Full Length Research Paper

Path analysis for yield traits in F_2 generation and molecular approaches for breeding rice tolerant to drought and submergence

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Drought and submergence are the two major limiting factors that reduce rice production. In this study, the relevance of yield traits through path analysis under drought and submergence conditions to improve grain yield of rice, from dry season 2014-2015 and genotypic analysis using SSR markers was evaluated, during 2015-2016. Path analysis indicated that the number of panicles/clusters had the highest and a direct positive effect on the grain yield, followed by the number of filled-grain/panicle, and the harvest index compared to other component traits. These traits could be used as selection criteria for high yield and drought tolerance in populations of rice. There were two markers including RM201 (210-225 bp) and RM219 (210-215 bp) chosen to select parents in backcrossing because production of polymorphic bands relevant to submergence and drought tolerance genes. By the BC_1F_1 and BC_2F_1 generations of the cross OM6162/Swarnasub1//OM6162, primers RM201 and RM219 were identified drought and submergence tolerant individuals. These lines will be used in breeding programme for release of both drought and submergence tolerant with considerable yield in next step. Findings of this study are promising to develop rice cultivars tolerant to both drought and submergence, and may therefore help to reduce detrimental impacts from climate changes to rice production.

Key words: Correlation, direct selection, grain yield, marker assisted selection.

INTRODUCTION

Rice is currently grown in varied environmental conditions where it shows different levels of response to abiotic stress, depending on the environmental condition of origin and cultivation (Rananwake and Hewage, 2014). The climate change, such as drought, flooding, salinity and high temperature have detrimental impacts on rice production, especially in developing countries. Abiotic stress as drought and submergence have been identified as the two constraints to cause most rice loss (Bernier et al., 2008; Devereux, 2007; Dey and Upadhyaya, 1996;

Gauchan and Pandey, 2012; Pandey and Bhadari, 2009; Venupeasad et al., 2007). Flood are major cause of low yields in rainfed lowed land areas of Mekong Delta Vietnam, which occupy more than 1 million ha. Excess water is a problem for about haft of rainfed areas. Rice production is damaged by both short-term submergence (up to 2 weeks) and by longer term stagnant flooding at water depth above 40 cm. Adaptability of rice to the drought and submergence stresses is the most important objective of the rice breeding program. Additionally, rice yield can be improved with a comprehensive combination of both conventional and molecular breeding techniques (Khush, 2005).

Marker-Assisted Selection (MAS) is a method proposed by Tanksley (1983) to investigate the introgression of tolerant genes (Melchinger, 1990). It includes the Marker-Assisted Evaluation of breeding materials, Marker-Assisted introgression, and Marker-Assisted pyramiding. To improve the selection for early generations, MAS can decrease the number of plants retained due to their early generation performance and can ensure a high probability of retaining superior lines (Eathington et al., 1997). The important prerequisites for successful selection of the early generation with MAS are the population size and heritability level of the selected traits (Lande and Thompson, 1990). Kuchel (2007) and Bonnett et al. (2005) noted that maximum grain in crops can be achieved at a much lower cost with the aid of MAS, compared with the conventional breeding. MAS have successfully introduced the bacterial blight resistance gene *Xa21* (Chen et al., 2000) and *waxy* gene (Zou et al., 2003) to target commercial rice cultivars.

Studies in genetics of rice showed that the submerged-tolerant trait of the FR13A variety is controlled by a polygene and effected by environment, designated *sub1* (Xu and Mackill, 1996). This gene was identified recently as an ethylene responsive like factor (ERF) and designated *Sub1A* (Xu et al., 2006). The most widely submerged-tolerant donors are IR64sub1 and Swanasub1. Swanasub1 was pyramided with the *sub1* gene for tolerance both drought and submergence. *Sub1* versions of popular rice varieties were developed through the Marker-Assisted Backcrossing (MABC) approach (Neeraja et al., 2007; Septinnighsih et al., 2009; Iftekharuddaula et al., 2015; Lang et al., 2015). Nguyen et al. (2004) developed 85 markers for mapping of QTL regions for drought tolerance in rice and identifying putative candidate genes. One QTL region controlling osmotic adjustment on chromosome 3 and 14 affects root traits which are located on chromosomes 1, 2, 4, 5, 6, 7, 8, 9, 10 and 12. In a previous study, Kumar et al. (2014) reported that two markers, RM201 and RM328, were linked with drought-tolerant genes (*qDTY_{1.1}, qDTY_{2.1},* and *qDTY_{3.1}*).

Path analysis appears as the best method to evaluate the relationship between yield and relevant traits (Board et al., 1997). Path analysis permits estimation of direct effects of various traits on yield as well as their indirect effects via other components traits. In crop breeding, the diallel theory which was first developed by Hayman (1954), is widely used for path analysis (Krisha Veni and Shobha Rani, 2005; Eradasappa et al., 2007). Path coefficient analysis partitions into direct and indirect matrix presenting correlation in a more meaningful way in breeding (Mohsin et al., 2009). The diallel analysis is useful to get information about the genetic structure of populations and helps to explore the genetic mechanism of various traits in crops plants such as rice (Griffing, 1956; Rahimi et al., 2010; Muthuramu et al., 210). In rice, information on correlation coefficient has been helpful as a basis for selection in a breeding programme.

Development of high yield cultivars that combine drought and submergence tolerance could be the ideal to reduce detrimental effects of climate change on rice production. IRRI has started drought and submergence breeding programs to develop germplasm for this target population (Kumar et al., 2008; Septiningsih et al., 2009). Therefore, the introduction of both drought and submerged-tolerant characteristics to target rice cultivars is an important task for rice breeders. Thus, the objectives of this study were (1) to clarify direct and indirect effects of yield traits under drought and submergence stresses and (2) to evaluate genotypic using SSR markers for background selection of drought and submergence tolerant.

MATERIALS AND METHODS

Plant materials

The materials consisted of 36 F_2 combinations by crossing of six parents IR64sub1, OMCS2000, OM6162, OM1490, Swarnasub1, and IR78933-B-24-BB-4 in a diallel mating design. The variety OM6162 was crossed with Swannasub1 which was used as the donor for both *qDTY* and *Sub1* genes to obtain a backcross population for MAS.

Path analysis

Evaluation of agronomic characters and grain yield of rice under drought stress

Seeds of the F_2 diallel lines were soaked, germinated in an incubator, and sown into plastic trays. After 15 days, they were transplanted into cement basins. The row-to-row and plant-to-plant space of 20 cm x 15 cm was maintained. Ten days after transplanting, water was not provided until flowering. Fertilizer was applied at the rate 100-40-30 kg of $N-P_2O_5-K_2O$ ha^{-1}. The record plant recovery for each entry followed the 0-9 score of the standard evaluation system (IRRI, 1996) with scores 0-3: tolerance and score 5-9: susceptible, and agro-morphological characters and grain yield were recorded.

Screening for submergence tolerance

Seeds of the F_2 diallel lines were soaked, germinated in an incubator, and sown into plastic trays. Ten-day-old seedlings were transplanted

using 1 plant/hill and with space of 20 x 15 cm in submergence tanks. At the seventh-day after transplanting, plants were completely submerged for 14 days at 10 cm water depth which was then increased by 10 cm at every 10-day interval. Finally, 50 cm water level was maintained up to the soft dough stage. Four plants were tagged for tiller counting. Surviving plants were counted just after the recession of water and their tillers were counted before and after submergence at 7-day intervals.

The standard evaluation system (SES) scores for submergence tolerance followed by IRRI (1988), 1 to 9 (1: all plants survive; 9: all plants completely dead).

Agro-morphological character evaluation

All agro-morphological traits including panicle/cluster, filled grain/panicle, the weight of 1000 grains (g), root length (cm), yield/cluster (g) were recorded. Biomass-weight of 10 plants harvested from each accession per replication was also recorded. Harvested plants were dried before weighing for calculating the Harvest Index as follows;

Harvest Index = Economic yield/Biological yield x 100

where economic yield is the total weight of grain harvest from 10 plants per accession per replication, and biological yield is the total grain weight and biomass from 10 plants per accession per replication.

The correlation coefficient (r) among traits was calculated by using SAS 9.1 program. The correlation coefficient is a measure of the association between two or more variables. It is a measure of symmetrical association between variables and does not measure the dependence of one variable on another.

Marker-Assisted selection

Microsatellite primers were used to survey polymorphism on the samples based on information of the gene mapping of Lang and Buu (2008). For submerged-tolerant genes, the molecular markers were evaluated based on the genetic mapping information of the International Rice Research Institute (IRRI) and the study of Lang et al. (2015). Sixteen microsatellite primers were selected from microsatellite primers mentioned above (Table 1).

In BC_1F_1 and BC_2F_2 generations, selection was initially carried out by markers through screening parental polymorphism at both *qDTY* and *Sub-1* loci.

DNA extraction

Leaves were collected 2-3 weeks after planting for extraction of DNA. Standard molecular grade chemicals and general techniques for preparing stock solutions, buffers, reagents, and equipment were followed according to Sambrook et al. (1989).

DNA extraction was prepared according to a method described by McCouch et al. (1997) and conducted at the Genetics and Plant Breeding Department of Cuu Long Rice Research Institute, Can Tho, Vietnam.

DNA quality was checked using 1% agarose (melting 3 g agarose in 300 ml TAE buffer). The mixture was heated in the microwave for 5-6 min and then cooled to around 55-60°C. This was then poured into a prepared electrophoresis box with combs. Gels were ready and the combs were removed after about 45 min. Seven microliters of DNA sample and 3 μl loading buffer (Tris 1M pH = 8.0, glycerol, EDTA 0.5 M pH = 8.0, xylene cyanol 0.2%, bromophenol blue 0.2% and distilled water) were mixed and placed in the wells. The electrophoresis program   was run at   70-80 V, 60 mA for 45 min or

until loading buffer dye moved far from the wells. Gel was then taken out and stained with ethidium bromide. The gel image was visualized under UV light.

Amplification of microsatellites and detection of their polymorphisms

PCR amplification was performed in a mixture of 10 mM Tris-HCl (pH=8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1 unit of TAKARA *Taq*, 4 nmole of dNTPs, 10 ρmole of primers, with 30 ng of genomic DNA per 25 μl using a thermal cycler 9600 (Perkin-Elmer, USA). The PCR reactions were denatured at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The final extension was at 72°C for 5 min. After PCR, 13 μl of loading buffer (98% formamide, 10 mM EDTA, and 0.025% bromophenol blue, 0.025% xylene cyanol) were added. Polymorphisms in the PCR products were detected by ethidium bromide staining after electrophoresis on 3% agarose gel.

RESULTS

Path analysis

The correlation coefficient was conducted out 6 to 36 crosses of diallel from the varieties IR64sub1, OMCS2000, OM6162, OM1490, Swarnasub1, and IR78933-B-24-BB-4 (Table 2). The trait of panicle/cluster showed a strong and positive association with root length, drought tolerant at seedling, and yield. Positive and high phenotypic correlations of yield with the number of panicles/cluster were obtained in this study. Wherever, negative significant association with 1000 weight was observed. Association among yield traits revealed that filled grains/panicle showed significant positive association with HI and root length, whereas, significant negative correlation was observed with the weight of 1000 grains and drought tolerance at the flowering stage. The number of filled grains/panicle also showed a strong positive and high phenotypic correlation on grain yield. The weight of 1000 grains was recorded to have a positive significant association with drought tolerance at the flowering stage and significant negative association was recorded with drought tolerance at the seedling stage. The weight of 1000 grains and submergence at seedling was not correlated with grain yield. Correlation between the HI and drought responses at the flowering stage was significantly negative, and phenotypic correlation of HI with yield trait was 0.69. Therefore, the HI could be used as a reliable criterion for improving yield. The trait root length exhibited a significant and positive association with drought tolerance and was associated with yield trait. The trait test for drought tolerance at the flowering stage showed a positive and significant association with drought tolerance at the seedling stage, significant negative association with yield, and strong positive and high phenotypic ccorrelation (0.65). Whereas, drought tolerance at the seedling stage did not show significant phenotypic association with yield.

Path analysis permits estimation of direct effects of

Table 1. The list of information molecular markers used in diagnosis of submergence and drought.

No.	Markers	Sequence code (5' - 3')	Chromosome	Repeating sequence
1	RM201	F: ctcgtttattacctacagtacc R: ctacctcctttctagaccgata	9	(CT)17
2	RM511	F: cttcgatccggtgacgacac R: aacgaaagcgaagctgtctc	12	(GAC)7
3	RM11125	F: ccaagaaccctagctccctctcc R: tcgacgagatcctcctcgtaaacc	1	(CT)22
4	RM10713	F: atgaacccggcgaactgaaagg R: ctggctccctcaaggtgattgc	1	(AGA)12
5	RM3252	F: ggtaactttgttcccatgcc R: ggtcaatcatgcatgcaagc	1	(CT)13
6	RM10115	F: acaagacgaggtaacacgcaagc R: gcgaaggatcaacgatgatatgg	1	(CTT)24
7	RM105	F: gtcgtcgacccatcggagccac R: tggtcgaggtggggatcgggtc	9	(CCT)6
8	RM219	F: cgtcggatgatgtaaagcct R: catatcggcattcgcctg	9	(CT)17
9	RM23662	F: gagaggacgatggcactattgg R: cgaggaacttgattcgcatgg	9	(GGC)10
10	RM23877	F: tgccacatgttgagagtgatgc R: tacgcaagccatgacaattcg	9	(CA)30
11	RM547	F: taggttggcagacctttcg R: gtcaagatcatcctcgtagcg	8	(ATT)19
12	RM249	F: ggcgtaaaggttttgcatgt R: atgatgccatgaaggtcagc	5	(AG)5A2(AG)14
13	RM24103	F: actgacgagagagacatggatgg R: ccggcacacaatgaataggg	9	(AC)17
14	RM25181	F: aaagagcttccctaatggcttcg R: gagagaatgacctctcccaagacc	10	(TTC)22
15	RM1125	F: ggggccagagttttcttcag R: gtacgcgcagaaaatgagag	10	(AG)12
16	RM328	F: catagtggagtatgcagctgc R: ccttctcccagtcgtatctg	9	(CAT)5

various traits on yield as well as their indirect effects via other component traits. The number of panicles/cluster was found to have a maximum direct positive effect on grain yield (Table 3), followed by the number of filled grains/panicle, HI and weight of 1000 grains, which indicated that these traits were contributors towards yield in these combinations, but there has not been stability in the results in various experiments or in different populations.

Marker assisted selection approach

A total of 16 markers were used to screen for drought and submergence in parent varieties (Table 1). The parental polymorphic survey was performed among the parental genotypes OM6162 and Swarnasub1. Two SSR markers RM201 and RM219 produced polymorphic bands (Figures 1 and 2). These markers clearly distinguished drought, submergence susceptible and tolerant parents. Homozygous plants were selected for backcrossing generation.

In the BC_1F_1 generation of OM6162/Swarnasub1//OM6162, plants were screened for drought tolerance by the robust tightly-linked marker RM201, a marker linked to drought tolerance QTL. There were two amplified bands, type P1 of the 225 bp band and type P2 of the 210 bp band. Out of 38 plants, eight lines showed "B" score similar homozygous donor allele by the 210 bp band, 15 lines showed heterozygous "H" score

Table 2. Correlations coefficients among the traits with yield of F_2 diallel generation.

Traits	Panicle/Cluster	FG	W-1000	HI	RL	SubS	DF	DS	Yield r	Pr
Panicle /Cluster	1	0.20	-0.28*	0.19	0.59**	-0.13	0.15	0.43**	0.78**	0.88
FG		1	-0.38*	0.68**	0.79**	-0.04	-0.68**	0.25	0.76**	0.67
W-1000			1	-0.19	-0.07	-0.04	0.82**	-0.39*	-0.24	0.37
HI				1	0.08	0.25	-0.17	-0.69**	-0.89**	0.69
RL					1	-0.04	0.28	0.37**	0.84**	-0.39
SubS						1	0.06	0.11	-0.14	-0.08
DF							1	0.99**	-0.68**	0.65
DS								1	-0.20	-0.19

**: significant at P<0.01. *: significant at P<0.05; r: correlations coefficients; Pr: phenotype correlation coefficients. DF: Drought at flowering stage; DS: Drought at seedling stage; RL: Root length; SubS: Submergence at seedling; HI: harvest index; W-1000: Weight of 1000 grains: FG: Filled grain/panicle.
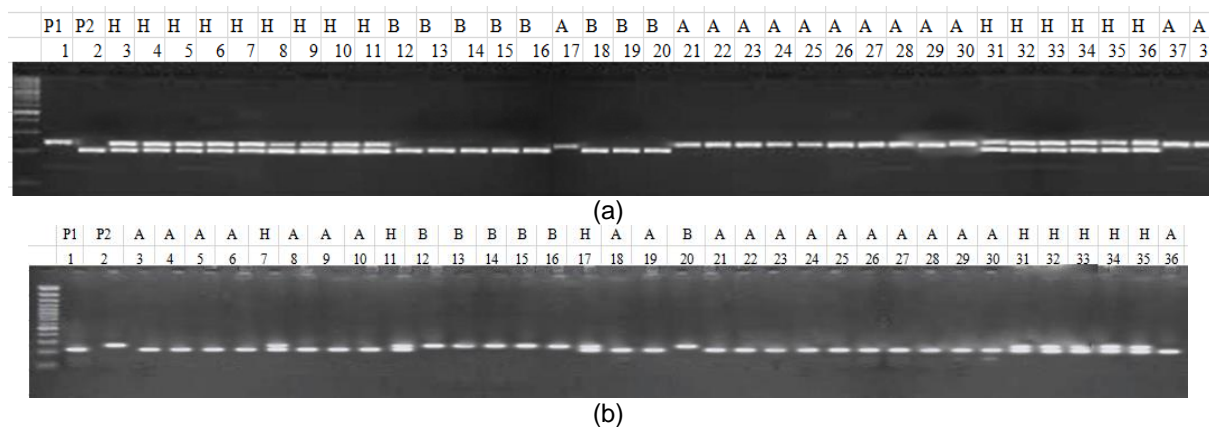
Table 3. Analysis of correlation system by path (path analysis) between the grain yield traits of rice in F2 diallel generation.

Traits	Panicle/Cluster	FG	W-1000	HI	RL	SubS	DF	DS	r total
Panicle /Cluster	0.85	0.15	-0.10	0.06	0.00	0.00	0.00	0.00	0.68
FG	0.19	0.67	-0.11	0.34	-0.14	0.07	0.11	-0.01	0.73
W-1000	-0.27	-0.20	0.46	-0.10	0.02	0.04	0.00	0.01	-0.21
HI	0.15	0.37	-0.06	0.59	-0.01	-0.01	0.00	0.02	0.41
RL	0.44	0.27	-0.02	0.01	-0.44	0.00	0.00	-0.01	0.52
SubS	-0.12	-0.03	-0.01	0.13	0.02	-0.06	-0.00	0.00	-0.16
DF	0.09	-0.31	0.29	-0.05	-0.07	0.00	0.00	-0.03	0.15
DS	0.16	0.11	-0.10	-0.41	-0.40	-0.10	0.01	-0.06	0.17

DF: Drought at flowering stage; DS: Drought at seedling stage; RL: Root length; SbS: Submergence at seedling; HI: harvest index; W-1000: Weight of 1000 grains: FG: Filled grain/panicle.

Figure 1. PCR profiles of some lines genotype in BC_1F_1 of OM6162/Swarnasub1//OM6162; (a) RM211 linked drought-tolerant gene: single band linked lane 2 as P1: the recipient parent (225 bp), P2: the donor parent (210 bp), A: similar homozygous recipient allele B: homozygous donor allele and H: double band indicated heterozygous allele; (b) RM219 linked submergence-tolerant gene: same as "a"; P1: the recipient parent (210 bp), P2: the donor parent (215 bp).

and 15 lines showed "A" score (Figure 1a). Thus, eight plants from cross OM6162/Swarnasub1//OM6162 were self-ed to develop BC_2. These plants with the "H" score for tightly linked marker were subjected for phenotypic selection.
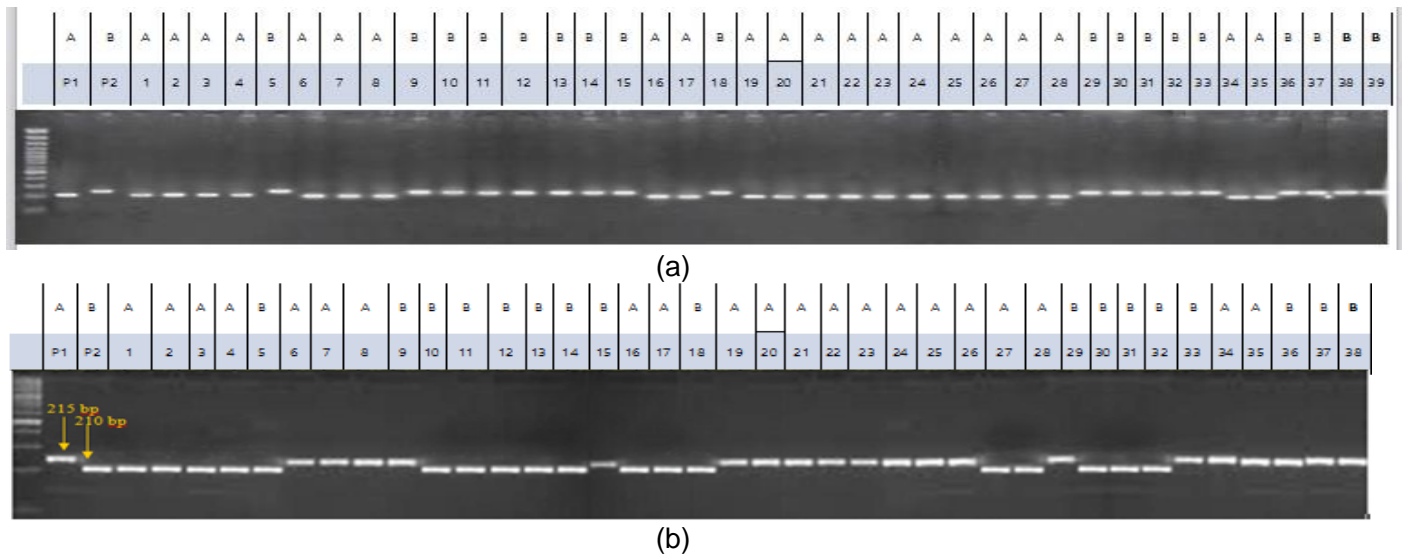
In the BC_2F_1 generation, segregation of plants into

(a)

(b)

Figure 2. PCR profiles of some lines genotype in BC$_2$F$_1$ of OM6162/Swarnasub1//OM6162; (a) RM211 linked drought-tolerant gene: single band linked lane 2 as P1: the recipient parent (210 bp), P2: the donor parent (2105 bp), A: similar homozygous recipient allele B: homozygous donor allele and H: double band indicated heterozygous allele; (b) RM219 linked submergence-tolerant gene: same as "a"; P1: the recipient parent (215 bp), P2: the donor parent (210 bp).

drought tolerant and susceptible can be seen clearly in the gel picture with linked RM201 by type P1 of 215 bp band and type P2 of 210 bp band. The 21 plants with " A" score similar similar homozygous recipient allele. Eighteen plants with "B" score as homozygous donor allele of Swarnasub1 were produced due to accidental failure of backcrossing (Figure 2a).

In the case of RM219, a marker linked to the submergence tolerance QTL *sub1*. there were two amplified bands, 210 bp, and 215 bp band. The twenty plants showed "A" score, 8 plants showed heterozygous "H" score and only six plants of OM6162/Swarnasub1//OM6162 had the band similar to Swarnasub1 variety. (Figure 1b). In the BC$_2$F$_1$ generation of OM6162/Swarnasub1//OM6162, the marker RM219 linked to type P1 of 210 bp band and type P2 of 215 bp band. A total of eighteen plants had homozygous donor allele as Swarnasub1 (Figure 2b).

DISCUSSION

The number of panicles/cluster was found to have a maximum direct positive effect on grain yield. The results of the importance of the direct effect of panicles per plant were reported by (Bagheri et al., 2011; Madhavilatha et al., 2005; Yogameenaskshi and Vivekanandan, 2010). Here, the analysis aimed to determine important traits directly correlated to the yield or indirectly through other traits because they are able to help improve rice yield. Vaishali (2003) showed that grain yield exhibited strong significant positive correlation with the number of productive tillers per plant. Significant genetic variability in some root traits

has been demonstrated and implicated for improving drought tolerance in crop plants (O'Toole and De Datta, 1986; Thangaraji et al., 1990; Sharma et al., 1994; Sinclair and Muchow, 2001). Jeena and Mani (1990) studied root traits and grain yield on some upland rice varieties and indicated that high root length density and root weight were important for breeding drought tolerance genotypes.

In summary, according to the principle of correlation, the system was evaluated by path analysis, and the results are displayed in Table 3. If the correlation coefficient among the cause and the result are equivalent to its direct value, the correlation can be explained as a really close relationship and direct selection through this traits. If the correlation is positive, but directly affected values are negative or negligible, the indirect values can be seen as the causes of the correlation. In this case, the indirect causes must be simultaneously considered in the selection. For example, for the length of roots, we must consider its indirect factors simultaneously if the traits for selection are a number of panicles/cluster, filled grains/panicle and drought tolerance at the flowering and seedling stages. Commonly, phenotype correlation of grain yield, yield, and drought related traits provides the information (to determine the direction of association (Sunderraj et al., 1972).

Molecular markers can be used in many steps of rice breeding program. Markers are also used to examine parental polymorphism with desirable genes and gene combinations. This approach has the potential to make parental selection more efficient, to expand the gene pool of modern cultivar and to speed up the development of new varieties. Lang and Buu (2008) studied that the

markers RM201 and RM328 were linked to drought-tolerant traits. Under drought stress treatment, it was confirmed that this root length QTL with target segment on chromosome 9 was segregated in the BC population of OM1490/WAB 880-1-38-18-20P1-HB; OM1490/WAB881 SG9, and OM4495/IR65195-3B-2-2-2-2 (Lang et al., 2013). If BC_1F_1 generation more than on individual satisfying the strong condition is found, selection between them can be performed on the basis of analysis of other marker loci to determine the most desirable individual for producing BC_2 (Tanksley et al., 1989). SSR marker, RM219 has been mapped for 3.4 cmRM219 to *sub 1* locus (Xu et al., 2004). Rathnayake et al. (2012) studied that 220 bp of allele of RM219 was used as diagnostic alleles or gel bands to monitor Sub-1 in IRRI119/Bw363 cross. For Swarna variety, a combination of three QTLs ($qDTY_{1.1,}$ $qDTY_{2.1,}$ and $qDTY_{3.1}$) was pyramided *Sub1*, the large effected QTL for tolerance of submergence (Kumar et al., 2014).

Exploitation of the initial materials are very important in breeding. Based on the drought and submergence tolerant gene are multi-gene, therefore evaluation of initial materials to select the parents serving studies of hybridization is urgently to select good hybrid material for achieving targets in breeding. Currently, at least one popular determined the usefulness of the two markers (RM201 and RM219) for selection both of submergence and drought tolerance genes. These evaluated lines with genotypes will be reference to pick for the next generation. At the same time, phenotypic testing of final products of the MAS exercise needs to be performed in order to confirm the transfer of QTL.

Conclusions

Most of the above traits showed that traits as root length, the number of panicles/cluster, and a number of filled grains/panicles at harvest had a strong and positive correlation with grain yield. Based on path analysis, trait number of filled grains/panicles, the number of filled-grain/panicle, and harvest index had strong and direct positive effect correlation with grain yield.

The present study established the utilization of marker assisted selection for developing new varieties by combinations between drought and submergence tolerance. Fortunately, both *qDTY* and *Sub1* can be combined in the same variety. These best lines will be used for development of further breeding. This type of variety was approached as a first step to develop new varieties for gathering genes of drought and submergence tolerance.

Abbreviations

MAS, Marker-assisted selection; **MABC,** marker-assisted backcrossing; **IRRI,** International Rice Research Institute; **SES,** standard evaluation system; **BC,** backcross.

Conflict of Interests

The authors have not declared any conflict of interest.

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