January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Committees: The Second International Conference on Genetic Resources and Biotechnology

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Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

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Phenotypic and Genetic Stability Evaluation of the Targeted *GA20ox-2* Gene Mutation in CRISPR/Cas9 Mutant Rice Derived from Mentong Cultivar

Aniversari Apriana[1, a)], Tri Joko Santoso[2], Atmitri Sisharmini[1], Reflinur[1], A. Dinar Ambarwati[1], Toto Hadiarto[1], Sustiprijatno[1] and Nuryati[1]

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Abstract. Lodging is a major constraint in cereal crops which lead to reduction of yield and grain quality. Therefore, lodging tolerant varieties have led to remarkable harvest index increases in cereal crops. Gibberellin (GA) is important endogenous hormones responsible to plant height trait and its deficiency in plants may cause dwarfism. In this study, a set of semidwarf F_3 mutant rice population generated from a cross between Mentong, an Indonesian local swamp rice cultivar (female parent exhibits tall plant height), and K-15, a CRISPR/Cas9-target mutant of *GA20ox-2* in Kitaake (donor parent), was subjected to assessment for their phenotypic and genotypic stability. A total of 19 mutant rice lines along with parents were planted in the greenhouse and some important agronomic traits (plant height, tiller number, and heading date) were observed to assess stability of the phenotype. The genetic or hereditary stability of the mutated sites on *GA20ox-2* gene was further confirmed by sequence comparison between targeted gene in mutant rice lines and their parents. Based on phenotype observation, all lines generally showed reduction in plant height (20.6–53.8%) compared to their female parent and had more tiller number (14.33–26.50) compared to their parents. Of these, three lines were phenotypically homogeneous, and were molecularly confirmed conferring the allele of the *GA20ox-2* which were homozygous. Our findings suggest that development of mutant rice using genome editing technology approach combined with conventional breeding would be useful to facilitate rapid and precise crop improvement in plant breeding program.

INTRODUCTION

Shorter plant height is an important trait in rice plant due to its reduction of lodging potential. Lodging in rice plants can reduce production, and can even lead to crop failure, if occurs during the flowering phase or the initial phase of grain filling. One of the factors that cause rice lodging is a relatively high posture. During heavy rain or strong winds, the plants can easily collapse.

Improving lodging resistance in rice breeding program could be a best strategy to overcome such a problem. The improvement of certain trait can be done by conventional breeding (crosses) and utilizing modern technology such as mutations, and even genetic engineering. Plant breeding to reduce the level of lodging with the strategy of shortening the plant posture has been carried out on food crops, such as wheat [1] and rice [2]. The mutation method that is considered effective for now is genome editing technology [3]. Since the discovery of genome editing technology with the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system in 2013, the method is considered to be simpler and more effective than the zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) systems. CRISPR/Cas system only requires RNA guidance to recognize specific and precise target loci. The activity of the Cas endonuclease enzyme can cause gene modification by cutting the target DNA and forming DNAs double-strand breaks (DSBs) which can stimulate

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in vivo DNA repair that can produce mutations in the targeted genes. Mutations that occur can be in the form of insertion, deletion or replacement of nucleotide sequences or bases [3]. The repairment of DNA that has been cut will be carried out randomly with a non-homologous end-joining (NHEJ) mechanism which can cause insertion or deletion of the target gene in the cell which has the potential to create gene mutations [3]. The application of CRISPR/Cas9 genome editing technology has been widely used in food crops, including the assembly of powdery mildew resistant wheat plants [4] and the development of a ripening delay in tomatoes [5].

It is known that one of the genes responsible for plant height is semidwarf gene (*SD1* or *GA20ox-2*) which encodes the GA20ox-2 enzyme [6]. This enzyme is a key enzyme in the biosynthesis of the gibberellin (GA20) growth hormone. It is known that there is a mutation of the *SD1* gene which causes the posture of the plant to become semidwarf. IR8 rice, which is the first semidwarf *indica* rice that has been able to increase yield, contains a mutation of 383 bp in the *GA20ox-2* gene, which causes GA20 production to decrease and affects the decrease in plant height [7]. Semidwarf character in plants have a pleiotropic effect on the higher number of tillers, higher number of panicles, higher number of spikelet per panicle, the leaves become straighter so that they can capture more sunlight, the harvest index is higher, and the plants become more responsive to nitrogen fertilization, so that plant productivity increases [8–10]. Rice production is not only determined by the number of panicles, grain weight, and the number of grains per panicle, but also by plant height and flowering time. The most important agronomic characters in rice are plant height, flowering time, and yield. Flowering time has a positive correlation with plant height, but has a negative relationship with grain yield per plant [11, 12].

Since 2016, the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) has developed lodging-resistant rice by applying genome editing technology with the CRISPR/Cas9 system [13]. CRISPR/Cas9 genome editing by targeting the *GA20ox-2* gene located on chromosome 1 has been successfully carried out in *japonica* rice plant cv. Kitaake and produced mutant lines of Kitaake (K15) that do not contain the transgene. The lines are then crossed with several varieties, including the local swamp rice Mentong (PR135) to improve the plant's height posture so that it is resistant to lodging. The aim of this study was to evaluate the phenotype of *GA20ox-2* mutant lines and the genetic stability of the introgression of the *GA20ox-2* mutant gene in F_3 generation. It is hoped that from this research lodging resistant rice lines from the *GA20ox-2* gene mutant can be obtained.

MATERIALS AND METHODS

The research was conducted at the laboratory and greenhouse of ICABIOGRAD, Bogor. The genetic material used for this activity was 19 mutant rice lines of F_3 generation derived from crosses between female parents Mentong (PR135) local swamp rice and male parents the mutant Kitaake (K15). The assembling of the *GA20ox-2* K15 mutant lines was described by Santoso *et al*. [14]. Mentong (PR135) was used as the control plant.

Planting the *GA20ox-2* Mutant Rice Lines

Control plants Mentong and 19 mutant rice lines were sown in seedlings container filled with sandy soil. Each mutant rice line was planted in 10 repetition (10 plants from 5 selected panicle). They were germinated for 21 days, and then transplanted into buckets filled with mud. The plants were maintained by watering, fertilizing, and controlling pests and diseases.

Observation and Analysis of Plant Phenotypic Data

Phenotypic observations in the form of agronomic characters were carried out on 10 plants from each mutant rice line. Observations consisted of plant height, number of tillers, and time of flowering. The phenotypes of the *GA20ox-2* mutant rice lines were selected based on the agronomic traits and compared to control plants. The mutant rice lines that have a shorter posture will be selected and will be used for testing in the next generation. The phenotypic character data were analyzed by comparing the mean value for each mutant line in each character and compared to their parents (control plants).

Analysis of Stability of the *GA20ox-2* Gene Mutation in Three Mutant Rice Lines of Mentong Derivative

DNA that has been isolated from three *GA20ox-2* mutant rice lines of Mentong-derived F_3 generation. The three lines were selected due to their homogeneous performance. They were subjected to PCR using specific primers designed to flank the targeted areas for mutation from the *GA20ox-2* gene sequence. The mutated target gene sequence is in the exon 1 from the *GA20ox-2* gene. The PCR reactions were carried out with a total volume of 20 µl for each reaction. The PCR reaction mixture consisted of 6 µl nuclease-free water, 10 µl KAPA2G Fast ReadyMix (Sigma-Aldrich, Germany), 1 µl (10 pmol) forward primer GA20 (5'-ATGTCTGTCCAGTGGCAACC-3'), 1 µl (10 pmol) reverse primer GA20 (5'- CACCATCGTTTTAATTACCCCATT-3'), and 1 µl of genomic DNA template.

The PCR program used to amplify the target DNA was initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 15 sec, annealing at 53°C for 15 sec, and primary extension at 72°C for 15 sec. Final elongation at 72°C for 5 min. The PCR was carried out in 35 cycles. PCR product was then electrophoresed on 1% agarose gel using TBE buffer. The PCR product was then sent to the 1st BASE Laboratory for sequencing. Sequencing analysis was performed using the available Clustal W Omega.

RESULTS AND DISCUSSIONS

ICABIOGRAD has carried out gene editing for *GA20ox-2* on the *japonica* rice cv. Kitaake using CRISPR/Cas9 technology, and resulted in the *GA20ox-2* Kitaake 15 (K-15) mutant rice line. Two gRNAs were designed from the upstream part of the *GA20ox-2* gene in exon 1, where gRNA1 was at position -16 to +4 (20 bp, sense) and gRNA2 at positions +43 to +63 (20 bp, antisense) [14]. The mutation occurred in the *GA20ox-2* gene sequence with 2-base adenine (A) insertion, causing a frameshift mutation, which led to changes in the amino acid composition in the conserved domain [15]. This change seemed to induce a semidwarf phenotype in rice lines. The *GA20ox-2* gene encodes the GA20ox-2 enzyme which is a key enzyme in the biosynthesis of the growth hormone (GA). This enzyme is involved in the final cycle of GA20 active biosynthesis [6]. GA20 growth hormone is involved in plant growth and development, among which it is responsible for the character of plant height [6].

The *GA20ox-2* K15 mutant rice line that does not carry transgene (*hpt* and *Cas9*) is used as a male parent (*GA20ox-2* mutant allele donor) in crossbreeding with the female parent of the local swamp rice (Mentong). The results of these crosses have now produced F_3 generation of *GA20ox-2* mutant lines derivative, where the F_2 generation of *GA20ox-2* mutant lines have been confirmed molecularly to be carrying the *GA20ox-2* mutant allele (data not shown).

Phenotypic Evaluation of the F_3 Generation of *GA20ox-2* Mutant Rice Lines

Phenotypic evaluation and genetic stability were carried out on the F_3 generation of the *GA20ox-2* mutant rice lines. The phenotypic observations and the flowering time generally showed a shorter plant posture compared to the control plants. The average height of control plants (Mentong) was 161.33 cm, while the plant height of the mutant rice lines ranged from 73.33 to 128.03 cm, which is a reduction in plant height posture by 20.6–53.8%. Line 8.4 was the line with the highest reduction in plant height (73.33 cm). The shorter posture of the mutant plants was probably due to the presence of *GA20ox-2* mutant allele which was successfully introgressed in the genome of the Mentong mutant lines. The mutation in the *GA20ox-2* gene sequence suggest the impairment of the biosynthesis of GA in plants, so that the plant growth process (i.e. plant height) is disrupted.

From the evaluated mutant lines, there were three lines that showed homogeneous phenotypes, namely lines 8.10, 8.17, and 8.18. The reduction in plant height of lines 8.10, 8.17, and 8.18 was 28.8%, 31.4%, and 30.9%, respectively, compared to the control plant (Fig. 1). Reduction of plant height posture demonstrates to be followed by an increase in the number of tillers and early maturity compared to the control plants. The mutant rice lines had a higher number of tillers of 14.33–26.50 compared to their parents (11.67 tillers). The lines 8.10, 8.17, and 8.18 had the tiller numbers of 23.1, 18.9, and 23.6, respectively. The increase in the tiller number compared to control plants ranged from 61.5 to more than 100% (Table 1). The increase in the tillers number in the *GA20ox-2* mutant rice lines was probably due to the pleiotropic effect of the expression of the *GA20ox-2* gene mutant. Similar findings have also been reported by Yu *et al*., Murai *et al*., and Hu *et al*. [10, 16, 17] where the Hikarishinseiki rice isogenic lines and the Kasalath lines containing *GA20ox-2* mutant gene (*sd1* allele) had a higher number of tillers. The flowering time characters of the mutant rice lines also showed early maturity, ranging from 45–67 days after sowing (DAS),

compared to their parents (70 DAS) (Table 1). According to Yu *et al*. [10], the *GA20ox-2* mutant rice which has a higher number of tillers and a higher number of spikelets per panicle is a pleiotropic effect of the *GA20ox-2* mutation.

TABLE 1. Agronomic characters and flowering time of the F_3 generation of *GA20ox-2* mutant rice lines of Mentong × Kitaake 15 (K-15).

Lines	Plant height (cm)	Number of tillers	Heading date (DAS)
Mentong (control)	161.33	11.67	70
8.1	75.93	17.93	45
8.2	91.98	20.13	54
8.3	95.11	17.4	54
8.4	73.33	20.13	56
8.5	87.31	23.72	56
8.10	**114.76**	**23.07**	**63**
8.11	84.38	19.27	54
8.12	89.65	26.5	46
8.13-1	84.7	14.63	54
8.13-2	75.8	14.33	56
8.14	85.87	15.33	69
8.16	81.35	19.00	67
8.17	**110.74**	**18.92**	**56**
8.18	**111.52**	**23.6**	**56**
8.19	77.4	16.67	52
8.20	112.5	9.93	67
8.21	74.53	14.6	52
8.22	128.03	9.27	56
8.24	78.91	22.67	56

Numbers in bold indicate lines that are phenotypically homogeneous. DAS = days after sowing.

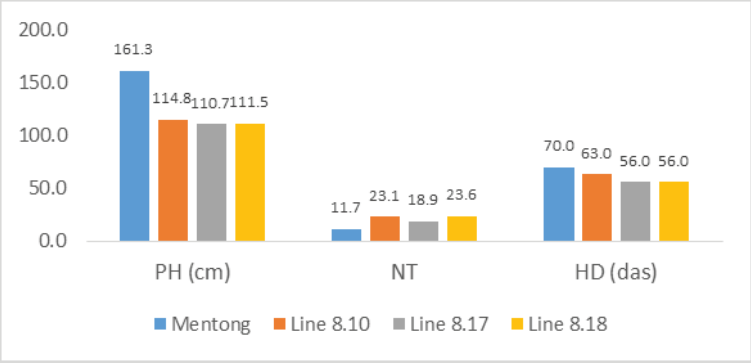

FIGURE 1. Agronomic characters and flowering time of three selected lines (8.10, 8.17, and 8.18) of *GA20ox-2* mutant rice lines and control (Mentong). PH = plant height, NT = number of tiller, HD = heading date, DAS = days after sowing.

The results of the observation on the yield components of *GA20ox-2* mutant lines showed that the panicle length of 19 lines tested did not show any difference compared to the control plants. Panicle length is one of the important characters in rice because it is associated with production (number of grains per panicle) [6]. Mutations in *GA20ox-2* gene are not considered to have negative effects on panicle length in the mutant rice lines. This is in line with the

research conducted by Tomita and Ishii [9] that rice lines carrying *GA20ox-2* mutant allele did not change panicle length character.

The observation on filled grain per panicles indicated that, in general, the F_3 generation of *GA20ox-2* mutant lines had more filled grain per panicle than control plants. From the lines tested, there were six lines that had a smaller number of filled grains than the control plants. Control plants (Mentong) had 114 grain-filled per panicle, while lines 8.1, 8.3, 8.11, 8.13-2, 8.21, and 8.24 had the number of filled grains ranged from 73.71–112.69 grains (Table 2). However, these six lines had the total number of grains per panicle relatively similar or more than the control plants, the same as the other tested lines (Table 2).

TABLE 2. Yield components of the F_3 generation of *GA20ox-2* mutant rice lines of Mentong × Kitaake 15 (K-15).

Lines	Length of panicle (cm)	Number of fertile seed per panicle	Number of sterile seed per panicle	Total number of grain per panicle	Percentage of fertile seed per panicle
Mentong (control)	25.02	114.00	20.80	134.80	84.57
8.1	21.10	86.07	66.20	152.27	56.52
8.2	22.62	203.47	99.00	302.47	67.27
8.3	26.82	73.71	108.93	182.64	40.36
8.4	22.16	127.81	58.63	186.44	68.56
8.5	25.36	134.00	45.69	179.69	74.57
8.10	**28.78**	**135.01**	**64.86**	**199.87**	**67.55**
8.11	24.74	112.69	92.44	205.13	54.94
8.12	23.26	122.50	70.75	193.25	63.39
8.13-1	23.28	152.71	67.93	220.64	69.21
8.13-2	23.93	96.33	21.67	118.00	81.64
8.14	25.98	126.00	38.50	164.50	76.60
8.16	24.70	118.90	40.00	158.90	74.83
8.17	**28.34**	**126.08**	**84.08**	**210.16**	**59.99**
8.18	**28.02**	**125.80**	**56.24**	**182.04**	**69.11**
8.19	21.97	142.00	57.33	199.33	71.24
8.20	27.89	163.29	142.57	305.86	53.39
8.21	21.21	78.67	70.60	149.27	52.70
8.22	27.57	142.00	146.07	288.07	49.29
8.24	21.82	93.14	96.93	190.07	49.00

Numbers in bold indicate lines that are phenotypically homogeneous.

In general, the F_3 generation mutant rice lines had a relatively high total number of grains (filled and empty grains) per panicle, which ranged from 118 to 305.86, compared to the control plants (134.80). This is in line with the statement from Yu *et al*. [10] that the *GA20ox-2* mutant rice plant has a pleiotropic effect on increasing the number of tillers and grains per panicle so that it can increase rice yields [10]. However, although the *GA20ox-2* mutant rice lines had a high total number of grains, the percentage of fertile grains was relatively low, which was only around 40.36–81.64%, while the percentage of fertile grains in control plants was 84.57%. This indicates that the mutant rice lines have a relatively high sterile seed. That high number of sterile seed of the mutant lines can be caused by early maturity of these lines. As the data obtained, the average mutant rice lines have an earlier flowering time compared to the control plants (Table 2). Early maturity is controlled by genetic and environmental factors. According to Abdullah [18], the nature of voidness can be caused by genetic or non-genetic factors. One of the reasons rice plants have a high sterile seed is due to an imbalance between the sink (big) and source (little). Rice plants that have a greater number of grain per panicle (sink), if the source does not support, such as wide leaves, thin leaves, horizontal leaves, wither quickly, and early maturity, they will reduce the formation of assimilates, so that they are not sufficient for filling the grain [18].

Among the *GA20ox-2* mutant rice lines tested, there were three lines that were phenotypically homogeneous (8.10, 8.17, and 8.18). These lines have the character of a shorter plant posture compared to the control plants, produce more tillers, and flowering earlier (Fig. 1), and have a higher number of filled grains per panicle, which is 125.80–135.01 (Fig. 2), compared to the control plants (114 filled grains per panicle). From the result, these lines will be used as materials for further testing.

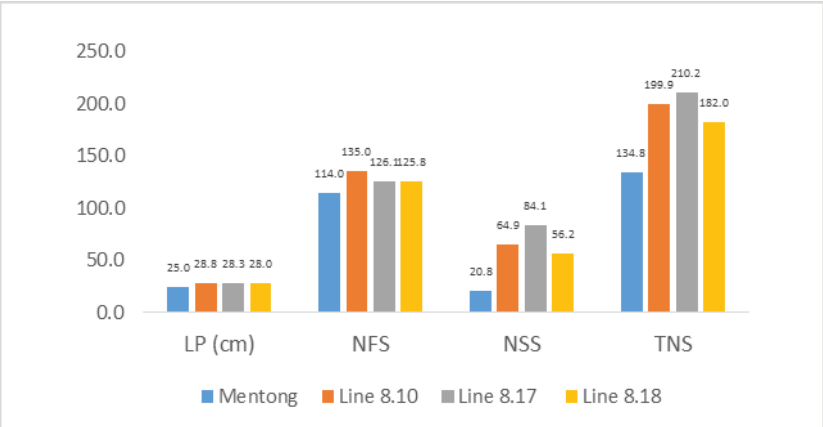

FIGURE 2. Yield components of the three selected lines (8.10, 8.17, and 8.18) of *GA20ox-2* mutant rice lines. LP = length of panicle, NFS = number of fertile seed, NSS = number of sterile seed, TNS = total number of seed.

Molecular Analysis of Three *GA20ox-2* Mutant Rice Lines

Sequencing analysis was performed on three selected mutant rice lines (8.10, 8.17, and 8.18) which were phenotypically homogeneous to determine the introgression of *GA20ox*-2 mutant allele originating from K-15 mutant line. Results of the alignment analysis of the *GA20ox-2* gene sequence in the mutation target section showed that the genome of the three mutant rice lines contained two adenine bases insertions which were exactly similar to the motif of the K-15 *GA20ox-2* gene sequence (Fig. 3). These results indicate that the introgression of the *GA20ox-2* mutant allele from K-15 has been stably inherited to the F_3 generation.

```
K-15            AATCTCAATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGACCCATGG
Mentong (WT)    AATCTCA-TGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCG-CCCATGG
8.10            AATCTCAATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGACCCATGG
8.17            AATCTCAATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGACCCATGG
8.18            AATCTCAATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGACCCATGG
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FIGURE 3. Alignment of the targeted DNA sequences of the three selected mutant lines (8.10, 8.17, and 8.18). The insertion of two adenine bases occurred in the targeted *GA20ox-2* gene sequence of the F_3 mutant rice lines. The letter A in red indicates the presence of adenine base insertion. WT = wild type (control).

CONCLUSION

F_3-*GA20ox-2* mutant lines of Mentong × Kitaake 15 (K-15) generally had a shorter phenotype, higher number of tillers, earlier in maturity, and higher number of filled grains panicle compared to the control plants. Evaluation of agronomic characters of the 19 mutant lines resulted in the three selected lines which were phenotypically homogeneous and had better performance. The introgression of the mutant allele of *GA20ox-2* gene has been stably inherited in the three selected F_3 mutant lines.

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