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Bioinformatics and Expression Analysis of *CPMAX2* in Citrange

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Abstract

Transgenic citrange lines with *rol*ABC genes behave rosette branching and extreme dwarfing. To explore the regulatory mechanism of plant hormones in axillary shoot growth of transgenic citrange, a full length cDNA of *CPMAX2* was cloned from citrange [*Citrus sinensis* (L.) Osb × *Poncirus trifoliate* (L.) Raf.] by RT-PCR in this study. The expression of *CPMAX2* was detected in axillary tender leaves of 3 transgenic citrange lines and the wild type. Additionally, we constructed an over-expression vector *CPMAX2*-pCAMBIA1301 for further study. The results showed that the cDNA sequence and its putative peptide sequence shared 99.66% and 99.14% of identity with its *Citrus sinensis* ortholog *MAX2*. The deduced amino acid sequence contained putatively one F-box and two LRR repeat domains that are highly conserved in *MAX2* genes. *CPMAX2* was obviously down-expressed in tender leaves of 3 transgenic citrange lines compared with the wild type. The results suggest *CPMAX2* play an important role in regulating the rosette shoot growth in transgenic citrange with *rol*ABC genes.

Keywords: *Citrus*; citrange; strigolactone; *CPMAX2* gene; axillary bud outgrowth

1. Introduction

Strigolactones (SLs), which have been identified in recent years, play a role in the synthesis of new endogenous hormones in roots (Gomez-Roldan et al., 2008) and are involved in the regulation of shoot branching by *MAX2* (more axillary growth locus 2) signal transduction (Nelson et al., 2011). *MAX2* is located within the nucleus of the F-box/leucine-rich repeat protein, which plays an important role in photomorphogenesis (Shen et al., 2007), abiotic stress (Bu et al., 2014) and strigolactone signal transduction (Chevalier et al., 2014). MAX2 interacts with the core SCF (S phase kinase-associated protein 1-cullin-F box) subunits SKP1 (S phase kinase-associated protein 1) and Cullin to degrade target proteins in plant, the action of which is enhanced by the mobile MAX signal and then plays a role in inhibiting the blooming of the axillary shoot (Stirnberg et al., 2007). Research has demonstrated that the signal intersection between SLs in rice and brassinosteroids (BRs) in rape distinctly regulates the development of the lateral branch through the same transcription factor, BES1 (bri1-EMS-suppressor 1). In addition, genetic and biochemical evidence demonstrates that BES1 interacts with MAX2 and acts as its substrate to regulate SL-responsive gene expression in rice. D (dwarf) 14, a putative receptor of SLs synthesis in the root of rice, can promote BES1 degradation by SCF^{max} (Wang et al., 2013). Another degraded target of SCF^{Max2} is D53 protein, which is a homologous protein of the SMXL (Suppressor of more axillary growth 2 1-like) family molecular chaperone. D53 is a substrate of the SCF (D3) ubiquitination complex and functions as a negative regulator in SL signaling (Jiang et al., 2013). TPRs (topless-related-proteins) interact with D53 in the cell nucleus and repress the downstream gene expression of SLs signaling. *d3* and *d53*

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are homologous genes of *MAX2* in rice, and the single mutant result in dwarf phenotype with a large number of tillers, respectively; d53-RNAi rice in the background of the *d3* or *d14* mutant was restored to nearly wild-type level (Zhou et al., 2013). *MAX*2 regulates and degrades a class of DELLA protein transcription factors and takes part in gibberellin signal transduction (Nakamura et al., 2013).

Lateral branch growth and development are synergistically regulated by multiple phytohormones. *MAX*2 might be the key factor involved in the signal cross-network of various plant hormones. *rolABC* genes were introduced into citrange; all transformed trees displayed dwarfing, increased lateral branching and reduced internode length (Hu et al., 2006). Transgenic citrange was used as an inter-rootstock, onto which we grafted Shatian pomelo (*Citrus grandis* [L.] Osbeck 'Shatian') and Dahong sweet orange. The dwarfing rate of the scions ranged from 12% to 30% after being grafted onto transgenic or inter-rootstock (Yuan, 2011). However, the dwarfing mechanism of *rol* genes remains unknown.

In this study, we cloned the *CPMAX2* gene and analyzed its expression in transgenic citrange with *rolABC* genes and successfully constructed the plant over-expression vector of *CPMAX2*. Consequently, the function of *CPMAX2* can be explored in future research.

2. Materials and methods

2.1. Plant materials and lateral shoot investigation

Seedling stem segments of Troyer citrange were infected with *Agrobacterium tumefaciens* carrying the pDN3514 plasmid with *rol*ABC genes. Clones B, D and E of transgenic citrange harboring *rol*ABC genes and control plants were potted in a greenhouse and grown under normal conditions at the National Centre for Citrus Improvement (Changsha, N 28°10′47.10″, E 113°04′36.88″), Hunan Province, China. After the shoots emerged in April 2014, ten branches were selected from every treatment, and two axillary leaves were used to isolate RNA.

A total of 30 annual shoots were selected from every treatment used for RNA isolation and investigation of the number of branches of spring shoots.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from each sample with Trizol (Invitrogen™) according to the manufacturer's instructions with some modification. After extraction, the RNA sample was dissolved in 15–20 µL of 0.1% diethyl pyrocarbonate (DEPC)-treated water. RNA was treated with DNase using the on-column Qiagen DNase Treatment (RNeasy MicroKit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration and A_{260}/A_{280} ratios were determined after DNase I treatment with a Shimadzu UV-1800 UV-VIS spectrophotometer (Shimadzu Corporation, Tokyo, Japan), and 1.1% agarose gel electrophoresis was conducted to visualize the integrity of the RNA. Only RNA samples with A_{260}/A_{280} ratios >1.9 were used for analysis. Total RNA was used to prepare the first-strand cDNA with oligo dT18 primer using the Reverse Transcriptase M-MLV kit (TaKaRa Corporation, Dalian, China). Twenty-five µL of the reaction mix was separately added to each 200 µL PCR tube according to manual. First placed 3 µL (≈2 µg) RNA, 11.5 µL DEPC H_2O in a microcentrifuge tube by 72 °C for 5 min, chilled on ice; next added 5 µL (5×) buffer, 3 µL (1 µmol · L^{-1}) dNTP, 0.5 µL (30 U · L^{-1}) RNase inhibitor, 0.5 µL (200 U · L^{-1}) M-MLVRTase, and 1.0 µL DEPC H_2O to the tube. Then incubated the reaction at 42 °C for 60 min, finally terminated the reaction by heating at 95 °C for 5 min.

2.3. Cloning of the CPMAX2 gene

The *CPMAX2* gene was cloned using the RT-PCR method; the 15 µL PCR reaction system included the following: ddH_2O 3.75 µL, $MgCl_2$ (25 µmol · L^{-1}) 1.5 µL, dNTPs (1 µmol · L^{-1}) 3 µL, forward and reverse primers (2.5 µmol · L^{-1}) 1.5 µL each, cDNA (20 ng · L^{-1}) 2 µL, and *Taq* DNA Polymerase (1 U · μL^{-1}) 0.25 µL. The PCR amplification program consisted of the following steps: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 1 min 30 s, 72 °C for 1 min, followed by 72 °C for 10 min.

2.4. Construction of the over-expression vector of the CPMAX2 gene

Bgl II and *Bst*E II restriction enzymes sites were introduced into the primers of cDNA full length amplification (Table 1), the amplified product was connected to pMD18-T vector according to the manual (TaKaRa Corporation, Dalian, China), *Bgl* II and *Bst*E II restriction enzymes were used to digest CPMAX2-T vector plasmid DNA by 37 °C for 3 h; the *CPMAX2* product was recycled from 1.0% agarose gel electrophoresis, and pCAMBIA 1301 was digested by the same method. Then, the *CPMAX2* product and pCAMBIA 1301 were joined by T4 DNA ligase by 16 °C for 12 h, which was transferred to DH5α *Escherichia coli*.

2.5. Phylogenetic analysis of CPMAX2 gene

The homologous sequence of the putative amino acid sequence of the *CPMAX2* gene was searched using the Blastp procedure, and then proteins were selected from 26 different plant species for the phylogenetic analysis of the CPMAX2 amino acid sequence using MEGA5.1 software with the Neighbor-joining method.

2.6. Quantitative real-time PCR

Primer pairs were designed with the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) for the genes; Quantitative real-time PCR (qRT-PCR) was performed on the ABI 7500 Real Time System (Applied Biosystems, Foster City, CA, USA) using the *β-actin* gene as the endogenous control. Briefly, the primers for the target gene and *β-actin* were diluted in SYBR Green (Power SYBR® Green PCR Mix) and 20 µL of