CONCURRENT SESSION 4

Induced Mutations for Traits that Affect Abiotic Stress Tolerance and Adaptation to Climate Change

Systematic Phenotype Analysis of *Arabidopsis Ds*-tagged Mutants to Unravel Gene Functions in Abiotic Stress Response as well as Growth and Development

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Abstract

By the availability of various mutant resources in *Arabidopsis*, it is now possible to investigate mutant lines for almost every gene. *Arabidopsis* is then, not only a model plant for plant research, but also a model species in which it is possible to carry out "saturation mutagenesis" for all genes, and to totally analyze each gene and mutant of one organism. One of the future goals of the "phenome" project is to collect information about the knockout-type mutant phenotypes for each *Arabidopsis* gene. We have generated thousands of *Dissociation* (*Ds*) transposon-tagged lines, which have a single insertion because of an advantage of the *Activator/Dissociation* (*Ac/Ds*) system, and deposited it to the RIKEN BioResource Center. In this resource, we selected 4,000 transposon-tagged lines with a transposon insertion in gene-coding regions, and systematically observed the visible phenotype of each line as a first step of phenome analysis. In total, about 200 clear visible phenotypes were classified into 43 categories of morphological phenotypes. Phenotypic images have been entered into a searchable database. Parallel to this, we have been selecting homozygous transposon-insertional plants, which would be useful resources to detect other phenotypes besides the visible ones. We are setting three categories of measurement to search various traits for total phenome analysis, such as physical, chemical or biological methods. Recently, we started to investigate biologically-measured phenotypes, which are stress-responsive or conditional phenotypes, using homozygous mutant resources. We are also collecting any mutant phenotype information from published reports in journal research activity to make a comprehensive phenotype database of *Arabidopsis* genes and mutants.

Introduction

Analysis of genetic mutations is an effective technique for investigating genetic function. Today, a wide variety of mutant organisms and cells created from gene silencing in model organisms is available for mass production, and great progress is being made in the use of tools for phenotype analysis [1–3]. RNAi gene silencing has been widely used in *Caenorhabditis elegans* and *Drosophila* [2,3]. In *Arabidopsis*, insertion mutations can be produced using transferred DNA (T-DNA) or transposons, making it possible to monitor the effects of changes in a single gene. Through self-pollination for maintaining progeny and through bulk storage of mutations in the form of seeds—not an option in animal models—it is now feasible to use insertion mutations to analyze every gene in the Arabidopsis genome. This makes *Arabidopsis* useful not only as a model organism for plant research, but also as the only multicellular organism in which it is currently possible to perform "saturation mutagenesis" to create knockout strains for each gene. Since the completion of sequencing of the *Arabidopsis* genome in 2000, an international team has been working to collect approximately 26,000 individual genes and to catalogue the functional genomics of the entire genome [4]. To contribute to this international project, we have generated transposon-tagged lines as a resource for *Arabidopsis* mutations, and are pursuing systematic phenotype analysis (phenome analysis). Since saturation mutagenesis is feasible in *Arabidopsis*, our goal is to prepare a gene encyclopedia that will catalogue the phenotypes for a variety of gene knockout strains.

Determining the insertion site for transposon insertion mutations

We generated transposon-tagged lines as a gene knockout mutant resource for research in functional genomics of *Arabidopsis.* In T-DNA insertion mutants, multiple T-DNA insertions have been reported [5]. However, using a transposon *Ac/Ds* system, it is possible to generate mutants with a high proportion of single-copy transposon insertions. This has the advantage of simplifying the production and subsequent genetic analysis of a single gene knockout system [6]. Using the transposon *Ac/Ds* system developed by Dr. N.V. Fedoroff et al., we have generated a total of 18,000 independent transposon-tagged lines [7–9]. In addition, we have used sequence analysis in the vicinity of the transposon to determine the transposon insertion site within the genome for each tag line, and are publishing the insertion site information (http://rarge.gsc.riken.go.jp/) [7–10]. The mutant lines included here have been deposited with the RIKEN BioResource Center (RIKEN BRC) for worldwide distribution (http://www.brc.riken.jp/lab/epd/).

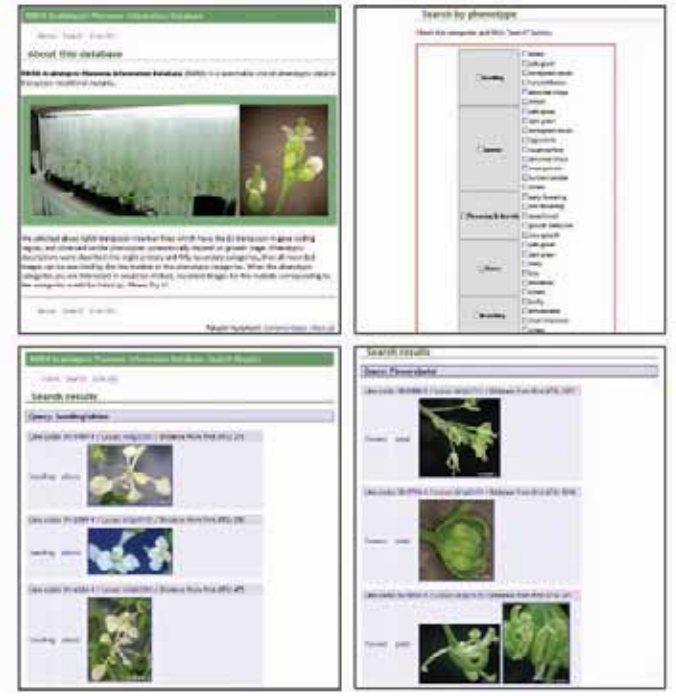

Figure 1 Top page of RAPID (RIKEN *Arabidopsis* Phenome Information Database) (upper left), search page of the database (upper right), and examples of search results (lower left and right).

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Phenotype analysis (phenome analysis) and creation of database

Next, as one method for implementing full-genome analysis that makes use of such a mutant resource, we reviewed the tagged lines that have been generated to date. We have selected the lines for which the transposon was inserted in the gene-coding region, and are conducting systematic phenotypic analysis (phenome analysis) for each line in approximately 4,000 genetic mutations. Up to this point, we have focused primarily on the morphological characteristics of external appearance (visible phenotypes). Through the stages of growth, we look at seedlings, leaves, stems, flowers, fruits (siliques), seeds, overall growth, and branching. We have established a total of eight categories and 43 detailed subcategories for classifying the phenotype data that we have obtained. Those mutations that show relatively clear aboveground morphological abnormalities with confirmed reproducibility are entered in a published database of mutations that permits searching by phenotypic category (RAPID: RIKEN *Arabidopsis* Phenome Information Database) (http://rarge.gsc.riken.jp/phenome/) (**Fig. 1**) [11].

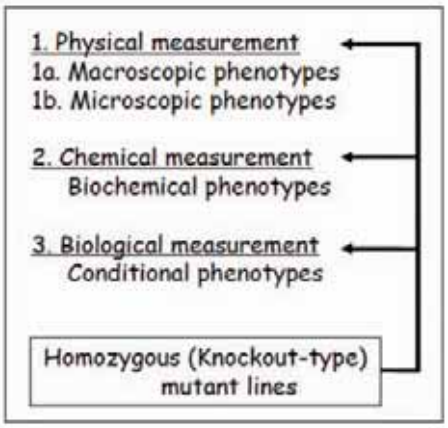

Figure 2 Application of mutant lines for various methods of phenotyping.

This database includes approximately 200 pages of image data, enabling users to search for a specific mutation by selecting the phenotypic category on the search screen. Search results are displayed as thumbnail images. One click enlarges the thumbnail to a full-size photograph, permitting the visual inspection of a mutation that would be quite difficult to describe in words. For each mutation that we observe, we indicate into which gene the transposon has been inserted, and look forward to progressively analyzing each mutant.

Phenotype parameters measured and the effective use of homozygous insertion mutants

The visible phenotypes currently entered in the database can be considered macroscopic phenotypes, representing a physically quantifiable portion of the data measured from phenome analysis. At the present stage, many mutant lines show no obvious morphological abnormalities in the visible phenotype, and no phenotype has been entered. For these lines, we are currently building up mutant lines with homozygous transposon insertion. We believe that these will be a novel resource for the gene knockout systems needed to investigate phenotypes that we have not monitored up to this point, including biochemical changes not externally visible as morphologic abnormalities (biochemical phenotypes), phenotypic response to stress (conditional phenotypes), and physical phenotypic qualities at the cellular level that cannot be determined without instruments (microscopic phenotypes) (**Fig. 2**). Recently, we started to investigate stress-responsive or conditional phenotypes from homozygous mutant resources. We are performing a high throughput stress examination using multi-titer plates to check germination and seedling growth under abiotic stress or abscisic acid treatment.

Homozygous mutant lines will also yield materials for gene knockout systems that not only can be applied one-by-one in phenotypic analysis, but also can be useful in generating double mutants and multiple mutants. In the future, we anticipate further progress in international cooperation in recording phenotypes in a format that increases the parameters for the variety of mutations in the entire *Arabidopsis* genome, and in methods

AGI code	Gene symbol		Homology	References	Phenotypes
At1g01290	CNX3		molybdopterin synthase	PNAS, 102, 3129-3134, 2005	sirtinol resistant
At1g02065	SPL8		plant-specific proteins that share a h	Plant Cell, 15, 1009-1019, 2003	strong reduction in fertility
At1g02450	NIMIN1		NPR1/NIM1-interacting protein	Plant Cell, 17, 1279-1291, 2005	hyperactivation of PR-1 gene expression after SA treatment b
At1g02910	LPA1		a chloroplast protein that contains tv	Plant Cell, 18, 955-969, 2006	high chlorophyll fluorescence phenotype
At1g03000	PEX6		encodes an apparent ATPase similar	PNAS, 101, 1786-1791, 2004	resistant to the inhibitory effects of IBA on root elongation an
At1g03160	FZL		a new plant-specific member of the (	PNAS, 103, 6759-6764, 2006	structures. fzl knockout mutants have abnormalities in chloro
At1g03310	ISA2	BE2	multimeric isoamylase	Plant J., 41, 815-830, 2005	Starch content is reduced
At1g03475	LIN2		coproporphyrinogen III oxidase	Plant J., 27, 89-99 , 2001	Lesion mimic mutant. The len2 mutant develops lesions on it
At1g04020	BARD1		BARD1, breast cancer associated	EMBO J., 25, 4326-4337, 2006	when challenged with the DNA crosslinking agent
At1g04120	MRP5		ABC Transporter; Putative lon	Plant Physiol., 134, 528-538, 2004	Decreased root growth; Increased lateral root formation
At1g04640	EMB1687		Mitochondrial Lipoyltransferase	Plant Physiol., 1351, 1206-1220, 2(	embryo lethality
At1g05470	CVP2		inositol polyphosphate 5' phosphatas	Plant Cell, 16, 1263-1275, 2004	an increase in free vein endings and a resulting open vein ne
At1g05630	At5PTase13		Inositol polyphosphate 5-phosphatas	Plant Physiol., 139, 1677-1691, 20(	At5PTase13 Deficiency Results in Abnormal Cotyledon Vein D
At1g06300	ACX3	IBR4	acyl-CoA oxidase	Plant J., 41, 859-874, 2005	decreased sensitivity to the inhibitory effect of IBA on root el
At1g06520	AtGPAT1		Membrane-bound glycerol-3-phospha	Plant Cell, 15, 1872-1887, 2003	atgpat1 Mutants Have Severely Reduced Male Fertility
At1g06950	Tic110		A multisubunit translocon of the inn	Plant J., 41, 412-428, 2005	embryo lethality
At1g07890	APX1		cytosolic ascorbate peroxidase	Plant J., 34, 187-203, 2003	Knockout-Apx1 plants were characterized by suppressed grow
At1g08090	NRT2.1	LIN1	putative high-affinity nitrate transpor	PNAS, 102, 13693-13698, 2005	Impaired in nitrate uptake; When the three nrt2.1 mutants v
At1g08260	TIL1	EMB2284	Catalytic Subunit of DNA Pol-epsilon	Plant Cell, 17, 3362-3377, 2006	Embryo defective, globular
At1g08430	ALMT1		a homolog of the wheat Al-activated	PNAS, 103, 9738-9743, 2006	when Al3+ was introduced into medium, the MT was much r
At1g08510	FATB		Acyl-Acyl CP Thioesterase	Plant Cell, 15, 1020-1033, 2003	Essential for Normal Seedling Growth
At1g08810	MYB60		MYB	Curr Biol., 14, 1739-1746, 2004	A null mutation in AtMYB60 results in the constitutive reduct
At1g08840	EMB2411		Helicase/Nuclease (Dna2)	Plant Physiol., 1351, 1206-1220, 2(	embryo lethality
At1g10270	GRP23		a pentatricopeptide repeat (PPR) pro	Plant Cell, 18, 815-830, 2006	the arrest of early embryo development
At1g10470	ARR4		Type-A Arabidopsis response regulato	Plant Cell, 16, 658-671, 2004	arr4 (reduced levels of the transcripts) and arr5 displayed sul
At1g10510	EMB2004		LRR Protein; Similar to Human CAR	Plant Physiol., 1351, 1206-1220, 2(	embryo lethality
At1g10860	SAR3	MOS3	similarity to human NUP96	Plant Cell, 18, 1590-1603, 2006	(Suppressors of axr1 Mutant) The primary root is shorter t
At1g10930	RecQl4A		RecQ (ATP-dependent 3' to 5' DNA h	Plant J., 43, 789-798, 2005	hypersensitive to UV light and MMS, and more resistant to m
At1g11680	CYP51A2	EMB1738	obtusifoliol 14-alpha demethylase	Plant Physiol., 138, 2033-2047, 20(	stunted hypocotyls, short roots, reduced cell elongation, and
At1g11720	AtSS3		starch synthase (SS) III	Plant Physiol., 138, 663-674, 2005	No obvious differences between mutant and wild type were o

Figure 3 Gene list describing the mutant phenotypes in Arabidopsis (part of the list).

for integrating that data and entering it in databases. We also look forward to providing thorough phenotypic data that will not only be useful in plant-related functional genomics, but that will also elucidate new gene-to-gene relationships and networks.

Extracting mutant phenotype information for an *Arabidopsis* gene encyclopedia

Our objective is to build a gene encyclopedia for the *Arabidopsis* genome/phenome by recording the phenotype for each gene mutants. In the previous section, we categorized the measurement parameters for obtaining phenotypic data using mutant resources. Additionally, mutant phenotypes have already been published in the literature for many of these genes. Including this published data, the groundwork has now been laid for generating a comprehensive mutant phenotype database. For reference, a mutant phenotype list has been published by Dr. D.W. Meinke [12]. We have combined this list with recent information from the literature, and are extracting phenotypic information to record for single genetic variations (**Fig. 3**). To date, mutant phenotypes have already been collected for approximately 1,700 *Arabidopsis* genes. In the future we plan to continue collecting phenotype information, both by the use of mutant resources for phenotype analysis and also by continuing to extract relevant data from the literature.

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Mutational Analysis to Dissect Oxidative and Abiotic Stress in *Arabidopsis thaliana*

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Abstract

A forward genetics approach was used to identify mutants more tolerant to oxidative stress. Chemically and T-DNA-mutagenized collections of *Arabidopsis thaliana* mutant lines were screened for survivors under conditions that trigger oxidative stress–induced programmed cell death (PCD). The fungal AAL-toxin triggers PCD through perturbations of sphingolipid metabolism in AAL-toxin-sensitive plants. While *Arabidopsis* is relatively insensitive to the toxin, the *loh2* mutant is sensitive to AAL-toxin due to knockout of a gene involved in sphingolipid metabolism. EMS mutagenesis of *loh2* resulted in second-site mutants that are more tolerant than *loh2* to the toxin. Nine of these mutants, named *atr* (**A**AL-**t**oxin-**r**esistant), were characterized towards their response to oxidative stress-induced cell death. Either application of the catalase inhibitor aminotriazole, leading to H_2O_2 accumulation was used, or paraquat, leading to superoxide radicals generation. Some mutants were more tolerant to aminotriazole, paraquat, or both herbicides. In another approach, T-DNA mutagenized wild type seeds were germinated on plant growth media supplemented with aminotriazole and one survivor was recovered. *Atr1, atr7* and *atr9*, with tolerance to both aminotriazole and paraquat, were studied in more details. They showed tolerance to paraquat at seedling stage as well as at rosette leaf stage. *Atr1* was subjected to microarray analyses at seedling stage under conditions that trigger cell death in *loh2* and no visible damage in *atr1*. While most of the genes showed similar expression pattern in both mutants, some genes were specifically regulated in *loh2* or *atr1*. These specifically regulated genes are potential targets for further functional studies. Downregulation of genes related to cell wall extension and cell growth in both mutants is consistent with the observed AT-induced growth inhibition in both mutants. It indicates that AT-induced oxidative stress influences two different processes: growth inhibition, observed in both mutants, and cell death, apparent only in *loh2*.

Introduction

Many unfavorable environmental factors, including drought, salinity, extreme temperatures and pollutants, result in rapid and sustained elevation of endogenous levels of reactive oxygen species (ROS), situation referred to as oxidative stress. In most cases, oxidative stress occurs as a result of both increased production and hampered detoxification of ROS. ROS, including hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\cdot-}$), and singlet oxygen ($^1O_2$), are not only toxic by-products of metabolism but also important modulators of a number of plant developmental processes, stress responses and programmed cell death (PCD) [1,2]. Examples of ROS-modulated developmental processes include embryo development, root hair growth, nucellar degeneration, maturation of tracheal elements and epidermal trichomes, formation of lace leaf shape, and leaf senescence [1]. Many of these processes are also associated with ROS-dependent PCD. ROS-induced PCD is also an important component of the hypersensitive response, a defence reaction in which plant cells in and around the site of pathogen infection die in order to physically restrict the spread of the pathogen [3]. While in the above examples cell death is beneficial and/or essential for plant development and survival, some necrotrophic pathogens can secrete toxins that cause cell death in healthy tissues so that the pathogens can feed on the dead tissues [4].

Biological effects of ROS signalling depend on several factors, including chemical identity of ROS, sites of ROS production, amounts and duration of the elevated ROS levels, and interaction with other signalling molecules like plant hormones, nitric oxide, and lipid messengers [1]. Signalling properties have been reported for hydrogen peroxide, superoxide radicals, singlet oxygen, and even for the most destructive and short-lived hydroxyl radicals [1]. In general, low doses of ROS may induce protective mechanisms resulting in stress acclimation, while higher doses of ROS can initiate PCD.

ROS are metabolized by the antioxidant system of the cell, comprised of antioxidant molecules and enzymes [5]. Catalase is the main H_2O_2-detoxifying enzyme, serving as a cellular sink for hydrogen peroxide, while superoxide dismutase is the only plant enzyme metabolizing superoxide radicals [1]. Important antioxidant enzymes are also ascorbate peroxidases, glutathione reductases, glutathione-S-transferases and glutathione peroxidases, monodehydroascorbate and dehydroascorbate reductases, peroxiredoxins, and others [1,5]. Reduction of catalase activity by gene silencing or by catalase inhibitor aminotriazole (AT) leads to increased endogenous H_2O_2 levels, oxidative stress and eventual cell death [6,7]. H_2O_2-dependent cell death is a programmed process, associated with specific alterations in gene expression, and can be compromised by increased CO_2 concentration in the air [6,8,9].

The fungal AAL-toxin triggers cell death through perturbations of sphingolipid metabolism in AAL-toxin-sensitive tomato [10]. The toxin inhibits ceramide synthase, a key enzyme in sphingolipid synthesis, which leads to accumulation of precursors and depletion of complex sphingolipids. Tomato plants sensitive to the AAL-toxin have a mutation in the Asc gene that is most likely a component of the ceramide synthase [11]. The *Arabidopsis thaliana loh2* mutant is more sensitive to the AAL-toxin than the wild type due to the knockout of a gene homologous to the tomato *Asc* gene [4]. Microarray analyses of AAL-toxin-induced cell death in *loh2* revealed induction of hydrogen peroxide-responsive genes and genes that are involved in the oxidative burst at early time points preceding visible cell death symptoms [4]. This indication of oxidative burst in AAL-toxin-treated plants was in agreement with previous studies demonstrating accumulation of reactive oxygen species in *Arabidopsis* plants treated with fumonisin B1 (FB1), an AAL-toxin analogue [12]. Moreover, a recently identified FB1- resistant mutant compromised in serine palmitoyl transferase, a key enzyme of *de novo* sphingolipid synthesis, failed to generate ROS and to initiate cell death upon FB1 treat-

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ment [13]. This paper describes a genetic approach carried out to isolate mutants more tolerant to oxidative stress and their characterization in respect to several types of reactive oxygen species-induced cell death.

Materials and Methods

Plant material, growth conditions and isolation of mutants

Forty thousand seeds from *Arabidopsis thaliana loh2* mutant, described earlier [4], were mutagenized with 0.1 - 0.3% ethane methyl sulfonate for eight hours. After extensive washing, the mutagenized seeds were planted on soil in pools and grown under standard greenhouse conditions (14 h light/10h dark period, photosynthetic photon flux density 400 μmol. m^{-2}. s^{-1}, 22°C and relative humidity 70%). Screening for resistance to AAL-toxin was done by plating the self-pollinated progeny seeds from M_1 plants on growth media containing 40 nM of AAL-toxin and grown in a climate room under the following conditions: 60 μmol. m^{-2}. s^{-1}, 22°C. AAL-toxin-resistant survivors were transferred to the greenhouse and seeds collected for further analysis. Screening for tolerance to AT was done by placing 8600 M_3 T-DNA activation tagged mutant lines obtained from the Ohio Arabidopsis Stock Center (CS21995) on 9 μM AT and one survivor isolated 10 days after germination.

DNA isolation and TAIL PCR, microarrays and bioinformatics analysis

DNA was isolated with *DNaesy* plant mini kit (Qiagen) according to the instructions of the manufacturer. TAIL-PCR was performed following the original protocol of [14,15] by using of 3 specific nested primers (SP1, SP2 and SP3: SP1 = TCCTGCTGAGCCTCGACATGTTGTC, SP2 = TCGACGTGTCTACATTCACGTCCA, SP3=CCGTCGTATTTATAGGCGAAAGC) and three arbitrary degenerated primers (AD1, AD2 and AD3: AD1= NTCGASTWTSGWGTT, AD2= NGTCGASWGANAWGAA, AD3= WGTGNAGWANCANAGA). Microarrays and bioinformatics analysis has been previously described [16].

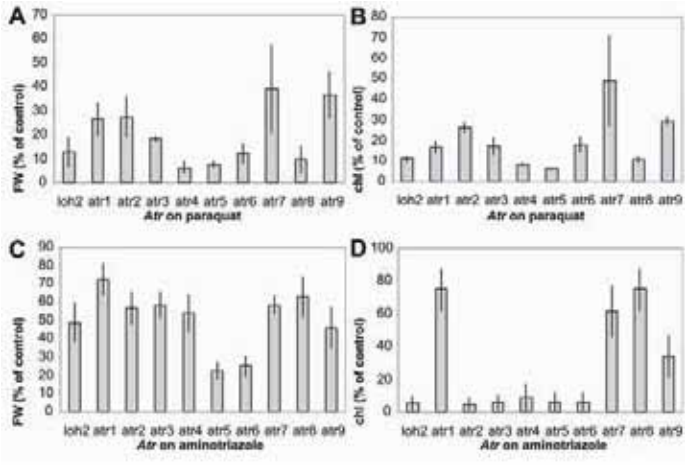

Figure 1 *Atr* mutants and their tolerance to reactive oxygen species-induced cell death. Seeds of nine *atr* mutants initially identified as more tolerant to AAL-toxin were plated on Murashige and Skoog (MS) media supplemented either with 0.5 μM paraquat (A, B) or with 7 μM aminotriazole (C, D) in order to assess their tolerance to cell death induced by superoxide radicals or hydrogen peroxide, respectively. Data represents the loss of fresh weight (FW) or chlorophyll (chl) of *atr* mutants grown on media supplemented with paraquat or aminotriazole and compared with *atr* mutants grown without paraquat and aminotriazole (controls). Samples for the measurements were collected one week after germination. Data are means of three measurements ±SD.

Evaluation of tolerance to oxidative stress and cell death assessment

Assessment for tolerance to ROS-induced programmed cell death was done by plating seeds from *loh2* and *atr* mutants on media containing either 7 μM AT or 0.5 μM paraquat and measuring the relative loss of fresh weight, chlorophyll, and visible cell death one week after germination. Chlorophyll content was measured photometrically as previously described [17]. In addition, plants were grown for four weeks to rosette leaf stage and sprayed with 15 μM paraquat. Visible damage, chlorophyll content and trypan blue staining for detection of dead cells was employed to evaluate the tolerance to paraquat-induced oxidative stress.

Results and Discussion

Isolation of mutants with enhanced tolerance to oxidative stress

Two approaches have been used to isolate mutants with enhanced tolerance to ROS-induced cell death. In the first approach, fungal AAL-toxin was used as an inducer of PCD and screening agent. Previous studies demonstrated that AAL-toxin leads to accumulation of H_2O_2, followed by transcriptional reprogrammeming and programmed cell death [4]. Moreover, comparative transcriptional analysis revealed a very similar expression pattern between AAL-toxin-treated plants and plants compromised in catalase activity [18]. The *loh2* mutant of *Arabidopsis* is sensitive to AAL-toxin due to knockout of a gene involved in sphingolipid metabolism [4]. Forty thousand seeds from *loh2* were chemically mutagenized with ethane methyl sulfonate, germinated on soil, self-pollinated and the resulting progeny plated on AAL-toxin-containing media in order to isolate mutants that are more tolerant to AAL-toxin than the original *loh2* background. While the wild type *Arabidopsis* is resistant to 200 nM AAL-toxin, the *loh2* mutant develops cell death symptoms at 20 nM AAL-toxin already and 40 nM of the toxin leads to lethality. Thirty independent survivors were isolated using a concentration of 40 nM AAL-toxin as a screening threshold. Nine of these mutants, named *atr* (AAL-toxin resistant), were selected for further analysis (**Fig. 1**). Genetic studies by crossing *atr* with the wild type and studying the progeny indicated that *atr* mutants were recessive (data not shown).

In the second approach, catalase inhibitor AT was used as an inducer of oxidative stress and screening agent. Previous results showed that AT added in plant growth media at concentrations of 7 μM to 9 μM, depending on the plant background, can inhibit catalase and lead to oxidative stress-dependent cell death in wild type plants [7]. A T-DNA activation-tagged mutant collection with 8,600 lines obtained from the Ohio Arabidopsis Stock Center (CS21995) was screened on media with AT. One mutant surviving the lethal AT concentrations was isolated. TAIL-PCR analysis has identified flanking DNA sequences around the T-DNA insert and revealed the position of the T-DNA on chromosome 2, between gene loci At2g27270.1 and At2g27280.1. Molecular analysis indicated presence of a single T-DNA insert.

Characterization of *atr* mutants for tolerance to ROS-inducing herbicides and expression analysis during AT-induced oxidative stress

Earlier studies indicated that the AAL-toxin causes induction of ROS-associated genes and H_2O_2 accumulation that precedes the cell death [4]. To investigate the link between AAL-toxin and oxidative stress, the nine *atr* mutants were also tested for tolerance to PCD induced by ROS-generating herbicides (**Fig. 1**). While AT leads to H_2O_2 accumulation, paraquat causes superoxide-dependent cell death [19]. Application of either AT or paraquat in plant growth media caused reduction in growth as measured by fresh weight loss (**Fig. 1**), reduction in total chlorophyll content (**Fig. 1**) and eventually death of *loh2* (**Fig. 2**). Some of the *atr* mutants were more tolerant to both paraquat and AT than *loh2*, as estimated by the lack of cell death, smaller decrease in fresh weight and more chlorophyll. Other mutants, however, were more tolerant either to AT or to paraquat, indicating the complexity of the cell death process. It could be that mutants more tolerant to both cell death stimuli are downstream of the convergence point of superoxide and hydrogen

peroxide-triggered signaling cascades. Three of the mutants with tolerance to both AT and paraquat, *atr1, atr7* and *atr9* were selected for further analysis. Interestingly, all three mutants grow slowly than *loh2* on normal media, which can be a 'trade-off' for their enhanced stress tolerance. In presence of AT or paraquat they show no visible damage while wild type plants die rapidly (**Fig. 2**). The tolerance of *atr1, atr7* and *atr9* towards paraquat was evident also at rosette leaf stage. Spraying with paraquat resulted in much less damage on the leaves of the mutants compared with the *loh2* control (**Fig. 3**). Preliminary results indicate that two of the oxidative stress-tolerant mutants are also more tolerant to chilling stress. Evaluation of other abiotic stress factors, currently going on, could further establish the link between oxidative and abiotic stress.

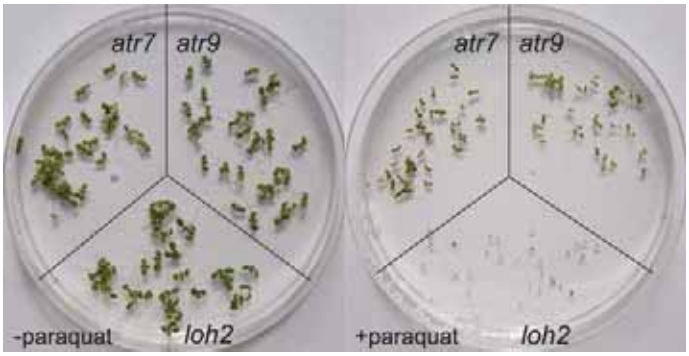

Figure 2 Oxidative stress-tolerant mutants *atr7* and *atr9* show enhanced tolerance to paraquat at seedlings growth stage. Oxidative stress-tolerant mutants *atr7* and *atr9*, and their parental line *loh2* were germinated without or with presence of 0.7 µM paraquat. Pictures were taken 5 days after germination.

Figure 3 *Atr1*, *atr7* and *atr9* show enhanced tolerance to superoxide radical-generating herbidice paraquat at rosette leaf stage. Four-weeks-old *atr1*, *atr7* and *atr9* and their respective control *loh2* were sprayed with 15 µM paraquat and pictures taken three days after treatment.

Microarray analysis of *atr1* and *loh2* on media with AT under conditions that trigger cell death in *loh2* and no visible damage in *atr1* at seedlings growth stage revealed that the majority of the genes are similarly induced or repressed in both mutants with only small sets of genes specifically regulated in *atr1* or *loh2* [16]. Most of the genes strongly

Table 1. AT-induced gene expression in *loh2* and *atr1*

Description	Gene Locus	*loh2*	*atr1*
high-affinity nitrate transporter NRT2	AT1G08090.1	8.21	83.81
putative isocitrate lyase	AT3G21720.1	9.81	2.64
similar to F-box protein family	AT2G16365.1	9.81	2.00
heat shock protein 17	AT3G46230.1	12.83	1.46
heat shock protein 17.6A	AT5G12030.1	8.65	0.89
extensin-like protein	AT5G46890.1	-27.96	-23.90
putative proline-rich protein	AT2G33790.1	-27.52	-30.62
extA (emb CAA47807.1)	AT5G46900.1	-21.92	-21.00
unknown protein	AT1G19900.1	-13.90	-9.89
cytochrome p450, putative	AT2G25160.1	-10.15	-4.22
glutathione transferase, putative	AT1G49860.1	3.51	4.01
glutathione transferase, putative	AT1G53680.1	-6.28	-6.39
glutathione transferase, putative	AT3G62760.1	-2.66	-2.41
glutathione transferase, putative	AT1G17190.1	-2.05	-2.51
thioredoxin, putative	AT1G45145.1	-2.98	-1.97
putative glutaredoxin	AT2G30540.1	2.12	2.10
glutaredoxin	AT4G15690.1	-0.96	-3.82
glutaredoxin homolog	AT4G15700.1	-1.06	-3.28
glutaredoxin	AT4G15670.1	-1.07	-3.17
glutaredoxin	AT4G15680.1	-0.96	-2.95
glutaredoxin	AT4G15660.1	-1.09	-2.66
type 2 peroxiredoxin, putative	AT1G65970.1	-2.91	-2.88
peroxiredoxin, putative	AT1G60740.1	-2.75	-2.33
copper/zinc superoxide dismutase (CSD2)	AT2G28190.1	-1.22	-2.17
monodehydroascorbate reductase, putative	AT3G09940.1	-4.88	-3.50
dehydroascorbate reductase, putative	AT5G36270.1	-2.94	-2.50
peroxidase, putative	AT1G24110.1	2.44	2.24
peroxidase family	AT4G16270.1	2.13	2.27
peroxidase, putative	AT1G05240.1	-11.85	-8.06
peroxidase, putative	AT4G26010.1	-11.12	-15.46
peroxidase, putative	AT1G34510.1	-9.94	-7.99
peroxidase, putative	AT5G67400.1	-9.11	-9.28
peroxidase, putative	AT2G39040.1	-8.26	-7.03
cationic peroxidase, putative	AT1G30870.1	-6.44	-6.64
peroxidase, putative	AT3G01190.1	-5.34	-3.20
peroxidase, putative	AT2G18980.1	-4.55	-3.98
peroxidase, putative	AT5G17820.1	-4.42	-3.23
peroxidase, putative	AT4G30170.1	-3.89	-3.48
peroxidase, putative	AT3G49960.1	-3.49	-4.25
peroxidase, putative	AT5G22410.1	-3.47	-5.35
peroxidase, putative	AT5G64100.1	-3.30	-2.64
peroxidase, putative	AT5G15180.1	-2.96	-3.48
peroxidase, putative	AT1G34330.1	-2.96	-2.25
peroxidase, putative	AT5G42180.1	-2.91	-2.29
anionic peroxidase, putative	AT1G14540.1	-2.49	-2.79
cationic peroxidase family	AT5G24070.1	-2.17	-2.14
peroxidase, putative	AT1G49570.1	-4.73	-1.73
peroxidase, putative	AT5G19890.1	-2.98	-1.74
peroxidase, putative	AT1G05260.1	-2.33	-1.39
anionic peroxidase, putative	AT1G14550.1	-1.23	-3.78

Arabidopsis thaliana loh2 and *atr1* mutants were grown on media without or with 7 µM AT, and samples for microarray experiments collected two days before cell death symptoms in *loh2*. The first 10 genes listed are the most induced or repressed genes; the other genes in the list encode for antioxidant enzymes and are regulated at least two-fold. Data are means of two biological replicates. Positive values indicate upregulated genes while negative values indicate downregulated genes.

downregulated in both mutants were related to cell wall extension and cell growth, in line with the similar AT-induced growth inhibition in both mutants. This indicates that two different pathways, one for modulating growth inhibition and second triggering cell death, are associated with AT-induced oxidative stress.

In this paper, we focused on the expression pattern of the antioxidant enzymes. Genes with regulation more than two-fold on average from the two biological repetitions are presented in Table 1. Majority of the antioxidant enzymes were repressed in both *loh2* and *atr1*. For example, 24 genes encoding for guaiacol peroxidases were regulated; 18 of them were repressed in both mutants, three were repressed only in *loh2*, one repressed only in *atr1*, and two induced in both mutants. One monodehydroascorbate reductase, one dehydroascorbate reductase, and two peroxiredoxins were regulated – all of them repressed in both mutants. 3 from four regulated glutathione transferases were repressed and one induced in both mutants. An exception from this general trend were glutaredoxins, as five out of six regulated genes were repressed only in *atr1*. The downregulation of majority of the antioxidant enzymes, although with unclear biological functions, may be related to the oxidative stress-induced repression of growth in both mutants.

Conclusion

Two approaches for isolation of mutants with enhanced tolerance to oxidative stress have been demonstrated. The approaches, based on the fungal AAL-toxin and on the catalase inhibitor AT, are suitable for screening chemical as well as T-DNA mutant lines. Some of the isolated mutants show enhanced tolerance to a number of factors causing oxidative stress-induced cell death, while other mutants show enhanced tolerance to limited or only one cell death trigger, indicating the complexity of the responses. AT causes two different effects: growth inhibition, evident in both the sensitive parental *loh2* line and the cell death-tolerant *atr* mutants, and cell death, evident only in the parental line. This notion is further supported by microarray analysis of *loh2* and *atr1*, revealing AT-dependent downregulation of growth associated genes in both *loh2* and *atr1*. The transcriptome analysis revealed also genes specifically regulated only in *loh2* or *atr1*. These genes are potential targets for further functional studies aimed at elucidating their role in the oxidative stress tolerance and cell death.

ACKNOWLEDGEMENTS

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Development of Salinity-tolerant Rice Varieties Using Biotechnological and Nuclear Techniques

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Abstract

A breeding programme using biotechnological and nuclear techniques was developed in order to obtain salinity-tolerant rice varieties, using Amistad-82 and Jucarito-104 rice varieties as donors. This study included the increasing genetic variability by means of somaclonal variation and in vitro mutagenesis with proton radiations, the establishment of culture medium for callus formation and plant regeneration, as well as the establishment of feasible salt tenors for in vitro selection and the identification of morphological markers for the early selection of salinity-tolerant lines. The selection was carried out under field conditions for four years. A methodology was established to obtain salinity-tolerant rice varieties using biotechnological and nuclear techniques and it was possible to release two salinity tolerant rice varieties that are being used in rice production.

Keywords:

Rice - Salinity - Somaclonal Variation - Mutation Induction - Protons

Introduction

Soil salinity is one of the most dangerous problems in the world. In Cuba, the inadequate rainfall distribution together with long periods of drought, some salt-polluted aquifers, as well as man´s misuse and mismanagement of land have multiplied saline areas up to 14% and the same amount is prone to become saline [1].

Rice (*Oryza sativa* L.) is one of the most important crops in the world. Rice is planted on about one tenth of the earth's arable land and it is the unique largest source of food energy to half of humanity [2, 3]. In Cuba, it is an essential cereal but its yielding is very low, taking into account that varieties are affected by several biotic and abiotic stresses, such as soil salinity and drought.

About 14% of the agricultural areas are affected by salinity in our country. Therefore, it is necessary to obtain salinity-tolerant rice varieties in order to increase production of this cereal in Cuba.

Commercial rice varieties are characterized by a high degree of genetic homogeneity [4]; however, a diverse genetic basis of breeding material would be advantageous when using genotypes resulting from induced mutations.

Biotechnological and nuclear techniques can be used along with traditional breeding methods in some breeding programmes. Rice mutation breeding could be considered especially successful to obtain new cultivars with good agronomic characteristics, as well as biotic stress resistance and/or abiotic stress tolerance, also to broaden crop genetic base [5].

A breeding programme using biotechnological and nuclear techniques was developed, in order to obtain salinity-tolerant rice varieties.

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Materials and Methods

Culture medium

To get the best culture medium for callus formation and plant regeneration, mature seeds of Amistad-82 (A-82) and Jucarito-104 (J-104) rice varieties were grown on a Murashige and Skoog medium [6], supplemented with different concentrations of 2,4-D and BAP, 30g refine sugar and vitamins. Callus formation was evaluated. After 30 days, calluses were transferred to a fresh medium with kinetin and IAA for plant regeneration.

Saline concentration in the culture medium

Mature seeds of A-82 and J-104 varieties were cultured in the best medium supplemented with different commercial salt concentrations, in order to establish the optimal concentration for callus formation and plant regeneration under saline conditions.

Increased genetic variability

Mature seeds of Jucarito-104 (J-104) rice variety (11.5% moisture content) irradiated with 20Gy protons at the Phasotron facilities (DUBNA) as well as those of Amistad-82 were grown in vitro on a Murachige & Skoog medium supplemented with 2 mg.L^{-1} 2,4-D, 2 mg.L^{-1} BAP and 4g.L^{-1} salt, in order to increase genetic variability. After 30 days, calluses were transferred to regenerated plant medium.

Such regenerated plants were planted under greenhouse conditions, and plant cycle, height, panicle number/plant, full grains/panicle as well as yield/plant were evaluated. A Multivariate Analysis was used to assess variability.

Selection

Seeds of each regenerated plant were sown in boxes containing inert substratum with saline water at an electrical conductivity of 8d.Sm^{-1}. After 15 days, root length and plant height of surviving plants were evaluated for an early selection. Plants selected were multiplied and sown in saline soil (4,000 to 16,000 ppm). Selection on saline soil was developed during four generations.

Plant cycle, number of tiller per plant, grain number per panicle, grain weight and yield were evaluated in the plants selected every selection cycle.

Results

Culture medium selected

The culture medium enabled to obtain a higher percentage of callus formation as well as more callus with buds and bud number per callus. In both varieties, the best combination was that containing 2 mg.L^{-1} 2,4D and 2 mg.L BAP^{-1} (**Table 1**).

It should be pointed out that by combining 2,4D and BAP, bud regeneration started when transferred to lighting conditions using the same means employed for callus formation.

When evaluating the effect of different salt concentrations in the callus formation and plant regeneration medium, a considerable decrement was observed with an increase of salt concentration in the culture medium as well as higher concentrations than 7 g.L^{-1} affect the process of plant regeneration considerably in both varieties; thus the concentration of 4 g.L^{-1} was selected (**Table 2**).

It can be observed that the variety J.104 is more susceptible to salinity than A-82, since plant regeneration is considerably affected with an increase of saline concentration in the culture medium. Considering the results, it was determined that a culture medium with 2 mg.L^{-1} 2,4D, 2 mg.L^{-1} BAP and 4 gL^{-1} salt should be used.

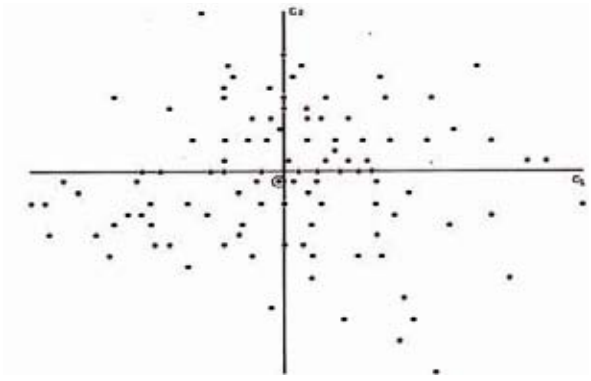

Figure 1 Space distribution of plants selected in vitro starting from Main Components Analysis using the characters: height, panicle number/plant, full grains/panicle and yield.

Table 1. Callus formation and plant regeneration of A-82 and J-104 rice varieties cultivated on a MS medium supplemented with different combinations of 2,4-D and BAP

Saline concentrations (g.L^{-1})		Callus formation (%)		Callus with shoots (%)		No. shoots/ callus	
2,4-D	BAP	A-82	J-104	A-82	J-104	A-82	J-104
1	-	100	100	1,0	2,0	1,0	1,0
2	-	100	100	2,0	-	1,0	-
3	-	100	100	1.0	-	1,0	-
1	1	99	100	28,1	12,0	4,5	3,2
1	2	100	98	22,0	10,3	9,0	2,8
1	3	100	100	41,0	20,5	13,4	6,2
2	1	100	99	2,5	2,0	1,0	1.0
2	2	100	100	58,3	49,2	16,,6	12,3
2	3	100	100	21,5	6,5	8,5	3,4
3	1	99	100	1,5	-	1,0	-
3	2	100	100	2,5	1,0	3,2	1,0
3	3	100	100	14,8	1,0	2,5	1,0

Table 2. Callus formation and plant regeneration of A-82 and J-104 rice varieties cultivated on a MS medium supplemented with 2 mg.L^{-1} 2,4-D and 2m.L^{-1} BAP and different commercial salt concentrations.

Saline concentrations (g.L^{-1})	Callus formation (%)		Callus with shoots (%)		No. shoots/ callus	
	A-82	J-104	A-82	J-104	A-82	J-104
0	100	100	40,3	48,9	12,5	13,2
1	92	85	30,1	21,5	5,3	1,0
4	66	26	15,5	2,5	1,5	1,0
7	25	5	-	-	-	-
10	3		-	-	-	-
13	-	-	-	-	-	-
16	-	-	-	-	-	-

Table 3. Number of plants selected in each selection cycle

Conditions	Saline concentrations (ppm)	Selected genotypes
In vitro selection	4,000	125
Screening in saline solution (8 d.Sm^{-1})	7,500	68
Selection in field conditions	4,000–16,000	36
Selection in field condition	4,000–16,000	18
Selection in field condition	4,000–16,000	10
Yield trial	4,000-7,000	2

Table 4. Main differences of mutants and somaclones with its respective donors

Character	Donor	Somaclon	Donor	Mutant
	A-82	LP-7	J-104	GINES
Days to maturity	111/126	135/150	133/150	119/137
Weigh of 1,000 grains	28	30	31	33
Grain number per panicle	35-42	67-75	31-47	68-73
Disease resistance	Susceptible Steneotarsonemus Spinki	Resistant Steneotarsonemus Spinki	Susceptible Pyricularia grisea	Middle resistant Pyricularia grisea
Yield in saline conditions (5-7 dSm/m)	1,8 t/ha	3,4 t/ha	1,4 t/ha	3,3 t/ha

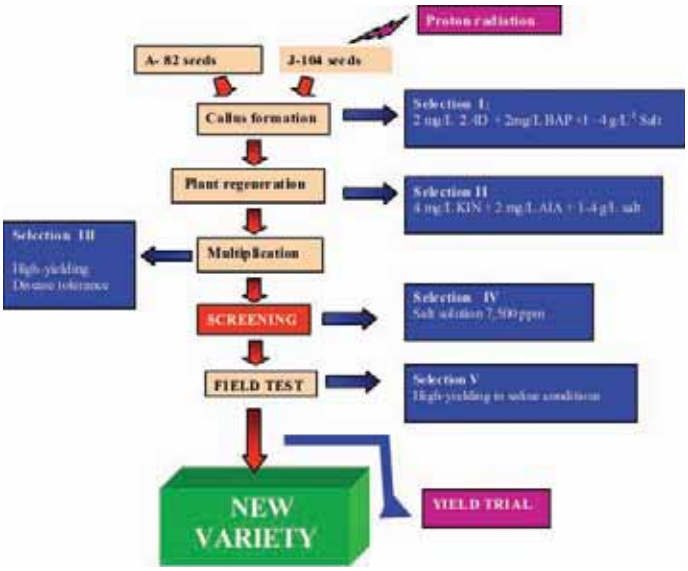

Figure 2 Methodology established for salinity breeding programme in rice using biotechnological and nuclear techniques.

Selection

The number of selected plants was diminished in each selection cycle (**Table 3**). Starting from the methodology employed, it could be recommended to register two new rice varieties for rice production, since they are salinity-tolerant, show good agronomic characteristics and also more tolerance to some diseases affecting the national rice production (**Table 4**).

Further research will give us insight to the feasibility of somaclonal variation and mutation induction with protons in rice genetic improvement, as well as to establish a methodology for obtaining salinity tolerant rice varieties using biotechnological and nuclear techniques (**Fig. 2**).

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Evaluation and Characterization of Mutant Cowpea Plants for Enhanced Abiotic Stress Tolerance

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Abstract

The objective of the project is to use the radiation-induced mutations in cowpea to improve cowpea varieties grown by resource-poor farmers in South Africa. The first aim of the project was to select mutant cowpea plants with improved levels of drought tolerance without alteration to the color of the testa or the growth form. It was demonstrated that it was possible to examine mutant lines at seedling stage in wooden boxes. Mature plants were screened in rain out shelters and physiological traits for drought stress were identified among the lines tested. Roots of mature plants were also assessed and variations observed could be correlated with drought tolerance. The data demonstrated that physiological methods can be used to screen mutants. The yield performance of some mutant lines proved to be outstanding under well-watered, as well as under drought stress conditions. The second aim was to further characterize the most promising mutant lines using molecular and physiological techniques. cDNA-Amplified Fragment Length Polymorphism showed differential gene expression at different time points of drought stress. The sequenced transcript derived fragments (TDF) showed high homology to expressed sequence tags of soybean, with a possible function in cell defense/resistance and most importantly, signal transduction. Reverse transcription PCR using a number of primers from published sequences, as well as from the TDF sequences, validated the differential gene expression obtained from the cDNA-AFLP display. The third aim was to evaluate selected mutants on station and at different communities. On station field trials were conducted at the ARC-VOPI's research farm under dry land as well as irrigation conditions for the last two seasons. The long term plan is to introgress the drought tolerance trait from the best mutant line into drought susceptible South African cultivars grown by resource-poor farmers.

Introduction

Vigna unguiculata (L.) Walp., commonly known as cowpea, is a grain legume that is grown mainly in Africa, Asia, and South America. Cowpea grain contains about 25% protein, making it extremely valuable where many people cannot afford animal protein foods [1]. Cowpea is a dual crop in Africa, where the nutritious tender leaves of the plant as well as the green pods are consumed [2]. As a drought-tolerant crop, cowpea is adapted to dry or arid environments where rainfall is low and erratic, soils less fertile and other crops habitually fail [3]. Another valuable characteristic is that cowpea fixes atmospheric nitrogen through its root nodules and can grow in poor soils with more than 85% sand, with less than 0.2% organic matter and low levels of phosphorus [4]. Several centers of domestication have been suggested for cowpea, such as Ethiopia, Central Africa, South Africa and West Africa, but the East and Southern Africa are considered as the primary region of diversity and West and Central Africa to be the secondary centers of diversity [5].

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Drought is a major constraint to agricultural production in many developing countries. Direct and indirect economic losses in the agricultural sector due to drought are huge. Moreover, the recent climatic changes necessitate the need to develop crops more tolerant to drought and to reduce poverty in the developing world. Significant potential exists for the improvement of crop productivity by selecting plants that are better equipped to cope with drought stress. Cowpea is an extremely resilient crop that is well known for its ability to survive under conditions of water stress and it plays an important role in regions where drought is the factor most limiting to crop yield [6]. One way to combat drought is to develop crops of agricultural importance that are more tolerant to drought stress by combining plant physiology and biotechnological techniques. A better understanding of the physiology and genetics of cowpeas under drought could lead to the improvement of its drought tolerance and water use, in order to improve yield. A multidisciplinary approach was thus initiated over the past years at ARC-VOPI in collaboration with IAEA, to improve cowpea by inducing mutations for enhanced drought tolerance.

Materials and Methods

Various cowpea mutant lines were screened and compared with control lines received from International Institute of Tropical Agriculture (IITA) in Nigeria. These lines comprised of the control lines IT96D-602 (drought-tolerant) and TVu7778 (susceptible), as well as the parent line of the mutants, IT93K129-4. This line was selected for its color, growth form and yield. Various gamma irradiation dosages between 0 and 300Gy were applied (n=100 seed) to IT93K129-4 to obtain a high frequency of gene mutation and chromosomal alterations. A total of 17,000 cowpea seeds were consequently irradiated using the optimal irradiation dosage of 180Gy. Aberrations that were observed include leaf mutation and chlorophyll deficiencies.

The wooden box procedure of Singh [7] was used for the screening of mutant seedlings. The calorimetric method of Bates [8] was used to determine the proline concentrations of freeze dried leaves. Leaves were collected early in the morning to determine relative water content (RWC) [9]. The root systems of the plants were evaluated using the root architecture box technique developed by Singh [7].

The Restriction fragment length polymorphysim (RAPD) technique was performed according to the method of Fall [10] and the amplified fragment length polymorphism (AFLP) modification version of Vos [11] was used.

Results and Discussion

The first aim of the project was to improve the drought tolerance and yield of cowpea plants without alteration to the color of the testa or the growth form, to such an extent that it could be used in marginal areas where rainfall is either scarce or unreliable. M_1 seed of IT93K129-4 were planted in the field, after which 8 230 M_2 true to type plants, that had survived the irradiation process and yielded seed, were selected. M_2 seeds were planted in wooden boxes in a greenhouse for early drought selec-

tion (891), as well as under a rain out shelter for late drought selection (1239). These lines were evaluated against IT96D-602 and TVu7778, and resulted in selection of 487 lines with higher levels of drought tolerance. Some of the mutant lines used a drought evasion method to survive, they produced seed before reaching the permanent wilting stage and although the plants did not recover after the stress period, viable seeds were produced, thus, ensuring the next generation. Other lines withstood the period of drought stress and were able to recover to such an extent that the plants were able to produce seed after being rewatered. Promising M_3 and M_4 lines were replanted in wooden boxes, in dry land trials in the field and under rain out shelters. Nine of the best performing lines were selected on the basis of yield in the field, 22 promising lines were identified in the rain out shelter trials and 36 in the wooden box trials [12].

The six best lines from all this screening methods were characterized by using physiological screening techniques conducted on drought-stressed greenhouse plants. The screening techniques included chlorophyll fluorescence, free proline, RWC and yield. After 12 days without water the plants started to react visibly to the drought stress condition. Some plants started to lose chlorophyll in their lower leaves, while others inclined their leaves away from the sun. The RWC of the stressed plants at this stage was between 80% and 90%. As the stress condition intensified the RWC dropped further and after 24 days without water the RWC of line Cp-m346 was as low as 65%, compared to the 75% of Cp-MA1 and IT96D-602. The more drought tolerant cowpea lines, Cp-m217 and IT96D-602, only started to produce proline in the latter part of the stress period, and at 24 days without water exhibited lower concentrations than the other lines tested [12].

The yield performance (number of seed) of the mutant lines Cp-m447, CpMA2 and Cp-m217 proved to be outstanding under well-watered conditions, as well as lines Cp-m447, Cp-m217 and Cp-m346 under drought-stress conditions. The plants were also evaluated according the mean seed weight. Line IT93K129-4 produced the heaviest seed under well-watered conditions, followed by Cp-MA2, Cp-m447 and Cp-m164, and under drought conditions lines IT93K129-4, Cp-m447 and Cp-m164 produced the heaviest seed. The ideal plant for subsistence farmers will be the one that produces a moderate to good yield under all conditions like lines Cp-m447 and CP-m217. The data demonstrated that Cp-m217 performed very well in terms of RWC, free proline concentration and yield [12].

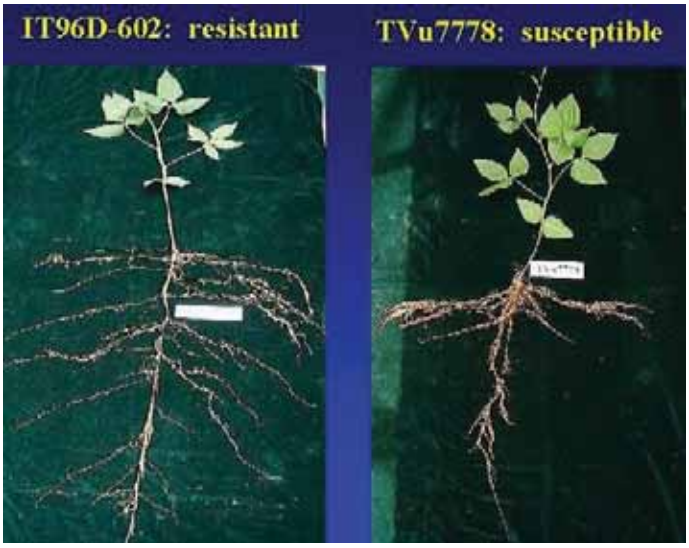

Figure 1 Root distribution patterns of the cowpea drought tolerant control line IT96D-602 and the more susceptible line TVu7778.

Roots of mature plants were also assessed using a pin-board root-box as a method for identifying the role of root characteristics in drought tolerance [7]. The variation observed between the drought-tolerant control (IT96D-602) and drought-sensitive control (TVu7778) indicates differences in the distribution of the roots, but not in total root length. The tendency of the drought-tolerant cultivar was to increase the amount of roots in the lower levels of the box, while the distribution of the roots of the sensitive cultivar was more at the top of the soil (**Fig. 1**, [12]). The distribution of the roots in the mutant plants was similar to that of the drought-tolerant cultivar, enabling them to access soil in the deep soil layers. This tendency was also observed by Matsui [13].

The first part of the project enabled the identification of a number of drought tolerant mutant lines based on data recorded for agronomic, morphological and physiological traits. The genomic knowledge for cowpea, with a chromosome number of 22 (2n=2x) and a genome size of ±600Mb, is very limited [14], thus the second aim of the project was to further characterize the most promising mutant lines using molecular techniques.

The first molecular analysis that was performed involved random RAPD studies. The RAPD technique utilizes low-stringency polymerase chain reaction (PCR) amplification with single primers of arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments [15]. Polymorphism was scored by looking at the banding patterns of the DNA fragments, as well as the number of bands per primers. Ten mutant cowpea lines were drought stressed for 21 days, together with the drought-tolerant line, IT96D-602, and parent line, IT93K129-4. RAPD analysis was conducted using various RAPD primers to screen the DNA samples from these lines. All of the primers amplified the DNA, but some (OPA08 and OPA10) did not show clear discrimination, and were therefore not used for subsequent experiments. The remaining 12 primers yielded either one or two polymorphic RAPD bands. Although the level of polyphorphism observed was very low, this analysis gave some indication of the genetic variation between the mutant and the control lines tested. The mutant lines Cp-m447 and Cp-m217 displayed different banding pattern with most of the primer tested when compared to the other lines (**Table 1**).

Table 1. Summary of the OPA and OPH primers used for RAPD analysis.

Primers	Lines & Controls								
	1	2	3	4	5	6	7	8	9
OPA-01		√		•	√				•
OPA-04				√		√	•		
OPA-08					Not clear				
OPA-10					Not clear				
OPA-12		√			? •				
OPA-19		√					√		
OPA-20	•				√				•
OPH-05					√				
OPH-08					√				
OPH-09					√				
OPH-11					√				
OPH-12		√		√					
OPH-14		√		•					
OPH-15			√				√	√	

1-10: 1: IT96D602; 2: IT93K129-4; 3: Cp-m26; 4: Cp-m164; 5: CP-m217; 6: Cp-m364; 7: Cp-m447; 8: Cp-MA1; 9: Cp-MA2. The blue tick and red dot were used as an indication of polymorphism.

The two mutant lines, Cp-m217 and Cp-m447, were subsequently grown in a greenhouse together with the parent line IT93K129-4. Samples were taken from the drought stressed plants at different time points to identify changes in gene expression by cDNA- AFLP transcript profiling. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA [11]. The cDNA-AFLP based marker system was used to detect polymorphism in the lines at the transcript level in response to the onset of drought stress. Primers representing EcoRI-ACT and MseI-CCT, gave reproducible profiles. A DNA fragment was found to be present in the mutant fingerprints but absent from the parental fingerprints. Band intensities, as well as presence and absence of the bands were scored. No polymorphic bands were observed between the lines at the 0 day time point which was evidence for the close homology of the genotypes. However, from the fourth to the 24th day of drought stress, there were clear differences in the transcript banding patterns between the mutant lines.

Nucleotide sequences of the transcript-derived fragments (TDF) were compared with nucleotide sequences of the expressed sequence tag (EST) databases by using the BLAST sequence alignment programme. Best database match and identity at the nucleotide level were obtained using the blastn and blastx programmes [16]. The first nine transcript-derived fragments of cowpea that were sequenced were found to have best matches with cDNA sequences cloned from soybean and common bean. Some of the identified transcripts exhibited similarity to published sequences, including a leucine rich protein, a NADH dehydrogenize subunit, a GTP binding protein and a transducin-like protein, indicating possible involvement in plant cell defense, energy and signal transduction.

Reverse transcription (RT)-PCR analysis was performed on six randomly selected TDF to verify the reliability of the cDNA-AFLP profile. A semi-quantitative PCR method was used to study the expression of some of the transcripts derived fragments. The 18S ribosomal RNA gene was used as an internal control. Primers (Cp-Mp56 & Cp-Mp60) were designed from the sequences of the TDF 56 and 60. As additional controls, primers from previously identified drought-induced Cowpea genes were also used to amplify the cDNA isolated for this experiment (Generation Challenge Programme: Cp-001, Cp-002, Cp-099). A similar expression pattern as with the cDNA-AFLP profile was observed, validating the cDNA-AFLP results.

The third aim was to evaluate the selected mutant lines on station and at different communities. Twelve mutant cowpea lines, together with drought tolerant line, IT96D-602, and parent line, IT93K129-4, were planted under dry land conditions at Kgora Resource Center near Mafikeng, North West Province. The majority of cowpea growers are women. They grow cowpea because it provides food for their families, and they can sell the grain in local market, or to traders, generating cash for household needs. Cowpea suffers heavily from insects, both in the field as well as when the grain is stored after harvest. The community members were thus trained in different production aspects such as soil preparation, fertilization and scouting for pests and diseases (**Fig. 2**). No significant differences were observed in yield between the different cowpea mutant lines. However, marked differences in growth habits were observed. Certain lines can only be used as a pulse crop, where the more spreading (indeterminate) growers can also be used as a leafy green vegetable. Being able to plant and evaluate the different lines themselves and not just being told that the one line is better than the other, was very important to the community members. They could not believe that the plants produced a good yield on soils without fertilizer.

The on station trials at ARC VOPI were planted in a randomized split plot design with two treatments, one irrigated and one dry land. Three replicates were included in every treatment and the lines were randomized within a treatment. On station field trials were conducted at the ARC-VOPI's research farm under dry land as well as irrigation conditions for the last two seasons. The mean yields for the cowpea during the last season varied between 112g and 862.23g in the dry land treatment, with a grand mean of 312g. There were three replicates in the treatment and 16 degrees of freedom. The mean yield for the cowpea varied between 93.9g and 310.2g in the irrigation treatment, with a grand mean of 186.6g. There were three replicates in the treatment and 16 degrees of freedom.

Figure 2 Community members were trained in different production facets.

The long term plan is to introgress the drought tolerance trait from the best mutant line into drought susceptible South African cultivars grown by the communities. This will also enable the identification and development of markers that are associated with drought tolerance to be further used in Marker-Assisted Selection in the existing cowpea breeding programmes.

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Radiation Induced *In Vitro* Mutagenesis, Selection for Salt Tolerance and Characterization in Sugarcane

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Abstract

Salinity is one the major environmental stresses affecting plant productivity. Combined use of mutagenesis and tissue culture can greatly facilitate the selection and isolation of useful tolerant lines. In the present study, *in vitro* mutagenesis was employed in the selection of salt tolerant lines in popular sugarcane (*Saccharum officinarum* L.) cv. CoC-671. Embryogenic cultures were gamma irradiated (10-50Gy) and challenged with different levels of NaCl (42.8 - 256.7 mM). Salt-stressed calli exhibited lower relative growth rate, decreased cell viability and higher levels of free proline and glycine betaine. The membrane damage (electrolyte leakage) was threefold more under salt stress compared to control. The ion levels were drastically affected under salt stress as leached out Na^+ and K^+ was much more than that retained in tissue in both adapted and unadapted callus cultures. The tolerance could also be related to the maintenance of better water status and a high to low level of K^+ to Na^+ under salinity stress, indicating that sugarcane can be a Na^+ excluder. Plant regeneration was observed in 10 and 20Gy irradiated calli up to 171.1 mM NaCl selection. A total of 147 plantlets were selected on different salt levels and the tolerant lines are being evaluated at field level. Molecular characterization using RAPD markers revealed genetic polymorphism among selected putative salt tolerant lines and control plants. In addition, plantlets regenerated form irradiated calli of sugarcane cv. CoC-671, Co 86032 and Co 94012 were field planted and agronomically desirable variants were identified for economic traits like cane yield and sucrose (Brix). The genetic stability of the variants is being evaluated at field level in M_3 generation. The proper evaluation of these variants for salinity tolerance may be useful for economic cultivation under the stress regime.

Introduction

Sugarcane (*Saccharum officinarum* L.) is an important agro-industrial sugar crop, contributing about 70% of the world sugar production. Being a typical glycophyte, it exhibits stunted growth or no growth under salinity, with its yield falling to 50% or even more of its true potential [1]. Somaclonal variation in combination with *in vitro* mutagenesis can be beneficial for the isolation of salinity and drought tolerant lines in a short duration employing *in vitro* selection. In sugarcane, studies have been conducted on isolating mutants resistant to red rot, water logging and delayed flowering [2,3] and salt tolerance [4]. In this study, results are presented on the Gamma-ray mutagenesis *in vitro*, followed by selection for salinity and drought tolerance and characterization of the putative salt and drought-tolerant regenerants of sugarcane cv. CoC-671.

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Materials and Methods

Establishment of embryogenic cultures, *in vitro* mutagenesis and selection

Embryogenic callus cultures of popular Indian sugarcane cultivars CoC-671, Co 86032 and Co 94012 were established [5] from young leaf explants on callus induction medium-CIM containing MS basal salts supplemented with 100 mg l^{-1} malt extract, 100 mg l^{-1} L-glutamine, 1000 mg l^{-1} casein hydrolysate, 50 ml l^{-1} coconut water, 2.0 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 2.0 g l^{-1} gelrite. The cultures were maintained through regular subcultures on fresh induction medium under a 16h photoperiod (30 µmol m^{-2} s^{-1} PFD) at 25±2°C and 70% RH.

Embryogenic calli were subjected to gamma radiation using ^{60}Co as a source in Gamma Cell 220 at dose rate of 9.6Gy/min. The irradiation doses were 10, 20, 30, 40 or 50Gy. Radiation treated calli were immediately cultured on CIM to eliminate the radiolysis hazards and subcultured for at least thrice, at monthly interval, on the same medium (CIM) before using for further studies. Survival percent of the calli was recorded in terms of White Proliferating Clumps (WPCs).

Gamma-irradiated sugarcane (cv. CoC-671) calli (200 mg) were cultured on CIM supplemented with different levels of salt-NaCl (42.8, 85.6, 128.3, 171.1, 213.9, 256.7, 299.5 or 342.2 mM). Callus growth was determined in terms of relative growth rate (RGR) after four weeks of culture on salt selection medium. The putatively tolerant calli were exposed for salt selection in subsequent cycles.

Salt stressed calli was used for the estimation of free proline, glycine betaine, membrane stability index in terms of electrolyte leakage, and Na^+ and K^+ as per the methods described earlier [5, 6].

Each treatment consisted of 15 calli (five per each 9.5cm dia. culture plate) and the values are given in the form of mean±standard error. Experiments consisting of treatments and control were replicated thrice and analysis of variance (ANOVA) was carried out using IRRISTAT programme.

Plantlets were regenerated after two to three weeks of transfer of salt selected calli on regeneration medium, i.e., CIM without 2,4-D. About 5cm long individual shoots were transferred on ½ MS medium with 2 mg l^{-1} NAA for rooting. The regeneration efficiency was expressed in terms of number of plantlets regenerated in a particular treatment of gamma irradiation and salt stress. The rooted plantlets were hardened in the green house.

The radiation induced plant population (derived from *in vitro* mutagenesis) of sugarcane cv. CoC-671, Co 94012 and Co 86032 were field planted and at maturity stage, data was collected on various agronomic traits including number of millable canes, stool weight, number of internodes, cane weight, cane diameter, H.R. Brix of sugarcane variant and control plants. The variants that performed better over checks are being field evaluated in M_3 generation under Rod Row Trial.

RAPD analysis

Genomic DNA was isolated from leaf tissue (50mg) of the selected tolerant lines. The OD of different samples was taken at 260 nm and the

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samples were then diluted so as to get the final concentration at 50ng/µl. The different components of PCR, viz. genomic DNA (50, 100, 150 or 200 ng), $MgCl_2$ (1.5, 2.5 or 3.5 mM) and *Taq* DNA polymerase (0.5 or 0.6U) and different annealing temperatures (35, 36, 37, 38, 39 or 40°C) were optimized to get appropriate amplification product.

Based on the previous investigations on RAPD analysis carried out with sugarcane embryogenic cultures and somaclones in this laboratory, the 60 decamer oligonucleotide primers from Operon Technology Inc., USA were considered. Among the primers screened, the best-suited nine primers (OPA-02, OPA-03, OPH-3, OPH-4, OPH-5, OPH-7, OPH-9, OPH-12 and OPH-19) that showed distinct banding pattern were selected for the present RAPD study.

PCR Amplification reactions were performed in a MJ Research, USA (PTC100) thermalcycler. The reaction conditions were initial denaturation at 94°C for five minutes, 40 cycles each consisting of denaturation step of one minute at 94°C, primer annealing at 37°C for 1.5 minutes, primer extension at 72°C for two minutes, and final extension step at 72°C for 10 minutes. The amplified products were subjected to agarose gel electrophoresis using 1.5% agarose and the gels were analyzed on a gel documentation system.

RAPD bands were scored as present (1) or absent (0). The data was used for similarity-based analysis using the programme NTSYS-Pc (version 2.02) developed by Rohlf [7]. Jaccards coefficient (F) was calculated using the programme SIMQUAL. Similarity coefficients were used to construct UPGMA (Unweighted Pair Group Method with Average) dendrogram.

Results and Discussion

The 20Gy irradiated cultures exhibited almost 50% survival response. Salt selections with 85.6 mM and above showed significantly lower relative growth rate as compared to control calli (**Fig. 1A, 1B**). Cell viability decreased drastically in salt-stressed calli (0.91±0.12) as compared to the control (53.16±0.39). Salt-stressed calli also exhibited higher levels of free proline and glycine betaine. In general, membrane damage rate in terms of electrolyte leakage was found to be more (almost three-fold) under salt stress (88.57±1.75) as against control condition (30.92±1.5). The ion levels were drastically affected under salt stress, as leached out Na^+ and K^+ was much more than that retained in tissue of both adapted and unadapted callus cultures. The sodium leached in both adapted and unadapted callus increased progressively with increasing salt concentration. Potassium, leached and retained, in both adapted and unadapted callus did not exhibit much variation. This accumulation of salt ions could play an important role in osmotic adjustment in stressed sugarcane cells. The tolerance could also be related to the maintenance of an ample water status and a high to low level of K^+ to Na^+ under salinity stress. Such a mechanism implies that sugarcane can be considered as a Na^+ excluder. In case of 10 and 20Gy irradiated calli, regeneration was observed up to 85.6 mM salt selection medium, whereas higher treatments (128.3 mM and beyond) exhibited browning initially. However, in the subsequent subcultures, regeneration was obtained in case of 10 and 20Gy irradiated calli on 128.3 and 171.1 mM salt selections. Higher gamma irradiation (40Gy) also showed regeneration but only with 85.6 mM salt selection. The unirradiated calli regenerated highest number of plantlets followed by 10 and 20Gy irradiated calli on salt selection. A total of 147 plantlets were selected on different salt levels.

Molecular characterization based on RAPD analysis revealed genetic polymorphism between the selected putative salt-tolerant lines from the control plants (**Fig. 1C**). RAPD analysis of the putatively tolerant regenerants resolved 72 scorable markers from nine out of 60 primers screened. On an average, each primer produced eight bands. The amplification products ranged from 0.1 kb to 2 kb. The primer OPH-05 produced maximum 10 bands, out of which three were polymorphic. The primer OPH-09 produced five polymorphic bands from a total of nine bands. An interesting observation was recorded in case of RAPD profile obtained from primer OPH-07 (**Fig. 1C**). An intense non-parental band was obtained among the selected drought-tolerant lines. But the band intensity decreased with increasing selection pressure of PEG. The genetic similarity between the control and salt-tolerant lines ranged between 0.63 and 0.80.

A wide range of mutations for morphological, quality and yield contributing characters were obtained through *in vitro* mutagenesis using gamma irradiation (**Fig. 2**). For morphological traits, mutation spectrum was broader in Co 94012, while for quality and yield traits, Co 86032 showed a wide range of mutations. A total of 44 clones were identified for various desirable agronomic traits. Clones (AKTS 2, 7, 11 of CoC 671; AKTS 22, 26, 27 of Co 86032; AKTS 36,38,39, 44 of Co 94012) performed better over respective checks for average cane weight and H.R. Brix (% sucrose).

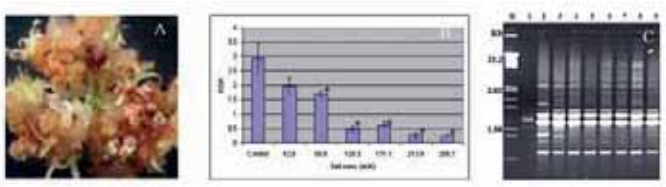

Figure 1 (A) 20Gy irradiated and 171.1 mM salt selected calli, **(B)** Relative growth rate of salt stressed calli. Asterisk indicates significant differences over control at P≤0.05, **(C)** RAPD profile obtained with primer OPH-7.

Figure 2 Field evaluation of Gamma-ray induced variants of sugarcane. **(A)** variants for canopy trait; **(B)** field view of irradiated plant population (M_2); **(C)** variant for no. of tillers and **(D)** variant for spineless sheath.

The crop improvement programme can be speeded up by combining the radiation mutagenesis with *in vitro* culture [8]. *In vitro* techniques allow for the rapid execution of propagation cycles of subculture aimed to separate mutated from non-mutated sectors [9]. Tissue culture induced variation may offer additional variation to that induced through mutagenesis and such a variation can be most effective if it is successfully associated with cellular level selection and handling of large populations for screening [10]. This approach has contributed to genetic improvement in several crop plants such as pineapple, banana and grape.

Detection of variants is of immense importance in order to utilize these lines in crop improvement. RAPD is widely used to study the variation at DNA level among the variants and technique has proved very sensitive for the characterization, salinity-tolerant plants in sugarcane. The salt-tolerant lines are being evaluated at the field level for their genetic stability. The proper evaluation of these radiation-induced variants tolerant to salinity may be useful for economic cultivation under stress conditions. The genetically stable variants for various economic traits may be released for commercial cultivation.

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Development of Salt-tolerant High-yielding Barley Lines via Crossing Between a Mutant Induced by EMS and a Local Cultivar

R A K Moustafa

Abstract

In the winter season of 2002/2003, a high-yielding barley mutant line and a local variety were hybridized to obtain the salt tolerance of the local variety and the high yield potential of the induced mutant in one genotype. The obtained hybrid grains were planted in the 2003/2004 growing season under normal field irrigation conditions to raise F_1 population, which was grown in the 2004/2005 season to advance F_2 generation under saline conditions at El-Fayoum experimental agriculture station belonging to the Nuclear Research Center. Phenotypic correlation coefficients between grain yield and its effective traits were estimated for F_2 plant population. Results revealed that the characters most strongly correlated with grain yield were found to be number of spikes and biological yield/plant. Therefore, these couple traits were used as a selection criterion to screen F_2 plant population in order to detect high yielding variants under salinity conditions. As a result, a considerable number of outstanding individual plants was selected from the large F_2 population and their grains were planted in 2005/2006 winter season to raise F_3 progeny rows. Superior plants from superior rows were selected and carried forward to the next winter season of 2006/2007 as F_4 single plant progenies along with the two parental genotypes and a suitable check. Obtained results indicated that mean values of yield and most of its components for the tested progeny lines were significantly (P=0.1) surpassed averages of the original parents and the check as well.

Keywords:

Barley – EMS – Crossing – Variation – Correlation – Selection – Salt tolerance

Introduction

Barley (*Hordeum vulgare*, L.) is one of the most important winter crops in Egypt and is used in many bakery preparations and energy-rich foods for human consumption. It is also grown for feeding animals and green forage. Additionally, barley grains can be used in the malting brewing industry. The national production of barley is generally low since it is mostly planted in the desert or on salt affected soils. The productivity of barley crop under these environments is very low and estimated at 1.26 t/ha versus 3.74 t/ha in the fertile lands of the Nile Valley [1]. However, about 420,000 h. of these fertile lands are damaged by excess soluble salts and exchangeable sodium accumulation [2]. Therefore, the present study aimed to create genetic variability and selection for salt-tolerant high-yielding barley lines derived from a manual crossing between a high-yielding mutant and a local cultivar tolerant to salinity, but lower in terms of yield potential.

Materials and Methods

A high-yielding mutant line namely Mutant 7 (Mut. 7) induced by a concentration of 0.15% EMS (4h treatment) was crossed with the local variety Giza 123 (G.123) in the 2002/2003 winter season with a view to combine in one genotype salt tolerance of G.123 and the high yield potential of Mut.7. The obtained hybrid grains were planted in the subsequent growing season of 2003/2004 under normal field irrigation conditions to raise F_1 population, which was grown in 2004/2005 winter season to advance F_2 generation under saline conditions at El-Fayoum experimental station belonging to the Nuclear Research Center (NRC).

Simple phenotypic correlation coefficient between grain yield and related traits were estimated for F_2 population to detect the most important traits that are associated with grain yield to be utilized as a criterion to select high-yielding variants with improving tolerance to salinity stress. F_2 selected plants were grown in the winter season of 2005/2006 to raise F_3 individual progeny rows. Superior plants from the best rows were selected and carried forward to the next season of 2006/2007 as F_4 progeny lines. At the end of the growing season, all developed progeny lines were screened and those of high mean productivity were harvested individually and kept for further evaluation.

Field experiments were carried out using a randomized complete block design. Both F_3 and F_4 progeny rows were grown in two replicates owing to the limited seeds number of their single plant parents. The experimental plot comprised two rows, 3m long and 20cm apart; spacing of plants was 10cm apart. Consequently, 120 individual plant per each progeny were developed either in F_3 or F_4 generations.

Table 1. Mechanical properties of El-Fayoum experimental soil

Depth (cm)	Particles size distribution%			Texture class
	Sand	Silt	Clay	
0-15	20.0	73.6	5.4	Silty loam
15-30	31.2	65.6	3.2	Silty loam
30-45	22.9	71.9	5.2	Silty loam
45-60	49.6	47.2	3.2	Sandy loam
60-75	20.3	75.4	4.3	Silty loam

Table 2. Chemical properties of El-Fayoum experimental soil

Depth (cm)	pH 1:2.5	Anions (meq/L)				Cations (meq/L)				SAR	Available, ppm		
		SO4=	Cl-	HCO3-	CO3=	K+	Na+	Mg++	Ca++		N	P	K
0-30	8.18	41.0	32.5	2.0	-	0.64	59.36	4.5	11	87	64.5	5.5	343.2

Soil analysis

2004/2005 and 2005/2006 growing seasons

Representative soil samples from El-Fayoum experimental site were taken to the depth of 75cm to determine some physical and chemical properties (**Tables 1 & 2** and **Fig. 1**). Soil analysis indicated that the site is silty loam texture with the exception of 45-60cm depth, whereas the texture is sandy loam. The concentrations of the available nitrogen and

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phosphorus are moderate, while, the available potassium is high. The site has ECe=9.21 (**Fig.1**) and irrigated with fresh water (Ecw=0.8 ds/m).

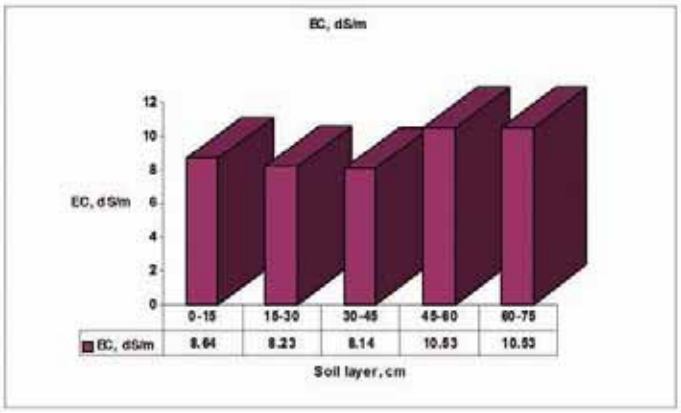

Figure 1 Average ECe of different layers of El-Fayoum saline soil.

2006/2007 growing season

The field experiment was carried out in another part of El-Fayoum site has an average ECe=12.7 ds/m. The aim was to evaluate F_4 progeny lines under higher salt pressure.

Data recorded

The studied traits in F_2-F_4 barley populations were plant height (cm), spike length (cm), number of spikes/plant, 100-grain weight (gm), biological yield /plant (gm), straw yield/plant (gm), harvest index and grain yield /plant(gm).

Statistical analysis

The data obtained were subjected to the proper statistical analysis of variance described by [3]. The L.S.D. test was used for comparison between means of F_4 progeny lines. Phenotypic correlation coefficients between yield and yield components were calculated according to [4].

Results and Discussion

As it will be known, yield is the ultimate criterion which a plant breeder has always to keep in view in his attempts to evolve improved types of any crop plant. However, yield itself is not a unitary character, but is the result of the interaction of a number of factors inherent both in the plant as well as in the environment in which the plant grows. It therefore becomes difficult to evaluate or select based on this complex trait directly. Accordingly, plant breeders may resort to more indirect methods such as determination of the association existing between other less variable characters and yield. Selection pressure may then be more easily exerted on any of the traits which show close association with yield [5]. On this basis, simple phenotypic correlation coefficients were estimated for F_2 barley population of the Mut.7 x G.123 cross to determine the most important traits that are associated with grain yield under salinity-stressed conditions at El-Fayoum environment. Data in **Table 3** indicates the presence of highly significant positive correlation between grain yield/plant and plant height, number of spikes/plant, biological yield /plant and 100-grain weight. These findings are in accordance with those previously obtained by several investigators studied the relation-

Table 3. Simple correlation coefficients between studied traits of F2 barley population of the cross Mut.7 x G.123 at El-Fayoum salt affected soil.

Characters	Plant height	Spike length	No. of spikes/ plant	100-grain weight	Biological yield/plant	Harvest index
Spike length	0.204*					
No. of spikes/plant	0.058	-0.16				
100-grain weight	0.258**	-0.031	0.316**			
Biological yield/plant	0.178	0.074	0.808**	0.332**		
Harvest index	- 0.218*	0.081	0.134	0.112	0.085	
Grain yield/plant	0.261**	0.165	0.803**	0.415**	0.786**	0.054

Table 4. Mean values of yield and yield components of F_4 progeny lines compared to their parental and the check genotypes under saline conditions at El-fayoum location.

Genotype	Spike length (cm)	No. of spikes/ plant	100-grain weight (g)	Biological yield/ plant (g)	Grain yield/ plant (g)	Straw yield/ plant (g)	Harvest index
Giza 123 (parent)	5.72	5.69	4.86	32.83	10.33	22.46	31.46
Mutant 7 (parent)	5.50	5.67	5.21	29.61	9.73	19.91	32.76
Giza 2000 (check)	6.14	6.02	5.41	31.73	8.92	22.76	28.11
Mut.7 x G.123 selected progeny lines							
No. 1	7.30	7.92	4.50	49.01	18.02	31.0	36.77
No. 2	7.05	14.67	5.92	50.04	17.38	32.68	34.75
No. 3	7.03	11.00	4.59	69.08	23.17	45.90	35.54
No. 4	6.45	12.56	4.83	66.75	23.55	43.18	35.18
No. 5	7.00	16.73	4.86	66.03	22.18	43.85	33.60
No. 6	7.00	15.14	4.26	65.13	20.67	44.51	31.65
No. 7	7.30	7.92	4.50	49.01	18.02	31.0	36.66
No. 8	7.75	10.68	4.80	61.31	17.26	44.09	28.12
No. 9	7.20	9.75	4.71	52.82	17.54	35.30	33.22
L.S.D							
0.05	0.582	3.399	1.042	2.341	0.800	1.897	3.20
0.01	0.822	4.796	1.470	3.303	1.128	2.677	3.147

ship between yield and yield components in barley under different environmental conditions [6, 7, 8, 9, 10, 11, 12, 13]. However, the two characters most strongly correlated with grain yield were found to be number of spikes/plant (r=0.803) and biological yield/plant (r=0.786). Estimate of correlation, between these two traits, however, was greater in magnitude than other two studied characters (**Table 3**). In this respect, [13] found high significant positive correlation between barley grain yield and both spikes number and biological yield/plant under stressed and non-stressed conditions, suggesting that selection for these two characters would be useful for increasing barley grain yield under different environments.

Based on the results of the correlation studies, all F_2 plant population was screened and resulted in selection a considerable number of outstanding variants showing high spike number, biological yield, and at the same time yielded more grains than the average of the best plants of the relaed tolerant parent cultivar by at least 25%. The selected plants were grown in F_3 as plant-progeny rows. Superior plants from superior rows were picked out and carried forward to the next growing season as F_4 progeny lines, which were screened, and eventually, the most promising lines retained the high yielding productive of their elite F_3 individuals were selected. Mean values of yield and its attributes for the progeny lines compared to the original parents Mut.7 and G.123 as well as G.2000 check cultivar are given in **Table 4**.

As shown in **Table 4**, means of yield and yield components of F_4 progeny lines exhibited marked increases, mostly reaching the limits of significance (P=0.01) over the averages of Mut.7 and G.123 cross parents and G.2000 the check variety. The exception was noticed for 100-grain weight of the progeny lines, were insignificantly decreased as compared to the averages of the parents and the check genotypes. These reductions, however, reached the limits of significance between the progeny line No.6 and the check (**Table 4**). The selected progeny lines will be further evaluated in multi-location trials under different saline-stressed environments to confirm their breeding values.

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Genetic Enhancement of Lentil (*Lens culinaris* Medikus) for Drought Tolerance through Induced Mutations

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Abstract

An attempt has been made to isolate a number of drought-tolerant mutants from four lentil cultivars, two small seeded (PL-639 and PL-406) and two bold seeded (K-75 and L-4076) groups by treating the seeds with physical (10, 20 and 30 kR of γ-rays) and chemical mutagens (0.04M of ethyl methane sulfonate and 0.05M of sodium azide) separately and in various combinations. The experiment was initiated during the winter season of 1999-2000 and carried over to advanced generations. The selection of environment (water stress or non-stress) for the development of drought-resistant varieties still remains controversial, however, the findings from present study suggest that materials ought to be tested in both stress and non-stress conditions so that the favourable alleles under drought can be maintained as well as selection response under favourable condition can be maximized. Yield under drought (Y_d), yield potential (Y_p), drought susceptibility index (S) and geometric mean (GM) were considered as the potential indicators for drought resistance of a family. Correlation coefficients between these parameters were calculated for selecting the parameter(s) which are more effective than others for screening the drought-resistant mutant line(s). It was observed that GM was positively and significantly correlated with both Y_d and Y_p, whereas it was negatively, but insignificantly correlated with S. There was significant, but negative correlation between S and Y_d, while no significant correlation between S and Y_p was observed. From the correlation studies it may be concluded that for the enhancement of yield potential under both the conditions, selection should be based on GM rather than on S. Because S is a better measure of drought tolerance than a measure of performance under stress, genotypes may be first selected on the basis of high GM and then on the basis of high yield under drought (Y_d). Twenty mutants lines selected on the basis of higher GM than their respective control, and were further evaluated for their yield performance under rainfed conditions and were subjected to drought tolerance tests through M_4 to M_6 generations. Three chemical tests, viz., nitrate reductase (NR) activity, protein content, and wax content were conducted and data were recorded on grain yield/plant. Nitrate reductase activity and wax content of most of the mutant lines were higher than their respective control and both were positively associated with grain yield, while protein content was lower in the mutant lines and was negatively associated with grain yield in that comparison. The lines showing higher nitrate reductase activity, wax content and grain yield appeared to be promising.

Introduction

Drought continues to be a challenge to agricultural scientists in general, and to plant breeders in particular, despite many decades of research. Breeding for drought tolerance involves identification and transfer of morpho-physiological and biochemical traits that may impart drought tolerance to high-yielding cultivars [1-3].

Research in the past has shown that plants tolerate drought stress to some extent by accumulating osmolytes [4-5]. Through a comparative analysis for drought tolerance, it was concluded that the drought tolerance seemed to be associated at least in part with its ability to maintain greater levels of amino acid pool, coupled with more pronounced reassimilation of ammonia [6]. A positive and significant correlation of nitrate reductase (NR) with protein accumulation and seed yield in different cultivars has been reported [7]. The importance of epicuticular wax content in relation to drought tolerance has also been discussed and analysed by several workers [8-10]. The highest osmotic adjustment along with high wax content was found responsible for two drought-tolerant accessions out of nine studied in lentil [11].

Looking at the importance of drought and lentil, the objectives of this paper is to find out the parameters to form the basis of selection and the environment under which the mutants/materials are to be screened for drought tolerance/resistance coupled with higher grain yield.

Materials and Methods

The experiments were carried out with the promising 20 mutant lines isolated from four lentil cultivars, two small seeded (PL-639 and PL-406) and two bold seeded (K.75 and L.4076) groups. The seeds of these cultivars were treated with physical (10, 20 and 30 kR of Gamma-rays) and chemical mutagens [0.04 M of ethyl methane sulphonate (EMS) and 0.05M sodium azide (SA)], separately and in various combinations, and were grown during the winter season of 1999-2000 as M_1 generation and carried over to advanced generations.

M_1 and M_2 generations were grown under moisture stress (rainfed) conditions while M_3 was grown under two environments viz. moisture stress (rainfed) and moisture non-stress (i.e. one supplemental irrigation just before blooming). A proportion of M_3 families were selected on a high geometric mean (GM). Within this group, a second selection was performed based on high yield under drought (Y_d) to ensure the maintenance of yield performance under stress [12]. Finally 20 mutant lines were selected and were subjected to drought tolerance tests through M_4 to M_6 generations. Three chemical tests, viz. NR activity in leaf samples was determined in vivo method as described by [13], total seed protein was estimated by using Micro-Kjeldahl method [14] and wax content through spectrophotometer following the standard procedures.

Results and Discussion

Drought tolerance in M_3 generation

One of the objectives of this investigation was to select plants for drought tolerance/resistance and the character(s) to form the basis for the selection. The magnitude of the induced genetic variability was assessed in the M_2 and was utilized for the selection of plants for further evaluation in M^3 generation. Further, these selected plants were grown under two environmental conditions as described above to assess the drought sus-

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ceptibility index. Drought susceptibility/resistance of a family in the field was assessed through the measurement of yield under moisture stress (Y_d), under moisture non-stress, i.e. full genetic yield potential (Y_p), drought susceptibility index (S) and geometric mean (GM). These were considered as the potential indicators for drought tolerance/ resistance of a family and therefore, calculations were made on these parameters. On the basis of these parameters (Y_d Y_p, S, GM) promising mutant lines were selected. But it was difficult to conclude which parameter(s) were more effective than others for screening the drought-resistant mutant line(s). To solve this problem, correlation studies were made for each cultivar between the drought parameters and shown in **Table 1**. All the combinations of these parameters showed highly significant correlations, except the correlation between S and Y_p and S and GM in all the four cultivars. In general, highest correlation coefficients were noticed between GM and Y_p in PL-639 (0.977), PL- 406 (0.973) and K.75 (0.961) and between Y_d and Y_p in L.4076 (0.933).

Table 1. Correlation between yield under stress (Y_d) and non-stress (Y_p), geometric mean (GM), and drought susceptibility index (S) for lentil cultivars PL- 639, PL- 406, K- 75 and L- 4076 in M_3 generation.

Traits	Correlation co-efficients			
	PL- 639	PL- 406	K. 75	L. 4076
Y_d & Y_p	0.682*	0.972*	0.855*	0.933*
S &Y_d	-0.219*	-0.243*	-0.329*	-0.224*
S & Y_p	0.167	-0.087	0.071	0.183
S & GM	-0.032	-0.123	-0.099	-0.008
GM &Y_d	0.756*	0.967*	0.928*	0.902*
GM &Y_p	0.977*	0.973*	0.961*	0.887*

* significant at the 0.05 probability level

Chemical test in M_4 to M_6 generations

A number of mutant lines were promising for drought tolerance/resistance and were selected on the basis of GM in M_3 generation (**Table 2**). Only 20 mutant lines (six each from PL-639 and L.4076; five from PL-406 and three from K.75) showing the highest GM within each cultivar were further evaluated through M_4 to M_6 generations. Three chemical tests viz. protein content, NR activity and wax content were conducted and the mean values of each test along with the yield per plant are presented in **Table 3**. All the mutant lines showed significant positive correlations between grain yield and NR activity (0.382) and between grain yield and wax content (0.466). It was observed that NR activity and wax content of most of the mutant lines were higher than their respective control, while the reverse was true for protein content. Out of 20 mutant lines screened through chemical tests, there were two mutant lines in PL-639 (T3-4 and T3-1) and one in PL-406 (T10-3) and one in L 4076 (T3-3) which showed higher values of all the tests as compared to their respective controls.

An overall observation suggested that some cultivars were more resistant to water stress than others, the reason being that at cellular level, plants tolerate drought stress to some degree by accumulating osmolytes [4-5] and most of these osmolytes are nitrogenous compounds, hence nitrogen metabolism is of utmost importance under stress conditions. In this study, NR activity was found positively correlated with the grain yield as was also reported by [7]. Both positive [15-16] and negative [17-18] correlations were noted between the protein content and grain yield. Induced mutants with increased, as well as reduced seed protein content were reported in different pulse crops [19]. In this study also, it was

Table 2. Promising mutant lines in each cultivar selected on the basis of geometric mean in M_3 generation.

Mutant line	Description	Y_p	Rank	Y_d	Rank	GM	Rank	S	Rank
PL- 639	Control	4.80	6	4.16	23	4.47	18	2.14	64
T2-3	3rd line of 10 kR	4.98	1	4.73	4	4.85	1	0.90	36
T11-6	11th line of SA + 20 kR	4.80	6	4.78	2	4.78	2	0.07	4
T11-1	1st line of SA + 20 kR	4.80	6	4.74	3	4.76	3	0.22	11
T9-6	6th line SA	4.86	2	4.63	9	4.74	4	0.85	35
T3-4	4th line of 20 kR	4.85	3	4.64	8	4.74	4	0.78	32
T3-1	1st line of 20 kR	4.74	9	4.70	6	4.71	5	0.45	16
PL- 406	Control	5.16	5	4.88	13	5.02	5	2.10	61
T2-6	6th line of 20 kR	5.18	4	5.10	3	5.13	1	0.39	17
T11-6	6th line of SA + 20 kR	5.10	6	5.04	4	5.11	2	0.29	14
T3-5	5th line of 20 kR	5.10	6	5.02	5	5.05	3	0.39	17
T9-3	3rd line of SA	5.18	4	4.89	12	5.03	4	1.42	50
T10-3	3rd line of SA + 10 kR	5.35	7	5.00	6	5.02	5	0.25	13
K. 75	Control	5.34	1	4.97	5	5.14	3	1.40	52
T10-4	4th line of SA + 10 kR	5.18	5	5.13	4	5.15	1	0.23	15
T2-7	7th line of 10 kR	5.30	2	5.21	1	5.25	2	0.41	19
T12-1	1st line of SA + 30 kR	5.16	6	5.14	3	5.14	3	0.09	10
L. 4076	Control	5.18	3	4.79	14	4.98	6	1.60	43
T2-1	1st line of 10 kR	5.20	2	5.10	1	5.14	1	0.52	21
T2-7	7th line of 10 kR	5.13	5	5.00	2	5.06	2	0.69	27
T3-3	3rd line of 20 kR	5.23	1	4.88	7	5.05	3	1.82	49
T3-4	4th line of 20 kR	5.10	6	4.93	4	5.01	4	0.90	34
T11-4	4th line of SA + 20kR	4.99	10	4.99	3	4.99	5	0.00	9
T11-5	5th line SA+20 kR	5.06	7	4.84	9	4.94	6	1.18	36

Table 3. Mean of protein content NR activity, wax content and grain yield per plant (averaged over M_4 to M_6 generations).

PL- 639 Control	24.15	1.82	146.22	4.12
T2-3	21.22	2.42	164.78	4.93
T11-6	24.06	2.30	152.63	4.71
T11-1	24.10	2.28	152.98	4.69
T9-6	23.98	2.38	162.94	4.75
T3-4	24.42	2.08	159.12	4.62
T3-1	24.16	2.20	164.62	4.73
PL- 406 Control	24.32	1.98	164.62	4.32
T2-6	23.90	2.60	173.15	4.88
T11-6	24.16	1.99	182.32	5.29
T3-5	24.06	2.73	182.48	4.76
T9-3	24.10	2.44	169.26	4.33
T10-3	24.42	2.82	184.82	5.73
K. 75 Control	25.48	2.28	152.24	4.06
T10-4	23.78	2.59	199.06	4.12
T2-7	23.92	2.31	183.31	5.34
T12-1	24.82	2.29	157.22	4.24
L. 4076 Control	24.08	1.98	160.35	4.19
T2-1	23.76	2.75	161.45	4.94
T2-7	23.10	2.49	187.12	4.89
T3-3	24.27	2.17	189.82	4.92
T3-4	22.58	1.92	179.28	4.69
T11-4	23.76	2.34	192.42	4.95
T11-5	23.84	2.23	181.32	4.78

observed that the protein content was negatively correlated with grain yield (-0.272). Unlike the protein content, there was significant positive correlation between grain yield and wax content (0.466). A stable cell membrane that remains functional during water stress appears central to adaptation to high temperatures and was found to be related to heat and drought tolerance [20-21]. A genotypic difference in thermo stability of membrane was observed by [22]. They concluded that a cell membrane was more thermo stable when it was covered with a sufficient quantity of wax.

Thus, it may be concluded that the selection of mutants/plants be done on the basis of higher NR activity, wax content and higher grain yield under drought (moisture stress) conditions.

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