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ORIGINAL PAPER

Changes in DNA-methylation during zygotic embryogenesis in interspecific hybrids of beans (*Phaseolus* ssp.)

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Abstract Hybrid embryos resulting from crosses between *Phaseolus* species often fail to reach maturity and some combinations frequently abort at early developmental stages. The genetic or molecular basis for these consistent developmental defects is at present not clear. However, an extremely complex genetic system, thought to be caused by major epigenetic changes associated with gene expression changes, has been shown to be active in plant species. We have investigated DNA methylation in two interspecific hybrids, *Phaseolus vulgaris* × *Phaseolus coccineus* and its reciprocal crosses, using methylation sensitive amplification polymorphism (MSAP). The potential use of MSAP for detecting methylation variation during embryogenesis in interspecific hybrids is discussed. Significant differences in the DNA methylation patterns were observed in abortive (interspecific hybrids) and non abortive (parental) genotypes. Taken together, our results strongly suggest that generalized alterations in DNA methylation profiles could play a causative role in early interspecific embryo abortion in vivo. A considerable change in the methylation pattern during embryogenesis could be involved in the disruption of the regulation or maintenance of the embryogenesis process of *Phaseolus* interspecific hybrids. The results also support the earlier hypothesis that DNA methylation is critical for the regulation of plant embryogenesis and gene expression.

Keywords DNA-methylation · MSAP · Interspecific hybrids · *Phaseolus vulgaris* L. · *Phaseolus coccineus* L.

Abbreviations

DAP	Days after pollination
MSAP	Methylation sensitive amplification polymorphism
RT–PCR	Reverse transcription polymerase chain reaction
SSR	Simple sequence repeats

Introduction

Beans are important grain legumes for direct human consumption in the world. Within the genus *Phaseolus*, the common bean (*Phaseolus vulgaris* L.) is the most important species widely distributed in the word and occupies more than 90% of production areas sown to *Phaseolus* species (Baudoin et al. 2001; Broughton et al. 2003; Graham et al. 2003). In many developing countries, such as in Latin America, and in Central and East Africa, beans are staple crops serving as the primary source of protein in the diet, mainly because of their protein and amino acid contents. In tropical regions, the common bean is characterized by low and unstable grain yields. This is due mainly to the crop's susceptibility to numerous pests and diseases.

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The agronomic characteristics of the common bean have been improved in plant breeding programs, but further improvement is needed for higher grain production, such as tolerance to environmental stress and resistance to pests and diseases. Interspecific hybridization has been widely used in crop species to broaden their genetic base by introducing genetic information from related wild or cultivated species (Dweikat 2005). Major production constraints, such as the lack of resistance to pests and diseases and the lack of improved varieties tolerant to abiotic stresses (poor soil, high temperature, drought, etc.), have led to the widespread adoption of interspecific hybrid among *Phaseolus* species. A secondary gene pool, such as *P. coccineus* L. and *P. polyanthus* Greenm. offers very good breeding potential (Broughton et al. 2003). *Phaseolus polyanthus* is well known for its resistance to ascochyta blight and Bean Golden Yellow Mosaic Virus (BGYMV), and *P. coccineus* is a source of resistance to anthracnose, root rots, white mold, BGYMV and Bean Yellow Mosaic Virus (BYMV) (Schmit and Baudoin 1992). The transfer of desirable traits from related *Phaseolus* species, such as *P. coccineus* to *P. vulgaris*, could greatly improve the agronomic characteristics of the common bean.

In plant breeding, the success of gene introgression via sexual hybridization depends on the phylogenetic relationships between species, opportunities for genetic recombination, and stability of the introgressed gene (Marfil et al. 2006). Some interspecific crosses have been attempted between *P. vulgaris* and *P. coccineus* or *P. polyanthus* (Hanson et al. 1993; Bianchini 1999; Beaver et al. 2005). Consistent success has been limited to crosses with *P. vulgaris* used as female parent but it was observed that descendants naturally reverted to the cytoplasm donnor parents after few generations (Baudoin et al. 2004). The reciprocal cross is generally unsuccessful although rare successes have been reported (Nguema et al. 2007). Abnormalities are frequently observed during embryo development and depend to a great extent on the compatibility between the crossed genotypes (Geerts et al. 2002).

Plant zygotic embryogenesis is controlled by many genes that are critical for normal embryo development, and the dysfunction of these genes can disrupt embryo formation and lead to seed abortion (Hoe Huh et al. 2008). Moreover, the differential expression of maternal and paternal genes plays a role in the reproductive barriers observed in the endosperm of interspecific crosses (Kinoshita 2007). Baudoin et al. (2004) therefore assumed that major genes have established a barrier between *Phaseolus* species during interspecific hybridization.

Epigenetic regulation is defined as a mechanism that controls gene expression without altering base sequence. DNA methylation is the major epigenetic modification and plays an important role in regulating gene expression during embryogenesis in eukaryotes (Eckardt 2006). DNA methylation is also known to be an essential component of the parental imprinting mechanism in mammals as well as in plants (Tilghman 1999).

Li (2002) and FitzGerald et al. (2008) suggested that DNA METHYLTRANSFERASE1 (MET1) is a central regulator of genes essential for seed development and viability. In *Arabidopsis thaliana*, genome-wide changes in DNA methylation affect the earliest stages of embryogenesis (Bushell et al. 2003). Mutants with loss-of-function mutations in MET1 display altered planes and cell divisions of both suspensor and embryo cells from the earliest stages of embryogenesis, and have reduced viability (Xiao et al. 2006). The authors found that MET1 regulate the expression of genes that influence cell identity during embryogenesis such as the YODA (YDA) gene, which encodes a mitogen-activated protein kinase involved in specifying embryo and suspensor cell identity (Lukowitz et al. 2004).

Based on a homology analysis of these *Arabidopsis thaliana* embryo genes and the *Phaseolus* ESTs dataset, we identified 22 partial mRNA sequences (ESTs) encoding to corresponding genes during *Phaseolus* seed development, such as the YDA gene (GenBank accession: FE709393).

Hybrids resulting from interspecific crosses in some species such as potato (Marfil et al. 2006), *Arabidopsis* (Bushell et al. 2003), barley (Kranthi Kumar and Subrahmanyam 1999) and cotton (Keyte et al. 2006), showed changes in the DNA methylation pattern compared with parental species, suggesting that methylation results in a post-zygotic barrier and the cessation of seed development through alteration in gene expression.

The objective of this research was to investigate the involvement of DNA methylation in embryogenesis dysfunction in *Phasolus* interspecific hybrids. We examine and compare changes in DNA methylation between reciprocal interspecific hybrids, *P. coccineus* (NI16) × *P. vulgaris* (NI637), and the two parents. We also provide an analysis of the presence and expression level of the YDA gene. This gene is examined because of its integral role in embryogenesis and the possibility that it is directly regulated by MET1 (Xiao et al. 2006).

Materials and methods

Plant material

Two parental genotypes *P. vulgaris* (cv, NI637) and *P. coccineus* (cv, NI16) and their hybrids, were maintained in pots placed in growth chambers under the following conditions: 27/23°C (day/night), 75% relative humidity and 12 h photoperiod. Different combinations were made

between the selected genotypes using either *P. vulgaris* or *P. coccineus* as female partner. Flowers were emasculated 1 day before opening and pollinated on the same day. For self-pollination, the same procedure was followed, but pollen from other flowers of the same plant was used. Seeds from the parental genotypes and the interspecific crosses were freshly harvested 3, 6, 8, 9, and 12 days after pollination (DAP).

Histological studies

Seeds from the parental genotypes and the interspecific crosses were freshly harvested and eventually nicked with a scalpel to facilitate penetration by the fixing solutions. The seeds were then embedded in Technovit 8100 resin for 2 days at 4°C. Sections 3 µm thick were cut on a Zeiss HM 360 microtome fitted with a tungsten-carbide knife. They were stained with an adapted Toluidine blue O and viewed with a Nikon Eclipse E800 fluorescence microscope.

DNA extraction and microsatellite analysis

DNA was prepared from seeds or leaves, as described by Murray and Thompson (1980). DNA quality and concentration were checked by electrophoresis in 1% agarose gels. The different genotypes were analyzed using two SSR markers, BM114 and BM141 (Tanaka et al. 1999). Amplification reactions were performed in a total volume of 50 µl consisting of 200 ng template DNA, 0.2 µM of each primer, 200 µM of each dNTP (Invitrogen), 1× PCR buffer and 0.5 unit *Taq* DNA polymerase (Invitrogen). PCR was carried out in an iCycler Thermal Cycler (Biorad, USA). The cycling program consisted of the following steps: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C, and a final extension step of 7 min at 72°C. The amplification products were analyzed and checked by electrophoresis in 3% (w/v) agarose gel.

Methylation sensitive amplification polymorphism

Genomic DNA was isolated from the young seeds 3–6 DAP of individual plants of the parental lines and interspecific hybrids. For methylation sensitive amplification polymorphism (MSAP), the protocol developed by Reyna-Lopez et al. (1997) and adapted for rice by Xiong et al. (1999) was followed. The isoschizomers *Hpa*II and *Msp*I were used as frequent cutters and *Eco*RI was used as rare cutter (Fig. 1). Genomic DNA (1 µg) was digested with 10 U *EcoR*I (Fermentas) in a final volume of 20 µl of the appropriate buffer overnight at 37°C. For the second digestion, 10 U *Msp*I or *Hpa*II (Fermentas) were used. Genomic digested DNA (300 ng) was simultaneously

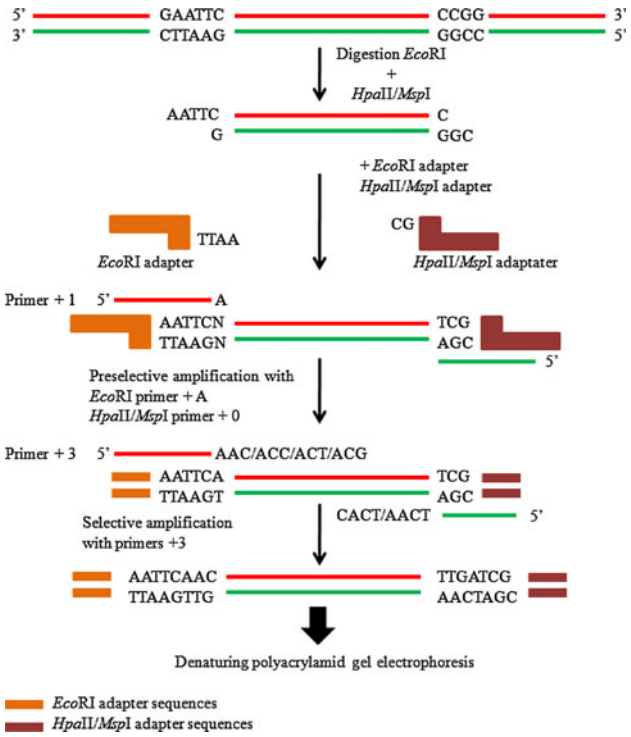

Fig. 1 Schematic diagram of the MSAP procedure

ligated to 100 pmol of H*pa*II/M*sp*I adapter and 10 pmol of *Eco*RI adapter (Table 1) in a total reaction volume of 20 µl with 1 U T4 DNA ligase (Fermentas) in 1× T4 DNA ligase buffer. The reaction was performed at 22°C for 2 h. The restriction-ligation samples were diluted with 140 µl H_2O prior to preselective polymerase chain reaction (PCR) amplification. Preamplification was performed using 4 µl dilute restriction and ligation product, 6 pmol of each preselective primer (Table 1), 1× PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, and 0.5 U *Taq* DNA polymerase (Invitrogen) in a total volume of 20 µl. The reaction entailed a 2 min hold at 72°C to ensure that the base-paired strand of the adapter was ligated by *Taq* polymerase, followed by 20 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, and finally, 30 min at 60°C. The product was diluted to 300 µl. The selective amplification was conducted in a volume of 20 µl using 5 µl dilute preamplification product, 1× PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 1 pmol of each selective primer (Table 1), and 0.5 U *Taq* DNA polymerase (Invitrogen). The amplification protocol involved the following: 1 cycle of 94°C for 5 min, 1 cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min, followed by 12 cycles with a 0.8°C decrease in annealing temperature per cycle, and 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, and a final extension of 60°C for 30 min. The amplification products were separated by electrophoresis in 6.5% denaturing polyacrylamide gel using an automated ALF express

Table 1 MSAP adapters and primers (primer combinations) used in this study

Name	Sequence or description
Adapters	
*EcoR*I-adapterI	5′-CTCGTAGACTGCGTACC
EcoRI-adapterII	5′-AATTGGTACGCAGTC
*Hpa*II/*Msp*I-adapterI	5′-GATCATGAGTCCTGCT
*Hpa*II/*Msp*I-adapterI	5′-CGAGCAGGACTCATGA
Preselective primers	
*EcoR*I + A	5′-GACTGCGTACCAATTCA
*Hpa*II/*Msp*I + 0	5′-ATCATGAGTCCTGCTCGG
Selective primers	
*EcoR*I + AAC	*EcoR*I + A + AC
*EcoR*I + ACC	*EcoR*I + A + CC
*EcoR*I + ACT	*EcoR*I + A + CT
*EcoR*I + ACG	*EcoR*I + A + CG
*Hpa*II/*Msp*I + TCAC	*Hpa*II/*Msp*I + 0 + TCAC
*Hpa*II/*Msp*I + TCAA	*Hpa*II/*Msp*I + 0 + TCAA

sequencer (Pharmacia Biotech). MSAP images were analysed with Allelinks™ analysis software.

RT–PCR

Total RNA was isolated from the seeds of the parents and their interspecific hybrids by the Trizol reagent (Invitrogen), following the manufacturer's protocol. For RT-PCR, 10 µg of total RNA was treated with 10 U of DNaseI (Fermentas) in DNAseI assay buffer (40 mM Tris–HCl, PH 7.5, 6 mM $MgCl_2$) 30 min at 37°C. The RNA was then purified by chloroform extraction and precipitated (1/10 volume 3 M NH_4OAC, two volumes ethanol) at −80°C for 1 h. The RNA pellet was suspended in 10 µl RNAse-free water. Five microgram of total RNAs from the developing seeds was reverse transcribed using the RevertAid™ H Minus M-MuLV Reverse Transcription 200 U/µl (Fermentas) at 42°C for 1 h. Fragments were amplified from these cDNA pools using *Taq* DNA polymerase (Invitrogen), following the manufacturer's procedures. An amplification of 18S was used as an internal control. Primers pairs were designed by Primer3 software and were as follows: for YDA, F-GGACTACACAAGGGGCAAAA and R-CCCTATTTGCATTCCTCGAA; and for 18S, F-TCGAAGCGATCTTTTCGTAGA and R-TTCTCAGTCGACTCGCTTTTT. PCR amplification was performed over 30 cycles using the following program: denaturing at 94°C for 30 s, annealing at 55–60°C for 30 s, and synthesis at 72°C for 30 s. An elongation step at 72°C for 5 min was conducted in the last cycle. Reactions were performed in a Minicycler (BioRad iCycler) thermal cycler. The experiments were repeated three times. The amplified PCR products (15 µl) were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer.

Results

Reciprocal hybridization between *P. vulgaris* and *P. coccineus*

A total of 451 *P. coccineus* (NI16) × *P. vulgaris* (NI637) crosses were made, with about 64% pod abortion occurring between 3 and 6 DAP, 30% between 8 and 9 DAP and 5% beyond 12 DAP (Table 2). Five mature pods supposed hybrids derived from these crosses were obtained. For reciprocal crosses, 387 hybridizations were performed. The rate of pod abortion was about 20% between 3 and 6 DAP, 21.5% between 8 and 9 DAP and 3.5% beyond 12 DAP, with 152 mature pods supposed hybrids obtained. Self-pollination of genotypes NI16 and NI637 was characterized by 26 and 8.3%, respectively, of pod abortion beyond 8 DAP. A low percentage of aborted pods (3–4%) was observed up to 3 days after self-pollination in *P. vulgaris* and *P. coccineus* genotypes (Table 2). From 6 DAP, a significant difference in percentage of pod abortion and seed development was observed between *P. vulgaris* and *P. coccineus*. In addition most of the pods aborted in *P. coccineus* registered at later stages (8, 9, and 12 DAP), although similar results were seen in *P. vulgaris* at early stages of seed development (3 and 6 DAP).

Table 2 Percentage of aborted pods in selfing and reciprocal interspecific crosses of *P. vulgaris* × *P. coccineus* at different stages of embryo development: early globular stage (3 DAP); globular stage (6 DAP); heart stage (8 DAP); torpedo stage (9 DAP); and cotyledonary stage (12 DAP)

Cross combinations	Number of flowers pollinated	Percentage of pods aborted at 3 DAP	Percentage of pods aborted at 6 DAP	Percentage of pods aborted at 8 DAP	Percentage of pods aborted at 9 DAP	Percentage of pods aborted at 12 DAP	Percentage of pods set
P. coccineus (NI16)	644	2.8	12.42	10.86	5.4	9.3	59.22
P. vulgaris (NI637)	501	3.75	2.9	1.66	0.83	0.44	90.22
P. coccineus (NI16) × *P. vulgaris* (NI637)	451	24.1	39.7	15.2	14.9	5	1.1
P. vulgaris (NI637) × *P. coccineus* (NI16)	387	7.14	12.5	11.6	9.9	3.4	55.46

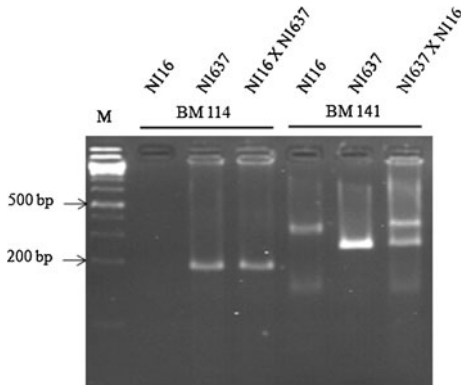

Fig. 2 SSR analysis of the parental and interspecific hybrids lines of *Phaseolus* species. PCR amplification of genomic DNA extracted from ovule (3–6 DAP). Fragment amplified by microsatellites primers BM 114 and BM 141. The amplification products were fractioned on 3% agarose gel electrophoresis

Overall, five seeds were obtained from the *P. coccineus* × *P. vulgaris* combinations in the present experiment and hundreds of seeds were obtained in the reciprocal crosses (only 10 seeds of supposed hybrids were used for this study). Some of the tested seeds did not germinate and some developed as paternal plants. SSR marker analysis was carried out to confirm the interspecific hybrids obtained for each cross (Fig. 2). After the seedlings formed roots, they were transferred into soil and grown under controlled conditions. The normal development of the seedlings continued for only 10–15 days for the *P. coccineus* × *P. vulgaris* hybrids and for 20–25 days for the reciprocal crosses after their transfer into soil. Thereafter, the seedlings ceased to grow and develop normally, and died.

Morphology of parents and interspecific hybrids

The interspecific hybrids displayed considerable variation in morphology, flowering time, and fertility compared with parental phenotypes. The parents were vigorous in growth, intermediated in height, profusely branched, and flowered abundantly. However, the interspecific hybrids displayed developmental abnormalities (Fig. 3). When *P. coccineus* was used as the female parent, the interspecific hybrid displayed a dwarf morphology. This plant stopped expanding new leaves and failed to bear flower buds after it developed 5–6 leaves. Similarly, in the reciprocal crosses, no mature hybrids plants were obtained.

Interspecific hybrids embryos development

To investigate the embryo tissue development in the developing seed of parental *P. vulgaris* (NI637), *P. coccineus* (NI16) and interspecific hybrids (Fig. 4), 77 ovules from different genotypes were examined. Embryological

Fig. 3 Phenotype of parents and hybrids at 40 days after germination. **a** *Phaseolus coccineus* (NI16); **b** *Phaseolus vulgaris* (NI637); **c** *Phaseolus coccineus* (NI16) × *Phaseolus vulgaris* (NI637); **d** *Phaseolus vulgaris* (NI637) × *Phaseolus coccineus* (NI16). *Scale bars* 20 cm

observations at 7 days after self-pollination indicated different embryo growth progressions among *Phaseolus* species. At 7 DAP, the embryos of *P. vulgaris* were already well developed and in the latter globular stage (Fig. 4a), whereas the *P. coccineus* embryos were at the early globular stage (Fig. 4b). These data suggested that, in self-pollinated flowers, embryo development is slower in *P. coccineus* than in *P. vulgaris* seeds. The basal cells of the suspensor of *P. coccineus* embryos were bigger and

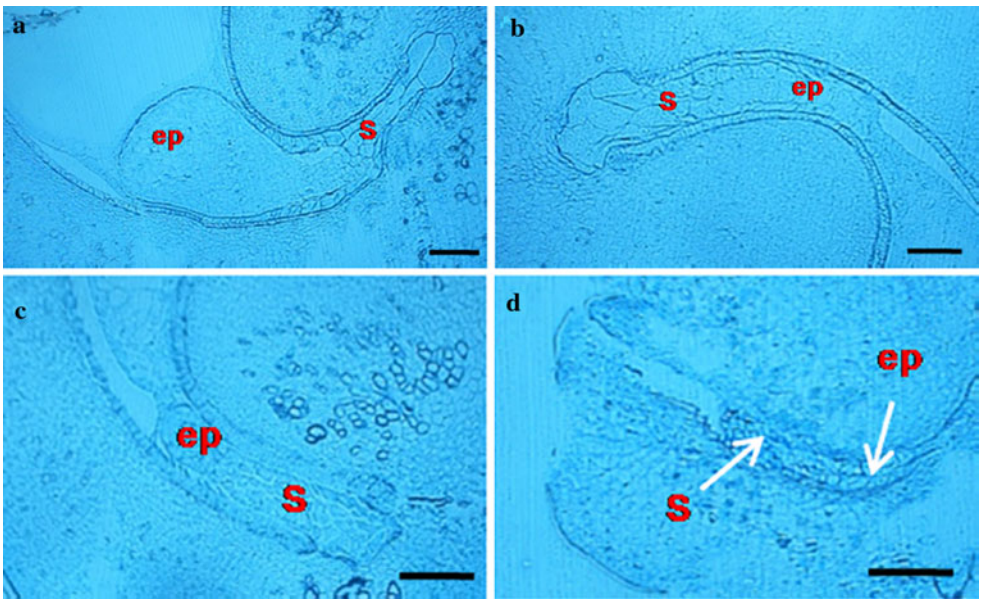

Fig. 4 Development of parental and interspecific hybrids of *Phaseolus* species embryos. Seeds from parental plants and hybrids were staged, fixed, embedded in resin, and sliced into sections (3 µm; see Material and methods). **a** Median longitudinal section in *P. vulgaris* NI637 (7 DAP) showing a latter globular embryo at the micropylar end; **b** Median longitudinal section in *P. coccineus* NI16 (7 DAP) showing an early globular embryo at the micropylar end; **c** Median longitudinal section in *P. vulgaris* NI637 × *P. coccineus* NI16 (7 DAP) showing delayed embryo growth; **d** Median longitudinal section in *P. coccineus* NI16 × *P. vulgaris* NI637 (7 DAP) showing embryo arrested growth at pre-globular stage. *ep* embryo proper; *s* suspensor. *Scale bars* 100 µm

contained larger vacuoles. We suggested a 2–3 day delay in embryo development between the two parental species. In general, embryo development was normal in the selfings of parental genotypes, and mature seeds were obtained. However, morphologically abnormal embryos were formed from crossing *P. vulgaris* with *P. coccineus*, particularly when *P. coccineus* was used as the female parent. Two classes of abnormal embryos could be clearly distinguished, the delayed (Fig. 4c) and the undeveloped (Fig. 4d). The seeds containing undeveloped embryos showed abnormalities mainly in the suspensor and embryo proper from the early steps of development. These abnormalities increased during seed development and led to embryo abortion before maturity. Similar observations were reported by Toussaint et al. (2004) and Geerts et al. (2002). According to these authors embryo abortion in *Phaseolus* interspecific hybrids is the result of poor coordination of the simultaneous development of embryo and endosperm, which could be related to a decrease in nutrient exchanges at the beginning of embryo development.

DNA methylation

To investigate the involvement of methylation in DNA *Phaseolus* embryogenesis, we initiated a comparative study between interspecific hybrids and parent genotypes. MSAP analysis which assesses cytosine methylation at specific restriction sites throughout the genome was employed (Baranek et al. 2010). The isoschizomeric methylation sensitive enzymes *Hpa*II and *Msp*I which cut at CCGG sites and show varing sensitivity to the presence of methyl residues were used for the MSAP analysis. *Hpa*II is sensitive to full methylation (both strands methylated) of either cytosine, but cleaves the hemimethylated external cytosine; *Msp*I is sensitive only to the methylation of the external cytosine (Roberts and Macelis 2001). The detection of methylated fragments in MSAP analyses required a comparison between DNAs digested with methylation-sensitive and insensitive-restriction enzyme isoschizomers. If the fragments from the *Hpa*II-digested sample or the *Msp*I-digested sample were detected in both parents, but not in the *Hpa*II-digested hybrid sample or the *Msp*I-digested hybrid sample, the genomic segments were considered to be methylated (M). Reciprocally, the genomic segments were considered to be demethylated (D). The percentage of methylation changes among parental and reciprocal crosses are shown in Table 3.

For MSAP analysis, eight pairs of selective primers were used and 547 fragments were amplified. Of these fragments, 179 (32.72%) were differentially amplified from the two digests between the parents and the hybrids (Fig. 5; Table 3). Therefore, on average, 32.72% (179 of 547) of the 5′-CCGG sites in the parental line genome were cytosine methylated in the leaves. The following types of

Table 3 Number of bands amplified using eight MSAP selective primer combinations in the parental genotypes and their interspecific hybrids of *Phaseolus* species

Oligonucleotides	Total number of bands in the two parents[a]	Total number of methylated sites[b]	Methylation alteration in the *P. coccineus* (NI16) × *P. vulgaris* (NI637) hybrid	Methylation alteration in the *P. vulgaris* (NI637) × *P. coccineus* (NI16) hybrid
*Eco*RI + ACC-*Hpa*II/*Msp*I + TCAA	94	26	2	1
*Eco*RI + ACC-*Hpa*II/*Msp*I + TCAC	72	17	1	0
*Eco*RI + ACT-*Hpa*II/*Msp*I + TCAA	69	24	2	1
*Eco*RI + ACT-*Hpa*II/*Msp*I + TCAC	34	13	0	3
*Eco*RI + ACG-*Hpa*II/*Msp*I + TCAA	78	29	4	0
*Eco*RI + ACG-*Hpa*II/*Msp*I + TCAC	91	35	1	3
*Eco*RI + AAC-*Hpa*II/*Msp*I + TCAA	68	24	2	1
*Eco*RI + AAC-*Hpa*II/*Msp*I + TCAC	41	11	1	0
Total	547	179	13	9

[a] Bands from four lanes were analyzed in each selective primer combination, two lanes for each parent (digested with either *Hpa*II or *Msp*I). Bands that were monomorphic (identical in both parents) were counted only once

[b] Bands were considered methylated if they showed polymorphism between the two isoschizomers. Bands with the same methylation pattern in both patterns were scored only once

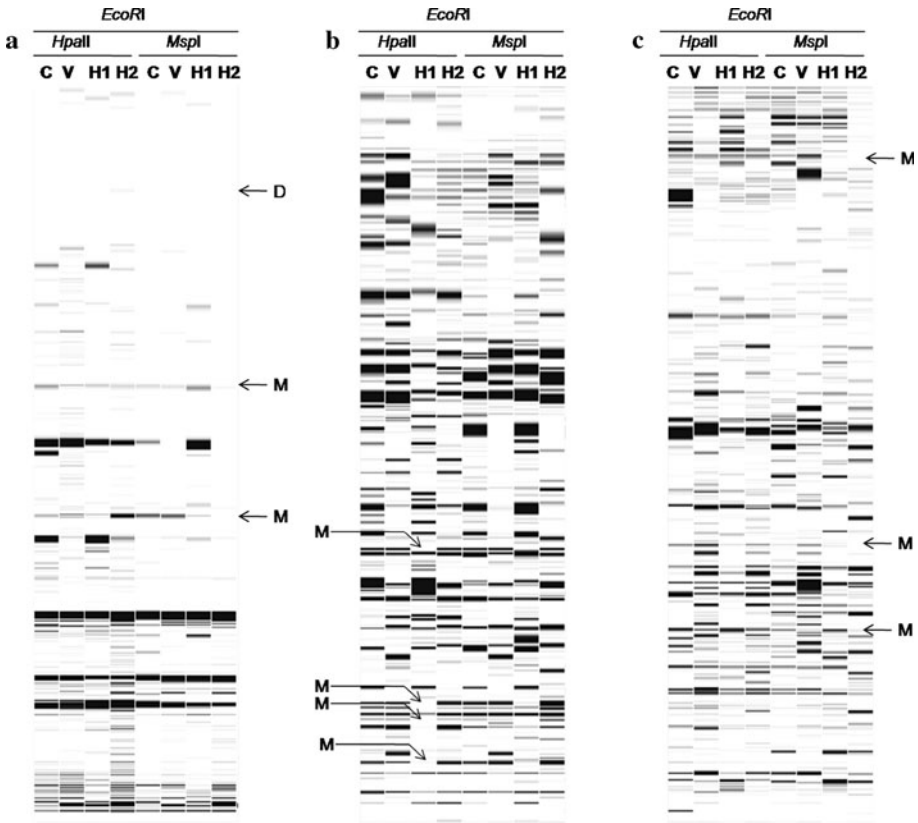

Fig. 5 MSAP fragment detected in *P. coccineus* NI16 (*C*), *P. vulgaris* NI637 (*V*), and the hybrids *P. coccineus* NI16 × *P. vulgaris* NI637 (*H*1), and *P. vulgaris* NI637 × *P. coccineus* NI16 (*H*2). The primer combination used was *Eco*RI + ACT-*Hpa*II/*Msp*I + TCAC (**a**), *Eco*RI + ACG-*Hpa*II/*Msp*I + TCAA (**b**), and *Eco*RI + ACG-*Hpa*II/*Msp*I + TCAC (**c**). The *arrows* indicate differential MSAP products resulting from methylation (*M*) or demethylation (*D*)

changes were observed: (i) full methylation of both cytosine residues at the recognition site, resulting in neither *Hpa*II nor *Msp*I cleavage; (ii) full demethylation of both cytosine residues at the recognition site, resulting in both *Hpa*II and *Msp*I cleavage; (iii) full methylation of the internal cytosine, resulting in *Msp*I cleavage, but not *Hpa*II cleavage; and (iv) hemimethylation of the external cytosine, resulting in cleavage by *Hpa*II, but not *Msp*I. Based on these MSAP profiles, the numbers of hemi-methylated and fully-methylated CCGG sites were calculated

(Table 3). The proportion of the modified (methylated or demethylated) fragments out of the total number of fragments indicated that the methylation pattern of the genomic DNAs changed in the two hybrids compared with the parent pattern. Thirteen bands (7.26% of all methylated sites), and nine bands (5.02% of all methylated sites) displayed methylation alteration in the *P. coccineus* × *P. vulgaris* (H1) and *P. vulgaris* × *P. coccineus* (H2) interspecific hybrids respectively. By comparing MSAP profiles using isoschizomers that differ in methylation sensitivity, a striking change in the methylation status of genomic DNAs in the interspecific hybrids was evident. Our MSAP results revealed an alteration in the methylation pattern in interspecific crosses (Table 3; Fig. 5). Therefore, epigenetic difference might contribute to the phenotype variation shown in interspecific hybrids.

Alteration of YDA gene expression in interspecific hybrids

To investigate whether dysfunction of *Phaseolus* seed development could be directly influenced by embryonic regulatory genes, RT–PCR analysis was performed with gene-specific primers on total RNA isolated from the ovules (3 DAP) of parent and hybrid seeds. In this study, we analyzed YDA gene expression, which plays a key part in regulating the first cell fate decision in plant embryogenesis, such as in *A. thaliana* (Lukowitz et al. 2004). As shown in Fig. 6, the expression of YDA is remarkably absent in interspecific hybrids seeds at 3 DAP. No significant changes in expression of the 18S were observed. We therefore suggest that the YDA gene is involved in the process of *Phaseolus* embryogenesis and is down regulated in aborting seeds (interspecific hybrids).

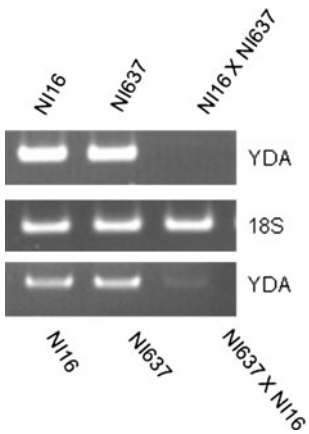

Fig. 6 Expression of the *YDA* gene in *Phaseolus* species and hybrids that regulate embryo cell fate specification. RNA was isolated from parental and interspecific hybrids seeds (3 DAP). YDA and 18S were amplified by RT–PCR as described in Material and methods

Discussion

The interspecific hybrids obtained between *Phaseolus* species display abnormalities during seed development. The mechanisms underlying this phenomenon are not understood. In plants, phenotypic variations have generally been found to be caused by epigenetic changes due to aberrant methylation states (Boyes and Bird 1991; Bender 2004).

Interspecific hybridization from different genera, such as *Arabidopsis*, *Oriza*, *Brassica* and *Phaseolus* in this study, commonly showed phenotypic instability, low fertility and low embryonic viability (Kakutani et al. 1996; Ronemus et al. 1996; Comai et al. 2000; Schranz and Osborn 2000). Accorded to Reddy et al. (2001), hybrid unviability could be attributed to the genomic imbalance or cytoplasmic incompatibility between two parental species.

In interspecific crosses, successful pod set was obtained in direct crosses involving *P. vulgaris* as the female parent and *P. coccineus* as the pollen parent. Nevertheless, 44.5% of these crosses led to abortion of the immature embryo, usually at the globular or early heart-shaped developmental stages, with most embryos aborting at 3–9 DAP. In contrast, the reciprocal crosses failed to set pods. Although fertilization occurs and embryos are formed, interspecific hybrid embryos are generally limited in their developmental potential. The particular stage at which the developmental arrest occurs is related to the interspecific combination (Sage and Webster 1990) and to the direction of the cross. The maternal parent seemed to play an important part in the degree of incompatibility, especially in the crosses using *P. coccineus* as the female parent. The maternal link to cross-compatibility has also been reported in the other interspecific crossing (Lelivelt 1993). More recently, Garcia et al. (2005), FitzGerald et al. (2008), and Hoe Huh et al. (2008) have all supported the hypothesis that the maternal genome plays the predominant role in controlling early seed development. The distinctive mechanism involves DNA methylation, which is maintained by MET1 as a central regulator of parentally imprinted genes (Bantignies and Cavalli 2006; Makarevich et al. 2006).

In general, both interspecific hybrids obtained in both combinations showed stunted growth morphology and loss of apical dominance (Nguema et al. 2007). Here, the phenotypes of interspecific hybrids are similar to the phenotypical abnormalities reported in DNA methyltransferase (MET1) and Histone (H1) antisense of *A. thaliana* plants (Kakutani 1997; Werzbicki and Jerzmanowski 2005) such as reduced size and number of leaves, and delay in flowering initiation.

It is possible that a change in the DNA methylation of the maternally derived genome causes the disruption of a defined

basic level of DNA methylation during embryogenesis and leads to a loss of interspecific hybrids embryonic potential. Thus, changes in the DNA methylation pattern could be part of the embryo abortion events in interspecific hybrids. Recent research on *A. thaliana* hybrids obtained between diploid *A. thaliana* and *A. arenosa* suggest that transposable elements (TEs) are associated with the failure of these hybrids (Hsieh 2009; Mosher and Melnyk 2010). These authors suggested that TEs in the embryo were hypermethylated, compared with vegetative tissues causing the production of siRNAs that might translocate in the sperm cells and cause transcriptional and post-transcriptional silencing of the transposable elements there. This would lead to the loss of parental genome integrity.

To test the hypothesis that developmental abnormalities such as embryo abortion in *Phaseolus* interspecific hybrids alters the epigenetic gene regulation affecting the level of gene expression, we investigated methylation patterns in interspecific hybrids using MSAP. Data obtained in this study suggest a difference in methylation patterns between parental and hybrids plants. The cytosine methylation level in all the hybrids tested differed from the level shown in parental plants, suggesting a modification in the level of cytosine methylation and alteration in DNA patterns. Altogether, three different digestion patterns were apparent: fragments were generated either by only *Eco*RI/H*pa*II, or by only *Eco*RI/M*sp*I, or by both couples of restriction enzymes. Thus, the *Phaseolus* hybrid differed in terms of methylation state and MSAP profiles from the respective parent plants.

In addition, the percentage of alteration in DNA methylation in interspecific hybrids varied between 7.26% (13 sites of changed methylation) and 5.02% (9 sites of changed methylation) in *P. coccineus* × *P. vulgaris* and their reciprocal crosses, respectively (Table 3). The present results showed conclusively a small difference (2.24%) in the methylation pattern among *Phaseolus* interspecific hybrids, whereas *P. coccineus* × *P. vulgaris* interspecific hybrids revealed a high percentage of aborted embryos (98.9%) at the early stages of embryogenesis compared with the reciprocal crosses (44.54%). The difference in DNA methylation in interspecific hybrids could possibly explain the difference in the loss of embryonic competence in interspecific hybrids.

The alteration in a few specific genes could lead to abnormal embryo development in *Phaseolus* interspecific hybrids. We suggest that only very few loci are associated with abnormal methylation and consequently with abnormal embryogenesis, and that this abnormal methylation occurs more frequently in *P. coccineus* × *P. vulgaris* interspecific hybrids than in other crosses.

Genomic and methylation alteration has been described in several hybrids, such as *Arabidopsis* allotetraploids (Madlung et al. 2002), wheat interspecific and intergeneric hybrids (Shaked et al. 2001), *Brassica napus* × *Orychophragmus violaceus* intergeneric hybrids (Cheng et al. 2002), *Oriza sativa* × *Oriza rufipogon* interspecific hybrids (Xiong et al. 1999) and interspecific hybrids *Solanum tuberosum* × *Solanum kurtzianum* (Marfil et al. 2006).

Wide crosses between different species of *Phaseolus* have been reported (Leonard et al. 1987; Kuboyama et al. 1991; Geerts et al. 2002). Our results are consistent with the hypothesis explored by other authors, such as Adams et al. (2000) and Marfil et al. (2006) that the frequent gene alteration and phenotypic abnormalities observed in interspecific hybrids could be explained by the simple assumption that many loci in these plants become methylated. We therefore suggest that the change in the DNA methylation pattern observed in two *Phaseolus* interspecific hybrids could be involved in modifying gene expression and epigenetic regulation.

A reproducible difference in the mRNA levels of the YDA gene between parents and interspecific hybrids developing seeds was reported. RT–PCR results showed that YDA is down regulated in interspecific hybrids. These results suggest that interspecific hybrid embryogenesis could be disrupted. The modified genomic DNA methylation status of interspecific hybrids could account for the change in YDA gene expression.

Conclusion and prospects

The development of interspecific hybridization leads to extensive alteration in gene expression, including gene silencing, part of which could be mediated through methylation. MSAP is a very powerful method for highlighting DNA methylation variation in *Phaseolus* interspecific hybrids compared with parental genotypes. *Phaseolus* interspecific hybrids present new MSAP fragments, accompanied by changes in the methylation level and pattern. The mechanisms whereby a new fragment occurs are unknown. It is possible however, that these newly generated bands might be derived from altered parental bands arising from epigenetic modifications such as cytosine methylation induced by interspecific hybridization.

The most important result obtained in this study was that the developmental abnormalities seen in the *Phaseolus* interspecific hybrids plants may be due to dysregulation of gene expression.

Regardless of the specific mechanism, further study is needed on the effects of change in DNA methylation patterns on the expression of the genes involved in *Phaseolus* embryogenesis using subtractive suppressive hybridization (SSH).

For a better understanding of how DNA methylation is involved in the post-hybridization barrier of *Phaseolus*

interspecific hybrids we postulated that the alteration (down/over-regulation) of some selected genes identified by SSH and involved in the *Phaseolus* embryogenesis process was due to hypermethylation or hypomethylation.

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