CAROLINA PEREIRA NASCIMENTO

FUNCTIONAL ANALYSIS OF THE THIOREDOXIN SYSTEM DURING SEED GERMINATION IN *Arabidopsis thaliana*

Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

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BIOGRAPHY

Carolina Pereira Nascimento, daugther of Dimas Antônio Moreira do Nascimento and Elza Maria Pereira do Nascimento, was born in Aiuruoca, Minas Gerais state, Brazil, on August 26th, 1991.

In 2012, she started undergraduate studies in Agronomy at the Universidade Federal de Viçosa (UFV), Minas Gerais State, Brazil, earning the bachelor's degree in January 2017.

She started her Master's Studies at the Universidade Federal de Viçosa (UFV) in March 2017, under supervision of Prof. Adriano Nunes Nesi.

ABSTRACT

NASCIMENTO, Carolina Pereira, M.Sc., Universidade Federal de Viçosa, March, 2019. **Functional analysis of the thioredoxin system during seed germination in *Arabidopsis thaliana.*** Adviser: Adriano Nunes Nesi.

A series of processes occurs during seed formation, including remarkable changes from early development to the end of germination. The changes associated with processes initiated mainly after seed imbibition are usually characterized by extensive changes in redox state of seed reserve proteins and of pivotal enzymes for protein mobilization and usage. Such changes in redox state are often mediated by Thioredoxins (Trxs), which are protein oxiredutases capable of catalyzing the reduction of disulfide bonds in target proteins, thereby regulating their structure and function. Here, we analyzed the previously characterized mutants of NADPH-dependent Trx reductase A and B (ntra ntrb), two independent mutant lines of mitochondrial thioredoxin o1 (trxo1) and two mutant thioredoxin h2 (trxh2) mutant lines. Our results indicate that plants deficient for the NADPH-dependent thioredoxin system are able to mobilize their reserves, but at least partially fail to use these reserves during germination, thereby leading to lower availability of energy substrates than wild type seeds. Trx mutants also show decreased activity of regulatory systems needed to maintain cellular homeostasis. Moreover, we observed reduced respiration in mutant seeds and seedlings, which in parallel with an impaired energy metabolism, disrupts core biological processes responsible for proper germination and early development of Trx mutants. In conclusion, the results suggest that the lack of thioredoxin induces a substantial adaptation in seeds and seedlings, which undergo a metabolic reprogramming to adapt to a new redox state.

RESUMO

NASCIMENTO, Carolina Pereira, M.Sc., Universidade Federal de Viçosa, março de 2019. **Análise funcional do sistema tiorredoxina durante a germinação de sementes de *Arabidopsis thaliana.*** Orientador: Adriano Nunes Nesi.

Uma série de processos ocorre durante a formação de sementes, incluindo mudanças notáveis que se estendem desde o início do desenvolvimento até o final da germinação. As mudanças associadas a processos iniciados principalmente após a embebição das sementes são geralmente caracterizadas por extensas alterações no estado redox das proteínas de reserva de sementes e de enzimas essenciais para mobilização e uso de proteínas. Tais alterações no estado redox são frequentemente mediadas por tiorredoxinas (Trxs), que são oxiredutases proteicas capazes de catalisar a redução de ligações dissulfureto em proteínas alvo, regulando assim a sua estrutura e função. Aqui, analisamos os mutantes previamente caracterizados de Trx redutase A e B (ntra ntrb) dependentes de NADPH, duas linhas mutantes independentes de tiorredoxina o1 mitocondrial (trxo1) e duas linhagens mutantes tiorredoxina h2 (trxh2) mutantes. Nossos resultados indicam que as plantas deficientes para o sistema de tiorredoxina dependente de NADPH são capazes de mobilizar suas reservas, mas são parcialmente falhas no uso destas reservas durante a germinação, o que leva a uma menor disponibilidade de substratos energéticos comparado ao controle, o tipo selvagem. Trx mutantes também mostraram diminuição da atividade dos sistemas regulatórios necessários para manter a homeostase celular. Além disso, observou-se uma baixa respiração em sementes e plântulas mutantes, que paralelamente a um metabolismo energético comprometido, prejudicam os processos biológicos centrais responsáveis pela germinação adequada e pelo desenvolvimento precoce de mutantes Trx. Em conclusão, os resultados sugerem que a falta de tiorredoxina induz à uma adaptação substancial em sementes e plântulas, envolvendo uma reprogramação metabólica para se adaptarem a um novo estado redox.

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1. Introduction

Seed germination is the first step toward ensuring successful plant establishment. In general, the germination *sensu strictu* begins with the absorption of water by the dry seed and ends with the emergence of the radicle (Rosental et al., 2014). Following the completion of germination, starts the post-germination, the period in which occurs the major mobilization of the reserves within the storage tissues or in the endosperm of the seed (Iglesias-Fernández et al., 2014; Ortiz-Espín et al., 2017). Lipids, proteins and carbohydrates (most frequently in the form of starch) can all be used as reserves during this moment, although lipids are considered the most widely distributed in nature (Eastmond et al., 2000; Graham, 2008). For instance, in oleaginous seeds, as in the case of *Arabidopsis thaliana* (Brassicacea), the main source of reserve are the fatty acids, found in the form of triacylglycerols (TAGs) (Eastmond et al., 2000; Zhou et al., 2014; Sew et al., 2016). The mobilization of TGAs, proteins and carbohydrates accumulated in the maternal tissues that feed the seed is decisive for the transition from seed to seedling (Sew et al., 2016; Silva et al., 2016). Once germination has started, the energy from the hydrolysis of these reserve compounds supports the heterotrophic growth of the seedling until it becomes photosynthetically self-sustaining (Iglesias-Fernández et al., 2014). In other words, the reserves need to be rapidly converted to soluble metabolites that can be transported throughout the seedling and used to fuel growth, which enables photoautotrophism to be achieved before reserves are exhausted (Pritchard Sarah et al., 2002; Graham, 2008). Therefore, the energy metabolism is essential to support germination and seedling growth (Silva et al., 2016).

A range of metabolic changes occurs during seed germination since a quiescent dry seed until a metabolically active state following water uptake (Fait et al., 2006; Wojtyla et al., 2016). In addition to the storage reserve mobilization, a number of other key biochemical and cellular events associated with germination are energy-demanding (Czarna et al., 2016; Wojtyla et al., 2016). Soon after imbibition, energy demands increase to support reserve mobilization, organelle repair (Narsai et al., 2011) and alterations in transcripts, proteins, and metabolites (Weitbrecht et al., 2011). Notably, most of the energy used to power seed germination and reserve mobilization seems to be supported by the respiration, which is initiated immediately upon rehydration (Sew et al., 2016). In accordance, previous transcriptomic data demonstrated the activation of genes encoding enzymes required to build the energy metabolism machinery in germinating seeds of Arabidopsis (Fait et al., 2006; Narsai et al., 2011; Sew et al., 2016). From this, the

respiratory associated transcripts of glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, and mitochondrial electron transport are up-regulated significantly (Narsai et al., 2011).

Apart from demands a high mitochondria activity, the changes associated with the processes initiated after seed imbibition are usually characterized by the production of large quantities of reactive oxygen species (ROS) (Bailly et al., 2008; Barba-Espín et al., 2011; Wojtyla et al., 2016). ROS have a dual action in seed physiology (Bailly et al., 2008). They function as signaling molecules in seed germination and dormancy, but also cause oxidative stress if present in high amounts, affecting seed vigor and longevity (Bailly et al., 2008; Hägglund et al., 2016). Therefore, germination and ROS homeostasis seem to be associated and, in that way, the success of seed germination can be influenced by the cellular redox status (Bailly et al., 2008; El-Maarouf-Bouteau and Bailly, 2008; Arc et al., 2011; Barba-Espín et al., 2011).

Seed development and germination are accompanied by an extensive change in the redox state of seed reserve proteins (Kobrehel et al., 1992; Buchanan and Balmer, 2005; Catusse et al., 2008; Hägglund et al., 2016; Buchanan, 2017). In cereals, it is well established that the major proteins of both starch endosperm and embryo are synthesized in the reduced or sulfhydryl (-SH) state and become oxidized to a more stable disulfide (S−S) state during maturation and drying. Upon germination, the proteins are converted back to the reduced (-SH) state to facilitate mobilization (Buchanan and Balmer, 2005; Catusse et al., 2008; Hägglund et al., 2016; Buchanan, 2017). This change in the redox state is often mediated by Thioredoxins (Trxs), a large family of thiol-disulfide oxireductases proteins capable of catalyzing the reduction of disulfide bonds in target proteins to regulate their structure and function (Meyer et al., 2012).

About 20 isoforms of Trxs occur in Arabidopsis (Geigenberger et al., 2017). Some of them occur exclusively in plastids, such as *f*1-2, *m*1-4, *x*, *y*1-2 and *z* isoforms, while Trx *o*1-2 are found in mitochondria and nucleus, and Trxs *h*1-8 are distributed between cytosol, nucleus, endoplasmic reticulum, and mitochondria (Thormählen et al., 2015; Geigenberger et al., 2017). The reduction of the different Trxs is performed by two distinct pathways in plants (Meyer et al., 2012; Thormählen et al., 2015; Geigenberger et al., 2017). In chloroplast, Trxs are reduced by light through the ferredoxin (Fdx)/thioredoxin reductase (FTR) system, while in extraplastidial compartments Trxs are reduced by NADPH via NADPH-Trx reductase A (NTRA) and B (NTRB) (Dietz and Pfannschmidt, 2011; Marchal et al., 2013; Geigenberger et al., 2017).

The NTR/Trx *h* system has been widely recognized as important regulator of seed germination (Renard et al., 2011; Guo et al., 2013; Hägglund et al., 2016). For instance, *trxh9* mutants in wheat were characterized by a delay in seed germination rate (Li et al., 2009), while overexpression of the *Triticum* spp. TRX *h*5 in barley accelerates germination by an increased solubilization of disulfide proteins (Cho et al., 1999; Wong et al., 2002; Li et al., 2009). Moreover, Trx also mediates redox changes of proteins during germination in seeds of the dicot *Medicago truncatula* (Alkhalfioui et al., 2007; Buchanan, 2017), demonstrating that Trx-linked redox changes take place more broadly in germinating seeds and are not restricted to cereals. Collectively, the results from this and other experiments made clear that, during germination, Trx *h* can: i) increase the susceptibility of storage proteins to proteolysis by breaking their intramolecular disulfide bonds (Guo et al., 2013); ii) change the activities of enzymes either directly by reduction of functional enzymes in starch and protein utilization or iii) indirectly by reductive inactivation of specific disulfide inhibitor proteins such as inhibitors of the starch-degrading enzymes α-amylase (Kobrehel et al., 1991).

It seems that the mitochondrial Trx system is also important for seed germination, given that both *ntra ntrb* double mutant and *trxo1* mutant present a delay in seed germination in Arabidopsis (Daloso et al., 2015). Moreover, previous results demonstrated that Trx *o1* transcript levels are particularly high in dry seeds and cotyledons in Arabidopsis (Ortiz-Espín et al., 2017). It is therefore suggested that Trx *o1*, as well as others mitochondrial Trxs, may resemble Trx *h* proteins in the regulation of seed germination. Given that seed germination demands a high mitochondria activity (Czarna et al., 2016; Heidorn-Czarna et al., 2018), it is reasonable to hypothesize that mitochondrial Trx system may act in seed germination by regulating mitochondrial enzymes (Hägglund et al. 2016).

It is also worth mentioning that the mitochondrial Trx system has an important role in energy metabolism by acting as an important regulator of the TCA cycle (Daloso et al., 2015) and of alternative oxidase (AOX) (Gelhaye et al., 2004) in plants. Thus, mitochondrial Trxs are responsible for the direct regulation of at least five TCA cycle associated enzymes, namely fumarase (FUM), succinate dehydrogenase (SDH), citrate synthase (CS), isocitrate dehydrogenase (IDH) and succinyl-CoA ligase (SCoAL) (Nunes-Nesi et al., 2013; Daloso et al., 2015). However, the mechanism that coordinates these metabolic variations is not yet completely understood and even less is known about the regulation of these enzymes by Trxs in imbibed seeds. It is also worth mentioning

that, in general, several enzymes of the TCA cycle are highly susceptible to oxidative stress (Winger et al., 2007; Obata et al., 2011), which probably involves the participation of Trx systems by mechanisms not yet fully understood.

Taken together, the above information corroborates with an important, though to some extent unknown, role of Trxs, possibly by redox regulation under various growth conditions. The mentioned results support the performance of mitochondrial Trxs in the regulation of redox status in seeds, by a mechanism that seems to involve metabolic regulation mediated by alterations mainly at the mitochondrial level. It is believed that the lack of thioredoxins and also of reductases of these thioredoxins cause disorder within the cells, being necessary the performance of other mechanisms of regulation.Thus the imbalance caused by the inefficient regulatory system will cause delays in the germination and development of these seeds and seedlings that in turn may reflect in parameters such as the production of these plants in later periods. These data reinforce, therefore, the need for a better understanding of the performance of mitochondrial Trxs in the germinative and post-germinative events. In view of these considerations, we investigate how the primary metabolism and physiological potential are impaired in independent mutant T-DNA insertion lines during germination and post-germination under optimal conditions. of *A. thaliana.* For this, six genotypes were used: WT (wild type), double mutant *ntra ntrb*, and *trxo1* and *trxh2* genotypes, where Trx *o*1 is found in mitochondria and Trx *h*2 in mitochondria and cytosol. By analyzing metabolic and physiological changes over time in *trx* mutant plants, we demonstrated that the mitochondrial Trx system is important for seed germination and reserve mobilization during post-germinative events in Arabidopsis.

Although we know the regulatory function of Trxs in modulating the cellular redox state, the exact mechanism connecting Trxs, the mobilization of reserves and respiration in dry seeds and in the early seedling development remains unclear. Here, we investigated how the primary metabolism, physiological and phenotypic aspects are altered in dry seeds and in the early stages of development of mutant plants with reduced expression of the mitochondrial Trx system. Our results demonstrate a delay in the germination of the mutants, especially for the double mutant plants that does not express in NTRA and NTRB, isoforms localized in both the cytosol and mitochondria. Double mutants presented a longer germination delay and even a longer delay in the early stages of development. Changes in respiratory metabolism and reserve utilization were observed in mutants compared to wild type. This can be attributed to a deactivation of enzymes

responsible for providing substrate for energy metabolism. The results are discussed in the context of the importance of regulation of cellular redox and current knowledge related to respiration and the use of reserves after seed germination.

2. Material and methods

2.1 Plant material and growth conditions

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study were of the Columbia (Col-0) background. The *ntra ntrb* double knockout mutant was previously described (Reichheld et al., 2007), while the two T-DNA insertion mutants in the *TRX o1* gene (At2g35010) from the Salk collection, *trxo1-1* (SALK_042792) and *trxo1-2* (SALK_143294), were described by Ortiz-Espín *et al.*, 2017. Moreover, the two T-DNA insertion mutants, in the second intron and in the third exon of *TRX h2* gene (At5g39950), *trxh2-1* (SALK_079516) and *trxh2-2* (SALK_079507), respectively.Seeds were surface sterilized and placed at 4°C for 3 days in the dark (stratification period) on 0.7% (w/v) agar plates containing one-half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose (Sigma-Aldrich; pH 5.7). Seeds were subsequently germinated and grown for 10 days at 22°C under neutral-day conditions (12-h light/12-h dark photoperiod at 22°C), 60% relative humidity with 150 µmol photons m^{-2} s^{-1}, 10-day-old seedlings were transferred to soil. Seeds from fully developed and ripened brown siliques of the wild-type and mutant plants were collected at the same time point for further analysis.

2.2 Evaluation of biometric parameters of seeds and siliques

To phenotype reproductive tissues, seeds were submitted to the procedure described above, and the seedlings were transferred to commercial substrate and kept in a growth chamber at 22°C ± 2°C, 60% relative humidity, and irradiance of 150 µmol photons m^{-2} s^{-1} with a photoperiod of 12 h of light and 12 h of dark for seed production. The number of siliques and branches was measured when most of the stem silica was yellow (senescent), according to the method of (Balazadeh et al., 2008) adapted. All siliques were collected at the same time after flowering and captured at the same magnification. Siliques from at least 10 plants per genotype were collected at the end of reproductive phase to determine the number of siliques per plant. Mature siliques were harvested and cleared with 0.2 M NaOH and 1% (w/v) sodium dodecyl sulfate (SDS)

solution to remove pigments (Barros et al., 2017). These siliques were used to obtain two parameters, silique lenght and number of seeds per silique. For this, images of at least 15 Arabidopsis siliques per genotype were taken with a digital camera (Canon Power- shot A650 IS) attached to a stereomicroscope (Supplemental Figure S1). The measurement of silique length and counting seeds were performed on the images using ImageJ software (Schneider et al., 2012). Seed weight was determined by weighing aliquots of a known number of seeds (about 500 seeds per aliquot). Total seed yield was determined by weighing seeds collected from at least 10 individual plants (Zeiss Stemi 2000-C). Photos of the seeds were also taken (Inverted Microscope Olimpus CKX41) to evaluate possible variations in their appearance (Supplemental Figure S3).

2.3 Seed germination assay

The studied seeds were surface sterilized by washing in 70% (v/v) ethanol for 5 min and then in 5% (v/v) household bleach for 10 min with mild rotation, followed by six rinses in sterile water. The sterilized seeds were then stratified for 3 days at 4°C in the dark on 0.7% (w/v) agar plates containing one-half-strength MS medium with additions as indicated. To determine the effects of sucrose, abscisic acid (ABA) and mannitol, the MS-agar plates were supplemented with 1% (w/v) sucrose, 2 µM ABA and 150 mM mannitol, respectively. Seeds were subsequently germinated for 3 days at 22°C under 12-h light/12-h dark photoperiod, 60% relative humidity, with 150 µmol photons m^{-2} s^{-1}. Germination tests were performed with six replicates per genotype in at least three different assays using at least 140 seeds for an individual assay on petri dishes. Radicle emergence was used as the criterion of seed germination.

2.4 Root length assay

Sterilized and stratified seeds were germinated on one-half-strength MS medium with or without 1% (w/v) sucrose as described above and grown in a vertical position for 10-14 days at 22°C, 12-h light/12-h dark photoperiod, with a light intensity of 150 µmol photons m^{-2} s^{-1} and 60% humidity. Seedling root length was measured every 48 h starting on the 3th day post exposure in the light. The images of whole seedlings on agar plates were captured and the root length was analyzed using ImageJ software (Schneider et al., 2012). At least 30 seedlings were analyzed per treatment and genotype.

2.5 Root and hypocotyl length assay in the dark

For measurement of hypocotyl and root lengths in the dark, the seeds were sterilized and stratified as previously described. After stratification in the dark, vertical plates with sterilized and stratified seeds were keep in the dark condition at 22˚C and 60% humidity. Root and hypocotyl lengths of dark grown seedlings were measured every 48 h starting on 3th days after the end of the stratification. The images of whole seedlings on agar plates were captured and the root length was analyzed using ImageJ software (Schneider et al., 2012). At least 30 seedlings were analyzed per treatment and genotype. Green light was used during evaluation, since it does not stimulate photomorphogenesis and photosynthesis (Johkan et al., 2012).

2.6 Seed collection for metabolic and respiration analyses

For metabolic analysis, the seeds used in this study were collected at the following time points: dry seeds (DS); seeds after 72 h of stratification (4 ºC, in the dark) and no imbibition in the light (0 h); seeds after 72 h of stratification followed by imbibition for 24, 48 and 72 h (at 22 ° C, 12-h light/12-h dark photoperiod, with a light intensity of 150 μmol photons m^{-2} s^{-1} and 60% humidity). For oxygen consumption measurements, the samples were collected from the same time points except for 72 h after imbibition. Moreover, stratification (4 °C for 3 days in the dark) and germination for biochemical analysis were performed on circles of Whatman paper in 8-cm Petri dish wetted with one-half-strength MS medium supplemented with 1% (w/v) sucrose (Sigma-Aldrich; pH 5.7) and without agar just to facilitate harvesting.

2.7 Determination of pyridine nucleotides

The pyridine nucleotide assays were based on the selective hydrolysis of the reduced forms (NADH and NADPH) in acid medium, and the oxidized forms (NAD^+ and $NADP^+$) in alkaline medium (Hajirezaei et al., 2002). Pyridine nucleotides were assayed using the phenazine methosulfate-catalyzed reduction of dichlorophenolindophenol in the presence of ethanol and alcohol dehydrogenase (for NAD^+ and NADH) or glucose 6-phosphate (G6P) and G6P dehydrogenase (for $NADP^+$ and NADPH) (Queval and Noctor, 2007). Imbibed seeds sampled at the time points described above were immediately frozen in liquid nitrogen and stored at –80º C until lyophilization for further analysis.

2.8 Determination of rates of oxygen consumption

The respiratory rate of dry seeds and imbibed seeds harvested at the indicated time points was measured using a Clark-type oxygen electrode (Chlorolab 2 System, Hansatech, Norfolk, UK). The electrode assembly was done according to Hapter, 2001. To the electrode disc preparation, the cathode was moistened with a drop of KCl (5%) and covered with teflon membrane to prevent bubbles. The instrument was calibrated using a solution of sodium dithionite until the curve forms a plateau which was considered as zero oxygen concentration. After 2.5 mL of mili-Q water was added to clean the chamber. Approximately 10 mg of dry seeds and imbibed seeds previously sterilized and stratified for 72 h (4 ºC, in the dark) were transferred to the chamber for O_2 consumption measurements with 2.5 mL of water per replicate. The sample measurement time was approximately 10 minutes.

2.9 Biochemical assays

Dry and imbibed seeds sampled at the time points described above were immediately frozen in liquid nitrogen and stored at –80 ºC until lyophilization for further analysis. Freeze-dried material was milled to a fine powder in a ball mill (MM2; Retsch, Hanover, Germany). About 7 mg of the fine powder was used for the extraction of the metabolites through a methanolic extraction procedure based on the gas chromatography-mass spectrometry (GC-MS) metabolite profiling protocol of Lisec *et al.*, 2006. The levels of starch, sucrose, fructose and glucose in the seed tissues were determined exactly as described previously (Fernie et al., 2001). Proteins, amino acids and malate were determined according to the methodologies present at Bradford, 1976, Cross *et al.*, 2006, and Nunes-Nesi et al., 2007.

2.10 Lipids

The samples were harvested at the indicated time points and lyophilized as described above. Total lipids were extracted using chloroform:methanol:0.1 M KCl (2:1:1, v/v/v) essentially as described (Vanhercke et al., 2013; Zhou et al., 2013) with some modification. Briefly, a known amount of freeze dried seed tissue was first homogenized in chloroform:methanol in a microfuge tube containing a metallic ball with a ball mill (MM2; Retsch, Hanover, Germany) for 3 min at 20 frequency/s, after which 0.1 M KCl was added. After beating again, the mixture was centrifuged for 5 min at 10000 g and the lower lipid phase collected in a new 1.5 mL pre-weighed microfuge tube. The

remaining phase was washed once with $CHCl_3$ and the lipid phase pooled with the earlier extract. Solvent of the lipid phase was completely dried in a laminar flow hood for approximately five hours, and further forty-eight hours at 60 ºC on a stove until constant mass. After drying, the microfuge tubes were weighed for a second time, and the difference of the two masses was used to measure the concentration of the lipids and the relative amounts based on the same dry weight of seed.

2.11 Statistical analysis

The experiments were conducted in a completely randomized design with 6–15 replicates of each genotype. Data were statistically examined by Tukey's test ($P<0.05$). All statistical analyses were performed using the algorithm embedded into Microsoft Excel.

3. Results

3.1. Arabidopsis mutants involved in extraplastidial trx system show altered growth phenotype

Several mutants impaired in TRX system were characterized in by a Arabidopsis delayed growth phenotypes (Meng et al., 2010). Thus we investigated whether mutations in the extraplastidial TRX system would affect plant growth. For that, we analyzed the mutants previously characterized NADP-TRX reductase *a* and *b* double knockout mutant (*ntra ntrb*) plant that does not express two NTR isoforms localized in the cytosol and mitochondria (Reichheld et al., 2007) and two independent lines that contained T-DNA elements inserted into the *TRX o1* gene (At2g35010) encoding a mitochondrial Trx from the Salk collection (Ortiz-Espín et al., 2017). In addition, we used two T-DNA insertion mutants, in the *TRX h2* gene (At5g39950), which encodes isoforms located in extraplastidial compartments (cytosol, mitochondria and endomembranes) (Traverso et al., 2013), described by Fonseca et al. 2017 (unpublished data). In an early experiment, mutant plants were grown side by side with their respective wild-type controls under neutral-day conditions (12-h light/12-h dark photoperiod) in order to evaluate their growth. Under this condition, *ntra ntrb* double knockout plants clearly displayed a growth retardation phenotype, while for the other mutants evaluated here the development was slightly less compromised when compared with their respective WT plants (Figure 1A). The *ntra ntrb* double knockout seedling showed delayed development until about 34 days

after germination (24 days after transplanting), whereas lines *trxh2* and *trxo1* at 38 days after germination (28 days after transplanting) already presented a similar development to the wild type. Notably, the differences gradually reduced along time in such a way that mutants did not show any obvious difference in vegetative growth phenotype compared with WT at 39 days after germination (which corresponds to the 28 days after transplanting in the figure). In spite of this acceleration at the end of vegetative growth, mutants also exhibited a significant delay in their reproductive development, which was more evident for the *ntra ntrb* plants (Figure 1B). Thus, it seems that the retardation in leaf growth and bolting is resulting in delayed growth of the inflorescence stems. There were no visible aberrant phenotypes in the mutants during reproductive period, and only the double mutant for thioredoxin reductase (*ntra ntrb*) presented a delay in the production and maturation of siliques (Fig. 1B).Altogether, these results are consistent with the important role of TRXs for plant development (Reichheld et al., 2010).

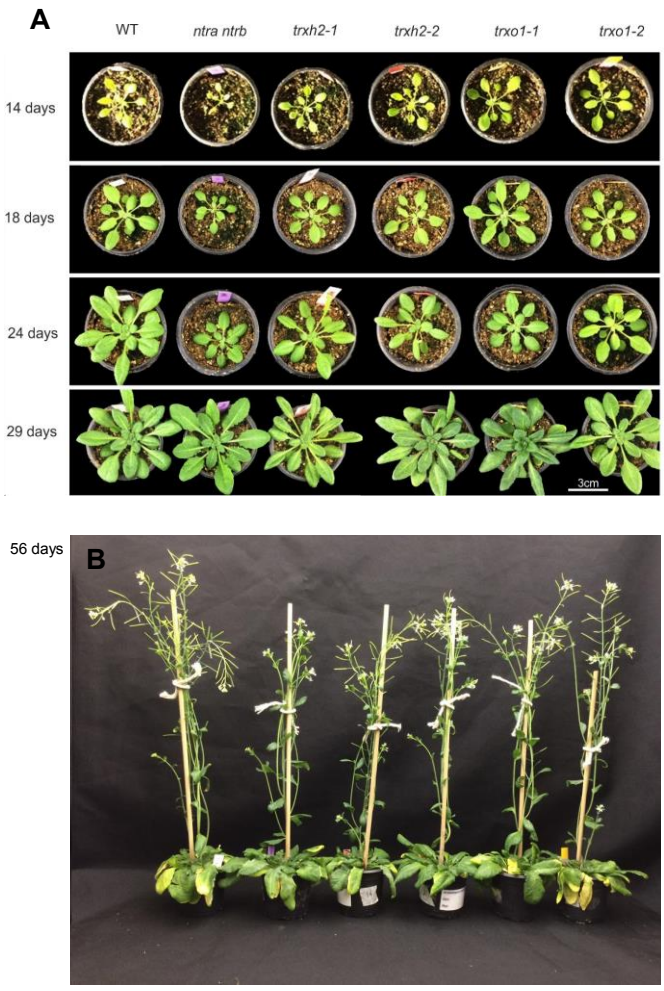

Figure1 **Growth phenotype of Arabidopsis *trx* mutants during plant development.** (A) Plants with 14, 18, 24 and 29 days after transplanting; (B) Plants with 56 days after transplanting. (The plants were germinated in medium containing sucrose for ten days. After this period they were transplanted to commercial substrate and grown at ~ 60% humidity and a photoperiod of 12 h of light and 12 h of dark. The transplant occurred 10 days after germination.

3.2. Seed and silique phenotype are altered in arabidopsis mutants involved in extraplastidial trx system

Given that the inactivation of TRX resulted in plant growth reductions, we asked whether this phenomenon is associated with seed performance. Under neutral-day conditions (12-h light/12-h dark photoperiod), all genotypes analyzed produced shorter siliques than WT plants (Table 1). Although not statistically significant, all the mutants also exhibited lower number of siliques per plant, which might be explained, at least partially, by the fact that the counting has been performed at the end of reproductive phase, showing that the accumulated energy obtained from photosynthesis and respiration was likely enough to recover the number of siliques. Moreover, *trxh2-2*, *trxo1-2* and *ntra ntrb* presented heavier seeds than those of wild-type plants, whereas only *ntra ntrb* double mutant showed a lower quantity of seeds per silique than wild type plants, confirming that extraplastidial system is important for seed production and quality in Arabidopsis.

Table I: Seed and silique phenotype observed in Arabidopsis *trx* mutants. Values presented are means ± SE. Values in boldface were determined by Student's *t* test to be significantly different ($P< 0.05$) from the wild type.

Parameters	Genotype					
	WT	trxh2-1	trxh2-2	trxo1-1	trxo1-2	ntra ntrb
Branches per plant	7.67 ± 0.42	7.20 ± 0.49	8.20 ± 0.57	6.80 ± 0.53	8.10 ± 0.35	8.80 ± 0.60
Number of siliques per plant	249.00 ± 14.84	236.10 ± 16.47	223.40 ± 6.54	237.90 ± 10.47	242.20 ± 7.59	236.40 ± 18.04
Number of seeds per silique	54.34 ± 0.81	53.65 ± 1.03	53.50 ± 1.11	52.58 ± 1.04	53.93 ± 0.90	**50.13 ± 1.27**
Seed weight (mg/1000seeds)	16.48 ± 0,32	16.57 ± 0.40	**18.45 ± 0,21**	16.19 ± 0.19	**19.07 ±0,37**	**17.75 ± 0.18**
Seed yield per plant	0.152 ± 0.009	0.154 ± 0.012	0.117 ± 0.041	0.153 ±0.010	0.155 ± 0.014	0.149 ± 0.010
Lenght silique (cm)	1.387 ± 0.030	**1.318 ± 0.026**	**1.309 ± 0.039**	**1.276 ± 0.033**	**1.282 ± 0.026**	**1.177 ± 0.026**
Lenght seed (cm)	0.0437 ± 0.001	0.0425 ± 0.001	0.0433 ± 0.001	0.0414 ± 0.001	0.0417 ± 0.001	**0.0410 ± 0.001**

3.3. Extraplastidial trx function is important for seed and root growth

To address the question of whether the extraplastidial TRX system has a functional role during the germination process of Arabidopsis, we characterized seeds with respect to germination efficiency and root growth. To this end, radicle emergence was scored daily in replicate experiments carried out in the presence and absence of 1% (w/v) sucrose, as well as in medium supplemented with mannitol (150 mM) and ABA (2 μM) (Figure 2). Analysis of germination in sucrose-free medium demonstrated that seeds of all evaluated mutant genotypes displayed a final germination percentage of nearly 100% (Figure 2A), making the assumption that all the seeds used were viable. Moreover, *ntra ntrb* double-KO plants displayed a small but significant reduction of germination in

sucrose-free medium, with no difference between the other mutants and WT under the same treatment (Figure 2B).

We further investigated whether germination would be differentially dependent on carbon by evaluating seed germination in the presence of an external carbon source. For that, we next compared seed germination rates of the mutants with those of WT in the presence of sucrose and mannitol, a sugar analogous that is not efficiently metabolized by plants. All mutant genotypes presented germination velocity and final percentage of germination clearly inferior to the wild type in the medium containing sucrose (Figures 2C and D, respectively), which indicates problems in the use of the sucrose available in the medium. For instance, in the presence of sucrose the germination of *ntra ntrb* mutants is strongly reduced (to about 45 %) with smaller reductions in *trxh2* (~ 25%) and *trxo1* (to ~ 10%) mutant lines compared with their WT counterparts (Figure 2D). By sharp contrast, only the *ntra ntrb* double mutant exhibited a delayed germination (~ 40%), while no differences were found for *trxo-1* mutants and both *trxh2* mutant lines displayed a mild increase in their germination rates in mannitol containing medium in comparison to the WT (Figure 2H). Given that double mutant plants displayed a reduced germination in both sucrose free and mannitol containing mediums, it can be suggested that reduced germination found in *ntra ntrb* mutants is only partly explained by the osmotic effect of sucrose.

Considering the close relationship between sugar and ABA signaling (Dekkers et al., 2008), the germination assay was carried out by applying exogenous ABA in order to determine if the deficient germination exhibited by the mutants in the presence of sucrose could be related to an altered ABA signaling. Confirming the inhibitory effect of ABA on germination (Penfield et al., 2006), all genotypes displayed mild reductions in their germination rates in the presence of ABA (Figure 2F). Moreover, only *ntra ntrb* mutants presented lower values of final percentage of germination in comparison to WT plants in medium supplemented with ABA (Figure 2H), indicating that *ntra ntrb* plants have higher sensitivity to the exogenous application of ABA. Collectively, germination data demonstrate that the inactivation of both *NTRA* and *NTRB* seems to be more severe than the individual absence of *TRX o1* or *TRX h2* since *ntra ntrb* mutants display retarded germination in all evaluated conditions (Figure 2) and is the only genotype producing wrinkle seeds (Supplemental Figure S2, Reichheld *et al.* 2007).

The impairment of early seedling growth in the TRX mutant plants was further investigated with root length analysis. Root growth was measured every two days in the

mutants and compared with that in WT (Supplemental Figure S3). Final root growth in seedlings was not affected in the mutants in sucrose-free medium relative to the WT (Supplemental Figure S3), whereas root growth was reproducibly reduced by up to 25% in the *ntra ntrb* double-KO plants compared with WT plants in presence of sucrose as found earlier (Reichheld et al., 2007; Daloso et al., 2015) (Supplemental Figure S3).

To provide additional information of the effect of sugars on early post-germinative growth of the *Trx* mutants, we measured both root and hypocotyl length in the dark in the presence and absence of 1% (w/v) sucrose (Supplemental Figure S4). The *ntra ntrb* double mutant presented significantly lower root and hypocotyl length in medium with sucrose, while *trxo1-1* showed significantly higher hypocotyl in the same condition. Moreover, the *trxo1-2* and *ntra ntrb* plants presented shorter hypocotyl in the dark when germinated in the medium without sucrose compared with the respective WT. Overall the mobilization of seed reserves was not constitute a problem for the mutants under study, and only the double mutant maintained a decrease in root and hypocotyl growth in medium with and without sucrose.

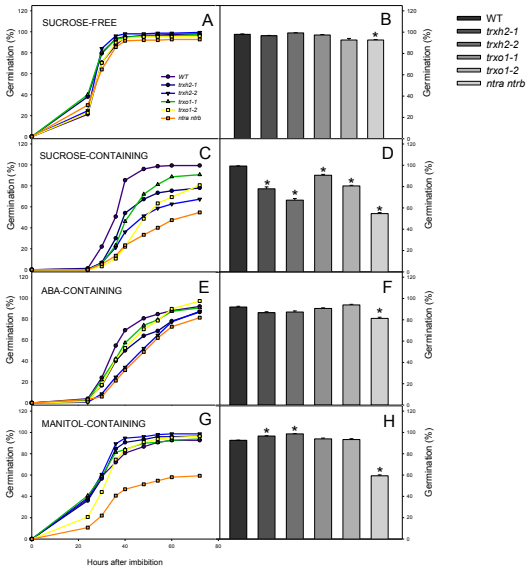

Figure 2 **Seed germination of Arabidopsis *trx* mutants.** (A) Seed germination in sucrose-free medium; (B) Final germination in sucrose-free medium; (C) Seed germination in sucrose-containing medium; (D) Final germination in sucrose-containing medium; (E) Seed germination in ABA-containing medium; (F) Final germination in ABA-containing medium; (G) Seed germination in mannitol-containing medium; (H) Final germination in mannitol-containing medium. The germination assays were performed with six replicates per genotype in at least three different assays using at least 6 seeds for an individual assay on petri dishes. Radicle emergence was used as the criterion of seed germination. Asterisks designate values that were determined to be significantly different (P<0.05) from the wild type by Student's t test.

3.4. Expression of trx genes during germination

To provide insights into dynamic changes in the expression of TRX-encoding genes under study during seed germination, we used high-resolution RNA-seq time courses (data derived from travadb.org; Klepikova et al., 2016). We examined the level of transcript of *TRXo1*, *TRX h2*, *NTRA* and *NTRB* in dry seeds and seeds collected after 24, 48 and 72 h after imbibition (HAI) in the light (Figure 3). In dried seeds the expression of the trxh2, trxo, ntra and ntrb genes was very low. During the early stages of imbibition, the reactivation of metabolism in the seed occurs, and this was reflected in the considerable increase in expression of all genes responsive to thioredoxin and reductase in study up to 24 hours. At 48 hours trxh2 and ntra maintained the increasing pattern of expression while trxo1 decreased expression and ntrb maintained. This proposes a greater cytosolic activity at this time, since NTRA is predominantly found in the cytosol. At 72 hours the expression of all genes studied was decreased. This variation in gene expression helps us to propose the predominance of some cellular processes according to their location.

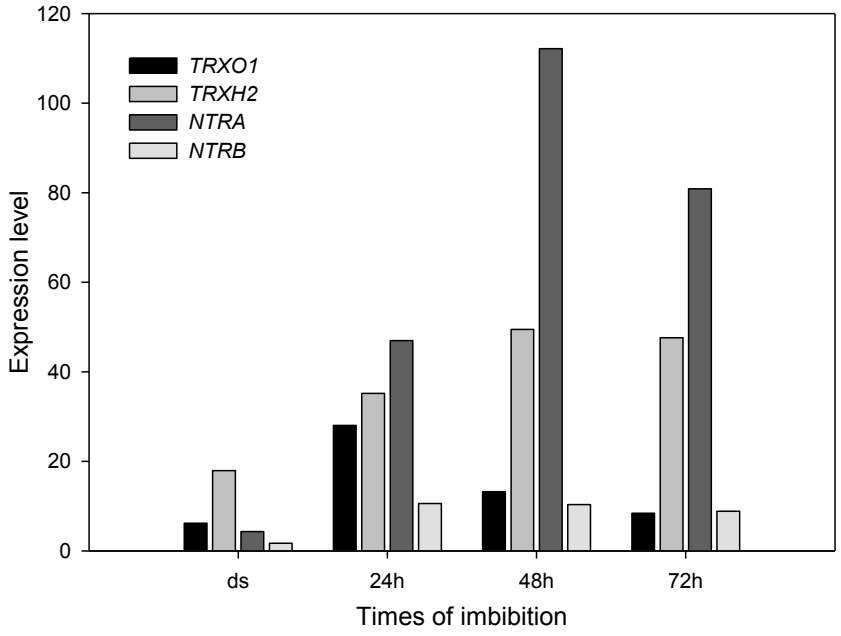

Figure 3 **Expression of genes responsive to thioredoxins and reductases of these**. These data were obtained using high-resolution RNA-seq time courses (data derived from travadb.org; Klepikova et al., 2016). The data refer to dry seeds (ds), with 24, 48 and 72 hours of imbibition in light (HAI).

3.5. Differential metabolic responses of arabidopsis trx mutants

The observed changes in seed performance and the seed germination phenotype previously commented prompted us to analyze metabolic changes over the time course of germination. For this purpose, we measured the levels of total starch, lipids, sugars, amino acids and protein on dry and imbibed seeds (in the light for 0, 24, 48 and 72 hours). In general, the mutants presented, compared to WT plants, a lower accumulation of sucrose, except in dry seeds, in which sucrose levels were higher in the two *trxo1* lines and *ntra ntrb* mutants, and seeds at 24 HAI, in which there was no significant difference between the genotypes (Figure 4B). Moreover, only *trxh2-1* mutants displayed significant lower sucrose levels at 0 HAI. It is known that sucrose is broken down in glucose and fructose either via invertase or sucrose synthase (SuSy) enzymes (Fernie et al., 2001). To evaluate the effectiveness of sucrose breakdown following germination in Trx mutants the contents of glucose and fructose were determined. These metabolites showed a similar pattern, their amounts were very low in dry seeds of all genotypes, with significant differences for mutants in the glucose content, and no detection of content in all mutants for fructose, and were increasing after the transition from vernalization to germinative conditions in all evaluated genotypes (Figure 4A and C). In spite of the similar pattern over the time, considerable variation between WT and mutant genotypes was detected in glucose and fructose levels at the given studied stages. All mutants displayed reduced content of glucose in dry seeds compared to WT. On the contrary, mutants displayed an overall higher increase in the levels of fructose and glucose after 24 and 48 HAI (except for *ntra ntrb* at 24 HAI) concomitant with a lower abundance of sucrose at 48 HAI, thus indicating a faster sucrose breakdown in the mutants in comparison to the WT during the early stages of germination. At 72 HAI, mutants exhibited significant lower levels of sucrose, glucose and fructose (except for glucose and sacarose in *ntra ntrb* mutants) than WT plants, indicating a faster utilization of sugars in the mutants.

In addition to the quantification of soluble sugars, we directly assessed starch content in dry seeds and following imbibition. No changes were observed in starch content between the genotypes in dry seeds and at 24 and 72 HAI (Figure 4D). Both *trxo-1* lines and *ntra ntrb* presented significantly less starch at 0 HAI, whereas lower amount of starch were found in all mutant genotypes at 48 HAI (not significant for *trxh2-1* and *ntra ntrb*) in comparison to WT seeds (Figure 4D). To gain a more complete perspective of the reserve accumulation and mobilization in the mutants, we also quantified lipids, the major storage reserve in oilseeds species such as Arabidopsis (Pritchard Sarah et al., 2002; Graham, 2008). Lower accumulation of total lipids were found in *trxh2-2* mutants

and *ntra ntrb* dry seeds compared with WT and, as might be expected, lipid levels progressively declined in all genotypes from 0 HAI onward (Figure 4D). In addition, the lipid content was significantly higher in *trxh2-1* and in both *trxo1* mutant lines at 48 HAI in relation to WT values, possibly indicating a compromised lipid breakdown in these mutants. It can be said that the content of lipids followed a patterning trend along the initial germination and development, and at 72 HAI no lipids were detected in the seedlings. Notably, malate levels were also greatly higher than in WT in all mutants under study at 48 HAI (Figure 4F). At dry seeds the mutants for *trxh-2* presented a discrepancy between the lines, and *trxh2-1* presented significantly lower malate contents whereas *trxh2-2* was higher in relation to WT. At 24 hours there was no difference for the mutants (except for *trxo1-1*). In addition, 0 HAI did not detect malate in any of the genotypes, and at 72 HAI this compound was not detected in both *trxh2* mutant lines, and *ntra ntrb* showed significantly less than WT.

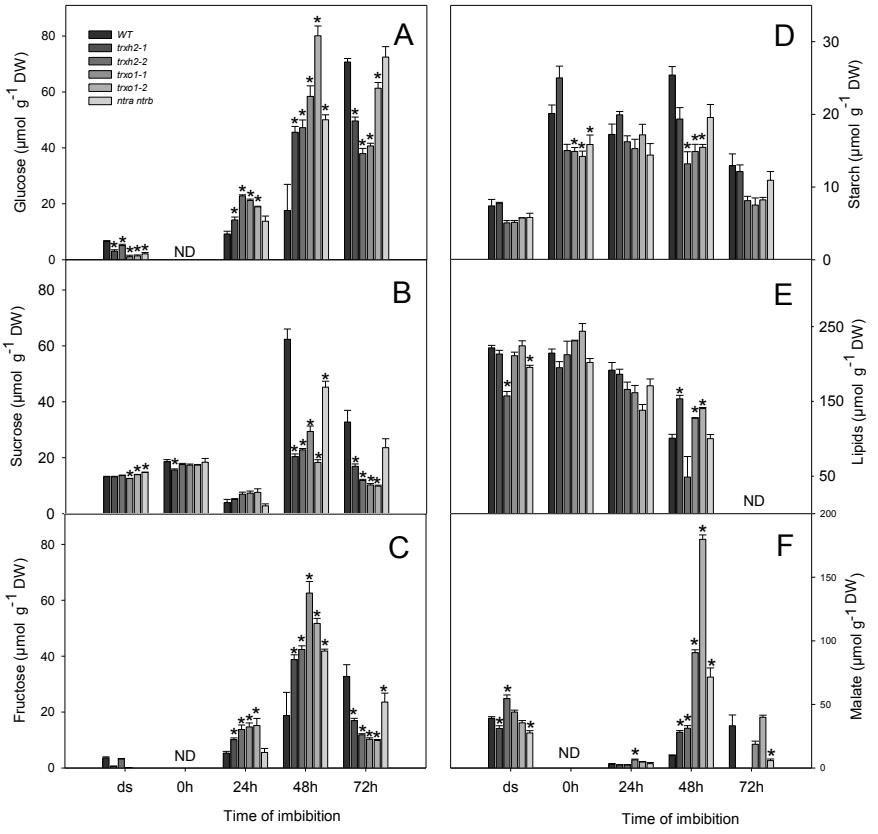

Fig.4 **Changes in the main carbon related compounds during seed germination of Arabidopsis *trx* mutants.** Levels of (A) Glucose; (B) Fructose; (C) Sucrose; (D) Starch; (E) Lipids; (F) Malate. The quantification was determined from dry seed (ds) and germinated seeds in sucrose-containing medium at 0, 24, 48 and 72 hours after imbibition. Asterisks designate values that were significantly different (P<0.05) from the wild type (WT) by Student's t test.

Overall, the levels of amino acids increased significantly in all genotypes under study at the onset of imbibition in the light (Figure 5A). In dry seeds and at 24 HAI the mutants exhibited amino acid concentration significantly higher, whereas all mutants had lower amounts of total amino acids at 72 HAI in comparison with the levels observed in the WT. At 48 HAIs only *trxh2-1*, *trxo1-1* and *trxo1-2* genotypes showed larger amounts of total amino acids. Given that many reserve proteins were demonstrated to be targets of the of TRX-mediated redox regulation (Tan-Wilson and Wilson, 2012), we next decided to examine the protein content in dry seeds and upon germination. Notably, *ntra ntrb* double mutant showed significantly lower protein content in dry seeds (Figure 5B). Considering the reduced levels of lipids (Figure 4E) and protein (Figure 5B) in the dry seeds of *ntra ntrb* plants, it can be inferred that the wrinkle phenotype previously commented (Supplemnetal Figure S2) is mostly likely associated with perturbation of the accumulation of storage components (proteins and lipids). The total protein content increased over time, with a large peak at 72 hours, both for the WT and for the mutants. Moreover, all mutants showed significantly higher protein content at 0 HAI compared with the corresponding WT. Elevated protein content was also observed in the mutants at 24 HAI (in both *trxh2*lines) and at 48 HAI (in *trxo1-2* and *ntra ntrb* mutants).

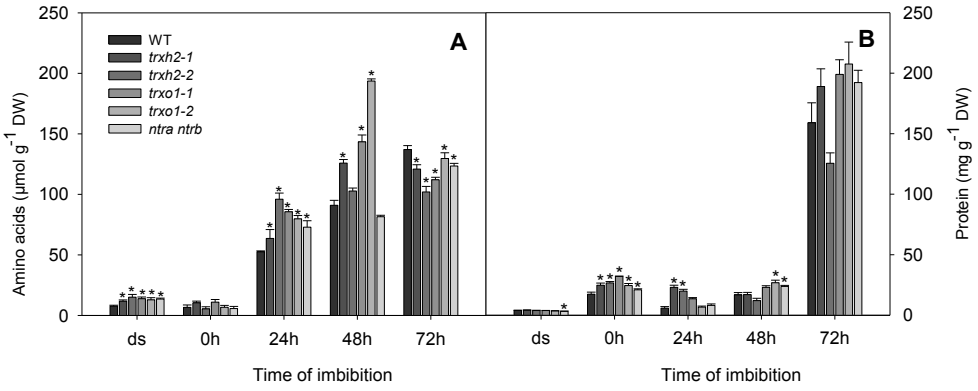

Fig. 5 **Changes in the main nitrogen related compounds during seed germination of Arabidopsis *trx* mutants. Levels of** (A) Amino acids; (B) Protein. The quantification was determined from dry seeds (ds) and germinated seeds in sucrose-containing medium at 0, 24, 48 and 72 hours after imbibition. Asterisks designate values that were determined to be significantly different (P<0.05) from the wild type (WT) by Student's t test.

3.6. Differential response of pyridine nucleotide content in arabidopsis trx mutants

We next decided to assay the levels of pyridine nucleotides in the five conditions evaluated here (Figure 6). In dry seeds, all genotypes, except of *trxo1-2* showed lower NADH contents and higher NAD^+ contents (Figure 6). At 0 HAI NAD^+ was not detected in any genotype, and at the same time the *ntra ntrb* genotype also presented no detected

amounts of NADH. The *ntra ntrb* double mutant maintained this pattern, presenting higher contents of NADH at 48 and 72 hours of imbibition in light. Mutants for thioredoxin *trxo1* showed higher NAD^+ contents at 24 and 72 hours of imbibition in light, while the *ntra ntrb* double mutant presented this behavior at 24 and 48 hours. The *trxh2* mutant lines presented higher contents of NAD^+ in dry seeds and with 72 HAI.

The content of NADPH presented a decreasing pattern in the WT, whereas in the mutants there was variation of this concentration. No NADPH was detected in seeds at 0 HAI. Interestingly, the double mutant *ntra ntrb* showed smaller amounts of this nucleotide at 24 HAI and a peak at 48 HAI compared with WT imbibed seeds. The *trxo1-2* genotype presented higher accumulations of NADPH at 24 and 48 hours. Still at 48h the mutant *trxh2-2* presented lower amount of this. At 72 HAI the *trxh2-1*, *trxh2-2* and *trxo1-1* genotypes showed inferior accumulation of NADPH. In dry seeds the genotypes *trxh2-2, trxo1-1* and double mutant ntra ntrb presented greater accumulation of $NADP^+$, whereas at 0 HAI only the *trxo1-1* genotype maintained this differential accumulation. At 48 hours, the mutant genotypes for thioredoxin h presented less amount of $NADP^+$, whereas the mutant genotype *trxo1-2* presented more amount of this. At 72 HAI all mutants accumulated less $NADP^+$, but only mutants *trxh2-1* and double mutant *ntra ntrb* showed significant values.

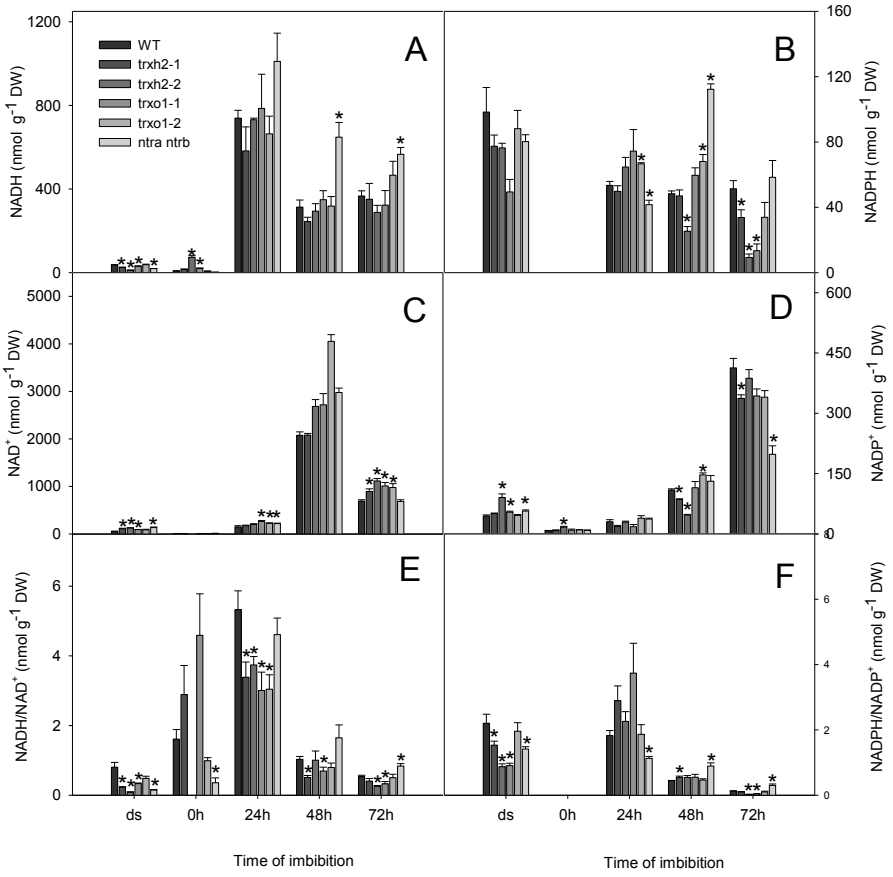

Figure 6 **Pyridine nucleotide levels and ratios during seed germination of Arabidopsis *trx* mutants.** (A) NADH content; (B) NAPH content; (C) NAD^+ content; (D) $NADP^+$ content; (E) $NADH/NAD^+$ ratio; (F) $NADPH/NADP^+$ ratio. The quantification was determined from dry seeds (ds) and germinated seeds in sucrose-containing medium with 0, 24, 48 and 72 hours after imbibition. Asterisks designate values that were determined to be significantly different ($P<0.05$) from the wild type (WT) by Student's *t* test.

3.7. The respiratory response of mitochondrial trx mutants

Considering the importance of thioredoxins in the regulation of some respiratory chain enzymes and of alternative oxidase (AOX), we evaluated respiration through the quantification of oxygen consumption in all genotypes. Regarding the changes in the respiration rates throughout development, we can evaluate an increasing pattern up to 24 hours of light exposure. At 0 HAI all mutants (except *trxh2-1* genotype) had a lower respiratory rate than the WT. At 24 hours, the *ntra ntrb* double mutant was the only one to keep the difference significantly lower compared to the WT. At 48 HAI respiration tended to decrease, but we can observe that this decrease was very smooth for the WT, whereas the mutants presented an abrupt drop in the respiratory rate. Respiration rates measured immediately at the dry seeds treatment (ds) were virtually invariant between the genotypes, however with the beginning of the imbibition and after development the

respiratory process became visibly important, indicating that mitochondrial trx deficiency has a greater impact on seed respiration.

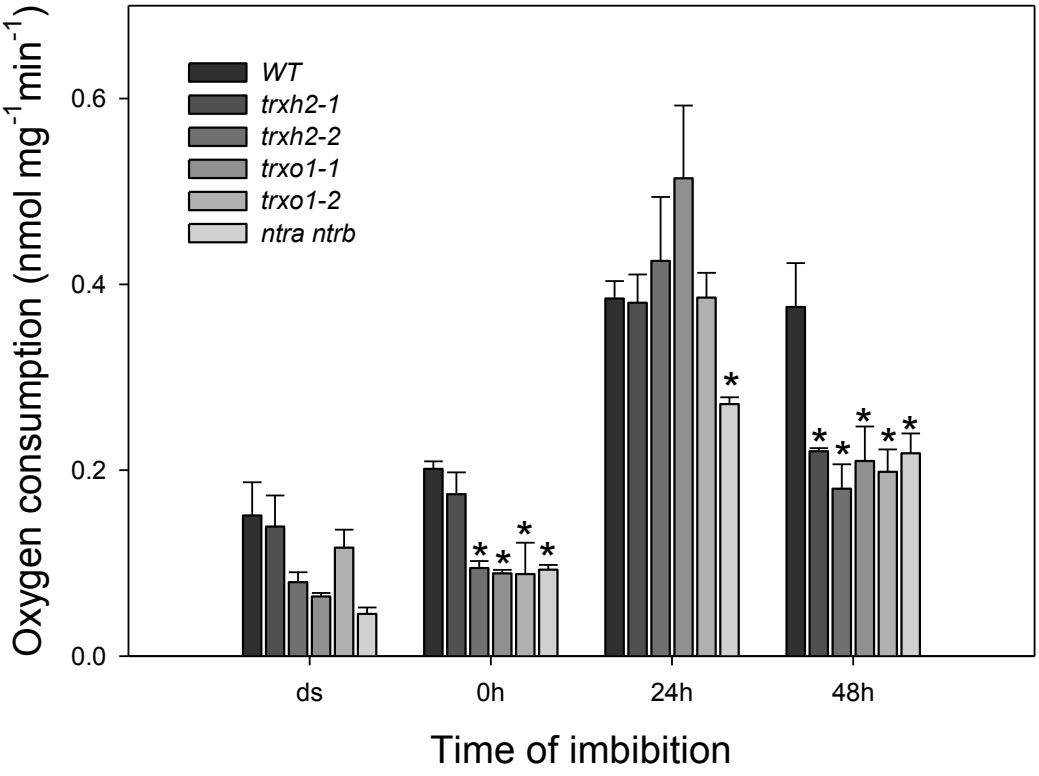

Fig.7 **Respiratory rate of Arabidopsis *trx* mutants**. The quantification was determined by Clark Electrode, from dry seeds (ds) and, germinated seeds in sucrose-containing medium at 0, 24, 48 and 72 hours after imbibition. Asterisks designate values that were determined to be significantly different (P<0.05) from the wild type (WT) by Student's *t* test.

4. Discussion

Seed germination is a complex process largely influenced by genetic factors as well as environmental conditions (Rosental et al., 2015). A growing body of evidence indicates that the germination of seeds is accompanied by extensive change in the cellular redox state (Alkhalfioui et al., 2007). Despite the presence of compelling evidence demonstrating the pivotal role of Trx system for regulation of ROS in seeds (Hägglund et al., 2016), our current understanding of the metabolic function of Trxs in regulating the redox status in seeds and upon germination remains far from complete. Here we provide further evidence of the importance of extraplastidial Trx system in contributing for primary metabolic processes that occur during seed germination in Arabidopsis.

4.1. Performance of extraplastidial thioredoxins in germination and the problems of their lack

Mobilization of protein, lipids, starch and others nutrients is particularly crucial at early stages of plant development (To et al. 2002; Tan-Wilson & Wilson 2012). There is evidence that such mobilization depends on the activation of enzymes responsible for the degradation and utilization of reserves and also of regulatory systems, such as the thioredoxin system. (Renard et al., 2011; Daloso et al., 2015). Taking into account these findings, we used mutants with reduced expression of the extraplastidial Trx system, and observed that Trx disruption leads to physiological dysfunctions manifested as (1) developmental delay in the early stages of the post-germination period (Figure1A) and (2) in the flowering period (Figure1B).

The initial period of germination demands high energy for activation of the seed metabolism and the posterior mobilization of reserves. In spite of the slight reduction in the germination rates of *ntra ntrb* mutants when deprived of an external carbon source during germination, our experiments demonstrate that *Trx* mutant seeds were able to fully germinate (Figure 2A and B) which indicates that all seeds genotypes were viable. On the other hand, we found significant differences in germination speed and final germination rate among genotypes when germination was carried out in sucrose-rich medium (Figure 2C and D). Despite the excess of exogenous metabolic substrates in sucrose-rich medium, the seed is still able to mobilize endogenous reserves, which to some extent disturbs physiological homeostasis and demands a greater degree of regulation. Excess sucrose in germination medium acts as an osmotic regulator and a carbon source (when it is broken down into glucose and fructose). In this context, we used mannitol to control for the osmotic effect. Mannitol is inert and non-toxic, and while it provides an osmotic effect analogous to that of sucrose, it is not metabolized as a carbon source (ÁVILA et al., 2007). Given that no differences were found in the germination rates in the presence of mannitol (except for *ntra ntrb*) (Figure 2G and H) it can be proposed that the osmotic effect was not responsible for the lower germination rate observed in Trx mutants in sucrose medium (Figure 2G and H). It is also known that excessive exogenous glucose is a signal for ABA accumulation (Arenas-Huertero et al., 2000; Price, 2003), which acts as a germination-inhibiting hormone. However, the germination patterns observed in sucrose-rich medium were not replicated in medium supplemented with exogenous ABA (Figure 2E and F), which can be considered as evidence that the effects of sucrose may not be attributed in

its entirety to ABA. Therefore, our results suggest (1) the lower germination rates of the mutants in sucrose-rich medium are related to the function of sucrose as a source of carbon, and (2) that mitochondrial Trx mutants struggle to utilize sucrose sources during germination (Figure 2C and D). The double mutant in general presented problems in germination under all conditions analyzed. Some aspects presented by the seeds of these mutants may be associated with lower germination rates under specific conditions. Another important factor that may explain the lower germination of the double mutants is the fact that the inactivation of both NTRA and NTRB reductases cause a decrease in cell division, leading to a delay in the emission of leaves and roots (Reichheld et al., 2007)

4.2. Metabolic reprogramming under thioredoxin system deficiency

In oleaginous plants such as Arabidopsis the main energy reserves are lipids. These lipid stores need to be mobilized from the endosperm to the embryo and converted into sugars for efficient seed availability. Seeds generally accumulate oils in the form of triacylglycerol, and the conversion of this compound into sucrose goes through a series of processes, which are in this order, β-oxidation, glyoxylate cycle, TCA cycle and gluconeogenesis (Eastmond and Graham, 2001). It is also known that TRXs are directly linked to the regulation of enzymes common to the glyoxylate cycle and TCA cycle (Graham, 2008; Geigenberger et al., 2017). The remaining energy stocks of plants are converted into soluble metabolites to be transported and support events such as growth and respiration (Eastmond and Graham, 2001). Our data show an accumulation of glucose at 24 and 48 HAI in the light, which indicates a problem in the degradation of this glucose (glycolysis) to provide energy during the development of the seedling. In line with these findings, we observed that all mutants display lower respiration that WT at 48 HAI (Figure 7), while only the double mutants were significantly different from WT at 24 hours. Taken together, these results provide evidence that Trx mutants display a disruption in glycolysis at 48HAI, as proposed by Wong et al., 2003 e Pérez-pérez et al., 2006. As expected, the effects were more pronounced in double mutants.

TRXs also act on nitrogen metabolism, thereby affecting synthesis, degradation and storage of proteins in the plant cell (Wong et al., 2004). This fact brings into light the importance of quantifying proteins and amino acids in seeds and seedlings. As expected, both proteins and amino acids accumulate during imbibition (Figure 5A and B). It can also be noted that there is an efficient conversion of amino acids into proteins, and at 72 HAI there is a greater trend towards proteins production. These findings may be explained

by the fact that at this point (72 HAI) the seedling's cellular components are under formation and require greater protein content.

By quantifying root growth in sucrose medium, we observed that the double mutants *ntra ntrb* display a reduced rate of root growth (Supplemental Figure S3 C and D). Some studies have shown a regulation of meristematic tissues by thioredoxin (Reichheld et al., 2007). Lacking two reductases of thioredoxins, NTRA NTRB, disrupts cell division and consequently leads to arrest of shoot and root growth, whereas mutants for only one of the reductases display normal growth (Reichheld et al., 2007). We speculate that the lack of both TRA reductase (A and B) causes a major deficiency in the TRX system dependent on NADPH. Our results go according to previous results, which leads us to propose that the near inactivation of the NADPH-dependent TRX system in the double mutants (ntra ntrab) disturbs the growth and development of the plants, whereas the lack of only one TRX can be compensated by the activation of other enzymes that have a similar regulatory function.

When germinated in the dark, Arabidopsis seeds rely exclusively on the mobilization of their endogenous reserves to execute germination and establishment processes, (Eastmond et al., 2000). We observed impaired root growth in *trxo1-1* and double mutants grown in sucrose medium, as compared to WT grown in the same conditions (Supplemental Figure S4 D). Similarly, our results also show deficient hypocotyl growth in *trxo1*-2 and the double mutants in sucrose-rich medium (Supplemental Figure S4 F). Only the double mutant genotype was unable to recover root and hypocotyl growth capacity in sucrose-free medium, which suggests that inactivation of the NADPH-dependent Thioredoxin system compromises the capacity of seeds to utilize endogenous substrates of some of our genotypes under study.

4.3. Effects of thioredoxins on tca cycle activity and respiration

In dry seeds the term promitocondria refers to mitochondria that display reduced metabolic activity, and that can be quickly activated a few hours after seed imbibition (Howell et al., 2006; Paszkiewicz et al., 2017). In Arabidopsis this activation occurs still in the stratification period, causing a gradual increase in respiratory activity (Paszkiewicz et al., 2017). At the time of 0 hours, which corresponds to the moment after the stratification of 72 hours in the dark at 4 ºC, the mutant seeds under study presented a compromised respiration. This may be associated with a slower pace of transition from

promitocondria to active mitochondria in Trx mutants. Double mutants exhibited deficient respiration between 24 and 48 hours when compared to WT. These findings may be explained by the fact that amino acid synthesis precursors are produced by glycolysis and the TCA cycle important steps in respiration and, the latter being directly regulated by thioredoxin. At 48 hours, all Trx mutants displayed poor respiration, which may be associated with inactivation of TCA cycle enzymes such as succinate dehydrogenase and fumarase, leading respiratory substrates to be used in other metabolic pathways. This activation and deactivation of the enzymes of the glyoxylate cycle leads to a remodeling of the energy flow that contains the substrates for respiration and other processes (Sweetlove et al., 2010)

Thioredoxins play an important role in regulating the status of several enzymes, such as MDH and citrate synthase (Kunze et al., 2006; Nunes-Nesi et al., 2013; Daloso et al., 2015; da Fonseca-Pereira et al., 2018). Our results point to an accumulation of NAD^+ in the mitochondria (Figure 6C), possibly due to deficient activity of TCA cycle dehydrogenases in the conversion of NADH to NAD^+. This would lead Trx mutants to display a lower NADH / NAD^+ ratio than wild type. The NADPH / $NADP^+$ ratio gives us the activity of the oxidative phosphate pentose pathway, where a higher concentration of NADPH means an efficient activity of this pathway. The oxidative phosphate pentoses pathway is regulated by thioredoxins in the plastids however it was not possible to observe a pattern in the NADPH/$NADP^+$ ratio specifically in this organelle (Figure 6 F).

Taken together, our results suggest that the mutants for the TRXs under study perform a metabolic reprogramming to maintain an energy metabolism capable of sustaining essential events such as germination and establishment, although delayed in relation to the wild type. Perhaps more importantly, we observed that TRX mutant plants exhibited poor respiration, which may also be related to metabolism and may be attributed to inefficient regulation of TCA cycle enzymes. In order to correctly assign the roles to known regulatory systems, in-depth studies with genes for TRXs and glutarredoxins are required, so it becomes possible to understand the specificities and redundancies of these different redox systems. Also with regard to the mobilization and use of reserves, more research is needed for more precise conclusions.

5. References

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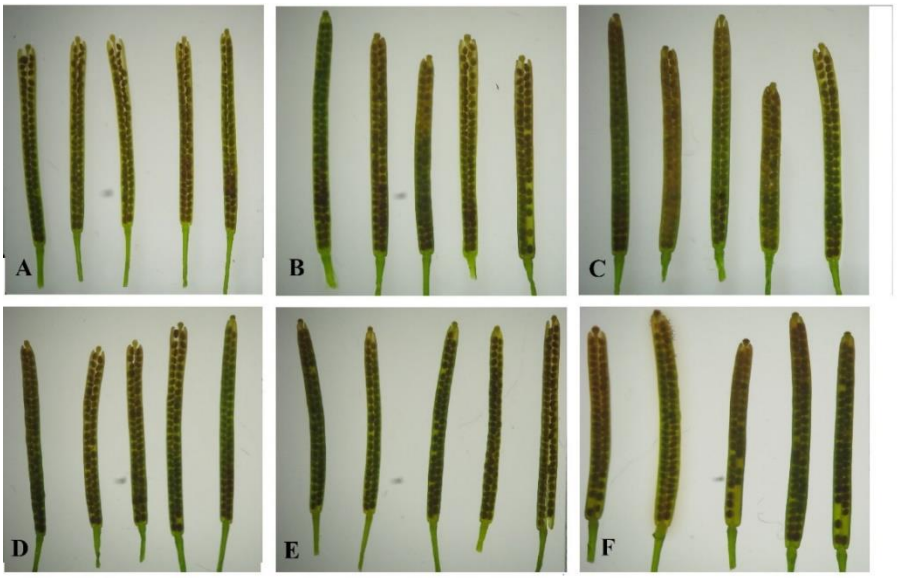
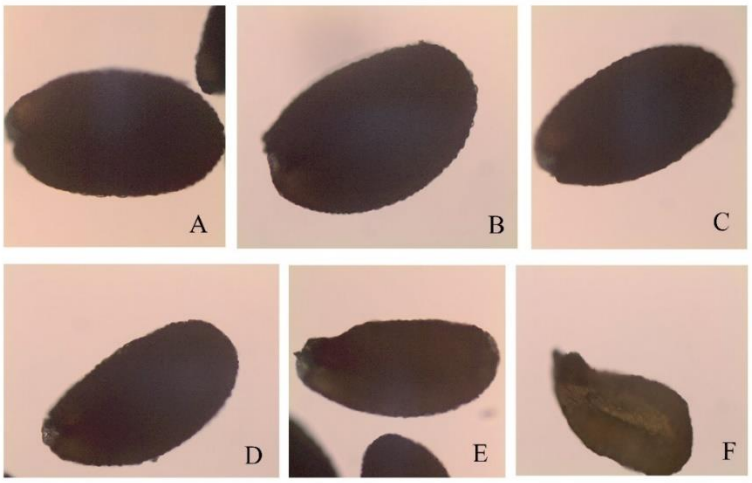
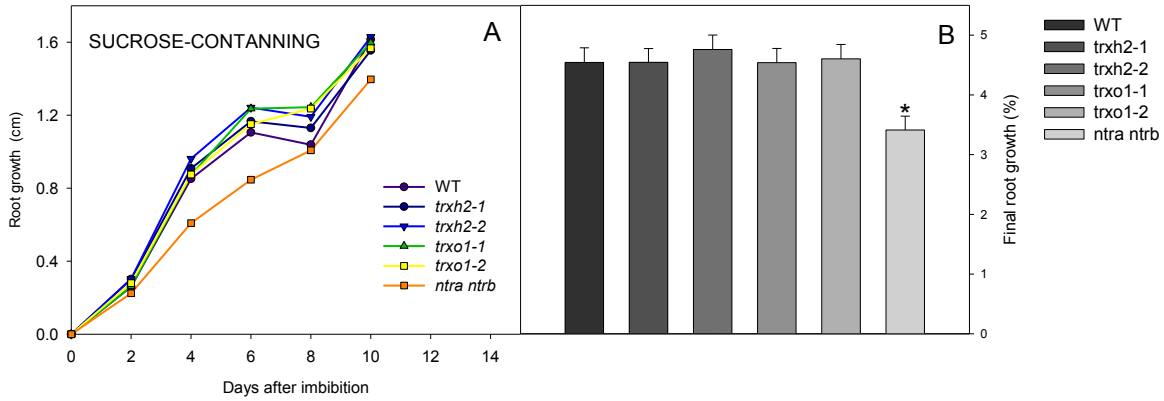
6. Supplemental figures



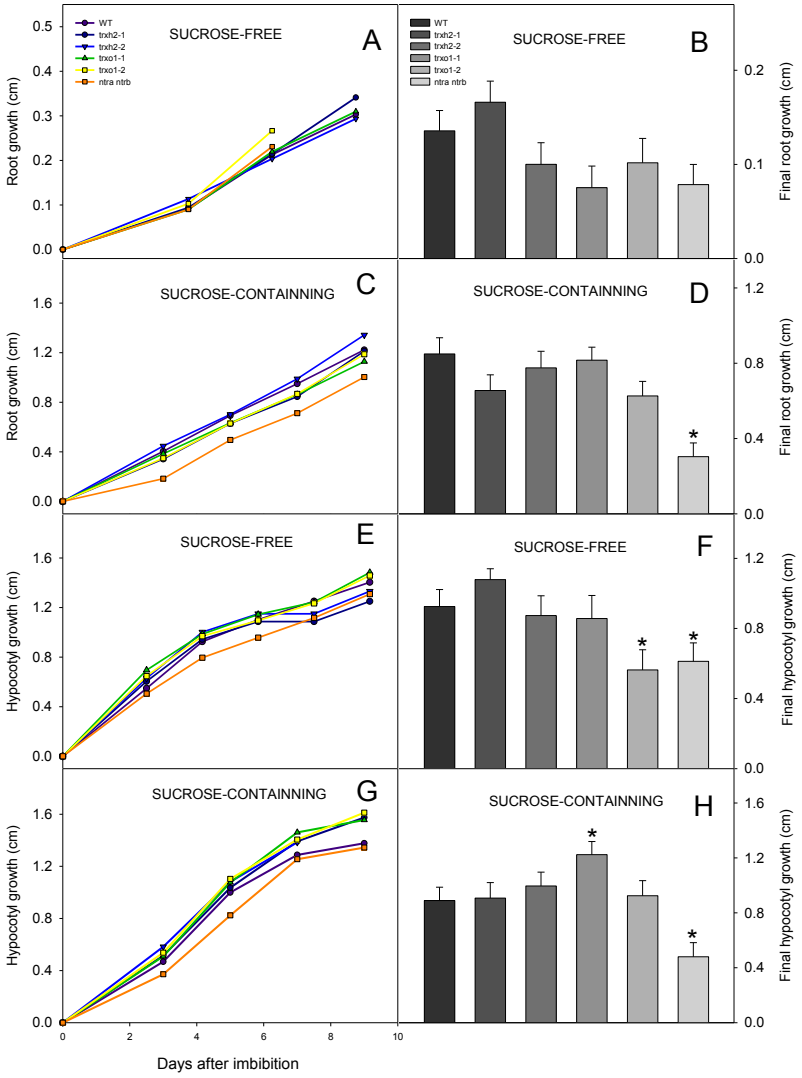
Supplemental Figure S1 **Seed development in siliques.** (A) Siliques and seeds wild type; (B) Siliques and seeds genotype *trxh2-1*; (C) Siliques and seeds genotype *trxh2-2*; (D) Siliques and seeds genotype *trxo1-1*; (E) Siliques and seeds genotype *trxo1-2*; (F) Siliques and seeds genotype *ntra ntra* double mutant. For obtain these photos on the digital camera (Canon Power- shot A650 IS), mature siliques were harvested and cleared with 0.2 M NaOH and 1% (w/v) sodium dodecyl sulfate (SDS) whose function is remove pigments.



Supplemental Figure S2 **Seed phenotype in Arabidopsis trx mutants**. (A) Seeds wild type; (B) Seeds genotype *trxh2-1*; (C) Seeds genotype *trxh2-2*; (D) Seeds genotype *trxo1-1*; (E) Seeds genotype *trxo1-2*; (F) Seeds genotype *ntra ntra* double mutant. The photos were made with dried seeds on the Inverted Microscope Olimpus CKX41.



Supplemental Figure S3 **Root growth of Arabidopsis *trx* mutants.** (A) Root growth in sucrose-free medium; (B) Final root growth in sucrose-free medium; (C) Root growth in sucrose-containing medium; (D) Final root growth in sucrose-containing medium. The data show the growth of Arabidopsis root with and without carbon source. The seeds were germinated on vertical plates in the dark and then transferred to a photoperiod of 12 h of light and 12 h of dark, and the measurements were made by 10 days in the sucrose-free medium and 14 days in the sucrose-containing medium. Root lengths were recorded every two days. Asterisks designate values that were determined to be significantly different (P<0.05) from the wild type by Student's t test.



Supplemental Figure S4 **Root and hypocotyl growth of Arabidopsis *trx* mutants germinated in dark conditions.** (A) Root growth in sucrose-free medium; (B) Final root growth in sucrose-free medium; (C) Root growth in sucrose-containing medium; (D) Final root growth in sucrose-containing medium; (E) Hypocotyl growth in sucrose-free medium; (F) Final hypocotyl growth in sucrose-free medium; (G) Hypocotyl growth in sucrose-containing medium; (H) Final hypocotyl growth in sucrose-containing medium. The seeds were germinated on vertical plates in the dark and then transferred to continuous darkness. Root lengths were recorded every two days. Asterisks designate values that were determined to be significantly different (P<0.05) from the wild type by Student's t test.