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Identification of Candidate Genes and Genome Engineering of Drought Tolerance in Bangladeshi Rice Varieties

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Submitted to the Department of Botany
University of Rajshahi, Bangladesh in Partial Fulfillment of the Requirements for the Degree
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THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Identification of Candidate Genes and Genome Engineering of Drought Tolerance in Bangladeshi Rice Varieties

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DECEMBER, 2008

DEDICATED

To my beloved parents
And teachers
Who are always busy
With creating
A congenial atmosphere
For acquiring
Knowledge

Identification of candidate genes and genome Engineering of drought tolerance in Bangladeshi Rice Varieties

A Thesis submitted
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DECLARATION

I hereby declare that the Ph.D. thesis entitled **"Identification of candidate genes and genome engineering of drought tolerance in Bangladeshi Rice Varieties"** submitted to the University of Rajshahi, Bangladeh from independent work carried out by me under supervision of **Dr Firoz Alalm, Dr Rafiul Islam**, Professors, Department of botany, University of Rajshahi. Bangladesh. and **Dr Philippe Herve,** Plant Biotechnologist, IRRI, Philippines. The Research work has conducted in the biotechnology Laboratory, International Rice Research Institute (IRRI) Philippines. Research work in abroad was continued with the postgraduate fellowship from **Third World Organization for Women in Science (TWOWS).** The thesis has not been formed previously the basis for the award of any degree.

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CERTIFICATION

We hereby certify that the thesis entitled "**Identification of candidate genes and genome engineering of drought tolerance in Bangladeshi Rice Varieties**" submitted by Sayeda Mahfuja Khatun in partial fulfillment for the requirements of the degree of Doctor of philosophy in the field of Plant Biotechnology, to the University of Rajshahi, Bangladesh. It is also certified that the research work embodied in this thesis is original and carried out by her during the period from January 2001 to December 2008 under our supervision in collaboration with IRRI(July,2004 to October,2007) and the University of Rajshahi, Bangladesh. The whole work or part of the work has not been submitted before as candidature for any other degree or diploma.

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Sayeda Mahfuja Khatun

CONTENTS

ABSTRACT

Drought is one of the major constrains to the productivity of rice in many parts of the world, particularly in South and South East Asia. Among the natural disaster drought is a common phenomenon in different cropping season and it causes vulnerable yield loss of rice in Bangladesh. The aim of this study was to identify candidate gene involves in the drought response mechanism of rice and to investigate suitable method for genome engineering for popular rice cultivars in Bangladesh. Dehydration responsive binding protein (DREB) is a group of transcription factor (cis-acting element) reported to be induced by abiotic stress and activate function/regulation of target genes involves to stress tolerance (drought, salt and freezing) mechanism in plants. Reverse transcription PCR analysis confirmed the presence of 14 genes of the OsDREBs gene family (including a new OsDREB1J) in BR-29, BR-28, and BR-11 rice genome. Experiments were carried out to understand physiological response against drought stress in the studied rice cultivars. Beside the physiological parameters and molecular analysis were carried out from drought stressed, and well watered plant tissues (reproductive organ). RT-PCR analysis was performed for three major tissues at reproductive stage of rice such as flag leaf, peduncle, and panicle. The results showed that interesting response to drought stress and response pattern varied across genotypes and tissues. Most of the gene expressed positively to the change of peduncle elongation rate, panicle exertion rate, and relative water content due to drought stress level. OsDREB1A, OsDREB1G, OsDREB1F and OsDREB2A found to be most drought responsive genes across the studied varieties and among them OsDREB1A identified to be most potential for over-expression through gene transformation method to enhance drought tolerance in rice. In order to develop suitable protocol for genome engineering, attempts were made to standardize criteria for successful gene transformation in rice. Different media compositions, genotypes, and explants were evaluated for efficient Agrobacterium mediated gene transformation. OsDREB1A gene was driven by stress inducible Lip-9 promoter introduced in to BR-29, BR-28 and BR-11 to make drought tolerant rice lines. A total number of 117 individual transgenic events were confirmed by genomic PCR of OsDREB1A. Southern blotting was revealed the transgenic integration. Molecular analysis of T_1 progeny demonstrated the stable integration, inheritance, and expression of the OsDREB1A gene. The subsequent segregating generation followed the mendalian 3:1 monogenic inheritance pattern of the transgene. In green house, transgenic plants growth (T_1 progeny) was

vigorous and showed normal flowering and grain setting. The transgenic lines were given drought stress in green house condition, showed enhanced drought tolerance than non-transgenic plants. These finding suggests that plants with enhanced drought tolerance developed by genome engineering may contribute to reduce the yield loss problem due to uneven drought in the Asian and South Asian countries including Bangladesh where the population consumes rice as staple food.

CHAPTER 1
INTRODUCTION

INTRODUCTION

1.1 General Introduction

Demand for food and fiber is determined by the number of people to be fed and clothed. According to the united nation organization (UNO), the worlds population is expected to rise to at least 9.3 billion by the year 2050 and global demographic trends are not expected to stabilized before 2100 (UNO, 2001). A special impact will be felt in developing countries, as projections indicate a rise in populations to 8.2 billion by 2050 from the present level of 4.9 billion. About 59% of the increase over the next 25 years will take place only in south Asia and sub- Saharan Africa where poverty and hunger are already whispered (James, 2000;Hossain, 2001). As a result, more food and fibers are needed to satisfy demand, these being the most basic human needs. An increase in food production of more than 50% is needed to meet the requirements of the projected population increase, which is one of the most formidable challenges for humankind. Moreover, food demand also increases from the changes in food habit of people (James, 2000 and UNO, 2001). This increased demand can only be achieved by diversifying crop production or by increasing crop productivity.

Rice, (*Oryza sativa* L) is one of world's most important food crops and the staple food for more than three billion people or over half the world's population (FAO, 2004). It is cultivated in about 11% of the world land. Present annual rice production is 598 million tons, which is produced from about 153.7 millions hectares of land (FAO, 2001). Rice represents about 23% of global caloric consumption whereas wheat and maize represent only 17 % and 9 %, respectively. Approximately 92 percent (perhaps more) of the world's rice produced and consumed in Asia, particularly in south and south East Asia, as a staple food, providing between 30-70 % of the necessary calories per person (Papademetriou, 1999; Kush, 2001). It provides 27 per cent of the dietary energy supply and 20 per cent of dietary protein intake in the developing world. Grown in at least 114 mostly developing countries, in Asia it covers half of the arable land used for agriculture in many countries (Cantrell and Hettel, 2004). This shows that production and consumption of rice is mostly confined to Asia where the population density is high.

As far as increased food production is concerned, this can be achieved either expanding cultivable lands or by increasing production per unit area. Evidence suggests that increased production per unit area is more likely than the expansion of land under cultivation (Paterson et al.,1991; Toennissen, 1991; Mannion, 1995). With growing urbanization and industrialization, the area of fertile agricultural land used for the production of food and fibers has been decreasing, due to the demand for housing, roads and factories. Moreover, the chances of increasing cultivable land are low in densely populated countries of the world, such as Bangladesh and China. This being the case, high yielding varieties with excellent grain quality, adaptable to adverse agro-climatic conditions such as drought, salinity, unsuitable soil structure, and nutrition deficiency with durable resistance to insects and pest are clearly required (Toennisen, 1991). In other words, food production increase must be achieved from less land with less labor, water fertilizer and pesticides, and the increase in production must be sustainable (Virmani, 1996). However, it is not an easy task to meet the demand for increased agriculture productivity with a decrease in the farmland available. Consequently, genetic improvement of crops represents one of the most important approaches to attain a balance between food production and population growth (Kush, 1997; Tinsley and McCouch, 1997). The combined application of conventional breeding molecular biology and biotechnology will help to relieve the task of increasing food production.

1.2 Constrains for Rice Production in its Ecosystems

The rice ecosystems are low land, upland flood prone or deep water, and irrigated ecosystems. They are mainly dependent on water regime, rainfall pattern, depth and duration of flooding and drainage. More than 50 % of world rice area is under irrigation and contributes to about 75% of the world rice production. In an irrigated ecosystem, rice productivity is high, and this is largely due to the adoption of modern rice technology by farmers. Upland ecosystem account for about 15% of the world rice production area but contribute only 5% of total rice production. Deep-water ecosystem account for about 10% of the world's rice crop area and contribution to total production is also 5%. As a result, rice production is always subject to drought, flood, and saline condition. Rainfed lowland ecosystems predominate in the worlds most densely populated rural regions, such as South and Southeast Asia. They contribute about 17% of the world rice production, from about 25% of the world rice land, which means that productivity lower in rainfed ecosystem than that irrigated. This low productivity is due to floods or drought

or both occur almost every year. Moreover, salinity (Flower et al.,2000; Koyama et al.,2001) causes low productivity in rainfed lowland ecosystems. It is clear from the above statistic that the production of rice per unit area is less in rainfed lowland, upland, and flood prone or deep-water ecosystems. One reason may be the lack of high yielding variety for these ecosystems. Therefore, rice production could be increased more efficiently in these ecosystems through the selection or development of appropriate varieties, as the production is poor or indigent.

Major environmental stresses are drought, high temperature, low temperature, flood, salinity, and high radiation (Boyer, 1982; Herdt, 1991) and account for about 85%of the total crop plant s production losses (Boyer, 1982). Among these, drought and high temperature considered major factors that limit the yield of crops (Boyer, 1982). Water is the most important factor than any other for the development of crops. Drought limits the yield at some stages of the crop cycle in rainfed and upland conditions. In upland and lowland areas, farmers generally do not have access to irrigation, resulting to low yield due to water stress caused by uneven distribution, and unpredictable and insufficient rainfall patterns (Hossain, 1996). More than one third of the South and South-East Asian rice lands are under rainfed lowland conditions, where drought and flood along with salinity hamper crop production at some stages of crop growth. In eastern India, farmers identified drought stresses as the foremost constrain to higher yield in upland and rainfed ecosystems (Herdt, 1996; Mackill, 1996). These ecosystems may experience frequent and severe water stress during the rice-growing period at any time. The rainfed lowland as well as uplands offers tremendous potential for increasing rice production and for making rice profitable (IRRI, 1982; Toennesson, 1991; Ribout et al.,1996). Productivity of rice could be increased in upland and rainfed ecosystems with the development of drought resistant varieties.

1.3 Rationale of the Work

Bangladesh is one of the most densely populated countries in the world. Present population is about 147 million and more than 900 people living on per square kilometer area. Rice occupies the central role of Bangladesh economy and agriculture, it accounts for nearly 18% of national GDP and provide about 70% of an average total caloric intake. The impact of drought spreads disproportionately among regions of Bangladesh. The High Barind Tract of northwest Bangladesh is relatively dry, receiving only 50 inches of rainfall annually occurring in June to October. The eastern districts, in contrast, receive more than 80 inches of rainfall (Figure 1.1)

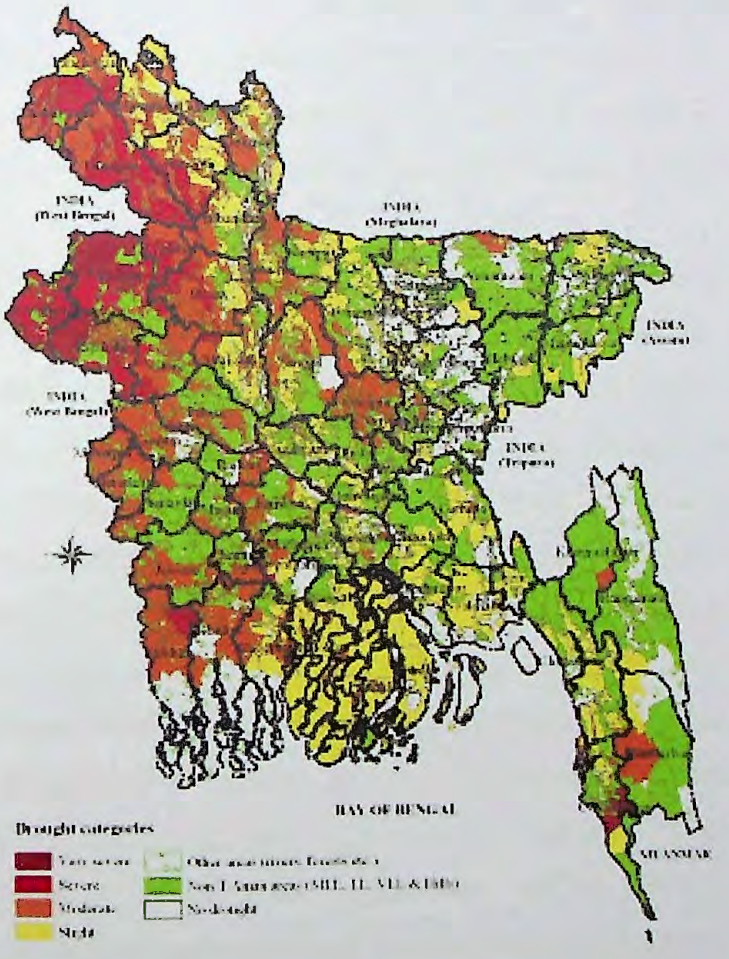


Figure 1.1 Map of Bangladesh showing drought prone areas.

Nevertheless, drought can hit both drought-prone and no drought-prone areas (Nur-E-Elahi et al.,1999; Saleh et al.,2000). Drought adversely affects all three rice varieties (aman, aus, and boro) grown in three different cropping seasons in Bangladesh. Droughts in March-April prevent land preparation and plowing activities from being conducted on time. As a result, broadcast aman, aus, and jute cannot be sown on schedule. Droughts in May and June destroy broadcast aman, aus, and jute plants. Inadequate rains in August delay transplantation of aman in high land areas, while droughts in September and October reduce yield of both broadcast and transplanted aman and delay the sowing of pulses and potatoes. Boro, wheat, and other crops grown in the dry season are also periodically affected by drought (Saleh and Bhuiyan, 1995). However, the loss of rice production is the most costly damage incurred by droughts in Bangladesh. BR-11, BR-28, and BR-29 are among the most widely grown cultivars in Bangladesh. BR-11 is grown during 'aman' season (summer to autumn/wet monsoon), the most widely planted season started at the onset of the monsoon in July. BR 28 and BR 29 are irrigated, dry-winter season crop that is transplanted in February. Hence, they were chosen for the present study.

Drought is a major limitation on rice production and several approaches have been suggested for enhancing drought tolerance of popular varieties. These approaches include conventional breeding, marker-aided selection to understand the physiological and molecular responses of stress related gene(s) to drought stress and genetic transformation.. Plants respond and adapt to stresses to survive under stress conditions at the molecular and cellular levels as well as at the physiological and biochemical levels. Therefore, environmental stresses can induce the expression of a large amount of genes. Among these are many transcription factors that regulate the expression of downstream genes by specifically binding to cis-elements or forming transcriptional complexes with other proteins. Expression of a variety of genes has been demonstrated to be induced by these stresses in a variety of plants (Ingram and Bartels, 1996; Shinozaki and Yamaguchi- Shinozaki, 1996, 2000; Thomashow, 1999). The cis- and trans-acting elements that function in stress-responsive gene expression have been precisely analyzed to elucidate the molecular mechanisms of gene expression in response to drought stress. The dehydration-responsive element (DRE) with the core sequence A/GCCGAC was identified as a cis-acting promoter element in regulating gene expression in response to drought, high salt, and cold stresses in Arabidopsis (Yamaguchi-Shinozaki and Shinozaki, 1994). Rice, an important crop plant, has also emerged as an ideal model

species for the study of crop genes because of its commercial value, relatively small genome size (approx. 430 Mb), diploid origin (2x=24), and close relationship with other important cereal crops. It is important to analyze the DRE/DREB (CRT/CBF) regulon in rice to understand the molecular mechanisms of stress tolerance and improve monocots with higher stress tolerance by gene transfer. In rice Some DREB homologues have been isolated and analyzed (Dubouzet et al.,2003).Based on these repots one of the aim of this research was to analyze the expression of DREB gene family under in some Bangladeshi rice variety under drought stress and well water condition.

Several different approaches have been attempted to improve the stress tolerance of plants by gene transfer (Shinozaki et al., 2003). The genes selected for transformation were those encoding enzymes required for the biosynthesis of various osmoprotectants. Other genes that have been selected for transformation include those that encode enzymes for modifying membrane lipids, LEA protein, and detoxification enzymes. In all these experiments, a single gene for a protective protein or an enzyme was over expressed under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter in transgenic plants, although several genes have been shown to function in environmental stress tolerance and response. The genes encoding protein factors that regulate gene expression and signal transduction and that function in stress responses may be useful for improving the tolerance of plants to stresses so can regulate many downstream genes involved in stress tolerance. DRE has been shown to function in gene expression in response to stress in tobacco plants, that suggests the existence of similar regulatory systems in tobacco and other crop plants (Yamaguchi-Shinozaki and Shinozaki, 1994). The DRE related motifs have been reported in the promoter region of cold-inducible *Brassica napus* and wheat genes (Jiang et al., 1996, Ouellet et al., 1998). CBF/DREB1 transcription factors were also identified in *B. napus*, wheat, rye, tomato, and rice, and all of them showed a rapid response to cold stress (Jaglo et al., 2001, Dubouzet et al., 2003). On the other hand, over expression of the *Arabidopsis* DREB1/CBF genes in transgenic *B. Napus* or tobacco plants induced expression of orthologs of *Arabidopsis* DREB1/CBF-targeted genes and increased the freezing tolerance of the transgenic plants (Jaglo et al., 2001, Kasuga et al., 1999). These observations suggest that the DRE/ DREB regulon can be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high salinity, and freezing stresses by gene transfer. Another aim of the present research to enhance

drought tolerance in BR-29, BR 28 and BR-11, the high yielding and popular varieties by over expression of DREB gene(s).

1.4 Objectives of the Work

Rice is not only an important crop but also a model monocot crop. It is crucial to analyze the DRE/DREB (CRT/CBF) regulon in rice to understand the molecular mechanisms of drought tolerance and produce drought tolerant rice cultivars. To examine the existence of the DRE/DREB regulon in Bangladeshi rice cultivar, rice DREB genes were analyzed to check their potentiality, Stress responsiveness, and function in the stress signal.

In the current study three widely cultivated Bangladeshi rice cultivars named, BRRI-11, BRRI- 28, and BRRI-29 were used for molecular analysis of DREB gene family under drought and for the development of drought tolerant through genetic engineering approaches.

Therefore, the present research project has been undertaken considering following objectives.

(A) 1. Characterization of gene expression of the DREB genes family by RT-PCR methods.

2. Identification of suitable candidate genes.

(B) 1. Optimization of genome engineering method for Bangladeshi rice varieties viz. BR11, BR29, and BR28.

2. Production and molecular characterization of transgenic plants to enhance drought tolerance in Bangladeshi rice variety by over expression of DREB gene(s) by Agrobacterium mediated gene transformation.

CHAPTER 2
REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 Pathway of Crop Genetic Improvement

Within the last 100 years, the world has seen the rise of genetics as a scientific discipline (1900s). Which includes the finding of DNA as the hereditary material (1944), the elucidation of the double helix structure of the DNA molecule (1953), the cracking of the genetic code (1966), the ability to isolate genes (1973), and the application of DNA recombinant techniques (from 1980 onwards). Methods of crop improvement have also changed dramatically throughout this century. Mass and pure line selection in landraces, consisting of genotype mixtures, was the popular breeding techniques until the 1930s for most crops. In the 1930s maize breeders started the commercial development of double cross hybrids that was followed by the extensive utilization of single crop hybrids since the 1960s (Troyer 1996). Pedigree, bulk, backcross, and other selection methods were also developed especially for self-pollinating crop species. Such scientific advances in plant breeding led to the so-called 'Green Revolution', one of the greatest achievements to feed the world in the years of the Cold War (Perkins, 1997). Owing to this agricultural betterment, cereal production, which accounts for more than 50% of the total energy intake of the world's poor, kept in pace with the high average population growth rate of 1.8% since 1950 (Daily et al.,1998). Today, 370 kg of cereals per person are harvested as compared to only 275 kg in the 1950s; i.e., in excess of 33% per capita gain. Similar progress in other food crops resulted in 20% per capita gains since the early 1960s. According to FAO (1995), there are 150 million fewer hungry people in the world today than 40 years ago, though there were twice as many human beings. Despite this splendid progress in crop productivity, even greater progress must be made in order to feed an additional two billion people by the early part of the 21st century (Anderson, 1996a). Around 800 million people are hungry today and another 185 million pre-school children are still malnourished owing to lack of food and water, or disease (Herdt, 1998). Hence as suggested by the Nobel Peace Laureate, Norman Bourlag (1997), new biotechniques, in addition to conventional plant breeding, are needed to boost yields of the crops that feed the world.

Careful choice of such biotechniques as well as a realistic assessment of their potential in crop improvement is needed to avoid not only the criticism of the anti-science lobbyists but also the distrust of pragmatic traditional breeders (Simmonds,

1997). For example, a World Bank panel recently released for discussion a well-based report concerning bioengineering of crops (Kendall et al.,1997). In this working paper, the panel members recommend, "to give priority to all aspects of increasing agricultural productivity in the developing world while encouraging the necessary transition to sustainable methods". Indeed, plant biotechnology has been regarded as a priority area for technology transfer (Altman and Watanabe, 1995), because genetically modified food, feed, and fibre are of vital concern to the developing world (Ives and Bedford, 1998). Therefore, the industrialized countries could share their biotechniques and avoid policies that do not allow the progress of agriculture in poor, non-industrialized parts of the world (Erbisch and Maredia, 1998), where this economic activity still provides 60 to 80% employment and 50% of national income (Anderson 1996). Such support will assist the developing world towards food self-reliance (Herdt, 1998), which will be very important to avoid hunger and keep peace in many regions of the tropics, where the agricultural sector remains the most important basis for economic growth. Furthermore, a wealthy society provides high living standards to its citizens.

Tissue culture was developed in the 1950s and became popular in the 1960s. Today, micro-propagation and in vitro conservation are standard techniques in most important crops, especially those with vegetative propagation. At the beginning of the 1980s genetic engineering of plants remained a promise of the future, although gene transfer had already been achieved earlier in a bacterium. The first transgenic plant, a tobacco accession resistant to an antibiotic, was reported in 1983. Transgenic crops with herbicide, virus or insect resistance, delayed fruit ripening, male sterility, and new chemical composition have been released to the market in last decade (NCGR, 1998; USDA-APHIS, 1997). In 1996, there were about 3 million ha of transgenic crops grown in the world (mainly in North America) whereas in excess of 34 million ha (a 12-fold addition) of transgenic crops will be harvested this year in North America, Argentina, China, and South Africa among other countries. Argentina is the leading developing country with an excess of 4 million ha of transgenic herbicide-resistant soybean. There are 4.4 million ha of transgenic corn (14% of total acreage), 5 million ha of transgenic soybean (20%), and 1.6 million ha of transgenic canola (42%) grown only in North America (Moore, 1998). It has been calculated that in 1998 US farmers are growing over

50% of their cotton fields with transgenic seeds, the largest percentage for any crop ever. Trees are the next target in the agenda of genetic engineering.

Allozymes were available as the first biochemical genetic markers in the 1960s. Population geneticists took advantage of such marker system for their early research. In the 1970s, restriction fragment length polymorphisms (RFLP) and Southern blotting were added to the toolbox of the geneticists. Taq polymerase was found in the 1980s, and the polymerase chain reaction (PCR) developed shortly afterwards. Since then, marker-aided analysis based on PCR has become routine in plant genetic research and marker systems have shown their potential in plant breeding (Paterson, 1997). Furthermore, new single nucleotide polymorphic markers based on high density DNA arrays, a technique known as 'gene chips' (Chee et al., 1996), have been developed. With 'gene chips', DNA belonging to thousand of genes can be arranged in small matrices s (or chips) and probed with labeled cDNA from a tissue of choice. DNA chip technology uses microscopic arrays (or micro-arrays) of molecules immobilized on solid surfaces for biochemical analysis (Lemieux et al., 1998; Marshall and Hodgson, 1998). An electronic device connected to a computer may read this information, which will facilitate marker-assisted selection in crop breeding. In summary, since Mendel's work on peas, there have been five eras in genetic marker evolution. Liu, 1997 describes the five eras as: morphology and cytology in early genetics (until late 1950s), protein and allozyme electrophoresis in the pre-recombinant DNA time (1960 - mid1970s), RFLP and minisatellites in the pre-PCR age (mid 1970s - 1985), random amplified polymorphic DNA, microsatellites, expressed sequence tags, sequence tagged sites, and amplified fragment length polymorphism in the Oligocene period (1986 - 1995) and complete DNA sequences with known or unknown function as well as complete protein catalogs in the current cyber genetics generation (1996 onwards). The driving force for such a development has been the scientific interest of human beings to understand and manipulate the inheritance of their own characters.

2.2 Recent Advances in Rice Biotechnology

Rice is the most important food crop in the world. Almost half of the world's population depends on rice as their staple food (Coffman and Juliano, 1987). So far, an advance in breeding of rice has kept pace with the growing demand, despite losses suffered because of various biotic and abiotic factors. Over the next 7 to 8 years, global rice plantings are predicted to remain static, which will result in a shortfall of about 130 million tones (Khush, 2001; Brookes and Barfoot, 2003). Therefore, conventional breeding methods need to be supplemented with recent achievements in rice biotechnology to meet the needs of the growing world population. Rice genetic transformation has taken rapid strides since the first transgenic rice plant was produced 15 years ago. During the last 10 years, tremendous progress has been made to develop a high-frequency, routine and reproducible genetic transformation protocol of rice either through direct DNA transfer or by Agrobacterium-mediated transformation technologies. Using these technologies, a number of important traits, including quality and nutritional value, have been addressed. These improvements could not be possible through the application of conventional breeding technologies. Rice reached another milestone by becoming the first crop plant to have its genome sequenced (Sasaki and Burr, 2000; Barry, 2001; Delseny, 2003; The Rice Chromosome 10 Sequencing Consortium, 2003; Sasaki et al.,2005). Besides its own improvement, the sequencing of rice genome opens up the possibility of improving other cereals such as maize and wheat as there is significant conservation of genes among cereals (Devos and Gale, 2000; Tyagi et al.,2004). It has been suggested that these advances in rice and other crops will realize a second green revolution through genetic engineering of food crops (Sakamoto and Matsuoka, 2004).

Most of these gains were not possible through conventional breeding technologies. Transgenic rice system has been used to understand the process of transformation itself, the integration pattern of transgene as well as to modulate gene expression. New molecular tools such as inducible expression of transgene and selectable marker-free technology help in producing superior transgenic product. It is also a step towards alleviating public concerns relating to issues of transgenic technology and gain regulatory approval. Knowledge gained from rice can also be applied to improve other cereals. The completion of the rice genome sequencing together with a rich collection of

full-length cDNA resources has opened up a plethora of opportunities, paving the way to integrate data from the large-scale projects to solve specific biological problems (Bajaj and Mohanti, 2005)

2.3 Transcription Factors as Regulators of Any Target Genes

In molecular biology, a **transcription factor** is a protein that regulates the binding of RNA polymerase and the initiation of transcription. A transcription factor binds upstream to either enhance or repress transcription of a gene by assisting or blocking RNA polymerase binding. Transcription factors are usually modular proteins, have several effect or domains (DNA binding & activator domain), and affect regulation of gene expression. The regulation of transcription factors is a highly complex process as it is dependent upon a number of events, most notable of which are the presence of other DNA binding proteins (including other transcription factors) as well as local chromatin structure. Initial models, based on *in vitro* experiments suggested that there is a definite assembly sequence of transcription factors dictated by the DNA sequence. It is, however, becoming increasingly obvious that the events leading to activation of transcription are dependent on a large number of factors and are highly intertwined. Furthermore epigenetic information present on DNA appears to play an important role in transcriptional activation.

There are three classes of transcription factors:

- General transcription factors are involved in the formation of a pre-initiation complex. The most common are abbreviated as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. They are ubiquitous and interact with the core promoter region surrounding the transcription start site(s) of all class II genes.
- Upstream transcription factors are proteins that bind somewhere upstream of the initiation site to stimulate or repress transcription.

Inducible transcription factors are similar to upstream transcription factors but require activation or inhibition.

The exquisite coordination of cellular processes needed to maintain life might be likened to a symphony, in which the many different instruments must play their parts in

time with all the others. The timing of gene expression is one of the great puzzles of understanding life: How does each gene get turned on and off at the right time? Although transcription factors are clearly an important part of the answer, they themselves are proteins—the products of genes that must be regulated by other transcription factors. The way out of this paradox is to remember that each organism does not arise from nothing, nor does it spring forth fully formed, with all of its parts fully functioning. Rather, it develops from a preexisting cell, with a specific set of transcription factors in place to turn on a specific set of developmental genes, many of which are themselves transcription factors that turn on other genes. Although this is, only the barest outline of an explanation that is still being worked out, it is clear that the pulsing interplay of transcription factors is a central feature of life's coordinated complexity (Miles, 2001). The example below (Figure 2.1) provides clear view of the regulation of transcription factors.

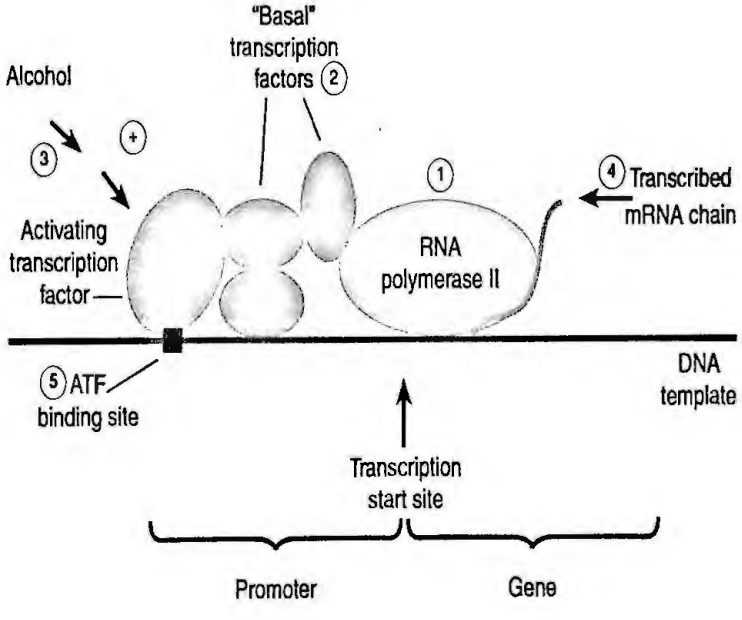


Figure 2.1 Regulation of gene expression through promoters and transcription factors. (1) The enzyme RNA polymerase II initiates transcription at a specific site in the promoter. (2) Certain 'basal' transcription factors control the binding of RNA polymerase to this site. Other proteins that bind to specific short DNA stretches in the promoter. And basal factors work together to activate or inhibit transcription. Drugs such as alcohol may modify the activity of those factors (3) here, alcohol is arbitrarily assumed to increase the activity of an activating transcription factor (ATF), resulting in (4) increased RNA synthesis from the alcohol responsive gene. (5) Genes lacking this particular ATF binding site would not respond to alcohol. (Source: Miles, 2001).

2.4 Transcription Factors that Mediate Abiotic Stress Response

The term abiotic stress refers to factors such as sub and supra optimal temperatures, excess salt (primarily NaCl) levels, reduced water availability leading to dehydration stress, excess water resulting in flooding stresses (which is associated with reduced oxygen supply leading to anaerobic stress as well) and oxidative stress (caused by low temperature stress, water stress, light stress, chemical stress etc).

Abiotic stresses adversely affect almost all major field grown plants belonging to varied ecosystem. Abiotic stress are the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Boyer, 1982; Bray et al.,2000). Drought and salinity are becoming particularly widespread in many regions, and may cause salinization of more than 50% of all arable lands by the year 2050. Abiotic stresses lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity (Wang et al., 2001a). Drought, salinity, extreme temperatures, and oxidative stress are often interconnected, and may induce similar cellular damage. For example, drought and/or salinization are manifested primarily as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano et al., 1999; Zhu, 2001). Oxidative stress, which frequently accompanies high temperature, salinity, or drought stress, may cause denaturation of functional and structural proteins (Smirnoff, 1998). Consequently, these diverse environmental stresses often activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001) and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes (Vierling and Kimpel, 1992; Cushman and Bohnert, 2000). The complex plant response to abiotic stress, which involves many genes and biochemical-molecular mechanisms, is schematically represented in Fig. 2.2. The ongoing elucidation of the molecular control mechanisms of abiotic stress tolerance, which may result in the use of molecular tools for engineer more tolerant plants, is based on the expression of specific stress-related genes. These genes include three major categories:

(i) those that are involved in signaling cascades and in transcriptional control, such as MyC, MAP kinases and SOS kinase (Shinozaki and Yamaguchi-Shinozaki 1997; Munnik *et al.* 1999), Phospholipases (Chapman, 1998; Frank et al.,2000), and transcriptional factors such as HSF, and the CBF/DREB and ABF/
ABRE families (Stochinger et al.,1997; Scho¨ffl et al.,1998; Choi et al.,2000; Shinozaki and Yamaguchi- Shinozaki, 2000);

(ii) those that function directly in the protection of membranes and proteins, such as heatshock proteins (Hsps) and chaperones, late embryogenesis abundant (LEA) proteins (Vierling, 1991; Ingram and Bartels, 1996; Tomashow, 1999; Bray et al.,2000), osmoprotectants, and free-radical scavengers (Bohnert and Sheveleva ,1998);

(iii) those that are involved in water and ion uptake and transport such as aquaporins and ion transporters (Serrano et al.,1999; Zimmermann and Sentenac, 1999; Blumwald, 2000). The readers can refer to many excellent reviews on this topic (Vierling, 1991; Ingram and Bartels, 1996; Bohnert and Sheveleva, 1998; Smirnoff, 1998; Tomashow, 1999; Blumwald, 2000; Bray et al.,2000; Cushman and Bohnert, 2000; Shinozaki and Yamaguchi-Shinozaki, 1997, 2000; Serrano and Rodriguez-Navarro, 2001; Zhu, 2002).

To maintain growth and productivity, plants must adapt to stress conditions and deploy specific tolerance mechanisms. Plant modification for enhanced tolerance is mostly based on the manipulation of genes that protect and maintain the function and structure of cellular components. In contrast to most monogenic traits of engineered resistance to pests and herbicides, the genetically complex responses to abiotic stress conditions are more difficult to control and engineer. Present engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, such as osmolytes and antioxidants, or that encode stress-tolerance-conferring proteins. The current efforts to improve plant stress tolerance by gene transformation have resulted in important achievements; however, the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side effects, make this task extremely difficult.

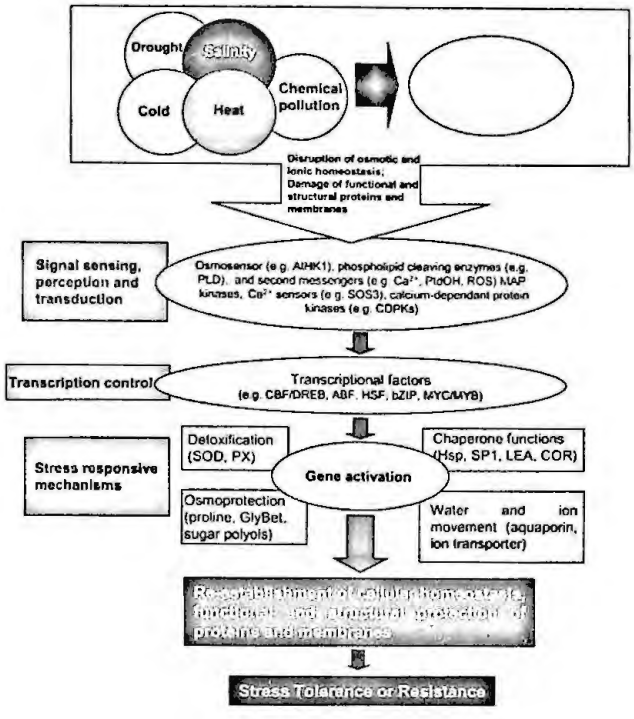


Figure 2.2 The complexity of the plant response to abiotic stress. Primary stresses, such as drought, salinity, cold, heat and chemical pollution are often interconnected, and cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals (e.g. osmotic and ionic effects, or temperature, membrane fluidity changes) trigger the downstream signaling process and transcription controls which activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the signaling and gene activation may ultimately result in irreversible changes of cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death.
(Source :Wang et al.,2003)

2.5 DREBs as Transcription factors and their significance in plant Stress Tolerance

Plant stress responses are regulated by multiple signaling pathways that activate gene transcription and its downstream machinery. Plant genomes contain a large number of transcription factors (TFs); for example, Arabidopsis has about 5.9% of its genome coding for more than 1,500 TFs (Riechmann et al.,2000). Most of these TFs belong to a few large multigene families, e.g. MYB, AP2/EREBP, bZIP and WRKY. Individual members of the same family often respond differently to various stress stimuli. On the other hand, some stress responsive genes may share the same TFs, as indicated by the significant overlap of the gene-expression profiles that are induced in response to different stresses (Bohnert et al.,2001; Seki et al.,2001; Chen et al.,2002; Fowler and Thomashow 2002; Kreps et al.,2002).

DREB transcription factors play key roles in plant stress signalling transduction pathway, and they can specifically bind to DRE/CRT element (G/ACCGAC) and activate the expression of many stress inducible genes. The dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) bind to DRE and CRT cis-acting elements that contain the same motif (CCGAC). Members of the CBF/DREB1

family, such as CBF1, CBF2, and CBF3 (or DREB1B, DREB1C, and DREB1A, respectively) are themselves stress-inducible. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of several genes such as rd29A, rd17, cor6.6, cor15a, erd10, kin1, kin2 and others in response to cold and water stress (Ingram and Bartels, 1996; Stockinger et al.,1997; Gilmour et al.,1998; Liu et al.,1998; Seki et al.,2001; Thomashow et al.,2001). Fig. 2.3 illustrates the regulatory function of DREB transcription factors. The study of DREB transcription factors suggests that a transcription factor may control the expression of many genes involved in correlated characters. In molecular breeding to improve crop stress-resistance, it may be a more effective strategy to improve or enhance the control capability of a key transcription factor for the ideal and multiple effects, compared with the transfer of a single functional gene for improving a single trait. If the regulatory ability of a key transcription factor is enhanced, it can activate the expressions of many target genes controlling correlated characters, and then make a fundamental improvement.

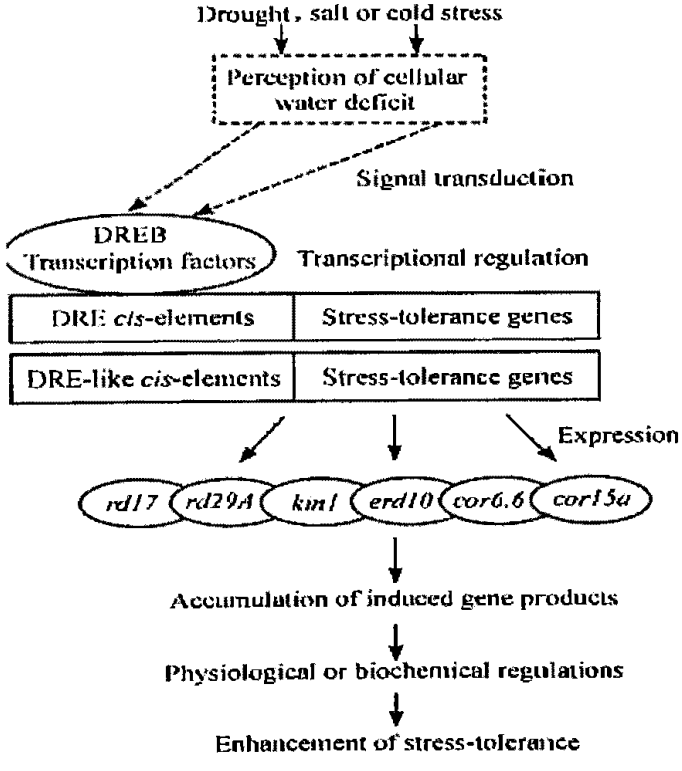


Figure 2.3 A stress-regulatable DREB transcription factor can activate the expression of DRE-element-containing target genes involved in plant tolerance to drought, high salt, and cold stress. (Source:Liu et al.,2000)

2.6 DREB Genes in Crop Gene Pool

Stress inducible regulatory genes that encode proteins which act as transcription factors (e.g. DREB genes from Arabidopsis) have been validated through over-expressed in transgenic plants, which show stress-tolerant phenotypes (Yamaguchi-Shinozaki, and Shinozaki, 2006). Thus, since regulatory genes appear to be an important component of plants response to environmental stresses, there have been many efforts to isolate abiotic stress-inducible transcription factors in other species, especially in important cereal crops.

In rice, one homolog, named OsDREB2A, was identified as a DREB2-type protein. Similar to Arabidopsis DREB2A, OsDREB2A was gradually induced by dehydration and high salinity stress, but hardly increased under cold stress (Dubouzet et al., 2003). A transcription factor, OsDREB1B, has been also isolated in rice, and shown to be over-expressed in transgenic Arabidopsis plants under cold and high-temperature and providing tolerance to those stresses (Qin et al., 2007b).

In wheat, the TaDREB1 was found to be induced by cold, salinity and drought, and was classified as a DREB2-type transcription factor based on phylogenetic analysis (Shen et al., 2003). Similarly, a gene named TdDRF1 has been isolated and characterized in durum wheat (T. durum) (Latini et al., 2007). This gene belongs to the DREB gene family and produces three forms of transcripts through alternative splicing. The truncated form TdDRF1.2 was at all times the most expressed and together with TdDRF1.3 transcript, it increased sharply after 4 days of dehydration, but then decreased at 7 days, whereas the TdDRF1.1 transcript was the least expressed overall and less variable with the duration of dehydration. In barley, a DREB2-type protein HvDRF1, was also reported to accumulate under drought and salt stresses and was involved in ABA-mediated gene regulation (Xue and Loveridge, 2004).

Qin et al., (2007a) cloned ZmDREB2A, a DREB2 homolog from maize, the transcripts were stimulated by cold, dehydration, salt and heat stresses in maize seedlings. Moreover, their research showed that constitutive or stress-inducible expression of ZmDREB2A resulted in improved drought stress tolerance. Finally, the authors indicated that over-expression of ZmDREB2A also enhanced thermo-tolerance in transgenic plants,

which suggests it may play a dual functional role in mediating the expression of genes responsive to both water and heat stress.

A cDNA with an open reading frame of 332 amino acids, encoding the transcription activation factor DREB2A, was cloned recently from pearl millet (*Pennisetum glaucum*), which is well known as one of the most stress tolerant food grain crop of the semi-arid tropics (Agarwal et al.,2007). Three DREB homologue genes (GmDREBa, GmDREBb and GmDREBc) have also been isolated from soybean (*Glycine max*), and all of them bound to the dehydratation-responsive element (Li et al., 2005). More recently, the function of these genes has been validated through their over-expression in transgenic soybean plants where they conferred drought and salinity tolerance (Chen et al., 2007).

The above results show a consistent presence and role of DREB genes in abiotic stress responses across species. This highlight that transcription factors are be ubiquitous in higher plants, and reinforces the value of using plant model systems for identifying useful genes that can be implemented in the germplasm enhancement of food staples. Hence, geneticists can search for more DREB homolog(s) through allele mining in crop species of the semi-arid tropics such as millets or sorghum (Sorghum bicolor). However, it is critically important that this type of work is carried out in close collaboration with physiologists to ensure accurate and precise testing of their performance under appropriate water deficit profiles and other stresses. When such work is carried out under a holistic approach, it is likely to open new paths for the genetic betterment of crop species for improved food security and enhanced livelihoods of poor farmers across the developing world.

2.7 Rice Genetic Engineering for Abiotic Stress Tolerance

It has been estimated that abiotic stresses such as drought, salinity, extreme temperatures, submergence and oxidative stress reduce crop yield worldwide by more than 50% (Bray et al., 2000). These stresses are often interconnected, cause similar cellular damage and activate similar cell-signalling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001; Zhu, 2003). Several proteins, antioxidants, and compatible solutes are induced because of abiotic stress responses. Many plant species have been produced over-expressing these compounds and tested for various abiotic

stresses, most prominent being salt/drought tolerance (Bajaj et al., 1999; Apse and Blumwald, 2002). Recently, the focus has been to engineer abiotic stress tolerance by over-expressing structural and regulatory proteins to activate the multistep cascade involved in stress response (Zhang et al., 2004). However, most of this research has been carried out in model plants such as *Arabidopsis* and tobacco. Therefore, the challenge is to translate the information obtained from model plants into commercial crop varieties (Zhang et al., 2004).

2.7.1 Tolerance to water deficiency

Drought is one of the most important factors that reduce rice yield world wide. The transgenic approach imparted tolerance to dehydration in transgenic model dicot species (Bajaj et al., 1999; Tyagi and Mohanty, 2000), have been extended to crop plants such as rice (Table 2.1). Further, many new genes have been isolated that provide water-deficit tolerance in model plants (Mukhopadhyay et al.,2004; Zhang et al.,2004). The dehydration responsive element (DRE) with core sequence A/GCCGAC has been identified as a *cis*-acting promoter element that activates expression of several genes under abiotic stress conditions (Yamaguchi-Shinozaki and Shinozaki, 1994). Dubouzet et al., (2003) isolated five rice DREB orthologs of *Arabidopsis* named as OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D, and OsDREB2A and analysed their function in *Arabidopsis*. The authors said that they generated transgenic rice over-expressing various OsDREB proteins in combination with several promoters and suggested that OsDREB1A could be very useful for producing transgenic dicot and monocot plants with higher tolerance to drought, salt and/or cold stresses. However, it should be noted that constitutive over-expression of these factors may cause aberrant phenotype (Shen et al., 2003), so these genes need to be expressed under regulated/inducible promoter.

2.7.2 Tolerance to Different a Biotic Stress.

Introduction by gene transfer of several stress-inducible genes has demonstrably enhanced abiotic stress tolerance in transgenic plants (Zhang et al., 2004; Bartels and Sunkar, 2005; Umezawa et al., 2006). These particular genes encode key enzymes regulating biosynthesis of compatible solutes such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines), and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose, galactinol, and raffinose).Recent

advances in transgenic rice tolerant to different abiotic stresses are summarized in table 2.1.

Table2.1: Recent advances in transgenic rice tolerant to different a biotic stresses.

Gene/function	Source	Performance of transgenic plant	References
GS2/glutamine synthase	Rice	Tolerant to salt and possibly chilling stresses	Hoshida et al. (2000)
OsCDPK7/calcium-dependent protein kinase	Rice	Tolerant to salt and drought stresses	Saijo et al. (2000)
OsMAPK5/MAP kinase	Rice	Inverse modulation of biotic and abiotic stress tolearance by MAP kinase	Xiong and Yang (2003)
YK1/rice homolog of maize HC toxin reductase	Rice	Tolerant to multiple abiotic stresses including salt and submergence	Uchimiya et al. (2002)
OsNHX1, AgNHX1/	Rice,	Tolerance to salt stress	Ohta et al. (2002);
Na^+/H^+ antiporter HVPIP2; 1/aquaporins	*Atriplex gmelini*, rice Barley	Differential response to salt-stress	Fukuda et al. (2004); Katsuhara et al. (2003); Lian et al. (2004)
Adc, Samdc/polyamine biosynthesis	Datura/oat/ tritordeum	Salt/drought tolerant	Roy and Wu (2001, 2002); Capell et al. (2004)
HVA1/LEA proteins	Barley, wheat	Tolerant to salt and dehydration stress	Cheng et al. (2002); Rohila et al. (2002); Babu et al. (2004)
codA/glycine betaine	*Arthrobactergl obiformis*	Tolerant to salt and/or drought stress	Sakamoto and Murata, (2002); Mohanty et al. (2002);Sawahel (2003)
OtsA, OtsB/trehalose biosynthesis	*E. coli*	Multiple abiotic stress tolerance such as salt, drought and cold	Garg et al. (2002); Jang et al. (2003)
p5cs/proline biosynthesis	Moth bean	Stress induced transgene expression	Su and Wu (2004); Hur et al. (2004)
pdc1, adc/pyruvate decarboxylase/alcohol dehydrogenase	Rice	Submergence tolerant	Quimio et al. (2000); Rahman et al. (2001)
AGPAT, SGPAT/fatty acids biosynthesis	*Arabidopsis*, spinach	Improved photosynthesis and growth at low temperatures	Ariizumi et al. (2002)
Cat/catalase	Wheat	Cold tolerant	Matsumara et al. (2002)
GST/ζGlutathione S-transferase	Rice	Enhanced germination and growth under low temperature	Takesawa et al. (2002)
Spl7/heat-stress transcription factor	Rice	Transformation of rice *spl7* mutant with *Spl7* show tolerance to heat stress	Yamanouchi et al. (2002)
hsp101, hsp17.7/heat-shock proteins	Rice	Tolerant to high temperatures	Katiyar-Aggarwal et al. (2003); Murakami et al. (2004)
naatA, naatB/biosynthesis of mugenic acid phytosiderophores	Barley	Tolerant to iron deficiency	Takahashi et al. (2001)

Abbreviations: adc, arginine decaboxylase; codA, choline oxidase; hsp, heat-shock protein; GPAT, glycerol-3-phosphate acyl transferase; LEA, late embryogenesis-abundant protein; MAPK, mitogen activated protein kinase; Naat, nicotianamine aminotransferase; OtsA and OtsB, E. coli trehalose biosynthesis genes encoding for trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP); pdc, puruvate decarboxylase; P5CS, Δ-pyrroline-5-carboxylste synthase; Spl, spotted leaf.

2.8 Recent Advances in Genome Engineering of Cereal Crops with DREB Genes to enhance Stress.

Drought, high salinity, and low temperature are three important abiotic stresses that are commonly encountered by plants growing in their native environments. Upon exposure to the stresses, many genes are induced and their products are thought to function as cellular protectants of stress-induced damage (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

Genetically engineered cultivars containing various gene constructs to enhance their performance under water stress are an important future strategy for facilitating the production of cereals and other crops in drought-prone environments (Hoisington and Ortiz, 2006). They provide an attractive and complementary option for improving a plant's performance under stress conditions. Particularly attractive is the single, dominant nature of the transgene that makes the transfer and maintenance of this system in any cultivar much easier than conventional sources based on polygene.

Crop engineering by inserting transcription factors will require both promoter and coding regions to ensure the functioning of those elements that govern cross-talk between different abiotic stresses and plant developmental "programs" (Bonhert et al., 2006). Transgenic rice with AtDREB1A or its rice orthologue OsDREB1A tested in pots showed improved tolerance to simulated drought, high-salt and low-temperature stresses (Yamaguchi-Shinozaki and Shinozaki, 2004). Enhanced content of Proline and soluble sugars was detected in the transgenic rice, together with altered transcript levels for various genes controlled by DREB1A. Likewise, the DREB1.CBF transcription factor was involved in cold-responsive expression in transgenic rice (Ito et al., 2006). Although these transgenic rice plants had improved tolerance to drought, high salt, and low-temperature stresses like the transgenic *Arabidopsis* plants. They showed growth retardation under normal growth conditions. Resolving this type of issue is fundamentally important for developing a viable transgenic drought tolerant new cultivar. Potential solutions include coupling DREB-like genes with a strong stress-inducible promoter to reduce the growth inhibition under unstressed conditions (Kasuga et al., 1999). Alternatively, conventional breeding approaches may be able to identify

background genotypes with good combining ability or modifier genes to counteract the yield penalty under optimum cropping conditions.

The Japan International Research Center for Agricultural Sciences (JIRCAS) has shared with some centers of the CGIAR gene constructs containing the AtDREB1A gene under the control of various promoters (Nakashima and Yamaguchi-Shinozaki, 2005). These transgenes were introduced into several crops with the expectation that AtDREB1A would recognize the DRE of endogenous genes and enhance stress responsiveness. For example, different transgenic groundnut (*Arachis hypogaea*) lines were produced by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), and the transgenic lines showed delayed wilting under simulated drought by 20 to 25 days compared with non-transgenic controls (Bhatnagar-Mathur et al.,2004, 2007). Similarly, transgenic potato (*Solanum tuberosum*) bearing the DREB1A gene driven by the stress-inducible *rd29A promoter* increased salt-stress and freezing tolerance concomitant to its copy number (Behnam et al.,2006, 2007), which shows the versatile use of DREB transgenic technology to improve crops for growing in stressful environments.

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

The present research work was conducted at the biotechnology laboratory, International Rice Research Institute Manila, Philippines. The whole research program was divided into a number of experiments to achieve the objectives. The material and methods described in this section are general application and more specific experimental techniques are described under the relevant section of experimental results. The present investigation in rice covers mainly molecular analysis of OsDREB gene family and gene transfer.

3.1 Plant Material

Three Bangladeshi modern rice varieties (BR 29, BR28, and BR 11, respectively) were collected from BRRI. And tested for embryogenic callus induction from matured seeds and immature embryos. Rice plant (*Oryza sativa* L.) of the indica genotypes were grown in the screen house under natural condition. Immature caryopsis collected of 8-10 days after anthesis with liquid endosperm was collected. The characteristic of features of the genotype used are discussed below.

3.1.1 The Characteristic of the Studied Rice Genotypes

3.1.1.1 BR-29

BR- 29 is a popular high yielding rice variety in Bangladesh and is cultivated as boro dhan. It is 95 cm tall, having 160 days life cycle and medium fine white grain. The average yield is 7.5 t/ha.

3.1.1.2 BR-28

Bangladesh Rice Research Institute released this variety in 1994 as boro dhan. It is 90 cm tall, having 140 days life cycle and medium fine white grain. The average variety yield of this variety is 5.0 t/ha.

3.1.1.3 BR-11

BR- 11 is known as Mukta an Aman variety of Bangladesh. BRRI released this variety in 1980. Since first release, BR11 is still the dominant variety in the T. Aman season, covering 47% area of rice land of Bangladesh. It is 115 cm tall, having 145 days life cycle and medium coarse grain. The average yield is 6.5 t/ha.

3.2 Drought- stress Treatment and Physiological Parameter

Three experiments (3 biological replications) were carried out to observe effect of drought stress on BR-29, BR-28 and BR-11 at reproductive stage, comparing with well watered plants.

Seeds of the rice varieties were planted in 10 litter pails and placed in a glass house. For each variety same number of plants used for well watered and drought stressed condition. For every experiment, 20 pails were taken for each variety (3 plants per pail); plants were grown in glass house and maintained under the same conditions. When the plant Interoricle length reached to the average length of three days before heading, (An initial study was carried out to estimate the average measurement of Inter-oricle length at reproductive stage) for half of the plant, water was drained out from 10 pails and the rest were given normal watering. This stage is considered 0-day stress and stress was applied up to 5 days then re-watered the plants. Different observations were done, samples of different tissue from drought stressed, and well-watered plants were collected.

3.2.1 Observations

3.2.1.1 RWC (Relative Water Content):

To estimate the level of stress RWC from flag leaf was calculated. For RWC at least 3 flag leaves from each variety were collected in the morning (8:00-9:00Am) in 3 pre weighted airtight 50 ml vial and placed beneath ice, brought back to the Laboratory as soon as possible. In the Laboratory, vials were weighed to obtain leaf sample weight (W), after which the samples were immediately hydrated to full turgidity for 6-8 h under normal room light and temperature. After 6-8 hours the samples were taken out of water and were well dried of any surface moisture quickly and lightly with filter paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80^0C overnight and weighed (DW).

(RWC (%) = [(W-DW) / (TW-DW)] x 100,

W =Sample fresh weight, TW= Sample turgid weight and DW= Sample dry weight.

3.2.1.2 Physiological Growth

Data of Interoricle Lenth (**IOL**), Peduncle elongation (PD **Elongation**) Fig .3 and Panicle exertion were recorded for every stressed level until maturity to compare growth of drought stressed plants with well-watered plants. Panicle fertility and 1000 grain weight was also been recorded for both conditions.

3.2.2 Sample Collection

To avoid sampling error tillers of average length were tagged three days before heading the tillers came to heading too early or too late were avoided to standardize the average elongation rate. Sample collection was started from three days before heading (presumed from a pre study measuring the peduncle elongation). Two type of samples were collected from both well watered and drought stressed plants.

1) Different tissues viz. Flag Leaf, Panicle, and Peduncle collected for all three studied varieties from drought stressed and well watered plant at different time point of drought stress. 2) Different zones i.e. division zone, elongation zone and mature zone of peduncle (Figure 4.10) for BR -29 under both well watered and drought stressed condition were also collected. Samples were cut from plant in the morning, immediately wrapped in labeled aluminum foil and placed in the liquid nitrogen. Collected samples were preserved in -80^{0}C for RNA extraction to study expression of drought responsive gene(s).

3.3 Bio-informatics

3.3.1 Bio-informatics of DREB gene family

Bio-informatics of DREB gene family: To identify members of the rice (Oryza sativa L. subsp. japonica) DREB family, multiple database searches were performed. First, the BLAST program (TBLASTN) available on the Rice Genome Database-japonica of the NCBI web search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. Based on this search, we identified DREB family genes in the rice genome. The cDNA coding regions for the OsDREB genes were predicted using the Rice Genome Automated Annotation System. After the IRGSP (International Rice Genome Sequencing Project, 2005) announced completion of a high-quality rice genome sequence, again the rice database using position-specific iterated BLAST (Altschul et al., 1997) program on the NCBI

Web site was also searched. In addition, the databases were surveyed of coding sequences from genes in the TIGR Rice Pseudomolecules for 12 chromosomes using a TBLASTN search at the TIGR Web site (http://tigrblast.tigr.org/euk-blast/index.cgi?project5osa1). Based on these searches, all members of OsDREB family from the current available genomic database were collected (Nakano et al., 2006)

Gene family detection, phylogenetic trees and primer design:

The protein sequences of Rice DREB were used as tblastn queries (http://www.ncbi.nlm.nih.gov/blast/) against the rice genomes. Sequence alignments and tree drawings were conducted by clustalw analysis (http://align.genome.jp/) and the tree view program for tree construction (Page, 1996). Table 4.1 representing lists of accession numbers for the rice genes used in this study.

3.3.2 Primers of Os DREB Genes for PCR and RT PCR Analysis

For gene-specific amplification of each gene by RT-PCR, a primer pair was designed based on the predicted exon–intron structure of the gene (Table 4.2). Where possible, the forward primer was derived from the second-last or third-last exon and the reverse primer was derived from the 3' untranslated (UTR) region (Table 4.2). Otherwise, forward primer was derived from 5' untranslated (UTR) region and reverse primer from first exanthema. Optimum number of cycles was selected to reveal any differences in gene expression among treatments.

Table 3.1 Primers of OsDreb gene family (and control genes) for detection of the genes and transcript analysis.

Gene Name	Forward Primer (F) 5'-3'	Reverse Primer (R) 5'-3'	Tm /oligo	cDNA PCR Product	%GC of cDNA PCR Product	Genomic PCR Product
OsDREB1A	TGCCTCAACTTCGCCGACTC	CCGAAAGTAGTGTCCGTACAG	64°C	477	65.2	477
OsDREB1B	CGATGGCGACGAAGAAGAAG	AGGAGGGAGAAATCTGGCAC	58.3	389	56.8	389
OsDREB1C	TCAGGGAGCCCAACAAGAAG	AACTGCTAGCTCCAACTCGCG	63.6	522	72.4	522
OsDREB1D	CAATTGATGACCTCCTCCGCG	TTTGGCTGACAGAACGGGTGC	64.5	466	71.2	466
OsDREB1E	ACCAAGTTCCAGGAGACGAG	AGGCATTGTCGTCGTTGGGTG	63.3	418	75.6	418
OsDREB1F	TCCTCTCATCGCACCACCAC	GGTAATGGTCATCGTCGTCG	63.6	697	73.2	697
OsDREB1G	CAAGTTCAAGGAGACGAGGC	CCATCGGGTAGCAAGGGAAG	63.1	398	74.4	398
OsDREB1H	CGCAAGCCACGACGACATAC	CCTTCCGCTTCTTCCGATCAC	60.1	503	57.9	503
OsDREB1I	AACTAGAGGAGATCACCGGCG	GGGAACACGGACGATGATGAC	65.1	454	78	454
OsDREB1J	GGCAGCAGTACAAGAAGCGG	CGTGTCGTAGTCCATTTCGCC	64.8	557	75.9	557
OsDREB2A	GGCAAGGGCAATGTATGGTC	CCTATTGACCCGCAGCATGAC	55.4	500	44.2	500
OsDREB2B	ACATTGTCAGCCAGCAGCGTG	CTAGCCGACCAAAGCACCAG	57.7	427	52	427
OsDREB2C	GCAGCAGCAAGTCACGCAAG	GATGATTTGGCGGTGGTGGTG	63.4	619	68.2	619
OsDREB2D	TTCTGTTCTCCTGCCACCACC	AACCTGTCTAGATGGCTGCC	59.8	395	65.5	395
GAPDH	TTCCCCCTCCAGTCCTTGCT	GCAGGAACCCTGAGGAGATC	62°C	329	60	697
LiP 9	AGAGCCTCCTCTCCAAGCTG	GCCTTGACCAATCTTGACACG	64°C	682	65	776

3. 4 Bacterial Strain and Plasmid

Two plant expression vectors were used for the present transformation studies. The *Agrobacterium* strain harbored the transformation vector was LBA4404. The plant expression vector named IRS-5 and IRS-147 was constructed, both contained the OsDREB1A fragment and the selective marker gene HpT that confers the resistance to hygromycin. The plasmid constructs contained the common selectable marker gene HpT driven by NOS promoter. In IRS-5, OsDREB1A was driven by the ubiquitin promoter and in plasmid IRS-147 the OsDREB1A was driven under the stress responsive Lip-9 promoter (Figure 3.1 and 3.2). Expression cassettes were obtained from JIRCAS, JAPAN for this study.

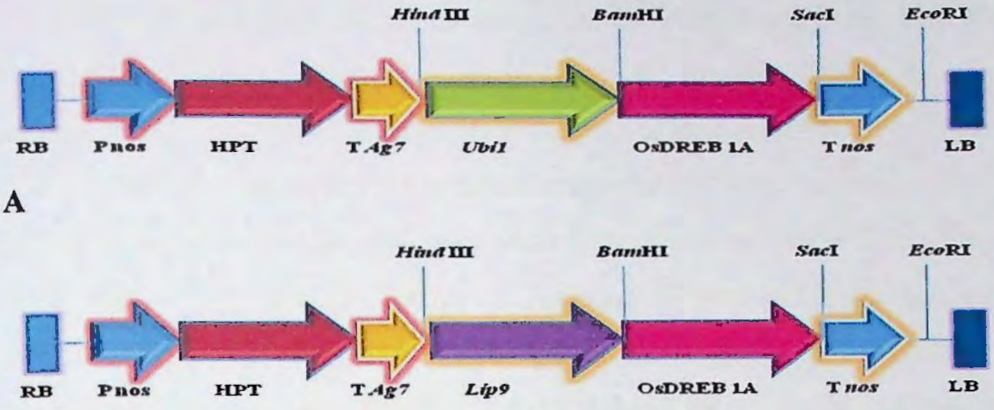


Figure3.1 Partial plasmid maps A) IRS5 (Ubi1::OsDREB1A). b) IRS147 (Lip9::OsDREB1A).

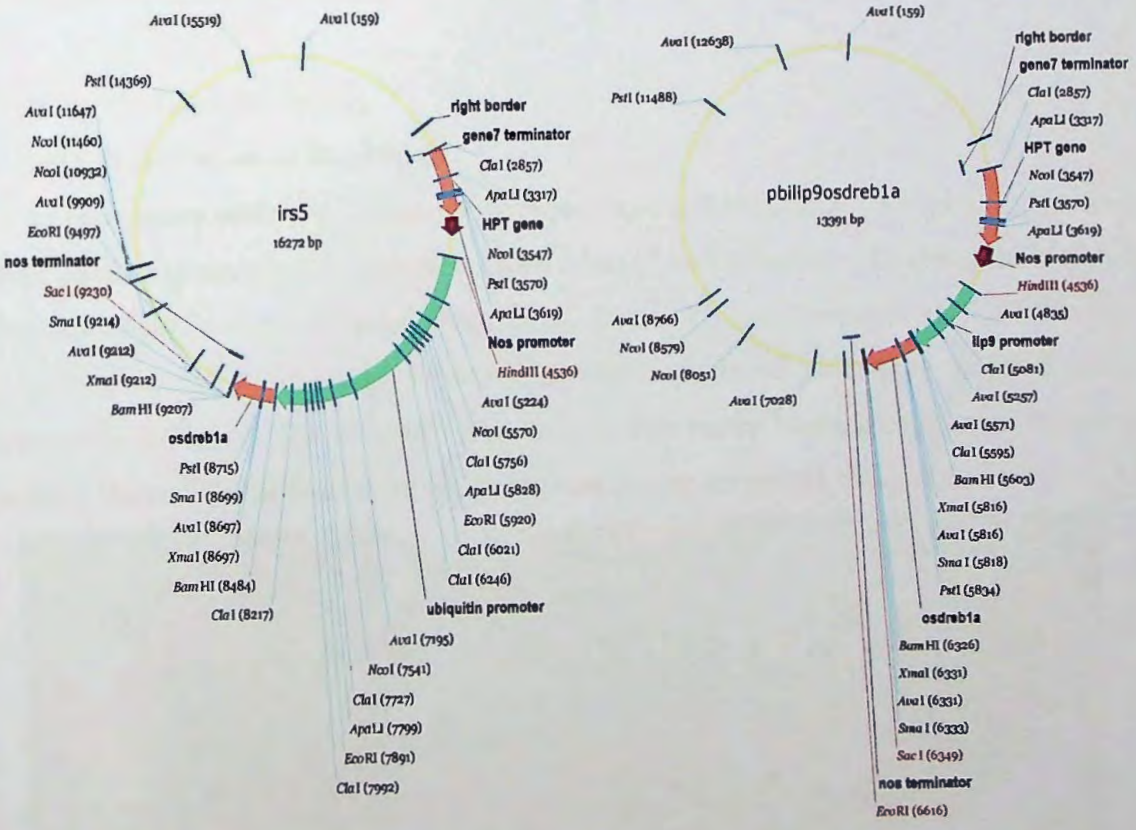


Figure 3.2 Restriction maps of plasmids. (A) Restriction map of plasmid IRS-5. (B) Restriction map of plasmid IRS-147.

3.5 Culture and Maintenance of Agrobacterium Strains

For single colonies, the strain with plasmid were streaked on YEP plates with 20 ug/ml Rifampicin and appropriate antibiotic for the plasmid. The culture was grown for 2 days at 28°C incubator. 2 ml of YEP Inoculated that contains 20 ug/ml of Rifampicin and appropriate antibiotics for plasmid maintenance with the strain. The culture was grown to early or middle stationary phase (usually overnight). The culture and sterile YEP with 50% glycerol Chilled on ice. An equal volume of the medium was added with glycerol to make the culture 25% glycerol. Culture and medium were mixed completely.The cells were transferred to a labeled 2 ml cryogenic vial and placed it in the freezer. The frozen culture can be stored indefinitely.

To recover the strain, simple scrape a bit of the frozen culture into plate medium containing the appropriate antibiotics. It is important to handle the culture quickly to prevent thawing and refreezing.

3.6 Genetic Transformation

3.6.1 Preparation of Explants

A. Immature embryo: Immature caryopsis was collected of 8-10 days after anthesis with liquid endosperm. The spikelets were selected and dehulled. The dehulled material then collected in a 50 ml tube (FIG.3.1 A, B and C) and treated with 50% Sodium hypochloride for 30 minutes. Then rinsed with autoclaved nano-pure distilled water repeatedly 5/6 times. The materials were air dried on sterile blotting paper and immature embryo isolated immediately for use as explant in transformation experiment

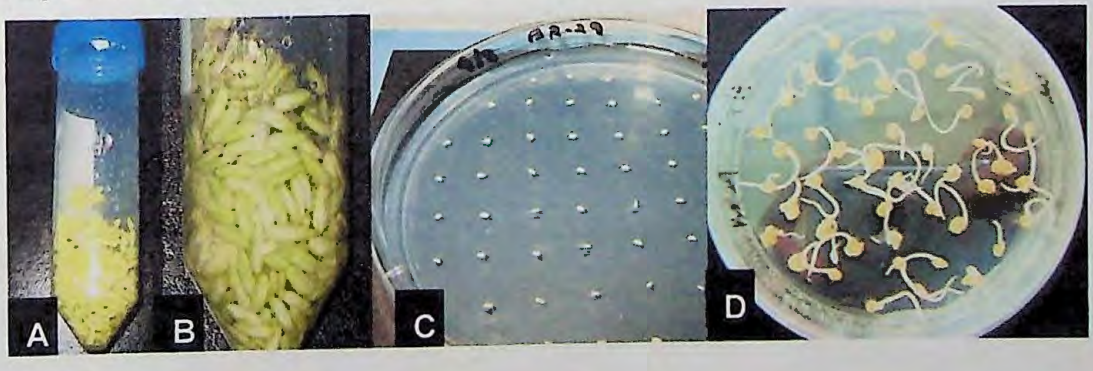


Figure 3.3 preparation of explant (A) and (B) immature seeds in 50ml tube, (C) isolated immature embryo (D) 7day old seedling with rising calli on semisolid medium.

B. 5-7 day old seedling from Matured seed: Mature seeds were selected care fully avoiding any lack/brown spots and then dehulled. Dehulled matured seeds were collected in conical flask and was treated with 50% Sodium hypo-chloride for 30 minutes with vacuum pressure, then rinsed with autoclaved nano-pure distilled water repeatedly 5/6 times, air dried on sterile blotting paper and placed on MS semisolid medium containing 2mg/ml 2-4D, Placed in lighted room maintaining temperature (31^0C) for 5-7 days. Seedlings having whitish rising calli were used as explant in transformation experiments (FIG. 3.3(D), 4.10)

3.6.2 Agrobacterium Mediated Gene Transformation

Immature Embryo Transformation - Co-cultivation: Infection medium was prepared followed by the previously described method (Hiei and Komari, 1994). Bacterial pellet was resuspended with infectiom medium (R200). Immature embryos were placed scuttellum side facing up on the medium containing N_6 major salts+B5 minor salts and vitamin+ 2 mg/l 2, 4-D + 1 mg/l NAA + 1 mg/l BA+ 500 mg/l l-Proline + 500 mg/l CasaminoAcid+ 20 g/l sucrose+ 10 g/l sucrose+100uM Acetosyringone. 5µl of infection medium was dropped on each embryos and incubated in dark for 5 days at 25°C.

Selection: After co-cultivation, elongated shoots were removed with scalpel. Separated embryos were placed on **Resting medium** (N_6 major salts+B5 minor salts and vitamin+ 1 mg/l 2,4-D + 1 mg/l NAA + 0.2 mg/l BA+ 500 mg/l l-Proline + 500 mg/l CasaminoAcid+300 mg/l L-glutamine +36 g/l D-mannitol + 20 g/l D-Maltose(2%) + 250 mg/l Claforan + 100 mg/l Carbenicillin) and incubed for 5 days at 30°C under continuous illumination at100 µmol/m/sec. After aresting period, each embryo transferred to the fresh selection medium containing 50mg/l Hygromycin and 250 mg/l Cefatoxim. The actively growing calli were selected and placed on second and third stage selection media every 10 days.

Regeneration and hardening: After 3 weeks of incubation on this medium, the surviving calli were transferred to pre-regeneration medium, including 250 mg/l Cefatoxim and 50 mg/l Hygromycin and incubated under continuous illumination for another 10 days. Proliferating calli with green spots were selected and transferred to

regeneration medium containing 50 mg/l Hygromycin (M4-50H) and 250 mg/l Cefotaxime for shooting and rooting. The rooted plants were transferred to Yoshida solution for hardening of the roots, and were established in the transgenic glass house in pots containing a mixture of vermiculite and soil.

3.6.3 Molecular Analysis of Putative Transgenic Plants

3.6.3.1 DNA extraction and PCR analysis

For PCR analysis, genomic DNA was extracted from the leaves of both transformants and non-transformed control plants with the method followed by standard IRRI protocol for rice genomic DNA extraction. PCR was performed using a GS1 G-storm PCR thermal cycler (UK) with 100 ng of DNA and oligonucleotide primers of the OsDREB1A gene (100 nM). The primers used are listed in table 3. Primers from both gene of interest (OsDREB1A) and selectable marker gene (Hygromycin phosphotransferase) used to check the integration of foreign gene in rice genome. The PCR amplification was carried out in 20µl reaction mixture using a PCR master kit (Promega) having 2mM MgCl2 and 100µM of a deoxyribonucleotide mixture and buffers at pH 8.3. The samples were denatured initially at 94oC for 5 min, followed by 30 cycles of 30 s denaturation at 94oC, 30 s of primer annealing at 55oC and 30 s of synthesis at 72oC, with a final extension step of 72oC for 5 min. The PCR products analyzed through electrophoresis of the samples on a 1.2% Agarose melted in 1x TAE gel followed by ethidium bromide staining and visualized under UV light in the gel documentation system.

Table 3.2 Primers for PCR and RT PCR analysis of transgenic plants.

Gene Name	Primer Name	Primer	Tm	Expected Product Size
Hygromycin phosphotransferase	Hph F	TACTTCTACACAgCCATC	52	837bp
	HpT R	TATGTCCTGCGGGTAAAT	52	
Oryza sativa dehydration responsive binding protein	OsDERB1A	ATGCTCATGGAGCCACCATC	50	396bp
Nos terminator	Nos	GTTGTAAAACGACGGCCAGT	52	

3.6.3.2 Southern Blot Analysis

For Southern blot analysis, total DNA was extracted from young leaves (~2 g) of putative transformants, as well as from non-transformed control plant, followed by rapid mini-prep method (Dellaporta et al., 1983). DNA (10–20μg) was digested with BamHI or HindIII, respectively, and fractionated on a 1.0% agarose gel by electrophoresis at 20 V/cm for 12 h. Southern hybridization was carried out as described by Reed and Mann, 1975. Shortly, digested DNA was electophorsed and transferred on the membrane, and fixed. Meanwhile, the wild type rice DNA and the expression vector were used as the negative and positive controls, respectively. The probe of OsDREB1A gene was synthesized and detected according to the labeled DIG DNA Labeling and Detection Kit instructions (Roche). The membrane with the fixed single strand DNA was immersed in pre- hybridization buffer. After prehybridization, the membrane was transferred to hybridization solution containing the labeled probe. A thermostable alkaline phosphatase (AP)-labelled OsDREB1A gene-specific probe was generated using a CDP-Star AlkPhos Direct Labelling Kit, following the manufacturer's instructions (GE Healthcare UK Ltd.). The membrane was hybridized 5min at room temperature with the probe. Hybridization signals were detected by chemiluminescence using CDPstar (Roche Diagnostics GmbH, Mannheim, Germany), followed by exposure to X-ray film (Hyperfilm ECL, GE Healthcare UK Ltd.).

3.6.3.3 Inheritance of Transgenes in T1 Generation

Selfed seeds from the primary transformant (T0) were collected and germinated. The T1 progeny plants were maintained in the glass house under controlled conditions. T_1 plants were tested for tolerance to drought. PCR of DNA and RT-PCR analyses of RNA from these plants were carried out as described earlier.

CHAPTER 4
RESULTS

RESULTS

Plants have a remarkable ability to cope with highly variable environmental stresses, including drought, cold, and soils with changing salt and nutrient concentrations (i.e. abiotic stress). Understanding a plant's response to a stress require a comprehensive evaluation of stress-induced changes in gene expression

This research work presents mRNA expression profiles of reproductive part of rice subjected to drought stress treatments. The presence and expression pattern of 14 members of the OsDREB gene family were studied in BR-29, BR 28 and BR 11 rice varieties. Besides these, experiments for optimization of genome engineering protocol and gene transformation were performed using the studied rice varieties. In gene transformation studies OsDREB1A Was used for over-expression in BR varieties to enhance drought stress response.

4.1 Characterization of the OsDREB genes family

4.1.1 Genome analysis

4.1.1.1. Bio informatics of OsDREB genes

Yamaguchi-Shinozaki and Shinozaki (2003) listed 13 DREB genes in the genome of the rice cv. Nipponbare: OsDREB1A-OsDREB1I and OsDREB2A-OsDREBD. These genes are classified into two groups based on similarities of the amino acid sequence of their binding domains. Two of these genes, OsDREB1A and OsDREB1I, are found on the same BAC clone, along with a third gene, which was not mentioned by previous, authors (Yamaguchi-Shinozaki and Shinozaki 1994, Shinozaki et al 2003, Dubouzet et al 2003). We named it **OsDREB1J** and included it this study (Table 4.1). All necessary information for the OsDREB genes i.e. genomic sequence accession numbers, mRNA and protein identification numbers, their location in the chromosome gathered from the database using different tools and all the information are shown in Table 4.1. The expressed sequence tags (ESTs) were obtained by blast against rice genome database.

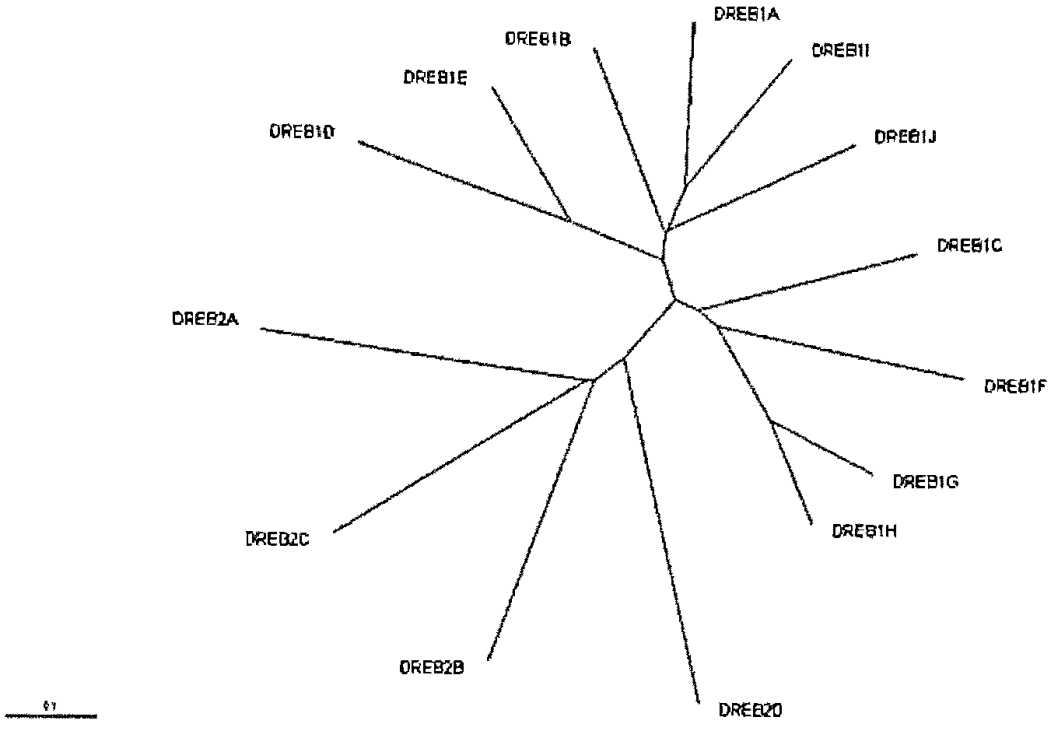


Figure 4.1 An unrooted phylogenetic tree of the DREB proteins in rice. The amino acid sequences were aligned using ClustalW and the phylogenetic tree was constructed using the NJ method.

Comparison of the deduced amino acid sequences of the ERF/AP2 DNA-binding domains of the OsDREB proteins in rice was done. A database search was carried out against rice DNA sequences with the TBLASTN program using the amino acid sequences of the ERF/AP2 domain. The sequence of the OsDREB proteins was aligned and an unrooted phylogenetic tree was constructed (Fig. 4.1) to study inter relationship of the genes according to gene function. Moreover, multiple alignments of OsDREB proteins showed consensus relation between the genes (Fig.4.2). The multiple alignments show similarity with *Oryza sativa* DREB (OsDREB) family members in both the overall amino-acid sequences and the secondary structure arrangement within the DNA-binding motifs. The capital letters indicate conserved amino acids in all proteins and small letters indicate conserved sequences in at least three genes. Dashes indicate gaps in the amino acid sequences introduced to optimize alignment. Black and light gray shading indicate identical and conserved amino acid residues, respectively.

Table 4.1 Summery of characterized and predicted OsDREB genes from *Oryza sativa* var. japonica.

Name of gene	Amino acid numbers	Accession number (Genomic sequence)	mRNA identification	Protein identification	Location in Chromosome	ESTs
OsDREB1A	238	AP006859	AK105599 AK071519 AF300970	AAN02486	9	leaf, stem, Inflorescence, seedling
OsDREB1B	218	AP008215	AF300972 AY785894	BAF25624 AAX28958	9	Inflorescence, stem, leaf
OsDREB1C	214	AP002839	AY327040	BAC2483	6	Root, leaf, inflorescence
OsDREB1D	253	AP002536	AY345235	BAD67857	6	leaf
OsDREB1E	242	AP004632	NM_001068949	BAD09738	8	Shoot, root, seedling
OsDREB1F	219	AP003448	AY345234	AAQ23984	1	leaf, stem, Inflorescence, root
OsDREB1G	219	AL606640	AY114110	CAD61655	4	Root
OsDREB1H	224	AP005775	AK106041 AK060550	BAD2923	2	Inflorescence, stem
OsDREB1I	238	AP004163(bases 20207-18000)	NM_001068950	NM-_001062415	8	callus, stem, leaf
OsDREB1J	286	AP006859	NM_001070246	NP_001063711	9	leaf, stem, Inflorescence
OsDREB2A	274	AP003301	AF300971 AK067313 AK059335 AK121956	AAN02487	1	Callus, inflorescence, leaf, stem
OsDREB2B	298	AC134928	AK099221 AK067313	AAV43805	5	Inflorescence, leaf, stem, callus, flower, root
OsDREB2C	230	AP001643	AK108143	BAC56005	8	Inflorescence
OsDREB2D	261	AC135924	AK102559 AK103822 AK109855	AAV44236	5	Stem, callus

Genes were predicated from the integrated results of the following: BLASTN2.0, BLASTX2.0, GENSCAN, Chris Burge, http://genes.mit.edu/GENSCAN.html), Fgenesh (http://www.softberry.com/), (http://www.ncbi.nlm.nih.gov/index.html)

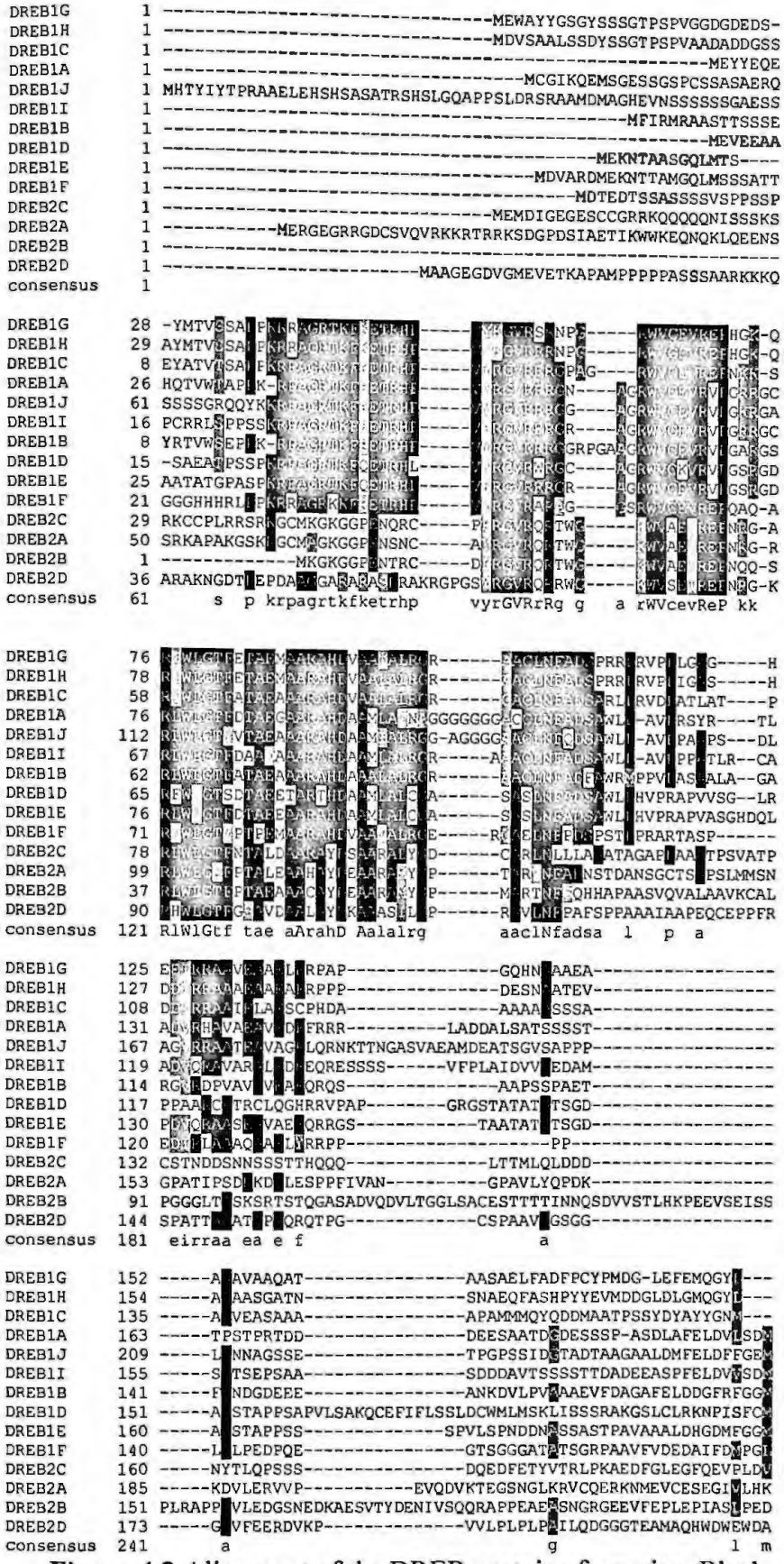


Figure 4.2 Alignment of the DREB proteins from rice. Black and light gray shading indicate identical and conserved amino acid residues, respectively

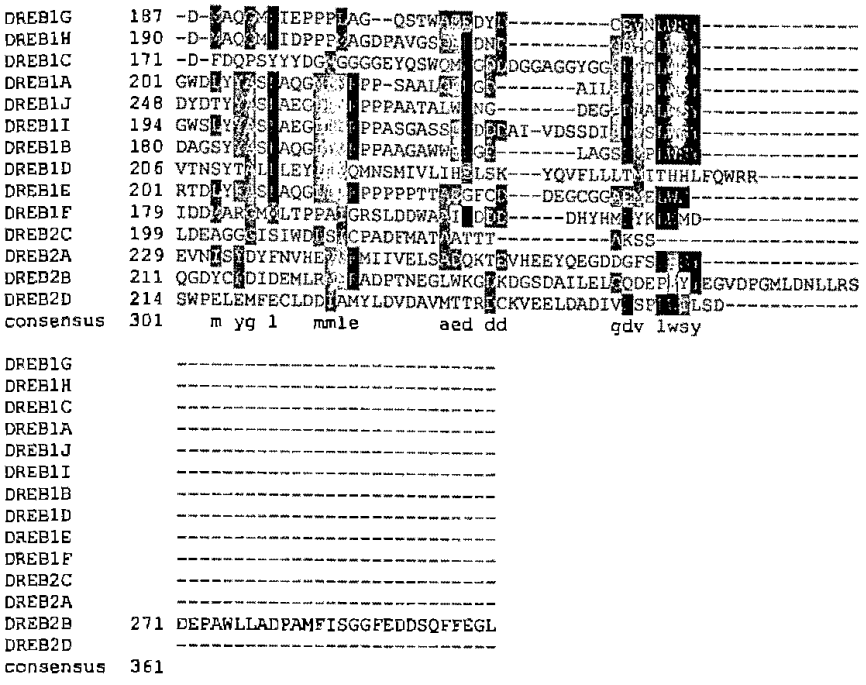


Figure 4.2 Continued Alignment of the DREB proteins from rice. Black and light gray shading indicate identical and conserved amino acid residues, respectively.

4.1.1.2 Detection of DREB Genes in Indica and Japonica Rice Genome

The presence of the 14 genes of OsDREB genes in BR29, BR 28 and BR 11, the most widely grown rice cultivars of the Rainfed ecosystem in Bangladesh were examined. Using the PCR primers listed in Table 4.2. The immediate parent cultivars were also used in this experiment for conformation of the presence or absence of any gene (Fig.4.3). Amplicons of the expected size were detected from all 14 genes in Nipponbare, but failed to detect OsDREB1D in BR29 (Figure 4.4). OsDREB1D was also undetectable in BG90-2/ BR51-46-5, one of the two parents of BR29 (Figure 1B). By contrast, all 14 genes were detected by PCR in both BR28 and BR 11, the second most widely grown cultivar in the Rainfed ecosystem, the most widely grown cultivar of the irrigated ecosystem. PCR primers amplifying other regions of the OsDREB1D gene also failed to produce an amplicon from BR29 genomic DNA, suggesting that the whole gene is absent from the genome. Nipponbare, the japonica rice cultivar was used as control and IR-64 was used as control from the group of indica cultivar. The whole experiment was done in two technical replicates.

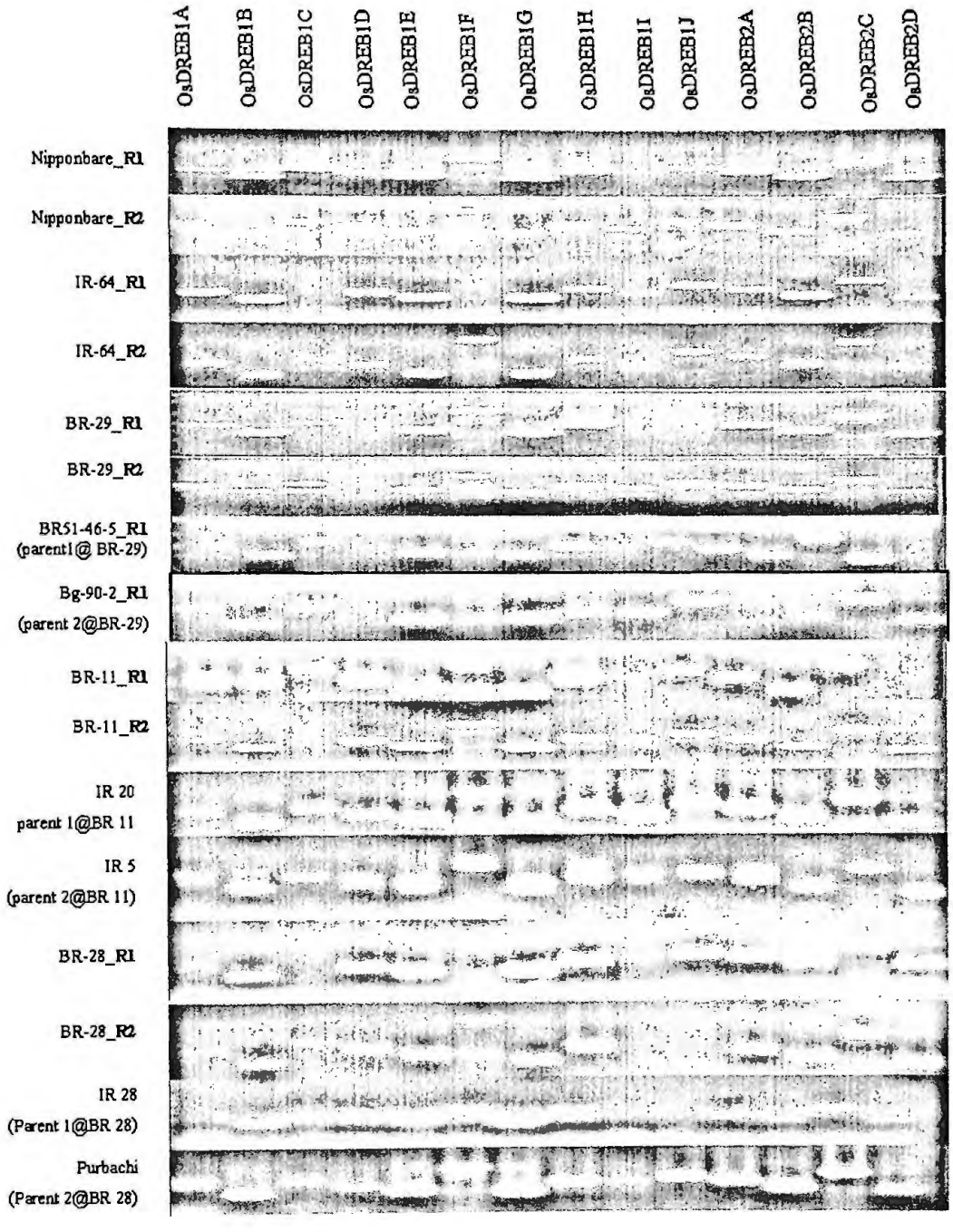


Figure 4.3 Detection of OsDREB genes in indica and japonica genomes by PCR analysis.

4.1.2 Transcript Analysis

4.1.2.1 Drought Stress Treatment of BR 29, BR 28 and BR 11.

To study the OsDREB genes response against drought stress, the overall plant behavior against drought stress was studied. Some physiological studies were conducted under certain stress level and in different tissues i.e. flag leaf (Fl), peduncle (Pd) and panicle (Pn).

In green house condition, the studied varieties were grown and stressed at flowering stage. Drought stress started at three days before heading by withdrawal watering and watering restored after 5 days of drought stress. Stressed level was measured by relative water content (RWC) of the Flag leaf. Elongation rate of peduncle, interoricle length and panicle elongation rate were also observed at each stressed level. For each physiological study, normally grown plant lines were used as control parameter. The experiment was done in triplicates and the same tissues were used in molecular analysis of transcripts to understand gene responses under same stress level.

4.1.2.1.1 Relative water content (RWC) as parameter of drought stress.

Relative water content was measured as a parameter of drought stress for BR 29, BR 28 and BR 11. The RWC value decrease from 90% to (30-40) % and tendency was increases immediately after rewatering. In most of the cases, RWC recovered about 30% after 24 hours of rewatering for all the studied varieties. The RWC value whereas the RWC of well watered showed almost similar values for all the studied time points (Fig.4.4). The measurement taken at certain time points and calculated RWC were identical for all of the studied rice varieties.

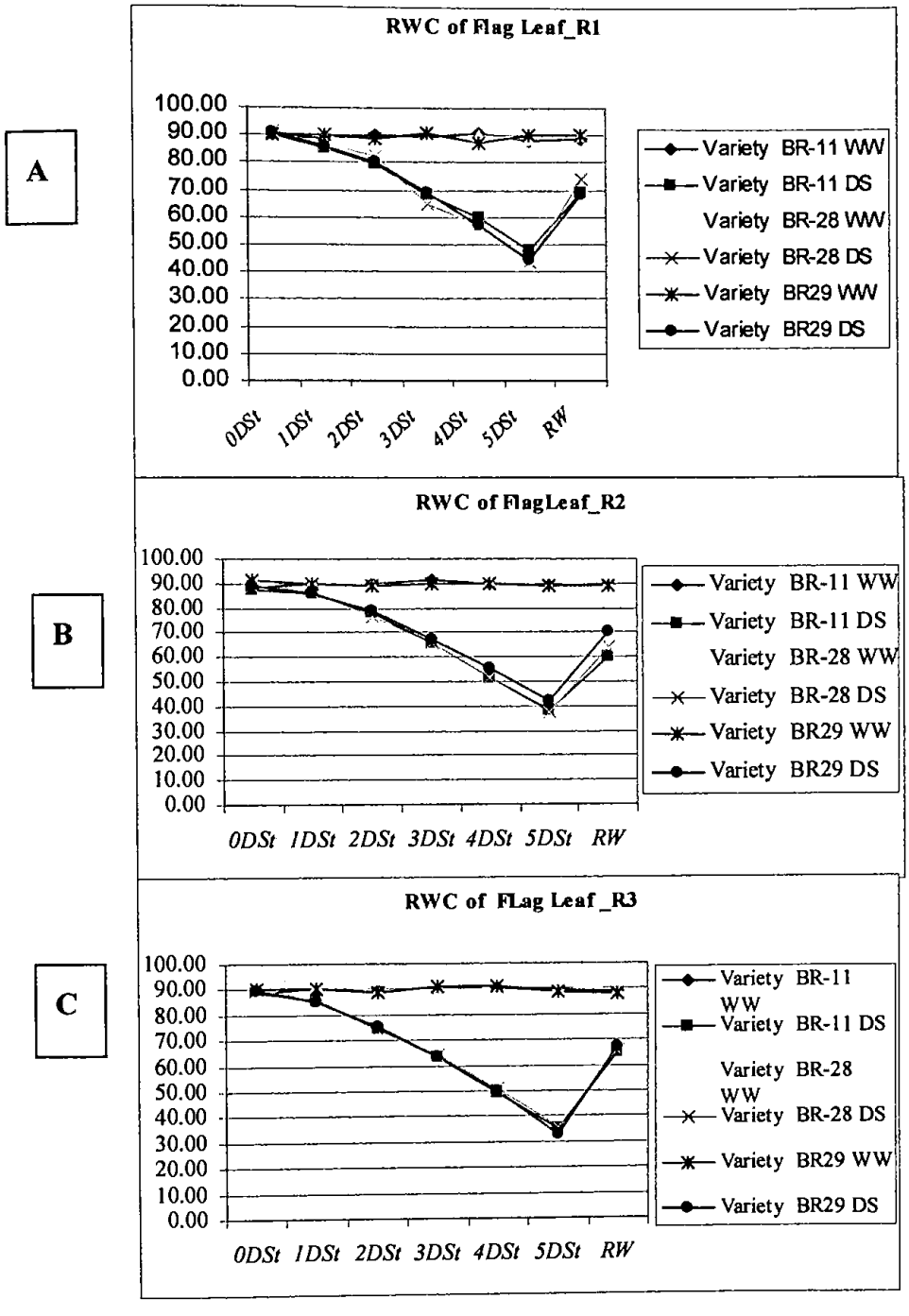
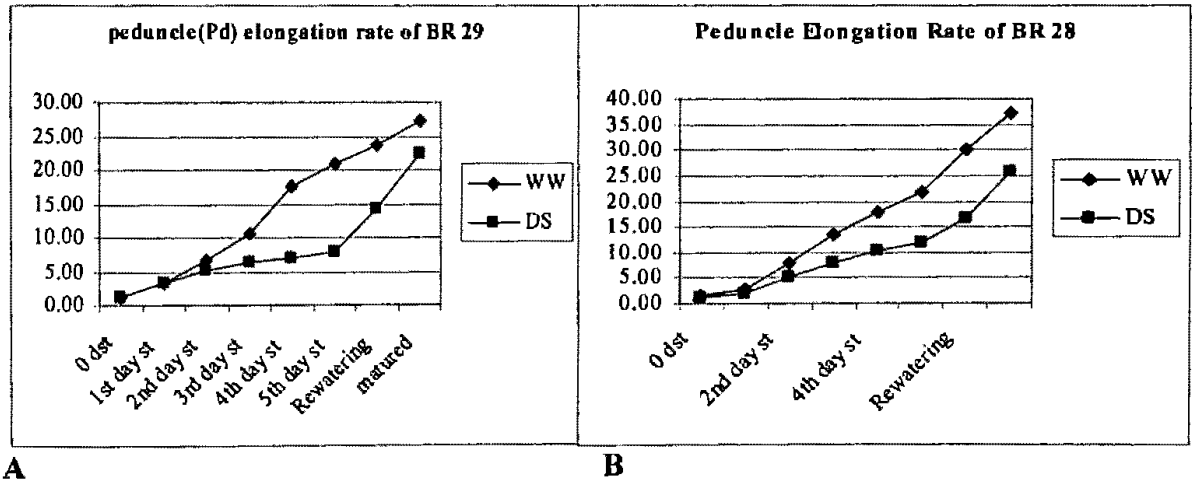


Figure 4.4 Relative water content (RWC) of flag leaf (%) at certain time points of drought condition. (A) Biological replication 1 (R1) (B) Biological replication 2 (R2) (A) Biological replication 3 (R3)

4.1.2.1.2 Drought Stress on Reproductive Organs of Rice Plant

The effect of drought stress measured for different reproductive organ developmental mechanism i.e. peduncle elongation, interoricle length elongation and panicle exertion rate the effect was negative compared to well watered plants.

Peduncle elongation rate decreased during drought stress and almost stopped at 5th day of stress for BR 29 and BR 11.but for BR 28 the reduction of the peduncle elongation was less significant. Though the elongation rate was not same as well watered but the tendency of elongate despite of drought stress was visible (Figure 4.5). The peduncle elongation rate was measured till maturity and results showed the fact that the plant recovery was not completely successful for the plants that experienced drought stress; and the plant remained relatively shorter causing delayed and incomplete panicle exertion (Fig.4.6). The changes of the interoricle-length elongation rate were similar for three BR rice varieties (Figure 4.7).



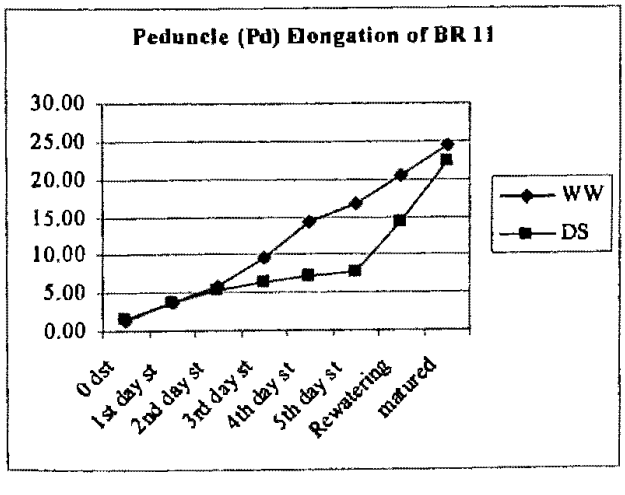


Figure 4.5 Peduncle elongation rate (cm) of (A) BR -29 (B) BR -28 and (C) BR -11 under certain drought stress condition. (Data is the mean value of three replicates).

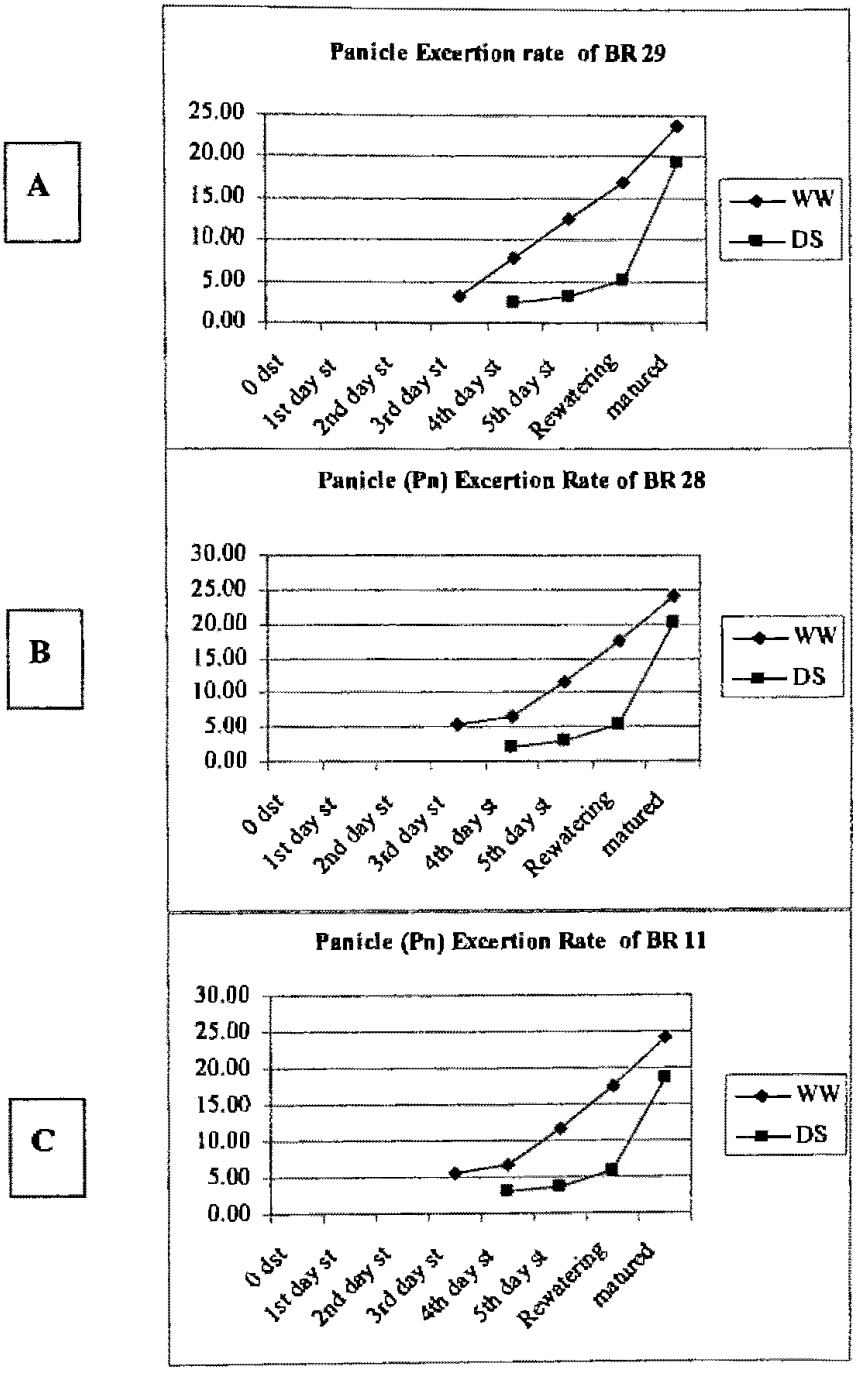
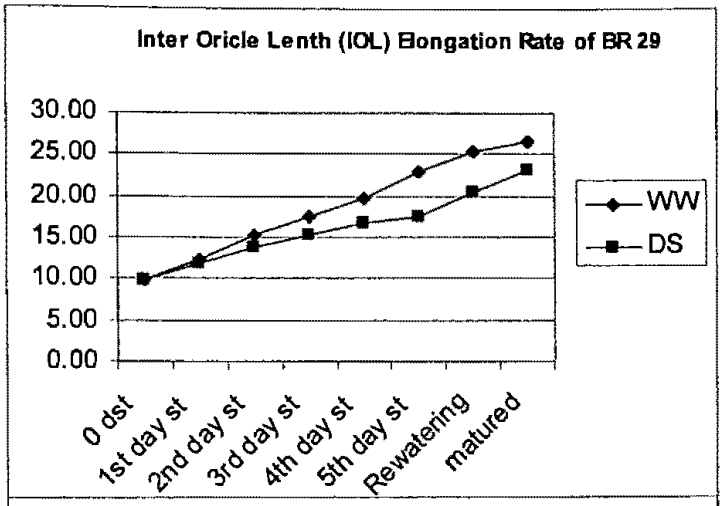
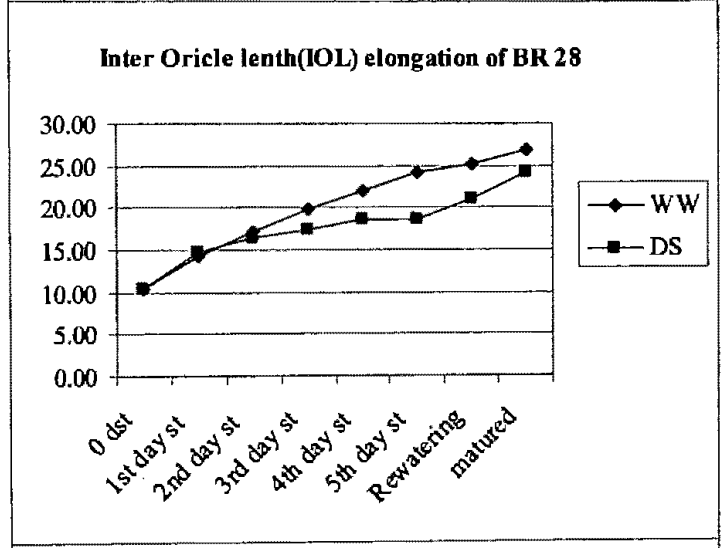


Figure 4.6 Panicle Exertion Rate (cm) of A) BR -29 (B) BR -28 and (C) BR -11 under certain drought stress condition (data is the mean value of three replicates).

A



B



C

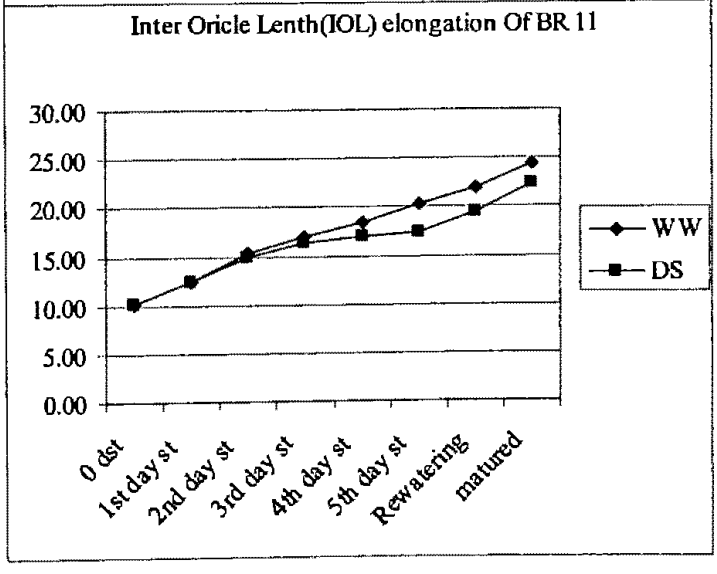


Figure 4.7 Interoricle Length elongation of A) BR -29 (B) BR -28 and (C) BR -11 under certain drought stress condition (data is the mean value of three replicates).

4.1.2.2 Expression of 14 genes in different tissues under certain drought stress conditions.

The expression of 14 OsDREB genes in different tissues of well watered, drought stressed, and rewattered plants in BR 29, BR 28 and BR11 by RT PCR analysis was conducted using the primers listed in Table 3.1. Except for OsDREB1D, the OsDREB genes of BR29 were expressed in at least one of the tissues (flag flag leaf blade, panicle, peduncle, etc). House keeping gene GAPDH was used as control by RT PCR analysis and a stress inducible gene Lip-9 was used as control of expression in different tissue.

More detail the expression of the 14 OsDREB genes was examined in three reproductive-stage organs in response to water stress and re-watering. The organs were flag leaf blade, panicle and peduncle (Figure 4.8, 4.9and 4.10). RT-PCR was conducted on replicated samples that were normalized for rRNA content. Several genes were activated by drought stress and down regulated by re-watering, whereas some other genes were down-regulated by drought stress and up-regulated by re-watering

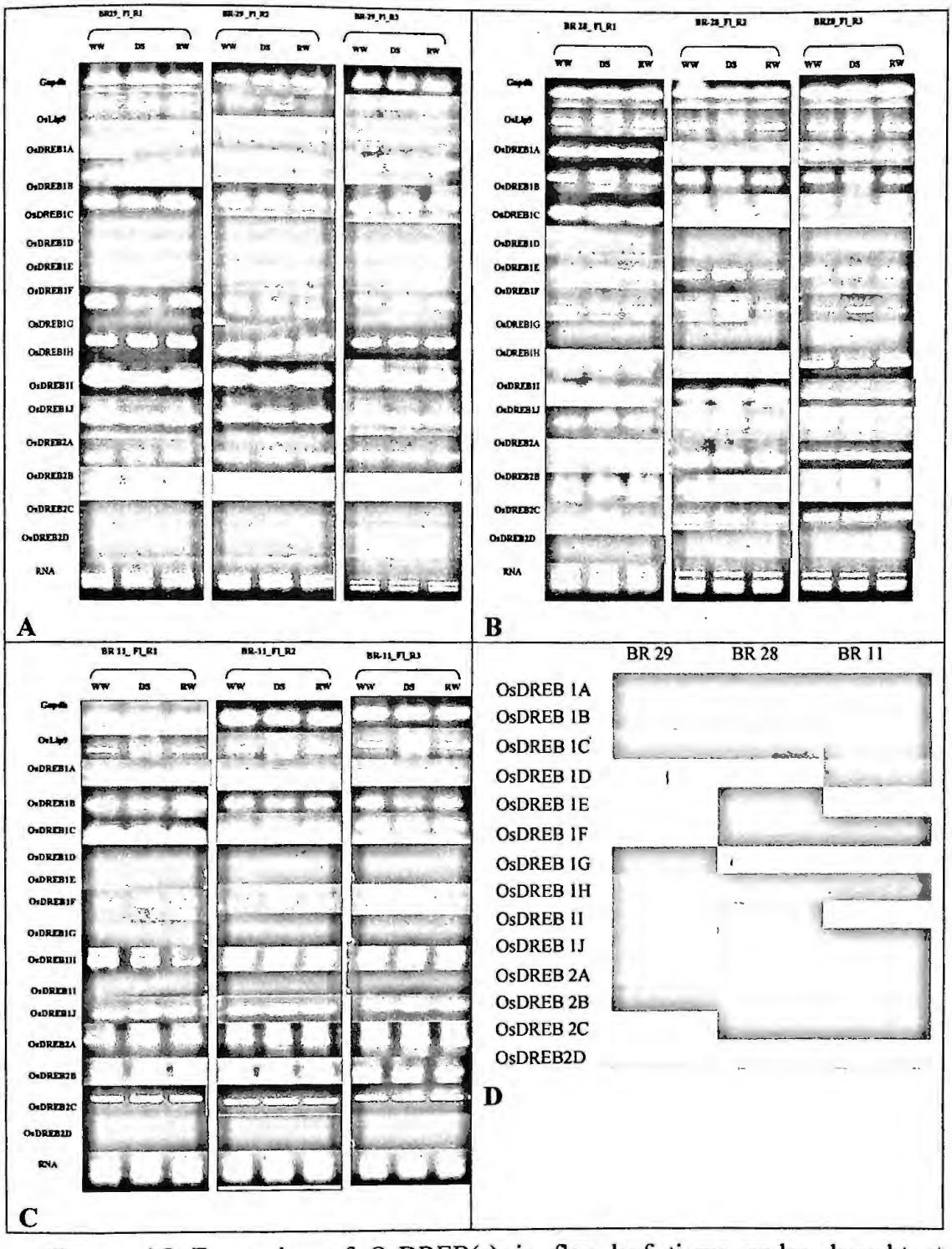


Figure 4.8 Expression of OsDREB(s) in flag leaf tissue under drought stress condition. (A) OsDREBs expression in well watered and drought stressed Flag leaf of BR 29 (B) OsDREBs expression in well watered drought stressed Flag leaf of BR 28 (C) OsDREBs expression in well watered drought stressed Flag leaf of BR 11 (D) OsDREBs expression atlas in BR 29 BR 28 And BR 11. Genes showed no difference in expression across treatments Genes showed no expression across treatments Genes showing different expression across treatments (WW-DS-RW).

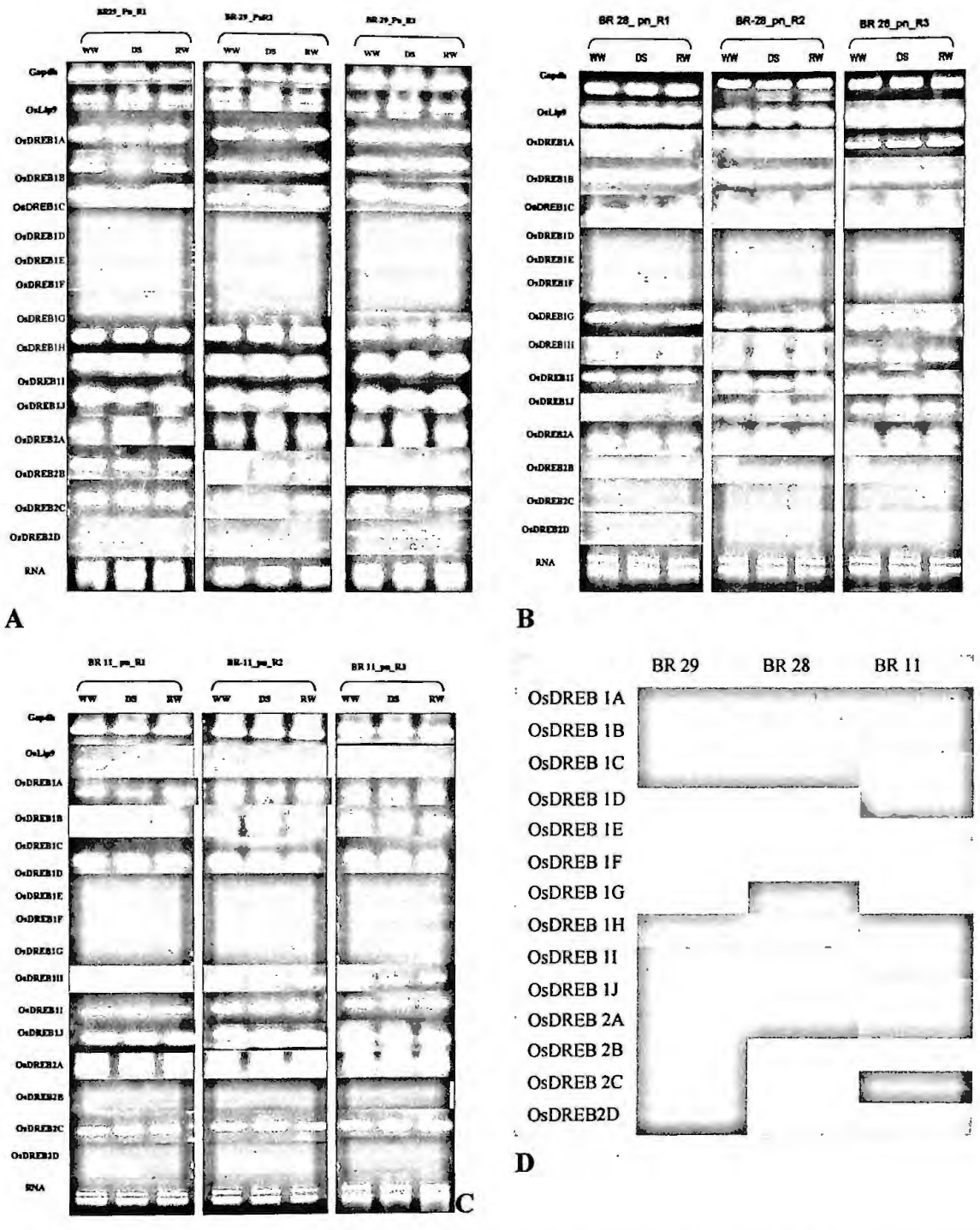


Figure 4.9 Expression of OsDREB(s) in panicle tissue under drought stress condition. (A) OsDREBs expression in well watered and drought stressed peduncle of BR 29 (B) OsDREBs expression in well watered drought stressed panicle of BR 28 (C) OsDREBs expression in well watered drought stressed panicle of BR 11 (D) OsDREBs expression atlas in BR 29 BR 28 And BR 11. Genes showed no difference in expression across treatments Genes showed no expression across treatments Genes showing different expression across treatments (WW-DS-RW).

The peduncle has rotational symmetry around its main axis and a clear developmental gradient along its axis, from the division zone and elongation zone to the maturation zone. Results showed that several genes were more highly expressed in the division zone than in the other two zones Fig 4.11, while others were most highly expressed in the embryo. The relative water content of the BR29 flag leaf blade declined gradually during drought-stress and recovered after re-watering (Figure 4.8). At the same time, the expression patterns of the 14 genes changed more markedly in the stressed tissues than in the well-watered tissues (Figure 4.8). Some genes responded to drought at a higher RWC than others. The decline in RWC was accompanied by a decline in peduncle elongation rate. The peduncle elongation rate under well-watered conditions was similar in BR11, BR28 and BR29, but only BR28 continued to elongate and to head under drought stress (Figure 4.5 and 4.6). By contrast, peduncle growth rate was halted in cultivars BR29 and BR11 and resumed only after re-watering. The pattern of OsDREB gene expression differed markedly among the three cultivars. Obtained data indicates that the peduncle elongation of BR28 is less drought-sensitive than those of BR11 and BR29. A possible explanation is that BR28 does not express OsDREB1A and OsDREB2B, whereas both of these genes are expressed in the peduncles of BR11 and BR29.

In the peduncle of BR29, OsDREB1A transcript levels increased substantially before heading under well-watered conditions, as the rate of peduncle elongation increased, but later declined as the elongation rate slowed. Under drought stress, there was a brief increase in OsDREB1A transcript levels while stress levels were low, but a marked decline followed from the third day as stress intensified; after re-watering transcript levels increased sharply. OsDREB1G transcript levels were fairly constant under well-watered conditions but declined markedly under drought stress, beginning at the third day; re-watering increased transcript levels. Similar results were obtained for OsDREB1I. By contrast, transcript levels for OsDREB1H and OsDREB1J, which were fairly constant under well-watered conditions, increased significantly from the second or third day of stress, and declined again on re-watering. Under well-watered conditions, OsDREB2A transcript levels increased significantly only at or after heading and then declined as the elongation decelerated, whereas they increased significantly after one day of stress and remained high until re-watering. OsDREB2B transcript levels increased significantly

from heading under well-watered conditions, but declined sharply from the third day of drought stress, only to increase again after re-watering. Transcript levels of OsDREB1B and OsDREB1C remained steady under both stressed and unstressed conditions.

The best correlations between peduncle elongation rate and OsDREB transcript levels were seen for OsDREB1A and OsDREB2B. As elongation rate is dependent on both cell division and cell elongation (Figure.4.10), we examined the distribution of OsDREB1A and OsDREB2B transcripts between the division, elongation and maturation zones (Figure.4.11) under well-watered, drought-stressed and re-watered conditions (Figure 4.12). OsDREB1A transcript abundance was highest in the division zone, intermediate in the elongation zone and lowest in the maturation zone, while OsDREB2B transcripts showed the reverse distribution. OsDREB2A expression showed a strong negative correlation with peduncle elongation rate (Figure.4.13). It was strongly expressed in the division and maturation zones but in these zones the negative correlation with elongation rate was absent; only in the elongation zone was the negative correlation observed.

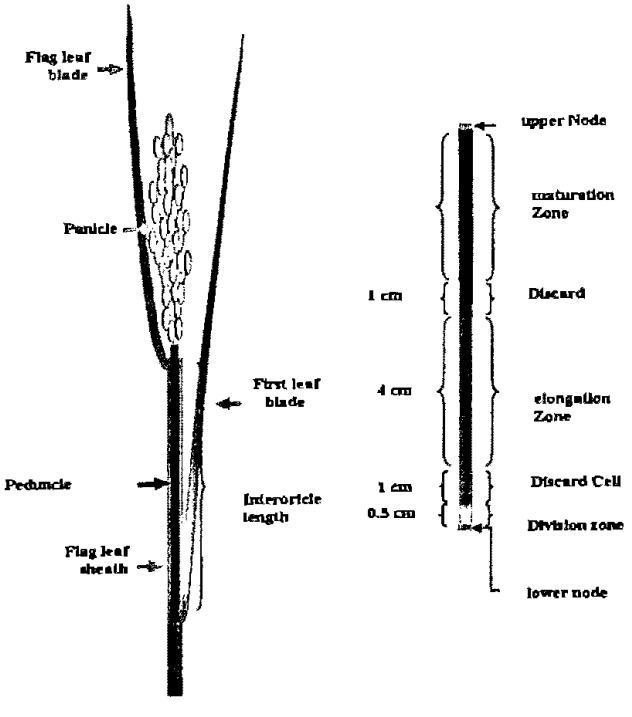


Figure 4.10 Different zones of reproductive stage of rice plant.

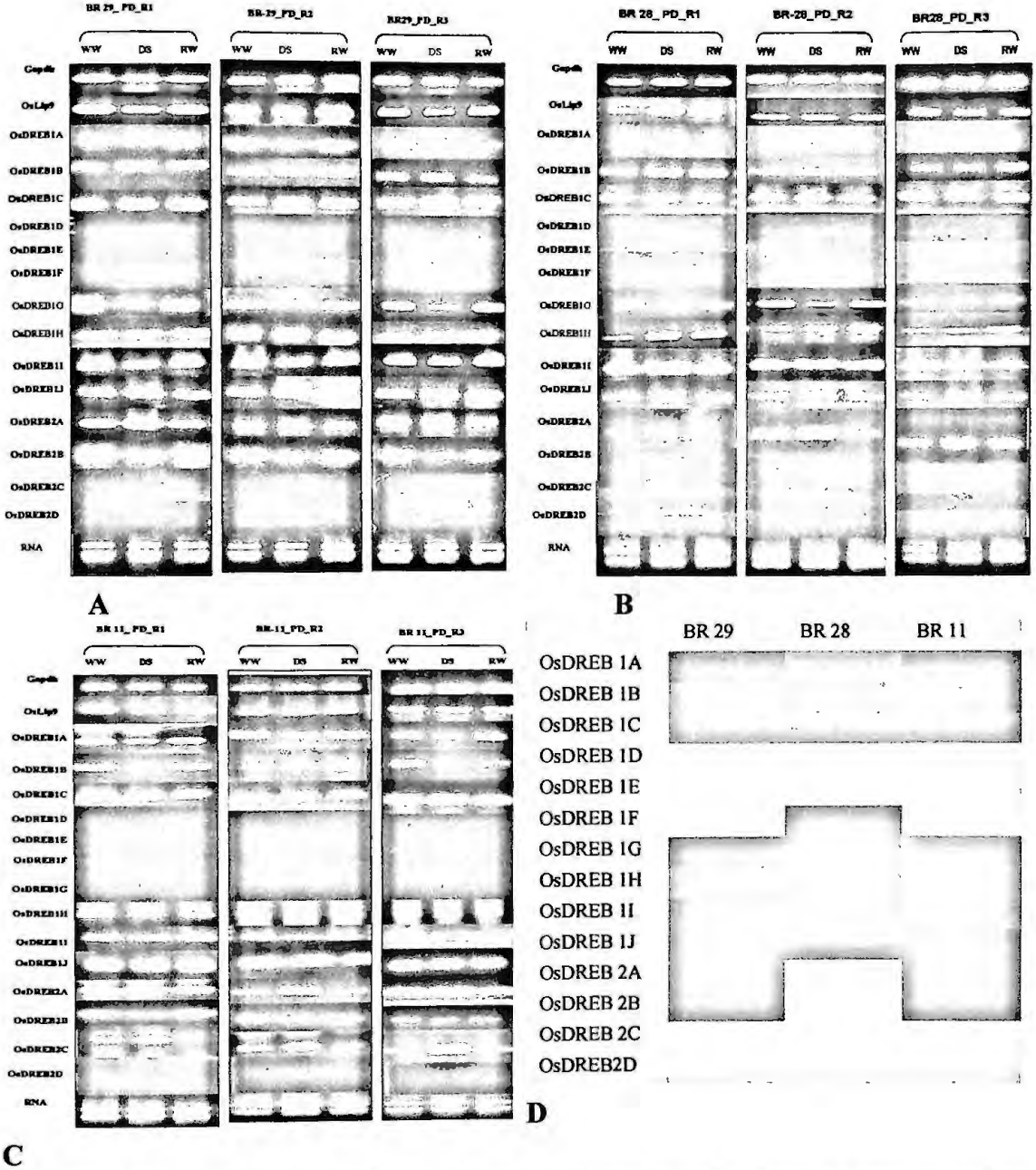


Figure 4.11 Expression of OsDREB(s) in peduncle tissue under drought stress condition. (A) OsDREBs expression in well watered and drought stressed peduncle of BR 29 (B) OsDREBs expression in well watered drought stressed peduncle of BR 28 (C) OsDREBs expression in well watered drought stressed peduncle of BR 11 (D) OsDREBs expression atlas in BR 29 BR 28 And BR 11 Genes showed no difference in expression across treatments Genes showed no expression across treatments Genes showing different expression across treatments(WW-DS-RW).

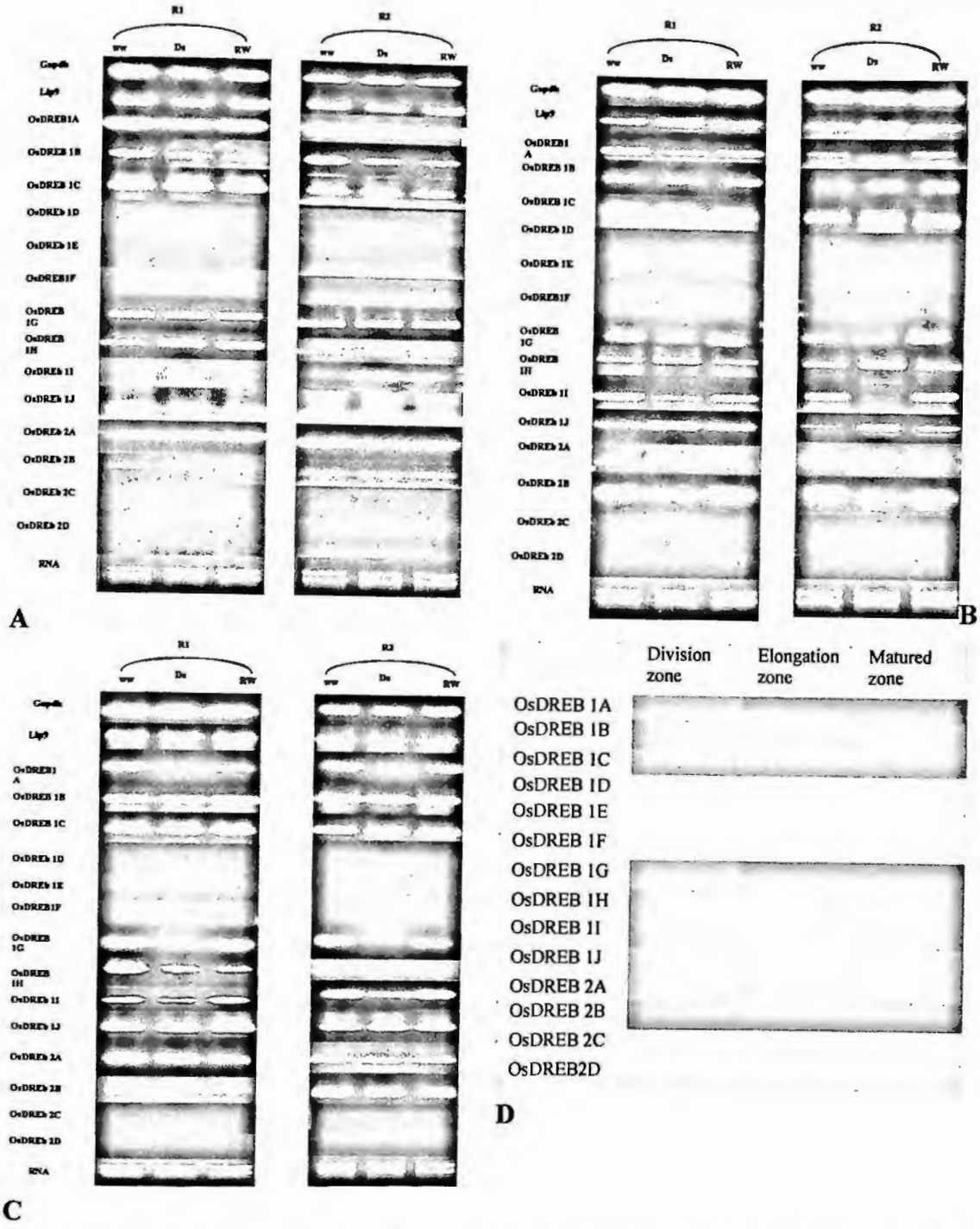


Figure 4.12 Expression of OsDREB(s) in different developing zones of BR 29 peduncle tissue under drought stress condition. (A) OsDREBs expression Of division zone of BR 29 peduncle (B) OsDREBs expression Of Elongation zone of BR 29 peduncle (C) OsDREBs expression Of Matured zone of BR 29 peduncle (D) OsDREBs expression atlas in division zone elongation zone and matured zone, Genes showed no difference in expression across treatments Genes showed no expression across treatments Genes showing different expression across treatments (WW-DS-RW).

The peduncles of BR29 were compared with respect to elongation rate and OsDREB transcript levels (Figure 4.13). The development of drought stress, as measured by the decline of the flag leaf relative water content, was almost identical in the three genotypes, but the progress of peduncle elongation was different in BR28 compared with BR11 and BR29. In drought-stressed BR28 the peduncle continued to elongate, albeit more slowly that in control plants, and heading occurred before re-watering, whereas in BR11 and BR29 elongation essentially ceased under stress, and heading occurred only after re-watering (Figure 4.13).

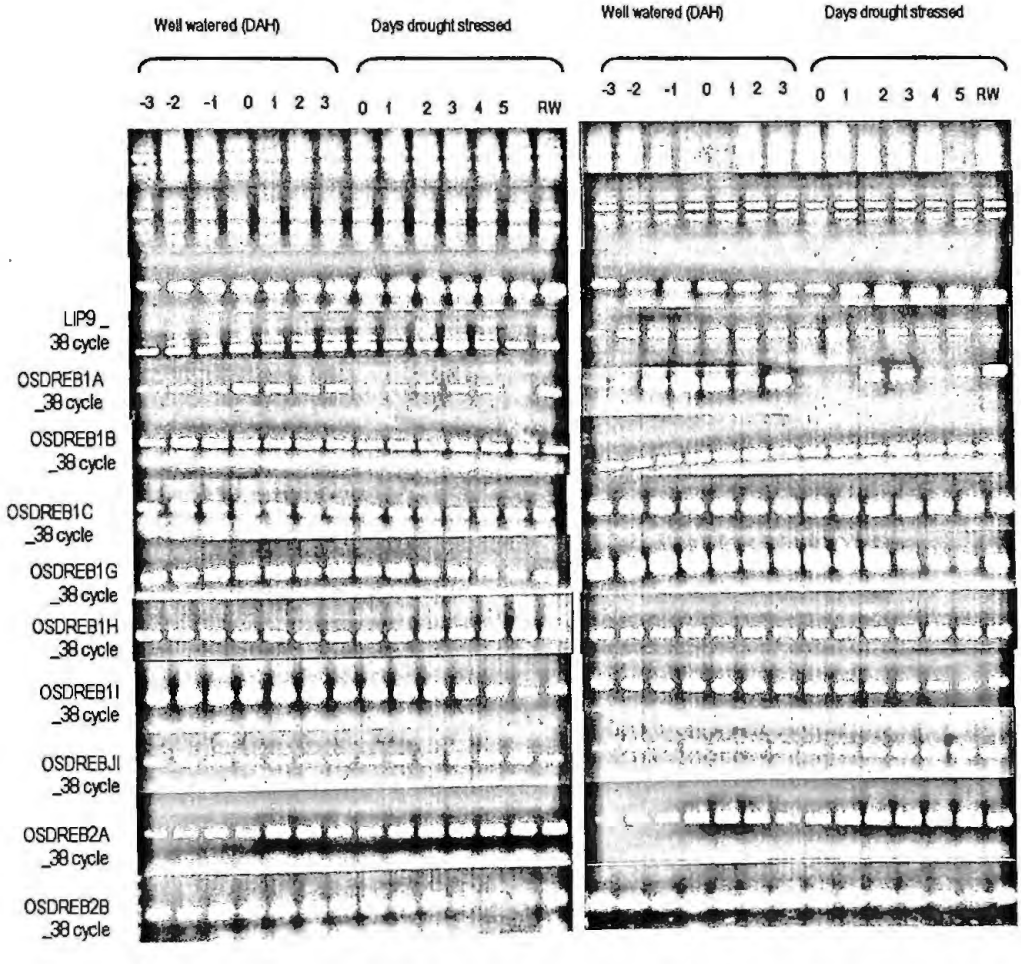


Figure.4.13 OsDREB(S) transcript level with peduncle elongation rate in BR -29.

By contrast, the pattern of OsDREB expression differed in all three genotypes (Figure 4.8-4.10). All three genotypes expressed OsDREB1B, OsDREB1C, OsDREB1H, OsDREB1I and OsDREB2A and did not express OsDREB1D, OsDREB1E and OsDREB2D. The differences were (i) only BR11 expressed OsDREB1F and OsDREB2C, (ii) only BR11 did not express OsDREB1G and OsDREB1J and (iii) only BR28 did not express OsDREB1A and OsDREB2B. Although this is a complex pattern of differential expression, it is intriguing that the reduced drought sensitivity of peduncle elongation in BR28 was associated with non-expression of the two genes shown above to be positively correlated with peduncle elongation. It should be noted that both OsDREB1A and OsDREB2B are expressed in the blade and sheath of the flag leaf of BR 28, suggesting that the non-expression of these genes in the peduncle are not due to the absolute loss of the expression of these genes.

4.2 GENE TRANSFORMATION OF BANGLADESH RICE VARIETIES

The main objective of these experiments was to optimize protocol and genotype as well as to select suitable explant for successful over expression of OsDREB1A gene to alter drought stress response. Three modern rice cultivars (BR-29, BR-28 and BR-11) from Bangladesh were used for genetic transformation of OsDREB1A gene. Two types of explants such as immature embryo and mature embryo were used for transformation experiments. In summary immature embryo as explant and BR -28 as genotype showed better plant regeneration as well as genetic transformation.

4.2.1. Protocol Optimization of Gene Transformation

Several experiments were conducted to optimize genetic transformation protocol for BR rice varieties. In these experiments different plasmids, explants and basal medium were used to standardize the criteria of successful and convenient genetic transformation in BR 29. In previous studies, BR 29 was reported (Datta *et al.*, 2003) to be a good material for callus induction and regeneration efficiency. Therefore, this variety was chosen to do preliminary experiments and to optimize a suitable protocol for gene transformation.

4.2.1.1 Agrobacterium-mediated Gene transformation of BR 29 using two types of explants in N_6-MS Basal media.

In the initial experiments, a composition of N_6-MS based media was used to compare two explants i.e. 5-day-old mature embryo derived seedling and matured embryo derived embryogenic calli (4-6 weeks after inoculation on callus induction medium).

With 5-day-old seedling, three experiments were conducted with both IRS 5 and IRS 147 plasmids. Callus induction was done using N_6 basal medium in combination with 2mg/l 2, 4-D, 300mg/l CasaminoAcid, 30 g/l sucrose and 10g/l D+-Glucose-Monohydrate. For Agrobacterium infection, a High concentration of sugar and low concentration of acetosyringone were used in N_6 basal media based liquid infection medium. The **infection medium** combines the following: N_6 salts and vitamins + 300 mg/l CasaminoAcid + 68.5 g/l Sucrose + 36g/l D+-Glucose-Monohydrate + 100 µM acetosyringone. Hygromycin was used for calli selection and a combination of Vancomycin and Cefotaxime used to control the growth of agrobacterium in **selection**

medium (N_6 salts and vitamins + 2mg/l 2, 4-D +30 g/l Sucrose + 300 mg/l CasaminoAcid +500 L-Proline + 300 mg/l Glutamine + 100mg/l Cefotaxime + 100mg/l Vancomycin+ 50mg/l Hygromycin). Developing resistant (Hygromycin) embryogenic calli (FIG.4.14) were transferred to **pre-regeneration medium** (N_6 salts and vitamins + 2 mg/l Kinetin+ 1 mg/l NAA + 5 mg/l ABA+ 30 g/l Sucrose + 300 mg/l CasaminoAcid +500 L-Proline + 300 mg/l Glutamine + 100mg/l Cefotaxime + 100mg/l Vancomycin+ 50mg/l Hygromycin) followed by **regeneration medium**(MS salts+30 g/l Sucrose + 30 g/l Sorbitol + 2 mg/l Kinetin+ 0.02 mg/l NAA+ 300 mg/l CasaminoAcid + 100mg/l Cefotaxime + 100mg/l Vancomycin+ 50mg/l Hygromycin). Summary results of this experiment shown in Table 4.2. The results showed poor efficiency for plasmid IRS-5 and no regeneration from plasmid IRS-147.

Table 4.2 Agrobacterium-mediated genetic transformation of BR 29 with IRS 5 and IRS 147 plasmid using 5 day old matured embryo derived seedling.

Plasmid	Experiment No.	Explants	Number of calli	OD 600 nm	Resistant Callus Events No	Regenerable Events No	Rooted Plantlet Events No	Transplanted Events No
IRS- 5	1	5 day old seedling	400	0.120	10	7	5	3
	2	5 day old seedling	400	0.096	13	7	6	2
	3	5 day old seedling	400	0.086	15	7	6	4
IRS-147	4	5 day old seedling	400	0.068	8	0	0	0
	5	5 day old seedling	400	0.065	6	0	0	0
	6	5 day old seedling	400	0.074	4	0	0	0

4.2.1.2 Gene Transformation of BR 29 using Embryogenic Calli (EGC) in N_6 –MS Basal Media.

Matured embryo derived (4-6 weeks after inoculation) embryogenic (EGC) calli were used in this experiment. 5 experiments were conducted with both plasmids. Same media composition and procedure of previous experiments were used in this experiment. No callus proliferation or regeneration was observed from this experiment. The results are shown in Table 4.2.

A simple modification of the protocol was done for EGC explants to obtain transgenic plant. The calli was air dried after agrobacterium infection (D) and compared with the original protocol based of moistened (M) calli without drying. But no improvement was observed. Results are shown in Table 4.3.

Table 4.3 Agrobacterium-mediated genetic transformation of BR 29 with IRS 5 and IRS 147 plasmids using matured embryo derived embryogenic calli (6 weeks after inoculation).

plasmid	Experiment No	Protocol	Number of calli	OD 600 nm	Resistant Callus Events No	Regenerable Events No	Rooted Plantlet Events No	Transplanted Events
IRS 5	1	EGC	320	0.084	0	0	0	0
	2	EGC	314	0.078	0	0	0	0
	3	EGC	300	0.096	0	0	0	0
	4	EGC	321	0.084	0	0	0	0
	5	EGC	281	0.068	0	0	0	0
IRS 147	1	EGC	300	0.081	0	0	0	0
	2	EGC	321	0.073	0	0	0	0
	3	EGC	308	0.094	0	0	0	0
	4	EGC	300	0.092	0	0	0	0
	5	EGC	312	0.065	0	0	0	0

Table 4.4 Agrobacterium-mediated genetic transformation of BR 29 with IRS 5 and IRS 147 plasmids using matured embryo derived embryogenic calli (6 weeks after inoculation). Using modification in inoculation process; drying (D) comparing with moistening (M).

plasmid	Experiment No	Protocol	Number of calli	OD 600 nm	Resistant Callus Events No	Regenerable Events No	Rooted Plantlet Events No	Transplanted Events
IRS 5	1(M)	EGC	298	0.072	0	0	0	0
	2 (M)	EGC	314	0.083	0	0	0	0
	3(M)	EGC	315	0.076	0	0	0	0
	4(M)	EGC	321	0.071	0	0	0	0
	1(D)	EGC	256	0.072	0	0	0	0
	2 (D)	EGC	274	0.083	0	0	0	0
	3(D)	EGC	341	0.076	0	0	0	0
	4(D)	EGC	338	0.071	0	0	0	0
IRS	1(M)	EGC	306	0.088	0	0	0	0
147	2 (M)	EGC	326	0.088	0	0	0	0
	3(M)	EGC	319	0.074	0	0	0	0
	4(M)	EGC	279	0.074	0	0	0	0
	1(D)	EGC	317	0.069	2	0	0	0
	2 (D)	EGC	321	0.069	3	0	0	0
	3(D)	EGC	328	0.079	I	0	0	0
	4(D)	EGC	286	0.079	3	0	0	0

In the preliminary experiment only 16 plants (Data not shown) of 9 event (Table 4.2) was obtained. The presence of the inserted gene was confirmed by PCR analysis of Hygromycin (Figure 4.15, A and OsDREB1A gene (Figure 4.15, B). These plants were transplanted and showed normal growth compared with control plant (Figure 4.14)

Fig 4.14 Gene transformation from matured embryo derived 5day old seedling (A) Callus induction (B-C) selection (D) Callus proliferation in pre-regeneration medium) (E) Friable embryogenic calli (F) Plant Regeneration (G) Survived shoots in Hygromycin containing rooting medium. (H) Putative T_0 OsDREB 1A lines having ripened grains in green house.

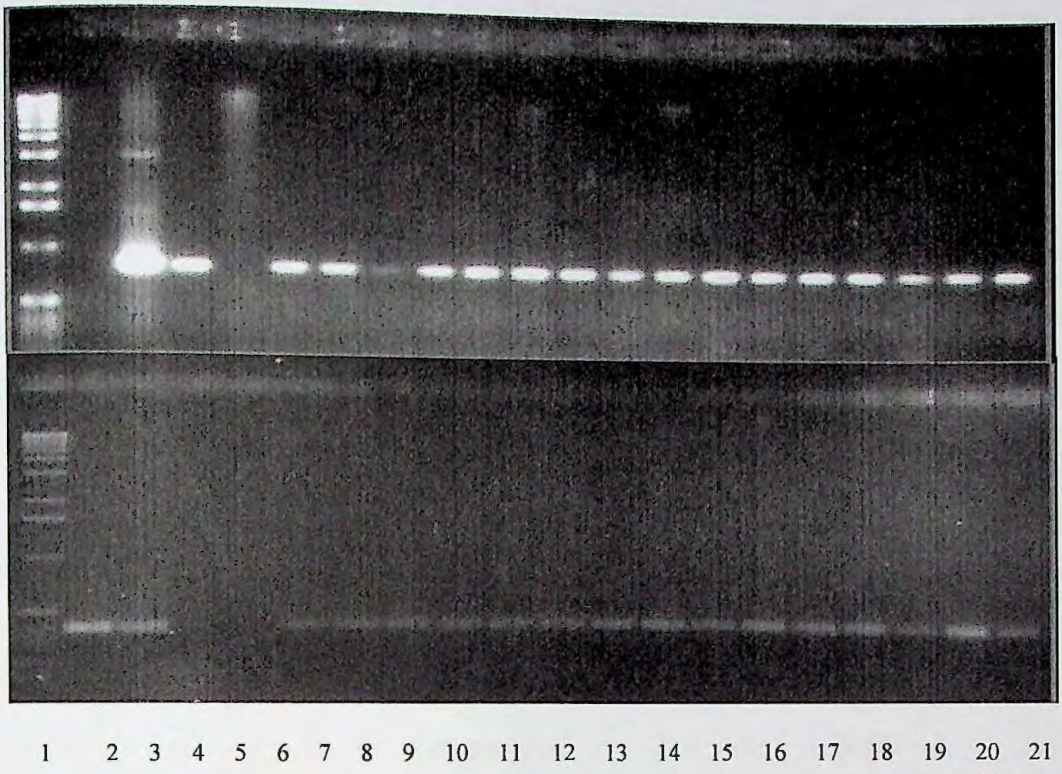


Figure 4.15 PCR detection of transgenic BR 29 plants plant using matured embryo as explant.

(A) PCR detection for the presence of *Hph* gene in putative transformant lane 1: 1Kb ladder, lane 2: water, lane 3:PGL2 =Plasmid. lane 4: IR-64-IRS145-001=Positive control, lane5: IR-64 (Negative control), lane 6:BR-29IRS-5-001A, lane7:BR-29IRS-5-001B, lane8:BR-29IRS-5-002A, Lane:10BR-29IRS-5-002B, Lane11:BR-29IRS-5-002C, Lane12:BR-29IRS-5-003B, Lane13; BR-29IRS-5-004, Lane 15; BR-29IRS-5-005A, Lane16:BR-29IRS-5-010A, Lane 17:BR-29IRS-5-010B, Lane 18:BR-29IRS-5-014A, Lane19:BR-29IRS-5-014B, Lane 20:BR-29IRS-5-015A, Lane 21:BR-29IRS-5-016A] product size 800bps.

(B) PCR detection of OsDREB1A in primary transformant [Lane 1: 1Kb Ladder, Lane 2: IRS145 (Plasmid), lane3: IR64-IRS145-004(positive control), Lane4: WATER, lane5: IR-64 (Negative control), lane (6-21): BR-29IRS-5-001A to BR-29IRS-5-016A] product size 396bps.

4.2.1.3 Agrobacterium-mediated genetic transformation of BR 29 using immature embryo in N_6-B5-MS basal media.

Because of poor transformation efficiency next the N6-B5-MS medium based protocol, modified from Hiei *et al.*, (1994) and Kumara *et al.*, (2005) were evaluated. In this experiment, immature embryos (6-8 days after anthesis) were used as explant.

Three experiments were done with IRS-147 plasmid and better regeneration as well as transformation was observed. Isolated immature embryo were co-cultivate for 5 days along with a droplet of **liquid infection medium** (Figure 4.16) containing *agrobacterium* on semisolid co cultivation medium (N_6 major salts+B5 minor salts and vitamin+ 2 mg/l 2, 4-D + 1 mg/l NAA + 1 mg/l BA+ 500 mg/l l-Proline + 500 mg/l CasaminoAcid+ 20 g/l sucrose+ 10 g/l sucrose+100uM Acetosyringone). Calli were recovered on **resting medium** (N_6 major salts+B5 minor salts and vitamin+ 1 mg/l 2,4-D + 1 mg/l NAA + 0.2 mg/l BA+ 500 mg/l l-Proline + 500 mg/l CasaminoAcid+300 mg/l L-glutamine +36 g/l D-mannitol + 20 g/l D-Maltose(2%) + 250 mg/l Claforan + 100 mg/l Carbenicillin).Resistant calli were selected using **selection medium** containing 50mg/ml Hygromycin. plantlets were obtained MS based **pre-regeneration** (MS salts and vitamins+2 mg/l Kin + 0.5 mg/l NAA+ 20 g/l D-Maltose(2%) + 10 g/l sorbitol+ 50mg/ml Hygromycin) and **regeneration medium** (MS salts and vitamins+2 mg/l Kin + 1 mg/l NAA +30 g/l sucrose+50mg/ml Hygromycin+ 250 mg/l Claforan)and withdrawn in rooting (MS0) medium. summary of the result is shown in Table (Table 4.5) Results showed that 22 events (total of three replicate of experiments) were obtained out of 300 explants.

Table 4.5 Agrobacterium-mediated genetic transformation of BR 29 using immature embryo (6 -8days weeks after anthesis).

Plasmid	Experiment No.	Number of explant	OD 600 nm	Resistant Callus Events No.	Regenerable Events No	Rooted Plantlet Events No.	Transplanted Events
IRS 147	1	100	0.3	37	12	7	7
	2	100	0.3	45	10	9	9
	3	100	0.3	34	5	6	6
Total		300		116	27	22	22

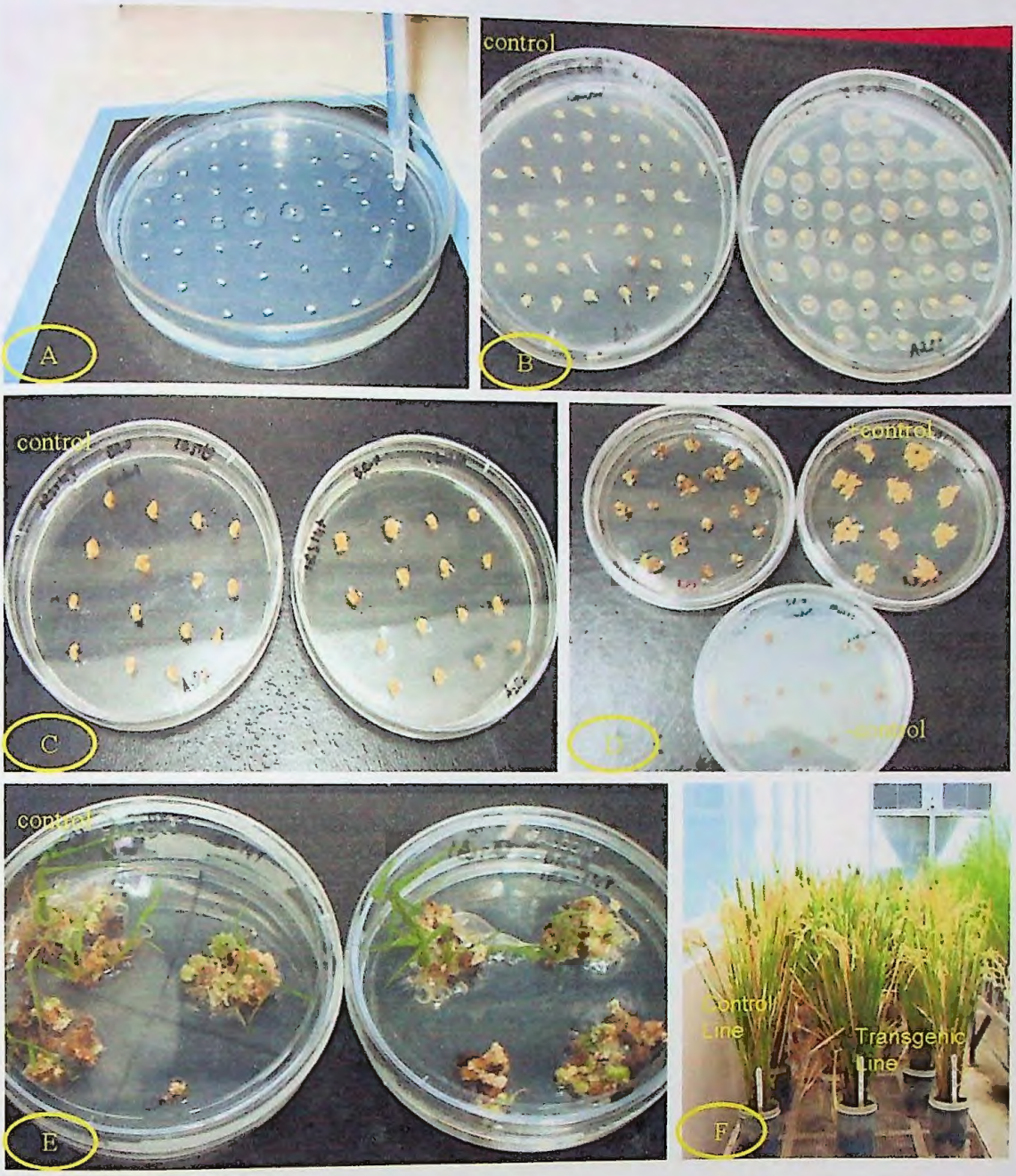


Figure 4.16 Gene transformation of BR 29 Immature embryo using droplet method of Agrobacterium infection. A) Agrobacterium infection B) Immature embryo after 5 days co cultivation, C) calli recovering stage in resting medium D) Transformed Calli selection in Hygromycin selection medium, +control is control calli in selection medium with out Hygromycin, - control is control calli in medium containing Hygromycin E) plant regeneration in deep Petridis F) Putative T_0 OsDREB 1A lines having ripened grains in green house.

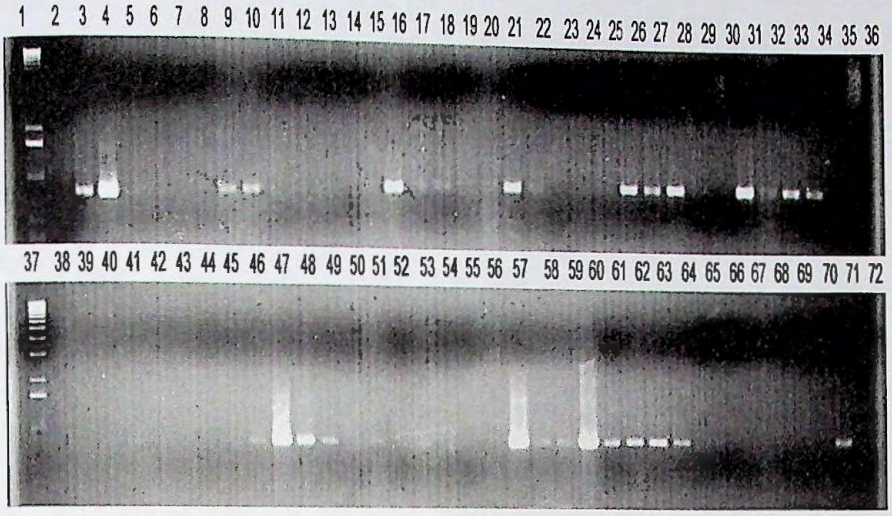


Figure 4.17 PCR for the presence of *Hph* marker gene in primary transformant (product size =0.8 KB) the positive and negative plant Identification from the gel picture is summarized in Table4.6

Table 4.6 Results of the presence of *Hph* gene in transgenic plant according to PCR analysis.

Lane No.	Plant ID	*Hph*	Lane No.	Plant ID	*Hph*	Lane No.	Plant ID	*Hph*
1	I kb ladder		40	BR29 -IRS147-014b	0	35	BR29 -IRS147-013a	0
2	neg control	0	41	BR29 -IRS147-014c	1	36	BR29-IRS147-013b	0
3	IR64IRS147017A	1	42	BR29 -IRS147-015a	0	51	BR29 -IRS147-018a	1
4	Plasmid	1	43	BR29 -IRS147-015b	0	52	BR29-IRS147-018b	0
5	BR29 -IRS147-003a	0	44	BR29 -IRS147-015c	0	53	BR29 -IRS147-018c	1
6	BR29 -IRS147-003b	0	45	BR29 -IRS147-016a	0	54	BR29 -IRS147-019a	0
7	BR29 -IRS147-003c	0	46	BR29 -IRS147-016b	1	55	BR29 -IRS147-19b	0
8	BR29 -IRS147-003a	0	47	BR29 -IRS147-016c	1	56	BR29 -IRS147-019c	0
9	BR29 -IRS147-004b	1	48	BR29 -IRS147-017a	1	57	BR29 -IRS147-020a	1
10	BR29 -IRS147-004c	1	49	BR29 -IRS147-017b	1	58	BR29-IRS147-020b	1
11	BR29 -IRS147-005a	0	50	BR29 -IRS147-017c	0	59	BR29 -IRS147-020c	1
12	BR29 -IRS147-005b	0	22	BR29 -IRS147-008c	1	60	BR29 -IRS147-021a	1
13	BR29 -IRS147-005c	0	23	BR29 -IRS147-009a	0	61	BR29-IRS147-021b	1
14	BR29 -IRS147-006a	0	24	BR29 -IRS147-009b	0	62	BR29 -IRS147-021c	1
15	BR29 -IRS147-006b	0	25	BR29 -IRS147-009c	0	63	BR29 -IRS147-022a	1
16	BR29 -IRS147-006c	1	26	BR29 -IRS147-010a	1	64	BR29-IRS147-022b	1
17	BR29 -IRS147-007a	1	27	BR29 -IRS147-010b	1	65	BR29 -IRS147-022c	0
18	BR29 -IRS147-007b	1	28	BR29 -IRS147-010c	1	66	BR29 -IRS147-023a	0
19	BR29 -IRS147-007c	0	29	BR29 -IRS147-011a	1	67	BR29 -IRS147-23b	0
20	BR29 -IRS147-008a	1	30	BR29 -IRS147-011b	0	68	BR29 -IRS147-023c	0
21	BR29 -IRS147-008b	1	31	BR29 -IRS147-011c	1	69	BR29 -IRS147-024a	0
37	1KB ladder		32	BR29 -IRS147-012a	1	70	BR29-IRS147-024b	0
38	BR29 -IRS147-013c	0	33	BR29 -IRS147-012b	1	71	BR29 -IRS147-024c	1
39	BR29 -IRS147-014a	0	34	BR29 -IRS147-012c	1	72	blank	

During the experiment the calli produced from each embryo were carefully kept separated Figure 4.14and 4.16) and plant obtained from one calli considered to be one event (From I cluster of regenerated calli maximum 3 plants (clones) were taken for further analysis to reduce work-load and costs of experiment. After successful regeneration, plantlets were transferred to yosida culture solution. After 2weeks, plant samples were collected for PCR analysis. The plants were transplanted in transgenic green house after the detecting the presence of marker gene (Hygromycin Phospho-transferase Gene) by PCR analysis (Figure 4.17, Table 4.6).Transgenic plant were

vigorous and uniform, and showed normal flowering and produced fertile seeds.

In these preliminary experiments for protocol optimization, the medium based on N_6-B5-MS was found to be suitable for callus induction and plant regeneration as well as transformation.

Further experiments were continued with N_6-B5-MS based protocol using plasmid IRS147 to optimize transformation efficiency of the three-studied variety. Moreover, two explants i.e. matured embryo and immature embryo were tested for optimal genetic transformation.

4.2.2 Transformation Efficiency of Different Rice Varieties and Explants.

Based on initial experiments above, further experiments were conducted with three popular rice varieties of Bangladesh i.e. BR-29, BR-28 and BR-11 were tested for their transformation efficiency. The medium compositions of N_6-B5-MS basal media were used to compare the efficiency of two different explants of the studied varieties. The whole experiment was replicated twice.

For mature and immature embryo two experiments were conducted for each variety .and each experiment started with 100 explants so the confirmed number of transgenic event also represents the genetic transformation efficiency.

4.2.2.1 Genetic Transformation from Immature Embryo (IE) of BR 29, BR 28 and BR 11.

Between two types of explants, immature embryo showed better plant regeneration as well as for transformation. Regeneration response and transformation efficiency was lowest in BR-11 and BR 29. The results of genetic transformation experiment for immature embryo are summarized in Table 4.7. The result showed that 35 events were obtained out of 200 explants for BR 28 whereas BR-29 and BR-11 produced 24 and 13 out of 200 explants, respectively.

Table 4.7 Genetic transformations from immature embryo (IE) of BR 29, BR 28 and BR 11.

Genotype	Experiment No.	Number of explant	Plasmid	strain Number	OD 600 nm	Resistant Callus Events No.	Regenerable Events No.	Rooted Plantlet Events No.	Transplanted Events
BR-29	1	100	IRS-147	LBA-4404	0.3	87	61	16	10
	2	100	IRS-147	LBA-4404	0.3	75	56	20	14
BR-28	1	100	IRS-147	LBA-4404	0.3	72	46	20	17
	2	100	IRS-147	LBA-4404	0.3	82	52	22	18
BR-11	1	100	IRS-147	LBA-4404	0.3	54	45	8	7
	2	100	IRS-147	LBA-4404	0.3	58	38	8	6

4.2.2.2 Gene Transformation from Matured Embryo (ME) of BR 29, BR 28 and BR 11

For matured embryo, BR-28 showed better response than the other two genotypes. Regeneration response and transformation efficiency was the same for BR 11 and BR 29. The results of genetic transformation experiment for Matured embryo are summarized in Table 4.8 result shows BR 28 produced 17 transgenic events. BR-29 11 and BR-11 10, respectively out of same number of explants (Table 4.9).

Table4.8 Genetic transformation from matured embryo (ME) of BR 29, BR 28 and BR 11.

Genotype	Experiment No.	Number of explant	Plasmid	Strain Number	OD 600 nm	Resistant Callus Events No.	Regenerable Events No.	Rooted Plantlet Events No.	Transplanted Events
BR-29	1	100	IRS-147	LBA-4404	0.3	64	51	8	6
	2	100	IRS-147	LBA-4404	0.3	79	56	6	5
BR-28	1	100	IRS-147	LBA-4404	0.3	74	40	11	9
	2	100	IRS-147	LBA-4404	0.3	69	44	9	8
BR-11	1	100	IRS-147	LBA-4404	0.3	42	39	6	5
	2	100	IRS-147	LBA-4404	0.3	55	36	5	5

Table 4.9 Integrated results of transformation efficiency for BR 29 BR 28 and BR 11 comparing the two explants (Explants immature and matured embryo).

Type of Explant	Genotype	Number of used Explants	Putative Transgenic Event	Efficiency
IE	BR 29	200	24	12
	BR 28	200	35	**17.5**
	BR 11	200	13	6.5
ME	BR 29	200	11	5.5
	BR 28	200	17	**8.5**
	BR 11	200	10	5

4.2.3 Molecular Analysis of putative Transformed Plants.

4.2.3.1 Integration of Foreign Gene

Putative transformed plants derived from Agrobacterium-mediated transformation were analyzed by PCR using appropriate sets of primer especially designed for gene amplification (Table 3.2). PCR product was electrophoresed with 1% agarose gel and visualized by UV. Primarily the t_0 plants did show the presence of *Hph* marker gene by PCR analysis. A total number of 124 plants of BR 28 from different experiments were analyzed after calli selection. Out of 124 plants 97 plants were found to be positive (Figure 4.19, Table 4.10) Among 147 plants of BR 29 obtained from different experiments 100 plants were found to be positive(Figure 4.18, Table 4.9) and out of 63 Hygromycin resistant plant of BR 11, 53 plants were positive for *Hph* marker gene(Figure 4.20, Table 4.11).

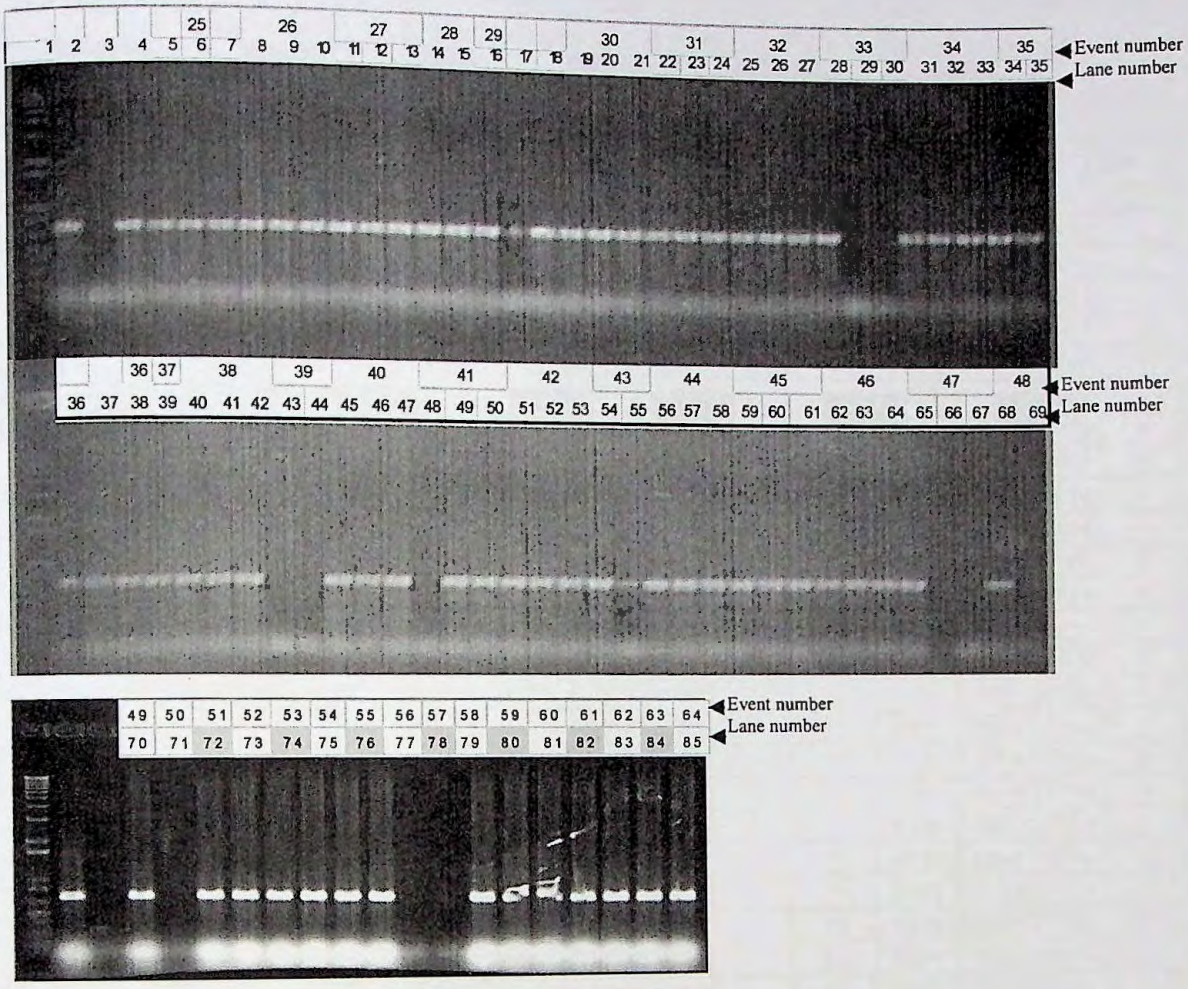


Figure 4.18 PCR detection for *Hph* gene (product size =0.8 KB) in putative transformant of BR 29. Detailed information is summarized in Table 4.10.

Table 4.10 Results of PCR analysis for the presence of *Hph* marker gene of primary transformant of BR 29 .This Table is summary of Figure 4.18

Lane	Plant ID		GOI (HPT)	Lane	Plant ID		GOI (HPT)	Lane	Plant ID		GOI (HPT)
1		1Kb ladder	ladder	30	BR29	IRS147-033C	0	59	BR29	IRS147-045A	1
2		IRS147	1	31	BR29	IRS147-034A	1	60	BR29	IRS147-045B	1
3		Neg control	0	32	BR29	IRS147-034B	1	61	BR29	IRS147-045C	1
4	IR64_IRS147-15		1	33	BR29	IRS147-034C	1	62	BR29	IRS147-046A	1
5	BR29	IRS147-025A	1	34	BR29	IRS147-035A	1	63	BR29	IRS147-046B	1
6	BR29	IRS147-025B	1	35	BR29	IRS147-035B	1	64	BR29	IRS147-046C	1
7	BR29	IRS147-025C	1	36	BR29	IRS147-035C	1	65	BR29	IRS147-047a	1
8	BR29	IRS147-026A	1	37	BR29	IRS147-036A	1	66	BR29	IRS147-047b	0
9	BR29	IRS147-026B	1	38	BR29	IRS147-036B	1	67	BR29	IRS147-047C	0
10	BR29	IRS147-026C	1	39	BR29	IRS147-037A	1	68	BR29	IRS147-048a	1
11	BR29	IRS147-027A	1	40	BR29	IRS147-038A	1	69	BR29	IRS147-048C	0
12	BR29	IRS147-027B	1	41	BR29	IRS147-038B	1	70	BR29	IRS147-049a	0
13	BR29	IRS147-027C	1	42	BR29	IRS147-038C	1	71	BR29	IRS147-050a	1
14	BR29	IRS147-028A	1	43	BR29	IRS147-039A	0	72	BR29	IRS147-051a	1
15	BR29	IRS147-028B	1	44	BR29	IRS147-039B	0	73	BR29	IRS147-052a	1
16	BR29	IRS147-029A	1	45	BR29	IRS147-040A	1	74	BR29	IRS147-053a	1
17	BR29	IRS147-029B	0	46	BR29	IRS147-040B	1	75	BR29	IRS147-054a	1
18	BR29	IRS147-029C	1	47	BR29	IRS147-040C	1	76	BR29	IRS147-055a	1
19	BR29	IRS147-030A	1	48	BR29	IRS147-041A	0	77	BR29	IRS147-056a	0
20	BR29	IRS147-030B	1	49	BR29	IRS147-041B	1	78	BR29	IRS147-057a	0
21	BR29	IRS147-030C	1	50	BR29	IRS147-041C	1	79	BR29	IRS147-058a	1
22	BR29	IRS147-031A	1	51	BR29	IRS147-042A	1	80	BR29	IRS147-059a	1
23	BR29	IRS147-031B	1	52	BR29	IRS147-042B	1	81	BR29	IRS147-060a	1
24	BR29	IRS147-031C	1	53	BR29	IRS147-043A	1	82	BR29	IRS147-061a	1
25	BR29	IRS147-032A	1	54	BR29	IRS147-043B	1	83	BR29	IRS147-062a	1
26	BR29	IRS147-032B	1	55	BR29	IRS147-043C	0	84	BR29	IRS147-063a	1
27	BR29	IRS147-032C	1	56	BR29	IRS147-044A	1	85	BR29	IRS147-064a	1
28	BR29	IRS147-033A	1	57	BR29	IRS147-044B	1				
29	BR29	IRS147-033B	0	58	BR29	IRS147-044C	1				

Figure 4.19 PCR detection for the presence of *Hph* gene in putative transformant of BR 28 (product size =0.8 KB). Detailed information is shown in Table 4.11.

Table 4.11 Results of PCR analysis for the presence of *Hph* marker gene of primary transformant of BR 28 .This Table is summary of Figure4.19.

Lane	Plant ID		GOI (HPT)	Lane	Plant ID		GOI (HPT)	Lane	Plant ID		GOI (HPT)	Lane	Plant ID		GOI (HPT)
1		1Kbladder	Ladder	33	BR28	IRS147-010C	1	65	BR28	IRS147-022B	1	98	BR28	IRS147-033C	1
2		IRS147	1	34	BR28	IRS147-011A	1	66	BR28	IRS147-023A	1	99	BR28	IRS147-034A	1
3		IR64_IRS147-1	1	35	BR28	IRS147-011B	1	67	BR28	IRS147-023B	1	100	BR28	IRS147-034B	0
4		Negative contro	0	36	BR28	IRS147-011C	1	69	BR28	IRS147-024A	0	101	BR28	IRS147-035A	1
5	BR28	IRS147-001A	1	37	BR28	IRS147-012A	1	70	BR28	IRS147-024B	0	102	BR28	IRS147-035B	0
6	BR28	IRS147-001B	1	38	BR28	IRS147-012B	1	71	BR28	IRS147-024C	1	103	BR28	IRS147-035A	0
7	BR28	IRS147-001C	1	39	BR28	IRS147-013A	1	72	BR28	IRS147-025A	0	104	BR28	IRS147-037a	0
8	BR28	IRS147-002A	1	40	BR28	IRS147-013B	1	73	BR28	IRS147-025B	0	105	BR28	IRS147-038a	0
9	BR28	IRS147-002B	1	41	BR28	IRS147-013C	1	74	BR28	IRS147-025C	0	106	BR28	IRS147-039a	1
10	BR28	IRS147-002C	1	42	BR28	IRS147-014A	1	75	BR28	IRS147-026A	0	107	BR28	IRS147-041a	1
11	BR28	IRS147-003A	1	43	BR28	IRS147-014B	1	76	BR28	IRS147-026B	0	108	BR28	IRS147-042a	1
12	BR28	IRS147-003B	1	44	BR28	IRS147-014C	1	77	BR28	IRS147-026C	0	109	BR28	IRS147-043a	1
13	BR28	IRS147-003C	1	45	BR28	IRS147-015A	0	78	BR28	IRS147-027A	1	110	BR28	IRS147-044a	0
14	BR28	IRS147-004A	1	46	BR28	IRS147-015B	0	79	BR28	IRS147-027B	1	111	BR28	IRS147-045a	1
15	BR28	IRS147-004B	1	47	BR28	IRS147-015A	1	80	BR28	IRS147-027C	1	112	BR28	IRS147-046a	1
16	BR28	IRS147-004C	1	48	BR28	IRS147-016B	1	81	BR28	IRS147-028A	1	113	BR28	IRS147-047a	1
17	BR28	IRS147-005A	1	49	BR28	IRS147-016C	1	82	BR28	IRS147-028B	1	114	BR28	IRS147-048a	1
18	BR28	IRS147-005B	1	50	BR28	IRS147-017A	1	83	BR28	IRS147-028C	1	115	BR28	IRS147-049a	1
19	BR28	IRS147-005C	1	51	BR28	IRS147-017B	0	84	BR28	IRS147-029A	1	116	BR28	IRS147-050a	1
20	BR28	IRS147-006A	0	52	BR28	IRS147-018A	1	85	BR28	IRS147-029B	1	117	BR28	IRS147-051a	1
21	BR28	IRS147-006B	0	53	BR28	IRS147-018B	1	86	BR28	IRS147-029C	1	118	BR28	IRS147-052a	1
22	BR28	IRS147-006C	0	54	BR28	IRS147-018C	1	87	BR28	IRS147-030A	0	119	BR28	IRS147-053a	1
23	BR28	IRS147-007A	1	55	BR28	IRS147-019A	1	88	BR28	IRS147-030B	0	120	BR28	IRS147-054a	0
24	BR28	IRS147-007B	1	56	BR28	IRS147-019B	1	89	BR28	IRS147-030C	0	121	BR28	IRS147-055a	1
25	BR28	IRS147-007C	1	57	BR28	IRS147-019C	1	90	BR28	IRS147-031A	1	122	BR28	IRS147-056a	1
26	BR28	IRS147-008A	1	58	BR28	IRS147-020A	1	91	BR28	IRS147-032A	1	123	BR28	IRS147-057a	1
27	BR28	IRS147-008B	1	59	BR28	IRS147-020B	1	92	BR28	IRS147-032B	1	124	BR28	IRS147-058a	1
28	BR28	IRS147-008C	0	60	BR28	IRS147-020C	1	93	BR28	IRS147-031A	1	125	BR28	IRS147-059a	1
29	BR28	IRS147-009A	1	61	BR28	IRS147-021A	1	94	BR28	IRS147-032A	1	126	BR28	IRS147-060a	1
30	BR28	IRS147-009B	1	62	BR28	IRS147-021B	1	95	BR28	IRS147-032B	1	127	BR28	IRS147-061a	0
31	BR28	IRS147-010A	1	63	BR28	IRS147-021C	1	96	BR28	IRS147-033A	1	128	BR28	IRS147-062a	1
32	BR28	IRS147-010B	0	64	BR28	IRS147-022A	1	97	BR28	IRS147-033B	1				

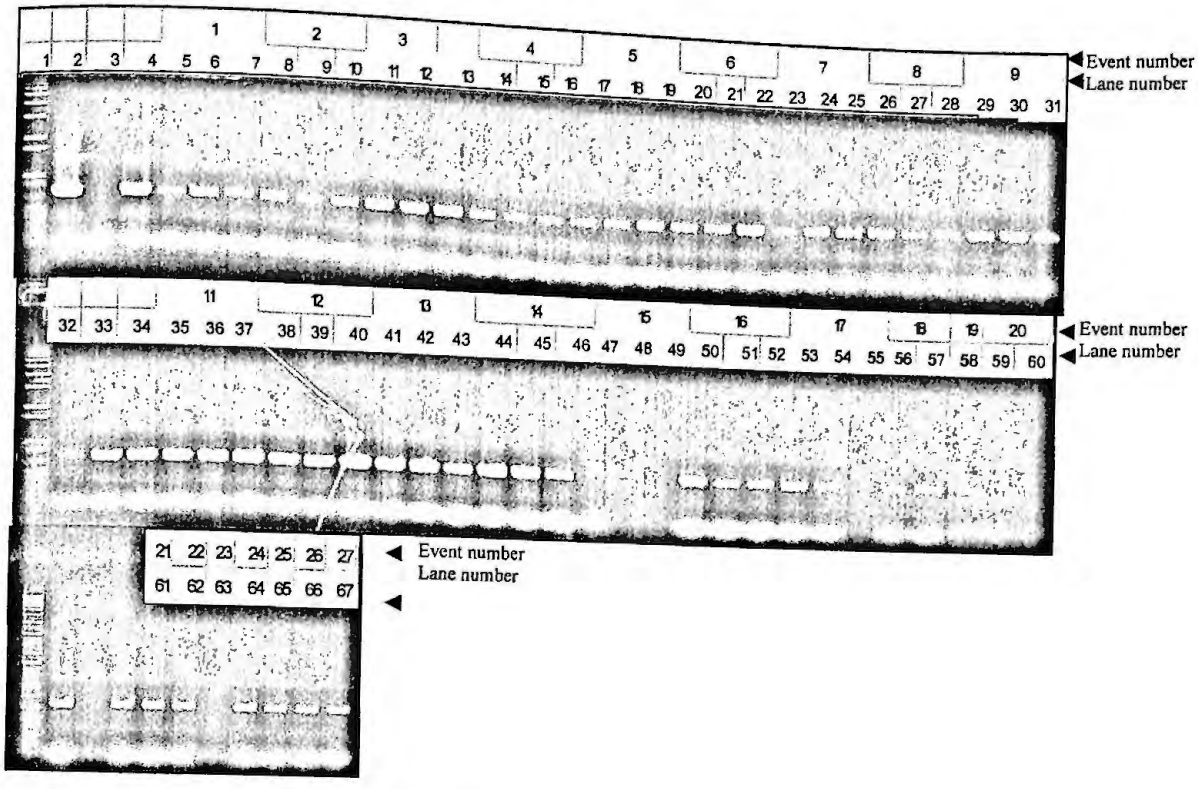


Figure 4.20 PCR detection for the presence of *Hph* gene in putative transformat of BR 11. Detailed information is shown in Table 4.12.

Table 4.12 Results of PCR analysis for the presence of *Hph* marker gene of primary transformant of BR 11 .This Table is summary of Figure 4.20.

Lane	Plant ID		GOI HPT	Lane	Plant ID		GOI HPT	Lane	Plant ID		GOI HPT
1		1Kb ladder	ladder	24	BR-11	IRS147-007B	1	47	BR-11	IRS147-015A	0
2		IRS147	1	25	BR-11	IRS147-007C	1	48	BR-11	IRS147-015B	0
3		Negetive control	0	26	BR-11	IRS147-008A	1	49	BR-11	IRS147-015C	0
4		IR64_IRS147-15	1	27	BR-11	IRS147-008B	1	50	BR-11	IRS147-016A	1
5	BR-11	IRS147-001A	1	28	BR-11	IRS147-008C	1	51	BR-11	IRS147-016B	1
6	BR-11	IRS147-001B	1	29	BR-11	IRS147-009A	1	52	BR-11	IRS147-016C	1
7	BR-11	IRS147-001C	0	30	BR-11	IRS147-009B	1	53	BR-11	IRS147-017A	1
8	BR-11	IRS147-002A	1	31	BR-11	IRS147-009C	1	54	BR-11	IRS147-017B	1
9	BR-11	IRS147-002B	0	32	BR-11	IRS147-010A	1	55	BR-11	IRS147-017C	0
10	BR-11	IRS147-002C	1	33	BR-11	IRS147-010B	1	56	BR-11	IRS147-018A	1
11	BR-11	IRS147-003A	1	34	BR-11	IRS147-010c	1	57	BR-11	IRS147-018B	1
12	BR-11	IRS147-003B	1	35	BR-11	IRS147-011A	1	58	BR-11	IRS147-019A	0
13	BR-11	IRS147-003C	1	36	BR-11	IRS147-011B	1	59	BR-11	IRS147-019B	0
14	BR-11	IRS147-004A	1	37	BR-11	IRS147-011C	1	60	BR-11	IRS147-020A	0
15	BR-11	IRS147-004B	1	38	BR-11	IRS147-012A	1	61	BR-11	IRS147-021A	1
16	BR-11	IRS147-004C	1	39	BR-11	IRS147-012B	1	62	BR-11	IRS147-022A	1
17	BR-11	IRS147-005A	1	40	BR-11	IRS147-012C	1	63	BR-11	IRS147-023A	0
18	BR-11	IRS147-005B	1	41	BR-11	IRS147-013A	1	64	BR-11	IRS147-024A	1
19	BR-11	IRS147-005C	1	42	BR-11	IRS147-013B	1	65	BR-11	IRS147-025A	1
20	BR-11	IRS147-006A	1	43	BR-11	IRS147-013C	1	66	BR-11	IRS147-026A	1
21	BR-11	IRS147-006B	1	44	BR-11	IRS147-014A	1	67	BR-11	IRS147-027A	1
22	BR-11	IRS147-006C	1	45	BR-11	IRS147-014B	1				
23	BR-11	IRS147-007A	1	46	BR-11	IRS147-014C	1				

4.2.3.2 Southern Blot Analysis of Well-Grown Transgenic Plants.

Primary confirmed plants were further confirmed by southern blot analysis. Genomic DNA (10-15ug) was digested with *EcoRI* (single cut).the results of southern blot analysis indicated the integration and copy number of transgene in T_0 plants.

For the preliminary experiments of transformation, 16 events were obtained. All plants were analyzed to compare copy number obtained from same callus derived plant .in most of the cases plants were obtained from same events showed same position and copy number of integration. Fig.4.21, Table 4.12 is the surmised results of primary southern blot analysis and copy number of the transgene No negative plant observed from PCR positive plants. A known plant carrying same foreign gene used as positive control and digested plasmid; IRS147 used as positive control (Figure 4.21, lane 33, 34)

For further experiments, one plant from one event was analyzed to confirm integration of transgene and copy number count. Primary transformants of BR 29, BR 28 and BR 11 confirmed by PCR analysis of *Hph* marker gene was subjected to southern blot hybridization. A single cut digestion method was used to detect copy number of integrated OsDREB1A transgene. For a suitable follow up of segregation pattern, transgenic plant carrying single copy of inserted gene of interest is important for inheritance analysis of gene of interest in next generation (T_1).

From 51 transgenic event of BR 28, 15 transgenic events were found to be single copy foreign gene insertion(Figure 23, Table 14) 7 and 5 from BR 29 and BR 11were found single copy gene insertion, respectively(Figure 22 and 24 ,Table 13 and 15).

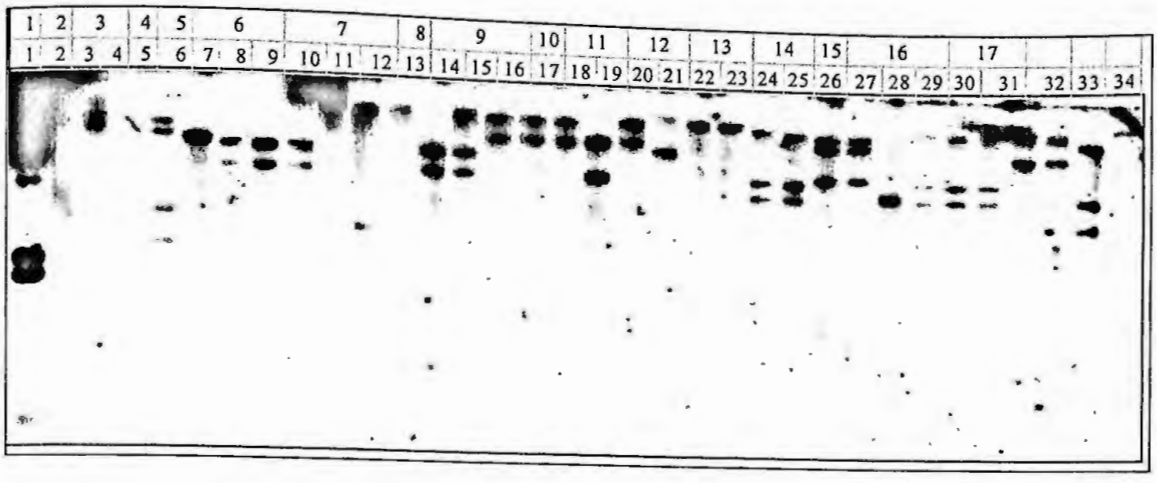


Figure 4.21 Southern blot analysis of BR 29 showing integration and copy number of OsDREB1A gene Detailed information is shown in Table 4.13.

Table 4.13 Result of southern blot analysis of primary positive BR 29 T_0 plants and copy number count. This Table is summary of Figure 4.21.

Lane No.	sample	Osdreb1a	copy no.	Lane No.	sample	Osdreb1a	copy no.
1	HIND III Lamda			18	BR29 -IRSI47-014c	1	2
2	neg control	0		19	BR29 -IRSI47-016b	1	2
3	BR29 -IRSI47-004b	1	3	20	BR29 -IRSI47-016c	1	2
4	BR29 -IRSI47-004c	1	1	21	BR29 -IRSI47-017a	1	1
5	BR29 -IRSI47-006c	1	4	22	BR29 -IRSI47-017b	1	1
6	BR29 -IRSI47-007a	1	1	23	BR29 -IRSI47-018a	1	3
7	BR29 -IRSI47-007b	1	2	24	BR29 -IRSI47-018c	1	3
8	BR29 -IRSI47-008a	1	2	25	BR29 -IRSI47-020a	1	3
9	BR29 -IRSI47-008b	1	2	26	BR29 -IRSI47-020b	1	3
10	BR29 -IRSI47-008c	1	2	27	BR29 -IRSI47-020c	1	2
11	BR29 -IRSI47-010a	1	2	28	BR29 -IRSI47-021a	1	3
12	BR29 -IRSI47-010b	1	1	29	BR29 -IRSI47-021b	1	3
13	BR29 -IRSI47-010c	1	2	30	BR29 -IRSI47-021c	1	3
14	BR29 -IRSI47-011a	1	3	31	BR29 -IRSI47-022a	1	3
15	BR29 -IRSI47-012a	1	2	32	BR29 -IRSI47-022b	1	3
16	BR29 -IRSI47-012b	1	2	33	IR64IRSI47016A	1	3
17	BR29 -IRSI47-012c	1	2	34	IRSI47	1	1

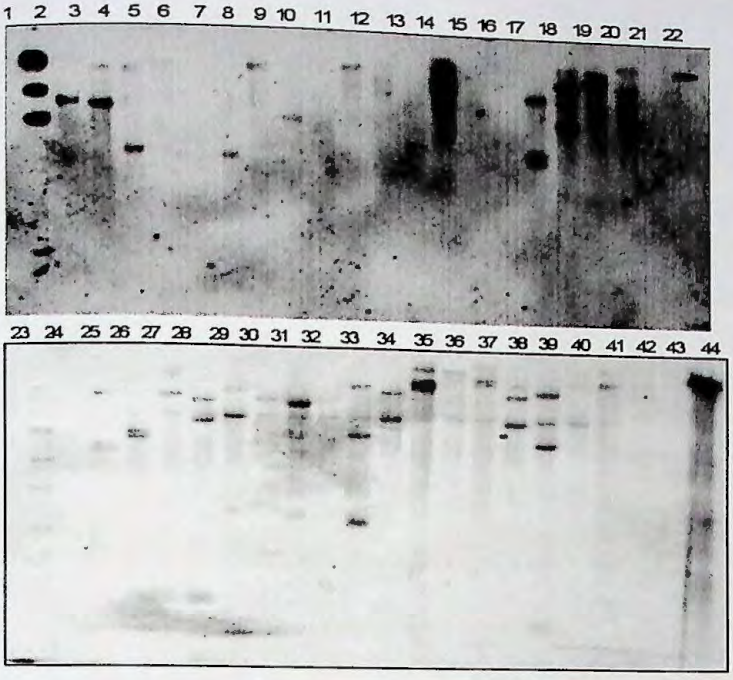


Figure 4.22 Southern blot analysis of BR 29 showing integration and copy number of OsDREB1A transgene Detailed information are shown in Table 4.14

Table 4.14 Result of southern blot analysis of primary positive BR 29 T_0 plants and copy number count. This Table is summary of Figure 4.22

Lane	Plant ID		Copy number	Lane	Plant ID		Copy number
1	Hindi iii lamda			23	ladder		
2	BR -29 IRS 147	26a	1	24	blank		
3	BR -29 IRS 147	27a	2	25	BR -29 IRS 147	25a	3
4	BR -29 IRS 147	28a	2	26	BR -29 IRS 147	31a	2
5	BR -29 IRS 147	29a	1	27	BR -29 IRS 147	36a	3
6	BR -29 IRS 147	31a	0	28	BR -29 IRS 147	42a	2
7	BR -29 IRS 147	32a	1	29	BR -29 IRS 147	44a	2
8	BR -29 IRS 147	33a	1	30	BR -29 IRS 147	45a	2
9	BR -29 IRS 147	34a	1	31	BR -29 IRS 147	50a	3
10	BR -29 IRS 147	36a	0	32	BR -29 IRS 147	51a	0
11	BR -29 IRS 147	37a	1	33	BR -29 IRS 147	52a	3
12	BR -29 IRS 147	40a	2	34	BR -29 IRS 147	54a	2
13	BR -29 IRS 147	42a	0	35	BR -29 IRS 147	55a	2
14	BR -29 IRS 147	43a	3	36	BR -29 IRS 147	58a	2
15	BR -29 IRS 147	44a	0	37	BR -29 IRS 147	60a	3
16	BR -29 IRS 147	45a	0	38	BR -29 IRS 147	61a	3
17	BR -29 IRS 147	46a	3	39	BR -29 IRS 147	62a	3
18	BR -29 IRS 147	47a	2	40	BR -29 IRS 147	63a	2
19	BR -29 IRS 147	48a	2	41	BR 29-IRS 147 22B	+contrl	2
20	IR 64_IRS147-116a		3	42	BR 29-IRS 147 22B	+ contrl	2
21	negative control		0	43	negative control	BR 29 -NT	0
22	plasmid	IRS 147	1	44	Plasmid	IRS 147	1

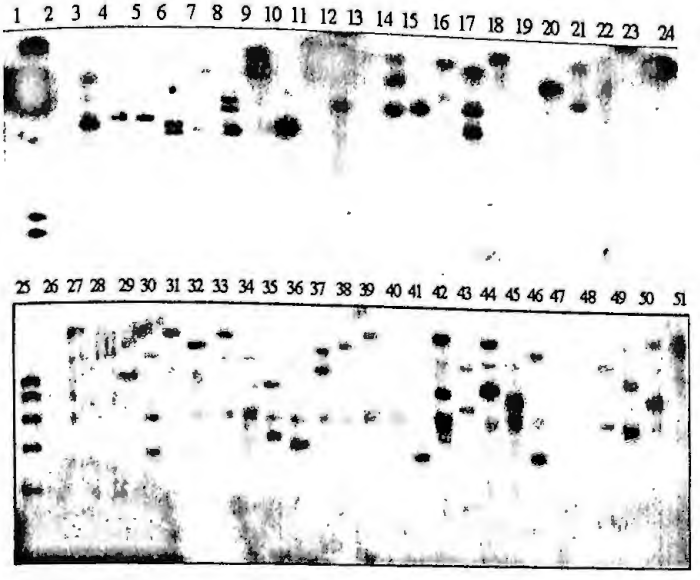


Figure 4.23 Southern blot analysis of BR 28 showing integration and copy number of OsDREB1A transgene Detailed information are shown in Table 4.15

Table 4.15 Result of southern blot analysis of primary positive BR 28 T_0 plants and copy number count. This Table is summary of Figure 4.23

Lane	Plant ID		Copy number	Lane	Plant ID		Copy number
	Hindi iii lamda			27	IR64-IRS-147	116A	3
2	negative control		0	28	BR28 -IRS147	27a	1
3	IR64_IRS147-	016A	3	29	BR28 -IRS147	30a	1
4	BR28 -IRS147	001a	1	30	BR28 -IRS147	33a	3
5	BR28 -IRS147	002a	2	31	BR28 -IRS147	35a	1
6	BR28 -IRS147	003a	2	32	BR28 -IRS147	37a	1
7	BR28 -IRS147	004a	2	33	BR28 -IRS147	39a	1
8	BR28 -IRS147	007a	4	34	BR28 -IRS147	41a	2
9	BR28 -IRS147	010a	2	35	BR28 -IRS147	42a	2
10	BR28 -IRS147	013a	1	36	BR28 -IRS147	43a	2
11	BR28 -IRS147	014a	2	37	BR28 -IRS147	45a	1
12	BR28 -IRS147	016a	3	38	BR28 -IRS147	46a	2
13	BR28 -IRS147	017a	1	39	BR28 -IRS147	47a	0
14	BR28 -IRS147	018a	3	40	BR28 -IRS147	48a	1
15	BR28 -IRS147	020a	1	41	BR28 -IRS147	49a	4
16	BR28 -IRS147	021a	2	42	BR28 -IRS147	50a	2
17	BR28 -IRS147	022a	3	43	BR28 -IRS147	51a	4
18	BR28 -IRS147	024a	1	44	BR28 -IRS147	52a	3
19	BR28 -IRS147	027a	0	45	BR28 -IRS147	53a	3
	BR28 -IRS147	028a	1	46	BR28 -IRS147	55a	0
21	BR28 -IRS147	029a	3	47	BR28 -IRS147	56a	1
22	BR28 -IRS147	030a	2	48	BR28 -IRS147	58a	2
23	BR28 -IRS147	032a	0	49	BR28 -IRS147	59a	3
24	plasmid	IRS147	1	50	BR28 -IRS147	61a	3
25	ladder			51	plasmid	IRS147	1
26	negative control		0				

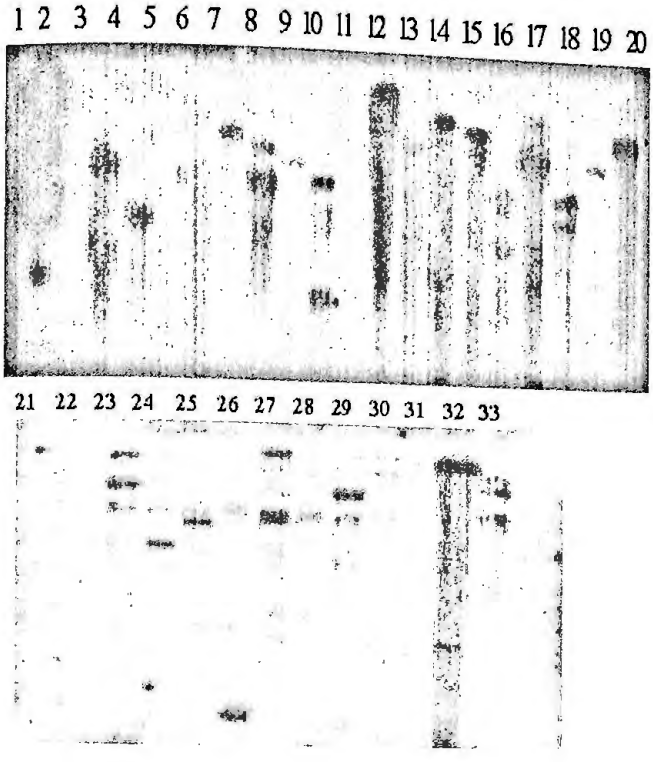


Figure 4.24 Southern blot analysis of BR 11 showing integration and copy number of OsDREB1A gene. Detailed information shown in Table 4.16

Table 4.16 Result of southern blot analysis of primary positive BR 11 T_0 plants and copy number count. This Table is summary of Figure 4.24

Lane	Plant ID	copy number	Lane	Plant ID	copy number
1	Hindi iii lamda		18	BR11 -IRS147-0018a	1
2	negative control	0	19	IRS147	1
3	IR64_IRS147-16A	3	20	Hindi iii lamda	
4	BR11 -IRS147-001a	1	21	neg control	0
5	BR11 -IRS147-002a	0	22	IR64 -IRS147-16 A	3
6	BR11 -IRS147-003a	0	23	BR11 -IRS147-002a	2
7	BR11 -IRS147-004a	1	24	BR11 -IRS147-003a	1
8	BR11 -IRS147-006a	2	25	BR11 -IRS147-009a	2
9	BR11 -IRS147-007a	1	26	BR11 -IRS147-0021a	3
10	BR11 -IRS147-008a	3	27	BR11 -IRS147-0022a	2
11	BR11 -IRS147-009a	0	28	BR11 -IRS147-0023a	2
12	BR11 -IRS147-010a	2	29	BR11 -IRS147-0024a	2
13	BR11 -IRS147-011a	3	30	BR11 -IRS147-0025a	0
14	BR11 -IRS147-012a	2	31	plasmid IRS147	1
15	BR11 -IRS147-013a	3	32	BR11 -IRS147-0026a	3
16	BR11 -IRS147-014a	2	33	BR11 -IRS147-0027a	1
17	BR11 -IRS147-017a	2			

4.2.4 Inheritance and expression of transgene in T_1 Generation

To investigate the inheritance pattern of the OsDREB gene, T_1 progenies seeds from 3 primary transformants from each variety (single copy, confirmed by southern blot analysis) were chosen and grown in the green house and were used for molecular analysis. Detailed PCR analysis was done for the detection of the presence of the gene using appropriate primer set (forward primer chosen from Nos terminator, Table 3.2). The PCR results are summarized in Table 4.16. The results of PCR analysis confirmed the stable integration of gene by the amplification of the DNA segment starting from gene of interest to NOS terminator sequences. The OsDREB 1A gene segregated in the T_1 progenies following a normal Mendelian fashion, and showed a monogenic ration of 3:1 (Table 4.17).

Table 4.17 Presence of transgene (OsDREB 1A) in T1 progeny of BR 29, BR 28 and BR 11 transgenic plant.

Line ID	Total Analyzed T_1 plants	Number of PCR +ve T_1 plants	Number of PCR -ve T_1 plants	% of PCR +ve T_1 plants	% of PCR -ve T_1 plants	Expected segregation ratio	χ^2 value (df=1)
BR29-026A-T1	158	117	41	117	25.94	3:1	3.00^{ns}
BR29-033A-T1	156	116	40	116	25.64	3:1	0.03^{ns}
BR29-037A-T1	155	122	33	122	21.29	3:1	0.56^{ns}
BR28-039A-T1	155	111	44	111	28.39	3:1	0.971^{ns}
BR28-020A-T1	181	125	56	125	30.94	3:1	3.40^{ns}
BR28-056A-T1	143	116	27	116	18.88	3:1	2.85^{ns}
BR 11 -IRS147-12A	64	39	25	39	39.06	3:1	4.92^{ns}
BR 11-IRS147-021A	169	121	48	121	28.40	3:1	1.04^{ns}
BR 11-IRS147-041A	147	102	42	95	35.37	3:1	1.35^{ns}

df=degree of freedom, ns=non significant at p=0.05

Figure 4.25 Drought stressed transgenic (T_1) plants of BR -29.
(A) Transgenic line) Non-transgenic line

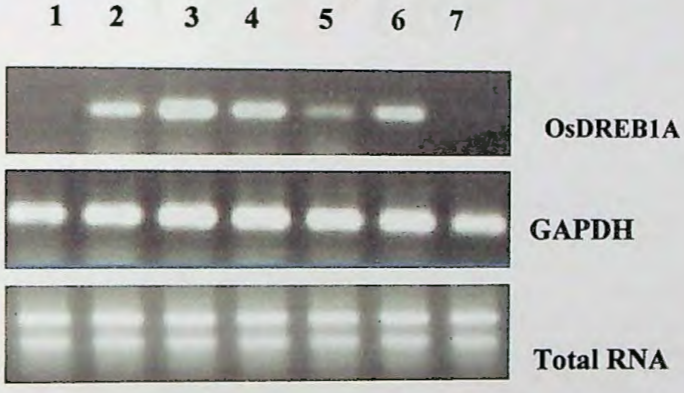


Figure 4. 26 Expression of transgene (OsDREB1A) in flag leaf of transgenic (T_1progeny) of 1) BR29 RS147-026A 2) BR29- RS147033A 3) BR28- RS147039A 4) BR28- RS147020A 5) BR 11-IRS147-041A 6) BR 11-IRS147-021A 7) Non-transgenic BR 29 plant (control)

Transgenic plan (T_1) grown normally in green house condition a given certain level of drought stress. Transgenic plants showed better tolerance to drought than non-transgenic plant (Figure 4.25). Two plants of each variety containing single copy inserted gene used to see the gene expression of transgene analyzed by RT PCR method. Except one, all inserted OsDREB1A gene expressed in all analyzed plants (Figure 4.26)

CHAPTER 5
DISCUSSION

DISCUSSION

The drought tolerance of plants, a complex property controlled by the multiple factors, is mainly genetically determined and simultaneously affected by environment. There are many functional genes and regulatory genes (e.g. transcriptional factors) regulating the plant response to drought stress, however, their expression levels are very low under normal conditions, therefore, the gene expression regulation system plays a vital role in drought tolerance. Compared with the transformation of single functional gene, the transformation of transcription activator is a more effective way to improve the drought tolerance in plants.

The dehydration responsive element binding proteins (DREB) are important transcription factors that induced a set of abiotic stress-related genes and impart stress endurance to plants. The DREB transcription factors could be dichotomized as DREB1 and DREB2, which are involved in two signal transduction pathways under low temperature and dehydration. They belong to the ERF (Ethylene Responsive Element Binding Factors) family of transcription factors. ERF proteins are a sub-family of the APETLA2 (AP2)/ethylene responsive element binding protein (EREBP) transcription factors that is distinctive to plants.

In the present investigation, *Oryza sativa* DREB genes (OsDREB) characterized for identification of drought responsive gene(s) in indica rice genome i.e. BR 29, BR 28, and BR 11. The bioinformatics of the 14 members of OsDREB family showed consensus similarity. Yamaguchi-Shinozaki and Shinozaki (2003) listed 13 DREB genes in the genome of the rice cv. Nipponbare: The 13 genes are as follows; **OsDREB1A**, OsDREB1B, OsDREB1C, OsDREB1D, OsDREB1E, OsDREB1F, OsDREB1G, OsDREB1H, **OsDREB1I**, OsDREB2A, OsDREB2B, OsDREB2C and OsDREB2D. Among them two genes, OsDREB1A and OsDREB1I, are found on the same BAC clone, along with a third gene was identified by bioinformatics, which was not mentioned by previous authors (Yamaguchi-Shinozaki and Shinozaki 1994, Shinozaki et al., 2003, Dubouzet et al., 2003). It was named **OsDREB1J** according to its predicted phylogenetic relationship and included it this study. The expression patterns of the total 14 genes under drought stress condition were analyzed in reproductive tissues by RT PCR method. Interesting expression pattern was observed under drought stress and

expression mottled among different genotypes and tissues. The relative water content of the BR29 flag leaf blade declined gradually during drought-stress and recovered after re-watering at the same time, the expression patterns of the 14 genes changed more markedly in the stressed tissues than in the well-watered tissues. Some genes responded to drought at a higher RWC than others did. The decline in RWC was accompanied by a decline in peduncle elongation rate. By contrast, the pattern of OsDREB expression differed in all three genotypes. All three genotypes expressed OsDREB1B, OsDREB1C, OsDREB1H, OsDREB1I and OsDREB2A and did not express OsDREB1D, OsDREB1E and OsDREB2D. The differences were (i) only BR11 expressed OsDREB1F and OsDREB2C, (ii) only BR11 did not express OsDREB1G and OsDREB1J and (iii) only BR28 did not express OsDREB1A and OsDREB2B. DREB2A reported to function mainly in ABA-independent water stress–inducible gene expression, since expression of DREB2A is strongly induced by drought and high-salinity stresses but not by ABA treatment (Liu et al., 1998).

In this study, the gene expression of peduncle tissue was compared with its growth rate under same condition. Interesting correlation was observed in BR 29 expression of OsDREB1A with the elongation rate of peduncle, suggesting the positive role of OsDREB1A in reproductive tissue development. In drought stressed BR 29 Peduncle expressions of OsDREB1A declines at the point of drought stress when elongation rate declines and this elongation rate declines in drought stress plant and results incomplete panicle exertion as well as reduced seed fertility. Certainly, more experiments should be carried out to investigate the expression patterns of OsDREB genes in the transgenic rice under drought stress.

This correlation confers the fact that the over-expression of OsDREB1A may play an important role for peduncle elongation during drought stress and help to avoid yield loss due to certain drought stress at reproductive stage. Kasuga et al., 1999; Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004 also reported the enhanced drought tolerance by over-expressing OsDREB1A gene in monocot and dicot plants. Yamaguchi-Shinozaki and Shinozaki, (1994) and Dubouzet et al., (2003), generated transgenic rice over-expressing various OsDREB proteins in combination with several promoters and suggested that OsDREB1A could be very useful for producing transgenic

dicot and monocot plants with have higher tolerant potentiality to drought, salt and/or cold stresses. Hence, OsDREB1A was chosen as candidate gene of genome engineering to enhance drought stress in the studied indica cultivars. Kasuga et al., (1999), describe the possible mechanism of drought tolarency by DREB1A. In brief, DREB1A binds to the *cis*-acting DRE and regulates the expression of many stress inducible genes under drought, salt, and cold stress in *Arabidopsis* (Liu et al., 1998). The expression of the transferred DREB1A gene and its stress inducible target genes were correlated in the transgenic plants under control conditions (Kasuga et al., 1999)

Gene transformation in Rice is efficiently mediated either through Agrobacterium or particle bombardment method. In 1994 in a landmark report, Hiei et al. (1994) provided unequivocal evidence for stable transformation of Japonica rice with Agrobacterium after molecular and genetic analysis of large numbers of R_0, R_1, and R_2 progenies. This report opened the possibility of using Agrobacterium for genetic transformation of recalcitrant cereals plants. A super binary vector in the Agrobacterium strain LBA4404 was demonstrated to be the most effective for transformation of all three-japonica cultivars tested. Agrobacterium-mediated transformation of rice has now emerged as a reliable and highly reproducible method for transferring genes of interest into the rice genome. The success of Hiei and colleagues ignited a significant interest in transforming other argonomically important crop species, such as maize, barley, and wheat. Using an approach similar to the one developed by Hiei and colleagues, maize transformation was accomplished with freshly isolated immature embryos (Ishida et al. 1996). In maize, transformation frequency was further improved with the addition of silver nitrate in the medium, modification of medium components, and optimization of co-culture and resting timing periods. For the first time in 2002, it was demonstrated that maize can be transformed by using a combination of standard binary vector and the antioxidant cysteine in the co-culture medium. Since the first report of Agrobacterium-mediated transformation of wheat in 1997, various factors that influence T-DNA delivery have been further investigated and modified (Jones et al. 2005). Following the success of Tingay and colleagues (Tingay et al. 1997), in Agrobacterium-mediated transformation of barley, a number of laboratories around the world reported the successful production of transgenic barley plants (Shrawat and Lörz, 2006). Therefore, optimization of parameters that are considered crucial for cereal transformation and screening of highly

competent explants and genotypes should broaden the scope for the genetic transformation with genes of interest.

In the present investigation, three widely cultivated Bangladeshi indica rice cultivars viz. BR 29, BR 28, and BR 11 were used for protocol optimization of genetic transformation and OsDREB 1A gene successfully transformed by Agrobacterium-mediated transformation method. Transgenic rice plants were regenerated that expressing OsDREB1A gene under the control of stress inducible Lip-9 promoter. The Lip -9 promoter used to express the OsDREB1A transformation, which have two distinctive advantages in the experimental system. One is that the gene originated from rice. Another advantage of this promoter is the ability to induce high level of expression under stressed condition. In earlier studies, constitutive over-expression of these factors reported to cause aberrant phenotype, stunted growth, and sterility so these genes need to be expressed under regulated/inducible promoter (Shen et al., 2003). In this present study, another plasmid was used where OsDREB1A driven by Ubiquitine (Ubi-1) promoter for standardization of gene transformation efficiency, but absolute sterility was observed in obtained transgenic lines. Putative transformed plant obtained using Lip-9: OsDREB1A (IRS147) was showed normal growth and fertility.

A total number of 117 primary transgenic independent events were produced from the three studied cultivars. PCR and southern blot analysis of the putative transformed plants confirmed the stable integration of OsDREB1A and HPT genes in the indica rice genomes. Genomic DNA digested with *EcoRI* and hybridized with the OsDREB1A probe revealed the copy number of inserted gene in each of the transformant, suggesting an independent nature of transgene integration in primary transformants. The phenotypic expression of the putative transformed plants were planted in to the green house and grown healthy, normal flowering and produced fertile seeds.

To confirm the definite nature of primary transformed plant, the inheritance pattern of the transgenes were analyzed in T_1 generation. In this segment of experiment, the plants showed single insertion of the gene of interest were selected from each studied cultivar for easier analysis of segregation pattern. The PCR of OsDREB1A (specific for construct sequence) indicated the transmission of the gene in the next generation with a

mendelian fashion. Segregation analysis of T_1 generation clearly showed a monogenic ratio 3:1 for OsDREB1A gene, suggesting that genes were stably integrated into the genome.

Over expression of DREB1A gene in various transgenic plant has been reported earlier (Kasuga et al., 1999; Seki et al., 2001; Fowler and Thomashow, 2002; Maruyama et al., 2004). They reported the stress-regulated expression of the DREB1A gene by the rd29A promoter produced plants with increased tolerance to freezing, salt, and drought stresses, without a drastic change in the normal phenotype of the transformed plants. In the present investigation, the results of mRNA expression by RT PCR analysis of T_1 plant revealed the successful function of OsDREB transgene amplifying specific fraction of sequence along with a part of Nos terminator sequences. Transgenic plant of T_1 generation also showed better tolerance to drought stress under green house condition than non-transgenic plants, suggesting over-expression of OsDREB1A a possible way to acquire enhanced drought tolerance similarly, over-expression of a rice gene, 35S:OsDREB1A, in transgenic *Arabidopsis* also increased drought tolerance (Dubouzet et al., 2003).

The stable inheritance and expression of foreign genes are of critical importance in the application of genetically engineered cereals to agriculture. Without any doubt, the problem of transgene silencing raises serious concerns regarding selection of transgenic lines for crop improvement with specific trait(s). Therefore, it now appears imperative that transgenic lines carrying gene(s) of economic importance need to be carefully tested for gene expression levels over many generations.

CHAPTER 6
SUMMARY

SUMMARY

The recent completion of the sequencing of the rice genome has opened up new avenues for gene regulation and transformation in rice. It has been debated that information gained from rice genome by utilizing ancestral system among the cereals would help genetically improve not only rice but also other cereals such as maize, wheat, and barley, which have significantly large genomes. It has been estimated that abiotic stresses such as drought, salinity, extreme temperatures, submergence and oxidative stress reduce crop yield worldwide by more than 50%. These stresses are often interconnected, cause similar cellular damage and activate similar cell-signalling pathways . The dehydration responsive element binding proteins (DREB) are important transcription factors that induce a set of abiotic stress-related genes and impart stress endurance to plants. Understanding of the stress response mechanism may facilitate candidate genes discovery and increased levels of stress tolerance can be engineered into plants by reprogramming the expression of endogenous genes.

In the present study,RT PCR method used for characterization of OsDREB genes for identification of appropriate drought stress responsive candidate gene(s). Three Bangladeshi indica rice cultivars BR 29, BR28, and BR 11 used to identify expression pattern of 14 members of OsDREB gene family under drought stress condition. The RT PCR results showed up and down regulation of genes due to drought stress. The expression pattern differs among genotypes and tissues. the physiological parameter of drought stress also compared with gene expression pattern. The results revealed drought responsiveness of several genes OsDREB1A, OsDREB 1F, OsDREB 1G, OsDREB 2A etc. among them, OsDREB 2A showed most significant correlation. Because of the previous reports of growth retardation of transgenic plant by transferring OsDREB 2A gene, the gene was not considered good candidate gene and OsDREB1A was selected as desired candidate gene for enhanced drought tolerance in the studied rice varieties.

How ever, the same cultivars used to optimize agrobacterium mediated gene transformation protocol. Gene transformation efficiency of mature and immature embryo

investigated for the studied cultivars. In addition, OsDREB 1A gene was introduced first time in the studied cultivar under the control of stress inducible Lip-9 promoter.

A number 138 putative primary Transgenes (T_0) regenerated after co cultivation and calli selection. The presence of the selectable marker gene gene was examined by PCR analysis. The results showed that, out of 138 events 117 T_0 events were positive for selectable marker gene. Further conformation was done by southern blot analysis using OSDREB1a gene as probe. The results of southern revealed the integration and copy number of OSDREB gene in the genome of primary transgenic plants.

In order to determine the stability and inheritance patterns of OsDREB 1A gene PCR was done with specific primer for the construct sequence .the results of PCR analysis of OsDREB1A gene revealed the transgene stability and inheritance patterns of OSDREB1A gene. Inheritance of the transgene in most of the progeny line followed the classical mendalian segragationratio3:1, which indicating mendalian inheritance as a single dominant locus.

Phenotypic evaluation under drought stress done for t1 plants and plant shoed better tolerance comparing with non-transgenic plant under certain drought stress level. The expression of transgene was also analysed by RT PCR method. The RT PCR results showed successful expression of transgene in transgenic plants. The results suggested that the expression of OsDREB 1A under control of Lip-9 promoter effectively enhanced drought stress in rice plant.

The OsDREB gene family characterization and genome engeeniaring with OsDREB1A to indica rice cultivars, which resulted better drought stress tolerance in rice plant. This finding suggested that the rice line with enhance capacity of drought tolerance by genome engendering may contribute to solve the uneven drought stress related yield loss problem of the population that consumes rice as a major staple food.

CHAPTER 7
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REFERENCES

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ABBREVIATIONS

BAP	:	6-benzylaminopirine
NAA	:	α-naphthelene acetic acid
0.1N	:	0.1 Normal
2,4-D	:	2,4-dichlorophenoxy acetic acid
BRRI	:	Bangladesh Rice Research Institute
bp	:	Base Pair
cm	:	Centimeter
DREB	:	Dehydration responsive binding protein
et al.	:	et alli and the rest
etc.	:	et cetra, and the rest
e.g.	:	Exempli gratia, for example
ESTs	:	Expressed sequence taq
Fig./s	:	Figure/Figures
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
gm	:	Gram (s)
Hr(s)	:	Hour (s)
HCl	:	Hydrochloric Acid
hph	:	Hygromycin Phosphotransferase
i.e.	:	id est-which to say in other words
IBA	:	indole-3-acetic acid
KIN	:	Kinetin (6- furfural amino purine)
$HgCl_2$	:	Marcuric Chloride
mRNA	:	Messenger Ribonucleic Acid
m	:	Meter
µM	:	Micro mole
µg	:	microgram
µ	:	Micron
mg	:	Milligram
mg/l	:	Milligram/litre
ml	:	Milliliter
min (s)	:	Minutes (s)
MS	:	Murashige and Skoog (1962) medium
viz	:	Namely
pH	:	Negative logarithm of hydrogen
No.	:	Number
%	:	Percentage
PCR	:	Polymorphic Chain Reaction
RT PCR	:	Revarse Transcription Polymorphic Chain Reaction
rRNA	:	Ribozomal Ribonucleic Acid
Sec.	:	Second (s)
SDS	:	Sodium Dodeccyle Sulphate
NaOH	:	Sodium hydroxide
Na_2-EDTA	:	Sodium Salt of Ferric Ethylene Diamine Tetra Acetate
Taq	:	Thermophilus Aquaticus DNA-Dependent Polymarase
TAE	:	Tris HCL/EDTA buffer
v/v	:	Volume by volume
cDNA	:	Complementary Deoxy Ribo Nucljeic Acid.