epigenomes

MDPI

Article

The Pea (*Pisum sativum* L.) Rogue Paramutation is Accompanied by Alterations in the Methylation Pattern of Specific Genomic Sequences

Tatiana E. Santo, Ricardo J. Pereira and José M. Leitão *

Laboratory of Genomics and Genetic Improvement, FCT, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal; ts.tatianasanto@gmail.com (T.E.S.); ricper1990@gmail.com (R.J.P.)

* Correspondence: jleitao@ualg.pt; Tel.: +351-289-800-939; Fax: +351-289-818-419

Academic Editor: Etienne Bucher

Received: 28 February 2017; Accepted: 11 May 2017; Published: 18 May 2017

Abstract: The spontaneous emergence among common pea (*Pisum sativum* L.) cultivars of off-type rogue plants exhibiting leaves with narrower and pointed leaflets and stipules and the non-Mendelian inheritance of this new phenotype were first described in the early 20th century. However, so far, no studies at the molecular level of this first identified case of paramutation have been carried out. In this study, we show for the first time that the pea rogue paramutation is accompanied by alterations in the methylation status of specific genomic sequences. Although, no significant differences were observed in the genome-wide DNA methylation in leaves of non-rogue cv. Onward in comparison to its rogue paramutant line JI2723, 22 DNA sequences were identified by methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) analysis as differentially methylated in the two epigenomes. Mitotically inherited through all leaf tissues, the differential methylation patterns were also found to be meiotically inherited and conserved in pollen grains for 12 out of the 22 sequences. Fourteen of the sequences were successfully amplified in cDNA but none of them exhibited significant differential expression in the two contrasting epigenotypes. The further exploitation of the present research results on the way towards the elucidation of the molecular mechanisms behind this interesting epigenetic phenomenon is discussed.

Keywords: paramutation; epigenetics; rogue plants; *Pisum sativum*; DNA methylation

1. Introduction

Among multiple self-fertilized pea (*Pisum sativum* L.) lines and cultivars occasionally emerge plants exhibiting an off-type (rogue) phenotype characterized by pointed and narrower leaflets and stipules, referred to by the nickname 'rabbit ears'.

The non-Mendelian inheritance of this phenotype was reported for the first time in the early 20th century by Bateson and Pellew [1,2], who observed that: (i) self-fertilized rogue plants, produce rogues exclusively; (ii) reciprocal crosses between true-to-type and rogues create plants that turn into rogues; and (iii) the F_2 produced by self-fertilization of F_1 plants, and all subsequent progenies, are exclusively constituted by rogues. In fact, this was the first analytical observation of a wider epigenetic phenomenon later termed 'paramutation' [3].

Although several cases of paramutation, as the *Kit* locus in mouse, have been reported in mammalians [4], so far, most paramutation phenomena have been identified in plants.

In maize, four loci involved in the regulation of the flavonoid biosynthesis: *r1* (red color), *b1* (booster1), *pl1* (plant color), *p1* (pericarp color), and a locus involved in the biosynthesis of phytic acid (*lpa1*, low phytic acid 1) were found to undergo paramutation [5–8].

Among the above mentioned maize loci, the most significant progresses have been achieved in the study of *b1* and *pl1* and the identified genes required for paramutation at these two loci: *mediator of paramutation* (*mop1* [9] and *mop2* [10]) and *required to maintain repression* (*rmr1* [11], *rmr2* [12], *rmr6* [13], and *rmr7* [14]) were found to encode proteins involved in small interfering RNA (siRNA) biogenesis and RNA directed DNA methylation (RdDM).

More recently, the *sulfurea* paramutation that affects pigment production in tomato (*Solanum lycopersicum*) was also associated to the production of siRNAs and increased methylation of the SLTAB2 locus promoter [15], adding new data for the previous reports of association of paramutation with alterations in the methylation pattern of specific DNA sequences.

In petunia, the paramutation-like reduction of the activity of a transgene was found associated with the hypermethylation of the promoter region of a homologous epiallele [16]. In maize, the hypermethylation of specific loci sequences correlates with different epigenetic states at the *p1* and *r1* loci [17–19], while the restoration of the wild dark color is associated with the hypomethylation of CHG sites at the 3′ regions of the *pl1* locus [12]. Also in maize, the paramutagenic *B′* epiallele of the locus *b1* is hypermethylated at the junction sequences between the required for paramutation hepta-repeats located 100 kb upstream of the locus [20], and in the paramutation induced by the presence of transgenic *b1* repeats, the increasing DNA methylation at the endogenous *b1* repeats correlates with stronger silencing of *b1* locus [21].

Contrarily to the above cited paramutation cases which have been object of systematic and intensive studies, since the pioneer studies of Bateson and Pellew [1,2] and the immediately following works of Brotherton [22,23], the study of the pea rogue paramutation was rarely resumed, and very few research data have been added. In fact, it was only confirmed the absence of differences between the chromosome complement of rogue and non-rogue plants [24], and that the reduced size of stipules and leaflets in rogue plants was due to lower number of cells and not to differences in cell size [25].

Herein, we report the results of our research aimed at to obtain a first insight of the association of the pea rogue phenotype with alterations in DNA methylation.

2. Results

2.1. Genetic Similarity between Rogue and Non-Rogue Epigenotypes

The plants of the paramutant line JI2723 exhibit the typical rogue phenotype, clearly evident when compared to the original non-rogue cv. Onward (Figure 1).

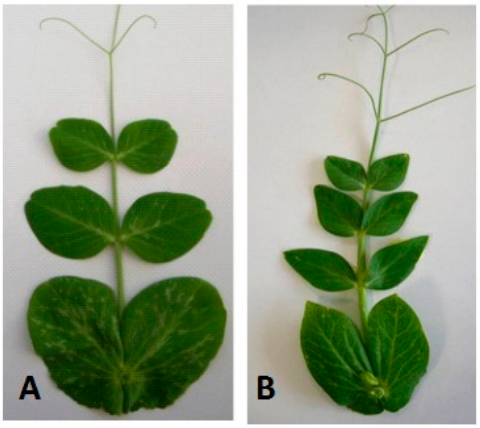


Figure 1. The pea rogue paramutation. (**A**) Typical leaf of cv. onward; (**B**) Typical leaf of the paramutant onward rogue line JI2723. Notice the characteristic narrower and pointed (rabbit ears) stipules and leaflets.

In order to rule out any previous misclassification and mishandling occurred during the propagation and manipulation of the plant material we performed comparative Random Amplified Polymorphic DNA (RAPD) and Single Sequence Repeat (SSR) analyses. The amplified 641 RAPD and five SSR markers were all found monomorphic between the cv. Onward and the rogue line JI2723 confirming the high genetic similarity between the two genotypes (Figures S1 and S2).

2.2. Genome-Wide Methylation Analysis

The absence of drastic differences in agarose gels (Figure 2A) between the restriction patterns of leaf genomic DNA of the rogue and respective non-rogue lines after digestion with the restriction enzymes HpaII and MspI suggests similar levels of genome methylation of the 5′-CCGG-3′ sequences in the two epigenomes.

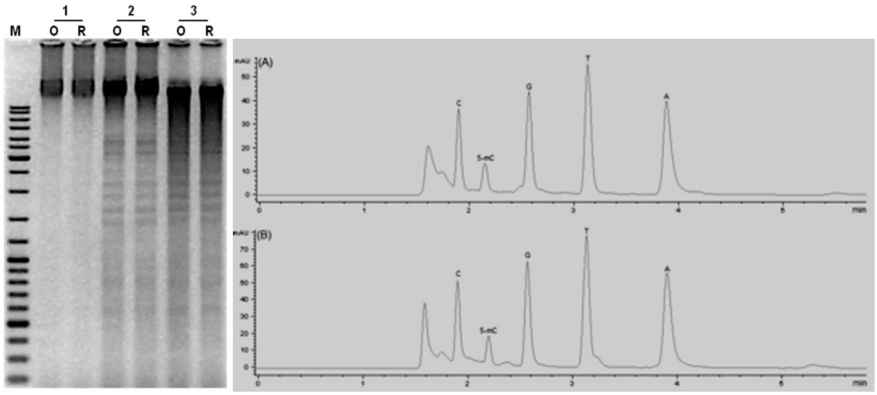


Figure 2. Genome-wide methylation analysis. (**Left**) HpaII and MspI digestion of total genomic DNA. (O) cv. Onward. (R) rogue line JI2723. (M) 100 bp ladder marker (1) Non-digested (control) genomic DNA; (2) HpaII-digested DNA; (3) MspI-digested DNA; (**Right**) Reverse phase high-performance liquid chromatography (RP-HPLC) chromatograms of hydrolyzed leaf DNA of: (Top) cv. Onward, (Below) rogue line JI2723. The peaks correspond, respectively, to cytosine (C), 5-methylcytosine (5-mC), guanine (G), thymine (T), and adenine (A).

Very preliminary and simply indicative, these results were further confirmed by reverse phase high-performance liquid chromatography (RP-HPLC). The analysis of the hydrolyzed genomic DNA of both epigenomes resulted in very similar chromatograms and identical relative amounts of methylated cytosine, with an estimated ratio between the areas of 5-methylcytosine and cytosine peaks of 0.3437 (standard error = 0.0173) for the non-rogue and 0.3366 (standard error = 0.0164) for the rogue plants (Figure 2B).

However, since these results do not discard the hypothesis that the rogue paramutation is accompanied, or even triggered, by alterations of the 5-cytosine methylation at specific genomic sequences a finer comparative methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) analysis was carried out.

2.3. MS-AFLP Analysis

The use of 64 primer combinations (Tables S1–S3) resulted in the amplification of 2338 MS-AFLP markers among which 22 (0.9%) were confirmed to be epi-polymorphic between the two epigenomes (Figure 3). Ten of these markers were amplified only in cv. Onward plants and the remaining 12 markers only in the rogue line JI2723.

The 22 epimarkers were successfully excised from the dried polyacrylamide gels, re-amplified, cloned, sequenced, and the respective sequences (Table S4) uploaded to genomic data bases (www.ncbi.nlm.nih.gov) with the GenBank accession numbers KF861513 to KF861534. Two markers,

AGG/AT_302_O and AAG/AA_197_R, arose from the same locus. The first marker, 302 bp long, was amplified from cv. Onward while the second (197 bp long) was amplified from the rogue plants in consequence of the differential methylation of an internal 5′-CCGG-3′ motif (Table S1).

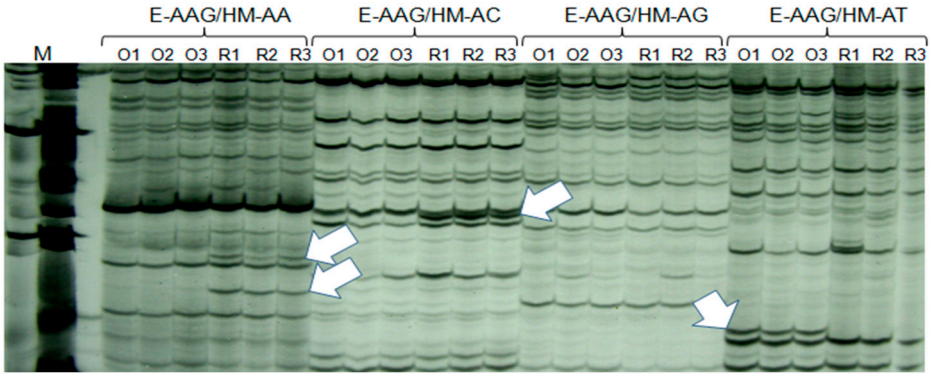
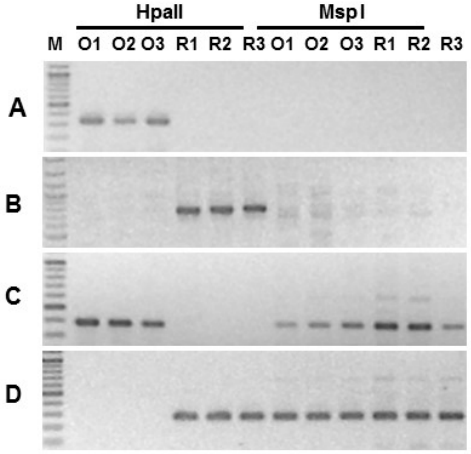


Figure 3. Partial view of a methylation-sensitive amplified fragment length polymorphisms (MS-AFLP) autoradiograph that confirms four previously identified epipolymorphisms. O1, O2, and O3 -three plants of cv. Onward. R1, R2, and R3 - three plants of rogue line JI2723. Arrows indicate polymorphic bands that result from the differential methylation of specific restriction sites in the two genomes.

The re-amplification of the 22 sequences on leaf DNA, performed as described in the Material and Methods section (Table S5), allowed the confirmation of the differential methylation of these sequences and the identification of the respective methylation patterns.

The analysis of the exhibited differential methylation showed that the paramutated phenotype is accompanied by methylation and demethylation processes (Figure 4, Table 1).



Methylation pattern	Association with paramutation	Number of sequences (%)
A	**CHG Methylation** (hemi- to full)	**6** (27.3)
B	**CHG Demethylation** (full to hemi-)	**4** (18.2)
C	**CpG Methylation** (to hemi- or full)	**4** (18.2)
D	**CpG Demethylation** (from hemi- or full)	**8** (36.4)

Figure 4. Confirmation of the differential methylation of specific DNA sequences in cv. Onward (plants O1, O2, and O3) and rogue line JI2723 (plants R1, R2, and R3). (**Left**) Examples of the four possible amplification patterns (agarose gels). (**Rigth**) Correspondence between amplification profiles, methylation patterns and number (frequency) of observed cases among the 22 DNA sequences.

2.4. Differential Methylation in Pollen DNA

The analysis of the identified sequences in pollen DNA, showed that 12 of the 22 sequences conserved the differential methylation pattern exhibited in leaf DNA, 6 sequences exhibited novel but still different methylation patterns, while the remaining 4 sequences exhibited identical methylation patterns in both epigenotypes (Tables 1 and 2, Table S6).

Table 1. Alteration of the methylation pattern associated with 'rogue' paramutation in leaf DNA *.

	CHG Methylation	CHG Demethylation	CpG Methylation	CpG Demethylation
Maintained in pollen DNA	ACA/AG_560_ O **AGC/AT_466_O** **	**ACA/AG_735_R** ACT/AC_451_R **AGG/AG_705_R**	**AAG/AG_366_O** **AGC/AT_134_O**	**AAG/AC_613_R** **ACA/AA_749_R** **AGC/AA_202_R** AGG/AC_384_R **TA/CA_260_R**
Altered in pollen DNA	**AAC/AA_300_O** ACG/CC_81_O **AGG/AT_139_O** AGG/AT_302_O	**ACT/CA_584_R**	**ACT/AG_449_O** ACA/CT_546_O	AAC/AA_174_R AAG/AA_197_R **AAG/AA_325_R**

* The suffix O or R indicates the origin (cv. Onward or "rogue" line JI2723) of the MS-AFLP product. ** Sequences highly similar to expressed sequence tags (EST) and that amplify in cDNA, are in bold.

Table 2. Methylation patterns in leaves and pollen.

Sequence	Methylation Pattern (Leaves) *	Methylation Pattern (Pollen) *
AAC/AA_174_R	D	Non-methylated
AAC/AA_300_O	A	C
AAG/AA_197_R	D	Fully CHG methylated
AAG/AA_325_R	D	Non-methylated
AAG/AC_613_R	D	D
AAG/AG_366_O	C	C
ACA/AA_749_R	D	D
ACA/AG_560_O	A	A
ACA/AG_735_R	B	B
ACA/CT_546_O	C	Non-methylated
ACG/CC_81_O	A	C
ACT/AC_451_R	B	B
ACT/AG_449_O	C	D
ACT/CA_584_R	B	D
AGC/AA_202_R	D	D
AGC/AT_134_O	C	C
AGC/AT_466_O	C	C
AGG/AC_384_R	D	D
AGG/AG_705_R	B	B
AGG/AT_139_O	A	C
AGG/AT_302_O	A	C
TA/CA_260_R	D	D

* Letters from A to D correspond to the differential methylation patterns in Figure 4.

2.5. Expression of the Differentially Methylated Sequences

The Basic Local Alignment Search Tool (BLAST) analysis against the GenBank (the National Center for Biotechnology Information, NCBI) and *Medicago trucatula* databases showed that 13 sequences have expressed homologs in *Medicago truncatula* or *Cicer arietinum*. Identical analysis against the *Pisum sativum* unigenes database allowed the identification of four additional sequences (Table S7).

A new set of primers were designed for quantitative real-time RT-PCR (RT-qPCR) analysis and validated by common PCR amplification of genomic DNA and complementary DNA (cDNA). Eight sequences, including the putatively unexpressed five sequences, were successfully amplified in genomic DNA but not in cDNA. The remaining 14 sequences amplified in both kinds of DNA and their relative expression was further assessed by RT-qPCR. Surprisingly, in spite of the differential methylation, no significant differences were found in the expression of any of these sequences in the contrasting epigenotypes (Table S7).

3. Discussion

The main aim of the here reported research was to get a first insight into the molecular mechanisms that underlie the rogue (rabbit ears) paramutation in peas (*Pisum sativum* L.).

Contrarily to the study of other plant paramutation systems, particularly in maize, in which during the last decades significant research progress has been achieved—including the identification of multiple genes involved in paramutation—the study of the rogue paramutation in pea was almost limited to the analysis of the inheritance of the paramutated phenotype and cytogenetic observations.

The association of plant paramutation with DNA-methylation has been established in multiple plant paramutation systems [12,15–21]. Bearing in mind these results we carried out a comparative analysis of the genome-wide 5-cytosine methylation and a random identification of specific differentially methylated sequences in cv. Onward vs. its rogue paramutated line JI2723. Twenty-two sequences were identified as differentially methylated in the two epigenotypes. The analysis of the methylation status of the terminal 5′-CCGG-3′ motifs in these sequences revealed that the occurrence of the rogue paramutation was accompanied by methylation and demethylation of specific sequences.

In the large majority of the cases, the methylation polymorphisms were revealed by the presence of very clear PCR bands vs. total absence of the same PCR products, suggesting a strong mitotic inheritance of these epigenetic marks throughout all leaf tissues.

An indication of meiotic, and probably transgenerational, inheritance of these epigenetic marks arose from the observed conserved differential methylation in pollen DNA exhibited by 12 out of the 22 sequences (Figure 4, Tables 1 and 2). Nevertheless, the RT-qPCR analysis showed that the differential methylation does not result in differential expression of the identified sequences.

With this study, it was established for the first time that—as in other paramutation phenomena—the pea rogue paramutation is accompanied with alterations in the methylation pattern of specific genomic sequences. However, additional studies are needed to unveil the biological consequences of the identified differential methylation. For the moment, we can only speculate that the observed alterations in DNA methylation, and eventual modifications in chromatin conformation, probably spread over larger genomic regions encompassing the identified sequences, and eventually affect the expression of other, surrounding, genes. The fast accumulation of genomic data is expected soon to allow the identification of the 22 sequences in the *Pisum* genome, permitting the assessment of the methylation status and expression of neighboring genes.

4. Material and Methods

4.1. Plant Material

Seeds of pea cv. Onward (line JI2722) and of the derived from this cultivar rogue line JI2723, identified by Dr. Stig Bixt (University of Lund, Sweden), were kindly provided by Dr. Mike Ambrose, John Innes Centre, UK, and multiplied at the University of Algarve, Country. The successive progenies were used for experimental work.

After germination in petri dishes, the young seedlings were transplanted to pots containing peat:vermiculite (1:1) mixture inoculated with macerated *Rhizobium* nodules and grown under controlled greenhouse conditions.

4.2. DNA Extraction

DNA was extracted from leaf and from pollen grains and quantitatively and qualitatively analyzed as previously described [26].

4.3. RAPD and SSR Analyses

The assessment of true-to-typeness and genetic similarity of the studied epigenotypes was performed by RAPD analysis using 60 Operon Technologies primers (kits AL, AM, AN, and M; Operon Technologies Inc., Alameda, California, United States) and by the analysis of five microsatellite

loci: A9, AA219, AB146, AC58, and AD146 [27]. PCR amplifications and agarose and polyacrylamide gel electrophoresis were carried out as previously described [28,29].

4.4. HpaII and MspI Restriction Analysis of Genomic DNA

Equal amounts of leaf genomic DNA of three plants per epigenotype were pooled apart and 2 µg of each DNA-bulk was used for restriction analysis.

The analyses were performed using the isoschizomeric restriction enzymes, HpaII and MspI, which recognize the same restriction sequence (5′-CCGG-3′) but are differentially sensitive to DNA (cytosine) methylation [30]. Digestions were performed overnight at 37 °C with 30 U of each enzyme in 20 µL reaction volume. The reactions were stopped by heating the samples at 65 °C for 20 min, and the digestion products were analyzed by agarose (0.8%) gel electrophoresis, for 3 h at 4 V/cm.

4.5. HPLC Analysis

Genomic DNA from cv. Onward and line JI2723 were pooled as above described. Ten micrograms of each DNA bulk were precipitated overnight with cold ethanol and, after centrifugation at 13,000 rpm for 10 min at 4 °C, the pellet was air dried and hydrolyzed in 50 µL of 70% perchloric acid for 1 h at 100 °C. The pH of the reaction was adjusted to approximately 5.0 with 10 M KOH and the supernatant was collected after centrifugation. The precipitate was washed with 50 µL HPLC grade water (Chromasolv, Honeywell Riedel-de Haën, Seelze, Germany) and, after new centrifugation, the two supernatants were combined and dried in a SpeedVac concentrater (Savant DNA120, ThermoFisher Scientific, Waltham, MA, USA) . The dried pellets were dissolved in 100 µL of the HPLC grade water and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through 0.2 µm pore size syringe filters (Nalgene, Thermo Fisher Scientific, Waltham, MA, USA).

The hydrolyzed samples were analyzed by reverse phase chromatography (Kinelex 2.6UC18 100A, 150 × 4.60 mm column) in an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) at room temperature, using 0.05 M ammonium formate (pH 4.5) in 12% methanol as mobile phase at a flow rate of 0.8 mL/min and 278 nm detection wavelength.

The retention time of each base was determined with commercially available standards of thymine (Sigma-Aldrich, St. Louis, MO, USA), adenine (Sigma-Aldrich, St. Louis, MO, USA), guanine (AppliChem, Darmstadt, Germany), cytosine (AppliChem, Darmstadt, Germany), and 5-methylcytosine (Alfa Aesar, Haverhill, MA, USA) at 50 mg/L. The genome-wide cytosine methylation of cv. Onward and respective rogue variant line JI2723 were computed as the ratio of the area of the 5-methylcytosine vs. the area of the cytosine peak.

4.6. MS-AFLPs Analysis

Methylation-sensitive amplified fragment length polymorphism (MS-AFLP) analyses were performed using the restriction enzymes HpaII and MspI [31,32]. Three bulks of leaf material of three plants each were used for analysis of each epigenotype.

One hundred-twenty-five nanograms (125 ng) of genomic DNA of each bulk of three plants were digested with 1.25 U of the endonucleases HpaII or MspI for 2 h at 37 °C in 21 µL total volume. After inactivation of the enzymes for 20 min at 65 °C, and correction of the buffer (Tango, Thermo Fisher Scientific, Waltham, MA, USA) to the double (×2) concentration, the samples were digested under the same conditions with 1.25 U of EcoRI and reactions stopped by new incubation at 65 °C for 20 min. For ligation of the restricted DNA to adapters (Table S5), an equal volume of a solution containing 5 pmol of EcoRI-adapter and 50 pmol of HpaII/MspI-adapter, 0.5 U of T4 DNA ligase (Thermo Fisher Scientific, Waltham, MA, USA) and 2× ligase buffer was added to each inactivated restriction reaction and the reaction was left to proceed overnight at room temperature.

The adapter, the pre-amplification, and the selective primers for EcoRI generated restriction ends were the same used in standard AFLP analysis [33]. The adapter for ligation to HpaII and MspI restriction ends and the respective pre-amplification and selective primers, were the same

used by other authors [34] (Tables S1 and S2). Further MS-AFLPs procedures were performed as previously described for standard AFLPs [35]. Selective amplifications were performed using 64 primer combinations (Table S3). The excision from gels, re-amplification, and cloning of polymorphic bands were performed as previously described [36].

The identified epi-polymorphic DNA sequences and putative protein products were successively BLAST (Nucleotide-Nucleotide BLAST and Protein-Protein BLAST) analyzed against the genome databases: NCBI [37]; *Medicago truncatula* [38] and *Pisum sativum* unigenes [39] (last access - 03.03.2017) .

4.7. Confirmation of the Differential Methylation

For confirmation of the differential methylation, new (17–20 bp) primers were designed spanning over the terminus of the MS-AFLP adapter and the first nucleotides of the identified sequences. The amplifications were performed using restricted genomic DNA ligated to MS-AFLP adapters.

Amplifications were carried out in 15 µL reaction mixtures containing 1.5 µL of 1:50 diluted MS-AFLP pre-amplification product, 1× Dream Taq DNA polymerase buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.16 mM of each dNTP, 0.4 µM of each primer (Sigma-Aldrich, St. Louis, MO, USA), and 0.6 U of Dream Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), using the PCR program: initial step of 1.5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 59 to 68 °C (depending on the primers), and 1min at 72 °C, and a final extension at 72 °C for 10 min. The amplification products were analyzed on 2% agarose gels.

4.8. Meiotic Inheritance of Differential Methylation

Late floral buds were collected from cv. Onward and six JI2723 (rogue) plants. The flowers were manually open and the mature pollen from two groups of three plants of the same phenotype was shaken out into a mortar and checked by stereomicroscopy for contamination with another tissue. Liquid nitrogen was carefully added to the mortar and the pollen grains homogenized with a pestle until a fine powder was obtained. The genomic DNA was extracted, digested with the restriction enzymes HpaII and MspI, and ligated to MS-AFLP adapters as described above. The methylation status of the epi-polymorphic sequences was assessed in pollen DNA as described for leaf DNA.

4.9. RNA Extraction and cDNA Synthesis

Leaf tissue from pea plants grown under rigorously controlled identical conditions was collected, immediately frozen in liquid nitrogen, and used for total RNA extraction with Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Extractions were performed from pools of leaves of three different plants. Total RNA concentration and RNA integrity were assessed, respectively, by NanoDrop 2000c spectrophotometry ((Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis in the presence of formamide.

The messenger RNA (mRNA) was isolated using the kit PolyATtract® mRNA Isolation System III (Promega, Madison, WI, USA) according to the manufacturer's instructions. Purity, concentration, and quality of the eluted mRNA were determined as above described for total RNA.

The first-strand cDNA was synthesized by reverse transcription with RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). As template of 200 U of M-MuLV reverse transcriptase was used 1µg of mRNA was used (Thermo Fisher Scientific, Waltham, MA, USA) and 5 mM of oligo-dT primer T12MN (Sigma-Aldrich, St. Louis, MO, USA) in a final volume of 20 µL. The obtained cDNA samples were diluted 1:1000.

4.10. RT-qPCR

RT-qPCR analyses were performed in tree replicates of independent biological samples, each constituted by leaf tissue of three plants, using a Bio-Rad IQCycler (Icycler) Real-Time Quantitative

Thermal Cycler (Bio-Rad, Hercules, CA, USA). Reaction mixtures consisted of 7.5 μL iQ SYBR Green (Bio-Rad, Hercules, CA, USA) mix, 0.4 μM of primers and 1 μL of cDNA in a final volume of 15 μL.

β-Tubulin 2 (GenBank:X54845.1) was used as reference gene (primers: 5′-TGGTCAGCTTAAC TCTGAT-3′ and 5′-CTGCTGAGAGCCTCTAG-3′). The data analysis was performed using the IQCycler software (Bio-Rad, Hercules, CA, USA).

Supplementary Materials: The following are available online at www.mdpi.com/2075-4655/1/1/6/s1.

Acknowledgments: Tatiana Espírito Santo was the recipient of the Ph.D. fellowship SFRH/BD/47450 /2008 and José Leitão of the sabbatical grant SFRH/BSAB/716/2007, both awarded by Portuguese Foundation for Science and Technology. The research work was funded by the "Programa Plurianual" of the same foundation. We are grateful to Vicky Chandler for hosting José Leitão as a visiting scholar (BIO5, University of Arizona, USA), to Mike Ambrose (John Innes Institute, UK) for the kindly provided pea lines JI2722 and JI2723.

Author Contributions: Tatiana E. Santo as performed most of the research work and analysis of data. Ricardo J. Pereira has confirmed and completed multiple research results. José M. Leitão has conceived and designed the experiments, analyzed the data, and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Bateson, W.; Pellew, C. On the genetics of "rogues" among culinary peas (*Pisum sativum* L.). *J. Genet.* **1915**, 13–36. [CrossRef]
2. Bateson, W.; Pellew, C. The genetics of "rogues" among culinary peas (*Pisum sativum*). *Proc. R. Soc. Lond.* **1920**, *91*, 186–195. [CrossRef]
3. Brink, R.A. Paramutation at the R Locus in Maize. *Cold Spring Harb. Symp. Quant. Biol.* **1958**, *23*, 379–391. [CrossRef] [PubMed]
4. Rassoulzadegan, M.; Grandjean, V.; Gounon, P.; Vincent, S.; Gillot, I.; Cuzin, F. RNA-mediated non-Mendelian inheritance of an epigenetic change in the mouse. *Nature* **2006**, *441*, 469–474. [CrossRef] [PubMed]
5. Arteaga-Vazquez, M.A.; Chandler, V.L. Paramutation in maize: RNA mediated trans-generational gene silencing. *Curr. Opin. Genet. Dev.* **2010**, *20*, 156–163. [CrossRef] [PubMed]
6. Hollick, J.B. Paramutation and development. *Annu. Rev. Cell. Dev. Biol.* **2010**, *26*, 557–579. [CrossRef] [PubMed]
7. Erhard, K.F.; Hollick, J.B. Paramutation: A process for acquiring trans-generational regulatory states. *Curr. Opin. Plant Biol.* **2011**, *14*, 210–216. [CrossRef] [PubMed]
8. Pilu, R. Paramutation: Just a curiosity or fine tuning of gene expression in the next generation? *Curr. Genom.* **2011**, *12*, 298–306. [CrossRef]
9. Alleman, M.; Sidorenko, L.; McGinnis, K.; Seshadri, V.; Dorweiler, J.E.; White, J.; Sikkink, K.; Chandler, V.L. An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* **2006**, *442*, 295–298. [CrossRef] [PubMed]
10. Sidorenko, L.; Dorweiler, J.E.; Cigan, A.M.; Arteaga-Vazquez, M.; Vyas, M.; Kermicle, J.; Jurcin, D.; Brzeski, J.; Cai, Y.; Chandler, V.L. A dominant mutation in mediator of paramutation2, one of three second-largest subunits of a plant-specific RNA polymerase, disrupts multiple siRNA silencing processes. *PLoS Genet.* **2009**, *5*, e1000725. [CrossRef] [PubMed]
11. Hale, C.J.; Stonaker, J.L.; Gross, S.M.; Hollick, J.B. A novel Snf2 protein maintains trans-generational regulatory states established by paramutation in maize. *PLoS Biol.* **2007**, *5*, e275. [CrossRef] [PubMed]
12. Barbour, J.-E.R.; Liao, I.T.; Stonaker, J.L.; Lim, J.P.; Lee, C.C.; Parkinson, S.E.; Kermicle, J.; Simon, S.; Meyers, B.C.; Williams-Carrier, R.; et al. Required to maintain repression2 is a novel protein that facilitates locus-specific paramutation in maize. *Plant Cell.* **2012**, *24*, 1761–1775. [CrossRef] [PubMed]
13. Erhard, K.F., Jr.; Stonaker, J.L.; Parkinson, S.E.; Lim, J.P.; Hale, C.J.; Hollick, J.B. RNA polymerase IV functions in paramutation in *Zea mays*. *Science* **2009**, *323*, 1201–1205. [CrossRef]
14. Stonaker, J.L.; Lim, J.P.; Erhard, K.F., Jr.; Hollick, J.B. Diversity of Pol IV function is defined by mutations at the maize *rmr7* locus. *PLoS Genet.* **2009**, *5*, e1000706. [CrossRef] [PubMed]

15. Gouil, Q.; Novák, O.; Baulcombe, D.C. SLTAB2 is the paramutated SULFUREA locus in tomato. *J. Exp. Bot.* **2016**, *67*, 2655–2664. [CrossRef]
16. Meyer, P.; Heidmann, I.; Niedenhof, I. Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. *Plant J.* **1993**, *4*, 89–100. [CrossRef]
17. Das, O.P.; Messing, J. Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. *Genetics* **1994**, *136*, 1121–1141. [PubMed]
18. Sidorenko, L.V.; Peterson, T. Transgene-Induced Silencing Identifies sequences involved in the establishment of paramutation of the maize *p1* gene. *Plant Cell.* **2001**, *13*, 319–335. [CrossRef] [PubMed]
19. Walker, E.L.; Panavas, T. Structural features and methylation patterns associated with paramutation at the *r1* locus in *Zea mays*. *Genetics* **2001**, *159*, 1201–1215. [PubMed]
20. Haring, M.; Bader, R.; Louwers, M.; Schwabe, A.; van Driel, R.; Stam, M. The role of DNA methylation, nucleosome occupancy and histone modifications in paramutation. *Plant J.* **2010**, *63*, 366–378. [CrossRef] [PubMed]
21. Belele, C.L.; Sidorenko, L.; Stam, M.; Bader, R.; Arteaga-Vazquez, M.A.; Chandler, V.L. Specific tandem repeats are sufficient for paramutation-induced trans-generational silencing. *PLoS Genet.* **2013**, *9*, e1003773. [CrossRef] [PubMed]
22. Brotherton, W., Jr. Further studies on the inheritance of rogue types in garden peas (*Pisum sativum*). *J. Agric. Res.* **1923**, *24*, 815–852.
23. Brotherton, W., Jr. Gamete production in certain crosses with rogues in peas. *J. Agric. Res.* **1924**, *28*, 815–852.
24. Bunten, I. A preliminary report on the chromosome complement of "rabbit eared rogues" in culinary peas (*Pisum sativum* L.). *Am. J. Bot.* **1930**, *17*, 139–142. [CrossRef]
25. Pyke, K.A.; Hedley, C.L. Aspects of the rogue phenotype of peas. *Plant Sci. Lett.* **1984**, *35*, 87–90. [CrossRef]
26. Pereira, G.; Marques, C.; Ribeiro, R.; Formiga, S.; Dâmaso, M.; Tavares Sousa, M.; Farinhó, M.; Leitão, J.M. Identification of DNA markers linked to an induced mutated gene conferring resistance to powdery mildew in pea (*Pisum sativum* L.). *Euphytica* **2010**, *171*, 327–335. [CrossRef]
27. Loridon, K.; McPhee, K.; Morin, J.; Dubreuil, P.; Pilet-Nayel, M.L.; Aubert, G.; Rameau, C.; Baranger, A.; Coyne, C.; Lejeune-Hènaut, I.; et al. Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). *Theor. Appl. Genet.* **2005**, *11*, 1022–1103. [CrossRef] [PubMed]
28. Elisiário, P.J.; Justo, E.M.; Leitão, J.M. Identification of mandarin hybrids by isozyme and RAPD analysis. *Sci. Hort.* **1999**, *81*, 287–300. [CrossRef]
29. Carlier, J.D.; Sousa, N.H.; Santo, T.E.; d'Eeckenbrugge, G.C.; Leitão, J.M. A genetic map of pineapple (*Ananas comosus* (L.) Merr.) including SCAR, CAPS, SSR and EST-SSR markers. *Mol. Breed.* **2012**, *29*, 245–260. [CrossRef]
30. Cervera, M.T.; Ruiz-Garcia, L.; Martinez-Zapater, J. Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers. *Mol. Gen. Genom.* **2002**, *268*, 543–552. [CrossRef] [PubMed]
31. Reyna-López, G.E.; Simpson, J.; Ruiz-Herrera, J. Differences in DNA methylation pattern are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol. Gen. Genet.* **1997**, *253*, 703–710. [CrossRef] [PubMed]
32. Xiong, L.Z.; Xu, C.G.; Saghai-Maroof, M.A.; Zhang, Q. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. *Mol. Gen. Genet.* **1999**, *261*, 439–446. [CrossRef] [PubMed]
33. Vos, P.; Hogers, R.; Bleeker, M.; Reijans, M.; van de Lee, T.; Hornes, M.; Frijters, A.; Pot, J.; Peleman, J.; Kuiper, M. AFLP: A new technique for DNA fingerprinting. *Nucl. Acids Res.* **1995**, *23*, 4407–4414. [CrossRef] [PubMed]
34. Baurens, F.-C.; Nicolleau, J.; Legavre, T.; Verdeil, J.-L.; Monteuuis, O. Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated in vitro with reference to leaf morphology as a phase change marker. *Tree Physiol.* **2004**, *24*, 401–407. [CrossRef] [PubMed]
35. Farinhó, M.; Carlier, J.; Svetleva, D.; Coelho, P.; Monteiro, A.; Leitão, J. Mapping of a locus for adult plant resistance to downy mildew in broccoli (*Brassica oleracea* convar. italica). *Theor. Appl. Genet.* **2004**, *109*, 1392–1398. [CrossRef] [PubMed]
36. Farinhó, M.; Coelho, P.; Monteiro, A.; Leitão, J. SCAR and CAPS markers flanking the *Brassica oleracea* L. Pp523 downy mildew resistance locus demarcate a genomic region syntenic to the top arm end of *Arabidopsis thaliana* L. chromosome 1. *Euphytica* **2007**, *157*, 215–221. [CrossRef]

37. Available online: www.ncbi.nlm.nih.gov (accessed on 3 February 2017).
38. Available online: www.jcvi.org/cgi-bin/medicago/overview.cgi (accessed on 3 February 2017).
39. Available online: www.coolseasonfoodlegume.org (accessed on 3 February 2017).