

Blocked pollen release prevents fruit formation in the halophyte *Elaeagnus angustifolia* in non-saline habitats

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Abstract

Background: Anther development affects the reproduction of flowering plants. Halophyte *Elaeagnus angustifolia* can bear fruits when grown in saline soils. However, no fruits are born in non-saline soils. The possible reasons and differences in *E. angustifolia* under two conditions were elucidated.

Results: We examined features including pollen vitality and germination, in situ pollen germination after natural and hand pollination, anthers after pollen release, and the transcriptome in anthers. No significant difference was observed in pollen vitality or stigma receptivity in *E. angustifolia* in non-saline vs. saline habitats. However, no pollen tubes were present in styles, and pollen grains were abundant in *E. angustifolia* anthers under non-saline conditions. Notably, many pollen tubes formed in styles of *E. angustifolia* after hand pollination in the non-saline habitat. And the differentially expressed genes in anthers from saline vs. non-saline habitats were mainly related to phytohormones, cell wall secondary thickening, transcription factors and ion transport.

Conclusions: *E. angustifolia* fail to form fruits in non-saline habitats due to poor anther pollen release. The induction and coordinated upregulation of genes related to anther cell wall formation and JA biosynthesis likely contribute to anther dehiscence in *E. angustifolia* in saline habitats, whereas anther dehiscence is blocked in plants grown in non-saline habitats.

Background

Over 800 million hectares of land worldwide are affected by salt, accounting for more than 6% of the world's total land area [1-3]. A large amount of saline soil is present in China, which seriously reduces agricultural production [4]. Salt also limits the survival and growth of trees by reducing root survival and hindering the absorption of water and mineral elements [5, 6]. Halophytes have developed mechanisms to tolerate salinity, including salt exclusion to prevent salt absorption (e.g., *Pistacia* genus) [7]; salt secretion outside the plant body through glands (e.g., *Tamarix* spp.) [8]; and salt dilution to maintain a lower salt content in the cytoplasm (e.g., mangrove *Bruguiera cylindrica*) [9]. Access to halophyte trees would be a benefit for cultivation to improve saline lands, develop saline agriculture, prevent soil erosion, and enhance urban afforestation in saline lands.

Elaeagnus angustifolia L., a member of the *Elaeagnaceae* family, can grow in a wide range of environmental conditions [10]. This tree is deciduous, with unique silver-gray scales covering the leaves, flowers, and newly grown branchlets. *E. angustifolia* flowers, which are bisexual, yellow on the inside, silvery white on the outside, and fragrant, form and open in May and June. *E. angustifolia* fruit is edible and sweet, with a single seed [11], and the roots undergo nitrogen fixation [12]. Since this tree is resistant to salt and drought stress and is strongly adaptable, it is widely used for urban and road greening. *E. angustifolia* can grow and complete its life cycle in saline soil [13]. Many studies have focused on the utilization of *E. angustifolia* fruits, flowers [14, 15], and plant extracts [16-18]. In addition, the growth performance and salt tolerance of *E. angustifolia* at the seedling stage were recently investigated [19, 20].

However, little is known about the reproductive development of *E. angustifolia* in saline environments, especially compared to non-saline environments.

Plants switch to the reproductive stage after completing vegetative growth when induced by environmental conditions such as low temperatures and a suitable photoperiod. Much progress has been made in uncovering the molecular mechanism of flower induction and initiation under these conditions [21-23]. In general, for non-halophytes, salinity inhibits plant growth and yields [24-26]. However, salinity has diverse effects on plant reproduction in halophytes. For example, the halophyte *Suaeda salsa* exhibits the highest biomass and yields when treated with high salinity during the reproductive phase [27-30]. NaCl (300 mM) treatment stimulates flowering in *Plantago crassifolia* but reduces seed number, with aborted seeds produced in half the spikes [31]. In the halophyte *Crithmum maritimum*, the inflorescence and flower number are significantly reduced by salinity treatment [32]. How specific plants adapt to high-salinity environments might be related to the ionic balance in leaves and roots, phytohormone balance in plant cells, and an efficient carbon supply [33-35].

Normal stamen development is important for completing the plant lifecycle, while defects in stamen development can lead to male sterility. Environmental factors such as drought and extreme temperature led to male sterility via inhibiting the development and function of anthers [36-40]. However, little is known about anther development in plants growing in a saline environment.

Anther development is a precise and complex process, especially the meiosis of the microspore mother cell to produce microspores and pollens. Many genes play pivotal roles in this process and are expressed at different developmental stages. The anther development F-box gene in rice (*OsADF*) is essential for tapetum cell development and pollen formation [41]. In Arabidopsis, the processes of anther differentiation, anther wall development, anther dehiscence and pollen fertility are regulated by numerous related genes such as *MYB* transcription factors, *RECEPTOR-LIKE PROTEIN KINASE2 (RPK2)*, and *CALCINEURIN B-LIKE10 (CBL10)* [42, 43]. Phytohormones are also involved in stamen development [44]. The Arabidopsis *ga1-3* mutant is male sterile with defective stamens and pollen development due to reduced gibberellic acid (GA) biosynthesis [45]. However, what genes are involved in the regulation of anther development in halophytes, and whether the expression levels of genes related to anther development are altered in halophytes grown in saline environments, remains unknown.

E. angustifolia grown in saline lands in Shandong, Xinjiang, Gansu, and Ningxia of China forms flowers and bears numerous fruits. However, *E. angustifolia* grown in non-saline lands in Shandong (such as Ji'nan) forms only flowers without fruits, unless it is treated with NaCl, in which case it also bears fruits. In the current study, to explore why *E. angustifolia* flowers but does not form fruits in non-saline habitats, we performed transcriptome analysis of *E. angustifolia* anthers from plants grown in two habitats using high-throughput Illumina RNA sequencing (RNA-seq). Our findings shed light on the relationship between salt tolerance and reproductive development in *E. angustifolia* and lay the foundation for identifying key factors specific to halophytes that contribute to anther development and fruit formation.

Results

***E. angustifolia* plants flower and bear fruits in the saline habitat and flower but do not bear fruits in the non-saline habitat**

We observed the flowering period and reproductive growth of *E. angustifolia* in two habitats. *E. angustifolia* flowers are bisexual, with 1–3 flower clusters per axil. The flowers are yellow on the inside, silvery white on the outside, and fragrant and are born from early May to late June. In both habitats, flower buds formed in *E. angustifolia*, and the flowers opened (Fig. 1a, b). After flowering, fruits bore in *E. angustifolia* in the saline habitat (Fig. 1c), one or two fruits each leaf axil. Whereas plants in the non-saline habitat had no fruits, and all of the flowers fell off of the plants (Fig. 1d).

***E. angustifolia* plants grown in two habitats do not exhibit significant differences in pollen viability or stigma receptivity**

To determine whether the lack of fruit in *E. angustifolia* in the non-saline habitat was caused by pollen vitality, we dyed pollen collected from *E. angustifolia* in both habitats with Alexander's solution. This stain dyes aborted pollen grains green and non-aborted pollen grains red. There was no difference in pollen vitality between the habitats: 80% of the pollen grains were viable (Fig. 2a, b). Pollen grains that germinated in vitro from plants grown in both non-saline and saline habitats were well developed, indicating they could germinate and form pollen tubes (Fig. 2c, d).

To determine whether insufficient pollen tube growth led to the lack of fertilization of *E. angustifolia* in the non-saline habitat, we determined the pollen germination rate and pollen tube length. As shown in Fig. S1, the pollen germination rate (Fig. S1 a) of *E. angustifolia* from both habitats was approximately 50%, and there was no significant difference in pollen tube length (Fig. S1 b) between habitats. These results indicate that the flowering and lack of fruit in *E. angustifolia* in non-saline habitats were not caused by pollen dysplasia.

To determine whether altered stigma receptivity prevents fruit formation in *E. angustifolia* in non-saline habitats, we measured peroxidase activity in stigmas, which represents stigma receptivity. As shown in Fig. 3b, *E. angustifolia* stigmas from the non-saline habitat were surrounded by blue staining and numerous bubbles, and there were no significant differences between these stigmas and stigmas from plants grown in the saline habitat (Fig. 3a). The stigmas of plants grown in both environments showed high receptivity, and the pollen vitality was high. These results indicate that pollen viability and stigma receptivity are not the reasons for the lack of fruit in *E. angustifolia* in the non-saline habitat.

Stigmas from the non-saline habitat lack pollen and pollen tubes under natural conditions but contain numerous pollen tubes after hand pollination

Under natural conditions, after self pollination, aniline blue staining of pistils revealed numerous pollen tubes in the styles and many pollen grains on the stigmas of plants grown in the saline habitat (Fig. 4a, d). By contrast, the styles of plants grown in the non-saline habitat had almost no pollen tubes in the

styles and no pollen grains on stigmas (Fig. 4b, e). These results suggest that due to blocked anther dehiscence, no pollen grains reach the stigmas of plants in the non-saline habitat under natural conditions. At 4 h after hand pollination, many pollen tubes formed in the styles and many pollen grains were present on the stigmas of *E. angustifolia* grown in the non-saline habitat (Fig. 4c, f).

Blocked anther dehiscence occurs in the non-saline habitat

To further investigate whether blocked anther dehiscence led to the lack of fruit formation in the non-saline habitat, we observed flowers after anther dehiscence in plants from both habitats. As shown in Fig. 5a, the anthers were pale with almost no pollen grains in *E. angustifolia* in the saline habitat, as many pollen grains were released to the surfaces of the petals. However, in the non-saline habitat, the anthers were dark yellow and contained many pollen grains, and no pollen grains were present on the surfaces of the petals (Fig. 5b).

To determine if the color difference of the anthers from saline vs. non-saline habitats was due to the presence of pollen grains, we observed anthers after pollen released by DIC microscopy. The anthers of plants grown in the saline habitat contained almost no pollen grains and had undergone dehiscence, with only a single layer of cells (Fig. 5c). By contrast, the anthers of plants grown in the non-saline habitat contained numerous pollen grains after anther dehiscence, and no obvious stomium was observed in the anthers (Fig. 5d). The anther sections of *E. angustifolia* before dehiscence in two habitats were further prepared, no significant difference in pollen amount in anthers of the two habitats, obvious crack in anthers from saline habitat were observed, while not in anthers from non-saline habitat (Fig. S2). It is speculated that the anthers of the *E. angustifolia* in saline habitat can dehisce and loose pollens when the pollen grains are mature. However, the pollination in *E. angustifolia* flowers in non-saline habitat was inhibited.

Na⁺ content increases in leaves but not flowers in the saline habitat

Significantly (27.4%) ($P < 0.05$) higher Na⁺ contents were detected in the leaves of *E. angustifolia* grown in the saline vs. non-saline habitat, whereas no significant difference in Na⁺ content was observed in the flowers of these plants (Fig. 6a). Moreover, there was no significant difference in K⁺ content in the leaves or flowers of *E. angustifolia* plants grown in the saline vs. non-saline habitat (Fig. 6b). However, plant reproduction was not inhibited, but rather was improved, by the increased Na⁺ content in leaves under saline conditions.

Sequence assembly and quantitative real-time PCR validation

To investigate the molecular mechanism involved in the differences in anther development and dehiscence in *E. angustifolia* under saline vs. non-saline conditions, we collected anthers during early development and subjected them to RNA-seq analysis. The number of clean reads obtained from the both groups is shown in Table S3. Genes with differential expression in anthers between the two groups were filtered based on an adjusted P value < 0.01 and $|\log_2(\text{fold change})| > 1.5$. The remaining genes were

analyzed to identify the differentially expressed genes (DEGs) that might lead to failed dehiscence in *E. angustifolia* anthers in the non-saline habitat.

To validate the quality of the data obtained by RNA-seq, were subjected 11 DEGs involved in anther development to quantitative real-time PCR (qPCR). The results from qPCR were consistent with the RNA-seq results, indicating that the RNA-seq data were reliable (Fig. 7) and could be used for further analysis.

Analysis and annotation of DEGs

To evaluate the DEGs in *E. angustifolia* anthers under saline vs. non-saline conditions, we performed differential expression analysis using DESeq2 [46]. In total, 8,817 genes displayed significantly different expression levels between the saline and non-saline habitats. Among these, 5,063 were upregulated and 3,754 were downregulated in *E. angustifolia* anthers under saline vs. non-saline conditions (Fig. S3 a).

To identify the likely biological pathways involving the DEGs in anthers between the two habitats, we performed functional analysis of the DEGs using the Kyoto Encyclopedia Genes and Genomes (KEGG) database [47]. In total, 1,919 DEGs were assigned to 116 KEGG pathways. These DEGs were mapped to many categories, such as “amino sugar and nucleotide sugar metabolism”, “biosynthesis of unsaturated fatty acids”, and “flavonoid biosynthesis” (Fig. S3 b). The differences in the pathways might be related to mechanisms used to ensure anther development in *E. angustifolia* in saline habitats. Furthermore, many other DEGs were involved in the categories “ABC transporters” and “starch and sucrose metabolism”, perhaps contributing to the anther dehiscence and pollination of flowers in *E. angustifolia* under saline conditions. During plant reproduction, plant hormone biosynthesis and signal transduction play crucial roles in development and plant responses to stress. Many of the DEGs were also assigned to the categories plant hormone biosynthesis and plant signal transduction.

DEGs involved in cell wall formation in *E. angustifolia* anthers

During cell and anther development, cell wall formation is fundamentally important for protecting the cells from adverse conditions, especially the formation of secondary cell walls (SCWs). In the present study, we identified DEGs related to cell wall modification in the anthers of *E. angustifolia* plants grown under saline vs. non-saline conditions. Thirteen DEGs associated with cell wall modification were upregulated in *E. angustifolia* anthers from the saline vs. non-saline habitat. In particular, three (Cluster-2772.54625, Cluster-2772.45882 and Cluster-2772.41972) genes were inducibly expressed under the saline habitat (Table S4). Actin and tubulin participate in anther development [48]. Six DEGs encoding tubulin and one DEG encoding actin were upregulated and four of them (Cluster-2772.60317, Cluster-2772.61547, Cluster-2772.59057 and Cluster-2772.85525) were inducibly expressed under salinity (Table S4).

Lipoxygenase (LOX) and allene oxide cyclase (AOC) are involved in anther dehiscence [49]. In *E. angustifolia* anthers, four DEGs encoding LOX and two DEGs encoding AOC were upregulated and DEGs (Cluster-2772.115415, Cluster-2772.82284 and Cluster-2772.46629) were inducibly expressed in the

saline environment (Table 1). Lignin deposited in endothecium cells is essential prior to anther dehiscence [50]. In *E. angustifolia* anther from the saline habitat, three DEGs encoding cinnamyl alcohol dehydrogenase (CAD) were upregulated and one (Cluster-2772.88336) was inducibly expressed in the saline environment (Table 1). In addition, two DEGs encoding E3 SUMO-protein ligase SIZ1 involved in the functional regulation of the endothecium in anthers [51, 52] were upregulated in *E. angustifolia* anthers under saline conditions. Notably, one DEG (Cluster-2772.16029) encoding receptor protein kinase (RPK), which plays a key role in anther dehiscence, was inducibly expressed in the saline environment [42, 43]. These results suggest that salinity induces or enhances the gene expression for cell wall formation in *E. angustifolia* anther to maintain anther dehiscence.

Table 1 Upregulated genes in *Elaeagnus angustifolia* anthers from the saline habitat related to the anther dehiscence pathway in conjunction with anther cell wall thickening and anther dehiscence based on RNA-seq.

Gene ID	Putative Function	Non-saline readcount	Saline readcount	log2FC	Regulated
lipoxygenase (LOX)					
ter-2.115415	linoleate 9S-lipoxygenase 5	0	31.23	7.63	up
ter-2.82284	linoleate 9S-lipoxygenase 6	0	27.59	7.45	up
ter-2.122397	linoleate 9S-lipoxygenase 6	0.28	34.69	6.8	up
ter-2.88203	linoleate 9S-lipoxygenase 6	2.54	35.48	3.79	up
allene oxide cyclase (AOC)					
ter-2.46629	allene oxide cyclase 2	0	130.73	9.69	up
ter-2.48391	allene oxide cyclase 3	553.73	1127.01	1.02	up
cinnamyl alcohol dehydrogenase (CAD)					
ter-2.88336	cinnamyl alcohol dehydrogenase 1	0	11.95	6.24	up
ter-2.89366	cinnamyl alcohol dehydrogenase 1	249.78	699.19	1.48	up
ter-2.4671	cinnamyl alcohol dehydrogenase 1	115.47	434.41	1.91	up
receptor protein kinase (RPK)					
ter-2.16029	receptor protein kinase 1	0	188.45	10.22	up
E3 SUMO-protein ligase SIZ1					
ter-2.41096	E3 SUMO-protein ligase SIZ1 OS=Arabidopsis thaliana GN=SIZ1 PE=1 SV=2	1.97	56.466	4.82	up
ter-2.69463	E3 SUMO-protein ligase SIZ1 OS=Arabidopsis thaliana GN=SIZ1 PE=1 SV=2	1.13	27.88	4.61	up

DEGs involved in hormone biosynthesis and signal transduction in *E. angustifolia*

Plant hormones play crucial roles in anther development. The expression levels of hormone-related genes correspond to higher concentrations of phytohormones or highly efficient signal transduction. To evaluate the possible reasons for the differences in anther development in *E. angustifolia* plants in non-saline vs. saline habitats, we identified three DEGs encoding the gibberellin receptor *GID1*, four DEGs encoding jasmonic acid-amino synthetase and two DEGs encoding auxin transporter-like protein, which were upregulated in plants grown in the saline environment. In particular, DEGs including Cluster-2772.3809, Cluster-25028.5, Cluster-2772.2343 and Cluster-2772.123259 were inducibly expressed in the saline environment (Table 2).

Table 2 Upregulated genes in *Elaeagnus angustifolia* anthers from the saline habitat related to hormone synthesis and signal transduction based on RNA-seq.

Gene ID	Putative Function	Non-saline readcount	Saline readcount	log2FC	Regulated
Gibberellin receptor <i>GID1</i>					
ter-2.3809	gibberellin receptor <i>GID1B</i>	0	122.30	9.60	up
ter-2.93098	gibberellin receptor <i>GID1</i>	109.87	224.51	1.03	up
ter-2.118690	gibberellin receptor <i>GID1B</i>	4.82	45.45	3.23	up
Auxin influx carrier (<i>AUX</i>)					
ter-28.5	auxin transporter-like protein 2	0	16.52	6.71	up
ter-2.5188	auxin transporter-like protein 5	28.69	104.27	1.85	up
Jasmonic acid-amino synthetase <i>JAR1</i>					
ter-2.2343	jasmonic acid-amino synthetase	0	42.63	8.08	up
ter-2.123259	jasmonic acid-amino synthetase	0	16.31	6.69	up
ter-26.0	jasmonic acid-amino synthetase	16.57	50.32	1.61	up
ter-2.111141	jasmonic acid-amino synthetase	21.51	148.69	2.78	up

DEGs encoding transcription factors in *E. angustifolia* anthers

NAC, WRKY, and MYB TFs are involved in anther dehiscence in Arabidopsis [53-55]. In *E. angustifolia* anthers from the saline habitat, eleven DEGs encoding NAC TFs, nine encoding WRKY TFs and five DEGs encoding MYB TFs were upregulated compared to anthers from the non-saline habitat (Table 3). Moreover, NAC domain-containing protein 82, MYB 44, MYB6, MYB39 and WRKY40 were inducibly expressed in *E. angustifolia* anthers from the saline habitat.

Table 3 Upregulated transcription factor genes in *Elaeagnus angustifolia* anthers from the saline habitat related to anther development and anther dehiscence based on RNA-seq.

Gene ID	Putative Function	Non-saline readcount	Saline readcount	log2FC	Regulated
transcription factor					
ter-2.44244	NAC domain-containing protein 82	0	219.81	10.45	up
ter-2.65220	NAC domain-containing protein 83	0.28	836.79	11.41	up
ter-2.111726	NAC transcription factor 29	9.44	117.49	3.63	up
ter-2.111729	NAC transcription factor 56	21.88	172.22	2.97	up
ter-2.0970	NAC domain-containing protein 72	6.29	43.42	2.78	up
ter-2.119476	NAC domain-containing protein 100	6.84	44.02	2.68	up
ter-2.115630	NAC domain-containing protein 21/22	33.18	207.40	2.64	up
ter-2.6230	NAC domain-containing protein 19	19.69	116.37	2.55	up
ter-2.57270	NAC domain-containing protein 14	114.54	674.13	2.55	up
ter-2.74382	NAC domain-containing protein 18	2.26	51.01	4.48	up
ter-2.70933	NAC transcription factor 25	1794.59	4527.70	1.33	up
transcription factor					
ter-2.31676	transcription factor MYB44	0	171.53	10.09	up
ter-2.96288	transcription repressor MYB6	0	83.87	9.05	up
ter-2.090	transcription factor MYB39	0	19.22	6.93	up
ter-2.55623	transcription factor MYB12	25.44	217.93	3.09	up
ter-2.87414	transcription factor MYB108	15.15	110.38	2.86	up
WRKY transcription factor					
ter-4331.1	WRKY transcription factor 40	0	57.76	8.52	up
ter-2.10112	WRKY transcription factor 21	0.84	53.12	5.96	up
ter-2.4494	WRKY transcription factor 15	2.61	76.79	4.88	up
ter-2.125225	WRKY transcription factor 33	3.15	50.99	4.01	up
ter-8261.1	WRKY transcription factor 56	6.87	52.44	2.93	up
ter-2.46955	WRKY transcription factor 2	10.53	58.85	2.47	up
ter-2.53116	WRKY transcription factor 19	32.04	150.26	2.22	up
ter-2.182	WRKY transcription factor 30	23.71	100.41	2.08	up
ter-2.13133	WRKY transcription factor 65	34.27	118.31	1.78	up

DEGs involved in sugar transport and metabolism in *E. angustifolia* anthers

Sugar transport and metabolism play important roles in plant growth and development, especially during anther development. We detected enhanced and inducible expression of sugar transporter genes in *E. angustifolia* anthers from the saline habitat (Table S5). Three DEGs encoding sucrose synthase, one encoding sucrose phosphate synthase 1F and ten DEGs encoding sugar transporters or sugar transport proteins were upregulated in the anthers of plants of the saline habitat. In addition, eight of them (Cluster-2772.44272, Cluster-2772.44273, Cluster-2772.122621, Cluster-2772.17932, Cluster-2772.26904, Cluster-2772.115155, Cluster-2772.44021 and Cluster-2772.44963) were inducibly expressed in the saline habitat.

DEGs involved in ion content and ROS scavenging in *E. angustifolia* anthers

Altered levels of ions, especially Na^+ and K^+ , are a critical indicator of plants grown in a saline environment. The transmembrane transport of ions occurs via transporters and channels located in the membrane, such as NHX (Na^+/H^+ antiporter), AKT (inward-rectifying K^+ channel), and KEA (K^+ efflux antiporter) proteins. In the anthers of plants from the saline habitat, four DEGs (Cluster-2772.37266, Cluster-2772.84379, Cluster-2772.125705, Cluster-2772.84388) encoding the Na^+/H^+ exchanger on the plasma membrane and tonoplast were upregulated, with at least a 2.34-fold (up to 11.97-fold) higher expression in anthers from the saline vs. non-saline habitat (Table 4). In addition, Cluster-5936.0 (encoding potassium channel AKT1) was upregulated 3.37-fold and Cluster-2772.33146 (encoding KEA) was upregulated 1.42-fold in the anthers of plants from the saline habitat. The expression levels of several reactive oxygen species (ROS) scavenging-related genes were also higher in *E. angustifolia* anthers from the saline habitat, including genes encoding catalase (CAT), peroxidase, and superoxide dismutase (SOD); the expression levels of these genes were at least 1.32-fold (up to 9.42-fold) higher in the anthers of plants from the saline vs. non-saline habitat. In particular, NHX6, four catalases, two PODs and one SOD were inducibly expressed the saline habitat (Table 4). Significant ($P < 0.05$) higher ROS content was detected in the anthers and the bloomed flowers of *E. angustifolia* grown in the saline vs. non-saline habitat, whereas no inhibition was observed in the flowers development of these plants (Fig. S4). The increased expression levels of ROS scavenging-related genes were likely responsible for the ROS homeostasis in *E. angustifolia* from saline habitat.

Table 4 Upregulated genes in *Elaeagnus angustifolia* anthers from the saline habitat related to ion transport and reactive oxygen species (ROS) scavenging based on RNA-seq.

Gene ID	Putative Function	Non-saline readcount	Saline readcount	log2FC	Regulated
Sodium ion transport					
ter-2.125705	Na ⁺ /H ⁺ exchanger (NHX6)	6	0	12.83	6.34 up
ter-2.37266	Na ⁺ /H ⁺ antiporter (NHX1)	1	16.25	150.05	3.20 up
ter-2.84379	Na ⁺ /H ⁺ exchanger (NHX2)	2	55.32	369.82	2.73 up
ter-2.84388	Na ⁺ /H ⁺ exchanger (NHX3)	3	10.24	52.07	2.34 up
Potassium transport					
ter-3.0	potassium channel AKT1-like	14.85	14.85	308.41	4.37 up
ter-2.33146	K ⁺ efflux antiporter 2	4.27	4.27	22.87	2.42 up
Catalase (CAT)					
ter-2.88012	catalase isozyme 1	0	0	40.70	8.01 up
ter-2.87140	catalase	0	0	6.91	5.45 up
ter-2.72350	catalase family protein	0	0	63.08	8.64 up
ter-2.46067	catalase	0	0	24.73	7.29 up
ter-2.72352	catalase family protein	1827.62	1827.62	4796.81	1.39 up
Peroxidase (POD)					
ter-1.1	peroxidase 27-like	0	0	19.25	6.93 up
ter-3.0	peroxidase 51	0	0	9.40	5.89 up
ter-2.78073	peroxidase 55-like	72.78	72.78	559.71	2.94 up
ter-2.116977	peroxidase 57-like	22.13	22.13	2151.71	6.60 up
ter-2.108903	peroxidase 31-like	148.97	148.97	1606.99	3.43 up
ter-3.0	peroxidase 3-like	12.29	12.29	163.92	3.73 up
ter-2.50083	peroxidase 29	71.46	71.46	179.25	1.32 up
ter-2.123580	peroxidase N1-like	3.395	3.395	53.65	3.98 up
ter-2.48594	cationic peroxidase 1-like	327.05	327.05	8882.93	4.76 up
Superoxide dismutase (SOD)					
ter-2.115555	Iron/manganese superoxide dismutases	0	0	108.42	9.42 up
ter-2.40719	superoxide dismutase 2Ba	158.33	158.33	830.60	2.39 up
ter-2.105888	Cu/Zn-superoxide dismutase	118.94	118.94	506.27	2.09 up
ter-	superoxide dismutase	370.62	370.62	1074.87	1.53 up

DEGs involved in ethylene biosynthesis and signal transduction in *E. angustifolia* anthers

Organs falling off of plants are closely related to the ethylene content and signal transduction in plants. In order to investigate the relationship between the flowers falling and the ethylene, the DEGs involving in hormone ethylene, such as ethylene biosynthesis and signal transduction, between anthers of two habitats were analyzed. Expression levels of three S-adenosyl-L-homocysteine hydrolase A encoding DEGs, three 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) encoding DEGs, and two 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) encoding DEGs were all down-regulated in the anthers in saline habitat vs non-saline habitat (Table S6).

Discussion

How are plant growth and reproduction affected when halophytes are grown in non-saline soil? In the present study, when the halophyte *E. angustifolia* was grown in the saline habitat of Dongying, Shandong province, China, it grew well, bloomed normally, and bore fruits from the remaining flowers, thus completing its life cycle (Fig. 1a, c), which also occurred in the saline habitat of the Halophyte Garden in Ji'nan, Shandong province. However, although *E. angustifolia* plants grown in the non-saline habitat of Shandong Ji'nan Spring Park formed flower buds, the flowers opened but all of them fell off of the plant, and no fruits were born (Fig. 1b, d). And the falling of flowers might relate to the relative higher ethylene biosynthesis in the anthers of *E. angustifolia* in non-saline habitat than in the saline habitat (Fig. S5). These results indicate that salinity is likely a clue signal promoting for the reproduction of *E. angustifolia*. Similarly, when the halophyte *S. salsa* was grown in the presence of a certain concentration of NaCl, the seed yield increased and the seeds were larger compared to plants grown in the absence of NaCl [30].

Blocked anther dehiscence inhibits fertilization of *E. angustifolia* in non-saline habitats

The development of male reproductive organs is reflected by pollen vitality. To explore whether flowers but no fruits formed in *E. angustifolia* plants grown in the non-saline habitat due to impaired pollen development, we examined pollen vitality in plants grown in both habitats. Alexander staining indicated that differences in pollen vitality were not the reason for the reproductive differences between the two habitats, as pollen vitality reached 80% under both conditions (Fig. 2a, b). An in vitro pollen germination assay showed that the pollen grains of *E. angustifolia* plants grown in the non-saline habitat were well developed, could germinate in vitro, and formed pollen tube (Fig. 2d). The pollen germination percentages in both habitats were >50% (Fig. S1 a), and there was no significant difference in pollen tube length between the two habitats (Fig. S1 b). These results indicate that the flowering and lack of fruit in *E. angustifolia* plants grown in the non-saline habitat was not caused by pollen dysplasia, as the pollen was well development and produced pollen tubes in vitro. Perhaps salinity helps maintain normal male reproductive development and allows *E. angustifolia* to finish its lifecycle. Indeed, *S. salsa* displayed enhanced anther development under NaCl vs. control conditions [56].

In addition to male reproductive organ development, plant reproduction is also influenced by the development of female reproductive organs [57]. We therefore examined the stigma receptivity of *E. angustifolia* plants from both habitats during the pollen vitality experiment. When the stigmas showed high receptivity (Fig. 3), the pollen also showed high vitality (Fig. 2), indicating that the pollen and stigma reached maturity simultaneously. However, there were no pollen tubes in the pistils of *E. angustifolia* plants from the non-saline habitat after natural pollination based on aniline blue staining (Fig. 4b), and no visible pollen grains on the stigmas were detected (Fig. 4e). Interestingly, following hand pollination, numerous pollen tubes formed in the styles of *E. angustifolia* plants from the non-saline habitat (Fig. 4c), with abundant pollen on the stigmas (Fig. 4f). Furthermore, many pollen grains were present in the anthers of *E. angustifolia* from the non-saline habitat after natural pollination (Fig. 5b, d), with no obvious cracks (Fig. 5d). By contrast, there were almost no pollen grains in the anthers of *E. angustifolia* plants from the saline habitat after anther dehiscence (Fig. 5a, c). Obvious crack in anthers of *E. angustifolia* in saline habitat were observed through the anther section results, while not in anthers of *E. angustifolia* in non-saline habitat (Fig. S2). It is speculated that the pollination in *E. angustifolia* flowers in non-saline habitat was inhibited.

We conclude that the stigma could accept pollen and could form pollen tubes in the styles and that the flowering and lack of fruit in *E. angustifolia* in the non-saline habitat is due to poor anther dehiscence, which prevents the pollen from being released, thus inhibiting fertilization. To explore the molecular mechanism underlying the differences in anther development in *E. angustifolia* from saline vs. non-saline habitats, we performed RNA-seq analysis of these tissues.

Ion and ROS balance in flowers promote anther development of *E. angustifolia*

In general, salinity affects the survival and inhibits the growth of trees due to reduced root growth and material absorption [5, 6]. Non-halophytes are sensitive to salt stress [58-60]. In addition, salinity inhibits the reproduction of non-halophytes by decreasing fertility and reproductive organ formation [61, 62]. Salt stress specifically affects male gamete development, typically resulting in high levels of microspore abortion and the induction of male sterility [63, 64]. Salt treatment reduces fertility in rice [65]. However, the growth of halophytes is significantly enhanced by the presence of moderate salinity. The seed number of the halophyte *Cakile maritima* significantly increased in plants treated with 50–100 mM NaCl, which was also optimal for vegetative growth [66]. In the halophyte *S. salsa*, vegetative growth [67] and reproductive growth [30] are enhanced when the growth medium contains a certain concentration of NaCl.

When grown in saline environments, plants induce measures to deal with the damage caused by salt ions [68]. Ion balance in plant cells is crucial for plant development under saline conditions, especially the maintenance of low levels of Na⁺ in plant cells. Crop yield decreases when higher concentrations of Na⁺ accumulate in plant cells [58]). Four genes encoding Na⁺/H⁺ exchangers were expressed at higher levels in *E. angustifolia* anthers from the saline vs. non-saline habitat (Table 4). Three (NHX1, NHX2, and NHX3) of them were localized in the tonoplast, the other (NHX6) was localized in vesicle membrane, and all were

associated with intracellular ion homeostasis and flowering [69]. Perhaps a constant concentration of Na^+ is maintained in *E. angustifolia* flowers in the saline habitat (Fig. 6a), thus plays important roles in improving anther development. Similarly, certain concentrations of salt ions (such as Na^+ and Cl^-) are maintained in the tissues of the halophyte *S. salsa* in the presence NaCl , which enhances reproduction [56]. Potassium plays a vital role in carbohydrate metabolism during plant reproduction [70]. We determined that a desirable concentration of K^+ was maintained in *E. angustifolia* flowers (Fig. 6b), perhaps due to the high expression of potassium channel gene *EaAKT1* (Table 4). Additionally, a desirable K^+ concentration is beneficial for sugar transport in *E. angustifolia* anthers. A sufficient supply of materials for reproductive growth in *E. angustifolia* is maintained under salinity conditions, as revealed by the increased expression of genes related to sucrose synthase and sugar transport (Table S5).

Finally, peroxidase plays vital roles in maintaining ROS homeostasis in plants, which is required for proper anther development. Arabidopsis mutants with non-functional *PEROXIDASE9* (*PRX9*) and *PRX40* exhibit male sterility [71]. In the current study, 18 DEGs related to the ROS scavenging system (such as catalase, peroxidase, and superoxide dismutase) were upregulated in *E. angustifolia* anthers in the saline vs. non-saline habitat, and some genes for ROS scavenging enzymes such as catalase isozyme 1, peroxidase 51 were induced by salinity (Table 4). These results indicate that the ROS scavenging enzymes of *E. angustifolia* are induced during growth under saline conditions, thus maintaining anther development.

Successful dehiscence depends on cell wall development related-gene expression

The process of anther dehiscence is affected by many factors and involves a series of steps, such as differentiation of the anther cellular layer, secondary cell wall thickening in the endothecium, dehydration, and stomium cell wall digestion [72]. In flowering plants, successful reproduction benefits from the establishment of the anther wall, which ensures the successful production and release of fertile pollen grains [73]. During anther development, lignin is deposited in endothecium cells in preparation for anther dehiscence. In Arabidopsis, CAD participates in lignin biosynthesis; mutants lacking a functional *CAD* gene exhibit failed anther dehiscence [50]. In *E. angustifolia*, three DEGs encoding CAD were upregulated in anthers under saline vs. non-saline conditions; in particular, cinnamyl alcohol dehydrogenase 1 was inducibly expressed upon exposure salinity (Table 1).

The thickening of the endothecium in anthers is facilitated by receptor protein kinase (RPK) activity. Indeed, Arabidopsis *rpk* mutants show male sterility due to disrupted metabolism in the tapetum, along with the suppressed expression of *RPK* [51]. Similarly, expression of *receptor protein kinase 1* (*RPK1*) was induced by salinity, which perhaps contributes to the successful dehiscence of *E. angustifolia* anthers in the saline habitat. Just before anther dehiscence, the septum and endothecium were degenerated with the regulation of *SIZ1* [52]. Indeed, the rice *siz1* mutant and plants lacking *SIZ1* activity exhibited a distinct phenotype including indehiscent anthers [52]. In *E. angustifolia*, two *SIZ1* isoenzymes were expressed at higher levels in the anthers of plants from the saline vs. the non-saline habitat (Table 1), which might contribute to anther development and guarantee successful anther dehiscence. Finally,

several genes related to cell wall modification were expressed at higher levels in *E. angustifolia* anthers from the saline vs. non-saline environment (Table S4). The high expression levels of these genes could facilitate anther development in *E. angustifolia*, ensuring the production of viable pollen and anther dehiscence.

Successful dehiscence involves TFs and hormone related-gene expression

Various TFs also play roles in anther dehiscence. For example, NAC TFs play important roles in the secondary thickening of cell walls, which is required for anther dehiscence in Arabidopsis [53, 74]. Indeed, the expression levels of 11 DEGs encoding NAC TFs were higher in *E. angustifolia* anthers from the saline habitat compared to the non-saline habitat (Table 3). Moreover, WRKY TFs play pivotal roles in fertility development. In Arabidopsis, male fertility is related to the expression level of *WRKY27*, as too high or too low a level of *WRKY27* expression leads to defective anther dehiscence [55]. In Arabidopsis, the development of stamens was also regulated by MYB TFs [54]. In *E. angustifolia* anthers from the saline habitat, nine DEGs encoding WRKY TFs and five DEGs encoding MYB TFs were expressed at higher levels compared to anthers from the non-saline habitat (Table 3). The successful anther development and dehiscence of *E. angustifolia* anthers in the saline habitat might be due, at least in part, to the relatively high expression levels of TF genes, particularly to inducible expression of NAC domain-containing protein 82, MYB 44, MYB6, MYB39 and WRKY40.

In flowering plants, anther development is influenced by auxin and GA contents [75, 76]. For example, auxin plays a vital role in the coordination of anther dehiscence [77]. In Arabidopsis, stamen elongation and anther dehiscence are impaired by altered auxin transport [78]. Perhaps the induced expression of genes related to GA (*gibberellin receptor GID1 B*) and auxin transport (*auxin transporter-like protein 2*) facilitate early anther development in *E. angustifolia* grown in the saline habitat (Table 2), allowing the plants to finish flowering and form fruits. Similarly, in the halophyte *S. salsa*, NaCl treatment promoted reproduction and increased the levels of auxin and GA in floral organs [79].

In Arabidopsis, anther dehiscence is also affected by a jasmonic acid (JA)-mediated pathway. JA biosynthesis is catalyzed by the enzyme jasmonic acid-amino synthetase (JAR) [49]. In addition, JA biosynthesis is correlated with auxin levels [80]. In *E. angustifolia*, two genes encoding JAR were expressed at higher levels and two genes encoding JAR were induced in anthers from the saline habitat (Table 2), suggesting that these genes are involved in the anther dehiscence of *E. angustifolia* in saline habitat.

Conclusions

In the saline habitat, *E. angustifolia* grew well, bloomed, and underwent successful pollination to form fruits. By contrast, in the non-saline habitat, the plants bloomed, but all of the flowers fell off of the plants. There was no significant difference in pollen vitality or stigma receptivity in *E. angustifolia* flowers between the two habitats. However, the anthers significantly differed in these habitats after natural pollination. Numerous pollen grains were observed in *E. angustifolia* anthers in the non-saline habitat

after pollen release. The lack of fruit in *E. angustifolia* in the non-saline habitat was due to poor pollen release, preventing the pollen from coming in contact with the stigma, thereby preventing pollination.

In the saline habitat, the reproductive development of *E. angustifolia* was better due to successful anther development, along with the induced and enhanced expression of genes related to anther cell wall thickening and JA biosynthesis; these genes function in anther development and tapetum degeneration. The induced and increased expression levels of genes encoding a Na^+/H^+ exchanger and potassium channel ensured that relatively low Na^+ and relatively high K^+ levels were present in anthers in the saline habitat. The increased Na^+ content in the leaves of *E. angustifolia* plants in the saline habitat possibly acts as a signal. On the one hand, Na^+ promotes the auxin-induced pathway in conjunction with JA biosynthesis, in which the anther dehiscence was properly regulated due to the induced and enhanced expression of genes such as *LOX*, *AOC*, and *JAR*. On the other hand, Na^+ induced or upregulated the expression of the related TF genes, such as those for MYB and NAC TFs, and the genes involving in the programmed thickening and degeneration of the anther wall, for example, the genes related to the lignin biosynthesis (*CAD*), the endothecium thickening (*RPK*), and the septum and endothecium degeneration (*SIZ1*), finally caused the anther dehiscence in *E. angustifolia* plants in the saline habitat. Conversely, the anther dehiscence was blocked in *E. angustifolia* plants in the non-saline habitat due to the lack of appropriate leaf Na^+ and induction of anther dehiscence-related genes, thereby preventing anther dehiscence and pollen release. These findings are incorporated into a working model describing how anther dehiscence is regulated in *E. angustifolia* in saline and non-saline habitats (Fig. 8). This study lays the foundation for further investigating the regulation of salinity responses in halophytes during reproductive development.

Methods

Samples

E. angustifolia trees (two separate individuals in each habitat with more than 10 years' age were selected, and they were grown naturally without artificial fertilization and artificial watering) in Ji'nan Quancheng Park and Halophyte Garden in Dongying, China were analyzed in this study. Ji'nan Quancheng Park (N 36°40'; E117°00') is a region with non-saline soils with a Na^+ content of ca. 0.014–0.015%, and mean annual precipitation of 650–700 mm, a relative lower pH and EC (6.9 and 29 mS cm^{-1}) and a higher organic matter content (16.7 g kg^{-1}) was detected (Table S1). Halophyte Garden (N 37°27'; E118°30') is located in a region with typical saline and alkaline soil, with a Na^+ content of ca. 0.5–1.2% and mean annual precipitation of 570–690 mm, a relative higher pH and EC (7.9 and 982 mS cm^{-1}) and a lower organic matter content (6.3 g kg^{-1}) was detected (Table S1). During the growth period of *E. angustifolia*, particularly the flowering and fruiting periods, there was no significant difference in temperature, mean rainfall, or relative humidity between Ji'nan Quancheng Park and Halophyte Garden in Dongying. The samples used in the present study were planted by Shandong Normal University with the permission of

Shandong province (China). The sample collection and the performance of experimental research on such plant were complied with the national guidelines of China.

Observing the flowering process in *E. angustifolia*

To identify the stage of reproductive growth that differs in *E. angustifolia* grown in the two habitats, flowers were observed during the reproductive period from April to June. The process of anthesis was observed every 3 days by visual observation and under a stereoscope (Nikon SMZ745T, Japan).

Analysis of pollen development in *E. angustifolia*

To determine whether pollen development and fertility differed between the two habitats, pollen grains were collected from fresh anthers immediately after anther dehiscence, and pollen viability was assessed as described in Alexander [81], allowing non-aborted and aborted pollen grains to be distinguished. Pollen germination was detected in vitro using 35 mm diameter plastic dishes containing germination medium [82]. After 2 h of incubation at 25 °C in a germination chamber, the pollen grains were observed under a microscope (Nikon 80i Eclipse, Japan). A pollen grain was considered to germinate with the pollen tube length was double the grain diameter. At least five duplicates were performed per experiment, and pollen grains in five fields of vision were counted in each dish.

Examining stigma receptivity in *E. angustifolia*

During the pollen viability and in vitro germination tests, ten flowers from plants in both saline and non-saline habitats were selected randomly, and the stigmas were tested immediately for peroxidase activity as follows. Intact pistils were excised, placed into depression slides, and immersed in a mixture of freshly made benzidine and H₂O₂ (1% benzidine in 60% ethanol, 3% hydrogen peroxide, and water, 4:11:22, by volume) to estimate stigma receptivity [83, 84]. In the presence of peroxidase, the benzidine is stained by oxidation when the hydrogen peroxide is broken down. If the stigma is receptive, it is surrounded by blue staining and many bubbles.

Observation of pollen tube growth in situ

To observe pollen tube growth in the style, after self pollination, the pistils of *E. angustifolia* under two habitats were excised, immersed in freshly prepared FAA (formalin: acetic acid: 50% ethanol, 1:1:18 by volume; FAA), and incubated for 24 h at 4°C. The samples were washed three times with PBS, incubated overnight in 8.0 M NaOH, and washed at least three times (1 h each time) with distilled water. The samples were stained in 0.1% aniline blue solution [85] for 30 min, loaded onto slides, and observed under a fluorescence microscope (ECLIPSE 80i, Nikon, Japan) with UV excitation of 330–380 nm.

Observation of pollen germination in situ after hand pollination of *E. angustifolia* in the non-saline habitat

For hand pollination, one day prior to anthesis, at 19:00 hours, flowers that were about to open were randomly selected. The anthers were artificially removed, and the emasculated flowers were placed over

soaked florist's foam at room temperature. The following day (anthesis), at 9:00 hours, the flowers were hand pollinated with a paintbrush loaded with pollen and incubated at room temperature. Previous work carried out in peach showed that emasculation did not interfere with hand pollination [86]. At 4 h after artificial pollination, the pistils were collected and fixed in freshly prepared FAA as described previously.

Observation of anthers after pollen release

After pollen release, anthers were collected from *E. angustifolia* grown in two habitats and fixed in freshly prepared FAA for 24 h. Following incubation in HCG solution [87] (chloral hydrate: glycerol: gum arabic: distilled water=100 g: 5 ml: 7.5 g: 30 ml) for 40 min, the samples were observed by differential interference contrast microscopy (DIC, ECLIPSE 80i, Nikon, Japan).

Quantification of ions in leaves and flowers

At the same stage of anther development before flower blooming, the leaves and the flowers of the same leaf axils were collected from *E. angustifolia* trees grown in saline and non-saline habitats and subjected to ion detection (Na^+ and K^+ content). Na^+ and K^+ extraction and measurement were performed as described by Guo et al. [56].

Quantification of ROS in anthers and flowers

To detect the ROS homeostasis in the flowers of *E. angustifolia* trees grown in saline and non-saline habitats, the anthers, flowers before bloom and in blooming were collected, and the ROS contents were detected using the Plant (ROS) Elisa Kit (RF1305).

Total RNA extraction, library construction, and sequencing

In order to obtain more related genes as possible that were participated in the regulation of the development and dehiscence of anthers in *E. angustifolia*, anther materials with earlier development stages were collected from *E. angustifolia* trees grown in saline and non-saline habitats and used for RNA sequencing. Three biological replicates were performed per treatment. Total RNA was extracted from anthers and the integrity and concentration of the RNA were measured as described by Guo et al. [56]. Sequencing libraries were generated using a NEBNext Ultra™ RNA Library Prep Kit for Illumina following the manufacturer's recommendations (NEB, USA). The final cDNA library was constructed using Illumina HiSeq™ 4000 at Novogene Technologies Co. Ltd., Beijing, China.

Transcriptome assembly and detection of differentially expressed genes

Final *de novo* transcriptome assembly was carried out based on the obtained clean reads using Trinity [88] with default parameters, followed by clustering with Corset [89]. The assembled unigenes, which were expressed in the anthers of *E. angustifolia* plants grown in saline and non-saline habitats, were annotated as previously described [56]. DEGs between anthers from saline vs. non-saline habitats were detected using DESeq. The threshold adjusted *P* values < 0.01 and $|\log_2(\text{fold change})| > 1.5$ was used to

judge significant differences in gene expression. A unigene was generated based on each “cluster”. The assembled sequences were mapped by performing BLAST searches against seven databases as described in Guo et al. [56]. The raw data of RNA-seq have been deposited in SRA database with accession number SRR11446326, SRR11446325, SRR11446324, SRR11446323, SRR11446322 and SRR11446321.

Quantitative real-time PCR

To verify the result of RNA-Seq, 11 DEGs annotated to pathways involved in anther development were selected and subjected to qPCR. The primers used for qPCR were designed using Beacon Designer software (version 7.0) and are shown in Table S2. The *ACTIN* gene (Gene id: cluster-2772.56507) was used as the internal standard. qPCR and analysis of the results were carried out as described by Guo et al. [56].

Statistical analysis

The results are presented as means \pm standard deviation (SD). Statistical analysis was carried out using SPSS software (version 17) and one-way ANOVA; different letters in the figures indicate a significant difference of the means (at $P < 0.05$) based on Duncan's test.

Abbreviations

CBL: CALCINEURIN B-LIKE; GA: gibberellic acid; RNA-seq: RNA-sequencing; DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia Genes and Genomes; qPCR: Quantitative real-time PCR; SCWs: secondary cell walls; LOX: Lipoxygenase; AOC: allene oxide cyclase; ACC oxidase: aminocyclopropane carboxylate oxidase; RPK: receptor protein kinase; NHX: Na⁺/H⁺ antiporter; AKT: inward-rectifying K⁺ channel; KEA: K⁺ efflux antiporter; PRX: PEROXIDASE; ROS: reactive oxygen species; CAT: catalase; SOD: superoxide dismutase; JA: jasmonic acid; JAR: jasmonic acid-amino synthetase; SD: standard deviation;

Declarations

Ethics approval and consent to participate

All experiments in the manuscript were performed at Shandong Provincial Key Laboratory of Plant Stress, and they are in compliance with relevant laws in China.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials that were analyzed in the current study could be available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author' contributions

JG and BW designed the experiments. JG performed the experiments. JG and BW wrote the manuscript. All authors have read and approved the manuscript.

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Figures



Figure 1

Elaeagnus angustifolia plants have flowers with fruits in the saline habitat and no fruits in the non-saline habitat. The plants were observed from early May to late June 2010–2016. (a) Flowers at the full-bloom stage in the saline habitat. (b) Flowers at the full-bloom stage in the non-saline habitat. (c) Branches with fruits in the saline habitat. (d) Branches with no fruits in the non-saline habitat.

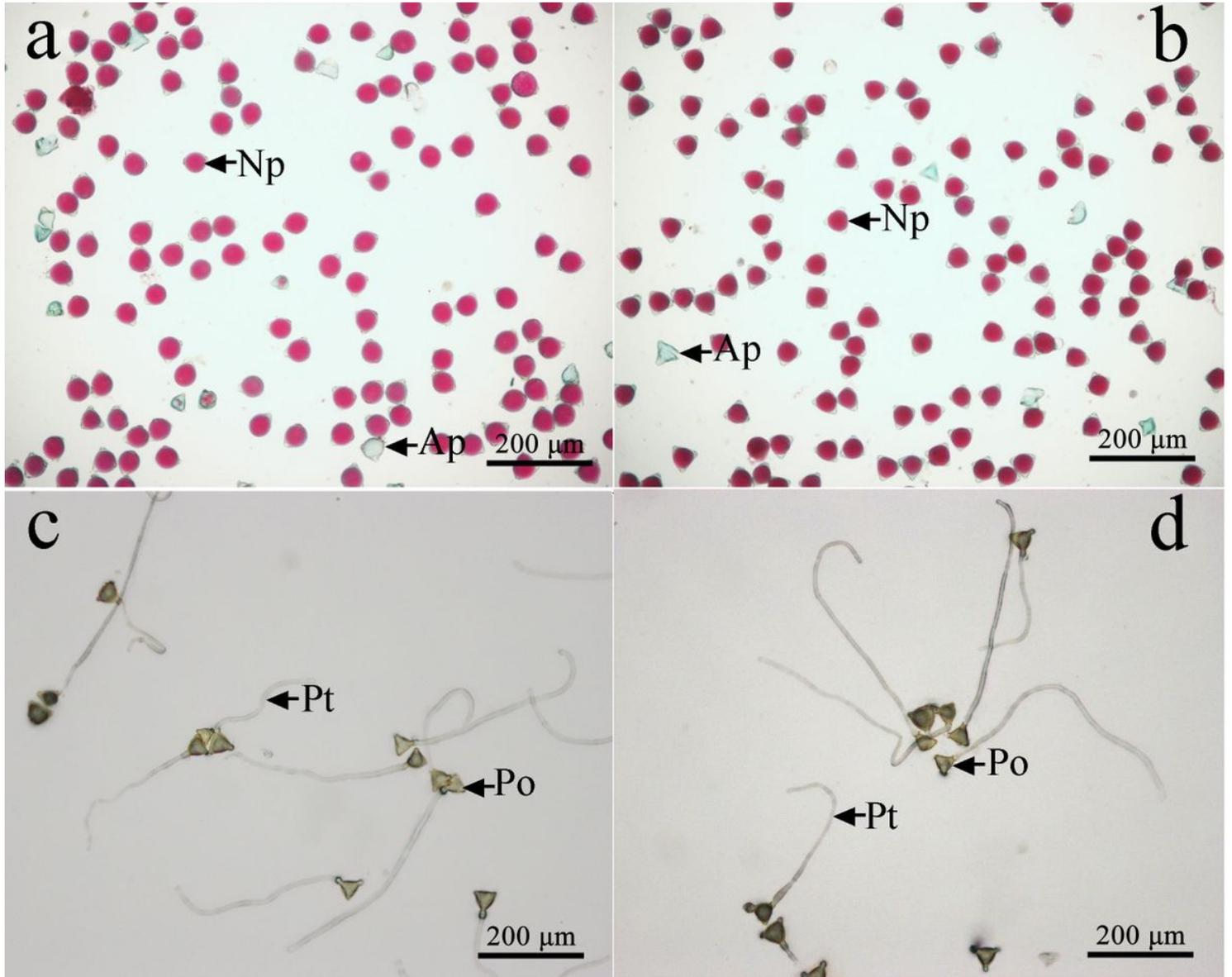


Figure 2

No significant difference in pollen viability and in vitro pollen germination in *Elaeagnus angustifolia* grown in saline (a, c) and non-saline (b, d) habitats. The aborted pollen grains were stained green, and the non-aborted pollen grains were stained red. (a) Staining of pollen grains from the saline habitat. (b) Staining of pollen grains from the non-saline habitat. (c) Pollen germination in vitro from the saline habitat. (d) Pollen germination in vitro from the non-saline habitat. Ap, aborted pollen; Np, normal pollen; Po, pollen; Pt, pollen tube. Bar, 200 μm.

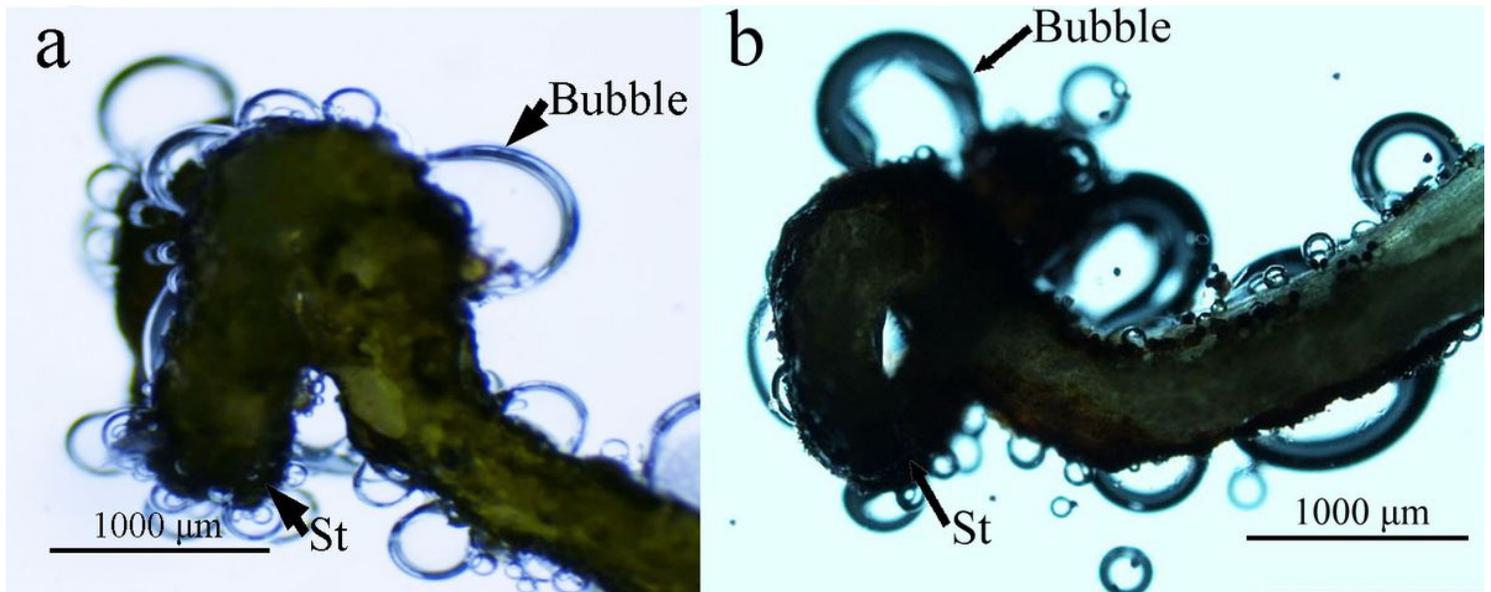


Figure 3

No significant difference in stigma receptivity in *Elaeagnus angustifolia* grown in saline (a) and non-saline (b) habitats. St, stigma. Bar, 1000 µm.

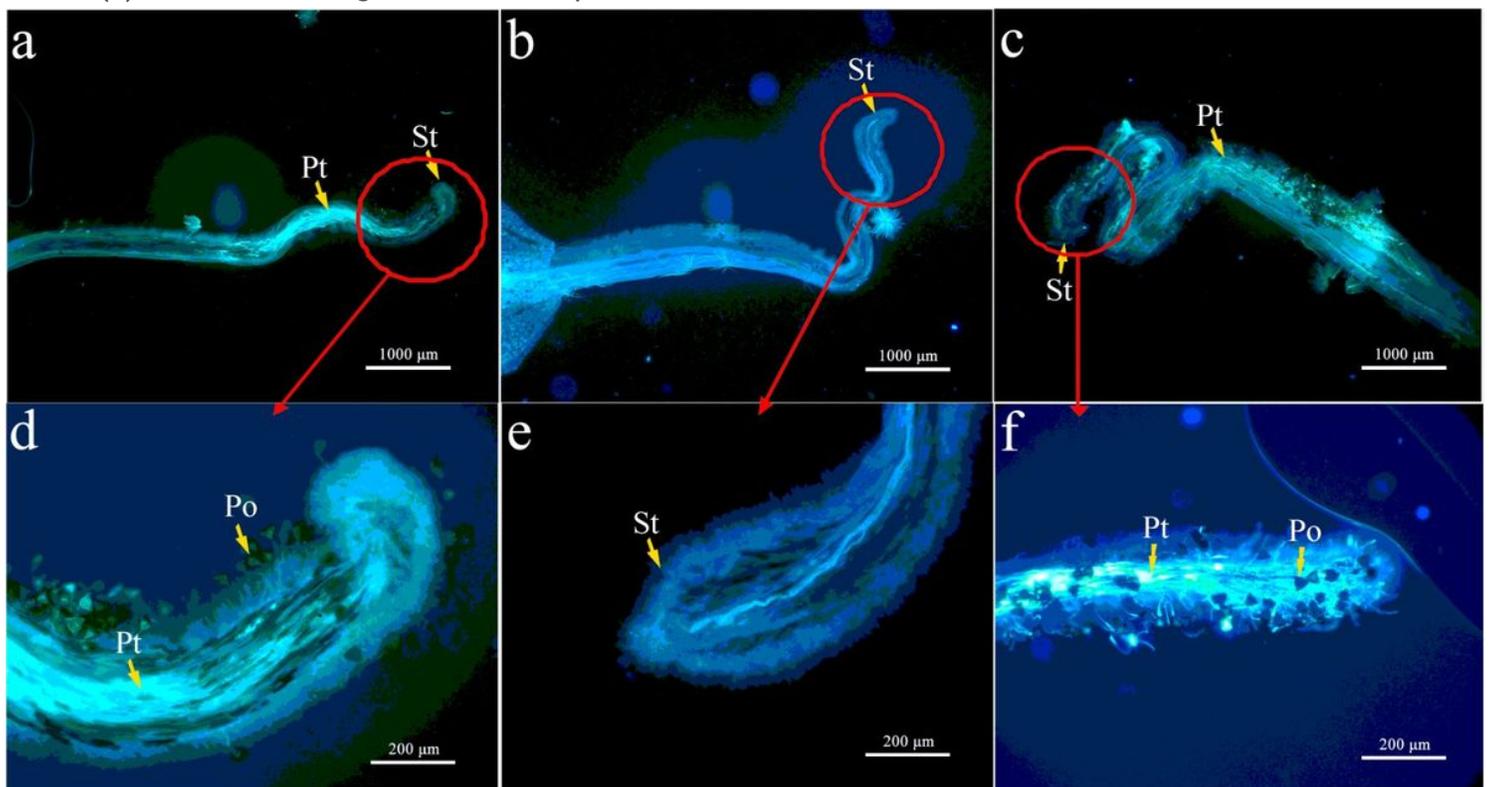


Figure 4

Stigmata of *Elaeagnus angustifolia* from the non-saline habitat lack pollen and pollen tubes under natural conditions but contain numerous pollen tubes after hand pollination. The styles were stained with aniline blue and observed under a fluorescence microscope. (a, d) *Elaeagnus angustifolia* pistils from the saline habitat after natural pollination. (b, e) *Elaeagnus angustifolia* pistils from the non-saline habitat after

natural pollination. Note that no pollen tubes were present in the style. (c, f) *Elaeagnus angustifolia* pistils from the non-saline habitat after hand pollination. St, stigma; Po, pollen; Pt, pollen tubes. Bar, 1000 μm (a, b and c), 200 μm (d, e and f).

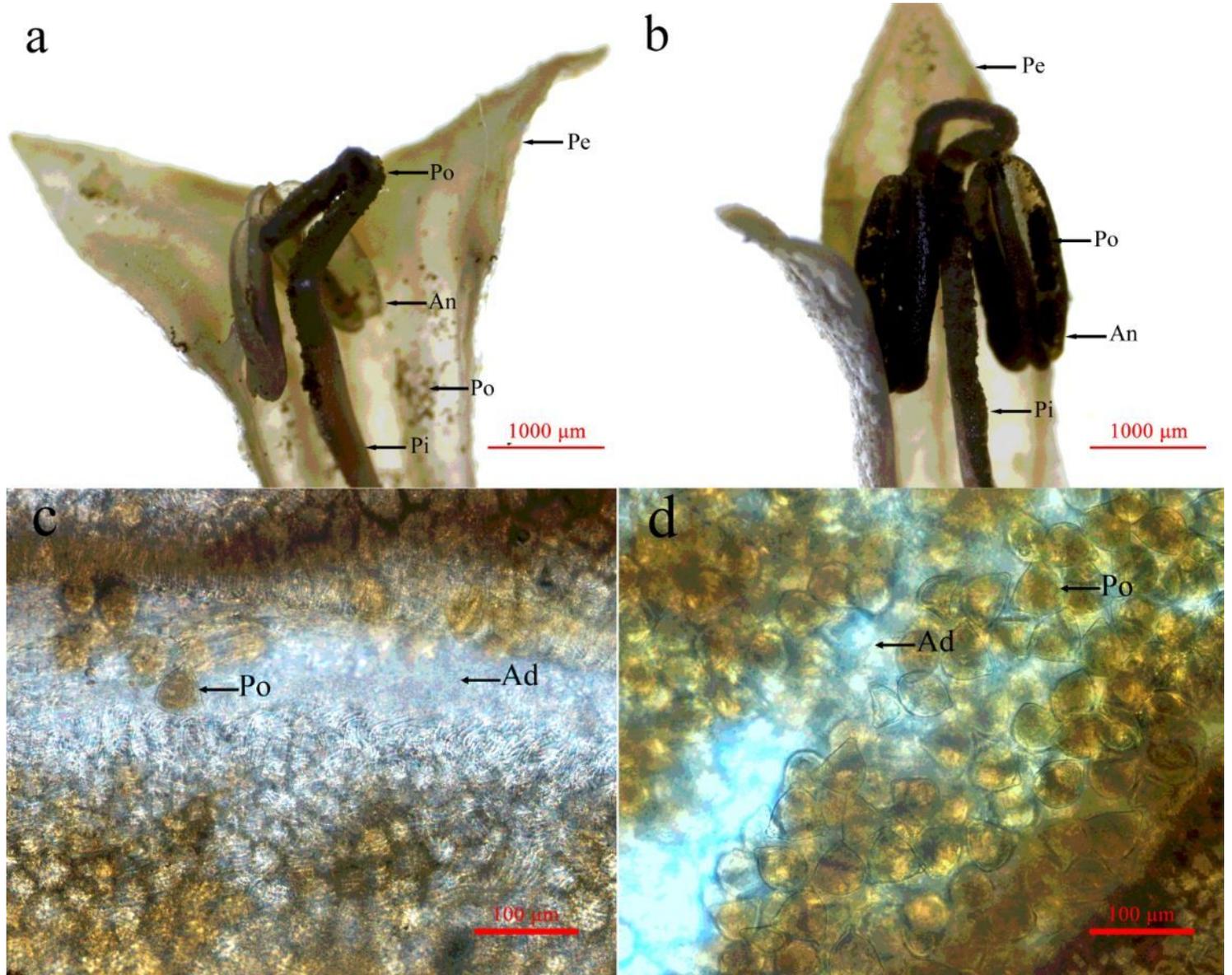


Figure 5

No pollen grains form in the anther in the saline habitat (a and c), whereas numerous pollen grains form in the non-saline habitat (b and d) of *Elaeagnus angustifolia* after anther dehiscence. Po, pollen; Pe, petal; An, anther; Pi, pistil; Ad, anther dehiscence. Bar, 1000 μm (a and b), 100 μm (c and d).

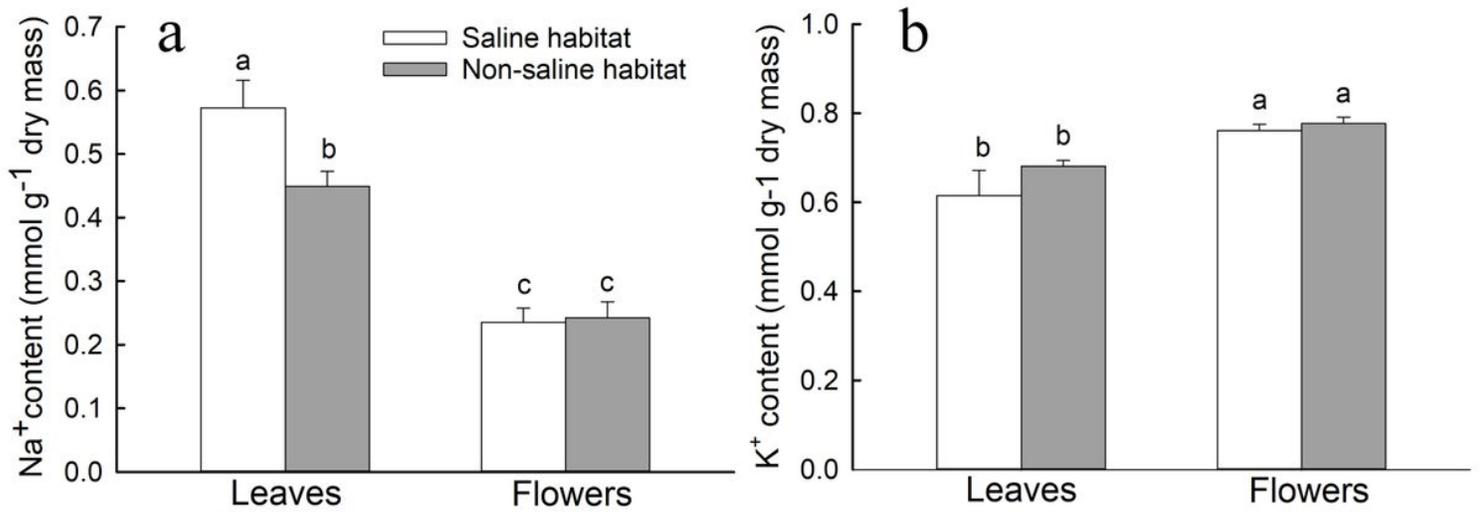


Figure 6

Na⁺ (a) and K⁺ (b) contents in the leaves and flowers of *Elaeagnus angustifolia* grown in the saline and non-saline habitat. Data are means (n=3) ± SD. Different letters indicate a significant difference at P<0.05.

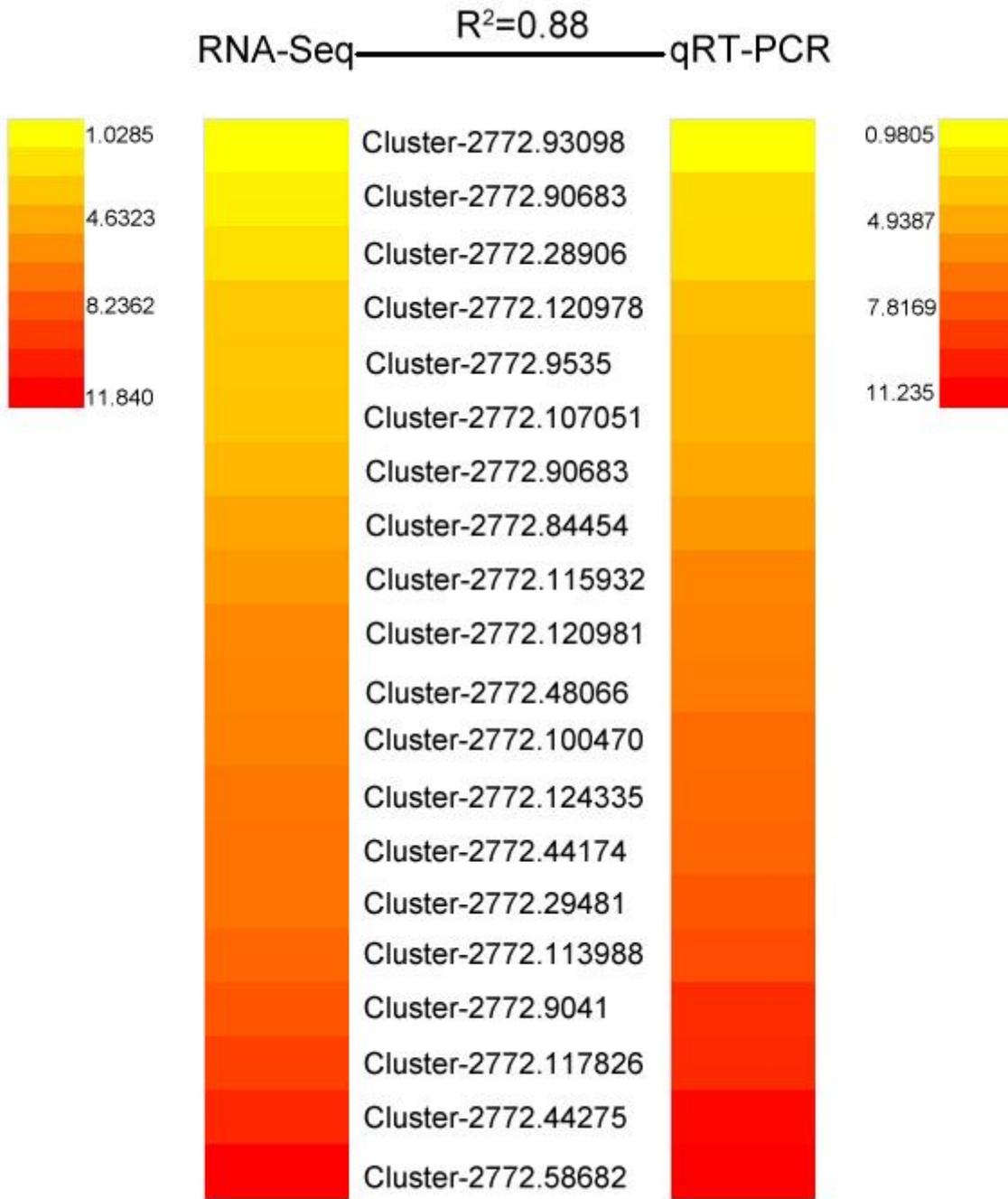


Figure 7

Validation of the RNA-seq results via quantitative real-time PCR (qPCR). The relative expression levels of 11 DEGs identified were analyzed by qPCR. The histogram displays the relative expression levels based on qPCR. Primers and annotations of the 11 DEGs are listed in Supplementary Table S2.

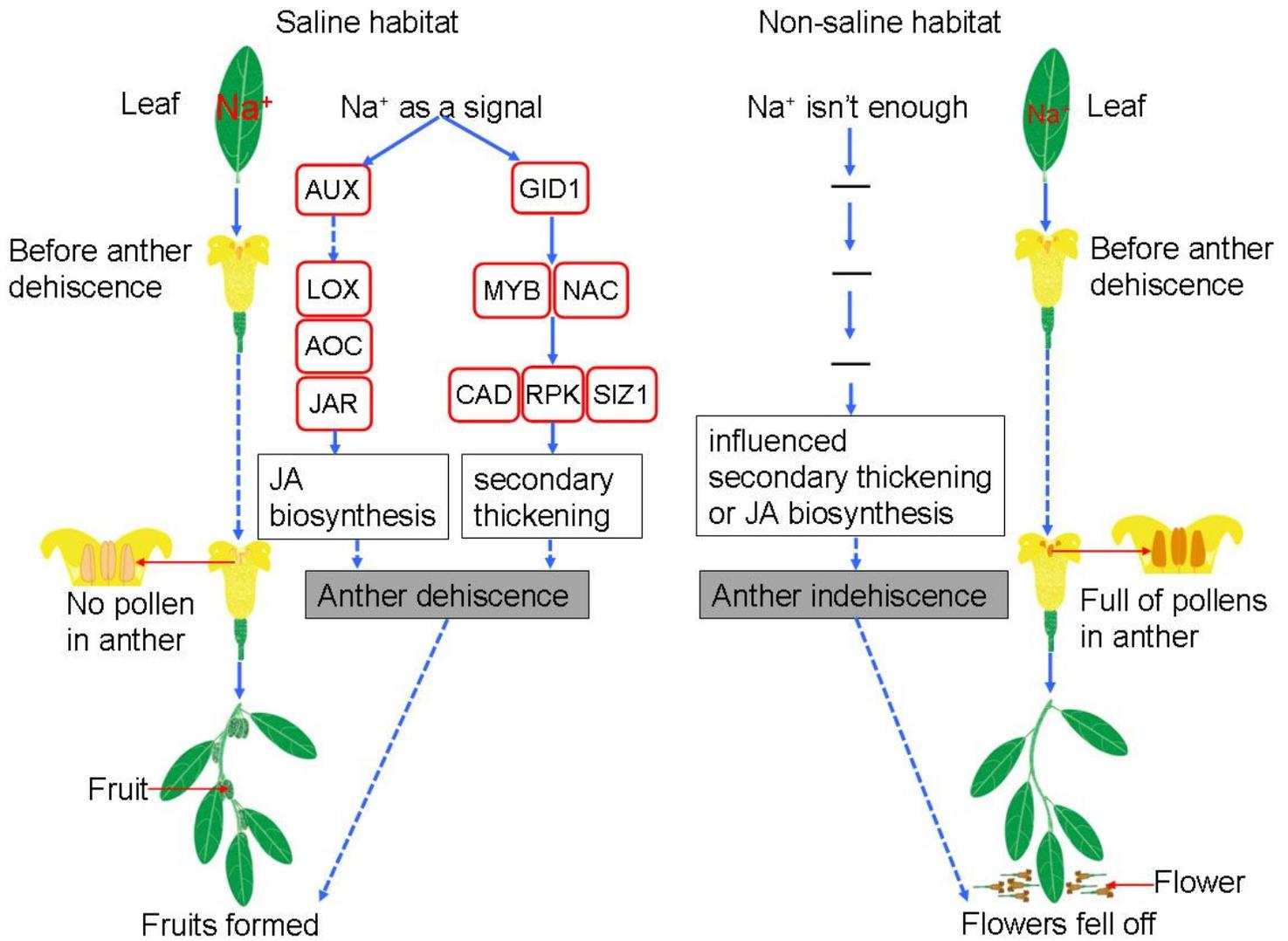


Figure 8

Working model describing the regulation of anther dehiscence in *Elaeagnus angustifolia* in saline and non-saline habitats. In *E. angustifolia* grown in a saline habitat, reproduction is completed due to successful anther dehiscence, which is mediated by the induced and enhanced expression of genes related to anther cell wall thickening and JA biosynthesis. An appropriate Na^+ content in the leaves acts as an inducing signal to enhance anther dehiscence by two possible pathways in *E. angustifolia* plants in the saline habitat. One is the auxin-induced pathway in conjunction with JA biosynthesis by inducing and up-regulating the expression of genes such as LOX, AOC, and JAR. The other pathway is the programmed thickening and degeneration of the anther wall via inducing or up-regulating the expression of genes involved in, for example, lignin biosynthesis (CAD), endothecium thickening (RPK), and septum and endothecium degeneration (SIZ1). By contrast, anther dehiscence was blocked in *E. angustifolia* plants in the non-saline habitat due to the lack of appropriate leaf Na^+ and induction of anther dehiscence-related genes, thereby preventing anther dehiscence and pollen release. The sizes of the symbols for Na^+ represent their relative contents in the leaves.

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