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

T. BASHANDY\textsuperscript{1} AND J. P. REICHHELD\textsuperscript{2}

1- Genetics Department, The New Valley Agriculture Faculty, Assiut University, Egypt

2- Laboratoire Génome et Développement des Plantes, Université de Perpignan, UMR CNRS-IRD-UPVD 5096, 52 avenue Paul Alduy, 66860 Perpignan, France

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 (GGGGACAAGTTTGTACAAAAAAGCAGGCTATCTCCGCTTGTGCTGTTTG CGACG) and attb2-NTRB-R (GGGGACCACCTTTGTAACAGAAAGC TGGTAGAT CCAATCTCTGTAAGTAATGC) and cloned into RNAi vector, pH7GW1WG2 (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’-CATGGATCTTCTCTACAGCTTC-3’, NTRB forward primer 5’-CGAAAGCTTTGCACGGCTTGGTG-3’, reverse primer 5’-GATCAATAACTCAATGACCT-3’ and Act2 forward primer 5’-GTATGGGAT 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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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.