The Plant Journal (2007) **50**, 858–872 doi: 10.1111/j.1365-313X.2007.03096.x

Arabidopsis Ovate Family Protein 1 is a transcriptional repressor that suppresses cell elongation

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Received 28 January 2007; accepted 9 February 2007.
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Summary

Transcription factors regulate multiple aspects of plant growth and development. Here we report the identification and functional analysis of a plant-specific, novel transcription factor in Arabidopsis. We isolated a dominant, gain-of-function mutant that displays reduced lengths in all aerial organs including hypocotyl, rosette leaf, cauline leaf, inflorescence stem, floral organs and silique. Molecular cloning revealed that these phenotypes are caused by elevated expression of the *Arabidopsis thaliana Ovate Family Protein 1* (*AtOFP1*). This mutant was designated as *Atofp1-1D*. We show that the altered morphology of *Atofp1-1D* mutant is caused by reduced cell length resulting from reduced cell elongation, and demonstrate that a mutant harboring a transposon insertion that disrupts the OVATE domain of AtOFP1 is indistinguishable from wild-type plants. Plants overexpressing other closely related *AtOFP* genes phenocopy plants overexpressing *AtOFP1*, implying a possible overlapping function among members of the *AtOFP* gene family. We found that AtOFP1 localizes in the nucleus, and that AtOFP1 functions as an active transcriptional repressor. Chromatin immunoprecipitation results indicated that *AtGA20ox1*, a gene encoding the key enzyme in GA biosynthesis, is a target gene regulated by AtOFP1. Consistent with this, exogenous gibberellic acid can partially restore defects in cell elongation in plants overexpressing *AtOFP1*, suggesting that such a reduced cell elongation is caused, in part, by the deficiency in gibberellin biosynthesis. Taken together, our results indicate that AtOFP1 is an active transcriptional repressor that has a role in regulating cell elongation in plants.

Keywords: AtOFP1, transcription factor, cell elongation, OVATE, gibberellin, Arabidopsis.

Introduction

Transcription factors regulate a wide variety of plant growth and developmental processes, including meristem function and maintenance, lateral organ specification, and leaf development and branching. Most, if not all, of these growth and developmental processes are thought to be due to up- or downregulation of specific gene(s) with cell/tissue/organ specificity by transcription factors.

Two of the best -characterized transcription factor families involved in plant growth and development are the auxin response factor (ARF) and Aux/IAA protein families. There are 22 genes in Arabidopsis that encode full-length ARF proteins (Guilfoyle and Hagen, 2001; Liscum and Reed, 2002; Remington *et al.*, 2004), and 29 genes in Arabidopsis encoding Aux/IAA proteins (Abel *et al.*, 1994; Liscum and Reed, 2002; Reed, 2001). In protoplast transfection assays, most of the ARF proteins have been shown to either activate or repress expression of auxin response genes containing TGTCTC auxin response elements (AuxREs) in their promoters (Tiwari *et al.*, 2003; Ulmasov *et al.*, 1997a, 1999a,b; Wang *et al.*, 2005). Most Aux/IAA proteins are thought to repress the expression of auxin response genes by forming heterodimic complexes with ARFs (Tiwari *et al.*, 2003; Ulmasov *et al.*, 1997a, 1999a). By analysis of loss-of-function and gain-of-function mutants, it has been demonstrated that the ARF and Aux/IAA families are involved in almost all aspects of plant growth and development, namely embryo patterning, vascular tissue formation, hypocotyl and root growth, lateral root and root hair formation, floral organ number and fertilization (Hardtke and Berleth, 1998; Hardtke *et al.*, 2004; Li *et al.*, 2004; Mattsson *et al.*, 2003; Okushama *et al.*, 2005; Sessions *et al.*, 1997; Tian *et al.*, 2004; Wilmoth *et al.*, 2005).

The formation and patterning of trichomes and root hair cells are thought to be regulated mainly by family of transcription factors that are different from ARFs and Aux/

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IAAs (reviewed by Schiefelbein, 2003). Transcription factors involved in these two processes include a WD-repeat protein, TRANSPARENT TESTA GLABRA (TTG; Galway *et al.*, 1994; Walker *et al.*, 1999), R2R3 MYB-type transcription factors GLABRA1 (GL1) (Oppenheimer *et al.*, 1991) and WEREWOLF (WER) (Lee and Schiefelbein, 1999), bHLH transcription factors GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3) (Payne *et al.*, 2000; Walker *et al.*, 1999; Zhang *et al.*, 2003), several small MYB proteins, TRYPTICHON (TRY), CAPRICE (CPC) and ENHANCER OF TRY AND CPC1 and 2 (ETC1 and ETC2; Kirik *et al.*, 2004a,b; Schellmann *et al.*, 2002; Wada *et al.*, 1997, 2002), and a homeodomain protein, GLABRA2 (GL2) (Masucci *et al.*, 1996; Rerie *et al.*, 1994). These transcription factors have opposite roles in the specification of trichomes and root hair cells. A R2R3 MYB-type transcription factor (WER or GL1), a bHLH transcription factor (GL3, EGL3 or an unknown bHLH protein) and a TTG form a complex to induce *GL2* expression which inhibits the formation of a root hair but promotes the formation of trichomes (reviewed by Schiefelbein, 2003). The small MYB transcription factors TRY, CPC, ETC1 or ETC2 could exert a lateral inhibitory effect on a neighboring cell (Hülskamp *et al.*, 1994; Kirik *et al.*, 2004a,b; Wada *et al.*, 1997).

By using a comprehensive list of proteins, domains and motifs to query the Arabidopsis sequence database, Riechmann *et al.* (2000) performed a genome-wide transcription factor analysis, and found that at least 1533 genes (approximately 5.9% of the total genes) from more than 30 families in Arabidopsis encode transcriptional regulators. Among them, many transcription factor families are found only in plants (Riechmann *et al.*, 2000). Yet transcription factors that do not share conserved domains or motifs may remain unresolved. For example, BES1 has been proved to be a plant-specific transcription factor that involves in brassinosteroid signaling, but it does not belong to any known transcription factor family (Yin *et al.*, 2005).

We report here the identification and characterization of another novel plant-specific transcription factor AtOFP1, a member of the *Arabidopsis thaliana* ovate family proteins (AtOFPs; Hackbusch *et al.*, 2005). The *OVATE* gene was first identified in tomato, in which a mutation led to a premature stop codon in the *OVATE* gene (Liu *et al.*, 2002) causing the transition of tomato fruit from round to pear-shaped. The tomato OVATE protein contains a C-terminal domain of approximately 70 amino acids, designated the OVATE domain, which is present exclusively in plant proteins. The OVATE domain was also known as the DUF623 domain (domain-of-unknown-function 623). There are 18 genes in the Arabidopsis genome that encode proteins with a predicted OVATE domain. However, little is known about the function of OVATE-containing proteins in plants, and their molecular mechanisms of action are unknown. Here we defined the OVATE-containing protein AtOFP1 as an active transcriptional repressor. We provide evidence that AtOFP1 has a role in regulating cell elongation by controlling the expression of *AtGA20ox1*, a gene that encodes a key enzyme in gibberellin biosynthesis.

Results

Isolation and phenotypic analysis of the Atofp1-1D *mutant*

A mutant with reduced hypocotyl length was identified by screening of an activation-tagged mutagenized population in Arabidopsis (Figure 1a,b). The population was generated in the *agb1-2* mutant background, a null allele of the Arabidopsis heterotrimeric G-protein β subunit (*AGB1;* Lease *et al.*, 2001; Ullah *et al.*, 2003), by transforming *agb1-2* mutants with the pSKI015 vector that contains four outward-facing 35S enhancers adjacent to the right border (Weigel *et al.*, 2000). After molecular cloning, we found that the *Arabidopsis thaliana Ovate Family Protein 1* (*AtOFP1*) was tagged (see below, Figure 2), thus this mutant, which is in the *agb1-2* background, was named *Atofp1-1D* (Figure 1).

Examination under the microscope revealed that the reduced hypocotyl length of the mutant was caused by reduced cell lengths (Figure 1c), indicating that cell elongation is affected in *Atofp1-1D* plants. Compared with wild-type or the parental *agb1-2* plants, *Atofp1-1D* plants also had reduced lengths of other aerial organs including cotyledons (Figure 1d), leaves (Figure 1e), floral organs (Figure 1f) and siliques (Figure 1g), and a reduced size of rosettes (Figure 1h). These results suggest that AtOFP1 has a role in regulating cell elongation.

Molecular cloning of AtOFP1

Co-segregation of the *Atofp1-1D* phenotype with BASTA resistance conferred by the pSKI015 vector indicated that there is only a single locus of T-DNA activation tagging insertion in the mutant. To identify the site of T-DNA insertion in the *Atofp1-1D* mutant, we used a plasmid rescue procedure (Weigel *et al.*, 2000). As shown in Figure 2(a), in *Atofp1-1D* mutants, the T-DNA was inserted in chromosome 5 at a position that is 2981 bp upstream of the start codon of gene locus At5g01850, and 4332 bp downstream of the stop codon of gene locus At5g01840. RT-PCR results indicated that the transcript level of gene locus At5g01840 (*AtOFP1*), but not of gene locus At5g01850, was elevated (Figure 2b). Quantitative real-time PCR revealed that the transcript level of gene locus At5g01840 increased approximately 16-fold in the *Atofp1-1D* mutant compared with wild-type Col-0 or the *agb1-2* mutant. These results indicate that the gene locus tagged by the T-DNA activation tagging insertion is most likely At5g01840.

A BLAST search indicated that At5g01840 is the gene locus for *AtOFP1*. AtOFP1 encodes a protein with a putative

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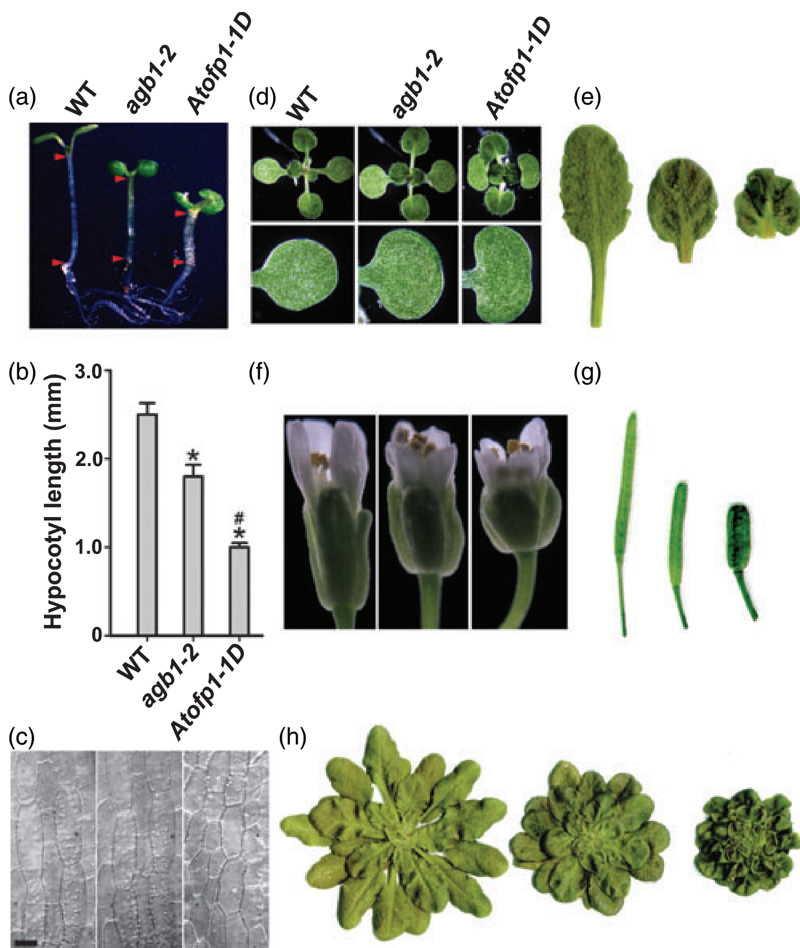

Figure 1. Phenotypes of *Atofp1-1D* mutants.
(a) Four-day-old seedlings of wild-type (WT, Col-0), *agb1-2* and *Atofp1-1D* mutants grown on MS/G plates under a 14/10 h (light/dark) photoperiod at relatively low intensity of light (60 μmol m^{-2} sec^{-1}) for easy visualization and quantification of the length of hypocotyls. Arrowheads indicate the top and base of hypocotyls.
(b) Hypocotyl lengths of 4-day-old wild-type (WT), *agb1-2* and *Atofp1-1D* seedlings. *Significantly different, $P < 0.05$, in comparison with WT. #Significantly different, $P < 0.05$, in comparison with *agb1-2* mutants.
(c) Hypocotyl epidermal cells in the wild-type (left), *agb1-2* (middle) and *Atofp1-1D* (right) seedlings shown in (a). Scale bar = 20 μm.
(d) Fourteen-day-old seedlings of wild-type (WT), *agb1-2* and *Atofp1-1D* seedlings grown under a 14/10 h (light/dark) photoperiod. Top: top view of the seedlings. Bottom: close-up view of cotyledons.
(e) Fully developed rosette leaves of wild-type (left), *agb1-2* (middle) and *Atofp1-1D* (right) plants.
(f) Floral organs of wild-type (left), *agb1-2* (middle) and *Atofp1-1D* (right) plants.
(g) Siliques of wild-type (left), *agb1-2* (middle) and *Atofp1-1D* (right) plants.
(h) Rosettes of 63-day-old plants of wild-type (left), *agb1-2* (middle) and *Atofp1-1D* (right) plants grown under a 8/16 h (light/dark) photoperiod.

nuclear targeting sequence at the N-terminal, and an OVATE domain that is conserved in all AtOFP members at the C-terminal (Figure 2c). The OVATE domain of the AtOFP1 protein also contains an LxLxL motif (Figure 2c) that is conserved in Aux/IAA and ERF transcription factors (Ohta *et al.*, 2001; Tiwari *et al.*, 2004).

Recapitulation of Atofp1-1D *phenotypes*

To confirm that the phenotypes observed in the *Atofp1-1D* mutant were indeed caused by elevated expression of *AtOFP1* transcript, we generated transgenic plants by transforming *agb1-2* and Col-0 plants with a binary vector containing the full-length open reading frame of *AtOFP1* driven by the strong CaMV 35S promoter (*35S:AtOFP1*). We found that overexpression of *AtOFP1* in both Col wild-type and *agb1-2* mutant background could recapitulate the *Atofp1-1D* phenotypes (Figure S1). Although the *Atofp1-1D* mutant was originally isolated from a mutagenized *agb1-2* mutant population, three lines of evidence suggest that AtOFP1 is not likely to be a component in the heterotrimeric G-protein signaling pathway. First, *agb1-2* mutants mainly have defects in cell division (Ullah *et al.*, 2003), whereas *Atofp1-1D* mutants mainly have defects in cell elongation. Both *agb1-2* mutants and plants overexpressing *AtOFP1* have short hypocotyls. However, we found that the short hypocotyl of *agb1-2* mutants was caused by reduced cell division (i.e. the number of epidermal cell from the base to the top of hypocotyl: Col 20.2 ± 1.1 versus *agb1-2* 10.6 ± 1.2), whereas the short hypocotyl of plants overexpressing *AtOFP1* in the wild-type background was caused by reduced cell elongation (i.e. *AtOFP1* overexpression lines have wild-type number of epidermal cells). Second, siliques in plants overexpressing *AtOFP1* in the Col background do not have blunt tips, a characteristic phenotype caused by the *agb1-2* mutation (Lease *et al.*, 2001; Ullah *et al.*, 2003). Third, the reduced length of aerial organs in *Atofp1-1D* mutants is not dependent on the loss-of-function of *AGB1*, because overexpression of *AtOFP1* in the Col background generates very similar phenotypes as in the *agb1-2* mutant background (Figure S1). Therefore, in our subsequent studies, we used transgenic plants overexpressing *AtOFP1* in the wild-type Col background. Overexpression of *AtOFP1* did not appear to affect root morphology (data not shown).

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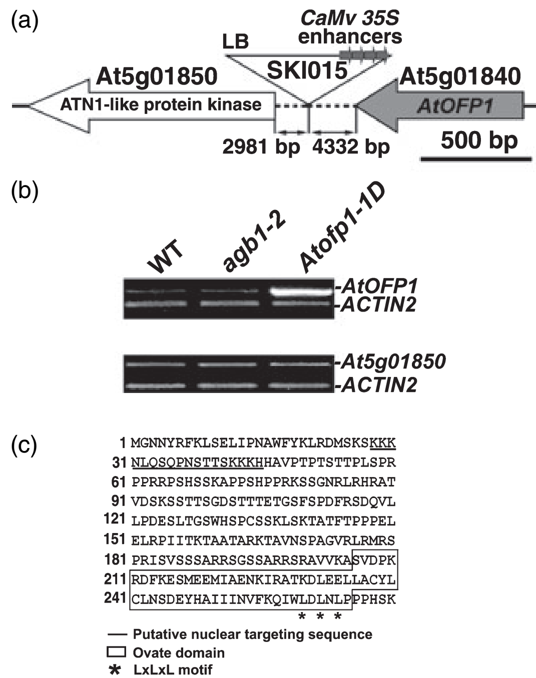

Figure 2. Molecular cloning of *AtOFP1*.
(a) Insertion site of the activation tagging T-DNA in the *Atofp1-1D* mutant. The orientation of the four 35S enhancer repeats in the T-DNA, which is 4332 bp downstream of the stop codon of the *AtOFP1* gene, is indicated by arrows.
(b) Transcript level of gene loci *AtOFP1* and *At5g01850* in wild-type, *agb1-2* and *Atofp1-1D* plants. RNA was isolated from 10-day-old light-grown seedlings. *ACTIN2* was used as a control in PCR reactions.
(c) Amino acid sequence of AtOFP1. The locations of the putative nuclear targeting sequence (underlined), the OVATE domain (boxed) and the LxLxL motif (asterisks) are indicated.

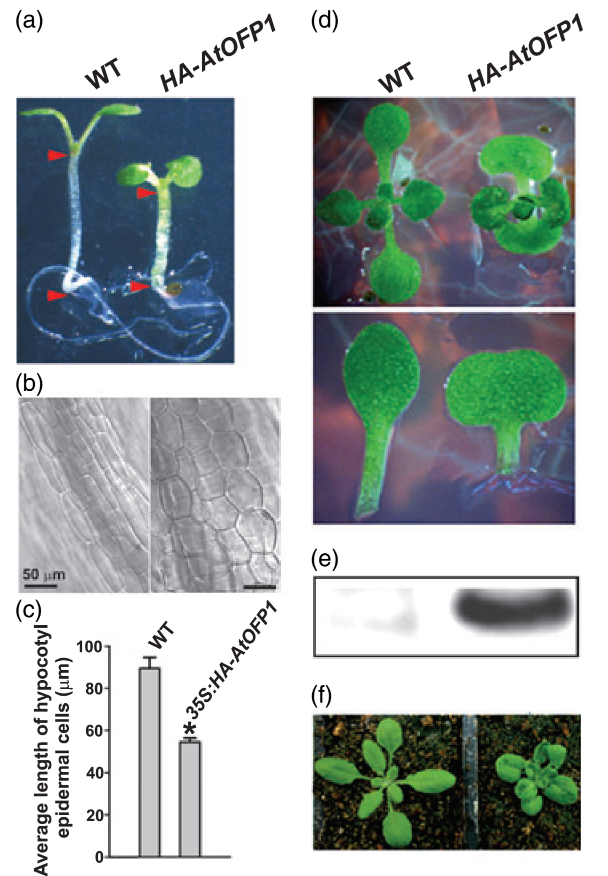

Figure 3. Phenotype of transgenic plants overexpressing *HA-AtOFP1* in wild-type background.
(a) Four-day-old *35S:HA-AtOFP1* and wild-type (WT) seedlings. Seedlings were grown under a 14/10 h (light/dark) photoperiod at relatively low intensity of light (60 μmol m^{-2} sec^{-1}) for easy visualization and quantification of the length of hypocotyls. Arrowheads indicate the top and base of hypocotyls.
(b) Hypocotyl epidermal cells in 4-day-old wild-type (left) and *35S:HA-AtOFP1* (right) seedlings. Bars = 50 μm.
(c) Average length of hypocotyl epidermal cells in 4-day-old wild-type (WT) and *35S:HA-AtOFP1* seedlings. *Significantly different from WT, $P < 0.05$.
(d) Ten-day-old wild-type (WT) and *35S:HA-OFP1* seedlings. Top: top view of the seedlings. Bottom: close-up view of the cotyledons.
(e) Expression of HA-AtOFP1 protein in wild-type (left) and *35S:HA-AtOFP1* (right) transgenic plants. Proteins were extracted from 10-day-old seedlings and 20 μg total proteins were loaded.
(f) Rosette of wild-type (left) and *35S:HA-OFP1* (right) plants.

In order to facilitate our studies on the AtOFP1 protein, we generated transgenic plants overexpressing AtOFP1 fused in-frame with an N-terminal HA tag (*35S:HA-AtOFP1*) in the wild-type Col background. Plants transformed with this construct recapitulated the phenotypes of *Atofp1-1D* mutants or plants overexpressing the intact form of *AtOFP1* (*35S:AtOFP1*) (Figure 3), indicating that the HA–AtOFP1 fusion protein can function effectively as the AtOFP1 protein. We found that overexpression of *AtOFP1* not only affects cell elongation in hypocotyl epidermal cells (Figure 3b,c), but also cell elongation in other rapidly elongating organs such as the leaf petiole (Figure S2). The elevated AtOFP1 protein level was correlated with the phenotypes observed in the transgenic plants (Figure 3e). Therefore, *35S:HA-AtOFP1* overexpression plants were used for most of our subsequent studies.

Loss-of-function mutants of AtOFP1

In order to further analyze the function of *AtOFP1*, we took a reverse genetics approach to isolate and characterize loss-of-function mutant alleles of *AtOFP1*. We first analyzed three independent T-DNA insertion mutant alleles of *AtOFP1* in Arabidopsis: SALK_11492, SALK_127550 and SAIL_904_F06 (Figure S3a). In this paper, these three *AtOFP1* mutant alleles are named *Atofp1-2*, *Atofp1-3* and *Atofp1-4*, respectively (Figure S3). *Atofp1-2* and *Atofp1-3* were used by Hackbusch *et al.* (2005) and were proposed to be loss-of-function mutant alleles of *AtOFP1*. Based on the study of these two alleles together with another T-DNA insertion allele, the authors suggested that *AtOFP1* is essential for male transmission and pollen function (Hackbusch *et al.*, 2005). However, our extensive analyses of these mutants suggested

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that neither of them is a loss-of-function mutant allele of *AtOFP1* (Figure S3). Moreover, we recovered plants homozygous for the *Atofp1-2* locus, and showed that the transcript level of *AtOFP1* was not affected in this mutant (Figure S3), and that plants homozygous for *Atofp1-2* locus had wild-type morphology (data not shown). We also analyzed the *Atofp1-3* mutant allele (Figure S3c), the second mutant allele used by Hackbusch *et al.* (2005). Again, based on our results, *Atofp1-3* is not a loss-of-function allele for *AtOFP1* (Figure S3c).

In the *Atofp1-4* mutant, the T-DNA is predicted to insert into the promoter region of the *AtOFP1* gene (Figure S3a), a location that is similar to that in the third allele used by Hackbusch *et al.* (2005). We recovered plants homozygous for the *Atofp1-4* locus. These plants had wild-type levels of *AtOFP1* transcripts (Figure S3d) and wild-type morphology (data not shown). Therefore, *Atofp1-4* is unlikely to be a loss-of-function mutant allele of *AtOFP1*. Because *Atofp1-2*, *Atofp1-3* and *Atofp1-4* mutants did not display any obvious morphological phenotypes, a role of *AtOFP1* in male transmission or pollen function cannot be established.

Having found that none of the above three alleles are loss-of-function mutant alleles for *AtOFP1*, we continued our efforts to seek and analyze other available mutant alleles of *AtOFP1*. We characterized a transposon insertion mutant SM_3_21689 (http://signal.salk.edu/cgi-bin/tdnaexpress). We named this mutant allele as *Atofp1-1*. In this allele, the transposon element was located 118 bp upstream of the stop codon of *AtOFP1* (Figure 4a), and is predicted to disrupt the OVATE domain of AtOFP1 protein (Figure 4a). The results from our RT-PCR analysis indicated that the full transcript of *AtOFP1* was undetectable in plants homozygous for the *Atofp1-1* allele (Figure 4b), suggesting that *Atofp1-1* is probably a loss-of-function mutant allele of *AtOFP1*. Identification of *Atofp1-1* as a potential loss-of-function mutant disfavors the possibility that *AtOFP1* has a role in male transmission or pollen function. *Atofp1-1* mutant plants had wild-type length of epidermal cells in the hypocotyl (Figure 4d). We have yet to detect any significant morphological phenotypes of *Atofp1-1* mutants. One interpretation of the lack of loss-of-function phenotypes is that *AtOFP1* may have an overlapping function with other members of *AtOFP* gene family, but another is that the mutants have very subtle phenotypes.

Having failed to identify obvious morphological phenotypes in loss-of-function mutants, we turned our attentions to gain-of-function transgenic lines. We reasoned that if *AtOFP* genes are redundant to each other , we would see similar phenotypes among plants overexpressing other *AtOFP* genes. We chose to overexpress *AtOFP2* and *AtOFP7*, and compare the phenotypes of plants overexpressing *AtOFP2* or *AtOFP7* with that of *35S:HA-AtOFP1* plants. AtOFP2 and AtOFP7 proteins not only have a conserved OVATE domain near their C-terminus, but also have a relatively higher similarity to AtOFP1 than to other AtOFP proteins (Figure S4a). In addition, both AtOFP2 and AtOFP7 also function similarly to AtOFP1 as active transcriptional repressors in protoplast transfection assays as discussed below (Figure 6b). Indeed, we found that plants overexpressing *AtOFP2* or *AtOFP7* have phenotypes similar to that of *35S:AtOFP1* plants (Figure S4b). These results support the notion that *AtOFP1* may have overlapping function with other members of the *AtOFP* family, such as *AtOFP2* and *AtOFP7*.

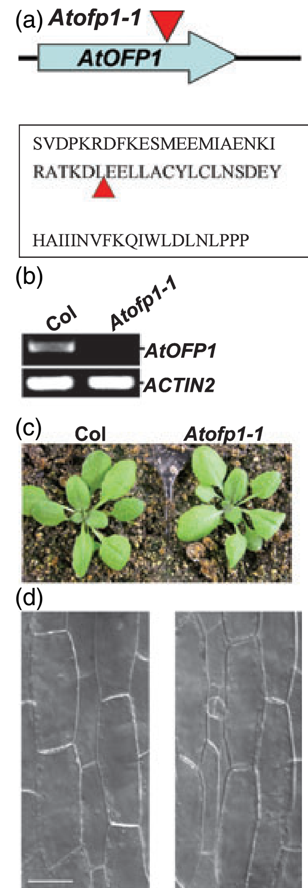

Figure 4. Identification of *Atofp1-1* transposon insertion mutant.
(a) The transposon insertion site in *AtOFP1*. The triangle indicates the position of transposon insertion in the *AtOFP1* gene (top). The transposon was inserted 118 bp upstream of the stop codon of *AtOFP1*. The transposon element is predicted to disrupt the OVATE domain of AtOFP1 (bottom).
(b) RT-PCR analysis of *AtOFP1* transcript in the *Atofp1-1* mutant. RNA was isolated from leaves collected from wild-type plants (Col) and plants homozygous for the *Atofp1-1* locus. The full-length *AtOFP1* transcript is undetectable in *Atofp1-1* mutants. *ACTIN2* was used as a control in each PCR reaction.
(c) *Atofp1-1* mutant plants have wild-type morphology. The plants shown are 20-day old plants grown under a 14/10 h (light/dark) photoperiod.
(d) *Atofp1-1* mutants have a wild-type length of hypocotyl epidermal cells. Images are from 4-day-old wild-type (left) and *Atofp1-1* (right) seedlings grown under a 14/10 h (light/dark) photoperiod. Bar = 20 μm.

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Expression of AtOFP1

To test the tissue and organ expression patterns of *AtOFP1*, we generated transgenic plants by transforming wild-type plants with an *AtOFP1* promoter:GUS (β-glucuronidase) fusion construct. We found that, in 4-day-old seedlings, *AtOFP1prom:*GUS was expressed mainly in the roots and lower part of hypocotyls, but not in root hairs, lateral root primordium or shoot (Figure 5a). *AtOFP1prom:*GUS was still expressed in the roots of 10-day-old seedlings including root hair (Figure 5b), but unlike the 4-day-old seedlings, it was also expressed in shoots, vasculatures and trichomes (Figure 5b). Thus, expression of *AtOFP1prom:*GUS appears to be developmentally regulated. In mature plants, *AtOFP1-prom:*GUS was expressed mainly in the inflorescence, especially flowers with mature pollen (Figure 5c), and the lower part of young siliques (Figure 5d).

In accordance with the tissue and organ expression patterns of the *AtOFP1prom:*GUS reporter, we found *AtOFP1* transcript in the roots of young seedlings and both roots and shoots of relatively old seedlings. In mature plants, transcripts of *AtOFP1* were detected in inflorescence stems, flowers and young siliques (Figure 5e).

AtOFP1 is a transcriptional repressor

The presence of a putative nuclear targeting sequence in AtOFP1 protein indicates that AtOFP1 is probably a nuclear protein. To test this, we generated transgenic plants by transforming wild-type Col plants with a binary vector that contains a full-length open reading frame of *AtOFP1* fused in-frame with *GFP*. Expression of the AtOFP1–GFP fusion protein was driven by *AtOFP1*'s native promoter. The transgenic plants had a similar phenotype to plants over-expressing the intact form of *AtOFP1* (*35S:AtOFP1;* Figure 5f), suggesting that the AtOFP1–GFP fusion protein functions efficiently as the AtOFP1 protein. By using these transgenic plants, we found that AtOFP1 is predominantly localized in the nucleus (Figure 5g).

In a transient expression system using protoplasts derived from Arabidopsis rosette leaves, we also observed a nuclear-localized AtOFP1–GFP fusion protein when the 35S

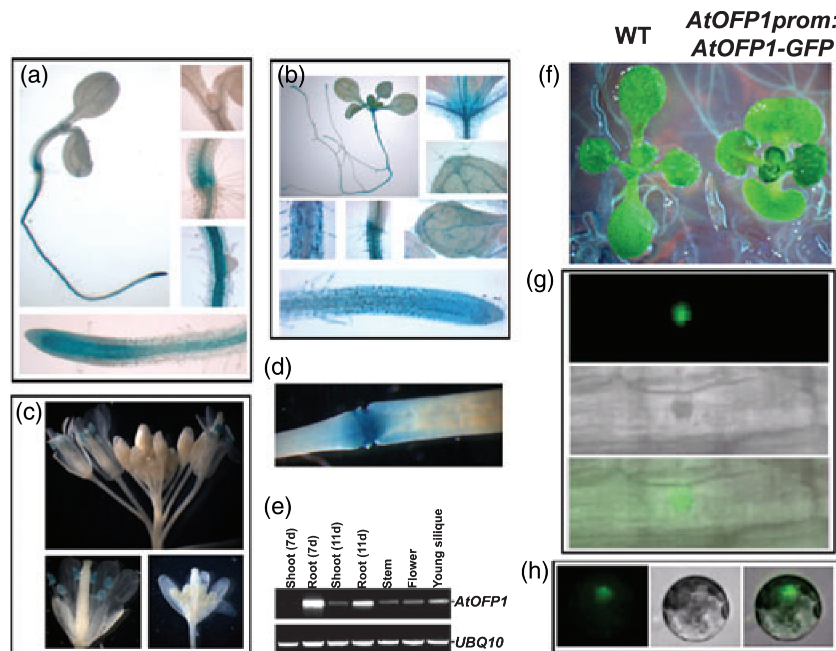

Figure 5. Expression of *AtOFP1*.
(a) Expression of *AtOFP1prom:*GUS in 4-day-old seedlings. Top left, whole seedling; top right, close-up views of shoot (top), hypocotyl (middle) and lateral root primordium (bottom). Bottom, root tip.
(b) Expression of *AtOFP1prom:*GUS in 10-day-old seedlings. Top left, whole seedling; top right, close-up views of shoot (top) and trichomes (bottom). Middle, close view of root (left), hypocotyl (middle) and leaf (right). Bottom, root tip.
(c) Expression of *AtOFP1prom:*GUS in inflorescences. Top, whole inflorescence. Bottom, close-up views of the staining in the most recently matured flower (left) and the flower next to it (right).
(d) Expression of *AtOFP1prom:*GUS in siliques.
(e) Expression of *AtOFP1* in young seedlings and various parts of adult plants. *UBQ10* was used as a control in each PCR reaction.
(f) Phenotype of plants expressing the AtOFP1–GFP fusion protein under the control of the *AtOFP1* native promoter.
(g) A root epidermal cell from a 7-day-old transgenic plant expressing the AtOFP1–GFP fusion protein under the control of the *AtOFP1* promoter. Top, image with GFP channel; middle, DIC image; bottom, merged images.
(h) A protoplast transfected with *35S:AtOFP1-GFP*. Left: image with GFP channel; middle, DIC image; right, merged images.

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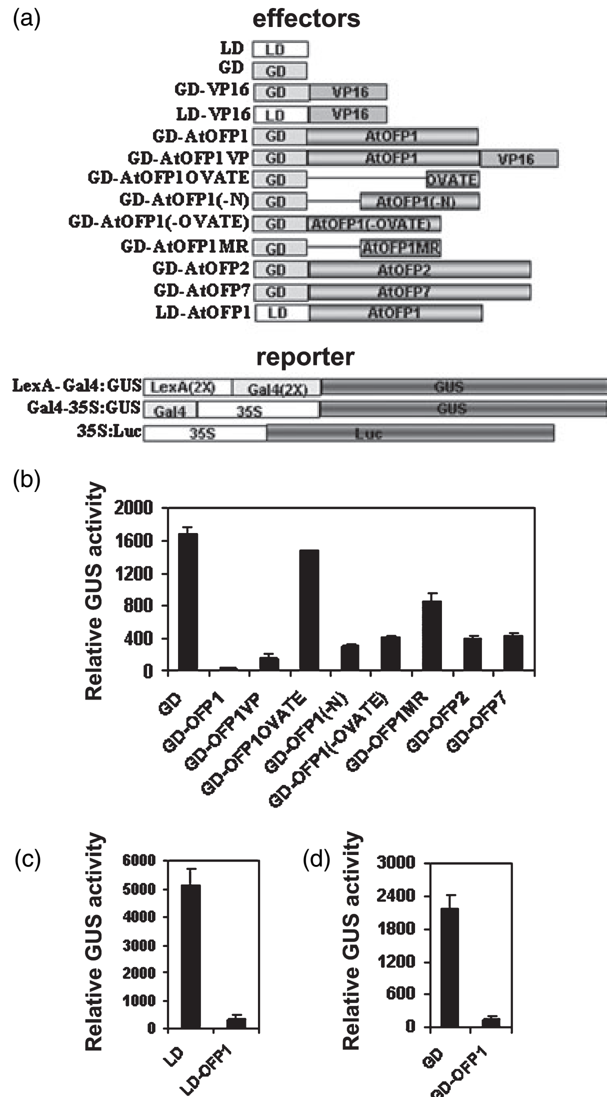

Figure 6. AtOFP1 is an active transcriptional repressor.
(a) Effectors and reporter constructs used in the transfection assays.
(b) AtOFP1 represses the expression of the reporter activated by a transactivator. Effector genes, transactivator and reporter genes were co-transfected into protoplasts derived from Arabidopsis rosette leaves. GUS activity was assayed after protoplasts had been incubated in darkness for 20–22 h. The expression of 35S:luciferase (Luc) was used to normalized the expression of the GUS reporter gene.
(c) Transfection assay in which GD-VP16 was used as transactivator and LD-fused AtOFP1 as effector.
(d) Transfection assay in which GD-AtOFP1 was co-transfected with the constitutively expressed reporter Gal4-35S:GUS in the absence of transactivator.

promoter was used to drive AtOFP1–GFP expression (Figure 5h), which is largely consistent with the results reported by Hackbusch *et al.* (2005), except that we did not observe a significant association of AtOFP1–GFP with the cytoskeleton. Such a discrepancy may be due to the differences in expression system used. For example, we used an Arabidopsis homologous expression system, whereas Hackbusch *et al.* (2005) used a tobacco heterologous expression system. Nonetheless, our results for localization of AtOFP1–GFP (expression driven by 35S promoter) in Arabidopsis protoplast are consistent with those in stable AtOFP1–GFP transgenic lines in which the expression of AtOFP1–GFP was driven by the *AtOFP1* native promoter (Figure 5g,h). In both cases, we found that AtOFP1–GFP is predominantly localized in the nucleus.

The presence of an LxLxL motif, a motif that is conserved in Aux/IAA and ERF transcription factors (Ohta *et al.*, 2001; Tiwari *et al.*, 2004), in the AtOFP1 protein indicates that AtOFP1 might function as an active transcriptional repressor. To test this possibility, we adopted a protoplast transfection system designed for study of the active transcriptional repression of Aux/IAA as described by Tiwari *et al.* (2001, 2004), but used homologous Arabidopsis protoplasts in our transfection assays instead of heterologous *Daucus carota* (carrot) suspension cell protoplasts. We first tested whether AtOFP1, if targeted to one DNA binding site in a promoter, could repress the activity of a transactivator targeted to another site in the promoter. In this transfection system, the expression of the GUS reporter gene is driven by the -46 35S promoter with both LexA and Gal4 DNA binding sites [LexA(2x)-Gal4(2x):GUS reporter gene]. The transactivator gene encodes a chimeric protein consisting of the LexA DNA-binding domain (DBD) or the *Saccharomyces cerevisiae* Gal4 DBD fused to the herpes simplex virus VP16 activation domain (LD-VP16 or GD-VP16) and is driven by 35S promoter. The effector gene encodes a chimeric protein consisting of Gal4 DBD or LexA DBD fused to AtOFP1 or truncated versions of AtOFP1. The effector genes and reporter gene are shown in Figure 6(a). The GUS reporter was co-transfected with both effector gene and the transactivator LD-VP16 or GD-VP16. As shown in Figure 6(b), co-transfection of the LD-VP16 transactivator gene and the effector gene encoding only the Gal4 DBD (GD) resulted in strong activation of the reporter gene, which was similar to that when LD-VP16 alone with the reporter gene were co-transfected (Tiwari *et al.*, 2004; data not shown). Co-transfection of LD-VP16 with an effector gene encoding Gal4 DBD fused to the AtOFP1 protein (GD–AtOFP1) resulted in a nearly complete repression of expression of the reporter gene (Figure 6b). Co-transfection of GD-VP16 with an effector gene encoding LexA DBD fused to the AtOFP1 protein (LD–AtOFP1) also resulted in a nearly complete repression of the expression of the reporter gene (Figure 6c). These results indicate that AtOFP1 can work both up- and downstream of an activator to repress reporter gene expression when it is targeted to the DNA binding site in the promoter.

To test whether AtOFP1, if targeted to one DNA binding site in the promoter, could repress the activity of a constitutively expressed reporter, an alternative reporter gene Gal4-35S:GUS (Figure 6a) was co-transfected with the effec-

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tor gene. As shown in Figure 6(d), AtOFP1 can also repress the expression of a constitutively expressed reporter gene.

We further tested whether the repression function of AtOFP1 is dominant over an activation domain. We fused AtOFP1 with Gal4 DBD at the N terminal and the VP16 activator at the C terminal (Figure 6a), then co-transfected it with reporter LexA(2x)-Gal4(2x):GUS and activator LD-VP16, and found that AtOFP1 could still repress reporter gene expression activated by an activator (Figure 6b).

To determine whether the LxLxL motif of AtOFP1 protein is required for repression as in Aux/IAA and ERF proteins (Ohta *et al*., 2001; Tiwari *et al*., 2004), truncated versions of AtOFP1 were fused to the Gal4 DBD and tested in co-transfection assays. Surprisingly, as shown in Figure 6(b), the effector gene encoding Gal4 DBD fused to an LxLxL-containing OVATE domain (GD–AtOFP1OVATE) only showed marginal repression of the expression of the reporter gene. An effector gene encoding Gal4 DBD fused with truncated version of AtOFP1 without the LxLxL-containing OVATE domain (AtOFP1–OVATE) was more efficient than that containing AtOFP1OVATE in repressing the reporter gene expression (Figure 6b). These results suggested that LxLxL motif contributes little to the repression function of AtOFP1. In accordance with this, when another two proteins in the AtOFP family, AtOFP2 and AtOFP7, which are closely related to AtOFP1 in protein sequence but lack the LxLxL motif (Figure S4a), were fused to Gal4 DBD (GD–AtOFP2, GD–AtOFP7), expression of the reporter gene was also repressed (Figure 6b).

In order to further determine the repression domain that is responsible for the repression function of the AtOFP1 protein, we prepared a further two truncated version of the AtOFP1 protein. First, we deleted the N-terminal portion of AtOFP1 and fused it with Gal4 DBD [GD–AtOFP1(-N)]. We found that this truncated AtOFP1 protein could still function as a potent repressor (Figure 6b), indicating the major repression domain is within the middle region of AtOFP1 (AtOFP1MR). To confirm this, we fused the middle region of AtOFP1 protein with Gal4 DBD (GD–AtOFP1MR), and tested its repression activity. As shown in Figure 6(b), the AtOFP1MR indeed represents a major repression domain of AtOFP1.

AtOFP1 regulates AtGA20ox1 *expression*

Having determined that AtOFP1 acts as an active transcriptional repressor, we wished to further determine the target genes whose expression is regulated by AtOFP1. Because plants overexpressing *AtOFP1* have reduced length in all aerial organs examined (Figures 1 and 3, Figure S1), and because plants significantly overexpressing *AtOFP1* showed dwarfism (Figure S1), we concluded that AtOFP1 may have a role in regulating the expression of genes encoding key enzymes in gibberellin (GA) biosynthesis, because GAs are the most important class of plant hormones known to regulate cell elongation. We hypothesized that one such gene may be *AtGA20ox1* (Kusaba *et al*., 1998; Tanaka-Ueguchi *et al*., 1998), because the expression of *AtGA20ox1* is downregulated in plants overexpressing *AtOFP1* (Hackbusch *et al*., 2005). Further, AtOFP1 interacts with KNOX proteins in a yeast two-hybrid assay (Hackbusch *et al*., 2005), while KNOX proteins suppress the expression of gibberellin 20-oxidase (Hay *et al*., 2002; Sakamoto *et al*., 2001). To test this directly, we used a chromatin immunoprecipitation (ChIP) assay to determine the association of AtOFP1 protein with the *cis*-acting regulatory sequence of *AtGA20ox1*. We used *35S:HA-AtOFP1* plants for our ChIP assay, because we have shown that the HA–AtOFP1 fusion protein is functional (Figure 3). Furthermore, the presence of the HA tag in the HA–AtOFP1 fusion protein allowed us to immunoprecipitate the HA–AtOFP1 fusion protein using anti-HA antibodies. The specificity of the HA antibodies has been tested (Figure 3e). By using anti-HA antibodies for ChIP, we detected a specific PCR product of the expected size amplified using primers specific to the promoter region or the first intron of *AtGA20ox1* (Figure 7a). These regions have been previously shown to be the binding sites for KNOX proteins (Chen *et al*., 2004; Sakamoto *et al*., 2001). We used anti-H3-K9 Ac antibodies to monitor our ChIP assays. As expected, a PCR product specific to the *ARPN* gene (At2g02850) was amplified (Figure 7b), consistent with previous findings by Tian *et al*. (2005). As a mock control, we used rabbit pre-immune sera, and did not

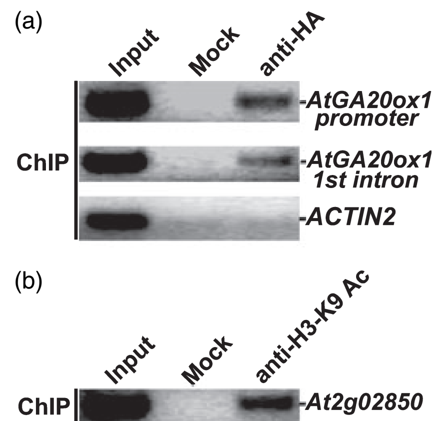

Figure 7. *AtGA20ox1* is a target gene for AtOFP1.
(a) Chromatin immunoprecipitation (ChIP) assay performed in *35S:HA-AtOFP1* plants using anti-HA antibodies. Rabbit pre-immune serum was used as a mock control. Primer sets specific for the promoter region or the first intron of *AtGA20ox1* were used in PCR reactions.
(b) ChIP assay using anti-acetyl-histone H3 (Lys9) rabbit antiserum (anti-H3-K9 Ac). Rabbit pre-immune serum was used as a mock control. The *At2g02850* locus is known to be modified by acetylation (Tian *et al*., 2005). This anti-H3-K9 Ac antibody-based ChIP was used to verify the ChIP protocol.

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detect any specific PCR products using the same sets of primers (Figure 7). Therefore, we conclude that *AtGA20ox1* is indeed a direct target gene of AtOFP1.

Partial rescue of 35S:HA-AtOFP1 *plants by* GA_3

Because we have shown that *AtGA20ox1* is a target gene of AtOFP1 and GA20 oxidase is the key enzyme for gibberellin biosynthesis, we would expect that exogenously applied active gibberellins, such as GA_3, would rescue the phenotype caused by the overexpression of *AtOFP1*. Therefore, we wished to test whether GA_3 could restore the reduced length of rapidly elongating organs including hypocotyl, leaf petioles and inflorescence stems in the *35S:HA-AtOFP1* plants to the wild-type level. As shown in Figure 8(a,b), exogenously applied GA_3 promotes hypocotyl elongation in a GA_3 dose-dependent manner. GA_3 at 1.0 μM could restore the length of the hypocotyl of *35S:HA-AtOFP1* plants to that of non-GA-treated wild-type plants (Figure 8a,b). Microscopic examination revealed that the increased length of the hypocotyl of *35S:HA-AtOFP1* plants by treatment with exogenous GA_3 was due to increased cell elongation in the hypocotyl epidermal cells (Figure 8c). Exogenous GA_3 also dramatically increased the length of leaf petioles (Figure S5) and inflorescence stems (Figure 8e and Figure S6), subsequently rescuing the dwarfism of *35S:HA-AtOFP1* plants (Figure 8e). However, exogenous GA_3 did not rescue the kidney-shaped cotyledons (Figure 8d and Figure S5) and the round shape of rosette and cauline leaves (Figure 8e and Figure S6) of *35S:HA-AtOFP1* plants, suggesting that regulation of the expression of genes encoding key enzymes in GA biosynthesis may only represent part of AtOFP1's function. Nonetheless, we demonstrated that exogenous GA_3 could efficiently restore the elongation of rapidly elongating organs (i.e. hypocotyl of seedlings and the inflorescence stem of mature plants) that determine the height of a plant, supporting the hypothesis that *AtGA20ox1* is a target gene of AtOFP1.

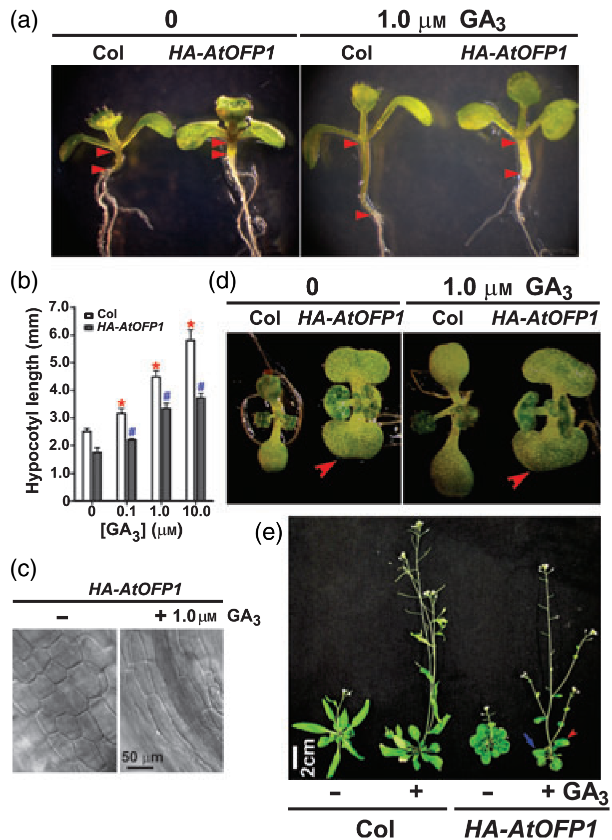

Figure 8. Partial rescue of *35S:AtOFP1* plants by exogenous GA_3.
(a) Exogenous GA_3 promotes hypocotyl elongation. Seeds of wild-type (Col) and *35S:HA-AtOFP1* plants were germinated on MS/G plates containing 1.0 μM GA_3. The seedlings were photographed 7 days later. Arrowheads indicate the top and base of hypocotyls.
(b) Hypocotyl lengths of *35S:HA-AtOFP1* seedlings in response to various concentrations of GA_3. Seeds were germinated on MS/G plates with GA_3 at the concentrations indicated. *Significantly different, $P < 0.05$, in comparison with no GA_3 treatment control in Col. #Significantly different, $P < 0.05$, in comparison with no GA_3 treatment control in *35S:HA-AtOFP1* seedlings.
(c) Exogenous GA_3 promotes hypocotyl epidermal cell elongation in *35S:HA-AtOFP1* seedlings. Images were taken from seedlings in (a). Bar = 50 μm.
(d) Exogenous GA_3 does not alter the cotyledon shape of *35S:HA-AtOFP1* seedlings. Arrowheads indicate the kidney-shaped cotyledons.
(e) Exogenous GA_3 promotes inflorescence stem elongation in *35S:HA-AtOFP1* plants. Arrows indicate the round rosette and cauline leaves.

Discussion

AtOFP1 is an active transcriptional repressor

Transcription factors play an important role in regulating plant growth and development. Various transcription factors have been identified through genetic screens. Here we identified AtOFP1 as a novel transcription factor that has a role in regulating cell elongation. The dominant mutant *Atofp1-1D* has a defect predominantly in cell elongation (Figure 1) caused by the overexpression of the *AtOFP1* gene (Figure 1 and Figure S1). We provide evidence that AtOFP1 is an active transcriptional repressor (Figure 6) that affects cell elongation in various rapidly elongating aerial organs (Figure 3 and Figure S1).

The presence of LxLxL, a motif conserved in Aux/IAA and repressor ERF proteins (Ohta *et al.*, 2001; Tiwari *et al.*, 2004) in the AtOFP1 protein (Figure 2c) protein led us to test whether AtOFP1 is a transcriptional repressor. We found that AtOFP1 localizes in the nucleus, and that AtOFP1 suppresses the expression of both a constitutively expressed reporter and a reporter gene activated by a transcriptional activator in a protoplast transient transfection system (Figure 6), indicating that AtOFP1 is indeed a transcriptional repressor. Surprisingly, we found that the LxLxL-containing OVATE domain contributed little to the transcriptional repression function of the AtOFP1 protein. AtOFP1 without the LxLxL-

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containing OVATE domain, as well as two closely related members of AtOFP family, AtOFP2 and AtOFP7, that lack the LxLxL motif in their OVATE domain, could still function as potent transcriptional repressors (Figure 6b). These results indicate that the transcription repression function of the LxLxL motif may not be conserved in the AtOFP1 transcription factor. Although we could not identify the exact amino acids responsible for the repression activity of AtOFP1 proteins, we found that the middle region of AtOFP1 protein probably represent the major repression domain of AtOFP1 (Figure 6). AtOFP1 and the closely related AtOFP2 and AtOFP7 may represent a new class of plant-specific transcription factors. The transcription repression or activation activity of other AtOFP proteins has yet to be determined.

How does AtOFP1 regulate gene expression?

The major function of a transcription factor is to regulate gene expression by directly or indirectly binding to the *cis*-acting regulatory element presented in the promoter region and other regions (such as the intron and UTR region) of the target gene. Three lines of evidence support the possibility that genes encoding key enzymes in gibberellin biosynthesis may be target genes of AtOFP1. First, plants overexpressing *AtOFP1* displayed reduced length in all rapidly elongating aerial organs examined, including hypocotyl, leaf petiole and inflorescence stems (Figures 1, 3 and 8; Figures S1, S2, S5 and S6), and plants expressing high levels of *AtOFP1* are dwarf (Figure S1). The reduced lengths in these organs are mainly due to reduced cell elongation (Figure 1, 3 and 8). Because gibberellins are the most important class of plant hormones known to promote cell elongation in various organs, it was reasonable to assume that defects in plants overexpressing *AtOFP1* could be due to defects in GA biosynthesis or signalling. Second, the reduced cell elongation in rapidly elongating aerial organs could be partially rescued by exogenous GA_3 (Figure 8 and Figures S5 and S6), indicating that the reduced cell elongation in plants overexpressing *AtOFP1* may be caused by GA biosynthesis deficiency. Because plants overexpressing *AtOFP1* had a nearly wild-type response to exogenous GA_3 (Figure 8 and Figures S5 and S6), AtOFP1 may have a more important role in regulating GA biosynthesis than in GA signaling. Third, overexpression of *AtOFP1* suppressed the expression of *AtGA20ox1* (Hackbusch *et al.*, 2005), a gene encoding a key enzyme in GA biosynthesis (Kusaba *et al.*, 1998; Tanaka-Ueguchi *et al.*, 1998). Our ChIP assay demonstrated that *AtGA20ox1* is indeed a target gene of AtOFP1 (Figure 7).

Transcription factors regulate the expression of target genes by binding either directly or indirectly to the *cis*-acting regulatory sequence of the target gene. Because AtOFP1 interacts with homeodomain transcription factors KNOX and BELL (Hackbusch *et al.*, 2005), and overexpression of *KNAT1* also suppresses expression of *AtGA20ox1* (Hay *et al.*, 2002), AtOFP1 and KNAT1 may function together in a transcription repression complex to regulate the expression of *AtGA20ox1*. Because KNOX proteins have been shown to directly bind to the promoter region and the first intron of the GA20 oxidase gene (Chen *et al.*, 2004; Sakamoto *et al.*, 2001), and AtOFP1 protein does not contain an apparent DNA binding domain, the current data support a model in which AtOFP1 regulates *AtGA20ox1* expression through binding to KNAT1. Because there are at least seven KNAT1-like proteins and 18 AtOFP proteins in Arabidopsis and each component could potentially form homodimers with itself and heterodimers with other closed related members (Hackbusch *et al.*, 2005), such a networking transcription repression complex offers a large capacity and flexibility in regulating the expression of *AtGA20ox1* or other target genes, and contributes to phenotypic plasticity.

Adding more complexity to such a networking transcription repression complex is the fact that the expression of *AtOFP1* is developmentally regulated (Figure 5a–e), and introduction of an extra copy of the *AtOFP1* gene (i.e. *AtOFP1promoter:AtOFP1* transgenic plants) resulted in pleiotropic phenotypes (Figure 5f). These results indicate that expression of the *AtOFP1* gene itself may be tightly regulated, which could offer another fine-tuning regulatory mechanism for this transcriptional repression complex.

Similarly, the expression of *KNAT1* also appears to be tightly regulated. For example, it has been shown that the expression of *KNAT1* is negatively regulated by a homeodomain transcription factor ASYMMETRIC LEAVES1 (AS1), and that the expression of *AS1* is in turn negatively regulated by another homeodomain transcription factor SHOOT MERISTEMLESS (STM; Byrne *et al.*, 2002).

Intriguingly, our results demonstrated that exogenous GA_3 could not restore non-rapidly elongating organs of plants overexpressing *AtOFP1* to wild-type morphology (Figure 8 and Figures S5 and S6). For example, the shapes of cotyledons, rosette and cauline leaves, floral organs and siliques of plants overexpressing *AtOFP1* remained largely unchanged upon exogenous GA_3 application. These organs are typically not directly involved in determining the height of Arabidopsis plants. In addition, the widths of epidermal cells in the hypocotyls of plants overexpressing *AtOFP1* are larger (Figure 3b). These results suggest that the regulation of *AtGA20ox1* expression by AtOFP1 may only represent a part of AtOFP1's function. It is likely that AtOFP1 also regulates the expression of other genes.

Possible overlapping function among members of the AtOFP *gene family*

At least some transcription factors have redundant function in regulating plant growth and development. One example is the Aux/IAA protein family. There are 29 genes in Arabidopsis encoding Aux/IAA proteins. So far, 13 different *aux/*

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iaa loss-of-function mutants have been identified and none of those single mutants displayed any obviously morphological phenotypes, and the *iaa5 iaa6 iaa19* triple mutant is still very similar to wild-type (Overvoorde *et al.*, 2005), indicating the existence of functional redundancy among members of Aux/IAA family.

There are 18 genes in Arabidopsis encoding AtOFP proteins. Three lines of evidence support the possibility that AtOFP1 and other AtOFP proteins may have overlapping functions. First, a possible loss-of-function mutation in *AtOFP1* does not confer significant defects in morphology (Figure 4). Second, plants overexpressing other *AtOFP* genes that are closely related to *AtOFP1* phenocopy the plants overexpressing *AtOFP1* (Figure S4). Third, like AtOFP1, AtOFP2 and AtOFP7 can function as transcriptional repressors (Figure 6b). As discussed above, such a functional redundancy among *AtOFP* genes may be required to achieve maximal regulatory flexibility of the transcriptional repression complex and phenotypic plasticity.

AtOFP proteins may also have some similarities with Aux/IAA proteins in terms of their mode of action. For example, neither AtOFP proteins nor Aux/IAA proteins contain an obvious DNA binding domain. Aux/IAA proteins repress gene expression via dimerization with ARF proteins. Similarly, AtOFP proteins may repress *AtGA20ox1* expression by forming a dimeric protein complex with a KNOX transcription factor, and AtOFP proteins thus function as transcriptional repressors. However, the transcriptional repression function of the LxLxL motif seen in Aux/IAA proteins does not appear to be well conserved in the AtOFP1 protein (Figure 6).

In summary, we have identified AtOFP1 as an active transcriptional repressor that regulates cell elongation. We demonstrated that AtOFP1 regulates cell elongation in part by directly controlling the expression of *AtGA20ox1*. Future studies should focus on the identification of other target genes the expression of which are regulated by AtOFP1, and on the exact role of AtOFP1 in the KNAT1-containing transcriptional repression complex regulating target gene expression.

Experimental procedures

Plant materials and growth conditions

All mutants, transgenic lines and wild-type are in ecotype Columbia background (Col-0). For plant transformation and protoplast transfection, approximately 20 Col-0 or *agb1-2* mutant (Ullah *et al.*, 2003) seeds were germinated and grown in 2 × 2 inch pots containing a moistened 1:3 mixture of Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd, www.sungro.com) and Metro-Mix 220 (W.R. Grace & Co., www.grace.com) with a 14/10 h (light/dark) photoperiod at approximately 120 μmol m^{-2} sec^{-1} at 23°C, unless specified otherwise. Plants that were about 5 weeks old with several mature flowers in the main inflorescence were used for plant transformation. Leaves from plants that were approximately 3–4 weeks old were used for protoplast isolation.

To produce seedlings for use in phenotypic analysis, Western blot or GUS activity assays, seeds were surface-sterilized and sown on MS/G plates consisting of half-strength Murashige & Skoog (MS) basal medium with vitamins (PlantMedia, www.plantmedia.com), 1% w/v sucrose and 0.6% w/v phytoagar (PlantMedia), pH adjusted to 5.7 with 1 N NaOH, cold-treated at 4°C in the dark for 2 days, then moved to 23°C, with a 14/10 h (light/dark) photoperiod either under relatively weak light (60 μmol m^{-2} sec^{-1}) for better visualizing and quantifying the length of hypocotyls, or at 120 μmol m^{-2} sec^{-1} for the GA_3 responsiveness assay described below.

Isolation of the Atofp1-1D mutant and molecular cloning of AtOFP1

agb1-2 mutants were mutagenized by transforming with the activation tagging vector pSKI015 (Weigel *et al.*, 2000). The *Atofp1-1D* dominant mutant was identified from approximately 10 000 mutant populations. A plasmid rescue technique was used to clone the *AtOFP1* gene locus. Genomic DNA (20 μg) isolated from the *Atofp1-1D* mutant was digested with restriction enzymes *Hin*dIII or *Pst*I that leave the right-border of the T-DNA intact, ligated, transformed into *Escherichia coli* DH5α, and the transformants selected on LB plates containing 100 μg ml^{-1} ampicillin. Three independent colonies were selected and the plasmid DNAs were sequenced using a T-DNA left-border primer (5′-TTGACAGTGACGACAAATCG-3′) and a right-border primer (5′-ATGTGATATCTAGATCCGAAAC-3′). Sequence analysis indicated that the activation tagging vector was inserted at an identical site in three colonies. The *Atofp1-1D* phenotypes were confirmed using a recapitulation experiment by transforming the *agb1-2* mutant and Col-0 with a binary expressing vector containing *35S:AtOFP1*.

Isolation of insertion mutants of AtOFP1

A transposon insertion mutant allele of *AtOFP1*, SM_3_21689, was identified by searching the Exon Trapping Insert Consortium (EXOTIC) database (http://www.jic.bbsrc.ac.uk/science/cdb/exotic/index.htm). This mutant was designated as *Atofp1-1*. The transposon insertion site is in the sole exon of the *AtOFP1* gene. The insertion was identified by PCR using an *AtOFP1*-specific primer (5′-TTATTTGGAATGGGGTGGTGGAAGA-3′) and the transposon element-specific primer (5′-TACGAATAAGAGCGTCCATTTTAGAGTGA-3′). Loss of detectable full-length *AtOFP1* transcript in the *Atofp1-1* mutant was verified by RT-PCR. T-DNA insertion mutants SALK_111492 and SALK_127550 were obtained from the Arabidopsis Biological Resources Center (Columbus, OH, USA) and from Dr Joachim Uhrig (University of Cologne, Germany). SALK_111492 and SALK_127550 were designated as *Atofp1-2* and *Atofp1-3*, respectively. The T-DNA insertion mutant SAIL_904_F06 was obtained from the Arabidopsis Biological Resources Center, and was named as *Atofp1-4* here. All insertion mutants were examined by PCR genotyping using *AtOFP1*-specific primers and primers specific for the transposon element or T-DNA left border. The full-transcript level of *AtOFP1* in each insertion line was examined by RT-PCR.

Plasmid construction

To generate *AtOFP1* (*At5g01840*), *AtOFP2* (*At2g30400*) and *AtOFP7* (*At2g18500*) constructs, the full-length open-reading frame (ORF) of *AtOFP1*, *AtOFP2* or *AtOFP7* were amplified by PCR using genomic

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DNA isolated from 10-day-old seedlings, because all of these genes contains a single exon . The PCR fragments were then cloned in-frame with an N-terminal HA, GD or LD tag into the pUC19 vector under the control of the double 35S enhancer promoter of CaMV followed by the translational enhancer from the 5′ leader of tobacco mosaic virus, and terminated by a 3′ untranslated region derived from the nopaline synthetase gene (Tiwari *et al.*, 2003). Constructs expressing truncated AtOFP1 were cloned by fusing corresponding domains of AtOFP1 in-frame with an N-terminal GD. Construct GD-AtOFP1VP was cloned by fusing AtOFP1 in-frame with GD at the N-terminal and VP16 at the C-terminal. *AtOFP1prom:*GUS was produced by replacing *GH3prom* in *GH3:*GUS constructs (Hagen *et al.*, 1991). AtOFP1–GFP constructs were cloned by fusing AtOFP1 in-frame with GFP, then sub-cloned into the pUC19 vector under the control of the *AtOFP1* promoter or the 35S promoter. Transactivator LD-VP16 and GD-VP16, and reporter Gal4(2x)LexA(2x):GUS, Gal4-35S:GUS and 35S:Luc constructs have been described previously by Tiwari *et al.* (2001, 2004). For plant transformation, corresponding constructs in pUC19 were digested with *Eco*RI, then sub-cloned into binary vector pPZP211.

Plant transformation and selection of transgenic plants

Plants that were about 5 weeks old were transformed with constructs in *Agrobacterium tumefaciens* by the floral dip method (Clough and Bent, 1998). T_1 seeds were selected on MS/G plates containing 50 $\mu g\ ml^{-1}$ kanamycin and 50 $\mu g\ ml^{-1}$ carbenicillin, and transgenic plants were transferred to soil. Phenotypes of transgenic plants overexpressing *AtOFP1*, *AtOFP2* or *AtOFP7* were visible in T_1 plants, and confirmed in the T_2–T_4 generations. The expression levels of the transgene were determined by RT-PCR or Western blot analysis as described below.

Expression of AtOFP1–GFP

The localization of AtOFP1–GFP in transgenic plants expressing AtOFP1–GFP under the control of the *AtOFP1* promoter was examined in 7-day-old seedlings. To examine the expression and localization of AtOFP1–GFP in protoplasts, *35S:AtOFP1-GFP* plasmid DNA in the pUC19 vector was transfected into protoplasts, and incubated for 20–22 h. The GFP fluorescence was examined and photographed using a Leica DM-6000B upright fluorescent microscope with phase and differential interference contrast (DIC) equipped with a Leica FW4000 digital image acquisition and processing system (Leica Microsystems, www.leica-microsystems.com).

Protoplast isolation, transfection and GUS activity assay

The procedures for protoplast isolation, transfection and GUS activity assays have been described previously (Tiwari *et al.*, 2003; Wang *et al.*, 2005). Briefly, protoplasts were isolated from rosette leaves collected from 3–4-week-old plants. Effector plasmids encoding the full-length or truncated protein of AtOFPs fused in-frame with GD were co-transfected with transactivator LD-VP16 and reporter LexA(2x)-Gal4(2x):GUS into protoplasts and incubated under darkness for 20–22 h. GUS activities were measured using a Fluoroskan Finstruments microplate reader (MTX Lab Systems Inc., www.mtxlsi.com). Expression of 35S:luciferase (Luc) was used to normalize the expression of the GUS reporter. Luciferase activities were measured using a microplate luminometer (Turner Designs, www.turnerdesigns.com) together with the Promega Steady-Glo luciferase assay system (http://www.promega.com/).

GA_3 treatment

To test the gibberellin responsiveness of plants overexpressing *AtOFP1*, wild-type Col and *35S:HA-AtOFP1* seedlings were grown MS/G plates supplemented with various concentrations of GA_3 (0, 0.1, 1.0 or 10 μM). The hypocotyl lengths were measured in 7-day-old, light-grown seedlings using a dissecting microscope. The hypocotyl epidermal cells were visualized using a Leica DM-6000B upright microscope with phase and DIC equipped with a Leica FW4000 digital image acquisition and processing system (Leica Microsystems). Both 7- and 11-day-old seedlings were photographed. For GA_3 spraying experiment, wild-type Col and *35S:HA-AtOFP1* plants were grown on MS/G plates plus 10 μM GA_3 for 7 days, transferred into soil, and sprayed with 10 μM GA_3 every 2 days until maturity.

Histochemical staining for GUS activity

Histochemical staining was used to examine the GUS activity using substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Rose Scientific Ltd, www.rosesci.com). The general procedure of histochemical staining for GUS activity has been described previously by Ulmasov *et al.* (1997b). The GUS activities of seedlings at various developmental stages and of organs from adult plants were examined.

RNA isolation and RT-PCR

Total RNA was isolated from seedlings or various parts of adult plants using TRIzol reagent (Invitrogen, http://www.invitrogen.com/). cDNA was synthesized using 1 μg of total RNA by oligo$(dT)_{20}$-primed reverse transcription, using THERMOSCRIPT reverse transcriptase (Invitrogen). *AtOFP1*-specific primers (5′-ATGGGT-AATAACTATCGGTTTAAGCT-3′ and 5′-TTATTTGGAATGGGGTG-GTGGAAGA-3′) were used to amplify the full-length ORF of *AtOFP1*. *ACTIN2* (*ACT2*) (amplified by primers 5′-CCAGAAGGATGCA-TATGTTGGTGA-3′ and 5′-GAGGAGCCTCGGTAAGAAGA-3′) or *UBIQUITIN10* (*UBQ10*) (amplified by primers 5′-GATCTTTGC-CGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAG-AAAGAAAGAGATAACAGG-3′) were used as controls in PCR reactions.

Western blotting

Anti-HA rabbit antisera (Abgent, www.abgent.com) was used to examine HA-AtOFP protein levels in the transgenic plants overexpressing *HA-AtOFP1*. Ten-day-old seedlings grown on MS/G plates were collected and frozen in liquid nitrogen. The frozen seedlings were ground directly in SDS sample buffer and boiled for 3 min (Laemmli, 1970). The whole-cell extracts were centrifuged at 10 000 rpm (8609 g) in a microcentrifuge for 3 min, and the supernatants were collected and used for Western blotting according to the procedure described by Wang *et al.* (2005).

Chromatin immunoprecipitation assay

The Chromatin immunoprecipitation (ChIP) assay was conducted according to the procedure described by Lawrence *et al.* (2004), and

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the well-established protocol (protocol reference: PROT12) described by Werner Aufsatz of the Gregor Mendel Institute of Molecular Plant Biology. This protocol is available at http://www.epigenome-noe.net/researchtools/protocol.php?protid=13. Briefly, about 1.5 g of 10-day-old *35S:HA-AtOFP1* seedlings were collected, cross-linked using 1% formaldehyde solution, ground with liquid nitrogen, sonicated using a Branson sonifier for 4×10 sec, 40% duty cycle, and 20% power. Soluble chromatin was subject to ChIP using anti-HA antibodies (Abgent), anti-acetyl-histone H3 (Lys9) rabbit antiserum (anti-H3-K9 Ac) (Upstate , catalog number 07-352, www.upstate.com) or rabbit pre-immune sera. Chromatin–antibody complexes were collected using salmon sperm DNA/protein A agarose (Upstate, catalog number 16–157). DNA–protein crosslinks were reversed at 65°C overnight. DNA was purified and used in PCR reactions. Primer pairs used for PCR were: AtGA20ox1promFW (5′-GTAGCAAGACTAATACGAGTCCG-3′) and AtGA20ox1promRV (5′-CAAGATTACTTAGTGGCTGGGTG-3′), AtGA20ox1intronFW (5′-ATTACTTCTGCGATGCGTTGGG-3′) and AtGA20ox1intronRV (5′-AGAAGCTCCATGATCTTCAGTG-3′), and At2g02850FW (5′-CCAAGGGAAGAGGCAGTGCATC-3′) and At2g02850RV (5′-TTGCCCATACTTAGAAGCAGCCAT-3′).

Acknowledgements

We thank Dr Alan Jones (University of North Carolina at Chapel Hill, USA) for his support of the early phase of the mutant screen work, Drs Tom Guilfoyle, Gretchen Hagen and Shiv Tiwari (University of Missouri-Columbia, USA) for providing transactivator and reporter constructs used in the protoplast transfection assays, the Arabidopsis Biological Resources Center, the Exon Trapping Insert Consortium and Dr Joachim Uhrig (University of Cologne, Germany) for providing Arabidopsis mutant seeds, Dr Zhenlan Liu for technical help with the ChIP assay, Tom Malcolm for technical help with the luciferase activity assay, and Dr George Haughn for critical reading of the manuscript and invaluable comments. Work in J.-G.C.'s lab is supported by grants from the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, the British Columbia Ministry of Advanced Education, and the University of British Columbia. Y.C. is supported by a scholarship from Northeast Agricultural University.

Upon request, all novel materials described in this paper will be made available in a timely manner for non-commercial research purposes.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Recapitulation of *Atofp1-1D* phenotypes

Figure S2. Comparison of epidermal cells in cotyledon, leaf blade and leaf petiole of wild-type Col-0 and plants overexpressing *AtOFP1*

Figure S3. T-DNA insertion mutants of *AtOFP1*

Figure S4. Overexpression of *AtOFP* genes

Figure S5. GA responsiveness of plants overexpressing *AtOFP1*

Figure S6. GA responsiveness of *35S:HA-AtOFP1* plants

This material is available as part of the online article from http://www.blackwell-synergy.com

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