Putative role of proteins involved in detoxification of reactive oxygen species in the early response to gravitropic stimulation of poplar stems

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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 signaling cascade leading to stem reorientation is not fully known especially in woody species in which primary and secondary growth occur. Several different second messengers and proteins have been suggested to be involved in signal transduction of gravitropism. Reactive oxygen species (ROS) have been implicated as second messengers in several plant hormone responses. It has been shown that ROS are asymmetrically generated in roots by gravistimulation to regions of reduced growth. Proteins involved in detoxification of ROS and defense were identified by mass spectrometry: i.e., Thioredoxin h (Trx h), CuZn superoxide dismutase (CuZn SOD), ascorbate peroxidase (APX2), oxygen evolving enhancer 1 (OEE1), oxygen evolving enhancer 2 (OEE2), and ATP synthase. These differentially accumulated proteins that correspond to detoxification of ROS were analyzed at the mRNA level. The mRNA levels showed different expression patterns than those of the corresponding proteins, and revealed that transcription levels were not completely concomitant with translation. Our data showed that these proteins may play a role in the early response to gravitropic stimulation.

Introduction

Plants can reorient their growth direction by sensing organ tilt relative to the direction of gravity. With respect to gravity sensing in gravitropism, the classic starch statolith hypothesis, i.e., that starch-accumulating amyloplast movement along the gravity vector within gravity-sensing cells (statocytes) is the probable trigger of subsequent intracellular signaling, is widely accepted.

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. How the displacement of amyloplasts might trigger a signaling cascade is still a matter of debate. Several different second messengers and proteins have been suggested to be involved in signal transduction of gravitropism.

Although ROS such as superoxide anions and \( \text{H}_2\text{O}_2 \) are generally considered to be toxic by products of respiration, recent evidence suggests that the production of ROS might be an integral component of intracellular signaling. Production of ROS occurs in response to many physiological stimuli such as during stomatal closure, adventitious root development and root gravitropism. The excess production of ROS under biotic and abiotic stresses causes oxidative damage to cellular compartments. Plants combat oxidative stress by inducing various protective enzymes and anti-oxidants. In mammalian cells, a variety of extracellular stimuli have been shown to induce a transient increase in the intracellular concentration of ROS, and specific inhibition of the ROS generation results in a complete blockage of stimulus-dependent signaling. ROS have been implicated as second messengers in several plant hormone responses. Joo et al. showed that ROS are asymmetrically generated in roots by gravistimulation to regions of reduced growth. A function for ROS in root curvature was reported by inhibiting cell growth, thus contributing to tropisms. Auxin also induced ROS production in roots and the auxin transport inhibitor \( \text{N}-1-\text{naphthylphthlamic acid} \) did not inhibit hydrogen peroxide (\( \text{H}_2\text{O}_2 \))-induced root curvature, leading to the suggestion that ROS play a role downstream of transport in auxin signaling and gravitropism.

However, the potential of ROS as a second messenger in gravitropism is still unclear. The implication of ROS in response to 45 min of inclination is consistent with the fact that we...
The mRNA levels of **Trx h**, **ATP synthase**, **OEE1**, **OEE2**, **CuZn SOD**, **APX2** showed different expression patterns than those of the corresponding proteins. The result of the present investigation is supported by the previously established concept that transcription patterns are not directly concomitant with protein expression levels.

**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. 2A). Increase in **Trx h** mRNA expression levels has also been observed following salt treatment of rice seedlings. Such regulation of Trxs suggests that redox balance is affected in the early step of gravi-stimulation. We previously observed that **Trx h** was also upregulated one week after stem inclination. This is in agreement with the finding that production of ROS is essential for auxin-induced gravitropic signaling in maize roots.

Interestingly, the energetic pathway seems to be affected by gravitational stimulation. The real-time quantitative RT-PCR analysis showed two significant increases of **ATPase** mRNAs: from 30 min and 3 h after gravistimulation, in basal internodes (Fig. 2C). After 45 min of inclination, among the few genes that were downregulated at the base of the stem, we have found mitochondrial **ATP synthase β** chain. Indeed, genes coding for subunits of **ATP synthase** increased in their transcript abundance following gravitational stimulation of Arabidopsis root.

Alternatively to the energetic hypothesis, it has been postulated that the **ATP synthase** might be a response related to the detrimental effect caused by an oxidative stress in the synthesis of ATP, because ATP synthase catalyzes key phosphorylation reactions associated with aerobic catabolism. In these past years, the involvement of oxidative burst plant defense genes in the gravitropic response has been evoked. The implication of ROS in response at the inclination is consistent with the fact that we identified a cytosolic CuZn SOD and APX at the base of the stem. These ROS serve as substrates in metabolism and act as signals during development. However, other evidence suggests that ROS play significant roles in intracellular signaling in gravitropism in maize. **CuZn SOD** plays a central role in protecting against oxidative stress. It is interesting that it showed a downregulation pattern in response to gravistimulation. Consistent with our results, CuZn SOD also has been shown to be downregulated at the protein level by several oxidative stresses. RT-PCR analysis showed significant increases of **CuZn SOD** mRNAs: from 20, 30, 45, 1 h and 3 h after gravistimulation, in basal internodes (Fig. 2E). This upregulation began 20 min after inclination and peaked at 30 min. It has

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**Results and Discussion**

ROS have been implicated as second messengers in several plant hormone responses. However, the excess production of ROS under biotic and abiotic stresses causes oxidative damage to cellular compartments. Plants combat oxidative stress by inducing various protective enzymes and anti-oxidants. In Poplar stem, a number of protein spots involved in detoxification of ROS and defense were identified previously: Thioredoxin h (**Trx h**) (spot n° 6), i.e., superoxide dismutase (CuZn SOD) (spot n° 2), ascorbate peroxidase (APX2) (spot n° 1), OEE1 (spot n° 4), OEE2 (spot n° 3), and ATPase (spot n° 5) (Table 2). These proteins were selected in order to investigate their expression patterns, in response to gravistimulation at the mRNA level for kinetics 0, 10, 20, 30, 45 min, 1 h, 3 h and 6 h (Fig. 2).
been shown that APX is highly sensitive to inactivation by ROS and is often insufficient to protect the photosynthetic apparatus from photoinhibition during severe drought stress. The absence of APX2 results in reduced photosynthetic activity, during light stress and altered stomatal responses. In our study the APX2 was present in the control but not detected after inclination 45 min. No upregulation of the transcripts APX2 occurred after inclination, as demonstrated by comparison with the control (Fig. 2B). APX2 expression significantly decreased at 20 min, 30 min, 3h and 6h after inclination. The downregulation of the transcripts OEE1 (which is bound to photosystem II) might also be interpreted as a response to oxidative stress caused by inclination. It has been shown that deviation from regular redox homeostasis can be sensed in chloroplasts and that specific chloroplast signals control nuclear gene expression. The decrease in OEE1 transcript (Fig. 2D) could be a consequence of such signaling and thus would impair oxygen-evolving activity and photosystem II stability. No upregulation or a slight decrease in OEE2 expression was observed at 1h and 6h after inclination (Fig. 2F). OEE2 was identified as the molecule inducibly phosphorylated by AtGRP-3 and WAK1. In addition, the phosphorylation of OEE2 was enhanced in Arabidopsis infected with avirulent Pseudomonas syringae. These data suggest that OEE2 is a molecule downstream of AtGRP-3/ WAK1, possibly in defense signaling. It is currently investigating whether AtGRP-3/ WAK1 signaling is related to the production of ROS such as H₂O₂ and O₂⁻ from chloroplasts under stress conditions.

Involvement of oxidative stress proteins in the gravitropic response was to some extent unexpected, although rapid non-pathogen-related induction of the oxidative stress is known to occur in response to wounding, extreme temperatures, UV irradiation, salt, and osmotic and mechanical stimulation. The oxidative stress proteins have not yet been considered to play a role in gravitropism, except for the recent study on role of auxin-induced ROS in root gravitropism. Joo et al. demonstrated that gravity-induced asymmetric ROS generation in roots of maize (Zea mays), unilateral application of ROS to vertical roots stimulated root bending, and scavenging of ROS by antioxidants inhibited root gravitropism. Theses results support our findings of involvement of oxidative stress genes in the gravitropic response. Our data showed an implication of ROS signaling in the decurving process via Trx h, Cu/Zn SOD, APX2, OEE1, OEE2, and ATPase. It was however not surprising that ROS could participate in gravitational stress signal integration, because ROS in plants act as regular signal transducers in various processes. How molecular mechanisms involving ROS was integrated into a physiological signal that leads to the gravitropic response remains to be elucidated.

### Materials and Methods

#### Plant materials

Hybrid poplar (Populus tremula x Populus alba), clone INRA n° 717-1-B4 was multiplied clonally in vitro on Murashige and Skoog medium, acclimatized in hydropony, and grown in a controlled environment growth chamber (16h 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. After one week on the device in a straight position, poplars showing 20 developed internodes were inclined at 35° from the vertical axis for 45 min, as described early by Azri et al. Starting from the base, internodes 1 to 5 (showing preponderant secondary growth) were harvested from inclined and non inclined plants, frozen in liquid nitrogen and stored at -80°C until protein and RNA extraction.

#### Protein extraction and 2-DE analysis

Trees were leaned for 45 min. Upright trees were used as controls. Two independent biological replicates were performed using five leaned trees and five upright trees. For each replicate, base internodes from five leaned and five upright trees were sampled. The internodes from 10 trees representing each sample type were pooled and ground in liquid nitrogen. From each pooled sample three replicate protein preparations were isolated. Total protein were extracted and identified as described early by Azri et al. The silver-stained gels were scanned using a densitometer GS-800 (Bio-Rad). Spot abundance was normalized as a relative volume to compensate for differences in sample loading and gel staining, using the PDQuest software. The spots whose quantity varied significantly after inclination were numbered (Fig. 1). After automated detection and matching, manual editing was performed. Triplicate gels using independent protein preparations were analyzed for each condition. Statistical analyses were performed using the Student’s t-test (p < 0.05).

#### RNA extraction and real-time RT-PCR experiments

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, 1h, 3h, and 6h as described earlier.

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**Table 1. List of primers used for RT-qPCR experiments**

| Gene     | Primer for            | Primer rev        | Size (bp) | Tm (°C) |
|----------|-----------------------|-------------------|-----------|---------|
| APX2     | GGA CGA TCA GAC ACC CAG AT | CCT TCT GGA GGT GGA TCA GA | 214       | 58      |
| Cu/Zn SOD | TGG CAC CAT CTT CTT GAC AC | TGA CAT TTC CCA GAT CAC CA | 215       | 59      |
| OEE2     | GCA GGC AGT ACA GGA AGA GG | TCA GAA CCT GAC CGG AGA AC | 248       | 61      |
| OEE1     | GTG GTG CCT TCT AGG ACC AG | TCG GAA CTC CCT CAG CAC TT | 179       | 61      |
| ATPase   | CAG TCA ATC CGG TTG GTT CT | TGG AGC CTC CCT ATG AAT TG | 243       | 59      |
| Trx h    | AGG GAA AGG GGT CTC AGA AA | ATT GCC TTC ACA TTC CAC TC | 178       | 55      |

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These proteins involved in detoxification of ROS. *Assigned spot number as indicated in Figure 1. *Assigned name: Blastp from the JGI database (http://genome.jgi-psf.org) in *Populus trichocarpa* species.

**Table 2.** Differentially expressed proteins following gravistimulation of polar stems was presented by Azri et al.4

| Spot number | Protein name | Organism | Assigned name | Method | Peptides matched | % Coverage | Exp. pl/Mr | Theor. pl/Mr | Score | Ratio I/C |
|-------------|--------------|----------|---------------|--------|-----------------|-----------|-----------|------------|-------|----------|
| 1           | L-ascorbate peroxidase (EC 1.11.1.11) | *Populus trichocarpa* v1.1 | >graili3.002811501 | MS/MS | 1               | 4         | 5.8/33.0  | 5.4/26.9   | 50    | 0.36     |
| 2           | CuZn-superoxide dismutase (EC 1.15.1.1) | *Populus trichocarpa* v1.1 | >eugene3.00700152 | MS/MS | 2               | 17        | 6.0/15.7  | 5.6/15.3   | 87    | 0.36     |
| 3           | 23 kDa Oxygene evolving enhancer protein 2 | *Populus trichocarpa* v1.1 | >estExt_f/enes14422/pC_L | MS/MS | 3               | 4         | 5.8/21.2  | 8.6/28.1   | 301   | 0.02     |
| 4           | ATP synthase beta chain mitochondrial (EC 3.6.3.14) | *Populus trichocarpa* v1.1 | >estExt_f/enes14422/pC_L | Maldi- Tof | 8              | 28        | 5.4/33.8  | 5.8/35.1   | 62    | 2.68     |
| 5           | Thioredoxin f | *Populus trichocarpa* v1.1 | >estExt_f/enes14422/pC_L | MS/MS | 4               | 36        | 5.1/13.1  | 5.6/12.6   | 128   | 0.02     |

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5. The oligonucleotides primers were constructed based on specific *Populus EST database* [http://genome.jgi-psf.org] the EST detected with the BLAST program. The reference genes 18S RNA and Ubiquitin transcripts (POPTR_0012s01250, Phytozome http://www.phytozome.net) were amplified using the primers 18SF 5'-CTT CCG GAT CCG AGT AAT GA-3', 18SR 5'-GGG TTC GAG TAC AAC AAA CA-3', and UbiF 5'-GGC CCC TCC TAG ACC CAT ATC AC-3', UbiR 5'-GGG TTC AGC TTC TTG CAC TC-3', respectively.

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Figure 2. Time course accumulation of transcripts Trx h (A), APX2 (B), ATPase (C), OEE1 (D), CuZn SOD (E) and OEE2 (F) after gravitational stimulus. Total RNAs were extracted from basal internodes of inclined plants for 0 (control), and 10, 20, 30, 45 min, 1 h, 3 h and 6h. The accumulation of relative transcripts was determined by RT-qPCR. Mean values (+SE) of three technical replicates are shown. For each time, one plant is analyzed. Similar results were obtained on a second series of plants (white histograms). Asterisks (*) represent Student’s t-test significant at p ≤ 0.05. Double asterisks (**) represent Student’s t-test significant at p ≤ 0.01. Asterisks (***) represent Student’s t-test significant at p ≤ 0.001.
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