HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in Arabidopsis

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Summary

In plants, organs are generated post-embryonically from highly organized structures known as meristems. Cell division in the meristem is closely integrated with cell fate specification and organ formation. The presence of multiple cyclin-dependent kinases (CDKs) and their partner cyclins reflects the complexity of cell cycle regulation within developmental contexts. The Arabidopsis genome encodes at least eight CDKs and 30 cyclins. However, no mutants in any CDKs have been reported, and the function of the great majority of these genes in plant development is unknown.

We show that HUA ENHANCER3 (HEN3), which encodes CDKE, a homolog of mammalian CDK8, is required for the specification of stamen and carpel identities and for the proper termination of stem cells in the floral meristem. Therefore, CDK8 plays a role in cell differentiation in a multicellular organism.

Supplemental data available online

Key words: Flower development, HEN3, Cyclin-dependent kinase

Introduction

The shoot apical meristem (SAM) serves as the ultimate source of the above-ground biomass in plants. Cells in the central zone of the SAM are stem cells that maintain the undifferentiated state, while cells in the peripheral zone participate in organ formation and eventually acquire various identities. Cell division in the meristem must be closely integrated with cell fate specification and organ formation. The SAM generates floral meristems after floral transition. A floral meristem, like the SAM, gives rise to lateral organs (sepals, petals and stamens), but unlike the SAM, terminates in a gynoecium. In Arabidopsis, the termination of stem cells in the floral meristem requires AGAMOUS (AG), a MADS-domain transcription factor (Bowman et al., 1989; Yanofsky et al., 1990). AG represses the expression of WUSCHEL (WUS), a stem cell maintenance gene, in a temporal manner in the floral meristem (Laux et al., 1996; Lenhard et al., 2001; Lohmann et al., 2001). AG also acts in floral organ identity specification. As a class C floral homeotic gene, AG specifies stamen identity together with the class B and SEPALLATA (SEP) genes and carpel identity together with the SEP genes (Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Pelaz et al., 2000).

In addition to AG, several genes that act in the control of stamen and carpel identities and floral determinacy have been identified through sensitized genetic screens. Recessive mutations in HUA1 and HUA2, hua1-1 and hua2-1, respectively, were isolated as enhancers of the weak ag-4 allele (Chen and Meyerowitz, 1999). hua1-1 hua2-1 double mutant flowers show partial carpel-to-sepal and occasional stamen-to-petal transformation, phenotypes that are indicative of partial loss of class C activity (Chen et al., 2002; Chen and Meyerowitz, 1999; Western et al., 2002). In a genetic screen in the hua1-1 hua2-1 background, recessive mutations in HUA ENHANCER1 (HEN1), HEN2, HEN4 and PAUSED (PSD), were found to enhance the weak floral homeotic phenotypes of hua1-1 hua2-1 flowers such that stamens are transformed to petals and carpels are partially transformed to sepals (Chen et al., 2002; Cheng et al., 2003; Li and Chen, 2003; Western et al., 2002). The HUA and HEN genes all appear to directly or indirectly promote the expression of AG – in hua1-1 hua2-1 hen mutants, AG RNA or protein is of lower abundance than in wild type or in hua1-1 hua2-1 (Cheng et al., 2003; Li and Chen, 2003). HUA1 and HEN4 physically interact in the nucleus, and together with HUA2 and HEN2 promote AG expression by preventing the production of alternative transcripts containing intron sequences (Cheng et al., 2003). HEN1 is required for microRNA accumulation and may modulate AG expression or activity through regulation of APETALA2, a class A gene (Park et al., 2002; Chen, 2004)). In this study, we show that HEN3, which encodes an E-type CDK, also acts in the AG pathway.

CDKs are a family of serine/threonine protein kinases that control cell cycle progression, and/or coordinate cell cycle progression with transcription regulation. For a fully active state, they require both the association with regulatory subunits, cyclins and the phosphorylation of a conserved threonine residue by CDK-activating kinases (CAKs) (reviewed by Kobor and Greenblatt, 2002; Morgan, 1997). Among the nine known CDKs (CDK1-CDK9) in vertebrates, Cdc2 (CDK1) and Cdk2 carry out central cell cycle functions (reviewed by Morgan, 1997). CDK4 and CDK6 are thought to integrate developmental signals and environmental cues into...
the cell cycle to drive cells through the G1-S transition. Cyclin D/CDK4 and cyclin D/CDK6 complexes phosphorylate the retinoblastoma protein (Rb), which renders Rb unable to associate with E2F and related transcription factors, thus allowing them to activate genes necessary for S-phase progression (reviewed by Harbour and Dean, 2000). Plants have both Rb-related (RBR) and E2F-related proteins (de Veylder et al., 2002; Huntley et al., 1998; Maricotti et al., 2002; Xie et al., 1996), but do not have orthologs of CDK4 or CDK6 (Dewitte and Murray, 2003; Vandepoele et al., 2002). Three CDKs, CDK7, CDK8 and CDK9, function in transcriptional regulation. CDK7 is in the TFIIH complex, where it phosphorylates the C-terminal domain (CTD) of the largest subunit of RNA polymerase II to promote transcription elongation (Kobor and Greenblatt, 2002). CDK9/cyclin T belons to the positive transcription elongation factor b (P-TEFb) complex, which phosphorylates the CTD to promote transcription elongation (de Falco and Giordano, 2002).

In mammalian cells, CDK8/cyclin C is a component of RNA polymerase II holoenzyme and serves as a repressor of transcription through two mechanisms. It phosphorylates the cyclin H subunit of TFIIH and this leads to the repression of the ability of TFIIH to activate transcription and the inhibition of the CTD kinase activity of CDK7 (Akoulitchev et al., 2000). It also phosphorylates the CTD of RNA polymerase II prior to the formation of the preinitiation complex to result in inhibition of transcription (Liao et al., 1995; Rickert et al., 1996; Sun et al., 1998). Although the developmental functions of CDK8 in higher eukaryotes are currently unknown, CDK8 proteins from yeast and *Dictyostelium* appear to act in cell differentiation in response to nutrient conditions. Srb10p, the yeast CDK8, regulates filamentous growth in response to nutrient limitation (Nelson et al., 2003). The *Dictyostelium* CDK8 is required for aggregation, which leads to sporulation, under starvation (Takeda et al., 2002).

The *Arabidopsis* genome encodes eight CDKs classified into the A, B, C and E types, and 30 cyclins classified into the A, B, C and D types, according to sequence similarity among themselves and with their mammalian counterparts (Vandepoele et al., 2002). Of the four types of CDKs, A and B types have been best studied in plants. A-type CDKs regulate both the G1-S and G2-M transitions, whereas B-type CDKs control the G2-M transition (Hemerly et al., 1995; Magyar et al., 1997; Porceddu et al., 2001). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). Among themselves and with their mammalian counterparts, CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002). CDKAs have the PSTAIRE cyclin-binding motif and are associated with D-type cyclins (Vandepoele et al., 2002).

**Materials and methods**

**Isolation of hen3 mutations**

*hual1-1 hua2-1* seeds were mutagenized as described (Chen et al., 2002). Single M2 families were screened and three independent lines with ag-like floral phenotypes were identified. The mutations were recovered from the fertile siblings. Complementation tests with plants heterozygous for each mutation showed that the three alleles are in the same gene, which we named *HEN3*. The three *hen3* lines were backcrossed to *hual1-1 hua2-1* three times to clean up the genetic background. The *hual1-1 hua2-1 hen3* triple mutants were then crossed to either *hual1-1 or hua2-1* to obtain *hual1-1 hen3* or *hual2-1 hen3* double mutants. The two double mutants were then crossed to each other to obtain *hen3* single mutants. In these crosses, molecular genotyping for *hual1-1 and hua2-1* (Chen and Meyerowitz, 1999; Li et al., 2001) facilitated the identification of plants of the expected genotypes.

**Map-based cloning of HEN3**

*hual1-1 hua2-1 hen3-1/+* plants (in the Landsberg ecotype) were crossed to *hual1-1 hua2-1(Col)*, a line in which the *hual1-1* and *hua2-1* mutations were introgressed into the Columbia background. Five hundred and two plants with the *hual1-1 hua2-1 hen3-1* phenotypes in the F2 population were used for mapping. *HEN3* was initially mapped close to *HEN4* on chromosome V with the markers used to isolate *HEN4* (Cheng et al., 2003). New markers were developed based on the Cereon Genomics SNPs and used to map *HEN3* to a region of ~150 kb covered by P1 clones MBK5, MG119, MLE2 and MBM17 (see Figure S1A at http://dev.biologists.org/supplemental). We sequenced 12 candidate genes in this region from *hual1-1 hua2-1 hen3-1* plants and found a C-to-T mutation that would result in a stop codon in AT5g63610. Sequencing of AT5g63610 from *hen3-2* and *hen3-3* plants uncovered separate G-to-A mutations that would result in R-to-K and G-to-R amino acid substitutions, respectively (Fig. 4B). A fragment covering AT5g63610 was amplified by PCR with primers MBK5p7 (5’ tggctcgggtggaattacaaag 3’) and MBK5p8 (5’ctgggtagaatagatagatacggcag 3’) (see Figure S1A at http://dev.biologists.org/supplemental), cloned into pPZP211 (Hadjukiewicz et al., 1994). The resulting plasmid, pHEN3g, was transformed into *hual1-1* and *hua2-1* and found to rescue the silique length defects of these plants (see Fig. S1B at http://dev.biologists.org/supplemental). To determine whether the clone also rescues the *hual1-1 hua2-1 hen3-1* floral homeotic phenotypes, a *hual1-1 hen3-1* plant hemizygous for the *HEN3* gene was crossed to *hual2-1 hen3-1*. F2 seeds were plated on Kanamycin medium to select for the HEN3 transgene and Kanamycin resistant F2 plants were transferred to soil and screened to identify those with floral phenotypes that resembled *hual1-1 hua2-1*. Three such plants were obtained from a total of 56 F2 plants. The three plants were then genotyped for *hual1-1* and *hua2-1* and confirmed to be homozygous for both mutations. Therefore, these plants were *hual1-1 hua2-1 hen3-1* triple mutants that carried the *HEN3* transgene. As they exhibited *hual1-1 hua2-1* floral phenotypes, the transgene rescued the floral homeotic phenotypes conferred by the *hen3-1* mutation.

**HEN3 plasmid construction**

The 5’ and 3’ ends of *HEN3* RNA were determined by 5’ and 3’ RACE, respectively. The GenBank Accession Number for *HEN3* cDNA is AY600243.

To generate a HEN3-GUS reporter construct, a GUS-NOS cassette was released from pBI121 by EcoRl/BamHI and cloned into pPZP211 to generate pPZP211-GUS. The *HEN3*-coding region plus 1.5 kb of sequences upstream of the start codon was amplified with primers MBK5p7 and MBK5p10 (5’aggacctgtgatgatgagacgg 3’) and cloned into pPZP211-GUS. The resulting plasmid pPZP211-HEN3-GUS was transformed into Ler and *hen3-1* plants. The construct largely rescued the *hen3-1* vegetative defects. The Ler transformants were used to determine the expression profiles of *HEN3* by GUS staining.
The HEN3-coding region plus 1.5 kb of sequences upstream of the start codon was cloned into pZP211-HA to provide an HA tag to the C terminus of HEN3. The resulting plasmid pZP211-HEN3-HA was transformed into hen3-1 plants and found to largely rescue the silique defects of hen3-1 (Fig. S1B at http://dev.biologists.org/supplemental). These hen3-1 HEN3-HA plants were used to immunoprecipitate HEN3-HA in the protein kinase assay.

RNA filter hybridization and scanning electron microscopy (SEM)

RNA filter hybridization was carried out as described (Li et al., 2001). Total RNA (40 μg) was used to detect AG and AP1 RNAs. PolyA+ RNA (1 μg) was used to detect HEN3 RNA. Hybridization signals were quantified with a phosphorimager.

SEM was carried out as described previously (Chen et al., 2002).

Protein expression in E. coli

The C-terminal domain (CTD) of the largest subunit of RNA polymerase II from Arabidopsis (Dietrich et al., 1990) was amplified by RT-PCR using primers CTDp1 (5′-CCTGGGATCCCTGGAGTTGCAAATCTCCTCTT-3′) and CTDp2 (5′-CCTGGGATCCCTGGAGTTGCAAATCTCCTCTT-3′). The amplified CTD fragment was cloned into the E. coli expression vector pRSETa to generate pRSETa-CTD. The His-tagged CTD polypeptide was expressed in E. coli (or anti-protein C as a control)-coupled matrix slurry and incubated (or anti-protein C as a control)-coupled matrix slurry and incubated. Finally, 40 μl of supernatant was added to 160 μl of 50% anti-HA mixture and precipitated by lysis and purification according to manufacturer’s instruction (Qiagen).

Histone H1 was purchased from Roche (CAT#6023549). His-GFP protein was used as a negative control, while histone H1 was used as a potential alternative substrate. The reaction was stopped by adding SDS-PAGE loading buffer and boiling for 5 minutes. Samples were analyzed by SDS-PAGE.

Results

HEN3 is required for cell fate specification in flowers

We performed a genetic mutagenesis screen in the hua1-1 hua2-1 background to search for genes acting in floral meristems to control stem cell termination and lateral organ identities. We isolated three recessive mutations in a gene we named HUA ENHANCER3 (HEN3), hen3-1, hen3-2, and hen3-3 all resulted in loss of reproductive organ identity and floral determinacy in the hua1-1 hua2-1 background. While the third whorl organs were stamens in wild-type and hua1-1 hua2-1 flowers, petals were the predominant type of organs in the third whorl of hua1-1 hua2-1 hen3 flowers (Table 1, Fig. 1A-D). Although stigmatic tissue was found on some fourth whorl organs in hua1-1 hua2-1 hen3-1 and hua1-1 hua2-1 hen3-2 flowers (Fig. 1C,D), all abaxial epidermal cells on these organs showed epicuticular striations similar to those on abaxial sepal cells (Fig. 1H). By contrast, the majority of epidermal valve cells on hua1-1 hua2-1 ovaries exhibited a smooth surface characteristic of normal ovary valve cells (Fig. 1G). The number of fourth whorl organs in hua1-1 hua2-1 hen3-1 and hua1-1 hua2-1 hen3-2 flowers was increased (Table 1). In addition, internal flowers were present in all hua1-1 hua2-1 hen3 flowers (Fig. 1E and data not shown). These phenotypes suggest that HEN3 acts in the specification of stamen and carpel identities and floral determinacy in Arabidopsis flower development. The hen3-2 allele is the most severe among the three in the floral phenotypes—staminoid petals were found in the third whorl of hua1-1 hua2-1 hen3-1 or hua1-1 hua2-1 hen3-2 but not hua1-1 hua2-1 hen3-3 flowers (Table 1). No organ identity or floral determinacy defects were obvious in hua1-1 hen3, hua2-1 hen3 or hen3 mutants (Fig. 1F; data not shown). Although siliques failed to develop, all hua1-1 hen3, hua2-1 hen3 double mutants and hen3 single mutants produced shorter siliques and stems than the corresponding HEN3 genotypes (Fig. S1 and data not shown).

Consistent with the floral phenotypes, we found that hua1-1 hua2-1 hen3-1 flowers were defective in the spatial control of AP1 expression and the temporal control of WUS expression in flower development. AP1 RNA is found throughout the floral primordium during stages 1-2 but is restricted to the outer two
floral whorls starting at stage 3 because of repression of its expression by AG in the inner two whorls (Gustafson-Brown et al., 1994; Mandel et al., 1992). At stage 3, AP1 RNA accumulation patterns in hua1-1 hua2-1 flowers were similar to those in wild type (Chen and Meyerowitz, 1999) (Fig. 2A). However, in hua1-1 hua2-1 hen3-1 stage 3 flowers, as in ag-3 flowers, AP1 RNA was found throughout the floral primordium (Fig. 2B). The ectopic expression of AP1 in the inner two floral whorls was found in hua1-1 hua2-1 hen3-1 flowers of all stages (data not shown). WUS is expressed in a few cells underneath the presumed stem cells in the floral meristem till stage 6, when AG acts to terminate its expression (Mayer et al., 1998). Although WUS RNA was not observed in stage 7 and older hua1-1 hua2-1 flowers (Fig. 2C), WUS RNA was readily
detected in stage 7 and older hua1-1 hua2-1 hen3-1 (Fig. 2D) and hua1-1 hua2-1 hen3-2 (data not shown) flowers.

**HEN3 acts primarily in the AG pathway in flower development**

As HEN3 acts similarly to AG in flower development, we asked whether HEN3 acts in the AG pathway or in a parallel pathway. If HEN3 acts strictly in the AG pathway, we would expect hua1-1 hua2-1 hen3-1 ag-1 flowers to be identical to ag-1 flowers in all phenotypes. Indeed, ag-1 was essentially epistatic to hua1-1 hua2-1 hen3-1 in that only perianth organs were present in hua1-1 hua2-1 hen3-1 ag-1 flowers (Fig. 1I). However, there appeared to be more whorls of petals than those of sepals in the quadruple mutant flowers. To investigate this further, we constructed the hen3-1 ag-1 double mutant and found that hen3-1 caused partial sepal-to-petal transformation in the fourth whorl of ag-1 flowers (Fig. 1J). This was probably the reason for the presence of more petals in the quadruple mutant. Therefore, HEN3 has functions overlapping with, but not identical to, those of AG in organ identity specification. In terms of floral determinacy, HEN3, as well as HUA1 and HUA2, appeared to act in the AG pathway as hen3-1 did not enhance ag-1 in this phenotype. AG and CLV1 both control floral determinacy by regulating the expression of WUS. However, the molecular mechanisms differ – while AG represses WUS in a temporal manner, CLV1 restricts the domain of WUS expression (Schoof et al., 2000). In fact, ag-1 enhances clv1-1 in floral determinacy defects (Clark et al., 1993). If HUA1, HUA2 and HEN3 act in the AG pathway in floral determinacy, we would expect hua1-1, hua2-1 and hen3-1 to enhance the severe clv1-1 allele in floral determinacy defects. Indeed, hua1-1 hua2-1 hen3-1 clv1-4 flowers showed dramatic enlargement of the floral meristem, such that a massive group of apparently undifferentiated cells accumulated in the center of the flower (Fig. 1L). This dramatic phenotype was not observed in either hua1-1 hua2-1 hen3-1 or clv1-4 (Fig. 1K) flowers.

Mutations in HEN1, HEN2, HEN4 and PSD all resulted in reduced AG expression in the hua1-1 hua2-1 background (Cheng et al., 2003; Li and Chen, 2003), which was consistent with the floral homeotic phenotypes caused by these mutations. The hen3 mutations lead to more severe loss-of-C-function phenotypes when compared with the other hen mutations. We performed RNA filter hybridization to determine whether the hen3-1 mutation also results in reduced AG RNA levels in the hua1-1 hua2-1 background. Three AG RNAs, RNA1, RNA2 and AG mRNA, can be detected in hua1-1 hua2-1 and hua1-1 hua2-1 hen3-1 flowers (Fig. 3A). RNA1 and RNA2 are second intron-containing AG RNAs that cannot generate the full-length AG protein (Cheng et al., 2003). Although mutations in HEN2, HEN4, HUA1 and HUA2 lead to increased abundance of RNA1 and RNA2, and a concomitant decrease in abundance of AG mRNA (Cheng et al., 2003), hen3-1 resulted in an increase in abundance in both the intron-containing RNAs and the mRNA in the hua1-1 hua2-1 background (Fig. 3A). The abundance of API (Fig. 3A) and AP2 (not shown) was also increased in hua1-1 hua2-1 hen3-1 flowers relative to hua1-1 hua2-1 flowers.

In situ hybridization showed that the timing and domain of AG mRNA accumulation in hua1-1 hua2-1 hen3-1 flowers were normal (data not shown). AG protein abundance was similar between hua1-1 hua2-1 and hua1-1 hua2-1 hen3-1 flowers (Fig. 3B).

**HEN3 encodes CDKE**

To begin to understand the molecular functions of HEN3, we cloned this gene with a map-based approach (see Materials and methods; Fig. S1). The predicted HEN3 (At5g63610) protein shares significant sequence similarity to plant and animal cyclin-dependent protein kinases (Fig. 4A,B), and was classified as an E-type CDK with a SPTAIRE cyclin binding motif in the kinase domain (Joubes et al., 2000; Vandepoele et al., 2002). HEN3 and related proteins from alfalfa and rice are currently the only members of the E class of plant CDKs (Fig. 4C), for which no cellular or developmental functions are known. Class E CDKs appear to be more related to CDK8 throughout the kinase domain than any other CDKs in metazoans (Fig. 4C). In vertebrates, CDK8, in association with cyclin C, phosphorylates the CTD of the largest subunit of RNA polymerase II (Liao et al., 1995; Rickert et al., 1996). Interestingly, HEN3 contains a SPTAIRE cyclin binding motif that differs from the SMSACRE motif from CDK8 but resembles the PSTAIRE motif from plant CDKs (Fig. 4B). Plant CDKAs are more closely related to human CDK1 (cdc2), CDK2 and CDK3 than to CDK8 (Fig. 4C).

To test whether HEN3 possesses protein kinase activity, we generated transgenic lines in which HEN3 was tagged with the HA epitope in a translational fusion under the control of the HEN3 promoter. The HEN3-HA transgene was able to rescue the hen3-1 silique (Fig. S1B at http://dev.biologists.org/supplemental) and stem elongation defect
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**Fig. 4.** HEN3 is closely related to CDK8. (A) A diagram of HEN3 protein with the serine-threonine kinase domain represented by the oval. (B) A Clustal W alignment of the kinase domains in HEN3 and human CDK8. Identical and similar amino acids are shaded. The amino acid positions and nature of the three hen3 alleles are indicated above the sequences. The cyclin-binding motifs of HEN3 and CDK8 are underlined. (C) A bootstrap consensus phylogenetic tree involving nine different human CDKs, CDK8 from *Drosophila* (DmCDK8), mouse (MmCDK8), *Dictyostelium* (DdCDK8) and yeast (Srb10p), three E type CDKs from plants, and the *Arabidopsis* CDKA (AtCDKA). Cdc2MsE and OsAAG46164 are CDKEs from alfalfa and rice, respectively. The tree was constructed using the neighbor joining method and the bootstrap consensus was generated from 1000 replications. The numbers represent the percentage occurrence of the nodes in the replications. Only the protein kinase domains from these proteins were used in the analysis. The CDK8 clade is indicated by the rectangle.

HEN3 is expressed in proliferating tissue

CDKs involved in cell cycle progression are preferentially expressed in dividing tissues (Dewitte and Murray, 2003). To determine if HEN3 is expressed preferentially in proliferating tissue, we generated a translational fusion of HEN3 to b-glucuronidase (GUS) under the control of the HEN3 promoter. This fusion construct was introduced into *hen3-1* and found to largely rescue the *hen3-1* vegetative phenotypes. Histochemical staining for GUS activity showed that HEN3 is expressed in young, presumably dividing, tissue, such as shoot and root tips, lateral root primordia, young leaves, and flowers (Fig. 2E-H). We performed in situ hybridization to determine the localization of HEN3 RNA in inflorescence meristems and in flowers. HEN3 RNA was present throughout the inflorescence meristem, the inflorescence stem and young flowers (Fig. 2I,J). The uniform presence of HEN3 RNA in every cell in inflorescence and floral meristems indicates that HEN3 RNA abundance is not regulated in a cell cycle dependent manner. The alfalfa CDKE gene is also expressed at a basal unchanged level throughout the cell cycle (Magyar et al., 1997). RNA filter hybridization showed that HEN3 RNA was...
Fig. 5. HEN3 has CTD kinase activity. HEN3-HA transgenic plants or control non-transgenic plants were immunoprecipitated with an anti-HA monoclonal antibody, and the immunoprecipitate was used in a kinase assay with 10 μg purified histone H1, 6xHis-CTD or 6xHis-GFP as substrates. The positions of the substrate proteins are illustrated by the asterisks. The amount of substrate proteins is identical in each lane. (A) The anti-HA (lanes 2) but not the anti-protein C (lanes 1) immunoprecipitate displays kinase activity on HEN3-HA transgenic plants (lanes 2). Histone H1 and 6xHis-GFP are not phosphorylated by the anti-HA immunoprecipitate. (B) The anti-HA immunoprecipitate from HEN3-HA transgenic plants but not from wild-type plants phosphorylates 6xHis-CTD.

Discussion
HEN3 and CDK8
Phylogenetically, HEN3 groups with CDK8 proteins from yeast, Dictyostelium, and metazoans. In mammals and yeast, CDK8 forms a complex with cyclin C and phosphorylates the CTD of the largest subunit of RNA polymerase II (Kobor and Greenblatt, 2002). The phosphorylation status of the CTD is dynamically controlled by kinases and phosphatases during transcription initiation and elongation and appears to dictate number or cell size. We measured the area of the fully expanded fifth leaf in wild-type and the three hen3 mutants. The most severe hen3-2 allele caused a nearly 50% reduction in leaf size (Fig. 6D). We next performed scanning electron microscopy to examine leaf adaxial epidermal cells in the four genotypes. Although leaf epidermal cells from the same leaf can vary greatly in cell size in all genotypes (hence the large standard error), the hen3 mutants had, on average, smaller cells than wild type (Fig. 6E). In the same unit area in a leaf, the hen3 mutants had more epidermal cells (Fig. 6F). Taking into account the differences in leaf size, these genotypes have roughly the same number of cells in the fifth leaves. Therefore, it appears that the reduction in leaf size caused by hen3 mutations is due to reduced cell expansion rather than cell division. Consistent with this, we did not observe any differences in the morphology of the SAM or leaf primordia between Ler and hen3-1 in longitudinal sections or by SEM (Fig. S2 at http://dev.biologists.org/supplemental). In situ hybridization showed that histone H4, a gene known to be expressed in a cell cycle-dependent manner, was expressed similarly in wild-type and hen3-1 SAMs, and leaf primordia (see Fig. S2 at http://dev.biologists.org/supplemental).

hen3 single mutant plants also exhibited shorter stems and siliques (see Fig. S1B at http://dev.biologists.org/supplemental). The valve cells of hen3 siliques were shorter than those in wild type (data not shown). Root growth on media with or without sucrose was similar in wild-type and hen3-1 plants (see Fig. S3 at http://dev.biologists.org/supplemental; data not shown).

Fig. 6. Effects of hen3 mutations on leaf size and leaf epidermal cell size. (A–C) Dissected rosette leaves 1-7 (arranged from left to right) from hen3 (top) and wild-type (bottom) plants that were grown side by side. (A) hen3-1 and wild type. (B) hen3-2 and wild type. (C) hen3-3 and wild type. Note that the first two leaves are not affected as much as the later leaves by the hen3 mutations. The hen3 mutations all lead to a reduction in leaf size. (D) Area (cm²) of the fully expanded fifth leaf from wild-type and hen3 genotypes. Ten leaves were measured for each genotype. (E) Surface area (μm²) of adaxial epidermal cells on the fifth leaf of wild-type and hen3 genotypes. The number of cells measured was 95, 141, 173 and 143 for wild type, hen3-1, hen3-2 and hen3-3, respectively. The large standard error is largely due to the intrinsic variation in cell size on the leaf epidermis. (F) Number of epidermal pavement cells in a 70,000 μm² area from similar positions in the fifth leaves of various genotypes. Measurements were performed on SEM images of three leaves from each genotype. The error bars in D–F represent standard errors.
Developmental roles of HEN3

HEN3 RNA and protein (HEN3-GUS) are present in proliferating tissue, where cells need to coordinate developmental events such as cell division, expansion and fate specification. HEN3 seems to be required for cell expansion in leaves and cell fate specification in floral meristems. Although a direct role of HEN3 in cell division is not obvious from the hen3 mutant phenotypes, its mammalian homolog CDK8 controls cell proliferation through negative regulation of TFIIH transcription activity (Akoulitchev et al., 2000). We speculate that HEN3 can be a potential link between cell division and cell fate specification in the floral meristem. For example, key regulators of cell division and cell fate specification may be coordinately regulated by HEN3 through either transcriptional regulation or phosphorylation, thus linking cell division control to cell fate specification. Alternatively, HEN3 activity may be regulated by the cell cycle, which ensures that the function of HEN3 in cell fate specification is integrated with cell division. Although HEN3 RNA or HEN3-GUS does not accumulate in a cell-cycle-dependent manner, HEN3 activity may be regulated by the cell cycle through its partner cyclins.

How might HEN3 be involved in cell expansion? Plant cell expansion can be influenced by many processes, such as cell wall relaxation or reorganization, endoreduplication, cytoskeleton remodeling and hormone signaling (reviewed by Clouse and Sasse, 1998; Cosgrove, 1993; Sugimoto-Shirasu and Roberts, 2003; Wasteney and Galway, 2003). HEN3 may directly or indirectly affect the expression or function of genes in any of these processes.

How HEN3 acts in the AG pathway is currently unknown. Our previous work showed that HUA1, HUA2, and HEN4 prevent transcription termination within the second intron of AG. Mutations in these genes result in reduced abundance of AG mRNA and increased abundance of AG RNA1 and RNA2, transcripts that terminate in the second intron. HEN3 is able to phosphorylate the CTD, which regulates transcription elongation and coordinates RNA processing with transcription elongation. Therefore, it is plausible that HEN3 is also involved in alternative transcript production from AG. However, the hen3-1 mutation leads to increased accumulation of not only AG RNA1 and RNA2 but also AG mRNA. This can be best explained by a role of HEN3 in repressing transcription, as has been demonstrated for mammalian and yeast CDK8 proteins. One possible explanation for the loss of C-function phenotypes caused by hen3 mutations is that the hen3 mutations lead to higher levels of RNAs from class A genes than from AG, such that A function wins over C function during flower development. Alternatively, HEN3 may play an indirect role in organ identity through its primary function in cell division or cell elongation. It is conceivable that missing cells or altered timing of cell elongation in organ primordia may influence their identities. However, we have not observed gross differences in developing organ primordia between wild-type and hen3 mutants. Finally, HEN3 may regulate class A or C proteins by phosphorylation. A recent study showed that the budding yeast cdc28 kinase, a key cell cycle regulator, phosphorylates not only proteins that drive cell cycle events but also other regulatory proteins to supposedly orchestrate global gene expression throughout the cell cycle (Ubersax et al., 2003). Another study demonstrated that Srb10, the budding yeast homolog of CDK8, regulates yeast filamentous growth in response to nutrient conditions by phosphorylating, and thus regulating, Ste12, a transcription factor required for filamentous growth (Nelson et al., 2003). It is possible that HEN3 acts as a class C gene by phosphorylating either class C or class A proteins.

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References


Clouse and Sasse, 1998; Cosgrove, 1993; Sugimoto-Shirasu and Roberts, 2003; Wasteney and Galway, 2003).


