Atom Indonesia

Journal homepage: http://aij.batan.go.id

Identification, Selection, and Response of Radiation Induced Towuti Mutant Rice (*Oryza Sativa* L.) in Drought Stress Conditions

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ARTICLE INFO

Article history:
Received 7 November 2021
Received in revised form 27 February 2022
Accepted 28 February 2022

Keywords:
Drought stress
Gamma-rays
Characters selection
Stay-green gene
Rice mutant

ABSTRACT

Climate change with the impact of drought stress has become a major environmental problem for rice (*Oryza sativa* L.). The use of gamma ray radiation at a dose of 300 Gy is one way to develop drought tolerant rice varieties with little change to the characteristics of the Towuti variety. However, research is still needed to determine its resistance to drought stress. This study aims to identify characters for selection, genotype selection, and determine the response of Towuti mutant rice to drought stress conditions. The characters that can be used to select rice genotypes under drought stress conditions are plant height, number of leaves, number of tillers, and SPAD chlorophyll value. The Towuti mutant has the best tolerance to drought stress compared to other genotypes. Tolerance to drought stress in the Towuti mutant is not caused by the stay-green gene.

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food of the Indonesian population [1]. According to the Central Statistics Agency (BPS) 2021 database [2], in the last ten years (2010–2020) Indonesia's population growth rate was 1.25 % per year. Indonesia's population is projected to reach 296 million by 2030, with a ratio of 3.4 % of the world's population. Population growth is increasing but paddy fields have decreased [3] and been converted into industrial, residential, and other non-agricultural areas [4]. Indonesia's harvested rice area was 16.11 million ha in 2018 and has decreased by 33.7 %, or around 10.68 million ha in 2019 [2].

One of the solutions is to utilize dry land which has potential to increase food productivity [5]. Dry land with drought stress conditions has a chance to be cultivated, including rice [6]. However, the plant life cycle from germination to harvest requires water [7,8]. The amount of water needed in each growth phase during its life cycle is different [9,10]. This is directly related to the process of physiology, morphology, and environmental factors [11,12].

Efforts to maintain productivity in drought stress conditions are to use drought tolerant varieties [13]. The availability of rice varieties that can adapt to dry land is very low in nature, so that the development of drought-tolerant rice becomes difficult. In addition, the available dry land types have very low productivity [14]. Therefore, the use of varieties that are adaptive to drought stress is a promising alternative for use in dry lands [15].

Variety development can be done by plant breeding methods [16,17]. Plant breeding is the activity of changing the genetic arrangement of individuals and plant populations for a purpose, so that more useful plant genetics are obtained [18,19]. Changes in the genetic structure of individuals and

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DOI: https://doi.org/10.17146/aij.2022.1198

populations of plants can be done by genetic mutations [20-22]. Plant genetic mutations can be induced by using mutagens such as gamma-ray irradiation [23,24]. Mutation breeding using gamma-ray irradiation is efficacious to improve plant varieties [25].

In previous studies, the expected genotype was produced from the induction of gamma-ray irradiation at 300 Gy on the Towuti variety from anther culture. However, these genotypes still need to be studied to determine their adaptation to drought stress. One of the challenges that makes breeding efforts for drought tolerant rice slow is the absence of screening techniques that are able to effectively select tolerant varieties. One method to predict whether a genotype is resistant to drought stress is to provide drought treatment directly or predict it with molecular markers (indirectly). Molecular selection using stay-green markers is expected to obtain drought-tolerant mutants more quickly and accurately [26,27]. The selection technique with molecular markers is not influenced by the environment because the test is based on plant genetics [28].

In a past report, a functional stay-green wheat mutant with further developed drought resistance was produced [27]. Stay-green mutations in various plant species have been accounted for by keeping up with leaf greenness longer than their wild-type partners during senescence [29-31]. Some 'useful stay-green' mutants can photosynthesize for longer and may consequently be relied upon to give a better return [32-34].

The photosynthesis activity of the chloroplast is one of the most delicate physiological mechanisms to drought stress, which harms the thylakoid layer, upsets its capacities, function, and at last declines photosynthesis and yield [35-37]. Hence, conservation of the photosynthesis mechanism assembly is an important strategy for improving photosynthesis activity under drought stress conditions. Leaf senescence in plants is an inside degeneration process, during which the photosynthesis mechanical assembly is decreased, thus causing plant death. The most noticeable apparent change in leaf senescence is related to a decrease in chlorophyll and a dynamic decrease in photosynthesis ability [38].

In recent years, the response of the Towuti mutant rice in drought stress condition is still unknown. Therefore, this study aims to identify the characteristics of selection, genotype selection, and determine the response of Towuti mutant rice (*Oryza sativa* L.) in drought stress conditions.

MATERIALS AND METHODS

Research was conducted at the Center for Application of Isotope and Radiation, National Nuclear Energy Agency of Indonesia (BATAN), Pasar Jumat, South Jakarta from May to November 2019. Molecular analysis was carried out at the Laboratory of BB Biogen, Bogor, West Java.

The rice genotype used was Towuti M4 seed (4^{th} generation mutant, irradiated at 300 Gy from anther culture). The comparison varieties used were Towuti and 8375 as tolerant controls. As for varieties, IR20 and IR64 were used as sensitive controls. The primers used in the molecular analysis can be seen in Table 1. OsJSG1 and OsJSG2 are located at the locus LOC4347672, as the specific location of the stay-green gene.

Table 1. List of primers used to detect stay-green gene.

Code Gene	Product Size (bp)	Primer (5' → 3')
OsJSG1	571 bp	F : GTGGTACAACAAGCTGCAGC
		R : CGACATTGCATGCGTGTAGG
OsJSG2	591 bp	F : GATCCGAGGGAGCAGACATG
		R : GGAAGTAGACCCACACCGTG

Remarks : F = forward; R = reverse; bp = base pair

Procedures

The experiment was planned under a randomized complete block design with two factors and three replications. The first factor was drought stress. The second factor was rice genotypes, i.e. Towuti 300 Gy, Towuti, 8375, IR64, and IR20. Planting was carried out in 12 plots in open fields with a system of 9 lines per plot with a spacing of 30 x 30 cm^2. The size of plot is 3 x 3 m^2, thus one plot consists of 81 plants. Towuti M4 was planted in 5 rows for each plot, while comparison varieties i.e. Towuti and 8375 (tolerant control) and IR20 and IR64 (sensitive control) were planted in 1 row, respectively. The total population of Towuti M4 was 540 plants, with 108 comparison plants per variety.

Three seeds from each rice genotype were planted in a seedling hole. In both control and drought stress treatments, applications of NPK fertilizer (16:16:16) were done twice, i.e. at 14 and 50 days after planting (dap) at the rate of 30.25 g per seedling hole.

Drought stress was imposed at 45 until 65 dap, observations of soil moisture content were carried out every 4 days for 20 days, at 12 different plots randomly. Before drought stress, field capacity was maintained at around 80-100 %. Plant maintenance included weeding and watering. Data

collected from this experiment included plant height (PH), number of leaves (LN), number of tillers (TN), scoring for leaf rolling (LR) and leaf drying (LD) referred to Standard Evaluation System for Rice [39], SPAD chlorophyll value (SCV), stomatal length to width ratio (SLWR), and molecular analysis.

Molecular analysis

Rice DNA was isolated from samples of young rice leaves using a modified cetyltrimethylammonium bromide (CTAB) method [40]. The rice leaf sample was ground until smooth by adding liquid nitrogen and 500 µL of extraction buffer and then put into a 2 mL tube. It was incubated to prevent degradation. DNA separation was carried out by adding 2 µL of marchapto ethanol per sample and then centrifuged at 12,000 rpm at 20 ℃ for 10 minutes. After centrifugation, two layers will be formed. The supernatant layer at the top was transferred to a new tube and then centrifuged again. At the DNA precipitation stage, sodium acetate and cold isopropanol were added so that the supernatant was recovered, then incubated in a freezer at 20 ℃ for one hour. Purification of the precipitated DNA produced a DNA pellet at the bottom of the tube, then the supernatant was discarded and the DNA pellet was air-dried for 3 minutes with the addition of 100 µL of Te buffer.

The quantity and quality of the isolated DNA were carried out with a Nanodrop 2000 Spectrophotometer machine to determine the concentration and purity of DNA. The results are recorded and analyzed to make a DNA template with a concentration of 10 ng/µL for use in polymerase chain reaction (PCR) analysis.

PCR analysis was carried out by mixing PCR master mix into a tube consisting of 1 µL forward and reverse primers, 5 µL/sample bioline, 3 µL ddH_2O/sample and 2 µL/sample DNA template, so the total volume of the solution is 11 µL/sample. PCR machine was programmed according to the required temperature, i.e. predenaturation of 95 °C for 5 minutes, 34 cycles including separation denaturation at a temperature of 95 °C for 30 seconds, primer annealing at 55 °C for 20 seconds, elongation of the primer extension at 72 °C for 30 seconds, and final elongation at 72 °C for 7 minutes. The results of the amplification were observed by electrophoresis on a 1.5 % agarose gel in TBE buffer and stained using the red gel.

Data analysis

Analysis of variance (ANOVA) for all traits was conducted on the obtained data to understand the performance of different rice genotypes under drought stress condition. Significant effects were tested further by using the Duncan test at $\alpha = 5$ %. SPSS 22 software was used for this analysis.

Using SPSS 22, data were analyzed by pearson correlation and Bartlett's test of sphericity to know the relationship between characters. Principal component analysis serves to identify characters that contribute significantly to variance using SPSS 22. Selection of drought tolerant rice genotypes using the preference selection index method using Microsoft Excel 2019 [41] based on the characters obtained from the principal component analysis. All genotypes were confirmed by molecular analysis using stay-green markers.

RESULTS AND DISCUSSION

Water plays an important role in the translocation of nutrients from roots to all parts of the plant, so a deficiency of water will affect the decline in photosynthesis, growth, and development of the plant [10]. The condition of soil moisture content during drought treatment can be seen in Fig. 1.

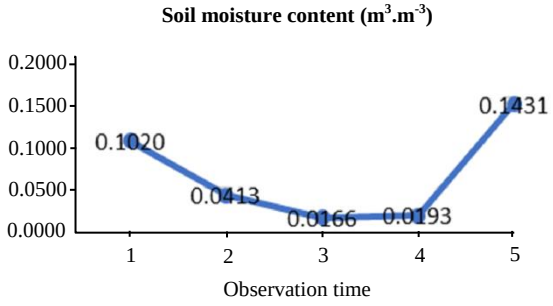

Fig 1. Soil moisture content during drought treatment. Observations are carried out every 4 days for 20 days of drought treatment, at 12 plots randomly.

Before the treatment, the soil moisture conditions ranged from 0.1904 to 0.2103 $m^3.m^{-3}$ with an average of 0.2004 ± 0.0258 $m^3.m^{-3}$, which equates to 100 % of the field capacity. Thus, the condition of soil moisture content during drought stress, which ranged from 0.0166 to 0.1431 $m^3.m^{-3}$ with an average of 0.0645 ± 0.0558 $m^3.m^{-3}$, was equivalent to 32.23 % of field capacity.

Analysis of variance

Analysis of variance showed significant effects on the characters of plant height (sig. 0.00), number of leaves (sig. 0.00), number of tillers (sig. 0.00), leaf rolling (sig. 0.045), leaf drying (sig. 0.011), and SPAD chlorophyll value (sig. 0.027), but had no significant effect on the characters of stomatal length to width ratio (sig. 0.371). Significant effects were tested further by using the Duncan tests at $\alpha = 5$ % (Table 2). Based on the ANOVA and Duncan test, non-significant characters were not continued for Pearson correlation analysis. This is because these characters do not show sensitivity to the given drought treatment, which means they cannot be used to select drought tolerant genotypes.

Table 2. Average and analysis of variance agronomic characters of rice genotypes in drought stress conditions.

Genotypes	PH	LN	TN	LR	LD	SCV	SLWR
Towuti 300 Gy	65.79a	45.11a	13.66a	3.45b	3.91a	37.95b	0.85a
Towuti	62.54ab	34.86b	11.18b	4.32ab	3.79a	38.09b	0.64a
8375	65.53a	26.82c	8.00cd	4.85a	3.15b	39.81a	0.75a
IR64	45.24c	20.07d	7.54d	3.89ab	3.52ab	38.83ab	0.66a
IR20	59.30b	32.31bc	9.94bc	3.74a	3.20b	39.33ab	0.73a

Remarks :
Numbers followed by the same letter in the same column are not significantly different at the 5 % level by Duncan's test. PH = plant height (cm), LN = number of leaves, TN = number of tillers, LR = leaf rolling, LD = leaf drying, SCV = SPAD chlorophyll value, SLWR = stomatal length to width ratio.

The genotype (G) × environment (E) interaction is very important in the process of selecting genotypes that are tolerant to drought stress conditions. Plants that have good adaptability (tolerant) will be able to grow and produce in extreme or drought-stressed environments even though their production decreases [42]. Characters that have a significant G × E interaction can be used as a basis for selection. Significant interactions show that these characters can give different responses to different environmental conditions [43]. The mechanism of adaptation between genotypes to drought stress shows different character responses in different environments, so characters that can show differences in each environment are very important.

Pearson correlation analysis and bartlett's test

A Pearson correlation analysis was carried out to see the close relationship between the observed characters. The correlation analysis was reported by Seyoum et al. (2012) and Vaisi & Golpavar (2013) which aims to obtain selection criteria in simultaneous selection [44,45]. The results of Pearson correlation analysis (Table 3) showed that the characters had a positively or negatively significant correlation ($p<0.05$) and can be used as a reference for conducting principal component analysis on the selection of drought tolerance characters [46].

Table 3. Pearson correlation analysis of rice genotypes characters in drought stress conditions.

Characters	PH	LN	TN	LR	LD	SCV
Plant Height	1.000	0.549**	0.555**	-0.214**	-0.293**	0.195**
Number of Leaves		1.000	0.779**	-0.298**	-0.176**	0.043^{ns}
Number of Tillers			1.000	-0.336**	-0.225**	0.055^{ns}
Leaf Rolling				1.000	0.226**	-0.502^{ns}
Leaf Drying					1.000	-0.204**
SPAD Chlorophyll Value						1.000

Remarks :
** = correlation is significant at the 0.01 level (2-tailed), ns = not-significant, PH = plant height (cm), LN = number of leaves, TN = number of tillers, LR = leaf rolling, LD = leaf drying, SCV = SPAD chlorophyll value.

Whether the correlation between variables was significant or not, it could be compared with the significance level of Bartlett's Test of Sphericity to the significance level used [47]. Based on the results of the analysis, it was found the value of significance ($p<0.05$) (data not shown). This indicates that the correlation between the variables is significant for principal component analysis to be carried out. Thus, principal component analysis will be carried out on all characters.

Principal component analysis

The general purpose of principal component analysis is to find a way to condense (summarize) the information contained in a number of original variables into a smaller set of new (component factors), composite dimensions or variables with a minimum loss of information [48]. The results of the principal component analysis showed that the first principal component explaining the variance was 43.18 %, and the second principal component was 18.72 % (Table 4). Cumulatively, the use of the 1st and 2nd components can explain 61.91 % of the variance.

According to Kaiser (1960) and Field (2009), not all factors are retained in an analysis, and there is debate over the criterion used to decide whether a factor is statistically important [49,50]. Eigenvalues associated with a variable indicate the substantive importance of that factor. Thus, it is recommended to retain all factors with eigenvalues greater than 1,

so that the selected characters in the two components above can describe the important characters.

Table 4. Principal component analysis on rice genotypes characters in drought stress conditions.

Characters	Component 1	Component 2
Plant Height	0.717	0.297
Number of Leaves	0.898	-0.028
Number of Tillers	0.905	0.018
Leaf Rolling	-0.49	-0.168
Leaf Drying	-0.262	-0.677
SPAD Chlorophyll Value	-0.036	0.83
Initial Eigenvalues	2.591	1.123
% of Variance	43.188	18.72
Cumulative %	43.188	61.908

If the high contribution to the 1st principal component is confirmed, then the selection of characters that have an important influence on the selection process can be made in the 1st principal component. The selected characters that have a high value (>0.5) in the 1st principal component are: plant height (0.717), number of leaves (0.898), number of tillers (0.905). The selected characters have a high value (>0.5) in the 2nd principal component. The character has a SPAD chlorophyll value (0.83). Thus, of the 6 characters used, when principal component analysis is carried out, it can be 4 characters (plant height, number of leaves, number of tillers, and SPAD chlorophyll value) that are able to explain 61.91 % of the variance.

Preference selection index

The proposed approach is new for the selection of materials. Material selection is mostly completed using multi-attribute decision making methods. A literature review clearly indicates that in all these existing multi-attribute decision making methods, it is necessary to assign relative importance between attributes or attributes weight and this requires many complex calculations. In the proposed method, it is not necessary to assign relative importance between attributes, but in this method, overall preference values of attributes are calculated using the concept of statistics. This method is useful when there is conflict in deciding the relative importance of attributes, and that is the benefit of the PSI method. Using the overall preference value, the preference selection index for each alternative is calculated, and the alternative with the highest value of PSI is selected as the best alternative [41].

Based on the results of the preference selection index (Table 5), it was found that the Towuti 300 Gy genotype was ranked first (0.987) regarding its tolerance to drought stress. The ranking was based on the character of plant height, number of leaves, number of tillers, and SPAD chlorophyll. The 2nd rank is occupied by the Towuti genotype (0.880). Interestingly, from the mutation induction of 300 Gy gamma-ray irradiation in the Towuti genotype, it was able to produce an expected genotype that was superior to its parent in terms of tolerance to drought stress.

Table 5. Preference selection index on rice genotypes in drought stress conditions.

Genotype	Index Value	Rank
Towuti 300 Gy	0.987	1
Towuti	0.880	2
8375	0.807	4
IR64	0.679	5
IR20	0.841	3

Molecular analysis using specific markers

Based on Table 5, it is known that Towuti 300 Gy has a superior response in terms of tolerance to drought stress. The resistance to drought stress will be confirmed through molecular analysis using specific stay-green markers. This analysis is intended to determine the cause of resistance to drought stress due to the influence of the stay-green gene on the genotype, or due to the influence of other genes.

Identification of molecular markers was carried out using two primers, and one primary polymorphism was obtained, namely OsJSG1. The OsJSG1 primer can show differences between Towuti 300 Gy and its parent (Towuti). Based on the amplification using PCR in Fig. 2, it can be seen that the rice DNA samples amplified using OsJGS1 showed a polymorphic banding pattern where the Towuti 300 Gy showed a different banding pattern from its parent, while the OsJSG2 primer showed a monomorphic banding pattern. This indicates that the drought-tolerant character of the Towuti 300 Gy is thought to be unrelated to the locus LOC4347672, the specific location of the stay-green gene. The size of the DNA banding pattern between the Towuti 300 Gy and its parent showed differences in the OsJSG1 primer, but the Towuti 300 Gy banding pattern was monomorphic with sensitive control.

The difference in polymorphic banding pattern on OsJSG1 primers between Towuti 300 Gy and its parent is that the former did not have drought-tolerant properties with the stay-green gene. The banding pattern is shown by the IR20 control plant as a sensitive control also produced the same DNA bands as the Towuti 300 Gy (Fig. 2.a). It can

be assumed that the genetic changes of the Towuti 300 Gy did not occur in the PCR amplification of the primary area.

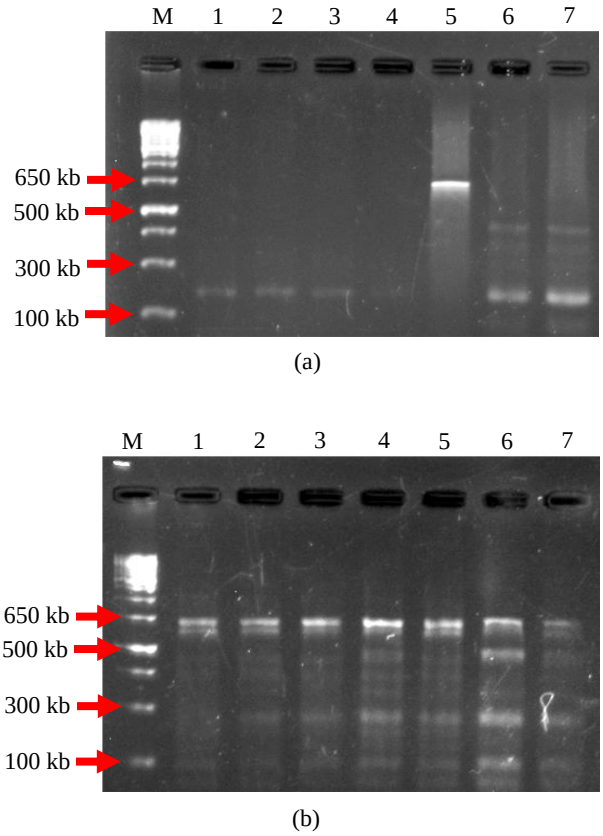

Fig. 2. PCR band pattern of rice genotype using primers a) OsJSG1 and b) OsJSG2 from locus LOC4347672. M = marker, kb = kilobase pairs, 1 = Lombok, 2 = 8375, 3 = IR20, 4 = IR64, 5 = Towuti, 6 = Towuti 200 Gy, 7 = Towuti 300 Gy.

The Towuti rice mutant showed smaller polymorphic DNA bands (<500 bp) than the parent plant (>500 bp). A genotype is called a mutant if its DNA shows differences from the control DNA [51]. The missing DNA bands in the rice mutant caused the cells to suffer direct damage due to gamma-ray irradiation treatment. This happens because of the breaking of the bonds of DNA structures [52].

CONCLUSION

The characters that can be used to select rice genotypes under drought stress conditions are plant height, number of leaves, number of tillers, and SPAD chlorophyll value. The Towuti mutant has the best tolerance to drought stress compared to other genotypes. Tolerance to drought stress in the Towuti mutant is not caused by the stay-green gene.

ACKNOWLEDGMENT

The authors would like to thank the Center for Application of Isotope and Radiation - National Nuclear Energy Agency (BATAN) and the Molecular Biology Laboratory (BB Biogen) for genetic material, technical advice, funding, and permission to use the company's research facilities for this research.

AUTHOR CONTRIBUTION

All authors contributed equally to the paper. Hasna Dama: Study conception and design, acquisition of data, data analysis and interpretation, manuscript drafting, critical revision. Syarifah Iis Aisyah: conceptualization and design of study, critical revision and interpretation of data. Sudarsono: conceptualization and design of study, critical revision and interpretation of data. Azri Kusuma Dewi: conceptualization and design of study, critical revision and interpretation of data. Kunto Wibisono: manuscript drafting, critical revision, data analysis and interpretation.

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