Evaluation of the dehydration-responsive element (DRE) – binding proteins (DREBs) technology in rice under water limited conditions

TOMOKO SAKAI

7206507

UNIVERSIDAD NACIONAL DE COLOMBIA

FACULTAD DE AGRONOMÍA

ESCUELA DE POSGRADO

PALMIRA

2009

Evaluation of the dehydration-responsive element (DRE) – binding proteins (DREBs) technology in rice under water limited conditions

TOMOKO SAKAI

7206507

Trabajo de grado para optar al título de Magister en CIENCIAS AGRARIAS, ÁREA DE ÉNFASIS EN FITOMEJORAMIENTO

DIRIGIDO POR:

César P. Martínez, Ph.D.

Centro Internacional de Agricultura Tropical – CIAT

Franco Alirio Vallejo C, Ph.D.

Universidad Nacional de Colombia sede Palmira

Zaida Lentini, Ph.D.

Universidad ICESI

UNIVERSIDAD NACIONAL DE COLOMBIA

FACULTAD DE AGRONOMÍA

ESCUELA DE POSGRADO

PALMIRA

2009

Nota de aceptación:

_____

_____

_____

_____

_____

(Firma del presidente del jurado)

_____

(Firma del jurado)

_____

(Firma del jurado)

Palmira, 29 Mayo de 2009

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Manabu Ishitani and all members of his team; Lucia Chávez, Andres Salcedo, Lorena López, Jorge Pachón, “Don” Martín Caña, Eliana Macea, Dr. Jagadish Rane, Eduardo Hernández, Campo Elias Manríque, Aflanio Benavides, Blanca Celón, Hugo Jaimes, Dr. Yoshimi Umemura, María Eugenia Recio, María del Carmen Hernández, Danilo Moreta, and Martín Emilio Rodriguez, for encouragement, guidance and suggestions.

I greatly acknowledge Dr. Zaida Lentini and colleagues of the Anther lab. at Centro Internacional de Agricultura Tropical (CIAT); Eddie Tabares, Gerardo Delgado, Manuel Quintero, Carlos Ordoñez, Tomas Agrono, Carlos Cruz, Eugenio Bolaños, for valuable advice, professional technical support and friendship.

Thanks to Myriam Cristina Duque, James Silva and Juan B. Cuasquer, for their helping with the statistical analysis.

Dr. César P. Martínez, Dr. Hernán Ceballos, and Dr. Franco Alirio Vallejo, I’m deeply grateful for their excellent supervision.

During these three years, I was kindly supported with a homely help by the Biotechnology unit at CIAT and a lot of “amigos” in CIAT, I’m grateful for their warm encouragements. Also I would like to express my gratitude to Universidad Nacional de Colombia sede Palmira and CIAT, which made this work possible.

I sincerely express my special thanks to Dr. Takumasa Kondo (Corpoica), for critical review of this manuscript.

Thanks to the Instituto Colombiano de Crédito Educativo y Estudios Técnicos en el Exterior (ICETEX), Colombia, for their aid to the postgraduate study program at Universidad Nacional, sede Palmira and for the financial support of this study.

La facultad y los jurados de tesis no se harán responsables de las ideas emitidas por el autor.

Articulo 24, resolución 04 de 1974

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ANNEX

SUMMARY

Reducing water consumption in crop production is now been generally recognized as an essential strategy for eco-efficient agriculture to meet the global shortage of water. Developing transgenic rice lines containing the DREB gene and evaluating grain yield and yield components under water-limited conditions is here considered as a fast and effective plant breeding strategy to develop drought-tolerant rice varieties in Latin America.

Candidate rice genotypes for genetic transformation were evaluated and selected. Curinga and CT6241 were selected based on their good performance under water-limited conditions. CICA8 and Palmar were also chosen as drought intolerant genotypes for further study.

A high-speed transformation protocol optimized for Nipponbare was tested to speed up the transformation process for selected rice genotypes. Transformed rice plants were obtained, and some critical details were identified for plant regeneration. Gene copy number and rearrangements in the transformed plant should be also considered to establish an effective transformation protocol.

The transgenic plants I-P-A-43, III-P-A-70-5 and VII-P-A-107-3 performed similarly as non-transgenic CT6241 under water-limited conditions at biosafety greenhouse. Performances of the three transgenic events were considered as promising DREB transgenic rice lines for future studies. On the other hand, to determine the relationship between field capacity and gene expression for DREB transgenic rice lines, future studies in this area are required for rice improvement in Latin America.

RESUMEN

Reducir el consumo de agua para la producción de productos agronómicos es reconocido generalmente como una estrategia esencial para mejorar la agricultura ante la escasez mundial de agua. La creación de líneas de arroz transgénico que contienen el gen DREB y la evaluación del rendimiento y componentes del rendimiento bajo condiciones limitadas de agua, se considera como una rápida y efectiva estrategia de fitomejoramiento, para desarrollar variedades de arroz que sean tolerantes a la sequía en América Latina.

Se evaluaron y seleccionaron genotipos de arroz candidatos para la transformación genética; Curinga y CT6241 fueron seleccionados por su buen desempeño bajo condiciones limitadas de agua. Las variedades CICA8 y Palmar también fueron seleccionadas como genotipos intolerantes a la sequía para futuros estudios.

Un protocolo de transformación de alta rapidez, optimizado para la transformación de la variedad de arroz Nipponbare, fue probado para disminuir el tiempo del proceso de transformación de algunos genotipos de arroz seleccionados. Plantas transformadas de arroz se obtuvieron, y algunos detalles críticos se identificaron para la regeneración de plantas transgénicas. Número de copias de genes y rearreglos genéticos en la planta transformada también se deben considerar para establecer un protocolo de transformación efectivo.

Lineas homozygotas derivadas de las plantas transgénicas I-P-A-43-3, III-P-A-70-5 y VII-P-A-107-3 respondieron de manera similar a la linea no transgénica CT6241, bajo condiciones de agua limitada en un invernadero de bioseguridad. Los tres eventos transgénicos mostraron características evaluadas que se consideraron como lineas promisorias de arroz transgénico DREB para estudios futuros. Por otro lado, para aclarar la relación entre la capacidad de campo y expresión de génes DREB en líneas transgénicas, se requieren más estudios en esta área para el mejoramiento de arroz en América Latina.

1. INTRODUCTION

Water deficit, more commonly referred to as 'drought', has been, and continues to be the most limiting factor affecting food production, especially in areas with inadequate agriculture water resources (Pantuwan *et al*. 2002; Lanceras *et al*. 2004; Yue *et al*. 2005; Xiao *et al*. 2008). Therefore, with the global shortage of water, reducing water consumption in crop production has now been generally recognized as an essential strategy for sustainable agriculture (Xiao *et al*. 2008).

Rice is one of the world's most important staple foods. Rice grain yield and yield components have been known to be highly influenced by water supply. There are numerous studies about drought tolerance in rice. Use of yield as an index for adaptation to drought stress in rice (Garrity and O'Toole 1994; Atlin 2001) may be considered as a reasonable approach, as grain yield is a major attribute of interest in most plant breeding programs (Pantuwan *et al*. 2004). However, drought tolerance is a complex trait that involves various aspects of developmental, physiological, biochemical, and molecular adjustments.

Plants respond to conditions of severe environmental changes or stresses (Mansfield 1987). Drought or high-salt conditions induce dehydration of plant cells, which may trigger physiological and biochemical responses against such stresses (Yamaguchi-Shinozaki and Shinozaki 1994), and a number of genes have been demonstrated to be important for tolerance to environmental stress in many plants (Ingram and Bartels 1996; Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000; Rabbani *et al*. 2003). The products of these genes are althought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in response to stress (Xiong *et al*. 2002; Shinozaki *et al*. 2003).

Yamaguchi-Shinozaki and Shinozaki (1994) reported that 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*. DREB transcription factors have also

been identified in *Brassica napus*, wheat, rye, tomato and rice, and all of them showed a good response to cold stress (Jaglo *et al*. 2001; Dubouzet *et al*. 2003). To overcome environmental limitations and improve crop yield under stress conditions, it is important to improve stress tolerance in crops (Shinozaki and Yamaguchi-Shinozaki 2000; Rabbani *et al*. 2003; Ito *et al*. 2006).

In recent years, plant transformation studies using *Agrobacterium tumefaciens* have been well recognized as one of the plant breeding methods not only in dicotyledonous plants, but also in monocotyledonous plants, such as rice. The rice (*Oryza sativa* cv. Nipponbare) genome has been sequenced and its relationships to other closely related important crops are being studied (International Rice Genome Sequencing Project 2005). An efficient *Agrobacterium*-mediated transformation system in the *Japonica* subspecies of rice established by Hiei *et al*. (1994) has greatly facilitated the application of this technology. Recently, Toki *et al*. (2006) reported a high-speed transformation system for rice of the *Japonica* cultivar. Most of the genetic transformation studies have been conducted on *Japonica* subspecies and not many on the *Indica* subspecies. Nevertheless more than 90% of the world rice supply comes from *Indica* varieties (Boriss 2006). Transformation efficiency factors in *Indica* and *Tropical Japonica* type of rice such as callus induction, antibiotic sensibility, and plant regeneration are highly dependent on the genotype; on the other hand, there has been very few transformation studies conducted on rice in Latin America. Consequently, more efficient and quick transformation protocols for *Indica* and *Tropical Japonica* varieties grown in Latin America are urgently needed.

Creating transgenic rice lines containing the DREB gene and evaluating the grain yield and yield components under water-limited conditions is here considered as a fast and effective plant breeding strategy to improve drought tolerant rice varieties in Latin America.

2. OBJECTIVES

2.1. Goal:

Evaluation of DREB gene in transgenic rice under water-limited conditions

2.2. Specific goals:

2.2.1. Establishment of drought screening protocols for rice in the field and screening of candidate rice genotypes for genetic transformation

2.2.2. Rice genetic transformation

2.2.2.1. Application of the high-speed transformation protocol reported by Toki *et al*. (2006) for selected rice genotypes in Latin America

2.2.2.2. Production and selection of homozygous DREB transgenic rice lines

2.2.3. Evaluation of homozygous DREB transgenic rice lines under water-limited conditions

2.2.3.1. Vegetative stage screening using Big Trays

2.2.3.2. Yield response of homozygous DREB transgenic rice lines

3. HYPOTHESIS

3.1. Null Hypothesis (H_0):

DREB transgenic rice does not show differences compared with non-transgenic rice.

3.2. Alternative Hypothesis (H_1):

DREB transgenic rice shows significant differences compared with non-transgenic rice.

4. MATERIALS AND METHODS

4.1. Establishment of drought screening protocols for rice in the field and screening of candidate rice genotypes for genetic transformation.

A total of six rice genotypes were used in this study (Table 4.1-1). The irrigated varieties CICA8 and Palmar (*Indica* type), and the upland line CT6241 (*Japonica* type), are three genotypes developed for Latin America. An upland NERICA variety generated by the Africa Rice Center (WARDA), Curinga (CT11251-7-2-M-M-BR1), a Brazilian commercial variety originated from CIAT in 2003 (Annual report of IP-4 project at CIAT, 2003), and Azucena (a *Japonica* rice of Philippine origin) were pre-selected as drought tolerant genotypes. All rice genotypes were tested under well-irrigatedand drought stress conditions, respectively.

Table 4.1-1. Background of rice genotypes used in field experiments

Common Name	Pedigree	Group	Origin	Cultivation	History
Palmar	P2231-F4-138-6-2-1	Indica	Venezuela	Lowland	Improved
CICA8	P918-25-1-4-2-3-18-1131-1	Indica	Colombia	Lowland	Improved
CT6241	CT 6241-17-1-5-1	Japonica	Latin America	Upland	Improved
Curinga	CT-11251-7-2-M-M-BR1	Tropical Japonica	CIAT	Upland	Improved
NERICA	NERICA WAB-788-54-1-1-2-HB	Japonica	Africa	Upland	Improved
Azucena	Traditional Land race	Japonica	Philippines	Upland	Traditional

Field experiments were conducted between August 2006 and January 2007 at the rice farm of the International Center for Tropical Agriculture (CIAT), located at Palmira, Valle del Cauca, Colombia, 03˚29'43.2"N, 76˚21'12.5"W, 995 m. The soil was slightly alkaline, low iron, clayey and classified as *Typic Pellustert*. Details of the soil physiological and chemical properties are shown in Table 4.1-2.

Table 4.1-2. Soil properties for field experiments

Property	
pH (1:1 water)	7.90
Organic matter (%)	2.52
Total N (%)	0.13
P-BrayII (mg/kg)	51.97
K (cmol/kg)	2.37
Zn (mg/kg)	5.93
Mn (mg/kg)	55.32
Fe (mg/kg)	0.81

Fifteen 23-days old seedlings for each rice genotype were transplanted into three-row plots, with a distance of 25 cm between the plants within a plot, and 40 cm between rows. Rice seedlings recovered from the transplanted seeds approximately two weeks after transplant (Fig. 4.1-1a).

Field experiments were carried out following a randomized complete block design with three replications. Each experimental plot was separated by a distance of 45 m from the neighboring plot. A well-irrigated plot (experiment 1) followed standard irrigation practices and served as the control treatment; and a water-limited plot (experiment 2) simulated drought stress using a rain-out shelter with minimum irrigation. The sizes of the experiments were 63 m^2 for experiment 1 and 200 m^2 for experiment 2. Each experiment plot was covered with nets to avoid damage and seed dissemination by birds. Two individual experiments were well-irrigated after plowing and harrowing for a month until transplanting in order to increase the availability of iron and other nutritional components in the soil. Additionally, a basic fertilization was applied. Its composition (per 10000 m^2) was as follows; 280Kg of urea; 240Kg mono-ammonium phosphate; 15Kg zinc sulfate, 110Kg potassium chloride; and 35Kg of microelements.

Water treatment of experiment 1 was surface-irrigated and kept under irrigated and normal optimum cultivation conditions. Experiment 2 was created an artificial drought stress condition stopping irrigation 26 days after transplant by draining out the water and keeping off rainfall using the shelter. Furthermore, in order to

prevent water movement from outside the experiment plot, a transparent vinyl sheet was placed to a depth of 60 cm into the soil (Fig. 4.1-1b). The water conditions in experiment 2 were as follows: the plot was irrigated 2-3 times (approximately 420 L water irrigation for 57.8 m^2) per week providing the plants a minimum amount of water with sprinklers, starting at the vegetative stage of growth. These water conditions were maintained until one week before harvest (Fig. 4.1-1e).

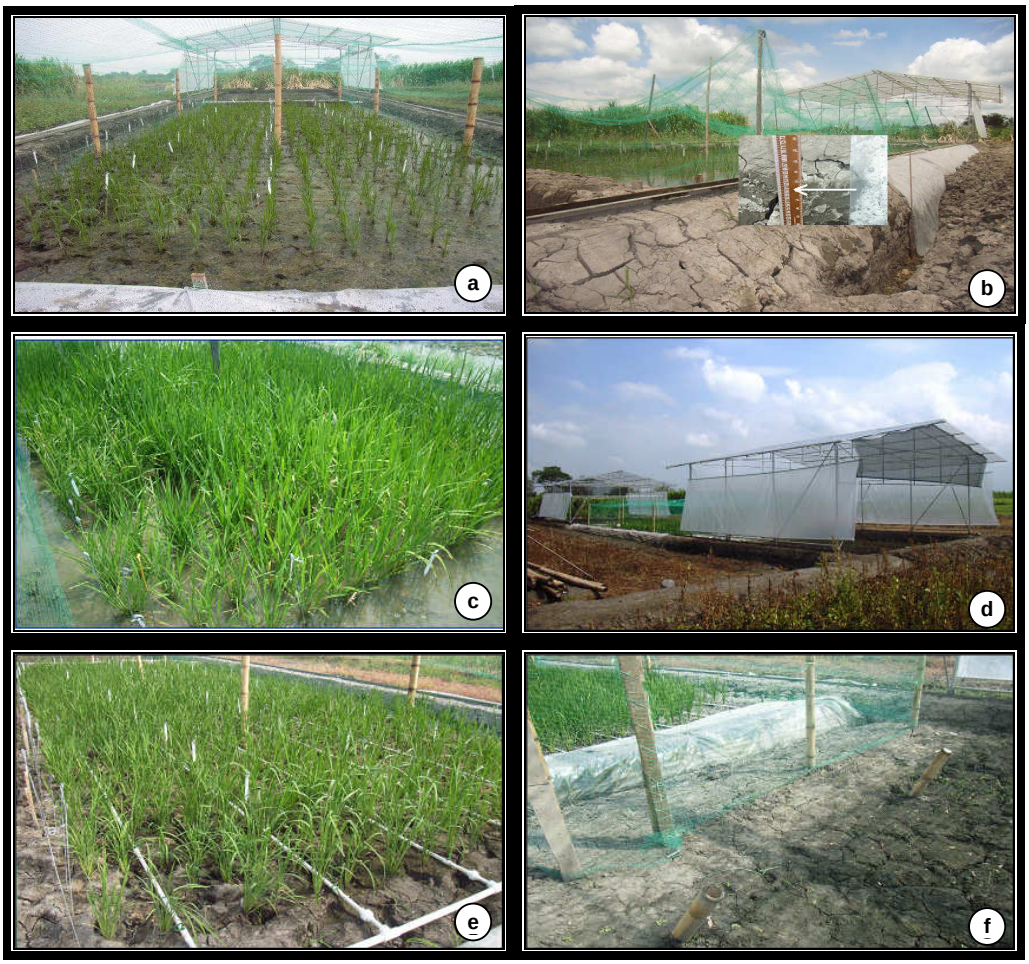

Fig. 4.1-1. Details of field experiments. a. Rice seedlings recovered from the transplanted seeds (29 days after transplant). Fifteen seedlings into three-row plots, with a distance of 25 cm between the plants within a plot, and 40 cm between rows; b. Transparent vinyl sheet was placed to a depth of 60 cm into the soil at experiment 2; c. Plot of experiment 1; d. Shelter to keep off rainfall; e and f. Plot of experiment 2.

Data collected from these experiments included yield, yield components, dry matter, flowering date, and plant height by measuring three plants located at the

center of each plot to avoid a border effect on experiments 1 and 2, respectively. Flowering dates were determined visually by measuring three plants that were selected at random and when these had 50% visible panicles. Plant height and panicle number were measured at about dough stage. All measured plants were harvested from each plot, and dried at 50^{o}C to determine their total dry matter. The percentage of filled grains was calculated by counting the filled and unfilled grains for each of the sampled panicles from the harvested plants. All grains were dried in a hot air oven at 50^{o}C for 7 days, and 1000 grains weight was calculated from the dry weight of filled grains divided by the total number of filled grains, then multiplied 1000 times.

Analysis of variance (ANOVA) based on a randomized complete block design was carried out for all characters. All statistical analyses were performed using SAS software (SAS Institute Inc. 2004, SAS/STAT®, 9.1).

4.2. Rice genetic transformation

4.2.1. Application of a high-speed transformation protocol reported by Toki *et al*. (2006) for selected rice varieties in Latin America.

Candidate rice genotypes for genetic transformation. A total of five rice genotypes were used in this study: CICA8, CT6241, Curinga and Palmar, which were selected in previous field experiments as candidate genotypes for genetic transformation. However, CICA8, CT6241, and Palmar were not included in this study because they have an efficient standardized genetic transformation protocol, and advanced transgenic lines have been produced from established protocols. However, there was no information about genetic transformation for Curinga, thus only Curinga was included for this study. Additionally, four different rice genotypes were included in this study due to their good agronomic performances in previous evaluations. Fedearroz50 (McNally *et al*. 2006) is an *Indica* type lowland rice that is cultivated widely in Colombia, and considered as a model rice genotype for *Indica* type transformation studies; two genotypes from Nicaragua, Inta Chinandega (CT12249-3-26-1-1P-1P) and CT15944-10-4-3-3 (Caiapo/ *O.glaberrima*), which showed good agronomic characteristics and high yield under drought stress condition in Nicaragua (Trouche *et al*. 2006). And the *Japonica* variety, Nipponbare was included in this study as a control for the high-speed transformation protocol studies (Table 4.2.1-1).

Table 4.2.1-1. Background of candidate genotypes for rice transformation

Common Name	Pedigree	Group	Origin	Cultivation	History
Curinga	CT-11251-7-2-M-M-BR1	Tropical Japonica	CIAT	Upland	Improved
CT15944	CT15944-10-4-3-3	Japonica	Nicaragua	Upland	Improved
Fedearroz50	FB0007-3-1-6-1-M	Indica	Colombia	Lowland	Improved
Inta Chinandenga	CT12249-3-26-1-1P-1P	Tropical Japonica	Nicaragua	Upland	Improved
Nipponbare	IRRI Collection No. PI 514663	Japonica	Asia	Lowland	Improved

Hygromycin (hyg.) resistance tests. Some Latin American rice genotypes are either highly susceptible or more tolerant to the standard hygromycin concentration of 30-50 mg/L usually used for most rice genotype worldwide (Tabares *et al*. 2007).

Hygromycin concentrations of 10, 30, 50, and 75 mg/L were tested to establish the appropriate concentration of hygromycin required in the selection medium. Inta Chinandega and Curinga were used for this study, and Nipponbare was tested as a control. The evaluation was carried out 3 weeks after the calli were transferred to a selection medium containing hygromycin. All tested medium also contained 500 mg/L cefotaxime sodium salts.

Rice genetic transformation. A large number of transgenic rice plants were generated at CIAT following a standardized protocol; for some *Indica* and upland rice in a period of about 3-4 months. In order to evaluate the possibility to speed up this process, a high-speed transformation protocol optimized for Nipponbare (Toki *et al*. 2006) was applied to compare with CIAT's methods (based on Lentini *et al.* 2003 with some modifications following Flórez 2003). Details of each protocol are described in the Table 4.2.1-2. To confirm and establish appropriate conditions for plant regeneration for selected rice genotypes, embryogenic calli (1-2 mm in diameter), which were derived from each rice genotype on two different calli induction procedures, were transferred to two types of plant regeneration media. Regeneration frequencies were evaluated approximately four weeks after treatment.

For rice transformation, mature healthy seeds were supplied by the Rice Program of CIAT. The protocol described by Toki *et al*. (2006) was followed with some modifications; nine-cm-diameter petri dishes were used and all dishes were sealed with surgical tape; embryogenic calli induction and hygromycin resistance calli selection were carried out at 29^{o}C, and hygromycin resistance calli were transferred to the regeneration medium and incubated at 26^{o}C.

Table 4.2.1-2. Details of CIAT and Toki (2006) protocols

	CIAT (Lentini *et al.* 2003)	Toki (2006)
Material	Mature seeds*	Mature seeds

Calli Induction	Material type	Isolated embryos*	Disinfected mature seeds
	Medium	NBA	N6D
	Temperature	26˚C	32˚C
	Light condition	Dark	Continuous illumination
	Duration	3-4 weeks	1-5 days
Sub-culture of Calli	Material type	Embryogenic calli (1-2 mm in diameter)	
	Medium	NBA	
	Temperature	26˚C	
	Light condition	Dark	
	Duration	3 days	
Pre-culture of *Agrobacterium*	Medium	LB liquid*	AB
	Temperature	27˚C*	28˚C
	Duration	24 hours with shaking (250rpm)*	3 days (Incubator)
Sub-culture of *Agrobacterium*	Material	10 ml of Pre-cultured *Agrobacterium**	
	Medium	30ml of NBA liquid*	
	Acetocyringone	100μM*	
	Temperature	26˚C*	
	Light condition	Dark*	
	Duration	2 hours with shaking (40rpm)*	
Infection	Materials	3 days sub-cultured Embryogenic calli (1-2 mm in diameter)*	1-5 days pre-cultured mature seeds
	Medium	2 hours sub-cultured *Agrobacterium* in NBA liquid*	AAM liquid
	Acetocyringone	200μM*	200μM
	$O.D._{600}$	0.5 - 1.0*	0.1
	Duration	10 minutes*	1.5 minutes
Co-Culture	Medium	NBA	2N6-AS
	Acetocyringone	100μM	100μM
	Temperature	21˚C	25˚C
	Light condition	Dark	Dark
	Duration	3 days	3 days
Calli Selection	Medium	NBA	N6D
	Temperature	26˚C	32˚C
	Light condition	Dark	Continuous illumination
	Duration	3-4 weeks	2-3 weeks
Regeneration	Medium	MSKA	R-III
	Temperature	26˚C	28˚C
	Light condition	Dim light	Continuous illumination
	Duration	3-4 weeks	3-4 weeks
Total Duration		10-13 weeks	6.5-8.5 weeks

*: Modified from Flórez 2003.

Plasmid constructions. *Agrobacterium* strain AGL1 and EHA105 containing *pCAMBIA*1305.2. (Jefferson *et al*. 1998) (Fig. 4.2.1) were tested to develop a quick and efficient transformation protocol.

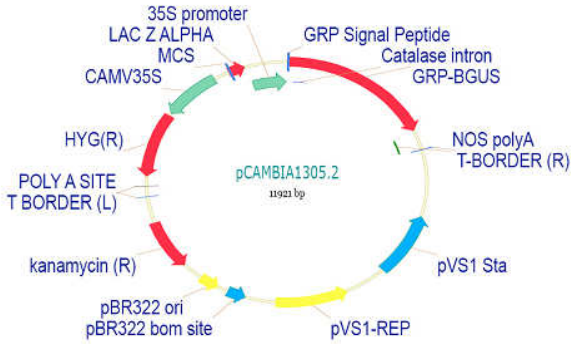

Fig. 4.2.1. Gene cassette construct maps of *pCAMBIA*1305.2. *HYG(R.)* Hygromycin resistance gene, *GRP-BGUS* GUSPlus™ gene.

Gus expression analysis. The transient *gus* gene expressions in the proliferated calli were confirmed by segments of hygromycin resistance calli incubated in X-glu solution containing *gus* assay buffer (Kosugi *et al*. 1990), 0.5 mg/ml X-glu (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide), 0.1% triton X-100 and 20% methanol. The reaction mixtures were incubated overnight at 37^{o}C. To stop the reaction, the materials were soaked in 70% ethanol and the blue staining was observed visually.

Molecular analyses of the transgenic rice plants. Genomic DNA was extracted from 15 mg of rice leaves according to the CTAB protocol modified by Lorieux *et al*. (2000). Confirmation by PCR for *pCAMBIA*1305.2. was performed using the specific primer pairs GusA (5'- CAA CAT CCT CGA TAG CA -3') and GusB (5'- GGT CAC AAC CGA GAT GTC CT -3'). The PCR reaction volume was 20µl, and its composition was as follows: 1x of PCR buffer; 1mM $MgCl_2$; 0.2mM each deoxynucleotide triphosphate; 0.4µM each olygonucleotide primer; 1µl of Taq polymerase (CIAT) and 100ng DNA extract. Reactions were followed by 35 cycles with 95^{o}C denaturation for 45 sec. (2 min. for the first cycle), annealing temperature of 56.2^{o}C for 45 sec. and extension at 74^{o}C for 60 sec. After cycling, final extension was held at 72^{o}C for 5 min. (MJ Mini Gradient Thermal Cycler, Bio-

Rad Laboratories, Inc.). Amplification products were then separated by electrophoresis using a 1.2% agarose gel (Invitrogen) with a TRIS-borate, EDTA buffer. These products were detected by staining the gel with ethidium bromide and photographed under UV light.

Regenerated transgenic plants were evaluated until maturity in a CIAT biosafety greenhouse.

4.2.2. Production and selection of homozygous DREB transgenic rice lines

T_0 Transgenic plants of CICA8, CT6241 and Palmar, which contain the *Lip9::AtDREB1A* and *Lip9::OsDREB1B* constructs were transformed by Dr. Lentini's group (Tabares *et al*. 2004), and Dr. Ishitani's team produced advanced generations of these transgenic lines. Seeds of T_2 transgenic lines that were determined as a single transgene insertion and with no rearrangements at T_0 generation by the southern blot analyses (Fory *et al*. 2005) were kindly provided by Dr. Ishitani from the Biotechnology unit of CIAT (Table 4.2.2); non-transgenic plants of each rice genotype were used as control. Dehulled seeds were first sterilized with 70% ethanol for one minute. Seeds were further sterilized with 2.5% sodium hypochlorite containing 1 drop of Tween 20 per 50 ml for 15 minutes, and then washed five times in sterilized water. This step was repeated once without Tween 20; sterilized seeds were placed on sterilized water solidified with 0.8% Gelrite® (SIGMA) and cultured under 12 hours photoperiod light at 24-26^{o}C for 7-10 days. Germinated seeds were transferred to MS medium containing 50 mg/L hygromycin and incubated at 24-26^{o}C under 12 hours photoperiod light for 2-3 weeks to test hygromycin sensitivity until non-transgenic seedlings died. Number of plants that survived the treatment was evaluated.

Table 4.2.2. Materials for T_2 homozygous selection

Genotype	Palmar		CICA8		CT6241	
Gene	AtDREB1A	OsDREB1B	AtDREB1A	OsDREB1B	AtDREB1A	OsDREB1B
Event	2	3	1	4	0	3
Line	2	8	6	16	0	6

4.3. Evaluation of homozygous DREB transgenic rice lines under water-limited conditions.

4.3.1. Vegetative stage screening of homozygous DREB transgenic rice lines using BigTrays.

The following experiments were conducted in a screenhouse at CIAT following recommended biosafety norms. Screenhouse experiments were conducted between August and October 2007 at the screenhouse *TypeII*-2 at CIAT.

Soil moisture was monitored using an ECH_2O soil moisture sensor (EC-5, Decagon Devices, Inc. USA). Well-irrigated conditions (experiment 3) were kept at all time at more than 85% field capacity (FC) by normal irrigation of the plot as a control treatment; water-limited conditions (experiment 4) were created under drought stress adjusted to 20-35% FC by stopping water supply and monitoring the soil moisture starting 2 weeks after transplants until one week before harvest, and then re-watering to bring back FC to more than 85% like in experiment 3 (Fig. 5.4-1).

All transgenic rice plants used in experiments 3-4, and in the greenhouse experiment (see section 4.3.2) incorporated the *Lip9::AtDREB1A* (I-P-A-43-3, III-P-A-70-5, VII-P-A-107-3, and IX-P-A-165-6), and *Lip9::OsDREB1B* (IX-P-B-212-5, and X-P-B-278-1) constructs, and originated from transformation studies into the Palmar variety by Dr. Lentini's group in 2004 (Tabares *et al*. 2004). These selected transgenic events are characterized by having a single transgene insertion and by the absence of rearrangements in the T_0 generation (Fory *et al*. 2005). Dr. Ishitani's team at CIAT carried out advance generations of these transgenic lines, and kindly provided a total of sixteen T_2 lines, of which all tested plants survived on hygromycin containing medium as homozygous lines; six of these lines were selected as independent lines for screenhouse experiments. In addition, non-transformed Palmar BCF962 (Palmar) was included in the experiments as a control. There were CICA8, CT6241 and Palmar transgenic plants containing

DREB genes, which were used in a previous experiment for selecting homozygous DREB transgenic rice lines. However, numbers of homozygous lines were successfully selected from Palmar only, and used in this study.

A soil mix was prepared by mixing CIAT soil with soil from Santander de Quilichao (SQ) as an iron source, and sand to improve soil permeability. The soil used in the experiments was prepared in a 2:1:1 ratio. Details of the soil physiological and chemical properties are shown in Table 4.3.1. This soil mix was ground using a grinding machine, before weighting. Weighted soil and sand were mixed in a soil mixer, and then sterilized by vapor. Sterilized soil was dried again, and a mixture of fertilizers as basic fertilization was applied using a soil mixer. Its composition (per 100Kg) was as follows; 10g of urea; 8g of mono-ammonium phosphate; 2.6g of zinc sulfate, 4.5g of potassium chloride; and 0.8g of microelements. Then the maximum soil moisture content (field capacity (FC)) was determined. One thousand kilograms of the soil mixture was used in each experiment.

Table4.3.1. Soil properties for screenhouse experiments

Property	
pH (1:1 water)	6.71
Organic matter (%)	3.13
Total N (%)	0.135
P-BrayII (mg/kg)	30.58
K (cmol/kg)	0.20
Zn (mg/kg)	6.34
Mn (mg/kg)	26.75
Fe (mg/kg)	8.24

Big circle shape trays (BigTrays, Fig. 4.3.1), each 2 m in diameter were designed to evaluate large numbers of plants simultaneously, by controlling the soil moisture more precisely and by avoiding soil moisture gradient. Both experiments 3 and 4 were carried out following a randomized complete block design with four replications.

Fourteen seedlings (15 days after sowing (DAS)) for each independent transgenic line and fifteen seedlings for non-transgenic Palmar were transplanted at 10 cm distance of each plant. Non-transgenic Palmar were also transplanted at the edge of trays to avoid a border effect on experiments.

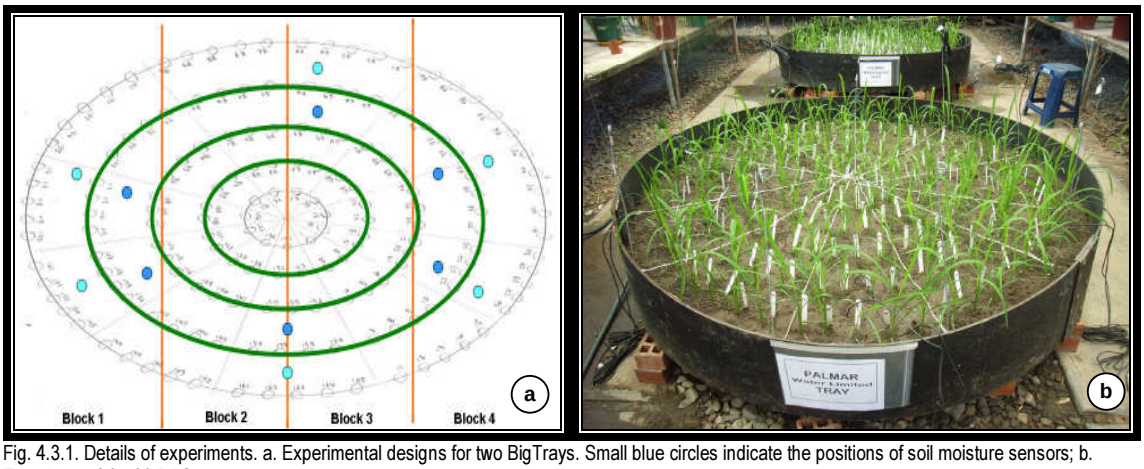

Fig. 4.3.1. Details of experiments. a. Experimental designs for two BigTrays. Small blue circles indicate the positions of soil moisture sensors; b. Experiment 4 for 38 DAS.

The following data was collected from these experiments: leaf temperature, difference of temperatures between leaf and screenhouse conditions, plant height, tiller number, leaf number, leaf rolling score, plant recovery score and biomass production. Leaf temperature and temperature difference was recorded just one time 45 days after transplanting (60 DAS). Plant height, tiller number and leaf numbers were measured weekly, starting at 15 days after transplants (30 DAS). Leaf rolling score was visually recorded with a scale from “0” to “9” at noon when symptoms appeared, and were recorded for a total of three times. A score “0” indicated no symptom of leaf rolling, and score “9” indicated complete leaf rolling. Plant recovery score was recorded every day from beginning of re-watering to before harvest. A rating of plant recovery score was visually estimated for each plant using a 0-9 scale, where score 0 was completely recovered (healthy) and 9 when it was not recovered. Leaf rolling and plant recovery score was recorded only for drought stress treated plants. Biomass production was weighted after harvest

immediately as fresh matter, and dried in a hot air oven at 50^{o}C for 7 days for total dry matter determination.

All data were analyzed separately using analysis of variance with SAS program.

4.3.2. Yield response of homozygous DREB transgenic rice lines

This experiment was conducted at the biosafety greenhouse at CIAT under complete biosafety norms.

In this experiment, transgenic Palmar plants I-P-A-43-3, III-P-A-70-5, VII-P-A-107-3, IX-P-A-165-6 (with the *Lip9::AtDREB1A* construct), IX-P-B-212-1, IX-P-B-239-5, X-P-B-278-1 and X-P-B-290-1 (with the *Lip9::OsDREB1B* construct) were selected as independent homozygous lines at T_2 generation to evaluate their yield response. In addition, non-transformed Palmar BCF962 (Palmar) and non-transformed CT6241-17-1-5-1 BCF1096 (CT6241) were included in the experiment as a control.

The greenhouse experiment was conducted between March and August 2008 at the biosafety greenhouse at CIAT. The soil used was a mix of soils which was prepared by using CIAT soil, SQ soil and sand in 2:1:2 ratio, and an adequate fertilization (Nitrogen, Phosphorus, Potassium, Zinc and Micronutrients) was applied in order to get healthy plants without symptoms of nutrient deficiencies. Its composition (per 100Kg) was as follows; 10g of urea; 8g of mono-ammonium phosphate; 2.6g of zinc sulfate, 4.5g of potassium chloride; and 0.8g of microelements. Details of the soil physiological and chemical properties are shown in Table 4.3.2. Soil preparation for this experiment was the same as of screenhouse experiments.

Table 4.3.2. Soil properties for greenhouse experiment

Property	
pH (1:1 water)	5.23
Organic matter (%)	2.08
Total N (%)	0.10
P-BrayII (mg/kg)	12.61
K (cmol/kg)	0.69
Zn (mg/kg)	2.47
Mn (mg/kg)	53.51
Fe (mg/kg)	23.58

Three seeds of transgenic rice from each independent line were sowed at two symmetrical hills (Fig. 4.3.2) in a long pail (36.5 cm diameter, 60 cm depth) containing 70kg of the soil mixture, and following a randomized complete block design with three replications for both control and drought stress treatments. Approximately two weeks after sowing, the healthiest plant per hill was selected and the remaining two were discarded.

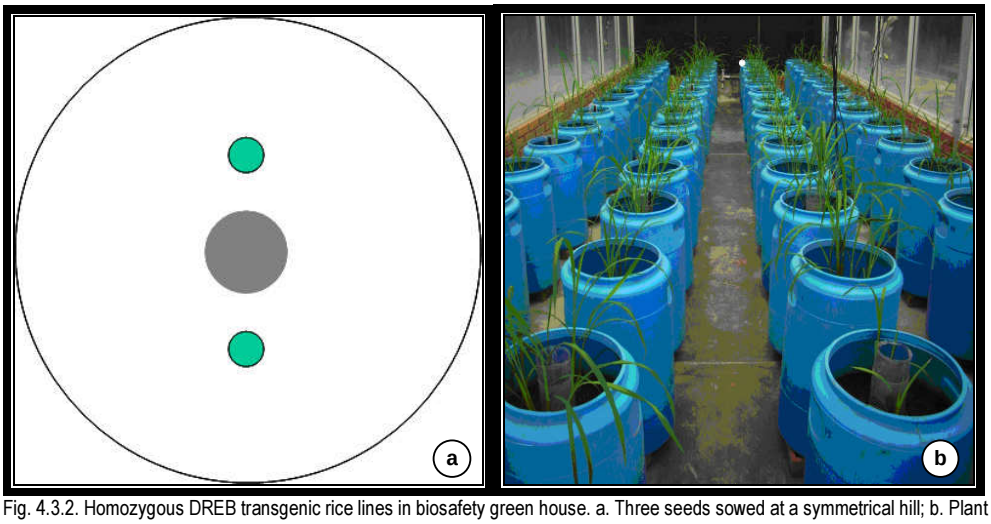

Fig. 4.3.2. Homozygous DREB transgenic rice lines in biosafety green house. a. Three seeds sowed at a symmetrical hill; b. Plant growth at 48 DAS.

Normal water supply for drought stress treatment was discontinued at 57 DAS to keep the soil moisture at 30-50% FC during the end of vegetative stage and the reproductive stage compared with the control (well watered) treatment, which was kept at 100% FC soil moisture during both vegetative and reproductive stages. Soil moisture was monitored using a ECH_2O soil moisture sensor (EC-5, Decagon Devices, Inc. USA).

Measurements at this experiment were the same as for the field experiments 1 and 2, described elsewhere.

Data was analyzed separately using analysis of variance (ANOVA). The subsequent multiple comparisons among the means of treatments, plants and treatments by plants interactions were examined based on the Ryan-Einot-Gabriel-

Welsch multiple range tests (herein referred as Ryan's multiple range tests). All statistical analysis were performed with SAS program (SAS Institute Inc.2004. SAS/STAT®, 9.1).

5. RESULTS AND DISCUSSION

5.1. Establishment of drought screening protocols for rice in the field and screening of candidate rice genotypes for genetic transformation

Palmira has a subtropical climate with 900-1000 mm precipitation per year. The difference between the maximum and minimum temperature ranged from 12^{o}C at the beginning of the field experiments to less than 8^{o}C by the time of flowering (Fig. 5.1-1). Amount of weekly total rainfall was high and well distributed during the reproductive stage, but rainfall declined by the time of crop maturity.

Water treatments had a significant effect on most traits except on panicle number per plant and dry matter; genotypes performed differently in terms of dry matter due to water treatment (Tables 5.1-1, and 5.1-2). Flowering dates were highly affected by the water treatments, genotypes and their interactions. Azucena, CICA8 and Palmar flowered around 100 DAS, and plant growth was delayed under water-limited conditions (Fig. 5.1-5). In contrast, Curinga, CT6241 and NERICA were not affected by the two water treatments.

Significant differences were observed for plant height (Fig. 5.1-6). In particular, Azucena showed a reduction of about 50 cm in plant height under water stress. Curinga, CT6241 and NERICA showed were less affected by both treatments.

Of all the traits, tiller number per plant was one of the most affected by the different water treatments (Fig. 5.1-3). Particularly, tiller number of CICA8 and Palmar under water-limited conditions was higher than normal irrigated conditions. Azucena and NERICA produced a small number of tillers at the two water treatments. These findings suggest that these genotypes have a strong dependence response.

No significant difference was observed for panicle number due to water treatments (Table 5.1-3), but there were differences in genotypes. CICA8, Curinga and CT6241 produced more panicles. Water treatments affected productive panicle

number, and significant differences were observed amongst genotypes. However, Ryan's multiple range tests did not detect significant statistical differences of their interactions for productive panicle number (Tables 5.1-1 and 5.1-7). These results probably indicate that a large tiller number was associated with panicle production amongst evaluated rice genotypes, and that genotypes respond differently to water treatmens. CICA8 and Palmar (which were developed for irrigation conditions) had more non-productive panicles than productive panicles. Curinga showed a high percentage of productive tillers than other tested genotypes under water-limited conditions (data not shown).

Significant differences were observed due to the different water treatments and/or amongst evaluated rice genotypes for both panicle length and panicle weight. However, the interactions, which evaluated plant genotypes by water treatments, were observed for panicle length only (Fig. 5.1-4, Tables 5.1-1 and 5.1-4). Panicle weight of all tested genotypes at normal irrigation conditions was heavier than those grown under water-limited conditions. CICA8 and Palmar were highly affected by the effect of water treatments.

No significant differences were observed for yield and yield components between rice genotypes and water treatments, except for percentage of filled grains (Tables 5.1-6, 5.1-8, 5.1-9; Fig. 5.1-7, 5.1-8). The effect of water treatments highly affected spikelets number. Curinga had more spikelets under the drought stress treatment (Table 5.1-8). There were differences in spikelets numbers due to genotypes. All tested rice genotypes produced more filled grains and high percentage of filled grains under normal irrigated conditions. The effect of water treatments shown for these two traits was particularly expressed in the varieties CICA8 and Palmar (Fig. 5.1-2 and 5.1-7). No significant differences were observed in Curinga and NERICA for filled grain number and percentage of filled grains due to water treatments.

Significant differences in the weight of filled grains were observed in Azucena, CICA8 and Palmar, due to water treatments (Table 5.1-5). There were difference in

thousand-kernel weights due to genotypes and water treatments; however, there were no significant differences due to their interaction (Table 5.1-9). Significant differences were observed for thousand-kernel weight in CICA8 and Palmar under water-limited conditions. This finding suggests that thousand-kernel weight is less affected by water treatments compared to other yield components. Yield components of CT6241, Curinga and NERICA were not affected by water treatments; however, Azucena, CICA8 and Palmar were highly affected by water treatments. Curinga showed the best yield performance amongst evaluated rice genotypes under drought stress conditions.

These results probably indicate that Curinga and CT6241 have a potential to perform similarly under both water treatments based on measured traits. Furthermore, these two genotypes showed a higher yield response than other tested genotypes under drought stress treatments. Azucena also responded well for some traits, however, flowering date and plant height of Azucena were undesirable. On the other hand, these results clearly indicate that CICA8 and Palmar are susceptible to water-limited conditions such as those imposed in experiment 1. Breeders may be able to discard a large number of drought susceptible lines from the breeding program and select only promising lines with vegetative drought resistance (Pantuwan *et al*. 2004). However, it is unclear whether DREB transgenic lines in those drought susceptible genotypes will yield well under water-limited conditions. Curinga and CT6241 were selected due to their good performance under water-limited conditions. CICA8 and Palmar were also chosen as drought intolerant genotypes for further study.

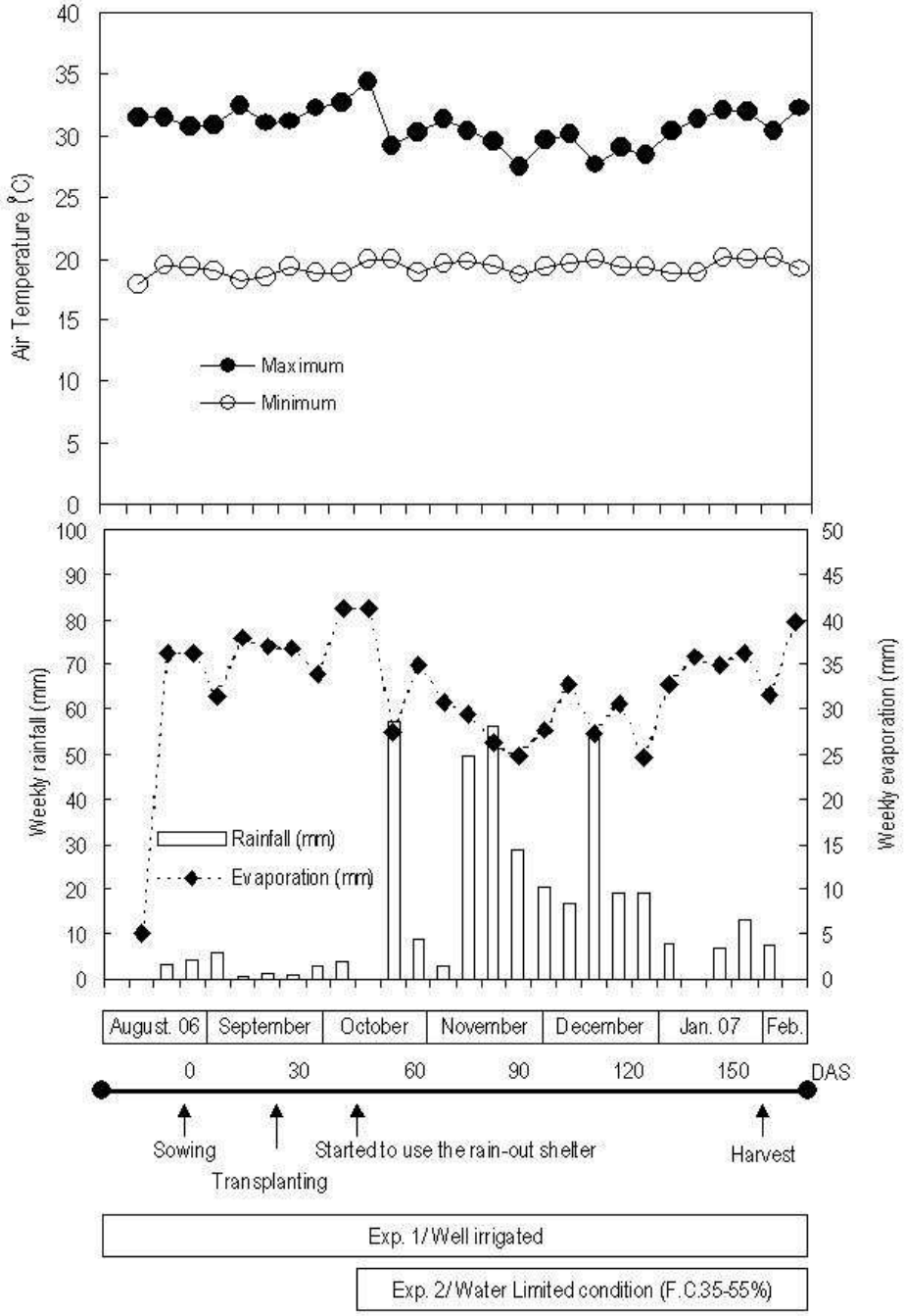

Fig. 5.1-1. Environmental conditions and time lines of field experiments.

Table 5-1.1. Significance for source of variations in measured traits at field experiments.

	Source of variations		
Trait	Water Treatment	Genotype	Water Treatment*Genotype
Dry matter	ns	ns	ns
Panicle number per plant	ns	*	ns
Panicle weight	**	ns	ns
Weight of filled grains	**	ns	ns
Yield per plant	**	ns	ns
Productive panicle number per plant	*	*	ns
Spikelets number per plant	**	*	ns
Thousand kernel weight	**	**	ns
Filled grains per plant	**	ns	*
Tiller number per plant	**	**	*
Panicle length	**	**	*
Flowering date	**	**	**
Plant height	**	**	**
Percentage of filled grains per plant	**	**	**

*: Significant at 0.05≤*P*≤0.01; **: Significant at *P*≤0.01; ns: No significant at *P*≥0.05.

Table 5.1-2. Dry matter of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated			Water-limited			Global		
Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	46.78±5.48	90.40	20.33	38.32±2.34	16.55	10.62	42.55±3.27	64.26	18.84
CICA8	49.19±3.17	30.17	11.17	31.03±3.57	38.40	19.97	40.11±4.58	126.38	28.03
CT6241	37.14±4.61	63.83	21.51	25.99±2.42	17.57	16.13	31.57±3.41	69.86	26.48
Curinga	34.39±4.22	53.60	21.29	28.29±3.12	29.37	19.16	31.34±2.71	44.37	21.25
NERICA	27.64±0.61	1.12	3.83	25.02±1.63	7.99	11.30	26.33±0.97	5.72	9.08
Palmar	40.62±6.02	108.77	25.68	30.59±2.97	26.59	16.86	35.60±3.74	84.31	25.79
Global	39.29±2.32	97.40	25.12	29.87±1.41	36.22	20.15	34.58±1.56	87.73	27.08

Table 5.1-3. Panicle number per plant of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated			Water-limited			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	10.22±0.94	2.70	16.09	8.22±0.22	0.15	4.68	9.22±0.62b	2.34	16.59
CICA8	19.00±3.05	28.00	27.85	12.89±0.80	1.93	10.77	15.94±1.96ab	23.17	30.19
CT6241	15.94±1.94	11.34	21.12	11.22±1.63	8.04	25.26	13.58±1.55ab	14.44	27.98
Curinga	15.11±1.86	10.48	21.42	14.89±1.71	8.79	19.91	15.00±1.13a	7.72	18.53
NERICA	11.28±0.89	2.40	13.73	8.39±0.69	1.45	14.37	9.83±0.82b	4.04	20.45
Palmar	14.17±2.26	15.36	27.67	13.11±1.55	7.29	20.59	13.64±1.25ab	9.39	22.47
Global	14.29±0.98	17.31	29.12	11.45±0.73	9.69	27.18			

Means within Global column followed by same letter are not different significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-4. Panicle weights of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	37.49±3.31	33.01	15.32	10.04±1.52	6.95	26.25	23.77±6.35	242.03	65.45
CICA8	45.01±8.26	204.70	31.79	5.74±0.84	2.13	25.40	25.38±9.53	545.29	92.02
CT6241	40.09±4.40	58.32	19.05	17.32±4.17	52.34	41.77	28.71±5.77	199.84	49.24
Curinga	40.61±3.86	44.71	16.47	21.87±1.80	9.76	14.29	31.24±4.60	127.18	36.10
NERICA	30.36±2.62	20.59	14.95	9.86±2.26	15.33	39.70	20.11±4.83	140.37	58.92
Palmar	35.18±6.77	137.74	33.36	8.81±1.75	9.19	34.40	21.99±6.67	267.33	74.34
Global	38.12±2.12	81.11	23.62	12.28±1.55	43.54	53.75			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-5. Weight of filled grains of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	35.31±3.79	43.28	18.63	7.60±1.32	5.24	30.14	21.45±6.45	249.78	73.68
CICA8	40.51±7.30	160.24	31.24	3.55±0.47	0.68	23.21	22.03±8.89	474.38	98.87
CT6241	36.74±4.20	53.03	19.82	14.85±3.74	41.97	43.62	25.80±5.50	181.71	52.25
Curinga	35.19±1.96	11.80	9.76	19.35±1.91	11.03	17.16	27.27±3.75	84.42	33.69
NERICA	27.68±2.19	14.47	13.74	8.33±2.33	16.29	48.45	18.00±4.55	124.57	62.00
Palmar	31.54±6.69	134.33	36.75	5.59±1.37	5.65	42.52	18.56±6.55	257.93	86.52
Global	34.49±1.91	66.32	23.61	9.88±1.51	41.38	65.12			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-6. Yield per plant (g) of six rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	35.31±3.79	43.28	18.63	7.60±1.32	5.24	30.14	21.45±6.45	249.78	73.68
CICA8	40.51±7.30	160.24	31.24	3.55±0.47	0.68	23.21	22.03±8.89	474.38	98.87
CT6241	36.74±4.20	53.03	19.82	14.85±3.74	41.97	43.62	25.80±5.50	181.71	52.25
Curinga	35.19±1.98	11.80	9.76	19.35±1.91	11.03	17.16	27.27±3.75	84.42	33.69
NERICA	27.68±2.19	14.47	13.74	8.33±2.33	16.29	48.45	18.00±4.55	124.57	62.00
Palmar	31.54±6.69	134.33	36.75	5.59±1.37	5.65	42.52	18.56±6.55	257.93	86.52
Global	34.49±1.91	66.32	23.61	9.88±1.51	41.38	65.12			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-7. Productive panicle number per plant of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	10.11±0.88	2.37	15.23	5.94±0.47	0.68	13.83	8.03±1.03a	6.43	31.58
CICA8	16.39±3.20	30.79	33.86	9.06±0.72	1.56	13.81	12.72±2.20a	29.07	42.38
CT6241	14.83±1.74	9.08	20.32	8.61±1.69	8.62	34.10	11.72±1.76a	18.70	36.89
Curinga	12.78±0.80	1.93	10.86	11.28±0.72	1.56	11.09	12.03±0.58a	2.07	11.97
NERICA	11.00±1.00	3.00	15.75	6.22±0.40	0.48	11.15	8.61±1.17a	8.24	33.34
Palmar	13.89±2.11	13.37	26.33	9.67±2.52	19.08	45.19	11.78±1.74a	18.33	36.35
Global	13.17±0.81	12.04	26.35	8.46±0.64	7.49	32.34			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-8. Spikelets number per plant of six-rice genotypes at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	1256.90±151.37	68739.8	20.86	581.44±116.43	40671.6	34.68	919.19±173.51a	180654.6	46.24
CICA8	2402.30±525.92	829787.1	37.92	655.39±115.19	39806.8	30.44	1528.90±458.87a	1263382.0	73.52
CT6241	1804.70±244.64	179547.1	23.48	974.00±164.66	81343.4	29.28	1389.40±227.81a	311386.0	40.16
Curinga	1428.90±47.53	6777.5	5.76	1109.10±93.26	26094.0	14.57	1269.00±85.48a	43847.3	16.50
NERICA	1324.90±126.95	48354.7	16.60	525.39±96.61	29174.3	32.51	925.14±192.68a	222771.7	51.02
Palmar	1792.20±317.88	303144.2	30.72	818.17±115.84	40261.1	24.52	1305.20±265.19a	421964.9	49.77
Global	1668.30±135.57	330840.3	34.48	777.24±65.57	77412.6	35.80			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.1-9. Thousand-kernel weight of six-rice genotype at field experiments.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Genotypes	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
Azucena	30.17±0.65	1.30	3.77	23.24±0.65	1.28	4.86	26.70±1.60a	15.46	14.72
CICA8	22.98±1.34	5.43	10.14	16.26±0.87	2.31	9.35	19.62±1.66b	16.67	20.81
CT6241	25.36±0.40	0.50	2.79	23.21±0.53	0.86	3.99	24.28±0.56a	1.93	5.73
Curinga	27.42±1.34	5.42	8.49	22.09±0.49	0.73	3.88	24.76±1.35a	11.01	13.40
NERICA	26.62±1.14	3.93	7.45	23.72±0.54	0.89	3.98	25.17±0.86a	4.46	8.39
Palmar	21.11±0.74	1.66	6.10	18.01±1.97	11.66	18.96	19.56±1.17b	8.22	14.66
Global	25.61±0.79	11.38	13.17	21.09±0.77	10.88	15.65			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

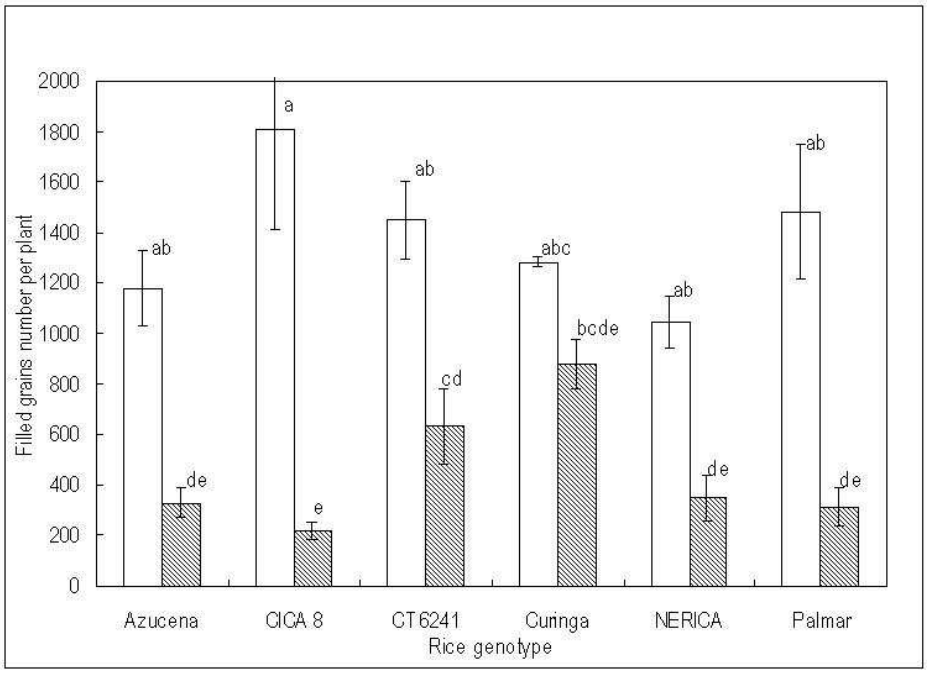

Fig. 5.1-2. Filled grains number per plant of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure represent standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

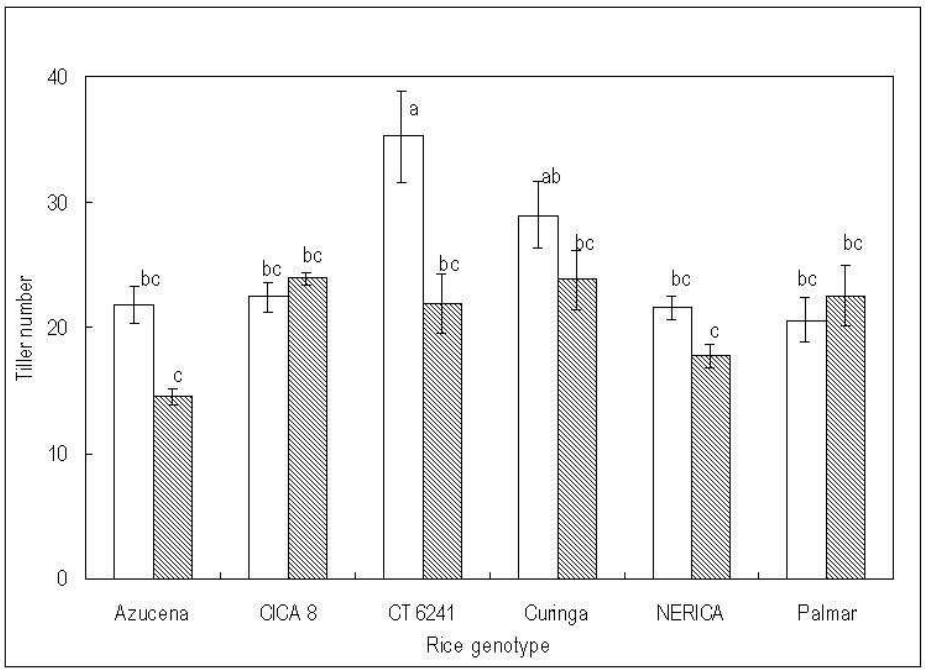

Fig. 5.1-3. Tiller number of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

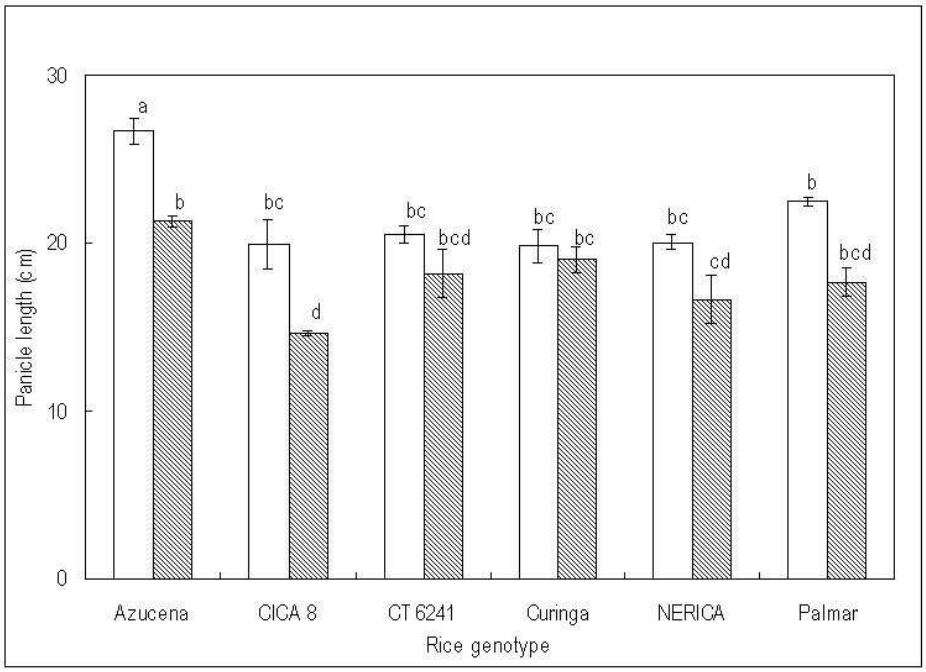

Fig. 5.1-4. Panicle length of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

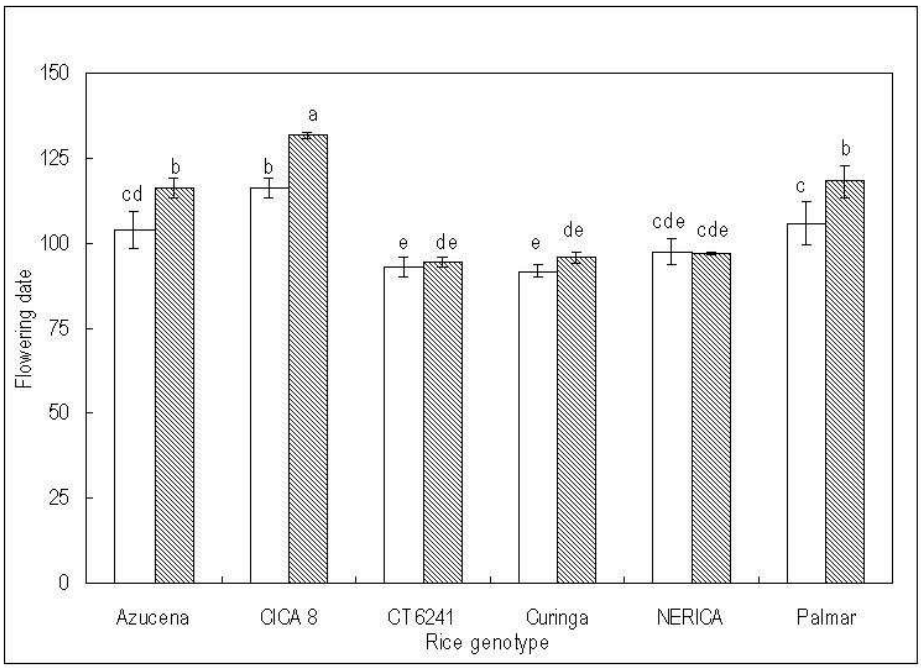

Fig. 5.1-5. Flowering date of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

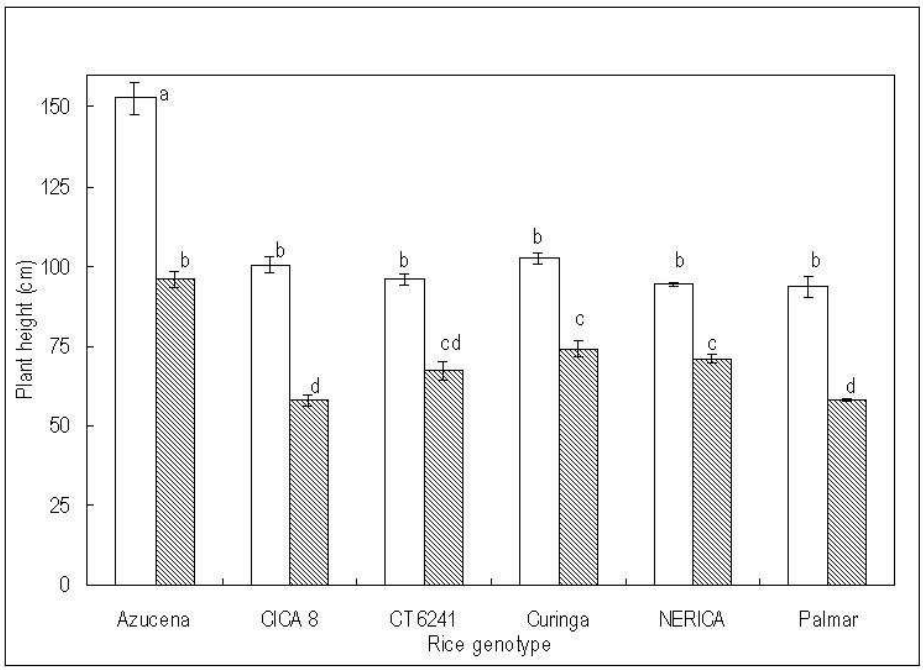

Fig. 5.1-6. Plant height of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

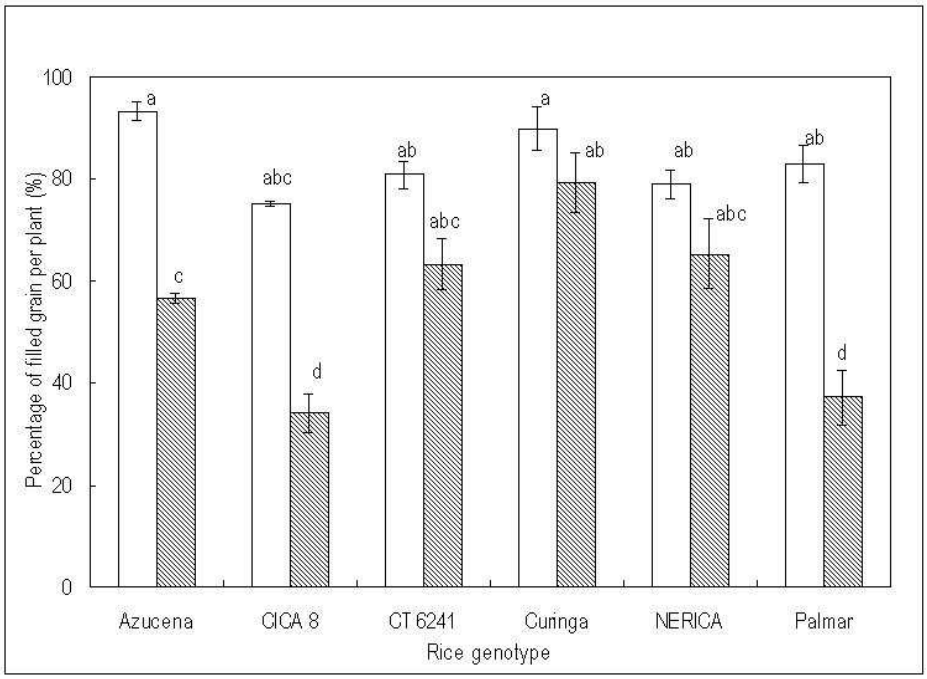

Fig. 5.1-7. Percentage of filled grains per plant of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

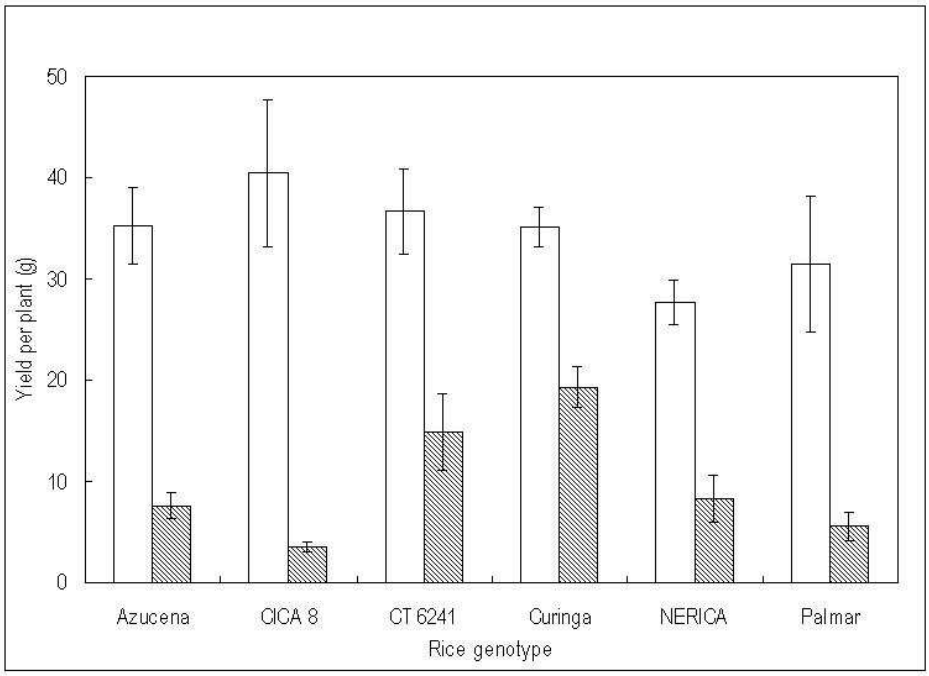

Fig. 5.1-8. Yield per plant (g) of six-rice genotypes at field experiments. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error.

5.2. Application of a high-speed transformation protocol reported by Toki *et al*. (2006) for selected rice varieties in Latin America.

All evaluated traits were highly affected by rice genotype (Table 5.2-1). Significant differences were observed due to rice genotypes and/or media; however, significant rice genotypes by media interactions were not observed for the number of green spots per calli, which generally regenerated into plants (Tables 5.2-1, 5.2-2.1, 5.2-2.2 and 5.2-2.3). A larger number of green spots per calli were observed for Curinga compared to other tested rice genotypes except Nipponbare. Curinga plants regenerated rapidly from induced calli at both temperatures. Moreover, the effects of regeneration temperatures by media interactions were not affected by the different varieties. However, albino plants were observed in regenerated Curinga plants (Tables 5.2-1, 5.2-3.1, 5.2-3.2 and 5.2-3.3; Fig. 5.2-1.1). This result probably indicates that Curinga calli have an ability to regenerate into plants at similar conditions following Toki's protocol. A large number of embryogenetic calli was recorded for CT15944 for all interactions (Fig. 5.2-2). However, embryogenetic calli of CT15944 mostly regenerated into roots at these evaluated mediums (Fig. 5.2-3). On the other hand, Toki's conditions regeneration frequency was unfavourable for Inta Chinandega. Induced calli of Inta Chinandega regenerated very few plants at MSKA medium; and, no regenerated plants were obtained with R-III medium, where calli death was caused by necrosis (Fig. 5.2-4). These results also confirmed that Toki's method is more efficient in terms of calli induction and plant regeneration for Nipponbare, and it was much better for Curinga than for CT15944.

Calli proliferation of Inta Chinandega was inhibited at 30 mg/L of hygromycin concentration (Fig. 5.2-5). Curinga was more sensitive to hygromycin; its calli proliferation was weak at 10 mg/L of hygromycin concentration (data not shown). In the case of Nipponbare and/or most rice genotypes, the optimal selection for transgenic plants can be obtained at a 30-50 ml/L hygromycin concentration.

Results probably indicate that the high sensitivity of Curinga to hygromycin may cause difficulty in the selection of agrobacterium-infected callus.

Curinga was selected due to the plant regeneration frequency and their favorable performance using Toki's method.

The protocol developed for Nipponbare (Toki *et al.* 2006) to reduce the time span for rice transformation using high temperature and continuous illumination for calli induction and selection was highly efficient; however, hygromycin resistant calli showed low stable *gus* expression (Fig. 5.3, and Table 5.2-4). In contrast, about 68 to 100% stable *gus* expressions were observed on hygromycin-resistant calli that followed CIAT's protocol independently of the *Agrobacterium* strain used. However, in a number of plants regenerated using Toki's protocol, *gus* expression and PCR positive plants were confirmed only in one-third of all regenerated plants. These results probably suggest that low temperature and dark conditions are key factors to establish an efficient protocol for Curinga. On the other hand, a large number of *gus*/PCR negative plants were observed, and this was probably due to the low hygromycin concentration in the regeneration stage. Curinga is highly susceptible to hygromycin, and a better solution is necessary to establish a genetic transformation protocol. Differences amongst cultivars and between *Agrobacterium* strains were found at two independent conditions. Hygromycin resistance calli were not obtained from Nipponbare, which transformed with *Agrobacterium* strain EHA105 following CIAT's transformation procedure. Furthermore, few or no T_1 seeds were obtained from transgenic plants. Curinga provided 1.1 to 9.7 g seed per transformed plant; however, all seeds that were harvested from transgenic Fedearroz50 were sterile. The reasons for this difference is unknown, but the selection of the bacterial strain and *Agro*-infection method might be relevant, as seen in various rice cultivars (Aldemita *et al*. 1996, Rashid *et al*. 1996, Hiei *et al*. 1997, Ishizaki *et al*. 2007). Gene copy number and rearrangements in the transformed plants should be also considered.

Table 5.2-1. Significance for source of variations in calli induction and plant regeneration for transformation candidate rice genotypes.

	Trait						
Trait	Green spot (Number)	Embryogenesis (Number)	Necrosis (Number)	Root (Number)	Plantlet (Number)	Regenerated Plant (Number)	Albino plant (Number)
Genotype	**	**	**	**	**	**	**
Calli Induction Temperature	ns	ns	*	ns	ns	ns	ns
Genotype *C*alliIndTemp*	ns	ns	*	ns	*	ns	ns
Regeneration Temperature	ns	*	ns	ns	ns	ns	ns
Genotype **RegTemp*	ns	ns	ns	ns	ns	ns	ns
CalliIndTemp **RegTemp*	ns	ns	ns	ns	ns	ns	ns
Genotype **CalliIndTemp* **RegTemp*	ns	ns	ns	ns	ns	ns	ns
Medium	**	ns	**	**	ns	ns	ns
Genotype *Medium	ns	**	**	**	ns	ns	ns
CalliIndTemp *Medium	ns	ns	ns	ns	ns	ns	ns
Genotype **CalliIndTemp* *Medium	ns	**	ns	ns	ns	ns	ns
RegTemp *Medium	ns	ns	ns	ns	*	*	ns
Genotype **RegTemp* *Medium	ns	ns	ns	ns	ns	ns	ns
CalliIndTemp **RegTemp* *Medium	ns	ns	ns	ns	ns	ns	ns
Genotype **CalliIndTemp* **RegTemp* *Medium	ns	*	ns	ns	ns	ns	ns

*: Significant at 0.05<*P*<0.01; **: Significant at *P*≤0.01; ns: No significant.
Abbreviations: *CalliIndTemp.*, Calli induction temperature; *RegTemp.*, Regeneration temperature.
Note: Green spot (Number): Calli on which green spots were observed, which generally regenerated into a plant. Embryogenesis (Number): Embriogenetic calli on which transparent color parts were observed. Necrosis (Number): Necrotic calli were considered those, which showed brown color parts. Roots (Number): Calli on which only root regeneration was observed. Plantlet (Number): Calli with more than one plantlet (without roots). Regenerated Plant (Number): Completely regenerated plantlet (with roots). Albino plant (Number): Calli with more than one albino plant (with roots). When several symptoms appeared simultaneously in the same calli, the observed predominant symptom in the calli was recorded. The calli was evaluated as a plantlet when at least one plantlet was observed at calli.

Table. 5.2-2.1. Number of green spots per calli of four-rice genotypes on MSKA regeneration medium at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CallilndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Curinga	0.00±0.00	0.00		2.00±2.00	0.00	223.60	1.00±1.00	10.00	316.20
28	INTA	0.00±0.00	0.00		2.00±2.00	0.00	223.60	1.00±1.00	10.00	316.20
28	Nipponbare	2.00±2.00	20.00	223.60	5.00±2.88	33.33	115.50	3.33±1.66	25.00	150.00
28	Global	0.50±5.00	5.00	447.20	2.10±0.96	17.54	199.00	1.28±0.54	11.47	264.20
32	CT15944	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Curinga	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	8.22±3.778	71.35	102.70	2.00±2.00	20.00	223.60	5.11±2.26	51.35	140.20
32	Global	2.05±1.19	28.36	259.10	0.50±5.00	5.00	447.20	1.27±0.65	16.87	321.50
Global	CT15944	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
Global	Curinga	0.00±0.00	0.00		1.00±1.00	10.00	316.20	0.50±0.50	5.00	447.2
Global	INTA	0.00±0.00	0.00		1.00±1.00	10.00	316.20	0.50±0.50	5.00	447.2
Global	Nipponbare	5.11±2.26	51.35	140.20	3.33±1.66	25.00	150.00	4.26±1.40	37.62	143.70
Global	Global	1.27±0.65	16.87	321.50	1.28±0.54	11.47	264.20	1.28±0.42	14.02	292.60

Abbreviations: *CallilndTemp.*, Calli induction temperature.

Table. 5.2-2.2. Number of green spots per calli of four-rice genotypes on R-III regeneration medium at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CallilndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	4.00±2.44	30.00	136.9	2.00±2.00	20.00	223.60	3.00±1.52	23.33	161.00
28	Curinga	6.00±4.00	80.00	149.1	12.44±5.09	129.87	91.60	9.22±3.23	104.81	111.00
28	INTA	0.00±0.00	0.00		4.00±4.00	80.00	223.60	2.00±2.00	40.00	316.20
28	Nipponbare	6.44±4.39	96.54	152.50	8.22±2.06	21.35	56.20	7.33±2.30	40.00	316.20
28	Global	4.11±1.58	50.30	172.50	6.66±1.87	69.91	125.40	5.38±1.22	60.24	144.00
32	CT15944	2.00±2.00	20.00	223.60	2.00±2.00	20.00	223.60	2.00±1.33	17.77	210.80
32	Curinga	2.00±2.00	20.00	223.60	5.33±2.26	25.55	94.8	3.67±1.52	23.33	131.70
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	4.22±2.59	33.58	137.20	14.50±5.02	126.25	77.50	9.36±3.16	100.37	107.00
32	Global	2.05±0.94	17.84	205.50	5.45±1.85	68.67	151.80	3.75±1.06	45.11	178.80
Global	CT15944	3.00±1.52	23.33	161.00	2.00±1.33	17.77	210.80	2.5±0.99	19.73	177.70
Global	Curinga	4.00±2.21	48.88	174.80	8.88±2.88	83.12	102.60	6.44±1.85	68.82	128.70
Global	INTA	0.00±0.00	0.00		2.00±2.00	40.00	316.20	1.00±1.92	20.00	447.20
Global	Nipponbare	5.33±2.43	59.20	144.30	11.36±2.76	76.55	316.20	8.34±1.92	73.86	103.00
Global	Global	3.08±0.92	34.28	189.90	6.06±1.30	67.89	135.90	4.57±0.81	52.68	158.07

Abbreviations: *CallilndTemp.*, Calli induction temperature.

Table. 5.2-2.3. Number of green spots per calli of four-rice genotypes on two regeneration media (Global) at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CallilndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	2.00±1.33	17.77	210.80	1.00±1.00	10.00	316.20	1.50±0.81	13.42	244.20
28	Curinga	3.00±2.13	45.55	225.00	7.22±3.11	96.91	136.30	5.11±1.90	72.17	166.20
28	INTA	0.00±0.00	0.00		3.00±2.13	45.55	225.00	1.50±1.09	23.94	326.20
28	Nipponbare	4.22±2.39	57.28	179.30	6.79±1.70	26.06	75.20	5.34±1.48	41.96	119.10
28	Global	2.36±0.87	30.28	238.70	4.44±1.11	48.60	156.90	3.36±0.71	39.98	188.10
32	CT15944	1.00±1.00	10.00	316.20	1.00±1.00	10.00	316.20	1.00±0.68	9.47	307.80
32	Curinga	1.00±1.00	10.00	316.20	2.66±1.38	19.25	164.60	1.83±0.85	14.59	208.40
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	6.22±2.26	51.08	114.90	8.25±3.29	108.40	126.20	7.23±1.95	76.62	121.00
32	Global	2.05±0.75	22.51	230.80	2.97±1.02	42.19	218.00	2.51±0.63	32.16	225.30
Global	CT15944	1.50±0.81	13.42	244.20	1.00±0.68	9.47	307.80	1.25±0.53bc	11.21	267.90
Global	Curinga	2.00±1.17	27.36	261.60	4.94±1.73	60.49	157.30	3.47±1.06b	45.02	193.30
Global	INTA	0.00±0.00	0.00		1.50±1.09	23.94	326.20	0.75±0.55c	12.24	466.50
Global	Nipponbare	5.22±1.61	52.38	138.60	7.55±1.86	66.34	107.80	6.36±1.23a	59.01	120.80
Global	Global	2.18±0.57	26.08	234.20	3.70±0.75	45.32	181.80			

Abbreviations: *CallilndTemp.*, Calli induction temperature. Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table. 5.2-3.1. Number of regenerated albino plants of four-rice genotypes on MSKA regeneration medium at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CallilndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	0.20±0.20	0.20	223.60	0.40±0.40	0.80	223.60	0.30±0.21	0.45	225.00
28	Curinga	1.20±1.20	7.20	223.30	0.40±0.24	0.30	136.90	0.80±0.59	3.51	234.20
28	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Global	0.35±0.32	1.81	385.30	0.21±0.21	0.28	254.30	0.28±0.16	1.05	363.30
32	CT15944	0.00±0.00	0.00		0.60±0.40	0.80	149.10	0.30±0.21	0.45	225.00
32	Curinga	0.80±0.49	1.20	136.90	0.20±0.20	0.20	223.60	0.50±0.26	0.72	170.00
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	0.00±0.00	0.00		0.40±0.40	0.80	223.60	0.20±0.20	0.40	316.20
32	Global	0.20±0.13	0.37	307.80	0.30±0.14	0.43	219.00	0.25±0.10	0.39	252.20
Global	CT15944	0.10±0.10	0.10	316.20	0.50±0.26	0.72	170.00	0.30±0.14	0.43	219.00
Global	Curinga	1.00±0.61	3.77	194.40	0.30±0.15	0.23	161.00	0.65±0.31	2.02	219.10
Global	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
Global	Nipponbare	0.00±0.00	0.00		0.22±0.22	0.44	300.00	0.10±0.10	0.21	435.90
Global	Global	0.27±0.16	1.07	377.30	0.25±0.09	0.35	231.90	0.26±0.09	0.71	317.10

Abbreviations: *CallilndTemp.*, Calli induction temperature.

Table. 5.2-3.2. Number of regenerated albino plants of four-rice genotypes on R-III regeneration medium at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CalliIndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	0.20±0.20	0.20	223.60	0.40±0.40	0.80	223.60	0.30±0.21	0.45	225.00
28	Curinga	0.20±0.20	0.20	223.60	0.00±0.00	0.00		0.10±0.10	0.10	316.20
28	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Global	0.10±0.06	0.09	307.80	0.10±0.10	0.20	447.20	0.10±0.06	0.14	378.90
32	CT15944	0.40±0.40	0.80	223.60	0.00±0.00	0.00		0.20±0.20	0.40	316.20
32	Curinga	0.40±0.40	0.80	223.60	1.40±0.74	2.80	119.50	1.00±0.47	2.22	149.10
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Global	0.25±0.17	0.61	314.60	0.35±0.22	0.97	282.30	0.30±0.14	0.77	294.30
Global	CT15944	0.30±0.21	0.45	225.00	0.20±0.20	0.40	316.20	0.25±0.14	0.40	255.50
Global	Curinga	0.40±0.30	0.93	241.50	0.70±0.42	1.78	191.10	0.55±0.25	1.31	208.40
Global	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
Global	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
Global	Global	0.17±0.09	0.35	339.60	0.25±0.12	0.58	341.10	0.20±0.07	0.46	341.30

Abbreviations: *CalliIndTemp.*, Calli induction temperature.

Table. 5.2-3.3. Number of regenerated albino plants of four-rice genotypes on two regeneration media (Global) at two different calli induction/regeneration temperatures.

		Regeneration Temperature								
		24-26 °C			28 °C			Global		
CalliIndTemp	Genotype	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
28	CT15944	0.20±0.13	0.17	210.80	0.40±0.26	0.71	210.80	0.30±0.14	0.43	219.00
28	Curinga	0.70±0.59	3.56	269.80	0.20±0.13	0.17	210.80	0.45±0.30	1.83	301.40
28	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
28	Global	0.22±0.15	0.94	432.80	0.15±0.07	0.23	317.70	0.19±0.08	0.59	405.10
32	CT15944	0.20±0.20	0.40	316.20	0.30±0.21	0.45	225.00	0.25±0.14	0.40	255.50
32	Curinga	0.70±0.36	1.34	165.60	0.80±0.41	1.73	164.60	0.75±0.27	1.46	161.10
32	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00	0.00	
32	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.10±0.10	0.20	447.20
32	Global	0.25±0.11	0.48	310.00	0.35±0.13	0.68	254.90	0.27±0.08	0.58	277.30
Global	CT15944	0.20±0.11	0.27	261.60	0.35±0.16	0.55	212.90	0.27±0.10ab	0.41	232.70
Global	Curinga	0.70±0.34	2.32	217.90	0.50±0.22	1.00	200.00	0.60±0.20a	1.63	212.80
Global	INTA	0.00±0.00	0.00		0.00±0.00	0.00		0.00±0.00b	0.00	
Global	Nipponbare	0.00±0.00	0.00		0.00±0.00	0.00		0.05±0.05b	0.10	624.50
Global	Global	0.22±0.09	0.70	374.00	0.24±0.07	0.46	284.20			

Abbreviations: *CalliIndTemp.*, Calli induction temperature. Different letters in the table denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

Table 5.2-4. Generation of transgenic plants following Toki and CIAT protocols.

Protocol	Toki (2006)					CIAT			
Genotype	Fedearroz50	Curinga		Nipponbare		Curinga		Nipponbare	
Agrobacterium strain	AGL1	AGL1	EHA105	AGL1	Control	AGL1	EHA105	AGL1	EHA105
Agrobacterium infected calli (A)	88	30	44	99	22	18	40	40	60
Hygromycin resistant calli†(B)	11	26	28	12	19	12	16	15	0
Percentage of Hyg. resistance calli proliferation (B)/(A)*100	12.50	86.67	63.64	12.12	86.36	66.67	40.00	37.50	0.00
Gus tested calli (C)	3	144	193	4	0	7	16	12	0
Gus expressed calli (D)	2	15	50	2	0	7	11	12	0
Gus expression efficiency (%=(D)/ (C) *100)	66.67	10.42	25.91	50.00	0.00	100.00	68.75	100.00	0.00
Regeneration tested calli (E)	63	130	124	63	36	10	8	NA	0
Plant regenerated calli‡ (F)	5	2	8	33	12	1	1	NA	0
Plant regeneration efficiency % (F) / (E)*100	7.94	1.54	6.45	52.38	33.33	10.00	12.50	0.00	0.00
Total regenerated plants	19	3	24	149	41	1	1	1	0
Number of transferred plants to greenhouse	9	3	24	26	2	1	1	1	0
Gus positive plants	5	0	7	18	0	0	0	0	0
PCR positive plants	3	0	6	12	0	0	0	0	0

NA; Not Available

†; Medium, which contained 50 ml/L (for Fedearroz50 and Nipponbare), 20 ml/L (for Curinga at Toki's protocol and Curinga/AGL1 at CIAT's protocol) or 10 mg/L (Curinga/EHA105 at CIAT's protocol) of Hygromycin and 500 ml/L of Cefotaximine.

‡; Medium, which contained 30 ml/L (for Fedearroz50 and Nipponbare) or, 5 ml/L (for Curinga) of Hygromycin and 250 ml/L of Cefotaximine.

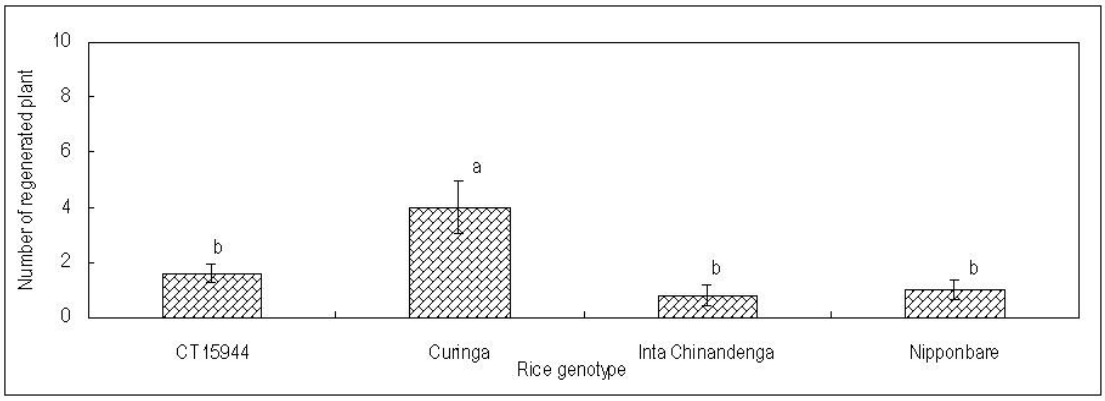

Fig. 5.2-1.1. Number of regenerated plants of four-rice genotypes (Global). Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

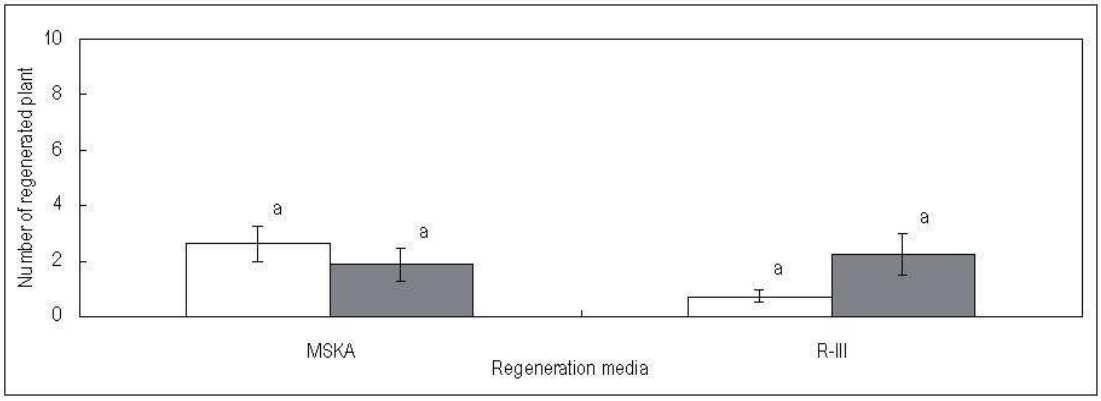

Fig. 5.2-1.2. Number of regenerated plants on two different regeneration media, and two different regeneration temperatures. White poles represent regeneration temperature at 24-26°C, dark poles represent regeneration temperature at 28°C. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

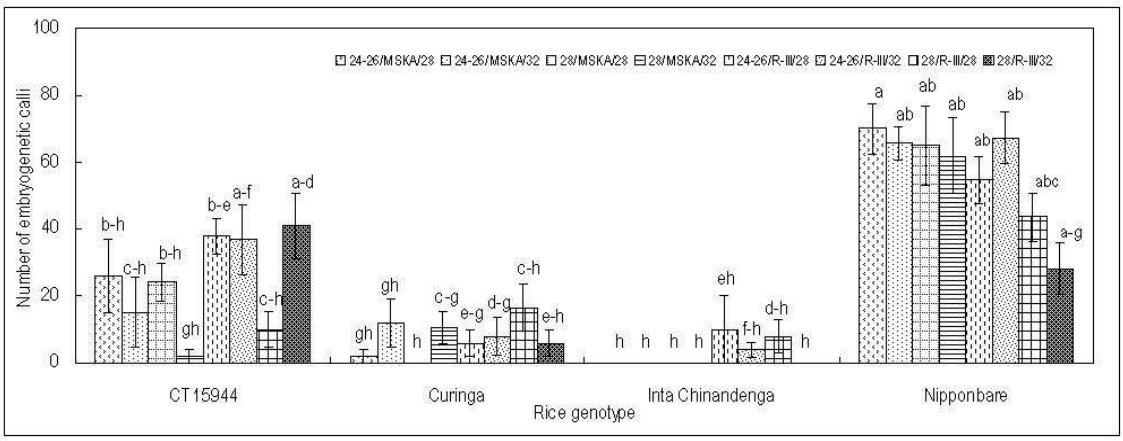

Fig. 5.2-2. Number of embryogenetic calli on two different regeneration media, and two different regeneration temperatures.
Key: The first number indicates the calli Induction temperature, the following number indicates the regeneration medium and the last number indicates the regeneration temperature. For example, 24-26/MSKA/28 refers to a calli induced at 24-26°C, then transferred to a MSKA medium to regeneration, and then incubated at 28°C. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

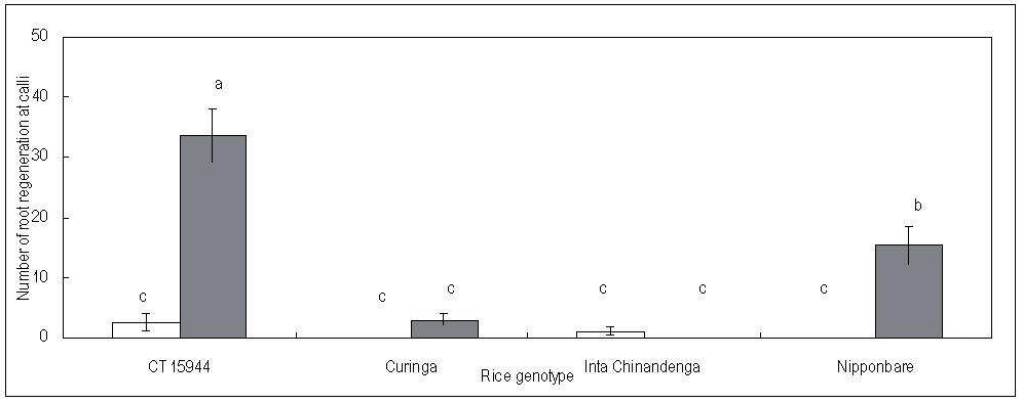

Fig. 5.2-3. Number of root regeneration per calli on two different regeneration media. White poles represents MSKA medium; dark poles represent R-III medium. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

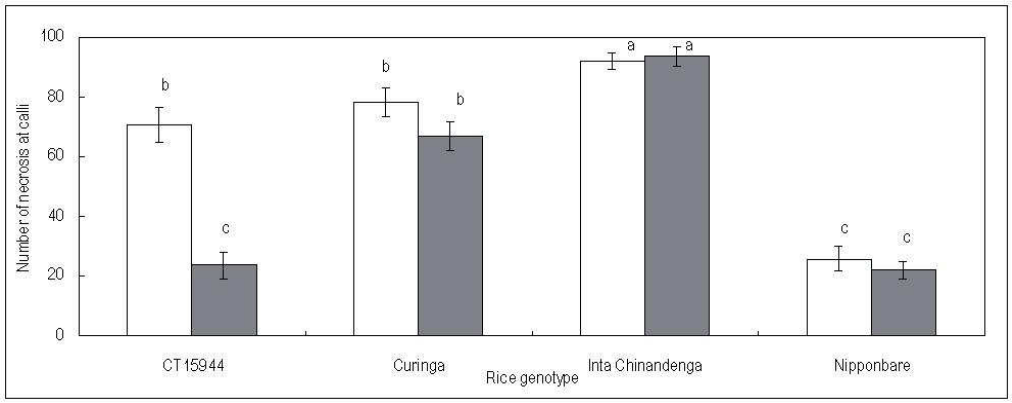

Fig. 5.2-4. Number of necrosis symptoms per calli on two different regeneration media. White poles represent MSKA medium; dark poles represent R-III medium. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

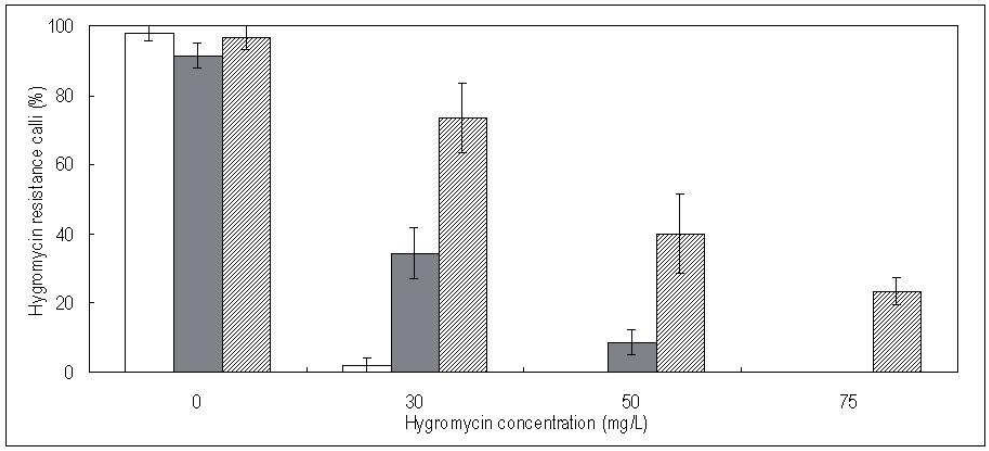

Fig. 5.2-5. Hygromycin resistance calli of three rice genotypes. White poles represent Curinga; dark poles represent CT15944, and striped poles represent Nipponbare. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

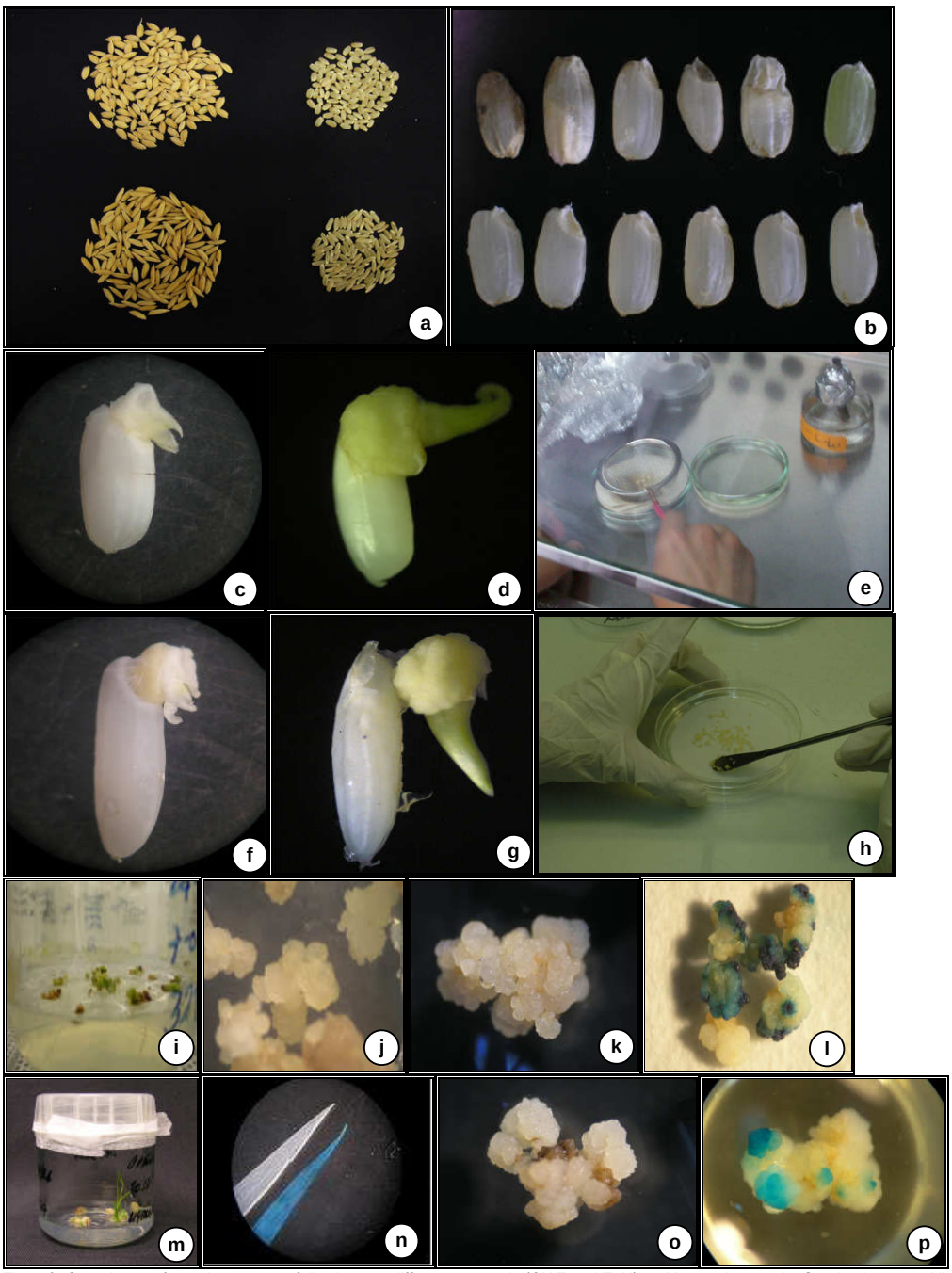

Fig. 5.3. Genetic transformation process following two different protocols (CIAT and Toki). a. Mature rice seeds. Seeds above are Nipponbare; seeds below are Fedezrroz50; b. Critical seed selection; c, d, f, and g. Seed germination following Toki's protocol, c and d are Nipponbare, f and g are Curinga, c and f are 2 days after sowing, d and g are 6 days after sowing; e. *Agrobacterium* infection following Toki's protocol; h. *Agrobacterium* infection following CIAT's protocol; i. Calli regeneration at regeneration medium; j. Calli proliferation of Curinga on hygromycin not containing medium; k. Hygromyicin resistance calli of Nipponbare; l. *gus* expressions on hygromyicin resistance calli of Nipponbare; m. plantlet regeneration of transformed calli of Curinga; n. *gus* expressions at transformed Fedezrroz50 leaves; o. hygromyicin resistance calli of Curinga; p. *gus* expressions on hygromyicin resistance calli of Curinga.

5.3. Production and selection of homozygous DREB transgenic rice lines

The success of contemporary breeding programs involving genetic engineering depends on the stability of transgene expression over many generations (Ukai 2003). Inserted genes following Mendelian inheritance are known in a large number of crops (Umbeck *et al*. 1989). Gahakwa *et al*. (2000) reported that stable transgene expression was observed at subsequent generations in a total eleven lines of evaluated transgenic rice.

Transgenic Palmar, transformed using the *Lip9::OsDREB1B* construct 212-1, showed completely hygromycin-resistant at T_2 seed generation; however, other tested transgenic lines also presented some hygromycin resistance (Table 5.3). Particularly, the transgenic CT6241, which had OsDREB1B 30-1, 30-2 and 30-3 were highly susceptible to hygromycin, although the germination was very high. On the other hand, albino plants and delayed germination were observed at transgenic CT6241 (data not shown). Transgenic CICA8 showed segregation between evaluated lines; completely hygromycin-resistant lines were not obtained.

These results suggest that more transgenic lines were necessary to obtain homozygous lines at T_2 generation. The segregation ratio of single copy is 3:1, and the probability of obtaining homozygous line at T_2 seed generation is 0.25. According to the calculation by Schwager *et al*. (1993), the sample size required to produce at least one homozygous line at T_2 generation, with a 0.95 probability, is eleven. The result suggests that the transgenic Palmar 212-1, which showed complete hygromycin-resistance, could be considered a homozygous line. This transgenic line is useful for future studies.

Table 5.3. Hygromycin resistance of T_2 transgenic plants.

Genotype	Gene	Line	Germination (%)	SE	Hygromycin resistance	Hygromycin susceptible
CICA8	AtDREB1A	59-1	87.33	5.98	12	24
CICA8	AtDREB1A	59-2	87.11	3.94	15	21
CICA8	AtDREB1A	59-3	76.67	7.24	15	21
CICA8	AtDREB1A	59-4	89.33	0.27	11	30
CICA8	AtDREB1A	59-5	62.50	4.17	8	10
CICA8	AtDREB1A	59-6	76.11	7.11	9	21
CICA8	OsDREB1B	37-1	89.17	7.86	26	18
CICA8	OsDREB1B	37-2	92.02	2.09	29	12
CICA8	OsDREB1B	37-3	92.48	4.73	30	14
CICA8	OsDREB1B	37-4	91.39	2.26	29	13
CICA8	OsDREB1B	37-5	82.54	7.01	13	11
CICA8	OsDREB1B	37-6	90.55	4.28	32	13
CICA8	OsDREB1B	41-1	73.78	5.77	14	14
CICA8	OsDREB1B	41-2	89.33	4.73	18	20
CICA8	OsDREB1B	41-3	79.44	5.53	10	20
CICA8	OsDREB1B	41-4	74.31	6.93	2	18
CICA8	OsDREB1B	41-5	88.10	0.79	14	13
CICA8	OsDREB1B	41-6	67.78	10.79	6	21
CICA8	OsDREB1B	44-2	75.93	3.03	12	10
CICA8	OsDREB1B	44-3	93.64	3.19	25	4
CICA8	OsDREB1B	44-5	96.00	4.00	34	15
CICA8	OsDREB1B	44-6	77.86	4.61	25	5
CICA8	OsDREB1B	43-5	82.00	5.83	26	7
CICA8 BCF078 (Control)			93.33	4.44	0	42
CT6241	OsDREB1B	47-6	95.56	2.72	31	8
CT6241	OsDREB1B	30-1	100.00	0.00	0	49
CT6241	OsDREB1B	30-2	95.56	4.44	0	47
CT6241	OsDREB1B	30-3	100.00	0.00	1	48
CT6241	OsDREB1B	25-1	96.00	4.00	16	24
CT6241	OsDREB1B	25-2	98.18	1.82	21	15
CT6241	OsDREB1B	47-6	97.50	2.50	31	8
CT6241-17-1-5-1 BCF1096 (Control)			98.61	1.39	0	45
Palmar	AtDREB1A	92-4	96.00	4.00	43	8
Palmar	AtDREB1A	107-4	94.00	4.00	36	14
Palmar	OsDREB1B	212-1	81.11	5.25	35	0
Palmar	OsDREB1B	155-1	80.29	6.98	24	14
Palmar	OsDREB1B	155-4	88.00	5.83	23	19
Palmar	OsDREB1B	302-1	82.18	7.40	21	17
Palmar	OsDREB1B	302-2	83.33	5.58	34	6
Palmar	OsDREB1B	302-3	84.17	5.83	23	7
Palmar	OsDREB1B	302-4	82.00	9.17	23	6
Palmar	OsDREB1B	302-5	93.33	3.33	18	2
Palmar BCF962 (Control)			76.00	5.10	0	38

5.4. Vegetative stage screening of homozygous DREB transgenic rice lines using BigTrays.

Air temperature was kept at around 30^{o}C during experiments 3 and 4. FC showed significant differences (P<0.05) between control and water-limited conditions starting at 45 DAS (Fig. 5.4-1).

ANOVA of data collected in two experiments indicated that water conditions effects were highly significant for plant height, leaf temperature, difference of temperatures between leaf and air, and biomass productions. Differences amongst independent transgenic lines were also highly significant for plant height, tiller number, plant recovery, and biomass production (Table 5.4-1). However, variation due to transformation of plants was not significant for leaf temperature, nor temperature difference. Leaf number was not significantly affected by water conditions in any transgenic lines (Table 5.4-2). Significant differences for the response of individual lines for leaf number and leaf rolling scores were not observed (data not shown).

There were significant differences amongst lines for tiller number. However, the evaluated transgenic lines (except IX-P-B-212-5) produced a similar number of tillers as the control plant. The effect of water treatment was not significant for tiller number amongst these evaluated transgenic lines (Table 5.4-3) except for IX-P-B-212-5.

Plants that were under water-limited conditions maintained a higher leaf temperature than plants under normal screenhouse conditions (Table 5.4-4); thus, temperature differences between leaf and surrounding air were significantly affected by water treatments (Table 5.4-5). The results probably indicate that the plants preserved water by shrinking their auricles, emitting only the heat by keeping their stomata opened in order to prevent water evaporation, under water-limited conditions. Leaf temperature is correlated with transpiration and transpiration is related to water loss from plants in the form of vapor. This is a

dominant process in plant-water relations because of the large volume of water involved and its controlling influence on plant water status (Kramer *et al*. 1995). Future studies are needed to elucidate this correlation.

Plant recovery score was significantly (P<0.01) affected amongst evaluated plants. However, Ryan's multiple range tests did not show significant differences between transgenic lines and Palmar, except for IX-P-B-212-5 (Fig. 5.4-2). These results suggest that the recovery of four transgenic events using the *Lip9*::*AtDREB1A* construct was similar to the non-transgenic Palmar when a discontinued drought stress was imposed.

Non-transgenic Palmar at normal irrigated conditions grew taller than other transgenic lines (Fig. 5.4-3, and 5.4-7). Significant differences in plant height were not observed amongst the transgenic lines transformed using the *Lip9*::*AtDREB1A* construct and Palmar, except VII-P-A-107-3 in experiment 4. Transgenic lines, based on the *Lip9*::*OsDREB1B* construct, grew dwarf at both water treatments. Plant height of IX-P-B-212-5 was not significantly affected by field capacity. These results suggest that I-P-A-43-3, III-P-A-70-5 and IX-P-A-165-6 showed similar plant height as non-transgenic Palmar under water-limited conditions. Furthermore, these three transgenic lines showed similar performances for plant height at both FC>85% and FC<20-35% soil moisture conditions.

Fresh matter showed significant differences amongst lines in the two water treatment interactions except for IX-P-B-212-5 (Fig. 5.4-4), although dry matter amongst evaluated lines was not significantly different using Ryan's multiple range tests under drought stress conditions (Fig. 5.4-5). Differences in the measurements between fresh matter and dry matter were significantly affected by the transgenic lines in the water treatment interactions, except in IX-P-B-212-5 (Fig. 5.4-6). The difference between fresh and dry matter also indicates efficient water use in the lines. The effect of water treatment was not significant for biomass production of

IX-P-B-212-5 in spite of having less biomass production than other transgenic lines.

On the other hand, the agronomic traits measured in these experiments such as plant height, tiller number and biomass production of the transgenic line IX-P-B-212-5 was not significantly influenced by water treatments. These results suggest that maybe the agronomic performances of IX-P-B-212-5 were related with the expression of the OsDREB1B gene. However, it is not clear that the OsDREB1B gene was consistently expressed at these soil moisture levels.

In general, independently of the DREB gene and genotype, transgenic plants were dwarf, highly sterile, and also showed growth delay under non-stressed growth conditions (Lee *et al*. 2004, Ito *et al*. 2006). Similar phenomena have been reported for transgenic *Arabidopsis*, tomato, tobacco and wheat overexpressing DREB1A/CBF3 or CBF1/DREB1B (Jaglo-Ottosen *et al*. 1998, Kasuga *et al*. 1998, Jaglo *et al*. 2001). Ito *et al.* (2006) reported that overexpression of the OsDREB1A and OsDREB1B proteins also caused growth delay under non-stress control conditions in transgenic rice. In the case of Palmar, T_0 transgenic plants were shorter and showed delayed flowering compared with non-transgenic plants (Fory *et al*. 2005). These phenotypic effects on plant development due to the DREB transgenes require more detailed analyses in the future.

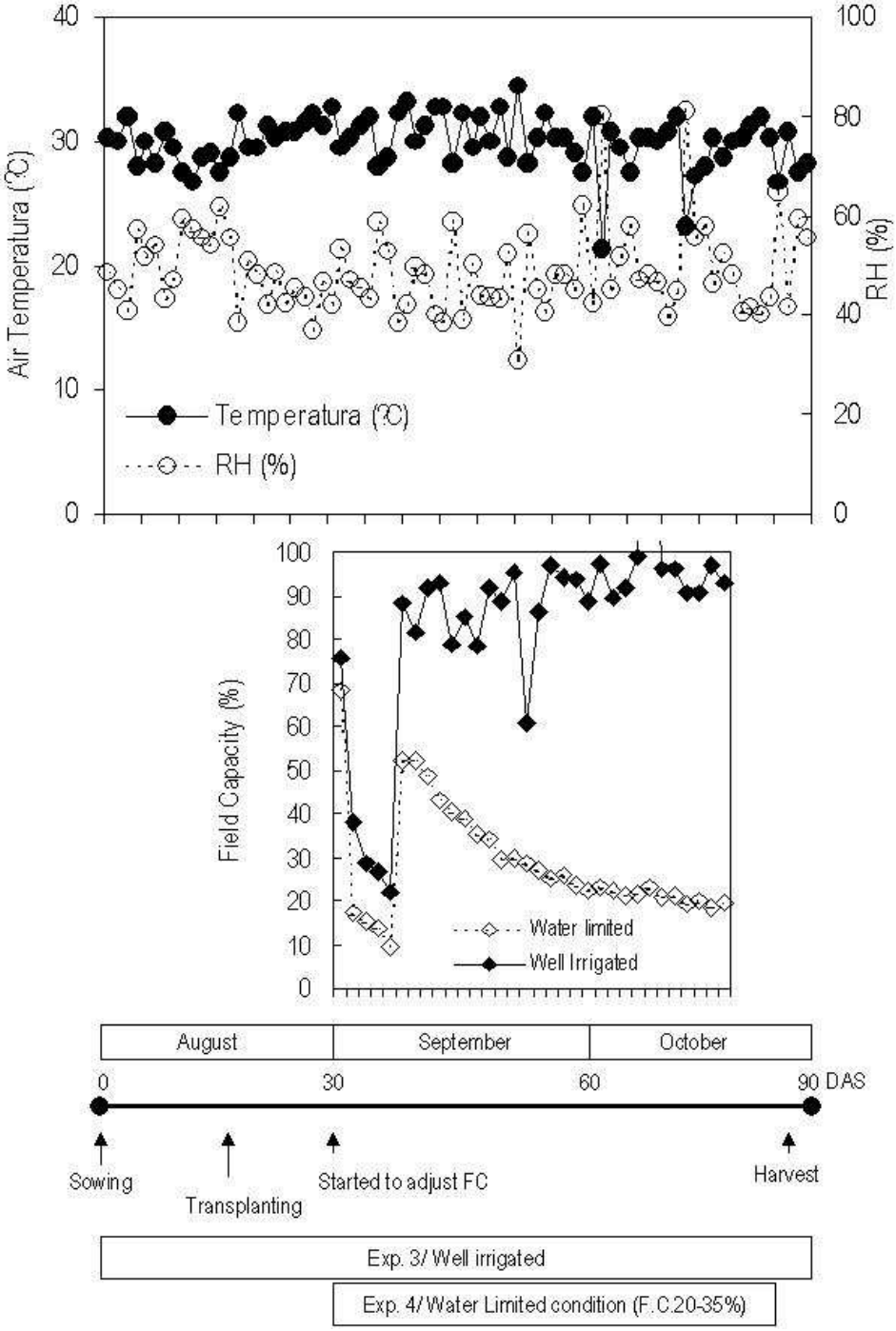

Fig. 5.4-1. Environmental conditions and time lines of screenhouse experiments

Table 5.4-1. Significance for source of variations in measured traits at screenhouse experiments.

Trait	Source of variations		
	Water Treatment	Transgenic Plan	WaterTreatment *Transgenic Plant
Leaf number	ns	ns	-
Leaf rolling	-	ns	-
Plant recovery	-	**	-
Tiller number	ns	**	ns
Leaf temperature	**	ns	ns
Temperatures difference	**	ns	ns
Plant teight	**	**	*
Fresh matter	**	**	**
Dry matter	**	**	**
Difference fresh-dry matters	**	**	**

*: Significant at $0.05 \leq P \leq 0.01$; **: Significant at $P \leq 0.01$; ns: No significant at $P \geq 0.05$; -: Data was recorded under water-limited conditions only.

Table 5.4-2. Leaf number of six independents Palmar homozygous DREB transgenic lines at screenhouse experiments

Transgenic Plant	Water Treatment								
	Well-irrigated			Water-limited			Global		
	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	8.88±0.14	1.41	13.04	8.68±0.11	0.89	10.9	8.78±0.09	1.16	12.2
III-P-A-70-5	9.06±0.14	1.39	13.0	9.04±0.10	0.73	9.5	9.05±0.09	1.05	11.3
VII-P-A-107-3	8.87±0.13	1.22	12.4	8.47±0.11	0.8	10.5	8.67±0.09	1.04	11.8
IX-P-A-165-6	9.03±0.14	1.35	12.8	8.95±0.10	0.76	9.8	8.99±0.09	1.05	11.4
IX-P-B-212-5	9.21±0.15	1.67	14.0	8.74±0.13	1.11	12.1	8.98±0.10	1.44	13.4
X-P-B-278-1	9.03±0.13	1.25	12.4	8.69±0.10	0.76	10	8.86±0.09	1.03	11.4
Palmar BCF962	9.09±0.12	1.16	11.9	8.81±0.10	0.75	9.8	8.95±0.08	0.97	11
Global	9.03±0.05	1.34	12.8	8.77±0.04	0.85	10.5	8.90±0.03	1.11	11.9

Table 5.4-3. Tiller number of six independents Palmar homozygous DREB transgenic lines at screenhouse experiments

Transgenic Plant	Water Treatment								
	Well-irrigated			Water-limited			Global		
	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	2.32±0.09	0.75	37.3	2.32±0.09	0.65	34.8	3.32±0.06c	0.7	36
III-P-A-70-5	3.07±0.08	0.6	25.2	3.10±0.08	0.52	23.3	3.08±0.06a	0.56	24.2
VII-P-A-107-3	2.44±0.07	0.42	26.5	2.56±0.06	0.35	23	2.50±0.05bc	0.38	24.8
IX-P-A-165-6	3.11±0.08	0.51	22.9	2.90±0.08	0.59	26.5	3.01±0.06ab	0.56	24.8
IX-P-B-212-5	1.50±0.06	0.33	38.0	1.51±0.06	0.33	37.9	1.50±0.04d	0.32	37.9
X-P-B-278-1	2.44±0.09	0.63	32.7	2.26±0.09	0.75	38.3	2.35±0.06c	0.7	35.5
Palmar BCF962	2.73±0.08	0.58	27.9	2.51±0.10	0.81	35.9	2.62±0.06abc	0.71	32
Global	2.52±0.04	0.79	35.4	2.45±0.04	0.79	36.3			

Different letters in the table denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

Table 5.4-4. Leaf temperature of six independents Palmar homozygous DREB transgenic lines at screenhouse experiments

	Water Treatment								
	Well-irrigated (b)			Water-limited (a)			Global		
Transgenic Plant	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	28.13±0.26	0.96	3.5	31.89±0.25	0.9	3	30.01±0.40	4.57	7.1
III-P-A-70-5	28.54±0.63	5.54	8.2	31.97±0.21	0.63	2.5	30.26±0.46	6.02	8.1
VII-P-A-107-3	29.09±0.40	2.19	5.1	32.20±0.37	1.78	4.1	30.59±0.41	4.43	6.9
IX-P-A-165-6	28.29±0.36	1.81	4.8	32.26±0.23	0.74	2.7	30.27±0.44	532	7.6
IX-P-B-212-5	28.61±0.57	4.59	7.5	31.96±0.31	1.19	3.4	30.16±0.47	5.8	8
X-P-B-278-1	28.64±0.62	5.33	8.1	32.24±0.35	1.7	4	30.49±0.50	6.95	8.6
Palmar BCF962	29.03±0.59	5.17	7.8	32.45±0.21	0.63	2.5	30.74±0.44	5.84	7.9
Global	28.62±0.19	3.56	6.6	32.16±0.10	1.04	3.2			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

Table 5.4-5. Temperature difference of six independents Palmar homozygous DREB transgenic lines at screenhouse experiments

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic Plant	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	-3.93±0.28	1.1	-27	-0.52±0.45	2.82	-322	-2.23±0.42	4.9	-99
III-P-A-70-5	-3.28±0.59	4.85	-67.0	-0.40±0.40	2.2	-371	-1.84±0.44	5.54	-128
VII-P-A-107-3	-2.97±0.40	2.29	-51	-0.32±0.49	3.16	-551	-1.70±0.40	4.42	-124
IX-P-A-165-6	-3.59±0.36	1.85	-38	0.14±0.35	1.75	947	-1.73±0.44	5.32	-134
IX-P-B-212-5	-3.44±0.43	2.61	-47.0	-0.70±0.45	2.47	-225	-2.17±0.41	4.39	-96
X-P-B-278-1	-3.17±0.63	5.61	-75	-0.09±0.51	3.69	2240	-1.54±0.51	7.22	-174
Palmar BCF962	-2.95±0.62	5.79	-82	0.37±0.35	1.89	375	-1.29±0.47	6.55	-198
Global	-3.33±0.18	3.36	-55	-0.18±0.16	2.53	-903			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

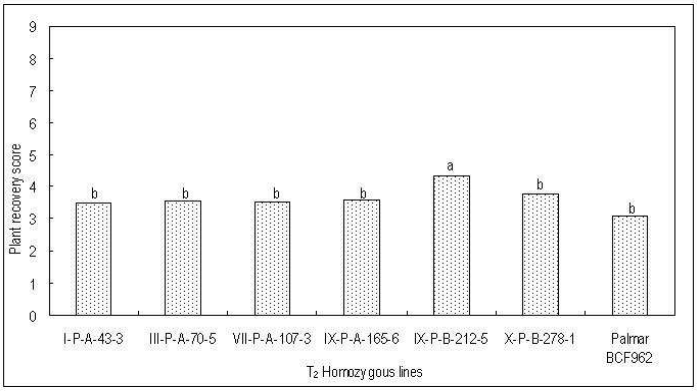

Fig. 5.4-2. Plant recovery score of six independents Palmar homozygous DREB transgenic lines which were imposed to drought stress. Different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

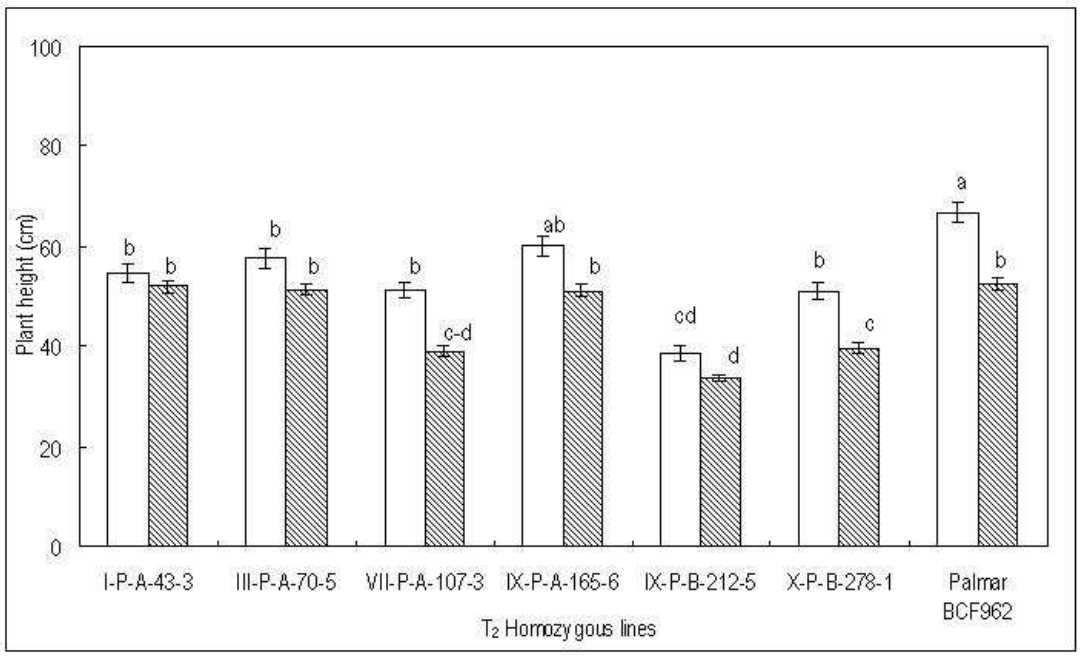

Fig. 5.4-3. Plant height of six independents Palmar homozygous DREB transgenic lines treated at two water conditions. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

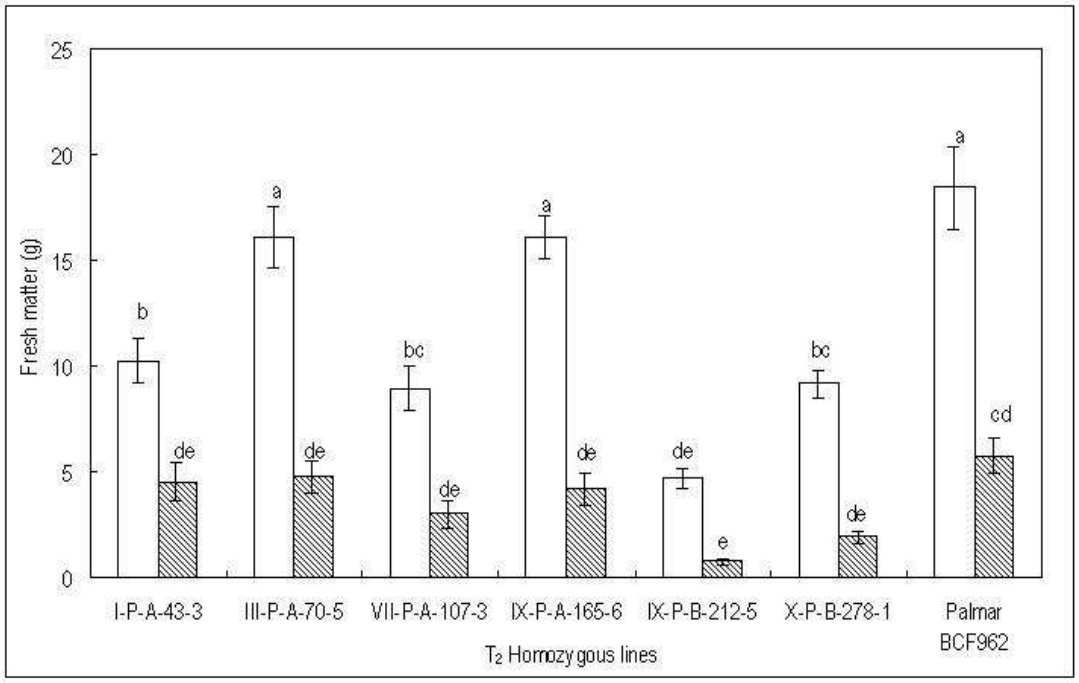

Fig. 5.4-4. Biomass production. Fresh matter of six independents Palmar homozygous DREB transgenic lines treated at two water conditions. White poles represent well–irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

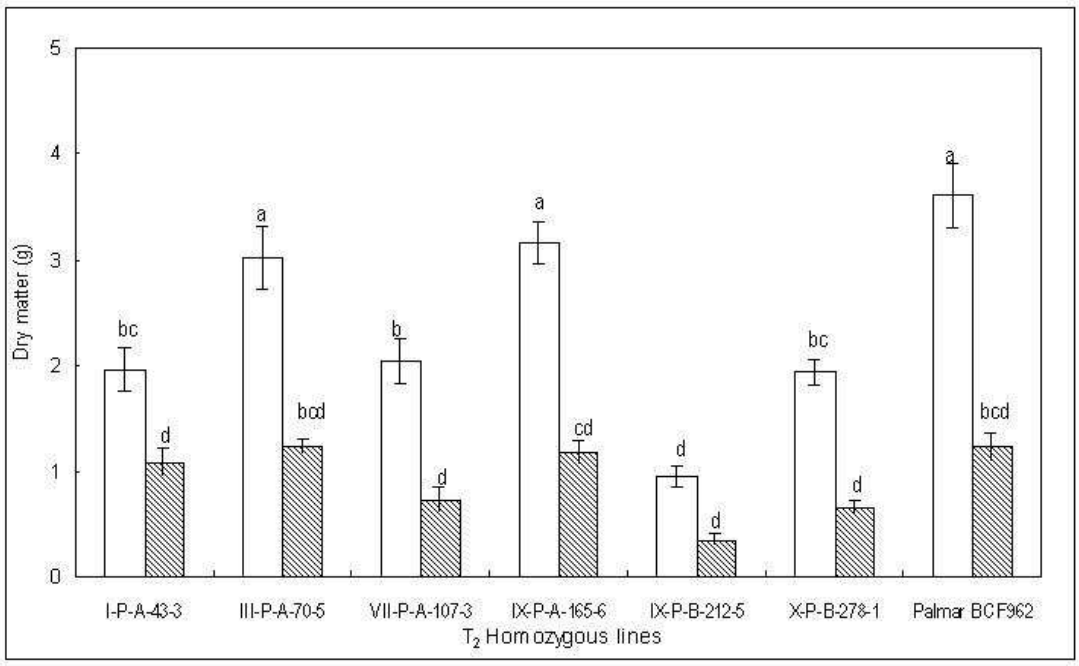

Fig. 5.4-5. Biomass production. Dry matter of six independents Palmar homozygous DREB transgenic lines treated at two water conditions. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

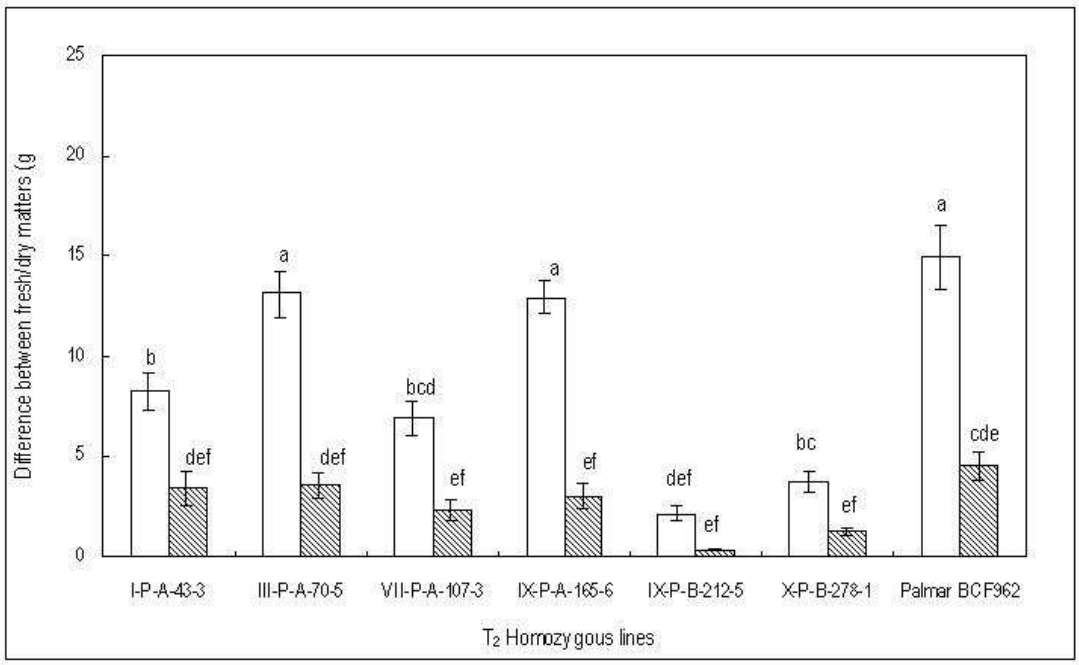

Fig. 5.4-6. Biomass production. Difference of fresh/dry matter of six independents Palmar homozygous DREB transgenic lines treated at two water conditions. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

Fig.5.4-7. Plant growth of six independents Palmar transgenic lines at 17 DAS. From left side, I-P-A-43-3, III-P-A-70-5, VII-P-A-107-3, IX-P-A-165-6, Palmar BCF962, IX-P-B-212-5 and X-P-B-278-1.

5.5. Yield response of homozygous DREB transgenic rice lines

Air temperature was kept at around 30^{o}C during greenhouse experiment. FC at water-limited condition was kept around 30-50% strarting 57 DAS until harvest (Fig. 5.5-1).

The effect of water treatments was significant for all traits except for thousand-kernel weight (Tables 5.5-1, and 5.5-2). ANOVA detected significant differences by water treatments for flowering date; however, there were no significant differences in transgenic lines for flowering date (Tables 5.5-1, and 5.5-3). Differences amongst independent transgenic lines were highly significant except for flowering date, filled grains number, percentage of filled grains and thousand kernel weights. Significant differences in the evaluated lines due to water treatments interaction were observed for panicle number, panicle weight, spikelets and weight of filled grains.

Significant differences were observed between water treatment and amongst evaluated lines; however, no significant line by water treatment interaction was observed for plant height and dry matter (Tables 5.5-7 and 5.5-8). Evaluated transgenic lines (except X-P-B-278-1) at normal irrigated conditions grew taller than in the water-limited conditions. I-P-A-43-3, III-P-A-70-5 and X-P-B-278-1 showed similar growth as CT6241 under drought stress conditions. IX-P-B-239-5 grew shorter than other transgenic lines. Dry matter production of plants transformed using the *Lip9::AtDREB1A* construct were higher than other transgenic plants, which were transformed using the *Lip9::OsDREB1B* construct. Significant differences for dry matter were not observed amongst transgenic lines that had the *AtDREB1A* gene and two non-transgenic plants. Furthermore, these transgenic plants produced more dry matter than CT6241 at water-limited conditions (Table 5.5-8).

Tiller number, panicle number and productive panicle number varied significantly between the water treatments and amongst transgenic lines. Significant plant by water treatments interaction was observed only in panicle number (Tables 5.5-9, 5.5-10 and Fig. 5.5-5). Tiller number was higher for all transgenic plants at water-limited conditions, except X-P-B-239-5 (Table 5.5-9). Plants transformed using the *Lip9::AtDREB1A* construct performed similarly as non-transgenic Palmar for number of tillers. Transgenic lines (except VII-P-A-107-3) produced more panicles at normal water level than water-limited conditions. VII-P-A-107-3 had a similar response as non-transgenic plants, with higher panicle number at water-limited conditions than the control treatment (Fig. 5.5-5). At water-limited conditions, no significant differences were observed for panicle number between CT6241 and transgenic lines except X-P-B-278-1; I-P-A-43-3, III-P-A-70-5 and VII-P-A-107-3 showed about the same number of panicle as CT6241 at water-limited conditions. The effect of water treatments was significant for productive panicle number. Few productive panicles were observed in all transgenic lines, transformed using the *Lip9::OsDREB1B* construct at both water treatments (Table 5.5-10). These results suggest that a large tiller number was not exactly associated with panicle production amongst evaluated transgenic lines. I-P-A-43-3 and VII-P-A-107-3 produced a percentage of productive tillers close to CT6241 in water-limited conditions.

Water treatments significantly affected panicle length and panicle weight of transgenic lines. All transgenic lines had larger panicles under normal irrigated conditions. Significant differences for panicle length at drought stress treatment were not observed for the four transgenic plants that carried the *AtDREB1A* gene and X-P-B-278 (Table 5.5-11). The plant by water treatment interaction was highly significant for panicle weight (Fig. 5.5-6). The effect of water treatments was significant for I-P-A-43 and III-P-A-70-5; however, no significant difference was observed for VII-P-A-107-3. The panicle weight of VII-P-A-107-3 was not affected by water treatments.

No significant difference amongst the evaluated transgenic lines by water treatments interaction was observed for yield and all yield components except spikelets number per plant (Tables 5.5-3, 5.5-4, 5.5-5, and 5.5-6, Fig. 5.5-2, 5.5-3, and 5.5-4). No significant difference was observed for thousand-kernel weight, although the line by water treatment interaction was highly significant for weight of filled grains.

All evaluated transgenic lines performed better under normal irrigated conditions. Four transgenic plants that carried the AtDREB1A gene had more spikelets compared with other transgenic lines transformed using the *Lip9::OsDREB1B* construct. I-P-A-43, III-P-A-70-5 and VII-P-A-107-3 showed similar performance as CT6241 for spikelets number and weight of filled grains at drought stress treatment. The effect of water treatments was significant for I-P-A-43-3 and III-P-A-70-5 (Table 5.5-4). The yield response of I-P-A-43-3 was closer to non-transgenic plants (Palmar and CT6241) under water-limited conditions (Table 5.5-5). Yield was highly affected by water treatments for evaluated transgenic lines, IX-P-A-165-6 and X-P-B-278-1 produced grain only under normal irrigation conditions.

These results may suggest that I-P-A-43, III-P-A-70-5 and VII-P-A-107-3 seem to perform as non-transgenic CT6241 under water-limited conditions. I-P-A-43 and III-P-A-70-5 responded better at the normal irrigation treatment, although, VII-P-A-107-3 was not significantly affected by water treatments for almost all measured traits. I-P-A-43 showed better response for the traits associated with yield such as percentages of productive tiller and filled grain, under water-limited conditions. III-P-A-70-5 produced non-bearing tiller; these results probably suggest that the transgenic line III-P-A-70-5 has more biomass production than other evaluated transgenic lines under water-limited conditions.

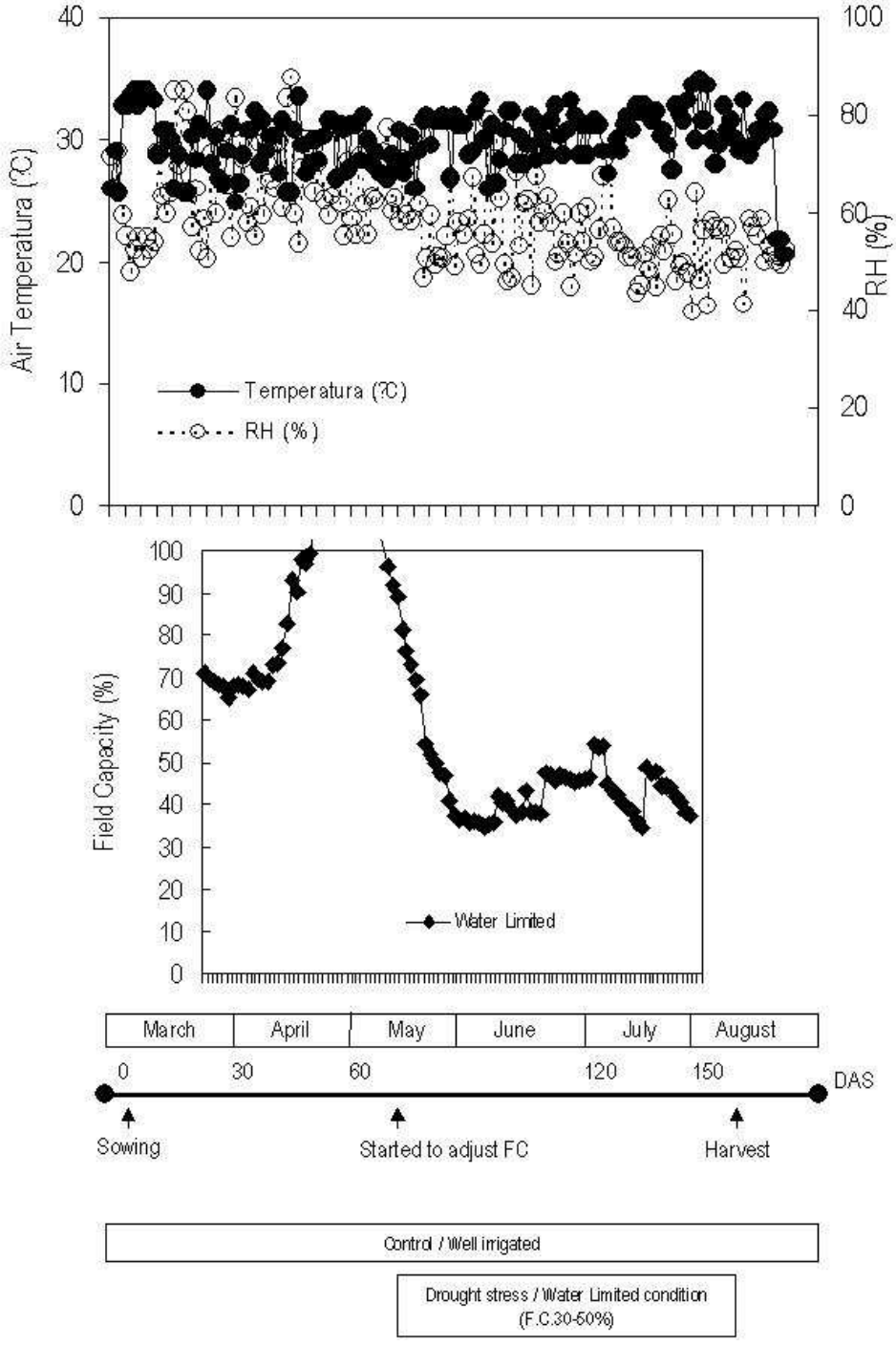

Fig. 5.5-1. Environmental conditions and time lines at greenhouse experiment.

Table 5.5-1. Significance for source of variations in measured traits at greenhouse experiment.

	Source of variations		
Trait	Water Treatment	Genotype	Water Treatment*Genotype
Thousand kernel weight	ns	ns	ns
Flowering date	*	ns	ns
Filled grains per plant	**	ns	ns
Percentage of filled grains per plant	**	ns	ns
Yield per plant	**	**	ns
Plant height	**	**	ns
Dry matter	**	**	ns
Tiller number per plant	**	**	ns
Productive panicle number per plant	**	**	ns
Panicle length	**	**	ns
Panicle number per plant	**	**	**
Panicle weight	**	**	**
Number of spikelets per plant	**	**	**
Weight of filled grains	**	**	**

*: Significant at 0.05≤P≤0.01; **: Significant at P≤0.01; ns: Not significant at P≥0.05.

Table 5.5-2. Thousand-kernel weight of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated			Water-limited			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	20.30±0.55	1.83	6.66	19.61±0.29	0.35	3.01	20.03±0.35	1.26	5.61
III-P-A-70-5	19.14±0.61	2.25	7.84	16.92±1.27	9.72	18.43	18.03±0.75	6.79	14.45
VII-P-A-107-3	18.18±0.95	5.52	12.93	17.72±0.83	4.16	11.51	17.95±0.60	4.46	11.76
IX-P-A-165-6	20.58±0.44	1.17	5.26	14.53±1.26	3.19	12.29	19.06±1.06	9.14	15.85
IX-P-B-212-1	19.69±0.23	0.33	2.93	19.64±8.49	288.53	86.48	19.67±3.10	96.36	49.90
IX-P-B-239-5	18.58±0.64	2.51	8.54	19.08±1.58	12.59	18.60	18.81±0.76	6.37	13.41
X-P-B-278-1	22.45±1.04	11.8	15.30	0	0.00	.	22.45±1.40	11.80	15.30
X-P-B-290-1	24.16±3.45	71.83	35.08	15.36±0.54	0.60	5.04	21.96±2.91	67.98	37.54
Palmar-NT	19.24±1.21	8.83	15.44	19.41±0.32	0.65	4.16	19.33±0.59	4.32	10.75
CT 6241-NT	30.30±3.51	74.06	28.40	23.78±1.64	16.25	16.95	27.04±2.09	52.64	26.83
GLOBAL	21.26±0.67	27.53	24.68	19.01±0.89	32.96	30.21	20.35±0.55	30.67	27.22

Table 5.5-3. Flowering date of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	103.47±1.02	2.99	1.67	121.63±8.37	420.46	16.86	112.55±4.85	282.37	14.93
III-P-A-70-5	103.98±1.02	6.34	2.42	113.76±3.46	71.96	7.46	108.87±2.26	61.70	7.22
VII-P-A-107-3	110.77±2.41	34.97	5.34	106.92±3.09	57.40	7.09	108.84±1.95	46.03	6.23
IX-P-A-165-6	100.46±0.53	1.73	1.31	120.63±6.14	150.95	10.19	108.52±3.99	159.75	11.65
IX-P-B-212-1	105.27±0.96	5.58	2.24	119.13±3.82	73.19	7.18	111.57±2.77	84.47	8.24
IX-P-B-239-5	109.88±2.57	39.87	5.75	115.30±2.25	30.64	4.80	112.59±1.82	40.05	5.62
X-P-B-278-1	103.53±0.71	3.03	1.68	135.00±10.00	200.00	10.48	111.40±5.51	242.91	13.99
X-P-B-290-1	109.91±2.45	36.23	5.48	120.88±4.72	111.43	8.73	114.90±2.94	95.53	8.51
Palmar-NT	102.30±0.79	3.80	1.91	109.76±0.81	3.95	1.81	106.03±1.24	18.68	4.08
CT 6241-NT	119.3±28.61	4912.20	58.75	97.20±1.32	10.48	3.33	108.25±14.05	2370.80	44.98
GLOBAL	106.89±2.75	456.54	19.99	114.23±1.76	161.79	11.14			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-4. Filled grain number (per plant) of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	1751.70±209.39	263079	29.28	368.50±145.35	126761	96.62	1060.10±241.34	698968	78.87
III-P-A-70-5	1663.50±121.93	89203	17.95	352.17±183.63	202324	127.72	1007.80±223.86	601493	76.95
VII-P-A-107-3	926.00±138.91	115790	36.75	329.50±100.36	60442	74.61	627.75±121.49	177145	67.05
IX-P-A-165-6	1595.70±190.09	216823	29.18	19.50±16.00	1536.7	201.03	807.58±254.42	776791	109.14
IX-P-B-212-1	583.67±49.36	14620	20.72	88.83±43.24	11223	119.25	336.25±80.89	78527	83.34
IX-P-B-239-5	447.83±62.85	23706	34.38	57.50±36.71	8085.9	156.39	252.67±68.31	56004	93.66
X-P-B-278-1	1260.80±67.46	27310	13.11	0	0.00	.	630.42±192.77	445968	105.93
X-P-B-290-1	464.83±83.79	42125	44.15	16.33±10.41	650.27	156.12	240.58±78.68	74303	113.30
Palmar-NT	1680.20±140.09	117761	20.42	778.17±67.06	26989	21.11	1229.20±154.83	287687	43.64
CT 6241-NT	1098.50±182.70	200283	40.74	542.50±120.53	87178	54.43	820.50±133.84	214974	56.51
GLOBAL	1147.30±75.68	343650	51.10	255.30±42.50	108402	128.96			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-5. Percentage of filled grains per plant of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	59.33±5.57	39.78	10.63	36.42±3.59	51.69	19.74	50.17±4.23	179.39	26.70
III-P-A-70-5	58.69±2.36	41.73	11.01	22.72±10.43	653.04	112.50	40.70±7.46	668.69	63.53
VII-P-A-107-3	70.93±5.43	177.49	18.78	28.34±8.33	416.83	72.03	49.64±7.98	764.72	55.71
IX-P-A-165-6	64.01±2.55	39.27	9.79	4.15±2.18	9.55	74.53	49.04±9.98	797.25	57.58
IX-P-B-212-1	53.73±2.95	52.46	13.48	29.78±11.02	485.59	74.01	44.15±5.86	344.03	42.01
IX-P-B-239-5	69.63±4.07	99.60	14.33	16.73±8.92	398.10	119.24	45.59±9.40	972.15	68.40
X-P-B-278-1	64.29±4.25	108.61	16.21	0.	0.00	.	64.29±4.25	108.61	16.21
X-P-B-290-1	74.56±4.97	148.72	16.36	13.64±1.48	4.41	15.40	59.33±10.62	902.12	50.62
Palmar-NT	63.11±2.08	26.15	8.10	41.98±3.76	85.16	21.98	52.55±3.78	172.31	24.98
CT 6241-NT	66.77±2.54	38.92	9.34	32.85±6.53	256.56	48.76	49.81±6.11	448.17	42.50
GLOBAL	64.50±1.29	101.36	15.61	27.79±2.94	355.94	67.89			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-6. Yield (g/plant) of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	35.47±4.26	109.35	29.48	10.83±2.65	28.14	49.01	25.61±4.81a	232.06	59.48
III-P-A-70-5	32.08±3.01	54.36	22.98	6.42±3.53	75.09	134.98	19.25±4.45abc	238.46	80.21
VII-P-A-107-3	17.37±3.38	68.74	47.74	5.95±1.92	22.22	79.22	11.66±2.53bcd	76.89	75.22
IX-P-A-165-6	32.53±3.38	68.57	25.45	0.80±0.5	0.50	88.39	24.60±5.75a	264.84	66.15
IX-P-B-212-1	11.50±0.99	5.93	21.18	3.18±1.33	7.16	84.22	8.17±1.55cd	24.15	60.15
IX-P-B-239-5	8.25±1.12	7.55	33.32	1.25±0.79	3.18	142.60	5.07±1.29d	18.41	84.66
X-P-B-278-1	28.18±1.87	21.07	16.29	0.	0.00	.	28.18±1.87a	21.07	16.29
X-P-B-290-1	10.12±1.70	17.49	41.33	0.75±0.05	0.01	9.43	7.78±1.97cd	31.29	71.95
Palmar-NT	32.58±3.81	87.47	28.70	15.17±1.47	12.96	23.74	23.88±3.27ab	128.38	47.46
CT 6241-NT	31.67±4.33	112.72	33.53	13.80±3.48	72.96	61.89	22.73±3.78ab	171.46	57.60
GLOBAL	23.98±1.60	154.75	51.89	7.64±1.14	53.54	95.74			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-7. Plant height of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	108.95±0.41	1.03	0.93	94.67±7.38	327.39	19.11	101.81±4.13a	204.92	14.06
III-P-A-70-5	107.98±1.23	9.19	2.81	95.5±25.61	3936.20	65.70	101.74±12.37a	1835.90	42.11
VII-P-A-107-3	82.55±4.90	144.14	14.54	70.43±4.08	100.31	14.22	76.49±3.54bcd	151.15	16.07
IX-P-A-165-6	103.95±1.82	20.00	4.30	75.03±7.68	354.00	25.08	89.49±5.75abc	398.05	22.29
IX-P-B-212-1	84.08±1.25	9.38	3.64	66.30±7.01	295.03	25.91	75.19±4.32bcd	224.62	19.93
IX-P-B-239-5	70.55±1.86	20.83	6.47	55.48±4.38	115.33	19.36	63.02±3.21d	123.80	17.66
X-P-B-278-1	97.30±2.00	24.06	5.04	98.12±3.65	79.92	9.11	97.71±1.98ab	47.44	7.05
X-P-B-290-1	74.98±3.57	76.60	11.67	69.13±7.03	320.33	25.89	72.06±3.97cd	189.76	19.12
Palmar-NT	112.22±1.41	12.02	3.09	94.70±0.76	3.53	1.98	103.46±2.75a	90.75	9.21
CT 6241-NT	112.95±2.70	44.03	5.87	99.43±2.57	39.63	6.33	106.19±2.07a	87.86	8.83
GLOBAL	95.55±2.11	268.25	17.14	81.88±3.44	712.69	32.60			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-8. Dry matter of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	28.78±3.55	76.02	30.29	19.03±2.29	31.72	29.59	23.91±2.49a	74.90	36.20
III-P-A-70-5	21.63±3.55	75.97	40.29	22.18±1.28	9.86	14.15	21.91±1.80a	39.10	28.54
VII-P-A-107-3	20.10±2.01	24.32	24.54	17.35±0.61	2.28	8.71	18.73±1.08a	14.16	20.09
IX-P-A-165-6	24.80±1.88	21.36	18.64	20.30±1.40	11.82	16.94	22.55±1.31a	20.61	20.13
IX-P-B-212-1	12.12±0.57	1.97	11.59	10.08±1.10	7.27	26.73	11.10±0.66bc	5.33	20.79
IX-P-B-239-5	7.80±1.08	7.02	33.96	5.18±0.29	0.52	13.88	6.49±0.66d	5.29	35.44
X-P-B-278-1	19.45±0.71	3.03	8.95	12.93±1.08	7.01	20.47	16.19±1.16ab	16.14	24.82
X-P-B-290-1	7.75±1.17	8.23	37.02	9.70±1.08	19.53	45.56	8.73±1.06cd	13.65	42.35
Palmar-NT	26.30±1.85	20.64	17.28	21.17±2.71	44.33	31.45	23.73±1.74a	36.72	25.53
CT 6241-NT	18.20±2.34	32.88	31.50	17.28±2.34	32.84	33.16	17.74±1.58a	30.10	30.92
GLOBAL	18.69±1.10	72.84	45.66	15.52±0.85	44.16	42.81			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-9. Tiller number (per plant) of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	13.5±1.87	21.10	34.03	17.33±2.45	36.27	34.74	15.42±1.58c	30.08	35.58
III-P-A-70-5	13.67±0.95	5.47	17.11	16.50±1.54	14.30	22.92	15.08±0.96c	11.17	22.16
VII-P-A-107-3	12.83±0.83	4.17	15.91	17.00±2.30	32.00	33.28	14.92±1.32c	21.17	30.85
IX-P-A-165-6	14.67±2.34	33.07	39.21	18.17±2.19	28.97	29.63	16.42±1.62c	31.54	34.21
IX-P-B-212-1	8.83±0.40	0.97	11.13	11.00±0.89	4.80	19.92	9.92±0.57b	3.90	19.92
IX-P-B-239-5	7.00±0.89	4.80	31.30	6.17±0.40	0.97	15.94	6.58±0.48a	2.81	25.47
X-P-B-278-1	10.33±0.66	2.67	15.80	13.17±1.24	9.37	23.24	11.75±0.79bc	7.66	23.55
X-P-B-290-1	6.33±0.80	3.87	31.05	9.33±1.17	8.27	30.81	7.83±0.81a	7.97	36.04
Palmar-NT	10.83±0.87	4.57	19.73	14.67±0.42	1.07	7.04	12.75±0.74bc	6.57	20.10
CT 6241-NT	9.50±0.42	1.10	11.04	10.50±0.42	1.10	9.99	10.00±0.32b	1.27	11.28
GLOBAL	10.75±0.49	14.53	35.46	13.38±0.66	26.41	38.40			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-10. Productive panicle number (per plant) of eight Palmar homozygous DREB transgenic plants at greenhouse experiment

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	8.17±1.01	6.17	30.41	3.67±1.49	13.47	100.08	5.92±1.09ab	14.45	64.24
III-P-A-70-5	7.83±0.87	4.57	27.28	2.50±1.28	9.90	125.86	5.17±1.09a	14.33	73.28
VII-P-A-107-3	7.67±0.61	2.27	19.64	4.00±1.15	8.00	70.71	5.83±0.83ab	8.33	49.49
IX-P-A-165-6	8.00±0.68	2.80	20.92	0	0.00	.	4.00±1.24ab	18.73	108.19
IX-P-B-212-1	5.67±0.66	2.67	28.82	1.83±0.83	4.17	111.34	3.75±0.77ab	7.11	71.12
IX-P-B-239-5	5.33±0.80	3.87	36.87	0.83±0.47	1.37	140.29	3.08±0.81ab	7.90	91.17
X-P-B-278-1	6.50±0.22	0.30	8.43	0	0.00	.	3.25±0.98b	11.66	105.06
X-P-B-290-1	5.00±0.81	4.00	40.00	0.33±0.21	0.27	154.92	2.67±0.81ab	7.88	105.26
Palmar-NT	7.33±0.61	2.27	20.53	5.83±0.47	1.37	20.04	6.58±0.43ab	2.27	22.86
CT 6241-NT	5.50±0.95	5.50	42.64	3.33±0.76	3.47	55.86	4.42±0.66ab	5.36	52.40
GLOBAL	6.70±0.26	4.32	31.00	2.23±0.34	7.13	119.57			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

Table 5.5-11. Panicle length of eight Palmar homozygous DREB transgenic plants at greenhouse experiment.

	Water Treatment								
	Well-irrigated (a)			Water-limited (b)			Global		
Transgenic line	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV	Mean±Std Error	Variance	CV
I-P-A-43-3	24.90±0.36	0.79	3.57	20.68±1.57	14.95	18.69	22.79±1.00a	12.01	15.20
III-P-A-70-5	24.22±0.29	0.51	2.96	21.21±2.27	31.09	26.29	22.71±1.18a	16.84	18.07
VII-P-A-107-3	22.39±0.72	3.12	7.89	20.91±0.95	5.48	11.20	21.65±0.61a	4.51	9.81
IX-P-A-165-6	24.11±0.28	0.50	2.92	20.73±1.65	10.99	16.00	22.75±0.83a	6.98	11.61
IX-P-B-212-1	18.63±0.31	0.61	4.20	14.97±1.07	5.75	16.03	16.97±0.75b	6.27	14.76
IX-P-B-239-5	17.19±0.62	2.37	8.95	15.34±0.68	2.79	10.88	16.26±0.52b	3.28	11.14
X-P-B-278-1	24.32±0.24	0.35	2.43	17.27±1.23	3.05	10.12	22.56±1.19a	11.36	14.94
X-P-B-290-1	18.69±0.64	2.49	8.45	15.59±1.45	10.61	20.89	17.28±0.85b	8.11	16.48
Palmar-NT	25.54±0.21	0.29	2.09	23.86±0.09	0.05	0.96	24.70±0.27a	0.92	3.89
CT 6241-NT	24.22±0.40	1.00	4.14	23.57±0.38	0.90	4.03	23.89±0.28a	0.98	4.15
GLOBAL	22.42±0.40	9.60	13.82	19.68±0.59	18.16	21.65			

Different letters in the table denote significant differences at 0.01≤P, as determined by Ryan's multiple range tests.

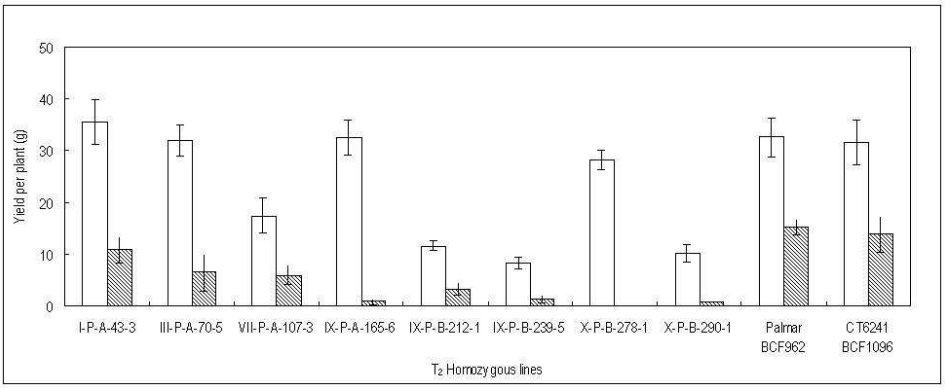

Fig. 5.5-2. Yield (g/plant) of eight Palmar homozygous DREB transgenic lines at greenhouse experiment. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

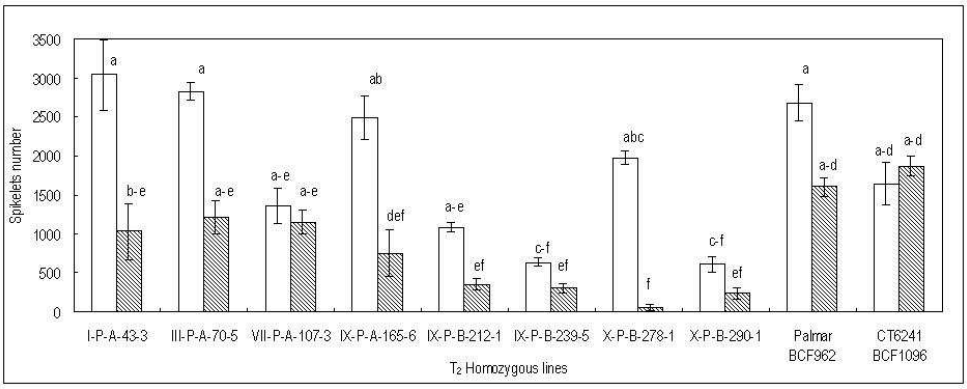

Fig. 5.5-3. Spikelets number (per plant) of eight Palmar homozygous DREB transgenic lines at greenhouse experiment. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests.

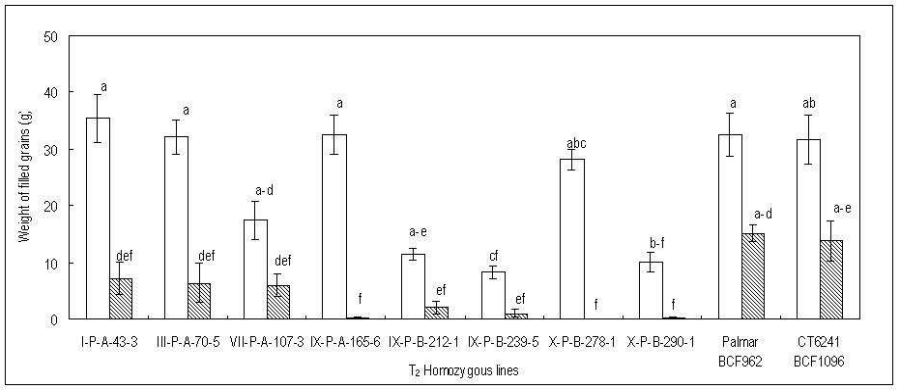

Fig. 5.5-4. Weight of filled grains of eight Palmar homozygous DREB transgenic lines at greenhouse experiment. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

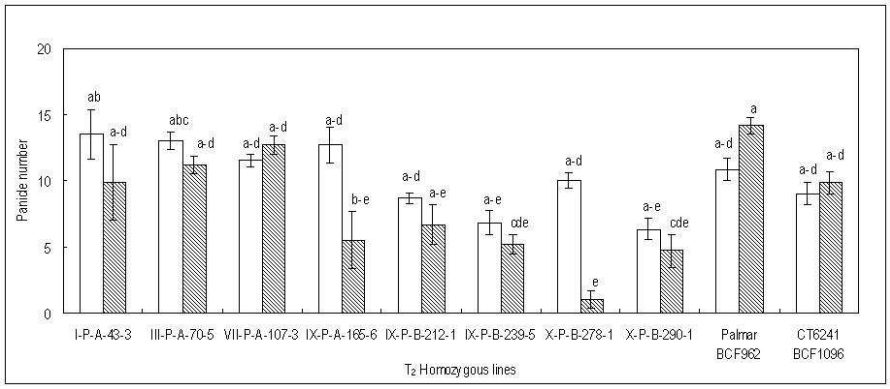

Fig. 5.5-5. Panicle number (per plant) of eight Palmar homozygous DREB transgenic lines at greenhouse experiment. White poles represent well-irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

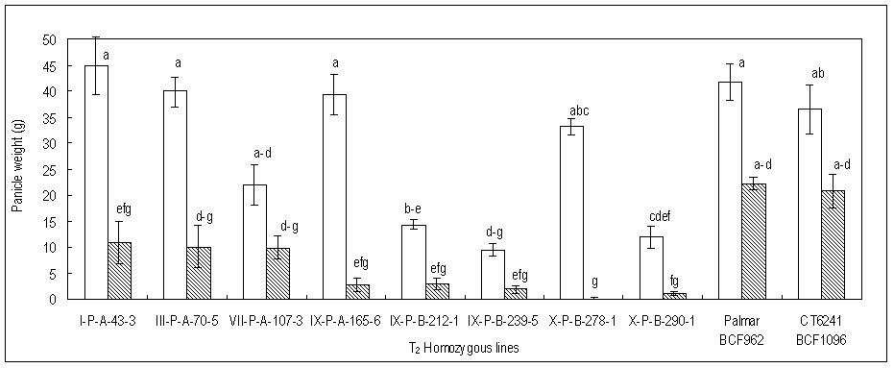

Fig. 5.5-6. Panicle weight of eight Palmar homozygous DREB transgenic lines at greenhouse experiment. White poles represent well–irrigated conditions, and striped poles represent water-limited conditions. Bars in the figure show the standard error; different letters in the panel denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests.

6. CONCLUSIONS

Protocols were established for screening rice genotypes for tolerance to water-limited conditions under field and greenhouse conditions. These protocols were successfully used to select the best genotypes for transgenic experiments. Tested rice genotypes responded differently when subjected to water-limited conditions. In experiments conducted under field conditions, Curinga and CT6241 performed much better for yield production under water-limited conditions than Azucena, CICA8, NERICA, and Palmar. Therefore, Curinga, CT6241, CICA8 and Palmar were selected for transformation with DREB 1 transcription factor. The first two are tolerant genotypes and the latter two are susceptible genotypes to water stress.

Toki's protocol for transformation was not adequate for transformation of Curinga; therefore, CIAT's protocol was used with some modifications. Few homozygous T_2 transformed lines derived from Palmar were available for evaluation under water-limited conditions in a screenhouse and the biosafety greenhouse.

Transformed T_2 lines responded differently to water-limited conditions; which affected all agronomic traits of genotypes used in this study. Under water-limited conditions none of the transformed lines performed better than non-transformed Palmar and CT6241; however, transformed lines I-P-A-43-3, III-P-A-70-5 and VII-P-A-107-3 did better than other transformed lines. The Performance of these three transgenic events suggests that they could be considered as promising materials for future studies. On the other hand, transgenic plants transformed using the *Lip9::OsDREB1B* construct did not show any advantage under water-limited conditions in the greenhouse experiment. However, the relation between field capacity and gene expression in the evaluated transgenic plants is still unclear. Water-limited conditions used in this study were probably not good enough to trigger expression of the lip9 promoter in the case of the OsDREB1B in Palmar.

Future studies in this area are essential for the development of rice varieties suitable for water-limited conditions in Latin America.

7. REFERENCES

Aldemita, R. R.; Hodges, T. K. (1996). *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties. Planta 199:612-617

Annual report of IP-4 project at CIAT, (2003). Executive summary

Atlin, G.N. (2001). Breeding for Suboptimal Environments. In: Shu, F., Jaya, B. (Eds.), Increased Lowland Rice Production in the Mekong Region. Proceeding of the International Workshop, Vientiane, Laos, 30 October-2 November 2000. ACIAR Canberra, p245-251

Boriss, H. (2006). Commodity Profile Rice. Agricultural Issues Center, University of California. Accessed December 2008, Available at: http://aic.ucdavis.edu/profiles/Rice-2006.pdf

Cuevas-Pérez, F. (1992). Rice in Latin America: Improvement, Management, and Marketing. Palmira, Colombia: Graphic Arts Unit, CIAT; p. 11-114.

Dubouzet, J.G.; Sakuma, Y.; Ito, Y.; Kasuga, M.; Dubouzet, E.G.; Miura, S.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J. 33: 751-763

Flórez Ramos, C. P. (2003). Desarrollo metodológico de la transformación genética de *Brachiaria humidicola* Stapf en fitomejoramiento. Tesis (Doctor en Ciencias Agropecuarias Area Agraria: Mejoramiento Genético, Agronomía y Producción de Semillas de Cultivos Tropicales). Universidad Nacional de Colombia (UNAL), Palmira, Valle del Cauca, CO. 208 p.

Fory, L.; Tabares, E.; Galindo, L. M.; Pachon, J.; Delgado, G.; Arango, C. Ordóñez; Yamaguchi-Shinozaki, K; Kumashiro, T; Ishitani, M.; Lentini, Z. (2005).

Foreign genes as novel sources for increased efficiency of water use in rice. CIAT IP-4 Annual Report: 22-29

Gahakwa, D.; Maqbool, S.B.; Fu, X.; Sudhakar, D.; Christou, P.; Kohli, A. (2000). Transgenic rice as a system to study the stability of transgene expression: multiple eterologous transgenes show similar behaviour in diverse genetic backgrounds. Theor Appl Genet. 101: 388-399

Garrity, D.P.; O'Toole, J.C. (1994). Screening rice for drought resistance at reproductive phase. Field Crops Res. 39: 99-110

Hiei, Y.; Ohta, S.; Komari, T.; Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271-282

Hiei, Y.; Komari, T.; Kubo, T. (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Mol. Biol. 35:205-218

Ingram, J.; Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 33: 857-865

International Rice Genome Project (2005). The map-based sequence of the rice genome. Nature 436:793-800

Ishizaki, T.; Kumashiro, T. (2007). Genetic transformation of NERICA, interspecific hybrid rice between *Oryza glaberrima* and *O. sativa*, mediated by *Agrobacterium tumefaciens*. Plant Cell Rep. 27: 319-327

Ito, Y.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. (2006). Functional Analysis of Rice DREB1/CBF-type Transcription Factors Involved in Cold-responsive Gene Expression in Transgenic Rice. Plant cell Physiol. 47: 141-153

Jaglo, K.R.; Kleff, S.; Amundsen, K.L.; Zhang, X.; Haake, V.; Zhang, J.Z.; Deits, T.; Thomashow, M.F. (2001). Components of the *Arabidopsis* C-repeat/dehydration-

responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. Plant Physiol. 127: 910-917

Jaglo-Ottosen, K.R.; Gilmour, S.J.; Zarka, D.G; Schabenberger, O.; Thomashow, M.F. (1998). *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280: 104-106

Jefferson, R.A.; Kilian, A.; Keese, P.K. (1998). Microbial genes for secreted beta-glucuronidases, gene products and uses thereof. CAMBIA, Molecular Technologies, Canberra, Australia. Accessed February 2007, Available at: http://www.cambia.org/daisy/cambia/materials/vectors/585.html#dsy585_Description

Kasuga, M.; Miura, S.; Shinozaki, K.; Yamaguchi-Shinozaki, K. (2004). A combination of the *Arabidopsis* DREB 1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol. 45: 346-350

Kosugi, S.; Ohashi, Y.; Nakajima, K.; Arai, Y. (1990). An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci. 70:133–140

Kramer, P.J.; Boyer, J.S. (1995). Water relations of plants and soils. Academic Press, San Diego, USA. : p201-207

Lafitte, H.R.; Price, A. H.; Courtois, B. (2004). Yield response to water deficit in an upland rice mapping population: associations among traits and genetic markers. Theor. Appl. Genet. 109: 1237-1246

Lanceras, J.C.; Pantuwan, G.; Jongdee, B.; Toojinda, T. (2004). Quantitative Trait Loci Associated with Drought Tolerance at Reproductive Stage in Rice. Plant Physiol. 135: 384-399

Lee, S.C.; Huh, K.W.; An, K.; An. G.; Kim, S.R. (2004). Ectopic Expression of a Cold inducible Transcription Factor, CBF1/DREB1b, in Transgenic Rice (*Oryza sativa* L.). Mol. Cells. 18:107-114

Lentini, Z.; Lozano, I.; Tabares, E.; Fory, L.; Domínguez, J.; Cuervo, M.; Calvert, L.; (2003). Expression and inheritance of hypersensitive resistance to rice hoja blanca virus mediated by the viral nucleocapsid protein gene in transgenic rice. Theor. Appl. Genet. 106: 1018-1026

Liu, Q.; Kasuga, M.; Sakuma, Y.; Abe, H.; Miura, S.; Yamaguchi-Shinozaki, K.; Shinozaki, K. (1998). Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low- Temperature-Responsive Gene Expression, Respectively, in Arabidopsis. Plant Cell. 10: 1391-1406

Lorieux, M.; Ndjiondjop, M. N.; Ghesquière, A. (2000). A first interspecific *Oryza sativa* x *Oryza glaberrima* Microsatellite-based Genetic Linkage Map. Theor Appl Genet. 100: 593–601

Mansfield, T.A. (1987). Hormones as regulators of water balance. In: Davies, R.D. (Ed) Plant Hormones and Their Role in Plant Growth and Development. Dordrecht, The Netherlands: Martinus Nijhoff Publishers; p. 411-430

McNally, K.L.; Bruskiewich, R.; Mackill, D.; Robin Buell, C.; Leach, J.E.; Leung, H. (2006). Sequencing Multiple and Diverse Rice Varieties. Connecting Whole-Genome Variation with Phenotypes. Plant Physiol. 141: 26-31

Moncada, P.; Martínez, C.P.; Borrero, J.; Chatel, M.; Gauch Jr, H.; Guimaraes, E.; Tohme, J.; McCouch, S.R. (2001). Quantitative trait loci for yield and yield components in an *Oryza sativa* x *Oryza rufipogon* BC_2F_2 population evaluated in an upland environment. Theor. Appl. Genet. 102: 41-52

Nishimura, N; Aichi, I; Matsuoka, M. (2007). A rapid protocol for Agrobacterium-medianted transformation in rice. Nature protocols 1(6): 2796-2802

Pantuwan, G.; Fukai, S.; Cooper, M.; Rajatasereekul, S.; O'Toole, J.C. (2002a). Yield response of rice (*Oryza sativa* L.) genotypes to different types of drought under rainfed lowlands Part1. Grain yield and yield components. Field Crops Res. 73: 153-168

Pantuwan, G.; Fukai, S.; Cooper, M.; Rajatasereekul, S.; O'Toole, J.C. (2002b). Yield response of rice (*Oryza sativa* L.) genotypes to drought under rainfed lowlands 2. Selection of drought resistant genotypes. Field Crops Res. 73: 169-180

Pantuwan, G.; Fukai, S.; Cooper, M.; Rajatasereekul, S.; O'Toole, J.C.; Basnayake, J. (2004). Yield response of rice (*Oryza sativa* L.) genotypes to different types of drought under rainfed lowlands 4. Vegetative stage screening in the dry season. Field Crops Res. 89: 281-297

Rabbani, M.A.; Abe, H.; Maruyama, K.; Khan, M.A.; Katsura, K.; Ito, Y.; Yoshiwara, K.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. (2003). Monitoring expression profiles of rice (*Oryza sativa* L.) genes under cold, drought and high-salinity stresses, and ABA application using both cDNA microarray and RNA gel blot analyses. Plant Physiol. 133: 1755-1767

Rashid, H.; Yokoi, S.; Toriyama, K.; Hinata, K. (1996). Transgenic plant production mediated by *Agrobacterium* in *Indica* rice. Plant Cell Rep. 15: 727-730

Schwager, S.J.; Mutschler, M.A.; Federer, W.T.; Scully, B.T. (1993). The effect of linkage on sample size determination for multiple trait selection. Theor Appl Genet. 86: 964-974

Septiningsih, E. M.; Prasetiyono, J.; Lubis, E.; Tai, T.H.; Tjubaryat, T.; Moeljopawiro, S.; McCouch, S.R. (2003). Identification of quantitative trait loci for yield and yield components in an advanced backcross population derived from the

Oryza sativa variety IR64 and the wild relative *O. rufipogon*. Theor. Appl. Genet. 107: 1419-1432

Shinozaki, K.; Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr. Opin. Plant Biol. 3: 217-223

Shinozaki, K.; Yamaguchi-Shinozaki, K.; Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Biol. 6: 410-417

Tabares, E.; Delgado, G.; Fory, L.; Salcedo. A.; Galindo, L. M.; Arango, T.; Ordóñez, C.; Ishitani, M.; Lentini, Z. (2004). Foreign genes as novel sources for increased efficiency of water use in rice. CIAT SB-2 Annual Report: 248-252

Tabares, E.; Sakai, T.; Delgado, G.; Salcedo. A.; Galindo, L.M.; Arango, T.; Ordóñez, C.; Ishitani, M.; Lentini, Z. (2007). Foreign genes as novel sources for increased efficiency of water use in rice. CIAT SB-2 Annual Report: 215-222

Thomashow, M.F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 50: 571-599

Toki, S. (1997). Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol. Bio. Rep. 15: 16-21

Toki, S.; Hara, N.; Ono, K.; Onodera, H.; Tagiri, A.; Oka, S.; Tanaka, H. (2006). Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. Plant J. 47: 969-976

Trouche, G.; Rojas, L.N.; Wong, Z.C.; Corrales, J. (2006). Fitomejoramiento participado del arroz de secano en Nicaragua: metodologias, resultados y lecciones aprendidas. Agronomia Mesoamericana 17 (3): 309-325

Ukai, Y. (2003). Plant Breeding (In Japanese). University of Tokyo; p. 386

Umbeck, P.; Swain, W.; Yang, N.S. (1989). **I**nheritance and Expression of Genes for Kanamycin and Chloramphenicol Resistance in Transgenic Cotton Plants. Crop.Sci. 29:196-201

Wang, M.B.; Upadhyaya, N.M.; Bretell, R.I.S.; and Waterhouse, P.M. (1997) Intron-mediated improvement of a selectable marker gene for plant transformation using *Agrobacterium tumefaciens*. J.Genetics and Breed. 51: 325-334

Xiao, B.Z.; Chen, X.; Xiang, C.B.: Tang, N; Zhang, Q.F.; Xiong, L.Z. (2008). Evaluation of Seven Function-Known Candidate Genes for their Effects on Improving Drought Resistance of Transgenic Rice under Field Conditions. Molecular Plant: 1-11

Xiong, L.; Schumaker, K.S.; Zhu, J.K. (2002). Cell signaling during cold, drought, and salt stress. Plant Cell 14 (Suppl. 1): S165-S183

Yamaguchi-Shinozaki, K.; Shinozaki, K. (1994). A novel *cis*-acting element in an Arabidopsis gene in involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6: 251-264

Yue, B.; Xiong, L.; Xue, W.; Xing, Y.; Luo, L.: Xu, C. (2005). Genetic analysis for drought resistance of rice at reproductive stage in field with different type of soil. Theor Appl Genet. 111: 1127-1136

Zeng, L.; Shannon, M.C. (2000). Salinity Effects on Seedling Growth and Yield Components of Rice. Crop Sci. 40: 996-1003

Annex A. Summary tables of evaluated traits of six-rice genotypes at field experiments

	Panicle number per plant		
Genotypes	Well-irrigated	Water-limited	Global
Azucena	b	b	b
CICA8	ab	ab	ab
CT6241	ab	ab	ab
Curinga	a	a	a
NERICA	b	b	b
Palmar	b	b	b
Global			

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

	Panicle weight			Weight of filled grains			Yield per plant			Productive panicle number per plant			Number of Spikelets per plant			Thousand kernel weight		
Genotypes	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
Azucena	a	b		a	b		a	b				a			a			a
CICA8	a	b		a	b		a	b				a			a			b
CT6241	a	b		a	b		a	b				a			a			a
Curinga	a	b		a	b		a	b				a			a			a
NERICA	a	b		a	b		a	b				a			a			a
Palmar	a	b		a	b		a	b				a			a			b
Global	a	b		a	b		a	b		a	b		a	b		a	b	

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

	Filled grains per plant			Tiller number per plant			Panicle length			Flowering date			Plant height			Percentage of filled grain per plant		
Genotypes	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
Azucena	ab	de		bc	c		a	b		cd	b		a	b		a	c	
CICA8	a	e		bc	bc		bc	d		b	a		b	d		abc	d	
CT6241	ab	cde		a	bc		bc	bcd		e	de		b	cd		ab	abc	
Curinga	abc	bcde		ab	bc		bc	bc		e	de		b	c		a	ab	
NERICA	ab	de		bc	c		bc	cd		cde	cde		b	c		ab	abc	
Palmar	ab	de		bc	bc		b	bcd		c	b		b	d		ab	d	
Global																		

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

Annex B. Summary tables of evaluated traits of six independents Palmar homozygous DREB transgenic lines at screenhouse experiments

	Tiller number			Leaf temperature			Temperature difference		
Transgenic Plant	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
I-P-A-43-3	c	c	c	b	a		a	b	
III-P-A-70-5	a	a	a	b	a		a	b	
VII-P-A-107-3	bc	bc	bc	b	a		a	b	
IX-P-A-165-6	ab	ab	ab	b	a		a	b	
IX-P-B-212-5	d	d	d	b	a		a	b	
X-P-B-278-1	c	c	c	b	a		a	b	
Palmar BCF962	abc	abc	abc	b	a		a	b	
Global				b	a		a	b	

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

	Plant height			Fresh matter			Dry matter			Difference fresh-dry matters		
Transgenic Plant	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
I-P-A-43-3	b	b		b	de		bc	d		b	def	
III-P-A-70-5	b	b		a	de		a	bcd		a	def	
VII-P-A-107-3	b	cd		bc	de		b	d		bcd	ef	
IX-P-A-165-6	ab	b		a	de		a	cd		a	ef	
IX-P-B-212-5	cd	d		de	e		d	d		def	ef	
X-P-B-278-1	b	c		bc	de		bc	d		bc	ef	
Palmar BCF962	a	b		a	cd		a	bcd		a	cbe	
Global												

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

Annex C. Summary tables of evaluated traits of eight Palmar homozygous DREB transgenic plants at greenhouse experiment

	Flowering date			Filled grains number per plant			Percentage of filled grain per plant		
Transgenic line	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
I-P-A-43-3	a	b		a	b		a	b	
III-P-A-70-5	a	b		a	b		a	b	
VII-P-A-107-3	a	b		a	b		a	b	
IX-P-A-165-6	a	b		a	b		a	b	
IX-P-B-212-1	a	b		a	b		a	b	
IX-P-B-239-5	a	b		a	b		a	b	
X-P-B-278-1	a	b		a	b		a	b	
X-P-B-290-1	a	b		a	b		a	b	
Palmar-NT	a	b		a	b		a	b	
CT 6241-NT	a	b		a	b		a	b	
GLOBAL	a	b		a	b		a	b	

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

	Yield per plant			Plant height			Dry matter			Tiller number per plant			Productive panicle number per plant			Panicle length		
Transgenic line	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
I-P-A-43-3			a			a			a			c			ab			a
III-P-A-70-5			abc			a			a			c			a			a
VII-P-A-107-3			bcd			bcd			a			c			ab			a
IX-P-A-165-6			a			abc			a			c			ab			a
IX-P-B-212-1			cd			bcd			bc			b			ab			b
IX-P-B-239-5			d			d			d			a			ab			b
X-P-B-278-1			a			abc			a			bc			b			a
X-P-B-290-1			cd			cd			cd			a			ab			b
Palmar-NT			ab			a			a			bc			ab			a
CT 6241-NT			ab			a			a			b			ab			a
GLOBAL	a	b		a	b		a	b		a	b		a	b		a	b	

Different letters in the table denote significant differences at 0.01≤*P*, as determined by Ryan's multiple range tests

	Spikelets number per plant			Weight of filled grains			Panicle number per plant			Panicle weight		
Transgenic line	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global	Well-irrigated	Water-limited	Global
I-P-A-43-3	a	bcde		a	def		ab	abcd		a	efg	
III-P-A-70-5	a	abcde		a	def		abc	abcd		a	defg	
VII-P-A-107-3	abcde	abcde		abcd	def		abcd	abcd		abcd	defg	
IX-P-A-165-6	ab	def		a	f		abcd	bcde		a	efg	
IX-P-B-212-1	abcde	ef		abcde	ef		abcd	abcde		bcde	efg	
IX-P-B-239-5	cdef	ef		cdef	ef		abcde	cde		defg	efg	
X-P-B-278-1	abc	f		abc	f		abcd	e		abc	g	
X-P-B-290-1	cdef	ef		bcdef	f		abcde	cde		cdef	fg	
Palmar-NT	a	abcd		a	abcd		abcd	a		a	abcd	
CT 6241-NT	abcd	abcd		ab	abcde		abcd	abcd		ab	abcd	
GLOBAL												

Different letters in the table denote significant differences at $0.01 \leq P$, as determined by Ryan's multiple range tests