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COMMUNICATION

Redox-control of chlorophyll biosynthesis mainly depends on thioredoxins

Andreas S. Richter[1], Juan M. Pérez-Ruiz[2], Francisco Javier Cejudo[2] and Bernhard Grimm[1]

1 Plant Physiology, Institute of Biology, Humboldt-Universität zu Berlin, Germany
2 Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas, Universidad de Sevilla, Spain

Correspondence
B. Grimm, Humboldt Universität zu Berlin, Lebenswissenschaftliche Fakultät, Institut für Biologie, AG Pflanzenphysiologie, Philippstrasse 13, Haus 12, 10115 Berlin, Germany
Fax: +49 30 2093 6337
Tel: +49 30 2093 6106
E-mail: bernhard.grimm@hu-berlin.de

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In order to maintain enzyme stability and activity, chloroplasts use two systems of thiol-disulfide reductases for the control of redox-dependent properties of proteins. Previous studies have revealed that plastid-localized thioredoxins (TRX) and the NADPH-dependent thioredoxin reductase C (NTRC) are important for the reduction of cysteine residues of enzymes involved in chlorophyll synthesis. Very recently, it was shown that the pale green phenotype of the *ntrc* mutant is suppressed when the contents of 2-cysteine peroxiredoxins (2CP) A and B are decreased. Here, we show that suppression of the *ntrc* phenotype results from a recovery of wild-type-like redox control of chlorophyll biosynthesis enzymes in *ntrc*/*2cp* mutants. The presented results support the conclusion that TRXs rather than NTRC are the predominant reductases mediating the redox-regulation of these enzymes.

Keywords: NADPH-dependent thioredoxin reductase C; redox-regulation; tetrapyrrole biosynthesis

Redox regulation is an essential post-translational mechanism to sustain cellular homeostasis in each organism, from bacteria and fungi to plants and animals. In plants, all sub-cellular compartments and organelles are equipped with a multifaceted repertoire of enzymes controlling the redox status of proteins. In general, thiol groups of cysteine residues can switch between the oxidized (-S-S-) and reduced (-SH) state. The reduction of intra- or intermolecular disulfide bonds is catalyzed by target specific redox-regulators. Chloroplasts comprise at least two systems of thiol-disulfide reductases for the control of redox-dependent protein stability and/or activity. First, the family of thioredoxins (TRX), which encompasses several members of the *f*, *m*, *x*, *y* and *z*-type, reduces target proteins. Oxidized plastidic TRXs are reduced by the ferredoxin-dependent thioredoxin reductase (FTR), using the electron donor ferredoxin, which is reduced through the photosynthetic electron transfer chain. Thus, the activity of TRXs is linked to the availability of light. In addition, plastids contain an NADPH-dependent thioredoxin reductase C (NTRC) [1], an NTR with a joint TRX domain, which shows high affinity for NADPH and, thus, is able to catalyze thiol-disulfide exchange reactions with target enzymes even in the absence of photosynthetic electron transfer, that is, darkness.

After the first description of functional NTRC, one of the intriguing questions was to which extent TRXs and NTRC functionally complement each other in the reduction of specific targets. NTRC was initially described to reduce 2-cysteine peroxiredoxins (2CP), thiol-dependent peroxidases of which the *Arabidopsis thaliana* chloroplasts contain two almost identical isoforms, A and B [2,3]. But NTRC was also shown to

Abbreviations
2CP, 2-cysteine peroxiredoxin; Chl, chlorophyll; CHLM, Mg-protoporphyrin IX methyltransferase; GLUTR, glutamyl-tRNA-reductase; TBS, tetrapyrrole biosynthesis.

be important for the acclimation of photosynthesis to varying growth light [4–6] and to function in redox-regulation of enzymes involved in starch [7,8] and tetrapyrrole biosynthesis (TBS) [9,10]. The *NTRC* knockout mutant (*ntrc*) shows a pale green phenotype, which was attributed to altered activities and stability of glutamyl tRNA-reductase (GLUTR) and Mg-protoporphyrin IX methyltransferase (CHLM), two important enzymes for the synthesis of heme and chlorophyll (Chl) [9]. However, the redox status of Chl synthesis enzymes, such as CHLM, was cumulatively affected in plants with deficiency of NTRC and three TRX *m* isoforms [11].

Recently, Pérez-Ruiz *et al.* [12] showed the suppression of Chl deficiency of *ntrc* in the background of *2CPA* and *B* deficient plants and suggested that the altered redox-balance in *ntrc* is the consequence of a re-distribution of electrons from TRXs to oxidized 2CPs, which accumulated in NTRC-deficient plants, so that targets of TRXs were not sufficiently reduced. The deficiency of 2CPs alleviates this re-distribution of electrons and results in a normal supply of reducing power from TRX to their targets. This report stimulated a debate about the division of labor between both types of reductases, NTRC and the multiple forms of TRX, as previous studies suggested at least a partial overlap in the targets of TRXs and NTRC. Given that different enzymes involved in TBS were shown to be targets for redox-regulation, we aimed to examine the observed compensatory impact of missing 2CP and NTRC on redox-dependent enzymes of TBS. Based on the model presented in [12], we intended to provide an explanation for a molecular mechanism that enables the maintenance of the Chl content in mutants deficient in NTRC and 2CPs in comparison to the single *ntrc* mutant.

Material and methods

Plant material

The genotypes used in this study were described in [12]. For the analysis of Chl, protein and RNA content as well as CHLM-activity assays plants were grown for 14 days on soil in short-day conditions at 100 µmol photons·m^{-2}·s^{-1}. Plants were harvested as pool of 10–15 seedlings per sample.

Molecular techniques

All analyses were essentially performed as described in [9]. qPCR analysis was performed using the primers listed in supplemental Table S1.

Results

The following mutants were applied to examine the physiological connection between NTRC and 2CP: the null mutant *ntrc*, a double mutant *ntrc*/*2cpa* (with a knock-down of *2CPA*), the double knockout mutant *ntrc*/*2cpb* and the triple mutant *ntrc*/*Δ2cp* (double null mutant NTRC and 2CPB and knock-down 2CPA) (Fig. 1A,B). In comparison to *ntrc*/*2cpb* and *ntrc*/*2cpa, ntrc*/*Δ2cp* contained more severely decreased 2CP content (Fig. 1B) [12]. The pale green pigmentation and growth retardation of the *NTRC* knockout mutant (Fig. 1A) was partially rescued upon knock-down of *2CPA* (*ntrc*/*2cpa*) and completely complemented in the double mutant *ntrc*/*2cpb* and the triple mutant *ntrc*/*Δ2cp* (Fig. 1A). In agreement, Chl contents of *ntrc* and *ntrc*/*2cpa* were significantly reduced compared to wild type (WT) plants, while they recovered to WT-like levels in *ntrc* lines lacking *2CPB* and *ntrc*/*Δ2cp* (Fig. 1C). The increasing Chl content correlated with weaker growth retardation of the different analyzed double mutants compared to *ntrc*.

As NTRC interacts with GLUTR and CHLM, respectively, and redox regulation of CHLM was shown to be crucial for its activity [13] and stability [9,11], we analyzed the amount of TBS enzymes in the mutants (Fig. 2). As confirmed by western blotting, all genotypes in the *ntrc* background lack NTRC (Fig. 1B). The content of glutamate-1-semialdehyde aminotransferase (GSAT) and CHL27, a subunit of the aerobic Mg protoporphyrin IX monomethylester (oxidative) cyclase, were not altered in the mutant lines. In contrast, the GLUTR and CHLM contents were reduced in *ntrc* and *ntrc*/*2cpa* compared to WT plants, while *ntrc*/*2cpb* and *ntrc*/*Δ2cp* accumulated more of these enzymes than *ntrc* (Fig. 2A). As revealed by AMS labeling, we found a WT-like redox-state of CHLM in the *ntrc*/*2cpb* and *ntrc*/*Δ2cp* mutants which is in agreement with the increased stability of the protein compared to *ntrc* or *ntrc*/*2cpa* (Fig. 2A).

Given that the redox status of CHLM was different in the mutants, we next intended to analyze the *in vivo* CHLM activity (Fig. 2B). The CHLM activity was lower in *ntrc* and *ntrc*/*2cpa* than in WT most likely due to the decreased CHLM content (Fig. 2B). In Agreement with this observation the WT-like CHLM activity in *ntrc*/*2cpb* and *ntrc*/*Δ2cp* correlated with the WT-like accumulation of CHLM (Fig. 2A). This finding confirms that redox regulation affects CHLM stability rather than its activity *in vivo*. The different degree in the recovery of a WT-like pigmentation and metabolic activity in Chl synthesis of *ntrc*/*2cpa* and *ntrc*/*2cpb* is worth to mention and remains to be analyzed in future (Fig. 1B).

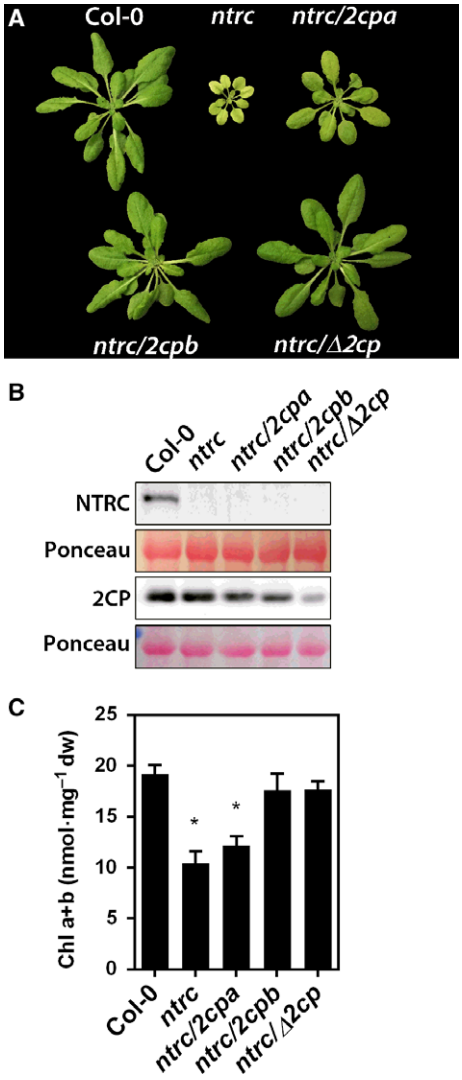

Fig. 1. (A) Phenotype of Arabidopsis wild-type and mutant lines grown for 6 weeks in short day conditions. (B) The contents of NTRC and 2CP in wild-type and mutant lines were determined by western blot probed with anti-NTRC and anti-2CP antibodies, as indicated. Even loading and transfer was checked by Ponceau staining. (C) Chlorophyll (Chl) content of genotypes shown in (A). Data given as mean ± SD of at least three biological replicates. *Significantly different to Col-0 with $P < 0.05$ (Student's *t*-test).

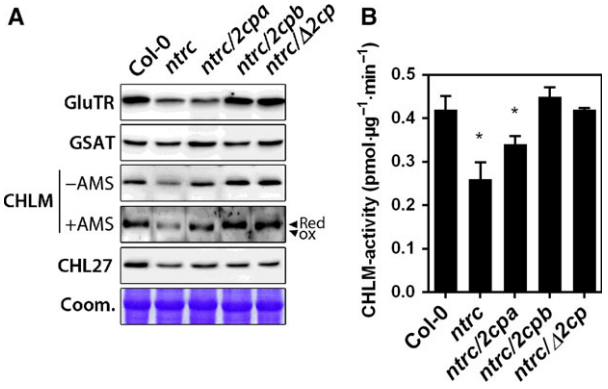

Fig. 2. (A) Protein content of selected TBS pathway enzymes in wild-type and mutant lines. Redox status of CHLM was visualized using AMS labeling of proteins extracted in the absence of reducing agents. (B) *in vivo* CHLM activity. Data given as mean ± SD of at least three biological replicates. *Significantly different to Col-0 with $P < 0.05$ (Student's *t*-test). n.s., nonspecific; red, reduced; ox, oxidized.

Although rather unexpected, the simultaneous knock-out/-down of *NTRC* and *2CPA/B* led to a full complementation of impaired Chl synthesis and the complete suppression of the pale green phenotype of *ntrc* in *ntrc/2cpb* and *ntrc/Δ2cp* (Fig. 1A). As the transcripts of *HEMA1* and *HEMA2* (for GLUTR) and *CHLM* accumulated to WT levels, the higher amounts of both enzymes in *ntrc/2cpb* and *ntrc/Δ2cp* was not a result of upregulated transcription (Fig. 3A). Hence, the different stability of GLUTR and CHLM could be only explained by a different degree of modified redox-status in *ntrc* and *ntrc/Δ2cp* mutants. In this line, we speculated that transcriptional upregulation of TRX genes might serve as a compensatory mechanism for the redox imbalance due to NTRC and 2CPA/B deficiency. In contrast to genes encoding the *f*- and *m*-type TRX variants, the genes encoding the *x*-, *y*- and *z*-type TRXs were moderately upregulated in *ntrc* and *ntrc/2cpa* (Fig. 3B). This upregulation is interpreted to be likely due to a secondary compensatory response as result of missing NTRC (Fig. 1). In summary, the *TRXs* were either expressed at WT level or were only slightly affected in the mutants with a combined lack of NTRC and one or both 2CPs (Fig. 3B). Based on the expression analysis, it is unlikely that the post-translational stability of GLUTR and CHLM as well as the suppression of the consequences of NTRC deficiency are explained by increased amounts of TRXs transcripts in *ntrc/2cpb* and *ntrc/Δ2cp*.

Discussion

Our results support the model proposed by Pérez-Ruiz *et al.* [12], and expanded the view on the regulatory action of the NTRC-2CP system to the stability and function of TBS enzymes. According to this model, oxidized 2CPs, which accumulate in plants devoid of NTRC, act as a relevant sink for electrons from TRXs. Thus, TRXs compensate the lack of NTRC for 2CP reduction on the cost of reducing equivalents needed for the reduction of other TRXs targets [12]. When the amount of 2CP decreases the compelled

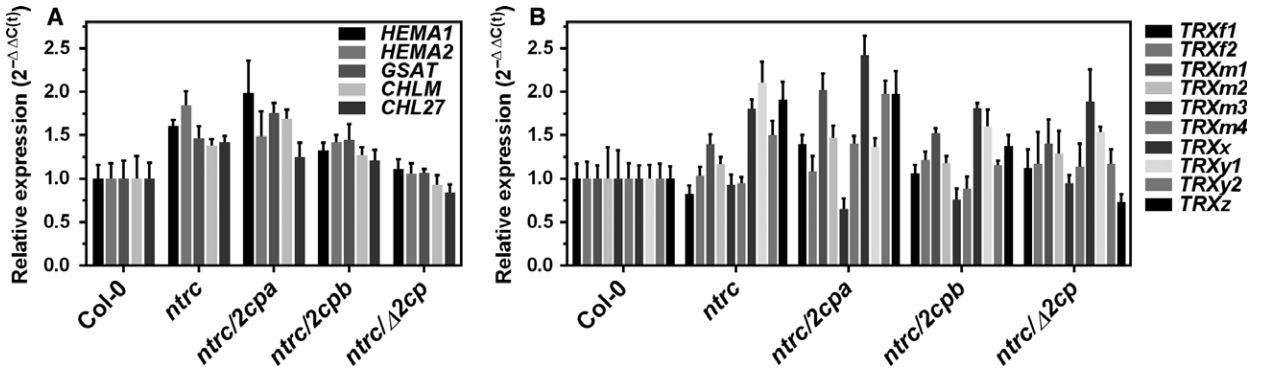

Fig. 3. Expression analysis of gene products involved in TBS pathway (A) and chloroplast localized thioredoxin isoforms (B). Plants were grown for 14 days on soil in short day conditions; qRT-PCR results are given as mean ± SD. of at least three biological replicates calculated as relative expression to *ACTIN* and Col-0 ($2^{-\Delta\Delta C(t)}$).

consumption of reducing capacity from TRXs decreases in the *ntrc/Δ2cp* mutant, TRXs remain available for redox-regulation of TBS and other metabolic pathways. Despite the published evidence for the interaction and impact of NTRC on the redox status of GLUTR, CHLM, and other TBS enzymes [9,10,13], we do not exclude to reconsider the function of NTRC on plastid-localized proteins. The new data correspond with a main TRX function for redox control of plastid-localized enzymes in Chl synthesis rather than NTRC. This conclusion is supported by the interaction of CHLM with *m*-type TRX [11] and the analysis of *TRXf1,2* mutant as well as *TRXm (1,2,4)* triple-silencing plants, which do show *ntrc*-like alteration of the redox state and de-stabilization of CHLM [11,14]. Additionally, a recovery of Chl content was also observed in older leaves of the *ntrc* mutant [4]. Although the demand for Chl is reduced compared to young developing tissues, the increased Chl content in older *ntrc* leaves could be also explained by an increased activity of the FTR system and a prevailing impact of TRX on the redox-regulation of TBS enzymes at a later stage of development. Increased accumulation of Chl was also achieved by overexpression of an NTRC protein with dysfunctional TRX domain ($NTRC_{SGPS}$) in the *ntrc* background [15]. As it was shown, the remaining functional reductase domain of $NTRC_{SGPS}$ was able to reduce TRX*f*. With the increased supply of reduced TRXs, more reducing power is available for redox-regulation of TBS enzymes by TRXs and the partial complementation of the Chl deficiency in *ntrc*/$NTRC_{SGPS}$ compared to *ntrc* alone could be explained. However, given that CHLM was found to be entirely in the reduced state throughout the day/night cycle in Arabidopsis WT plants [9], we do not exclude NTRC activity on CHLM in darkness. Then, a functional overlap would still exist between both disulfide reductases. In addition, the redox-dependent de-stabilization of GLUTR observed in *ntrc* is not visible in TRX *f*- and TRX *m*-deficient plants [11,14] suggesting that either NTRC or one of the other TRXs are more important to maintain GLUTR's redox-state.

Derived from the analysis of the *ntrc/Δ2cp* triple mutant, future conclusions should be drawn with caution. Despite the fact that a simultaneous defect of NTRC and 2CPs probably never occurs in WT plants, the presented results uncover interesting redistribution routes of electrons inside plastids *in vivo*. In summary, the presented results strongly support the potential of TRXs to mediate redox-regulation of TBS-pathway enzymes.

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Author contributions

ASR performed the research. ASR and BG wrote the manuscript. JMPR and JFC provided the plant material.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. qPCR primer used in the study.