INCOMPOSITA: a MADS-box gene controlling prophyll development and floral meristem identity in *Antirrhinum*

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Summary

INCOMPOSITA (INCO) is a MADS-box transcription factor and member of the functionally diverse StMADS11 clade of the MADS-box family. The most conspicuous feature of *inco* mutant flowers are prophylls initiated prior to first whorl sepals at lateral positions of the flower primordium. The developing prophylls physically interfere with subsequent floral organ development that results in aberrant floral architecture. *INCO*, which is controlled by *SQUAMOSA*, prevents prophyll formation in the wild type, a role that is novel among MADS-box proteins, and we discuss evolutionary implications of this function. Overexpression of *INCO* or *SVP*, a structurally related *Arabidopsis* MADS-box gene involved in the negative control of *Arabidopsis* flowering time, conditions delayed flowering in transgenic plants, suggesting that SVP and INCO have functions in common. Enhanced flowering of *squamosa* mutants in the *inco* mutant background corroborates this potential role of *INCO* as a floral repressor in *Antirrhinum*. One further, hitherto hidden, role of INCO is the positive control of *Antirrhinum* floral meristem identity. This is revealed by genetic interactions between *inco* and mutants of *FLORICAULA*, a gene that controls the inflorescence to floral transition, together with *SQUAMOSA*. The complex regulatory and combinatorial relations between *INCO*, *FLORICAULA* and *SQUAMOSA* are summarised in a model that integrates observations from molecular studies as well as analyses of expression patterns and genetic interactions.

Key words: MADS-box protein, Prophyll, Floral meristem identity, Floral architecture, *Antirrhinum majus*

Introduction

Plants can generate new organs post-embryonically throughout their life cycle, owing to the activity of meristems located at the tip of growing shoots (shoot apical meristem or SAM) and roots. Meristems are dynamic structures, in which mitosis continuously replenishes cells that are depleted by differentiation (Traas and Vernoux, 2002). The SAM undergoes several distinct changes of identity during development of flowering plants (Pidkowich et al., 1999). In the vegetative phase the SAM generates lateral leaves and axillary meristems. In the reproductive phase the SAM becomes an inflorescence meristem (IM) that will give rise to a number of lateral leaves and floral meristems (FM) in their axils, which initiate floral organs.

Vegetative and reproductive organs are arranged in a species-specific phyllotaxis (Reinhardt and Kuhlemeier, 2002). The switch from vegetative to reproductive growth is accompanied by a change of phyllotaxis in *Antirrhinum majus* (Carpenter et al., 1995). During the vegetative phase, the plants display decussate phyllotaxis, where the two leaves formed per node are positioned at opposite sides of the stem. After the transition to the reproductive phase, *Antirrhinum* plants switch to a spiral phyllotaxis, producing small leaf-like organs (bracts) at each node along the main (inflorescence) stem. Flowers arise in the axils of bracts and consist of four types of organs arranged in a whorled phyllotaxis. Five sepals in the calyx constitute the outer (first) whorl, followed by five petals (second whorl), four stamens and a stamenoid (third whorl), and two fused carpels in the inner (fourth) whorl. Mutations in two *Antirrhinum* genes, *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*), transform flowers into indeterminate axillary inflorescences with bracts arranged in spiral phyllotaxis (Coen et al., 1990; Huijser et al., 1992). The phenotype of *flo* and *squa* mutants indicates that both genes play a crucial role in the specification of the floral meristem. The transcript level of *SQUA* and *FLO* in *flo* and *squa* mutants, respectively, is unchanged, but after independent transcriptional induction, the SQUA and FLO functions converge in the control of flower development. This is revealed by the enhanced *squa* or *flo* mutant phenotypes when the respective *FLO* or *SQUA* functions are reduced (Carpenter et al., 1995). In this report, we provide genetic evidence that *INCOMPOSITA* (*INCO*) is an additional factor required, in cooperation with *FLO* and *SQUA*, for the control of floral meristem identity.

INCO, like SQUA, is a MADS-box transcription factor (Schwarz-Sommer et al., 1990). MADS-box genes constitute a large family, which, throughout plant evolution, have been recruited as transcriptional regulators controlling the

development of various plant structures and organs (Ng and Yanofsky, 2001). *inco* flowers display two extra organs, named prophylls or bracteoles (Bell, 1991; Weberling, 1989), which develop very close to the lateral sepals. We propose that *INCO* represses prophyll development in *Antirrhinum*, which is a novel function for a MADS-box transcription factor, and show that absence of this control results in impaired floral architecture.

Materials and methods

Genetic stocks and plant material

Antirrhinum plants were grown in the greenhouse at 18-25°C with additional light during winter. The wild-type lines JI98 (the progenitor of line 165E), *flo*-640 (Carpenter et al., 1995) and *flo*-662 (McSteen et al., 1998; Simon et al., 1994) were kindly provided by Rosemary Carpenter (John Innes Centre, Norwich, UK). The wild-type line Sippe 50 and the mutants, *def*-gli (Sommer et al., 1990), *inco*-pannosa and *inco*-deformis (referred to as *def*, *inco*-1 and *inco*-2, respectively) were obtained from the collection at the IPK, Gatersleben (Accession numbers MAM88, MAM162 and MAM161, respectively). *inco*-3 and *inco*-4 arose in a mutagenesis program, where the *inco*-1 allele has been targeted for transposon insertion (E. de Andrade Silva and Z.S.-S., unpublished). The *squa*-347 mutant has been previously described (Huijser et al., 1992). Because all *inco* mutant lines displayed identical phenotypes and lacked *INCO* expression (see Results), double mutants with *squa*-347 and *flo*-662 were constructed using the *inco*-1 allele. The presence of mutant alleles was confirmed by PCR and the phenotype of double mutants was corroborated by growing F3 populations of about 100 plants with defined genotypes.

Col0 *Arabidopsis thaliana* plants were transformed according to a dipping protocol (Clough and Bent, 1998). 35S::INCO was constructed by inserting the full-size cDNA into the *Bam*HI site of the pPCV072 vector (Koncz et al., 1990), and for 35S::SVP the *Xba*I site of pBAR-35S [modified from Becker et al. (Becker et al., 1992)] was used. The T2 progeny of several transgenic lines was grown in climate chambers at 20°C and 16 hours of light.

Microscopy

Scanning electron microscopy (SEM) was carried out with replicas of flowers and developing inflorescences as previously described (Green and Linstead, 1990).

The vascular skeleton was viewed under bright field after processing the tissues according to Candela et al. (Candela et al., 1999).

In situ hybridisation and northern blotting

In situ hybridisation with digoxigenin-labelled antisense RNA was performed as previously described (Bradley et al., 1993; Davies et al., 1996; Huijser et al., 1992). The *INCO* probe did not contain the MADS-box.

For northern blot analyses, 1 μg of mRNA was loaded per lane, transferred to nylon membranes and processed as previously reported (Sommer et al., 1990).

DNA preparation and PCR screening

Leaf samples were ground in liquid nitrogen and suspended in extraction buffer (250 mM TrisHCl, pH 7.5; 1% SDS; 25 mM EDTA, 250 mM NaCl). After phenol/chloroform extraction, the DNA was precipitated and the pellet was resuspended in TE buffer containing 5 μg/ml RNAseA. The screening procedure followed the protocol described by Keck et al. (Keck et al., 2003). Detailed information on PCR primers and PCR conditions for these and all other experiments performed in this report are available upon request.

Yeast methods

For yeast two-hybrid experiments the INCO or SQUA coding sequences were inserted into the *Eco*RI/*Sal*I site of the pGAD424 and pGBT9 (Clontech) vectors. INCOΔC (amino acids 1 to 206) was constructed by PCR amplification of the respective region of the INCO cDNA. This C-terminal deletion eliminates the transcriptional activator domain and prevents auto-activation in yeast. Ternary complex formation was tested with INCOΔMIK1/2 (amino acids 104 to 229 in the pGAD424 vector) using the full-size PLE cDNA (inserted into pGBT9) and the full-size DEFH200 cDNA (cloned into the *Eco*RI/*Sal*I site of the pTFT1 vector (Egea-Cortines et al., 1999). Yeast libraries, screening protocols and all other constructs are described elsewhere (Davies et al., 1996; Egea-Cortines et al., 1999).

Semi-quantitative assays for comparing the strength of INCO homodimerisation and heterodimerisation with several partners were performed by liquid *lacZ* assays (Kippert, 1995) using the SFY527 yeast strain. Activity in Miller units was calculated according the formula $(1000 \times A_{420} \times V_r)/(t \times V_c \times A_{600})$, where V_r=final reaction volume in ml; V_r=volume of culture assayed in ml; t=time in minutes. Average and standard deviation of four independent assays are shown in Table 1.

Results

DEFH70 corresponds to the *INCOMPOSITA* gene

DEFH70 is a MADS-box transcription factor first isolated during screening of an *Antirrhinum* genomic library for MADS-box genes and was also detected in yeast two-hybrid screens as a putative interacting partner of PLENA and DEFH200 (Davies et al., 1996). A reverse genetic approach (Keck et al., 2003) using sequence information of *DEFH70* identified two classical allelic mutants, *inco*-1 and *inco*-2 (Stubbe, 1966). Two additional *inco* alleles, *inco*-3 and *inco*-4, were obtained during a transposon mutagenesis program. All *inco* alleles carried CACTA-type transposons inserted in the gene (Fig. 1A). Among 162 *inco-4* plants, a wild-type revertant was isolated, in which the Tam7-like element had been excised leaving a footprint behind (not shown). The occurrence of four alleles, the genetic instability of *inco*-4 and the absence of *DEFH70* transcript in the mutants (Fig. 1B) provide the evidence that *DEFH70* corresponds to the *INCO* gene.

INCOMPOSITA belongs to the StMADS11 subfamily

Searches in databases showed that INCO shares 73.8% and 69.9% amino acid identity and 82.7% and 78.2% similarity with JOINTLESS (J) (Mao et al., 2000) and SHORT VEGETATIVE PHASE (SVP) (Hartmann et al., 2000), respectively (Fig. 2A,B). These genes belong to the *StMADS11* subfamily (Becker and Theißen, 2003), and perform very diverse functions in plant development; SVP is a floral repressor in *Arabidopsis* (Hartmann et al., 2000) and JOINTLESS organises the pedicel abscission zone in tomato (Szymkowiak and Irish, 1999).

Like *JOINTLESS,* the *INCO* gene consists of eight exons and seven introns (Fig. 1A), while *SVP* has nine exons. The INCO MADS-box is encoded by the first exon, a typical feature of MIKC type MADS-box genes.

INCO is expressed during early stages of organ initiation

Northern blot and RT-PCR analyses indicate that *INCO* mRNA is present in all organs during the vegetative and reproductive phases (not shown). *INCO* mRNA is detectable in situ in two

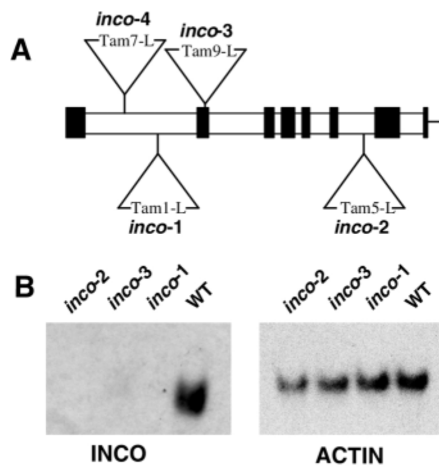

Fig. 1. (A) Structure of the *INCO* gene in the wild-type and in *inco* mutant alleles. Black boxes represent exons and white boxes are introns. Triangles show the position of transposable elements (Tam) in four mutant *inco* alleles. (B) Northern blot with mRNA isolated from wild-type and *inco* inflorescences probed with *INCO* cDNA and with *ACTIN* cDNA as loading control.

opposite domains of the SAM (Fig. 3A), corresponding to the position of incipient leaf primordia [P0 (Waites et al., 1998)]. Similarly, *INCO* transcript is present in initiating bract primordia in the IM (Fig. 3B). Meristematic cells within the apical dome of the SAM and IM, however, do not express *INCO*. *INCO* is transcribed in emerging axillary meristems during the vegetative phase and in floral meristems until early stage 3 (Fig. 3C-E). During stage 3, *INCO* mRNA disappears from the emerging sepal primordia and becomes more restricted to deeper layers of the floral meristem. Later, *INCO* expression is only detectable in developing and mature anthers as revealed by northern blot analysis and in situ hybridisation experiments (not shown).

In summary, *INCO* expression during vegetative and reproductive development displays striking similarities, although, as shown below, mutation of *INCO* phenotypically affects only reproductive development.

INCO represses prophyll development

The most conspicuous feature of *inco* mutants are two narrow, or filamentous structures beneath the two lateral sepals, outside the calyx, which are absent in the wild-type flower (Fig. 4) (Wilkinson et al., 2000). Occasionally, these organs reach the size of sepals, but in contrast to true sepals, and similar to bracts (Keck et al., 2003), they develop a glandular structure at their tip (Fig. 4D,E). In contrast to leaves and bracts with branched venation, however, the venation of prophylls resembles the parallel pattern observed in sepals (Fig. 4E,F). Mature flowers frequently display twisted or distorted petals, petaloid lateral sepals and petals that are partly fused to sepals (Fig. 4B,C). The lateral sepals are often smaller than in the wild type and are sometimes reduced to tiny narrow organs. The *inco* phenotype is variable, displaying nearly wild type to severely distorted petals and extra organs that are free-standing or fused to the adjacent lateral sepals. The phenotypes of different *inco* mutants are very similar, although in *inco*-4 flowers the additional two organs are sometimes positioned lower on the pedicel or very close to the subtending bract.

Comparison of developing wild-type and *inco* flowers by SEM revealed that the primordia of the additional two organs arise at stage 2, before the genuine sepals, very close to the position normally occupied by the two lateral sepal primordia (Fig. 5A,B). This resembles the position of prophylls ['the first leaf on the shoot' (Weberling, 1989)] in species in which their development is not suppressed. As in wild-type flowers, the two ventral (abaxial) sepal primordia and the dorsal organ initiate almost simultaneously in *inco* flowers. In species with suppressed prophyll development the order of sepal initiation and the position of the organs is the same as if prophylls were present (Weberling, 1989). The lateral positioning of the additional organs in the *inco* mutant and the initiation pattern of sepals in the wild type (as well as in the mutant) suggest that the two additional *inco* floral organs are prophylls, the development of which is suppressed by the *INCO* gene product in the wild type.

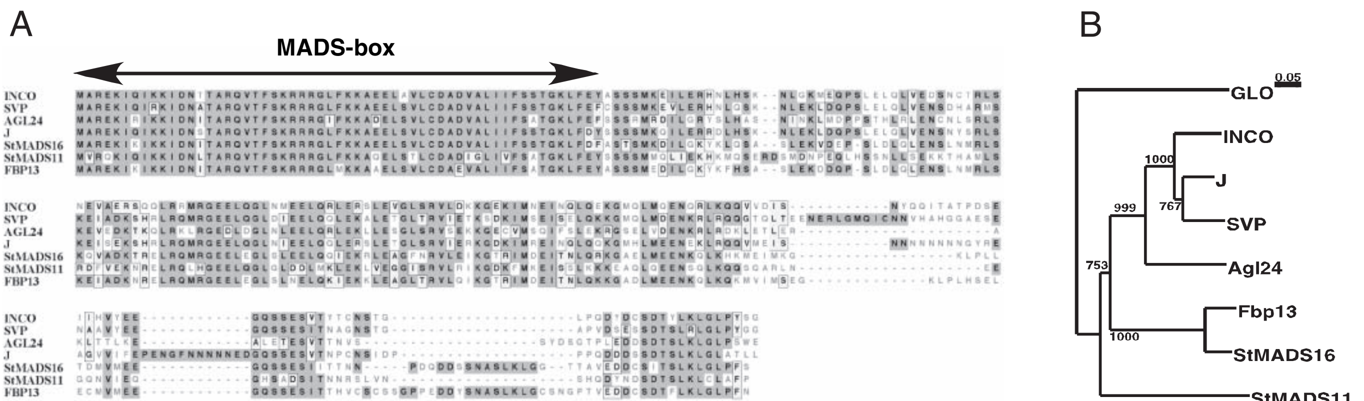

Fig. 2. The MADS-box protein INCO belongs to the StMADS11 subfamily. (A) The predicted amino acid sequence of INCO (AJ699174) is aligned with other plant MADS-box transcription factors. Identical amino acids are shown in shaded boxes, conservative changes by light shading and non-conserved position in light-grey capitals. (B) Phylogenetic tree generated with the ClustalW program using the first 180 amino acids (M, I and K domains) of the proteins. SVP (Q9FVC1) and AGL24 (CAB79364) are from *Arabidopsis*; FBP13 (AAK21250) from *Petunia*; J (Q9FUY6) from tomato; and StMADS11 (AAB94006) and StMADS16 (AAB94005) from potato. The tree was rooted with the *Antirrhinum* GLO (X68831) sequence (as closely or distantly related to the StMADS11 subfamily as members of any other MADS subfamily (Becker and Theißen, 2003); local bootstrap probabilities are indicated at the branching points.

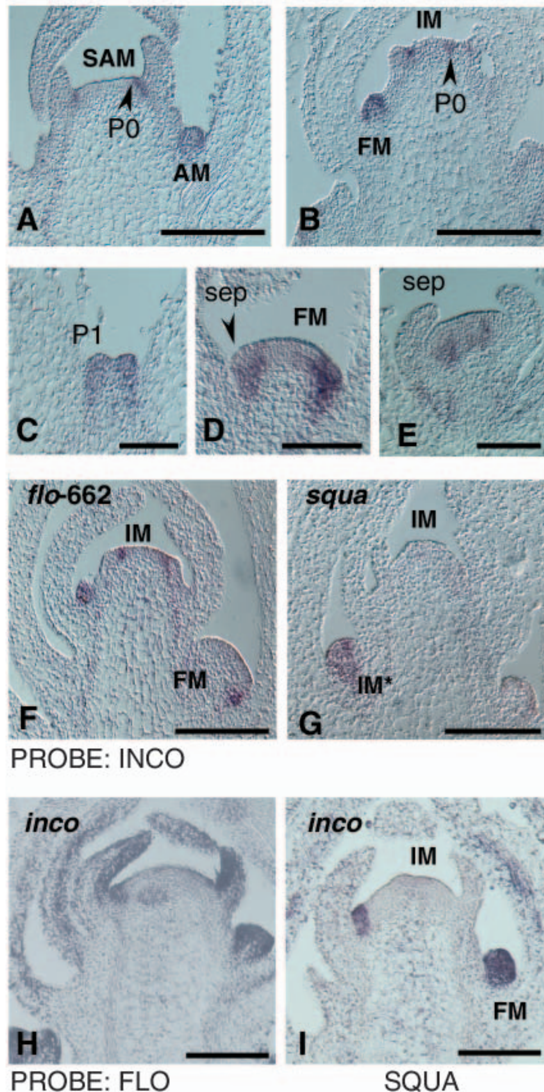

Fig. 3. In situ expression pattern of *INCO* in longitudinal sections of wild-type (A-E) and mutant (F,G) plants. (A,C) *INCO* accumulates in vegetative P0 and P1 primordia and in axillary meristems (AM) in wild-type seedlings. (B) *INCO* messenger in initiating bracts (P0) and in young floral meristems (FM) of a wild-type inflorescence. Signal is absent from the apical dome of vegetative (SAM) and inflorescence (IM) meristems. (D) Stage 2 floral meristem with emerging sepal primordia (sep). (E) Floral meristem at late stage 3. (F) *INCO* expression is not affected by the *flo*-662 mutation. (G) Reduced *INCO* expression in *squa* mutant inflorescences. Serial sections probed with *FLO* revealed signals as in wild type, proving that decreased *INCO* expression is not an artefact (not shown). IM* is an axillary inflorescence meristem. (H,I) In *inco* inflorescences, expression of the floral meristem identity controlling genes *FLO* and *SQUA* is comparable with wild type (not shown). Scale bars: 200 µm in A,B,F-I; 100 µm in C-E.

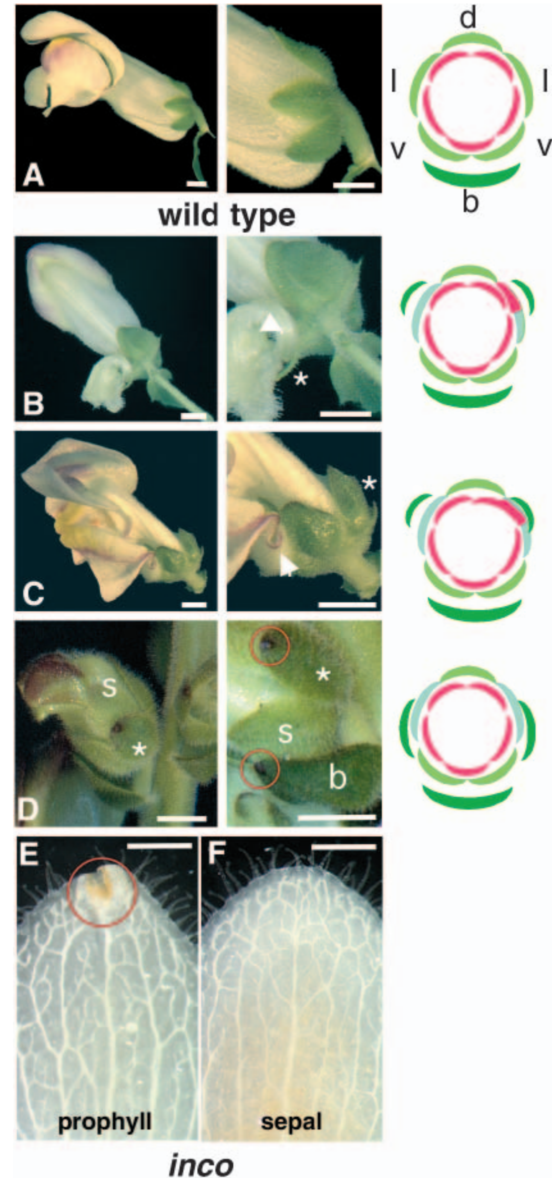

Fig. 4. Phenotypes of wild type (A) and *inco*-1 mutant flowers (B-F). The bottom regions of flowers in B-C are magnified on the right to show prophylls (indicated by asterisks) and fusion of petals and sepals (arrows in B,C). *inco* buds with large prophylls are depicted in D. Globular glands (circled) are present at the tip of prophylls and bracts, but not in sepals. The diagrams on the right schematically show the morphological changes. The positions of sepals in the wild type are designated by d (dorsal, i.e. adaxial), l (lateral) and v (ventral, i.e. abaxial). Size alteration and displacement of lateral *inco* sepals is highlighted in blue. (E,F) Vascular skeletons show that secondary veins develop parallel to (instead of branching from) the midvein in *inco* prophylls and sepals (parallel venation), and the glandular structure (circled) at the tip of prophylls. b, bract; s, sepal. Scale bars: 5 mm in A-D; 0.5 mm in E,F.

However, subsequent initiation of the lateral sepals is delayed in *inco*, and the organs are displaced towards the centre of the flower primordium (Fig. 5C,D). Frequent petaloidy of lateral sepals and fusions between sepals and petals are most likely the consequence of this displacement. The mechanical nature of these distortions is corroborated by the reduced frequency of lateral fusions between organs in whorls 1 and 2 and the lack of organ size reduction in whorl 1 of *def inco* double mutants, where whorl 2 is occupied by small sepaloid organs instead of the larger petals, owing to the homeotic defect caused by mutation in the *DEF* gene (Sommer et al., 1990) (Fig. 6A,B and Fig. 5G,H).

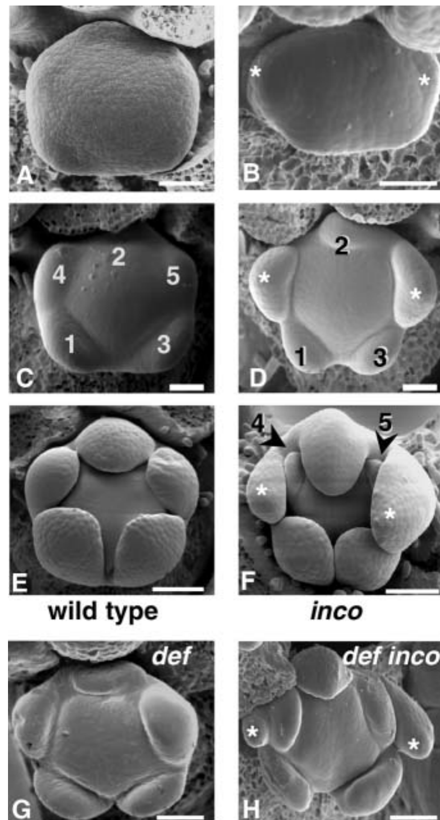

Fig. 5. SEM analysis of the ontogeny of wild-type (A,C,E) and *inco* floral meristems (B,D,F). The flowers shown in B are at late stage 2 and at early stage 3 in A, at stage 4 in C,D and at stage 5 in E-H [stages from Carpenter et al. (Carpenter et al., 1995)]. In *def* (G), second whorl organs are homeotically transformed to sepals and are smaller than in the wild type (see Fig. 6A,B), therefore size and position of sepals is less affected in *def inco* flowers (H). Prophylls are shown by an asterisk and numbers indicate the sequential order of appearance of sepal primordia. Scale bars: 50 µm in A,B; 100 µm in C-H.

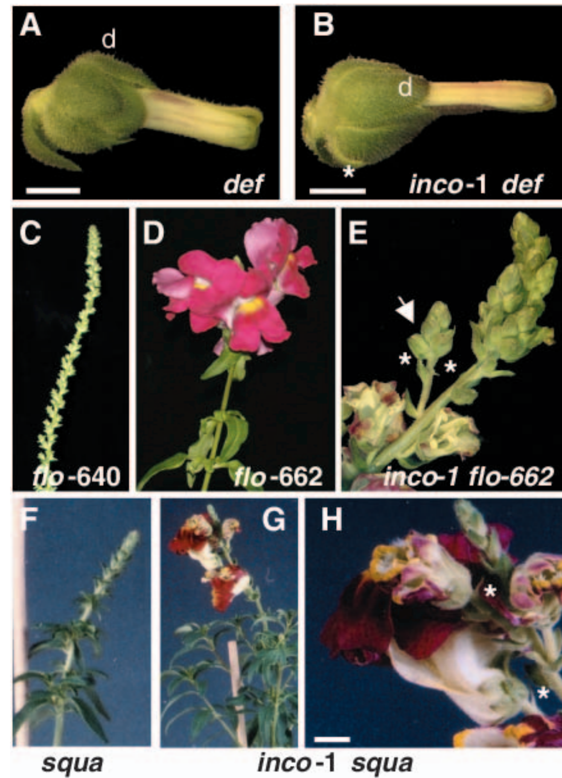

Fig. 6. Double mutant analyses with *inco*-1. (A) *def* flower with petals replaced by sepals. (B) *inco def* flower. In contrast to *inco* flowers (Fig. 4), lateral sepal size is hardly affected and no fusions occur to second whorl organs. d, dorsal sepal (C) Inflorescence of the strong *flo*-640 mutant compared with the weak *flo*-662 allele shown in D. (E) *inco flo*-662 inflorescence with axillary inflorescences instead of flowers (arrow). (G,H) Flower formation is enhanced in *inco squa* double mutants compared with *squa* (F) grown under identical conditions in segregating populations. Asterisks indicate prophylls. Scale bars: 1 cm.

The role of *INCO* in the control of floral meristem identity

Development of prophylls perhaps is due to some delay or incompleteness in determination of floral meristem identity. To test this possibility, double mutants between *inco* and mutants controlling *Antirrhinum* floral meristem identity such as *flo* and *squa* were constructed.

Severe *flo*-640 mutants (Fig. 6C) display bracts arranged in a spiral phyllotaxis that carry, instead of flowers, axillary inflorescences composed of bracts in their axils (Coen et al., 1990). This severe phenotype is not affected when combined with *inco* (not shown). However, in combination with the weak *flo*-662 allele, which displays wild-type-like inflorescences with flowers (McSteen et al., 1998), *inco flo*-662 double mutants exhibit inflorescences (Fig. 6D,E). Expression of *FLO* is not altered in the *inco* mutant (Fig. 3H), indicating that the enhanced *flo* mutant phenotype is not due to impaired transcriptional regulation of *flo*-662 in the double mutant. The synergistic effect of mutations in *inco* and *flo* thus suggests that the *INCO* and *FLO* functions converge in establishing the floral meristem. It is possible, that the role of INCO in preventing prophyll development is related at least in part to its function in the control of floral meristem identity. If so, then prolonged delay in floral determination should result in more leaf-like organs before sepal initiation. Frequent development of an additional filamentous leaf-like organ ('third prophyll') in the *inco flo*-662 double mutant (not shown) supports this assumption.

squa mutants are affected in the transition from inflorescence to floral meristem, similar to *flo* mutants, but *squa* deletion mutants occasionally produce flowers, which are frequently misshapen and are subtended by prophyll-like organs (Huijser et al., 1992). Intriguingly, *inco squa* plants produced more flowers and flowered earlier than *squa* (Fig. 6F,G), and *squa* plants heterozygous for *INCO* had a phenotype intermediate between *squa* and the *inco squa* double mutant, indicating a dose effect of *INCO*.

The partial epistasis of *inco* to *squa* might suggest that *INCO* in the *squa* mutant background prevents flower formation and that *SQUA* counteracts this negative influence in the wild type. Enhanced flowering tendency of *inco squa* double mutants, if interpreted as indication for improved floral determination (see above), should be accompanied by improved flower morphology. Yet, the morphology of *inco squa* double mutant

Table 1. Protein interactions in yeast

GAL4 binding domain	GAL4 activation domain		
	INCO	*lacZ* assay*	SVP (*Arabidopsis thaliana*)
INCOΔC[†]	+	1.40±0.09	Not tested
SVP (*Arabidopsis thaliana*)	Not tested	Not tested	–
SQUA	+	10.83±1.19	+
AP1 (*Arabidopsis thaliana*)	Not tested	Not tested	+
SEP1 (*Arabidopsis thaliana*)	+	Not tested	+
SEP2 (*Arabidopsis thaliana*)	+	Not tested	+
DEFH200	+[‡,§]	17.83±0.75	+
DEFH84	+	18.14±0.51	Not tested
DEFH72	+	Not tested	Not tested
PLE	+[‡]	17.98±0.42	+
SQUA	SQUA	1.07±0.33	
INCOΔC[†]	SQUA	13.65±1.05	

*Miller units (see Materials and methods)
[†]C-terminal deletion derivative that does not activate transcription in yeast
[‡]Described by Davies et al. (Davies et al., 1996)
[§]Higher order complexes with INCOΔMIK1/2 and PLE

flowers (Fig. 6H) remains similar to *squa* flowers, and they even contain a 'third prophyll' sometimes. This suggests that promotion of flowering, floral determination and prophyll initiation are not fully linked.

In the *squa* mutant, *INCO* transcript was not detectable in P0 bract primordia (Fig. 3G), in accordance with the presence of prophylls on the long pedicel of occasionally forming *squa* flowers (Huijser et al., 1992). *SQUA* is thus an activator of *INCO* expression. The level of *INCO* expression was slightly reduced in secondary inflorescence meristems (Fig. 3G), suggesting that *SQUA* is not absolutely necessary to establish and maintain *INCO* transcription in reproductive axillary meristems.

In summary, the functional relations of INCO, FLO and SQUA in the control of floral meristem identity, promotion of flowering and prophyll formation are complex, and the role of INCO in these processes cannot be simply reduced to the control of floral meristem identity.

Protein interactions

Given the influence of *INCO* on floral meristem identity, we asked whether INCO interacts with MADS-box transcription factors involved in the same process. Yeast two-hybrid screens showed that INCO heterodimerises with several other MADS-box proteins such as SQUA and the so-called identity mediating (Im) proteins DEFH200, DEFH84 and DEFH72 (Table 1), the orthologues of the *Arabidopsis* AP1 and SEPALLATA (SEP) proteins, respectively (Egea Gutierrez-Cortines and Davies, 2000). Interestingly, according to semi-quantitative assays, heterodimer formation between INCO and SQUA, as well as with several other MADS-box proteins, is favoured compared with INCO homodimer formation (Table 1). It is likely therefore, that INCO homodimerisation is prevented in vivo by interactions with other proteins. As expression of all potential INCO heterodimerisation partners tested is controlled by *SQUA* (Davies et al., 1996), INCO homodimerisation appears to be favoured in the *squa* mutant background.

SVP is the closest *Arabidopsis* relative of the INCO protein (Fig. 2) and, similar to INCO, SVP interacts with AP1, SEPALLATA1 (SEP1) and SEP2, as well as with the respective *Antirrhinum* proteins (Table 1). The similarity in protein interactions is in line with the observed common features of *Arabidopsis* plants overexpressing SVP or INCO (see below). In contrast to INCO, however, SVP cannot homodimerise and cannot activate transcription in yeast on its own. In fact, the knockout phenotypes of *inco* or *svp* mutants differ, in part perhaps as a consequence of these differences.

INCO interacts with the floral organ identity MADS-box protein PLENA (PLE), and we also observed higher order complexes (e.g. ternary) between INCO, PLE and DEFH200 (Table 1). This could suggest that INCO is involved, together with PLE, in a developmental control function in stamens, in agreement with the expression of *INCO* mRNA in mature anthers. *inco* mutant stamens are not visibly affected in their development under our growth conditions, suggesting that other factors can mask here the role of INCO.

Ectopic expression of *INCO* and *SVP* in *Arabidopsis* represses flowering

The observation that heterodimer formation is favoured over INCO homodimerisation prompted us to determine whether an excess of the *INCO* gene product resulting in over-production of INCO in the plant has some developmental consequences. *Arabidopsis* plants expressing the 35S::INCO transgene were generated to address this issue.

35S::INCO transgenic plants showed dramatic delay in the floral transition compared with wild-type plants (Fig. 7A). Their flowers displayed leaf-like features, such as branched trichomes on sepals, petals and carpels (Fig. 7B-D). Early arising flowers were more severely affected than later ones, and initiated inflorescences within the gynoecium (not shown). All changes observed point to incomplete floral transition in the presence of the 35S::INCO transgene. This is in line with the observed enhancement, compared with *squa*, of flowering in *inco squa* double mutants and supports the idea that, in the *squa* mutant, flowering is prevented by an excess of INCO, e.g. by formation of the INCO homodimer. In fact, *Arabidopsis svp* mutants flower earlier than wild type, suggesting that SVP prevents floral transition (Hartmann et al., 2000). In agreement with this function, and also with the yeast experiments, the behaviour of 35S::SVP and 35S::INCO in transgenic plants is similar (Fig. 7A,D), suggesting some common molecular and functional properties of the proteins. However, the *svp* and *inco*

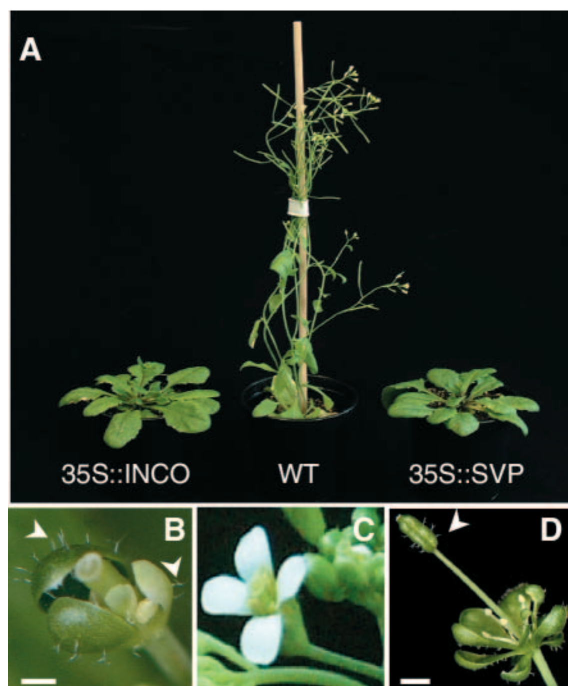

Fig. 7. Phenotypes of transgenic *Arabidopsis* plants overexpressing INCO and SVP. (A) Delay of flowering in 35S::INCO and 35S::SVP plants (about 27 rosette leaves when bolting after 32 days of growth) compared with wild type (WT, about 17 rosette leaves when bolting after 22 days of growth). 35S::INCO (B) and 35S::SVP (D) flowers with vegetative characters such as branched trichomes on sepals, petals, carpels (arrowheads) and sepaloid (green) petals compared to wild type (C). A more aberrant early flower is shown in D and a less affected later arising one in B. Scale bars: 1 mm.

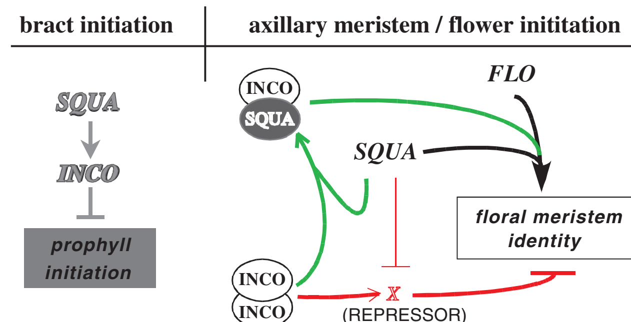

Fig. 8. Genetic control of *Antirrhinum* floral meristem identity and prophyll initiation by INCO and SQUA. Arrows indicate promoting functions and bars show negative effects; neither of these is meant to be direct. Proteins are shown as ovals. For simplicity, we neglect the option that INCO and SQUA are likely to interact with other MADS-domain proteins. The control of prophyll development by *SQUA* and *INCO* is highlighted by grey on the left. The arrows merging in the *FLO* control show convergence of processes promoting flower formation. Green shows potential activation by preference for heterodimerisation of INCO with SQUA. Red (and thin lines) suggests a possible mechanism that can counteract the negative influence of the INCO homodimer on flowering in the wild type. Reinforced heterodimerisation of INCO with SQUA supersedes repression of flowering by INCO. This negative influence, therefore, is relevant only in the *squa* mutant background.

knockout phenotypes differ, indicating that this common potential is exploited in different ways in *Arabidopsis* and *Antirrhinum*. Thus, as previously noticed, the bona fide function of MADS-box proteins cannot be fully deduced from overexpression in transgenic plants in the absence of observations with loss-of-function mutants (Davies et al., 1999).

Discussion

Functional diversity among members of the StMADS11 group

MADS-box genes, whose protein products belong to a particular subfamily, frequently reveal similar spatial and temporal expression patterns and perform similar control functions (Theißen et al., 1996). This ‘rule’ does not hold true for the StMADS11 group, the members of which participate in diverse functions and display broad, but distinct, expression patterns.

The tomato MADS-box gene *JOINTLESS* is expressed in all tissues tested by northern hybridisation, but has a defined role in the establishment of the abscission zone on pedicels only (Mao et al., 2000).

SVP, a negative regulator of flowering time in *Arabidopsis*, is expressed in reproductive meristems in a pattern similar to *INCO* (Hartmann et al., 2000). Interestingly, overexpression of *SVP* or *INCO* in *Arabidopsis* prolongs the vegetative phase and prevents flowering suggesting potential functional similarities. *INCO*, like *SVP*, could either directly repress the vegetative to floral transition in *Arabidopsis*, or could indirectly interfere with the function of proteins controlling flowering. As several regulators of *Arabidopsis* flowering time are MADS-box proteins (reviewed by Becker and Theißen, 2003), reinforced interactions in the presence of excess of SVP or INCO might result in their depletion and inactivation. In spite of the potential to interfere negatively with flowering in *Arabidopsis* and in apparent contrast to *svp* mutants, *inco* mutants have no obvious flowering time defects. In fact, as discussed below, the negative role of INCO is masked by competitive interaction with other proteins, such as SQUA, in the wild-type plant.

AGL24 and SVP are close *Arabidopsis* relatives within the StMADS11 group (Fig. 2B). The *AGL24* gene is abundantly expressed in *Arabidopsis* in all meristems, except for the floral, where its expression is limited to a single cell layer (Yu et al., 2004). When overexpressed, *AGL24* promotes flowering (Michaels et al., 2003), in apparent contrast to *INCO* and *SVP*, which repress flowering (see above). Furthermore, in the wild type, *AGL24* negatively controls floral meristem identity as demonstrated by the rescued, wild-type like phenotypes of double mutants of *agl24* with *lfy* (Yu et al., 2004). By contrast, defects in *FLO* (the orthologue of *LFY*) in *flo* mutants are enhanced by *inco*, suggesting a positive role of *INCO* in the control of *Antirrhinum* floral meristem identity, as discussed below. Thus, the role of *INCO* in floral meristem identity control is distinctively different from that of either *SVP* or *AGL24*.

Last but not least, *INCO* is a crucial control gene for repression of prophyll development, a function that has not been reported yet for MADS-box genes. Thus, the StMADS11 clade unites members with a variety of different functions:

some are unique to individual members, some are shared among them and some others have opposite functions.

Prophylls: some plants have them and some do not

The term prophyll is applied to the leaf or leaves at the first (proximal) node on the shoot, distinguishable in shape and arrangement from other leaf organs. Bracteoles, which are small leaf-like organs developing between a subtending bract and the calyx of the flower, are therefore prophylls (Bell, 1991). Prophylls possibly protected floral buds and reproductive organs in ancestors of modern angiosperms, and their function was taken over by the calyx during evolution. The evolutionary ancient origin of prophylls is supported by their presence in the 'living fossil' *Amborella*, where female and male flowers contain two prophylls placed close to, or within, the flower (Endress and Igersheim, 2000). Similar to *inco* mutant flowers *Amborella* prophylls can fuse with the nearest sepals.

Prophylls became integrated into the calyx of some species during evolution and in some others prophylls were 'lost' (Weberling, 1989). Indicative for the former case are flowers where lateral sepals (the genuine position of the prophylls) initiate first, while evolutionary loss (degeneration by suppression) of prophylls is suggested by the abaxial-dorsal-abaxial sepal initiation pattern, before lateral sepals appear. In *Antirrhinum*, the order of sepal initiation in the wild type indicates that prophylls were not integrated into the calyx and the development of prophylls in the *inco* mutant suggests that *INCO* was recruited during evolution to prevent their development. This interpretation correlates well with the lateral position of *inco* prophylls beneath the calyx, the emergence of their primordia before those of ventral and dorsal sepals, and the maintenance of the principal order of floral organ initiation in the *inco* mutant. Alternatively, it is possible that the *inco* mutation affects the timing of lateral sepal initiation and causes a heterochronic homeotic defect. In this scenario, the lateral sepals initiate first, at a time when floral identity is not fully established, and hence they acquire an intermediate bract/sepal fate. The pair of lateral organs that develop subsequent to the ventral and dorsal sepals correspond then to extra sepals, which, in the mature calyx, occupy the position of lateral sepals. The developmental role of *inco* thus would be to prevent premature lateral sepal initiation. However, it is difficult to relate displacement of the two first initiated (transformed) sepals outside of the calyx and initiation of two extra organs to a homeotic defect alone. Therefore we favour the 'prophyll hypothesis', where the extra *inco* organs are prophylls rather than homeotically transformed lateral sepals.

Several species of the Scrophulariaceae lack prophylls, like *Antirrhinum*, but their presence is common as well (Heywood, 1998), for example, in two of the English *Verbascum* species (Stace, 1997) and in the Chinese *Mimulus bracteosus*. It will be interesting to elucidate whether presence and absence of prophylls in a species is associated with changes at an *INCO*-like locus.

Prophyll development and floral architecture

Various theories assume that organ initiation is regulated by the geometry of the apex and by mechanical forces (tension and compression) that act on meristem surfaces (Reinhardt and Kuhlemeier, 2002). According to the theory of the 'first available space', based on microsurgical manipulations, the timing and positioning of organ initiation is regulated by the availability of the minimal free area on the meristem surface, at a minimal distance from the summit and from pre-existing primordia (Snow and Snow, 1931; Snow and Snow, 1933). In other interpretations, space itself is not decisive; a new primordium emerges at the position of weakest inhibition where the most recently formed primordium is the strongest source of inhibition (Tooke and Battey, 2003; Wardlaw, 1949). Indeed, in *inco* mutants, development of lateral sepal primordia is significantly delayed, owing to the aberrant initiation of prophyll primordia, but the temporal order and the principal site of their initiation are not affected. Initiation of prophylls thus does not seem to interfere with auxin redistribution, shown to be a decisive factor in the maintenance of phyllotaxis (Reinhardt et al., 2003).

However, the presence of prophylls has severe consequences for the overall architecture of the flower, in that lateral sepal primordia are forced towards the developing petal primordia in the second whorl and, perhaps owing to consumption of cells by the prophylls, lateral sepals are frequently smaller than the corresponding wild-type sepals. Chimeric sepaloid-petaloid organs develop frequently, or, if contact is established to the petal primordia, sepals and petals can fuse. Such anomalies were also observed in sunflower, where applying mechanical stress during development resulted in large bracts instead of the dyad (bract/floret) structure (Hernandez and Green, 1993). The mechanical nature of these alterations in the *inco* mutant is corroborated by the lower frequency of size reduction of organs and of fusions between first and second whorl organs in *inco def* double mutants, where the size of second whorl primordia is reduced, owing to their homeotic transformation to sepaloid organs. Thus, repression of prophyll initiation by *INCO* is a prerequisite for establishment of the wild-type floral architecture.

INCO is a novel component of *Antirrhinum* floral meristem identity control

The phenotype of *inco* with the lack of repression of prophyll development and the disordered development of floral organs in *inco* mutants resembles the phenotype of rarely forming *squa* flowers. In fact, we found that during the time of bract initiation, *SQUA* is a direct or indirect activator of *INCO* expression (Fig. 8, left).

During flower formation, however, *INCO* expression is less dependent on *SQUA* and the relation between their functions becomes more complex. This is revealed by the observation that *squa inco* plants produce more flowers than do *squa* plants, suggesting that INCO in the absence of SQUA prevents reproductive axillary meristems to become flowers. INCO, therefore, is a repressor of flower development, although other factors may be involved in addition, as *squa* mutants can flower, albeit at low frequency. The fact that *inco* mutants do not flower more abundantly than wild type can be explained when assuming that *INCO* promotes expression or function of a repressor of flowering whose effect on flower formation is counteracted by *SQUA* (Fig. 8, thin red lines). This function is most probably performed by INCO as homodimer, the existence of which is supported by yeast two-hybrid experiments and whose negative influence on flowering is

manifested in transgenic *Arabidopsis* plants overexpressing *INCO*. The observed dependence of flowering on *INCO* dose in the *squa* mutant background would thus indicate that the amount of homodimer that can form in an *inco*/*INCO* heterozygote is not fully sufficient to promote the function of the putative repressor of flowering. However, the possibility that, in addition to (or instead of) the INCO homodimer, INCO in association with other proteins performs this repressor function cannot be excluded.

Somewhat surprisingly, *INCO* is also a positive factor in the control of floral meristem identity. This is uncovered in *inco flo*-662 double mutants, where *inco* enhances the otherwise not manifested meristem identity defect in the weak *flo*-662 mutant. In this respect, the influence of *INCO* is comparable with the enhancement of the *flo*-662 mutant phenotype in the background of *squa* [see Introduction and Carpenter et al. (Carpenter et al., 1995)]. Thus, in the presence of *SQUA*, *INCO* acts together with *FLO* to promote flower development.

Given that wild-type plants flower in spite of INCO expression and hence potential repression of flowering, we have to explain how INCO can become a positive factor in flowering. One appealing assumption is that the INCO/SQUA heterodimer (and/or heterodimerisation with proteins whose expression is controlled by *SQUA*) performs the promoting function, and that in the presence of SQUA heterodimerisation is favoured compared with INCO homodimerisation (Fig. 8, green lines). This would deteriorate the repressive function of the INCO homodimer in the wild type, which is in favour of promotion of flowering. In fact, at least in yeast, the SQUA/INCO interaction (and the interaction of INCO with several other *SQUA*-controlled proteins) is stronger than INCO homodimerisation. In addition, impaired floral determination of transgenic *Arabidopsis* plants expressing excess of INCO shows that disturbing the balance in favour of INCO, and hence facilitating homodimer formation, enhances the negative effect of INCO. The overlap of the *SQUA* and *INCO* expression patterns in initiating floral primordia is in agreement with the potential for protein-protein interaction between the INCO and SQUA proteins in planta.

Intriguingly, in the presence of sufficient *SQUA* and *FLO* function in the wild type, the role of *INCO* in the control of floral meristem identity appears superfluous, and the lack of *INCO* in the *inco* mutant manifests itself in prophyll development only. Possibly, suppression of prophyll development by *INCO* is a relatively novel function acquired by a MADS-box protein with the potential to interfere with the floral transition. The complex relations to *SQUA* and *FLO* were perhaps established to prevent this interference.

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