

station ALOHA, samples were amplified by polymerase chain reaction (PCR) with a nested set of degenerate *nifH* primers^{19,20}. The resulting PCR products were cloned and sequenced to characterize the *nifH* sequence diversity. Detailed descriptions of the methods used for the PCR amplification, cloning and sequencing are found in ref. 19.

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Correspondence and requests for materials should be addressed to J.P.M. (j.montoya@biology.gatech.edu).

Control of phyllotaxy by the cytokinin-inducible response regulator homologue *ABPHYL1*

Anna Giulini*, Jing Wang & David Jackson

Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA

* Present address: Department of Plant Production, University of Milan, Via Celoria 2, 20133 Milan, Italy

Phyllotaxy describes the geometric pattern of leaves and flowers, and has intrigued botanists and mathematicians for centuries^{1,2}. How these patterns are initiated is poorly understood, and this is partly due to the paucity of mutants³. Signalling by the plant hormone auxin appears to determine the site of leaf initiation; however, this observation does not explain how distinct patterns of phyllotaxy are initiated⁴. *abphyl1* (*abph1*) mutants of maize initiate leaves in a decussate pattern (that is, paired at 180°), in contrast to the alternating or distichous phyllotaxy observed in wild-type maize and other grasses⁵. Here we show that *ABPH1* is homologous to two-component response regulators and is induced by the plant hormone cytokinin. *ABPH1* is expressed in the embryonic shoot apical meristem, and its spatial expression pattern changes rapidly with cytokinin treatment. We propose that *ABPH1* controls phyllotactic patterning by negatively regulating the cytokinin-induced expansion of the shoot meristem, thereby limiting the space available for primordium initiation at the apex.

Named after their ability to promote proliferation of cultured cells, the cytokinins have diverse roles in shoot morphogenesis^{6,7}. Cytokinin signals are perceived by trans-membrane histidine kinase receptors, which regulate gene expression through transcription factors known as response regulators (reviewed in refs 8, 9). Although the molecular details of this pathway have been described in detail, the precise biological functions of the response regulators are unknown, as loss-of-function mutants do not have striking phenotypes^{10–13}. Here we show that the phyllotaxy mutant *abph1* is caused by a loss-of-function mutation in a cytokinin-inducible response regulator.

abph1 mutants can have opposite leaf (decussate) phyllotaxy throughout their life cycle, and also initiate ears in opposite pairs (Fig. 1a, b). The altered phyllotaxy originates at the shoot apical meristem (SAM) by the initiation of leaf primordia in opposite pairs (Fig. 1c, d)^{5,14}. Our reference *abph1* allele, *abph1-0*, arose spontaneously in breeding stocks⁵. We screened for putative tagged alleles in progeny from crosses of mutant plants onto *Mutator* (*Mu*) or *Suppressor-mutator* transposon lines, and recovered four novel alleles. Co-segregation analysis using one of the *Mu* alleles, *abph1-mum181*, identified a 4-kilobase (kb) *Mu7* hybridizing band in 50 mutant individuals but not in any wild-type siblings or progenitors (not shown). We cloned this band from a sub-genomic library, and amplified the flanking sequences by polymerase chain reaction (PCR). The products contained an entire open reading frame arranged in five predicted exons, with the *Mu7* transposon 3 bp downstream of the predicted start codon (Fig. 1e).

We used this candidate gene as a probe to characterize additional *abph1* alleles. Southern blotting revealed that the three other transposon alleles were deleted for the cloned flanking region (Supplementary Fig. S1b); *Mu* transposons are known to give rise to deletions¹⁵. We were unable to amplify the intact coding region from the *abph1-0* reference allele (supplementary data, Fig. S1d) or to detect transcript from this allele on northern blots (not shown) or by PCR with reverse transcriptase (RT-PCR) (Fig. 2a). We obtained

an additional allele from F. Salamini, and detected a single-nucleotide substitution at the 5' junction of the first intron, which led to mis-splicing or non-splicing of this intron (Supplementary Fig. S1e). In summary, we found molecular lesions in six independent *abp1* alleles, confirming that the cloned gene corresponds to *ABPH1*. Each of the alleles is likely to be a null, as we failed to detect normal *ABPH1* transcript in any of them.

The predicted coding sequence of *ABPH1* was identical to *Zea mays* response regulator 3 (ZmRR3)¹⁶, which is related to a

cytokinin-inducible response regulator, although expression of that gene was not inducible by cytokinin in leaves. As our first description of *ABPH1* pre-dates this description, we continue to use the name *ABPH1* (ref. 5). The predicted protein product is 15 kDa and is a member of the 'A' class of response regulators that contains a receiver domain but no DNA-binding output domain. An alignment with CheY, a bacterial response regulator, is shown in Fig. 1f, with invariant Asp and Lys residues marked¹⁷. Phylogenetic analysis failed to detect obvious orthology relationships between members

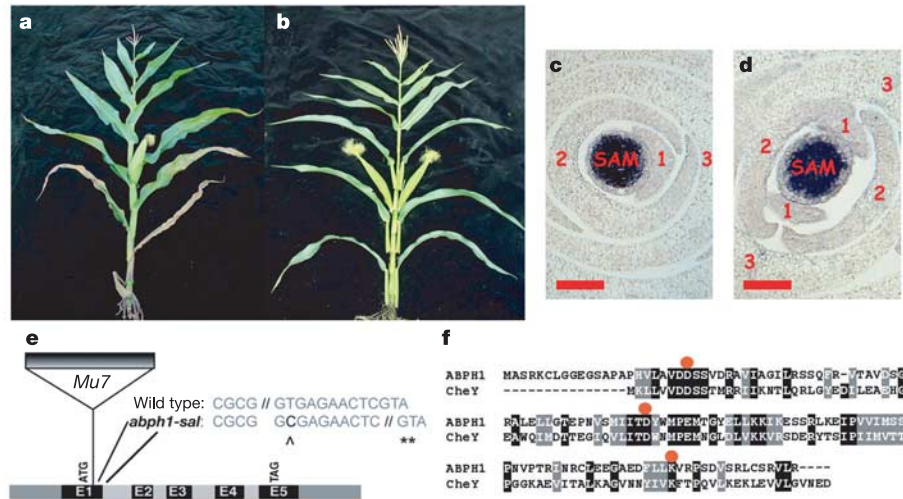


Figure 1 *abp1* phenotypes and gene isolation. **a, b**, Adult normal (**a**) and *abp1* mutant plant (**b**) showing decussate phyllotaxy and two prominent ears in the mutant; some leaves were removed for clarity. **c, d**, The decussate phyllotaxy initiates at the SAM, as seen in transverse section of normal (**c**) and *abp1* mutant (**d**) apices, hybridized *in situ* with *knotted1* probe. Leaf primordia are numbered (with the youngest as 1). Scale bar,

100 μ m. **e**, Map of the *ABPH1* locus showing exons (E1–E5), start ATG and stop TAG codons, the position of the *Mu7* transposon in the *abp1-181* allele, and the sequence of the exon1–intron1 junction in the wild type and *abp1-sal* allele. Asterisks indicate the new intron splice donor site in *abp1-sal*. **f**, Alignment of *ABPH1* and CheY protein sequences, with invariant Asp and Lys residues marked.

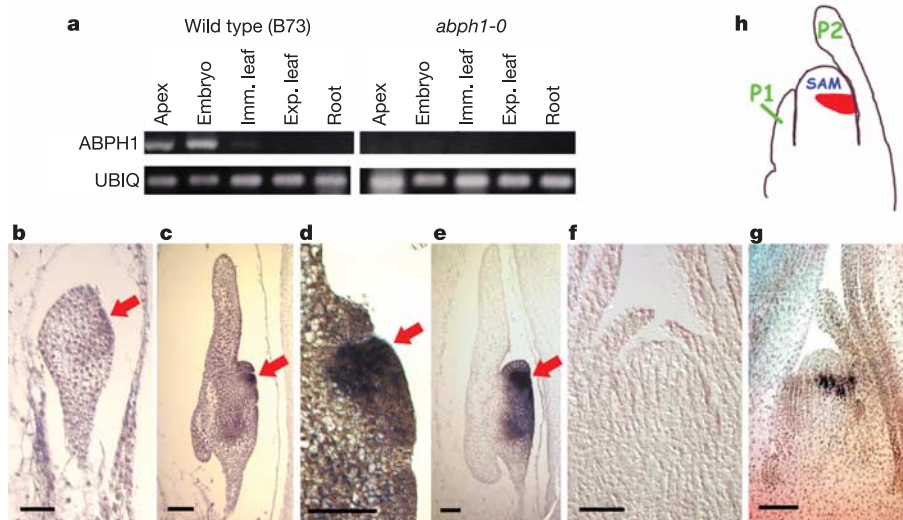


Figure 2 *ABPH1* gene expression. **a**, RT-PCR analysis shows strongest expression of *ABPH1* in shoot apex or embryo, very weak expression in immature (Imm.) leaf, and no expression in expanded (Exp.) leaf or in root. No expression was detected in *abp1-0* mutants. Ubiquitin (UBIQ) control amplifications show equal loading. **b**, *In situ* hybridization shows weak *ABPH1* expression in a transition stage embryo at the site of SAM initiation. **c–e**, Expression is stronger in the coleoptilar stage SAM (**c**), magnified in

(**d**), and persists in the first leaf stage SAM (**e**). Expression of *ABPH1* in **b–e** is marked by red arrows. **f–h**, In 2-week-old seedlings, no expression was detected in a control *abp1* mutant apex (**f**), and expression overlaps with the incipient leaf primordium in a normal apex (**g**), diagrammed in **h**. P1 and P2 indicate plastochron 1, 2 leaf. Scale bars: 50 μ m (**b, d**); 100 μ m (**c, e–g**).

of the maize and *Arabidopsis* A class response regulator gene families, suggesting that expansion of this gene family may have occurred after the monocotyledon–eudicotyledon divergence¹⁶.

We examined expression of *ABPH1* by RT–PCR. A transcript was present in shoot apex and embryo extracts, was very weak in immature leaf, and not detected in expanded leaf or root. No transcript was detected from the *abph1-0* allele (Fig. 2a). The development of *abph1* mutants first deviates from normal by initiation of a larger SAM in the embryo⁵. Using *in situ* hybridization, we first detected weak *ABPH1* expression at the site where the SAM forms on the flank of the transition stage embryo (Fig. 2b). By the coleoptilar stage (Fig. 2c, d), expression was stronger and again concentrated in the SAM region, and it persisted in the SAM at the first leaf stage (Fig. 2e). As expected, an *abph1* mutant apex had no hybridization signal (Fig. 2f). Later in development, in 2-week-old seedlings that had initiated around 10–15 leaves, *ABPH1* expression was restricted to a small domain of the SAM at the site of the incipient leaf primordium (Fig. 2f, g; see also Supplementary Fig. 2A). However, it did not label the entire incipient leaf primordium, which is marked by KN1 downregulation that fully encircles the SAM¹⁸. An *Arabidopsis* A class response regulator gene is also expressed in the SAM region and its expression correlates with SAM initiation in culture^{19,20}, suggesting that these genes may have similar functions. However, mutations in some of the *Arabidopsis* A class response regulators have relatively subtle effects on morphology, suggesting distinct functions or a higher degree of redundancy in *Arabidopsis* compared to maize¹³. *ABPH1* was also expressed in the female ear inflorescence¹⁶ (Supplementary Fig. 2B), although *abph1* mutants have no obvious phenotype in ears (not shown).

We next asked whether *ABPH1* was induced by cytokinin, as

suggested by homology^{21–23}. Seedling shoots, excised at the root–shoot junction, were incubated in water or in cytokinin for 30–120 min, then dissected into to a ‘shoot’ fraction (apical and axillary shoot meristems, stem and 4–5 young leaf primordia) or a ‘leaf’ fraction (expanding and mature leaves). *ABPH1* expression was induced in the shoot fraction within 30 min of cytokinin treatment, but not in the leaf fraction (Fig. 3a). *ABPH1* expression level was on average threefold and eightfold greater after 30 and 120 min cytokinin treatment, respectively, compared with water controls (mean of eight samples each, using from 10^{−4} M to 10^{−6} M kinetin or benzyladenine).

To investigate whether the localized pattern of *ABPH1* expression in the seedling SAM responded to cytokinin, we performed *in situ* hybridization on cytokinin-treated shoots. After 30 min cytokinin treatment, the *ABPH1* expression domain expanded to form a stripe across the entire SAM (Fig. 3b, compare to Fig. 2g). Longer cytokinin treatment led to additional induction of expression in young leaf primordia and vascular strands within the stem (Fig. 3c), although *ABPH1* expression was not induced in the central zone at the apex of the SAM. Although we do not fully understand the significance of these patterns, they suggest that endogenous *ABPH1* expression in the SAM is regulated either directly or indirectly by cytokinins.

The A class response regulators are thought to act as negative feedback regulators of cytokinin signalling^{8,9}. Therefore *ABPH1* may function by negatively regulating cytokinin signalling in the SAM. To test this hypothesis, we investigated whether we could phenocopy *abph1* mutants by culturing wild-type embryos in the presence of cytokinin. The youngest embryos that we could isolate were at the first leaf stage, and these were cultured in the presence or absence of cytokinin for 5 days, then fixed and cleared and the shoot meristems were measured. As expected, embryos grown on cytokinin showed reduced root growth (Fig. 3d, e)⁶. They also developed significantly larger shoot meristems, on average one-third wider than normal (Fig. 3f, g). The average SAM width for embryos grown without cytokinins was 100.3 ± 5.9 μm, and after growth on 10^{−6} or 10^{−7} M kinetin it was 134.7 ± 12.6 μm (*n* = 15, difference significant at 0.1% level). Interestingly, this increase in SAM size is about the same as in *abph1* mutants⁵. We did not observe changes in phyllotaxy in cultured embryos, which may be because the youngest embryos that we could culture had already initiated the first leaf; thus their phyllotactic pattern was already established.

Our results indicate that cytokinin response regulator signalling controls the establishment of phyllotactic pattern in maize. These findings are consistent with studies linking SAM size with cytokinin concentration²⁴, and confirm a link between cytokinins, meristem size and phyllotaxy that was suggested by studies of *altered meristem programming1* mutants^{25,26}. As the defect in SAM size was phenocopied by culturing embryos on exogenous cytokinin, the simplest model for *ABPH1* function is that it acts in the embryo to restrict cytokinin-induced SAM proliferation. *ABPH1* therefore seems to regulate phyllotaxy by controlling the available signalling space in the meristem, consistent with repressor field theories of phyllotaxy or with biophysical models (reviewed in refs 4, 27–29). *ABPH1* may act upstream of the recently described auxin transport mechanism for determination of leaf position⁴, by controlling the dimensions of the morphogenetic domain in which the spatial coordinates of auxin transport are established. Later in development, localized *ABPH1* expression appears to overlap with that of the putative auxin efflux carrier PIN-FORMED1 at the incipient leaf initiation site⁴, suggesting that cytokinin signalling may also have a more specific role in determination of leaf position. This implies an interaction between cytokinin and auxin signalling, as already described for shoot regeneration⁶. Although future studies are required to test this hypothesis, the discovery of a role for cytokinin response regulation in phyllotaxy brings us a step closer to understanding the origin of these fascinating patterns. □

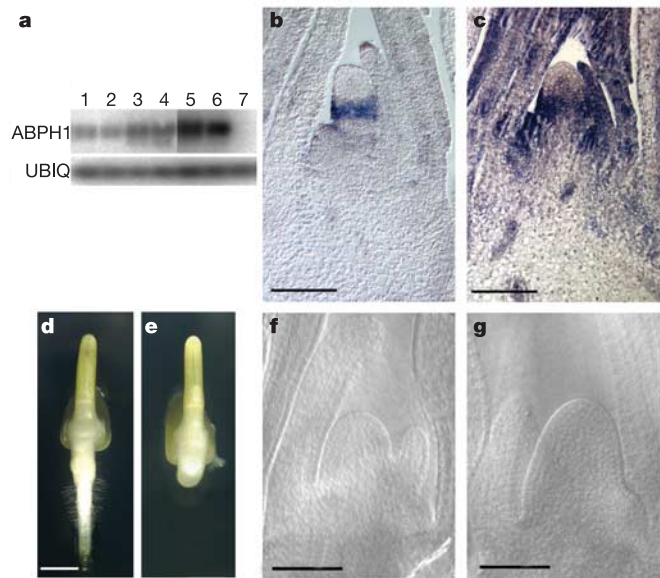


Figure 3 Effect of cytokinin on *ABPH1* expression and shoot meristem size. **a**, Southern blot of an RT–PCR gel of *ABPH1* expression in shoot apex at time zero or 120 min water control (lanes 1, 2), after 30 min treatment with 10^{−4} M or 10^{−5} M kinetin (lanes 3, 4), or after 120 min kinetin treatment (lanes 5, 6). *ABPH1* was not induced in leaf tissues even after 120 min cytokinin treatment (lane 7). The *UBIQUITIN* (*UBIQ*) control is also shown. **b, c**, In shoots treated with cytokinin, the expression domain of *ABPH1* mRNA expands after 30 min (**b**) or 120 min (**c**) treatment. Cytokinin also affects SAM development. **d, e**, Embryos excised at 16 days after pollination were cultured for 6 days in the absence (**d**) or presence (**e**) of 10^{−6} M kinetin. **f, g**, Clearing of the shoot apex revealed that the shoot meristem was bigger in cytokinin-treated embryos (**g**) compared with untreated embryos (**f**). Scale bar: 5 mm (**d, e**); 100 μm (**f, g**); 200 μm (**b, c**).

Methods

Plant materials

Plants were grown in the greenhouse or in the field under standard conditions. We used the B73 wild-type line. For cytokinin induction experiments, 2-week-old seedlings were excised at the shoot–root junction, and stood in water supplemented with different concentrations of cytokinin. After treatments, the seedlings were dissected into a shoot fraction (apical and axillary shoot meristems, stem and 4–5 young leaf primordia) or a leaf fraction (expanding and mature leaves). For embryo culture experiments, pollinated ears at either 13 days after pollination (first leaf stage) or 16 days after pollination (second to third leaf stage) were sterilized in 30% commercial bleach solution for 20 min then rinsed five times with sterile water. The embryos were dissected out and cultured on maize embryo culture medium (1 × Murashige and Skoog salts, 1 × Gamborg's vitamins, 2% sucrose, 0.7% agar, pH 5.7) in some cases with the addition of cytokinin (kinetin, 10^{-5} , 10^{-6} or 10^{-7} M). The embryos were cultured at 28 °C in the dark for 5–7 days then fixed and cleared and the shoot meristems were measured as described⁵.

Molecular biology

Standard protocols were used for maize DNA isolation and Southern blotting³⁰. For RT–PCR analysis, poly(A⁺) RNA was isolated using an Oligotex messenger RNA mini kit (Qiagen) according to the manufacturer's protocol. The primers ZmRR3f (GATGGCGAGCCGCAAGTGT) and ZmRR3r (AATGCCGCTGCTACAGCTACCA) were used to amplify *ABPH1* transcripts using the one step RT–PCR kit (Qiagen). The control primers Ubi 5' (TAAGCTGCCGATGTGCCTGCGTCCG) and Ubi 3' (CTGAAAGACAGAACATAATGAGCACAG) were used to amplify control ubiquitin transcripts. PCR conditions were 94 °C, 15 s, 62 °C, 15 s, 72 °C, 45 s, with a 1 °C reduction in annealing temperature per cycle, touching down at 56 °C annealing, followed by 15 additional cycles. The PCR cycle number was limited to ensure semi-quantitative amplification, and no PCR product was visible on the ethidium-bromide-stained agarose gels. The gels were Southern blotted and probed with an *ABPH1* or *UBIQUITIN* probe. *ABPH1* transcript levels were estimated using a phosphoimager (Fuji) and were normalized against the respective ubiquitin values. *In situ* hybridizations were performed as described⁵.

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Correspondence and requests for materials should be addressed to D.J. (jackson@cshl.edu).

Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB

Sirith Douma¹, Theo van Laar¹, John Zevenhoven¹, Ralph Meuwissen¹, Evert van Garderen² & Daniel S. Peeper¹

¹Division of Molecular Genetics, and ²Department of Experimental Animal Pathology, The Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1066 CX, The Netherlands

Metastasis is a major factor in the malignancy of cancers, and is often responsible for the failure of cancer treatment. Anoikis (apoptosis resulting from loss of cell–matrix interactions) has been suggested to act as a physiological barrier to metastasis; resistance to anoikis may allow survival of cancer cells during systemic circulation, thereby facilitating secondary tumour formation in distant organs^{1–3}. In an attempt to identify metastasis-associated oncogenes, we designed an unbiased, genome-wide functional screen solely on the basis of anoikis suppression. Here, we report the identification of TrkB, a neurotrophic tyrosine kinase receptor^{4,5}, as a potent and specific suppressor of caspase-associated anoikis of non-malignant epithelial cells. By activating the phosphatidylinositol-3-OH kinase/protein kinase B pathway, TrkB induced the formation of large cellular aggregates that survive and proliferate in suspension. In mice, these cells formed rapidly growing tumours that infiltrated lymphatics and blood vessels to colonize distant organs. Consistent with the ability of TrkB to suppress anoikis, metastases—whether small vessel infiltrates or large tumour nodules—contained very few apoptotic cells. These observations demonstrate the potent oncogenic effects of TrkB and uncover a specific pro-survival function that may contribute to its metastatic capacity, providing a possible explanation for the aggressive nature of human tumours that overexpress TrkB.