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NADPH Thioredoxin Reductase C is localized in Plastids of Photosynthetic and Non-photosynthetic Tissues and is involved in lateral root formation in *Arabidopsis thaliana*

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Synopsis

NTRC functions in maintaining redox homeostasis of chloroplasts and heterotrophic plastids of Arabidopsis. Leaf-specific expression of NTRC was sufficient to restore leaf and root growth, whereas root-specific expression of NTRC was not. The results emphasize the function of chloroplasts not only as source of carbon and energy, but also of signaling molecules for development of heterotrophic organs.

ABSTRACT

Plastids are organelles present in photosynthetic and non-photosynthetic plant tissues. Whilst it is well known that thioredoxin-dependent redox regulation is essential for leaf chloroplast function, little is known of the redox regulation in plastids of non-photosynthetic tissues, which cannot use light as direct source of reducing power. Thus, the question remains whether redox regulation operates in non-photosynthetic plastid function and how it is integrated with chloroplasts for plant growth. Here we show that NADPH-thioredoxin reductase C, NTRC, previously reported as exclusive to green tissues, is also expressed in non-photosynthetic tissues of *Arabidopsis thaliana*, where it is localized to plastids. Moreover, we show that NTRC is involved in maintaining the redox homeostasis of plastids also in non-photosynthetic organs. To test the relationship between plastids of photosynthetic and non-photosynthetic tissues, transgenic plants were obtained with redox homeostasis restituted exclusively in leaves or in roots, through the expression of NTRC under the control of organ-specific promoters in the *ntrc* mutant. Our results show that fully functional chloroplasts are necessary and sufficient to support wild type rate of root growth and lateral root formation. In contrast, fully functional root amyloplasts are not sufficient for root, or leaf, growth unless chloroplasts are functional.

INTRODUCTION

Redox regulation based on disulfide-dithiol interchange of key cysteine residues of regulatory enzymes is a rapid and reversible mechanism, which allows the control of metabolic fluxes and its adjustment to ever changing environmental constraints. This is a universal type of regulation, present in all types of organisms, from bacteria to plants and animals (Buchanan and Balmer, 2005; Meyer et al., 2009). Thioredoxins (TRXs), small proteins of 12-14 kDa with a conserved WC(G/P)PC active site, play a central role in redox regulation. In its reduced state, the TRX is able to reduce disulfides of target proteins so that its own active site becomes oxidized to a disulfide. Thus, for a new catalytic cycle, oxidized TRX needs to be reduced in a reaction catalyzed by NADPH-dependent thioredoxin reductase (NTR). Therefore, in all types of organisms the maintenance of the redox status includes a two-component system formed by NTR and TRX, the so-called NADPH-TRX system (NTS), which uses NADPH as the source of reducing power and, in eukaryotic cells, is localized to the cytoplasm and mitochondria (Jacquot et al., 2009).

Although the NTS is universal, plants have several characteristics that make them unique organisms concerning redox regulation. While in bacteria, yeast and animals NTR and TRX are encoded by 1-2 genes, the plant genomes so far sequenced reveal the presence of two genes encoding NTR, stated NTRA and NTRB, but a large number of up to eleven genes encoding the *h*-type (*h* for heterotrophic) TRXs (Gelhaye et al., 2005; Meyer et al., 2005). In plants, as in other eukaryotes, the NTS is localized in mitochondria and cytoplasm, NTRA being the major cytosolic isoform, whereas NTRB is more abundant in mitochondria (Laloi et al., 2001; Reichheld et al., 2005). Regarding *h*-type TRXs, most of them are predicted to be localized to the cytoplasm, but alternative localization to mitochondria (Gelhaye et al., 2004) and nucleus (Serrato et al., 2001; Serrato and Cejudo, 2003) have been reported. Even a double targeting to nucleus and mitochondria has been described for a novel *o*-type TRX from pea (Marti et al., 2009). Moreover, in cereal seeds cells that suffer oxidative stress during development and germination NTR is also accumulated in the nucleus and has been proposed to play an antioxidant function based on its ability to reduce 1-Cys PRX (Pulido et al., 2009).

A remarkable characteristic of redox regulation in plants and algae is the presence of a specific and complex set of TRXs in chloroplasts. These include types *f*, *m*, *x* and *y*

(Collin et al., 2003), and additional TRXs and TRX-like proteins more recently identified such as HCF164 (Motohashi and Hisabori, 2006), CDSP32 (Broin et al., 2000), TRX *z* (Arsova et al., 2010; Chibani et al., 2010) or the family of atypical TRXs stated ACHT (Dangoor et al., 2009). The chloroplast also contains a specific system for TRX reduction, which is dependent on ferredoxin (Fd) reduced by the photosynthetic electron transport chain and an Fd-dependent TRX reductase (FTR) (Schürmann and Buchanan, 2008). Therefore, redox regulation in chloroplasts has been considered to rely on reduced Fd, thus being light-dependent, in contrast with redox regulation in heterotrophic organisms, and non-photosynthetic plant tissues, which use NADPH as source of reducing power. In chloroplasts NADPH is produced during the day as the final product of the photosynthetic electron transport chain in a reaction catalyzed by Fd-$NADP^+$oxidoreductase (FNR) (Ceccarelli et al., 2004; Lintala et al., 2007), and also during the night by the oxidative pentose phosphate pathway (Neuhaus and Emes, 2000). Therefore, the use of reduced Fd, but not NADPH, for redox regulation in this organelle was considered to be due to the lack of an enzyme able to use NADPH rather than to the lack of NADPH itself. This view of the redox regulation of the chloroplast changed after the discovery of a novel bimodular enzyme named NADPH-thioredoxin reductase C, NTRC, which is localized in chloroplasts (Serrato et al., 2004). NTRC is composed of NTR and TRX domains and conjugates both activities to efficiently reduce 2-Cys PRXs using NADPH as source of reducing power (Moon et al., 2006; Pérez-Ruiz et al., 2006; Pérez-Ruiz and Cejudo, 2009). Hence, NTRC allows the use of NADPH to maintain redox homeostasis in the chloroplast (Spínola et al., 2008).

The severe phenotype of an Arabidopsis NTRC knock out mutant, which is highly dependent on photoperiod and darkness (Pérez-Ruiz et al., 2006; Lepistö et al., 2009), shows that the function of NTRC is very important for plant growth and development. Moreover, the comparison of the *ntrc* mutant with mutants lacking TRX *x* and 2-Cys PRX suggested that NTRC is the principal reductant of 2-Cys PRX in the chloroplast (Pulido et al., 2010). Among the redox-regulated processes of the chloroplast, in which NTRC plays a role, some are dependent on its ability to reduce 2-Cys PRX, such as chlorophyll synthesis (Stenbaek et al., 2008; Stenbaek and Jensen, 2010). However, the phenotype of the *ntrc* mutant is more severe than the phenotype of the 2-Cys PRX double mutant, suggesting that NTRC has additional functions, which are independent of 2-Cys PRX reduction (Pulido et al., 2010). Some of these functions have already been identified and include aromatic amino acid and auxin synthesis (Lepistö et al.,

2009) and starch biosynthesis, since NTRC is involved in the redox regulation of ADP-glucose pyrophosphorylase (AGPase) (Michalska et al., 2009).

Interestingly, redox regulation of AGPase was severely affected not only in leaves of the Arabidopsis NTRC knock out mutant, but also in roots. This finding revealed the involvement of NTRC in redox regulation in plant heterotrophic tissues and therefore implied the localization of NTRC in these tissues. Thus, the first objective of this work was to establish the pattern of expression of the *NTRC* gene and the subcellular localization of the enzyme in Arabidopsis plants. Our results show a pattern of broad expression of the *NTRC* gene in both photosynthetic and non-photosynthetic tissues, and the localization of the enzyme in any type of plastids. This localization of NTRC led to the hypothesis that the enzyme functions as a general molecular switch able to convert NADPH into redox signals in plastids and, thus, might serve to integrate redox regulation between photosynthetic and non-photosynthetic tissues. However, whilst the function of the chloroplast for plant growth is well known, very little is known about the function of non-green plastids. To gain insight into the relative function of root amyloplasts in plant growth, as compared to chloroplasts, we have constructed transgenic Arabidopsis plants expressing NTRC exclusively in leaves or in roots. The phenotypes of these plants show the supreme importance of the chloroplast for the growth of non-photosynthetic plant tissues, including roots. In contrast, root amyloplasts have a low impact on root growth.

RESULTS

The *NTRC* gene is expressed in photosynthetic and non-photosynthetic tissues of Arabidopsis plants

To establish the pattern of expression of the *NTRC* gene in Arabidopsis, the content of transcripts in different organs of mature plants was analyzed by qPCR. This analysis confirmed the expected high expression of this gene in leaves and also revealed a high content of transcripts in stems and flowers, whereas in roots the presence of *NTRC* transcripts was detected at a much lower level (Fig. 1A), a pattern which was confirmed with data from Genevestigator (Suppl. Table 1). In agreement with this pattern of expression, Western blot analysis showed a high content of the NTRC protein in leaves, stems and flowers, and a much lower content in roots (Fig. 1B). Furthermore, this

analysis confirmed the absence of NTRC in any of the organs of the *ntrc* knock out mutant (Fig. 1B).

To further analyze the pattern of expression of the *NTRC* gene in Arabidopsis, a 1.05-kbp fragment containing its putative promoter (Suppl. Fig. 1) was transcriptionally fused to the β-glucuronidase (GUS) reporter gene and introduced into Arabidopsis wild type plants. GUS staining of transgenic lines confirmed the high level of expression driven by the *NTRC* gene promoter in green tissues such as cotyledons and first leaves of seedlings grown under long-day (Fig. 2A) or short-day conditions (Fig. 2B). Expression was high in leaf mesophyll cells in agreement with the high content of *NTRC* transcripts in leaves, but GUS staining revealed a higher expression associated with the vascular tissue (Fig. 2C), as well as in stem guard cells (Fig. 2E). *NTRC* promoter driven expression was also detected in roots (Fig. 2D and G) and hypocotyls (Fig. 2G), staining being associated to the vascular tissue and the base of hypocotyls diverging to the cotyledons (Fig. 2F). GUS staining was also analyzed in reproductive organs of the transgenic plants, which showed the expected high expression of *NTRC* in green tissues, such as sepals of inflorescences and flowers (Fig. 2H, I, L). In addition, GUS staining revealed *NTRC* expression in stigma (Fig. 2I, J), anthers (Fig. 2I, K), siliques and silique petioles (Fig. 2M, N).

NTRC is localized in plastids of photosynthetic and non-photosynthetic tissues in Arabidopsis plants

Once established the broad distribution of *NTRC* expression in photosynthetic and non-photosynthetic tissues of Arabidopsis plants, a set of new transgenic lines was designed to analyze the subcellular localization of the enzyme. To that end, a translational fusion of Arabidopsis NTRC, including its signal peptide, with GFP was expressed in Arabidopsis wild type and *ntrc* mutant plants under the control of the CaMV 35S or the *NTRC* gene promoter. The expression of NTRC-GFP in *ntrc* mutant plants partially complemented the mutant phenotype (Suppl. Fig. 2), thus showing the functionality of the fusion protein. Confocal microscopy analysis of the transgenic plants revealed coincidence of the green fluorescent signal, corresponding to NTRC-GFP, with chlorophyll red fluorescence in leaf mesophyll and guard cells of plants expressing the fusion protein under the CaMV 35S promoter (Fig. 3A, C) or the *NTRC* promoter (Fig. 3B, D), and revealed the presence of the protein in stromules, which were clearly

labeled in lines expressing the NTRC-GFP fusion protein under the 35S promoter (Fig. 3C, arrow).

The analysis of the green fluorescence signal in primary and secondary roots of transgenic plants showed the localization of NTRC in amyloplasts either in plants expressing the fusion protein under the 35S (Fig. 3E) or the *NTRC* promoter (Fig. 3F), in contrast with the diffuse signal observed in transgenic plants expressing GFP not fused to NTRC (Fig. 3G). The analysis of petals (Fig. 3H) and anthers (Fig. 3I) showed the localization of NTRC in plastids in these tissues. Finally, hypocotyls of etiolated plants showed the localization of NTRC in etioplasts (Fig. 3J), indicating the localization of NTRC in plastids of photosynthetic and non-photosynthetic tissues of Arabidopsis plants, regardless of growth under light or dark conditions. Therefore, the analysis of plants expressing the GUS reporter gene under the *NTRC* promoter in conjunction with plants expressing the NTRC-GFP fusion protein under the 35S and *NTRC* promoter allow the conclusion that *NTRC* is widely expressed in both photosynthetic and non-photosynthetic tissues, with the enzyme being localized to plastids.

NTRC is involved in maintaining the redox homeostasis of plastids from photosynthetic and non-photosynthetic tissues

Previous studies carried out with the aid of different Arabidopsis mutants showed that the redox status of the chloroplast 2-Cys PRX is essentially controlled by NTRC (Pulido et al., 2010). The finding of the localization of NTRC in plastids of non-photosynthetic tissues of Arabidopsis plants suggested that this enzyme might also be involved in the maintenance of the redox homeostasis of these plastids. This possibility was tested by the analysis of the redox status of the 2-Cys PRX in different tissues of wild type and *ntrc* mutant plants. Western blot analysis, under reducing conditions, confirmed the expected expression of 2-Cys PRX in organs with green tissues such as leaves, stems and flowers, and revealed the presence of the enzyme, though at lower level, in roots (Fig. 4A). These results are in agreement with the content of transcripts of 2-Cys PRXs A and B genes obtained from Genevestigator, which showed lower expression of both genes in roots than in leaves, stems and flowers (Suppl. Table 1). Gel electrophoresis under non-reducing conditions showed the decreased content of monomeric 2-Cys PRX, indicative of unbalanced redox status, in any of the organs analyzed of the NTRC knock

out mutant as compared with wild type plants (Fig. 4B). Therefore, these results show that NTRC is involved in the maintenance of the redox homeostasis of chloroplasts and non-photosynthetic plastids.

Restitution of chloroplast, but not of amyloplast, redox homeostasis is sufficient for wild type level root growth

The finding that NTRC is involved in redox regulation in plastids from photosynthetic and non-photosynthetic tissues led us to test whether the enzyme has any function integrating redox regulation in both types of plastids, and the contribution of each of them to plant growth. To this end, we took advantage of the Arabidopsis NTRC knock out mutant, which was used to generate plants expressing NTRC exclusively in leaves or in roots with the aim of restituting NTRC-dependent redox regulation in either photosynthetic or in non-photosynthetic organs. For leaf-specific expression, the Arabidopsis NTRC cDNA was expressed in the *ntrc* mutant background under the control of the *Rbcs* gene promoter (Donald and Cashmore, 1990), whereas the promoters of the phosphate transporter genes *Pht1,2* and *Pht1,3* (Mudge et al., 2002) were used for root-specific expression. In parallel, Arabidopsis transgenic plants expressing the GUS-reporter gene under the control of these promoters confirmed the expected pattern of expression exclusive in leaves, for the *Rbcs* promoter, or in roots, for the *Pht1,2* and *Pht1,3* promoters (Suppl. Fig. 3).

Of the transgenic plants obtained for each construct, two representative lines of each promoter (termed #1 and #2) were chosen for further analysis. In addition, for comparative purposes, transgenic plants expressing NTRC in leaves and roots, under the CaMV 35S promoter, or transformed with the empty vector, were also included. The Western blot analysis of extracts from leaves and roots shows the expected content of NTRC - high in leaves and low in roots - in wild type plants, whilst NTRC was undetectable in either organ of the *ntrc* mutant or the mutant transformed with the empty vector (Fig. 5A). Plants expressing NTRC under the CaMV 35S promoter were obtained either in the wild type or in the *ntrc* background. A line in the wild type background (WT35S_NTRC) showing high expression in leaves and low in roots, and a line in the mutant background (*ntrc*35S_NTRC) with the opposite pattern, high expression in roots and low in leaves, were chosen (Fig. 5A). Finally, among the transgenic plants with organ-specific promoters, those expressing NTRC under the *Rbcs*

promoter accumulated NTRC exclusively in leaves, line #1 showing higher content than line #2, whereas transgenic plants with the *Pht1,2* promoter showed root-specific accumulation of NTRC (Fig. 5A). However, the *Pht1,3* promoter, which produced the expected high expression of NTRC in roots, was not specific since, although at a low level, the enzyme was also detected in leaves (Fig. 5A). To test the functionality of NTRC in the transgenic lines, the redox status of 2-Cys PRX was determined. Fig. 5B shows the expected low content of monomeric 2-Cys PRX, indicative of unbalanced redox status, in leaves and roots of the *ntrc* mutant, as compared to the wild type plants. A high level of monomeric 2-Cys PRX in leaves and roots was recovered in transgenic plants expressing NTRC under the 35S promoter, the level in roots being higher in the *ntrc*35S_NTRC line in agreement with the higher content of NTRC in roots of these plants. Similarly, transgenic lines expressing NTRC under the *Pht1,3* promoter recovered the redox status of 2-Cys PRX in leaves and roots (Fig. 5B), in agreement with the presence of NTRC in roots but also in leaves (Fig. 5A) of these plants. The redox status of the 2-Cys PRX was restored in leaves of plants expressing NTRC under the *Rbcs* promoter, but not under the *Pht1,2* promoter, in agreement with the presence or absence, respectively, of NTRC in leaves of these plants (Fig. 5A, B). Similarly, plants showing leaf-specific expression of NTRC, under the control of the *Rbcs* promoter, showed almost undetectable amounts of monomeric 2-Cys PRX in roots, which was recovered in plants with root-specific expression of NTRC, with the *Pht1,2* promoter (Fig. 5B). Therefore, these results show that the redox status of the 2-Cys PRXs in leaves and roots is highly dependent of the presence of NTRC in these organs and thus confirm the functionality of NTRC in the transgenic lines under analysis.

The phenotype of these transgenic plants was thereafter analyzed with the aim of testing the effect of NTRC-dependent redox regulation of plastids from leaves and roots, respectively, on plant growth. Plants were grown under short-day conditions, which were previously described to cause a more severe phenotype on the *ntrc* mutant (Pérez-Ruiz et al., 2006; Lepistö et al., 2009). Under these conditions, the NTRC knock out plants showed the characteristic inhibition of growth, with the corresponding lower leaf and root fresh weight (Fig. 6A-C). As expected, the constitutive expression of NTRC, under the 35S promoter, in leaves and roots of the *ntrc* mutant background, recovered the wild type phenotype in terms of both leaf and root fresh weight (Fig. 6A-C). In agreement with these results, plants expressing NTRC under the *Pht1,3* promoter, which contained NTRC both in leaves and roots, showed also recovery of the wild type

phenotype with respect to both leaf and root growth (Fig. 6A-C), although these plants displayed a different distribution of NTRC, with a higher content in roots than in leaves, as compared to the wild type plants (Fig. 5A). Interestingly, the expression of NTRC exclusively in leaves was sufficient to completely recover wild type phenotype in terms of leaf and root fresh weight (Fig. 6A-C). In sharp contrast, the expression of NTRC exclusively in roots resulted in a slight increase of leaf and root fresh weight as compared to *ntrc* mutant plants (Fig. 6A-C).

Chloroplast redox homeostasis is essential for root growth and lateral root formation

The poor effect of root-specific expression of NTRC on root growth suggests that amyloplast function required fully functional chloroplasts. As a well-recognized function of photosynthesis is to provide sucrose as source of carbon and energy for sink organs, we analyzed in more detail the rate of root growth and the effect of the addition of sucrose to the growth medium (Fig. 7). The rate of root growth was lower in NTRC knock out plants than in wild type plants, and feeding with sucrose had a slightly positive effect on both types of plants. As expected, constitutive expression of NTRC in leaves and roots, under the 35S or the *Pht1,3* promoter, recovered wild type level of root growth rate. The expression of NTRC exclusively in leaves was sufficient to recover wild type level of root growth despite the fact that root amyloplasts show signs of redox unbalance (Fig. 5B). Notably, expression of NTRC exclusively in roots, under the *Pht1,2* promoter, was not sufficient to recover wild type level of root growth regardless of the addition of sucrose to the medium. Therefore, the reestablishment of the redox homeostasis of root amyloplasts is not sufficient for recovery of root growth, whereas functional chloroplasts are necessary and sufficient to recover root growth independently of the redox status of the root amyloplasts.

The weak effect of exogenous sucrose on root growth in plants expressing NTRC exclusively in roots suggested that chloroplasts provide something else than carbon and energy for root growth. To further analyze this possibility we studied root phenotypes caused by NTRC deficiency. During early seedling growth the *ntrc* mutant shows a slow rate of root growth, but also less abundant lateral roots than the wild type plants (Fig. 8A, B). As lateral root formation is highly influenced by auxins, this phenotype of the *ntrc* mutant suggests the involvement of NTRC in auxin signaling.

Treatment with exogenous indole-3-acetic acid (IAA) exerted a similar inhibitory effect on root growth in wild type and *ntrc* mutant plants (Fig. 8C), showing that NTRC deficiency does not affect auxin sensitivity. We then analyzed the content of lateral roots in Arabidopsis lines with organ-specific expression of NTRC to determine the function of chloroplasts and amyloplasts on lateral root formation. As expected, expression of NTRC in leaves and roots, under the 35S or the *Pht1,3* promoter, rescued wild type level of lateral root formation (Fig. 8B). Interestingly, leaf-specific expression of NTRC, with the *Rbcs* promoter, was sufficient to recover wild type level of lateral roots, whereas root-specific expression of NTRC, with the *Pht1,2* promoter, was not (Fig. 8B). Inhibition of root growth in response to exogenous IAA treatment was similar for all transgenic lines under analysis (Suppl. Fig. 4), showing no alteration of auxin sensitivity. Therefore, restitution of chloroplast redox homeostasis is sufficient for lateral root formation even in plants with impaired amyloplasts.

An alternative pathway for redox regulation is expressed in roots

The recovery of the wild type rate of root growth and lateral root formation in plants expressing NTRC exclusively in leaves indicates that the presence of NTRC in root amyloplasts is not essential for root growth, thus suggesting the presence of an alternative pathway for redox regulation in amyloplasts. Redox regulation by this pathway would require the transfer of electrons from NADPH, generated from sugars by the oxidative pentose phosphate pathway, to amyloplast TRXs with the participation of FNR, Fd and FTR. To test for the presence of this alternative pathway in roots, the content of the corresponding gene transcripts was analyzed by qPCR in wild type and *ntrc* mutant plants. Of the four genes encoding *FNR* in Arabidopsis, *FNR1* and *FNR2* showed leaf-specific expression, whereas *RFNR2* was expressed at higher level in roots and *RFNR1* showed a poor expression in both tissues (Fig. 9A). No significant difference of transcript content was observed in wild type and mutant plants (Fig. 9A). In agreement with these results, Genevestigator data (Suppl. Table 2) show higher expression of *FNR1* and *FNR2* genes in leaves than in roots, whereas *RFNR2* gene is expressed at higher level in roots. However, the Genevestigator data predicts higher expression of the *RFNR1* gene than determined in our qPCR-based analysis (Fig. 9A). Genes encoding Fd (*Fd1* and *Fd2*), the catalytic (*FTRB*) and regulatory (*FTRA*) subunits of FTR and the two genes encoding type-*f* TRXs (*Trxf1* and *Trxf2*), which were chosen

as example of TRXs, showed higher expression in leaves but were also detected in roots (Fig. 9B-D), in agreement with Genevestigator data (Suppl. Table 2). The analysis of expression of these genes in the *ntrc* mutant revealed higher expression of *Fd2*, *FTRA* and the two genes encoding TRX *f* in leaves, but not in roots (Fig. 9 A-D). Therefore, genes encoding the alternative pathway for redox regulation are expressed in roots though at lower level than in leaves, like the *NTRC* gene.

DISCUSSION

NTRC is important for redox homeostasis of plastids of photosynthetic and non-photosynthetic tissues

The recent finding that the redox regulation of AGPase was severely altered in roots of the Arabidopsis NTRC knock out mutant (Michalska et al., 2009) implied the presence of NTRC in roots, in contrast with the previously established view of NTRC as an enzyme exclusive to photosynthetic tissues (Serrato et al., 2004; Moon et al. 2006; Lepistö et al., 2009). Thus, the first objective of this work was to establish the pattern of expression of NTRC in Arabidopsis mature plants. Both qPCR and Western blot analyses, as well as Genevestigator data, confirmed the expression of NTRC in organs with photosynthetic cells, and revealed the presence of the enzyme, though at a lower levels, in roots (Fig. 1). This broad pattern of expression was confirmed by $NTRC_{pro}$-GUS transgenic lines, which showed high level of expression driven by the *NTRC* gene promoter in green tissues but also in root or hypocotyl, in which it is associated to the vascular tissue. Moreover, the analysis of these plants revealed a more unexpected expression in tissues of reproductive organs, such as stigma and anthers. Therefore, although *NTRC* is expressed at high level in photosynthetic tissues, in agreement with previous reports (Serrato et al., 2004; Moon et al., 2006; Alkhalfioui et al., 2007; Lepistö et al., 2009), the different approaches carried out in this work reveal a broad pattern of expression in either photosynthetic and non-photosynthetic tissues for this gene.

Different studies based on Western blot analysis of purified chloroplasts from rice leaves (Serrato et al., 2004), Arabidopsis plants expressing an NTRC-GFP fusion protein (Moon et al., 2007), or immunogold labeling (Pérez-Ruiz et al., 2009) clearly indicated that NTRC is a chloroplast-localized enzyme, in agreement with the high-level

of expression of the gene in green tissues and the function of the enzyme in chloroplast-localized processes such as chlorophyll and starch synthesis (Stenbaek et al., 2008; Stenbaek and Jensen, 2010; Michalska et al., 2009). However, the finding of *NTRC* expression in non-photosynthetic tissues raised the question of the subcellular localization of the enzyme in these tissues. This question was addressed by the generation of Arabidopsis lines expressing the NTRC-GFP fusion protein, which confirmed the localization of the enzyme in chloroplasts and showed the localization of NTRC in plastids of any of the non-photosynthetic tissues analyzed including roots, hypocotyls, anthers and petals (Fig. 3).

The presence of NTRC in plastids of non-photosynthetic tissues suggested that redox regulation might be an important component for the control of the metabolic pathways in these organelles, and that NTRC, which is able to use NADPH for redox regulation, may play a central function in these plastids with no photochemical reactions. Although 2-Cys PRXs were described as chloroplast-localized enzymes (Baier and Dietz, 1997), Western blot analysis under reducing conditions showed that these enzymes are also present in non-photosynthetic tissues of Arabidopsis plants (Fig. 4A). Moreover, expression in non-photosynthetic tissues of the genes encoding 2-Cys PRXs was confirmed by Genevestigator data (Suppl. Table 1), thus indicating that the redox state of these proteins may be taken as marker of the redox homeostasis of non-photosynthetic plastids. In chloroplasts, 2-Cys PRXs were proposed to be reduced by CDSP32 (Broin et al., 2002; Rey et al., 2005) and TRX *x* (Collin et al., 2003), but NTRC appeared to be the main reductant of this enzyme *in vivo* (Pulido et al., 2010). Indeed, Western blot analysis under non-reducing conditions showed a lower content of the monomeric form of the 2-Cys PRX in the *ntrc* mutant not only in leaves but in any of the other tissues here analyzed (Fig. 4B), thus indicating the involvement of NTRC in the maintenance of the redox status in plastids from either photosynthetic and non-photosynthetic tissues. These results are in agreement with the previous finding that NTRC is involved in the redox regulation of AGPase in roots (Michalska et al., 2009) and emphasize the notion that redox regulation occurs in plastids of non-photosynthetic tissues.

NTRC is a redox switch able to convert NADPH into redox signal

In chloroplasts, redox regulation relies on Fd reduced by the photosynthetic electron transport chain. This is the source of reducing power to the FTR/TRX system, which thus participates in the control of the redox status of the numerous TRX targets so far identified (Buchanan and Balmer, 2005). As a complementary pathway for chloroplast redox regulation, NTRC allows the use of NADPH, produced both by the oxidative pentose phosphate pathway and the photosynthetic electron transport (Spínola et al., 2008). In contrast to the thorough knowledge of redox regulation in chloroplasts, very little is known about the function and redox regulation of plastids in heterotrophic tissues. Plastids from some non-photosynthetic tissues, such as amyloplasts of cereal endosperm, are specialized in starch synthesis (Tetlow et al., 2008). However, recent proteomic analyses suggest that these plastids perform a complex diversity of metabolic pathways (Balmer et al., 2006a, b; Dupont, 2008). The finding of a complete Fd/FTR/TRX system in amyloplasts isolated from wheat endosperm and the identification of TRX targets in this organelle suggested that redox regulation may be an important component of the regulation of starch synthesis, but also of amino acid and lipid biosynthesis (Balmer et al., 2006a). Moreover, the presence of TRX *y* in Arabidopsis roots (Collin et al., 2004), and of TRXs *f* and *m* in pea roots and flowers (Barajas-López et al., 2007; Traverso et al., 2008) lend further support to the notion that redox regulation is a relevant aspect of plastid function in non-photosynthetic tissues. The identification of TRX *y* targets in Arabidopsis roots suggests that metabolic pathways including amino acid, lipid and phenylpropanoid metabolism, protein degradation and folding and the response to oxidative stress are redox-regulated processes (Marchand et al., 2010).

The localization of NTRC in plastids of photosynthetic and non-photosynthetic tissues of Arabidopsis reported here (Fig. 3) supports the notion of plastid redox regulation as a general phenomenon in plants. Since heterotrophic plastids lack photochemical reactions, reducing power to support redox regulation in these plastids relies entirely on NADPH produced from sugars by the oxidative pentose phosphate pathway (Kammerer et al., 1998; Neuhaus and Emes, 2000). The biochemical properties of NTRC, including its high affinity for NADPH and the presence of a TRX domain at the C-terminus, allow us to propose that NTRC acts as a redox switch able to convert reducing power in the form of NADPH, which might be indicative of the capacity to perform biosynthetic metabolism, into a redox signal through the thiol groups of its TRX domain. Thus, NTRC may constitute a direct pathway for redox homeostasis in

heterotrophic plastids (Fig. 10). However, transfer of electrons from NADPH to Fd, catalyzed by FNR, is also possible in heterotrophic plastids, as suggested by the high expression of the *RFNR2* gene in roots (Fig. 9A), in agreement with the previous report of a root-specific form of this enzyme (Oji et al., 1985). Though at a lower level than in leaves, qPCR analyses showed the expression in roots of genes encoding Fd, the catalytic and regulatory subunits of FTR and TRX *f1* and TRX *f2* (Fig. 9B-D), a pattern confirmed by the Genevestigator data (Suppl. Table 2). Therefore, the Fd/FTR/TRX pathway might be also operative in non-photosynthetic plastids (Fig. 10), as it is in chloroplasts. The fact that plants that lack NTRC in root amyloplasts, but express the enzyme in chloroplasts, show wild type levels of root growth, suggests that the Fd/FTR/TRX pathway compensates for NTRC deficiency in these organelles, although the roots of these plants will probably have unbalanced metabolite levels. The identification of specific targets of NTRC and the different TRXs of root amyloplasts will help to establish the metabolic processes which depend on NTRC or the Fd/FTR/TRX pathway.

Chloroplast function is sufficient to support root growth in plants with impaired amyloplast redox homeostasis

It has long been known that light, as the primary source of energy for plants, is used for the production of photosynthates in chloroplasts of source tissues, which are transported to sink tissues to support their growth. Therefore, growth of heterotrophic tissues is highly dependent on photosynthetic ones. Moreover, it has been proposed that photosynthates form part of a signaling network integrating both photosynthetic and non-photosynthetic tissues (Koch, 1996; Paul and Foyer, 2001). Based on the finding of an Fd/TRX system in wheat amyloplasts, Balmer et al. (2006a) proposed that TRX might act integrating redox regulation between both types of organelles, but due to the scarce knowledge of redox regulation in heterotrophic tissues this proposal has not been tested yet. The presence of NTRC in both photosynthetic and heterotrophic plastids and the unique properties of the enzyme serving as a switch of NADPH into redox signal suggest that NTRC might be important for such integration.

In this work, we have taken advantage of the severe phenotype of the Arabidopsis NTRC knock out mutant to address whether NTRC functions in coordinating plastid redox regulation between photosynthetic and heterotrophic tissues

and the relevance of each type of plastid for plant growth. Leaf-specific expression of NTRC restored redox homeostasis in leaf chloroplasts and recovered wild type leaf and root phenotypes. In contrast, restitution of redox homeostasis exclusively in roots was insufficient to recover leaf growth and remarkably was also insufficient for recovery of root growth and lateral root formation. Therefore, these results establish that chloroplast function is necessary and sufficient to reach wild type rate of root growth, thus emphasizing the essential function of chloroplasts for growth of heterotrophic tissues.

The poor growth of roots of transgenic lines expressing NTRC exclusively in roots might be due to deficiency of sucrose supply for amyloplast function since these plants have impaired chloroplast redox homeostasis. However, external addition of sucrose to the culture medium exerted a poor effect on root growth in these plants thus showing that leaf chloroplast function is required to provide other metabolites and/or signaling molecules, besides sucrose, for root growth. In this regard it should be mentioned that NTRC deficiency causes decreased auxin content (Lepistö et al., 2009). Moreover, the *ntrc* mutant shows not only slower root growth but also impairment of lateral root formation (Fig. 8), which is a process profoundly affected by shoot-derived auxins at early stages of seedling growth (Bhalerao et al., 2002). The lateral root formation phenotype of the NTRC-deficient mutant was rescued by expression of NTRC exclusively in leaves, but not in roots (Fig. 8B), thus showing that the recovery of the redox homeostasis of the chloroplast is sufficient to rescue lateral root formation regardless of amyloplast function. These results point to chloroplasts as source of signaling molecules important for development of heterotrophic organs and are in agreement with the model proposed by Ljung et al. (2005) according to which IAA synthesized in leaves at early stages of seedling development are important for lateral root formation, before roots gain more competence for auxin synthesis. Deficiency of NADP-dependent thioredoxin and glutathione systems, as occurs in the triple mutant *ntra ntrb cad2*, affects auxin signaling. This triple mutant shows lower auxin content, which causes a severe phenotype including defects of secondary root growth, vasculature and pin-like phenotype (Bashandy et al., 2010). In contrast, the auxin-related phenotype of the *ntrc* mutant is less-severe affecting only lateral roots. Because NTRC deficiency affects auxin synthesis (Lepistö et al., 2009) but not auxin sensitivity (Fig. 8C), the lateral root formation phenotype of the *ntrc* mutant might indicate a lower content of auxins, but more work is still needed to test this possibility.

In conclusion, the results presented in this report show that NTRC is broadly expressed in all plant tissues, localizes to plastids, and is involved in the maintenance of plastid redox homeostasis of photosynthetic and heterotrophic tissues. The biochemical properties of NTRC allowed us to propose its function as a redox switch converting NADPH into redox signal, thus enabling a direct use of NADPH for redox regulation. The finding that restitution of chloroplast redox homeostasis is sufficient to recover root-related phenotypes in plants with impaired amyloplast redox homeostasis emphasizes the essential role of the chloroplast for the growth of heterotrophic tissues. Consequently, green plastids are not only a source of carbon and energy, but also of signaling molecules, which may be important in coordinating the growth of photosynthetic and heterotrophic organs of the plant.

METHODS

Plant material and growth conditions

Arabidopsis thaliana wild type (ecotype Columbia), *ntrc* mutant, line SALK_012208 (Serrato et al., 2004), and the transgenic plants generated in this work were grown in soil supplemented with Hoagland medium in culture chambers under long-day (16 h light/8 h darkness) or short-day (8 h light/16 h darkness) conditions at 22ºC during the light and 20ºC during darkness. The light intensity was set at 140 µmol m^{-2} s^{-1}. For root growth experiments seeds were surface sterilized (3 min in 70% (v/v) ethanol followed by 4 min in 30% bleach), placed on plates containing 1 x MS media solidified with 0.8% agar and stratified for 2 days at 4ºC. Plates were oriented vertically and incubated in a growth chamber under short-day conditions. Seven-day-old seedlings were transferred onto fresh MS plates supplemented with 30 mM sucrose or 30 mM mannitol for 14 additional days. For auxin treatments seedlings grown on MS media for five days were transferred to media containing indole-3-acetic acid (IAA) for an additional period of 3 days. Root length and number of lateral roots were measured with the ImageJ software.

qPCR and Western blot analysis

Total RNA (1 µg) was extracted from tissues dissected from mature plants grown for 48 days using Trizol™ and retro-transcribed by means of QuantiTect™ RT-kit (Qiagen). Real time PCR was performed in a total reaction volume of 20 µL containing primers (4 µM each), cDNA (40 ng) and 10 µL of iQ™ SYBR Green Supermix (Bio-Rad). Results obtained from 3 independent biological samples (3 analytical replicates each) are represented as $2^{-\Delta\Delta CT}$ (Threshold Cycle) as described by Livak and Schmittgen (2001). *Ubiquitin 10* was used as reference gene. Gene specific primers used are described in Suppl. Table 3. Fluorescence of PCR products was determined continuously by the iQ5 cycler (Bio-Rad).

Western blot analysis was performed as previously described (Kirchsteiger et al., 2009) using as probes previously described antibodies (anti-NTRC, anti-NTRB and anti-2Cys Prx). For optimized resolution, SDS-PAGE was performed at 12-15% Acrylamide/Bisacrylamide gels.

Generation of Arabidopsis transgenic lines expressing the NTRCpro:GUS gene

The NTRCpro:GUS gene was constructed in the binary vector pGII0229 (Hellens et al., 2000) by a three-step process. First, a 276-bp fragment containing the nopaline synthase gene terminator (NOSter) was amplified by PCR from the pBI121 plasmid using oligonucleotides F-NOSter-NotI (5'-AATTGCTACCGCGGCCGCGAATTT-3') and R-NOSter-SacI (5'-CAGTGAGCTCCCGATCTAGTAACATAGAT-3') introducing *Not*I and *Sac*I restriction sites, underlined, used to clone the NOSter fragment into the pGII0229 vector. The *NTRC* gene (At2g41680) is separated of the flanking At2g41690 gene by approx. 2.7 kbp. Both genes are transcribed in opposite directions so that this 2.7-kbp sequence, which shows an even distribution of putative *cis*-acting elements (Suppl. Fig. 1), may contain the promoters of both genes. Thus to analyze the putative *NTRC* promoter, avoiding as much as possible interference with the flanking At2g41690 gene promoter, we selected a 1.05-kbp fragment upstream the *NTRC* gene coding sequence (Suppl. Fig. 1). This fragment was amplified from Arabidopsis (ecotype Columbia) genomic DNA with oligonucleotides FpNtrc-HindIII (5'GTAAGCTTCACGCGTCTGTAAAT-3') and RpNtrc-XbaI (5'-GGTCTAGAATTTTTTTTGATTGCCTTACC-3') introducing *Hind*III and *Xba*I restriction sites, underlined, which were used to insert the fragment into the pGII0229-NOSter plasmid. Finally, the *UidA* gene from *E. coli*, encoding the GUS-reporter

enzyme was amplified with oligonucleotides F-GUS-XbaI (5'-AACACGGGGGACTCTAGAGGATCC-3') and R-GUS-NotI (5'-ACGCGGCCGCAGTTGTTGATTCATTGTTT-3') introducing *Xba*I and *Not*I sites, underlined, which were used to transcriptionally fuse the GUS-reporter gene to the *NTRC* promoter. The final construct was sequenced and introduced into *Agrobacterium tumefaciens* (C58pMP90). Arabidopsis plants were then transformed by the floral dip method (Clough and Bent, 1998), and homozygous plants for the transgene were selected.

Histochemical GUS staining

Histochemical detection of GUS activity was performed using the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; Jefferson et al., 1987), by vacuum-infiltrating seedlings in assay buffer (50 mM sodium phosphate pH 7.0, 0.2% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 10 mM EDTA) containing 0.05% X-gluc. Samples were incubated at 37ºC overnight wrapped in aluminium foil to keep them in the dark. Green tissues were cleared with ethanol prior to observation.

Generation of Arabidopsis transgenic lines expressing the NTRC-GFP fusion protein

Subcellular localization of NTRC was analysed in Arabidopsis transgenic plants expressing the NTRC-GFP fusion protein. Given the putative presence of a transit peptide at the N-terminus of NTRC, the GFP was translationally fused at the C-terminus of the enzyme. To this end, the cDNA encoding GFP was digested from the peGFP plasmid (kindly provided by Prof. Thomas Roitsch, Graz University, Austria) by digestion with *Sma*I and *Xba*I. These restriction sites were then used to clone this fragment into the pBIBA7 vector (Becker, 1997) to generate the pBIBA7-GFP plasmid. Then, the full-length coding sequence of Arabidopsis NTRC was amplified by PCR from plasmid pUNI_U14278 (TAIR Accession Sequence 504962595) with oligonucleotides Ntrc-F-KpnI (5'-CAGGTACCATGGCTGCGTCTCC-3') and Ntrc-R-SmaI (5'-GACCCGGGa**TCA**TTTATTGGCCTCAATG-3') introducing *Kpn*I and *Sma*I restriction sites (underlined), the mutation of the stop codon TGA to TCA (bold) and the

insertion of an additional nucleotide (lower case) to keep the coding frame. The NTRC fragment was then introduced in the pBIBA7-GFP plasmid to generate the pBIBA7-NTRC-GFP construct for expression under the CaMV 35S promoter. For expression of the NTRC-GFP fusion protein under the control of the *NTRC* gene promoter the *Xba*I-*Not*I fragment containing the *UidA* gene coding sequence, in the NTRCpro:GUS construct described above, was replaced by an *Xba*I-*Not*I fragment containing the coding sequence of the NTRC-GFP fusion protein. All plasmids were checked by sequencing and introduced into *Agrobacterium tumefaciens* (C58pMP90) to transform Arabidopsis plants by the floral dip method, as above indicated.

Generation of Arabidopsis transgenic lines with leaf and root-specific expression of NTRC

The *Pht1,2* (2000-bp), *Pht1,3* (1647-bp) and *Rbcs1A* (1700-bp) promoters were PCR amplified from wild type Arabidopsis (ecotype Columbia) genomic DNA using specific primers with GATEWAY™ tails. The forward primers contain the AttB1 tail (5´-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3'), and the reverse primers contain the AttB2 tail (5´- GGGGACCACTTTGTACAAGAAAGCTGGGT-3'). Specific sequences for each oilgonucleotide were: *Pht1,2*-Fw (5´-TAGGATCCGATCACTATACAACTCTGC-3´), *Pht1,2*-Rev (5´-GAGGTACCTCTCTTGTCTTTCC-3´), *Pht1,3*-Fw (5´-TAGGATCCTAATGAGTATAAGAG-3´), *Pht1,3*-Rev (5´-CTGGTACCTCTCCTATTTTGCAC-3´), *Rbcs*-Fw (5´-TGGGATCCTGAGTCTCAAAGTGGC-3´) and *Rbcs*-Rev (5´-TGGTACCTCTTCTTTACTCTTTG-3´). All PCR products were introduced into the GATEWAY™ pDONR207 (Invitrogen) vector using BP Clonase, generating promoter entry clones. The promoter fragments were then transferred into the pGWB3 and pGWB1 destination vectors (Nakagawa et al., 2007) using LR Clonase II (Invitrogen). Restriction sites *Bam*HI and *Kpn*I (underlined) were incorporated into the specific sequence primer to facilitate subsequent cloning.

The full-length NTRC cDNA from Arabidopsis (DNA stock no. U-14278), was amplified by PCR with oligonucleotides (5´- CAGGTACCATGGCTGCGTCTC-3´ and 5´- GAGAGCTCTCATTTATTGGCCTCA-3´), which added *Kpn*I and *Sac*I restriction sites, underlined, at the 5´and 3´ends, respectively. The fragment was cloned into the

pGEMt vector (Promega) which was sequenced in both strands. The NTRC cDNA was digested with *Kpn*I and *Sac*I and fused to the *Kpn*I/*Sac*I site of a binary vector pGWB1 to yield plasmids pGWB1-*Pht1,2*-AtNTRC, pGWB1-*Pht1,3*-AtNTRC and pGWB1-*Rbcs*-AtNTRC.

The constructs were integrated into the Arabidopsis T-DNA insertion mutant SALK_012208 (*ntrc*) by *A. tumefaciens* (C58pMP90) mediated floral dip procedure (Clough and Bent, 1998) and transgenic seedlings were selected on half-strength MS medium containing 20 mg·L^{-1} hygromycin. Several independently transformed plants were obtained with each construct. An empty vector transgenic line, which showed no difference to wild-type plants, was used as control.

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AUTHOR CONTRIBUTIONS

F.J.C., designed the research; K.K. J.F., B.P. and M.G. performed research; all authors analyzed data; and F.J.C. wrote the paper.

Suppl. Figure 1. Scheme showing the position of the *NTRC* gene (Atg41680) and the flanking At2g41690 gene in Arabidopsis and distribution of possible *cis*-acting elements.

Suppl. Figure 2. Characterization of transgenic plants expressing the NTRC-GFP fusion protein or GFP under the control of the CaMV 35S promoter in wild type and *ntrc* mutant backgrounds.

Suppl. Fig. 3. Histochemical localization of GUS expression under the control of organ-specific promoters.

Suppl. Fig. 4. Effect of IAA treatment on root growth inhibition.

Suppl. Table 1. Expression of genes encoding NTRC, 2-Cys PRX A and 2-Cys PRX B in different Arabidopsis organs based on data obtained from Genevestigator.

Suppl. Table 2. Expression of genes encoding the alternative pathway for redox regulation in root amyloplasts based on data obtained from Genenvestigator.

Suppl. Table 3. Gene-specific oligonucleotides used for qPCR analysis.

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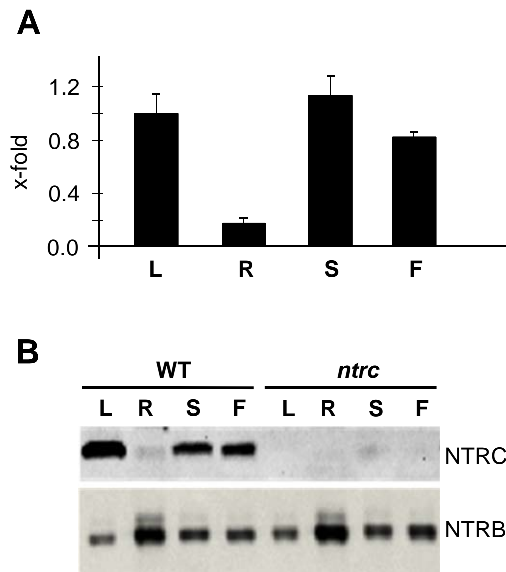

Figure 1

Figure 1. Expression pattern of NTRC in Arabidopsis.

(A) qPCR analysis of *NTRC* transcripts in different organs of Arabidopsis wild type plants. The amount of transcripts in each organ was represented as arbitrary units relative to the level in leaves, which was set to 1.0. Analysis was performed three times on two independent biological samples and the mean values ± standard errors are indicated. **(B)** The amount of NTRC protein was determined by Western blot probed with an anti-NTRC polyclonal antibody. Samples (30 µg of protein) were subjected to SDS-PAGE, transferred onto nitrocellulose membranes and probed with anti-NTRC or anti-NTRB antibodies, to test even protein loading, as indicated. Arabidopsis wild type and *ntrc* mutant plants were grown under long-day conditions for 48 days. RNA, for qPCR, and protein, for Western blot analysis, were extracted from leaves (L), roots (R), stems (S) and flowers (F).

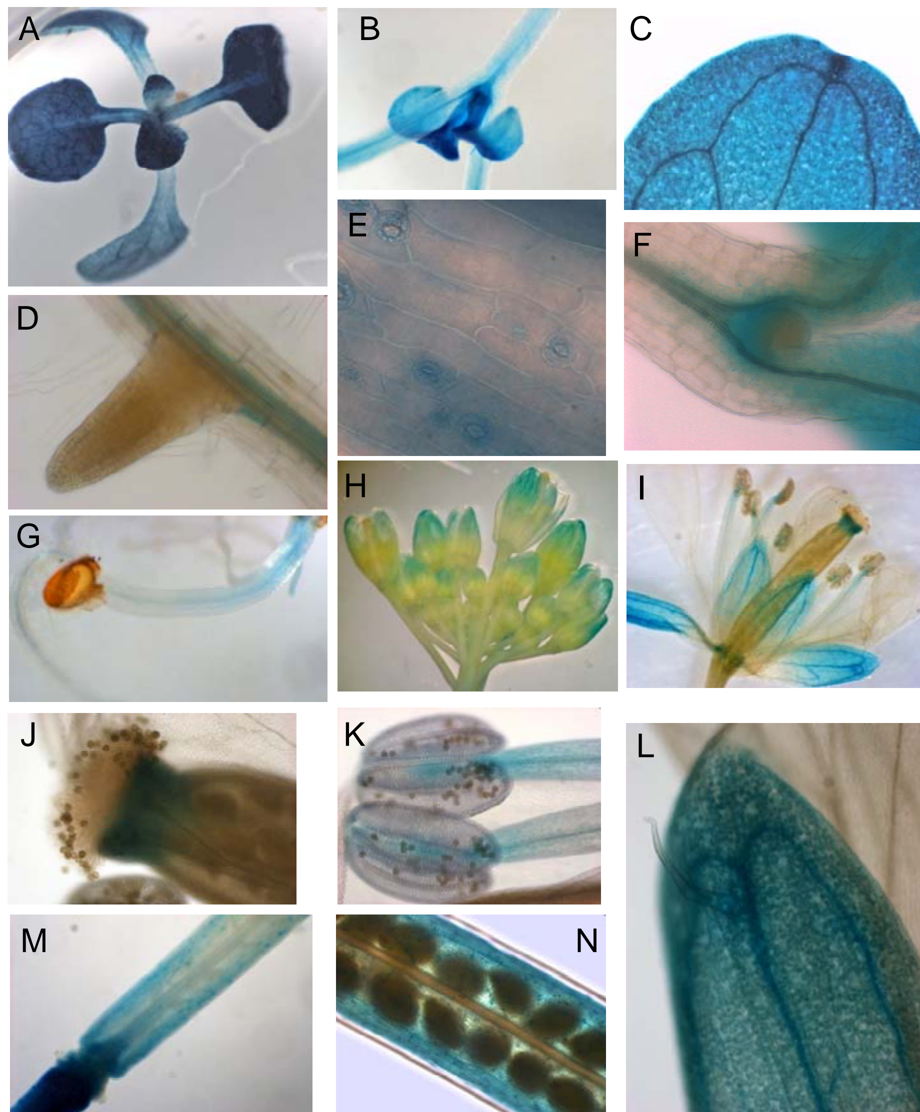

Figure 2

Figure 2. Histochemical localization of GUS expression in Arabidopsis plants transformed with the NTRCpro-GUS reporter gene.

GUS staining of ten-day-old seedlings grown under long-day conditions (**A, C, D, E**) or under short-day conditions (**B, F**). (**G**) Etiolated seedlings showing GUS staining in root and hypocotyl. GUS staining of inflorescence (**H**), flower (**I**), stigma (**J**), anthers (**K**), sepal (**L**), silique petiole (**M**) and silique (**N**) of Arabidopsis plants grown for 42 days under long-day conditions.

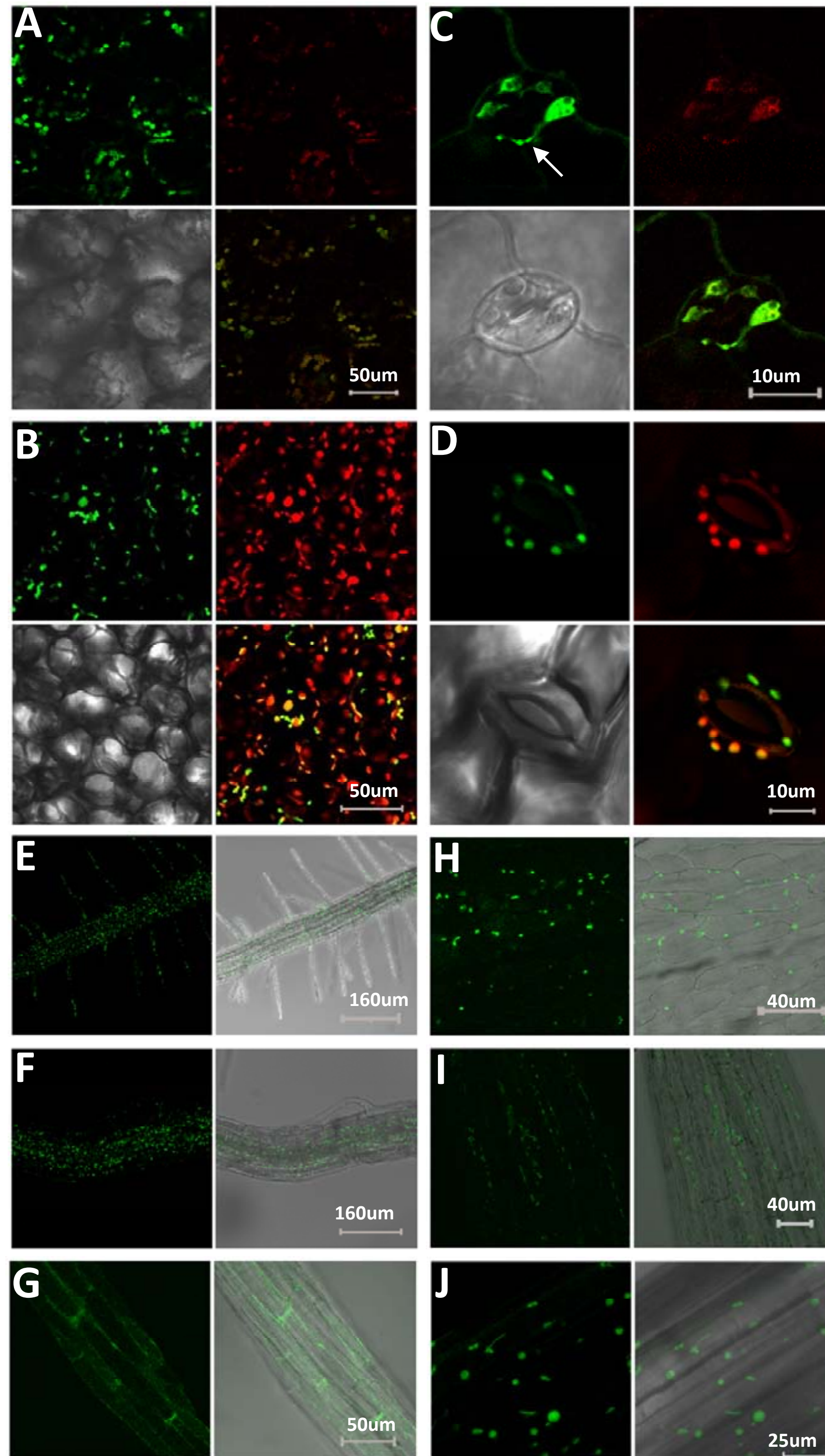

Figure 3

Figure 3. Subcellular localization of NTRC in Arabidopsis plants.

A-D, Confocal microscopy micrographs of mesophyll **(A, B)** and guard cells **(C, D)** of Arabidopsis plants transformed with the NTRC-GFP fusion protein expressed under the CaMV 35S promoter **(A, C)** or the *NTRC* gene promoter **(B, D)**. Plants were grown for 18 days. **E-J** Subcellular localization of NTRC in non-photosynthetic tissues of Arabidopsis. Confocal microscopy micrographs showing plastid localization of NTRC in roots of five-day-old seedlings with the NTRC-GFP fusion protein expressed under the CaMV 35S promoter **(E)** or the *NTRC* gene promoter **(F)**, and a root of plants expressing the GFP protein not fused to NTRC **(G)**. Plastid localization of NTRC in petal **(H),** anther **(I)** of 48-day-old plants, and hypocotyl of five-day-old Arabidopsis seedlings grown under darkness **(J)** and expressing the NTRC-GFP fusion protein under the 35S promoter. Red, chlorophyll autofluorescence; green, GFP fluorescence. Arrow indicates a chloroplast stromule. Magnifications are indicated in bars.

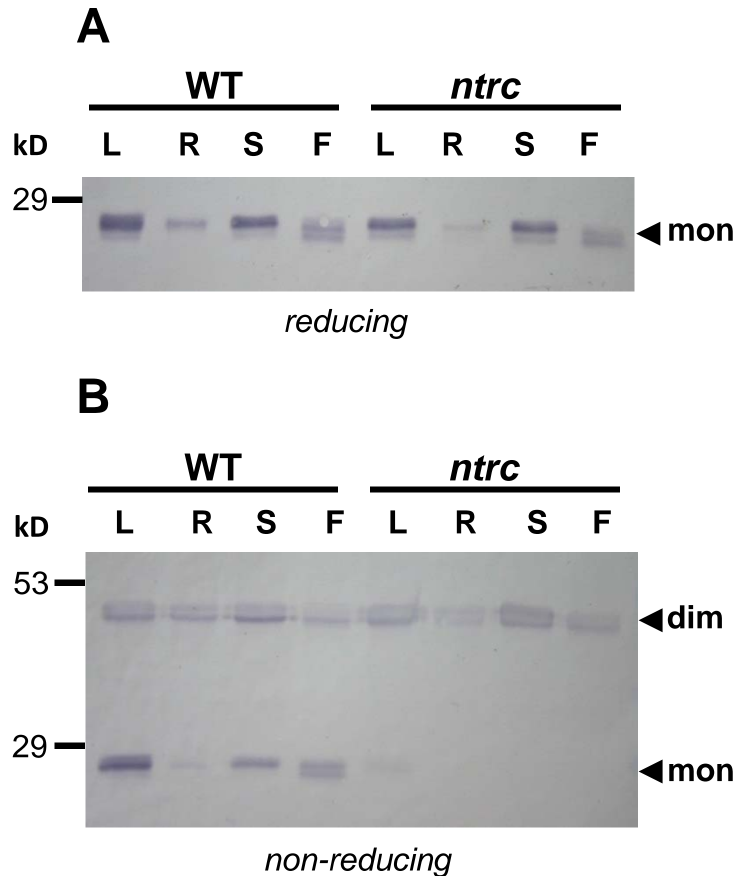

Figure 4

Figure 4. Unbalanced redox status of 2-Cys PRXs in photosynthetic and non-photosynthetic tissues of *ntrc* mutant plants.

Protein extracts from leaves (L), roots (R), stems (S) and flowers (F) of Arabidopsis wild type and *ntrc* mutant plants were subjected to SDS-PAGE under reducing (7.5 µg of protein loaded) **(A)** or non-reducing conditions (15 µg of protein loaded) **(B)**, as indicated, electrotransferred to nitrocellulose sheets, and probed with anti-2-Cys PRX antibodies. mon indicates the monomeric; dim, the dimeic form of the enzyme. Molecular mass markers (kD) are indicated on the left.

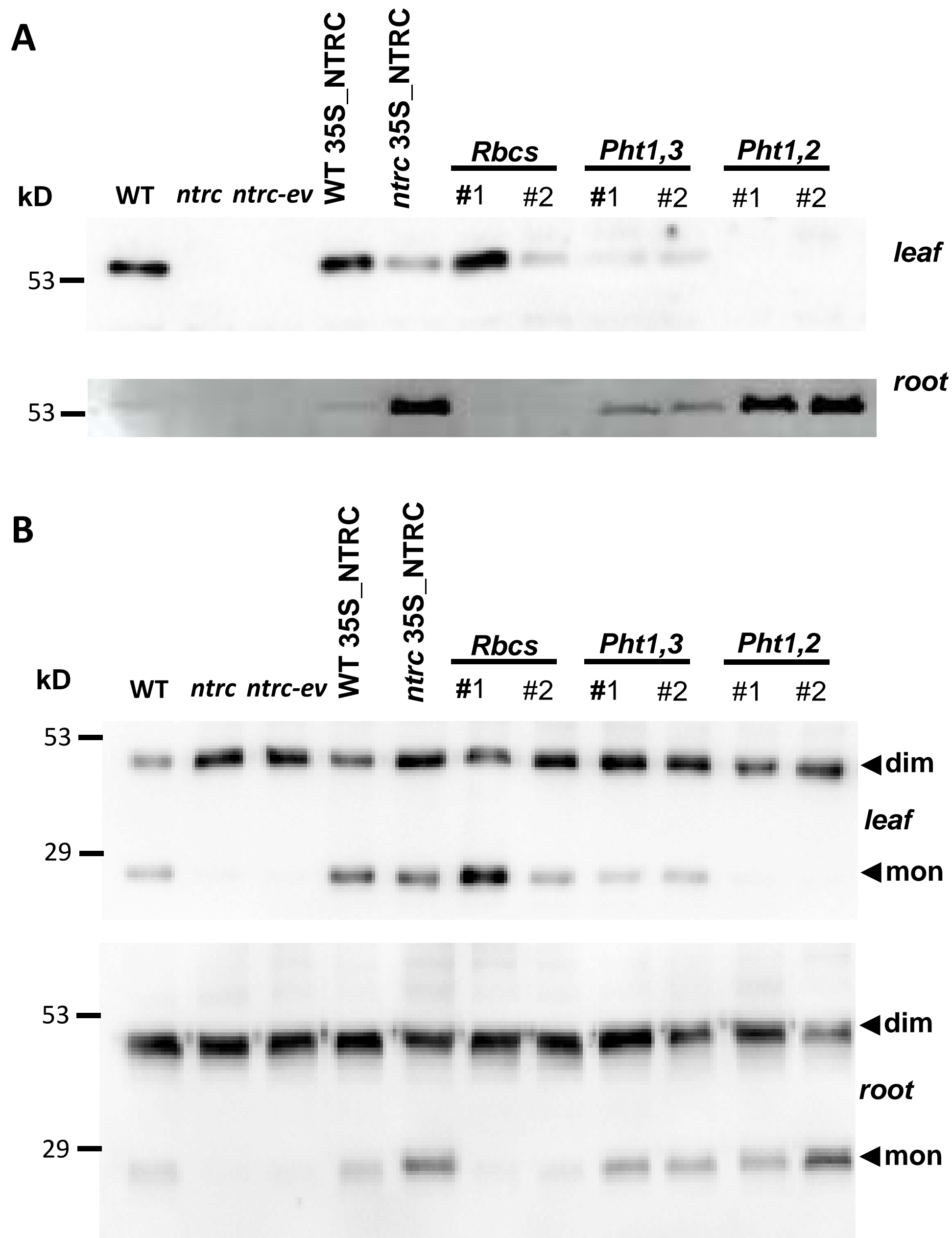

Figure 5

Figure 5. Effect of the presence of NTRC in leaves and roots on the redox status of 2-Cys PRX.

(A) Western blot analysis of the content of NTRC in leaf (30 µg of protein) and root (50 µg of protein) extracts from wild type, *ntrc* mutant, *ntrc* mutant transformed with the empty vector (*ntrc-ev*) and transgenic lines expressing NTRC under the CaMV 35S, *Rbcs*, *Pht1,3* and *Pht1,2* promoters, as indicated, in the *ntrc* mutant background.

Proteins were subjected to SDS-PAGE under reducing conditions, electrotransferred to nitrocellulose sheets, and probed with anti-NTRC antibodies. **(B)** Aliquots (15 μg of protein) of the same protein samples indicated above were subjected to SDS-PAGE under non-reducing conditions, eletrotransferred to nitrocellulose sheets, and probed with anti-2-Cys PRX antibody. mon, indicates monomeric; dim, dimeric form of the enzyme. Molecular mass markers (kD) are indicated on the left.

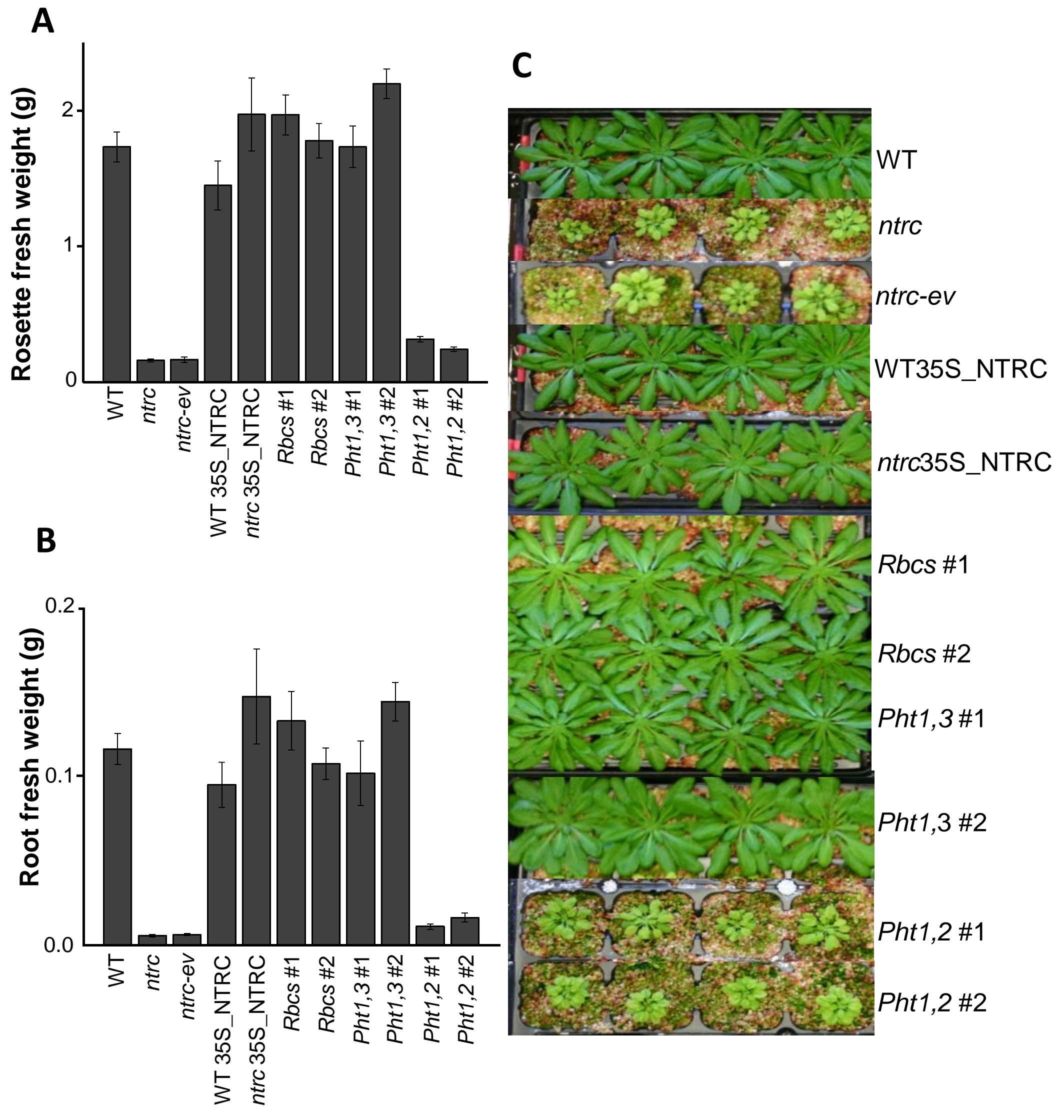

Figure 6

Figure 6. Effect of NTRC expression in photosynthetic and non-photosynthetic tissues on plant growth.

Wild type, *ntrc* mutant and the different transgenic lines, as indicated, were grown under short-day conditions for 53 days and rosette leaves **(A)** or roots **(B)** from seven plants were dissected and weighed. Mean values ± standard errors are shown. The experiment was repeated at least three times with similar results and a representative one is shown. **(C)** Photographs of representative plants of each of the lines under analysis.

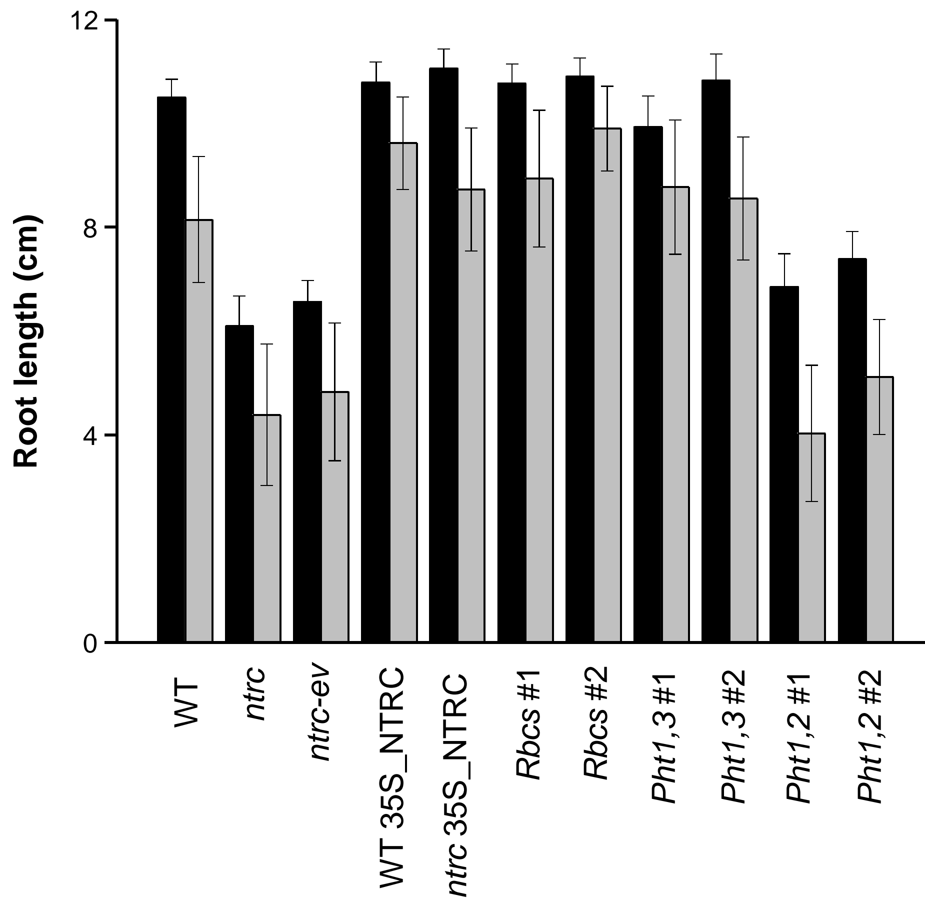

Figure 7

Figure 7. Effect of NTRC expression in photosynthetic and non-photosynthetic tissues on root growth.

Root length for each of the Arabidopsis lines, as indicated, was determined as follows. Seven-day-old seedlings grown in absence of any added sugar were transferred onto fresh MS plates supplemented with 30 mM, final concentration, of sucrose (black bars) or mannitol (grey bars) for 14 additional days under short day conditions on vertical-oriented plates. Assays were repeated three times with at least 21 plants per treatment. Mean values ± standard errors are shown.

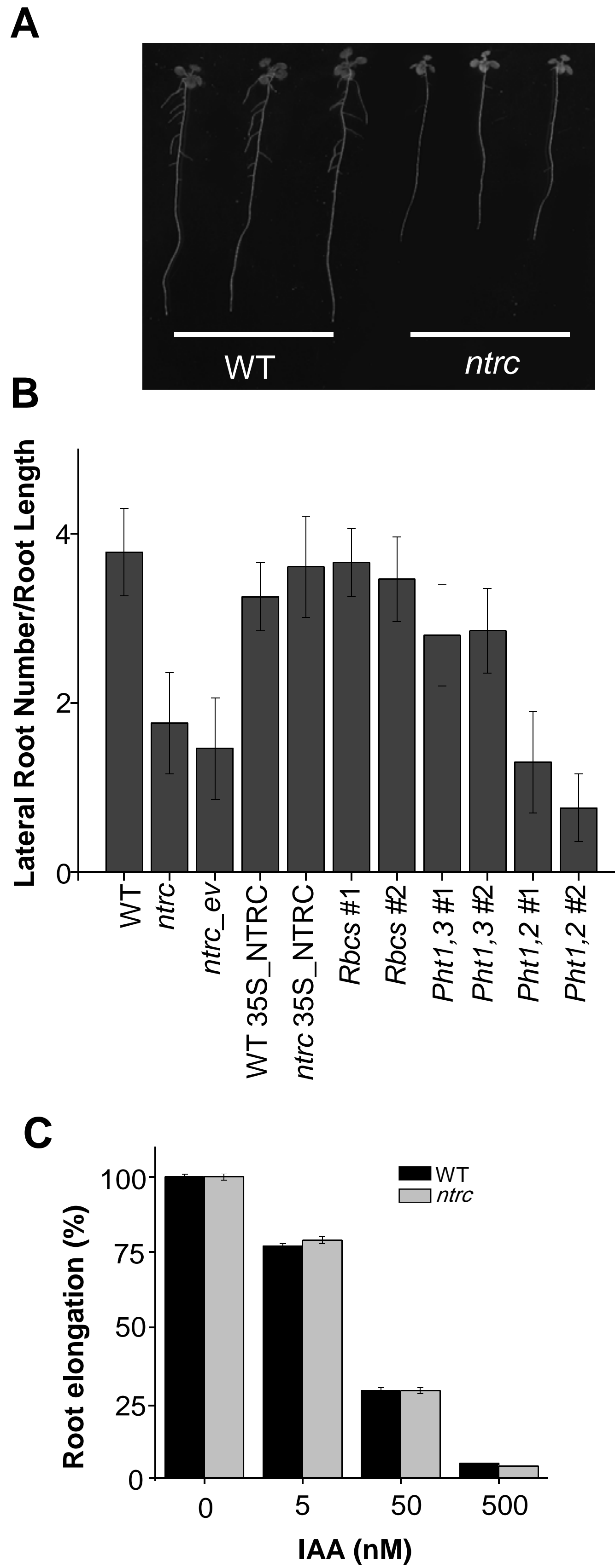

Figure 8

Figure 8. NTRC is involved in lateral root formation in Arabidopsis seedlings.

(A) Images of eleven-day-old WT and *ntrc* mutant seedlings, as indicated, grown under long day conditions. **(B)** Quantification of the number of lateral roots related to root length of seedlings of the different Arabidopsis lines, as indicated, grown for 11 days under long day conditions**. (C)** Five-day-old WT and *ntrc* mutant seedlings grown on MS media were transferred to media supplemented with increasing concentrations of IAA for an additional period of 3 days. Root length was expressed as a percentage of root elongation of untreated seedlings, which was considered 100%. Assays were repeated three times with at least 21 plants per treatment. Mean values ± standard errors are indicated.

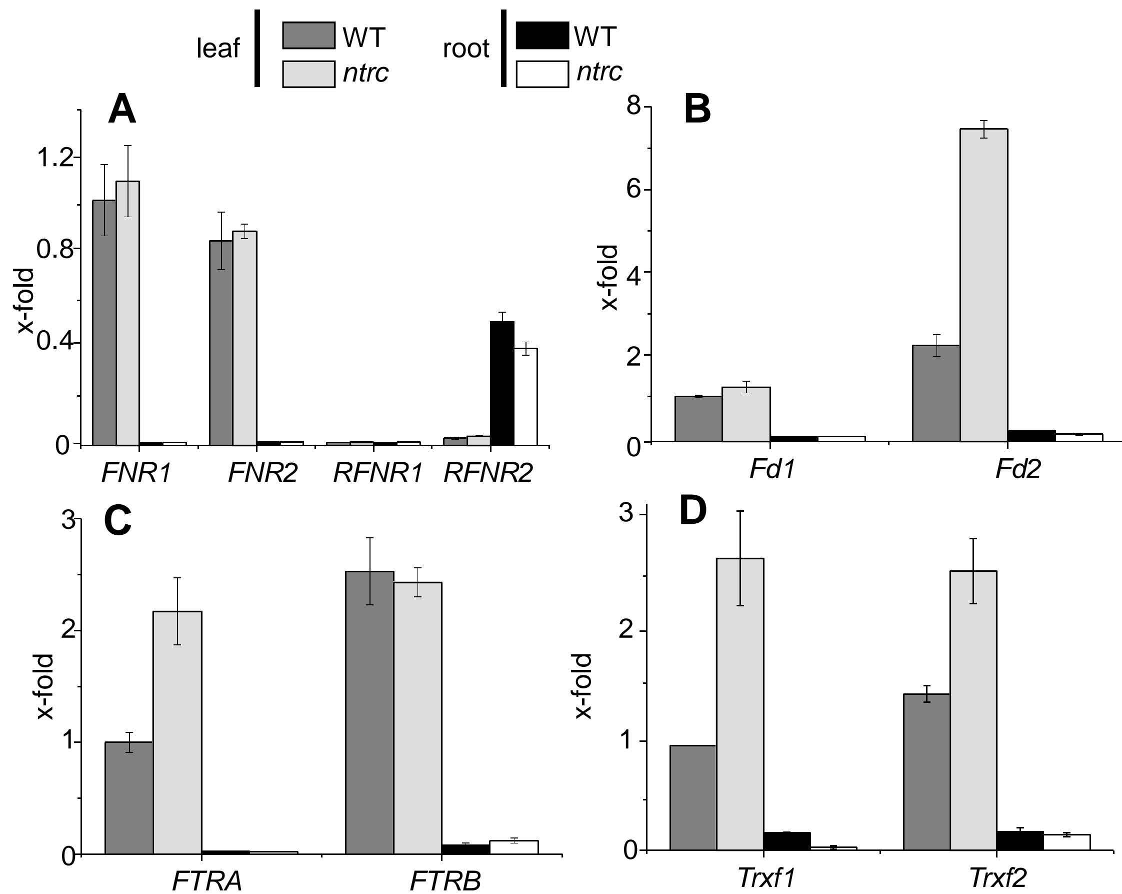

Figure 9

Figure 9. Expression of FNR, Fd, FTR and type-*f* TRX in leaves and roots of Arabidopsis wild type and *ntrc* mutant plants.

qPCR analysis of transcripts of genes encoding FNR **(A)**, Fd **(B)**, FTR **(C)** and TRX *f* **(D)** in leaves and roots of Arabidopsis wild type and *ntrc* mutant plants, which were grown during 14 days under short day conditions on plates containing MS medium supplemented with sucrose. The amount of transcripts was represented as arbitrary units relative to the level of one of the genes for each family in leaves, which was set to 1.0. Analysis was performed three times on two independent biological samples and the mean values ± standard errors are indicated.

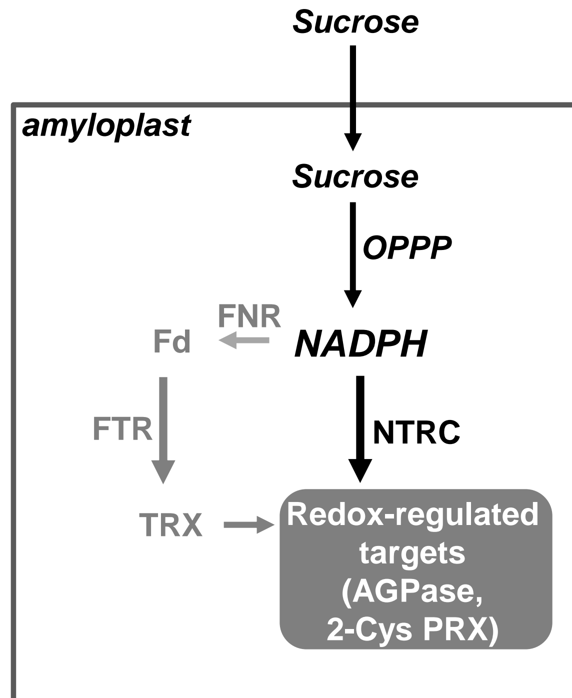

Figure 10

Figure 10. Schematic representation of alternative pathways for redox regulation in root amyloplasts.

In the absence of photochemical reactions, redox regulation in root amyloplasts depends on NADPH produced from sucrose by the oxidative pentose phosphate pathway (OPPP). Whilst NTRC is able to directly use NADPH for redox regulation, the alternative pathway requires the reduction of Fd catalyzed by FNR and the transfer of reducing power to amyloplast TRXs catalyzed by FTR. Evidence has been reported showing NTRC-dependent redox regulation in amyloplasts of AGPase (Michlaska et al., 2009) and 2-Cys PRX (this work).