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The *Malus carotenoid cleavage dioxygenase* 7 is involved in stress response and regulated by *basic pentacysteine* 1

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ABSTRACT

Strigolactones have multiple roles for plants, including repression of the development of lateral buds and lateral roots, and improvement of stress tolerance, while the knowledge about the regulation of strigolactone synthesis is rare. The *Malus* genus is important resource of apple's rootstocks, thus study on strigolactone synthesis in *Malus* is helpful to realize the signal communication between apple scion and rootstock. Here, we detected the strigolactone contents of seven *Malus* species and confirmed the synthesis of strigolactones widely existed in *Malus*. Then the putative strigolactone synthesis gene, carotenoid cleavage dioxygenase 7 (*CCD7*), and its promoter sequence were cloned. The sequences were quite conserved and some stress responsive *cis*-acting elements were predicted in the promoters. The *CCD7's* expressions and its promoter's activity decreased under drought, waterlogging or methyl jasmonate (MeJA) treated conditions. By yeast one-hybrid screening, it was found that one transcriptional factor (TF) basic pentacysteine 1 (*BPC1*) had the ability of binding to *CCD7's* promoter. Transient overexpression of *BPC1* repressed the activity of *CCD7* promoter. The expressions of *BPC1* were increased when *Malus* suffered from the stresses, and it was believed that *BPC1* was one of the potential regulator of *CCD7's* expression and strigolactone synthesis.

1. Introduction

Strigolactones are newly discovered plant hormones that can inhibit the shoot branching (Gomez-Roldan et al., 2008). They were first identified in research projects with root parasitic plants (Cook et al., 1966). Seeds of those plants did not germinate in the absence of strigolactones (Yoneyama et al., 2010). More than eleven naturally biosynthesized strigolactones had been identified, with all sharing similar chemical structures (Kim et al., 2010), and synthesized through the carotenoid cleavage pathway (Matusova et al., 2005). Strigolactones are synthesized mainly in the root and then transported through the xylem (Kohlen et al., 2011). Strigolactones also play important roles in lateral root growth. Besides arrest of the lateral root's formation (Ruyter-Spira et al., 2011), strigolactones also enhanced the elongation of root hair (Kapulnik et al., 2011a), by increasing the synthesis of downstream ethylene (Kapulnik et al., 2011b). Strigolactones even function in leaves. Strigolactone synthesis and signaling mutants showed delayed senescence phenotype whether related to normal aging or stress (Liu et al., 2013). The light-harvesting ability in leaves was positively regulated by strigolactones (Mayzlish-Gati et al., 2010). Strigolactones influenced not only the development process but also the stress tolerance. Both the synthesis and signalling mutants were hypersensitive to the drought and salt stress than the wild-type plants (Van Ha et al., 2014), and they also presented less resistance to the foliar fungal pathogens (Torres-Vera et al., 2014), which suggested that strigolactones could positively regulate both the abiotic and biotic stress tolerance. Strigolactones are also the key signals in the symbiosis between arbuscular mycorrhizal fungi (AMF) and their host (Akiyama et al., 2005). Because approximately 80% of land plants serve as AMF hosts, strigolactones are likely to synthesize not only in the host of parasitic plants but also in most terrestrial species.

Strigolactones are categorized as apocarotenoids, and β-carotene is their natural substrate for synthesis (Ruyter-Spira et al., 2013). After β-carotene is isomerized by *DWARF 27* (*D27*), 9-*cis*-β-carotene is formed and is cleaved by *CCD7* and then by *carotenoid cleavage cioxygenase 8* (*CCD8*) (Seto et al., 2014). The first active strigolactone, carlactone, was synthesized after the two cleavage steps (Alder et al., 2012). The gene More Axillary Growth 1

Abbreviations: AbA, aureobasidin A; AMF, arbuscular mycorrhizal fungi; BPC1, basic pentacysteine 1; CaMV, cauliflower mosaic virus; CCD7, carotenoid cleavage dioxygenase 7; CCD8, carotenoid cleavage dioxygenase 8; D14, DWARF 14; D27, DWARF 27; GFP, green fluorescence protein; GUS, β-glucuronidase; MAX1, more axillary growth 1; MAX2, more axillary growth 2; MeJA, methyl jasmonate; NSP, nodulation signaling pathway; ORF, open reading frame; TF, transcriptional factor.

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(MAX1) operates downstream of carlactone and is likely responsible for modifications to strigolactones (Cardoso et al., 2014). Three genes involved in the signalling pathway of strigolactones were identified. The first, More Axillary Growth 2 (MAX2), encoded an ubiquitin E3 ligase of F-box family protein that controls various physiological processes associated with strigolactones (Nelson et al., 2011). The second, *DWARF 14* (*D14*), encoded an α/β hydrolase that interacts with strigolactones and MAX2, and is the possible receptor of strigolactones (Nakamura et al., 2013). Thirdly, *DWARF 53* was the first target protein of the D14-SCF^{MAX2} complex and its degradation led to the repression of lateral shoot growth (Zhou et al., 2013).

Along with identification of these genes, more researches have focused on determining their regulators. Lack of phosphorus or nitrogen improved the strigolactone contents in many species (Yoneyama et al., 2012), and it is likely due to induced expression of strigolactone synthesis genes (Umehara et al., 2010). Two GRAS-type TFs, Nodulation Signaling Pathway 1 and Nodulation Signaling Pathway 2, modulate D27's expression and final strigolactone contents, although no direct evidence has been determined that they can bind to D27's promoter region (Liu et al., 2011).

The major researches of strigolactones were limited in some model species, such as Arabidopsis, rice and tomato. Thus, to increase our understanding of strigolactones in *Malus* genus, we investigated the relative contents of strigolactone analogs and analysed the *CCD7* gene and its promoter in some *Malus* species. Meanwhile, the response of *CCD7's* expression to various stresses were tested and the TF regulating *CCD7* promoter's activity was screened.

2. Materials and methods

2.1. Plant materials and stress treatments

Seeds of seven *Malus* species (*M. sieversii*, *M. prunifolia*, *M. hupehensis*, *M. baccata*, *M. xiaojinensis*, *M. toringoides*, and *M. micromalus*) were stored in moist sand at 4 °C for 60 d to break dormancy. The germinated seeds were sowed into plastic pots and grew in the greenhouse. The seedlings of 90 d old were used for the following experiments. Tissue samples from three-year-old *M. baccata* trees were used for analysis of tissue-specific gene expressions. Drought stress was imposed by withholding irrigation since treatment. The entire pots were submerged into water for waterlogging treatment. By simultaneously spraying and irrigating with 50 μM MeJA (Sigma, US) solution, the seedlings were induced to mimic biotic stress. The seedlings with normal irrigation every day were as control. The seedlings' roots were cleaned and harvested 0, 24, 48, and 72 h later respectively.

2.2. Germination bioassay of the strigolactones

The method was according to Zhang et al. (2013) and Rubio-Moraga et al. (2014) with modifications. The *Malus* seedlings were separated into root, stem phloem, stem xylem and leaf. The separated tissues were dried in the vacuum freeze dryer (Labogene, Denmark) and grinded into powder with mortar. The powder of 0.1 g was placed into 50 ml centrifuge tube and 10 ml methanol (Sigma, US) was added. After 30 min extraction by ultrasonic and 2 min centrifugation at 10,000 × *g*, the extracted supernatant was ready for test. The small round disks (diameter of 8 mm) of glass fiber filter were placed in the petri dish and 20 μl of the extract was added. After volatilization of methanol, around 50 sunflower broomrape (*Orobanche cumana*) seeds were placed on the disk and 40 μl diluted water was added. The 1 μM GR24 (Chiralix, Netherland) solution or diluted water replaced extracts as control. The Petri dish was sealed and placed in the incubator at 25 °C to induce germination. The germination rates were counted 10 d later by dissecting microscope (Olympus, Japan).

2.3. Sequence clone and real-time PCR

Total RNA of root was extracted by the CTAB method (Chang et al., 1993), and the first-strand cDNA was synthesized with the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, US) by the manufacturer's protocol. Genomic DNA was also isolated by the CTAB method (Porebski et al., 1997). Primers for sequence clone and real-time PCR were designed (Table S1) by the Primer Premier software (Premier Biosoft, US). The PCR for sequence clone was performed in 25 μl solution containing 1 μg of cDNA or DNA, 0.2 μM of specific primers, 0.2 mM dNTPs, and 1 unit of Ex-Taq DNA polymerase (Takara, Japan). The reaction procedures were one cycle of 95 °C/10 min, 38 cycles of 95 °C/45 s, 50–60 °C/45 s, 72 °C/2 min, and followed a final 72 °C/10 min. For real-time PCR, the first-strand cDNA was synthesized by the PrimeScript RT Reagent Kit (Takara, Japan) and diluted to 150 ng μl^{-1}. The reference gene used was *EF1α* (Wang et al., 2014). Real time PCR was performed by a Bio-Rad iQ™5 platform (Bio-Rad, US) and SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (Takara, Japan). The PCR procedures were 95 °C/3 min, then 40 cycles of 95 °C/20 s, 53 °C/20 s, and 72 °C/20 s, followed by melting curve analysis of 95 °C/15 s, 53 °C/60 s, and ramping by 0.2 °C s^{-1} to a final 95 °C.

2.4. Sequence analysis and predictions of cis-acting elements

The blastn and tblastn program were conducted in the apple genome database (http://www.rosaceae.org/). The sequences were aligned by the MUSCLE program within MEGA software (Tamura et al., 2011). The aligned sequences were shown and prediction of molecular masses and isoelectric points was performed by DNAMAN software (Lynnon, Canada). The domain prediction was based on the Pfam database (Finn et al., 2014). Phylogenetic trees were constructed per the Neighbor-Joining method (Bootstrap method with 1000 replications, Poisson model) by MEGA. Putative *cis*-acting elements of the promoter were predicted by the database of Plant *cis*-Acting Regulatory Elements (Lescot et al., 2002)

2.5. Assays of promoter activity

To determine *MbCCD7* promoter's activity under different stresses, *MbCCD7* promoter was inserted into the 5′ end region of β-glucuronidase (GUS) gene of pCambia0390 vector. This constructed vector was transformed into *Agrobacterium tumefaciens* EHA105 strain, and was further transiently transformed into the *M. baccata* root through the vacuum infiltration method (de Oliveira et al., 2009). The agrobacterium was cultivated to OD_{600} 1.0 at 28 °C with shaking of 200 g. After centrifugation of 5 min at 5000 × *g*, the pellets were resuspended with equal volume of solution containing 10 mM MgCl, 10 mM 2-(*N*-morpholino) ethanesulfonic acid, and 100 μM acetosyringone. The *Malus* roots were immersed in the inoculum and vacuum-infiltrated for 30 min. The residual inoculum in the root surface was removed and the roots were cultivated in the medium for 72 h. The MS agar medium with 20% (w:v) PEG 6000 was used for drought treatment; the MS agar medium with 50 μM MeJA was used to mimic the biotic stress; the alternative MS liquid medium was used for waterlogging treatment; and the normal MS medium was used as control. The GUS activity was assayed as described by Jefferson et al. (1987).

To determine *CCD7* promoter's activity under overexpression of BPC1, the open reading frame (ORF) of *MbBPC1* was inserted into the 3′ end region of cauliflower mosaic virus (CaMV) 35S promoter in pCambia2300 vector. The negative control was the empty pCam-