Cytosine methylation levels in the genome of *Stellaria longipes*

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Environment-induced alteration of DNA methylation levels was investigated in *Stellaria longipes* (Caryophyllaceae). Total cytosine methylation levels were measured using HPLC in 6 genets representing two ecotypes (alpine and prairie) grown in short day photoperiod and cold temperature (SDC) and long day photoperiod and warm temperature (LDW) conditions. The levels of methylated cytosine were 16·54–22·20% among the three genets from the alpine and 12·62–24·70% in the three prairie genets when they were grown in SDC conditions. After the plants were moved to the LDW conditions, all of the three genets from the alpine showed decreasing levels of DNA methylation up to 6 days of growing in LDW. When the plants continued to grow in LDW for 10 days the average methylation level in the prairie genotypes showed no significant change. Cytosine methylation level was also detected in *HpaII* and *Sau3AI* restriction sites using the coupled restriction enzyme digestion and random amplification (CRED-RA) procedure, in which 15 random primers were used. Fifty per cent of the amplified bands with either or both of these two restriction sites were identified as being methylated in an alpine genotype (1C) and approximately 66% were found to be methylated in a prairie genotype (7C). It was observed that the change in growing conditions from SDC to LDW induced a decrease of methylation levels in *HpaII* sites.

1. Introduction

DNA methylation, typically in the residues of cytosine, is an important post-replicative modification. The most important biological significance of this modification event is that it is involved in gene regulation. This includes inhibition and activation of gene transcription via methylation and demethylation respectively in promoter regions. It has been shown that the synthesis of rRNA in pea (Watson *et al* 1987), production of ethylene in *Bryonia dioica* (Galaud *et al* 1993a, b) and transcriptional regulation of nuclear genes for photosynthesis in non-green plant cells (Ngernprasirtsiri *et al* 1989) are related to cytosine methylation levels. More direct evidence for the role of DNA methylation in gene inactivation was obtained from assessment of the effect of demethylation, induced by a demethylation drug, 5-azacytosine (5-azaC), that rapidly activated T-DNA genes which had been methylated following integration (Klaas *et al* 1989). In *B. dioica*, demethylation caused by mechanical rubbing or by 5-azacytosine (5-azaC) induced an increase of peroxidase activity and ethylene production (Galaud *et al* 1993b).

DNA methylation has been found to be a heritable phenotype (Messeguer *et al* 1991) and an example of the site specificity and pattern of methylation was shown to be directly controlled by a specific gene (Cai *et al* 1996). However, the levels of DNA methylation in plants is also influenced by environmental stresses. It was reported that mechanical stress greatly reduced the cytosine methylation level in *B. dioica* (Galaud *et al* 1993a) while higher growing temperature increased cytosine methylation levels of the 35S promoter region of a maize A1 gene construct in transgenic *Petunia* (Meyer *et al* 1992). It is interesting that correlating with these changes was the alteration of plant phenotypes. For example, transient decline of cytosine methylation level in *B. dioica* was followed by growth inhibition of

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internodes, and the transgenic *Petunia* responded to the induced cytosine methylation in the transgene construct by changing the flower colour which was conferred by the maize A1 gene. The most recent studies in *MET1* antisense transgenic plants and *ddm1* mutants of *Arabidopsis* have provided some strong evidence showing that some gene expression changes are directly regulated by cytosine methylation status. In a constructed strain containing the *ddm1* hypomethylation mutation and a methylated and silenced PAI2 tryptophan biosynthetic gene (MePAI2), Jeddeloh *et al* (1998) found that the methylated PAI2 gene that is silenced, conferring a blue flourescent phenotype, is progressively demethylated in the *ddm1* mutant, resulting in progressive loss of the blue flourescent colour. In the *MET1* antisense overexpressed transgenic plants, Finnegan *et al* (1998) reported that the reduction of DNA methylation level caused by antisense gene expression promoted earlier flowering in *Arabidopsis* plants. These features implicate that DNA methylation may be involved in regulation of some environment stress-responsive variations in natural populations. Furthermore, DNA methylation may be associated with a regulation mechanism to coordinate phenotypic plasticity.

The "influence" of phenotypic plasticity at developmental, ecological and evolutionary levels has been well discussed (Bradshaw 1965; Jain 1979; Chinnappa and Morton 1984; Schlichting 1986; Sultan 1987; Macdonald *et al* 1988; Stearns 1989; Emery *et al* 1994a). Although most of the evidence concerning plasticity has been obtained from morphological observation, plasticity is considered to be associated with fundamental physiological processes. Brown (1983) reported that application of gibberellin could reproduce the field-induced plastic changes in the leaf form of *Plantago paradoxa* and, hence, suggested that plasticity was regulated through changes in endogenous gibberellin levels that are environmentally induced. Macdonald *et al* (1986) explored the role of gibberellin and abscisic acid in regulating the plasticity of stem elongation in *Stellaria longipes* (Caryophyllaceae) in response to temperature and photoperiod. Emery (1995) focused on the role of ethylene in phenotypic plasticity by comparing the ethylene levels responding to wind stress, and the effects of ethylene levels on the stem elongation in two ecotypes (alpine dwarf form with low plasticity and prairie tall form with high plasticity) of *S. longipes*. Emery *et al* (1994b) suggested that ethylene may be involved in regulating the stem elongation plasticity. More recently, Kathiresan *et al* (1998) demonstrated that ACC synthase genes are differentially regulated by photoperiod and temperature in the alpine and prairie ecotypes. We suggest that stress-induced alteration of the physiological process involved in the regulation of phenotypic plasticity may be associated with activation or inactivation of some genes, which are perhaps directly regulated by DNA methylation. In the present study, we measured total cytosine methylation levels in the genome of *S. longipes* and the cytosine methylation associated with the restriction sites of two enzymes: *HpaII* and *Sau3AI*. We also investigated the effect of two contrasting growing conditions on the methylation levels in alpine and prairie ecotypes.

2. Materials and methods

2.1 *Plant materials*

The plant materials used for this study included six genotypes representing two ecotypes: 1C, 1D and 1L from alpine tundra (Plateau Mountain, Alberta) and 7B, 7C and 7H from prairie (Chain Lakes, Alberta). All these plants were maintained in a greenhouse at the University of Calgary, Calgary, Alberta. Plants from these two ecotypes were grown in growth chambers (Conviron, Winnipeg, Canada) at two different conditions: short-day cold (SDC) 8°C at night, 5°C day, 8 h photoperiod for a minimum of 60 days (minimum cold days required for LDW induction of stem elongation) and long-day warm (LDW) 8°C night, 22°C day, 16 h photoperiod. For a time course assay to record the effect of LDW, the plants were moved from the SDC chamber to an LDW chamber and the leaf and stem tissues were collected after 3, 6 and 10 days.

2.2 *Isolation of genomic DNA and CRED-RA test of DNA methylation*

The genomic DNA was extracted from leaf and stem tissue following the procedure described by Durham *et al* (1992) and modified by Li *et al* (1994). One gram plant tissue was ground to a fine powder in liquid nitrogen. The powder was suspended in 15 ml of ice cold extraction buffer (0·1 M Tris-HCl, pH 8·0, 50 mM EDTA, pH 8·0, 0·5 M NaCl and 10 mM 2-mercaptoethanol) and then, adding 1 ml of 20% SDS, the solution was incubated in a 65°C water bath for 10 min. Subsequently the solution was mixed with 5 ml of 3 M potassium acetate (pH 5·2) and left on ice for 20 min. With centrifuging at 25,000 *g* for 20 min, the supernatant was poured through 4 layers of microcloth into a fresh tube and the DNA was precipitated overnight at 5°C with 10 ml isopropanol. The DNA was pelleted by centrifuging at 20,000 *g* for 15 min and resuspended into 0·5 ml of 50 mM Tris-HCl/10 mM EDTA (pH 8·0). After two rounds of phenol/chloroform (1 : 1) extraction, the DNA solution was passed through Sephacryl S-400-HR column (Sigma). DNA methylation in *S. longipes* was tested using coupled restriction enzyme digestion and random

amplification (CRED-RA) method described by Cai *et al* (1996). Two restriction endonucleases *HpaII* and *Sau3AI* (Pharmacia), both sensitive to cytosine methylation in their restriction site, were used to digest genomic DNA prior to and/or following PCR amplification. The following random primers UBC153, 156, 157, 173, 174, 184, and 188 (Biotechnology Research Centre, University of British Columbia, Vancouver), OPA01, A02, A03, A09, A10, A19 and A20 (Operon Technologies Inc., CA, USA) were used individually for amplification of genomic DNA. PCR was conducted in Robo Cycler 40 (Stratagene) as described by Cai *et al* (1994). The amplified DNA was separated in 1·5% agarose gel in 1 × TAE and stained with ethidium bromide. Methylation is recognized as the DNA bands present in the amplified DNA profile from digested genomic DNA template but absent after post-amplification.

2.3 *HPLC analysis of methylated cytosine content in the S. longipes genome*

DNA was hydrolyzed to nucleosides using DNase I and snake venom phosphodiesterase (BRL) as described by Kemp and Sutton (1976). The nucleosides were purified and separated according to Galaud *et al* (1993a), using a Waters Bondpak reverse phase C_{18} HPLC column attached to a Waters model 600 HPLC unit. Detection of 5 nucleosides was made photometrically at 270 nm in the integrator (Philips SP8-400 UVVIS spectrophotometer) and quantification was performed after calibration with pure standards of the five nucleosides (Sigma). Measurement of cytosine methylation level for each sample was repeated twice.

3. Results and discussion

The methylated cytosine levels were measured using HPLC in six genets from two ecotypes growing in two environmental conditions: short day photoperiod and cold temperature (SDC) versus long day photoperiod and warm temperature (LDW). Table 1 presents the data obtained from the HPLC measurement of the cytosine methylation level in the samples collected from the plants growing for 60 days in the SDC and those from plants in 6 days of LDW treatment. Under the SDC conditions, 16·54–22·20% of genomic cytosines were found methylated in the genets from the alpine ecotypes and 12·62–24·70% were methylated in the genets from the prairie ecotypes. Under the LDW conditions, however, the methylated cytosine levels were lower in all the three genets from the alpine ecotype (9·70, 13·14, 14·00%) when compared to the levels measured in SDC. In the prairie ecotype, only one genet showed lower (12·75%) methylated cytosine levels in response to change in growing conditions from SDC to LDW while the other two genets exhibited slight increase (15·67, 25·62%) in the methylated levels. The change of the average methylation level from SDC to LDW is statistically significant (*t* test) in the alpine ecotype but not in the prairie ecotype. Therefore, down-regulation of genomic cytosine methylation by the LDW conditions can be recognized as a feature in the alpine ecotype but not in the prairie ecotype.

A time course assay from 0 to 10 days was done to study the effect of the LDW conditions. The average level of the methylated cytosine among three genets from the alpine ecotype greatly decreased (by 33·3%) in response to the alteration of growing conditions from SDC to LDW (3 days) and this decreased level was maintained for up to 6 days in LDW (figure 1). When the plants continued to grow in LDW for 10 days, the methylation level ascended to a point higher than the level in SDC. These results demonstrate that the methylation level in this ecotype is down-regulated only in the early days of LDW exposure. In contrast, the average cytosine methylation level in the prairie ecotype showed no obvious change when exposed to the LDW from 0 to 10 days (figure 1). However, the level decreased in one genet (7B) under LDW exposure from 3 to 10 days. The value detected for the plants under SDC was 19·9% while the values noted for the plants exposed to LDW respectively are 9·4% at day 3, 12·8% at day 6 and 9·7% at day 10 (data not shown). In contrast to this genet, the methylation level showed a slight increase in another genet (7H) and showed no change in 7C under LDW. This indicates a variable pattern of response of the genomic methylation levels in the prairie genotypes.

Table 1. Cytosine methylation levels* measured in 6 genets (6 ramets per genet) from two ecotypes (1D, 1L, 1C alpine and 7B, 7H, 7C prairie) of *S. longipes* grown in SDC (60 days) and LDW (6 days) conditions.

	Methylated cytosine (%)	
Genet	SDC	LDW
1D	16·54	13·14
1L	17·07	9·70
1C	22·20	14·00
7B	19·86	12·75
7H	12·62	15·62
7C	24·70	25·62

*The HPLC measurement of all samples were repeated and the mean values between two repeated measurements are given. The methylation level is calculated as % mdC = mdC/(dC + mdC) × 100, in which, mdC is methylated deoxycytidine and dC is deoxycytidine.

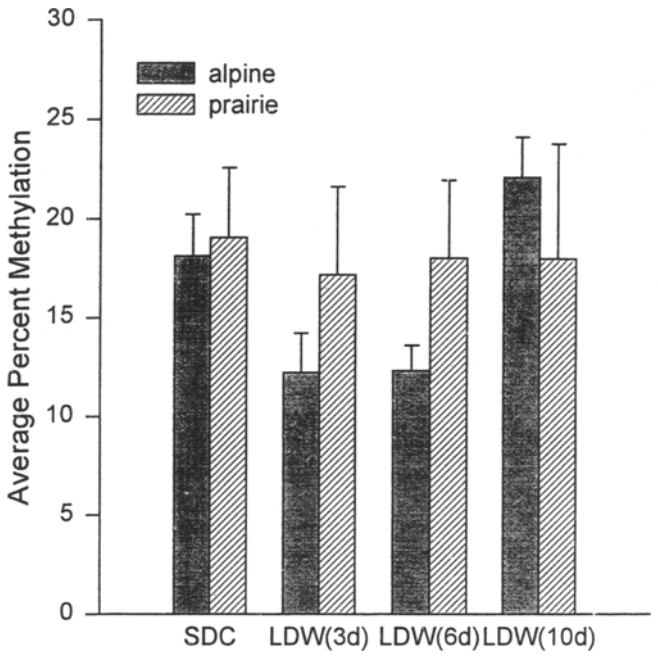

Figure 1. Changes in the average methylation levels in alpine and prairie ecotypes of *S. longipes* in a time course of long day photoperiod and warm temperature (LDW) and short day photoperiod and cold temperature (SDC). The plant samples were collected at 0 days (SDC), 3 days in LDW, 6 days in LDW and 10 days in LDW. The error bars represent standard errors. The bars represent average values from three genets (six ramets per genet).

The cytosine methylation in the *S. longipes* genome was also detected in this study using the CRED-RA procedure (Cai *et al* 1996). This experiment was an attempt to detect the cytosine methylation associated with some particular DNA sequences so as to explore a possible relationship between methylation/demethylation and gene expression that is associated with the two ecotypes. We used two restriction enzymes, *HpaII* and *Sau3AI*, respectively recognizing CCGG and GATC sites, coupled with 17 random primers. Both of these enzymes are sensitive to cytosine methylation within their restriction sites and thus cannot digest the DNA when these cytosines are methylated. Therefore, cytosine methylations in these restriction sites can be recognized by comparing the random amplified products from the undigested DNA template and digested template as well as the double-digested product (Cai *et al* 1996). Digestion with *HpaII* can be used to detect methylation typically in CpG islands that are usually associated with transcriptionally active DNA. In this study, the CRED-RA test was conducted in two genotypes – 1C and 7C. These two genotypes respectively represent two typically different ecotypes – alpine, with lower phenotypic plasticity, and prairie, with higher phenotypic plasticity. The data are presented in table 2. In total 110 amplified bands were visualized when the 1C DNA was amplified using the 15 random primers. Among these, 82 bands were found containing *HpaII* or *Sau3AI* restriction sites and about 50% of the restriction sites were identified as being methylated. When the 7C DNA was amplified using these random primers, 108 bands were detected and 90 bands contained either or both of the restriction sites in which 66·3% were identified as being methylated. The CRED-RA test provided a direct evidence showing that cytosine methylation patterns in some restriction sites have been differentiated between the samples from SDC and those from LDW. In the CRED-RA test of genotype 1C DNA using an UBC157 primer, for instance, a *HpaII* site in the amplified band with 600 bp size was found

Table 2. The random amplified bands, restriction sites and methylations in the genomic DNA from two ecotypes, alpine (1C) and prairie (7C) in *S. longipes*.

DNA sample	Primer	RE	Total amplified bands	Bands with restriction sites	Bands with methylation
1C	UBC155	*HpaII*	10	2	2
7C	"	"	9	6	4
1C	UBC156	*HpaII*	9	5	3
7C	"	"	9	7	4
1C	UBC153	*HpaII*	8	5	4
7C	"	"	6	4	2
1C	UBC157	*HpaII*	7	5	5
7C	"	"	9	5	4
1C	UBC173	*HpaII*	9	8	7
7C	"	"	9	6	4
1C	UBC174	*HpaII*	6	3	2
7C	"	"	9	4	0
1C	UBC184	*HpaII*	7	3	3
7C	"	"	8	4	4
1C	UBC188	*HpaII*	5	5	3
7C	"	"	5	4	2
1C	OPA1	*HpaII*	7	4	0
7C	"	"	6	4	1
1C	OPA3	*HpaII*	7	4	2
7C	"	"	7	3	2
1C	OPA9	*HpaII*	7	7	2
7C	"	"	8	7	5
1C	OPA10	*HpaII*	6	2	1
7C	"	"	6	5	4
1C	OPA1	*Sau3AI*	7	3	0
7C	"	"	6	3	1
1C	OPA2	*Sau3AI*	8	7	4
7C	"	"	9	6	3
1C	OPA3	*Sau3AI*	7	6	4
7C	"	"	7	7	5
1C	OPA4	*Sau3AI*	6	4	1
7C	"	"	5	5	2
1C	OPA7	*Sau3AI*	2	2	1
7C	"	"	3	2	2
1C	OPA19	*Sau3AI*	5	5	3
7C	"	"	6	5	4
1C	OPA20	*Sau3AI*	2	2	2
7C	"	"	3	3	2

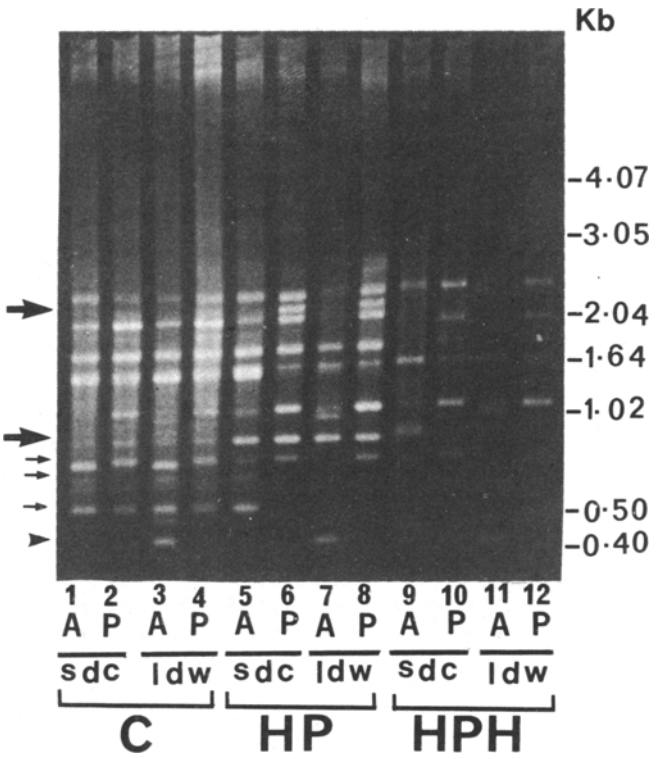

Figure 2. DNA profile of two *S. longipes* genets, 1C (A, alpine) and 7C (P, prairie) respectively growing in short day cold (sdc) and long day warm (ldw) conditions, amplified using UBC157 coupled with *HpaII* digestion, showing methylation differences between two different conditions. Lanes 1–4, DNA amplified from undigested template (C); lanes 5–8, DNA amplified from *HpaII* digested template (HP); lanes 9–12, DNA amplified from *HpaII* digested template and subjected to further digestion after amplification (HPH). In these groups, the samples were loaded in the following order: 1C-SDC (lanes 1, 5 and 9), 7C-SDC (lanes 2, 6 and 10), 1C-LDW (lanes 3, 7 and 11) and 7C-LDW (lanes 4, 8 and 12). The DNA was separated in 1 × TAE solution containing 1·5% agarose. The arrowhead points to the band showing a different pattern between sdc and ldw when amplified from the undigested template. The large arrows point to the bands distinguishable between the DNA samples amplified from digested and undigested templates and the small arrows point to the bands differentiated between samples from sdc and ldw when amplified from the digested templates, indicating methylation change due to alteration of growing conditions.

methylated in the sample from SDC, but not methylated in that from LDW (figure 2, small arrow). This suggests that cytosine methylations or demethylations in some particular sites of *S. longipes* genomic DNA can be induced by changing the growing conditions. It is very interesting that, when comparing the methylation changes occurring in *HpaII* sites and those in *Sau3AI* sites in response to the change from SDC to LDW, the methylation levels of *HpaII* sites both in 1C and in 7C DNA tend to be lower in LDW than those found in SDC. For 1C DNA, approximately 64% of the amplified bands with either or both restriction sites were identified as being methylated in the SDC sample but only 48% were methylated in the LDW. A CRED-RA test of 7C DNA showed 61% methylation in the SDC sample and only about 53% methylation in LDW sample (table 3). However, the reverse result was observed for *Sau3AI* which showed slightly higher methylation levels in LDW than in SDC. This may imply that LDW conditions may induce demethylation in some *HpaII* sites. Cytosine methylation and demethylation in CpG islands located in promoter regions have been associated with regulation of transcriptional activity and it has been confirmed that methylation of the CpG in promoters inhibits transcription indirectly via a methyl-CpG binding protein (Boyes and Bird 1991). Therefore environment-induced cytosine methylation and demethylation in CpG islands may be particularly interesting for the mechanism of regulating the plasticity in *S. longipes*.

It should be pointed out that, in the CRED-RA test, some random amplified bands were found specific to the LDW sample even in undigested DNA. One of these bands is shown in figure 2 (arrowhead). Also some bands were exclusively amplified in the digested DNA samples both from SDC and from LDW and two of them are shown in figure 2 (large arrows). The banding pattern differentiation between SDC and LDW samples may be due to structural alterations in the DNA template caused by the environmental conditions because some secondary structures of a DNA template can inhibit amplification by the obstruction of primer annealing or hindrance of strand extension. In some cases, digestion

Table 3. The amplified bands with restriction sites and the percentage of bands with methylations respectively in *HpaII* and *Sau3AI* sites detected in the DNA samples from the alpine (1C) and prairie (7C) ecotypes of *S. longipes* under SDC and LDW conditions.

	HpaII			*Sau3AI*		
Sample	Bands with restriction sites	Bands with methylation	Methylation (%)	Bands with restriction sites	Bands with methylation	Methylation (%)
1C-SDC	53	34	64·15	28	15	53·57
1C-LDW	54	26	48·15	28	16	57·18
7C-SDC	59	36	61·02	31	19	61·29
7C-LDW	59	31	52·54	31	21	67·77

of DNA templates using restriction enzymes or using chemicals such as DMSO can help to remove these secondary structures so that the normal amplification can be restored. This may be the reason why some bands were exclusively amplified in the digested DNA template samples. It has been suggested that some DNA structural changes may be the consequence of DNA methylation itself (Hodges-Garcia and Hagerman 1992). It is also proposed that alteration of DNA structure caused by methylation may affect the gene expression. If this is true in the *S. longipes* genome, then the environment-induced DNA structure changes in *S. longipes* may be important in controlling phenotypic plasticity in this species.

Both the alpine and prairie forms of *S. longipes* can respond to the LDW condition by increasing their sizes in plastic traits, e.g., internode length and leaf length. However, the amount of phenotypic plasticity is different between these two ecotypes. The alpine form has a low level of phenotypic plasticity while that of the prairie form is much higher (Emery *et al* 1994a, b). The present study provides a clue suggesting that the low phenotypic plasticity in the alpine form may be related to the down regulation of methylation level in the early stages of LDW since it has been demonstrated that the environment-induced decrease of DNA methylation level can partially inhibit internode elongation (Galaud *et al* 1993a). It is however difficult to explain the pattern of environment-induced changes of DNA methylation levels in the prairie ecotype since the methylation levels were variable in different genets. It is very likely that the ecotypes in the two habitats have evolved quite differently. The harsh alpine tundra habitat with a short growing season is stable without any temperature fluctuations and the ecotypes with no competition are less plastic. The large temperature fluctuations, and competition with neighbouring plants in the prairie habitat may have moulded the prairie ecotypes to be plastic and heterogeneous in many respects. Detection of DNA methylation in some particular positions such as the *HpaII* restriction sites seems more important because it may provide a means to approach the effects of DNA methylation on expression of genes functioning in the regulation of phenotypic plasticity. Our further work will focus on the role of methylation in regulating the activity of specific genes for ethylene biosynthesis and stem elongation plasticity in *S. longipes*.

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