

Increased population epigenetic diversity of the clonal invasive species *Alternanthera philoxeroides* in response to salinity stress

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Epigenetic modification can change the pattern of gene expression without altering the underlying DNA sequence, which may be adaptive in clonal plant species. In this study, we used MSAP (methylation-sensitive amplification polymorphism) to examine epigenetic variation in *Alternanthera philoxeroides*, a clonal invasive species, in response to salinity stress. We found that salinity stress could significantly increase the level of epigenetic diversity within a population. This effect increased with increasing stress duration and was specific to particular genotypes. In addition, the epigenetic modification of young plants seems less sensitive to salinity than that of mature plants. This elevated epigenetic diversity in response to environmental stress may compensate for genetic impoverishment and contribute to evolutionary potential in clonal species.

Key words: DNA methylation, salinity stress, developmental stage, treatment duration, alligator weed

INTRODUCTION

Epigenetic modification is the covalent modification of DNA and chromatin, and can influence gene expression without altering the underlying DNA sequence (Holliday, 2006; Richards, 2006; Jones, 2012). Genome-wide patterns of epigenetic modification change dynamically and coordinately at defined stages of development (Feng et al., 2010; Law and Jacobsen, 2010; Feil and Fraga, 2012; Hu et al., 2014). In natural populations, epigenetic variation among individuals is a significant source of phenotypic variance within and among populations (Medrano et al., 2014; Kooke et al., 2015; Liu et al., 2015; Foust et al., 2016). Recently, epigenetics has attracted considerable interest in plant ecological genetics because it can affect ecological-adaptive potential in plant species. Numerous studies have provided new insights into epigenetic responses to environmental stimuli (Labra et al., 2002;

Aina et al., 2004; Downen et al., 2012; Sani et al., 2013; Radford et al., 2014). It has been proposed that plants can adjust epigenetic modification in response to stress, which could be adaptive as it may generate phenotypic variation by changing gene expression (Castonguay and Angers, 2012; Douhovnikoff and Dodd, 2014).

DNA methylation is an important type of epigenetic modification, and frequently occurs at position 5 of the cytosine ring, converting cytosine to 5-methylcytosine (Sahu et al., 2013). Stress-induced methylation rearrangement has been proposed to contribute to coping with severe environmental stress in plants (Angers et al., 2010; Zhang et al., 2010; Downen et al., 2012; Radford et al., 2014). For example, in the model species *Arabidopsis thaliana*, high salinity induced global hypermethylation at CG sites (Wibowo et al., 2016), and the methylation level at CHH sites (where H is A, C or T) was found to increase at higher temperature (Dubin et al., 2015). In non-model plants, environmental stimuli such as drought (Labra et al., 2002), heavy metals (Aina et al., 2004) and cold stress (Steward et al., 2002) also caused a change in methylation level throughout the genome and at specific loci. These stress-induced methylation changes may

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be targeted specifically to a certain stress-related signal pathway, or to stress-related genes, thus leading to a set of defense responses to resist environmental stress (Wada et al., 2004; Choi and Sano, 2007; Secco et al., 2015; Wibowo et al., 2016). Additionally, methylation changes may raise the probability of nonspecific epimutations and increase the epigenetic diversity among individuals under stress. Similar to DNA mutation, increased epimutations may have adaptive significance because they broaden the range of variation that natural selection can act upon (Verhoeven et al., 2010). As a result, environmentally induced methylation changes, whether or not they target functional genes, may play an important role in the plant stress response.

The complex interactions between DNA sequence and DNA methylation make it difficult to separate epigenetic from genetic variation in genetically diverse populations (Bossdorf et al., 2008). However, detecting independent epigenetic variation is less complicated in populations that lack genetic variation, or in asexual lineages (Verhoeven et al., 2010). For asexual lineages, their adaptive potential to environmental change tends to be severely limited because of the lack of genetic variation that is normally associated with sexual reproduction (Verhoeven et al., 2010). It is thus surprising that asexual reproduction is widespread in plants, some of which are successful invasive species that grow in broad geographic areas with heterogeneous environments and show considerable tolerance to environmental stress (Silvertown, 2008). There are indications that clonal plants, especially the clonal invasive species, may have compensatory mechanisms to generate phenotypic variation, for instance via epigenetic modification. In addition, epigenetic variation may accumulate rapidly across generations in asexual clones due to the absence of genetic recombination. Stable epigenetic variation can generate heritable phenotypic variation, allowing plants to respond to environmental challenges (Castonguay and Angers, 2012; Douhovnikoff and Dodd, 2014; Verhoeven and Preite, 2014). Furthermore, epigenetic variation may be particularly important to the evolutionary potential of asexual species if the adaptive epigenetic variation can be stably transmitted across generations (Bossdorf et al., 2008; Kronholm and Collins, 2016).

Alligator weed, *Alternanthera philoxeroides*, is an invasive clonal plant and has been considered as an ideal species for ecological epigenetics, in which epigenetic variation can be studied independently from genetic variation. Alligator weed is native to South America (Julien, 1995) but has now become an invasive species in many countries, causing huge economic loss and ecological damage in the invaded areas (Wang and Wang, 1988). Natural populations in China are dominated by asexual reproduction (Sainty et al., 1997; Sosa et al., 2008) and are highly genetically uniform (Geng et al., 2016). Despite their

uniform genetic background, alligator weed populations have developed high adaptability and vigorous expansion capability. Individuals not only can grow under different water gradients (Geng et al., 2007), but also are tolerant of extreme climatic variables (Julien, 1995), high salinity (Longstreth et al., 2004), heavy metals (Naqvi and Rizvi, 2000) and herbicides (Eberbach and Bowmer, 1995). It is therefore presumed that epigenetic variation plays an important role for alligator weed adapting to highly heterogeneous habitats and stress conditions.

In this study, we used alligator weed as a model to investigate epigenetic variation in response to salinity stress. Both seedlings and mature plants of different genotypes were exposed to high salinity to evaluate epigenetic modification in response to environmental stress. Amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) markers were used to assess genetic variation and methylation variation, respectively. Three key questions of this study are as follows. First, can salinity stress induce significant DNA methylation changes? Second, if so, are these changes consistent across different genotypes? Third, how are epigenetic changes affected by stress duration and plant developmental stage (i.e., young and mature individuals)?

MATERIALS AND METHODS

Plant material Alligator weed is a clonal plant species and most individuals in China share one single multi-locus genotype. However, there is considerable genetic diversity within natural populations in other areas (e.g., the USA and Argentina) (Geng et al., 2016). In this study, we used six genotypes, namely KM, N20, N23, N26, N28 and N25, of which KM was collected from Kunming, China and the other five from the USA (Geng et al., 2016). All six genotypes were cultivated in the laboratory.

Experimental design The aim of this study was to examine the effect of salinity stress on epigenetic variation in different genotypes of alligator weed. We also wanted to know whether any such effect depends on plant developmental stage. Thus, we used a factorial design with three factors: genotype (six genotypes), salinity stress (treatment vs. control) and plant developmental age (young vs. mature plants). There were five replications for each treatment combination, totally including 120 plant individuals. This experiment was performed in Yunnan University (E102°42'31", N25°3'37"), Kunming. First, clonal offspring for each genotype were derived from short stem-fragments of mother plants, and grown in plastic pots (21 cm diameter × 15 cm height) with a homogeneous mixture of vermiculite and sand (1:1). Each pot was fertilized with 4 g of commercial slow-release compound fertilizer containing 15:11:13 of N/P/K

(Osmocote, Scotts Company, Marysville, OH, USA). The pots were labeled and then watered with tap water every two days. Next, when the young seedlings were established and had produced two or three pairs of leaves, 20 healthy individuals for each genotype (120 individuals in total) were randomly assigned to four different treatment groups (i.e., Control-Young, Stress-Young, Stress-Mature and Control-Mature). The plants in Control groups were watered as above with tap water throughout the experiment, while the plants in the Stress-Young group were exposed to salinity stress from the very beginning of the experiment by adding 200 ml NaCl solution (0.2 M) every two days. In contrast, the plants in the Stress-Mature group were grown under control conditions for one month (i.e., until they became mature plants) and then were switched to salinity stress conditions. The salinity stress lasted for 30 days, and then all plants were switched to control conditions for one week. Newly produced and fully expanded leaves were collected for epigenetic analysis. After sampling, all plants were harvested at the end of the experiment. Each plant was separated into above-ground and below-ground parts, which were oven-dried at 80 °C for 48 h and then weighed for dry mass.

Genetic and epigenetic DNA sampling For genetic background analysis, two leaves were collected and dried in sealed plastic bags with silica gel. For epigenetic analyses, leaves were collected from the Young groups and Mature groups under different schedules. Specifically, for the Young groups, epigenetic samples were collected from newly produced leaves after 30 days of stress duration. For the Mature groups, epigenetic samples were collected at two time points (i.e., seven and 30 days after the beginning of stress treatment), resulting in two levels of stress duration. Altogether, we had 30 samples for genetic analysis (6 genotypes $\times$ 5 offspring replications) and 180 samples for epigenetic analysis (6 genotypes $\times$ 3 stress levels (Stress-Young-30-Day, Stress-Mature-7-Day, and Stress-Mature-30-Day) $\times$ 2 treatments (stress and control) $\times$ 5 replications). There were 210 DNA samples in total.

AFLP and MSAP analysis Total genomic DNA of each sample was extracted with the TIANGEN (Beijing, China) Plant Genomic DNA Kit following the manufacturer's protocol. The AFLP technique was used to determine the genetic background information for each genotype. The process was performed according to the method of Vos et al. (1995) with some modifications (Gao et al., 2010). Nine EcoRI/MseI primer combinations were used for fragment amplification: AGG/CAA, AGC/CAA, AAC/CTT, ACA/CTA, CAA/CAT, AGC/CTT, AGC/CTA, AGG/CTT, AGG/CAT. The final PCR products were separated on 6% denaturing polyacrylamide gels, which were then silver-stained and scanned for further data

scoring.

MSAP fingerprinting was used to examine the epigenetic variation of alligator weed in response to salinity stress. MSAP analysis followed the protocol described by Portis et al. (2004), which is similar to AFLP but the 'frequent cutter' MseI was replaced by methylation-sensitive restriction enzymes HpaII and MspI. These isoschizomers both cleave 5'-CCGG sequences but have different sensitivities to the methylation patterns of cytosine (Schulz et al., 2013): HpaII can recognize the methylated external cytosine on a single strand but cannot cut sequences with methylated cytosines on both strands, whereas MspI can cleave the sequence with methylated internal cytosines but cannot cut if the external cytosine is methylated. Consequently, different patterns of fragments appear on the polyacrylamide gels, indicating the epigenetic variation among individuals.

Ten EcoRI + HpaII/EcoRI + MspI primer combinations were selected to conduct MSAP amplification: AAG/TCC, ACA/TCG, ACT/TCT, ACC/TGA, AGA/TTC, AGG/TTG, AAC/TCT, AAG/TTC, AAC/TGA, AAC/TCAA. Amplified fragments were separated on 6% sequencing gels and silver-stained. To assess reproducibility and minimize experimental error, MSAP reactions were repeated twice for each sample using independent DNA isolations.

Data analysis For AFLP and MSAP results, the sequencing gels were inspected visually and fragments were scored as present (1) and absent (0) in the data sets. Visually poor-quality samples were excluded from scoring and only reproducible fragments were scored. All scoring was performed by the same person in the absence of information on sample identities. The AFLP results were scored as a binary matrix following a conventional protocol. The MSAP results were somewhat more complicated. The status of each MSAP site was determined by comparing the EcoRI/HpaII and EcoRI/MspI fragment profiles: fragments are present in both profiles (epi-loci type:11), fragments are present in the EcoRI/HpaII profile but absent from EcoRI/MspI (epi-loci type: 10), fragments are present in EcoRI/MspI but absent from EcoRI/HpaII (epi-loci type: 01), and fragments are absent from both profiles (epi-loci type: 00). The first three conditions represent different methylation status while the last indicates a complicated state that could reflect either methylation variation or DNA sequence mutation. Here, we used clonal offspring as the plant material, and presumed that there was no genetic variation within each genotype. Accordingly, we included the last fragments status (epi-loci type: 00) as part of the data sets and considered these fragments as methylated. The raw MSAP data were transformed into a binary matrix for further analysis using the 'Mixed Scoring 2' method (Schulz et al., 2013), which is based on R statistical software (version 3.2.3, R Core Team, 2015, <http://cran.r-project.org/>)

src/base/R-3/R-3.2.3.tar.gz).

The binary data sets from AFLP and MSAP were analyzed in GenALEx6.59 (Peakall and Smouse, 2012). Genetic diversity and epigenetic diversity within each genotype $\times$ treatment combination were calculated, including the percentage of polymorphic loci, Shannon's diversity index and Nei's unbiased gene diversity (uHe). Hierarchical AMOVA was performed to test the significance of epigenetic differentiation among treatments and genotypes in each group (i.e., Young-30-Day; Mature-7-Day and Mature-30-Day). In the AMOVA calculation, the probability of non-differentiation ($\Phi_{PT} = 0$) was estimated over 9,999 permutations. To investigate the effect of stress on methylation status, the fragment patterns were compared between the stressed plants and the control plants within and among genotypes based on the raw MSAP data sets.

Shannon's diversity index of MSAP data, and above-ground and below-ground biomass data, was analyzed using two-way ANOVA with two main factors, salinity

stress (two levels) and genotypes (six levels), in order to test the influence of salinity stress on epigenetic diversity and plant growth. Means of treatments were compared using Tukey's HSD tests at a 5% probability. The biomass data were log-transformed if necessary to meet assumptions of homoscedasticity, and all data were analyzed with R statistical software (version 3.2.3, R Core Team, 2015).

RESULTS

Genetic variation within and among genotypes A total of 563 AFLP bands were scored from the nine primer combinations. The AFLP results showed that each genotype had a unique multi-locus profile. In contrast, for each genotype, the five clonal offspring were genetically uniform, as revealed by AFLP markers. Specifically, Nei's index of genetic diversity (uHe) and Shannon's diversity index within each genotype were close to zero at the genotype level (Supplementary Table S1). These

Table 1. Results of analysis of variance (ANOVA) of *Alternanthera philoxeroides* showing the effect of stress treatment (salinity stressed, control) and genotype on Shannon's diversity index, above-ground biomass and below-ground biomass in young and mature plants (treated for seven and 30 days)

Group	Effects	df	SS	MS	F value	P value
<i>Shannon's diversity index</i>						
Stress-Young-30-Day	Stress treatment	1	0.064	0.064	29.458	0.000
	Genotype	5	0.199	0.040	18.406	0.000
	Stress $\times$ genotype	5	0.097	0.019	9.011	0.000
Stress-Mature-7-Day	Stress treatment	1	0.151	0.151	54.821	0.000
	Genotype	5	0.067	0.013	4.844	0.000
	Stress $\times$ genotype	5	0.037	0.007	2.716	0.019
Stress-Mature-30-Day	Stress treatment	1	1.550	1.550	229.704	0.000
	Genotype	5	0.103	0.021	3.045	0.009
	Stress $\times$ genotype	5	0.115	0.023	3.403	0.004
<i>Above-ground biomass</i>						
Stress-Young-30-Day	Stress treatment	1	13.727	13.727	352.351	0.000
	Genotype	5	6.466	1.293	33.195	0.000
	Stress $\times$ genotype	5	0.930	0.186	4.776	0.004
Stress-Mature-30-Day	Stress treatment	1	6.460	6.460	104.092	0.000
	Genotype	5	3.759	0.752	12.115	0.000
	Stress $\times$ genotype	5	0.851	0.170	2.741	0.043
<i>Below-ground biomass</i>						
Stress-Young-30-Day	Stress treatment	1	4.862	4.862	382.418	0.000
	Genotype	5	4.963	0.993	78.079	0.000
	Stress $\times$ genotype	5	0.182	0.037	2.868	0.036
Stress-Mature-30-Day	Stress treatment	1	0.828	0.828	22.960	0.000
	Genotype	5	5.111	1.022	28.340	0.000
	Stress $\times$ genotype	5	0.071	0.014	0.396	0.847

data indicated that the clonal offspring of each genotype were genetically homogeneous and that all MSAP polymorphisms within genotypes can be interpreted as true epigenetic variation rather than genetic mutation.

Effect of salinity stress Salinity stress had a significant effect on plant growth (i.e., biomass) and methylation variation in alligator weed. Both above-ground and below-ground biomass were significantly decreased by salinity stress (Table 1, Fig. 1). The raw MSAP data were used to compare the methylation variation between control and stress groups. We observed obvious changes in methylation status in all genotypes, with the propor-

tion of changed loci ranging from 6.28% to 8.91%. We had expected that salinity stress might induce reproducible changes in certain epigenetic loci that are involved in functional gene expression of stress resistance-related pathways. Both methylation and demethylation occurred in stress groups compared to control groups; however, we did not find consistent epigenetic modification changes at such loci across different individuals even within the same genotype (Fig. 2). In contrast, we found significantly higher levels of epigenetic diversity within genotypes in stress groups (Fig. 3, Table 2).

Similar results were found in AMOVA analysis, which suggested that a large proportion of epigenetic variation

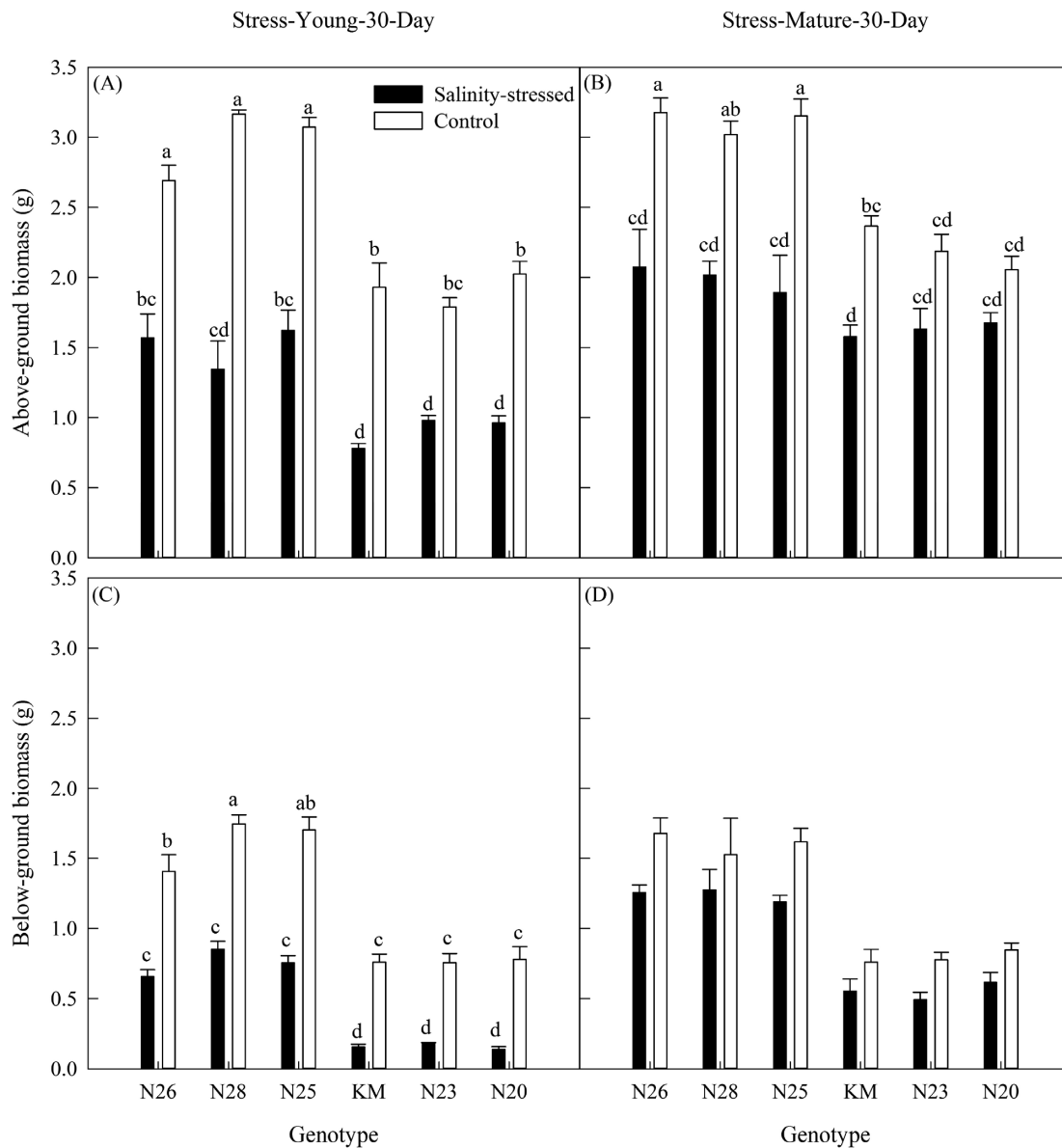


Fig. 1. Above- and below-ground biomass of young plants and mature plants. Values represent means $\pm$ SE ($n = 5$). Different superscript letters indicate means that are significantly different at $P < 0.05$ based on Tukey's pair-wise comparisons. Two means that have the same letter, or no superscript letters, are not significantly different from each other at the 5% significance level.

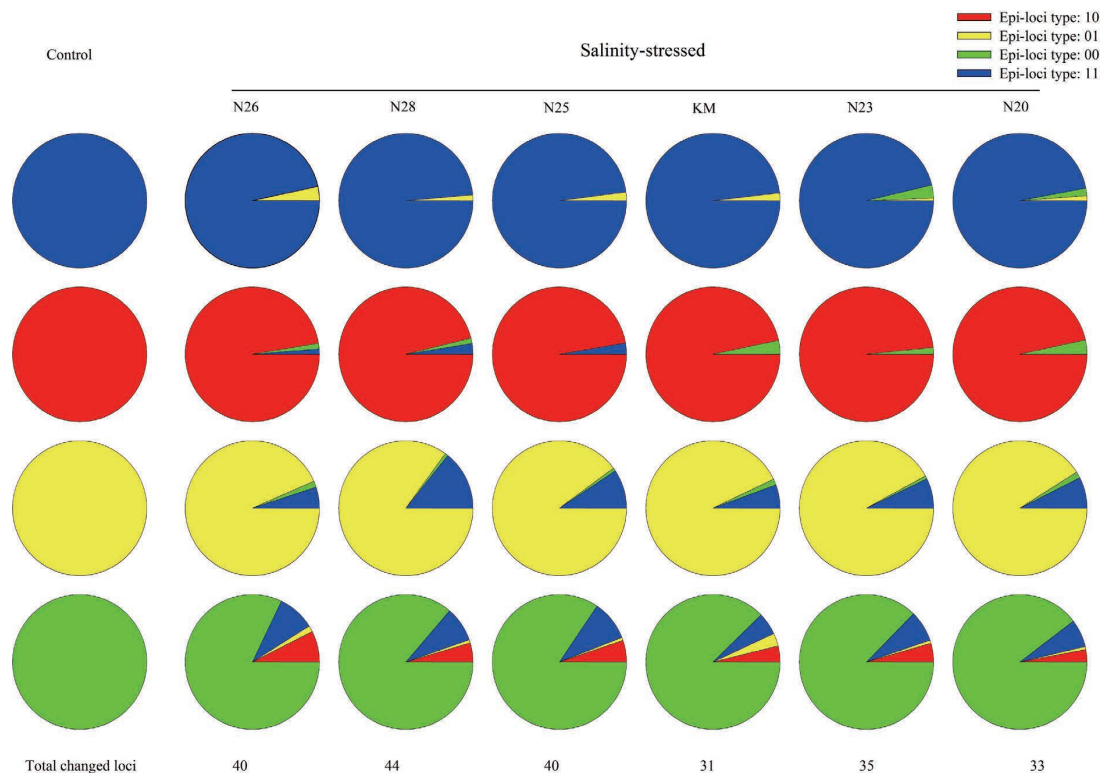


Fig. 2. Methylation status change after salinity stress in mature plants treated for 30 days. The color code indicates the different methylation states (epi-loci type of MSAP profile). We only show the proportion of each epi-loci type; the percentage of epi-loci types in control plants was 100%. Note that the numbers of loci were variable in each genotype.

resided among different treatments rather than different genotypes within a treatment (Table 3). Additionally, the effect of salinity stress on epigenetic variation increased with increasing stress duration: across all genotypes, longer stress duration produced higher epigenetic diversity in the stressed plants. On average, plants that were treated for 30 days had higher epigenetic diversity indices (percentage of polymorphic loci, uHe and Shannon's diversity index) than those that were treated for seven days (Table 2).

Effect of plant developmental stage We found that epigenetic modification in young plants was less sensitive to salinity stress than that in mature plants. AMOVA results suggested that salinity stress had no significant effect on the structuring of epigenetic diversity in young plants (Table 3). The percentage of polymorphic loci, Nei's diversity index and Shannon's diversity index of the stressed plants ranged from 0.20–4.45%, 0.001–0.015 and 0.001–0.020, respectively, which were similar to the control (Table 2). Tukey's HSD tests on Shannon's diversity index showed that salinity treatment only affected the plants of genotype N26 in the Young groups, and had no effect on the other genotypes (Fig. 3A), which indicated a significant salinity $\times$ genotype interaction (Table 1). In contrast, salinity stress for 30 days significantly

increased Shannon's diversity index across all genotypes in the Mature groups, as described above.

Effect of genotypic diversity In this study, we used six different genotypes to examine epigenetic change in response to salinity stress. We did find some evidence that different genotypes had contrasting sensitivity to salinity stress in plant growth and epigenetic modification. For example, in the Mature groups, high salinity only reduced the above-ground biomass in genotypes N26, N28, N25 and KM (Fig. 1B), but had no significant effect on below-ground biomass ($P = 0.847$) (Fig. 1D). Shannon's diversity indices of both the Stress-7-Day and the Stress-30-Day groups were significantly influenced by genotype and salinity treatment according to two-way ANOVA analysis (Table 1). Stress duration had the greatest effect on plants from genotype N28, while genotype N23 was the least affected by stress duration among all genotypes (Fig. 4). Tukey's HSD tests showed that salinity stress had no effect on genotypes N26 and N23 in the Stress-7-Day group (Fig. 3B), whereas high salinity significantly affected all genotypes in the Stress-30-Day group (Fig. 3C). In addition, genotype N28 was the most variable with 44 methylation sites showing changed patterns, while genotype KM changed the least with 31 altered methylation sites (Table 4).

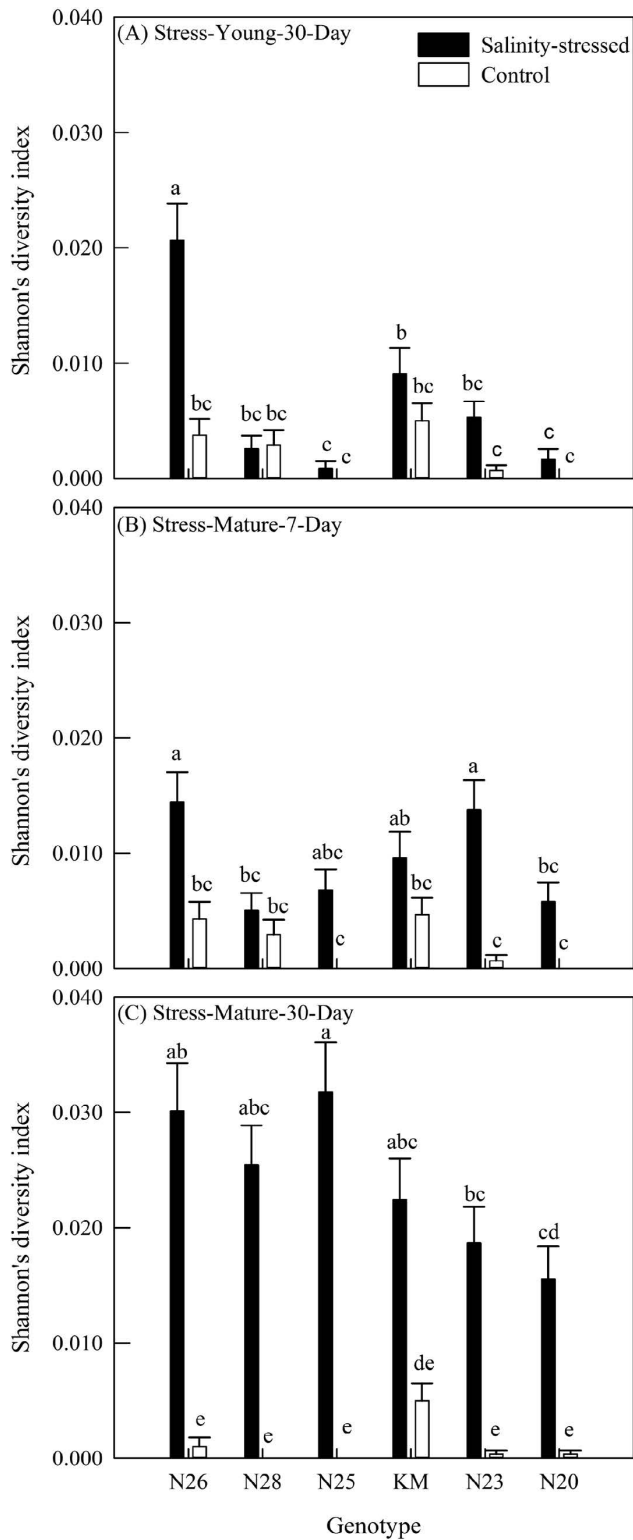


Fig. 3. Shannon's diversity index of *Alternanthera philoxeroides* based on MSAP loci. (A) Stress-Young-30-Day; (B) Stress-Mature-7-Day; and (C) Stress-Mature-30-Day. Values represent means $\pm$ SE (n = 988). Different superscript letters indicate means that are significantly different at $P < 0.05$ based on Tukey's pair-wise comparisons. Two means that have the same letter are not significantly different from each other at the 5% significance level.

Table 2. Epigenetic diversity of each genotype $\times$ treatment (salinity stress, and young/mature plants) based on MSAP loci: Stress-Young-30-Day, Stress-Mature-7-Day and Stress-Mature-30-Day

Genotype	Treatment	Percentage of polymorphic loci	Shannon's diversity index	uHe
<i>Stress-Young-30-Day</i>				
N26	Salinity-treated	4.45	0.021	0.015
	Control	0.81	0.004	0.003
N28	Salinity-treated	0.61	0.003	0.002
	Control	0.51	0.003	0.002
N25	Salinity-treated	0.20	0.001	0.001
	Control	0.00	0.000	0.000
KM	Salinity-treated	1.72	0.009	0.007
	Control	1.21	0.005	0.003
N23	Salinity-treated	1.52	0.005	0.003
	Control	0.20	0.001	0.000
N20	Salinity-treated	0.40	0.002	0.001
	Control	0.00	0.000	0.000
Mean	Salinity-treated	1.48	0.007	0.005
	Control	0.46	0.002	0.001
<i>Stress-Mature-7-Day</i>				
N26	Salinity-treated	3.34	0.014	0.010
	Control	0.91	0.004	0.003
N28	Salinity-treated	1.21	0.005	0.003
	Control	0.51	0.003	0.002
N25	Salinity-treated	1.52	0.007	0.005
	Control	0.00	0.000	0.000
KM	Salinity-treated	1.92	0.010	0.007
	Control	1.11	0.005	0.003
N23	Salinity-treated	3.04	0.014	0.010
	Control	0.20	0.001	0.000
N20	Salinity-treated	1.32	0.006	0.004
	Control	0.00	0.000	0.000
Mean	Salinity-treated	2.06	0.009	0.006
	Control	0.46	0.002	0.001
<i>Stress-Mature-30-Day</i>				
N26	Salinity-treated	5.36	0.030	0.023
	Control	0.20	0.001	0.001
N28	Salinity-treated	5.47	0.025	0.018
	Control	0.00	0.000	0.000
N25	Salinity-treated	5.36	0.032	0.024
	Control	0.00	0.000	0.000
KM	Salinity-treated	4.15	0.022	0.017
	Control	1.21	0.005	0.003
N23	Salinity-treated	3.64	0.019	0.014
	Control	0.10	0.000	0.000
N20	Salinity-treated	3.24	0.016	0.011
	Control	0.10	0.000	0.000
Mean	Salinity-treated	4.54	0.024	0.018
	Control	0.27	0.001	0.001

Table 3. AMOVA results of *Alternanthera philoxeroides* based on MSAP loci

Group	Variance components	Est. var.	% Total	P	Phi-statistics
Stress-Young-30-Day					
Among treatments		0.000	0	0.946	PhiRT = -0.193
Among genotypes within treatment		82.447	98	0.000	PhiPR = 0.976
Within genotypes		2.050	2	0.000	PhiPT = 0.971
Stress-Mature-7-Day					
Among treatments		107.766	85	0.002	PhiRT = 0.850
Among genotypes within treatment		16.220	13	0.000	PhiPR = 0.851
Within genotypes		2.833	2	0.000	PhiPT = 0.978
Stress-Mature-30-Day					
Among treatments		107.593	81	0.002	PhiRT = 0.814
Among genotypes within treatment		18.686	14	0.000	PhiPR = 0.762
Within genotypes		5.850	4	0.000	PhiPT = 0.956

Table 4. Numbers and percentage of changed and stable epi-loci after salinity treatment in mature plants treated for 30 days

Genotype	Total cases	Changed epi-loci	Changed %	Stable epi-loci	Stable %
N26	494	40	8.10	454	91.90
N28	494	44	8.91	450	91.09
N25	494	40	8.10	454	91.90
KM	494	31	6.28	463	93.72
N23	494	35	7.09	459	92.91
N20	494	33	6.68	461	93.32

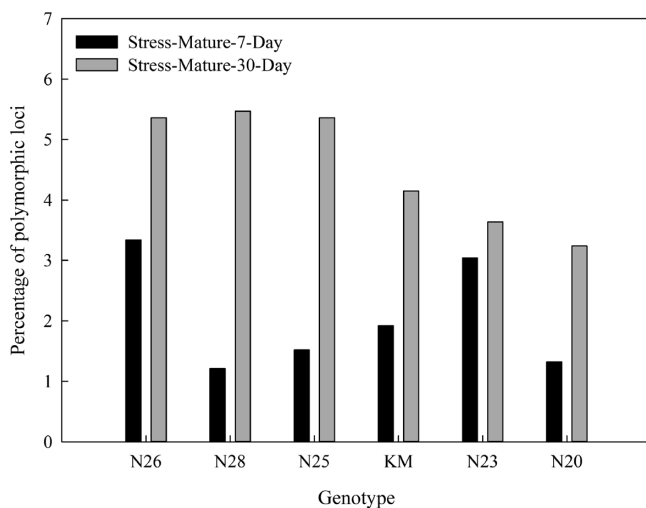


Fig. 4. Percentage of polymorphic loci in mature plants treated for seven days and 30 days.

DISCUSSION

In this study, we examined the effect of salinity stress on epigenetic variation in the clonal plant alligator weed. Although the effect of salinity stress is qualita-

tively variable depending on the specific genotype, stress duration and plant developmental stage, a clear and consistent pattern is that salinity stress produced significantly higher epigenetic diversity among clonal offspring within genotypes. This effect was more obvious in mature plants than young plants, and increased with prolonged stress duration. Given the limited genetic variation in this clonal plant, our results suggested that elevated epigenetic diversity in response to environmental stress compensates for genetic impoverishment and contributes to evolutionary potential in alligator weed.

Effect of salinity stress on epigenetic variation We found that salinity stress could induce methylation alterations in alligator weed, which is consistent with previous studies in other plant species (Labra et al., 2002; Mason et al., 2008; Bonasio et al., 2010). In rice (*Oryza sativa*), salt-induced methylation changes were detected in roots in both salt-sensitive and salt-tolerant genotypes. Most methylation variation remained even after recovery, implying its stability in the present generation (Wang et al., 2015). After stressing *Jatropha* seedlings (*Jatropha curcas* L.) by different concentrations of NaCl solution, Mastan et al. (2012) found global hypermethylation in both roots and leaves, and an increased methylation polymorphism level. In addition, methylation changes have been reported in response to heavy metals (Aina et al., 2004), cold temperature (Xie et al., 2015), viral infection (Choi and Sano, 2007; Mason et al., 2008) and water deficit (Verkest et al., 2015). Methylation alterations detected under stress may be beneficial for plants coping with environmental changes as the methylation difference induced by stress may represent candidate epigenetic markers, which previously have been suggested to be linked to plastic phenotypic variation (Lukens and Zhan, 2007; Gao et al., 2010). Phenotypic differences between individuals may have adaptive significance by enabling a

population to maintain itself in a heterogeneous environment.

Our study innovatively found significantly higher levels of epigenetic diversity in stressed plants within genotypes. These epigenetic changes were not consistent across different individuals or genotypes; in other words, they were not occurring at specific loci. It seems that the elevated epigenetic diversity may instead result from random permutation. DNA methylation in plants is known to be maintained and modulated by molecular machines including several enzymes like MET, CMT, DNMT2 and DRM (Choi and Sano, 2007; Furner and Matzke, 2011). A possible explanation is that salinity stress interferes with the activity of these functional enzymes, resulting in a higher epimutation rate. This stress-epimutation hypothesis is supported by another finding in our data. Specifically, we found that the effect of salinity stress on epigenetic changes was reinforced by extended stress duration. The higher epigenetic diversity in long-term stress (i.e., 30 days) may result from the progressive accumulation of epimutations during DNA replication. These stress-induced random epigenetic modifications may persist or spread with somatic cell proliferation, resulting in higher levels of epigenetic diversity within genotypes in long-term stress duration. Researchers have reported that increasing genome methylation results in the inhibition of expression of some genes and reduces energy consumption to maintain growth and development (Kovalchuk et al., 2004). Although these random epigenetic modifications are not necessarily associated with salinity resistance-related pathways, they may act like random genetic mutations in adaptive evolution. As there is nearly no genetic differentiation in alligator weed in China, the observed epigenetic diversity in a stressful environment may compensate for genetic impoverishment, contributing to its evolutionary potential and thus guaranteeing vigorous invasiveness in this invasive species (Schlichting, 1986; Sultan, 2004).

Effect of plant developmental stage Our results confirmed that plants at different developmental stages had differential tolerance to stress. Generally, the epigenetic diversity within genotypes seems less sensitive to salinity stress in young seedlings than in mature plants. This pattern could also be explained by the stress-epimutation hypothesis. As the young seedlings were relatively small in size, the salinity stress would have had a stronger effect on plant growth in young seedlings than in mature plants. For example, salinity stress decreased greatly both the above-ground and the below-ground biomass of young seedlings (Fig. 1A and 1C). In contrast, plant growth was more tolerant to stress in mature plants. Thus, in the same period of stress duration (i.e., 30 days), the number of cell divisions

would be much lower in young seedlings than in mature plants. It is therefore not surprising that we observed a lower accumulation of epigenetic change in young seedlings, producing different sensitivity to salinity stress at different plant developmental stages.

Different responses among genotypes We observed different sensitivity to salinity stress among six genotypes and the stress-induced epigenetic diversity increased only in stressed seedlings of genotype N26, while plants of all genotypes showed a significant elevation of epigenetic diversity in the Stress-Mature group. The methylation status changed inconsistently at some loci across different individuals. Such differences are probably related to the genetic variation between genotypes. As the activity of DNA methyltransferase is usually regulated by gene expression, methyltransferase may have varied activities in different genotypes, leading to diverse methylation variation in response to stress. Furthermore, we found that methylation status differed between genotypes at certain loci. If these epigenetic differences could be stably maintained when the stress was eliminated (Verhoeven et al., 2010; Wang et al., 2015) and transmitted across generations (Crews et al., 2007; Boyko and Kovalchuk, 2008), they would represent candidate epigenetic alleles that could influence the evolutionary trajectory between two closely related genotypes. For clonal reproductive species, epigenetic variation between genotypes is especially interesting because it potentially provides new and independent heritable phenotypic variation, opening a potential new pathway of rapid evolution even without DNA sequence variation (Bossdorf et al., 2008).

Implications for adaptive evolution of alligator weed Epigenetic variation is considered to play a vital role in population evolution because of its higher mutation rate than genetic variation (Ossowski et al., 2010; Becker et al., 2011; Schmitz et al., 2011) and its capacity to regulate gene expression. Populations established by asexual reproduction are often characterized by extremely low levels of genetic diversity within and among populations, which may limit their adaptive potential in fluctuating environments. Diverse epigenetic variation may compensate for low genetic variation and contribute to adaption in asexually reproducing populations. If epigenetic variation accumulates rapidly in asexual populations, thereby producing heritable phenotypic variation that is independent of genetic variation, the evolutionary constraints in genetically impoverished populations might be alleviated significantly. In other words, epigenetic variation may have important impacts on clonal populations. Our results on epigenetic variation of alligator weed in response to different salinity treatments support this hypothesis. For alligator weed, flexible epigenetic variation is particularly important during plant

development. The adaptive potential in novel and heterogeneous environments, which may be closely related to epigenetic variation, could be one of the reasons that alligator weed, with its low genetic diversity, expanded rapidly in the areas it invaded.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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