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ARTICLE ADDENDUM

Photosynthetic activity of cotyledons is critical during post-germinative growth and seedling establishment

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ABSTRACT

Thioredoxins (Trxs) play a relevant role in thiol-dependent redox regulation, which allows the rapid adaptation of chloroplast metabolism to unpredictable environmental conditions. In chloroplasts, Trxs use reducing equivalents provided by photoreduced ferredoxin (Fdx) via the action of a ferredoxin-thioredoxin reductase (FTR), thus linking redox regulation to light. In addition, these organelles contain an NADPH-thioredoxin reductase, NTRC, with a Trx domain at the C-terminus. NTRC efficiently reduces 2-Cys peroxiredoxins (Prxs), hence having antioxidant function. However, NTRC also participates in the redox regulation of processes, such as starch and chlorophyll biosynthesis, which are known to be regulated by Trxs. Thus, the question arising is whether there is a cross-talk between the 2 redox systems. Arabidopsis mutants simultaneously devoid of NTRC and Trx *x* or Trxs *f* show a dramatic growth inhibition phenotype, indicating that NTRC is required for the function of these unrelated Trxs. Remarkably, both the *ntrc-trxx* double mutant and, to a higher extent, the *ntrc-trxf1f2* triple mutant show high mortality at the seedling stage, which is rescued by sucrose. These findings show the relevant role of redox regulation for chloroplast performance and uncover the key function of cotyledons chloroplasts at the transition to autotrophic metabolism during seedling establishment.

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Chloroplasts, the organelles in which light energy is converted in organic material, are the source of metabolic precursors for plant growth. Therefore, the deep influence of light on chloroplast performance and, hence, on plant development is not surprising. Central to the metabolic plasticity of chloroplasts is thiol-dependent redox regulation of enzyme activity, a regulatory mechanism in which the protein disulphide reductase activity of thioredoxins (Trxs) plays an important role.[1] While in heterotrophic organisms Trxs are reduced by NADPH with the participation of an NADPH-thioredoxin reductase (NTR), the complex set of chloroplast Trxs are reduced by photosynthetically reduced ferredoxin (Fdx) with the participation of ferredoxin-thioredoxin reductase (FTR).[2] Therefore, by using photoreduced Fdx as source of reducing power the FTR-Trxs system links chloroplast redox regulation to light. Virtually any process taking place in this organelle is redox sensitive, the different types of Trxs showing specific functions. While Trxs *f* are considered to participate in redox regulation of metabolic pathways, such as the Calvin-Benson cycle enzymes, Trx *x* has been proposed to have antioxidant function.[3]

This classical view of chloroplast redox regulation was modified by the discovery of an NTR with a joint Trx domain at the C-terminus, termed NTRC,[4] which is exclusively found in organisms that perform oxygenic photosynthesis.[5] Based on the finding that NTRC is a very efficient reductant of the thiol-dependent peroxidase 2-Cys peroxiredoxin (2-Cys Prxs), it was proposed the participation of NTRC in the antioxidant defense mechanism of the chloroplast.[6] However, Arabidopsis mutants devoid of NTRC show growth retard phenotype[6,7] and low efficiency of light energy utilization,[8,9] suggesting additional functions for this enzyme. In line with this notion, it was shown that NTRC participates in redox regulation of previously identified Trx-regulated pathways such as starch[10,11] and chlorophyll biosynthesis,[12,13] hence raising the question of the relationship of NTRC and the Fdx/FTR/Trxs redox system in chloroplast redox regulation.

This issue is currently being addressed by genetic approaches. In this regard, it is worth mentioning that Arabidopsis single mutants lacking specific isoforms of chloroplast Trxs such as those of the types *x*,[14] *f*,[15-17] or *m*[18,19] show almost wild type phenotype, which is in contrast with the important function assigned to these Trxs based on biochemical analyses.[20] Interestingly, mutants combining the deficiencies of NTRC and chloroplast Trxs, such as the *ntrc-trxf1* mutant, show a very severe phenotype,[21] suggesting that both systems have overlapping functions in chloroplast redox regulation. In support of this notion, Arabidopsis mutants devoid of NTRC and FTR are not viable.[22] Our finding that Arabidopsis mutants lacking NTRC and Trx *x*, and, to a higher extent, mutants lacking NTRC and Trxs *f*1 and *f*2 show a very severe growth inhibition phenotype,[23] indicate that the deficiency of NTRC impairs

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the activity of chloroplast Trxs. While these findings further support the notion that the 2 chloroplast redox systems act concertedly, the molecular basis of this interaction remains unknown. One possibility is that NTRC and the different Trxs modulate the activity of common targets. If this is the case, the deficiency of one of the systems could be counteracted by the other while the simultaneous deficiency of both systems would cause the severe impairment of redox regulation of these targets. However, the light-dependent reduction of fructose 1,6-bisphosphatase (FBPase), a well-established redox regulated enzyme of the Calvin-Benson cycle, was more affected in the *ntrc* than in the *trxx* and *trxf1f2* mutants despite the fact that the enzyme was reduced by Trxs *f*, and less efficiently by Trx *x*, but not by NTRC *in vitro*.[23] These results suggest that NTRC may exert an indirect effect on the redox regulation of FBPase. Since NTRC is the most efficient reductant of 2-Cys Prxs *in vivo*,[14] an additional possibility to be taken into account is that the lack of NTRC affects hydrogen peroxide homeostasis provoking oxidative stress, which would affect Trx activity. However, the biochemical basis of the functional relationship of NTRC, 2-Cys Prxs and the Trxs remains to be elucidated.

The *ntrc-trxx* and the *ntrc-trxf1f2* mutants show a severe growth inhibition phenotype; moreover, a remarkable feature of the phenotype of these mutants was the low number of individuals that reached the adult stage when grown on soil, the mortality of the *ntrc-trxx* and *ntrc-trxf1f2* seedlings being of approx. 50% and 95%, respectively.[23] These results not only show the profound relationship of NTRC and functionally unrelated Trxs *x* and *f*, they also highlight the relevance of redox regulation on chloroplast performance at early stages of development. Seedling establishment, defined as the formation of the first true leaves, was delayed in *ntrc-trxx* and *ntrc-trxf1f2* surviving seedlings. In line with these results, seedlings of these mutant lines germinated in synthetic media without exogenous carbon source displayed impaired root growth. This phenotypic effect was partially rescued by sucrose, indicating that the photosynthetic activity of cotyledon chloroplasts is critical to reach autotrophic growth and for the development of new tissues such as roots and true leaves.

Therefore, the impairment of the chloroplast redox network in the *ntrc-trxx* and, more severely, in *ntrc-trxf1f2* mutants causes growth retard but not lethality at the adult phase of development. In contrast, this impairment of the redox network is critical for post-germinative seedling establishment. The analysis of soil-germinated seedlings of the *ntrc-trxf1f2* triple mutant (Fig. 1A) shows short hypocotyls and expanded cotyledons, indicating that the developmental program of photomorphogenesis is not affected by the deficient chloroplast redox regulation in these mutants. However, these seedlings are unable to generate the new organs and undergo progressive cotyledon bleaching (Fig. 1A), suggesting that the photosynthetic performance of the cotyledon chloroplasts is not sufficient to provide the sucrose required for further development of the new tissues.[24] In contrast with the wild type (Fig. 1B), chloroplasts of the *ntrc-trxf1f2* seedlings show symptoms of thylakoid dismantling and the presence of clear regions in the stroma indicating active degradation of chloroplast structures (Fig. 1C-E). Moreover, chloroplasts in advanced degree of degeneration with the appearance of gerontoplasts[25] were also detected at this stage of development (Fig. 1E). Chloroplast

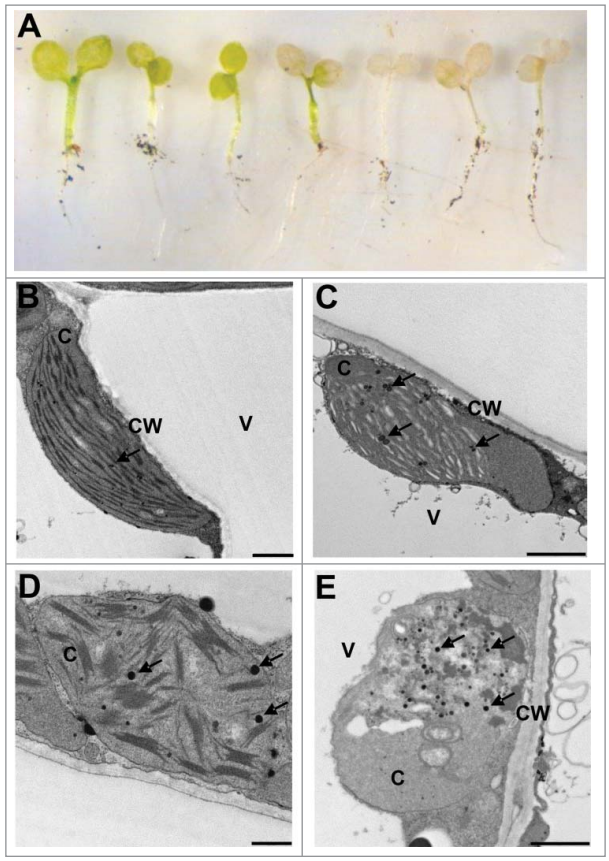

Figure 1. Cotyledon chloroplast structure of wild type and *ntrc-trxf1f2* bleaching seedlings. (A) Seeds of *ntrc-trxf1f2* triple mutant were allowed to germinate on soil and seedlings at different stages of bleaching are shown. (B-E) Electron transmission microscopy analysis of chloroplast structure from wild-type plants (B) and the mutant line seedlings (C-E). Plants were germinated on soil for 10 days, and seedlings were collected just before the appearance of the first true leaves and fixed in glutaraldehyde. Transmission electron microscopy analysis was performed as previously reported.[23] Bars represent 1 μm (B, C, E) and 0.5 μm (D). c, chloroplasts; cw, cell wall; v, vacuoles. Arrows indicate plastogobules.

degeneration is characterized by the increase in the number and size of plastoglobules and an increase of plastoglobule attachment (Fig. 1C-E), which is indicative of oxidative stress.[26] This feature suggests that once autotrophic growth is arrested by the deficiency of the redox system of cotyledon chloroplasts, these cells suffer increasing oxidative stress and normal development of the new organs, leaves and roots, is inhibited. The dramatic effect of the lack of the redox systems here analyzed at the seedling stage uncovers the relevance of redox regulation for chloroplast performance, which is critical for the transition to autotrophic growth at the seedling stage.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Abbreviations

Fdx	ferredoxin
FTR	ferredoxin-dependent thioredoxin reductase
NTRC	NADPH-dependent thioredoxin reductase C
Prx	peroxiredoxin
Trx	thioredoxin

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