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Evolution of buffering in a genetic circuit controlling plant stem cell proliferation

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Abstract

Precise control of plant stem cell proliferation is necessary for the continuous and reproducible development of plant organs^{1,2}. The peptide ligand CLAVATA3 (CLV3) and its receptor CLV1 maintain stem cell homeostasis within a deeply conserved negative feedback circuit^{1,2}. In *Arabidopsis*, *CLV1* paralogs also contribute to homeostasis, by compensating for the loss of *CLV1* through transcriptional upregulation³. Here we show that compensation^{4,5} operates in diverse

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Author Contributions

D.R.L. performed tomato CRISPR and *Arabidopsis* molecular and phenotypic analyses, prepared figures, and wrote the manuscript. C.X. performed tomato CRISPR and complementation experiments, genetic and phenotypic analyses, prepared figures and helped edit the manuscript. C.T.K. performed tomato CRISPR experiments, genetic, RNAseq, and phenotypic analyses. C.S. generated *Arabidopsis* CRISPR knockouts, performed plant transformations, genetic and phenotypic analyses. E.D.A performed maize CRISPR experiments, genetic and phenotypic analyses. L.L. performed maize RNAseq analyses. J.M. performed synteny and CLE clustering analyses. Z.H.L performed tomato RNAseq analyses. D.S.J. performed *Arabidopsis* genetic and phenotypic analyses. J.V.E performed tomato transformations. D.P.J. conceived and supervised the maize research and edited the manuscript. M.E.B. conceived, designed and guided synteny and CLE clustering experiments, and helped prepare figures and write the manuscript. Z.L.N conceived and supervised the *Arabidopsis* research and wrote and edited the manuscript. Z.B.L conceived and led the research, supervised and performed experiments, and wrote the manuscript.

lineages for both ligands and receptors, but while the core CLV signaling module is conserved, compensation mechanisms have diversified. Transcriptional compensation between ligand paralogs operates in tomato, facilitated by an ancient gene duplication that impacted the domestication of fruit size. In contrast, we found little evidence for transcriptional compensation between ligands in Arabidopsis and maize, and receptor compensation differs between tomato and Arabidopsis. Our findings show that compensation among ligand and receptor paralogs is critical for stem cell homeostasis, but that diverse genetic mechanisms buffer conserved developmental programs.

Editorial Summary

A study of a stem cell receptor-ligand signaling module across tomato, maize and Arabidopsis identifies different genetic mechanisms of compensation that contribute to homeostasis.

Keywords

Robustness; Buffering; Redundancy; Compensation; Stem Cells; Meristem; Tomato; Arabidopsis; Maize; CRISPR/Cas9

Plant development is driven by the replenishment of stem cells in growing apices known as meristems. In shoot meristems the receptor kinase CLV1 and its ligand CLV3 function in a negative feedback circuit that dampens stem cell proliferation by regulating expression of the stem cell promoting transcription factor WUSCHEL (WUS)^{1,2}. This core CLV signaling module is deeply conserved. Mutations in orthologs in the distantly related plants Arabidopsis, maize, rice, and tomato all cause similar stem cell over-proliferation, resulting in meristem enlargement and excess organs^{1,2}. Mutations that partially disrupt CLV signaling have been important in domestication, making the CLV-module an attractive crop improvement target^{1,6}. However, both the CLV3-EMBRYO SURROUNDING REGION (CLE) ligands and their receptors are part of large gene families⁷, suggesting that CLV signaling and the phenotypic consequences arising from its perturbation could be influenced by widespread redundancy and compensation.

In Arabidopsis, stem cell homeostasis is mediated both through CLV-WUS negative feedback, and through genetic buffering by *CLV1* paralogs³. The severity of the *clv1* phenotype is buffered by the paralogous BARELY ANY MERISTEM (BAM) receptors through an “active compensation” mechanism^{3,4}. In active compensation, genes change their behavior to compensate for genetic or environmental perturbation, like the loss of a paralog. In contrast, in passive compensation, paralogs do not change their behavior under perturbation, and are closer to being truly redundant⁴. Passive compensation between paralogs is often assumed, but active compensation between paralogous genes is widespread in yeast^{8,9}. In the case of the Arabidopsis *BAMs*, their expression levels increase, and their expression domains change when *CLV1* is compromised, compensating actively for *CLV1* loss³. It is unclear whether there is similar active compensation between CLE ligands, or whether compensation mechanisms are as conserved as the core CLV-module^{4,7}.

Our previous work suggested that there is active compensation between the tomato *CLV3* ortholog, *SICLV3*, and another *CLE*, *SICLE9*. The stem-cell repressive activity of *SICLV3* requires arabinosylation of the mature dodecapeptide⁶. *SICLV3* expression increases 15-fold in arabinosyltransferase enzyme mutants, consistent with loss of stem cell homeostasis due to disrupted negative feedback. Interestingly, *SICLE9* expression also increases substantially⁶. *SICLE9* is the closest paralog of *SICLV3*, and thus might be functionally similar to *SICLV3* (Fig. 1a)⁷. Therefore, *SICLE9* represented a good candidate for an active *SICLV3* compensator in tomato.

To dissect the relationship between *SICLE9* and *SICLV3*, we first phenotyped *slclv3* homozygous null mutants generated by CRISPR/Cas9, and quantified expression of both genes in meristems. As expected, *slclv3* mutants developed enlarged meristems, fasciated stems, and increased floral organ and fruit locule number (Fig. 1b)⁶. Notably, both *SICLV3* and *SICLE9* were upregulated more than 40-fold in *slclv3* meristems, well beyond the meristem size increase (Fig. 1c). This suggested *SICLE9* repressed stem cell proliferation alongside *SICLV3*. However, *slcle9* null mutants resembled wild type plants, with a subtle effect on locule number (Fig. 1d, e, Supplementary Fig. 1a, Supplementary Table 1). We therefore generated *slclv3 slcle9* double mutant plants, and they were dramatically more fasciated than *slclv3* mutants, with thicker stems, more leaves, and a remarkably enlarged primary shoot meristem (Fig. 1f–h, Supplementary Table 2). Side shoots showed similar phenotypes, and developed severely fasciated flowers and fruits with twice as many locules as *slclv3* mutants (Supplementary Fig. 1b–d). Notably, a third *SICLE* homolog (*SICLE3*) was upregulated threefold in *slclv3* mutants (Supplementary Fig. 1c). However, null mutations in *SICLE3* did not further enhance *slclv3 slcle9* double mutants, nor increase locule number in the *slcle9* background (Supplementary Fig. 1, Supplementary Table 1). Thus, even though *SICLE3* is upregulated in *slclv3* mutants, *SICLE3* shows no evidence of compensation. Thus, loss of *SICLV3* triggers an active compensation mechanism⁴, where upregulation of *SICLE9* buffers stem cell homeostasis in tomato.

The discovery of active *CLE* compensation in tomato prompted us to ask if similar mechanisms existed in other plants. Notably, *CLV* receptor and ligand mutant phenotypes in *Arabidopsis* suggested *CLE* compensation. *Arabidopsis clv1 bam1/2/3* quadruple mutants, in which all *CLV3* receptor function is lost, exhibit extreme meristem over-proliferation, well beyond *clv3* mutants³. This phenotypic similarity to tomato *slclv3 slcle9* double mutants suggested additional *CLE* genes could buffer stem cell homeostasis in *Arabidopsis*^{3,10}. However, unlike tomato, there are no close *CLV3* paralogs in *Arabidopsis* (Fig. 1a). Therefore, to identify putative *CLE* compensators, we selected 18 meristem-expressed *CLE* genes (Supplementary Fig. 2a, Supplementary Table 3, Methods), and measured their expression in wild type and *clv3* inflorescence apices (Fig. 2a). As in tomato, *CLV3* expression rose dramatically (>100-fold) in *clv3* mutants (Fig. 2a). However, none of the other *CLE* homologs increased more than two-fold. We confirmed these findings using transcriptome data, which identified no other upregulated *CLEs* (Supplementary Fig. 2b, Supplementary Table 4, Methods). Therefore, if any *Arabidopsis* *CLEs* buffer against *clv3* disruption it is primarily through a passive compensation mechanism⁴, involving one or more *CLE* genes with little change in their expression.

To test for passive *CLE* compensation in Arabidopsis, we took a multiplex CRISPR/Cas9 approach. None of the existing *cle* null mutants are fasciated or have increased locule number^{11,12}, *CLV3* has no close paralogs, and our transcriptomics yielded no clear compensator candidates (Fig. 1a, Fig. 2a, Supplementary Fig. 2b, Supplementary Table 4). This lack of clear candidates makes dissecting passive *CLE* compensation gene-by-gene challenging, requiring many mutant combinations, further complicated by linked *CLE* genes. We therefore used multiplex CRISPR/Cas9 to simultaneously mutate 11 Arabidopsis *CLEs* with known repressive activity in peptide assays¹³, in a *clv3* mutant background. Notably, homozygous *clv3 cle* multi-gene mutants (hereafter referred to as *cle dodeca*, see Methods), in which 9 mutations disrupt the *CLE* peptide (Supplementary Fig. 2c), showed dramatic enhancement of stem fasciation, meristem size, and flower production over *clv3* single mutants (Fig. 2b–g, Supplementary Table 2). However, this enhancement was not nearly as extreme as in *clv1 bam1/2/3* quadruple receptor mutants, and locule number was only subtly affected. This suggests additional *CLEs*, beyond those targeted here, buffer stem cell homeostasis (Fig. 2h, Supplementary Fig 3a–b, Supplementary Table 1,2). Thus, unlike in tomato where a single *CLE* paralog compensates through active upregulation, many *CLEs* work together to compensate passively for the loss of *CLV3* in Arabidopsis⁴.

We next examined the relative contributions of *CLV1* versus *BAM* receptors to compensation in both Arabidopsis and tomato. We found that in quadruple *clv1 bam1/2/3* receptor mutants' locule number and vegetative meristem size were both considerably increased relative to both *clv1 clv3* and *clv3 bam1/2/3* quadruple mutants (Supplementary Fig. 3). This demonstrates that in Arabidopsis passive *CLE* compensation is mediated by shared *CLV1* and *BAM* receptor function. In contrast, as tomato *SICLE9* and *SICLV3* are close paralogs, we hypothesized that active compensation might rely on *SICLV1* (Fig. 3a). We tested this by generating *slclv1 slclv3* double mutants, where fasciation was dramatically enhanced, approaching the severity of *slclv3 slcle9* mutants. This contrasts with Arabidopsis, where *clv1* does not enhance *clv3* (Fig. 3b, c, Supplementary Fig. 1d, 4b, d; Supplementary Table 1)¹⁴. We also generated *slclv1 slcle9* double mutants, and found that *slcle9* did not enhance *slclv1* (Supplementary Fig. 4d, Supplementary Table 1). We then analyzed the transcriptome profiles from vegetative and transition meristems of wild type, *slclv1* and *slclv3* single mutants, and *slclv1 slclv3* and *slclv3 slcle9* double mutants, showing that *SICLV3*, *SICLE9* and *SIWUS* were all upregulated to similar levels in both double mutants (Fig. 3d, Supplementary Table 5). These double mutants shared a significant overlap of differentially expressed genes (87.6% in transition meristems) compared to *slclv3* and each other (Fig. 3e). Additionally, CRISPR/Cas9-generated null mutations in *SICLV2* (encoding the ortholog of the co-receptor *CLV2*) did not enhance *slclv3*, similar to Arabidopsis *clv2 clv3* mutants (Supplementary Fig. 4)¹⁵. These analyses show that active *SICLE9* compensation in tomato acts primarily through *SICLV1*, whereas passive *CLE* compensation in Arabidopsis requires multiple receptor paralogs.

Since *CLE* compensation is active in tomato and passive in Arabidopsis, we next asked if there were similar differences in *CLV* receptor signaling. In Arabidopsis, the *BAM* receptors are upregulated when *CLV1* is compromised in an active compensation mechanism³. However, transcriptome profiling in tomato showed that none of the four *BAM* (*SIBAM*) homologs¹⁶ were dramatically upregulated in *slclv1* or *slclv3 slcle9* meristems, suggesting

the lack of an active receptor compensation mechanism (Supplementary Fig. 4c). Nonetheless, we tested for active compensation genetically by disrupting the only *SIBAM* that was upregulated more than 1.5-fold in mutant meristems, *SIBAM4* (Supplementary Fig. 4c). We could not distinguish *slbam4* single mutants from wild type, and locule number in *slclv1 slbam4* double mutants was the same as in *slclv1*. Similarly, *slbam1* single mutants and *slbam1 slbam4* double mutants had wild type locule numbers, and locule number in *slclv1 slbam1 slbam4* triple mutants remained indistinguishable from *slclv1* (Supplementary Fig. 4, Supplementary Table 1). This contrasts with Arabidopsis, where locule number in *clv1* mutants is enhanced step-wise by the *bam* mutants^{3,17}. However, our results indicate some receptor compensation, as all available receptor mutant combinations show weaker fasciation than *slclv3 slcle9* mutants (Supplementary Fig. 4d, Supplementary Table 1). Thus, additional receptors, potentially including SIBAMs, likely contribute to SICLV3 and SICLE9 signaling beyond SICLV1, however their relative contributions appear to be different from the active compensation observed in Arabidopsis.

The elevated expression of *SICLE9* in *slclv3* mutant meristems nearly matches *SICLV3* levels in wild type, however compensation only partially masks *slclv3* stem cell homeostasis defects, suggesting that expression differences, barring possible differences in expression domains, are not responsible for the limited efficiency of *SICLE9* compensation (Supplementary Table 5). Peptide sequence differences likely limit compensation efficiency; four amino acid substitutions distinguish SICLE9 and SICLV3 dodecapeptides and synthetic SICLE9 peptides are less potent than SICLV3⁶. To test this genetically, we expressed the SICLE9 dodecapeptide in the context of the *SICLV3* gene. Whereas *slclv3* mutants were nearly fully rescued when transformed with a genomic construct containing the *SICLV3* coding region with native upstream and downstream regulatory sequences (*gSICLV3^{SICLV3}*), fasciation could not be complemented by replacing the SICLV3 dodecapeptide with that of SICLE9 (*gSICLV3^{SICLE9}*) (Fig. 4a,b). Thus, active compensation efficiency is dampened by weaker activity of SICLE9 peptide.

The tomato domestication mutation *fasciated* (*fas*) disrupts the promoter of *SICLV3*, reducing expression and promoting a moderate increase in locule number⁶. We hypothesized that *SICLE9* compensation might mitigate the severity of this weaker natural *slclv3* allele. Supporting this, *SICLE9* expression increased four-fold in *fas* meristems and still compensated, as locule number was higher in *fas slcle9* double mutants compared to *fas* alone (Fig. 4c,d). However, this enhanced fasciation did not reach the severity of *slclv3* single mutants, indicating the *SICLE9* compensation mechanism may scale with SICLV3 dosage. Notably, *fas* was a major contributor to increasing fruit size during domestication⁶, and our results suggest the impact of *fas* on locule number might have been too extreme, were it not for active compensation by *SICLE9*.

To investigate the origin of *SICLE9* compensation, we traced the syntenic blocks containing *SICLV3* and *SICLE9* through eudicot evolution. Both blocks were found in the order Solanales, throughout the Solanaceae family and extending to the Convolvulaceae, as represented by *Ipomoea trifida*, the progenitor of sweet potato (Fig. 4e). Outside the Solanales, only *CLV3*-like genes were found in syntenic blocks, indicating that *SICLE9* and *SICLV3* originated from a Solanales-specific duplication event, although we cannot exclude

the possibility of two independent duplications, specific to the Solanaceae and *Ipomoea*^{18,19}. Interestingly, following the emergence of *SICLE9*, synteny within the *SICLV3* block degraded faster than within the *SICLE9* block²⁰, leaving *SICLE9* as a clearer *CLV3* syntenic ortholog than *SICLV3* (Fig. 4e). Once emerged, *SICLE9*-like genes underwent a dynamic history of retention, duplication and loss, marked by two *SICLE9*-like fragments in pepper, and independent losses of *SICLE9* from potato and eggplant (Fig. 4e, Supplementary Table 6). A broader CLE clustering supported the orthology of Solanales *CLV3*-like genes, and showed that the pepper *SICLE9*-like sequences did not fall into any subcluster, supporting their identity as pseudogenes (Fig. 4f). Thus, *SICLE9*-like genes likely emerged more than 30 million years ago, prior to Solanaceae diversification. Critically, active compensation mediated by *SICLE9* could only have arisen after *SICLE9* emerged, and is specific to tomato and, potentially, its relatives in the Solanales.

Broader CLE clustering showed that the grasses, which are monocots separated from eudicots by ~150 million years²¹, typically harbor two closely related *CLV3*-like genes in their genomes, represented by the rice stem cell regulators *FON2* and *FCP1* (Fig. 4f)²². These paralogs originated from a monocot-specific duplication event, independent from the duplication leading to *SICLV3* and *SICLE9* (Fig. 4e,f)⁷. This mirroring between grasses and tomato led us to ask whether *CLV3*-like duplication in monocots also led to the evolution of active compensation. We mutated the maize orthologs of *FON2* (*ZmCLE7*) and *FCP1* (*ZmFCP1*) (Fig. 4g). *ZmfcP1* mutants are fasciated²³, but this phenotype was suppressed when introgressed into the standard B73 genotype (Fig. 1e, Fig. 4h,i). In contrast, *Zmcle7* null mutants had strongly fasciated ears (Fig. 4f–i). Notably, expression profiling of *Zmcle7* inflorescence meristems showed that only *ZmCLE7* and *ZmFCP1* were significantly upregulated among more than 50 maize *CLEs* (Fig. 4j, Supplementary Table 7). Significantly, ear fasciation was enhanced in double mutants, suggesting *ZmFCP1* partially compensates for *Zmcle7*. However, unlike *slcle9* and *slclv3* in tomato, inflorescence transition meristems from *ZmfcP1* and *Zmcle7* single mutants were each increased in size compared to WT, and double mutants were additive. Thus, although maize shows molecular hallmarks of active compensation, with another *ZmCLE* upregulated in *Zmcle7* mutants, our comparisons of the single and double mutants suggest a passive mechanism, in which these *CLE* paralogs could have redundant, but unequal, roles in stem cell homeostasis (Fig. 4h–i)²³.

We have discovered the independent evolution of CLE compensation in the monocots and the eudicots, driven by independent gene duplication events. Given the distant relationships between Arabidopsis, maize, and tomato, the genetic buffering of stem cell homeostasis uncovered in these species may reflect a formative feature of meristem biology. Interestingly, compensation is only partial, with compensators being less potent than the primary gene, paralleling principles of paralog compensation in yeast⁸. Partial compensators, like tomato *SICLE9*, the Arabidopsis *BAMs*, and maize *ZmFCP1* could function both to buffer stem cell homeostasis and provide developmental flexibility²⁴, and could participate in meristem size changes that occur during developmental transitions²⁵. They could also have as yet undiscovered primary roles in other contexts, as is likely for the *BAMs*²⁶, maintaining subsidiary roles in shoot meristems. Similarly, paralogous gene pairs in yeast are rarely truly redundant^{27,28}. While the core CLV-module is deeply conserved, our

work shows that CLV compensation mechanisms, which shaped at least one domestication event, are diverse. This genetic complexity of CLV-module compensation, which could contribute to the tolerance of CLV-WUS feedback to changes in CLV3 expression²⁹, identifies an underappreciated barrier to modification of these genes for crop improvement^{1,30}. Our work provides a roadmap to dissect the genetic complexity underlying CLV compensation in other plants, and also compensation mechanisms involving other gene families in different developmental programs, which will be important for intelligent manipulation of plant development to enhance crop productivity.

Online Methods

Plant materials and growth conditions

Seeds of tomato cultivar M82 and derived CRISPR mutants used for plant phenotyping were directly sown and germinated in soil on 96-cell plastic flats and grown as previously³¹. Arabidopsis plants were grown under continuous light conditions at 25°C. The mutant *clv1-101*, *clv3-9*, *bam1-4*, *bam2-4*, and *bam3-2* alleles in the isogenic Col-0 background used in this study are from a previous report and were genotyped as therein⁴. Double and higher order mutants not generated using CRISPR were created by standard crossing and appropriate genotypes were selected using gene specific mutant genotyping primers. Maize CRISPR/Cas9-derived mutants and wild-type segregants were sown directly on soil and grown under standard long-day greenhouse conditions (16-h light/8-h dark photoperiod) or in the field.

Plant phenotyping

Tomato meristem imaging and size measurement were performed as previously described^{7,32}. Briefly, hand-dissected tomato meristems at late vegetative and transition meristem (11 and 13 days after germination) were captured on a Nikon SMZ1500 microscope. Arabidopsis inflorescences apices images were also captured similarly. To quantify meristem width in Arabidopsis, 5-week-old inflorescence meristems from Col-0 (WT), *clv3-9* (*clv3*), and *cle dodeca* were removed, hand dissected, and fixed in FAA (2% formaldehyde, 5% acetic acid, 60% ethanol) overnight at 4°C. Tissue was dehydrated in an ethanol series (70%, 80%, 95%, 100%) for 30 min each at room temp and cleared in methyl salicylate overnight. Meristems were mounted in methyl salicylate in a glass bottom petri dish (MatTek #P35G-1.5–10-C) and imaged on an inverted Zeiss LSM 710 confocal microscope. Signal corresponds to structural autofluorescence following excitation with a 488nm Argon laser and emission detected in two broad windows (green: 504–597nm, red: 629–731nm). Images were edited and processed using ImageJ (v.2.0.0-rc-68/1.52e) where gamma was adjusted (0.5) in order to ensure complete delineation of the L1 layer of the inflorescence meristems (intensity data were not used for any downstream analysis). Measurements were made in ImageJ spanning the width of the meristem where the first primordia were visible on each side. As *clv1 bam1/2/3* quadruple mutants rarely make a main inflorescence, vegetative meristems from seedlings were compared across genotypes. Seedling meristem perimeter, width, and height in Arabidopsis, were analyzed in 10 day old seedlings grown on ½ MS media plates lacking sucrose for Col-0 (WT), *clv3-9* (*clv3*), *clv1-101* (*clv1*), *cle dodeca*, *clv1-101 clv3-9* (*clv1 clv3*), *bam1/2/3*, *clv1-101 bam1/2/3* (*clv1*

bam1/2/3), and *clv3–9 bam123 (clv3 bam1/2/3)*. Plants were hand dissected to expose meristems and fixed in FAA and mounted as described above for the inflorescence meristems. The shoot apical meristems were imaged with an inverted Zeiss LSM 710 confocal microscope following the same procedure as for the inflorescence meristems. SAM measurements were made in Image J. The perimeter measurement spanned the entirety of the meristem excluding primordia. The width was measured spanning the width of the meristem where the first primordia were visible on each side while the height was measured spanning from the outer top portion of the meristem to the bottom portion where the meristem and primordia differentiate. (N>10 for each genotype imaged). For Arabidopsis locule number quantifications the primary inflorescence of independent individuals per genotype were analyzed and mature, opened flowers were counted. Specific N values differ, see figures for numbers. For *clv1 bam1/2/3* mutant plants the main inflorescence rarely bolts and flowers were counted from lateral shoots as necessary. To quantify Arabidopsis flower production, all mature flowers (fully open and with perianth organs abscised), were counted on the main inflorescence stems by eye. Arabidopsis stem fasciation measurements were taken with a digital SPI caliper (model 15–719-8) 70 millimeters up from the rosette of 25 day old plants. For quantification and imaging of maize meristems and ears, the following genotypes were obtained from a segregating F2 population from a cross between *Zmcle7* and *Zmfcp1* CRISPR/Cas9-generated mutants: B73 (WT), *Zmcle7 Zmfcp1/+ (Zmcle7)*, *Zmcle7/+ Zmfcp1 (Zmfcp1)* and *Zmcle7 Zmfcp1*. Apices were dissected and imaged directly in the scanning electron microscope (Hitachi) or fixed and cleared for measuring, as described for Arabidopsis.

CRISPR/Cas9 constructs for generating tomato, *Arabidopsis* and maize *CLE* mutants

To generate CRISPR/Cas9 mutants in tomato, a binary plasmid was built containing a functional Cas9 driven by a constitutive promoter (CaMV 35S) and two guide RNAs (gRNA) each driven by the Arabidopsis *U6 (AtU6)* promoter using Golden Gate cloning^{31,33,34}. The final binary vectors were introduced into the M82 tomato line by *Agrobacterium tumefaciens*–mediated transformation as previously described^{33,35}. First-generation (T₀) transgenic plants were transplanted in soil and grown under greenhouse conditions. Genotyping of CRISPR/Cas9-generated mutations was performed as previously described³¹. Stable non-transgenic, homozygous plants were used for phenotyping and crosses. The CRISPR/Cas9 construct for producing the Arabidopsis *cle dodeca* mutant was built using the pCUT vector system³⁶. Twelve 20 bp gRNA target sites were selected upstream of the dodecapeptide coding region in the genomic sequence of each target *CLE* gene. Three separate gRNA arrays gene were synthesized by GeneArts (Thermofisher) as groups of 4 *AtU6::gRNA* tandem constructs³⁶, which were cloned together by restriction enzyme digestion into the recipient GeneArts pMA plasmid to generate a single vector hosting 12 gRNA units. The 12-stacked gRNA unit was then cloned into the pCUT4 binary vector as described in³⁶. A separate set of 12-stacked gRNA units were cloned into the pCUT6 binary vector. *clv3–9* plants were transformed with the pCUT4 CRISPR binary construct by floral dipping and T₁ transgenic seed derived was selected on B5 media lacking sucrose and containing 100mg/L hygromycin. T₁ plants were screened for editing efficiency by sequencing of *CLE* gene PCR products from leaf DNA. Plants were scored as having efficient editing by confirmation of overlapping sequencing traces originating at –3 position

from the PAM site. As no obvious phenotypes were observed in T₁ plants and no homozygous mutants in the T₁ were expected³⁶, T₂ seed was sown on selective B5 media, DNA was collected from T₂ plants, each targeted *CLE* gene was amplified via PCR, and products were directly sequenced via Sanger sequencing. We noted that some gRNAs from the pCUT4 set did not appear to work and a heterozygous pCUT4 T₂ line was transformed with the pCUT6 gRNA set to target the remaining *CLE* genes and potentially obtain larger deletion mutations. From T₂ of this transformation, higher order *CLE* mutant combinations were identified that contained lower order homozygous fixed alleles and from those plants the next generation was screened on hygromycin-containing B5 media, and basta, to identify heterozygous *Cas9* transgenic plant lines. A select line was propagated to the T₃ generation and subjected to additional rounds of sequencing. As no obvious phenotypes arose in the T₃, this process was continued until homozygous mutants were selected for all *CLE* genes by the T₆ generation. Plants lacking *Cas9* were confirmed by PCR and screening on both basta and hygromycin plates. *CLE* genes from *Cas9*-free mutants were re-sequenced in independent plants to assure fixed mutations, and seed was propagated from a single fixed mutant plant. To generate CRISPR/Cas9 mutants in maize genes *ZmFCP1* and *ZmCLE7*, a binary plasmid was built containing a monocot-optimized Cas9 driven by maize *UBI* (*ZmUBI1*) promoter with two gRNAs under and introduced into the maize genotype Hi-II by *A. tumefaciens*-mediated tissue culture transformation³⁷. Maize calli were genotyped by PCR at the target sites of both genes and those carrying mutant alleles were selected for plant regeneration. T₀ plants carrying mutant alleles were then backcrossed two to three times to B73 to segregate away the transgene.

RNA extraction and quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) for both tomato and Arabidopsis was performed as previously described³². Briefly, total RNA from vegetative meristems of tomato plants and dissected shoot apices from inflorescences of *Arabidopsis* was extracted with the PicoPure RNA Extraction kit and the RNeasy mini kit (Qiagen), respectively. One microgram of total RNA was treated with DNase I and used for cDNA synthesis with a SuperScript III reverse-transcriptase kit (Invitrogen). qPCR was performed using gene-specific primers (Supplementary Table 8) in the iQ SYBR Green SuperMix (Bio-Rad) reaction system on the CFX96 Real-Time system (Bio-Rad), following the manufacturer's instructions.

Meristem transcriptome profiling

Total RNA from tomato vegetative and transition meristems was extracted using the PicoPure RNA Extraction kit (Arcturus) from 20–40 meristems per replicate for each genotype, yielding 200–1000ng RNA. RNA sequencing libraries were prepared using the Kapa mRNA Hyper prep kit (Roche). The quality of each RNA sequencing library was tested using a Bioanalyzer 2100 (Agilent). Paired-end 75-base sequencing was performed on the Illumina HiSeq sequencing platform. Two biological replicates were used for all library constructions^{7,32}. For maize RNAseq, inflorescence meristems (~0.5 mm) from a segregating population were dissected from growing ears (2–7 mm length). Total RNA was extracted using Direct-zol RNA kit (Zymo Research) and sequenced by Illumina NextSeq 500 platform at the Cold Spring Harbor Laboratory Genome Center (Woodbury, NY). Reads for the wild-type tomato M82 and *slc1v* mutants were trimmed by quality using

Trimmomatic v0.32 (parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:40:15:1:FALSE LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:50)³⁸ and aligned to the reference genome sequence of tomato (SL3.00)³⁹ using Tophat2 v2.1.1 (parameters: -b2-very-sensitive -read-mismatches 2 -read-edit-dist 2 -min-anchor 8 -splice-mismatches 0 -min-intron-length 50 -max-intron-length 50000 -max-multihits 20)⁴⁰. Alignments were sorted with samtools⁴¹ and gene expression quantified as unique read pairs aligned to reference annotated gene features (ITAG3.2) using HTSeq-count v0.6.08 (parameters: -format = bam -order = name -stranded = no -type = exon -idattr = Parent)⁴². Maize RNA-seq data was trimmed with Trimmomatic v0.36 (parameters: ILLUMINACLIP:./TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:50)³⁸ and aligned to reference genome (B73 RefGen v3)⁴³ using Tophat2 v2.1.1 (parameters: -b2-sensitive -read-mismatches 2 -read-edit-dist 2 -min-anchor 8 -splice-mismatches 0 -min-intron-length 50 -max-intron-length 50000 -max-multihits 20)⁴⁰. Aligned reads were then sorted with samtools⁴¹ and gene expression quantified as unique read pairs aligned to reference annotated gene features in the maize (B73 AGPv3.22) using HTSeq-count v0.6.08 (parameters: -format = bam -order = name -stranded = no -type = exon -idattr = Parent)⁴². All statistical analyses of gene expression were conducted in R⁴⁴. Significant differential expression between meristem stages in wild-type M82 and maize inflorescence ear tips was identified with edgeR⁴⁵ using 4-fold change, average 1 CPM, and FDR 0.10 cutoffs or 2-fold change, average 5 CPM, and FDR 0.10, respectively⁴⁶.

Quantification and statistical analysis

For tomato and locule number quantifications, at least 3 primary or secondary inflorescences from 3 individuals per genotype were analyzed. For tomato and maize meristem measurements at least 5 independent plants were analyzed per genotype, and 10–15 independent plants used for Arabidopsis fixation and inflorescence meristem imaging. For Arabidopsis seedling meristem measurements 11 to 16 independent seedlings were analyzed per genotype. For Arabidopsis carpel number quantifications, N>140 per genotype were counted, with the exception of *clv1 bam1/2/3* mutants which have reduced flower production owing to extreme stem overgrowth (N= 26). For qRT-PCR experiments, two biological and three technical replicates were analyzed per experiment. Statistical analysis was performed using two-tailed, two-samples Student's *t*-test and one-way anova with Tukey test (alpha = 0.05). Raw data and specific number of plants (N), meristems, flowers or fruits (n) is shown in Supplementary Tables 1 and 2. RNAseq differential expression analysis for tomato and maize is shown in Supplementary Tables 5 and 7. All raw data are provided in Supplementary Tables 1–8.

Transgenic complementation of *SICLV3* and *SICLE9*

The genomic DNA sequences of *SICLV3* (*gCLV3^{gCLV3}*: 3,261 bp in total with 1,995 bp upstream and 666 bp downstream). To mutate *SICLV3* dodecapeptide into *SICLE9* within *gCLV3^{gCLV3}* (*gCLV3^{gSICLE9}*) PCR products were amplified from pDONOR221-*gCLV3^{gCLV3}* and a vector containing the genomic region of *SICLE9* (pDONOR221-*gCLE9*) with overlapping primers (Supplementary Table 8) by using KOD Xtreme™ Hot Start DNA Polymerase (Merck). The resulting PCR products were digested with DpnI (NEB) and transformed into DH5a competent cells. Sanger sequencing confirmed pDONOR221-

gCLV3^{gCLV3}, *gCLV3^{gSICLE9}* and colonies were recombined into binary vector pGWB401⁴⁷ for transgenic complementation.

CLE clustering

We constructed Hidden Markov Models (HMM) particular to each CLE cluster defined by Goad *et al.*, 2017⁷. We generated HMMs that included all angiosperm CLE sequences in a particular cluster, as well as Brassicaceae, Solanaceae and monocot-specific HMMs. We searched Brassicaceae, Poaceae and Solanaceae genomes with these HMMs and used the retrieved sequences (E<0.001) in downstream clustering analyses. For the clustering analysis focused on CLV3 and SICLE9, we included only those CLE pro-peptide sequences in cluster 1D, as well as those sequences identified in our synteny analysis (Supplementary Table 6). We submitted either all full CLE pre-propeptide sequences from Brassicaceae and Solanaceae, or only cluster 1D CLE pre-propeptide sequences, to an all-by-all BLAST using the MPI Bioinformatics Toolkit⁴⁸, and the results were visualized by clustering using CLANS ver 1.0⁴⁹. The resulting clusters were named according to the conventions set by Goad *et al.*, 2017⁷. Based on the clusters formed in the above analysis, the full pre-propeptide translations of CLE genes from clusters 1D1 and 1D2 were chosen for further analysis.

SICLV3, *SICLE9* synteny analysis

To find genomic regions orthologous to the tomato *SICLE9/SICLV3* regions in each target species, the peptide sequences of each of the 4 genes flanking *SICLE9* and *SICLV3* were used to run CoGe BLAST⁵⁰ on the target species' genome using the *tblastn* search algorithm. Groundcherry genomic fragments were obtained from Lemmon *et al.*, 2018⁵¹. For each search, the genomic regions that contained the three best matches were compared to *SICLE9* and *SICLV3* regions using CoGe GEvo⁵⁰ at a scale of 160,000 bp centered on the matched gene, using the (B)LastZ: Large Regions algorithm with a score threshold of 3,000. If two or more genes in this region aligned to genes in the tomato *SICLE9* or *SICLV3* regions, it was considered syntenic.

CLE peptide collection from syntenic regions

For each syntenic region match, GEvo alignment parameters were adjusted to detect CLEs, which are often missed by the default parameters. Two strategies were employed: First, the (B)LastZ: Large Regions algorithm with a reduced score threshold of 2,000, and second, the BlastN: Small Regions algorithm with a mismatch penalty reduced to -1. If either of these strategies found an alignment to *SICLE9* or *SICLV3* in the syntenic region, that portion of sequence was extracted and aligned to both *SICLE9* and *SICLV3* using MAFFT ver. 7.313⁵² using the L-INS-i algorithm and BLOSUM45 scoring matrix. From those individual alignments, we attempted to extract a CLE peptide translation, over the full pre-propeptide if possible, or just the dodeca region if alignment quality was poor. The *CaCLE9* pseudogene was identified by aligning the pepper genomic region syntenic to the *SICLE9* region using MAFFT ver. 7.313⁵² with a lowered gap offset value of 0.001. This aligned *SICLE9* to an unannotated region of the pepper genome at chromosome 6 starting at position 9321808. In this alignment, the sequences that underlie the *SICLE9* exons share ~80% nucleotide identity, but the pepper sequence has stop codons in all three reading frames, and a portion

of what aligns to the *SICLE9* dodecapeptide is deleted. Further analysis with Eukaryotic GeneMark.hmm version 3.47⁵³ accurately predicted the three *SICLE9* exons but predicted no exons in the orthologous pepper region. To verify genome assembly integrity at this locus, the region was amplified from pepper genomic DNA (Supplementary Table 8), and Sanger sequenced, which had no discrepancies to the assembly sequence. All efforts to find a similar feature in the potato genome failed, which suggests that the entire *CLE9* coding region is absent in potato.

Data software and availability

Raw data for all quantifications is included as Supplementary Tables. All RNAseq data from tomato are available in NCBI. Tomato SRA project and BioProject accession are SRP161864 and PRJNA491365, respectively. Maize SRA projects and Bioproject accessions are SRR7970748, SRR7970747, SRR7970749, SRR7970750 and PRJNA494874, respectively. RNA-seq data from Arabidopsis was obtained from Klepikova *et al.*, 2015⁵⁴ and Mandel *et al.*, 2016⁵⁵.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Buffering of stem cell homeostasis in tomato depends on transcriptional compensation from *SIC9*.

a, Clustering of CLE proteins from Brassicaceae and Solanaceae. **b**, Wild type (WT) and *slclv3* tomato inflorescences. Red arrowheads, branches. White arrowheads in insets, locule number. **c**, qRT-PCR in vegetative meristems of *SICLV3* and *SIC9*, normalized to *SIUBIQUITIN* (*SIUBI*). Mean $\pm$ SEM; two biological with three technical replicates (n = 30 meristem per replicate). **d**, Representative inflorescence of *slcle9*. Red arrowhead, branch. **e**, Quantification and distribution of locule number in WT, *slclv3* and *slcle9* (n = 105, 43 and 166). **f**, Side and top-down view of *slclv3 slcle9*. White arrowhead, Apex; white dotted circle, meristem. **g**, Primary meristems from WT, *slclv3*, *slcle9*, and *slclv3 slcle9*. **h**, Quantification of meristem width and height from WT single and higher-order mutants (n= 5, 17, 5, 12 and 7). Boxplots, 25th–75th percentile; whiskers, full data range; center line, median. One-way anova and Tukey test; letters represent significance groups at p < 0.05 in e,h. Scale bars, 1 cm in b, d, f; 100 μ m in g.

Fig. 2. Arabidopsis stem cell homeostasis is controlled by multiple redundant CLEs.

a, qRT-PCR of *CLV3* and 18 additional *CLEs* from inflorescence apices (dashed circles in pictures) of WT and *clv3* mutants. Normalized to *AtUBI*. Mean $\pm$ SEM; two biological with three technical replicates ($n = 40$ per replicate). **b and c**, Representative stems and quantification of stem width from WT, *clv3* and *cle dodeca* ($n = 20, 20$ and 20). **d and e**, Confocal micrographs of vegetative and inflorescence meristems and meristem size quantification from WT, *clv3* and *cle dodeca* ($n = 26, 30$ and 29). **f and g**, Top-down view of inflorescences and flower numbers from WT, *clv3*, *cle dodeca* ($n = 47, 104$ and 173). **h**, quantification of locule number from WT, *clv3*, *cle dodeca* and *clv1 bam1/2/3* ($n = 149, 199, 199$ and 26). Boxplots, 25th–75th percentile; whiskers, full data range; center line, median. One-way anova and Tukey test; letters represent significance groups at $p < 0.05$ in **c**, **e**, **g** and **h**. Scale bars, $300 \mu\text{m}$ in **a**; 1 cm in **b**; $50 \mu\text{m}$ and $100 \mu\text{m}$ in **d**; 1 cm in **f**.

Fig. 3. SICL9 compensation acts primarily through the receptor kinase SICLV1.

a, A proposed model showing buffering of stem cell homeostasis by SICL9 acts primarily through SICLV1 when SICLV3 is compromised, achieving partial suppression of *SIWUS* through negative feedback. **b**, Side and top-down view of *slclv1 slclv3* showing enlarged meristem flanked by multiple fasciated floral buds (dashed circle). **c**, Quantification of locule number from *slclv3* ($n = 43$) and *slclv1 slclv3* ($n = 24$). **d**, Fold change in expression of *SICLV3*, *SICLE9* and *SIWUS* relative to wild-type RNA-seq from *slclv1*, *slclv3* and *slclv1 slclv3* and *slclv3 slclv1* mutants (mean $\pm$ SD). Data are from 2 biological replicates (4-

fold change, 1 cpm cutoff, FDR < 0.10). **e**, Venn diagrams of RNA-seq data of differentially-expressed genes (DEGs) comparing the indicated mutant genotypes for vegetative and transition stages of meristem maturation. Boxplots, 25th–75th percentile; whiskers, full data range; center line, median. *** $P = 5 \times 10^{-22}$, two-tailed, two-sample t-test in **c**. *** $P < 0.001$ of 1,000 simulations from random sampling in **e**. Scale bars, 2 cm in **b**.

Fig. 4. Buffering impacted tomato domestication alongside a dynamic evolution of active compensation in flowering plants.

a, Representative inflorescences from *slc1v3* T₀ plants *gSICLV3^{SICLV3}* and *gSICLV3^{SICLE9}*.

b, Quantification of locules from *gSICLV3^{SICLV3}* (n = 38) and *gSICLV3^{SICLE9}* (n = 28).

Blue and red dashed lines, mean values WT and *slc1v3*. **c**, qRT-PCR of *SICLE9* from reproductive meristems of WT and *fas*. Mean ± SEM; two biological and three technical replicates (n = 30 per replicate). **d**, Quantification and distribution of locule number in *fas* (n = 59) and *fas slc1e9* (n = 59). **e**, Syntenic analysis of *SICLV3* and *SICLE9* chromosomal

blocks. Red triangles, *SICLE9*-like fragments with partial dodecapeptides. **f**, Clustering analysis of cluster 1D⁷ containing *SICLE9*-like sequences. **g**, Gene models, gRNAs target sites (red arrows), primers (black arrows), and *ZmCLE7* and *ZmFCP1* CRISPR/Cas9 alleles. **h**, Representative ears and ear primordia from WT, *ZmfcP1*, *Zmcle7* and *ZmfcP1 Zmcle7*. **j**, Fold-change of *ZmCLE7*, *ZmFCP1*, *ZmWUS1* and *ZmWUS2* relative to wild-type (blue line) in RNA-seq from *Zmcle7* (mean $\pm$ SD; two biological replicates, 2-fold change, 5 cpm cutoff, FDR < 0.10). **k and l**, Representative micrographs and quantification of inflorescence transition meristem size from WT, *ZmfcP1*, *Zmcle7* and *ZmfcP1 Zmcle7* (n = 7, 15, 15 and 7). Boxplots, 25th–75th percentile; whiskers, full data range; center line, median. ***P = 1×10^{-21} , **P = 0.005; two-tailed, two-sample t-test in **b**, **d**. One-way anova and Tukey test; letters, significance groups at p < 0.05 in **l**. Scale bars, 1 cm in **a**, **h**; 500 μ m in **j** and 100 μ m in **k**.