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Research Article

Gene Pyramiding for Brown Planthopper Resistance-related Traits, Early Maturity and Aroma of Rice Assisted by Molecular and Phenotypic Markers

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ABSTRACT

The brown planthopper (BPH)-resistant rice, early maturity, and aromatic are expected by both farmers and consumers. The traits have been combined through gene pyramiding and some promising rice genotypes obtained. However the genetic control of BPH resistance and maturity is quantitatively inherited, it is therefore both molecular and phenotypic assessments would be tremendously helpful in selecting promising genotypes. The study aimed to obtain genotypes with such valuable traits. Rice lines were analyzed using molecular markers i.e., RM586, RM589, RM8213 (BPH resistant gene markers); RM7610 and RM19414 (early maturity markers), and IFAP (Internal Fragrant Antisense Primer) for detecting aromatic, and INSP (Internal Non-fragrant Sense Primer) for non-aromatic rice. Phenotypic assessment was performed for brown planthopper resistant-related traits, such as chlorophyll content, stomatal conductance, and trichome density. Other evaluations were heading date and aroma (using 1.7% KOH solution). Results showed that molecular markers for evaluating BPH resistance genes (Bph3, Bph4, Qbph4, and Bph17), aroma (fgr gene), and heading date (Hd2 and Hd3 genes) could differentiate genotypes, and they serve as perfect markers, except for heading date markers. Seven genotypes i.e., #2, #3, #4, #5, #6, #10, and #11 were related to all traits expected based on molecular marker analysis. Meanwhile, genotypes #1, #2, #4, #6, and #11 were similar to their parents based on phenotypic analysis. Pyramiding program based on molecular and phenotypic markers enables us to combine three valuable traits into one rice genotype as presented in this study.

Keywords: Chlorophyll content; Rice; Stomatal conductance; Trichome density.

1. Introduction

Rice is a major staple food and strategic commodity which plays important roles to support food security in Indonesia and other Asian countries. The steady growing of human population (BPS, 2020) and economic prosperity may increase the demand for rice especially in Asian countries. In order to fulfil a variety of rice demand which is growing every year (0.3%; Yanuarti and Afsari, 2016), rice lines with superior traits should be developed. Gene pyramiding, an effort to combine many valuable traits derived from various superior genotypes into one genotype (Francis *et al*., 2013) is a promising approach to breed multiple traits.

Nowadays pyramiding program has been the main focus of rice genetic improvement in some laboratories. The ultimate goal of our gene pyramiding is to obtain rice that resistant to BPH, early maturity and also aromatic. From previous pyramiding effort, eleven promising genotypes were obtained from some hybridizations of PP51 (Pandanwangi/PTB-33) x CAKA283 (Ciapus/KA). Pandanwangi is well known aromatic cultivar in West Java, selected from rice lines from Cianjur, with good taste. PTB-33 is one of the most resistant genotype against brown planthopper, has *bph2*, *Bph3*, and QTLs (Jairin *et al*., 2007; Yadavalli *et al*. 2012). Ciapus is a high yielding cultivar, released in 2003. Meanwhile, Kitaake is japonica rice, originated from Japan, is neutral to photoperiod

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changes and has very short life cycle and as source of early maturity genes.

As far as we concern, BPH (*Nilaparvata lugens* Stal.) is a major important pest in rice (Wei *et al.*, 2009). BPH has a capacity in changing its biotype rapidly, because it has a large variation in their virulence genes, rice crop which previously resistant will be slowly turned to susceptible (Tanaka, 1999). Genetic analysis found that resistance to BPH is controlled by many genes. To date, 29 major BPH resistance genes have been known, however only four genes namely *Bph14*, *Bph26*, *Bph17* and *bph29* have been successfully cloned (Hu *et al.*, 2016). In addition, development of aromatic rice to meet demand for high-quality rice is considered necessary. Aromatic has been recognized as a high value-added trait (Cruz and Khush, 2000) and demand of aromatic rice is increasing nowadays. The fragrance of rice shows an important role in affecting the market value and consumers' preference. Other, the development of early maturity rice is highly demanded by farmers since it will save more time, provide opportunity to grow other rice crop, optimize use of land, and as an effective way to escape from drought or other abiotic stresses.

Considering the above effort, evaluation of pyramiding program is important to be done in order to find the right genotype(s) for the next step pyramiding programs. The evaluation can be conducted using phenotypic and genotypic analysis. Phenotypic evaluation needs to be supported by the use of molecular markers. Currently molecular marker is one of the technologies which is beneficial in the development of crop plants, for example, it can increase the reliability. In addition the application of molecular markers is not affected by environment, pleiotropic, cell type or tissue, plant growth stage, and the phenomenon of epistasis (Bahagiawati, 2012). Molecular marker technology assists the breeding process because pyramiding genes can speed up selection cycles process (Lan and Chao, 2011). Phenotypic and molecular markers evaluation for gene pyramiding in rice has been conducted for bacterial leaf blight (Chukwu *et al.*, 2019), eight grain yield-related QTLs (quantitative trait loci; Zong *et al.*, 2012), *cry1Ac* (insect resistance) and lysine rich protein (Liu *et al.*, 2016), blast, bacterial blight and BPH resistance genes in restorer lines (Ji *et al.*, 2016). Meanwhile, just recently QTLs for salinity, drought and submergence have been successfully pyramided into cv. Improved White Ponni (Muthu *et al.*, 2020). These pyramiding efforts are valuable for creating new cultivars. However, our efforts are different with the above-mentioned experiments. We were focusing on the development of rice genotypes having resistant to BPH, early maturity and aromatic which is expected to have a significant contribution to our farmers and consumers. Therefore an evaluation for these traits in the early generation is very essential to be done in order to find out the promising genotypes with three valuable traits for next breeding program.

2. Materials and Methods

2.1 Genetic materials

Eleven genotypes (genotypes ≠1 to #11) obtained from previous gene pyramiding program (Tambunan *et al.*, 2019) were used as genetic materials. Those genotypes were derived from hybridization between PP51 (Pandanwangi/PTB-33) X CAKA283 (Ciapus/Kitaake). PP51 and CAKA283 have been selected from hybridization between Pandanwangi (aromatic) x PTB33 (BPH resistant) and Ciapus (high yielding) x Kitaake (early maturity), respectively. Three parental genotypes (Kitaake, Pandanwangi, and PTB33) were also used as the check. The plants were properly grown and maintained in the field with sufficient water, fertilizers and pest and disease control according to integrated crop management (Abdulrachman *et al.*, 2013)

2.2 Molecular analysis

For molecular analysis, genomic DNA was isolated from young leaves using CTAB method (Dellaporta *et al.*, 1983) with a slight modification. The quality and quantity DNA were checked by using spectrophotometer (*Rayleigh* UV-9200). DNA quantity was measured at a wavelength of 260nm and 280nm in spectrophotometer. DNA amplification was done using PCR (polymerase chain reaction) machine (*Mastercycler Epgradient, Eppendorf*). The component for PCR reaction is template DNA (1µL), Forward and Reverse primers (each 1µL), Go Taq Green Master Mix (9.5µL) with total volume 12.5µL.

PCR product was electrophoresed using 1.5-3.0% agarose gel 0.5x TBE buffer at 75V for 70-90 min. Gel agarose was then immersed in 0,2µg/ml Ethidium-Bromide solution for 15-20 min. DNA visualization was performed using gel documentation system (G-Box, Syngene) and estimation of the DNA band size was aided using GeneTools (Syngene).

SSR markers i.e., RM586 and RM589 with PCR product 271bp and 186bp, respectively, were applied. These markers were linked to *Bph3* resistance gene (Jairin *et al.*, 2007) and also RM8213 with PCR product 177bp (Sun *et al.*, 2005). For detecting early maturity, RM7610

(Moeljopawiro *et al.*, 2010) and RM19414 (Anas and Carsono, 2010), with PCR product 133bp and 504bp, respectively, were employed. To detect fragrant rice, we applied IFAP (Internal Fragrant Antisense Primer) for detecting aromatic (257bp) and INSP (Internal Non-fragrant Sense Primer) for non-aromatic (355bp). These markers were applied for aromatic detection because the pattern result can be seen clearly (Bradbury *et al.*, 2005).

Molecular analysis was done by comparing the banding pattern of DNA on progeny with its parental descriptively.

2.3 Phenotypic evaluation

Measurement of phenotypic data was recorded on BPH resistant traits including: chlorophyll content (*Chlorophyll Content Meter* CCM200 Optisciences), stomata conductance (in mmol m^{-2} s^{-1}, *Leaf Porometer* from Decagon Devices Inc.) and trichome density (binocular microscope, model BM-180 SP). Two agronomic traits i.e., aromatic (sensory test) and heading date (days after transplanting) which represent early maturity were also recorded. Aroma was detected by sensory test using 1.7% KOH following Sood and Siddiq (1978). Six panellists were subjected to detect aroma. Scoring was employed from no aroma (0) to strong aroma (3). The genetic distance among genotypes with their parents was measured based on some phenotypic traits including chlorophyll content, stomata conductance and trichome density (BPH resistance related traits) using UPGMA (the Unweighted Pair-Group Method of Arithmetic Average) Cluster analysis in the NTSYS-PC program version 2.0.

3. Results

Based on DNA visualization, all three markers that are associated with the BPH resistance showed polymorphism as detected by GeneTool (Fig. 1, 2 and 3). Visualization of agarose gel electrophoresis using RM586 showed band size at 271bp, while using RM589 and RM8213, DNA fragment size at 186bp and 177bp were obtained, respectively. DNA fragment size of pyramided genotypes was also similar with that of PTB-33 as a BPH resistant donor parent.

Meanwhile, DNA patterns of 11 pyramided genotypes and their parents are presented in Fig. 4. All progenies were aromatic, as seen in Fig. 4 (from genotypes #1 to #11No contradiction between molecular testing (Fig. 4) and sensory test (Table 1) for all progenies, although with different intensity of aroma (from score 1.0 to 2.0), with a bit lower with cv. Pandanwangi (their parent).

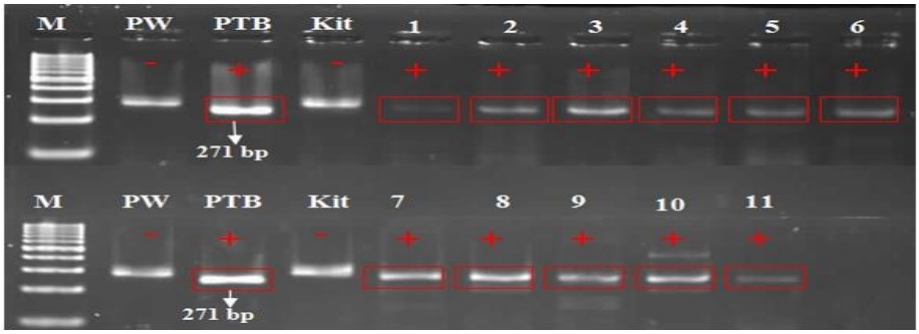


Fig. 1. DNA profile of RM586 SSR marker of pyramided genotypes along with their parents. M = 100 bp DNA Ladder; PW = Pandanwangi; Kit = Kitaake; ☐ = DNA fragment with 271bp size. + = Band pattern accordance with the target. - = DNA fragment not accordance with the target expected.

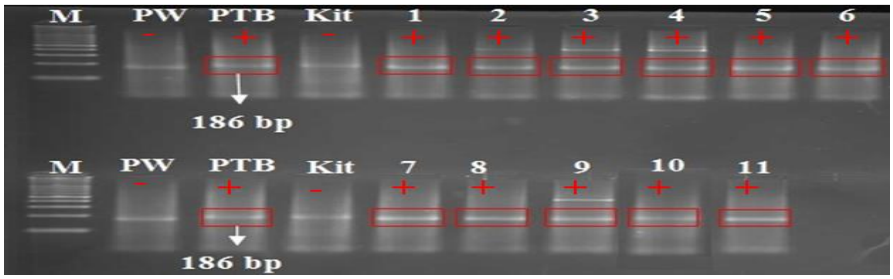


Fig. 2. DNA profile of RM589 SSR marker of pyramided genotypes along with their parents. M = 100bp DNA Ladder ; PW = Pandanwangi; Kit = Kitaake; ☐ = DNA fragment with 186bp size; - = Band pattern not accordance with the target expected.

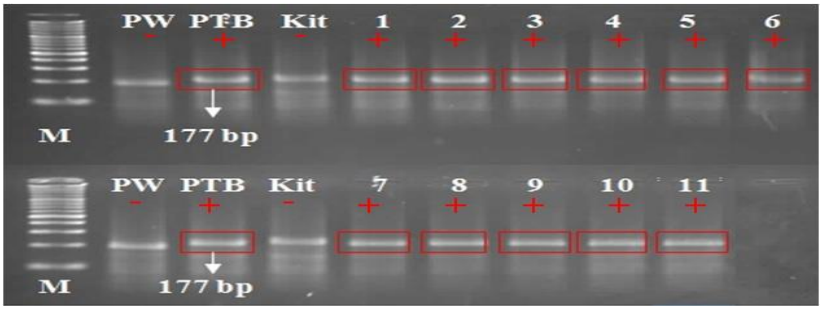


Fig. 3. DNA profile of RM8213 SSR marker of pyramided genotypes along with their parents. M = 100bp DNA Ladder; PW = Pandanwangi; Kit = Kitaake; ☐ = DNA fragment with 177bp size; - = Band pattern not accordance with the target expected.

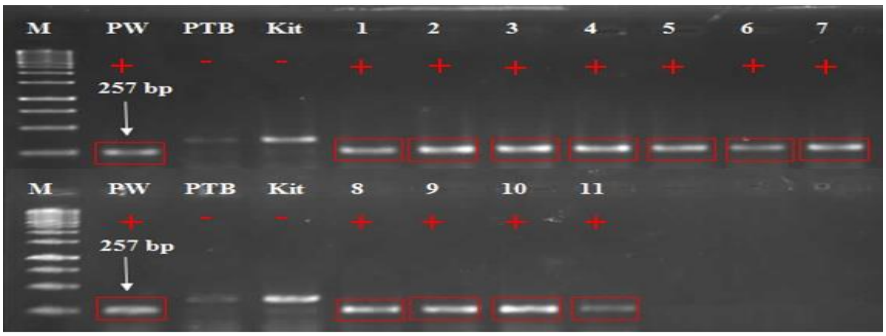


Fig. 4. DNA fragment of aromatic marker of pyramided genotypes along with their parents. Note: M = DNA ladder 1 kb; ☐ = DNA fragment with 257bp size. + = correspond to aromatic marker; - = not correspond to aromatic marker. PW= Pandanwangi, PTB= PTB33 and Kit= Kitaake.

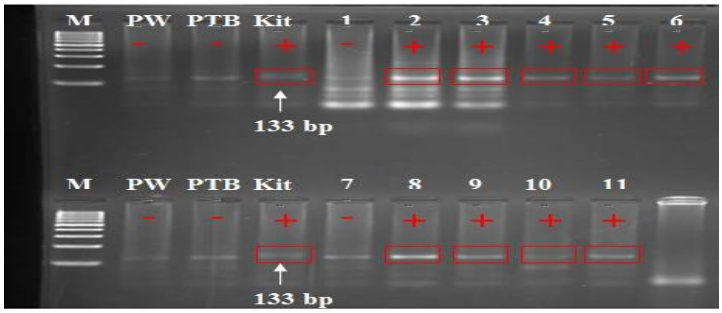


Fig. 5. DNA fragment of RM7601 SSR marker of pyramided genotypes along with their parents. M = 100bp DNA Ladder; PW = Pandanwangi; Kit = Kitaake; ☐ = DNA fragment with 133bp; + = correspond to aromatic marker; - = DNA fragment not correspond to the target expected.

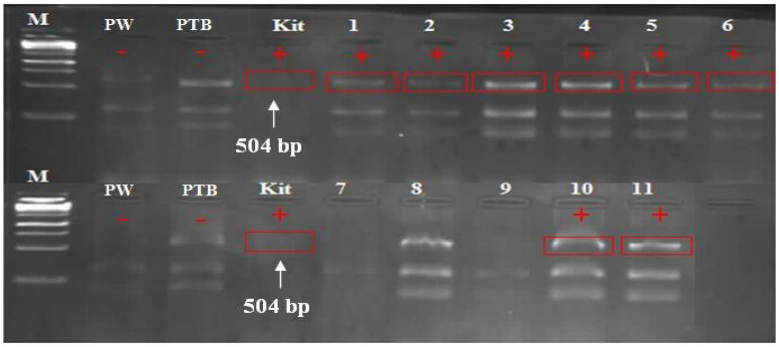


Fig. 6. DNA fragment of RM19414 SSR marker of pyramided genotypes along with their parents. M = 100bp DNA Ladder; PW = Pandanwangi; Kit = Kitaake; ☐ = DNA fragment with 504bp; + = DNA fragment correspond to the expected band; - = DNA fragment not correspond to the band expected.

Table 1. Evaluation of aroma compound using sensory test (1.7% KOH) on 11 pyramided genotypes and their parents

No.	Geno-type	Aromatic (+) or Non-aromatic (-)	Score	Category
1	#1	+	1.40	Aromatic
2	#2	+	2.00	Aromatic
3	#3	+	1.80	Aromatic
4	#4	+	1.40	Aromatic
5	#5	+	1.00	Aromatic
6	#6	+	1.60	Aromatic
7	#7	+	1.20	Aromatic
8	#8	+	1.80	Aromatic
9	#9	+	1.80	Aromatic
10	#10	+	1.20	Aromatic
11	#11	+	1.40	Aromatic
12	Pandan wangi	+	2.25	Aromatic
13	PTB33	-	0	Non-aromatic
14	Kitaake	-	0	Non-aromatic

Notes: + = aromatic rice; - = non-aromatic rice; score >1 = aromatic rice

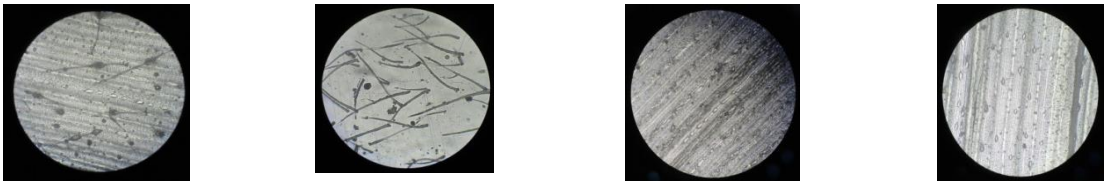
For heading date which has been well-known as quantitative trait with many genes involved (Wei *et al*., 2020), both molecular markers RM76021 and RM19414 could amplify DNA fragments which are similar with those of Kitaake. RM7601 (Moeljopawiro *et al*., 2010) was supposed to link with *Hd2* gene, meanwhile, RM19414 with *Hd3* gene *(*Anas and Carsono, 2010). Pyramided genotypes with similar DNA fragment size as Kitaake's fragment were genotypes #2, #3, #4, #5, #6, #8, #9, #10, #11 (Fig. 5). DNA amplification using RM19414 with PCR product size was 504bp was found on some genotypes #1, #2, #3, #4, #5, #6, #10, and #11 (Fig. 6).

PTB33 had chlorophyll content and trichome density was much higher than that of other genotypes (Table 2, Fig. 7). Meanwhile, stomata conductance of pyramided genotypes #1, #5, #6 and #11 was higher than those of progenies, and their parents (Table 2).

Table 2. Chlorophyll content, stomata conductance and density of trichome

Genotypes	**Chlorophyll content (CCI)**	**Stomata conductance (mmol m^{-2} s^{-1})**	**Trichome density**	**Aromatic with its score**	**Heading date (DAT)**
≠1	17.78	56.56	10	Aromatic (1.40)	79
≠2	13.47	37.11	9	Aromatic (2.00)	87
≠3	16.97	43.67	7	Aromatic (1.80)	80
≠4	14.36	37.44	9	Aromatic (1.40)	79
≠5	15.74	45.44	3	Aromatic (1.00)	85
≠6	14.03	53.33	6	Aromatic (1.60)	85
≠7	16.33	37.22	7	Aromatic (1.20)	88
≠8	16.40	34.78	1	Aromatic (1.80)	87
≠9	17.48	38.89	3	Aromatic (1.80)	92
≠10	15.68	37.56	1	Aromatic (1.20)	91
≠11	13.26	52.67	3	Aromatic (1.40)	94
Pandanwangi	12.34	36.73	0	Aromatic (2.25)	68
PTB33	19.92	45.33	20	Non-aromatic (0)	71
Kitaake	11.70	36.94	0	Non-aromatic (0)	31

Notes: CCI = Chlorophyll content index; mmol m^{-2} s^{-1} is the measure of the rate of passage of carbon dioxide (CO_2) entering, or water vapor exiting through the stomata of a leaf. DAT= days after transplanting.



(a) **(b)** **(c)** **(d)**

Fig 7. Trichome density of some rice genotypes (a) Genotype #6, (b) PTB-33 (BPH resistant) (c) Pandanwangi (BPH susceptible) (d) Kitaake (BPH susceptible).

4. Discussion

Evaluation of Pyramided Genotypes using Molecular Markers

Developing multiple traits in rice has been conducted by many researchers all over the world (Ji *et al.*, 2016; Lan and Chao, 2011). Improving rice resistant to BPH through introgression of BPH resistance genes derived from PTB33 has been applied in this experiment. BPH resistance genes found in PTB33 are *bph2* and *Bph3* (Santhanalakshmi *et al.*, 2010; Velusamy *et al.*, 2016), *Bph4* (Jairin *et al.*, 2007), *Bph32* (Ren *et al.*, 2016), *Bph4*, *Qbph4, Qbph17* (Nugaliyadde *et al.*, 2007), and supposed unknown other QTLs. PTB33 has been well-known as donor parent for BPH resistance in rice breeding (Jairin *et al.*, 2007; Ren *et al.*, 2016; Jiang *et al.*, 2018) and BPH resistant genes from PTB33 (derived from a hybridization of Pandanwangi x PTB33) have been inherited to other rice lines. In our cases BPH resistant genes of PTB33 have been transmitted to the all pyramided genotypes. This study is in accordance with Nugaliyadde *et al.* (2007) who found that the PTB33 had multiple BPH resistant genes with dominant trait inherited to the next generation (F_1 and F_2 progenies) from a hybridization between PTB33 and TN1 (Taichung Native 1). *Bph3, Bph4, Qbph4, Qbph17*, and *Bph32* were dominance genes found in PTB33. Some promising lines resulted from some research above would be beneficial, thus they will contribute to the genetic improvement of rice crop resistant to BPH.

For aroma assessment, it was found that all progenies had equal DNA fragment with cv. Pandanwangi which has been well-known as superior aromatic rice. PCR product produced by cv. Pandanwangi and 11 pyramided genotypes was 257bp, thus indicating as homozygous aromatic genotypes (Fig. 4), which is a bit far as we expected. It is supposed to be non-aromatic genotype (heterozygote constitution) since they were derived from a hybridization between aromatic rice PP51 (Pandanwangi/PTB33) x non-aromatic rice CAKA283 (Ciapus/Kitaake).

This genetic inheritance is surprising since eleven pyramided genotypes derived from hybridization between aromatic PP51 (Pandanwangi/PTB33) and non-aromatic CAKA283 (Ciapus/Kitaake). It is likely that aroma (derived from Pandanwangi) is not controlled by a single recessive gene. However some studies found that the aromatic trait is controlled by a single recessive gene (Sun *et al.*, 2008; Patil and Patil, 2012). But Fitzgerald *et al.* (2008) argued that any other *fgr* gene controlling aroma in rice. This study founds that about 15 genotypes of rice from South East Asia that are not associated with *fgr* allele but they were categorized as aromatic rice due to the accumulation of 2-AP compounds. One of the rice varieties tested was a local Indonesian rice cv. Pandanwangi, which used in this study. Based on this study it can be assumed that there are other allele that can accumulate 2-AP compounds or may other mutated gene drives the accumulation of the 2-AP. Our result found that all pyramided genotypes had aromatic compound as detected by KOH 1.7% (Table 1) and molecular markers developed by Bradbury *et al.* (2005), suggesting there is other allele controlling the aroma for the case of Pandanwangi as a parent (genetic background).

Other researchers found that aroma is controlled by QTLs, quantitative trait loci (Lorieux *et al.*, 1996; Pachauri *et al.*, 2010). Three QTLs have been mapped, viz. q*aro*3-1, q*aro*4-1 and q*aro*8-1 which is located on chromosome 3, 4 and 8, respectively. Many studied revealed that volatile compounds present in the leaf and rice grain, 2-acetyl-1-pyrolline (2-AP) is a key compound that presents in all aromatic rice cultivars (Pachauri *et al.*, 2010; Fitzgerald *et al.*, 2008). Other case, Sun *et al.* (2008) revealed that aromatic trait did not found on F_1 progeny from crosses between non-aromatic and aromatic genotype, but 3 : 1 (non-aroma versus aroma) segregation ratio observed in the F_2, indicating a recessive gene controlling aroma. This condition is not in accordance with our case, in which hybridization between aromatic rice versus non-aromatic one obtaining all homozygous aromatic rice. Pachauri *et al.* (2010) found a significant variation in the type and intensity of aroma in the different groups of aromatic rice varieties, suggesting involvement of additional chemical compounds in varying proportions.

Evaluation of pyramided genotypes by phenotypic characterization

Phenotypic evaluation found that all pyramided genotypes did not express heading date (flowering time) really similar with heading date of Kitaake, suggesting many genes controlling this quantitative trait (Hu *et al.*, 2015). *Hd2* and *Hd3* are major genes controlling the trait. Around 255 QTLs distributed widely across the Asian rice genome. 128 QTLs have been identified by previous study such as *Hd1*, *Hd6*, *Hd3a*, *Ghd7*, *DTH8* and *RFT1*, and other 127 QTLs were detected in different chromosomal regions than heading date genes (Hori *et al.*, 2015). Due to so many QTLs are involved for heading date, it is difficult to obtain rice genotype with heading date is very close to Kitaake.

According to finding in chlorophyll content and trichome density in which PTB33 had chlorophyll

content and trichome density was much higher than that of other genotypes (Table 2, Fig. 8). Watanabe and Kitagawa (2000) found that chlorophyll content, protein content of leaves and photosynthetic rate of two susceptible rice genotypes reduced when feeding to BPH. Changes in chlorophyll content may occur as a result of BPH infection since BPH is a sucking the phloem sap, thus rice leaf turn become yellow. However in our research, feeding to BPH is not conducted, but our data provide important insight of physiological characteristic of rice plant resistant to BPH.

In addition, Wang *et al*. (2008) proved that there was a very significant decrease in the chlorophyll content of rice genotype that susceptible to BPH, while it did not occur in resistant cultivars after invested by BPH. Presumably due to chlorophyll content in resistant cultivars is higher than that of the susceptible one physiologically. High chlorophyll content is likely to be able to support rice growth and development although rice plant has invested by BPH.

High chlorophyll content and trichome (pubescent leaves) density (Hu *et al*., 2013) may be beneficial as defense mechanism against BPH infestation. Genotypes #1, #3, #4, #5, #6, #7, #9 and #11 had chlorophyll content, stomata conductance and trichome density that inherited from PTB33, although their phenotype was not exactly similar with those of PTB33 since other genes/alleles derived from other parents (Pandanwangi, Ciapus and Kitaake). These lines need some backcrosses program to cv. Pandanwangi or Ciapus for accumulating favorable genes in one genotype. Hybridization to other superior genotype is also recommended in order to combine other valuable traits.

Furthermore trichome density was negatively correlated with BPH (Chandramani *et al*., 2009) suggesting the more the trichome density the lower the number of BPH number on rice plant. It is possible that trichome density may inhibit BPH's feeding behaviour. Resistance mechanisms based on the density of trichomes is an antixenosis resistance in which rice plant is not chosen by BPH as host for feeding, pecundity and development.

According to the data on Table 2 and Table 3, it can be seen that #1, #2, #4, #6, and #11 are genotypes that resemble to the PTB33. These genotypes will be further evaluated. Meanwhile, stomata conductance of pyramided genotypes #1, #5, #6 and #11 was higher than those of progenies, and their parents (Table 2), indicating the potential of these genotypes as resistant rice genotype particularly for BPH. It has been known that Nitric Oxide (NO), a compound that involved in many physiological processes including the opening and closing of stomata. NO levels can incline due to eating or sucking activity of BPH in rice. Increased levels of NO in plants may cause a decreasing in stomata conductance thus, consequently the presence of plant water loss. However, genotypes that highly resistant to BPH they possessed high stomata conductance ability which may help to slow the water shortages (Liu *et al*., 2011).

5. Conclusion

Simple sequence repeats markers found 11 pyramided genotypes with BPH resistance genes (*Bph3*, *Bph4*, *Qbph4* and *Bph17*) and aroma (*fgr* gene) and early maturity (*Hd2* and *Hd3* genes). Seven genotypes (#2, #3, #4, #5, #6, #10, and #11) were related to all traits expected based on molecular marker analysis. Meanwhile genotypes #1, #2, #4, #6, and #11 were similar with their parents based on phenotypic analysis. Gene pyramiding program assisted by molecular and phenotypic markers open the possibility to combine three valuable traits into one rice genotype as presented in this study. Further assessment on their benefits will be soon conducted in order to contribute to rice breeding program in Indonesia.

6. Acknowledgment

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