**PEPPER, a novel K-homology domain gene, regulates vegetative and gynoecium development in Arabidopsis**

Juan José Ripoll, Cristina Ferrándiz, Antonio Martínez-Laborda, Antonio Vera

Abstract

Pistil final morphology relies on floral meristem homeostasis, proper organ specification and regional differentiation. These are developmental processes in which sophisticated signaling networks are being uncovered. However, further elements for fine-tuning adjustment still remain to be disclosed. At the molecular level, posttranscriptional modulators may fit such a profile. In this work, we describe the characterization of **PEPPER** (**PEP**), a novel **Arabidopsis** gene encoding a polypeptide with K-homology (KH) RNA-binding modules, which acts on vegetative growth and pistil development. **PEP** was initially identified as one of the gene functions affected in a complex mutant carrying a chromosomal reorganization, which exhibits aberrant phyllotaxy and small fruits with supernumerary carpels. In contrast, plants carrying single-gene **pep** null mutations exhibit subtle morphological alterations. Individuals bearing a stronger-than-null allele present a phenotype comprising leaf alterations, phyllotactic errors and sporadic presence of fruits with multiple valves. Accordingly, dynamic **PEP** expression was detected in all major organs examined. Complementation experiments with a **PEP** genomic clone confirmed a role for **PEP** as a regulator in vegetative and reproductive development. Moreover, our genetic studies suggest that **PEP** interacts with element(s) of the **CLAVATA** signaling pathway.

© 2005 Elsevier Inc. All rights reserved.

Keywords: **Arabidopsis thaliana; PEP; Organogenesis; Pistil; Fruit; KH domain; RNA-binding protein**

Introduction

One of the most complex developmental processes in flowering plants is the morphogenesis of pistil or gynoecium, the female reproductive structure, as judged by the increasing number of gene products known to be required for correct patterning and the specific cell types produced. Pistils arise at the center of floral meristems (FM), stem cell systems homologous to the shoot apical meristem (SAM) from which they emerge. Therefore, gynoecium ontogeny must rely first on correct meristem homeostasis. A pool of undifferentiated cells must be preserved while a constant supply of their descendants are incorporated into the new organs (Carles and Fletcher, 2003). In the dicotyledonous **Arabidopsis thaliana**, the **WUSCHEL (WUS)-CLAVATA (CLV)** pathway is crucial to maintain such a balance. The homeodomain protein WUS confers meristematic identity and promotes **CLV3** expression at the stem cell domain (Schoof et al., 2000). **CLV3**, a small secreted polypeptide (Rojo et al., 2002), acts as a ligand for a heterodimer formed by receptor-like proteins **CLV1** and **CLV2** (Clark et al., 1997; Jeong et al., 1999; Trotochaud et al., 2000). In this way, **CLV** genes relay a signal that indirectly represses **WUS** in a negative feedback loop, thus limiting the stem cell population (Schoof et al., 2000). Indeed, **clv** mutants display an augmented **WUS** domain which leads to an increased proliferative cell population, enlarged meristems and supernumerary floral organs, namely carpels (Clark et al., 1993, 1995; Kayes and Clark, 1998; Schoof et al., 2000).

**Arabidopsis** floral organs arise in concentric whorls with four sepals, four petals, six stamens and a central gynoecium with two fused carpels. According to the refined version of the well established ABC model, organ identity in each whorl is determined by the combinatorial action of specific transcription factors, most of which belong to the MADS-box type. Hence, coordinated activity of redundant **SEPALLATA (SEP)** genes (Pelaz et al., 2000; Ditta et al., 2004) and the C-class gene...
AGAMOUS (AG) determine carpel identity in the innermost position (recently reviewed in Jack, 2004). A key additional role of AG is to attain FM determinacy, in contrast to indeterminate growth in the SAM (Bowman et al., 1989; Steeves and Sussex, 1989). Thus, WUS and the FM identity gene LEAFY (LFY) cooperate activating AG, which in turn represses WUS, terminating floral stem cell maintenance (Lenhard et al., 2001; Lohmann et al., 2001). Deficiencies in represses gene LEAFY induce indeterminate growth in the SAM (Bowman et al., 1989; AG). 1% sucrose) and transferred to soil after 2 weeks. Plates were supplemented with 3:3:1 potting soil (3:3:1), watered with ATM medium (Kranz and Kirchheim, 1987);

HUA2 redundant genes encoding RNA-interacting proteins: HUA1, HUA2, HUA ENHANCER2 (HEN2) and HEN4 (Chen and Meyerowitz, 1999; Western et al., 2002; Cheng et al., 2003). In fact, genetic and biochemical experiments have revealed their roles facilitating the processing of AG pre-mRNA (Cheng et al., 2003).

Following acquisition of carpel identity and termination of FM activity, carpel congenital fusion gives rise to the Arabidopsis closed gynoecium, which includes a short style topped by stigmatic tissue, and an ovary with two valves separated by two vertical medial furrows, the repla. The latter define the position of two outgrowths which fuse postgenetically creating the septum (Bowman et al., 1999; Ferrándiz et al., 1999). This internally divides the ovary into two locules where ovules arise (Bowman et al., 1999). Genetic control of this scheme of regionalization and specification of distinct tissues is exerted by a number of genes that determine or respond to apical–basal and abaxial–adaxial domains, in connection with hormonal action (Eshed et al., 1999; Ferrándiz et al., 1999; Nemhauser et al., 2000; Dinney and Yanofsky, 2004).

In spite of the great progress in elucidating the regulation of the above developmental processes, there are still many gaps in our knowledge. An obvious way to gain further insight is the characterization of novel modulators. Here, we report on PEPPER (PEP), an Arabidopsis gene that encodes a putative RNA-binding protein with three KH domains which participates in pistil development, affecting carpel determinacy seemingly through the CLAVATA pathway. pep mutations also impinge on diverse aspects of vegetative growth. Consistent with this, dynamic PEP expression was detected in all major organs examined.

Materials and methods

Plant material, growth conditions and clones

Seeds were germinated directly in a mixture of vermiculite, perlite and potting soil (3:3:1), watered with ATM medium (Kranz and Kirchheim, 1987) or on GM medium plates (Germination Medium: 0.5× Murashige-Skoog with 1% sucrose) and transferred to soil after 2 weeks. Plates were supplemented with 50 µg/ml kanamycin or 20 µg/ml hygromycin when antibiotic selection was applied. The resulting plants were grown at 20–22°C in a growth chamber with continuous cool-white fluorescent light.

Accessions Wassilewskija (Ws-2) and Columbia (Col-0) were used as the wild-types. The pep-1 mutation was isolated in the Ws-2 background during a screening of a T-DNA collection obtained at the laboratory of J. Paz-Ares (Centro Nacional de Biotecnologia, C.S.I.C., Madrid, Spain) with the pGKBS plasmid conferring kanamycin resistance (Bouchez et al., 1993), and was backcrossed five times to Col-0. The T-DNA alleles pep-2, pep-3 (Basta selectable, SAIL; Sessions et al., 2002), pep-4 and pep-5 (kanamycin selectable, SALK; Alonso et al., 2003) are in the Col-0 background and were obtained from public domain collections, corresponding to lines F09_517, G12_1249, N055265 and N081555, respectively. All insertions were confirmed by PCR amplification and sequencing with T-DNA-specific primers which, together with proper genomic oligonucleotides, served as genotyping sets to distinguish between wild-type alleles and mutant alleles carrying fused T-DNA-plant genomic sequences (see Table S1 for oligonucleotide sequences). T-DNA mutants were backcrossed to Col-0 wild-type prior to further study (pep-2 and pep-4, three times; pep-1 and pep-5, twice). For each allele, distinct F2 populations (four different families, 200 individuals each) were examined, and resistance due to the selectable marker (Basta or kanamycin) was observed to be transmitted as a simple dominant character (see Table S2). Co-segregation of resistance and mutant phenotype was established for each allele by studying at least 12 F2 families (40 individuals each) derived from F2 mutant plants grown on non-selectable media. All F2 progenies were composed of resistant plants displaying the corresponding mutant phenotype. Nevertheless, all phenotypes were finally observed in plants grown in non-selectable media.

The mutations clv2-1 and clv3-2 (lines N46 and N8066, respectively; NASC, The European Arabidopsis Stock Centre, Nottingham, UK) are in the Landsberg erecta (Ler) background, and were backcrossed three times to Col-0 prior to genetic crossings. Plants devoid of the erecta (er) mutation were used for subsequent studies. The CLV3 gene lies very close to the ER locus (~1 cm) on the chromosome 2 (Clark et al., 1995); however, recombinant clv3-2 ER plants were obtained during introgression into the Col-0 background.

Atg26000 cDNA clones (RAFL08-13-K20, RAFL09-64-E03; Seki et al., 1998, 2002) were obtained from RIKEN Institute (Tsukuba, Japan). Sequence searches and multiple protein alignments were obtained using the BLAST and Clustal X programs, respectively.

Genetic linkage analysis

Homozygous pep-1 mutant plants (Ws-2 background) were used as pollen donors in crosses with Ler wild-type individuals. Two hundred mutant plants from the resulting F2 populations were assayed for linkage to polymorphic markers CIW7 (chromosome 4, TAIR database; Table S1) and MN5 (chromosome 5, this work; Table S1).

Genetic interactions

Homozygous single mutants were cross-fertilized and double mutants were identified among the F2 segregants by a differential phenotype and/or genotyping. When possible, antibiotic selection was used to bias certain genotypes. Thus, to study heterozygous clv mutants in a pep-4 homozygous background, F2 seeds were germinated in kanamycin, particular plants were PCR identified as pep-4 homozygous individuals and their phenotypes were analyzed. Next, their progeny were tested for inheritance of kanamycin resistance and the Clv- phenotype. All phenotype comparisons were undertaken with plants grown in the absence of the antibiotic.

TAIL-PCR, RT-PCR and SQRT-PCR

Plant DNA was extracted as in Li et al. (1999). Cloning of pep-1 genomic sequences adjacent to T-DNA was conducted by TAIL-PCR (Thermal asymmetric interlaced PCR) according to Liu et al. (1995). Nested oligonucleotides corresponding to both left and right border sequences of the T-DNA were employed as specific primers (Table S1).

For RT-PCR, polyA mRNA was extracted from 100 mg of plant tissue with a commercial kit (Dynabeads Oligo (dT)25, Dynal Biotech, Norway) according to the manufacturer’s instructions. RNA samples were treated extensively with RNase-free Dnase I, and first strand was synthesized with 1 µg of polyA mRNA using M-MuLV reverse transcriptase (Roche Diagnostics) and Rnase inhibitor (Roche Diagnostics) in a 20-µl reaction. Two microliters of the reaction mixture was subsequently subjected to PCR. The RT-PCR runs were 35 cycles with...
each cycle at 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 0.5 min, with a final cycle of 72°C for 7 min.

Semiquantitative RT-PCR (SQRT-PCR) was carried out as outlined above except that starting material was 5 μg of total RNA extracted with the Rneasy Mini Kit (Qiagen Inc.) and only 20 cycles were executed. Every reaction was diluted (1/250) and 1 μl was gel-separated, transferred to a nylon membrane (Hybond-N, Amersham) and subjected to chemiluminescent detection with a digoxigenin-labeled probe (Roche Diagnostics) according to the manufacturer’s instructions.

Histological analysis

For light microscopy, tissue was vacuum infiltrated with FAE (45% ethanol, 5% glacial acetic acid, 1.85% formaldehyde, 1% Triton X-100) for 45 min and fixed for 2 h at room temperature, dehydrated through a graded ethanol series (70%, 80%, 90%, 95% with cosine and 95%) and embedded in JB4 resin (Electron Microscopy Sciences). Sections 3–4 μm thick were cut with a Microm HM350S microtome, stained in 0.1% toluidine blue and observed using an ECLIPSE E800 microscope (Nikon) equipped with a COLORVIEW-III digital camera (Nikon).

For scanning electron microscopy (SEM), samples were treated with methanol for 10 min and incubated in ethanol (100%) prior to critical point drying with CO₂ (Electron Microscopy Sciences EM5850). Specimens were sputter-coated with gold (Balzers SCD004) and analyzed in a JSM-640 JEOL scanning electron microscope.

For in situ hybridization, the protocol in Ferrándiz et al. (2000) was followed. A 368 base pair (bp) PEP cDNA fragment, flanking the first intron, was synthesized (see Table S1), and cloned into the pGEM-T plasmid (Promega). Two micrograms of SauI linearized plasmid was used as template for a DIG-labeled antisense riboprobe. A sense DIG-labeled riboprobe was generated after restriction with ApaI.

GUS staining

Samples were treated for 15 min in 90% ice-cold acetone, and then washed for 5 min with washing buffer (25 mM sodium phosphate; 5 mM ferrocyanide; 5 mM ferricyanide; 1% Triton X-100) and incubated overnight at 37°C with staining buffer (25 μM sodium phosphate; 5 μM ferrocyanide; 5 μM ferricyanide; 1% Triton X-100; 1μM X-Gluc). For thin sections, tissue was stained and then fixed with FAE and dehydrated in an ethanol series, as described above, and embedded in Paraplast Plus (Kendall). Eight micrometer cross-sections were obtained and photographed as described for light microscopy.

Transgenic constructs

For genetic complementation of the pep mutants, a wild-type PEP genomic clone was introduced into pep-2 mutant plants. A proofreading cocktail of DNA polymerases (High Fidelity, Roche Diagnostics) was employed to amplify a genomic complementing clone from BAC F20B18, consisting of a 5111 bp fragment containing the entire At4g26000 (included in primer sequences; Table S1), thereby fusing the coding region.

For scanning electron microscopy (SEM), samples were treated with methanol for 10 min and incubated in ethanol (100%) prior to critical point drying with CO₂ (Electron Microscopy Sciences EM5850). Specimens were sputter-coated with gold (Balzers SCD004) and analyzed in a JSM-640 JEOL scanning electron microscope.

In either case, the binary vector was introduced into A. tumefaciens (EHA 105 strain, bearing the pSOUP helper plasmid; Hoekema et al., 1983) and the plants were infected using a floral dip procedure (Clough and Bent, 1998). Hygromycin-resistant transformants were selected and their progenies were collected for testing antibiotic resistance, phenotype analyses and/or GUS staining in the T₂ and subsequent generations.

Results

Characterization of pep-1, a mutation affecting pistil development

We analyzed an Arabidopsis T-DNA mutagenized population, screening for mutants defining novel gene functions for pistil and/or fruit development. A mutant harboring aberrant small fruits was selected for further characterization. It was designated pepper-1 (pep-1) because some mutant fruits resembled small bell-peppers (Fig. 1). The phenotype, which cosegregated with antibiotic resistance, was totally penetrant and transmitted in a simple recessive pattern (Table S2). Reciprocal crosses with its wild-type ancestor (Ws-2) indicated that the pep-1 mutation does not exhibit gametophyte lethality (Fig. S1). Introggression into the Col-0 background did not reveal fundamental modifications of the phenotypes described below.

Close inspection showed that most mutant fruits (>70%) consist of more than two valves (three to six, average 3.08 ± 0.92; Figs. 1A–C and Figs. S1 and S2). In addition, a large proportion of bicarpellate fruits exhibit valve asymmetric growth, with valve tissue removed basally (Fig. 1D), which is not rare in fruits with extra-valves (Figs. 1B and C; Table S3). No extra-organs were detected in other floral whorls.

Morphological alterations were visible early during pep-1 pistil development. During stage 9, wild-type gynoecium appeared as an open cylinder (Fig. 1E) with apparent stigmatic papillae at stage 10 (Fig. 1F). About stage 12, bicarpellar condition and apical closure were evident (Figs. 1G and H) (Bowman, 1994; Ferrándiz et al., 1999). Postgenital fusion of the septum occurred during stages 9–12, dividing the mature ovary into two lateral locules where the ovules develop (Fig. S1) (Bowman et al., 1999). In contrast, supernumerary carpels in pep-1 gynoecia were apparent from stage 9 (Fig. 1I). Stigma and style development was delayed in the mutant, as well as complete postgenital fusion of the solid style (Figs. 1J–L), that occasionally failed to join entirely.

Cross-sections of pep-1 fruits showed wild-type valve and replum tissue organization. When three or more valves formed, the number of locules increased concomitantly, and frequently unfused septa were observed, especially in the upper half of the ovary (Fig. S1). Other less frequent pep-1 attributes included long basal internodes (gynophores) and two fruits in the same floral receptacle (see Figs. S1 and S2, and Table S3).

The pep-1 mutant exhibits abnormalities in vegetative growth

The phenotype of pep-1 plants is not confined to the gynoecium as it comprises a number of vegetative abnormalities. Thus, rosette overall size was reduced, their leaves being pointed and narrower compared to the wild-type (Figs. 4A and C). The pep-1 plants showed abnormal phyllotaxy, with
irregular internode length (compare Figs. 4D and F; see also Fig. S1). Therefore, two or more flowers and/or secondary inflorescences may arise from the same point on the shoot (Fig. 4D, see also Fig. S1 and Tables S3 and S4). Additional alterations included flowers subtended by cauline leaves (Fig. S1 and Table S4). Since phyllotaxy errors are frequently associated with meristem alterations, we examined SAMs in the wild-type and pep-1 mutants (10 plants each). However, no significant size or morphology differences were observed (not shown).

The pep-1 mutant harbors a complex chromosomal rearrangement

Cloning of genomic regions adjacent to the T-DNA insertion sites by the TAIL-PCR method (Liu et al., 1995) revealed the presence of sequences from chromosomes 4 and 5. Likewise, genetic mapping of the pep-1 mutation with polymorphic markers (see Materials and methods and Table S1) yielded total linkage to the same chromosomes, which suggested the presence of a reciprocal translocation (Coe and Kass, 2005) as that depicted in Fig. 2A. In addition, heterozygous PEP/pep-1 plants exhibited semisterility (half of the progeny was not viable early in development; Fig. 2B), which is a hallmark of this sort of chromosome aberrations (Coe and Kass, 2005; Griffiths et al., 2005). The requirement of a balanced reciprocal translocation for viability may explain why pep-1 behaves as a single insertion T-DNA mutation totally linked to antibiotic resistance, segregating in an apparent Mendelian ratio of 3:1. Altogether, these findings indicate that pep-1 represents a rather complex mutation whose phenotype may be the result of varied genetic effects.

Phenotypes of single-insertion T-DNA mutants for At4g26000, a gene containing KH motifs

A detailed analysis of the pep-1 chromosome rearrangement was beyond the scope of this article. Nonetheless, in order to identify genes whose mutations might be contributing to the pep-1 phenotype, we obtained T-DNA lines involving genes...
potentially affected by the translocation. Careful examination of diverse homozygous single insertion mutants did not yield any conclusive result (not shown). However, our attention was drawn by a previously annotated gene, At4g26000, which encodes a polypeptide with RNA-binding KH domains (see Fig. 5 below). This gene is transcriptionally null in pep-1, since a T-DNA insertion is located just 18 bp upstream of its initiation codon (Figs. 5A and B). Four homozygous lines for single insertions along the At4g26000 sequence (Fig. 5A) were studied and tentatively named pep-2 through pep-5 as a result of the phenotype examination and assays described below. Therefore, the At4g26000 gene will be hereafter referred to as PEP.

At first sight, homozygous plants for the pep-3, pep-4 and pep-5 alleles did not differ remarkably from their wild-type ancestor Col-0. Nevertheless, more thorough examination showed recessive, mild, identical phenotypes consisting of phylotactic alterations reminiscent of those previously observed in pep-1, although with lower expressivity (Figs. S1 and S2, and Table S4). Occasionally, asymmetry in valve growth and unfused carpels were also observed (Fig. 3H, see also Fig. S2 and Table S3).

By contrast, plants carrying the stronger pep-2 allele showed a more evident, recessive mutant phenotype. In pep-2 plantlets, leaf emergence was clearly delayed with respect to the wild-type (Figs. 3G and K; Table 1). Although other pep alleles behaved in a similar manner (Table 1), this heterochronic phenotype was most obvious in pep-2 plants (Figs. 3G and K). The pep-2 leaves were pointed and showed prominent marginal protrusions, with a conspicuous venation superimposed on a paler green mesophyll (Fig. 3B). This phenotype was somewhat mitigated as the leaf lamina expanded, yet remained clearly distinguishable. In agreement with pep-2 being the strongest allele among those affecting solely the PEP...
Gene, pep-2/pep-4 transheterozygous plants displayed a pep-2 rosette phenotype (Fig. S3E).

The pep-2 plants displayed the same phyllotactic alterations observed in pep-3, pep-4 and pep-5 although at a higher frequency (Fig. S2 and Table S4). Most interestingly, a low proportion of pep-2 fruits (~1%) showed extra-valves (Fig. 3A and Table S3). Fruits with basally removed valve tissue and other aberrations previously observed in pep-1 also appeared occasionally (Fig. S3I, Fig. S2 and Table S3).

Genetic complementation of pep mutants

The phenotypic traits described above were clearly evocative of those observed in pep-1 plants, bearing a chromosomal rearrangement. Therefore, it seemed reasonable to envision PEP gene dysfunction as a contributor to that complex mutant phenotype. Bearing this in mind, we crossed pep-2 to pep-1 and analyzed their F1 progeny. Unlike PEP/pep-1 plants, which appeared entirely wild-type (Fig. 3D), transheterozygous pep-1/pep-2 rosettes (Fig. 3C) also displayed a leaf mutant phenotype (Figs. 3B–D). Most noticeably, pep-1/pep-2 plants presented numerous phyllotactic alterations (not shown) and three or more valves in most (circa 70%) of their fruits (Figs. 3F and J; Tables S3 and S4). Siliques attained a normal size, comparable to three-valve fruits eventually occurring in pep-2 (Fig. 3A). These results were consistently reproduced, whereas more than 50 PEP/pep-1 plants examined were morphologically indistinguishable from the wild-type, thus reinforcing the view of PEP as a determinant of the pep-1 phenotype.

To provide conclusive evidence that impaired function of this gene is responsible for the Pep- phenotype, we performed a transgenic complementation analysis. The conspicuous and fully penetrant mutant phenotype of pep-2 rosette leaves was chosen to monitor phenotypical rescue. Plants homozygous for pep-2 were transformed with a genomic fragment containing the entire PEP gene (PEP::gPEP). Multiple independent transformants were obtained, selecting hemizygous transgenic lines bearing a single T-DNA insert (3:1 segregation of hygromycin resistance). In 21 out of 24 independent T1 plants, the pep-2 mutant phenotype was restored to wild-type, with restoration cosegregating with the T-DNA in the T2 and subsequent generations. Thus, pep-2 plants transformed with the PEP::gPEP construct showed wild-type leaf morphology and time of emergence (Figs. 3G and K; Table 1). This result unambiguously confirmed that At4g26000 is PEP and that the genomic clone used is fully functional in planta.

In the above transgenic assay, the possible role of PEP as a regulator of pistil development could not be assessed due to the low penetrance of gynoecial phenotypes in pep-2 mutants. To address this issue, PEP::gPEP transgenic line was crossed to pep-1. As described above, the pep-1 mutants bear a chromosome rearrangement, its phenotype very probably being the outcome of a complex combination of mutations. Hence, it came as no surprise to observe only partial rescue in homozygous pep-1 plants harboring the PEP::gPEP transgene (Fig. 4, Tables S3 and S4). Nevertheless, weakening of the pep-1

Table 1

<table>
<thead>
<tr>
<th>Average number of rosette leaves in wild-type (Col-0) and pep mutantsa</th>
<th>10 das</th>
<th>12 das</th>
<th>14 das</th>
<th>16 das</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (n = 20)</td>
<td>4.55 ± 0.88</td>
<td>5.90 ± 0.64</td>
<td>8.20 ± 0.41</td>
<td>9.30 ± 0.47</td>
</tr>
<tr>
<td>pep-2 (n = 20)</td>
<td>2.20 ± 0.61</td>
<td>4.35 ± 0.74</td>
<td>6.00 ± 0.72</td>
<td>8.30 ± 0.65</td>
</tr>
<tr>
<td>pep-4 (n = 20)</td>
<td>3.30 ± 0.97</td>
<td>4.90 ± 0.85</td>
<td>7.65 ± 0.67</td>
<td>9.00 ± 0.85</td>
</tr>
<tr>
<td>pep-2 PEP::gPEP (n = 20)</td>
<td>4.30 ± 0.57</td>
<td>5.30 ± 0.73</td>
<td>8.05 ± 0.51</td>
<td>9.00 ± 0.64</td>
</tr>
</tbody>
</table>

a n, number of plants examined in each strain at every measuring time.

pep-2 PEP::gPEP are pep-2 plants carrying the rescue construct (T1 generation).

Leaf numbers were determined from wild-type and mutant plants grown simultaneously under the same conditions on GM plates without antibiotics.

* Values represent the mean number ± standard deviations. For data comparison, SPSS12.0 statistical program was used to carry out a Student’s t test (P ≤ 0.05; confidence interval, 95%).
phenotype was evident. Rosette phenotype was alleviated to some extent (Figs. 4A–C), phyllotactic distortion clearly being less pronounced (Figs. 4D–F). Likewise, pep-1 overall reduction in fruit size (Figs. 4D and G), though not completely restored to wild-type levels (Figs. 4F and I), was significantly mitigated by the presence of the transgene (Figs. 4E and H). Of note was the fact that this also held true for the occurrence of fruits with extra-valves, which was greatly reduced although not abolished (Figs. 4G and H, Table S3). These results emphasize the preceding complementation assays and strongly suggest the participation of PEP in pistil development.

PEP encodes a putative RNA-binding polypeptide with three KH domains

The PEP gene coding sequence is organized in six exons (Fig. 5A), and the information available from diverse databases (NCBI, http://www.ncbi.nlm.nih.gov/; TAIR, http://www.arabidopsis.org/) predicts a transcription unit of 2590 nucleotides (nt), including a 24 nt leader sequence, and a 304 nt long 3' untranslated region (UTR). The PEP cDNA was deduced to encode a polypeptide of 495 amino acids with a calculated molecular mass of 54.02 kDa. These structural features were corroborated through the analysis of full-length cDNA clones (see Materials and methods).

As mentioned above, the most noteworthy feature revealed by structural analysis of the PEP polypeptide was the presence of three type-1 K-homology RNA-binding domains (KH) (Adinolfi et al., 1999). Initially identified as three repeats in the human heterogeneous nuclear ribonucleoprotein-K (hnRNP-K) (Siomii et al., 1993), the evolutionary conserved KH module is one of the major categories of eukaryotic RNA motifs (Fedoroff, 2002; Lorkovic and Barta, 2002), and has been described in proteins involved in virtually every step of RNA metabolism (Anantharaman et al., 2002; Makeyev and Liebhaber, 2002). This motif encompasses a region of about 70 amino acids in length including a core consensus sequence V/IGXXGXXI (Grishin, 2001), which is conserved in PEP KH domains (Fig. 5C). These are highly homologous to KH motifs of poly(C) binding proteins (PCBP), including the founding member hnRNP-K (Makeyev and Liebhaber, 2002). Similarly, the distribution of two KH domains very close to each other at the N-terminus, and a third one distantly located, shows a strong parallelism to known animal KH-containing polypeptides as PCBPs and Nova proteins (Fig. 5C) (Makeyev and Liebhaber, 2002; Musunuru and Darnell, 2004).

In rice (Oryza sativa), a probable orthologous gene was found (Q9AY47) which encodes a protein approaching 48% identity and 75% similarity to PEP, tentatively named OsPEP (Fig. 5D). Homology searches also showed that PEP shares an overall identity of 42.4% with Arabidopsis FLOWERING LOCUS K (FLK), its closest paralog, which is involved in flowering time regulation (Lim et al., 2004; Mockler et al., 2004). FLK also has a rice homolog sharing 43% identity (Lim et al., 2004). The three KH domains are situated at equivalent positions in the four genes. Interestingly, the degree of conservation between PEP and FLK is lower than those observed between each of them and their respective rice orthologs, suggesting that duplication took place before angiosperm divergence, and perhaps reflecting the preservation of their functions across monocot and dicot lineages.

Mutations in the PEP gene

T-DNA insertions in pep-4 and pep-5 alleles interrupt exons 2 and 3, respectively, and most probably represent complete loss-of-function mutations since no transcription could be revealed by RT-PCR (Figs. 5A and B and results not shown). By contrast, inserts in pep-2 and pep-3, both at the very 3’ end of the coding region, do not abolish transcription (Figs. 5A and B and results not shown). At present, whether pep-3 corresponds to a hypomorphic or a null mutation remains unclear since its phenotype is indistinguishable from those of pep-4 and pep-5 null mutations.

On the other hand, pep-2 causes a stronger phenotype than complete loss-of-function alleles although it is not a transcriptional knock-out. Since pep-2 is not dominant, it might not be interfering with the wild-type allele. Thus, pep-2 may represent an example of recessive interfering mutation (Strader et al., 2004) with a mild neomorphic effect. Interestingly, heterozygous pep-2/pep-4 plants display a pep-2 phenotype (Fig. 3E).

The T-DNA insert in pep-2 creates a premature stop codon, removing the last six amino acids of the ORF, including a predicted tyrosine phosphorylation site which might account for abnormal regulation. Additionally, sequences in the T-DNA likely provide a polyadenylation signal to support the stability of the mRNA and translatability of that slightly shorter protein (not shown).

Dynamic expression of PEP along vegetative and reproductive development

PEP expression was initially studied by means of semiquantitative RT-PCR (SQR-PCR) experiments. Consistent with the pleiotropic effects of its mutants, PEP transcript was detected in all major organs examined (Fig. 6A), which suggests that PEP may function in regulating multiple developmental pathways.
The PEP expression pattern was then analyzed in detail by driving β-glucuronidase (GUS) gene expression from the PEP promoter (see Materials and methods). The same expression pattern was verified in four independent transgenic lines. To validate our histochemical expression studies, we performed in situ hybridization (Fig. S3), confirming that our PEP::GUS constructions faithfully reflect the endogenous pattern of PEP transcriptional expression.

Histochemical analysis of 3-day-old seedlings did not reveal PEP expression in the SAM (not shown) although it was detected throughout the whole leaf primordia and young developing leaves (Fig. 6B). As leaves developed, the expression domain of PEP became circumscribed to the adaxial side (Fig. 6C), finally being restricted to the veins in fully expanded leaves (Fig. 6D), which matches the relatively mild expression found by SQRT-PCR in this organ (Fig. 6A). Likewise, root staining was confined to the vasculature (Fig. 6E).

Inflorescences were also examined, expression being observed in floral buds (not shown) and young developing flower organs. Intense staining in perianth organs and stamens (Figs. 6G and H) became restricted to the vascular system in later postanthesis stages (Figs. 6J, K and M). Thus, in fully mature stamens, PEP expression is absent from the anther and conspicuous in the center of the stamen filament (Figs. 6K and M), most likely corresponding to its vasculature.

Pistil and fruit PEP expression was also highly dynamic. Strong staining was detected throughout the young pistil (Figs. 6G and H). Apical stigma was the first structure devoid of GUS activity during postanthesis fruit maturation (Fig. 6F). This was in sharp contrast with the style, where PEP promoter activity remained robust until later stages in fruit development (Figs. 6J and K and Fig. S3), whereas it progressively faded away from most of the ovary region (Figs. 6I and K and Fig. S3).

Histological sections of fully developed fruits showed that remaining GUS activity was also associated with the vascular
system (Fig. 6L), in consonance with temporal and spatial changes of PEP expression in other organs. No GUS activity was found in the transmitting tract. Conversely, this was surrounded by a ring of PEP-driven activity corresponding to the style vasculature (see inset in Fig. 6L). The GUS signal in the style was connected to the medial bundles (Fig. 6L and Fig. S3) which run towards the base of the ovary to reach the gynophore, where staining was clearly visible (Fig. S3) probably due to the presence of a ring of vascular bundles (Bowman et al., 1999). In fully matured fruits, we observed short stretches of GUS staining projecting from the medial bundle (Fig. S3), which may correspond to isolated strands of vascular tissue present in the funicula connecting the ovules (Bowman et al., 1999).

Relationship of PEP with the CLV pathway

CLV genes regulate the size of meristems, their mutations leading to an increased number of valves in the gynoecium (Carles and Fletcher, 2003). This prompted us to construct clv pep double mutants to explore whether PEP might be acting on the same genetic pathway. We made use of the null pep-4 allele lacking fruits with multiple valves. The utilized clv alleles are in the Ler accession and they were backcrossed three times to Col-0 prior to crossings with pep-4 to avoid background influences. Likewise, to rule out effects of the erecta (er) mutation (Torii et al., 2003), homozygous ER F2 segregants were selected for phenotypic comparisons (see Materials and methods).

clv3-2 is a null mutation causing a dramatic fruit phenotype (Clark et al., 1995; Fletcher et al., 1999). As Fig. 7A illustrates, the vast majority of fully developed clv3-2 fruits in an ER background are relatively straight. Aside from a modest thickening at the uppermost portion, their habitual strong club-shaped appearance (Clark et al., 1995) is greatly reduced. In contrast, clv3-2 pep-4 siliques displayed pronounced bending and swelling at the apical zone, to a certain extent restoring the club-shaped aspect (Fig. 7B). Moreover, clv3-2 pep-4 double mutant plants showed a modest increase in the average number of valves (5.14 ± 0.81) with respect to the single clv3-2 mutant (4.11 ± 0.80; Table S5).

The study of double mutants with clv2-1, a null allele with intermediate severity (Jeong et al., 1999), yielded a similar result and, on average, gynoecium/fruit morphology was less affected in clv2-1 single mutants than in clv2-1 pep-4. Fruits of clv2-1 single mutants lacking the er mutation exhibited a phenotype weaker than previously described (Kayes and Clark, 1998), being long, straight, not or very mildly club-shaped (Figs. 7E and F), and showing a clearly discernible style region (Figs. 7C, E and F). They presented multiple valves, many of which did not extend to the end of the gynoecium (Fig. 7C) (Kayes and Clark, 1998). In our conditions, clv2-1 valves often appeared wrinkled, particularly at the top (Figs. 7C, E and F), although much less than in the clv2-1 pep-4 double mutant (Figs. 7D, G and H). clv2-1 pep-4 fruits grew straight except at the apex, where they frequently bent and thickened (Figs. 7D, G and H). Wrinkled valve tissue covered most of the gynoecium uppermost region, leading to a marked reduction of the style (Figs. 7G and H). In both clv2-1 and clv2-1 pep-4 gynoecia, cells of valveless regions lacked stomata (not shown) and, as previously reported (Kayes and Clark, 1998), they were similar to those in wild-type repla. In the double mutant, moderate increments in average valve number and the incidence of basally removed valve tissue were observed (Table S5).

In both clv3-2 and clv2-1 mutant backgrounds, pep-4 seems to foster an enhancement of the Clv- phenotype. To obtain further insight into possible PEP interactions with CLV genes, we also examined heterozygous CLV3/clv3-2 and CLV2/clv2-1 mutants in a pep-4 background. A moderate increment in the occurrence of fruits with supernumerary valves was observed in CLV/clv pep-4 plants compared to CLV/clv individuals in an otherwise wild-type background (Table S6). These results suggest that PEP may positively influence flower determinacy.
by affecting some component(s) of the CLV pathway. Nevertheless, the slight enhancement observed in the homozygous clv3-2 and clv2-1 mutant phenotypes caused by PEP loss-of-function also suggests that PEP participates in an independent pathway(s).

Discussion

PEP, a new Arabidopsis developmental regulator encoding a KH protein

This work documents the characterization of PEP, an Arabidopsis gene encoding a putative RNA-binding polypeptide with K-homology (KH) domains. We provide compelling evidence supporting the involvement of PEP in vegetative and reproductive development.

Although much of the coordination required by morphogenetic programs is exerted at the transcription level, a feasible source of new regulatory elements are the genes involved in the vast array of posttranscriptional modifications that most transcripts undergo before becoming fully operative (Anantharaman et al., 2002; Cheng and Chen, 2004). The Arabidopsis genome contains hundreds of genes that potentially encode RNA interacting polypeptides (The Arabidopsis Genome Initiative, 2000) and very few of them have been functionally characterized. Among them, Arabidopsis contains a sizeable family of 27 members with RNA-binding KH motifs (Chekanova et al., 2002; Lorkovic and Barta, 2002).

Mutations in KH motif containing genes provoke conspicuous developmental phenotypes in various organisms as Caenorhabditis elegans (Lee and Schedl, 2001), Drosophila melanogaster (Nabel-Rosen et al., 2002) and vertebrates (Zorn and Krieg, 1997). Notably, the severe human disorder X-linked mental retardation (FMR1) and Krieg, 1997). Notably, the severe human disorder X-fragile syndrome (ANX1, HUA2 and HEN4, are phenotypically normal (Chen and Meyerowitz, 1999; Cheng et al., 2003). Specifically, KH-containing proteins have undergone lineage-specific expansions in eukaryotes which may lead to a wider functional versatility (Anantharaman et al., 2002) but also to redundancy (Pickett and Meeks-Wagner, 1995). FLK represents an obvious candidate to explore redundancy with PEP although we found no evidence of overlapping functions between both genes (our unpublished results).

In this context, the initially identified pep-1 mutant, harboring a complex chromosomal rearrangement, has been instrumental in uncovering the role of PEP in vegetative and gynoecium development. Genetic and molecular analysis of pep-1 might be considered analogous to second-site mutagenesis efforts for unraveling gene functions that otherwise would remain overlooked.

pep-2 may be a recessive interfering allele

The pep-2 allele is not transcriptionally null, it is stronger than amorphic alleles and exhibits a dominant effect in transheterozygous pep-2/pep-4 individuals. Given that it does not behave as a dominant allele in a wild-type background, it seems more appropriate to designate it as recessive interfering. Recessive stronger-than-null mutations in the gibberellin signaling gene SLEEPY1 (SLY1) have been recently denominated in this way (Strader et al., 2004). The conceptual pep-2 protein lacks the last six amino acids of its C-terminus, thus losing a tyrosine phosphorylation site which might account for abnormal regulation. In animal KH proteins, multiple phosphorylation sites are known to be modified in response to diverse signals, allowing them to sense changes in their environment and facilitating cross-talk between kinase-mediated and nucleic acids processes (Makeyev and Liebhaber, 2002; Bomsztyk et al., 2004). Knowledge of the precise mechanism of action awaits further molecular characterization but we postulate that pep-2 interferes with other functionally redundant activities.

PEP is required for normal vegetative and reproductive development

Phenotypic and genetic complementation studies of pep mutants point to a role for PEP in diverse aspects of plant development. Consistently, PEP is expressed in many organs and tissues, suggesting that it may function in regulating multiple developmental pathways. Moreover, PEP expression profiles suggest a broader role than revealed by mutant phenotypes. For example, no developmental defect is observed.
in roots, stamens or perianth floral organs. \textit{PEP} might be particularly necessary during active growth periods since it is widely expressed in young growing organs, after which it is progressively restricted to the vasculature. Among others, class III HD-Zip genes \textsc{phabulosa} (\textit{PHB}), \textsc{phaviourita} (\textit{PHV}) and \textsc{revoluta} (\textit{REV}) exhibit similar mRNA expression, although they are expressed in the SAM (McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003). One of their functions is the establishment of adaxial identity of lateral organs (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003). Like those genes, \textit{PEP} is transiently expressed in leaf and floral organ adaxial sides, although no sign of polarity disturbance was detected in its mutants.

Abnormalities shown by \textit{pep} mutants, including multicarpellar gynoecia, are frequently associated to meristem alterations. However, no appreciable size or structural changes were visible in \textit{pep} single mutant meristems. In \textit{pep} FMs, a mild increase in the stem-cell population might be compensated by a faster recruitment into lateral organs, thus maintaining meristem size.

\textbf{PEP functions in gynoecium morphogenesis}

A role for \textit{PEP} in gynoecium determinacy is supported by the genetic complementation studies of \textit{pep} mutants, occasional occurrence of \textit{pep-2} fruits with extra-valves and genetic interactions with \textit{clv} mutants. In this respect, the observed semidominance of \textit{clv} mutations in a \textit{pep} mutant background fits with a positive regulatory role for \textit{PEP} in the \textit{CLV} pathway, although at which point(s) of the pathway \textit{PEP} exerts its action remains to be elucidated. A direct interaction with any of the \textit{CLV} genes cannot be excluded, but it seems unlikely since the semidominant effect appears to be common to both \textit{clv2} and \textit{clv3} mutants. Conceivable targets of \textit{PEP} include additional downstream regulators of the pathway, such as the negative effectors \textit{kapp} (Trotochaud et al., 1999) and \textit{poltergeist} (\textit{POL}) (Yu et al., 2003).

Although a negative regulator of the \textit{CLV} pathway, \textit{POL} presents some parallelism with \textit{PEP} in that their loss-of-function mutants are nearly wild-type in appearance, providing, however, recessive partial suppression of \textit{Clv}\textsuperscript{–} phenotypes (Yu et al., 2000). Based on broad expression profiles and genetic analyses, \textit{POL} was proposed to regulate multiple signaling pathways, including two \textit{CLV} routes modulating stem cell identity, \textit{WUS}\textsuperscript{–}-dependent and \textit{WUS}\textsuperscript{–}-independent, respectively (Yu et al., 2003). Another meristem regulator, \textit{ultrape- talai} (\textit{ULTI}), was also shown to act through two distinct routes, the \textit{AG} pathway at the FM, and a \textit{WUS}\textsuperscript{–}-independent one at the SAM (Fletcher, 2001; Carles et al., 2004, 2005).

We have also observed that complete loss-of-function of \textit{PEP} enhances the phenotype of \textit{clv3-2} and \textit{clv2-1} plants, including a slight increment in the average number of valves. This could be interpreted as a \textit{pep} effect on a separate pathway. In the FM, spatial restriction of \textit{WUS} by the \textit{CLV} pathway is followed by repression of \textit{WUS} expression by \textit{AG} (Lenhard et al., 2001; Lohmann et al., 2001). A participation of \textit{PEP} in both processes would account for our results.

In addition, \textit{PEP} seems to affect the length of valves and might regulate the proportion of the gynoecium occupied by valve tissue. Multiple functions are not infrequent for RNA-binding proteins. For example, \textit{hua1} and \textit{hua2} affect \textit{C} function during flower development, yet \textit{hua1 hua2} double mutants display a series of vegetative phenotypes (Chen and Meyerowitz, 1999). Moreover, \textit{hua2} has been recently identified as a repressor of the floral transition (Doyle et al., 2005). PEP-related PCBP-type KH proteins participate in a vast array of biological processes (Makeyev and Liebhaber, 2002), thereby making conceivable a multifunctional role for \textit{PEP}.

\textbf{Acknowledgments}

We thank H. Alonso-Cantabrana and I. Ochando for critical reading of the manuscript, J.L. Micol and L.V. López-Llorca for research facilities, J. Paz-Ares and the NASC for providing seeds, and M.A. Climent and A. Baso for technical assistance. This work was supported by research grants BIO2002-04083-C03-03 from the Ministerio de Educación y Ciencia of Spain to A.M.L., and GV99-142-1-05 from the Generalitat Valenciana to A.V. J.J.R. was a recipient of a doctoral fellowship from Ministerio de Educación y Ciencia of Spain.

\textbf{Appendix A. Supplementary data}

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.037.

\textbf{References}


