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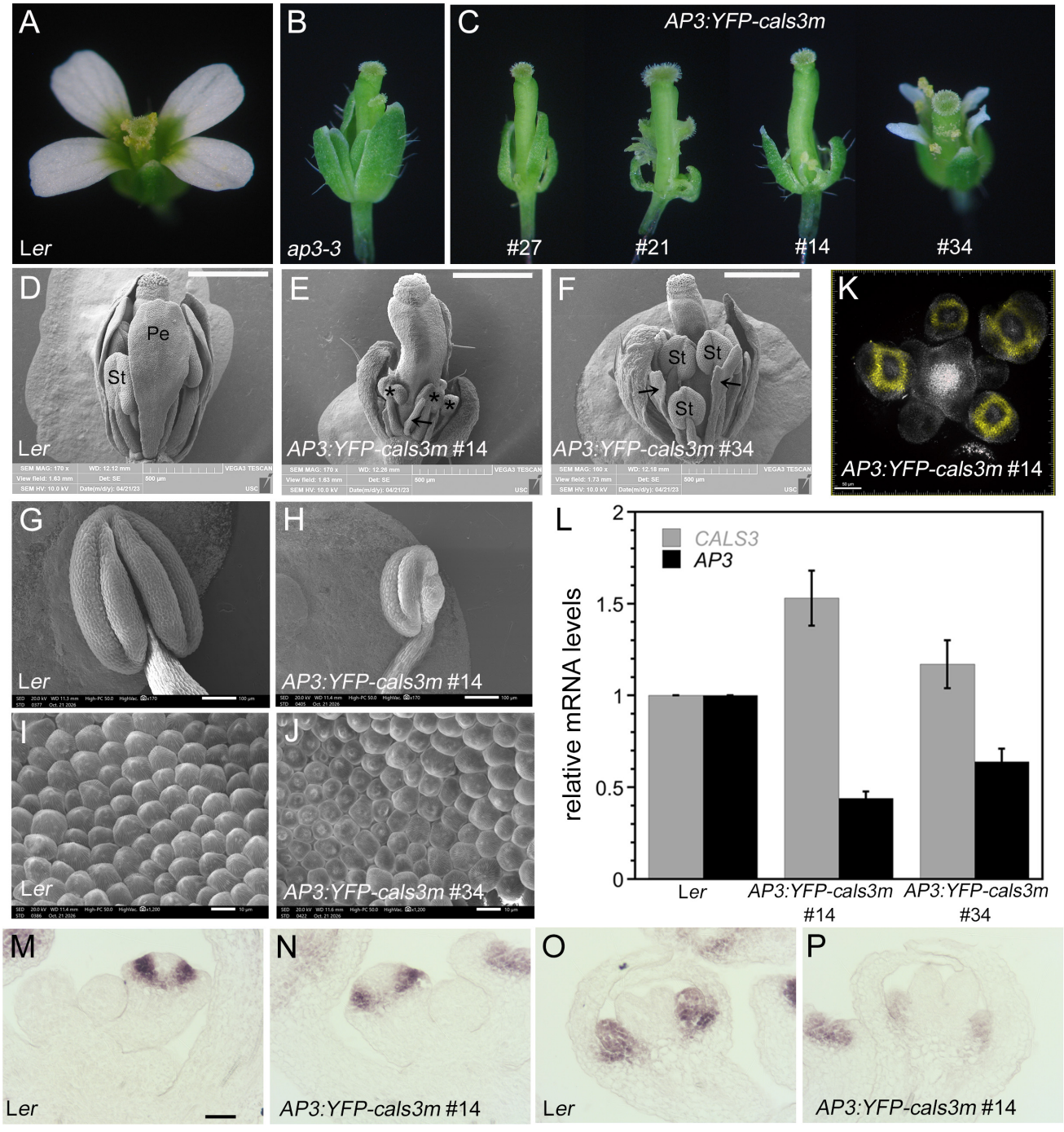
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Symplastic signaling regulates floral organogenesis in *Arabidopsis thaliana*

Beth A Krizek
Biological Sciences, University of South Carolina
To whom correspondence should be addressed: krizek@sc.edu

Abstract

Openings in the plant cell wall called plasmodesmata (PD) connect the cytoplasm of plant cells and allow for direct cell to cell movement of molecules. Callose deposition at PD reduces permeability and transport. To investigate whether movement through PD is required for floral organogenesis in Arabidopsis, I created *AP3:YFP-cals3m* transgenic plants in which a constitutively activated version of a callose synthase gene is expressed under the control of the promoter of the floral homeotic class B gene *APETALA3* (*AP3*) (AT3G54340). *AP3:YFP-cals3m* plants produced flowers that either lacked second and third whorl organs or exhibited reduced growth and development of these organs. This work suggests that floral organ development requires symplastic transport.



Organ types	Ler	AP3:YFP-cals3m #14	AP3:YFP-cals3m #34
Whorl 1			
Se	4.03	4.08	4.02
total	4.03	4.08	4.02
Whorl 2			
Pe	4.03		
Pe-like			4.1
filament		3.97	0.03
total	4.03	3.97	4.13

Whorl 3			
St	5.38	0.22	6.08
St-like	0.02	6.28	0.02
filament	0.02	0.18	0.05
Ca/St		0.02	
total	5.42	6.7	6.15
Whorl 4			
Ca	2	2	2
total	2	2	2
Total all whorls	15.48	16.75	16.3

Figure 1. *AP3:YFP-cals3m* flowers show disruptions primarily in the second and third whorls:

(A-C) Pictures of flowers from wild type (L*er*) (A), *ap3-3* (B), and *AP3:YFP-cals3m* lines 27, 21, 14, and 34 (C). (D-F) Scanning electron micrographs of a L*er* flower showing a second whorl petal (Pe) and a third whorl stamen (St) (D), *AP3:YFP-cals3m* line 14 flower with a filament in the second whorl (arrow) and stamenoid organs (asterisks) in the third whorl (E), and an *AP3:YFP-cals3m* line 34 flower with petal-like organs in the second whorl (arrows) and stamens (St) in the third whorl (F). (G-H) Scanning electron micrographs of a L*er* anther (G) and *AP3:YFP-cals3m* line 14 anther (H). (I-J) Scanning electron micrographs of adaxial cells on a L*er* petal (I) and *AP3:YFP-cals3m* line 34 petal-like organ (J). (K) Confocal image of an *AP3:YFP-cals3m* line 14 inflorescence showing expression of YFP-cals3m in the second and third whorls of young flower primordia. (L) Bar graph showing *CALS3* and *AP3* mRNA expression in L*er*, *AP3:YFP-cals3m* lines 14 and 34 inflorescences. The data are presented as means ± SD. (M-P) In situ hybridization showing *AP3* mRNA expression in stage 3 (M, N) and stage 6 (O, P) L*er* and *AP3:YFP-cals3m* flowers. The size bars correspond to 500 µm in D-F, 100 µm in G and H, 10 µm in I and J, and 50 µm in K and M. M-P are shown at the same magnification.

Table 1: Floral organs in L*er* and *AP3:YFP-cals3m* lines 14 and 34

Description

During reproductive development in Arabidopsis, flower primordia are initiated around the periphery of the dome shaped inflorescence meristem. These flower primordia give rise to floral organ primordia in four concentric rings or whorls that adopt one of four fates (sepal, petal, stamen or carpel) based on distinct combinations of floral organ identity gene activities as described by the ABCE model [reviewed in (Krizek and Fletcher 2005)]. Sepal identity is specified in the outermost first whorl by the combined activity of class A and E genes, petal identity is specified in the second whorl by the combined activity of class A, B, and E genes, stamen identity is specified in the third whorl by the combined activity of class B, C, and E genes, and carpel identity is specified in the fourth whorl by the combined activity of class C and E genes. Mutations in class A, B, and C genes result in homeotic transformations in floral organ identity in two adjacent whorls of the flower. A wild-type Arabidopsis flower contains four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl, and two fused carpels in the fourth whorl (Fig. 1A). Mutations in the class B gene *APETALA3* (*AP3*) (AT3G54340) result in flowers with sepals in whorls one and two and carpels in whorls three and four (Fig. 1B) (Jack et al. 1992).

Cell fate decisions during flower development depend on positional information, some of which may be conveyed through the cell-to-cell movement of molecules via openings in the plant cell wall called plasmodesmata (PD). PD are plasma membrane-lined intercellular channels that create cytoplasmic continuity between plant cells [reviewed in (Brunkard et al. 2015)]. Both small molecules (e.g. hormones, metabolites, ions) and macromolecules (e.g. RNAs, proteins) can move through PD [reviewed in (Gallagher et al. 2014)]. Cells connected by PD form the symplast. Because PD are dynamic and can be modified structurally to alter their permeability, symplastic domains can change during plant development. Deposition of β-1,3-glucan (callose) at PD decreases their permeability and results in reduced trafficking rates while degradation of callose at PD enhances transport (Bucher et al. 2001; Simpson et al. 2009).

Changes in PD permeability regulate developmental transitions, morphogenesis and/or differentiation [reviewed in (Bahafid et al. 2026)]. Trafficking through PD is known to regulate several aspects of reproductive development including floral induction and embryo sac development (Liu et al. 2013; Yoo et al. 2013; Qin et al. 2023). In addition, two

transcription factors: the floral meristem identity protein LEAFY (LFY) (AT5G61850) and the class C floral organ identity protein AGAMOUS (AG) (AT4G18960) can move between cells (Urbanus et al. 2010; Sessions et al. 2000). However, it is not known whether cell-to-cell movement is required for the function of these transcription factors during flower development or whether signaling within the symplast is critical for floral organogenesis.

A tool for altering movement through PD is a constitutively active version of *CALLOSE SYNTHASE 3* (*CALS3*) (AT5G13000) (i.e. *cals3m*) which increases callose deposition at PD and thus decreases PD permeability (Vatén et al. 2011). I used misexpression of *cals3m* to investigate the importance of symplastic signaling during flower development by generating transgenic plants in which *cals3m* is expressed as a fusion with yellow fluorescent protein (YFP) under the control of the *AP3* promoter The *AP3* promoter is active in the second and third whorls of stage three flowers (time of sepal initiation) and remains active in developing petal and stamen primordia (Jack et al. 1992). The *AP3* promoter is also active at the base of first whorl sepals (Weigel and Meyerowitz 1993).

The most common defects in *AP3:YFP-cals3m* transgenic plants occur in the second and third whorls of the flower. Second and third whorl organs were either missing, reduced in size, or altered in morphology. In T1 *AP3:YFP-cals3m* lines with the strongest phenotypes, such as line 27, there was a loss of second and third whorl organs and narrow sepals (Fig. 1C). In some lines, such as line 21, there were outgrowths from the central carpel in addition to reductions in sepal number and size (Fig. 1C). No seed was recovered from these lines with strong phenotypes. Homozygous *AP3:YFP-cals3m* line 14 flowers produced filaments in place of petals in the second whorl and small malformed stamens and stamen-like organs in the third whorl (Fig. 1C, D, E; Table 1). The anthers of these stamen-like organs have typical anther epidermal cells but often lack four well-defined locules and are much reduced in size (Fig. 1G, H). In addition, the sepals of *AP3:YFP-cals3m* line 14 were reduced in size and did not fully enclose the developing buds, and carpels were misshapen with a curved or bumpy appearance (Fig.1E). While homozygous *AP3:YFP-cals3m* line 14 plants did not produce seed, seed could be obtained from hemizygous individuals. Hemizygous individuals produce flowers with small petal-like organs in the second whorl and normal stamens in the third whorl, very similar to the phenotype of flowers of *AP3:YFP-cals3m* line 34 homozygous individuals (Fig. 1C, F; Table 1). Examination of the epidermal cells of these petal-like organs revealed that they were like those of wild type petals in shape and epicuticular markings (Fig. 1I, J). While floral organ numbers in whorls one, two, and four were similar in L*er* and *AP3:YFP-cals3m*, the number of third whorl stamens/stamen-like organs were slightly increased in *AP3:YFP-cals3m* lines 14 and 34 compared to L*er* (Table 1).

To confirm that the *AP3:YFP-cals3m* transgene conferred increased expression of *CALS3*, I measured *CALS3* mRNA levels in wild type (L*er*) and *AP3:YFP-cals3m* inflorescences by RT-qPCR. *CALS3* expression was elevated in both *AP3:YFP-cals3m* line 34 (1.2-fold) and line 14 (1.5-fold) with higher expression in line 14 consistent with the more severe floral phenotypes (Fig. 1L). In addition, the spatial pattern of YFP-cals3m protein was investigated by confocal microscopy. YFP signal was detected in whorls two and three of stage 3-5 flower primordia, as expected from the activity of the *AP3* promoter (Fig. 1K). As the most severe defects of *AP3:YFP-cals3m* transgenic plants were observed in the second and third whorls where *AP3* acts to specify petal and stamen identity, I examined *AP3* expression by RT-qPCR and in situ hybridization. *AP3* mRNA levels were reduced in inflorescences of both *AP3:YFP-cals3m* line 14 (0.44-fold) and line 34 (0.64-fold) (Fig. 1L). The spatial domain of *AP3* expression was similar in stage three L*er* and *AP3:YFP-cals3m* line 14 flowers but the signal intensity appeared slightly reduced in *AP3:YFP-cals3m* line 14 compared to L*er* (Fig. 1M, N). The difference in *AP3* mRNA signal between *AP3:YFP-cals3m* line 14 and L*er* was more dramatic in stage six flowers, and the domain of *AP3* expression was also reduced in size (Fig. 1O, P). Reduced *AP3* expression could indicate that regulators of *AP3* expression require symplastic transport for their activity either because of their direct movement or the generation of signaling molecules that move through PD. Alternatively, the reduced levels of *AP3* mRNA might be a consequence of cosuppression by the *AP3* promoter used in the transgene. However, the increased *CALS3* expression and observed YFP signal in *AP3:YFP-cals3m* inflorescences (Fig. 1K, L) indicate that the transgene is expressed and thus likely not subject to cosuppression.

Reduced *AP3* expression seems unlikely to be responsible for the second and third whorl defects observed in the *AP3:YFP-cals3m* transgenic lines, as the floral organ phenotypes are distinct from those in strong and weak *ap3* alleles. In strong *ap3-3* mutants, second whorl petals are converted to sepals and third whorl stamens are converted to carpels (Fig. 1B) (Jack et al. 1992). In *ap3-1*, a weak temperature sensitive allele, second whorl petals are converted to sepals while third whorl organs are stamens, carpelloid stamens or unfused carpels in plants grown at 25^{o}C (Bowman et al. 1989). At 15^{o}C, *ap3-1* flowers contain sepalloid petals in the second whorl and stamens in the third whorl. The sepalloid petals in *ap3-1* are slightly reduced in size compared to wild-type petals and have sepal-like epidermal cells. In contrast, second whorl organs in *AP3:YFP-cals3m* flowers do not exhibit any aspects of sepal identity, and third whorl organs do not

exhibit characteristics of carpel identity. Thus, the results presented here suggest that the defects in floral organ initiation in strong *AP3:YFP-cals3m* lines and the altered growth and morphology of second and third whorl organs in weak *AP3:YFP-cals3m* lines likely result from decreased PD permeability. This could result from decreased movement of hormones, nutrients, and/or transcription factors that promote floral organ growth and development. While interpretations of this study are limited by the absence of data directly showing increased callose deposition at PD or decreased PD permeability in developing flower primordia, the unique defects in *AP3:YFP-cals3m* flowers suggest that signaling through PD plays an important role in floral organ initiation, growth and morphogenesis.

Methods

Plant growth conditions

Plants were grown on a soil mixture of Sunshine Mix #1:perlite:vermiculite (8:0.5:1) in 16 hour days (100-200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 22°C.

Plasmid construction and plant transformation

A 1.7kb *AP3* promoter was initially subcloned into the HindIII/BamHI sites of pLITMUS28 and subsequently cloned into the AatII/XhoI sites of pMCS-YFP-GW. *cals3m* was then cloned into *AP3*/pMCS-YFP-GW using Gateway technology. The *AP3:YFP-cals3m* plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101. L*er* plants were transformed with this Agro strain by vacuum infiltration (Bechtold and Pelletier 1998).

Gene expression analyses

RNA was extracted from inflorescences using TRIzol (Life Technologies) and treated with DNase while on an E.Z.N.A. Plant RNA spin column (Omega Bio-Tek). First-strand cDNA synthesis was performed using LunaScript RT SuperMix Kit (New England Biolabs). qPCR reactions were performed on a BioRad CFX Connect using Luna Universal qPCR Master Mix (New England Biolabs) and the following primers: CALS3-F and CALS3-R and AP3-F and AP3-R. Data analyses were carried out using PCR-Miner, a software program that calculates Ct and amplification efficiencies (Zhao and Fernald 2005). Normalization was performed using AT5G15710 as a reference gene as it is stably expressed throughout development and under a variety of different environmental conditions (Czechowski et al. 2005). Fold differences were calculated using the Pfaffl method (Pfaffl 2001). Three biological replicates and three technical replicates were used in each experiment. For in situ hybridization, inflorescences were fixed, embedded, sectioned, hybridized and washed as described in (Wu and Wagner 2012). The *AP3* probe was made using the Riboprobe in vitro transcription T7 system (Promega) and DIG-UTP (Roche) on an *AP3* template derived from PCR with primers AP3-T7 and AP3-BK1.

Scanning electron microscopy

Tissue for SEM was fixed, dehydrated, dissected and coated as previously described (Krizek 1999). SEM analyses were performed on a Tescan Vega-3 SBU Variable Pressure SEM or a JEOL IT510 SEM.

Confocal microscopy

Flowers were dissected from live inflorescences using a 26-gauge needle. Inflorescences were transferred to a coverslip onto which a 24 well adhesive silicone isolator (Grace Bio-Labs) had been placed and filled with approximately 10µl of 0.8% agarose/0.5x MS salts. Confocal image stacks were acquired using a Leica TCS SP8X confocal microscope with a 40x water-immersion lens. A 514nm laser line was used to excite YFP and a 640nm laser line was used to excite chlorophyll. Fluorescence was detected with a 520-560nm (YFP) or a 650nm long pass filter (chlorophyll).

Reagents

Gene	Primer name	Primer Sequence
CALS3	CALS3-F	5'-ACCCTAGCACAAAATCTGGAC-3'
	CALS3-R	5'-TTTGGTTTTCTGGCTTTCCTTC-3'
AP3	AP3-F	5'- CGAATGCAAGAAACCAAGAGG -3'

	AP3-R	5'- GAATGTCAAGCTCGTCCAAAC -3'
	AP3-T7	5'-CATAATACGACTCACTATAGGGTTATTCAAGAAGATGGAAGGTAATGAT-3'
	AP3-BK1	5'-GATCTGTACCAAACTATTTCTG-3'

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