The Cyclin-Dependent Kinase Inhibitor KRP6 Induces Mitosis and Impairs Cytokinesis in Giant Cells Induced by Plant-Parasitic Nematodes in Arabidopsis

Paulo Vieira, Annelies De Clercq, Hilde Stals, Jelle Van Leene, Eveline Van De Slijke, Gert Van Isterdael, Dominique Eeckhout, Geert Persiau, Daniël Van Damme, Aurine Verkest, José Djair Antonio de Souza, Nathalie Glab, Pierre Abad, Gilbert Engler, Dirk Inzé, Lieven De Veylder, and Janice de Almeida Engler

INTRODUCTION

The establishment and maintenance of the nematode feeding site by root-knot nematodes (RKNs) is a complex process associated with significant changes in gene expression of both the plant and the plant-parasitic nematode (Jammes et al., 2005; Caillaud et al., 2008; Gheysen and Mitchum, 2009). Previous efforts have aimed to enhance our understanding of the molecular mechanisms driving feeding cell development induced by these sedentary nematodes (Meloidogyne spp.). The complexity of plant–RKN interaction is well demonstrated by the number of host genes involved, and transcriptional data showing extensive crosstalk between the various host molecular pathways (reviewed by Gheysen and Fenoll, 2002; Gheysen and Mitchum, 2009). Because RKN infection leads to the formation of multinucleated giant cells through synchronous nuclear divisions in the absence of cytokinesis, the specific involvement of the host cell cycle machinery appears to be a major driver for the formation of these specialized feeding cells (de Almeida Engler et al., 1999; de Almeida Engler and Gheysen, 2013). Therefore, knowledge of the plant cell cycle machinery is fundamental for understanding comparable events occurring during nematode feeding site development in plant roots. Previously, infection of Arabidopsis thaliana reporter lines carrying cell cycle markers like cyclin-dependent kinases (CDKs) and their regulatory cyclin subunits (CYCs) illustrated that there is early stimulation of the host cell cycle machinery at the nematode feeding site (Niebel et al., 1996; de Almeida Engler et al., 1999). More recently, a detailed characterization of plant genes directly involved in endocycle, such as CCS52A, CCS52B, DEL1, and RHL1, has been reported for RKN, as well as for cyst nematodes (de Almeida Engler et al., 2012; Vieira et al., 2013a). Giant cells make use of this alternative cell cycle process during which mitosis is repressed in favor of continued DNA replication, resulting in polyploid cells. The involvement of cell cycle and endocycle genes in proper nematode feeding site establishment illustrates
the need for both cell cycle types for successful RKN reproduction (de Almeida Engler et al., 2012).

Different types of CDK/CYC complexes account for the correct temporal and unidirectional ordering of cell cycle events (Inzé and De Veylder, 2006). The model plant species Arabidopsis encodes up to 12 CDKs and 49 CYCs that have been categorized into different classes according to their sequence similarity (Vandepoele et al., 2002; Wang et al., 2004; Menges et al., 2005). Plants possess six types of CDKs. The A-type CDKs are the most closely related to the mammalian CDK1 and CDK2 because they contain the characteristic PSTAIRE amino acid sequence in their cyclin binding domain. In association with the D-type cyclins (CYCDs), the CDKA/CYCD complexes are believed to regulate the G1-to-S transition through phosphorylation of the retinoblastoma-related protein (De Veylder et al., 2007). The G2-to-M transition most probably requires A- and plant-specific B-type CDKs, as well as A- and B-type cyclins to form the mitotic CDK/CYC complexes (Inzé, 2005; De Veylder et al., 2007).

Inhibitory proteins regulate CDK/CYC activity. KRP proteins are a family of CDK inhibitors identified in plants. They are distantly related to the Kip/Cip family of animal CDK inhibitors, designated Kip-related proteins (KRPCs) (De Veylder et al., 2001) or interactors of Cdc2 kinases (Wang et al., 1997, 1998; Lui et al., 2000). They are generally believed to specifically interact with and inhibit A-type CDKs and CYCDs (Wang et al., 1998; De Veylder et al., 2001), although some family members might interact with B-type CDKs as well (Nakai et al., 2006; Pettkó-Szandtner et al., 2006). The level of inhibition of this KRP family of proteins seems to be concentration dependent (Verkest et al., 2005a), differentially affecting the cellular DNA content. Low KRP2 levels increase DNA content, while high levels decrease DNA content (Verkest et al., 2005b; Weinl et al., 2005). Sequence alignment and specific temporal and spatial expression patterns during cell cycle progression and plant development (Menges and Murray, 2002; Ormenese et al., 2004; Menges et al., 2005) point to a functional difference among the various members of the KRP family (Kim et al., 2008; Jégu et al., 2013; Jun et al., 2013; Wen et al., 2013). Recently, KRPCs have been linked to different physiological processes. KRP6 and KRP7 have been reported to be involved in the control of Arabidopsis male gametogenesis (Kim et al., 2008); Guérinier et al. (2013) have shown that recombinant KRP6 and KRP7 can be phosphorylated by SNF1-Related protein Kinase-1, providing a possible connection between energy sensing and cell proliferation. KRP2 influences lateral root density in an auxin-dependent manner, whereas KRP5 appears to be limiting for primary root growth (Sanz et al., 2011; Wen et al., 2013). In addition to their role in CDK/CYC inhibition, some KRPCs, like KRP5, may serve other functions regulating gene transcription involved in cell wall organization (Jégu et al., 2013).

Although the link between RKN infection and cell cycle activity for nematode feeding site formation is well recognized, the molecular mechanisms employed by nematodes to exploit the host plant remain ambiguous. Recent work has shown that ectopic KRP1, KRP2, and KRP4 expression led to a drastic reduction in gall size by inhibiting mitosis (Vieira et al., 2012, 2013b). Here, we found that KRP6 is highly expressed during nematode feeding site ontogenesis and studied the role of KRP6 during cell cycle progression in Arabidopsis and nematode feeding site development induced by RKN. Unexpectedly, we found that overexpression of KRP6 leads to multinucleated cells in Arabidopsis cell cultures and root cells, therefore stimulating and not inhibiting mitotic activity, as seen for other Arabidopsis KRP genes. We further show that overexpression of KRP6 increases mitotic activity in the nematode-feeding site, yet strongly interfered with nematode reproduction. Collectively, these findings point toward the involvement of KRP6 in the multinucleate and acytokinetic state of RKN-induced giant cells.

RESULTS

KRP6 Expression in Developing Galls Induced by Meloidogyne incognita

During our analysis of promoter activity of the seven KRP genes in Arabidopsis, we observed that KRP6 was strongly expressed in galls induced by RKN (Vieira et al., 2013b). Therefore, we analyzed a β-glucuronidase (GUS) reporter line in detail and performed in situ transcript localization of KRP6 in galls induced by M. incognita at early, intermediate, and later developmental stages (7, 14, and 21 d after nematode inoculation [DAI], respectively) (Figure 1). At 7 DAI, galls are in the mitotic phase, whereas at 14 DAI, nuclei in giant cells enlarge and show intense DNA biosynthesis, most likely via endoreduplication. At later developmental stages (21 up to 60 DAI, when the nematode life cycle ends with egg laying), no mitotic activity is observed either in giant or neighboring cells and DNA biosynthesis is significantly decreased or not occurring in giant cells (de Almeida Engler et al., 1999). Our analysis showed KRP6 expression in the root apex and the vascular tissue of noninfected roots (Figures 1A and 1B) and in galls at different developmental stages (Figures 1C to 1F). High promoter activity was observed in cells of the root vascular tissue showing a dense cytoplasm surrounding the nematode during migration and during the initial feeding site activation (Figure 1C). At 7 DAI, high KRP6 transcript levels were detected in giant and in neighboring cells (Figure 1G), which corresponds to high mitotic activity during gall development. Throughout gall expansion (up to 14 DAI) KRP6 expression decreased in giant cells but persisted in mitotic neighboring cells (Figures 1E and 1H), and at 21 DAI the promoter activity (Figure 1F) and transcript levels (Figure 1I) decreased in the whole gall.

GFP-KRP6 Localization and Dynamics during Nematode Feeding Site Development

Having analyzed promoter activity and performed transcript localization, additional localization and dynamics in the 3SS:GFP-KRP6 line (hereafter named KRP6OE) was followed in galls by confocal microscopy. GFP-KRP6 was detected in nuclei of both giant and neighboring cells at early stages (2 DAI) of gall development (Figure 2A), while at later stages (21 to 40 DAI), fluorescence was weak (Figure 2B) or below the detection level in giant cells (Figure 2C). Free GFP lines (3SS:GFP) were used as controls and showed constant fluorescence intensity levels throughout gall development (Supplemental Figure 1).
GFP-KRP6 Localizes to the Nucleus during Interphase and Its Overexpression Leads to Multinucleated Cells in Arabidopsis Cell Cultures

To study the dynamics of KRP6 protein localization during mitotic progression, we generated Arabidopsis cell cultures coexpressing 35S:GFP-KRP6 and 35S:RFP-TUA2, a microtubule marker (Figure 3). During time-lapse analysis throughout mitosis, GFP-KRP6 was localized within the nucleus in interphase and early prophase cells (Figure 3A). The GFP signal dispersed into the cytoplasm from late prophase (Figure 3B) until metaphase (Figures 3C and 3D) and anaphase (Figure 3E). During telophase, when the phragmoplast is formed, and the following cytokinesis, GFP-KRP6 relocated to the newly formed daughter nuclei (Figures 3F to 3J). Similar observations for GFP-KRP6 localization during mitosis were made in root cells of KRP6OE stable transgenic seedlings (Supplemental Figure 2).

Strikingly, in ~5% of the cells, overexpression of GFP-KRP6 led to the formation of polynucleate cells with up to 20 unequally sized nuclei (Figure 4; Supplemental Movie 1). Such nuclei were never seen in Arabidopsis control lines that overexpressed GFP with a nuclear localization signal or RFP-TUA2 alone.

To investigate the formation of these multinucleate cells coexpressing GFP-KRP6 and RFP-TUA2, we followed the course of cytokinesis using four-dimensional confocal time-lapse microscopy. Figure 5 presents a single projection of a Z-stack of three cells that divide synchronously with two nuclei in metaphase and two nuclei in anaphase (Figure 5A, asterisks). Originally, two nuclei were present in the upper left cell and one single nucleus in the other two cells (Figure 5A). The microtubular organization of the different spindles and phragmoplasts as visualized by RFP-TUA2 appeared normal in these cells (Figures 5A to 5D, asterisks). However, upon nuclear reformation, it became apparent that nuclear material was unevenly distributed relative to the expanding phragmoplast and that instead of two daughter nuclei, multiple nuclei formed accumulating GFP-KRP6 (Figures 5C to 5L). Often, some of these micronuclei fused to form larger nuclei (Figures 5D to 5G, 5K,
Overexpression of phase (Figure 6B). and/or the unequal separation of chromosomes during teloanaphase (Figure 6A, upper arrows; Supplemental Movie 3) could result from the observed lagging chromosomes during equal size (Supplemental Movie 2). The formation of micronuclei led to the formation of multinucleated cells with nuclei of unequal size (Supplemental Movie 2). Asterisks, giant cell; n, nematode. Bars = 20 μm.

and 5L, arrowheads). Finally, the overexpression of GFP-KRP6 led to the formation of multinucleated cells with nuclei of unequal size (Supplemental Movie 2). The formation of micronuclei could result from the observed lagging chromosomes during anaphase (Figure 6A, upper arrows; Supplemental Movie 3) and/or the unequal separation of chromosomes during telophase (Figure 6B).

**Overexpression of KRP6 Accelerates Entry into Mitosis, yet Delays Mitosis Progression and Exit**

To assess the consequences of overexpressing KRP6 on cell cycle progression, we synchronized the GFP-KRP6-expressing Arabidopsis culture by aphidicolin treatment, which blocks cell cycle progression specifically at early S phase. After removal of the inhibitor, cell cycle progression was monitored by counting the number of cells entering S phase through bromodeoxyuridine (BrdU) labeling of de novo-synthesized DNA (BrdU labeling index) and by scoring the metaphase/anaphase index (M/A index) (Figure 7A). Cells that overexpressed GFP-KRP6 entered S phase earlier than the control cells (PSB-D), as shown by the BrdU labeling index that peaked 1 h earlier in cells ectopically expressing KRP6 (Figure 7A). Entry into mitosis came 2 h earlier than in the control cells, as shown by the M/A index that peaked at 13 h, instead of 15 h in untransformed cells (Figure 7A). Also, the G2-M phase CDKB1;1 protein marker accumulated more rapidly in the 35S:GFP-KRP6 cell line than in the wild-type cells, while the CDKA;1 protein levels remained constant in both lines (Figure 7B). Although cells entered mitosis more rapidly, progression through mitosis was transiently arrested after metaphase-to-anaphase transition, as indicated by a slower decay in M/A index than that in the wild type (Figure 7A). These observations indicate that KRP6 overexpression leads to a faster entry into mitosis, but delays mitosis progression and exit. Similar observations of a shift in cell cycle progression, accompanied by a faster increase of CDKB1;1 protein level, were observed in a second cell culture overexpressing KRP6, obtained independently from the first culture (Supplemental Figure 3).

**KRP6 Overexpression Leads to Increased Mitotic Activity in Gall Tissues, Strongly Interfering with Nematode Reproduction**

To evaluate the effects of ectopic KRP6 expression on M. incognita–induced gall ontogeny, independent KRP6OE Arabidopsis lines were generated (Supplemental Figure 4), showing a serrated leaf phenotype and the expected nuclear localization of the KRP6 protein. For RKN infection, one out of the 10 KRP6OE generated lines was chosen to perform a detailed microscopic analysis during different feeding site developmental stages (7, 14, and 40 DAI). Remarkably, overexpression of KRP6 resulted in more nuclei in giant cells (Supplemental Figure 5A), as well as proliferation of the neighboring cells (Figures 8A to 8C compared with wild-type galls in Figures 8D to 8F). Therefore, ectopic KRP6 expression promotes mitotic activity in galls. Furthermore, in ~10% of the gall sections, we observed the formation of multiple aggregated micronuclei in giant cells (Supplemental Figures 5C to 5D,I), whereas in the wild type such nuclear organization was not detected (Figures 8D to 8F; Supplemental Figure 5B). Subsequently, 3D confocal projections of serial optical sections of whole KRP6OE galls at different developmental stages (7, 14, and 40 DAI; Figures 8G to 8I) were generated and compared with wild-type galls (Figures 8J to 8L). These observations clearly show the large number of nuclei in each giant cell and the increased number of neighboring cells, confirming augmented nuclear mitotic activity (Figures 8G and 8H). At later stages of gall development, KRP6OE nuclei were mostly clustered in all giant cells (Figure 8I). Electron microscopy analysis of gall sections confirmed the increased nuclei number in giant cells of the KRP6OE line (Supplemental Figure 6). Furthermore, nuclei in giant cells of the KRP6OE appeared more lobed compared with the wild type (Supplemental Figures 6D and 6B).

Statistical analysis revealed that the giant cells in the KRP6OE line were significantly smaller than those of the wild-type giant cells, at each time point after nematode infection (Figure 9A). Simultaneously, nuclear surface measurements in giant cells of the KRP6OE line revealed that nuclei were smaller than in the
Due to the convoluted shape and clustering of nuclei in older galls (40 DAI; Figures 8I and 8L), measurements were not performed at this stage. The nuclear size in wild-type giant cells gradually increased, whereas nuclei in giant cells of the KRP6OE line remained small (Figure 9B).

Ultimately, long-term infection tests showed that ectopic expression of KRP6 affected nematode development and reproduction (Figures 9C and 9D). Although there were not more galls in the KRP6OE line compared with the wild type, nematodes clearly showed a delay in development (Figure 9D). Consequently, a significant decrease in reproduction was observed, as seen by the strong decrease in the number of egg masses (Figure 9C).

Ectopic KRP6 Expression Affects Gall Ploidy Levels

To evaluate the effect of KRP6 overexpression on the nuclear ploidy levels, flow cytometric measurements were performed on noninfected roots (Supplemental Figure 7), gall-less roots (Figure 9E), and on galls (Figure 9F) of the wild type and KRP6OE. Ploidy levels in gall-less roots in wild-type plants ranged from 2C to 16C, whereas few nuclei above 8C were detected in comparable root samples of the KRP6OE line (Figure 9E). Ploidy levels in wild-type galls ranged from 2C to up to 64C (Figure 9F) and more than 50% of the nuclei showed 2C DNA content, most likely due to the large number of cells neighboring the giant-feeding cells. Ploidy levels of nuclei in wild-type galls above 16C most likely represent nuclei derived from giant cells. In galls overexpressing KRP6, the number of nuclei with a 16C and 32C ploidy level was strongly reduced, and no nuclei with a 64C DNA content were detected (Figure 9F). These data demonstrate that the ploidy levels are decreased in the KRP6OE line.

KRP6 Is Required for Normal Gall Development

To analyze the need for KRP6 expression during gall development, we analyzed gall morphology in two available T-DNA insertion lines (krp6KO). The first has an insertion in the third exon (SAIL_54B_B03) and the second a T-DNA insertion just before the start codon (ATG) (SALK_142997) (Supplemental Figure 8). Both lines were devoid of KRP6 transcripts (Supplemental Figure 8C). Nematodes were able to induce galls and reproduce in krp6KO roots (Supplemental Figures 8D and 8E). However, clear cellular abnormalities were observed during gall development (7, 14, and 21 DAI) as observed in both mutant lines by the increased cell wall stub frequency in giant cells compared with the wild type (Figure 10; Supplemental Figures 8G to 8I). Likewise, mature giant cells in krp6KO lines were significantly smaller than those in wild-type giant cells (Figure 10; Supplemental Figure 8F) and contained fewer and more dispersed nuclei, rather
DISCUSSION

Strong KRP6 Expression Is Associated with the Mitotic State in Galls

Targeting cellular mechanisms through modulation of host cell cycle components is a common feature adopted by several plant pathogens (Doonan and Sablowski, 2010; Wildermuth, 2010). The RKN Meloidogyne spp induces the formation of multinucleated giant cells through activation of host cell cycle machinery and inhibition of cytokinesis (de Almeida Engler et al., 1999, 2012). Here, we show that KRP6 promoter activity and high transcript levels are present mainly at early stages of gall formation (up to 14 DAI) associated with their mitotic activity phase. Consistent with this, GFP-KRP6 protein accumulates in nuclei of giant cells during this stage of high mitotic activity. GFP-KRP6 dynamics studies revealed that once the mitotic phase ended (21 to 40 DAI), GFP-KRP6 fluorescence became weak or below the detection levels within the giant cell nuclei. KRP6 protein levels remained high in cells neighboring giant-feeding cells until later stages (21 DAI) of gall development, possibly associated with their proliferative status during gall expansion. By contrast, localization and dynamics studies of GFP-KRP2 in galls illustrated that at an early phase of gall development, protein levels were low in giant cell nuclei (Vieira et al., 2013b), most likely allowing ongoing mitosis of giant and neighboring cells. This mitotic phase would rely on the presence of proteins like KRP6 to stimulate mitosis and possibly inhibit cytokinesis. Once the mitotic activity phase ceased, nucleus expansion takes place in giant cells and KRP2 fluorescence increased, while the KRP6 signal faded. This new gall status possibly reflects a shift from the mitotic phase to the endocycle phase. We observed that this transition involves giant cell nucleus enlargement and slowing of neighboring cell division. Although promoter activity of KRP2 and KRP6 is high in galls, the observed differences in protein levels suggest that nematodes are able to regulate their expression during parasitism. Additionally, it is possible that KRP2 and KRP6 levels might affect the cell cycle in a different way in giant cells.

KRP6 Overexpression Leads to Acceleration of G1-to-S and G2-to-M Transitions

The KRP gene family has been associated with inhibition of cell division and endoreduplication (Verkest et al., 2005b; Wang et al., 2007). Apparently in contrast to its supposed role as cell cycle inhibitor, KRP6 overexpression led to accelerated G1-to-S and G2-to-M transitions in cell cultures. Previously, KRP1 has been found to mediate the transport of CDKA;1 into plant nuclei (Zhou et al., 2006), reminiscent of the situation in mammals, where Cip/Kip proteins have been suggested as assembly and nuclear targeting factors of D-type cyclin complexes to the nucleus (LaBaer et al., 1997; Cheng et al., 1999). In addition, p21Cip1 promotes nuclear accumulation of CYCD1 by binding to its phosphorylated form to prevent its nuclear export (Alt et al., 2002). Analogously, the accelerated G1-S and G2-M transition in KRP6-overexpressing cultures may be explained if KRP6 functions through activation of CDK/CYCD complexes by promoting their assembly or stability, or their targeting to the nucleus, resulting in more efficient phosphorylation of target proteins. In accordance with a role as a nuclear targeting factor, KRP6 specifically localizes in the nucleus and might facilitate the transport of CYCDs into the nuclear compartment. There is evidence that increased CYCD/CDKA;1 activity may indirectly trigger G1-S phase transition and mitosis. D-type cyclins were shown to accelerate the cell cycle in cultured tobacco cells through the enhancement of G1/S entry and progression through S and G2 phases (Koroleva et al., 2004). A triple CYCD3KOkO mutant displays decreased mitosis and increased endoreduplication in leaves (Dewitte et al., 2007), whereas overexpression of CYCD3;1 in developing trichomes leads to division of the normally unicellular trichomes (Schnittger et al., 2002).

KRP6 Expression in Giant Cells Is Needed for Multinucleation and Inhibition of Cytokinesis

Together, our analyses of cell cultures and gall sections illustrated that the increased CDK activity mentioned above might be linked with the occurrence of multinucleate cells. Also, ectopic KRP6 expression in nematode feeding sites triggered an increase of mitotic activity. Cells overexpressing KRP6 entered mitosis earlier than normal cells, although progression through mitosis was slowed down. These effects were revealed by the increased number of nuclei in Arabidopsis suspension cells as well as in giant cells and by the abnormal proliferation of neighboring cells in galls. As a result, galls within the KRP6OE line were bigger than in KRP1-, KRP2-, or KRP4-overexpressing lines, which exhibited a reduced giant cell size and neighboring cell number severely affecting gall size (Vieira et al., 2012, 2013b). This differential gall morphology between the previously analyzed KRP1OE lines and KRP6OE suggest a distinct function of the latter during feeding site development.
High mitotic activity in giant cells within KRP6OE roots was also prolonged compared with wild-type feeding cells, possibly facilitating their multinucleate status. The observation of accelerated G1-S and G2-M transitions in cells with ectopic KRP6 expression as well as the increased CDKB1;1 protein levels provide further evidence that mitotic activity is enhanced in plant cells upon KRP6 overexpression. In addition, ectopic KRP6 expression led to the formation of multinuclei of unequal sizes in cultured cells as well as in nematode-induced giant cells. The augmented CDK activity during mitosis could lead to an accelerated nuclear division followed by inhibited cell division as seen by the later decelerated mitotic activity. This effect might be true as well for giant cells, given the high KRP6 expression.

Metaphase-to-anaphase transition requires the removal of cohesins that glue chromosomes together, a process that involves the activation of Aurora kinases. Recently, treatment of tobacco (Nicotiana tabacum) Bright Yellow 2 with the Aurora kinase inhibitor hesperadin has been found to induce the formation of micronuclei because of lagging chromosomes during the metaphase-to-anaphase transition (Kurihara et al., 2006). Multinucleate cells with two or more nuclei of unequal size, as observed here in KRP6OE cultured cells, and in nematode-infected root cells, has been similarly reported to occur upon the expression of indestructible CYCB1;1 in tobacco Bright Yellow 2 cells (Criqui et al., 2001; Weingartner et al., 2004). Destruction of both cohesins and CYCBs is essential to proceed into anaphase and is controlled through the activation of the anaphase-promoting complex/cyclosome (APC/C) at the spindle checkpoint (Peters, 2006). The resemblance of the phenotype of the KRP6 over-expression lines to that of hesperadin-treated and CYCB1;1-stabilized cells strongly suggests a link between KRP6 and APC/C activity. APC/C activity is at least in part regulated by CDK phosphorylation of several of its subunits (Harper et al., 2002; Peters, 2006). Therefore, increased KRP6 levels might interfere with CDK-dependent APC/C activation, delaying mitosis progression and inhibiting cytokinesis.
The assessment of KRP6 loss-of-function lines showed that, although giant cells were smaller, nematode reproduction was similar to the wild type. The induction of more cell wall stubs between nuclei as observed in a fraction of giant cells of the krp6KO lines suggests a stimulation of cytokinesis. Nematode secretions combined with the lack of KRP6 protein might prevent formation of complete cell walls between nuclei in giant cells. Combining our observations of the effects of KRP6 overexpression and loss of function, our data indicate that KRP6 expression on the one hand might stimulate nuclear division and on the other hand inhibits cytokinesis, both needed to stimulate multinuclear giant feeding cell formation in galls.

**Ectopic KRP6 Expression Disrupts Gall and Nematode Development and Affects Ploidy in Nematode Feeding Sites**

It is likely that a balance of the mitotic cycle followed by an increase in ploidy during nematode infection ultimately support giant cell fate and maintenance, and consequently nematode development and reproduction (de Almeida Engler et al., 2012). A fluctuation in expression of KRP genes is recognized to affect the endoreduplication process in plant cells (De Veylder et al., 2001; Zhou et al., 2003; Roeder et al., 2010). A decline of nuclear size was detected in giant cells overexpressing KRP6. By comparing the DNA content of galls of the KRP6OE line (2C to 32C) with the wild type (2C to 64C), a noteworthy reduction of giant cell nuclei ploidy levels was observed in gall tissue overexpressing KRP6. It is tempting to suggest that lower ploidy dramatically influenced giant cell size. Indeed, giant cells, the sole food source for the feeding nematode, were undersized in the KRP6OE line compared with the wild type. Nevertheless, the global gall size in the KRP6OE line was similar to the wild type due to the elevated number of neighboring cells, which ultimately determined gall size and shape. The extension of this mitotic status most likely delayed the onset of the endocycle in the small-sized giant cells of the KRP6OE line, thus also affecting the completion of RKN life cycle. These data confirm that the multinucleate state in giant cells is not sufficient to drive giant cell expansion and nematode reproduction (de Almeida Engler et al., 1999, 2012). A phase governed by the endocycle seems to be essential for giant cell expansion (de Almeida Engler et al., 2012; Vieira et al., 2013a).

**Concluding Remarks**

This work points to an unexpected role for KRP6 during mitosis, suggesting that not all KRPs regulate the cell cycle in the same manner. Whereas KRPs such as KRP1 and KRP2 are associated with inhibition of mitosis and consequently cell division, here we illustrate that upon its overexpression, KRP6 can accelerate entry into mitosis, delaying mitosis progression and exit. These observations are supported by plant cells presenting an amplified cell cycle, like giant-feeding cells induced by plant-parasitic nematodes. We found that KRP6 expression parallels the induction of a mitotic state in plant and giant cells prompting their

**Figure 6.** Micronuclei Form upon Overexpression of GFP-KRP6 in Arabidopsis Suspension Cells.

(A) Confocal Z-projection (18 images, 21.15 μm) of a telophase cell. The nuclear envelope reforms around the separated daughter nuclei and around lagging chromosomes (upper arrows). Lagging chromosomes likely result in the formation of the micronuclei (lower arrows).

(B) Single-plane confocal section of a telophase cell overexpressing GFP-KRP6 and TUA2-RFP. A ring-like phragmoplast indicated by the RFP-labeled microtubule separates reforming micronuclei at either side of the forming cell plate. 

Bars = 10 μm.

**Figure 7.** Effect of Ectopic Expression of KRP6 on Cell Cycle Progression of Cultured Arabidopsis Cells.

(A) BrdU labeling index (dashed lines) and M/A index (solid lines) indicating the percentage of cells that are in S phase and in metaphase and anaphase after aphidicolin synchronization of PSB-D (blue lines) and 35S:GFP-KRP6 (green lines) cell cultures.

(B) Total protein extracts of PSB-D and transgenic Arabidopsis cultures overexpressing GFP-KRP6 after release from the aphidicolin block, analyzed hourly by immunoblot analysis with antisera against CDKA;1 and CDKB1;1.
Figure 8. Ectopic KRP6 Expression Increases Mitotic Activity and Affects the Size of M. incognita–Induced Giant Cells.

(A) to (F) Histological analysis of wild-type galls compared with KRP6-overexpressing lines at different stages after nematode infection (7, 14, 21, and 40 DAI). Bright-field images of longitudinal gall sections stained with toluidine blue. Increased mitotic activity is observed in nuclei of giant cells, as well as in neighboring cells (A) to (C) compared with the wild-type gall (D) to (F). Giant cell size is also reduced in the KRP6OE line.

(G) to (I) KRP6OE galls at 7, 14, and 40 DAI. 3D confocal projections of serial optical sections of cleared whole-mount gall samples. Increased mitotic activity is observed in nuclei of giant cells as well in neighboring cells.

(J) to (L) Wild-type galls at 7, 14, and 40 DAI. White circles delimit giant cells. Asterisks, giant cell; n, nematode; NCs, neighboring cells. Bars = 50 μm in (A) to (F) and 20 μm in (G) to (L).
Figure 9. Ectopic KRP6 Expression Drastically Affects the Size and Ploidy Levels of *M. incognita*-Induced Giant Cells.

(A) Giant cell surface (μm²) of wild-type and KRP6OE galls was measured at different stages after nematode infection (7, 14, 21, and 40 DAI). Asterisks denote significant differences between the genotypes at that time point after nematode infection (P < 0.0001, ANOVA with Tukey-Kramer test).  
(B) Measurements (μm²) of giant cell nuclei in wild-type and KRP6OE at 7, 14, and 21 DAI. Asterisks denote significant differences between the genotypes at that time point after nematode infection (P < 0.0001, ANOVA with Tukey-Kramer test).  
(C) Infection tests by *M. incognita* show that the number of galls and egg masses decreased compared with the wild type. Asterisks indicate values that were significantly different from the wild type at P < 0.05 (Student’s t test).  
(D) Acid fuchsin staining show that galls in the KRP6OE line are larger even when giant cells are smaller compared with the wild type. In most galls, females have not reached maturity, consequently did not produce eggs. G, gall; n, nematode. Bars = 50 μm.
multinucleate and acytokinetic state. It is appealing to hypothesize that the irregular-sized nuclei and nuclear fusion observed in cell cultures with ectopic KRP6 expression might help to explain the multiple nuclear sizes observed in giant cells in wild-type plants. Functional analysis shows that the lack of KRP6 in nematode-induced giant cells leads to an increase in cell wall stubs, suggesting a stimulation of cytokinesis. Conversely, ectopic KRP6 expression induced mitosis and inhibited cytokinesis, as well as endoreduplication in plant cells, and led to small multinucleated giant cells. The endogenous high KRP6 expression in wild-type galls might therefore stimulate mitosis in giant cells and induce ectopic neighboring cell division. Together, our data support the view that KRP6 might play a role in regulating feeding cell growth, multinucleation, and the acytokinetic state of these giant-feeding cells. Thus, plant-parasitic nematodes might have evolved the competence to exploit plant cell cycle genes like KRP6 to the benefit of gall establishment.

**METHODS**

**Constructs, Plant and Cell Suspension Growth, and Transformation**

*Arabidopsis thaliana*, genotype Columbia-0 (Col-0), was used as the wild-type control. For construction of the KRP6pro:GUS line, the intergenic region (up to maximum 2 kb) was amplified from Arabidopsis genomic DNA. The corresponding PCR fragment was cloned into pDONR207 entry vector (Invitrogen) by BP recombination cloning and subsequently transferred into the pKGWFS7 destination vector (Karimi et al., 2002) by LR cloning, resulting in a transcriptional fusion between the KRP6 promoter and the EGFP-GUS fusion gene. Single T-DNA insertion mutant lines of KRP6 (SAIL_54B_B03 and SALK_142997) were obtained from the ABRC.

*Arabidopsis* cell cultures (2 d old) were stably transformed by *Agrobacterium tumefaciens* cocultivation according to Van Leene et al. (2007). The 3SS:GFP-KRP6-containing plant transformation vector pK7WG2-KRP6 was obtained by Gateway LR reaction (Invitrogen) of pEntryL1L2-KRP6 with the destination vector pK7WG2 (Karimi et al., 2002). The KRP6-coding region was amplified by PCR and cloned into the Gateway pDONR221 vector (Invitrogen), resulting in pEntryL1L2-KRP6. The 3SS:GFP-TUA2-containing plant transformation vector was constructed as described (Van Damme et al., 2004). To obtain the double (3SS:GFP-KRP6 × 3SS:GFP-TUA2) cell transformation vector, PSB-D cell suspensions of Arabidopsis were maintained as described (Van Leene et al., 2007). Kanamycin-resistant T3 overexpressing lines (3SS:GFP-KRP6) were evaluated based on protein localization and plant phenotype. Seeds from Arabidopsis transgenic lines and wild-type Col-0 were surface-sterilized for 10 min in 5% NaOCl, followed by four washes with 95% ethanol, and dried overnight. Seeds were kept in a growth chamber with a 16-h-light/8-h-dark photoperiod at 21°C:18°C, respectively.

**Histochemical GUS Staining and mRNA in Situ Hybridization Assays**

Promoter KRP6pro:GUS activity was monitored at different time points after nematode infection (7, 14, and 21 DAI) as previously described by de Almeida Engler et al. (1999). To avoid diffusion of the GUS precipitate, galls were fixed in 2% glutaraldehyde ON and were embedded in Technovit 7100, sectioned (3 μm), and microscopically analyzed by dark-field optics.

Gene-specific sense and antisense probes of KRP6 were generated as previously described (de Almeida Engler et al., 2009). In situ hybridization procedure was performed essentially as described by de Almeida Engler et al. (2009). Images were taken with a digital Axiocam (Zeiss) with standard dark-field optics.

**DNA and RNA Extraction and Analyses of KRP6 Knockout and Overexpressing Lines**

Genomic DNA extraction and PCR for amplification of T-DNA insertions in krp6KO mutant lines was done using the Extract-N-Amp Plant PCR kit (Sigma-Aldrich) and primers listed in Supplemental Table 1. Total RNA of krp6KO and KRP6OE lines was extracted from 7-d-old whole seedlings, using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer’s instructions. The RNA was treated with RQ1 RNase-free DNase (Promega) before reverse transcription. cDNA was prepared using 1 μg treated RNA and added to RT reactions using a SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) using a mix of oligo(dT) and random primers.

![Figure 10.](image-url) **Figure 10.** Histological Analysis of the krp6KO Line of *M. incognita*-Induced Galls in *Arabidopsis* Roots.

Bright-field images of longitudinal sections of galls stained with toluidine blue in krp6KO (SAIL_548_B03) and in wild-type lines at different stages after nematode infection (7, 14, and 21 DAI).

(A) to (C) Galls in the krp6KO mutant line. Note the presence of cell wall stubs (red arrow) in (B) and (C).

(D) to (F) Wild-type galls. Asterisks, giant cell; n, nematode. Bars = 50 μm.

Almeida Engler et al. (1999). To avoid diffusion of the GUS precipitate, galls were fixed in 2% glutaraldehyde ON and were embedded in Technovit 7100, sectioned (3 μm), and microscopically analyzed by dark-field optics.

Gene-specific sense and antisense probes of KRP6 were generated as previously described (de Almeida Engler et al., 2009). In situ hybridization procedure was performed essentially as described by de Almeida Engler et al. (2009). Images were taken with a digital Axiocam (Zeiss) with standard dark-field optics.
random hexamer primers. PCR amplifications were performed with specific primer pairs (Supplemental Table 1).

Gall Morphological Analyses

Transgenic seedlings (T3) KRP6OE were used for plant-RKN studies. Transgenic and wild-type Arabidopsis Col-0 seeds were surface sterilized for 10 min in 5% NaOCl, followed by four washes with 95% ethanol and dried overnight. Seeds were placed into sterile Petri dishes on 1% Murashige and Skoog germination medium (Duchefa) containing 1% sucrose and 0.8% plant cell culture-tested agar (Sigma-Aldrich), supplemented with the appropriate antibiotics. Plantlets were grown vertically to allow roots to grow at the surface, with a 16-h-light/8-h-dark photoperiod at 21°C/18°C respectively. Roots were infected and collected at 7, 14, 21, and 40 DAI and fixed in 2% glacial acetic acid in 50 mM PIPES buffer, pH 6.9, and then dehydrated and embedded in Technovit 7100 (Heraeus Kulzer), as described by the manufacturer. Embedded roots and gall tissues were sectioned (5 µm) and stained in 0.05% toluidine blue and mounted in Depex (Sigma-Aldrich). Microscopy observations were performed using bright-field optics, and images were performed with a digital camera (AxioCam; Zeiss).

In Vivo Confocal Microscopy Analyses

Arabidopsis cells were applied to a chambered cover glass system (Lab-Tek) and immobilized on a thin layer of 0.8% (w/v) low melting point agarose (Invitrogen) in MSMO medium. Cells were imaged with a Zeiss confocal fluorescence microscope 100X with software package LSM 510 version 3.2 (Zeiss), equipped with a 63× water corrected objective (numerical aperture of 1.2). Dual GFP and red fluorescent protein (RFP) fluorescence was imaged in a multichannel setting with 488- and 543-nm laser source for GFP and RFP excitation, respectively. Emission fluorescence was captured by sequential frame-scanning mode alternating GFP fluorescence via a 500- to 530-nm band-pass emission filter and RFP via a 560-nm long-pass filter. Images were recorded using 3× digital zoom. Image analysis and Z-stack projection were done with the LSM software (Zeiss). Noninfected KRP6OE T3 transformants selected on kanamycin were transferred to half-strength Murashige and Skoog plates after selection. Roots were imaged 7 d after transfer to half-strength Murashige and Skoog plates using an inverted confocal microscope (model LSM510 META; Zeiss).

Observation of the nuclei in the nematode feeding sites was performed in nematode infected KRP6OE line and Arabidopsis seedlings harboring free GFP (35S:GFP) lines. Galls at various time points after infection (2 to 40 DAI) were dissected from roots and embedded in 5% agar. Fresh thick sections of 50 to 100 µm (2 to 14 DAI) or 150 to 200 µm (14 to 40 DAI) were prepared with a Microm HM650V Vibratome (Walldorf). Whole roots and fresh slices were immediately observed using an inverted confocal microscope (model LSM510 META; Zeiss). GFP was excited with a 488-nm argon laser, and fluorescence was captured in lambda mode using 499- to 550-nm bandwidth.

Protein Extraction, SDS-PAGE, and Immunoblotting

Cell suspension cultures were harvested by filtration on a sintered glass filter, used immediately or snap-frozen in liquid nitrogen, and stored at -70°C. Total protein extracts were prepared as described by Van Leene et al. (2007). Equal amounts of total protein were separated on 12% SDS-PAGE gels, blotted onto Immobilon-P membranes (Millipore), and blocked in 3% skim milk in Tris-buffered saline and 0.1% Triton X-100. For immunodetection, polyclonal anti-CDKA;1 and CDKB1;1 rabbit antisera were used as primary antibodies, followed by anti-rabbit IgG coupled to horseradish peroxidase (GE-Healthcare) as secondary antibodies. Protein gel blots were developed by chemiluminescent detection (Perkin-Elmer).

Synchronization of Arabidopsis Cell Cultures, BrdU Labeling, and Mitotic Index Determination

For aphidicolin synchronization, 800 mL cell suspension culture was grown in a 2-liter Erlenmeyer flask by adding 134 mL saturated culture to 670 mL MSMO medium (4.43 g/liter MSMO [Sigma-Aldrich], 30 g/liter sucrose, 0.5 mg/liter α-naphthaleneacetic acid, and 0.05 mg/liter kinetin, pH 5.7, adjusted with 1 M KOH and 5 mg/mL 1-aphidicolin to a final concentration of 6.66 µg mL⁻¹ for 21.5 h. After removal of aphidicolin by washing cells five times with MSMO medium, the cells were resuspended in fresh MSMO medium. Samples were harvested hourly and cell cycle progression was analyzed by microscopy.

For determination of the M/A index, cell samples were taken hourly, fixed 3:1 (v/v) ethanol/acetic acid mixture and washed with 70% ethanol (v/v). DNA was stained with 1 µg mL⁻¹ 4',6-diamidino-2-phenylindole (Sigma-Aldrich) and observed by epifluorescence microscopy. For determination of BrdU incorporation, cells were immediately labeled with the BrdU-labeling reagent and incubated for 1 h at 25°C. Afterwards, cells were fixed in 3.7% formaldehyde in PEG buffer [50 mM piperazine-N,N'-bis(ethanesulfonic acid), 2 mM MgSO4, 5 mM EGTA, and 2% glycerol, pH 6.8] and processed for immunostaining with monoclonal BrdU antibody (GE-Healthcare). For each time point, at least 400 nuclei were scored for mitotic figures and BrdU labeling.

Giant Cell and Nucleus Surface Measurements

Two to three largest giant cells per gall were selected and analyzed at different time points after nematode infection (7, 14, 21, and 40 DAI), and the surface was measured using the AxioVision LE (Zeiss) software. A minimum of 60 giant cells was measured, for root sections of each time point studied. The nucleus surface was measured at 7, 14, and 21 DAI from a minimum of 80 nuclei of KRP6OE and wild-type lines, using the AxioVision LE software.

Whole-Mount Analysis of Cleared Nuclei Stained by Propidium Iodide

Nuclear analysis of cleared feeding sites was performed as described (Vieira et al., 2012). Cleared samples were analyzed with a Zeiss LSM 510 META confocal microscope. Propidium iodide excitation was performed with the 543-nm line of a HeNe laser. Stacks were generated from ~50 images of 1-µm optical slice thickness and represented as maximum brightness projections.

Electron Microscopy of Galls

Root galls of Arabidopsis Col-0 and KRP6OE lines were dissected at 14 and 21 DAI and fixed in a mixture of 1.5% glacial acetic acid and 3% parafomaldehyde in 10 mM PBS containing 150 mM NaCl (pH 7.2) for 3 h at room temperature. After several washes in PBS buffer, fixed galls were incubated in 0.5M NH4Cl for 1 h, dehydrated in graded ethanol series, embedded in acrylic resin LR White (Sigma-Aldrich), and polymerized overnight at 60°C. Ultrathin sections were collected on parlodion-coated nickel grids, treated with 0.1 M HCl for 10 min, and washed at least twice with double distilled water. Samples were observed under a Philips 400T electron microscope.

Flow Cytometry Analyses

Plant roots were chopped with a razor blade in 400 µL 45 mM MgCl2, 30 mM sodium citrate, 20 mM MOPS, pH 7.0, and 0.1% Triton X-100
The following materials are available in the online version of this article.

Supplemental Figure 1. Free-GFP Protein Expression during Gall Development.

Supplemental Figure 2. GFP-KRP6 Localization in Arabidopsis during Cell Division.

Supplemental Figure 3. Effect of Ectopic Expression of KRP6 on Cell Cycle Progression in an Arabidopsis Cell Culture Independently Obtained from the Transgenic Culture Used in Figure 7.

Supplemental Figure 4. GFP-KRP6 Overexpression Causes Leaf Serration in Seedlings.

Supplemental Figure 5. Micronuclei Form within 10% of KRP6OE Giant Cells.

Supplemental Figure 6. Electron Micrographs of Giant Cells of Galls in Arabidopsis Wild-Type Plants Compared with KRP6OE Line.

Nematode Infection Tests

Three-week-old Arabidopsis wild-type and KRP6OE seedlings were inoculated on Murashige and Skoog medium on Petri dishes, containing five plants per plate and six replicates per line. Seedlings were grown vertically with a 16-h-light/8-h-dark photoperiod at 21°C/18°C, respectively. One root tip of each plant was inoculated with ~100 surface-sterilized, freshly hatched second stage juveniles (J2s) of Meloidogyne incognita as previously described (de Almeida Engler et al., 1999). Six to seven weeks after infection, the number of galls and egg masses were counted on each plate and compared with the control plants. Data shown represents means ± SD from two independent biological experiments, in which a minimum of 60 seedlings of each line were evaluated for nematode infection.

Acid Fuchsin

The acid fuchsin staining was performed for nematode visualization within the roots. The infected roots (40 DAI) were fixed and stained for 5 h in a solution of equal parts of 95% ethanol and glacial acetic acid, containing 17.5 mg/mL acid fuchsin. Root tissue was destained by soaking in a solution of chloral hydrate (0.2 g/mL in water) for 16 h. After rinsing the roots several times with tap water, roots containing nematodes were stored in acidi-

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number At3g19150 (KRP6).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. List of Primers.


ACKNOWLEDGMENTS

We thank Danny Geelen for constructs, Sylvie Coutuer, Evelien Mylle, and Tom Beeckman for help with microscopy, and Sophie Pagnotta for help with electron microscopy. This work was supported by grants from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) (“Generisch Basisonderzoek aan de Universiteiten” no. 20193). A.D.C. and A.V. are indebted to the IWT for a predoctoral fellowship. D.V. is a postdoctoral fellow of the Research Foundation-Flanders. L.D.V. acknowledges the Research Foundation Flanders (FWO, G.022.10N) for financial support. P.V. was supported by a doctoral scholarship from Fundação para a Ciência e Tecnologia, from Portugal (SFRH/BDE/41339/2007). The work was supported by CAPES/COFECUB Project Sv 683/10. This research was funded by the Interuniversity Attraction Poles Programme (IAP P7/29 “MARS”) initiated by the Belgian Science Policy Office.

AUTHOR CONTRIBUTIONS


Received April 9, 2014; revised April 9, 2014; accepted May 28, 2014; published June 24, 2014.

REFERENCES


