which have more root length showed high root volume by water displacement measurements for Parambuvattan, Karuthamodan and Chuvanna Modan of 78.33 ml, 123.33 ml and 120.00 ml. Other than these three Karanavara also showed highest root volume of 163.33 ml. This may be because of more number of root hairs and root thickness which was in

confirmation with the experiments conducted by Zuno-Altoveros *et al.* (1990) to determine the root volume of some selected upland and lowland varieties. They found that Rikuto Norin12, a Japanese upland variety had very high root volume and a lowland variety, IR20 had low root volume because of more number of root hairs and root thickness.

5.2.3. Total root dry weight

Even though there was significant difference between maximum root length and total root volume between control and treatment in genotypes there was less significant variation in root dry matter content. Chuvanna Modan, Karuthamodan, Jyothi and Parambuvattan showed 10.78 g, 10.07 g, 7.54 g and 7.35 g under stress and 9.47 g, 8.19 g, 6.04 g and 5.51 g under watered condition respectively. This indicated increase in root length apportioning of photosynthates is less in roots under water stress but usually root dry matter content will be more in well watered condition than stress condition (Table 7). This was supported by the work of Sorte *et al.* (1992) i.e., 74 per cent reduction in root weight when soil moisture was reduced by 44 per cent.

5.2.4. Root to shoot ratio

Under drought stress conditions roots induce a signal cascade to the shoots *via* xylem causing physiological changes eventually determining the level of adaptation to the stress.

When the root dry matter content increases proportionally root to shoot ratio increases. Chuvanna Modan, Parambuvattan and Karuthamodan showed high root to shoot ratio of 0.72, 0.48 and 0.41 which indicated Chuvanna Modan and Parambuvattan having very high amount of dry matter content in roots comparatively with shoot dry wt. (Table 8). This view was supported by the work of Wu and Cosgrove, (2000) *i.e.,* when water availability is limited, the root: shoot ratio of plants

increases because roots are less sensitive than shoots to growth inhibition at low water potentials

5.2.5. Stomatal conductance

Another mechanism of drought avoidance in the rice is quick stomatal closure which acts to reduce water loss (O'Toole and Cruz, 1980). The stomata of rice plants close noticeably in response to a reduction in leaf water potential causing marked reduction in photosynthetic rate (Hirasawa *et al*., 1999).The results on genotypic variation in relative transpiration by Cabuslay *et al*. (1999) suggested that, drought tolerant genotypes maintained fairly open stomatas under stress. Stomatal conductance has a positive relationship with yield. Stomatal conductance regulates the entry of CO_2 into the plant and there by the accumulation of dry matter in the plant (Taiz and Zeiger, 1991). Fayez (2000) reported that higher stomatal conductance results in high yield under good irrigation system.

Chuvanna Modan has shown phenotypically no wilting symptoms when all the genotypes were showing wilting. The Chuvanna Modan has highest stomatal conductance of 1.11 μ mol of CO_2 m^{-2} s^{-1} under stress and 1.18 μ mol of CO_2 m^{-2} s^{-1} under control condition respectively, whereas in susceptible genotype it was 0.02 μ mol of CO_2 m^{-2} s^{-1} under water stress. This showed that drought tolerance of Chuvanna Modan may be due to good stomatal conductance due to continuous extraction of moisture. This was in confirmation with the reports of Cabuslay *et. al.* (1999) where high transpiration rate under conditions of water deficit also implies high stomatal conductance, which is associated with continued water extraction (Table 9).

5.2.6. Transpiration rate

Transpiration is a vital process in the life cycle of plants, which gives cooling effect besides promoting water and nutrient absorption (O'Toole and De Datta,

1986). The rate of water intake is determined largely by the rate of water loss by transpiration (Kramer, 1937) and is most sensitive to water stress (Hsiao, 1973). Relative transpiration during water deficit was highly and positively correlated with relative leaf area (Cabuslay *et al*., 1999), which is expected because the leaf is the organ of transpiration.

Chuvanna Modan was showing high transpiration rate both in stress and control condition (10.06 m mol of H_2O m^{-2} s^{-1} and 13.37 m mol of H_2O m^{-2} s^{-1}) respectively compared to other genotypes because of more stomatal conductance and hence did not showed any wilting symptom (Table 10). High transpiration in Chuvanna Modan is due to more water mining properties. This was supported by the findings of Cabuslay *et al*. (1999) i.e., high transpiration rate under conditions of water deficit also implies high stomatal conductance, which is associated with continued water extraction.

5.2.7. Electrical Conductivity

Electrical conductivity depends on the salt content. If the salinity is more EC value will be higher and due to water stress the membrane integrity will be lost and there will be leakage of osmolytes and salt through membrane. In susceptible genotypes integrity of the membrane will be lost and shows high EC values (Table 11).

Jyothi was showing high EC value of 157.00 d $Sm^{-1}$and 111.37 d Sm^{-1} under water stress and in normal condition which shows high membrane leakage and the plants were prone to wilting. Even Navara black, Kavunginpoothala and Kanali were also showing high EC values 153.97 d Sm^{-1}, 145.73 d Sm^{-1} and 129.23 d Sm^{-1} under stress which showed that they are susceptible to drought but Chuvanna Modan was showing lowest EC value of 54.30 d Sm^{-1} but the tolerance and susceptibility of the genotypes can be identified only through Membrane Stability index which can be calculated by the treatment and control EC values. Chuvanna Modan was showing

highest MSI value of 43 and Parmbuvattan was showing least MSI value of 18 which again indicates their tolerant and susceptible nature. The tolerance and susceptibility of genotypes by EC and MSI were confirmed by the findings of (Bano and Iqbal, 2009). They reported that water stress induced changes in membrane stablity index of four different wheat (*Triticum aestivum* L.) accessions (011251, 011417, 011320 and 011393) were determined in a pot study under natural condition during the wheat-growing season. Sampling was done 3, 6 and 9 days after induction of water stress. There was a marked decrease in membrane stability index occurred under water stress. Accession 320 showed maximum decrease in membrane stability index under water stress.

5.3. Extraction and quantification of total proteins

The TCA-Acetone process removes most secondary compounds, particularly lipids, phenolic compounds and pigments. A finer tissue powder results in a more thorough removal of secondary compounds. Finely powdered tissue is subjected to extensive cleanup with 10% (wt/vol) TCA/acetone and acetone plus either 0.07% DTT. Typically, after two washes with TCA/acetone and acetone containing 0.07% DTT, the tissue pellet should ideally be white or lightly coloured (Wang *et. al.,* 2014).

The concentration of the protein was quantified by z-Lowry's method by using spectrophotometer at 660 nm which ranges from 16-32mg/g of sample

5.4. Protein profiling by SDS-PAGE analysis

The SDS-PAGE profile of the proteins extracted from the two genotypes under water stress and control conditions were compared. In all the four samples many protein bands per lane were observed in the size range from 6.4 kD to 201.2 kD. The susceptible and tolerant genotypes were found to have almost similar banding pattern of proteins except for few bands and there is little variation of protein

regulation between those similar proteins. Under water stress four proteins of size 20 kD, 34.4 kD, 38 kD and 44 kD were expressed in Chuvanna Modan (Plate 8). 20 kD, 38 kD and 44 kD were differential bands whereas 34.4 kD was showing down regulation condition under water stress. One of the differentially regulated proteins of size 44 kD from Chuvanna Modan was sequenced by MS-MALDI TOF analysis.

In a similar manner there were three bands which were showing differential and up regulations in Parambuvattan treatment and control condition. The proteins with molecular weight of 20 kD and 33 kD in Parambuvattan treatment has shown upregulation and differential expression of protein respectively. In Parambuvattan under control condition one protein band with molecular weight 23 kD has shown differential pattern of protein expression whereas in stress condition same band was absent in Parambuvattan. 33 kD protein which was differentially expressed was sent for sequencing by MALDI-TOF/MS analysis.

5.5. Peptide mass fingerprinting by MALDI-TOF/MS

The two prominent bands of 44 kD and 33 kD in Chuvanna Modan and Parambuvattan which clearly depicted the expression in control and treatment after imposition of stress. The 44 kD and 33 kD proteins were digested with trypsin into peptides before embedding into a matrix made of aromatic compounds. A laser beam ionised the matrix along with the peptides, evaporated them and made to travel along a tube. The time of flight of each peptide is directly proportional to the molecular mass of the peptides, the data was fed to the computer and the peptide mass fingerprint was generated.

MALDI-TOF peptide mass finger printing (PMF) is the fastest and the cheapest method of protein identification promoting the characterisation of PR proteins in many plants like *Zea mays* (Campo *et al.,* 2004), *Oryza sativa* (Kim *et al.,* 2004) and *Medicago truncatula* (Colditz *et al.,* 2004)

5.6. *In-silico* analysis

Analysis of the peptide mass fingerprint of the two prominent bands of size 44 kD and 33 kD in the Mascot Server Software compared the data with all other protein sequences in NCBI database. Thus the two prominent bands of size 44 kD and 33 kD were found to be highly similar to the glycosyltransferase At5g03795 isoform X2 and kinesin like protein NACK-1 like isoform X1 respectively. Glycosyltransferases are enzymes that establish natural glycosidic linkages on a wide range of small and macromolecules including cell wall components, natural products, other saccharides, proteins and even nucleic acids. They catalize the transfer of saccharide moieties from activated nucleotide sugar to a nucleophilic glycosyl acceptor molecule (Williams, 2009). Kinesins are the protein move along microtubule and are powered by the hydrolysis of adenosine triphosphate (ATP). The active movement of kinesins supports several cellular functions including mitosis, meiosis and transport of cellular cargo, such as in axonal transport. Most kinesins walk towards the positive end of a microtubule, which, in most cells, entails transporting cargo from the centre of the cell towards the periphery (Vale, 2009)

5.7. 2D gel electrophoresis

In tolerant genotype, Chuvanna Modan treatment four differentially and up regulated protein spots were detected i.e., spots 1 and 2 were up regulated, spots 3 and 4 were differential spots. It indicates four different types of proteins expressed under water stress and these proteins may be responsible for the drought tolerance in tolerant genotype whereas in susceptible genotype, Parambuvattan treatment one differentially expressed protein spot was detected In the similar manner Parambuvattan treated plants four proteins spots were disappeared which were present in Parambuvattan control. These findings correlated with the findings of (Parker *et al.,* 2006). They analyzed rice leaf protein which resulted in the separation of approximately 2500 protein species of which 32 were observed to be significantly regulated by salinity; so far 11 of these proteins have been identified by tandem mass

spectrometry which indicated that only those 32 proteins were regulated under salt stress.

5.8. Peptide mass fingerprinting by MALDI-TOF/MS

Analysis of the peptide mass fingerprint of all the four spots in Mascot Server Software compared the data with all the protein sequences in NCBI database.

Spot 1 and 2 from drought tolerant Chuvanna Modan have shown highly similar to the protein ribulose bisphosphate carboxylase small subunit present in plants and green algae, raising the possibility that these subunits may regulate the structure or function of Rubisco. Studies of interspecific hybrid enzymes have indicated that small subunits are required for maximal catalysis and, in several cases, contribute to CO_2/O_2 specificity (Spreitzer, 2003). The clear function of small subunit is not yet known.

Spot 3, presents Protein tyrosine phosphatases At 1g050000 are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Protein tyrosine (pTyr) phosphorylation is a common post-translational modification that can create novel recognition motifs for protein interactions and cellular localization, affect protein stability, and regulate enzyme activity. As a consequence, maintaining an appropriate level of protein tyrosine phosphorylation is essential for many cellular functions. Tyrosine-specific protein phosphatases catalyse the removal of a phosphate group attached to a tyrosine residue, using a cysteinyl-phosphate enzyme intermediate. These enzymes are key regulatory components in signal transduction pathways (such as the MAP kinase pathway) and cell cycle control, and are important in the control of cell growth, proliferation, differentiation, transformation, and synaptic strengthening (Dixon, 1998).

Spot 4 has shown similarity with Photosystem II stability/assembly factor HCF136 which plays crucial role in photosynthesis i.e., it encodes a hydrophilic protein localised in the lumen of stroma thylakoids and it acts as assembly factor of PS II. It also essential for assembly of the PS II reaction center in *Arabidopsis thaliana* (Plucken, 2002).

Summary

6. SUMMARY

The study entitled “Identification and Characterization of traditional rice genotypes for drought tolerance through proteomic approach.” was conducted as part of the M.Sc programme at the centre for Plant Biotechnology and Molecular Biology, College of Horticulture, during the period of 2013-2015. The objective of the study to phenotype and genotype land races of rice for drought tolerance through physiological characterization and further proteome analysis through SDS-PAGE and 2D gel electrophoresis and identification of the differentially expressed proteins by MS/MALDI-TOF followed by in-silico analysis.

Genotypes were screened at IRRI leaf rolling score 7.0 and the genotype Chuvanna Modan which took 25 days to show wilting symptoms at the IRRI leaf rolling score of 7.0 was identified as drought tolerant genotype and the genotype Parambuvattan took 11 days to wilt was identified as susceptible genotype which were selected for proteomic analysis.

After identification of the tolerant and susceptible genotypes for drought the plants were anlaysed by phenotypical observations and characterized which phenotypic character is responsible for drought tolerance in rice genotypes But all the characters analysed for the drought study showed positive results for the tolerant Chuvanna Modan but the susceptible genotype Parambuvattan showed some variations.

Phenotypic parameters in Chuvanna Modan recorded root length, root volume, root dry wt. and root to shoot ratio of 92.62 cm, 120.00 ml, 10.78 g and 0.72 whereas Stomatal Conductance and transpiration rate was 1.11 µ mol of CO_2 m^{-2} s^{-1},and 10.06 m mol of H_2O m^{-2} s^{-1} because of better water mining trait. Phenotypic parametres in Parambuvattan recorded root length , root volume, root dry wt. and root to shoot ratio of 93.33 cm, 78.33 ml, 5.35 g and 0.48 respectively

Whereas stomatial Conductance and transpiration rate of 0.02 μ mol of CO_2 m^{-2} s^{-1}, and 0.65 m mol of H_2O m^{-2} s^{-1}.

Total protein was extracted from the leaf samples collected. Concentration of the extracted proteins was high in the range of 16-32 mg/g as quantified by z-lowrys', method. The normalized proteins of 1.5mg/ml concentration were analysed by SDS-PAGE technique to obtain the protein profile of different genotypes. The leaf samples were collected after plants reached IRRI leaf score of 7.0.

The protein profiles were distinct for each of the two different genotypes with a maximum of 20 protein bands per lane. In Chuvanna Modan genotype showed differential proteins of size 20 kD, 38 kD and 44 kD and 34.4 kD protein was down regulated. In Paramuvattan genotype - 20 kD protein was up regulated 23 kD and 33 kD protein were differentially regulated.

The two prominent bands at 44 kD and 33 kD were cut from the gel and sequenced by MS/MALDI-TOF to obtain the peptide mass finger print. The data was further analysed by MASCOT server Software and protein bands were found to be homologous to the glycosyl transferase and Kinesin NACK-1 like protein. In Chuvanna Modan protein glycosyl transferase along with other proteins has some mechanisms for better tolerance under water stress condition and in Parambuvattan Kinesin NACK-1 like protein was differentially regulated. By 2D gel electrophoresis in Parambuvattan 4 differential and upregulated spots were detected. Spot 1 and 2 were identified as ribulose bisphosphate carboxylase small subunit, spot 3 was identified as Protein tyrosine phosphatases and spot 4 was identified as Photosystem II stability/assembly factor HCF136.

- Genotypes identified may be used for Molecular breeding to enhance the drought tolerance in high yielding and popular varieties which are susceptible to water stress

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ANNEXURE-I

Chemicals for SDS-PAGE analysis

Acrylamide-Bisacrylamide stock

29.2 g of acrylamide and 0.8 g of N' N' Bismethlene acrylamide was dissolved in 80 ml of distilled water and made up to 100 ml. The solution was the filtered and stored at 4°C in dark up to 30 days.

Separating gel buffer (pH - 8.8)

22.7 g of 1.875M Tris base was dissolved in 80 ml of distilled water, the pH was adjusted to 8.8 with IN HC1 and the volume was made up to 100 ml with distilled water. The solution was stored at 4°C.

Stacking gel buffer (pH - 6.8)

7.26 g of 0.6M Tris base was dissolved in 60 ml of distilled water, the pH was adjusted to 6.8 with 1N HCl and the volume was made up to 100 ml with distilled water. The solution was then stored at 4°C.

10 percent Sodium Dodecyl Sulphate (SDS)

1 g of SDS was dissolved in distilled water and the volume was made up to 10 ml with distilled water. The solution was stored at room temperature.

10 per cent Ammonium Persulphate (APS)

0.1g APS was dissolved in 1 ml of distilled water to obtain 10 per cent of APS.

Tank Buffer

192mM Glycine – 14.4 g

25mM Tris base – 3.0 g

0.1 per cent SDS – 1.0 g
Distilled water - 1 L

All the components are mixed and made up to 1 L. The buffer can be stored at 4 ^{0}C and warmed to 37 ^{0}C before use. The same buffer can be used 2-3 times for running the gel.

Sample buffer

0.125 M Tris HCl (pH-6.8) -1.25 ml

Glycerol -1.0 ml

2-mercaptoethanol -0.1 ml

Bromophenol blue - 0.1 **g**

10 per cent SDS - 2 ml

Made up to 10 ml with distilled water.

Preparation of the gel mixture

Components	12 per cent Separating gel	4 per cent Stacking gel
Distilled water	13.5 ml	6.15 ml
Acrylamide stock	15.83 ml	1.34 ml
1.875 M Tris HC1 buffer	10 ml	
0.6 M Tris HC1 buffer		2.5 ml
10 per cent SDS	0.4 ml	0.05 ml
10 per cent APS	0.2 ml	80 µl
TEMED	0.02 ml	16 µl
Total	40 ml	10 ml

ANNEXURE-II

Solutions for Silver Staining

Fixing solution

Add 30 per cent ethanol and 10 per cent acetic acid.

Pretreatment solution

0.02 per cent sodium thiosulphate prepared by adding 0.02 per cent sodium thiosulphate in 100 ml of distilled water.

Developing solution

250 µl of formaldehyde was added with 3 g of sodium carbonate and 0.5 mg of sodium thiosulphate. The volume was made up to 100 ml with distilled water.

Stop solution

4 per cent Tris was prepared and added with 2 per cent acetic acid.

ANNEXURE-III

Solutions for Coomassie Staining

Protein staining solution

Coomassie brilliant blue R 250 dye - 0.1 g

Methanol - 50 ml

Acetic acid - 10 ml

Distilled water - 40 ml

The dye was first dissolved in methanol and all other components were added. Every time fresh preparation of the dye solution was prepared.

Destaining solution

Methanol - 30 ml

Acetic acid - 7ml

Distilled water - 53 ml

ANNEXURE-IV

Protein extraction:

A) TCA extraction solution

10% TCA: 10g
0.07% DTT: 0.07g
Made upto 100ml with acetone

B) Sample washing buffer

0.07% DTT: 0.07g
Made upto 100ml with aceone
Stored I glassbottle at -20oC

C) Lysis buffer:

9M Urea: 5.4g
CHAPS 4%: 0.4g
DTT 1%: 0.1g
pH 3-10 ampholytes-250µl
35mM Tris base: 0.0424g

Lysis buffer was made up to 10ml with milliQ water and filtered through 0.2µm pore size membrane. Small aliquots were made and stored at -80oC. Sample buffer once thawed cannot be refrozen for further use.

ANNEXURE-V

One dimensional focussing

A) Rehydration buffer:

Urea: 12g
CHAPS: 0.5g
Bromophenol blue: few grains
Double distilled water: 25ml
Ampholyte pH 3-10
DTT: 0.1g

B) Equilibration buffer I:

6M Urea: 3.6g
30% w/v Glycerol: 3ml
2% w/v SDS: 0.2g
1% w/v DTT: 0.1g
1.5mM Tris HCl buffer pH 8.8:

C) Equilibration buffer II:

6M Urea: 3.6g
30% w/v Glycerol: 3ml
2% w/v SDS: 0.2g
Iodoacetamide: 0.25g

Identification and characterization of traditional rice genotypes for drought tolerance through proteomic approach

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The study on "Identification and characterization of traditional rice genotypes for drought tolerance through proteomic approach'' was carried out during 2013-2015. Traditional rice genotypes were collected from RARS, Pattambi and Ambalavayal which was further screened and evaluated for drought tolerance. The identified tolerant and susceptible genotypes was further analysed by 2D gel electrophoresis. Based on the morpho-physiological characters, the plants which showed wilting symptoms were screened according to international standard i.e., IRRI leaf score 7.0. The genotype which showed no wilting symptom even after 90% of the plants were showing wilting was identified as tolerant and the plant which showed early wilting symptom was identified as susceptible genotype.

The rice genotypes were transplanted in polybags and water stress was imposed to each genotype at reproductive stage (visual panicle initiation stage). The genotype Chuvanna Modan has taken 25 days to wilt and the genotype Parambuvattan has taken only 10 days for wilting according to IRRI leaf rolling score 7.0. After 90% of plants reached IRRI score 7.0 phenotypic observations were recorded for each genotype. The morphological observations evaluated in each genotype were water mining traits like root length, root volume, root dry weight and root to shoot ratio and the physiological observations evaluated were measurement of Electrical Conductivity (EC), measurement of Stomatal conductance and transpiration rate using Infra red gas analyser (IRGA).

The morphological observations recorded for the most tolerant, Chuvanna Modan at IRRI score 7.0 has shown root length, root volume, root dry wt and root to shoot ratio of 92.67 cm, 120 ml, 10.78 g, 0.72 respectively whereas the most susceptible genotype Parambuvattan recorded 93.33 cm, 78.33 ml, 5.35 g, 0.48 respectively. Similarly, the physiological observations like Stomatal Conductance, Transpiration rate and EC for the Chuvanna Modan were 1.11 μ mol of CO_2 m^{-2} s^{-1},

10.06 m mol of H_2O m^{-2} s^{-1} and 54.30 d Sm^{-1} whereas 0.02 µ mol of CO_2 m^{-2} s^{-1}, 0.65 m mol of H_2O m^{-2} s^{-1} and 108.04 d Sm^{-1} for Paramabuvattan

Leaf samples collected from Chuvanna Modan and Parambuvattan were further subjected for proteome analysis through SDS and 2D gel electrophoresis. The proteins extracted through TCA/Acetone precipitation were analyzed through SDS PAGE and identified a total of 7 differentially expressed proteins. In Chuvanna Modan genotype proteins of size 20 kD, 38 kD and 44 kD were expressed differentially and 34.4 kD protein was down regulated. In Parambuvattan genotype - 20 kD protein was up regulated 23 kD and 33 kD protein was differentially regulated. The differential bands of size 44 kD and 33 kD bands from Chuvanna Modan and Parambuvattan respectively were sequenced by MALDI-TOF/MS (Sandor Proteomics, Hyderabad). 44 kD band was identified as glycosyltransferase At5g03795 isoform X2, 33 kD band as kinesin-like protein NACK1-like isoform X1 and an uncharacterized protein LOC105957228.

Many proteins of same molecular weight will be accumulated, in single band of SDS. Hence to highly resolve the protein based on isoelectric point and on molecular weight two dimensional (2D) gel electrophoresis was carried out to differentiate the proteins present in a single band. The total proteins from tolerant and susceptible genotypes were subjected to 2D gel electrophoresis to identify and characterize the up regulated, down regulated and differentially expressed proteins for drought tolerance. Totally four differentially expressed protein spots were identified in Chuvanna Modan. The differential protein spots were sequenced by MALDI-TOF/MS (Sandor Proteomics, Hyderabad). Spot 1 and 2 were identified as ribulose bisphosphate carboxylase small subunit, spot 3 was identified as Protein tyrosine phosphatases and spot 4 was identified as Photosystem II stability/assembly factor HCF136.