DOWN-REGULATION OF *NTR* GENES BY RNAi IN THE *cad2* MUTANT IMPAIRS PLANT DEVELOPMENT OF *Arabidopsis thaliana*

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The responses and the adaptation of plants to environmental stress require several defense mechanisms. The redox signaling pathways are strongly involved in these responses, detoxifying accumulated reactive oxygen species and regulating several developmental processes (Xing *et al*., 2005; Reichheld *et al*., 2007; Benitez-Alfonso *et al*., 2009; Bashandy *et al*., 2010; Cheng *et al*., 2011; Marchal *et al*., 2014). Major actors are NADPH-dependent glutathione (GSH)/ glutaredoxin system (NGS) and the NADPH-dependent thioredoxin (TRX) system (NTS), which are acting in the cellular redox signaling by modulating the redox state of thiol groups of many proteins (Buchanan and Balmer, 2005; Rouhier *et al*., 2008; Montrichard *et al*., 2009). Several genetic studies aiming to identify functions of TRX and Glutaredoxin (GRX) in single mutants have been performed. Such approaches have assigned some functions for specific glutaredoxin and thioredoxins (reviewed by Rouhier *et al*., 2015). Nevertheless, not clear phenotypes have been detected, for most cytosolic TRX and GRX, which can be attributed to functional redundancies between members of large multigenic families (Meyer *et al*., 2008). Several studies on the interplay between NTS and NGS pathways have been performed in different organisms by association of different mutants involved in these two pathways (Carmel-Harel and Storz, 2000; Kanzok *et al*., 2001; Gelhaye *et al*., 2003; Koh *et al*., 2008). In *Arabidopsis thaliana*, different phenotypes have emerged and are affected in several plant developmental functions (Reichheld *et al*., 2007; Marty *et al*., 2009; Bashandy *et al*., 2010) e.g., by crossing of *ntra ntrb* double mutant (inactivated in both the two genes NTRA and NTRB (encoding cytosolic and mitochondrial thioredoxin reductases (NTR) the main reducer of cytosolic and mitochondrial TRXs) with different mutants are affected in GSH1 gene (control of the first step of the glutathione biosynthesis) and having decreased quantities of GSH like *rml1* mutant, which has about 3% GSH and unable to form roots (Vernoux *et al*., 2000), the triple *ntra ntrb rml1* mutant produced an additive shoot meristemless phenotype (Reichheld *et al*., 2007). Furthermore, when the *ntra ntrb* double mutant was crossed with *cadmiumsensitive2* (*cad2*) mutant, which has about 30% of glutathione, the resultant

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triple mutants has a pin-like phenotype and is obviously unfertile. This phenotype is linked to perturbation in auxin level and transport, which can affect the meristem development (Bashandy *et al*., 2010). Nevertheless, a major problem that limits the use of these plants in further investigations is that the mutant is unfertile. In order to solve this problem would be to isolate less affected and fertile homozygote mutant plants. This can be done by using RNA interference as a convenient strategy for down regulation of gene expression, which can efficiently induce reduction of mRNA level through post-transcriptional process by forming double stranded RNA (dsRNA) in cell which causes the specific degradation of target mRNA (Carthew, 2001; Baulcombe, 2004). In this study we have generated *cad2* NTR *RNAi* lines that can alternatively be used to produce less affected and fertile phenotype.

MATERIALS AND METHODS

Plant Materials

Seedlings and plants of *Arabidopsis thaliana* mutants (*ntra*, *ntrb* and *cad2*) having genetic background of ecotype Col-0 described by Reichheld *et al*. (2007) and Howden *et al*. (1995) were used for this work. For *in vitro* seedling growth, seeds were surface sterilized and grown on 0.5x Murashige and Skoog (MS) medium, including Gamborg B5 vitamins supplemented with 1% sucrose, and 0.8% plant agar. For growth in soil, seeds were sown in pots containing a mixture of soil and vermiculite (3:1, v/v) and irrigated with water. The growth conditions of the green house were 22°C and 70% humidity under a 16 h light (4000 lux)/8-h-dark regime.

Generation of RNAi lines

A 552 bp fragment of the NTRA cDNA (containing fully conserved sequences with *NTRB* gene) was amplified by PCR using primers attb1-NTRB-F (GGGGACAAGTTTGTACAAAAAAGCAGGCTATCTCCGCTTGTGCTGTTTGCGACG) and attb2-NTRB-R (GGGGACCACTTTGTACAAGAAAGCTGGGTAGAT CCAATCTCTTGTAAGTAATGC) and cloned into *RNAi* vector, pH7GWIWG2 (II) (Karimi *et al*., 2002), using the Gateway (Invitrogen) direct recombination system. The induced construct was verified by restriction enzyme digestion, direct PCR and followed by sequencing. The resulting construct was introduced into *Agrobacterium tumefaciens* GV3101. Then, in order to decrease the level of NTR in presence of low level of GSH the *cad2* mutant was transformed with *Agrobacterium via* floral dip method (Clough and Bent, 1998). T1 seedlings were selected *in vitro* on half minerals of MS medium supplemented with 30µg/ml hygromycin. Hygromycin-resistant seedlings were then transferred to soil.

Gene Expression Analysis by Reverse transcription PCR (RT-PCR)

Total RNA was extracted from leaves using TRIzol reagent according to the manufacturer's protocol. The cDNAs were synthesized by using Super Script

first-strand synthesis system and an oligo (dT) primer as described by the manufacturer's protocol (First-Strand RT-PCR kit, ProSTAR; Stratagene). Twenty five cycles of PCR was performed as described by Laloi *et al*. (2004) by using the following specific oligonucleotide primers: NTRA forward primer 5′-GCAAAATGTGTTGGATCTCAATGAG-3′, reverse primer 5′-CATGGATCCTTCTCCTACAGCTTC-3′, NTRB forward primer 5′-CGAAAGCTTTGCACGGCTTGGTGGTG-3′, reverse primer 5′-GATCAATCAACAATAACTCAATGACCT-3′ and Act2 forward primer 5′-GTTAGCAACTGGGAT GATATGG-3′, reverse primer 5′-AGCACCAATCGTGATGACTTGCCC-3′. PCR fragments were detected by GelRed (Biotium) staining and visualized with U-Genius (Syngene). Sequence data for NTRA, NTRB, GSH1 and ACT2, can be found in the GenBank/EMBL data libraries under accession numbers NP 179334 (At2g17420), NP 195271 (At4g35460), NP 194041 (At4g23100) and NP 850611 (At3g18780), respectively.

Protein extraction and western blot analysis

Protein was extracted from grounded leafs in liquid nitrogen and melted in extraction buffer (25 mM Tris-HCl, pH 7.6, 75 mM NaCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40). After centrifugation (15 min, 13,000 rpm, 4°C), protein concentrations were determined by using Bradford. Proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membrane. Protein gel blots were hybridized with anti-NTRB antibodies which interact with both NTRA and NTRB proteins as described by Reichheld *et al*. (2005).

RESULTS AND DISCUSSION

Selection and phenotyping of RNAi transgenic plants

In order to obtain close similar phenotype to the triple *ntra, ntrb* and *cad2* mutant that are fertile, we performed RNAi technique. Due to the high nucleotide similarity between NTRA and NTRB we decided to partially inactivate the gene expression of both NTR genes by RNA interference by using RNAi construct harbouring two head-to-tail copies of NTRA gene in the *cad2* mutant (Fig. 1A, 1B). Seeds were collected and cultured on selective medium (MS supplemented with 30 μg/ml hygromycin) to select transformed plants that then were transferred to soil. We obtained several different phenotypes some of them looks fertile but show decrease of apical dominance (Fig. 2-b). Other plants are highly affected and produced smaller siliques containing no/or fewer mature seeds (Fig. 2 c, d).

Reduced expression of NTR in RNAi plants

RT-PCR was used to determine the expression of NTR in several *RNAi* lines which produced from down regulation of

NTR genes in *cad2* mutant. Total RNA was isolated from leaves of *RNAi* lines, *cad2* and *ntra, ntrb* mutations, then RT-PCR analysis with specific NTRA and NTRB primers have been performed. We observed that, the expression of NTR is very weak in lines number 1 and 2, but it is more slightly affected in lines number 3 and 5, and it looks intermediate in lines number 4 and 6 (Fig. 3A). Comparing this expression level with the shape of obtained phenotypes we found that, the expression level of both NTRs especially NTRA decreased with increasingly severe phenotype. Furthermore, western blot analysis of total protein from *cad2, ntra* and *ntrb* mutants and the same *RNAi* lines has been done in presence of anti-NTRB antibodies, which recognize both NTRA and NTRB proteins (Reichheld *et al*., 2005). In agreement with the expected molecular mass of the cytosolic isoform of NTRB, a single band of 35 kDa is revealed in the cytosolic fractions of *cad2* mutant and is not detected in the *ntra ntrb* mutant (Fig. 3B). In other hand, *RNAi* lines showed that the most severe phenotype is correlated with the most pronounced reduction of the NTR protein level, while the plants having a weak phenotype have also lower decrease of the NTR expression (Fig. 3B).

According to the meristematic phenotype of the *ntra ntrb cad2* mutant suggests that downstream targets of TRX and/or GRX are implicated. Such targets are likely under a redox control performed by both TRX and GRX, or by a TRX that can be reduced alternatively by the NTS or the NGS. Obviously, the research of such targets is limited by the fact that we do not know which redoxins are involved in the phenotype. In order to isolate downstream actors of the phenotype we need to isolate revertant of the *ntra ntrb cad2* mutant. Nevertheless, a major problem that could limit the use of this approach is that the mutant is unfertile and that the mutagenesis should be performed on heterozygote plants. The identification of reverted mutants would be very complex. An alternative way to solve this problem is to isolate less affected mutants leading to fertile homozygote plants. For this purpose, we have generated *cad2 NTR RNAi* lines that could alternatively used. While, we have used RNAi-mediated gene silencing as an effective tool which can efficiently induce reduction of mRNA level, as it was also suggested by several studies in different plants (Chuang and Meyerowitz, 2000; Miki *et al*., 2005; Travella *et al*., 2006; Jiang *et al*., 2013; Dang *et al*., 2014). This approach could efficiently facilitate more investigations on the crosstalk between these two thiol reduction systems. We found strong correlation between decreased level of mRNA and increased severity of phenotypes, which is in agreement with the results obtained by Chuang and Meyerowitz (2000) and Wang *et al*. (2005). Completely inactivation of NTR and combination with presence of approximately 30% GSH of wild-type in *cad2* mutant, lead to very severe phenotype including pin like phenotype and unfertile flowers (Bashandy *et al*., 2010). In this aggressive phenotype was never seen in

RNAi lines, this may be due to the RNAi is not fully blocking the mRNA at the same level in the two *NTRA* and *NTRB* genes and/or the appearance of this phenotype needs to fully inactivate the *NTR* genes in presence of low level of GSH. We should realize that, the variation in the degree of silencing which observed in *RNAi* lines is very useful and reliable tool to go for more investigations on the crosstalk between the two thiol pathways.

SUMMARY

The NADPH-Thioredoxin System (NTS) and NADPH Glutathione System (NGS) are the two major thiol reduction systems that play a key role in the maintenance of cellular redox homeostasis and several plant developmental processes. Crosstalk between these two thiol reduction systems has been studied by associating TRX reductase (*ntra ntrb*) and glutathione biosynthesis *(cad2*) mutations. Triple *ntra ntrb cad2* mutant revealed a new phenotype related to flower meristem development. Unfortunately, this mutant is unfertile and therefore it cannot be maintained at a homozygous stage. In this study, we used the RNAi technique to obtain close similar phenotype to this mutant, but that are fertile. RNAi strategy is performed by down-regulating the expression of both *NTR* genes by introducing RNAi construct harbouring two head-to-tail copies of the *NTRA* gene in the genetic back-ground of the *cad2* mutant. The transformed plants obtained exhibit attenuated phenotypes compared to the *ntra ntrb cad2* mutant. Remarkably, no plants exhibit the characteristic pin-like phenotype of the *ntra ntrb cad2* mutant was obtained. However, some plants looks fertile but show a decrease of the apical dominance. Others are more affected and show unfertile flowers. Our data show that the *RNAi* strategy is an efficient strategy to generate fertile plants with down-regulated NTS and NGS reduction systems and to investigate the crosstalk between these two thiol systems.

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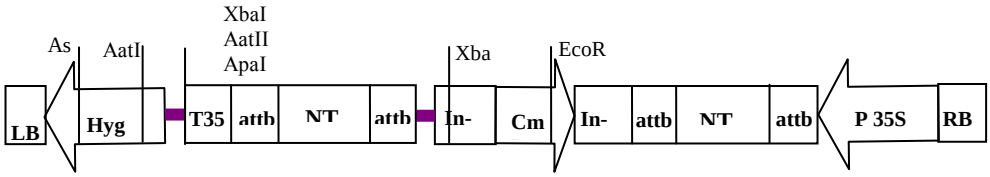
A



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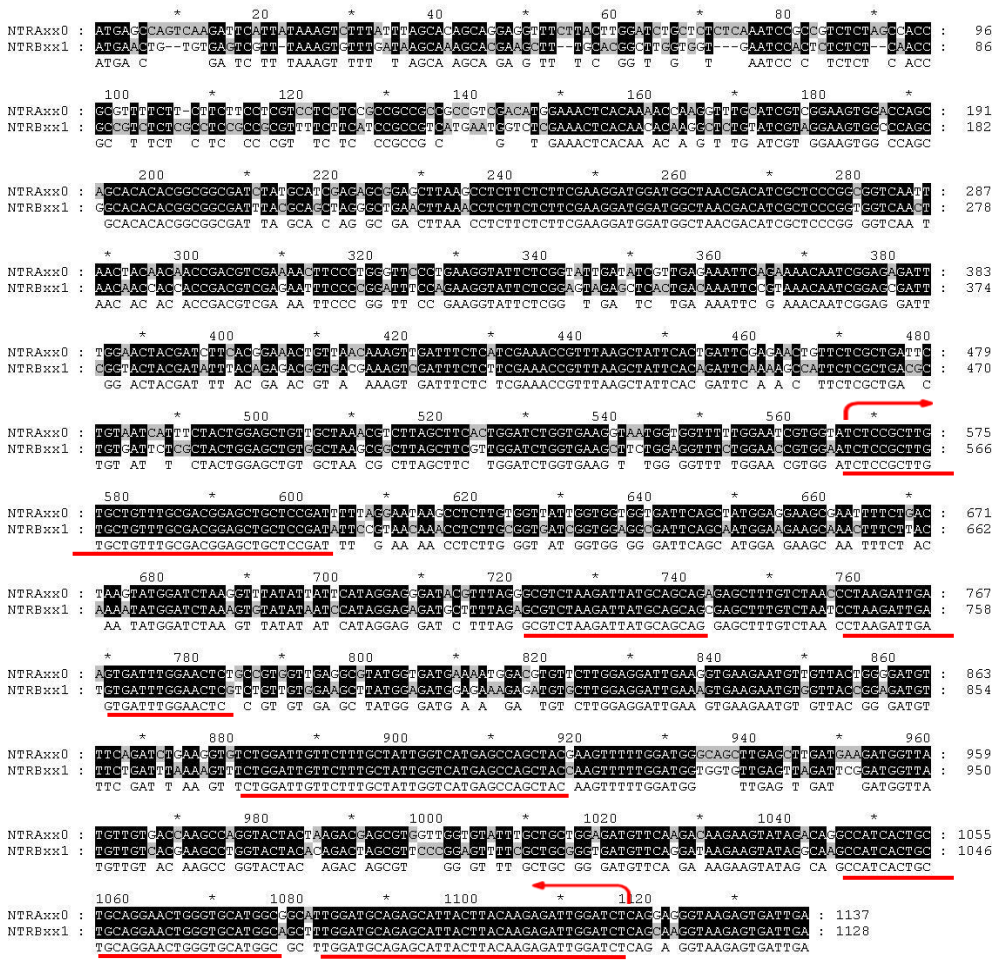

Fig. (1): RNAi strategy to inactivate NTR genes. (A) RNAi construct harbouring two copies of *NTRA* gene. (B) Nucleotidic alignment of NTRA and NTRB CDS. Oligonucleotides used to amplify PCR fragment are represented by arrows. Identical stretches >24 nt are represented by red bars.

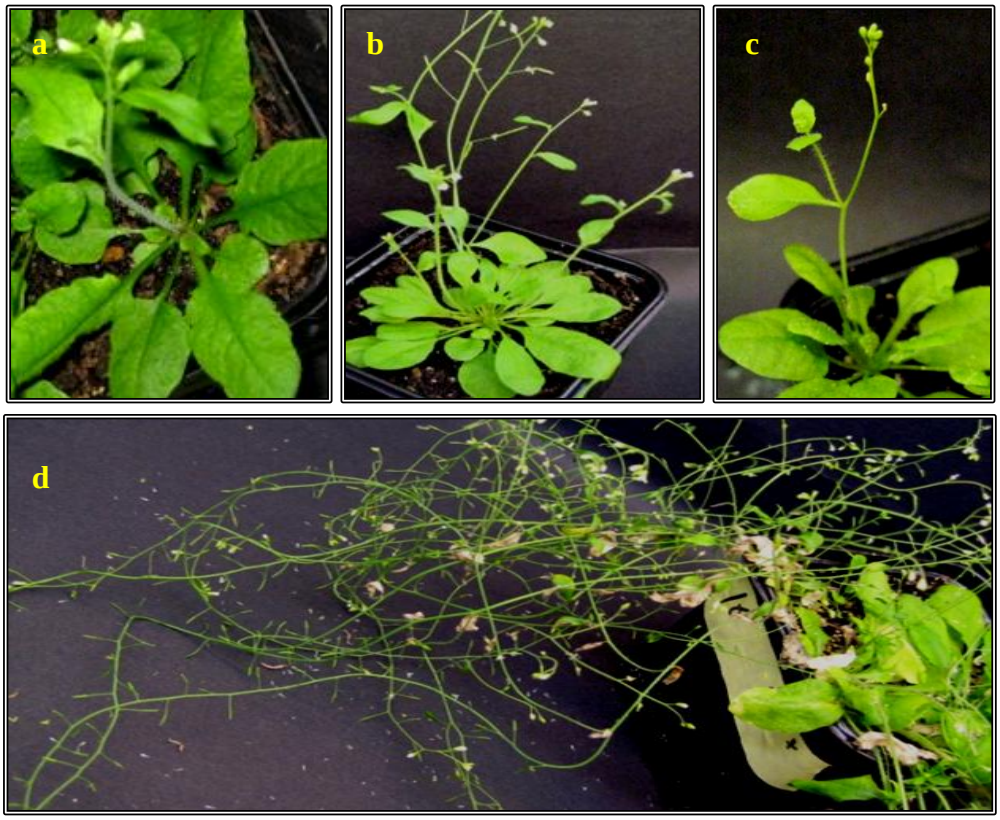

Fig. (2): Phenotypes of the *RNAi* plants. (a) *cad2* mutant. (b, c and d) *RNAi* plants.

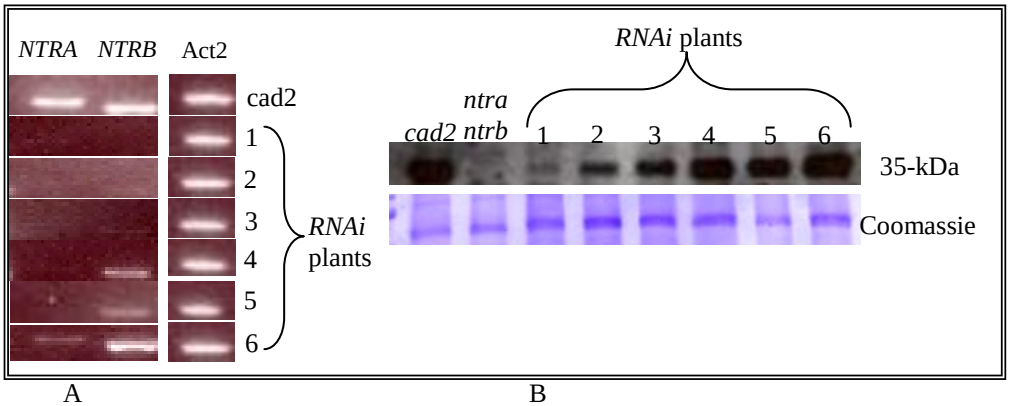

Fig. (3): NTR expression in *RNAi* plants. (A) RT-PCR expression analysis of *NTRA*, *NTRB* and *ACT2* (reference gene) genes. 25 cycles of PCR were performed on cDNA prepared from RNAs of different *RNAi* plants (1, 2, 3, 4, 5 and 6) and *cad2* mutant. (B) Western blot analysis of protein extracts were probed with antibodies directed against NTRB. Fractions were prepared from *cad2*, *ntra ntrb* mutants, and different *RNAi* plants (1, 2, 3, 4, 5 and 6).The position of the 35-kDa NTRB protein band reacting with antibody.