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# Overexpression of an *EIN3-binding F-box protein2-like* gene caused elongated fruit shape and delayed fruit development and ripening in tomato

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ABSTRACT

Ethylene signaling converges on the ETHYLENE-INSENSITIVE3 (EIN3)/EIN3-like (EIL) transcription factors to regulate a wide range of developmental processes in plants. EBF1/2 (EIN3-binding F-box protein 1 and 2) negatively regulate the ethylene signaling pathway by mediating the degradation of EIN3/EIL proteins. We uncovered previously that *SlEBF1* and *SlEBF2* are involved in ethylene response, plant senescence, and fruit ripening in tomato. The present study reports on the identification of a novel tomato F-box gene, designated as *SlEBF2-like* due that its encoded protein is greater similarity to SlEBF2. The *SlEBF2-like* promoter region contains three ethylene-response elements (EREs). *SlEBF2-like* is upregulated by ethylene and downregulated by ethylene inhibitors in tomato seedlings. It is dynamically expressed in flowers during bud-to-anthesis and anthesis-to-post-anthesis transitions, and at the onset of fruit ripening, suggesting its role in these situations where ethylene is required for flower opening and fruit ripening. *SlEBF2-like* overexpression leaded to fruit elongation, caused ripening and color change to start from fruit bottom and expand gradually to the pedicel, and strongly delayed fruit development and ripening in tomato. Our study indicates that the novel *EBF* gene, *SlEBF2-like*, is involved in fruit development and ripening via regulating the ethylene response in tomato.

## 1. Introduction

The ubiquitin proteasome system (UPS) mediates specific protein degradation and plays an important role in all eukaryotes that allows cells to respond rapidly to signal molecules and changes in environmental conditions [1–3]. The ubiquitination of target proteins depend on a total of three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligases) [4]. E1 and E2 carry out the activation and conjugation of the ubiquitin moieties and are relatively nonspecific, whereas E3 enzymes are responsible for the recognition of specific target proteins for ubiquitination [5]. These E3 enzymes can be classified into several large families. One major E3 type is the SCF ubiquitin-ligase complex, which is best-characterized and formed by four primary subunits: Skp1, Cullin, Rbx1, and F-box protein [6–8]. In the SCF complex, Cullin and Rbx1 form a core scaffold, and Skp1 connects the scaffold to an F-box protein. The F-box protein performs the crucial role of delivering specific substrates to the SCF complex for proteolysis [6,9,10].

F-box protein is a kind of protein family with a conserved F-box domain of 40–50 amino acids at the N-terminus for interacting with the Skp1 subunit, and one or several highly variable protein–protein interaction domains at the C-terminus for the specific substrate recognition [1,8,11]. F-box superfamily is one of the largest gene family in plants, for example, more than 698, 337, and 858 putative F-Box genes in *Arabidopsis thaliana*, poplar (*Populus trichocarpa*), and rice (*Oryza sativa*) genomes, respectively [8,12]. These F-box genes have been discovered to control many crucial processes in plants, such as embryogenesis, hormonal responses, seed germination, seedling development, lateral root formation, flowering time, senescence, disease resistance, and abiotic stress responses [10,13–15].

Plant hormones regulate a wide variety of plant processes and act to integrate diverse environmental cues with endogenous growth programs. So far it has become clear that the F-box protein-dependent UPS plays a prominent regulatory role in most hormone biology, including hormone perception, repression of hormone signaling pathways, degradation of hormone specific transcription factors, and regulation of hormone biosynthesis [13]. Ethylene is a very important gaseous plant hormone involved in a wide range of plant development processes, including germination, hypocotyl and root elongation, flowering, fruit ripening, organ senescence, and responses to abiotic stresses and pathogen attack [16,17]. Through the extensive characterization of *Arabidopsis* mutants with altered ethylene responses, the ethylene signaling pathway has been partially defined in its upstream part as a linear transduction pathway [16,18]. Briefly, ethylene perception by five

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ethylene receptors blocks CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) activation thus allowing ETHYLENE INSENSITIVE 2 (EIN2) to promote ethylene responses via activating the downstream ETHYLENE INSENSITIVE 3/EIN3-likes (EIN3/EILs) transcription factors [16]. EIN3/EILs are key positive switch for activating the transcription of ethylene response factors (ERFs), which regulates the expression of genes involved in the response to ethylene [7,19]. It is well established that two F-box proteins, EIN3-binding F-box protein 1 and 2 (EBF1 and EBF2), play an important role in regulating ethylene responses through directly binding their targets EIN3/EILs for ubiquitination in *Arabidopsis* [7,20–22].

Tomato (*Solanum lycopersicum*) is the most important model plant for studying fleshy fruit development and ripening [23]. Since ethylene is the main trigger of climacteric fruit ripening, it is necessary to study the functions of ethylene-related genes for uncovering the molecular regulatory mechanism of fruit development and ripening in tomato. Two tomato F-box genes, *SlEBF1* and *SlEBF2*, were reported previously by us. Silencing *SlEBF1* and *SlEBF2* expression caused constitutive ethylene response phenotype, and accelerates plant senescence and fruit ripening in tomato [24]. Following the release of the complete tomato genome sequence, we found a novel F-box protein with the greatest similarity to SlEBF2 in tomato. But it is unclear whether this F-box protein also plays a role in controlling fruit development and ripening in tomato. This study identified the novel tomato F-box gene, designated as *SlEBF2-like*, and established its expression profile in different tomato tissues and at various stages of flower and fruit development. Overexpression of *SlEBF2-like* leaded to fruit elongation, caused abnormal color transformation during ripening process, and strongly delayed fruit development and ripening in tomato. Our study indicates that *SlEBF2-like* gene is involved in fruit development and ripening.

## 2. Materials and methods

### 2.1. Plant materials and growth condition

Tomato (*Solanum lycopersicum* cv. MicroTom) plants were grown under the following conditions: 14/10 h day/night cycle, 25/20 °C day/night temperature, 65% humidity, and 250 μmol $m^{-2} s^{-1}$ light intensity. The roots, stems, leaves, flowers, and fruits were collected from 8-week-old tomato plants. Samples taken from different parts (ovary, stamen, petal and sepal) of flowers were harvested at bud (−2 dpa; days post anthesis), anthesis (0 dpa), and post-anthesis (4 dpa) stages. The developmental stages of tomato fruits investigated in this study were 4 dpa (just after fruit set), 8 dpa (early immature green), immature green, mature green, breaker, orange and ripening.

### 2.2. Gene cloning and sequence analysis

The partial sequence of *SlEBF2-like* was obtained through TBLASTN analysis (protein to translated nucleotide database) against the Solanaceae Genome Network tomato unigene database (https://solgenomics.net) with the known *Arabidopsis* AtEBF1/2 and tomato SlEBF1/2 amino acid sequences. Subsequently, the full-length cDNA of this gene was isolated from tomato fruits using rapid amplification of cDNA ends (RACE) (Clontech Laboratories, Inc.) according to the manufacturer's instructions. The encoding sequence was analyzed using a translation tool (http://web.expasy.org/translate/). Amino acid sequence alignments were performed using ClustalX 2.1 (http://www.clustal.org/), and the result was displayed using Genedoc 2.7.0. The protein subcellular localization was predicted by cNLS Mapper [25], LocTree3 [26] and WoLF PSORT (http://www.genscript.com/wolf-psort.html). The F-box domains and leucine-rich repeats (LRRs) motifs were analyzed using the SMART (http://smart.embl-heidelberg.de). The cis-acting regulatory elements of ethylene response elements (EREs) in the promoter were identified by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Phylogenetic analysis was performed using the Neighbor-Joining approach by MEGA7.0 [27]. GenBank accession numbers for the sequences analyzed are as follows: *Arabidopsis thaliana* AtEBF1 (NP_565597), AtEBF2 (NP_197917), AtCOI1 (coronatine insensitive 1) (NP_565919), AtFKF1 (Flavin-binding kelch repeat F-box protein 1) (AAF32298), AtTIR1 (transport inhibitor response 1) (NP_567135), AtZTL (ZEITLUPE, Flavin-binding kelch repeat F-box protein 2) (NP_568855), *Nicotiana attenuate* NaCOI1 (OIT06933), NaTIR1 (OIT05652), NaEBF1 (OIT39126), NaEBF2 (OIT29606), *Glycinemax* GmCOI1 (AAZ66745), GmFKF1 (ABD28287), *Ipomoea nil* InZTL (ABC25060), InFKF1 (AIZ66163), *Capsicum annuum* CaCOI1 (AFM82491), *Solanum lycopersicum* SlEBF1 (ACS44349), SlEBF2 (ACS44350), SlEBF2-like (MG727556), SlCOI1 (AAR82926), SlTIR1 (NP_001234673).

### 2.3. Subcellular localization in tobacco protoplast

The coding sequences of *SlEBF2* and *SlEBF2-like* excluding stop codons were amplified from tomato cDNA using specific primers (see Supplementary Table S1 in the online version at DOI:10.1016/j.plantsci.2018.04.016). The PCR products were cloned as a C-terminal fusion in frame with the green fluorescent protein (GFP) into the pGreen vector under the transcriptional control of the cauliflower mosaic virus 35S (CaMV 35S) promoter and the nopaline synthase (nos) terminator, respectively. A 0.2 ml protoplast suspension of tobacco (*Nicotiana tabacum*) BY-2 cells was transfected with 50 μg shared salmon sperm carried DNA and 30 μg of 35S:*SlEBF2*:*GFP*, 35S:*SlEBF2-like*:*GFP* or 35S:*GFP* (control) plasmids according to the method described by Wang et al. [28]. Then the transfected protoplasts were incubated for 16 h at 25 °C, and the fluorescence signals were detected by the confocal microscopy. All transient expression assays were performed at least three times.

### 2.4. Ethylene and auxin treatments

To perform phytohormone treatments, the tomato seeds were germinated and grown in Murashige and Skoog (MS) culture medium, and the 21-d-old light-grown seedlings were incubated in 50% MS buffer containing corresponding hormone or inhibitor as described by Wang et al. [28]. For ethylene and auxin treatments, the following treatments were employed: 100 μmol/L of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) for 8 h, 100 μmol/L ethylene inhibitor $AgNO_3$ or aminoethoxyvinylglycine (AVG) for 1 h, 20 μmol/L indole-3-acetic acid (IAA) for 1 h, 25 μmol/L auxin inhibitor 1-*N*-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) for 3 h, respectively. The corresponding control experiments (mock treatment) were carried out concomitantly. Treated tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Each treatment was performed in three replicates.

### 2.5. Abiotic stress treatments

For drought stress treatments, the 30-d-old tomato plants were stopped watering. When the leaves showed serious wilting, the leaves of drought stress plants and control plants supplied water normally were collected, respectively. Subsequently, the drought treatment plants were supplied water again, and the leaves of 2 h, 6 h and 24 h were collected during the recovery process. For salt treatments, the 21-d-old light-grown tomato seedlings grown in 1/2 MS culture medium were incubated in 50% MS buffer containing 200 mmol/L NaCl for 10 h. The mock treatments were performed concomitantly. For flooding treatments, the 21-d-old seedlings were submerged with water for 1 h and 3 h. All Materials collected were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Each treatment was performed in three replicates.