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Research Article

ROLE OF ISSR MOLECULAR MARKER IN ANALYSIS OF GENETIC DIVERSITY OF MUTANT *Glycine soja* THROUGH MUTATION INDUCTION WITH COMBINATION OF GAMMA RAY IRRADIATION AND EMS (ETHYL METHANE SULFONATE)

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ABSTRACT. Mutation induction in soybeans is performed to achieve a high degree of genetic diversity as a basis for plant breeding in an attempt to obtain desired varieties. It is performed physically using gamma rays and chemically using EMS mutagen. This research was intended to figure out the effectiveness of the combination of chemical (EMS) and physical mutagens (gamma rays) in the induction of genetic diversity in black soybeans. Mutation detection can be carried out with molecular markers to characterize the genetic diversity of a crop. The mutation induction method employed in this research involved EMS and gamma-ray treatments. This research made use of a 5-primer ISSR molecular marker. The UBC 810, UBC 811, UBC 812, UBC 828, and K18 primers used had successfully amplified and demonstrated genetic diversity in the black soybeans which were subjected to gamma-ray irradiation and EMS administration. The study results indicate that gamma-ray irradiation treatment yielded a higher extent of polymorphism than did control and EMS treatment, thus fit to be applied to candidate parent plants for further treatments

Keywords: *mutation induction, gamma rays, EMS, ISSR, black soybeans*

INTRODUCTION

The black soybean variety Detam is a soybean variety with higher protein levels and antioxidant properties in comparison to other black soybean variants. For this reason, this variety was selected for this research. The chemical contents in soybeans are highly beneficial to health, but this benefit must come with an array of problems, including a limited amount of production and the fact that this crop applies self-pollination [1, 2].

In heterozygous, self-pollinated plants, heterozygosity reduction to a half and homozygosity increase by twofold occur every single time self-pollination takes place. Continuous self-pollination will lead to increased homozygous loci and shrunken heterozygosity [3]. As a result, genetic diversity and the number of soybean varieties become low [4]. Plant breeding in soybean crops by conventional crossing techniques or genetic engineering, thus, assumes much importance. Assembly of new varieties will need populations with a high degree of genetic diversity which may be retrieved through the introduction, crossing, mutation, and genetic transformation. Soybean plants' small

flower size causes hybridization to be difficult and costly and necessitates mutagenesis induction to expand genetic variability promptly [5].

Improved soybean variety assembly can be performed with physical mutation technology such as gamma-ray irradiation and with chemical compound ethyl methanesulfonate (EMS) [6, 7]. A wide variety of mutation techniques can be used to expand plant genetic diversity, thus elevating the chance of selection success for prospective lines that meet the purpose of plant breeding [8,9,10,11].

Influence of gamma rays on soybeans has been investigated in a study with the results that 1,000 Gy of gamma-ray irradiation improved antioxidant activity (42.74%), genistein content (21.47 mg/kg), daidzein content (34.51 mg/kg), total phenolic content (2.423 mg kat/g), and total tannin content (1,182) [12]

The highest rate of genetic variation in the M2 generation of soybeans is indicative that 200 Gy of gamma-ray irradiation is effective in inducing genetic variety [13]

The results of a study on *Vigna unguiculata* show that EMS mutagen treatment was higher in effectiveness and efficiency in inducing mutation than was gamma-ray irradiation treatment or the combination of both [14].

Gamma-ray irradiation treatment for soybeans at a low dosage of 20 Gy could improve tolerance to drought and minimize yield loss under the condition of water scarcity [15].

A study's results show that the two gamma-ray irradiation dosages applied (0.2 kGy and 0.4 kGy) produced significant decreases in the larger part of agronomy and morphology properties evaluated in the M1 population. Differing results of the use of 0.2 kGy and 0.4 kGy irradiation were observed in the M2 population with increased yield and significant yield components in the three soybean varieties [16].

Molecular characterization may streamline plant selection processes. One of the molecular markers frequently employed to detect polymorphism for its advantages is Inter Simple Sequence Repeat (ISSR). Usage of molecular markers is an effective technique in genetic analyses and has been widespread in plant breeding programs [17,18, 19]. ISSR is capable of polymorphism detection without the need of identifying the base sequence of the plant genome between repeated base sequences as long as the repeated base sequences are widely and evenly representative throughout the entire genome [19, 20, 21].

MATERIALS AND METHODS

Tools

The tools used were mortar and pestle, tip size of 1000 µl and 200 µl, micropipette, micro 22 R Hettich centrifuge, water bath, refrigerator, autoclave, microwave, plate chamber electrophoresis, vortex, spindown, Genesys 10 UV Spectrophotometer, Macro Vue-20 Hoefer UV transilluminator (Gel Doc), and PCR (Master Cycler Gradient Eppendorf). The materials used for DNA sampling were the leaves of the Dering-1 soybean variety.

Materials

The leaves of the Detam-1 soybean variety. The materials used for DNA isolation were as follows: liquid nitrogen, CTAB extraction buffer, phenol:chloroform:isoamilalkohol (PCI) = 25:24:21, chloroform: isoamilalkohol (CI) = 24:1, ammonium acetate, absolute

ethanol, 70% ethanol, Tris-EDTA buffer (TE buffer), distilled water, ethidium bromide, loading dye, DNA marker, 10x buffer taq, dNTP, MgCl2, primer, taqDNA polymerase, and ddH_2O.

Code A was for UBC 810 with the sequence 5'-GAGAGAGAGAGAGAGAT-3' at annealing temperature 50 ºC, code B for UBC 811 with the sequence 5'-GAGAGAGAGAGAGAGA-3' at annealing temperature 50 ºC, code C for UBC 812 with the sequence 5'-GAGAGAGAGAGAGAGAA-3' at annealing temperature 50 ºC, code D for UBC 828 with the sequence 5-TGTGTGTGTGTGTGTG-3' at annealing temperature 50 ºC, and code E for K18 with the sequence 5-DVHCACACACACACACACA-3' at annealing temperature 54 ºC.

Mutation induction

Mutations control treatment T0: seeds were submerged in phosphate buffer at pH 7 for 6 hours; T1: seeds were planted without submersion; T2: seeds were irradiated at 1,000 Gy; T3: seeds were submerged in phosphate buffer at pH 7 for 6 hours, then in 1% EMS for 6 hours; T4: seeds were irradiated at 1,000 Gy, then submerged in phosphate buffer at pH 7 for 6 hours and in 1% EMS for 6 hours.

The seeds of black soybeans were submerged in phosphate buffer for 6 hours, then in 1% EMS for another 6 hours. The seeds were then rinsed with distilled water to remove mutagen residues (Narayanan and Konzak, 1969). In control treatment (0% EMS), seeds were submerged in phosphate buffer at pH 7 for 6 hours. This treatment was administered at room temperature. For the irradiation, a total of 200 seeds were used, which were then inserted into brown paper envelopes. Gamma-ray irradiation was performed at 1,000 Gy through Gamma Chamber Type 4000-A irradiator. Gamma-ray irradiation on the black soybeans was carried out at BATAN, Jakarta.

The mutated seeds were then tested for viability with a rolled paper test. The seeds for DNA isolation were planted in soil in polybag, then young leaf samples were extracted 15 days after planting.

DNA Amplification Using PCR

The pure isolated DNA whose concentration was known was used as a PCR template. DNA Amplification was performed with Polymerase Chain Reaction (PCR). Amplification was carried out on a reaction mixture of 12.5 µl, containing 7.5 µL of PCR mix, 2.5 µl of ddH_2O, and 1.5 µl and 1 µl (10-100 ng/µl) of soybean genomic DNA each. PCR reaction using a programmed PCR machine was carried out in 30 cycles. The PCR program was set at 95 °C for 3 minutes of preheating, 30 cycles at 95 °C for 1 minute of denaturation, at 50 °C for 1 minute of annealing, and at 72 °C for 2 minutes of extension. The last extension was done for 4 minutes at 72 °C. The PCR amplification product was visualized on 1% agarose gel.

Data Analysis

Data from the amplicon band visualization were given scores with the Gel Analyzer software (Lazar, 2010). Each band seen in the gel was considered as an allele; DNA bands with the same rate of the movement were considered as one allele. In the case of DNA bands with the same rate of movement, each band that looked the size of the target was given a score of 1, while an invisible band was given a score of 0. Differently put, the scoring was binary. Scoring data were then analyzed with the UPGMA (Unweight-Pair

Group Method with Arithmetic Means) software based on the Jaccard genetic similarity index on PAST (Paleontological Statistics Software Package for Education and Data Analysis). The analysis results are presented in the form of a dendrogram and genetic similarity matrix. Scoring data were analyzed by the PAST software to determine the main allele frequency values, genetic diversity, polymorphic information content (PIC), and the resulting heterozygosity.

RESULTS AND DISCUSSION

The results of the black soybean viability testing in sprout percentages are presented in Fig 1. From the mutation induction, it was found that EMS mutagen treatment and the combined gamma-ray and EMS mutagen treatment produced low levels of viability, meaning that the two treatments resulted in mutants with higher levels of lethality. This is in line with previous results showing that the higher the mutagen dosage applied, the higher the lethality [4] [22].

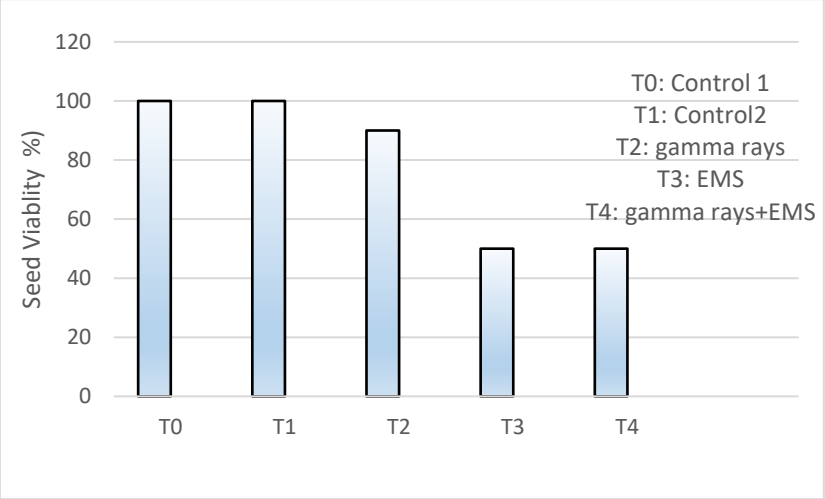

***Fig. 1**. Viability of mutant Glycine soja in gamma-ray and EMS induction*

The results of the black soybean DNA and quantification of the total genomes with a spectrophotometer are presented in Table 1. The DNA purity, as shown in Table 1, it determines with calculate the deep absorbance rate at A260 and A280 wavelengths (A260 to A280 ratio). A DNA molecule is pure if the absorbance rate ranges from 1.8 to 2.0 [23]. From the electrophoresis, the whole genome was successfully isolated in the product at 1,500 bp. The DNA concentration suggests that the quantity of successfully isolated DNA ranged between 1,184 and 4,035 ng/µL. The recommended quantity of template (for the reaction at 50 µL) is 50–250 ng for the DNA genome (https://barricklab.org/twiki/bin/view/Lab/ProtocolsStandardPCR).

Table 1. *Quantitative whole genome using nano spectrophotometer*

Sample	Abs 260	Abs 280	260/280	Con (ng/µL)
TO	74,09	47,37	1,56	3704,5
T1	74,09	47,37	1,45	4035,2
T2	35,49	17,84	1,99	1774,6
T3	59,43	39,42	1,70	2971,6
T4	23,68	11,81	2,06	1184,0

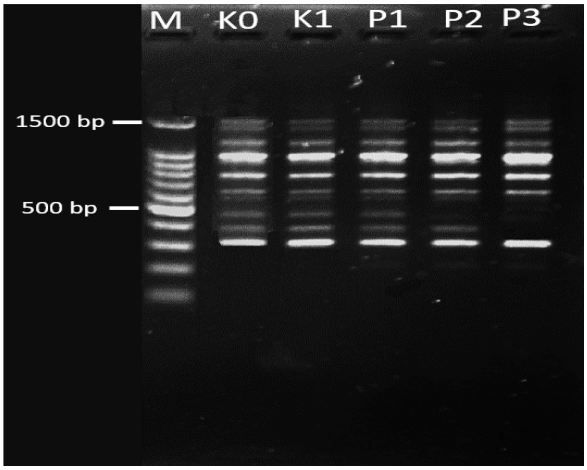

Fig. 2. *Pattern of DNA band amplified with the UBC 810 ISSR primer. M = marker, KO = control 1, K1 = control 2, P1 = gamma-ray irradiation at 1,000 Gy, P2 = 1% EMS administration for 6 hours, P3 = gamma-ray irradiation at 1,000 Gy + 1% EMS administration for 6 hours*

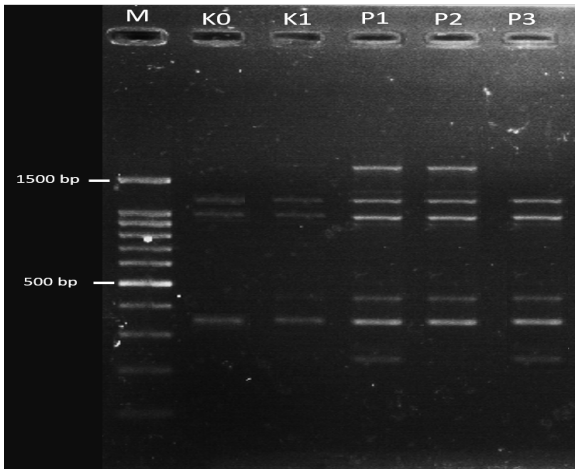

Fig. 3. *Pattern of DNA band amplified with the UBC 811 ISSR primer. M = marker, KO = control, K1 = control 2, P1 = gamma-ray irradiation at 1,000 Gy, P2 = 1% EMS administration for 6 hours, P3 = gamma-ray irradiation at 1,000 Gy + 1% EMS administration for 6 hours*

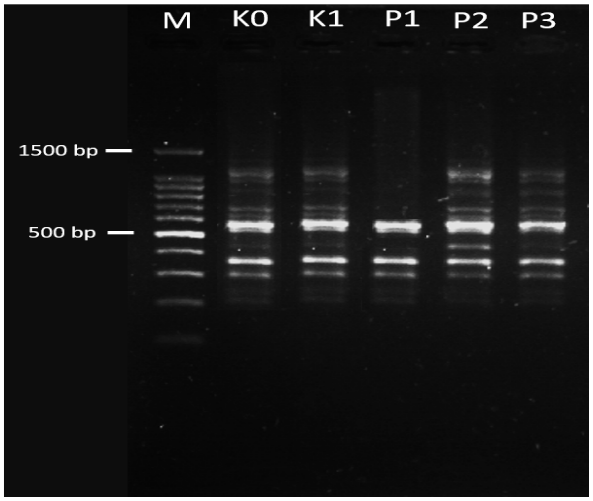

***Fig. 4**. Pattern of DNA band amplified with the UBC 812 ISSR prier. M = marker, KO = control, K1 = control 2, P1 = gamma-ray irradiation at 1,000 Gy, P2 = 1% EMS administration for 6 hours, P3 = gamma-ray irradiation at 1,000 Gy + 1% EMS administration for 6 hours*

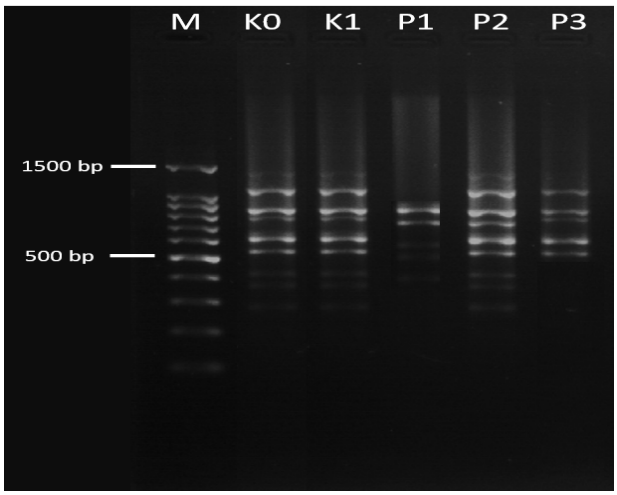

***Fig. 5.** Pattern of DNA band amplified with the UBC 828 ISSR primer. M = marker, KO = control, K1 = control 2, P1 = gamma-ray irradiation at 1,000 Gy, P2 = 1% EMS administration for 6 hours, P3 = gamma-ray irradiation at 1,000 Gy + 1% EMS administration for 6 hours*

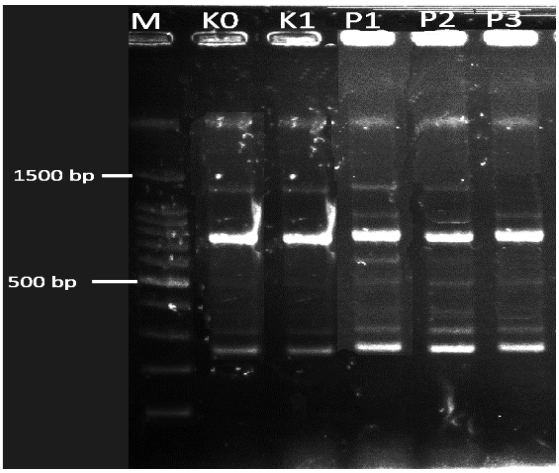

Fig. 6. *Pattern of DNA band amplified with the K18 ISSR primer. M = marker, KO = control, K1 = control 2, P1 = gamma-ray irradiation at 1,000 Gy, P2 = 1% EMS administration for 6 hours, P3 = gamma-ray irradiation at 1,000 Gy + 1% EMS administration for 6 hours*

The patterns of the DNA bands amplified with some ISSR primers in some mutant samples are presented in Figures 2–6. Figures 2–6 show the use of the UBC 810, UBC 811, UBC 812, UBC 828, and K18 primers, respectively. The five ISSR primers had succeeded in amplifying the mutant samples' DNA and produced varying band patterns. Based on the DNA band profiles in some primers, the P1 treatment, in which gamma-ray irradiation was applied, demonstrated a differing band pattern from control and other mutation treatments (Figures 4 and 5). This shows that mutagenesis by gamma rays is more likely to produce diversity and deviance than is one by control [24]. The mutation is an inheritable alteration in the DNA structure which is either spontaneous or induced. Spontaneous mutations have been observed in natural populations over a long period spanning many years. Spontaneous mutants are selected by farmers or breeders to be propagated and grown as new cultivars and as parents in cross-breeding programs.

Table 2*. Polymorphism band produced by ISSR primers in mutant soybeans through gamma rays and EMS induction*

Primer Name	Sequence (5'-3')	Annealing Temp. (C°)	Size (bp)	Amplified Bands	Polymorphic Bands	P%
P1						
UBC 810	GAGAGAGAGAGAGAGAT	50	320-1520	7	1	14
UBC 811	GAGAGAGAGAGAGAGA	50	250-1700	6	3	100
UBC 812	GAGAGAGAGAGAGAGAA	50	200-550	5	3	66,66
UBC 828	TGTGTGTGTGTGTGTG	50	650-900	4	2	50
K18	DVHCACACACACACACACA	54	260-1400	8	4	50
P2						
UBC 810	GAGAGAGAGAGAGAGAT	50	320-1520	7	1	14
UBC 811	GAGAGAGAGAGAGAGA	50	350-1250	5	2	50
UBC 812	GAGAGAGAGAGAGAGAA	50	300-1400	7	3	42
UBC 828	TGTGTGTGTGTGTGTG	50	300-1400	5	1	20
K18	DVHCACACACACACACACA	54	260-1400	6	1	16
P3						
UBC 810	GAGAGAGAGAGAGAGAT	50	320-1520	6	2	33,33
UBC 811	GAGAGAGAGAGAGAGA	50	250-1250	4	1	25
UBC 812	GAGAGAGAGAGAGAGAA	50	300-1100	6	2	33,33
UBC 828	TGTGTGTGTGTGTGTG	50	550-1200	4	0	0
K18	DVHCACACACACACACACA	54	260-800	4	2	50

The polymorphism of the band patterns generated by the ISSR primers in some mutant soybeans is presented in Table 2. In the P1 treatment, in which gamma-ray irradiation was applied, amplification took place at DNA lengths of 200–1,520 bp, with the highest amplification occurring in the UBC 812 primer at 66.66%.

***Table 3**. ISSR primer amplification, % polymorphism of mutant soybean through gamma rays and EMS induction*

Primer Name	Total Number Band	Number Polymorphic Band	PIC	EMR	MI	RP
UBC 810	10	7	1.84	70	128.8	18.4
UBC 811	6	3	1.46	18	26.4	8.8
UBC 812	12	7	1.60	84	134.4	19.2
UBC 828	13	12	1.07	156	168,0	14.0
K18	10	9	1.08	90	97.2	10.8

*PIC: Polymorphic Information Content, EMC: Effective Multiple Ratio, MI: Marker Index, RP: Resolution Power

Table 3. demonstrates the effectiveness of the ISSR primers in amplifying and detecting mutation-induced diversity. The potential of the five primers to amplify DNA as shown by the most informative primer identification table can be discovered from the PIC (Polymorphic Information Content) values, starting from 1.07 to 1.8. High PIC values indicate that the primers were good for genetic diversity [25]. EMR (Effective Multiple Ratio) used to determine the number of polymorphic fragments in the sample observed. The higher the EMR, the more effective the primer was effective in producing polymorphic fragments. MI (Marker Index) was used to determine the primer index in producing polymorphic fragments (Varshney et.al., 2007). RP (Resolution Power) value used to determine the power required to produce clear fragments. The higher the RP value, the better the primer at producing clear fragments (Prevost dan Wilkenson, 1999).

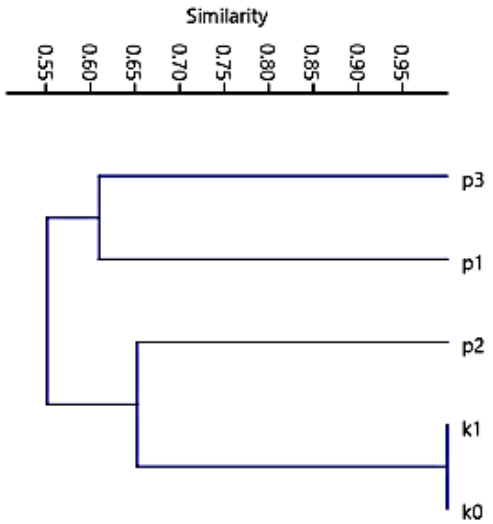

***Fig. 7**. Dendrogram of Mutants from Gamma Rays and EMS Induction (K0 and K1: control, P1: gamma-ray irradiation, P2 : EMS, P3: gamma-ray irradiation and EMS)*

The dendrogram and mutant classification by polymorphism band similarity is presented in Figure 7. Mutation treatment using EMS shows a cluster that grouped with control. This means that the DNA band profile in the mutant resulted was not much different from the control group with a similarity index of 0.65. The next cluster of gamma-ray irradiation treatment and combined gamma-ray irradiation and EMS treatment was distinguishable from the control group. This indicates that gamma-ray irradiation treatment was more effective in inducing differences in DNA band based on the ISSR molecular marker. This strays from the results of a study on rice paddy which shows that chemical mutagens were more effective than were gamma rays. However, this finding is in agreement with another study's results that gamma-rays treatment was more effective in reducing the percentages of sprouting and life survival and seedling height than was EMS treatment [26]. In the M2 generation of *Capsicum annum*, the gamma-rays treatment caused the proportion of chlorophyll mutants to rise higher than those rendered by EMS. In general, mutation frequency was higher in EMS treatment than was in gamma-rays treatment. The 30 mM EMS treatment was more effective in inducing mutation of desire at the highest frequency [27].

The results of a study on *Vicia faba* show that EMS was higher in mutation induction effectiveness than was gamma-rays with the following trend: EMS > gamma rays + EMS > gamma rays. However, in terms of mutagenic efficiency by lethality, gamma rays were higher than EMS with the following trend: gamma rays > gamma rays + EMS > EMS. Mutagenic effectiveness and efficiency were both found highest at the lowest dosages of the two mutagens. For both chemical and physical mutagens alike, higher dosages are for mutation induction with lower effects of lethality or damage (Khursheed, Shahnawaz., 2018).

CONCLUSION

The UBC 810, UBC 811, UBC 812, UBC 828, and K18 primers were successful in amplifying and demonstrating genetic diversity in black soybeans that were subjected to mutagenesis by gamma-ray irradiation and EMS. The results show that gamma-ray irradiation treatment generated amplified bands that differed from those generated in control and EMS treatment. The idea for future research fit for producing prospective parents for further treatments.

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