Bioinformatics and Expression Analysis of CPMAX2 in Citrange

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Abstract

Transgenic citrange lines with rolABC 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 trifoliata (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 rolABC 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-Roldán 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 morphogenesis (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 SCFmax (Wang et al., 2013). Another degraded target of SCFmax 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...
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). MAX2 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. MAX2 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 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 rolABC genes. Clones B, D and E of transgenic citrange harboring rolABC 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°3′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 A260/A280 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 A260/A280 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 H2O 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 H2O to the tube. Then incubated the reaction by heating 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: ddH2O 3.75 μL, MgCl2 (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 BstE 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 BstE II restriction enzymes were used to digest CPMAX2-T vector plasmid DNA by 37 °C for 3 h; the CPMAX2 product was recycled from 0.1% 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
the reaction mix was added to each 200 μL PCR tube according to the manufacturer’s instructions; the following were also included: forward and reverse primers 1.5 μL (2.5 μmol·L⁻¹) each (Table 1), cDNA 1.5 μL, ddH₂O 5.5 μL. Every treatment was replicated 3 times and there was an NTC control. Reactions were performed by an initial incubation at 95 °C for 30 s, and then cycled at 95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s for a total of 40 cycles. The steps were conducted to collect the fluorescent signal during extension at 72 °C; output data were generated by the 7500 System software, and the relative expression was calculated using the 2⁻ΔΔCt method (Kenneth and Thomas, 2001).

3. Results

3.1. Cloning and sequence analysis of the CPMAX2 gene

The length of CPMAX2 was 2 297 bp (Fig. 1). According to the analysis of the sequence of CPMAX2 using the biological information method, the cDNA sequence included: forward and reverse primers 1.5 μL (2.5 μmol·L⁻¹) each (Table 1), cDNA 1.5 μL, ddH₂O 5.5 μL. Every treatment was replicated 3 times and there was an NTC control. Reactions were performed by an initial incubation at 95 °C for 30 s, and then cycled at 95 °C for 5 s, 55 °C for 30 s and 72 °C for 30 s for a total of 40 cycles. The steps were conducted to collect the fluorescent signal during extension at 72 °C; output data were generated by the 7500 System software, and the relative expression was calculated using the 2⁻ΔΔCt method (Kenneth and Thomas, 2001).

3.3. Phylogenetic analysis of the CPMAX2 gene

The phylogenetic tree showed that there were obvious differences among Citrus sinensis × Poncirus trifoliata, Populus euphratica and Vitis vinifera (7 different branches were clustered from 23 other plants). Citrange, sweet orange, clementine mandarin, and trifoliate orange clustered on the first branch (Fig. 5); citrange is the hybridization progeny of trifoliate orange and sweet orange; however, the variation of the amino acid sequence of CPMAX2 was not obvious, which indicated the main genetic information originated from sweet orange. Gossypium raimondii and G. hirsutum clustered on the second branch, Ricinus communis and Jatropha curcas on the third, Arabidopsis thaliana, Brassica napus and B. rapa on the fourth, Cucumis melo and C. sativus on the fifth, Malus × domestica, M. hupehensis, M. baccata, Prunus persica, P. mume on the sixth, and Nicotiana tomentosiformis, Solanum tuberosum and S. lycopersicum on the seventh.

3.4. CPMAX2 gene expression analysis in transgenic citrange with rolABC genes

Quantitative RT-PCR showed that CPMAX2 was obviously down-expressed in tender leaves of 3 transgenic citrange lines compared with the wild type (Fig. 6). The trunk base developed a large number of lateral branches after rolABC genes were transferred into Troyer citrange. Observing the annual shoot growth of transgenic and wild type citrange, we found that all plants investigated were able to sprout spring and summer shoots. Three transgenic citrange lines had more axillary branches than the wild type. The E line had the maximum lateral shoots with an average of 3.5 lateral shoots; by contrast, the wild type only had an average of 1.9 lateral shoots (Fig. 7).

The results indicated that CPMAX2 expression played an important function in citrange lateral growth; however, there was no evidence of a relationship between lateral shoot number and CPMAX2 expression value. It is possible to attribute the dramatic changes in plant hormones to the transference of the rol genes. The mechanism underlying lateral shoot growth of transgenic citrange with rolABC genes is suggested as follows: CPMAX2 down-expression blocks SL signal transduction. Then, the ability to suppress lateral shoots is decreased; the balance between auxin, cytokinin and gibberellin, etc. promotes lateral germination, resulting in the growth of rosette branches of transgenic citrange with rolABC genes.

### Table 1 The primers of CPMAX2 cloning and qRT-PCR analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPMAX2</td>
<td>TTTAGATCTTTTTTACCTGCCCACCT</td>
<td>TCCGGTTACCGGAAAAATCGTTCCATGCTCT</td>
<td>Full-length cDNA amplification</td>
</tr>
<tr>
<td>CPMAX2</td>
<td>ACCAAGTGGTTGAAAGTGATTGG</td>
<td>ATTGGCCTTTGGGTTGGAAGGG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>β-actin</td>
<td>CAGACGAGGTAGTGGGTGTTGG</td>
<td>ATGACGACTTGGTGGGGGAGG</td>
<td>qRT-PCR</td>
</tr>
</tbody>
</table>

Fig. 1 Electrophoresis of RT-PCR products for cloning of the CPMAX2 gene. 1, 2: RT-PCR products of CPMAX2; M: DL5000 DNA marker.
Fig. 2 Analysis of the amino acid sequence of CPMAX2 from citrange

Red represents F-box domain, blue represents two LRR repeat domains.
**Fig. 3** Electrophoresis results of *CPMAX2*-pCAMBIA 1301 digest by *Bgl* II and *Bst* EI I
1: Enzyme-digestion of *CPMAX2*-pCAMBIA 1301; M: DL5000 DNA marker.

**Fig. 4** *CPMAX2*-pCAMBIA1301 vector
The promoter: CaMV 35 S; Terminator: NOS.

**Fig. 5** Phylogenetic analysis of MAX2 proteins from 26 different plant species
The value of the evolutionary tree represents bootstrap support values.
4. Discussion

F-box proteins exist widely in plants. These proteins participate in plant hormone signal transduction, self-incompatibility, floral organ development, and biological stress (Bi et al., 2006). The structural domain of the F-box protein where it conjugates with Skp1 or proteins similar to the Skp1 combined region of the SCF (Skp1-Cullin-F-box protein) composite body, which is located in the N-terminal protein, includes about 40–50 amino acids. There is no strictly conserved sequence (only the residues of several amino acids are conservative); location 9 amino acid sequence is usually leucine or methionine, location 10 corresponds to proline, location 17 corresponds to different forms of isoleucine or valine, location 21 to leucine or methionine, and position 33 to serine or cysteine (Kipreos and Pagano, 2000). In this study, the CPMAX2 amino acid sequences contain the typical F-box domain. The 21st amino acid residues were transformed into isoleucine, the 33rd amino acid was transformed into valine, and other conservative sequences were unchanged. Previous studies have suggested that the F-box domain combines with other protein C-terminal motifs to play a role, and its functional mechanism is related to target proteins for degradation through the proteasome pathway to regulate the expression of downstream genes (Cheng et al., 2011), and then impacts the properties of plants.

MAX2 was the first identified SL signal transduction element in Arabidopsis thaliana. max2 mutants enhance the phenotype of Arabidopsis lateral branches, and grafting experiments show that it is the regulation factor of the SL response gene (Stirnberg et al., 2002). F-box proteins of MAX2 encode one of the SCF E3 ubiquitin ligase complex subunits; this is expressed primarily in the xylem parenchyma cells (Stirnberg et al., 2007). Transgenic citrange with rolABC genes had rosette-type branch, dwarfing, shorter internodes, and qRT-PCR confirmed 3 transgenic, healthy axillary leaves and a significant decrease in CPMAX2 gene expression; these results indicate that the SL signal participated in bud germination and growth regulation. Therefore, the possible mechanism is that rol gene expression results in a weaker SL signal, which inhibits the downstream expression of response genes, hinders the function of SLs in inhibiting bud germination, promotes large axillary bud germination, resulting in rosette-type branches and dwarf plant type. Moreover, MAX2 expression may simply be an important factor in plant bud germination.

Many years of observations on the morphological and physiological characteristics of transgenic citrange with rolABC genes have indicated that clones B, D, and E have more lateral shoots compared with the wild type, and the E line has the maximum number of lateral branches. The zeatin content increased significantly in the middle leaf of the spring shoot of transgenic citrange (Hu et al., 2006), and the IAA and GA3 contents were significantly decreased in the apical bud (Yuan, 2011). In this study, the authors found CPMAX2 down-expression in 3 transgenic citrange lines, but there was no evidence of a significant correlation between lateral shoot growth and CPMAX2 expression. It is speculated that unbalanced plant hormones resulted in differences in rolABC gene insert location and copy number in 3 transgenic lines. In addition, the SL signal interacts with other plant hormones and synergistically regulates axillary shoot germination and growth. Studies on Arabidopsis, rice, and Petunia plants have confirmed that auxin, cytokinin, gibberellin, brassinolide and other plant hormones are involved in the regulation of lateral bud germination (Ferguson and Beveridge, 2009; Muller and Leyser, 2011; Bennett and Leyser, 2014). However, the collaborative regulation mechanism is still unclear. In future studies, the authors will focus on transferring CPMAX2 into transgenic citrange with rolABC genes, observing the axillary bud germination and branching characteristics of transgenic citrange, analyzing the expression of CPMAX2, the various genes involved in plant hormone biosynthesis, and signal transduction factors, to explore the regulatory mechanism of lateral shoot growth and plant type by studying CPMAX2 gene function and related genes involved in other plant hormones.

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References


