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[biodiv] Submission Acknowledgement

1 pesan

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3 Oktober 2020 20.30

Balas Ke: Ahmad Dwi Setyawan <editors@smujo.id>

Kepada: Reny Herawati <reny.herawati@unib.ac.id>

Reny Herawati:

Thank you for submitting the manuscript, "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potential of Lines from Single Crossing Bengkulu Local Rice Varieties" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: <https://smujo.id/biodiv/authorDashboard/submission/6884>

Username: renywati

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

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Reny Herawati <reny.herawati@unib.ac.id>

[biodiv] Editor Decision

4 pesan

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4 November 2020 11.32

Reny Herawati:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potential of Lines from Single Crossing Bengkulu Local Rice Varieties".

Our decision is to: Decline Submission

Note: We have invited c. 20 experts but no one wants to review. So, please make your "own-review" by sending your paper to at least two reviewers, and one professional language editor; then sending us your final revised paper along with comments from the two reviewers (incl. name & email address) and language editing certificate.

Smujo Editors
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T-Identification of drought tolerant and molecular analysis of DREB2A and BADH2 genes and yield potential of lines from singl.doc
700K

Reny Herawati <reny.herawati@unib.ac.id>
Kepada: Smujo Editors <editors@smujo.id>

5 November 2020 12.02

Dear editor team,

Thank you for the quick response, we will comply as suggested by the editor. We will send the results as soon as possible

Best regards,

Dr. Reny Herawati et al

[Kutipan teks disembunyikan]

Reny Herawati <reny.herawati@unib.ac.id>
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25 November 2020 13.02

Dear editorial team,
as previously suggested, we attached the submit the final manuscript and supporting documents previously requested (proofreading, reviewer, certificate, and turnitin check).

We include 2 reviewers, namely:

1. Ir. Suprayogi, M.Sc., Ph.D (Rice Breeding from University of Jenderal Soedirman) [email:suprayogi@unsoed.ac.id](mailto:suprayogi@unsoed.ac.id)
2. Dr. Ir. Heni Safitri, M.Si (Rice Breeding, Research from Balai Besar Penelitian Tanaman Padi, Sikamandi) [email:henisafitri2@gmail.com](mailto:henisafitri2@gmail.com)

We hope that our manuscript can be processed in the biodiversity journal. We look forward hearing from you soon

1 Identification of drought tolerant and molecular analysis of DREB2A 2 and BADH2 genes and yield potential of lines from single crossing 3 Bengkulu local rice varieties

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10 **Abstract.** Screening in the seedling stage of 39 progeny of F6 lines to drought stress was carried out in the greenhouse. Drought tolerant
11 and sensitive varieties of IR 20 and Salumpikit, respectively, were used as control plants. The methods for traits identification of leaf
12 curled, dried, and recovery ability after exposure to severe drought for two weeks was following the Standard Evaluation System (SES)
13 developed by IRRI. Molecular analysis to detect the presence of the DREB2A gene was carried out by PCR amplification of genomic
14 DNA using forward- and reverse- oligonucleotide primers of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA,
15 respectively, while for BADH2 gene using forward- and reverse- oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and
16 TGTCCCCAGCTGCTTCATCC, respectively. Molecular markers of DREB2A and BADH2 genes were also identified in 39 tested
17 lines with approximately 250 and 2300 bp length, respectively. This study concluded that the progeny of F6 lines generating from the
18 crossing of local varieties of IR7858 and IR148 is the potential to become a drought-tolerant variety of upland rice. Line numbers BKL2
19 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 tonnes/ha. These line has the potential to be developed on
20 rainfed lowland rice or dry land because it has drought resistance.

21 **Keywords:** BADH2, DREB2A, drought tolerance, gene identification, yield potential

22 **Running title:** *Identification of DREB2A and BADH2 genes for drought tolerant*

24 INTRODUCTION

25 The development of upland rice variety is an alternative to increase national rice production in Indonesia because the
26 extensification of lowland rice is increasingly difficult. This strategy is carried out through optimizing the use of
27 uncultivated lands, where most of them have the potential for upland rice cultivation (Center for Research and
28 Development 2006). The use of superior varieties, which has higher yields and tolerance to various obstacles so that it can
29 adapt well to climate change, is urgently needed to support efforts to increase rice yields in the dry land. Anticipating the
30 impact of climate change on sustainable agricultural systems is carried out to produce technological innovations that are
31 able to overcome and suppress the impacts caused. These technological innovations include superior varieties of drought-
32 tolerant rice. Genetic improvement to obtain superior varieties that are adaptive to the conditions of drought stress is an
33 essential priority in rice breeding programs.

34 ~~Assembling~~ **Breeding** drought-tolerant rice varieties can be done through crossbreeding, which combines the ~~resistant~~
35 ~~tolerant~~ traits of the parents with other crops that have a high yield. Molecular marker technology can help selection ~~more~~
36 ~~accurately~~ than conventional. One of the markers related to drought tolerance is the QTL marker (quantitative trait locus)
37 12.1. The International Rice Research Institute (IRRI) had crossed the Vandana variety of Indian rice with Way Rarem
38 from Indonesia. One of the filial is a crossing number of IR148+, which is derived from IR crossing 79971-B-369-B-B
39 (Mulyaningsih et al. 2010). The crossing population has been showed ~~to~~ contain QTL 12.1 markers. The location of
40 markers is on chromosome 12, between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of
41 these markers can maintain yields in conditions of severe drought stress during the ~~reproductive~~ stage before flowering. In
42 normal conditions, the marker QTL 12.1 did not have a significant effect on some of the parameters observed (Bernier et
43 al. 2007).

44 The DREB2 gene controls drought stress in plants (Matsukura et al. 2010; Srivastav et al. 2010; Akhtar et al. 2012;
45 Huang et al. 2018). DREBs (Dehydration Responsive Element Bindings) are transcription factor proteins (TFs) that are
46 very important in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The
47 homology of the DREB2 gene in rice is DREB2A (Sakuma et al. 2002). Some of the DREB2A target genes are MT2A,
48 At1g69870, At3g53990, At1g22985, RD29A, LEA14, At2g23120 [9], RD29B, At1g52690, RD17 (Sakuma et al. 2006;

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49 Qin et al. 2011), AtHsfA3, HSP18.2, and Hsp70 (Qin et al. 2011). The importance of the DREB2A gene is because it can
50 be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-resistant genes.

51 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). The reports
52 of previous studies indicate that osmoprotectant substances, namely glycine betaine, plays an essential role in cell
53 stabilization by balancing the structure of the protein quaternary and membrane structure against the adverse effects of
54 salinity (Sakamoto and Murata 2000; Saxena et al. 2019). Besides, it facilitates osmotic adjustment by lowering the
55 internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO
56 complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et
57 al. 2020). The positive effects of exogenous application of glycine in plants that grow on salinity stress have been shown to
58 protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

59 Betaine aldehyde dehydrogenase (BADH) is known as a key enzyme for biosynthesis of glycine betaine. Many
60 researchers have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity,
61 dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objective of the study was to identify
62 drought-tolerant traits and molecular analysis of DREB2A and BADH2 genes the progeny lines of F6 lines resulted from
63 the crossing of local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL
64 on chromosome

65 MATERIALS AND METHODS

66 The experiments were conducted at the University of Bengkulu. Screening study was done in the greenhouse of
67 Agricultural Faculty from Februari to April 2020, while molecular analysis was done in the laboratory of the Department
68 of Biology from May to July 2020. Plant materials were using the progeny of 39 lines that selected from F6 generations
69 resulted from the single crossing of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of
70 IR7858 and IR148+ QTL positive on chromosome 12.1 (Mulyaningsih et al. 2010). Var. Salumpikit and IR 20 as drought-
71 tolerant and sensitive control varieties (Table 1).

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Table 1. Selected F6 lines for traits and molecular identification of drought-tolerant genes of DREB2A and BADH2

| Lines number | Genotype | Initial Crossing | Line number | Genotype | Initial Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

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Screening of drought-tolerant rice of 39 F6 lines was carried out following the standard Evaluation System (SES)
developed by IRRRI (2002). The drought-susceptible variety (IR20) and local drought-tolerant variety (Salumpikit) were
used as control. The test was carried out following the method of Kumar et al. (2015); Swain et al. (2017); Herawati et al.
(2017). Plastic tubs sizes of 40 cm x 25 cm x 20 cm was filled with soil. Then, each tub was planted ten family lines and
two control varieties. Each line was sown for 20 seeds in a row. Seedlings were watered intensively in 2 weeks after
planting. After this stage, watering was stopped until the sensitive plants dried. Drought tolerance assessment was carried
out based on the SES methods, as described in Table 2. Trait responses of the seedlings were recorded, then seedlings

Comment [H5]: Salinity or drought? This manuscript talk about drought, not salinity.

Comment [H6]: This table make the reader confused. Make it one row one line!

Comment [H7]: Many identic crossing. What the differences?

Comment [H8]: In the first sentence in this paragraph tell the reader that the test according to SES.

83 were watered intensively for the next ten days. Recovery ability was recorded following the methods of SES, as described
84 in Table 2.

85 Genomic DNA was isolated from fresh leaves at 14 days after treatment (DAT). Fragments of 0.1 g of rice leaf were
86 ground in the mortar by adding liquid nitrogen. Isolation of total DNA was carried out by modifying the protocols of
87 Wizard's Genomic DNA Purification Kit. The ground leaf was put into a 2 ml tube, then 600 µl of Nuclei Lysis Solution
88 was added, followed shaking by vortex for 3 seconds. Then, the solution was heated in a water bath at 65°C for 15
89 minutes. RNase of 3 µl was added followed incubation at 37 °C for 15 minutes. Then, 200 µl Precipitation Solution
90 was added, and the microtubes were centrifuged for 3 minutes at 13,000 rpm. The supernatants were removed to a 1.5 ml
91 tube, and 600 µl of isopropanol was added. The microtubes were further centrifuged for 1 minute at room temperature.
92 The solution was discarded, and DNA remained on the bottom of microtubes was air-dried for 15 minutes. DNA
93 Rehydration Solution of 100 µl was added and further incubated at 65°C for 1 hour or at 4°C for one night. The total
94 isolated DNA is used as a template DNA for PCR amplification of DREB2A and BADH2 genes.

95 PCR amplification of the DREB2A gene using forward- and reverse- oligonucleotide primers of
96 CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
97 Lathif et al. 2018). While the amplification of the BADH2 gene was using forward- and reverse- oligonucleotide primers
98 of GGCCAAGTACCTCAAGGCG and TGTCCCCAGCTGCTTCATCC, respectively (Robin et al. 2003). The PCR
99 mixtures, including the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix), and Taq polymerase,
100 were developed in the thermocycling. The program was started with denaturation temperature at 94oC for 5 minutes,
101 followed by 35 cycles of denaturation at 94oC for 1 minute, annealing at 59oC for 2 minutes, and extension at 72oC for 2
102 minutes, and the final extension at 72oC for 10 minutes. PCR amplification products were subjected to electrophoresis in
103 agarose gel 1% of TBE buffer to identify successful amplifications. The gel from electrophoresis was immersed in EtBr
104 1% for 10 minutes, rinsed with ddH2O for 5 minutes, and visualized under UV transilluminator light.

105 In the season in 2020, a yield test of selected superior lines was carried out in March-July 2020 in Semarang Village,
106 Bengkulu City. The materials used in this study were 16 selected superior lines in the F7 generation. The experiment was
107 carried out on a plot measuring 8 m x 6 m with a spacing of 20 x 20 cm, and 1 seed was planted. Fertilize twice, the first
108 fertilization at the age of 14 days after planting (HST) with a dose of 150 kg/ha of Urea, 100 kg/ha SP36 and 100 kg/ha
109 KCl. The second fertilization at the age of 30 HST with a dose of 100 kg/ha Urea, 100 kg/ha SP36 and 100 kg/ha KCl.
110 Intensive control was carried out against weeds, pests and diseases. Observation of the agronomic characters of 10
111 plant/plot samples taken from each line number. The characters observed included plant height, number of panicles/hill,
112 panicle length, number of filled grains/panicle, percentage of empty grain/panicle, 1000 grain weight, grain weight per hill,
113 and yield per plot.

114 **RESULTS AND DISCUSSION**

115 **Identification of drought tolerant traits**

116 Screening of F6 lines at the seedling stage was carried out to select the drought-tolerant rice (Kumar et al. 2015; Swain
117 et al. 2017; Herawati et al. 2017). Drought tolerant assessment following the methods of SES was done by comparing the
118 treated plants with control varieties of Salumpikit and IR20 (Table 2). The symptoms were identified after exposed to
119 drought stress for 14 days, including leaf curling, leaf drying, and ability to recover (Figure 1). The criteria of 39 F6 lines
120 were identified as highly to rather tolerant, tolerant, and moderately tolerant to drought for a total number of 11, 19, and 9
121 lines, respectively (Table 3). The scores of dry leaf of the 30 lines with highly to rather a tolerance and tolerance were 0-1
122 which recovery ability was 90 to 100%, while the scores of the rest nine lines with moderate tolerance were 3-5 which
123 recovery ability was 70 to 90% (Table 4, Figure 1).

124 **Table 2.** Traits identification of selected F6 lines based on criteria description of SES developed by IRRI (2002)

Comment [H9]: Put it in "Material and Method"

| Score | Criteria | Description | | |
|-------|----------------------|---------------------------------|---|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate tolerant | Leaves fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |
| 7 | Moderate susceptible | Leaf margins touching (O-shape) | More than 2/3 of all leaves fully dried | 20-39% of plants recovered |
| 9 | Susceptible | Leaves tightly rolled (V-shape) | All plants apparently dead. Length in most leaves fully dried | 0-19% of plants recovered |

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Table 3. Screening of the 39 F6 lines for resistant traits and molecular identification of gene markers of DREB2A AND BADH2

| Line number | Genotype | Crossing | The score of rolling leaf | The score of drought leaf | Score of recovery | Criteria | DREB2A genes | BADH2 genes |
|-------------|------------|--------------------|---------------------------|---------------------------|-------------------|----------|--------------|-------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | RT | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | RT | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | RT | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | + | + |
| S | Salumpikit | Control variety | 1 | 1 | 1 | RT | | |

HT=High Tolerant (6 lines); RT=Rather Tolerant (5 lines); T= Tolerant (19 lines); MT= Moderate Tolerant (9 lines); + = gene was identified

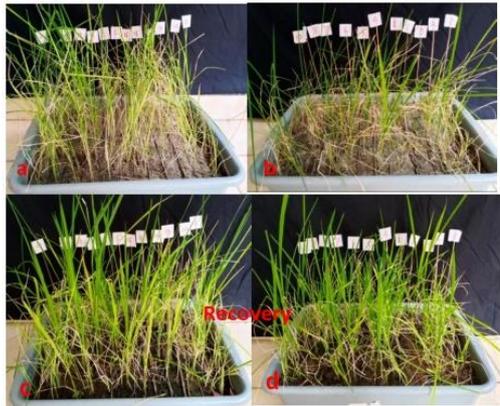
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Table 4. The distribution of 39 F6 lines for identification of drought-tolerant

| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------------|-----------|-------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Rather Tolerant | Tolerant | Moderate Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | ■ | | | | ■ | | | | |
| 19 | | ■ | | | | ■ | | | |
| 9 | | | ■ | | | | ■ | | |
| IR20 | | | ■ | | | | | | |
| Salumpikit | ■ | | | | | | | | |

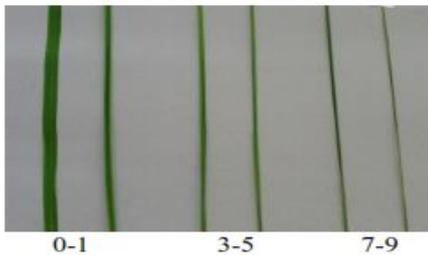
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Comment [H10]: Cluster analysis is needed for grouping the lines according to agronomic and drought tolerance traits.



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Figure 1. Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c dan d)



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Figure 2. Description of rolling leaves based on SES Method; scores 0-1: started rolling to form V; 3-5: rolling to form V and U inside leaves; 7-9: rolling leaves totally

Molecular identification of drought tolerant genes

Molecular analysis using PCR products showed that the DREB2A gene was visualized in 39 selected lines with the marker sizes approximately 250 bp (Tawfik et al. 2016; Lathif et al. 2018) (Figure 3). It proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1, which are drought-tolerant generated F6 lines that are the potential to be drought tolerant. This evidence proved that drought tolerance in rice plants is controlled by the DREB2A genes (Akhtar et al. 2012; Huang et al. 2018).

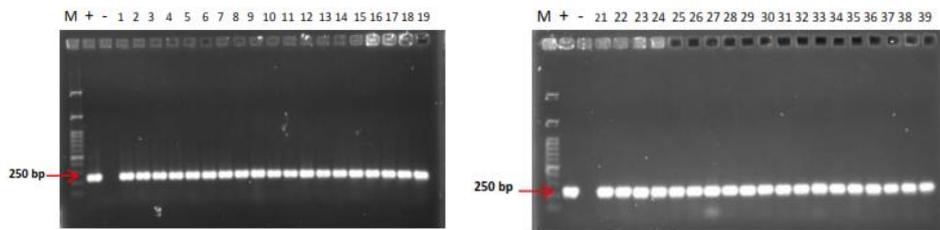


Figure 3. PCR amplification of DREB2A (250 bp) on 39 selected lines from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - =negative control)

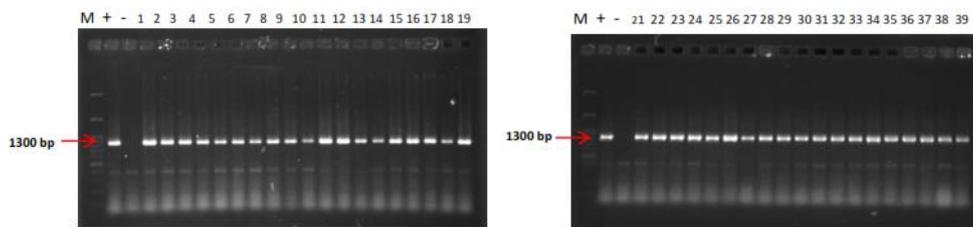


Figure 4. PCR amplification of BADH2 (1300 bp) on 39 selected lines generated from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - = negative control)

Successful use of molecular markers that control complex traits for obtaining drought-tolerant superior rice varieties has been reported by Lanceras et al. (2004). Some of the traits that have been studied include the yield, root length, root thickness, leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017), and osmotic adjustment (Zivcak et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is known as a key enzyme for the biosynthesis of glycine betaine. Many researchers have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). Visualization of the BADH2 gene 39 selected lines showed a marker with a size approximately 1300 bp (Shrestha 2011; Hasthanasombut et al. 2011) (Figure 4).

Performance of agronomic characters, yield and yield potential of superior lines

The appearance of agronomic characters, yields and yield potential of the 16 superior lines tested are presented in Table 5. Almost all tested lines have shown uniformity as shown by the lowest average plant height appearance, namely 101.1 and the highest is 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the 8th generation (F7). The highest average number of panicles/hill was 14.7 and the lowest was 6.5. however, the panicle length ranges from 24.61 - 27.6 cm. The number of filled grains/panicles ranged from 99.5 - 150.07, while the percentage of empty grains rice was categorized as low based on the SES IRRI (2012), which ranged from 9.87% - 26.66%. This led to variations in grain weight per hill, which was around 19-35.5 grams/hill .

Table 5. The performance of agronomic characters, yields and yield potential of superior lines

| Assesion | Plant height | Number of Panicle/hill | Panicle length (cm) | Number of fill grains | % of empty/panicle | 1000 grains weight (gram) | grains weight/hill (gram) | Yield/p lot(1x1 m ²) (gram) | Yield pote ntial (ton/ ha) |
|------------------------------------|--------------|------------------------|---------------------|-----------------------|--------------------|---------------------------|---------------------------|---|----------------------------|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |

Comment [H11]: Which lines among the 39 lines used??? The lines name were different.

| | | | | | | | | | |
|-----------------|--------------|--------------|--------------|----------------|---------------|--------------|--------------|------|-------|
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

The yield of grain per plot varies from the lowest was 458 grams, and the highest, which was 1210 grams. If seen from the appearance of agronomic characters, the high grain yield was supported by the characters of the large number of panicles, the low percentage of empty grain, and the weight of 1000 grains. The length of the panicles did not show any significant variation, which the range between 24.61-27.6 cm (Table 5).

Discussion

Seedlings' responses to drought stress were identified after 14 days without water. The tolerant lines continued to grow well, vigorous, and leaves remained fully open, whereas the moderate tolerant lines were dried on leaf tips (Figure 1). Kumar et al. (2014) reported that leaf rolling was delayed in drought-tolerant rice genotypes. Leaf rolling in rice plants was induced by loss of turgor and low osmotic regulation. Delayed leaf rolling in the tolerant genotype showed that the turgor remained normal, and the plants were protected from dehydration (Figure 2). Leaf rolling is one of the mechanisms of plants to adjust the water potential, which enables the plants to absorb groundwater in drought stress conditions (Bunnag and Pongthai 2013; Swain et al. 2017).

Swain et al. (2017) reported that during the drought conditions and the level of groundwater was below 30 cm depth, of the 78 lines of drought-tolerant assessments were identified that 30 lines were scored of 1, and 48 lines were scored of 3. Of these 78 assessments, 13 lines produced more than 1-ton grain/ha, tolerant lines (CR 143-2-2) produced more than 2.7 tons grain/ha, while sensitive plants as a control (IR20) did not produce any.

Leaf rolling can reduce leaf surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This condition will help plants to survive in a certain period when the availability of water in the environment decreases. The genes in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the leucine zipper class IV transcriptional factor homeodomain. Overexpression of these genes results in leaf curling on the adaxial side, whereas suppression of this gene causes leaf rolling on the abaxial side (Zou et al. 2011). Delaying leaf rolling indicates that a plant effort to maintain turgor and avoid dehydration. Water shortages tend to have lower rates of leaf rolling, which appeared in the plants that have tolerant criteria with a Score of 1 (Table 3). It allowed the plant to survive to drought at the low water potential of leaf tissue (Sevanto 2018). Plants were recovery after passing through a period of drought indicated the ability of plants to improve their metabolic system (Bian et al. 2017; Wang et al. 2019).

Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the ancestors with other varieties that have high productivity. The use of molecular marking technology can help selection more accurate and faster. One of the markers related to drought tolerance is the QTL (quantitative trait locus) 12.1, which has been produced through the crossing of Vandana varieties of Indian rice and Way Rarem variety from Indonesia (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study. This marker can maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL 12.1 did not have a significant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

DREBs (Dehydration Responsive Element Bindings) are transcription factor proteins (TFs) that are very important in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The DREB2A gene is essential as a regulator of drought-responsive genes, making it a marker of drought stress-tolerant.

Transcription factors in DREB2A are involved in the regulatory mechanisms that exist in some plants in response to drought, salinity, and heat stress (Lata and Prasad 2011; Mizoi et al. 2012). There are five DREB2 genes in the rice genome, including OsDREB2A, OsDREB2B, OsDREB2C, OsDREB2E, and OsABI4 (Matsukura et al. 2010; Srivastav et al. 2010). Expression of OsDREB2A in rice is caused by water deficit and exogenous ABA application, which can result in increased drought stress (Cui et al. 2011). The OsDREB2B transcript has a functional and non-functional form. It is marked during drought conditions, and consecutively can increase drought tolerance through alternative splicing induced by its pre-mRNA (Matsukura et al. 2010). All of these results indicate that OsDREB2s also play an essential role in the regulation of drought tolerant.

Huang et al. (2018) identified a new transcription factor gene such as DREB2, namely OsDRAP1 (Responsive Drought Genes AP2/EREBP), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly regulated by drought at DK151, showing its role in drought tolerance rice.

237 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous
238 studies indicated that osmoprotectant substances, namely glycine betaine, plays an essential role in cell stabilization by
239 balancing the structure of the protein quaternary and membrane against the adverse effects of salinity (Sakamoto and
240 Murata 2000; Saxena et al. 2019). Besides, it facilitates osmotic adjustment by reducing the potential of internal osmotic
241 that contributes to the ability of plant cells tolerant to water stress. It also stabilizes the PSII and RuBisCO complexes
242 during photosynthesis under stress conditions (Holmström et al. 2000; Huang et al. 2020). These are the positive effects
243 of exogenous betaine glycine application in plants that grow under the pressure of salinity or drought stress. Plant cells can
244 be protected from adverse effects of salinity induced oxidative stress by exogenous application of glycine betaine (Demiral
245 and Türkan 2004; Saxena et al. 2019).

246 The results of the PCR amplification of 39 selected lines for drought tolerance using DREB2A and BADH2 primers
247 are presented in Table 3. All tested lines showed positive, containing both genes and had criteria with varying degrees
248 from the results of the drought test in the seedling stage. Although the results of the molecular study showed positive
249 results as a drought-tolerant marker gene in the seedling stage, evaluation at the productive stage needs to be done to
250 obtain more accurate data. Drought-tolerant plants can adapt to drought conditions, which are shown on high grain. The
251 use of superior varieties is the most efficient technology to increase rice yield with low-cost production in the dry land.
252 Therefore, developing a superior variety by crossbreeding is needed to produce superior potential lines. Before releasing a
253 new superior variety, potential selected lines need to be tested in various locations.

254 The agronomic performance and yield of 16 superior lines showed that all lines had reached homozygous in the 8th
255 generation (F₇), where the plant height showed a relatively low standard deviation in all lines. The number of panicles
256 ranging from 14.7 had a high yield potential, while the number of filled grains was 150.07 (Table 5). The new paradigm of
257 new rice breeding is the number of productive tillers between 8-12 tillers/hill with the number of grains/panicles ranging
258 from 150-200 grains (Peng and Khush 2003). Peng et al. (2008) stated that in the new type of rice breeding avoid extreme
259 traits such as 200-250 grain/panicle which can produce panicles with low seed filling. Therefore, the increase in the second
260 generation of new types of rice has been modified by IRRI to 150 grains/panicle. Several lines have a potential yield of
261 more than 10 tonnes/ha, namely lines with the accession number BKL1 B-1-259-1 and BKL1 B-3-261-3 have yield
262 potential of 10.05 tonnes/ha and 10.08 tonnes/ha, respectively. Meanwhile, the lines with the BKL2 B-2-264-6 and BKL4
263 B-1-268-10 numbers had a potential yield of more than 12 ton /ha, namely 12.1 and 12.06 tons/ha, respectively (Table 5).
264 These lines have the opportunity to be developed on dry land or as rice on rainfed land because the lines tested were
265 identified as drought resistance (Table 3).

266 ACKNOWLEDGEMENTS

267 Appreciation and thank are expressed to the Directorate of Research and Community Service, Ministry of Education
268 and Culture Republic of Indonesia that provided funding by National Competitive Applied Research with Contract
269 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amandement Contract Number: 165/SP2H/LT/DRPM/2019. Also,
270 thank Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of the Department of Crop
271 Production at the University of Bengkulu for their help to facilitate this research.

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Identification of drought tolerant and molecular analysis of DREB2A and BADH2 genes and yield potential of lines from single crossing Bengkulu local rice varieties

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Manuscript received: 03 10 2020. Revision accepted: 2016.

Abstract. Screening in the seedling stage of 39 progeny of F6 lines to drought stress was carried out in the greenhouse. Drought tolerant and sensitive varieties of IR 20 and Salumpikit, respectively, were used as control plants. The methods for traits identification of leaf curled, dried, and recovery ability after exposure to severe drought for two weeks was following the Standard Evaluation System (SES) developed by IIRI. Molecular analysis to detect the presence of the DREB2A gene was carried out by PCR amplification of genomic DNA using forward- and reverse- oligonucleotide primers of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively, while for BADH2 gene using forward- and reverse- oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and TGTCCCCAGCTGCTTCATCC, respectively. Molecular markers of DREB2A and BADH2 genes were also identified in 39 tested lines with approximately 250 and 2300 bp length, respectively. This study concluded that the progeny of F6 lines generating from the crossing of local varieties of IR7858 and IR148 is the potential to become a drought-tolerant variety of upland rice. Line numbers BKL2 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 tonnes/ha. These line has the potential to be developed on rainfed lowland rice or dry land because it has drought resistance.

Keywords: BADH2, DREB2A, drought tolerance, gene identification, yield potential

Running title: *Identification of DREB2A and BADH2 genes for drought tolerant*

INTRODUCTION

The development of upland rice variety is an alternative to increase national rice production in Indonesia because the extensification of lowland rice is increasingly difficult. This strategy is carried out through optimizing the use of uncultivated lands, where most of them have the potential for upland rice cultivation (Center for Research and Development 2006). The use of superior varieties, which has higher yields and tolerance to various obstacles so that it can adapt well to climate change, is urgently needed to support efforts to increase rice yields in the dry land. Anticipating the impact of climate change on sustainable agricultural systems is carried out to produce technological innovations that are able to overcome and suppress the impacts caused. These technological innovations include superior varieties of drought-tolerant rice. Genetic improvement to obtain superior varieties that are adaptive to the conditions of drought stress is an essential priority in rice breeding programs.

Assembling drought-tolerant rice varieties can be done through crossbreeding, which combines the resistant traits of the parents with other crops that have a high yield. Molecular marker technology can help selection more accurately than conventional. One of the markers related to drought tolerance is the QTL marker (quantitative trait locus) 12.1. The International Rice Research Institute (IRRI) had crossed the Vandana variety of Indian rice with Way Rarem from Indonesia. One of the filial is a crossing number of IR148+, which is derived from IR crossing 79971-B-369-B-B (Mulyaningsih et al. 2010). The crossing population has been showed to contain QTL 12.1 markers. The location of markers is on chromosome 12, between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of these markers can maintain yields in conditions of severe drought stress during the reproductive stage before flowering. In normal conditions, the marker QTL 12.1 did not have a significant effect on some of the parameters observed (Bernier et al. 2007).

The DREB2 gene controls drought stress in plants (Matsukura et al. 2010; Srivastav et al. 2010; Akhtar et al. 2012; Huang et al. 2018). DREBs (Dehydration Responsive Element Bindings) are transcription factor proteins (TFs) that are very important in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The homology of the DREB2 gene in rice is DREB2A (Sakuma et al. 2002). Some of the DREB2A target genes are MT2A, At1g69870, At3g53990, At1g22985, RD29A, LEA14, At2g23120 [9], RD29B, At1g52690, RD17 (Sakuma et al. 2006;

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49 Qin et al. 2011), AtHsfA3, HSP18.2, and Hsp70 (Qin et al. 2011). The importance of the DREB2A gene is because it can
 50 be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-resistant genes.

51 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). The reports
 52 of previous studies indicate that osmoprotectant substances, namely glycine betaine, plays an essential role in cell
 53 stabilization by balancing the structure of the protein quaternary and membrane structure against the adverse effects of
 54 salinity (Sakamoto and Murata 2000; Saxena et al. 2019). Besides, it facilitates osmotic adjustment by lowering the
 55 internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO
 56 complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et
 57 al. 2020). The positive effects of exogenous application of glycine in plants that grow on salinity stress have been shown to
 58 protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

59 Betaine aldehyde dehydrogenase (BADH) is known as a key enzyme for biosynthesis of glycine betaine. Many
 60 researchers have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity,
 61 dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objective of the study was to identify
 62 drought-tolerant traits and molecular analysis of DREB2A and BADH2 genes. The progeny of F6 lines resulted from the
 63 crossing of local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL on
 64 chromosome 12.1.

65 MATERIALS AND METHODS

66 The experiments were conducted at the University of Bengkulu. Screening study was done in the greenhouse of
 67 Agricultural Faculty from Februari to April 2020, while molecular analysis was done in the laboratory of the Department
 68 of Biology from May to July 2020. Plant materials were using the progeny of 39 lines that selected from F6 generations
 69 resulted from the single crossing of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of
 70 IR7858 and IR148+ QTL positive on chromosome 12.1 (Mulyaningsih et al. 2010). Var. Salumpikit and IR 20 as drought-
 71 tolerant and sensitive control varieties (Table 1).

72 **Table 1.** Selected F6 lines for traits and molecular identification of drought-tolerant genes of DREB2A and BADH2

| Lines number | Genotype | Initial Crossing | Line number | Genotype | Initial Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

75 Screening of drought-tolerant rice of 39 F6 lines was carried out following the standard Evaluation System (SES)
 76 developed by IRR (2002). The drought-susceptible variety (IR20) and local drought-tolerant variety (Salumpikit) were
 77 used as control. The test was carried out following the method of Kumar et al. (2015); Swain et al. (2017); Herawati et al.
 78 (2017). Plastic tubs sizes of 40 cm x 25 cm x 20 cm was filled with soil. Then, each tub was planted ten family lines and
 79 two control varieties. Each line was sown for 20 seeds in a row. Seedlings were watered intensively in 2 weeks after
 80 planting. After this stage, watering was stopped until the sensitive plants dried. Drought tolerance assessment was carried
 81 out based on the SES methods, as described in Table 2. Trait responses of the seedlings were recorded, then seedlings
 82

Comment [LC8]: Ada yang missing di sini "among" ...

Comment [LC9]: On chromosome 12.1 (?)

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Comment [LC14]: population

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83 were watered intensively for the next ten days. Recovery ability was recorded following the methods of SES, as described
 84 in Table 2.

85 Genomic DNA was isolated from fresh leaves at 14 days after treatment (DAT). Fragments of 0.1 g of rice leaf were
 86 ground in the mortar by adding liquid nitrogen. Isolation of total DNA was carried out by modifying the protocols of
 87 Wizard's Genomic DNA Purification Kit. The ground leaf was put into a 2 ml tube, then 600 µl of Nuclei Lysis Solution
 88 was added, followed shaking by vortex for 3 seconds. Then, the solution was heated in a water bath at 65°C for 15
 89 minutes. RNase of 3 µl was added followed incubation at 37 °C for 15 minutes. Then, 200 µl Precipitation Solution
 90 was added, and the microtubes were centrifuged for 3 minutes at 13,000 rpm. The supernatants were removed to a 1.5 ml
 91 tube, and 600 µl of isopropanol was added. The microtubes were further centrifuged for 1 minute at room temperature.
 92 The solution was discarded, and DNA remained on the bottom of microtubes was air-dried for 15 minutes. DNA
 93 Rehydration Solution of 100 µl was added and further incubated at 65°C for 1 hour or at 4°C for one night. The total
 94 isolated DNA is used as a template DNA for PCR amplification of DREB2A and BADH2 genes.

95 PCR amplification of the DREB2A gene using forward- and reverse- oligonucleotide primers of
 96 CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
 97 Lathif et al. 2018). While the amplification of the BADH2 gene was using forward- and reverse- oligonucleotide primers
 98 of GGCCAAGTACCTCAAGGCG and TGTCCCCAGCTGCTTCATCC, respectively (Robin et al. 2003). The PCR
 99 mixtures, including the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix), and Taq polymerase,
 100 were developed in the thermocycling. The program was started with denaturation temperature at 94°C for 5 minutes,
 101 followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 2 minutes, and extension at 72°C for 2
 102 minutes, and the final extension at 72°C for 10 minutes. PCR amplification products were subjected to electrophoresis in
 103 agarose gel 1% of TBE buffer to identify successful amplifications. The gel from electrophoresis was immersed in EtBr
 104 1% for 10 minutes, rinsed with ddH₂O for 5 minutes, and visualized under UV transilluminator light.

105 In the season in 2020, a yield test of selected superior lines was carried out in March-July 2020 in Semarang Village,
 106 Bengkulu City. The materials used in this study were 16 selected superior lines in the F7 generation. The experiment was
 107 carried out on a plot measuring 8 m x 6 m with a spacing of 20 x 20 cm, and 1 seed was planted. Fertilize twice, the first
 108 fertilization at the age of 14 days after planting (HST) with a dose of 150 kg/ha of Urea, 100 kg/ha SP36 and 100 kg/ha
 109 KCl. The second fertilization at the age of 30 HST with a dose of 100 kg/ha Urea, 100 kg/ha SP36 and 100 kg/ha KCl.
 110 Intensive control was carried out against weeds, pests and diseases. Observation of the agronomic characters of 10
 111 plant/plot samples taken from each line number. The characters observed included plant height, number of panicles/hill,
 112 panicle length, number of filled grains/panicle, percentage of empty grain/panicle, 1000 grain weight, grain weight per hill,
 113 and yield per plot.

114 RESULTS AND DISCUSSION

115 Identification of drought tolerant traits

116 Screening of F6 lines at the seedling stage was carried out to select the drought-tolerant rice (Kumar et al. 2015; Swain
 117 et al. 2017; Herawati et al. 2017). Drought tolerant assessment following the methods of SES was done by comparing the
 118 treated plants with control varieties of Salumpikit and IR20 (Table 2). The symptoms were identified after exposed to
 119 drought stress for 14 days, including leaf curling, leaf drying, and ability to recover (Figure 1). The criteria of 39 F6 lines
 120 were identified as highly to rather tolerant, tolerant, and moderately tolerant to drought for a total number of 11, 19, and 9
 121 lines, respectively (Table 3). The scores of dry leaf of the 30 lines with highly to rather a tolerance and tolerance were 0-1
 122 which recovery ability was 90 to 100%, while the scores of the rest nine lines with moderate tolerance were 3-5 which
 123 recovery ability was 70 to 90% (Table 4, Figure 1).

124 **Table 2.** Traits identification of selected F6 lines based on criteria description of SES developed by IRRI (2002)

| Score | Criteria | Description | | |
|-------|----------------------|---------------------------------|---|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate tolerant | Leaves fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |
| 7 | Moderate susceptible | Leaf margins touching (O-shape) | More than 2/3 of all leaves fully dried | 20-39% of plants recovered |
| 9 | Susceptible | Leaves tightly rolled (V-shape) | All plants apparently dead. Length in most leaves fully dried | 0-19% of plants recovered |

Comment [LC24]: treatment apa ya? Apakah maksudnya *of 14 day-old seedlings (?)

Comment [LC25]: Cuttings

Comment [LC26]: in the presence of

Comment [LC27]: Eppendorf tube

Comment [LC28]: vortexing

Comment [LC29]: incubated

Comment [LC30]: and

Comment [LC31]: by

Comment [LC32]: and centrifuged

Comment [LC33]: DNA pellet was allowed to remain sit in the bottom ..

Comment [LC34]: and let to air-dried

Comment [LC35]: hapus

Comment [LC36]: used

Comment [LC37]: used

Comment [LC38]: hapus

Comment [LC39]: hapus

Comment [LC40]: Fertilizer application was done twice, ...

Comment [LC41]: Hati2.. karena 'fertilization' artinya penyerbukan

Comment [LC42]: Fertilizer application

Comment [LC43]: Hati2 .. karena 'control sudah digunakan di atas untuk varietas .. tapi maknanya berbeda.

Comment [LC44]: observed characters

Comment [LC45]: bukanlah semua galur, termasuk varietas control, juga diberi treatment

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Comment [LC48]: rather (?) tolerant, and tolerant .

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Comment [LC50]: tolerant

Comment [LC51]: the

Table 3. Screening of the 39 F6 lines for resistant traits and molecular identification of gene markers of DREB2A AND BADH2

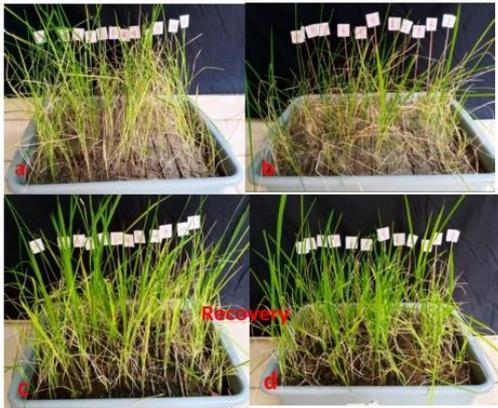
| Line number | Genotype | Crossing | The score of rolling leaf | The score of drought leaf | Score of recovery | Criteria | DREB2A genes | BADH2 genes |
|-------------|------------|--------------------|---------------------------|---------------------------|-------------------|----------|--------------|-------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | RT | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | RT | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | RT | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | + | + |
| S | Salumpikit | Control variety | 1 | 1 | 1 | RT | | |

HT=High Tolerant (6 lines); RT=Rather Tolerant (5 lines); T=Tolerant (19 lines); MT=Moderate Tolerant (9 lines); + = gene was identified

144 **Table 4.** The distribution of 39 F6 lines for identification of drought-tolerant
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| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------------|-----------|-------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Rather Tolerant | Tolerant | Moderate Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | ■ | | | | ■ | | | | |
| 19 | | ■ | | | | ■ | | | |
| 9 | | | ■ | | | | ■ | | |
| IR20 | | | ■ | | | | | | |
| Salumpikit | ■ | | | | | | | | |

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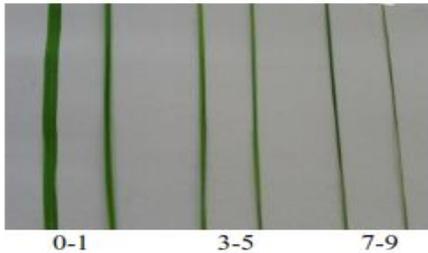


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Figure 1. Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c dan d)



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Figure 2. Description of rolling leaves based on SES Method; scores 0-1: started rolling to form V; 3-5: rolling to form V and U inside leaves; 7-9: rolling leaves totally

Molecular identification of drought tolerant genes

Molecular analysis using PCR products showed that the DREB2A gene was visualized in 39 selected lines with the marker sizes approximately 250 bp (Tawfik et al. 2016; Lathif et al. 2018) (Figure 3). It proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1, which are drought-tolerant generated F6 lines that are the potential to be drought tolerant. This evidence proved that drought tolerance in rice plants is controlled by the DREB2A genes (Akhtar et al. 2012; Huang et al. 2018).

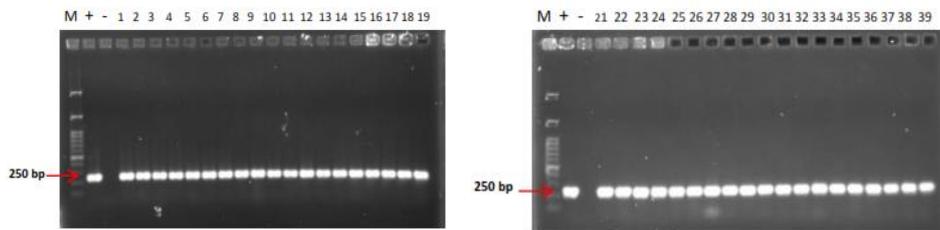


Figure 3. PCR amplification of DREB2A (250 bp) on 39 selected lines from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - =negative control)

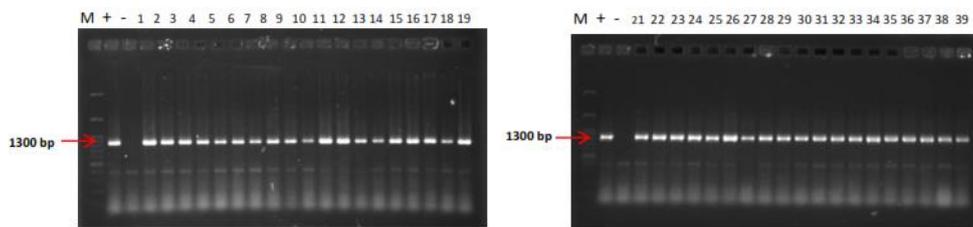


Figure 4. PCR amplification of BADH2 (1300 bp) on 39 selected lines generated from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - = negative control)

Successful use of molecular markers that control complex traits for obtaining drought-tolerant superior rice varieties has been reported by Lanceras et al. (2004). Some of the traits that have been studied include the yield, root length, root thickness, leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017), and osmotic adjustment (Zivcak et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is known as a key enzyme for the biosynthesis of glycine betaine. Many researchers have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). Visualization of the BADH2 gene 39 selected lines showed a marker with a size approximately 1300 bp (Shrestha 2011; Hasthanasombut et al. 2011) (Figure 4).

Performance of agronomic characters, yield and yield potential of superior lines

The appearance of agronomic characters, yields and yield potential of the 16 superior lines tested are presented in Table 5. Almost all tested lines have shown uniformity as shown by the lowest average plant height appearance, namely 101.1 and the highest is 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the 8th generation (F7). The highest average number of panicles/hill was 14.7 and the lowest was 6.5. however, the panicle length ranges from 24.61 - 27.6 cm. The number of filled grains/panicles ranged from 99.5 - 150.07, while the percentage of empty grains rice was categorized as low based on the SES IRRI (2012), which ranged from 9.87% - 26.66%. This led to variations in grain weight per hill, which was around 19-35.5 grams/hill .

Comment [LC52]: hapus

Comment [LC53]: langsung menggunakan istilah F7 generation akan lebih pas

Table 5. The performance of agronomic characters, yields and yield potential of superior lines

| Assesion | Plant height | Number of Panicle/hill | Panicle length (cm) | Number of fill grains | % of empty/panicle | 1000 grains weight (gram) | grains weight/hill (gram) | Yield/p lot(1x1 m ²) (gram) | Yield pote ntial (ton/ ha) |
|---|--------------|------------------------|---------------------|-----------------------|--------------------|---------------------------|---------------------------|---|----------------------------|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |

| | | | | | | | | | |
|-----------------|--------------|--------------|--------------|----------------|---------------|--------------|--------------|------|-------|
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

191 The yield of grain per plot varies from the lowest was 458 grams, and the highest, which was 1210 grams. If seen from
 192 the appearance of agronomic characters, the high grain yield was supported by the characters of the large number of
 193 panicles, the low percentage of empty grain, and the weight of 1000 grains. The length of the panicles did not show any
 194 significant variation, which the range between 24.61-27.6 cm (Table 5).

Comment [LC54]: Seen from the agronomic characters, ..

Comment [LC55]: hapus

Comment [LC56]: hapus

Comment [LC57]: hapus

195 Discussion

196 Seedlings' responses to drought stress were identified after 14 days without water. The tolerant lines continued to grow
 197 well, vigorous, and leaves remained fully open, whereas the moderate tolerant lines were dried on leaf tips (Figure 1).
 198 Kumar et al. (2014) reported that leaf rolling was delayed in drought-tolerant rice genotypes. Leaf rolling in rice plants
 199 was induced by loss of turgor and low osmotic regulation. Delayed leaf rolling in the tolerant genotype showed that the
 200 turgor remained normal, and the plants were protected from dehydration (Figure 2). Leaf rolling is one of the mechanisms
 201 of plants to adjust the water potential, which enables the plants to absorb groundwater in drought stress conditions
 202 (Bunnag and Pongthai 2013; Swain et al. 2017).

203 Swain et al. (2017) reported that during the drought conditions and the level of groundwater was below 30 cm depth,
 204 of the 78 lines of drought-tolerant assessments were identified that 30 lines were scored of 1, and 48 lines were scored of
 205 3. Of these 78 assessments, 13 lines produced more than 1-ton grain/ha, tolerant lines (CR 143-2-2) produced more than
 206 2.7 tons grain/ha, while sensitive plants as a control (IR20) did not produce any.

Comment [LC58]: hapus

207 Leaf rolling can reduce leaf surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This
 208 condition will help plants to survive in a certain period when the availability of water in the environment decreases. The
 209 genes in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the
 210 leucine zipper class IV transcriptional factor homeodomain. Overexpression of these genes results in leaf curling on the
 211 adaxial side, whereas suppression of this gene causes leaf rolling on the abaxial side (Zou et al. 2011). Delaying leaf
 212 rolling indicates that a plant effort to maintain turgor and avoid dehydration. Water shortages tend to have lower rates of
 213 leaf rolling, which appeared in the plants that have tolerant criteria with a Score of 1 (Table 3). It allowed the plant to
 214 survive to drought at the low water potential of leaf tissue (Sevanto 2018). Plants were recovery after passing through a
 215 period of drought indicated the ability of plants to improve their metabolic system (Bian et al. 2017; Wang et al. 2019).

Comment [LC59]: hapus

216 Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the
 217 ancestors with other varieties that have high productivity. The use of molecular marking technology can help selection
 218 more accurate and faster. One of the markers related to drought tolerance is the QTL (quantitative trait locus) 12.1, which
 219 has been produced through the crossing of Vandana varieties of Indian rice and Way Rarem variety from Indonesia
 220 (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study. This marker can
 221 maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL
 222 12.1 did not have a significant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

Comment [LC60]: parents (?)

223 DREBs (Dehydration Responsive Element Bindings) are transcription factor proteins (TFs) that are very important in
 224 regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The DREB2A gene is
 225 essential as a regulator of drought-responsive genes, making it a marker of drought stress-tolerant.

226 Transcription factors in DREB2A are involved in the regulatory mechanisms that exist in some plants in response to
 227 drought, salinity, and heat stress (Lata and Prasad 2011; Mizoi et al. 2012). There are five DREB2 genes in the rice
 228 genome, including OsDREB2A, OsDREB2B, OsDREB2C, OsDREB2E, and OsABI4 (Matsukura et al. 2010; Srivastav et
 229 al. 2010). Expression of OsDREB2A in rice is caused by water deficit and exogenous ABA application, which can result
 230 in increased drought stress (Cui et al. 2011). The OsDREB2B transcript has a functional and non-functional form. It is
 231 marked during drought conditions, and consecutively can increase drought tolerance through alternative splicing induced
 232 by its pre-mRNA (Matsukura et al. 2010). All of these results indicate that OsDREB2s also play an essential role in the
 233 regulation of drought tolerant.

234 Huang et al. (2018) identified a new transcription factor gene such as DREB2, namely OsDRAP1 (Responsive Drought
 235 Genes AP2/EREBP), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly
 236 regulated by drought at DK151, showing its role in drought tolerance rice.

237 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous
238 studies indicated that osmoprotectant substances, namely glycine betaine, plays an essential role in cell stabilization by
239 balancing the structure of the protein quaternary and membrane against the adverse effects of salinity (Sakamoto and
240 Murata 2000; Saxena et al. 2019). Besides, it facilitates osmotic adjustment by reducing the potential of internal osmotic
241 that contributes to the ability of plant cells tolerant to water stress. It also stabilizes the PSII and RuBisCO complexes
242 during photosynthesis under stress conditions (Holmström et al. 2000; Huang et al. 2020). These are the positive effects
243 of exogenous betaine glycine application in plants that grow under the pressure of salinity or drought stress. Plant cells can
244 be protected from adverse effects of salinity induced oxidative stress by exogenous application of glycine betaine (Demiral
245 and Türkan 2004; Saxena et al. 2019).

246 The results of the PCR amplification of 39 selected lines for drought tolerance using DREB2A and BADH2 primers
247 are presented in Table 3. All tested lines showed positive, containing both genes and had criteria with varying degrees
248 from the results of the drought test in the seedling stage. Although the results of the molecular study showed positive
249 results as a drought-tolerant marker gene in the seedling stage, evaluation at the productive stage needs to be done to
250 obtain more accurate data. Drought-tolerant plants can adapt to drought conditions, which are shown on high grain. The
251 use of superior varieties is the most efficient technology to increase rice yield with low-cost production in the dry land.
252 Therefore, developing a superior variety by crossbreeding is needed to produce superior potential lines. Before releasing a
253 new superior variety, potential selected lines need to be tested in various locations.

254 The agronomic performance and yield of 16 superior lines showed that all lines had reached homozygous in the 8th
255 generation (F₇), where the plant height showed a relatively low standard deviation in all lines. The number of panicles
256 ranging from 14.7 had a high yield potential, while the number of filled grains was 150.07 (Table 5). The new paradigm of
257 new rice breeding is the number of productive tillers between 8-12 tillers/hill with the number of grains/panicles ranging
258 from 150-200 grains (Peng and Khush 2003). Peng et al. (2008) stated that in the new type of rice breeding avoid extreme
259 traits such as 200-250 grain/panicle which can produce panicles with low seed filling. Therefore, the increase in the second
260 generation of new types of rice has been modified by IRRI to 150 grains/panicle. Several lines have a potential yield of
261 more than 10 tonnes/ha, namely lines with the assesion number BKL1 B-1-259-1 and BKL1 B-3-261-3 have yield
262 potential of 10.05 tonnes/ha and 10.08 tonnes/ha, respectively. Meanwhile, the lines with the BKL2 B-2-264-6 and BKL4
263 B-1-268-10 numbers had a potential yield of more than 12 ton /ha, namely 12.1 and 12.06 tons/ha, respectively (Table 5).
264 These lines have the opportunity to be developed on dry land or as rice on rainfed land because the lines tested were
265 identified as drought resistance (Table 3).

Comment [LC61]: hapus

Comment [LC62]: they

266 ACKNOWLEDGEMENTS

267 Appreciation and thank are expressed to the Directorate of Research and Community Service, Ministry of Education
268 and Culture Republic of Indonesia that provided funding by National Competitive Applied Research with Contract
269 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amandement Contract Number: 165/SP2H/LT/DRPM/2019. Also,
270 thank Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of the Department of Crop
271 Production at the University of Bengkulu for their help to facilitate this research.

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Molecular analysis of DREB2A and BADH2 genes and yield potential derived from single-cross of Bengkulu local rice varieties

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Manuscript received: 03 10 2020. Revision accepted: 20.

Abstract. This study aims to identify drought-tolerant and molecular analysis of DREB2A and BADH2 genes and yield potential from single-crossing varieties of rice in Bengkulu. The sensitive varieties of IR 20 and Salumpikit were used as control plants in the screening and seedling stages of the 39 F6 progeny lines, which were carried out in the greenhouse. In addition, the Standard Evaluation System (SES) developed by IRRI was used to the recovery ability of the curled and dried leaves after two weeks. The molecular analysis used to detect the presence of the DREB2A gene was carried out by PCR amplification and genomic DNA using the forward and reverse oligonucleotide primers consisting of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively. Meanwhile, for the BADH2 gene, the forward and reverse oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and TGTCCCAGCTGCTTCATCC were used respectively. Molecular markers of DREB2A and BADH2 genes were also identified in 39 tested lines with approximately 250 and 2300 bp length. This result showed that the progeny of F6 lines generated from crossing local varieties of IR7858 and IR148 is potential to becoming a drought-tolerant variety of upland rice. Line numbers BKL2 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 tonnes/ha and can be developed on rain field, low or dry land due to its drought resistance.

Keywords: BADH2, DREB2A, drought tolerance, gene identification, yield potential

Running title: *Identification of DREB2A and BADH2 genes for drought tolerant*

INTRODUCTION

Upland rice cultivation is an alternative means rice production used to increase the yearly number of rice in Indonesia, which has significantly decreased due to the rising difficulty associated with the extensification of lowlands. According to the Center for Research and Development (2006), this technique is carried out by optimizing the use of uncultivated lands, where the majorities have the potential for upland rice cultivation. The use of superior varieties with higher yields and tolerance to various obstacles is urgently needed to support efforts to increase rice yields in the dry land. Furthermore, it is important to anticipate the impact of climate change on sustainable agricultural systems to produce technological innovations that can overcome and suppress the impacts caused, such as the superior varieties of drought-tolerant rice. The genetic improvement to obtain superior varieties that are adaptive to the conditions of drought stress is an essential priority in rice breeding programs.

Crossbreeding is used to assemble drought-tolerant rice varieties, which combines the resistant traits of the parents with other high yield crops. Molecular marker technology helps in selecting more accurate than conventional areas, and one of the markers related to drought tolerance is the QTL marker (quantitative trait locus) 12.1. Furthermore, the International Rice Research Institute (IRRI) crossed the Vandana variety of Indian rice using Way Rarem from Indonesia, such as the filial with crossing number of IR148+, derived from IR crossing 79971-B-369-B-B (Mulyaningsih et al., 2010). The crossing population contains QTL 12.1 markers, with the location on chromosome 12, as well as between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of these markers maintains yields before flowering and during severe drought stress at the reproductive stage. In normal conditions, the marker QTL 12.1 had an insignificant effect on some of the parameters observed (Bernier et al. 2007).

According to Matsukura et al. (2010), Srivastav et al. (2010), Akhtar et al. (2012), and Huang et al. (2018) the DREB2 gene controls drought stress in plants. DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). Sakuma et al. (2002) stated that the homology of the DREB2 gene in rice is DREB2A. Some of the DREB2A target genes are MT2A, At1g69870, At3g53990, At1g22985, RD29A, LEA14, At2g23120, RD29B, At1g52690, RD17 (Sakuma et al. 2006; Qin et al. 2011), AtHsfA3, HSP18.2, and Hsp70 (Qin et al. 2011). The importance of the DREB2A gene is because it can be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-resistant genes.

51 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous
 52 studies carried out by Sakamoto & Murata (2000) and Saxena et al. (2019) indicated that osmoprotectant substances, such
 53 as glycine betaine, plays an essential role in cell stabilization by balancing the structure of the protein quaternary and
 54 membrane structure against the adverse effects of salinity. Besides, it facilitates osmotic adjustment by lowering the
 55 internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO
 56 complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et
 57 al. 2020). The positive effects and exogenous application of glycine in plants that grow on salinity stress have been shown
 58 to protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

59 Betaine aldehyde dehydrogenase (BADH) is a key enzyme for biosynthesis of glycine betaine. Several studies have
 60 reported the accumulation ability of glycine betaine and BADH1 gene expression in tolerating salinity, dryness, and low
 61 temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objective of this study is to identify drought-tolerant traits and
 62 molecular analysis of DREB2A and BADH2 genes using the progeny of F6 lines from the crossing of local varieties
 63 (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL on the chromosome 12.1.
 64
 65

MATERIALS AND METHODS

66 This research was carried out at the University of Bengkulu, with drought evaluation conducted at the greenhouse of
 67 Agricultural Faculty from February to April 2020, while molecular analysis was done in the laboratory of the Department
 68 of Biology from May to July 2020. The plant materials used are the 39 lines selected from F₆ populations leading to the
 69 single cross of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ QTL
 70 positive on chromosome 12.1 (Mulyaningsih et al. 2010). Var. Salumpikit and IR 20 were used as drought-tolerant and
 71 sensitive control varieties respectively (Table 1).
 72

73 **Table 1:** The selected F6 lines for traits and molecular identification of drought-tolerant genes of DREB2A and BADH2
 74

| Lines number | Genotype | Crossing | Line number | Genotype | Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

75
 76 The standard Evaluation System (SES) developed by IRRI (2002) was used to evaluate the drought-tolerant of 39 F6
 77 lines with the drought-susceptible variety (IR20), and local drought-tolerant variety (Salumpikit) used as control.
 78 Furthermore, the test was carried out in accordance with Kumar et al. (2015), Swain et al. (2017), and Herawati et al.
 79 (2017) methods with plastic tubs sizes of 40 cm x 25 cm x 20 cm filled with soil. Each tub was planted using ten family
 80 lines and two control varieties, with each line sown for 20 seeds in a row. After planting, the seedlings were intensively
 81 watered for 2 weeks with the plants allowed to dry to determine its sensitivity. Drought tolerance assessment was carried
 82 out based on the SES methods, as shown in Table 2. Furthermore, the trait responses of the seedlings were recorded,
 83 followed by intensive watering for the next ten days. Recovery ability was recorded following the methods of SES, as
 84 shown in Table 2.

85 Genomic DNA was isolated from fresh leaves 14 days after treatment (DAT) with cuttings of 0.1 g of rice leaf ground
 86 in the mortar by adding liquid nitrogen. The total DNA was isolated by modifying the protocols of Wizard's Genomic
 87 DNA Purification Kit, and the ground leaf was put into a 2 ml eppendorf tube, before adding and shaking 600 µl of Nuclei
 88 Lysis Solution for 3 seconds. In addition, the solution was heated in a water bath at 65°C for 15 minutes, followed by the
 89 addition and incubation of 3 µl RNase at 37 ° C for 15 minutes. This was followed by the addition of 200 µl Precipitation
 90 Solution, with the microtubes centrifuged for 3 minutes at 13,000 rpm. The supernatants were collected into a 1.5 ml tube,
 91 before the addition of 600 µl of isopropanol. Furthermore, the microtubes were further centrifuged for 1 minute at room
 92 temperature with the solution discarded, while the remaining DNA on the bottom of microtubes was air-dried for 15
 93 minutes. DNA Rehydration Solution of 100 µl was added and further incubated at 65°C for 1 hour or at 4°C for one night.
 94 The total isolated DNA is used as a template for PCR amplification of DREB2A and BADH2 genes.

95 PCR amplification of the DREB2A gene was determined using the forward and reverse oligonucleotide primers of
 96 CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
 97 Lathif et al. 2018). Meanwhile, the amplification of the BADH2 gene was carried out using forward- and reverse-
 98 oligonucleotide primers of GGCCAAGTACCTCAAGGCG and TGTCCCCAGCTGCTTCATCC (Robin et al. 2003).
 99 The PCR mixtures, which comprise of the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix),
 100 and Taq polymerase, were developed during the thermocycling process. The program was carried out using a denaturation
 101 temperature at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 2
 102 minutes, and extension at 72°C for 2 minutes, and the final extension at 72°C for 10 minutes. PCR amplification products
 103 were subjected to electrophoresis in agarose gel 1% of TBE buffer to identify successful amplifications. The gel from
 104 electrophoresis was immersed in EtBr 1% for 10 minutes, rinsed with ddH2O for 5 minutes, and visualized under UV
 105 transilluminator light.

106 A yield test of selected superior lines was carried out from March-July 2020 in Semarang Village, Bengkulu City. The
 107 materials used in this study were 16 selected superior lines in the F₇ generation with the experiment carried out on a plot
 108 measuring 8 m x 6 m, a spacing of 20 x 20 cm, and by planting a seed. Fertilizer application was done twice, the first
 109 fertilizer was carried out at the age of 14 days after planting (HST) with a dose of 150 kg/ha of Urea, 100 kg/ha SP36 and
 110 100 kg/ha KCl, and the second fertilizer was carried out at the age of 30 HST with a dose of 100 kg/ha urea, 100 kg/ha
 111 SP36 and 100 kg/ha KCl. Furthermore, intensive control was carried out against weeds, pests and diseases, while
 112 observation of the agronomic characters of 10 plant/plot samples was taken from each line number. The observed
 113 characters included plant height, number of panicles/hill, panicle length, number of filled grains/panicle, percentage of
 114 empty grain/panicle, 1000 grain weight, grain weight per hill, and yield per plot.

115 RESULTS AND DISCUSSION

116 Identification of drought-tolerant traits

117 Screening of F₆ lines at the seedling stage was carried out to select the drought-tolerant rice (Kumar et al., 2015; Swain
 118 et al. 2017; Herawati et al. 2017). Furthermore, Table 2 shows the drought-tolerant assessment carried out with the SES
 119 methods by comparing the treated plants with control varieties of Salumpikit and IR20. The symptoms, such as leaf
 120 curing, drying and recovery ability, were identified after exposure to drought stress for 14 days, as shown in Figure 1. The
 121 criteria of 39 F₆ lines were identified as highly to moderately tolerant drought for a total number of 11, 19, and 9 lines,
 122 respectively, as shown in Table 3. The scores of dry leaf of the 30 lines with high tolerant level were 0-1 with recovery
 123 ability of 90 to 100%, while the scores of the rest nine lines with moderate tolerant were 3-5, with recovery ability of 70 to
 124 90% as shown in Table 4 and Figure 1.

125
 126 **Table 2:** Traits identification of selected F₆ lines based on criteria description of SES developed by IRRI (2002)
 127

| Score | Criteria | Description | | |
|-------|----------------------|---------------------------------|--|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate tolerant | Leaves fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |
| 7 | Moderate susceptible | Leaf margins touching (0-shape) | More than 2/3 of all leaves fully dried | 20-39% of plants recovered |
| 9 | Susceptible | Leaves tightly rolled (V-shape) | All plants were dead. Length in most leaves thoroughly dried | 0-19% of plants recovered |

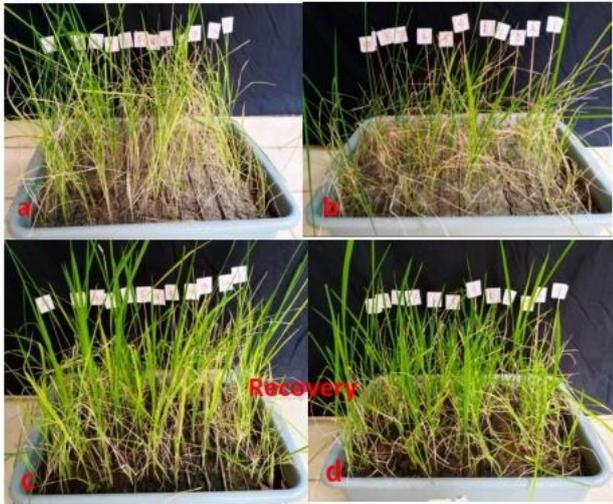
128
 129
 130
 131 **Table 3:** Screening of the 39 F₆ lines for resistant traits and molecular identification of gene markers of DREB2A AND BADH2

| Line number | Genotype | Crossing | The score of rolling leaf | The score of drought leaf | Score of recovery | Criteria | DREB2A genes | BADH2 genes |
|-------------|------------|--------------------|---------------------------|---------------------------|-------------------|----------|--------------|-------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | RT | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | RT | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | RT | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | + | + |
| S | Salumpikit | Control variety | 1 | 1 | 1 | RT | | |

HT=High Tolerant (6 lines); RT=Rather Tolerant (5 lines); T= Tolerant (19 lines); MT= Moderate Tolerant (9 lines); + = gene was identified

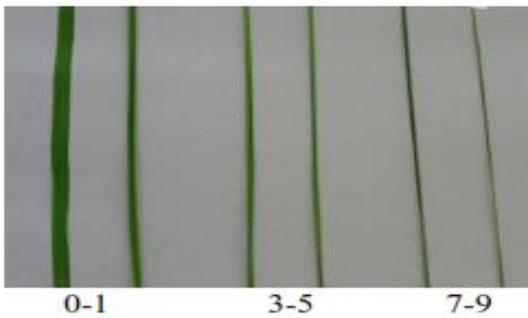
Table 4: The distribution of 39 F6 lines for identification of drought-tolerant

| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------------|-----------|-------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Rather Tolerant | Tolerant | Moderate Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | ■ | | | | ■ | | | | |
| 19 | | ■ | | | | ■ | | | |
| 9 | | | ■ | | | | ■ | | |



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Figure 1: Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c and d)

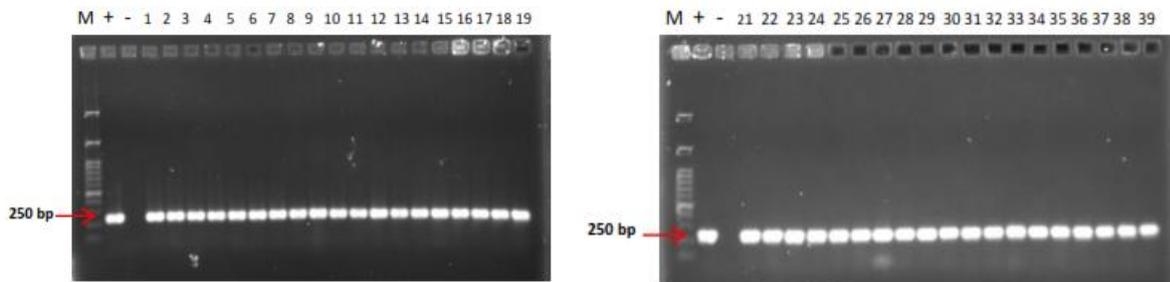


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Figure 2: Description of rolling leaves based on SES Method; scores 0-1: started rolling to form V; 3-5: rolling to form V and U inside leaves; 7-9: rolling leaves totally

Molecular identification of drought-tolerant genes

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The molecular analysis using PCR products showed that the DREB2A gene was visualized in 39 selected lines with the marker sizes approximately 250 bp as shown in Figure 3 (Tawfik et al. 2016; Lathif et al. 2018). This proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1, which are drought-tolerant generated from F6 lines. This evidence proved that drought tolerance in rice plants is controlled by the DREB2A genes (Akhtar et al. 2012; Huang et al. 2018).



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Figure 3: PCR amplification of DREB2A (250 bp) on 39 selected lines from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, += positive control, -=negative control)

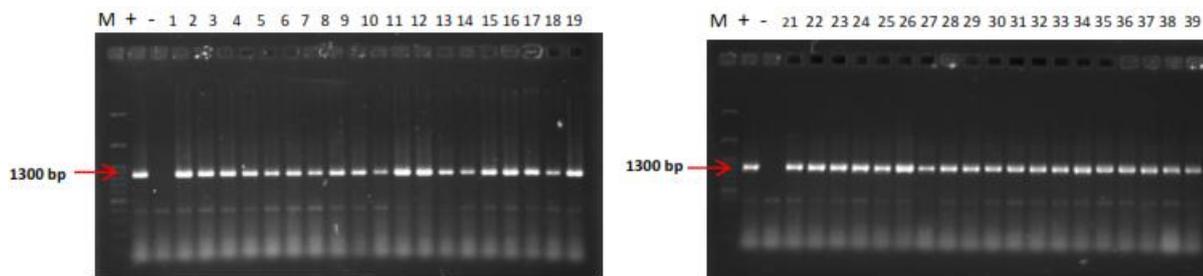


Figure 4: PCR amplification of BADH2 (1300 bp) on 39 selected lines generated from a single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - = negative control)

The successful use of molecular markers that control complex traits for obtaining varieties of drought-tolerant superior rice have been reported by Lanceras et al. (2004). Some of the studied traits include the yield, root length, thickness, leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017) and osmotic adjustment (Zivcak et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is a key enzyme for the biosynthesis of glycine betaine. This is because several studies have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). Furthermore, the visualization of the BADH2 gene 39 selected lines showed a marker with a size of approximately 1300 bp (Shrestha 2011; Hasthanasombut et al. 2011) as shown in Figure 4.

Performance of agronomic characters, yield and yield potential of superior lines

The appearance of agronomic characters and yield potential of the 16 tested superior lines are shown in Table 5. Almost all tested lines have indicated uniformity as shown by the lowest and highest average plant height, of 101.1 and 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the F₇ generation. Furthermore, the highest and lowest average number of panicles/hill were 14.7 and 6.5, with the length ranging from 24.61 - 27.6 cm. Furthermore, the number of filled grains/panicles ranged from 99.5 - 150.07, while the percentage of empty grains rice was categorized as low based on the SES IRRI (2012), which ranged from 9.87% - 26.66%. This led to variations in grain weight per hill, which was approximately 19-35.5 grams/hill.

Table 5: The performance of agronomic characters, yields and yield potential of superior lines

| Assesion | Plant height | Number of Panicle/hill | Panicle length (cm) | Number of fill grains | % of empty/panicle | 1000 grains weight (gram) | grains weight/hill (gram) | Yield/p lot(1x1 m ²) (gram) | Yield potential (ton/ha) |
|------------------------------------|--------------|------------------------|---------------------|-----------------------|--------------------|---------------------------|---------------------------|---|--------------------------|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

The yield of grain per plot varies from the lowest at 458 grams to the highest, at 1210 grams. When determined from the appearance of agronomic characters, the high grain yield was supported by the characters of a large number of

183 panicles, the low percentage of empty grain, and the weight of 1000 grains. The length of the panicles did not show any
184 significant variation and range from 24.61-27.6 cm, as shown in Table 5.

185 Discussion

186 Seedlings' responses to drought stress were identified after 14 days without water. Furthermore, the tolerant lines
187 continued to grow adequately and leaves remained fully open, whereas the moderate tolerant lines were dried on leaf tips,
188 as shown in Figure 1. Kumar et al. (2014) stated that leaf rolling was delayed in drought-tolerant rice genotypes and
189 induced by loss of turgor as well as low osmotic regulation. Delayed leaf rolling in the tolerant genotype indicated that the
190 turgor remained normal, and the plants were protected from dehydration, as shown in Figure 2. According to Bunnag &
191 Pongthai (2013) and Swain et al., (2017), leaf rolling is one of the mechanisms used by plants to adjust the water
192 potential, and enable them to absorb groundwater in drought stress conditions. Swain et al. (2017) reported that during
193 drought conditions, the level of groundwater was below 30 cm depth, of the 78 lines of drought-tolerant assessments.
194 Furthermore, 30 and 48 lines were scored by 1, and 3, respectively. Out of these 78 assessments, 13 and tolerant (CR 143-
195 2-2) lines produced more than 1-ton grain/ha, and 2.7 tons grain/ha, while sensitive plants as a control (IR20) did not
196 produce any.

197 Leaf rolling can reduce the surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This
198 condition helps plants to survive in a certain period when the availability of water in the environment decreases. The genes
199 in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the leucine
200 zipper class IV transcriptional factor homeodomain. According to Zou et al. (2011), overexpression and suppression of
201 these genes lead to leaf curling on the adaxial and abaxial sides of the plants. Delay in leaf rolling indicates a plant effort to
202 maintain turgor and avoid dehydration. Water shortages tend to have lower rates of leaf rolling, which appears in plants
203 with tolerant criteria score of 1, as shown in Table 3. It allowed the plant to survive to drought at the low water potential of
204 leaf tissue (Sevanto, 2018). Furthermore, plants' recovery after passing through a period of drought, thereby indicating the
205 ability of plants to enhance their metabolic system (Bian et al. 2017; Wang et al. 2019).

206 Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the
207 parents with other varieties that have high productivity. The use of molecular marking technology can help hasten the
208 selection process, thereby making it accurate and faster. One of the markers related to drought tolerance is the QTL
209 (quantitative trait locus) 12.1, which was produced by crossing the Vandana varieties of Indian rice and Indonesian Way
210 Rarem (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study due to its ability to
211 maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL 12.1
212 had an insignificant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

213 DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating
214 the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The DREB2A gene is essential as a
215 regulator of drought-responsive genes, making it a marker of drought stress-tolerant. The transcription factors in DREB2A
216 are involved in the regulatory mechanisms that exist in some plants in response to drought, salinity, and heat stress (Lata
217 and Prasad 2011; Mizoi et al. 2012). There are five DREB2 genes in the rice genome, including OsDREB2A, OsDREB2B,
218 OsDREB2C, OsDREB2E, and OsABI4 (Matsukura et al. 2010; Srivastav et al. 2010). Expression of OsDREB2A in rice is
219 caused by water deficit and exogenous ABA application, which leads to increased drought stress (Cui et al. 2011). The
220 OsDREB2B transcript has a functional and non-functional form marked during drought conditions. Consequently, it can
221 increase drought tolerance through alternative splicing induced by its pre-mRNA (Matsukura et al. 2010). All of these
222 results indicate that OsDREB2s also play an essential role in the regulation of drought tolerance.

223 Huang et al. (2018) identified a new transcription factor gene such as DREB2, namely OsDRAP1 (Responsive Drought
224 Genes AP2/EREBP), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly
225 regulated by drought at DK151, thereby showing its role in drought tolerance rice. According to osmotic adjustment in
226 cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous studies indicated that
227 osmoprotectant substances, namely glycine betaine, plays an essential role in cell stabilization by balancing the structure of
228 the protein quaternary and membrane against the adverse effects of salinity (Sakamoto and Murata 2000; Saxena et al.
229 2019). In addition, it facilitates osmotic adjustment by reducing the internal occurrence possibility that contributes to the
230 ability of plant cells to be tolerant to water stress. It also stabilizes the PSII and RuBisCO complexes during
231 photosynthesis under stress conditions (Holmström et al. 2000; Huang et al. 2020). These are some of the positive effects
232 of the application of exogenous betaine glycine in plants that grow under the pressure of salinity or drought stress. Plant
233 cells can be protected from adverse effects of salinity induced oxidative stress by exogenous application of glycine betaine
234 (Demiral and Türkan 2004; Saxena et al. 2019).

235 The PCR amplification of 39 selected lines for drought tolerance using DREB2A and BADH2 primers are shown in
236 Table 3. All tested lines showed positive, containing both genes and had criteria with varying degrees from the results of
237 the drought test in the seedling stage. However, the results of the molecular study showed positive results as a drought-
238 tolerant marker gene in the seedling stage, evaluation at the productive stage needs to be carried out to obtain more
239 accurate data. Drought-tolerant plants can adapt to drought conditions, which are shown on high grain. The use of superior
240 varieties is the most efficient technology to increase rice yield with low-cost production in the dry land. Therefore,

241 developing a superior variety by crossbreeding is needed to produce superior potential lines. Furthermore, before releasing
242 a new superior variety, potential selected lines need to be tested in various locations.

243 The agronomic performance and yield of 16 superior lines showed that all lines reached homozygous in the F₇
244 generation, where the plant height had a relatively low standard deviation. The number of panicles ranging from 14.7 had a
245 high yield potential, with the value of a filled grain of 150.07, as shown in Table 5. The new paradigm of rice breeding is
246 the number of productive tillers between 8-12 tillers/hill with the grains/panicles ranging from 150-200 (Peng and Khush
247 2003). Peng et al. (2008) stated that in the new type of rice breeding avoid extreme traits, such as 200-250 grain/panicle
248 which can produce panicles with low seed filling. Therefore, the increase in the second generation of new types of rice has
249 been modified by IRRI to 150 grains/panicle. Several lines have a potential yield of more than 10 tonnes/ha, such as those
250 with the assesion number BKL1 B-1-259-1 and BKL1 B-3-261-3 to yield potential of 10.05 tonnes/ha and 10.08
251 tonnes/ha, respectively. Meanwhile, the lines with the BKL2 B-2-264-6 and BKL4 B-1-268-10 numbers had a potential
252 yield of more than 12 ton /ha, namely 12.1 and 12.06 tons/ha, as shown in Table 5. These lines have the opportunity to be
253 developed on dry land or on rainfed land because they were identified as drought resistance, as shown inTable 3.

254 ACKNOWLEDGEMENTS

255 The authors are grateful to the Directorate of Research and Community Service, Ministry of Education and Culture
256 Republic of Indonesia for funding this research through the National Competitive Applied Research with Contract
257 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amendment Contract Number: 165/SP2H/LT/DRPM/2019. The
258 authors are also, grateful to the Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of
259 the Department of Crop Production at the University of Bengkulu for their help in facilitating this research.

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Best regards,

Reny Herawati, et al

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Reny Herawati <reny.herawati@unib.ac.id>
Kepada: Smujo Editors <editors@smujo.id>

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Dear editor,

We have submitted revision by sistem since 25 Nov 2020, but we don't have any notif until now...

[Kutipan teks disembunyikan]



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[biodiv] New notification from Biodiversitas Journal of Biological Diversity

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Smujo Editors <smujo.id@gmail.com>

8 Januari 2021 16.31

Balas Ke: Smujo Editors <editors@smujo.id>

Kepada: Reny Herawati <reny.herawati@unib.ac.id>

You have a new notification from Biodiversitas Journal of Biological Diversity:

There is new activity in the discussion titled "Supporting documents previously requested (proofreading, reviewer, certificate, and turnitin check)" regarding the submission "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potential of Lines from Single Crossing Bengkulu Local Rice Varieties".

Link: <https://smujo.id/biodiv/authorDashboard/submission/6884>

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[biodiv] Editor Decision

1 pesan

Smujo Editors <smujo.id@gmail.com>
Balas Ke: Smujo Editors <editors@smujo.id>
Kepada: Reny Herawati <reny.herawati@unib.ac.id>

13 Januari 2021 22.53

Reny Herawati:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potential of Lines from Single Crossing Bengkulu Local Rice Varieties".

Our decision is: Revisions Required

Smujo Editors
editors@smujo.id

Reviewer A:

Dear Athors

After reviewing the manuscript, i reached the decision that:

1. The manuscript fulfill the science contribution and significances, therefore it feasible for publishing in Biodiversitas Journal.
2. There are still many comments that should be clariffied (see the reviewed version of the manuscript), and and the revision should be resubmitted.
3. Pay attention in the results parts concerning the references!
4. Thank you

Recommendation: Resubmit for Review

Reviewer C:

This is an extensive work with highly sufficient data for publication as a full research article. The methods are appropriate, sophisticated and robust. The results are well presented and discussion is comprehensive. The novelty of the research lies on the drought tolerance genes found to be present on the advanced rice lines tested in this study, but there are some issues that need to be explained regarding the association between tolerance markers and the phenotypic drought tolerance level observed in the seedling stage, as commented in the manuscript. In addition, many grammatical and editorial corrections are suggested. These issues need to be solved before further process of publication

Recommendation: Revisions Required

Identification of *DREB2A* and *BADH2* genes, and yield potential from single-crossing varieties of rice in Bengkulu, Indonesia

Abstract. This study aims to identify drought-tolerant and molecular analysis of *DREB2A* and *BADH2* genes, as well as yield potential from single-crossing varieties of rice in Bengkulu. The sensitive varieties of IR 20 and Salumpikit were used as control plants in the screening and seedling stages of the 39 F6 progeny lines, which were carried out in the greenhouse. In addition, The Standard Evaluation System (SES) developed by IRRI was used to evaluate the recovery ability of the curled and dried leaves after two weeks. The molecular analysis used to detect the presence of the *DREB2A* gene was carried out by PCR amplification of the genomic DNA using the forward and reverse oligonucleotide primers consisting of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively. Meanwhile, for the *BADH2* gene, the forward and reverse oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and TGTCCCAGCTGCTTCATCC were used respectively. Molecular markers of *DREB2A* and *BADH2* genes were also identified in 39 tested lines with approximately 250 and 2300 bp length. This result showed that the progeny of F6 lines generated from the crossing of the local varieties of IR7858 and IR148 is potential to become a drought-tolerant variety of upland rice. Line numbers BKL2 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 t_{onnes} ha⁻¹ and can be developed on rain field, low or dry land due to its drought resistance.

Keywords: *BADH2*, *DREB2A*, drought tolerance, gene identification, yield potential

Running title: Identification of *DREB2A* and *BADH2* genes for drought tolerant

INTRODUCTION

Upland rice cultivation is an alternative means rice production used to increase the yearly rice production number of rice in Indonesia, which has significantly decreased due to the rising difficulty associated with the extensification of lowlands. According to the Center for Research and Development (2006), this technique is carried out by optimizing the use of uncultivated lands, where the majorities have the potential for upland rice cultivation. The use of superior varieties with higher yields and tolerance to various obstacles is urgently needed to support efforts to increase rice yields in the dry lands. Furthermore, it is important to anticipate the impact of climate change on sustainable agricultural systems by producing technological innovations that can overcome and suppress the impacts caused, such as by assembling the superior varieties of drought-tolerant rice. The genetic improvement to obtain superior varieties that are adaptive to the conditions of drought stress is an essential priority in rice breeding programs.

Crossbreeding is used to assemble drought-tolerant rice varieties, which combines the resistant traits of the parents with other high yield crops. Molecular marker technology helps in selecting more accurate than conventional areas, and one of the markers related to drought tolerance is the QTL marker (quantitative trait locus) 12.1. Furthermore, the International Rice Research Institute (IRRI) crossed the Vandana variety of Indian rice using Way Rarem from Indonesia, such as the filial with crossing number of IR148+, derived from IR crossing 79971-B-369-B-B (Mulyaningsih et al., 2010). The crossing population contains QTL 12.1 markers, with the location on chromosome 12, as well as between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of these markers maintains yields before flowering and during severe drought stress at the reproductive stage. In normal conditions, the marker QTL 12.1 had an insignificant effect on some of the parameters observed (Bernier et al. 2007).

According to Matsukura et al. (2010), Srivastav et al. (2010), Akhtar et al. (2012), and Huang et al. (2018) the *DREB2* gene controls drought stress in plants. DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). Sakuma et al. (2002) stated that the homologs of the *DREB2* gene in rice is *DREB2A*. Some of the *DREB2A* target genes are *MT2A*, *At1g69870*, *At3g53990*, *At1g22985*, *RD29A*, *LEA14*, *At2g23120* [9], *RD29B*, *At1g52690*, *RD17* (Sakuma et al. 2006; Qin et al. 2011), *AtHsfA3*, *HSP18.2*, and *Hsp70* (Qin et al. 2011). The importance of the *DREB2A* gene is

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50 because it can be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-
51 resistant genes.

52 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous
53 studies carried out by Sakamoto & Murata (2000) and Saxena et al. (2019) indicated that osmoprotectant substances, such
54 as glycine betaine, plays an essential role in cell stabilization by balancing the structure of the protein quaternary and
55 membrane structure against the adverse effects of salinity. Besides, it facilitates osmotic adjustment by lowering the
56 internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO
57 complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et
58 al. 2020). The positive effects and exogenous application of glycine in plants that grow on salinity stress have been shown
59 to protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

60 Betaine aldehyde dehydrogenase (BADH) is a key enzyme for biosynthesis of glycine betaine. Several studies have
61 reported the accumulation ability of glycine betaine and *BADH1* gene expression in tolerating salinity, dryness, and low
62 temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objective of this study is to identify drought-tolerant traits and
63 molecular analysis of *DREB2A* and *BADH2* genes in using the progeny of F6 lines derived from the crossing of local
64 varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL on the chromosome.

65 MATERIALS AND METHODS

66 Plant materials

67 This research was carried out at the University of Bengkulu, with screening conducted at the greenhouse of
68 Agricultural Faculty from February to April 2020, while molecular analysis was done in the laboratory of the Department
69 of Biology from May to July 2020. The plant materials used are the progeny of 39 lines selected from F6 generations
70 leading to the single crossing of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858
71 and IR148+ QTL positive on chromosome 12.1 (Mulyaningsih et al. 2010). Table 1 shows the var. Salumpikit and IR 20
72 as drought-tolerant and sensitive controls.

73 **Table 1:** Selected F6 lines for traits and molecular identification of drought-tolerant genes of *DREB2A* and *BADH2*

| Lines number | Genotype | Initial Crossing | Line number | Genotype | Initial Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

77 Drought tolerance screening

78 The standard Evaluation System (SES) developed by IRRI (2002) was used to screen the drought-tolerant rice of 39 F6
79 lines with the drought-susceptible variety (IR20), and local drought-tolerant variety (Salumpikit) used as control.
80 Furthermore, the test was carried out in accordance with Kumar et al. (2015), Swain et al. (2017), and Herawati et al.
81 (2017) methods with plastic tubs sizes of 40 cm x 25 cm x 20 cm filled with soil. Each tub was planted using ten family
82 lines and two control varieties, with each line sown for 20 seeds in a row. After planting, the seedlings were intensively
83

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84 watered for 2 weeks with the plants allowed to dry to determine its sensitivity. Drought tolerance assessment was carried
 85 out based on the SES methods, as shown in Table 2. Furthermore, the trait responses of the seedlings were recorded,
 86 followed by intensive watering for the next ten days. Recovery ability was recorded following the methods of SES, as
 87 shown in Table 2.

88 DNA extraction

89 Genomic DNA was isolated from fresh leaves 14 days after treatment (DAT) with fragments of 0.1 g of rice leaf
 90 ground in the mortar by adding liquid nitrogen. The total DNA was isolated by modifying the protocols of Wizard's
 91 Genomic DNA Purification Kit, and the ground leaf was put into a 2 ml tube, before adding and shaking 600 µl of Nuclei
 92 Lysis Solution for 3 seconds. In addition, the solution was heated in a water bath at 65°C for 15 minutes, followed by the
 93 addition and incubation of 3 µl RNase at 37 ° C for 15 minutes. This was followed by the addition of 200 µl Precipitation
 94 Solution, with the microtubes centrifuged for 3 minutes at 13,000 rpm. The supernatants were collected into a 1.5 ml tube,
 95 before the addition of 600 µl of isopropanol. Furthermore, the microtubes were further centrifuged for 1 minute at room
 96 temperature with the solution discarded, while the remaining DNA on the bottom of microtubes was air-dried for 15
 97 minutes. DNA Rehydration Solution of 100 µl was added and further incubated at 65°C for 1 hour or at 4°C for one night.
 98 The total isolated DNA is used as a template for PCR amplification of *DREB2A* and *BADH2* genes.

99 DNA amplification and gel electrophoresis

100 PCR amplification of the *DREB2A* gene was determined using the forward and reverse oligonucleotide primers of
 101 CCTATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
 102 Lathif et al. 2018). Meanwhile, the amplification of the *BADH2* gene was carried out using forward- and reverse-
 103 oligonucleotide primers of GGCCAAGTACCTCAAGGCG and TGTCCCCAGCTGCTTCATCC (Robin et al. 2003).
 104 The PCR mixtures, which comprise of the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix),
 105 and Taq polymerase, were developed during the thermocycling process. The program was carried out using a denaturation
 106 temperature at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 2
 107 minutes, and extension at 72°C for 2 minutes, and the final extension at 72°C for 10 minutes. PCR amplification products
 108 were subjected to electrophoresis in agarose gel 1% of TBE buffer to identify successful amplifications. The gel from
 109 electrophoresis was immersed in EtBr 1% for 10 minutes, rinsed with ddH₂O for 5 minutes, and visualized under UV
 110 transilluminator light.

111 Field experiment and yield potential evaluation

112 A yield test of selected superior lines was carried out from March-July 2020 in Semarang Village, Bengkulu City. The
 113 materials used in this study were 16 selected superior lines in the F7 generation with the experiment carried out on a plot
 114 measuring 8 m x 6 m, a spacing of 20 x 20 cm, and by planting a seed. The first fertilization process was carried out twice
 115 after planting (HST) for 14 days with a dose of 150 kg ha^{-1} of Urea, 100 kg/ha SP36 and 100 kg/ha KCl. The second
 116 fertilization was carried out at the age of 30 HST with a dose of 100 kg/ha urea, 100 kg/ha SP36 and 100 kg/ha KCl.
 117 Furthermore, intensive control was carried out against weeds, pests and diseases, while observation of the agronomic
 118 characters of 10 plant per plot samples was taken from each line number. The characters observed included plant height,
 119 number of panicles/hill, panicle length, number of filled grains/panicle, percentage of empty grain/panicle, 1000 grain
 120 weight, grain weight per hill, and yield per plot.

121 **RESULTS AND DISCUSSION**

122 **Identification of drought-tolerant traits**

123 Screening of F6 lines at the seedling stage was carried out to select the drought-tolerant rice (Kumar et al., 2015; Swain
 124 et al. 2017; Herawati et al. 2017). Furthermore, Table 2 shows the drought-tolerant assessment carried out with the SES
 125 methods by comparing the treated plants with control varieties of Salumpikit and IR20. The symptoms, such as leaf
 126 curing, drying and recovery ability, were identified after exposure to drought stress for 14 days, as shown in Figure 1. The
 127 criteria of 39 F6 lines were identified as highly to moderately tolerant drought for a total number of 11, 19, and 9 lines,
 128 respectively, as shown in Table 3. The scores of dry leaf of the 30 lines with high tolerance level were 0-1 with recovery
 129 ability of 90 to 100%, while the scores of the rest nine lines with moderate tolerance were 3-5, with recovery ability of 70
 130 to 90% as shown in Table 4 and Figure 1.

131 **Table 2:** Traits identification of selected F6 lines based on criteria description of SES developed by IRRI (2002)

| Score | Criteria | Description | | |
|-------|-------------------|--------------------------------|---------------------------------------|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate tolerant | Leaves fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |

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| | | | | |
|---|----------------------|---------------------------------|--|----------------------------|
| 7 | Moderate susceptible | Leaf margins touching (0-shape) | More than 2/3 of all leaves fully dried | 20-39% of plants recovered |
| 9 | Susceptible | Leaves tightly rolled (V-shape) | All plants were dead. Length in most leaves thoroughly dried | 0-19% of plants recovered |

Table 3: Screening of the 39 F6 lines for resistant traits and molecular identification of gene markers of *DREB2A* and *BADH2*

| Line number | Genotype | Crossing | The score of rolling leaf | The score of drought leaf | Score of recovery | Criteria | DREB2A genes | BADH2 genes |
|-------------|------------|--------------------|---------------------------|---------------------------|-------------------|----------|--------------|-------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | RT | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | RT | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | RT | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | RT | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | RT | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | + | + |
| S | Salumpikit | Control variety | 1 | 1 | 1 | RT | | |

HT=High Tolerant (6 lines); RT=Rather Tolerant (5 lines); T= Tolerant (19 lines); MT= Moderate Tolerant (9 lines); += gene was identified

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Comment [L12]: Many mistake in deterring the drought tolerance criteria, especially 3 for rather tolerance according to the table 2. This changes will influence the decision of identification. Please clarify.

Comment [L13]: The controls showed a positive (IR 20) band, and according to the SES, why the control (Salumpikit) is Tolerant? The interpretation should be added in the results section.

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Table 4: The distribution of 39 F6 lines for identification of drought-tolerant

| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------------|-----------|-------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Rather Tolerant | Tolerant | Moderate Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | █ | | | | █ | | | | |
| 19 | | █ | | | | █ | | | |
| 9 | | | █ | | | | █ | | |
| IR20 | | | █ | | | | | | |
| Salumpikit | █ | | | | | | | | |

Comment [L14]: The tolerance criteria here is different from in table 2 (especially here 1 is rather tolerant whereas in table 2, 1 is tolerant and 3 is rather tolerant), which is the right one? Because it will make differences in the identification at table. Please clarify.

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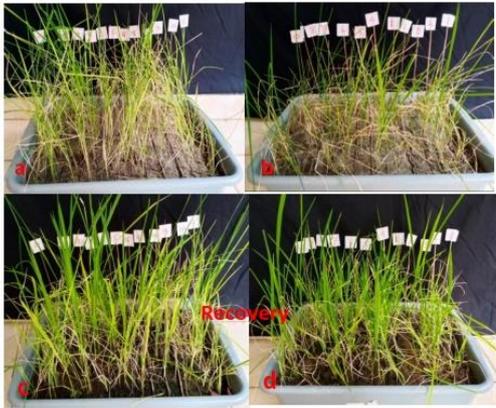


Figure 1: Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c and d)

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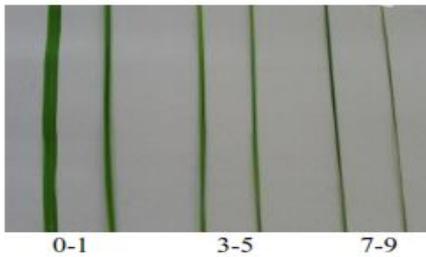


Figure 2: Description of rolling leaves based on SES Method; scores 0-1: started rolling to form V; 3-5: rolling to form V and U inside leaves; 7-9: rolling leaves totally

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Molecular identification of drought-tolerant genes

The molecular analysis using PCR products showed that the *DREB2A* gene was visualized in 39 selected lines with the marker sizes approximately 250 bp as shown in Figure 3 (Tawfik et al. 2016; Lathif et al. 2018). This proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1, which are drought-tolerant generated from F6 lines. This evidence proved that drought tolerance in rice plants is controlled by the *DREB2A* genes (Akhtar et al. 2012; Huang et al. 2018).

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Comment [L15]: The same comments: Why p the references here? Does it mean that this work(molecular identification) belong to them (people in references)? Not belong to the investigator? Please clarify!

Comment [L16]: The same comment with abo

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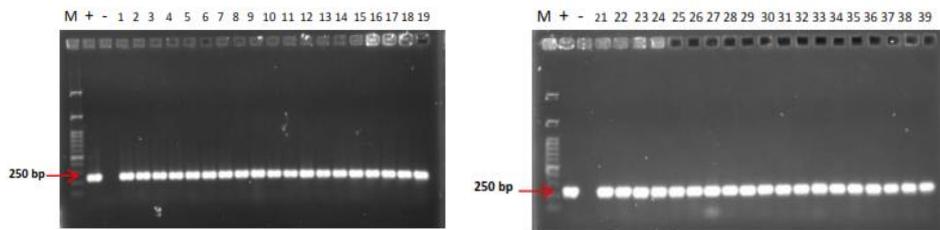


Figure 3: PCR amplification of *DREB2A* (250 bp) on 39 selected lines from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - =negative control)

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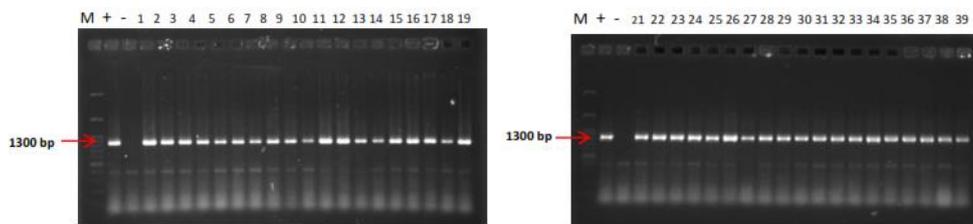


Figure 4: PCR amplification of *BADH2* (1300 bp) on 39 selected lines generated from a single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - = negative control)

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The successful use of molecular markers that control complex traits for obtaining varieties of drought-tolerant superior rice have been reported by Lanceras et al. (2004). Some of the studied traits include the yield, root length, thickness, leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017) and osmotic adjustment (Zivcak et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is a key enzyme for the biosynthesis of glycine betaine. This is because several studies have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). Furthermore, the visualization of the *BADH2* gene 39 selected lines showed a marker with a size of approximately 1300 bp (Shrestha 2011; Hasthanasombut et al. 2011) as shown in Figure 4.

Comment [L17]: This should be put on the discussion section. Results should present the data interpretation.. for the example, how is the band profile? Good or not? Which line is positive? Why control has also positive band? What is the indication?

Performance of agronomic characters, yield and yield potential of superior lines

The appearance of agronomic characters and yield potential of the 16 tested superior lines are shown in Table 5. Almost all tested lines have indicated uniformity as shown by the lowest and highest average plant height appearance, of 101.1 and 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the 8th generation (F7). Furthermore, the highest and lowest average number of panicles *per* hill were 14.7 and 6.5, with the length ranging from 24.61 - 27.6 cm. Furthermore, the number of filled grains/panicles ranged from 99.5 - 150.07, while the percentage of empty grains rice was categorized as low based on the SES IRRI (2012), which ranged from 9.87% - 26.66%. This led to variations in grain weight per hill, which was approximately 19-35.5 grams *per* hill.

Table 5: The performance of agronomic characters, yields and yield potential of superior lines *in the field experiment*.

| Assesion | Plant height | Number of Panicle <i>per</i> hill | Panicle length (cm) | Number of fill grains | % of empty/panicle | 1000 grains weight (g ₁₀₀₀) | grains weight <i>per</i> hill (gram) | Yield/p lot(1x1 m ²) (g ₁₀₀₀) | Yield potential (t ₁₀₀₀ ha ⁻²) |
|---|--------------|-----------------------------------|---------------------|-----------------------|--------------------|---|--------------------------------------|---|---|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |

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|-----------------|--------------|--------------|--------------|----------------|---------------|--------------|--------------|------|-------|
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

The yield of grain per plot varies from the lowest at 458 grams to the highest, at 1210 grams. When determined from the appearance of agronomic characters, the high grain yield was supported by the characters of a large number of panicles, the low percentage of empty grain, and the weight of 1000 grains. The length of the panicles did not show any significant variation and range from 24.61-27.6 cm, as shown in Table 5.

DISCUSSION

Seedlings' responses to drought stress were identified after 14 days without water. Furthermore, the tolerant lines continued to grow adequately and leave remained fully open, whereas the moderate tolerant lines were dried on leaf tips, as shown in Figure 1. Kumar et al. (2014) stated that leaf rolling was delayed in drought-tolerant rice genotypes and induced by loss of turgor as well as low osmotic regulation. Delayed leaf rolling in the tolerant genotype indicated that the turgor remained normal, and the plants were protected from dehydration, as shown in Figure 2. According to Bunnag & Pongthai (2013) and Swain et al. (2017), leaf rolling is one of the mechanisms used by plants to adjust the water potential, and enable them to absorb groundwater in drought stress conditions. Swain et al. (2017) reported that during the drought conditions, the level of groundwater was below 30 cm depth, of the 78 lines of drought-tolerant assessments. Furthermore, 30 and 48 lines were scored by 1, and 3, respectively. Out of these 78 assessments, 13 and tolerant (CR 143-2-2) lines produced more than 1-ton grain/ha, and 2.7 tons grain/ha, while sensitive plants as a control (IR20) did not produce any.

Leaf rolling can reduce the surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This condition help plants to survive in a certain period when the availability of water in the environment decreases. The genes in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the leucine zipper class IV transcriptional factor homeodomain. According to Zou et al. (2011), overexpression and suppression of these genes lead to leaf curling on the adaxial and abaxial sides of the plants. Delay in leaf rolling indicates a plant effort to maintain turgor and avoid dehydration. Water shortages tend to have lower rates of leaf rolling, which appears in plants with tolerant criteria score of 1, as shown in Table 3. It allowed the plant to survive to drought at the low water potential of leaf tissue (Sevanto, 2018). Furthermore, plants were recovery after passing through a period of drought, thereby indicating the ability of plants to enhance their metabolic system (Bian et al. 2017; Wang et al. 2019).

Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the ancestors with other varieties that have high productivity. The use of molecular marking technology can help hasten the selection process, thereby making it accurate and faster. One of the markers related to drought tolerance is the QTL (quantitative trait locus) 12.1, which was produced by crossing the Vandana varieties of Indian rice and Indonesian Way Rarem (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study due to its ability to maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL 12.1 had an insignificant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The DREB2A gene is essential as a regulator of drought-responsive genes, making it a marker of drought stress-tolerant. The transcription factors in DREB2A are involved in the regulatory mechanisms that exist in some plants in response to drought, salinity, and heat stress (Lata and Prasad 2011; Mizoi et al. 2012). There are five DREB2 genes in the rice genome, including OsDREB2A, OsDREB2B, OsDREB2C, OsDREB2E, and OsABI4 (Matsukura et al. 2010; Srivastav et al. 2010). Expression of OsDREB2A in rice is caused by water deficit and exogenous ABA application, which leads to increased drought stress (Cui et al. 2011). The OsDREB2B transcript has a functional and non-functional form marked during drought conditions. Consequently, it can increase drought tolerance through alternative splicing induced by its pre-mRNA (Matsukura et al. 2010). All of these results indicate that OsDREB2s also play an essential role in the regulation of drought tolerant.

Huang et al. (2018) identified a new transcription factor gene such as DREB2, namely OsDRAP1 (Responsive Drought Genes AP2/EREBP), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly regulated by drought at DK151, thereby showing its role in drought tolerance rice. According to osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous studies indicated that osmoprotectant substances, namely glycine betaine, plays an essential role in cell stabilization by balancing the structure of

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245 the protein quaternary and membrane against the adverse effects of salinity (Sakamoto and Murata 2000; Saxena et al.
246 2019). In addition, it facilitates osmotic adjustment by reducing the internal occurrence possibility that contributes to the
247 ability of plant cells to be tolerant to water stress. It also stabilizes the PSII and RuBisCO complexes during
248 photosynthesis under stress conditions (Holmström et al. 2000; Huang et al. 2020). These are some of the positive effects
249 of the application of exogenous betaine glycine in plants that grow under the pressure of salinity or drought stress. Plant
250 cells can be protected from adverse effects of salinity induced oxidative stress by exogenous application of glycine betaine
251 (Demiral and Türkan 2004; Saxena et al. 2019).

252 The PCR amplification of 39 selected lines for drought tolerance using *DREB2A* and *BADH2* primers are shown in
253 Table 3. All tested lines showed positive, containing both genes and had criteria with varying degrees from the results of
254 the drought test in the seedling stage. However, the results of the molecular study showed positive results as a drought-
255 tolerant marker gene in the seedling stage, evaluation at the productive stage needs to be carried out to obtain more
256 accurate data. Drought-tolerant plants can adapt to drought conditions, which are shown on high grain. The use of superior
257 varieties is the most efficient technology to increase rice yield with low-cost production in the dry land. Therefore,
258 developing a superior variety by crossbreeding is needed to produce superior potential lines. Furthermore, before releasing
259 a new superior variety, potential selected lines need to be tested in various locations.

260 The agronomic performance and yield of 16 superior lines showed that all lines reached homozygous in the 8th
261 generation (F₇), where the plant height had a relatively low standard deviation. The number of panicles ranging from 14.7
262 had a high yield potential, with the value of a filled grain of 150.07, as shown in Table 5. The new paradigm of rice
263 breeding is the number of productive tillers between 8-12 tillers per hill with the grains/panicles ranging from 150-200
264 (Peng and Khush 2003). Peng et al. (2008) stated that in the new type of rice breeding avoid extreme traits, such as 200-
265 250 grain/panicle which can produce panicles with low seed filling. Therefore, the increase in the second generation of
266 new types of rice has been modified by IRRI to 150 grains/panicle. Several lines have a potential yield of more than 10
267 tonnes/ha, such as those with the accession number BKL1 B-1-259-1 and BKL1 B-3-261-3 to yield potential of 10.05
268 tonnes/ha and 10.08 tonnes/ha⁻¹, respectively. Meanwhile, the lines with the BKL2 B-2-264-6 and BKL4 B-1-268-10
269 numbers had a potential yield of more than 12 tonnes/ha⁻¹, namely 12.1 and 12.06 tonnes/ha⁻¹, as shown in Table 5. These lines
270 have the opportunity to be developed on dry land or as rice on rainfed land because the lines tested were identified as
271 drought resistance, as shown in Table 3.

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272 ACKNOWLEDGEMENTS

273 The authors are grateful to the Directorate of Research and Community Service, Ministry of Education and Culture
274 Republic of Indonesia for funding this research through the National Competitive Applied Research with Contract
275 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amendment Contract Number: 165/SP2H/LT/DRPM/2019. The
276 authors are also, grateful to the Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of
277 the Department of Crop Production at the University of Bengkulu for their help in facilitating this research.

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Molecular analysis of DREB2A and BADH2 genes and evaluation of yield potential of single-crossing varieties of rice in Bengkulu, Indonesia

Abstract. This study aimed to identify drought-tolerant tolerance and molecular analysis characteristics of DREB2A and BADH2 genes and yield potential of single-cross varieties of rice in Bengkulu. The drought sensitive varieties of IR 20 and Salumpikit were used as (as the control plants) and 39 F6 progeny lines were used in the screening at seedling stages of the 39 F6 progeny lines, which were carried out in the greenhouse. In addition, the Standard Evaluation System (SES) developed by IRRRI was used to assess the recovery ability of the curled and dried leaves after two weeks tested varieties/lines. The molecular analysis used to detect the presence of the DREB2A gene was carried out by PCR amplification in the genomic DNA using the forward and reverse oligonucleotide primers consisting of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively. Meanwhile, for the BADH2 gene, the forward and reverse oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and TGTCCCCAGCTGCTTCATCC were used, respectively. Molecular markers of DREB2A and BADH2 genes were also identified in 39 tested lines with approximately 250 and 2300 bp length. This result showed that the progeny of F6 lines generated from crossing local varieties of IR7858 and IR148 are potential to becoming as drought-tolerant upland rice varieties of upland-rice. Line numbers BKL2 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 tonnes/ha and can be developed on rain-fed, lowland or dryland due to its drought resistance tolerance.

Keywords: BADH2, DREB2A, drought tolerance, gene identification, yield potential

Running title: Identification of DREB2A and BADH2 genes for drought tolerant

INTRODUCTION

Upland rice cultivation is an alternative strategy for rice production to increase the annual yearly number of rice production in Indonesia, which has been significantly decreasing during the last decade due to the rising difficulty associated with the extensification of lowlands. According to the Center for Research and Development (2006), this technique is carried out by optimizing the use of uncultivated lands, where the majorities have which are the potential for upland rice cultivation. The use of high yielding superior varieties with higher yields and tolerance to various obstacles is urgently needed to support efforts to increase rice yields in the dryland. Furthermore, it is important to anticipate the impact of climate change on sustainable agricultural systems to produce technological innovations that can overcome and suppress the impacts caused, such as the use of superior varieties drought-tolerant rice varieties. The genetic improvement to obtain produce superior varieties that are adaptive to the drought stress condition of drought stress is an essential priority in rice breeding programs.

Crossbreeding is used to assemble drought-tolerant rice varieties, which combines the resistant traits of the parents with other high yield cropping varieties. Molecular marker technology can be used to more accurately selecting the desirable traits, helps in selecting more accurate than conventional area through marker assisted selection (MAS), and one of the markers related to drought tolerance is the QTL marker (quantitative trait locus) 12.1. Furthermore, the International Rice Research Institute (IRRI) crossed the Vandana variety of Indian rice using Way Rarem from Indonesia, such as which generated the filial with crossing number of IR148+, derived from IR crossing 79971-B-369-B-B (Mulyaningsih et al., 2010). The crossing population contains QTL 12.1 markers, with the location on chromosome 12, as well as between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of these markers maintains yields before flowering and during severe drought stress at the reproductive stage. In normal conditions, the marker QTL 12.1 had an insignificant effect on some of the parameters observed (Bernier et al. 2007).

According to Matsukura et al. (2010), Srivastav et al. (2010), Akhtar et al. (2012), and Huang et al. (2018) the DREB2 gene controls the drought stress tolerance in plants. DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). Sakuma et al. (2002) stated that in rice, the homology of the DREB2 gene in rice is homolog to DREB2A. Some of the DREB2A target genes are MT2A, At1g69870, At3g53990, At1g22985, RD29A, LEA14, At2g23120 [9], RD29B, At1g52690, RD17 (Sakuma et al. 2006; Qin et al. 2011), AtHsfA3, HSP18.2, and Hsp70 (Qin et

Comment [A1]: This is an extensive work with highly sufficient data for publication as a full research article. The methods are appropriate, sophisticated and robust. The results are well presented and discussion is comprehensive. The novelty of the research lies on the drought tolerance genes found be present on the advanced rice lines tested in this study, but there are some issues that need to be explained regarding the association between tolerance markers and the phenotypic drought tolerance level observed in the seedling stage, as commented in the manuscript. In addition, many grammatical and editorial corrections are suggested. These issues need to be solved before further process of publication.

Comment [A2]: The title need to be made representing the content of the article. This on section not to.

Comment [A3]: This confusing in meaning, please revise

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al. 2011). The ~~importance of the~~ DREB2A gene is important because it can be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-~~resistant-tolerance~~ genes.

Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous studies carried out by Sakamoto & Murata (2000) and Saxena et al. (2019) indicated that osmoprotectant substances, such as glycine betaine, plays an essential role in cell stabilization by balancing the structure of the protein quaternary and membrane structure against the adverse effects of salinity. Besides, it facilitates osmotic adjustment by lowering the internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et al. 2020). The positive effects and exogenous application of glycine in plants ~~that grown on~~ under salinity stress condition have been shown to protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

Betaine aldehyde dehydrogenase (BADH) is a key enzyme for biosynthesis of glycine betaine. Several studies have reported the accumulation ability of glycine betaine and BADH1 gene expression in tolerating salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objectives of this study ~~is-were to identify~~ identification of drought-~~tolerant-tolerance~~ traits and molecular analysis of DREB2A and BADH2 genes using the progeny of F6 lines derived from the crossings of local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL on the chromosome.

MATERIALS AND METHODS

This research was carried out at the University of Bengkulu, ~~with~~. The screening of the rice lines was conducted at the ~~greenhouse~~ Greenhouse of Agricultural Faculty, Universitas Bengkulu, from February to April 2020, while molecular analysis was done in the laboratory of the Department of Biology from May to July 2020. The plant materials used ~~are~~ were the ~~progeny-progenies~~ of 39 lines selected from F6 generations leading to the single crossing of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ QTL positive on chromosome 12.1 (Mulyaningsih et al. 2010). Table 1 shows the var. Salumpikit and IR 20 as drought-tolerant and sensitive controls.

Table 1: Selected F6 lines for traits and molecular identification of drought-tolerant genes of DREB2A and BADH2

| Lines number | Genotype | Initial Crossing | Line number | Genotype | Initial Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

The standard Evaluation System (SES) developed by IRRI (2002) was used to screen the drought-tolerant rice ~~of from~~ 39 F6 lines with the drought-susceptible variety (IR20), and local drought-tolerant variety (Salumpikit) used as control. Furthermore, the test was carried out in accordance with Kumar et al. (2015), Swain et al. (2017), and Herawati et al. (2017) methods with plastic ~~tubs-trays sizes~~ of 40 cm x 25 cm x 20 cm size filled with soil. Each tub was planted using ten family lines and two control varieties, with each line sown for 20 seeds in a row. After planting, the seedlings were intensively watered for 2 weeks with the plants allowed to dry to determine its sensitivity. Drought tolerance assessment

Comment [A5]: This is confusing, please revise

Comment [A6]: This Table is not containing of the two varieties but also the 36 F6 lines tested. Please revise appropriately.

85 was carried out based on the SES methods, as shown in Table 2. Furthermore, the trait responses of the seedlings were
 86 recorded, followed by intensive watering for the next ten days. Recovery ability was recorded following the methods of
 87 SES, as shown in Table 2.

88 Genomic DNA was isolated from fresh leaves 14 days after treatment (DAT) with ~~fragments a weight~~ of 0.1 g of rice
 89 leaf ~~was added with liquid nitrogen and then ground using a mortar by adding liquid nitrogen~~. The total DNA was isolated
 90 by modifying the protocols of Wizard's Genomic DNA Purification Kit, and the ground leaf was put into a 2 ml tube,
 91 ~~before adding and shaking~~ added with 600 µl of Nuclei Lysis Solution ~~and then shaken~~ for 3 seconds. In addition, the
 92 solution was heated in a water bath at 65°C for 15 minutes, ~~and followed by then addition added and incubation of with~~ 3 µl
 93 RNase ~~and incubated~~ at 37 °C for 15 minutes. This was followed by the addition of 200 µl Precipitation Solution, ~~and~~ the
 94 microtubes ~~containing the mixture were~~ centrifuged for 3 minutes at 13,000 rpm. The supernatants were collected into a
 95 1.5 ml tube, ~~before and then addition added with~~ 600 µl isopropanol. Furthermore, the microtubes were further
 96 centrifuged for 1 minute at room temperature, ~~then the solution supernatant was~~ discarded while the remaining DNA on
 97 the bottom of microtubes was air-dried for 15 minutes. DNA Rehydration Solution of 100 µl was added and further
 98 incubated at 65 °C for 1 hour or at 4°C for ~~overnight~~. The total isolated DNA ~~was~~ used as a template for PCR
 99 amplification of DREB2A and BADH2 genes.

100 PCR amplification of the DREB2A gene was determined using the forward and reverse oligonucleotide primers of
 101 CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
 102 Lathif et al. 2018). Meanwhile, the amplification of the BADH2 gene was carried out using forward- and reverse-
 103 oligonucleotide primers of GGCCAAGTACCTCAAGGCG and TGTCCCCAGCTGCTTCATCC (Robin et al. 2003).
 104 The PCR mixtures, which comprise of the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix),
 105 and Taq polymerase, were developed during the thermocycling process. The program was carried out using a denaturation
 106 temperature at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 2
 107 minutes, and extension at 72°C for 2 minutes, and the final extension at 72°C for 10 minutes. PCR amplification products
 108 were subjected to electrophoresis in ~~an~~ agarose gel ~~of 1% on~~ TBE buffer to identify successful amplifications. The gel
 109 from electrophoresis was immersed in EtBr 1% for 10 minutes, rinsed with ddH2O for 5 minutes, and visualized under UV
 110 transilluminator light.

111 A yield ~~performance~~ test of ~~selected superior lines was carried out from March-July 2020~~ in Semarang Village,
 112 Bengkulu City. The materials used in this study were 16 selected superior lines in the F7 generation with the experiment
 113 carried out on a plot measuring 8 m x 6 m, a spacing of 20 x 20 cm, and by planting ~~a seed~~. The first fertilization process
 114 was carried ~~out twice after planting (HST) for 14 days~~ with a dose of 150 ~~kg ha⁻¹~~ of Urea, 100 ~~kg ha⁻¹~~ SP36 and 100 ~~kg ha⁻¹~~
 115 ~~KCl~~. The second fertilization was carried out at the age of 30 HST with a dose of 100 ~~kg ha⁻¹~~ urea, 100 ~~kg ha⁻¹~~ SP36
 116 and 100 ~~kg ha⁻¹~~ KCl. Furthermore, intensive control was carried out against weeds, pests and diseases, while observation
 117 of the agronomic characters of 10 plant ~~plot⁻¹~~ samples was taken from each line number. The characters observed included
 118 plant height, number of panicles ~~hill⁻¹~~, panicle length, number of filled grains ~~panicle⁻¹~~, percentage of empty grain ~~panicle⁻¹~~,
 119 ~~1~~, 1000 grain weight, grain weight per hill, and yield per plot.

Comment [A7]: 100 mg?

Comment [A8]: From where were superior lines selected? From the previous screening? The time overlap with the time of screening, and before the molecular analysis, so how you selected the lines? Please clarify.

Comment [A9]: Only one seed was planted? Please clarify, and the reasons?

Comment [A10]: When and when????

120 RESULTS AND DISCUSSION

121 ~~Identification Assessment of drought-tolerant tolerance traits –level~~

122 Screening of F6 lines at the seedling stage was carried out to select the drought-tolerant rice (Kumar et al., 2015; Swain
 123 et al. 2017; Herawati et al. 2017). ~~Furthermore~~, Table 2 shows the drought-~~tolerant-tolerance~~ assessment carried out with
 124 the SES methods by comparing the treated ~~plants lines~~ with control varieties of Salumpikit and IR20. The symptoms, such
 125 as leaf curling, drying and recovery ability, were identified after exposure to drought stress for 14 days, as shown in Figure
 126 1. The criteria of 39 F6 lines were identified as highly to moderately tolerant ~~drought to drought, for a total number~~ of 11,
 127 19, and 9 lines, respectively, as shown in Table 3. The scores of dry leaf of the 30 lines with high tolerance level were 0-1
 128 with recovery ability of 90 to 100%, while the scores of the rest nine lines with moderate tolerance were 3-5, with recovery
 129 ability of 70 to 90% as shown in Table 4 and Figure 1.

130
 131 **Table 2:** Traits identification of selected F6 lines based on criteria description of SES developed by IRRI (2002)
 132

| Score | Criteria | Description | | |
|-------|------------------------------------|--|--|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves are healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves are folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate ly tolerant | Leaves are fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |
| 7 | Moderate ly susceptible | Leaf margins are touching (0-shape) | More than 2/3 of all leaves are fully dried | 20-39% of plants recovered |

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|---|-------------|--|--|
| 9 | Susceptible | Leaves <u>are</u> tightly rolled (V-shape) | All plants were <u>are</u> dead. Length in most leaves 0-19% of plants recovered thoroughly dried |
|---|-------------|--|--|

Comment [A11]: What length?

Table 3: Screening of the 39 F6 lines for resistant-drought tolerance traits and molecular identification of gene-molecular markers of DREB2A AND and BADH2 genes.

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| Line number | Genotype | Crossing | The score of <u>leaf rolling leaf</u> | The score of <u>drought leaf drying</u> | Score of recovery | Criteria | DREB2A genes | BADH2 genes |
|-------------|------------|--------------------|---------------------------------------|---|-------------------|----------|--------------|-------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | T | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | RT | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | RT | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | RT | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | RT | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | T | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | T | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | + | + |
| S | Salumpikit | Control variety | 1 | 1 | 1 | RT | | |

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HT=Highly Tolerant (6 lines); RT=Rather Tolerant (5 lines); T= Tolerant (19 lines); MT= Moderate Tolerant (9 lines); += gene was identified/present.

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Table 4: The distribution of 39 F6 lines for identification of drought-tolerant lines

| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------------|-----------|---------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Rather Tolerant | Tolerant | Moderately Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | ■ | | | | ■ | | | | |
| 19 | | ■ | | | | ■ | | | |
| 9 | | | ■ | | | | ■ | | |
| IR20 | | | ■ | | | | | | |
| Salumpikit | ■ | | | | | | | | |

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Comment [A12]: IR was used as the drought susceptible control variety but it was observed; Moderately Susceptible'. Please explain the reason

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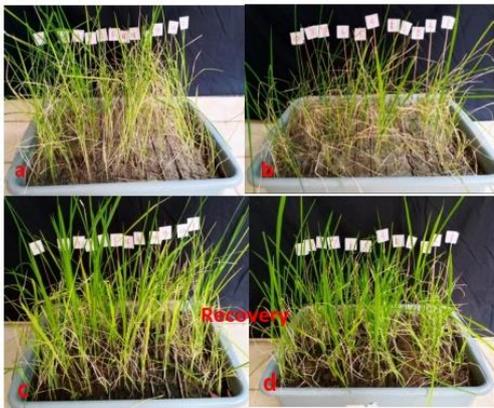


Figure 1: Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c and d)

Comment [A13]: Please indicate a, b, c, and d

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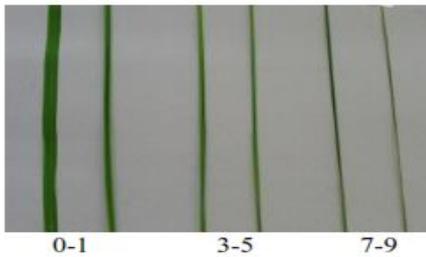


Figure 2: Description of rolling leaves based on SES Method; scores 0-1: started rolling to form V shape; 3-5: rolling to form V and U shapes inside leaves; 7-9: rolling leaves are rolling totally

Molecular identification of drought-tolerant tolerance genes

The molecular analysis using PCR products separated on agarose gel electrophoresis showed that the DREB2A gene was visualized present in the 39 selected lines with the marker sizes approximately 250 bp as shown in Figure 3 (Tawfik et al. 2016; Lathif et al. 2018). This proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1, which are drought-tolerant generated from F6 lines. This evidence proved that drought tolerance in rice plants is controlled by the DREB2A genes (Akhtar et al. 2012; Huang et al. 2018).

Comment [A14]: Are these figure generated in the results of the preent study?? Or of the these authors? Please be clear.

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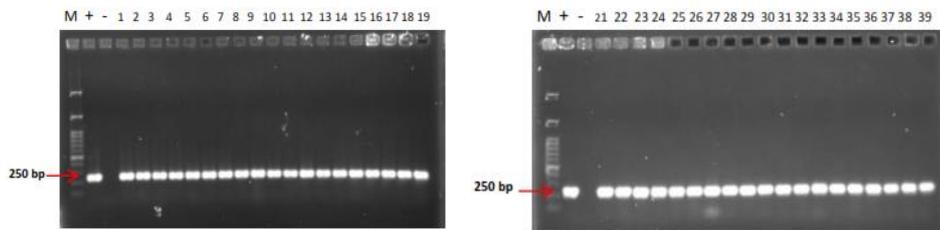


Figure 3: PCR amplification of DREB2A (250 bp) on 39 selected lines from single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - =negative control)

Comment [A15]: Mention the name of the local variety

Comment [A16]: Mention the names of check varieties

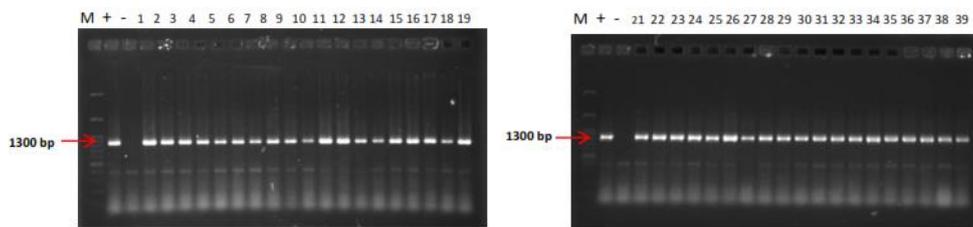


Figure 4: PCR amplification of BADH2 (1300 bp) on 39 selected lines generated from a single crossing of local variety with IR 20 and Salumpikit varieties (M= DNA ladder of 100 kb, + = positive control, - = negative control)

Comment [A17]: Mention the names of local variety and check/control varieties.

Comment [A18]: All tested lines contained both genes, while their tolerance level were varied: Moderately tolerant, tolerant, rather tolerant, and highly tolerant. WHY would you expect that the two markers bands are related to drought tolerance genes? Or there would be other factors that may contribute to the tolerance, a= in addition to the two gene? Please explain.

Comment [A19]: Drought???

The successful use of molecular markers that control complex traits for obtaining varieties of drought-tolerant superior rice varieties have been reported by Lanceras et al. (2004). Some of the studied traits included yield, root length, thickness, leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017) and osmotic adjustment (Zivcak et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is a key enzyme for the biosynthesis of glycine betaine. This is because several studies have reported the accumulation of glycine betaine and BADH1 gene expression for tolerance to salinity, dryness, and low temperatures (Lapuz et al. 2019; Kahraman et al. 2019). Furthermore, the visualization of the DNA marker linked to BADH2 gene was present on the 39 selected lines with the marker size of approximately 1300 bp (Shrestha 2011; Hashtanasombut et al. 2011) as shown in Figure 4.

Performance of agronomic characters, yield and yield potential of superior lines

The appearance performance of agronomic characters and yield potential of the 16 tested superior lines are shown in Table 5. Almost all tested lines have indicated uniformly performed as shown by the lowest and highest average plant height appearance range of 101.1 and 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the 8th generation (F7). Furthermore, the highest and lowest average number of panicles hill⁻¹ were 14.7 and 6.5, with the panicle length ranging range from of 24.61 - 27.6 cm. Furthermore, the number of filled grains panicles⁻¹ ranged from 99.5 - 150.07, while the percentage of unfilled grains rice was from 9.87% - 26.66%, which are categorized as low based on the SES IRRI (2012), which ranged from 9.87% - 26.66%. This led to variations in grain weight per hill (- which was approximately 19-35.5 grams hill⁻¹).

Comment [A20]: Is this ranged considered homogen?

Table 5: The performance of agronomic characters, yields and yield potential of superior rice lines

| Accession | Plant height | Number of Panicle hill ⁻¹ | Panicle length (cm) | Number of fill grains | % of unfilled grain panicle ⁻¹ | 1000-grain weight (gram) | grains weight hill ⁻¹ (gram) | Yield/pot (t/ha) | Yield potential (ton/ha) |
|---|--------------|--------------------------------------|---------------------|-----------------------|---|--------------------------|---|------------------|--------------------------|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |

| | | | | | | | | | |
|-----------------|--------------|--------------|--------------|----------------|---------------|--------------|--------------|------|-------|
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

The grain yield of grain per plot varied from the lowest at 458 grams to the highest at 1210 grams. When determined from the appearance of the agronomic characters that supports the observed high grain yield were the high supported by the characters of a large number of panicles, the low percentage of unfilled grain, and the high weight of 1000-grain weight. The length of the panicles did not show any significant variation and ie ranged from 24.61-27.6 cm as shown in Table 5.

Discussion

Seedlings' responses to drought stress tolerance were identified after 14 days without water after the stress treatment. Furthermore, after which, the tolerant lines continued to grow adequately normally and their leaves remained fully open, whereas the moderately tolerant lines experienced the drying/dried of the leaf tips as shown in Figure 1. Kumar et al. (2014) stated that leaf rolling was delayed in drought-tolerant rice genotypes and induced by loss of turgor as well as low osmotic regulation. Delayed leaf rolling in the tolerant genotype indicated that the turgor remained normal, and the plants were protected from dehydration, as shown in Figure 2. According to Bunnag & Pongthai (2013) and Swain et al. (2017), leaf rolling is one of the mechanisms used by plants to adjust the water potential, and enable them to absorb groundwater in drought stress conditions. Swain et al. (2017) reported that during the drought conditions, the level of groundwater was below 30 cm depth, of the 78 lines of drought-tolerant assessments. Furthermore, 30 and 48 lines were scored by 1, and 3, respectively. Out of these 78 lines assessments assessed, 13 lines and the tolerant (CR 143-2-2) variety produced more than 1 ton grain/ha and 2.7 tons grain/ha, respectively, while the sensitive plants as a control variety (IR20) did not produce any produced no grain at all.

Leaf rolling can reduce the surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This condition help plants to survive in a certain period when the availability of water in the environment decreases. The genes in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the leucine zipper class IV transcriptional factor homeodomain. According to Zou et al. (2011), overexpression and suppression of these genes lead to leaf curling on the adaxial and abaxial sides of the plants. Delay in leaf rolling indicates a plant effort to maintain turgor and avoid dehydration. Water shortages tend to have lower rates of leaf rolling, which appears in plants with tolerant criteria score of 1, as shown in Table 3. It allowed the plant to survive drought at the low leaf tissue water potential of leaf tissue (Sevanto 2018). Furthermore, the plants were recovery recovered after passing through a period of drought, thereby indicating the ability of plants to enhance their metabolic system (Bian et al. 2017; Wang et al. 2019).

Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the ancestors with other varieties that have high productivity. The use of molecular marking marker technology can help hasten-fasten the selection process, thereby making it accurate and faster. One of the markers related to drought tolerance is the QTL (quantitative trait locus) 12.1, which was produced by crossing the Vandana varieties of Indian rice and Indonesian Way Rarem (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study due to its ability to maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL 12.1 had an insignificant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The DREB2A gene is essential as a regulator of drought-responsive genes, making it a marker of drought stress-tolerant tolerance. The transcription factors in DREB2A are involved in the regulatory mechanisms that exist in some plants in response to drought, salinity, and heat stress (Lata and Prasad 2011; Mizoi et al. 2012). There are five DREB2 genes in the rice genome, including OsDREB2A, OsDREB2B, OsDREB2C, OsDREB2E, and OsABI4 (Matsukura et al. 2010; Srivastav et al. 2010). Expression of OsDREB2A in rice is caused by water deficit and exogenous ABA application, which leads to increased drought stress (Cui et al. 2011). The OsDREB2B transcript has a functional and non-functional form marked during drought conditions.

242 Consequently, it can increase drought tolerance through alternative splicing induced by its pre-mRNA (Matsukura et al.
243 2010). All of these results indicate that OsDREB2s also play an essential role in the regulation of drought
244 ~~tolerant~~tolerance.

245 Huang et al. (2018) identified a new transcription factor gene such as DREB2, namely OsDRAP1 (Responsive Drought
246 Genes AP2/EREBP), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly
247 regulated by drought at DK151, thereby showing its role in drought tolerance in rice. According to osmotic adjustment in
248 cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous studies indicated that
249 osmoprotectant substances, namely glycine betaine, plays an essential role in cell stabilization by balancing the structure of
250 the protein quaternary and membrane against the adverse effects of salinity (Sakamoto and Murata 2000; Saxena et al.
251 2019). In addition, it facilitates osmotic adjustment by reducing the internal occurrence possibility that contributes to the
252 ability of plant cells to be tolerant to water stress. It also stabilizes the PSII and RuBisCO complexes during
253 photosynthesis under stress conditions (Holmström et al. 2000; Huang et al. 2020). These are some of the positive effects
254 of the application of exogenous betaine glycine in plants that grow under the pressure of salinity or drought stress. Plant
255 cells can be protected from adverse effects of salinity induced oxidative stress by exogenous application of glycine betaine
256 (Demiral and Türkan 2004; Saxena et al. 2019).

257 The PCR ~~amplification assay~~ of 39 selected lines for drought tolerance using DREB2A and BADH2 primers are shown
258 in Table 3. All tested lines showed positive results, ~~they containing-contained~~ both genes and had the criteria of varying
259 degrees from the results of the drought tolerance level in the seedling stage evaluation. However, the results of the
260 molecular analysis showed positive results as a drought-tolerant marker gene in the seedling stage, then evaluation at the
261 productive stage needs to be carried out to obtain more accurate data. Drought-tolerant plants can adapt to drought
262 conditions, which are shown by high grain. The use of superior varieties is the most efficient technology to increase rice
263 yield with low-cost of production in the dry-land. Therefore, developing a superior variety by crossbreeding is needed to
264 produce superior potential lines. Furthermore, before releasing a new superior variety, potential selected lines need to be
265 tested in various locations (multi-location trials/MLT).

266 The agronomic performance and yield of 16 superior lines showed that all lines reached homozygous in the 8th
267 generation (F₇), where the plant height had a relatively low standard deviation. The number of panicles ranging ranged
268 from 14.7 had a high yield potential, with the value of a filled grain of 150.07, as shown in (Table 5). The new paradigm of
269 rice breeding is the number of productive tillers between 8-12 tillers ~~Ahill^{±1}~~ with the grains number /panicles-1 ranging
270 from 150-200 (Peng and Khush 2003). Peng et al. (2008) stated that in the new type of rice variety, breeding programmes
271 avoid extreme traits, such as 200-250 grain /panicle^{±1} which can produce panicles with low seed filling. Therefore, the
272 increase in the second generation of new types of rice has been modified by IRRI to 150 grains /panicle^{±1}. Several lines
273 have a potential yield of more than 10 tonnes/ha, such as those with the ~~assesion-accession~~ number sBKL1 B-1-259-1 and
274 BKL1 B-3-261-3 to yield potential of 10.05 tonnes/ha and 10.08 tonnes/ha, respectively. Meanwhile, the lines with the
275 BKL2 B-2-264-6 and BKL4 B-1-268-10 numbers had a potential yield of more than 12 ton /ha, namely 12.1 and 12.06
276 tons/ha, as shown in Table 5. These lines have the opportunity to be developed on dry-land or as rice on rainfed land
277 because the lines tested were identified as drought ~~resistance~~tolerant, as shown in Table 3.

Comment [A21]: This is not clear in meaning, please revise

Comment [A22]: Yes, in Figure 4, all tested lines contained both genes, while their tolerance levels were varied: Moderately tolerant, tolerant, rather tolerant, and highly tolerant. WHY would you expect that the two markers bands are related to drought tolerance genes? Or there would be other factors that may contribute to the tolerance, a= in addition to the two gene? Please explain.

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278 ACKNOWLEDGEMENTS

279 The authors are grateful to the Directorate of Research and Community Service, Ministry of Education and Culture
280 Republic of Indonesia for funding this research through the National Competitive Applied Research with Contract
281 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amendment Contract Number: 165/SP2H/LT/DRPM/2019. The
282 authors are also, grateful to the Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of
283 the Department of Crop Production at the University of Bengkulu for their help in facilitating this research.

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Identification of drought tolerant markers, *DREB2A* and *BADH2* genes, and yield potential from single-crossing varieties of rice in Bengkulu, Indonesia

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Abstract. This study aimed to identify drought-tolerance and molecular characteristics of *DREB2A* and *BADH2* genes, as well as yield potential from single-crossing varieties of rice in Bengkulu. The drought sensitive varieties of IR20 and Salumpikit (as the control plants) and 39 F6 progeny lines were used in the screening at seedling stages in the greenhouse. The Standard Evaluation System (SES) developed by IRRRI was used to assess the recovery ability of tested varieties/lines. The molecular analysis used to detect the presence of the *DREB2A* gene was carried out by PCR amplification in the genomic DNA using the forward and reverse oligonucleotide primers consisting of CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively. Meanwhile, for the *BADH2* gene, the forward and reverse oligonucleotide primers of GGCCAAGTACCTCAAGGCGA and TGTCCCCAGCTGCTTCATCC were used respectively. Molecular markers of *DREB2A* and *BADH2* genes were identified in 39 tested lines with approximately 250 and 2300 bp length. This result showed that the progeny of F6 lines generated from the crossing of the local varieties of IR7858 and IR148 are potential as drought-tolerant upland rice varieties. Line numbers BKL2 B-2-264-6 and BKL4 B-1-268-10 have a potential yield of more than 12 t ha⁻¹ and can be developed on rain-fed, lowland or dry land due to its drought tolerance.

Keywords: *BADH2*, *DREB2A*, drought tolerance, gene identification, yield potential

Running title: *Identification of DREB2A and BADH2 genes for drought tolerant*

INTRODUCTION

Upland rice cultivation is an alternative strategy to increase the annual rice production in Indonesia, which has been significantly decreasing during the last decade due to the increasing in the conversion of lowland. According to the Center for Research and Development (2006), this is carried out by optimizing the use of uncultivated lands, which are potential for upland rice cultivation. The use of high yielding superior varieties with tolerant to various obstacles is urgently needed to support efforts to increase rice yield in the dry land. Furthermore, it is important to anticipate the impact of climate change on sustainable agricultural systems by producing technological innovations that can overcome and suppress the impacts caused, such as by assembling the superior varieties of drought-tolerant rice. The genetic improvement to produce superior varieties that are adaptive to the drought stress conditions is an essential priority in rice breeding programs.

Crossbreeding is used to assemble drought-tolerant rice varieties, which combines the resistant traits of the parents with other high yield varieties. Molecular marker technology can be used to more accurately selecting the desirable traits, through marker assisted selection (MAS), and one of the markers related to drought tolerance is the QTL marker (quantitative trait locus) 12.1. Furthermore, the International Rice Research Institute (IRRI) crossed the Vandana variety of Indian rice using Way Rarem from Indonesia, which generated the filial with crossing number IR148+, derived from IR crossing 79971-B-369-B-B (Mulyaningsih et al., 2010). The crossing population contains QTL 12.1 markers, with the location on chromosome 12, as well as between SSR markers RM28048 and RM 511 (Mc Couch et al. 2002). The presence of these markers maintains yields before flowering and during severe drought stress at the reproductive stage. In normal conditions, the marker QTL 12.1 had an insignificant effect on some of the parameters observed (Bernier et al. 2007).

According to Matsukura et al. (2010), Srivastav et al. (2010), Akhtar et al. (2012), and Huang et al. (2018) *DREB2* gene controls the drought stress tolerance in plants. DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). Sakuma et al. (2002) stated in rice, the *DREB2* gene is homolog to *DREB2A*. Some of the *DREB2A* target genes are *MT2A*, *At1g69870*, *At3g53990*, *At1g22985*, *RD29A*, *LEA14*, *At2g23120*, *RD29B*, *At1g52690*, *RD17* (Sakuma et al. 2006; Qin et al. 2011), *AtHsfA3*, *HSP18.2*, and *Hsp70* (Qin et al. 2011). *DREB2A* gene is important because

52 it can be used as a regulator of various types of drought-responsive genes, making it a marker of drought stress-tolerant
53 genes.

54 Osmotic adjustment in cells is the primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous
55 studies carried out by Sakamoto & Murata (2000) and Saxena et al. (2019) indicated that osmoprotectant substances, such
56 as glycine betaine, plays an essential role in cell stabilization by balancing the structure of the protein quaternary and
57 membrane structure against the adverse effects of salinity. Besides, it facilitates osmotic adjustment by lowering the
58 internal osmotic potential, which contributes to the ability to tolerate water pressure, stabilizing the PSII and RuBisCO
59 complexes in the process of photosynthesis under seizure conditions (Holmström et al. 2000; Wang et al. 2019; Huang et
60 al. 2020). The positive effects and exogenous application of glycine in plants grown under salinity stress condition have
61 been shown to protect plant cells (Demiral and Türkan 2004; Saxena et al. 2019).

62 Betaine aldehyde dehydrogenase (BADH) is a key enzyme for biosynthesis of glycine betaine. Several studies have
63 reported the accumulation ability of glycine betaine and *BADH1* gene expression in tolerating salinity, dryness, and low
64 temperatures (Lapuz et al. 2019; Kahraman et al. 2019). The objective of this study were identification of drought-
65 tolerance traits and molecular analysis of *DREB2A* and *BADH2* genes in the progeny of F6 lines derived from the crossing
66 of local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of IR7858 and IR148+ positive QTL on the
67 chromosome.

68 MATERIALS AND METHODS

70 Plant materials

71 This research was carried out at the University of Bengkulu. The screening of the rice lines was conducted at the
72 Greenhouse of Agricultural Faculty from February to April 2020, while molecular analysis was done in the laboratory of
73 the Department of Biology from May to July 2020. The plant materials used were the progenies of 39 lines selected from
74 F6 generations from single crossing of Bengkulu local varieties (Sriwijaya and Bugis) with drought-tolerant varieties of
75 IR7858 and IR148+ QTL positive on chromosome 12.1 (Mulyaningsih et al. 2010). Table 1 shows the selected F6 lines
76 for traits and molecular identification of drought-tolerant genes of *DREB2A* and *BADH2*, and var. Salumpikit and IR 20 as
77 drought-tolerant and sensitive controls.

78 **Table 1:** Selected F6 lines for traits and molecular identification of drought-tolerant genes of *DREB2A* and *BADH2*
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| Lines number | Genotype | Initial Crossing | Line number | Genotype | Initial Crossing |
|--------------|------------|--------------------|-------------|------------|--------------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 22 | 259-6 | Bugis x IR7858 |
| 2 | 260.A3.2 | Bugis x IR7858 | 23 | 259-9 | Bugis x IR7858 |
| 3 | 260.A3.2 | Bugis x IR7858 | 24 | 259-15 | Bugis x IR7858 |
| 4 | 262.A1.4-2 | Bugis x IR148 | 25 | 260-21 | Bugis x IR7858 |
| 5 | 262.A1.4-3 | Bugis x IR148 | 26 | 260-26 | Bugis x IR7858 |
| 6 | 260.A3.2 | Bugis x IR7858 | 27 | 262-43 | Bugis x IR148 |
| 7 | 262.A1.4-4 | Bugis x IR148 | 28 | 262-48 | Bugis x IR148 |
| 8 | 260.A3.2 | Bugis x IR7858 | 29 | 255-59 | Sriwijaya x IR148 |
| 9 | 262.A1.4-5 | Bugis x IR148 | 30 | 253-2 | Sriwijaya x IR148 |
| 10 | 262.A1.4-6 | Bugis x IR148 | 31 | 259-17 | Bugis x IR7858 |
| 11 | 251-17 | Bugis x IR148 | 32 | 259-3 | Bugis x IR7858 |
| 12 | 248-14-1 | Bugis x IR7858 | 33 | 254-54 | Sriwijaya x IR148 |
| 13 | 249-15-1 | Bugis x IR7858 | 34 | 258-60 | Sriwijaya x IR7858 |
| 14 | 250-16 | Bugis x IR148 | 35 | 255-56 | Sriwijaya x IR148 |
| 15 | 247-13 | Bugis x IR7858 | 36 | 262-44 | Bugis x IR148 |
| 16 | 269-11 | Sriwijaya x IR7858 | 37 | 262-46 | Bugis x IR148 |
| 17 | 248-14-2 | Bugis x IR7858 | 38 | 259-18 | Bugis x IR7858 |
| 18 | 249-15-2 | Bugis x IR7858 | 39 | 259-4 | Bugis x IR7858 |
| 19 | 267-9-1 | Sriwijaya x IR148 | I | IR20 | Control variety |
| 20 | 267-9-2 | Sriwijaya x IR148 | S | Salumpikit | Control variety |
| 21 | 259-1 | Bugis x IR7858 | | | |

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82 Drought tolerance screening

83 The standard Evaluation System (SES) developed by IRRI (2002) was used to screen the drought-tolerant rice from 39
84 F6 lines with the drought-susceptible variety (IR20), and local drought-tolerant variety (Salumpikit) used as control.
85 Furthermore, the test was carried out in accordance with Kumar et al. (2015), Swain et al. (2017), and Herawati et al.

86 (2017) methods with plastic trays of 40 cm x 25 cm x 20 cm size filled with soil. Each tub was planted using ten family
 87 lines and two control varieties, with each line sown for 20 seeds in a row. After planting, the seedlings were intensively
 88 watered for 2 weeks with the plants allowed to dry to determine its sensitivity. Drought tolerance assessment was carried
 89 out based on the SES methods, as shown in Table 2. Furthermore, the trait responses of the seedlings were recorded,
 90 followed by intensive watering for the next ten days. Recovery ability was recorded following the methods of SES, as
 91 shown in Table 2.

92
 93 **Table 2:** Traits identification of selected F6 lines based on criteria description of SES developed by IRRI (2002)
 94

| Score | Criteria | Description | | |
|-------|----------------------|-------------------------------------|---|----------------------------|
| | | Leaf Rolling | Leaf Drying | Recovery Ability |
| 0 | Highly Tolerant | Leaves are healthy | No symptoms | 100 % plant recovered |
| 1 | Tolerant | Leaves start to fold (shallow) | Slight tip drying | 90-99% of plants recovered |
| 3 | Rather Tolerant | Leaves are folding (deep V-shape) | Tip drying extended up to ¼ | 70-89% of plants recovered |
| 5 | Moderate tolerant | Leaves are fully cupped (U-shape) | One-fourth to 1/2 of all leaves dried | 40-69% of plants recovered |
| 7 | Moderate susceptible | Leaf margins are touching (O-shape) | More than 2/3 of all leaves fully dried | 20-39% of plants recovered |
| 9 | Susceptible | Leaves are tightly rolled (V-shape) | All plants are dead. Length in most leaves thoroughly dried | 0-19% of plants recovered |

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 96 **DNA extraction**

97 Genomic DNA was isolated from fresh leaves 14 days after treatment (DAT) with a weight of 100 mg of rice leaf was
 98 added with liquid nitrogen and then ground using a mortar. The total DNA was isolated by modifying the protocols of
 99 Wizard's Genomic DNA Purification Kit, and the ground leaf was put into a 2 ml tube, added with 600 µl of Nuclei Lysis
 100 Solution and then shaken for 3 seconds. In addition, the solution was heated in a water bath at 65°C for 15 minutes, and
 101 then added with 3 µl RNase and incubated at 37 ° C for 15 minutes. This was followed by the addition of 200 µl
 102 Precipitation Solution, and the microtubes containing the mixture were centrifuged for 3 minutes at 13,000 rpm. The
 103 supernatants were collected into a 1.5 ml tube, and then added with 600 µl of isopropanol. Furthermore, the microtubes
 104 were further centrifuged for 1 minute at room temperature, then the supernatant was discarded while the remaining DNA
 105 on the bottom of microtubes was air-dried for 15 minutes. DNA Rehydration Solution of 100 µl was added and further
 106 incubated at 65°C for 1 hour or at 4°C for overnight. The total isolated DNA was used as a template for PCR amplification
 107 of *DREB2A* and *BADH2* genes.
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109 **DNA amplification and gel electrophoresis**

110 PCR amplification of the *DREB2A* gene was determined using the forward and reverse oligonucleotide primers of
 111 CCTCATTGGGTCAGGAAGAA and GGATCTCAGCCACCCACTTA, respectively (Jadhao et al. 2014; Kumar 2016;
 112 Lathif et al. 2018). Meanwhile, the amplification of the *BADH2* gene was carried out using forward- and reverse-
 113 oligonucleotide primers of GGCCAAGTACCTCAAGGCG and TGTCGCCAGCTGCTTCATCC (Robin et al. 2003).
 114 The PCR mixtures, which comprise of the DNA template, forward and reverse primers, buffer, dNTP (nucleotide mix),
 115 and Taq polymerase, were developed during the thermocycling process. The program was carried out using a denaturation
 116 temperature at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 59°C for 2
 117 minutes, and extension at 72°C for 2 minutes, and the final extension at 72°C for 10 minutes. PCR amplification products
 118 were subjected to electrophoresis in an agarose gel of 1% on TBE buffer to identify successful amplifications. The gel
 119 from electrophoresis was immersed in EtBr 1% for 10 minutes, rinsed with ddH₂O for 5 minutes, and visualized under UV
 120 transilluminator light.
 121

122 **Field experiment and yield potential evaluation**

123 A yield performance test of selected superior lines on previous experiments was carried out from March-July 2020 in
 124 Semarang Village, Bengkulu City. The materials used in this study were 16 selected superior lines in the F7 generation
 125 with the experiment carried out on a plot measuring 8 m x 6 m, a spacing of 20 x 20 cm, and by planting a seed. The first
 126 fertilization process was carried out at the age 14 days with a dose of 150 kg ha⁻¹ of Urea, 100 kg ha⁻¹ SP36 and 100 kg ha⁻¹
 127 KCl. The second fertilization was carried out at the age of 30 HST with a dose of 100 kg ha⁻¹ urea, 100 kg ha⁻¹ SP36 and
 128 100 kg ha⁻¹ KCl. Furthermore, intensive control was carried out against weeds, pests and diseases, while observation of the
 129 agronomic characters of 10 plant per-plot samples was taken from each line number. The characters observed included
 130 plant height, number of panicles per-hill, panicle length, number of filled grains per-panicle, percentage of empty grain
 131 per-panicle, 1000 grain weight, grain weight per-hill, and yield per-plot.

133 **Identification of drought-tolerance level**

134 Screening of F6 lines at the seedling stage was carried out to select the drought-tolerant rice. Table 2 shows the drought
 135 tolerance assessment carried out with the SES methods by comparing the treated lines with control varieties of Salumpikit
 136 and IR20. The symptoms, such as leaf curling, drying and recovery ability, were identified after exposure to drought stress
 137 for 14 days, as shown in Figure 1. The criteria of 39 F6 lines were identified as highly to moderately tolerant to drought of
 138 6, 5, 17, and 11 lines, respectively, as shown in Table 3. The scores of dry leaf of the 30 lines with high tolerance level
 139 were 0-1 with recovery ability of 90 to 100%, while the scores of the rest nine lines with moderate tolerance were 3-5, with
 140 recovery ability of 70 to 90% as shown in Table 4 and Figure 1.

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 142 **Table 3:** Screening of the 39 F6 lines for drought tolerance traits and identification of molecular markers of *DREB2A* and *BADH2*
 143 genes
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| Line number | Genotype | Crossing | The score of leaf rolling | The score of leaf drying | Score of recovery | Criteria | DREB2A gene | BADH2 gene |
|-------------|------------|--------------------|---------------------------|--------------------------|-------------------|----------|-------------|------------|
| 1 | 262.A1.4-1 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 2 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 3 | 260.A3.2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 4 | 262.A1.4-2 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 5 | 262.A1.4-3 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 6 | 260.A3.2 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 7 | 262.A1.4-4 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 8 | 260.A3.2 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 9 | 262.A1.4-5 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 10 | 262.A1.4-6 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 11 | 251-17 | Bugis x IR148 | 3 | 3 | 3 | RT | + | + |
| 12 | 248-14-1 | Bugis x IR7858 | 1 | 1 | 1 | T | + | + |
| 13 | 249-15-1 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 14 | 250-16 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 15 | 247-13 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 16 | 269-11 | Sriwijaya x IR7858 | 1 | 1 | 1 | T | + | + |
| 17 | 248-14-2 | Bugis x IR7858 | 0 | 0 | 0 | HT | + | + |
| 18 | 249-15-2 | Bugis x IR7858 | 3 | 3 | 3 | MT | + | + |
| 19 | 267-9-1 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 20 | 267-9-2 | Sriwijaya x IR148 | 1 | 1 | 1 | T | + | + |
| 21 | 259-1 | Bugis x IR7858 | 3 | 3 | 3 | MT | + | + |
| 22 | 259-6 | Bugis x IR7858 | 1 | 1 | 1 | T | + | + |
| 23 | 259-9 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 24 | 259-15 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 25 | 260-21 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 26 | 260-26 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 27 | 262-43 | Bugis x IR148 | 0 | 0 | 0 | HT | + | + |
| 28 | 262-48 | Bugis x IR148 | 1 | 1 | 1 | T | + | + |
| 29 | 255-59 | Sriwijaya x IR148 | 3 | 3 | 3 | RT | + | + |
| 30 | 253-2 | Sriwijaya x IR148 | 5 | 5 | 5 | MT | + | + |
| 31 | 259-17 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 32 | 259-3 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| 33 | 254-54 | Sriwijaya x IR148 | 3 | 3 | 3 | RT | + | + |
| 34 | 258-60 | Sriwijaya x IR7858 | 0 | 0 | 0 | HT | + | + |
| 35 | 255-56 | Sriwijaya x IR148 | 0 | 0 | 0 | HT | + | + |
| 36 | 262-44 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 37 | 262-46 | Bugis x IR148 | 5 | 5 | 5 | MT | + | + |
| 38 | 259-18 | Bugis x IR7858 | 5 | 5 | 5 | MT | + | + |
| 39 | 259-4 | Bugis x IR7858 | 3 | 3 | 3 | RT | + | + |
| I | IR20 | Control variety | 5 | 5 | 5 | MT | - | - |

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| Line number | Genotype | Crossing | The score of leaf rolling | The score of leaf drying | Score of recovery | Criteria | DREB2A gene | BADH2 gene |
|-------------|------------|-----------------|---------------------------|--------------------------|-------------------|----------|-------------|------------|
| S | Salumpikit | Control variety | 1 | 1 | 1 | T | + | + |

HT=Highly Tolerant (6 lines); T= Tolerant (5 lines); RT=Rather Tolerant (17 lines); MT= Moderate Tolerant (11 lines); + = gene was present

Table 4: The distribution of 39 F6 lines for identification of drought-tolerant lines

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| The number of Lines | Drought Response | | | | The score of Recovery Ability | | | | |
|---------------------|------------------|-----------------|---------------------|-------------|-------------------------------|---|---|---|---|
| | Highly-Tolerant | Rather Tolerant | Moderately Tolerant | Susceptible | 1 | 3 | 5 | 7 | 9 |
| | (score 0-1) | (score 3) | (score 5) | (Score 7-9) | | | | | |
| 11 | ■ | | | | ■ | | | | |
| 17 | | ■ | | | | ■ | | | |
| 11 | | | ■ | | | | ■ | | |
| IR20 | | | ■ | | | | | | |
| Salumpikit | ■ | | | | | | | | |

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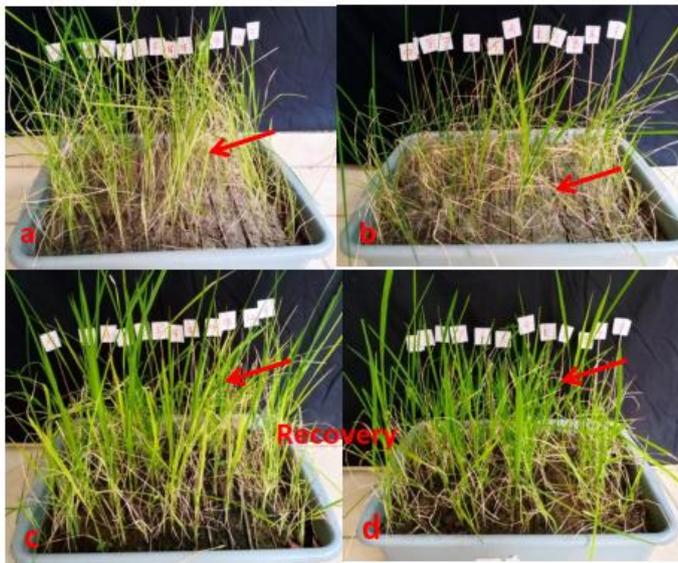


Figure 1: Responses of F6 progeny lines to drought observed on drying leaf (a and b) and recovery ability (c and d)

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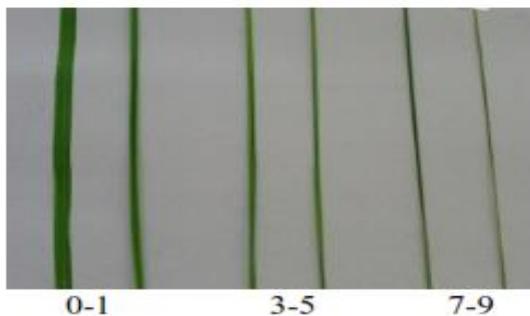


Figure 2: Description of rolling leaves based on SES Method; scores 0-1: start rolling to form V shape; 3-5: rolling to form V and U shapes inside leaves; 7-9: leaves are rolling totally

Molecular identification of drought tolerance genes

The molecular analysis using PCR products separated on agarose gel electrophoresis showed that the *DREB2A* gene was present in the 39 selected lines with the marker sizes approximately 250 bp as shown in Figure 3. This proves that the progeny of F6 lines originated from the parents IR148 + carrying QTL 12.1 and IR7858-1 are drought-tolerant. This evidence proved that drought tolerance in rice plants is controlled by the *DREB2A* genes. The visualization of the *BADH2* gene 39 selected lines showed a marker with a size of approximately 1300 bp as shown in Figure 4.

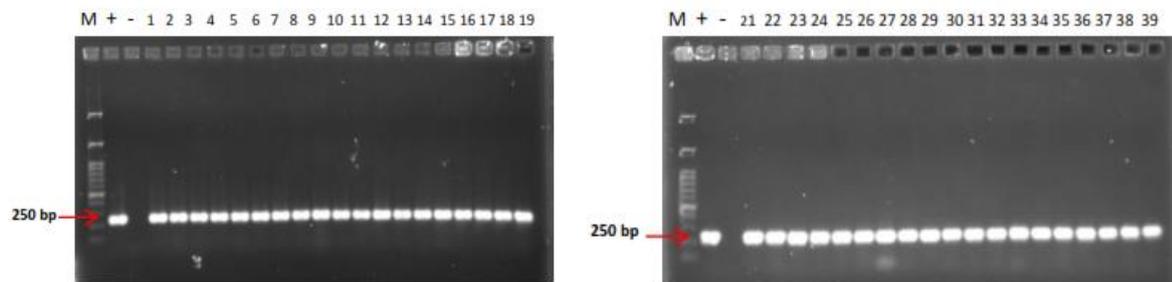


Figure 3: PCR amplification of *DREB2A* (250 bp) on 39 selected lines with Salumpikit and IR20 as positif and negative control respectively (M= DNA ladder of 100 kb)

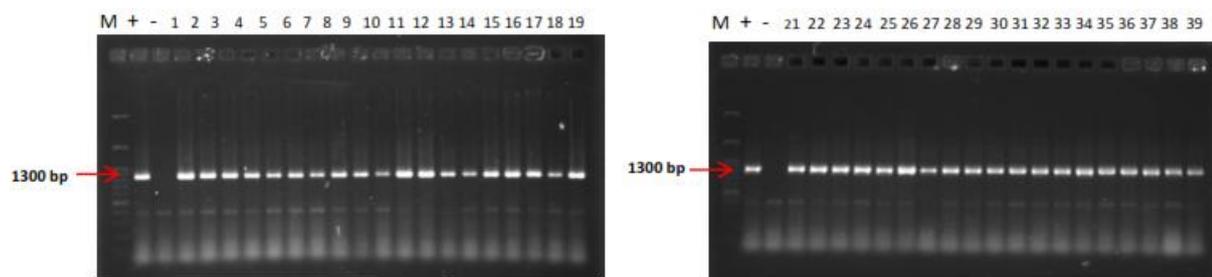


Figure 4: PCR amplification of *BADH2* (1300 bp) on 39 selected lines with Salumpikit and IR20 as positif and negative control respectively (M= DNA ladder of 100 kb)

Performance of agronomic characters, yield and yield potential of superior lines

The performance of agronomic characters and yield potential of the 16 tested superior lines are shown in Table 5. Almost all tested lines uniformly performed as shown by plant height range of 101.1 and 140.5 cm with a standard deviation of 1.52- 4.37. This shows that all tested lines were homozygous in the 8th generation (F7). Furthermore, the highest and lowest average number of panicles per-hill were 14.7 and 6.5, with the panicle length range of 24.61 - 27.6 cm. Furthermore, the number of filled grains per-panicles ranged from 99.5 - 150.07, while the percentage of unfilled grains was from 9.87% - 26.66%, which are categorized as low based on the SES IRRI (2012). This led to variations in grain weight per-hill were 19-35.5 g per-hill.

Table 5: The performance of agronomic characters, yields and yield potential of superior lines in the field experiment

| Accession | Plant height | Number of Panicle per-hill | Panicle length (cm) | Number of fill grains | % of unfilled gran per-panicle | 1000 grains weight (g) | grains weight per-hill (g) | Yield per-plot (1x1 m ²) (g) | Yield potential (t ha ⁻¹) |
|---|--------------|----------------------------|---------------------|-----------------------|--------------------------------|------------------------|----------------------------|--|---------------------------------------|
| X ± SD (Mean ± standard deviation) | | | | | | | | | |
| BKL3-R51-1-253-18 | 113.1 ± 1.91 | 7.6 ± 1.35 | 26.08 ± 2.13 | 115.71 ± 26.70 | 11.06 ± 7.81 | 28.4 ± 1.26 | 19.0 ± 3.71 | 512 | 5.12 |
| BKL3-R51-3-255-20 | 130.7 ± 2.87 | 9.9 ± 3.14 | 26.02 ± 1.94 | 150.07 ± 40.63 | 13.19 ± 5.81 | 27.4 ± 2.98 | 32.5 ± 15.89 | 519 | 5.19 |
| BKL4-R51-1-256-21 | 105.4 ± 1.64 | 10.88 ± 2.15 | 24.77 ± 2.99 | 112.5 ± 30.22 | 17.96 ± 10.97 | 28.6 ± 1.89 | 29.2 ± 11.29 | 478 | 4.78 |
| BKL4-R51-2-257-22 | 107.3 ± 2.58 | 8.5 ± 1.65 | 25.58 ± 1.99 | 111.28 ± 29.26 | 17.95 ± 8.19 | 27.9 ± 2.13 | 21.6 ± 8.43 | 431 | 4.31 |
| BKL4-R51-3-258-23 | 101.1 ± 1.79 | 7.9 ± 1.72 | 25.05 ± 2.62 | 111.86 ± 40.49 | 12.29 ± 8.76 | 28.5 ± 2.27 | 18.7 ± 5.59 | 520 | 5.2 |
| BKL1 B-1-259-1 | 111.6 ± 2.27 | 11.6 ± 1.95 | 24.61 ± 1.63 | 120.89 ± 30.07 | 12.71 ± 6.88 | 27.6 ± 1.84 | 31.8 ± 9.54 | 1005 | 10.05 |
| BKL1 B-2-260-2 | 115.8 ± 3.67 | 10.8 ± 2.25 | 25.11 ± 2.17 | 112.93 ± 28.54 | 11.14 ± 6.15 | 26.6 ± 1.65 | 26.2 ± 5.61 | 712 | 7.12 |
| BKL1 B-3-261-3 | 117.3 ± 2.45 | 14.7 ± 3.53 | 25.45 ± 2.23 | 110.04 ± 29.74 | 9.87 ± 6.34 | 27.6 ± 1.26 | 33.6 ± 12.87 | 1038 | 10.38 |
| BKL2 B-1-262-4 | 140.5 ± 3.24 | 12.8 ± 3.43 | 27.05 ± 2.25 | 105.92 ± 26.76 | 26.66 ± 9.4 | 29.00 ± 1.94 | 28.1 ± 7.25 | 719 | 7.19 |
| BKL2 B-2-263-5 | 123.7 ± 2.31 | 13.4 ± 3.81 | 26.07 ± 2.47 | 103.57 ± 29.42 | 24.87 ± 10.84 | 28.8 ± 1.68 | 35.5 ± 22.14 | 750 | 7.5 |
| BKL2 B-2-264-6 | 119.2 ± 1.55 | 12.0 ± 4.89 | 24.92 ± 1.57 | 110.5 ± 34.75 | 14.06 ± 8.33 | 29.2 ± 3.01 | 28.7 ± 13.71 | 1210 | 12.1 |
| BKL3 B-1-265-7 | 108.0 ± 2.00 | 14.3 ± 2.58 | 25.63 ± 1.68 | 99.5 ± 19.76 | 16.93 ± 7.26 | 28.4 ± 1.84 | 28.2 ± 6.23 | 653 | 6.53 |
| BKL3 B-2-266-8 | 107.1 ± 1.52 | 11.1 ± 1.79 | 27.6 ± 1.93 | 138.46 ± 34.52 | 21.94 ± 6.96 | 28.8 ± 1.39 | 36.7 ± 9.26 | 667 | 6.67 |
| BKL3 B-3-267-9 | 112.3 ± 3.37 | 12.20 ± 3.29 | 25.68 ± 2.67 | 110.10 ± 39.48 | 22.56 ± 14.07 | 26.80 ± 1.87 | 19.1 ± 8.57 | 458 | 4.58 |

| | | | | | | | | | |
|-----------------|--------------|-------------|--------------|----------------|---------------|-------------|-------------|------|-------|
| BKL4 B-1-268-10 | 127.3 ± 4.37 | 12.1 ± 3.38 | 25.55 ± 2.37 | 124.71 ± 36.6 | 21.72 ± 11.07 | 27.2 ± 1.93 | 31.9 ± 11.2 | 1206 | 12.06 |
| BKL4 B-3-270-12 | 112.3 ± 4.03 | 6.5 ± 1.35 | 26.46 ± 2.63 | 108.32 ± 27.01 | 19.08 ± 11.18 | 28.4 ± 1.50 | 16.6 ± 5.62 | 640 | 6.4 |

187 The grain yield per-plot varied from the lowest at 458 g to the highest, at 1210 g. The agronomic characters that
188 supports the observed high grain yield were the high number of panicles, the low percentage of unfilled grain, and the high
189 1000 grains weight. The length of the panicles did not show any significant variation and i.e range from 24.61-27.6 cm, as
190 shown in Table 5.

191 Discussion

192 Seedlings' responses to drought stress tolerance were identified after 14 days after the stress treatment. Afterwhich, the
193 tolerant lines continued to grow normally and their leaves remained fully open, whereas the moderately tolerant lines
194 experienced the drying of leaf tips as shown in Figure 1. Kumar et al. (2014) stated that leaf rolling was delayed in
195 drought-tolerant rice genotypes and induced by loss of turgor as well as low osmotic regulation. Delayed leaf rolling in
196 the tolerant genotype indicated that the turgor remained normal, and the plants were protected from dehydration, as shown
197 in Figure 2. According to Bunnag & Pongthai (2013) and Swain et al. (2017), leaf rolling is one of the mechanisms used
198 by plants to adjust the water potential, and enable them to absorb groundwater in drought stress conditions. Swain et al.
199 (2017) reported that during the drought conditions, the level of groundwater was below 30 cm depth, of the 78 lines of
200 drought-tolerant assessments. Furthermore, 30 and 48 lines were scored by 1, and 3, respectively. Out of these 78 lines
201 assessed, 13 line and the tolerant (CR 143-2-2) variety produced more than 1 and 2.7 t grain ha⁻¹ respectively, while the
202 sensitive control variety (IR20) produced no grain at all. The IR20 variety is often used as a check for drought sensitivity,
203 but our results show that IR20 was categorized as moderate in the drought stress treatment at the seedling phase. It is
204 necessary to review the sensitivity and adaptability in the seedling phase.

205 Leaf rolling can reduce the surface area exposed to sunlight, thereby reducing the rate of transpiration in plants. This
206 condition help plants to survive in a certain period when the availability of water in the environment decreases. The genes
207 in rice plants that play a role in this process are the Roc5 (Rice outermost cell-specific genes) genes that encode the leucine
208 zipper class IV transcriptional factor homeodomain. According to Zou et al. (2011), overexpression and suppression of
209 these genes lead to leaf curling on the adaxial and abaxial sides of the plants. Delay in leaf rolling indicates a plant effort to
210 maintain turgor and avoid dehydration. Water shortages tend to have lower rates of leaf rolling, which appears in plants
211 with tolerant criteria score of 1, as shown in Table 3. It allowed the plant to survive to drought at the low leaf tissue water
212 potential (Sevanto, 2018). Furthermore, the plants recovered after passing through a period of drought, thereby indicating
213 the ability of plants to enhance their metabolic system (Bian et al. 2017; Wang et al. 2019).

214 Drought-tolerant rice varieties can be generated through crossbreeding by combining the tolerant character of the
215 ancestors with other varieties that have high productivity. The use of molecular marker technology can help fasten the
216 selection process, thereby making it accurate and faster. One of the markers related to drought tolerance is the QTL
217 (quantitative trait locus) 12.1, which was produced by crossing the Vandana varieties of Indian rice and Indonesian Way
218 Rarem (Mulyaningsih et al. 2010). One of the lines is IR148+, which was used as a parent in this study due to its ability to
219 maintain rice yield in severe drought stress at the reproductive phase before flowering. The molecular marker of QTL 12.1
220 had an insignificant effect on some of the parameters observed under normal conditions (Bernier et al. 2007).

221 DREBs (Dehydration Responsive Element Bindings) are essential transcription factor proteins (TFs) used in regulating
222 the expression of drought-responsive genes (Lata and Prasad 2011; Fujita et al. 2013). The *DREB2A* gene is essential as a
223 regulator of drought-responsive genes, making it a marker of drought stress-tolerant. The transcription factors in *DREB2A*
224 are involved in the regulatory mechanisms that exist in some plants in response to drought, salinity, and heat stress (Lata
225 and Prasad 2011; Mizoi et al. 2012). There are five *DREB2* genes in the rice genome, including *OsDREB2A*, *OsDREB2B*,
226 *OsDREB2C*, *OsDREB2E*, and *OsABI4* (Matsukura et al. 2010; Srivastav et al. 2010). Expression of *OsDREB2A* in rice is
227 caused by water deficit and exogenous ABA application, which leads to increased drought stress (Cui et al. 2011). The
228 *OsDREB2B* transcript has a functional and non-functional form marked during drought conditions. Consequently, it can
229 increase drought tolerance through alternative splicing induced by its pre-mRNA (Matsukura et al. 2010). All of these
230 results indicate that *OsDREB2s* also play an essential role in the regulation of drought tolerance.

231 Huang et al. (2018) identified a new transcription factor gene such as *DREB2*, namely *OsDRAP1* (Responsive Drought
232 Genes *AP2/EREBP*), located in the introgressed segment on chromosome 8 of DK151 and whose expression is highly
233 regulated by drought at *DK151*, thereby showing its role in drought tolerance rice. Osmotic adjustment in cells is the
234 primary response of plants to drought (Zivcak et al. 2016; Blum 2017). Previous studies indicated that osmoprotectant
235 substances, namely glycine betaine, plays an essential role in cell stabilization by balancing the structure of the protein
236 quaternary and membrane against the adverse effects of salinity (Sakamoto and Murata 2000; Saxena et al. 2019). In
237 addition, it facilitates osmotic adjustment by reducing the internal osmotic potential that contributes to water stress
238 tolerance in plant cells. It also stabilizes the PSII and RuBisCO complexes during photosynthesis under stress conditions
239 (Holmström et al. 2000; Huang et al. 2020). These are some of the positive effects of the application of exogenous betaine
240 glycine in plants that grow under the pressure of salinity or drought stress. Plant cells can be protected from adverse effects
241 of salinity induced oxidative stress by exogenous application of glycine betaine (Demiral and Türkan 2004; Saxena et al.
242 2019).

243 The successful use of molecular markers that control complex traits for obtaining drought-tolerant superior rice
244 varieties have been reported by Lanceras et al. (2004). Some of the studied traits included yield, root length, thickness,
245 leaf curl, stomata sensitivity (Price et al. 2002; Courtois et al. 2003; Swain et al. 2017) and osmotic adjustment (Zivcak
246 et al. 2016; Blum 2017; Kahraman et al. 2019). Betaine aldehyde dehydrogenase (BADH) is a key enzyme for the
247 biosynthesis of glycine betaine. This is because several studies have reported the accumulation of glycine betaine and
248 *BADH1* gene expression for tolerance to salinity, drought, and low temperatures (Lapuz et al. 2019; Kahraman et al.
249 2019). DNA marker linked to *BADH2* gene was present on the 39 selected lines with the marker size of approximately
250 1300 bp (Shrestha 2011; Hasthanasombut et al. 2011) as shown in Figure 4.

251 The PCR assay of 39 selected lines for drought tolerance using *DREB2A* and *BADH2* primers are shown in Table 3. All
252 tested lines showed positive results, they contained both genes and had the criteria of varying degrees of drought level in
253 the seedling stage evaluation. However, the molecular analysis showed positive results as a drought-tolerant marker gene
254 in the seedling stage, then evaluation at the productive stage needs to be carried out to obtain more accurate data, due to
255 many genes contribute to regulate drought responsive gene expression. Drought-tolerant plants can adapt to drought
256 conditions, which are shown by high grain. The use of superior varieties is the most efficient technology to increase rice
257 yield with low-cost of production in the dry land. Therefore, developing a superior variety by crossbreeding is needed to
258 produce superior potential lines. Furthermore, before releasing a new superior variety, potential selected lines need to be
259 tested in various locations (multi-location trials/MLT).

260 The agronomic performance and yield of 16 superior lines showed that all lines reached homozygous in the 8th
261 generation (F₇), where the plant height had a relatively low standard deviation. The number of panicles ranged from 14.7
262 had a high yield potential, with the value of a filled grain of 150.07 (Table 5). The new paradigm of rice breeding is the
263 number of productive tillers between 8-12 tillers per-hill with the grains number per-panicles ranging from 150-200 (Peng
264 and Khush 2003). Peng et al. (2008) stated that in the new type rice variety breeding programmes avoid extreme traits,
265 such as 200-250 grain per-panicle which can produce panicles with low seed filling. Therefore, the increase in the second
266 generation of new types of rice has been modified by IRRI to 150 grains per-panicle. Several lines have a potential yield of
267 more than 10 t ha⁻¹, such as those with the accession number BKL1 B-1-259-1 and BKL1 B-3-261-3 to yield potential of
268 10.05 t ha⁻¹ and 10.08 t ha⁻¹, respectively. Meanwhile, the lines with the BKL2 B-2-264-6 and BKL4 B-1-268-10 numbers
269 had a potential yield of more than 12 t ha⁻¹, namely 12.1 and 12.06 t ha⁻¹, as shown in Table 5. These lines have the
270 opportunity to be developed on dry land or as rice on rainfed land because the lines tested were identified as drought
271 tolerant as shown in Table 3.

272

ACKNOWLEDGEMENTS

273 The authors are grateful to the Directorate of Research and Community Service, Ministry of Education and Culture
274 Republic of Indonesia for funding this research through the National Competitive Applied Research with Contract
275 Number: 165/SP2H/AMD/LT/DRPM/2020 base on Amendment Contract Number: 165/SP2H/LT/DRPM/2019. The
276 authors are also, grateful to the Head of Research and Community Board, Dean of the Agricultural Faculty, and Head of
277 the Department of Crop Production at the University of Bengkulu for their help in facilitating this research.

278

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RENY HERAWATI, ALNOPRI, MASDAR, MARULAK SIMARMATA, SIPRIYADI, MIMI SUTRAWATI:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potensial of Lines from Single Crossing Bengkulu Local Rice Varieties".

Our decision is to: Accept Submission

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19 Januari 2021 14.43

Balas Ke: Smujo Editors <editors@smujo.id>

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RENY HERAWATI, ALNOPRI, MASDAR, MARULAK SIMARMATA, SIPRIYADI, MIMI SUTRAWATI:

The editing of your submission, "Identification of Drought Tolerant and Molecular Analysis of DREB2A and BADH2 Genes and Yield Potensial of Lines from Single Crossing Bengkulu Local Rice Varieties," is complete. We are now sending it to production.

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