Plant Molecular Biology Reporter
https://doi.org/10.1007/s11105-021-01289-4

ORIGINAL ARTICLE

Ectopic Expression of *Erigeron breviscapus JAZ1* Affects JA-Induced Development Processes in Transgenic Arabidopsis

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Received: 19 December 2019 / Accepted: 22 March 2021

Abstract

The jasmonate ZIM-domain (JAZ) proteins are repressors that function in the regulation of plant growth, development, and response to stimulation of different signals in the JA signaling pathway. *Erigeron breviscapus* is characteristic of sporophyte self-incompatibility (SSI). However, whether JA signaling is involved in regulation of development processes in *E. breviscapus* is unclear. In this study, the JAZ homolog *EbJAZ1* was isolated and characterized from *E. breviscapus*. EbJAZ1 was localized to the nucleus, and expressed in roots, stems, leaves and flowers. Ectopic expression of *EbJAZ1* in Arabidopsis resulted in shorter filament and silique length, and lower seed fertility. In addition, MeJA-induced root growth inhibition was compromised in transgenic plants. Further qRT-PCR analysis indicated that expression patterns of marker genes for *VSP1*, *VSP2*, *JAZ1*, *JAZ5*, *JAZ8*, *JAZ10*, *MYC2*, and *bHLH17* were downregulated in transgenic plants compared to wild-type, suggesting that EbJAZ regulates the development of flower organs, seed fertility, and primary root growth through the JA signaling pathway. Thus, our results indicate that EbJAZ1 is one of the important regulators possibly involved in SSI and other developmental processes in *Erigeron breviscapus.*

Keywords *Erigeron breviscapus* · JAZ · Jasmonate pathway · Sterility · Root length

Key message
- EbJAZ1 is one of the important regulators involved in developmental processes in *Erigeron breviscapus*.
- The ectopic expression of EbJAZ1 can regulate the development of flower organs, seed fertility, and primary root growth through the JA signaling pathway.

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Introduction

*Erigeron breviscapus i*s a famous Chinese herb in Asteraceae, which has obvious effects on the treatment of cardiovascular and cerebrovascular diseases (Pharmacopoeia of the People's Republic of China 2010). Scutellarin is one of the main active components in *E. breviscapus*. At present, there are more than 30 Chinese patent medicines extracted from *E. breviscapus*, with an annual output value of more than 5 billion yuan. Because of its clear active ingredients and strong pharmacological activity, *E. breviscapus* is anticipated to become an international plant medicine in the foreseeable future. Accumulating evidence has shown that jasmonic acid (JA), a powerful and effective inducer, has been widely used to improve the active components of medicinal plants (Lin et al. 2007; Ma et al. 2015; Liu et al. 2008; Huang et al. 2014a, b; Wang et al. 2019). For example, methyl jasmonate (MeJA enhanced the activity of cinnamate-4-hydroxylase (C4H), coumarin CoA ligase (4CL), and stilbene synthetase STS) to increase stilbene-like compounds in *Polygonum multiflorum* (Liu et al. 2016). In addition, MeJA could improve

Published online: 19 February 2022

the biosynthesis of triterpenoid saponins in suspension cells of *Panax notoginseng* (Hu et al. 2008). Recent study has also shown that MeJA-induced activities of the signal pathway in *E. breviscapus* mainly led to re-programming of metabolism and cell activity involved in flavonoids (Chen et al. 2015). However, whether JA is also involved in regulation of other biological processes in medicinal plant is still poorly understood.

Plant growth and development are regulated by coordinating different plant hormones and environment factors. JA has been implicated directly in primary root elongation (Hou et al. 2010; Withers et al. 2012; Debora et al. 2015), anthocyanin accumulation, trichome initiation (Qi et al. 2011; Boter et al. 2015; Xie et al. 2016), stamen development (Cheng et al. 2009; Shan et al. 2015; Zhai et al. 2015), and stress responses (Hu et al. 2013; Wang et al. 2019; Zhang et al. 2019). In the JA signaling pathway, jasmonate ZIM-domain proteins (JAZs) can inhibit plant root elongation and regulate plant fertility and anthocyanin accumulation by interacting with downstream transcription factors MYCs, MYBs, and MBW complex, respectively. When JA was absent, jasmonate ZIM-domain protein (JAZ) protein can interact with transcription factors (MYCs, MYBs, and MBW) resulting in transcription factors not being able to bind to the promoter of downstream target genes, thus inhibiting the function of transcription factors. When JA was present, the functions of the SCF(COI1) ubiquitin ligase and the 26S proteasome were activated. It can specifically degrade JAZ protein through ubiquitination and release the original interacting transcription factors MYCs, MYBs, and MBW. These transcription factors bind to downstream target genes and regulate the expression of target genes (Yan et al. 2009; Sheard et al. 2010; Howe and Yoshida 2019).

Previously, we found that *E. breviscapus* has sporophyte self-incompatibility (SSI) characteristics by self-pollination and cross-pollination (Zhang et al. 2015). Further comparative transcriptomics identified some candidate genes (e.g., *MLPK*, *ARC1*, *CaM*, *EXO70A1*) that may be related to SSI of *E. breviscapus*, although their function has to be verified yet (Zhang et al. 2015). However, recent studies found that the S-RNase controlling the gametophytic self-incompatibility (GSI) in pear (*Pyrus bretschneideri*) and apple (*Malus domestica*) could stimulate JA production by entry of both self and non-self S-RNase into pollen tubes (Shi et al. 2017; Gu et al. 2019). Thus, it is interesting to investigate whether JA could have a role in sporophyte self-incompatibility (SSI) in addition to GSI. To this end, it is the genes associated with the JA signaling pathway that should be characterized in terms of SSI species. In this study, we characterized a gene encoding JAZ protein in *E. breviscapus,* a typical SSI plant species, and found that JA signaling is also involved in sterility and root growth in *E. breviscapus*.

Materials and Methods

Materials and Plant Growth Conditions

The phytohormone derivative MeJA was purchased from Sigma (St. Louis, Missouri, USA). Ex Taq DNA polymerases and SYBR Green Master Mix were purchased from Takara Biotechnology (Dalian, China), and other common chemicals were obtained from Shanghai Sangon (Shanghai, China). The seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) and EbJAZ1-overexpressing *Arabidopsis* lines were grown on Murashige and Skoog (MS) medium with 0.7% (w/v) agar plates, and MeJA-treated plants were grown in media containing 25 μM or 50 μM MeJA. These plates with seeds were placed in an artificial growth chamber at 22 °C under a 10-h light/14-h dark photoperiod. Seedlings were used to measure root length, and RNA was extracted for qRT-PCR.

Bioinformation Analysis

The coding sequence (CDS) of *EbJAZ1* was obtained from *E. breviscapus* genomes database (www.herbal-genome.cn). JAZ protein sequences from other species were acquired from publicly available databases (https://www.arabidopsis.org/; https://www.ncbi.nlm.nih.gov/). Nucleotide and amino acid sequences were analyzed using Clustal Omega (www.clustal.org/omega) and a phylogenetic tree constructed using MEGA 6.06 software (www.megasoftware.net).

Vector Construction and Plasmid Transformation

The *EbJAZ1* CDS was amplified using primers are list in Supplementary Table 1 that contained BamHI/Xbal restriction endonuclease sites to assay the subcellular localization of EbJAZ1. The amplified *EbJAZ1* CDS was inserted into the pocA30-35S::GFP vector to generate the recombinant plasmid to construct the model structure diagram. To generate transgenic plants overexpressing *EbJAZ1*, the *EbJAZ1* CDS were amplified with primers containing BamHI/Sall restriction endonuclease sites and inserted into the pocA30-CaMV-35S vector. The plasmids were transformed into *A. tumefaciens* EHA105 by freeze thawing, and the plasmids were transformed into *Col-0* plants by the floral dipping method. Kanamycin-resistant plants were selected, and their T2 progenies were propagated for analysis.

Subcellular Localization Analysis

The recombinant pocA30-CaMV-35S*::EbJAZ1*-GFP and the control pocA30-CaMV-35S::GFP plasmids were transformed into *A. tumefaciens* EHA105, and cultured overnight at 28° C. *A. tumefaciens* was harvested by centrifugation

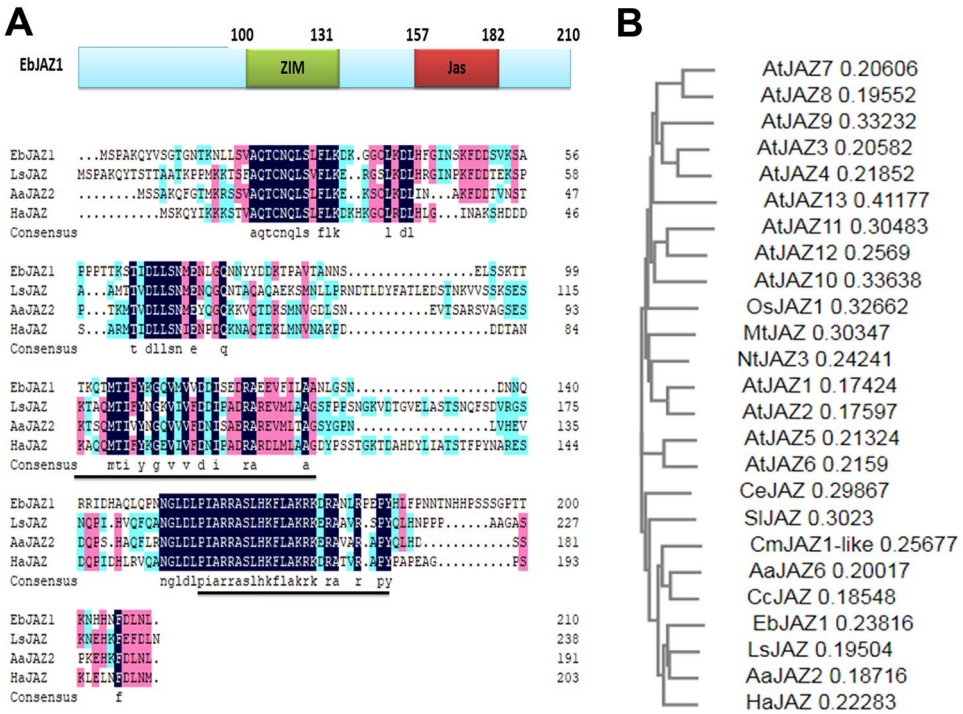


Fig. 1 Sequence analysis of EbJAZ1. **a** Amino acid sequence alignment of JAZ proteins from *Lactuca sativa* (LsJAZ, XP_023734481.1), *Artemisia annua* (AaJAZ2, PWA92554.1), and *Helianthus annuus* (HaJAZ, XP_022033525.1). The conserved ZIM and JAS domains are indicated. **b** Phylogenetic tree of EbJAZ1 and JAZs from other plant species, including Arabidopsis (AtJAZ1, AEE29814.1; AtJAZ2, ANM59944; AtJAZ3, AEE76018; AtJAZ4, AEE32304; AtJAZ5, AEE29581; AtJAZ6, AEE35326; AtJAZ7, AEC08997; AtJAZ8, AEE31184; AtJAZ9, AEE35101; AtJAZ10, AED91867; AtJAZ11, AEE77795; AtJAZ12, AED92902.1; AtJAZ13, XP_002885495.1), *Lactuca sativa* (LsJAZ, XP_023734481.1), *Artemisia annua* (AaJAZ2, PWA92554.1; AaJAZ6, PWA80949.1), and *Helianthus annuus* (HaJAZ, XP_022033525.1), *Nicotiana tabacum* (NtJAZ3, BAG68657.1), *Cynara cardunculus* (CcJAZ, XP_024969891.1), *Coffea eugenioides* (CeJAZ, XP_027168422.1), *Chrysanthemum x morifolium* (CmJAZ1-like protein, QBK47074.1), *Medicago truncatula* (MtJAZ, XP_003595354.1), *Solanum lycopersicum* (SlJAZ, XP_004252407.1), *Oryza sativa* (OsJAZ1, Q7XEZ1)

and resuspended in infiltration liquid media containing 0.15 mM acetosyringone, 10 mM $MgCl_2$, and 10 mM MES-KOH at pH 5.6. Leaf epidermis cells of *N. benthamiana* were imaged using a confocal laser scanning microscope (Olympus, Tokyo, Japan). The localization patterns of the GFP-tagged fusion protein were observed at 450 nm excitation and 490 nm emission, and samples were stained with DAPI to visualize the nuclei.

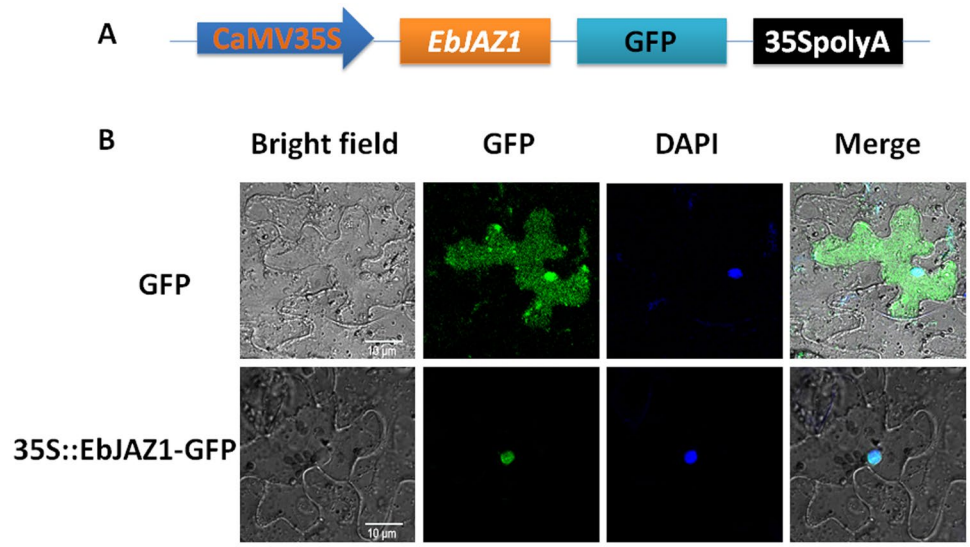


Fig. 2 Sub-cellular location of EbJAZ1. **a** The schematic of GFP-tagged EbJAZ1 protein (35S::EbJAZ1-GFP). **b** *35S::GFP* (control) and *35S::EbJAZ1-GFP* constructs were introduced *Nicotiana benthamiana* leaves. Merge, GFP, DAPI and bright field images

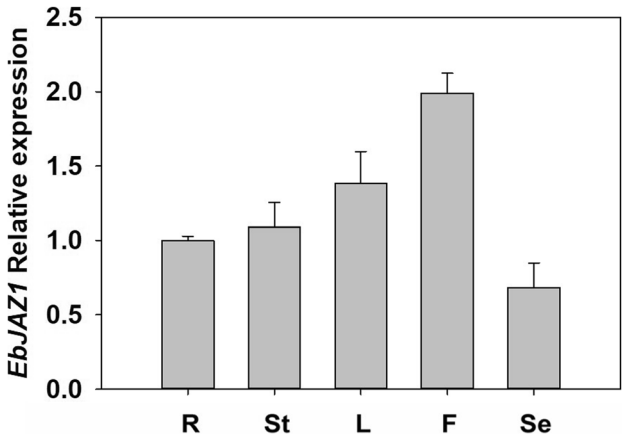


Fig. 3 qRT-PCR analysis of the tissue-specific expression patterns of EbJAZ1 in *E. breviscapus*. Abbreviations are as follows: R: roots; St: stems; L: leaves; F: flowers; Se: seeds

EbJAZ1 Expression Analysis in *E. breviscapus*

In order to investigate the expression patterns of *EbJAZ1* in *E. breviscapus*, total RNA first-strand cDNA was synthesized using the M-MuLV reverse transcriptase (Fermentas, EU) with oligo (dT) 18 primers. qRT-PCR was performed using SYBR Green Master Mix on a Roche Light-Cycler 480 real-time PCR machine, following the manufacturer's protocol. The primers used in the qRT-PCR assay are listed in Supplementary Table 1. *EbACTIN2* was used as the internal control, and expression data was analyzed based using the comparative $2^{-\Delta\Delta CT}$ method.

MeJA Treatment Assay

For MeJA treatment, 25 µM and 50 µM MeJA was added to the MS agar medium. Wild-type and transgenic lines seeds were grown at 22 °C in MS-only, 25 µM, and 50 µM MeJA media with agar, where the MS medium served as the control. Root lengths of the seedlings were measured after 15 days of treatment.

RNA Extraction and qRT-PCR

Wild-type and transgenic lines cultivated for 13 days in MS media were subsequently treated with 50 µM MeJA or water for 8 h. Total RNA was extracted from wild-type and transgenic *Arabidopsis* lines using the TRIzol reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed as described previously (Hu et al. 2012). Briefly, first-strand cDNA was synthesized from 1.5 µg DNase-treated RNA in a 20-µl reaction using M-MuLV reverse transcriptase (Fermentas, EU) with oligo (dT) 18 primers. qRT-PCR was performed using SYBR Green Master Mix on a Roche Light-Cycler 480 real-time PCR machine, following the manufacturer's instructions. Measurements were taken from at least three biological replicates for each sample, and at least two technical replicates were conducted for each biological replicate. *ACTIN1* was used as the control, and primers used in this experiment are listed in Supplementary Table 1.

Fig. 4 Overexpression of *EbJAZ1* affects fertility in Arabidopsis. ▶ **a** qRT-PCR characterization of *EbJAZ1* expression in three independent transgenic lines (*35S::EbJAZ1*-L1, *35S::EbJAZ1*-L2, and *35S::EbJAZ1*-L3). **b** The fertility of transgenic lines and wild type cultivating in soil for 35 days (top) and 60 days (bottom). **c** Characterization of filament length and pollen granules in transgenic lines and wild type. **d** Silique phenotype on main stem of transgenic and wild type. **e** Seed number per silique of transgenic lines and wild type. **f** Silique length of transgenic lines and wild type. Error bars indicate SDs (*$P < 0.05$)

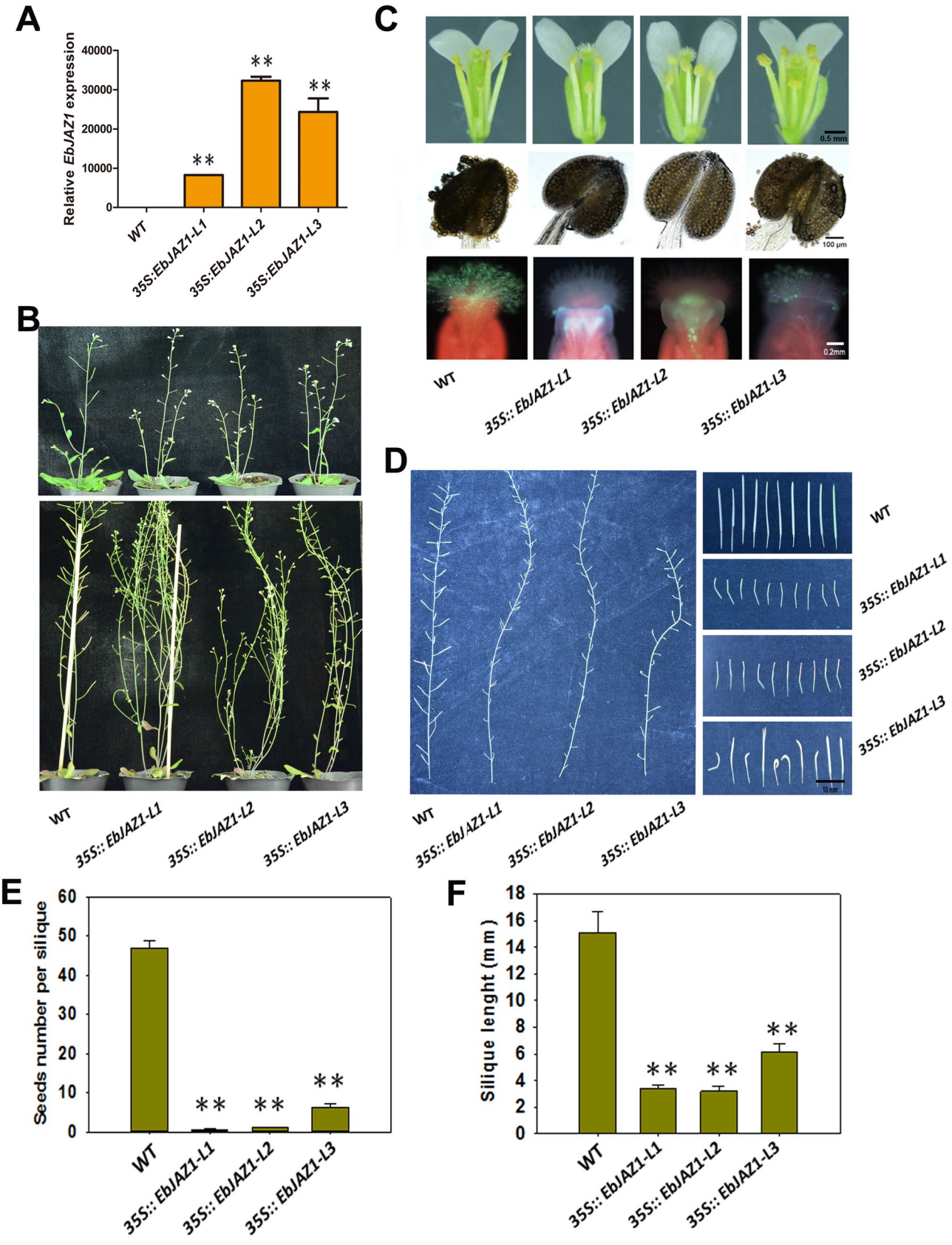
Results

Sequence and Phylogenetic Analysis of *EbJAZ1*

The homolog gene encoding jasmonate ZIM-domain proteins (JAZs) was cloned from *E. breviscapus*, and designated as *EbJAZ1*. *EbJAZ1* has a 633-bp CDS that encodes a protein with 220 amino acids. EbJAZ1 contains ZIM and JAS domains that are similar to those of *Lactuca sativa* LsJAZ, *Artemisia annua* AaJAZ2, and *Helianthus annuus* HaJAZ (Fig. 1a). Among the JAZs, the JAZ domain was highly conserved which displays an average amino acid sequence identity of 80%, as described by Thines et al. 2007. The phylogenetic analysis indicated that EbJAZ1 clusters more closely with LsJAZ and AaJAZ2, while clusters loosely with AtJAZs and OsJAZ1 from *Arabidopsis thaliana* and *Oryza sativa* (Fig. 1b).

Subcellular Localization and Tissue-Specific Expression Patterns of *EbJAZ1*

To investigate the subcellular localization of EbJAZ1, we transiently expressed GFP-tagged EbJAZ1 in *N. benthamiana* leaves to observe the localization of the GFP using confocal laser canning microscopy (Fig. 2a). The EbJAZ1-GFP fusion protein was observed solely in the nucleus, whereas control GFP protein was found in both nucleus and cytoplasm (Fig. 2b). Subsequently, the expression levels of *EbJAZ1* in different *E. breviscapus* tissues were examined by qRT-PCR. The results showed that *EbJAZ1* was expressed in all tissues, with higher levels of expression in flower and lower levels of expression in seeds (Fig. 3).


A
Relative EbJAZ1 expression
40000
30000
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0
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WT
35S:EbJAZ1-L1
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35S:EbJAZ1-L3
B
WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3
C
0.5 mm
100 µm
0.2mm
WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3
D
WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3
10 mm
WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3
E
Seeds number per silique
60
50
40
30
20
10
0
**
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**
WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3
F
Silique lenght (mm)
18
16
14
12
10
8
6
4
2
0
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WT
35S:: EbJAZ1-L1
35S:: EbJAZ1-L2
35S:: EbJAZ1-L3

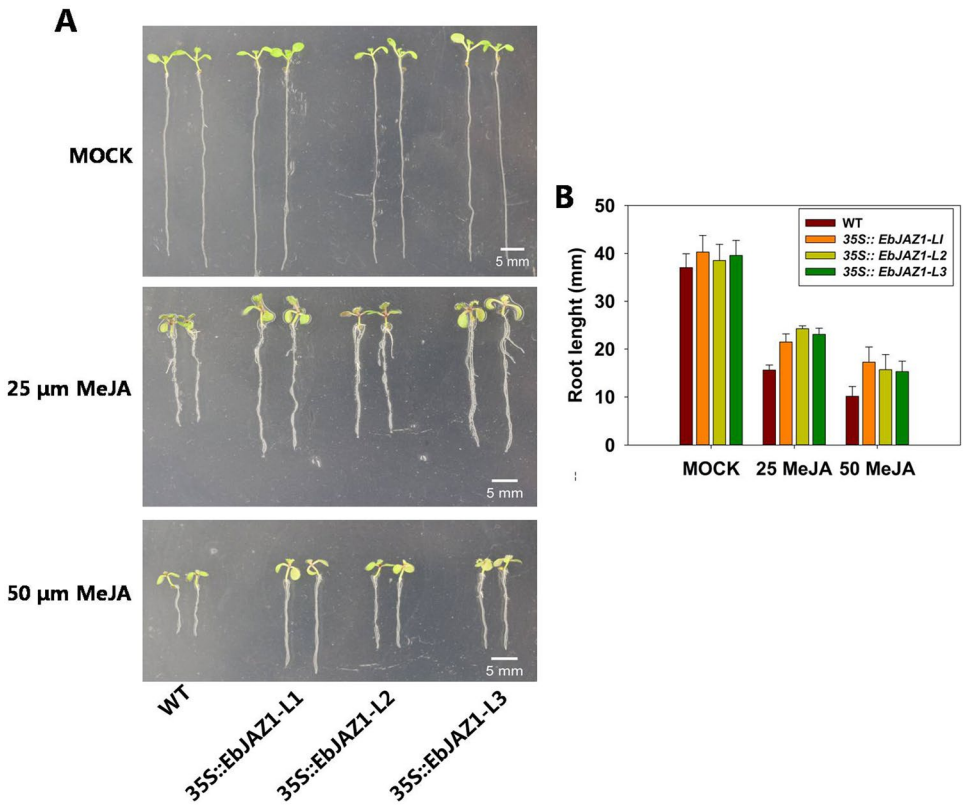

Fig. 5 Overexpression of *EbJAZ1* affects MeJA-mediated root growth in Arabidopsis. **a** Root growth of wild-type and transgenic lines. **b** Root length of wild-type and transgenic lines. For each biological replicate, we examined three times as technical replicates. Data are means ± SDs ($n = 10$). Asterisks are significant different between treatments at $P < 0.05$)

Overexpressing *EbJAZ1* in Arabidopsis Leads to Sterility

To explore whether plant fertility is regulated by *EbJAZ1*, *Arabidopsis* plants overexpressing *EbJAZ1* were constructed by transforming the recombinant plasmid into wild-type plants. Three transgenic lines (*35S::EbJAZ1-L1*, *35S::EbJAZ1-L2*, and *35S::EbJAZ1-L3*) were generated, showing that *EbJAZ1* was highly expressed in three independent transgenic lines, but not in wild-type plants (Fig. 4a). Phenotype observation showed that the number of lateral branches of *35S::EbJAZ1-L1*, *35S::EbJAZ1-L2*, and *35S::EbJAZ1-L3* were more than of wild type, indicating that *EbJAZ1* is possibly involved in the regulation of lateral branch development in *Arabidopsis thaliana* (Fig. 4b). Simultaneously, we found that *35S::EbJAZ1-L1* and *35S::EbJAZ1-L2* plants which did not have pollen were almost completely sterile, while *35S::EbJAZ1-L3* carrying few pollen granules was semi-sterile; this result also was confirmed by staining assay. We observed few normally germinated pollen on the stigma of *35S::EbJAZ1-L3* that showed that the pollen viability of *35S::EbJAZ1* was normal, while there was no pollen on the stigma of *35S::EbJAZ1-L1* and *35S::EbJAZ1-L2* (Fig. 4b, c). These results indicated that EbJAZ1 regulates anther dehiscence. Furthermore, the silique length in *35S::EbJAZ1-L1*, *35S::EbJAZ1-L2*, *35S::EbJAZ1-L3* was significantly shorter than that in wild type, while the seed number per silique was significantly less in those transgenic lines compared to that in wild type (Fig. 4d–f). In addition, there was no significant difference in filament length between transgenic plants and wild-type plants (Fig. 4c). These results suggest that overexpression of *EbJAZ1* possibly disrupts the anther dehiscence, thus leading to sterility in the transgenic Arabidopsis plants.

Overexpressing *EbJAZ1* in Arabidopsis Affects MeJA-Dependent Root Growth Inhibition

It has been confirmed that JAZ-MYC2 transcriptional regulatory cascade is involved in JA-mediated root growth responses. Therefore, we examined root length changes treated with MeJA (Fig. 5a). The root length in transgenic lines was similar with wild-type plants in normal media. However, when cultured in 25 and 50 μM MeJA media, MeJA-induced root growth inhibition could be alleviated in three independent transgenic plants (Fig. 5a, b), indicating that overexpresing *EbJAZ1* in Arabidopsis can reduce sensitivity to MeJA and negatively regulate JA response.

To verify whether increased root growth in transgenic plants is associated with the regulation of signaling pathway, we further examined the expression of several downstream target genes in the JA signaling pathway, including *VSP1*, *VSP2*, *JAZ1*, *JAZ5*, *JAZ8*, *JAZ10*, *MYC2*, and *bHLH17*. The results showed that the expression levels of those

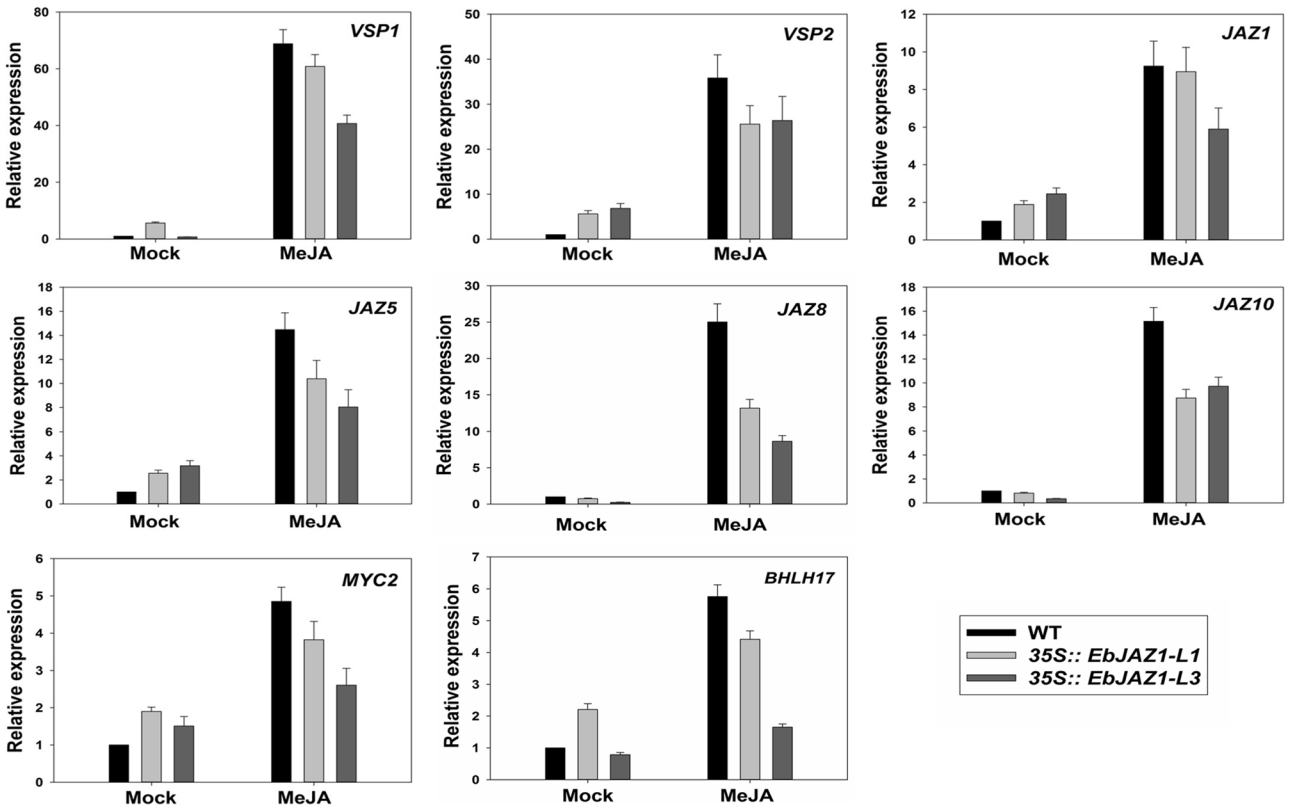

Fig. 6 qPCR analysis of genes involved in JA downstream signaling. The expression levels of AtJAZ1, AtJAZ5, AtJAZ8, AtJAZ10, AtVSP1, AtVSP2, AtMYC2, and AtBHLH17 in 35S::EbJAZ1-L1, 35S::EbJAZ1-L3, and wild-type plants were examined after treating with 50 μM MeJA for 8 h. Error bars denote SDs (*P < 0.05)

JA-responsive genes were downregulated in *35S::EbJAZ1-L1* and *35S::EbJAZ1-L2* seedlings compared to the wild type when treated with 50 μM MeJA (Fig. 6). These results indicated that *EbJAZ1* inhibits the expression of genes involved in JA downstream signaling.

Discussion

The jasmonate ZIM-domain (JAZ) proteins are repressors that function in the regulation of plant growth, development, and response to stimulation of different signals in the JA signaling pathway. In this study, EbJAZ1 was cloned from *E. breviscapus*, and first characterized in medicinal plants in terms of its roles in development. EbJAZ1 is located in the nucleus and has complete and conserved characteristics of the JAZ domain (ZIM and Jas) (Figs. 1 and 2), which is consistent with the reports in *Arabidopsis*, *Salvia miltiorrhiza*, and *Pohlia nutans* (Thines et al. 2007; Pei et al. 2018; Liu et al. 2019). Further tissue-specific expression showed that EbJAZ1 was expressed in all tissues, and the expression level in flowers was relatively high, while that in seeds was relatively low (Fig. 3), suggesting that it might be involved in the growth and development of these tissues.

In *Arabidopsis*, MYB transcription factors 21, 24, 57, and 108 positively regulate the fertility of plants in functional redundancy. In the *myb21myb24* mutant, the length of filaments was significantly shorter than that of stigma. Later studies found that this process depends on the interaction between JAZs and MYBs in the JA signaling pathway. In addition, it was found that JAZs can also interact with the bHLH transcription factor MYCs and/or MYB/bHLH complex to regulate the filament growth and seed development (Mandaokar et al. 2006; Song et al. 2011; Qi et al. 2015). In this study, the ectopic expression of *EbJAZ1* in Arabidopsis resulted in shorter filament and silique length and reduced seed fertility (Fig. 4), suggesting that *EbJAZ1* may play an important role in SSI of *E. breviscapus*. We speculate that overexpression of *EbJAZ1* resulted in the incomplete degradation of JAZ proteins by endogenous JA, which depressed the activation of MYBs and MYCs to regulate downstream response genes, thus resulting in Arabidopsis sterility. Among the 12 JAZs in Arabidopsis, JAZ1, 8, and 11 have been proven to be able to interact with MYB21 and 24 (Song et al. 2011).

Unfortunately, EbJAZ1 did not interact with Arabidopsis MYB21 and 24 (data not shown). Because EbJAZ1 clusters loosely with all Arabidopsis JAZs in a genetic relationship (Fig. 1b), there is a possibility that EbJAZ1 may interact with other Arabidopsis MYBs that have not been characterized. Further transcriptomics and yeast screening assay will confirm this hypothesis. Meanwhile, previous studies have shown that plant branching is mainly regulated by abscisic acid (ABA), auxin, strigolactones, and cytokinin (Zhu and Wagner 2019). JAZ protein was the key regulator of the JA signal, which affects the function of JA. In this study, we found that overexpression EbJAZ1 can promote plant branching (Fig. 4b). JA cross talks with ABA, auxin, and cytokinin signaling to regulate many physiological growth and development processes of plants, such as seed germination, plant freezing tolerance, and accumulation of secondary metabolites (Pan et al. 2020; Hu et al. 2017). EbJAZ1 possibly affects the function of ABA, auxin, and cytokinin in regulating branching through JA signaling.

MYCs are negative regulators of root elongation in Arabidopsis. There was no significant change in the root length of a single *mycs* mutant, but the root length was significantly longer in multiple myc2/3/4/5 mutants. It has been confirmed that JA negatively regulates root elongation through the interaction between JAZs and MYCs and inhibits the transcription activity of MYCs (Fernández-calvo et al. 2011; Debora et al. 2015). Here, ectopic expression of *EbJAZ1* in Arabidopsis showed insensitivity to JA (Fig. 5). Similarly, EbJAZ1 did not interact with any of Arabidopsis MYC2, 3, 4, and 5 the same as MYBs mentioned above (data not shown). However, qRT-PCR results in this study showed that the expression levels of marker genes in *EbJAZ1* transgenic lines were higher than those in wild type (Fig. 6), which was consistent with the phenotype (Fig. 5). Some studies have shown that root length is negatively correlated with the expression of *JAZ1*, *8*, *10*, and *VSP2* in JA-treated and untreated plants (Fernández-calvo et al. 2011; Dombrecht et al. 2007).

Taken together, ectopic expression of *EbJAZ1* in Arabidopsis can regulate development of flower organs, seed fertility, and primary root growth through the JA signaling pathway, suggesting that EbJAZ1 may be indirectly involved in the SSI of *E. breviscapus*.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11105-021-01289-4.

Author Contribution YRH and SCY conceived and designed the research; MC and MY conducted the experiments; WZ, SMH, and YCL analyzed the data and created the figures; GHZ, BH, and FW performed the statistical analysis; MC and WF wrote the manuscript. All authors read and approved the manuscript.

Funding This work was supported by the National Natural Sciences Foundation of China (Nos. 81760692, 81960684), the Major Science and Technique Programs in Yunnan province (No. 2017ZF002), and the special fund project for basic research of local universities in Yunnan Province (No. 2018FH001-011).

Declarations

Conflict of Interest The authors declare no competing interests.

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