Expression of the Arabidopsis Mutant abi1 Gene Alters Abscisic Acid Sensitivity, Stomatal Development, and Growth Morphology in Gray Poplars[^1](C)


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The consequences of altered abscisic acid (ABA) sensitivity in gray poplar (Populus × canescens [Ait.] Sm.) development were examined by ectopic expression of the Arabidopsis (Arabidopsis thaliana) mutant abi1 (for abscisic acid insensitive1) 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 nonmutant ABI1 or wild-type 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 wild-type 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⁺ antagonized the enhanced shoot growth. Thus, we provide evidence that ABA acts as negative regulator of shoot growth in nonstressed poplars by restricting ethylene production. Furthermore, we show that ABA has a role in regulating shoot branching by inhibiting lateral bud outgrowth.

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 (Arabidopsis thaliana), protein phosphatases (Mg²⁺-dependent Ser/Thr phosphatase type 2C [PP2C]) are key components in this regulatory network, acting as negative regulators of ABA responses (Merlot et al., 2001; Schweighofer et al., 2004). ABI1 (for Abscisic Acid Insensitive1) 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

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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 analyzing the roles of ABA at the whole-plant level.

Plants with altered ABA physiology, e.g. defects in ABA metabolism and ABA signaling, have attracted much attention in recent years. Beside the Arabidopsis mutants ab1-1 and ab1-2, 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 PP2Cs 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 defense hormone influencing resistance against pathogens, for instance via ABI1-dependent signaling (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 signaling pathway, acts along with ABA in preparing autumnal bud set (Rohde et al., 2002; Rutting 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 Gray poplar (Populus × canescens [Ait.] Sm.) with altered sensitivity to ABA by ectopic expression of the Arabidopsis mutant ab1 gene. The reduced ABA sensitivity affected stomata regulation and development and growth morphology, and interfered with ethylene production.

RESULTS

Expression of the Arabidopsis Mutant ab1 and Wild-Type ABI1 Genes in Poplar

Poplars that stably expressed the mutant Arabidopsis ab1 gene were generated to study woody plants with reduced sensitivity to ABA. Poplars expressing wild-type Arabidopsis ABI1 and wild-type poplars served as controls. Previous analysis in Arabidopsis has revealed an essential requirement for nuclear localization of the mutant ab1 protein to confer insensitivity toward ABA responses (Moes et al., 2008). The analysis also showed that protein fusion of ab1 with GFP and GUS still conferred ABA insensitivity, albeit at a reduced level.

Such GUS fusions were introduced into poplars for facile detection of ab1 and ABI1 expression. Seven to nine independent transformants were obtained for each gene construct expressed under the constitutive 35S promoter. Integration of both gene constructs into the poplar genome was verified by PCR analysis of genomic DNA using ABI1-specific full-length primers (Fig. 1A). No putative ABI1 homolog was detected with this primer pair in wild-type poplar. Protein expression of the ab1 and ABI1 genes was proven in six randomly selected transformants ab1 (1–3) and ABI1 (1–3) by immunoblot analysis using anti-GUS as specific probe for the ab1-GUS and ABI1-GUS fusion proteins. A polypeptide with an expected Mr of approximately 125 kD was detected in all selected transformants, indicating the presence of proteins corresponding to the ab1-GUS or ABI1-GUS gene (Fig. 1B). histochemical staining of leaves for GUS activity confirmed the expression of ab1-GUS and ABI1-GUS genes in all cell types and tissues (Fig. 1C).

Reduced Sensitivity to ABA in ab1 Poplars

Poplar plants expressing the Arabidopsis mutant ab1 gene showed a very strong tendency to wilt when transferred from growth conditions with high relative humidity (RH; approximately 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 wild-type poplars (abi1 [1]: −0.96 ± 0.05 MPa, abi1 [2]: −0.8 ± 0.04, wild type: −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 Murashige and Skoog (MS) medium at near humidity saturation (RH >90%). Under these conditions, all plants showed vigorous growth, and no differences in xylem water potential were detectable between abi1, ABI1, and wild-type poplars (Table I).

Table 1. Midday water potentials (Ψ, MPa) and EERs (pmol d⁻¹ g⁻¹ fresh weight) in wild-type, ABI, and abi1 poplars grown at ≥90% RH

<table>
<thead>
<tr>
<th>Water Potential and Ethylene Emission</th>
<th>Wild Type</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>Ψₓ₀</td>
<td>−0.72 ± 0.09</td>
<td>−0.7 ± 0.05</td>
<td>−0.78* ± 0.03</td>
<td>−0.74 ± 0.05</td>
<td>−0.73 ± 0.06</td>
<td>−0.62* ± 0.04</td>
<td>−0.66 ± 0.03</td>
</tr>
<tr>
<td>EER</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.3</td>
<td>2.1 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>4.9* ± 0.6</td>
<td>4.9 ± 0.9</td>
<td>4.4* ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± si (water potential: n ≥ 6; EERs: n = 3 samples/3 plants per sample; significant difference at P ≤ 0.05*; significantly different from wild-type and ABI plants at P ≤ 0.05; Student’s t test).

To further test whether the abi1 insertion into the poplar genome effected the sensitivity to ABA, the influence of exogenously applied ABA on different ABA-related physiological traits was determined in abi1, ABI1, and wild-type 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 wild-type 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 (Fig. 2). Lateral bud outgrowth was completely blocked in wild-type 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 (Fig. 3, A–F). Furthermore, ABA accelerated the abscission of old petioles in wild-type and ABI1 poplars, contrary to abi1 poplars where no such ABA-triggered abscission of petioles was observed (Fig. 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 wild-type poplars. The former histometrical analysis of stomatal apertures gave strong evidence that stomatal development is altered in abi1 poplars in favor of larger stomata (Fig. 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 wild-type poplars confirmed the increased stomatal size in abi1 poplars (Fig. 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 6 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 \(\text{abi1}\) poplars (Fig. 5). Interestingly, stomatal apertures in leaves from ABA-treated \(\text{abi1}\) poplars were similar to those observed in \(\text{ABI1}\) and wild-type poplars (compare with Fig. 2).

### Enhanced Ethylene Emission in \(\text{abi1}\) 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 (GA\(_1\), GA\(_4\), GA\(_8\), GA\(_9\), and GA\(_34\)) were determined in \(\text{abi1}\), \(\text{ABI1}\), and wild-type 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 to 3 times higher in \(\text{abi1}\) poplars than in \(\text{ABI1}\) or wild-type poplars (except nonsignificant increase in \(\text{abi1}\) [2]; Table I). No differences in EER could be detected when comparing \(\text{ABI1}\) with wild-type poplars. It is important to note that enhanced EER in \(\text{abi1}\) poplars were independent from plant water status as xylem water potentials were similar in \(\text{abi1}\), \(\text{ABI1}\), and wild-type poplars (except a less negative water potential in \(\text{abi1}\) [2]; Table I).

In addition, we analyzed the major endogenous gibberellin levels (Eriksson et al., 2000) from leaves, shoots, and roots. In contrast to ethylene, neither GA\(_1\), GA\(_4\), GA\(_8\), GA\(_9\), nor GA\(_34\) levels differed in \(\text{abi1}\), \(\text{ABI1}\), and wild-type poplars (data not shown).
Morphological Analysis of abi1, ABI1, and Wild-Type 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 (Fig. 6A). Wild-type poplars, in contrary, had stunted shoots, larger leaves, and a well-developed root system (Fig. 6C). The growth morphology of ABI1-expressing poplars was comparable to that of wild-type poplars except for a slightly enhanced height growth and somewhat longer internodes (Fig. 6B). Quantitative analysis of different morphological traits in abi1, ABI1, and wild-type poplars revealed these alterations in more detail (Table II). Internode length, height growth, leaf, and root weight differed significantly when comparing abi1 poplars with ABI1 or wild-type poplars. abi1 poplars showed enhanced height growth and longer internodes but lower leaf and root weight than wild type. ABI1 poplars also showed enhanced height growth and longer internodes in comparison to wild-type 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 wild-type poplars. A microscopic analysis of shoot segments revealed the formation of longer parenchyma cells in abi1 poplars, indicating enhanced longitudinal cell expansion (abi1: 106 ± 5 μm, ABI1: 53 ± 3 μm, wild type: 61 ± 3 μm; mean ± se).

To test whether enhanced ethylene formation contributes to the altered growth morphology in abi1 poplars, plants were additionally grown on MS medium supplemented with Ag3+, which is recognized as a specific inhibitor of ethylene perception (Beyer, 1976). Plants were exposed only to very low amounts of AgNO3 (10 μM) to prevent toxic effects of this inhibitor. The morphological analysis of Ag3+-treated abi1 poplars revealed a significant reduction in height growth and internode length in relation to nontreated abi1 poplars (Table II), thus indicating partial restoration of the wild-type phenotype. The leaf and root weights of abi1 poplars were not clearly affected by Ag3+ treatment. To exclude that a potential toxicity of Ag3+ contributed to the reduced height growth and internode length of abi1 poplars, ey experiments were performed with wild-type poplars. Ag3+ treatment of these plants had no negative effects on their growth morphology (Table II). Height growth and internode length increased in Ag3+-treated wild-type poplars and their leaf and root weights were higher in comparison to nontreated wild-type poplars. A toxic effect of the Ag3+ 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 the integration of signals resulting from drought, high salinity, as well as low temperature (Christmann et al., 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). In this study, Gray poplar was transformed with the dominant Arabidopsis mutant abi1 gene to alter ABA sensitivity in this woody model plant. Poplar transformed with the Arabidopsis wild-type ABI1 gene and wild-type 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 homolog could be detected in wild-type 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 signaling in both plant species via a conserved mechanism. Competition between mutant abi1 and...
nonmutant 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 signaling via competition with potential ABI1 homologs.

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 RH 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 wild-type poplars but not in abi1 poplars. This demonstrates reduced sensitivity to ABA in abi1 poplars and additionally emphasizes the role of ABA in controlling lateral bud dormancy.

Increased Stomatal Size in abi1 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 this study, we provide evidence that such a wilty phenotype is not only due to a defect in stomata regulation, but also to alterations in stomatal development. The increased stomatal size in abi1 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 abi1 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 (Solanum tuberosum), where endogenous 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 abi1 poplars we demonstrated that the increased stomatal size could be restored to wild-type levels, following the addition of ABA to developing leaves. This ABA-induced reversion of the increased stomatal size in abi1 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 adaptive responses of stomatal development.

Altered Growth Morphology and Ethylene Formation in abi1 Poplars

Morphological alterations in abi1-expressing poplars became evident, when comparing the growth habitus of these plants with that of ABI1 and wild-type poplars. A morphological analysis revealed enhanced shoot growth in abi1 poplars but retarded leaf and root development. The promotion of shoot growth resembles the situation in ABA-deficient mutants of tomato (Solanum lycopersicum) or abi1-transgenic potato, where similar growth characteristics have been

Figure 6. Growth habitus of poplar plants grown for 45 d 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, Wild-type poplars with stunted shoots, large leaves, and well-developed roots. [See online article for color version of this figure.]
observed (Jones et al., 1987; Chen et al., 2003; Noël et al., 2005). Other reports, however, describe reduced shoot growth in abi1-transgenic tobacco (Nicotiana tabacum; 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 (1) differences in plant water status that result from poor stomatal regulation, (2) the use of plants in different developmental stages in the different studies, or (3) species-dependent effects. In this study, differences in plant water status could be excluded as the poplar plants were grown at close to RH 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).

Our 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+ restored, at least partly, wild-type shoot growth characteristics. Toxic effects could be excluded as cause for the restoration of wild-type shoot growth since Ag+ treatment did not reduce shoot growth in wild-type poplars. Gibberellins seemed not to be involved in the enhanced shoot growth as abi1 poplars did not contain elevated levels of bioactive gibberellins (GA1 and GA3).

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) 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.

### Table II. Morphological characteristics of abi1, ABI1, and wild-type poplar grown for 45 d 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 ± s.e. (n = 10 plants; significantly different from wild-type plants at P ≤ 0.05*/0.005**; significantly different from ABI1 plants at P ≤ 0.05*/0.005**; significantly different from corresponding wild-type or abi1 lines at P ≤ 0.05*/0.005**; Student's t-test). FW, Fresh weight.

<table>
<thead>
<tr>
<th>Poplar Plants</th>
<th>Internode Length</th>
<th>Height Growth</th>
<th>Leaf Weight</th>
<th>Root Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm</td>
<td>mm d−1</td>
<td>mg FW</td>
<td>mg FW</td>
</tr>
<tr>
<td>Wild type</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 ± 0.4***</td>
<td>2.7 ± 0.1***</td>
<td>5.9 ± 0.6**</td>
<td>10.1 ± 1.4*</td>
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<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 ± 0.4***</td>
<td>2.6 ± 0.1***</td>
<td>9.6 ± 0.9**</td>
<td>16.9 ± 2.3*</td>
</tr>
<tr>
<td>Wild type/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 ± 0.10**</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 ± 0.10**</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 ± 0.10**</td>
<td>6.3 ± 0.5***</td>
<td>16.1 ± 1.4</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Transformation and Micropropagation

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

Verification of Transformation via PCR Analysis

To verify integration of transferred abi1 and ABI1 sequences into the poplar genome, genomic DNA was isolated from leaf tissue with Phytopure plant genome, genomic DNA was isolated from leaf tissue with Phytopure plant DNA extraction kit (Amersham Biosciences) following the manufacturer’s instructions. Transgene DNA was amplified by PCR using Arabidopsis (Arabidopsis thaliana) ABI1 full-length primers (forward 5’-attgaggagagttccagcgattc-3’ and reverse 5’-ttgcattgtccgctcgagagctc-3’), resulting in an amplicon with a length of 1.3 kb. In controls no product was amplified.

Immunochromeprotein 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 (Laemmli, 1970) and blotted onto polyvinylidene difluoride membranes. The membranes were probed with an anti-GUS antibody (Invitrogen) and gold-labeled secondary antibody (British Biocell).

ABA Sensitivity Assay

Nodal shoot segments with a single lateral bud and petiole were cut from intact plants. Shoot segments were incubated under physiological conditions (photosynthetic at 16 h with approximately 100 μmol m⁻² s⁻¹ PPFD) for 3 weeks on half-concentrated MS medium supplemented or not with 50 μM ABA. Outgrowth of lateral buds and abscession 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 s⁻¹ PPFD) to achieve full stomatal 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 Axiopt) and a digital image analysis system (Zeiss Axiocam). Measurements of stomatal pore areas of fully opened stomata were also used to estimate differences in stomatal size between abi1, ABI1, and wild-type poplars.

Histochemical Staining for GUS Activity

Detection of 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 ferrocyanide, 0.5 mM ferricyanide, 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 ninth internode, fixed with 3% (w/v) formaldehyde in phosphate-buffered solution, dehydrated in a graded series of ethanol, and embedded in Technovit methacrylate resin. Transverse sections (6 μm) were stained with Giemsa’s stain. Cell lengths were measured using a light microscope (Zeiss Axiopt) and a digital analysis system (Zeiss Axiolab Vision).

Hormone Analysis

Ethylene biosynthesis was measured on intact plants (45-d-old) kept for 24 h under standard conditions (photosynthetic at 16 h with approximately 100 μmol m⁻² s⁻¹ PPFD) in gas-tight glass containers. Each container harbored 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) equipped with a HayeSep Q column (3 m one-eighth inch i.d. particle size: 80/80 mesh, Supelco) and a flame ionization detector. Synthetic air (21% [v/v] O₂, 4.5, 79% [v/v] N₂, 5.0, Basi Schöberl) served as carrier gas, and the temperatures of the flame ionization detector 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 (C₂H₄, 99.95% purity; Air Liquide) with synthetic air. All measurements were repeated twice. 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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Expression of abi1 in Poplar