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Phenotypic plasticity of *Thellungiella salsaginea* in response to saline stress

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ABSTRACT

Background: Theoretical and empirical studies have shown that phenotypic plasticity can contribute to plant fitness by augmenting the ability of a plant to adapt to or tolerate novel conditions. Genetic analysis of *Thellungiella salsaginea* (salt cress, Brassicaceae) with neutral nuclear markers revealed no genetic variation within or among populations despite the wide variety of phenotypes in this species. Phenotypic variation is likely due to plasticity.

Goal: To examine the characteristics of phenotypic plasticity and its intrinsic constraint (i.e. trait integration) of several phenotypic traits in response to salt stress in populations of *T. salsaginea* with a homogeneous, neutral genetic background.

Organism: *Thellungiella salsaginea* is a halophyte that is widely distributed in the saline regions of northern China.

Methods: Seeds were collected from seven natural populations at distantly separated locations along a saline gradient. Plants were grown from seeds in a growth chamber, and growth and physiological traits were measured under conditions of saline stress. Experiments were performed in a greenhouse at Lanzhou University, China.

Results: All traits exhibited considerable plasticity in response to the various levels of salinity. Moreover, various patterns in plasticity were found among the seven populations. The degrees of trait integration were relatively low (mean difference of the correlation coefficients = 0.711). Moreover, the integration patterns varied between individual traits and treatments among populations. Thus, the ability of this species to adapt should be attributed to high plasticity and low integration, as well as to the various patterns of plasticity and integration among populations, which probably resulted from epigenetic changes among the populations.

Keywords: growing traits, phenotypic integration, phenotypic plasticity, physiological traits, population, *Thellungiella salsaginea*.

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INTRODUCTION

Phenotypic plasticity in plants is a quality that gives rise to a range of alternative morphological phenotypes and physiological variation in response to different environmental conditions (Schlichting and Levin, 1986; West-Eberhard, 1989; Poorter and Nagel, 2000; Stenstrom *et al.*, 2002; Griffith and Sultan, 2005). Differences in phenotypic plasticity among plant species are related to differences in their ecological distributions and fitness for survival in diverse habitats (Cordell *et al.*, 1998; Sultan *et al.*, 1998; Valladares *et al.*, 2000). Theory also predicts that natural selection, acting on genetic variation in a species that occupies different habitats, affects the phenotypic plasticity within and between populations (Schlichting and Levin, 1986; Poorter and Nagel, 2000; Stenstrom *et al.*, 2002; Griffith and Sultan, 2005). This has been confirmed in controlled experiments comparing phenotypic plasticity among populations of a widely distributed species (Ryser and Eek, 2000; Weinig, 2000). Thus, species that can inhabit diverse environments may increase their fitness locally during the adaptation of populations to specific levels of biotic and abiotic stress. Locally adapted traits are then observed in species with a wider space scale as their phenotypic plasticity, with the mean values of specific traits differing between populations (Edelist *et al.*, 2006; Richards *et al.*, 2006; Muth and Pigliucci, 2007; Lowry *et al.*, 2009).

The patterns of correlation among a suite of plastic traits (trait integration) could influence the phenotypic responses of plant species to various environments because different traits often respond to the same environmental stimuli in different ways (Pigliucci, 2003). Recently, it has been argued that trait integration could be an internal constraint to phenotypic plasticity (Schlichting, 1989; Gianoli and Palacio-López, 2009). The variation in phenotypic integration among populations may result in differential selection pressures on particular traits and plant fitness (Schlichting, 1989). This condition might eventually lead to patterns of phenotypic plasticity of individual traits and genetic differentiation among populations being highly correlated (Schlichting, 1989; Valladares *et al.*, 2000; Elberse *et al.*, 2003; Bloor and Grubb, 2004; Sardans *et al.*, 2006; Nicotra *et al.*, 2007; Gianoli and Palacio-López, 2009). Similarly, trait integration has been found to be significantly different among populations whose phenotypes are differentiated (Schlichting, 1989; Sardans *et al.*, 2006; Nicotra *et al.*, 2007). Despite some recent research (Reimann and Breckle, 1995; Rosenthal *et al.*, 2002; Lexer *et al.*, 2003; Karrenberg *et al.*, 2006; Nicotra *et al.*, 2007; Richards *et al.*, 2008, 2010, 2012; Walls, 2010), few studies have addressed inter-population phenotypic plasticity and phenotypic integration of plants.

In the present study, salt cress, *Thellungiella salsaginea* (Brassicaceae), was used as a model species to quantify plasticity in growth and physiological traits in response to salt stress. Phenotypic integration among populations sampled from a natural saline gradient was also analysed. *Thellungiella salsaginea* has good sodium/potassium homeostasis, which allows it to accumulate less sodium and retain more potassium (Inan *et al.*, 2004; Volkov and Amtmann, 2006). It has been used as a model species in the study of the molecular and physiological mechanisms of plants (Bressan *et al.*, 2001; Zhu, 2001; Volkov and Amtmann, 2006). It is widely distributed throughout central Asia, northern China, and North America, where it occupies diverse saline habitats (Zhou *et al.*, 1987). The morphology of this species varies greatly with respect to the basal branches of the main stem, leaf shape, and capsule length (Zhou *et al.*, 1987). However, the different populations may have resulted from a very recent expansion because genetic analysis with neutral nuclear markers has revealed no distinct between-population differences (B.Q. Yao *et al.*, unpublished data).

The aim of the present study was to determine whether this species adapted to novel conditions because of phenotypic plasticity. We wished to address three questions by

measuring the phenotypic variation of seven populations collected from geographically distinct habitats along a salt stress gradient. First, do *T. salsaginea* populations differ in growth and physiological traits in response to variations in saline stress? If so, are the traits and the degree of phenotypic plasticity correlated with environmental variables across the geographic range from which the populations were drawn. Second, do the patterns of trait integration (both within and among growing and physiological trait groups) differ among populations? Third, we test for the presence of significant correlations between the main plastic traits and whole-plant biomass [a possible proxy for fitness (e.g. Tucić and Stojković, 2001; Schlichting and Smith, 2002; Johnston *et al*., 2004)] because any such links may indicate potential trends in the selective changes that plastic traits within this species might undergo (e.g. Elberse *et al*., 2003).

MATERIALS AND METHODS

Study species and populations

Thellungiella salsaginea is widely distributed in the saline regions in northern China (Zhao and Li, 1999). Leaf shape, branch and capsule length vary greatly within and among natural populations (B.Q. Yao *et al*., unpublished data). However, morphological variation in natural populations is difficult to quantify because their growth stages and ecological habitats are usually inconsistent. We therefore collected seeds from seven populations; each population consisted of at least five individuals. The populations were sampled from mild, semi-arid, and arid regions between 114°25.399′E and 118°22.188′E longitude, and 34°49.064′N and 39°21.079′N latitude. The sampled habitats (all loam soil) differed with respect to soil salinity, although most were characterized by high salinity (mean $EC_{1:5} > 1\ dS \cdot m^{-1}$) (Table 1). The second-generation seeds collected from plants that had been grown from the original seed collected from the field were used to avoid maternal effects of local environmental factors in the experimental process. Thus, plants from the seeds of each individual from each local population were maintained separately in growth chambers until the second generation.

Experimental design

The experiment was conducted in growth chambers from April to June 2008. The plants in growth chambers were exposed to different levels of saline stress while all other environmental factors were kept uniform to monitor phenotypic plasticity. A photosynthetic photon flux density (PPFD) of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was provided by cool-white fluorescent bulbs in 16 h photoperiods. Air temperature was maintained between 23°C and 28°C during the day and between 15°C and 18°C at night. Relative humidity fluctuated between 45% and 85%.

The seeds selected from each population were vernalized at 4°C in the dark for 10 days to prevent dormancy and induce synchronous germination. Four seeds were sown into vermiculite in a pot measuring $5 \times 4.8 \times 4.8$ cm. Twenty-five replicate pots were prepared for each treatment and population. Two weeks after germination, the seedlings were thinned to leave one uniform, healthy seedling in each pot. All the plants were watered at 2-day intervals with a nutrient solution containing 1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.625 mM KH_2PO_4, and micronutrients (Arteca and Arteca, 2000).

Table 1. Description of local climate and climatic variability based on data collected from meteorological stations nearest the habitat of *Thellungiella salsaginea*

Province	Population (code)	Latitude, longitude	Altitude (m)	$EC_{1:5}$ ($dS \cdot m^{-1}$)	pH	Mean annual rainfall (mm)	Mean monthly rainfall (mm)		
							Max.	Min.	CV
Tianjin	Wuqing (WQ)	39°21.079′N, 117°03.776′E	1	1.29	7.73	706	216	4	0.92
Hebei	Yanshan (YS)	38°00.983′N, 117°15.728′E	3	8.65	7.03	852	362	6	1.05
	Raoyang (RY)	38°15.419′N, 115°44.920′E	31	1.22	7.30	796	252	8	1.03
Shandong	Dongying (DY)	37°24.849′N, 118°22.188′E	9	4.62	7.40	670	210	5	0.90
	Liaocheng (LC)	36°24.257′N, 116°01.758′E	34	0.28	8.03	419	132	15	0.79
Henan	Lankao (LK)	34°49.064′N, 114°47.462′E	54	2.43	7.60	668	242	12	1.01
	Fengqiu (FQ)	34°57.902′N, 114°25.399′E	77	1.37	7.50	616	205	10	1.02

Note: $EC_{1:5}$ = electrical conductivity of soil (all soils are loam soils measured with a soil suspension at a 1:5 soil-to-water ratio by weight); CV = coefficient of variation of mean values.

In the experimental treatments, the plants were exposed to a simulated salinity gradient comprising three salt concentrations, including a control with no salt: 0 mM NaCl, 300 mM NaCl, and 500 mM NaCl (this last salt concentration is higher than the mean annual salinities of all local habitats of the populations under study, except for populations YS and DY). The salt solution was mixed with other basic nutrient elements and was then applied to the 30-day-old seedlings. With the exception of biomass, all traits were measured on 45-day-old individuals.

Physiological traits

The maximal net photosynthetic rate and stomatal conductance of the most recent fully expanded leaves from five randomly selected individuals were measured at a PPFD of 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a Li-6400 portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA) equipped with a light source (6400–02B LED, LI-COR). The chlorophyll fluorescence values of these individuals were subsequently recorded with a portable modulated fluorometer (FMS-2, Hansatech Instruments Ltd., King's Lynn, Norfolk, UK). After allowing the leaves to adapt to dark for 30 min, the minimal fluorescence value (Fo) was measured by applying a low-intensity 650-nm red light source. The maximal fluorescence value (Fm) was determined by flashing a saturating light pulse of 3500 $mmol \cdot m^{-2} \cdot s^{-1}$. The maximum quantum efficiency was calculated as Fv/Fm = (Fm – Fo)/Fm. The leaf Na^+

and K^+ contents were determined with an atomic absorption spectrophotometer (Hitachi 180-80, Japan) as described by Inan *et al*. (2004).

Morphological and growth traits

Total leaf area, expanded leaf length, fresh and dry weights of leaves, specific leaf area (SLA), and leaf succulence were recorded after measuring the gas exchange parameters. Total leaf area was measured with an LI-3000 area meter (LI-COR). Specific leaf area ($cm^2 \cdot g^{-1}$) was calculated as the ratio of leaf area to leaf dry weight on the same leaf that photosynthesis was measured. Leaf succulence ($H_2O\ g \cdot cm^{-2}$) was calculated as the ratio of leaf water content to leaf area.

The shoot and root biomass was determined on days 30 and 45. All roots, including fine roots, were retained for weighing by soaking pots in water for 5 min. Afterwards, the root mass was washed under running tap water to remove vermiculite granules. The roots and shoots were then dried at 65°C for 72 h to a constant weight. Relative growth rate is expressed by the following equation:

$$\text{relative growth rate} = \ln (W_1 - W_0)/t_1 - t_0,$$

where W_0 and W_1 are the total dried weights of plants at times t_0 and t_1 respectively, and t_0 and t_1 are the ages at harvest (i.e. 30 and 45 days, respectively). The data for all morphological and growth traits, except SLA, were sampled from 15 individual plants randomly selected from the replicates for each treatment.

Statistical analysis

The responses to the treatments were analysed using ANOVA (SAS PROC GLM; SAS Inc., Cary, North Carolina, USA), with populations and salt treatments as random and fixed factors, respectively. The raw data for total leaf area, SLA, and stomatal conductance were log-transformed to normalize the distributions. Redundancy analysis (RDA) (Canoco) was performed to investigate the influence of the local environmental parameters, i.e. mean soil electrical conductivity (EC), pH, and mean annual rainfall (Table 1) on each level of salinity, on the mean of each trait, and its plasticity, respectively.

Comparisons of trait plasticity at the population level in the three salinity treatments were tested by principal components analysis (PCA) of the coefficients of variation (CV; standard deviation/mean) of each trait. Canoco for Windows (v.4.5) (Ter Braak and Smilauer, 1998) was used (Table 2). The trait integration was also assessed (Table 3) as described by Nicotra *et al*. (2007: 139–140). The traits were categorized into growth (G) and physiological (P) trait groups. A 3×3 correlation matrix between the three saline treatment pairs and the traits categorized into three groups (individual and combined trait groups), viz. G, P, and G:P, was constructed for each population (Table 3). For example, the correlation coefficients between the growth trait total biomass and all other traits in the growth group under saline treatments 1 and 2 were calculated. Thereafter, the differences in the correlation coefficients of these treatments were averaged to obtain the mean value, which is entered into the matrix of Table 3 in row 'G', column '1 vs. 2'. Each cell in the matrix thus presents the mean difference of the correlation coefficients of all the members of the trait groups G and P or combined group G:P. Similar matrices were derived for each population. The means of rows and columns in each matrix were also determined. Friedman's rank sum tests were

performed on each matrix and on all the pooled data [SAS PROC FREQ, with the Cochran-Mantel-Haenszel (CMH) statistic] to determine the differences of the correlation coefficients between trait groups and between treatments, respectively. In addition, type I linear regressions (SPSS 13.0) were performed between the total biomass (as an index of fitness) and the trait's root–shoot ratio, stomatal conductance, and SLA of all the measured individuals sampled from the growth chamber.

RESULTS

Plasticity in response to salinity stress

Significant interactions, including the population–salt interaction (ANOVA), indicated that the populations differed in the extent to which trait plasticity responded to saline stress (Table 2). Except for total leaf number, the growth characteristics varied significantly among populations according to the levels of salinity in treatments (Table 2). The total biomass and relative growth rate decreased with an increase in salinity (Fig. 1). The WQ and LK populations, which were exposed to saline stress of 300 mM and 500 mM NaCl respectively, exhibited negative growth rate. This result may be due to these plants losing their senescent leaves after extended exposure to high levels of NaCl, a process previously observed in many halophytes (Flowers *et al.*, 1986).

All growth and physiological traits were strongly affected by saline stress (Table 2). Except for the WQ population, which exhibited an anomalous response, the root–shoot ratio of the other *T. salsaginea* populations increased significantly with an increase in salinity; total leaf number, total leaf area, and SLA declined rapidly in all populations

Table 2. Results of ANOVA (*F*-values) of the growth and physiological traits of plants from the seven populations

	Population	Salinity	Population × salinity	Model R^2
Growth traits				
Total biomass	27.75***	22.17***	3.21**	0.787
Relative growth rate	6.16***	24.06***	2.76**	0.613
Total leaf area	10.80***	39.26***	2.15*	0.706
Total leaf number	1.23 $^{N.S.}$	17.78***	2.03*	0.433
Root–shoot ratio	18.70***	30.48***	3.78**	0.762
Expanded leaf length	8.08***	12.26***	2.49**	0.443
Specific leaf area	3.33**	16.51***	3.31**	0.540
Physiological traits				
Maximal photosynthetic rate	20.08***	360***	13.54***	0.941
Stomatal conductance	11.09***	350***	8.46***	0.932
Fv/Fm	0.623 $^{N.S.}$	5.177*	0.609 $^{N.S.}$	0.022
Leaf succulence	1.37 $^{N.S.}$	21.53***	2.79**	0.511
Leaf Na^+ content	12.01***	2713***	23.67***	0.989
Leaf K^+ content	8.36***	719***	4.89***	0.961

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $^{N.S.}$ $P > 0.05$.

(except for WQ and LK) with an increase in salinity (Fig. 1). The maximal photosynthetic rate, stomatal conductance, leaf K^+ content, and Fv/Fm decreased sharply along the saline stress gradient. However, all the Fv/Fm values remained above 0.8 (data not shown), indicating that the structural integrity of photosystem II was unaffected by the salinity treatments. Leaf succulence increased to between 0 and 300 mM NaCl, and decreased to between 300 and 500 mM NaCl. Leaf Na^+ content generally increased with an increase in saline stress.

Differences in phenotypic plasticity

ANOVA showed that the populations exhibited significant differences for most of the traits at all levels of saline stress, except for Fv/Fm (Table 2). However, RDA revealed only very weak correlations between local environmental factors and the population trait means (Monte Carlo test: first axis $P = 0.488$, all axes $P = 0.382$). These results, therefore, provide no evidence of local adaptation.

Analyses of the CV values showed that trait plasticity differed significantly among the seven populations for all traits (Friedman test, $P = 0.0099$). Moreover, the relative ranking of the degree of plasticity for each population differed among the traits (Fig. 2). The variation in trait plasticity for net photosynthetic rate, stomatal conductance, and Na^+ content was marked; however, LC exhibited low plasticity (Fig. 2). The PCA results (Fig. 3, eigenvalue of 0.926 for the first axis) were used to group the populations according to their trait plasticity (Fig. 3). The RY, LC, DY, and FQ populations exhibited relatively low plasticity in relative growth rate, total biomass, total leaf number, and total leaf area compared with the other populations but higher plasticity in Na^+ and SLA. These four populations were further divided into two sub-groups: (i) RY and LC populations, which exhibited relatively low plasticity in net photosynthetic rate and stomatal conductance, and (ii) FQ and DY populations, which showed high plasticity in root–shoot ratio and SLA and low plasticity in K^+.

The RDA of the CVs showed that, for each population, there was no significant relationship between the growth and physiological traits and the environmental variables that describe the natural habitat (Monte Carlo test: first axis $P = 0.206$, all axes $P = 0.226$). Therefore, the patterns of these plastic responses cannot be explained simply in terms of habitat or original climate.

The matrix of the mean differences of the correlation coefficients was derived from the correlations of the traits measured under different saline treatments (Table 3). For the WQ population, the mean difference in the coefficients for the growth traits of total biomass and other growth-related traits under saline treatment 1 (0 mM NaCl) versus the same traits under saline treatment 2 (300 mM NaCl) is 0.747, which was treated as a measure of trait integration. Various patterns in plasticity among the seven populations were revealed. When averaged with all the changes in the correlation coefficients, these patterns provided a high mean value of 0.711. Furthermore, no overall differences were observed (Friedman's rank sums: CMH statistic = 3.14, $P = 0.791$).

The different populations presented various patterns of trait integration. The mean changes in the correlation coefficients averaged for all paired combinations of saline treatments showed that the YS population had a significantly reduced mean change in correlation coefficients between traits and trait groups, averaged across treatments, of 0.693 ($P = 0.049$). This population also exhibited the most significant change in the combined

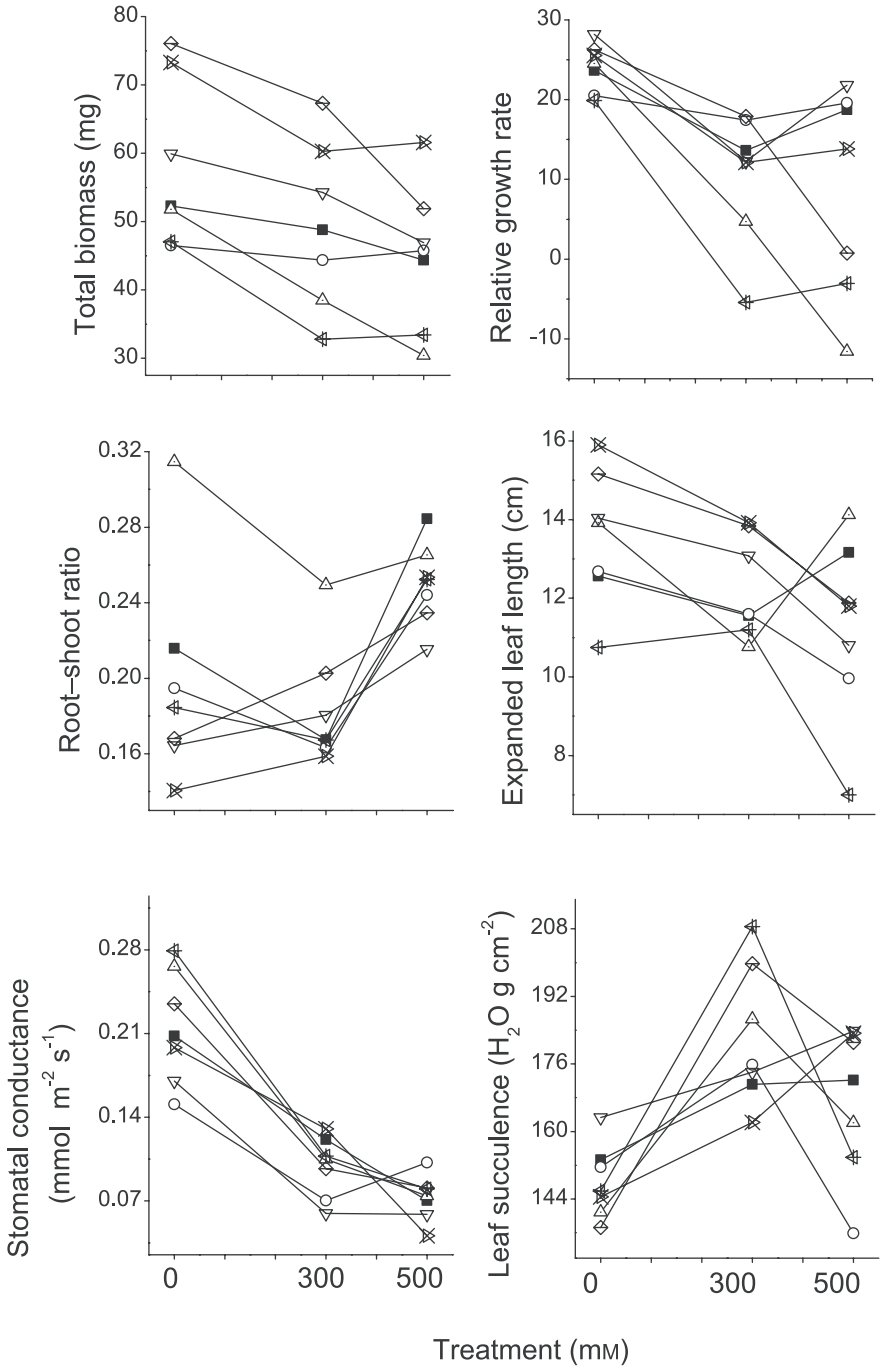
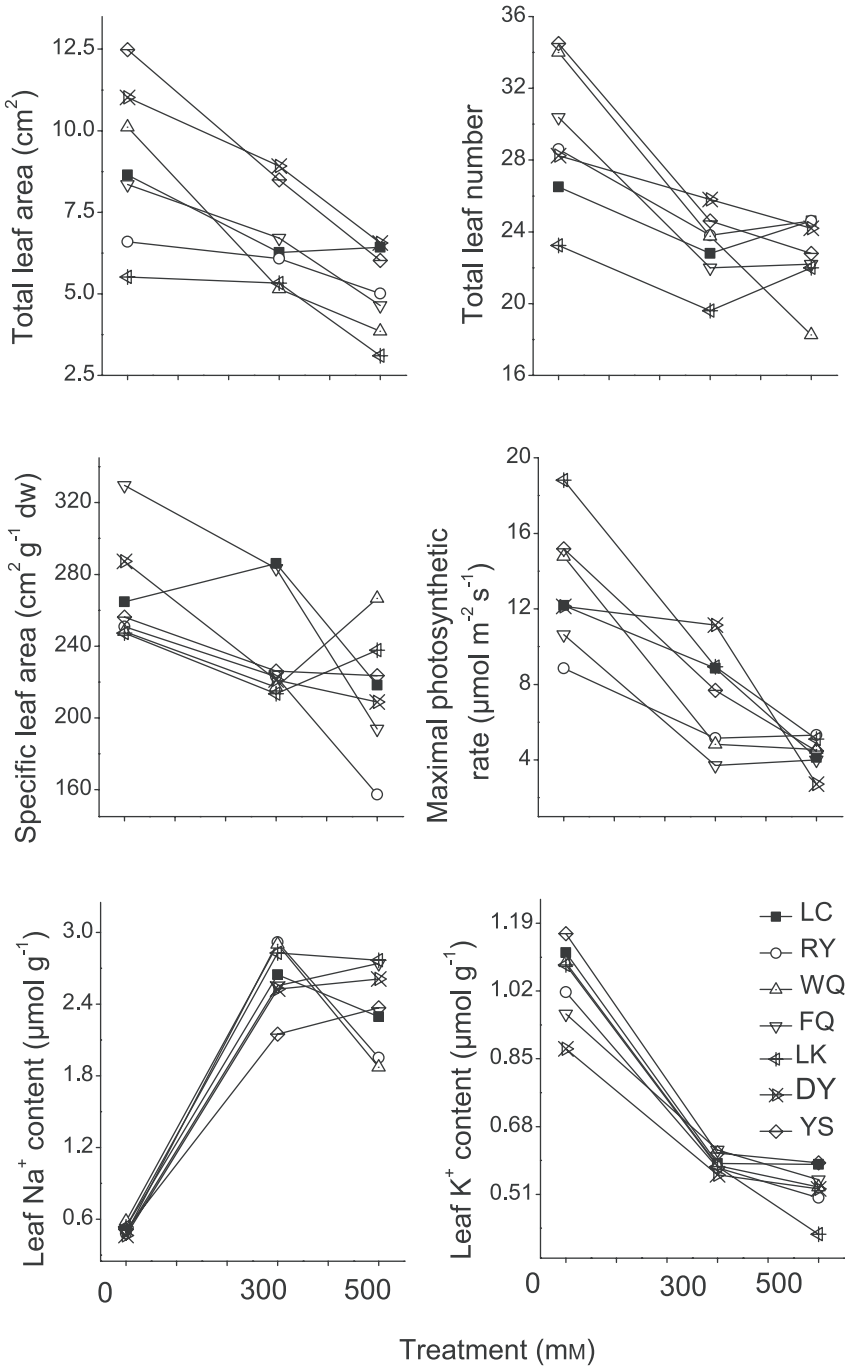


Fig. 1. Mean values of the traits in the three saline treatments. Populations: LC = Liaocheng; RY = Raoyang; WQ = Wuqing; FQ = Fengqui; LK = Lankao; DY = Dongying; YS = Yanshan.


Total leaf area (cm^2)
12.5
10.0
7.5
5.0
2.5
Total leaf number
36
32
28
24
20
16
Specific leaf area (cm^2 g^{-1} dw)
320
280
240
200
160
Maximal photosynthetic rate (μmol m^{-2} s^{-1})
20
16
12
8
4
Leaf Na$^+$ content (μmol g^{-1})
3.0
2.4
1.8
1.2
0.6
Leaf K$^+$ content (μmol g^{-1})
1.19
1.02
0.85
0.68
0.51
LC
RY
WQ
FQ
LK
DY
YS
0
300
500
Treatment (mM)

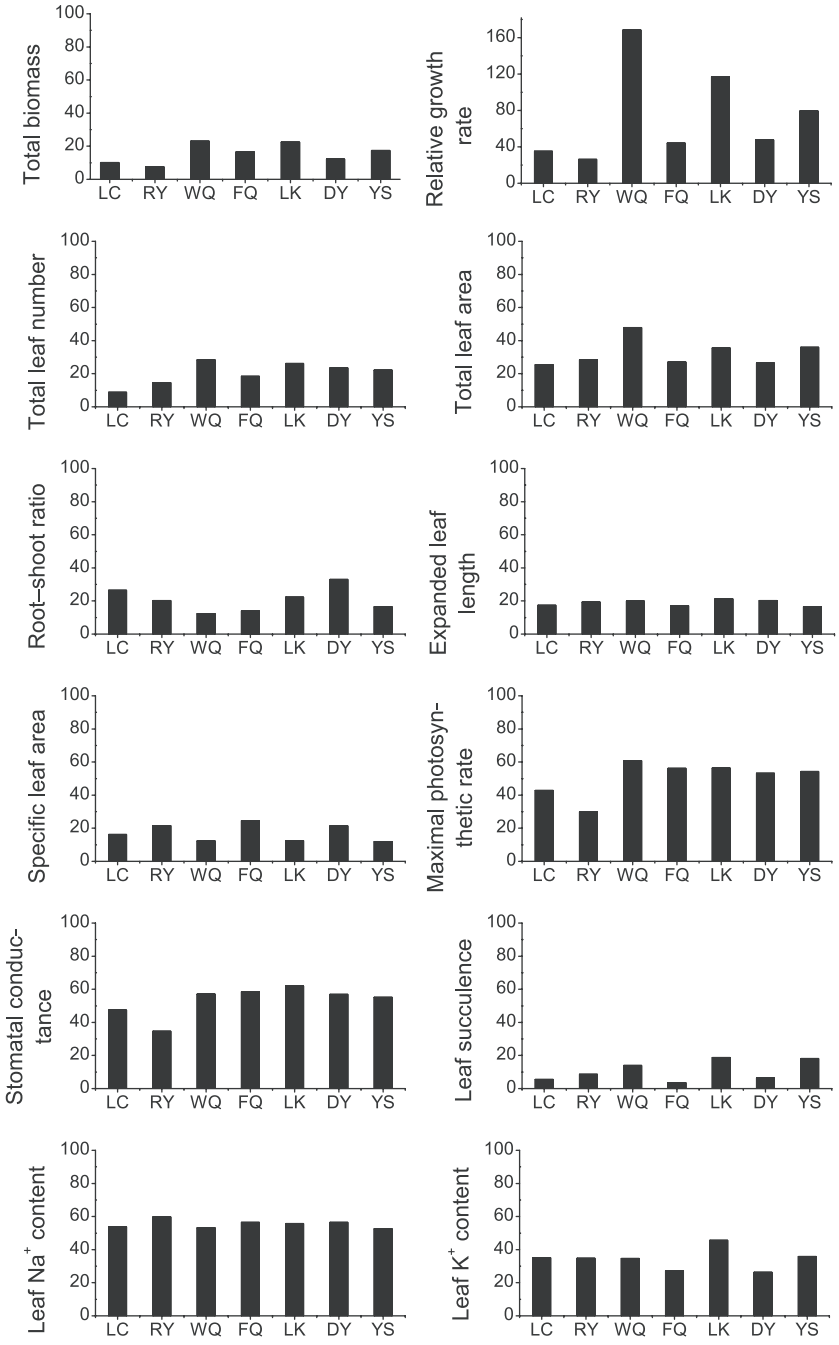


Fig. 2. Plasticity of the plant traits of the seven populations grown in different saline treatments. The coefficients of variation (CV, %) are shown for the trait plasticity of the populations. The populations are arranged according to the soil salinity in the field (from the lowest to highest EC values). Populations: LC = Liaocheng; RY = Raoyang; WQ = Wuqing; FQ = Fengqui; LK = Lankao; DY = Dongying; YS = Yanshan.

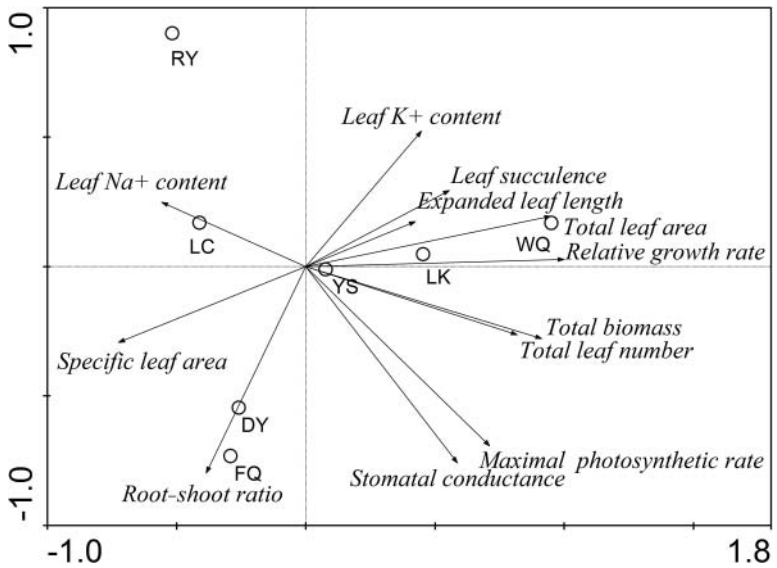


Fig. 3. PCA of plant trait plasticity (CV) in response to the different saline treatments. Open circles and arrows denote populations and traits, respectively. The first axis accounts for 92.6% of the variation among the populations. Populations: LC = Liaocheng; RY = Raoyang; WQ = Wuqing; FQ = Fengqui; LK = Lankao; DY = Dongying; YS = Yanshan.

growth and ecophysiological traits (i.e. G:P), whereas the LK population exhibited the most significant change in the growth traits (G), and the least change in the physiological traits (P). Several significant differences were observed in the combined datasets of trait groups across populations and saline treatments (Friedman's rank sums: CMH statistic = 3.14, $P = 0.013$). Furthermore, no significant differences were observed in plasticity in the three possible treatment transitions either for individual populations or for all pooled data (Friedman's rank sums: CMH statistic = 1.81, $P = 0.404$).

Correlations between trait plasticity and fitness

The potential relationship between fitness and plasticity in response to variations in saline stress was determined by evaluating the relationship between total biomass (index of fitness) and the traits root–shoot ratio, stomatal conductance, and SLA (Fig. 4). These traits were selected because they accurately reflect the ability of plants to tolerate salt. The regression between root–shoot ratio and total biomass revealed a significant negative slope for the control group ($y = -74.02x + 72.76$, $R^2 = 0.1011$, $P = 0.035$) but no significant relationship with either the 300 mM or 500 mM saline treatment ($P = 0.913$ and 0.550, respectively). Among the populations that received the highest saline treatment, fitness decreased with an increase in the rate of stomatal conductance ($y = -275.9x + 66.49$, $R^2 = 0.1943$, $P = 0.005$) and SLA ($y = -0.13x + 74.58$, $R^2 = 0.1868$, $P = 0.006$). For stomatal conductance and SLA, the regression slopes showed a tendency towards increasing negativity as the saline stress increased from 0 mM to 500 mM NaCl (Fig. 4).

Table 3. Average magnitudes of differences in correlation coefficients of traits within the growth (G) and physiology (P) (excluding Fv/Fm) categories or between (G:P) trait categories

		1 vs. 2	1 vs. 3	2 vs. 3	Mean	Row rank sum
Wuqing (WQ)	G	0.629	0.578	0.506	0.571	1.0
	P	0.810	0.811	0.906	0.842	2.3
	G:P	0.890	0.813	0.777	0.827	2.7
	Mean	0.776	0.734	0.730	0.747	$P = 0.097$
	Column rank sum	2.3	2.0	1.7		
	$P = 0.717$					
Raoyang (RY)	G	0.570	0.599	0.757	0.642	2.0
	P	0.862	0.273	0.711	0.616	1.7
	G:P	0.652	0.542	0.796	0.663	2.3
	Mean	0.695	0.471	0.755	0.640	$P = 0.717$
	Column rank sum	2.0	1.3	2.7		
	$P = 0.264$					
Fengqiu (FQ)	G	0.462	0.608	0.885	0.652	1.7
	P	0.899	0.876	0.378	0.718	2.0
	G:P	0.907	0.847	0.816	0.857	2.3
	Mean	0.756	0.777	0.693	0.742	$P = 0.717$
	Column rank sum	2.3	2.0	1.7		
	$P = 0.717$					
Yanshan (YS)	G	0.452	0.367	0.502	0.440	1.0
	P	0.758	0.769	0.634	0.720	2.3
	G:P	1.032	0.754	0.967	0.918	2.7
	Mean	0.747	0.630	0.701	0.693	$P = 0.049$
	Column rank sum	2.3	1.7	2.0		
	$P = 0.717$					
Dongying (DY)	G	0.771	0.424	0.592	0.596	1.7
	P	1.056	0.956	0.478	0.830	2.3
	G:P	0.770	0.901	0.741	0.804	2.0
	Mean	0.866	0.760	0.604	0.743	$P = 0.717$
	Column rank sum	2.7	2.0	1.3		
	$P = 0.264$					
Liaocheng (LC)	G	0.654	0.538	0.484	0.559	1.0
	P	0.825	0.856	0.629	0.770	2.0
	G:P	0.881	0.939	0.696	0.839	3.0
	Mean	0.787	0.778	0.603	0.722	$P = 0.097$
	Column rank sum	2.3	2.7	1.0		
	$P = 0.097$					
Lankao (LK)	G	0.604	0.868	1.013	0.828	2.7
	P	0.589	0.308	0.628	0.508	1.0
	G:P	0.808	0.555	0.813	0.725	2.3
	Mean	0.667	0.577	0.818	0.687	$P = 0.097$
	Column rank sum	1.7	1.3	3.0		
	$P = 0.097$					

Note: Columns (e.g. 1 vs. 2) represent the average change in the correlation between pairs of treatments. Treatment 1 (control): 0 mM NaCl; Treatment 2: 300 mM NaCl; Treatment 3: 500 mM NaCl. The *P*-values associated with rank sums were obtained using Friedman's rank sum tests. The *P*-values of the test for differences among columns and rows are provided in the second column and last column, respectively.

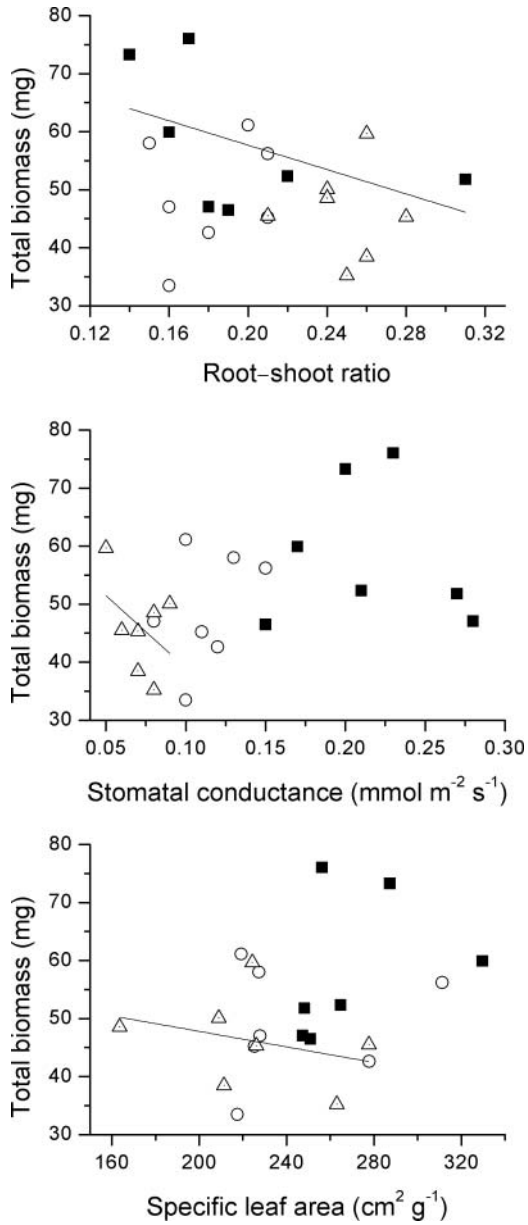


Fig. 4. Relationship between total biomass (a proxy for fitness) and root–shoot ratio, stomatal conductance, and specific leaf area for seven populations of *T. salsaginea*. Each point represents the mean data determined in each population. The solid lines represent the statistically significant regression lines. Saline treatments: ■, 0 mM NaCl; ○, 300 mM NaCl; △, 500 mM NaCl.

DISCUSSION

Variation in phenotypic plasticity and phenotypic integration

The extent of phenotypic plasticity depends largely on the magnitude of habitat heterogeneity (Balaguer *et al.*, 2001; Grant *et al.*, 2005; Rozendaal *et al.*, 2006). Although the means of individual traits showed significant inter-population variation, RDA did not reveal any significant correlation between the traits and the local habitats. The plasticity patterns of these populations may have resulted from the combined effects of several factors rather than from a single, local environmental factor such as salinity or rainfall (Debat and David, 2001; Schlichting and Smith, 2002; Nicotra *et al.*, 2007).

Recent studies (Bloor and Grubb, 2004; Griffith and Sultan, 2006; Deng *et al.*, 2008) have shown that, when plants suffer abiotic stress, plasticity in several traits might occur in plants at the expense of plasticity in other traits. Similarly, the physiological traits observed in this study were more flexible on average than the growth traits (Fig. 2). This phenomenon may have resulted from the physiological traits first developing plasticity in response to saline stress, with the growth traits developing plasticity later. Different populations may therefore acquire their overall plasticity in different ways; this condition could explain the inconsistent ranking in plasticity in terms of saline stress.

Although phenotypic integration is sometimes confused with adaptation or a constraint on future evolution on earth (Pigliucci, 2003), the integration patterns of plastic traits are certainly closely related to the ecological and evolutionary history of plant species as well as plasticity patterns (Murren, 2002; Pigliucci, 2003; Monteiro *et al.*, 2005; Parsons and Robinson, 2006; Nicotra *et al.*, 2007). Nicotra *et al.* (2007) claimed that the observed patterns of integration and plasticity in *Pelargonium australe* might reflect its biogeography and history. Our results show that, on average, relatively large differences exist between most individual correlation coefficients, whether between traits or between treatments (Table 3). Such finding suggests a low degree of integration in plasticity among traits (Nicotra *et al.*, 2007). Furthermore, no significant differences were observed among the three saline treatments when the data for all the traits were pooled. In contrast, significant differences were observed in overall plasticity among the traits and trait groups when the datasets for treatments were combined, as in the patterns of *P. australe* observed by Nicotra *et al.* (2007). Therefore, the ability of *T. salsaginea* to colonize and survive in a wide range of highly saline habitats may have contributed to the high trait plasticity observed among treatments, the low degree of trait integration, and the varied patterns of plasticity among individual traits and trait groups. In addition, the changes in the correlation coefficients of the trait groups were significantly different in the YS population ($P < 0.05$) drawn from the locations with the highest salinity. This phenomenon might have resulted from the YS population being subjected to more favourable conditions and growing better under the two saline stress treatments rather than in harsher conditions compared with its local habitat. Thus, the degree of trait integration in the growth traits was significantly enhanced (Fig. 1; Table 3).

Adaptive plasticity

Adaptive plasticity, which is defined in this study as a phenotypic response to an environment, enhances plant function and fitness in the environment (Sultan, 1987). Salt in soil water inhibits plant growth through two physiological mechanisms (Munns, 2005): (1) by reducing the

ability of plants to retain water, resulting in the osmotic or water-deficit effect; and (2) by salt entering the transpiration stream, which eventually injures the cells in the transpiring leaves because of an ion-excess effect. Plants may avoid or allay this damage by decreasing stomatal conductance (Lovelock and Ball, 2002) and SLA (Inan *et al*., 2004), as shown in our experiments on *T. salsaginea* (Fig. 1). However, such phenotypic responses to the harsh environmental circumstances result in restricted plant growth because of the reduced CO_2 availability and the decreased leaf area. Plants may also implement other adaptive strategies to maintain their survival under conditions of severe saline stress. For example, the allocation of additional biomass and energy-to-root growth (Fig. 1, Table 2) or leaf fall off could constrain Na^+ accumulation to the belowground part of the plant to avoid being transported to the aboveground part (Vera-Estrella *et al*., 2005; Volkov and Amtmann, 2006).

The differences between the regression slopes of the relationship between fitness and a plastic trait in populations from various environments have been used to assess whether plastic responses are adaptive or natural selection is likely to favour plasticity (Weinig *et al*., 2004; Caruso *et al*., 2006; Nicotra *et al*., 2007). In this study, the regression slopes of total biomass (a proxy of fitness) versus stomatal conductance and SLA varied widely among the populations across the salinity gradient (Fig. 4). Stomatal conductance and SLA generally declined, and the slopes shifted from positive to negative with an increase in saline stress. These findings indicate that these plastic traits are adaptive. Furthermore, both stomatal conductance and SLA exhibited a significant negative correlation with total biomass in the 500 mM NaCl treatment, thus indicating that the magnitude of total biomass increased with the decrease in the aforementioned traits. This result further demonstrates the adaptive strategies of *T. salsaginea* under high saline stress.

Adaptive plasticity should promote congruity between the novel environment and trait expression, thus improving the performance of a population in novel habitats (Ghalambor *et al*., 2007). Furthermore, among individuals that colonize a new territory, natural selection favours those with the most appropriate plastic traits (West-Eberhard, 2003). Developing a plastic phenotype is an important adaptive characteristic for a population or species and may ultimately bring about genetic differentiation in a heterogeneous environment. In contrast, a loss of trait plasticity would lead to genetic similarity within a population or species and consequently constrain its ability to further colonize new heterogeneous environments (Pigliucci *et al*., 2006). Thus, adaptive evolution might occur among populations in heterogeneous habitats and finally result in genetic homogeneity and heterogeneity within and between populations, respectively (Pigliucci *et al*., 2006). However, the genetic analysis of *T. salsaginea* with neutral nuclear markers revealed no genetic variation within or among populations; instead, diverse morphological variations were observed (D. Shi *et al*., unpublished data). Therefore, the observed high amount of phenotypic plasticity (Table 2) and low degrees of phenotypic integration might have resulted from epigenetic variation by, for example, DNA methylation, chromatin remodelling, histone deacetylation, position effects, and small RNAs interference (Schrey *et al*., 2013). Phenotypes or physiological expressions are affected by changes in heritable transcriptions (Arney and Fisher, 2004). Overall, this finding suggests that *T. salsaginea* is also a good model for the study of the molecular mechanism of phenotypic plasticity and integration in a relatively homogeneous neutral genetic background. Further evidence of these possible epigenetic changes has been collected in our laboratory.

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