Plant Cell Rep (2013) 32:453–463
DOI 10.1007/s00299-012-1378-x

ORIGINAL PAPER

DR5 as a reporter system to study auxin response in *Populus*

Yiru Chen · Yordan S. Yordanov · Cathleen Ma · Steven Strauss · Victor B. Busov

Received: 16 August 2012 / Revised: 19 November 2012 / Accepted: 12 December 2012 / Published online: 3 January 2013

Abstract
Key message **Auxin responsive promoter DR5 reporter system is functional in Populus to monitor auxin response in tissues including leaves, roots, and stems.**
Abstract We described the behavior of the DR5::GUS reporter system in stably transformed *Populus* plants. We found several similarities with *Arabidopsis*, including sensitivity to native and synthetic auxins, rapid induction after treatment in a variety of tissues, and maximal responses in root tissues. There were also several important differences from *Arabidopsis*, including slower time to maximum response and lower induction amplitude. Young leaves and stem sections below the apex showed much higher DR5 activity than did older leaves and stems undergoing secondary growth. DR5 activity was highest in cortex, suggesting high levels of auxin concentration and/or sensitivity in this tissue. Our study shows that the DR5 reporter system is a sensitive and facile system for monitoring auxin responses and distribution at cellular resolution in poplar.

Keywords *Populus* · Auxin · DR5 · Wood formation · Phloem · Adventitious roots

Communicated by S. Merkle.

Y. Chen · Y. S. Yordanov · V. B. Busov (✉)
Michigan Technological University, School of Forest Research and Environmental Science, 1400 Townsend Drive, Houghton, MI 49931, USA
e-mail: vbusov@mtu.edu

C. Ma · S. Strauss
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331-5752, USA

Introduction

Auxin is a key regulator in plant growth and development (Vanneste and Friml 2009; Benjamins and Scheres 2008; Teale et al. 2006). Its concentrations are precisely controlled through intricate interplay between its transport and metabolism (Normanly 1997). Auxin regulatory roles are mediated through establishment of auxin maxima and gradients which are typically localized to single or small numbers of cells, and thus very difficult to study with standard biochemical techniques that employ whole tissue/organ extracts. The need for cellular resolution in measurements of auxin concentrations has led to the development of several alternative techniques and approaches, including immunolocalization (Schlicht et al. 2006), ultrafine sampling using cryo-sectioning (Uggla et al. 1996, 1998, 2001; Tuominen et al. 1997; Tuominen et al. 2000; Hellgren et al. 2004), and reporter systems that are responsive to auxin (Sabatini et al. 1999; Ulmasov et al. 1997b; Li et al. 1999; Mathesius et al. 2000; Oono et al. 1998). The reporter system has been most widely adopted and extensively used of these methods (Chandler 2009; Perrine-Walker et al. 2010).

Auxin is involved in many aspects of tree biology and environmental response, including wood formation (Druart and Johansson 2007; Moyle et al. 2002), dormancy (Baba et al. 2011) reaction wood formation (Sundberg et al. 1994), response to abiotic stresses (Popko et al. 2010; Junghans et al. 2006; Teichmann et al. 2008), and interaction with microbes (Felten et al. 2009). Characterization of auxin concentrations at a cellular level, however, has been rare in woody plants. Cryo-sectioning through the radial sequence of the woody tissue yields sufficient sampling material to measure auxin concentrations using standard analytical techniques (Tuominen et al. 1997; Hellgren et al. 2004; Uggla et al. 1996; Tuominen et al. 2000).

However, this approach requires specialized expertise and instrumentation, which is often not readily available. Furthermore, it can only be applied to large organs like stems of trees, able to yield sufficient tissue for biochemical extractions. These problems are of course, compounded where auxin concentrations need to be measured in relation to complex spatial and temporal changes in plant development. Thus, reporter systems provide an important alternative method, as shown in *Populus* based on the soybean GH3 promoter (Teichmann et al. 2008).

Two major approaches have been employed to generate auxin-responsive reporter systems. One is based on the large segments of the promoter of auxin-inducible genes like soybean GH3 (Bierfreund et al. 2003; Staswick et al. 2005; Ishizaki et al. 2012). This system has been used successfully in a number of species to dissect involvement of auxin in various developmental processes (Sorin et al. 2005; Teichmann et al. 2008; Bierfreund et al. 2003). However, native promoters contain multiple regulatory elements that can respond to a diversity of signals (Li et al. 1999; Hagen and Guilfoyle 2002; Liu et al. 1994; Khan and Stone 2007). Therefore, it may be difficult to uncouple the auxin-inducibility from the effect of other interfering factors. Alternatively, an artificial auxin-responsive promoter has been developed based on the discovery and characterization of an auxin response elements (AuxREs), which consist of the TGTCTC sequence and typically found in the promoters of auxin-inducible genes (Ballas et al. 1993). A synthetic auxin-responsive promoter called DR5 contains a minimum promoter fused to seven AuxRE repeats (Ulmasov et al. 1997a). The DR5 promoter driving reporter genes like β-glucuronidase (GUS), green fluorescent protein (GFP) or red fluorescent protein (RFP) has been widely utilized in *Arabidopsis*, maize, soybean, and tomato to study auxin distribution and response at a cellular level (Sabatini et al. 1999; Gallavotti et al. 2008b; Chaabouni et al. 2009). However, its utility in woody perennial species like *Populus* has not been demonstrated. We show that this reporter system is effective in *Populus*, and can be used to study the role of auxin in secondary woody growth.

Materials and methods

Plant material and growth conditions

Poplar hybrid plants (INRA 717 1-B4 *Poplulus tremula* × *alba)* were used for transformation. The wild-type refers to untransformed 717 *P.tremula* × *alba.* Forty- to fifty-day-old, in vitro grown poplar plantlets served as explant sources. The plants were multiplied by in vitro micropropagation as previously described (Leple et al. 1992). The micro-cuttings were grown in the hormone free ½ strength Murashige and Skoog medium (½MS) (Murashige and Skoog 1962). The rooted plants remained in the same medium at 25 °C under a 16-h photoperiod (fluorescent tubes (TL70, F25T8/TL735, Philips) at a photon flux density of 45 μE m^{-2} s^{-1}). To study DR5 behavior in woody stems, the 7-week-old in vitro plants were then transplanted into soil (Sunshine LC1 complete soil) under greenhouse condition. The 5-month-old plants with about 35 internodes were harvested for analyses. For the experimental treatments, three transgenic events with at least two biological replicates were used.

Construct preparation

The auxin responsive promoter DR5 was fused with the GUSPlus reporter gene using the Gateway cloning system. The DR5 sequence plus minimal cauliflower mosaic virus 35S promoter sequence was PCR-amplified from the DNA fragment of the vector pUC19 harboring 7 × DR5:GUS (a gift from Prof. Tom J. Guilfoyle, University of Missouri) with the following primers (attB4 and attB1sites are underlined): dr5forward 5′-*GGGGACAAGTTTGTACAAAA AAGCAGGCT*ATGCTGTACCCGATCAACAC- 3′, and dr5 reverse 5′-*GGGGACTGCTTTTTTGTACAAACTTGGT A*ATTGTAATTGTAAATAG-3′. The PCR product was purified from agarose gel using the QIAquick gel extraction kit (Qiagen) and inserted into the pDONR221-P4-P1R vector through BP reaction (Invitrogen). The open reading frame of GUSPlus gene was amplified from the pCAMBIA vector (http://www.cambia.org) with the following primers (attB1 and attB2 sites are underlined): gusplus forward 5′-*GGGGACAAGTTTGTACAAAAAAGCAGGCT*ATGCTG TACCCGATCAACAC-3′, gusplus reverse 5′-*GGGGACC ACTTTGTACAAGAAAGCTGGGT*TCAGTTCAGTTCTTG TAGCCGAAATCTG-3′. The amplified fragment was cloned into pDONR221-P1-P2 vector through BP reaction (Invitrogen). All entry clones were sequenced to verify that no mutations were introduced by the PCR amplification. The DNA fragments were then transferred from the pDONR221 entry clone into the destination binary vector ph7m24gw (Karimi et al. 2002) using LR recombination reactions (Invitrogen). The binary vector ph7m24gw contains the hygromycin B resistance (Hyg) gene fused to nopaline synthase (nos) promoter and terminator. The resulting constructs were again sequence verified.

Transformation

The vector of DR5::GUSPlus was introduced into the *Agrobacterium* strain AGL1 (Lazo et al. 1991) using the freeze and thaw method (Holsters et al. 1978) The plant transformation was previously described (Filichkin et al. 2006) with modifications. *Agrobacterium* cells harboring the binary vector was grown for 24 h in Luria Bertani (LB)

medium supplemented with 50 mg/l rifampicin, 100 mg/l carbenicillin, and 50 mg/l spectinomycin on an orbital shaker at 28 °C and 250 rpm. The cells were centrifuged in LB medium at 3,500 rpm for 30–40 min to get the cell pellet and then re-suspended in *Agrobacterium* induction medium (IM) to achieve an OD600 nm of 0.5–0.6. The leaf discs and wounded internodes were co-cultured in the *Agrobacterium* suspension for 1 h. The inoculated explants were co-cultivated in callus-induction medium (CIM) [MS supplemented with 10 µM naphthaleneacetic acid (NAA) (Sigma) and 5 µM N6-isopentenyl adenine (2ip) (Sigma)] at 22 °C in darkness for 2 days. Explants were then washed four times in sterile deionized water and once with wash solution containing 250 mg/l timentin. Then, explants were transferred to CIM containing 10 mg/l hygromycin, 200 mg/l cefotaxime, and 200 mg/l timentin for 21 days to select transformed calli. The explants with calli were transferred to the shoot induction medium (SIM) [MS supplemented with 0.4 µM TDZ] containing 20 mg/l hygromycin, 200 mg/l cefotaxime, and 200 mg/l timentin for 2–3 months following several subcultures to select transformed shoots. For shoot elongation, the explants were transferred on to MS medium containing 0.1 M 6-benzylaminopurine (BAP) (Sigma), 20 mg/l hygromycin, 200 mg/l cefotaxime and 200 mg/l timentin. To induce rooting, the regenerated shoots were then transferred onto ½MS medium supplemented 0.5 µM indole-3-butyric acid (IBA) (Sigma) and 2.5 mg/l hygromycin. To ensure transformation events were independent, a single regenerating shoot per individual explant, termed a transgenic "event" below, was propagated. A total of 36 events were recovered and each event was PCR verified for the presence of the transgene using the following primers: dr5gus-forward 5′-AA ACTAGGATGTATCGCAGC-3'; dr5gus-reverse 5′-GTA ATTGTAATTGTAAATAG-3′.

Auxin treatments

For exogenous auxin treatment experiment, events 10, 12, and 38 were used. Leaf, stem, and root tissues from 6 week-old in vitro grown plants were collected from the three selected events. The freshly cut leaves, stems and roots were first floated in ½MS liquid medium for 6 h to deplete the endogenous auxin. The tissues were then transferred into ½MS liquid medium (pH 5.8) containing IAA or NAA at various concentrations. After the designated treatment time, the tissues were rinsed with distilled water before further analysis.

Histochemical and fluorometric assay of GUS expression

For histochemical GUS staining of the in vitro plants, the tissues were incubated at 37 °C in a solution containing 50 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA, 0.12 % Triton, 0.4 mM ferrocyanide, 0.4 mM ferricyanide, 1.0 mM 5-bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-Gluc) (Gold Biotechnology). The staining time varied between 2 and 12 h depending on the tissue type. For the histochemical GUS staining of stems from greenhouse grown plants, hand sections were prepared according to the method described by Hawkins et al. (2002) with slight modifications. The freshly hand cut stem sections were pretreated in 50 mM sodium phosphate buffer (pH 7.0) containing 4 % acetone for 5–30 min to prevent a possible wounding response. Vacuum infiltration for 5 min was applied to the stem samples in staining solution. After staining, whole plants or tissues were rinsed with distilled water and then transferred to 70 % ethanol for de-staining. The whole plants and leaves were viewed and photographed directly with a digital camera (Canon EOS 350D), and the root samples were viewed and photographed with a Leica M26 dissecting microscope equipped with a SONY 3CCD DKC-5000 camera. The wood sections were viewed under a Nikon (ECLIPSE E400) microscope and images were captured using a Leica DFC295 digital camera.

Fluorometric MUG assays were performed following: http://www.markergene.com/product_sheets/pis0877.pdf (Marker Gene Technologies, Inc.) with modifications. Harvested material was placed in 2.0 ml microcentrifuge safe-lock tubes and frozen in liquid nitrogen. The tissues were homogenized in a Tissuelyser (Qiagen). After homogenization, we added 200–300 µl of extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1 % Triton X-100, 0.1 % SDS, and 10 mM β-mercaptoethanol). For the greenhouse material, PVP40 (2 %) was used. The mixture was then centrifuged at 13,000 rpm at 4 °C. The supernatant was removed, frozen in liquid nitrogen, and stored at −80 °C. Aliquots of the supernatant (25 µl) were added to 1 ml GUS assay buffer (2 mM 4-methylumbelliferyl-beta-D glucuronide (Sigma) and 10 mM β-mercaptoethanol in extraction buffer), incubated at 37 °C for 5, 35 and 95 min. Samples (200 µl) of this reaction were mixed with 800 µl stop solution (0.2 M Na_2CO_3), and fluorescence measured with excitation at 365 nm and emission at 455 nm in a TKO 100 Mini-fluorometer (Hoefer). Protein content was measured at 595 nm using the Quick Start Bradford Protein Assay Kit (Bio-Rad). GUS activity as determined by MUG assays are presented as pmol 4-MU/min/mg protein.

Results

Regeneration and selection of transgenic plants

A total of 36 independent transgenic events were recovered and PCR verified for the presence of the reporter transgene.

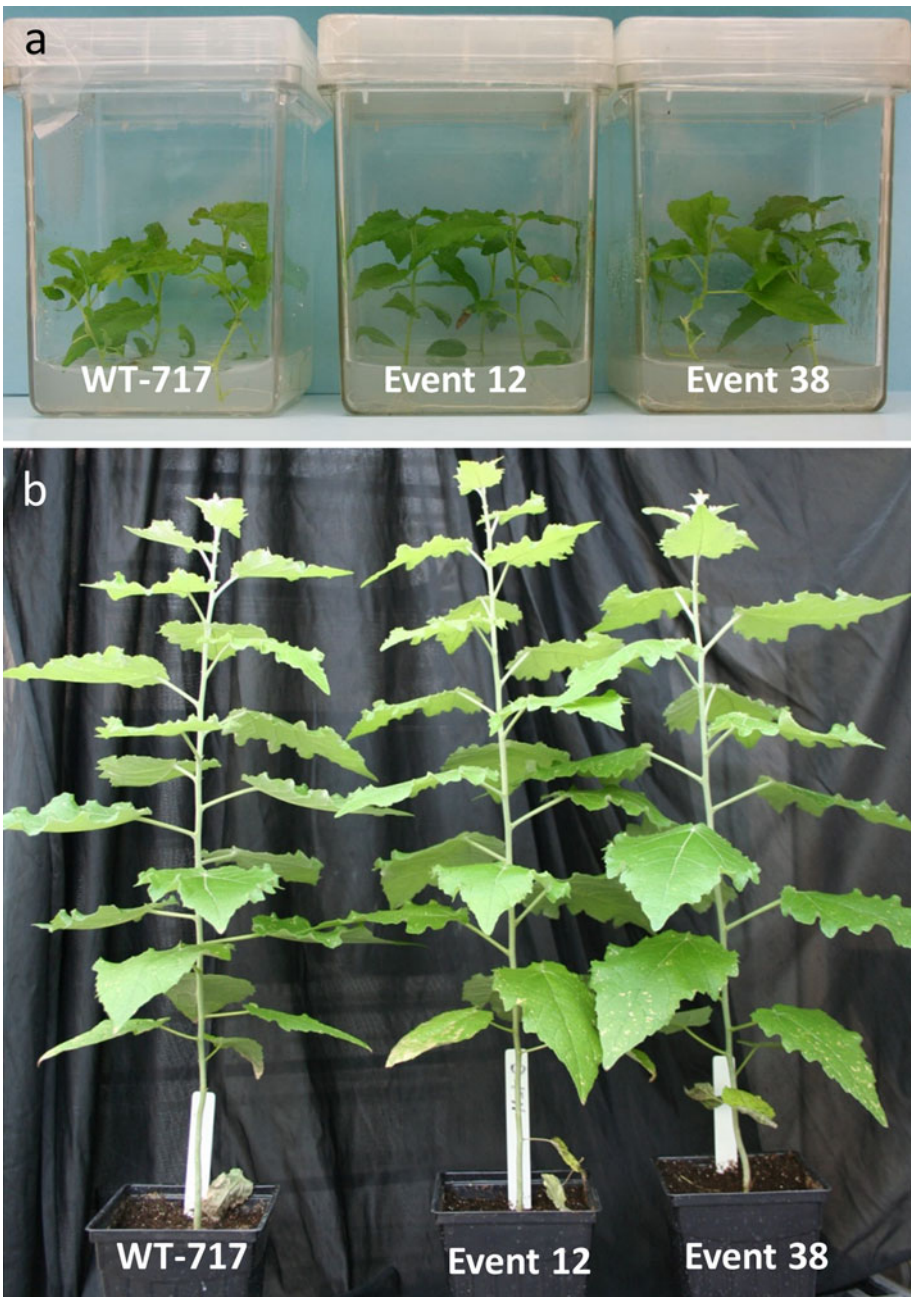

Fig. 1 DR5::GUS transgenic poplars are aphenotypic under in vitro and greenhouse conditions. Events 12 and 38 were shown as 10 weeks after in vitro (**a**) 3-month greenhouse (**b**) growth. No significant phenotypic differences were observed when compared with WT

We selected randomly 14 events for further characterization. The DR5 transgenic plants did not show any differences with respect to their growth and development when compared with wild type both in vitro and after 3 of greenhouse growth (Fig. 1). The levels of GUS activity among the 14 events varied significantly (Fig. 2). Two lines (8 and 39) showed no detectable GUS activity. Typically, in untreated plants, weak GUS activity was detected in the leaf and root vasculature; however, a strong GUS signal was observed in the primary root tip and at the sites of lateral root formation (Fig. 3a, c, e). Overnight treatments with 10 μM 1-naphthaleneacetic acid (NAA) elicited strong GUS activity in leaves, roots and stems (Fig. 3b, d, f). Three events (10, 12, and 38) with moderate levels of GUS expression, representative tissue localization, and high auxin inducibility were selected for further analysis.

DR5 is auxin-inducible

We measured DR5 activity in response to treatments with the main form of the native auxin, indole 3-acetic acid (IAA), as well as with the more biologically active synthetic analog 1-naphthaleneacetic acid (NAA) at two concentrations (0.1 and 10 μM) and in three tissues (leaves, stems and roots). DR5 was induced in all tested tissues by both hormones (Fig. 4). Overall DR5 was most responsive

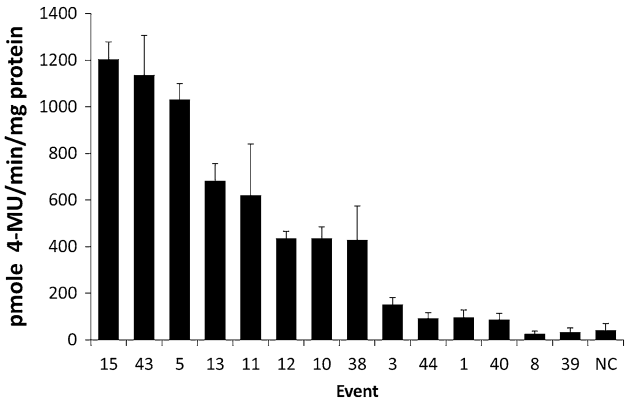

Fig. 2 GUS activity in apical shoots of transgenic *Populus* plants transformed with DR5::GUS. NC (negative control) corresponds to non-transformed WT plants. The *bars* show mean and standard error from three biological replications. All events, except 8 and 39 showed statistically significant higher GUS expression compared to WT (Student's *t* test, $P < 0.05$)

to auxin in roots, where we observed highest induction for both IAA and NAA at all concentrations. Leaves also showed high DR5 induction, with NAA being more effective at lower concentrations, but NAA induction decreased at high concentrations. Interestingly, stems were somewhat recalcitrant to the auxin treatments. Statistically significant high induction ($P < 0.05$) was observed only at the high concentration of the NAA treatment.

Temporal and concentration dynamics of auxin inducibility

We investigated the temporal dynamics and concentration range of DR5 auxin inducibility at NAA concentrations ranging from 0.01 to 100 μM. GUS activity increased with increasing NAA concentration up to 0.1 μM. However, further increases in the concentration of the hormone led to reduction in induction amplitude (Fig. 5). We also followed the temporal dynamics of the induction from 1 to 24 h. For all organs, induction could be detected as early as 1 h, but continued to increase throughout the studied period (Fig. 6). Temporal inductive kinetics for different organs was comparable in all time periods except for 4 and 16 h when the induction in roots was significantly higher than leaves but not significantly different from stems (Fig. 6).

Tissue-specific activity of DR5 under non-inductive conditions

We measured the activity of DR5 in a variety of untreated tissues; GUS activity was detected in all tissues, although the level was highly variable. The highest GUS activity was measured in young leaves, primary stems, phloem/bark, and root tips. DR5 activity was lower in the apex, woody stems, matured leaves, and xylem (Fig. 7).

DR5::GUS activity changes during the transition from primary to secondary growth

Perennial woody plants have primary (elongation) growth at the tip of the stem (internodes 1-9 in *Populus*), followed by a transition to secondary (lateral) growth, resulting in production of wood. Auxin is a well-known regulator of secondary woody growth, thus we used DR5 to visualize auxin concentrations and response in the developmental transition from primary to secondary growth at four locations on the stem. During primary growth (5th internode), GUS staining was localized in the pith, the protoxylem, and cortex. No significant staining was detected in the epidermis, phloem fibers, and cambium (Fig. 8a, b). At the transition zone between primary and secondary growth (10th internode), the most intense GUS signal was localized in the protoxylem and cortex (Fig. 8c, d). In portions of the stems with established secondary growth (20th internode), the overall intensity of GUS staining decreased as compared to that observed in the 5th and 10th internodes. The strongest GUS signal was detected in the cortex (Fig. 8e, f). Staining was absent from the epidermis, phloem fibers, cambium and xylem. Patches of cells displaying GUS stains could also be observed in the cambial zone (Fig. 8f).

Discussion

Reporter systems based on the auxin-inducible promoters have become a powerful tool for monitoring auxin concentrations and sensitivity at a cellular resolution (Ulmasov et al. 1997b; Sabatini et al. 1999; Benkova et al. 2003; Heisler et al. 2005). Although these systems have been most widely used in *Arabidopsis*, they have been also successfully employed in other commonly studied herbaceous plants (Yamamoto et al. 2007; Gallavotti et al. 2008a; Li et al. 1999; Mathesius et al. 2000), as well as in phylogenetically primitive plants such as *Physcomitrella* (Bierfreund et al. 2003) and *Marchantia* (Ishizaki et al. 2012). The DR5 system has been widely adopted as it contains auxin response regulatory elements exclusively, thus providing a high level of stringency with respect to inferences about auxin concentration and sensitivity.

We found a number of similarities to system function between poplar, *Arabidopsis,* and other herbaceous plants. DR5 was highly auxin responsive in *Populus*; both native (IAA) and synthetic (NAA) auxins elicited a strong activation of the DR5 promoter. Furthermore, auxins like NAA evoked a much stronger response than the native IAA. In *Arabidopsis* 4-ClIAA, MeIAA and NAA produced much

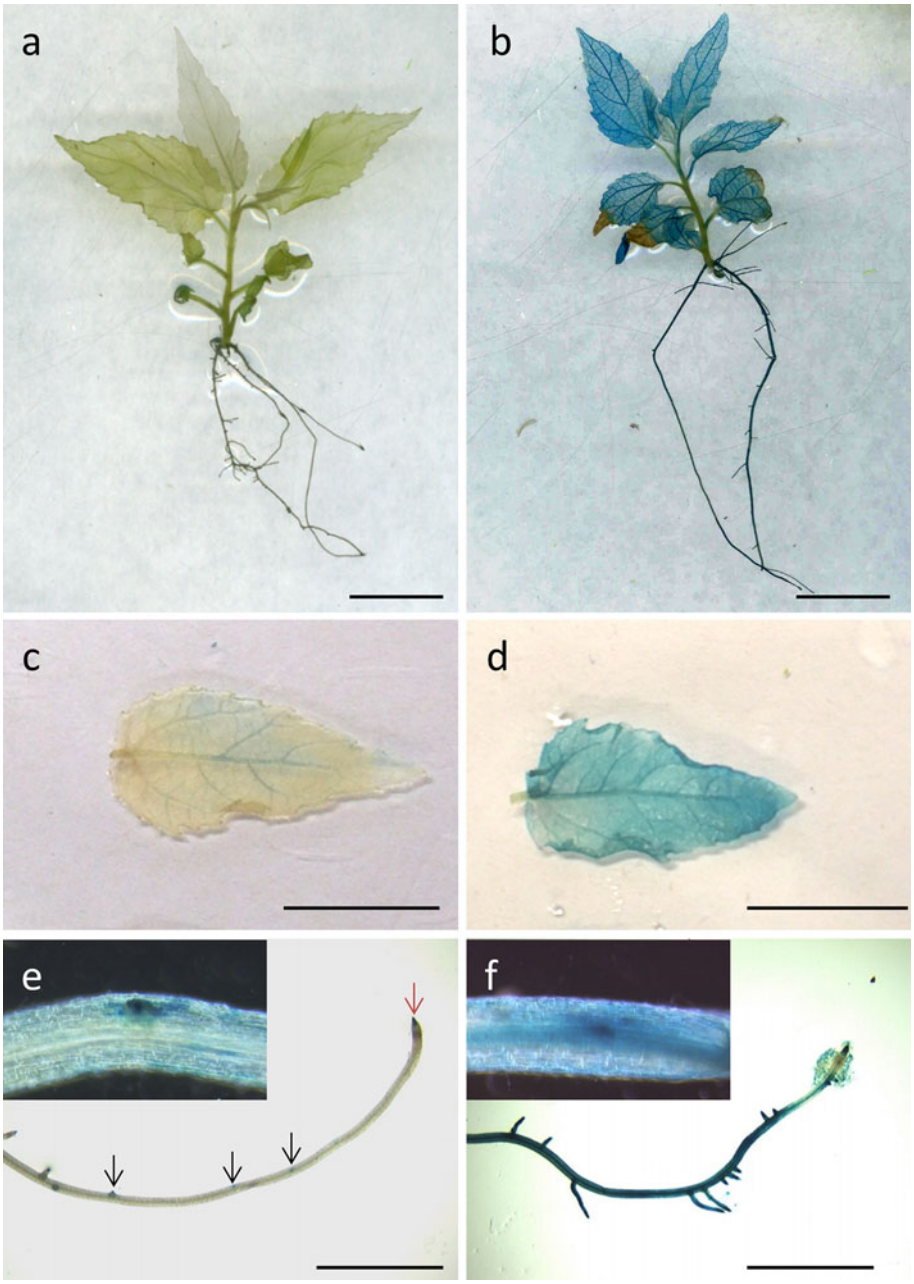

Fig. 3 GUS staining in tissues of *Populus* transformed with DR5::GUS with and without auxin treatment. The plantlets were incubated in ½ MS liquid medium without (**a**, **c**, and **e**) and with (**b**, **d**, and **f**) 1μM NAA for 4 h. Histochemical GUS staining was visibly increased after auxin treatment in leaf (**c**, **d**) and root (**e**, **f**). In roots the GUS staining was found in the root tip (*red arrow*) of the main root and the lateral root formation sites (*black arrows*). *Insets* in **e** and **f** show close-up views of lateral root initiation sites

stronger DR5 activation compared to IAA (Bai and Demason 2008). Presumably, this is because these auxin analogs are more hydrophilic, which allow for better membrane permeability and thus a stronger induction of gene expression (Delbarre et al. 1996; Yamamoto and Yamamoto 1998; Armstrong et al. 2004). As in *Arabidopsis*, the highest auxin response was measured in roots (Petersson et al. 2009), occurred as early as 1 h post treatment, and the response increased with increasing auxin concentrations (Nakamura et al. 2003).

The behavior of the DR5 reporter system also differed in poplar in several significant ways. Although the promoter was highly inducible, the amplitude of induction in *Arabidopsis* (~50-fold) (Ulmasov et al. 1997b) was much higher than what we measured in *Populus* (~8-fold), and stems were particularly recalcitrant. This may be associated with decreased permeability of the hormone through the woody stem tissues of poplar. We also found a slower response to the hormone in *Populus*. In *Arabidopsis*, the response maximum occurs within hours, while in *Populus* we could not detect saturation of the response even at the end of the studied period (24 h). We do not believe that the observed difference results from differences in the signal transduction pathway that leads to the activation of the promoter. DR5 is composed of AuXRE that serve as binding sites to AUXIN RESPONSE TRANSCRIPTION

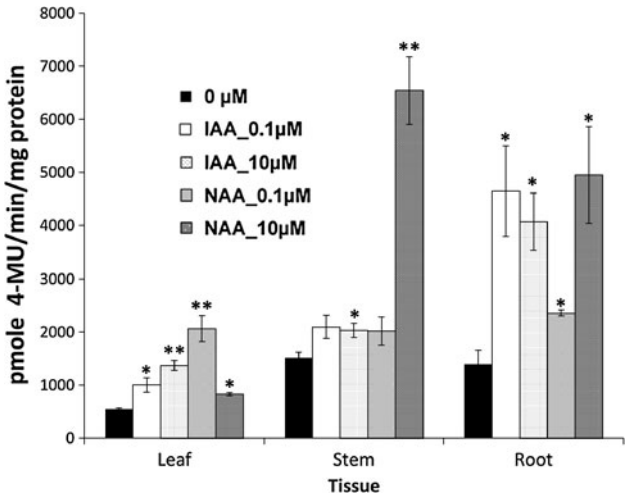

Fig. 4 Auxin induction of GUS in leaves, stems and roots of DR5::GUS-transformed poplar plants. Leaf, stem and root tissues of 6 weeks old in vitro grown plants from events 10, 12, and 38 were treated with IAA (0, 0.1 and 10 µM) and NAA (0, 0.1 and 10 µM) for 2 h. All sampled tissues were depleted from endogenous auxin by floating on ½ MS media for 6 h prior to treatments. GUS activities were measured by MUG assay (see "Materials and methods" for more detail). Leaves used in this experiment were young (LPI3). For each event, tissues were collected from three plants. The bars show mean and standard error from three events. '*' indicates a significant difference at $P < 0.05$ and '**' $P < 0.01$ compared to untreated tissues and as determined by a Student's *t* test

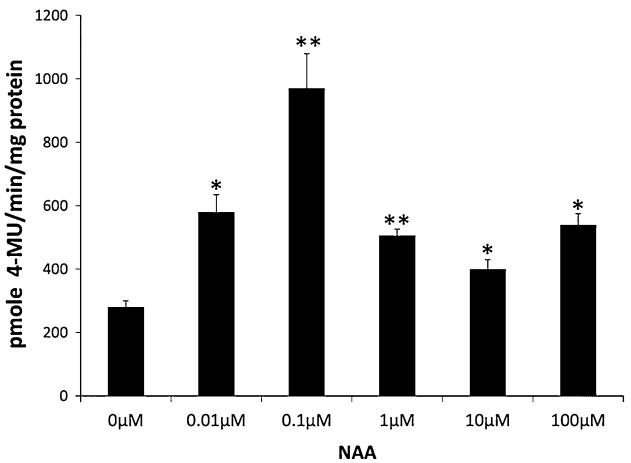

Fig. 5 GUS induction by various auxin concentrations in leaves of DR5::GUS poplar transgenics. Leaves from 6 weeks old in vitro transgenic poplar plants were treated by NAA with different concentrations (0.01, 0.1, 1, 10, and 100 µM) for 2 h. GUS activity at each concentration was measured by MUG assay (see "Materials and methods" for more detail). Leaves used in this experiment were young (LPI3). For each event, tissues were collected from three plants. The *bars* show mean and standard error from three events. '*' indicates a significant difference at $P < 0.05$ and '**' $P < 0.01$ as compared to untreated sample and determined by Student's *t* test

FACTORS (ARFs) (Ulmasov et al. 1997b), and genome-wide analyses have shown a close relationship of *Populus* and *Arabidopsis* ARFs (the family is somewhat expanded

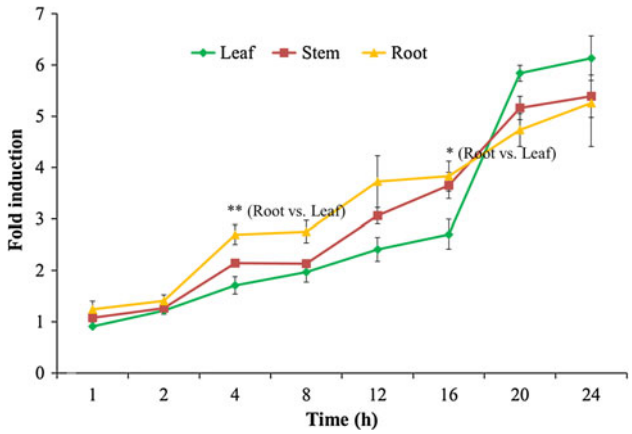

Fig. 6 Time course of GUS auxin induction in DR5::GUS poplar transgenics. Leaves, stems and roots from 6 weeks old transgenic poplar plants were treated with NAA (10 µM) continuously for 24 h. Leaves used in this experiment were young (LPI3). GUS activity at each time point of 1, 2, 4, 8, 12, 20 and 24 h after the treatment were measured by MUG assay (see "Materials and methods" for more detail). The *bars* show mean and standard error from three events. Fold induction is the relative GUS level as compared to untreated samples. '*' indicates a significant difference at $P < 0.05$ and '**' $P < 0.01$ as indicated on the graph and determined by Student's *t* test

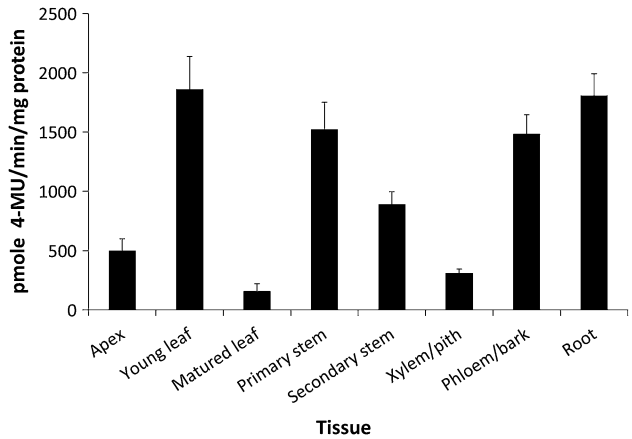

Fig. 7 GUS expression in untreated tissues of DR5::GUS *Populus* under greenhouse condition. GUS activities were measured by MUG assay in the apex, the young leaves [leaf plastochron index (LPI) = 3], mature leave (LPI = 20), young stem (the 5th internode), stems undergoing secondary woody growth (20th internode), xylem, phloem/bark, and root. For each event, tissues were collected from two plants. The *bars* show mean and standard error from three events

in size in *Populus* and ARFs with activation functions predominantly contribute to this expansion) (Kalluri et al. 2007). As discussed above, a possible reason for the differential response is the presence of thick secondary walls, particularly in the stem tissues which are less permeable to the hormone, and may thus delay and attenuate responses. However, we do not believe this can fully explain the differences seen, as *Physcomitrella*, which does not produce secondary walls, also showed a similar delayed

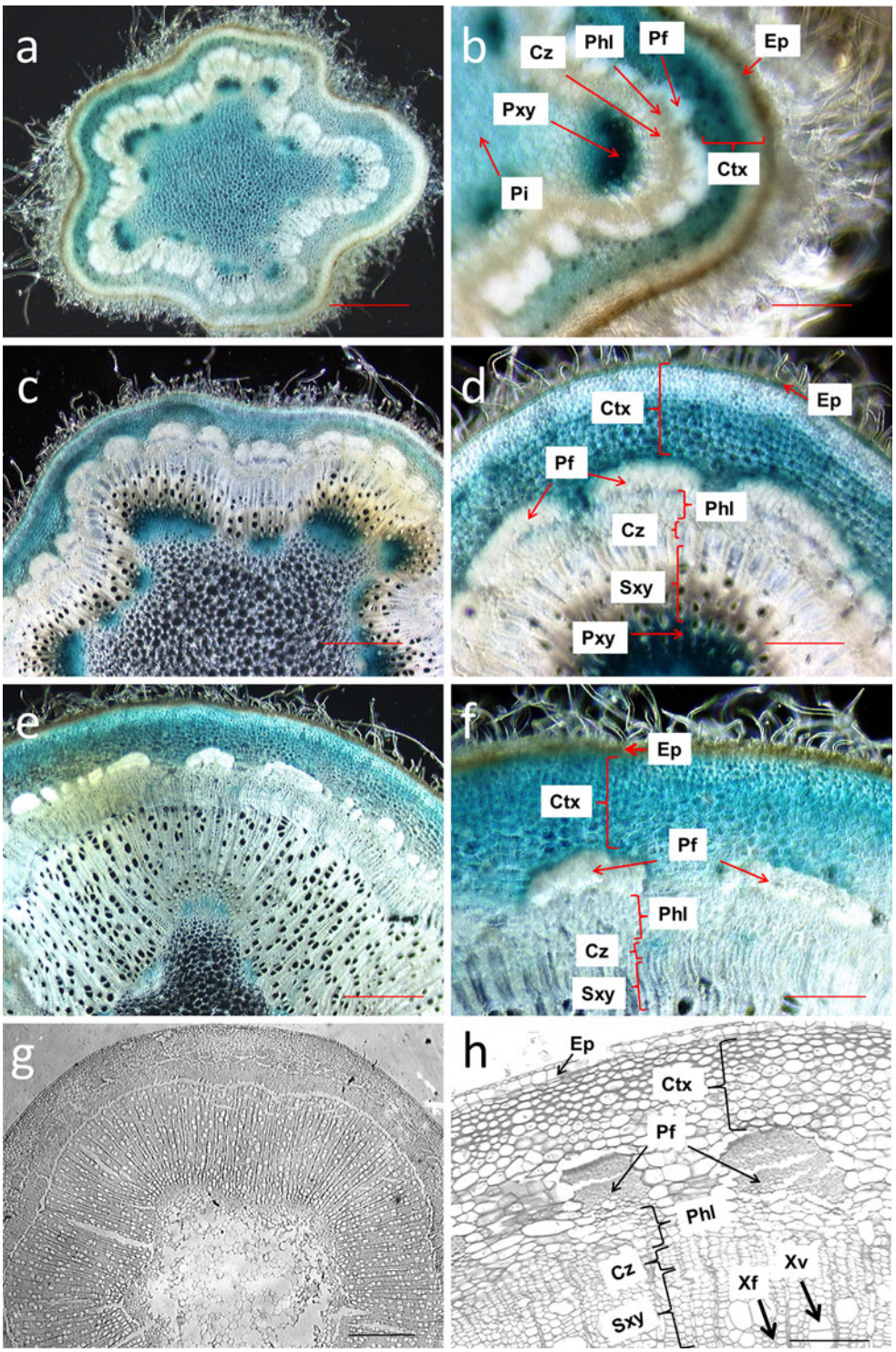

Fig. 8 Localization of GUS in stem cross sections along the developmental gradient from primary to secondary growth in DR5::GUS transformed transgenic poplars. Stems were harvested in September. Hand sections were prepared from 5th (**a**, **b**), 10th (**c**, **d**), and 20th (**e**, **f**) internodes in the middle between the subtending leaves and then subjected to anatomical and histochemical analysis as described in the text. 15 μm thick sections from 20th internode are also shown (**g**, **h**). The *scale bar* represents 500 μm in **a**, **c**, **e**, **g** and 100 μm in **b**, **d**, **f**, and **g**. *Ep* epidermis, *Ctx* cortex, *Phl* phloem, *Pf* phloem fiber, *Pi* pith, *Xv* xylem vessel, *Xf* xylem fiber, *Cz* cambial zone, *Sxy* secondary xylem, and *Pxy* protoxylem

response of DR5 to auxin treatment as compared to *Arabidopsis*. Another possible explanation is that DR5 contains mutagenized AuxRE-binding sites, which were added because they gave better auxin inducibility in *Arabidopsis* than the native AuxRE (Ulmasov et al. 1995). The performance of these AuxRE elements compared to the native AuxRE may not be as efficient outside of *Arabidopsis*, but this has not yet been tested. Regardless of the observed differences, our study shows canonical DR5 auxin responsiveness in *Populus* and, therefore, DR5:GUS appears to be a useful system to study its concentration and response.

Young, actively dividing tissues are typically the main source of auxin and DR5 is highly active in these tissues (Barlier et al. 2000). Consistent with this observation, we found that young leaves and stem sections below the apex showed much higher DR5 activity than older leaves and stems undergoing secondary growth. This is consistent with the properties of the intact GH3 promoter, where highest reporter gene activity was found in the upper portions of the stem, below the apex (Teichmann et al. 2008). Similarly in *Arabidopsis* and maize, young and actively dividing leaves displayed the highest levels of DR5 activity (Cheng et al. 2006; Mikkelsen et al. 2000). In roots, DR5 activity was highly localized to the main root tip and the sites of lateral root initiation and emergence. The same pattern was observed in *Arabidopsis* roots and, is consistent with the known flow and function of auxin in roots. The root tip is the main hub that redirects basipetal auxin flow, and auxin concentrations in tip of the main root are typically high (Petersson et al. 2009). In addition, high, localized auxin concentration in the root differentiation zone is known to be associated with lateral root initiation (Casimiro et al. 2001; Dubrovsky et al. 2011) and emergence (Swarup et al. 2008).

It has long been known that auxin is a major regulator of wood formation (Digby and Wareing 1966; Savidge 1988; Snow 1935), however, a detailed and precise understanding of its regulatory roles is still lacking. In contrast to the widely held belief that auxin maxima in the woody stems occurs in vascular cambium (Uggla et al. 1996, 1998, 2001; Tuominen et al. 1997; Groover and Robischon 2006; Groover et al. 2010; Spicer and Groover 2010), our results show that DR5 activity was highest in the cortex. One explanation may be that DR5 does not correctly predict auxin concentration/sensitivity. Although some authors have suggested that DR5 can respond to signals other than auxin (Nakamura et al. 2003; Nemhauser et al. 2004), a large body of literature shows that DR5 is indeed a reliable measure of both auxin sensitivity and concentration (Grieneisen et al. 2007; Sabatini et al. 1999; Blilou et al. 2005; Benkova et al. 2003). This agrees with our work, as we have shown that DR5 is highly auxin-inducible in poplar by both native and synthetic auxins. Thus, a more plausible reason for the cortical-dominant expression is that published studies of auxin concentrations in cambium had focused on a very narrow region encompassing vascular cambium and few subtending phloem and xylem cell layers, but have never focused on the cortical tissue. We did not histologically detect any GUS signal in this zone. However, our quantitative analysis encompassing the cambium together with the subtending secondary xylem and phloem did detect DR5-driven GUS expression. This would suggest of lower or saturated auxin sensitivity/responsiveness in this zone. Decreased or saturated auxin responsiveness was also proposed to be at least one of the reasons for no correlation between the auxin concentrations and expression of auxin-responsive genes in these tissues (Nilsson et al. 2008). Nevertheless, our finding that the overall auxin level/responsiveness in the woody stems is much higher in the cortex than in the vascular cambium is clear, and agrees with similar study in poplar (Teichmann et al. 2008). The GH3 auxin-inducible promoter fused to GUS gene showed a very similar pattern of activity as shown in our studies (Teichmann et al. 2008).

We have shown that the DR5 reporter can be used for studying distribution of auxin sensitivity and the involvement of auxin in growth and development in poplar, the model woody plant. It can therefore be a useful tool for a wide array of physiological studies, including of wood development and tension wood formation, as well as for photo- and gravitropism, root growth, and adventitious root development. The transgenic line produced is available for distribution to the research community upon request.

Acknowledgments This work was supported in part by grants from the US Department of Energy (DOE), Poplar Genome Based Research for Carbon Sequestration in Terrestrial Ecosystems (DE-FG02-06ER64185, DE-FG02-05ER64113), the Consortium for Plant Biotechnology Research, Inc. (GO12026-203A), the United States Department of Agriculture (USDA) CSREES, the USDA-NRI Plant Genome program (2003-04345) and USDA CSREES, the Biotechnology Risk Assessment Research Grants Program (2004-35300-14687), Plant Feedstock Genomics for Bioenergy: A Joint Research Program of USDA and DOE (2009-65504-05767), industrial members of the Tree Genomics and Biosafety Research Cooperative at Oregon State University.

References

Armstrong JI, Yuan S, Dale JM, Tanner VN, Theologis A (2004) Identification of inhibitors of auxin transcriptional activation by means of chemical genetics in Arabidopsis2. Proc Natl Acad Sci USA 101(41):14978–14983

Baba K, Karlberg A, Schmidt J, Schrader J, Hvidsten TR, Bako L, Bhalerao RP (2011) Activity-dormancy transition in the cambial meristem involves stage-specific modulation of auxin response in hybrid aspen. Proc Natl Acad Sci USA 108(8):3418–3423

Bai F, Demason DA (2008) Hormone interactions and regulation of PsPK2: GUS compared with DR5: GUS and PID : GUS in Arabidopsis thaliana1. Am J Bot 95(2):133–145

Ballas N, Wong LM, Theologis A (1993) Identification of the auxin-responsive element, Auxre, in the primary indoleacetic acid-inducible gene, Ps-Iaa4/5, of Pea (*Pisum sativum*). J Mol Biol 233(4):580–596

Barlier I, Kowalczyk M, Marchant A, Ljung K, Bhalerao R, Bennett M, Sandberg G, Bellini C (2000) The SUR2 gene of Arabidopsis thaliana encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proce Natl Acad Sci USA 97(26): 14819–14824

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol 59:443–465

Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115(5):591–602

Bierfreund NM, Reski R, Decker EL (2003) Use of an inducible reporter gene system for the analysis of auxin distribution in the moss *Physcomitrella patens*. Plant Cell Rep 21(12):1143–1152

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. Nature 433(7021):39–44

Casimiro I, Marchant A, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inze D, Sandberg G, Casero PJ, Bennett M (2001) Auxin transport promotes Arabidopsis lateral root initiation. Plant Cell 13(4):843–852

Chaabouni S, Jones B, Delalande C, Wang H, Li ZG, Mila I, Frasse P, Latche A, Pech JC, Bouzayen M (2009) Sl-IAA3, a tomato Aux/IAA at the crossroads of auxin and ethylene signalling involved in differential growth1. J Exp Bot 60(4):1349–1362

Chandler JW (2009) Local auxin production: a small contribution to a big field. Bioessays 31(1):60–70

Cheng YF, Dai XH, Zhao YD (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in Arabidopsis. Gene Dev 20(13): 1790–1799

Delbarre A, Muller P, Imhoff V, Guern J (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198(4):532–541

Digby J, Wareing PF (1966) The effect of applied growth hormones on cambial division and the differentiation of the cambial derivatives. Ann Bot 30(3):539–548

Druart N, Johansson A (2007) Environmental and hormonal regulation of the activity-dormancy cycle in the cambial meristem involves stage-specific modulation of transcriptional and metabolic networks. Plant J 50:557–573

Dubrovsky JG, Napsucialy-Mendivil S, Duclercq J, Cheng Y, Shishkova S, Ivanchenko MG, Friml J, Murphy AS, Benkova E (2011) Auxin minimum defines a developmental window for lateral root initiation. New Phytol 191(4):970–983

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legue V (2009) The ectomycorrhizal fungus Laccaria bicolor stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signaling. Plant Physiol 151(4):1991–2005

Filichkin SA, Meilan R, Busov VB, Ma C, Brunner AM, Strauss SH (2006) Alcohol-inducible gene expression in transgenic Populus. Plant Cell Rep 25(7):660–667

Gallavotti A, Yang Y, Schmidt RJ, Jackson D (2008a) The relationship between auxin transport and maize branching. Plant Physiol 147(4):1913–1923

Gallavotti A, Yang Y, Schmidt RJ, Jackson D (2008b) The relationship between auxin transport and maize branching. Plant Physiol 147(4):1913–1923

Grieneisen VA, Xu J, Maree AFM, Hogeweg P, Scheres B (2007) Auxin transport is sufficient to generate a maximum and gradient guiding root growth. Nature 449(7165):1008–1013

Groover A, Robischon M (2006) Developmental mechanisms regulating secondary growth in woody plants. Curr Opin Plant Biol 9:55–58

Groover AT, Nieminen K, Helariutta Y, Mansfield SD (2010) Wood formation in Populus

Genetics and genomics of Populus. In: Jansson S, Bhalerao R, Groover A (eds) Plant genetics and genomics: crops and models, vol 8, Springer New York, pp 201–224

Hagen G, Guilfoyle T (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol Biol 49(3–4):373–385

Hawkins S, Pilate G, Duverger E, Boudet A, Grima-Pettenati J (2002) The use of GUS histochemistry. In: Chaffey N (ed) Wood formation in trees: cell and molecular biology techniques. Taylor & Francis, London, pp 271–297

Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the Arabidopsis inflorescence meristem. Curr Biol 15(21):1899–1911

Hellgren JM, Olofsson K, Sundberg B (2004) Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. Plant Physiol 135(1):212–220

Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163(2):181–187

Ishizaki K, Nonomura M, Kato H, Yamato KT, Kohchi T (2012) Visualization of auxin-mediated transcriptional activation using a common auxin-responsive reporter system in the liverwort Marchantia polymorpha. J Plant Res 125(5):643–651

Junghans U, Polle A, Duchting P, Weiler E, Kuhlman B, Gruber F, Teichmann T (2006) Adaptation to high salinity in poplar involves changes in xylem anatomy and auxin physiology. Plant Cell Environ 29(8):1519–1531

Kalluri UC, Difazio SP, Brunner AM, Tuskan GA (2007) Genome-wide analysis of Aux/IAA and ARF gene families in Populus trichocarpa. BMC Plant Biol 7:59

Karimi M, Inze D, Depicker A (2002) GATEWAY((TM)) vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7(5):193–195

Khan S, Stone JM (2007) Arabidopsis thaliana GH3.9 in auxin and jasmonate cross talk. Plant Signal Behav 2 (6):483–485

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in Agrobacterium. Biotechnology 9(10):963–967

Leple JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L (1992) Transgenic poplars—expression of chimeric genes using 4 different constructs. Plant Cell Rep 11(3):137–141

Li Y, Wu YH, Hagen G, Guilfoyle T (1999) Expression of the auxin-inducible GH3 promoter GUS fusion gene as a useful molecular marker for auxin physiology. Plant Cell Physiol 40(7):675–682

Liu ZB, Ulmasov T, Shi X, Hagen G, Guilfoyle TJ (1994) Soybean GH3 promoter contains multiple auxin-inducible elements. Plant Cell 6(5):645–657

Mathesius U, Weinman JJ, Rolfe BG, Djordjevic MA (2000) Rhizobia can induce nodules in white clover by "hijacking" mature cortical cells activated during lateral root development. Mol Plant Microbe Interact 13(2):170–182

Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from Arabidopsis catalyzes the

conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J Biol Chem 275(43):33712–33717

Moyle R, Schrader J, Stenberg A, Olsson O, Saxena S, Sandberg G, Bhalerao RP (2002) Environmental and auxin regulation of wood formation involves members of the Aux/IAA gene family in hybrid aspen. Plant J 31(6):675–685

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with Tohaoco tissue cultures. Physiol Plant 15 (3):473–497

Nakamura A, Higuchi K, Goda H, Fujiwara MT, Sawa S, Koshiba T, Shimada Y, Yoshida S (2003) Brassinolide induces IAA5, IAA19, and DR5, a synthetic auxin response element in arabidopsis, implying a cross talk point of brassinosteroid and auxin signaling1. Plant Physiol 133(4):1843–1853

Nemhauser JL, Mockler TC, Chory J (2004) Interdependency of brassinosteroid and auxin signaling in Arabidopsis. Plos Biol 2(9):1460–1471

Normanly J (1997) Auxin metabolism. Physiol Plant 100(3):431–442

Oono Y, Chen QG, Overvoorde PJ, Kohler C, Theologis A (1998) Age mutants of Arabidopsis exhibit altered auxin-regulated gene expression. Plant Cell 10(10):1649–1662

Peret B, Larrieu A, Bennett MJ (2009) Lateral root emergence: a difficult birth. J Exp Bot 60(13):3637–3643

Perrine-Walker F, Doumas P, Lucas M, Vaissayre V, Beauchemin NJ, Band LR, Chopard J, Crabos A, Conejero G, Peret B, King JR, Verdeil JL, Hocher V, Franche C, Bennett MJ, Tisa LS, Laplaze L (2010) Auxin carriers localization drives auxin accumulation in plant cells infected by Frankia in *Casuarina glauca* Actinorhizal nodules. Plant Physiol 154(3):1372–1380

Petersson SV, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JY, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K (2009) An auxin gradient and maximum in the Arabidopsis root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. Plant Cell 21(6):1659–1668

Popko J, Hansch R, Mendel RR, Polle A, Teichmann T (2010) The role of abscisic acid and auxin in the response of poplar to abiotic stress. Plant Biol (Stuttg) 12(2):242–258

Sabatini S, Beis D, Wolkenfelt H, Murfett J, Guilfoyle T, Malamy J, Benfey P, Leyser O, Bechtold N, Weisbeek P, Scheres B (1999) An auxin-dependent distal organizer of pattern and polarity in the Arabidopsis root. Cell 99(5):463–472

Savidge RA (1988) Auxin and ethylene regulation of diameter growth in trees. Tree Physiol 4(4):401–414

Schlicht M, Strnad M, Scanlon MJ, Mancuso S, Hochholdinger F, Palme K, Volkmann D, Menzel D, Baluska F (2006) Auxin immunolocalization implicates vesicular neurotransmitter-like mode of polar auxin transport in root apices. Plant Signal Behav 1(3):122–133

Snow R (1935) Activation of cambial growth by pure hormones. New Phytol 34(5):347–360

Sorin C, Bussell JD, Camus I, Ljung K, Kowalczyk M, Geiss G, McKhann H, Garcion C, Vaucheret H, Sandberg G, Bellini C (2005) Auxin and light control of adventitious rooting in Arabidopsis require ARGONAUTE1. Plant Cell 17(5):1343–1359

Spicer R, Groover A (2010) Evolution of development of vascular cambia and secondary growth. New Phytol 186(3):577–592

Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, Suza W (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. Plant Cell 17(2):616–627

Suer S, Agusti J, Sanchez P, Schwarz M, Greb T (2011) WOX4 imparts auxin responsiveness to cambium cells in Arabidopsis. Plant Cell 23(9):3247–3259

Sundberg B, Tuominen H, Little C (1994) Effects of the indole-3-acetic acid (IAA) transport inhibitors N-1-naphthylphthalamic acid and morphactin on endogenous IAA dynamics in relation to compression wood formation in 1-year-old *Pinus sylvestris* (L.) shoots. Plant Physiol 106(2):469–476

Swarup K, Benkova E, Swarup R, Casimiro I, Peret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K, Jones JD, Taylor CG, Schachtman DP, May S, Sandberg G, Benfey P, Friml J, Kerr I, Beeckman T, Laplaze L, Bennett MJ (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol 10(8):946–954

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol 7(11):847–859

Teichmann T, Bolu-Arianto WH, Olbrich A, Langenfeld-Heyser R, Gobel C, Grzeganek P, Feussner I, Hansch R, Polle A (2008) GH3:GUS reflects cell-specific developmental patterns and stress-induced changes in wood anatomy in the poplar stem. Tree Physiol 28(9):1305–1315

Tuominen H, Puech L, Fink S, Sundberg B (1997) A radial concentration gradient of indole-3-acetic acid is related to secondary xylem development in hybrid Aspen. Plant Physiol 115(2):577–585

Tuominen H, Puech L, Regan S, Fink S, Olsson O, Sundberg B (2000) Cambial-region-specific expression of the Agrobacterium IAA genes in transgenic aspen visualized by a linked uidA reporter gene. Plant Physiol 123(2):531–541

Uggla C, Magel E, Moritz T, Sundberg B (2001) Function and dynamics of auxin and carbohydrates during earlywood/latewood transition in Scots pine. Plant Physiol 125(4):2029–2039

Uggla C, Mellerowicz EJ, Sundberg B (1998) Indole-3-acetic acid controls cambial growth in Scots pine by positional signaling. Plant Physiol 117(1):113–121

Uggla C, Moritz T, Sandberg G, Sundberg B (1996) Auxin as a positional signal in pattern formation in plants. Proc Natl Acad Sci USA 93(17):9282–9286

Ulmasov T, Hagen G, Guilfoyle TJ (1997a) ARF1, a transcription factor that binds to auxin response elements. Science 276(5320): 1865–1868

Ulmasov T, Liu ZB, Hagen G, Guilfoyle TJ (1995) Composite structure of auxin response elements. Plant Cell 7(10):1611–1623

Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997b) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9(11):1963–1971

Vanneste S, Friml J (2009) Auxin: a trigger for change in plant development. Cell 136(6):1005–1016

Yamamoto M, Yamamoto KT (1998) Differential effects of 1-naphthaleneacetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of Arabidopsis, aux1. Plant Cell Physiol 39(6): 660–664

Yamamoto Y, Kamiya N, Morinaka Y, Matsuoka M, Sazuka T (2007) Auxin biosynthesis by the YUCCA genes in rice. Plant Physiol 143(3):1362–1371