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EFFICIENT INDICA RICE ANTHER CULTURE DERIVED FROM THREE-WAY CROSSES

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SUMMARY

Low response of *indica* rice to anther culture (known as recalcitrant) is a major problem to develop new varieties through this technique. Improvement of anther culture response of some indica rice varieties by crossing them with a high *in vitro* culture responding is necessary to investigate. Previous studies reported that an *indica* rice variety Gadjah Mungkur had high *in vitro* culturability. The objective of this research was to determine anther culturability of six F1 hybrid crosses from *indica* rice genotypes derived from three-way crosses involving Gadjah Mungkur. Completely randomized design with 25 replications was used in this research. The results showed that high percentage of callus (> 40%) and plant regeneration rate (14.49-36.94%) were successfully obtained in all F1s derived from three-way crosses involving Gadjah Mungkur. All tested F1 hybrids also showed high anther culturability or culture efficiency ranged from 2.4-10.8%. In this study, 749 green plants were generated with the percentage of survived plant after acclimatization ranging from 88-95%. The percentage of doubled haploids was 39.7% from the total number of green plants.

Key words: Androgenesis, high culturability, doubled haploids, rice breeding

Key findings: This study revealed that high anther culturability can be achieved by three-way crosses involving certain *indica* rice genotypes that have high *in vitro* culturability, such as the variety Gajah Mungkur. This information is important because *indica* rice typically produces low green plant regeneration and is recalcitrant to anther culture.

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INTRODUCTION

Rice is an important staple food consumed by more than half of the world's population (FAO, 2014; Seck *et al*., 2012). Increase in rice demand is consistent with human population growth. Therefore, rice breeding to obtain new high yielding varieties is always needed (Wang *et al*., 2015). However, breeders are not only required to develop high yielding varieties but also high tolerance to various environmental stresses as well as good quality rice (Custodio *et al*., 2016).

Rice breeding programmes are directed to the formation of a pure line. In conventional breeding, it takes eight to ten years to obtain pure lines and release new varieties. Rice anther

culture produces pure lines as fully homozygous lines in just one generation compared to that of conventional breeding (Dewi and Purwoko, 2001; Dewi and Purwoko, 2011; Germana, 2011a; Mishra and Rao, 2016). Another advantage of this technique compared to conventional breeding is that it can facilitate the selection of the quantitative characters because it does not interfere with the dominant-recessive relationship (Dewi *et al*., 1996).

Although anther culture has been used in rice breeding, the success of the technique depends on the genotype used (Bojwani and Dantu, 2013; Grewal *et al*., 2006; Silva, 2010; Tapingkae *et al*., 2012). Rice subspecies *indica* has been identified and reported as one of the genotypes recalcitrant to anther culture unlike *japonica* rice. Problems such as low callus induction, low regeneration rate, and the high percentage of albinism presented new challenges in utilizing this technique in *indica* rice breeding (Herath and Bandera, 2011; Safitri *et al*., 2016; Sripichitt *et al*., 2000). However, application of polyamines in anther culture of *indica* rice improves androgenesis by inhibiting early senescence of the cultured anther, enhancing callus formation and plant regeneration (Dewi and Purwoko, 2008).

Recalcitrance to anther culture limits the exploitation of *indica* rice genotypes in developing new rice varieties. A previous report described that an *indica* rice line IRAT 112 was introduced from Kenya and has been released in Indonesia since 1994 as the variety Gadjah Mungkur. This variety showed high anther culturability (Sasmita *et al*., 2002) and higher response to callus induction and plantlet regeneration in embryo culture than subspecies *japonica* Taipei 309 (Hidayat, 2008). Gadjah Mungkur also possesses early maturity (90-95 days), blast resistant and drought tolerant characters (Harahap *et al*., 1995). Therefore, utilizing Gadjah Mungkur in rice breeding programs including *indica* rice using anther culture technique warrants further study. This study was carried out to determine the anther culturability of *indica* rice genotypes derived from three-way crosses involving Gadjah Mungkur.

MATERIALS AND METHODS

Experimental materials

Anther donor plants used in this study were six F1 hybrids derived from three way cross breeding. The seeds were obtained from the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD). They consisted of six cross breeding population (F1s), i.e. (1) DR7 = Inpari 18/B12825E-TB-1-25// Gajah Mungkur, (2) DR8 = Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur, (3) DR9 = Inpari18/IR83140-B-11-B//Gadjah Mungkur, (4) DR10 = Inpari 22/IR87705-14-11-B-SKI-12//Gadjah Mungkur, (5) DR11 = Inpari 22/IR83140-B-11-B//Gadjah Mungkur, (6) DR12 = Inpago 8/B12825E-TB-1-25//Gadjah Mungkur. Donor plants were grown in the green house in August 2015. Young panicles were harvested at booting stage, when the distance between flag leaf and second penultimate leaf were 7-10 cm. Cold temperature pretreatment was conducted by incubating panicles in a refrigerator at a temperature of 5 °C for eight days prior to anther culture (Dewi *et al*., 1996).

Experimental procedures

a. Callus induction

Callus induction medium in this study was N6 medium (Chu, 1978) enriched with 65 g. L^{-1} sucrose 2.0 mg.L^{-1} NAA, 0.5 mg.L^{-1} kinetin, 10^{-3} M putrescine, 3 gL^{-1} Phytagel ® (modified by Dewi *et al*., 2004). After cold pretreatment, panicles were removed from the sheath leaf and were surface sterilized with 20% commercial bleach containing 5.25% NaClO for 20 minutes. Then the panicles were rinsed two times with sterile water in the laminar airflow cabinet. Spikelets with anther containing microspores at mid-to-late uninucleate stage of development, in which the anther and filament length was no more than half the length of the spikelet, were selected from the midle portion of the panicles. Each spikelet was picked up by the uncut end using a forcep and anthers were dropped on the callus induction medium by tapping the forcep on the rim of the petridish. Each experimental

unit was a Petri dish containing 25 spikelets (approximately ± 150 anthers). Petridishes containing anthers were then sealed with plastic wrapper, then incubated in the dark at 25 ± 2^0C to induce callus from the microspores. Callus formation was observed within 4 to 12 weeks after anthers were plated.

b. Regeneration and rooting

The androgenic calli of 1–2 mm diameter were transferred onto regeneration media to induce shoots. Regeneration medium was MS medium enriched with 30 $g.L^{-1}$ sucrose, 0.5 $mg.L^{-1}$ NAA, 2.0 $mg.L^{-1}$ kinetin, 10^{-3} M putrescine, and 3 $g. L^{-1}$ Phytagel®. Green shoots 3-5 cm in size were then transferred onto a test tube containing MS medium enriched with 30 gL^{-1} sucrose, 0.5 $mg.L^{-1}$ IBA, 3 gL^{-1} Phytagel® to induce root formation (Dewi *et al*., 2004). The cultures were kept under light conditions (1600-1800 lux) with a temperature of 25 ± 2 ^{0}C during plant regeneration and rooting.

c. Experimental design and data collection

This experiment was arranged in a completely randomized design with 25 replications. The single factor used was anther donor plants of 6 genotypes (F1 hybrids) derived from three way crosses involving Gadjah Mungkur. Data collection included number of anther plated, the onset of callus initiation, number of callus, number of callus produced plantlets, number of callus produced green plantlets, number of callus produced albino plantlets, callus percentage, percentage of green plantlets, percentage of albino plantlets, regeneration rate, and culture efficiency. Callus percentage, regeneration ability, and culture efficiency are calculated based on the equations as follows:

$$\%\ \text{of callus} = \frac{\text{number of callus}}{\text{number of anther plated}} \text{x } 100$$

$$\text{Regeneration rate} = \frac{\text{number of plants recovered (green + albino)}}{\text{number of callus plated}} \text{x } 100$$

$$\%\ \text{of callus produced green plants} = \frac{\text{number of green plants}}{\text{number of callus plated}} \text{x } 100$$

$$\%\ \text{of callus produced albino plants} = \frac{\text{number of albino plants}}{\text{number of callus plated}} \text{x } 100$$

$$\text{Culture efficiency} = \frac{\text{number of green plants}}{\text{number of anther plated}} \text{x } 100$$

Data analysis

Statistical analysis of data were performed using variance analysis. Square root transformation was applied to normalize data. The differences between treatment average were tested by Duncan Multiple Range Test (DMRT). Analysis were carried out using the Statistical Tool for Agricultural Research (STAR) version 2.0.1.

RESULTS AND DISCUSSION

Callus induction

Six *indica* rice genotypes (F1 hybrids) derived from three-way crosses in this experiment were able to produce callus. Generally, the onset of callus formation ranged from 37.4 to 43.2 days after anther plating (Table 1). DR11 genotype (F1 from Inpari 22/IR83140-B-11-B//Gadjah Mungkur) required more time for callus formation which was significantly different from

other genotypes including DR7 (F1 from Inpari 18/B12825E-TB-1-25//Gajah Mungkur), DR8 (F1 from Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur) and DR12 (F1 from Inpago 8/B12825E-TB-1-25//Gadjah Mungkur). Those genotypes took less than 40 days for callus formation. Despite of the fact that there were differences among the genotypes, time of callus formation was in the range reported by Dewi *et al*. (2004) in which the time of callus formation ranged between 21-56 days after plating. Since the anther culture activities were aimed at accelerating the process of plant breeding (Germana, 2011a, Purwoko *et al*., 2010), the rapid response would be beneficial for reducing the time required in the laboratory.

Callus initiation process was characterized by swelling of anthers, afterwards callus emerged through anther locule that has been split-opened (Figure 1a). This mechanism is due to the osmotic pressure caused by high levels of sucrose or other carbon source in the media. This suggested that callus formation was not derived from somatic tissue, but from microspore inside the anther (Germana, 2011b). The individual callus regenerated from microspores can be separated easily. Similar finding has been reported from anther culture of tomato (Segui-Simarro and Nuez, 2005).

There were genotype effects on type of callus and callus induction ability among F1 hybrids (Table 1 and Figure 1). Observation showed that some calli showed white color, while others were creamy color or slightly transparent (Figure 1a and b). Previous reports revealed that calli with white color regenerated albino plants, while creamy color callus regenerated green plants (Dewi *et al*., 2004, Safitri *et al*., 2010). Naik *et al*. (2017) mentioned that only the creamy color compact callus was able to pass through the regeneration stage into plants, while the watery callus was unable to regenerate. Interestingly, according to observation of this study, the white callus was also able to generate green plants (Figure 1c).

Indica rice is recalcitrant to anther culture. Among 7 genotypes of *indica* rice used in anther culture by Chen and Lin (1976), achieve almost no callus or very small number of callus was produced (0.0-8.9%) and also the calli were not able to survive in the regeneration media. However, the results of this study was different with the above observation. The callus induction frequency of six F1 hybrids derived from three way crosses of *indica*/*indica*//*indica* involving Gadjah Mungkur was high and ranged from 43.5-107% (Table 1). DR8 genotype (F1 of Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur) showed the highest percentage of callus production. The responsive genotype could produce more than one callus from one anther (Figure 1a). In this study, Gadjah Mungkur was the male parent in the crosses and was a good donor parent as characterized by high callus induction response. The result was consistent with previous report in rice anther culture conducted by Sasmita *et al*. (2002). It was reported that percentage of callus generated from Gadjah Mungkur alone was 32.1%, whereas in another experiment when used the varietyas the male and female donor parent in single cross breeding, F1 hybrids showed 28.2% and 21.5% of callus induction, respectively. Result of Yan *et al*. (1996) supported the present study that callus induction ability was more determined by nuclear genes.

Plant regeneration

Plant regeneration usually started 15 days after embryogenic callus is transferred to the regeneration medium (Medhabati *et al*., 2014). Based on the number of callus producing plant and percentage of callus producing green plants, DR7 genotype (F1 from Inpari 18/B12825E-TB-1-25//Gajah Mungkur) showed the highest response in plant regeneration among other genotypes (Table 2). Combining ability analysis revealed that nucleus and cytoplasmic genes play an important role in the *in-vitro* plant regeneration of rice (Peng and Hodges, 1989). Moreover, Yan *et al*. (1996) using incomplete diallel analysis reported that callus induction was determined by genes in the nucleus than from cytoplasmic (the maternal effects), but the regeneration of green plants was more influenced by maternal effects.

The regeneration rate will determine the number of plants regenerated, and describe as number of plants produced compared to the number of callus plated in regeneration media. In this study, DR7 genotype (F1 from Inpari

18/B12825E-TB-1-25//Gajah Mungkur) also showed the highest regeneration rate (36.9%), while DR8 genotype (F1 from Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur) was the lowest (14.5%) (Table 2). This is interesting, since DR8 genotype had the highest callus induction capability among tested genotypes (Table 1), but had the lowest capability in planlet regeneration. Dewi *et al*. (2004) reported that the high rate of callus induction was not always accompanied by a high rate of plant regeneration, because calli were not always capable of regenerating plant. Nevertheless, regeneration rate of six genotypes of three way crosses in this study was quite high, ranged from 14.5 to 36.9%. Previously, in rice anther culture involving single cross of *indica* x *japonica* parents, regeneration rate was only between 0-13,8% (Safitri *et al*., 2010), while on *indica* aromatic rice the regeneration rate was less than 10% (Cha-um *et al*., 2009).

In this study, number of regenerated green plants ranged from 3.6 to 16.0 (Table 2.). DR7 genotype (F1 from Inpari 18/B12825E-TB-1-25//Gajah Mungkur) showed the highest average number of green plant (16.0 plants), while DR11 genotype (F1 from Inpari 22/IR83140-B-11-B//Gadjah Mungkur) was the lowest (3.6 plants). DR7 genotype also showed high percentage of green plants (61.8%). This result showed that DR7 genotype had better capability to produce high number of green plant compared to other genotypes. When compared to previous studies, which used a single cross from the same donor parent but without Gadjah Mungkur, F1 hybrids *indica* rice genotypes derived from three-way crosses involving Gadjah Mungkur was more responsive to callus induction and green plant regeneration. A study conducted by Gunarsih *et al*. (2016) had significantly lower callus induction (ranged from 3.2-10.2) response as well as number of green plant obtained (ranged from 0.2-1.1). Thus, the result implied that the use of Gadjah Mungkur which is known as *indica* rice with high culturability as donor parent might increase both callus induction and number of green plants.

In this study the average number of callus producing albino plants did not differ among genotypes tested and ranged between 2.0-3.8 (Table 2). However, based on number of callus plated, the percentage of callus producing albino plants ranged from 0.2-4.6. Albinism is the main problem, both in anther and microspore culture of cereal crops (Thorp and Andersen, 2009). High percentage of albino regenerants will limit the number of plants in later study. Albinism was due to deletion in plastid genome, and also chloroplast biogenesis was blocked which causes in failure to properly develop chlorophyll-containing photosystem in thylakoid (Kumari *et al*., 2009). Some reports indicated that albino plant formation was controlled by the genes on chromosomes 9 and 10 (Mishra and Rao, 2016; Silva, 2010).

DR7 genotype (Inpari 18/B12825E-TB-1-25//Gajah Mungkur) had the highest regeneration ability (36.9%) and low albino plantlets regeneration (38.2%). Meanwhile, DR8 genotype (F1 from Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur) had the lowest regeneration ability (14.5%), and the highest percentage of albino plants (62.7%) (Table 2). Similar to Mishra *et al* (2015), it is shown that albinism may still depend on the genotypes used and does not always follow the same pattern.

Percentage of green plants regenerated is very important because it will determine the number of new lines which will be generated from anther culture. Niroula and Bimb (2009) found that the frequencies of albino plants were always higher than that of green plants. In this study, the percentage of albino regenerants was not significantly different among the genotypes tested and varied between 38.2 to 62.7% (Table 2).

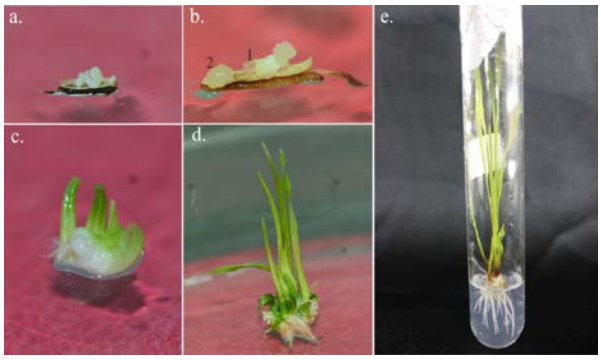

Figure 1. Callus induction to plantlet regeneration in rice anther culture. (a) callus formed from microspore and emerged from split-opened anther locule, (b) two types of callus, i.e. white callus (1) and cream-colored callus (2) might be emerged from one anther, (c) callus regenerated into plants, (d) plantlets ready to transfer to the rooting medium, (e) plantlets in the rooting medium with well-formed roots is ready to acclimatized and transferred to pots in the green house.

Table 1. Callus induction ability of *indica* rice genotypes derived from three-ways crosses.

Genotypes	The onset of callus initiation (days)	No. of callus	Callus percentage
DR7	38.0^{b}	87.2^{b}	58.6^{b}
DR8	37.8^{b}	164.1^{a}	107.0^{a}
DR9	40.1^{ab}	78.8^{b}	55.5^{bc}
DR10	40.1^{ab}	66.0^{b}	43.6^{bc}
DR11	43.2^{a}	67.8^{b}	43.5^{c}
DR12	37.4^{b}	82.7^{b}	55.5^{bc}

* Data has been transformed by square root transformation. Numbers in the same column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$. DR7 = Inpari 18/B12825E-TB-1-25/ Gajah Mungkur; DR8 = Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur; DR9 = Inpari 18/IR83140-B-11-B//Gadjah Mungkur; DR10 = Inpari 22/IR87705-14-11-B-SKI-12//Gadjah Mungkur; DR11 = Inpari 22/IR83140-B-11-B//Gadjah Mungkur; DR12 = Inpago 8/B12825E-TB-1-25//Gadjah Mungkur.

Table 2. The plant regeneration ability of *indica* rice derived from three-way cross

Genotypes	PR*	CPP*	CPG*	CPA*	PCPG*	PCPA*	NP*	GP*	PG*	AP*	PA*
DR7	36.9^{a}	8.8^{a}	5.0^{a}	3.8^{a}	6.3^{a}	4.6^{a}	29.4^{a}	16.0^{a}	61.8^{a}	13.4^{a}	38.2^{b}
DR8	14.5^{c}	5.9^{ab}	2.4^{bc}	3.6^{a}	1.7^{d}	2.4^{b}	22.4^{ab}	6.3^{bc}	37.3^{b}	16.1^{a}	62.7^{a}
DR9	23.3^{bc}	5.0^{b}	2.2^{bc}	2.8^{a}	3.1^{bc}	3.6^{ab}	16.5^{b}	6.6^{bc}	45.4^{ab}	9.9^{a}	54.6^{ab}
DR10	22.1^{bc}	4.6^{b}	2.6^{c}	2.0^{a}	4.2^{b}	3.2^{ab}	14.1^{b}	7.9^{b}	60.9^{a}	6.2^{a}	39.1^{b}
DR11	24.4^{ab}	4.3^{b}	1.4^{c}	2.9^{a}	3.3^{bc}	0.2^{c}	15.2^{b}	3.6^{c}	43.4^{ab}	11.6^{a}	56.6^{ab}
DR12	26.2^{ab}	5.3^{b}	2.4^{bc}	2.9^{a}	2.8^{cd}	4.4^{ab}	20.8^{ab}	7.7^{b}	51.3^{ab}	13.1^{a}	48.7^{ab}

* Data has been transformed by square root transformation. Numbers in a column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$. PR = % plant regeneration rate; CPP = no. of callus produced plants; CPG = no. of callus produced green plants; CPA = no. of callus produced albino plants; PCPG = % of callus produced green plants; PCPA = % of callus produced albino plants; NP = no. of total plants (albino + green); GP = no. of green plants; PG = percentage of green plants; AP = no. of albino plants; PA = percentage of albino plants

Compared to several reports on the use of anther culture for *indica* rice breeding, result in this study had better performance in term of green plant regeneration. Gunarsih *et al*. (2016) found the highest percentage of albino plants reached 93.1%, while Dewi *et al*. (2009) even obtained 100% albino plants. In rainfed rice landrace of East Kalimantan, the highest percentage of albino plants reached 95.7% (Nurhasanah *et al*., 2016). Previously, Dewi and Purwoko (2008) reported that rice anther culture medium containing putrescine increased green plant regeneration from 0% to 52.6% and increased ratio of green/albino plants from 0 to 3.3% in rice subspecies *indica* Krowal. Therefore, this study confirmed that in *indica* rice anther culture, putrescine helped in increasing number of green plantlets but could not be used to nullify albinism.

Culture efficiency

Anther culture-related production efficiency is expressed by the ratio of green plants against number of callus generating green plant, and the percentage of green plants compared to the number of anthers plated (Zhang, 1989). Ratio of green plants to number of callus generating green plants ranged 1.1-2.2 (Table 3). DR7 genotype (F1 from Inpari 18 B12825E-TB-1-25//Gajah Mungkur) showed the highest anther culture efficiency (10.8%), and was significantly different from the other genotypes. DR11 genotype (F1 from Inpari 22/IR83140-B-11-B//Gadjah Mungkur) resulted in the lowest efficiency (2.4%) and was not significantly different from DR8 and DR9 genotypes. The high culture efficiency of DR7 genotype was due to the high green plant regeneration, and by higher regeneration of green plants than the regeneration of albino plants. This result was better than that reported by Safitri *et al*. (2016) where the culture efficiency was only 0-3.1%.

Table 3. Green plant regeneration efficiency of *indica* rice genotypes derived from three-way crosses.

Genotypes	Ratio of green plant to no. of generating green plants*	Culture efficiency* (%)
DR7	2.2 a	10.8a
DR8	1.1 b	4.1bc
DR9	1.8 ab	5.0bc
DR10	1.8 ab	5.2b
DR11	1.2 b	2.4c
DR12	2.1 ab	5.2b

* Data has been transformed by square root transformation. Numbers in a column followed by the same letter are not significantly different by DMRT at $P \leq 0.05$.

Table 4. Number of plantlets acclimatized and doubled haploid production.

Genotypes	No. of green Plants				
	Acclimatization	No. of survive plant	% Survivor	Doubled haploid	% Doubled haploid
DR7	243	231	95.1	112	48.5
DR8	107	97	90.7	33	34.0
DR9	102	95	93.1	42	44.2
DR10	124	111	89.5	33	29.7
DR11	51	45	88.2	9	20.0
DR12	122	114	93.4	46	40.4
Total	749	693	91.7	275	39.7

Plants obtained from anther culture may include haploid, diploid or certain plants with various ploidy level (Dewi and Purwoko, 2011). Haploid plants can be easily distinguished because it has relatively short plant height and more number of tillers (Bishnoi *et al*., 2000). In this study, 693 green plants were successfully acclimatized in the green house (Table 4). The success rate of acclimatization activities overall reached 91.7% and ranged between 88.2 to 95.1%. DR7 genotype (F1 from Inpari 18/B12825E-TB-1-25//Gajah Mungkur) showed the highest success acclimatization rate (95.1%) followed by DR12 genotype (F1 from Inpago 8/B12825E-TB-1-25//Gadjah Mungkur). Doubled haploid plants produced from this study were 275 plants (39.7%). These plants need to be characterized further to determine their agronomic performance.

CONCLUSION

Six *indica* rice F1 hybrid genotypes derived from three ways crosses involving Gadjah Mungkur can be categorized to have high anther culturability based on callus induction, plant regeneration, and efficiency in culturability. DR8 genotype (F1 from Inpari 18/IR87795-14-11-b-ski-12//Gajah Mungkur) showed the highest callus induction ability, while DR7 genotype (F1 from Inpari 18/B12825E-TB-1-25//Gajah Mungkur) showed the highest plant regeneration ability. Out of 693 green plant that survived, 275 plants were doubled haploid. Further evaluation of the doubled haploid plants is needed to determine the agronomic characters, yield and resistance to biotic and abiotic stress.

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