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Sadaf Khan

University of Nebraska - Lincoln

Julie M. Stone

University of Nebraska-Lincoln, jstone2@unl.edu

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Arabidopsis thaliana GH3.9 influences primary root growth

Sadaf Khan and Julie M. Stone

Department of Biochemistry, Plant Science Initiative, University of Nebraska-Lincoln,
N230 Beadle Center, 1901 Vine Street, Lincoln, NE 68588-0664, USA

Corresponding author – Julie M. Stone, email jstone2@unl.edu

Abstract

Auxins regulate a complex signal transduction network to direct plant development. Auxin-responsive genes fit into three major classes: the so-called auxin/indole-3-acetic acid (*Aux/IAA*), the *GH3*, and the small auxin-up RNA (*SAUR*) gene families. The 20-member *Arabidopsis thaliana* *GH3* gene family has been subdivided into three groups. In vitro studies have shown that most Group II members function as IAA-amido synthetases to conjugate amino acids to the plant hormone auxin. Here we report the role of a previously uncharacterized *GH3* gene family member, *GH3.9*, in root growth. Unlike most other Group II family members, *GH3.9* expression was repressed by low concentrations of exogenous IAA in seedlings. Transgenic plants harboring a *GH3.9* promoter::reporter gene construct indicate that *GH3.9* is expressed in the root-hypocotyl junction, leaves and the shoot apical meristem of young seedlings, in mature embryos, and in the root vascular tissue. Expression was also observed in lateral root tips when seedlings were treated with exogenous IAA. Inverse PCR was used to identify an activation tagged T-DNA insertion in chromosome 2 near the 5'UTR region of At2g47750 (*GH3.9*). Plants homozygous for the T-DNA insertion (*gh3.9-1* mutants) had reduced *GH3.9* expression, no obvious effects on apical dominance or leaf morphology, greater primary root length, and increased sensitivity to indole-3-acetic acid (IAA)-mediated root growth inhibition. Additional T-DNA insertion alleles and transgenic plants with reduced *GH3.9* transcript levels due to RNA-interference (RNAi) also showed these same phe-

notypes. Our results provide new information on the function of *GH3.9* in roots where it is likely to control auxin activity through amino acid conjugation.

Keywords: auxin, plant hormones, primary root growth, T-DNA insertion mutant

Abbreviations:

AuxRE	Auxin-responsive element
GUS	β -Glucuronidase
IAA	Indole-3-acetic acid
MeJA	Methyl jasmonate
RNAi	RNA interference
SAUR	Small auxin up RNA
UTR	Untranslated region
X-Gluc	5-Bromo-4-chloro-3-indolyl- β -D-glucopyranoside

Introduction

Plant development is influenced by endogenous factors such as plant hormones, and external factors such as intensity and quality of light, and water and nutrient availability. Plant hormone biosynthesis, degradation, redistribution, and conjugation to other molecules facilitate plants' adaptation to these various environmental conditions. A more complete understanding of the complex interplay between the major phytohormones and their effects on plant development is greatly needed.

Auxins are a group of plant hormones that govern key processes such as root and shoot morphology, cell

division, cell proliferation, gravitropism, and phototropism (Woodward and Bartel 2005). Therefore the expression of many genes is regulated by these phytohormones (Goda et al. 2004). Auxin-responsive genes have been divided into three major classes: the so-called auxin/indoleacetic acid (*Aux/IAA*), the *GH3*, and the small auxin-up RNA (*SAUR*) gene families (Guilfoyle et al. 1998; Liscum and Reed 2002). The first member of the *GH3* gene family to be identified was isolated from soybean and shown to be specifically up-regulated by auxin (Hagen and Guilfoyle 1985, 2002; Hagen et al. 1991). The *Arabidopsis thaliana* *GH3* gene family is composed of 20 members that encode proteins capable of conjugating plant hormones such as auxin, jasmonic acid, and salicylic acid to amino acids. Accumulating evidence indicates that *GH3* family members function to regulate levels of biologically active hormones through amino acid conjugation, thereby targeting them for degradation/storage in the case of auxins and activating them in the case of jasmonates (Staswick et al. 2002, 2005). Based on their functions and sequence similarities the *GH3* family members have been subdivided into three groups: I-III. In *A. thaliana* there are two Group I genes, *JAR1/FIN219* and *DFL2* (Hsieh et al. 2000; Staswick et al. 2002; Takase et al. 2003), eight Group II genes, including *YDK1* and *DFL1* (Nakazawa et al. 2001; Takase et al. 2004), and 10 Group III genes (Staswick et al. 2005). *GH3* homologues are found in other plant species. The recently classified rice *GH3* gene family has members corresponding to Groups I and II, but Group III members were not reported (Jain et al. 2006; Terol et al. 2006). The *GH3* families have not been extensively studied in species other than rice and *Arabidopsis*.

The *A. thaliana* Group I member *JAR1* (*GH3.11*) encodes a protein that conjugates methyl jasmonate (MeJA) to amino acids (Staswick and Tiryaki 2004). Those Group II members that have been tested for activity are indole-3-acetic acid (IAA) amido synthetases, capable of conjugating amino acids to the auxin IAA (Staswick et al. 2002, 2005). This conjugation of IAA to amino acids or other small molecules is vital for the maintenance of appropriate free (active) IAA levels in plants (Normanly and Bartel 1999; Woodward and Bartel 2005). The Group II members are named *GH3.1*, *GH3.2*, *GH3.3*, *GH3.4*, *GH3.5*, *GH3.6*, *GH3.9*, and *GH3.17* (Hagen and Guilfoyle 2002; Staswick et al. 2005). The biochemical activities of proteins encoded by the 10 Group III genes are as yet unknown.

With some notable exceptions, the physiological roles of the *GH3* family members remain a mystery. The physiological functions of only a few Group II mem-

bers have been reported in the literature, perhaps as a result of genetic redundancy, or the subtlety of phenotypes associated with altered expression of these genes. Even though Group II family members might act similarly at the biochemical level, differential expression could account for the complex variation in phenotypes described (see below).

The dominant activation-tagged T-DNA insertion mutants *df1-D* and *ydk1-D*, with insertions in the promoters of the *GH3.6* and *GH3.2* genes, respectively, were identified in mutant screens for altered hypocotyl elongation under varying light regimes. While uncovered in a similar mutant screen, the mutants are phenotypically distinct. The *df1-D* mutant had short hypocotyls under all light conditions tested but displayed normal etiolated seedling morphology in darkness (Nakazawa et al. 2001). The *ydk1-D* mutant also exhibited short hypocotyls in light but in darkness as well. In addition, the plants showed epinastic and small rosette leaves with reduced apical dominance, and short primary root lengths (Takase et al. 2004). The *ydk1-D* mutant had shorter roots than wild type at all auxin concentrations tested, but no significant difference in sensitivity to auxin-mediated root growth inhibition (Takase et al. 2004). In contrast, the dominant *df1-D* mutant was resistant to IAA-mediated root growth inhibition compared to wild type, and did not display a short-root phenotype (Nakazawa et al. 2001; Staswick et al. 2005). Thus, these two dominant mutations, which over express closely related *GH3* Group II-family members, display distinctly different phenotypes.

Loss-of-function mutations in *GH3* Group II-family members have also been reported. A putative knockout mutation in *GH3.2* (*YDK1*) showed no obvious hypocotyl or root phenotypes (Takase et al. 2004). Plants homozygous for T-DNA insertions in *GH3.1*, *GH3.2* (*YDK1*), *GH3.5* (*AtGH3a*), and *GH3.17* did not show significant differences in root growth rates, but did display altered IAA sensitivity in root inhibition assays. The *gh3.1-1*, *gh3.17-1*, and *gh3.5-1* mutants all showed increased IAA sensitivity compared to wild-type controls, whereas the enhanced sensitivity of the *gh3.2-1* mutant was small compared to the others (Staswick et al. 2005). These observations, i.e., enhanced IAA sensitivity in loss-of-function mutants and resistance in a gain-of-function mutant (*df1-D*), are consistent with the supposition that most Group II *GH3* members function by conjugating auxins to amino acids, thereby reducing free (active) auxin pools. If these Group II *GH3* genes have overlapping functions, then the phenotypic variation observed in different mutants might be explained by differences in either tissue specificities and/or phyto-

hormone regulation (i.e., inducibility by auxin or other hormones).

We have investigated the physiological roles of a previously uncharacterized *GH3* gene, Group II member *GH3.9*. A T-DNA insertion was found in chromosome 2 near the 5'UTR region of At2g47750 (*GH3.9*). Plants homozygous for the T-DNA insertion, additional T-DNA insertion alleles, and transgenic plants with reduced *GH3.9* expression due to RNA-interference displayed greater primary root length and showed increased sensitivity to auxin-mediated root growth inhibition in seedlings. Unlike several other Group II-family members, *GH3.9* is not auxin-inducible in seedlings, and reduced *GH3.9* expression does not result in defects in apical dominance or altered leaf morphology. Our results provide new information on the physiological role of an additional Group II *GH3* family member.

Materials and methods

Plant materials, growth conditions, genetic analyses, and hormone treatments

All genotypes were in the *A. thaliana* Columbia (Col) ecotype background. The *gh3.9-1* mutant was isolated from seed pools (CS21195) of plants transformed with pSKI015 activation tagging vector (Weigel et al. 2000), and T-DNA insertion lines SALK_005057, SALK_005056, and WiscDsLox419D07 (<http://www.signal.salk.edu>) were obtained from the Arabidopsis Biological Resource Center (ABRC, OHIO State University, OH, USA). Dr. Paul E. Staswick (University of Nebraska-Lincoln, Lincoln, NE, USA) kindly provided *gh3.17-1* and *jar1-1* mutant seeds.

For plate-based assays, seeds were surface-sterilized in 3% sodium hypochlorite and 0.02% (v/v) Tween-20 for 15 min, washed several times with sterile water, incubated at 4°C for one day in 0.1% agarose, and sown on Murashige-Skoog (MS) media plates supplemented with 2% sucrose and 0.6% phytagar (Gibco-BRL, Gaithersburg, MD, USA) plus or minus various supplements. For root growth phenotypic analyses plates were incubated vertically in a growth chamber (Percival AR36L; Percival, Perry, IA, USA) at 22°C, 70% RH and $\sim 130 \mu\text{E m}^{-2} \text{s}^{-1}$ under cool white fluorescent lights supplemented with incandescent lamps with a 12 h photoperiod. Auxin sensitivity assays were performed on plates supplemented with 5 μM indole-3-acetic acid (IAA, No. 102037, ICN Biochemicals, Cleveland, OH, USA) as described (Tiryaki and Staswick 2002). Jasmonate sensitivity assays were performed on plates sup-

plemented with 10 μM methyl jasmonate (MeJA, No. 392707, Sigma-Aldrich, Milwaukee, WI, USA) as described (Staswick et al. 1992). Primary root length was determined (in mm) daily after emergence of the root for a period of 10 days using calipers.

For seed collection, genetic crosses and RNA and DNA isolation from adult tissue, plants were grown in soil (Metro-Mix 360; Scotts, Maryville, OH, USA) in a walk-in growth chamber at 24°C, 70% RH and $\sim 130 \mu\text{E m}^{-2} \text{s}^{-1}$ under cool white fluorescent lights with a 16 h photoperiod.

The *gh3.9-1* mutant (female) was backcrossed to the wild-type Col-0 parent (male), and two F1 progeny were allowed to self fertilize. The resulting two independent F2 populations were analyzed for the segregation of primary root length phenotype, and the data was subjected to chi-square goodness of fit analyses. PCR-based genotyping (described below) was used to verify the recessive nature of the *gh3.9-1* associated long-root phenotype.

Exogenous IAA treatment of seedlings was performed as described (Takase et al. 2004). Briefly, 4–6 leaf stage Col-0 or *GH3.9* promoter:: β -glucuronidase (GUS) transgenic seedlings were treated by immersing in aqueous solutions with 0, 0.01, 0.1 or 100 μM IAA for 60 min. RNA was isolated immediately after treatment using Qiagen mini RNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions followed by reverse transcription and polymerase chain reaction (RT-PCR) (described below).

Retrieval of plant DNA flanking the T-DNA insertion by inverse PCR

The plant DNA flanking the T-DNA insertion in *gh3.9-1* was identified by inverse PCR (iPCR) using oligonucleotide primers corresponding to regions of the pSKI015 T-DNA insertion region as described with modifications (Zhou et al. 1997). Specifically, genomic DNA (7 μg), isolated from leaf tissue using Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences, Piscataway, NJ, USA), was restriction enzyme digested in a 100 μl reaction with 10 U *EcoRI* enzyme (Promega, Madison, WI, USA) overnight at 37°C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with ethanol. Digested DNA (560 ng) was self-ligated in a 100 μl reaction at 16°C using 10 U of T4 DNA ligase and used as a template for the PCR reaction. PCR was performed for 40 cycles (94°C 1 min, 50.5°C 1 min, and 72°C 4 min) in a reaction containing 200 μM dNTPs, 1X TaqPlus Precision buffer, 1.25 U TaqPlus Precision polymerase mix

(Stratagene, La Jolla, CA, USA) and 100 nM oligonucleotide primers. Oligonucleotide primers were ACTRB5': 5'-GTTTCTAGATCCGAAACTATCAGTG-3' and T3: 5'-AATTAACCCTCACTAAAGGGAACAAAAG-3'. The resulting PCR product was purified using a PCR purification kit (Qiagen, Valencia, CA, USA), subcloned into pGEMTeasy vector (Promega, Madison, WI, USA) and subjected to DNA sequencing with T7 promoter and SP6 promoter oligonucleotide primers (UNL Genomics Core Facility).

PCR-based genotyping

The genotypes of the parents and F2 progeny from a backcross between a *gh3.9-1* homozygous T-DNA insertion mutant and wild-type Col-0 were verified by PCR-based genotyping. Genomic DNA was isolated from 100 mg leaf tissue using the Dellaporta method (Dellaporta et al. 1983) and subjected to PCR using *GH3.9*-specific oligonucleotide primers flanking the T-DNA insertion (p50 F: 5'-CCTTGATTGACTTGGTGTAG-3' and p50 R: 5'-ggatccTCTTTAATACACTGTCGTG-3') to detect a wild-type allele and p50 F and a T-DNA specific oligonucleotide primer TR2: 5'-TACGACGGATCGTAATTTGTCG-3' to detect a T-DNA insertion allele. The lowercase letters in the p50 R oligonucleotide primer represents an engineered *Bam*HI restriction enzyme site, and the TR2 primer corresponds to the left border region of the activation tagging T-DNA vector pSKI015. PCR was performed for 40 cycles in a 25 µl reaction containing 1X high fidelity PCR buffer, 1.25 U Eppendorf Triple Master mix enzyme (VWR International, West Chester, PA, USA), 50 µM dNTPs, and 500 nM oligonucleotide primers. A wild-type allele yielded a *GH3.9*-specific 2.6 kbp PCR product with p50 F and p50 R oligonucleotide primers and 94°C 30 s, 55°C 30 s, and 72°C 2.5 min cycling conditions. A T-DNA insertion allele yielded a 1.5 kbp PCR product with p50 F and TR2 oligonucleotides and 94°C 30 s, 55°C 30 s, and 72°C 1.5 min cycling conditions. PCR products were visualized after electrophoresis on 1% agarose gels.

Generation of *GH3.9* RNA-interference (RNAi) and *GH3.9* promoter::*GUS* gene fusion transgenic lines

An RNA-interference (RNAi) approach was used to generate transgenic lines with reduced expression of *GH3.9*. A *GH3.9*-specific region was cloned in two orientations in the binary vector pFGC5941 designed to produce double-stranded RNA in a hairpin structure for efficient gene silencing (Wesley et al. 2001). Total RNA was isolated from mature Col-0 plants using

Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions, and RNA concentration was determined spectrophotometrically. Reverse transcription was performed for 1 h at 42°C in a 20 µl reaction containing 2 µg total RNA, 500 ng random hexamer oligonucleotide primers, 40 U RNasin (Promega, Madison, WI, USA), 500 µM dNTPs, and 40 U M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA), then diluted to 50 µl. Polymerase chain reaction (PCR) was performed for 40 cycles (94°C 30 s, 58°C 30 s, and 72°C 40 s) in a 50 µl reaction containing 1X PCR buffer, 1 mM MgCl₂, 50 µM dNTPs, 500 nM each of Ri 50F and Ri 50R oligonucleotide primers, and Taq polymerase. The forward primer Ri 50F: 5'-tctagaggcgcgccGGAGCATAACGTTAGC-CAAG-3' and reverse primer Ri 50R: 5'-ggatccatttaaatTCATGGAACCCAAGTCGGG-3' amplify a 410 bp gene-specific region of *GH3.9* (Figure 1). Lowercase letters represent the engineered restriction enzyme sites, *Xba*I and *Asc*I in Ri 50F and *Bam*HI and *Swa*I in Ri 50R. The PCR product was ligated into the pGEMTeasy vector (Promega, Madison, WI, USA) and verified by DNA sequencing with T7 promoter and SP6 promoter oligonucleotide primers (UNL Genomics Core Facility). The *GH3.9*-specific region was first isolated by restriction enzyme digestion with *Asc*I and *Swa*I, purified on an agarose gel, and ligated into the *Asc*I/*Swa*I digested RNAi vector pFGC5941. Next, the *GH3.9*-specific region was first isolated by restriction enzyme digestion with *Xba*I and *Bam*HI, purified on an agarose gel, and ligated into the *Xba*I/*Bam*HI digested pFGC5941 vector containing the *Asc*I/*Swa*I fragment. The resulting pFGC5941-RNAiGH3.9 construct was used to generate transgenic plants (see below).

To examine the tissue-specific and developmental expression of *GH3.9*, transgenic plants expressing a *GH3.9* promoter::β-glucuronidase (*GUS*) fusion construct were generated. The wild-type *GH3.9* promoter region containing 1192 bp upstream of and 33 bp downstream of the ATG translation start codon was amplified by PCR from Col-0 genomic DNA. The PCR reaction was performed for 40 cycles (94°C 30 s, 55°C 30 s, 72°C 1.5 min) in a 50 µl reaction containing 1X high fidelity PCR buffer, 1.25 U Eppendorf Triple Master mix enzyme (VWR International, West Chester, PA, USA), 100 µM dNTPs, and 500 nM oligonucleotide primers new p50 F: 5'-ggatccGAGGTGAATTCATTAACACGG-3' and new p50 R: 5'-ggatccCCTACACTGTCGTGATCAAGCTTC-3'. Lowercase letters represent the engineered *Bam*HI restriction enzyme site and bold letters in the new p50 R oligonucleotide primer represent extra bases added to create an in-frame fu-

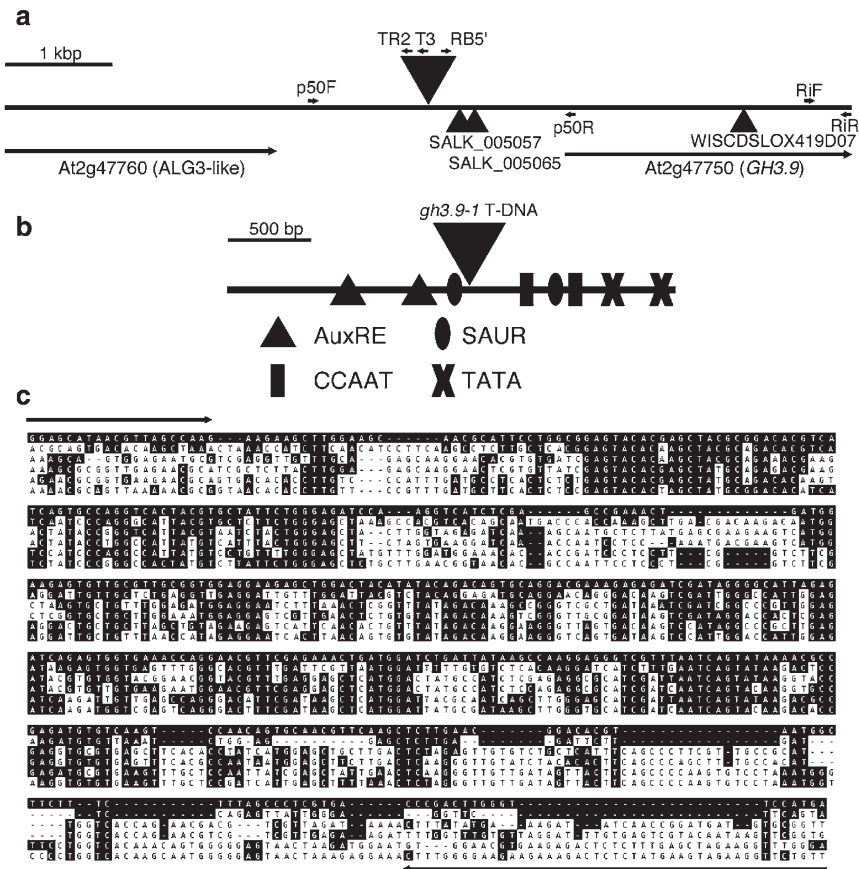


Figure 1. Schematic representation of the T-DNA insertions in the *gh3.9* mutants, the relative positions of various promoter elements in the *GH3.9* upstream region, and a nucleotide alignment of several Group II *GH3* cDNAs highlighting the region used for gene silencing by RNA-interference, semi-quantitative RT-PCR and a hybridization probe. **a**) The original *gh3.9-1* mutant was found to have an activation tagging T-DNA insertion (derived from pSKI015—*large triangle*) inserted in Chromosome 2 in the intergenic region between *At2g47750* and *At2g47760* (coding regions from the translation start to translation stop sequences are represented by *arrows*). Positions of additional T-DNA insertions corresponding to SALK_005057, SALK_005065 and WISCD-SLOX419D07 lines are indicated by *small triangles*. Oligonucleotide primer annealing sites are indicated with *small arrows*. Oligonucleotide primers corresponding to the T3 promoter (T3) and the right border region of pSKI015 (ACTRB5'; RB5') were used for retrieval of the plant DNA flanking the activation tagging T-DNA by inverse PCR. For PCR-based genotyping p50 F, p50 R (gene-specific primers) and TR2 (a T-DNA-specific primer) were used. Oligonucleotide primers Ri 50F (RiF) and Ri 50R (RiR) were used for semi-quantitative RT-PCR and to generate the *GH3.9* RNAi gene silencing construct and hybridization probe for Northern blot analyzes. **b**) Schematic representation of the intergenic region from the stop codon of *At2g47760* to the start codon of *GH3.9* and selected *cis* regulatory elements. The locations of the basal promoter elements (TATA and CCAAT boxes) and auxin-responsive *cis* elements (AuxRE:TGTCTC and SAUR:CATATG) are shown relative to the T-DNA insertion in the original *gh3.9* mutant (*large triangle*). **c** Regions at the 3' end of the coding sequences of cDNAs corresponding to the closely related Group II *GH3* family members (*GH3.9*, *GH3.17*, *GH3.2/YDK1*, *GH3.3*, *GH3.5/AtGH3a*, and *GH3.6/DFL1*, top to bottom) were aligned by the Clustal method using DNASTar software (Madison, WI). Nucleotides that match the sequence of *GH3.9* are shaded in *black*. Annealing sites for oligonucleotide primers (Ri 50F and Ri 50R) for gene-specific, semi-quantitative RT-PCR and to generate the RNAi construct and Northern blot hybridization probe are indicated by *arrows*.

sion to the *GUS* gene in pCAMBIA3301. The PCR product was ligated into the pGEMTeasy vector (Promega, Madison, WI, USA) and sequenced. Next, the cloned fragment in pGEMTeasy was isolated by restriction enzyme digestion with *Bam*HI and ligated into the *Bam*HI-digested pCAMBIA3301 vector. Correct orientation was verified by restriction enzyme mapping and DNA sequencing. The resulting pCAMBIA3301-*GH3.9*

promoter construct was used to generate transgenic plants (see below).

The pFGC5941-RNAiGH3.9 and pCAMBIA3301-*GH3.9* promoter constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101. Transgenic plants were generated in the Col-0 ecotype by the floral dip method (Clough and Bent 1998). The pFGC5941 and pCAMBIA3301 vectors carry genes that confer re-

sistance to the herbicide Basta™. Therefore, primary transformants were selected by spraying plants with 1:100 dilution of the herbicide Finale™ (AgrEvo Environmental Health, Montvale, NJ, USA). Plants homozygous for the T-DNA insertion were identified in subsequent generations.

The *GH3.9* RNAi lines were used for Northern (RNA) blot analysis and hormone sensitivity root growth assays. The *GH3.9* 'promoter':*GUS* lines were used for determining tissue and developmental expression patterns by histochemical staining using 5-bromo-4-chloro-3-indolyl- β -glucuronic acid (*X*-Gluc) as a substrate (Jefferson 1987).

Semi-quantitative RT-PCR and Northern (RNA) blot analyses

For semi-quantitative RT-PCR to determine whether *GH3.9* is auxin-responsive, total RNA was isolated from 4 to 6 leaf stage Col-0 seedlings treated with IAA as described above using the Qiagen mini RNAeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions, and RNA concentration was determined spectrophotometrically. Reverse transcription was performed as described above except that 2.4 μ g total RNA was used in each reaction. PCR was performed for 30 cycles (94°C 30 s, 58°C 30 s, 72°C 40 s) in a 25 μ l reaction containing 1X Econo Taq PCR buffer, 1.25 U Eppendorf Triple Master mix enzyme (VWR International, West Chester, PA, USA), 50 μ M dNTPs, and 500 nM Ri 50F and Ri 50R oligonucleotide primers. As loading controls, the reverse-transcribed RNA was also subjected to PCR using an 18S primer pair (Ambion, Austin, TX, USA) and primers to *ACTIN2* (At3g18780) *ACT2* F: 5'-GTGCCAATCTACGAGGGTTTC-3' and *ACT2* R: 5'-CAATGGGACTAAACGAAAA-3' for 25 cycles. The RT-PCR reactions (15 μ l) were electrophoretically resolved by agarose gel electrophoresis.

The total RNA blot representing transcripts from different *A. thaliana* plant organs was kindly provided by Steve Pechous (National Center for Biotechnology Information, Bethesda, MD, USA). To determine *GH3.9* expression in different genotypes and RNAi transgenic lines, total RNA was isolated from tissue comprising both mature and immature siliques and some flowers using an SDS-phenol extraction procedure followed by LiCl precipitation essentially as described (Downing et al. 1992). Total RNA (15 μ g) was electrophoresed on a 1.2% formaldehyde-agarose gel and blotted overnight in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) to a Hybond-N mem-

brane (Amersham Biosciences, Piscataway, NJ, USA). Radio-labeled hybridization probe was generated by performing PCR for 20 cycles (95°C 30 s, 50°C 45 s, and 72°C 1 min) in a reaction containing 500 nM dNTPs (minus dCTP), 2.5 mM MgCl₂, 500 nM Ri 50R oligonucleotide primer, and α -[³²P] dCTP. The RNA blot was hybridized in formamide buffer (5X SSPE, 50% formamide, 5X Denhardt's and 1% SDS solution) at 42°C overnight then washed under high stringency conditions in 2X SSC and 1% SDS solution two times for 5 min each at room temperature followed by 85 min at 65°C (Ausubel et al. 2006) and exposed to a phosphor-imager screen (Biorad, Hercules, CA, USA). The blot was stripped in 0.1X SSC and 0.1% SDS and hybridized with a radiolabeled *UBQ5* probe. A 250 bp fragment of *UBQ5* was amplified by PCR using *UBQ5* F: 5'-GTGGTGCTAAGAAGAGGAAGA-3' and *UBQ5* R: 5'-TCAAGCTTCAACTCCTTCTTT-3' oligonucleotide primers and cloned into pGEMTeasy vector (Promega, Madison, WI, USA). This cloned fragment was used as a template to generate radiolabeled probe by PCR as described for the *GH3.9* hybridization probe using the *UBQ5* R oligonucleotide primer.

Results

The GH3 Group II member, GH3.9, does not appear to be induced by exogenous auxin and is developmentally regulated

Inverse PCR was used to identify and activation tagged T-DNA in chromosome 2 in the promoter region of *GH3.9* between the 3'UTR of At2g47760 (*ALG3*-like gene) and the 5'UTR of At2g47750, *GH3.9* (Figure 1a). *GH3.9* belongs to the Group II class of the *GH3* gene family of proteins that are proposed to act as IAA amido synthetases to conjugate auxin to amino acids (Staswick et al. 2002, 2005). As several Group II members have been shown to be "auxin-responsive", the promoter region was searched for hormone-responsive *cis* regulatory elements using the PLACE database (Higo et al. 1999). Figure 1b shows the positions of auxin-response elements (AuxRE), small-auxin upregulated elements (SAUR), CCAAT box motifs and TATA box motifs relative to the T-DNA insertion in *gh3.9-1*. As the *A. thaliana* *GH3* family members share high sequence similarity, a multiple alignment of the Group II member cDNAs was performed to identify the most divergent region to perform *GH3.9*-specific semi-quantitative RT-PCR and generate a *GH3.9*-specific hybridization probe for Northern blot analysis and an RNAi construct (Figure 1c).

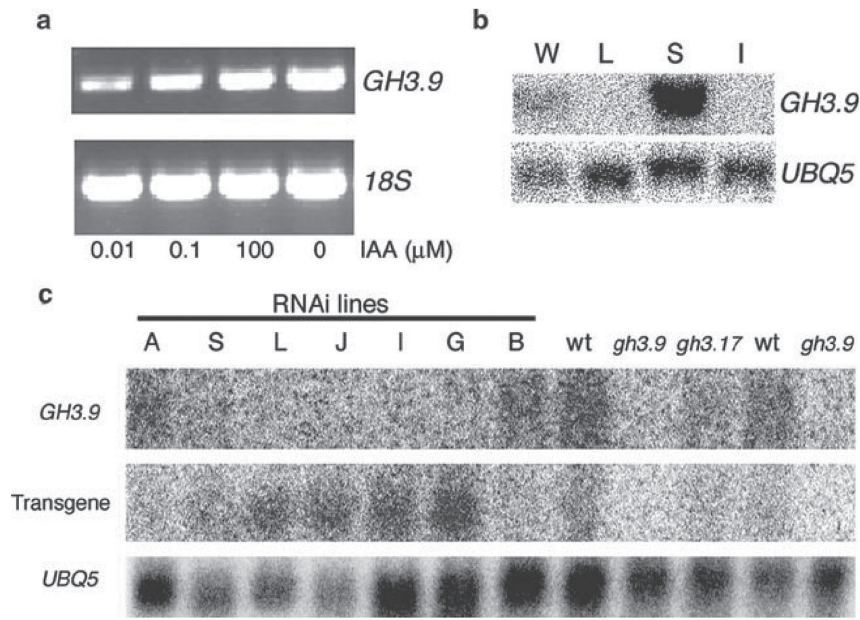


Figure 2. Steady-state *GH3.9* transcript levels are not increased by exogenous auxin in seedlings, are expressed in a tissue-specific manner and are reduced in the *gh3.9* mutant and RNAi gene silencing transgenic lines. **a)** Semi-quantitative RT-PCR was used to determine whether exogenous indole-3-acetic acid (IAA) increased *GH3.9* expression. Seedlings were mock treated (0) or incubated with increasing concentrations of IAA (0.01, 0.1, and 100 μ M). Similar results were obtained in three independent experiments, using *18S* or *ACTIN2* as RNA loading controls. *GH3.9* expression is suppressed at low concentrations of exogenous IAA. **b)** Northern (RNA) blot analysis of *GH3.9* (At2g47750) gene in wild-type Col-0 plants. Total RNA was isolated from whole plant (W), leaves (L), siliques (S), and inflorescences (I). The same 410 bp exon region of *GH3.9* used to generate the RNAi construct was used as a hybridization probe. Expression of the *UBQ5* gene was used as a loading control. *GH3.9* transcript accumulates more in siliques compared to whole plant and is undetectable in leaves or inflorescences. **c)** Northern (RNA) blot analysis of *GH3.9* (At2g47750) in wild-type (*wt*) Col-0, *gh3.9*, *gh3.17*, and transgenic RNAi lines A, S, L, J, I, G, and B. Total RNA was isolated from 1 g silique when siliques were largely mature. Fifteen micrograms (15 μ g) of total RNA was loaded in each lane. *GH3.9* transcript is not detectable or reduced in the *gh3.9* mutant and moderately expressed in the *gh3.17* mutant compared to wild-type Col-0. T3 transgenic RNAi lines have variable levels of suppression of *GH3.9*, which is inversely correlated with the expression of the transgene. *GH3.9* transcript level in *gh3.17* is very similar to Col-0. Detection of *UBQ5* transcript was used as a control.

We examined the expression of *GH3.9* in *A. thaliana* tissues by semi-quantitative RT-PCR and Northern blot analyses. *GH3.9* expression was undetectable by Northern blot analysis of mature leaf tissue (Figure 2b and data not shown). However, RT-PCR was sufficiently sensitive to detect *GH3.9* in seedlings. The RT-PCR should detect only *GH3.9* and not other Group II-family members based on the specificity of the oligonucleotide primers used (Figure 1c). To determine whether *GH3.9* is indeed an auxin-responsive gene, seedlings were treated with various concentrations of exogenous IAA. Auxin treatment failed to enhance *GH3.9* expression and actually somewhat repressed expression (~70% of untreated as determined by semi-quantitative RT-PCR) at low IAA concentrations (Figures 2a, 3e, f). Our data is consistent with publicly available microarray expression data suggesting that *GH3.9* is repressed by exogenous IAA application at low concentrations and unaffected at high concentrations (Zimmermann et al. 2004).

To learn whether *GH3.9* is expressed in a developmental or tissue-specific manner, Northern RNA blot analysis was performed on total RNA isolated from different tissues of wild-type Col-0 including leaves, flowers, siliques, and whole plants. We observed greater expression of *GH3.9* in siliques compared to the whole plant, whereas no detectable expression was observed in leaves or flowers (Figure 2b). These results are consistent with publicly available microarray data (Zimmermann et al. 2004; Toufighi et al. 2005).

The steady-state level of *GH3.9* transcripts is reduced in the *gh3.9-1* mutant and lines where *GH3.9* was targeted for silencing by RNAi. *GH3.9* expression is reduced to ~50% of the level in wild type in the *gh3.9-1* mutant. We observed a range of suppression in the RNAi lines (~60–90% of wild-type levels). In addition, we performed semi-quantitative RT-PCR several times with similar results (data not shown). Although the signal for *GH3.9* is very low (particularly as compared to that for *UBQ5*), we chose to show a Northern blot, be-

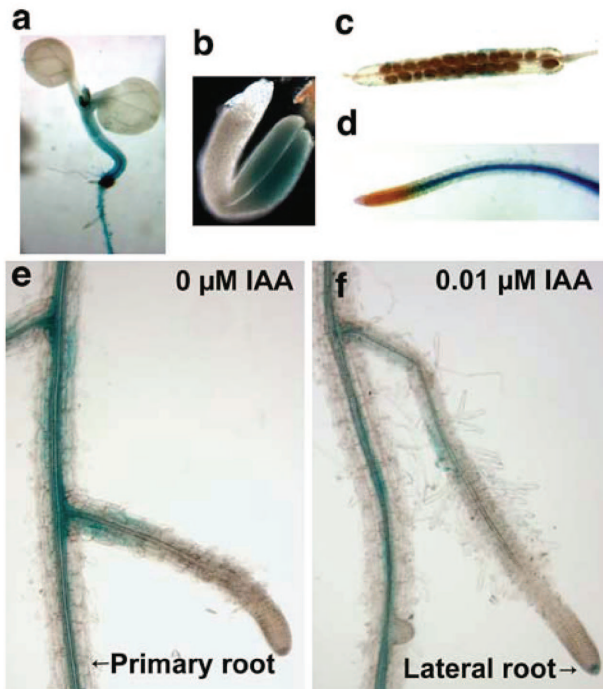


Figure 3. *GH3.9* promoter::*GUS* transgenic plants indicate that *GH3.9* is expressed in tissue-specific and developmentally regulated manners. Representative examples of histochemical detection of GUS activity in (a) a young seedling, (b) a mature embryo, (c) a mature silique, (d) a primary root, (e and f) primary and lateral roots untreated or treated with 0.01 μM IAA, respectively.

cause the correlation between transgene expression and *GH3.9* suppression is evident. RNAi lines G, J, L, and S showed significantly reduced *GH3.9* expression compared to RNAi lines A and B that was inversely correlated with transgene expression levels (Figure 2c). The level of suppression generally correlated with the strength of the long-root phenotype. For example, we saw the least suppression in *GH3.9* RNAi line B, and this line had the shortest roots (closer to wild type) in all experiments (Figures 4, 6a). Because *GH3.17* is the closest gene family member to *GH3.9*, we also analyzed the expression of *GH3.9* in the mutant *gh3.17-1*. In the mutant *gh3.17-1*, *GH3.9* transcript accumulated to similar levels to wild-type Col-0 (Figure 2c).

Further support for tissue specificity and developmental control of *GH3.9* expression comes from histochemical staining of *GH3.9* promoter::*GUS* transgenic plants. Strong GUS expression at the root-hypocotyl junction, young leaves and the shoot apical meristem, but only weak expression in cotyledons of young seedlings were observed (Figure 3a). The strong silique *GH3.9* expression seen by Northern blot (Figure 2b) can primarily be attributed to weak, but detectable, GUS expression in the outer wall of the fruit bodies or siliques and significant expression in mature embryos prior to

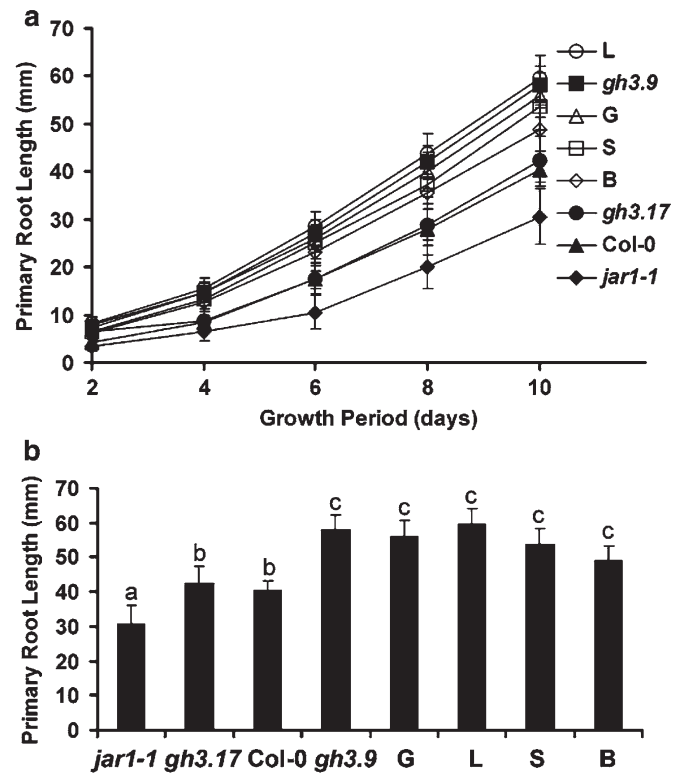


Figure 4. The *gh3.9-1* mutant and *GH3.9* RNAi lines display long primary roots compared to wild-type Col-0 and the *gh3.17-1* and *jar1-1* mutants when grown vertically on Murashige-Skoog (MS) media. **a**) A line graph depicting primary root lengths determined over a period of 10 days after seed germination. Homozygous T3 generations of transgenic RNAi lines were analyzed, and data is combined from three independent experiments. Error bars represent 95% confidence levels. **b**) A bar graph depicts the primary root lengths for the analyzed genotypes at the end of the experiment (day 10). Error bars represent 95% confidence levels. Significant differences between genotypes, determined by two-sample equal variance one-tailed Student's *t*-tests, are indicated by different alphanumeric symbols ($\alpha = 0.05$). Different letters represent significant differences.

germination (Figure 3b, c). In roots, the promoter was active in the vascular tissue of the root elongation zone (Figure 3d). Exogenous IAA treatment caused reduced promoter activity in the vascular tissue (consistent with the RT-PCR results, Figures 2a, 3e, f). Interestingly, IAA treatment triggered lateral root tip expression (Figure 3e, f), with only 13% of untreated lateral roots ($n = 131$) and 57% of treated lateral roots ($n = 76$) showing any expression at the tip. We failed to detect any changes in either the levels or the distribution of GUS activity in response to exogenous 10 μM MeJA treatment (data not shown). Therefore *GH3.9* expression is developmentally regulated. Moreover, *GH3.9* appears to respond to exogenous auxin, but by repression rather than induction, and a change in the tissue localization in lateral roots.

GH3.9 affects primary root growth and GH3.9 gene expression correlates with a long primary root phenotype

We observed consistently longer primary roots in *gh3.9-1* seedlings compared to wild-type Col-0 seedlings (Figure 4). Because the T-DNA insertion in *gh3.9-1* is derived from the activation tagging vector pSKI015, we expected that the long-root phenotype might be dominant gain-of-function (Weigel et al. 2000). Therefore, the *gh3.9-1* mutant was backcrossed to wild-type Col-0, and the primary root lengths were analyzed for two independent F2 populations. The wild-type (short) and mutant (long) root phenotypes were distinguished by the value of the longest Col-0 root. The long-root phenotype segregated in a 3:1 (short root: long root) ratio ($\chi^2 = 0.10$, $n = 118$), as would be expected if the long-root phenotype were conferred by a single recessive mutation, rather than by a gain-of-function mutation. Furthermore, the *gh3.9-1* mutant showed a reduction in the *GH3.9* transcript, rather than an increase in RNA levels (Figure 2c), thereby confirming the notion that the T-DNA causes a loss-of-function mutation. PCR-based genotyping was consistent with this finding (data not shown). As further verification that the long-root phenotype was due to *GH3.9* disruption, additional homozygous T-DNA insertion alleles were tested. SALK_005057 and SALK_005065 have pROK2-derived T-DNA sequences (Alonso et al. 2003) inserted 302 and 415 bp, respectively, removed from the site of the pSKI015-derived T-DNA insertion in *gh3.9-1* and WiscDsLox419D07 has pDS-LOX-derived T-DNA sequences (Medberry et al. 1995) in exon 3 of the coding region of *GH3.9* (Figure 1a). Homozygous insertion mutants for two of these alleles also exhibited the long-root phenotype (Table 1), and the heterozygous WiscDsLox line segregated for the long-root phenotype (data not shown). Moreover, *GH3.9* RNAi lines also had longer primary roots compared to wild-type Col-0 seedlings. Mutation of the Group II *GH3* gene family member most closely related to *GH3.9*, *GH3.17*, had no significant effect on primary root length, while mutation of a Group I *GH3* gene family member, *GH3.11* or *JAR1*, resulted in shorter primary roots than Col-0, *gh3.9-1*, *gh3.17-1*, and *GH3.9* RNAi lines (Figure 4). These results indicate that *GH3.9* influences primary root length, and that the long-root phenotype correlated with reduced *GH3.9* expression in *gh3.9-1* and *GH3.9* RNAi lines. Rates of root elongation in the *gh3.9-1* mutant and *GH3.9* RNAi lines were similar to wild-type seedlings in the early stages of root growth and became accelerated with time (Figure 4a, Table 2). No significant difference in lateral root numbers were observed for the *gh3.9-1* mutant and wild type.

Table 1. Primary root length determined for *GH3.9*-related T-DNA insertion lines

Genotypes	No. seedlings ^a	Root length (mm) ^b
Col-0	16	50.9 (7.9) ^{1,3}
<i>gh3.9-1</i>	18	59.4 (8.5) ²
SALK_005065	17	65.4 (9.0) ²
SALK_005057	16	59.0 (9.6) ^{2,3}

^a The number of seedlings tested per genotype

^b The mean primary root length determined 10 days after seed germination and error at 95% confidence intervals (in parentheses) are shown. Significant differences between genotypes, determined by paired, one-tailed Student's *t*-tests are indicated by different superscripted numbers ($\alpha = 0.1$)

Table 2. Primary root growth rates for various genotypes

Genotype	Root growth rate (mm day ⁻¹) ^a		
	Days 4–6	Days 6–8	Days 8–10
Col-0	4.45	5.22	6.27
<i>gh3.9-1</i>	6.14	7.49	8.00
RNAi-L	6.46	7.64	7.86
RNAi-G	5.58	7.09	7.95
RNAi-S	5.93	6.17	8.10
RNAi-B	5.15	6.19	6.67
<i>gh3.17-1</i>	4.38	5.57	6.78
<i>jar1-1</i>	2.04	4.72	5.30

^a Primary root lengths were determined over a 10-day period after seed germination. The average root growth rate (mm day⁻¹) is shown for various time intervals

GH3.9 has moderate influence on sensitivity to jasmonate-mediated and auxin-mediated root growth inhibition

Several *A. thaliana* *GH3* family member mutants have observable phenotypes, including altered sensitivity to plant hormones such as IAA and MeJA (Staswick et al. 1992, 2005; Tiryaki and Staswick 2002; Staswick and Tiryaki 2004). Therefore *gh3.9-1* and the *GH3.9* RNAi lines were tested for sensitivity to MeJA- and IAA-mediated root growth inhibition. The concentrations used in the experiments shown were based on these standardization experiments, information for *GH3.9* expression available from the Genevestigator microarray expression database, and published literature characterizing the other gene family members. The data is shown for concentrations that gave reproducible results and are consistent with those used by other researchers (Staswick et al. 1992, 2005; Tiryaki and Staswick 2002; Staswick and Tiryaki 2004). The MeJA-insensitive *jar1-1* mutant and

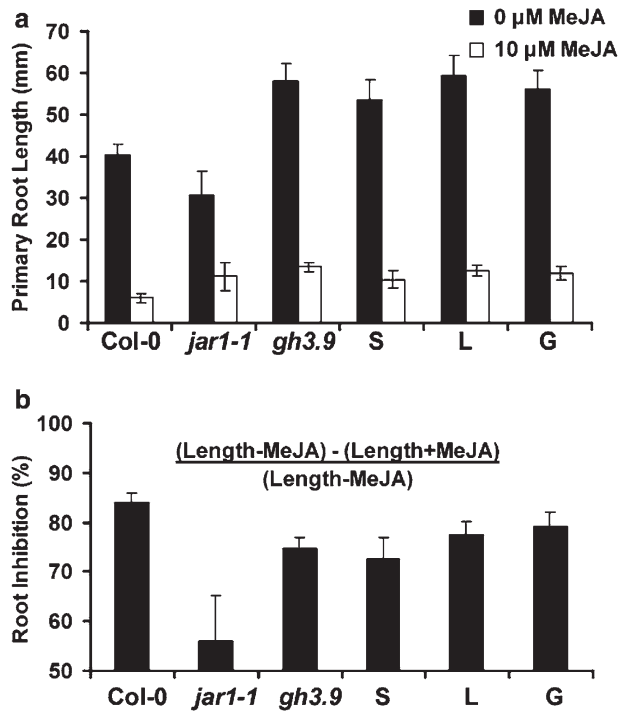


Figure 5. The *gh3.9-1* mutant and the *GH3.9* RNAi transgenic lines are moderately resistant to root growth inhibition by methyl jasmonate (*MeJA*). **a)** The mean root length at 10 days after seed germination is indicated for seedlings grown in the absence of exogenous *MeJA* (black bars) and the presence of 10 μ M *MeJA* (white bars). Error bars represent 95% confidence levels. The *jar1-1* mutant was used as a control for *MeJA* resistance. **b)** The *MeJA*-induced root inhibition (%) was calculated as the difference in root length in the presence and absence of *MeJA* divided by the root length in the absence of *MeJA*; $[(\text{length} - \text{MeJA}) - (\text{length} + \text{MeJA})] / (\text{length} - \text{MeJA})$. Data represent 14–20 individual seedlings of each genotype.

the auxin-sensitive *gh3.17-1* were used as controls. In the presence of 10 μ M *MeJA* and 5 μ M IAA, primary root growth of wild-type Col-0 seedlings was dramatically inhibited (Figures 5a, 6a). Root growth inhibition (percent) was calculated to compensate for the fact that *gh3.9-1* and the *GH3.9* RNAi lines had long primary roots on un-supplemented media (Figures 4, 5a, 6a).

The *gh3.9-1* mutant and the *GH3.9* RNAi lines were moderately resistant to *MeJA*, as they differ from the highly sensitive Col-0 and the resistant *jar1-1* mutant (Figure 5b). The *gh3.9-1* mutant and the *GH3.9* RNAi lines were moderately sensitive to IAA, as they displayed greater sensitivity than wild-type Col-0, but less sensitivity than *gh3.17-1* (Figure 6b).

Discussion

Auxin synthesis, degradation, conjugation, and transport control free auxin levels and distribution

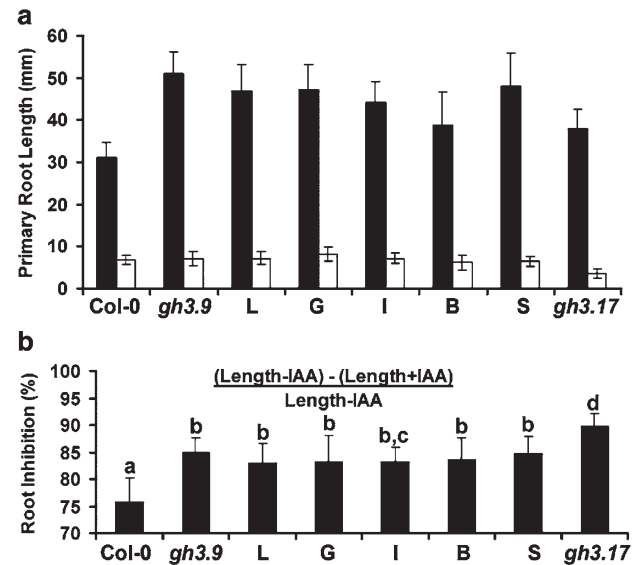


Figure 6. The *gh3.9-1* mutant and the *GH3.9* RNAi transgenic lines are moderately sensitive to root growth inhibition by IAA. **a)** The mean root length at 10 days after seed germination is indicated for seedlings grown in the absence of exogenous IAA (black bars) and the presence of 5 μ M IAA (white bars). Error bars represent 95% confidence levels. The *gh3.17-1* mutant was used as a control for IAA sensitivity. **b)** The IAA-induced root growth inhibition (%) was calculated as the difference in root length in the presence and absence of IAA divided by the root length in the absence of IAA; $[(\text{length} - \text{IAA}) - (\text{length} + \text{IAA})] / (\text{length} - \text{IAA})$. Error bars represent 95% confidence levels. Significant differences between genotypes, determined by two-sample equal variance one-tailed Student's *t*-tests, are indicated by different alphanumeric symbols ($\alpha = 0.05$). Different letters represent significant differences. Data represent two independent experiments with 15–30 individual seedlings of each genotype.

within plant tissues to establish basic plant architecture by regulating cell division, cell fate, and pattern formation. Recent insights into the molecular mechanisms regulating auxin transport were provided by studies using auxin transport inhibitors, transport mutants, and reporter genes. These studies established a key role for the auxin-inducible auxin efflux carrier proteins (PINs) and *PIN* gene transcription regulators (PLTs) in controlling auxin flux to maintain an auxin maximum at the root tip (Sabatini et al. 1999; Friml et al. 2002, 2003; Aida et al. 2004; Blilou et al. 2005). Much still remains to be uncovered regarding other aspects of auxin physiology including synthesis, degradation, and conjugation.

GH3.9 (At2g47750) belongs to the 20-member *A. thaliana* *GH3* gene family. Some *GH3* family members have been shown to encode proteins that can adenylate and conjugate plant hormones to amino acids in vitro (Staswick et al. 2002, 2005; Staswick and Tiriyaki 2004). One of the two Group I *GH3* gene family members, *JAR1*/

FIN219/GH3.11, functions as a JA (jasmonic acid)-amido synthetase to activate JA (Staswick and Tiriyaki 2004), and all eight Group II-family members with the exception of *GH3.9* (due to low recombinant GST protein levels) can function as indole-3-acetic acid (IAA)-amido synthetases in vitro (Staswick et al. 2005). A similar function has been proposed for *GH3.9*, because it is capable of adenylating auxin in vitro (Staswick et al. 2002). In contrast to MeJA, which is activated by amino acid conjugation, amino acid-conjugated forms of IAA appear to be inactive. Consistent with the hypothesis of Staswick et al. that Group II *GH3* members conjugate IAA to amino acids to reduce the availability of free (active) auxin, reduction-of-function mutations in some of these genes result in increased sensitivity to IAA-mediated root growth inhibition (Staswick et al. 2005), and a gain-of-function mutation in one of these genes confers resistance to IAA-mediated root growth inhibition (Nakazawa et al. 2001).

We discovered the *gh3.9-1* mutant in a screen of activation tagged populations for fumonisin B1-resistant (*fbr*) mutants (Stone et al. 2000). The original mutant had both the *fbr* phenotype and long primary roots. Individual F2 progeny from a backcross of *gh3.9-1* to wild type were identified that retained the *fbr* phenotype, but lacked the T-DNA insertion. Therefore, we concluded that the *fbr* phenotype was unlinked to the T-DNA insertion, but the long-root phenotype was linked and segregated as a single recessive trait. Contrary to our expectation that the adjacent pSKI015-derived activation tagging T-DNA would bestow a dominant phenotype, the *gh3.9-1* mutant identified in our study appears to be a loss-of- or reduction-of-function allele, unlike the dominant activation tagged mutations identified for the *GH3* Group II family members *YDK1* (*GH3.2*) and *DFL1* (*GH3.6*). *GH3.9*-related T-DNA insertion alleles and RNAi lines with reduced *GH3.9* expression also displayed the long-root phenotype. Lateral root numbers were unaffected in the *gh3.9-1* mutant, consistent with published reports for mutations in other closely related gene family members (Staswick et al. 2005).

GH3 family members share high sequence similarity and may have undetectable or subtle phenotypes when mutated due to functional redundancy. However, some *GH3* genes have been uncovered in mutant screens. For example, mutations in the Group I *GH3.11/JAR1/FIN219* gene, which encodes a JA-amido synthetase, are pleiotropic, although there is allelic variability. *GH3.11* mutations confer resistance to MeJA-mediated root growth inhibition, suppress the constitutive photomorphogenesis phenotype of a *cop1* mutant, and display a long-hypocotyl phenotype in far-red light (Staswick et al. 1992, 2002; Hsieh et al. 2000).

An activation tagged dominant mutation in Group II *YDK1/GH3.2* identified in a mutant screen for altered hypocotyl elongation also had short primary roots and reduced apical dominance, but no obvious morphological phenotypes were observed in the presumed knockout mutant (Takase et al. 2004). More comprehensive investigation, however, revealed the *gh3.2-1* mutant to be moderately sensitive to auxin-mediated root growth inhibition (Staswick et al. 2005). Likewise, *gh3.1-1*, *gh3.17-1*, and *gh3.5-1* mutants had normal plant architecture, wild-type root growth rates, but showed increased IAA sensitivity (Staswick et al. 2005). Therefore, the *gh3.9-1* mutant shares the normal leaf, shoot, or flower morphology and IAA sensitivity phenotype with several Group II loss-of-function mutants (Figure 5). In contrast, the homozygous *gh3.9-1* T-DNA insertion line and the *GH3.9* RNAi lines with reduced *GH3.9* expression had a long-root phenotype (Figure 4), which was not seen in *gh3.1-1*, *gh3.2-1*, *gh3.17-1*, and *gh3.5-1* loss-of-function mutants, nor the gain-of-function *df1-D* mutant (Nakazawa et al. 2001; Staswick et al. 2005). The failure to observe a root length phenotype in these mutants suggests that *GH3.9* serves a distinct function in roots. The opposite root length phenotypes in the dominant *ydk1-D* and the recessive *gh3.9-1* suggest that these two *GH3* genes may have overlapping functions or expression patterns.

In primary literature and publicly available microarray gene expression databases, six of the eight Group II *GH3* genes, *GH3.1*, *YDK1* (*GH3.2*), *GH3.3*, *GH3.4*, *GH3.5* (*AtGH3a*), and *DFL1* (*GH3.6*), were reported to be induced by exogenous (and presumably endogenous) auxin (Nakazawa et al. 2001; Tanaka et al. 2002; Goda et al. 2004; Takase et al. 2004; Staswick et al. 2005). However, the two closely related Group II *GH3* genes, *GH3.9*, and *GH3.17*, do not appear to be auxin responsive (Staswick et al. 2005). We investigated whether steady-state *GH3.9* transcript levels were altered in seedlings treated with exogenous IAA and found that *GH3.9* was downregulated by exposure to low, physiological concentrations of IAA (Figure 2a) (Ostin et al. 1998). Our results are consistent with publicly available microarray gene expression data indicating that *GH3.9* is repressed by exogenous IAA (Genevestigator; <http://www.genevestigator.ethz.ch/>). Moreover, phylogenetic analyses indicate that *AtGH3.9* and rice *OsGH3.11* are orthologues, and *OsGH3.11* expression was also unaffected or repressed by exogenous auxin (Jain et al. 2006; Terol et al. 2006).

Repression by low auxin concentrations was somewhat surprising, because the *GH3.9* upstream region contains *cis* regulatory elements that confer auxin responsiveness: the auxin-response element (AuxRE: TGTCTC) and SAUR element (CATATG) (Figure 1b).

However, AuxREs function in auxin response when they are adjacent to a constitutive or coupling element to create composite AuxRE elements (Guilfoyle et al. 1998), and similar sequences were not detected in the *GH3.9* promoter region. ARFs (auxin-response factors) bound to AuxREs interact with Aux/IAA repressor proteins to keep auxin-responsive genes silenced when auxin concentrations are low (Tian et al. 2004; Tiwari et al. 2004). Some of these ARFs have been shown to be posttranscriptionally cleaved by microRNAs to release repression of certain *GH3* genes (Mallory et al. 2005). The ARFs that might bind to the *GH3.9* promoter are not known. Perhaps, they are not targets of these miRNAs, or the miRNAs that target them do not accumulate in response to auxin.

Some evidence exists that Group I *GH3.11/JAR1/FIN219* (which functions in JA conjugation) is induced by auxin (Hsieh et al. 2000). Cross talk between the signal transduction pathways regulated by various plant hormones, such as auxins and jasmonates, is further exemplified by the identification of an *auxin resistance 1 (axr1)* allele in a mutant screen for MeJA resistance (Tiryaki and Staswick 2002). Because we observed short roots in the MeJA-resistant *jar1-1* mutant (Figure 4), we also tested *gh3.9-1* for sensitivity to MeJA. We found that *gh3.9-1* and the *GH3.9* RNAi lines displayed moderate resistance to MeJA compared to the control *jar1-1* (Figure 5). Because several *GH3* genes are apparently repressed by MeJA (Genevestigator; <http://www.genevestigator.ethz.ch/>), it would be interesting to determine whether other loss-of-function mutants have jasmonate-related phenotypes.

The lack of observable phenotype in tissues other than roots is likely due to tissue- and development-specific expression of *GH3.9* and other Group II family members. *YDK1/GH3.2* that influences both hypocotyl elongation and root development is expressed primarily in flowers and roots (Takase et al. 2004). Whereas the Northern blot was informative (Figure 2b), the *GH3.9* promoter::GUS reporter construct provides a more detailed picture. For example, the dominant expression in siliques can primarily be attributed to expression in the mature embryos (Figure 3b, c). The relatively low expression in other tissues is due to very specific tissue localization.

Relating the observed expression patterns with respect to auxin and root physiology offers some clues as to the function of *GH3.9* and the long-root phenotype. Free auxin levels are typically monitored by reporter genes driven by multimerized auxin-responsive elements (DR5::GUS or DR5::GFP). The *GH3.9* promoter::GUS expression patterns tend to be oppo-

site of those observed with these reporters. For example, high free auxin levels are found at the tips of cotyledons and roots of mature embryos, the base of trichomes, vasculature and hydathodes of leaves, the primary root apex and emerging lateral roots (Sabatini et al. 1999; Friml et al. 2002, 2003; Aloni et al. 2003; Ottenschlager et al. 2003; Lin and Wang 2005). Our *GH3.9* promoter::GUS expression is most evident in areas with low free auxin, which might be explained by a combination of factors—the repression of *GH3.9* by physiological concentrations of auxin and the presumed function of the protein in conjugating free auxin. *GH3.9* promoter-driven expression was present in the proximal region of the root where cell elongation and expansion takes place, but noticeably absent in the root apex where active cell division occurs, free auxin is maximum, and auxin-inducible genes predominate (Figure 3d). This pattern of expression in roots is consistent with the pattern of auxin-related (not necessarily auxin-responsive) gene transcript accumulation determined by microarray-based transcriptome analyzes (Birnbaum et al. 2003). Root elongation rates in the *gh3.9-1* mutant and RNAi lines are similar to wild-type seedlings in the early stages of root growth and become accelerated with time (Figure 4a, Table 2). These results are in agreement with *GH3.9* functioning in cells undergoing elongation, where the *GH3.9* promoter is active, as opposed to in cells that are actively dividing. Exogenous IAA repressed *GH3.9* expression in the cell elongation and expansion zone and triggered lateral root tip expression, a major perturbation in root auxin homeostasis in lateral roots. Perhaps, the efflux proteins are redistributing auxin in an attempt to maintain appropriate levels at the root tip.

While much has been learned about *GH3* gene family function since the discovery of the enzymatic activity associated with these proteins, much remains to be learned regarding the individual roles of these proteins in plant development. For example, the phenotypic variability observed in loss-of-function mutants suggests that we still need to explore how each gene is connected to other hormone pathways involved in plant developmental processes.

It is intriguing that *GH3.9* is not induced by auxin like other family members, and loss-of-function mutants exhibit only moderate IAA sensitivity. The long primary root phenotype and the moderate MeJA resistance have not been reported for mutations in any other family members. This indicates that whereas *GH3.9* shares some of the features of its family members and might be acting in pathways common to other *GH3* genes, it clearly has a divergent role in root development.

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