



Original Research Article

Application of molecular markers to improve drought tolerance and yield enhancement in upland rice

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Abstract: Upto now, a lot of drought related genes were cloned and individual gene showed positive effects under controlled stress experiments, but were not effective in the field. However, the progresses by conventional breeding approaches were achievable as some drought varieties have been released to the farmers in the recent years. Although, this is not adequate to cope up with the future demand of high yield for rice, as drought seems to spread to more regions and seasons across the country. Therefore, marker assisted selection came into lime light for accelerating and giving pace to plant breeding. The identification of QTLs with a major effect on grain yield raises a new hope of improving grain yield under drought through marker assisted breeding. The availability of the major effect QTL for drought tolerance, a theoretical frame-work for marker assisted selection and the existence of intolerant varieties that are widely accepted by farmers provides an opportunity to develop cultivars that would be suitable for larger areas of drought prone rice. In case of (Sarjoo - 52 × Birsa Gora) × Sarjoo - 52, two drought tolerant genes were incorporated. Only those plants were therefore selected which showed polymorphism for the genes *MQTL_{1.1}* and *qDTY_{3.2}*. Thus, plant number SA - D - 1, SA - D - 7, SA - D - 8, SA - D - 9, SA - D - 13, SA - D - 27 and SA - D - 56 were selected. These lines have been subjected to further breeding and trial tests. Agronomic performances and physiological behavior of these lines are also under track. The results showed that the variety Sarjoo - 52 could be efficiently converted to a drought tolerant variety in a backcross generation followed by selfing and selection, involving a time of two to three years. Polymorphic markers for foreground and background selection were identified for the high yielding variety to develop a wider range of drought tolerant variety to meet the needs of farmers in the drought-prone regions. This approach demonstrates the effective use of marker assisted selection for a major QTL in a molecular breeding program.

Key Words: *Oryza sativa*, drought, marker assisted selection, QTLs, foreground selection, background selection.

Introduction

Cereals have played a significant role in the evolution of human civilization. Rice (*Oryza sativa* L.) is the staple food of more than three billion people in the world, most of them living in Asia. In 2011, IRRI reported that the world produced 661 million tons of rough rice from 155.7 million ha of area. Of this total, Asian farmers produced around 600 million tons, which represents more than 90% of global rice. India and China together accounted for 341 million tons, with India producing 148 million tons. Rice is cultivated under diverse ecologies ranging from irrigated to rainfed upland to rainfed lowland to deep water. Irrigated rice accounts for 55% of world area and about 75% of total rice production. Rainfed lowland represents about 25% of total rice area, accounting for 17% of world rice production. Upland rice covers 13% of the world rice area and accounts for 4% of global rice production. Deepwater rice,

although it has less area, meets the need of around 100 million people. In India, the total area under irrigated, rainfed lowland and upland rice is 22.0, 14.4, and 6.3 million ha, respectively (Singh, 2009).

Studies on the plant response to water stress are becoming increasingly important, as most of climatic change scenarios suggest an increase in aridity in many areas of the globe (Petit *et al.*, 1989). On a global basis, drought (assumed to be soil and/ or atmospheric water deficit) in conjunction with high temperature and radiation, possess the most important environmental constraints to plant survival and to crop productivity (Boyer, 1982). As irrigation water is not adequate as per crop requirement, the possible solutions to improve field productivity are i) environment control development i.e. improve plant living environment to fit the

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needs of crop, this includes technologies which reduce soil and water loss, decrease soil water evaporation, increase and maximize the use of soil water storage, collect non cultivate field run offs and use them as irrigation supplement. ii) Approach of biological water saving i.e. modify plant to adapt the dry environment; this includes genetic modification of plant, physiological regulation and application of crop complementary effort. It is stated that management practices can contribute to increase yield in moisture stress environments but major progress will be realized through genetic improvement and therefore through plant breeding and molecular breeding, it would be better to develop drought tolerant varieties than to irrigate drylands.

Despite the importance of drought as a constraint, little effort has been devoted to developing drought-tolerant rice cultivars. In drought years, high yielding varieties inflict high yield losses, leading to a sudden decline in the country's rice production. Farmers of drought-prone areas require varieties that provide them with high yield in years of good rainfall and sustainable good yield in years with drought. The earlier approach of improving grain yield under drought through selection on secondary traits such as root architecture, leaf water potential, panicle water potential, osmotic adjustment, and relative water content (Fukai *et al.*, 1999; Price and Courtois, 1999; Jongdee *et al.*, 2002; Pantuwan *et al.*, 2002) did not yield the expected results to improve yield under drought. Breeders and physiologists practiced selection for secondary traits as several earlier studies reported low selection efficiency for direct selection for grain yield under drought stress (Rosielle and Hamblin, 1981; Blum, 1988; Edmeades *et al.*, 1989). Similarly, at the molecular level, initial efforts in rice were devoted to mapping of QTLs for secondary drought-related traits such as root morphology and osmotic adjustment (Yadav *et al.* 1997; Kamoshita *et al.* 2002; Babu *et al.* 2003). Because QTLs for secondary traits are not linked to direct yield increase under drought, marker-assisted selection for such QTLs has not been successfully used to improve yield under drought stress in rice.

Up to now, a lot of drought related genes were cloned and individual gene showed positive effects under controlled

stress experiments, but were not effective in the field. However, the progresses by conventional breeding approaches were achievable as some drought varieties have been released to the farmers in the recent years. Although, this is not adequate to cope up with the future demand of high yield for rice, as drought seems to spread to more regions and seasons across the country. Therefore, marker assisted selection came into lime light for accelerating and giving pace to plant breeding.

Mapping studies are performed to detect linkage of a molecular marker to a gene affecting a trait of interest. It then becomes possible to select for the desirable allele of those genes based on marker genotype rather than, or in addition to, field phenotype (Jongdee *et al.*, 2002). This technique, known as marker assisted selection (MAS), is theoretically more reliable than selection based solely on phenotype, as a marker tightly linked to the desirable gene would represent selection with a heritability of near unity for that specific gene (Bernardo, 2002). Marker-assisted selection may be useful to improve traits that are either controlled by a few genes or where phenotypic evaluation is difficult/ costly to perform. The relative difficulty associated with drought-resistance phenotyping suggests that there is scope for the use of MAS in breeding for drought resistance (Bernardo, 2002). The advantages of MAS for rice breeding include:

- Lower population sizes than conventional breeding that require less genotyping.
- The existence of widely-grown rice cultivars ("mega varieties") that possess the essential features of high grain quality, yield and local adaptation, but lack specific traits such as stress tolerance.
- Existence of major QTLs and major genes that could add value to many elite rice cultivars.
- Minimization of linkage drags surrounding the locus being introgressed.
- Rapid breeding of new genotypes with favorable traits.

The effectiveness of MAS depends on the availability of closely linked markers and/or flanking markers for the target locus, the size of the population, the number of backcrosses and the position and number of markers for background selection (Frisch *et al.* 1999a; Frisch and Melchinger 2005). MAS has previously been used in rice breeding to incorporate the bacterial blight resistance

gene *Xa21* (Chen *et al.* 2000) and *waxy* gene (Zhou *et al.* 2003) into elite varieties.

The identification of QTLs with a major effect on grain yield raises a new hope of improving grain yield under drought through marker assisted breeding. The availability of the major effect QTL for drought tolerance, a theoretical frame-work for marker assisted selection and the existence of intolerant varieties that are widely accepted by farmers provides an opportunity to develop cultivars that would be suitable for larger areas of drought prone rice (Mackill, 2006).

Materials and Methods

The present investigation was conducted during three seasons i.e. 2010, 2011 and 2012 at Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and off season, 2010- 2011 at CRRI, Cuttack, Odisha. Sarjoo - 52, derived from T (N) 1 × Kashi was notified in 1982 for general cultivation. It takes approximately 130-133 DAS to harvest. Sarjoo - 52 is an irrigated, semi dwarf (98 cm) and erect type. Grains are long, bold, AWP and white. It is moderately resistant to BLB. It is reported to yield 50-60 Q/ha. It is mainly grown in Uttar Pradesh. Birsa Gora, a germplasm collection of Gora was used as donar parent. Birsa Gora was notified in 1993 as an upland rice variety. It takes about 95-100 days to mature. It is a tall type (165-180 cm) whose grains are medium bold and red. The variety is moderately resistant to major diseases and pests and resistant to drought. Its average yield is 18-20 quintals/hectare. The variety is mostly grown in Bihar and Jharkhand. The seeds of drought tolerant variety (Birsa Gora) and drought susceptible variety (Sarjoo - 52) were sown in raised nursery beds in the last week of June, 2010 at the Research Farm, Banaras Hindu University. Twenty one days old seedlings were transplanted in the well puddled field at a spacing of 30 × 15 cm between row to row and plant to plant, respectively with row length of 3 m in a crossing block on three different dates at the interval of seven days in three replications. Standard agronomic practices were followed to raise good crop. Five rows of drought tolerant variety (male parents) were transplanted in separate block on different dates at the interval of 7 days to synchronize the flowering for making the crosses.

Half of the F₁ seeds of the cross with their parents were transplanted at the Research Farm at Central Rice Research Institute (C.R.R.I.), Cuttack, at the spacing of 30 × 15 cm between row to row and plant to plant, respectively with a row length of 3.0m in three replications.

Screening of the F₁s was done at different growth stages i.e., seedling stage and vegetative stage. Depending upon the screening test, backcrosses with drought susceptible parents were done. Seeds from the cross and F₂ seeds were harvested separately. Marker validation and hybrid confirmation were done before commencing marker assisted selection. F₁ plant progeny, along with the parents were grown to raise F₂ generation. Compact family randomized block design with three replications was followed. Drought susceptible and drought tolerant plants were screened on the basis of leaf rolling and were harvested separately. Fresh crosses were also made to get F₁ seeds.

BC₁F₁ along with the parents were also grown in compact family randomized block design in three replications. Drought tolerant and drought susceptible plants were selected and selfed to produce BC₁F₂ seeds for marker assisted selection (MAS) and harvested separately. *Sample Size* - 20 plants in parents F₁s and 50 plants in F₂ per replication. Marker assisted selection was applied for selecting drought tolerant plants. Plant progenies derived from the tolerant plants were selfed to produce BC₁F₂ generation for marker assisted selection (MAS). Similar to that of moisture non-stress condition plant progenies derived from the tolerant plants were selfed to produce BC₁F₂ generation for marker assisted selection (MAS).

Young leaves were collected from 20-25 days old seedlings and immediately stored in -20°C till further processing. The DNA was extracted following CTAB extraction method (Doyle and Doyle, 1987). Polymerase chain reaction was performed to selectively amplify *in vitro* a specific segment of the total genomic DNA to a billion fold (Mullis *et al.*, 1986). The most essential requirement of PCR is the availability of a pair of short (typically 20- 25bp nucleotides) primers having sequence complementary to either end of the target DNA segment (called template DNA) to be synthesized in large amount. The components of the PCR reaction were first

added in a sterilized 1.5ml microcentrifuge tube thoroughly in a sequence as mentioned in table 3.3 and then mixed thoroughly by vortexing. To each PCR tubes (0.2ml), 14 μ l of reaction mixture was distributed, and finally template DNA of individual rice genotypes was added. The tubes containing reaction mixture were placed in the wells of the thermal cycler block (Eppendorf Thermo – cycler, USA) and amplification reaction was carried out with the thermal cycler programme. The amplified DNA fragments generated through SSR primers were resolved through electrophoresis in 2.5% agarose gel prepared in TAE [242g Tris – base; 57.1ml glacial acetic acid and 100ml 0.5 M EDTA (pH 8.0) bring final volume to 1000ml] buffer. Ethidium bromide solution at a final concentration of 0.03ng/ μ l was added to the agarose solution.

For electrophoresis, 15 μ l of the PCR product was mixed with 2 μ l of 6x loading dye (0.25% bromophenol blue in 30% glycerol) and loaded in the slot of the agarose gel. In order to determine the molecular size of the amplified products, each gel was also loaded with 6 μ l of 50 bp DNA size marker (Fermentas, USA). Gel electrophoresis was performed at a constant voltage of 65V for about 3.5 hours. Finally, the gels were visualized under a UV light source in a gel documentation system (Gel Doc™ XR +, BIO RAD, USA) and the images of amplification products were captured and stored in a computer for further analysis and future use.

SSR markers linked to the QTLs for drought tolerance on various linkage groups were used for foreground selection to select the individuals presumably having the donor allele. Particular target (drought tolerance QTL) was flanked by these markers. The tighter the markers are linked to the QTL, the greater the chance that the QTL mapped between a pair of flanking marker has indeed been transferred. Therefore, phenotypic testing of final products of the MAS exercise needs to be performed in order to confirm the transfer of drought tolerance QTL. At the same time selected markers unlinked to drought tolerance have been used to select those individuals with minimal linkage drag (background selection).

Results

Parental polymorphism survey

Initially, parental polymorphism survey was performed among parental genotypes, Sarjoo – 52 (drought susceptible) and Birsa Gora (drought tolerant). 19 SSR markers were used to validate for the drought tolerance in the parental lines. Out of 19 SSRs, only 10 SSRs produced reproducible and polymorphic bands. These markers clearly distinguished drought susceptible and tolerant parents. In rice, hundreds of microsatellite markers were developed which are publicly available and are being used for MAS, gene tagging, mapping and phylogenetic studies.

Hybrid confirmation in F_1 's

Oryza sativa is basically a self-pollinated crop, with limited degree of outcrossing (< 0.5%). The factors limiting the receptivity of rice flowers to outcrossing include a short style and stigma (1.5 to 4 mm in combined length), short anthers, limited pollen viability and brief period between opening of florets and release of pollen (between 30 seconds and 9 minutes) (Morishima, 1984; Oka, 1988). It therefore became essential to confirm the true type hybrid condition in the F_1 s. RM 212 - RM 3825 (linked with $MQTL_{1.1}$) and RM 60 - RM 22 (linked with $qDTY_{3.2}$) which is co - dominant in nature, gave promising results. Heterozygous plants were selected for further generation advancement and backcrossing.

Production of BC_1F_1 generation

Five plants in F_1 generation of the cross Sarjoo - 52 \times BG were back crossed with the recurrent parent Sarjoo - 52 to produce around 240 seeds at Agricultural Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi.

Marker assisted foreground selection in BC_1F_1 and production of BC_1F_2 generation

BC_1F_1 plants were screened for the presence of $MQTL_{1.1}$ and $qDTY_{3.2}$ with the linked and validated SSR markers RM 212 - RM 3825 and RM 60 - RM 22, respectively from the cross (Sarjoo - 52 \times BG) \times Sarjoo - 52. SSR markers used in the study are co-dominant in nature therefore, in BC_1F_1 population, two types of banding patterns were amplified i.e., homozygous susceptible type and heterozygous types. The segregation of BC_1F_1 plants into drought tolerant and susceptible can be seen clearly in the

representative gel picture of screening of 101 BC₁F₁ plants for *MQTL_{1.1}* and *qDTY_{3.2}* with linked molecular marker RM 212 – RM 3825 and RM 60 – RM 22 (Fig 1 & Fig 2).

Production of BC₁F₂ generation

Since, flanking markers were used in the study and two gene introgression was done, emphasis was given on the selection of only those plants which exhibited heterozygous banding pattern for both the gene loci i.e. *MQTL_{1.1}* and *qDTY_{3.2}*. Thus plant number SA- D- 7, SA – D – 24, SA – D -31, SA – D -39 and SA – D - 73 from the cross (Sarjoo - 52 × BG) × Sarjoo - 52 were selected. Based on agronomic performance and drought related traits, three top performers were selected and allowed to self to produce BC₁F₂ seeds.

Marker assisted foreground and background selection in BC₁F₂ generation and production of BC₁F₃ seeds

Foreground selection: BC₁F₂ plants from the cross (Sarjoo - 52 × BG) × Sarjoo - 52 were screened with *MQTL_{1.1}* and *qDTY_{3.2}* with the linked and validated markers. (Figure 3 & 4).

Background selection: The gene positive plants in BC₁F₂ generation were taken for background selection with polymorphic primers (Fig 5). After, background selection in BC₁F₂, 67 loci became homozygous out of 74 polymorphic loci between Sarjoo-52 and Birsa Gora + *qDTY_{3.2}* in plant no. SA – D – 10 – 9 of the cross (Sarjoo – 52 × Birsa Gora) × Sarjoo – 52. Maximum genome recovery was observed to be about 84.1% in BC₁F₂ with *qDTY_{3.2}*.

Discussion

Marker assisted foreground selection was proposed by Tanksley (1983) and investigated in the context of introgression of tolerant genes by Melchinger (1990). If in BC₁ generation more than one individual satisfying the strongest condition is found, selection between them can be performed on the basis of analysis of other marker loci (located either on the carrier or on non carrier chromosome) to determine the most desirable individual for producing BC₂ (Tanksley *et al.*, 1989).

The success of marker assisted backcross breeding (MAB) depends upon several factors, including the distance

between the closest markers and the target gene, the number of target genes to be transferred, the genetic base of the trait, the number of individuals that can be analyzed and the genetic background in which the target gene has to be transferred, the type of molecular marker(s) used and available technical facilities (Weeden *et al.*, 1992; Francia *et al.*, 2005). Identification of molecular markers that should co-segregate or be closely linked with the desired trait (if possible, physically located beside or within genes of interest) is a critical step for the success of MAB. The most favourable case for MAB is when the molecular marker is located directly within the gene of interest (direct markers). MAB conducted using direct markers is called gene assisted selection (Dekkers, 2003). Alternatively, the marker is genetically linked to the trait of interest. Before a breeder can utilize linkage – based associations between a trait and markers, the associations have to be assessed with a certain degree of accuracy so that marker genotypes can be used as indicators or predictors of trait genotypes and phenotypes.

The lower the genetic distance between the marker and the gene, the more reliable is the application of the marker in MAS. However only in few cases will the selected marker allele be separated from the desired trait due to a recombination event (appearance of false positives). The presence of a tight linkage between desirable trait(s) and a molecular marker(s) may be useful in MAS to increase gain from selection. Based on the studies by Lee (1995) and Ribaut *et al.* (2002), it could be generalized that whenever a target gene is introduced for the first time from either wild or an adapted germplasm, flanking markers as close as 2cM is considered an ideal option, while in the transfer of the same target gene in subsequent phases from elite into elite lines, positioning the flanking markers nearby might be effective in reducing the required size of the backcross population.

MAS have generated a good deal of expectations, which in some cases has led to over optimism and in others to disappointment because many of the expectations have not yet been realized. Although documentation is limited the current impact of MAS on products delivered to farmers seems to be small. New developments and improvements in marker

technology, the integration of functional genomics with QTL mapping and the availability of more high density maps are the other factors that will greatly affect the efficiency and effectiveness of QTL mapping and MAS in the future. The development of high density maps that incorporate new marker types, such as single nucleotide polymorphism (SNPs) and expressed sequence tags (EST) will provide researchers with a great arsenal of tools for QTL mapping and MAS.

Conclusion

In case of (Sarjoo - 52 × Birsa Gora) × Sarjoo - 52, two drought tolerant genes were incorporated. Only those plants were therefore selected which showed polymorphism for the genes *MQTL_{1.1}* and *qDTY_{3.2}*. Thus, plant number SA - D - 1, SA - D - 7, SA - D - 8, SA - D - 9, SA - D - 13, SA - D - 27 and SA - D - 56 were selected. These lines have been subjected to further breeding and trial tests. Agronomic performances and physiological behavior of these lines are also under track. The results showed that the variety HUR 3022 could be efficiently converted to a drought tolerant variety in a backcross generation followed by selfing and selection, involving a time of two to three years. Polymorphic markers for foreground and background selection were identified for the high yielding variety to develop a wider range of drought tolerant variety to meet the needs of farmers in the drought-prone regions. This approach demonstrates the effective use of marker assisted selection for a major QTL in a molecular breeding program.

References

1. Babu RC, Nguyen BD, Chamarker V, Shanmugasundaram P, Chezian P, Jeyaprakash P, Ganesh SK, Palchamy A, Sadasivam S, Sarkarung S, Wade LJ and Nguyen HT, Genetic analysis of drought resistance in Rice by molecular markers, Association between Secondary Traits and Field Performance, *Crop sci*, 43, 1457- 1469, 2003.
2. Bernardo R, 2002, Breeding for quantitative traits in plants, Stemma Press, Woodburn, Minnesota,
3. Blum A, Plant Breeding for Stress Environments, CRC Press, Boca Raton, FL, 1988.
4. Boyer JS.1982. Plant productivity and environment. *Science*. 218: 443.
5. Chen S, Lin XH, Xu CG, Zhang Q. Improvement of bacterial blight resistance of 'Minghui 63', an elite restorer line of hybrid rice, by molecular marker assisted selection. *Crop Sci*. 40(1): 239- 244. 2000.
6. Dekkers JCM, commercial application of marker and gene assisted selection in livestock: strategies and lessons, Paper presented at the 54th annual meeting of the European association for animal production, Rome, Italy, 31st August - 3rd September.
7. Edmeades GO, Bolanos J, Lafitte HR, Rajaram S, Pfeiffer W and Fischer RA. Traditional approaches to breeding for drought resistance in cereals, In, Baker, F,W,G, (ed,), Drought Resistance in Cereals, CAB International, Wallingford, Oxon, UK, 27-52, 1989.
8. Francia E, Tacconi G, CrosattiC, Barabaschi D, Bulgarelli D, Dall'Aglio E and Vale G, Marker assisted selection in crop plants. *Plant Cell Tissue Organ Cult*, 82, 317- 342, 2005.
9. Frisch M, Bohn M and Melchinger AE. Minimal sample size and optimal positioning of flanking markers in marker assisted backcrossing for transfer of a target gene. *Crop sci*. 39: 967- 975.1999a.
10. Frisch M and Melchinger AE. Selection theory for Marker assisted backcrossing. *Genet*. 170: 909- 917. 2005.
11. Fukai S, Pantuwan G, Jongdee B and Cooper M, Screening for drought resistance in rainfed lowland rice, *Field Crop Res*, 64, 61-74, 1999.
12. Jongdee B, Fukai S and Cooper M, Leaf water potential and osmotic adjustment as physiological traits to improve drought tolerance in rice, *Field Crop Res*, 76, 153-163, 2002.

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