DNA damage and hypermethylation of rapeseed plants in response to salt stress

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Abstract

The genetic damages and DNA methylation changes induced by salt stress in rapeseed (*Brassica napus* L.) plants were evaluated by AFLP, SRAP and MSAP markers. AFLP and SRAP analysis showed that the DNA damage caused by 10-1000 mM sodium chloride was dose-dependent and the mutation effect in the genome was random, since the same salt concentration treatment in individual plants led to a different AFLP and SRAP banding patterns. Three band types were recovered in MSAP analysis, and extensive changes between band types were observed. After 10-200 mM salt treatment, an increase of 0.2-17.6% methylated CCGG sites was detected. Nine methylation related fragments were also sequenced, and one sharing high sequence homology with ethylene responsive element binding factor (ERF) was identified. These results demonstrated clear DNA genetic and epigenetic alterations in plants as a response to salt stress, and these changes may underlie a mechanism for plants adaptation under salt stress.

Key words: *Brassica napus* L., DNA-methylation, Genetic variation, MSAP, Salt stress

Introduction

Rapeseed is an important oil crop worldwide, and also a promising crop for biofuel production (Wang, 2005). However, salinity, an increasingly serious problem in some major productive regions, is a major environmental factor impeding its extension of agricultural acreage (Serrano et al., 1994). Therefore, breeding salt tolerant variety is an argent task to meet the world's edible oil and biofuel consumption. In spite of its importance, the genetic mechanism of salt tolerance in rapeseed is largely unknown. In this study, we employ the molecular marker AFLP (Vos et al., 1995) and SRAP (Li and Quiros, 2001) to investigate the genetic damage caused by salt stress, and MSAP (Xiong et al., 1999) to access the extent and pattern of DNA methylation changes under saline condition in rapeseed.

Materials and methods

Plant material and salt treatment

The rapeseed cv. 'Westar' was used as plant material. Seeds were first germinated at 25°C for 2 d in a Petri dish (9.0 cm) containing distilled water. Then, uniformly germinated seeds were transferred to a new dish with testing solution of 0, 10, 20, 50, 100, 150, 200, 300, 400, 500, 750 and 1000 mM NaCl, respectively. After additional three days of growth, the length of plantlet (hypocotyls + root) was measured. All tests were conducted on 50 plantlets/dose and replicated three times.

DNA was isolated from plantlets using the SDS method. Molecular analyses were performed on plants grown at each concentration of salt treatments and the control.

Molecular markers assay

AFLP was performed as described in Vos et al. (1995) with minor modification. SRAP assay was carried out following Li and Quiros (2001). MSAP analysis was performed as described previously (Lu et al., 2006). All PCR products were then resolved on 6% denaturing polyacrylamide gels and visualized by silver staining system (Promega, USA).

Cloning and sequencing of MSAP fragments

Target MSAP fragments were excited from the polyacrylamide gels, cloned into a pGEM-T vector (Promega, USA), and sequenced at both strands at BGI (Beijing, China). Homology search and sequences analysis were performed at NCBI website (http://www.ncbi.nlm.nih.gov).

Data analysis

The effects of plant growth inhibition by salt stress were analyzed by ANOVA statistical test using SAS program (Version 6.12, SAS Institute Inc., Cary, NC, USA).

For AFLP and SRAP analysis, each band was scored as '1' for presence and '0' for absence in the sequencing gel. The binary matrix was used for genetic clustering (UPGMA method) by employing software NTSYS-pc 2.1 (Exeter Software, NY, USA).

As for MSAP analysis, the methylation levels (hypo- and hyper- methylation) were evaluated based on the different digestion patterns of the two methylation sensitive isoschizomers, *Msp*I and *Hpa*II. All methylaiton changes at the CCGG site in treated samples versus control were calculated and summarized.

Results

Effect of salt stress on plant growth

Low concentration of salt (10-100 mM) greatly promoted plant growth, and the maximum plant growth was observed at a salinity of 50 mM. However, significant growth retardation was recorded starting with 200 mM concentration of salt, and higher salinity (> 400 mM) completely inhibited plant growth.

Salt stress induced genetic variation

12 DNA samples from plantlets exposing to various salt concentrations (0-1000 mM) were analyzed by 8 AFLP primer combinations, which produced a total of 232 bands. Of these, 68 bands were polymorphic. This set of data was used for genetic distance/salt concentration plotting (Fig.1). A significant correlation between salinity level and genetic variation was observed ($r = 0.8108$, $P < 0.05$), indicating that the effect of salt induced DNA damage is dose dependent.

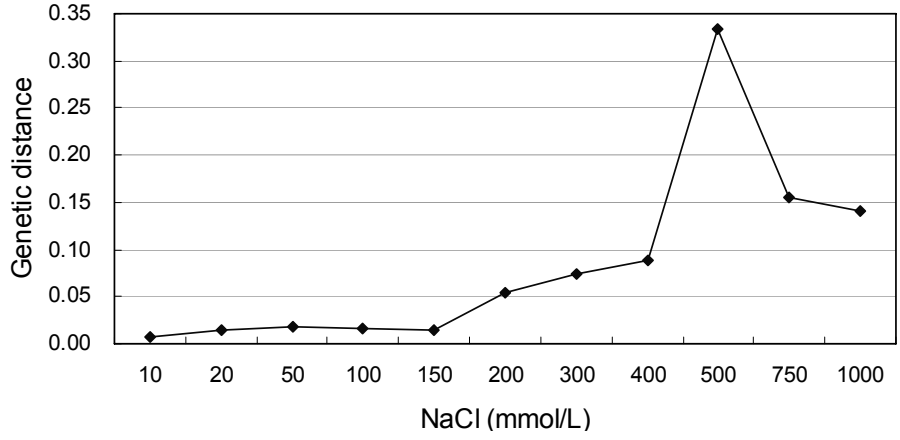

Fig.1 Relationship between genetic variation and salinity levels in rapeseed seedlings

To investigate if there exist any 'hot spot' of salt induced mutation in the rapeseed genome, we further analyzed groups of five plants for each of six treatments (0, 50, 100, 200, 400 and 1000 mM salt) with 171 AFLP markers and 52 SRAP markers resolved by each of 4 primer combinaitons. Genetic clustering showed that totally different banding patterns were observed in each of the five plants treated with each salt concentrations, and each plants treated with the same concentration of salt were clustered into different groups (Fig.2), indicating that there were no preferential mutation sites in the genome.

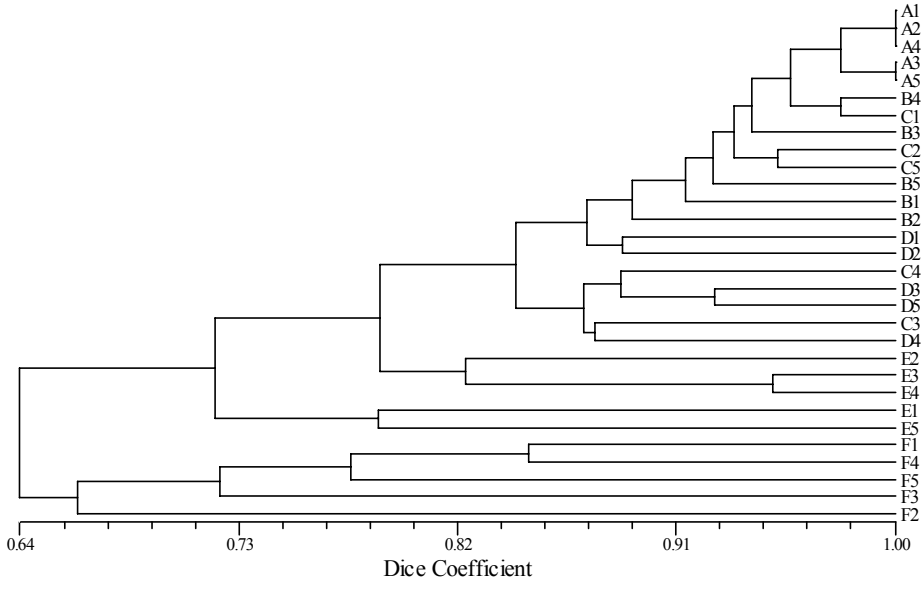

Fig.2 Dendrogram obtained from AFLP and SRAP analysis showing genetic differences due to DNA sequence changes. Alphabet letters A, B, C, D, E and F indicate salt concentrations of 0, 50, 100, 200, 400 and 1000 mM respectively, with a number showing different individuals within the same treatment.

DNA methylation changes

DNA isolated from pools of control and treated (0-200 mM) rapeseed plants were analyzed by 10 MSAP primer pairs, which yielded a total of 174 bands. For each of the samples analyzed, three types of MSAP bands were observed. Type I MSAP bands were present in both digestions of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I; Type II bands were present in *Eco*RI/*Hpa*II

but not in *Eco*RI/*Msp*I, and type III bands were present in *Eco*RI/*Msp*I but not in *Eco*RI/*Hpa*II.

By comparing the different digestion pattern of isoschizomers *Hpa*II and *Msp*I, the proportion of cytosine methylated CCGG sites can be roughly estimated by dividing the number of type II and III bands with the total number of bands (I+II+III). In normal condition (i.e. treated with distilled water), only 26.6% of CCGG sites were methylated in the rapeseed genome. However, this value increased to 26.8-44.2% when exposed to various levels of salinity (Table 1).

Table 1 A summary of type I, II and III bands for rapeseed seedling treated with different concentration of salt.

Banding patterns	Salt concentration (mM)						
	0	10	20	50	100	150	200
Type I	94	92	93	97	97	92	93
Type II	25	26	24	20	27	24	24
Type III	9	10	10	41	45	49	49
Total	128	128	127	158	169	165	166
Methylation polymorphism (%)	26.6	28.1	26.8	38.6	42.6	44.2	44.0

There were eight major classes of banding patterns, in which at least two bands were present (Table 2). Classes A, B and C were nonspecific MSAP bands that were present in all samples, and the additional five classes of banding patterns reflected various kinds of DNA methylation alterations among all samples. The observed methylation changes after salt treatment included appearance and disappearance of type I, II and III bands, as well as exchanges of type I/type II or type I/type III bands (Table 2).

Table 2 Typical AFLP-banding patterns and their respective abundance in different salt concentrations

Banding patterns	Salt concentration (mM)							No. of bands
	0	10	20	50	100	150	200	
A	I	I	I	I	I	I	I	87
B	II	II	II	II	II	II	II	18
C	III	III	III	III	III	III	III	38
D	II	II	II	I	II	II	II	3
E	III	III	III	-	III	III	III	3
F	I	III	III	III	III	III	III	2
G	III	III	III	-	-	III	III	2
H	-	-	-	I	I	-	I	3
Others								19

Sequence analysis

Nine salt-induced methylation polymorphic fragments were recovered and sequenced. BLAST search result showed that two of them (GenBank access. No. EF061314 and EF061315) are highly homologous to particular functional genes, ethylene responsive element binding factor (ERF) in *Brassica oleracea* (E-value of 1e-61) and copia-like retrotransposon *AtRE1* gene in *Arabidopsis* (E-value of 4e-11), respectively.

Discussion

By sequencing and BLASTn search, we have identified a methylation polymorphic fragment (EF061315) that is highly homologous to ERF, which was of particular interest. ERFs are members of a novel family of transcription factors that are specific to plants. In *Arabidopsis*, the *AtERF* genes were differentially regulated by ethylene and by abiotic stress conditions, such as wounding, cold, high salinity, or drought (Fujimoto et al., 2000).

In conclusion, salt stress caused extensive DNA damage and hypermethylation in the rapeseed genome. Several salt stress-related fragments (genes) were also identified. These will open new window for a better understanding of the plant stress adaptation mechanism. Further study on how these genes function in plant stress adaptation would be worthwhile.

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