PETAL LOSS, a trihelix transcription factor that represses growth in Arabidopsis thaliana, binds the energy-sensing SnRK1 kinase AKIN10

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Abstract

Organogenesis in plants involves differential growth. Rapidly growing primordia are distinguished from the meristem and each other by slower growing boundaries. PETAL LOSS (PTL) is a trihelix transcription factor of Arabidopsis that represses growth in boundaries between newly arising sepals. To identify partners involved in this growth limitation, a young inflorescence cDNA library was screened by yeast two-hybrid technology with PTL as bait. The most frequent prey identified was AKIN10, the catalytic α-subunit of the Snf1-related kinase1 (SnRK1). Interaction was mapped to the C-terminal (non-kinase) half of AKIN10 and the N-terminal portion of PTL. Binding of PTL was specific to AKIN10 as there was little binding to the related AKIN11. The interaction was confirmed by co-immunoprecipitation in vitro. Fluorescently tagged products of 35S:YFP-AKIN10 and 35S:CFP–PTL also interacted when transiently expressed together in leaf cells of Nicotiana benthamiana. In this case, most of the cytoplasmic AKIN10 was preferentially moved to the nucleus where PTL accumulated, possibly because a nuclear export sequence in AKIN10 was now masked. During these experiments, we observed that AKIN10 could variably accumulate in the Golgi, shown by its co-localization with a tagged Golgi marker and through its dispersal by brefeldin A. Tests of phosphorylation of PTL by AKIN10 gave negative results. The functional significance of the PTL–AKIN10 interaction remains open, although a testable hypothesis is that AKIN10 senses lower energy levels in inter-sepal zones and, in association with PTL, promotes reduced cell division.

Key words: AKIN10, Arabidopsis, Golgi, PETAL LOSS, SnRK1, transcription factor, trihelix.

Introduction

Organogenesis in plants is associated with the lateral outgrowth of primordia from the periphery of meristems. In the shoot apical meristem, boundaries develop between leaf primordia and the meristem as the leaves arise. In the flower meristem, similar boundaries occur between newly arising whorls of floral organs and the meristem, as well as between organs of the same type within whorls. A common property of boundary regions is a reduced rate of cell proliferation (Breuil-Broyer et al., 2004). This is associated with reduced levels of the growth-promoting hormone auxin (Heisler et al., 2005) and brassinosteroids (Gendron et al., 2012).
The genetic control of boundary formation has been investigated. In most cases, the genes identified encode transcription factors (reviewed by Žádníková and Simon, 2014). One class encodes NAC transcription factors, involving three CUP-SHAPED COTYLEDON (CUC) genes in Arabidopsis (Aida et al., 1997). The expression of CUC1 and CUC2 is downregulated by auxin (Furutani et al., 2004; Heisler et al., 2005) and brassinosteroids (Gendron et al., 2012). This does not occur in boundary regions where hormone levels are lower, thus allowing their probable function to repress cell division.

**PETAL LOSS (PTL)** is a boundary gene of Arabidopsis involved in promoting boundaries between developing sepals (Griffith et al., 1999). It encodes a transcription factor of the trihelix family, and is most strongly expressed between newly arising sepals (Brewer et al., 2004). Loss of PTL function results in some outward overgrowth of the inter-sepal zone (Lampugnani et al., 2012). On the other hand, ectopic expression of PTL leads to suppression of growth in any tissue where it is abnormally present (Brewer et al., 2004). Thus, PTL apparently normally limits cell division in the inter-sepal zone, acting to maintain an appropriate boundary. Genetic studies of interactions between PTL and CUC1 indicated that CUC1 functions differently, repressing upward rather than outward growth in the inter-sepal zone (Lampugnani et al., 2012). Similar genetic investigation of PTL interactions with RABBIT EARS (RBE), a zinc-finger transcription factor active in perianth development, suggested that it also represses tissue growth in this region, although acting independently of PTL (Lampugnani et al., 2013).

Despite its name, it seems likely that PTL influences petal initiation only indirectly. Some petals are initiated in early-formed flowers in *ptl* null mutant plants indicating that PTL function is not absolutely required (Griffith et al., 1999). Also, *PTL* is not expressed in petal anlagen or primordia (Lampugnani et al., 2012). Genetic interactions with auxin transport mutants revealed that loss of PTL function sensitizes the flower primordium to distortions in auxin dynamics such that almost no petals are formed in double mutants (Lampugnani et al., 2013). Significantly, generation of auxin in the PTL expression zone can rescue petal initiation in *ptl* mutant plants. It may be that overgrowth of the inter-sepal zone in *ptl* mutants is responsible for secondarily disrupting the movement of auxin as a petal initiation signal.

During a search for partners of the PTL protein in defining the inter-sepal zone, we uncovered a specific protein–protein interaction with the energy-sensing kinase AKIN10. AKIN10 acts as a global sensor of energy deprivation, downregulating energy-requiring metabolism and triggering the activation of catabolic processes (Baena-González et al., 2007). AKIN10 encodes a kinase α-subunit of Snf1-related kinase 1 (SnRK1), and is also known as *KIN10*, *SnRK1.1* and *SnRK1a1*. Snf1 itself is a sugar-non-fermenting protein kinase of yeast that plays a parallel role in regulating metabolism. The process is highly conserved, as equivalent AMP-activated kinases (AMPK1/2) also occur in mammals and are closely related in structure and function to Snf1 and SnRK1 (Polge and Thomas, 2007; Hedbačker and Carlson, 2008; Halford and Hey, 2009; Ghillebert et al., 2011; Hardie, 2012; Crozet et al., 2014). AKIN10, and another SnRK1 kinase α-subunit gene AKIN11, are expressed ubiquitously and at similar levels in Arabidopsis, although a third α gene, AKIN12, is expressed at lower levels and only in pollen, developing embryos, and seeds (Schmid et al., 2005). AKIN10 is activated by specific upstream kinases in response to energy deprivation (Sugden et al., 1999). This activation is inhibited by trehalose-6-phosphate, a signal of energy sufficiency (Zhang et al., 2009). AKIN10 can then phosphorylate key metabolic enzymes to immediately influence their activity. In addition, it has a longer-term effect on global gene expression through its regulation of specific transcription factors, including several basic leucine zippers that bind G boxes in promoter regions of upregulated target genes (Baena-González et al., 2007). The mechanism of activation of these bZIPs by AKIN10 is not clear, but AKIN10 has been shown to phosphorylate and activate FUSCA3 (FUS3), a transcription factor of the B3 class involved in embryo maturation (Tsai and Gazzarrini, 2012).

In the present study, AKIN10 and PTL were shown to bind each other in yeast, in vitro, and following transient expression in planta. In the latter, much of the cytoplasmic AKIN10 was relocalized to the nucleus, presumably bound to PTL, which accumulates there. The functional significance of this interaction was explored.

**Materials and methods**

**Preparation of cDNA library**

Wild-type Columbia (Col-0) plants were grown at 20 °C under short days (9 h light/15 h dark) for 30 d, and then transferred to long days (16 h light/8 h dark) for 14 d. Primary inflorescence tips with buds no older than stage 5 (Smyth et al., 1999) were dissected from surrounding leaves and the basal stem using a 26-gauge needle and snap frozen in liquid N₂. Total RNA was extracted using an RNasy Plant Minikit (Qiagen) and treated with DNase I (Ambion). A cDNA library was generated using a Clontech Matchmaker Library Construction and Screening kit. Activation domain (AD)-tagged cDNA inserts in plasmid pGADT7-Rec were generated (~2.2×10⁴ clones) and amplified in yeast strain AH109.

**Yeast two-hybrid experiments**

The Clontech Matchmaker Two-Hybrid System 3 was used. For library screening, binding domain (BD)-PTLAC1 bait (plasmid pGBKTT7) with the C-terminal AD removed (Kaplan-Levy et al., 2014) in cells of yeast strain Y187 were mated with the AD-library prey (plasmid pGADT7) in strain AH109, and approximately 4×10⁵–1×10⁶ diploids selected for each mating. 3-Amino-1,2,4-triazole (3-AT; Sigma) was included to suppress leaky growth of His3p and any activation by bait sequences, with the required concentration determined by trial runs. A concentration of 2.5 mM 3-AT was found to be sufficient to inhibit auto-activation by full-length BD–PTL. For pairwise interaction tests, a full-length clone of the protein-coding sequence of AKIN10 (At3g01090.2) was obtained from the Arabidopsis Biological Resource Center (U24028; see Supplementary Table S1 at JXB online for primer sequences). Full-length AKIN11 coding sequence (At3g29160) was obtained from the inflorescence cDNA library by reverse transcription (RT)-PCR and 5’-rapid amplification of cDNA ends. Truncations and deletions

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of PTL used to locate sites of interaction have been described elsewhere (Kaplan-Levy et al., 2014). Activation was quantified using triplicate technical assays of α-galactosidase activity of the MELI reporter in liquid cultures.

Co-immunoprecipitation

Full-length PTL protein tagged with the c-Myc epitope and AKIN10 protein tagged with haemagglutinin were generated from inserts in pGBK7 and pGADT7 plasmid clones, respectively (made for yeast two-hybrid screening), using rabbit reticulocytes (TnT T7 coupled system, Promega). AKIN10 was labelled with 35S-methionine (ICN) during synthesis. 35S-AKIN10 was incubated without and with Myc–PTL, and PTL was immunoprecipitated using Myc antibody following the instructions for the Matchmaker Co-IP kit (Clontech).

Generation and expression of pAKIN10:GUS reporter lines

The 1035bp region from the stop codon of the upstream gene (At3g01100.1) to the first methionine codon in the second exon of AKIN10 (as in splice form At3g01090.2) was amplified from genomic DNA (see Supplementary Table S1 for primer sequences) and translationally fused with the first methionine of the β-glucuronidase (GUS) gene in plasmid pRITA. This full-length insert was labelled pAKIN10a:GUS. Three smaller versions of the insert were generated, carrying sequences from the 5′ end to the transcription start site of AKIN10 (526bp, pAKIN10b:GUS), from the end of the 3′-untranslated region of the upstream gene to the transcription start site (232bp, pAKIN10c:GUS), or from this start site to the 3′ end (509bp, pAKIN10d:GUS). The inserts were excised with NotI and inserted into pMLBART, transferred to Agrobacterium tumefaciens strain AGL1, and transformed into Columbia wild-type plants. For each insert, 12 independent T1 transformants were selected that yielded consistent GUS staining levels and patterns in at least seven transformants using a method reported previously (Brewer et al., 2004). To avoid product leaching, 6 mM potassium ferri- and ferrocyanide was included.

Generation and transient expression of fluorescently tagged versions of AKIN10 and PTL

To test the interaction between AKIN10 and PTL proteins, our modification of intracellular localization (MILo) method was used (Kaplan-Levy et al., 2014). This involves tagging potential partner proteins that occupy different cellular compartments with different fluorochromes. If the fluorescent tag of one protein is moved from one cellular compartment into that occupied by the other protein, this indicates an interaction. To test the interaction between AKIN10 and PTL proteins, our modification of intracellular localization (MILo) method was used (Kaplan-Levy et al., 2014).

Kinase assay

PTL protein tagged with the c-Myc epitope was generated from the pGBK7 plasmid clone using a rabbit reticulocyte system as above. The AKIN10 sequence was inserted into plasmid pQE30 (see Supplementary Table S1 for primer sequences), expressed in Escherichia coli strain M15 cells, and tagged with a 6×His epitope purified using nickel beads (Ni-NTA kit; Qiagen). The two protein preparations were incubated together with [γ-32P]ATP (ICN) and separated by 10% SDS-PAGE. Prior to Western blot transfer, the gel was wrapped in Clingwrap and autoradiographed. The gel was then blotted and the membrane treated with mouse monoclonal anti-Myc antibody (Co-IP kit; Clontech), followed by rabbit anti-mouse antibody tagged with horseradish peroxidase, which was detected by luminol chemiluminescence using an ECL Prime Western Detection kit (Amersham). A duplicate gel run in parallel was similarly blotted, treated with mouse monoclonal anti-His (Qiagen), and visualized as above.

Results

PTL binds AKIN10 in yeast and in vitro

To search for partners of the PTL protein, yeast two-hybrid screens were performed. A cDNA library was made from dissected inflorescences with buds no older than stage 5 (Smyth et al., 1990) and combined with the yeast GAL4 AD. This was co-expressed with BD–PTLAC1 as bait, in which the endogenous PTL activation region at the C terminus had been deleted (Fig. 1A). An initial screen was carried out under stringent selective conditions for HIS3 activity (25 mM 3-AT). Prey inserts of 12 strongly growing colonies were sequenced, and nine contained sequences encoding AKIN10 (At3g01090) (Fig. 1B). Three other screenings of the same library using the same bait under more relaxed selection conditions (5, 1, and 0 mM 3-AT), and choosing colonies of a range of sizes, also yielded predominantly AKIN10 clones. Of the inserts in 48 colonies sequenced, eight were AKIN10, representing two different cDNA insertions, commencing at codon E243 or L302, the latter downstream of the kinase domain (Fig. 1B). When inserts of a larger sample of 447 clones from these three screenings were probed with an AKIN10-specific sequence, 97 (22%) hybridized.

To further test the PTL–AKIN10 interaction, prey containing full-length coding sequences of AKIN10 (from splice variant 2) was generated (AD–AKIN10) and tested for interaction with full-length BD–PTL as bait. As this bait retained the PTL AD, we used 25 mM 3-AT to prevent growth of control strains carrying the bait alone. BD–PTL and AD–AKIN10 showed strong growth, confirming their interaction
The reverse combination, with BD–AKIN10 as bait and full-length AD–PTL as prey, also activated reporter genes, although somewhat more weakly (Fig. 2A).

To locate the region within PTL that interacts with AKIN10 in yeast, several partially deleted versions (Fig. 1A) were tested as bait against AD–AKIN10 prey. Whereas deletions of the C-terminal region from codon 417 (ΔTC), or the mid-region (codons 205–416, ΔM), still showed a strong interaction, loss of the N-terminal region (up to codon 205, ΔN) did not (Fig. 2A). Thus, in yeast cells the interaction domains fall in the N-terminal third of PTL (Fig. 1A).

PTL and AKIN10 also interacted in vitro. This was established by co-immunoprecipitation of 35S-labelled AKIN10 with epitope-tagged Myc–PTL when challenged with an anti-Myc antibody (Fig. 2B).

**AKIN11 interacts specifically with AKIN10**

AKIN11 (At3g29160) encodes an alternative α-subunit of SnRK1. Its product is closely related to AKIN10, showing 82% identity and 90% similarity at the protein level (excluding exon 1 present in At3g01090.2). Both genes are ubiquitously expressed and at similar levels (Schmid et al., 2005). However, AKIN11 was not detected in our screens, although we confirmed the presence of AKIN11 transcripts in the inflorescence cDNA pool by RT-PCR. Thus, we were interested to examine whether AKIN11 was also able to interact with PTL.

A full-length open reading frame of AKIN11 was cloned into the prey vector (AD–AKIN11) and combined with PTL bait lacking the activation region (BD–PTLΔC1). Growth of yeast cells carrying both constructs was assessed on selective medium through the activity of the α-galactosidase protein in the host yeast strain Y187 (Fig. 2C). AD–AKIN11 activation was more than 10 times lower than for AD–AKIN10, and only slightly higher than the negative control. Thus, a strong interaction with PTL is specific to AKIN10.

AKIN11 is expressed in developing tissues and overlaps with PTL expression domains in floral primordia

For AKIN10 and PTL to physically interact, the transcripts and hence proteins must accumulate in the same cells (if they do not move between cells). To determine the tissue expression pattern of AKIN10 in developing buds, the upstream promoter region (including the 3′-untranslated region of the adjacent gene) and transcribed AKIN10 sequences down to

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*Fig. 1.* Maps of PTL and AKIN10, showing the results of yeast two-hybrid interactions. (A) Map of the PTL protein, showing the conserved trihelix DNA-binding domains, the central dimerization domain, and two terminal domains. BD–PTLΔC1, used in the library screen, removes a C-terminal activation region. The extent of other deletions used in yeast two-hybrid tests, and the results of these tests with AD–AKIN10, are shown. (B) Map of the AKIN10 protein, with functional domains predicted by Pfam. These are a kinase domain that includes a phosphorylated threonine at codon 198 (green), a ubiquitin-associated domain (UBA), and a C-terminal kinase-associated domain (KA1). The last 15 residues closely match a conserved nuclear export sequence (NES) (Kazgan et al., 2010). Its sequence in Arabidopsis (AKIN10/11/12), yeast (SNF1), and human (AMPKα1/2) proteins is shown, with conserved bulky hydrophobic amino acids (L, I, F, V, and M) indicated in red, and the predicted amphipathic α-helical region underlined in AKIN10. The overall map is based on splice form At3g01090.2, which includes an additional 5′ exon. The first methionine of the product of two other splice variants, At3g01090.1 and At3g01090.3, is indicated (M24). The extent of two truncated cDNA clones from a cDNA library that interacted with PTL in yeast cells is shown. Blue bars indicate the regions of AKIN10 and PTL required for their interaction.
the methionine in the second exon (codon 24) were translationally fused with GUS (Fig. 3A) and inserted into plants. Regions both upstream and downstream of the transcriptional start site were found to be required for strong expression (Fig. 3A and Supplementary Fig. S1 at JXB online).

AKIN10 was widely expressed, especially in newly developing tissues (Fig. 3B–F). It was only weakly expressed in the inflorescence meristem and bud primordia up to stage 2, except for the epidermis. Later, it was more strongly and evenly expressed throughout buds as floral organs developed (sepals from stage 3; petals and stamens from stage 5). As the organs grew out from stage 7, expression remained relatively high, although at lower levels in mature tissues.

By contrast, PTL expression in inflorescences was more restricted (Fig. 3G) (Brewer et al., 2004). It was not expressed in the inflorescence meristem itself. In flower primordia, it was expressed in regions associated with reduced growth, at first on each side of the flower primordium from stage 1, and then internal to the anlagen of the lateral sepals at stage 2 (Fig. 3G, arrows). Its strongest expression occurred as four spots around the equator of the bud between newly arisen sepal primordia from stage 3 to stage 6. From stage 6 to stage 9, it was also expressed at the basal margins of developing sepals. All these regions corresponded with significant AKIN10 expression (Fig. 3B–F), although AKIN10 expression also occurred more widely.

AKIN10 accumulates in the cytoplasm (including the Golgi) and variably in nuclei when transiently expressed in tobacco leaves

Before testing if PTL and AKIN10 proteins associate when both are transiently expressed in plant tissues, the cellular localization of AKIN10 was examined, as this is the subject of conflicting reports (Fragoso et al., 2009; Bitrián et al., 2011; Tsai and Gazzarrini, 2012; Williams et al., 2014). The fluorescently tagged construct 35S:YFP–AKIN10 was Agrobacterium tumefaciens infiltrated into leaves of N. benthamiana and expression was observed by confocal microscopy. Significant accumulation was observed close to the plasma membrane, as well as more widely in the cytoplasm (Fig. 4A). Nuclear accumulation was also consistently seen. This was somewhat variable in relative intensity among experiments, although replicate leaves within an experiment gave closely similar results. Nuclear localization was not anticipated because AKIN10 carries a conserved nuclear export sequence (NES), as found in orthologous Snf1 and AMPK kinases from yeast and mammals (Fig. 1B) (Kazgan et al., 2010).

In some cells, fluorescent punctate bodies were visible in the cytoplasm (Fig. 4A, D). On occasion, they were seen to move rapidly along linear pathways, and this behaviour, in addition to their number and size, suggested that they were Golgi bodies. To test this, we co-infiltrated 35S:YFP–AKIN10 with d35S:G–ck, a fluorescent CFP marker specific for Golgi apparatus (Fig. 4B, E) (Nelson et al., 2007). Merged images showed that the punctate bodies coincided with the Golgi (Fig. 4C, F; Supplementary Movie S1). Furthermore, the bodies were lost if the excised infiltrated leaf segments
were incubated in brefeldin A, a specific disruptor of Golgi structure (Satiat-Jeunemaitre and Hawes, 1992), before viewing (Fig. 4G–I). Thus, AKIN10 can accumulate in the Golgi when transiently expressed in leaf pavement cells.

PTL and AKIN10 also interact when transiently co-expressed in tobacco leaves

35S:YFP–AKIN10 is found predominantly in the cytoplasm, whereas PTL accumulates in the nucleus (Kaplan-Levy et al., 2014). To test if the two proteins bind each other, we examined their intracellular localization when co-expressed in the same cell to determine if they both now occupied the same compartment.

To differentially tag PTL and to ensure its strong nuclear localization, we added CFPN7 (CFP carrying the strong nuclear localization sequence N7; Cutler et al. 2000) to generate 35S:CFPN7–PTL. First, we showed that 35S:CFPN7 alone (i.e., without the PTL sequence) (Fig. 5B) did not affect localization of the YFP fluorescence pattern associated with AKIN10 (Fig. 5A, compare with Fig. 4A). However, when PTL was added (35S:CFPN7–PTL) (Fig. 5D), the YFP–AKIN10 fluorescence was now much stronger in the nucleus (Fig. 5C). To model the natural situation more closely, we generated a 35S:CFP–PTL construct without the added N7 NLS, thus relying on the endogenous NLS sequences in PTL (Fig. 5E) (Kaplan-Levy et al., 2014). In this case, too, the PTL sequences were able to localize much of the otherwise cytoplasmic AKIN10 to the nucleus (Fig. 5F). The conclusion is that AKIN10 and PTL proteins interact when both are transiently expressed in N. benthamiana leaves, and that the AKIN10 protein is predominantly carried into and retained in the nucleus as a consequence.

Phosphorylation of PTL is not detected in vitro

As a kinase, AKIN10 may bind PTL in order to phosphorylate it. To test this, AKIN10 protein, tagged with a 6×His epitope tag, was produced in E. coli and purified. It was then combined with a preparation of PTL protein tagged with a c-Myc epitope made using a rabbit reticulocyte translation system, and the combination was supplied with [γ-32P]ATP. Phosphorylation of AKIN10 was required for its activation, and phosphorylation of a protein band the size of AKIN10 was clearly apparent in this system (Fig. 6A, C). However, there was no indication of label associated with any protein similar in size to epitope-tagged PTL (Fig. 6B).
**Discussion**

**PTL and AKIN10 proteins specifically bind each other**

We have shown that the PTL protein binds AKIN10 under three conditions: in yeast, *in vitro*, and when co-expressed in leaf pavement cells of *N. benthamiana*. The functional significance of this interaction was tested in several ways.

One possibility is that PTL is a substrate of the AKIN10 kinase. However, tests of phosphorylation of PTL by AKIN10 proved negative, although AKIN10 itself was probably phosphorylated in the *in vitro* test system used. PTL lacks the strict consensus recognition sequence of AMPK/SnRK1 serine/threonine kinases (Weekes et al., 1993), and it may instead act as a bridge between AKIN10 and another component that is phosphorylated. A second transcription factor, FUSCA3, also binds AKIN10 in its C-terminal half (Tsai and Gazzarrini, 2012). In this case, phosphorylation of FUSCA3 by AKIN10 has been demonstrated, and a functional link was shown to be likely, as the growth consequences of overexpression of AKIN10 were reduced in plants that had lost FUSCA3 function. Similar tests of the effect of loss of PTL function in AKIN10 overexpressing plants would be worthwhile.

Functional significance was indicated by the finding that PTL bound AKIN10 but not AKIN11 in yeast two-hybrid tests. This specificity may reflect functional differences and depend on sequence divergence in the two α-kinase subunits. Both AKIN10 and AKIN11 carry strongly conserved ubiquitin-binding domains just downstream of the kinase domain (Farráš et al., 2001), and kinase-associated domains (KA1) near the C terminus that interact with the β-subunits. They also share an NES at the C terminus (Fig. 1B; Kazgan et al., 2010). However, there is a region of around 130 aa between the ubiquitin-binding domains and KA1 domains (Fig. 1B) that has lower sequence conservation (64% identical, 72% similar) (Bhalerao et al., 1999) that may be responsible for the PTL binding specificity. Even so, in other yeast two-hybrid tests, AKIN10 and AKIN11 bind equally well to most members of the domain of unknown function DUF581 family of proteins that contain a putative C4 zinc finger (Nietzsche et al., 2014). Furthermore, functional studies of AKIN10 and AKIN11 suggest that they act redundantly in energy sensing.
O’Brien et al. (Baena-González et al., 2007), although consequences of their overexpression may differ somewhat (Williams et al., 2014). Further comparison of AKIN10 and AKIN11 binding with PTL in vitro and in transient expression assays are needed. It would also be worthwhile testing the specificity of AKIN10 and AKIN11 binding to other trihelix proteins, related to PTL but differing in the N-terminal binding region (Kaplan-Levy et al., 2012, 2014).

If AKIN10 and PTL together regulate inter-sepal zone growth, then similar single mutant phenotypes would be expected. Loss-of-function phenotypes of AKIN10 have not been reported to date, and T-DNA insertion null mutants were not available at the time of this study (Tsai and Gazzarrini, 2012). Knockdown of AKIN10 function by RNA interference did not show an obvious abnormal phenotype (Baena-González et al., 2007), although subtle petal defects may have been missed. Examination of akin10 loss-function effects on petal initiation, and interactions with ptl loss-of-function mutants, are clearly a high priority for future work.

PTL and AKIN10 each downregulate growth

Despite the lack of any firmly established functional significance, a link between the role of AKIN10 in sensing reduced energy levels with consequent downregulation of growth and the established role of PTL in repressing growth remains a realistic hypothesis.

PTL normally dampens cell division as loss of its function leads to a 35–40% increase of the inter-sepal zone (measured radially) during early stages of flower development without any increase in cell size (Lampugnani et al., 2012). Conversely, ectopically expressed PTL blocks cell division, even when overexpressed in its usual locations (Brewer et al., 2004). AKIN10, too, may act to dampen growth. Whereas downregulation of AKIN10 expression does not seem to affect morphogenesis, its upregulation results in delays to flowering and other phase transitions (Baena-González et al., 2007), as well as growth defects in cotyledon and floral organ morphogenesis (Tsai and Gazzarrini, 2012). It is interesting that disruption of trehalose-6-phosphate levels, a signalling molecule acting negatively on SnRK1 and directly upstream of it (Zhang et al., 2009), also disrupts site-specific growth. Enzymatic degradation of trehalose-6-phosphate is encoded by the RAMOSA3 (RA3) gene in maize, and ra3 mutants show increased axillary development within inflorescences (Satoh-Nagasawa et al., 2006). RA3 is specifically expressed nearby, and the additional T6P accumulating in ra3 mutants might strongly inhibit SnRK1, thus allowing...
AKIN10 can accumulate in the Golgi as well as more widely in the cytoplasm and nuclei.

In this study, we confirmed that AKIN10 is more strongly expressed in newly arising and growing organs than in mature differentiated tissues (Bitrián et al., 2011; Williams et al., 2014). Our inflorescence results also extend the observation made in tomato (Pien et al., 2001) that a kinase subunit gene (LeSNF1) is not expressed significantly in the shoot meristem except for the epidermis.

The intracellular localization of AKIN10 has been variously reported. Bitrián et al. (2011) inserted a large genomic fragment including tagged AKIN10 coding sequence into transgenic Arabidopsis plants by recombining. Although the results were reported at low optical resolution, fluorescence in most growing tissues seemed strongest in the region of the plasma membrane and in a cytoplasmic cloud surrounding the nucleus. Usually, it was not clearly apparent inside the nucleus itself. A similar lack of nuclear accumulation was reported in transgenic Arabidopsis plants expressing 35S:AKIN10 (Williams et al., 2014). Our results, following transient expression in mature leaves of N. benthamiana, revealed accumulation at the plasma membrane and more widely in the cytoplasm, and some nuclear accumulation. Cytoplasmic and nuclear localization has also been reported in similar transient expression studies in intact leaves (Tsai and Gazzarrini, 2012; Williams et al., 2014), leaf protoplasts (Cho et al., 2012), and onion epidermal cells (López-Paz et al., 2009). Thus, nuclear accumulation has been seen consistently in transient assays but less so in stable transgene experiments, and such differences may result from higher transient expression levels resulting in cytoplasmic saturation and consequent movement into the nucleus.

We have evidence that small fluorescent punctate bodies seen by us and others in 35S:AKIN10 transient expression experiments (López-Paz et al., 2009; Tsai and Gazzarrini, 2012; Williams et al., 2014) reflect Golgi bodies. We found that their occurrence is variable between cells and may reflect differing physiological conditions. Consistent with this, Williams et al. (2014) reported that similar bodies (‘small puncta’) developed rapidly in tissues damaged by cutting in both transient and stable expression assays. Tsai and Gazzarrini (2012) observed similar bodies but did not identify them, although they excluded plastids. This is in contrast to a report that 35S:AKIN10–GFP in transgenic Arabidopsis plants is associated with chloroplasts (Fragoso et al., 2009), although different expression systems were involved. Williams et al. (2014) also excluded plastids, but they further excluded Golgi, peroxisomes, and mitochondria. Our finding of co-localization of punctate bodies with Golgi is inconsistent with this. It may be that rapid movement of the particles, apparent in time-course images (see Supplementary Movie S1), has masked their correspondence with a Golgi marker.

It is possible that AKIN10 trafficking in Golgi is simply an abnormal consequence of high levels of expression driven by 35S regulation. This is consistent with their apparent absence in transgenic plants expressing tagged AKIN10 under its endogenous promoter (Bitrián et al., 2011). Alternatively,
such intracellular localization may reflect a natural process only detectable under high expression levels. If this is the case, one possibility is that AKIN10 is normally delivered by the Golgi to the plasma membrane to be associated with β-subunits of SnRK1 that are N-myristylated specifically at this location (Pierre et al., 2007).

Does PTL alter the functioning of AKIN10 by influencing its intracellular localization?

We found that PTL bound AKIN10 and largely sequestered it to the nucleus in transient expression studies. Similar results have been reported for the FUSCA3 transcription factor (Tsai and Gazzarrini, 2012) and for the DUF581 family of nuclear proteins (Nietzsche et al., 2014). A potential mechanism is that PTL and FUS3 at least, by binding to the C-terminal half of AKIN10, mask the conserved NES in this region, thus helping retain it in the nucleus.

In yeast, it is significant that activation of the cytoplasmic α-subunit Snf1 by glucose limitation results in its move to the nucleus in association with the β-subunit Gal83 (Hedderick and Carlson, 2006), where they may regulate expression of many target genes.

However, it is not yet known if nuclear relocalization of AKIN10 has a similar functional significance. AKIN10 clearly has cytoplasmic functions. First, it seems likely that AKIN10 has a similar functional significance. AKIN10 clearly has cytoplasmic functions. First, it seems likely that AKIN10 can undergo a specific interaction with AKIN10 in the nucleus, but further studies are required to establish if this is part of a joint signalling process that dampens growth in the inter-sepal zone where energy is potentially limiting.

Supplementary data

Supplementary data are available at JXB online. Supplementary Table S1. Primers used in the study. Supplementary Fig. S1. GUS reporter gene expression in flowers and inflorescences driven by AKIN10 regulatory sequences. Supplementary Movie S1. Time-lapse series of a confocal optical section through a leaf of Nicotiana benthamiana transiently expressing 35S:YFP–AKIN10 and 35S:CFP–G-ck Golgi markers. Images were collected at 30 s intervals over 5 min.

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