The Role of the Cell Cycle Machinery in Resumption of Postembryonic Development

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Cell cycle activity is required for plant growth and development, but its involvement in the early events that initiate seedling development remains to be clarified. We performed experiments aimed at understanding when cell cycle progression is activated during seed germination, and what its contribution is for proper seedling establishment. To this end, the spatial and temporal expression profiles of a large set of cell cycle control genes in germinating seeds of Arabidopsis (Arabidopsis thaliana) and white cabbage (Brassica oleracea) were analyzed. The in vivo behavior of the microtubular cytoskeleton was monitored during Arabidopsis seed germination. Flow cytometry of Arabidopsis germinating seeds indicated that DNA replication was mainly initiated at the onset of root protrusion, when germination reached its end. Expression analysis of cell cycle genes with mRNA in situ localization, β-glucuronidase assays, and semiquantitative reverse transcription-polymerase chain reaction showed that transcription of most cell cycle genes was detected only after completion of germination. In vivo green fluorescent protein analysis of the microtubule cytoskeleton demonstrated that mitosis-specific microtubule arrays occurred only when the radicle had started to protrude, although the assembly of the microtubular cytoskeleton was promptly activated once germination was initiated. Thus, seed germination involves the synthesis and/or activation of a reduced number of core cell cycle proteins, which only trigger DNA replication, but is not sufficient to drive cells into mitosis. Mitotic divisions are observed only after the radicle has protruded and presumably rely on the de novo production of other cell cycle regulators.

Seed germination is the process by which the plant embryo resumes growth after a period of quiescence. Under favorable conditions, rapid growth of the embryo culminates in rupture of the covering layers and emergence of the radicle, which is considered the completion of germination. At this stage, the decision of individual embryo cells to reenter the cell cycle or to remain arrested is crucial to determine seedling formation. The building of plant shape and function depends on the ability of the embryo cells to resume division and differentiate. Understanding how the cell cycle genes work at this particular phase of plant development might help to clarify the cellular and structural events that bring a quiescent embryo to a metabolically active plant.

Cell cycle is a coordinated cyclic series of events, occurring between the end of subsequent cell divisions, by which cellular material is duplicated and divided between daughter cells. Thus, cell cycle consists of two major events, DNA replication (S phase) and mitosis (M phase) separated by two gap phases, G1 and G2 (for review, see Dewitte and Murray, 2003). The G1 phase is assumed to be the gate through which most cells resume cell cycle progression after a nonproliferative period. Additionally, G1-to-S transition is the central target of the cross-talk between cell cycle activation/progression and environmental signals (for review, see Rossi and Varotto, 2002). Taking into account that seed germination implies the resumption of cell cycle progression after a period of quiescence, entry into G1 and activation of the G1-to-S switch may represent an important control in early seedling development.

The progressive passage through the various phases of the cell cycle is controlled by a conserved mechanism based on sequential transient formation and activation of complexes between cyclin-dependent kinases (CDKs) and their activating subunits, the cyclins (CYC; for review, see De Veylder et al., 2003). The activity of CDK/CYC complexes relies on multiple controls exerted by intra- and extracellular signals applied at multiple levels, including transcription, translation, phosphorylation, protein destruction,
The cytoskeleton plays an important role in the preparation of cell division and cell elongation (Hasezawa and Kumagai, 2002). Microtubules (MTs) are heterodimeric polymers of α- and β-tubulins in association with other proteins known as MT-associated proteins (MAPs; Lloyd and Hussey, 2001). β-Tubulin accumulation has been extensively studied in relation to seed germination both with immunofluorescence microscopy and western-blot analysis (de Castro et al., 1995, 2000, 2001; Görnik et al., 1997; Fujikura et al., 1999; Jing et al., 1999). A correlation between the rate of germination and the rate of β-tubulin accumulation in imbibed tomato (Lycopersicon esculentum) seeds has been demonstrated (de Castro et al., 1995). We analyzed how the microtubular cytoskeleton is associated with seed germination. In higher plant cells, MTs show dynamic structural changes during cell cycle progression and play significant roles in cell morphogenesis (Azimzadeh et al., 2001). The occurrence of specific arrays, such as the spindle, the preprophase bands, and the phragmoplast, can be considered as exclusive markers for mitosis. On the other hand, the visualization of cortical MT (CMT) arrays allows the fast identification of elongating cells. Therefore, an alternative experimental approach, based on the in vivo imaging of the microtubular cytoskeleton, was undertaken to determine whether mitotic cell divisions are implicated in the germinative process. The dynamic reorganization of MTs during germination was monitored by using the MT-binding domain (MBD) of the MAP4 gene fused to the green fluorescent protein (GFP) gene. MAP4 is a MT-associated protein abundantly produced in all actively dividing cells of vertebrates,

Table 1. Cell cycle genes whose expression was analyzed during the seed germination process

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Cell Cycle Expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKA;1</td>
<td>A-type CDK; interacts with CYCA, CYCB, and CYCD cyclins</td>
<td>Constitutive</td>
<td>Mironov et al. (1999)</td>
</tr>
<tr>
<td>CDKB1;1</td>
<td>B-type CDK; mitosis-specific CDK, unique to plants</td>
<td>G2/M</td>
<td>Mironov et al. (1999)</td>
</tr>
<tr>
<td>CYCA2;1</td>
<td>A-type mitotic cyclin</td>
<td>G2/M</td>
<td>Menges et al. (2003)</td>
</tr>
<tr>
<td>CYCB1;1</td>
<td>B-type mitotic cyclin</td>
<td>M</td>
<td>Menges et al. (2003)</td>
</tr>
<tr>
<td>CYCD4;1, CYCD5;1, CYCD6;1</td>
<td>D-type cyclins; transcription of many CYCD</td>
<td>G1/S</td>
<td>Menges et al. (2003)</td>
</tr>
<tr>
<td>CKS</td>
<td>CDK-docking protein; interacts with CDKA and CDKB</td>
<td>Constitutive</td>
<td>Menges et al. (2003)</td>
</tr>
<tr>
<td>CDC6</td>
<td>Protein of the DNA replication licensing complex</td>
<td>G1/S</td>
<td>Menges et al. (2003)</td>
</tr>
<tr>
<td>CDC7</td>
<td>Kinase required for triggering DNA replication</td>
<td>G1/S</td>
<td>Nishitani and Lygerou (2002)</td>
</tr>
<tr>
<td>KRP1</td>
<td>CDKA;1 inhibitor; possible role in nondividing and dividing cells</td>
<td>G2/M</td>
<td>Menges and Murray (2002)</td>
</tr>
<tr>
<td>KRP2</td>
<td>CDKA;1 inhibitor; possible role during reactivation of cell division in early G1 phase</td>
<td>G1</td>
<td>Menges and Murray (2002)</td>
</tr>
<tr>
<td>E2Fa/E2Fb</td>
<td>Interacts with DP proteins forming transcription factors essential for the production of S-phase regulators</td>
<td>G1/S</td>
<td>Menges et al. (2003)</td>
</tr>
</tbody>
</table>
promotes MT polymerization, and has large MT-stabilizing activity (for review, see Wasteneys, 2002).

Although seed germination has been thoroughly analyzed from the physiological and biochemical point of views, the molecular mechanisms that directly link cell cycle with seed germination have been very poorly investigated. With the exception of a number of studies describing changes in cell cycle protein abundance during germination, the molecular aspects of the cell cycle during seed germination have been almost completely ignored. Here, seeds of Arabidopsis and white cabbage (Brassica oleracea) were chosen to monitor cell cycle-associated modifications during the transition from the quiescent dry state to the first divisions. As introduced above, many highly interesting Arabidopsis genes involved in the regulation of the cell cycle have been isolated and partially characterized during the last years. Expression studies carried out with many of these cell cycle genes on tissues of different Arabidopsis organs allow anticipation of possible involvement of cell cycle genes in early seedling development. By investigating cell cycle-related events, we wanted to determine whether cell cycle is implicated in the molecular accomplishments that lead to early seedling formation.

RESULTS

Nuclear DNA Content of Embryo Cells from Germinating Seeds of Arabidopsis

Flow cytometry was performed to monitor DNA replication and to identify the position of Arabidopsis embryo cells within the cell division cycle, while the seed germinated. The histograms from nuclei of dry seeds showed one large peak, corresponding to the 2C DNA content (Fig. 1A). In the same histogram, a very small peak could be observed with slightly more fluorescence representing the nuclei with 3C DNA content. This peak represents the relative DNA content of nuclei from endosperm cells. Upon 8 h of imbibition in water, a peak corresponding to 4C nuclei became visible. However, a large increase in the frequency of 4C nuclei was only observed 40 h after imbibition (HAI), just before or coinciding with root protrusion, also defined as the completion of germination (Fig. 1A).

Endoreduplication seemed to be initiated only between 40 to 48 HAI, when an 8C population started to be detected. At this stage, endoreduplication is normally restricted to the cotyledon cells.

The fraction of nuclei from which the DNA had replicated can be expressed as the ratio of 4C + 8C to the total amount of embryo nuclei [4C + 8C/(2C + 4C + 8C)]. This coefficient can be used as a direct measure of the nuclear DNA replication activity upon seed imbibition (Fig. 1B). The results illustrate that the percentage of embryo nuclei from dry seeds subject to DNA replication was negligible (0.7%). Following soaking, DNA replication increased up to 5% to 7% of the nuclei. A more remarkable increase in the fraction of nuclei going through S phase was only visible at 40 HAI. Thus, a major transition through S phase toward G2 was detected at about the moment when the radicle started to protrude (Fig. 1B).

Expression of Cell Cycle Control Genes by Promoter-Reporter Gene Analysis in Transgenic Arabidopsis Seeds

β-Glucuronidase (GUS) enzymatic assays were performed to assign the spatial and temporal expression profiles of major cell cycle regulators during seed germination in Arabidopsis. Seeds of the Arabidopsis mutant lines expressing the GUS gene under the control of the CDKA;1, CDKB1;1, CYCA2;1, CYCB1;1, CYCD4;1, and CKS1 gene promoters were imbibed in water and screened for GUS expression. In Arabidopsis seeds, radicle protrusion was initiated on average 1.75 d (42 h) after imbibition.

CDKA/CYCA complexes have been reported to be essentially implicated in G2-to-M transition, but at least in some cases A-type cyclins seem to be components of CDK/CYC complexes that commit a cell to enter S phase (for review, see Rossi and Varotto, 2002; Table I). For CDKA;1 and CYCA2;1, the corresponding GUS activity was already observed throughout the embryo of dry seeds (data not shown). During germination, GUS activity was slowly turned off in most...
embryo tissues (Fig. 2, A–E). In germinated seeds and young seedlings, CDKA;1 and CYCA2;1 promoter activities became restricted to dividing tissues (the root and shoot meristems and the vascular tissue; Fig. 2, B, and D and E, respectively). Similarly, GUS expression driven by the CYCD4;1 promoter was also observed in dry seeds. Although weakly at this stage, GUS expression spread in a patchy pattern

Figure 2. Promoter activity of cell cycle genes in Arabidopsis seeds and young seedlings as revealed by GUS enzymatic assays. A and B, Promoter activity of CDKA;1 in seeds imbibed for 1 and 2 d, respectively. C to E, Histochemical GUS detection of CYCA2;1 promoter activity in seeds imbibed for 1, 2, and 3 d, respectively. F to I, GUS expression driven by the CYCD4;1 promoter in dry seeds (F), and seeds imbibed for 2 (G) or 3 d (H and I). J and K, CDKB1;1 promoter activity in young seedlings (2 DAI and 3 DAI, respectively). L to R, CYCB1;1 promoter activity in dry seeds (L) and young seedlings 2 DAI (M), 3 DAI (N), 4 DAI (O), 5 DAI (P), and 6 DAI (Q and R). S and T, CKST promoter activity in germinating seeds, 1 DAI (S) and 3 DAI (T). Bar = 75 μM.
through the radicle tip and a few cells of the upper part of the radicle (Fig. 2F). In seeds imbibed for 1 d, GUS activity driven by the CYCD4;1 promoter slowly increased in the radicle meristem but still remained present in a number of other radicle cells (data not shown). Once germination was completed, GUS expression was exclusively confined to the radicle meristem (Fig. 2G). As the seedling grew older, CYCD4;1 promoter activity strongly increased in the radicle meristem and was also detected in the shoot apical meristem (SAM; Fig. 2, H and I).

CYCB1;1 and CDKB1;1 are mitotic cell cycle activators, whose expression is confined to actively dividing meristems (Table I). GUS activity was absent in dried seeds of pCDKB1:GUS and in seeds imbibed for 24 h (data not shown). Immediately after the root started to protrude, CDKB1;1 promoter became active in the SAM and the radicle tip (Fig. 2I). In young seedlings, 3 d after imbibition (DAI), GUS expression had increased, but remained localized in the meristematic regions of the shoot and root and in a few vascular cells (Fig. 2K). Additionally, as reported previously, the GUS expression pattern revealed that CDKB1;1 promoter was active in the stomata cells of the cotyledons (Boudolf et al., 2004). Also, CYCB1;1 promoter was only active after radicle protrusion and absent in the dry transgenic seeds and seeds imbibed for 24 h (data not shown). After root emergence, GUS expression was restricted to a very few cells of the radicle (Fig. 2, M and N). Only 2 to 3 d after germination, CYCB1;1 promoter activity was easily detectable in the root tip and SAM (Fig. 2, O and P). In young seedlings, CYCB1;1 promoter was strongly active during lateral root formation (Fig. 2, Q and R).

CKS1 promoter activity was detected in most embryo tissues before root protrusion (data not shown). After 2 DAI, CKS1 promoter activity was gradually switched off in most embryo tissues and activity was mainly localized in the cotyledons at the moment of root protrusion (Fig. 2S). After germination, GUS activity became restricted to the cotyledons and vascular tissue of the root (Fig. 2T). CKS1 promoter activity was never observed in the shoot or root meristems.

Cell Cycle Gene Expression Analysis by mRNA in Situ Hybridization

Extensive mRNA in situ localizations of several Arabidopsis cell cycle genes (CYCB1;1, CYCD5;1, CYCD6;1, E2Fa, E2Fb, CDC6, CDC7, CKS1, KRP1, KRP2, and the histone H4 gene) were performed in germinating seeds of white cabbage to determine precisely their spatial and temporal expression profiles during seed germination and early seedling development (Fig. 3). Radicle protrusion in white cabbage seeds was initiated just after 24 HAI in water.

Simultaneously, the expression patterns of CDKA;1, CDKB1;1, E2Fa, CDC6, and CDC7 genes were also analyzed in germinating seeds of Arabidopsis by whole-mount mRNA in situ hybridization. The results confirmed those obtained with in situ hybridization in cabbage seeds and the promoter-GUS fusion data.

From all genes analyzed by mRNA in situ hybridization, CDC6 transcripts were expressed the most early. This gene was expressed just prior to and during root protrusion in a clear patchy pattern throughout the radicle meristem (Fig. 3, A and B). At later stages, its expression was also observed in the epidermis of the radicle, the epidermis of the hypocotyl, and in the vascular tissue (data not shown). The truly early expression of this gene might be justified by its involvement in DNA replication. Shortly after germination, CDC6 was highly expressed in the root and hypocotyl, demonstrating that CDC6 expression was activated early during protrusion, as shown by the expression studies in cabbage. In older seedlings, expression was nearly absent, suggesting that CDC6 expression is strictly controlled during initial seedling development.

KRP1 was expressed in the epidermal tissues of the young seedling, predominantly in the SAM region, but also in the hypocotyl (Fig. 3, C and D). Expression could not be detected in the cotyledons (Fig. 3C). Like KRP1, CDC7 expression in germinating seeds was restricted to the epidermis of the hypocotyl and to the base of the cotyledons (data not shown). CDC7 was very weakly expressed at the early stages of seedling development for both cabbage and Arabidopsis.

KRP2 transcripts were detected just after the radicle had emerged from the seed coat. KRP2 was strongly expressed in the epidermis of all seedling organs (Fig. 3, E–H). Frequently, KRP2 transcripts accumulated in a patchy pattern, with nonuniform zones of high expression alternating with zones without expression (Fig. 3E). Moreover, KRP2 expression was very early visible throughout the epidermis of leaf primordia (Fig. 3F). Similarly, also CKS1 mRNA transcripts were confined to the embryoid epidermal cell layers and frequently a patchy expression pattern was observed (Fig. 3, I and J).

E2F genes code for transcription factors with an important role in the activation of S phase-specific genes (De Veylder et al., 2003). In germinating embryos, E2Fa gene expression seemed to be excluded from the root meristem, whereas transcripts were present nearly ubiquitously in the root (Fig. 3, K and L) and hypocotyls (data not shown). In both tissues, E2Fa expression was strongest in a restricted number of vascular cells (Fig. 3M).

D-type cyclins are able to integrate extracellular signals to mediate the progression from G1-to-S phase. In germinating seeds of cabbage, CYCD5;1 gene expression could only be observed in the endosperm of both dry and imbibed seeds (data not shown). Expression in the embryo tissues might only be initiated later in development. Likewise, also CYCD6;1 and E2Fb mRNA transcripts accumulated in the endosperm cells (data not shown). In addition, CYCD6;1 and E2Fb were expressed in the embryo epidermis and in

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a restricted number of cells of the vascular tissues (Fig. 3, N and O, and P and Q, respectively). In these tissues expression was only established after protrusion, whereas in the endosperm corresponding transcripts were present at all stages.

The tissue distribution pattern of CYCB1;1 mRNAs in cabbage coincided with the GUS pattern observed in transgenic seeds of Arabidopsis harboring the GUS gene under control of the CYCB1;1 promoter. Expression of this mitotic cyclin was mainly confined to the meristematic regions, both in the shoot and the root (Fig. 3, R and S). Clear in situ signals were also seen in the pericycle cells, which will probably form lateral roots (data not shown). Also, histone H4 mRNA was found in the main meristematic regions of the young seedling (data not shown). The expression of these genes could not be detected during germination but only 12 h after radicle protrusion.
Whole-mount hybridizations with CDKA;1 and CDKB1;1 mRNA probes illustrate that the CDKA;1 mRNA distribution pattern was very similar to the GUS expression pattern observed in young transgenic seedlings of Arabidopsis that harbor the GUS gene under control of the CDKA;1 and CDKB1;1 promoters (data not shown).

**Analysis of Cell Cycle Gene Transcript Profiles by Reverse Transcription-PCR**

To further validate our expression studies, the mRNA levels of key cell cycle genes were further analyzed in Arabidopsis seeds by semiquantitative reverse transcription (RT)-PCR. Five time points during seed imbibition were selected covering the complete seed germination and early seedling development. Specific transcript accumulation patterns were identified for CDKA;1, CDKB1;1, CYCD4;1, and CYCB1;1 (Fig. 4). Expression of CYCD4;1 increased abruptly during the first 24 h of seed soaking, and an expression peak was registered at 3 DAI, after which the transcript levels clearly decreased. CDKA;1 showed a very similar profile of gene expression, but the increase and decrease of its expression levels were less abrupt. Based on their expression profile, these cell cycle regulators might be essential for resumption of embryo growth, early during germination.

By contrast, the expression levels of CYCB1;1 and CDKB1;1 increased more evenly. CDKB1;1 transcript levels steadily increased during the initial 4 d of seed imbibition. This increase was slightly more abrupt between day 2 and day 3, coinciding with radicle protrusion. CYCB1;1 expression was nearly absent during the first 2 d after seed imbibition. Between day 3 and day 4, the expression levels increased rapidly. This expression peak might relate to the initiation of cell division events. Thereafter, the expression of this cyclin remained unchanged. These expression results largely corroborate the profiles revealed by tissue expression analysis.

Transgenic Arabidopsis MAP4-GFP plants were used as a living model system to monitor the in vivo dynamics of MTs within epidermal embryo cells, while the seed germinates and seedling growth is initiated. Based on our observations, organized MTs seemed to be absent in dry seeds but were rapidly assembled immediately after soaking. The visualization of living embryo cells implied that the seeds had to be soaked; so, the visualization of embryos of dry seeds was obviously unfeasible. After 45 min of imbibition in water, a reduced number of randomly aligned short MTs could be observed (Fig. 5A). At 6 to 8 HAI, the number of MTs had increased, but the arrays remained short and irregularly oriented. The number and size of the MT arrays steadily increased as the seeds were imbibed (Fig. 5B). By following the in vivo behavior of MTs in living epidermal cells of several germinating seeds between 10 and 20 HAI, we found that at this stage certain MTs were highly dynamic, while others remained remarkably stable in the cell over large periods. Moreover, the reorientation of MT arrays was not synchronized among neighboring cells, meaning that even when adjacent cells were subjected to the same external signals, the reorientation of MTs probably depended on each cell autonomously.

Until 24 HAI, the MT arrays were randomly aligned in all embryo cells (Fig. 5C). In general, approximately 24 HAI, MTs started to lose their seemingly free organization and progressively aligned in transverse arrays. This orchestrated array organization seemed to be preceded by a noteworthy increase in the number of MTs.

Gradually, the transverse-oriented MTs of some embryo cells were replaced by newly organized longitudinal arrays. After 48 HAI, when the radicle had already protruded the seed coat, the majority of the cells presented a very specific orientation of the CMTs (Fig. 5D). At this stage, the MT arrays of the majority of the cells were perpendicular to the root/hypocotyl axis, indicating that most cells were elongating. In the radicle, MTs were seemingly less organized in the meristematic region, whereas with increasing distance from the root tip, transverse arrays became more highly aligned. At this stage, although root protrusion had been accomplished, mitotic figures could only be observed in the root cap layer (Fig. 5F). In the hypocotyl, the CMTs presented various orientations from random to transverse and, in some cases, helical. Random orientation was particularly obvious in the top of the hypocotyl, close to the shoot apex.

In the cotyledons, as in other embryo tissues, the dispersed CMTs gradually aligned into transverse arrays (Fig. 5E). These MT arrays, once organized, retained the same transversal positioning throughout further development. No obvious differences in the length and number of MT arrays were noticed between cotyledons and other embryo tissues throughout germination.
The fluorescence intensity in the cells of the root/hypocotyl transition zone was significantly lower than that of other embryo tissues (Fig. 5G). Exhaustive observations revealed that this region had fewer MT arrays (data not shown).

Dry MAP4 transgenic seeds were also imbibed in water containing cycloheximide (CHX). The dynamic changes in MT distribution were then followed in vivo, showing that although this drug prevents protein synthesis, CHX-treated seeds displayed a normal organization of MT arrays. In seeds imbibed for 30 min in water containing CHX, a small number of randomly aligned short MTs were observed, both in the cotyledons and radicle (Fig. 5H). Time-sequence observations demonstrated that CHX treatment did not alter the cellular MT network and assembly of MT arrays. An increasing number of MTs was still detectable after embryo cells were imbibed for more than 60 min in CHX-containing water (Fig. 5, I and J). Although similar in number and still highly dynamic, these arrays remained much shorter than those of untreated seeds. Monitoring the behavior of MTs from CHX-treated seeds demonstrated that although protein synthesis is blocked, the assembly of MT arrays in germinating seeds is not halted.

**DISCUSSION**

Seed germination is a complex physiological process triggered by the imbibition of water and the release of quiescence mechanisms by appropriate signals. We investigated cell cycle-associated events to illustrate whether cell cycle regulators are implicated in the
events leading to early seedling formation. Although Arabidopsis has been generally accepted as the most important model organism for plant molecular biological research, its use for studying seed germination encounters some practical disadvantages, mainly related to the small size of the seeds. Therefore, some experiments were performed with white cabbage. These two species are ontogenetically closely related, and the morphology of the seeds is comparable. Previous hybridization experiments in Arabidopsis and rape (Brassica napus) seeds have revealed that the expression patterns correlate well between the two species, indicating that the Arabidopsis and Brassica sp. share high gene sequence homology (Girke et al., 2000).

Flow cytometry has demonstrated that embryos of dry seeds contain almost exclusively nuclei with 2C DNA content. This observation indicates that most embryo cells are arrested in the G1 phase of the cell cycle, as corroborated by both quantitative and cytological analyses of DNA synthesis in germinating embryos of tomato or faba bean (Vicia faba; Liu et al., 1994; Groot et al., 1997; Fujikura et al., 1999; de Castro et al., 2000). Additionally, the nonexistence of DNA replication during the 1st hour of imbibition has been demonstrated by the absence of bromodeoxyuridine incorporation into DNA from seeds imbibed for 3 h (de Castro et al., 2000). During imbibition, DNA synthesis seems to be activated first in the radicle tip and then to extend toward the cotyledons, resulting in an increased number of nuclei in G2 (Groot et al., 1997).

In root tips of white cabbage, the onset of DNA replication precedes root protrusion (Górnik et al., 1997). We demonstrate that in samples from entire Arabidopsis seeds, a clear increase in 4C DNA content, concomitantly with a decrease in 2C DNA, was observed mainly at the moment of radicle emergence. However, in both species, breaking of the seed coat correlates with the rise of an 8C peak. The discrepancy between cabbage and Arabidopsis in the temporal up-regulation of DNA synthesis in relation to embryo protrusion might be caused by the sampling method because in cabbage DNA content was measured in radicle tips, whereas in Arabidopsis the full seed was used. It should not be excluded that radicle tips from Arabidopsis exhibit replication levels very different from those in other embryo tissues, such as in tomato seeds (Liu et al., 1994).

Resumption of DNA replication after imbibition of tomato seeds might be correlated with an increase in β-tubulin accumulation and its assembly into MT cytoskeleton networks (Groot et al., 1997; de Castro et al., 1998). In germinating tomato and cucumber (Cucumis sativus) seeds, β-tubulin accumulates prior to the onset of DNA replication and both events precede radicle protrusion (Jing et al., 1999; de Castro et al., 2000). This evidence is in contradiction with similar work on fava bean in which β-tubulin levels remain constant throughout germination and MTs may be depolymerized in the seeds until visible germination. Moreover, in germinating faba bean, MT polymerization shortly precedes an increase in cellular DNA content (Fujikura et al., 1999). In Arabidopsis, MT assembly might not be temporally related to DNA replication. The formation of a MT cytoskeleton is initiated shortly after soaking in water, whereas a large increase in the fraction of nuclei going through S phase is just detected at the onset of root protrusion. Therefore, DNA replication and β-tubulin accumulation seem not to be associated directly. Our results illustrate that the formation of a proper MT network might be essential for radicle protrusion, whereas DNA replication may only significantly contribute to postgerminative growth. In Arabidopsis, a substantial increase in nuclear DNA synthesis indicates imminent radicle protrusion.

The onset of radicle protrusion is also marked by a rapid increase in cell cycle gene transcription as evidenced by our expression analysis results (Fig. 6). The promoter activity of CDKA;1, CDKB1;1, CYCB1;1, CYCA2;1, CYCD4;1, and CKS1 genes has been characterized in detail by GUS activity detection assays. Based on these assays, two classes of cell cycle genes can be defined: one class with genes whose promoter activity is just activated after germination (CDKB1;1 and CYCB1;1), and the other with the cell cycle activators from which the associated GUS activity can be detected in dry seeds and throughout germination. Interestingly, this latter class gathers core cell cycle genes implicated in the resumption of cell cycle after a nonproliferative period (CDKA;1 and CYCD4;1). However, GUS activity in dry seeds might not be directly related with the expression of cell cycle genes, but rather result from the activity of residual extant GUS proteins that remained in the dry seed after desiccation. This hypothesis is sustained by the presence of GUS activity in most of the dry embryo tissues instead of a more specific tissue pattern, as expected for the activity of most cell cycle gene promoters. Indeed, after imbibition, GUS activity in pCDKA;1::GUS, pCYCA2;1::GUS, or pCYCD4;1::GUS seeds appears to be gradually reallocated and becomes much more specific to meristematic tissues. Thus, the GUS analysis reveals a clear correlation between root protrusion and initiation or reestablishment of cell cycle gene promoter activity. As a clear example, CYCB1;1 promoter activity is not observed prior to completion of germination. CYCB1;1 is a mitotic cyclin whose expression has been previously demonstrated to be confined to dividing tissues, suggesting that its regulation might be one of the factors for the activation of cell division in some developmental programs, such as the resumption of postembryonic growth. On the contrary, CKS1 promoter activity was not detected in meristematic tissues. The preferential accumulation of GUS activity in the cotyledons could indicate that CKS1At plays a role in the endocycle. However, CKS1 overproduction in Arabidopsis has no effect on the endoreduplication pattern (De Veylder et al., 2001b).

The tissue expression analysis observed after GUS activity assays is largely corroborated by the transcript accumulation profiles revealed by RT-PCR analysis.
This technique was chosen to confirm and/or complement the tissue expression analysis in Arabidopsis because of its high sensitivity. Expression of CYCD4;1 and CDKA;1 was found to increase abruptly from the instant seed imbibition was initiated until 3 DAI. Based on these expression profiles, both CYCD4;1 and CDKA;1 seem to be necessary for early seed germination and the resumption of embryo growth, being probably implicated in reestablishment of cell cycle activity and preparation for G1-to-S transition. CDKA/CYCD complexes act at G1-to-S transition, being essential for the production of S-phase activators (De Veylder et al., 2003). Plant CYCD proteins seem to play a special role in the response to external signals and their integration into the cell developmental and positional contexts. Therefore, this group of plant cyclins might also play a key role in the perception of the environmental context and initiation of post-embryonic growth. The expression profile of CYCD4;1 indicates that its gene product is necessary for cell cycle progression to be restarted in the germinating seed. In germinating embryo axes of maize (Zea mays), CYCD protein levels remain constant for approximately 6 h and then decrease to almost disappear 24 HAI, still before radicle protrusion (Cruz-García et al., 1998). This accumulation pattern suggests that CYCD is implicated in the early resumption of cell cycle activity in the embryo cells of germinating seeds. Whether this protein is synthesized de novo in the germinating maize embryo or its presence during early germination results from its accumulation in the seed prior to desiccation remains to be determined. According to our results on mRNA accumulation of CYCD4;1, transcription of this gene is activated early during Arabidopsis seed germination.

Similarly, the expression profile of CDKA;1 also suggests that the corresponding protein is implicated in the early reactivation of cell cycle progression in germinating seeds. However, CDKA;1 transcript levels do not necessarily correlate with CDKA;1 protein activity. During cell cycle progression, transcript and protein levels remain constant, while CDKA;1 kinase activity increases throughout S and G2 and peaks at early M phase (Mironov et al., 1999). In maize, a CDKA-like protein has been identified that is present both in dry and germinating embryo axes (Herrera-Teigeiro et al., 1999). Maize CDKA protein levels remained constant through imbibition, even when germination was accelerated by addition of exogenous cytokinins and kinase activity increased, showing that CDKA activity in germinating maize is unrelated to its protein level (Herrera-Teigeiro et al., 1999). Thus, CDKA;1 mRNA accumulation early during Arabidopsis seed germination does not necessarily reflect an involvement of the CDKA;1 protein in resuming cell cycle activities in germinating embryos.

The RT-PCR results show an expression outbreak of CYCB1;1 and CDKB1;1 that coincides with radicle protrusion, in contrast with that of CDKA;1 and CYCD4;1. CYCB1;1 and CDKB1;1 are required to trigger entry into mitosis, and their activity fairly reflects their temporal expression pattern (Mironov et al., 1999). Therefore, the expression peak of both genes at the moment of radicle protrusion is an indication of a temporal relation between mitosis and embryo emergence.

The spatial pattern of cell cycle gene expression was also extensively analyzed by mRNA in situ localization. For all the genes, transcript accumulation was detected only during or after growth of the radicle through the
seed coat, evidencing that the de novo production of most cell cycle proteins mainly takes place after germination is accomplished. Thus, cell division activity per se does not seem to be essential for the initiation of the seed germination process but is required to allow further postgerminative development. This conclusion corroborates previous data revealing that chemical block of replication does not halt radicle protrusion in cabbage, although DNA replication starts roughly halfway through the completion of germination (Görnick et al., 1997). Additional evidence for a minor role of the cell cycle machinery in seed germination comes from the observation that transgenic plants overproducing cell cycle inhibitors or cell cycle dominant-negative proteins have normal rates of seed germination (L. De Veylder, unpublished data). The large number of cell cycle-related genes expressed in the epidermal cell layers of the young seedlings leads us to the question of whether the epidermis plays a particular role in early seedling development. A possible explanation comes from evidence that the epidermis is the first tissue to become metabolically active during germination (Mansfield and Briarty, 1996).

In germinating embryos, the rapid organization of a MT cytoskeleton might be required to accomplish the large rates of cell elongation that precede embryo emergence (de Castro et al., 2000). Monitoring the behavior of MTs in germinating seeds revealed that cells in division and elongation zones present different spatial MT patterning. Cells at the elongation zone have transverse CMT arrays, which are characteristic of longitudinal cellular expansion, whereas proliferation regions exhibit randomly aligned MTs. Although the assembly of the MT cytoskeleton is quickly activated once germination is triggered, mitosis-specific arrays seem to occur only when the radicle has protruded the seed coat. Therefore, we postulate that prompt assembly of the microtubular network is a preparation step toward elongation, and Arabidopsis radicle protrusion is probably accomplished through cell enlargement. Additionally, by using a protein synthesis inhibitor drug, we demonstrated that dry seeds contain already the required set of proteins necessary to initiate MT assembly. This evidence largely corroborates the results obtained previously in faba bean that showed that tubulins are stored in a diffuse form in dry seeds and are later organized as randomly oriented bundles, whereas characteristic cortical and mitotic configurations are most abundant at the moment of radicle protrusion (Fujikura et al., 1999). Therefore, we conclude that early steps in germination involve the assembly and reorganization of cytoskeleton elements from preexisting protein subunits.

Two-dimensional gel electrophoresis has been used to analyze the seed proteome and the changes in protein abundance in germinating Arabidopsis seeds (Gallardo et al., 2001). Protein extracts from dry and imbibed seeds (1–3 d) have been compared to follow the changes in protein production during seed germination. Among the proteins identified whose expression levels are associated with germination, none belongs to the group of cell division-associated proteins, with the exception of the cytoskeleton-associated proteins such as β-2 tubulin and actin-7. These proteomic studies revealed the absence or very low abundance of the main core cell cycle proteins during the initial stages of seedling development. Furthermore, most of the proteins identified are already present in dry seeds and their abundance remains constant throughout the germination process, revealing that germination is associated with modifications in the abundance of only a limited number of proteins and supporting the idea that dry seeds are essentially ready to germinate. Thus, resumption of metabolic activity during germination may rely essentially upon proteins that are stored during seed maturation.

CONCLUSION

Until now, the contribution of cell cycle regulators to seed germination has been largely neglected, and the molecular aspects concerning the involvement of cell cycle in that important developmental process have been only very poorly characterized. We show that a limited set of cell cycle proteins might be activated very early in the embryo to promote cell cycle progression, allowing a number of embryo cells to initiate DNA replication after only a few hours of imbibition. However, our gene expression results indicate that the de novo production of most cell cycle proteins mainly takes place after root protrusion. Therefore, we postulate that resumption of DNA replication prior to radicle protrusion is induced either by limited protein synthesis from stored mRNAs or by activation of cell cycle proteins already present in the dry seeds. This reduced core of cell cycle proteins initiates DNA replication but is not sufficient to drive cells into mitosis. Entry into M phase occurs only after radicle protrusion, as demonstrated by confocal observations, and might depend on the de novo production of specific cell cycle activators.

MATERIALS AND METHODS

Seed Germination

Two replicates of approximately 100 seeds of Arabidopsis (Arabidopsis thaliana L. Heynh.) or white cabbage (Brassica oleracea L. cv Bartolo) were imbibed on triple layers of filter paper saturated with distilled water, which were placed inside 145-mm petri dishes. The seeds were germinated at 22°C to 24°C under a 16-h-light/8-h-dark regime for the time periods specified (DAI or HAI). Seeds were regarded as having completed germination when 1 mm of the radicle had protruded through the seed coat.

Flow Cytometry

Relative DNA content of the seed nuclei was measured in Arabidopsis seeds imbibed from 0 to 72 h. Suspensions of intact nuclei were collected at an 8-h interval. Samples of at least 20 seeds were chopped with a razor blade in ice-cold nucleus isolation buffer (10 mM MgSO4 × 7H2O, 50 mM KCl, 5 mM HEPES, 1 mg mL−1 DTT, and 2.5 mg mL−1 Triton X-100, 1% [w/v] poly(vinylpyrrolidone-40) on ice. The suspension was sieved through an 88-µm
nylon mesh. After the samples had been digested with RNAse for 30 min at room temperature, they were stained with propidium iodide (1 mg mL\(^{-1}\)) for 15 min. DNA analyses were performed with an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami) equipped with an argon ion laser at 488 nm. The amount of DNA, proportional to the red fluorescent signal, is expressed as arbitrary C values, in which 1C represents the amount of DNA of the unreplicated haploid chromosome. The number of nuclei present in each peak of the histogram (2C, 3C, 4C, 6C, and 8C) was analyzed by measuring the peak area. Histograms were processed with the ModFit LT software (Verity, Turramurra, Australia; http://www.ampl.com.au/vsh_modfit.htm) for data analysis and correction of the background noise, and the volume of each histogram was calculated.

Construction of the Chimeric CYCD4:1 Promoter-GUS and CKSI Promoter-GUS Genes

The CYCD4:1 promoter of Arabidopsis (1,189 bp) was isolated by PCR amplification of a genomic DNA template with the primers GCCCTGACTGGGAAAGAACAGCATTATTCGGTGG and GGCCTAGCAGACAGACAGTTGTTCATGTAAGAAAATATTGCCC. The amplified promoter fragment was cloned into the pGUS1 vector upstream from the uidA (Escherichia coli) coding sequence, to produce the pGUSCYCD4:1 plasmid. The CYCD4:1 promoter-GUS-3’octopine synthase (3’OCS) cassette was excised from the pGUSCYCD4:1 and ligated into the pBluescript binary vector. Similarly, the Arabidopsis CKSI promoter (889 bp) also was isolated by PCR amplification of genomic DNA template with specific primers. The amplified promoter fragment was cloned into the pGUS1 vector upstream from the GUS coding sequence, producing the pGUSCKSI plasmid. The CKSI promoter-GUS-3’OCS cassette was excised from the pGUSCKSI and ligated into the pBluescript binary vector.

Plant Transformation

The pGUSCYCD4:1 and pGUSCKSI constructs in the pBluescript binary vector were mobilized into Agrobacterium tumefaciens C58C1R<sup><sub>2</sub></sup> (pMP90) and then introduced into Arabidopsis (ecotype Columbia) with the floral dip transformation method (Clough and Bent, 1998). Several independent transgenic lines were analyzed in the T2 generation, and one representative line containing a single copy of the respective transgenes was chosen for subsequent detailed GUS analysis.

Cell Cycle Gene Promoter-GUS Analysis

GUS expression was analyzed in germinating seeds of Arabidopsis lines expressing the GUS gene under the control of specific cell cycle promoters. In addition to the two GUS lines described above, this study was extended to the other cell cycle promoter-GUS lines available in our laboratory, namely those harboring the CDKA;1 promoter (Hemerly et al., 1993), the CDKB1;1 promoter (de Almeida Engler et al., 1999), the CYCA2;1 promoter (Busseins et al., 2000), and the CYCA2;1 promoter (Ferreira et al., 1994), and the CYCA2;1 promoter (Busseins et al., 2000). Seeds were imbibed in water for 1, 2, or 3 d (up to 7 d in a few cases) and subjected to GUS enzymatic assays. Dry seeds were imbibed in buffer containing the GUS substrate for 30 min and immediately peeled. After peeling, the embryos were checked for GUS and then further incubated in buffer containing GUS substrate up to 16 h. In the cases for which GUS expression is described as being present in the dry seed, GUS staining was observed immediately after the seeds had been peeled. In general, the patterns observed did not change by longer incubation time.

The histochemical GUS detection was carried out according to standard protocols. Seed embryos and young seedlings were incubated in 0.1% (w/v) acetone for 2 h at 4°C. After washing in phosphate buffer, the material was immersed in the enzymatic reaction mixture (1 mg mL\(^{-1}\) of 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-glucuronic acid, 2 mM ferricyanide, and 0.5 mM ferrocyanide in 100 mM phosphate buffer, pH 7.4). The reaction was carried out at 37°C in the dark for 4 h to overnight. Upon reaction, the material was cleared with chloroalcoholenol (chloral hydrate/phenol/lactic acid 2:1:1 [v/v/v]) and observed under stereo- and conventional light microscopy.

Gene Transcript Localization by mRNA in Situ Hybridization

mRNA in situ localizations were performed in white cabbage imbibed in water for 0, 12, 24, 36, or 48 h, transferred to fixative, and peeled. Dry seeds (time point 0) were placed directly in fixative (3% [w/v] glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2), without any pretreatment, and subsequently peeled. After seed coat removal, all the seeds were transferred to fresh fixative and incubated for an additional 12 to 16 h (at 4°C). Fixed seeds were dehydrated through standard ethanol series and embedded in paraffin. Embedded tissues were sliced into serial 10-μm sections and attached to coated microscope slides. \(\delta\)-UTP-labeled sense (control) and antisense RNAs of CYCRI;3, CYCD3;3, CYCD6;1, E2Fa, E2F1a, CDC6, CDC7, CKSI, KRP1, KRP2, and H4 genes were generated by in vitro transcription with Sp6 or T7 RNA polymerases, according to the manufacturer’s protocol (Roche Diagnostics, Brussels).

Plant material was hybridized overnight at 42°C with the appropriate antisense and control \(5\)-labeled mRNA probes (5 × 10<sup>6</sup> cpm per slide). After hybridization, the slides were washed in 2 × SSC (1 × SSC, 150 m mM NaCl, Na<sub>2</sub>-citrate, pH 7.0) at room temperature for 1 h and in 0.1 × SSC/0.1% (w/v) formamide at 42°C for 1 h. All posthybridization procedures, including RNase treatment and washes, were performed as described by de Almeida Engler et al. (2001). Signal detection was achieved by autoradiography, using Kodak (Rochester, NY) NBT film emulsion. After the slides had been developed, they were stained in 0.1% (w/v) toluidine blue and mounted in DePex mounting media (BDH, Poole, England). Autoradiographs were taken under bright- and dark-field illumination with a Diaplan microscope (Leitz, Heerbrugg, Switzerland).

Whole-Mount mRNA in Situ Analysis

Germinating seeds and young seedlings of Arabidopsis (ecotype Landsberg erecta) were fixed in phosphate-buffered saline solution containing 0.1% (v/v) Tween 20, 0.08 mM EDTA, 10% (v/v) dimethylsulfoxide, and 5% (w/v) paraformaldehyde. After dehydration, the samples were stored at −20°C until hybridization. RNA probes of CDK1;3, CDK1;3, CDK1;3, E2Fa, CDC7, and CDC6 were labeled with digoxigenin (DIG) by using a nucleic-acid-labeling kit according to the manufacturer’s instructions (Roche Diagnostics). Prehybridization and hybridization were carried out as described by de Almeida Engler et al. (1998) with minor modifications. During hybridization, samples were incubated in hybridization solution (50% [v/v] formamide, 5 × SSC, 50 μg mL<sup>−1</sup> heparin) containing the DIG-labeled probes (approximately 30 ng mL<sup>−1</sup> kb<sup>−1</sup>) and 5 ng mL<sup>−1</sup> herring sperm DNA for 16 h at 60°C. Posthybridization washes were conducted in 2 × SSC for 30 min at 45°C, and twice in 0.2 × SSC for 45 min at 62°C. For signal detection, samples were equilibrated in phosphate-buffered saline containing 0.1% (v/v) Tween 20 (PBST) for 5 min and blocked for 1 h in PBST containing 2% (w/v) bovine serum albumin. The tissue was then incubated for 16 h in PBST containing a sheep anti-DIG alkaline phosphatase-conjugated Fab antibody (Roche Diagnostics) diluted at 1:2,000. Detection solution was prepared by adding 5 μL nitroblue tetrazolium chloride (100 mg mL<sup>−1</sup> stock solution) and 3.75 μL 5-bromo-4-chloro-3-indolyl-phosphate (50 mg mL<sup>−1</sup> stock solution; both from Roche Diagnostics) per mL detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 0.1% [v/v] Tween 20). Formation of the stable blue/purple color precipitate took up to 16 h. The reaction was stopped by washing in PBST with 20 mM EDTA, pH 8.0. After staining, the material was cleared with chloroalcoholenol and microscopically observed and imaged.

RT-PCR Analysis

Total RNA was extracted from dried mature seeds and seeds at different stages of germination (1, 2, 3, and 4 DAI). Seeds of Arabidopsis (ecotype Columbia; 300 mg initial weight) were imbibed during the necessary time and promptly ground in liquid nitrogen. RNA was extracted as described by Chang et al. (1993) with minor modifications. After warm extraction with the appropriate buffer (2% [w/v] cetyltrimethylammonium bromide, 2% [w/v] polyvinylpyrrolidone, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 2.0 mM NaCl, 0.05% [w/v] spermidine), the RNA was purified with chloroform: isoamylalcohol (24:1), followed by precipitation with 10 mM LiCl. The RNA pellet was then redissolved in SSPE (1 mM NaCl, 0.5% [w/v] SDS, 10 mM Tris-HCl, pH 8.0, 1 M EDTA, pH 8.0) and again purified with a mixture of chloroform: isoamylalcohol (24:1). After ethanol precipitation for 2 h at −20°C, the RNA samples were resuspended in nuclease-free water. Two micrograms of each sample were reverse transcribed into cDNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Semiquantitative RT-PCR amplification of the CDNA was carried out with the ABI Prism 7900
real-time PCR machine (Applied Biosystems, Foster City, CA), with gene-specific primers (forward and reverse, respectively): 5'-ATATCTTT-TACGATCTCCCGTGA-3' and 5'-GAGTTTTTGTCCGCGATCAAT-3' for CDAK1; 5'-TTCCAGAATGTICCTACGGTTTTG-3' and 5'-GAACTCCTT-TACCTTTTACGAGGAAG-3' for CDK8; 5'-GCAAAAGACCCTGACGAGCAATGAG-3' and 5'-CTTGAATCTCCTCCGAGAAGCAGAC-3' for CYCB1; 5'-TGTAATATCATTGCAGGAAATGC-3' and 5'-ATGGTGGACAGAATCCTCGCATTGA-3' and 5'-AGCTTGTTCACGCCAGAATTG-3' for ubiquitin14 gene (UBQ14). PCR products were detected directly by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to double-stranded DNA (SYBR Green PCR core reagents; Applied Biosystems). Fluorescence values were recorded during every cycle and represented the amount of product amplified to that point in the amplification reaction.

The amount of target CDNA used for PCR was standardized by quantification of UBQ14 transcripts present in all the samples. However, it must be emphasized that ubiquitin levels are not absolutely stable during germination. The UBQ14 relative expression ratio between germinating and dry Arabidopsis seeds is approximately 2.4, as detected by CDNA microarray analysis (L. van der Geest, personal communication). This fluctuation was not considered, and, therefore, the values presented must be regarded as fairly accurate. This analysis does not intend to precisely quantify transcript levels on seeds, but to illustrate the transcription profile of main cell cycle genes during germination.

To facilitate the comparison between the different mRNA levels of the four genes analyzed, all values were adjusted to a relative final value of 100 (time point 4 d). This adjustment allowed the graphic representation of transcript fluctuation from the four genes in a single graph and the easy comparison of the respective profiles.

Confocal Microscopy

Arabidopsis (ecotype C24) transgenic seeds carrying a chimeric gene containing the GFP gene fused to the MBD of MAP4, under the control of the 35S promoter, were kindly provided by M. Karimi (Ghent University). Transgenic seeds expressing 35S-GFP-MDB were imbedded in water, as described above, and carefully peeled at different time points. For time sequence observations, the seed embryos were sealed in an approximately 2-mm-thick slide chamber (Lab-Tek, Christschurch, New Zealand) containing 1.5% (w/v) low-melting-point agarose (Sigma-Aldrich, St. Louis). This environment allowed the normal development of seed embryos for at least 48 h, as confirmed by the imaging of freshly peeled seeds after imbibition in water for the same time period. The rearrangement of the Mts was recorded at the peripheral epidermal cell layer of the embryo organs. Images were obtained by confocal laser scanning microscopy LSM510 (Zeiss) with an argon laser for 488 nm excitation, a 505 to 530 nm emission filter, and a water immersion 63× Plan-Apochromat objective, with exception of Figure 5G, which was obtained with a 10× Plan-Neofluar objective. Images of embryo epidermal cells are presented as single sections or as Z-stacks of consecutive sections as stated in the figure legend.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

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