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An Enhancer Trap Line Associated with a D-Class Cyclin Gene in Arabidopsis¹

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In yeast and animals, cyclins have been demonstrated to be important regulators of cell cycle progression. In recent years, a large number of A-, B-, and D-class cyclins have been isolated from a variety of plant species. One class of cyclins, the D-class cyclins, is important for progression through G1 phase of the cell cycle. In Arabidopsis, four D-class cyclins have been isolated and characterized (*CYCLIN-D1;1*, *CYCLIN-D2;1*, *CYCLIN-D3;1*, and *CYCLIN-D4;1*). In this report we describe the characterization of a fifth D-class cyclin gene, *CYCLIN-D3;2* (*CYCD3;2*), from Arabidopsis. An enhancer trap line, line 5580, contains a T-DNA insertion in *CYCD3;2*. Enhancer trap line 5580 exhibits expression in young vegetative and floral primordia. In line 5580, T-DNA is inserted in the first exon of the *CYCD3;2* gene; in homozygous 5580 plants *CYCD3;2* RNA is not detectable. Even though *CYCD3;2* gene function is eliminated, homozygous 5580 plants do not exhibit an obvious growth or developmental phenotype. Via in situ hybridization we demonstrate that *CYCD3;2* RNA is expressed in developing vegetative and floral primordia. In addition, *CYCD3;2* is also capable of rescuing a yeast strain that is deficient in G1 cyclin activity.

In yeast and animals, heterodimeric cyclin/cyclin-dependent kinase complexes are essential regulators of cell cycle progression. These complexes consist of a cyclin-dependent Ser/Thr kinase subunit (Cdk) and a regulatory cyclin subunit whose protein levels oscillate during the cell cycle due to regulated synthesis and degradation. The major cell cycle control points of cyclin-Cdk complexes are during late G1 phase and at the G2 to M phase transition. In animals, cyclins and Cdks are redundantly encoded and different cyclin-Cdk combinations function to regulate different cell cycle checkpoints. For example, in animals, A- and B-class mitotic cyclins together with Cdk1 regulate the G2 to M transition, whereas cyclin D together with Cdk2, Cdk4, or Cdk5 functions to control passage through G1.

Plants, like animals, contain multiple cyclin and Cdk genes. In plants, cyclins similar to animal cyclin A, cyclin B, cyclin D, and cyclin H (or cyclin C [Mironov et al., 1999]) have been isolated (for review, see Renaudin et al., 1996). At present more than 60 cyclin genes have been isolated from a variety of plant species. The majority of these genes were isolated by homology to previously isolated cyclins. A subset, however, was isolated based on the ability to complement a yeast strain that is deficient in G1 cyclin activity (Dahl et al., 1995; Soni et al., 1995).

In animals, D-class cyclins function during G1 and thus are thought to be important for reentry of quiescent cells (G0) into the cell cycle. Formation of an active cyclin D-Cdk complex during G1 is postulated to be dependent on signals that stimulate cell growth

(e.g. hormones, mitogens, and growth factors). D-class cyclins in Arabidopsis have been shown to be responsive to Suc and cytokinins (Soni et al., 1995; De Veylder et al., 1999). One D-class cyclin from Arabidopsis, *CYCLIN-D3;1* (*CYCD3;1*) is induced by cytokinins and stimulation of cell division by cytokinins can be overcome by ectopic expression of *CYCD3;1* under the control of a heterologous promoter (Riou-Khamlichi et al., 1999). This demonstrates that D-class cyclins from plants, like those from animals, are responsive to extrinsic signals that stimulate cell division. A second Arabidopsis D-class cyclin, *CYCLIND2;1* (*CYCD2;1*), when ectopically expressed in tobacco, results in a faster growth rate and larger plants (Cockcroft et al., 2000).

At present, four D-class cyclins have been isolated from Arabidopsis and these genes have been divided into four subgroups referred to as *CYCLIN-D1;1* (*CYCD1;1*), *CYCD2;1*, *CYCD3;1*, and *CYCLIND4;1* (*CYCD4;1*). All four Arabidopsis D-class cyclins contain a 105-amino acid domain called the cyclin box that is conserved in all eukaryotic cyclin proteins and is postulated to encode a Cdk-binding domain (Jeffrey et al., 1995). In addition to the cyclin box, all plant D-class cyclins contain a short domain with similarity to the LxCxE motif that is capable of being bound by plant retinoblastoma (Rb) and Rb-related proteins (Huntley et al., 1998). Rb is a well-characterized recessive mammalian oncoprotein that has been demonstrated to be important in the G1 to S cell cycle transition. Although retinoblastoma-related proteins (RBRs) have been isolated from a number of plant species (de Jager and Murray, 1999), it is not known if the plant RBRs function in cell cycle control, but the fact that overexpression and antisense inhibition of an Arabidopsis RBR results in fasciated and distorted

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meristems suggests that plant RBRs might function as cell cycle regulators (Inze et al., 1999).

In this report we describe the isolation and characterization of a fifth D-class cyclin gene from Arabidopsis. This gene was isolated from an enhancer trap line that exhibits expression in vegetative and floral primordia (Campisi et al., 1999). By sequence, this gene is closely related to *CYCD3;1* and thus has been designated *CYCLIND3;2* (*CYCD3;2*). Analysis of this enhancer trap line demonstrates that the T-DNA is inserted in the middle of the first exon of *CYCD3;2* and has created a loss-of-function allele. No obvious mutant phenotype is evident, however, suggesting that cyclin D genes from plants, like those in other kingdoms, function redundantly.

RESULTS

Enhancer Trap Line 5580

To identify genes that function during the early stages of Arabidopsis flower development based on

expression pattern, we generated more than 11,000 enhancer trap lines and determined the staining pattern in the inflorescence for all lines (Campisi et al., 1999). One line, 5580, exhibited expression in seedlings and inflorescences during the early stages of leaf and flower development (Fig. 1). To analyze the staining patterns in more detail, we sectioned β -glucuronidase (GUS)-stained seedlings and inflorescences and viewed them via dark field microscopy. In the seedling, GUS activity is detected in the shoot apical meristem and in young leaf primordia. No GUS activity is detected in mature leaves, hypocotyl, or roots (Fig. 1, A and C). In the inflorescence, GUS activity is detected in the inflorescence stem and in the shoot apex. GUS activity is detected at a low level in the pedicel and at a higher level throughout young flowers until floral stage 5 (Smyth et al., 1990; Fig. 1, B and D). Beginning at stage 6, GUS activity decreases in the sepals, but remains high in the stamens and carpels. During stages 7 to 8 GUS activity remains high in the carpels and stamen filaments, but

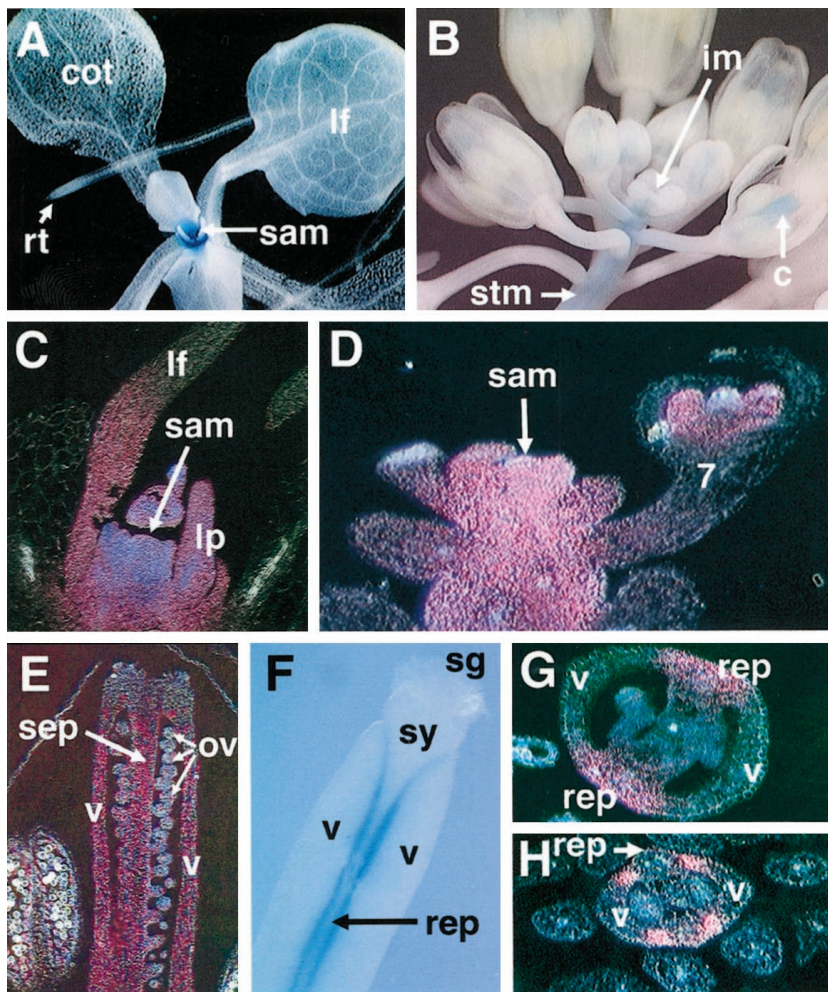


Figure 1. Enhancer trap expression pattern in line 5580. A, GUS activity pattern in a whole-mount 5580 seedling. GUS activity is detected at high levels in the shoot apical meristem (sam) and in young leaf primordia. No GUS activity is detected in the cotyledons (cot), leaves (lf), or roots (arrow points to a root tip [rt]). B, GUS activity pattern in a whole mount 5580 inflorescence. GUS activity is detected in the inflorescence stem (stm) and in carpels (c) from stages 6 to 12. The location of the inflorescence meristem (im) is indicated. C, GUS-stained and sectioned 5580 seedling viewed under dark field. GUS activity is detected at high levels in the shoot apex (sam) and in young leaf primordia (lp). In an older leaf, GUS activity is detected only in the basal region of the leaf (lf). D, GUS-stained and sectioned 5580 inflorescence viewed under dark field. GUS activity is detected in the inflorescence stem (sam) and throughout flower primordia through stage 5. Beginning at stage 6, GUS activity fails to be detected in the sepals, but is still expressed at high levels in the petals, stamens, and carpels. GUS activity is detected at a low level in the pedicel. Number refers to floral stage. E, Section through a 5580 gynoecium at stage 11. GUS activity is detected in the septum (sep) and ovary valves (v) of the carpel. GUS activity is not detected in the ovules (ov). F, Whole mount of a GUS-stained 5580 carpel at stage 14. GUS activity is detected in the cells at the border of the valves (v) and replum (rep), in the valve margin. GUS activity is not detected in the style (sy) of stigma (sg). G, Cross section through a stage 12 carpel. GUS activity is detected at high levels in the replum (rep). GUS activity is not detected in the carpel valve (v), septum, or ovule. H, Cross section through a stage 14 carpel. GUS activity is detected in four domains of expression, in the cells of the valve margin.

DNA flanking the right border of line 5580 was isolated by thermal asymmetric interlaced (TAIL) PCR (Liu et al., 1995). BLAST searches with the flanking TAIL PCR sequence revealed a strong similarity to cyclins from animals, plants, and fungi (Fig. 2). The highest level of similarity was with D-class cyclins from plants (Renaudin et al., 1996; De Veylder et

[illegible]

al., 1999). To further characterize this cyclin gene, genomic and cDNA clones were isolated. Sequencing of a 1.4-kb cDNA revealed the presence of a single long open reading frame that encodes a putative 367-amino acid protein (Fig. 2B). Throughout its length this protein exhibits similarity to D-class cyclins. This protein is most similar to cyclin D3 proteins from tobacco, tomato, pea, alfalfa, and Arabidopsis (Soni et al., 1995). Among characterized Arabidopsis cyclins, the 5580 cyclin is most similar to *CYCD3;1* (51% identical, 74% similar; Soni et al., 1995; Riou-Khamlichi et al., 1999). The cyclin associated with the 5580 insertion, however, exhibits the highest level of identity (62%) and similarity (76%) to an uncharacterized Arabidopsis cyclin D3 identified in the Arabidopsis Genome Initiative (accession no. AL132978, chromosome 3). Because of the similarity of the 5580 cyclin to cyclins in the D3 class, the cyclin associated with the 5580 insertion has been named *CYCD3;2*.

Similar to other D-class cyclins, *CYCD3;2* conserves the 105-amino acid cyclin box (amino acids 94–199; single underline in Fig. 2B). The cyclin box is postulated to encode a domain that interacts with cyclin-dependent kinases (Jeffrey et al., 1995). *CYCD3;2* also contains an LxCxE Rb interaction motif (amino acids 19–23; double underline in Fig. 2B).

To determine the map position of *CYCD3;2*, a polymorphism was identified between Arabidopsis ecotypes Landsberg and Columbia and *CYCD3;2* was mapped using a population of recombinant inbred lines (Lister and Dean, 1993) to chromosome 5 between markers g2368 and m555. The region of chromosome 5 containing *CYCD3;2* (TAC clone K3G17) was subsequently sequenced by the Arabidopsis Genome Initiative.

The sequence of the TAIL-PCR fragment reveals that the T-DNA in line 5580 is inserted 5' to the cyclin box in the first exon, between amino acids 74 and 75 in the *CYCD3;2* protein (Fig. 2A). It is curious that 43 bp of the TAIL-PCR fragment adjacent to the right border of the T-DNA derive from the T-DNA containing plasmid or from the *CYCD3;2* gene. BLAST searches with this sequence did not indicate any strong similarity to known sequences. Presumably this 43-bp sequence is an artifact of the T-DNA integration process.

To determine copy number of the T-DNA in the 5580 line we analyzed segregation of kanamycin resistance. The original line segregated approximately 2:1 (kanamycin-resistant:kanamycin-sensitive); this ratio suggests that likely there is only a single segregating T-DNA insertion. This conclusion was supported by Southern-blot analysis using probes within the T-DNA (data not shown). To ensure that the flanking Arabidopsis genomic DNA that we isolated was due to insertion of an intact T-DNA in the *CYCD3;2* locus, we performed a PCR assay to test for linkage of the 5580 staining pattern, kanamycin resistance, and presence of flanking *CYCD3;2* se-

quences. DNA was prepared from 20 individual homozygous 5580 plants; each of these plants was kanamycin resistant and exhibited the 5580 enhancer trap GUS activity pattern described above. PCR reactions were performed using a T-DNA-specific primer in combination with a *CYCD3;2*-specific primer. A 450-bp band was amplified from all 20 5580 DNA samples, but not from control wild-type Col plants, demonstrating that kanamycin resistance and the 5580 enhancer trap staining pattern cosegregate 100% of the time with the *CYCD3;2* gene.

CYCD3;2 Is Expressed in Actively Dividing Cells in Seedlings and Inflorescences

We determined the spatial and temporal expression pattern of *CYCD3;2* by RNA gel-blot and in situ hybridization. Although the *CYCD3;2* RNA expression pattern is similar to the 5580 enhancer trap pattern, it is not identical. It is likely that the difference is due to the orientation of the T-DNA in the *CYCD3;2* gene.

To determine the tissue distribution of the *CYCD3;2* RNA we performed RNA gel blots with total RNA from roots, seedlings, leaves, siliques, and inflorescences (Fig. 3A). *CYCD3;2* is expressed at high levels in shoots and inflorescences and at lower levels in leaves, siliques, and roots. To determine the spatial and temporal expression pattern of *CYCD3;2* RNA, we performed in situ hybridization in wild-type seedlings and inflorescences using a DIG-labeled antisense *CYCD3;2* probe (Fig. 4). In the seedling, *CYCD3;2* RNA is expressed at high levels in vegetative shoot apical meristem and in young leaf primordia (Fig. 4, A and C). As the leaves enlarge and differentiate, the levels of *CYCD3;2* RNA decrease to background levels. In the inflorescence, *CYCD3;2* is expressed in three types of cells: (a) young flower primordia and developing floral organs, (b) microspores and megaspores, and (c) vasculature in the main stem, pedicel, and floral organs. *CYCD3;2* is specifically expressed in the shoot apical meristem and throughout flowers through stage 3 (Smyth et al., 1990; Fig. 4D). Beginning at stage 4, two changes in the pattern occur (Fig. 4F). First, in sepal primordia, there is a reduction in the level of expression and the number of cells that express *CYCD3;2* RNA. Second, expression in whorls 2 and 3 becomes patchy with clusters of cells expressing at a lower relative level. At stage 6, *CYCD3;2* is expressed in the developing carpels, stamens, and petals located in the inner three whorls of the flower (Fig. 4G). In the first whorl sepals, however, *CYCD3;2* expression is detectable at the base of the sepals, but expression in more apical cells is not detectable. During stages 7 and 8, *CYCD3;2* is expressed at high levels throughout the petals (Fig. 4, H and I), and expression remains detectable in petals through stage 12. The expression pattern of *CYCD3;2* in petals is in sharp contrast to

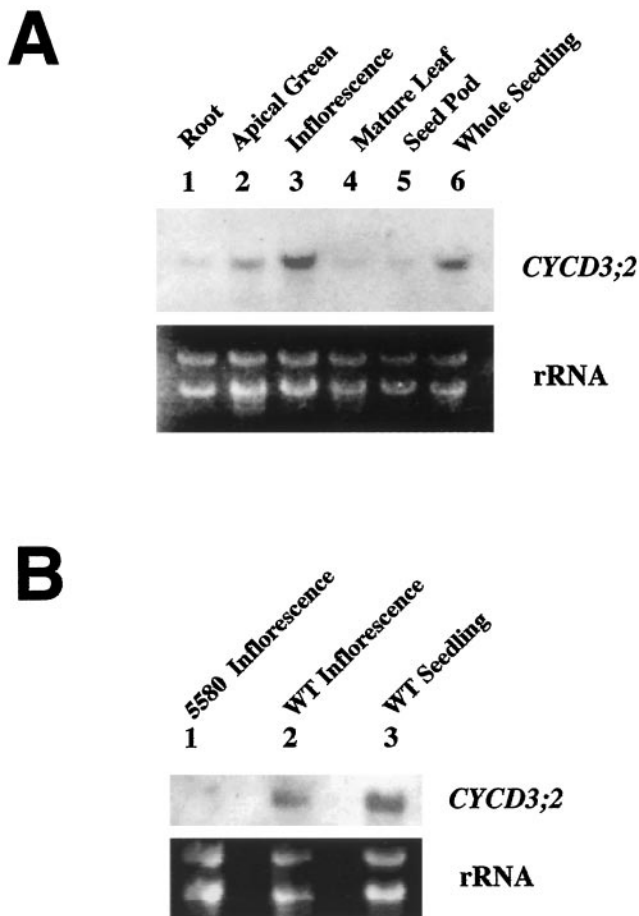


Figure 3. RNA gel blots. A, RNA gel blot of total RNA from various Arabidopsis tissues hybridized with a *CYCD3;2* probe (top). Lane 1, Root RNA; lane 2, RNA from apical green seedling tissue; lane 3, inflorescence RNA; lane 4, mature leaf RNA; lane 5, silique RNA; lane 6, whole seedling RNA. Highest levels of *CYCD3;2* expression are detected in inflorescence and seedling RNA; lower levels are detected in mature leaf, seedling leaf, and root RNA. Bottom, Ethidium bromide-stained gel showing ribosomal RNA as a loading control. B, top, RNA gel blot of total RNA from homozygous 5580 inflorescences (lane 1), wild-type seedlings (lane 2), and wild-type inflorescences (lane 3). The T-DNA in line 5580 is located in the first exon of the *CYCD3;2* gene. No *CYCD3;2* is detectable in RNA isolated from 5580 homozygous inflorescences. Bottom, Ethidium bromide-stained gel showing ribosomal RNA as a loading control.

the pattern in sepals, in which *CYCD3;2* is not detectable after stage 6. Beginning at stage 8, the ovules begin to develop and *CYCD3;2* is expressed throughout the developing ovules (Fig. 4, J and K). As the carpel differentiates during stages 9 through 12, *CYCD3;2* RNA is detectable in carpel walls (valves), the stigma, and the septum, but not in the style (Fig. 4K). Expression in the ovules is comparatively strong and is detected throughout the funiculus, integuments, and developing megaspore. In the stamens, *CYCD3;2* RNA is detectable at high levels in the vasculature of the stamen filaments, as well as in the developing microspores (Fig. 4I).

Comparison of the *CYCD3;2* RNA in situ expression pattern with the 5580 enhancer trap GUS expression pattern reveals that the two patterns are similar, but not identical. There are two major differences in the patterns. First, the 5580 enhancer trap exhibits high levels of expression in the inflorescence stem, but *CYCD3;2* is not expressed at a high level in the stem. Second, during the late stages of carpel development, *CYCD3;2* is expressed in the ovules, transmitting tract, valves, and stigma, whereas 5580 is expressed primarily in the replum and septum. The differences in the two expression patterns likely are due to the orientation of the T-DNA in the *CYCD3;2* gene (see "Discussion").

Plants Homozygous for the 5580 T-DNA Insertion Fail to Express *CYCD3;2* RNA, But Appear Phenotypically Normal

Because the T-DNA is inserted in the first exon of the *CYCD3;2* gene we were interested in knowing if the insertion affected the levels of *CYCD3;2* RNA. To test this we isolated RNA from inflorescences of plants homozygous for the 5580 enhancer trap line. We performed an RNA gel blot that contained inflorescence RNA from homozygous 5580 plants, as well as RNA from wild-type seedlings and inflorescences (Fig. 3B). Although expression of *CYCD3;2* was detected in the wild-type RNA lanes, no expression was detected in the homozygous 5580 RNA lane. This strongly suggests that the T-DNA insertion has eliminated RNA expression of the *CYCD3;2* gene, likely creating a loss-of-function *cycd3;2* allele.

Despite the fact that the 5580 homozygotes are RNA nulls for the *CYCD3;2* gene, plants homozygous for the 5580 T-DNA insertion do not exhibit obvious developmental or growth defects. There is no evidence of elevated embryo lethality, the seeds germinate at the same time as wild-type seedlings, and they possess a normal morphology. Leaf and root shape is normal in seedlings. Flowering time and flower morphology are also not affected. Overall plant size and growth rate appear normal. In summary, *cycd3;2* mutants do not exhibit an obvious mutant phenotype.

CYCD3;2 Is Functional in Yeast

To test whether *CYCD3;2* encodes a functional cyclin we tested whether *CYCD3;2* could complement a yeast strain deficient in G1 cyclin activity. In yeast, the G1 cyclins are redundantly encoded by the *CLN1*, *CLN2*, and *CLN3* genes. The yeast strain BF305-15d-21 contains insertions in the endogenous *CLN1* and *CLN2* genes, whereas *CLN3* is expressed under the control of a Gal inducible promoter. Thus BF305-15d number 21 grows well on Gal, but is inviable on Glc due to the lack of G1 cyclin activity (Fig. 5; Xiong et al., 1991). The *CYCD3;2* gene was cloned behind a

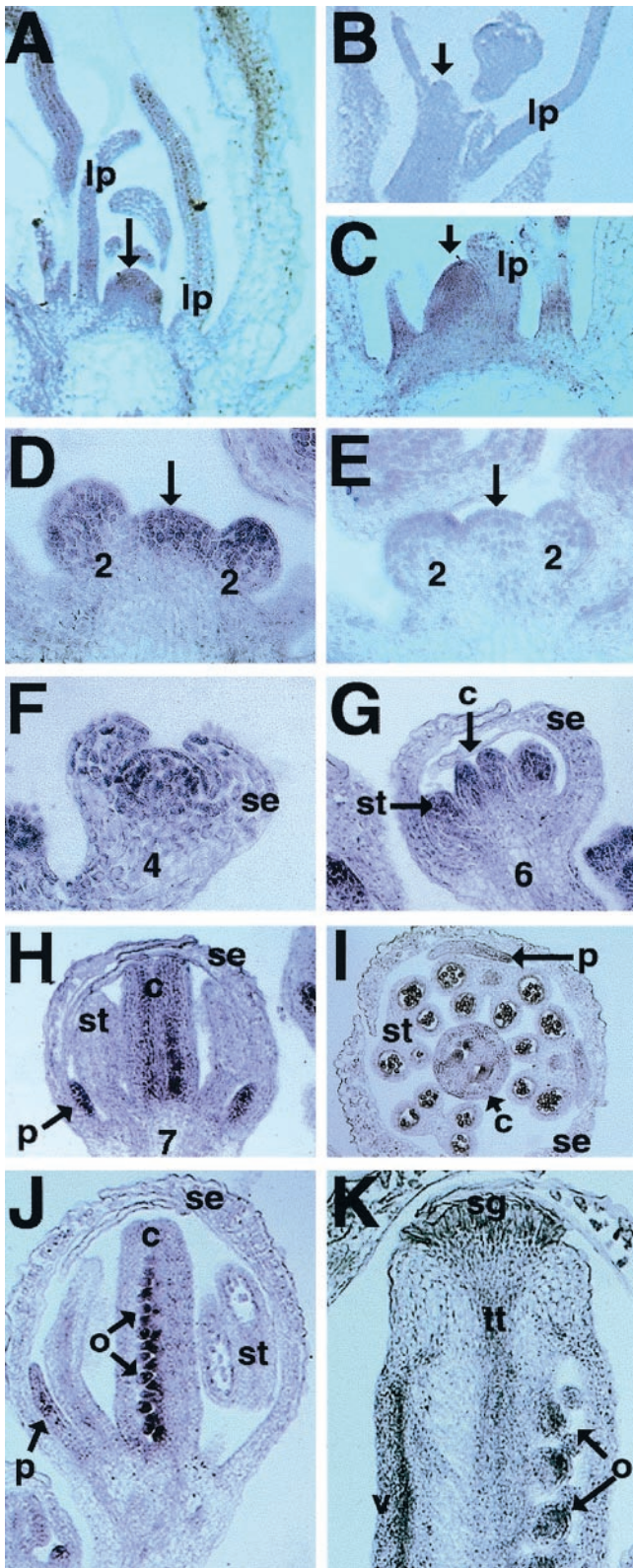


Figure 4. *CYCD3;2* RNA is expressed in proliferating cells of the shoot during vegetative and reproductive phases. The *CYCD3;2* RNA expression pattern was examined in seedlings (A–C) and inflorescences (D–K). A *CYCD3;2* antisense probe was used in A, C, D, and F through K and a *CYCD3;2* sense (control) probe was used in B and E.

strong promoter in a yeast vector and transformed into BF305-15d-21. Expression of the *CYCD3;2* gene in BF305-15d number 21 conferred on this strain the ability to grow on Glc (Fig. 5), demonstrating that *CYCD3;2* encodes a functional cyclin and is capable of complementing the defects of a yeast strain deficient in G1 cyclin activity.

DISCUSSION

In this study we report the isolation and characterization of a D-class cyclin gene, *CYCD3;2*, from *Arabidopsis*. By sequence comparison, *CYCD3;2* is most similar to the D3 subclass of cyclin genes. D-class cyclins from animals and plants are important for the passage through G1 of the cell cycle. In animal systems, a number of D-class cyclins have been demonstrated to be responsive to hormones and growth factors that signal quiescent cells to reenter the cell

E. A, Longitudinal section through a seedling showing the shoot apex (marked by arrow) and young leaf primordia (lp). *CYCD3;2* RNA is detected throughout the shoot apex and young leaf primordia, but as the leaves enlarge and differentiate, the *CYCD3;2* RNA is no longer detectable. B, *CYCD3;2* sense control. Longitudinal section through a seedling. The shoot apex (marked by arrow) and leaf primordia (lp) are indicated. No signal above background is detected in this section. C, Higher magnification longitudinal section through a seedling showing a shoot apex (marked by arrow) and leaf primordia (lp). D, Longitudinal section through a flowering shoot apex. The shoot apex is marked with an arrow. Two stage-2 flowers are indicated. *CYCD3;2* RNA is detected in the apical-most four to five cell layers of the shoot apex and throughout stage 2 flowers. E, *CYCD3;2* sense control. Longitudinal section through a flowering shoot apex. The shoot apex (marked by arrow) and stage 2 flowers are indicated. No signal above background is detected in this section. F, Longitudinal section through a stage 4 flower. *CYCD3;2* RNA is expressed throughout whorl 2, 3, and 4 anlagen. In the first whorl sepals (se), the levels of expression are lower and *CYCD3;2* RNA is not detected in every cell. G, Longitudinal section through a stage 6 flower. *CYCD3;2* RNA is expressed throughout developing stamens (st) and carpels (c) in whorls 3 and 4. Although some *CYCD3;2* RNA is detected at the base of the first whorl sepals, most sepal cells do not express detectable levels of *CYCD3;2*. H, Longitudinal section through a stage 7 flower. *CYCD3;2* RNA is expressed in most cells of the developing fourth whorl carpel (c). In the third whorl stamens (st), *CYCD3;2* RNA is detected at a low level in the stamen filament, but is not detected in anthers. *CYCD3;2* is expressed at high levels throughout second whorl petals. At stage 7, *CYCD3;2* is no longer detectable in first whorl sepals. I, Cross section through a stage 9 flower. *CYCD3;2* RNA expression is detected in microspores of the stamen (st), in stamen filaments, in developing ovules, in some cells of the carpel wall (c), and at the base of the petals (p). *CYCD3;2* RNA is not detectable in sepals (se) or in the connective of the stamen. J, Longitudinal section through a stage 9 flower. *CYCD3;2* RNA expression is detected at high levels in developing ovules (o) and in petals (p). *CYCD3;2* RNA is also detected in microspores of the stamen (st), in stamen filaments, and in some cells of the carpel wall (c). K, Longitudinal section through the apical region of a stage 12 carpel. *CYCD3;2* RNA expression is detected at high levels in developing ovules (o), in the stigma (sg), in the transmitting tract (tt), and in subepidermal cells of the valves (v). Numbers refer to floral stage (Smyth et al., 1990).

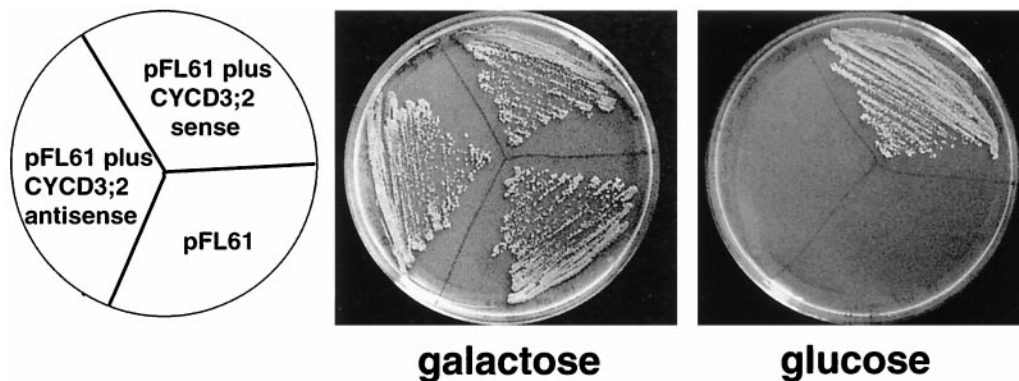


Figure 5. *CYCD3;2* is functional in yeast. The yeast strain BF305-15d-21 contains mutations in two G1 cyclins (*CLN1* and *CLN2*) and has a third G1 cyclin (*CLN3*) under the control of the *GAL1* promoter. In the presence of Gal, *CLN3* is expressed and BF305-15d-21 is viable, whereas in the presence of Glc *CLN3* is not expressed and the cells are inviable because they lack G1 cyclin activity. When a sense version of *CYCD3;2* is expressed in BF305-15d-21, it allows the strain to grow on Glc, demonstrating that *CYCD3;2* can rescue the G1 cyclin deficiency. Expression of an antisense copy of *CYCD3;2* or the pFL61 vector alone does not lead to rescue when grown on Glc.

cycle. Support for the hypothesis that plant D cyclins function similarly comes from experiments that demonstrate that a second member of the cyclin D3 subclass in Arabidopsis, *CYCD3;1*, is responsive to the cell division stimulatory effects of cytokinins; ectopic expression of *CYCD3;1* results in a stimulation of cell division in the absence of cytokinin (Riou-Khamlichi et al., 1999). Although it is not presently known if *CYCD3;2* is responsive to cytokinins or other mitogens, we have demonstrated that the *CYCD3;2* is capable of rescuing a yeast strain that is deficient in G1 cyclin activity and thus encodes a functional cyclin. Determination of the cell cycle expression profile of *CYCD3;2* will provide more definitive information about whether *CYCD3;2* functions during G1.

Despite the fact that *CYCD3;2* appears to encode a functional cyclin, elimination of *CYCD3;2* function does not result in a dramatic loss-of-function phenotype in Arabidopsis plants. The T-DNA in enhancer trap line 5580 is inserted in the first exon of *CYCD3;2*, 5' to the conserved cyclin box. *CYCD3;2* RNA is not detected in RNA isolated from homozygous 5580 inflorescences, suggesting that the T-DNA insertion has disrupted transcription of the *CYCD3;2* gene. Homozygous 5580 plants, however, do not exhibit an obvious growth or developmental phenotype. We grew homozygous 5580 plants only under standard laboratory growth conditions; perhaps under alternate growth conditions (e.g. short-day grown, low or high temperature) or stress conditions (e.g. drought, nutrient limitation, high photon flux density), a mutant phenotype will be observed.

The failure to observe a mutant phenotype in 5580 homozygous plants most likely is due to the redundancy of the cyclin D gene family in Arabidopsis. This is the fifth published member of the cyclin D gene family in Arabidopsis (Soni et al., 1995; De Veylder et al., 1999). In addition to the published sequences, four additional genes with similarity to D-class cyclins

have been isolated in the Arabidopsis Genome Initiative (chromosome 1, accession no. AC002062; chromosome 3, accession no. AL132978; chromosome 4, accession no. AC005275; and chromosome 5, accession no. AL162508). The gene most similar to *CYCD3;2* is the uncharacterized gene on chromosome 3, which likely is the third member of the cyclinD3 subclass in Arabidopsis.

If there is redundancy among the D-class cyclins, the relationship among the various members is complex because these genes are fairly divergent in sequence and are expressed in different temporal and spatial patterns. The sequence identity among the D-class cyclins from Arabidopsis is less than 65% in all cases. In addition, the tissue-specific expression patterns for the characterized D-class cyclins from Arabidopsis are not identical. Based on RNA gel blots, *CYCD3;1* is expressed at higher levels in roots than leaves and flowers, *CYCD1;1* is expressed at similar levels in roots, leaves, and flowers, and *CYCD2;1* is expressed in leaves and roots (Soni et al., 1995). In situ hybridization indicates that *CYCD4;1* is expressed in actively dividing cells in developing vascular tissue, during early stages of embryogenesis, in fertilized ovules, and in lateral root primordia (De Veylder et al., 1999). By contrast, RNA gel blots and in situ hybridization demonstrate that *CYCD3;2* is expressed at highest levels in developing shoot and flower primordia. The fact that *CYCD3;2* exhibits a spatial expression pattern different from the other characterized D class cyclins, yet loss of *CYCD3;2* function does not lead to a mutant phenotype, suggests that other D cyclins function redundantly with *CYCD3;2*. The functional role of the D-class cyclins will become clearer as the four as yet uncharacterized cyclin D genes are molecularly characterized and when loss-of-function alleles become available in the other D-class cyclin genes and multiple mutant strains are constructed and analyzed.

The spatial and temporal RNA expression pattern of *CYCD3;2*, as determined by in situ hybridization, is not identical to the GUS activity pattern in enhancer trap line 5580. The expression patterns of the enhancer trap and *CYCD3;2* are largely overlapping, but there are clear differences. In the seedling, the 5580 enhancer trap and *CYCD3;2* are expressed in the vegetative shoot apex, as well as in developing leaf primordia, but only *CYCD3;2* is expressed in roots. In the inflorescence, *CYCD3;2* RNA is detected at a high level in the cells of the shoot apex and at a low level in the vasculature of the inflorescence stem. By contrast, the 5580 enhancer trap is expressed at high levels throughout the inflorescence stem, in nonvascular, as well as vascular cells. 5580 and *CYCD3;2* are expressed in all cells of the flower primordia during early floral stages (stages 1–4). By stage 7, expression of 5580 and *CYCD3;2* is detectable in the petals, stamens, and carpels, but not in the sepals. The biggest difference between the 5580 and *CYCD3;2* patterns is the late expression pattern in stamens and carpels. *CYCD3;2* is expressed at high levels in developing ovules and pollen grains, as well as in the vasculature of the stamen filaments. By contrast, the 5580 enhancer trap line exhibits a dynamic expression pattern in the carpel; specifically, enhancer trap expression is detected in the valves, septum, and replum, but expression is not detected in the ovules.

The difference between the 5580 and *CYCD3;2* expression patterns likely is due to the orientation of the T-DNA in the *CYCD3;2* gene. The T-DNA in the enhancer trap vector pD991 contains the minimal –60 cauliflower mosaic virus (CaMV) 35S promoter fused to *uidA*, adjacent to the right T-DNA border (Fig. 2A). In line 5580, the right border is adjacent to the 3' end of the *CYCD3;2* gene and the left border is adjacent to the 5' end of the *CYCD3;2* gene; as a result the –60 CaMV promoter is in an inverted orientation compared with the direction of transcription of *CYCD3;2*. In addition, the minimal promoter in the T-DNA is more than 5 kb away from the promoter of the *CYCD3;2* gene, but is only 1.5 kb away from the 3' end of *CYCD3;2*. As a result of the orientation and position of the minimal promoter in the *CYCD3;2* gene, it is not unreasonable to expect that some of the transcriptional signals in the *CYCD3;2* promoter might be too distant to activate the minimal –60 CaMV promoter. In a similar manner, it is possible that enhancers associated with the gene 3' to *CYCD3;2* might be directing transcription from the minimal promoter in the enhancer trap. *CYCD3;2* is present on clone K3G17; annotation of this clone indicates that there is a gene about 1.5 kb 3' to *CYCD3;2*. This adjacent gene is transcribed in the opposite direction from *CYCD3;2* so it is the 3' end of this gene that is adjacent to the 3' end of *CYCD3;2*. This adjacent gene encodes a putative protein with no significant homology to proteins in databases. At present it is unclear whether part of the pattern de-

tected in the 5580 enhancer trap, in particular the late carpel pattern, is directed by transcriptional control elements in this adjacent gene.

MATERIALS AND METHODS

Isolation of Enhancer Trap Line 5580

Line 5580 was initially selected from the enhancer trap collection (Campisi et al., 1999) because it exhibited a GUS expression pattern during early floral stages. Seeds were collected from the original T1 plant that exhibited the 5580 enhancer trap pattern. Homozygous 5580 lines, which segregated 100% for kanamycin resistance, were identified in the T2 and used for subsequent studies.

GUS Staining

GUS activity patterns in inflorescences and seedlings were determined as described previously (Campisi et al., 1999).

PCR Assay To Establish Linkage between *CYCD3;2* and *NPTII*

Genomic DNA was isolated from 20 homozygous kanamycin-resistant plants that exhibited the 5580 enhancer trap pattern (Campisi et al., 1999). To establish that a T-DNA carrying the *NPTII* gene is associated with the T-DNA insertion in the *CYCD3;2* gene we performed PCR reactions using primers 86 (specific for the right T-DNA border sequence: 5'-TCGGGCCTAACTTTGGTG-3') and primer 142 (specific for *CYCD3;2* sequence: 5'-TCACAGG-GTGCATTCTCC-3', from bp 800–783 of genomic sequence in Fig. 2). In all 20 cases a 450-bp band was amplified demonstrating that all plants that are kanamycin-resistant and exhibit the 5580 GUS activity pattern also contain a T-DNA insertion in the *CYCD3;2* gene.

Cloning Flanking DNA and Isolation of *CYCD3;2*

Flanking DNA for line 5580 was isolated by TAIL PCR (Liu et al., 1995). Degenerate primer AD2 [5'-NGTCGA(G/C)(A/T) GANA(A/T) GAA-3'] and right border-specific primers 123 (5'-GCATGCAAGCTTGGCACTGG-3'), 124 (5'-TGAGACCTCAATTGCGAGC-3'), and 86 (see above) were used in three rounds of TAIL-PCR. The TAIL PCR fragment was subcloned into pGEM7z(+) and sequenced. The TAIL PCR fragment was used as a probe to screen a floral cDNA library (from ecotype Landsberg *erecta*) in λ gt10 (Yanofsky et al., 1990) and a genomic library (from ecotype Columbia) in λ ZAP (Stratagene, La Jolla, CA). The longest cDNA clone (1,380 bp) was subcloned into the *EcoRI* site of pGEM7z(+) to create plasmid pD1230. A 1,986-bp genomic clone was excised in vivo from λ ZAP to create plasmid pD1240. Genomic and cDNA clones were completely sequenced. Comparison of the genomic sequence with the TAIL PCR fragment indicated the presence of 43 bp of DNA (5'-TTCACCGAATAAAACAGCCA-CGAACATATATCATCGCATAGGG-3') that derived nei-

ther from the *CYCD3;2* gene nor from pD991, the vector used to generate the enhancer trap lines; presumably this sequence results from an artifact of the T-DNA integration process.

In Situ Hybridization

To construct a plasmid suitable for in situ hybridization, the *CYCD3;2* cDNA was PCR amplified with primers 140 (5'-TCACTGTAGAATCCGTCG-3') and 143 (5'-GGAGCTATTGAGCTGTC-3') and cloned into pGEM-T (Promega, Madison, WI) to generate pD1381. To generate a sense (control) probe, pD1381 was linearized with *NdeI* and transcribed with SP6 RNA polymerase to generate a 1,075-base probe. To generate an antisense *CYCD3;2* probe, pD1381 was linearized with *XhoI* and transcribed with T7 RNA polymerase to generate an 830-base probe. DIG-labeled probes were made using a kit from Roche-BM (Basel) following the manufacturers instructions. In situ hybridization was carried out according to standard protocols (Long et al., 1996; Long and Barton, 1998).

RNA Gel Blots

Total RNA was isolated from various plant tissues via the hot phenol method (Nagy et al., 1988). To examine the tissue-specific expression pattern of *CYCD3;2*, 15 µg of each RNA was loaded after treating with glyoxal. For the 5580 blot, 15 µg of RNA from 5580 inflorescences was run on an RNA gel blot with 15 µg of RNA from wild-type Columbia seedlings and inflorescences. Both blots were probed with the *CYCD3;2* cDNA (a random prime labeled 1.4-kb *EcoRI* fragment from pD1230). Filter was exposed to x-ray film for 2 weeks prior to development.

Map Position

A comparison of the cDNA sequence (from the Landsberg *erecta* ecotype) with the genomic sequence (from the Columbia ecotype) revealed the presence of a number of polymorphisms in these sequences. One polymorphism resulted in the presence of a *BglIII* site in the genomic DNA in Landsberg, but not in Columbia (at nucleotide 211 of the sequence in Fig. 2). A cleaved-amplified polymorphic sequence marker (Konieczny and Ausubel, 1993) utilizing this site was created by using primers 140 (5'-TCACTGTAGAATCCGTC-3') and 148 (5'-GGATTCGTTTCGTTTCC-3'), which flanked the *BglIII* site. Using this *BglIII* polymorphism, the presence and absence of this *BglIII* site was scored in a population of 31 recombinant inbred lines (Lister and Dean, 1993) and the *CYCD3;2* gene was shown to be linked to markers on the bottom of chromosome 5 on the recombinant inbred map (at position 131.1). The Arabidopsis Genome Initiative subsequently sequenced the region containing *CYCD3;2* (on clone K3G17) and the closest RI marker is CD34, which maps at 133.4 cM.

Rescue of G1 Cyclin-Deficient Yeast

The *CYCD3;2* cDNA was cloned into the yeast transformation vector pFL61 in the sense and antisense orienta-

tions to create plasmids pD1242 (sense) and pD1243 (antisense). pD1242, pD1243, and the pFL61 vector were transformed into the *Saccharomyces cerevisiae* strain BF305-15d-21 (MATa *leu2-3 leu2-112 his3-11 his3-15 ura3-52 trp1 ade1 met14 arg5, 6 GAL1::CLN3 HIS3::cln1 TRP1::cln2*; Xiong et al., 1991) via electroporation (Becker and Guarente, 1991). The pFL61 plasmid contains a *URA3* marker. Transformations were plated on uracil dropout plates containing raffinose plus Gal. Individual transformants were patched onto uracil dropout plates containing either Glc or raffinose plus Gal. Yeast cells that contain the *CYCD3;2* sense plasmid grew on Glc plates, but yeast cells that contain either the *CYCD3;2* antisense plasmid or pFL61 control plasmid grew only on the raffinose plus Gal plates. To confirm that the rescued colonies contained the pD1242 *CYCD3;2* sense plasmid, DNA from seven transformants was isolated via the smash and grab technique (Strathern and Higgins, 1990) and transformed into *Escherichia coli* TB1 cells. Colony PCR with *CYCD3;2*-specific primers 136 (5'-CCATTGCTCTTAGACCTCC-3') and 143 (5'-GGAGCTATTGAGCTGTC-3') demonstrated that all seven yeast transformants that grew on Glc plates contained the pD1242 *CYCD3;2* sense plasmid.

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