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Research article

Overexpression of alfalfa *Orange* gene in tobacco enhances carotenoid accumulation and tolerance to multiple abiotic stresses

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ABSTRACT

The multifunctional Orange (Or) protein plays crucial roles in carotenoid homeostasis, photosynthesis stabilization, and antioxidant activity in plants under various abiotic stress conditions. The *Or* gene has been cloned in several crops but not in alfalfa (*Medicago sativa* L.). Alfalfa is widely cultivated across the world; however, its cultivation is largely limited by various abiotic stresses, including drought. In this study, we isolated the *Or* gene from alfalfa (*MsOr*) cv. Xinjiang Daye. The amino acid sequence of the deduced MsOr protein revealed that the protein contained two trans-membrane domains and a DnaJ cysteine-rich zinc finger domain, and showed a high level of similarity with the Or protein of other plants species. The MsOr protein was localized in leaf chloroplasts of tobacco. The expression of *MsOr* was the highest in mature leaves and was significantly induced by abiotic stresses, especially drought. To perform functional analysis of the *MsOr* gene, we overexpressed *MsOr* gene in tobacco (*Nicotiana benthamiana*). Compared with wild-type (WT) plants, transgenic tobacco lines showed higher carotenoid accumulation and increased tolerance to various abiotic stresses, including drought, heat, salt, and methyl viologen-mediated oxidative stress. Additionally, contents of hydrogen peroxide and malondialdehyde were lower in the transgenic lines than in WT plants, suggesting superior membrane stability and antioxidant capacity of TOR lines under multiple abiotic stresses. These results indicate the *MsOr* gene as a potential target for the development of alfalfa cultivars with enhanced carotenoid content and tolerance to multiple environmental stresses.

1. Introduction

Alfalfa (*Medicago sativa* L.) is an excellent perennial legume and is widely cultivated across the world. Alfalfa is an important agricultural crop and is also used in animal husbandry, especially in arid and semi-arid regions (Wang et al., 2015b). In the semi-arid Loess Plateau region of China, alfalfa represents an important ecological function and is used as the main forage crop for grain for green project which was designed to convert unsustainable farmland to grassland or forestland (Yuan et al., 2014; Wang et al., 2017). However, cultivation of alfalfa on marginal lands under drought and salt stress results in a severe reduction in its yield and quality (Li et al., 2014; Wang et al., 2017). With continued global climate change, abiotic stresses such as drought, heat, and salt stress are expected to become more severe and frequent (Li et al., 2012b; IPCC, 2014). Current crop production cannot meet the increasing demand for high quality crops (Wang et al., 2015c). Therefore, developing alfalfa varieties with high quality and greater tolerance to multiple environmental stresses is urgently needed.

Carotenoids are a class of multifunctional pigments and nutrients and are essential for plant growth, development, and environmental

Abbreviations: Or, Orange; TOR, transgenic tobacco plants expressing alfalfa *Or* gene; WT, wild-type; PC, positive control; ROS, reactive oxygen species; PSY, phytoene synthase; CHY-β, β-carotene hydroxylase; *LCY-ε*, lycopene ε-cyclase; MMLV, Moloney murine leukemia virus; MDA, Malondialdehyde; GFP, green fluorescent protein; qRT-PCR, quantitative reverse transcriptase PCR; MV, methyl viologen; RWC, relative water content; PSII, photosystem II; H_2O_2, hydrogen peroxide; TBA, thiobarbituric acid; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; DAPI, 4′, 6-diamidino-2-phenylindole; *Fv/Fm*, maximum quantum yield of PSII; OEE2-1, oxygen-evolving enhancer 2–1

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adaptation as well as for optimal human health (Esteban et al., 2015). Carotenoids are critical components of photosynthetic and antioxidant systems in plant cells (Ramel et al., 2013; Zarco-Tejada et al., 2013; Zhou et al., 2015; Hou et al., 2016). During photosynthesis, carotenoids harvest light energy and protect photosynthetic organelles from excess light energy via the xanthophyll cycle (Förster et al., 2011; Nisar et al., 2015). Carotenoids function as potent antioxidants and efficiently scavenge intracellular reactive oxygen species (ROS) to prevent oxidative stress (Havaux, 2014). Carotenoids also act as substrates of apocarotenoids, including vitamin A, and plant hormones, such as strigolactones and abscisic acid (ABA), which play important roles in germination, growth, branching, and stress tolerance of plants (Khosla and Nelson, 2016). Because of their diverse roles, carotenoids represent key compounds for breeding crops with enhanced nutritional content and environmental adaptation (Esteban et al., 2015; Kim et al., 2018). Significant efforts have been made to improve the process of carotenogenesis in crops via the metabolic engineering of carotenoid biosynthetic genes, such as phytoene synthase (*PSY*), β-carotene hydroxylase (*CHY-β*), and lycopene ε-cyclase (*LCY-ε*) (Ye et al., 2000, Fraser et al., 2002, Han et al., 2008, Kim et al., 2012a, 2012b, 2013).

The *Orange* (*Or*) gene is crucial for carotenoid biosynthesis and accumulation. Nucleotide sequence of the *Or* gene is highly conserved in cauliflower (*Brassica oleracea* var. *botrytis*), sweetpotato (*Ipomoea batatas*), melon (*Cucumis melo* var. *cantalupensis*), and *Arabidopsis thaliana* (Lu et al., 2006; Bai et al., 2014; Kang et al., 2017; Kim et al., 2018). The Or protein uniquely enhances carotenoid accumulation by stimulating the formation of a metabolic sink for carotenoid accumulation rather than by directly increasing carotenoid biosynthesis (Lopez et al., 2008; Li et al., 2012a). Overexpression of the cauliflower *Or* gene in potato (*Solanum tuberosum*) under the control of a tuber-specific promoter induces the formation of chromoplasts with significant levels of β-carotene in transgenic potato tubers (Lopez et al., 2008). Previously, we showed that the overexpression of the sweetpotato *Or* gene significantly increases β-carotene and total carotenoid contents in sweetpotato calli and storage roots as well as in alfalfa leaves (Kim et al., 2013; Park et al., 2015; Wang et al., 2015c). Moreover, the Or protein contains a DnaJ cysteine-rich zinc-binding domain and exhibits notable holdase chaperone activity. Under heat and oxidative stress, the Or protein directly interacts with PSY, a key limiting enzyme in carotenoid biosynthesis, and prevents its degradation (Park et al., 2016). Notably, expression of the *Or* gene is induced by various environmental stresses and confers plants with enhanced tolerance against drought, heat, salt, and oxidative stress (Kim et al. 2013, 2018; Wang et al., 2015c). Taken together, these observations suggest the use of the *Or* gene as an efficient molecular tool for breeding crop plants with outstanding nutritional quality and adaptability to multiple abiotic stresses. However, the *Or* gene of alfalfa is not yet cloned, despite the importance of alfalfa as a forage crop on marginal lands.

In this study, we isolated the *Or* gene from alfalfa (*MsOr*) cv. Xinjiang Daye, and investigated its expression profile under various environmental stress conditions. To clarify the role of *MsOr* in tolerance to abiotic stress, we generated transgenic tobacco (*Nicotiana benthamiana*) lines overexpressing *MsOr* under the control of the cauliflower mosaic virus (CaMV) *35S* promoter via *Agrobacterium*-mediated transformation. Our results showed that overexpression of the *MsOr* gene improved the carotenoid content as well as the environmental adaptability of transgenic tobacco plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Alfalfa cv. Xinjiang Daye and tobacco plants were used in this study. Plants were grown in plastic pots (12 cm upper inner diameter × 9 cm lower inner diameter × 11 cm height) filled with soil, with one plant per pot. Pots were maintained in a growth chamber at 25 °C temperature, and under a 16 h light/8 h dark photoperiod, 60% relative humidity, and 200 μmol $m^{-2} s^{-1}$ light intensity. One-month-old healthy plants were used for further analysis.

2.2. Gene cloning and phylogenetic analysis

Total RNA was extracted from healthy leaves of 1-month-old alfalfa plants using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I to remove any contaminating genomic DNA. Total RNA (2 μg) was used for first-strand cDNA synthesis using Moloney murine leukemia virus (MMLV) reverse transcriptase (TOPscript™ RT DryMIX), according to the manufacturer's instructions. To isolate *MsOr*, a pair of forward and reverse primers (*MtOr*-F/R; Table S1) were designed on the basis of the sequence of the *Or* gene of *Medicago truncatula* (TA4160_3880), a close relative of alfalfa. The coding sequence (CDS) of *MsOr* was amplified from the first-strand cDNA using SolGent™ Pfu-X DNA Polymerase (Solgent, Daejeon, Korea). The purified PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

Nucleotide sequence of *MsOr* was compared with that of the other *Or* gene family members via online BLAST searches in UniProt and the National Center for Biotechnology Information (NCBI). The published *Or* sequences were translated to amino acid sequences using BioEdit. Alignments of the predicted amino acid sequences were conducted using BioEdit and BoxShade server. A phylogenetic tree was constructed with 1000 iterations using the neighbor-joining method in Molecular Evolutionary Genetics Analysis (MEGA) version 6.

2.3. Vector construction

The vector used to overexpress *MsOr* in tobacco was constructed using Gateway® cloning (Invitrogen), as described previously (Kim et al., 2013). Briefly, the *MsOr* CDS was amplified with primers containing *attB* sites (attB-*MsOr*-F/R; Table S1). Linear fragments flanked by *attB* sequences were used to construct the entry vector *MsOr*-pDONR207 (Invitrogen) using BP Clonase (Invitrogen). The pDONR207-*MsOr* plasmid was cloned into the plant expression vector (pGWB5), containing the CaMV *35S* promoter upstream of the *green fluorescent protein* (*GFP*) gene, using LR Clonase (Invitrogen). The resulting overexpression construct (pGWB5-*MsOr-GFP*) was transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method (Hofgen and Willmitzer, 1988), which was used for subcellular localization analysis and plant transformation.

2.4. Subcellular localization of MsOr

A. tumefaciens GV3101 strain carrying the pGWB5-*MsOr-GFP* vector was infiltrated into the young leaves of 4-week-old tobacco plants. Infected plants were grown at 25 °C and under a 16 h light/8 h dark photoperiod for 3 days. Then, infiltrated sections of leaves were cut, and nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). The stained leaf sections were visualized under a laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) as described previously (Kang et al., 2017).

2.5. PCR and gene expression analysis

Healthy leaves of 1-month-old alfalfa and tobacco plants were used for the extraction of total RNA, as described above. For semi-quantitative and quantitative expression analysis of the *MsOr* gene in alfalfa and transgenic tobacco plants, 2 μg of total RNA was used for first-strand cDNA synthesis using reverse transcriptase PCR (RT-PCR) Kit (TOPscript™ RTDry MIX). Quantitative RT-PCR (qRT-PCR) was performed in a CFX Real-Time PCR Detection System (Bio-Rad, USA) using fluorescent BRYT Green® Dye (GoTaq® qPCR Master Mix, Promega, Beijing, China), according to the manufacturer's instructions. The gene