Introgression of semi-dwarf gene in Kalanamak rice using marker-assisted selection breeding

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Kalanamak is an important aromatic rice variety in India. Tall stature of Kalanamak causes lodging due to which its yield and other characters severely declines. Introgression of the semi-dwarfing gene (sd_1) from CSR10 was performed with the help of marker-assisted breeding. Backcross-derived plants were characterized for semi-dwarf nature. Improved Kalanamak lines were analysed for the sd_1 gene and to check the presence of aroma, sensory analysis test and amplification with betaine aldehyde dehydrogenase 2 (*badh* 2) derived primer was performed. Improved versions of Kalanamak rice lines were either on par or superior in terms of yield, grain type and cooking quality with reduced height implicating the potentiality of marker-assisted backcross breeding for improvement of this rice variety.

Keywords: Aromatic rice, grain quality, lodging resistance, semi-dwarf gene.

AMONG scented rice, Kalanamak (kala = black husk; namak = salt) is a non-basmati aromatic, small and medium-sized grain rice with strong and pleasant aroma. Presently, cultivation of this rice variety has been decreasing due to several biotic and abiotic stresses, including lodging. The tallness of plants is an important factor that causes susceptibility to lodging and ultimately causes a reduction in yield[1]. The semi-dwarfing gene in rice (sd_1) is one of the most important genes deployed in modern rice breeding. Its recessive character results in a shortened culm with improved lodging resistance. The sd_1 gene has been cloned from rice and its dominant allele encodes gibberellin 20 oxidase-2 (GA20ox-2); a deletion or substitution mutation in this gene results in loss of its function. Thus plants showed deficiency of growth hormones, which control plant height[2]. PCR-based marker for sd_1 gene has been developed[2]. Varieties with sd_1 gene had better yields and lodging resistance[3]. Other dwarfing genes have also been used in the breeding programme, but many of them cause floret sterility, unusual plant and grain development[4]. Due to quality, aroma and palatability of Kalanamak rice, its price and demand in the global market are still high[5]. The candidate gene responsible for aroma, is *badh2* (*fgr*), a recessive gene, located on chromosome 8, encodes betaine aldehyde deydrogenase homologue 2 (BADH2). It is a key enzyme regulating 2-acetyl 1-pyrroline (2 AP) production[6,7] and 2 AP is mainly responsible for aroma in rice[8,9] which is found in all plant parts of fragrant rice varieties, except the roots[10].

Several methods are available to improve rice varieties, such as conventional breeding methods, molecular breeding, mutation breeding and transgenic approach. However, conventional breeding methods are time-consuming and influenced by the environment, while transgenic approach has biosafety issues in the present scenario. Molecular breeding involves the use of molecular markers that are economical, not influenced by the environment, accurate and requires less time. Several molecular breeding programmes in rice have been undertaken successfully for incorporation of semi-dwarf character without the loss of aroma[11]. Therefore, the present study was aimed at the introgression of sd_1 gene through marker-assisted selection breeding into the genetic background of Kalanamak rice, with its natural grain and cooking quality traits.

Materials and methods

Plant material and breeding strategy

Kalanamak rice was taken as the recurrent parent and CSR10 as the donor parent for the generation of F_1 plants. The F_1 plants were backcrossed with the

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Table 1. PCR primers used for marker-assisted selection in transferring semi-dwarf character in Kalanamak rice

Marker/primer pair	Primer sequence (5′–3′)	Chromosome	Reference
SD1 (semi-dwarfing)	F-CACGCACGGGTTCTTCCAGGTG R-AGGAGAATAGGAGATGGTTTACC	1	2
BADH2 (aroma)	F-TGCTCCTTTGTCATCACACC R-TTTCCACCAAGTTCCAGTGA	8	43

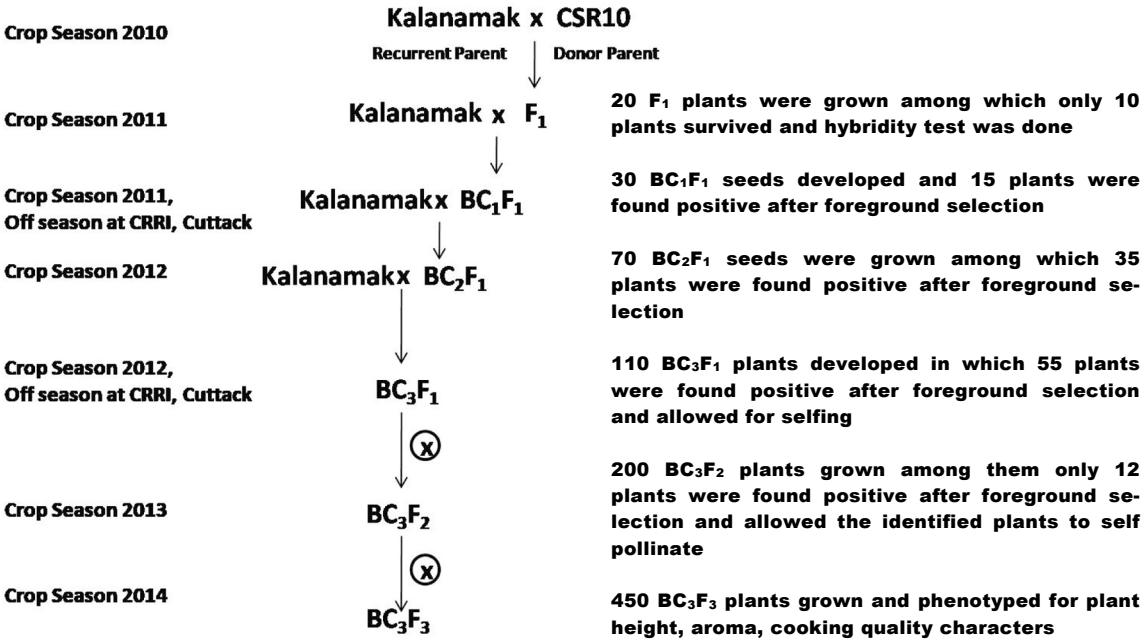

Figure 1. Schematic diagram of introgression of dwarfing gene in Kalanamak rice from the donor CSR10 through marker-assisted backcross breeding.

recurrent parent and positive BC_1F_1 plants having *sd*$_1$ and *badh2* genes were selected by PCR-based molecular markers (Table 1). Positive BC_1F_1 plants were again backcrossed with the recurrent parent for the development of BC_2F_1 generation and positive BC_2F_1 was again backcrossed for the development of BC_3F_1 generation. Selfing was carried out for the development of the BC_3F_2 generation. Positive BC_3F_2 plants were selfed to obtain BC_3F_3 progenies (improved lines; Figure 1). Further, selected (semi-dwarf and aromatic) backcrossed progenies, along with recurrent parent Kalanamak, were evaluated in random block design (RBD). Thirty plants (3 × 10 plants/per plot) were randomly selected for each tested line and scored for agronomic traits, viz. plant height (cm), effective tillers/plant, panicle length (cm), number of filled grains/panicle, spikelet fertility (%), 1000-grain weight (g), grain yield/plant and grain cooking quality traits.

DNA isolation and molecular marker analysis

Total genomic DNA from young leaves was isolated[12] and checked on 0.8% agarose gel. PCR was performed in a PTC-100 thermal cycler (BioRad, USA). For amplification with SD1 and BADH2 primer pairs (Table 1), PCR mixtures containing 50 ng of template DNA, 5 pmol of each primer, 0.05 mM of dNTPs (MBI, Fermentas, USA), 10× PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$) and 0.5 U of *Taq* DNA polymerase (Merck India Pvt Ltd, New Delhi, India) in a reaction volume of 20 µl were used. Template DNA was initially denatured at 94°C for 5 min followed by 35 cycles of PCR amplification with the following parameters: 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min of primer extension at 72°C followed by final extension of 72°C for 7 min. The amplified products were electrophoretically resolved on a 4% agarose (MetaPhor^{TM}) gel (Table 1).

Sensory analysis test for aroma

Aroma was tested by sniffing following a KOH-based method[13]. The sample was scored on a 0–3 scale – score 0 for the absence of aroma, 1 for mild aroma, 2 for strong aroma and 3 for very strong aroma. Evaluation of aroma in the samples was carried out by an expert panel of five individuals.

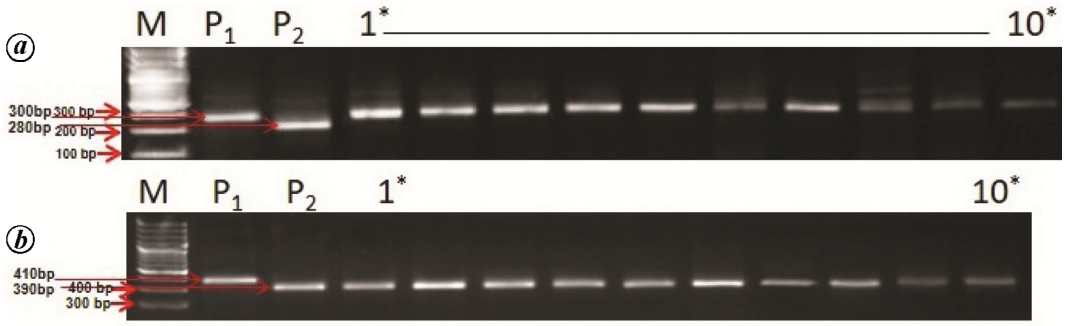

Figure 2. PCR analysis of the sd_1 gene with SD1 primer (***a***) and the *badh2* gene with BADH2 primer (***b***) in parental lines (P1, CSR10 and P2, Kalanamak) and improved lines. Lane M, Molecular weight marker and lanes 1–10, Selected improved line individuals.

Grain and cooking quality test

The grains harvested from improved lines along with the parents (Kalanamak and CSR10) were analysed for physico-chemical characteristics like grain shape, dimensions of milled and cooked rice. For measuring the kernel length and breadth before and after cooking, three fully developed wholesome milled rice kernels were chosen and measurements made using a photo-enlarger.

Statistical analysis

Statistical analysis for agronomic traits, grain and cooking quality was done using Online Statistical Analysis Tool (OPSTAT) software: statistical version for agricultural work[14].

Results

Marker-assisted selection

Marker-assisted selection was conducted on F_1 plants derived from the cross between Kalanamak and CSR10. Backcross generations developed by the crossing of positive F_1 with the recurrent parent were also tested for the sd_1 and *badh2* genes. The amplified product with SD1 primer from the donor line was 300 bp while in the recipient parent it was about 280 bp, which could be easily resolved on 4% agarose gel. Plants having both the bands in BC_1F_1 (300 and 280 bp) were selected for further experiments (Figure 2 *a*). The *badh2* allele was amplified with 390 bp product size in Kalanamak and 410 bp product size in CSR10 (Figure 2 *b*). Required alleles at the sd_1 and *badh2* loci were determined in the next generation. Positive BC_2F_1 plants were selected and backcrossed again for the generation of BC_3F_1, which produced 200 BC_3F_2 individuals. Ten best semi-dwarf plants having aroma were identified from BC_3F_2 generation and selfed to produce BC_3F_3 generation (improved line). Improved lines were analysed for homozygous allele, agronomic performance, grain cooking quality and aroma. Only semi-dwarf improved plants were selected for agronomic and quality analysis. The genotyping results presented in Figure 2 *a* and *b* are BC_3F_3 improved lines having homozygous allele.

Evaluation of agronomic performance in backcross lines

Data on the agronomic performance of selected improved lines of Kalanamak × CSR10 with respect to traits like plant height (cm), number of tillers, panicle length (cm), spikelet/panicle, grain/panicle, spikelet fertility (%), 1000-grain weight (g), and yield were recorded (Table 2). Plants having the sd_1 gene in backcross generation showed significantly reduced plant height (significant at CD = 0.05) in comparison to Kalanamak. A reduction of 17%–30% was observed in improved lines. Minimum plant height of 109 cm was observed in KC-4-7 (improved line). Improved lines produced more number of tillers with the higher number of spikelets and grain per plant (Table 2), and collectively contributed to the increase in grain yield/plant. A significant increase in the number of tillers/plant was observed in the line KC-4-10 producing up to 16 tillers in comparison of Kalanamak (11 tillers). Maximum number of spikelets/plant and grains/plant was found in KC-4-7 (227.66 and 210.00 respectively). However, maximum spikelet fertility percentage was found in line KC-4-4 (92.280). Improved lines of Kalanamak also had increased 1000-grain weight in line KC-4-1 (22.66 g) in comparison to Kalanamak (12.66 g). The highest grain yield /plant was found in line KC-4-7 (67.763 g). Selected plants of improved lines showed better agronomic traits with shorter plant height and higher grain yield per plant (Figure 3 *a* and *b* and Table 2).

Grain and cooking quality of improved lines

Improved lines also showed variation in husk colour. In Kalanamak, husk colour is black whereas CSR10 has a

Table 2. Agronomic performance and quality characteristics of improved lines derived from Kalanamak × CSR10

Improved lines	PH (cm)	NT	PL	S/P	G/P	SF (%)	1000-grain weight	GY/P	KLBC (mm)	KLAC (mm)	KBBC (mm)	KBAC (mm)	Aroma
KC-4-1	110 ± 0.57	14.33 ± 0.33	21.33 ± 0.33	213.33 ± 3.33	193.33 ± 1.66	88.54 ± 0.07	22.66 ± 0.33	61.000 ± 0.577	8.74 ± 0.11	11.16 ± 0.14	1.90 ± 0.00	2.26 ± 0.03	2
KC-4-2	111.66 ± 0.33	13.33 ± 0.33	24.00 ± 0.57	208.33 ± 4.41	192.66 ± 1.45	88.49 ± 0.26	21.00 ± 0.57	53.367 ± 0.037	8.70 ± 0.10	10.72 ± 0.27	1.80 ± 0.01	2.36 ± 0.08	3
KC-4-3	121.33 ± 0.33	15.33 ± 0.33	22.66 ± 0.33	203.33 ± 3.33	185.00 ± 2.88	90.20 ± 2.88	21.66 ± 0.88	61.373 ± 0.037	8.33 ± 0.88	10.69 ± 0.08	1.82 ± 0.02	2.36 ± 0.03	2
KC-4-4	120.66 ± 0.33	16.33 ± 0.33	21.66 ± 0.33	198.00 ± 0.57	180.00 ± 2.88	92.280 ± 0.02	21.00 ± 0.57	61.423 ± 0.155	8.36 ± 0.33	10.91 ± 0.30	1.82 ± 0.01	2.20 ± 0.05	2
KC-4-5	121.66 ± 0.33	14.33 ± 0.33	22.66 ± 0.33	197.33 ± 0.66	182.33 ± 1.20	91.65 ± 0.18	20.33 ± 0.33	53.173 ± 0.031	8.37 ± 0.06	10.76 ± 0.06	1.86 ± 0.02	2.30 ± 0.05	2
KC-4-6	110.33 ± 0.33	13.33 ± 0.33	21.66 ± 0.33	197.0 ± 00.57	177.66 ± 1.45	90.16 ± 0.03	21.00 ± 0.57	49.763 ± 0.008	8.80 ± 0.05	11.36 ± 0.07	1.91 ± 0.00	2.34 ± 0.031	3
KC-4-7	109.33 ± 0.33	15.66 ± 0.33	24.33 ± 0.33	227.66 ± 1.45	210.00 ± 5.77	90.25 ± 0.06	20.66 ± 0.33	67.763 ± 0.157	8.23 ± 0.08	11.15 ± 0.08	1.88 ± 0.00	2.23 ± 0.08	3
KC-4-8	125.33 ± 0.33	14.33 ± 0.33	23.33 ± 0.33	221.33 ± 0.88	196.66 ± 0.33	89.60 ± 0.05	21.00 ± 0.57	59.163 ± 0.023	8.27 ± 0.034	10.70 ± 0.07	1.823 ± 0.04	2.30 ± 0.00	2
KC-4-9	128.33 ± 0.33	15.66 ± 0.33	22.66 ± 0.33	221.00 ± 0.57	203.66 ± 1.85	89.23 ± 0.02	20.66 ± 0.33	65.697 ± 0.151	8.25 ± 0.03	10.46 ± 0.00	1.79 ± 0.06	2.13 ± 0.01	2
KC-4-10	126.66 ± 0.33	16.66 ± 0.33	23.33 ± 0.33	214.66 ± 0.33	196.66 ± 3.33	88.29 ± 0.04	21.33 ± 0.33	69.787 ± 0.052	8.20 ± 0.02	10.76 ± 0.02	1.79 ± 0.02	2.25 ± 0.07	3
KC-4-11	123.33 ± 0.33	15.66 ± 0.33	24.33 ± 0.33	214.00 ± 0.57	193.00 ± 2.08	91.43 ± 0.09	20.66 ± 0.33	62.363 ± 0.042	8.44 ± 0.30	10.98 ± 0.13	1.83 ± 0.02	2.20 ± 0.05	3
KC-4-12	116.33 ± 0.33	14.33 ± 0.33	21.66 ± 0.33	210.33 ± 0.33	192.33 ± 1.20	90.44 ± 0.05	20.33 ± 0.33	56.343 ± 0.194	8.53 ± 0.33	10.71 ± 0.11	1.84 ± 0.026	2.36 ± 0.33	2
KC-4-13	120.33 ± 0.33	15.66 ± 0.33	22.66 ± 0.33	203.66 ± 4.17	182.33 ± 1.45	87.88 ± 0.05	21.00 ± 0.57	59.767 ± 0.088	8.29 ± 0.04	10.77 ± 0.01	1.81 ± 0.049	2.10 ± 0.058	2
KC-4-14	121.33 ± 0.33	15.66 ± 0.33	20.33 ± 0.33	199.00 ± 0.57	181.66 ± 0.88	91.35 ± 0.08	19.667 ± 0.33	55.893 ± 0.019	8.51 ± 0.19	10.58 ± 0.07	1.85 ± 0.03	2.23 ± 0.03	3
KC-4-15	110 ± 0.57	15.33 ± 0.33	21.33 ± 0.33	207.66 ± 5.04	187.66 ± 1.45	89.96 ± 1.2	18.66 ± 0.33	53.727 ± 0.038	8.76 ± 0.13	10.63 ± 0.08	1.80 ± 0.00	2.30 ± 0.04	3
Kalanamak	154 ± 0.57	11.66 ± 0.88	25.00 ± 0.57	136.66 ± 1.66	111.66 ± 1.66	78.41 ± 0.107	12.66 ± 0.33	16.530 ± 0.017	7.46 ± 0.08	8.73 ± 0.12	1.71 ± 0.00	1.86 ± 0.03	3
CD at 0.05	24.11	1.15	1.096	7.7947	6.20	0.967	1.40	0.501	0.250	0.39	0.08	0.159	
SE (d)	11.75	0.56	0.53	3.79	3.02	0.47	0.68	0.244	0.12	0.19	0.03	0.07	
SE (m)	8.31	0.39	0.37	2.68	2.12	0.33	0.48	0.173	0.08	0.13	0.02	0.05	
CV	11.89	4.61	2.88	2.27	1.99	0.64	4.13	0.528	1.77	2.18	2.59	4.23	

PH, Plant height; NT, No. of tillers; PL, Panicle length (average of three panicles); S/P, Spikelet/panicle; G/P, Filled grains/panicle; SF, Spikelet fertility; TW, 1000-grain weight; GY/P, Grain yield/plant; KLBC, Kernel length before cooking; KLAC, Kernel length after cooking; KBBC, Kernel breadth before cooking; KBAC, Kernel breadth after cooking; Aroma: 0, Absent; 1, Mild; 2, Strong and 3, Very strong. CD, Crtical difference at 0.05 SE (d): Standard error of difference, SE (m), Standard error of mean; CV, Coefficient of variance and ± (x), Standard deviation.

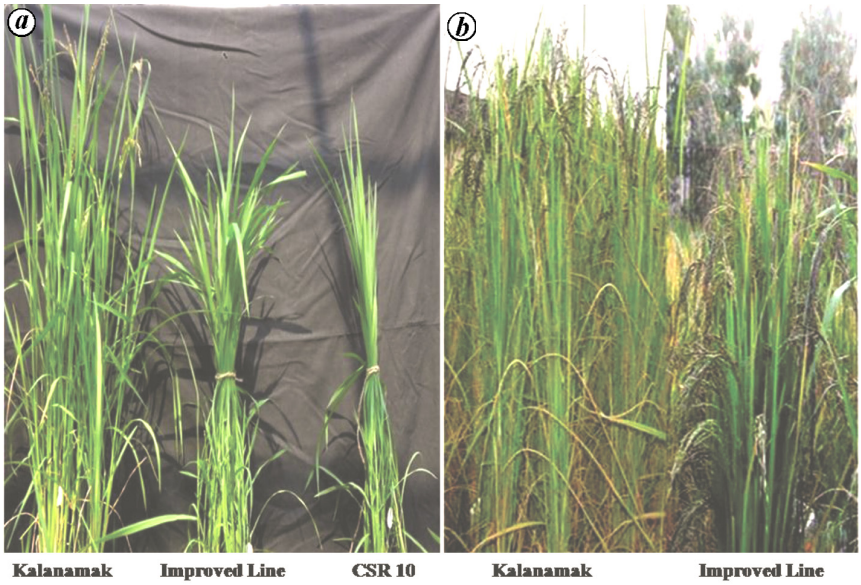

Figure 3. Morphological variation among the Kalanamak, CSR10 and improved line at flowering (***a***) and between the Kalanamak and improved line at maturity stage (***b***).

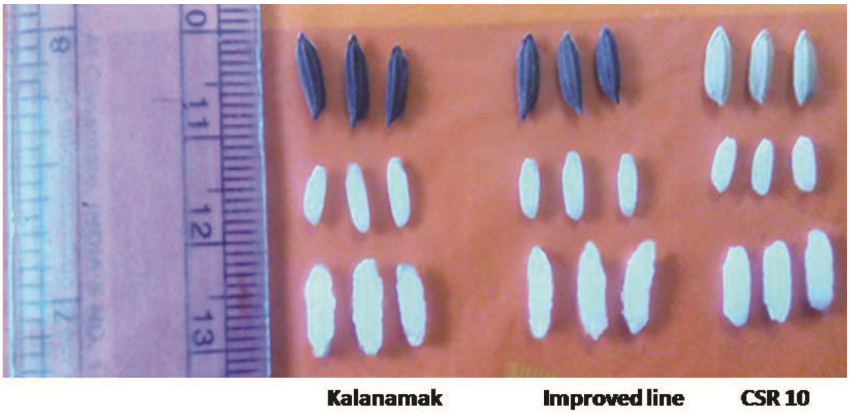

Figure 4. Variation in seed husk colour (top row), dehusked seeds (middle row) and cooked rice (bottom row) of improved line and parents (Kalanamak and CSR10).

yellow colour however, improved lines showed brown to black colour (Figure 4). Kernel length and breadth of improved lines also increased significantly (significant at CD = 0.05) in comparison to Kalanamak rice. In line KC-4-5, kernel length and breadth (KLBC and KBBC) of rice grain before cooking was 8.80 and 1.91 mm respectively, while in Kalanamak, it was 7.46 and 1.71 mm respectively. Similarly, in line KC-4-1, kernel length and breadth (KLAC and KBAC) after cooking was 11.16 and 2.26 mm respectively, while in Kalanamak rice it was 8.73 and 1.86 mm respectively (Table 2).

Aroma analysis by sensory test

The polished and cooked kernels of Kalanamak, CSR10 and selected improved lines were analysed for aroma by the panel of five individuals, including a specialized biochemist (Table 2). Most of the backcrossed seeds scored 2 but some had a score of 3, similar to Kalanamak, confirmed by the sensory test using milled rice seeds.

Discussion

Kalanamak, a non-basmati aromatic cultivar is one of the most prized rice varieties of Asia. Most of scented rice varieties have several undesirable traits such as lodging, low yield, vulnerability to pests and diseases[15]. These characteristics of Kalanamak rice reduce its agronomic value. The main reason for the reduction in yield of Kalanamak rice is its tall stature that causes lodging. The tall stature also limits optimum use of nitrogen fertilizer and causes problems in mechanical harvesting. Tall stature increases respiration, reduces translocation and causes chlorosis, which affect plant growth and development[16]. Semi-dwarf rice varieties can evade the damage caused by wind and rain due to their short height[17].

Therefore, there is a need to introgress semi-dwarfing gene into Kalanamak rice to overcome yield loss without losing its aromatic characteristics. In the present study, sd_1 gene from CSR10 was transferred in recurrent parent Kalanamak with the help of marker-assisted backcross breeding. Conventional breeding is more time-consuming and selection is based on phenotypic data, which require more number of plants[17]. Presently, marker-assisted selection (MAS) is based on the selection of plants with the help of molecular markers. These molecular markers reduce the selection cycle and are also precise and robust in nature. MAS selection requires screening of a small population size, thus ultimately reducing the time involved. Pyramiding of many target traits into the same cultivar is possible by MAS. There are many successful examples in the rice breeding programme, where MAS has been used for the improvement of agronomic traits as well as for disease resistance[18–25], for semi-dwarfing traits[26]. Basavaraj *et al.*[27] successfully improved parental lines of hybrid Pusa RH10 with the help of marker-assisted backcross breeding (MABB) using foreground selection and background selection. They reported that in two backcross generations, recurrent parent genome was recovered up to 97.30%. Several studies in support of MABB such as biotic stress, abiotic stress and semi-dwarfing genes in rice have been successfully performed[28–33]. Genetic basis of plant height in rice can be well understood by partitioning plant height into panicle length and length of upper internodes of rice[34]. In rice, molecular mapping of sd_1 gene has been reported[35] which is located on chromosome 1. For the selection of backcross plants having semi-dwarf allele, SD1 primer pair was used[2]. Our results are consistent with previous study of sd_1 allele transfer[36]. In an earlier study, gene for aroma along with disease resistance and submergence tolerance has been also transferred[37]. Earlier sd_1 gene was used as semi-dwarfing character in rice through breeding programmes[38,39]. Phenotyping for plant height was done at late developmental stage that reduce errors in the experiment for plant height observation[2]. Kovi *et al.*[40] also developed backcross population by making crosses between Zhenshan 97 and Pokkali, for the transfer of semi-dwarfing gene where Zhenshan 97 was used as the recurrent parent. Rajpurohit *et al.*[11] also pyramided three genes, two genes for bacterial blight resistance genes (*xa21* and *xa13*) and a semi-dwarfing gene (sd_1) in type 3 Basmati from a rice cultivar PR106-P2 using MAS. In the present study, we successfully introduced sd_1 gene into the Kalanamak rice.

Aroma is a key trait in scented rice due to BADH (betaine aldehyde)[41–43]. In rice, BADH has two homologs, BADH1 and BADH2, both having different expression patterns. While the expression of BADH2 is constitutive, that of BADH1 is not. BADH2 has the greatest affinity for 4-aminobutyraldehyde, which can spontaneously cyclize to form Δ1-pyrroline from which the fragrant compound 2-AP is formed in rice. Accumulation of *ν*-guanidinobutyraldehyde (GGBald) results in the formation of 2-AP in fragrant rice[41]. In the present study aroma analysis in the introgressed line (BC_3F_3) was carried out by aroma (*badh2*) gene specific primer coupled with sensory analysis test. The *fgr* gene is located on the long arm of chromosome 8, and codes for the enzyme BADH, that also renders the *badh2* gene product nonfunctional and leads to synthesis of 2-AP. Fragrance in rice appears to be due to the accumulation of 4-aminobutyraldehyde/Δ1-pyrroline, which is a precursor of 2-AP[41]. Aroma-specific, primer (badh2)-amplified 390 bp bands in BC_3F_3 generation were selected for sensory analysis test and in most positive backcross generation, plant scoares were 2 and in some cases 3. The above results indicate the presence of *badh* gene, which is responsible for the expression of 2-AP and finally leads to fragrance in rice[44].

Conclusion

In the present study we introgress the semi-dwarf gene (sd_1) into the background of Kalanamak rice with the help of marker assisted breeding to minimize the losses due to lodging. The improved lines are short in height, either on par or superior in agronomic performance, grain and cooking quality traits compared to the recurrent parent Kalanamak rice. Further improved semi-dwarf Kalanamak rice variety will help farmers in reducing losses due to lodging. The improved lines will also be valuable as donors for semi-dwarfing gene for the pyramiding of semi-dwarfing character in aromatic rice breeding programmes.

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