KNUCKLES (*KNU*) encodes a C2H2 zinc-finger protein that regulates development of basal pattern elements of the *Arabidopsis* gynoecium

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Summary

Flowers of the parthenocarpic *knuckles* mutant are conditionally male sterile and contain ectopic stamens and carpels that originate from placental tissue within developing gynoecia. The mutation was mapped to a 123 Kb interval on chromosome 5 using molecular markers. All aspects of the *knuckles* phenotype could be complemented by a genomic fragment from the region which contained the annotated *MAC12.2* gene. A guanine to adenine transition within a predicted C2H2 zinc finger-encoding region of *MAC12.2* causes the second of two critical zinc-binding cysteine residues to be replaced by a tyrosine. Transgenic plants in which translational fusions of the GUS reporter to KNUCKLES were driven by the presumptive *KNUCKLES* promoter indicate that the gene is expressed first in developing carpel primordia, and later in stamens and ovules of flower buds. In situ hybridization experiments showed a broader pattern of transcript localization, suggesting that post-transcriptional regulatory mechanisms may limit KNUCKLES protein accumulation and localization. Based on genetic evidence and the presence of a carboxy-terminal motif demonstrated by others to function as an active repression domain, we propose that KNUCKLES might function as a transcriptional repressor of cellular proliferation that regulates floral determinacy and relative size of basal pattern elements along the proximo-distal axis of the developing *Arabidopsis* gynoecium.

Key words: C2H2 zinc finger, KNUCKLES, Parthenocarpy, Floral development, Fruit development

Introduction

The primordia of plant lateral organs arise from the flanks of pluripotent meristems. After specification, organogenesis proceeds from the coordination of two complementary processes: cellular proliferation and differentiation. How these processes are coordinated, and how positional information is generated and regional identity maintained over the course of organ development, are fundamental questions which genetic studies of flower and fruit development have begun to address.

Arabidopsis thaliana floral meristems each give rise to four concentric whorls of determinate lateral organs: four sepals, four petals, six stamens, and two fused carpels (the gynoecium). Third- and fourth-whorl floral organs contain the reproductive male (micro-) and female (mega-) gametophytes, respectively. Each megagametophyte or embryo sac is enclosed by a specialized sporophytic structure, the ovule, which may give rise to a seed if fertilized.

After anthesis (pollen shedding) and fertilization, the gynoecium lengthens considerably and develops into a seed-containing silique, the *Arabidopsis* fruit (Fig. 1A). Elongation of the silique is accomplished primarily by longitudinal expansion of cells of the exocarp, schlerenchyma, and endocarp layers, whereas cell division accounts for most growth within the mesocarp (Vivian-Smith and Koltunow, 1999). A dehiscence zone allows for separation of the valves from the replum and seed dispersal (Ferrandiz et al., 1999). In the absence of fertilization, the gynoecium may undergo restricted post-anthesis elongation, but it remains a determinate organ that eventually abscises. Parthenocarpic *Arabidopsis* mutants, in which the gynoecium undergoes post-anthesis fruit development without fertilization, have been isolated (Ito and Meyerowitz, 2000; Vivian-Smith et al., 2001). The application of exogenous plant growth regulators to emasculated flowers induces fertilization-independent fruit development in *Arabidopsis* (Vivian-Smith and Koltunow, 1999), and transgenic Solanaceae producing elevated levels of auxin within placental tissue and ovules are also parthenocarpic (Rotino et al., 1997; Ficcadenti et al., 1999). It is likely that parthenocarpic mutants either produce abnormal levels of growth regulators or respond inappropriately to these compounds.

Regulation of floral organ specification can be explained in terms of the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). The model posits three functional classes of regulators (A, B, and C) expressed in partially overlapping domains. Where expression of the B-function MADS box genes *APETALA3* (*AP3*) (Jack et al., 1992) and *PISTILLATA* (*PI*) (Goto and Meyerowitz, 1994) overlaps that of the C-function MADS box gene *AGAMOUS* (*AG*) (Yanofsky et al., 1990), stamens form in the third whorl. AG alone specifies

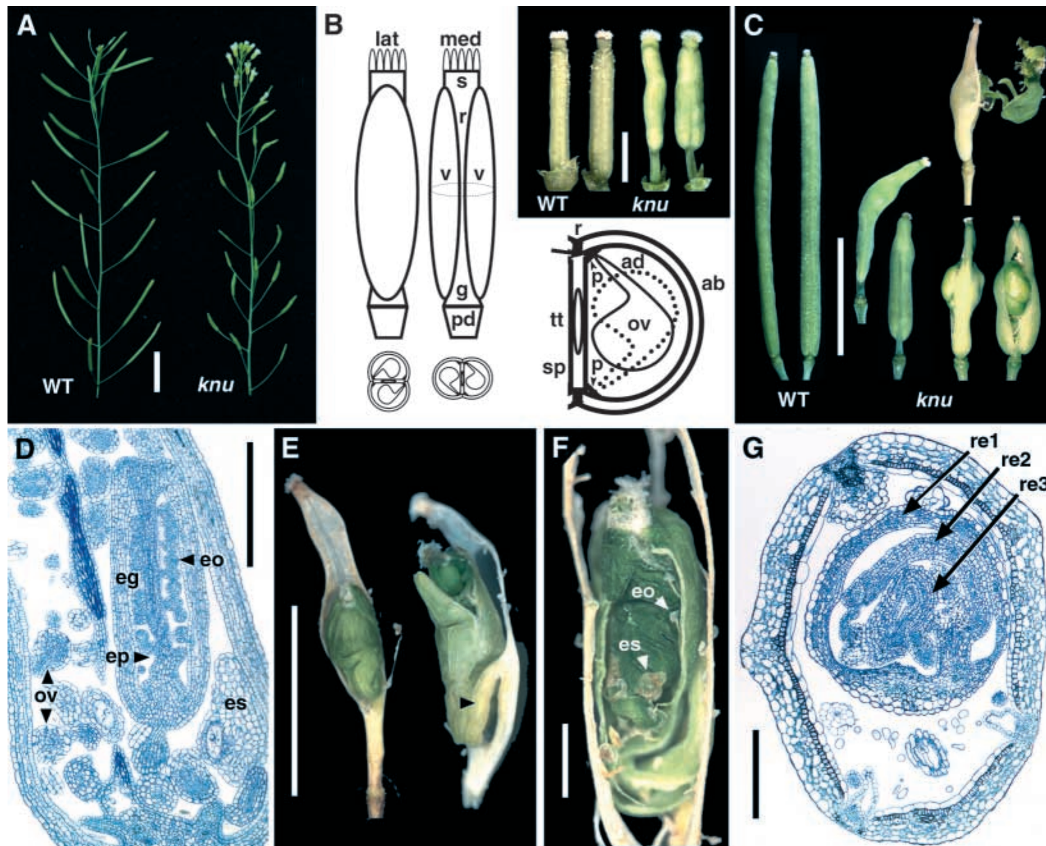

Fig. 1. Features of wild-type and *knuckles* gynoecia and siliques from plants grown at 25°C. (A) Inflorescences. (B) Drawing depicting wild-type anthesis-stage gynoecium, lateral and medial views, and cross-sectional view through single locule of the ovary. Inset: comparison of wild-type and *knu* gynoecia at anthesis. (C) Wild-type (left) and *knu* siliques (right) with examples of protruding ectopic structures (far right). (D) Longitudinal section through immature *knu* silique showing ectopic stamen and gynoecium. (E) Partially dissected dehiscent *knu* siliques showing a pellet-like knuckle at left with a teased out structure at right where a secondary ectopic gynoecium is emerging from the first. The arrowhead indicates stem-like umbilicus that attaches knuckle to the replum and pedicel. (F) Replum of dehiscent *knu* silique with knuckle dissected to show desiccated ovules of primary iteration and ectopic stamens of the secondary iteration. (G) Cross-section through tricarpelloid *knuckles* silique showing knuckle with arrows indicating at least three iterations of carpel tissue. Scale bars: 10 mm in A; 1 mm in B,G; 5 mm in C,F; 100 μm in D; 200 μm in E. ab, abaxial; ad, adaxial; eg, ectopic gynoecium; eo, ectopic ovule; ep, ectopic primordia/um; es, ectopic stamen; g, gynophore; lat, lateral; med, medial; ov, ovule; pd, pedicel; p, placenta; re, reiteration; r, replum; sp, septum; s, style; tt, transmitting tract; v, valve; wt, wild-type.

carpel development and regulates floral meristem determinacy; consequently *ag* mutants display a flower-within-flower phenotype (Bowman et al., 1989; Bowman et al., 1991).

A tenet of the ABC model is that A- and C-class regulators are antagonistic, and thus have cadastral as well as organ identity functions (Gustafson-Brown et al., 1994; Drews et al., 1991). Other, purely cadastral genes do not themselves confer primordium identity but mutations in them lead to homeotic transformations of floral whorl organs because the expression of floral organ identity genes extends beyond wild-type whorl boundaries. Recessive mutations at the *SUPERMAN* (*SUP*) locus, for example, lead to an extension of B-class gene expression and result in supernumerary stamen production at the expense of fourth whorl carpel development (Bowman et al., 1992; Sakai et al., 1995). It is theorized that SUP exerts its control over the boundary between whorls three and four by regulating the balance of cellular proliferation between meristematic regions fated to give rise to stamens or carpels (Sakai et al., 1995; Sakai et al., 2000). SUPERMAN is a C2H2 zinc finger protein and possesses additional putative motifs typical of a transcriptional regulator.

The floral meristems of plants triply mutant for A-, B- and C-class genes are indeterminate and produce whorls of leaf-like structures neither recognizable nor functioning as floral organs (Bowman et al., 1991). This homeotic phenotype substantiates a long-held belief that floral organs, including the two fused carpels that comprise the whorl four gynoecium, are modified leaves. Like leaves, the carpels may be described in terms of three developmental axes along which pattern elements or specific tissue types differentiate: adaxial-abaxial, medial-lateral, and basal-apical (Fig. 1B, drawing). Because of congenital fusion, the adaxial face of each carpel is inside the gynoecium, whereas the abaxial surfaces make up its exterior. Four narrow bands of placental tissue form along the interior of the gynoecium where the adaxial faces of the two carpels meet and fuse. The gynoecium is bisected into two locules by a false septum of adaxial origin that fuses post-genitally. Two placentae develop in each carpel and thus two rows of ovules form on opposite sides of each locule, interdigitating as they grow toward one another (Fig. 1B). Abaxial-adaxial polarity in carpel development is regulated redundantly by CRABS CLAW (CRC), KANADI (KAN), and GYMNOS (GYM) (Bowman and Smyth, 1999; Eshed et al., 1999).

The replum defines the medial axis of the bilaterally symmetrical ovary. The replum remains attached to the plant after ripening and dehiscence of the lateral valves that enable seed release (Fig. 1B). SPATULA (SPT) and other factors are required for complete development of medial tissues (Alvarez and Smyth, 1999; Heisler et al., 2001; Alvarez and Smyth, 2002). Carpel pattern elements occur as follows along the

basal-apical axis: gynophore, ovary, style, and stigma (see drawing, Fig. 1). Mutations at the *ETTIN* locus have been shown to alter the development of these elements along the proximo-distal or basal-apical axis, such that apical elements extend basally at the expense of ovary development (Sessions and Zambryski, 1995; Nemhauser et al., 2000).

We isolated a recessive, conditionally male-sterile *Arabidopsis* mutant, *knuckles* (*knu*). A fraction of *knu* flowers produce ectopic stamens and carpels in a reiterating pattern from placental tissue near the base of a primary fourth-whorl carpel, and this indeterminacy appears to be necessary for parthenocarpic silique development. We report the identification of the *KNUCKLES* locus that, like *SUPERMAN*, encodes a small protein containing a single C2H2 zinc finger and probably functions as a transcriptional repressor. KNU likewise appears to have a cadastral regulatory function in the developing flower. Our observations indicate that KNU expression occurs early in the development of the gynoecium and persists near its base until after ovule primordia appear. KNU suppresses overgrowth of basal gynoecial structures such as the nectaries and gynophore to allow for full development of the ovaries, and prevents the placentae therein from acquiring floral meristem identity.

Materials and methods

Mutagenesis and screening

Plant material used for the mutagenesis was of the Ws ecotype containing the MEA::GUS reporter construct described in Luo et al. (Luo et al., 2000). (However, the *knu* phenotype can be crossed away from the transgene, and the GUS staining pattern conditioned by the reporter is unaffected by the presence of the mutation.) Approximately 15,000 seeds were treated with 150 µL ethyl methanesulfonate (Sigma) in an aqueous volume of 35 mL for 16 hours. Seeds were then washed with water for ten hours in a 50 mL Falcon tube (hourly changes) before being collected on a filter and allowed to dry overnight in a fume hood. M_1 seeds were divided between 60 pots, and the bulk harvest from each of these pots was treated as an M_2 family. Three pots per M_2 family were sown with approximately 200 seeds each for subsequent screening.

Histology

Buds, flowers and siliques were fixed, embedded and sectioned essentially as described in Koltunow et al. (Koltunow et al., 1998). Sections were usually stained in 0.1% toluidine blue in 0.02% sodium carbonate and photographed under bright field on a Zeiss Axioplan microscope using a Spot digital camera (SciTech Pty). Lactophenol clearing of whole-mount tissues was allowed to proceed for 3-5 hours at room temperature prior to microscopy.

Mapping of the *knuckles* mutation

Ws plants homozygous for the recessive *knu* mutation were crossed with L*er*. F_2 progeny of this cross were scored for the presence of knuckled siliques. Mutant F_2 progeny comprised only 8% rather than the expected 25% of this mapping population, probably as a result of reduced male and female fertility, and decreased viability of homozygous *knu* seeds. Genomic DNA was prepared from leaf tissue using the protocol of Edwards et al. (Edwards et al., 1991). Preliminary mapping using published SSLP markers (Bell and Ecker, 1994) indicated that the *knu* mutation was linked to NGA151 on chromosome 5. The Cereon database of DNA polymorphisms between the Columbia and L*er* ecotypes (maintained by The *Arapidopsis* Information Resource, http://www.arabidopsis.org) was used to design a series of insertion/deletion (INDEL) PCR markers flanking NGA151. In all, 24 primer pairs were tested; half were polymorphic between WS and L*er*. Markers were named according to the AGI genomic clone they overlapped. The nearest left marker at which heterozygosity was detected was MXE10a, amplified by the primer set MXE10a-F (5′-GCG CTT AAC AAC GGT TTG TTG-3′) and MXE10a-R (5′-CAT TTG GGT GCC TGC ACA TTG-3′) and based on CER457604. The nearest heterozygous marker flanking the mutation on the right was F18O22b, which also overlaps the P1 clone MUA22. This marker corresponds to CER478399 and is amplified by the primer set F18O22b-F (5′-CTT GAA ACT TGA AAG CAA ACC AG-3′) and F18O22b-R (5′-GGG CCT AAA AAT TGT AAC TGT AG-3′). These markers defined an interval of 123 kb spanned by three AGI P1 clones: MXE10 (AB011484), MAC12 (AB005230) and MUA22 (AB007650).

Complementation of the *knuckles* mutant

Twenty-five overlapping genomic subclones derived from three AGI P1 clones spanning the 123 kb *knu* interval were produced in the pGEM derivative pSHUTTLE (Wang et al., 1998). Insert ends were sequenced to verify the identity of the inserts before further subcloning of the genomic fragments into the binary T-DNA vector pWBVec8 (Wang et al., 1998). *knu* seedlings grown at 16°C were transformed via the floral dip method (Clough and Bent, 1998). After dipping, plants were covered and kept at room temperature overnight, then returned uncovered to a 16°C growth chamber until seeds were harvested. Transgenic seedlings were selected on MS agar plates supplemented with 20 mg/L hygromycin and 150 mg/L Timentin. Between 5 and 40 transgenic seedlings harboring each construct were transplanted to soil and grown at non-permissive temperatures for evaluation of phenotype.

Of 15 hygromycin-resistant *knu* seedlings containing the p8MUASAL1-3 construct produced, five were wild-type in appearance. The remaining 10 displayed a spectrum of weak *knu* phenotypes characterized by reduced knuckling and (in all but two plants) partial restoration of fertility. The genomic insert in this clone is an 8751 bp *Sal*I fragment subcloned from the P1 clone MUA22 (AB007650) from an area of overlap with the P1 MAC12 (AB005230). It contains two annotated genes, *MAC12.2* (At5g14010, GI 18417266) and *MAC12.3* (At5g14000, GI 18417263). PCR products from the coding region of each gene were amplified from wild-type WS and *knu* seedlings and sequenced using BIG DYE Version 3.0 dideoxy terminators. Upon identification of the mutation in *MAC12.2*, further subcloning of the complementing fragment was performed to separate the two genes. The insert in pMUASAL1H-5 is a *Hin*dIII fragment of 5092 bp containing the *MAC12.2* coding region and 2010 bp of 5′ and 2596 bp of 3′ sequence. Half of the transgenic *knu* plants harboring this construct were wild-type in appearance, and half displayed weak *knu* phenotypes. None of the *knu* transgenics harboring a *Hin*dIII fragment containing the adjacent *MAC12.3* gene (pMUASAL1H-2) were complemented. The smallest segment of genomic DNA confirmed to be capable of complementing *knu* was cloned into pWBVec8 as a *Hin*dIII-*Pst*I fragment. In this construct, p8KNU, sequence 3′ to the *KNU* coding region was reduced to 1403 bp.

5′ and 3′ RACE

The GeneRacer kit for full-length, RNA ligase-mediated rapid amplification of 5′ and 3′ cDNA ends (Maruyama and Sugano, 1994; Schaefer, 1995; Volloch et al., 1994) (Superscript II RT version) and the TOPO TA cloning kit (both from Invitrogen Life Technologies) were used essentially as per the manufacturer's instructions. Template for cDNA synthesis was total RNA extracted from wild-type Ws inflorescence apices composed of pre-anthesis flower buds, prepared with an Rneasy Plant Mini kit (Qiagen). An on-column RNase-free DNase protocol was performed. For 5′ RACE the following KNU-specific primers were used in combination with those provided by the manufacturer of the GeneRacer kit: MAC12.2RTR (5′-TCG TCT TCT

TCC ATA ACG CC-3′) and MAC12.25R2 (5′-GTA GAA CTT TCG AGG ACA GTA CTG-3′). 3′ RACE primers were: MAC12.2ZF1 (5′-CAG TAC TGT CCT CGA AAG TTC-3′) and 12.2GR3PR (5′-CTC AAG CTC TCG GCG GTC ACC AAA A-3′).

In situ hybridization

Templates for generation of RNA probes were the plasmids XKNUX-4 and ICRTR-2. The full-length *KNU* insert in XKNUX-4 was amplified by PCR from the p8MUASAL1H-5 plasmid using the primer set XhoIKNUATG (5′-CCC CTC GAG CCC ATG GCG GAA CCA CCA CCG TC-3′) and 3′KNUXbaI (5′-GGG TCT AGA TAA CTT ATA AAC GGA GAG AAA-3′) and cloned into pGEM-T Easy (Promega). The 204 bp of *KNU* sequence inserted into pICRTR-2 was amplified by PCR from pMUASAL-1 using the primer set MAC12.2IC (5′-CAA CAA CAC GTT TCT TCG TCC-3′) and MAC12.2RTR (5′-TCG TCT TCT TCC ATA ACG CC-3′) and cloned into pGEM-T Easy. Plasmids were sequenced to verify identity and orientation of inserts. The full-length XKNUX-4 probe template was restricted with *Xba*I or *Sal*I, and DIG-labelled probes produced with SP6 (sense) or T7 (antisense) RNA polymerases, respectively, as per the manufacturer's instructions. The pICRTR-2 template was restricted with *Nco*I or *Nde*I, and the RNA polymerases SP6 (sense) and T7 (antisense) used respectively to generate probes. Samples of the probes were electrophoresed in TAE-agarose gels and capillary blotted on nylon membrane to check for integrity or extent of carbonate hydrolysis and success of labelling before being used in hybridization experiments. The in situ hybridizations were performed as described previously (Tucker et al., 2003) except that once probes were added to the formamide-based hybridization solution and cover slips applied, the slides were heated at 80°C for 2 minutes prior to hybridization overnight at 42°C.

cDNA synthesis and RT-PCR

Total RNA was prepared from freshly harvested floral tissues using Trizol reagent (Invitrogen), and treated with RQ1 RNase-free DNase (Promega). Some of each preparation (2 µg) was used as template for first-strand cDNA synthesis with an oligo dT primer and Thermoscript (Invitrogen) in 20 µL reactions. *KNUCKLES* and *β-tubulin* cDNAs were amplified in separate, otherwise identical 50 µL PCR reactions containing 2.5 µL first-strand reaction, 10 mM Tris pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 µM forward and reverse primers, and 2.5 units AmpliTaq (Applied Biosystems). After an initial denaturation step of 5 minutes at 94°C, reactions were subjected to amplification cycles consisting of 30 seconds at 94°C, 30 seconds at 56°C, and 1 minute at 72°C, with a final incubation of 10 minutes at 72°C. Thirty and 40 cycles of amplification were performed on *β-tubulin* and *KNUCKLES* reactions, respectively. *β-tubulin* (At5g23860) primers were essentially as described in Tucker et al. (Tucker et al., 2003), and provided a necessary control for the efficacy of the DNase treatment because *KNUCKLES* does not contain introns. *KNUCKLES* was amplified with MAC12.2ZF1 and MAC12.2RTR. Sequenced plasmid templates were used as positive amplification controls. Samples were electrophoresed on a 3% TAE agarose gel loaded to normalize the *β-tubulin* band across lanes.

Construction of the *KNUCKLES:GUS* fusion plasmids and GUS staining of transgenic floral tissues

In p12.2GUS2-1, a pBI101.2 derivative, the 2010 bp of upstream sequence present in the complementation construct pMUASAL1H-5 and all but 19 bp of the annotated coding sequence of *KNU* was fused in-frame to the *E. coli uidA* (GUS) gene. In p8KNUGUS the full-length *KNU* coding sequence is fused to GUS, whereas in p8KPGUS, GUS alone is placed under transcriptional control of KNU 5′ and 3′ sequence.

p12.2GUS2-1 was constructed as follows: pMUASAL-1, a pSHUTTLE derivative and the source of the complementing insert in p8MUASAL1-3, was prepared from a dam-strain of *E. coli* and restricted with the enzymes *Hin*dIII and *Xba*I. The desired fragment was then subcloned into pBI101.2 (Jefferson et al., 1987) cut with the same restriction enzymes.

We subsequently produced two additional binary T-DNA constructs containing the GUS reporter: p8KNUGUS and p8KPGUS. The *Hin*dIII-*Pst*I restriction fragment present in p8KNU was cloned into pALTER-1 (Promega), and the phosphorylated oligonucleotides MutKNUATG (5′-GGT GGT TCC GCC ATG GTT GAG AGG TTG TTA AGC-3′) and MutKNUXbaI (5′-CAA AAC AGA GAA GAA AGT CTA GAT AAC TTA TAA ACG-3′) were used to introduce an *Nco*I site overlapping the *KNU* start and a methylation-insensitive *Xba*I site four nucleotides downstream of the *KNU* stop codon with the Altered Sites II kit (Promega). The doubly mutant insert was then cloned into a version of the pBluescript II KS+ (Stratagene) in which the *Xba*I site had been destroyed by ligation to the adjacent *Spe*I site, to create pBSΔSXKNU-5. The GUS reporter gene from pCAMBIA1381xa was used as a template to amplify a modified version of the gene with *Nco*I and *Xba*I sites, and this was sequenced and cloned into pBSΔSXKNU-5 using the same sites to create pBSKPGUS-1. A version of the *KNU* coding sequence lacking the stop codon was amplified by PCR to incorporate *Nco*I sites overlapping its start and immediately 3′ of its final codon. The amplified fragment was cloned, sequenced, and then subcloned in front of GUS in pBSKPGUS-1 to create pBSKNUGUS. Finally, the *KNU* cassette containing GUS and the full-length *KNU:GUS* fusion were cloned into pWBVec8 as *Hin*dIII-*Not*I fragments to create the binary T-DNA constructs p8KPGUS and p8KNUGUS, respectively. These constructs were transformed into *knu* and wild-type Ws plants.

GUS staining of tissues from plants harbouring the constructs were performed in tissue culture dishes containing the staining buffer described in Vielle-Calzada et al. (Vielle-Calzada et al., 2000). Samples were routinely incubated at 37°C for 16-20 hours. Subsequent clearing of tissues consisted of treatment with a 1:1 mixture of acetic acid and ethanol for 1-2 hours followed by mounting in an ovule-clearing solution of (v/v) 20% lactic acid and 20% glycerol, or direct mounting in the latter solution, depending upon extent of dissection and type of tissue to be examined. Observations described herein are based on an examination of 24 p12.2GUS2-1 T_1 transgenics and five representative T_2 seedling sets derived from these. The p8KNUGUS and p8KPGUS results are based on 16 and 17 T_1 plants, respectively.

Results

Developmental abnormalities of *knuckles* flowers and fruits include production of ectopic floral organs, parthenocarpy, and conditional male sterility

The EMS mutant *knuckles* was isolated in a screen for M_2 plants displaying reduced fertility and abnormal silique morphology. The mutant is essentially male-sterile when raised at standard *Arabidopsis* growth room temperatures (22-25°C), but it sets a reduced number of seeds when cross-pollinated by wild-type. *knu* plants continue to flower long after wild-type Ws plants have senesced, probably as a consequence of the inability of the mutant to self-pollinate at non-permissive reproductive temperatures (Fig. 1A). In spite of this male sterility, a variable fraction of unpollinated *knu* flowers give rise to short, frequently kinked siliques, each with a single bulge that becomes more pronounced as the silique ages. This enlarged kink can be discerned even from the exterior of an anthesis-stage gynoecium (Fig. 1B, inset), and after parthenocarpic silique development resembles the knuckle of a human finger. The cause of the bulge is an internal green

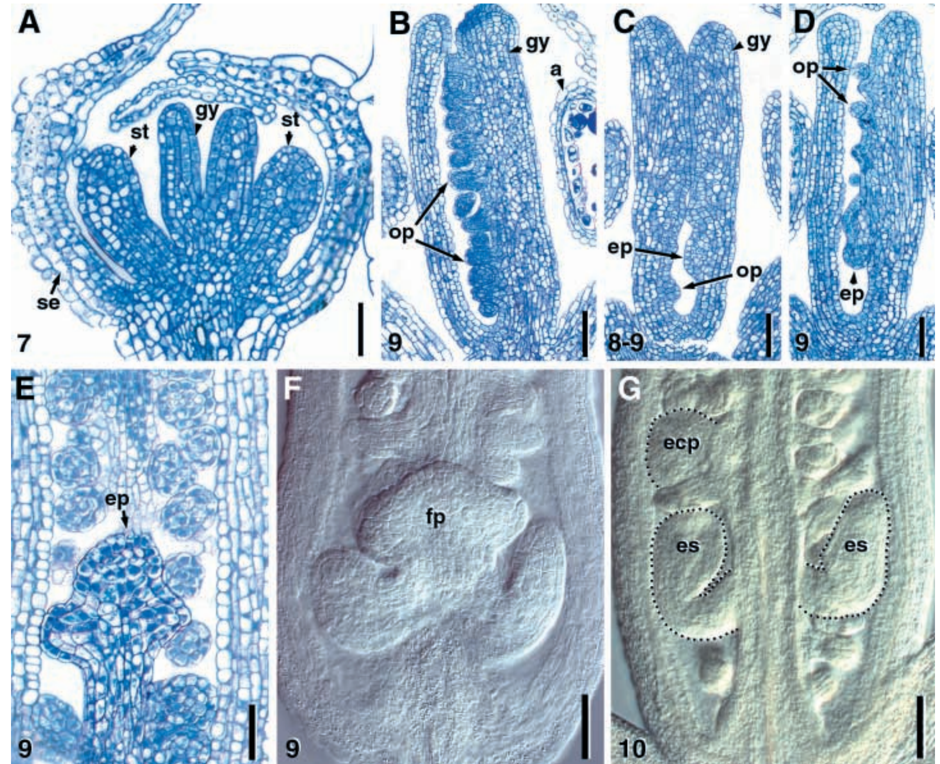

Fig. 2. Ectopic floral organs arise from the placentae. (A-E) Longitudinal sections of floral buds, whereas F and G are whole-mounts where images were captured using Nomarski optics. Stages of floral development are indicated in the bottom-left of each panel. (A) Early gynoecium initiation in a wild-type Ws flower bud. (B) Wild-type Ws gynoecium with ovule primordia. (C) *knu* gynoecium showing ectopic primordium developing from placental tissue. (D) Developing ectopic organs inside the basal one-third of a primary *knu* pistil. (E) Ectopic primordium interspersed with developing ovules inside a primary *knu* pistil. (F) Ectopic floral primordium developing inside primary *knu* pistil. (G) Ectopic carpel and stamen primordia developing inside primary *knu* pistil. Scale bars: 25 µm in A,E; 50 µm in B-D,F,G. a, anther; ecp, ectopic carpel primordium; ep, ectopic primordium; es, ectopic stamen; fp, floral primordium; gy, gynoecium; op, ovule primordium; se, sepal; st, stamen.

ectopic mass that may increase in size and density with time; and in some siliques splits the valve/replum boundaries, continuing to grow outside the silique (Fig. 1C).

In vitro culture (not shown), dissections and sectioning of these knuckle structures from flower buds and earlier stages of silique development revealed that they represent an indeterminate repetition of ectopic stamens and carpels (Fig. 1D-G). Ectopic carpel structures may remain green for the life of the *knu* mutant plant, even after ripening and abscission of primary valve tissues, and stay firmly attached to the replum by a fused vascular network that might extend to the pedicel (Fig. 1E,F). The cross-section of a knuckled silique shown in Fig. 1G reveals at least three reiterations of carpelloid tissue. We have elected to describe these ectopic, partly carpelloid growths as ‘knuckles’ to differentiate them from the superficially similar ‘carpel-like structures’ (CLSs) that sometimes develop in *bel1* siliques (Modrusan et al., 1994).

The ectopic floral organs of the *knu* mutant are of placental origin. We used bright field microscopy and Nomarski optics to examine thin sections and lactophenol-cleared gynoecia of wild-type and mutant plants as shown in Fig. 2A-G. Ectopic organs arose from placental tissues in the basal third or half of the developing gynoecium. Unlike *bel1* mutants they were not derived from ovule primordia. Abnormalities in carpel development were first observed around stage 8 in the basal portion of *knu* carpels, with the proliferation of placental tissue adjoining the zone of ovule primordia formation (Fig. 2C). The planes and patterns of cell division in ectopic primordia differed from those found in later developing ovules (Fig. 2C,D). Within a primary carpel, we observed that ectopic organ development was often more advanced than that of surrounding ovules. The ectopic structure in Fig. 2E appears to be composed of three primordia and is significantly larger than surrounding ovule primordia. We believe that the ectopic structure in the gynoecium from a stage 9 bud shown in Fig. 2F is an ectopic floral meristem flanked by two developing stamens. Within the primary *knu* gynoecium pictured in Fig. 2G well-developed ectopic stamens are intermingled with developing ovules from which inner and outer integuments have recently been initiated. The dome of an ectopic gynoecium is also evident. Although ectopic organs tended to originate in the basal third to half of the *knu* silique, the presence of ovules basal to the ectopic floral organs indicated that meristematic activity was not merely a consequence of direct whorl 4 indeterminacy.

Ectopic floral organ growth in the *knu* mutant is frequently reiterative, each new iteration originating from the placental tissue in the basal portion of a developing carpel of the preceding iteration. Growth of the stamens of the first ectopic iteration inside primary mutant carpels begins before initiation of first-iteration ectopic carpels and more proximal to the base of the primary gynoecium. The first set of ectopic stamens (a variable number, between 1 and 5 have been observed) are exterior to the knuckle, whereas all succeeding iterations of ectopic stamens occur layered and compressed between reiterating carpels within this structure. Instances in which ectopic stamens developed in the absence of a knuckle were not uncommon, but the development or initiation of ectopic stamens appeared to be a prerequisite for development of the knuckle, as the latter have not been seen to exist alone. When 265 mature *knu* siliques from plants grown at 25°C were dissected and examined, 86% contained ectopic stamens, and 53% had produced knuckles. In this same set of observations, 11% of siliques possessed a third (usually small) valve and consequently a trifurcated replum. All of these tricarpelloid siliques (30) contained a knuckle. Knuckled siliques have been

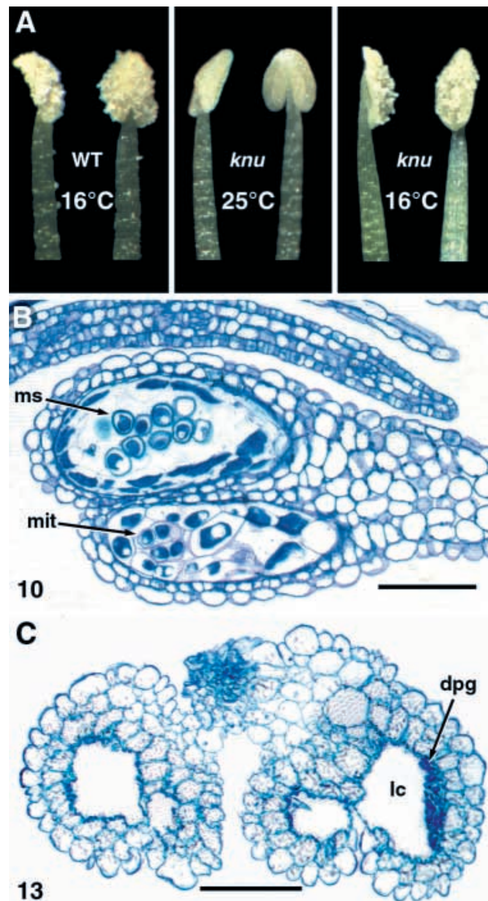

Fig. 3. Pollen development is restored and anthers dehisce in *knuckles* plants grown at 16°C. (A) Morphological comparison of wild-type and *knu* anthers at the time of flower opening taken from plants grown at 25°C and 16°C. Lateral and adaxial views are shown. (B) Section through *knu* anther from an early stage 10 flower taken from a plant grown at 25°C, showing post-tetrad microspores and a semi-intact tetrad. At this stage *knu* anthers and developing pollen were indistinguishable from wild-type. (C) Cross-section through *knu* anther taken from the open flower of a plant grown at 25°C. Stamen is non-dehiscent and mature pollen grains are absent. Scale bars: 50 μm in B,C. dpg, degenerating pollen grains; lc, locule; ms, microspore; mit, microspore tetrad.

observed to contain only a single knuckle, and this ectopic structure was always confined to one locule of the silique unless the septum was ruptured as a consequence of knuckle growth.

We investigated the effects of reduced growing temperature on the knuckling phenotype and fertility of our mutant. When 169 mature siliques from *knu* plants grown at 16°C were examined, 30% were found to contain knuckles. We also observed that male fertility was partially restored and that anthers dehisced in *knu* plants grown at 16°C (Fig. 3A). Sections of anthers from stage 10 buds dissected from *knu* mutants grown at reproductively non-permissive temperatures showed that microspores often formed (Fig. 3B) as in wild-type anthers (not shown); however, at flower maturity, indehiscent anthers did not contain recognizable pollen grains but a degenerated mass on the inside of the anther wall (Fig. 3C). Numbers of seeds formed in siliques of self-pollinated *knu* mutants grown at 16°C ranged from 0 to 51, with a mean of 23 (±13) seeds/silique (n=100 siliques). By contrast, 13 to 65 seeds were found in siliques of self-fertilized Ws plants grown simultaneously at 16°C, with a mean of 47 (±10) seeds/silique (n=100 siliques).

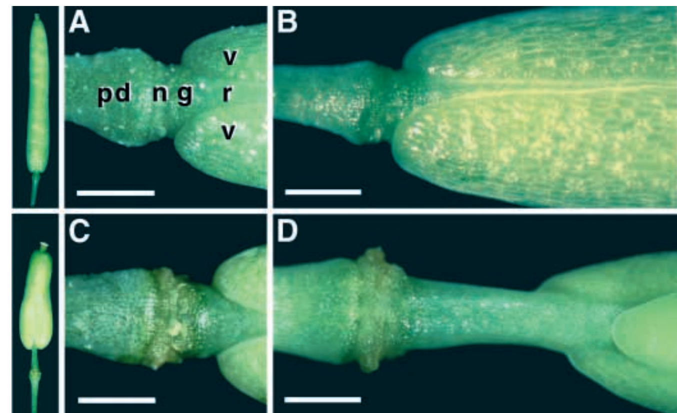

Fig. 4. Comparison of basal regions of wild-type and *knuckles* siliques grown at 25°C. Whole green wild-type and *knu* siliques are shown far left in vertical orientation. (A,B) Details of the basal portions of two wild-type siliques, and C and D show details of two *knu* siliques in plants grown at 25°C, in horizontal orientation. *knu* siliques show exaggerated nectary tissue relative to wild-type. The gynophore is extended in D and the green silique has three valves. Scale bars: 500 μm in A-D. g, gynophore; n, nectaries; pd, pedicel; r, replum; v, valve.

The *knu* mutant is weakly parthenocarpic because gynoecia that develop a knuckle when grown at 22-25°C increase in length and breadth in the absence of pollination or seed set and attain an average length of 5.8 mm (n=46) compared with an average length of 4.1 mm in the absence of a knuckle (n=174). A silique length of 5.8 mm slightly exceeds the minimum elongation criteria for parthenocarpy specified by Vivian-Smith et al. (Vivian-Smith et al., 2001) for parthenocarpy in *Arabidopsis* at 5.5 mm. However, no effort was made to allow for the pronounced curvature or kinking of the knuckled siliques, thus our measured average length of 5.8 mm underestimates parthenocarpic expansion in the axial dimension. Radial expansion of knuckled siliques also occurs, particularly in the vicinity of the knuckle, where the valve bulges outwards. Parthenocarpic *knu* siliques are dehiscent at maturity, although premature splitting along the valve/replum boundary of an affected locule may occur when ectopic organs are present.

The basalized phenotype of the *knuckles* gynoecium suggests that KNUCKLES may establish or maintain a boundary restricting gynophore development

Apart from the ectopic growth of stamens and carpels within the primary gynoecium, the most striking phenotype of the *knu* mutant grown at 22-25°C is the formation of 'basalized' gynoecia in which the gynophore, a basal pattern element, may extend apically to replace a portion of the ovary (Fig. 4). When *knu* plants were grown at 16°C, the basal-most portion of the ovary was reduced rather than replaced: the replum failed to bifurcate and the septum did not expand, but valves with an

exaggerated radial curvature differentiated in this region and ovules capable of fertilization and development into seeds were also present. The dried replum of a 16°C-grown dehiscent *knu* silique resembled an oar, and the detached valves were spoon-like (Fig. 5A,B). *knu* plants grown at 16°C also produced siliques in which the basal portion of the ovary was reduced, but in the absence of seed set the relative difference between expansion of basal and more distal portions of the ovary was less pronounced. In cross-section the extended gynophore produced by a fraction of *knu* siliques that developed at the non-permissive reproductive temperature possessed a ring of vascular tissue similar to wild-type gynophore and typical of stem. It may appear that bifurcation of the replum occurs as a result of internal pressure from ectopic organ growth, but it is important to note that apically-shifted replum bifurcation occurs even in the absence of knuckle formation. Although the position at which bifurcation of the replum and normal expansion of the septum began varied slightly from silique to silique, the positioning of the origin of the knuckle relative to the bifurcation was non-random. These morphological observations of the mutant may be taken to imply that the basalization and knuckling phenotypes are separate developmental consequences of the loss of KNU-mediated basal domain maintenance. Alternatively, the knuckling phenotype might have a stochastic relationship to gynoecial basalization, which seems plausible given that even consecutive siliques from the same inflorescence stem may be affected to sharply varying degrees.

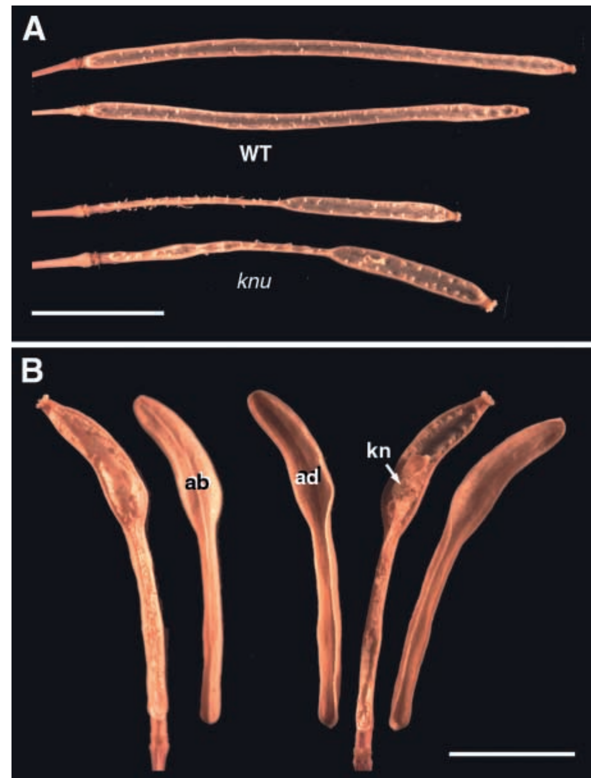

Fig. 5. Wild-type and *knuckles* silique phenotypes at 16°C. (A) Wild-type and *knu* replae from dehiscent siliques grown at 16°C. (B) Features of a single 16°C-grown dehiscent *knu* silique. Left, one valve removed to expose bulging septum of un-knuckled side of the replum, abaxial (ab) side of valve visible. Right, both valves removed, with knuckled side of replum and inner (adaxial, ad) faces of spoon-like valves visible. Knuckle (kn) indicated by arrow. Note that seeds have been removed. Scale bars: 5 mm in A,B.

KNUCKLES is *MAC12.2* and encodes a C2H2 zinc finger protein

PCR-based INDEL markers were used to map the *knu* mutation to a 123 Kb interval on chromosome 5. A genomic fragment capable of complementing all aspects of the *knu* mutant phenotype was identified and two candidate genes were sequenced from mutant and wild-type templates. A mutation was identified in the putative coding sequence of *MAC12.2*, whereas no changes were found in the other candidate gene. *MAC12.2* from *knu* contains a guanine to adenine transition such that a TGT codon is changed to TAT, and would result in the substitution of a tyrosine for a cysteine residue.

MAC12.2 is predicted to be a small C2H2 zinc finger protein (Miller et al., 1985) of 161 amino acids. The cysteine residue replaced in the *knu* mutant is the second of two required for zinc binding and thus is potentially critical for function of the zinc finger as a DNA binding or protein-protein interaction domain (Fig. 6A). In addition to the single zinc finger, MAC12.2 contains an EAR-like active repression domain as described by Hiratsu et al. (Hiratsu et al., 2002) at its carboxy terminus (Fig. 6B).

The annotated *MAC12.2* coding sequence in the GenBank database is predicted to be 486 bp in length, and contains no introns. Because the annotation was not substantiated by an EST, RACE PCR was used to identify the 5′ and 3′ limits of *MAC12.2* transcription. Our results indicate that transcription probably starts at –111 relative to the annotated start codon, a finding in agreement with the average dicot 5′ UTR length of 98 nucleotides determined by Kochetov et al. (Kochetov et al., 2002). 3′-RACE experiments identified multiple polyadenylation sites downstream of the putative translation stop codon, the longest of which occurred at +676 relative to the first base of the translation start codon. There are precedents for multiple polyadenylation sites in plant genes (Hunt, 1994), but the presence of several stretches of AU-rich sequence within this region could potentially lead to shortened 3′-RACE PCR artifacts. Henceforward we refer to *MAC12.2* as *KNUCKLES*.

Transcription of *KNUCKLES* during floral development

Preliminary characterization of *KNU* expression by RT-PCR indicated that the gene was transcribed most strongly in flower buds (Fig. 7). The patterns of *KNU* gene expression examined by in situ hybridization during floral development were identical in both mutant and wild-type plants. Experiments utilized two different probes. In all cases *KNU* transcripts were detected in a wide range of cell types comprising the floral organs. Fig. 8 shows data derived from in situ analysis of wild-type plants. *KNU* mRNA was detected in the sepals and pedicels of stage 6 floral buds. *KNU* transcripts were absent in the developing petal and stamen primordia at this stage, but transcripts were localized in a small region towards the base of the developing carpel primordium (Fig. 8A). Later in development, *KNU* transcripts were evident in cells of all floral organs (Fig. 8B-E) including male and female gametophytes.

A

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                  F/YXCXXCXXXFXXXXXLXXHXXXHXXXXX
KNUCKLES            FPCQYCPRKFYTSQALGGHQNAHKRERAAAR   38-68/161
SUPERMAN            YTCSFCKREFRSAQALGGHMNVHRRDRARLR   47-77/204
At5g48890.1         FPCLFCSRKFHSSQALGGHQNAHKKERTAAR   35-65/173
AtZFP10             YTCSFCRREFKSAQALGGHMNVHRRDRARLK   39-69/304
ZAT11               FECKTCNKRFSSFQALGGHRASHKKPKLTVE   47-77/178
                    5 * 4* 53*35 ****** 4 *55454 34
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B

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KNUCKLES            LDLSLRL     155-161/161
SUPERMAN            LDLELRL     195-201/204
At5g48890.1         LDLSLHL     165-173/173
AtZFP10             LDLELRL     290-296/304
ZAT11               LDLNLTP     172-178/178
                    *** *45
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Fig. 6. Motif sequence comparisons between KNUCKLES and other *Arabidopsis* C2H2 zinc finger proteins. (A) Alignment of C2H2 zinc fingers and adjacent basic residues. The first line consensus shown in red was taken from Evans and Hollenberg (Evans and Hollenberg, 1988). Cysteine and histidine residues responsible for zinc binding are highlighted in yellow. The sub-motif QALGGH is present in a majority of plant zinc finger proteins (Takatsuji, 1999). At5g48890.1, a protein of unknown function, is 77% identical to KNUCKLES through the aligned region. AtZFP10 has been studied by Dinkins et al. (Dinkins et al., 2002), and is part of a family of approximately 30 small, single-fingered proteins identified by those authors and Tague and Goodman (Tague and Goodman, 1995). (B) Alignment of EAR and EAR-like motifs. ZAT11 contains a consensus EAR motif (L/FDLNL/FXP) as defined by Ohta et al. (Ohta et al., 2001), whereas the others are EAR-like as described by Hiratsu et al. (Hiratsu et al., 2002). Residues conserved in all five proteins shown in blue and indicated below alignment by (*); 5/5 conservative by (5), and 4/5 conserved or conservative by (4), 3/5 conserved or conservative by (3). Numbers at right indicate position of motif relative to total protein length. Accession numbers as follows: KNUCKLES (NP_196905), SUPERMAN (S60325), At5g48890.1 (NM_124266), AtZFP10 (AAC23644), ZAT11 (F84792).

Transcripts were absent after fertilization in developing seeds (not shown).

We have noted that the *knu* mutant exhibits pronounced male and partial female sterility in addition to the formation of the knuckle from basal placental regions of the carpel when it is grown at 25°C. Developmental defects were not seen in other regions of the flower where in situ experiments indicated *KNU* transcript was found. Therefore, the KNU protein may only be produced and/or transported to a subset of locations where *KNU* transcript accumulates. Other proteins with redundant function might substitute for KNU in organs that develop normally in the *knu* mutant.

Expression patterns of KNUCKLES:GUS fusions indicate post-transcriptional regulation of *KNU*

Gene constructions encoding translational fusions of *KNU* to the *uidA* (GUS) gene of *E. coli* were made in order to examine the developmental pattern of KNU protein expression within specific floral organs. p8KNUGUS contained the entire coding sequence of *KNU* linked to GUS. p12.2GUS2-1 encoded a fusion that lacked the last 6 amino acids of the EAR-like domain from the C-terminal portion of the protein. A third construct, p8KPGUS, completely lacked *KNU* coding sequences and the GUS gene was flanked both 5′ and 3′ by KNU untranslated sequences. Fig. 9 shows the structure of these chimeric fusions, the complementation constructs upon which they were based, and a schematic summary of GUS expression in organs at particular stages.

Wild-type plants containing p8KNUGUS and p12.2GUS2.1

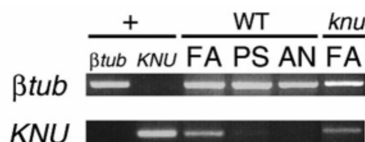

Fig. 7. RT-PCR demonstrates that *KNUCKLES* is transcribed at low levels in green flower buds but not in anthesis-stage flowers. Ethidium bromide-stained 3% TAE agarose gel. First row, 869 bp fragment of *β-tubulin* transcript cDNA amplified after 30 cycles. Second row, 332 bp *KNU* transcript cDNA fragment amplified after 40 cycles, loaded 2:1 relative to volumetric proportion of 50 μL β-tubulin reaction (β*tub*). Positive control amplifications from plasmid templates are denoted (+). Tissue sampled included: floral apices, including green buds up to floral stage 11 (FA), stage 12 buds with petals showing (PS), and open flowers at stage 13 with dehisced anthers (AN), in addition to floral apices from the *knuckles* mutant (*knu*).

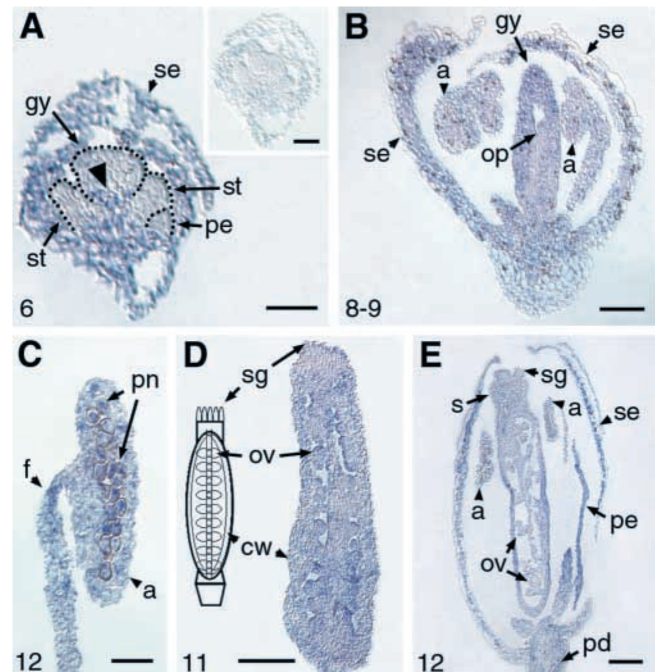

Fig. 8. *KNUCKLES* mRNA localization in developing wild-type *Arabidopsis* flowers. (A-E) Sectioned flower buds or floral organs from buds at the stages indicated at bottom-left of each panel were probed with the short (3′) antisense *KNU* probe, except the section inset in A, which was probed with sense *KNU* probe as a control. Hybridization of *KNU* transcripts and antisense probe is indicated by formation of a blue-purple precipitate. Scale bars: 20 μm in A, inset; 50 μm in B,C; 100 μm in D,E. a, anther; cw, carpel wall; f, filament; gy, gynoecial primordium or gynoecium; ov, ovule; op, ovule primordium; pd, pedicel; pe, petal; pn, pollen; se, sepal; st, stamen primordium or stamen; sg, stigma; s, style.

A. PLASMID (Backbone)	COMPLEMENTATION Full	Partial	Un	Total
p8MUASAL1H-5 (pWBVec8)	6	5	6	17
p8KNU (pWBVec8)	22	15	5	45
p8KNUNX (pWBVec8)	5	6	3	14

B. PLASMID (Backbone)	GUS STAINING Stage 6 buds	Stage 9 buds	Ovules	Leaf/ Stem
p8KNUGUS (pWBVec8)				
p8KPGUS (pWBVec8)				
p12.2GUS2-1 (pBI101.2)				

Fig. 9. Complementation and GUS reporter constructs and their effects in transgenic *Arabidopsis*. (A) Complementation constructs. Plasmids listed far left. Binary T-DNA parent plasmids shown in parentheses. Centre, inserts are depicted as coloured bars. *KNU* coding sequences are shown in red, and flanking genomic sequences are shown in yellow. Far right, hygromycin-resistant *knu t*ransgenics were scored as being fully or partially complemented or uncomplemented based on restoration of fertility and morphological criteria. (B) GUS reporter constructs. The *uidA* gene is shown as a blue bar. Far right, cartoons representing stage 6 and 9 flower buds, early ovules, and leaf and stem are shaded blue to approximate patterns of GUS staining at the stages depicted in multiple independent lines containing each construct. Scale in kilobases (Kb). H, *Hin*dIII; N, *Nco*I; P, *Pst*I; X, *Xba*I; X^m, methylation-sensitive *Xba*I site.

protein fusions exhibited similar patterns of GUS staining during development even though p12.2GUS2.1 lacked a portion of the EAR-like domain of the KNU protein. Transgenic plants containing these constructs showed GUS staining in specific cell types of developing gynoecia, stamens and ovules. This pattern was more restricted than the general distribution of *KNU* transcription observed using in situ hybridisation (Fig. 8). The major difference between p8KNUGUS and p12.2GUS2.1 GUS staining patterns was that in p12.2GUS2.1 plants sepals were unstained (Fig. 10A), whereas persistent GUS staining was evident in a faint pattern in and around vascular tissue of the sepals of all 16 plants containing p8KNUGUS (Fig. 10B). This may relate to the 3′ *KNU* sequences lacking in p12.2GUS2.1.

The expression of GUS in plants containing p8KPGUS was more general than that of the two translational fusions but still not as general as the pattern of *KNU* transcript distribution. GUS expression was strong in vascular tissues and the majority of the 17 transgenics showed GUS staining in leaves and stems (Fig. 10C). These data suggest that 3′ sequences flanking the *KNU* coding sequence may contain elements that direct transcription in the vascular tissue of sepals, leaves and stems. Because p8KNUGUS and p8KPGUS constructions differ only in that GUS in p8KNUGUS is fused to the full-length KNU coding sequence, KNU appears to be subject to some form of post-transcription regulation that limits protein accumulation in particular tissues.

Expression of KNUCKLES:GUS constructs correlate with the *knuckles* floral phenotype

The patterns of GUS expression in developing stamens and carpels of wild-type transgenic plants containing p8KNUGUS, p12.2GUS2.1 and p8KPGUS were conserved, and importantly were restricted to a small number of cell types. Staining first appeared in the fourth whorl of stage 6 buds as a central spot (Fig. 10D). This stained area of the primordium increased in size as the gynoecium differentiated (Fig. 10E), but as the gynoecial cylinder lengthened through stages 7 and 8, staining remained strong only at its base (Fig. 10F). At stage 9, when ovule primordia arose, staining of the gynoecium was concentrated at two spots at the base of the carpel that probably corresponded to the most basal portions of the developing valves (Fig. 10G,I).

Shortly after GUS stain was first observed in the gynoecial primordium, it became evident in developing anthers and was prevalent in the stamen predominantly in the developing pollen of anthers at floral stage 9 (Fig. 10F,H). GUS was evident throughout male gametophyte development but there was virtually no detectable staining in third-whorl organs at anthesis. During ovule development, GUS was observed in the archesporial cells. Expression persisted in the megaspore mother cell (Fig. 10J) and was evident during meiosis (Fig. 10K) and the mitotic events of embryo sac formation (Fig. 10L). GUS was evident in mature embryo sacs after fusion of the polar nuclei and absent after fertilization (not shown). Staining of internal portions of the style and of the stigmatic papillae was seen at the apex of the gynoecium as it matured during floral stages 10-13. Little or no staining of gynoecial tissues was observed beyond stage 13 (anthesis). Given the general pattern of *KNU* transcript accumulation, the conservation of specific cellular staining patterns during stamen and gynoecia development in plants containing the three constructs suggests that mechanisms limiting protein accumulation in these organs might involve 5′ sequences flanking the *KNU* protein coding region as they are common to all three constructs. Early and persistent GUS staining near the base of the carpels late into floral development is consistent with the basalized syndrome of alterations to ovary development seen in the *knu* mutant. The expression in developing male and female gametophytes is also consistent with the defects in male and female fertility observed in the *knu* plants. The role of *knuckles* in female gametophyte development will be investigated elsewhere.

GUS staining patterns of *knu* transgenics harboring KNU:GUS constructs support hypotheses about the role of KNU in gynoecial development

The p8KPGUS and p8KNUGUS constructs were transformed

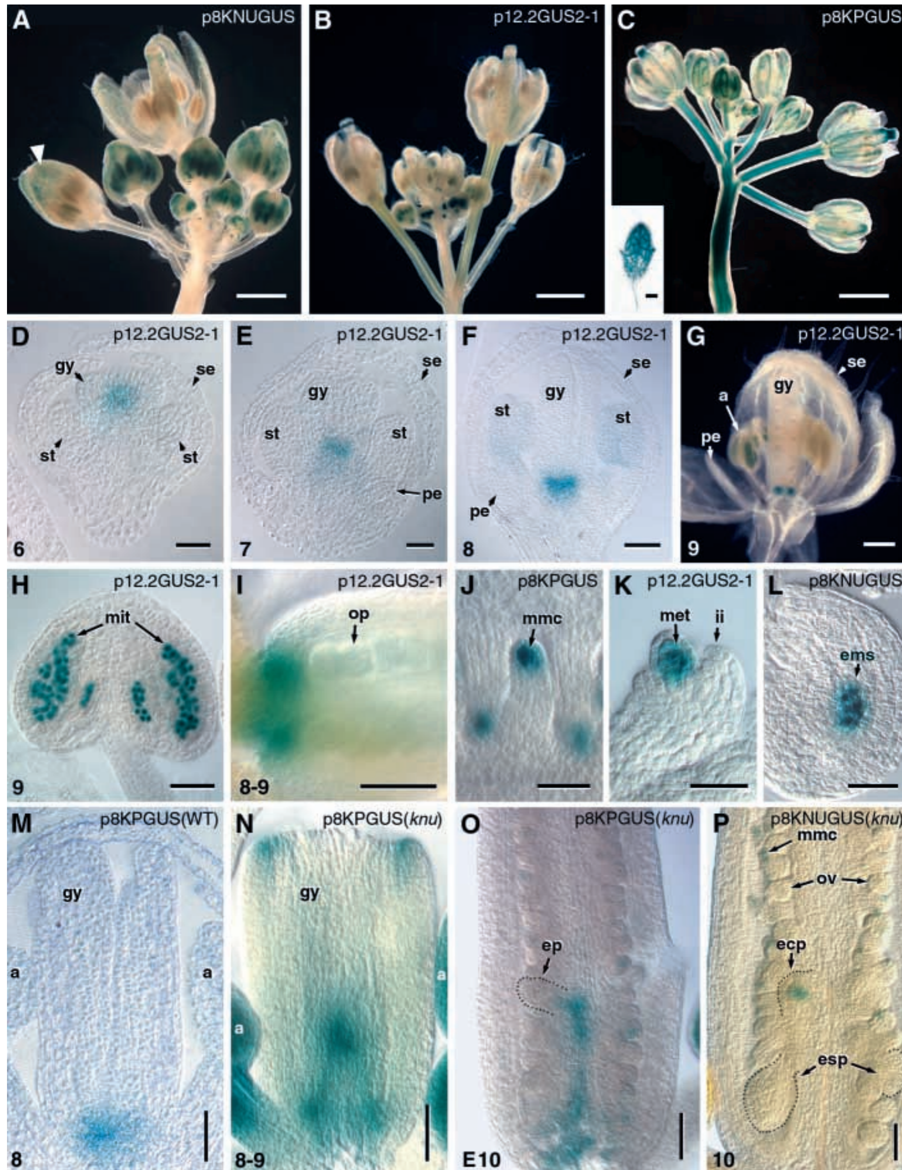

Fig. 10. Expression of KNU:GUS constructions in wild-type and *knu* genetic backgrounds. The three GUS constructions shown in Fig. 9 were introduced into *Arabidopsis* wild-type (A-M), and *knu* mutant backgrounds (N-P). Patterns of GUS activity were examined in whole-mounts or thin sections (D-F,M). (A-C) Inflorescences. (D-G) Representative expression in developing flower buds. (H) Detail of an anther; I-L show details of developing ovules. Images B,D-I,K were taken from primary transgenics, and the remainder from homozygous plants. The introduced construct is named in the top right-hand corner of each panel, and the developmental stage in the bottom-left corner (where relevant). Scale bars: 25 μm in D-F,H-L,M,N; 50 μm in O,P; 100 μm in G; 1 mm in A-C, inset. a, anther; ecp, ectopic carpel primordium; ep, ectopic primordium; esp, ectopic stamen primordia; ems, embryo sac; gy, gynoecium or gynoecial primordium; ii, inner integument of ovule; mmc, megaspore mother cell; met, megaspore tetrad; mit, microspore tetrad; op, ovule primordium; ov, ovules; pe, petal; se, sepal; st, stamen or stamen primordium.

into homozygous *knu* plants, and floral tissues derived from the resulting primary transgenics were subjected to GUS staining. Neither construct was able to complement *knu*. Expression in developing male and female gametophytes remained unchanged. Interestingly, the GUS staining pattern was expanded markedly in early developing gynoecia of *knu* p8KPGUS transgenics compared with transgenic gynoecia of wild-type plants harboring the same construct (Fig. 10N). Instead of the polarized basal expression typically seen in wild-type (Fig. 10M), staining extended into a larger proportion of basal gynoecial cells that overlapped the region where ectopic organ initiation and growth were observed in the mutant.

The p8KNUGUS fusion protein was expressed similarly in developing *knu* gynoecia. Expression in ectopic floral organs occurred in a developmental pattern comparable to that in stamens and carpels from wild-type plants, confirming that these ectopic floral organs have the same identity and capacity to accumulate KNU as their primary counterparts in the third and fourth whorls (Fig. 10P). The GUS staining patterns observed in the *knu* background are readily explained if KNU functions to limit proliferation in those cells where it is expressed.

Discussion

The complex *knuckles* phenotype may be indicative of a parthenocarpic mechanism common to diverse species

The fruit phenotype of tomato plants in which transcription of *TM29*, a presumptive *SEPALLATA3* (Pelaz et al., 2000) homolog, was downregulated (Ampomah-Dwamena et al., 2002), is similar to the *knu* silique abnormalities described here. Petals and stamens of flowers from these plants were transformed toward a sepaloid identity, and both stamens and carpels were infertile. Ovaries nevertheless produced parthenocarpic fruits from which additional inflorescence shoots might emerge, reiterating simple leaves, abnormal floral organs, and parthenocarpic fruits. The aberrant TM29-suppressed tomato fruits described by Ampomah-Dwamena et al. (Ampomah-Dwamena et al., 2002) and our observations of *knu* siliques suggest a regulatory linkage between ectopic organs originating within the gynoecium, determinacy defects, and parthenocarpy. Ectopic floral structures might engender a parthenocarpic phenotype by producing growth regulators or inappropriate proliferative signals counterfeiting those that normally occur only at pollination or fertilization. If the presence of ectopic organs within ovaries is not merely correlated with but has a causal relationship to parthenocarpy, an alternative means is suggested by which seedless fruit could be engineered in

diverse species of horticultural value, particularly those fruits that are derived from post-pollination development of gynoecial structures.

The reiterating structure of the knuckle suggests a role for KNU in the regulation of determinacy

The floral phenotype of *agamous* mutants is the transformation of third- and fourth-whorl organs to petals and sepals, respectively, as well as a loss of fourth-whorl determinacy such that a flower-within-flower pattern of reiterated floral whorls is produced: (sepal, petal, petal)$_n$ (Bowman et al., 1991). Our observations of the indeterminate floral phenotype of *knu* can be interpreted to mean that KNU is involved in the determinacy-promoting activity of AG, perhaps as a negative regulator of a proliferative or non-differentiative signal or a stem cell-promoting factor like WUSCHEL (WUS) (Lenhard et al., 2001).

Alvarez and Smyth (Alvarez and Smyth, 1999) observed a loss of floral determinacy similar to the ectopic organ phenotype of *knu* in plants homozygous for *crc-1* and heterozygous for *ag-1*. Like *knu*, *crc* mutants also produce many tricarpelloid gynoecia, an observation that has been attributed to a loss of determinacy (Bowman et al., 1999). CRC is a member of the YABBY family, as is INNER NO OUTER (INO), a regulator of ovule integument development (Villanueva et al., 1999). Interestingly, SUP, which is structurally similar to the KNU protein, has been shown by Meister et al. (Meister et al., 2002) to regulate outer integument growth by negatively regulating *INO* transcription. The exaggerated nectaries of *knu* flowers (Fig. 4C,D) suggest that KNU might co-regulate development of this tissue with CRC because *crc* mutant flowers lack nectaries altogether.

A model explaining the positioning of the knuckle along the proximo-distal axis of the placentae in relation to the ovules could be formulated as follows: KNU is essential for maintaining aspects of a basal domain or boundary in the developing gynoecium, such that floral meristematic activity within the placentae is suppressed and the determinacy of the flower is maintained. If no, or insufficient, or insufficiently active KNU protein is made (the latter being most likely for the allele described here, as our experiments indicate that *knu* is transcribed), placental or pre-placental tissue could proliferate to initiate an adventitious floral meristem before ovule primordia are generated. Ovules develop asynchronously from the parietal placentae (Gaiser et al., 1995), and basipetally, such that the most developmentally advanced ovules occur at the apical end of the gynoecium. KNU expression would be expected to overlap a region of potential competence to produce floral meristem in addition to ovule primordia, and ectopic floral meristem development would not preclude later ovule induction from more basal placental tissue.

KNUCKLES might be an active repressor of transcription regulating cellular proliferation during floral development relative to a hypothetical fourth whorl/fifth whorl boundary

The recessive *knu* phenotype, characterised by the production of ectopic floral organs, genetically defines the KNU protein as a repressor of non-ovule floral organ development within the context of the placentae of the pre-stage-9 gynoecium. Similarly, the homozygous *sup* phenotype is production of additional whorls of stamens in an inappropriate floral context (Bowman et al., 1992). Recent evidence indicates that SUP could be an active transcriptional repressor. Hiratsu et al. (Hiratsu et al., 2002) have identified an EAR-like transcriptional repression motif near the carboxy terminus of SUPERMAN. Dathan et al. (Dathan et al., 2002) found that basic residues flanking the SUP zinc finger domain on either side were important to stabilization of its interaction with an oligonucleotide target. In addition to a zinc finger flanked by small clusters of basic amino acids, the KNU protein is predicted to encode a consensus-matching carboxy-terminal EAR-like motif nearly identical to that found at a similar position in SUP. The presence of a zinc finger and an EAR-like motif provide additional if indirect evidence that KNU is a transcriptional repressor.

Evidence has accumulated that SUP is a negative regulator of cellular proliferation, and that the cadastral effects of *sup* on floral development result from an overproliferation of the third whorl at the expense of the fourth (Sakai et al., 2000). In the developing ovule, SUP negatively regulates adaxial growth of the outer integument. Hiratsu et al. (Hiratsu et al., 2002) found that *Arabidopsis* plants overexpressing full-length SUP were severely dwarfed because of a decrease in cell number rather than cell size. Our observation that a GUS reporter under the control of *KNU* flanking regulatory sequences engenders a larger population of stained cells in *knu* vs wild-type transgenics implies that KNU also regulates cellular proliferation in the basal gynoecial tissues where it is normally expressed.

Broadly interpreted phenotypic similarities between the *sup* and *knu* mutants as well as the existence of shared protein motifs lead us to speculate that KNU has a role in determining or maintaining a boundary between the fourth whorl of the floral meristem and the parietal placentae which arise from the developing gynoecium. Palaeobotanical studies indicate that the ovule (an integumented megasporangium) evolved prior to the carpel, and as Bowman et al. (Bowman et al., 1999) have pointed out, seeds and therefore ovules are not inventions of the angiosperms. Unlike the sepals, petals, stamens and carpels of angiosperm flowers, ovules are not thought to be derived from leaves (reviewed by Robinson-Beers et al., 1992). The placentae which give rise to the ovules in *Arabidopsis* are intimately associated with medial adaxial tissue of the carpels, but in the Solanaceous plant *Petunia hybrida* the placentae arise separately, from the central region of the floral meristem. Angenent and Colombo (Angenent and Colombo, 1996) have concluded that this central meristematic region represents an additional whorl. The demonstration that ovule and carpel development are genetically separable in *Petunia*, together with the characterization of MADS box genes (*FLORAL BINDING PROTEIN 7* and *11*) that might be thought of as providing D function (Angenent et al., 1995, Colombo et al., 1995), lends credence to the idea that ovules could be considered fifth-whorl organs. It is possible that one regulatory function of KNU is analogous to that proposed for the SUP protein (Sakai et al., 2000). KNU might act to maintain a proliferative balance between the meristematic tissues on either side of a developmentally discontinuous fourth whorl/placental ‘whorl’ boundary, because in the *knu* mutant organs normally present

only in the third and fourth floral whorls are repeated from the parietal placentae that in *Arabidopsis* would compose this hypothetical fifth whorl.

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