Summary  GH3 genes related to the auxin-inducible Glycine max (L.) Merr. GmGH3 gene encode enzymes that conjugate amino acids to auxin. To investigate the role of GH3 enzymes in stress responses and normal wood development, *Populus × canescens* (Ait.) was transformed with the promoter-reporter construct GH3::GUS containing a GH3 promoter and the 5′ UTR from soybean. β-Glucuronidase (GUS) activity was present in the vascular tissues of leaves and in developing lateral roots and was inducible in silent tissues by external auxin application. A decrease in GUS activity from the stem apex to the bottom corresponded to decreases in auxin concentrations in these tissues. High auxin concentration and high GH3::GUS activity were present in the pith tissue, which may provide storage for auxin compounds. GH3 reporter was active in ray cells, paratracheal parenchyma cells, maturing vessels and in cells surrounding maturing phloem fibers but not in the cambium and immature phloem, despite high auxin concentrations in the latter tissues. However, the GH3 promoter in these tissues became active when the plants were exposed to abiotic stresses, like bending or salinity, causing changes in wood anatomy. We suggest that adjustment of the internal auxin balance in wood in response to environmental cues involves GH3 auxin conjugate synthases.

Keywords: auxin, development, GH3, Populus, salt stress, tension wood, xylem.

Introduction

Auxins have also been implicated in adaptation of plants to stress. Auxin concentrations decreased in response to salt stress in Zea mays L. coleoptiles, Lycopersicon esculentum Mill. roots and developing xylem of *Populus* (Naqvi and...
onies were selected on YEB plates (Koncz et al. 1994) containing 100 µg ml \(^{-1}\) carbenicillin, 10 µg ml \(^{-1}\) gentamycin and 100 µg ml \(^{-1}\) rifampicin (Duchefa, Haarlem, The Netherlands). Plasmid DNA was isolated by alkaline lysis (modified after Birnboim and Doly 1979) and checked by restriction analysis. The stress-induced turnover of endogenous auxins catalyzed by GH3 enzymes is likely to be an adaptive strategy to coordinate growth and stress resistance (Park et al. 2007).

In the poplar genome, 10 GH3 genes encoding putative auxin amido conjugate synthases have been annotated (Tuskan et al. 2006; www.jgi.doe.gov/poplar). To investigate the expression of GH3 auxin conjugate synthases during wood differentiation and stress responses, we generated transgenic poplar (Populus \(\times\) canescens (Ait.)) expressing the GUS gene under the control of the heterologous soybean GH3 promoter. Our aim was to establish this GH3::GUS construct as a tool for investigation of woody plants, and to evaluate the results as an indicator of a common mechanism in poplar and other species. Several independent lines were produced and validated by comparing GUS staining with auxin concentrations along the apical and radial stem axes. Analysis of GUS staining in cross sections of the stem revealed cortex and pith as major sites of GH3::GUS activity and the cambium as being responsive to environmental stresses.

Materials and methods

Cloning of GH3::GUS into a plant binary vector

The GH3::GUS gene, containing the promoter and the 5′ UTR of the soybean GH3 gene (Hagen et al. 1991) was cloned into the EcoRI site of the binary vector pPCV002 (Koncz et al. 1994). After verification of the correct orientation of the GH3::GUS insert by sequence analysis, the construct pPCV002-GH3::GUS was transformed into a plant binary vector pPCV002-GH3::GUS harboring the neomycin phosphotransferase II (Birnboim and Doly 1979) and checked by restriction analysis. The stress-induced turnover of endogenous auxins catalyzed by GH3 enzymes is likely to be an adaptive strategy to coordinate growth and stress resistance (Park et al. 2007).

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pPCV002 harbors the neomycin phosphotransferase II (NptII) gene, which confers kanamycin resistance as a selectable marker of plant transformants. GH3 promoter sequences in poplar (Populus trichocarpa Torr. & A. Gray) were analyzed with the PLACE program (www.dna.affrc.go.jp/PLACE/signalscan.html; Prestridge 1991, Higo et al. 1999).

Plant transformation

Stem explants of \(P. \times\) canescens, a hybrid of \(P. alba\) L. \(\times\) \(P. tremula\) L. (Clone INRA717 1-B4) were used for transformation as described by Tzfira et al. (1997). Stem internodes of micropropagated \(P. \times\) canescens were cut longitudinally into segments of 1-cm length and co-cultivated with Agrobacterium tumefaciens GV3101 carrying the construct pPCV002-GH3::GUS. Explants were washed in sterile water supplemented with 500 µg ml \(^{-1}\) cefotaxime (Duchefa) and transferred to regeneration medium containing 50 µM kanamycin (Duchefa) for selection and 250 µg ml \(^{-1}\) cefotaxime to inhibit bacterial growth. The medium was changed every two weeks. Shoots from callus or directly from the explants were separated and rooted on regeneration medium containing 50 µM kanamycin and 250 µg ml \(^{-1}\) cefotaxime. After two weeks, surviving shoots were placed on regeneration medium with 100 µM kanamycin for more intensive selection. For experimental analyses, plantlets were micropropagated (see below).

To confirm transformation, genomic DNA was extracted with the nonionic detergent cetyltrimethylammonium bromide (CTAB; Roth, Karlsruhe, Germany) (Ausubel et al. 2001). After treatment with RNase A and proteinase K, the genomic DNA was extracted with phenol/chloroform and used for PCR and Southern blot analyses. The presence of the transgene was shown by PCR analysis using primers specific for the transferred GH3 promoter (5′-GAAGAGGCTTTTCAATAGCC-3′ and 5′-TTGCACGAGAAACAGTG-3′). Each PCR was performed with a 3-min initial denaturation step at 94 °C; 37 cycles of 20 s at 94 °C; 2 min at 55 °C; and 1 min at 72 °C. Because PCR analysis may give false positives due to the presence of persisting Agrobacterium, genomic Southern blots were performed to confirm the PCR results. Twenty µg of genomic DNA was digested with Sscl, separated by agarose gel electrophoresis and transferred on Hybond-N* membrane (Amersham, Little Chalfont, U.K.) by vacuum blotting (Vacuum Blotter Model 785, BIORAD). Hybridization was done with a 32P-labeled fragment of the GH3::GUS transgene using ULTRAHyb Hybridization Buffer (Ambion, Austin, TX) according to the manufacturer’s instructions. Hybridized filters were analyzed with a phosphor imager (BAS 1500, Fujiﬁlm) and the software AIDA (Image Analyzer, Raytest, Berlin, Germany).

Experimental treatments and harvest

Transgenic lines were multiplied by in vitro micropropagation (Leplé et al. 1992). Depending on the experimental requirements, plants were grown in soil (Frühstorfer Erde Typ N, Industrie-Erdenwerk Archut, Lauterbach-Wallroda, Germany) or in aerated hydroponic culture with Long Ashton macro-nutrient solution (Hewitt and Smith 1975), at 22 °C in a 16-h photoperiod with photosynthetic active radiation of about 100 µmol m\(^{-2}\) s\(^{-1}\).

Reaction wood was induced in 4-month-old plants with a stem diameter of 0.008 to 0.01 m and a height of about 0.80 m. Stems were bent by tightening a string between tree top and bottom so that the stem formed a 60° angle with the perpendicular at a height of 25 cm below the apex, and so that a stem area, containing secondary xylem, formed an angle of about 120° at a distance of 25 cm from the apical meristem. Plant material was harvested from upright control stems and stems

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bent for 24 h and 2 weeks and used for analyses of GUS staining and auxin concentrations.

Plant material for auxin measurements was harvested from plants with the same heights and stem diameters as plants used for GUS staining. For investigation of the axial pattern of auxin, stem sections were taken at 0.05, 0.40 and at about 0.75 m below the apex. Only the bark and the developing xylem were harvested to avoid biasing the data because of the increasing proportion of mature wood toward the base of the tree. For analysis of the radial distribution of auxin, different tissues were sampled from the lower portion of the stem where secondary xylem was present. The bark was peeled off and the surface of the bark facing the stem was scraped with a razor blade. Anatomical analysis with a microscope showed that the harvested tissue consisted of phloem and cambium cells. The remaining tissue of the peeled bark contained epidermis, cortex and secondary phloem fibers and is hereafter termed cortex (see Appendix). The peeled wood was also scraped with a razor blade to yield developing xylem. To obtain the pith, the stem was cut in half and the pith was separated from the mature xylem. The identity of all tissues was checked by microscopy (see Appendix). Hand sections were prepared, tissues stained with toluidine blue and viewed with the aid of a microscope (Axioplan, Zeiss). Photographs were taken at 100× magnification with a digital camera (Nikon CoolPix 4500).

To study the effects of salt stress, plants grown in aerated hydroponic culture were exposed for 1 week to 25 mM NaCl, followed by 1 week to 50 mM NaCl and finally for 2 weeks to 75 mM NaCl. Control plants were supplied with Long Ashton solution without NaCl. To mark the beginning of the stress treatments, the stem was punctured with a needle to injure the xyleogenic cambium (Dünisch et al. 1999). The wound reaction of the cambium was observed in stem cross sections and used to determine the starting point of the stress treatment.

**GUS staining**

Localization and intensity of GUS activity were investigated in intact leaves and roots as well as in 2-mm-thick stem cross section by a staining procedure. The tissues were infiltrated under vacuum for 15 min with GUS buffer (100 mM NaPO₄, pH 7.0, 10 mM Na₂EDTA, 0.05% Triton X-100) containing 1 mg ml⁻¹ 5-bromo-4-chloro-3-indole β-D-glucuronic acid (Duchefa, Haarlem, The Netherlands) and then incubated in the dark at 37 °C for 24 h. Pigments were removed in a series of ethanol treatments (30, 70 and 96% (v/v) ethanol) with gentle shaking. Subsequently, the samples were rehydrated by incubation in 70% and finally 30% (v/v) ethanol. Leaves were viewed and photographed directly. Root samples were viewed with the aid of a stereo microscope (Stemi SV11, Zeiss, Oberkochen, Germany). From paraffin-embedded stem tissue, 25-µm sections were prepared and viewed with the aid of a Zeiss Axioplan microscope. Photographs were taken at 20× (stereo microscope) and 100×, 200× and 400× magnification (microscope) with a digital camera (Nikon CoolPix 4500).

To induce GUS activity in the transgenic plants, tissues of transgenic plants were treated with auxin (10 µM 1-naphthylene acetic acid (1-NAA) in 0.1 M phosphate buffer, pH 5.8) for 48 h in darkness, washed with H₂O and assayed for GUS. Stem segments were assayed for GUS and then fixed in 2% formaldehyde, 5% acetic acid, 63% ethanol (FAE); modified after Sanderson (1994), dehydrated in an ethanol/isopropanol series (modified after Gerlach 1977) and embedded in Rotiplast with Roti-Histol (Roth, Karlruhe, Germany) according to the manufacturer’s instructions. Sections (25-µm thick) were cut with a sliding microtome (Reichert-Jung) and mounted on gelatine-coated-slides. The paraffin was removed with a Roti-Histol/isopropanol series and sections were viewed with the aid of a Zeiss Axioplan microscope.

**Auxin measurements**

Plant hormones were extracted as described by Schmelz et al. (2004), with some modifications. Fifty mg of frozen finely ground plant material were weighed and transferred to a 2-ml tube. One ml of diisopropylamine containing 50 ng D₅-IAA (Eurisotop, Freising, Germany) as an internal standard was added. The mixture was sonicated for 15 min, 1 ml of chloroform was added followed by a second 15-min sonication. For detection, phytohormones were converted to their pentafluorobenzyl esters (Müller and Brodschelm 1994) by adding 17 mg pentafluorobenzyl bromide (Sigma) to a final concentration of 33 mM and incubating at 60 °C for 1 h. After evaporation in a nitrogen stream, the residue was dissolved in 1 ml of diethyl ether and filtered. To enhance recovery of the pentafluorobenzyl esters, the sample tube was washed with 1 ml of n-hexane which was filtered and combined with the diethyl ether filtrate. The combined filtrate was evaporated in a stream of nitrogen.

The vapor phase extraction was carried out at 270 °C for 5 min with argon as carrier gas. The phytohormone derivatives were loaded on a SuperQ column (100 × 4.6 mm SDB-L, Strata, pore size 26 nm; Phenomenex, Aschaffenburg, Germany) and eluted with 3 ml of n-hexane and 3 ml of ethyl acetate. The solution was concentrated by rotary evaporation and then dissolved in 40 µl of dichloromethane and subjected to gas chromatography coupled to mass spectrometry.

A Polaris Q mass selective detector connected to a trace gas chromatograph (ThermoFinnigan) equipped with a capillary Rtx-5MS column (15 m × 0.25 mm, 0.25 µm coating thickness; Restek, Bad Homburg, Germany) was used, with helium as the carrier gas (1 ml min⁻¹). The temperature gradient was 100 °C for 1 min, 100–300 °C at 8 °C min⁻¹ and 300 °C for 5 min. The phytohormone derivatives were detected by negative chemical ionization with ammonia as ionization gas. Quantification of free auxin was based on the ions m/z 179 (D₅-IAA; R₁ = 17.78 min) and 174 (IAA; R₁ = 17.82 min).

**Results**

**GH3::GUS is functional in poplar**

We cloned the GH3::GUS promoter reporter construct into the pPCV002 vector, which provides kanamycin selection, and transformed poplar stem explants. From 74 regenerated plants...
12 lines were confirmed by genomic Southern analysis to contain stable integrations of the GH3::GUS construct in their genome (data not shown). All lines had a normal phenotype and showed, except one line, consistent histochemical staining patterns (Figures 1–3). Roots exhibited weak GUS activity in the vascular cylinder and strong activity at the sites of lateral root initiation (Figure 1G).

Leaves of poplar showed strong GUS staining in the vascu-

![Figure 1. GUS staining in leaves and roots of *Populus × canescens* transformed with GH3::GUS. (A) Leaves and (E) root of wildtype *P. × canescens* without auxin treatment; (B) leaves and (F) roots of wildtype *P. × canescens* treated with 10 µM 1-NAA for 48 h; (C) leaves and (G) roots of GH3::GUS transformed *P. × canescens* without auxin treatment; and (D) leaves and (H) roots of GH3::GUS transformed *P. × canescens* treated with 10 µM 1-NAA for 48 h. Scale bar: A–D, 0.5 cm; and E–H, 0.1 cm.](image1)

![Figure 2. Sensitivity of GH3::GUS in *Populus × canescens* leaves. Leaves were incubated with the indicated concentrations of IAA for 48 h and subjected to GUS staining. Panels A and B represent two independent lines of transgenic GH3::GUS *P. × canescens*. Scale bar: 0.5 cm.](image2)
lar tissues and in all tissues after auxin treatment (Figures 1C and 1D), indicating that the GH3::GUS construct was inducible. Induction of GUS was detected at IAA concentrations as low as 1 to 5 nM (Figure 2), and intense GUS staining was observed in tissues treated with up to 2.5 mM IAA (Figure 2), confirming that the promoter reporter construct, which contains three auxin responsive elements (Hagen et al. 2004), is auxin-responsive in a dose-dependent manner. *In silico* analyses of poplar IAA amido conjugate synthases revealed that the poplar GH3 promoter sequences contained between one and four auxin response elements (Table A1).

GH3::GUS reflects developmental patterns in the poplar stem

To investigate the activation of GH3::GUS in poplar stems during differentiation, we analyzed the histochemical staining patterns in stem sections taken at increasing distances from the apical meristem (Figure 3). Below the apical meristem (0.05 m), in the zone where the poplar stem exhibited elongation growth (Gross et al. 2002), single vascular bundles could be distinguished but the vascular cambium had not yet formed (Figure 3A). At this position GH3::GUS activity showed a relatively homogeneous distribution over the whole cross section, as indicated by the similar intensity of GUS staining in most cells of this young part of the stem, with slightly higher staining intensities in the epidermis and in vessels of the metaxylem.

At a distance of 0.25 m from the apical meristem, the vascular cambium was fully developed and the formation of wood was observed as well as the presence of immature, primary phloem fibers (Figure 3B). Compared with the apical section, the distribution and intensity of GUS staining was less homogeneous. Epidermis, cortex, phloem fibers and developing secondary xylem exhibited the highest GUS activity. Closer inspection at higher magnification revealed that cambial and young secondary phloem tissues were only slightly stained (Figure 4A). Pronounced GH3::GUS activity was detected in paratracheal parenchyma cells and the ray cells (Figure 4B) as well as in the perimedullary zone, the outermost region of the pith (Figure 4C).

Staining patterns similar to those observed at a distance of 0.25 m from the apical meristem, the vascular cambium was fully developed and the formation of wood was observed as well as the presence of immature, primary phloem fibers (Figure 3B). Compared with the apical section, the distribution and intensity of GUS staining was less homogeneous. Epidermis, cortex, phloem fibers and developing secondary xylem exhibited the highest GUS activity. Closer inspection at higher magnification revealed that cambial and young secondary phloem tissues were only slightly stained (Figure 4A). Pronounced GH3::GUS activity was detected in paratracheal parenchyma cells and the ray cells (Figure 4B) as well as in the perimedullary zone, the outermost region of the pith (Figure 4C).

Staining patterns similar to those observed at a distance of 0.25 m from the apical meristem, were found at distances of 0.50 and 0.75 m from the apex, but the staining intensity decreased significantly toward the stem base (Figures 3C and 3D). At a distance of 1 m from the stem apex, only epidermis and cortex showed GH3::GUS activity (Figure 3E). Auxin measurements in top, middle and basal stem sections showed that apical stem regions contained higher auxin concentrations than basal stem regions (Figure 5).

In addition to the vertical pattern, GH3::GUS activity showed a pronounced radial pattern with strong staining in the cortex, developing xylem and pith, less staining in the cambium, and no significant staining in mature xylem fibers and vessels (Figure 3). Determination of auxin concentrations in individual stem tissues by GC-MS-measurements showed that auxin was present in all tissues (Figure 6). The pattern of
GH3::GUS activity corresponded with the GC-MS measurements except for one significant area—the cambium + phloem had the highest measured auxin concentrations but barely any detectable GUS staining. This observation was unexpected and striking because the endogenous auxin concentrations were higher than 1 µM based on GC-MS analysis (Figure 6) and the water content of these tissues was about 90%. Because we observed no inhibitory effect of high auxin concentrations on GH3::GUS activity and induction of activity at IAA concentrations as low as 1–5 nM (Figure 2), the auxin concentration appears to be more than sufficient to induce the GH3 promoter. This finding indicates that the GH3-promoter is unresponsive to auxin in the vascular cambium of vertically oriented poplar stems.

GH3::GUS activity reflects changes in auxin physiology during tension formation

Because auxin plays a role in reaction wood formation, GH3::GUS poplars were used to investigate whether stem bending activates GUS activity. A shift in auxin distribution with higher concentrations in the tension-wood-forming side than on the opposite side has been considered an important factor in the regulation of tension wood formation. Because of contradictory data in the literature (Morey and Cronshaw 1968, Timell 1986, Little and Savidge 1987, Sundberg et al. 1994, Moyle et al. 2002, Hellgren et al. 2004), we investigated whether the GH3::GUS promoter-reporter system would point...
to a role of auxin conjugation during tension wood formation. The GUS histochemical assay revealed a clear difference in staining intensity between the tension-wood-forming upper side and the opposite lower side of poplar stems after subjecting stems to bending for 24 h or 2 weeks (Figures 7A–H). After bending the stems for 24 h, tension wood had not yet developed but GUS staining indicated that changes in auxin physiology had already occurred. Although the epidermis, cortex, phloem fibers and cambium on the tension-wood-forming side exhibited distinct GH3::GUS activity (Figure 7A), there was no clearly discernable GH3::GUS activity on the opposite side (Figure 7B). After bending the stems for 2 weeks, the formation of tension wood, with the characteristics of high cambial activity and decreases in vessel density and size, was clearly visible (Figure 7C). At this time, the cortex and phloem fibers showed only weak GUS staining, whereas staining in the cambium was more intense and extended into the developing xylem (Figures 7C and 7G, the latter panel shows the cambial area at higher magnification), whereas the opposite side still showed no distinct GUS staining (Figure 7H).

The GUS staining pattern in the cambial zone and the adjoining developing xylem differed from that observed in the control upright trees (Figure 3). Cambial cells in the process of forming tension wood were well stained (Figure 7G), whereas the cambium of upright control trees showed weak GH3::GUS activity, suggesting that the GH3 promoter in the cambium was stress responsive.

The GH3::GUS reporter is activated in the cambium in response to salt stress

We have previously shown that salinity leads to severe depletion (~80%) in auxin in developing xylem but with little loss in auxin conjugates (Junghans et al. 2006). Analysis of GH3::GUS activity in poplar exposed to salt stress revealed a loss of GUS staining intensity in the cortex; however, the salinity treatment caused significant staining in the cambium and adjoining phloem as well as in the ray cells (Figure 8B). In untreated controls, these tissues showed almost no GUS staining (Figures 8A and 3B–E).

Discussion

Stress adaptation of trees is an important process that involves decreases in growth and reallocation of resources to defense mechanisms. It has recently been suggested that readjustment of the internal hormonal balance involves GH3 auxin conjugate synthase as a key factor in coordinating these responses in Arabidopsis (Park et al. 2007). In woody plants, secondary stem growth is a major sink for resources and it is known that auxin plays a decisive role in controlling seasonal wood formation, though its action is not yet fully understood (Little and Savidge 1987, Little et al. 1990). If trees are exposed to salinity, or drought-induced osmotic stress, or if they are in the process of forming reaction wood, the developmental pattern of wood reveals distinct alterations involving changes in auxin physiology and auxin-responsive genes (Schrader et al. 2003, 2004).
The GH3::GUS promoter reporter fusion has previously been used to monitor distribution of physiologically active auxin at the cellular level in several higher plants (clover, soybean, Lotus, tobacco) as well as in an ancient moss species, Physcomitrella patens (Larkin et al. 1996, Li et al. 1999, Bierfreund et al. 2003, Pacios-Bras et al. 2003). We found the reporter in leaf veins and at sites of lateral root initiation, as has also been shown in clover and soybean expressing GUS under the control of the GH3 promoter and in Arabidopsis transformed with DR5::GUS (Mathesius et al. 1998, Avsian-Kretchmer et al. 2002, Pacios-Bras et al. 2003, Ashley et al. 2006, Scarpella et al. 2006). These results indicate that the heterologous soybean promoter is regulated by a common mechanism in different species.

Because GH3::GUS was more sensitive than DR5::GUS, Bierfreund et al. (2003) proposed that DR5 may be more specific to trace changes in auxin concentrations and considered GH3::GUS more suitable for identifying changes in auxin sensitivity. DR5 is a synthetic promoter containing tandem repeats of the auxin-responsive element TGTCTC also present in the natural GH3 promoter (Ulmasov et al. 1997). GH3 contains additional regulatory elements (Li et al. 1999, Hagen and Guilfoyle 2002) and its expression strength is regulated by transcription factors, some of which underlie developmental and environmental control (Strathmann et al. 2001, Heinenkamp et al. 2004).

We demonstrated for the first time activity of GH3::GUS in ray cells and in the perimedullary zone of the pith (Figures 4B and 4C). A novel finding was the presence of auxin in these tissues suggesting that rays and pith may have functions in auxin homeostasis or storage, or may play a role in auxin supply to the stem. In conclusion, the observed cell-specific patterns and their shifts in response to abiotic stresses demonstrate that GH3 auxin amido conjugate synthases play an important role in cell differentiation processes and adaptation.

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References


Appendix

Table A1. Auxin response elements in the promoter sequence of poplar GH3 genes. To identify auxin response elements in poplar GH3 promoter sequences, the DNA sequences 2000 bp upstream of the transcription start site of poplar GH3 genes (Tuskan et al. 2006, www.jgi.doe.gov/poplar) were analyzed using the PLACE program (www.dna.affrc.go.jp/PLACE/signalscan.html). The ARFART:ARF (auxin response factor) binding site found in the promoter of primary/early auxin response genes of Arabidopsis thaliana and of AUXREPSIAA4:AuxRE (auxine responsive element) of pea PS-IAA4/5 gene are shown.

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<th>Gene</th>
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<th>Location</th>
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<th>Type of AuxRE</th>
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Figure A1. Poplar tissues harvested for auxin measurements. (A) Cross section of poplar stem, (B) stem section after removal of bark, (C) mature wood after removal of developing xylem, (D) bark containing phloem and secondary cambium, (E) bark after removal of primary phloem and secondary cambium, (F) cross section of inner stem part and (G) inner stem part after removal of pith. Scale bar: 100 µm.