Development 126, 315-323 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV0206

Control of phyllotaxy in maize by the *abphyl1* gene

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Accepted 3 November; published on WWW 14 December 1998

SUMMARY

Organogenesis in plants occurs at the shoot apical meristem, a group of indeterminate stem cells that are organized during embryogenesis. Regulated initiation of leaves or flowers from the shoot meristem gives rise to the familiar geometric patterns observed throughout the plant kingdom. The mechanism by which these patterns, termed phyllotaxies, are generated, remains unclear. Maize plants initiate leaves singly, alternating from one side to the other in a regular pattern. Here we describe a recessive maize mutant, *abphyl1*, that initiates leaves in opposite pairs, in a pattern termed decussate phyllotaxy. The decussate shoot meristems are larger than normal throughout development, though the general structure and organization of the meristem is not altered. *abph1* mutants are first distinguished during embryogenesis, prior to true leaf initiation, by a larger shoot meristem and coincident larger expression domain of the homeobox gene *knotted1*. Therefore, the *abph1* gene regulates morphogenesis in the embryo, and plays a role in determining the phyllotaxy of the shoot.

Key words: *Zea maize*, Phyllotaxy, a*bphyll* (*abph1*), Meristem, Shoot development

INTRODUCTION

Leaves are initiated on the flanks of the shoot apical meristem, a group of indeterminate stem cells at the growing tip of the shoot (Meyerowitz, 1997; Sussex, 1989). Shoot meristems have a layered structure which is maintained by the propensity of cells to divide within the layer. Independent of this level of organization is the classification of zones in the meristem that differ both structurally and functionally. In the central zone, cells are less densely cytoplasmic and divide relatively infrequently, this zone serves to maintain the meristem as an indeterminate structure. In the peripheral zone on the flank of the meristem, the rate of cell division increases as cells are incorporated into primordia.

Leaf primordia normally arise in regular geometric arrays that are determined by a combination of genetic, environmental and developmental factors (Marc and Hackett, 1991; Schwabe, 1984; Steeves and Sussex, 1989). Leaf arrangements can be classified as one of two types. In whorled phyllotaxy, discrete leaf initiation cycles occupy the whole circumference of the meristem and produce a set number of leaf primordia per whorl. When a single leaf is initiated per whorl, the pattern is alternate or distichous, and when two leaves are initiated per whorl the pattern is termed opposite and decussate. The other type of leaf arrangement is spiral, in which initiation events generate single primordia that do not occupy the whole meristem circumference, and are separated by an angle of approximately 137º from the previous primordium. Although we understand little about how these different patterns are generated, our understanding of the regulation of shoot meristem activity in general has advanced rapidly in recent years by genetic analysis in *Arabidopsis*, maize and other model systems.

Genes that are required for vegetative shoot meristem initiation and/or maintenance include the homeobox gene *knotted1* in maize (Kerstetter et al., 1997), and its *Arabidopsis* homolog *SHOOTMERISTEMLESS* (Long et al., 1996), *ZWILLE* (Moussian et al., 1998), *NO APICAL MERISTEM* (Souer et al., 1996) and *WUSCHEL* (Laux et al., 1996). Mutations in the *CLAVATA* loci have opposing phenotypes in that they result in enlargement of the shoot meristem, and the *clv1* or *clv3* and *stm* mutations dominantly suppress each other, suggesting a balanced quantitative relationship between the gene products (Clark et al., 1996). This observation led to a model proposing that *STM* promotes meristem cell fate and *CLV* promotes the incorporation of cells into primordia, such that a balance of these gene products is required for normal meristem function (Clark et al., 1997). *CLV1* encodes a putative leucine rich repeat receptor kinase (Clark et al., 1997). *Arabidopsis* leaves are arranged in a spiral phyllotaxy, but some *clv* mutants, as well as a number of other *Arabidopsis* mutants, have altered patterns of leaf initiation. In these mutants there is sporadic or irregular leaf initiation rather than a shift to a different phyllotactic pattern (Leyser and Furner, 1992; Medford et al., 1992; Clark et al., 1993, 1995).

The mechanisms that regulate the positioning of primordia in discrete phyllotactic patterns have been studied most intensively through surgical experiments (reviewed in (Medford, 1992; Sussex, 1989). These studies suggest that positioning of primordia is determined by morphogenetic fields in the apex, containing inhibitory signals emanating from the central zone of the meristem as well as from preexisting primordia. According to this model, primordia are positioned at a point that is furthest from the apex and from existing primordia. An alternative view is that biophysical forces in the apex determine organ initiation sites (reviewed by Green, 1987). Support for the latter has come from recent studies showing that application of expansin, a protein that promotes cell wall expansion, to the shoot apex causes localized outgrowths and in some cases can alter the phyllotactic pattern in tomato apices (Fleming et al., 1997; Reinhardt et al., 1998). The models for phyllotactic pattern are generally used to explain the propagation of established patterns, and with a few exceptions do not explain how phyllotaxy arises de novo (Green, 1994).

In maize, as in all grasses, leaves are initiated singly in an alternate or distichous phyllotaxy (Fig. 1A) (Arber, 1925; Barnard, 1964). Variants of maize with altered patterns of leaf initiation have been described, though the inheritance of these characters was non Mendelian, and this phenomenon was therefore termed the abphyl (for **ab**errant **phyl**lotaxy) syndrome (Greyson and Walden, 1972; Greyson et al., 1978). In this paper we report a new recessive mutation of maize, *abphyl1* (*abph1*), that leads to the initiation of leaves in opposite and decussate phyllotaxy. Decussate phyllotaxy is commonly found in other plant species in the Dicotyledon group, but never in the grasses which are in the Monocotyledon group.

MATERIALS AND METHODS

Plant growth and mapping of *abph1*

Seed from plants showing opposite leaf phenotypes were originally obtained as a gift from Dr M. Menzi, Swiss Federal Research Station for Agronomy, Zurich-Reckenholz. The heritability of the opposite leaf phenotype had not been characterized in detail. Seedlings and plants for meristem and leaf measurements were from lines introgressed for two generations into the B73 inbred line, and were grown in the greenhouse under controlled conditions.

For mapping, DNA was prepared from tissue pools from 25 mutants (homozygotes) or wild-type siblings (heterozygotes) from back cross populations segregating in different inbred lines, digested with a range of restriction enzymes and subjected to Southern analysis using maize RFLP probes. Initially, linkage of the mutation was mapped to two RFLP markers on the short arm of chromosome 2, UMC 6 and UMC 131. Subsequent mapping using DNA prepared from individual mutant or normal sib plants placed the *abph1* gene very close (0 recombinants/60) to the RFLP probe UMC 34. (http://www.agron.missouri.edu/).

Scanning electron microscopy

Two-week-old seedlings were dissected by carefully removing the leaves and leaf primordia until the shoot meristem was visible. Impression casts were made using dental impression medium (Exaflex, GC Dental Industrial Corp.). Once set, the casts were removed and filled with 2 ton epoxy resin (ACE Hardware) which was left to set overnight. The resin replicas were removed, mounted on stubs and sputter coated before viewing in the SEM. The replica technique was not suitable for small embryos, so these were dissected out from the seed and fixed in 4% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) then dehydrated through an ethanol series, desiccated in a critical point dryer, mounted on stubs and coated and observed in the SEM.

Meristem measurements

For shoot meristem measurements, longitudinal slices about 1 mm thick were cut from shoot apices of 2-week-old seedlings and fixed in FAA (10% formalin, 45% ethanol, 5% acetic acid) overnight, dehydrated through an ethanol series to 100% ethanol and passed into methyl salicylate (2 parts ethanol: 1 part methyl salicylate, 1 part ethanol: 2 parts methyl salicylate, 100% methyl salicylate, twice, 1 hour each change) followed by direct observation of the meristem using Nomarski optics and measurement with the aid of a reticle. The width of the meristem dome just above the most recently initiated leaf primordium was measured. Root apices were measured following fixation, embedding in paraffin and sectioning; the width of the quiescent center was measured (Feldman, 1993). Inflorescence meristem diameters were measured in the SEM from replica casts.

In situ hybridization

This was performed as previously described for sections (Jackson et al., 1994). The cyclin 1b probe (Renaudin et al., 1994) was transcribed from a PCR fragment that was generated using two gene specific primers, TGTAATACGACTCACTATAGGGCGGTCTGTAGAATGTGT, and TGTCCCGTGTCTTTTCAAGG, that amplified approximately 650 bp at the 5′ end of the cDNA and did not contain the conserved cyclin box (Renaudin et al., 1994). The downstream primer was tailed with the T7 polymerase primer sequence to allow direct in vitro transcription of the PCR product.

For whole-mount in situ hybridization, pollinated ears were harvested at set days after pollination and the embryos were dissected out and were pricked on the scutellum side with a fine tungsten needle to aid probe and antibody penetration (embryos which were not pricked only rarely gave good hybridization signals) then fixed for 2-4 hours in freshly depolymerised 4% formaldehyde (Sigma) in PBS. The whole-mount procedure following fixation was as described by Rosen and Beddington (1993). Embryos probed with control sense strand probes gave no signal (not shown).

RESULTS

The *abph1* mutation causes alterations in vegetative phyllotaxy and segregates as a single recessive locus

The *abph1* trait arose spontaneously and seed was given to us by M. Menzi of the Swiss Federal Research Station for Agronomy, Zurich-Reckenholz. The phenotypic analysis was performed on segregating populations from plants back crossed twice into the B73 maize inbred line (Pioneer Hi-Bred International). Similar segregation ratios and phenotypes were observed in other genetic backgrounds (data not shown). Approximately 50% of *abph1* mutant seedlings had altered phyllotaxy in the first nodes compared to normal seedlings, and the majority of these seedlings were decussate (Table 1, Fig 1B). Decussate plants initiated two fertile ears (female inflorescence shoots) at a single node, in contrast to normal plants which initiate only one ear per node (Fig. 1G). The lower branches of the tassel, or male inflorescence, also showed decussate phyllotaxy in *abph1* plants, whereas in normal plants the first tassel branches were initiated singly (Fig 1G). The number of nodes initiated by decussate *abph1* plants was the same as in their normal siblings, therefore doubling the total number of leaves per plant. Closer observation of older *abph1* decussate shoots revealed that the opposite pair of leaves was indeed inserted at the same node rather than the plants having an alternation of long and short internodes that could give the false impression of decussate phyllotaxy.

Table 1. Frequencies of different *abph1* seedling phenotypes in back cross populations

Cross	Decussate phenotype	Mild phenotype	Others§	Normal phyllotaxy
abph1/abph1 × +/+	0	0	0	216‡
abph1/abph1 × *abph1*/+	59*	66	10	130
	(............................*abph1* total = 135............................)			

*Some of these reverted to normal phyllotaxy. About 20% of mutant plants in these families were decussate throughout their development.
§Includes irregular (presumed spiral) and twin seedlings.
‡In this genetic background a 'twin midrib' on the first leaf is seen in approximately 0.1% of seedlings.
Numbers are the sums from five representative families.

Some decussate seedlings spontaneously reverted to normal, distichous phyllotaxy. This change in phyllotaxy, however, was not abrupt. Rather, an intermediate node with a pair of adjacent, fused leaves was usually observed between the decussate and distichous nodes (Fig. 1C). Other mutant seedlings showed a mild phenotype consisting of a wide first leaf with two midribs, followed by normal phylotaxy in subsequent nodes (Fig. 1F). The twin midrib leaves were often split down the middle between the midribs, suggesting that in fact they represented two leaves that had partially fused along their margin. We interpret the 'twin midrib' leaves and partially fused leaves observed in mild and reverting plants, respectively, as resulting from the simultaneous initiation of two leaf primordia that fused because they were initiated adjacent to each other rather than opposite. Once the phyllotaxy reverted from decussate to normal, it remained normal for the rest of the life of the plant; we never observed a plant that switched from alternate back to decussate phyllotaxy.

Other phenotypes observed at a much lower frequency included 'twin shoots' and 'twisted plants' (Table 1). The twin shoots occurred when a decussate shoot appeared to split in two, and this was always accompanied by a reversion to normal phyllotaxy in both new shoots. We also observed occasional stunted, twisted plants that appeared to have spiral phyllotaxy (not shown).

To follow the heritability of these traits, decussate abphyl plants were crossed into a range of standard maize inbred lines. Self pollination revealed that the abphyl phenotype segregated as a single locus recessive trait. *abph1* was mapped using bulked segregant analysis (Michelmore et al., 1991) to chromosome 2S, bin 04. This map location has been confirmed by linkage to other known mutations on chromosome 2S (data not shown).

abph1 seedlings showed a range of phenotypes, described above, and we showed that all of these are due to the same mutation. First, self or test crossing of plants from any phenotypic class gave progeny that showed the full range of phenotypes associated with the *abph1* mutation, in similar ratios (data not shown). Second, plants from the different phenotypic classes were all homozygous for a restriction fragment length polymorphism closely linked to *abph1*. These findings indicated that the different phenotypes were manifestations of the same mutation, and also that the mild and revertant phenotypes were not the result of a heritable reversion event at the *abph1* locus.

In addition to the change in phyllotaxy, decussate *abph1* plants had narrower leaves compared to their normal siblings. We measured the width of each fully expanded leaf from eight plants of each class; *abph1* decussate phenotype, *abph1* revertant phenotype and normal sib. As shown in Fig. 2, the width of each leaf blade in decussate plants was significantly narrower than their normal siblings, however the shape of the curve plotted through the mean width of successive leaves was similar for each class (Fig. 2). The narrower leaves of decussate plants had normal marginal saw tooth hairs (Scanlon et al., 1996), and therefore did not lack a marginal domain. Rather, they had fewer lateral veins, whose spacing was not significantly different from their normal siblings (not shown). In *abph1* plants which reverted to normal phyllotaxy, the leaf width also was close to normal, especially in later nodes, at the stage where the shoot meristem size of these seedlings was found to be indistinguishable from that of their normal siblings (Table 2).

Decussate *abph1* seedlings have a larger shoot meristem and altered expression of *knotted1*

To visualize the pattern of leaf initiation at the shoot meristem, we used replica scanning electron microscopy (SEM) (Williams and Green, 1988). In the normal maize vegetative apex, leaf primordia are initiated singly on the flank of the shoot meristem, each one opposite the previous leaf. As the leaf primordium enlarges, its base spreads to fully encircle the apex (Fig. 3A). In a decussate *abph1* apex, leaves were initiated in perfectly matched opposite pairs, and each leaf primordium wrapped around the apex only halfway (Fig. 3B). Occasionally, the apex of a seedling with opposite and decussate phyllotaxy was found to be reverting to normal phyllotaxy when dissected

Table 2. Meristem sizes in *abph1* and normal siblings

Phenotype	Genotype	SAM diameter* (µm)	RAM diameter‡ (µm)	IM diameter‡ (µm)
Normal	*abph1*/+	160.3 (14.1)	201.9 (16.7)	380 (65)
Decussate	*abph1/abph1*	214.0 (24.5)	203.9 (32.3)	360 (51)
Mild or revertant	*abph1/abph1*	159.8 (12.3)	ND	ND

Values are the mean (standard deviation)
SAM, shoot apical meristem; RAM, root apical meristem; IM, ear inflorescence meristem.
*>16 individuals, ‡>10 individuals, ND = not done.

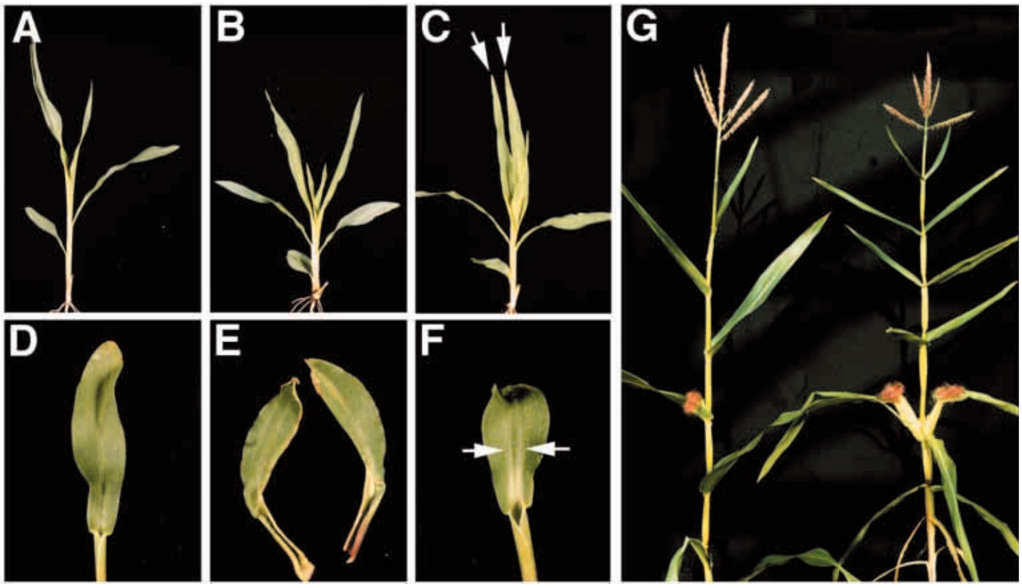

Fig. 1. Altered phyllotaxy in maize plants carrying the *abph1* mutation. (A-C) Seedling phenotypes. (A) a normal maize seedling has alternate or distichous phyllotaxy, with leaves being initiated singly and opposite to the previous leaf. (B) A seedling carrying the *abph1* mutation that has decussate phyllotaxy, with leaves initiated in opposite pairs. (C) An *abph1* seedling reverting from decussate to normal phyllotaxy. Note the presence of a pair of fused leaves (arrows) between a node initiating two opposite leaves and a node initiating a single leaf. (D-F) First leaf phenotypes; (D) a normal first leaf with a single prominent midvein; (E) a decussate pair of first leaves from an *abph1* seedling; (F) a first leaf showing two midveins (arrowed) from a mild phenotype *abph1* seedling. Note that decussate *abph1* seedlings can also have first leaves that are partially fused (as shown in B). (G) Adult plant phenotypes, normal plant on the left, decussate *abph1* plant on the right. The normal plant has alternate phyllotaxy and a single prominent ear shoot (visible by the brown color of the ear silks). The decussate plant has two opposite ear shoots, as well as opposite and decussate leaves at every node and the first tassel (male inflorescence) branches are also decussate. Leaves in a decussate pair always have the same width and length, however the perspective of the photograph can give the false impression that one is shorter.

to the meristem. In this case, one or two nodes after the last decussate node consisted of leaf primordia that were forked at their tips (Fig. 3C). We assume that these primordia resulted from a pair of leaves that were initiated adjacent to each other rather than opposite, causing them to fuse along one edge. These primordia likely gave rise to the partially fused leaves seen at maturity in plants undergoing phyllotactic reversion. *abph1* shoot apices from seedlings of the mild phenotypic class, or from seedlings that had already reverted to normal phyllotaxy appeared morphologically normal (not shown).

The SEM analysis also indicated that the shoot apical

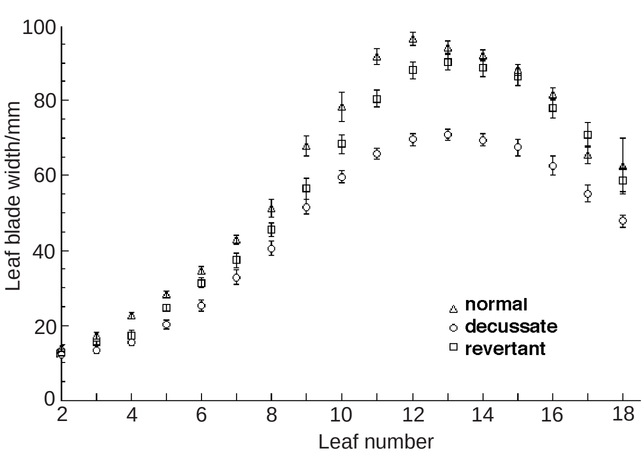

Fig. 2. Leaf width measurements of normal and *abph1* plants. The mean width of each fully expanded leaf from leaf 2 to the last initiated leaf is plotted. The width of every leaf blade from eight plants in each class was measured at the mid point of the blade. Error bars represent the standard error.

meristem of decussate seedlings was larger than that of their normal siblings. We measured the difference in shoot meristem size using cleared apices from decussate, revertant and normal sibling seedlings. The decussate *abph1* shoot meristem was on average about one third wider than that of normal siblings (Table 2). The significant range in shoot meristem size in each class is due to the normal size fluctuation during the leaf initiation cycle (Abbe et al., 1951). The shoot meristems of *abph1* seedlings that had reverted to normal phyllotaxy were the same size as those of normal siblings. Shoot meristem sizes from *abph1* heterozygous seedlings were not significantly different from those of homozygous wild-type siblings (not shown). There was no difference in root meristem size between decussate *abph1* plants and their normal siblings (Table 2), indicating that the effects of the *abph1* mutation were specific to the shoot. We also found no differences in the diameter of the seedling roots or the arrangement or number of lateral roots from normal or decussate seedlings (data not shown). To examine any possible effect of the *abph1* mutation on shoot meristems initiated postembryonically, we measured lateral (ear) inflorescence meristem sizes from decussate *abph1* plants and normal siblings, and found no significant difference (Table 2). In addition, flowers from *abph1* plants were normal with respect to organ number and arrangement (data not shown), indicating that *abph1* specifically affects the primary embryonic shoot apical meristem, and does not affect shoot meristems initiated postembryonically.

To determine how the *abph1* mutation affects pattern formation in the shoot apical meristem, we used the homeobox gene *knotted1* (*kn1*) as a marker. Normally, *kn1* is expressed throughout the shoot meristem but is down regulated in a group of cells on the flank of the meristem opposite the most recently initiated leaf, marking the position where the next leaf will be

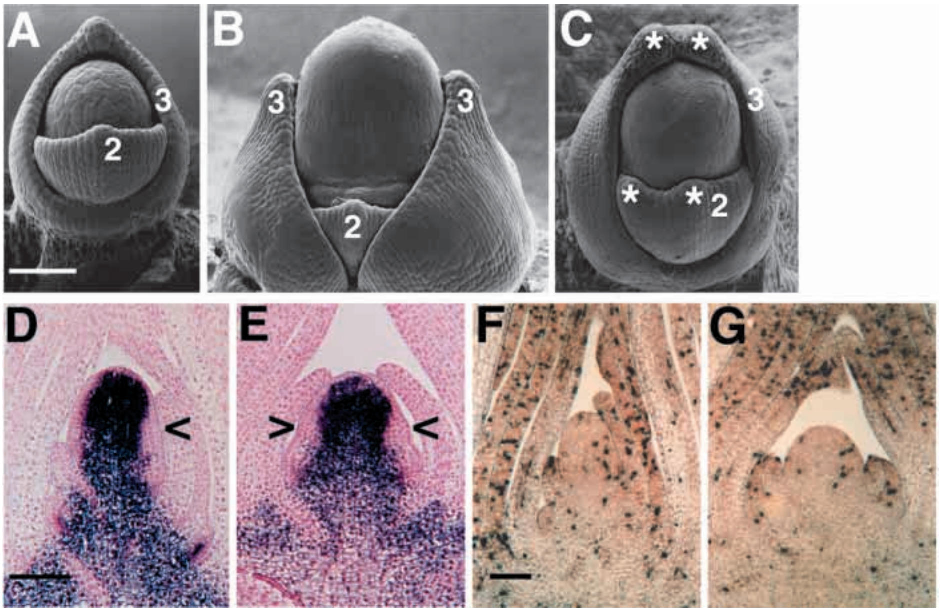

Fig. 3. Initiation of the altered phyllotaxy in *abph1* plants by the shoot apical meristem. (A-C) Scanning electron micrographs of replica casts of normal (A), and *abph1* (B,C) apices from two-week-old seedlings. (A) Normal shoot apex showing the shoot apical meristem (the central dome of tissue) and P2 and P3 leaf primordia (the most recently initiated primordium, the P1, is obscured). (B) A decussate *abph1* shoot apex showing the pair of P3 leaves which are perfectly matched. Only one P2 leaf is visible; the other one is behind the meristem. The ridge of tissue just above the P2 primordium corresponds to the abutted margins of the P1 primordia. (C) An *abph1* shoot apex that is reverting from decussate to normal phyllotaxy. All the leaves dissected from this seedling were opposite and decussate, though at the meristem the phyllotaxy had switched to alternate, and the leaf primordia had split tips (asterisks). Scale bar, 100 µm. (D,E) Sections through shoot apices hybridized in situ to the *kn1* probe. (D) A normal shoot apex shows down regulation of *kn1* expression in a group of cells on the flank of the meristem opposite the most recently initiated leaf, and therefore corresponding to the position of the next leaf initiation event (arrowed). (E) In a decussate *abph1* apex, *kn1* down regulation (arrowed) is evident on both sides of the meristem, corresponding to the positions where the next two leaf primordia will arise. Scale bar, 100 µm. (F,G) Expression of maize cyclin 1b mRNA by in situ hybridization to normal and *abph1* decussate apices, respectively. In both cases the frequency of labelled cells was very low in the central zone compared to the peripheral zone or to the leaf primordia. Scale bar, 100 µm.

initiated (Fig. 3D). Down regulation of *kn1* spreads to encircle the meristem, resulting in a croissant shaped domain lacking *kn1* that corresponds to the presumptive leaf founder cells (Jackson et al., 1994). In meristems from decussate *abph1* seedlings, *kn1* expression was down regulated equally on both sides of the meristem, revealing a pre-pattern that predicts the future phyllotaxy of the shoot (Fig. 2E). Therefore, the *abph1* mutation acts in the shoot meristem to affect *kn1* expression prior to the initiation of leaf primordia. The analysis of *kn1* expression also revealed that the layered structure of the meristem is not altered in *abph1* mutants; KN1 mRNA is expressed throughout the meristem corpus but is not detected in the tunica or L1 layer (Jackson et al., 1994), and this pattern is unaffected in decussate *abph1* meristems (Fig. 3E). In contrast, KN1 protein is also detected in the tunica or L1 cells of normal meristems, and this was the case in *abph1* meristems (not shown).

Shoot meristems are organized into a central zone, which typically has a low mitotic index, and a peripheral zone, from which cells are incorporated into primordia (Wardlaw, 1957). As a probe to reveal relative rates of cell division in the different zones of the meristem, we used a maize cyclin probe that is in a class of genes with predicted function in G2. Related genes in *Antirrhinum* have tightly restricted mRNA accumulation at a specific phase of the cell cycle (Fobert et al., 1994), so the frequency of cells that express this mRNA can be used as an indication of the mitotic index within a particular tissue. Probing apices with the maize cyclin 1b probe revealed

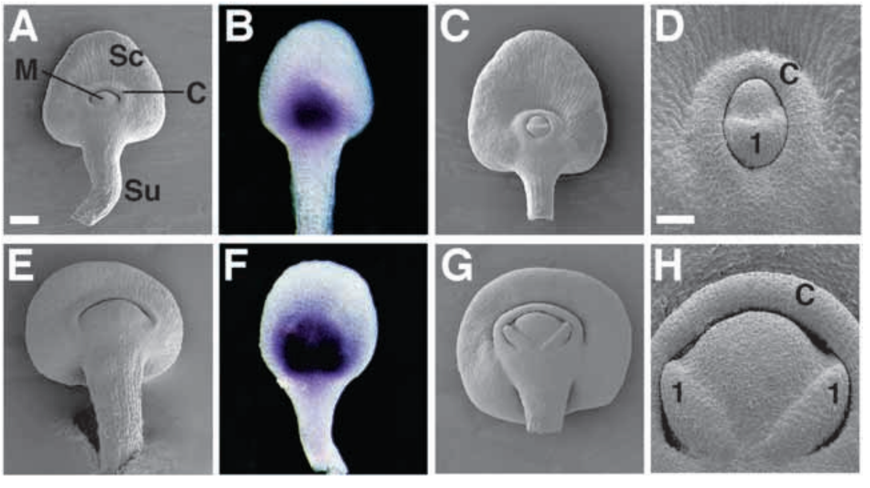

Fig. 4. Development of normal and *abph1* embryos. (A-D) Normal embryos; (E-H) the corresponding stages of *abph1* embryos. (A) Normal and (E) *abph1* embryos at the coleoptilar stage. Sc, scutellum; M, shoot meristem; Su, suspensor; C, coleoptile primordium. The dome of cells bounded by the coleoptile primordium, presumably the shoot meristem, is larger in the *abph1* embryo. (B,F) Whole-mount in situ hybridizations of embryos at the same stage as those in A and E, hybridized with the *kn1* probe. (C,G) Normal and *abph1* embryos at the first leaf stage. The normal embryo is initiating a single first leaf primordium. The *abph1* embryo is initiating a pair of first leaf primordia perpendicular to the normal plane. (D,H) Close up of the meristem region in C and G. C, coleoptile; 1, first leaf primordia. Scale bars A,C,E,G, 100 µm; D,H, 50 µm.

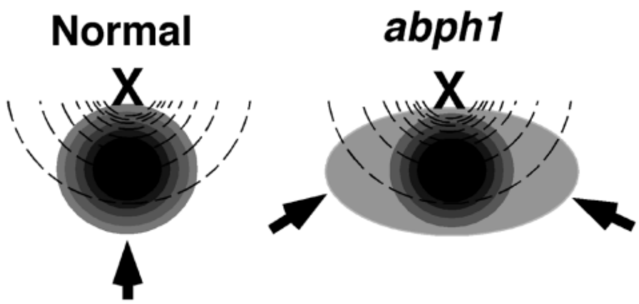

Fig. 5. A model to explain the initiation of decussate phyllotaxy in *abph1* embryos. The shaded area represents the embryonic shoot apical meristem, prior to the initiation of true leaves. The gradient of shading represents the inhibitory effect of the meristem center on leaf initiation. X marks the site of initiation of the coleoptile, and the concentric dashed circles represent a field of inhibition emanating from this primordium. In the normal apex, the region of the meristem furthest from the zones of inhibition is opposite to the coleoptile and this is the site where the first leaf would initiate (arrowed). In the *abph1* shoot meristem, which is considerably broader than normal, there are two zones where inhibition is lowest (arrowed). These are arranged perpendicular to the normal axis of leaf initiation, and correspond to the sites of leaf initiation in *abph1* embryos.

a similar pattern in both normal and decussate *abph1* apices. Only rarely did cells within the central zone label for cyclin 1b mRNA, whereas an increased frequency of labeling was seen in the peripheral zone of the meristem and in newly initiated leaf primordia (Fig. 2F,G). Therefore, both normal and *abph1* decussate meristems had a reduced frequency of cells expressing this cyclin gene, and presumably a reduced rate of cell division, within the central zone of the meristem.

Initiation of altered phyllotaxy in *abph1* embryos

The consistent effect of the *abph1* mutation on the first leaves indicated that it acts during embryogenesis, where the shoot apical meristem and the first true leaf primordia are initiated. To follow the development of embryos of known genotype, decussate *abph1* plants with two ear shoots were pollinated on the same day with normal (B73) pollen on one ear and with pollen from an *abph1* mutant plant on the other ear. Developing kernels were sampled from both ears at set times (days after pollination) and embryos dissected out and prepared either for SEM or for whole mount in situ hybridization. Embryos were staged according to morphological landmarks as described by Abbe and Stein (1954); Clark and Sheridan (1991).

After fertilization, the maize embryo first develops into a club shaped structure comprising an apical region that will give rise to the embryo proper and the suspensor, which acts as a conduit for maternally supplied nutrients. Five to seven days after fertilization, the apical part of the embryo flattens into a structure called the scutellum, which is homologous to the cotyledon or seed leaf (Arber, 1925; Randolph, 1936). At this time, the shoot meristem becomes visible as a small dome of cells on the face of the scutellum, and the coleoptile primordium initiates on the apical side of the shoot meristem (Fig. 4A). The coleoptile is thought to be an outgrowth of the scutellum, and forms a protective, ensheathing structure surrounding the embryonic shoot (Arber, 1925; Randolph, 1936). The first true leaf is initiated opposite the point where the coleoptile was initiated (Fig. 4C,D), and hereby forms the first sign of alternate phyllotaxy; from this point on all future leaves will be initiated in an alternating or distichous pattern.

The major embryonic structures were initiated at similar times in *abph1* embryos compared to normal embryos. In *abph1* embryos, the coleoptilar stage embryo was distinguished by a larger dome of tissue, presumably the shoot meristem, on the face of a broader and more rounded scutellum (Fig. 4E). The larger dome, presumed to be the shoot meristem, was observed in all *abph1* embryos that we examined (greater than one hundred observed in the SEM). A single coleoptile primordium encircled the dome and was therefore broader than normal. The mature coleoptile in *abph1* seedlings was also broader and often had an increased number of prominent veins but was otherwise normal (not shown). Following initiation of the coleoptile, the *abph1* shoot meristem initiated two opposite true leaf primordia perpendicular to the usual plane of phyllotaxy (Fig. 4G,H). This first indication of decussate phyllotaxy was observed in almost all *abph1* embryos at this stage (46/48 *abph1* embryos observed in the SEM). Therefore, the initiation of decussate phyllotaxy at the first node was a more reproducible aspect of the *abph1* phenotype than the maintenance of altered phyllotaxy in the *abph1* seedling, which was detected in approximately 50% of seedlings in this genetic background (Table 1). We assume therefore that the decussate pair of first leaf primordia often fused along one edge, either con- or postgenitally resulting in what appeared to be a single first leaf with two midveins, corresponding to the mild *abph1* phenotype described earlier. Therefore the ‘mild’ phenotype could be thought of as a reversion of phyllotaxy from decussate to normal that occurred at the first node, equivalent to the phenotypic reversion observed in later nodes.

To confirm that the larger dome structure seen in *abph1* embryos had meristem identity, we again used *kn1* as a molecular marker, because the onset of *kn1* expression in the maize embryo is coincident with the first histological changes associated with shoot meristem formation (Smith et al., 1995), and loss-of-function mutations in the *SHOOTMERISTEMLESS* gene, an *Arabidopsis* homolog of *kn1,* fail to initiate or maintain the shoot meristem (Clark et al., 1996, Long et al., 1996). Using whole-mount in situ hybridization, we showed that the domain of *kn1* expression was indeed larger in *abph1* embryos compared to their normal siblings and was coincident with the larger dome of tissue observed in the SEM (Fig. 4B,F). Therefore *abph1* embryos initiated a larger dome of tissue that by position, morphology and *kn1* expression appeared to have shoot meristem identity, and this larger meristem almost invariably initiated a decussate pair of first leaf primordia.

DISCUSSION

abph1 mutants have altered phyllotaxy and a larger shoot meristem

Although phyllotactic patterns have been described for centuries, little is known about the mechanisms involved in the initiation of these patterns. We have described a new recessive mutation, *abph1,* that leads to the formation of a larger shoot meristem and almost invariably causes the initiation of a decussate pair of first leaf primordia. In our studies, the frequency of *abph1* shoots remaining decussate throughout their development was about 20%, though this frequency has been as high as 90% in field trials of *abph1* lines in different genetic backgrounds (M. Menzi, pers. comm.). Although we do not understand the mechanism for reversion of phyllotaxy, the fact that reversion occurs indicates that the mode of action of *abph1* is unlikely to reflect an underlying mechanism for counting the number of leaf primordia initiated per node. Instead, the initiation of the decussate phyllotactic pattern is probably due to the altered morphology of *abph1* embryos, and in particular the presence of a larger shoot meristem domain (discussed later). The shoot apical meristems of decussate *abph1* seedlings are normal in terms of their layered cellular structure, but are consistently larger than normal. In addition, the expression of a cyclin marker gene shows that *abph1* meristems are correctly organized into central and peripheral zones, with a characteristic low mitotic index in the central zone. Shoot meristems initiated after embryogenesis, such as lateral inflorescence or floral meristems, are normal both in phyllotaxy and size, indicating that the effects of the *abph1* mutation are specific to the primary shoot apical meristem. Despite the striking change in phyllotaxy, *abph1* embryos initiate a single scutellum, an organ that is homologous to the cotyledon (Arber, 1925; Randolph, 1936), therefore the effects of the *abph1* mutation are unrelated to this fundamental difference between dicots and monocots.

The relation between primordium and meristem size

The leaves of decussate *abph1* plants were narrower than normal, an observation that is consistent with the relatively modest increase in meristem size despite the doubling of the number of leaves initiated per node. At two weeks after germination, when the maize shoot has initiated about 12 nodes, the meristem of decussate seedlings was about one third wider than normal, and therefore its circumference would also be only one third longer than normal. It is therefore not surprising that each leaf in the decussate pair is narrower than those of the normal siblings, since each primordium is initiated from only half the circumference of the meristem, compared to normal primordia that are initiated from the whole circumference. Based on this, one would predict that each decussate leaf primordium arises from a smaller number of founder cells, compared to normal primordia, and this is supported by preliminary clonal analysis data (D. Jackson and S. Hake, unpublished).

Despite the significant differences in leaf width between decussate and normal plants, the pattern of change in leaf width is similar. In normal maize plants, the leaf blade width increases progressively in successive nodes, peaks around leaves 12-14, then decreases. These changes probably reflect ontogenetic changes in meristem size and leaf initiation rate. During maize seedling development, the shoot meristem size increases gradually following each leaf initiation event (Abbe et al., 1951). Since the maize leaf is initiated as a ring of founder cells that surrounds the apex (Poethig, 1984), the increase in leaf width up to node 12 is likely due in part to the initiation of leaf primordia from a successively larger meristem. However, in later nodes, the time between leaf initiation events, the plastochron, shortens significantly (Abbe et al., 1951), so perhaps leaf width decreases at these nodes because the meristem has less time to recover in size before initiating the next leaf. In any case, it is significant that the pattern of change in leaf width was the same in *abph1* decussate shoots compared to normal siblings, indicating that this specific aspect of meristem programming is unaffected by the *abph1* mutation.

The effect of the ratio between meristem size and primordium size on phyllotaxy has been followed in a number of plants, and it is generally recognized that within a given system this ratio may correlate with the phyllotactic pattern. For example, in many dicots that switch from decussate phyllotaxy to spiral during their development, there is a corresponding increase in meristem size (Carr, 1984). What is not clear from these earlier studies is cause and effect; does the phyllotaxy follow from a change in meristem size, or is it the change in phyllotaxy that governs the size of the meristem? Our studies of the *abph1* mutation indicate that the meristem is larger in the embryo before the first decussate pair of leaves is visible, suggesting in this case that the phyllotaxy follows from the geometry of the meristem. However, a caveat with this hypothesis is that we do not know at what stage the positions of the leaf primordia are determined in the maize embryo; in other plants it is thought to be about half a plastochron before their emergence (Lyndon, 1982; Snow and Snow, 1933). Therefore we cannot rule out the possibility that the meristem in the *abph1* embryo is larger because more leaf initiation sites have been determined.

Mechanisms for determining phyllotaxy

In *abph1* plants, we observed a strong correlation between meristem size and phyllotaxy. The shoot meristem is larger while initiating and maintaining decussate phyllotaxy, but returns to normal size when the phyllotaxy reverts to normal. Furthermore, when decussate *abph1* shoots split to form two twin shoots, the phyllotaxy of each new shoot is alternate, and we assume this change in phyllotaxy results from smaller meristems formed during the twinning event.

Two main hypotheses have been proposed to explain the generation and maintenance of phyllotactic patterns. The first proposes that biophysical forces and tissue mechanics combine to promote morphogenesis through buckling of the tissue in predictable patterns (Green, 1987). Our observations of a correlation between meristem size and phyllotaxy are consistent with these ideas, and suggest a homeostatic mechanism balancing meristem size and phyllotaxy that could work through biophysical principles. A second model to explain the generation of phyllotaxy is the field model (Schoute, 1913; Wardlaw, 1949). This hypothesis proposes that leaf position is determined by inhibitory fields, presumably biochemical in nature, emanating from existing primordia and from the apex of the shoot meristem itself (Wardlaw, 1949). According to this model, the initiation of a different phyllotactic pattern in *abph1* embryos may be a consequence of the increase in size of the shoot meristem, within which the inhibitory fields act to determine the position of leaf primordia. This is diagrammed in Fig. 5, where the simultaneous initiation of two primordia from the *abph1* shoot meristem is modeled. Although one possibility is that the larger meristem would lead to the initiation of a single large primordium, the model shows how the concentration of a putative inhibitor could fall below a critical threshold level in two opposite regions of the meristem, leading to the simultaneous initiation of two primordia. In fact, Fig 5. could also represent the biophysical model if one assumes that the inhibitory fields are biophysical in nature.

The role of *abph1* in normal maize development

In addition to producing a wider shoot meristem, the *abph1* embryo is characterized by a wider scutellum and coleoptile, suggesting a more general role of the *abph1* gene in controlling embryonic growth. The *abph1* gene presumably plays a role in controlling the axial growth of several shoot structures in the embryo, however, it does not appear to affect all embryonic organs, for example the embryonic root develops into a normal primary root in *abph1* seedlings.

Other mutations that increase the size of the shoot apical meristem in the embryo and have effects on phyllotaxy in the vegetative and floral shoots have been described (Clark et al., 1993, 1995; Leyser and Furner, 1992; Medford et al., 1992). However, when these mutations affect the vegetative phyllotaxy they cause it to become irregular, rather than changing it to a new regular pattern. Mutations that resemble *abph1* more closely but affect floral organ number rather than leaf phyllotaxy include *perianthia* and *wiggum* (Running et al., 1998; Running and Meyerowitz, 1996). The mechanism of action of these mutations is probably different to *abph1*; *pan* leads to an increase in the number of outer whorl floral organs without affecting meristem size, and in *wig* flowers, although the width of the floral meristem is larger, floral organs are not initiated in a predictable pattern. We believe the *abph1* mutation is unique because it is suggestive of a function that is specific to the establishment of pattern during embryogenesis, since in revertant seedlings the shoot meristem size returns to normal. The maintenance of a larger shoot meristem and decussate phyllotaxy in some *abph1* plants does not necessarily reflect a function for the *abph1* gene during postembryonic shoot development, rather this maintenance may be due to a homeostatic mechanism that could be biophysical or biochemical in nature. Therefore the function of *abph1* may be more intimately associated with the establishment of the initial shoot apical meristem domain and of phyllotactic pattern, rather than being involved in the control of shoot meristem size throughout the life of the plant. Isolation and characterization of the *abph1* gene should clarify its role in shoot development, and also help determine if it is involved in the evolution of phyllotactic patterns.

We would like to thank Drs M. Menzi and M. Frei for sending families of abphyl seed, the UC Berkeley SEM facility for training, Professor Paul Green for advice on the replica technique and many discussions on phyllotaxy, Drs Richard Greyson and David Walden for discussions on the abphyl syndrome, and members of the Hake laboratory for comments on the manuscript. D. J. was supported by a NATO postdoctoral fellowship and by USDA support to S. H. (Agricultural Research Service Project 5335-21000-007-00D).

This paper is dedicated to the memory of Paul Green, for his warm friendship and stimulating discussions in plant development.

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