# Candidate genes for drought tolerance and improved productivity in rice (*Oryza sativa* L.)

M S Vinod[1], Naveen Sharma[2], K Manjunatha[1], Adnan Kanbar[3], N B Prakash[4] and H E Shashidhar[1,*]

[1] *Marker-Assisted Selection Lab, Department of Genetics and Plant Breeding, University of Agricultural Sciences, Bangalore 560 065, India*

[2] *Biometrics and Bioinformatics Unit, International Rice Research Institute, Los Banos, Philippines*

[3] *Department of Agronomy, Faculty of Agriculture, University of Damascus, Damascus, Syria*

[4] *Department of Soil Science and Agricultural Chemistry, University of Agricultural Sciences, Bangalore 560 065, India*

**Corresponding author* (*Fax, 91-80-23330277; Email, heshashidhar@rediffmail.com)*

Candidate genes are sequenced genes of known biological action involved in the development or physiology of a trait. Twenty-one putative candidate genes were designed after an exhaustive search in the public databases along with an elaborate literature survey for candidate gene products and/or regulatory sequences associated with enhanced drought resistance. The downloaded sequences were then used to design primers considering the flanking sequences as well. Polymerase chain reaction (PCR) performed on 10 diverse cultivars that involved *Japonica*, *Indica* and local accessions, revealed 12 polymorphic candidate genes. Seven polymorphic candidate genes were then utilized to genotype 148 individuals of CT9993 × IR62266 doubled haploid (DH) mapping population. The segregation data were tested for deviation from the expected Mendelian ratio (1:1) using a Chi-square test (<1%). Based on this, four candidate genes were assessed to be significant and the remaining three, as non-significant. All the significant candidate genes were biased towards CT9993, the female parent in the DH mapping population. Single-marker analysis strongly associated (<1%) them to different traits under both well-watered and low-moisture stress conditions. Two candidate genes, *EXP15* and *EXP13*, were found to be associated with root number and silicon content in the stem respectively, under both well-watered and low-moisture stress conditions.

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## 1. Introduction

Drought is one of the major limitations to food production worldwide and is endemic particularly in the semi-arid tropics. Improving drought tolerance and productivity is one of the most difficult tasks for cereal breeders. The difficulty arises from the diverse strategies adopted by plants themselves to combat drought stress depending on the timing,

**Keywords.** Candidate gene; mapping population; polymerase chain reaction; single marker analysis.

Abbreviations used: ABA, Absiscic acid; ABRE, absiscic acid responsive element; CG, candidate gene; DH, doubled haploid; LMS, low-moisture stress; MRL, maximum root length; NOP, number of panicles; NOT, number of tillers; PCR, polymerase chain reaction; PHT, plant height; PL, panicle length; PVC, polyvinyl chloride; QTL, quantitative trait loci; RCBD, randomized complete block design; RN, root number; RV, root volume; SDW, shoot dry weight; Si, silicon; SiS, silicon content in stem; SiST, total silicon content in stem; SW, stem weight; TIGR, The Institute for Genomic Research; TPL, total plant length; WW, well-watered.

severity and stage of crop growth. Compounding the problem further are the many loci that show efficacy only in a subset of circumstances (Lebreton *et al* 1995; Ribaut *et al* 1996, 1997; Tunistra *et al* 1996; Nguyen *et al* 2004). Drought-stress in plants stimulates the activity of several genes and the function of their gene products have been predicted from sequence homology with known proteins. Candidate genes (CGs) are sequenced genes of known biological function associated with the manifestation of the trait. They may be structural genes or genes in a regulatory or biochemical pathway which affect trait expression. One CG hypothesis states that "a significant proportion of the quantitative trait loci (QTL) affecting trait variation are in fact CGs associated with that trait" (Rothschild and Soller 1997). The CG approach involves choosing the CG, obtaining primer sequences to amplify the gene, uncovering polymorphism, developing a convenient procedure for large-scale genotyping, identifying a population for association studies, carrying out an association study of the CG with trait phenotype and verifying the uncovered associations. The CG approach has been utilized successfully to determine the biotic and abiotic characters in rice and other cereals (Faris *et al* 1999; Ramalingam *et al* 2003; Zheng *et al* 2003).

In the present study, CGs directly related to drought resistance and productivity at the morpho-physiological, phonological, biochemical, genetic and phenotypic levels were identified and polymerase chain reaction (PCR) primers designed. These were used to genotype 10 diverse cultivars of rice, comprising *Japonica, Indica,* a local variety and a transgressant to determine their genetic make-up for drought tolerance. Among the diverse genotypes 12 out of the total 21 CGs designed were polymorphic. Seven polymorphic CGs were then used for genotyping the CT9993 × IR62266 doubled haploid (DH) mapping population. The resultant data were tested for deviation from the expected Mendelian ratio using a Chi-square test, and trait association was carried out using single-marker analysis. Major emphasis was laid on selecting CGs specific for root characters.

## 2. Materials and methods

### 2.1 *Plant material*

Standard checks such as Moroberekan, Azucena, CT9993, IR50, IR64, IR20, IR62266, Budda, MM125, BIRB16-1 (transgressant derived from Budda/IR64 cross), along with 148 DH lines from a CT9993/ IR62266 mapping population constituted the study material.

### 2.2 *Phenotyping for shoot and root morphological traits*

The experiment was performed at Main Research Station, University of Agricultural Sciences, Bangalore. Phenotyping for shoot and root morphological traits was carried out in gray, open-ended polyvinyl (PVC) pipes measuring 100 cm in length and 18 cm in diameter in a randomized complete block design (RCBD). The PVC pipes were filled to 1.5 cm from the top with sandy loam soil and well-decomposed farmyard manure in a ratio of 4:1 (Shashidhar *et al* 1999). The soil was filled in three installments, the lowest layer was compacted, the central layer less compacted and the top layer had no compaction. Three seeds per DH line were directly sown in each pipe and plants were thinned after 10 days taking care to maintain one plant per genotype in each pipe. To obtain well-watered (WW) conditions, the genotypes were watered once in four days and for low-moisture stress (LMS) conditions once in 8 days. The plants were grown for up to 75 days before their roots were sampled. Sampling involved cutting the shoots at the base and submerging the pipes containing soil and root portions in water (overnight) and then carefully easing the soil out of the pipe before washing the roots clear of soil. The clean plant was collected in poly bags for recording observations. Observation consisted of plant height (PHT) in cm, number of tillers (NOT), number of panicles (NOP), panicle length (PL) in cm, total plant length (TPL) in cm, maximum root length (MRL) in cm, root number (RN), root volume (RV) in cc, stem weight (SW) in g, shoot dry weight (SDW) in g, silicon content in stem (SiS) and total silicon content in stem (SiST).

### 2.3 *Estimation of silicon content*

Two days before the analysis of silicon (Si) uptake, the culm, leaves and whole grain of the rice plant was dried in an oven. The powdered sample (culm or leaf or grain; 0.3 g) was microwave digested in a mixture containing 3 ml of nitric acid (63%), 3 ml hydrogen peroxide (30%) and 2 ml of hydrofluoric acid (46%). The digested samples were then diluted to 40 ml with boric acid (4%). The Si concentration in the digested sample was determined by the colorimetric molybdenum blue method. To 11.5 ml of water, 0.1 ml of sample was added, followed by 6 ml hydrochloric acid (0.2 N), 0.8 ml ammonium molybdate tetrahydrate (10%), 0.8 ml tartaric acid (20%) and 0.8 ml reducing agent (Ma *et al* 2002). The Si content was estimated thrice for each sample and the mean value computed.

### 2.4 *Isolation of DNA*

Genomic DNA was extracted from leaf tissues of 25-day old seedlings of the plant material used for the study, by the modified cetyl trimethyl ammonium bromide method (Caw and Oard 1997). DNA was quantified at 260 nm using an UV spectrophotometer.

### 2.5 *Designing the CGs*

To find the putative genes, the public database was exhaustively searched for CG products and/or regulatory sequences important for enhanced drought resistance or improved productivity along with an elaborate literature survey. The putative gene sequences were downloaded from the National Centre for Biotechnology Information (NCBI) database. The CGs were specifically looked for elements like MYB, MYC, abscisic acid responsive element (ABRE) etc. (using alignment software clustlW) which respond to absiscic acid (ABA) and different stimuli of abiotic stresses, responsible for the genetic makeup of drought resistance in plants. nBLAST of the CG was carried out to identify similarities across species and to pick up the specific BAC/PAC clone. These were then located physically on the annotated chromosome using the database of The Institute for Genomic Research (TIGR).

### 2.6 *Designing PCR primers*

The downloaded CG sequences were used to design primers considering the flanking sequences as well, by means of a primer designing software (Primer D, version 2). While designing the primers, all necessary conditions were adhered to.

### 2.7 *Standardization of primer annealing condition*

The primer annealing temperature of all CGs were calculated using the common formula 2(A+T) + 4(G+C). Based on this, the reaction profiles of all CGs were designed. But only a handful of them (*EXP15, EXP13, EXP2, CIS, RAB, JPF*) showed amplification at a single temperature, while the remaining CGs either showed non-specific amplification or no amplification. To circumvent this, a step-up PCR amplification profile was utilized which was identical to the normal single temperature profile in all aspects (denaturation and extension) except for the annealing temperature. In the step-up PCR, the annealing temperature increased by 1°C per cycle for 8–9 cycles. Once the specific annealing temperature of the primers was attained, the thermal cycler was programmed to repeat it for the remaining 29–30 cycles. This ensured that at lower temperatures the 20–25 mer long primers would easily bind to the template DNA to show amplification during the initial cycles. The problem of non-specific bands did not arise because the latter part of the annealing temperature profile was at a high temperature, which was calculated based on the length, purine and pyrimidine content of the primers. This relatively high temperature ensured that only those strands, in which the primers had been completely/tightly bound, were replicated.

### 2.8 *PCR reaction conditions*

PCR was performed in a total volume of 20 $\mu$l containing 10X PCR buffer (1X contains 10 mM Tris Cl, pH 8.8 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$), 15 pmol of each primer (Sigma Aldrich, USA), 40 ng of rice genomic DNA, 80 $\mu$M of each of the four dNTPs and 1 unit of Taq polymerase (Bangalore Genei, India) with mineral oil overlay. Thermal conditions such as initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 72°C for 1 min and a final extension of 72°C for 5 min were common to all. Variations were observed only for the annealing temperature. Amplified products were resolved on 8% polyacrylamide gels with silver staining (Panaud *et al* 1996).

### 2.9 *Statistical analysis*

The data was subjected to individual ANOVA based on RCBD over the two moisture regimes to assist the variability among the DH lines. The Chi-square test ($\chi^2$) was performed to examine the fit between the marker allele contribution of the CT9993 and IR62266 genomes in the DH lines. Single-marker analysis was carried out for the CG data with markers as the classifying variable to identify QTLs linked to root, shoot-related traits and Si accumulation pattern under WW and LMS conditions using the SAS software (SAS. V 6.12, Cary, North Carolina, USA).

## 3. Results and discussion

Of the 21 CGs screened from the 10 diverse cultivars including the local varieties and a transgressant, 12 CGs were polymorphic (table 1). Polymorphism was observed not only between the *Japonica* and *Indica* cultivars but also within for some of the CGs (table 2). Among the local cultivars, Budda showed a banding pattern identical to the *Japonica* cultivars and MM125 to the *Indica* cultivars for most of the CGs. In case of the transgressant, 7 CGs showed a banding profile similar to the Budda (female parent) and 5 CGs to IR64 (male parent) (table 2). With the polymorphism between the cultivars clearly established 7 polymorphic CGs were used to genotype the CT9993 × IR62266 DH mapping population of 145 lines evaluated under contrasting moisture regimens using PVC pipes.

Analysis of variance revealed significant differences. Wide variability was observed for all the traits (data not shown). The 7 CGs used to screen the DH mapping population were subjected to Chi-square test based on the equal contribution of both CT9993 and IR62266 marker alleles. While, 4 CGs (*EXP15, CIS, KCDL* and *LTP*) showed Skewedness towards CT9993 (the female parent), 3 CGs (*RAB21, CRTDRE* and *EXP13*) were non-significant (table 3).

**Table 1.** List of polymorphic candidate genes, their annealing temperatures and their putative function in rice.

| Candidate Gene | Annealing temperature and PCR profile | Gene action/phenotypic expression | GI No. |
|---|---|---|---|
| *EXP15, EXP2* and *EXP13* | 54°C for 1min, repeat for 35 cycles | They are cell elongation proteins<br>Their root-specific expression facilitates loosening of cell wall, hence root length | 16517040<br>13184872<br>16517036 |
| *RAB21* | 54°C for 1min, repeat for 35 cycles | These genes are responsive to absiscic acid.<br>The activity of and RAB16A promoter was induced by ABA and osmotic adjustment | 20316 |
| *RAB16* | 54°C for 1min, repeat for 35 cycles | Stresses in various tissues of vegetative and floral organs. | 16874549 |
| *CIS* | 54°C for 1min, repeat for 35 cycles | *Cis* acting element of gene pwsi 18 promoter showed transient expression in response to water stress which makes wsi gene express during water deficit | 8096460 |
| *P5CS* | 52°C + 1°C rise per cycle for 9 cycles for 1min, 60°C for 1min, repeat for 30 cycles | Induced by dehydration, ABA and high salt environments. Responsible for the production of proline which acts as osmo-protectant during abiotic stress | 2081611 |
| *RWC3* | 52°C + 1°C rise per cycle for 9 cycles for 1min, 60°C for 1min, repeat for 30 cycles | They are water-channel proteins, which are permeable to water and are root-specific | AC003802 |
| *CRTDRE* | 52°C + 1°C rise per cycle for 9 cycles for 1min, 59°C for 1min, repeat for 29 cycles. | C-repeat/drought-responsive element binding factor helps in the up-regulation of CRT/DRE genes in response to low temperature and water deficit | 8698574 |
| *LTP* | 52°C+ 1°C rise per cycle for 8 cycles for 1min, 58°C for 1min, repeat for 30 cycles. | This lipid transfer protein (LTP) is ABA induced and responsive to environmental factors such as water, salt or cold | 13184872 |
| *KCDL* | 55°C + 1°C rise per cycle for 7 cycles for 1min, 60°C for 1min, repeat for 29 cycles | This water stress-inducible protein is responsive to water stress | 1661161 |
| *OSMOTIN* | 52°C + 1°C rise per cycle for 9 cycles for 1min, 60°C for 1min, repeat for 30 cycles | This is a stress-inducible gene | 1196834 |

**Table 2.** Banding profile of the polymorphic CGs in diverse cultivars of rice.

| | *Japonica* | | | *Indica* | | | | Local | | Transgressant | | Position on chromosome | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Candidate Gene | Moro-berekan | Azucena | CT9993 | IR50 | IR64 | IR20 | IR62266 | Budda | MM125 | BIRB 16–1 | Expected size in bp | No. | Cm |
| *EXP15* | 1 | 3 | 1 | 3 | 3 | 3 | 3 | 1 | 3 | 3 | 1350 | 1 | 121.3 |
| *EXP2* | 4 | 3 | 3 | 1 | 1 | 3 | 3 | 3 | 1 | 3 | 800 | 1 | 140.5 |
| *EXP13* | 3 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 1 | 1500 | 1 | 122.1 |
| *RAB21* | 1 | 2 | 3 | 4 | 4 | 3 | 4 | 1 | 4 | 2 | 700 | 2 | - |
| *JPF* | 1 | 1 | 3 | 2 | 2 | 2 | 3 | 1 | 2 | 1 | 650 | 10 | - |
| *CIS* | 3 | 2 | 2 | 1 | 1 | 1 | 1 | 3 | 1 | 1 | 1252 | 1 | - |
| *P5CS* | 1 | 1 | 1 | 1 | 1 | 1 | 3 | 3 | 1 | 3 | 2060 | 1 | 143.7 |
| *RWC3* | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 3 | 2 | 1 | 1879 | 3&7 | 14.4/53.4 |
| *CRTDRE* | 3 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 946 | 6 | 12.9 |
| *LTP* | 3 | 3 | 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 690 | 1,7&10 | 140.5/11 7.7 /31.4 |
| *KCDL* | 4 | 4 | 3 | 4 | 4 | 1 | 1 | 4 | 1 | 4 | 954 | 6 | 12.3 |
| *OSMOTIN* | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 1 | 1 | 400 | 1 | 5.6 |

Scoring pattern: Top band – 1; bottom band – 3; both bands – 2; between 1 and 3 – 4.

**Table 3.** Chi-square test of seven candidate genes on a CT9993/IR62266 DH mapping population of rice.

| CG | $\chi^2_{1:1}$ | Probability | Skewedness towards parent |
|---|---|---|---|
| *RAB21* | 0.34 | 0.561 | – |
| *EXP15* | 27.37 | 0.000 | CT9993 |
| *CIS* | 11.59 | 0.001 | CT9993 |
| *KCDL* | 24.01 | 0.000 | CT9993 |
| *CRTDRE* | 1.99 | 0.158 | – |
| *EXP13* | 0.01 | 0.934 | – |
| *LTP* | 8.45 | 0.004 | CT9993 |

**Table 4.** Single-marker analysis of CGs in a CT9993/IR62266 DH mapping population under contrasting moisture regimens in rice

| Condition | CG | Trait | Pr > *F* | $R^2$ |
|---|---|---|---|---|
| | *LTP* | Total plant length (TPL) | 0.0003 | 10.67 |
| | | Root number (RN) | 0.0003 | 10.72 |
| | | Maximum root length (MRL) | 0.0028 | 7.37 |
| | | Plant height (PHT) | 0.0056 | 6.37 |
| | | Number of productive tillers (NPT) | 0.002 | 6.5 |
| WW | *KCDL* | Maximum root length (MRL) | 0.0080 | 5.8 |
| | *EXP15* | Number of tillers (NOT) | 0.0002 | 10.8 |
| | | Root number (RN) | 0.0092 | 5.6 |
| | *EXP13* | Stem weight (SW) | 0.005 | 5.58 |
| | | Shoot dry weight (SDW) | 0.01 | 4.5 |
| | | Silicon content in Stem (SiS) | 0.01 | 4.4 |
| | | Total silicon content in Stem (SiST) | 0.01 | 4.48 |
| | *RAB21* | Plant height (PHT) | 0.009 | 4.9 |
| | | Maximum root length (MRL) | 0.001 | 7.3 |
| | *Exp15* | Panicle length (PL) | 0.004 | 5.97 |
| LMS | | Root number (RN) | 0.01 | 4.6 |
| | *CIS* | Root volume (RV) | 0.01 | 4.1 |
| | | Root number (RN) | 0.01 | 4.42 |
| | *EXP13* | Silicon content in Stem (SiS) | 0.01 | 4.44 |

Single-marker analysis performed on the phenotypic and genotypic data generated revealed strong association (< 0.01) of four CGs, *LTP, KCDL, EXP13* and *EXP15* with certain shoot characters viz*.,* TPL, PHT, number of productive tillers, NOT, SW, SDW, SiS and SiST. Root traits such as RN and MRL were found to be associated with the CGs (table 4) under WW conditions. Similarly, under LMS conditions, four CGs (*RAB21, EXP15, CIS* and *EXP13*) were associated (< 0.01) with shoot traits such as PHT, PL, SiS and root characters such as MRL, RN and RV (table 4).

All the polymorphic CGs were validated using the 10 diverse cultivars. The phenotypic data were generated over a period of one year involving two seasons under both WW and LMS conditions. The results obtained were consistent with the above findings. The polymorphism observed within the *Japonica* and *Indica* cultivars for the 12 CGs suggests that the mechanism for drought tolerance/resistance is genotype specific. In the CT9993/IR62266 DH mapping population, it was observed that two CGs showed an association with the same trait under both the experimental conditions. *EXP15* was associated with RN (0.0092 and 0.01) and *EXP13* with SiS (0.01) under WW and LMS conditions, respectively. *EXP13* was also found to be associated with SiST under WW conditions.

Root characters are difficult to evaluate as the process involves destructive sampling of the genotype. The beneficial effects of Si are many, and include alleviation of abiotic and biotic stresses in higher plants such as rice (Epstein 1999; Ma *et al* 2001), but no genes encoding Si content have been isolated in rice. Thus *Exp15* and *Exp13* can be used in marker-assisted selection programmes to screen the genotypes for root and Si characters/traits, respectively, thereby reducing the time and labour involved. QTL mapping has been a very popular and powerful tool to assign specific positions to genes contributing to traits. These are still considered statistical entities and some loci span large chromosomal segments (Dupuis and Siegmund 1999). Mapping CGs in relation to other molecular markers will take us one step closer to assigning a biological meaning to markers employed for marker-assisted selection programmes.

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