Journal of Plant Physiology 170 (2013) 958–964

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Functional Biotechnology

Functional characterization of a novel tropinone reductase-like gene in *Dendrobium nobile* Lindl

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ARTICLE INFO

Article history:
Received 21 September 2012
Received in revised form 31 January 2013
Accepted 5 February 2013
Available online 6 April 2013

Keywords:
Dendrobium nobile
Functional analysis
Secondary metabolism
Substrate specificity
Tropinone reductase

ABSTRACT

Dendrobium nobile, a herbal medicine plant, contains many important alkaloids and other secondary metabolites with pharmacological and clinical effects. However, the biosynthetic pathway of these secondary metabolites is largely unknown. In present study, a cDNA sequence (*DnTR2*) that encodes a peptide with high similarity to known tropinone reductase (TR) was cloned from *D. nobile* Lindl. Sequence comparison and phylogenetic analysis showed that DnTR2 was evolutionarily distant from those well-characterized subgroups of TRs. qRT-PCR revealed that *DnTR2* was expressed constitutively in all three vegetative organs (leaves, stems and roots) and was regulated by methyl jasmonate (MeJA), salicylic acid (SA) and nitrogen oxide (NO). Catalytic activity analysis using recombinant protein found that DnTR2 was not able to reduce tropinone, but reduced the two structural analogs of tropinone, 3-quinuclidinone hydrochloride and 4-methylcyclohexanone. Structural modeling and comparison suggested that the substrate specificity of TRs may not be determined by their phylogenetic relationships but by the amino acids that compose the substrate binding pocket. To verify this hypothesis, a site-directed mutagenesis was performed and it successfully restored the DnTR2 with tropinone reduction activity. Our results also showed that the substrate specificity of TRs was determined by a few residues that compose the substrate binding pocket which may have an important role for directed selecting of TRs with designated substrate specificities.

Introduction

Short chain dehydrogenase/reductase (SDR) superfamily is a group of NAD(P)H-dependent oxidoreductases that typically consist of 250–350 amino acid residues (Oppermann et al., 2003). SDRs differ greatly in the substrate specificity and subsequently in their function. However, SDRs have very similar three dimensional structures with two conserved domains, an N-terminal core domain and a C-terminal domain. The N-terminal core domain, which includes most of the polypeptide, adopts a Rossmann fold and contains a conserved G-X3-G-X-G motif for NAD(P)H-binding (Persson et al., 1991). The C-terminal domain, which protrudes from the core domain, contains several amino acids that are crucial for substrate binding (Oppermann et al., 2003). SDR binds its substrate at the deep cleft between the N-terminal core domain and the small lobe.

Tropinone reductases (TRs), members of the SDR superfamily, use the NADPH as coenzyme to reduce tropinone. Previous studies showed that there were two kinds of TRs, namely TRI and TRII, which differed from each other by the stereo specificities of their reduction products. TRI reduces tropinone into tropine, the pre-product of hyoscyamine, whereas TRII reduces tropinone into pseudotropine (Nakajima et al., 1998). TRs were found mainly in plants within or closely related to the family of Solanaceae (Hashimoto et al., 1992; Kai et al., 2009; Kaiser et al., 2006; Keiner et al., 2002; Nakajima et al., 1993; Portsteffen et al., 1994; Rothe and Dräger, 2002). Recently, TR homologues were also found in other plant families, such as *Arabidopsis thaliana*, *Brachypodium distachyon*, *Vitis vinifera*, and *Cochlearia officinalis*. Of these TR homologues, only CoTR from *C. officinalis* (a species of the Brassicaceae) has been proven with tropinone reduction activity (Brock et al., 2008), whereas *Arabidopsis* AtSDR does not process a tropinone reduction activity (Brock et al., 2008). However, some TR homologues (such as AtSDR) are functional reductases with other substrates than tropinone (Brock et al., 2008), implying functional divergence of TR-like short-chain dehydrogenases in higher plants.

Dendrobium nobile, a species of important herbal medicine plant, contains many medically important secondary metabolic components, *e.g.* dendrobine and bioactive polysaccharides (Luo et al.,

Abbreviations: DnTR2, *D. nobile* tropinone reductase 2; IPTG, isopropyl-β-D-1-thiogalactopyranoside; MeJA, methyl jasmonate; qRT-PCR, quantitative reverse-transcriptase-polrmerase chain reaction; SA, salicylic acid; SDR, short-chain dehydrogenase; SNP, sodium nitroprusside; TR, tropinone reductase.

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http://dx.doi.org/10.1016/j.jplph.2013.02.007

2009; Wang et al., 2010), and has been developed into many products for health care. However, little information is available about the genes that are involved in the secondary metabolism of *D. nobile* at present. Previously, we constructed a cDNA library of *D. nobile* to assist cloning the genes that may function in *D. nobile* secondary metabolism (Chen et al., 2013). During analysis the expressed sequence taqs (ESTs) of *D. nobile*, we identified several cDNA fragments that encode peptides that share high sequence similarities with putative TRs encoded by *B. distachyon*, *V. vinifera* and *A. thaliana*. One of the TR-like genes of *D. nobile* (*DnTR1*) encodes a functional TR enzyme that is able to reduce tropinone into tropine (Chen et al., 2013). In order to understand diversification of the SDR superfamily, in the present study we further cloned and functionally characterized another *D. nobile* TR-like gene (named as *DnTR2*).

Materials and methods

Plant growth conditions

Dendrobium nobile plants were grown in moss media under the temperature of 22 °C during the day and 18 °C during the night with the relative humidity and photoperiod set to 75% and 12:12 (day: night), respectively.

External plant hormones treatments

For plant hormones treatment experiments, six-months-old plants were selected and sprayed with 100 mM methyl jasmonate (MeJA), 1% (m/v) salicylic acid (SA), 100 mM sodium nitroprusside (SNP; exogenous NO donor), or distilled water as a negative control for 1 min. After the treatment, plants were transferred to a growth chamber with the same growth conditions. Leaves, stems, and roots were collected separately at 0, 4, 8, 16, and 24 h post each treatment, and frozen in liquid nitrogen for further analysis. Each treatment contained 12 plants with three biological repeats.

Cloning of DnTR2

Total RNA was isolated from *D. nobile* plant using an AxyPrep Multisource Total RNA Miniprep Kit (Axygen Biosciences, Hangzhou, China) following the manufacturer's instructions. The first strand of cDNA was synthesized using an iScript™ cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) with Oligo $dT_{(18)}$ as primer. cDNA was used as template to amplify the full-length coding region of *DnTR2* with the primer set: TR1F (5′- ATG GCC GGA GGA GGA GG-3′) and TR1R (5′- TCA AGC ACC TAA GGT CCT-3′), designed according to EST sequence data from a CDNA library. The amplified fragment was cloned into the pMD18-T vector (TaKaRa, Dalian, China) and recombinant clone was named as pMD:DnTR2. Its nucleotide sequence was deposited in GenBank under the accession number of JQ063459.

Recombinant protein expression and purification

The *DnTR2* coding region was amplified from pMD:DnTR2 with the primer set of pTR1F (5′-CAT ATG GCC GGA GGA GGA GG-3′) (*Nde*I site underlined) and pTR1R (5′-CTC GAG TCA AGC ACC TAA GGT CCT-3′) (*Xho*I site underlined) and inserted into the pET-28a vector (Novagen, Darmstadt, Germany). The resulting plasmid, pET:DnTR2, was used for protein expression. DnTR2 was expressed as a fusion peptide with N-terminal 6 × His-tag in *E. coli* strain BL21(DE3) at 37 °C for 4 h with the presence of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Recombinant DnTR2 was purified as described previously (Cheng et al., 2011). Protein concentration was determined using a Bio-Rad protein assay kit I (Bio-Rad) as instructed. Purified protein was finally concentrated with an Amicon Ultra-15 Centrifugal Filter Unit (Millipore, Billerica, MA, USA) to a desired concentration; and stored at −80 °C.

Protein blot analysis

Protein blot analysis was performed as described previously using antibodies against histidine tag (Zuoyang Bio., Beijing, China) (Chen et al., 2013).

Site-directed mutagenesis

The mutagenesis was performed using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene). In detail, the pET:DnTR2 plasmid, that contain wild type of DnTR2, was amplified using the oligonucleotides DnTR2-Y201V-D (5′-ATCAATTCTG TTTCACCATG GGTAATCAAG ACTTCACTCG TAAAC-3′) and DnTR2-Y201V-R (5′-GTTTACGAGT GAAGTCTTGA TTACCCATGG TGAAACAGAA TTGAT-3′) which were designed to mutate the Tyr-201 (TAT) to Valine (GTA). Plasmid that contained the designed mutation was verified by DNA sequencing and transformed into *E. coli* BL21 (DE3) for protein over-expression. Protein expression and purification were performed as described above for wild-type DnTR2.

Enzyme assays

Enzyme activity was assayed as described previously with a few modifications (Hashimoto et al., 1992). In detail, enzymatic assay was performed at 25 °C in a total of 1 mL 100 mM sodium phosphate (pH 8.0) reaction buffer that contained 1–400 μg protein, 200 mM NADPH, 5 mM substrate (0.01–25 mM for determination of K_M values). Three substrates, including tropinone (Sigma–aldrich, St. Louis, MO, USA), 3-quinuclidinone hydrochloride (Damas-beta, Basel, Switzerland), and 4-methylcyclohexanone (Sigma–aldrich) were tested. As negative control, boiled proteins were added instead of native DnTR2. All these assays were repeated three times. The kinetic constant of the enzyme was determined using a non-linear regression of the Michaelis–Menten equation (Kou et al., 2005) using Sigma Plot enzyme kinetics module 9.0.

qRT-PCR analysis

qRT-PCR was carried out to investigate *DnTR2* transcript expression in roots, stems and leaves of *D. nobile* plants as well as the transcriptional response to the plant hormones as described previously (Chen et al., 2013) on a CFX96™ Real-Time PCR detection System. PCR mixtures were prepared using an iQ™ SYBR Green Supermix kit (Bio-Rad) with the primer set QTR2F (5′-CTC TCG GGC GTA TCG GTG AG-3′) and QTR2R (5′-TCT TCA ACA ATG GCA TAC CC-3′). The amplification condition was set as follow: 95 °C denaturation for 20 s, 40 cycles of 95 °C denaturation for 15 s, 58 °C annealing for 15 s, and 72 °C elongation for 15 s. As an internal control for normalization of *DnTR2* expression levels, a 196 bp fragment of *D. nobile* actin gene (*DnActin7*, GenBank acc no. JQ040497) was amplified as using primers set actinF (5′-GAT GTG CAG AGG TGC TTT TC-3′) and actinR (5′-GCT TCT CCT TGA TAT CTC GAA-3′). Each sample was run in triplicate.

Sequence analysis

DNAMAN version 6.0.3.40 software (Lynnon Corporation, QC, Canada) was used for protein sequences assembling. Phylogenetic tree was constructed with the neighbor joining method using MEGA 5.0 software (Tamura et al., 2007). The Bootstrap confidence limits were obtained by 1000 replicates.