Plant Science 258 (2017) 21–28

Contents lists available at ScienceDirect

Plant Science

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

An event of alternative splicing affects the expression of the *NTRC* gene, encoding NADPH-thioredoxin reductase C, in seed plants

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ARTICLE INFO

Article history:
Received 17 October 2016
Received in revised form 31 January 2017
Accepted 2 February 2017
Available online 7 February 2017

Keywords:
Algae
Alternative splicing
Cyanobacteria
NADPH thioredoxin reductase C
Phylogenetic analysis
Plant

ABSTRACT

The *NTRC* gene encodes a NADPH-dependent thioredoxin reductase with a joint thioredoxin domain, exclusive of photosynthetic organisms. An updated search shows that although most species harbor a single copy of the *NTRC* gene, two copies were identified in different species of the genus *Solanum*, *Glycine max* and the moss *Physcomitrella patens*. The phylogenetic analysis of NTRCs from different sources produced a tree with the major groups of photosynthetic organisms: cyanobacteria, algae and land plants, indicating the evolutionary success of the *NTRC* gene among photosynthetic eukaryotes. An event of alternative splicing affecting the expression of the *NTRC* gene was identified, which is conserved in seed plants but not in algae, bryophytes and lycophytes. The alternative splicing event results in a transcript with premature stop codon, which would produce a truncated form of the enzyme. The standard splicing/alternative splicing (SS/AS) transcripts ratio was higher in photosynthetic tissues from *Arabidopsis*, *Brachypodium* and tomato, in line with the higher content of the NTRC polypeptide in these tissues. Moreover, environmental stresses such as cold or high salt affected the SS/AS ratio of the NTRC gene transcripts in *Brachypodium* seedlings. These results suggest that the alternative splicing of the *NTRC* gene might be an additional mechanism for modulating the content of NTRC in photosynthetic and non-photosynthetic tissues of seed plants.

1. Introduction

Redox regulation based on the modulation of enzyme activity through the dithiol-disulfide interchange of selected, and usually well-conserved, cysteine residues is a regulatory mechanism universally distributed in all kinds of organisms from bacteria and fungi to animals and plants, in which thioredoxins (Trxs) play a central role [1]. In contrast with heterotrophic organisms, the gene family of Trxs in photosynthetic organisms is highly complex, in particular the chloroplast contains a large number of Trx and Trx-like polypeptides [2]. Moreover, while in heterotrophic organisms and non-photosynthetic plant tissues the reducing power required for redox regulation is provided by NADPH with the participation of a NADPH-dependent Trx reductase (NTR) [1,3], chloroplast redox regulation relies on ferredoxin (Fdx) reduced by the photosynthetic electron transport chain and requires the participation of a Fdx-dependent Trx reductase (FTR), which is specific of these organelles [4].

The classic model of chloroplast redox regulation is based on the function of the Fdx-FTR-Trx redox system [4]. According to this model, redox regulation in this organelle depends of photosynthetically reduced Fdx, so that the regulation of chloroplast metabolism is directly linked to light. This notion was modified upon the discovery of NTRC, a NADPH-dependent Trx reductase with a joint Trx domain at the C-terminus, which is localized in plastids [5,6]. NTRC shows high affinity for NADPH [7], hence allowing the use of this source of reducing power for chloroplast redox regulation, as in heterotrophic organisms. It was found that NTRC is an efficient reductant of 2-Cys peroxiredoxins (Prxs) [8–10] and, based on these results, an antioxidant function for this enzyme was proposed [8,11,12]. Indeed, comparative biochemical analyses with plastidial Trxs such as Trx *x* and CDSP32 (Chloroplast Drought-induced Stress Protein of 32 kDa) showed that NTRC is the most efficient reductant of 2-Cys Prxs [8], a notion which was further confirmed by *in vivo* analysis of *Arabidopsis* mutants lacking either NTRC or Trx *x* [13].

In line with the initial proposal of the antioxidant function of NTRC, further evidence showed that an *Arabidopsis* mutant devoid of NTRC is hypersensitive to either abiotic [5,14] or biotic [15,16] stresses. In addition, the *ntrc* mutant shows a characteristic pheno-

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http://dx.doi.org/10.1016/j.plantsci.2017.02.001
0168-9452/

type of growth inhibition and pale green leaves due to decreased content of chlorophylls [5,8,17], suggesting that the function of NTRC may be broader than previously anticipated. In support of this notion, it has been reported the participation of NTRC in redox regulation of the biosynthesis of chlorophyll [18,19] and starch [20,21], as well as in the regulation of ATP synthase [22]. The severe growth inhibition phenotype of an *Arabidopsis* mutant combining the deficiencies of NTRC and Trx *f*1 [23] and the lethal phenotype of *Arabidopsis* mutants simultaneously lacking FTR and NTRC [24] indicate the overlapping function of the NTRC and the Fdx-FTR redox systems. This notion is further supported by the finding that NTRC interacts with redox-regulated enzymes of the Calvin-Benson cycle and several Trxs [25]. Interestingly, the *ntrc* mutant showed impairment of the redox state of ADP-glucose pyrophosphorylase (AGPase) in leaves but also in roots [20], suggesting the expression of the gene also in this non-photosynthetic plant organ. A more in-depth analysis confirmed the wide pattern of expression of the *NTRC* gene in *Arabidopsis*, the enzyme being localized in any kind of plastid, not only in chloroplasts [6].

The *NTRC* gene was identified by searching the gene family of NTRs in *Arabidopsis* and rice, the only plants whose genomes were available at the time [5]. These analyses revealed that the *NTRC* gene is exclusive of oxygenic photosynthetic organisms including some, not all, cyanobacteria [26], algae [27,28] and plants, which contain a single copy of the gene [5,26]. Initial analyses revealed similar levels of transcripts of the *NTRC* gene in roots and shoots of rice seedlings whereas the protein was more abundant in shoots [5]. A more in-depth analysis confirmed higher levels of expression of the *NTRC* gene, and higher content of the NTRC protein, in photosynthetic tissues from *Arabidopsis* [6]. It is estimated that more than 60% of the intron-containing genes undergo alternative splicing in plants [29]. In particular, genome-wide analyses showed that at least 42% of the intron-containing genes of *Arabidopsis* undergo alternative splicing [30] whereas 128 events of alternative splicing were identified in *Brachypodium distachyon* by homology mapping of assembled expressed sequence tags [31]. Indeed, alternative splicing is proposed to play a relevant role in adjusting gene expression to plant development and adaptation to the environment [32–36].

In this work we have addressed the possibility that alternative splicing might affect the expression of the *NTRC* gene in plants. Based on sequence and RT-PCR analyses we identified an event of alternative splicing in the expression of the *NTRC* gene, which is conserved in seed plants, but not in the moss *Physcomitrella patens* nor in algae. The alternatively spliced transcripts would produce a truncated form of the NTRC protein lacking the whole Trx domain and part of the NTR domain, thus presumably lacking any biological activity. The analysis of *NTRC* alternative splicing in *Arabidopsis*, tomato and *Brachypodium* suggests that it may affect the level of the protein in non-photosynthetic tissues.

2. Materials and methods

2.1. Plant material

Brachypodium distachyon seeds were sterilized by immersion in 3% (v/v) NaOCl for 30 min. Then seeds were washed twice with sterile distilled water, once with 0.01 M HCl, and five more times with sterile distilled water. Tomato (*S. lycopersicum*) seeds were sterilized by successive immersions in 70% ethanol for 1.5 min and 1.5% NaOCl supplemented with 10% (v/v) Tween 20 for 25 min. Seeds were then washed three times with sterile distilled water. *A. thaliana* seeds were sterilized in a vacuum chamber in the presence of 100 mL sodium hypochlorite supplemented with 1.5% (v/v) HCl. Sterile *Brachypodium* and tomato seeds were allowed to germinate in Petri dishes on filter paper soaked in sterile distilled water. Seeds were incubated at 4 °C during two days in darkness, and then at room temperature under a long-day (16 h light, 8 h darkness) photoperiod. *Arabidopsis thaliana* was grown as previously reported [6]. For cold treatments *Brachypodium* seedlings were grown for 4 days under darkness at 25 °C and then for an additional day at 4 °C. For salt treatments, *Brachypodium* seedlings grown for 4 days under long-day photoperiod on filter paper soaked with water were incubated in the presence of 170 mM NaCl for an additional day.

2.2. RNA extraction and RT-PCR analysis

Total RNA was extracted using TRIsure™ reagent (BIOLINE). cDNA synthesis was performed with 1 μg of total RNA using the Maxima first strand cDNA synthesis kit (THERMO-SCIENTIFIC) according to manufacturer's instructions. PCR was performed with the GoTaq DNA polymerase (PROMEGA) using as template the first strand cDNA reactions. Full-length NTRC cDNA cloning from *Brachypodium distachyon* was carried out with the iProof High-Fidelity DNA polymerase (BIORAD) with oligonucleotides described in Table S1. Oligonucleotides used to search for alternative splicing in the *NTRC* gene from *Brachypodium distachyon* are described in Table S2 in Supplementary material, and for analysis of the intron 4 conserved alternative splicing events in Table S3 in Supplementary material.

2.3. Protein extraction and Western blot analysis

For protein extraction, plant tissues were ground with a mortar and pestle in liquid nitrogen. Extraction buffer (100 mM Tris-HCl pH 7.9, 10% (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1% (v/v) protease inhibitor cocktail for plant cell and tissue extracts (SIGMA–ALDRICH)) was immediately added, the sample given a swirl on a vortex, and centrifuged at 13,500*g* at 4 °C for 20 min. Total protein content was quantified using the Bradford reagent (BIORAD) and proteins were subjected to SDS-PAGE, under reducing conditions. Western blots were performed as previously described [37] and probed with the anti-NTRC antibody, which was raised by our group by immunization of rabbits with the purified Trx domain of NTRC, which does not cross react with any other Trx [5].

2.4. Bioinformatic tools

NTRC gene sequences were searched in available databases National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/) and Phytozome v.11 (http://phytozome.jgi.doe.gov/pz/portal.html). Multiple sequence alignment was performed with Clustal Omega available at the European Institute for Bioinformatics (EBI) (www.ebi.ac.uk). Phylogenetic analysis was performed with the program ClustalW2 Phylogeny and phylogenetic tree was visualized with software TreeView v.1.6. Gene structure was designed with Fancygene v1.4 (http://bio.ieo.eu/fancygene/).

3. Results

3.1. The NTRC gene is duplicated in some plant species

While cyanobacteria, algae and most plants contain a single copy of the *NTRC* gene, two copies were identified in different species of the genus *Solanum,* such as cultivated tomato (*Solanum lycopersicum*), wild tomato (*Solanum pennellii*) and potato (*Solanum tuberosum*), *Glycine max*, and the moss *Physcomitrella patens.* A phylogenetic analysis (Fig. 1), based on the comparison of NTRC amino acid sequences, clustered the *NTRC* genes into the three major groups of photosynthetic organisms: cyanobacteria, algae and land plants. Among the land plants, the phylogeny of the *NTRC* genes