AFLP and AMP Fingerprints as Markers to Evaluate Genetic Differences between *Medicago truncatula* Line Jemalong and 2HA, a New Line Produced by *in vitro* Culture Selection

R.R. Irwanto[1,*] & R. J. Rose[2]

[1]School of Life Sciences & Technology, Institut Teknologi Bandung
[2]School of Environmental & Life Sciences, The University of Newcastle, Australia
*E-mail : rina@sith.itb.ac.id

Abstract. A new line, *Medicago truncatula* cv. Jemalong 2HA (herein known as 2HA) has been developed via repetitive regeneration and selection of *M. truncatula* cv. Jemalong. During somatic embryogenesis, 2HA produces 500 times more embryos than its progenitor, Jemalong. It is interesting to study if those two lines are isogenic or has genetic differences. The main objectives of the study was to evaluate the genotypic differences between Jemalong and 2HA also to evaluate the methylation event in 2HA utilized two DNA fingerprinting techniques, i.e AFLP fingerprints (Amplified Length of Polymorphism) and AMP (Amplified Methylation Polymorphism). The results showed that AFLP analysis using eight primers combinations could not detect any differences between Jemalong and 2HA. However, using AMP methylation sensitive primers it could detect 15 polymorphisms out of 840 markers. These results lead to a conclusion that Jemalong and 2HA are isogenic lines. 2HA may have higher regeneration capacities due to methylation process which occurs during the production of 2HA through repetitive regeneration cycles.

Keywords: *AFLP; AMP; methylation; M. truncatula cv. Jemalong 2HA somatic embryogenesis.*

Abstrak. Line baru, *Medicago truncatula* cv. Jemalong 2HA (selanjutnya disebut 2HA), telah dikembangkan melalui regenerasi dan seleksi line Jemalong dalam kultur secara berulang. Pada proses pembentukan embrio somatik, 2HA menghasilkan 500 kali lebih banyak embrio dibanding dengan Jemalong. Menarik untuk diteliti apakah kedua line tersebut isogenik ataukah terdapat perbedaan genetik antara dua line tersebut. Penelitian ini bertujuan untuk meneliti perbedaan genotipik antara Jemalong dengan 2HA serta mempelajari kemungkinan adanya metilasi pada 2HA. Hasil penelitian menunjukan, Hasil AFLP (Amplified Length of Polymorphism) dengan menggunakan kombinasi delapan primer tidak mendeteksi adanya perbedaan antara Jemalong dengan 2HA, tetapi dengan menggunakan AMP dengan primer sensitif metilasi menunjukan 15 polimorfisme dari 840 marker yang dihasilkan. Kesimpulan dari penelitian ini adalah Jemalong dan 2HA adalah isogenik. 2HA mempunyai kemampuan regenerasi lebih tinggi diduga karena proses metilasi pada waktu proses seleksi berulang di dalam kultur.

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Kata Kunci: *AFLP; AMP; metilasi; M. truncatula cv. Jemalong; 2HA; embrio somatik.*

1 Introduction

Medicago truncatula cv Jemalong 2HA (2HA), herewith called 2HA line, has been established in the lab of Plant Sciences, The University of Newcastle, Australia, via a cycle of somatic embryogenesis and subsequent recurrent selection of seed grown plants [1]. The "new line" has an increased regeneration capacity, producing 500 times more somatic embryos than its progenitor line, *Medicago truncatula* cv Jemalong. If excised leaf segments of Jemalong and 2HA are grown in media containing NAA and BAP, two or three weeks later, the leaf tissue develop calli. Both lines, give a similar response. However, 4 weeks later, 2HA shows a different response, i.e 2HA produce embrios while in Jemalong fewer embryos is produced such as seen in Figure 1.

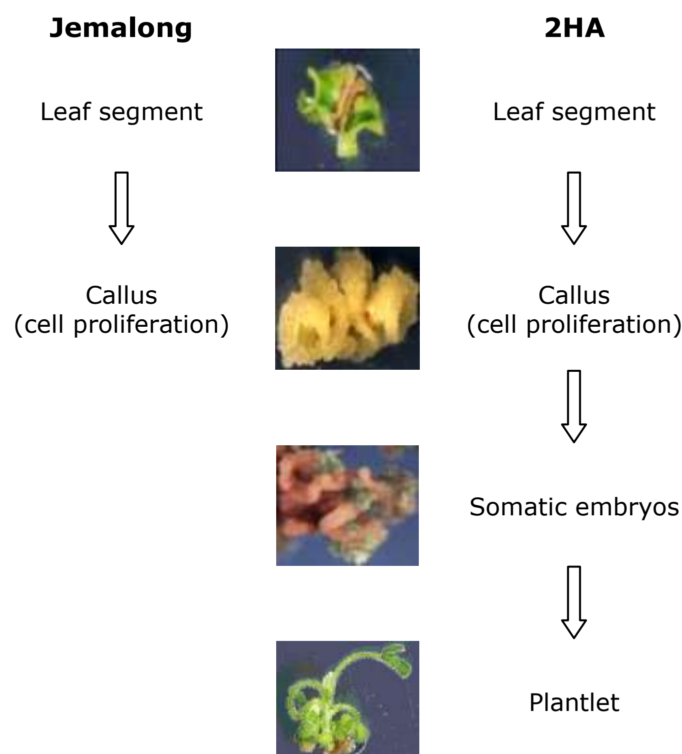

Figure 1 The response of Jemalong and 2HA tissue to culture on P4 media containing the hormones NAA (10 µM) and BAP (4 µM).

Despite the advantage of a high frequency somatic embryogenesis regeneration system, plantlets derived from a cycle of *in vitro* culture might exhibit somaclonal variation which is often heritable. It has been reported that chromosomal aberrations, DNA amplification, transposable elements, polyploid or aneuploid cells can cause somaclonal variations in long term cell culture [2,3]. It is also believed that DNA methylation changes play a role in somaclonal variation [4]. DNA methylation is an epigenetic event that affects cell function by altering gene expression and refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferase (DNMT), to the 5-carbon of cytosine in a CpG dinucleotide [5].

An important question that arises here is whether a new line, 2HA produce by culture selection has any rearrangements or genomic modifications related to the acquisition of enhanced somatic embryogenesis that can be detected using molecular approaches such as DNA fingerprinting methods.

AFLP as a fingerprinting analysis can be useful for analyzing genotypic differences and to locate and isolate mutations. AFLP also has the capacity to detect numerous independent genetic loci [6]. There are advantages of the AFLP technique compared to other fingerprinting techniques such as RFLP (Restriction-fragment-length-polymorphisms) or RAPD (Random-amplified-polymorphic DNA) i.e the number of polymorphisms per reaction is much higher and it is not necessary to predetermine genomic DNA sequences. However, AFLP might not be detect DNA methylation. In order to initiate epigenetic studies of the level of DNA methylation in two lines of *Medicago truncatula*, the methylation-sensitive amplification polymorphism (AMP) technique was utilized. Therefore, the objective of this study was to examine the genotypic differences between Jemalong and 2HA using the Amplified Fragment Length Polymorphism (AFLP) technique and to evaluate the possibility of methylation process in 2HA using the methylation sensitive amplification polymorphism (AMP).

2 Material and Methods

2.1 Plant Material

Medicago truncatula var Jemalong and 2HA lines were maintained under partially-controlled glasshouse conditions at 18-32^{0}C with a 14 h photoperiod provided by incandescent lights. The original *Medicago truncatula* (Family Leguminosae) obtained from the *Medicago* Genetic Resources Centre SARDI, Waite Precinct, The University of Adelaide. Plants were watered twice a day by an automatic dripper system and nutrients containing N, P, K and trace elements and plants were also watered with a liquid fertilizer every fortnight.

2.2 Genomic DNA Isolation for AFLP Analysis

Genomic DNA was extracted from 50-100 mg fresh weight of leaf tissue using a modified CTAB method [7]. Fresh leaves were quickly frozen and ground in liquid nitrogen to a very fine powder using a mortar and pestle. The powder was then suspended in lysis buffer containing 1% CTAB, 5% (w/v) polyvinyl pyrrolidone, 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-Cl, pH 8.0 and 350 mM 2-mercaptoethanol. The suspension was then extracted by 15% (w/v) SDS and *RNase* (optional) and incubated at 65^0C for 30 min. After an extraction in phenol: chloroform (1:1), the suspension was centrifuged at 4000 X g for 10 min. The top layer, which contained the DNA, was placed in a new tube and emulsified by adding an equal volume of chloroform and centrifuging at 10,000–13,000 rpm for 10 min to precipitate DNA. The top layer was then removed, and transferred to a fresh tube and added with 1/10 volume of 3M sodium acetate and 2 volume of ethanol 95% (v/v) and stored overnight at -20^0C. DNA was recovered by centrifuging the samples for 5 min and then the DNA was precipitated by adding 95% (v/v) ethanol. The DNA pellet was then washed in 70% ethanol and dried in speedvac. The pellet was resuspended in 20-30 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

2.3 Genomic DNA Isolation for Methylation Experiment

DNA for methylation experiment was extracted using the procedure described in [8]. Approximately 1g of young leaf tissues were ground frozen in liquid nitrogen and then put into Falcon tube containing 3 ml lysis buffer (it has been added with 0.6% sodium sulphite). The lysis buffer made from 200 mM Tris Cl pH 7.5; 50 mM EDTA, 2M NaCl, 2% CTAB and 0.6 ml of 5% (w/v) sarkosyl. 5 ml of phenol:chloroform was added into a Falcon tube and then the tube was inverted approximately 60 times.

The Falcon tube was spun at 3,000 rpm at 4^0C for 10 min and then 2-2.5 ml of the aqueous phase containing DNA was taken out and put into a new Falcon tube. Next, 1.2 ml of 100% isopropanol was added and the tube was inverted gently to precipitate the nucleic acids. The tube was left five min at room temperature (RT) and then inverted a few more times and spun for 5 min at 3,000 rpm at RT.

The pellet was transferred into an Eppendorf tube and washed by 70% (v/v) ethanol and left overnight at 4^0C. The pellet washed again in 70% (v/v) ethanol and spun at 3,000 rpm for five min. After the ethanol was removed, 400-800 µl TE buffer was added. DNA was purified and precipitated by chloroform extraction.

2.4 Amplified Fragment Length Polimorphisms (AFLP)

AFLP analysis was carried out with the AFLP Core Reagent Kit and Starter Primer Kit (Life Technologies Inc.) using the manufacturer's instructions and as described by [6]. The AFLP technique involves three major steps: restriction endonuclease digestion of the DNA and ligation of adapters; amplification of the restriction fragments and gel analysis of the amplified fragments.

M. truncatula DNA was digested with *Eco*RI and *Mse*I at 37^0C for 2 h, then heated to 70^0C for 15 min to inactivate the enzyme. The *Eco*RI and *Mse*I adapters were ligated to the ends of the restricted fragments. The digested and ligated DNA was preamplified by 20 PCR cycles (94^0C for 30 s, 56^0C for 60 s, 72^0C for 60 s). Selective amplification was conducted by PCR using aliquots of the preamplification fragments which were diluted 50-fold. ATP [γ^{33}P] labeled *Eco*RI and unlabeled *Mse*I primers were amplified by PCR using one cycle at 94^0C for 30 s, 65^0C for 30 s and 72^0C for 60 s, followed by lowering the annealing temperature each cycle 0.7^0C for twelve cycles. This gave a touchdown phase of 13 cycles. Then 23 cycles were performed at 94^0C for 30 s; 56^0C for 30s and 72^0C for 60 s. The samples were denatured at 90^0C for 5 min and then quickly cooled in ice. Pre-amplification and amplification were performed in a GenAmp^{R}PCR System 9700 (Applied Biosystems, California, USA).

Twelve primer combinations of *Eco*RI and *Mse*I, provided in the kit, were used for selective amplification. These primers contained a core sequence, restriction site and additional selective nucleotide at the 3' end (A and C for *Eco*RI and *Mse*I primers respectively). The samples were analyzed on a denaturing 6% polyacrylamide DNA sequencing gel containing 7.5 M urea. Electrophoresis was performed for 3-4 h in an electrophoresis chamber (BIO-RAD Protean ^{Tm} II, California, USA). The gel was dried in a gel dryer (Model 583, BIO-RAD, California, USA) at 60^0C for 2 h. Autoradiography was performed by exposing Kodak Bio Max MR-2 film (Eastman Kodak Co., New York, USA) to the dried gel for 1-3 days.

2.5 Amplified Methylation Polymorphism Analysis

The methylation sensitive amplification polymorphism was done in Biochemistry & Molecular Biology Lab, University of Queensland. Basically, the method was similar to AFLP, except it was utilized *Hpa*II restriction enzymes whose sensitive to methylation. *Hpa*II does not cleavage when 3'C residue is either 5-methylcytosine or the C residue is 4-methylcytosine [9] The procedure was based on [10] with modification.

Two digestion reactions were set up at the same time for genomic DNA sample. The first reaction, the genomic DNA was digested used *Eco*RI and *Mse*I. In the second reaction the genomic DNA was digested with *Eco*RI (Gibco BRL) plus *Hpa*II (Gibco BRL) in a final volume of 50 µL containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl for 5 h at 37°C. The digestion reactions were then ligated to the adapters by adding 10 µl of ligation mixture containing 1× T4 DNA ligase buffer (Gibco BRL), T4 DNA ligase (Gibco BRL), *Eco*RI adapter (5'-CTCGTAGACTGCGTACC-3'/3'-CATCTGACGCATTGGTTAA-5'). The ligation reaction was incubated at 23°C for 5 h. The digestion and ligation reactions were stopped by incubating at 65°C for 10 min.

The preamplification reaction was performed by using 5 µl of the above ligation product with *Eco*RI primer with one selective base (*Eco*RI+1), *Eco*R I+1 primer 5'-GACTGCGTACCAATTCA-3';1×PCR buffer (Gibco BRL), *Taq polymerase* (Gibco BRL), 1.5 mM $MgCl_2$ and 0.4 mM dNTP's in a final volume of 50 µl. Reaction conditions were 20 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 2 min.

Selective amplification was conducted in volumes of 20 µl. For selective amplification the preamplified mixtures were diluted 1:25 from their original volume with TEA. The volume of 5 µl of these diluted samples was mixed with *Eco*RI with two selective bases with two or three selective bases end-labelled with 0.8 µCi [α^{32}P] ATP, 1× PCR buffer, *Taq DNA polymerase* and 1.5 mM $MgCl_2$. Fragments present in these mixtures were amplified for 1 cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. In the following 12 cycles the annealing temperature of 65°C was lowered by 0.7°C each cycle, followed by 24 cycles with the following program: 94°C for 30 s, 56°C for 1 min and 72°C for 2 min with a final extension at 72°C for 5 min. All amplification reactions were conducted in a Perkin Elmer 9600 thermocycler (Perkin Elmer).

The MSAP products were mixed with an equal volume of tracking dye (98% formamide, 10 mM EDTA pH 8.0, 0.1% bromophenol blue and 0.1% xylene cyanol) denatured at 95°C for 3 min and immediately cooled on ice. Aliquots (5 µl) of each reaction were electrophoresed on 6% denaturing polyacrylamide gel containing 8 M urea and 1× TBE. Gels were run at 60 W for 2 h, dried and exposed to Kodak BioMax X-ray film for 2–4 days before being developed.

3 Results and Discussion

3.1 Are there any Large Scale Genomic Differences between Jemalong and 2HA?

AFLP was used to assess the 2HA genotype relative to Jemalong. Since the ability to detect polymorphism depends on the primer combination used, primer selection was utilized to obtain the most informative AFLP patterns. In this study, 12 out of 64 possible primer combinations provided in the Kit, were initially examined by the ATP [γ^{33}P] labeled or the silver staining method. In the screening of 12 primer combinations, it was found that some AFLP markers were easily scored while others were ambiguous and were not useful as genetic markers due to unclear bands. Primer pairs that produced only a few bands were excluded for further analyses. Therefore, only eight primer combinations were used for genomic analysis. The average number of bands ranged between 44 and 73 for an individual primer pair. Nearly all of the bands were between 80 and 600 bp.

AFLP banding patterns were scored manually and each locus was treated as a separate character. As observed in all AFLP fingerprints, the bands were distributed evenly from the top to the lower region of the gel. Table 1 summarizes the average number of bands in AFLP fingerprints from 8 different primer combinations. The highest number of bands was found to be 73 with primer combination E-AAG/M-CTG, whereas the least number of bands 44 was obtained with primer combinations E-ACC/M-CTC.

Table 1 The number of fragments amplified with different primer combinations using AFLP analysis. Data were obtained from 1-3 AFLPs fingerprints per primer pair. E : ***Eco***RI; M : ***Mse***I

Primer pair combinations	**Average number of bands**
E-AGC/M-CTG	55
E-AGC/M-CAG	66
E-AAG/M-CTG	73
E-AGG/M-CAA	49
E-AGG/M-CAT	47
E-AGG/M-CTA	63
E-ACC/M-CTC	44
E-AGG/M-CAC	52

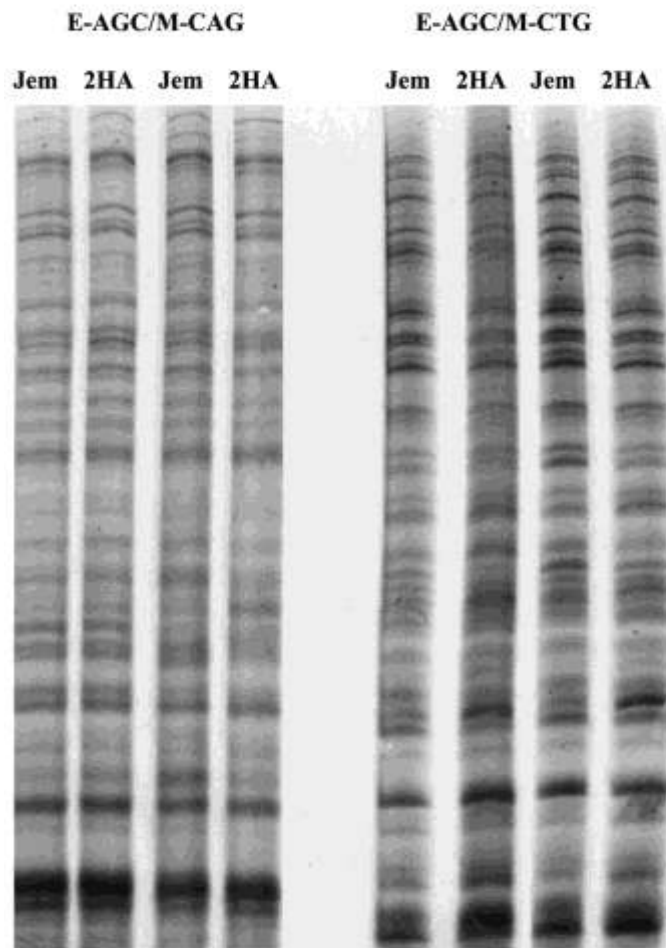

Figure 2 AFLP fingerprint pattern for Jemalong and 2HA using primer combinations E-AGC/M-CAG; E-AGC/M-CTG. Jemalong and 2HA show the same AFLP patterns. Duplicates represent different plants.

Figure 2 shows typical results which clearly showed the similarity between samples of both Jemalong and 2HA using the primer pairs E-AGC/M-CAG and E-AGC/M-CTG. These results were also supported by fingerprint patterns using 6 primer combinations E-AAG/M-CTG, E-AGG/M-CAA, E-AGG/M-CAT, E-AGG/M-CTA, E-ACC/M-CTC E-AGG/M-CAC (data not shown). The AFLP data (Figure 1) indicated that there were no large scale genomic differences between Jemalong and 2HA that could account for their different regenerability.

Plant genomes are large and in most plants, approximately 20 % of total DNA are highly repetitive DNA, i.e. 10^5-10^6 copies [3]. The failure of AFLP analysis to indicate any polymorphism associated with phenotypic variation could be due to the small genome portion covered by AFLP markers such as also found in *Asparagus* [11]. AFLP could also miss important variations such as genomic mutations. Nevertheless, the AFLP data indicated that point mutations or epigenetic changes were more likely to be the cause of the regenerability differences between Jemalong and 2HA

In comparison to A17, another line of *Medicago truncatula* var Jemalong that showed low rates of embryo formation [12] the AFLP fingerprints showed 93-97 % of the A17 bands were shared with Jemalong and 2HA (Table 2). In addition, Southern Blot utilizing the *MtSERK1* probe [13] also shows that Jemalong and 2HA have the same banding pattern which means there was no rearrangement in relation to *MtSERK1* and other members of *SERK* family. *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK)* was recently shown to enhance *Arabidopsis* somatic embryo development [14]. The factors that affect the different regenerability between Jemalong and 2HA is still unclear from AFLP study. However, epigenetic process such as DNA methylation could have occurred in 2HA.

Table 2 Six primer combinations were tested for selective amplification of DNA fragments in three lines of *Medicago truncatula* : Jemalong, 2HA and A17. Using AFLP analysis, the results show a few differences between A17 compared to Jemalong and 2HA.

Primer Pairs Combination	Total Amplified Bands	Polymorphism Bands
E-AGC/M-CTG	50	0
E-AGG/M-CAA	58	5
E-AGG/M-CAT	50	2
E-AGG/M-CTA	82	3
E-ACC/M-CTC	55	5
E-AGC/M-CAG	74	1
Total	**369**	**16**

3.2 Are there Methylation Changes in 2HA?

To examine methylation patterns between Jemalong and 2HA, DNA was extracted from leaves according to the protocol in 1.2. Amplified Methylation Polymorphism analysis (AMP) carried out with labeled methylation sensitive primers using methylation sensitive primers and restriction enzyme (+/- *Hpa*II digestion). The methylation pattern testing was done in the laboratory of A/Prof Bernie Carrol (unpublished method) and the result was analysed by Steve

Fletcher and A/Prof Bernie Carrol, Biochemistry & Molecular Biology, University of Queensland. Analysis was carried out with 12 primer combinations. Typical results are shown in Figure 3.

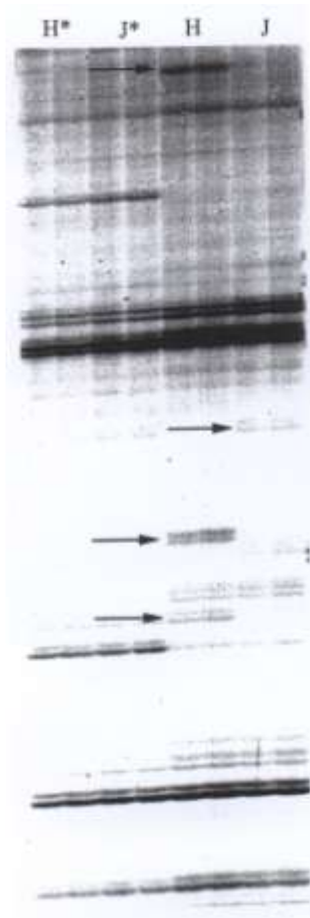

Figure 3 A part of a gel where there were methylation differences with one of the primer combination analysed by Amplified Methylation Polymorphism analysis (AMP). The banding pattern for 2HA and Jemalong was identical when undigested with *Hpa*II (H*, J*) but varied after *HpaII* digestion (H,J). H*=2HA undigested; J*=Jemalong undigested; H=2HA Hpa II digested; J=Jemalong *Hpa* II digested. (Analysis by Steve Fletcher and Bernie Carroll, Biochemistry and Molecular Biology, University of Queensland)

Figure 3 shows that the banding pattern for 2HA and Jemalong was identical when undigested with *Hpa*II (H*, J*) but varied (arrows) after digested by *Hpa* II (H, J). In this analysis widespread polymorphism was detected, i.e. 15 polymorphisms out of 840 markers.

In culture, callus consists of cells of various size and developmental potentialities [15]. During embryogenesis, cells gradually lose some of their developmental potential and become committed to develop along specific pathway [16]. Only cells which become determined to follow the somatic embryogenesis pathway form somatic embryos and in 2HA this increased propensity for somatic embryogenesis. The ability of cultures to respond to stress is at least partly responsible for their tolerance of genome amplifications and their adaptation to the varying environmental conditions [17].

Previous studies using *Medicago truncatula* lines with different capacities to produce somatic embryos showed that the demethylating drug 5-azacytidine inhibited regeneration capacity in the embryonic line of *M. truncatula*. They also suggested that disruption of somatic embryogenesis competence to be correlated with rDNA demethylation and the production of somatic embryos depended on a certain level of DNA methylation [18]. Methylation study in a Siberian ginseng culture using the methylation-sensitive amplification polymorphism (MSAP) technique found that hypermethylation of DNA occurred in non embryonic callus [19].

Therefore, it is reasonable to suggest that Jemalong and 2HA are near isogenic plants, except that 2HA has a different capacity to produce embryos in culture. The capacity to produce embryos in 2HA could be related to epigenetic processes, such as methylation, rather than genetic differences. The process might have occurred during the establishment of the 2HA line, which involved an initial regeneration cycle.

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