Characterization of the DNA Methylation Activity by Gene Expression Analysis in *Fragaria vesca*

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Abstract

DNA methylation represents one of the key processes that play an important role in the transcriptional control of gene expression. The role of cytosine methylation in plant development has been demonstrated. For this purpose gene expression analysis has been widely applied. The present work was directed to demonstrate how nitrate based manuring could induce DNA methylation in *Fragaria vesca*. Differential display analysis was used for this purpose. Two *Fragaria vesca* genotypes were analyzed. For each genotype a total of 40 plants were used. Plants were divided into two groups of twenty plants each. The first group was treated with a concentrated solution of calcium nitrate, the second was treated using an organic nitrogen solution at the same molar concentration. Plant material was sampled six times. The first sampling was before treatment, and subsequent sampling was done respectively 1, 3, 7, 15 and 20 days after the first one. For each genotype, leaves and roots were sampled and stored in liquid nitrogen. Genomic DNA was isolated and analyzed by applying isoschizomers to evaluate the extent of DNA methylation. This showed an increase of the DNA methylation in plants treated with calcium nitrate. Total RNA was separately purified from root and leaf and, in order to isolate differentially expressed genome fractions, cDNA was obtained. To generate differentially expressed sequences between different treatments and between sampling times, random and specific primers were applied. 62 differential fragments were isolated and sequenced. All sequences were compared to the database to evaluate similarity with previously isolated genes. Sequence analysis showed that 55% could be classified as ESTs. The other sequences showed homologies with genes related to abiotic stress. In particular it was possible to show a reduction of transcriptomic activity in the sample treated with calcium nitrate. Sequence analysis demonstrated that gene fractions isolated from plants treated with nitrate showed high homology with glycosylase, a gene related with DNA methylation repair action.

INTRODUCTION

DNA methylation is a conserved epigenetic modification of the genome. The methyl moiety on the base generally contributes to transcriptional repression by preventing activators from binding to their target, or by favouring the formation of inactive chromatin (Bird and Wolffe, 1999).

In prokaryotes, DNA methylation is important for DNA repair and replication, and in recognition and protection of self DNA (Noyer-Weidner et al., 1993). In eukaryotes, DNA methylation plays important roles in gene repression, genome organization and stability, genomic imprinting, and other developmental aspects (Bird, 2002), and in contrast glycosylase/lyase causes DNA demethylation. Plant improvement depends on generating phenotypic variation and selecting for characteristics that are heritable. Classical genetics and early molecular genetics studies on single genes showed that differences in chromatin structure, especially cytosine methylation, can contribute to heritable phenotypic variation and gene silencing (Cao et al., 2002).

Understanding molecular mechanisms underlying the manure-gene expression interaction is of primary importance in devising strategies to control physiological

processes. For this purpose, gene expression analysis is widely applied. One of the most powerful techniques for such analysis is differential display were cDNA is reverse-transcribed from mRNA isolated from different tissues.

For this reason, in this study the methylation induced by different manuring practices is described.

MATERIALS AND METHODS

Plant Material

Two *Fragaria vesca* genotypes obtained from a specific breeding programme were utilized (PZ99C101 and PZ99SP7.1). For each genotype 40 plants were used. All plants were cultivated for at least 50 days with the same manure protocol. In this period only organic manure was applied. After this period the plants of each genotype were split in two different groups. Twenty plants were treated with a high concentration solution of calcium nitrate, the others were manured with the same nitrogen amount but using only organic manure. Plant material was sampled six times. The first sampling was before treatments commenced, the following samplings were done respectively at 1, 3, 7, 15 and 20 days after the first one. For each sampling time and for each genotype 3 nitrate treated plants and 3 organic manured plants were collected. From each plant we separately collected roots and leaves and then stored them in liquid nitrogen.

DNA Extraction and Digestion

DNA was extracted from each sample as described by Martelli et al. (2002). DNA was analyzed by applying isoschizomers able to detect methylated DNA.

RNA Extraction and cDNA Synthesis

Sampled tissues were used for the RNA extraction. Total RNA was extracted starting from 2 g of tissue applying an extraction buffer: 0.2 M Tris HCl pH 8.5; 0.35 M NaCl; 7 M Urea; 0.2 M EDTA pH 8; 2% (w/v) SDS. Particular attention was paid during the cleaning step to obtain highly purified RNA; cDNA synthesis was carried out following the protocol described in Bachem et al. (1996).

cDNA Analysis

Fragaria vesca cDNAs obtained were analyzed by PCR applying random primers (Table 1). PCR reactions were carried out in 50 µl final volumes using 0.5 units of Taq polymerase, 10 mM Tris-HCl pH 8.1, 50 mM KCl, 250 mM dNTPs, 3 mM $MgCl_2$ and 2 mM primers, 0.5 ng of cDNA for a total of 45 cycles applying the following thermal profile: 94°C for 1.10 min, 46°C for 1.40 min, and 72°C for 2.0 min. The final extention step was done at 72°C for 5 min. Amplified fragments were separated through 1.4% agarose gel stained with ethidium bromide. All PCR reactions were repeated three times to confirm their repeatability (Fig. 1a).

Elution, Cloning and Sequencing

Differentially expressed amplicons obtained were excised from the gel and eluted using "Quantum Prep Freeze'N Squeeze DNA Gel Extraction Spin Column" (Biorad) and then reamplified (Fig. 1b). Recovered fragments were cloned using pGEM-T Easy Vector Systems (Promega). Cloned cDNAs were then sequenced using a Sequenase kit applying Sp6 primer. Sequences were analyzed applying FASTA and BLASTP softwares and compared with the EMBL Nucleotide Sequence Database. GENESCAN software was also used to locate CDS (Coding Sequence) regions in the sequences isolated.

RESULTS AND DISCUSSION

The procedure described was useful to obtain information about the physiological process mediated by DNA methylation and induced by nitrate manure. The isoschyzomer analysis showed different levels of methylated DNA between the organic and mineral

treatment. The procedure described for RNA extraction and cDNA synthesis proved to be very useful to isolate good quality RNA and to generate cDNA from the tissues analyzed. From applying the primers, 62 differentially expressed sequences were isolated (Fig. 1). From these 43 fragments were cloned and sequenced and the other 19 were discarded in consideration of the differences between isolated and amplified fragments. Fragment analysis showed a large reduction of the amplicons in the nitrate treated samples. This result could be ascribed to a gene silencing action mediated by methylated DNA. The homology analysis of the expressed fragments isolated showed the presence, starting from the second sampling time, of glycosylase activity in samples from the mineral treatment. This result suggests that the glycosylase action to repair methylation could be considered as a physiological answer to abiotic stress resulting from a high quantity of nitrate.

CONCLUSIONS

The experimental methods applied here were shown to be suitable to study the effect of mineral fertilization on the physiological response and the experimental design is appropriate to improve knowledge of specific physiological mechanisms. The results obtained showed that an incorrect manure management could produce a reduction of the physiological plant activities. The effects of a reduction of the metabolic profile can be a reduction of productivity and low fruit quality. The information obtained could be useful to devise crop management protocols directly to stabilize the production and to increase fruit quality.

Literature Cited

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Tables

Table 1. Primer applied.

Code	Sequence	bp	CG %
MG 102	5'-CGCTTCGGGT-'3	10	70
MG 104	5'-GTCGCCTGAG-'3	10	70
MG 107	5'-CCCGTTAAGG-'3	10	60
MG 108	5'-CCGGTTCCAG-'3	10	70
MG 109	5'-GACGGAGGTC-'3	10	70
MG 111	5'-GGGCGAGTGC-'3	10	80
MG 113	5'-ACGGGCGCTC-'3	10	80
MG 115	5'-CGGACCGCGT-'3	10	80
MG 119	5'-GCATGGTAGC-'3	10	60
MG 130	5'-CGGTTAGACG-'3	10	60
RS 1001	5'-GCCATCACCGGCCAG-'3	15	73
RS 1003	5'-AGCGCCGACAGGTGC-'3	15	73
RS 1004	5'-GCATAGGCAGAAGGG-'3	15	60
RS 1005	5'-TGACCCCTCATGACG-'3	15	60
RS 1011	5'-GCATAGGCAGAAGGG-'3	15	60

Figures

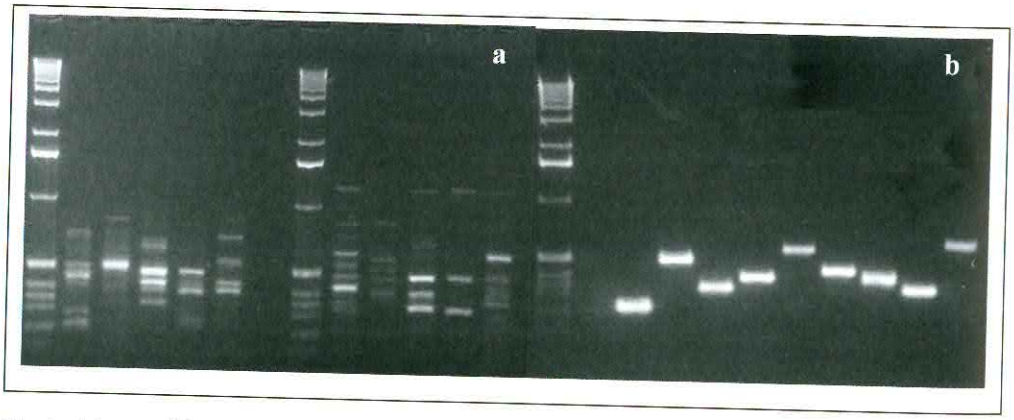

Fig. 1. (a) amplification fragments obtained applying RS1001 primer; (b) eluted fragments obtained by RS1001 primer.