Regulation of Vegetative Phase Change in *Arabidopsis thaliana* by Cyclophilin 40

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During its development, a plant shoot progresses from a juvenile to an adult phase of vegetative growth and from a reproductively incompetent to a reproductively competent state. In *Arabidopsis*, loss-of-function mutations in *SQUINT* (*SQN*) reduced the number of juvenile leaves and had subtle effects on inflorescence morphology but had no effect on flowering time or on reproductive competence. *SQN* encodes the *Arabidopsis* homolog of cyclophilin 40 (CyP40), a protein found in association with the Hsp90 chaperone complex in yeast, mammals, and plants. Thus, in *Arabidopsis*, CyP40 is specifically required for the vegetative but not the reproductive maturation of the shoot.

All plants go through qualitatively different stages of vegetative development before they enter the reproductive phase (*1*, *2*). In some species, the vegetative phases are marked by dramatic differences in a large number of traits, whereas in others the transition is more subtle (*3*, *4*). This phenomenon is known as heteroblasty (*5*) or phase change (*6*). Mutations affecting phase-specific features of vegetative development have been identified in a number of plants, but only one gene involved in the regulation of this process has been identified on a molecular level (*7*). Thus, the molecular mechanism of phase change remains almost completely unknown.

In *Arabidopsis*, leaves produced during the development of the rosette can be distinguished from one another by their size and shape (*8*), number of hydathodes (*9*), and pattern of trichome (leaf hair) distribution (*10*, *11*). The first two leaves are small and round, have only a few hydathodes, and do not produce trichomes on their abaxial (lower) surface. As the shoot develops, leaf size increases, the leaf blade becomes elliptical and more serrated, hydathode number increases, and leaves begin to produce abaxial trichomes. To identify genes that regulate this transition, we screened for mutations that cause these changes to occur precociously, focusing in particular on the production of abaxial trichomes, because this trait is relatively easy to score and quantify. Three mutant alleles of *SQN* were identified in this screen. Two of these alleles were induced in the Columbia (Col) ecotype, one by ethyl methane sulfonate (*sqn-1*) and the other by carbon ionization (*sqn-3*). A third allele (*sqn-2*) was induced by transferred DNA (T-DNA) transformation in a Wassilewskije background and was crossed six times to Col before being used for the studies described here. In a Col background, these alleles were phenotypically identical.

The effect of *sqn* mutations on leaf identity was particularly obvious in leaves one and two, because these leaves are normally quite different from all other rosette leaves. In mutant plants, leaves one and two were elliptical rather than round and were larger and more serrated than the first two leaves of wild type (WT) plants (Fig. 1, A and B); leaves at other positions on *sqn* shoots also resembled leaves at higher nodes on WT plants. Col plants normally begin producing abaxial trichomes on leaf 5 and do not produce trichomes under leaf one or two (*10*, *11*). In contrast, *sqn* plants usually produced abaxial trichomes on leaf three and produced abaxial trichomes on leaf two when grown in high light (Fig. 1B and Table 1). *sqn* mutations also accelerated the normal increase in both adaxial (upper leaf surface) and abaxial trichome density on successive rosette leaves (Fig. 1E). These changes were accompanied by a decrease in total leaf number that was largely attribut-

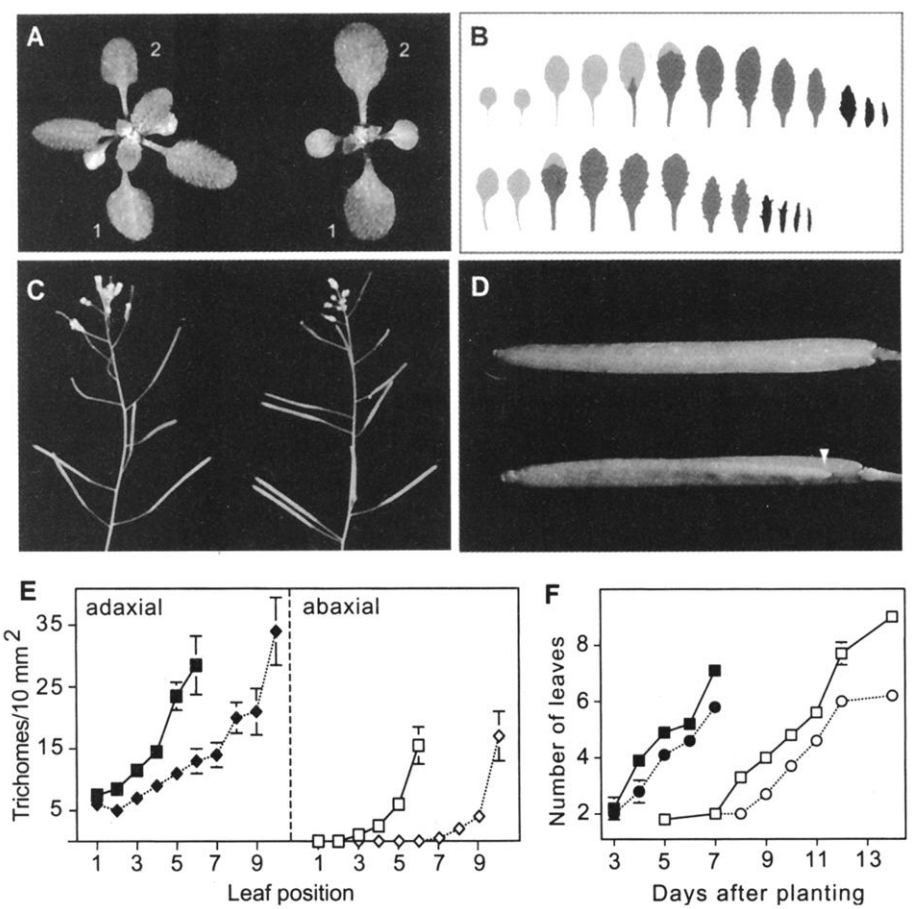

Fig. 1. Phenotype of *sqn* and Col plants. (**A**) Col (left) and *sqn-1* (right) plants at 8 days after planting. (**B**) Rosette and inflorescence leaves from Col (top) and *sqn-1* (bottom) plants. Light gray, no abaxial trichomes; dark gray, abaxial trichomes; black, inflorescence leaves. (**C**) Col (left) and *sqn-1* (right) inflorescences. (**D**) Col (top) and *sqn-1* (bottom) siliques. The arrowhead indicates an abnormal third carpel in the *sqn-1* silique. (**E**) Trichome density on the adaxial and abaxial surfaces of Col (diamonds) and *sqn-1* (squares) plants. (**F**) The rate of leaf initiation (solid symbols) and leaf emergence (open symbols) in Col (squares) and *sqn-1* (circles) plants.

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able to a reduction in the number of leaves lacking abaxial trichomes (that is, juvenile leaves) (Table 1); *sqn* did not affect, or only slightly reduced, the number of leaves with abaxial trichomes (adult leaves). Thus, *sqn* accelerated vegetative phase change rather than causing a homeotic transformation of juvenile leaves into adult leaves. In addition, *sqn* disrupted the phyllotaxis of the inflorescence (Fig. 1C) and increased carpel number in the first few siliques (Fig. 1D). These floral phenotypes were displayed by 88 to 100% of mutant plants, but their expressivity was weak and variable. Although the effect of *sqn* mutations on vegetative and reproductive morphology was recessive, their effect on abaxial trichome production was semidominant; on average, *sqn*/+ plants produced abaxial trichomes 0.5 leaves earlier than did *sqn/sqn* plants. Because these mutations probably produce a loss of function, this observation suggests that *SQN* may be haplo-insufficient for abaxial trichome production.

Mutant seedlings initially appeared "eyelike" (Fig. 1A) because immature leaves in the rosette were unusually small relative to leaves one and two. To determine the basis for this phenotype, we examined the effect of *sqn-1* on the rate of leaf initiation and leaf emergence (*12*) (Fig. 1F). We found that *sqn-1* had no effect on the initiation or time of emergence of the first two leaves but transiently delayed leaf production after the initiation of leaf two; *sqn-1* had no effect on the rate of leaf initiation or leaf emergence after this point. *sqn-1* also caused rosette leaf production to cease earlier than normal. Thus, the unusual morphology of *sqn-1* seedlings is attributable to a transient delay in leaf initiation after the production of leaf two, whereas the reduced leaf number of mature *sqn-1* plants is due both to this early delay and to a precocious cessation of leaf production.

To determine whether *sqn-1* accelerated reproductive phase change in the same way in which it accelerated vegetative phase change, we examined its effect on flowering time and its interaction with a *35S::LFY* transgene. In WT plants, *35S::LFY* induces flowering immediately after plants have made the transition to the adult vegetative phase (*13*, *14*), which implies that vegetative phase change is accompanied by a change in the reproductive competence of the shoot. We found that *sqn-1* had no effect on flowering time in either the absence or the presence of *35S::LFY* (Table 1). *sqn-1; 35S::LFY* plants flowered at exactly the same time as *35S::LFY* plants. These results suggest that *SQN* is primarily involved in regulating vegetative phase change, and has no effect on the reproductive competence of the shoot.

SQN was mapped to chromosome 2 and cloned using a map-based approach (*15*). Hybridization of bacterial artificial chromosomes (BACs) to Southern blots of genomic DNA from *sqn-3* demonstrated that this allele is a ≈10-kb deletion encompassing four predicted genes, one of which was found to be mutant in *sqn-1* and *sqn-2*.

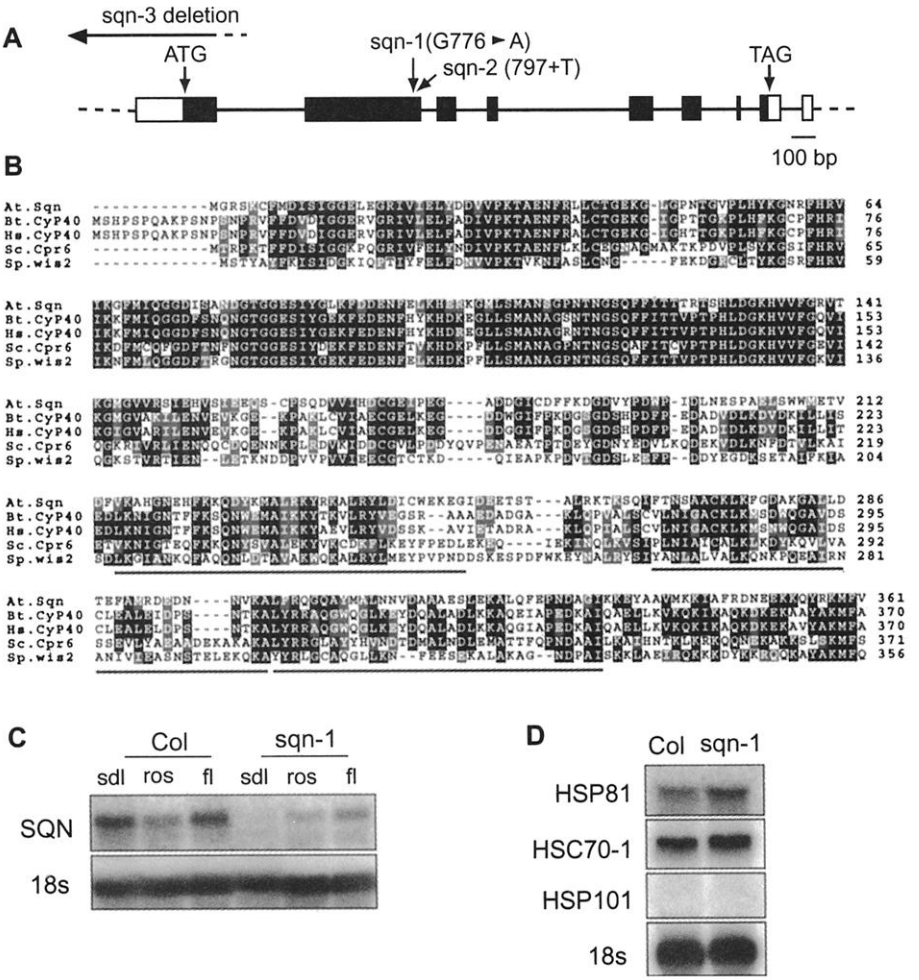

Fig. 2. Molecular analysis of *SQN*. (**A**) Exon-intron structure of *SQN*. The locations of the mutations in *sqn-1*, *sqn-2*, and *sqn-3* are indicated. *sqn-1* is a G → A transition, and *sqn-2* is a single base (T) insertion that introduces stop codons at amino acid positions 207 (*sqn-1*) and 220 (*sqn-2*). (**B**) Alignment of SQN with other known CyP40 amino acid sequences (*37*). The underlined segments represent the position of the TPR repeats described in (*17*). Gray shading indicates similar residues; black shading indicates identical residues. (**C**) Northern analysis of *SQN* expression in 5-day-old seedlings (sdl), 21-day-old rosettes (ros), and flower buds (fl) of WT and *sqn-1*. *SQN* mRNA is significantly reduced in *sqn-1* plants. (**D**) Northern analysis of the expression of *HSP* genes in WT and *sqn-1* seedlings. There is 1.5 times more *HSP81.2*, *HSP81.3*, *HSP81.4*, and *HSC70-1* mRNA in *sqn-1* than in WT seedlings. This difference was computed after normalizing the *HSP81* and *HSC70-1* band intensities to the 18*S* ribosomal RNA signal.

Table 1. The effect of *sqn-1* and *35S::LFY* on vegetative development and flowering time. Values are the mean ± SE.

Genotype*	Rosette leaves	Leaves without abaxial trichomes	Leaves with abaxial trichomes	Days to flowering†	*n*‡
+/+	9.4 ± 0.3	4.5 ± 0.2	4.9 ± 0.4	22.8 ± 0.4	11
sqn/sqn	6.4 ± 0.2	1.8 ± 0.1	4.6 ± 0.2	23.0 ± 0.3	25
35S::LFY/+; +/+	7.6 ± 0.7	5.6 ± 0.4	2.0 ± 0.8	23.0 ± 0.8	5
35S::LFY/+; *sqn/sqn*	6.0 ± 0.2	2.5 ± 0.2	3.5 ± 0.3	22.4 ± 0.3	22

*Siblings from the cross *sqn-1*/+ × *35S::LFY*/+; *sqn-1*/+ Genotypes were determined by progeny testing. †Flowering is defined as the opening of the first floral bud on the primary inflorescence. ‡Number of plants.

A 1313–base pair (bp) cDNA (GenBank accession number AY026065) corresponding to this gene rescued the mutant phenotype of *sqn-1* and *sqn-3* when introduced into plants under the regulation of the CaMV 35*S* promoter, confirming that it corresponds to *SQN*. RNA blots probed with this cDNA (Fig. 2C) revealed a single 1.3-kb transcript that was present at low levels in WT plants and was significantly reduced in *sqn-1*. Different amounts of *SQN* mRNA were detected in seedlings, adult leaves, and floral buds, but it is not clear whether this reflects a tissue-specific pattern of expression or the difference in the ages of the tissues (immature juvenile leaves and floral buds versus expanded adult leaves) used in this experiment.

SQN consists of nine exons (Fig. 2A) and is predicted to encode a 361–amino acid protein that is 48% identical and 64% similar to bovine CyP40 (Fig. 2B). *SQN* is the only *CyP40*-like gene in the *Arabidopsis* genome. Like other CyP40 proteins, SQN has an NH_2-terminal peptidyl prolyl isomerase domain that is very similar to low-molecular-weight cyclophilins (*16*). In the COOH-terminal half of the predicted protein, the most highly conserved domains correspond to the three tetratricopeptide repeats (TPRs) (*17*) that are required for the interaction of CyP40 with Hsp90 (*18*, *19*, *20*), and are sufficient for the function of the *CyP40* genes *Cpr7* (*21*) in *Saccharomyces cerevisiae* and *wis2* (*22*) in *Schizosaccharomyces pombe*.

In yeast, the CyP40-related gene *CPR7* negatively regulates the expression of the heat-shock protein (HSP) gene *HSP104* (*23*). To determine whether Cyp40 has a similar function in *Arabidopsis*, we studied (*24*) the thermotolerance of *sqn-1* and its effect on the expression of HSP genes that are transcribed under non-stress conditions (*HSP81.2*, *HSP81.3*, *HSP81.4*, and *HSC70-1*) (*25*, *26*) and one that is only transcribed in response to heat shock (*HSP101*) (*27*). In three independent experiments, Northern analysis revealed a small but reproducible increase (1.2 to 1.8 times) in *HSP81* and *HSC70-1* expression in *sqn-1* but no increase in the expression of *HSP101* (Fig. 2D). This minor change in HSP gene expression did not produce a change in the thermotolerance of mutant seedlings. Thus, in *Arabidopsis*, CyP40 negatively regulates the transcription of some HSPs.

CyP40 and the immunophilin, FK506-binding protein 52 (FKBP52), were originally discovered in unactivated steroid receptor complexes in mammals (*17*, *28*) and compete for a binding site on Hsp90 in both animals (*29*–*31*) and plants (*19*). It is believed that these immunophilins function as protein chaperones either on their own or in association with Hsp90, because they interact directly with a number of signaling molecules (*32*) and are present in varying amounts in steroid receptor complexes isolated from different tissues (*33*). Alternatively, they may be involved in the subcellular trafficking of Hsp90 complexes (*34*). In yeast, the loss of all 12 cyclophilin and FK506-binding proteins produces slow growth but is not lethal (*35*). Mutations in an *Arabidopsis* homolog of FKPB52, *PASTICCINO1*, have a highly pleiotropic phenotype (*36*), suggesting that this protein is critical for many aspects of plant growth and development. In contrast, CyP40 appears to have a more specific role in *Arabidopsis* development. The *sqn* mutant phenotype suggests that CyP40 plays a major role in promoting the expression of the juvenile phase of vegetative development and is involved to a more limited extent in regulating the positioning of floral buds, floral morphogenesis, and the expression of HSPs. This phenotype is consistent with the hypothesis that CyP40 regulates the activity of specific signaling pathways. Future studies should reveal the components of these pathways and provide insight into the biochemical function of Cyp40 in *Arabidopsis*.

References and Notes

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12. The stocks used in this study were homozygous for a *LFY::GUS* transgene, which is expressed transiently early in leaf development and is useful for identifying young leaf primordia. To determine the rate of leaf initiation, seedlings were harvested at different times after planting, stained for β-glucuronidase activity, and decolorized in 70% ethanol. The number of leaf primordia was then counted with the aid of a dissecting microscope. The rate of leaf emergence was measured by repeatedly counting the number of visible leaves on a single group of plants.
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15. *SQN* was mapped to the 1.5-centimorgan region between markers *mi398* and *THY1* on chromosome 2, using an F2 mapping population of 778 plants from a cross between *sqn-1* (Col) and Landsberg *erecta*. Polymerase chain reaction (PCR)–based markers were designed using end sequences from BACs spanning this region and were mapped relative to the *sqn-1* mutation. These markers localized *sqn-1* to a region between the Sp6 end of BAC F1E8 and the Sp6 end of BAC F9O13. BACs spanning this region were hybridized to genomic DNA from the three existing *sqn* alleles digested with various restriction enzymes in order to identify allele-specific polymorphisms. F19G14 revealed Eco RV and Xcm I polymorphisms in *sqn-3*. Further characterization of this allele by Southern analysis demonstrated that it is a deletion of ≈10 kb, which encompasses four predicted open reading frames (GenBank accession numbers AAD41984.1, AAD41983.1, AAD41982.1, and AAD41985.1). Sequencing of reverse-transcribed PCR products from AAD41985.1 in *sqn-1* and *sqn-3* revealed point mutations in this gene. cDNAs corresponding to this gene were isolated by probing a seedling library. The longest of these cDNAs was cloned behind the CaMV 35*S* promoter in pCAMBIA 3300 and was introduced into *sqn-1* and *sqn-3* plants by T-DNA transformation. This transgene rescued the *sqn* mutant phenotype in 10 of 10 transgenics.
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37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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