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Expression of the Arabidopsis mutant abi1 gene alters ABA sensitivity, stomatal development and growth morphology in Grey poplars (Populus x canescens (Ait.) Sm.)

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SUMMARY

The consequences of altered ABA sensitivity in grey poplar development (*Populus x canescens* (Ait.) Sm.) were examined by ectopic expression of Arabidopsis mutant *abi1* gene. The expression resulted in an ABA-insensitive phenotype revealed by a strong tendency of *abi1* poplars to wilt, impaired responsiveness of their stomata to ABA, and an ABA-resistant bud outgrowth. These plants therefore required cultivation under very humid conditions to prevent drought stress symptoms. Morphological alterations became evident when comparing *abi1* poplars with poplars expressing Arabidopsis non-mutant *ABI1* or wild type (wt) plants. *abi1* poplars showed increased stomatal size, enhanced shoot growth and retarded leaf and root development. The increased stomatal size and its reversion to the size of wt plants by exogenous ABA indicate a role for ABA in regulating stomatal development. Enhanced shoot growth and retarded leaf and root development support the hypothesis that ABA acts independently from drought stress as a negative regulator of growth in shoots and as a positive regulator of growth in leaves and roots. In shoots, we observed an interaction of ABA with ethylene: *abi1* poplars exhibited elevated ethylene production, and the ethylene perception inhibitor Ag⁺ antagonised the enhanced shoot growth. Thus, we provide evidence that ABA acts as negative regulator of shoot growth in non-stressed poplars by restricting ethylene production. Furthermore, we show that ABA has a role in regulating shoot branching by inhibiting lateral bud outgrowth.
INTRODUCTION

The plant hormone abscisic acid (ABA) controls various aspects of plant development. It integrates environmental stress factors, such as drought, cold and rising temperatures, with the metabolic and the developmental program of the plant, it controls seed dormancy and fine-tunes plant growth through a regulatory circuit with other plant hormones (Leung and Giraudat, 1998; Rock, 2000).

The signal transduction pathway triggering ABA-related responses comprises ABA-receptors, several intracellular messengers and a set of signal and transcriptional regulators (Himmelbach et al., 2003; Christmann et al., 2006; Wasilewska et al., 2008). In Arabidopsis, protein phosphatases (Mg\(^{2+}\)-dependent serine / threonine phosphatase type 2C; PP2Cs) are key components in this regulatory network acting as negative regulators of ABA-responses (Merlot et al., 2001; Schweighofer et al. 2004). ABI1 and the highly homologous ABI2 belong to the group of these PP2Cs. The PP2Cs derived from these two genes regulate numerous ABA responses such as stomatal closure, seed dormancy and vegetative growth. The mutant proteins abi1 and abi2, bearing a single amino acid exchange in their PP2C domain, confer a dominant ABA-insensitive Arabidopsis phenotype with impaired stomatal closure, reduced seed dormancy and changes in seedling development (Merlot et al., 2001). The Arabidopsis mutants abi1-1 and abi2-1 (Koornneef et al., 1984) and expression of the mutant proteins in heterologous plant systems (Armstrong et al., 1995; Noël et al., 2005) provide attractive models, not only for studying cellular ABA signal transduction but also for analysing the roles of ABA at the whole plant level.

Plants with altered ABA physiology, e.g. defects in ABA metabolism and ABA signalling, have attracted much attention in recent years. Beside the Arabidopsis mutants abi1-1 and abi2-1, numerous other mutants and transgenic lines have been described in herbaceous species (Finkelstein and Rock, 2002). Functional studies of ABA biosynthesis and response mutants provided new insights into molecular aspects of ABA functioning, thus helping to dissect the complexity of its mode of action. These mutagenic and genetic approaches have confirmed the classical roles of ABA in stress physiology and seed development and revealed further implications in hormone balancing (Koornneef et al., 1982), assimilate partitioning (Koornneef et al., 1989) and even in promoting vegetative development (Barrero et al., 2005). Moreover, ABA plays a key role in responses to drought stress. Saez et al. (2006) have generated ABA-hypersensitive drought-avoidant
Arabidopsis mutants by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1, indicating that these mutants could provide an approach for improving crop performance under drought stress conditions. In addition, ABA has been shown to be a defence hormone influencing resistance against pathogens, for instance via ABI1- and ABI4-dependent signalling (Kaliff et al., 2007).

In contrast to herbaceous plants, less attention has been paid to molecular aspects of ABA functioning in trees. This does not come as a surprise because ABA-related mutants are very rare among woody species and transgenic approaches are considerably more laborious than in Arabidopsis. There is, nonetheless, a growing interest in exploring ABA functioning in perennial plants because of their unique ability to cope with contrasting growth conditions throughout the annual seasons. For instance, in an ABA-deficient genotype of birch (Betula pubescens Ehrh. hibernifolia) it was shown that ABA participates in the accurate timing of cold acclimation, probably by triggering RAB (responsive to ABA) protein expression (Rinne et al., 1998), and in transgenic poplar it was demonstrated that ABI3, a component of the ABA signalling pathway, acts along with ABA in preparing autumnal bud set (Rohde et al., 2002; Ruttink et al., 2007).

Although these studies have revealed important aspects of ABA functioning in woody plants, our knowledge of ABA’s role in trees is far from complete. There is, for instance, a great deal of uncertainty concerning the actual influence of ABA on tree growth and concerning the interaction between ABA and other growth regulators in this process (Lachaud et al., 1999). The phytohormones auxin, cytokinin and gibberellins are well-known for their important functions in controlling longitudinal shoot growth (Eriksson et al., 2000) and shoot branching (Shimizu-Sato and Mori, 2001; Ongaro and Leyser, 2008). In contrast we know little about the involvement of ABA in these processes, which are major determinants of tree architecture and hence canopy structure. In Arabidopsis, hypersensitivity to ABA reduces shoot branching, suggesting a role for ABA in maintaining axillary bud dormancy, and hence in shoot architecture (Pei et al., 1998).

To analyze the role of ABA in trees, we generated Grey poplar (Populus x canescens (Ait.) Sm.) with altered sensitivity to ABA by ectopic expression of the Arabidopsis mutant abi1 gene. The reduced ABA sensitivity affected stomata regulation and development, growth morphology and interfered with ethylene production.
RESULTS

Expression of the Arabidopsis mutant *abi1* and wild-type *ABI1* genes in poplar

Poplars that stably expressed the mutant Arabidopsis *abi1* gene were generated to study woody plants with reduced sensitivity to ABA. Poplars expressing wild-type Arabidopsis *ABI1* and wt poplars served as controls. Previous analysis in Arabidopsis has revealed an essential requirement for nuclear localization of the mutant abi1 protein to confer insensitivity towards ABA responses (Moes et al., 2008). The analysis also showed that protein fusion of abi1 with GFP and β-glucuronidase (GUS) still conferred ABA-insensitivity, albeit at a reduced level.

Such GUS fusions were introduced into poplars for facile detection of *abi1* and *ABI1* expression. Seven to nine independent transformants were obtained for each gene construct expressed under the constitutive 35S promotor. Integration of both gene constructs into the poplar genome was verified by PCR analysis of genomic DNA using *ABI1* specific full-length primers (Figure 1 A). No putative *ABI1* homologue was detected with this primer pair in wt poplar. Protein expression of the *abi1* and *ABI1* genes was proven in six randomly selected transformants (abi1 (1-3) and ABI1 (1-3)) by immunoblot analysis using anti-β-glucuronidase as specific probe for the abi1-GUS and ABI1-GUS fusion proteins. A polypeptide with an expected molecular weight of approximately 125 kD was detected in all selected transformants, indicating the presence of proteins corresponding to the *abi1-GUS* or *ABI1-GUS* gene (Figure 1 B). Histochemical staining of leaves for β-glucuronidase activity confirmed the expression of *abi1-GUS* and *ABI1-GUS* genes in all cell types and tissues (Figure 1 C).

Reduced sensitivity to ABA in *abi1* poplars

Poplar plants expressing the Arabidopsis mutant *abi1* gene showed a very strong tendency to wilt when transferred from growth conditions with high relative humidity (RH; approx. 90 %) to growth conditions with lower RH values. Therefore, all our efforts to adapt such plants to growth on soil at ambient air humidity failed. We were only able to grow a few *abi1* expressing plants in soil at a RH of 80 % after step wise adaptation over several months. The midday water potential in these plants was significantly lower than in wt poplars (*abi1* (1): -0.96 ± 0.05 MPa, *abi1* (2): -0.8 ± 0.04, wt: -0.67 ± 0.03; mean ± SE; n > 10 leaves; *P* < 0.01) and leaves had a slightly xeromorphic appearance (data not shown). To prevent such drought symptoms, all further experiments described here were carried out
with plants growing in glass containers on half concentrated MS medium at near humidity saturation (RH > 90 %). Under these conditions, all plants showed vigourous growth, and no differences in xylem water potential were detectable between abi1, ABI1 and wt poplars (Table 1).

To further test whether the abi1 insertion into the poplar genome affected the sensitivity to ABA, the influence of exogenously applied ABA on different ABA-related physiological traits was determined in abi1, ABI1 and wt poplars. In particular, we tested the reaction of stomatal aperture and lateral bud dormancy upon external application of ABA. Stomatal aperture decreased significantly by about 30% in wt and ABI1 poplars after treatment of excised leaves with 200 µM ABA for 3 h, whereas stomatal aperture in abi1 poplars did not respond to this treatment (Figure 2). Lateral bud outgrowth was completely blocked in wt and ABI1 poplars when excised shoot segments were incubated on MS medium supplemented with 50 µM ABA. In abi1 poplars, however, lateral bud outgrowth of shoot segments was not inhibited by this treatment even though subsequent growth of lateral shoots was strongly impaired (Figure 3 A-F). Furthermore, ABA accelerated the abscission of old petioles in wt and ABI1 poplars, contrary to abi1 poplars where no such ABA-triggered abscission of petioles was observed (Figure 3 A-F).

**Altered stomata morphology in abi1 poplars**

The strong tendency of abi1 poplars to wilt and their failure to adapt to growth environments with RH values lower than 80 % prompted us to conduct a more detailed study of stomatal morphology in abi1, ABI1 and wt poplars. The former histometrical analysis of stomatal apertures gave strong evidence that stomatal development is altered in abi1 poplars in favour of larger stomata (Figure 2). Since this histometrical analysis was performed with intensely watered and illuminated leaves with fully opened stomata (except for ABA treatments), the measurements of maximal achievable stomatal aperture areas can be considered a good estimate of stomatal size. A representative light microscopic inspection of leaves from abi1, ABI1 and wt poplars confirmed the increased stomatal size in abi1 poplars (Figure 4).

To further check whether reduced sensitivity to ABA is the cause of altered stomata development in abi1 poplars, stomata were analyzed in leaves developed from abi1 shoot segments grown on MS medium initially supplemented with 50 µM ABA. After six weeks on MS medium without further ABA supply, stomatal apertures were determined on
intensely irrigated and illuminated leaves with fully opened stomata. These measurements revealed a significant reduction of stomatal apertures in leaves from ABA-grown shoots, indicating an ABA-triggered decrease in stomatal size in *abi1* poplars (Figure 5). Interestingly, stomatal apertures in leaves from ABA-treated *abi1* poplars were similar to those observed in *ABI1* and wt poplars (compare Figure 2).

**Enhanced ethylene emission in *abi1* poplars**

To test the effect of reduced ABA sensitivity on other plant growth regulators, ethylene emission rate (EER) and the tissue content of different gibberellins (GA₁, GA₄, GA₈, GA₉, and GA₃₄) were determined in *abi1*, *ABI1* and wt poplars. EER measurements were carried out on the whole plant level to prevent artificial ethylene emission induced by mechanical injuries after dissection of single plant organs. EERs were significantly 2 - 3 times higher in *abi1* poplars than in *ABI1* or wt poplars (except non-significant increase in *abi1* (2); Table 1). No differences in EER could be detected when comparing *ABI1* with wt poplars. It is important to note that enhanced EER in *abi1* poplars were independent from plant water status as xylem water potentials were similar in *abi1*, *ABI1* and wt poplars (except higher water potential in *abi1* (2); Table 1). In addition, we analyzed the major endogenous gibberellin levels (Eriksson et al., 2000) from leaves, shoots and roots. In contrast to ethylene, neither GA₁, GA₄, GA₈, GA₉, nor GA₃₄ levels differed in *abi1*, *ABI1* and wt poplars (data not shown).

**Morphological analysis of *abi1*, *ABI1* and wt poplars**

Ectopic expression of *abi1* in poplar caused a distinct change in growth morphology of these plants, characterized by a tall habitus with elongated shoots, smaller leaves and a less developed root system (Figure 6 A). Wt poplars, in contrary, had stunted shoots, larger leaves and a well developed root system (Figure 6 C). The growth morphology of *ABI1* expressing poplars was comparable to that of wt poplars except a slightly enhanced height growth and somewhat longer internodes (Figure 6 B). Quantitative analysis of different morphological traits in *abi1*, *ABI1* and wt poplars revealed these alterations in more detail (Table 2). Internode length, height growth, leaf and root weight differed significantly when comparing *abi1* poplars with *ABI1* or wt poplars. *abi1* poplars showed enhanced height growth and longer internodes but lower leaf and root weight than wt. *ABI1* poplars also showed enhanced height growth and longer internodes in comparison to wt poplar but these differences were much less pronounced than those observed in *abi1* poplars. Leaf and
root weight in ABI1 poplars showed no differences to wt poplars. A microscopic analysis of shoot segments revealed the formation of longer parenchyma cells in abil poplars indicating enhanced longitudinal cell expansion (abi1: 106 ± 5 µm, ABI1: 53 ± 3 µm, wt: 61 ± 3 µm; mean ± SE).

To test whether enhanced ethylene formation contributes to the altered growth morphology in abil poplars, plants were additionally grown on MS medium supplemented with Ag$^{3+}$, which is recognised as a specific inhibitor of ethylene perception (Beyer, 1976). Plants were exposed only to very low amounts of AgNO$_3$ (10 µM) to prevent toxic effects of this inhibitor. The morphological analysis of Ag$^{3+}$-treated abil poplars revealed a significant reduction in height growth and internode length in relation to non-treated abil poplars (Table 2), thus indicating partial restoration of the wt phenotype. The leaf and root weights of abil poplars were not clearly affected by Ag$^{3+}$-treatment. To exclude that a potential toxicity of Ag$^{3+}$ contributed to the reduced height growth and internode length of abil poplars, control experiments were performed with wt poplars. Ag$^{3+}$-treatment of these plants had no negative effects on their growth morphology (Table 2). Height growth and internode length increased in Ag$^{3+}$-treated wt poplars and their leaf and root weights were higher in comparison to non-treated wt poplars. A toxic effect of the Ag$^{3+}$- treatment can therefore be excluded.
DISCUSSION

Reduced ABA sensitivity in abi1-expressing poplars

ABA is known to play a major role in seed development (Finkelstein et al., 2002), growth (Cheng et al., 2002; LeNoble et al., 2004; Lin et al., 2007), stomatal movements (Schroeder et al., 2001b), and in the integration of signals resulting from drought, high salinity as well as low temperature (Christmann et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). In the present study, Grey poplar was transformed with the dominant Arabidopsis mutant abi1 gene to alter ABA sensitivity in this woody model plant. Poplar transformed with the Arabidopsis wildtype ABI1 gene and wt poplar served as controls. Stable integration and expression of these genes could be verified by PCR with gene specific primers and protein analysis. Interestingly, no putative ABI1 homologue could be detected in wt poplar by PCR, indicating limited nucleotide sequence similarity between the Arabidopsis abi1 / ABI1 gene and the corresponding poplar gene. This observation is consistent with the wide structural divergence among PP2Cs (Leung et al., 1994; Meyer et al., 1994; Shiozaki et al., 1994; Schweighofer et al., 2004). Nevertheless, poplars expressing the abi1 gene exhibited an ABA-related phenotype, reminiscent of the ABA-insensitive phenotype of the dominant Arabidopsis mutant abi1-1 (Koornneef et al., 1984). This finding indicates that the abi1 gene interferes with ABA signalling in both plant species via a conserved mechanism. Competition between mutant abi1 and non-mutant ABI1 proteins for common binding sites is discussed as a cause for such interfering effect (Wu et al., 2003). A similar situation might be supposed for poplar where ectopic expression of the dominant abi1 gene might interfere with ABA signalling via competition with potential ABI1 homologues.

The ABA-related phenotype of abi1 expressing poplars became evident through their strong tendency to wilt when transferred to growth conditions with ambient air humidity. Cultivation of transgenic plants with this phenotype was therefore only possible at close to relative humidity-saturation. Physiological analysis of ABA-related traits in these plants revealed an impaired ability to respond to exogenous ABA, thus yielding further evidence for reduced ABA sensitivity of abi1 poplars. The tested traits - stomatal closure and lateral bud dormancy - depend on the action of ABA and may therefore be considered as useful indicators of modified ABA sensitivity (Schroeder et al., 2001a; Shimizu-Sato and Mori, 2001). Even though in the case of lateral bud dormancy some uncertainty exists concerning the actual role of ABA, our results clearly show that exogenous ABA maintains lateral bud...
dormancy in ABI1 and wt poplars but not in abil poplars. This demonstrates reduced sensitivity to ABA in abil poplars and additionally emphasizes the role of ABA in controlling lateral bud dormancy.

**Increased stomatal size in abil poplars**

The wilty phenotype of plants with impaired ABA biosynthesis or sensitivity has been exclusively linked to the failure of stomata to close in response to ABA (Koornneef et al., 1984; Rousselin et al., 1992; Armstrong et al., 1995). In the present study, we provide evidence that such a wilty phenotype is not only due to a defect stomata regulation, but also to alterations in stomatal development. The increased stomatal size in abil poplars, indicated by exceptionally large stomatal apertures, hampers stomatal control of water loss and may thus contribute to the strong tendency to wilt in these lines. This could additionally explain why abil poplars failed to adapt to growth conditions with ambient air humidity.

Increased stomatal size and a wilty phenotype have also been reported for transgenic potato, where endogenous applied ABA was inactivated by expression of an ABA-binding antibody fragment (Strauß et al., 2001). Interestingly, stomata in these plants were still able to close in response to environmental stimuli, thus providing evidence that in this case the increased stomatal size is the cause for the wilty phenotype. In further experiments with abil poplars we demonstrated that the increased stomatal size could be restored to wt levels, following the addition of ABA to developing leaves. This ABA induced reversion of the increased stomatal size in abil poplars supports the hypothesis that ABA participates in the control of stomatal development. Thus, ABA might function not only as short time regulator of stomatal closure but also as long term signal in adaptational responses of stomatal development.

**Altered growth morphology and ethylene formation in abil poplars**

Morphological alterations in abil-expressing poplars became evident, when comparing the growth habitus of these plants with that of ABI1 and wt poplars. A morphological analysis revealed enhanced shoot growth in abil poplars but retarded leaf and root development. The promotion of shoot growth resembles the situation in ABA-deficient mutants of tomato or abil-transgenic potato, where similar growth characteristics have been observed (Jones et al., 1987; Chen et al., 2003; Noël et al., 2005). Other reports, however, describe
reduced shoot growth in *abi1*-transgenic tobacco (Armstrong et al., 1995) or ABA-deficient mutants of Arabidopsis (LeNoble et al., 2004) and tomato (Sharp et al., 2000). Explanations for these conflicting results might be related to (i) differences in plant water status that result from poor stomatal regulation, (ii) the use of plants in different developmental stages in the different studies or (iii) species-dependent effects. In the present study, differences in plant water status could be excluded as the poplar plants were grown at close to relative humidity-saturation and water potentials were similar in all lines. Consequently, the morphological alterations observed in *abi1* poplars may be clearly considered as a direct effect of altered ABA sensitivity.

The strongly enhanced shoot growth in *abi1* poplar contrasted sharply with the concomitant inhibition of leaf and root development, thus indicating that growth processes in these plant organs are differently affected by ABA. With respect to reduced ABA sensitivity of *abi1* poplars, this result suggests a role of ABA as a negative regulator of shoot growth, in contrast to a role as a positive regulator of leaf and root development. Such differential growth responses are not unprecedented, as similar results have been reported in previous studies on ABA-deficient mutants of tomato (Sharp et al., 2000; Chen et al., 2003). The specific reason for these contrasting growth responses is so far unknown but a hormonal interaction of ABA with ethylene might play a role. Both synergistic and antagonistic growth effects of ABA and ethylene are described and the specific growth response may depend on the tissue or plant organ that is targeted by these hormones (Beaudoin et al., 2000; Ghassemian et al., 2000).

The present data demonstrated an elevated ethylene formation in *abi1* poplars, testifying for a strong functional interaction between ABA and ethylene. This observation is in line with studies on ABA-deficient tomato and Arabidopsis mutants where low ABA levels coincided with elevated ethylene biosynthesis rates (Tal et al., 1979; Rakitina et al., 1994; Sharp et al., 2000; LeNoble et al., 2004). Interestingly, shoot growth is inhibited in these ABA-deficient herbaceous mutants, contrary to *abi1* poplars where elevated ethylene biosynthesis correlated with enhanced shoot growth. This enhanced shoot growth appeared to be a direct consequence of elevated ethylene biosynthesis as treatments of *abi1* poplars with the ethylene perception inhibitor Ag$^{3+}$ restored, at least partly, wt shoot growth characteristics. Toxic effects could be excluded as cause for the restoration of wt shoot growth since Ag$^{3+}$ treatment did not reduce shoot growth in wt poplars. Gibberellins seemed not to be involved in the enhanced shoot growth as *abi1* poplars did not contain elevated levels of bioactive gibberellins (GA$_1$ and GA$_8$).
Even though ethylene is generally considered to be a growth inhibitor (Abeles et al., 1992; Hussain et al., 1999; Sharp, 2002) an opposite effect is not unexpected. Indeed, ethylene has been reported to stimulate hypocotyl elongation of Arabidopsis seedlings (Smalle et al., 1997; Saibo et al., 2003), longitudinal shoot growth of semi-aquatic plants (Voesenek and Van der Veen, 1994) and radial shoot growth in trees (Eklund and Klintborg, 2000). Interactions with other hormonal regulators, e.g. ABA, might be crucial for such growth stimulation by ethylene (Pierik et al., 2006). Our results provide strong evidence for such a functional interaction as they indicate a role for ABA in negative control of poplar shoot growth via restriction of ethylene biosynthesis.
MATERIALS AND METHODS

Transformation and micropropagation

The construction of vectors used for poplar transformation is described in detail by Moes et al. (2008). Poplar plants (Populus x canescens (Ait.) Sm., clone 717-B4, Institute de la Recherche Agronomique, Nancy, France) were transformed with Agrobacterium tumefaciens strain GV3101 by stem-internode transformation, and regenerated as described by Leplé et al. (1992). Wt and selected transgenic lines were amplified by micropropagation as described by Leplé et al. (1992) on half-concentrated Murashige-Skoog (MS) medium (Murashige and Skoog, 1962), in 1 l glass containers under standard conditions (photoperiod of 16 h with approximately 100 µmol m⁻²sec⁻¹ PPFD; room temperature). Plants for phenotypic analysis were grown for 45 days under these conditions.

Verification of transformation via PCR analysis

To verify integration of transferred abi and ABI1 sequences into the poplar genome, genomic DNA was isolated from leaf tissue with Phytopure plant DNA extraction kit (Amersham Biosciences, Freiburg, Germany) following the manufacturer’s instructions. Transgene DNA was amplified by PCR using A. thaliana ABI1 full length primers (forward 5'-atggaggaagtatctccggcgatc-3' and reverse 5'-gctcttgagtttcctccgaggcttc-3') resulting in an amplicon with a length of 1.3 kb. In controls no product was amplified.

Imunochemical protein analysis

Expression of the abi1-GUS and ABI1-GUS fusion proteins were proven by immunoblot analysis. SDS-soluble protein was extracted from frozen leaf tissue as previously described by Arend et al. (2004). Equal amounts of protein (50 µg) were separated by SDS-gel electrophoresis (Laemmeli 1970) and blotted onto PVDF membranes. The membranes were probed with an anti-GUS antibody (Invitrogen, Karlsruhe, Germany) and gold-labeled secondary antibody (British Biocell, UK).

ABA sensitivity assay

Nodial shoot segments with a single lateral bud and petiole were cut from intact plants. Shoot segments were incubated under standard conditions (photoperiod of 16 h with approximately 100 µmol m⁻²sec⁻¹ PPFD) for three weeks on half-concentrated MS
medium supplemented or not with 50 µM ABA. Outgrowth of lateral buds and abscission of petioles were documented photographically.

**Stomatal aperture assays and estimation of stomatal size**

Measurements of ABA-induced stomatal closure were performed on detached leaves in a solution consisting of 10 mM KCl and 10 mM MES / TRIS, pH 6.2. The leaves were kept for 3 h in the light (approximately 100 µmol m⁻² sec⁻¹ PPFD) to achieve full stomata opening. Where specified, leaves were supplemented with 200 µM ABA to induce stomatal closure. Stomatal apertures were measured by determining the stomatal pore area using a light microscope (Zeiss Axiophot, Oberkochen, Germany) and a digital image analysis system (Zeiss Axio Vision, Oberkochen, Germany). Measurements of stomatal pore areas of fully opened stomata were also used to estimate differences in stomatal size between abi1, ABI1 and wt poplars.

**Histochemical staining for GUS activity**

Detection of β-glucuronidase (GUS) activity was performed as described by Jefferson et al. (1987) with some modifications: leaf tissue was fixed in 80% (v/v) ice-cold acetone, rinsed in 50 mM sodium phosphate, pH 7.0 and then incubated in GUS assay buffer (50 mM sodium phosphate, pH 7.0, 0.1% (v/v) Triton X-100, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) for 12 h at 37°C.

**Microscopic analysis of shoot segments**

Shoot segments were taken from the 5th internode, fixed with 3% (w/v) formaldehyde in phosphate-buffered solution, dehydrated in a graded series of ethanol and embedded in Technovit methacrylate resin. Transversal sections (6 µm) were stained with Giemsa’s stain. Cell lengths were measured using a light microscope (Zeiss Axiophot, Oberkochen, Germany) and a digital analysis system (Zeiss Axio Vision, Oberkochen, Germany).
**Hormone analysis**

Ethylene biosynthesis was measured on intact plants (45-days-old) kept for 24 h under standard conditions (photoperiod of 16 h with approximately 100 μmol m$^{-2}$sec$^{-1}$ PPFD) in gas tight glass containers. Each container harboured three plants and a small amount of liquid, half-concentrated MS medium to ensure sufficient water and nutrient supply. At the end of the incubation period two gas samples (2 x 10 ml) were collected from each container. All gas samples were analyzed immediately for their ethylene concentration with a gas chromatograph (GC 8A, Shimadzu Deutschland GmbH, Duisburg, Germany) equipped with a HayeSep Q column (3 m 1/8” i.d. particle size: 60/80 mesh, Supelco, Deisenhofen, Germany) and a flame ionization detector (FID). Synthetic air (21 % (v/v) O$_2$ 4.5, 79 % (v/v) N$_2$ 5.0, Basi Schöberl, Rastatt, Germany) served as carrier gas, and the temperatures of the FID and the column oven were 250°C and 50°C, respectively. Calibration was performed using a range of different ethylene gas concentrations made by mixing pure ethylene (C$_2$H$_4$, 99.95% purity; Air Liquide, Griesheim, Germany) with synthetic air. All measurements were repeated two times. Quantitative determination of endogenous gibberellins was performed as described by Lange et al. (2005).

**Water potential**

Midday water potentials were measured in excised shoots using a Scholander Pressure Bomb (Scholander et al., 1964).

**Statistical analysis**

All comparisons of parameter means were analyzed using *Student's* t-test. Differences between parameter means were considered significant at $P < 0.05$.

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FIGURE LEGENDS

Figure 1. Stable transformation of Arabidopsis genes abi1 and ABI1 in poplar and their ectopic expression. A, Amplification of abi1 / ABI1 transgenes (analysis of five independent abi1 / ABI1 transformants) via PCR with ABI1 specific primers. wt: wildtype control. B, Immuno-blot analysis of abi1- GUS and ABI1-GUS protein expression (analysis of three independent abi1 / ABI1 transformants) with anti GUS antibody. C, Histochemical detection of abi1-GUS and ABI1-GUS protein expression (analysis of three independent abi1 / ABI1 transformants).

Figure 2. Effect of ABI1 and abi1 expression on ABA promotion of stomatal closure. (■) control, (□) 200 µM ABA. Values are mean ± SE (n = 10 leaves / 60 stomata per leaf, P ≤ 0.05*/0.005**, Student's t test).

Figure 3. Lateral bud outgrowth of short shoot segments grown for three weeks on half-concentrated MS medium supplemented with ABA (50 µM) or without ABA. A and B, Poplars expressing abi1. Outgrowth of lateral buds is not inhibited by ABA. C and D, Poplars expressing ABI1. Outgrowth of lateral buds is inhibited by ABA. E and F, Wt poplars. Outgrowth of lateral buds is inhibited by ABA. (Arrows point to old petioles still clinging on the stem axis. Asterisks indicate old petioles separated from the stem axis. Inserts in (D) and (F) show dormant lateral buds.)

Figure 4. Typical stomata in wt, ABI1 and abi1 poplars (leaf tissue double-stained with anilin-blue and safranin; bar 10 µm).

Figure 5. Aperture of fully opened stomata of abi1 poplars grown for five weeks on half-concentrated MS medium supplemented initally with 50 µM ABA (■) or without ABA (■). Values are mean ± SE (n = 10 leaves, P ≤ 0.005**, Student's t test).

Figure 6. Growth habitus of poplar plants grown for 45 days on half-concentrated MS medium with high air humidity (RH > 90%). A, Poplars expressing abi1 with elongated shoots, small leaves and less developed roots. B, Poplars expressing ABI1 with short shoots, large leaves and well developed roots. C, Wt poplars with stunted shoots, large leaves and well developed roots.
Table 1. Midday water potentials ($\Psi_{xyl}$, MPa) and ethylene emission rates (EER, pmol day$^{-1}$ g$^{-1}$ FW) in wt, ABI and abi1 poplars grown at $\geq$ 90% RH. Values are means ± SE (water potential: $n \geq 6$; ethylene emission rates: $n = 3$ samples / 3 plants per sample; significant difference at $P \leq 0.05^*$; significantly different from wt and ABI plants at $P \leq 0.05^\dagger$; Student's t test).

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>ABI1 (1)</th>
<th>ABI1 (2)</th>
<th>ABI1 (3)</th>
<th>abi1 (1)</th>
<th>abi1 (2)</th>
<th>abi1 (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Psi_{xyl}$ mean</td>
<td>-0.72</td>
<td>-0.7</td>
<td>-0.78 *</td>
<td>-0.74</td>
<td>-0.73</td>
<td>-0.62 *</td>
<td>-0.66</td>
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<tr>
<td></td>
<td>SE</td>
<td>0.09</td>
<td>0.05</td>
<td>0.03</td>
<td>0.05</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>EER mean</td>
<td>1.9</td>
<td>1.7</td>
<td>2.1</td>
<td>1.2</td>
<td>4.9 $^\dagger$</td>
<td>4.9</td>
<td>4.4 $^\dagger$</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
<td>0.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Table 2. Morphological characteristics of *abi1*, *ABI1* and wt poplar grown for 45 days on half-concentrated MS medium. An additional set of *abi1* poplars were grown on half-concentrated MS medium supplemented with 10 µM AgNO₃. Values are means ± SE (n ≥ 10 plants; significantly different from wt plants at $P \leq 0.05^* / 0.005^{**}$; significantly different from *ABI1* plants at $P \leq 0.05^† / 0.005^{††}$; significantly different from corresponding wt or *abi1* lines at $P \leq 0.05^{◊} / 0.005^{◊◊}$; Student's t test).

<table>
<thead>
<tr>
<th>poplar plants</th>
<th>internode length (mm)</th>
<th>height growth (mm d⁻¹)</th>
<th>leaf weight (mg FW)</th>
<th>root weight (mg FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>3.3 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>16.1 ± 1.7</td>
<td>31.4 ± 4.8</td>
</tr>
<tr>
<td>ABI1 (1)</td>
<td>5.2 ± 0.2 **</td>
<td>1.5 ± 0.1 **</td>
<td>19.9 ± 1.9</td>
<td>38.3 ± 6.3</td>
</tr>
<tr>
<td>ABI1 (2)</td>
<td>5.4 ± 0.3 **</td>
<td>1.5 ± 0.1 **</td>
<td>20.9 ± 2.7</td>
<td>37.8 ± 7.4</td>
</tr>
<tr>
<td>ABI1 (3)</td>
<td>4.3 ± 0.2 **</td>
<td>1.2 ± 0.1 **</td>
<td>22.2 ± 1.7 *</td>
<td>65.8 ± 5.5 **</td>
</tr>
<tr>
<td>abi1 (1)</td>
<td>10.3 ± 0.3 ** ††</td>
<td>2.7 ± 0.1 ** ††</td>
<td>5.9 ± 0.6 ** ††</td>
<td>10.1 ± 1.4 * ††</td>
</tr>
<tr>
<td>abi1 (2)</td>
<td>9.5 ± 0.4 ** ††</td>
<td>2.5 ± 0.1 ** ††</td>
<td>7.3 ± 0.8 ** ††</td>
<td>13.1 ± 1.7 * ††</td>
</tr>
<tr>
<td>abi1 (3)</td>
<td>10.7 ± 0.3 ** ††</td>
<td>2.6 ± 0.1 ** ††</td>
<td>9.6 ± 0.9 ** ††</td>
<td>16.9 ± 2.3 †</td>
</tr>
<tr>
<td>wt / AgNO₃</td>
<td>6.2 ± 0.5 ♦</td>
<td>1.7 ± 0.2 ♦</td>
<td>48 ± 3.9 ♦</td>
<td>403 ± 69 ♦</td>
</tr>
<tr>
<td>abi1 (1) / AgNO₃</td>
<td>8.2 ± 0.2 ♦</td>
<td>1.6 ± 0.1 ♦</td>
<td>5.4 ± 0.5</td>
<td>18 ± 1.7 ♦</td>
</tr>
<tr>
<td>abi1 (2) / AgNO₃</td>
<td>7.9 ± 0.4 ♦</td>
<td>1.6 ± 0.1 ♦</td>
<td>5.9 ± 0.7</td>
<td>16.4 ± 1.7</td>
</tr>
<tr>
<td>abi1 (3) / AgNO₃</td>
<td>7.8 ± 0.2 ♦</td>
<td>1.3 ± 0.1 ♦</td>
<td>6.3 ± 0.5 ♦</td>
<td>16.1 ± 1.4</td>
</tr>
</tbody>
</table>
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