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

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Abstract

The *NTRC* gene encodes a NADPH-dependent thioredoxin reductase with a joint thioredoxin domain, exclusive of photosynthetic organisms. An updated search of *NTRC* genes 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.

Highlights

- The *NTRC* gene is duplicated in some plant species.
- The *NTRC* gene undergoes alternative splicing in seed plants.
- The standard spliced *NTRC* transcript is more abundant in photosynthetic tissues.

Keywords: Algae, alternative splicing, cyanobacteria, NADPH thioredoxin reductase C, phylogenetic analysis, plant

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 phenotype 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 4ºC 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 25ºC and then for an additional day at 4ºC. 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^{TM} 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, and for analysis of the intron 4 conserved alternative splicing events in Table S3.

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 ºC 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 Fancygenev1.4 (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 distinguished the clusters corresponding to Bryophytes (*P. patens*, *Sp. fallax*), Lycophytes (*Se. moellendorffii*), and seed plants (Euphyllophytes), in which monocots and dicots were also clearly clustered (Fig. 1). The fact that the second copy of the *NTRC* gene was present in distant species including the moss *P. patents* suggests that they arose from independent duplication events.

3.2. The NTRC *gene undergoes alternative splicing, which is conserved in seed plants*

Our initial attempts to clone NTRC cDNAs from rice yielded clones encoding the full-length protein that was biochemically studied and reported [5], but also a number of clones with premature stop codons, which were not further analyzed. It is now well established that alternative splicing affects the expression of a large number of genes in plants [29, 36]. Moreover, the genome-wide analysis in maize showed that predominant developmental splicing events are tissue-specific [38], indicating that alternative splicing may be important for plant development. Thus, the finding of variable sequences among the rice NTRC cDNAs suggested that the *NTRC* gene might undergo alternative splicing. To test this possibility, we have performed an analysis of *NTRC* genes from different photosynthetic organisms, including the plants *Brachypodium distachyon*, *Oryza sativa* and *Arabidopsis thaliana*, the moss *Physcomitrella patens* and the algae *Chlamydomonas reinhardtii*. The gene structure, formed by ten exons separated by nine introns, is conserved in *NTRC* genes from all photosynthetic eukaryotes under study (Fig. 2). However, the length of the *NTRC* genes from different species shows a high variability (Table S4), while the sizes of the coding sequences and the corresponding deduced proteins is conserved, hence indicating high variability of the extensions of the intronic sequences (Fig. 2).

To search for alternative splicing events in the *NTRC* gene, we analyzed NTRC cDNAs from *Brachypodium distachyon* using as templates RNAs isolated from leaves and mature seeds. Most of the cDNA sequences obtained (cDNA_2 in Fig. S1) encoded the expected full-length NTRC protein showing a high similarity with the rice enzyme [5]. In addition, cDNA clones were found (cDNA_1 in Fig. S1) containing a 23-bp insertion localized at the junction of intron 4 and exon V, thus suggesting a possible

alternative splicing event retaining the 23-bp sequence at the 3' side of intron 4 (Fig. 3a). According to these data, two transcripts of the *NTRC* gene are generated due to the use of a different splice acceptor site. The transcript produced by standard splicing (SS) encodes the full-length version of NTRC, while the transcript produced by alternative splicing (AS), includes an additional 23-bp sequence, changing the reading frame and resulting in a premature stop codon (underlined in Fig. 3a). Therefore, the AS transcript would produce a truncated version of NTRC lacking the Trx domain and a fragment at the C-terminus of the NTR domain, though the FAD and NADPH binding motifs and the active site of this domain would be maintained in the truncated variant (Fig. 3a). To analyze the presence of alternatively spliced mRNAs of the *NTRC* gene, oligonucleotides were designed at the 3'-end of exon IV and the 5´-end of exon V (Fig. 3a, arrowheads) and tested with *Brachypodium* cDNA clones as templates. The PCR amplifications from cDNA clones 1 and 2 respectively produced fragments of the size expected for SS and AS mRNAs (Fig. 3b), indicating that oligonucleotides at these position of the NTRC gene may allow detection of alternatively spliced transcripts by RT-PCR (see below).

Once identified this putative AS event in the *NTRC* gene from *Brachypodium*, we tested the degree of conservation of this post-transcriptional process in *NTRC* genes from eukaryotic photosynthetic organisms. Interestingly, the intron-exon junction containing the 23-bp sequence of the AS transcripts showed a high degree of conservation in *NTRC* genes from different plant sources, being completely conserved around the two putative splicing sites (Fig. 4a). In contrast, no conservation was observed when *NTRC* gene sequences from the green algae *C. reinhardtii* and the two *NTRC* genes from the moss *Physcomitrella patens* were compared (Fig. 4b), thus indicating that the AS event may be exclusive of seed plants.

Finally, we searched for additional events of alternative splicing affecting the expression of the *NTRC* gene in *Brachypodium*. The RT-PCR analysis with pairs of oligonucleotides flanking every intron of the *NTRC* gene showed additional bands for introns 1, 3 and 7 when RNAs from roots were used as templates (Fig. S2). The sizes of these bands indicate the presence of *NTRC* transcripts that retain these introns, all of them containing premature stop codons. However, the occurrence of these AS events was clearly less abundant than the one occurring at intron 4 (see below) and were not analyzed further.

3.3. The level of alternative splicing of the NTRC *gene is tissue-specific and is altered in response to abiotic stress*

The high conservation of the 23-bp sequence undergoing AS in *NTRC* genes of seed plants suggested that AS may influence the level of NTRC polypeptide in the different plant tissues. Thus, we performed RT-PCR analysis, using the oligonucleotides described above (Fig. 3a, b), to determine the relative content of SS and AS transcripts in RNA isolated from photosynthetic and non-photosynthetic tissues of *Arabidopsis* and *Brachypodium*, which were chosen as representatives of dicot and monocot plants, respectively. In addition, this analysis was extended to tomato (*S. lycopersicum*), one of the plants containing two copies of the *NTRC* gene, to test whether or not the two copies of the gene are actively expressed and undergo alternative splicing. These analyses confirmed the presence of both SS and AS *NTRC* transcripts in leaves and roots from *Arabidopsis* plants. However, while the SS transcript was slightly less abundant than the AS transcript in roots, in leaves it was approx. 5-fold more abundant (Fig. 5a). Despite loading higher amounts of protein extracts from roots (25 μg) than from leaves (12.5 μg), the NTRC signal in western blots was clearly more intense in leaves (Fig. 5a), thus showing that the NTRC protein is more abundant in leaves than in roots, as previously reported [6].

Since tomato (*S. lycopersicum*) contains two copies of the *NTRC* gene, on chromosomes 4 and 10, respectively, oligonucleotides were designed to specifically detect transcripts of each of them. RT-PCR analyses with total RNA isolated from root, hypocotyl and cotyledons revealed that the two *NTRC* genes are expressed in these tissues of tomato seedlings (Fig. 5b). Moreover, both the SS and AS transcripts for *NTRC* genes located on chromosomes 4 and 10 were detected in all the tissues analyzed indicating that the two genes from tomato undergo alternative splicing (Fig. 5b). Remarkably, the *NTRC* gene located on chromosome 10, but not the one on chromosome 4, showed significantly higher levels of SS transcripts in cotyledons (3.4-fold) and hypocotyls (2.3-fold) (Fig. 5b). Again, despite loading higher amounts of protein extracts from roots (25 μg) than from cotyledon and hypocotyl (12.5 μg), the NTRC signal in western blots was clearly more intense in photosynthetic tissues (Fig. 5b).

A similar approach was used to analyze the pattern of expression of the *NTRC* gene in *Brachypodium* seedlings. In line with the results obtained with *Arabidopsis* and tomato, the SS and AS transcripts were detected in *Brachypodium* seedlings roots and shoots, but the SS transcripts were 5-fold more abundant than the AS transcripts in the shoot, which contained higher levels of NTRC (Fig. 5c). In contrast, similar levels of SS and AS transcripts were detected in roots, in line with the lower content of NTRC in this tissue (Fig. 5c). Finally, alternative splicing of the *NTRC* gene was analyzed in *Brachypodium* seeds after 5 days of germination. In accordance with the low content of NTRC polypeptide, as detected in western blots (Fig. 5c), the ratio of SS/AS transcripts was lower in this tissue (Fig. 5c).

Different environmental stresses have been shown to affect alternative splicing in plants [32-36]. Thus, it was tested whether the alternative splicing event of the *NTRC* gene is affected by stresses, such as cold and salt. For cold treatments, 4-day-old etiolated *Brachypodium* seedlings, grown at 25ºC, were incubated at 4ºC during 24 h. First, an effect of darkness on alternative splicing of the *NTRC* gene was noticed since the SS/AS transcript ratios (5.0 in shoots, 1.4 in roots, Fig. 5c) were decreased in etiolated seedlings (2.0 in shoots, 0.3 in roots, Fig. 6a). Cold treatment promoted increased SS/AS *NTRC* transcript ratio both in shoots and roots (Fig. 6a), which was reflected in a higher amount of NTRC protein in roots but not in shoots (Fig. 6a). Finally, 4-day-old *Brachypodium* seedlings grown under long-day conditions were subjected to salt treatment (24 h at 170 mM NaCl), which caused a slight increase of the SS/AS *NTRC* transcript ratio in roots and, to a higher extent, in shoots (Fig. 6b); however, no significant differences of the content of the NTRC polypeptide were observed in response to salt (Fig. 6b).

4. Discussion

The search of genes encoding NTR in plants led to the identification of the *NTRC* gene, which encodes a NTR with a joint Trx domain and is exclusively found in oxygenic photosynthetic organisms [5, 39]. The comparison of NTRC with NTRs from different sources clearly showed the phylogenetic relationship of plant NTRC with those of cyanobacteria, hence defining a novel group of genes encoding bimodular enzymes composed of NTR and Trx domains [5]. Taking advantage of the increase of genome sequences available from photosynthetic organisms, in this work we have

performed a phylogenetic analysis of the *NTRC* gene. This analysis confirmed the previously established conclusion that the *NTRC* gene is present in some, but not all, cyanobacteria and in any type of eukaryotic photosynthetic organism so far sequenced from algae to land plants. Interestingly, cyanobacterial strains that lack NTRC, such as *Synechococcus*, show a prokaryotic-type strategy to cope with oxidative stress consistent in high resistance to hydrogen peroxide, which is based on high catalase activity and 2-Cys Prxs insensitive to overoxidation [26]. In contrast, cyanobacterial strains equipped with NTRC, such as *Anabaena*, are highly sensitive to hydrogen peroxide, hence showing a eukaryotic-type strategy based on lower catalase activity and 2-Cys Prxs that undergo overoxidation [26]. The presence of NTRC in all eukaryotic photosynthetic organisms is indicative of the success of this redox system in the evolution of algae and land plants. Indeed, the phylogenetic tree constructed with NTRC sequences (Fig. 1) defines the major groups of photosynthetic organisms; cyanobacteria and algae, which are more closely related, and land plants in which the clades formed by Bryophytes (such as *P. patens*) and Lycophytes (such as *Se. moellendorffii*) could be clearly distinguished from Euphyllophytes, thus indicating the central position of NTRC in the evolution of photosynthetic eukaryotes.

In contrast with our previous statement that NTRC is encoded by a single-copy gene in photosynthetic organisms, the availability of more genome sequences reveals the presence of two copies of the gene in some plant species. The fact that species containing two copies of the *NTRC* gene, like the moss *Physcomitrella* and plant species such as *G. max* and different species of the genus *Solanum*, are phylogenetically distant suggests that the two copies of the *NTRC* gene were produced by duplications that occurred independently during the evolution of these species. Here, we show that the two *NTRC* genes of tomato (*S. lycopersicum*) are actively expressed in all the tissues analyzed (Fig. 5b); however, western blot analyses of protein extracts from tomato identified a single band (Fig 5b) like in Arabidopsis or *Brachypodium* (Fig. 5a, c), which have a single copy of the *NTRC* gene. The deduced polypeptides of the two *NTRC* genes from tomato show a high identity at the amino acid level, most differences being observed in the N-terminal side putatively encoding the transit peptide (Fig. S3). Accordingly, the deduced molecular weight of the full-length NTRC encoded by the gene on chromosome 4 is 59.2 kDa, while the one encoded by the gene on chromosome 10 is 58.9 kDa. It is thus expected that the anti-NTRC antibody, which was raised

against the enzyme from rice, efficiently cross-reacts with either of the two tomato enzymes, which should be detected as a single band in Western blot analyses (Fig. 5b).

Sequence comparison of NTRC cDNA clones from *Brachypodium* identified a 23-bp fragment insertion, which was localized at the 3'-end of the junction of intron 4 and exon V (Fig. S1, Fig. 3a). This finding suggested the presence of two splice acceptor sites at this intron-exon junction producing two types of transcripts (Fig. 3a), which can be readily detected by RT-PCR analysis (Fig. 3b). The open reading frame of the SS transcript is translated into the full-length version of NTRC from *Brachypodium* with an expected molecular weight of 56.8 kDa. However, the AS transcript contains a premature stop codon and, thus, would produce a truncated version of the protein with an expected molecular weight of 29.8 kDa, lacking the Trx domain and the C-terminal side of the NTR domain (Fig. 3a). It has been reported that the expression of the complete NTR domain of NTRC in the *ntrc* mutant of *Arabidopsis* did not restore the wild type phenotype [40]. Therefore, it is unlikely that the truncated version of NTRC produced by alternative splicing, in which the NTR domain is not complete, has any activity. Moreover, attempts to produce the truncated version of NTRC in *E. coli* were unsuccessful (results not shown). Altogether, these results strongly suggest that the AS transcript of the *NTRC* gene produces a non-sense mRNA or a truncated polypeptide likely unable to display any enzyme activity.

Interestingly, the AS event identified in *NTRC* gene from *Brachypodium* is highly conserved in *NTRC* genes from seed plants, but not in the moss *P. patens* nor in algae (Fig. 4). The RT-PCR analyses with total RNA isolated from photosynthetic and non-photosynthetic tissues of *Arabidopsis*, tomato and *Brachypodium* (Fig. 5a-c) shows that the SS/SA transcript ratio was higher in photosynthetic tissues, in line with the higher content of NTRC in these organs, whereas it was lower in non-photosynthetic tissues, which contain lower levels of the protein. The high degree of conservation of the AS event in *NTRC* genes from seed plants, in conjunction with the changes of SS/AS ratio in photosynthetic and non-photosynthetic tissues from different plants species, including the two copies of the gene in tomato, suggests a role for this alternative splicing event in the regulation of the *NTRC* gene in seed plant tissues. In support of this notion, environmental cues such as light (compare Fig. 5c, Fig. 6a), cold (Fig. 6a) or high salt treatment (Fig. 6b) affect the SS/AS ratio of the *NTRC* gene in *Brachypodium* seedlings (Fig. 6a, b), cold promoting the highest increase of the SS/AS ratio (Fig. 6a). Among these environmental conditions cold treatment led to the highest

increase of the SS/AS ratio (Fig. 6a), which was reflected in a higher content of the NTRC polypeptide in roots but not in shoots (Fig. 6a). This finding suggests that alternative splicing of the *NTRC* gene may be relevant for regulating the amount of NTRC in tissues containing a low content of the protein, such as roots, while in photosynthetic tissues, in which NTRC is more abundant [5, 6], other mechanisms may be more important. However, more work is needed to reach a conclusion since the knowledge of the mechanisms regulating the expression of the *NTRC* gene in plants is still very limited.

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Supplementary data

Figure S1. Alignment of *NTRC* cDNA sequences cloned from seeds and leaves of *Brachypodium distachyon* with the sequence available in the *Brachypodium* database (cDNA-db).

Figure S2. Identification of additional alternative splicing events in the *NTRC* gene from *Brachypodium.*

Figure S3. Amino acid sequence comparison of the NTRC proteins encoded by *NTRC* genes on chromosomes 4 and 10 from *L. lycopersicum.*

Table S1-S3. Oligonucleotides used in this study.

Table S4. Sizes of the *NTRC* genes, coding sequences and number of amino acids of the deduced NTRC polypeptide from different organisms.

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LEGENDS FOR FIGURES

Fig. 1 Phylogenetic tree of NTRCs from different sources. The phylogenetic analysis was performed with full-length NTRC amino acid sequences using the program TreeView v. 1.6 for visualization. The three clusters identified are marked in red (cyanobacteria), green (algae) and blue (plants). The accession numbers of the sequences are: *Anabaena* (BAB72694.1), *Tolypothrix* (WP_045869140.1), *Synechocystis* (WP_009632856.1), *V. carteri* (XP_002948592.1), *C. reinhardtii* (XP_001689807.1), *C. vulgaris* (BAH29954.1), *O. lucimarinus* (XP_001422184.1), *P. patens* Chr20 (XP_001753500.1), *P. patens* Chr23 (XP_001785248.1), *Sp. fallax* (Sphfalx0006s0389.1.p) *Se. moellendorffii* (XP_002990200.1), *V. vinifera* (XP_010653766.1), *T. cacao* (XP_007028668.1), *G. max* Chr02 (XP_003519084.1), *G. max* Chr10 (XP_003535866.1), *A. thaliana* (NP_565954.1), *S. tuberosum* Chr04 (XP_006350177), *S. tuberosum* Chr10 (XP_006351311.1), *S. lycopersicum* Chr04 (XP_010319181.1), *S. lycopersicum* Chr10 (XP_004249259.1), *S. pennellii* Chr04 (XP_015071469.1), *S. pennellii* Chr10 (XP_015055743.1), *O. sativa* (XP_006658913.1), *B. distachyon* (XP_003562531.1), *Z. mays* (NP_001136660.1).

Fig. 2 Structure of *NTRC* genes from different organisms. Gene structures were deduced using the software Fancygen. Coloured rectangles, exons; lines, introns. The accession numbers of the sequences are: *C. reinhardtii* (GeneID:5715808), *P. patens* Chr20 (GeneID:5916755), *P. patens* Chr23 (GeneID:5948454), *O. sativa* (GeneID:102716057), *B. distachyon* (GeneID:100829068), *A. thaliana* (GeneID:818766). The two copies of the *NTRC* gene in *P. patens*, on chromosomes 20 and 23, are indicated.

Fig. 3 The *NTRC* from *B. distachyon* undergoes alternative splicing. **a**, Scheme showing the structure of the *NTRC* from *B. distachyon* indicating exonic regions (green rectangles) with roman numerals. Exons encoding the NTR and Trx domains of NTRC are indicated. Nucleotide sequence at the intron 4 (black), exon V (green) junction is shown. The 23-bp sequence skipped by the standard splicing (SS) or retained by alternative splicing (AS) is underlined and the two AG acceptor sites are marked in bold. The frame of the AS transcript generates a premature stop codon (underlined),

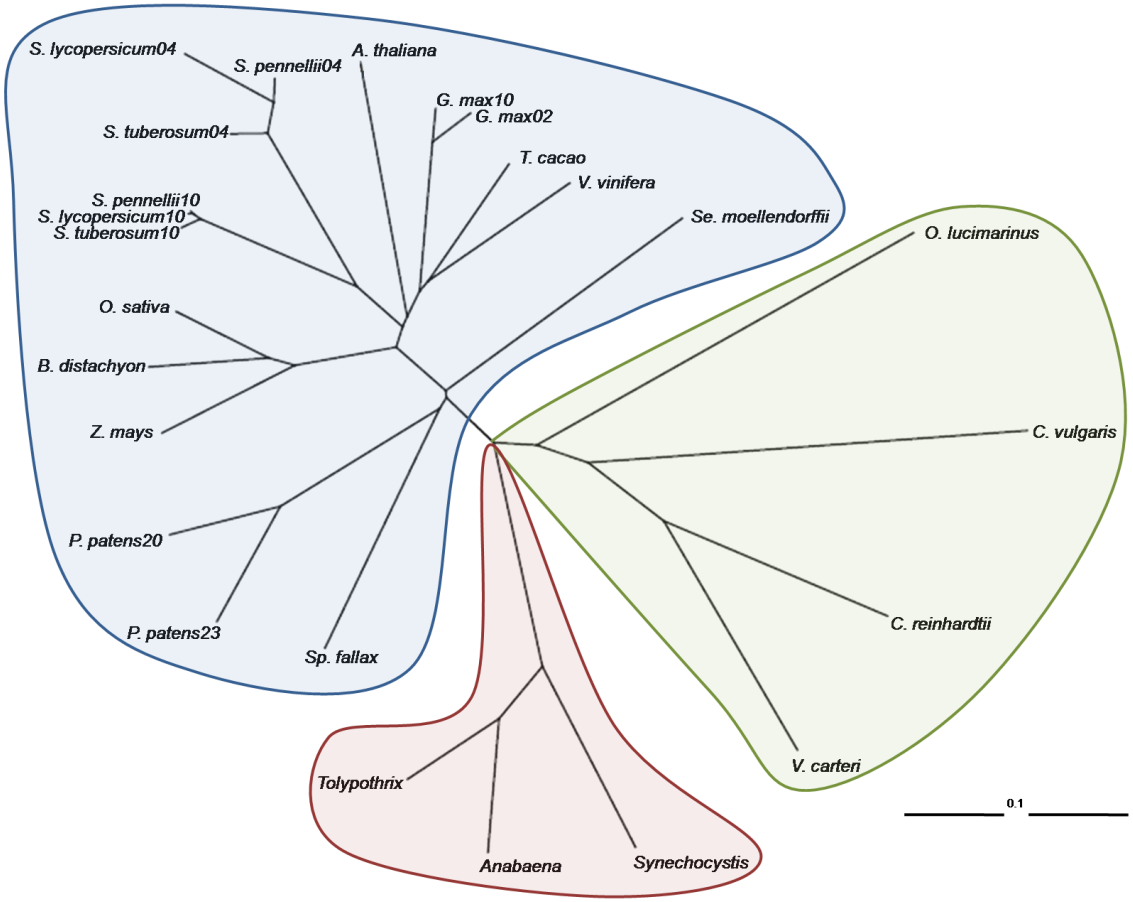
which would produce a truncated version of NTRC. The approximate location of the NTR domain active site is marked by a double C, the FAD binding motif by inverted triangles, and the NADPH motif by triangles. **b**, PCR amplified fragments with the oligonucleotides in positions indicated by red arrowheads in **a** and using as templates cDNA clones 1 and 2 described in Fig. S1. The expected size of the fragment from the standard splicing (SS) and alternative splicing (AS) transcripts are 265 bp and 288 bp, respectively.

Fig. 4 Alignment of nucleotide sequences at the intron 4, exon V junction of the *NTRC* genes from different organisms. The 23-bp fragment (underlined) at alternative splicing site is highly conserved in *NTRC* genes from seed plants (**a**), but not from algae and bryophytes (**b**). Alignment of intronic (black) and exonic (green) sequences was performed with the program ClustalOmega. The accession numbers of the sequences are: *G. max* Chr02 (GeneID:100794561), *G. max* Chr10 (GeneID:100819254), *T. cacao* (GeneID:18598885), *V. vinifera* (GeneID:100262307), *S. lycopersicum* Chr04 (GeneID:101266017), *S. lycopersicum* Chr10 (GeneID:101254347), *S. tuberosum* (GeneID:102585390), *A. thaliana* (GeneID:818766), *Z. mays* (GeneID:100216789), *O. sativa* (GeneID:102716057), *B. distachyon* (GeneID:100829068), *C. reinhardtii* (GeneID:5715808), *P. pantens* Chr20 (GeneID:5916755), *P. pantens* Chr23 (GeneID:5948454).

Fig. 5 Analysis of alternative splicing of the *NTRC* gene in photosynthetic and non-photosynthetic tissues of *Arabidopsis*, *Brachypodium* and tomato. RT-PCR analysis was performed with pairs of oligonucleotides designed at the intron 4-exon V junction (as indicated by red arrowheads in Fig. 3a) of the *NTRC* genes from *A. thaliana* (**a**), *L. lycopersicum* localized on chromosomes 4 and 10 (**b**), and *B. distachyon* (**c**). Fragments corresponding to standard splicing (SS) and alternative splicing (AS) are indicated with their sizes. Band intensities were quantified (ScionImage) and the ratio SS/AS transcripts are indicated under each sample. The content of NTRC protein in these tissues, as indicated, was analyzed by Western blotting of protein extracts (12.5 µg from leaf and cotyledon; 25 µg from root, hypocotyl and seed), which were subjected to SDS-PAGE, electrotransferred to nitrocellulose sheets, and probed with the anti-NTRC

antibody, which was raised by our group [5]. C, cotyledon; H, hypocotyl; L, leaf; R, root; Se, seed; Sh, shoot.

Figure 6. Effect of abiotic stresses, cold and salt, on the alternative splicing of the *NTRC* gene in *Brachypodium* seedlings. **a**, Etiolated *Brachypodium* seedlings grown during 4 days at 25ºC under darkness were incubated for an additional day in the same conditions (U) or at 4ºC (Cold). **b**, Non-etiolated *Brachypodium* seedlings grown during 4 days at 25ºC under long-day (16 h light-8 h darkness) photoperiod were incubated for an additional day in the same conditions (U) or in the presence of 170 mM NaCl (NaCl). After treatments shoots and roots were dissected. RT-PCR analysis was performed with pairs of oligonucleotides designed at the intron 4-exon V junction of the *NTRC* gene from *B. distachyon*. Fragments corresponding to standard splicing (SS) and alternative splicing (AS) are indicated with their sizes in base pairs (bp). Band intensities were quantified (ScionImage) and the ratio SS/AS transcripts are indicated under each sample. The content of NTRC protein in these tissues, as indicated, was analyzed by Western blotting of protein extracts (12.5 µg from shoots; 25 µg from roots), which were subjected to SDS-PAGE, electrotransferred to nitrocellulose sheets, and probed with the anti-NTRC antibody. Even loading and transfer was checked by Ponceau staining. Molecular masses in kDa are indicated.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S. lycopersicum10
S. tuberosum10
Se. moellendorffii
O. lucimarinus
O. sativa
B. distachyon
Z. mays
C. vulgaris
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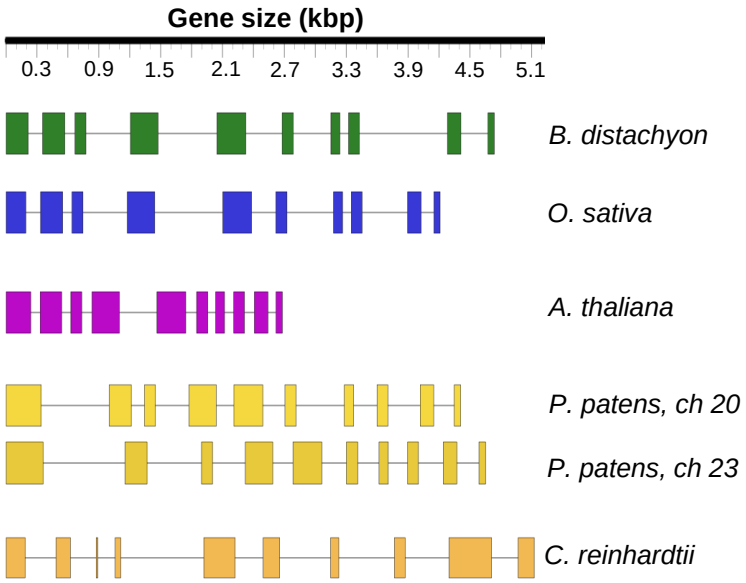
Synechocystis
0.1

Figure 1


Gene size (kbp)
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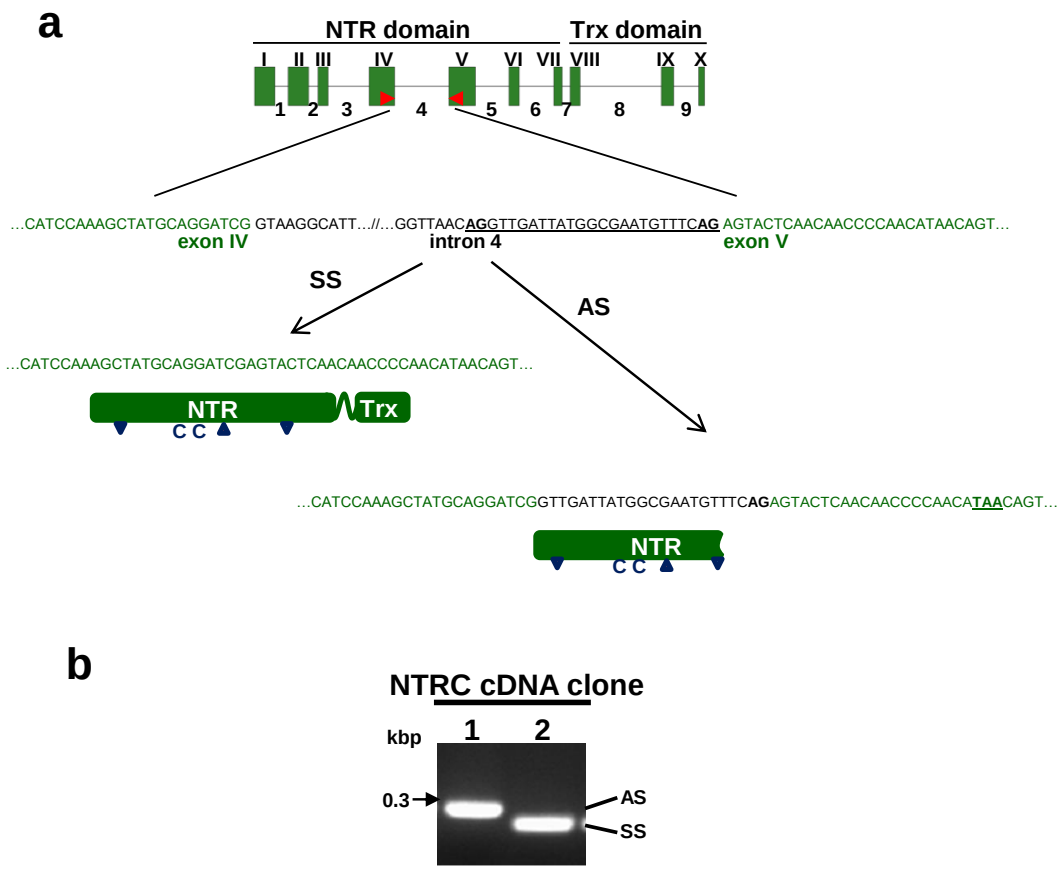
0.9
1.5
2.1
2.7
3.3
3.9
4.5
5.1
B. distachyon
O. sativa
A. thaliana
P. patens, ch 20
P. patens, ch 23
C. reinhardtii

Figure 2


a
NTR domain
Trx domain
I II III IV V VI VII VIII IX X
1 2 3 4 5 6 7 8 9
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exon IV
intron 4
exon V
SS
AS
…CATCCAAAGCTATGCAGGATCGAGTACTCAACAACCCCAACATAACAGT…
NTR
Trx
C C
…CATCCAAAGCTATGCAGGATCGGTTGATTATGGCGAATGTTTCAGAGTACTCAACAACCCCAACATAACAGT…
NTR
C C

b
NTRC cDNA clone
kbp
1
2
0.3
AS
SS

Figure 3

a

```
G.max10                 ACTCCCTGAATGTTTGTTTAACAGGTTGATTATCACGAATGTTTCAGAGTGTTTGACAAT
G.max02                 ACTCCCTGAATGTTTGTTTAACAGGTTGATTATCACGAATGTTTCAGAGTGTTTGACAAT
T.cacao                 ACTCCCTGAATGTTTGTTTAACAGGTTAATTATCGCGAATGTTTCAGAGTTTACAACAAT
V.vinifera              ACTCCTTGAATGTTTGTTTAACAGGTTGATTATGGCGAATGTTTCAGAGTTCACAACAAT
S. lycopersicum04       CTCCCTG-CATGGTTGTTTAACAGGTTGATTATCGCGAATGTTTCAGAGTTTTTAACAGT
S. lycopersicum10       CTCCCTGAAATGTTTGTTTAACAGGTTGATTATGGGGAATGTTTCAGAGTTTTCAACAAT
S.tuberosum10           CTCCCTGAAATGTTTGTTTAACAGGTTGATTATGGGGAATGTTTCAGAGTTTTCAACAAT
A.thaliana              ACACCCTGAGTGTTTTTTTAACAGGTTGGTTATGGCGAATGTTTCAGAGTGATCAACAAT
Z.mays                  ATTCGCTGGATGTTTGGTTAACAGGTTGATTATGGCGAATGTTTCAGAGTACTCAACAAC
O.sativa                ATTCCCTGGATGTTTGGTTAACAGGTTGATTATGGCGAATGTTTCAGAGTACTCAACAAT
B.distachyon            ATTCCCTGGATGTTTGGTTAACAGGTTGATTATGGCGAATGTTTCAGAGTACTCAACAAC
                           *      ** **  **********  ****   **************     ***
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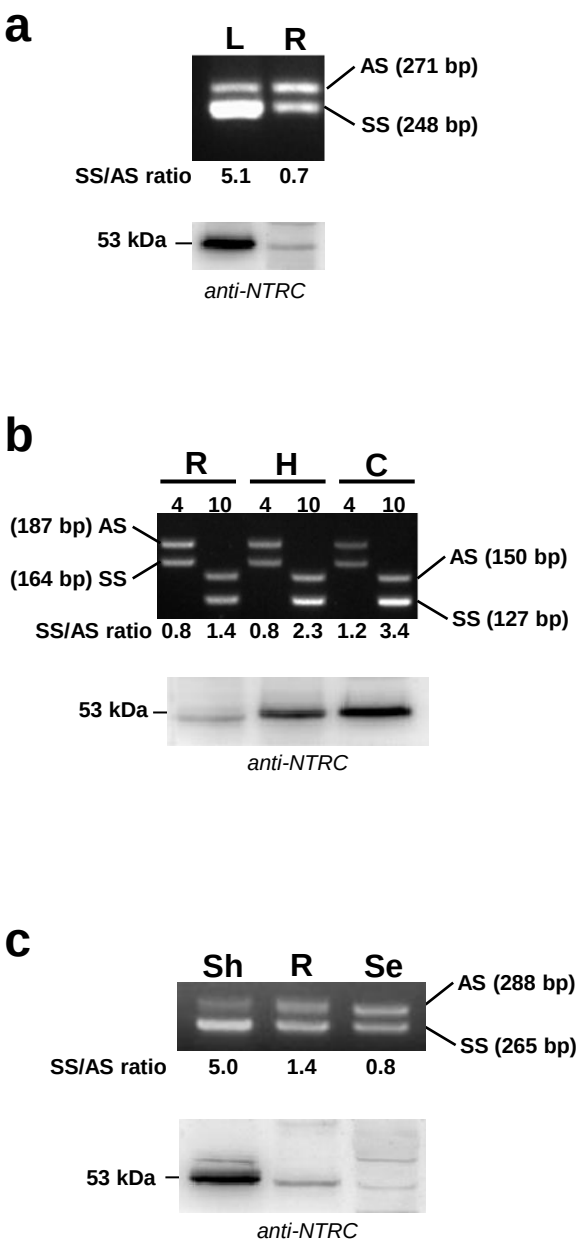
b

```
C. reinhardtii          TGTGCCCTCCCTCTCT----CGCTCTCGCTCTCCCTGCTCCCCCT-CCTCCTCCTCCTCC
P.patens20              TTCTGTTTGCTGATCTGAATGTTTAATGATCATTTTTGTGATGGAATTCCAGAGTTCTTA
P.patens23              TTGTGGATGTTTATTTTTTTTCCTGATGAACGTTCTTGTTATGGAATTTCAGAGTTATCA
B.distachyon            TTTCAATTCCCTGGATGTTTGGTTAACAGGTTGATTATGGCGAATGTTTCAGAGTACTCA
A.thaliana              TTTCAACACCCTGAGTGTTTTTTTAACAGGTTGGTTATGGCGAATGTTTCAGAGTGATCA
S. lycopersicum10       TTTCAACTCCCTGCATGGTTGTTTAACAGGTTGATTATCGCGAATGTTTCAGAGTTTTTA
                        *             *       *          *              *    *  *
```

Figure 4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b
R
H
C
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(187 bp) AS
(164 bp) SS
AS (150 bp)
SS (127 bp)
SS/AS ratio 0.8 1.4 0.8 2.3 1.2 3.4
53 kDa
anti-NTRC
c
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R
Se
AS (288 bp)
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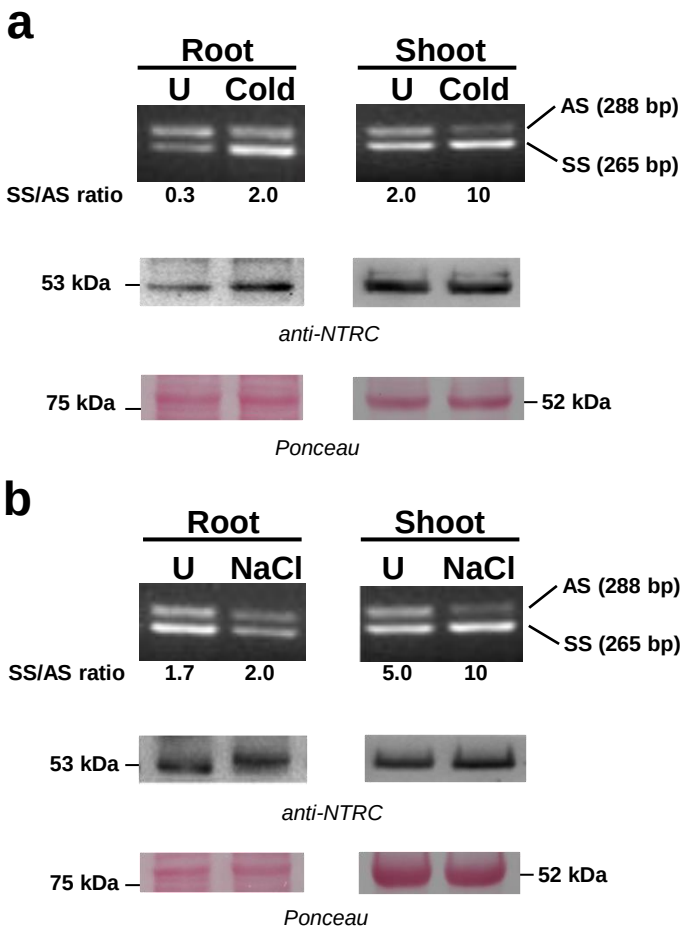
53 kDa
anti-NTRC

Figure 5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Root
Shoot
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Cold
U
Cold
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SS (265 bp)
SS/AS ratio
0.3
2.0
2.0
10
53 kDa
anti-NTRC
75 kDa
52 kDa
Ponceau
b
Root
Shoot
U
NaCl
U
NaCl
AS (288 bp)
SS (265 bp)
SS/AS ratio
1.7
2.0
5.0
10
53 kDa
anti-NTRC
75 kDa
52 kDa
Ponceau

Figure 6