Gene 627 (2017) 49–56

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

A novel *GhBEE1-Like* gene of cotton causes anther indehiscence in transgenic *Arabidopsis* under uncontrolled transcription level

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ARTICLE INFO

Keywords:
Brassinosteroids
GhBEE1-Like
Anther dehiscence
Cotton
Arabidopsis

ABSTRACT

Male-sterile lines are very important for selective breeding, and anther dehiscence defect is an effective way to generate male-sterile lines. Although several bHLH-family proteins in *Arabidopsis* have been characterized, little is known about the role of bHLH-family proteins in cotton. Here, we isolated a novel bHLH protein from cotton (*Gossypium hirsutum*), named *GhBEE1-Like*. Protein domain analysis showed that GhBEE1-Like contained a basic domain and an HLH domain. Subcellular localization analysis revealed that GhBEE1-Like was a nuclear-localized protein. Expression pattern analysis showed *GhBEE1-Like* was highly expressed in floral organs, and its expression was induced by the active brassinosteroid (BR) substance 24-epi-BL. *GhBEE1-Like* overexpression in *Arabidopsis* resulted in two types of transgenic lines, one with normal anther dehiscence and the other with defective anther dehiscence. Semi-qRT-PCR and qRT-PCR analyses revealed that *GhBEE1-Like* transcript levels acted as a check-point determining how anther dehiscence proceeds in these transgenic lines; regulated transcript levels result in normal anther dehiscence, whereas uncontrolled transcript levels lead to anther indehiscence. These results suggest that *GhBEE1-Like* plays an important role via its accumulation in regulating anther dehiscence. Therefore, controlling the level of *GhBEE1-Like* expression in cotton could be a convenient tool for generating male-sterile lines to use in selective breeding.

1. Introduction

Brassinosteroids (BRs) are a group of polyhydroxylated plant-specific steroidal hormones and were first isolated from *Brassica napus* pollen (Grove et al., 1979). BRs are regarded as a class of essential plant hormones that regulate multiple physiological functions, including reproductive processes, photomorphogenesis, root development, seed germination, cell division and elongation, guard cell development, vascular-differentiation, senescence, and responses to various biotic and abiotic stresses (Clouse and Sasse, 1998; Li and Chory, 1999; Choudhary et al., 2012; Sreeramulu et al., 2013; Sharma et al., 2015).

The function of BRs in reproductive processes has been extensively reported. One distinguishing role of BRs relates to flowering time: BRs have been found to promote flowering by up-regulating the expression levels of the *LD* and *FCA* genes, which are involved in suppressing the central floral repressor gene *FLC* (*FLOWERING LOCUS C*) (Domagalska et al., 2010; Li et al., 2010). In *Arabidopsis*, *bri1* mutants exhibit a weak late flowering phenotype but do not show altered *LD* or *FCA* gene expression (Domagalska et al., 2007; Shakoor et al., 2010). Moreover, BES1, a key transcription factor in the BR signal transduction pathway, has been shown to regulate the expression of *ELF6* and *REF6*, which acts as a repressor of the *FLC* gene, and these genes are involved in controlling flowering time (Yu et al., 2008). These data suggest the existence of BR induction of the flowering pathway that is independent the *LD* and *FCA* genes. BRs are also essential for male fertility in plants. Studies in *Arabidopsis* indicate that BR mutants, such as *cpd*, *bin2*, and *bri1-201*, have little or no male fertility (Szekeres et al., 1996; Bouquin et al., 2001; Li et al., 2001). In the BR-deficient mutant *cpd*, male sterility is likely caused by pollen tube elongation failure (Szekeres et al., 1996). In addition, studies of the BR-deficient mutant *cpd* and the

Abbreviations: BR, brassinosteroid; BRs, brassinosteroids; bHLH, basic helix-loop-helix; Col-0, Columbia-0; 24-epi-BL, 24-epi-brassinolide; GFP, green fluorescent protein; dpa, days post-anthesis; JA, jasmonic acid; GAs, gibberellins

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http://dx.doi.org/10.1016/j.gene.2017.06.007
Received 26 February 2017; Received in revised form 12 May 2017; Accepted 5 June 2017

BR signalling mutant *bri1-116* have revealed that BRs control male fertility via regulating the expression of key genes involved in *Arabidopsis* anther and pollen development (Ye et al., 2010). Moreover, studies on another BR-deficient mutant, *dwf4*, have revealed that male sterility may be caused by significantly reduced filament elongation that results in pollen not being delivered to the stigma (Kim et al., 2005).

Basic helix-loop-helix (bHLH) family proteins contain approximately 60 amino acids and possess two highly conserved and distinct domains: the basic domain at the N-terminal end and the HLH domain carrying two α-helices linked by a variable loop at the C-terminal end (Ellenberger et al., 1994). The basic domain is responsible for binding to a DNA promoter element termed the E-box, whereas the HLH domain is involved in homo- or heterodimerization. bHLH proteins are generally classified into two categories based on the diverse functions of the basic domain: DNA-binding and non-DNA-binding bHLH proteins (Jones, 2004). Many bHLH proteins have been implicated in BR signalling. Studies suggest that BES1, a key transcription factor in the BR signalling pathway that also contains a bHLH-like domain, binds to the *SAUR-AC1* promoter, interacting with another bHLH protein, BIM1 (BES1-INTERACTING MYC-LIKE), indicating that *SAUR-AC1* is a BR up-regulated gene (Yin et al., 2005). BEE1 (BR Enhanced Expression 1), BEE2, and BEE3 are bHLH transcription factors and are products of BR early-response genes. Genetic analysis has found that BEE1, BEE2, and BEE3 are functionally redundant positive regulators of the BR signalling pathway (Friedrichsen et al., 2002). Moreover, the regulatory E-box element is recognized by bHLH proteins and is predominantly present in the promoter regions of many BR response genes (Nemhauser et al., 2004; Vert et al., 2005).

In the *Arabidopsis* genome, there are approximately 162 bHLH proteins, and they can be further divided into 21 subfamilies (Toledo-Ortiz et al., 2003). Previous studies have implicated bHLH proteins in a wide range of plant developmental and physiological processes, especially in the BR signal transduction pathway. Three bHLH proteins (*BEE1*, *BEE2*, and *BEE3*) are related to BR early-response genes and are involved in events downstream of the BR receptor BRI1. For different combinations of single or double mutants, *bee1*, *bee2*, and *bee3* have not been found to display any notable phenotypes. However, triple mutants for these genes have been documented to show both seedling and floral phenotypes similar to those of known BR mutants (Friedrichsen et al., 2002). CESTA (CES), a bHLH transcription factor, acts as an activator of BR biosynthesis through an interaction with its homologue, BEE1, and directly binds to a G-box motif in the promoter of CPD, which is a crucial gene for BR biosynthesis. The dominant mutant of the *CESTA* gene, *cesta-D*, shows enhanced hypocotyl growth. These plants have serrated, outwardly curved and epinastic rosette leaves on elongated petioles and have markedly increased numbers of inflorescences and rosette leaves (Poppenberger et al., 2011). BEE and BES1-INTERACTING MYC-LIKE (BIM) proteins could interact with *PHYTOCHROME RAPIDLY REGULATED 1* (*PAR1*) to form a network that positively regulates the responses involved in shade-avoidance syndrome in *Arabidopsis*. Genetic analysis revealed that *bee123* and *bim123* mutants display defective hypocotyl elongation when perceiving shade, and the subsequently reduced BEE and BIM activities could result in a dwarf rosette phenotype and reduced responses to shade (Cifuentes-Esquivel et al., 2013). The *HAF* gene (*HALF FILLED*) encodes a bHLH transcription factor closely related to *BEE1* and *BEE3* and has been shown to have overlapping expression patterns in the reproductive tract. In addition, *haf*, *bee1*, *bee3* triple mutants have impaired pollen tube growth. Genetic analysis found that *HAF* promotes pollen and carpel growth and controls cell death within the reproductive tract. More importantly, minimal pollination assays revealed that *HAF* is sufficient and necessary to promote efficient fertilization (Crawford and Yanofsky, 2011).

Here, we identified a bHLH transcription factor that is a product of a gene orthologous to *AtBEE1* in *G. hirsutum*, named *GhBEE1-Like*. *GhBEE1-Like* is a BR response gene and is up-regulated by treatment with the active BR substance 24-epi-BL. An analysis of its tissue expression patterns showed that *GhBEE1-Like* was highly expressed in floral organs such as anthers and petals. The subcellular localization analysis of GhBEE1-Like shows that it is a nuclear localization protein, suggesting that it acts as a transcription factor. Semi-qRT-PCR and qRT-PCR analyses showed that two types of phenotypes were associated with *GhBEE1-Like* expression level in *Arabidopsis* plants. In type one, the transgenic *Arabidopsis* showed the regulated transcription of *GhBEE1-Like* and had normal anther dehiscence; in type two, the transgenic lines showed significantly elevated *GhBEE1-Like* expression levels, and the normal process of anther dehiscence failed, resulting in the production of deformed seeds in the siliques. Our results presented here shed light on how GhBEE1-Like, a bHLH transcription factor in cotton, regulates anther dehiscence in plants and might lay the foundation for creating male-sterile lines to use in future genetic engineering-based modifications of cotton.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, *Arabidopsis thaliana* ecotype Columbia-0 (Col-0). Col-0 seeds were sown on solid MS medium after surface-sterilization and were cold-treated for 2 days at 4 °C in the dark to synchronize germination. The MS plates mentioned above were then moved to a growth chamber. Eleven days later, the Col-0 seedlings were transferred to pots with a soil mixture containing nutritional soil and vermiculite (v/v = 2) and grown in a growth chamber, and adult *Arabidopsis* were used for transformation one month later. The growth chamber had a constant temperature of 22 °C and a 16 h light/8 h dark photoperiod.

Transgenic seeds of the T_0 generation were sown on solid MS medium that contained kanamycin (50 μg L^{-1}) and cefotaxime sodium (250 μg L^{-1}). Twelve days later, kanamycin-resistant seedlings (T_1 generation) with two true leaves and a longer primary root were selected from the non-resistant seedlings and were transplanted into a soil mixture for growth. One month later, phenotypes, including the shoot tissues and siliques from the same location on each transgenic line and from Col-0 plants of the same age, were photographed (Canon, EOS 70D). At the same time, the phenotype of the flowers and anthers of the transgenic lines and Col-0 plants were observed using a stereomicroscope (Motic, Moticam Pro 285A). Moreover, the seeds in siliques at the same location on transgenic lines and Col-0 plants at maturity were also observed using the stereomicroscope.

CCRI24, which is a fine, easily transformed short-season variety of Upland cotton cultivated by Institute of Cotton Research, Chinese Academy of Agricultural Sciences, were sown in pots containing nutritional soil and grown in a growth chamber. The cotton leaves of trefoil stage seedlings were quickly collected, frozen in liquid nitrogen and further stored at −80 °C for RNA isolation and *GhBEE1-Like* cloning. The growth chamber had a constant temperature of 28 °C and a 16 h light/8 h dark photoperiod.

2.2. RNA extraction and cDNA synthesis

Total RNA was isolated from various tissue samples using the RNAprep Pure Plant Kit (TIANGEN, China) according to the manufacturer's instruction. For synthesizing first-strand cDNA, the PrimeScript® RT Reagent Kit along with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) was used in accordance with the manufacturer's protocol, and the cDNAs were used as templates in the following RT-PCR and qRT-PCR reactions.

2.3. Cloning of the GhBEE1-Like gene and transformation into Arabidopsis

To produce *35S::GhBEE1-Like* transgenic *Arabidopsis* lines, the full-length *GhBEE1-Like* coding sequence was amplified from *G. hirsutum*