

# The *Arabidopsis* nectary is an ABC-independent floral structure

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## SUMMARY

In contrast to the conservation of floral organ order in angiosperm flowers, nectary glands can be found in various floral and extrafloral positions. Since in *Arabidopsis*, the nectary develops only at the base of stamens, its specification was assayed with regard to the floral homeotic ABC selector genes. We show that the nectary can form independently of any floral organ identity gene but is restricted to the ‘third whorl’ domain in the flower. This domain is, in part, specified redundantly by *LEAFY* and *UNUSUAL FLORAL ORGANS*. Even though nectary glands arise from cells previously expressing the B class

genes, their proper development requires the down-regulation of B class gene activity. While *CRABS CLAW* is essential for nectary gland formation, its ectopic expression is not sufficient to induce ectopic nectary formation. We show that in *Arabidopsis* multiple factors act to restrict the nectary to the flower, and surprisingly, some of these factors are *LEAFY* and *UNUSUAL FLORAL ORGANS*.

Key words: Nectary, flower development, *CRABS CLAW*, *Arabidopsis thaliana*

## INTRODUCTION

Nectaries are secretory structures that produce nectar, a carbohydrate-rich solution composed mainly of sugars. Nectar generally serves as a reward for pollinators or for protectors (e.g., ants) against herbivores, or in carnivorous plants as a lure for animal prey (Fahn, 1988; Schmid, 1988; Owen and Lennon, 1999). Owing to the importance of nectaries in pollination, their location within flowers is under selection so that their relative position within the flower ensures the deposition of pollen on the stigma by a particular pollinator.

In the Brassicaceae, the floral nectary is located such that the nectary glands are positioned at the base of stamen filaments (Knuth, 1908; Arber, 1931a; Norris, 1941; Davis et al., 1986) and the nectar is composed mainly of glucose and fructose (Davis et al., 1994). In *Arabidopsis*, the nectary glands subtend the abaxial side of the stamen filaments (Davis, 1994) and exhibit different morphology and physiology depending upon their location (Davis et al., 1998). In comparison to the medial glands, the lateral glands are larger, always innervated with sieve tube members, produce more nectar and have a higher glucose to fructose ratio. In *Arabidopsis*, *CRABS CLAW* (*CRC*) is the only gene known to be essential for nectary formation; in *crc* mutants, no nectary glands develop (Bowman and Smyth, 1999). *CRC* encodes a putative transcription factor and is expressed in the nectary throughout its development, consistent with a role in the specification and/or differentiation of the nectary.

Within the angiosperms, the relative order of floral organs (sepals, petals, stamens and carpels) along the receptacle is invariant, leading to the suggestion that the genetic program that specifies the identity of these organs is conserved

(Bowman, 1997; Ambrose et al., 2000; Kyoizuka et al., 2000). In contrast, the location of nectaries within flowers is variable, with nectaries arising at any position along the receptacle or associated with any of the four floral organs (Brown, 1938). For example, in species of the Brassicaceae the nectary is found at the base of the stamens (Knuth, 1908; Arber, 1931a; Norris, 1941; Davis et al., 1986), in the Solanaceae, the nectary is found at the base of the gynoecium, and in species of the Malvaceae, nectary glands are found on the abaxial side of the involucre bracts as well as the adaxial side of sepals (Butler et al., 1972). Studies using *Arabidopsis* have shown that the four groups of floral organs (sepals, petals, stamens and carpels) are specified by three classes of genes; class A [*APETALA1* (*API*) and *APETALA2* (*AP2*)], class B [*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)] and class C [*AGAMOUS* (*AG*)] (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). A class genes alone specify sepals, A and B class genes specify petals, B and C specify stamens and C class alone specifies carpels. Most of these genes belong to the group encoding MADS box transcription factors and have been shown to specify the identity of floral organs in other plant species also (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Ambrose et al., 2000). Based on preliminary observations of nectaries in some of the floral homeotic mutants, it has been suggested that the nectary is position dependent and not dependent on the presence of stamens (Bowman et al., 1991; Davis et al., 1993; Bowman and Smyth, 1999).

The objective of this study is to investigate the influence of the floral homeotic genes and regulators of floral development on the formation of the nectary. Results from our analyses demonstrate that *CRC* is necessary but not sufficient for

nectary formation, suggesting other factors are responsible for nectary formation along with *CRC*. In *Arabidopsis* the nectary is formed in the third whorl and is position dependent, being independent of both the ABC floral homeotic genes and the type of organ occupying the whorl in which the nectary resides. Factors required for both formation of third whorl nectary promotion and its restriction to this domain include upstream regulators of floral meristem architecture and organ whorl boundary maintenance.

## MATERIALS AND METHODS

### Growth conditions, marker lines

All mutant and transgenic lines were analyzed in the Landsberg *erecta* background, unless otherwise noted. Plants were grown in an 18-hour light/6-hour dark regime. The enhancer trap line *ET668* was a gift from Hong Ma. YJ73, YJ86 and YJ103 were generated as described by Eshed et al. (Eshed et al., 1999). *FUL::GUS* (Gu et al., 1998) and *SHP2::GUS* (Columbia) (Savidge et al., 1995) were kindly provided by Marty Yanofsky. TJ2791 (Columbia) (Campisi et al., 1999) was a gift from Tom Jack. *KNAT2::GUS* (C-24) was a gift from Jan Döckx. A599-1 (Columbia) was a gift from Gary Drews. *CRC::GUS* and *SHP1::GUS* lines contain transcriptional fusions of approximately 8 kb and 3.5 kb of sequence, respectively, 5' of the putative translational start site with the  $\beta$ -glucuronidase (GUS) reporter gene.

### Microscopy

Anatomical and SEM analyses were carried out according to the method of Baum and Rost (Baum and Rost, 1996) and Siegfried et al. (Siegfried et al., 1999), respectively. During the infiltration step, tissue was allowed to infiltrate for 2 weeks at 4°C to alleviate the problem of tissue separating from the plastic during sectioning. Samples were first stained for GUS activity (McConnell and Barton, 1998) and then cleared in 70% ethanol overnight and observed using a Zeiss dissecting stereoscope or processed as above prior to sectioning. Sections were photographed using dark-field optics on an Olympus Vanox photomicroscope. For bright-field microscopy, tissue sections were stained using the PAS reaction (Baum and Rost, 1996). SEM images were captured electronically and compiled in PhotoShop (Adobe).

### In situ hybridization

In situ hybridizations were performed as previously described (Vielle-Calzada et al., 1999). *CRC*, *AP3* and *PI* probes were generated as previously described (Bowman and Smyth, 1999; Jack et al., 1992; Goto and Meyerowitz, 1994).

### Genotyping *lfy-6* and *ufo-2*

Both alleles were identified using CAPS markers according to the method of Lee et al. (Lee et al., 1997), Michaels and Amasino (Michaels and Amasino, 1998) and Blazquez et al. (Blazquez et al., 1997).

## RESULTS

### Development and morphology of nectaries

The nectary in *Arabidopsis* is composed of two distinct parts: the nectary glands themselves (from which nectar is secreted) and a ridge of tissue connecting each of the glands (Fig. 1A,B). The nectary occupies the entire circumference of the receptacle situated within the third whorl. Nectary glands are positioned on the abaxial side of each of the six stamens, of which two

occupy lateral positions and four medial positions. The medial glands are oblong and smaller than the lateral ones. The lateral glands consist of two oblong confluent parts or are sometimes large and disk-shaped, the width of which is slightly greater than the diameter of the stamen filaments (present study) (Davis, 1994). The epidermal cells of the glands have a distinct cuticle with a reticulate pattern of thickenings (Fig. 1C). The ridge of tissue between the lateral and medial glands (Fig. 1D,E), as well as the region between the medial glands does not exhibit this cuticle pattern, but these regions are considered part of the nectary on the basis of the pattern of *CRC* expression (Bowman and Smyth, 1999). Modified stomata (i.e., permanently open) (Davis and Gunning, 1993) can be found on all lateral glands on the abaxial side near the tip and on the abaxial side of most of the medial glands (Fig. 1C).

Each nectary gland is composed of two tissues: the epidermis (composed of guard cells and non-guard cells) and parenchyma tissue of the nectary gland body (Fig. 1E-G). The parenchyma cells are densely cytoplasmic with starch containing plastids (Fig. 1F). Within the basal region near the receptacle of the lateral and some of the medial glands are sieve tube members that are connected to the phloem of the receptacle vascular bundles (Fig. 1G). The estimated number of cells in a lateral nectary gland at anthesis is approx. 2000 and in a medial gland is approx. 400.

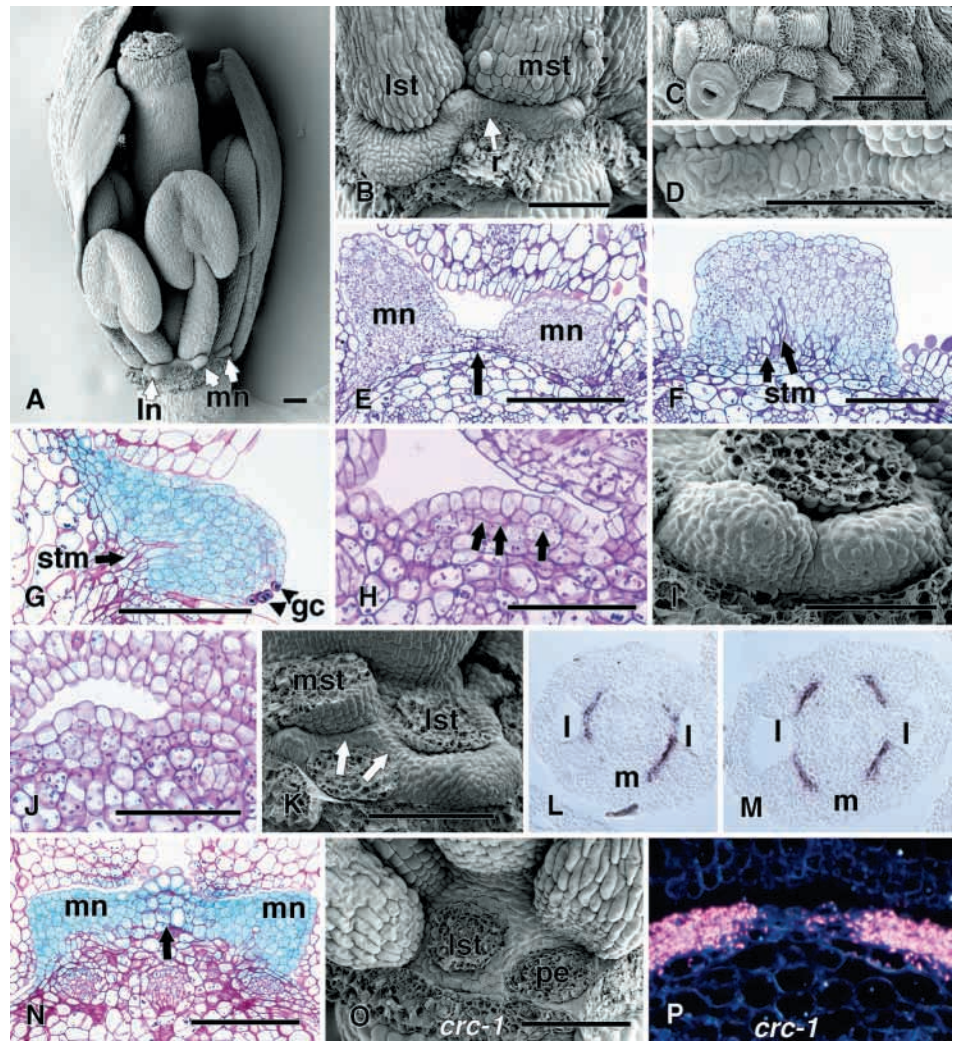
The first morphological signs of nectary gland formation appear during stage 9 (stages according to Smyth et al.) (Smyth et al., 1990). At this stage, all other floral organs have begun to acquire their characteristic morphology. The glands originate from periclinal divisions in the L2 layer of the receptacle (Fig. 1H) with the lateral glands initiating before the medial glands. A lateral gland initiates as two protrusions by two localized regions of cell divisions, followed by further divisions in an expanded domain (Fig. 1I,J). Prolonged cell divisions in two localized regions will result in two oblong confluent glands, while a short period of localized division, followed by divisions in an expanded area would produce a disc-shaped organ. Concomitant with the formation of the glands is the differentiation of an accompanying ridge of tissue connecting the glands. Topographically, the ridge of tissue starts at the medial gland, continues underneath the associated filament and then down the receptacle to connect with the lateral gland (Fig. 1K). The curved nature of the ridge reflects the fact that the medial and lateral stamens are not located in the same plane along the receptacle. Stomata become evident by stage 10 (Fig. 1K) followed by the first signs of cuticle formation. The presence of starch in the parenchyma cells is evident from the earliest signs of nectary gland development (Fig. 1H,J).

### Molecular markers of nectary development and the role of *CRC*

Since nectary glands are small and variable in their development, both morphological and molecular markers were utilized in our analyses (Table 1). Two marker lines, *CRC::GUS* and *ET668*, were chosen to represent the early and middle stages of nectary development, respectively. GUS staining of line *ET668* is confined to the nectary from the time of gland initiation and expression continues beyond anthesis through stage 17 (at which stage all sepals, petals and stamens have abscised but the fruit is still green). Expression is in both the gland and the ridge of tissue between lateral and medial



**Fig. 1.** SEM (A-D,I,K and O) and sections (E-H,J,L-N and P) of nectary development. (A) *Arabidopsis* flower before anthesis, stage 12-13 highlighting nectary glands (arrows). (B) Higher magnification of A showing lateral and medial nectary glands with intervening ridge (r) of tissue (arrow). (C) Close-up of lateral nectary gland showing reticulate pattern of the cuticle and modified stomata. (D) Close-up of medial nectary gland and intervening medial region without characteristic nectary gland cuticle. (E) Transverse section of two medial nectary glands and intervening region (arrow). (F,G) Transverse and longitudinal section of a lateral nectary gland, respectively. Arrows indicate sieve tube members (stm) and arrowheads indicate guard cells (gc). In G the nectary gland is expressing *CRC::GUS*. In E and F starch appears as dark grains within the cells of the nectary gland and intervening region. (H) Transverse section of the flower receptacle of a stage 9 flower. Arrows point to newly formed periclinal divisions of the L2 layer. (I) Morphology of a young lateral nectary gland from a stage 10 flower. (J) Transverse section of a stage 9-10 flower receptacle showing the early developmental anatomy of a lateral nectary gland. The gland is composed of two small protrusions from the receptacle. (K) Close-up of a medial and lateral nectary gland with intervening ridge of tissue (arrows) from a stage 13 flower. (L,M) Two serial consecutive sections of a stage 7/8 flower showing *CRC* expression. There is no expression in the medial domain in the region between the two medial stamens. (N) Transverse section of two medial nectary glands taken just within the receptacle of a stage 12 flower. Receptacle tissue is present in between the two glands and is expressing *CRC::GUS* (arrow). (O) A stage 13 *crc* mutant flower. The nectary glands are absent. (P) Transverse section through a stage 13 flower receptacle of a *crc-1* mutant showing three cell layers expressing *CRC::GUS* (red crystals), there is no expression in the middle of the medial domain. In, lateral nectary gland; mn, medial nectary gland; lst, lateral stamen; mst, medial stamen; l, lateral domain; m, medial domain; pe, petal. Scale bars, 100  $\mu$ m for A,B,D-H,K,N and O; 50  $\mu$ m for I and J; 20  $\mu$ m for C.



glands with faint staining detected between medial glands. Plants carrying the *CRC::GUS* transgene show staining in the nectary anlagen of stage 7/8 flowers with continued expression throughout the nectary beyond stage 17. While *GUS* is also detected in the carpels, *CRC* was chosen as it has been shown to be required for nectary development (Bowman and Smyth, 1999). *CRC* expression is initially detected in two crescent-shaped domains with no expression in the region in between the medial nectary glands (i.e., the medial domain; Fig. 1L,M). By stage 10, expression is present throughout the nectary glands, including the accompanying ridges of tissue, and the medial domain in between each of the two pairs of medial nectary glands (Fig. 1N). No visible signs of nectary gland differentiation are evident in *CRC* mutants (Fig. 1O) (Bowman and Smyth, 1999). Expression of *CRC::GUS* in flowers of *crc-1* at anthesis is in two horseshoe-shaped domains, similar to the pattern observed in stage 7/8 of wild-type flowers (Fig. 1P).

Within the receptacle, expression is restricted to three cell layers deep and 4-6 cell layers high. Carpel expression of the *CRC::GUS* marker line is similar to the previously described expression pattern of the gene (Bowman and Smyth, 1999).

Other marker lines exhibiting nectary expression were examined (Table 1) and their expression patterns define three categories: (1) expression initiates when glands develop and terminates by anthesis (*KNAT2::GUS* and *AG::GUS*); (2) expression initiates when glands develop and continues past anthesis (*SHP1::GUS*, *SHP2::GUS* and *FUL::GUS*) and (3) expression initiates late in nectary gland development and is maintained in glands of anthesis stage flowers (YJ103, YJ86, YJ73, TJ2791 and A5991-1). These expression patterns likely reflect developmental stages of nectary gland differentiation. Nectary expression in all of the marker lines examined is abolished in *CRC* mutants, suggesting that *CRC* acts upstream of these genes in the nectary.

**Table 1. Genes expressed in the nectary glands**

Gene/enhancer trap	Nectary expression pattern (duration)
<i>KNAT2::GUS</i>	Stage 9 to 14
<i>AG::GUS</i>	Stage 9, decreasing by stage 13
<i>SHP1::GUS</i>	Stage 9, continuing past stage 14
<i>SHP2::GUS</i>	Stage 9, continuing past stage 14
<i>AGL8::GUS</i>	Stage 9, continuing past stage 14
YJ-103	Light expression in stage 13-14 flowers
YJ-86	Stage 12 becoming restricted to the tip of the outgrowth during stage 14
YJ-73	Stage 13, continuing past stage 14
TJ-2791	Stage 10, continuing past stage 14
a599-1	Stage 11, continuing past stage 14
<i>CRC::GUS</i>	Starts in the nectary anlagen of stage 7/8 flowering the continuing beyond stage 17
ET668	Stage 9 continuing beyond stage 17

Since *CRC* has been shown to be required for nectary gland formation (Bowman and Smyth, 1999), we tested whether its misexpression is sufficient to induce ectopic nectaries. No ectopic nectaries were observed in *35S::CRC* plants, and the nectary in these flowers appeared relatively normal. This indicates that *CRC* is necessary but not sufficient for nectary formation, implying the existence of other factors that promote (or inhibit) nectary development.

### Nectary development is independent of other floral organ identities

To examine the possible relationships between a specific organ identity and nectary formation in *Arabidopsis*, a variety of mutant backgrounds with altered floral organ identity were examined for the presence of a nectary. The morphology, anatomy and gene expression patterns of nectary markers in the various mutant backgrounds were analyzed, along with a functional assay for nectar secretion. The various backgrounds along with the summarized results are presented in Table 2. For the sake of characterization, nectary glands were defined as receptacle outgrowths with expression of both marker lines *ET668* and *CRC::GUS*. Unless otherwise mentioned, the outgrowths had the unique cuticular thickening and modified stomata typical of the wild-type nectary.

To examine whether stamen formation is a prerequisite for nectary development, their presence was assayed in mutant flowers where stamens are replaced by other floral organs. Flowers of both *pi-1* and *ap3-3* have sepals in the first two whorls, followed by fused third and fourth whorl carpels (floral formula=se, se, ca, ca) (Bowman et al., 1989; Jack et al., 1992). Nectary glands in *pi-1* mutants are located in the region between the second whorl sepals and the central carpels, and are oblong in shape even in the lateral domain (Fig. 2A). *pi-1* flowers always have lateral glands and sometimes possess medial glands; the glands are reduced in size when compared to wild type. *ap3-3* flowers generally have larger glands than those found in *pi-1* (Fig. 2B). Most *ap3-3* flowers have both lateral and medial glands though not all glands have stomata. Unlike *pi-1* flowers, *ap3-3* flowers have disk-shaped as well as oblong lateral glands. In *ag-1* flowers ([se, pe, pe]<sub>n</sub>) (Bowman et al., 1989), nectary glands are present between the second and third whorl petals (Fig. 2C). The morphology of the glands is variable, being either a bulb-like or disk-like with multiple glands sometimes occurring on one side of the receptacle (Fig. 2F). The presence of stomata is also variable. In the inner

**Table 2. Floral plan and nectar secretion in different genetic backgrounds**

Genotype/allele	Reference	Floral plan	Nectar secretion
Wild type		Se Pe nSt Ca	Yes
<i>crc-1</i>	a	Se Pe St Ca	No
<i>ap1-1</i>	b	Se – nSt Ca	Yes
<i>ap2-2</i>	c	Ca – nSt Ca	Sometimes
<i>pi-1</i>	d	Se Se nCa Ca	Infrequent
<i>ap3-3</i>	e	Se Se nCa Ca	Infrequent
<i>ag-1</i>	d	(Se Pe n*Pe) <sub>n</sub>	No
<i>ap2-2 pi-1</i>	c	Ca/Lv** – n – Ca	No
<i>ap2-2 ag-1</i>	c	(Ca/Lv St/Pe n*St/Pe) <sub>n</sub>	No
<i>pi-1 ag-1</i>	c	(Se Se) <sub>n</sub>	No
<i>ap3-3 ag-1</i>	f	(Se Se) <sub>n</sub>	No
<i>ap2-2 pi-1 ag-1</i>	c	(Ca/Lv – n – ) <sub>n</sub>	No
<i>lfy-6</i>	g	Se Se nCa Ca	No
<i>ufo-2</i>	h	Se Se nCa Ca	No
<i>sup-1</i>	i	Se Pe nSt nSt...	Yes
<i>35S::AP3</i>	j	Se Pe nSt St/Ca	Yes
<i>35S::PI</i>	k	Se/Pe Pe nSt Ca	No
<i>35S::B<sup>‡</sup></i>	k	Pe Pe nSt St	No
<i>35S::B<sup>‡</sup> ap2-2</i>	k	St St nSt St	No
<i>AP3::AG</i>	l	Se St nSt Ca	Yes
<i>35S::UFO</i>	m	Se/Pe Pe nSt nSt	Yes
<i>35S::UFO pi-1</i>	m	Se Se nCa nCa	No
<i>35S::UFO ap2-2</i>	f	Ca – nSt nSt	Yes
<i>35S::LFY</i>	n	Se Pe nSt Ca	Yes
<i>35S::CRC</i>	o	Se Pe nSt Ca	Yes
<i>35S::B<sup>‡</sup> ufo-2 lfy-6</i>	f	Se/Pe Pe Pe/St St	No
<i>ap1-1 35S::UFO</i>	f	Se – nSt nSt	Sometimes
<i>ap1-1 35S::CRC</i>	f	Se – nSt Ca	Sometimes
<i>35S::UFO 35S::CRC</i>	f	Se/Pe Pe nSt nSt	Yes

Se, sepals; Pe, petals; St, stamens; Ca, carpels; n, nectary; Ca/Lv, carpelloid leaves; St/Pe, staminoid petals; Se/Pe, sepaloid petals; St/Ca, stamens and carpels; Pe/St, petaloid stamens

\*Nectary outgrowths are not found in every reiteration; \*\*the lateral first whorl organs are leaf-like and are located farther down on the pedicel and are not transformed into carpels; <sup>‡</sup>*35S::B* is used to denote both transgenes; *35S::AP3* and *35S::PI*.

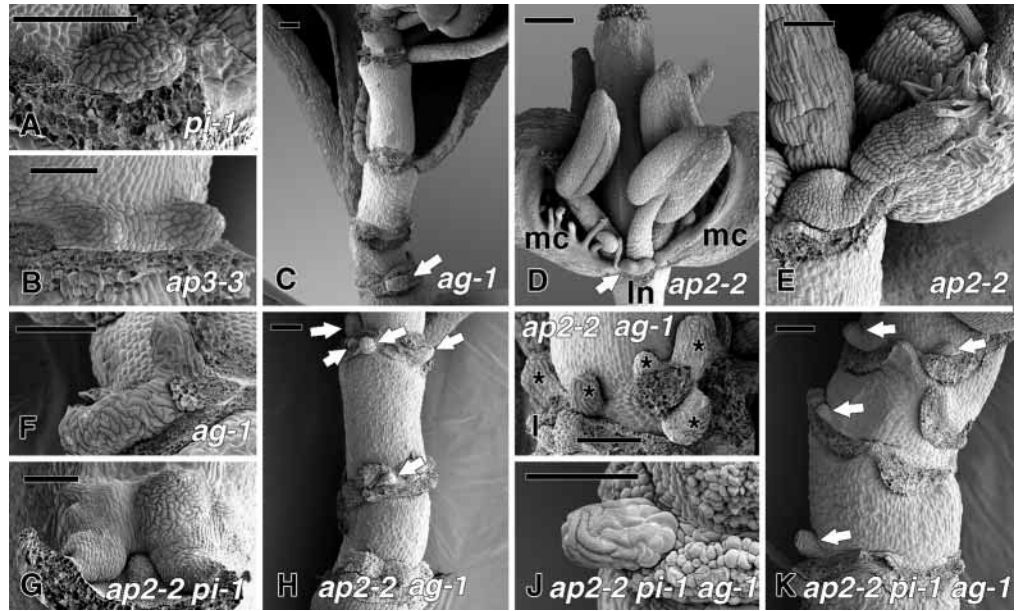
a, Alvarez and Smyth, 1999; Bowman and Smyth, 1999; b, Irish and Sussex, 1990; Bowman et al., 1993; c, Bowman et al., 1991; d, Bowman et al., 1989; e, Jack et al., 1992; f, present study; g, Weigel et al., 1992; h, Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; i, Bowman et al., 1992; Schultz et al., 1991; j, Jack et al., 1994; k, Krizek and Meyerowitz, 1996; l, Jack et al., 1997; m, Lee et al., 1997; n, Weigel and Nilsson, 1995; o, Bowman and Smyth, 1999, Eshed et al., 1999.

‘flowers’ (reiterations), glands may be present or not. Thus, the nectary develops in the third whorl irrespective of whether this whorl is occupied by stamens, sepals or petals.

In *ap2-2* flowers, carpels occupy the first whorl, second whorl organs fail to develop, a reduced number of stamens occupy the third whorl and carpels occupy the fourth whorl such that in many flowers, no second or third whorl organs are present (ca, –, st, ca) (Bowman et al., 1991). However, even in these cases, nectary glands are present in the lateral positions between the two medial first whorl carpelloid organs (Fig. 2D). Sometimes nectary glands develop along the margin of the first whorl medial organs instead of between them (Fig. 2E). The morphology of the glands is variable; they either resemble wild-type lateral glands or exhibit an aberrant morphology (Fig. 2E). In *ap2-2 pi-1* flowers the first whorl lateral organs are leaf-like and all other organs are carpels that tend to fuse to one another (lv/ca, –, ca, ca) (Bowman et al., 1991). Nectary glands are found interior to the lateral first whorl organs. Here



**Fig. 2.** SEM of nectary glands from the ABC single, double and triple mutants. (A) *pi-1*, (B) *ap3-3*, (C) *ag-1*, (D,E) *ap2-2*, (F) an example of a disk-shaped nectary in *ag-1*, (G) *ap2-2 pi-1*, (H,I) *ap2-2 ag-1*, (J,K) *ap2-2 pi-1 ag-1*. White arrows and black asterisks indicate nectary glands; ln, lateral nectary gland; mc, medial carpel. Scale bars, 100  $\mu$ m in A-C,E-K; 250  $\mu$ m in D.



too, the glands are variable in shape (Fig. 2G) and stomata may be absent. Thus, the presence of nectary glands does not require the concomitant development of a third whorl organ.

In *ap2-2 ag-1* double mutants the coordination between whorls and organ primordia initiation is partially restored. Thus *ap2-2 ag-1* flowers have a floral ground plan of carpelloid leaves in the first whorl followed by two whorls of staminoid petals, and this pattern is reiterated in internal whorls ([ca/lv, st/pe, st/pe]<sub>n</sub>) (Bowman et al., 1991). In these double mutants, more nectary glands per reiteration are present compared to *ag-1* mutant alone (Fig. 2H,I). The morphology of the glands is variable, from short oblong structures to disk-shaped glands.

Flowers lacking both B and C functions (*pi-1 ag-1*; *ap3-3 ag-3*) are composed of only sepals (Bowman et al., 1989; Jack et al., 1997). No signs of nectary formation were found in flowers of either of these genotypes. Based on the pattern of primordia initiation and the presence of internodal elongation between every two whorls, it is suggested that these flowers are composed of first and second whorls only (Bowman et al., 1991). Thus, the absence of a nectary could be considered either as synergism between the single mutants or as absence of the region of the floral meristem from which the nectary arises.

When A, B and C class activities are all compromised, as in *ap2-2 pi-1 ag-1* flowers, all flower organs are carpelloid leaf-like organs (Bowman et al., 1991). The floral ground plan was interpreted to be (ca/lv, –, –), such that the second and third whorl organs fail to develop and the flower consists of reiterations of carpelloid leaf-like structures occupying repeated first whorl positions. In addition, the organs tend to fuse to one another during ontogeny, both within whorls and between whorls. As a consequence, reiterations appear as rings around the receptacle when the organs are removed. In this triply mutant background, nectary glands are observed in some of the reiterations, generally appearing at least in the lateral domain in the first reiteration (Fig. 2K). The nectary glands were smaller and less frequent than those found in the doubly mutant *ap2-2 ag-1* flowers, and glands sometimes lacked stomata (Fig. 2J). That the *ap2-2 pi-1 ag-1* triple mutant has

nectaries implies that nectaries can develop independently of stamens, in particular, and of other floral organs in general and, furthermore that nectary development can be independent of the ABC homeotic selector genes. However, the lack of nectaries in *pi ag* and *ap3 ag* double mutants and their presence in the *ap2 pi ag* triple mutant implicates roles for the ABC genes in patterning the floral whorls.

While nectary formation is separable from stamens, it is still possible that the presence of stamens can promote nectary formation. To examine this possibility, flowers with multiple whorls of stamens were studied. Plants carrying both *35S::AP3* and *35S::PI* transgenes (referred to as *35S::B* hereafter) produce flowers with two outer whorls of petals and two inner whorls of stamens (pe, pe, st, st) (Krizek and Meyerowitz, 1996). In these flowers nectary glands are located subtending third whorl stamens but are not found subtending stamens occupying the fourth whorl (Fig. 3A). Likewise, in *ap2-2 35S::B* flowers, in which all four floral whorls are occupied by stamens (Krizek and Meyerowitz, 1996), nectary glands are restricted to the third whorl (albeit at a lower frequency than in *35S::B* flowers) and not in other floral whorls (Fig. 3B). Expressing *AG* under the control of the *AP3* promoter also results in the ectopic development of stamens (se, st, st, ca) (Jack et al., 1997), and again, nectary glands only subtend the third whorl stamens in these flowers.

### Nectary formation is position dependent

The genotypes discussed above demonstrate that nectary and stamen formation are separable. Moreover, they suggest that the nectary forms in the third whorl of the flower, irrespective of the floral organ which occupies the third whorl. This interpretation could explain why no nectary glands are formed in *pi-1 ag-1* and *ap3-3 ag-3* double mutants since the third whorl does not appear to develop in flowers of this genotype. Therefore we examined genotypes in which ectopic third whorls develop. In *superman-1* (*sup-1*) flowers, the boundary between the third and fourth whorls is disrupted such that the third whorl is reiterated multiple times (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). In *sup-1* flowers,

nectary glands subtend all stamens (Table 2). In addition, the glands subtending the outermost stamens are greatly enlarged relative to wild-type nectary glands (Fig. 3C). A similar pattern was seen in flowers of plants ectopically expressing *UNUSUAL FLORAL ORGANS (UFO)* which will be described later.

Thus, we conclude that nectary development is tightly associated with a region of the flower that in wild-type will give rise to the third whorl (referred to simply as the 'third whorl' hereafter), but independent of the presence of any type of floral organ.

### Complex relationships between the B class genes and nectary gland morphology

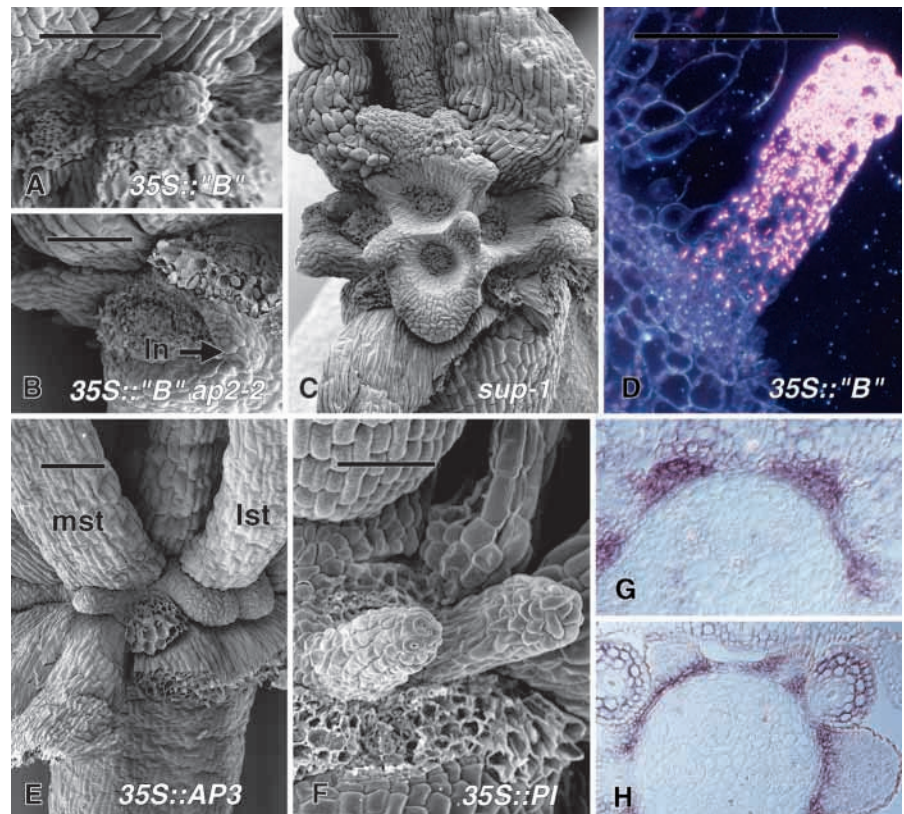
While nectary gland formation does not depend on stamen development, in wild-type flowers, absence of a stamen correlates with aberrant morphology of the associated gland. When a lateral stamen is missing, the associated gland is altered to resemble a medial gland in appearance, e.g., fig. 1B in Bowman and Smyth (Bowman and Smyth, 1999). Similarly, in B class mutants, stamens are not present and most nectary glands have a medial-gland-like appearance. Nectaries of plants carrying the *35S::B* transgenes are also altered compared to wild type. In these plants, the lateral glands are usually oblong and display a mosaic of cell types. The basal portions of these structures resemble stamen filaments, based on the appearance of the cuticle. However, the apical regions exhibit an appearance similar to wild-type nectary glands, based on their cuticular thickenings and the presence of open stomata. In addition, while *ET668* is expressed throughout wild-type nectary glands, its expression is limited to the apical end of *35S::B* nectary glands (Fig. 3D). Not all *35S::B* flowers have medial glands but in cases where they are present their morphology resembles that of the lateral glands.

To further understand the influence of the B class genes on nectary development, plants ectopically expressing either *AP3* or *PI* individually were studied. In plants ectopically expressing *AP3*, nectary glands appear like those observed in wild-type flowers though they are slightly misshapen (Fig. 3E). In plants ectopically expressing *PI* there are one or two nectary glands, oblong in shape, in each of the lateral domains and generally none in the medial domains (Fig. 3F). The glands have a mosaic appearance, as do the nectary glands of *35S::B* flowers. The characteristic nectary epidermal cuticular thickening is restricted to the apical region while the basal region has a cuticle similar to filaments. In addition, *ET668* is expressed only at the tips of the glands. Since it is suggested that *AP3* and *PI* act as a heterodimer (Jack et al., 1994; Riechmann et al., 1996), our results suggest that in wild-type flowers *AP3* is

expressed in the nectary glands while *PI* is not. Consistent with this hypothesis, *AP3* is expressed throughout nectary primordia but its expression subsequently becomes restricted to the base of the nectary glands (Fig. 3G,H). In contrast, *PI* mRNA was not detected in the wild-type nectary (not shown). This result is congruous with the observation that ectopic *PI* expression results in nectary glands that have a mosaic appearance: the base has filament characters while the apical end has nectary characters. These results suggest that while the nectary is part of the third whorl where both B class genes are normally expressed early during flower development, *PI* and to some extent *AP3*, are actively suppressed in cells giving rise to nectary glands.

### *LFY* and *UFO* are required for the establishment of the third whorl

*LEAFY (LFY)* and *UFO* have been implicated as positive regulators of *AP3* and *PI* and in establishing the whorled pattern of the flower (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Lee et al., 1997; Percy et al., 1998; Honma and Goto, 2000). In the flowers of *lfy* mutants the outer four organs are whorled and sepal-like, while the second and third whorl organs arise in a spiral phyllotaxis and are mosaics with



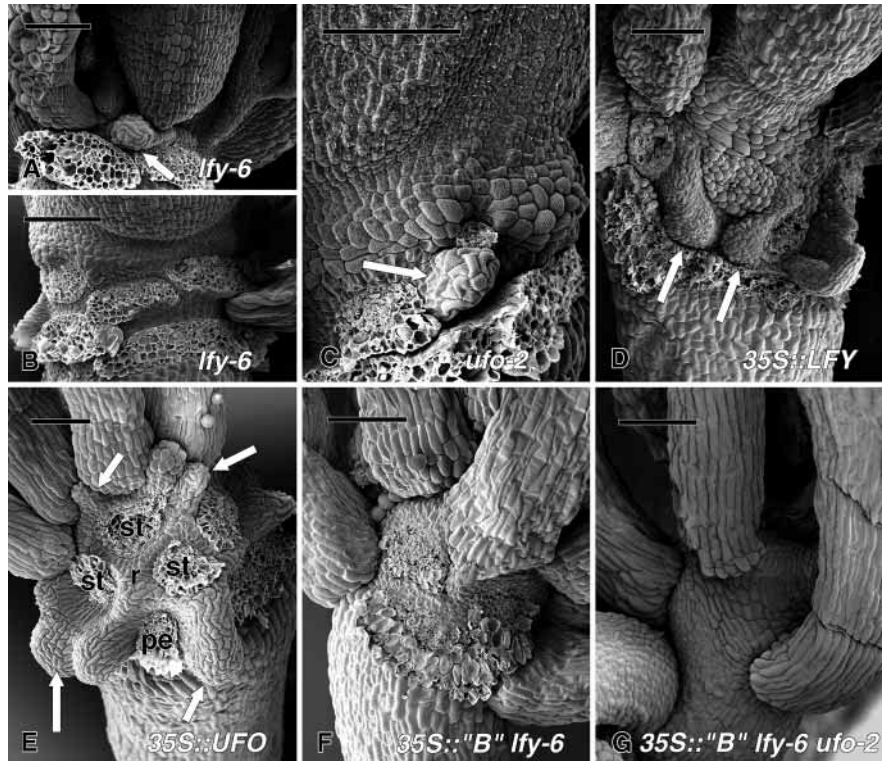
**Fig. 3.** The effects of B class genes and the third whorl on nectary gland development as analyzed using SEM and sectioned material. (A,B) SEM of nectary glands from *35S::AP3 35S::PI* (*35S::B*) expressed in wild type and *ap2-2* mutant, respectively. (C) Lateral nectary glands from a *sup-1* mutant. (D) Longitudinal section of a lateral nectary gland from a plant expressing *35S::AP3 35S::PI*. *ET668* GUS expression is mainly seen at the tip of the nectary gland. (E,F) SEM of nectary glands from wild-type plants expressing *35S::AP3* and *35S::PI*, respectively. (G,H) In situ localization of *AP3* mRNA in young and older nectary glands, respectively, as seen in transverse section. ln, lateral nectary gland; mst, medial stamen; lst, lateral stamen; Scale bar, 100 µm in A-E and 50 µm in F.



characteristics of sepals and carpels (Huala and Sussex, 1992; Weigel et al., 1992). Flowers of *ufo* mutants have characteristics of *lfy*, *ap3* and *pi* flowers, with sepals, petals, stamens, carpels, filaments, or mosaic combinations of these organ types being produced in the second and third whorls (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). Simultaneous ectopic expression of *AP3* and *PI* in *lfy* or *ufo* mutants restores petals and stamens, yet, phyllotaxy in *lfy* flowers, and the elimination of filamentous organs in *ufo* flowers, is not completely corrected (Krizek and Meyerowitz, 1996). Since the nectary is associated with the third whorl of the flower and not simply with the presence of stamens, we investigated the effects of *UFO* and *LFY* loss- and gain-of-function alleles on third whorl establishment, as assayed by the presence of nectary glands.

In *lfy-6* flowers, nectary glands, if present, are found in the lateral domain, are reduced in size, resemble those found in *pi-1* flowers and do not possess modified stomata (Fig. 4A,B). In *ufo-2* flowers, glands are found in the lateral domain and resemble those of *pi-1* and *lfy-6* flowers (Fig. 4C). In general, more glands are found in *ufo-2* flowers than in *lfy-6*. In *lfy-6 ufo-2* double mutant flowers, no evidence of nectary development is observed. Ectopically expressing *LFY*, using the CaMV 35S promoter, in wild-type plants produces flowers with a normal floral ground plan but the nectary glands exhibit normal to extremely aberrant morphology (Fig. 4D).

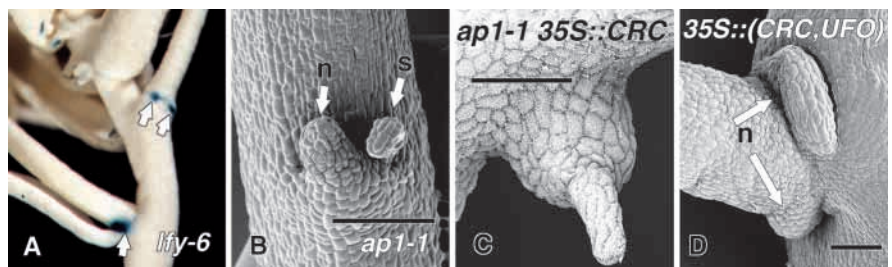
Flowers of *35S::UFO* plants have sepals or petaloid sepals in the first whorl, followed by petals in the second whorl, and usually two whorls of stamens (Lee et al., 1997). Interior to the stamens, occasional reduced carpels are present. Nectary glands subtend both the third and fourth whorl stamens (Fig. 4E) in a manner reminiscent of *sup-1* flowers. The amount of nectary gland tissue is greatly increased such that the glands surround the base of the stamens in the lateral domain whereas glands in the fourth whorl cover all areas of the exposed receptacle. These results suggest that ectopic expression of *UFO* is sufficient for the reiterative development of the third whorl. This activity of *UFO* is independent of B class activity, since in *35S::UFO pi-1* flowers, where multiple whorls of



**Fig. 4.** SEM of nectary glands from gain- and loss-of-function alleles of *LFY* and *UFO*. (A, B) *lfy-6*, (C) *ufo-2*, (D) *35S::LFY*, (E) *35S::UFO*, (F) *lfy-6 35S::AP3 35S::PI* (*35S::B*), (G) *lfy-6 ufo-2 35S::AP3 35S::PI*. st, stamen; pe, petal, r, nectary ridge; arrows, nectary glands. Scale bars, 100 µm.

unfused carpels form interior to the second whorl, all of the unfused carpels are subtended by nectary glands (Table 2).

To further characterize the involvement of *UFO* and *LFY* in establishing the 'third whorl' domain of the flower, their activity was compromised, either singly or doubly, in the presence of ectopic B class activity. In *lfy-6 35S::B* flowers, petals and stamens were restored but formation and normal morphology of nectary glands were not (Fig. 4F). Similarly, while ectopic expression of B class genes in a *ufo-2* background restores the development of petals and stamens, it fails to rescue nectary development. Finally, in *lfy-6 ufo-2* flowers ectopic B class gene expression restores petal and stamen development, but no evidence of nectary development is observed (Fig. 4G). It should be noted that it is formally possible that the function of the 35S promoter is sufficient to restore partial B class function (e.g. restoration of stamens)



**Fig. 5.** Ectopic nectary-like glands and misexpression of *CRC*. (A) Misexpression of *CRC::GUS* at the base of flower pedicels in a *lfy-6* mutant plant (arrows). (B) Ectopic nectary-like structure and stipule-like structure along the pedicel of an *ap1-1* primary flower. (C) An ectopic nectary-like structure located at the base of a flower pedicel on the abaxial side of an *ap1-1 35S::CRC* plant. (D) Ectopic nectary-like structures positioned at the base of a flower pedicel in a *35S::CRC 35S::UFO* plant. n, nectary gland-like structure; s, stipule-like structure. Scale bars, 100 µm.

but is insufficient to restore other functions (e.g. nectary development). Thus, a partially redundant role for *UFO* and *LFY* in the establishment of the third whorl is implicated, and this function is separable from the activation of B class genes.

### Multiple factors restrict the nectary to the flower

While in *Arabidopsis*, the nectary is flower specific, in other species nectaries may develop outside the flower. In our investigation of the nectary in *Arabidopsis* floral mutants, ectopic GUS staining was detected in *CRC::GUS* and *ET668* lines in locations other than the nectary (and carpel tissues in the case of *CRC::GUS*). In backgrounds of *ap1-1*, *lfy-6*, *ufo-2*, *35S::UFO*, and multiple combinations thereof, misexpression of *CRC::GUS* was observed at the base of the flower pedicel (Fig. 5A). In *ap1-1 lfy-6* and *ap1-1 ufo-2* double mutants, nectary outgrowths were found in multiple whorls of the flower. However, in these plants, the normal whorled phyllotaxy of the flower was replaced by a spiral arrangement of organs and it was difficult to assign the nectaries to a specific whorl. In addition, in some cases, nectary outgrowths were also located on the stem in these genotypes.

To test whether *CRC* is limiting in *ap1-1* and *35S::UFO* plants, *35S::CRC ap1-1* and *35S::CRC 35S::UFO* plants were examined. The addition of *CRC* caused ectopic structures to occasionally develop at the base of flower pedicels in positions corresponding to where *CRC* was misexpressed in *ap1-1* and *35S::UFO* plants. These ectopic structures expressed *CRC::GUS* and *ET668* and had a rudimentary nectary cuticle but no stomata were observed (Fig. 5C,D). It can be concluded that *CRC* is limiting for the differentiation of nectary characteristics but not for the formation of nectary glands. While these results do not represent a thorough survey for suppressors of nectary formation, they provide a glimpse of the complex mechanisms that restrict the nectary to a specific position.

Lastly, in *ap1-1* plants expression of *CRC::GUS* and *ET668* was also found half way up along the pedicel of the primary flower. The tissues along the pedicel that ectopically express these nectary marker lines also exhibit morphological characteristics of nectary glands. The nectary gland-like structures along the pedicel have modified stomata and an immature nectary gland cuticle (Fig. 5B). However, secreted nectar has never been observed around these structures. In most cases, stipule-like structures are found accompanying these nectary-like structures. Since first whorl organs are often separated from the other floral organs in *ap1-1* mutants as a result of pedicel elongation (Bowman et al., 1993), these structure may represent displaced first whorl organs, with nectaries in their axils suggesting that the nectaries themselves could be third whorl nectaries that are displaced along the pedicel. Alternatively, the structures could be ectopic reduced bract-like organs with axillary nectaries. Arber (Arber, 1931b) notes that similar stipule-like structures, sometimes referred to as squamules, likely represent the stipules for the non-existent bracts in other Brassicaceae species.

## DISCUSSION

### Wild-type nectaries and the role of *CRC*

The nectary of *Arabidopsis* flowers is composed of a ring of receptacle tissue out of which arise six glands on the abaxial side of the stamens. Because mutations in *CRC* result in the

loss of nectary glands (Bowman and Smyth, 1999), we believe its expression provides a reliable marker for identifying cells specified to become the nectary. Based on *CRC* expression in stage 7-8 flowers, the nectary develops from two crescent-shaped groups of cells occupying the region between the petals and stamens in the third whorl of the flower. At later stages, *CRC* expression defines a ring of tissue around the entire circumference of the receptacle. One interpretation is that the initial expression pattern in two crescent-shaped regions represents the initial allocation of cells to be specified as the nectary, and subsequently, cells in the regions between the two sets of medial nectary glands are recruited in a *CRC*-dependent manner to become nectary tissue.

Presently, *CRC* is the only gene known to be absolutely required for nectary gland formation in *Arabidopsis*; in all known alleles of *CRC* mutants, nectary glands are absent (Bowman and Smyth, 1999). The expression of *CRC* precedes any visible signs of nectary gland development and continues to be expressed in the nectary gland after nectar secretion ceases. Ectopic expression of *CRC* throughout the plant does not induce ectopic nectaries, and in these plants, the placement and morphology of the nectary glands is normal. Therefore, *CRC* is required but is not sufficient for nectary formation. As *CRC::GUS* is detected in nectary gland-less *crc* mutants, other genetic factors acting upstream of *CRC* specify where in the plant nectary tissue arises. Subsequently, *CRC* expression is required for the recruitment of receptacle cells to be included in the nectary and the differentiation of the nectary tissues.

In addition to the morphological differences between the nectary and its surrounding tissue, expression patterns of several genes also demarcate the nectary from the surrounding receptacle. For example, *ET668* and *CRC::GUS* are expressed in the nectary during all stages of its development but not in the receptacle. The expression patterns of the marker lines, in regards to the nectary (except *KNAT2::GUS*) are restricted to the nectary gland and not the receptacle and may reflect developmental stages of nectary gland development. *KNAT2::GUS* is also expressed in the receptacle suggesting that it may have a more general role. While many of the genes identified as expressed in nectaries in *Arabidopsis* are transcription factors (Table 1), *NECTARIN 1* from *Nicotiana* sp. and *NECTARIN1* from *Brassica campestris* L. ssp. *pekinensis* encode nectary expressed proteins likely required during aspects of nectar secretion (Carter et al., 1999; Song et al., 2000). While all nectary gene expression observed in *Arabidopsis* is dependent of *CRC* activity, whether any of these genes are direct targets of *CRC* remains to be determined.

### Nectaries are independent of other floral organs and linked to the third whorl

The presented results demonstrate that nectary identity is independent of floral ABC gene function except where they appear to canalize proper flower architecture and the formation of the third whorl nectary zone. In the floral ABC single, double and triple mutants (except *pi-1 ag-1* and *ap3-3 ag-1*), nectary glands are present even though the identity of the other floral organs is altered. This is exemplified in homeotic mutants in which the third whorl is occupied by organs other than stamens, and yet nectary glands are still present. In *pi-1* and *ap3-3* flowers, the third whorl is occupied by carpels rather than stamens, and nectary glands are still present. Likewise in



*ag-1* and *ap2-2 ag-1* double mutants in which the third whorl organs are petals and staminoid-petals, respectively, nectary glands are present, albeit their morphology and physiology is altered. Furthermore, in flowers in which stamens develop in ectopic positions, the location of the nectary is not altered. Wild-type plants ectopically expressing *AP3* alone or in combination with *PI* produce stamens in the third and fourth whorls, but nectary glands are only present at the base of the third whorl stamens. Changing the identity of the second whorl petals to stamens by ectopically expressing *AG*, or changing the identity of all whorls to stamens by ectopically expressing the B class genes in an *ap2-2* mutant background does not change the location of the nectary; in all these genotypes the nectary is only found in the third whorl. Conversely, in *sup-1* mutants in which the third whorl is reiterated (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995) nectary glands are found subtending all stamens. This suggests that in *Arabidopsis*, genetic factors associated with the third whorl are responsible for the initial placement of the nectary and that the subsequent development and morphology of the nectary glands is under control of *CRC*-dependent genetic programs. Thus, nectary development is independent of the presence or identity of the floral organs. That nectaries develop in *lfy ap1* plants, in which flower meristem specification is compromised, further supports the independence of nectary development from the specification of floral organ identity.

#### ***UFO*, *LFY* and *AP1* act to promote floral third whorl nectary growth and repress extrafloral nectaries**

*UFO* and *LFY* appear to act upstream to help establish the third whorl tissues from which stamens and nectary glands arise. Because the nectary is present in the fourth whorl of *35S::UFO* and *35S::UFO pi-1* plants but is not present in the fourth whorl of plants expressing *35S::B*, we propose that *35S::UFO* reiterates the third whorl and is not merely activating B class genes as previously proposed (Lee et al., 1997; Samach et al., 1999). Thus, gain-of-function *UFO* alleles appear to result in ectopic development of additional third whorls with the accompanying nectary growth zone, similar to that observed in *sup* mutants (Schultz et al., 1991; Bowman et al., 1992). Conversely, loss-of-function *ufo* alleles, when combined with *lfy* mutations result in a loss of the nectary, perhaps as a result of failure to establish the third whorl. Ectopically expressing the B class genes in *ufo lfy* flowers restores stamens and petals but not the nectary, indicating that *UFO* and *LFY* act to promote third whorl establishment independent of the activation of B class genes. How these interactions are accomplished at the molecular level is as of yet unclear.

That the third whorl does not appear to develop in *pi ag* and *ap3 ag* double mutants, implicates the B and C class genes in the establishment of the third whorl. A role in floral meristem determinacy has been noted for the B class genes previously. For example, B class loss-of-function mutations result in a reduction in the number of third and/or fourth whorl organs in both *Arabidopsis* and *Antirrhinum* (Bowman et al., 1989; Sommer et al., 1990; Bowman et al., 1991; Tröbner et al., 1992; Jack et al., 1992). Conversely, additional third (or interior) whorl organs develop when B class genes are ectopically expressed interior to the third whorl, either by means of transgenes or due to *sup* mutations (Schultz et al., 1991; Bowman et al., 1992; Krizek and Meyerowitz, 1996). The role

of *AG* in the formation of the third whorl is only uncovered when B class activity is also compromised (Bowman et al., 1991). Loss of A class activity also results in alterations in the formation of organ primordia in the outer whorls (Komaki et al., 1988; Kunst et al., 1989; Irish and Sussex, 1990; Bowman et al., 1991; Bowman et al., 1993). In addition, *AP2* is absolutely required for second whorl organ primordia formation in a *ag pi* background and appears to suppress third whorl formation in this background (Bowman et al., 1991). However, the suppression of third whorl formation by *AP2* is context dependent since additional third whorls do not form in outer whorls of *35S::UFO ap2-2* mutants. Thus, all three classes of floral homeotic genes appear to have complex roles in patterning floral organ numbers and/or whorls, in addition to their roles in specifying identity. These two activities, however, are largely independent as exemplified by alleles in which the roles can be genetically separated.

Since *CRC* is not sufficient for nectary development, there must be other factors that either suppress or promote nectary formation. Three factors that restrict the expression of *CRC* within the flower are *AP1*, *LFY* and *UFO*. In *ap1-1*, *lfy-6*, *ufo-2* and *35S::UFO* plants, *CRC* is expressed in the axils of the pedicels while it is not expressed there in wild-type plants. However, *CRC* is only limiting for the differentiation of nectary characteristics in these backgrounds, implying other as yet unidentified genetic components necessary for the formation of ectopic nectaries. That nectary tissue differentiates in these backgrounds, but not in wild type, when *CRC* is ectopically expressed implies that these genes also regulate other factors required for nectary development. In some species of *Capparidaceae*; phylogenetically closely related to the *Brassicaceae*, extrafloral nectaries develop in the axils of the pedicels (Zimmerman, 1932) in a position similar to that in the above *Arabidopsis* mutant genotypes, and it is tempting to speculate that a similar genetic mechanism is responsible for the formation of extrafloral nectaries in these species.

#### **Nectary evolution**

Unlike the floral organs, whose relative positions are conserved across angiosperms, the nectary is not located in the same position in all plants, consistent with our results that the nectary is not specified by the combinatorial action of the ABC genes. The ABC model of floral organ identity specification appears to hold for widely divergent species within the eudicots as well as grasses, and this genetic network canalizes floral architecture to some extent, resulting in a conserved floral organ order (Bowman, 1997; Ambrose et al., 2000; Kyoizuka et al., 2000). Since the nectary is independent of the ABC floral homeotic genes it is not constrained by the limitations of the ABC genetic network, and thus, it is potentially 'free' to move about the flower over evolutionary time in response to selection imposed by interactions with pollinators. Consistent with this the nectary can be found on the receptacle associated with any of the floral organs or associated with any of the floral organs themselves in various taxa (Brown, 1938). However, the question then remains as to what specifies a nectary? Ectopic expression of *CRC* does not produce ectopic nectaries suggesting that other factors are required. In *Arabidopsis*, those factors are associated with the third whorl which is, in part, established by the action of *UFO* and *LFY*. In other species nectaries are located elsewhere within the flower or even

outside the flower and it is believed both floral and extrafloral nectaries arose independently a number of times (Brown, 1938). Given this diversity of nectary morphology and location within the plant, it is likely that the mechanism for specifying the nectary in *Arabidopsis* is just one of a number of mechanisms for specifying the nectary position within a plant and, additionally, it is not clear whether *CRC* is universally used to promote nectary differentiation.

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