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WAG2 represses apical hook opening downstream from gibberellin and PHYTOCHROME INTERACTING FACTOR 5

Björn C. Willige, Eri Ogiso-Tanaka, Melina Zourelidou and Claus Schwechheimer*

SUMMARY

When penetrating the soil during germination, dicotyledonous plants protect their shoot apical meristem through the formation of an apical hook. Apical hook formation is a dynamic process that can be subdivided into hook formation, maintenance and opening. It has previously been established that these processes require the transport and signaling of the phytohormone auxin, as well as the biosynthesis and signaling of the phytohormones ethylene and gibberellin (GA). Here, we identify a molecular mechanism for an auxin-GA crosstalk by demonstrating that the auxin transport-regulatory protein kinase *WAG2* is a crucial transcription target during apical hook opening downstream from GA signaling. We further show that *WAG2* is directly activated by PHYTOCHROME INTERACTING FACTOR 5 (PIF5), a light-labile interactor of the DELLA repressors of the GA pathway. We find that *wag2* mutants are impaired in the repression of apical hook opening in dark-grown seedlings and that this phenotype correlates with GA-regulated *WAG2* expression in the concave (inner) side of the apical hook. Furthermore, *wag2* mutants are also impaired in the maintenance or formation of a local auxin maximum at the site of *WAG2* expression in the hook. WAG2 is a regulator of PIN auxin efflux facilitators and, in line with previous data, we show that this kinase can phosphorylate the central intracellular loop of all PIN-FORMED (PIN) proteins regulating apical hook opening. We therefore propose that apical hook opening is controlled by the differential GA-regulated accumulation of *WAG2* and subsequent local changes in PIN-mediated auxin transport.

KEY WORDS: *Arabidopsis*, WAG2 protein kinase, Apical hook, Auxin transport, Gibberellin

INTRODUCTION

Gibberellins (GA) are phytohormones that regulate a number of physiological responses in plants such as germination, elongation growth, greening, flowering time as well as apical hook formation (Achard et al., 2003; Alabadí et al., 2004; Gallego-Bartolomé et al., 2011; Ueguchi-Tanaka et al., 2007; Vriezen et al., 2004). All GA responses known to date are mediated by DELLA proteins, which have emerged as key repressors of the GA pathway that regulate different classes of transcriptional regulators such as the light-labile PHYTOCHROME INTERACTING FACTORs (PIFs; namely PIF1, PIF3, PIF4 and PIF5) and the related but light-stable bHLH transcription factors ALCATRAZ and SPATULA (Arnaud et al., 2010; de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolomé et al., 2011; Gallego-Bartolomé et al., 2010).

GA controls DELLA protein abundance by binding to the GIBBERELLIN INSENSITIVE DWARF 1 (GID1) receptors and then promotes DELLA protein degradation via E3 ubiquitin ligases such as *Arabidopsis* $SCF^{SLEEPY1(SLY1)}$ (Ueguchi-Tanaka et al., 2007). Therefore, GA and DELLAs antagonistically control the activity of their downstream regulators, e.g. the DNA-binding activity of PIFs is repressed by DELLA interactions and GA relieves this DELLA-imposed restraint by promoting DELLA degradation (de Lucas et al., 2008; Feng et al., 2008). At the same time, PIF abundance is also downregulated by light as light-induced phytochrome-interactions promote PIF protein degradation (Bauer et al., 2004; Castillon et al., 2009; Lorrain et al., 2008; Monte et al., 2004; Park et al., 2004; Shen et al., 2005). As such, the repression of PIFs by DELLAs is particularly important when PIFs are abundant, notably in the dark, whereas phytochrome-dependent PIF degradation may be the predominant pathway controlling PIF abundance and activity in the light (Leivar and Quail, 2011). Although two recent reports reveal the identity of PIF transcription factor target genes using chromatin immunoprecipitation at the genome-wide level (Hornitschek et al., 2012; Oh et al., 2009), only a few studies have examined direct PIF targets in a biological context (Cheminant et al., 2011; Franklin et al., 2011; Gallego-Bartolomé et al., 2011; Li et al., 2012; Richter et al., 2010; Sun et al., 2012).

The phytohormone auxin regulates a large number of growth processes in plants such as tissue differentiation, organ formation and tropic responses (Teale et al., 2006). At the cellular level, auxin responses are controlled by the auxin response factor transcriptional regulators and by the inhibitory auxin-labile AUX/IAA proteins (Chapman and Estelle, 2009). At the organismal level, the polar transport of auxin within the plant plays a pivotal role in the control of plant development and differentiation. Cell-to-cell polar auxin transport is mediated by the AUXIN-RESISTANT 1/LIKE-AUX 1 (AUX1/LAX) auxin import carriers and PIN-FORMED (PIN) auxin efflux facilitators that may function together with MULTIDRUG RESISTANCE/PHOSPHOGLYCOPROTEIN (MDR/PGP) transporters (Kleine-Vehn and Friml, 2008). Particularly instructive for the understanding of polar auxin transport is the localization of PIN auxin efflux facilitators that are polarly distributed in the plasma membrane of many cells in the growing plant. In *Arabidopsis thaliana*, at least five members of the PIN protein family with in part specific and in part overlapping biological functions have been implicated in PIN-dependent polar auxin transport, PIN1, PIN2, PIN3 and PIN4, as well as PIN7 (Blilou et al., 2005). Although auxin transport itself cannot be directly visualized, models for

Department of Plant Systems Biology, Center for Life and Food Sciences, Technische Universität München, Emil-Ramann-Strasse 4, 85354 Freising, Germany.

*Author for correspondence (claus.schwechheimer@wzw.tum.de)

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auxin transport by far and large support the notion that the knowledge on PIN polarity is sufficient to predict cell-to-cell auxin transport and to explain auxin-dependent growth processes (Grieneisen and Scheres, 2009; Wisniewska et al., 2006).

AGC kinases are a family of serine/threonine kinases that share homology with the well-characterized protein kinase A, protein kinase G and cAMP-dependent kinase from animals (Galván-Ampudia and Offringa, 2007). In *Arabidopsis*, a subset of the AGC kinases, PINOID (PID), PID2, WAG1 and WAG2 have been directly implicated in the control of auxin transport. PIDs and WAGs form a subgroup within the AGCVIII family and at least PID and the WAGs regulate PIN polarity by phosphorylating PINs at conserved phosphorylation sites (Cheng et al., 2008; Dhonukshe et al., 2010; Friml et al., 2004; Huang et al., 2010; Santner and Watson, 2006). The four members of the D6 PROTEIN KINASE (D6PK) subgroup of AGCVIII also regulate auxin transport and phosphorylate PINs; they have, however, been proposed to regulate PIN auxin transport activity rather than PIN polarity (Dhonukshe et al., 2010; Zourelidou et al., 2009). Interestingly, the blue light receptors PHOTOTROPIN1 (PHOT1) and PHOT2, which promote the auxin transport-dependent hypocotyl bending in response to lateral light, are also AGCVIII kinases (Christie et al., 2011; Galván-Ampudia and Offringa, 2007).

A number of growth and developmental processes in plants are regulated by auxin as well as by GA. To date, only a few studies have elucidated crosstalk mechanisms that can explain aspects of the interdependency of the two hormone pathways (Björklund et al., 2007; Frigerio et al., 2006; Fu and Harberd, 2003; Jacobs and Case, 1965; Scott et al., 1967; Willige et al., 2011). Auxin stimulates GA biosynthesis and it can be proposed that auxin promotes growth at least in part by increasing GA hormone synthesis and thereby degradation of the GA-labile DELLA growth repressors (Frigerio et al., 2006), e.g. the cessation of root growth in the absence of auxin transport from the shoot and the accumulation of DELLA proteins in the root may be explained through this mechanism (Fu and Harberd, 2003). Inversely, evidence for a control of auxin transport and signaling by GA has also been provided. Auxin transport in the stem is less efficient in GA signaling mutants and GA deficiency reduces PIN protein abundance, possibly by targeting PINs for degradation in the vacuole (Willige et al., 2011).

The development of the apical hook is a specific example for a process that is controlled by auxin transport as well as by GA signaling (Gallego-Bartolomé et al., 2011; Lehman et al., 1996; Li et al., 2004; Zádníková et al., 2010). The apical hook is formed during skotomorphogenesis of dicotyledonous seedlings and it protects the sensitive shoot apical meristem while the hypocotyl is growing through the soil to reach the light. Apical hook development can be divided into the hook formation, hook maintenance and hook opening phase (Raz and Ecker, 1999; Zádníková et al., 2010). The local accumulation of auxin is necessary for the formation and maintenance of the apical hook to restrict the elongation of cells in the concave (inner) side of the hook, which induces asymmetric growth in the apical part of the hypocotyl (Kuhn and Galston, 1992; Raz and Ecker, 1999). Recently, the auxin influx facilitators AUX1 and LAX3, as well as the auxin efflux facilitators PIN1, PIN3, PIN4 and PIN7, were identified as being necessary for normal hook development (Vandenbussche et al., 2010; Zádníková et al., 2010). In particular, the loss of PIN3 activity impairs the establishment of the asymmetric auxin maximum in the apical hook (Friml et al., 2002; Zádníková et al., 2010).

Like auxin transport, GA biosynthesis and signaling are prerequisite for the formation and maintenance of the apical hook (Achard et al., 2003; Alabadí et al., 2004; An et al., 2012; Gallego-Bartolomé et al., 2011; Vriezen et al., 2004): although the loss of GA biosynthesis in the *ga1* mutant results in the loss of hook formation, the weaker DELLA gain-of-function mutant *gai*-1 is able to form a weak apical hook that opens prematurely. By contrast, DELLA loss-of-function mutants form an exaggerated hook, indicating that DELLA protein function is required to control the degree of hook formation in the wild type. From two recent studies, it has emerged that GA controls the transcription of the ethylene biosynthesis genes *AMINOCYCLOPROPANE CARBOXYLIC ACID SYNTHASE 5* (*ACS5*) and *ACS8*, of the ethylene response regulator *HOOKLESS 1* (*HLS1*), as well as that of the auxin efflux facilitators *PIN3* and *PIN7* (An et al., 2012; Gallego-Bartolomé et al., 2011). The mode of transcriptional GA control could be elucidated for two of these GA target genes by chromatin immunoprecipitation (ChIP) where it was shown that *ACS8* and *HLS1* expression is directly controlled by the DELLA interactors PIF5 and ETHYLENE INSENSITIVE 3/EIN3-LIKE1 (EIN3/EIL1), respectively.

We have examined genes of the auxin transport machinery with regard to their transcriptional regulation by GA. In this study, we identified the AGC kinase *WAG2* as a GA-regulated auxin transport gene. We further found that *WAG2* represses apical hook opening in dark-grown *Arabidopsis* seedlings and that *WAG2* is expressed in a GA-regulated manner in the concave side of the apical hook. We further identified the DELLA interactor PIF5 as a transcriptional regulator that activates *WAG2* expression by binding to a G-box motif in the *WAG2* promoter. Finally, in line with previous data on the role of WAG2 as regulator of auxin transport polarity (Dhonukshe et al., 2010), our data suggest that WAG2 may control apical hook opening by phosphorylating PIN proteins and by influencing the formation or maintenance of a specific local auxin maximum in the apical hook.

MATERIALS AND METHODS

Biological material

Unless otherwise stated in the text, mutants and transgenic lines used in this study have been previously described: *ga1* (Willige et al., 2007); *gai*-1 (Peng et al., 1997); *rga*-24 *gai*-t6 (King et al., 2001); *sly1*-10 (McGinnis et al., 2003); *DR5:GUS* (Ulmasov et al., 1997); *DR5rev:GFP* (Friml et al., 2003); *pif1*, *pif3*, *pif4*, *pif5* and *PIF5ox* (Fujimori et al., 2004; Huq et al., 2004; Khanna et al., 2007; Leivar et al., 2008; Lorrain et al., 2008; Monte et al., 2004); *PIN1:GFP, pin3* and *PIN3:GFP* (Benková et al., 2003; Zádníková et al., 2010); *wag1* and *wag2* (Santner and Watson, 2006).

Growth conditions and physiological experiments

Seeds were stratified for 3 days at 4°C, exposed to white light for 8 hours and grown in the dark at 21°C. *ga1* mutant seeds were incubated for 5 days at 4°C in 100 μM GA3 to induce germination. The seeds were then thoroughly washed five times in water to remove excess GA before exposure to light for 20 hours. To measure growth parameters, all seedlings were grown on vertically oriented plates containing half-strength MS medium without sucrose. At the indicated time points, plates were scanned to determine apical hook angles or hypocotyl lengths using the ImageJ software (NIH). The angle of a completely closed apical hook was defined as 180°, whereas the angle of a fully opened hook was defined as 0°.

Cloning and transgenic material

To generate *WAG1pro:GUS* and *WAG2pro:GUS*, 2.5 kb and 2.9 kb promoter fragments were amplified from Col-0 genomic DNA using the primers WAG1pro-LP/RP and WAG2pro-LP/RP, respectively. These sequences were inserted as *Eco*RI-*Nco*I fragments into pCAMBIA1391Z. At least six transgenic lines were generated in the Col-0 ecotype and

analyzed. Plant transformations were performed as previously described (Clough and Bent, 1998). The gene fragments of the cytoplasmic loops of PIN1, PIN2, PIN3, PIN4 and PIN7 were amplified by RT-PCR with specific PINLOOP-FW and PINLOOP-RV primers from *Arabidopsis thaliana* (Columbia) mRNA. The *WAG2*-coding sequence was amplified by PCR from cDNA clone U84295 (ABRC). The kinase-dead WAG2 (*WAG2in*) coding sequence was created using overlap extension PCR with the primers (WAG2mut5a and WAG2mut3a). The Gateway system-compatible fragments were then cloned into the expression vector pDEST15 (Invitrogen) to generate *GST:PIN* and *GST:WAG2*. See supplementary material Table S1 for a list of primers.

Quantitative real-time PCR

Extraction of total RNA and cDNA synthesis was conducted as described previously (Richter et al., 2010). The cDNA equivalent of 42 ng of total RNA was used in a 10 μl PCR reaction on a CFX96 Real-Time System Cycler with iQ SYBR Green Supermix (Bio-Rad). A 40-cycle two-step amplification protocol (10 seconds at 95°C, 30 seconds at 60°C) or a 40-cycle three-step amplification protocol (20 seconds at 95°C, 20 seconds at 58.5°C, 25 seconds at 72°C) was used for all measurements. Primer sequences are listed in supplementary material Table S1. Unless otherwise stated, the average and the standard error of at least three technical replicates pooled from three biological replicates are shown.

Biochemical analyses

Protein extraction and immunoblotting were performed as previously described (Willige et al., 2011). Anti-GFP for detection of PIN1:GFP (1:3000; Invitrogen) or for detection of DR5:GFP (1:4000; Roche), anti-PIN3 (1:3000; NASC) and anti-RGA [1:1000 (Willige et al., 2011)] were used for immunoblotting. For ChIP, seedlings were grown in the dark for 3 days. ChIP quantitative RT-PCR was performed and analyzed as described previously (Oh et al., 2007; Fode and Gatz, 2009). Anti-HA agarose and anti-HA antibody (Roche) were used for precipitation and detection of PIF5:HA. The in vitro phosphorylation assays were conducted as described previously (Zourelidou et al., 2009).

Cell biological and histological analyses

For GUS staining, etiolated seedlings were fixed in heptane for 15 minutes and incubated in GUS staining solution [100 mM Na-phosphate buffer (pH 7.0), 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$, 0.1% Triton X-100, 1 mg/ml X-Gluc]. GUS-stained seedlings were photographed using a Leica MZ16 stereo-microscope with a PLAN-APOX1 objective (Leica). Microscopy of PIN1:GFP, PIN3:GFP and DR5rev:GFP was performed on 2- to 4-day-old etiolated seedlings using an Olympus BX61 fluorescence microscope with a digital camera XM10 (Olympus) or a FV1000/IX81 laser scanning confocal microscope.

RESULTS

WAG2 is a GA-regulated AGC protein kinase

We have previously shown that *Arabidopsis* GA biosynthesis and pathway mutants are deficient in polar auxin transport (Willige et al., 2011). In search for possible molecular mechanisms that underlie the control of auxin transport by GA, we also examined the GA-dependent transcriptional regulation of genes encoding AGCVIII kinase family members that have been or may be implicated in the regulation of polar auxin transport as well as auxin transport proteins (Fig. 1A). To this end, we tested seedlings of the GA biosynthesis mutant *ga1* for GA-dependent changes in transcript abundance by quantitative real time (qRT)-PCR. This experiment identified *WAG2* as the gene whose transcript abundance is most strongly increased in response to GA3 treatment (Fig. 1A). As light-regulated signaling by the PIF transcription regulators may interfere with GA signaling, we were next interested in examining GA-regulation of *WAG2* in dark-grown seedlings to eliminate a possible co-regulation of *WAG2* expression by these two stimuli. In addition, this experiment revealed a strong

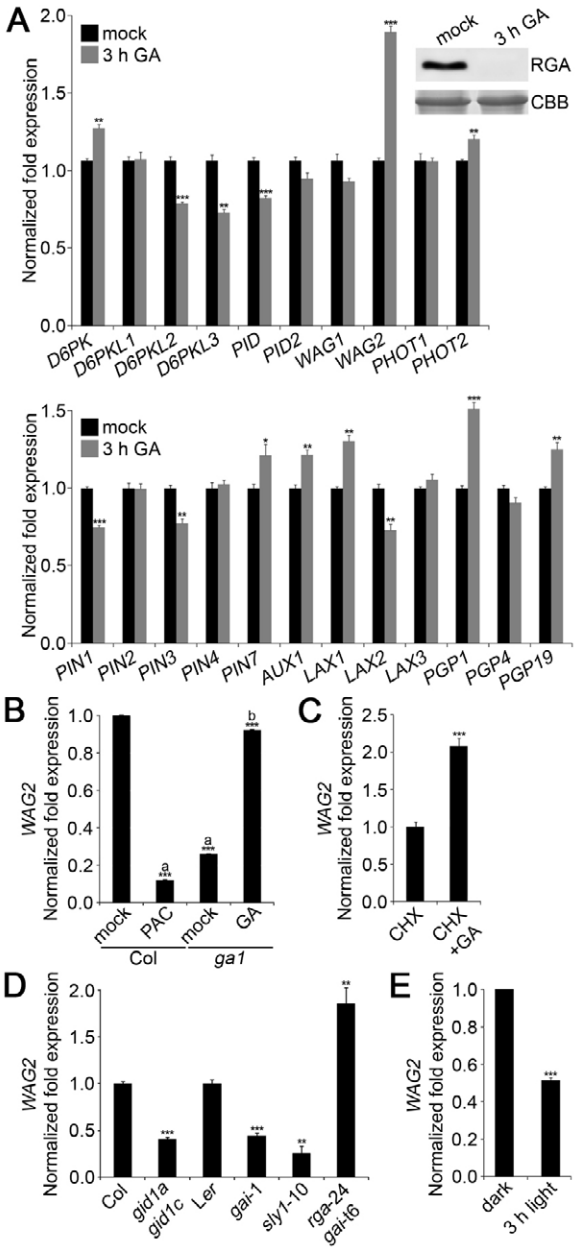

Fig. 1. *WAG2* transcript abundance is regulated by GA and light. (**A**) qRT-PCR of biologically characterized AGC kinase and auxin transporter genes of 7-day-old light-grown seedlings following a 3 hour GA3 (100 μM) treatment. The inset with an immunoblot with anti-RGA antibody of 45 μg total protein extracts indicates the efficiency of the GA3 treatment at the analyzed time point. (**B**) qRT-PCR analysis of *WAG2* after GA3 (1 μM) treatment of 4-day-old dark-grown *ga1* seedlings or wild-type (Col) seedlings grown in the presence of the GA biosynthesis inhibitor paclobutrazol (PAC). (**C**) qRT-PCR analysis of short-term GA3 (100 μM, 1 hour)-treated dark-grown seedlings in the presence of the protein biosynthesis inhibitor cycloheximide (CHX, 50 μM). (**D**) Basal expression levels of *WAG2* in different GA signaling mutants as detected by qRT-PCR. (**E**) qRT-PCR analysis of dark-grown seedlings and of dark-grown seedlings that were transferred to white light (150 μM m^{-2} s^{-1}). Shown are the average and the standard deviation of two biological replicates. For all experiments, *UBC21* was used for normalization. Asterisks indicate the significances (Student's *t*-test: *0.01<*P*<0.05; **0.001<*P*<0.01; ***P<0.001). In B, a and b indicate the comparisons to Col (mock) and *ga1* (mock), respectively.

GA-dependent increase in *WAG2* transcript abundance in dark-grown *ga1* seedlings, and inversely a decrease in *WAG2* abundance in wild-type seedlings that had been depleted from GA by treatment with the GA biosynthesis inhibitor paclobutrazol (PAC) (Fig. 1B). As we observed that the GA-mediated regulation of *WAG2* transcription can also take place in the presence of the protein synthesis inhibitor cycloheximide (CHX), we concluded that the GA response is dependent on a protein synthesis-independent mechanism such as GA-induced DELLA protein degradation (Fig. 1C). We then found that *WAG2* abundance is reduced in other GA pathway mutants such as *gid1a gid1c*, *gai*-1 and *sly1*-10, which are known to accumulate DELLA repressors and to be increased in the DELLA loss-of-function mutant *rga*-24 *gai*-t6 (Fig. 1D) (Peng et al., 1997). Finally, we found that *WAG2* transcript abundance is reduced after the transfer of dark-grown seedlings to light, suggesting that *WAG2* transcription is induced by light-labile regulators such as the PIFs (Fig. 1E). In summary, we concluded that *WAG2* expression is regulated by DELLAs and may be downstream from PIFs, their light-labile interaction partners.

WAG2 is a repressor of apical hook opening

To gain an insight into the possible role of GA-regulated *WAG2* transcription, we phenotyped previously characterized *wag2* mutants (Santner and Watson, 2006). As *WAG2* expression is elevated in dark-grown seedlings, we paid particular attention to the effects of the loss of *WAG2* function during skotomorphogenic growth and noticed with interest a decreased apical hook angle in 4-day-old *wag2* mutants (Fig. 2A). At the same time, we did not observe any other phenotypes in dark-grown seedlings, such as changes in hypocotyl elongation (supplementary material Fig. S1). Interestingly, we measured an increased opening of the apical hook only in 3- and 4-day-old *wag2* mutant seedlings (during the hook opening phase), and not in 2-day-old seedlings during the hook formation phase, suggesting that *WAG2* is required for the repression of hook opening (Fig. 2B). As *WAG2* had previously been found to genetically interact with its closest paralog, *WAG1*, in the control of root growth, we also included *wag1* single and *wag1 wag2* double mutants in our analysis (Santner and Watson, 2006). However, we found no evidence for a contribution of *WAG1* to the apical hook opening phenotype of *wag2*, neither in the *wag1* single mutant nor in the *wag1 wag2* double mutant (Fig. 2A,B). We thus conclude that WAG2 represses apical hook opening in dark-grown seedlings and that WAG2 functions independently from WAG1 in this process.

We also tested whether GA is required and sufficient for the normalization of apical hook development in *wag2* and *wag2 ga1* seedlings. The GA-deficient *ga1* mutant fails to form an apical hook and therefore apical hook formation requires GA treatments. In a comparison of GA-treated *ga1* and *wag2 ga1* mutants, we noted with interest that the normalization of the *ga1* phenotype by GA was impaired in the presence of the *wag2* mutation, indicating that *WAG2* represses apical hook opening in dark-grown seedlings downstream from GA (Fig. 2C).

In order to gain insight into the tissue-specific expression pattern of *WAG2* and to understand the divergent role of *WAG1* in apical hook opening, we generated transgenic *Arabidopsis* lines that express the GUS reporter under control of *WAG1* and *WAG2* promoter fragments (2.5 kb and 2.9 kb, respectively), *WAG1pro:GUS* and *WAG2pro:GUS*. Our analysis of the *WAG1* and *WAG2* expression patterns indicated a specific expression of *WAG2pro:GUS* but not of *WAG1pro:GUS* in the concave side of

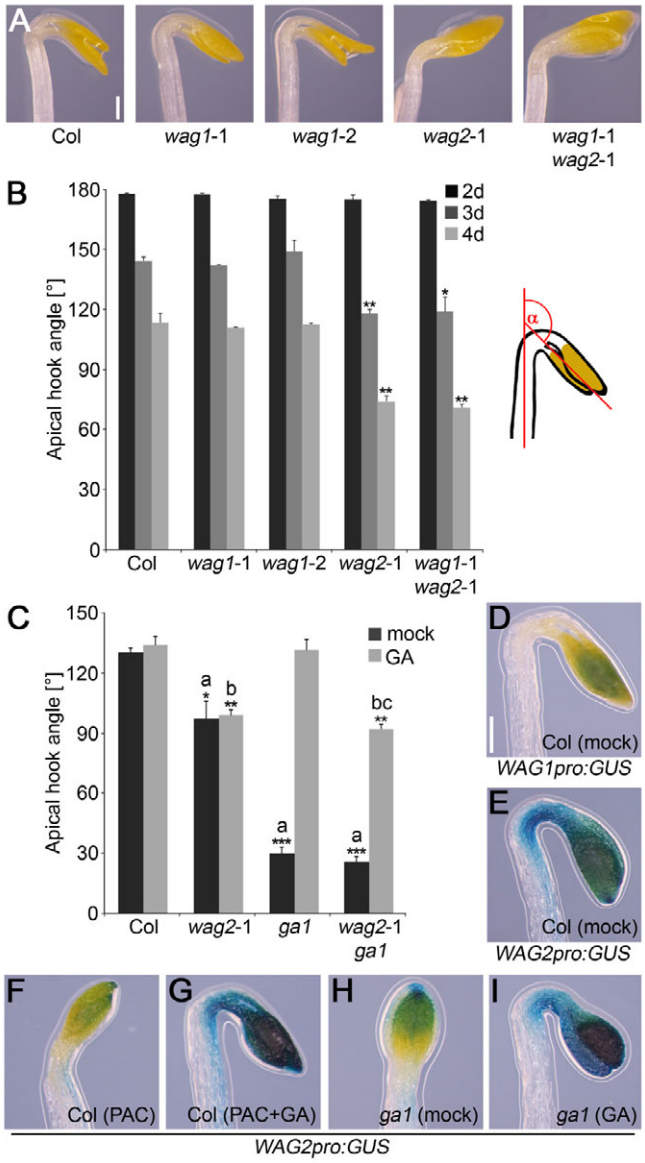

Fig. 2. Apical hook opening is accelerated in *wag2* mutants. (**A**) Representative photographs of the apical hook of 4-day-old dark-grown seedlings of the genotypes indicated in the figure. Scale bar: 200 μm. (**B**) Quantification of the apical hook angle of 2-, 3- and 4-day (d)-old dark-grown seedlings of the *wag1* and *wag2* genotypes indicated in the figure reveals an accelerated hook opening in *wag2* mutants when compared with the wild type or *wag1* mutants. Shown are the averages and the standard deviations of two biological replicates using minimum 20 seedlings per genotype per experiment. Asterisks indicate the significance (Student's *t*-test: *0.01<*P*<0.05; **0.001<*P*<0.01) in comparison with the wild type at the specific time point. (**C**) Quantification of the apical hook angle in 3.5-day-old seedlings grown in the absence and presence of 1 μM GA3. Shown are the average and the standard deviation of two biological replicates (*n*>20). Asterisks indicate the significances (Student's *t*-test: *0.01<*P*<0.05; **0.001<*P*<0.01; ***P*<0.001). a, b and c indicate the comparison with Col (mock), Col (GA) or *ga1* (GA), respectively. (**D**,**E**) Photographs of representative 3-day-old dark-grown transgenic *WAG1pro:GUS* and *WAG2pro:GUS* seedlings. (**F**,**H**) *WAG2pro:GUS* activity following PAC treatment of the wild type (F) or in the GA-deficient *ga1* background (H). (**G**,**I**) *WAG2pro:GUS* expression following GA treatment of PAC-treated seedlings (G) or *ga1* mutants (I). Scale bar: 200 μm.

the apical hook (Fig. 2D,E). The expression of *WAG2pro:GUS* in the apical hook was GA regulated as shown after crossing *WAG2pro:GUS* into the GA-deficient *ga1* background or treatment of *WAG2pro:GUS* lines with the GA biosynthesis inhibitor paclobutrazol (PAC) (Fig. 2F-I). *WAG2pro:GUS* expression thus mirrors the expression of the *WAG2* gene as observed in our qRT-PCR experiments (Fig. 1) and reveals the specific expression of *WAG2* in the concave side of the apical hook, which is of particular interest with regard to the phenotype of the *wag2* mutant. At the same time, the apparent absence of *WAG1pro:GUS* expression in the apical hook, even after extended staining for GUS reporter activity, is consistent with our finding that *WAG1* does not contribute to the *wag2* hook phenotype.

WAG2 is regulated by PIF5

As *WAG2* expression is controlled by light and GA, we reasoned that *WAG2* expression may be controlled by PIF transcription factors. This hypothesis was particularly intriguing as PIF5 had previously been reported to regulate apical hook maintenance (Gallego-Bartolomé et al., 2011; Khanna et al., 2007). We used qRT-PCR to examine the expression of *WAG2* in previously characterized *pif1*, *pif3*, *pif4* and *pif5* single, double and quadruple mutants (Fig. 3A). This analysis revealed a strongly decreased *WAG2* transcript abundance in *pif5* mutants. At the same time, *WAG2* transcript levels were increased in *pif4* mutants but slightly decreased in *pif4 pif5* (*pif45*) double mutants. These findings are consistent with the notion that PIF5 is an activator and PIF4 is a repressor of *WAG2* expression. Although *WAG2* expression was unaltered in *pif1* and *pif3* single mutants, *WAG2* expression was decreased in *pif1 pif3* (*pif13*), suggesting that PIF1 and PIF3 may be functionally redundant activators of *WAG2* expression. Importantly, our analysis of an overexpressor line of PIF5 (*PIF5ox*) supported the concept of PIF5 being an activator of *WAG2* expression (Fig. 3B). The results from the qRT-PCR analysis were confirmed when we examined the expression of *WAG2pro:GUS* transgenes in the *pif5* and *PIF5ox* backgrounds, respectively (Fig. 3C). Owing to an apparent linkage between the *WAG2pro:GUS* transgene and the *PIF5* locus, we had to use a different *WAG2pro:GUS* parental line (line #2) for the cross with *pif5*, which exhibited a lower (line 2) basal *WAG2pro:GUS* expression level than our *WAG2pro:GUS* reference line (line 1). Regardless of this fact and consistent with our qRT-PCR data, we found that *WAG2pro:GUS* expression in the apical hook is reduced in the *pif5* mutant background and increased in *PIF5ox* when compared with their respective wild-type segregants (Fig. 3C).

We next examined the contribution of PIF5 to apical hook opening. In agreement with the previously published study, we detected also in our experimental conditions decreased and increased apical hook angles in *pif5* and *PIF5ox* lines, respectively (Fig. 3D; supplementary material Fig. S2). These findings are thus consistent with the notion that the reduced and increased *WAG2* transcript abundance in *PIF5* loss-of-function mutants and overexpression lines may be causative for their differential apical hook phenotype.

To examine whether *WAG2* is a direct transcription target of PIF5, we performed ChIP experiments followed by qRT-PCR with dark-grown seedlings using *PIF5ox*, which expresses an HA-tagged PIF5. Our binding site searches identified one G-box as putative PIF-binding site in the promoter of *WAG2*. Our ChIP experiment then revealed that this site is specifically bound by PIF5:HA, thus PIF5 may be a direct regulator of *WAG2* (Fig. 3E). Consistent with the differential transcriptional regulation of *WAG2* and *WAG1*, we did not find G-boxes in the promoter of *WAG1*.

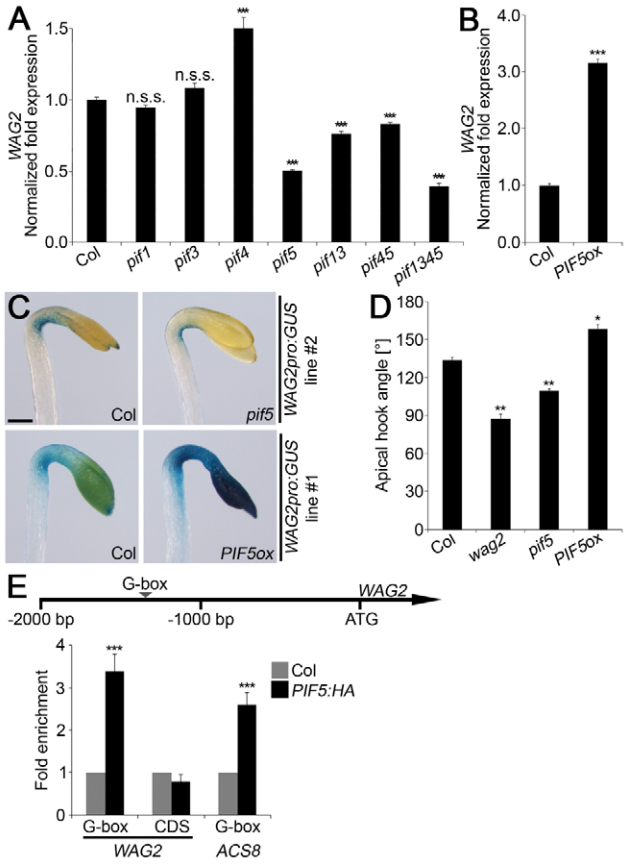

Fig. 3. *WAG2* expression is PIF dependent. (**A**,**B**) qRT-PCR analysis of 3-day-old dark-grown *pif* mutant and PIF5 overexpression (PIF5ox) seedlings. *UBC21* (A) and *AT3G50685* (B) were used for normalization. (**C**) *WAG2pro:GUS* expression in wild-type and the *pif5* or *PIF5ox* background. Owing to the apparent linkage of *WAG2pro:GUS* (line #1) to the *PIF5* locus, a different WAG2pro:GUS transgenic line (line #2) had to be used for the cross with *pif5*. To account for the variability in the basal *WAG2pro:GUS* expression between these lines, wild-type segregants of either cross were analyzed and used for comparisons with the respective *PIF5* mutant or overexpressor. (**D**) Apical hook phenotype of 3.5-day-old seedlings of the genotypes indicated in the figure. Shown are the average and the standard deviation of two biological replicates (n>20). (**E**) Scheme of the *WAG2* promoter and ChIP of PIF5:HA from dark-grown *PIF5ox Arabidopsis* seedlings. A region in the *WAG2*-coding sequence (CDS) was used as negative control. The previously reported binding site in the *ACS8* promotor was used as a positive control. Asterisks in all experiments indicate the significances in comparison with Col (Student's *t*-test: n.s.s., not statistically significant; *0.01<P<0.05; **0.001<P<0.01; ***P<0.001).

WAG2 is required for a local auxin maximum in the apical hook

The analysis of mutants of the PIN auxin efflux facilitators implicated PIN1, PIN3, PIN4 and PIN7 in the maintenance of the apical hook (Zádníková et al., 2010). These analyses also identified *pin3* as the single *PIN* gene mutant with the strongest apical hook phenotype. As WAG2 is an established regulator of PIN polarity (Dhonukshe et al., 2010), a link between *WAG2* function and auxin transport regulation appeared reasonable. We therefore tested the genetic interaction between *WAG2* and *PIN3* in *wag2 pin3* double mutants and compared their apical hook phenotype with that of the single mutants. This analysis revealed an enhancement of the apical

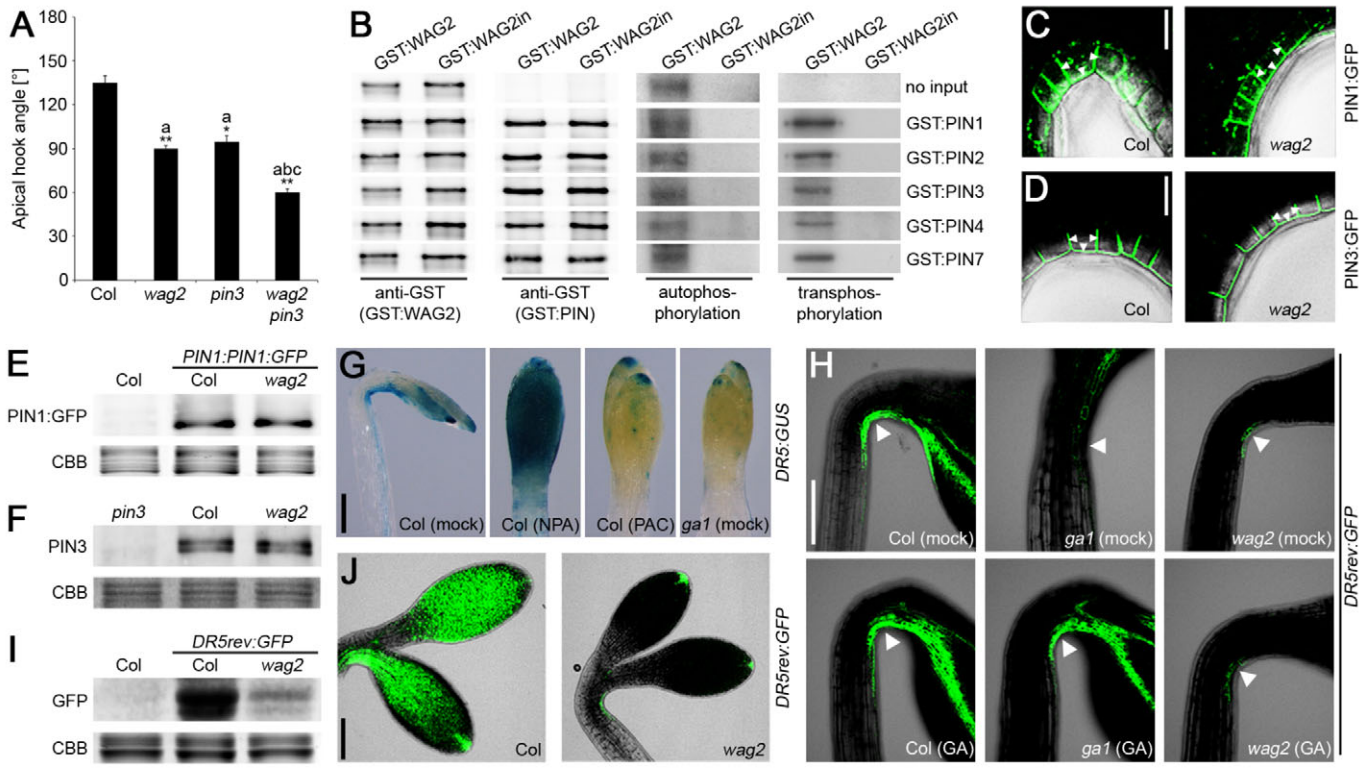

Fig. 4. WAG2 phosphorylates PIN proteins and regulates the apical hook auxin maximum. (**A**) Apical hook opening phenotype of 3.5-day-old seedlings. Shown is the average and the standard deviation of two biological replicates (n>20). Asterisks indicate the significances (Student's t-test: *0.01<P<0.05; **0.001<P<0.01). a, b and c indicate the comparison with Col, to wag2 or to *pin3*, respectively. (**B**) Phosphorylation experiment of recombinant GST-tagged PIN fragments by recombinant GST-tagged WAG2 or kinase-dead WAG2in. The two panels on the left represent immunoblots to demonstrate equal loading of the purified proteins. The two panels on the right are autoradiographs that reveal autophosphorylation of the active GST:WAG2 but not of GST:WAG2in and transphosphorylation of GST-tagged cytoplasmatic loops of PINs by GST:WAG2. (**C**,**D**) Confocal images of the concave apical hook region of (C) PIN1:PIN1:GFP- and (D) PIN3:PIN3:GFP-expressing, 3.5-day-old skotomorphogenic wild-type and *wag2* mutants. Arrowheads indicate the polar distribution of PIN:GFP proteins. Scale bars: 25 μm. (**E**) Immunoblot with anti-GFP antibody of 15 μg total protein extracts prepared from the cotyledons and 1-2 mm of the apical hypocotyl of 3.5-day-old dark-grown PIN1:PIN1:GFP-expressing seedlings. (**F**) Immunoblot with anti-PIN3 antibody of 15 μg membrane protein extracts from the cotyledons and 1 to 2 mm of the apical hypocotyl of 3.5-day-old dark-grown seedlings. (**G**) Photographs of shoot apices of *DR5:GUS*-expressing wild-type and *ga1* 3.5-day-old dark-grown seedlings grown, where indicated, on 1 μM NPA- or 1 μM PAC-containing media. Scale bar: 200 μm. (**H**) Confocal images of the apical hook of *DR5rev:GFP*-expressing 3.5-day-old dark-grown wild-type, *ga1* and *wag2* mutants. Scale bar: 200 μm. (**I**) Immunoblot with anti-GFP antibody of 15 μg total protein extracts prepared from the cotyledons and 1 to 2 mm of the apical hypocotyl of 3.5-day-old skotomorphogenic *DR5rev:GFP*-expressing seedlings. Arrowheads indicate the position of the apical hook auxin maximum. (**J**) Confocal images of the cotyledons of *DR5rev:GFP*-expressing 3.5-day-old skotomorphogenic wild-type and *wag2* seedlings. Scale bar: 200 μm.

hook opening defect in the double mutant when compared to the single mutants suggesting that *WAG2* and *PIN3* act together to repress apical hook opening (Fig. 4A). As it is known that also other PINs participate in apical hook opening, it can of course be envisioned that other PINs are involved in WAG2-regulated hook development.

It has previously been reported that WAG2 phosphorylates PIN2 and thereby controls PIN2 polarity in cortical root cells (Dhonukshe et al., 2010). As PIN2 is not expressed in the apical hook (Zádníková et al., 2010) and as we had observed an enhancement of the apical hook phenotype in the *wag2 pin3* double mutant, we questioned whether WAG2 may phosphorylate PIN3 and other members of the PIN protein family besides PIN2. Using recombinant PIN fragments that correspond to their intracellular cytoplasmic loop, we could demonstrate that recombinant WAG2 phosphorylates – in addition to PIN2 – PIN1, PIN3, PIN4 and PIN7, at least in vitro, thus all PINs with an established role in cell-to-cell auxin transport and apical hook development (Fig. 4B). We subsequently examined wild-type and *wag2* apical hooks to find out whether the loss of *WAG2* had a consequence on the polarity or distribution of PIN1:GFP and PIN3:GFP. This analysis did not reveal any striking differences in PIN distribution or polarity in the epidermis and cortex of the apical hook, suggesting that changes in PIN polarity may not be responsible for the *wag2* apical hook phenotype (Fig. 4C,D). At the same time, our observations by epifluorescence microscopy indicated that PIN1:GFP and PIN3:GFP are strongly expressed in the inner tissue of the apical hook (supplementary material Fig. S3). Unfortunately, specifically in the apical hook region and in contrast to the remaining hypocotyl, these endodermis and stele cell layers are not amenable to confocal microscopy analysis. As we cannot image PIN proteins in this region, we are also unable to draw any conclusions on the effect of WAG2 on PIN protein behavior, e.g. polarity. To test whether changes in PIN protein abundance are the cause for the changes in apical hook formation in *wag2*, we also performed immunoblots to detect PIN1 and PIN3 in wild-type and *wag2*

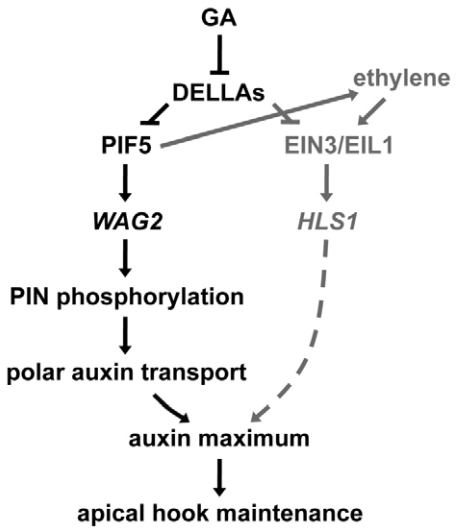

Fig. 5. Model for the proposed role of GA in apical hook opening. GA promotes the degradation of DELLA proteins. The DELLA repressors impair PIF5 activity, which is a transcriptional regulator of the AGCVIII kinase gene *WAG2* and of the ethylene biosynthesis gene *ACS8*. WAG2 phosphorylates PIN proteins and by this means the kinase regulates polar auxin efflux and the auxin (signaling) maximum at the concave side of the apical hook. This maximum prevents premature hook opening by impairing the elongation of cells localized at this position. Additionally, the DELLA proteins impair and ethylene promotes the transcriptional activity of EIN3/EIL1. EIN3 and EIL1 induce the expression of *HLS1*, which regulates the auxin signaling maximum in the apical hook by an unknown mechanism. Findings presented by Gallego-Bartolomé et al. (Gallego-Bartolomé et al., 2011) and An et al. (An et al., 2012) are depicted in gray.

seedlings (Fig. 4E,F). As PIN protein levels were identical in both genotypes, we concluded that changes in PIN protein abundance are not the cause for the apical hook phenotype of *wag2*.

We next examined auxin distribution in the apical hook region using the well-established DR5 auxin (response) reporters. *DR5:GUS*, as well as *DR5rev:GFP* expression analysis revealed a strong signal of the reporters in the concave side of the apical hook in dark-grown *Arabidopsis* seedlings (Fig. 4G,H). In agreement with previous reports, the lateral apical hook expression pattern of *DR5:GUS*, as well as apical hook formation were absent in seedlings that had been treated with the auxin transport inhibitor NPA or the GA biosynthesis inhibitor PAC (Fig. 4G,H) (Gallego-Bartolomé et al., 2011; Lehman et al., 1996; Zádníková et al., 2010). In addition, when we introduced *DR5:GUS* and *DR5rev:GFP* into the *ga1* mutant, the DR5 signal for the apical hook was not visible, unless the *ga1* mutant had been treated with GA (Fig. 4G,H). These findings confirmed the recently established observations from other laboratories showing that auxin transport and GA biosynthesis are not only required for apical hook formation but also for the formation of a lateral auxin maximum that correlates with apical hook formation (Gallego-Bartolomé et al., 2011; Zádníková et al., 2010). Importantly, our analysis of DR5rev:GFP in the *wag2* mutant by confocal microscopy, as well as by immunoblotting revealed a strong reduction of the DR5:GFP signal in the concave side of the apical hook of *wag2* seedlings and, as we noted with interest, also in their cotyledons (Fig. 4H-J). Interestingly, the difference in *DR5rev:GFP* expression was more prominent in 4-day-old seedlings than in 2-day-old seedlings (supplementary material Fig. S4). As GA treatments did not restore or influence the DR5rev:GFP signal in the *wag2* mutant, this analysis suggests that WAG2 positively regulates the lateral auxin maximum in the apical hook (Fig. 4H). In view of the previously established function of WAG2 as an auxin transport regulatory kinase, we are tempted to speculate that this is the consequence of altered or reduced auxin transport in *wag2* mutants.

DISCUSSION

The present study reveals the role of WAG2 as a repressor of apical hook opening in dark-grown *Arabidopsis* seedlings. We show that *WAG2* is specifically expressed in the concave side of the apical hook and that *wag2* mutants open their apical hook faster than the wild type. At least two pieces of evidence point to a role for WAG2 in controlling auxin transport. First, we show that *wag2* mutants fail to establish the strong lateral auxin maximum that is characteristic for the apical hook. Second, and as already shown in a previous study for the auxin efflux facilitator PIN2, we show that WAG2 phosphorylates all PINs – at least in vitro. As WAG2 had previously been implicated in the regulation of PIN polarity by others (Dhonukshe et al., 2010; Ding et al., 2011; Sorefan et al., 2009), we examined whether changes in PIN polarity are causative for the *wag2* phenotype and the apparent changes in auxin accumulation in the apical hook. However, we could not find any evidence for a hook maintenance phase-specific or WAG2-dependent change in the polarity of PIN1 and PIN3 when examining GFP-tagged variants of these two PINs. At the same time, our microscopic analysis also indicated that *PIN1* and *PIN3* are expressed in the inner tissue of the hypocotyl and the apical hook but, unfortunately, the inner tissues of the apical hook are not amenable to confocal studies. In addition, this has been reported in different biological contexts by others; based on our experiments, we cannot draw any conclusions on the role of WAG2 in controlling PIN protein polarity (Dhonukshe et al., 2010; Ding et al., 2011; Sorefan et al., 2009). Previously published studies have shown that auxin transport from the endodermis to the outer tissues is dynamically controlled during phototropism and gravitropism. Similar auxin tranport mechanisms may govern apical hook formation and therefore an analysis of PIN polarity in the inner tissues of the apical hook would be highly desirable but can at present not be realized. It also did not escape our attention that DR5 auxin responses are strongly reduced in *wag2* cotyledons. As auxin transported from the cotyledons may be required for the establishment of the auxin maximum in the apical hook, it may well be that the loss of the apical hook auxin maximum is a consequence of the reduced auxin levels in the cotyledons that we infer from the reduced *DR5rev:GFP* expression in this tissue. As several reports point at a role of PIF transcription factors in the control of auxin biosynthesis and response (Franklin et al., 2011; Hornitschek et al., 2012; Li et al., 2012; Nozue et al., 2007; Sun et al., 2012), the correlation of *WAG2* expression and the apparent auxin maximum in the apical hook could also be the consequence of a direct regulation of auxin distribution via the PIFs. As the local auxin maximum is strongly reduced in *wag2* mutants, the maintenance of this auxin maximum should, however, be *WAG2* dependent, either through the postulated role of WAG2 in controlling auxin transport or another role of WAG2 on auxin biosynthesis and signaling.

Our interest in *WAG2* had arisen from our observation that *WAG2* abundance is increased after GA treatment. In recent years, different families of transcription factors have been reported that are controlled by DELLA repressors of the GA pathway (Schwechheimer, 2011).

We inferred from our observation that *WAG2* expression is controlled by light that *WAG2* is downstream from the light-labile PIF transcription factors. Comparing *WAG2* expression patterns in different *pif* mutant backgrounds and subsequent ChIP analyses identified PIF5 as one PIF transcription factor that activates *WAG2* expression downstream from GA, DELLAs and light. The role of PIF5 as a regulator of *WAG2* is also supported by the apical hook phenotype of *pif5* mutants and *PIF5* overexpressors.

Based on the close relationship between WAG2 and WAG1, and the fact that both kinases had previously been shown to act in a functionally redundant manner in the control of root growth, we also examined the functional redundancy between the two kinases as repressors of apical hook opening. Interestingly, we found no evidence for a role of *WAG1* in apical hook formation. Most striking is our observation that *WAG1* is not expressed in the apical hook region and that the *WAG1* promoter, in contrast to the WAG2 promoter, does not contain any G-boxes, which are known binding sites for PIF transcription factors.

In summary, our results propose a model whereby PIF5, the activity of which is regulated by GA, DELLAs, and light, controls *WAG2* expression in dark-grown seedlings. We further propose that the tissue-specific expression and activity of *WAG2* in the concave side of the dark-grown seedling alters PIN polarity or PIN activity by direct PIN phosphorylation and that thereby WAG2 contributes to the regulation of a lateral auxin maximum that is required for apical hook maintenance in dark-grown seedlings (Fig. 5). Interestingly, we also observed that *WAG2* expression in the concave side of the dark-grown seedling can be detected throughout dark-grown seedling development whereas the phenotype of *wag2* mutants is restricted to the apical hook opening phase. Therefore, our results also suggest that other regulatory mechanisms must exist that control hook formation.

Importantly, DELLA proteins have most recently also been shown to repress the ethylene signaling proteins EIN3 and EIL1 (An et al., 2012). Ethylene promotes apical hook formation and *ein3 eil1* mutants are insensitive to the ethylene effect on apical hook formation. Ethylene and GA control the expression of the apical hook regulatory protein HLS1 and this control is achieved through inhibitory interactions of the GA-labile DELLAs with EIN3 and EIL1, direct regulators of *HLS1* expression. Importantly, this study also suggests that, besides the ethylene/GA-dependent regulation of *HLS1*, a GA-controlled auxin transport-dependent process must exist that modulates hook curvature through asymmetric auxin accumulation. In the context of this very recent study, the GA-dependent control of *WAG2* expression is a good candidate for this elusive auxin transport regulatory mechanism (Fig. 5).

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Competing interests statement
The authors declare no competing financial interests.

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