Development of new aromatic rice lines with high eating and cooking quality

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Abstract Rice is the staple food of about half of the world's population. Preferred by consumers, aromatic rice is special rice with great commercial value. Cooking and eating quality and aroma are major grain qualities favored by most consumers. Currently, most of the available aromatic varieties have low yield with some undesirable agronomic traits. Thus, there is an urgent need to develop better aromatic rice varieties. This work aims to identify rice germplasm lines having good grain quality and to develop new varieties with desirable traits. Thirty-six out of 188 germplasm lines were identified to have *betaine aldehyde dehydrogenase 2* (*badh2*) controlling aroma and were determined for their 2-acetyl-1-pyrroline (2AP) contents. Then, 17 of these lines were identified to have alleles for low amylose content and low gelatinization temperature, controlled by *waxy* and *starch synthase IIa* (*SSIIa*) respectively, suggesting that they were aromatic rice lines with high cooking and eating quality. A total of 158 F_7 recombinant inbred lines (RILs) generated from five crosses of selected germplasm lines were planted for phenotypic and yield observation, resulting in 27 F_8 RILs selected for yield evaluation and genotyping. Finally, four out of the seven F_9 aromatic RILs showed high yield, high 2AP production, and low amylose content, in agreement with their genotypes. The other 3 F_9 RILs were aromatic rice lines with high amylose content and high yield. Because consumers' preference for grain quality varies depending on regions and ethnic groups, the high-yielding aromatic RILs generated from this study can be used to increase yield of Thai rice and to raise market value and farm profit.

Keywords: germplasm, functional marker, aromatic rice, *badh2*, *waxy*, *SSIIa*, RILs[1]

1. Introduction

Rice is one of the most important food crops because it is the staple food of about half of the world's population. For Thailand, rice is our main food and also a major export product (Limjumroon 2017). Aromatic or fragrant rice is special rice sold at a premium price because it is preferred by consumers. Fragrance is an economically important grain quality of rice (Addison *et al*. 2020). Highly favored by consumers, aroma property is an important objective for rice breeding in several countries (Wang *et al*. 2010; Shao *et al*. 2011; He and Park 2015).

There are several desirable agronomic traits in rice including high grain quality, high yield, resistance to diseases and pests, and resistance to undesirable environmental factors. In general, aromatic rice varieties are susceptible to pests and diseases, low-yield, tall-statured and susceptible to lodging, and affected by some abiotic and biotic stresses (Ahn *et al*. 1992; Mathure *et al*. 2011). With demand for aromatic rice expected to rise in the future, and varieties available at the present time having low yield and undesirable agronomic traits, there is an urgent need for the development of better aromatic rice varieties.

The grain quality of rice affecting its acceptability by consumers can be sorted into two main groups; grain appearance and cooking and eating qualities. The appearance quality is determined by grain length, width, length-width ratio, and translucency of the endosperm. The cooking and eating quality traits include volume expansion, fluffiness, cooked kernel elongation, firmness/stickiness, gelatinization temperature, mouth feel and a pleasant aroma (Juliano and Villareal 1993; Amarawathi *et al.* 2008). Although consumers' preference for grain

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quality varies depending on regions and ethnic groups, cooking and eating quality and aroma are major grain qualities favored by most consumers.

Aroma of rice grain is a quality trait which directly affects the consumers' choice and marketability. It has been reported that 2-acetyl-1-pyrroline (2AP) was a major factor of the aromatic qualities in fragrant rice varieties due to a mutation in Betaine aldehyde dehydrogenase 2 (*BADH2*) (Buttery and Ling 1983; Bradbury *et al.* 2005; Bradbury *et al.* 2008). However, some aromatic rice varieties do not contain the mutated alleles of this gene, suggesting the presence of other genes responsible for their aroma (Fitzgerald *et al*. 2008). Aroma quality also depends on the cultivation process and environmental conditions. Environmental conditions such as temperature, relative humidity, moisture content and pH during flowering to maturity stages highly affect aroma quality. In addition, stress during cultivation, such as drought and salinity stress increase 2AP content in rice grains. Thus, rice aroma depends on both genetic and environmental factors (Itani *et al*. 2004; Mo *et al*. 2015; Prodhan and Shu 2020).

Starch is a key factor affecting cooking and eating quality of rice grain. Cooking and eating quality is largely determined by the starch structure of the endosperm, a major edible part of rice grains (Liu *et al.* 2019). Apparent amylose content (AAC) and gelatinization temperature (GT) are two main parameters used to evaluate the starch properties (Bao *et al*. 2006). AAC is a major factor controlling characteristics of post-cooked rice grain. Based on ACC contents, rice variety may be classified under five different classes including, waxy (0-2%), very low (3-9%), low (10-19%), intermediate (20-25%), and high amylose content (>26%) (Kumar *et al*. 1986). Amylose content in rice is controlled by granule-bound starch synthase encoded by the *Waxy* (*Wx*) gene (Nelson *et al.* 1962). Several alleles of *Wx* have been identified in rice accessions having different levels of amylose content (Liu *et al.* 2019). GT is a physical property of rice amylopectin affecting cooking time of milled rice grain measured as the alkali spreading value. The GT of rice flour is controlled by the *alk* locus, which has been co-mapped to the *starch synthase IIa (SSIIa)* locus (Bao *et al*. 2006).

Most of the grain quality traits are controlled by quantitative trait loci (QTLs), making it difficult for breeders to select desirable plants by conventional methods due to the lack of discrete phenotypic classes in the segregating progeny and tedious methodologies for quality testing. In addition, these traits are affected by environments (Amarawathi *et al.* 2008). Functional markers are developed from DNA polymorphisms within the genes that cause phenotypic trait variations (Kumar *et al*. 2012). Functional markers are directly linked to the allele of the target traits (Andersen and Lübberstedt 2003). Therefore, for marker-assisted breeding, functional markers are better than random DNA markers such as simple sequence repeat (SSR) and single nucleotide polymorphism (SNP). Several genes controlling desirable agronomic traits such as fragrance, high eating and cooking quality, high yield, and biotic and abiotic resistances were used as functional markers for rice breeding programs (Wanchana *et al.* 2003; Bradbury *et al*. 2005; Bao *et al*. 2006; Ingvardsen *et al.* 2008; Ji *et al*. 2010; Jin *et al.* 2010; Gao *et al*. 2012; Kim *et al.* 2016).

Germplasms are important resources for crop breeding. Rice germplasm with important agronomic traits is crucial for development of new varieties with better traits. However, it is difficult to identify varieties with several desirable agronomic traits. Functional markers can be used to facilitate identification of rice germplasm having positive alleles of genes controlling target traits. These germplasms can be used for development of better varieties. In addition, genotyping with genes controlling desirable agronomic traits can be used for genetic diversity, enabling breeders for efficient utilization of germplasm resources and effective breeding systems (Bora *et al.* 2016).

This present work identified rice germplasms having good grain quality using functional markers controlling eating and cooking quality traits. Some of these germplasms were used to develop RILs which were planted in fields for yield and grain quality evaluations. The resulting new aromatic varieties with high quality and yield could be used to raise market value and farm profit.

2. Materials and methods

2.1. Plant materials

A total of 188 rice accessions including Thai and exotic rice genotypes (Appendix A) were used for genotyping using a functional marker, *badh2*, controlling fragrance. The Thai accessions including upland and low land rice genotypes were collected nationwide. Most of the germplasm were obtained from Genebank of the Department of Agriculture, National Plant Genetic Resources Center, Thailand. Some were obtained from Thai farmers. The others were the exotic rice genotypes obtained from the International Rice Research Institute (IRRI). The details of each accession are described in Appendix A. For genotyping, 10-15 seeds of each accession were grown in a greenhouse at National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Khlong Luang, Pathum Thani, Thailand. First, these plants were used for genotyping to identify accessions having a positive allele for *badh2*. Then, the selected rice accessions were used for genotyping with the other functional markers including *waxy*, and *SSIIa* .

For development of fragrant rice lines with desirable agronomic traits, the four fragrant accessions; PTT1, Pin Ka sat3 (Pin3), Hawm nin-132, and Azucena were crossed with 3 non-fragrant rice lines; Koshihikari, Nipponbare, and SPR91062-5-PTT-1-2-1 (B11) to produce F_1 from five crosses. PTT1 and Pin3 are developed *indica* Thai fragrant elite rice lines (http://www.riceland.co.th>engpathum, https://www.thairicedb.com/rice-detail.php?id=33). Hawm Nin-132 is an *indica* color rice lines having high nutrition value (http://www.gonkham.com/2018/pages/rice_homNil.php). Azucena is an aromatic tropical *japonica* rice with good cooking and eating quality (Lapitan *et al.* 2007). Koshihikari and Nipponbare are *japonica* varieties with high yield. In addition, Koshihikari is a rice variety with high eating quality and good adaptation to different environments, tolerance to pre-harvest sprouting, and cold tolerance during the booting stag (Kobayashi *et al.* 2018). B11 is Thai high-yielding breeding line. The F_1 plants from these crosses were planted and selfed to produce F_2 seeds. About 600-1000 F_2 seeds of each cross were planted at BIOTEC, or at Khlong Luang rice research center for selection. In each generation, 52 plants from each line were planted, and plants with desirable agronomic traits were selected and selfed, and kept selection up to F_7 generation. 158 F_7 recombinant inbred lines (RILs) (Appendix B) were planted for phenotypic and yield observation at Khlong Luang rice research center, dry season 2019. Then, 27 selected F_8 RILs were used for intra-station yield evaluation at Khlong Luang rice research center, wet season 2019. These selected F_8 RILs were planted in two experiments, Ex1 and Ex2, with 16 and 11 lines, respectively. These plants were genotyped using three functional markers: *badh2*, *waxy*, and *SSIIa*. Then, seven F_9 RILs were selected and planted for yield evaluation, genotyping using the three functional markers, 2AP determination, and phenotyping for grain quality traits including amylose content and gelatinization temperature. The procedures used in this study are presented in Fig. 1.

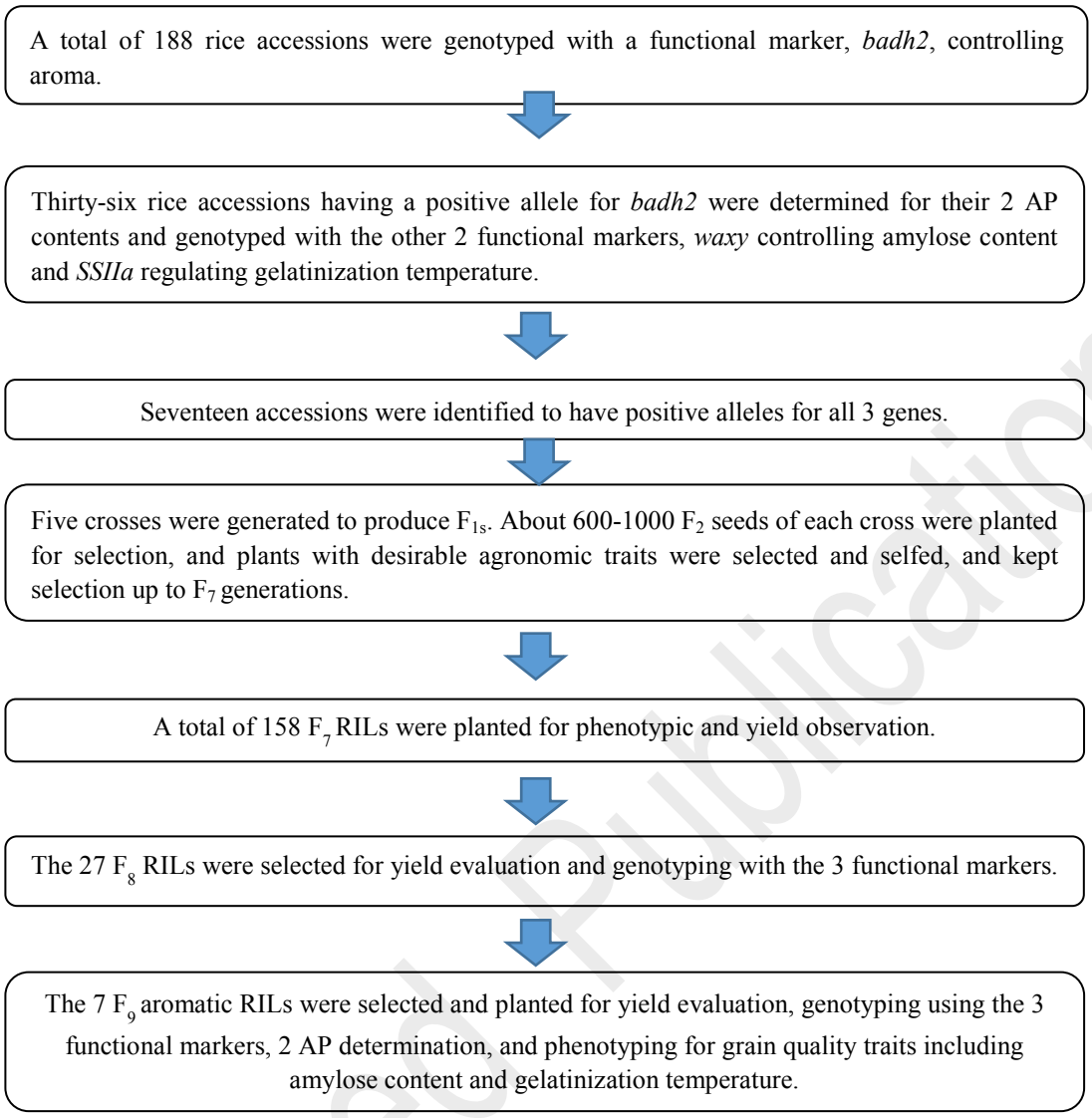

Fig. 1 Procedural scheme used in this study

2.2. Experimental design

For phenotypic and yield observation, the selected 158 F_7 RILs were grown one seedling per hill, at a hill spacing of 20.0 cm×20.0 cm at Khlong Luang rice research center in 2019. For each line, six rows, with 26 plants per row (6×26 plants) were planted. Harvesting areas were 4×24 plants inside each plot. The results obtained were used to select 27 F_8 RILs for yield evaluations in wet season, 2019, using 2 experiments (Ex) with 16 lines in Ex1 and 11 lines in Ex2. In each experiment, PTT1, a fragrant elite rice line, and RD 31, a high-yielding line, were included as standard check varieties. The results obtained were used to select 7 F_9 RILs having positive allele for *badh2* for yield evaluation in dry season, 2020, using PTT1 as a standard check variety. The experiments for yield evaluations in both years, 2019 and 2020, were conducted at Khlong Luang rice research center using a randomized complete block design with three replications, one seedling per hill, at a hill spacing of 20.0 cm×20.0 cm. For each replication, 6 rows, with 26 plants per row (6×26 plants) were grown for each line. For all experiments for yield evaluation, agronomic traits such as plant height, day to flowering, panicles per plant, grain number per panicle, spikelet fertility, and grain weight per plant were measured. Fertilizers (N-P-K), 6-20-0, 46-0-0, and 0-0-60 were added 187.5, 16.25, and 81.25 kg ha^{-1}, respectively at 2 days after transplanting, and 46-0-0 was added 81.25 kg ha^{-1} at 40 days after transplanting. Crop management was conducted according to the standard cultural practices. Fields were flooded at 5-10 cm depth until two weeks after flowering, when surface water was removed.

2.3. Sampling and measurements

Grain yields were measured from 96 plants (4×24) inside each plot and adjusted to standard moisture content of 0.14 g H_2O g^{-1}. Agronomic traits, including number of spikelets per panicle and spikelet fertility (100×filled spikelet number/total spikelet number, %) were determined from 10 plants of each plot, one panicle per plant. Numbers of panicles per plant were determined from active tillers of each plant from 10 plants. Plant height was measured from the plant base to the tip of the highest leaf or panicle using 10 plants. Data were analyzed using analysis of variance and means of varieties were multiple comparisons based on Duncan at the 0.05 probability level for each line using IBM® SPSS® Statistics version 26 software.

2.4. Molecular analysis

Genomic DNAs were isolated from fresh leaves using the Cetyl-trimethylammonium bromide (CTAB) method (Murray and Thompson 1980). To identify positive alleles for desirable agronomic traits, DNA samples from 10 individuals of each RIL were pooled and tested along with positive and negative DNA samples. Functional markers used were developed from *badh2*, *waxy*, and *SSIIa*, which are key genes controlling eating and cooking quality in rice. *Badh2* is a gene controlling aroma (Bradbury *et al*. 2005). Allele-specific amplification used 4 primers providing 580 bp as a positive control for each sample, and 355 and 257 bp for a non-fragrant allele and a fragrant allele, respectively, allowing simple analysis for an 8 bp deletion using agarose gels (Bradbury *et al*. 2005). *Waxy* is a gene influencing amylose content. A functional marker based on G/T SNP was designed. The amplification products of 228 and 425 bp were detected in genotypes having amylose content less than 15.6%, whereas only 425 bp control band were detected in genotypes having amylose content more than 20.1% (Gao *et al*. 2012). *SSIIa* is a gene associated with gelatinization temperature (GT), and GC/TT SNP was reported to differentiate rice lines with high or intermediate GT from those with low GT in about 90% of the cases. In addition, using 4 primers in a single PCR reaction, this marker can be surveyed on a large scale (Bao *et al.* 2006). Details of the tested primers were presented in Table S3 and PCR conditions were performed as previously reported (Tongmark *et al.* 2021). Genotyping of rice germplasm and RILs were conducted as indicated in Fig 1.

2.5. Phenotyping for grain quality traits

2-Acetyl-1-pyrroline in the headspace of rice were determined in the germplasm having positive allele for *badh2*, and in the F_9 RILS using SPME/GC-MS following the protocol previously described (Grimm *et al.* 2001). Rice samples (0.75 g) were weighed into 20 ml headspace vials. One hundred microliter (100 μL) of deionized (DI) water was added to the sample by pipetting onto the rice kernels. A total of 20 μL) of 2,4,6-Trimethylpyridine (TMP) at the concentration of 1 ppm were used as the internal standard. The vial was closed immediately using a metal screw cap with PTFE/white silicone septum. All sample preparation and injection were performed using the SPME tool on the PAL3 system (RSI model, CTA Analytics AG, Switzerland). The sample vial was incubated at 80°C for 25 min. Then, the headspace of the sample was extracted using the 50/30 μm DVB/Carboxen/PDMS SPME Fiber (Supelco, Bellefonte, PA) for 15 min at 80°C. The SPME was desorbed on a gas chromatography (7890D, Agilent, USA). The GC inlet was controlled at 270°C for 2 min in splitless mode . The GC oven temperature was held for 1 min at 50°C, and then ramped to 250°C at 10°C min^{-1}. Then, the temperature was ramped to 280°C at 20°C min^{-1}, and held for 10 min. A GC capillary column was DB-1MS (30 m×0.25 mm×0.25 μm) with helium as the carrier gas using a constant flow at 1 mL min^{-1}. The tandem mass spectrometer (GC-MS/MS, 7000D, Agilent, USA) was performed in multiple reaction monitoring (MRM) mode. Transfer line temperature, ion source temperature, and quadrupole temperature were set at 290, 240, and 180°C, respectively. For 2-acetyl-1-pyrroline (2-AP) and TMP, the precursor ions were 111 m/z and 121 m/z, respectively. The collision energy conditions of 111 m/z and 121 m/z were 15 and 30 eV, respectively. For 2-AP, a quantitative ion (Q1) was 83 m/z, and a qualitative ion (Q2) was 82 m/z. For TMP, a quantitative ion (Q1) was 79 m/z, and a qualitative ion (Q2) was 77 m/z. The authentic 2-AP standard was used to generate a calibration curve, normalized with TMP. Quantitative analysis was performed using Quantitate Mass Hunter Analysis (Agilent, USA).

Amylose content and alkali spreading value (ASV) were determined using the procedure of Juliano (1971) and Little (1958), respectively, with some modification as previously reported (Amarawathi *et al.* 2008).

3. Results

3.1. Identification of germplasm having positive alleles for grain quality traits and their 2AP contents

To identify rice genotypes having positive alleles for grain quality traits, first, the 188 rice accessions were genotyped with a functional marker controlling aroma, *badh2*. The results showed that 36 rice accessions had a positive allele for fragrance (Table 1; Appendix A). These rice accessions included both landrace and improved breeding lines. They were upland, low land, and some color rice lines. Then, the rice accessions having a positive allele for *badh2* were genotyped with the other two functional markers, *waxy* controlling amylose content and *SSIIa* regulating gelatinization temperature. The results showed that 17 rice accessions not only had a positive allele for *badh2* but also for *waxy* and *SSIIa*, suggesting that they are aromatic rice lines with high eating and cooking quality. A total of 14 out of the 17 were landraces, and the other three were improved breeding lines including PTT1, RD39, and Khao Jow Hawm Suphan Buri (Table1). In addition, 2AP was detected in most of these accessions. Several of them had high 2AP contents such as KDML105, 258, Hawm Nin-132, Hawm Nin-Plueak Khao Ton Khiao, Bueng Choo Bang Puey, Dam Lung, Azucena, and Pin Ka sat3 (Table 1). However, several of these accessions possessed undesirable agronomic traits, such as photosensitivity, late flowering, and tall plant type.

3.2. Development of aromatic rice lines having high eating and cooking qualities

To develop aromatic rice lines having high eating and cooking qualities, results from genotyping, phenotyping, and important agronomic traits of these germplasm lines were used to select plants used as parental lines. Most of the generated crosses were *japonica*×*indica* aiming for high heterosis resulting from inter-subspecies crosses. Five crosses; Koshihikari×Hawm Nin-132, Nipponbare×Hawm Nin-132, PTT1×Pin Ka sat3, B11×Azucena, and Azucena×Pin Ka sat3, were generated to produce five different F_1s. The resulting F_1 plants were planted and selfed to produce F_2 populations. These populations were planted and selected and kept selection to produce RILs having desirable agronomic traits. A total of 158 F_7 RILs were planted for phenotypic and yield observation at Klong Luang rice research center in dry season, 2019. The results showed that several lines have higher yield compared with high-yielding standard varieties (Appendix B). PTT1 and RD31 were used as high-yielding aromatic and non-aromatic standard varieties, respectively. Most of the RILs generated from the cross of Nipponbare×Hawm Nin-132 had higher grain weight per plants and higher seed setting rate compared to other RILs generated from *japonica*×*indica* crosses**.** Several RILs generated from PTT1×Pin Ka sat3, the only *indica* x *indica* cross, were selected up to this F_7 generation due to their important agronomic traits, such as yield and plant types. These plants had wide ranges of grain weight per plant and seed setting rate (Appendix B). The results from yield observation of the 158 F_7 RILs were used to select 27 F_8 RILs having high yield with desirable agronomic traits for yield evaluation in wet season, 2019. Two experiments, ex1 and ex2, with three replications each, were planted at Khlong luang rice research center. These plants were genotyped using functional markers for *badh2*, *waxy*, and *SSIIa*. The results showed that most of these lines have higher yields compared to high-yielding standard varieties. In addition, several of these lines had positive alleles for *bahd2*, *waxy*, and *SSIIa* (Tables 2 and 3).

To focus on the development of new aromatic rice lines with high yield and high grain quality, the results were used to select 7 F_9 IRLs having positive allele for *bahd2* from the three crosses for yield evaluation, phenotyping of grain quality, and genotyping. The selected lines must have at least the positive allele for *badh2* controlling fragrance. The yield evaluations were conducted at Khlong Luang rice research center in dry season, 2020. The results showed that all of the selected lines had higher yields compared to PTT1, an aromatic Thai elite line, a standard check variety. Most of them also had higher grain number per panicle, and higher grain weight than PTT1. In addition, most of them were taller than PTT1. However, several of them had lower seed setting rates (Table 4). Pictures of some RILs are presented in Fig. 2.

For phenotyping of grain quality of the seven F_9 RILs including determination of 2AP, amylose content, and ASV, the results showed that most of these lines had higher 2AP than that of PTT1. Four of them had low amylose content. The other three had high amylose content. Most of the RILs having low amylose content had similar ASV with PTT1 (Table 5). For genotyping of the seven F_9 RILs, all of the tested RILs had positive alleles for *badh2*, same as the results of their previous generation. All of the RILs having low amylose content had positive alleles for *waxy* and *SSIIa*, similar to PTT1. All of the three RILs having high amylose content had negative alleles for *waxy*, and two of the three also had negative alleles for *SSIIa*. Four of them had positive alleles for all three tested genes (Table 5; Fig. 3).

4. Discussion

Rice germplasm with important agronomic traits is critical for development of new varieties with better traits. Grain quality is a key breeding goal in rice due to high levels of consumer's demand and economic growth. In India, thirty-eight rice germplasm accessions were investigated, and germplasm having good grain quality and cooking properties were identified (Singh *et al.* 2012). In addition, using grain chemical analysis combined with genome-wide association study (GWAS), germplasm lines having desirable grain quality traits were identified from USDA rice mini-core collection representing the world wide rice germplasm lines (Song *et al.* 2019). Accordingly, in this study, using functional markers, 17 out of 188 germplasm lines were identified to have positive alleles for *badh2*, *waxy*, and *SSIIa* controlling fragrance, amylose content, and gelatinization temperature, respectively, suggesting that they were aromatic rice lines having high eating and cooking quality. Most of these rice accessions were landraces, and only three were improved breeding lines. Most of the landraces are photosensitive, except all accessions with the name starting with Hawm Nin. All the three improved breeding lines are photo-insensitive.

To develop high yield and high grain quality aromatic rice lines with agronomic desirable traits, some of these germplasm lines were used to produce RILs. It is generally believed that inter-subspecific crosses have stronger heterosis than intra-subspecific crosses (Fu *et al.* 2014), and *japonica* has better eating and cooking quality compared to *indica* (Sun *et al.* 2012). Therefore, most of the crosses generated were *indica-japonica* crosses. The RILs from these crosses were selfed and selected up to F_7 generation based on their yield and other important agronomic traits such as plant height, flowering time, and plant types. Then, yield observation and genotyping using functional genes controlling grain quality, including fragrance, amylose content, and gelatinization temperature, were used to select F_8 RIL lines. Finally, F_9 RILs lines having at least a positive allele for fragrance were used for yield evaluation, determination of 2AP, amylose content, and gelatinization temperature, and genotyping using the functional gene markers controlling cooking and eating quality.

In F_7 yield observation, RILs were selected from the five crosses. The number of selected RILs was determined by their performance in previous generations. Interestingly, most of the F_7 RILs generated from an inter-subspecific cross, Nipponbare×Hawm Nin132, showed higher yield compared to most of the other RILs and both of the high yielding standard varieties, particularly compared to F_7 RILs generated from Koshihikari×Hawm Nin132. In addition, they had higher grain number per plant, higher rate of seed setting, and 1000-grain weight, compared to most of the other RILs. Accordingly, F_8 RILs generated from this cross had higher yield. Similar results were reported that grain yield/plant was positively and significantly correlated with spikelet fertility (Pratap *et al.* 2018). Grain size, grain number per panicle, and panicle number per plant are major components of rice grain yield (Kim *et al.* 2016). Previous studies reported that seed sterility is a major problem in hybrids generated from *indica-japonica* crosses (Guo *et al.* 2016; Ouyang and Zhang 2018; Zhang 2020). However, this problem was not observed in the RILs generated from this cross. Unfortunately, results from genotyping indicated that these RILs did not have a positive allele for fragrance. Thus, they were not included further. However, these lines can be used in the breeding program for high-yielding rice.

F_7 RILs generated from PTT1×Pin Ka Sat3 showed a wide range of grain weight per plant and seed setting rate. Although PTT1 x Pin Ka Sat3 was an *indica-indica* cross, several of the RILs generated from this cross had high grain weight per plant, suggesting that they had high yield. Several of them were selected for yield evaluation and genotyping in F_8 generation.

All the selected F_9 RILs had positive alleles for fragrance. However, only four out of seven had positive alleles for all three tested genes. The results from genotyping were concordant with the results from the determination of 2AP, amylose content, and alkali spreading value (gelatinization temperature). Eating and cooking quality is mainly affected by amylose content, gel consistency, and gelatinization temperature (Tian *et al.* 2009; Nakata *et al.* 2018). In addition, cooked rice texture is affected by postharvest processing, the method of cooking, and the *indica*/*japonica* subspecies (Champagne *et al.* 1998; Li *et al*. 2016; Li and Gilbert 2018). Both of our F_9 RILs generated from Koshihikari×Hawm Nin, an *indica* x *japonica* cross, had negative alleles for *waxy* and *SSIIa*. Concordantly, they had high amylose content. The 4 F_9 RILs, 3 from *indica -indica* and 1 from *indica-japonica*, had high levels of 2AP, and low amylose content in agreement with their genotypes. However, quantity of aroma depends on genotype, environment, and interaction between genotype and environment (Gaur *et al.* 2016). Environment factors such as temperature, storage time, planting density, and harvest time affect quantity of aroma. In addition, soil type, abiotic stress, and milling process also affect quantity of aroma (Goufo *et al.* 2010; Hashemi *et al.* 2013). These factors should be considered for the production of aromatic rice.

All the selected F_9 RILs had higher 2AP contents and higher yield than PTT1, a standard check variety. All the 4 F_9 RILs having positive alleles for all three tested genes had similar days to flowering but had 7-21% higher yield than the standard check variety, PTT1. Similarly, previous study reported the development of an aromatic high-yielding basmatic rice variety having 7-15% higher yield than local existing varieties, Super Basmati and Basmati 515, respectively (Akhter *et al*. 2019). Some of the RILs had unexpected genotypes and phenotypes different from their parents. These could be due to contamination during cross-pollination to generate F1, or during selection processes. The resulting RILs were diverse, in terms of genotypes and phenotypes, including their yield and eating and cooking quality. Because aroma and eating and cooking quality depend on consumer preference, therefore the RILs generated from this study can be beneficial to various consumers and can be used to increase farm profits.

5. Conclusion

This study identified aromatic rice germplasm lines using a functional gene marker, *bahd2*. Several of them also had positive alleles for *waxy* and *SSIIa*, controlling amylose content, and gelatinization temperature, suggesting that they were aromatic rice with high cooking and eating quality. Some of these germplasm lines were used to produce RILs. Several of these RILs were aromatic rice lines with desirable agronomic traits, which could be benefit to farmers and consumers.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Table 1 Genotypes and 2AP contents of rice accessions having positive allele for *badh2*

No.	Line/variety	2AP (ppm)	Genotypes		
			badh2	*waxy*	*SSIIa*
1	Khao Niaw Dam	0.34 ± 0.08 ^{n}	P	P	P
2	Ruang Diaw	0.66 ± 0.04 $^{i-m}$	P	N	P
3	Gam	0.48 ± 0.04 $^{k-n}$	P	P	P
4	Non Rai	0.00 ± 0.01 ^{o}	P	N	P
5	Khao Ruang Yao	0.90 ± 0.16 $^{f-j}$	P	P	P
6	Niaw Dam Noi	0.49 ± 0.14 $^{k-n}$	P	P	P
7	Chao Kao	0.69 ± 0.06 $^{i-l}$	P	P	P
8	Niaw Di Tah Ke	1.11 ± 0.17 $^{d-g}$	P	P	P
9	Bueng Choo Bang Puey	1.29 ± 0.03 ^{cde}	P	N	N
10	Jao Tep Pah Rat	0.41 ± 0.03 ^{lmn}	P	N	N
11	Sai Rai	1.20 ± 0.04 $^{c-f}$	P	N	N
12	Dawk Pud Rai	0.83 ± 0.11 $^{g-j}$	P	N	N
13	Jao Tah Hin Ngom	1.10 ± 0.07 $^{d-g}$	P	N	N
14	Dam Lung	1.39 ± 0.04 ^{bcd}	P	N	H
15	Gam Noi	0.93 ± 0.31 $^{f-j}$	P	P	N
16	Hawm Nin-Plueak Muang	0.00 ± 0.00 ^{o}	P	P	P
17	Kahp Yao	0.00 ± 0.00 ^{o}	P	P	P
18	Gam Piak Khahw	0.65 ± 0.22 $^{j-m}$	P	P	N
19	Hawm Nin-Plueak Khao Ton Khiao	1.33 ± 0.25 ^{cde}	P	P	P
20	Hawm Nin-caps	1.05 ± 0.15 $^{e-h}$	P	P	P
21	Hawm Nin-132	1.42 ± 0.3 ^{abc}	P	P	P
22	Khao Niao Luam Pua	0.52 ± 0.02 $^{k-n}$	P	P	N
23	Jao Khao	0.96 ± 0.01 $^{f-i}$	P	N	P
24	Nahm Sa Gui	0.76 ± 0.11 $^{h-k}$	P	N	P
25	Lok Daeng Pat Ta nee	0.78 ± 0.08 $^{h-k}$	P	N	N
26	Khao Luang San Pa Tong	0.68 ± 0.04 $^{i-l}$	P	P	P
27	Dawk Pah Yawm	0.00 ± 0.00 ^{o}	P	N	P
28	KDML105	1.65 ± 0.48 ^{ab}	P	P	P
29	PTT1	0.64 ± 0.06 $^{j-m}$	P	P	P
30	Azucena	1.14 ± 0.17 $^{c-f}$	P	N	N
31	Pin Ka sat3	1.09 ± 0.12 $^{d-g}$	P	N	P
32	RD39	0.00 ± 0.00 ^{o}	P	P	P
33	Suphan Buri90	0.00 ± 0.00 ^{o}	P	N	P
34	Hawm Chon La sit	0.00 ± 0.00 ^{o}	P	P	N
35	258	1.68 ± 0.11 ^{a}	P	P	P
36	Khao Jow Hawm Suphan Buri	0.37 ± 0.01 ^{mn}	P	P	P
PCK	KDML105	1.65 ± 0.48 ^{ab}	P	P	P
NCK	RD31	0.01 ± 0.00 ^{o}	N	N	N

2AP, 2-acetyl-1-pyrroline content (ppm); N, negative-allele; P, positive-allele; PCK, positive-allele check variety; NCK, negative-allele check variety. Different of lowercase letters within the same column show significant difference at the 0.05 level.

Table 2 Intra-station yield evaluation, Ex1, of selected F_8 RILs at Khlong Luang rice research center, wet season, 2019, and their genotypes for *badh2*, *waxy* and *SSIIa*

Source	Parents	Height (cm)	50%DTF (days)	No. of panicle/ plant	Grain yield (kg/ha)	Genotypes		
						badh2	*waxy*	*SS IIa*
F_7-No. 9	Koshihikari×Hawm Nin-132	113 ± 5.9 ^{fgh}	91 ± 0.6 ^{abc}	11 ± 0.0	4924 ± 348.3 $^{d-h}$	P	N	N
F_7-No. 10	Koshihikari×Hawm Nin-132	115 ± 4.2 $^{d-g}$	91 ± 0.6 ^{abc}	11 ± 0.0	4678 ± 187.4 ^{i}	N	N	N
F_7-No. 19	Nipponbare×Hawm Nin-132	112 ± 4.6 ^{ghi}	82 ± 0.0 ^{g}	11 ± 0.6	5161 ± 91.5 $^{c-g}$	N	N	P
F_7-No. 21	Nipponbare×Hawm Nin-132	114 ± 2.5 $^{e-h}$	82 ± 0.0 ^{g}	10 ± 0.0	5257 ± 478.1 $^{b-f}$	N	N	P
F_7-No. 23	Nipponbare×Hawm Nin-132	113 ± 3.8 $^{f-i}$	82 ± 0.0 ^{g}	11 ± 0.6	5213 ± 286.5 $^{b-g}$	H	P	P
F_7-No. 25	Nipponbare×Hawm Nin-132	114 ± 1.7 $^{d-g}$	82 ± 0.0 ^{g}	10 ± 0.6	5820 ± 202.6 ^{bc}	N	N	P
F_7-No. 27	Nipponbare×Hawm Nin-132	115 ± 0.6 $^{d-g}$	82 ± 0.6 ^{g}	10 ± 0.6	5122 ± 323.1 $^{b-e}$	N	N	P
F_7-No. 28	Nipponbare×Hawm Nin-132	117 ± 1.0 $^{c-g}$	82 ± 0.0 ^{g}	10 ± 0.6	5032 ± 354.0 $^{d-h}$	N	N	P
F_7-No. 29	Nipponbare×Hawm Nin-132	115 ± 3.5 $^{d-g}$	82 ± 0.6 ^{g}	10 ± 0.6	5620 ± 207.9 ^{bcd}	N	N	P
F_7-No. 31	Nipponbare×Hawm Nin-132	118 ± 3.1 $^{b-f}$	82 ± 0.0 ^{g}	11 ± 0.6	5388 ± 359.9 $^{b-e}$	N	N	P
F_7-No. 59	PTT1×Pin Ka sat3	122 ± 3.2 ^{abc}	92 ± 0.0 ^{ab}	10 ± 0.6	6413 ± 854.2 ^{a}	P	N	H
F_7-No. 60	PTT1×Pin Ka sat3	116 ± 2.6 $^{d-g}$	91 ± 0.0 ^{bc}	11 ± 0.6	5965 ± 454.2 ^{b}	P	N	N
F_7-No. 94	PTT1×Pin Ka sat3	120 ± 0.6 ^{bcd}	89 ± 0.0 ^{e}	10 ± 0.0	4485 ± 90.2 ^{ghi}	P	P	P
F_7-No. 95	PTT1 x Pin Ka sat3	126 ± 1.5 ^{a}	92 ± 2.3 ^{a}	11 ± 0.0	5542 ± 450.4 ^{bcd}	P	P	P
F_7-No. 97	PTT1×Pin Ka sat3	119 ± 2.5 $^{b-e}$	91 ± 0.0 ^{bc}	11 ± 0.0	5493 ± 553.0 ^{bc}	P	P	P
F_7-No. 128	B11×Azucena	108 ± 2.1 ^{i}	83 ± 0.6 ^{g}	11 ± 0.6	4427 ± 336.6 $^{e-i}$	P	P	P
	PTT1 (PCK)	115 ± 1.2 $^{d-g}$	90 ± 0.6 ^{cd}	11 ± 0.0 ^{a}	4573 ± 457.9 $^{f-i}$	P	P	P
	RD31 (NCK)	124 ± 2.5 ^{ab}	89 ± 0.6 ^{de}	11 ± 0.0 ^{a}	4317 ± 176.7 ^{hi}	N	N	P

50% DTF, days to heading at 50% flowering; N, negative-allele; P, positive-allele; H, heterozygous (PCK), positive-allele check variety; (NCK), negative-allele check variety.

Different lowercase letters within the same column show significant difference at the 0.05 level.

Table 3 Intra-station yield evaluation, Ex2, of selected F_8 at Khlong Luang rice research center, wet season, 2019, and their genotyping using *badh2*, *waxy and SSIIa*

Source	Parents	Height (cm)	50% DTF (days)	No. of panicle/ plant	Grain yield (kg/ha)	Genotypes		
						badh2	*waxy*	*SSIIa*
F_7-No. 20	Nipponbare×Hawm Nin-132	117 ± 1.7 ^{b}	82 ± 0.6 ^{e}	11 ± 0.0 ^{bc}	6278 ± 33.2 ^{ab}	N	N	P
F_7-No. 22	Nipponbare×Hawm Nin-132	118 ± 1.7 ^{b}	82 ± 0.0 ^{e}	11 ± 0.6 ^{d}	6767 ± 34.6 ^{a}	N	N	P
F_7-No. 24	Nipponbare×Hawm Nin-132	117 ± 3.5 ^{b}	83 ± 0.6 ^{e}	10 ± 0.0 ^{cd}	5852 ± 434.9 ^{bc}	N	N	P
F_7-No. 26	Nipponbare×Hawm Nin-132	118 ± 3.2 ^{b}	83 ± 0.6 ^{e}	10 ± 0.6 ^{bc}	5788 ± 174.7 ^{bcd}	N	N	P
F_7-No. 30	Nipponbare×Hawm Nin-132	118 ± 2.3 ^{b}	83 ± 0.0 ^{e}	11 ± 0.6 ^{b}	6297 ± 408.7 ^{ab}	N	N	P
F_7-No. 32	Nipponbare×Hawm Nin-132	116 ± 2.3 ^{b}	82 ± 0.6 ^{e}	11 ± 0.0 ^{b}	6132 ± 334.8 ^{ab}	N	N	P
F_7-No. 93	PTT1×Pin Ka sat3	116 ± 2.3 ^{b}	89 ± 1.2 ^{c}	11 ± 0.0 ^{b}	5171 ± 269.9 ^{cde}	P	P	P
F_7-No. 96	PTT1×Pin Ka sat3	129 ± 2.3 ^{a}	91 ± 0.6 ^{ab}	11 ± 0.0 ^{b}	5756 ± 163.7 ^{bcd}	P	N	P
F_7-No. 100	PTT1×Pin Ka sat3	117 ± 1.5 ^{b}	92 ± 1.0 ^{a}	11 ± 0.0 ^{b}	4662 ± 621.2 ^{e}	P	N	P
F_7-No. 135	Azucena×Pin Ka sat3	116 ± 1.7 ^{b}	87 ± 1.2 ^{d}	11 ± 0.0 ^{b}	4838 ± 405.0 ^{e}	P	P	H
F_7-No. 13	Koshihikari×Hawm Nin-132	119 ± 0.0 ^{b}	91 ± 0.0 ^{ab}	11 ± 0.0 ^{b}	6292 ± 440.5 ^{ab}	P	N	N
	PTT1 (PCK)	118 ± 1.0 ^{b}	92 ± 0.0 ^{a}	12 ± 0.6 ^{a}	4991 ± 456.3 ^{de}	P	P	P
	RD31 (NPK)	125 ± 1.5 ^{a}	90 ± 0.6 ^{bc}	12 ± 0.0 ^{a}	4829 ± 576.6 ^{e}	N	N	N

50% DTF, days to heading at 50% flowering; N, negative-allele; P, positive-allele; H, heterozygous (PCK), positive-allele check variety; (NCK), negative-allele check variety.

Different lowercase letters within the same column show significant difference at the 0.05 level.

Table 4 Yield and yield components of selected F_9 RILs planted at Khlong Luang rice research center, dry season, 2020

No.	Parents	Height (cm)	50% DTF (days)	No. of panicle/ plant	No. of spikelet/ panicle	1000-Grain weight (g)	Spikelet fertility (%)	Grain yield (kg/ha)
1	Koshihikari x Hawm Nin-132	107 ± 2.1 c	93 ± 0.6 a	11 ± 1.0 ab	186 ± 20.8	25.64 ± 1.8 a	72.1 ± 8.9 d	5181 ± 100.7 b
2	PTT1×Pin Ka sat3	123 ± 2.6 a	92 ± 0.0 a	10 ± 0.6 b	193 ± 22.8	25.85 ± 0.4 a	81.1 ± 4.0 b	5367 ± 210.0 ab
3	PTT1×Pin Ka sat3	121 ± 2.5 a	92 ± 1.2 b	11 ± 0.6 b	196 ± 27.3	26.84 ± 1.2 a	71.6 ± 6.0 d	4865 ± 125.0 c
4	B11×Azucena	115 ± 3.5 b	91 ± 1.5 a	11 ± 0.6 ab	151 ± 13.5	26.01 ± 0.1 a	88.7 ± 4.9 a	5194 ± 69.9 b
5	PTT1×Pin Ka sat3	115 ± 1.2 b	92 ± 1.0 a	11 ± 0.6 b	189 ± 26.3	27.01 ± 0.6 a	76.0 ± 10.6	5486 ± 110.6 a
6	PTT1×Pin Ka sat3	126 ± 1.0 a	92 ± 1.0 a	11 ± 0.6 b	189 ± 21.2	26.82 ± 1.7 a	78.7 ± 4.8 c	5342 ± 143.2 ab
7	Koshihikari ×Hawm Nin-132	115 ± 3.2 b	89 ± 0.0 c	11 ± 0.0 ab	206 ± 25.4	24.63 ± 1.7 a	80.8 ± 6.1 b	5356 ± 180.9 ab
PTT1		107 ± 3.2 c	92 ± 1.2 a	12 ± 0.0 a	112 ± 18.9	24.22 ± 3.8 a	86.8 ± 5.5 a	4529 ± 112.0 d

50% DTF, days to heading at 50 %flowering, Different lowercase letters within the same column show significant differences at the 0.05 level.

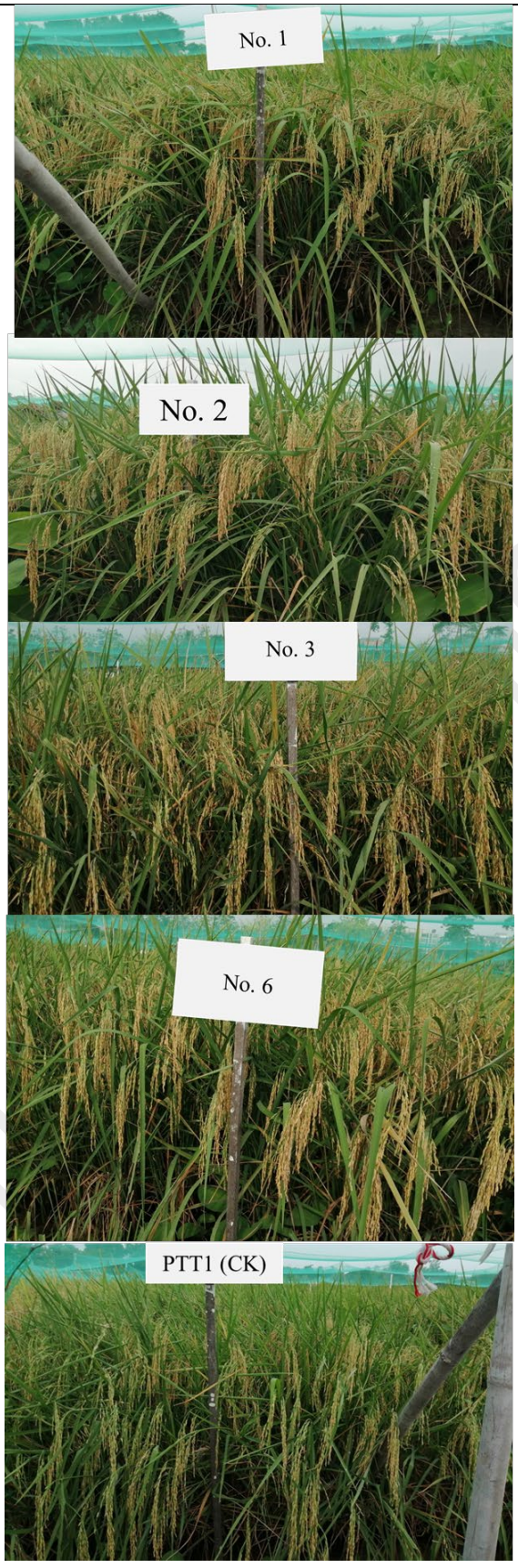

Fig. 2 Samples of the selected aromatic RILs and PTT1, an aromatic high-yielding standard variety.

Table 5 2AP, physicochemical Characterization, and genotyping using *badh2*, *waxy* , *SSIIa* of selected F_9 RILs planted at Khlong Luang rice research center, dry season, 2020

No.	Parents	2 AP (ppm)	Physicochemical Characterization		Genotypes		
			Amylose content (%)	ASV	*badh2*	*waxy*	*SSIIa*
1	Koshihikari×Hawm Nin-132	0.65 ± 0.10 ^{d}	28.46 ± 0.04 ^{a}	5.0 ± 0.00	P	N	N
2	PTT1×Pin Ka sat3	1.51 ± 0.70 ^{ab}	15.29 ± 0.01 ^{e}	7.0 ± 0.00	P	P	P
3	PTT1×Pin Ka sat3	1.29 ± 0.34 ^{abc}	15.16 ± 0.04 ^{f}	7.0 ± 0.00	P	P	P
4	B11×Azucena	1.60 ± 0.18 ^{a}	14.22 ± 0.04 ^{g}	7.0 ± 0.00	P	P	P
5	PTT1×Pin Ka sat3	1.26 ± 0.11 ^{abc}	16.71 ± 0.02 ^{d}	6.0 ± 0.00	P	P	P
6	PTT1×Pin Ka sat3	0.99 ± 0.27 ^{bcd}	26.31 ± 0.05 ^{c}	7.0 ± 0.00	P	N	P
7	Koshihikari×Hawm Nin-132	0.80 ± 0.16 ^{cd}	26.81 ± 0.01 ^{b}	5.0 ± 0.00	P	N	N
	PTT1 (CK)	0.64 ± 0.06 ^{d}	14.05 ± 0.01 ^{h}	7.0 ± 0.00	P	P	P

2AP, 2-acetyl-1-pyrroline content (ppm); ASV, alkali spreading value ; .N, negative-allele; P, positive-allele; (CK), check variety, Different lowercase letters within the same column show significant difference at the 0.05 level, ASV not significant at $p<0.05$.

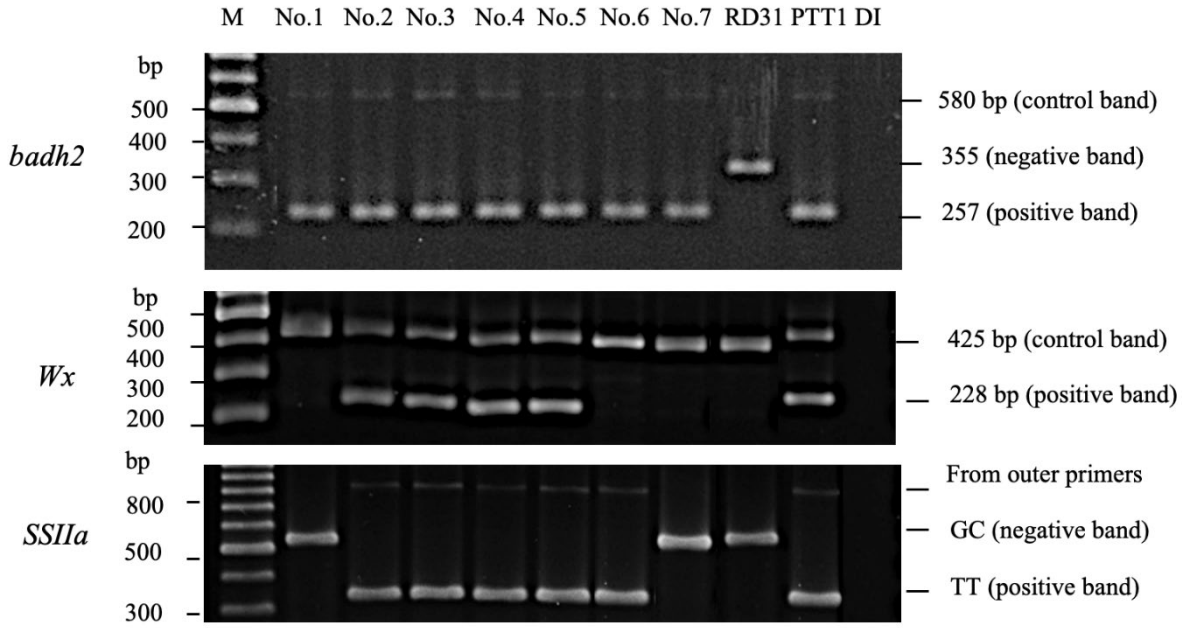

Fig. 3 Genotype of F_9 RILs planted at Khlong Luang rice research center, dry season, 2020. Banding patterns were detected using markers for *badh2*, *Wx* and *SSIIa*. M is 1000 bp Marker. DI is deionized water for negative control of PCR, and RD31 was used as negative control for *badh2*, *Wx* and *SSIIa* markers.