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Analysis of the Arabidopsis *superman* allelic series and the interactions with other genes demonstrate developmental robustness and joint specification of male–female boundary, flower meristem termination and carpel compartmentalization

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• **Background and Aims** *SUPERMAN* is a cadastral gene controlling the sexual boundary in the flower. The gene's functions and role in flower development and evolution have remained elusive. The analysis of a contrasting *SUP* allelic series (for which the names *superman*, *superwoman* and *supersex* have been coined) makes it possible to distinguish early vs. late regulatory processes at the flower meristem centre to which *SUP* is an important contributor. Their understanding is essential in further addressing evolutionary questions linking bisexuality and flower meristem homeostasis.
• **Methods** Inter-allelic comparisons were carried out and *SUP* interactions with other boundary factors and flower meristem patterning and homeostasis regulators (such as *CLV*, *WUS*, *PAN*, *CUC*, *KNU*, *AG*, *AP3*/*PI*, *CRC* and *SPT*) have been evaluated at genetic, molecular, morphological and histological levels.
• **Key Results** Early *SUP* functions include mechanisms of male–female (sexual) boundary specification, flower mersitem termination and control of stamen number. A *SUP*-dependent flower meristem termination pathway is identified and analysed. Late *SUP* functions play a role in organ morphogenesis by controlling intra-whorl organ separation and carpel medial region formation. By integrating early and late *SUP* functions, and by analyzing in one single experiment a series of SUP genetic interactions, the concept of meristematic 'transference' (cascade) – a regulatory bridging process redundantly and sequentially co-ordinating the triggering and completion of flower meristem termination, and carpel margin meristem and placenta patterning – is proposed.
• **Conclusions** Taken together, the results strongly support the view that *SUP*(-type) function(s) have been instrumental in resolving male/female gradients into sharp male and female identities (whorls, organs) and in enforcing flower homeostasis during evolution. This has probably been achieved by incorporating the meristem patterning system of the floral axis into the female/carpel programme.

Key words: Arabidopsis, allelic series, carpel, evo-devo, flower meristem determinacy, flower homeostasis, flower pattern, meristematic 'cascade'/transference, pistillody/carpelloidy, placenta, stamen, *SUPERMAN gene: superman, clark-kent*/*superwoman, supersex, AG, CLV, CRC, CUC2, KNU, PAN, SPT, WUS*.

INTRODUCTION

Arabidopsis flowers produce fixed numbers of sepals, petals, stamens and carpels as distinct and successive whorls. The floral meristem (FM) becomes determinate after the initiation of carpels, when it ceases to maintain its stem cell population. In this process, also known as FM termination, the expression of *WUS*, a homeodomain protein with a central role in stem cell activity, is controlled through a sequence of processes that initiate and subsequently maintain stem cell arrest at the flower meristem centre (FMC) (see also Prunet *et al*., 2008); we label the process the 'triggering and completion of FM termination'.

There are spatial and/or temporal control regulators of FM termination (Prunet *et al*., 2009): an altered spatial control results in an increased stem cell population, enlarged meristem and supernumerary organs (i.e. an altered homeostasis); a prolonged maintenance of stem cells produces a delay in FM termination with supernumerary whorls and organs (i.e. flower indeterminacy). This study primarily focuses on several factors of determinacy that control reproductive organ identities and the corresponding boundaries in the FMC space. Importantly, meristem termination processes and boundary establishment are interdependent (Rast and Simon, 2008) and trigger female programme development (Prunet *et al*., 2009). One question this study addresses is how the sequence of meristematic and differentiation processes is orchestrated in space and time. Meristematic modularity or 'transference of meristematic capacity' (Mathews and Kramer, 2012) is likely to account for the way meristematic 'cascading' is subtended by genetic-level

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modularity. The concept relies on the co-option of meristematic programmes [termination–(re)initiation)] through identity switches deployed during development, and is reflected in new trait acquisition by organs and tissues.

In this context, the timely separation of the stamen whorl from the carpel whorl at the FMC is the critical early event in a hermaphrodite flower. The homeotic *MADS* domain family genes *AP3*, *PI* (B function) and *AG* (C function), respectively, determine stamen (B + C) and carpel (C) whorl identities. *AG* is thus the master player in reproductive organ formation. Loss of *AG* function results in an indeterminate flower in which sepals and petals are produced repeatedly. Therefore, *AG* is also required for FM termination, the gene being expressed at high levels in an inner sub-domain of whorl 4 (Prunet *et al*., 2009). By shutting off the stem cell factor *WUS* (Lenhard *et al*., 2001; Lohmann *et al*., 2001), *AG* integrates stem cell regulation with the general floral patterning and female programme initiation (Bowman *et al*., 1989; Yanofsky *et al*., 1990; Sablowski, 2007*a*, *b*; Das *et al*., 2009; Prunet *et al*., 2009).

Loss of class B gene function feminizes the stamen whorl by producing carpel-like structures and causes premature FM termination immediately above the third whorl (Bowman *et al*., 1991; Jack *et al*., 1992). Conversely, ectopic expression of B genes results in partial indeterminacy, with stamens replacing carpels, a phenotype similar to that resulting from loss of function in the sexual boundary gene *SUPERMAN* (*SUP*) (Jack *et al*., 1994; Krizek and Meyerowitz, 1996). More precisely, mutant flowers of the best characterized allele, *sup-1*, produce extra whorls of stamens at the expense of the gynoecium (Bowman *et al*., 1992).

Thus, the correct control of FM termination requires both class B and C gene functions (Davies *et al*., 2006).

The cadastral gene *SUP* is necessary for the proper development of reproductive organs (Schultz *et al*., 1991; Bowman *et al*., 1992). The gene encodes a transcriptional repressor with a C2H2-type Zn finger motif, a serine/proline-rich domain, a basic domain and a leucine zipper-like domain with a C-terminal EAR-like active repression motif (Sakai *et al*., 1995; Ito *et al*., 2003; Table 1). Consistent with its role in separating stamens from carpels, *SUP* is expressed (Ito *et al*., 2003) at stages 3–5 in the boundary region between whorls 3 and 4 as a ring, in the inner part of whorl 3 and in stamen primordia. By excluding class B genes from the FMC, *SUP* is insulating *AG*, allowing more specific control of determinacy and carpel identity at the FMC.

This is the simplified picture. As a matter of fact, there are at least three additional layers to this picture that this report is addressing in the frame of the concept of 'meristematic cascading/transference' mentioned above, and, ultimately, FM homeostasis. First, the nature of the *sup* phenotype and functional models have largely been based on one allele, *sup-1*, a nonsense mutation. Other alleles have been reported (Schultz *et al*., 1991; Bowman *et al*., 1992; Gaiser *et al*., 1995; Jacobsen and Meyerowitz, 1997, and references therein), several of which exhibit phenotypes departing from *sup-1* (Table 1). Such alleles mimic *CLV* pathway alterations affecting RLK (receptor-like kinase) receptors, a HSP90-like chaperone or a kinase-associated protein phosphatase (KAPP) (Clark *et al*., 1993; Ishiguro *et al*., 2002; Tichtinsky *et al*., 2003). The question then is whether and, if so how, *SUP* integrates the *WUS* repression system controlled by partly redundant CLV and AG pathways (Prunet *et al*., 2009). Secondly, *SUP* is also expressed at later stages: in adaxial and distal parts of anthers at stages 5–7 (in connective, vasculature) and at stages 9–12 (14) in the septum and developing ovules (in funiculi, integuments) (Ito *et al*., 2003). Thirdly, other members of the *SUP* gene family, such as *RABBIT EARS* (petal–stamen boundary; Takeda *et al*., 2004) and *KNUCKES* (*KNU*; carpel–placenta boundary; Payne *et al*., 2004), represent flower- and whorl/organ-specific boundary regulators. For example, *knu* mutants produce reiterated stamen–carpel structures at the level of the placenta.

A shortcut view to the above considerations is that *WUS* regulation at the FMC can be seen as a co-ordination between whorls 3 and 4: in whorl 3, class B genes induce *SUP* to control cell growth (proliferation rates and differentiation, with models according to Davies *et al*., 1999; Sakai *et al*., 2000; Prunet *et al*., 2009); in whorl 4, *AG* regulates stem cell activity and cell differentiation via *KNU* (Sun and Ito, 2010). Class B and C proteins participate in quartet MADS protein complexes where their relative abundance determines the balance of male and female organs/identities (Liu and Mara, 2010; Smaczniak *et al*., 2012).

Taken together, these data suggest that the analysis of *SUP* functions should consider both early and late stages of reproductive organ development in an attempt to reveal possible interactions between as yet unlinked processes (FM patterning and termination, and sexual organ identity and differentiation).

To help clarify some of these questions, we (1) made a comparative genetic, morphological, cellular and molecular analysis of the chosen *sup* alleles and (2) dissected the combined early and late effects of *SUP* mutations in a spatial–temporal manner. To do so, we performed crosses between allelic classes, between *sup* alleles and mutations in genes affecting reproductive organ identity and flower determinacy, as well as crosses to reporter genes and *in situ* hybridization with some of these genes.

We show that *SUP* contributes to an integrative process of meristematic capacity transference in which FMC, carpel and placenta formation offer new opportunities to question theories on the origin of bisexuality in angiosperms (Frohlich and Parker, 2000; Theissen *et al*., 2002; Baum and Hileman, 2006) and the making of the robust modern eudicot flower through developmental homeostasis.

MATERIALS AND METHODS

Plant growth, crosses and line isolation

Mutants lines were obtained from the Nottingham Arabidopsis Stock Center (SIGnAL T-DNA-Express, http://www.arabidopsis.org), the INRA deVersailles stock (http://dbsgap.versailles.inra.fr) and the Meyerowitz lab (California Institute of Technology, CA, USA), unless stated otherwise (see Table 1). The reporter lines *pAP3::GUS*, *pAGi::GUS* and *pWUS::GUS* were provided by the Meyerowitz lab. Plants were grown as reported in Prunet *et al*. (2008) and Ito *et al*. (2003).

Desired mutant combinations were identified among phenotypic categories in the F_2 segregants. Genotypes were confirmed by monitoring Mendelian ratios, by progeny testing, PCR analysis and sequencing. Observed averages were compared using the *t*-test.

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TABLE 1. *Contrasting SUP allelic series used in this study with ecotype, mutation position, protein domain/motif affected and source of material and information*

Allele	Ecotype	Associated mutation	SUP putative protein	Reference
Class 1, *superman*				
sup-1 (EMS)	L*er*	Nonsense mutation at amino acid 22*	Possibly an N-terminal 22 amino acid truncated protein	Sakai *et al.* (1995); this study
sup-3 (EMS)	Col (NASC; CS6227)	Missense mutation at amino acid 63 (Gly > Asp; GGT > GAT)	Altered protein in conserved QALGGH ZnF motif†	Bowman *et al.* (1992); Sakai *et al.* (1995)
sup-6	Ws	Missense mutation at amino acid 108 (Pro > His; CCT > CAT)	Altered protein in Ser/Pro-rich domain	This study
Class 2, *superwoman* (including *clark-ken*t and *fon, flower organ number*, mutants) (Huang and Ma, 1997; Jacobsen and Meyerowitz, 1997)				
sup-eA31	Ler	*SUP* methylation (Southern blot *Bgl*II digestion and sequencing the region 31–354, cf. Jacobsen and Meyerowitz, 1997)	No significant reduction in transcript level observed‡ (protein quantity or quality?)	Ac/Ds line, Kidner C, CSHL. This study§
Class 3, *supersex*				
sup-5 (EMS)	Ler	Deletion starting before the ATG, from –46 to + 568 (614 nucleotides)	A truncated transcript encompassing the 5'-UTR + 88 nucleotide intronic + 47 nucleotides of coiled-coil/leucine zipper (456 nucleotides). Not in frame	Gaiser *et al.* (1995); Jacobsen and Meyerowitz (1997)

Three distinct phenotypic classes and the corresponding genetic alterations are presented within the context of already published alleles. *SUP* alleles are in three distinct ecotypes, the results indicating no major ecotype effects (i.e. SUP modifiers). The structure of the gene is sketched below, with the ORF (blue box), intron (grey box), *Bgl*II restriction site and the relative position of the mutations in the gene with the corresponding SUP protein domains indicated.

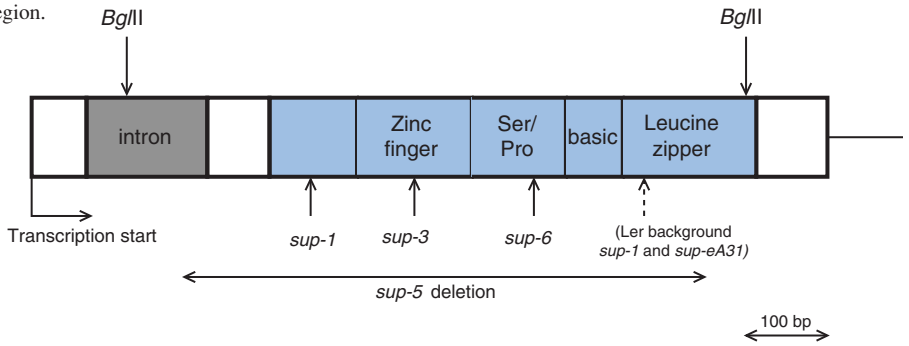
**sup-4* (Col) and *sup-2* (Col) have stop codons at the same position in the gene (Sakai *et al.*, 1995). A missense variant mutation at amino acid 156 (Val > Met; GTG > ATG) in the coiled-coil region has been registered in the L*er* background, including *sup-1* and *sup-eA31*. This amino acid substitution does not seem to affect *SUP* function.

†The mutation abolishes *SUP* DNA binding activity (Dathan *et al.*, 2002);

‡Reduced amounts of *SUP* transcript are reported in *SUP* epi-alleles (*clark-kent* type; Jacobsen and Meyerowitz, 1997).

§The bisulfite sequencing was targeted to the region surrounding the 5' *Bgl*II site (323 nucleotides, including the CT-rich region). Methylation systematically targeted CHH positions. The methylation rate was on average 10 % higher in mutant compared with wild-type samples. In the mutant, nine cytosines were exclusively methylated (including the *Bgl*II site; 15–60 % range) and six motifs with up to 3-fold higher methylation rates were registered in the mutant. Four of the motifs contained some of the exclusively methylated cytosines.

Mutant from root transformation regenerants; Ds is not linked to the mutation. *Bgl*II restriction fragment of 805 nucleotides spanning the intron to the 3'-end region.



PCR, RT-PCR and sequencing of amplification products

RNA isolation and amplification conditions were as described in Breuil *et al.* (2004) and Prunet *et al.* (2008). The following primers were used: *SUP* (SUP4, TTGTGAACT TCGAATCCTTTATGG and SUP9, GATATATCTTAGATT TTTCCCAGGGG), *AP3* (Jack *et al.*, 1992), *CLV1* (Clark *et al.*, 1993), CRC and SPT (Alvarez and Smyth, 1999), *CUC2* (Kamiuchi *et al.*, 2014), *KAPP* (Williams *et al.*, 1997), *KNU* (Payne *et al.*, 2004), *PAN* (Das *et al.*, 2009) and *WUS* (Lohmann *et al.*, 2001).

Sequencing was performed on an ABI PRISM® 3100 Genetic Analyzer sequencer (Perkin Elmer, USA), according to the manufacturer's instructions. Computer-based sequence analysis was performed using the VectorNTI® Suite (Informax, USA) and Sequencher 4.0 (Gene Codes Corporation, Ann Harbor, MI, USA) software. Multiple alignments were obtained using ClustalX and edited with SeqVu (The Garvan Institute of Medical Research, Australia).

Bisulfite sequencing

Eight *sup-eA31* plants with checked phenotype and six wild-type plants were analysed. DNA extraction and quantification were performed with the DNeasy plant mini kit Qiagen 69106 and DropSENSE 96 TRINEAN, respectively. A 500 ng aliquot of DNA was treated with bisulfite according to the 'Cells-to-CpG' Applied Biosystems 4445555 instruction kit, with the following time and temperature conditions: 2 min at 95 °C, nine series of 2 h at 75 °C and 2 min at 95 °C. The reaction products were purified according to the Cells-to-CpG protocol. The

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PCRs were performed on the purified DNA with TAKARA RR006A Extaq with the following designed oligos: SP Sup1 Bis2, GCGTTTAAGAATTAGTTTTTTTTATAGTTTTAAT; and ASP Sup1 Bis2, CAAAAACTACAAATATAAAA TCTT AATAACCATAAAAACC. The PCR products were purified and sequenced with the ASP Sup1 Bis2 oligo by GATC company.

The methylation profiles were determined with the BISMA program (http://services.ibc.uni-stuttgart.de/BDPC/BISMA/).

SUP antibodies

Antigenic specific motifs, eventually discriminating between alleles, were searched including two of the closest *SUP* homologues (At2g37740 and At4g17810). Prediction of the secondary structure was based on DSC, PHD and SOPM methods, and physico-chemical profiling (antigenicity, solvent accessibility and degree of hydrophilicity) was performed with Clustal WProtein.

Four synthetic antigenic peptides were designed (Prosite data and procedures according to http://npsa-pbil.ibcp.fr). Two were common to the three *SUP* genes (amino acids 191–204 and 80–100) and two were SUP-specific, one in the ZnFinger motif (amino acids 18–31; RTSPWSYGDYDNCQ) and the second in the LeuZipper domain (amino acids 136–153; XSPKSKH TPENACKTKKSSX). Antisera were raised in rabbits and mice by ChangBioscience, Eurogentec and/or Covalab).

None of the tested antibodies, including the commercial brand (Santa Cruz Biotech), worked with plant extracts, despite consistent reactions in bacterial samples.

Inflorescence (cut flower) culture and bromodeoxyuridine (BrdU) incorporation and immunohistochemical detection were as reported in Breuil-Broyer *et al*. (2004).

In situ *hybridization and GUS staining*

Tissue preparation was as described by de Almeida-Engler *et al*. (2009). Tissue sections were 10 µm thick. Probes for *in situ* hybridization were transcribed using the digoxigenin labelling mix (Boehringer, Germany) as described in Prunet *et al*. (2008). β-Glucuronidase (GUS) activity was assayed according to Jefferson and Wilson (1991).

Microscopy

Plants were observed with a Leica MZFLIII stereomicroscope coupled to a DC300F digital camera (Leica Microsystems, Germany). For morphological analyses, imaging of reproductive structures was systematically performed to ensure homogenous and repeated analysis capacity. Images were processed with the FW4000 software (Leica). *In situ* hybridization signals were visualized using a Nikon epifluorescence microscope (Optiphot-2, Nikon, Japan) coupled with an AxioCam MRc digital camera (Carl Zeiss, Germany). Images were processed with the AxioVision software (Zeiss). All figures were composed with Adobe Photoshop 7.0 (Adobe Systems, USA), and certain pictures of flowers were treated to remove unrelated background.

RESULTS

Most of what is known about *SUP* has been determined through a systematic analysis of one allele, *sup-1* (Bowman *et al*., 1992; Sakai *et al*., 1995, 2000; Ito *et al*., 2003). Therefore, one might miss some key aspects of the gene's functions because a detailed comparative analysis of allelic types has not been performed. For example, there are *sup-2* and *sup-3* alleles (with flowers similar to *sup-1*, i.e. excess of stamens and almost no female organs). *sup* (epi)alleles and *sup-5* showing an excess of carpels have been reported (Gaiser *et al*., 1995; Huang and Ma, 1997; Jacobsen and Meyerowitz, 1997). Table 1 gives an inventory of chosen alleles by classifying them into three groups: *superman*, *superwoman* and *supersex*. To generate a meaningful comparison of the chosen alleles, we performed one large and comprehensive series of crosses of *sup* alleles to mutants altered in reproductive organ identity and flower determinacy (Tables 2 and 3). Therefore, some of these crosses are repetitions of past results, including *ag*, *clv*, *pan* or class B gene overexpressors.

So far, it is not clear, mainly due to the absence of effective SUP antibodies (either commercial or home-designed; see the Materials and Methods), whether the effects of the various alleles within the series are due to variant SUP proteins and/or to the levels of their production and/or degradation. Furthermore, the formation of numerous and variable stamen/carpel chimeric structures raises questions about their origins and precise natures. The dominant idea is that stamen–carpel mosaics in *sup-1* 'result from congenital fusion of carpel primordia and innermost presumptive stamen primordia' (Bowman *et al*., 1992; for a contrasting view, see Ronse De Craene *et al*., 2011).

sup *alleles define three distinct mutant classes with partial indeterminacy*

By comparing the alleles at the transcriptional and morphological levels (i.e. the levels of indeterminacy and sexual boundary effects, and typology and nature of chimeric organs), three contrasting classes have been defined. Table 1 summarizes the position and nature of the chosen mutations. Tables 2 and 3 present the genetic and morphological analyses of five *sup* alleles, alone and in various crosses.

All sup *alleles produce* SUP *transcripts*. The generated cDNA samples were visualized by RT-PCR, cloned and sequenced. *SUP* cDNA fragments from the wild type and all alleles, except for *sup*-5, migrate as two different size products of 700 and 800 nucleotides, respectively (Fig. 1; also see Ito *et al*., 2003). The largest transcript containing the intronic sequence is the most abundant form, indicating facultative splicing and a putative role for *SUP* mRNA in the global *SUP* functions. Translation of *SUP* sequences (tBlastN in TAIR and CBI) identified a putative product (in direct 2 frame) (IYLRFFPGEKSEFI FSDLTNTLLLTDVPQIITSTILIIFTIFLSSIAKKKELAH) in the 5'-untranslated region (UTR) that had no hits with TAIR data resources. PCR amplification of *sup-5* produced a 289 nucleotide truncated transcript in low amounts (<10 % of wild-type levels). We checked whether the *sup-5* transcript sequence and stretches with 50 bp overlaps (Table 1; Supplementary

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TABLE 2. *Morphological analysis of single and double mutants assessing male, female and chimeric traits, and their proportions*

Allele and crosses	No. of flowers (% male flowers)	Stamens/ flower (s.d.)	Free stamens (%)	Fused stamens (%)	Pistillody (%) w/m/s	Carpels/ flower (s.d.)*	Genetic interactions, observations†
WT	50 (0)	5·7 (0·7)	100	0	0	2 (0)	
sup-1	39 (84)	13·2 (1·6)	85	4	8 w	0·1	33 % perfect flowers
sup-3	32 (94)	11·9 (2·1)	85	4·5	6·7 w	0·1	22 % perfect flowers
sup-6	30 (96)	10·4 (1·9)	81	3·5	14 w	0	33 % perfect flowers
sup-5	31 (0)	11·5 (2·1)	63	40	8 m, s, w	3·2 (0·8)	Phenocopy 35S::KAPP
sup-eA31	33 (0)	7·5 (1)	71	5	9 m, s, w	2·9 (1)	Phenocopy *clv1-6*
ap3-3	25 (0)	2·2 (1·6)	0	54	100	3 (0·9)	(anti-*sup-1*)
35S::AP3 ap3-3‡	31 (87)	11·7 (1·4)	91	9·5	18 w, m	0·6	6 % perfect flowers, phenocopy *sup-1*
clv1-6	25 (0)	6·1 (0·3)	100	0	0	2·6 (0·7)	
35S::KAPP§	42 (0)	13·6 (1·7)	71	17	11 m, s, w	3·4 (1·5)	Phenocopy *sup-5 sup-1*; *clv1-6 sup-5*
clv1-6 sup-1	38 (0)	16·4 (0·4)	80	17	3 w (m, s)	1·6 (1·2)	Synergistic St, additive Ca; enhanced pistillody
clv1-6 sup-5	31 (0)	14·4 (2)	68	34	5 m, s	4·6 (1·3)	Synergistic
sup-5 sup-1	48 (0)	13·6 (2)	80	12	2 m, s	2·8 (1)	Additive
sup-5 sup-3	57 (0)	12·6 (1·4)	80	17	6 m, s	1·6 (1·2)	Weak synerg· St/additive Ca; dose effect??
sup-5 sup-6	34 (0)	10 (1·5)	81	11	15 w, m	0·6 (0·7)	Weak epistatic (to weak additive Ca?)
eA31 sup-5	56 (0)	7·8 (1·8)	85	9	10 w,m	2·8 (0·9)	eA31 epistatic
eA31 sup-1	59 (0)	7·8 (2·5)	86	8	3 w, m, s	2·1 (0·6)	eA31 partly epistatic
eA31 sup-3	43 (0)	6·3 (1)	91	2·5	2 w	2·5 (0·8)	'Near wild-type'
eA31 sup-6	38 (0)	6 (0·3)	82	0	0	2 (0·2)	'Wild type'

Corresponding phenocopy candidates are evaluated· The general trend is a partial, limited degree of indeterminacy. Counts were performed on flowers 3–10 of the main inflorescence. w, weak; m, medium; s, strong; s.d., standard deviation.

All flowers were counted for flower organ numbers. Sepals and petals were removed and each flower was photographed to record the organ phenotypes at the FMC only.

*Female fertility: 0·1–0·5 % of flowers form single carpels in *sup-1; sup-3* – 1 silique/500 flowers; 20–30 % of misshapen carpels in *sup-5* produce low seed set; 70 % of carpels are normal in *epi-supA31*, average seed set.

†*t*-test performed between the wild type and mutants, single or double, and in pair-wise comparisons of mutants. No statistically significant differences were registered in the categories corresponding to epistatic, 'near wild-type' and 'wild-type' genetic interactions (alpha risk of 0·05). Ca, carpel; St, stamen.

‡Ectopic expression of *AP3* results in the persistent expression in whorl 4 of the endogenous *PI* gene (Jack *et al*., 1994). Similar figures where produced in *p35S::AP3*/*PI* flowers (not shown).

§The published *35S::KAPP* line caused phenotypes similar to weak loss-of-function *clv1* alleles (cf. Williams *et al*., 1997).

TABLE 3. *Analysis of genetic interactions between* SUP *with* CRC, SPT, PAN *and* KNU *as evaluated at the flower meristem centre (FMC)*

Allele	No. of flowers	Stamens/ flower	Free St (%)	Fused St (%)	Pistillody (%) w/m/s	Carpels/ flower	Observations
Wild-type*	50	5·7 (0·7)	100	0	0	2 (0)	
sup-1	(E) 42	15 (2·4)	91	2	6·6 w	0·2 (0·5)	15 total ReprOrg
	(L) 58	10·8 (1)	87	2·7	7·6 w	0·1	
sup-5	21	13·7 (3·2)	60	40	11 s, m, w 2:1:1	2·9 (1)	16·7 total ReprOrg
knu sup-5†	28	17 (2·4)	89	20	9·5 s, m, w	3·6 (1·2)	Internal mass prolif.; carpel reiterations
pan-3 sup-1‡	31	10·3 (1·7)	80 (95)	3·7	16 leafy$_{wm}$	2 (1·2)	Ca restoration; wh5 structures
pan-3 sup-5	(E) 25	7·3 (1·4)	84 (94)	2	10 leafy	3·5 (1·4)	Ca restoration; wh5 carpel reiter.; most carpels WT
	(L) 20	8·9 (1·6)	80	16	0	2·1 (0·8)	
crc-1 knu§	25	5·8 (0·6)	100	0	0	2·6 (0·7)	Fifth whorl, reiterated stamens and carpels
crc-1 sup-1	49	14·7 (1·6)	90	3·3	7·4 leafy^{var}	0·04	*sup-1* epistatic; leafy pistillody, sterile
crc-1 sup-5¶	53	10·6 (1·5)	55	43	4 leafy	0·8 (0·5)	Attenuation of *sup-5* strength, similar to *sup-3*/*6*; sterile
spt-2 sup-1	(E) 30	11·2 (1·6)	88	2·1	5·3 leafy	0	*sup-1* epistatic; Sterile
	(L) 36	10·6 (1·2)	87	2·6	8·5 leafy	0·06	
spt-2 sup-5	(E) 31	10·3 (0·8)	76	22	11 leafy^{spt}	0·9 (0·9)	Attenuation of *sup-5* strength, similar to *sup-3*/*6*;
	(L) 25	8·5 (0·6)	74	27	15 leafy$_{msw}$	1	pistil apically unfused, fewer ovules, sterile

Early and late flower phenotypes were observed in certain mutant combinations. Parameters as in Table 2.

Ca, carpel; E, first ten flowers; L, late flowers. Pistilody levels, w, weak; m, medium; s, strong; Prolif, proliferation; ReprOrg, reproductive organs; St, stamen; wh, whorl.

**spt*, *crc*, *knu*: same flower formula as the wild type; in our conditions in *knu*, 11 % of siliques were tri-carpelloid, 86 % of pistils contained ectopic stamens and 53 % produced knuckles (carpel-like structures) that were derived from placental tissues (Payne *et al*., 2004).

†*knu sup-1* – strong indeterminate male flower (Fig. 7A, B).

‡*pan-3*: average of Se5 P5 St5; no changes in carpel number and morphology (Running and Meyerowitz, 1996).

§Gynophore is present on most primary carpels; 64 % of outbreaking structures in whorl 5 show elongated internodes with reiterated stamens and carpels.

¶Extensive organ fusions occurred.

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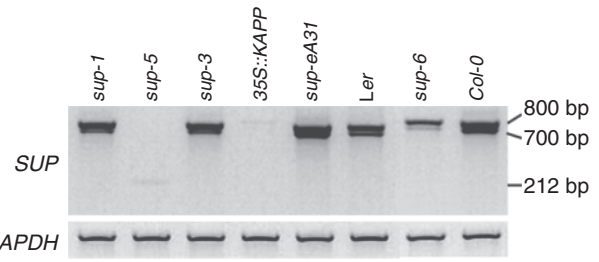

FIG. 1. Transcript analysis in the *sup* allelic series. RT-PCR products with primers SUP 9 and the 3'-UTR; the amplification products have been sequenced and correspond to spliced (lower band) and non-spliced (upper band) transcripts in all alleles, except *sup-5*. The latter produces a single transcript as a result of a large deletion in the gene (Table 1). In quantitative terms, similar levels of transcripts are generated, except for *sup-5*, with strongly reduced amounts of cDNA. In the transgenic line *35S::KAPP*, essentially no *SUP* transcript is observed.

Data Information S1) could generate (cryptic) stem–loop precursors or mature microRNAs, but no such evidence was found when blasting on TAIR (http://ensembl.gramene.org/Arabidopsis_thaliana).

Contrasting morphologies in sup *allelic classes* superman, superwoman *and* supersex *indicate distinct mechanisms of FM termination*. The results of morphological analysis are summarized in Table 2. They focus on changes of reproductive organ number and morphology. *sup* heterozygotes for the various alleles in the wild-type background were scored phenotypically as wild type, indicating no co-suppression and negative dominant effects or haploinsufficiency. These results do not entirely agree with those of Jacobsen and Meyerowitz (1997) reporting semi-dominance in *clk-3* and *sup-5* heterozygotes, but are in conformity with *sup-5* data from Gaiser *et al.* (1995) and Huang and Ma (1997).

Class 1, **sup-1, sup-3, sup-6** *(***superman***)*. These alleles produce partially indeterminate (imperfect) male flowers (on average, a doubling of stamen number as compared with the wild type) (Table 2). Extranumerary stamens form 1–2 additional subwhorls (i.e. stamens are not formed simultaneously in the mutants). In the first (sub-)whorl, 7–8 stamens are produced instead of six, corresponding to an increase in lateral stamen number. Depending on the allele, 84–94 % of class 1 *sup* flowers have stamens or slightly aberrant stamens filling up the entire FMC space (i.e. are male flowers, Fig. 2A–H; in B, the very centre space remains unspecified), the rest producing carpelloid structures at their centre (see below). Overall, the fertility of these mutants is very low (0·1–0·2 % of the flowers set seeds).

Phenocopy of class 1 phenotypes. The *p35S::AP3 ap3* genotype (Table 2; also compare with *ap3* and with flowers in *p35S::AP3/PI* plants (Krizek and Meyerowitz, 1996; Yang *et al*., 2003) has a similar phenotype, including pistillody in stamens produced at the FMC (see below; Bowman, 1994). The same phenotype has been reported in *pAP3::AG ag-3* (Jack *et al*., 1997).

Taken together, results with class 1 support the SUP 'boundary model': SUP prevents the spread of *AP3* expression into whorl 4 (Sakai *et al*., 2000; Yun *et al*., 2002).

In summary, class 1 alleles have a common trend: an indistinct male–female boundary (carpel formation is occasional), suggesting that indeterminacy is linked to the systematic expanding of class B gene function at the FMC, i.e. FM termination is delayed (see also Schultz *et al*., 1991, Bowman *et al*., 1992, Breuil-Broyer *et al*., 2004). The main differences concern the relative strength of indeterminacy, with *sup-1* > *sup-3* > *sup-6*, and indicate a quantitative effect on determinacy. The above suggests that the putative defective SUP proteins produced in *sup-3* and *sup-6* (Table 1) have some biological activity. Amino acid substitutions in *sup-3* and *sup-6* and their similar phenotypes indicate that SUP ZnFinger-like and Serine/Proline-rich domains are involved in sexual boundary function (Table 1). We conclude that the boundary function requires an intact SUP protein.

Class 2, **sup-epiA31** *(***sup-eA31***;* **superwoman***; Table 1)*. This class is phenotypically similar to the *clark-kent* epiallele type and exhibits an altered *SUP* genomic DNA methylation profile (Table 1; Supplementary Data Information S1). All flowers are bisexual, with stamens on a single whorl (average of 7·5) and whorl 4 indeterminate (approx. 3 carpels per flower) (Table 2; similar figures were reported in the *fon1* and *clk* series of mutants, Huang and Ma, 1997; Jacobsen and Meyerowitz, 1997). Seventy per cent of the carpels were wild type, and thus partly phenocopy weak *clavata* alleles (Diévart *et al*., 2003). However, carpels were frequently open, and fusion of stamens and carpels has been observed (Table 2; Fig. 2I–K).

This phenotype indicated that the sexual boundary was only slightly affected as compared with class 1 alleles, with flower indeterminacy resulting in increased carpel number. Such a phenotype could result from a reduction by > 50 % of *SUP* expression (remember, there was no phenotype in plants heterozygous for *SUP*) and/or from an altered protein. *sup-eA31* is the only allele to produce an increased level of indeterminacy in whorl 4 mainly, i.e. after sexual boundary setting and during carpel initiation.

Class 3, **sup-5** *(***supersex***)*. All flowers in class 3 are bisexual and exhibit whorl 3 and 4 indeterminacy, i.e. more stamens (average of 12·5) and carpels (average of 3) than in the wild type (Table 2). Similar figures for *sup-5* were reported in Jacobsen and Meyerowitz (1997). The majority of carpels were short, misshapen and exhibited various alterations such as club-shaped pistils. Fifty per cent of the carpels were apically open, suggesting medial region defects with frequent callus-like proliferation masses along an uneven and split replum (Fig. 2L, Q).

sup-5 produces a truncated transcript and is a strong allele: the sexual boundary function was delayed and diminished, there being a pronounced carpel number increase.

Phenocopy of *sup-5*. The *sup-5* phenotype appears to combine most of the class 1 and class 2 traits (stamen number increase, i.e. extended class B + C gene domain, but also carpel number increase in whorl 4, i.e. an extended class C gene domain). *sup-5* is largely phenocopied by *35S::KAPP* (Table 2; Fig. 5B–D).

KAPP is a 2C type kinase-associated protein phosphatase capable of modulating CLV1–CLV3 complex endocytosis

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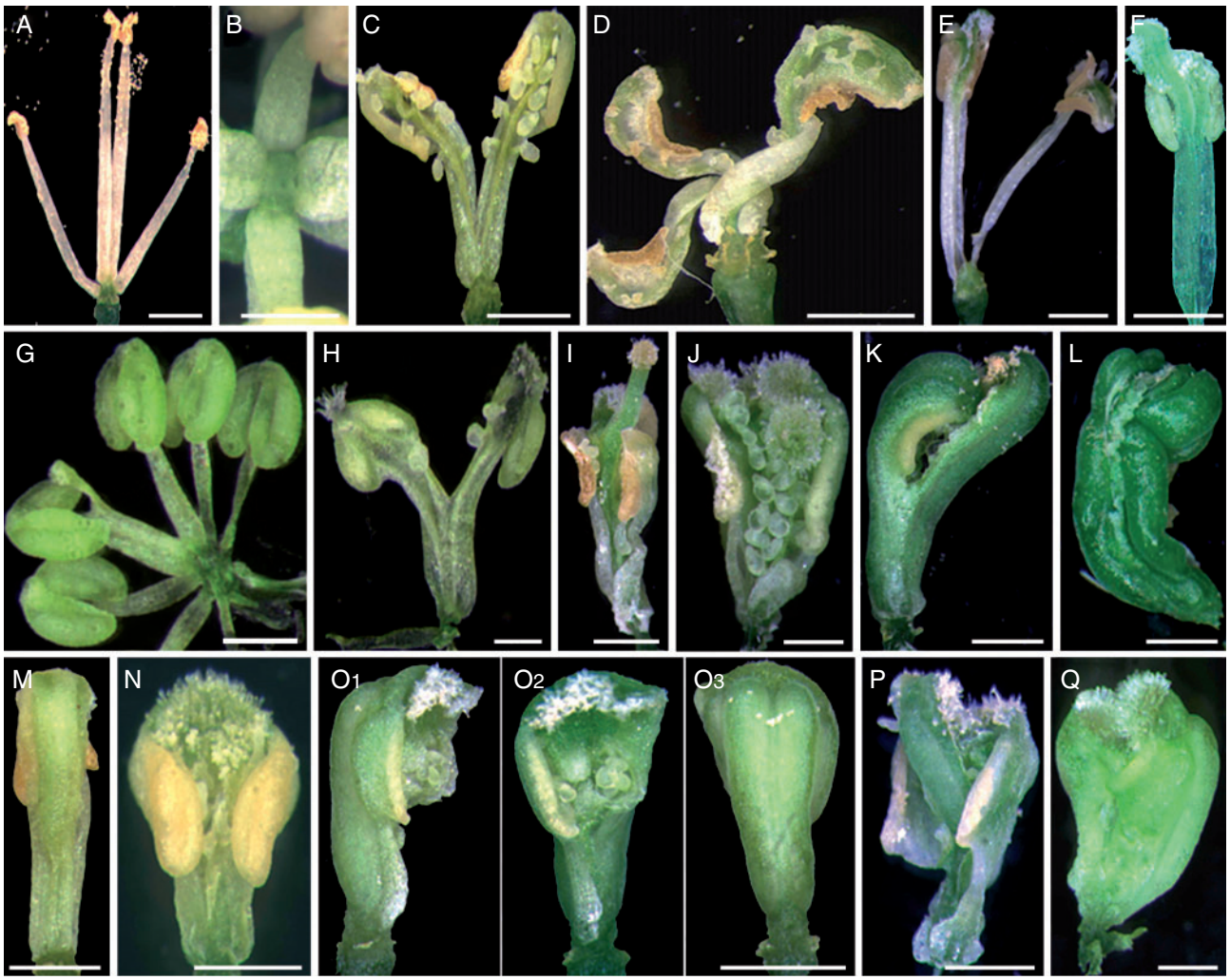

FIG. 2. Morphological analysis of *SUP* alleles – mutations of *SUP* produce a limited degree of indeterminacy. The alleles are: *sup-1* (A–D), *sup-3* (E, F), *sup-6* (G, H), *sup-eA31* (I–K) and *sup-5* (L–Q). Only structures at the FMC are shown, with most of the stamens removed. Stamen abscission zones and nectaries can be seen (A, D, H, K, M, O). The centre-most region of the FMC can produce normal stamens, i.e. perfect male flowers (A), remain free, i.e. residual apex (B), or exhibit feminized stamens (see below). Only centre-most stamens feminize (i.e. pistillody, *sensu* Ronse De Craene *et al*., 2011; see text). The weak to strong feminization series is illustrated: the identity of distinct stamen parts can be traced to colour codes, i.e. silverfish for filaments (D, F, G, H, O1), greenish for connective, brown-yellowish for pollen sacs (D, F, I–K, M-Q). Thecae (pollen sacs) are less susceptible to feminization (C, E, G–K, M–Q). Group 1 alleles (*sup-1*, *-3* and *-6*). Weak to medium pistillody is observed. The weakest phenotypes show stamens tending to enlarge and stay greenish, with the apical region of the stamen bulging (G). In weak phenotypes, stamens produce stigmatic-like tissue apically (E, F, H) and ectopic ovules can be seen in various positions (C, D, F, H). The series illustrates the remarkable feminization potential of the connective (C–H) and of the filament that becomes wider and shorter (B–H). Groups 2 and 3 (*sup-eA31* and *sup-5*). The carpel-like structures in (J–L, M–O, Q) have a narrow base and strongly expand apically. Medium to strong pistillody is the rule. The stamen-derived structures contain recognizable pollen sacs and filaments (I, J, M–P). An irregularly shaped pistil with extra valves and proliferating medial ridge in (L). Considering organ polarity, on the adaxial side, expanded connectives produce rows of ovules along the vasculature (C, J), resembling placental arrangements. Sometimes style–stigma structures (E, H, I) are observed. On the abaxial side, connectives and filaments can produce one to several valve-like structures (K, M, O, Q). The (O) series represents the same stamen observed from lateral (O1), adaxial (O2) and abaxial (O3) positions. On (strongly) expanded connectives, pollen sacs are visible as disporangiate (E, H, J, M, N, P) and monosporangiate arrangements (C, D, H, M, O). The latter indicate asymmetric transformations. In (P), the adaxial side of a feminized connective produced a secondary 'carpel'. Scale bars = 500 μm.

through dephosphorylation (Trotochaud *et al*., 1999). Plants overexpressing *35S:KAPP* showed stronger indeterminacy compared with *sup-5* (for both stamen and carpel numbers; Table 2), while chimeric organs and organ fusions were similar in *sup-5* and *35S:KAPP* flowers. Such phenotypes are reminiscent of strong *clv* alleles (Dievart *et al*., 2003). This suggests that this genotype opposes, or at least dampens, CLV signalling (Williams *et al*., 1997).

We subsequently investigated *SUP* expression in these ectopic *KAPP* plants and show that *SUP* transcription was abolished in the corresponding *35S:KAPP* flowers (Fig. 1). *SUP* appears therefore as one of multiple and redundant regulatory networks of stem cell signalling and homeostasis operating in the FM (Tichtinsky *et al*., 2003) that require *KAPP* function. This is supported by the phenotypic indeterminacy strength scale *35S:KAPP* > *sup-5* ≥ *sup-1* > *clv1-6* (Table 2),

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cautiously acknowledging the limits of ectopic gene expression data interpretation.

In summary, the *sup-5* data support the 'cell proliferation control' model in which *SUP* function consists of controlling the balance of cell proliferation and differentiation at the FMC (whorl 3 vs. whorl 4) (Sakai *et al*., 2000).

In conclusion, in the analysed allelic series, the allelic strength of indeterminacy is *sup-1*/*sup-5* > *sup-3* > *sup-6* > *sup-eA31*. The large deletion of *sup-5* and the N-terminal truncation in *sup-1* produce the strongest phenotypes. Of note, *sup-1* produced significant transcript levels, suggesting that *sup-1* transcripts with an early stop codon are not degraded as often occurs by nonsense-mediated decay, and that *sup-1* translation might be re-initiated from a downstream methionine. This suggests that *sup-1* may not be null. In addition, *sup-5* and *sup-eA31* show that in the corresponding mutants the boundary activity can be (partly) dissociated from other *SUP* functions.

Crosses between allelic classes inform on structural and functional SUP *features*

Heteroallelic combinations sup[a]/sup[b] were generated, and the results of the morphological analysis are presented in Table 2 and Supplementary Data Fig. S2. The results indicated that *sup-5* truncated transcripts do not operate through a co-suppression mechanism or through a dominant-negative effect in diallelic contexts.

Crosses between class 1 and class 3 alleles showed a range of situations, from additivity to epistasis, confirming the allelic force series for class 1: *sup-1* > *sup-3* > *sup-6*. The following characteristics of diallelic combinations have been observed:

1. *sup-5 sup-1* showed additive effects with, as a result, the highest indeterminacy levels in the *sup* context (and phenotypically the closest to the transgenic *35S::KAPP* line). Comparing total stamen number in *sup-1* and *sup-5*, *sup-1* produced more stamens (+2), but the overall number of reproductive organs in the two alleles was rather similar in terms of number of primordia formed at the FMC (Tables 2 and 3), suggesting that the two alleles have an equivalent strength of overall indeterminacy. However, based on their contrasting phenotypes and corresponding DNA alterations, *sup-1* and *sup-5* are candidates for alleles lacking a functional SUP protein (Table 1). Therefore, *sup-5 sup-1* could represent the equivalent of a null 'allele'. If this is the case, we cannot exclude the fact that the altered *SUP* transcripts in *sup-5* could operate through effects at the RNA level. Finally, the heteroallelic heterozygote phenotype suggests that boundary formation and FM termination represent variations on the same theme. This remains to be further explored.
2. *sup-5 sup-3* showed weak synergistic effects on stamen number (whorl 3) at the expense of whorl 4 carpel number, suggesting sexual boundary sliding towards the FMC (expansion of B activity, delay in B exclusion) as compared with *sup-5* alone. One likely hypothesis is that one dose of a putatively altered SUP-3 protein in the ZnFinger motif could compensate for some of the whorl 4 defects in a *sup-5* context and leave more room for stamen production.
3. *sup-5 sup-6* showed partial *sup-6* epistasis, indicating that one dose of the putatively altered SUP-6 protein in the Ser/Pro-rich domain could compensate for most *sup-5* defects in whorl 4. Interestingly, siliques in the double mutant were frequently helicoidal, indicating substantial asymmetric valve growth (Supplementary Data Fig. S2E, F) and a role which *SUP* could have in the formation of the carpel medial zone. In comparison with *sup-5 sup-3*, the results indicate that a functional ZnFinger motif is determinant for a functional SUP.
4. *e-supA31* (class 2) showed (partial) epistasis over *sup-5* (class 3) and *sup-1* (class 1) in the corresponding double allelic combinations, and almost full complementation to the wild type when crossed into class 1 *sup-3* and *sup-6* alleles (Table 2; Supplementary Data Fig. S2C, D, G). The latter cases suggest that, in contrast to the monoallelic contexts, these diallelic combinations produced sufficient amounts of functional SUP protein to complement most of the specific allelic defects. Although we cannot exclude enhanced instability of the epi-allele in inter-ecotype crosses (reduction of methylation levels), we interpret this as suggesting that functional SUP acts as a dimer. The corresponding diallelic (hetero)dimers with *sup-3* and *sup-6* are likely to produce distinct levels of inter-molecular complementation.

In the light of the above considerations, we focused the analysis on *sup-1* and *sup-5*.

Early SUP functions – linking sexual boundary and FM processes

To investigate the dual role *SUP* plays in sexual boundary formation and FM termination, crosses to a series of reporters (including *AP3*, *AG* and *WUS* reporters), to a *clv* null allele and to several other regulators of FM patterning and termination were performed. In addition, *WUS* and a general boundary marker, *CUC2*, were analysed by *in situ* hybridization, and BrdU labelling patterns were observed in parallel.

(i) Linking sexual boundary and FM termination

AP3 *is expressed at the FMC in* sup *mutants*. Exclusion of class B genes from whorl 4 by *SUP* is necessary for floral determinacy; their ectopic expression at the FMC somehow opposes FM termination. *AP3* expression is altered in *sup-1* (Bowman *et al*., 1992). We wanted to compare *sup-1* and *sup-5* by observing *pAP3:GUS* and *pAGi::GUS* expression in the two alleles. *pAP3::GUS* early patterns showed that the gene was not expressed in whorl 4 in the wild type, but was systematically present at the FMC in *sup-1* and *sup-5* at flower developmental stages 4–6(7) (Fig. 3A–F). In comparison, the *pAGi::GUS* pattern was unchanged in *sup* alleles at these early stages (not shown), suggesting that *AG* expression *per se* was not altered in *sup* flowers as compared with the wild type (also shown by *in situ* hybridization; Sakai *et al*., 1995). This means that altered levels of class B vs. class C genes are present at the FMC at stages at which the acquisition of whorl identity and organ initiation occur. The data are in agreement with the morphology of class 1 alleles, which clearly indicated that most flowers produced stamens at their FMC. Therefore, this pattern of class B

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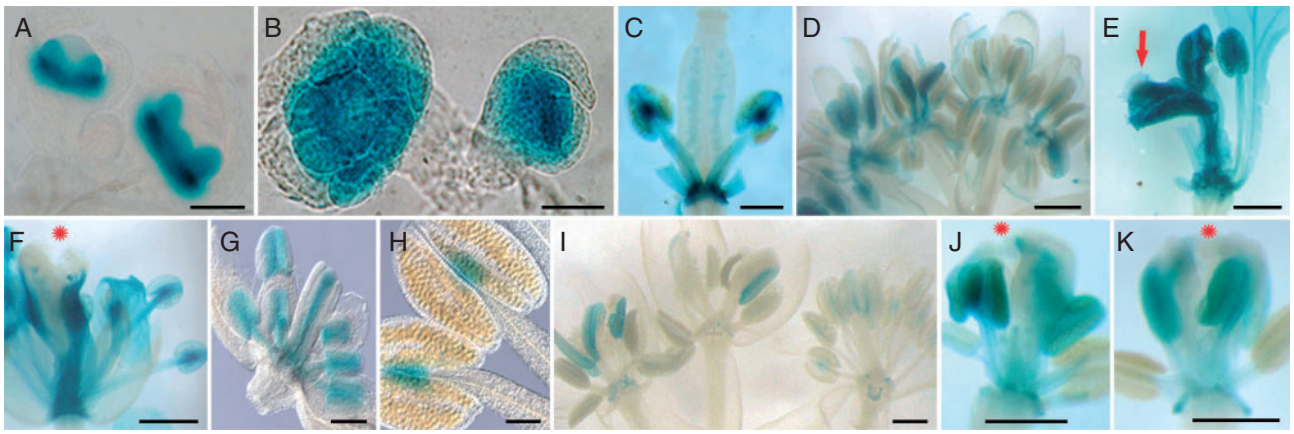

FIG. 3. Class B and C gene-driven GUS reporter expression analysis in *sup* alleles. *pAP3::GUS* patterns in early stages of the wild type (A; stages 4–5/6) and *sup-5* (B; stages 6–8), and late stages in the wild type (C; stage 12), *sup-1* (D, E; stages 10–12/13) and *sup-5* (F; stage above 12). Note that whorl 4 is not stained in the wild type. *pAGi::GUS* pattern in the wild type (G, H; stages 8/9 and 12, respectively), *sup-1* (I; stages 10–12) and *sup-5* (J, K; stage 12). Note that patterns differ exclusively in structures developing at the FMC; stamens exterior to the region express a wild-type reporter profile. Connective tissues in the wild type and connective- and filament-derived structures in the mutants are sites with persistently high GUS expression levels. Pistillody ranges from weak to medium. Asymmetric staining is frequently observed (I, K), most carpelloid tissues in adaxial positions (asterisk) showing no reporter expression. The arrow in (E) indicates stigmatic tissues. Scale bars = 50 µm (A, B), 100 µm (H), 200 µm (C, E–G, I–K) and 400 µm (D).

gene expression can fully explain the class 1 phenotype, but not class 2 and class 3 phenotypes.

New redundancy at the FMC: a SUP-dependent mechanism of FM termination. The FM stem cell system depends on two independent pathways, the *CLV–WUS* and the *AG–WUS* pathways (Sun and Ito, 2015): the former spatially restricts and maintains the stem cell population, while the latter controls the temporal arrest of the stem cell activity. Thus the level and timing of *WUS* repression is paramount for FM homeostasis. Since *SUP* is primarily pointing to class B gene activity alterations, it is not clear whether and how the *SUP*–class B gene system is targeting *WUS*. To that end, we have analysed *WUS* expression in the mutants and made crosses of *sup* alleles into mutants of the *CLV* signalling system.

WUS *expression is temporally, but not spatially, altered in* sup-1 *and* sup-5 *mutants*. *WUS* expression patterns inform on spatial (extent of cell proliferation) and temporal (delayed termination) control of the FM. To evaluate the situation in *sup*, *in situ* hybridization with *WUS* as probe showed that *WUS* expression is prolonged in the FM to late stage 6 in *sup-1* and *sup-5* as compared with the wild type, where the signal was detected up to stage 5/6 (Fig. 4A–H). We concluded that FM termination is slightly delayed in *sup* mutant flowers due to an altered *WUS* signalling. Prolonged *WUS* expression can explain that extra organs are sequentially produced in *sup* alleles, but it does not allow understanding of the morphological differences between *sup-1* and *sup-5* alleles.

sup *crosses to* clavata1 *and* squint *mutants show strong synergic interactions*. *CLV1* and *SQN* are more general regulators of FM stem cell homeostasis loops (Prunet *et al*., 2008). The morphology of single mutant/transgenic line flowers indicated the following phenotypic strength series: *35S::KAPP* > *sup-5* ≥ *sup-1* > *clv1-6* (Table 2).

sup-1 and *sup-5* crossed into the *clv1-6* null allele resulted in more severe phenotypes than any of the single mutants (Table 2). In these mutants the apical region of carpel or carpelloid structures was strongly expanded, with abundant stigmatic tissues being produced (Fig. 5A–I). However, some important differences need to be acknowledged: the double *clv1-6 sup-1* mutant showed synergistic effects on stamen number (with stamens at the FMC exhibiting stronger levels of pistillody), while carpel number was intermediate (additive) with respect to single mutant phenotypes. In contrast, the morphology of *clv1-6 sup-5* was consistent with synergistic effects for both stamen and carpel numbers. The carpelloid structures had a lamellar shape with few if any medial ridge tissues. In the *sup-5* context, the strength of the observed indeterminacy was *sup-5 clv1-6* > *35S::KAPP* > *sup-1 sup-5* (Table 2), indicating a combined effect of (partly) independent pathways operating through *SUP* and *CLV1* regulatory networks.

We refined the analysis by performing crosses with the *clv3-1* mutant. The resulting double mutant phenotypes were extreme indeterminate flowers (loss of determinacy), the phenotypic strength series being *clv3-1 sup-1* ∼ *clv3-1 sup-5* > *clv3-1 35S::KAPP* (Supplementary Data Fig. S3). The result suggests that *CLV*, *KAPP* and *SUP* overlap in qualitatively distinct manners. Concerning the *AG–SUP* interaction, *sup ag* double mutants show synergistic effects including fasciation at the FMC (i.e. loss of determinacy; Bowman, 1994; Huang and Ma, 1997; Prunet *et al*., 2009; data not shown), pointing to an *AG*-independent mechanism of FM termination control by *SUP*.

That *sup-5* acts in a broader regulatory context than *sup-1* was further substantiated by phenotypes generated in crosses with mutants altered in *SQUINT*. *SQN* is expected to activate *AG* and modulate stem cell termination through the *CLV* pathway (Prunet *et al*., 2008). In *sqn-4 sup-1* and *sqn-4 sup-5*, the mild effects of *sqn-4* on flower morphology were strongly enhanced by *sup* alleles, with *sup-1* impacting on stamen number (Prunet *et al*., 2008) and *sup-5* on carpel number and carpel reiterating axes (extra whorls of carpels; Fig. 5P).

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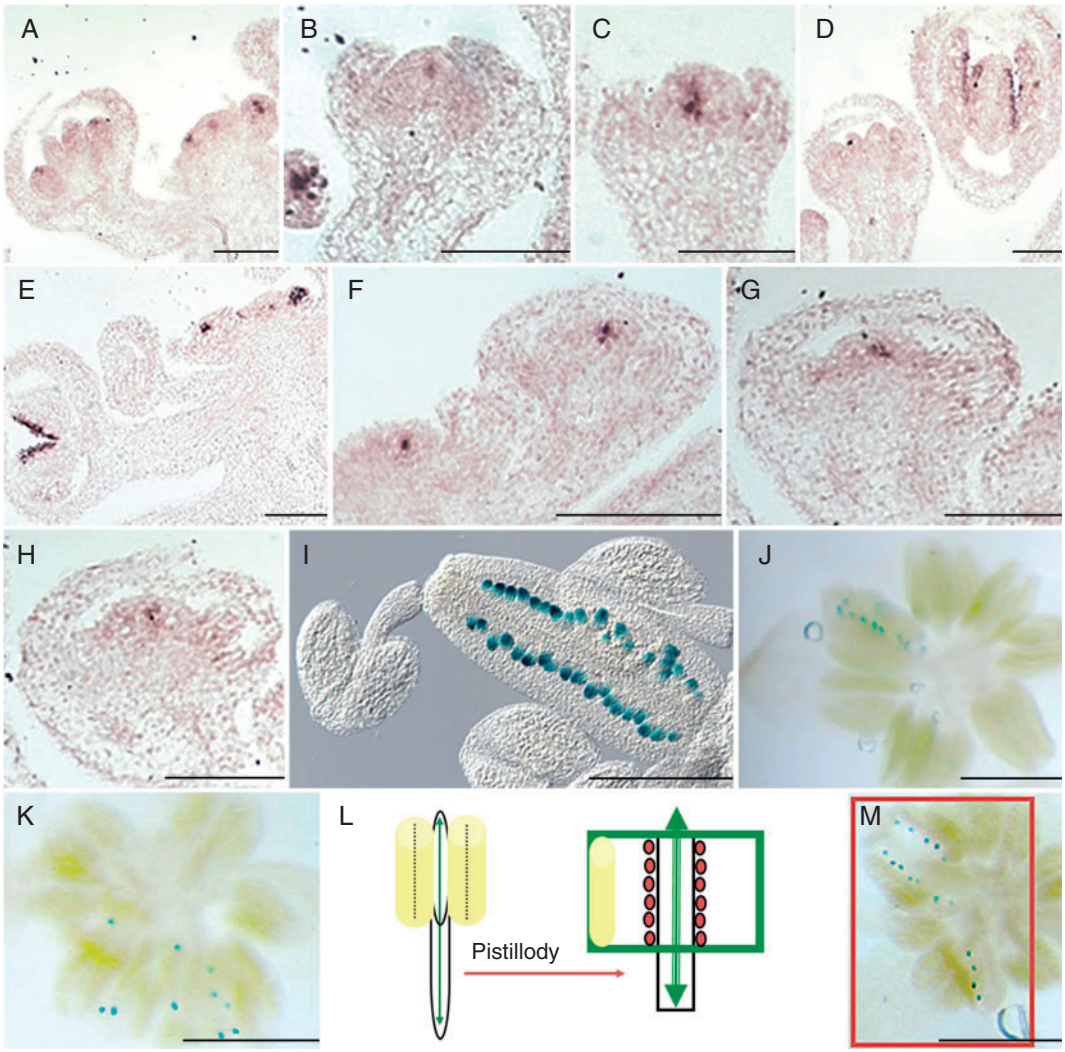

FIG. 4. *WUS* expression patterns in early and late stages of flower development in *sup* alleles. *In situ* hybridization in the wild type (A–D), with two distinct sections at stage 5–6 (B, C), and in *sup-5* (E–H), with two serial sections at late stage 6 (G, H). The following structures are visible: in (A), SAM, stage 1 and 2, and stage 9/10 buds; in (D), stages 6/7 and 12, the latter with the dehiscence zone labelled; in (E) SAM, stages 3, 4 and 10, the latter with the dehiscence zone labelled; in (F) FM and stage 6. Similar patterns have been observed in *sup-1* (not shown). *pWUS::GUS* expression in ovules of a wild-type pistil (I; stage 9/10), *sup-1* (J) and *sup-5* (K) both at stage 10. Note that such ovules are produced in stamens with medium pistillody strength. The sketch (L) illustrates stamen morphology and pistillody leading to the simultaneous production of ovules and pollen sacs in mono- and bi-sporangiate arrangements (M). Scale bars = 50 µm (A, I) and 500 µm (J, K, M).

Taken together, these results point to the fact that *SUP* works in synergy with more general factors of FM termination, indicating indirect genetic interactions between those factors and a *SUP*–class B gene-dependent mechanism of FM termination. Thus, two FM-specific termination pathways are at work: a class C gene- and a class B gene-dependent pathway.

(ii) Linking sexual boundary and FM patterning

SUP, CUC2 *and* PAN. The above results prompted us to investigate the interaction of *SUP* contrasting alleles with more general meristem patterning and boundary regulators, such as *CUC2*, and with FM patterning factors, such as *PAN*. The question is whether *SUP*, in addition to specifying the sexual boundary and stamen number in whorl 3, is also affecting FMC patterning.

Differential expression of CUC2 *in* sup-1 *and* sup-5. *CUC2* is a *NAC* gene family member expressed in all flower organ boundaries, in the medial region of stage 8 carpels and in septum tissues (Kamiuchi *et al.*, 2014, and references therein). The *CUC* genes are redundantly required for carpel margin formation and fusion (Nikovics *et al.*, 2006). In *sup-1*, the *CUC2* expression pattern is similar to that of the wild type, except that the gene is transiently misexpressed at the FMC up to flower stage 6 (Breuil-Broyer *et al.*, 2004; Fig. 6M–T). Alternatively, the signal marked the boundary of a strongly diminished whorl 4. In *sup-5*, the *CUC2* signal was faint at the perianth level and erratic to absent in presumptive central whorls of the FM. Thus, *CUC2* expression is altered differently in *sup-1* and *sup-5*, and represents an important indication that the observed patterns affect, albeit in distinct ways, FM indeterminacy.

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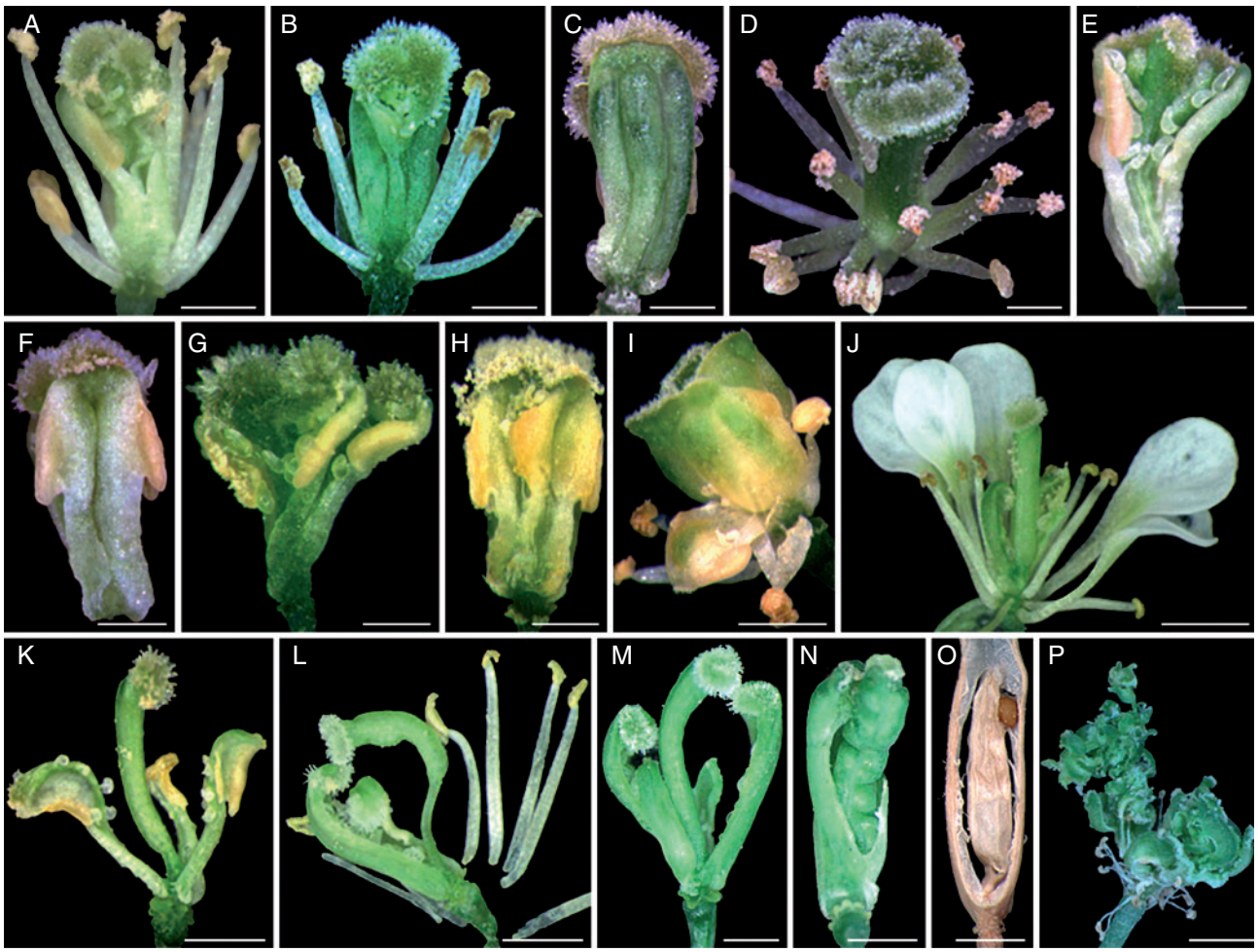

FIG. 5. FM termination alterations in *sup-5* phenocopies and crosses of *sup* mutants with *clv* and *pan*. Stamen, carpel and carpelloid structures in *sup-5* (A), *p35S::KAPP* (B, C), *clv1-6 sup-1* (D–G) and *clv1-6 sup-5* (H, I). Note the broad range of carpelloid morphologies at the FMC that combine pistillody (medium to strong), organ fusion and strong expansion of apical stigmatic tissues. *clv1-6* enhances pistillody strength in the *sup-1* background. In *clv1-6 sup-5*, the medial region was reduced or absent. Organ fusions were similar to those observed in the *sup-5* single mutant. Partial restoration of whorl 4 and formation of whorl 5 carpels or carpelloid tissues in *pan1 sup-1* (J–L) and *pan1 sup-5* (M–O). Note that most primary carpels lack medial ridges (L–N), the leafy type pistillody (K), and seed setting in a whorl 5 gynoecium (N, O). *sqn-4 sup-5* exhibited strong indeterminacy, with reiterated and elongating axes bearing carpelloid structures (P). Scale bars = 500 μm.

To see whether these *CUC2* patterns correlate with changes in cell division patterns at the FMC, we have analysed the topographical distribution of BrdU-labelled cells in the wild type, *sup-1* and *sup-5*. In *sup-1*, at early stages 4–6, BrdU labelling profiles were similar to those of the wild type: clear-cut stretches of unlabelled cells (boundaries) separate successive whorls of stamen initials (Breuil-Broyer *et al*., 2004). Subsequently, during organ morphogenesis (stages 8–14), such boundaries became narrower and more irregular (Breuil-Broyer *et al*., 2004). Occasionally, BrdU-labelled cells could be observed in epidermal and sub-epidermal layers of such boundaries.

In *sup-5*, only sepal–FM and petal–FM boundaries were easy to distinguish at the early stages 4–6, the rest of the meristem consisting of proliferating cells spread across the FM (almost all cells of the FM have incorporated BrdU during the pulse). Subsequently, growing stamen, carpel and carpelloid primordia (strong label) were not separated by stretches of unlabelled cells, as shown in the analysed serial sections (Fig. 6A–L).

In brief, BrdU labelling patterns confirmed the observed *CUC2* hybridization profiles. The results showed that *sup-5* flowers lacked proper general and specific boundary functions at the level of whorls 3 and 4, with, as a consequence, no apparent cell division domains at the FMC, i.e. rather homogenous BrdU incorporation/cell (over)proliferation patterns on both sides of the putative whorl 3–whorl 4 boundary.

Taken together, *CUC2* and BrdU patterns represent so far the most refined molecular and cellular results that best discriminate between *sup-1* and *sup-5* alleles.

PERIANTHIA (PAN)–SUP *interactions in FM homeostasis*. Because *SUP* controls cell growth redundantly with *AG* and *CLV*, interactions with other FM patterning factors, such as *PAN*, could contribute to better understand the role that class B and C genes play in FMC homeostasis. *PAN*, a bZIP transcription factor, is a flower (pre-)pattern gene that controls flower organ number (Running and Meyerowitz, 1996) and regulates

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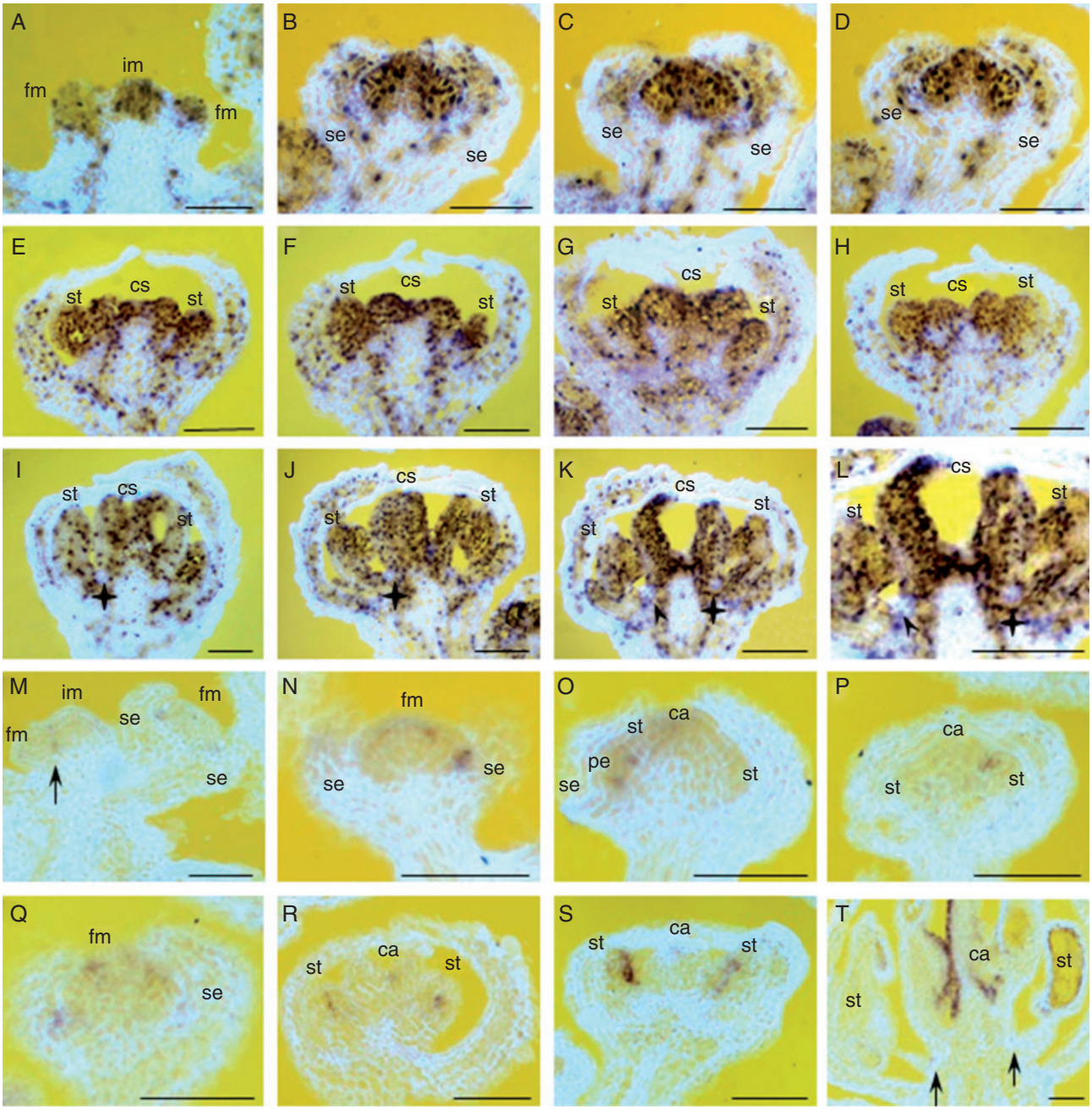

FIG. 6. Cell proliferation patterns in the FM and at inter-whorl and inter-organ boundaries in *sup-5* observed by BrdU incorporation and *CUC2-1 in situ* hybridization. BrdU labelling was monitored on (A) shoot apex, (B–D) three consecutive sections of stage 4–5 flowers, (E, F) two consecutive sections of stage 6 flowers, (G, H) two consecutive sections of stage 8 flowers and (I–L) older flower buds (from stages 10 to 12). *CUC2-1* expression was characterized on (M) inflorescence meristems, (N–Q) stage 3–5 flowers and (R–T) stage 8–12 flowers. (L) Close-up view of (K). Arrowheads indicate non-dividing cells organized as a true boundary, stars indicate non-dividing cells islets at the base of stamens and arrows point to small groups of cells at the base of, and between, stamens that weakly express *CUC2-1*. All flowers are oriented with the apex of the flower towards the top. im, inflorescence meristem; fm, floral meristem; se, sepal, pe, petal; st, stamen; cs, chimeric carpel–stamen structure. Scale bars = 60 µm.

stem cell fate by positively controlling *AG* expression (Das *et al*., 2009). This activity is spatially restricted to the centre-most region of the *AG* expression domain. *PAN* also exhibits enhanced indeterminacy in double mutants with *SEUSS* (fifth whorl formation) and contributes to the development of the carpel margin meristem from the medial region (Wynn *et al*., 2014). *pan* mutations restore fourth whorl carpels to flowers of the *sup-1* single mutant (Running and Meyerowitz, 1996). Reduction in *AG* expression caused by the absence of *PAN* could lead to increased or prolonged *WUS* expression, and thus to an increase in the size of this region (Das *et al*., 2009).

In crosses with *pan-3*, *pan-3 sup* doubles had fewer extra stamens and the innermost stamens showed stronger levels of pistillody with leafy appearance (Table 3; Fig. 5J–O). They

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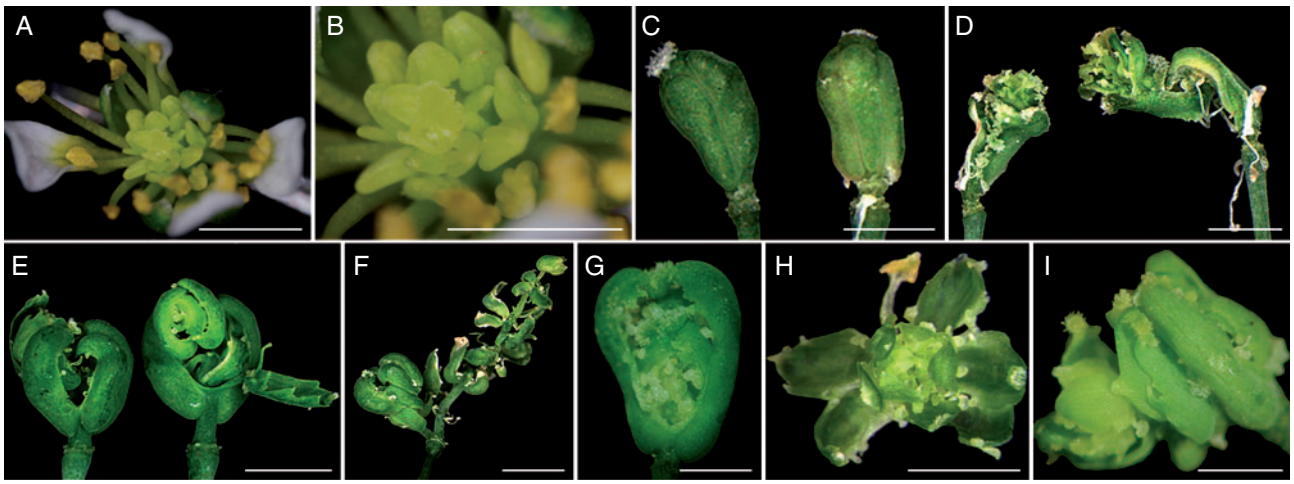

FIG. 7. The meristematic continuity process revealed in morphological traits of double mutant combinations of *SUP*, *KNU*, *CRC* and *PAN*. The double mutatnts are *knu1 sup* (A–D), *crc1 knu1* (E, F) and *crc1 pan-3* (G–I). In *knu1 sup-1* (A, B), the *sup-1* phenotype was strongly enhanced, stamens being produced in extravagant numbers. Similar trends of indeterminacy consisting of reiteration of leafy carpelloid structures inside supra-nummerary primary carpels were observed. In *knu1 sup-5* (C, D), fused or unfused primary carpels can form with proliferative masses along medial ridges. In the other combinations, carpels are not fused. Reiterated carpel whorls can form on elongated axes, with occasional stamen formation. Scale bars = 1 mm.

produced extra carpels at the FMC, frequently positioned on a gynophore. Thus, *pan-3* counteracted most of the *sup-1* effects by restoring a male–female boundary (i.e. somehow diminishing B expansion), increasing carpel number and consequently female fertility. In *pan-3 sup-5*, the same tendencies were observed. In 66 % of the flowers, the restored carpels were located inner to a fourth whorl of primary open carpels, many with a narrow base and a leafy appearance (Fig. 5M–O), i.e. they corresponded to an additional whorl 5. While *sup-5* showed expanding proliferation capacity in whorl 3 and 4, *pan-3 sup-5* flowers suffered a reduction in whorl 3 to the benefit of whorl 4 (i.e. a likely impact on class B gene function levels). The results indicated that the *pan-3* mutation can restore a bona fide, albeit loose, carpel boundary in *sup-5*. Furthermore, *pan-3* also counteracted *sup-5* effects on organ fusion, suggesting additional links to the *CUC2* boundary system explored above.

The results revealed contrasting roles of *PAN* and *SUP* in stamen and carpel whorls. Sexual boundary restoration in the double mutant was associated with extra whorls of carpels formed in the intercarpelary space (also see Prunet *et al*., 2009). This is important because the effects of *PAN* and *SUP* mutant combinations at the centre-most region of whorl 4 allow separation of the boundary setting from FM termination processes. The results further indicate that regulators of FM patterning (*PAN*), boundary (*SUP*) and reproductive organs/meristem identity (B/C) are components of FMC patterning and flower homeostasis mechanisms. They also contribute to proper carpel identity, considered as a developmental unit homologous to the leaf (Ferrandiz *et al*., 2010).

Linking early and late SUP functions – co-ordinating meristematic territories at the FMC

Since *SUP* activity interferes with both FM patterning and homeostasis, and with general boundary and whorl 4 functions, some kind of integration between these processes is expected to occur in co-ordinating FM termination, carpel initiation and carpel margin meristem/placenta formation. The boundary factors *SUP* and *KNUCKLES* (*KNU*), belonging to the same gene family, might play such a role. *KNU* has a carpel–placenta boundary function, i.e. the next boundary in space and time at the FMC. The early spatial–temporal expression profiles of *SUP* and *KNU* are complementary. *KNU* expression is initiated at stage 6 at the FMC (Ito *et al*., 2003; Payne *et al*., 2004; Sun *et al*., 2009). *SUP* and *KNU* expression patterns overlap during carpel morphogenesis, in the carpel margin meristem region (placenta, septum).

In the context of carpel initiation and development, a *SUP–KNU* boundary system might depend on interactions with early carpel-specific factors, such as *CRABS-CLAW* (*CRC*) and *SPATULA* (*SPT*), known to promote lateral vs. medial zones patterning in the gynoecium (Alvarez and Smyth, 2002).

SUP *and* KNU *are bridging the meristematic capacity in space and time*. So far, the analysis concentrated on processes that initiate FM termination. We wanted to understand how FM termination is maintained. The question is whether *SUP* and *KNU* boundary factors are central players in the initiation/maintenance of FM termination. Genetic relationships of *AG*, *KNU* and *WUS* suggest that these genes function in the same pathway in floral meristem control, *KNU* being a key link in the feedback regulation of *WUS* by *AG* (Sun *et al*., 2009).

Crosses of *knu1* to *sup-1* and *sup-5* resulted in synergistic effects. *knu1* mutant flowers produced ectopic stamens and carpels in a reiterating pattern inside fourth whorl carpels (Payne *et al*., 2004). In *knu1 sup-1*, strong indeterminate male flowers were observed (Fig. 7A, B) corresponding to an enhanced *sup-1* phenotype. In *knu1 sup-5*, carpel number was increased (Table 3), and reiterated carpelloid structures, increased internal mass and callus-like tissues were produced inside the primary carpels (Fig. 7C, D). The synergistic effects in *knu1 sup-5* indicated independent but co-ordinated mechanisms linking the control of FM termination and subsequent development events

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leading to medial region formation. The production of abundant internal tissues and callus in *knu1 sup-5* further indicated that *SUP* and *KNU* jointly control that region. One can hypothesize that *SUP* and *KNU* are bridging over early (sexual boundary and FM termination initiation as illustrated by *knu1 sup-1*) and late (FM termination maintenance and medial region formation as illustrated by *knu1 sup-5*) meristematic functions. In other words, *SUP* and *KNU* redundantly overlap to initiate and maintain FM determinacy.

Linking FM termination and carpel compartmentation – unravelling the FMC meristematic continuity. The above hypothesis questions whether proper termination of the FM is required for formation of carpel primordia and carpel margin meristem. To understand the possible role played by the carpel polarity genes *CRC* and *SPT* in FM termination maintenance, we crossed *sup* and *knu* to *crc* and *spt* mutants. Of note, *CRC* and *SPT* were shown to pattern the gynoecium through indirect genetic interactions (Alvarez and Smyth, 2002): *CRC* is an early component of carpel identity and abaxial polarity (Alvarez and Smyth, 2002), while *SPT* is essential in carpel adaxial polarity and carpel margin meristem formation (Alvarez and Smyth, 2002; Nahar *et al*., 2012). Similar results were obtained with the respective double mutant combinations (Table 3; Supplementary Data Fig. S4). Only results concerning *crc sup* are presented below.

In *crc1 sup-1* double mutants, *sup-1* was epistatic to *crc1* (Table 3; Supplementary Data Fig. S4B, C), which is an expected outcome when considering the *sup-1* phenotype and that the spatial–temporal expression profiles of *SUP* and *CRC* do not overlap. However, in *crc sup*/+ backgrounds, indeterminacy resulted in both an increase in carpel number and mild whorl 5 phenotypes (Fig. S4I–K).

In *crc1 sup-5*, mutant flowers exhibited reduced indeterminacy for stamens and, to a larger extent, for carpels as compared with *sup-5* (Table 3; Supplementary Data Fig. S4D, E), i.e. the attenuation of *sup-5* allelic strength, the phenotype being similar to a class 1 weak *sup* allele. Note the enhanced leafy-type pistillody traits. Thus, the *crc1 sup-5* phenotype indicated that (late) *SUP* and *CRC* functions overlap and participate in the FM termination–carpel patterning switch. An active *KNU* might confer the bridging context.

To explore this possibility, we analysed the *knu1 crc1* double mutants: strong indeterminacy was observed, with supranumerary (whorls of) stamens and carpelloids inside the primary carpels (Table 3; Fig. 7E, F). Thus, *crc* enhanced the *knu* phenotype, indicating synergistic effects between *CRC* and *KNU* in defining meristematic territories within the carpel through genetically independent and complementary genetic pathways. Of note, a similar phenotype was produced in *crc1 pan-3* double mutant flowers (Fig. 7G–I).

SUP and *KNU* have an apparently opposite relationship to *CRC* and *SPT*. We hypothesize that *SUP* and *KNU* are primary FMC components of FM termination initiation, *KNU* operating through the *AG* pathway and *SUP* through an independent route. Since *sup-1*, but not *knu-1*, is epistatic to *crc*, *KNU* is likely to bridge over FM termination initiation and maintenance, with *CRC* and *SPT* having joint and redundant effects in the maintenance process (note that *crc spt* double mutants are indeterminate, exhibiting extra carpels and stamens inside the primary carpel whorl (Alvarez and Smyth, 1999).

In summary, the results reveal that the proposed *SUP–KNU* sequence controlling the transition 'FMC termination to carpel patterning' is orchestrated in time and space with the differential contribution of carpel patterning factors.

Pistillody and chimeric stamen–carpels: CUC2 *differential expression and altered class B/C gene balance in* sup-1 *and* sup-5. Stamens that produce ectopic ovules and/or stigmatic tissues undergo a feminization process called pistillody (*sensu* Ronse De Craene *et al*., 2011). In the study, *sup-1* stamens produced ectopic ovules and/or stigmatic tissues, showing various degrees of filament, connective and/or apical region expansion. Connective and filaments (single-veined tissues; Hufford, 1996) were the most responsive to feminization. Laminar filaments, bulging of the apical region of the connective and disporangiate stamens illustrate some basal angiosperm traits (Hufford, 1996). Feminization was observed at stage 8 or later. The study concluded that in this *sup* class background the primary identity of supernumerary organs at the FMC was stamen.

Similar results were obtained in the analysed allelic series. Approximately 10 % of the stamens in class 1 exhibited weak pistillody (Table 2; Fig. 2A–H). Such bisexual organs producing mega- and microspores at the same time were positioned at the FMC only. In class 2 and class 3 mutants, pistillody was estimated at 5 and 8 %, respectively, of the total number of stamens, ranging from weak to strong (Table 2; Fig. 2I–Q). Again, pistillody occurred in the innermost (whorl of) stamens. In medium to strong cases, connective and filament tissues formed (partial) valve structures. Those structures were frequently aberrant stamen–pistil chimeras with proliferative medial tissues (Fig. 2; Supplementary Data Fig. S5L–P). For example, in *sup-5*, two-thirds of whorl 4 organs were stamen–carpel mosaics that were present in all flowers.

We conclude that pistillody and stamen–carpel fusions are primarily late developmental events and that it is rather difficult to distinguish between cases of strong pistillody and stamen–carpel fusions. Post-genital fusion of stamens and carpels is well documented in cases of synorganization (Endress, 2006; Ronse De Craene *et al*., 2011).

On the basis of these results, we wanted to investigate inter- and intra-whorl boundary defects further in stamen–carpel chimeras and class B gene expression patterns at later stages of flower development. The role of the epidermal cell layer in post-genital fusions has been described (Endress, 2006).

Altered and differential CUC2 *expression correlates with organ fusion in* sup-5. As reported in an earlier section, no or erratic *CUC2* signal at boundaries between and within reproductive organ whorls was observed in *sup-5* flower tissues during all stages of flower development. Proper signals were registered in stamens (locule boundaries) and in carpel internal tissues (Fig. 6, late stages), suggesting that *sup-5* did not affect *CUC2* during organ formation, i.e. during proper determination and differentiation of stamen and gynoecium compartments. The inter-organ and inter-whorl boundary defects in *CUC2* expression could explain the observed extensive organ fusions in *sup-5* because cell proliferation was maintained in boundary regions in the mutant and thus affected proper spacing between whorls and between organs within a whorl (Fig. 6I–L, S, T). Mainly

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epidermal cell layers were not labelled, suggesting that organ fusions are essentially post-genital events, as illustrated in Supplementary Data Fig. S5; wild-type in (A–C), *sup-5* in (H–P) and *sup-1* (D–G) for comparison.

AP3 *and* AG *genes are misexpressed in chimeric organs at FMC*. The profiles of *pWUS::GUS*, *pAP3::GUS* and *pAGi::GUS* reporters were observed in *sup* mutants at a range of developmental stages. *pWUS::GUS* patterns in ovule primordia and in stamen dehiscence zones were normal in all mutants, including ectopic and chimeric tissues or organs (Fig. 4, late stages). With *pAP3::GUS* and *pAGi::GUS*, while normal patterns were observed in stamens produced in whorl 3 and in most extra stamens, enhanced expression of *AP3* and *AG* reporters occurred in all feminized stamens (i.e. in expanding connective and filaments and in abnormal chimeric anther parts) and were easily detected beyond developmental stage 8 (Fig. 3, late stages) (see the Discussion regarding quartet complex balance/dosage).

Such aberrant patterns could be at the origin of various levels of pistillody produced at the FMC in both *sup-1* and *sup-5* mutants. Pistillody and chimeric organs occur in *35S:AP3* and *p35S:AP3/PI* (Krizek and Meyerowitz, 1996; this work). We concluded that an altered balance of class B/C gene activity was present during organ formation in the mutants, affecting reproductive organ traits alone or in combination with other carpel development factors expressed at the centre-most region of the FM.

In summary, the combined effects of altered B and C balance during reproductive organ initiation/maintenance with differential patterns of *CUC2* expression between the wild type and each of the two alleles are likely to explain most of the morphological particularities of *sup-1* and *sup-5* alleles.

DISCUSSION

Over the last decade, the known functions of *SUP* have been cadastral (sexual boundary), FM termination and ovule integument differentiation (Schultz *et al*., 1991; Bowman *et al*., 1992; Gaiser *et al*., 1995; Sakai *et al*., 2000; Prunet *et al*., 2009). Our results add to the list of described *SUP* functions, which have included phenotypes range from *superman*, to *superwoman* to *supersex*. This range of morphologies and double mutant phenotypes mimic some traits of basal angiosperm and even more archaic features (Hufford, 1996; Endress, 2001, 2004; Rudall and Bateman, 2010), and are essential in further addressing evolutionary questions of bisexuality, FM termination and flower homeostasis.

The allelic series we analyse – five alleles with differing strengths, due to defined alterations in SUP protein domains or in the *SUP* transcript – shows that *SUP* fulfils multiple functions during flower development, starting with stage 6 (also see Huang and Ma, 1997; Sakai *et al*., 2000) and extending to the medial region of the gynoecium and to ovule development (see Gaiser *et al*., 1995). *SUP* helps to determine the sequence of reproductive organ identities and meristematic specialization events at the FMC by the following functions.

1. Early *SUP* functions: linking mechanisms in FM termination (*a*) to male–female (sexual) boundary setting (i.e. B function exclusion, resulting in stamen–carpel identity specification) and (*b*) to stamen number control (i.e. lateral stamen reduction from four to two) and organ separation. *SUP* control of FM termination is linked to general meristem genes (such as *WUS*, *CLV*, *CUC2* and *SQN*) and to FM patterning genes (such as *PAN*, *AG* and class B genes).
2. Late *SUP* functions: contributing to medial region formation through carpel/placenta boundary control (in conjunction with *KNU*, *CRC* and *SPT*) and class B/C gene balance for the proper differentiation of carpel meristematic regions and stamen region differentiation.

Models to explain *SUP* functions that refer to ‘boundary’ vs. ‘cell proliferation’ control processes (Sakai *et al*., 2000; Prunet *et al*., 2009) only address the early set of *SUP* functions. Our results explore a more inclusive view of *SUP* actions. They are supported by espression patterns of *WUS*, *AP3* and *AG*, and by BrdU cell division profiles on both sides of the sexual boundary. Accordingly, *SUP* sets the correct level of cell allocation between whorls 3 and 4 and contributes to the control of the class B/C MADS-box gene balance during both early (FM termination) and late (stamen and carpel tissue determination and differentiation) stages. According to the gradient model of Liu and Mara (2010), in the quartets of MADS protein complexes, the effects of class B and C genes are strongly dose dependent (Smaczniak *et al*., 2012), because class B and C MADS-box proteins compete for the same partners. The relative abundance of class B and C proteins in such complexes in both early and late stage flowers was demonstrated by *in planta* formation of organ-specific quaternary complexes (Smaczniak *et al*., 2012). The results showed a flexible composition of MADS complexes, including the AP3, PI, AG and SEP3 complex (see also Chanderbali *et al*., 2010). This can explain the large morphological variations observed between and within *sup* alleles (see below). Of note, ectopic *WUS* expression in whorl 3 (resulting in out of balance B/C expression due to increased *AG* in a domain where it is normally present) produces carpelloid stamens (Lenhard *et al*., 2001; Lohmann *et al*., 2001).

In this context, the *AG*, *SUP* and *CLV* pathways appear to function at least partially independently to repress *WUS* (the end-point of regulatory loops required for stem cell production, maintenance/homeostasis and arrest), because the effects of *ag*, *sup* and *clv1* mutations on floral meristem determinacy are additive to synergic in double mutant combinations (Clark *et al*., 1993; Lohmann *et al*., 2001; Bowman *et al*., 1992; Prunet *et al*., 2008; this work; also see Liu *et al*., 2011). In such combinations, the partial indeterminacy observed in the single mutants becomes a more dramatic loss of determinacy.

For example, *sup-5* and *35S::KAPP* affect meristematic functions in at least partly distinct ways compared with those involving the CLV cascade. Interestingly, it has been proposed that strong *clv* alleles alter additional RLK signalling cascades within meristems (Diavert *et al*., 2003), while KAPP has been shown to interact *in vitro* with sub-sets of plant RLKs (Braun *et al*., 1997). *SUP* therefore may act on FM termination through RLK signalling cascades different from *CLV*, implying redundant signalling control of FM termination. Thus, the results of the *SUP* allelic series provide further evidence that the determination of floral stem cell fate is a highly redundant process, requiring interactions and integration between domain- and/or

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stage-specific factors with diverse floral patterning functions, resulting in developmental homeostasis.

The comparison of *sup-1* and *sup-5* alleles has been very informative on early vs. late *SUP* functions, but the question of which allele is the neomorph remains open. *sup-1* and *sup-5* have similar effects on *WUS*, but cause different changes in the expression of *CUC2*, a meristem and general boundary factor. In *sup-5*, in contrast to *sup-1*, indeterminacy affects both whorls 3 and 4, pointing to a partial dissociation of sexual boundary from FMC growth control, with extension to carpel medial region control.

In *sup-1*, strong sexual boundary effects with direct consequences on indeterminacy link class B genes and *WUS* expression control: *SUP* control of FM termination is mediated by class B genes. Of note, in *p35S::AP3 p35S::PI* (Krizek and Meyerowitz, 1996), *AP3* early expression was present in the whole of the FM, with the exception of a small region at the very centre of the FM. A similar pattern has been reported in *sup-1* (Bowman *et al*., 1992; also see Sakai *et al*., 1995, 2000), which leaves room for occasionally producing carpel structures in class 1 alleles (Tables 2 and 3). An expansion/maintenance over time of this FMC region lacking class B gene expression could explain class 2 and 3 phenotypes. In addition, in several mutants, the centre-most subdomain of whorl 4 was shown to correspond to an AG-free sector with phenotypes showing indeterminacy (Prunet *et al*., 2008; Das *et al*., 2009).

We argue that the sub-domain of whorl 4 is the domain where a reprogramming of meristematic activities can take place. Therefore, the completion of *WUS* repression by *KNU, CRC* and *SPT* is imperative for the proper development of carpel margin meristems (their initiation, positioning and patterning; Liu and Franks, 2015). Of note, carpel margin meristems share regulatory mechanisms with FMs, including *CUC*, *PAN* and *STM* (Mathews and Kramer, 2012; Liu and Franks, 2015). In brief, the whorl 4 centre-most region may have an open developmental fate characterized by a co-ordinated meristematic transference process: from floral meristem to carpel margin meristem to placenta. The process is illustrated in Fig. 8.

Furthermore, the allelic *sup* series shows that the centre-most stamens, under altered (higher) B + C gene expression levels, alone or in combination with other factors, can express two sexual fates at the same time. Pistillody in Arabidopsis *SUP* mutants consists of a redifferentiation process of stamen parts (i.e. a late *SUP* control process; Ronse De Craene *et al*., 2011). Carpeloid stamens not only produce ectopic ovules, but also express a broad range of carpel traits. Laminate stamens, as reported here, mimic stamen morphologies known in basal angiosperms (Hufford, 1996; Endress, 2001). Since carpels are recognized as laminar or originally urceolate structures, stamens and carpels are often considered homologous (homoplastic) structures (Endress, 2006).

With weak to strong levels of pistillody as a recurrent allelic trait, *SUP* offers an experimental opportunity to test hypotheses on the origin of bisexuality in angiosperms. Among such theories, Mostly Male Theory (MMT) and Out of Male/Out of Female Theory (OOM/OOF) posit the resolution of B and C regulatory functions as central for the evolutionary change from uni- to bisexuality (Frohlich and Parker, 2000; Theissen *et al*., 2002; Baum and Hileman, 2006; Tavares *et al*., 2010). For example, male gymnosperm cones share genetic features with perianth organs and stamens, supporting the MM and OOM hypotheses (Chanderbali *et al*., 2010). Importantly, both hypotheses can be tested experimentally.

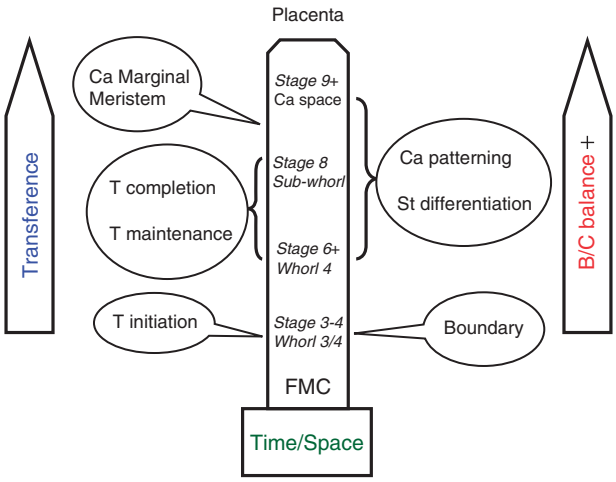

FIG. 8. Sketch of early and late *SUP* functions encompassing meristematic transference and class B/C gene balance control. The left side of the diagram illustrates the modularity of meristematic capacity (*sensu* Mathews and Kramer, 2012). The co-option (relocation/reactivation) of meristematic genetic module(s) in distinct identity contexts is illustrated by the sequence FMC–medial ridge/placenta (–and ultimately ovule). We posit that *SUP* and *KNU* are bridging agents in the way class B and C genes, *WUS*, *CUC* and others are deployed during reproductive organ formation. For example, FM termination is a necessary condition for carpel initiation followed by the onset of the meristematic programme on the adaxial carpel blade. This corresponds to an axilary meristem, *CUC 2*, *KNOX*, etc. genes (cf. Mathews and Kramer, 2012) operating in conjunction with carpel module-specific identity factors (*CRC*, *SPT*). The right side of the diagram introduces other facets of *SUP* functions operating through the class B and C balance calibration in setting the male–female boundary, in properly specifying the connective and contributing to other mechanisms such as organ polarity control (+ on the figure). The cross-talk between these processes remains to be understood. Flower development is shown through stages 3 to beyond stage 9. At stages 3–4, *SUP* expression is induced in whorls 3 and 4, the FM homeostasis being under the control of the *CLV–WUS* feedback loop. The sexual boundary and the pattern of stamen number are being established. At stage 6, class B and C gene territories are refined, the FM termination being initiated/triggered (Ti) through independent mechanisms by *AG*, *SUP* and *KNU*, together with other FM patterning factors (*PAN*, *SQN*, *CRC*, etc) to extinct *WUS*. Subsequently FM termination needs to be maintained (Tm). This is consistent with *SUP* expression starting at stage 3 and *KNU* expression being initiated at stage 6. *WUS* extinction is gradual in the wild type and arrests at stages 6. At stage 8, in the centre-most region of whorl 4, FM termination is enforced by *KNU* and *CRC*/*SPT* genes (Tc, termination reaches completion), a condition required for carpel early patterning.At stage 9 and later, *KNU* and *SUP* contribute to carpel compartmentalization by controlling the formation of the carpel margin region and meristem. Placenta formation can now proceed. Ca, carpel, FMC, flower meristem centre; St, stamen. T, flower meristem termination triggering/initiation, maintenance, completion.

Based on *SUP* data, we propose an evo-devo scenario that is a combination of MMT and OOM/OOF models, reconciled by the fact that the control of the class B/C homeotic gene balance is required during both early and late stages of flower development. There are at least four steps in the process. Steps 1 and 2 fulfil MMT conditions (i.e. ectopic female identity expression, with carpels originating through stamen transformation), while step 3 rather corresponds to OOM/OOF (i.e. a homeosis process).

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1. In the ancestor, the male 'flower' is indeterminate (identity controlled by continuing B + C expression) and the female 'flower' is indeterminate (due to continuing C expression), corresponding to simple uniaxial strobilus type axes (Endress, 2004; Rudall and Bateman, 2010). In basal angiosperms, sporophylls are elongated axes: laminar stamens with thick connectives and incompletely closed carpels lacking synorganization are predominant (Hufford, 1996; Endress, 2004). Features intermediate between inflorescence and flower highlight the role of heterotopy (i.e. gender expression) and heterochrony (i.e. internode length) in flower evolution (Ruddal and Bateman, 2010).
   Mutations in *SUP* exhibit several of the features described above. Genetic interactions of *SUP* with FM patterning and homeostasis genes probably indicate some of the contributors to those changes (see step 4 below).
2. Pistillody appears, ectopic ovules being produced on stamens. In alloplasmic wheat, for example, pistillody is caused by alterations to the class B gene expression patterns in appropriate genetic backgrounds (Hama *et al*., 2004). More generally, the heterotopic expression of female gender on the axis of the opposite sex suggests that the centre-most region of the FMC has acquired new gene expression capabilities, resulting in the novel structures produced there (Mathews and Kramer, 2012).
3. *SUP* function sets up a sexual boundary (class B gene expression exclusion). Despite the fact that imperfect bisexual flowers can develop in the absence of *SUP* (as in our allelic series), variable male/female gradients are transformed by *SUP* into two separate states, male and female. A fluctuating, labile sexual boundary becomes sharp and precisely positioned.

   Alterations in *SUP* activity in Arabidopsis probably reflect vestiges of the weaker developmental homeostasis of early angiosperm flowers. Studied ANITA grade plants exhibit overlapping 'fading borders' in the flower (Chanderbali *et al*., 2010). Canalization of ancestral transcriptional networks (of class B gene function in particular) has been considered of paramount importance for flower diversification (Chanderbali *et al*., 2010), and also for the evolution of enforced flower homeostasis. *SUP* is a candidate canalization factor for increased developmental robustness (Mestek Boukhibar and Barkoulas, 2016) of the male–female boundary resulting in spatially discrete and strictly controlled B function expression at the FMC. Studies in ANITA grade plants would be very important to observe expression patterns of *SUP* family genes.
4. From precise boundary to FM homeostasis, i.e. the optimization of the reproduction process by further reinforcing developmental robustness. A SUP (-type) control of precise sexual boundary is likely to have facilitated additional refinements of flower development, i.e. 'co-opting the meristem patterning system' at the centre of the floral axis into the gynoecium programme(Parcy *et al*., 1998; Endress, 2006).

   This has most probably been achieved by integrating regulatory mechanisms of organ identity with FMC and flower whorl patterning and determinacy (thus with both temporal and spatial changes in the activity of the FM). The result is the production of robust male and female modules. The early and late control functions of *SUP*, as reported here, support a scenario in which the formation of reproductive meristematic tissues and their boundaries, and their developmental fates are co-ordinated from the flower to the placenta to the ovule. We show that developmental robustness in Arabidopsis flowers depends on the combined effects of at least two regulatory gene networks. As a result, double mutant combinations are necessary and sufficient to produce extreme (synergic) morphological aberrations.

It is possible that alterations in *SUP* activity (or SUP-type functions) might have been involved in the origin of flowers, in particular, in flower bisexuality, and/or *SUP* might have been important only in the increased developmental homeostasis observed in eudicots and many monocots, as compared with ANITA grade flowers.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Supplementary information 1: *SUP* gene structural features and the *sup-5* deletion. Figure S1: *SUP* methylation pattern in wild-type and mutant *epi-A31*. Figure S2: morphology of reproductive structures produced at the FMC in *sup* interallelic crosses. Figure S3: FM termination analysed by flower morphology in *clv3-1 sup* doubles. The trend is loss of floral meristem determinacy. Figure S4: role of *SUP* in carpel compartmentalization. Figure S5: alterations in male–female boundary and intra- and inter-whorl organ separation in *sup* mutants.

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