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ORIGINAL ARTICLE

The branch-thorn occurrence of *Lycium ruthenicum* is associated with leaf DNA hypermethylation in response to soil water content

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Abstract

Background *Lycium ruthenicum* is an eco-economic shrub which can exist in two forms, thorny and thornless under varying soil moisture conditions. The aim of this study was to determine if the two forms of *L. ruthenicum* were influenced by soil water content (SWC) and to test the three-way link among SWC, occurrence of branch-thorn and DNA methylation modification.

Methods and results Here, pot experiment was carried out to reveal the influence of SWC on the occurrence of branch-thorn and then paraffin sections, scanning electron microscope and methylation-sensitive amplification polymorphism(MSAP) analysis were used to determine the three-way link among SWC, branch-thorn occurrence and DNA methylation. The results showed that (a) soil drought promoted the development of thorn primordium into branch-thorn and (b) branch-thorn covered axillary bud to protect it against drought and other stresses; (c) the branch-thorn occurrence response to drought was correlated with hypermethylation of CCGG sites and (d) thorny and thornless plants of a clone were distinguished successfully based on the MSAP profiles of their leaves.

Conclusions Branch-thorns of the *L. ruthenicum* clone, which occurred in response to drought, covered axillary buds to protect them against drought and other stresses; thorn primordium of the clone did not develop into branch-thorn under the adequate soil moisture condition. The occurrence and absence of the branch-thorns were correlated with the hyper- and hypomethylation, respectively. We proposed that the branch-thorn plasticity might be an adjustment strategy for the environment, which seems to support the theory of "Use in, waste out".

Keywords Epigenetic · Black wolfberry · Phenotypic plasticity · Thorn · Axillary bud

Introduction

Trees are sessile organisms subjected to environment fluctuations over their long lifetime. Tree's capacity to rapidly acclimate to climate change will be crucial for their survival [1]. Phenotypic plasticity refers to the ability of a genotype to express different phenotypes in different environments [2]. Recently, DNA methylation has been proposed as a source of plant phenotypic plasticity but little is known in trees [1]. Moreover, DNA methylation was reported to enhance drought tolerance of plants via regulating gene expression [3]. Hypermethylation was found dominant in the drought-susceptible rice genotypes, while de-methylation events were usually found to be presented in drought-tolerant rice genotypes when under drought condition [4, 5]. In short, DNA methylation changes of plant are not only responsive to the soil water content but also related to its phenotypic plasticity.

Lycium ruthenicum, a pioneer tree species of desert, is regarded as an excellent eco-economical tree species for the control, development and utilization of both sandy land and saline–alkali land [6–9]. Due to the self-incompatibility of *L. ruthenicum* [10], the genotypes of its seedlings cannot be identical [11]. However, the micro-propagated plants from a single *L. ruthenicum* donor should have an identical genotype. Interestingly, we found that the plants of a *L. ruthenicum* clone show typical thorn-phenotypic plasticity in response to soil water content. The clonal plants under the condition of 40 ~ 60% field capacity (FC) produced plenty of

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thorns and the clonal plants without any visible thorns were found under the condition of 80% FC.

In summary, we speculated that the *L. ruthenicum* thorns occur in response to drought and the thorns could protect them against drought, however, the *L. ruthenicum* thorns disappear in response to a long-term non-drought condition. This is similar to "Use in, waste out" which was reasonable from the epigenetic view [12]. Furthermore, we proposed that DNA methylation variation response to soil water content might account for the thorn-phenotypic plasticity of the clonal *L. ruthenicum* plants. This study aims to verify the above hypothesis. Thus, pot experiment was carried out to reveal the influence of soil water content on the occurrence of branch-thorn and then paraffin sections, scanning electron microscope and methylation-sensitive amplification polymorphism(MSAP) analysis were used to determine the three-way link among soil water content, branch-thorn occurrence and DNA methylation modification. This is the first report of completely inhibiting the development of plant thorn primordium. Also, we did not find any report in regarding the relationship between the thorn-phenotypic plasticity and DNA methylation. The study proposed a hypothesis model of the thorn-phenotypic plasticity, enriched the correlation between tree phenotypic plasticity and DNA methylation, provided foundation for revealing the epigenetic mechanism of the thorn phenotypic plasticity and shined plant evolution.

Materials and methods

Plant materials

In vitro micropropagated plants derived from *L. ruthenicum* seedling G [11] were used as original materials. Stems of the healthy in vitro plants were cut into explants of 1 ~ 2 cm and then the explants were inoculated on 1/2 MS media without PGR [11] for both shooting and rooting. The stem explants produced roots and shoots quickly on the 1/2 MS media, and then the new shoots were cut into explants and inoculated on the fresh 1/2 MS media for shooting and rooting again. After several rounds of micro-propagations, the stronger in vitro plantlets were transplanted into pot with a mixture of sterile humus and sphagnum moss (1:1) according to our previous report [11]. During the whole process of acclimatization, the pots were watered to FC above 100%.

Pot experiment

Pot experiment was done to aim to reveal if thorn occurrence of the clone G were influenced by soil water content. In order to determine FC, the soil (humus: sphagnum moss = 1:1) was taken from the pots using cutting rings. The cutting rings with soil were saturated with water for 24 h until all the air in the soil pores was replaced by water. Thereafter, the cutting rings were placed on dry river sand for 48 h. The cutting rings with soil were then weighed (C) and their soil were completely dried at 105 °C for 24 h [13]. The cutting rings with completely dried soil were also measured (D). The FC of the soil was calculated using the following equation: $\frac{C-D}{D-G}\times 100\%$. The G of the equation is the weight of the cutting rings. After acclimatization, the *L. ruthenicum* uniform plantlets without thorns in pots were selected for water control experiments. Briefly, the pot plants were irrigated at three levels of soil water content (80%, 60% and 40% of FC) daily. Weighing method was used to keep the set soil water content of each treatment every day and the other cultivation conditions of each treatment were the same. Pots with *L. ruthenicum* plants were placed in the greenhouse of *Shenyang Agricultural University* (41° 49′ 25″ N; 123° 34′ 10″ E, 60 m above sea level) with a temperature of 25 ± 2 °C. After 45 days of soil water control experiments, thorn occurrence of the new shoots from the three treatments was observed.

Paraffin sections and scanning electron microscopy of thorns

The *L. ruthenicum* upper 1 ~ 7 stem nodes of both the thorny plants under 40 ~ 60% FC and the thornless plants under 80% FC were used for paraffin sections and scanning electron micrograph (SEM) to reveal structure and development of the thorns or thorn primordiums, and to investigate possible function of the thorns. Moreover, stem nodes with lignified thorn were used for paraffin section. According to the conventional paraffin section method, the samples were fixed in FAA fixative solution for 24 h and longitudinally cut through the thorns (thorn primordium) and axillary buds (axillary meristem) with a thickness of 8 ~ 10 μm [14]. The tissue sections were stained with saffron solid-green for 1 ~ 2 h. Observation of the paraffin section was performed with microscope of Leica DM3000 (Leica Microsystems, Germany). Moreover, leaf axils of the stem nodes were observed using cold field-emission scanning electron microscope of HITACHI Regulus 8100 after sampling and other treatments according to a previous report [15].

MSAP analysis

Plant leaves are easy to be taken and usually chosen as materials for MSAP analysis [16–18]. Thus, DNA methylation in the expanded leaves of the thornless (80% FC) and thorny plants (40 and 60% FC) were analyzed by MSAP method (Fig. S1) to reveal the relationship between thorn-phenotypic plasticity/soil water content and DNA methylation of *L. ruthenicum*,. For convenience, the samples were renamed as follows: 80WC (expanded leaves of the thornless

plants under the condition of 80% FC), 60YC (expanded leaves of complete-thorn branches under the condition of 60% FC), 601YC (expanded leaves of thorny stem nodes of partial-thorn branches under the condition of 60% FC), 40YC (expanded leaves of complete-thorn branches under the condition of 40% FC) and 401YC (expanded leaves of thorny stem nodes of partial-thorn branches under the condition of 40% FC). Genomic DNA was extracted from the 80WC, 60YC, 601YC, 40YC and 401YC using the NuClean PlantGen DNA Kit e139634 (Kangwei Century Biotechnology Co. Ltd.) according to the instructions. Two replicate extractions from all the samples were performed. DNA purity, integrity and concentration were assessed as our previous report [16]. Two replicate MSAP analyses of the above five samples were performed. Thereafter, the thorny-plant-specific MSAP site was verified using 31 thornless plants and 19 thorny plants as materials. All the adapters and primers used in MSAP were custom synthesized from GENEWIZ, Inc. The T4 ligase and restriction enzymes *Eco*RI, *Hap*II and *Msp*I were purchased from New England Biolabs Inc. The MSAP method using capillary electrophoresis (CE) of our previous report [11] was followed with the selective primer combinations in Table S1. Only *Eco*RI + 3 primers were 5′-end-labeled using TAMARA, FAM or HEX (GENEWIZ, Suzhou, China) to allow product detection during CE on an ABI 3730XL [19]. The pre-amplification and selective amplification reaction methods are following the PCR thermal cycler conditions in a previous report [16].

Data analysis

The scored MSAP bands were transformed into a binary character for the absence (0) or presence (1). The levels of cytosine (CCGG sites) methylation and locus-specific methylation differences between any two samples were subjected to statistical analysis using one-way ANOVA of LSD method (2-tailed, $P \leq 0.05$) by software SPSS ver. 20.0 [19]. Each locus-specific methylation alterations between any two samples were subjected to statistical analysis using single sample t-test (2-tailed, $P \leq 0.05$ and 0.01). Furthermore, the principal coordinate analysis (PCA) and UPGMA cluster analysis (Jaccard) of MSAP profiles were carried out by software MVSP ver. 3.22 [11, 18]. We generated primary binary data matrices by software Gene Marker V2.2.0. First, all MSAP sites that showed a monomorphic pattern or a 'Suspected' by Gene Marker V2.2.0 in only one sample were excluded from the binary data matrices [20]. Second, the remaining binary data matrices were transformed into quaternion matrices [11]. Third, the thornless- or thorny-plant specific MSAP site(s) were found [17]. Finally, the specific MSAP marker was verified in more thornless- and thorny-plants.

Results

Thorns occur in response to soil drought

Interestingly, the clonal plants of *L. ruthenicum* showed thorn-phenotypic plasticity. All the micro-propagated plants in vitro or during acclimatization did not produce visible thorns regardless of development stages. Moreover, almost all the transplanted plants of G under the condition of 80% FC did not produce visible thorns (thornless plants). However, the plants under drought stress (60% and 40% FC) developed visible thorns (thorny plants, Fig. 1). The same plants under the condition of 60% or 40% FC had two types of branches: branches with thorns at all the stem nodes (complete-thorn branches); branches with visible thorns at partial stem nodes (partial-thorn branches). Notably, all the leaf axils of the complete-thorn branches produced thorn but the upper four to five leaf axils did not show the visible thorns, however, the visible thorns will appear one by one with the development going on. Meanwhile, the frequencies of the two types of branches for each thorny plant were random and the average frequency of the complete-thorn branches was 54.77%. All the tested plants in this study are from a donor (Fig. S1). Nevertheless, their thorn occurrences are significantly different among plants under different conditions and even among branches of the same plants, which is a typical example of phenotypic plasticity. These indicated that soil drought promoted the occurrence of thorns in the clonal plants. However, all the seedlings of *L. ruthenicum* under 40 ~ 80% FC in greenhouse produced thorns at each

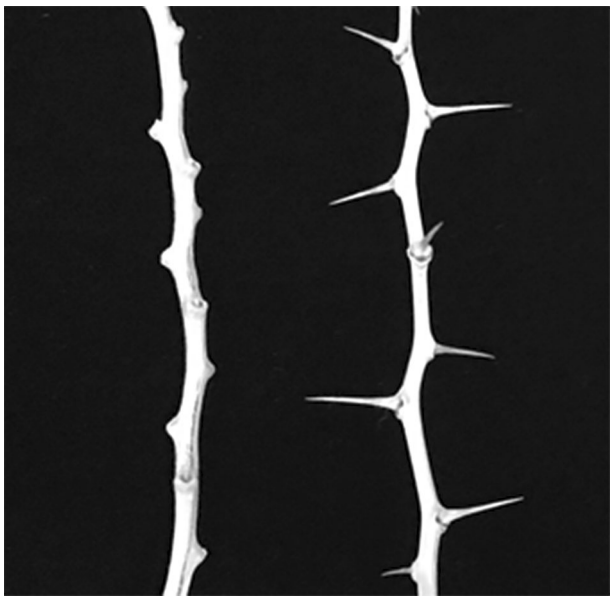

Fig. 1 Stems of thornless plant (left) and thorny plant (right) of *L. ruthenicum*

leaf axil and did not show the phenotypic plasticity in our experiment. In summary, we proposed that both high humidity environment of the in vitro culture and adequate water supply during the acclimatization & pot experiment might account for the thorn absence of the clonal *L. ruthenicum* plants under the 80% FC.

Branch-thorns cover and protect axillary buds

Paraffin sections and scanning electron microscopy showed that thorn primordium but not developing thorn was found in the thornless plant of this study (Fig. 2a). The development of axillary bud is delayed as well as that of thorn in the thornless plant (Fig. 2a). However, after loss of their shoot tips, the lateral branches of the thornless plants were normally produced. The thorn primordium and axillary meristem of *L. ruthenicum* coexist closely (Fig. 2a) and the first task of thorn seems to be covering the axillary bud (Fig. 2b, c). The axillary bud covered by the thorn was more developed and showed obvious leaf primordium (Fig. 2b, c). Also, slit exists between the axillary bud and outside (Fig. 2b, c). In our opinion, *L. ruthenicum* thorns, which cover the corresponding axillary buds, can resist drought by both reducing the water transpiration of the axillary buds and preventing animals from feeding. *L. ruthenicum*, a pioneer tree species of desert, is a typical drought resistant species. Thus, the fact of thorn covering axillary buds is probably the evolutionary result of adapting to arid desert environment. Moreover, both the developmentally delayed axillary buds of the thornless plants and the axillary buds covered by the thorns of the thorny plants might explain why the axillary buds of *L. ruthenicum* are invisible to the naked eye. Longitudinal paraffin sections indicated that the structure of thorns is similar to that of stems (Fig. 2d) and vascular bundles of thorns directly connected with stems (Fig. 2e). Thus, thorn of *L. ruthenicum* is belonging to the category of branch-thorn. However, there is usually tiny thorn invisible to the naked eye (Fig. 2f) but not thorn primordium in the thornless axil of the partial-thorn branch, that is to say, the thornless axil thorns of the partial-thorn branches (Fig. 2f) are not same to the counterparts of the thornless plants. Thus, the expanded leaves at stem nodes with visible thorns of the thorny plants and expanded leaves of the thornless plants were compared to reveal the relationship between DNA methylation and the thorn occurrence & absence.

DNA metylation alterations response to drought was related to the branch-thorn occurrence

Selection of suitable primer pairs for MSAP analysis

Fourteen primer pairs (Table S1) were used as our previously described [11]. Using the 14 primer pairs, we scored a total of 1727 and 597 clear bands from the five types of leaves in the first and second replication, respectively (Table S2, 3). Of the 1727 bands, 972 were polymorphic in either double digestion with a total methylation polymorphism frequency of 56.28% (Table S2). Also, the total methylation polymorphism frequency for the second replication was 61.43% (981/1597) (Table S3). The total methylation polymorphism frequency is high and the 14 primer combinations all gave polymorphic bands for the five samples.

Differences in cytosine methylation level existed among the five types of leaves

Of the CCGG sites assessed in the 80WC, 19.58% are fully methylated at the internal cytosines (CG), 11.40% are hemi-methylated at the external cytosines (CNG) and 20.22% are both fully methylated at the internal cytosines and hemi-methylated at the external cytosines (CG and CNG, Table 1). The average total cytosine methylation level in *L. ruthenicum* is 52.94% (Table 1), which is similar to that of *L. ruthenicum* [11] but higher than that in most reported higher plants [16]. Compared with the leaves of the thornless plant (80WC), all four types of leaves of the thorny plants (60YC, 40YC, 601YC, 401YC) showed more or less alterations in three types of detectable cytosine methylation levels (Table 1). Notably, the levels of both hemi-methylation at the external cytosines and total cytosine methylation of 80WC are lower than those of 60YC, 40YC, 601YC and 401YC (Table 1). However, only the level of total cytosine methylation in 401YC was significantly higher than that in 80WC (Table 1). These indicate that the expanded leaves of thornless *L. ruthenicum* plant are associated with a lower level of total cytosine methylation in CCGG sites. Meanwhile, the presence of the visible branch-thorns is associated with the higher level of total cytosine methylation in the corresponding expanded leaves of *L. ruthenicum*. In addition, the global DNA methylation level increased with decreasing of soil water content (Table 1). To sum up, the high global DNA methylation level in response to drought (40 ~ 60% FC) was related to the branch-thorn occurrence; the low DNA methylation level in response to non-drought (80% FC) was correlated with the branch-thorn absence.

Locus-specific methylation alterations among the five types of leaves

All scorable MSAP sites in the tested samples versus the other samples were classified into six major patterns: CG, CNG, both CG and CNG hypomethylation and hypermethylation (Table S4). Single sample t-test indicated that only partial locus-specific alterations are significant; however, all the six MSAP patterns of locus-specific alterations occurred between any two of the tested samples

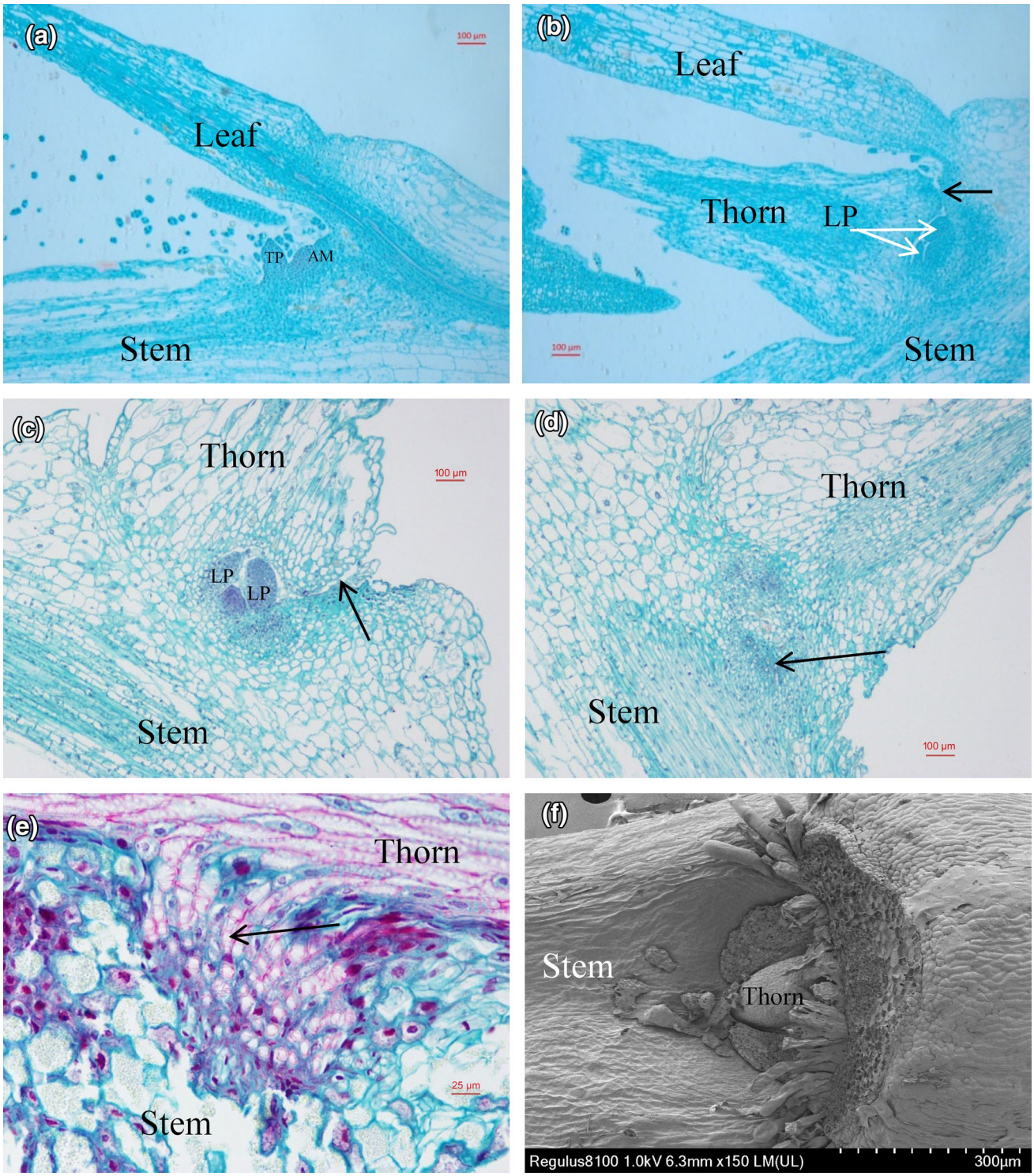

Fig. 2 Paraffin sections and SEM of *L. ruthenicum* thorns and axillary buds. **a** Paraffin section of stem node showed the thorn primordium (TP) and axillary meristem (AM) of the thornless plant, **b**–**f** are from the thorny plant. **b** Paraffin section of upper stem node without visible thorn. **c** Paraffin section of stem node with visible thorn. *LP* leaf primordium. Black arrows indicate slits between the axillary buds and outside (**b**, **c**), **d** Paraffin section of stem with young thorn. **e** Paraffin section of stem with lignified thorn; Black arrows indicate vascular bundles (**d**, **e**). **f** SEM of thornless axil of the partial-thorn branch

Table 1 Cytosine methylation level in leaves of thornless plant (80 WC), leaves of complete-thorn branches (60 YC, 40 YC) and leaves of partial-thorn branches (601 YC and 401 YC) based on MSAP analysis using 14 primer pairs

Sample (leaves)	Total bands	Unmethylated CCGG sites	Methylated CCGG sites			
			CG (%)	CNG (%)	CG and CNG (%)	Total (%)
80WC	1402.50	48.80 ± 0.61^{a}	19.58 ± 0.72^{a}	11.40 ± 0.96^{a}	20.22 ± 1.07^{a}	51.20 ± 0.61^{b}
60YC	1403.50	47.25 ± 0.26^{ab}	19.74 ± 0.34^{a}	12.83 ± 1.45^{a}	20.18 ± 1.53^{a}	52.75 ± 0.26^{ab}
601YC	1383.00	47.10 ± 0.66^{ab}	19.10 ± 1.31^{a}	12.46 ± 0.59^{a}	21.34 ± 1.37^{a}	52.90 ± 0.66^{ab}
40YC	1375.50	46.51 ± 0.94^{ab}	19.54 ± 5.90^{a}	12.09 ± 1.20^{a}	21.87 ± 3.76^{a}	53.49 ± 0.94^{ab}
401YC	1366.50	45.66 ± 0.94^{b}	19.34 ± 1.35^{a}	12.67 ± 0.46^{a}	22.32 ± 2.35^{a}	54.34 ± 0.94^{a}
Total mean	1386.20	47.06 ± 0.42	19.46 ± 2.92	12.29 ± 0.37	21.19 ± 0.80	52.94 ± 0.42

Data within column followed by the same letter are not significantly different at the 0.05 level by LSD

(Table S4). Totally, all the patterns of locus-specific alterations significantly existed between the leaves of the thornless plant (80WC) and the tested leaves of the thorny plant (60YC, 40YC, 601YC and 401YC), between the tested leaves of the thorny plants under the condition of 40% and 60% FC, and among all the tested leaves of the thorny plants (Table 2). Furthermore, all the frequencies of locus-specific alterations between the leaves of the thorny plants under the same FC (46 vs. 46) are lower than those of the forgoing comparisons. However, all the frequencies of locus-specific alterations (46 vs. 46) but not *Both Hyper* are significant at 0.05 levels (Table 2). Although only the frequencies of both *Total Hyper* and *Total* between the leaves of the thrornless plant and the thorny plants are significantly higher than those between the leaves of the thorny plants under the same FC (LSD of Table 2), not only the single types of locus-specific alterations but also the total locus-specific alteration between the leaves of the thornless plant and the thorny plants is the most obvious (Table 2). The four comparisons arranged in descending order according to the total frequencies of locus-specific alterations are as follows: WC vs. YC, 60 vs. 40, YC vs. YC, 46 vs. 46 (Table 2). That is to say, the more different factors between the tested samples the higher frequency of locus-specific alterations you get. The total frequency of locus-specific alterations between the leaves of the thorny plants under the same FC is 25.30% and that under different FC is 27.51% (Table 2). Thus, it was speculated that cytosine methylation modification of 2.21% (27.51–25.30%) MSAP sites would change in response to FC alterations in the study. Under the condition of different FC, the frequencies of locus-specific alterations among the leaves of the thorny plants and between the leaves of the thornless plant and the thorny plants are 27.51% and 29.59%, respectively (Table 2). We speculated that cytosine methylation modification of 2.08% (29.59–27.51%) MSAP sites is correlated with the branch-thorn occurrence of *L. ruthenicum*. Thus, about 29 (1386.2×2.08%) MSAP sites are theoretically related to the occurrence of the branch-thorn. Totally, all types of the locus-specific methylation alterations between the thorny and thornless plants were the highest; locus-specific methylation alterations in response to different FC were related to the branch-thorn plasticity of the *L. ruthenicum* clone.

Table 2 Average alterations of cytosine methylation patterns between leaves of the thornless plant and thorny stem nodes (WC vs. YC), between leaves of thorny stem nodes under the same FC (46 vs. 46), between leaves of thorny stem nodes under the condition of 40% and 60% FC (60 vs.40), and among all the leaves of thorny stem nodes (YC vs. YC)

Pattern	Comparison [frequencies (%)]				
	WC vs. YC	46 vs. 46	60 vs. 40	YC vs. YC	Total mean
CG Hyper	$6.62 \pm 1.36^{a**}$	$5.60 \pm 0.59^{a*}$	$5.88 \pm 0.53^{a**}$	$5.78 \pm 0.43^{a**}$	6.12 ± 1.59
CG Hypo	$5.90 \pm 1.23^{a**}$	$5.33 \pm 0.44^{a*}$	$5.14 \pm 0.28^{a**}$	$5.20 \pm 0.03^{a**}$	5.48 ± 0.31
CNG Hyper	$8.28 \pm 2.55^{a**}$	$6.68 \pm 0.66^{a*}$	$7.89 \pm 1.70^{a**}$	$7.49 \pm 1.42^{a**}$	7.80 ± 0.54
CNG Hypo	$6.74 \pm 0.71^{a**}$	$6.27 \pm 0.78^{a*}$	$6.85 \pm 0.03^{a**}$	$6.66 \pm 0.23^{a**}$	6.69 ± 0.33
Both Hyper	$1.30 \pm 0.11^{a**}$	0.87 ± 0.13^{a}	$1.30 \pm 0.32^{a**}$	$0.98 \pm 0.22^{a**}$	1.10 ± 0.08
Both Hypo	$0.74 \pm 0.02^{a**}$	$0.56 \pm 0.01^{a**}$	$0.71 \pm 0.05^{a**}$	$0.66 \pm 0.04^{a**}$	0.67 ± 0.06
Total Hyper	$16.20 \pm 1.30^{a**}$	$13.15 \pm 0.20^{b**}$	$14.80 \pm 1.49^{ab**}$	$14.25 \pm 1.20^{ab**}$	15.03 ± 0.53
Total Hypo	$13.38 \pm 0.50^{a**}$	$12.15 \pm 0.34^{a*}$	$12.70 \pm 0.36^{a**}$	$12.52 \pm 0.24^{a**}$	12.87 ± 0.25
Total	$29.59 \pm 1.03^{a**}$	$25.30 \pm 0.54^{b*}$	$27.51 \pm 1.23^{ab**}$	$26.77 \pm 0.92^{ab**}$	27.90 ± 0.70

Data within line followed by the same letter are not significantly different at the 0.05 level by LSD

*Difference at 0.05 level by single sample t-test

**Difference at 0.01 level by single sample t-test

Epigenetic divergence among the thornless and thorny plants

It was found that Jaccard similarity coefficient among the five types of leaves ranged from 0.727 (80WC vs. 401YC) to 0.801 (60YC vs. 601YC) (Table S5). The above similarity coefficients are much lower than the counterparts of normal clones from plant tissue culture [11]. This indicated that the five types of expanded leaves are epigenetically diverged more from each other although they are from the same donor plant, and further confirmed the relationship between DNA methylation and the branch-thorn occurrence or FC of *L. ruthenicum*. Cluster analysis showed that all the five samples are clustered into two groups, with the leaves of all the thorny plants in one group and leaves of the thornless plant in another group (Fig. 3a). The PCA further validated the results of cluster analysis (Fig. 3b). However, the five types of leaves were not clustered into three groups according to the FC. These results consist with the above findings in both global and locus-specific cytosine methylation alterations. Taken together, the analysis revealed that the expanded leaves of the thornless plant have diverged more from the expanded leaves of the thorny plants of the same clone. That is to say, the thornless and the thorny plants of a *L. ruthenicum* clone can be successfully distinguished by MSAP analysis using the expanded leaves as materials, which proved the relationship between the leaf DNA methylation modification of CCGG sites and the branch-thorn plasticity of the *L. ruthenicum* clone again.

Thorny/thornless plant specific MSAP marker

Pattern of a MSAP site was found exclusively in the leaves of the thornless plant based on the quaternion matrices of replications. The CCGG site of H4-4 (54 bp) in the leaves of the thornless plant was not methylated at the external cytosines (01) but that in the leaves of all the thorny plants were hemi-methylated at the external cytosines (00). After testing on a larger *L. ruthenicum* population (19 thorny plants and 31 thornless plants), we found that the hypomethylation of the external cytosines in H4-4 sites (01) is stably and specifically associated with the expanded leaves of the thornless *L. ruthenicum* plants. This robustly indicated that DNA methylation modifications associated with branch-thorn occurrence are not completely random. Also, the specific MSAP marker might be used to explore the mechanism of the thorn-phenotypic plasticity.

Discussion

The genotype of *L. ruthenicum* clone from a donor should be theoretically identical. The genotypes of its seedlings, however, could not be identical because of self-incompatibility [21]. Nevertheless, the clonal plants but not seedlings of *L. ruthenicum* showed difference in the occurrence of the thorns. This is a typical example of plant phenotypic plasticity. All the pot clonal plants under the conditions of 40–60% FC produced the visible thorns, and the pot clonal plants without any visible thorns were found under the condition of 80% FC, which indicated that the thorns of *L. ruthenicum* clone occur in response to drought; however, the thorns of the micro-propagated plants could be successfully inhibited by sufficient soil water. The above findings are similar with several previous reports which found that spine mass ratio of *Prosopis alpataco* R.A. Philippi [22], three *Berberis* species [23], juvenile *Acacia tortilis* [24], *Acacia xanthophloea* [25], *Acacia drepanolobium* [25, 26] and *Balanites glabra* [25] increased in response to drought [22], fire [23] or browsing [24–26]. However, the spines (modified leaves) [27] of the previous reports are distinct from the *L. ruthenicum* thorns which refer to axillary shoots ending in a sharp hard tip [28]. To best of our knowledge, this is the first report of completely inhibiting the occurrence of visible thorns

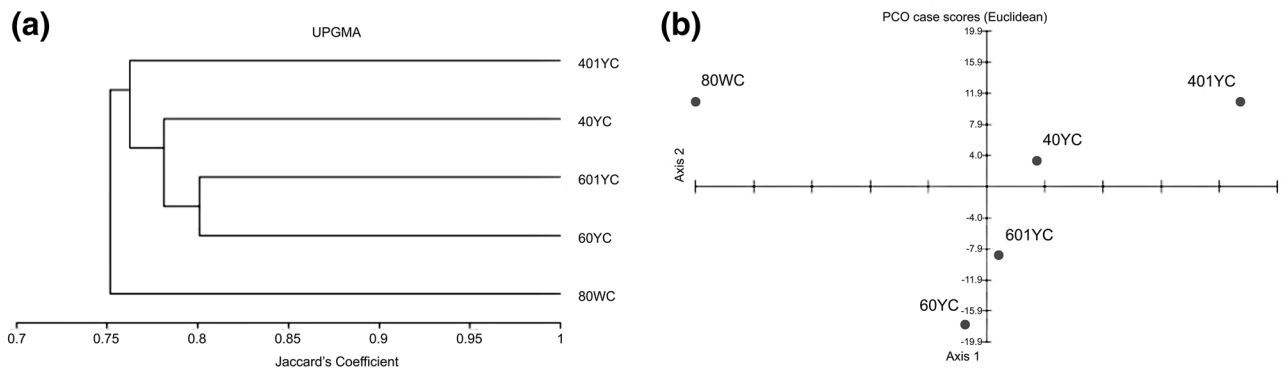

Fig. 3 Dendrogram illustrating coefficient similarities among five samples of *L. ruthenicum* by the UPGMA cluster analysis (**a**), and associations among the five samples revealed by PCA (**b**) based on the MSAP profiles

in plants. *L. ruthenicum* is a pioneer tree species of desert and its axillary buds are invisible to the naked eye. Paraffin sections showed that the axillary buds of the thorny *L. ruthenicum* are covered by the thorns (Fig. 2b, c), which indicates that thorns of *L. ruthenicum* might play an important role in protecting axillary buds. We proposed that the fact of *L. ruthenicum* thorns covering axillary buds might be an evolutionary result of adapting to arid desert environment because it seems that the thorns could not only reduce the water evaporation but also prevent browsing or other mechanical damage of the axillary buds. In our experiment, the thornless plants appeared in the in vitro and pot micropropagated population but not in the pot seedling population of *L. ruthenicum*. We proposed that both high humidity environment of the in vitro culture and adequate water supply during the acclimatization & pot experiment lead to de-evolution and thus thorn primordium does not develop into visible thorn (Fig. 2a). Interestingly, the development of axillary meristem and thorn meristem appears to be synchronized because the development of axillary buds as well as that of thorns is delayed in the thornless plant. All in all, the *L. ruthenicum* branch-thorns occur in response to drought and protect the corresponding axillary buds against drought stress; the branch-thorns but not thorn primordium of the *L. ruthenicum* clone disappear during a long-term condition of adequate water supply. It seems to support the interesting theory of "Use in, waste out".

Phenotypic plasticity, one particularly important class of ecologically traits, refers to the ability of a genotype to express different phenotypes in different environments [2]. Here, the *L. ruthenicum* thorns of a clone show typical phenotypic plasticity. The relationship between DNA methylation and leaf morphology in heterophyllous *Ilexaquifolium* and *Acacia mangium* trees has been studied [17, 29]. The results of *Ilexaquifolium* support the emerging three-way link among herbivory, leaf phenotypic plasticity and DNA methylation alteration in plants [29]. However, the relationship between DNA methylation and branch-thorn phenotypic plasticity is yet to be reported. Here, the MSAP analysis using the expanded leaves of the thorny (80% FC) and thornless (40% and 60% FC) *L. ruthenicum* as materials was carried out. The results showed that (1) global DNA methylation level of the thorny plants is higher than that of the thornless plant; (2) global DNA methylation level increased with decreasing of FC and about 2.21% MSAP sites change in response to FC but (3) the frequency of locus-specific methylation alterations between the thornless and the thorny plants is the highest, about 29 MSAP sites are theoretically correlated with branch-thorn occurrence of *L. ruthenicum*; (4) all the thorny plants are clustered into one group and the thornless plant into another group; (5) one thornless plant-specific MSAP marker of hypomethylation was found in the study. The above findings indicated that hypermethylation events are dominant in leaves of the *L. ruthenicum* clone under the condition of the lower FC, which is similar to the result of drought-susceptible rice which showed that DNA hypermethylation was dominant when under drought condition [4]. Furthermore, the findings also suggested that the visible thorns are associated with CCGG hypermethylation in the expanded leaves. It is contrary to the result of *Ilexaquifolium* which suggested that the genome of a prickly leaf was significantly demethylated in relation to the nearest nonprickly leaf on the same branchlet [29]. However, the *L. ruthenicum* thorns which refer to lateral shoots ending in a sharp hard tip are different from prick of *Ilex aquifolium* on leaf margin. Locus-specific methylation modification of leaf was highly correlated with occurrence of the visible thorn at the same stem node of *L. ruthenicum*. The thorny and thornless clonal plants of *L. ruthenicum* were distinguished successfully based on the MSAP profiles. Also, the locus-specific methylation alterations between the thorny and thornless plants did not occur completely randomly across the genome and a reliable thorny/thornless-plant specific MSAP marker was found in a very strict way. Epigenetic variation, such as DNA methylation, is correlated with both phenotype [19, 30, 31] and phenotypic plasticity [2, 32, 33] and leads to evolutionary events [12, 34, 35]. These findings indicated that DNA hypermethylation in response to drought might lead to the branch-thorn occurrence of *L. ruthenicum* and DNA hypomethylation in response to a long-term in vitro and pot non-drought could inhibit the occurrence of the visible branch-thorn. Thus, we propose that the permanent thornless plants will appear soon in *L. ruthenicum* population under the condition of sufficient soil water.

We proposed a hypothesis model of *L. ruthenicum* thorn-phenotypic plasticity based on the above findings (Fig. 4). The soil water content/FC might affect branch-thorn occurrence of the *L. ruthenicum* clone through DNA methylation of CCGG sites. Both the occurrence (Fig. 4a) and absence (Fig. 4b) of the visible branch-thorn response to different FC are illustrated in the model. Based on the model, we speculate that (a) branch-thorn occurrence of *L. ruthenicum* (desert pioneer tree) probably is an important adaptation mechanism for long-term drought and possibly other environmental stresses; (b) branch-thorn disappearance of the *L. ruthenicum* plants after long-term in vitro culture is an adjustment strategy for their long-term adaptation to humid environment. This seems to support the interesting theory of "Use in, waste out".

Conclusions

The clonal *L. ruthenicum* in this study showed thorn-phenotypic plasticity. Almost all the transplanted plants under the condition of 80% FC did not produce visible thorns

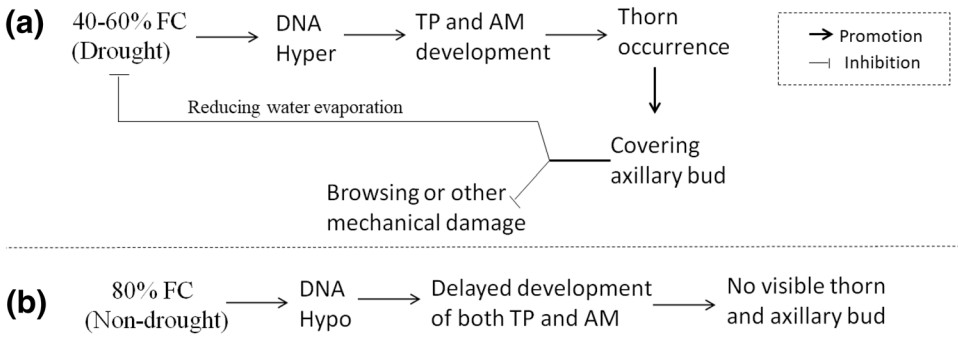

Fig. 4 Hypothesis model of the *L. ruthenicum* thorn-phenotypic plasticity. The hypermethylation events of the pot plants under drought stress (40% and 60% FC) are dominant. Thus, both TP and AM continue to develop so that the thorn can cover the developed auxiliary bud. As a result, thorns of the *L. ruthenicum* not only prevent browsing or other mechanical damage of the axillary buds but also improve the drought resistance of the whole plants by reducing water evaporation of the axillary buds (**a**). On the contrary, after a long-term in vitro culture and the transplanting, the pot plants under non-drought stress (80% FC) do not produce visible branch-thorns (**b**). *Hyper* hypermethylation, *Hypo* hypomethylation, *TP* thorn primordium, *AM* axillary meristem

but those under the condition of 60% and 40% FC developed visible thorns on both the complete-thorn branches (54.77%) and the partial-thorn branches (45.23%). Paraffin sections and SEM showed that thorn primordium but not developing thorns was found in the thornless plants; thorns of *L. ruthenicum* are belonging to the category of branch-thorns and the task of thorns seem to be covering and protecting the axillary buds. MSAP analyses indicated that branch-thorn absence of *L. ruthenicum* clone was correlated with low level of global DNA methylation in the leaf and a specific MSAP marker of hypomethylation. Meanwhile, the visible branch-thorn of *L. ruthenicum* clone was related to high level of global DNA methylation in leaf and a specific MSAP marker of hypermethylation. Locus-specific methylation modification of leaf was highly correlated with occurrence of the branch-thorn at the same stem node and the thorny and thornless plants of *L. ruthenicum* were distinguished successfully based on the MSAP profiles. Taken together, the *L. ruthenicum* clone showed thorn-phenotypic plasticity response to soil water content and the plasticity is correlated with DNA methylation of CCGG sites. It was proposed that the permanent thornless plants will appear soon in *L. ruthenicum* population under the condition of sufficient soil water. This is the first report that supports three-way link among soil water content, occurrence of branch-thorn and DNA methylation modification of plant. In addition, a hypothesis model of the *L. ruthenicum* thorn-phenotypic plasticity was proposed in the study, which not only provides foundation for revealing the epigenetic mechanism of branch-thorn phenotypic plasticity response to soil water content but also shines plant evolution.

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Data availability The datasets used and/or analyzed during the current study are all in the supplementary materials.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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