Variation in Growth Rate between Arabidopsis Ecotypes Is Correlated with Cell Division and A-Type Cyclin-Dependent Kinase Activity

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We used a kinematic analysis to investigate the growth processes responsible for variation in primary root growth between 18 ecotypes of Arabidopsis. Root elongation rate differed 4-fold between the slowest (Landsberg erecta, 71 μm h⁻¹) and fastest growing line (Wassilewskija [Ws]; 338 μm h⁻¹). This difference was contributed almost equally by variations in mature cortical cell length (84 μm [Landsberg erecta] to 237 μm [Ws]) and rate of cell production (0.63 cell h⁻¹ [NW108] to 1.83 cell h⁻¹ [Ws]). Cell production, in turn, was determined by variation in cell cycle duration (19 h [Tsu] to 48 h [NW108]) and, to a lesser extent, by differences in the number of dividing cells (32 [Weiningen] to 61 [Ws]). We found no correlation between mature cell size and endoreduplication, refuting the hypothesis that the two are linked. However, there was a strong correlation between cell production rates and the activity of the cyclin-dependent kinase (CDKA). The level of the protein could explain 32% of the variation in CDKA. Therefore, it is likely that regulators of CDKA, such as cyclins and inhibitors, are also involved. These data provide a functional link between cell cycle regulation and whole-plant growth rate as affected by genetic differences.

The rate at which plants grow is an important agronomic trait in cultivated plants, as well as an adaptive trait under natural growth conditions. Therefore, the physiological characteristics associated with fast and slow growth have been extensively investigated (for an overview, see Lambers et al., 1998). They involve the acquisition of growth-supporting substances (photosynthesis and nutrient uptake) and their utilization (anatomy, chemical composition, cell division, and cell expansion). In contrast to the multitude of physiological investigations into the basis of growth rate differences, genetic studies are scarce.

Arabidopsis, the model plant for genetic research, has been collected from a wide range of habitats distributed primarily over most of the northern hemisphere. Genetic differences between local populations (commonly called ecotypes, despite that this term does not conform strictly to its ecological definition [Pigliucci, 1998]) are presumably associated with adaptation to the prevailing environmental conditions. Numerous ecotypes were collected by the pioneers of Arabidopsis research and have since been used to investigate a wide range of physiological processes, through comparisons between ecotypes and by genetic mapping using recombinant inbred lines (Alonso-Blanco and Koornneef, 2000). With some notable exceptions (Li et al., 1998), little is published about differences in the rate at which Arabidopsis ecotypes grow when compared under standardized laboratory conditions.

There are two complementary views on how root growth is regulated (Silk, 1984). The spatial model describes at what rate division and expansion occur as a function of position along the root axis. Root elongation rate in this model is determined by the size of the growth zone and local rates of expansion. According to this view, cell division merely subdivides cellular space provided by the expansion process. In an alternate manner, a cellular model can be adopted whereby cell production in the meristem drives growth by producing the cells that will subsequently expand to reach a given mature size (Fiorni et al., 2000). Data obtained from analyses of ontogenetic acceleration of root elongation rate (Beemster and Baskin, 1998), inhibition of root elongation in response to the stp1 mutation, and to externally applied hormones (Beemster and Baskin, 2000) are easiest explained in terms of a cellular model, implying a crucial role for cell division in determining organ growth rates. Contradictory to this proposition are observations from γ-radiated seedlings and transgenic lines overexpressing cell cycle genes that show that cell division and organ growth can be partly uncoupled (Haber, 1962; Hemerly et al., 1995; De Veylder et al., 2001a). However, evidence from...
transgenic plants overexpressing other cell cycle

genes conclusively shows that modulated cell divi-
sion directly affects organ growth rates (Doerner et

al., 1996; Cockcroft et al., 2000; De Veylder et al.,

2001b). Based on detailed analyses of such transgenic

lines, as well as wild-type plants responding to en-
vironmental stimuli, we are now starting to unravel
the mechanism by which cell division activity in
higher plants organs is controlled and how this is
related to growth.

To study the regulation of plant growth rate in
Arabidopsis, we adapted the existing kinematic
framework for quantifying cell division and expan-
sion in the growth zone of the primary root tip
(Beemster and Baskin, 1998). Root tips and interca-
lary meristems of monocotyledonous leaves grow
essentially linearly, and growth occurs in a well-
defined region. Therefore, they are ideal model sys-
tems for investigating the relationship between cell
division and growth rate. The growth of these organs
can be analyzed in terms of a single representative

 cell file. In such a file, cell production resulting from

co-occurring division and expansion is restricted to
the apical region bordering the quiescent center (root
tips; Fig. 1) and the basal region bordering the junc-
tion with the stem (monocotyledonous leaves). In
these meristems, the production of new cells causes a
flux of cells that increases with distance from the
quiescent center and leaf base (Beemster and Baskin,
1998). At the end of the meristem, cells stop dividing,
but continue to elongate, often at higher rates than in
the meristem (Beemster and Baskin, 1998). Cells will
reach the end of the growth zone, where cell expa-
sion also stops and they have reached their final size.

In such a linear system, adopting the cellular view,

the steady-state rate at which an organ grows (E) is
determined by the final length cells reach when ex-
iting the growth zone (l\text{mat}; primarily determined in

the elongation zone) and the number of cells produced
in the meristem per unit of time (P) according to:

\[ E = l\text{mat} \times P \] (Eq. 1)

The cell production rate (P), in turn, is a function of
the number of cells in the meristem (N\text{div}) and their
average cell cycle duration (T\text{c}):

\[ P = N\text{div} \times \frac{\ln(2)}{T\text{c}} \] (Eq. 2; Ivanov, 1994)

Thus, we can decompose root elongation rate in three
functional parameters, l\text{mat}, N\text{div}, and T\text{c}. What could
be the mechanisms that regulate these parameters?
Mature cell size has frequently been correlated with

DNA content in Arabidopsis (Melaragno et al.,
1993; Kondorosi et al., 2000) and other plants as well
as in animal species (Cavalier-Smith, 1978). The DNA
content of individual cells can be increased above the
base 2C (G1) and 4C (G2) levels by the process of
endoreduplication, a modified cell cycle where cells
go through S-phase but then bypass mitosis to go
directly into G1. We hypothesize that cell cycle reg-
ulation controls the degree of endoreduplication,
which, in turn, determines mature cell size. As ma-
ture cell size partly determines organ growth rate
(Eq. 1), this would be a first link between cell cycle
regulation at the molecular level and root elongation
rates. A second link was recently demonstrated in

Zea mays leaves that respond to changes in
water deficit and temperature. The growth of such
leaves was closely correlated with cell division ac-

tivity. It is interesting that local rates of cell division
were linearly related to the activity of the cyclin-
dependent kinase (CDKA) (Granier et al., 2000).
This leads to the model where CDKA controls cell pro-
duction rates and, thereby, the second component
that determines growth rate. The aim of this article is
to explore these putative roles of cell cycle regulation
in mediating genetically determined growth rate dif-
ferences. We report that variation in mature cortical

cell length, average cell cycle duration, and number of
dividing cells all contribute to differences in the
elongation rate of the primary root between 18
ecotypes of the model plant Arabidopsis. Moreover,
we demonstrate the absence of a correlation between
endoreduplication and mature cell length, while con-
firming the relationship between CDKA and cell
production.

RESULTS

Variation in Root Elongation Rate

For our analysis we selected 18 ecotypes of Ar-

bidopsis, comprising the most commonly used labora-
tory strains and some that originated from contrast-

ing growth habitats (Table I). Under our growth
conditions (see “Materials and Methods”), we readily
observed large differences in whole-plant growth
rates between these lines (data not shown). These differences were reflected by the rate of growth of their primary root as determined by measurements of the daily length increments (Table II). In contrast to the situation on Hoagland medium (Beemster and A. von Raden, 1998), the primary root of most ecotypes grew at a constant rate on 1:1:1:1:1 (Table II). Although part of the difference was associated with the ability of the fastest lines to accelerate, there was also a nearly 3-fold difference in growth rate between steady-state growing ecotypes (71 \mu m h^{-1} [Ler] to 201 \mu m h^{-1} [Wt]; Table II).

**Relationships between Root Elongation Rate, Cell Production, and Mature Cell Length**

To investigate the relationship between cell cycle regulation and plant growth rates, we first determined the respective contributions of mature cell size...
and cell production, focusing on the cortical cell files. Under steady-state growth, root elongation rate is the product of cell production and mature cell size (see Eq. 1). Under conditions where the root accelerates, a small fraction of the cell production is “invested” in the expansion of the meristem (Beemster and Baskin, 1998). As mentioned, this was the case for the three fastest growing ecotypes, Ws, Est, and Tsu. To correct for this phenomenon, we calculated the rate of change of the number of cells in the region that represents the meristem on d 9. This factor was calculated from the cell length data from d 7 and 11 and was included in the total cell production (for a detailed description of this procedure, see Beemster and Baskin, 2000). From the thus obtained cell production rate and the number of dividing cells, the cell cycle duration, $T_c$, was calculated (Eq. 2).

To investigate the correlation between growth parameters, the data were log transformed and used for multiple linear regression analysis (see “Materials and Methods”). The data showed that the regression model containing cell production rate and mature cell length accounted for 99% of the variance in root elongation rate (Fig. 2A; $R^2$ value in Table III). Both parameters contributed, given that both partial regression coefficients were significantly different from 0 ($C_1$ and $C_2$ in Table III). The data for the three non-steady (and fastest) growing ecotypes slightly deviated from the linear relationship (Fig. 2A). This is due to the extra cells produced for the expansion of the meristem, and causes the $R^2$ to be 0.99 instead of 1.00. When analyzed separately, the model with cell production (ranging from 0.63 [Nw] to 1.83 cells h$^{-1}$ [Ws]; Table II) accounted for 47% of the variance in root elongation rate, whereas the model with mature cortical cell length (ranging from 84 [Lcr] to 237 $\mu$m [Ws-3]; Table II) could explain 62% of variation in growth rate ($R^2$ in Table III). For both explanatory variables, the correlation with root elongation rate was highly significant (Fig. 2, B and C; P values in Table III). Hence, variation in mature cell size accounted for a little over one-half and, consequently, cell production for a little less than one-half of the variation in root elongation rate. There was no significant correlation between cell production and mature cell size, implying that cell growth in the elongation zone is independent from cell production in the meristem.

Relationship between Cell Production, Number of Dividing Cells, and Cell Cycle Duration

The number of dividing cells in each file and average cell cycle duration determine cell production (Eq. 2). After linearizing this relationship by log transformation (Eq. 4), it was possible to further analyze the role of these two cell division parameters in cell production by means of multiple linear regression analysis. Given that the number of dividing cells and the average cell cycle duration are the only two parameters that determine overall cell production, the regression model including them both accounted for all variation in cell production (Fig. 3A; $R^2$ of 1.00 in Table IV). The model with the number of dividing cells ($N_{div}$, ranging from 32 [Wei] to 61 [Ws]; Table II) individually accounted for 37% of the variance in cell production (Table IV). The model with average cell cycle duration ($T_{c'}$ ranging from 18.6 h [Tsu] to 47.7 h...
Table III. Multiple linear regression analysis of the relationship between root elongation rate (E) and cell production (P) and mature cell length (l_{mat})

<table>
<thead>
<tr>
<th>Model</th>
<th>R^2</th>
<th>C_0^a</th>
<th>C_1^a</th>
<th>C_2^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log (E) = C_0 + C_1 log (P) + C_2 log (l_{mat})</td>
<td>0.99</td>
<td>0.12</td>
<td>0.85</td>
<td>0.94</td>
</tr>
<tr>
<td>Log (E) = C_0 + C_1 log (P)</td>
<td>0.47</td>
<td>2.18</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Log (E) = C_0 + C_1 log (l_{mat})</td>
<td>0.62</td>
<td>0.0</td>
<td>–</td>
<td>1.0</td>
</tr>
</tbody>
</table>

^a Significance: * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001; not significant (N.S.).

[Table II] explained 66% of the variation in cell production (Table IV). The correlation was highly significant for both models (Fig. 3, B and C; P values in Table IV), implying that both parameters play a role in determining differences in cell production and, thus, in root elongation rates. There was no correlation between average cell cycle duration and the number of dividing cells, implying that these two cell production parameters are independently regulated (data not shown).

Relationship between Mature Cell Size and DNA Content

To test if the observed variation in mature cortical cell size was correlated with nDNA content, we performed flow cytometry on the roots of all ecotypes. For this analysis, nuclei were isolated from whole root systems, which predominantly consist of mature tissue. Flow diagrams typically contain peaks corresponding to 2C, 4C, 8C, and, in some cases, 16C DNA content, whereby 2C DNA content corresponds to cells in G1. The 4C population encompasses “normal” cells in G2 and cells that have gone through a single round of endoreduplication and are in G1. Therefore, only the 8C and 16C populations contain cells that must have all undergone endoreduplication. The fraction of cells in these two populations varied from only 1% [Nd] to nearly 40% [Nw]; Fig. 4), indicating a considerable variation in the degree of endoreduplication. However, in contrast to the hypothesis, we did not find a correlation between nDNA content and mature cortical cell length (Fig. 4A), indicating that these parameters were independent.

Relationships between CDKA, Cell Division, and Endoreduplication

The current model for the molecular regulation of cell division involves the regulation of CDKA (Mironov et al., 1999). In corn leaves, the rate of cell division was tightly correlated with the spatial distribution of CDKA histone H1 kinase activity (Granier et al., 2000). The Arabidopsis root apical meristem is too small (500 and 700 μm for Col and C24, respectively; Beemster and Baskin, 1998; De Veylder et al., 2001b) to enable sampling of subsec-

DISCUSSION

Our results highlight the large inherent variation in growth between Arabidopsis ecotypes. As a first step resolving the relationship between cell cycle regulation and growth, we investigated the cellular basis of the observed growth rate differences, using the primary root as a model system. The data presented show that the differences are not attributable to a single growth parameter. Rather, variation in cell production and mature cell length contribute roughly equally to the total variation of root elongation rate. The rate of cell production, in turn, was primarily correlated with the average cell cycle duration, although the number of dividing cells also
contributed. It is significant that we could not discover any correlation between variations in mature cell length, cell cycle duration, and the number of dividing cells, which suggests that they are all independent. For several reasons, correlation between cell production in the meristem and cell expansion in the elongation zone could be expected. First, the two processes might compete for limited resources. Second, under the spatial hypothesis, the process of cell expansion is independent upon cell division (Fiorani et al., 2000). Therefore, increasing cell division activity would not affect overall growth, but instead lead to smaller cells being produced. It could have been that differences in "general vigor" genes would affect both processes in a similar way. However, the observed independence implies that under the conditions used the two processes do not seem to compete for limited resources. Given that we analyzed genetic differences, these results also mean that separate genes probably regulate individual growth parameters. This result agrees with the observations that individual growth parameters are differentially affected by auxin, cytokinin, and the stp1 mutation (Beemster and Baskin, 2000) and by overexpression of the cell cycle gene CKS1 (De Veylder et al., 2001b). Independence of cell production and mature cell length shows that high cell production does not result in a smaller cell size. This would be predicted from a spatial model where growth results from expansion and cell division merely subdivides existing cellular compartments (Green, 1976). Therefore, the cellular model that includes cell division as a driving process is consistent with our observations. This validates the use of Equations 1 and 2 as a basis for the kinematic analysis performed here.

### Kinematic Methodology

To enable multiple linear regression analysis for estimation of the relative contribution of various growth parameters to variation in root elongation rate between ecotypes, a sufficient number of lines needed to be analyzed. We arbitrarily chose 18, which proved to be sufficient. It is unfortunate that the kinematic method developed earlier for Arabidopsis roots is rather laborious, as it entails time-lapse and cell length measurements on each replicate root (Beemster and Baskin, 1998). The amount of time required for this analysis probably explains why only a limited number of treatments have been analyzed to date. Automation may ultimately help to resolve this bottleneck. However, to reduce the amount of work involved with the manual analysis, we decided to omit the time-lapse analysis and base the calculations on cell length profile and overall root elongation rate only, analogous to Baskin et al. (1995). Crucial for this approach is the determination of the basal margin of the meristem. We defined it as the position where cortical cells reach a length of 40 μm, a value representative for earlier experiments (Beemster and Baskin, 1998, 2000; De Veylder et al., 2001b). How sensitive are the results for fallacies of this assumption? Published data for the Col ecotype (Beemster and Baskin, 1998) show that the cell length of 40 μm occurs in a region where cell length changes rapidly over a relatively small distance. Based on
these data, we can estimate the consequences of over-
and underestimation of the length at which cells
leave the meristem. If cortical cells stop dividing at a
length of 20 μm, one-half of what we expected, the
meristem would have been about 100 μm shorter,
resulting in an underestimation of the number of
meristematic cells by 6% (49 rather than 52). In a
converse manner, when cells were to leave the mer-
istem at a length of 80 μm, or double the expected
value, the meristem would have been roughly 150
μm longer but contain only 4% more cells (54 cells).
As average cell cycle duration was derived from
overall cell production, which is not affected by the
choice of the meristem boundary, and the number of
meristematic cells, the errors involved in this param-
eter are similarly small. Therefore, an important
aspect to this paper is that it presents a less laborious
alternative to the full-blown kinematic analysis,
which is relatively accurate, particularly with respect
to the most important cell division parameters. How-
ever, there are some trade-offs. It is obvious that by
using a predefined cell length for estimating the basal
margin of the meristem, the actual size of cells exiting
the meristem cannot be determined, nor is estimation
of the physical size of the meristem very accurate (see
above). As the total number of cells in the growth
zone as a whole is independently estimated, the
choice of the margin of the meristem partitions the
same cells between meristem and elongation zone.

Relative to the number of cells in the meristem, the
number of cells in the elongation zone (Nel) is rather
small. Therefore, the same absolute difference in cell
number will create larger relative errors. In the above
element, overestimating the size of the meristem by
three cells (cells are 20 μm rather than 40 μm) when
they exit the meristem will result in underestimation
of the number of rapidly elongating cells by 16% (16
instead of 19). In a similar manner, underestimating
the number of meristematic cells by two (if cells
leaving the meristem are 80 μm) will cause overesti-
mation of the number of rapidly elongating cells by
14% (16 instead of 14). Residence time in the elonga-
tion zone can be calculated the ratio between Nel and
P (Beemster and Baskin, 1998). Because P is indepen-
dent of the size of the meristem, the accuracy of the
calculated residence time is affected to the same ex-
tent as Nel. In conclusion, this approach is relatively
accurate in terms of meristem parameters, whereas
the precision of elongation zone parameters is
compromised.

Molecular Basis of Differences in Cell Production

As a second step toward linking cell cycle regu-
lation to organ growth rates, we tested if the correla-
tion between cell division and kinase activity in the
response to environmental conditions observed in
corn leaves (Granier et al., 2000) could also be vali-
dated by genetic variations and in Arabidopsis roots.
The data suggest that this is the case. In fact, we
recently found that even in transgenic lines that are
inhibited in cell production as a consequence of the
overexpression of the cell cycle gene CKS1At (De
Veylder et al., 2001b), kinase activity and cell produc-
tion are reduced simultaneously (G.T.S. Beemster

### Table IV. Multiple linear regression analysis of the relationship between cell production rate (P) and
number of dividing cells (N div) and average cell cycle (T c)

<table>
<thead>
<tr>
<th>Model</th>
<th>R²</th>
<th>C₀a</th>
<th>C₁a</th>
<th>C₂a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log (P) = C₀ + C₁ log (N div) + C₂ log (T c)</td>
<td>1.00</td>
<td>-0.19***</td>
<td>1.02***</td>
<td>-0.99***</td>
</tr>
<tr>
<td>Log (P) = C₀ + C₁ log (N div)</td>
<td>0.37</td>
<td>-1.8**</td>
<td>1.1**</td>
<td>-</td>
</tr>
<tr>
<td>Log (P) = C₀ + C₁ log (T c)</td>
<td>0.66</td>
<td>1.5***</td>
<td>-</td>
<td>-1.0***</td>
</tr>
</tbody>
</table>

a Significance: * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001; not significant (N.S.).

Figure 4. The absence of a relationship between endoreduplication and mature cortical cell size (A) and CDKA (B) in the roots of 18 Arabidopsis ecotypes. The fraction of endoreduplicated cells was estimated from 10 complete root systems from which the nuclei were isolated and analyzed by flow cytometry. Mature cortical cell length was determined as the average over all positions in the mature part of the root (see “Materials and Methods”).
pressed in C24 background (Hemerly et al., 1995). In these plants, kinase activity in the root tips was, in contrast to what was seen at the whole-plant level, not higher than wild type (G.T.S. Beemster and L. De Veylder, unpublished data). It may well be that when the same construct is overexpressed in an ecotype with intrinsically low and rate-limiting CDKA expression levels, it will result in increased kinase activity and cell division. Nevertheless, additional mechanisms are likely to operate in plants with relatively high CDK levels. It can be presumed that many of the CDK interacting proteins could fulfill this role. It has been demonstrated that overexpression of B- and D-type cyclins can enhance plant growth rates, presumably by increasing cell production in the meristems (Doerner, 1994; Cockcroft et al., 2000). In a similar manner, overexpression of the CDK inhibitor KRP2 was shown to inhibit kinase activity and cell division rates in Arabidopsis leaves (De Veylder et al., 2001a).

Endoreduplication and Mature Cell Size

Our data shows that between the 18 ecotypes analyzed, there was large variation in mature cortical cell size. We investigated the hypothesis that differences in cell size would be associated nDNA content, determined by the number of endoreduplication cycles each cell undergoes. However, we found no correlation between the fraction of cells that had undergone endoreduplication and mature cortical cell length. This is in contrast with other observations in Arabidopsis on leaf epidermal cells (Melaragno et al., 1993), hypocotyls (Gendreau et al., 1997), and trichomes. In addition, we recently reported that overexpression of the cell cycle gene KRP2 in Arabidopsis results in a large increase in the size of most cell types, whereas nDNA content in the same leaves was reduced (De Veylder et al., 2001a). Together, these data refute the conception that endoreduplication would drive or is even required for obtaining a large cell size. Rather, it seems that endoreduplication and mature cell size are genetically independent phenomena in these ecotypes. As for the regulation of the endoreduplication process itself, our data showed no correlation between CDKA kinase activity and endoreduplication levels (Fig. 4B). This could be explained in two ways. First, the kinase activity signal associated with endoreduplication is independent of and obscured by the much higher activity associated with cell production. Second, it is conceivable that endoreduplication is regulated by other CDKs such as CDKB (Yoshizumi et al., 1999) or depends upon the relative activity of a combination of different CDK types. Further research targeted directly at the regulation of this process should deliver more insight as to the validity of each of these explanations. With regard to the molecular mechanism that determines mature cell size, the processes asso-

and L. De Veylder, unpublished data). Therefore, we conclude that evidence is mounting to support a pivotal regulatory role for kinase activity in plant growth regulation, in response to the environment (Granier et al., 2000) and genetic predisposition (this paper). Important in this context is how CDKA is regulated. Our data show a significant correlation between CDKA protein levels and kinase activity, implicating differences in transcription or protein stability. However, there was also considerable variation in CDKA levels that resulted in similar kinase activities (Fig. 5B), suggesting that CDKA levels could be limiting in some, but not all ecotypes. The latter situation would be analogous to that of tobacco (Nicotiana tabacum) Bright Yellow 2 suspension cultures, where most of the CDKA protein was found to be in its inactive, monomeric form (Porceddu et al., 2001). This could well explain the absence of a growth stimulatory effect when CDKA was overex-

Figure 5. Cell production rates are correlated with kinase activity and CDKA levels in the root tip of 11 Arabidopsis ecotypes. A, Relationship between kinase activity (A) and cell production (P); regression, $P = -0.47 + 0.7 \times A$; $R^2 = 0.56, P < 0.001$, B, Relationship between CDKA levels (C) and kinase activity (regression, $C = 0.02 + 0.19 \times A$; $R^2 = 0.32, P = 0.09$). Kinase activities were calculated relative to the average of all samples from each individual experiment and then averaged between two replicate experiments. Error bars denote s.e. ($n = 2$). CDKA levels were determined by measuring the integrated intensity on western blots. The regressions were obtained from multiple linear regression analysis.

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associated with cell wall formation and expansion are obvious candidates. Most progress in this field has been made with regards to expansins. They have first been shown to be correlated with cell expansion (Cosgrove and Li, 1993), and more recently, it was also demonstrated that their overexpression alters plant growth, a change that is correlated with differences in mature cell size (Cho and Cosgrove, 2000). Therefore, it would be interesting to investigate to what extent differences in mature cell length observed here are associated with the activity and expression levels of these proteins.

Prospects

With the availability of the complete genome sequence of Arabidopsis (The Arabidopsis Initiative, 2000) and several mapping populations (Alonso-Blanco and Koornneef, 2000), it seems feasible to map and clone the genes that are responsible for the observed variations in growth parameters. One interesting aspect from such an approach is that from all the genes that potentially affect plant growth rate in some way, it would identify the ones that actually do so in natural conditions. However, the fact that such genes result in variation in cell cycle or cell expansion should not be taken as evidence that they are directly involved in these processes. It is very well possible that they involve crucial upstream events that, in turn, impact on particular growth processes. Finding the genes and functions that underlie variation in growth characteristics is going to be an exciting challenge for future research. Such work holds great promise for improvements of growth characteristics of agronomically important crop species.

MATERIALS AND METHODS

Root Growth and Microscopy

Seeds of each of 18 ecotypes (see Table I) were sterilized in 12.5% sodium hypochloride for 10 min, and eight were subsequently plated approximately 1 cm from the edge of each of 10 replicate square (9 × 9 cm) petri dishes (Greiner Biochemicals, Flacht, Germany), containing 1× Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands), 1 g L⁻¹ Suc (Merck Eurolab, Darmstadt, UK), and 0.8 g L⁻¹ plant tissue culture agar (Lab M, Bury, UK). After sowing, the plates were stored in a refrigerator at 4 °C for 3 d, and were subsequently placed near vertically in a growth chamber set at 22°C and with constant light (PAR = 80 µE m⁻² s⁻¹) supplied by a bank of cool-white fluorescent tubes. Directly after germination, the position of the root tip was marked daily on the back of the dishes with a razorblade. At d 7, 9, and 11, 10 roots were harvested from two representative plates. These roots were mounted on a microscope slide in the same culture medium without the agar. Two strips of scotch tape were used as spacers to reduce pressure from the coverslip. Cortical cells were visualized using differential interference contrast optics of a microscope (DMLB; Leica, Wetzlar, Germany) fitted with a 20× lens (HL PL Fluotar; Leica, n.a. = 0.50), and an imaging system encompassing a CCD camera (4910 CCIR; Cohu, San Diego) and frame grabber board (LG3 CCIR; Scion, Frederick, MD) fitted in a PentiumII PC running the image analysis program ScionImage (WinNT version b3b; Scion). Using this imaging software, a series of overlapping images spanning 2 to 3 mm of the root apex, covering the entire growth zone and well into the mature region were obtained. The remaining plates were digitized at a resolution of 5.9 pixels mm⁻¹ using a flatbed scanner (Scanjet 4C/T; Hewlett-Packard, Palo Alto, CA). On the latter images, the growth rate of individual roots was determined from daily length increments, i.e. its length between subsequent marks, divided by the corresponding time interval. These rates were subsequently averaged for all observed roots (E).

Kinematic Analysis

In case root growth was steady state, the cell length profile of only d 9 roots was determined. In case root growth accelerated over time, this was also done for d 7 and 11. For this, a composite image was created by pasting overlapping sections together using the imaging program Photoshop (version 5.0; Adobe Systems, Mountain View, CA). On these composite images the length of all cortical cells was measured per file and was expressed as a function of their position relative to the base of the quiescent center. The data of all files of a root were combined and interpolated into 25-µm spaced points using a kernel smoothing routine described earlier (Beemster and Baskin, 1998) implemented as a macro in Excel (version 97; Microsoft, Redmond, WA). From these data, the number of meristematic cells (Ndiv) as well as the mature cell length (lmat) was estimated for each root individually. To this end, the base of the meristem was defined as the position where the cells reach a length of 40 µm, a value typically observed in earlier experiments (Beemster and Baskin, 1998, 2000; De Veylder et al., 2001b). Average cell division rates and cell cycle duration were calculated using the equations described earlier (Beemster and Baskin, 2000).

Multiple Linear Regression Analysis

The relationships between growth parameters were investigated by multiple linear regression analysis. For this, we log transformed the data, effectively changing Equations 1 and 2 into Equations 3 and 4, respectively:

\[ \log(E) = C_0 + C_1 \log(l_{mat}) + C_2 \log(P) \]  
(Eq. 3)

\[ \log(P) = C_0 + C_1 \log(N_{div}) + C_2 \log(T_c) \]  
(Eq. 4)

The regression analysis itself was performed using the add-in analysis toolpack of Excel (Microsoft).

Flow Cytometry

To determine DNA content of root cells, approximately 25 complete root systems of 9-d-old seedlings were har-
vested after the plates were scanned for evaluation of root elongation rate (see “Root Growth and Microscopy”) and prepared by chopping the roots in 300 μL of Galbraith buffer (Galbraith et al., 1991) using a razor blade. The resulting solution was filtered using a 30-μm mesh after which 1 μL of a 1 mg mL⁻¹ stock of 4',6-diamidino-2-phenylindole was added. The DNA content distribution was analyzed with the aid of a flow cytometer and accompanying software (Bryte HS and WinBryte, respectively; Bio-Rad, Hercules, CA).

CDKA and Concentration Assays

Histone H1 kinase activity was analyzed in two separate experiments on samples of 5-mm tips from approximately 250 roots grown on 10 plates as described above. These were cut, immediately frozen, and stored in liquid nitrogen-cooled 2-mL Eppendorf tubes. Protein extracts were prepared by grinding the frozen tissue in a cooled ball mill (mm20; Retsch, Haan, Germany), after which the protein fraction was isolated by centrifuging in a homogenization buffer (50 mM Tris-HCL, pH 7.2, 60 mM β-glycerophosphate, 15 mM nitrophenylphosphate, 15 mM EGTA, 15 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM vanadate, 50 mM NaF, 20 μg ml⁻¹ soybean trypsin inhibitor, 100 μM benzamidine, 1 mM phenylmethylsulfonylfluoride, and 0.1% Triton X-100). Total protein concentration in the extracts was determined with a protein assay kit (Bio-Rad, Hercules, CA). CDKA complexes were purified from total plant extracts by affinity binding to p9ckS⁻Sepharose beads, which specifically bind A-type CDKs (Stals et al., 2000). Otherwise, the protocol described earlier was followed (Hemerly et al., 1995). To ascertain fidelity of the kinase data, we repeated the kinase assay twice in independent experiments.

CDKA protein levels were determined by western-blot analysis. For this, total protein extract was denatured for 10 min at 95°C in SDS loading buffer, separated on a 12.5% SDS-PAGE gel, and blotted onto a nitrocellulose membrane (Hybond-C⁺; Amersham Pharmacia Biotech, Piscataway, NJ). Filters were blocked overnight with 2% milk powder in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), washed five times with PBST, and probed for 2 h with anti-CDKA;1 (diluted 1/5,000 in blocking solution). The blots were rinsed five times with PBST and were incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated antibodies (Amersham Pharmacia Biotech) diluted in blocking solution. The membranes were washed five times with PBST, and signals were developed using a chemiluminescent detection kit (PerkinElmer Life Sciences, Boston). The integrated intensity of the bands was measured correcting for background intensities by image analysis using the image analysis program ScionImage.

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LITERATURE CITED


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