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Short communication

Putative involvement of Thioredoxin *h* in early response to gravitropic stimulation of poplar stemsWassim Azri^{a,*}, Nicole Brunel^{b,c}, Jérôme Franchel^{b,c}, Ichrak Ben Rejeb^a, Jean-Pierre Jacquot^d, Jean-Louis Julien^{b,c}, Stéphane Herbette^{b,c}, Patricia Roeckel-Drevet^{b,c}^a Laboratoire de Biologie et Physiologie Végétales, Département de Biologie, Faculté des Sciences de Tunis, campus universitaire, 1060, Tunis, Tunisia^b Clermont Université, Université Blaise Pascal, UMR547 PIAF, BP 10448, F-63000 Clermont-Ferrand, France^c INRA, UMR547 PIAF, F-63100 Clermont-Ferrand, France^d Interaction arbres microorganismes, Unité Mixte de Recherches, Faculté des Sciences, Université Henri-Poincaré-Nancy I – INRA (UMR 1136), BP 239, 54506 Vandoeuvre Cedex, France

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ABSTRACT

Gravity perception and gravitropic response are essential for plant development. In herbaceous species, it is widely accepted that one of the primary events in gravity perception involves the displacement of amyloplasts within specialized cells. However, the early signaling events leading to stem reorientation are not fully known, especially in woody species in which primary and secondary growth occur. Thirty-six percent of the identified proteins that were differentially expressed after gravistimulation were established as potential Thioredoxin targets. In addition, Thioredoxin *h* expression was induced following gravistimulation. *In situ* immunolocalization indicated that Thioredoxin *h* protein co-localized with the amyloplasts located in the endodermal cells. These investigations suggest the involvement of Thioredoxin *h* in the first events of signal transduction in inclined poplar stems, leading to reaction wood formation.

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Introduction

It is widely accepted that one of the primary events in gravity sensing in vascular plants involves the displacement or settling of dense starch-containing amyloplasts, which can occur in seconds to minutes depending on the plant tissue (Blancaflor and Masson, 2003). Studies with herbaceous plants (Kiss et al., 1997; Fukaki et al., 1998) provide evidence that perception of gravity in younger stems is mediated by sedimentable amyloplasts located in the endodermal starch sheath cells along the stem axis. In trees, sedimentable amyloplasts in the endodermal cells may play a role in gravity perception, leading to secondary xylem formation, eccentric growth and reaction wood formation in gravi-stimulated tree stems (Nakamura et al., 2001). How the displacement of amyloplasts might trigger a signaling cascade is still a matter of debate (Perera et al., 2006). Several different second messengers and proteins have been suggested to be involved in signal transduction of gravitropism (Chang et al., 2003; Perera et al., 2006; Azri et al., 2009). Reactive oxygen species (ROS) are possible second messengers, since Joo et al. (2001) have reported that production of ROS is essential for auxin-induced gravitropic signaling in maize

roots. Redox-dependent regulators are central and flexible mechanisms to control metabolic and developmental activities of the cells. Thioredoxins (Trxs) are 12 kDa proteins that contribute to the redox control by dithiol/disulfide exchange. In plants, a large number of genes encode Trxs, and 19 different isoforms have been identified in *Arabidopsis thaliana* and were grouped in six subfamilies: the Trxs *f*, *h*, *m*, *o*, *x* and *y* (Buchanan and Luan, 2005). Recently, plastidial thioredoxins from the z-type have been described (Chibani et al., 2011). Trxs *f*, *m*, *x* and *y* are localized in chloroplasts, whereas Trxs *o* are found in mitochondria. Trx *m* was detected in amyloplasts from wheat starchy endosperm (Balmer et al., 2006). Thioredoxins *h* were first found in the cytoplasm compartment, then some isoforms were purified from mitochondria and endoplasmic reticulum, and nuclear localization has also been reported (Buchanan and Balmer, 2005). Trxs *h* are encoded by a multigenic family of 8 genes in *A. thaliana*, and at least five in *Populus* sp. (Gelhay et al., 2004). Trxs *h* are involved in multiple processes, such as reserve breakdown that sustains early seedling growth of germinating cereal seeds (Wong et al., 2002), or self-incompatibility (Cabrilac et al., 2001), in carbon and nitrogen metabolism (Wong et al., 2003). They are also implicated in the cellular protection against oxidative stress, in particular during seed desiccation and germination (Serrato and Cejudo, 2003). Furthermore, Trxs *h* are electron donors to several enzymes involved in the protection against oxidative stress such as

* Corresponding author. Tel.: +33 21697243698.

E-mail addresses: azwassim@yahoo.fr, azwassim@voila.fr (W. Azri).

Table 1
Putative or established Thioredoxin targets among differentially expressed proteins expressed in poplar stems after gravi-stimulation.

Putative or established Thioredoxin targets			
40S ribosomal protein S12	2	GSH-dependent dehydroascorbate reductase 1 (EC 1.8.5.1)	1
Actin 3	1	HSP90 co-chaperone p23	2
Adenosine kinase	2	Malate dehydrogenase, NAD-dependent (EC 1.1.1.37)	1
ATP synthase, beta subunit (EC 3.6.3.14)	1	Molecular chaperones HSP70 superfamily	1
Tubulin alpha-5 chain	1	Nucleoside-diphosphate kinase 1 (EC 2.7.4.6)	1
Beta tubulin	1	Oxygen Evolving Enhancer 1	1
Calreticulin 1	2	Oxygen Evolving Enhancer 2	2
Chaperonin, Cpn60/Hsp60p (3.6.4.9)	1	Pyruvate dehydrogenase E1 Beta subunit isoforme 1 (EC 1.2.4.1)	1
Chlorophyll A/B binding protein precursor	2	RuBisCO large subunit (EC 4.1.1.39)	1
CuZn-superoxide dismutase (EC 1.15.1.1)	1	S-adenosylmethionine synthetase (EC 2.5.1.6)	1
Elongation factor 1-alpha	1	Translationally controlled tumor protein	1
Glutathione s-transferase (EC 2.5.1.18)	1	Triosephosphate isomerase (EC 5.3.1.1)	1

The complete list of differentially expressed proteins following gravistimulation of poplar stems was presented by Azri et al. (2009). Among these proteins, some were putative or established Thioredoxin targets according to Buchanan and Balmer (2005) [1] and/or Montrichard et al. (2009) [2].

peroxiredoxin, methionine sulfoxide reductase, and glutathione reductase (Rouhier et al., 2001; Gelhaye et al., 2003; Jung et al., 2002). In addition to their role as antioxidant, Trxs *h* was proposed to be involved in modulating redox-dependent signaling cascades (Dietz, 2003).

Starting from the observation that many proteins involved in gravitropic response are potential Thioredoxins targets, we investigated the expression and localization of thioredoxin *h* in the early response to gravitropic stimulation in poplar stems.

Materials and methods

Plant material and culture conditions

Hybrid poplar (*Populus tremula* × *Populus alba*), clone INRA n^o. 717-1-B4 was multiplied clonally *in vitro* on Murashige and Skoog medium (Murashige and Skoog, 1962), acclimatized in hydropony (Herbette et al., 2004), and grown in a controlled environment growth chamber (16 h photoperiod at 60 μmol m⁻² s⁻¹, 22 °C/18 °C (day/night) and 70% of relative humidity). At the 14 internodes stage, the poplars showing straight stems were transferred on a new device for tilting (Azri et al., 2009).

After one week on the device in a straight position, poplars showing 20 developed internodes were inclined at 35° from the vertical axis for 0, 10, 20, 30, 45 min, 1 h, 3 h, and 6 h as described earlier by Azri et al. (2009). Starting from the base, internodes 1–5 (showing preponderant secondary growth) were harvested from inclined and non-inclined plants, frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

RNA extraction and real-time RT-PCR experiments

For each time of the kinetics, the basal portions of two stems were used. Total RNA was extracted according to the method of Chang et al. (1993) and then treated with RNase-free RQ1 DNase (Promega, Charbonnières-les-Bains, France). RNA concentration and quality were determined at 260 and 280 nm using the NanoDrop1000™ spectrophotometer (Thermo Fisher Scientific, Wilmington, U.S.A.) and checked by agarose gel electrophoresis.

The real-time RT-PCR amplifications were performed according to Mai et al. (2009). Trx *h* transcripts were detected by amplifying 178 bp with primers TrxF 5'-AGGGAAAAGGGTCTCAGAAA-3' and TrxR 5'-ATTGCCTCCACATTCCAATC-3'. These primers detected transcripts corresponding to the Trx *h* protein that was upregulated in basal internodes of *Populus tremula* × *Populus alba* after 1 week inclination (Azri et al., 2009) and similar to POPTR.0005s25420.1 (Phytozome <http://www.phytozome>.

Net - JGI v2.2). The reference genes 18S RNA and Ubiquitin transcripts (POPTR.0012s01250, Phytozome) were amplified using the primers 18SF 5'-CTTCGGGATCGGAGTAATGA-3', 18SR 5'-GCGGAGTCCTAGAAGCAACA-3', and UbiF 5'-CCC GGCTCTAACCATATCCA-3', UbiR 5'-GGGTCCAGCTTCTTGACAGTC-3', respectively. The reference genes were combined into an index using the BestKeeper software tool (<http://www.wzw.tum.de/gene-quantification/bestkeeper.html>) (Pfaffl et al., 2004). Target gene abundance was conventionally normalized using this BestKeeper Index (I) using the delta-delta method mathematical model (McMaugh and Lyon, 2003).

Histochemical and immunolocalization analyses

After 45 min inclination, basal stem portions were prepared for histochemical analysis as described previously (Azri et al., 2009). Briefly, stems portions were fixed in a solution containing 3.7% (v/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol, for 4 h at 4 °C. The samples were then dehydrated and embedded in L.R.-White resin (Sigma–Aldrich). Semi-thin sections were performed and the Periodic acid/Schiff (PAS) reaction was used to detect starch and polysaccharides (Jensen, 1962). Stained sections were dried, mounted in Eukitt (Kindler GmbH & Co, Freiburg, Germany) and examined under a Zeiss Axioplan 2 microscope. Data were recorded

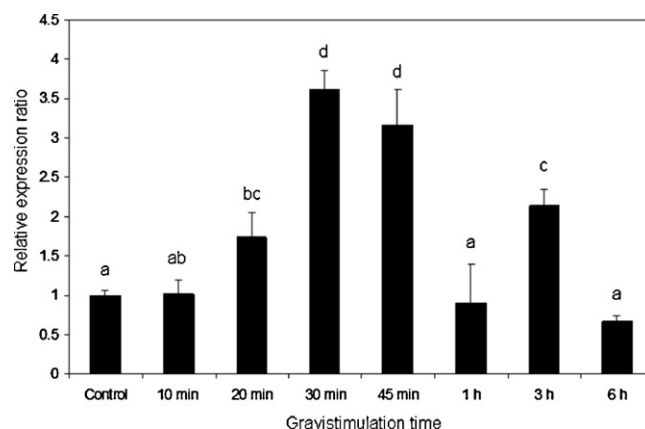


Fig. 1. Time course accumulation of Trx *h* after gravitational stimulus. Total RNAs were extracted from basal internodes of inclined plants for 0 (control), 10, 20, 30, 45 min, 1 h, 3 h and 6 h. The accumulation of relative transcripts was determined by RT-qPCR. Mean values (+SE) of three technical replicates are shown. For each time of the kinetics, one plant is analyzed. Similar results were obtained on a second series of plants (data not shown). Data were analyzed using ANOVA (Stagraphics Plus, version 5.1). Different letters indicate significant differences ($P < 0.05$) for Fisher's LSD pairwise comparisons.

using a digital camera (AxioCam HR, Zeiss) with AxioVision digital imaging software.

Samples embedded in L.R.-White resin for histochemical analysis were also used for *in situ* immunolocalization as described by Dusotoit-Coucaud et al. (2010). The primary antibody directed against the protein Trx *h1* (Rouhier et al., 2001) from *Populus tremula* X *Populus tremuloides* (similar to POPTR_0005s25420.1) was provided by Dr. Rouhier at the University Henry Poincaré, Nancy, France. The goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma, Saint-Quentin Fallavier, France) was used as secondary antibody. For the revelation, sections were incubated with chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, Marnes-la-Coquette, France). Color development was stopped by washing in H₂O. Sections were then mounted onto microscope slides, air dried and covered with cover slips for microscopy using Eukitt mounting medium.

Results and discussion

The data presented here suggest a role of Trxs *h* in the early response to gravistimulation of poplar stems, in relation with the amyloplast-linked mechanism of gravity sensing.

Earlier expression studies carried out at the proteomic level on proteins from control and inclined poplar stems showed that about 40% of the proteins analyzed undergo significant changes following gravi-stimulation (Azri et al., 2009). The development of proteomic tools led to the identification of many Trxs *h* potential targets (Yano et al., 2001; Maeda et al., 2003; Marx et al., 2003; Yamazaki et al., 2004).

Here, we observed that among 60 spots that were successfully identified, 36 spots were potential targets of Trxs, according to the listings established by Buchanan and Balmer (2005) and Montrichard et al. (2009). These 36 spots represented 24 proteins (Table 1). Among the 24 proteins listed, 5 are plastidial proteins.

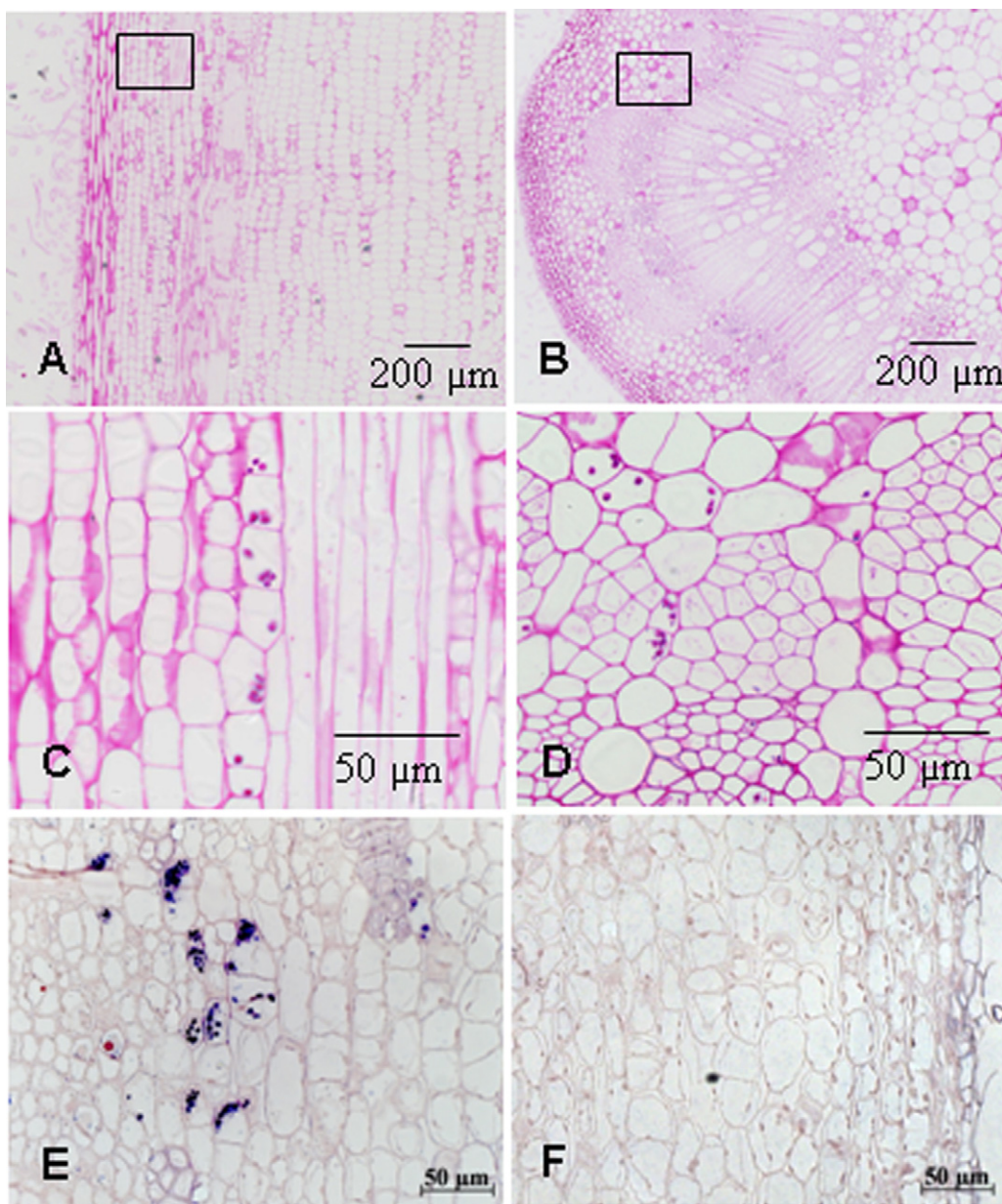


Fig. 2. Sections of poplar stem (4th internode) inclined during 45 min. Longitudinal sections (A, C) or transversal sections (B, D) were stained using Periodic acid/Schiff (PAS) reaction to detect starch and polysaccharides. Photographs C and D are respectively enlarged views of the photographs A and B (black rectangle area). Transversal sections were probed with the antibody anti-Trx *h1* (E) or incubated without primary antibody as a negative control (F).

Previous reports have listed chloroplastic proteins as cytosolic thioredoxin targets. Yamazaki et al. (2004), investigating possible cytosolic thioredoxin targets in *A. thaliana* cells, found some chloroplast proteins and suggested that they came from plastid contaminants. The observation that most proteins regulated after gravi-stimulation are probable Trxs *h* targets was also made in *Populus euphratica* Oliv. upon heat stress (Ferreira et al., 2006). The proteins, potentially regulated both by gravi-stimulation and Thioredoxins, are involved in various processes (Table 1, Azri et al., 2009), among which are cytoskeleton organization and biogenesis (actin-3, Tubulin-alpha-5-chain, Beta-tubulin). The displacement of amyloplasts following gravi-stimulation may modify the cell wall-plasma membrane-cytoskeleton structural continuum and trigger signaling cascades (Perera et al., 2006). We assume that Trx *h* may have a particular role in signal transduction after amyloplast displacement in the cytosolic compartment.

Trx *h* expression was induced by gravitropic stimulation. Indeed, the real-time quantitative RT-PCR analysis showed two significant increases of Trx *h* mRNAs: from 20 to 30–45 min, and 3 h after gravistimulation, in basal internodes (Fig. 1). An increase in Trx *h* mRNA expression levels has also been observed following salt treatment of rice seedlings (Zhang et al., 2011). Such regulation of Trxs suggests that redox balance is affected in the early step of gravi-stimulation. We previously observed that Thioredoxin *h* was also expressed one week after stem inclination (Azri et al., 2009). This is in agreement with the finding that production of ROS is essential for auxin-induced gravitropic signaling in maize roots (Joo et al., 2001, 2005).

Using the Periodic acid/Schiff (PAS) reaction to detect starch and polysaccharides, we observed amyloplast sedimentation at the base of starch sheath cells of poplar stems (Fig. 2A and C). In agreement with our observations, Nakamura et al. (2001) found that the upright reorientation of woody stems of Japanese Cherry was correlated with the sedimentation of the amyloplasts at the base of endodermal cells. Using an *in situ* immunolocalization approach, Trx *h1* co-localized with the amyloplasts of endoderm cells of the stem (Fig. 2E). A control section incubated without primary antibody (Fig. 2F) confirmed the specificity of the signal. The endoderm, which contains the amyloplasts, has been suggested as essential for shoot gravitropism (Fukaki et al., 1998). Since Trx *h* are expected in the cytoplasm compartment, the co-localization of Trx *h1* with the amyloplasts might indicate that Trx *h* targets are found in the outer membrane of the amyloplasts envelope. These targets may be essential for transduction of the gravitropic signal. Isolation and characterization of proteins from this outer envelope that interact with Trx *h* could aid in understanding the signaling cascade following amyloplast displacement. We cannot exclude the possibility that the antibody anti-Trx *h1* detected the presence of Trx *h* inside the amyloplasts. To date, only Trx *m* has been detected in isolated amyloplasts from wheat endosperm (Balmer et al., 2006). Thus, Trxs could act as regulatory links between the redox signal generated by gravitropic stimulation (Joo et al., 2001) and metabolic processes in amyloplasts. For example, Kolbe et al. (2005) have shown that cytoplasmic trehalose metabolism and sugar utilization in amyloplasts are linked *via* Thioredoxin-mediated redox transfer.

In conclusion, our results support the involvement of Thioredoxin *h* in the early response to gravitropic stimulus leading to reaction wood formation and poplar stem reorientation. Further studies need to be done to discriminate among the Thioredoxin *h* isoforms involved. Indeed, Trxs *h* form a large and diverse group of protein disulfide reductases, but the function of each isoform is still unclear (Gelhay et al., 2004). The next step would be to identify the components interacting with amyloplast associated-Thioredoxin in order to dissect the events linked to gravity sensing.

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