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Molecular cloning, characteristics and low temperature response of raffinose synthase gene in *Cucumis sativus* L.

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ABSTRACT

Raffinose synthase (RS, EC2.4.1.82) is one of the key enzymes that channels sucrose into the raffinose family oligosaccharides (RFOs) biosynthetic pathway. However, the gene encoding RS is poorly characterized in cucumber (*Cucumis sativus* L.), which is a typical RFOs-translocating plant species. Here we isolated the gene encoding RS (*CsRS*) from the leaves of cucumber plants. The complete cDNA of *CsRS* consisted of 2552 nucleotides with an open reading frame encoding a polypeptide of 784 amino acid residues. Reverse transcription-polymerase chain reaction and RNA hybridization analysis revealed that expression of *CsRS* was the highest in leaves followed by roots, fruits, and stems. The RS activity was up-regulated and the raffinose content was high in the leaves of transgenic tobacco with over-expression of *CsRS*, while both the RS activity and the raffinose content decreased in the transgenic cucumber plants with anti-sense expression of *CsRS*. The expression of *CsRS* could be induced by low temperature and exogenous phytohormone abscisic acid (ABA). In cucumber growing under low temperature stress, *CsRS* expression, RS activity and raffinose content increased gradually in the leaves, the fruits, the stems and the roots. The most notable increase was observed in the leaves. Similarly, the expression of *CsRS* was induced in cucumber leaves and fruits with 200 μM and 150 μM ABA treatments, respectively.

Introduction

Raffinose family oligosaccharides (RFOs), such as raffinose (*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 ↔ 2)-*O*-β-D-fructofuranoside) and stachyose, are major soluble galactosyl-Suc carbohydrates in seeds, roots and tubers of many plant species (Avigad and Dey, 1997). Photo assimilation is exported exclusively in the form of sucrose in many plant species, including tomato, *Arabidopsis*, maize, tobacco and soybean (Hu et al., 2009). However, in Cucurbitaceae, Lamiaceae, Oleaceae and other plant families, raffinose and stachyose are the predominant carbohydrates translocated in the phloem. Besides their roles in carbon storage and transportation in plants, RFOs can also act as compatible solutes for plants' tolerance against abiotic stresses (Bachmann et al., 1994; Haritatos et al., 1996; Taji et al., 2002). RFOs are found to increase cold tolerance during the process of cold acclimation (Koster and Lynch, 1992; Bachmann et al., 1994; Castonguay et al., 1995; Gilmour et al., 2000). Raffinose can also act as osmoprotectants and stabilizes the chloroplast thylakoid membrane or photo system II during a freeze–thaw cycle (Hincha, 1990; Knaupp et al., 2011). Raffinose is found to accumulate in vegetative tissues under drought stress to increase drought tolerance (Taji et al., 2002). Furthermore, raffinose and galactinol also play a role in the cellular metabolism as they protect the photosynthesis of chloroplasts from reactive oxygen species (ROS) caused by salinity, chilling, and drought (Nishizawa et al., 2008).

In many plant species, phytohormone abscisic acid (ABA) can induce similar cellular responses caused by abiotic stresses such as drought, cold and salinity (Kahn et al., 1993; Hasegawa et al., 2000; Bray, 2002). ABA can increase the carbohydrate level in stressed plants by regulating the enzymes involved in carbohydrate biosynthesis (Kobashi et al., 1999; Taji et al., 2002; Blöchl et al., 2005). The accumulation of raffinose and stachyose as well as other sugars is observed in the ABA treated somatic embryos of alfalfa (Blöchl et al., 2005) and peach fruit (Kobashi et al., 1999). However, the molecular mechanism of ABA induction on RFOs metabolism is still elusive.

Abbreviations: ABA, abscisic acid; *CsRS*, raffinose synthase gene in cucumber; FW, fresh weigh; GolS, galactinol synthase; α-Gal, α-galactosidase; HPLC, high performance liquid chromatography; PPFD, photosynthetic photon flux density; RACE, rapid amplification of cDNA ends; RFOs, raffinose family oligosaccharides; RS, raffinose synthase; RT-PCR, reverse transcription-polymerase chain reaction; STS, stachyose synthase.

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RFOs are synthesized from sucrose by the addition of activated galactose moieties donated from galactinol (Peterbauer and Richter, 2001). Galactinol is formed by galactinol synthase (GolS; EC 2.4.1.123) from UDP-Gal and *myo*-inositol. Trisaccharide raffinose and tetrasaccharide stachyose are the products of this biosynthetic pathway and these reactions are mediated by raffinose synthase (RS; EC 2.4.1.82) and stachyose synthase (STS, EC 2.4.1.67), respectively (Peterbauer et al., 2002a,b). Thus, a set of galactosyl transferases is involved in the biosynthesis of RFOs (Peterbauer and Richter, 2001). RS is one of the key enzymes that can channel sucrose into the RFO biosynthetic pathway. Unlike GolS and STS, RS has not been well studied, probably because it is the most labile enzyme of the pathway (Peterbauer et al., 2002a). RS is first isolated from *Vicia faba* seeds (Lehle and Tanner, 1973) and crude RS enzyme is first extracted from the leaves of *Ajuga reptans* (Bachmann et al., 1994). Recently, several genes encoding RS have been reported in patent (Oosumi et al., 1998; Watanabe and Oeda, 1998), and a number of gene sequences have been deposited in GenBank. Although the molecular and biochemical characterizations of RS in pea (Peterbauer et al., 2002a) and rice (Li et al., 2007) have been studied in part, many characteristics of RS remain poorly described.

Cucumber (*Cucumis sativus* L.) is a typical RFOs-translocating plant (Gross and Pharr, 1982; Pharr et al., 1985; Hu et al., 2009). It is widely cultivated as one of the most important vegetable crops in China. However, the gene encoding RS in cucumber is poorly characterized. In this report, we identified a putative RS cDNA in cucumber (*CsRS*). We further characterized *CsRS* including *CsRS* homological analysis, spatiotemporal expression, over-expression in tobacco, anti-sense expression in cucumber, responses to low temperature and exogenous ABA, content of raffinose and RS activity.

Materials and methods

Plant samples and chemical materials

Cucumber cultivar Guonong No. 25 (a cold-tolerant cultivar) was used in this study. Seeds (obtained from Professor Huazhong Ren at China Agricultural University) were sowed in 20 cm × 20 cm plastic pots containing peat–vermiculite (2:1, v/v) in a greenhouse at China Agricultural University in Beijing. Seedlings were grown under 25–28 °C/14–18 °C (day/night) and a 14 h light/10 h dark, with a photosynthetic photon flux density (PPFD) of 500–600 μmol m^{-2} s^{-1}. Raffinose, sucrose, stachyose, galactose, fructose, glucose and galactinol were purchased from Sigma (USA).

CsRS gene cloning and phylogenetic analysis

Total RNA was extracted from 200 mg fresh cucumber leaves by Trizol reagent (Invitrogen, USA) and used as the template for the synthesis of single-strand cDNA using PowerScript™ Reverse Transcriptase and SMARTTMIII Oligonucleotide/CDSIII3′ primers (Clontech, USA). The partial sequence of *CsRS* was amplified with the primers 5′-GAAGGAAGTTTCAGGTCTGCCAT-3′ (forward) and 5′-CCTATGGAGTTTTGCTTTGTGCC-3′ (reverse) based on the published coding sequence of raffinose synthase (RS) in cucumber (*CsRS*) (GenBank accession number: E15707). The PCR mixture (50 μL) contained 1 μg template cDNA, 0.2 mM of dNTP mix, 10 μM of each primer, 5 μL of 10× PCR buffer and 2.5 units of *Taq* DNA polymerase (TaKaRa, Japan). PCR amplification was accomplished in 30 cycles at 94 °C for 45 s, 57 °C for 45 s and 72 °C for 3 min. PCR products were cloned into the pGEM-T Easy vector (Promega, USA) and sequenced.

The cDNA ends were amplified using the rapid amplification of cDNA ends (RACE)-PCR kit (Invitrogen, USA) according to the protocol provided by the manufacturer. For 3′ RACE, the RNA was reverse-transcribed with an oligo-(dT) adaptor primer and subsequently used as the template for further PCR. Two pairs of primers were used for PCR amplification. First forward primer was 5′-TCGCGATTGTTTGTTTGAAGACCCT-3′ and second forward primer was 5′-CGCAACCAATGCTTTTCACAATACTCA-3′, both reverse primers were complementary to the adaptor primer (CDSIII/3′PCR universal primer). For 5′ RACE, the RNA was reverse-transcribed with a specific reverse primer (5′-AAGACGGCAGGGCATTT-3′). After that an oligo-(dC) anchor sequence was added to the 3′ end of the reaction products using a 5′ RACE kit (Invitrogen, USA). The partial *CsRS* gene from the 5′ end was then amplified by a pair of PCR primers: the gene specific reverse primer (5′-TGCATCAACAACTTTCGACGAACCACT-3′) and the 5′ RACE adaptor primer. PCR was performed according to the manufacturer's instruction. All PCR products were purified, then cloned into pGEM-T Easy vector (Promega, USA), respectively, and sequenced.

The sequence alignment and the phylogenetic tree were constructed by DNAman 4.0 and NCBI web (http://www.ncbi.nlm.nih.gov).

Vector construction and Agrobacterium-mediated transformation

The cDNA of *CsRS* was inserted into the expression vector P-super 1300 (kindly provided by Dr. Dapeng Zhang in Tsinghua University, Beijing, China) which transformed from pCambia 1300, at *Xba*I and *Kpn*I sites in either the sense orientation (sense-P-super 1300-*CsRS*) or the anti-sense orientation (antisense-P-super 1300-*CsRS*). The resultant plasmids (Figs. 3A and 4A) were transformed into *Agrobacterium tumefaciens* strain LBA4404, respectively. The sense-vector was extracted and transformed into tobacco (*Nicotiana tabacum* L. cv. Yunyan 85) using the leaf disk transformation method. The antisense-vector was extracted and transformed into cucumber cultivar Guonong No. 25 using the fresh expanding cotyledon disk transformation method (Mohiuddin et al., 1997). Briefly, the well-expanded cotyledons of 3-day-old were cut into two parts and placed upside down on the polarization medium (MS medium supplemented with 0.5 mg L^{-1} 6-BA, 1 mg L^{-1} abscisic acid (ABA) and 2 mg L^{-1} $AgNO_3$, pH 5.7–5.8). After 1 day of cultivation in the dark, the cotyledons were incubated with *A. tumefaciens* cells harboring antisense-P-super 1300-*CsRS* in MS liquid medium for 12 min at 28 °C. The cotyledons were then transferred to the polarization medium and co-cultured for 2 days at 28 °C in the dark. Then they were selected on polarization medium containing 10 mg L^{-1} hygromycin and 500 mg L^{-1} carbenicillin. Three weeks later, the shoots were transformed to the rooting medium (MS medium supplemented with 10 mg L^{-1} hygromycin and 200 mg L^{-1} carbenicillin) for root growth. After the root grew well in the rooting medium, the transgenic plants were transferred to phytotron, under a 12 h photoperiod (600 μmol m^{-2} s^{-1} PPFD) at 25 °C (day) and 18 °C (night).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), RNA hybridization and PCR-based DNA hybridization analysis

Total RNA extracted from the leaves, the fruits, the roots, and the stems, respectively, was used as the template for single-strand cDNA synthesis using PowerScript™ Reverse Transcriptase. Total RNA was treated with DNase to remove traces of DNA. Single-strand cDNA was used for semi-quantitative RT-PCR with 18s rRNA as a control. The primers used for PCR amplification of the *CsRS* were 5′-CCCTTCTTTTAGTTTTTTGGGTTTG-3′ (forward) and 5′-GCCTCTTTAACAAGTGCAAACGGAT-3′ (reverse). Thermal cycling