NAC Transcription Factor SPEEDY HYPONASTIC GROWTH Regulates Flooding-Induced Leaf Movement in Arabidopsis

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INTRODUCTION

Root flooding (waterlogging) in rosette plants like Arabidopsis thaliana causes an upward leaf movement, called hyponastic growth, to reestablish contact with air and photosynthetic gas exchange (Pierik et al., 2005; Jackson, 2008; Vashisht et al., 2011). The upward leaf movement is triggered by unequal cell elongation at the basal (proximal to the shoot) petiole region, where abaxial cells extend more than adaxial cells, resulting in increased petiole angle. The gaseous phytohormone ethylene (ET; C2H4), which regulates many plant developmental processes (Schaller and Kieber, 2002; Lin et al., 2009; Stepanova and Alonso, 2009; Schaller, 2012), has been indicated as the primary trigger of several waterlogging or whole-plant submergence-induced physiological and morphological acclimations in plants, including hyponastic growth (Cox et al., 2003; Milenaar et al., 2005; Voesenek et al., 2003, 2006; Bailey-Serres and Voesenek, 2008; Jackson, 2008). In the semiaquatic dicot Rumex palustris, the ET level increases 20-fold within the first hour of waterlogging (Banga et al., 1996). The hyponastic growth response to waterlogging is notably fast in R. palustris with a lag phase of only 1.5 to 3 h and the response being completed after 7 h, depending on the initial leaf angle (Cox et al., 2003). A comparably fast hyponastic growth response to flooding was observed in the Columbia-0 (Col-0) accession of Arabidopsis (Millenaar et al., 2005). Recently, a study by Polko et al. (2012) showed that ET-mediated hyponasty in Arabidopsis involves the reorientation of cortical microtubules at the abaxial side of the petiole from longitudinal to transverse; thus, ET is associated with tissue-specific changes in the arrangement of cortical microtubules along the petiole and most likely ET also triggers local stimulation of cell expansion upon waterlogging.

ROTUNDIFOLIA3 (ROT3) in Arabidopsis encodes the cytochrome P450 enzyme CYP90C1, which catalyzes the C-23 hydroxylation of various brassinosteroids (BRs). ROT3 is involved in polar cell elongation, and it has recently been shown that rot3 mutants have reduced hyponastic growth upon ET treatment, as well as low-light treatment and heat treatment, which both also induce hyponastic growth. Treatment with brassinazole, an inhibitor of BR biosynthesis, reduces the ET-induced increase of the petiole angle, revealing a modulatory role of BRs in petiole angle establishment (Polko et al., 2013). In addition to BRs, other phytohormones like auxin and gibberellins can act as positive regulators of hyponastic leaf growth, while abscisic acid functions as a negative regulator (Polko et al., 2011). Furthermore, the defense-related hormones methyl jasmonate and salicylic acid have been shown to act as positive and negative modulators, respectively, of ET-induced hyponastic leaf growth (van Zanten et al., 2012). Flooding triggers and accelerates leaf senescence in many plant species, including tobacco (Nicotiana tabacum), tomato (Solanum lycopersicum), sunflower (Helianthus annuus), and maize (Zea mays), possibly because of the reduced production of cytokinin, a phytohormone that delays senescence, in flooded roots (Burrows and Carr, 1969; Trought and Drew, 1980; VanToai et al., 1994; Huynh et al., 2005). In line with this model, expression...
of the rate-limiting enzyme of cytokinin biosynthesis (i.e., isopentenyl transferase) from a senescence-induced promoter enhanced flooding tolerance in Arabidopsis (Zhang et al., 2000). Taken together, although various molecular players affecting the adaptive leaf growth response to waterlogging have been identified in recent years, an integrated view of the underlying regulatory networks is currently missing.

ET regulates two important molecular processes during hyponastic leaf growth, namely, rapid acidification of the apoplastic to reduce cell wall rigidity and enhance cell wall extensibility and the upregulation of the expression of various EXPANSIN genes, which encode cell wall–loosening enzymes. Four sequence-related expansin protein families are currently distinguished in plants: EXPANSIN A (EXPA), EXPB, EXPANSIN-LIKE A (EXLA), and EXLB (Kende et al., 2004). In R. palustris, Rp-EXPA1 mRNA levels increased ninefold relative to air-exposed control plants with a subsequent increase in expansin activity (Neeburg et al., 2005). A phylogenetic comparison of Rp-EXPA1 with putative orthologs in rice (Oryza sativa), Arabidopsis, and Regnellodium diphylum revealed high similarity of Rp-EXPA1 to At-EXPA8 and At-EXPA2 from Arabidopsis, Os-EXPA2, Os-EXPA2, Os-EXPA3, Os-EXPA4, and Os-EXPA10 from rice, and Rd-EXPA1 from R. diphylum. These expansin genes are induced during flooding–induced growth responses (Cho and Kende, 1997; Kim et al., 2000). Furthermore, several members of the XYLOGLUCAN ENDOGLYCOSYLASE/HYDROLASE (XTH) gene family are upregulated by submergence, including XTH23 from Arabidopsis (Lee et al., 2011). XTHs are another class of cell wall–modifying proteins that contribute to loosening cell walls during cell expansion (Rose et al., 2002; Nishitani and Vissenberg, 2007; Van Sandt et al., 2007).

The biosynthesis of ET involves the conversion of Met to S-adenosyl-L-Met (S-AdoMet) by the enzyme S-AdoMet synthetase. S-AdoMet is then further converted to 1-aminocyclopentane-1-carboxylic acid (ACC) by ACC synthase (ACS), followed by subsequent metabolism of ACC to ET by ACC oxidase (ACO) (Yang and Hoffmann, 1984). In all plant species studied so far, ACS is encoded by a multigene family, and the Arabidopsis genome contains nine authentic ACS genes that exhibit distinct expression patterns throughout plant development and in response to stresses (reviewed in Lin et al., 2009). Similarly, ACO is encoded by multiple genes in plants (ACO1 to ACO5 in Arabidopsis), and expression patterns differ between the various ACO genes. In R. palustris, Rp-ACO transcript level is strongly induced by ET treatment or submergence, and ACO enzyme activity in shoots increases twofold within 24 h of submergence, suggesting that ET formation during submergence is due to a rise of ACO protein level. Notably, the increased expression of Rp-ACO was particularly evident in petioles, where most of the cell elongation occurs during submergence (Vriese et al., 1999). In Arabidopsis, the ACO2 gene is involved in endosperm cap weakening and rupture during seed germination (Linkies et al., 2009). In addition to the direct activation of genes involved in ET biosynthesis, altered expression of perception–signaling-related genes and transcription factors such as ETHYLENE RESPONSE2, ETHYLENE INSENSITIVE2 (EIN2), and EIN3 under submergence (Lee et al., 2011) may contribute to enhanced hyponastic leaf growth; however, this has not been tested so far.

NAC (for NAM, ATAF1, ATAF2, and CUC2) domain transcription factors (TFs) represent a plant-specific transcription regulatory family encoded by ~100 to 150 genes in all higher plants sequenced to date (Ooka et al., 2003; Nuruzzaman et al., 2010; Purakank et al., 2013; Singh et al., 2013), with 106 genes present in the genome of Arabidopsis. NAC TFs typically contain a highly conserved N-terminal DNA binding domain and a divergent C-terminal part (Olsen et al., 2005). NAC TFs play important roles in various biological processes, including senescence (Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2010, 2011; Lee et al., 2012; Hickman et al., 2013; Rauf et al., 2013), the response to abiotic and biotic stresses (Tran et al., 2004; Hu et al., 2006; Jensen et al., 2008; Wang et al., 2009; Wu et al., 2009; Jeong et al., 2010), leaf development (Berger et al., 2009), secondary wall biosynthesis (Zhang et al., 2010, 2011), regulation of Fru sensitivity (Li et al., 2011), regulation of iron homeostasis (Ogo et al., 2008), and others. The NAC TF VASCULAR-RELATED NAC-DOMAIN INTERACTING2 (ANAC083) integrates abscisic acid signaling with leaf senescence (Yang et al., 2011) and negatively regulates xylem vessel formation (Yamaguchi et al., 2010). The NAC TF JUNG-BRUNNEN1 (ANAC042) positively regulates plant longevity, most likely by reducing the cellular hydrogen peroxide level (Wu et al., 2012), and plays a role in acquired thermotolerance (Shahnejat-Bushehri et al., 2012). In wheat (Triticum aestivum), the NAC TF NAM-B1 was shown to affect senescence and nutrient remobilization during grain maturation (Uauy et al., 2006).

Here, we report the physiological function of the NAC TF SPEEDY HYponastic GROWTH (SHYG; ANAC047; At3g04070). We demonstrate that SHYG mediates waterlogging-induced hyponastic leaf growth by directly regulating the expression of ACO5 involved in ET biosynthesis.

RESULTS

SHYG Encodes a Submergence-Induced NAC TF

The TF SHYG (ANAC047) belongs to group III of the NAC family, which also includes the functionally characterized stress-related TFs ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR1 (ATAF1), AT-NAP (for NAC-LIKE, ACTIVATED BY APETAL3/PISTILLATA), and RESPONSIVE TO DESICICATION26 (Fujita et al., 2004; Guo and Gan, 2006; Lu et al., 2007). SHYG encodes a 359-amino acid protein with a calculated molecular mass of 40.8 kD. SHYG contains a NAM domain (pfam02365) at its N terminus. Its C-terminal part (Olsen et al., 2005). NAC TFs play important roles in various biological processes, including senescence (Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2010, 2011; Lee et al., 2012; Hickman et al., 2013; Rauf et al., 2013), the response to abiotic and biotic stresses (Tran et al., 2004; Hu et al., 2006; Jensen et al., 2008; Wang et al., 2009; Wu et al., 2009; Jeong et al., 2010), leaf development (Berger et al., 2009), secondary wall biosynthesis (Zhang et al., 2010, 2011), regulation of Fru sensitivity (Li et al., 2011), regulation of iron homeostasis (Ogo et al., 2008), and others. The NAC TF VASCULAR-RELATED NAC-DOMAIN INTERACTING2 (ANAC083) integrates abscisic acid signaling with leaf senescence (Yang et al., 2011) and negatively regulates xylem vessel formation (Yamaguchi et al., 2010). The NAC TF JUNG-BRUNNEN1 (ANAC042) positively regulates plant longevity, most likely by reducing the cellular hydrogen peroxide level (Wu et al., 2012), and plays a role in acquired thermotolerance (Shahnejat-Bushehri et al., 2012). In wheat (Triticum aestivum), the NAC TF NAM-B1 was shown to affect senescence and nutrient remobilization during grain maturation (Uauy et al., 2006).

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We analyzed public transcriptome data and found that SHYG expression increased 4.3-fold in rosettes of fully submerged Arabidopsis plants within 7 h (Lee et al., 2011), indicating that it might act as a transcriptional regulator in waterlogging- or submergence-controlled processes. Indeed, as we show below, SHYG plays an important role in waterlogging-induced hyponastic leaf movement, a function not reported for any of the other group III NACs.

SHYG Regulates the Expression of ACO5

First, to identify genes downstream of SHYG, we expressed it from an estradiol-inducible promoter (Zuo et al., 2000) and performed
Figure 1. SHYG Acts Upstream of ACO5.

(A) ACO5 and SHYG expression, determined by qRT-PCR in 2-week-old β-estradiol-treated (1, 2, 4, and 6 h) SHYG-IOE seedlings, relative to the wild type. (B) Immunodetection of ACO protein after β-estradiol induction using anti-ACC antibody (top panel). RbcL, ribulose-1,5-bis-phosphate carboxylase/oxygenase large subunit (loading control; bottom panel). (C) ACO5 expression in 35S:SHYG-GFP, 35S:SHYG, and shyg-1 and shyg-2 lines compared with the wild type. (D) SHYG transactivates the ~1.87-kb (three SHYG binding sites), but not the 169-bp (no binding site), ACO5 promoter. Luciferase signal was determined 8 and 16 h after transfection. Data in (A), (C), and (D) represent means ± SE (n ≥ 3). (E) EMSA. SHYG-GST protein binds to 40-bp double-stranded oligonucleotide containing SHYG BS-1 of ACO5 promoter (lane 2), while no binding occurs in the absence of SHYG-GST protein (lane 1) or when nonlabeled competitor is added (lane 3). For EMSA, we used 1 µL (0.05 pmol/µL) labeled probe in 20 µL of total volume. Competitor was used in 100-fold molar excess. (F) ChIP-qPCR on 35S:SHYG-GFP Arabidopsis plants. Data are the means ± sd (two biological replicates each with three technical replicates). Enrichment of sequences at BS-1, BS-2, and BS-3 of the ACO5 promoter was quantified by qPCR. Negative control (Neg cont): qPCR on a promoter (CLAVATA1; At1g75820) lacking SHYG binding sites.
expression profiling 5 h after β-estradiol treatment, using 14-d-old transgenic seedlings (SHYG-IOE). Only few genes robustly increased upon SHYG induction, including ACO5 (4.3-fold), encoding an uncharacterized enzyme involved in ET biosynthesis (see Supplemental Table 1 online). ACO5 transcript abundance and ACO5 protein level increased upon SHYG induction in a time-dependent manner (Figures 1A and 1B). ACO5 mRNA was also more abundant in Arabidopsis 35S:SHYG overexpressors than in Col-0 wild-type plants (Figure 1C) and showed reduced levels in two homozygous T-DNA insertion lines, shyg-1 and shyg-2 (Figure 1C; see Supplemental Figures 1A to 1C online). These observations are consistent with the model that SHYG acts upstream of ACO5 in a regulatory cascade. SHYG fused to green fluorescent protein (GFP) accumulated in nuclei of transformed Arabidopsis plants (see Supplemental Figure 2 online), consistent with its function as a TF.

**SHYG Directly Binds to the ACO5 Promoter**

To further test the SHYG-ACO5 regulatory cascade, we next performed transactivation assays using Arabidopsis mesophyll cell protoplasts cotransfected with a 35S:SHYG effector plasmid and a reporter construct (pACO5-fLUC) carrying a fusion between the ACO5 promoter (1.87 kb) and firefly lucerase (fLUC) as a reporter for transcriptional activation. As shown in Figure 1D, SHYG strongly transactivated ACO5 expression 8 and 16 h after transfection.

To investigate whether ACO5 is a direct target gene of SHYG, we analyzed the SHYG DNA binding sequence. Sequence alignment of SHYG with Ta-NAC69 (Xue et al., 2006) revealed 90% amino acid identity in their DNA binding domains, while little sequence identity (19%) exists between their C-terminal domains. Considering the fact that SHYG and Ta-NAC69 have very high amino acid sequence similarity in their DNA binding domains, we assumed that these proteins may share similar DNA binding specificity. Previously, two high-affinity binding sites of Ta-NAC69 (site I, mwkmCGTtmmnnyACGtmay; site II, rswkvtynnnnnnACGwcwct) were identified through in vitro binding site selection, followed by extensive mutation analysis of the two binding sites (Xue et al., 2006). To test the DNA binding affinity of SHYG to Ta-NAC69 binding sites, we determined the binding activity of SHYG toward Ta-NAC69 binding site I (SO1) and site II (SO39). In addition, to learn more about the specificity of binding, we also included the following sequences in our analysis: SO1m (mutated SO1 motif) that appears to confer no Ta-NAC69 binding affinity; SO39h that contains a sequence similar to Ta-NAC69 binding site II; ANAC019 binding site (ANAC019S); 35S motif-1 (–139 to –110) from the cauliflower mosaic virus (CaMV) 35S promoter that contains a weak Ta-NAC69 binding site I; and 35S motif-2 containing the sequence of aggctag, which is the protected site of At-NAM (Duval et al., 2002). As shown in Table 1, the binding affinities of SHYG to Ta-NAC69 sites SO1 and SO39 were highly similar to that of Ta-NAC69 (Xue et al., 2006). SHYG also showed binding toward the ANAC019 binding site and the CaMV 35S motif-1, but with higher binding affinity. In contrast with Ta-NAC69, SHYG better tolerated mutations within the two consensus motifs (SO39h and ANAC19S).

With these binding sequence data, we identified three potential SHYG binding sites (BS) within the ACO5 promoter 170 bp (BS-1, TCAACTG[8n]TCAGTTTTT), 206 bp (BS-2, CATAACGTA[6n]AACGATAAA), and 1719 bp (BS-3, AACCTTT[6n]TCAGTTTTT) upstream of the translation initiation codon (see Supplemental Figure 3 online). However, the predicted affinity of BS-3 is low, as the reduction in the spacer length in Ta-NAC69 binding site II markedly reduces the binding affinity (Xue et al., 2006). Transactivation

**Table 1. Comparison of Binding Specificity of SHYG and Ta-NAC69**

<table>
<thead>
<tr>
<th>Motifs</th>
<th>Synthetic Oligonucleotide Sequence</th>
<th>RBA (%)</th>
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<tbody>
<tr>
<td>Ta-NAC69 DNA binding site I</td>
<td>ggagatcCGTggcagatACGtaactgtagta</td>
<td>100 ± 3.1</td>
</tr>
<tr>
<td>SO1</td>
<td>ggagatcCGTccacagaaactgtagta</td>
<td>0</td>
</tr>
<tr>
<td>SO1m</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ta-NAC69 DNA binding site II</td>
<td>gagggtttaagtttaacACGtcctctagtg</td>
<td>94.2 ± 3.5</td>
</tr>
<tr>
<td>SO39</td>
<td>tagtaacagaaatgtacACGcaactgccc</td>
<td>32.8 ± 1.7</td>
</tr>
<tr>
<td>SO39h</td>
<td>gaggtttaagtttaacACGtcctctagtg</td>
<td>94.2 ± 3.5</td>
</tr>
<tr>
<td>ANAC019 binding site</td>
<td>gaggccctctctctctacAGCgatgtgtagtg</td>
<td>16.9 ± 1.2</td>
</tr>
<tr>
<td>ANAC19S</td>
<td></td>
<td></td>
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<tr>
<td>CaMV 35S motifs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35S motif-1</td>
<td>aagaagaCGTtccacaccACGctcctcaag</td>
<td>10.3 ± 1.0</td>
</tr>
<tr>
<td>35S motif-2</td>
<td>ccaagtacgtaagggagtacacacactccc</td>
<td>0</td>
</tr>
</tbody>
</table>

Relative binding activity (RBA) is expressed as percentage relative to SO1. Values are means ± SD of three assays. Bases matching with the high-affinity binding sequences of Ta-NAC69 site I (mwkmCGTtmmnnyACGtmay) or site II (rswkvtynnnnnnACGwcwct) are in bold. The NAC core binding site is shown as capital letters. Substituted bases of SO1 (SO1m) are underlined. SO39h contains a sequence similar to Ta-NAC69 binding site II. ANAC19S represents the ANAC019 binding site. The as-1 site of the CaMV 35S promoter in 35S motif-2 (–87 to –58) is shown in italics. The nucleotide positions of the 35S motif-1 in the 35S promoter are from –139 to –110. The data indicate that the high-affinity binding sites of SHYG can be modified from the Ta-NAC69 binding sites to mwkmCGTt(6-n)yACGtmay (site I) and rswkvty(8-n)ACGwct (site II), where d = a, g, or t; h = a, c, or t; k = g or t; m = a or c; r = a or g; s = g or c; v = a, c, or g; w = a or t; y = c or t.
capacity of SHYG toward ACO5 was strongly reduced when all three binding sites were deleted (Figure 1D). Electrophoretic mobility shift assays (EMSAs) confirmed binding of recombinant SHYG-glutathione S-transferase (SHYG-GST) fusion protein to BS-1 (Figure 1E), which has a better match to the preferred binding sequences of Ta-NAC69 than BS-2 and BS-3. Finally, to demonstrate in vivo binding of SHYG to the ACO5 promoter, we performed chromatin immunoprecipitation (ChIP) and determined enrichment of ACO5 promoter fragments in the precipitated chromatin by quantitative PCR (qPCR) with primers spanning the three predicted SHYG binding sites. We detected clear enrichment of BS-1 and BS-2, whereas no binding to BS-3 was observed (Figure 1F).

**SHYG Regulates Waterlogging-Induced Hyponastic Leaf Growth**

Although SHYG transcript abundance increased in plants submerged for several hours (see above), a physiological function for this NAC TF was not known. As root flooding leads to hyponastic leaf growth in soil-grown plants in *Arabidopsis*, we thought to investigate whether SHYG has a function in this adaptive growth response. To this end, 30-d-old plants grown in individual pots in soil were placed in a flat plastic tank and roots were flooded by filling the tank with water up to a level slightly below the pot’s upper edge (for details, see Methods). Shoots remained in ambient air. For control treatments, plants were put inside the tanks without flooding. We followed hyponastic leaf movement in Col-0 wild-type plants and the two shyg T-DNA insertion mutants using a time-lapse video setup. While hyponastic petiole growth was clearly detected in the Col-0 wild type, it was strongly impaired in shyg-2 (Figures 2A and 2B; see Supplemental Movies 1 and 2 online) and shyg-1 (see Supplemental Figure 4 and Supplemental Movie 2 online) over an 8-h waterlogging time span, revealing SHYG’s critical function in this physiological response. This conclusion was further supported by the fact that waterlogging-induced hyponastic growth was strongly stimulated compared with Col-0 wild-type plants in 35S:SHYG lines (Figures 2A and 2B; see Supplemental Movie 1 online). The petiole bending response was stimulated by ACC spray but diminished by treatment with aminoisobutyric acid (AIB), which blocks ACO activity and, hence, ET biosynthesis, and silver nitrate (AgNO₃), an ET receptor.
antagonist that minimizes ET perception (Guo and Ecker, 2003; Ouaked et al., 2003; see Supplemental Figure 5A online), strongly supporting the model that SHYG acts through ET and likely through ACO5. Similarly, treatment with another ET receptor antagonist, 1-methylcyclopropene, prevented submergence-induced hyponasty in the strongly responding Col-0 accession and reduced it in the weaker responding accession Landsberg erecta (Millenaar et al., 2005).

**SHYG Acts through ACO5**

To substantiate our premise that waterlogging-induced petiole growth is controlled via the SHYG-ACO5 regulatory cascade, we identified two homozygous T-DNA insertion mutants, aco5-1 and aco5-2 (see Supplemental Figures 1D and 1E online), with low ACO5 expression (see Supplemental Figure 1F online). Waterlogging-induced hyponastic growth was strongly reduced in both mutants and correlated to the level of residual ACO5 expression (Figures 2C and 2D; see Supplemental Figure 1F and Supplemental Movie 3 online), indicating that ACO5 has a unique function in this process. ACO5 deficiency was virtually overcome by treating plants with the ET-releasing agent ethephon (see Supplemental Figure 5B online), a response blocked

**Figure 3.** Hyponastic Leaf Movement in SHYG Inducible Lines.

(A) SHYG-IOE plants. Petioles reorient to a more vertical position upon waterlogging after pretreatment with either 10 μM β-estradiol (EST; four rosette sprays during 48-h prior waterlogging), ACC, or both, while this response is diminished after pretreatment either with AIB or AgNO₃.

(B) Leaf reorientation kinetics in SHYG-IOE line. Means ± se (n = 5). Pretreatments were performed using 10 mM each chemical 4 h prior to waterlogging.

Deletion of the ACO5 gene was virtually overcome by treating plants with the ET-releasing agent ethephon (see Supplemental Figure 5B online), a response blocked

**Figure 4.** Petiole Epidermal Cells.

(A) Microscopic view of abaxial epidermal imprints of petiole segments proximal (1.4 mm) to the stem (leaf No. 9) before (−WL) and after 8 h of waterlogging (+WL), WT, the wild type. Bar = 100 μm.

(B) to (E) Epidermal cell lengths of petiole regions of wild-type, 3SS:SHYG, and shyg-2 plants, before [B] and [C] and after [D] and [E] waterlogging. Data points represent means ± se (n = 8) of epidermal cell lengths of the first 5.4-mm adaxial and abaxial petiole surfaces.

(F) Expression of EXPA11 and EXPA8 in abaxial and adaxial proximal parts of the leaf petiole after waterlogging compared with control. Error bars represent means ± se (n = 4).
by the simultaneous application of AgNO₃ (see Supplemental Figure 5B online).

Next, we tested whether SHYG indeed functions via activation of ACO5 and to this end established 35S:SHYG/aco5-1 and 35S:SHYG-GFP/aco5-2 lines. We found waterlogging-induced hyponastic leaf movement to be strongly impaired in both aco5 mutant backgrounds (Figures 2E and 2F; see Supplemental Movie 4 online); the lack of petiole bending was reversed by treatment of plants with ethephon, a response that was blocked by AgNO₃ (see Supplemental Figure 5C online). Finally, we tested the effect of waterlogging on SHYG-IOE lines. Although root flooding alone triggered hyponastic leaf growth, as expected, this response was enhanced by treatment with β-estradiol or ACC (Figures 3A and 3B); the strongest growth effect was observed when β-estradiol and ACC were applied together to waterlogged plants, a reaction almost completely diminished in the presence of AIB or AgNO₃ (Figures 3A and 3B). Collectively, our data show that ACO5 is critically important for SHYG-mediated petiole bending during root flooding.

Localized Cell Expansion Is Modified by SHYG Expression

Waterlogging-induced hyponastic leaf growth requires localized expansion of abaxial cells of the basal (shoot-proximal) petiole segment, while cell elongation in adaxial petiole cells is not affected by waterlogging (Polko et al., 2012). We determined the length of epidermal cells in petioles of wild-type, shyg, and 35S:SHYG plants. Whereas cell length in the abaxial and adaxial petiole epidermis did not significantly differ before waterlogging (Figures 4A to 4C), abaxial cells elongated more in 35S:SHYG than wild-type petioles after root flooding, but abaxial cell elongation was reduced in shyg-2 petioles (Figures 4A to 4D). As expected, adaxial cell length remained unaffected by root flooding in all three genotypes (Figure 4E). Thus, the level of SHYG expression is critically important for root waterlogging-induced localized cell expansion at the petiole base.

Expansins are secreted proteins playing critical roles during cell expansion (Cosgrove, 2000). EXPANSIN A11 (EXPA11) is a marker for ET-induced cell elongation in Arabidopsis petioles (Millenaar et al., 2005; Polko et al., 2011), and we therefore tested its expression in the SHYG-modified plants. Upon root flooding, EXPA11 expression increased in shoot-proximal abaxial petiole segments of wild-type plants, as expected. Notably, EXPA11 expression in abaxial petiole cells increased to higher levels in the 35S:SHYG overexpressor plants but remained low in shyg-2 (Figure 4F). Expression of EXPA11 remained largely unaffected in adaxial cells. EXPAS8, a submergence-induced gene (Lee et al., 2011), behaved similarly (Figure 4F).

We also tested SHYG expression in the proximal segment of the leaf petiole using the ProSHYG-GUS lines. As seen in Supplemental (B) Venn diagram showing an overview of EXP and XTH genes that were significantly differentially expressed in 35S:SHYG and shyg-2 mutant lines compared with the wild type. (C) The heat map indicates expression ratios of EXP and XTH genes in 35S:SHYG and shyg-2 compared with the wild type. Expression ratios (log2): red, increased expression (between 0 and +2); blue, reduced expression (between 0 and −2).
Waterlogging Triggers SHYG Expression

Global expression profiling revealed elevated transcript levels of many genes, including SHYG, in fully submerged plants (Lee et al., 2011). To test whether enhanced SHYG expression is due to promoter activation, we tested waterlogging-dependent GUS activity in SHYG promoter–GUS fusion (ProSHYG:GUS) lines. GUS staining in petioles and the rosette center was weak in non-stressed plants. However, GUS activity was strongly elevated after 8 h of waterlogging (Figures 6A and 6B), when petiole angle was also increased in these lines (Figure 6A; see Supplemental Figure 7 online), indicating a positive feedback loop connecting SHYG expression with ET level, most likely via ACO5.

We next tested the effect of ET released from β-estradiol–induced SHYG-IOE seedlings on SHYG expression in neighboring ProSHYG:GUS lines (pooled in a tightly sealed deep-well plate to ensure the capture of ET produced by SHYG:IOE plants; see Supplemental Figure 8 online). As seen in Figures 6C and 6D, β-estradiol treatment strongly enhanced GUS activity in ProSHYG:GUS lines in a time-dependent manner. This induction was not observed in mock-treated seedlings or when ET synthesis was blocked by AIB in β-estradiol–treated samples.

Natural Variation of the SHYG-ACO5 Regulatory Cascade

To assess whether there exists natural variation of the SHYG-ACO5 regulatory cascade, we tested the response to root flooding in accession Cvi-0, which shows a low tolerance to complete submergence, and compared it with Col-0, an accession reported to exhibit moderate submergence tolerance (Vashisht et al., 2011).
Leaf movement was much less pronounced in Cvi-0 than in Col-0 (see Supplemental Figure 9 online). Expression of SHYG and ACO5 (and EXPA8) increased upon waterlogging in the proximal petiole segments of Col-0 but remained largely unaffected in Cvi-0, in accordance with its weak growth response (see Supplemental Figure 9 online). As expected, expression of these genes was not affected by waterlogging in the distal petiole segments.

**DISCUSSION**

NAC TFs have been shown to be involved in many developmental and stress-related processes in plants (Tran et al., 2004; Jensen et al., 2008; Ogo et al., 2008; Berger et al., 2009; Kim et al., 2011; Wu et al., 2009, 2012; Balazadeh et al., 2010; Li et al., 2011; Zhong et al., 2011; Lee et al., 2012). Here, we report the biological function of SHYG, a group III NAC TF from *Arabidopsis*, in root flooding–induced hyponastic leaf growth. Expression of SHYG in shoots of soil-grown plants is low to moderate before root waterlogging but rapidly increases upon waterlogging as well as ACC treatment. As we demonstrate here, SHYG directly regulates the expression of ACO5 by binding to its promoter, in accordance with the observation that root flooding triggers ET formation (as reviewed in Bailey-Serres and Voessele, 2008; Jackson, 2008). Loss of SHYG function strongly diminishes waterlogging-induced hyponastic leaf growth (Figures 2A and 2B), and a loss of ACO5 has a similar effect (Figures 2C and 2D).

Notably, when SHYG was overexpressed from the CaMV 35S promoter in the absence of a functional ACO5 gene (in aco5-1 and aco5-2 mutant backgrounds) waterlogging-induced hyponastic leaf movement was strongly impaired (Figures 2E and 2F), revealing ACO5 to be a key element of this physiological response under the direct regulation of SHYG. Therefore, our data provide firm evidence for a central role of SHYG as an upstream regulator of waterlogging-induced hyponasty by influencing ET biosynthesis through direct transcriptional activation of an ET biosynthesis gene. Our data indicate that other ACO genes in *Arabidopsis* do not compensate for the lack of ACO5 in this physiological response. An important aspect of our findings is that SHYG expression itself is stimulated by ACC/ET, thereby constituting a positively acting ET-SHYG-ACO5 activator loop for rapid petiole cell expansion upon root flooding.

Upon flooding, ACC accumulates in the anoxic roots and after transport via the xylem to the shoot ET is liberated by the action of ACO (Bradford and Yang, 1980). Generally, ACO is not considered to be rate-limiting; thus, ACC arriving at the shoot might be expected to be readily converted to ET. However, as we demonstrate, ACO5 expression is barely induced in the proximal petiole segment upon root flooding in accession Cvi-0, which shows a weak hyponastic growth response to waterlogging, while it is strongly induced in accession Col-0, which exhibits a much more pronounced hyponastic response to root flooding (see Supplemental Figure 9 online). This observation and the fact that the strength of the hyponastic growth response correlates with the level of residual ACO5 expression in the two aco5 T-DNA insertion mutants (Figures 2C and 2D; see Supplemental Figure 1F online) points to rate-limiting expression (and potentially activity) of ACO during waterlogging-induced hyponastic leaf growth. Notably, SHYG did not modulate expression of any of the nine ACO genes in the *Arabidopsis* genome in our experimental setting, indicating that SHYG-mediated enhanced ET formation upon waterlogging does not involve these genes. However, submergence-triggered increases of ACO gene expression have been observed (Peng et al., 2005; Lee et al., 2011), leaving open the possibility that other transcriptional regulators control their expression. Currently, however, no single ACO gene has been functionally connected to hyponastic leaf growth.

Hyponastic leaf movement is due to a locally restricted expansion of cells in the proximal, abaxial petiole region, while cells at the adaxial petiole side remain largely unaffected with respect to cell length upon root flooding. Compared with the wild type, we found increased and decreased abaxial cell expansion, respectively, in SHYG overexpressors and shyg mutants, a phenotype consistent with the macroscopic leaf movement response. In contrast with the locally restricted cell elongation, however, expression of both SHYG and ACO5 did not appear to be regionally distinct at the abaxial and adaxial petiole sides (see Supplemental Figure 6 online), suggesting that also ET formation may not be locally different between the two petiole sides.

Previously, it was shown in *Arabidopsis* that ET triggers elevated expression of EXPA11 at the proximal abaxial but not adaxial petiole side (Polko et al., 2012), which is consistent with our observation of the differential cell elongation responses during waterlogging. We observed a similar transcriptional response for EXPA11 as well as for EXPA8 in wild-type (Col-0) *Arabidopsis* plants; notably, compared with wild-type plants, expression of the two genes at the abaxial petiole side was stimulated in SHYG overexpressors, while it was reduced in the shyg-2 mutant; no difference in EXPA8 and EXPA11 expression was observed at the adaxial petiole side. Thus, although expression of SHYG itself lacks local specificity (as does ACO5), genes involved in cell expansion are likely to be of functional relevance for the upward directed petiole bending (EXPA8 and EXPA11) and maintain their
locally distinct patterns of expression upon SHYG overexpression. Currently, it remains an open question whether EXPA8 and EXPA11 are direct transcriptional output genes of SHYG or whether additional TFs are involved in regulating these genes during waterlogging. Of note, however, EXPA8 is an ortholog of Rp-EXP1A1, a marker gene for submergence-induced leaf hyponasty in *R. palustris* (Vreeburg et al., 2005), indicating regulatory and functional conservation of the gene networks controlling this physiological response.

As we show in Figure 5, several other EXP genes as well as XTH genes are modulated by SHYG, which most likely contributes to abaxial petiole cell expansion upon waterlogging. However, the contribution of each individual gene to hyponastic leaf growth has so far not been analyzed; it is possible that several of the EXP/XTH genes affected by SHYG work in concert to contribute to cell expansion during waterlogging-triggered leaf hyponastic leaf growth. In addition, our data reveal several other genes rapidly modulated by SHYG after its induction by estradiol in SHYG-IOE lines (see Supplemental Table 1 online). However, their functional involvement in hyponastic leaf growth remains open at present.

A further important question that remains to be addressed in future experiments is which upstream transcriptional regulators control the expression of SHYG. A possible candidate is EIN3, a master transcriptional regulator of ET-controlled processes (Roman et al., 1995; Chao et al., 1997). Of note, an EIN3 core binding site is located ~580 bp upstream of the SHYG start codon (data not shown), and ChIP-seq studies have revealed binding of EIN3 to the SHYG promoter (Chang et al., 2013), indicating that both TFs constitute a transcriptional cascade with SHYG as an output regulator of the EIN3 TF. However, a detailed functional analysis will be required to unravel in detail under which physiological conditions and in which cells EIN3 regulates SHYG expression.

Collectively, our data suggest a regulatory model (Figure 7) whereby SHYG plays a decisive role in regulating root waterlogging-induced leaf movement by directly or indirectly stimulating localized cell expansion at the abaxial petiole side through direct activation of AC05 and ET formation. The control circuit involves an intrinsic ET-SHYG-AC05 activator loop that accelerates cell expansion after an initial trigger and underlies the rapid petiole growth response that becomes already evident 90 min after starting root flooding (Figure 2). Our data also suggest natural variation of SHYG, AC05, and EXPA8 gene expression, which is in accordance with the differential hyponastic leaf response observed in Col-0 (relatively strong responder) versus Cvi-0 (weak responder; see Supplemental Figure 9 online), providing further evidence for the biological importance of the SHYG-AC05 regulatory interaction.

Future work will have to unravel the peculiarities of the signaling circuitry that fine-tunes SHYG expression at the cell level and the details of the downstream gene network(s) that governs local cell expansion to drive directional leaf movement. The SHYG-AC05 regulatory system appears to be distinct from the regulatory networks controlled by the *SNORKEL1* (*SK1*) and SK2 genes in deepwater rice, both of which encode ET response factor TFs that regulate internode elongation to keep leaves above the rising water surface and avoid tissue anoxia. Both SK genes are upregulated by ET. Experimental evidence shows an involvement of gibberellic acid in the elongation process controlled by SK1 and SK2 (Hattori et al., 2009, 2011). Flash flood is another type of stress that appears suddenly and often damages rice seedlings. Tolerance to flash flood is achieved by temporally restricting growth (up to a few weeks), leading to stunted seedlings. When flood waters recede, seedlings restart growth. The ET response factor TF Submergence-1A has been shown to be a central regulator of submergence tolerance (Xu et al., 2006; Jung et al., 2010). Within the Arabidopsis NAC family, several other NACs besides SHYG, including, for example, ANAC013, ANAC084, ANAC091, and ATAF1, are induced by submergence (Lee et al., 2011). It appears that plants have evolved different strategies and molecular mechanisms to combat threats posed by waterlogging or submergence.

**METHODS**

**General**

Standard molecular techniques were employed as reported (Sambrook et al., 2001; Skirycz et al., 2006). Chemicals and reagents were obtained from Sigma-Aldrich, Fluka, and Roche Diagnostics. For sequence and expression analyses, the following tools were used: eFP browser (www.bar.utoronto.ca/ epft/cgi-bin/epfWeb.cgi), GENEVESTIGATOR (www.genevestigator.com), the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the Arabidopsis Information Resource (www.Arabidopsis.org), the European Bioinformatics Institute (http://www.ebi.ac.uk/), and the Plant Transcription Factor Database (http://plntdfb.bio.uni-potsdam.de/v3.0/).

**Plants**

Seeds of *Arabidopsis thaliana*, accessions Col-0 and Cvi-0, were obtained from the Arabidopsis thaliana Resource Centre for Genomics (Institut National de la Recherche Agronomique, France; http://dbgap versailles.inra. fr/publiclines/). For growth under long-day conditions, seedlings were grown in soil (Einheitserde GS90; Gebrüder Patzer) in a climate-controlled chamber with 16-h day/length provided by fluorescent light at ~100 µmol m⁻² s⁻¹, and day/night temperatures of 20/16°C and RH of 60/75%. After 2 weeks, seedlings were transferred to a growth chamber (16-h day, 120 µmol m⁻² s⁻¹) with day/night temperature of 22/16°C and 60/75% RH. For growth on nutrient agar medium (1% Murashige and Skoog and 1% Suc, pH 5.8), the Arabidopsis seeds were surface-sterilized using sodium hypochlorite solution (10%) and sown on the plates. The vertically oriented plates were stored for 2 d under vernalization conditions and then transferred to long-day growth conditions (16 h light at 22°C and 8 h dark at 18°C). Seed stocks of the SALK (http://signal.salk.edu/cgi-bin/tdnaexpress) T-DNA insertion lines shyg-1 (SALK-066615) and aco5-1 (SALK-042400) and the GABI-Kat (http://www.gabikat.de) insertion lines shyg-2 (GK-343D11) and aco5-2 (GK-119A07) were obtained from the Nottingham Arabidopsis Stock Centre (http://Arabidopsis.info). T-DNA insertion sites and homozygosity were confirmed by PCR-based genotyping (see legend to Supplemental Figure 1 online). Primer sequences are listed in Supplemental Table 2 online. qRT-PCR was performed to check for transcript abundance. Plant 3SS:SHYG (BASTA) was crossed with aco5-1 (Kan¹). Plant 3SS:SHYG-GFP (Kan¹) was crossed with aco5-2 (sulfadiazine)².

**DNA Constructs**

Constructs were generated by PCR. Construct generation strategies and primer sequences are listed in Supplemental Table 2 online. Amplicons generated by PCR were checked for correctness by DNA sequence analysis (Eurofins MWG Operon). Constructs were transformed into Arabidopsis Col-0 via Agrobacterium tumefaciens-mediated transformation.

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**References:**


Expression Profiling by qRT-PCR

RNA extraction, synthesis of cDNA, and qRT-PCR were done as described (Caldana et al., 2007; Balazadeh et al., 2008, 2010). Genes included in the qRT-PCR platforms and primer sequences are given in Supplemental Table 3 online. Primers were designed using QuantPrime (Arvidsson et al., 2008; www.quantprime.de). PCR reactions were run on an ABI PRISM 7900HT sequence detection system (Applied Biosystems Applera).

Affymetrix ATH1 Microarray Hybridization

The RNeasy plant mini kit (Qiagen) was used to extract total RNA from two biological replicates of 14-d-old SHYG-IOE seedlings treated with 10 µM β-estradiol (Sigma-Aldrich) for 5 h or ethanol (0.1%) for control. Preparation and hybridization to the Arabidopsis ATH1 array were performed by ATLAS Biolabs. Data analysis was performed as reported (Balazadeh et al., 2010). Microarray data (SHYG-IOE-5 h) are available from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE38721.

Identification of SHYG Binding Sites

The DNA binding activity of SHYG-CELD fusion protein was determined as described previously (Xue, 2002, 2005), using 1 pmol biotin-labeled synthetic oligonucleotides that were immobilized in the wells of streptavidin-coated eight-well strips and binding/washing buffer (25 mM HEPES/KOH, pH 7.0, 50 mM KCl, and 2 mM MgCl2) containing 0.5 mM DTT, 0.15 mM sheared herring sperm DNA, 0.3 mg ml⁻¹ BSA, 10% glycerol, and 0.025% Nonidet P-40. DNA binding assays with a biotin-labeled single-stranded oligonucleotide or a biotin-labeled double-stranded oligonucleotide without a target binding site were used as controls.

Transactivation Assay

Transactivation assays in Arabidopsis mesophyll cell protoplasts were performed as described (Wu et al., 2012; Rauf et al., 2013), with 35S:SHYG and ProACOS:FLuc as effector and reporter plasmids, respectively. The 35S: RLuc (for Renilla luciferase) vector (Licauisi et al., 2011) was used for normalization against transformation efficiency. FLuc and RLuc were assayed using the Dual Luciferase Reporter Assay System (Promega). Six micrograms of DNA was used for protoplast transformation. Readings were performed 8 and 16 h after transfection using a GloMax 2020 Luminometer (Promega). Data were collected as ratio (FLuc activity:RLuc activity).

EMSA

Recombinant SHYG-GST protein for EMSA experiments was expressed in Escherichia coli as described (Dortay et al., 2011). Protein purification for EMSA was performed using a 1-mL GSTrap HP column (GE Healthcare) coupled to an Akta-Purifier FPLC system (GE Healthcare). EMSA was described as performed (Wu et al., 2012) using the Odyssey Infrared EMSA Kit (LI-COR). 5'-DY682-labeled DNA fragments were purchased from Eurofins MWG Operon. Sequences of labeled DNA fragments and unlabeled competitors are given in Supplemental Table 2 online.

Immunoblot

Fourteen-day-old seedlings were incubated in liquid Murashige and Skoog medium containing 10 µM β-estradiol (control treatment; 0.1% ethanol). The seedlings were kept on a rotary shaker for 1, 2, 4, and 6 h, harvested, and after removal of the roots immediately frozen in liquid nitrogen. Total protein was extracted from seedlings essentially as described (Martinez-García et al., 1998). Proteins (15 µg) were separated on 12% polyacrylamide SDS gels and transferred to Protran nitrocellulose membrane (Whatman). Immunoblot analysis was performed as described (Dortay and Mueller-Roeber, 2010) using goat polyclonal IgG primary antibody directed against ACO (aE-18; Santa Cruz Biotechnology) and IRDye800CW donkey anti-goat IgG secondary antibody (LI-COR). Signal intensities were analyzed at 800 nm using the Odyssey Infrared Imaging System (LI-COR).

In Vivo Binding of SHYG to the ACOS Promoter

ChIP followed by qPCR (ChIP-qPCR) was performed with whole shoots from short-day-grown, 35-d-old Arabidopsis plants expressing GFP-tagged SHYG protein from the CaMV 35S promoter (35S:SHYG-GFP). Wild-type plants were used as negative control. ChIP was performed as described (Kaufmann et al., 2010) using an anti-GFP antibody. The experiment was run in two independent replications with three technical replications per assay. Equal amounts of starting plant material and ChIP products were used for PCR reactions. Primers used for qPCR flanked the SHYG binding site within the ACOS promoter. As a negative control, we used primers annealing to a promoter region of an Arabidopsis gene (At1g75820) lacking a SHYG binding site. Primers are listed in Supplemental Table 2 online. ChIP-qPCR data analysis was performed as described (Wu et al., 2012).

Waterlogging Experiments

Seedlings were grown in soil in a climate-controlled chamber with an 8-h light period (short-day condition) provided by fluorescent light at ~100 µmol m⁻² s⁻¹ and day/night temperatures of 20/16°C and RH of 60/75%. After 2 weeks, seedlings were transferred to a growth chamber with a 16-h light period (long-day condition) provided by fluorescent light at 120 µmol m⁻² s⁻¹ and day/night temperatures of 22/16°C and 60/75% RH. For all waterlogging experiments, 30-d-old plants were used. Root flooding was started (t = 0 h) 6 h after the beginning of the photoperiod to prevent against the effect of diurnal leaf movement. Plants grown individually in pots (height 5.6 cm) with organic potting mix were placed inside plastic tanks (45 cm:27 cm:5 cm, length:width;height) and were exposed to flooding with tap water by filling up to 5 cm such that the upper water level always remained below the pot upper edge. For control treatments, plants were put inside the tanks without flooding. To assess natural variation of waterlogging-induced hyponastic leaf movement in Arabidopsis, accession Cvi-0 (low tolerance to flooding) was compared with Col-0 (intermediate tolerance) (Vashisht et al., 2011).

Treatment with ET Synthesis Inducers and Signaling Modifiers

To determine whether ACC or 2-chloroethylphosphonic acid (ethephon) promotes waterlogging-induced hyponastic leaf movement, shoots were sprayed with 10 mM each compound (in aqueous solution containing 0.1% Tween 20) 4 h before flooding. The aqueous solution containing 0.1% Tween 20 was used in control treatments. To test the effect of ET biosynthesis and signaling inhibitors on waterlogging-induced leaf movements, plants were sprayed with 10 mM AIB (ACO inhibitor) or 10 mM AgNO₃ (ET signaling inhibitor). To evaluate whether AgNO₃ reverses ethylene-promoted waterlogging-induced hyponastic leaf movement, plants were sprayed with 10 mM ethephon and 10 mM AgNO₃ in combination. All chemicals were from Sigma-Aldrich.

Petiole Angle Measurements

In the intact plants, leaf hyponastic movements were recorded using high-resolution digital images of rosette leaves captured using time-lapse photography on a Color Box charge-coupled device Camera XCD21 (X-Core), at a rate of one frame per 20 s. Quantitative measurements of petiole angle kinetics from the adaxial surface of the petiole relative to the horizontal plane were obtained on the digital images by drawing a straight line from the petiole base using the freeware algorithm ImageJ version
Epidermal Cell Length Measurements

Epidermal imprints along the abaxial and adaxial epidermal surfaces of leaf petioles before and after waterlogging were obtained by agarose impressions (Mathur and Koncz, 1997). To this end, 1-cm-long proximal petiole regions close to the stem were placed in 3% molten agarose and carefully removed from the solidified carrier before taking microscopic photographs. When dried, the thin film of gel was placed onto a microscopic slide and observed under an M125 stereomicroscope (Leica). Cell length measurements were performed on 5-mm-long segments of the abaxial and adaxial surfaces of the leaf petiole proximal to the stem using laser absorption spectrometry Live-Measurement software (Leica). To allow calculations of average cell sizes relative to the distance from the stem along the petiole, each cell was assigned to a 200-μm class, depending on its position relative to the most proximal part of the petiole.

GUS Assays in Twinned SHYG-IOE and Pro_{SHYG}:GUS Seedlings

Two-week-old SHYG-IOE and Pro_{SHYG}:GUS seedlings were pooled together in a tightly sealed deep-well plate to ensure the capture of ET produced by SHYG-IOE plants inside the wells after β-estradiol induction (see Supplemental Figure 8 online). SHYG expression in SHYG-IOE seedlings was induced by 10 μM β-estradiol for 0, 6, 12, and 24 h (mock treatment: 0.1% ethanol). GUS activity in the Pro_{SHYG}:GUS seedlings was determined histochemically and quantified by spectrophotometric assay. AIB (10 mM) was used to block ACO activity.

Other Methods

The histochemical assay for GUS was performed according to Jefferson et al. (1987). Quantitative GUS assays using p-nitrophenyl glucuronide were performed as described (Wilson et al., 1992; Subramaniam et al., 2009). For GUS staining in petioles of Pro_{SHYG}:GUS rosette leaves (see Supplemental Figure 6A online), waterlogging was performed for 8 h. After GUS staining, rosettes were submerged in 70% ethanol to remove chlorophyll. Free-hand cross section through the petiole region proximal to the base was performed by cutting with a sharp razor blade. Sections were cleared in 8:2:1 (chloral hydrate:glycerol:water) before microscopy analysis.

Microscopy

Distribution of SHYG-GFP fusion protein was analyzed by confocal fluorescence microscopy using an Eclipse E600 microscope (Nikon).

Statistical Analyses

Statistical analyses were performed using Student’s t test embedded in the Microsoft Excel software. Only the return of P < 0.05 was taken as statistically significant.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SHYG (At3g04070), ACO5 (At1g77330), At-EXP A8 (At2g0610), and At-EXP A11 (At1g20190). Microarray data (SHYG-IOE-S5) are available at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) under accession number GSE38721.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Confirmation of T-DNA Insertions in shyg-1, shyg-2, aco5-1, and aco5-2 Mutants.

Supplemental Figure 2. Nuclear Localization of SHYG-GFP Fusion Protein in Transgenic Arabidopsis.

Supplemental Figure 3. Schematic Presentation of Predicted SHYG Binding Sites in the ACO5 Promoter and Recombinant SHYG-GST Protein Used for EMSA.

Supplemental Figure 4. Hyponastic Leaf Growth in 4-Week-Old Wild-Type and shyg-1 Mutant Plants upon Waterlogging.

Supplemental Figure 5. Hyponastic Leaf Movement in SHYG- and ACO5-Modified Plants.

Supplemental Figure 6. Gene Expression in Proximal Petiole Region.

Supplemental Figure 7. Quantitative Measurements for Absolute Angles of Leaf Petiole Kinetics Obtained Using a Time-Lapse Digital Camera Setup.

Supplemental Figure 8. Schematic Representation of the Experimental Setup of the Twinned-Seeding Experiment.

Supplemental Figure 9. Hyponastic Leaf Movement in Arabidopsis Accessions Col-0 and Cvi-0.

Supplemental Table 1. Genes Affected by SHYG.

Supplemental Table 2. Constructs and Primer Sequences.

Supplemental Table 3. Sequences of Primers Used for Expression Profiling by qRT-PCR.

Supplemental Movie 1. Time-Lapse Video of Waterlogging-Induced Hyponastic Leaf Growth in 35S:SHYG, Wild-Type, and shyg-2 Plants.

Supplemental Movie 2. Time-Lapse Video of Waterlogging-Induced Hyponastic Leaf Growth in shyg-1, Wild-Type, and shyg-2 Plants.

Supplemental Movie 3. Time-Lapse Video of Waterlogging-Induced Hyponastic Leaf Growth in aco5-1, aco5-2, and Wild-Type Plants.


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AUTHOR CONTRIBUTIONS

B.M.-R. initiated the research. B.M.-R., S.B., M.R., and M.A. designed the experiments. M.R. and M.A. conducted the experiments. G.-P.X. performed the binding site selection assay. J.F. provided expertise in time-lapse video imaging. B.M.-R., M.R., and S.B. prepared the article with input from all the authors.
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NAC Transcription Factor SPEEDY HYponastic Growth Regulates Flooding-Induced Leaf Movement in Arabidopsis
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