Overexpression of plastidial thioredoxins f and m differentially alters photosynthetic activity and response to oxidative stress in tobacco plants

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INTRODUCTION

Thioredoxins (Trxs) are ubiquitous and evolutionarily conserved enzymes of ca. 12 kDa catalyzing the reduction of disulfide bonds through a redox-active dithiol CxxC motif (Arnér and Holmgren, 2000). Trxs, discovered 50 years ago in bacteria, cover functions as redox carriers in numerous physiological processes such as DNA synthesis, sulfur assimilation or regulation of transcription factors (Arnér and Holmgren, 2000). In plants, two plastidial Trx types, named Trx f and Trx m, were primarily identified as light-dependent regulators of enzymes related to photosynthetic processes and carbon metabolism (Jacquot et al., 1978; Wolskiuk et al., 2007). Their denomination was based on in vitro ability to activate by reduction fructose-1,6-bisphosphatase (FBPase) and NADP-dependent malate dehydrogenase (NADP-MDH), respectively. These Trxs are reduced by the ferredoxin/thioredoxin system (Wolskiuk and Buchanan, 1977). A third plant Trx type located in cytosol was identified later (Wolskiuk et al., 1979). This type named Trx h is reduced by cytosolic NADPH thioredoxin reductase (Jacquot et al., 1994) and participates in various processes such as mobilization of seed reserves (Kobrehel et al., 1992) and responses to oxidative stress (Verdoucq et al., 1999).

Whereas most organisms possess a low number (two or three) of Trxs achieving multiple functions, plants display a remarkable diversity of these reductases. A survey of genomic and EST sequences from Arabidopsis and other species revealed the presence of almost 50 genes encoding Trx or Trx-like proteins in higher plants (Meyer et al., 2005). On the basis of gene and peptide sequences, other Trx types, o, x, y, and z, have been defined and in Arabidopsis, Trx f, h, m and y include 2, 9, 4, and 2 isoforms, respectively (Meyer et al., 2005; Lemaire et al., 2007). This outstanding diversity raised the question of functional specialization or redundancy. Genetic studies revealed...
that several plant Trxs possess specific and unique physiological functions in development, metabolism and stress responses. For instance, Arabidopsis mutants deficient in Trx b9 display impaired root and leaf development. This cytosolic Trx is associated with plasma membrane and presumed to participate in cell-to-cell communication processes (Meng et al., 2010). In Arabidopsis plants lacking plastidial Trx x, chloroplast biogenesis is inhibited revealing a critical role of this Trx (Aronova et al., 2016). Trx z has been proposed to regulate transcription via a redox control of plastid-encoded plastid RNA polymerase. In other respects, Trx b5 is required for the response to vitamin, a fungal toxin inducing programmed cell death in sensitive plants (Sewat and Wolpert, 2007).

Other Trxs participate in plant responses to the oxidative stress conditions resulting from environmental constraints (Vieira Dos Santos and Rey, 2006), mainly due to their ability to provide reducing power to peroxiredoxins (Prxs) and methionine sulfoxide reductases (MSRs), enzymes reducing organic peroxides and repairing oxidized proteins, respectively. Thus, CDSP32 (chloroplastic drought-induced stress protein of 32 KDa), a double module Trx initially isolated in potato plants subjected to water deficit (Rey et al., 1998) supplies Prxs and MSRs with electrons (Broun et al., 2002; Rey et al., 2005; Tarrago et al., 2010). Another plastidial Trx-like protein, NAPDH thioseredoxin reductase C (NTRC), uses NAPDH to reduce 2-Cys Prxs and has been proposed as a protection system against oxidative damage (Ferré-Piérola et al., 2006). Trx x and y are also presumed to participate in responses to oxidative stress based on their ability to reduce Prxs and MSRs in vitro (Collin et al., 2003, 2004; Navrot et al., 2006; Vieira Dos Santos et al., 2007), but evidence for such a function in plants is still scarce. Very recently, we showed that Trx y2 maintains growth under high light and long day in Arabidopsis, likely through electron supply to plastidial MSRs (Laugier et al., 2013). Note that the other Trx y isoform, y1, which is specifically expressed in non-photosynthetic organs (Collin et al., 2004) could also fulfill a protective function in seeds, in which MSRs likely play a key role in preserving longevity (Chabreil et al., 2011).

Although Trx f and m have been the first Trxs discovered in plants, the knowledge concerning their physiological functions is only emerging. Based on biochemical studies, Trxs f and m are presumed to regulate photosynthesis and carbon metabolism although Trx f seems more efficient than Trx m to redox regulate most enzymes involved in these processes. Trx f specifically activates glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and fructose-bisphosphate aldolase (FBPase), and controls the activity of other redox-sensitive enzymes like NADP-MDH and glucose-6-phosphate dehydrogenase (G6PDH; Collin et al., 2003; Lemaire et al., 2007; Mazié et al., 2009; Né et al., 2009). Similarly, Trx m reduces enzymes involved in carbon metabolism and catabolism such as NADP-MDH and G6PDH, but also regulates the activity of enzymes involved in antioxidant mechanisms like Prxs and MSRs (Collin et al., 2003; Vieira Dos Santos et al., 2007).

Among Trx m isoforms, Trx m3 displays highly distinct properties, since it cannot reduce known Trx targets (Collin et al., 2003; Vieira Dos Santos et al., 2007). This isoform, expressed in non-green plastids of meristems and organ primordia, could be involved in redox regulation of symplastic permeability (Benitez-Alfonso et al., 2009). Using RNA-interference, Chi et al. (2008) showed that rice plants knockdown for Trx m expression display abnormal chloroplast development and impaired growth.

In contrast, no obvious phenotype was observed in Arabidopsis plants lacking either Trx m1 or Trx m4 (Laugier et al., 2013). But, most interestingly, Trx m4-deficient mutant plants specifically display strongly increased cyclic photophosphorylation electron transfer around PSI (Courteille et al., 2013). Only very recent papers brought information regarding the physiological function of Trx f. In pea plants displaying a dramatically reduced Trx f transcript level due to silencing, no phenotype was noticed (Liao et al., 2012). Similarly, no change was found in growth and photosynthesis in Arabidopsis knockout lines for Trx f, but reduced light-activation of ADP-glucose pyrophosphorylase (AGPase) in leaves accompanied by a decrease in starch accumulation was observed in these mutants (Thomé et al., 2013). Consistently, we observed that transplastomic tobacco plants overexpressing Trx f show a strong increase in starch content (Sanz-Barrio et al., 2013).

The data gained in various species indicate that Trx f is involved in the regulation of starch metabolism, whereas the role of Trx m seems more complex. In this work, we compare the phenotypes of tobacco plants overexpressing either Trx f or Trx m with regards to growth, photostimulated metabolism, activation and content of Trx targets and response to oxidative stress. We show that overexpression of Trx m leads to delayed growth, reduced pigment content and impaired photosynthetic activity. Further, we found a differential behavior of plants overexpressing Trx f or Trx m exposed to oxidative stress conditions, revealing that Trx m very likely displays, compared to Trx f, a broader range of physiological functions.

### MATERIALS AND METHODS

#### PLANT MATERIAL AND STRESS TREATMENTS

Nicotiana tabacum L. plants, cv Petit Havana (Wt and transplastomic lines), were sown and grown on compost in phytocon 40-day-old plants. For whole plant treatments, young well-expanded leaves from 35- to 40-day old plants.

Photosynthetic and biochemical analyses were carried out on young well-expanded leaves from 35- to 40-day-old plants. Photosynthetic acti sa treatment was carried out by exposing 35-day-old tobacco plants grown under standard conditions to high light intensity (950μmol photons m⁻² s⁻¹) and a 25°C/19°C (day/night) temperature regime for standard conditions. Transplastomic plants overexpressing either Trx f or Trx m were generated and characterized as reported in Sanz-Barrio et al. (2013). Photosynthetic and biochemical analyses were carried out on young well-expanded leaves from 35- to 40-day-old plants.

Photosynthetic actin and content of Trx targets and response to oxidative stress. We show that overexpression of Trx m leads to delayed growth, reduced pigment content and impaired photosynthetic activity. Further, we found a differential behavior of plants overexpressing Trx f or Trx m exposed to oxidative stress conditions, revealing that Trx m very likely displays, compared to Trx f, a broader range of physiological functions.

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Ascorbate (AsA) was analyzed by HPLC as described by Havaux et al. (Sigma). The concentration of oxidized glutathione (GSSG) was calculated as \( (F_{m}' - F_w) - 1 \), where \( F_{m}' \) is the maximal fluorescence level in the dark.

**ASSAYS**

**Photosynthetic measurements**

Chlorophyll fluorescence parameters were measured using a PAM-2000 modulated fluorometer (Walz Effeltrich, Germany) as previously described (Havaux et al., 2000). A saturating pulse of white light was applied on leaf and measurements were recorded during actinic light illumination (from 25 to 2,500 μmol photons m⁻² s⁻¹). The PSII photochemical efficiency (\( \Phi_{PSII} \)) during actinic illumination was estimated by calculating \( \Delta F/F_{m}' \), where \( \Delta F \) is the steady-state chlorophyll fluorescence level and \( F_{m}' \) is the maximal level. NPQ (non-photochemical quenching) reflects the dissipation of absorbed light energy from PSII as heat. Total ascorbate was measured in the same volume following reduction of dehydroascorbic acid into ascorbic acid using 10 mM TCEP for 2 h in the dark at 25°C. AsA was detected at 245 nm in sulfuric acid-acidified water (pH 2.5) at a retention time of 1 min under a 0.65 ml min⁻¹ flow. Quantification of AsA amount was based on peak area and calibration was performed using AsA from Sigma.

**Glutathione content**

Samples in buffer B were immediately alkylated with monobromoacetone or N-acetyl-L-cysteine and 0.15% trifluoroacetic acid (TFA). After incubation for 30 min in the dark, the content in glutathion (GSH) and GSSG was determined using spectrophotometrically and calculated according to Lichtenthaler (1987).

**Analysis of chlorophyll content**

One-cm diameter leaf disks were collected from fully expanded mature leaves and immediately frozen in liquid nitrogen and stored at −80°C until use. Leaf disks were crushed in 1 ml 80% acetone. After storing overnight in the dark at 4°C and centrifugation (14,000 g, 10 min), the content in chlorophylls a and b was measured spectrophotometrically and calculated according to Lichtenthaler (1987).

**Glutathione content**

Three tobacco leaf disks of 1 cm diameter (about 100 mg) were ground to a fine powder in liquid nitrogen and extracted in 1 ml 6.3 mM diethylenetriamine-pentaacetic acid (DTPA), 40 μM N-acetylcysteine and 0.15% trifluoroacetic acid (TFA). After centrifugation (15,000 g, 10 min), the supernatant containing non-protein thiols was filtered on 0.2 mm nylon membrane. 125 μl were added to 225 μl buffer A (6.3 mM DTPA, 0.2 M 4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid, pH 8.2) or buffer B (buffer A + 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride, TCEP). For measurements of GSH content, samples in buffer B were immediately alkylated with monobromoacetone and NADPH-dependent malate and glyceraldehyde-3-phosphate dehydrogenase activities in tobacco leaf crude extracts were assayed spectrophotometrically as previously described in Rey et al. (2005). The protein content was determined using the BC Assay Kit (Bio-Rad Laboratories). Leaf pieces were blended in liquid nitrogen and the powder revealed at 680 nm using the "Odyssey Infrared Imager" from LiCor.

**Protein extraction, SDS-PAGE and western analysis**

Leaf pieces were blended in liquid nitrogen, and the powder was used to prepare soluble proteins as described in Rey et al. (2005). The protein content was determined using the BC Assay Reagent (Interchim, Montluçon, France). Proteins were separated using SDS-PAGE and Coomassie Brilliant Blue staining of gels was carried out to check quality of protein extracts. For immunoblot analysis following electrophoresis, proteins were electro-transferred unto nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA) and Red Ponceau staining was achieved to ensure equal loading in all lanes. Western analysis was carried out using primary antibodies raised in rabbit against NtTrx m (1:5,000; Sanz-Barrio et al., 2011), AtMSRB1 and AtMSRB2 (1:1,000; Laugier et al., 2010), poplar PrxQ (1:2,000; Rouhier et al., 2004) and catalase (1:1,000; Agrisera, Vännäs, Sweden). Western analysis of Z-Cys Prx abundance and redox status was performed as in Rey et al. (2007). Bound antibodies were detected using either an anti-rabbit IgG alkaline phosphatase conjugate (Sigma-Aldrich) or a goat anti-rabbit "Alexa Fluor® 680" IgG from Invitrogen diluted 1:10,000. When using the latter, antibodies were revealed at 600 nm using the "Odyssey Infrared Imager" from LiCor.

**NADP-MDH and NADP-GAPDH activity assays**

NADP-dependent malate and glyceraldehyde-3-phosphate dehydrogenase activities in tobacco leaf crude extracts were assayed spectrophotometrically as previously described in Keryer et al. (2004) and Marri et al. (2009), respectively. Extractable enzymatic activities were measured on aliquots of freshly prepared extracts and maximal activities were measured after pre-treatment (reductive activation) of extracts with 25 mM DTT for 20 min at room temperature.

**MSR activity assay**

Maximal MSR activity in tobacco leaf extracts was assayed by monitoring the reduction of the synthetic substrate, dabsyl-MetO, in the presence of DTE (Viera Dos Santos et al., 2005; Laugier et al., 2010). After blending leaves and suspension in extraction buffer, the content in soluble proteins was determined as above. The reaction mixture contained 15 mM HEPES pH 8, 10 mM MgCl₂, 30 mM KC1, 20 mM DTE, 0.25 mM dabsyl-MetO and 30 or 300 μg soluble proteins. After incubation for 3 h at 37°C, stopping using an ethanol-acetate buffer (50:50) and centrifugation, a supernatant aliquot was loaded on a C18 reverse phase 5.5 µm, 3 mm x 50 mm column SunFire™ (Waters, Milford, MA, USA) to separate dabsyl-MetO and dabsyl-Met.
RESULTS

GROWTH CHARACTERISTICS OF TOBACCO PLANTS OVEREXPRESSING

Trx f OR Trx m

The transplastomic tobacco plants studied in this work were generated as described previously (Sanz-Barrio et al., 2013) by inserting tobacco Trx f or Trx m sequences (GenBank Acc. N° HQ338526 and HQ338525, respectively) without predicted transit peptides in the chloroplast genome under the control of the psbA regulatory sequence. Compared to Wt, the tobacco transformant lines termed Trx f and Trx m were shown to contain strongly increased Trx protein levels (at least 20 times more for Trx f and 15 times for Trx m; Sanz-Barrio et al., 2013 and data not shown). The first phenotype analysis revealed that compared to Wt and Trx f plants, Trx m plants display some delay (2–3 days) in germination in in vitro conditions, a pale-green phenotype and a delay of few days in flowering when grown in greenhouse conditions (Sanz-Barrio et al., 2013). In the present work, we further investigated the growth parameters of the transplastomic lines grown on compost in phytotron conditions. We noticed that plants overexpressing Trx m do not exhibit any delay in germination in these conditions, but show a slower growth compared to Wt and those overexpressing Trx f (Figures 1A–C). Thus, at 40 days, the height stem of Trx m plants (15.3 ± 2.1 cm) is significantly lower than that of Wt plants (22.9 ± 6.3 cm), whereas the growth of Trx f plants is slightly altered, their height stem being 19.3 ± 5 cm (Figure 1C). The slower growth of Trx m plants is associated with some delay (ca. 3 days) in flowering, but the plant size at this developmental stage is very similar to that of Wt and Trx f plants (data not shown), as previously observed (Sanz-Barrio et al., 2013). We determined the leaf specific weight and did not notice any difference for the three lines (Table 1). Regarding the dry matter percentage, we observed a slightly, but significantly higher ratio in Trx f plants (Table 1).

Table 1 | Leaf specific weight, dry matter percentage and chlorophyll content in 40-day old Wt and transplastomic tobacco plants.

| Genotype | Wt | Trx f* | Trx m* |
|----------|----|--------|--------|
| Leaf specific weight (mg FW cm−2) | 214 ± 0.9 | 208 ± 1.6 | 210 ± 1.7 |
| % Dry matter | 10.3 ± 0.8 | 11.4 ± 1.1* | 10.2 ± 1.3 |
| Chl a (μg cm−2) | 44.6 ± 1.4 | 41.1 ± 1.0** | 30.9 ± 1.3*** |
| Chl b (μg cm−2) | 316 ± 1.7 | 289 ± 1.0** | 23.4 ± 0.3*** |
| Chl a/b | 13.0 ± 0.6 | 12.2 ± 1.1 | 78 ± 0.3*** |
| Chl a/Chl b | 2.4 ± 0.2 | 2.4 ± 0.2 | 3.1 ± 0.1*** |

Chlorophyll content was measured in young well-expanded leaves of 40-day old Wt and transplastomic tobacco plants. Chlorophyll data are mean values ± SD from five independent measurements per plant and genotype. Leaf specific weight and dry matter data are mean values ± SD from eight independent measurements per plant and genotype. Each measurement was carried out using three 1 cm leaf disks. Wt, wild-type; Trx f* and Trx m* plants overexpressing Trx f or Trx m, respectively. Values significantly different from Wt values with *p < 0.05, **p < 0.01, and ***p < 0.001 (t-test).

This characteristic could originate from the much higher starch content measured in this line (Sanz-Barrio et al., 2013). We then measured chlorophyll content in fully expanded leaves (Table 1) and observed that Trx f plants display a pigment content slightly, but significantly, lower than that of Wt (41.1 ± 1.0 against 44.6 ± 1.4 μg cm−2). This difference has not been observed when plants were grown in greenhouse under longer photoperiod and higher temperature (Sanz-Barrio et al., 2013). Thus, we presume that this phenotype feature originates from the different environmental conditions used in the present study. Consistent with visual observations, a chlorophyll content reduced by more than 30%...
We then investigated the photosynthetic properties of transplastomic tobacco plants. Overexpression of plastidial thioredoxins (Trxs) modulate the activity of known targets in planta in a similar manner. Excess Trxs were very strongly lowered to 40 and 30% of the wild-type (Wt) value for MDH and GAPDH, respectively (Table 1), due to a chlorophyll a/b content reduced to a larger extent (42% compared to Wt) than that of chlorophyll b (26%; Table 1).

**Photosynthetic Properties of Tobacco Plants Overexpressing Trx f or Trx m**

We then investigated the photosynthetic properties of transplastomic tobacco plants and first measured maximal PSI photosynthetic efficiency parameter, $F_{v}/F_{m}$, which reflects the PSI capacity to reduce the primary $Q_{b}$ electron acceptor. We observed a typical value close to 0.8 in Wt plants (0.793 ± 0.0007). A slightly altered $F_{v}/F_{m}$ value (0.766 ± 0.017) was recorded in the Trx f line, whereas this fluorescence parameter was noticeably reduced in plants overexpressing Trx m (0.692 ± 0.020). These data reveal impairment in PSI functioning in plants accumulating Trx m. Photosynthetic electron transport activity was also estimated by measuring $Phi_{PSI}$, a chlorophyll fluorescence parameter indicative of the efficiency of PSI photochemistry as a function of light intensity. While only slightly decreased in Trx f plants, PSI photochemical performance appeared more impaired in plants overexpressing Trx m (Figure 2B). Thermal dissipation of absorbed light energy, expressed by the NQI of chlorophyll fluorescence coefficient, was also recorded in the different lines (Figure 2C). A noticeable difference was detected between Wt and Trx + transplastomic lines since they displayed a similar ability to recover photosynthesis and PSI photochemical efficiency following light irradiation.

**Activity of Trx Targets Involved in Carbon Metabolism**

To get evidence that overexpression of plastidial Trxs has a functional impact on plants on Trx target proteins, we first measured maximal NADP-dependent MDH and GAPDH in transplastomic lines (Figure 3A,B). Both Trx f and Trx m + plants show similar marked changes in enzymatic activities. Extractable leaf activities were very strongly lowered to 40 and 30% of the Wt value for MDH and GAPDH, respectively (Figures 3A,B, left panels). Conversely, leaf MDH and GAPDH capacities corresponding to the maximal activities measured in extracts chemically reduced with DTT, which allows Trx reduction but not direct Trx-target activation, were substantially increased in both transplastomic lines (Figures 3A,B, right panels). These findings show that overexpressed Trxs modulate the activity of known targets in planta. Further, they indicate that both Trx f and m, when they are in large excess, regulate NADP-dependent MDH and GAPDH enzymes in a similar manner.

**Peroxidases and MSR Abundance**

Peroxidases are ubiquitous thiol-based peroxidases detoxifying hydrogen and organic peroxides. In plants, several Trx types such as NTRC, Trx x, CDSP32 and Trx y supply with electrons the main Trx plastidial type, 2-Cys Prx, and another isoform, PrxQ (Dietz, 2011). We thus investigated the abundance of these Prxs in tobacco plants by performing Western analysis. First, we confirmed the high abundance of Trx f and m in transplastomic lines (Figure 4A). The antibodies raised against Arabidopsis 2-Cys Prx served as controls for Western analysis.

**FIGURE 2** | Photosynthetic fluorescence parameters of transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Maximal photosystem II photochemical efficiency in tobacco plants ($F_{v}/F_{m}$). (B) Effective photosystem II yield ($Phi_{PSII}$). (C) Non-photochemical quenching (NPQ). Light response curves are shown for $Phi_{PSII}$ (B) and NPQ (C). Measurements were achieved in leaves from 35-day-old Wt and transplastomic tobacco plants grown in standard conditions. Data are mean values ± SD from at least five independent measurements per genotype. Wt, wild-type; Trx f and Trx m +, plants overexpressing Trx f or Trx m, respectively. ***Significantly different from Wt with $p < 0.001$ (t-test).
and poplar PrxQ homologues specifically revealed bands with the expected molecular masses in tobacco extracts (Figure 4B). No change was detected in the amount of the two plastidial Prxs in Trx\(^+\) plants (Figure 4B). We also used antibodies raised against overoxidized 2-Cys Prxs, an inactivated form possibly involved in signaling processes related to redox homeostasis (Rey et al., 2007). Whereas there was no change in 2-Cys Prx abundance in plants overexpressing Trx \(\text{f}^+\) or Trx \(\text{m}^+\) compared to Wt, we observed a more variable amount of overoxidized 2-Cys Prx in the same leaf extracts. We noticed that such a variation occurred within the same genetic background (Figure 4B). A quantitative analysis, performed on data gained from six independent plants per line (data not shown), lead us to conclude that there was no significant variation in the 2-Cys Prx redox status in transplastomic tobacco plants.

Methionine sulfoxide reductase enzymes, which repair oxidized methionine in proteins, are also well-known targets of Trxs involved in plant responses to environmental constraints (Laugier et al., 2010). The abundance of plastidial MSRs was investigated in tobacco plants using sera raised against Arabidopsis MSRB1, MSRB2 and poplar MSRA4. MSRA4 antiserum recognized two bands at ca. 25 kDa in tobacco extracts (Figure 4C), corresponding to the reduced and oxidized forms of plastidial MSRA as observed in other species (Vieira Dos Santos et al., 2005; Bouchenak et al., 2012; Marok et al., 2013). No substantial change was observed regarding the abundance of MSRA4 forms in Trx-overexpressing plants compared to Wt. For MSRB1, no unambiguous signal could be detected in tobacco extracts due to poor cross-reactivity of the serum generated against Arabidopsis homologue (data not shown). Like in Arabidopsis extracts, a faint band at ca. 15 kDa was revealed for MSRB2, next to a lower non-specific and intense band (Laugier et al., 2010). Again, overexpression of either Trx \(\text{f}^+\) or Trx \(\text{m}^+\) was not found to result in any substantial change in the amount of this plastidial MSR isoform. Altogether, Western data reveal that the levels of some major Trx targets involved in antioxidant mechanisms are not modified when Trx \(\text{f}\) and \(\text{m}\) are overproduced.

**LEAF MSR CAPACITY**

We then measured the MSR enzymatic capacity in leaf extracts from tobacco plants using dabsyl-MetO, a substrate mimicking peptide-bound MetO (Figure 4D). In Wt, a maximal activity in the range of 22 pmol Met. mg prot\(^{-1}\) min\(^{-1}\) was measured in the presence of reductant. Note that this value is noticeably lower than that measured in Arabidopsis, ca. 30 pmol Met. mg prot\(^{-1}\) min\(^{-1}\) (Laugier et al., 2010), but higher than that recorded in barley, ca. 10 pmol Met. mg prot\(^{-1}\) min\(^{-1}\) (Marok et al., 2013). In Trx \(\text{f}^+\) plants, a rather similar value was found, ca. 25 pmol Met. mg prot\(^{-1}\) min\(^{-1}\). In sharp contrast, a twice higher value (46 pmol Met. mg prot\(^{-1}\) min\(^{-1}\)) was measured in plants overexpressing Trx \(\text{m}^+\). These data, showing a more elevated MSR capacity...
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FIGURE 4 | Abundance of proteins involved in antioxidant mechanisms and leaf peptide methionine sulfoxide reductase enzymatic capacity in leaf extracts from transplastomic tobacco plants overexpressing Trx f or Trx m. (A) Western analysis of Trx f and Trx m abundance. (B) Western analysis of the abundance and redox status of plastidial 2-Cys peroxiredoxin (2-Cys Prx) and of the abundance of peroxiredoxin Q (PrxQ). (C) Western analysis of the abundance of plastidial methionine sulfoxide reductases A4 and B2, MSRA4 and MSRB2, respectively. (D) Peptide methionine sulfoxide reductase enzymatic capacity measured using HPLC in crude leaf extracts using dabsyl-MetO, a synthetic substrate mimicking peptide-bound MetO. Data are mean values ± SD from at least five independent measurements per genotype. *Significantly different from Wt with p < 0.01 (t-test). (E) Western analysis of catalase abundance. Similar Western data were obtained using leaf extracts from two independent plants originating from at least two distinct growth experiments. Wt, wild-type; Trx f + and Trx m +, plants overexpressing Trx f or Trx m, respectively.

Catalase in Trx m + plant extracts, reveal that in planta Trx m, but not Trx f, very likely regenerates and sustains the activity of plastidial MSRs.

Catalase Abundance
The previous data revealed that Trx m likely provides reducing power to MSRs. MSRs are enzymes repairing oxidized proteins, but could also play a more general antioxidant function since their action results in ROS scavenging at the expense of NADPH (Moskovitz et al., 1997). To further analyze a putative function of plastidial Trxs in the control of redox homeostasis in plant cells, we analyzed leaf catalase abundance using Western blot analysis. Catalase is one major enzymatic system responsible for H2O2 scavenging in plant cells. We did not notice any substantial difference in catalase amount in plants overexpressing either Trx f or Trx m compared to Wt (Figure 4E), likely indicating no important change in the catalase-based capacity for detoxifying H2O2 in these lines.

Content and Redox Status of Glutathione and Ascorbate
We then investigated whether non-enzymatic antioxidant systems could be altered in transplastomic tobacco lines and measured the leaf content in GSH and ascorbate, which are abundant soluble antioxidants fulfilling key roles in redox homeostasis (Noctor and Foyer, 1998). In young well-expanded leaves of 40-day-old Wt tobacco plants, a GSH content of ca. 0.90 μmol g FW−1 was measured, the proportion of reduced form being 91% (Figures 5A,B). In Trx f + plants, the content and percentage values were very similar: 0.95 μmol g FW−1 and 94%, respectively. Interestingly, in plants overexpressing Trx m, whereas the proportion of reduced GSH was not modified (93%), the content, 0.75 μmol g FW−1, was significantly lower (ca. −20%) than in Wt and Trx f + plants (Figures 5A,B). Regarding ascorbate, both Wt and Trx f + plants display very similar total contents (0.82 and 0.85 μmol g FW−1, respectively) and proportions of reduced form (83 and 86%, respectively) in young well-expanded leaves (Figures 5C,D). In contrast, the total ascorbate content, 0.65 μmol g FW−1, was significantly lower in Trx m + plants, but with an unchanged redox status (82%) compared to the two other lines (Figures 5C,D). Taken together, these data reveal that GSH and ascorbate pools are not altered when Trx f is overexpressed, but significantly and similarly modified in Trx m + plants. Indeed, these plants exhibit a decrease of ca. 20% in the total content of ascorbate and GSH without any noticeable change in redox status.

Tolerance to Phototoxic Treatment of Tobacco Plants Overexpressing Trx f or Trx m
Oxidative-stress tolerance of transplastomic tobacco plants was monitored by exposing them to a treatment combining high light and low temperature, conditions known to generate loss of photosynthetic membranes within chloroplasts due to phototoxic damage. Thirty-day old plants grown in standard conditions were transferred to a light intensity of 950 μmol photons m−2 s−1 at
structures are damaged (Maxwell and Johnson, 2000). As previously observed in control conditions (Figure 2), Wt and Trx f plants displayed close values. Maximal PSII photosynthetic efficiency (Fv/Fm) was measured 5 h after MV treatment. Similarly to the results reported above for photoxidative damage, PSII photosynthetic efficiency was significantly impaired in Wt and Trx f plants (Fv/Fm) values reduced by ca. 20 and 49%, respectively), whereas almost no change was observed in Trx m plants (Figure 7B). To further evaluate the tolerance of transplastomic tobacco plants to methyl viologen (MV), a redox-cycling herbicide that generates superoxide radicals by accepting electrons from PSI and transferring them to oxygen (Balbis et al., 1989), MV-mediated oxidative damage was assessed in whole plants sprayed with MV, and visual symptoms were registered 2 days following treatment. Wt and Trx f plants were severely affected by MV treatment, whereas necrotic lesions were more limited in Trx m plants (Figure 7A and data not shown). Maximal PSII efficiency (Fv/Fm) was measured 5 h after MV treatment. Similarly to the results reported above for photoxidative damage, PSII photosynthetic efficiency was significantly impaired in Wt and Trx f plants (Fv/Fm) values reduced by ca. 20 and 49%, respectively), whereas almost no change was observed in Trx m plants (Figure 7B). To further evaluate the tolerance of transplastomic tobacco plants to methyl viologen (MV), a redox-cycling herbicide that generates superoxide radicals by accepting electrons from PSI and transferring them to oxygen (Balbis et al., 1989), MV-mediated oxidative damage was assessed in whole plants sprayed with MV, and visual symptoms were registered 2 days following treatment. Wt and Trx f plants were severely affected by MV treatment, whereas necrotic lesions were more limited in Trx m plants (Figure 7A and data not shown). Maximal PSII efficiency (Fv/Fm) was measured 5 h after MV treatment. Similarly to the results reported above for photoxidative damage, PSII photosynthetic efficiency was significantly impaired in Wt and Trx f plants (Fv/Fm) values reduced by ca. 20 and 49%, respectively), whereas almost no change was observed in Trx m plants (Figure 7B). To further evaluate the tolerance of transplastomic tobacco plants to
MV, membrane damage was estimated by measuring ion release from control and treated leaf disks. Following incubation on MV, the level of electrolyte leakage from Trx f disks was relatively high and comparable to that of Wt (ca. 60% of the total electrolyte content, Figure 7C). In contrast, plants overexpressing Trx m displayed a much lower level of ion release (25%). Altogether, these results signify that overexpression of Trx m, but not of Trx f, increases tolerance to MV.

**DISCUSSION**

**SPECIFICITY OF Trx m FUNCTION**

In contrast to other organisms, where only two or three multi-functional Trx isoforms are present (Ritz et al., 2000; Garrido and Grant, 2002), plants display a great diversity of Trxs with physiological functions remaining elusive for many of them. Our work based on a genetic strategy contributes to decipher the role of Trx m in planta. Clear differences have been observed between plants overexpressing either Trx m or Trx f. Trx m+ plants exhibit reduced growth and impaired photosynthesis (Figures 1,2), but increased tolerance to oxidative treatments (Figures 6,7). In comparison, Trx f+ plants are almost indistinguishable from Wt, in full agreement with previous data (Sanz-Barrio et al., 2013) and others gained in Arabidopsis mutants knockout for Trx f (Thormählen et al., 2013) and in Trx f RNAi pea plants (Luo et al., 2012). Consistently, in heterologous complementation assays in yeast, Arabidopsis Trx m, but not Trx f, confers tolerance to oxidative stress (Issakidis-Bourguet et al., 2001). Taken together, these reports indicate that Trxs f and m fulfill distinct and non-overlapping physiological functions very likely through a marked specificity towards their targets in plants.

In the last years, knowledge has been acquired about the essential roles of some plant Trxs, particularly plastidial isoforms. Some are critical such as Trx z which is needed for plastid development and seedling viability (Arsova et al., 2010). NTRC-KO Arabidopsis plants display pale phenotype, impaired photosynthesis and sensitivity to oxidative stress, high temperature and prolonged darkness (Pérez-Ruiz et al., 2006; Lepistö et al., 2009; Chae et al., 2013). Other plastidial Trxs play more specialized functions such as CDSP32, which prevents oxidative damage during environmental constraints (Broin et al., 2002; Rey et al., 2005) and Trx y2, which maintains growth under high light conditions (Laugier et al., 2013). In other respects, cytosolic Trx h1 participates in responses to salt treatment (Zhang et al., 2011) and Trx h5 in sensitivity...
to a fungal toxin (Sweat and Wolpert, 2007). Our present work reveals that Trx m fulfills essential functions in photosynthetic processes and in stress tolerance. Noteworthy, down-regulation of Trx m gene expression in rice also leads to impaired growth and reduced chlorophyll content in control conditions (Chi et al., 2008). This further argues for a function of Trx m as a central actor controlling photosynthesis. However, no obvious phenotype was recorded in Arabidopsis mutants deficient for either Trx m3 or Trx m4 (Courtelle et al., 2013; Laugier et al., 2013). Since these Trxs share, with Trx m2, very similar sequence and biochemical properties (Collin et al., 2003), we hypothesize that the three isozymes could play overlapping functions in Arabidopsis.

**PHENOTYPE OF Trx m**

**TOBACCO UNDER CONTROL CONDITIONS**

Compared to Wt and Trx f plants, Trx m plants grown in control conditions display modified chlorophyll composition and impaired PSII activity (Figure 2, Table 1). Trx m overexpression in tobacco (Table 1), like Trx m deficiency in rice (Chi et al., 2008), leads to lower chlorophyll content and increased Chl a/Chl b ratio. Luo et al. (2012) showed that pea plants silenced for both genes encoding Trx f and m exhibit reduced chlorophyll content and a much higher level of the oxidized form of CHL a, a subunit of magnesium chelatase, an enzyme essential for chlorophyll biosynthesis and regulated by Trx (Ilegami et al., 2007). Moreover, Luo et al. (2012) reported that the expression of numerous genes involved in tetrapyrrole biosynthesis was also strongly altered in these pea plants. Therefore, we can hypothesize that expression and redox activation of enzymes participating in chlorophyll synthesis are modified in Trx m tobacco due to the large Trx excess, leading to change in pigment content. In other respects, one LHCI1 protein has been identified as a Trx target in spinach thylakoid membranes (Baltzer et al., 2006). Interestingly in Trx m plants, we recorded a much lower content in Chl b (Table 1), which is more specifically associated with LHc proteins, and preliminary proteomic analyses on transplastomic lines indicate that LHc proteins are less abundant compared to Wt and Trx f+ (unpublished data). All these data prompt us to propose that Trx m might be involved in the regulation of the light capture process via the control of the abundance and/or redox status of LHc proteins. We previously reported that Trx m+ plants are characterized by the absence of cyclic electron transfer via the NDH pathway (Courtelle et al., 2013). Thus, the suppression of this pathway might modify the NADPH/ATP ratio within plastids and finally impair the whole photosynthetic process. This hypothesis is not supported by the fact that tobacco plants knockout for the whole NDH complex do not exhibit any noticeable phenotype in control conditions (Horvath et al., 2006). Nonetheless, as the other cyclic electron pathway via the proton gradient regulation (PGR) complex is also negatively regulated by Trx m4 in in vitro experiments (Courtelle et al., 2013), we cannot exclude that inhibition of both cyclic NDH and PGR pathways due to high Trx m abundance results in substantial impairment of photosynthetic processes. In other respects, in control conditions no obvious difference between the two transplastomic lines has been noticed regarding the activities in leaf extracts of two enzymes involved in carbon metabolism, NADP-MDH and NADP-GAPDH (Figure 3). Based on all these data, we conclude that the phenotype of Trx m+ plants is probably not linked to changes in carbon metabolism, but more likely to modifications in photochemical processes from light capture to electron transfer.

Interestingly, plants overexpressing Trx m display reduced contents in ascorbate and GSH (ca. –20% compared to Wt and Trx f+ plants), with no modification in redox status (Figure 5). This reveals that Trx m exerts a specific control on the amount of these compounds through mechanisms remaining to be delineated. Regarding AsA, redox regulation of enzymes involved in biosynthesis and regeneration pathways could account for the modified content. Indeed, dehydroascorbate reductase (DHAR), the enzyme regenerating AsA from its oxidized form, has been identified as a Trx target in several reports (Marchand et al., 2004; Hagglund et al., 2008; Montrichard et al., 2009) and reduction of DHAR by Trx is known to activate the enzyme (Dixon et al., 2002). Consequently, overexpression of Trx m in tobacco might trigger activation of DHAR and lead to increased AsA recycling, thus explaining the need for a reduced pool of AsA in Trx m+ tobacco. But note that in tobacco plants overexpressing DHAR, increased AsA recycling is associated with increased AsA content (Chen et al., 2003). Currently, there is no evidence for a possible redox regulation of enzymes involved in GSH metabolism (Montrichard et al., 2009). It is worth mentioning that the increase in AsA content due to DHAR overexpression in tobacco plants is accompanied by a GSH pool increased in the same range (Chen et al., 2003). Further studies are needed to investigate whether the decreased GSH content in Trx m+ plants is linked to modified AsA level or is a direct consequence of Trx m overexpression. Whether the modified GSH and AsA pools in Trx m+ plants lead to the observed growth and photosynthesis phenotype remains also unclear. Nonetheless, these data unveil a new role for Trx m in plant cell redox homeostasis through regulation of the content in major soluble antioxidants.

**TOBACCO UNDER OXIDATIVE TREATMENTS**

Compared to Wt and to Trx f+, Trx m+ plants are more tolerant to conditions generating oxidative damage, induced by either MV or high light combined to low temperature (Figures 6, 7), revealing a role of Trx m in the protection of plastidial structures. Accordingly, the abundance of Trx m1 is strongly up-regulated in cold-stressed Arabidopsis plants and this Trx has been proposed to preserve photosynthetic apparatus (Goudas et al., 2006). The tolerance of Trx m+ plants is nonetheless rather puzzling when taking into consideration their reduced chlorophyll content (by more than 40% for Chl b) since an Arabidopsis mutant lacking Chl b is much more sensitive to photoinhibitory treatments, partly due to increased single oxygen production (Dall’Osto et al., 2010). Thus, the tolerance of Trx m+ tobacco plants is not directly linked to chlorophyll content, but to other mechanisms also counterbalancing the deleterious effects due to Chl b shortage. Based on our data, we presume that neither NQO, nor soluble antioxidants constitute primary determinants in this stress tolerance. Indeed, in Trx m+ plants, NQO is not modified (Figure 2) and both AsA and GSH pools are reduced by ca. 20% (Figure 5), with no change in redox status. In numerous cases, there is a positive correlation between stress tolerance level and AsA content. For instance, an Arabidopsis mutant deficient in AsA is highly sensitive to environmental constraints (Conklin et al., 1996) and tobacco plants with increased...
AsA content are more tolerant to high light (Chen and Gallie, 2008). Regarding GSH, as reviewed very recently (Zagorchev et al., 2013), complex and contradictory data have been reported since tobacco plants with decreased GSH reductase activity are more sensitive to oxidative stress (Ding et al., 2009), but plants displaying elevated GSH biosynthesis capacity show high sensitivity to light (Creissen et al., 1999).

Trx m plants display reduced PSI activity in control conditions (Figure 2), and surprisingly this activity is much less affected under oxidative conditions compared to Wt (Figures 6,7). Exposure to environmental constraints generally impairs PSI at the D1 protein level due to increased production of reactive oxygen species and inhibits PSI repair via the suppression of the synthesis of this subunit (Takahashi and Murata, 2008). Thus we can speculate that the excess of Trx m is associated with preservation of PSI structure in stressed conditions. In connection with this hypothesis, it is worth mentioning that Trx has been proposed to regulate D1 antioxidative apparatus. We recently reported that both plastidial Trx m and f immunoblot analysis. Furthermore, we have analyzed the plastidial Trx m abundance and/or redox status of the main plastidial Prxs in Trx m plants (Figure 4). In contrast, we observed a twice higher MSR enzymatic capacity in Trx m plants than in Trx f and Wt plants with no change in protein abundance (Figure 4). This signifies that Trx m very likely constitutes a physiological electron donor to MSRs. This is consistent with our previous work showing that while MSR capacity is slightly lowered in Arabidopsis plants deficient for Trx f, it is significantly reduced in mutants lacking various Trx m types (Laugier et al., 2013). MSRs are key enzymes repairing oxidized proteins and possibly scavenging ROS via MetO (Moskovite et al., 1997). In plants, they are involved in the protection against environmental constraints (Romero et al., 2004; Laugier et al., 2010) and in seed longevity (Châtelain et al., 2013). Altogether, these data give high credence for a role of the Trx m/MSR system in the tolerance of Trx m plants to oxidative treatments.

To conclude, the complex phenotype of transplastomic tobacco Trx m plants indicates that this Trx is very likely a central actor in plant cell redox homeostasis. In contrast to other types like f, the m type could regulate in planta numerous redox-based processes in photosynthesis and antioxidant responses. Further investigations, based for instance on co-immunoprecipitation methods to isolate Trx m partners in plant extracts (Rey et al., 2005), are needed to unveil the target proteins and the mechanisms underlying the physiological function(s) of this Trx type.

ACKNOWLEDGMENTS

We are very grateful to P. Henri for valuable assistance in growth of tobacco plants and to the Groupe de Recherche Appliquée en Phytotechnologie (CEA, IBER, SIVME, GRAP) for technical assistance with controlled growth chambers.

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Conflicts of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2013; accepted: 12 September 2013; published online: 16 October 2013.

Citation: Rey E, Sanz-Barrio R, Innocenti C, Kumar A, González L, Rumeau P, Farran I (2013) Overexpression of plastid thioredoxin f and m differentially alters photosynthetic activity and response to oxidative stress in tobacco plants. Front. Plant Sci. 4:313. doi: 10.3389/fpls.2013.00313

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

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