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Gene dosage effect of WEE1 on growth and morphogenesis from arabidopsis hypocotyl explants

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• *Background and Aims* How plant cell-cycle genes interface with development is unclear. Preliminary evidence from our laboratory suggested that over-expression of the cell cycle checkpoint gene, *WEE1*, repressed growth and development. Here the hypothesis is tested that the level of WEE1 has a dosage effect on growth and development in *Arabidospis thaliana*. To do this, a comparison was made of the development of gain- and loss-of-function *WEE1* arabidopsis lines both *in vivo* and *in vitro*.
• *Methods* Hypocotyl explants from an over-expressing *Arath;WEE1* line (*WEE1*^{oe}), two T-DNA insertion lines (*wee1-1* and *wee1-4*) and wild type (WT) were cultured on two-way combinations of kinetin and naphthyl acetic acid. Root growth and meristematic cell size were also examined.
• *Key Results* Quantitative data indicated a repressive effect in *WEE1*^{oe} and a significant increase in morphogenetic capacity in the two T-DNA insertion lines compared with WT. Compared with WT, *WEE1*^{oe} seedlings exhibited a slower cell-doubling time in the root apical meristem and a shortened primary root, with fewer laterals, whereas there were no consistent differences in the insertion lines compared with WT. However, significantly fewer adventitious roots were recorded for *WEE1*^{oe} and significantly more for the insertion mutant *wee1-1*. Compared with WT there was a significant increase in meristem cell size in *WEE1*^{oe} for all three ground tissues but for *wee1-1* only cortical cell size was reduced.
• *Conclusions* There is a gene dosage effect of *WEE1* on morphogenesis from hypocotyls both *in vitro* and *in vivo*.

Key words: *Arabidopsis thaliana*, cell cycle, development, growth, hypocotyl, tissue culture, *WEE1*.

INTRODUCTION

The cell cycle is regulated by protein kinase complexes which, in their minimal configuration, consist of a Ser/Thr cyclin dependent kinase (CDK), and a regulatory cyclin subunit (Norbury and Nurse, 1992). CDK activity is also subject to negative regulation imposed by specific inhibitory kinases. In fission yeast, at the G_2/M transition, MIK1/WEE1 kinases act as inhibitors by phosphorylating Tyr15 of the CDK and, in animals, there is a further involvement of the Thr14/Tyr15 MYT1 kinase (Russell and Nurse, 1987; Mueller *et al.*, 1995). Neither MIK1 nor MYT1 features in the arabidopsis genome but WEE1 kinase has been cloned in a range of higher plants (reviewed by Shimotohno and Umeda, 2007).

Expression of arabidopsis *WEE1* (*Arath;WEE1*) is highest in proliferative regions of the plant (Sorrell *et al.*, 2002), including young roots (Rhee *et al.*, 2003) (Supplementary Data Fig. S1). However, the expression of a *WEE1*p::*GUS* construct in root tips was only detected occasionally as a faint signal under normal growth conditions (De Schutter *et al.*, 2007). Moreover, a T-DNA insertion mutant developed normally, raising doubts about *WEE1*'s role in normal cell cycles. Possible functional redundancy of negative G_2/M regulators could explain the apparently normal development of the T-DNA insertion line. However, the expression of the tomato *WEE1* homologue, *Solly;WEE1* in BY-2 cells delayed entry of cells into mitosis (Gonzalez *et al.*, 2007) and over-expression of *Arath;WEE1* driven by a strong activatable promoter resulted in arrest of root growth and a block on cells at G_2 (De Schutter *et al.*, 2007).

Unpublished observations in our laboratory indicated that over-expression of *Arath;WEE1* repressed growth and development of arabidopsis seedlings and that over-expression of tobacco *WEE1* delayed entry into mitosis in synchronized BY-2 cells. Given these negative effects of plant *WEE1* on growth and development we decided to test the genetic basis of these responses by expanding the analysis to include two T-DNA insertion lines for *WEE1*. In particular, we analysed

the gene dosage effects of *WEE1*, in the series *WEE1* over-expressors, wild type (WT) and *wee1* insertion mutants, testing the morphogenetic competence of arabidopsis hypocotyls *in vitro* and seedling growth and development *in vivo*. We used the Inoue *et al.* (2001) grid system that tests the responses of explanted hypocotyls to two-way auxin and cytokinin gradients. Qualitative data are consistent in showing excess *WEE1* represses growth and morphogenesis from hypocotyls *in vitro*. Quantitative data at specific kinetin/naphthyl acetic acid (NAA) combinations, confirmed *WEE1*'s repressive effects which were reversed in the T-DNA insertion lines *in vitro*. Further morphogenetic data *in vivo* showed a similar trend in adventitious-root formation.

MATERIALS AND METHODS

Arabidopsis lines

Arath;WEE1 was expressed in the BIN HYG TX vector (Gatz *et al.*, 1992) under an attenuated form of the 35S promoter and transformed into *Arabidospis thaliana* ecotype Columbia using the floral dip method (Clough and Bent, 1998) and selected on hygromycin. The full open reading frame of *Arath;WEE1* was PCR amplified from seedling cDNA using primers FW 5'-AGGCCCGGGCTCGAGATGTTCGAGAAGAACGG-3' and RV5'-GCACACTAGTCGACTCAACCTCGAATCCTAT-3', that included *Sma*I and *Sal*I sites respectively. The PCR product was cloned and fully sequenced to ensure errors had not been introduced by the PCR. Primers for measuring expression of *Arath;Act2* were as described in Sorrell *et al.* (2002). Over-expression of the *Arath;WEE1* was checked by semi-quantitative RT-PCR (as described below) using primers Atwee1F 5'-AGCTTGTCAGCTTTGCCT-3' and Atwee1R 5'-CGTGCATCCCTCCTTCTTCTACT-3'. The number of PCR cycles for the target gene was optimized with the specific gene primer pairs and each cDNA batch. Dilutions of the cDNA were run with each experiment to ensure that the PCR reaction was in the linear exponential phase and that product fluorescence [as measured by ethidium bromide fluorescence using a Gene Genius Bioimaging System (Syngene Ltd)] was linear. Relative cDNA amounts were normalized using 18S rRNA target primers as described previously (Price *et al.*, 2008), again optimizing cycle number for each cDNA batch. Two lines over-expressing *Arath;WEE1* (*WEE1*^{oe} lines #58 and #61) which showed strong expression of the transgene by RT-PCR were selected for further experiments. RT-PCR to demonstrate the expression of Arath;WEE1 in root tips used primers as described in Sorrell *et al.* (2002). One primer from each pair spanned an intron junction and thus avoided the risk of amplification from residual contaminating genomic DNA.

Homozygous T-DNA insertion lines for *Arath;WEE1* (*wee1-1* and *wee1-4*) were obtained from the GABI-Kat collection of T-DNA insertion lines (Rosso *et al.*, 2003) (GABI_270E05;GABI_006C10).

Culture of hypocotyls on grids: qualitative assessment

Arabidopsis seedlings were grown aseptically on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in a Sanyo–Gallenkamp arabidopsis chamber with 16 h light (fluence rate = 300 μmol m^{-2} s^{-1}) and 8 h dark at 21 °C. Five-millimetre-long segments from the middle of hypocotyls were excised from 14-d-old seedlings and cultured using a two-way grid system. Hypocotyls were cultured on a modified MS medium: 4·3 g l^{-1} MS salts (Duchefa Biochemie, Melford Laboratories) with Gamborg's vitamins (Sigma-Aldrich), 2 % sucrose and 0·8 % agar (DifcoTM) adjusted to pH 5·7 (Inoue *et al.*, 2001). Sterilin® plates (5 × 5 squares) enabled a two-way increasing concentration range of cytokinin (kinetin, *x*-axis) and auxin (NAA, *y*-axis] to be established. The exogenous concentration of the plant growth regulators ranged from 25 ng mL^{-1} to 300 ng mL^{-1}. The grids were cultured as above and analysed 30 d later. Three replicate experiments were performed. Hypocotyl explants were scored for growth of callus and presence or absence of shoots and/or roots using a dissecting microscope (Nikon Z-100).

Culture of hypocotyls in Petri dishes: quantitative assessments

Replicate hypocotyls of each line ($n = 25$) were excised aseptically and cultured on Petri dishes containing MS medium modified as above and supplemented with selected concentrations of NAA and kinetin as above. After 30 d, cultures were scored as above and the area of the callus was determined by image analysis (SigmaScanPro®).

Analysis of root and root apical meristem (RAM) phenotypes

Seeds were sown aseptically 1·5 cm apart on 200-mm square Petri dishes containing MS medium and stratified for 48 h (5 °C). Seedlings were grown vertically as described above. Primary root length was measured daily for 16 d. A regression analysis was applied to each sub-set of data using Minitab version15. Seedlings were also fixed in 3 : 1 absolute ethanol : glacial acetic acid and Feulgen stained (Armstrong and Francis, 1985). Using a stereo dissecting microscope (Nikon Z100), primary root length, the number of lateral root primordia and number of lateral roots were recorded. Additionally, hypocotyls were excised from the main root system and the number of adventitious roots forming on them was scored similarly.

Seventeen-day-old seedlings were used for the RAM analyses. Roots were fixed and mounted on slides in 8 : 3 : 1 chloral hydrate : distilled water : glycerol taking care to gently apply a coverslip (Perilli and Sabatini, 2010). Cell length, breadth and number were measured in three tissues of the RAM – epidermis, cortex and stele – using a ZeissAxiophot set for DIC (differential interference contrast) interfaced to image analysis software [PixiLINK (C) Capture S.E.]. Measurements were taken along longitudinal files of cells of epidermis, cortex and mid-stele until the parameter spanned a cell that suddenly increased its cell length/width substantially compared with the previous one. For all genotypes, and for all tissues, this was between a 1·40- and a 1·95-fold increase. This was the transition point beyond which those cells began to elongate substantially and is taken to be the basipetal border of the promeristem for each tissue.

Cell-doubling times (CDTs)

Primary root tips of 12-d-old seedlings were exposed to a 0·125 % (w/v) solution of colchicine (Sigma). At 30-min intervals seedlings were fixed in 3 : 1 absolute ethanol : glacial acetic acid, hydrolysed in 5 M HCl for 20 min at 25 °C and stained with Feulgen and monolayers of RAMs were prepared in Acetic Orcein stain (Armstrong and Francis, 1985). CDTs were calculated from the linear rate of metaphase accumulation (linear regression coefficient) using the formulae of Clowes (1976) and methods of Evans *et al.* (1957).

RESULTS

Altering the expression of WEE1 *affects morphogenesis from hypocotyls* in vitro

In a previous report, De Schutter *et al.* (2007) were unable to generate a line constitutively over-expressing *WEE1* using the 35S promoter. To study the potential effects of *Arath;WEE1* expression on arabidopsis growth and development, we have been able to generate transgenic lines in which *Arath;WEE1* expression is driven by an attenuated CaMV 35S promoter (Gatz *et al.*, 1992). Two lines showing a high level of expression of *Arath;WEE1* (*WEE1*^{oe} #58 and #61) were selected by RT-PCR (Fig. 1). Compared with WT, the level of *WEE1* expression was 10- and 54-fold higher in lines #58 and #61, respectively (Fig. 1). Given the reported negative effects of a strong constitutive expression of *WEE1* described by De Schutter *et al.* (2007), we chose line #58, showing weaker over-expression, for most of the measurements reported here, although comparative phenotypic data for both lines #58 and #61 indicate very similar responses between these genotypes. We further employed two T-DNA insertion lines for *WEE1*, *wee1-1*and *wee1-4*. The former has a T-DNA insertion in the seventh intron and has been characterized by De Schutter *et al.* (2007) as a line lacking *WEE1* transcription. The latter originated from the GABI-Kat collection of T-DNA insertion lines (Rosso *et al.*, 2003) and carries an insertion in the 5'-UTR. Neither of these lines exhibited WEE1 kinase activity either in the presence or absence of hydroxyurea (Lentz Grønlund, 2007), a drug known to cause transcriptional up regulation of *WEE1* and induction of WEE1 kinase activity in wild-type arabidopsis (De Schutter *et al.*, 2007; Lentz Grønlund, 2007).

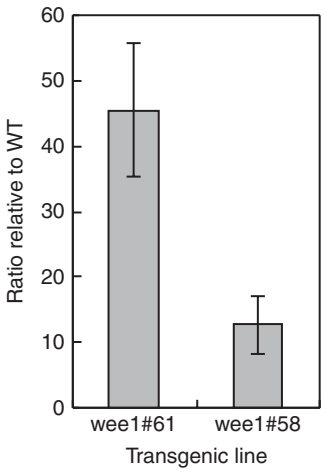

FIG. 1. Molecular characterization of *WEE1*^{oe} genotypes: semi-quantitative RT-PCR of *Arath;WEE1* showing increased expression in the two transgenic lines (#58 and #61) compared with WT (mean ± s.e., $n = 3$).

In initial experiments, we applied the Inoue *et al.* (2001) tissue culture system comprising a two-way grid of increasing auxin (NAA) and cytokinin (Kin) concentrations ranging from 25 to 300 ng mL^{-1} (Supplementary Data Fig. S2). From here on, these are represented by a simplified notation. For example, 25NAA/50Kin represents 25 ng mL^{-1} of NAA and 50 ng mL^{-1} of kinetin. Hypocotyl culture was performed with the two independent *WEE1* over-expressing lines but as there was no significant difference in the *in vitro* response between the two lines, results are from one set of grids (*Arath;Wee1*^{oe} #58).

In the absence of NAA there was neither growth nor morphogenesis in cultured hypocotyls in any of the genotypes (observations not shown) indicating an auxin requirement for arabidopsis hypocotyls in culture, confirming the results of Inoue *et al.* (2001). A minimum level of NAA (25 ng mL^{-1}) was required for callus induction from hypocotyls in all genotypes. In the absence of kinetin, callus formed providing NAA was added (25NAA/0Kin), but morphogenesis did not occur (observations not shown).

In general, levels of morphogenesis in WT hypocotyls increased when both NAA and kinetin concentrations were raised, confirming the results of Inoue *et al.* (2001) (Supplementary Data Fig. S2). However, hypocotyls from *WEE1*^{oe} (*Arath;WEE1* over-expressing) plants exhibited poor morphogenetic responses compared with WT at ≥100 NAA. In other words, a repression of growth and morphogenesis in hypocotyls of *WEE1*^{oe} was more acute at a threshold ≥100 NAA.

The grid responses for the T-DNA insertion lines were more similar to WT than the *WEE1*^{oe} (Supplementary Data Fig. S2) but overall there was a higher level of morphogenesis in *wee1-1* and *wee1-4* compared with WT. This is consistent in showing a *WEE1* gene dosage effect on morphogenetic competence of cultured hypocotyls at NAA concentration ≤50 NAA albeit more so for *wee1-1* than *wee1-4*.

Following this initial qualitative analysis across a wide range of NAA/Kin concentrations, we selected three combinations of exogenous NAA/Kin concentrations from the grid system that induced typical differential responses of hypocotyls of *WEE1*^{oe} relative to WT and the two T-DNA insertion lines for a more rigorous quantitative analysis using 25 replicates per genotype. These were: firstly, 25NAA/25Kin and, secondly, 50NAA/200Kin for which callus growth was not evident in *WEE1*^{oe}; thirdly, 200NAA/50Kin in which WT, *wee1-1* and *wee1-4* exhibited morphogenesis but *WEE1*^{oe} did not; and, finally, 300NAA/300Kin in which morphogenesis was strong in WT, *wee1-1* and *wee1-4* but less so in *WEE1*^{oe} (Supplementary Data Fig. S2).

At 25NAA/25Kin, there was a significant increase in the number of shoots produced by the *wee1-1* and *wee1-4* hypocotyls compared with WT ($P < 0·02$; Fig. 2A). In addition, compared with WT there was a significant increase in the number of roots produced in *wee1-1* and *wee1-4* ($P \leq 0·05$; Fig. 2B). Apart from *WEE1*^{oe}, the other genotypes produced callus but

wee1-1 and *wee1-4* produced a significantly greater area of callus compared with WT (Fig. 2C; $P \leq 0{\cdot}05$). At 50NAA/200Kin, a significantly greater number of shoots, but not roots, was produced by *wee1-1* and *wee1-4* hypocotyls ($P < 0{\cdot}002$) (Fig. 2A, B). Note the null growth response of *WEE1*^{oe} hypocotyls at ≤50 NAA (Fig. 2A–C) confirming the original qualitative grid observations.

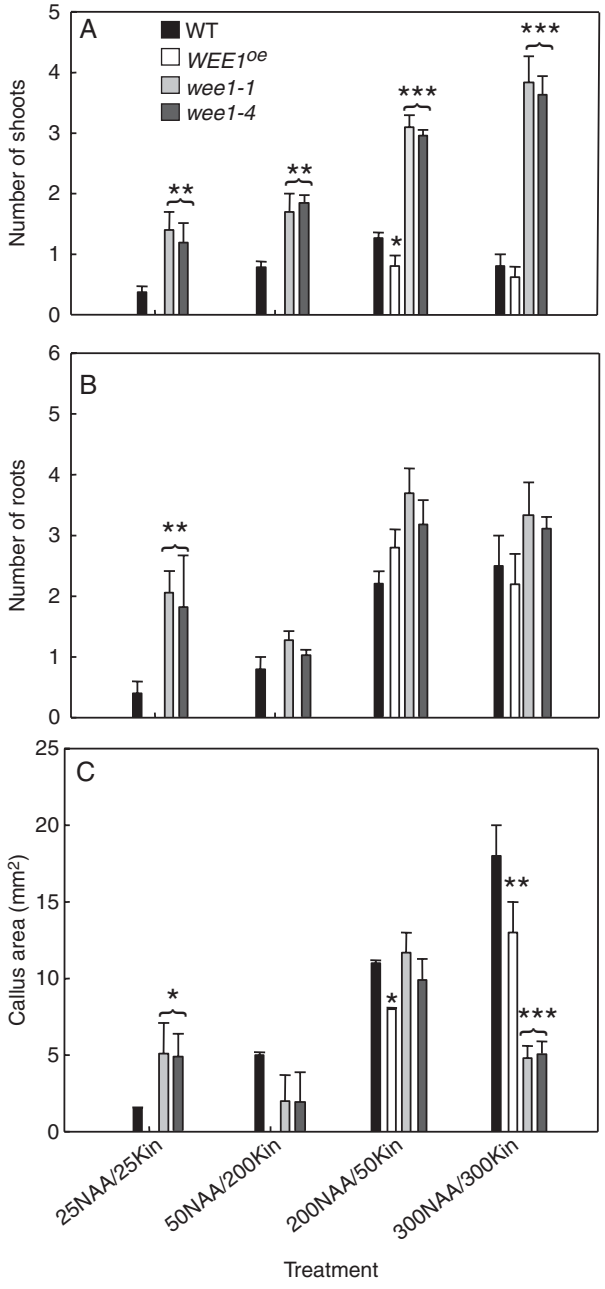

FIG. 2. Differential growth and morphogenetic responses from hypocotyls *in vitro*. Mean number (± s.e.) of (A) shoots (B) roots (C) callus area in 30-d-old cultures of 14-d-old hypocotyls of wild type (WT), over-expressing *Arath;WEE1* line #58 (*WEE1*^{oe}), and the T-DNA insertion lines for WEE1, *wee1-1* and *wee1-4*, cultured on MS medium supplied with a range of concentrations of kinetin (Kin ng mL^{-1}) and naphthyl acetic acid (NAA ng mL^{-1}) for 30 d. Significance levels are *P*-values from Student's *t*-tests between each genotype and WT: *, $P < 0{\cdot}05$; **, $P < 0{\cdot}02$; ***, $P < 0{\cdot}001$ ($n = 25$).

At 200NAA/50Kin, the number of shoots forming in *wee1-1* and *wee1-4* was significantly higher than WT ($P < 0{\cdot}001$; Fig. 2A). In *WEE1*^{oe}, shoots formed at this combination but the mean number was significantly lower than WT ($P < 0{\cdot}05$). At this NAA/Kin combination there were no significant differences in rooting frequency between genotypes (Fig. 2B). At 300NAA/300Kin, significantly more shoots formed in the loss-of-function *wee1-1* and *wee1-4* genotypes compared with WT ($P < 0{\cdot}001$; Fig. 2A) but there was no significant difference in the number of shoots that formed in *WEE1*^{oe} compared with WT ($P > 0{\cdot}05$; Fig. 2A). Regarding roots, there were no significant differences between genotypes at this NAA/Kin combination ($P > 0{\cdot}05$; Fig. 2B). Callus growth was significantly less in *wee1-1* and *wee1-4* ($P < 0{\cdot}001$), and so too for *WEE1*^{oe} compared with WT ($P \leq 0{\cdot}02$) (Fig. 2C).

Overall, the quantitative data show that hypocotyls from *wee1-1* and *wee1-4* were more competent to root (at 25NAA/25Kin) and shoot (in all combinations) than WT. In contrast, hypocotyls from *WEE*^{oe} plants neither grew nor initiated morphogenesis at 25NAA/25Kin or 50NAA/200Kin; *WEE*^{oe} cultures were only able to exhibit morphogenesis once the concentration of NAA was raised to 200 and 300 ng mL^{-1}. In summary, a *WEE1* gene dosage effect on morphogenetic competence in both root and shoot production was revealed at 25NAA/25Kin and on shooting in all combinations.

Altering WEE1 *expression in arabidopsis seedlings affects root growth and developmental responses in 10-d-old seedlings*

Repression of growth and morphogenesis by *WEE1*^{oe}, contrasting with promotion in *wee1-1* and *wee1-4* hypocotyl explants prompted an analysis of the root phenotype in both genotypes. Thus, primary root length and the number of lateral roots that formed per unit length of primary were examined in 10- or 24-d-old seedlings of each genotype.

Daily measurements established that root elongation was significantly reduced in *WEE1*^{oe} compared with WT (Fig. 3A) whereas primary root length per unit time in *wee1-1* and *wee1-4* was no different from WT (Fig. 3B, C). Note the virtually identical phenotypic response of #58 and #61, and, *wee1-1* and *wee1-4* (Fig. 4B). Root length is the result of cell division in the RAM and elongation growth of cells displaced from the RAM.

To examine the proliferative contribution to this elongation response, we measured CDTs in the RAM of *WEE1*^{oe} #58, *wee1-1* and WT. Compared with WT there was an over 4-fold lengthening in the CDT in *WEE1*^{oe} (#58) but there was no clear alteration of CDT in *wee1-1* (Fig. 3D). Note that the method used to estimate CDTs relies on temporal accumulation of cells in metaphase per unit time following continuous exposure of roots to colchicine. The method does not lead to definitive measures of rates of cell division in meristems but is used here for comparative purposes. Overall the data are consistent in showing that *WEE1* gain-of-function led to a reduction but not complete inhibition of root growth compared with WT, whereas loss-of-function had a null effect (Fig. 3).

Scoring the number of lateral root primordia and number of emerged lateral roots in 10-d-old seedlings revealed a

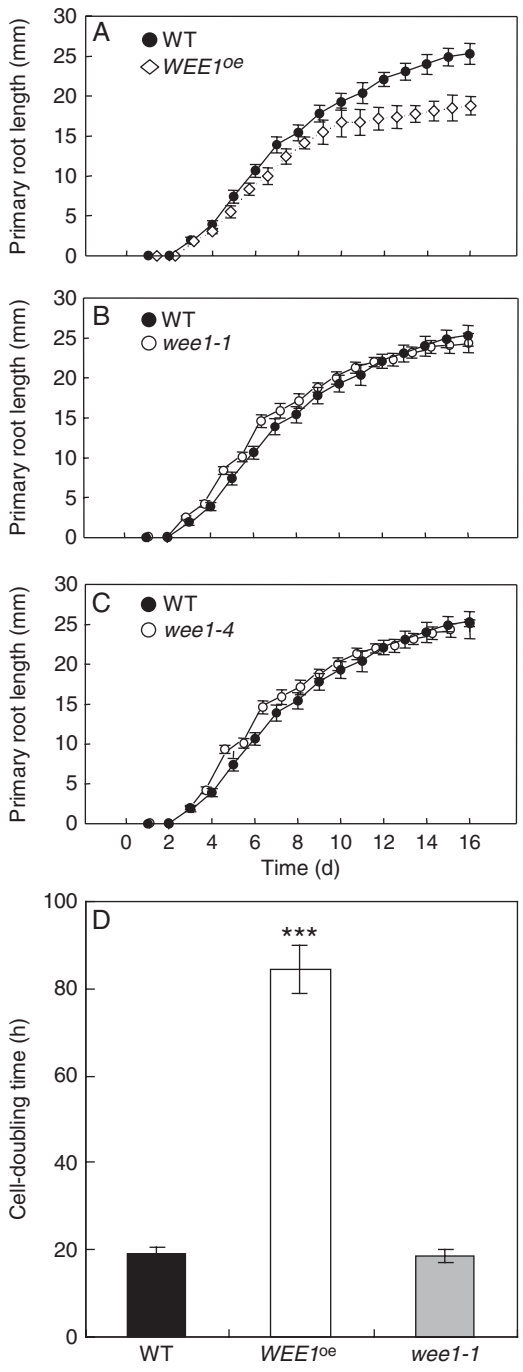

FIG. 3. Elongation and cell cycle response in *WEE1*^{oe} and *wee1* mutants compared with wild type (WT). Relationship between mean (± s.e.) primary root length (mm) and time (d) following germination in arabidopsis genotypes: (A) *WEE1*^{oe} (line #58), (B) *wee1-1*; (C) *wee1-4* at 21 °C (n = 10–15). (D) Mean cell-doubling time in the RAM for WT, *WEE1*^{oe} and *wee1-1*. Significance levels are *P*-values from Student's *t*-tests between each genotype and WT ($n = 6 \pm$ s.e.): ***, $P < 0{\cdot}001$.

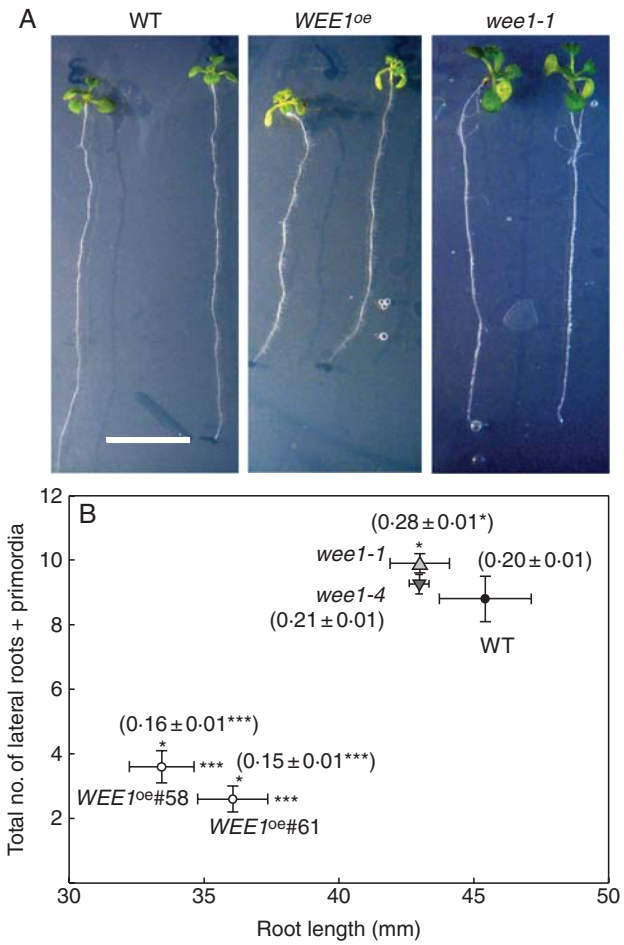

FIG. 4. WEE1 represses lateral root formation: *WEE*^{oe} inhibits and loss-of-function alleles show increased lateral root formation. (A) Phenotypes of 10-d-old arabidopsis seedlings: wild type (WT), *WEE1*^{oe} (line #58) and *wee1-1* (scale bar = 10 mm). (B) The relationship between mean total number of lateral roots and lateral root primordia (± s.e.) and mean primary root length (mm ± s.e.) for 10-d-old seedlings grown at 20 °C: wild type (WT), two *WEE1* over-expressing lines (*WEE1*^{oe}), #58 and #61, and two T-DNA insertion lines *wee1-1* and *wee1-4*. Significance levels are *P*-values from Student's *t*-tests between each genotype and WT. Root length was significantly reduced in *WEE1*^{oe} #58 and #61 compared with WT (*$P < 0{\cdot}05$), but was not significantly different from WT ($P > 0{\cdot}05$) in *wee1-1* and *wee1-4*. Numbers of laterals and primordia were significantly different from WT in *WEE1*^{oe} #58 and #61 (***, $P < 0{\cdot}001$) and *wee1-1* (**, $P < 0{\cdot}02$). Mean (± s.e.) rate of lateral root formation per millimetre of primary is indicated in brackets above each symbol (n =10; *, $P < 0{\cdot}05$; ***, $P < 0{\cdot}001$).

significant reduction in root length in both *WEE1*^{oe} lines, and also the rate of lateral root formation was significantly lower in both *WEE1*^{oe} genotypes (Fig. 4B). Conversely, compared with WT there were significant increases in the number of laterals that formed per millimetre of primary in *wee1-1* (Fig. 4B). Hence, compared with WT, the greater morphogenetic competence of *wee1-1* and *wee1-4* hypocotyls *in vitro* was also evident *in vivo* regarding increased numbers of secondary roots per unit length of primary. To check on the relative persistence of the phenotypes, the analysis was repeated on 17-d-old *WEE1*^{oe} *#58* and *wee1-1* seedlings. Compared with WT, the rate of lateral root formation was significantly lower ($P < 0{\cdot}02$) in the *WEE1*^{oe} *#58* genotype (Fig. 5A, B). However, there was no significant difference between *wee1-1* and WT ($P > 0{\cdot}05$; Fig. 5A, B).

Given that hypocotyls were the source of explants in the grid experiments, we tested whether the null phenotype of the insertion mutants also applied to adventitious root formation

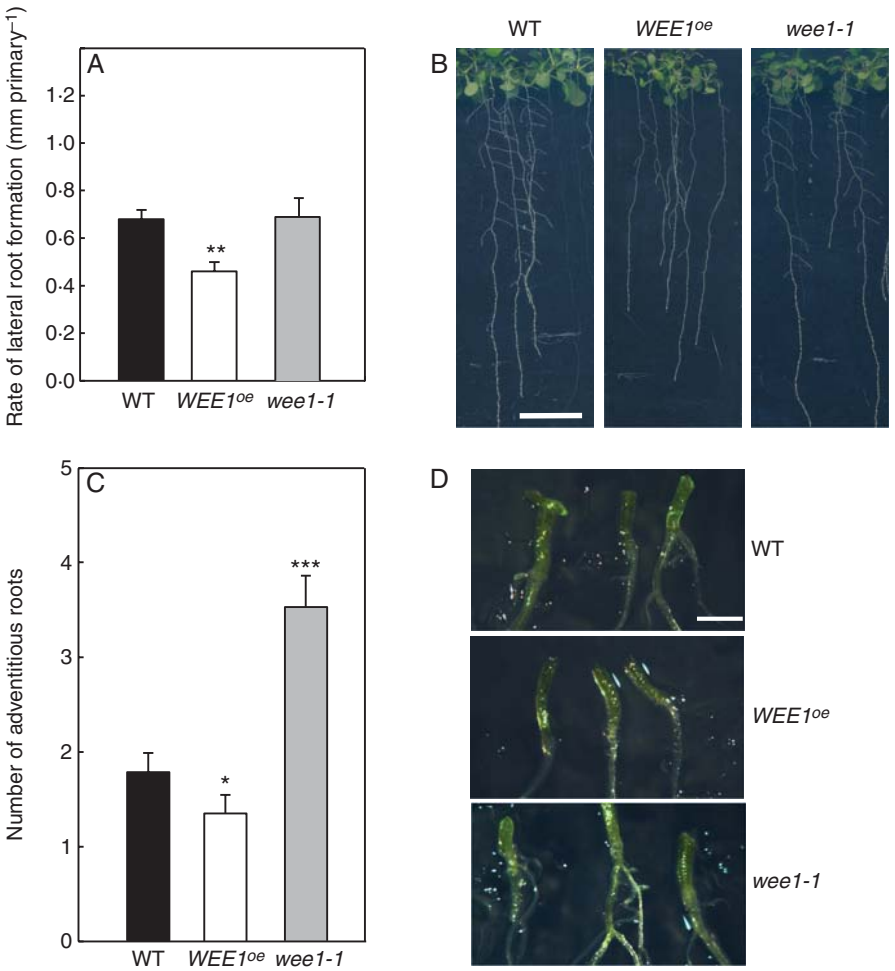

FIG. 5. Differential lateral root and adventitious root phenotypes of wild type (WT), *WEE*^{oe} #58, and the T-DNA insertional line, *wee1-1*: (A) mean (± s.e.) rates of lateral root formation per millimetre of primary root; (B) primary and lateral roots in 17-d-old seedlings (scale bar = 10 mm); (C) mean number and (D) images of adventitious roots from hypocotyls of 24-d-old seedlings (scale bar = 1 mm). Significance levels are *P*-values from Student's *t*-tests between each genotype and WT: *, $P < 0{\cdot}05$; **, $P < 0{\cdot}02$; ***, $P < 0{\cdot}001$ ($n = 25$).

from hypocotyl explants. Compared with WT, there was a significant decrease in the number of adventitious roots recorded in *WEE1*^{oe} in 24-d-old seedlings (Fig. 5; $P < 0{\cdot}05$). Conversely, this score was significantly higher for *wee1-1* compared with WT (Fig. 5; $P < 0{\cdot}001$). Hence a gene dosage effect on adventitious root formation is clearly observable from hypocotyls, supporting the results obtained from hypocotyls cultured on the grids.

In summary, there were subtle phenotypes in the lines in which *WEE1* expression was perturbed. The *in vivo* lateral root phenotypic analysis revealed no consistent differences between the *WEE1* loss-of-function genotype in seedlings compared with WT. However, analysis of hypocotyls *in vivo* revealed a greater capacity to form adventitious roots in *wee1-1* (matching their greater morphogenetic competence *in vitro*) compared with WT. Conversely *WEE1*^{oe} was less able to form lateral or adventitious roots compared with WT.

WEE1 *over-expression increases cell size and cell number in the RAM*

A hallmark feature of *Arath;WEE1* is that it induced a longer cell size when over-expressed in fission yeast (e.g. Sorrell *et al.*, 2002). Given *WEE1*'s repressive effect on root growth we next tested its effect on cell size in the RAM. Here, we measured cell length and cell width in the ground tissues of the RAM – epidermis, cortex and mid-stele – identified as spanning from the root cap/apical meristem junction to the transition point when cells in each tissue begin an irreversible mode of cell elongation (see Dello Ioio *et al.*, 2007).

In *WEE1*^{oe} RAMs, cell length and cell width were significantly longer in all three meristematic tissues compared with WT (Fig. 6 A–F, H) and there was a significantly greater number of cells in epidermal, cortical and stelar lineages of the RAM in WEE1^{oe} compared with WT (Fig. 6G). Although, in *wee1-1* RAM, cell length and cell breadth tended to be smaller in each tissue it was only significantly

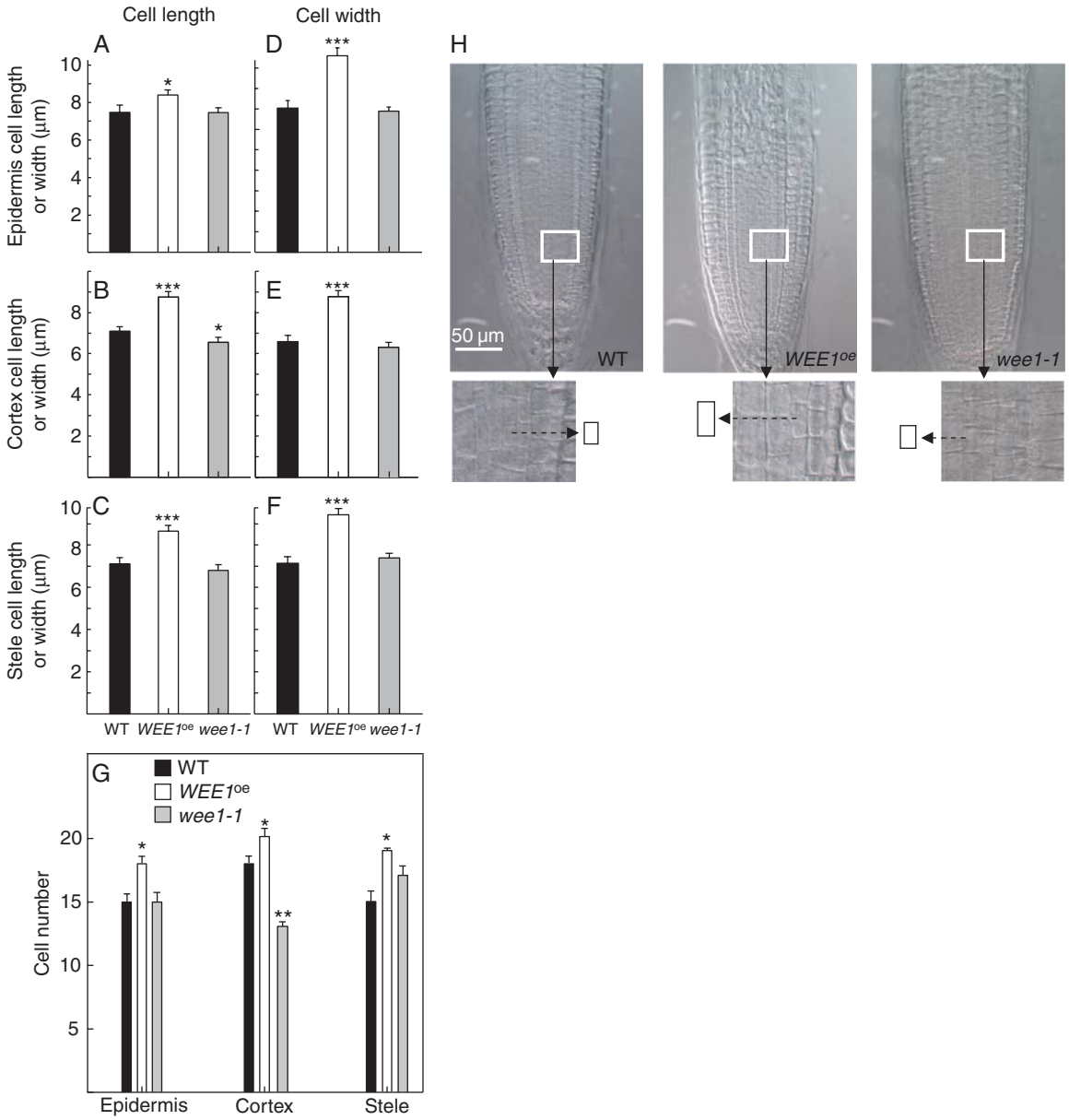

FIG. 6. Measurements of cell size and cell number in WT, *WEE1*^{oe} #58 and *wee1-1* root meristems: mean ± s.e. cell length (A–C) and breadth (D–F) in epidermis, cortex and stele meristems of wild type (WT), the T-DNA insertional line *wee1-1* and in *WEE1*^{oe} together with mean ± s.e. meristem cell file number × 10^{-3} for each genotype (G). (A–F) Unpaired Student's *t*-tests: $25 \leq n \leq 95$; (G) paired Student's *t*-tests (***, $P < 0{\cdot}001$; *, $P < 0{\cdot}05$); (H) representative 17-d-old WT, *WEE1*^{oe} #58 and *wee1-1* whole root tips imaged by DIC microscopy. Magnified sections are shown below, with the size of an individual cell illustrated next to each image, indicating the enlargement of stelar cell size in *WEE1*^{oe} compared with WT.

so for cell length in the cortex (Fig. 6B, H). Consequently, cell number in the epidermal and stelar lineages did not differ appreciably in *wee1-1* compared with WT, although there were significantly fewer cells in the cortical lineage (Fig. 6G). Hence, a cellular gain-of-function and loss-of-function *WEE1* cellular phenotype was restricted to the cortex.

DISCUSSION

Our data are consistent in showing a repression of growth and morphogenesis from hypocotyls *in vitro* when *WEE1* is over-expressed. In support of this, the loss of function *wee-1-1* and *wee1-4* mutant hypocotyls showed a greater capacity for morphogenesis *in vitro* at specific NAA/Kin combinations. In other words, for hypocotyls *in vitro*, an abundance and significant loss of *WEE1* transcripts is consistent with repressed or enhanced growth/morphogenesis, respectively.

Cultured hypocotyls from *Arath;WEE1* over-expressing plants exhibited neither root nor shoot morphogenesis at NAA concentrations of $\leq$100 ng mL^{-1} and *WEE1*^{oe} retarded root growth. When *WEE1* is over-expressed in tobacco BY-2 cells, G_2 is delayed (Gonzalez *et al.*, 2007). Moreover, *WEE1* over-expression caused a marked lengthening of mean CDT in RAMs of arabidopsis. Taken together, these

independent experiments show that elevated expression of *WEE1* can suppress or delay mitosis, slows cell division and root growth and represses morphogenesis both *in vivo* and *in vitro*. That the repression of morphogenesis in the *WEE1*^{oe} could be partially reversed by exogenous NAA *in vitro* ($\geq$200 ng mL^{-1}) suggests that *WEE1* might be regulated by an auxin signal transduction pathway. Certainly, auxin-regulated SCF complexes serve to remove negative regulators in more defined genetic pathways (e.g. Gray *et al.*, 2001). Note that local production and subsequent accumulation of auxin in pericycle cells converts them into founder cells for lateral root morphogenesis (Dubrovsky *et al.*, 2009). Also, in a global transcriptional analysis of cell cycle genes in arabidopsis it was suggested that the down-regulation of *WEE1* might be important for lateral root initiation (de Almeida Engler *et al.*, 2009). Hence, the arrival of auxin and the down-regulation of *WEE1* in pericycle cells that then divide may be more than coincidental.

In vivo, *WEE1*^{oe} repressed primary root growth and decelerated the rate of lateral initiation in seedlings. However, there was no consistent effect of WEE1 loss of function on seedling root growth. That the *in vivo* data for roots did not entirely support the *in vitro* data for hypocotyls should not be too surprising given that different tissue systems were assessed. However, *in vitro*, *wee1-1* hypocotyls (the organs used in the grids experiments) did exhibit a significantly higher frequency of adventitious roots compared with WT, whilst *WEE1*^{oe} hypocotyls formed significantly fewer adventitious roots. Thus, these observations are consistent with a gene dosage effect of *WEE1* in arabidopsis hypocotyls. Hence, we suggest that *WEE1* might have a role at a growth/developmental interface in addition to its role in cell-cycle checkpoints. Clearly, the primary effect of *WEE1* over-expression is to slow down cell division as evidenced by markedly lengthened cell-doubling times (CDTs) and the failure of *WEE1*^{oe} hypocotyls to exhibit anything other than poor callus growth *in vitro* (at $<$100 ng NAA). Hence, the simplest explanation for these results is a dampening of cell division and a consequential lack of morphogenesis *in vitro*.

At the cellular level in the RAM, *WEE1* over-expression induced larger cells in the meristem, whereas in *wee1-1* cell size was clearly reduced in the cortex. Thus, *Arath;WEE1* over-expression in arabidopsis induces an increased cell size in the RAM as it does in fission yeast (Sorrell *et al.*, 2002). This increase in cell size occurred alongside an increased cell number in cell lineages up to the transition point of each ground tissue of the RAM but occurred alongside substantially slower CDTs and slower rates of primary elongation compared with WT. In contrast, cortical cell size and cortical cell number in the RAM was reduced in *wee1-1* compared with WT. This reduction in cortical cell size and number has no effect either on rate of primary root elongation or mean CDT in the RAM of *wee1-1* compared with WT. Much conflicting literature exists on cell size and organ growth in plants and hence clear rules have not been established regarding meristem cell size and rates of root elongation (for example, see Barlow and Rathfelder, 1984; Beemster *et al.*, 2003). Our data indicate a *WEE1* dosage effect on cortical cell size in RAMs of arabidopsis which is also consistent with the known effect of *WEE1* in enlarging cells in tomato fruits (Gonzalez *et al.*, 2007).

Perturbation of cell cycle genes does not always affect development despite effects at a cellular level. For example, a dominant negative *Arath;CDKA1;1* allele expressed in tobacco resulted in fewer but larger cells, whilst development of those plants was unaffected (Hemerly *et al.*, 1995). Plants expressing a dominant negative allele of *Arath;CDKB1;1* were also normal, but again with fewer and larger cells with long G_1 phases (Porceddu *et al.*, 2001). Indeed, these data support the organismal theory of development where cell division is merely a functional consequence of inherent developmental programmes (Kaplan and Hagemann, 1991). On the other hand, perturbation of other cell-cycle genes has profound effects on development. For example, disruption of *CDKB2* interfered with normal cell cycle progression and induced severe defects in the shoot apical meristem of arabidopsis (Andersen *et al.*, 2008). *CDKB2* featured as a vital cog in a gene expression network that included *WUSCHEL* and *SHOOTMERISTEMLESS*, genes that are essential for normal function of the vegetative shoot apical meristem (Andersen *et al.*, 2008). Also, over-expression of *KRP1* (an inhibitor of CDK activity) in arabidopsis, resulted in a distinct reduction in the frequency of lateral roots (Himanen *et al.*, 2002). Our data are consistent with a perturbation of *WEE1* having consequent effects on development. Indeed, our observations support the conclusion of Wang *et al.* (2007), from their studies of KRPs, that cell division, growth and development are intrinsically intertwined. However, given that the effects are subtle, this argues for a network of control including other positive and negative regulators probably acting in a partially redundant fashion.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: expression of *Arath;WEE1*, and *ACT2* in arabidopsis seedlings and root tips. Figure S2: over-expression of *Arath;WEE1* represses morphogenesis in cultured hypocotyls.

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