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ENHANCEMENT OF FLOOD TOLERANCE IN A HIGH YIELDING RICE VARIETY 'AMARA' BY MARKER ASSISTED SELECTION

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SUMMARY

Rice crops in coastal irrigated lowland areas are adversely affected by flash flooding or stagnant flooding. The challenges facing rice production in these areas are becoming even more complicated due to climate change and poor drainage systems. Development of submergence tolerant rice varieties by incorporation of *Sub1A* into widely-adopted varieties using marker assisted backcross breeding would enhance the productivity in flood-prone areas. The aim of this study was to introgress *Sub1A* into the widely-adopted high-yielding variety Amara (MTU1064) using Swarna-Sub1 as the donor. The marker RM 464 was used as the foreground marker to select heterozygous alleles of donor segment of the *Sub1* locus up to the BC_2F_2 generation. Plants were selected by phenotypic screening of 2500 plants of BC_2F_2 generation by imposing artificial flooding at 15 days after transplanting for 10 days followed by stagnant flooding (20-50 cm depth of water) up to harvesting stage. Forty-eight families of BC_2F_3 generation exhibited high survival (> 75%) under flash floods cum stagnant flooding out of 120 families. Thirty-one single plants were selected from these 48 families using intragenic foreground markers. Recombinant selection of 16 plants using RM8300 (downstream of *Sub1*) and 14 plants with RM23788 (upstream of *Sub1)* was performed. The *Sub1* introgression lines of Amara performed well after flash flooding for 2 weeks followed by stagnant flooding during wet season of 2013. Marker assisted selection was successfully implemented to improve Amara (MTU1064) for flash flood tolerance and stagnant flooding to give sustainable productivity even under adverse climatic conditions provoked by recent trends of climatic change.

Key words: Rice, submergence tolerance, stagnant flooding, marker assisted selection

Key findings: Developed *Sub1* introgression lines of Amara (MTU1064) are performing well under both flash floods and stagnant flooding and these lines would give sustained yields under either type of flooded conditions in Andhra Pradesh. *Sub1* lines with moderate shoot elongation could survive sequential flash floods followed by prolonged stagnant flooding prevailing due to adverse effects of climate change.

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INTRODUCTION

Cultivation of rice in flood-prone areas constitute nearly 35% of the total rice area, covering 47 million ha in Asia and contributing only ~25% of global rice production, which is far behind the production of irrigated ecosystem. Rice is the staple food crop of India cultivated in an area of 40-42 M ha and contributes to 26% of global rice production (Ismail *et al*., 2012). Rice crops often suffer with many biotic and abiotic stresses resulting in low yields in India. Frequent floods are the major constraint in reducing productivity in 5 M ha of the cultivated area in India. Flood prone area in coastal Andhra Pradesh is about 1,200,000 ha and is increasing year by year due to unpredicted cyclonic rains being experiences since past 5 years. Research efforts on development of flood tolerant varieties unabated as rice crop is quite often prone to submerged condition from sowing to harvesting stage due to adverse effects of climate change. Recent climatic trends lead to flooding of rice followed by prolonged stagnant flooding (Mackill *et al*., 2010; Singh *et al*., 2011). *Sub1A-1* is a primary determinant of submergence tolerance (Xu *et al*., 2006) on chromosome 9 was introgressed into mega varieties like Swarna, Samba Mashuri, IR64, CR1009 (Neeraja *et al*., 2007; Septiningsih *et al*., 2009).

Sub1 versions of mega varieties tolerates flash floods up to 2 weeks by quiescence strategy but are vulnerable to stagnant flooding (30-50 cm water depth) and lodging of the crop. Varieties tolerating stagnant flooding, adapts escape strategy with elongation ability but does not suitable under flash floods (Reddy *et al.*, 2010; Singh *et al*., 2011). Longer-term partial or stagnant flooding is also common in low-lying areas hinders tillering and plant growth. This results in lodging and poor grain quality. Both flash floods and stagnant flooding situations are becoming quite common due to unpredicted cyclonic rains and the situation is further aggravated by ill drained condition resulting in substantial reduction in yield.

The 'ideotype' for most flood prone areas is submergence tolerance (survival under water) with partial elongating ability (Sarkar *et al*., 2009), particularly for areas where water stagnates in the field following complete submergence. To date 3 QTLs on chromosomes 1, 3 and 12 that regulate the shoot elongation have been identified (Hattori *et al*., 2007; 2008). Some rice genotypes grown in rainfed flood prone areas have both quiescence and elongation, the 2 opposite strategies work together by which rice adapts to short as well as long term flooding (Sarkar and Bhattacharjee, 2011).

The aim of this study was to incorporate Sub1A into adopted semi tall variety Amara (MTU1064) for improvement of submergence tolerance for flash floods and stagnant flooding.

MATERIALS AND METHODS

Generation of the advanced breeding lines

Amara (MTU 1064) rice variety developed from cross combination of PLA 1100/MTU 1010 by Andhra Pradesh Rice Research Institute, Maruteru of Acharya N G Ranga Agricultural University and it was released in the year 2009. This variety can tolerate 7-10 days flash floods at tillering stage and suitable for cultivation in low lying areas where stagnant flooding prevails. But it is vulnerable to flash floods of 2 weeks at seedling stage and tillering stage. The aim of this project was to generate breeding lines with Sub1 tolerating sequential flash floods followed by stagnant flooding by incorporating *Sub1A* from Swarna-Sub1 into Amara in the wet season of 2009. The generation of a backcross population started from dry season of 2009 by crossing F_1 with recurrent parent MTU1064. Two successive backcrosses were made and the material was advanced to study as advanced backcross population described in Figure 1.

DNA Isolation and PCR assay

Healthy leaf samples were collected at 40 days after sowing for DNA extraction. The total genomic DNA was isolated from the leaf samples of rice using the method described by (Zheng *et al*., 1995) with some modifications. The quality and quantity of DNA was estimated

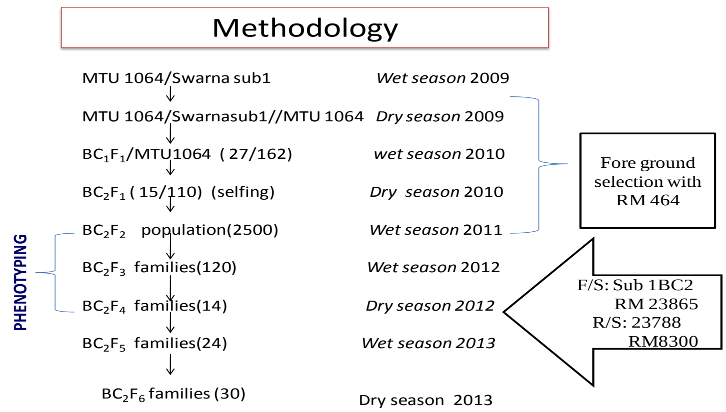

Figure 1. Schematic advanced backcrosses (R/S: Recombinant selection)

using ND 8000 eight-channel spectrophotometer. PCR reaction mixture comprises of 1 µl of 10X buffer with $MgCl_2$, 0.5 µl of dNTPs (2.5 m M. L^{-1}), 1 µl (5 µ molar) each of forward and reverse primers, 1 µl Taq DNA polymerase (0.5 U/µl), 3 µl of template DNA (10 ng/µl) and 2.5 µl of sterilized distilled water accounting to final volume of 10 µl. Amplification of microsatellite markers was performed using Eppendorf thermo cycler, with initial denaturation at 94 °C for 5 mins, followed by 35 cycles of denaturing at 94 °C for 0.5 min, annealing at 55 °C for 0.5 min, extension at 72 °C for 1.0 min and 7 min at 72 °C for the final extension . The PCR products were subjected to electrophoresis on 3% agarose gel stained with ethidium bromide (10 mg/ml) at 100 volts for 1.5 hr in 1X TBE buffer. A 100 bp ladder (Genei) was used for appropriate sizing of the products. The gel images were captured under UV light using Ingenius gel doc system.

Marker assisted selection

Marker assisted selection to introgress *Sub1* into MTU1064 was adopted using microsatellite markers. Parental polymorphism survey was carried out between MTU1064 and Swarana-Sub1 using markers saturated at Sub1 region of chromosome 9 and selected RM464, RM23865 and Sub1 BC2 as foreground markers. RM23788 and RM8300 were identified as recombinant markers on both sides of *Sub1* region for precise selection without linkage drag of donor parent. Fifty-seven polymorphic markers covering all the 12 chromosomes were selected for back ground selection to assess recovery of the recurring parent (Table 1). Graphical genotyping of advanced back cross families were performed using GGT 2.0 version (Van Berloo, 1999).

Phenotypic selection for flood tolerance

Phenotypic selection based on morphological characters of recurring parent MTU1064 after confirming the presence of *Sub1* was employed up to BC_1F_2 generation. Advanced backcross families from BC_2F_2 generation were transplanted in submergence pond along with recurring parent MTU1064 and donor Swarna-Sub1 to screen for flood tolerance at different stages as mentioned below. Each line was transplanted in 4 rows at spacing of 20 x 15 cm and both the parents were repeated for 4 times in augmented design.

Flash floods were imposed by complete submergence of advanced back cross families of BC_2F_2, BC_2F_3 and BC_2 F_4 generations for 10 days at 15 days after transplanting and submergence duration was prolonged to 14 days to screen BC_2F_5 and BC_2F_6 generations. Water depth of 40-50 cm was maintained in cement lined submergence pond immediately after recede of water from complete submergence (i.e., from 26 days after transplanting for BC_2F_2, BC_2F_3 and BC_2 F_4 and from 30 days after transplanting for BC_2F_5 and BC_2F_6 generations)

Table 1. List of SSR markers used for background selection.

Chromosome number	Number of markers used	Details of markers used
1	6	RM10270, RM094 RM212, RM5919, RM246,RM6141
2	4	RM13616,RM3858,RM497, RM20
3	4	RM14482, RM168, RM1350 RM4404
4	4	RM307, RM335, RM 8213, RM303
5	2	RM169, RM 163
6	6	RM225, RM549, RM 3, RM 400, RM 20634, RM439
7	4	RM 6097, RM180, RM 320, RM 5720
8	3	RM6925, RM5432, RM149
9	10	RM 23778, RM23788, RM23805, RM23865, Sub1BC2,RM23877, RM 23887, RM 8300, RM219, RM 566
10	5	RM 216, RM 5708, RM 304, RM6100, RM 477
11	4	RM 286, RM229, RM 206, RM 5766
12	5	RM 2529, RM 2854, RM 12, RM 1227, RM 2197
Total	57	

Table 2. List Summary results of Sub1 confirmed advanced back cross lines of BC_2F_6 generation during dry season of 2013-14.

Advanced breeding line	Flowering time	Plant height (cm)	Number of ear bearing tillers per plant	Panicle length (cm)	Grain yield per plant (g)	% of genome recovery of recurring parent MTU1064
2244-47-15-6-77	109	117.8	10	28.1	27.3	84.3
2244-119-59-63-40	110	114.8	11	27.9	22.6	94.6
2244-39-20-59-2	118	124.4	11	29.7	26.1	84.4
MTU1064	114	116.8	9	29	22	
Swarna-Sub1	106	113.8	11	27	13.6	

up to the harvesting stage. This method is followed to screen *Sub1* introgression advanced back cross lines for both flash floods and stagnant flooding as Swarna-Sub1 tolerates flash floods only and recurring parent MTU1064 tolerates stagnant flooding.

Plant survival (%) in each generation was recorded at 10 days after de-submergence to assess plant recovery. Plant height was measured from 5 randomly selected plants in each family before submergence and immediately after de-submergence. Total shoot elongation under submergence was calculated by subtracting plant height before submergence from plant height after de-submergence. Plant survival (%) more than 75% and moderate shoot elongation (20-30 cm) were used as criteria to select advanced generation families tolerating both floods for genotyping as per Toojinda *et al*. (2003). Screening of advanced lines at 14 days after sowing for 14 days of complete submergence in trays was adopted for identification of lines with submergence tolerance at seedling stage.

Data on flowering time, plant height, number of ear bearing tillers per plant and grain yield per plant were collected on 5 randomly selected plants in each advanced back cross families of BC_2F_5 and BC_2F_6 generations (Table 2).

RESULTS AND DISCUSSION

Foreground selection for *Sub1* in BC_1F_1 generation was performed using RM464 a *Sub1* linked marker and 27 plants were selected out of 162 plants genotyped. These selected 27 plants were used to generate BC_2F_1 population and fifteen single plants with Sub1 from BC_2F_1were selected using RM464 out of 110 plants genotyped in the year 2010. Thus foreground and phenotypic selection was employed to generate advanced back cross families up to BC_2F_1 generation by using fore ground marker RM464 at 7.2 Mb region on chromosome 9 and phenotypic characters of recurring parent MTU1064. One hundred twenty single plants were selected by phenotypic screening of 2500 population of BC_2F_2 generation by complete submergence at 15 days after transplanting for 10 days followed by stagnant flooding (30-50 cm depth of water) up to harvesting stage during wet season of 2011 to select advanced back cross line tolerating both flash floods and stagnant flooding.

The *Sub1* gene confirming flash flood tolerance adapts quiescence strategy with minimal shoot elongation and Sub1 versions like Swarna-Sub1 are unable to survive under stagnant flooding (Reddy *et al.*, 2010). To select genotype surviving under flash floods cum stagnant flooding, there is need to identify plants having *Sub1* coupled with moderate shoot elongation. To select such plants, 120 BC_2F_3 families evaluated under complete submergence followed by stagnant flooding during wet season of 2012. Evaluated advanced back cross families of BC_2F_3 generation exhibited wide range of variation for plant survival % (0 to 100) with mean of 88.5 and total shoot elongation under submergence (20-48.6 cm) with a mean of 29.0 (Table 3). Forty eight families of BC_2F_3 generation exhibiting plant survival percentage ranging from 75 to 100 and total shoot elongation ranging from 20-30 cm were selected for genotyping to identify plants with Sub1 gene possessing moderate shoot elongation. Selected 31 single plants from the BC_2F_3 generation having *Sub1* gene out of 5280 plants genotyped using both *Sub1* BC2 and RM23865 as foreground markers (Figure 2). Recombinant selection of 16 plants using RM8300 (6.6 Mb) at downstream and 14 plants with RM23788 (4.2 Mb) at up stream of Sub1A helped in precise selection for *Sub1A* region. These selected single plants were screened for submergence at seedling stage at 14 days after sowing for confirmation of flood tolerance as depicted in Figure 3.

Table 3. Summary of 120 BC_2F_3 families evaluated in wet season of 2012.

	Plant survival (%)	Total shoot elongation cm
Mean	88.5	29.0
Range	0-100.0	20-48.6
MTU1064	66.2	23.6
Swarna-Sub1	0	0

Figure 2. Survived families of BC_2F_4 generation after14 days of submergence at 14 days seedling stage in comparison with lodged recurrent parent (MTU 1064) and donor (Swarna-Sub1).

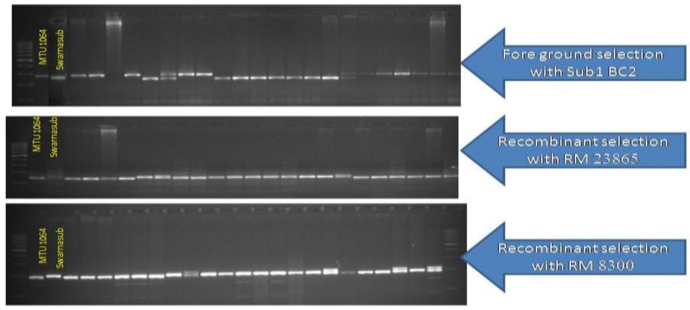

Figure 3. Gel images of BC_2F_3 families selected by fore ground and recombinant selection during the wet season of 2012.

In dry season of 2012, selected 14 families of BC_2F_4 generation were phenotypically screened for flood tolerance and back ground selection using 57 polymorphic markers covering 12 chromosomes was performed besides foreground and recombinant selections. Two BC_2F_4 lines 2244-39-20 (95.6%) and 2244-119-83 (91.3%) showed higher plant survival (%) than corresponding recurring parent MTU1064 (68.0%) and donor Swarna-Sub1 (36.0%) under 10 days complete submergence at 15 days after transplanting coupled with stagnant flooding up to 50 cm water depth till harvesting stage were identified during dry season of 2012 (Table 4). Lower plant survival (%) of Swarna-Sub1 was observed as it does not tolerate stagnant flooding immediately after recede of water from complete submergence. These results showed enhanced flood tolerance of *Sub1* introgression lines of MTU1064 for both flash floods and stagnant flooding due to presence of Sub1 from donor and favorable alleles of stagnant flooding from recurring parent. Graphical genotyping of these families revealed percentage of genome recovery of MTU1064 as 84.4% and 81.0% to the advanced lines 2244-39-20 and 2244-119-83 respectively. Selected 24 single plants out of the 7 families pertaining to BC_2F_4 generation based on foreground, recombinant and phenotypic selection under flash floods cum stagnant flooding situation were advanced to study BC_2F_5 generation.

Four developed breeding lines of MTU1064 with *Sub1* exhibited higher plant survival (%) more than 75% coupled with moderate shoot elongation and gave better yield than the recurring parent under flash floods followed by stagnant flooding during wet season of 2013 out of 24 lines tested (Table 5). Where flash floods imposed at 15 days after transplanting for 2 weeks followed by stagnant flooding of 30-40 cm water depth and natural flash floods were prevailed for 7 days at 70 days after transplanting. These results indicated that the developed breeding lines could sustain sequential floods leading to sustainable yields under adverse climatic conditions of recent trend of climate change.

Thirty single plants from the 4 families were studied in dry season of 2013-14 under normal condition for phenotypic characters and to assess recovery of genome through back ground selection. Results of back ground selection using graphical genotyping revealed that breeding line 2244-119-59-63-40-1 of BC_2F_6 generation exhibited 94.6% recovery of recurring parent with single plant yield on par with recurring parent. Graphical representation of the selected breeding line was depicted in Figure 4. Whereas advanced breeding lines 2244-47- 15-6-77 and 2244-20-59-2 exhibited higher yield than recurring parent with plant genome recovery of 84.3% and 84.4% respectively.

So far, *Sub1* introgression varieties like Swarna-Sub1, Samba Mahsuri-Sub1, IR64-Sub1 tolerates flash floods only and these Sub1 versions are vulnerable to stagnant flooding. Introgression of the Sub1 into moderately elongating varieties is one of the best strategies to develop climate resilient rice varieties tolerating sequential flash floods cum stagnant flooding.

Table 4. Summary results of selected BC_2F_4 lines during *dry season of* 2012-13 under flash floods followed by stagnant flooding.

S. No.	Code	Plant survival (%)	Total shoot elongation under submergence (cm)	% of genome recovery of MTU1064	No of plants selected with double recombinants
1	2244-39-2	70.2	25	82.9	-
2	2244-39-3	58.6	26.3	87.7	-
3	2244-39-4	40.5	20.6	80.7	-
4	2244-39-5	54.3	22.3	80.5	-
5	2244-39-6	55.1	26.3	76.9	-
6	2244-39-8	68.9	22.6	80.8	-
7	2244-39-10	70.6	23.6	87.8	3
8	2244-47-15	72.7	22	81.2	1
9	2244-39-19	62	22	82.3	1
10	2244-39-20	95.6	25	84.7	7
11	2244-119-10	75	31	75.5	1
12	2244-119-59	86.2	26.3	74.2	4
13	2244-119-83	91.3	22	81	7
14	2244-119-85	48.2	21.3	66.4	-
15	MTU 1064	68	24.3		
16	Swarna-Sub1	12	18.3		

Table 5. Summary results of Sub1 confirmed advanced breeding lines of BC_2F_5 generation during wet season of 2013 under flash floods followed by stagnant flooding.

Advanced breeding line	Plant survival (%)	Total shoot elongation (cm)	Flowering time	Plant height (cm)	Number of ear e tillers per plant	Grain yield per plant (g)
2244-47-15-6	75.7	37	138	114	13	60
2244-119-83-65	91.7	35	136	116.4	8.6	46
2244-119-59-63	75.8	28	137	110.8	13	37.4
2244-39-20-59	100	38.4	137	121.4	8.6	36
MTU1064	63.9	38.4	133	114	13.8	36
Swarna-Sub1	0	0	0	0	0	0

Genetic improvement of Amara (MTU1064) variety for flood tolerance resulted in enhancement of flash flood tolerance for 2 weeks followed by stagnant flooding besides seedling stage tolerance by incorporation of *Sub1* through marker assisted selection. Developed advanced breeding lines with Sub1 expressed higher plant survival, grain yield compared to recurring parent even under complete submergence for 2 weeks at vegetative stage followed by continuous stagnant flooding and flash floods at booting stage for 7 days were caused by successive cyclonic rains of 'Phailon', 'Helin' and 'Lehar' cyclones during wet season of 2013 in coastal Andhra Pradesh. Combining submergence tolerance and stagnant flooding tolerance would address flood intensified situation by incorporation of *Sub1* into cultivars with moderate elongation (Mackill *et al*., 2010; Vergara *et al*., 2014). Our developed breeding line of MTU1064 with *Sub1* possessing moderate shoot elongation could withstand sequential flash floods followed by stagnant flooding under adverse climatic conditions ultimately results in realization of sustainable productivity in flood prone areas.

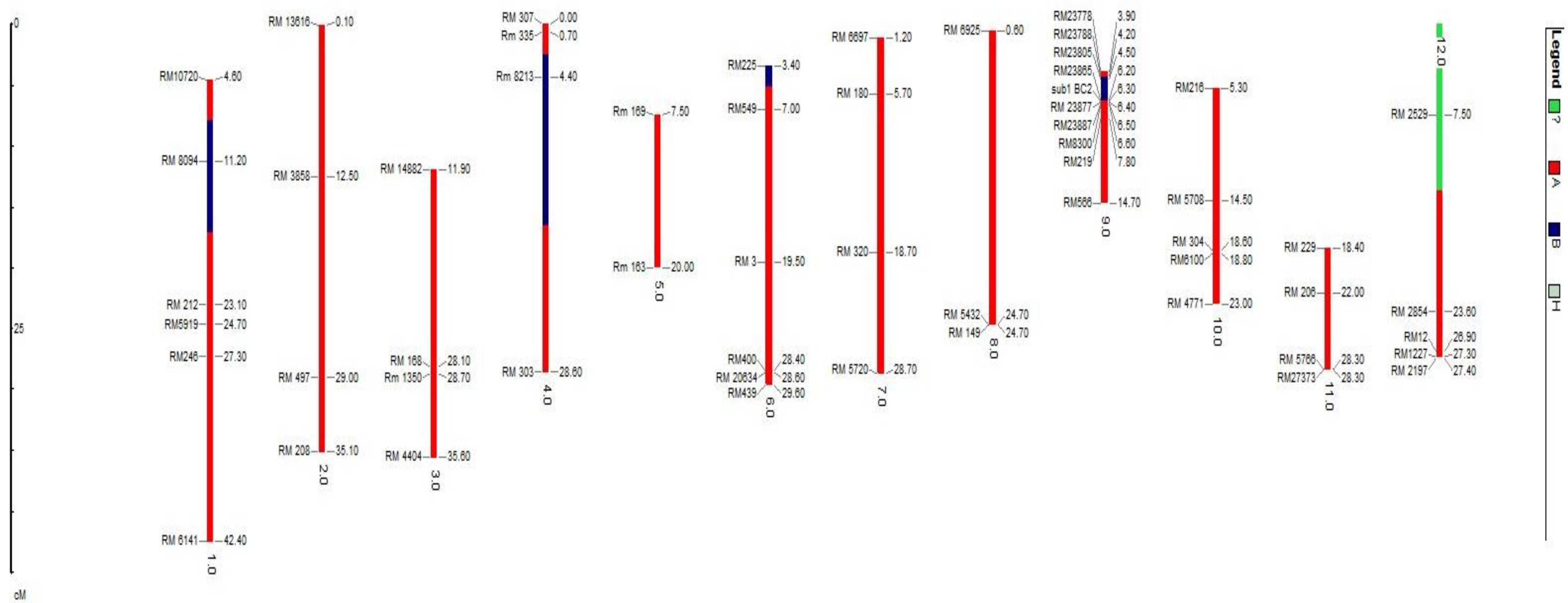

Figure 4. Graphical representation of developed *Sub1* introgression breeding line of MTU1064 at BC_2F_6 generation during dry season of 2013.

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