K-homology Nuclear Ribonucleoproteins
Regulate Floral Organ Identity and Determinacy in Arabidopsis

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Abstract

Post-transcriptional control is nowadays considered a main checking point for correct gene regulation during development, and RNA binding proteins actively participate in this process. Arabidopsis thaliana FLOWERING LOCUS WITH KH DOMAINS (FLK) and PEPPER (PEP) genes encode RNA-binding proteins that contain three K-homology (KH)-domain, the typical configuration of Poly(C)-binding ribonucleoproteins (PCBPs). We previously demonstrated that FLK and PEP interact to regulate FLOWERING LOCUS C (FLC), a central repressor of flowering time. Now we show that FLK and PEP also play an important role in the maintenance of the C-function during floral organ identity by post-transcriptionally regulating the MADS-box floral homeotic gene AGAMOUS (AG). Previous studies have indicated that the KH-domain containing protein HEN4, in concert with the CCCH-type RNA binding protein HUA1 and the RPR-type protein HUA2, facilitates maturation of the AG pre-mRNA. In this report we show that FLK and PEP genetically interact with HEN4, HUA1, and HUA2, and that the FLK and PEP proteins physically associate with HUA1 and HEN4. Taken together, these data suggest that HUA1, HEN4, PEP and FLK are components of the same post-transcriptional regulatory module that ensures normal processing of the AG pre-mRNA. Our data better delineates the roles of PEP in plant development and, for the first time, links FLK to a morphogenetic process.

Author Summary

Unlike animals, angiosperms (flowering plants) lack a germline that is set-aside early in embryo development. Contrariwise, reproductive success relies on the formation of flowers during adult life, which provide the germ cells and the means for fertilization. Therefore, timing of flowering and flower organ morphogenesis are critical developmental operations that must be finely regulated and coordinated to complete reproduction.
Arabidopsis thaliana FLOWERING LOCUS WITH KH DOMAINS (FLK) and PEPPER (PEP) encode two KH-domain RNA-binding proteins phylogenetically related to human proteins characterized by their high developmental versatility. FLK and PEP modulate the mRNA expression of the MADS-box gene FLOWERING LOCUS C, key in flowering control. In this work we have found that FLK and PEP also play a pivotal role in flower organogenesis by post-transcriptionally regulating the MADS-box floral organ identity gene AGAMOUS (AG). Interestingly, FLK and PEP physically interact with proteins involved in AG pre-mRNA processing to secure correct AG function in the floral meristem and flower. Taken together, our results reveal the existence of a post-transcriptional regulatory activity controlling key master genes for floral timing and flower morphogenesis, which might be instrumental for coordinating both developmental phases.

Introduction

Development of multicellular organisms relies on exquisitely controlled transcriptional and post-transcriptional regulatory actions to govern gene expression and accurately respond to endogenous and environmental fluctuations. As exemplified in the reference plant Arabidopsis thaliana (Arabidopsis hereafter), reproductive success in angiosperms largely depends on two developmental events that initiate the reproductive phase: floral timing and flower morphogenesis. Upon flowering, the shoot apical meristem (SAM) transforms into an inflorescence meristem (IM) which will give rise to floral meristems (FMs) [1]. FM identity genes, such as LEAFY (LFY) [2] and APETALA1 (API) [3], are crucial in activating the floral homeotic genes that specify identity of concentric whorls of organs in the Arabidopsis flower [1]. According to the ABC(E) model [4–6], the class A genes AP1 and AP2 specify sepals and, together with the B function genes PISTILLATA (PI) and AP3, contribute to petal identity. Co-expression of B-genes and the C-function gene AGAMOUS (AG) confer male stamen identity, while AG alone specifies female carpels, defining the pistil or gynoecium situated in the innermost whorl. The model also establishes mutual antagonism between A and C activities and requirement of the E activity, represented by the redundant SEPALLATA function [4–9]. With the exception of AP2 (an AP2/EREBP) [10,11], all floral homeotic genes encode type II MADS-box transcription factors, a lineage comprising central regulators in most aspects of plant development [9,12,13].

In addition to floral organ identity, AG plays a crucial role in FM determinacy by repressing the homeobox stem-cell-identity gene WUSCHEL (WUS) [14,15]. WUS and LFY activate AG, which in turn, represses WUS both directly and through the activation of the transcriptional repressor KNUCKLES (KNU) [16], resulting in consumption of the stem cell niche [16–21]. Otherwise, continuing cell proliferation leads to an indeterminate pattern of alternating whorls of sepals and petals, as described in strong ag mutants [22].

Whereas transcriptional control of gene expression is key to development, it is nowadays widely accepted that post-transcriptional operations are crucial to secure proper gene regulation. For example, mounting evidence indicates that mRNA processing steps, such as splicing and polyadenylation, usually proceed co-transcriptionally in a tightly coordinated manner to ensure correct gene activity [23–25]. RNA-binding proteins from multifunctional ribonucleoprotein (RNP) complexes coat nascent transcripts to regulate different aspects of mRNA synthesis, affecting thus, the final levels of gene expression [26, 27].

It has been shown that, in addition to its transcriptional control, post-transcriptional regulation is essential to secure correct AG function during flower development, in particular AG
intron 2 processing [28]. So far three Arabidopsis RNA-binding proteins (RNPs) were found to facilitate this process: HUA1, a nuclear CCCH-type zinc-finger protein [29], the RPR-domain (Regulation of nuclear pre-mRNA) protein HUA2 [30], and HUA ENHANCER 4 (HEN4), containing 5 K-homology (KH) domains and one of the few KH proteins functionally characterized in Arabidopsis [31,28]. Interestingly, hua1 hua2 hen4 triple mutants displayed stamen and carpel homeotic transformations, and loss of flower determinacy as a result of the reduced levels of mature AG mRNA. The fact that HUA1 binds to the AG pre-mRNA and physically associates with HEN4, suggests that both proteins belong to the same RNP regulatory complex [28].

Named after the human heterogeneous nuclear ribonucleoprotein K (hnRNP K) [32], the KH domain is an ancient RNA-binding module present in proteins whose disruption causes important developmental alterations in animals, including human syndromes as fragile-X [33,34], metastasis and cancer progression [35]. The hnRNP K is also representative of the remarkably versatile poly(C)-binding proteins (PCBP), characterized by a stereotypical triple-KH-domain configuration. PCBP play roles in multiple developmental processes in animal systems, from erythropoiesis to neuronal differentiation [36-40]. The KH domain also provides a structural basis for protein-protein interactions, which most likely contributes to the multifunctionality of PCBP [36,41].

In contrast, very little is known about plant PCBP-type hnRNPs and their relevance to plant development or morphogenesis is largely unexplored. So far, only two canonical PCBP-type hnRNP encoding genes, FLOWERING LOCUS WITH KH DOMAINS (FLK) [42,43] and PEP-ER (PEP) [44], have been characterized in Arabidopsis to some extent. FLK promotes flowering in the autonomous pathway by negatively regulating the MADS-box floral repressor FLOWERING LOCUS C (FLC) [42,43,45]. PEP was originally described to interact with element(s) of the WUS pathway [44] and more recently we found that PEP is a positive regulator of FLC activity, hence antagonizing with FLK [46]. In line with this, the late flowering phenotype of flk plants (due to elevated levels of FLC) is rescued in the flk pep background [46]. However, in spite of the fact that PEP is expressed in FM and developing flowers, pep, flk or pep flk double mutants lack conspicuous floral defects, probably reflecting the compensation by overlapping activities [43,44,46].

In this work, we have functionally investigated the magnitude of PEP and FLK roles in flower patterning. Our genetic and molecular analyses place PEP as a positive regulator of the floral C-function by facilitating AG pre-mRNA processing and preventing premature polyadenylation in the large second intron. Here we also show that FLK also contributes to maintain the C-function. Furthermore, we provide evidence that PEP and FLK interact with the previously identified AG mRNA processing factors HUA1 and HEN4, strongly suggesting that all these proteins likely work together as components of a common post-transcriptional regulatory activity. Identifying PEP and FLK as new regulators of AG, broadens the scope of the developmental functions played by plant PCBPs, as they impinge upon the control of master regulatory genes, in this case AG, central during reproductive development.

Results

Genetic interactions with HEN4, HUA1 and HUA2 uncover the contribution of PEP to maintain the floral C-function

As mentioned above, PEP is expressed in FM and developing flowers, but pep flowers are largely normal. Thus, to test whether the role of PEP in floral patterning was masked by redundant gene activities, we combined the null pep-4 allele [44] (pep hereafter) with mutations in HEN4, HUA1 and HUA2, genes that encode post-transcriptional regulatory proteins [28].
Fig 1. PEP regulates flower reproductive organ identity and determinacy. A) Scanning Electron Microscopy micrograph (SEM) of a post-anthesis wild-type flower. The different parts of the pistil have been artificially colored: stigma (yellow), style (purple), ovary (green), gynophore (g, orange), pt, petal; st, stamen; s, sepal. B-F) Post-anthesis flowers of different mutant backgrounds. Some outer organs were removed to better show petaloid stamens in the third
HEN4 is a KH paralog relatively distant to PEP [31]. Unlike 
hen4-2 (hen4 hereafter) and pep single mutants (S1A Fig.) [28,44], ~ 10% of 
hen4 pep flowers exhibited petaloid stems
(Fig. 1A, 1B, and S2B-S2D Fig.). Similarly, hua1-1 mutants (hua1 hereafter) appeared normal
(S1B Fig.) [30], but hua1 pep double mutants displayed abundant petaloid transformations in
the third whorl (40% of the flowers examined; Fig. 1C). We could not obtain hua4 hua1 pep triple
hyomozygous mutants implying that PEP becomes essential in the hua4 hua1 background.
This was noteworthy since hen4 hua1 double mutants flowers look wild-type [28]. Strikingly,
introducing only one pep allele into hen4 hua1 plants (hen4 hua1 pep/+) led to conspicuous fi-

ded alterations including petaloid stems in all flowers (Fig. 1D).

Loss of HUA2 does not cause any obvious floral phenotype [30] and, although HUA2 and
PEP interact during floral timing, hua2-4 pep flowers are normal [46]. However, this might not be surprising as our data indicate that the hua2-4 allele is leaky (S3 Fig.). We therefore used the null hua2-7 allele (hua2 hereafter, unless it is specified otherwise). Double mutants hua1 hua2 showed a variety of flower defects, including stamen-to-petal transformations (Fig. 1E and S1 Table), as reported for hua1 hua2-1 [30]. Unexpectedly, we were unable to isolate hua2 pep or hua1 hua2 pep individuals and only hua1 hua2 pep/+ plants were identified among the progeny. This background was sterile and showed a significant enhancement of the hua1 hua2 floral phenotype, including stronger petaloid transformations (Fig. 1E-I and S1 Table).

Dramatic alterations of fruit morphogenesis in pep, hua and hen mutant combinations

The fruit derives from the fertilized gynoecium carpels, whose formation, in turn, almost entirely depends on C-function [7,47]. We therefore decided to use carpel and fruit development as readout of how pep, hua, and hen mutant combinations affect C-function.

Although fruits in some of the mutant backgrounds were slightly shorter but normal looking (100% in hen4 pep, 20–60% in hua1 pep), we detected pistils with very distorted development, such as unfused carpels, and reduced style and stigma (S1D Fig.). In certain combinations, the apical portion of carpels was pointed with areas of white or pale green tissue conformed by smaller fringe cells as those in the apex of wild-type sepals (Fig. 1K, 1L, 1N, 1O and S2F, S2G Fig.).

The hua1 hua2 double mutant presented shorter pistils broadened at the tip [30] (Fig. 1M and S1D Fig.). However, hua1 hua2 pep/+ pistils were on average much shorter and crumpled (S1D Fig.). Indeed, close inspection of severely affected gynoecia in hua1 hua2 pep/+ by scanning electron microscopy (SEM) revealed that the carpel epidermis, rather than the wild-type characteristic vertical files of smooth cells (Fig. 1P), showed a wide range of epidermal cell sizes with epicuticular wax crenulations, including sepal-like giant cells [48-50] (Fig. 1N, 1Q, 1R).
These alterations are typical of carpel-to-sepal transformation and were also seen in additional pep mutant combinations (Fig. 1K, 1L and S2H-S2K Fig.).

We detected that a significant percentage of hua1 pep pistils (40%) developed supernumerary valves (S1 Fig. and S2L Fig.). This trait is typical of loss of meristem determinacy and it was further enhanced in hua1 hua2 pep/+ (Fig. 1O and S1 Table). Terminal hua1 pep flowers, and at least a quarter of the hua1 hua2 pes/+ flowers exhibited conspicuously long gynophores and gynoecia that, strikingly, contained additional flowers inside. These basically consisted of petals and sepaloid gynoecia recapitulating the sepaloid features seen in the fourth whorl (Fig. 1S, 1T and S2M, S2O Fig. and see below). This phenotype, never observed in hua1 hua2 flowers (S1 Table), was reminiscent of that of ag mutants and also resembled the loss of HEN4 in the hua1 hua2 background [28]. In hen4 hua1 pep/+ a significant fraction of flowers (25%) contained supernumerary sepaloid valves (S2N Fig.), reflecting certain loss of determinacy in this genotype.

Overall, these results indicate that PEP, in collaboration with HUA and HEN genes, act as a positive regulator of the floral C-activity to, therefore, secure the downstream developmental programs depending on this function, such as fruit development.

The mutant combinations described above exhibit very similar developmental defects. Moreover, gene dosage effects in hua1 hen4 pep/+ and hua1 hua2 pep/+ plants illustrate the sensitivity of such backgrounds to PEP activity. These findings strongly suggest that PEP shares redundant developmental functions with HUA1, HUA2 and HEN4 despite their protein structural disparity. Accordingly, hen4 hua1 hua2/+ pep/+ plants showed very dramatic floral alterations (Fig. 1U-W and S2P-S2R Fig.). Hence, these factors were tentatively included in a common gene activity abbreviated as HUA-PEP along this work.

The lack of HUA-PEP activity causes sepaloid transformations in ful gynoecia

The MADS-box regulatory gene FRUITFULL (FUL) [51] is crucial for valve formation during ovary patterning, and it does so, in part, by preventing valves from adopting valve margin identity through the negative regulation of valve margin identity genes [52–56]. Upon fertilization, ful lignified valve cells remain small, arresting stomata development and silique growth. However, replum cells develop normally leading to a characteristic zig-zag configuration of this tissue in ful fruits [51] (Fig. 2A, 2B and S4A Fig.). Additionally, ful siliques show elongated styles [57] (Fig. 2A and S4A, S4K Fig.).

To get more insights into the role of HUA-PEP activity during pistil development, we decided to characterize the behavior of hua-pep activity mutants in the ful background.

The ful-1 hua1 fruit was virtually identical to that of ful-1 plants [51] (ful hereafter; S4A Fig.). In contrast, ful pep, ful hua2 and ful hua1 pep siliques were progressively longer and showed shorter styles (S4A Fig.). In such backgrounds, valve epidermal cells were elongated and streaked, along with interspersed stomata. These phenotypes indicated that valves took onto sepaloid identity (Fig. 2B and S4B Fig.). In ful hua1 hua2, gynoecia were smaller and replum cells remained small as in wild-type unpollinated pistils [58], abolishing the characteristic zig-zag shape (Fig. 2A, 2B and S4A Fig.). Fertility in ful hua1 hua2 plants was severely reduced.

ful hua1 hua2 pep/+ plants were phenotypically identical to hua1 hua2 pep/+. In such combinations, we also found new floral organs developing inside swollen gynoecia that were often seating on long gynophores (Fig. 2E and S4A, S4E, S4I, S4L, S4M Fig.).

The glucuronidase (GUS) reporter harbored by the ful-1 transposon reflects the native expression pattern of FUL [51]. Pistils of ful or wild-type-looking heterozygous ful/+ plants displayed characteristic GUS activity in the valves, style and nectaries [51] (Fig. 2C and S4D, S4G...
Fig 2. The loss of HUA-PEP activity is epistatic over the ful phenotype. A) SEM images of the top portion of a ful fruit. The typical long style and wide zig-zag replum were suppressed in ful hua1 hua2 and ful hua1 hua2 pep/+ pistils, and sepaloid giant cells were observed on the valve surface. Simple or branched trichomes (white and yellow arrows, respectively) were occasionally observed on the surface of ful hua1 hua2 pep/+ pistils. B) SEM images of the abaxial ovary surface in wild-type (WT) and different mutant backgrounds. Observe interspersed stomata (arrows) in a ful hua1 hua2 pep/+ panel. C-F) GUS reporter whole-mount staining (ful-1) in ful (C), ful hua1 hua2 (D), ful hua1 hua2 pep/+ (E) pistils and wild-type sepal (F). Observe long gynophores and full petaloid conversion of stamens in (E). Scale bars: 100 μm (A, B), except 10 μm in the last B panel (ful hua1 hua2 pep/+ genotype), and 1 mm (C-F). r, replum; v, valve.

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In *ful hua1 hua2*, strong GUS signal was detected in nectaries and apical territory preserving style identity, whereas valves presented a more irregular pattern (Fig. 2D and S4H Fig.). The GUS-staining pattern of *ful hua1 hua2 pep/+* in the fourth whorl organs had little resemblance to that of a gynoecium, except in nectaries and style vestiges, notably evoking *FUL* expression in the sepal vasculature [51] (Fig. 2E, 2F and S4E, S4F, S4I Fig.).

Next, we treated flowers with the lignin-specific dye phloroglucinol. Mature wild-type fruits showed preferential staining in the valve margin, whereas in *ful* mutants valves were ectopically lignified [52,53] (S4J, S4K Fig.). Nonetheless, in equivalent flowers from *ful hua1 hua2 pep/+* plants, lignification in the presumptive gynoecium was nearly restricted to branched red lines with striking resemblance to lignified sepal vasculature (S4L-S4N Fig.). Altogether, genetic and histochemical analyses indicate that the *HUA-PEP* gene activity is required to prevent gynoecium tissues from adopting sepaloid fate independently of their original identity (valve, valve margin), highlighting the role played by *PEP* to preserve carpel identity.

**PEP is a positive regulator of AG functional mRNA levels**

To determine whether *PEP* impinges upon *AG* regulation and therefore C-function, we measured mRNA levels from wild-type and mutant flower buds by quantitative PCR (qPCR). In consonance with the phenotypes described above, relative expression of *AG* decreased significantly in *hua1 pep* and *hua1 hua2* double mutants, and reduced even further in *hua1 hua2 pep/+* plants (Fig. 1X).

To investigate whether somehow *PEP* (and *HUA*) control A and B function, we measured the transcript levels of the homeotic A- and B-class genes *AP1* and *PI*, respectively. Results were inconclusive because, although expression of both genes declined moderately in some mutant strains (S5 Fig.), no morphological evidence of altered A- or B-floral functions was observed in any of the *hua-pep* mutant combinations examined. Therefore, our molecular and genetic data suggest that, in contrast to the C-function, it appears that *HUA-PEP* gene activity has little or no role in regulating A- and B-functions.

*AG* triggers several reproductive developmental programs in part by activating additional regulators that perform different subsets of its functions. For example, *SPOROCYTELESS* (*SPL*) stimulates stamen development, including organ identity [59–61], whereas the zinc-finger gene *KNU* cooperates with *AG* to repress *WUS* [19,21]. Consistently, *SPL* and *KNU* expression decreased markedly in *hua1 pep* and *hua1 hua2* double mutants, and *hua1 hua2 pep/+* plants (S6A, S6B Fig.). Accordingly, *KNU* gene expression monitored by a GUS-reporter construct was found to be less intense in *hua1 pep* developing flower organs, as compared to the wild type (S6C-S6H Fig.).

Interestingly, we found that the mutant phenotype of *hua1 pep* plants was completely rescued by increasing the dosage of *AG* gene with a genomic construct able to complement *ag* mutants [62] (S7 Fig.), thus reinforcing our hypothesis that *AG* functions depend on *HUA-PEP* activity genes. Collectively, these results might explain the organ identity and determinacy defects seen in *pep hen hua* combos and further support *PEP* as a positive regulator of *AG*.

**PEP prevents AP1 expansion to inner whorls**

One of the functions of *AG* is to prevent *AP1* expression in the two inner whorls of organs where stamens and carpels normally form [8]. To examine the expression of *AP1* we used the genomic GFP (green fluorescent protein)-based reporter *gAP1::AP1-GFP*, that largely mirrors endogenous *AP1* expression [62]. As expected, in the wild type *AP1*-GFP signal was detected in sepals but absent in pistils (S8 Fig.). However, a number of *hua1 pep* pistils showed *AP1*-GFP fluorescence (Fig. 3A-D). These results are coherent with earlier work showing *AP1*...
Fig 3. Detection of the AP1-GFP protein in *hua1 pep gynoecia*. A) Apical region of a mildly affected gynoecium with recognizable pistil morphology. Specific AP1-GFP signal is detected in some style cells. B) Fourth whorl organs of a pre-anthesis flower displaying a severe sepaloid phenotype. C) Adaxial (inner) view of a manually open pistil with severe sepaloid transformations, but containing some developing ovules (do). D) Detail of a fourth whorl organ from panel D showing nuclear-localized AP1-GFP. A cell has been outlined with a dotted line and the nucleus marked with an arrow. Scale bars: 25 μm (A), 50 μm (B and C) and 10 μm (D).

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mRNA ectopic expression in inner whorls of hua1 hua2 and hua1 hua2 hen4 [28,30], and underscore the importance of PEP as a regulator of the C-function during flower organogenesis.

**PEP overexpression impairs flower morphogenesis**

Our loss-of-function genetic analyses show that components of the HUA-PEP function are redundantly required for the floral C-function. So we asked whether PEP alone could compensate for the deficiency in members of this activity. To test this idea, a 35S::PEP overexpressing construct [46] was introduced into the hua1 hua2 background. Strikingly, PEP overexpression, instead of rescuing, dramatically enhanced the hua1 hua2 mutant phenotypes. Homozygous hua1 hua2 35S::PEP flowers were sterile, and exhibited much stronger stamen-to-petal and carpel-to-sepal transformations than in hua1 hua2, as well as frequent severe indeterminacy defects, a trait never observed in hua1 hua2 plants (Fig. 4A-D, 4F-H, S9A-S9E Fig. and S1 Table). In line with the strong phenotypes observed, the levels of AG, KNU and SPL mRNAs in hua1 hua2 35S::PEP plants were significantly lower than those of hua1 hua2 mutants (Fig. 4I and S9K, S9L Fig.).

We ruled out any RNA silencing effect as hua1 hua2 35S::PEP plants showed much higher PEP mRNA levels than wild-type individuals (S10D Fig.). Rather, PEP protein overproduction might exceed a certain critical threshold, leading to the strong phenotypes observed. Consistent with this idea, hemizygous hua1 hua2 35S::PEP/+ plants produced PEP mRNA levels higher than those of the wild type, yet much lower than in homozygous hua1 hua2 35S::PEP plants (S10D Fig.), and did not show the severe floral phenotypes of the latter, being indistinguishable from hua1 hua2 individuals (S10E-J Fig. and S1 Table).

Although, PEP overexpression in hen4, hua1 and hua2 single mutant backgrounds did not result in noticeable morphological alterations (S10A-C Fig.), we speculated whether excess of PEP was critically detrimental in more compromised conditions. In line with this interpretation, PEP overexpression in the wild-type looking hen4 hua1 plants [28] led to the same developmental abnormalities previously described for the strong deficient hua-pep backgrounds. A significant number of hen4 hua1 35S::PEP flowers (~65%) displayed severe indeterminacy, closely resembling ag flowers (Fig. 4E and S9F-J Fig.). It is worth mentioning that this phenotype never occurred in hen4 hua1 pep/+, indicating that PEP gain-of-function has a stronger impact on floral determinacy in hen4 hua1 than reducing PEP activity, similarly as described for hua1 hua2 background (S1 Table).

**PEP secures correct AG function by facilitating pre-mRNA processing**

Mutations in HEN4, HUA1 and HUA2 led to a gradual decrease of AG mRNA levels concomitant with the accumulation of aberrant transcripts incorrectly terminated at the large second intron [28]. To test whether PEP impacts on this process, we carried out qPCR assays using intronic primers situated near the exon2/intron2 junction (Fig. 4J and S2 Table). The relative abundance of a PCR product increased progressively in various hua-pep mutant strains, notably in hua1 hua2 pep/+ and hua1 hua2 35S::PEP individuals, whereas it was barely detectable in the wild type (Fig. 4J). These values negatively correlated with the levels of correctly spliced AG transcript in the mutant backgrounds under study, and unambiguously indicated that altering levels of PEP has an important impact on the accumulation of these transcript species.

To examine transcript structure, polyadenylated RNA from hua1 hua2 pep/+ and hua1 hua2 35S::PEP plants was subjected to 3' RACE (Rapid Amplification of cDNA Ends). Several products were obtained corresponding to transcripts comprising correctly spliced exons 1 and 2 followed by a variable stretch of nucleotides of intron two (105–368 nt), after which premature cleavage and polyadenylation events took place (S11A, S11B Fig.). These transcripts miss
Fig 4. PEP overexpression impairs flower morphogenesis and AG pre-mRNA processing. A-D) hua1 hua2 35S::PEP flowers. E) hen4 hua1 35S::PEP flower. In both genotypes, loss of determinacy was frequent. All flowers displayed severely transformed petaloid stamens and sepaloid carpels. F-H) SEM micrograph of a hua1 hua2 35S::PEP flower (G), and close-up views of the fourth whorl organ abaxial surface (F) and inner additional whorl organ (H), respectively. Sepaloid traits were found in these gynoecia. I, J) Relative expression levels of AG mRNA (I) and AG transcripts including intron 2 sequences (J), in wild-type plants (WT) and diverse hua-pep mutant backgrounds, monitored by qPCR. In (J), a diagram of the AG gene is shown below. Purple boxes denote exons whereas intronic regions are colored in orange. Relative positions of forward (black arrow) and reverse (red arrow) primers are indicated. To increase annealing specificity, the forward primer sequence was split between exons 1 and 2. Error bars, SD. Asterisks indicate statistically significant differences from hua1 hua2 plants (**P < 0.01). Scale bars are 1 mm (A-E, G) and 100 μm (F, H).

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the last 6 exons, lacking the ability to encode a functional AG polypeptide. In plants, three polyadenylation signals define the site of processing: the far upstream element (FUE), the near upstream element (NUE), and the cleavage element (CE) [63]. Inspection of such RACE products revealed the presence of FUE, NUE and CE elements properly situated, strongly suggesting their implication in the premature termination event [63] (S11A, S11B Fig.).

**FLK: an additional component of the HUA-PEP activity**

FLK is expressed in all major organs, yet its loss of function did not cause any visible defect [42,43]. FLK interacts with PEP and HUA2 during flowering time regulation [46] but its possible role in flower morphogenesis has not yet been studied.

To explore FLK activity during flower development and to determine whether FLK participates in the HUA-PEP function, the null flk-2 mutant [43] (flk hereafter) was crossed to different hua-pep mutant combinations, flk hen4 double mutant flowers were wild-type in appearance (S12A, S12B Fig.). Unlike hua1 pep (Fig. 1 and S1 and S2 Fig.), flk hua1 and flk pep double mutant flowers also looked essentially normal (S12C Fig.) [46]. In contrast, flk pep hua1/+ plants showed some aberrant gynoecia, and petaloid stamens (S12D-F Fig.). Interestingly, stamen identity in the flk pep background, therefore, is sensitive to HUA1 gene-dosage since this trait is never observed in pep hua1/+, nor in flk pep flowers.

Next, the flk mutant was crossed to hua1 hua2 plants, a sensitized background repeatedly used to uncover gene activities involved in flower organ identity and determinacy [28,64–66, this work]. The resulting flk hua1 hua2 triple mutants were easily identified because of their conspicuous flower defects. flk hua1 hua2 flowers had two sets of petals and were “stamenless” (Fig. 5A and S12G Fig.), thus lacking fertilization and fruit set. Besides, flk hua1 hua2 gynoecium development was severely distorted with obvious sepaloid attributes (Fig. 5B, 5C). Nevertheless, the most defining feature was again the occurrence of indeterminate flowers (>50%) (Fig. 5B, 5D, 5E and S12H Fig.). As indicated above, hua1 hua2 flowers never show this severe developmental alteration, underscoring the contribution of the flk mutation to debilitate the floral C-function.

Our qPCR gene expression data backed up the hypothesis of FLK as part of the HUA-PEP activity. In flk the expression levels of AG, KNU and SPL remained unaltered when compared to those of the wild type, whereas in flk hua1 hua2 significantly dropped, being even lower than in hua1 hua2 individuals (Fig. 5F and S12I Fig.). This result substantiates the floral defects detected. Conversely, levels of AG transcripts containing intron 2 sequences increased in flk hua1 hua2 (Fig. 5G), suggesting an influence of FLK on AG post-transcriptional regulation. Indeed, we performed 3’ RACE assays for RNA from flk hua1 hua2 and identified new aberrant transcripts indicating premature cleavage and polyadenylation within the large intron 2. As described above, polyadenylation signals were found around the presumptive maturation site (S11C Fig.). Altogether, these results strongly support FLK as an additional component of the HUA-PEP activity.

**The components of the HUA-PEP activity physically associate**

As mentioned in the introduction, RNA binding proteins participate in multimeric RNP complexes to perform their regulatory functions [36,41]. Our genetic and expression analyses indicated that genes of the HUA-PEP activity act in concert during floral organogenesis, which makes reasonable their interplay at the protein level. Nuclear localization of their products has been demonstrated [28,42,43,46-67] and, importantly, physical interaction between HEN4 and HUA1 has been already established [28]. Moreover, HEN4 was also computationally predicted to interact with PEP and FLK [68]. We therefore, conducted in vivo bimolecular fluorescence
Fig 5. Loss of FLK dramatically enhances the floral phenotypes of hua1 hua2 plants. A) flk hua1 hua2 flower with all stamens converted into petals. B) Gynoecium with a long gynophore. A sepaloid valve was manually removed to better observe a new flower developing inside. C) SEM image of a sepaloid.

**AG**

| Relative mRNA expression |
|--------------------------|
| WT | flk | hua1 hua2 | flk hua1 hua2 |
| 1  | 1.25 | **0.25** | **0.25** |

**AG intron 2**

| Relative RNA expression |
|-------------------------|
| WT | hua1 hua2 | flk hua1 hua2 |
| 5  | 30 | **30** |

**Note:** The ** symbols indicate statistical significance.
complementation (BiFC) assays in tobacco leaves using PEP, FLK, HEN4 and HUA1. Reconstituted yellow fluorescent protein (YFP) was detected in leaf cell nuclei when FLK-PEP, HEN4-PEP and HUA1-PEP interactions were assayed, respectively (Fig. 6 and S13 Fig.). Similarly, robust nuclear interaction was seen when FLK was tested against HUA1 and HEN4 (Fig. 6 and S13 Fig.). The HUA1-HEN4 BiFC interaction was used as a positive control (S13 Fig.). All associations were tested in both directions, thus endorsing specificity of the interactions (Fig. 6 and S13 Fig.).

We were also able to confirm in vivo protein homodimerization of PEP and FLK in our assays, corroborating the publicly available in silico data [68] (S13 Fig.). Homodimer formation was also seen in HUA1 BiFC experiments (S13 Fig.). These associations were further verified in yeast-two-hybrid assays (Y2H; S14 Fig.).

In a subset of our BiFC assays we detected, in addition to clear signal in the nuclei, specific cytoplasmic fluorescence (Fig. 6 and S13 Fig.). KH-domain containing proteins, particularly PCBPs, are known to participate in numerous RNA processing events in the nucleus and in the cytoplasm (RNA transport, stability, translation) [36–38]. Therefore, this extranuclear signal might reflect additional regulatory roles for PEP and FLK in this cell compartment.

Taken together, these results strongly suggest that PEP, FLK, HUA1 and HEN4 proteins physically associate likely reflecting their participation in common multimeric complexes involved in pre-mRNA processing. Additionally, these data further reinforce the assumption of FLK as a new partner of the HUA-PEP activity.

Discussion

PEP and FLK were previously identified to control flowering time through regulation of the FLC gene [46,42]. Now, our analyses show that PEP and FLK also play a key role in the specification of flower organ identity as components of the post-transcriptional machinery that ensures normal processing of the AG pre-mRNA. Genetic, functional and molecular interactions with additional RNA-binding proteins previously established as AG regulators [28] led us to define HUA-PEP as a common gene activity comprising HUA1, HUA2, HEN4, PEP and FLK.

**PEP** is a positive regulator of C-function activity during flower morphogenesis

We have demonstrated that PEP is functionally linked to the AG pre-mRNA processing pathway. Whereas hua1, hua2 and hen4 single mutants are phenotypically wild-type [28, this work], when these same mutants were combined with pep, we observed developmental abnormalities consistent with reduced C-function activity. Moreover, hua1 hua2 double mutant flower defects [30] were dramatically enhanced when combined by plants that were heterozygous for a mutation in PEP (hua1 hua2 pep/+), illustrating dosage-effects among HUA-PEP genes as previously reported for HUA1, HUA2 and HEN4 [28]. The intensity of these floral phenotypes correlated with a reduction in AG mRNA levels. As a result, the A-function gene API, which is normally expressed in whorls 1 and 2, was ectopically expressed in the inner whors of hua1 pep flowers, consistent with a compromised C-function and the A-C antagonism [4,8]. The sepaloid transformations seen in gynoecium tissues when HUA-PEP genes
Fig 6. The hnRNPs PEP and FLK physically interact with HUA1 and HEN4. BiFC visualization of protein dimerization (yellow fluorescence) in *Nicotiana benthamiana* leaf cells agroinfiltrated with plasmids encoding fusion proteins. In each test, the first protein was fused to the C-terminal fragment of the YFP (YFPct), and the second protein to the N-terminal portion (YFPnt), respectively (see Materials and Methods section).

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were mutated in the ful background provided further evidence for the critical contribution of PEP to carpel identity.

Loss of PEP contributed to reduce the floral C-function activity. Surprisingly, PEP overexpression in hua1 hua2 and hua1 hen4 also caused a dramatic enhancement of flower mutant phenotypes. Although this might seem unexpected, there are many examples in which loss- and gain-of-function result in the same phenotypical alterations. Loss and overexpression of bancal, encoding a Drosophila homologue of vertebrate hnRNP K, generates appendage developmental defects [69]. In Xenopus embryos, both reduction and overexpression of the KH gene Mex3b, involved in neural plate formation, led to downregulation of target genes [70]. In Arabidopsis, increasing or reducing the expression of kinase-encoding genes FAB1A/B elicits the same pleiotropic alterations, which are attributed to perturbations in the protein complexes in which they participate [71].

However, PEP overexpression in wild-type or single hua-pep mutant backgrounds rendered normal flowers, suggesting certain buffering capacity against PEP excess. Nevertheless, simultaneous inactivation of various HUA-PEP components (hua1 hua2 or hen4 hua1) when PEP is overexpressed might aggravate a detrimental excess of PEP, by likely disrupting protein stoichiometric equilibria [72]. In line with this hypothesis is the fact that hemizygous hua1 hua2 35S::PEP/+ plants, expressing higher levels of PEP than the wild type but much less than homozygous hua1 hua2 35S::PEP plants, do not differ from hua1 hua2 double mutants.

**FLK is a member of the HUA-PEP gene activity**

Our analyses have also uncovered a role for FLK in plant morphogenesis. FLK participates in the HUA-PEP activity during C-function maintenance. The genetic interaction between flk, pep, hua1 and hua2, the phenotypic similarities between flk hua1 hua2 (Fig. 5) and hen4 hua1 hua2 [28], the gene expression analyses, as well as FLK physical associations, firmly support this conclusion.

FLK represses FLC and thus promotes flowering whereas PEP and HUA2 are FLC activators [42,43,46,73,74]. During flower morphogenesis, however, FLK and PEP promote flower morphogenesis through the positive regulation of AG (this work). Taking into consideration the promiscuity of RNA-binding proteins, it is very plausible that components of the HUA-PEP activity might be participating in functionally distinct complexes. This is not unprecedented. For example Arabidopsis SR (serine/arginine rich) factors and the hnRNP AtGRP8 exhibit antagonistic and cooperative effects during circadian regulation [75]. Also, closely related MADS-box genes AGAMOUS-LIKE 24 (AGL24) and SHORT VEGETATIVE PHASE (SVP) accelerate and delay flowering, respectively. Later, AGL24 and SVP cooperate with AP1 to downregulate AG during first stages of floral development [76–78]. Similarly, FUL-SVP replaces FLC-SVP heterodimers countering the repressive effect of the latter on flowering time [79]. Moreover, AG and AP3/PI participate in the same protein complexes to specify stamen anlagen. However, many genes promoting carpel development that are induced by AG are, on the contrary, repressed by AP3/PI [80]. Functional versatility of the HUA-PEP activity, in turn, might be very advantageous to provide regulatory flexibility to modulate the highly dynamic and complex networks governing reproductive development.

**Association of HUA-PEP proteins**

PEP and FLK physically associate, as well as with HUA1 and HEN4, indicating that, probably, they all participate in common regulatory complexes. HUA2, however, might affect AG independently since no physical interaction between HUA2 and any other HUA-PEP component described here could be detected in a recent Y2H screen [81]. Formally, HUA2 molecular
interactions might be mediated through HUA-PEP factors yet to be identified. We observed stronger phenotypes in hua-pep backgrounds when HUA2 was mutated. These results might be explained with the existence of two complementary subactivities: one incorporating the HUA2 function and another one comprising the remaining identified HUA-PEP factors. Simultaneous disruption of both complexes might account for more profound phenotypic defects. Lethality in hua2 pep mutants substantiates this notion.

PEP and FLK secure AG expression by mediating correct RNA processing

Our molecular analyses of hua-pep mutants are coincident with previous work showing accumulation of transcripts retaining intronic sequences at the expense of the functional AG mRNA [28]. A large intron where important regulatory motifs reside is a feature shared by AG, FLC and other MADS-box genes, that is conserved across species [82–87]. However, nascent transcripts are vulnerable to premature processing and large introns might increase the risk of cryptic signals recognizable by the splicing and/or polyadenylation machineries [88,89].

Transcript maturation mainly proceeds co-transcriptionally, increasing the fidelity of the process [24,90,91]. Altering PEP and FLK expression in the hua1 hua2 background had a profound effect on the accumulation of AG intron-retaining transcripts. Remarkably, FLC intron-containing transcripts also increased in pep plants [46]. We propose that the HUA-PEP proteins assist transcription elongation by “hiding” cryptic signals in the nascent RNA (Fig. 7A–C). Otherwise, these sites could be accessible to the corresponding processing machinery, giving rise to non-functional or prematurely terminated transcripts (Fig. 7D). Our hypothesis is consistent with the recent characterization of mammal PCBPs as global regulators of alternative polyadenylation. Knock down of PCBPs actually favors usage of cryptic intronic sites [40]. Interestingly, hnRNP K suppresses usage of a premature polyadenylation site for NEAT1, a long non coding RNA (lncRNA) operating in nuclear paraspeckles (ribonucleoprotein bodies) formation, thus increasing the ratio of the long effective transcript [92].

By sequestering intronic polyadenylation motifs, PEP (and the remaining HUA-PEP factors) may also facilitate correct splicing, as documented for other PCBPs [36,41]. The U1 snRNP (U1), in addition to its splicing role, protects pre-mRNAs from premature termination at intronic polyadenylation sites [88,89], raising the attractive possibility of a connection with the HUA-PEP gene activity. The carboxyl-terminal (CTD) domain of eukaryotic RNA polymerase II coordinates transcription and transcript maturation [93]. The Arabidopsis KH protein SHINY1 (SHI1) interacts with a phosphatase that dephosphorylates particular residues in CTD, downregulating transcription of abiotic stress-related genes by preventing 5’ capping [94,95]. Uncovering new functional and molecular relationships among distinct HUA-PEP components will certainly provide a better understanding of the developmental programs regulated by this activity (floral timing; flower patterning) and the importance, at the regulatory level, of multifunctional plant PCBP-type hnRNPs.

Materials and Methods

Plant material

This work was carried out with the Arabidopsis thaliana Columbia (Col-0) accession as the wild type. Strains previously obtained in other accessions were backcrossed at least five times into Col-0 before any further experiment. Plant materials used in this study were pep-4 [44], flk-2 [43], hua2-4 [73]; hua2-7 [74], 35S::PEP [46], and ful-1 [51]. gAG::AG-GFP and gAP1:: AP1-GFP [62] were provided by Gerco Angenent and Richard Immink (Wageningen
Fig 7. The HUA-PEP activity facilitates pre-mRNA processing of target genes. A) As the RNA polymerase (RNA pol II) activity progresses, the HUA-PEP hnRNP complex coats the nascent transcript, still chromatin-associated, thus sequestering intronic cryptic sites (ICS) from cleavage and polyadenylation. B) The elongation complex reaches the distal terminal cleavage and polyadenylation site (CPS), where correct termination occurs. C) Adequate intron excision and 3’ maturation take place. D) Conversely, an altered HUA-PEP activity does not prevent the RNA 3’ processing machinery to access cryptic motifs in the elongating transcript, producing thereby a prematurely terminated and ineffective RNA.

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University, The Netherlands). *hen4-2* [28], *hua1-1* and *hua2-1* [30] were provided by Xuemei Chen (UC Riverside, USA). *KNU::GUS* [16] was provided by Anna M. Koltunow (CSIRO, Adelaide, Australia). Information about all primers used in this work and molecular genotyping can be found in S2 Table. Plants were grown in MS plates or soil as previously described [44].
Microscopy and histology
Phloroglucinol lignin staining [96,97] and GUS assays were performed essentially as described [44,96,97]. All GUS analyses, except in the case of ful-1/+, were performed in homozygous lines. Whole-mount pictures were taken under a Nikon SMZ1500 stereomicroscope. Histological sections (8 μm) were photographed under bright-field or dark-field illumination using a Nikon E800 microscope. In both cases Nikon Digital Camera DXM1200f was used operated by the ACT-1 2.70 program. Scanning electron microscopy (SEM) was according to [44]. For confocal laser scanning microscopy, all analyses were performed in homozygous lines. Samples were pre-treated with methanol/acetone (1:1 v/v) solution for 30 minutes at -20°C, and subsequently rinsed in PBS buffer (1.94 mM K2PO4; 8.06 mM Na2PO4; 2.7 mM KCl, 0.137 mM NaCl, pH 7.4) to be observed under a Leica TCS SPE confocal microscope. Pictures were taken with the LAS AF program.

Quantitative RT-PCR and RACE
For quantitative RT-PCR (qPCR), 5 μg of total RNA was extracted from young flower buds until stage 9, treated with DNase I, and used for cDNA synthesis with an oligo(dT) primer and RevertAid Premium Reverse Transcriptase (Thermo Scientific) following the manufacturer’s instructions. Subsequently, for each qPCR reaction, 0.5 μl of the cDNA was used as template. Relative changes in gene expression levels were determined using the LightCycler 1.5 system with the LightCycler FastStart DNA amplification kit according to the manufacturer (Roche Diagnostics). RNA levels were normalized to constitutively expressed genes OTC (ORNITINE TRANSCARBA-MILASE) [98] and ACT2 [99], and the corresponding wild-type levels, as previously reported [46,100]. Each experiment was undertaken using three biological replicates with three technical replicates each. The standard deviation was calculated in Microsoft Excel. Statistical significance was estimated by the Student’s t-test according to [101] (**P < 0.01, ***P < 0.001).

For 3’ rapid amplification of cDNA ends (3’ RACE), 5 μg of young flower bud total RNA was reverse transcribed using Maxima Reverse Transcriptase and the adaptor oligo d(T)-anchor (kit 5’/3’ RACE, Roche Diagnostics) as a primer. Then, AG cDNAs were amplified with High Fidelity PCR Enzyme Mix (Thermo Scientific) using forward primers situated in the exon 2 (S2 Table) and the PCR anchor (Roche Diagnostics) as a reverse primer hybridizing with the adaptor sequence, thus ensuring that only polyA-containing sequences were amplified. Amplified products were cloned into pSC-A plasmids and sequenced with M13F and M13R primers. Sequences were analyzed using CLUSTAL-W aligning [102].

Protein interactions
For bimolecular fluorescence complementation (BiFC), coding sequences of all genes under study were amplified from their respective cDNAs using Phusion Taq-polymerase (NEB). The corresponding primer sequences (S2 Table) were designed for cloning the resulting PCR amplicons via Gibson DNA assembly method [103], and cloned into both the pBJ36-SPYNE and pBJ36-SPYCE plasmids, containing N-terminal (nt) and C-terminal (ct) halves of the yellow fluorescent protein (YFP), respectively (YFPnt and YFPct) [104]. The 35S::SPYNE and 35S::SPYCE cassettes were then cloned via NolI into the binary vectors pGreen0229 and pGreen0179 [105], respectively. Transformed AGL-0 Agrobacterium tumefaciens cells were used to infect Nicotiana benthamiana leaves. YFP reconstituted fluorescence was visualized 72 h after inoculation under a Nikon Eclipse TE2000-U epifluorescence microscope. The reciprocal BiFC assays were also performed obtaining the same results as shown in Fig. 6 and S13 Fig. As negative controls, Nicotiana leaves were co-infiltrated with the corresponding recombiant YFPct construct and the empty YFPnt version, yielding no signal in any case.
For yeast two-hybrid assays, the cDNAs for PEP, FLK, HEN4 and HUA1 genes were amplified with the proof-reading Phusion Taq-polymerase (New England Biolabs, Inc.) using the corresponding primers (S2 Table). The resulting products were cloned into the pB42AD (+Trp) and pGilda (+His) vectors via Gibson DNA assembly procedure [103]. The integrity of constructs was checked by sequencing. The yeast strain EGY48 (-Ura) was cotransformed with the corresponding combinations of pGilda and pB42AD constructs. Empty vectors were used as negative controls. Positive colonies were selected on solid media (-Ura, -His, -Trp +glucose). Induction for testing protein–protein association was assayed growing the resulting yeast strains on plates or liquid in the presence of galactose and raffinose (DB Falcon). X-gal was used for colorimetric assays on plates, and ONPG (2-Nitrophenyl β-D-galactopyranoside, SIGMA) for β-galactosidase liquid experiments. The Clontech protocol book was followed for all these procedures.

Supporting Information

S1 Fig. Flower phenotypes of single mutants and mutant combinations between pep and hen4, hua1 and hua2. A–C) Flowers and young pollinated pistils of hen4, hua1 and hua2–7, respectively. Front sepals and petals were manually removed to show wild-type looking stamens. D) Siliques/gynoecia from wild-type (WT) and different hua-pep mutants. Scale bars: 1 mm. (TIFF)

S2 Fig. Additional flower phenotypes of mutant combinations between pep and hen4, hua1 and hua2. A) Post-anthesis wild-type flower after removing some outer organs. B–D) SEMs of the adaxial side of a hen4 pep anther partially transformed into a petal-like organ. The transformed organ retains staminoid features, and even pollen production (B). The apical portion shows petaloid histology (C), whereas normal anther cells occur at the base (D). E, F) Top portion of apically open hua1 pep (E) and hua1 hen4 pep/+ (F) pistils. Observe little stigmatic development, absent style, and white pointed tip (arrow). G) SEM of the apical portion of a hua1 hua2 pep/+ gynoecium. H–K) SEMs of wild-type valve epidermal layer (H), close-up view of hua1 pep valve territory showing irregular striated cells (J) and wild-type sepal (K). L) hua1 pep pistil with supernumerary valves (arrows), open at the top and containing residual style and stigma tissues. M) A hua1 pep gynoecium. A fourth whorl organ was manually removed to show developing floral organs inside (white arrow) together with normal ovules (yellow arrow). N) SEM of a hen4 hua1 pep/+ gynoecium displaying an extra valve (white arrow). O) A hua1 hua2 pep/+ gynoecium in which a valve-like organ was manually removed to show additional flower organs inside. P–R) Flower phenotypes of hua1 hen4 hua2/+ pep/+ plants. Scale bars: 1 mm (A, F, L–R), 500 μm (E), 100 μm (B, G, J), 10 μm (H, I, K) and 2 μm (C, D). (TIFF)

S3 Fig. hua2–4 is a leaky allele. Expression levels of HUA2 mRNA from 16-day-old wild-type (WT, Col-0) and hua2–4 mutant individuals monitored by quantitative RT-PCR (qRT-PCR). Error bars, standard deviation (SD). (TIFF)

S4 Fig. Fruit phenotypes resulting of combining hua-pep mutants with the ful background. A) SEM images of fruits/gynoecia from ful and hua-pep mutant combinations. In ful hua2 and ful pep plants the style was shortened and stripes of longer cells occurred on the valve, likely explaining a modest increase in size with respect to ful fruits. This trend was enhanced in the ful hua1 pep triple mutant. These plants produced longer fruits with thinner repla (r) and further reduction of the style territory. In ful hua1 hua2 these latter traits were even further pronounced but gynoecia were smaller due to associated fertility problems. In ful hua1 hua2 pep/+, where
long gynophores are common, gynoecia appear bulged as a consequence of additional floral organs growing inside. B, C) Details of valve territory of ful hua2 (B) and ful hua1 hua2 (C) flowers. D, E) Whole-mount FUL GUS-staining (ful-1) in ful/+ wild-type looking pistil (D) and ful hua1 hua2 pep/+ gynoecium (E). F-I) FUL GUS staining in cross-sections of wild-type sepal (F) and fully developed fruits of ful (G), ful hua1 hua2 (H), and ful hua1 hua2 pep/+ (I) mutants. In ful, characteristic valve staining (G) can be appreciated. In (H) and (I) GUS signal is faint, essentially coinciding with the vasculature. Big cells (bc) appear on the outer surface (H), like in sepals (F). Inner additional floral organs (fo) can be seen in ful hua1 hua2 pep/+ (I). J-N) Whole-mount lignin-specific staining with phloroglucinol. Mature wild-type (J) and ful (K) siliques. In wild-type red phloroglucinol staining is limited to dehiscence zones whereas in ful staining is detected in valves due to the ectopically lignification of this territory. In ful hua1 hua2 pep/+ (L, M), lignification is largely coincident with the vascular system, closely resembling that of a wild-type sepal (N). Dissection of the fourth whorl reveals the presence of additional floral organs inside (M). Observe staining domains connected to basal structures as the long gynophore and the floral stalk (M, red arrows). Scale bars: 1 mm (A, D, E, J-M), 100 μm (B, C, F-I) and 500 μm (N).

S5 Fig. Expression of AP1 and PI genes in hua-pep mutant backgrounds. Relative mRNA expression of AP1 (A) and PI (B) in the wild type (WT) and diverse mutant backgrounds, monitored by qPCR. Error bars, SD. Asterisks indicate statistically significant differences from hua1 hua2 plants (*P < 0.05, **P < 0.01).

S6 Fig. The mRNA expression of SPL and KNU is regulated by the HUA-PEP activity. A, B) mRNA expression levels of SPL (A) and KNU (B), respectively, in the wild type (WT) and diverse hua-pep mutant backgrounds, monitored by qPCR. Error bars, SD. C-H) KNU::GUS [16] expression pattern in wild-type and hua1 pep mutant backgrounds. Longitudinal sections (C, D, F, G) and whole-mount (E, H) staining. C) Stage 6 wild-type flower showing GUS signal in a central spot between growing carpel primordia, as previously reported [16]. F) No detectable GUS signal was seen in stage 6 hua1 pep flowers. D) In wild-type stage 8 flowers strong staining also appears in stamens (white arrows). G) Stage 8 hua1 pep flower. GUS signal is absent at the base of the fourth whorl and substantially less intense in stamens (yellow arrows). E) Apex of a wild-type pistil during stage 12. GUS expression is detectable in ovules and internal sections of the style. H) Stage 12 hua1 pep pistil showing a significant reduction in reporter expression (yellow line). Scale bars: 50 μm.

S7 Fig. Increasing AG gene-dosage rescues the wild-type floral phenotype in the hua1 pep mutant. A) hua1 pep mutant flower in which sepals and petals have been manually removed to show petaloid conversions of stamens (red arrow). B) A heavily distorted hua1 pep gynoecium. C, D) Flower and silique, respectively, of a triple homozygous hua1 pep gAG::GFP plant. In (C) some outer organs were manually removed to visualize wild-type looking organs inside. Scale bars: 1mm.

S8 Fig. Detection of the AP1-GFP fusion protein in wild-type plants by confocal microscopy. A) Inflorescence showing signal in sepals. B) Magnification of one of the flowers from (A). C) AP1-GFP signal in stage 12 pistil. Autofluorescence was arbitrarily displayed in blue or red. Scale bars: 75 μm (A,C) and 25 μm (B).
S9 Fig. Reproductive phenotypes of PEP overexpression in the hua1 hua2 and hen4 hua1 backgrounds. A-E) SEM images. Adaxial surface of wild-type anther (A) and petal (B), and hua1 hua2 35S::PEP third whorl organ (C). In hua1 hua2 35S::PEP style and stigma were dramatically reduced and apical closure usually failed (D), as well as flower determinacy (E). F-J) Flower phenotypes of hua1 hen4 35S::PEP recapitulating the mutant traits appearing in the previous genotype. K, L) Relative expression levels of SPL (K) and KNU (L) mRNA in the wild type (WT) and diverse mutant backgrounds, monitored by qPCR. M) Scale bars: 10 μm (A-C), 100 μm (D, J), 1 mm (E-H) and 500 μm (I). Error bars, SD. (TIFF)

S10 Fig. The floral phenotypes of hua1 hua2 35S::PEP plants are not the result of PEP silencing. A) Double homozygous hen4 35S::PEP flower. B) Double homozygous hua1 35S::PEP flower. C) Double homozygous hua2 35S::PEP flower. D) PEP mRNA levels were monitored by qPCR in the wild type and hua1 hua2-4 plants hemizygous (hua1 hua2-4 35S::PEP/+ ) or homozygous (hua1 hua2-4 35S::PEP) for the 35S::PEP construct, respectively. Transgenic plants produced much higher levels of PEP transcript than the wild type. We used the hua2-4 allele [73] (SALK_032281) containing caulimoviral 35S promoter sequences, potentially able to trigger silencing of overexpressor lines driven by the same promoter element [Daxinger et al., 2008 in S2 Table]. E-G) The phenotype of hua1 hua2-4 gynoecia (E) was not modified in hua1 hua2-4 35SPEP/+ plants (F), but appeared dramatically enhanced in triple homozygous hua1 hua2-4 35S::PEP plants (G). H-J) Similar results were obtained with the hua2-7 allele [74] (SAIL_314_A08) lacking 35S sequences in its T-DNA. H) hua1 hua2-7 gynoecium. I) hua1 hua2-7 hua1 hua2-7 35S::PEP/+ gynoecium. J) hua1 hua2-7 35S::PEP gynoecium. In panels (A-C) some outer organs were manually removed to visualize inner organs. Scale bars: 1 mm. Error bars, SD. (TIFF)

S11 Fig. Sequence scheme of prematurely processed AG transcripts identified by RACE. DNA sequence corresponding to exon 2 appears as white upper-case letters boxed in black. Intron 2 sequence is shown as lower-case black letters. Cleavage site is indicated (A in red). Putative cis-elements associated to cleavage and polyadenylation are depicted boxed in blue (FUE), green (NUE) and yellow (CE), respectively [63]. A) Some prematurely processed transcripts identified in the hua1 hua2 pep/+ mutant background. B) An aberrantly processed transcript identified in the hua1 hua2 35S::PEP mutant background. The most abundant version of FUE in plants (UUUGUU boxed in blue), and a canonical AAUAAA sequence element for NUE (green) are indicated [63]. C) Prematurely terminated transcripts identified in the flk hua1 hua2 mutant background. (TIFF)

S12 Fig. Interactions of flk with other hua-pep mutants. Floral phenotypes and RNA expression data. A) Wild-type looking flk hen4 double mutant flower. Some outer organs were manually removed to show normal looking stamens. B) Young developing flk hen4 fruit. C) Wild-type looking flk hua1 double mutant flower. Some outer organs were manually removed to show normal looking stamens. D-F) SEM images of flk pep hua1/+ flower organs. D) Corrugated pistil. E) A flower after removing most organs around the pistil, except three third whorl organs with obvious petaloid traits. F) Close-up view of the adaxial surface of a petaloid area in a third whorl organ in (E) showing conically shaped cells. G, H) SEM images of flk hua1 hua2 flower organs. G) Petaloid conically shaped cells on the adaxial surface of a third whorl organ. H) A flower in which a fourth whorl organ was manually removed to uncover additional flowers inside. I) Relative mRNA expression of SPL and KNU in the wild type (WT) and diverse...
mutant backgrounds, monitored by qPCR. Error bars, SD. Scale bars: 1 mm (A, B, C, H), 200 μm (E, F), 10 μm (D, G).

S13 Fig. Additional transient BiFC experiments. Visualization of YFP reconstitution (yellow fluorescence) in *Nicotiana benthamiana* leaf cells agroinfiltrated with plasmids encoding fusion proteins. The first 5 interactions show the reciprocal assays of those depicted in Fig. 6 (the first protein fused to the YFPct, and the second protein to the YFPnt). The interaction between HEN4 and HUA1 [28] was confirmed in both directions and used as a positive control. The ability of PEP, FLK and HUA1 to homodimerize was also verified. HEN4 was unable to homodimerize, thus providing a negative control. As further negative controls, *Nicotiana* leaves were co-infiltrated with the corresponding recombinant YFPct construct and the empty YFPnt version. The reciprocal assays were also performed. No signal was detected in any case. In merged visible+YFP fluorescence pictures, blue background has been used to increase the contrast.

S14 Fig. Yeast two hybrid assays to test protein-protein interactions between PEP, FLK, HEN4 and HUA1. Protein interactions for PEP (A), FLK (B), and HUA1 (C). Induced yeast X-Gal plate assays are shown on the top of each panel. Y2H liquid assays using the reagent ONPG are shown below. Error bars indicate standard deviation (SD).

S1 Table. Loss and overexpression of *PEP* enhance the mutant phenotypes of *hua1 hua2* flowers. For each genotype approximately 75 flowers from 5 different plants were examined. Flowers were collected from top, bottom and intermediate zones of each plant. Petaloid transformations of stamens were arbitrarily classified as mild (totally or partially transformed lateral stamens), intermediate (transformed lateral stamens and partially converted medial stamens), and strong (all indistinguishable from second whorl petals). In *hua1 hua2*, pistils with four valves were extremely scarce whereas in the other two genotypes were easily found.

S2 Table. Oligonucleotides, genotyping and additional references.

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Author Contributions
Conceived and designed the experiments: ERC JJR AML MFY AV. Performed the experiments: ERC JJR AA LJB AV. Analyzed the data: ERC JJR AA AML MFY AV. Contributed reagents/materials/analysis tools: AML MFY AV. Wrote the paper: ERC JJR AML MFY AV.

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