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Identification of Heading Date Six (*Hd6*) Gene Derived from Rice Mutant Varieties

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Abstract

Genes which were associated with flowering time to indicate the early maturity is known as heading date (*Hd*). Heading date six (*Hd6)* gene was identified from rice mutant varieties were Atomita 2, Atomita 3, Atomita 4, Bestari, Cilosari, Diah Suci, Sidenuk, Kahayan, Mayang, Meraoke, Mira-1, Pandan Putri, Superwin, Suluttan Unsrat 1, Suluttan Unsrat 2, Winongo, Woyla, Yuwono, while the rice var. Nipponbare was used as a positive control. All of rice mutant varieties derived from mutation induction by the dose of 0.2 kGy. The aim of this experiment was to find out the data base of mutant varieties which could be used as parent material with earlier maturity trait genetically. To obtain the DNA of plants, young leaves of each variety were extracted by liquid nitrogen, and then lysis and extracted by Kit Plant Genomic DNA. The amplification of DNA with 7 primers of *Hd6* conducted of 40 cycles by PCR and were continues to separated by 1 % agarose. The results were shown that the rice Mira-1 and Bestari varieties obtained from mutation of Cisantana highly different from one to another on 7 primers of *Hd6* used. Mayang variety from mutation of cross breeding between Cilosari and IR64, Pandan putri from Pandan wangi and Woyla from mutation of cross breeding from Atomita 2 and IR64 were highly different with those of their parents. Identification of *Hd6* gene on Sidenuk variety was shown the same bands pattern with Nipponbare as control positive toward all primers used, this variety would be better for earlier maturity parent material compared to others. The information could be useful for breeding programs aiming to develop early maturing widely adaptive and high yielding rice cultivars.

How to Cite

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INTRODUCTION

Rice is staple food for Indonesian people. The population of Indonesia is 254 million people and needs 35.56 million ton rice, however, supply from national rice production lower than rice consumption. The problem is not only rice production, but also increasing of the population about 1.49 % per year. According to Nugraha et al. (2014), rice production already decline 14.06 % in 2013 compared to 2012. To reach the national rice needs, development of rice with early maturity trait is the right step to fulfill rice consumption. One of treat to increase the rice production was Harvesting Index Program (IP 400) which was released by the government several years ago. To obtain IP400, improvement of rice has been done by crossbreeding long time ago, however, this method that only reveal the nature of the two parents (Dadang et al, 2013).

Induction mutation by gamma rays was successfully used for generating novel characteristics of direct importance in rice production and of potential quality and nutritional value (Ichitani et al, 2014; Parry et al, 2009; Oladosua et al, 2016). According to the data base of Atomic Energy Agency (IAEA), 3,222 officially released mutant varieties worldwide in more than 200 crop species (FAO/IAEA, 2015). National Nuclear Energy Agency (NNEA/BATAN) has gamma irradiator facilities which were used for mutation of plants, and had released 20 rice varieties, among others were Atomita 2, Atomita 3, Atomita 4, Bestari, Cilosari, Mira -1, Sidenuk, Suluttan Unsrat 1 and Suluttan Unsrat 2. The maturity age of those varieties varies from 103 to 120 days. The trait of BATAN rice varieties was high productivity, tolerant to pest and diseases, and earlier mature than their parent plants.

Genes which were associated with flowering time to indicate the early maturity is known as heading date (*Hd*) (Takahashi and Shimamoto, 2011; Higashi and Izawa, 2011). Heading date, *Hd* is one of key factors determining adaptation to different cultivation areas and cropping seasons. Several heading date-related genes have been identified and isolated throughout 12 chromosomes by some researchers (Chen et al, 2014;. Hori et al, 2015; Lee and An 2015; Zhang et al, 2015; Zhan et al, 2015). Study on the structure of genes associated with response to photoperiod on flowering of rice has been reported by Nakamichi 2015 and Yoshitake et al, 2015, which is triggered by day length (Ichitani et al, 2014) and is regulated by many genes (Itoh and Izawa 2013; Tsuji et al 2011), and Ebana et al. (2011) also reported to detect the eight gene loci of *Hd* including *Hd6*.

Mapping of *Hd1*, *Hd2* and *Hd3* have successfully carried out by Yamamoto *et.al* in Shang et al. (2012). They reported that a dominant gene *Hd* with photoperiod sensitive found in *Hd1*, *Hd2* , *Hd3*, *Hd5*, *Hd6*, Se -1, Se - 3 (t), Se - 4 (t), E1 and the other group were recessive photoperiod sensitive. Heading date 6 (*Hd6*) with locus ID Os03g762000 encodes a CK2 α-subunit and was identified by crossing Nipponbare and Kasalath (Ogiso, 2010 and Matsubara, 2014). Heading date 6 and *Hd1* genes are short day activators and long day repressor respectively. This genes are also flowering activator of OsG1, constitutive repressor of OsPhyB and SE5 which could be elucidated by gamma rays (Matsubara 2014). Identification of *Hd6* by using primers designed based on base pairs of exons of chromosome 3 will get the precision of plant breeding improvement (Hasan et al., 2015, Bertrand and Mackill, 2008) and by knowing of containing *Hd6* of rice mutant varieties genetically, it would be valuable for parent material with earlier mature trait as the objective of this experiment.

Identification of heading date six genes in rice mutant varieties have never been studied, from this study, it would be differentiate between parents and their mutants and different among tested rice varieties genetically, and also could be used for earlier mature parent plant genetically. This analysis can lead to the development rice mutant varieties for next generation sequencing particularly from within the varieties. Beside that, it would be understood the mutation occur in exon of rice mutant varieties, and allow us to evaluate and analyze variations for their similarity, and the differences reveal potential functional information. The objective of this experiment to find out the data base of heading date gene of mutant varieties will answer these challenges and making it useful for development of early maturity seed.

METHODS

Plant Materials

Rice varieties used for this study were Atomita 2, Atomita 3, Atomita 4, Bestari, Cilosari, Cisantana, Diah suci, IR64, Kahayan, Mayang, Meraoke, Mira -1, Pandan putri , Pandan wangi, Sidenuk, Sullutan Unsrat 1 Sullutan unsrat 2, Superwin, Woyla, Winongo, Yuwono, which were Batan rice varieties released from 1982 to 2012, those varieties from induction mutation by gamma irradiation. Nipponbare rice variety was used as a positive control and the rice local plant

as a negative control. Leaves of plants were taken from the plants grown in the greenhouse at PAIR - BATAN Jakarta in 2014. Chemicals for PCR used were from Qiagen GmbH , Germany.

Plant genomic DNA extraction

Young and fresh leaves of all parents and individuals of rice mutant varieties were stored at -70°C until used for DNA extraction. DNA was isolated from the frozen leaves with grounded by mortar and pestle under liquid nitrogen. BIO-SPIN Kit Plant Genomic DNA extraction from Bioer Technology Co. Ltd , China was used for extraction. Fine powder was added with 450 µl LP buffer, then mixed throughly, and incubated at 65°C for 15 minutes. After cooling at room temperature, the solution added with 150 µl DA buffer, and mixed throughly, then incubated for 5 minutes in ice, and spin at 12.000 rpm with using spin coloumn centrifuge. The solution was then removed to 1.5 ml new tube and added with 750 µl of P binding buffer and spin again at 6000 rpm for 1 minute, sediment in the coloumn then added with 500 µl G binding buffer and centrifuge again for 30 seconds. The sediment then washed with 600 µl washing buffer, centrifuge at 10.000 rpm for 30 seconds, washing twice, and added with 100 µl elution buffer to obtain the DNA solution.

Polymerase Chain Reaction (PCR)

The base sequences were used as the primer for this experiment displayed in Table 1.

The primers in Table1 was designed based on the sequence in exon of chromosome 3. PCR reactions were performed in a 25 ul reaction volume which was consisting of 2.5 ul 10x buffer, 1.5 ul of 25 mM MgCl2, 5 ul of 5Q, 1UL of 10 mM dNTP mix, 0.75 ul of primer R, 0.75 ul of primer F (each primer in Table 1), 0.25 ul of Taq polymerase enzyme 5U/ul, 8.25 ul of DEPC and 5 ul of rice DNA. PCR reaction was carried out with the conditions (i) the denaturation at 94°C for 5 minute, (ii) denaturation during 1 minute at 94 °C, annealed for 1 minute at the temperature of 55°C, extension 72°C for 2 minutes, extended extension 72 °C for 7 minutes, the number of cycles were 40 cycles. After amplification, 6 ul of products was combined with 1 ul of a loading buffer and analyzed directly on 1 % agarose and its running in electrophoresis for 45 minutes by using 100 bp DNA ladder.

Table 2 was displayed the characters of each rice mutant varieties to show the origin of each rice parent plant with their mutant varieties, and the differences in between of variety, especially harvesting age of variety.

Rice mutant varieties of Bestari, Mira-1, Pandan putri, Sidenuk, Suluttan unsrat 1, Suluttan unsrat 2 and Yuwono obtained by mutation of their parents respectively, and rice mutant varieties of Kahayan, Mayang, Meraoke, Woyla and Winongo were obtained from mutation induction of crossing between Atomita with IR64 by gamma rays dose of 0.2 kGy at Centre for Isotopes and Radiation Application (CIRA/PAIR), National Nuclear Energy Agency (NNEA/BATAN) Jakarta. The irradiation was conducted on about 100 gram rice seeds placed in plastic bag and irradiated by gamma rays in Gamma Chamber, irradiated seeds then were planted in the field to obtain of M1. Seeds from harvesting of M1 then were planted in the field to obtain M2 and selec-

Table 1. Sequence of *Hd6* gene used as primers

Sequence of primers	Annealing temp (°C)	Target amplicon Length (bp)
Hd6-1, F: CGCCGCCTCTATCTATCTCC *Hd6*-1, R: GTAGGTAGCACAGCCAGC	59	381
Hd6-2, F: TTTTCCCTGACCTGATGTT *Hd6*-2, R: GTCTTCATTGAGCCTTCA	53	430
Hd6-3, F: TTAACAGTGAGCAGGATGA *Hd6*-3, R: TGAAGGCTCAATGAAGACC	53	430
Hd6-4, F: ATTTGCCCTTAATCCTGT *Hd6*-4, R: AGCAATACGTGAACCGAT	52	420
Hd6-5, F: TGCTTACTAATGCGTCAA *Hd6*-5, R: AGTTTGATTCTGGCCTCT	53	380
Hd6-6, F: GGACTAAGGCAGATGTCA *Hd6*-6, R:ACCCCGACAGATAATATTTGTTACA	53	560
Hd6-7, F: GCAATAGATAGAAACCCTT *Hd6*-7, R: GGTTGCGTTTGATGAATG	52	365

Table 2. Agronomic traits of rice mutant varieties

Variety	Parent plant	Plant age (days)	Plant height (cm)	Amilose (%)	Production (ton/ha)
Bestari	0.2 kGy Cisantana	115 - 120	100 - 115	20.62	6.56
Kahayan	0.2 kGy – F1 Atomita 4 x IR64	110 - 115	96 - 105	22.00	6.50
Mira-1	0.2 kGy Cisantana	115 - 120	105 - 110	19.00	6.29
Mayang	0.2 kGy – F1 Cilosarix IR64	115 - 120	90 - 100	20.38	6.29
Meraoke	0.2 kGy - F1 Atomita 4 x IR64	110 - 120	120 - 125	22.10	5.00 – 7.00
Pandan putri	0.2 kGy PW 1-PsJ	115 - 120	140	23.00	6.50
Sidenuk	0.2 kGy Diah suci	103	104	20.60	6.90
Suluttan Unsrat 1	0.2 kGy Super win	112	101	21.90	7.30
Suluttan Unsrat 2	0.2 kGy Super Win	111	99	21.40	7.10
Woyla	0.2 kGy – F1 Atomita 2 x IR64	105 - 115	115 - 120	22.10	5.00 – 7.00
Winongo	0.2 kGy – F1 Atomita 3 x IR64	110 - 115	110 - 115	19.00	6.00
Yuwono	0.1 kGy IR64	110 - 115	95 - 105	22.69	9.00

Source : BATAN, description of rice mutant varieties from mutation breeding

tion based on the target traits. The main target traits were high productivity, resistant to pest and diseases, earlier mature than their parent plants and good quality. To obtain the mutant lines with that triats, the agronomic characters like plant heigh, the number of productive tillers, flag leave lenght, flag leaf erect, grain size, grain weight of 1000 grain, early flowering, were observed one by one plant and selected by pedigree. The homogenous lines were then tested to yield trials, yield in multi-location, tested to resistant to pest and diseases. Research conducted 3 replicates and analyzed in a randomized block design. The research to obtain new varieties of each rice Batan varieties was performed by every rice breeders.

RESULTS AND DISCUSSION

The use of molecular and genomic tools for mutant screening and characterization also enabled mutation breeding to embrace and utilize the very recent findings and technological innovations in plant genomics and molecular biology research. Forward genetics works starting from traits (phenotypes) in the field to genes and is the typical approach in plant breeding, genetics and genomics studies. Plant development patterns can also be significantly altered by mutations in gene coding regions. For example, homeobox genes in plants are involved in meristem maintenance and the development of lateral organs (Shu et al., 2011).

In plants, the timing of floral transition has a direct impact on reproductive success. The variation of flowering date of rice could be used for grouping of level grain maturity. According to Tasliah et al. (2011), the age grouping of rice based on the General Guidance of IP 400 is as follows : ultra early maturing age (≤ 85 days), super early maturing age (85 – 94 days), very early maturing age (95 – 104 days), early maturing age (105 – 124 days), moderate (more than 125 days) and extreme maturing (more than 165 days). Based on that grouping, agronomic traits of Batan's rice mutant varieties shown in Table 2 that the age of all rice mutant varieties were from 105 to 125 days, and included to early maturing age.

Mutation techniques have proven particularly useful in traditional variety improvement, because this technique had succeeded in improving their yield, disease resistant, earlier maturity, while keeping their quality characters unchanged. To identified of maturity of rice mutant varieties

genetically we conducted using heading date six (*Hd6*) gene as displayed in Figure 1 for *Hd6*-6.

Physically or chemically induced mutations occur randomly across the whole genome and within any locus or gene. This is a very important feature of induced mutagenesis, because it not only provides the probability of generating mutations for any gene of interest, but enables the development of multiple mutations for any target gene in a predictive manner. Figure 1 can be seen that rice varieties were very different with their parent plants, except Sidenuk (18) variety has no different with Diah suci (10) as its parent plant. Rice Cisantana (9) was a parent of Mira-1 (15) and Bestari (7) varities. Mutation induction by the dose of 0.2 kGy had different effected in between of both varieties, the same phenomenon also detected between Suluttan unsrat 1 (19) and Suluttan unsrat 2 (20) from mutation induction of Superwin (21) rice variety. Mutations of different categories, as well as those found in different locations, can have distinct consequences for the function of a given gene. Therefore, the effects of any gene mutation on an organism will vary, depending upon where the mutation occurs.

Figure 2 shown the band size of rice mutant varieties by *Hd6*-7, it was shown that all rice mutant varieties and their parents have the same band at 365 bp, the position of the primer *Hd6*-7 designed was in exon 10 of chromosome 3. From this figure it could be explained that irradiation by the dose of 0.2 kGy has no effect on exon 10 of rice mutant varieties and the maturity between parents and their mutant varieties were similar.

Different types of changes in SSRs such as band disappearance and shifted band position were observed among irradiated plants compared to their parent plant (Table 3). Nipponbare was japonica rice variety containing the early maturity gene, it was clear band appeared in 381 bp. Bestari and Mira-1 varieties were from mutation induction of Cisantana variety, in between of varieties which shows that Mira-1 highly different with their parent compared to Bestari. The similar phenomenon also found in Pandan putri with its parent Pandan wangi, and Yuwono from mutation of IR64, Mayang from mutation of crossing Cilosari and IR64, and also Woyla from mutation of crossing of Atomita 2 and IR64 at *Hd6*-4, *Hd6*-5 and *Hd6*-7. Sidenuk rice which

Figure 1. The amplification of rice mutant varieties with *Hd6*-6
(1.DNA ladder; 2.Nipponbare; 3.Local; 4.Atomita 2; 5.Atomita 3; 6.Atomita 4; 7.Bestari; 8.Cilosari; 9.Cisantana; 10.Diah suci; 11.IR64; 12.Kahayan; 13.Meraoke; 14.Mayang; 15.Mira-1; 16.Pandan putrid; 17.Pandan wangi; 18.Sidenuk; 19.Suluttan unsrat1; 20.Suluttan unsrat2; 21.Superwin; 22.Winongo; 23.Woyla and 24.Yuwono)

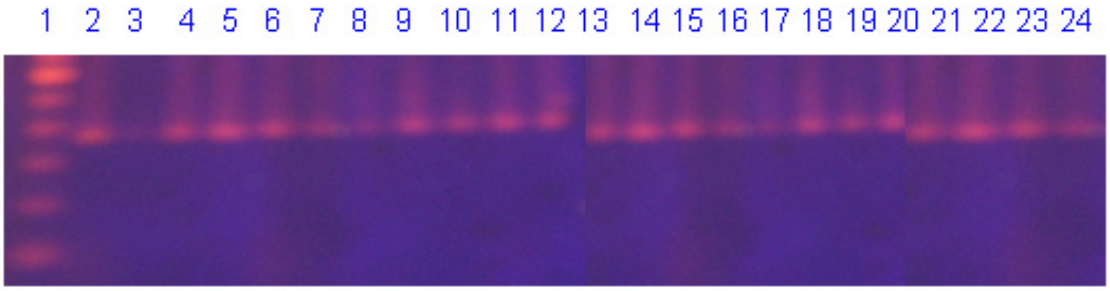

Figure 2. The amplification of rice mutant varieties with *Hd6*-7
(1.DNA ladder; 2.Nipponbare; 3.Local; 4.Atomita 2; 5.Atomita 3; 6.Atomita 4; 7.Bestari; 8.Cilosari; 9.Cisantana; 10.Diah suci; 11.IR64; 12.Kahayan; 13.Meraoke; 14.Mayang; 15.Mira-1; 16.Pandan putrid; 17.Pandan wangi; 18.Sidenuk; 19.Suluttan unsrat1; 20.Suluttan unsrat2; 21.Superwin; 22.Winongo; 23.Woyla and 24.Yuwono)

Table 3. The presence of *Hd6*-1, *Hd6*-2, *Hd6*-3, *Hd6*-4, *Hd6*-5, *Hd6*-6, *Hd6*-7 genes in rice mutant varieties

Variety	Primer						
	H6-1	*H6*-2	*H6*-3	*H6*-4	*H6*-5	*H6*-6	*H6*-7
Nipponbare	+	+	+	+	+	+	+
Local	-	-	-	+	-	+	-
Bestari Mira-1	-	+	+	+	+	+	+
Parent (Cisantana)	-	-	+	-	+	-	+
	+	+	+	+	+	+	+
Sidenuk	+	+	+	+	+	+	+
Parent (Diah Suci)	+	+	+	+	+	+	+
Pandan putri	-	-	-	+	+	-	+
Parent (Pd wangi)	-	-	+	+	+	+	+
Suluttan unsrat 1	+	+	-	+	+	+	+
Sulutttan unsrat 2	+	+	+	+	+	+	+
Parent(Superwin)	+	+	+	+	+	-	+
Yuwono	+	-	-	+	+	-	+
Parent(IR64)	+	+	+	+	+	+	+
Mayang	-	-	-	+	+	-	+
(Cilosari X	+	-	+	+	+	+	+
IR 64)	+	+	+	+	+	+	+
Kahayan	+	+	+	+	+	-	+
Meraoke	+	+	+	+	+	-	+
(Atomita4 X	+	-	+	+	+	+	+
IR 64)	+	+	+	+	+	+	+
Winongo	+	+	+	+	+	-	+
(Atomita3 X	+	-	+	+	+	+	+
IR 64)	+	+	+	+	+	+	+
Woyla	+	-	-	+	+	-	+
(Atomita2 X	-	+	+	+	+	+	+
IR64)	+	+	+	+	+	+	+

was a result of mutation induction of Diah suci shown had no differences between them, they were positive toward all primers used (from *Hd6*-1 to *Hd6*-7). Furthermore, Kahayan from mutation of crossing Atomita 3 with IR64, Winongo from crossing Atomita 3 with IR64 were shown the similar characters with their parent plant in *Hd6*-3, *Hd6*-4, *Hd6*-5 and *Hd6*-7. Gamma rays is a physical mutagenic, producing free radicals from water radiolysis, free radicals is an unstable spur which would attack the chemical bonds randomly including chromosome and DNA of plants. The existence of genetic differences between the parents with their mutant can be assumed that the plants have been mutated. Mutations can occur in the form of deletion, substitution, and transvertion of some base pairs or more. Shang et al. (2012) has successful to identified *Hd*(t) gene in chromosome 10 of mutant rice, he found that there was no base point mutation in this promoter region, but there were a T base-pair deletion and early stopped coding at 60 and 67 bp respectively.

Mutation induction of Cisantana rice had been changing the genetic of Mira-1 variety which was shown has no bands at *Hd6*-1, *Hd6*-2, *Hd6*-4 and *Hd6*-6, contrastingly, Bestari variety negative at *Hd6*-1. Mayang rice variety was also significant different with Cilosari and IR64 as their parent, which was shown at *Hd6*-1, *Hd6*-2, *Hd6*-3 and *Hd6*-6. Naito et a.l (2005) reported that no DNA fragment was amplified with primer sets designed between the first exon and the third exon, perhaps due to a sequence rearrangement around this region in gamma irradiated of pollen *Arabidosis thaliana*. He also found that 33 bp of the first exon from 215th to 247th base pair were deleted and 253 bp in whole second exon were inverted. According to Cecchini in Morita et al. (2009), deletion occurred at 5 kbp when *Arabidopsis thaliana* irradiated by gamma rays. Morita

also mentioned the result of Takano's research that deletion was obtained at 8, 10, 11 dan 33 bp respectively, however, Sato et al. (2006) obtained 2 bases and 4 bases substitution respectively. The mutation induction of Hitomobore and Nipponbare Japonica rice reported that deletion with size 1 bp and 3 bp at GA 3ß-hidroksilase (GA3ox2) and deletion of 4 and 16 bp at ent-kaurenoic acid oxidase (KAO) gene. Sequencing toward WX gene also appeared deletion 2 bp (wx-g1) and 6 bp (wx-g3) at exon 2 and exon 5 which was affected to premature of stop codon. The deletion of wx-g3 from codon 200 to position of 202 shown was affected by changes of amino acid leusin-leusin-cystein to arginin. He also mention about 7 rice mutant related to semi dwarf gene showed that 1 mutant occurred at 1 bp deletion at frame shift

Mutations can be classified based on the extent of the DNA sequence affected by the mutational event as either small-scale mutation involving one or a few nucleotides, or large-scale mutations where the chromosomal structure is affected. Small DNA changes may be further classified into point mutations, deletions and insertions. Deletion leads to a dominant mutation which may be more common is that in most white grained rice varieties carrying the mutant allele rc (a 14-bp deletion) of the Rc gene, another 1-bp deletion in the rc gene reverses the frame shift and generates a pseudo-wild type red rice mutant. Moreover Shu et al. (2011) mentioned that sequence analysis of the entire coding region of mutant d6-ID6 revealed deletions (~700 bp) spanning the entirety of exon 1 and a portion of the 5' upstream region in OSH15, and normal growth was restored to mutants transformed with wild-type OSH15. These findings demonstrated that the d6-type dwarf phenotype in rice was due to the loss of function of OSH15.

Simple sequence repeats (SSRs) or microsatellites are random tandem repeats of short nucleotide motifs (1–6 nucleotides long). Di-, tri- and tetra-nucleotide repeats, e.g., (GT)n, (AAT)n and (GATA)n, are widely distributed throughout the genomes of plants and animals. The copy number of these repeats varies among individuals and is a source of polymorphism in plants. SSR markers are characterized by their hyper-variability, reproducibility, co-dominant nature, locus-specificity, and random genome-wide distribution in most cases. SSR assays require only very small DNA samples and low start-up costs for manual assay methods (Jiang in Al-Khayri et al., 2015).

The pictures of some rice mutant varieties and their parent plant was displayed as below in Figure 3

From the result of preview researchers as has been earlier explained and associated with BATAN rice mutant varieties had shown that some varieties significantly differ from their parent plants. The mutation of double stranded DNA will recover through two ways which is ho-

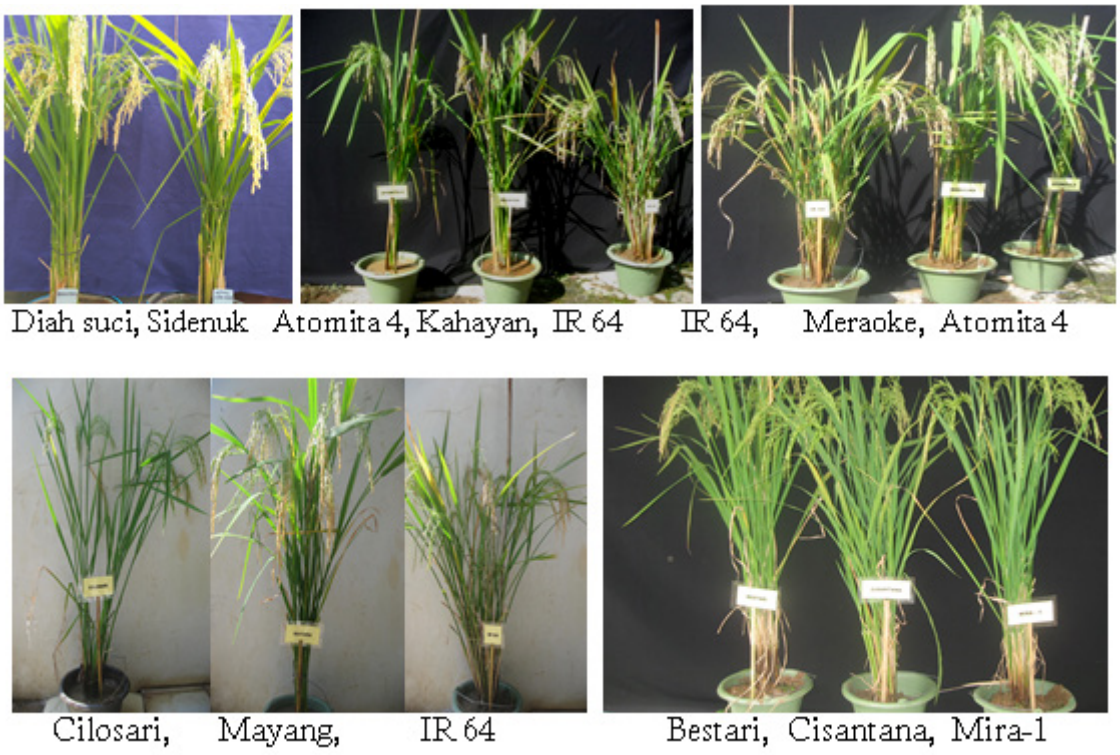

Figure 3. The performance of rice mutant varieties and their parent plant

molog recombinant (HR) with error-free repair pathway and non homolog end joining (NHEJ) with error-prone repair path*way* will produce varies of mutation (Tanaka et al., 2010). It is assumed that rice mutant varieties also have deletion and substitution at each exon which was identified, and needs for further verified and it will be useful for development of early maturity seeds.

CONCLUSION

Rice mutant varieties of Sidenuk was positive toward seven primers of *Hd6* gene used, which was identical with early maturity trait and only one primer was negative in Bestari, Kahayan, Mayang, Meraoke and Winongo, varieties. Those of varieties were the best superior seed to be used as early maturity seeds.

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