

Developmental steps in acquiring competence for shoot development in *Arabidopsis* tissue culture

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Abstract *Arabidopsis* shoots regenerate from root explants in tissue culture through a two-step process requiring preincubation on an auxin-rich callus induction medium (CIM) followed by incubation on a cytokinin-rich shoot induction medium (SIM). During CIM preincubation, root explants acquire competence to respond to shoot induction signals. During CIM preincubation, pericycle cells in root explants undergo cell divisions and dedifferentiate, losing the expression of a pericycle cell-specific marker. These cells acquire competence to form green callus only after one day CIM preincubation and to form shoots after 2–3 days CIM preincubation. Reversible DNA synthesis inhibitors interfered with the acquisition of competence to form shoots. Genes requiring CIM preincubation for upregulation on SIM were identified by microarray analysis and included *RESPONSE REGULATOR 15 (ARR15)*, *POLYGALACTURONASE INHIBITING PROTEIN 2 (PGIP2)* and *WUSCHEL (WUS)*. These genes served as developmental markers for the acquisition of competence because the CIM preincubation requirements for *ARR15* and *PGIP2* upregulation correlated well with the acquisition of competence to form green callus, and the CIM preincubation requirements for *WUS* upregulation matched those for shoot formation. Unlike *ARR15*, another cytokinin inducible, A-type ARR gene, *ARR5*, was upregulated on SIM, but the induction did not require CIM preincubation. These findings indicate that competencies for various events associated with shoot regeneration are acquired progressively during CIM preincubation, and that a set of genes, normally upregulated on SIM, are repressed by a process that can be relieved by CIM preincubation.

Keywords Competence acquisition · Shoot regeneration · Response regulator · Pericycle · Dedifferentiation

Abbreviations

APH	Aphidicolin
ARR	<i>Arabidopsis</i> response regulator
CIM	Callus induction medium
DTA	Diphtheria toxin chain A
FDR	False discovery rate
GUS	β -glucuronidase
HU	Hydroxyurea
LRP	Lateral root primordium
MUG	4-Methylumbelliferyl β -glucuronide
NAA	1-Naphthalene acetic acid
NPA	<i>N</i> -1-naphthylphthalamic acid
PI	Propidium iodide
RT-PCR	Reverse transcriptase polymerase chain reaction
SIM	Shoot induction medium
YFP	Yellow fluorescent protein

Introduction

Plant organs can be regenerated from vegetative tissue in culture by treatment with the appropriate plant hormones. Species, cultivars and varieties differ in the efficiency of organ formation and whether organogenesis is direct or indirect (Hicks 1994), i.e., whether callus production is required for shoot or root induction. Shoot or root organogenesis from *Convolvulus* leaf explants or tobacco pith are examples of indirect organogenesis in which prior callus production is required (Thorpe 1993). In general, callusing is undesirable for the regeneration of elite material because of the genetic instability associated with the undifferentiated growth (Lee and Phillips 1988). Hence, there is interest in reducing the

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callusing stage and in understanding the critical events during callus formation that empower shoot formation.

In *Arabidopsis*, procedures for the production of shoots from root and/or hypocotyl explants typically involve indirect organogenesis (Valvekens et al. 1988). Explants are preincubated on an auxin-rich callus induction medium (CIM) and then are transferred to a cytokinin-rich shoot induction medium (SIM) for shoot formation. During CIM preincubation, root explants “acquire competence” to respond to shoot induction signals and to form shoots during subsequent incubation on SIM (Christianson and Warnick 1983). What the acquisition of competence is in cellular or molecular terms is not known. It has been generally thought that preincubation on CIM promotes dedifferentiation of tissues that ultimately redifferentiate into organs (Gautheret 1966; Hicks 1980).

Shoot regeneration has many features in common with lateral root primordia (LRP) formation. Lateral roots derive from the pericycle and more specifically from cell files adjacent to the xylem poles (Casimiro et al. 2003). LRPs in *Arabidopsis* arise acropetally along the root from clusters of about 11 pericycle founder cells (Laskowski et al. 1995). Sites of LRP formation are marked by clusters of shorter cells formed by anticlinal divisions that are both polarized and asymmetric occurring in pericycle cell files adjacent to the xylem pole and in the two flanking cell files (Casimiro et al. 2003). Later stages of LRP development involve periclinal divisions, cell differentiation and activation of the root meristem (Malamy and Benfey 1997).

Lateral root formation can be dramatically induced by auxin (Celenza et al. 1995; Torrey 1950). The formative divisions of the LRP depend on basipetal transport of auxin (Bhalerao et al. 2002; Casimiro et al. 2001), and Himanen et al. (2002) used this information to establish a synchronous lateral root inducible system. They applied the auxin transport inhibitor, NPA (*N*-1-naphthylphthalamic acid) to block lateral root initiation and subsequently administered the auxin, NAA (1-naphthalene acetic acid), to reactivate the process. By observing a *CycB1*;1-GUS reporter, they found that auxin activated the entire pericycle which was blocked at the G1–S boundary.

Cytokinin, on the other hand, has an inhibitory effect on LRP formation (Aloni et al. 2006; Kuroha and Satoh 2007; Li et al. 2006; Werner et al. 2001, 2003). Werner et al. (2001, 2003) genetically engineered tobacco and *Arabidopsis* plants to overexpress cytokinin oxidase to reduce the levels of endogenous cytokinin. In doing so, they found increased lateral root formation indicating cytokinins inhibited root branching. Li et al. (2006) found that cytokinins interfere with LRP initiation by blocking the G2–M transition. They argued that cytokinins reduce the levels of cyclins required for the transition (CYCA2;1, CYCB1;1, CYCB2;1 and CYCB2;3). Of interest they found that once

LRPs had been established that cytokinins no longer had an inhibitory effect.

The contrasting roles of cytokinins and auxin in LRP formation creates an interesting framework for the study of shoot regeneration in which root explants are treated sequentially with an auxin-rich CIM followed by a cytokinin-rich SIM. In this report, we characterized events that occur during CIM preincubation and their effects on the gene expression program that unfolds subsequently during shoot development on SIM. To do so, we identified genes that require CIM preincubation for expression and used these genes to mark developmental events that occur during the acquisition of competence. We found that various competencies are progressively acquired during preincubation on CIM and that some of these events are associated with cell cycle transitions.

Materials and methods

Plant material and RNA extraction

Plant material (*Arabidopsis thaliana*) and procedures for RNA extraction are described in Che et al. (2006).

Microarray and statistical analysis

Gene expression profiling using Affymetrix *Arabidopsis* (ATH1) DNA chips and statistical analysis were as described in Che et al. (2006). To identify genes upregulated during the acquisition of competence, a single contrast of time point means (4 days CIM vs. 0 time) was implemented for each gene as part of our analysis of variance. Gene-specific *t*-tests with 4 degrees of freedom each were used to identify genes downregulated in the CIM dropout experiment. In all cases *P*-values were converted to *q*-values using the method of Storey and Tibshirani (2003) to obtain approximate control of the false discovery rate.

Semiquantitative RT-PCR analysis was performed as described in Che et al. (2006). The primers used for these analyses were as follows:

At1g74890: ARR15RTF 5'-GTATAGAACAATGTA TGATA-3', ARR15RTR 5'-TAGACTCTAATTTGATC CTC-3'; At2g40670: ARR16RTF 5'-GCTTCTGCAGTTC ATGAGATGACA-3', ARR16RTR 5'-CTTGTGTCAAA GTGACAACAGCAG-3'; At3g48100: ARR5F 5'-CTCA CGAGTCACGATCCTACTC-3', ARR5R 5'-GGTTCT ATCAGCAAAAGAAGCC-3'; At4g17980: NAMCIMF 5'-GAGTTGCCAGGTAAATCTTT-3', NAMCIMR 5'-CTTCTTAACACGCGACATA-3'; At1g75040: PR5rtF 5'-TTCATCACAAGCGGCATTGC-3', PR5rtR 5'-GATC CTCCGGATGGTCTTAT-3'; AT1G52400: BGL1rtF 5'-AGCATTTTCAGGTTGAAGGAG-3', BGL1rtR 5'-AGTAA GTTGTGACTGACTTG-3'; At5g57260: BGL2F 5'-AAC

CACACAGCTGGACAAAT-3', BGL2R 5'-GTATCAGT GGTGGTGTCACT-3'; At3G62950: GLUTLrF 5'-ATGG AGAGAATAAGAGATTT-3', GLUTLrR 5'-ATATTTA TCATCTATGCGCT-3'; At3G62960: GLUTF 5'-ATGGA CAAGGTTATGAGAAT-3', GLUTR 5'-CTAGTTATGA AATGACTGAT-3'; At5G24580: COPPERF 5'-CAAAT GAGAGGGGTCCAAACA-3', COPPERR 5'-ATAGCCG TCATTTTCATTGTCTC-3'; At3G54490: rbprF 5'-AACT TTCCCGATTGAAGAT-3', rbprR 5'-CAACGAATTTG CCTTTCTTC-3'; At1G72110: 72110rtF TAGCATACTG GTGACTGGTCA-3', 72110rtR 5'-AATGCCCTCGGGAT CACATGT-3'; At5g06870: PGIPIF 5'-AAGCTTACAG GTCCGATACC-3', PGIPIR 5'-CCACACAGTCTGTTA TAGCT-3'; At2g17950: WUSrtR 5'-GCTAGTTCAGACG TAGCTC-3', WUSrtF2 5'-TGGATCTATGGAACAAGA CT-3'; At5g53950: CUC2F 5'-AAGCTCCAAGGATGA ATGGGT-3', CUC2R 5'-GACGGCTGAATGAGTTAAC GT-3'; At3g62250: UBQ5F 5'-CTTGAAGACGGCCGTA CCCTC-3', UBQ5R 5'-CGCTGAACCTTTCAAGATCC ATCG-3'.

Protein extraction

Arabidopsis seedlings (0.1 g) were frozen in liquid N₂ and homogenized with an Eppendorf pestle in 1.5 ml microcentrifuge tubes. The resulting powder was extracted at 4°C with 0.3 ml of extraction buffer (0.1 M Hepes–KOH pH 7.0, 20 mM 2-mercaptoethanol, 0.1 mg/ml phenylmethanesulphonylfluoride (PMSF), 0.1% (v/v) Triton X-100, 1 mM EDTA, and 20% (v/v) glycerol). The mixture was immediately centrifuged at 12,000g for 15 min at 4°C. The supernatant was recovered and frozen in liquid N₂ and the pellet was discarded. Protein concentrations were determined by the Bradford method (BioRad, Hercules, CA, USA) using bovine serum albumin as a standard (Bradford 1976).

GUS constructs

An *ARR15* promoter:GUS construct was generated as follows: 1.2 kb *ARR15* promoter was amplified using a genomic DNA template and following primers:

ARR15pF 5'-ccaagcttgggCGATTGGTTTTGTTTTA TTTTGTGTTTGA AAAA-3';
ARR15pR 5'-gctctagagcTGTTTTCTCTCGGGAAA GTAAACAACAAAAC-3'.

A transcriptional fusion linking the *ARR15* promoter to GUS was created by inserting the promoter into *HindIII* and *XbaI* sites of pCAMBIA3300 GUS vector. GUS expression was localized in whole mounts or in histological sections as described in Che et al. (2006).

GUS activity was determined in extracts with a fluorometric assay essentially as described by Jefferson (1987). Plant

samples were collected in triplicate and protein extracts prepared as described above. Ten microliter of protein extract were mixed with 50 µl GUS assay buffer [2 mM 4-methylumbelliferyl β-glucuronide (MUG) in extraction buffer] and incubated in 37°C. Aliquots of 10 µl were removed at timed intervals (generally 5, 15, 25, 35 and 45 min), and reactions were terminated by adding 90 µl of 0.2 M Na₂CO₃. The fluorescent product was quantified using a multi-detection microplate reader (BIO-TEK, Model Synergy HT). Excitation and emission wavelengths were 365 and 455 nm, respectively. Tissues from regenerated non-transformed plants were used to quantify background GUS activity.

GFP constructs

The enhancer-trap line J0121 expressing GFP in the pericycle and cell-wall GFP marker line CS84726 were obtained from the Arabidopsis Biological Resource Center (ABRC). For GFP visualization *Arabidopsis* root explants were stained for 2–10 min in µg/ml propidium iodide (PI) (Sigma, St Louis, MO, USA) rinsed and mounted in 0.7% low melting agarose with a #1 coverslip. Imaging was performed using Leica TCS/NT (Exton, PA) upright confocal system with 40× oil lens. GFP and PI fluorescence was excited with argon (488 nm) and krypton (568) lasers, respectively, along with DD 488/566 filter. Emission was filtered by using either 475–575 nm (for green) or 590+ nm (for red) band-pass filter. The confocal Z-stacks were aligned by using either Adobe Photoshop or MetaMorph imaging system.

Results

Proliferation and dedifferentiation of pericycle cells

A standard protocol for regenerating shoots from root explants of *Arabidopsis* seedlings involves preincubation on CIM (Valvekens et al. 1988) during which time explants acquire competence to form shoots on SIM. During the induction of LRPs, treatment of *Arabidopsis* roots with auxin stimulates anticlinal cell divisions in the pericycle layer of the root producing shorter cells (Himanen et al. 2002). Likewise, during CIM preincubation, pericycle cells in root explants undergo anticlinal divisions giving rise to shorter cells followed by periclinal cell divisions producing additional cell layers (Fig. 1a, b). At various points along the roots, more extensive cell divisions lead to the formation of cell masses or callus foci. The process appears much like lateral root formation, however, the cell masses at 4 days CIM are less well formed and spaced.

During CIM preincubation, a fundamental change that occurs in the pericycle is the loss of pericycle-specific markers. J0121 is an enhancer-trap line that uses GAL4-

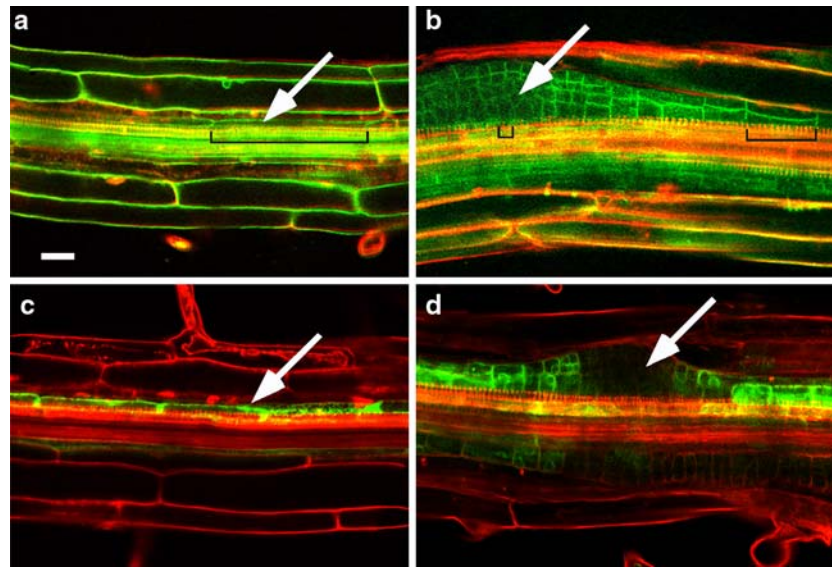


Fig. 1 Cell proliferation and dedifferentiation during CIM preincubation. Confocal microscopy of root explants preincubating on CIM. **a** CS84726 is a cell wall marker that highlights root cell outlines with GFP. At 0 days CIM, the pericycle (*arrow*) is only one cell layer thick, and in this region of the root, the cells are very long (*bracket*). Root explants are counter-stained with propidium iodide. **b** At 4 days CIM, pericycle cells have undergone some anticlinal and periclinal cell divi-

sions, and at sites where cell masses or foci of callus form (*arrow*), cells are shorter (*brackets*) and may be more than one cell-layer thick. **c** J0121 is a pericycle cell marker that marks xylem-pole pericycle cells with GFP (Laplaze et al. 2005). At 0 days CIM, the GFP-marked pericycle cells constitute a single-layered cell file adjacent to the xylem pole (*arrow*). **d** At 4 days CIM, GFP fluorescence is extinguished in cell masses (*arrow*). Bar 20 μ m

mediated transactivation to express GFP specifically in the xylem-pole adjacent cell files of the pericycle (Laplaze et al. 2005) (Fig. 1c). We found that the GFP marker in J0121 was expressed in the pericycle, but was extinguished during CIM preincubation in the cell masses presumably derived from this cell layer. As a result, the cell masses were unmarked by the pericycle-specific marker (Fig. 1d). Note that pericycle-specific marker expression persisted outside of the cell masses even though these cells had undergone anticlinal, and at least one periclinal division. Thus, our findings indicate that extinguishment of this marker is not a prelude to early divisions of the pericycle, but occurs with more extensive cell proliferation in cell masses, some of which give rise to organs.

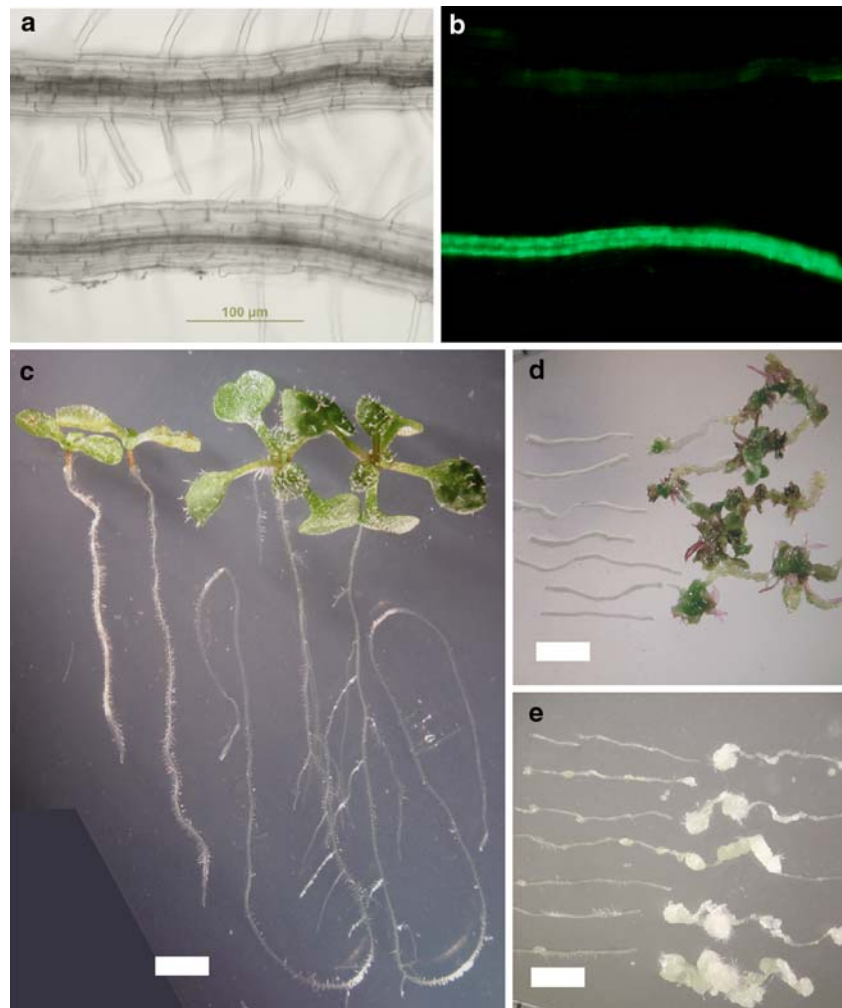
It appears that the loss of the pericycle-specific marker in cell masses is a process of pericycle cell dedifferentiation, rather than the proliferation of non-pericycle derived cells. Founder cells for LRP formation have been shown by others to be located in the pericycle by tracing the continuity of a cell lineage marker generated by heat shock excision of a transposon that activates the expression of a YFP marker gene (Kurup et al. 2005). Pericycle cells adjacent to the xylem poles are required for lateral root formation as found by Laplaze et al. (2005) who ablated these cell files by transactivating a diphtheria toxin chain A (DTA) effector transgene in the J0121 enhancer trap line. DTA is a ADP-ribosylase that targets eukaryotic elongation factor 2, and inhibits translation in a cell autonomous manner (Czako et al. 1992). We obtained a comparable, transactivatable

DTA effector gene construct (pSDM7021) from *R. Offringa* (Weijers et al. 2003), introduced it into *Arabidopsis* and then transferred the construct into J0121 by crossing. We found that the DTA transgene did, indeed, ablate xylem-pole adjacent pericycle cell files as indicated by loss of GFP marker expression (Fig. 2a, b). F1 progeny seedlings expressing the construct produced roots that outwardly appeared normal, but were shorter and did not form lateral roots (Fig. 2c). Roots from the affected seedlings were explanted and subjected to our standard tissue culture regime for regenerating shoots. We found that pericycle ablation eliminated shoot regeneration on SIM (Fig. 2d) and callus formation on CIM (Fig. 2e). The latter was best demonstrated by continuing preincubation of root explants on CIM for 18 days at which time there was ample callus formation on wild type roots, but almost none on roots from seedlings expressing the pericycle-specific DTA transgene (Fig. 2e). We concluded that the xylem pole-adjacent cells of the pericycle are required, and are the likely source of cells for callus formation on CIM and for shoot formation on SIM as suggested by Atta et al. (2004).

Time course for competence acquisition during CIM preincubation

To define events during CIM preincubation as root explants acquire competence for shoot formation, samples were preincubated for various times in a culture scheme in which all samples were preincubated for the same total period of

Fig. 2 Effect of ablation of xylem-pole pericycle cells by the action of a pericycle-specific diphtheria toxin chain A (DTA) transgene. **a** Light microscope and **b** epifluorescence images of roots from F1 progeny from a cross of DTA-1 \times J0121 (*upper*) and wild-type (*lower*) seedlings. **c** F1 progeny expressing the DTA transgene (two seedlings on left) and wild type (two seedlings on right). Seedlings are 14-day-old. **d** Shoot regeneration in root explants from DTA-1 \times J0121 F1 (*left*) and wild-type (*right*) seedlings after 4 days preincubation on CIM and 14 days incubation on SIM. **e** Callus formation in root explants from DTA-1 \times J0121 F1 (*left*) and wild-type (*right*) seedlings after 18 days incubation on CIM. Bars in C, D and E = 2 mm



time either on CIM or on basal B5 medium (Fig. 3a). Without CIM preincubation, explants failed to form shoots efficiently on SIM (0 days, Fig. 3b, d). However, after only two days of CIM preincubation, explants rapidly acquired competence to form shoots, and competence continued to increase for the next two days (Fig. 3b). To demonstrate that CIM preincubation did not simply represent physical carryover of hormones from the CIM medium, explants were transferred from CIM to SIM after only 10 min CIM preincubation. Short exposure to CIM did not have a significant effect on green callus or shoot formation (10 min, Fig. 3b, c). We found that with only one day CIM preincubation, root explants formed green callus (after subsequent SIM incubation) as abundantly as they did when forming shoots (Fig. 3c, d). Therefore, we concluded that root explants first acquire competence to produce green callus, and then they acquire competence to form shoots.

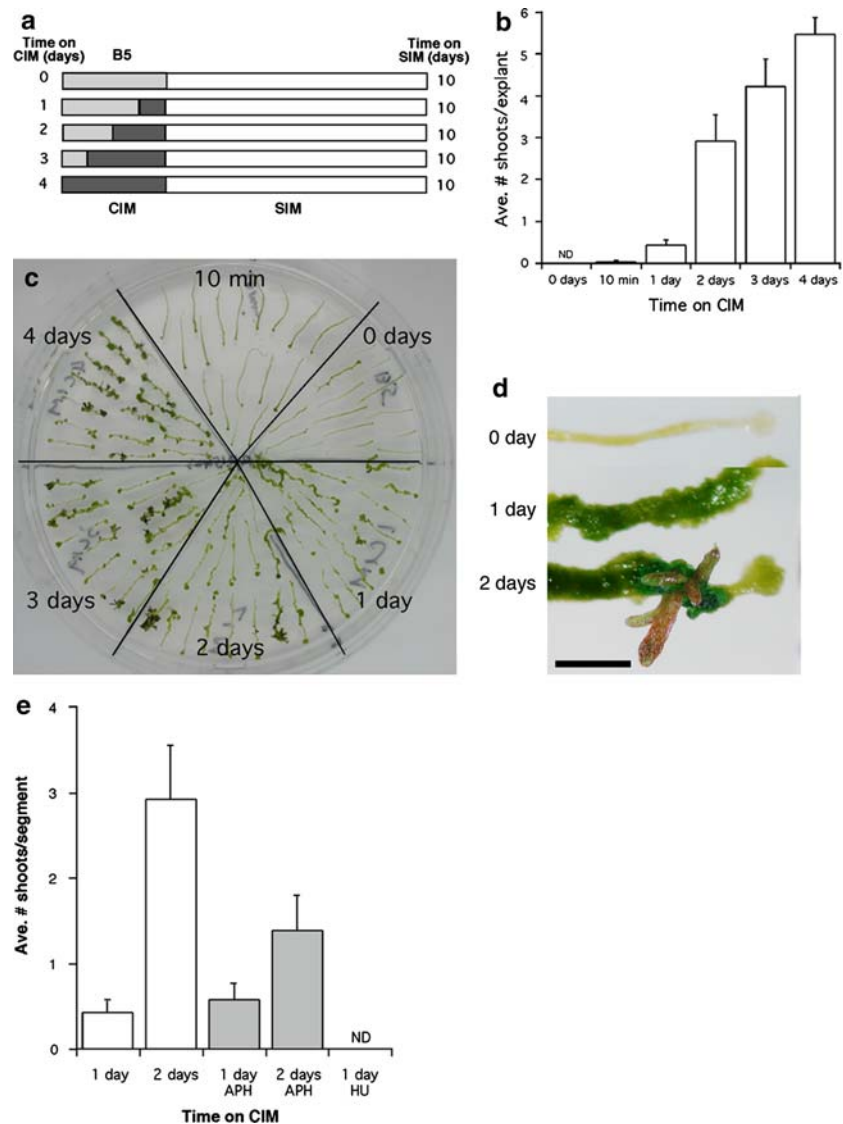
Effects of cell cycle inhibitors and on shoot regeneration

To determine whether cell cycling is required during CIM preincubation for the acquisition of competence to form

shoots, root explants were treated with reversible cell cycle inhibitors. Root explants were preincubated on CIM containing either 5 μ g/ml aphidicolin (APH) or 100 mM hydroxyurea (HU) to inhibit DNA synthesis. HU inhibits ribonucleotide diphosphate reductase, APH is a DNA polymerase α and δ inhibitor and both block the cell cycle in the G1-S transition (Planchais et al. 2000). Explants were transferred to SIM without inhibitors and scored for shoot formation 10–14 days later. To avoid long-term cytotoxic effects, explants were preincubated no longer than two days in the presence of the inhibitor. However, as a control, explants were incubated for more than a week on these inhibitors to show that the concentrations were effective in blocking cell divisions and callus growth (data not shown).

Treatment with 5 μ g/ml APH for two days during preincubation on CIM significantly reduced shoot formation during subsequent incubation on SIM (Fig. 3e). Similar treatment with APH for one day had less effect because fewer shoots were formed in the untreated control with only one day CIM preincubation. However, treatment with 100 mM HU was harsher because 1 day HU treatment during CIM preincubation blocked all subsequent shoot

Fig. 3 Effect of CIM preincubation period length on green callus and shoot formation. **a** CIM preincubation period was varied between 0 and 4 days before transferring explants onto SIM for 10 days. (Explants were cultured on B5 medium without hormones when they were not preincubated on CIM, such that explants were all cultured for the same period of time.) **b** Average number of shoots formed in three replicate cultures after 10 days incubation on SIM depended on the time of CIM preincubation. Error bars represent \pm SE. **c** Tissue culture dish showing explants after 10 days on SIM that had been preincubated previously on CIM for various times as indicated. **d** Close-up of root explants after 14 days incubation on SIM. Number of days of CIM preincubation is indicated. Bar 1 mm. **e** Effect of reversible cell cycle inhibitors on shoot formation. Explants were preincubated on CIM for times indicated in the presence or absence of 5 μ g/ml aphidicolin (APH) or 100 mM hydroxyurea (HU). Number of shoots per explant scored in three replicate samples with no less than 20 root segments per sample. Error bars are \pm SE



formation (Fig. 3e). A two-way ANOVA was conducted to demonstrate the significance of the differences in time ($P = 0.00635$) and inhibitor treatments ($P = 0.02410$) and of the interaction between time and treatment ($P = 0.01266$). We conclude that the G1–S transition in pericycle cells during CIM preincubation is required for explants to acquire full competence to efficiently form shoots on SIM. Beckman et al. (2001) demonstrated that pericycle cells leaving the root meristem are G1 arrested, and cells in presumptive LRP sites progress into G2. Thus, most of the pericycle cells from which regenerating shoots are derived are G1-arrested cells, and apparently, auxin treatment during CIM preincubation is a necessary step to engage that cell population.

Impact of CIM dropout on gene expression

In other studies, Che et al. (2002, 2006) described gene expression changes during shoot development; therefore, we

investigated how the gene expression program was affected by omitting CIM preincubation. For this “CIM dropout” experiment, we preincubated explants as above on basal B5 medium without hormones for the same period of time as normal CIM preincubation. The dropout explants and normal CIM preincubated controls were then transferred to SIM, further incubated for 6 days at which time samples were taken for RNA extraction. The experiment was analyzed as a randomized block design with three biological replicates per treatment. The impact of the CIM dropout was profound in the sense that 57 genes were downregulated 20-fold or more when controlling the false discovery rate (FDR) at the 0.02 level (Storey and Tibshirani 2003) (Table 1). The five top genes include an AAA-type ATPase family protein (At3g28510), NAM (no apical meristem)-like transcription factor (At4g17980), IAA-amido synthase (At2g23170), a subtilisin-like serine protease (At1g01900) and cytokinin oxidase-like protein (At3g63440). Of possible consequence

Table 1 Expression changes in genes most highly affected in CIM dropout experiment

Locus ID	FC ^a	P values ^b	q values ^b	Normally upregulated on SIM ^c	Gene descriptions
At3g28510	261.7	1.03E-06	2.50E-04		AAA-type ATPase family protein
At4g17980	249.2	9.93E-06	4.82E-04		NAM (no apical meristem)-like transcription factor
At2g23170	133.5	1.95E-03	4.62E-03		IAA-amido synthase
At1g01900	121.6	5.08E-05	7.94E-04		Subtilisin-like serine protease
At3g63440	115.5	1.20E-04	1.11E-03		Cytokinin oxidase-like protein
At3g55720	91.7	3.98E-05	7.09E-04		Unknown
At5g55450	89.2	1.23E-06	2.57E-04		Lipid transfer protein (LTP) family protein
At5g13320	53.5	3.32E-04	1.75E-03		GH3 family protein
At3g60420	53.5	6.35E-04	2.41E-03		Unknown
At1g75040	47.9	7.57E-06	4.41E-04	X	Pathogenesis-related protein 5 (PR-5)
At2g18660	47.4	1.93E-03	4.59E-03		Expansin-related protein 3 precursor (EXPR3)
At2g30750	45.8	2.07E-03	4.80E-03		Putative cytochrome P450
At1g52400	41.8	5.19E-04	2.18E-03	X	***β-glucosidase
At3g57260	40.3	4.92E-06	4.04E-04	X	Beta-1, 3-glucanase 2 (BG2) (PR-2)
At3g62950	40.3	2.06E-03	4.79E-03	X	Glutaredoxin-like protein
At3g56400	39.9	3.45E-05	6.92E-04		WRKY4 transcription factor
At5g60020	39.4	5.46E-04	2.24E-03		Laccase-like protein (LAC2-4)
At3g52770	39.3	3.35E-05	6.82E-04		Hypothetical
At5g59320	38.6	5.41E-04	2.23E-03		Lipid transfer protein 3 (LTP3)
At2g40750	37.0	2.87E-03	5.89E-03		WRKY family transcription factor
At1g30100	35.5	4.53E-03	7.92E-03		9-cis-epoxycarotenoid dioxygenase
At5g22860	35.3	3.73E-03	6.97E-03		Serine carboxypeptidase S28 family protein
At2g41510	34.5	1.31E-03	3.63E-03		Putative cytokinin oxidase
At5g46590	33.8	1.31E-02	1.66E-02		NAM (no apical meristem)-like transcription factor
At1g70830	32.5	2.75E-06	3.36E-04		Bet v I allergen Family protein
At2g24850	30.6	2.24E-03	5.03E-03		Putative tyrosine aminotransferase
At3g10080	30.5	3.77E-04	1.87E-03		Germin-like protein
At3g62960	30.3	6.09E-03	9.72E-03	X	Glutaredoxin-like protein
At1g66690	29.4	6.02E-04	2.36E-03		SAM:carboxyl methyltransferase protein
At5g24580	28.6	4.26E-04	1.99E-03	X	Copper-binding family protein
At3g54490	27.2	1.80E-04	1.31E-03	X	RNA polymerase 24kDa subunit-like protein
At1g72110	26.8	8.70E-03	1.25E-02	X	Unknown
At1g09950	26.5	8.38E-04	2.80E-03		Transcription factor-related protein
At2g18060	26.3	1.31E-03	3.33E-03		NAM (no apical meristem)-like transcription factor
At2g04450	26.2	1.54E-03	3.99E-03		MutT/nudix family protein
At1g71380	26.0	1.99E-05	5.76E-04		Glycosyl hydrolase family 9 protein
At1g59500	25.8	4.24E-04	1.98E-03		IAA-amido synthase that conjugates Asp to auxin
At1g17420	25.8	2.45E-03	5.32E-03		Lipoxygenase
At5g19800	25.3	1.24E-04	1.12E-03		Proline-rich extensin-like protein
At5g50740	25.3	2.91E-03	5.92E-03		Copper chaperone (CCH)-related protein
At5g06870	25.0	3.09E-05	6.65E-04	X	Polygalacturonase inhibiting protein 2 (PGIP2)
At5g22460	24.6	2.20E-03	4.98E-03		Esterase/lipase/thioesterase family protein
At1g54020	23.5	3.05E-04	1.68E-03		Myrosinase-associated protein
At5g10760	23.4	1.39E-03	3.75E-03		Aspartyl protease family protein
At4g00080	23.3	2.95E-05	6.47E-04		Invertase/pectin methylesterase inhibitor
At4g23140	23.3	1.51E-03	3.95E-03		receptor-like protein kinase 5 (RLK5)
At5g52760	22.9	1.10E-03	3.28E-03		Heavy-metal-associated domain-containing protein

Table 1 continued

Locus ID	FC ^a	<i>P</i> values ^b	<i>q</i> values ^b	Normally upregulated on SIM ^c	Gene descriptions
At3g57460	22.4	2.28E-03	5.08E-03		<i>N</i> -arginine dibasic convertase
At1g25510	22.3	1.01E-02	1.38E-02		Aspartyl protease family protein
At2g26400	21.9	2.47E-05	6.06E-04		Acireductone dioxygenase (ARD/ARD') protein
At2g17950	21.7	1.61E-03	4.09E-03	X	WUSCHEL homeodomain transcription factor
At1g02450	21.5	9.64E-03	1.33E-02		NPR1/NIM1-interacting protein
At3g47480	21.3	5.36E-06	4.18E-04		Calcium-binding EF hand family protein
At1g75880	20.7	8.38E-03	1.21E-02		Family II extracellular lipase 1 (EXL1)
At1g51000	20.6	4.87E-03	8.32E-03		Unknown
At5g15160	20.1	4.42E-03	7.80E-03		bHLH family transcription factor
At1g74890	20.0	1.49E-04	1.22E-03	X	Response regulator 15 (ARR15)

Experiment was conducted by comparing estimated mean level of expression for explants preincubated for 4 days on CIM with those that were not. Shown are the genes in which the fold change (FC) was 20-fold or more when controlling the false discovery rate (FDR) at the 0.02 level

^a Fold change in the estimated mean level of expression in the sample preincubated on CIM compared to sample not preincubated on CIM

^b *P*- and *q*-values for the comparison between experiments performed with and without CIM preincubation

^c Gene normally upregulated during incubation on SIM indicated with an X

to shoot formation was the fact that three *NO APICAL MERISTEM*-like transcription factor genes (At4g17980, At5g46590 and At2g18060) and *WUSCHEL* (*WUS*, At2g17950) homeodomain factor gene were all more than 20-fold downregulated in the absence of CIM preincubation. *WUS* is required for specifying the stem cell niche in the shoot meristem (Baurle and Laux 2005; Laux et al. 1996; Mayer et al. 1998). Other genes of note are those normally upregulated on SIM (indicated by X's, Table 1). Topping this list are genes encoding pathogenesis-related protein 5 (PR5, At1g75040), β -glucosidase (At1g52400), β -glucanase (At3g57260), glutaredoxin (At3g62950), copper-binding family protein (At5g24580), RNA polymerase 24KD subunit (At3g54490), an unknown protein (At1g72110), polygalacturonase inhibiting protein (At5g06870), *WUS* (At2g17950) and *ARABIDOPSIS RESPONSE REGULATOR 15* (*ARR15*, At1g74890). Thus, this analysis yielded a wealth of genes, which can be used as developmental markers to study the role of CIM preincubation.

Developmental markers for the acquisition of competence

Of the genes most highly downregulated in the CIM drop-out experiment, *ARR15*, encoding an A-type response regulator, caught our attention because the regulation of this gene had been well studied by others (Kiba et al. 2002, 2003). *ARR15*, like other A-type response regulator genes, such as *ARR5*, is rapidly induced in seedlings by cytokinin (Brenner et al. 2005). In our system, both A-type ARRs were upregulated during incubation on SIM, a cytokinin-rich medium (Fig. 4a). *ARR5* had a fairly high basal level of expression, however, as reported by Che et al. (2002; 2006),

it was upregulated 3–4 fold on SIM. *ARR15* was upregulated more than 10-fold (Fig. 4a), but the upregulation was fairly gradual, taking days, not hours to reach peak levels (Fig. 4b). Although both A-type response regulator genes were upregulated during incubation on SIM, only *ARR15* was dependent on CIM preincubation (Table 1). To determine whether the dependency of *ARR15* on CIM preincubation is a transcriptional phenomenon, we developed an *ARR15* promoter:GUS reporter construct and introduced it into transgenic plants. We observed that the promoter construct was upregulated on SIM and required CIM preincubation for upregulation (Fig. 4c). Therefore, we concluded that the major effect of CIM preincubation on subsequent *ARR15* expression is, at least, partly transcriptional.

We examined the time course by which explants acquire competence to upregulate *ARR15* expression following transfer to SIM (Fig. 5a). As with shoot formation, explants were preincubated on CIM for various periods of time, then transferred to SIM. Explants were analyzed for MUG expression six days later on SIM (Fig. 5b). Also, as before, all experimentals and controls were preincubated for the same amount of time (4 days), and to do so, explants were preincubated on basal B5 medium when not on CIM. Root explants from the transgenic plants gained full competence to express the *ARR15* promoter:GUS reporter construct within one day of CIM preincubation (Fig. 5b). The various cell cycle inhibitors, including HU that blocked shoot formation when administered during CIM preincubation, were ineffectual in blocking *ARR15* promoter expression (Fig. 5b).

CIM preincubation was also needed to accumulate *ARR15* RNA transcripts as assayed by semiquantitative

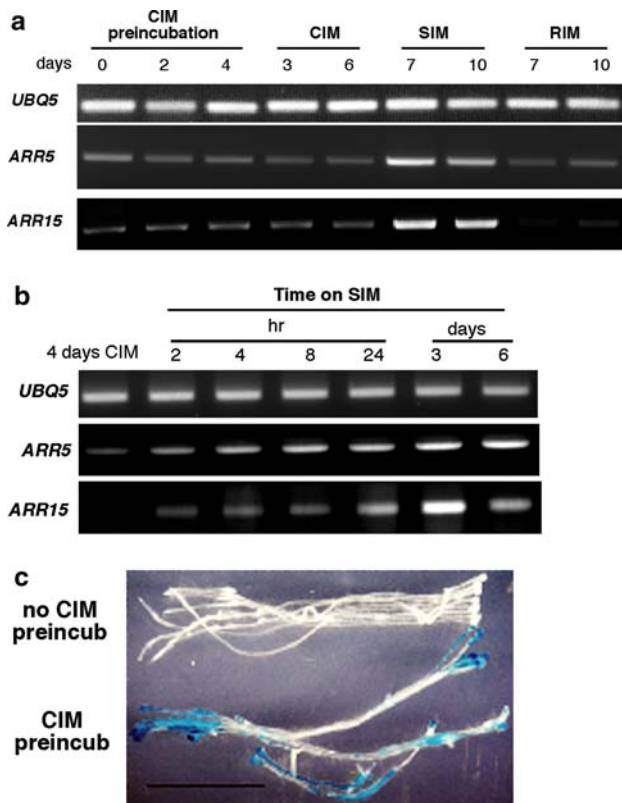


Fig. 4 Expression profiles of *ARR5* and *ARR15* during normal shoot regeneration. **a** Root explants were preincubated on CIM for four days and then transferred to fresh CIM, SIM or RIM (root induction medium). RNA was extracted at various times and analyzed by semiquantitative RT-PCR analysis. **b** Time course for the expression of *ARR5* and *ARR15* during incubation on SIM as analyzed by semiquantitative RT-PCR analysis. *UBIQUITIN5* (*UBQ5*) was used as a control. **c** GUS expression in roots from transgenic seedlings bearing *ARR15* promoter:GUS constructs. Top group of explants were preincubated for four days on B5 medium without hormones, while group below were preincubated for four days on CIM. Both were transferred to SIM and subsequently incubated on SIM for 6 days before histochemical staining

RT-PCR (Fig. 6). *ARR15* required one day CIM preincubation for high-level transcript accumulation on SIM. Thus, the CIM preincubation time required for *ARR15* expression closely corresponds to the acquisition of competence to form green callus. As indicated above, however, not all SIM upregulated A-type ARR were dependent on CIM preincubation. *ARR5*, which is also upregulated during transfer to SIM, did not require CIM preincubation (Fig. 6). Several other genes that were highly dependent on CIM preincubation and upregulated during SIM incubation (At1g75040, At1g52400, At3g57260, At3g62950, At3g62960, At5g24580, At3g54490, At1g72110, At5g06870) required only one day CIM preincubation (Fig. 6). Thus, many SIM upregulated genes that are highly dependent on CIM preincubation required only one day of CIM preincubation, similar to the time required for green

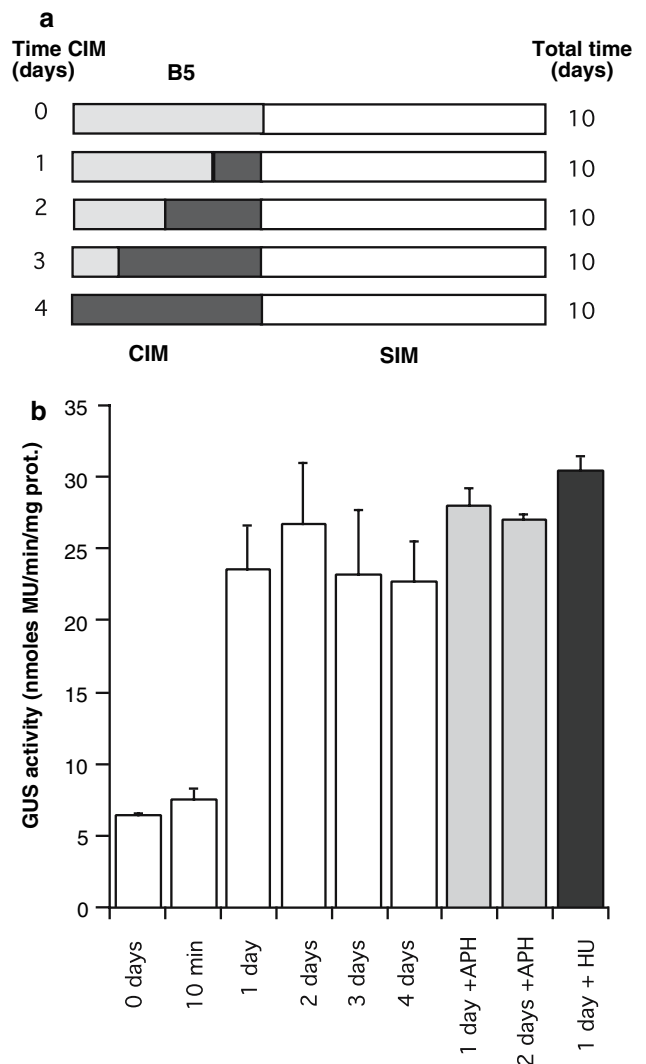


Fig. 5 Effect of CIM preincubation period length on *ARR15* promoter activity. **a** CIM preincubation period was varied between 0 and 4 days before transferring explants onto SIM for 6 days. **b** Mean GUS activity as determined in MUG assays from three replicate samples of explants from *ARR15* promoter:GUS seedlings after 6 days incubation on SIM. Explants were also preincubated on CIM in the presence or absence of 5 µg/ml aphidicolin, APH, or 100 mM hydroxyurea, HU (shaded bars). Error bars are ± SE

callus formation. One important exception was *WUSCHEL* (*WUS*, At2g17950) that required longer CIM preincubation. *WUS* expression during SIM incubation rose with 3–4 days CIM preincubation (Fig. 6). Therefore, *WUS* expression comes the closest of the genes on the CIM drop-out list (Table 1) in representing the CIM preincubation time required for shoot development.

We searched further down the list of genes dependent on CIM preincubation for other genes associated with shoot apical meristem formation and noted *CUP SHAPED COTYLEDON2* (*CUC2*, At5g53950). *CUC2*, an important shoot meristem identity gene, which acts with *CUC1* in activating

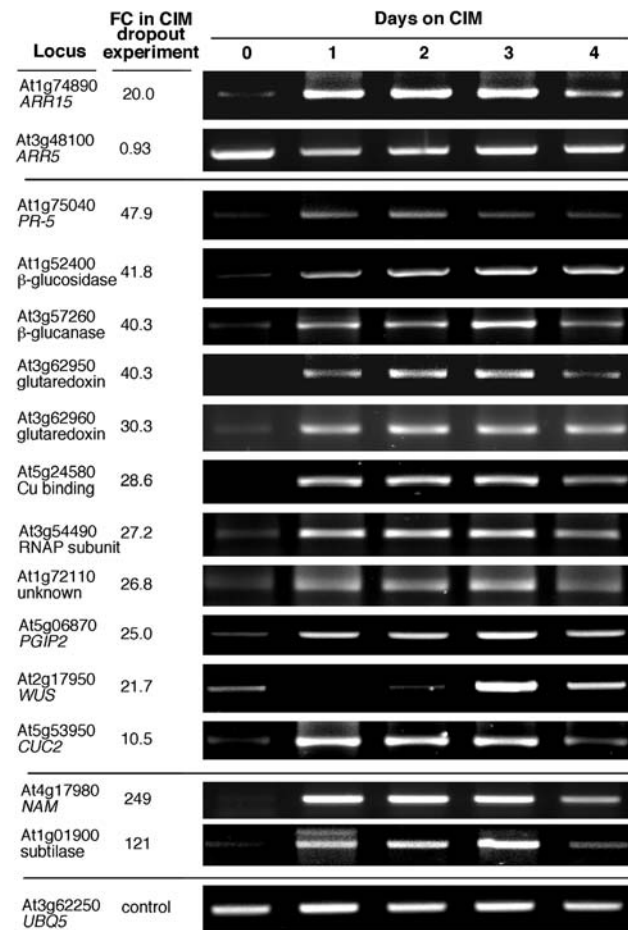


Fig. 6 CIM preincubation time required for upregulation of developmental marker genes on SIM. CIM preincubation period was varied between 0 and 4 days before transferring explants onto SIM for 6 days. Semiquantitative RT-PCR analysis of the expression of genes requiring CIM preincubation from the CIM dropout experiment described in Table 1. FC is fold change

the expression of *SHOOT MERISTEMLESS (STM)* (Dai-mon et al. 2003) and in establishing boundaries in organ formation (Breuil-Broyer et al. 2004). *CUC2* is highly

upregulated during shoot development and incubation SIM (Che et al. 2002, 2006) but like most other SIM upregulated genes, *CUC2* only requires a single day of CIM preincubation (Fig. 6). Thus, not all meristem identity genes have the same CIM preincubation requirements as does shoot development.

We examined preincubation time requirements for other genes that were highly CIM dependent, but not normally upregulated on SIM. A *NO APICAL MERISTEM*-like (*NAM*) gene (At4g17980) and a subtilisin-like serine protease (subtilase) gene (At1g01900) were not appreciably upregulated during SIM (Che et al. 2002, 2006), however, these genes were highly dependent on CIM preincubation (Fig. 6). But like *ARR15* these genes required only one day CIM preincubation to acquire competence for high level SIM expression.

Discussion

Competence to form shoots on SIM is acquired progressively during CIM preincubation (Fig. 7). We described different events that were used to monitor progress during the period of competence acquisition, and these included shoot and green callus formation and the expression of various marker genes. Competence to express *ARR5*, an A-type response regulator that is upregulated during shoot development and functions as a feedback inhibitor of cytokinin responses (To et al. 2004) did not require CIM preincubation. Competence to form green callus and to express another marker gene *ARR15*, an A-type response regulator that also functions as a feedback inhibitor (Kiba et al. 2003), was acquired within one day preincubation without the need for cell divisions. Competence to form shoots necessitated two or more days of CIM incubation and required cell cycle transit beyond the G1–S boundary. Banno et al. (2001) found that the upregulation of *ENHANCER OF SHOOT*

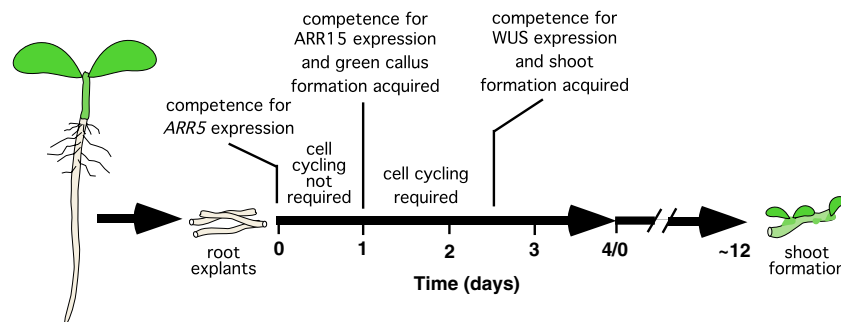


Fig. 7 Events during the acquisition of competence for shoot regeneration. *Arabidopsis* shoots are regenerated from root explants through a process that involves preincubation CIM followed by incubation on SIM, which leads to green callus formation and shoot development. During CIM preincubation competence to express different develop-

mental marker genes and undergo various developmental step during subsequent SIM incubation are acquired progressively. Cell cycle progression during CIM preincubation is required for acquisition of some competencies, but not others

REGENERATION1 (ESR1) during shoot development required CIM preincubation. (*ESR1* is not included on the *ATH1* gene chip and was not included in our analysis.) *ESR1* may play an important role in shoot development because overexpression of *ESR1* in transgenic plants increases the efficiency of shoot regeneration.

One model that might account for the effect of CIM preincubation on the different developmental markers is that expression of markers is under repression and that the mechanism of repression is inactivated during CIM preincubation. *ARR15* would be a prime candidate for gene under repressive control, while *ARR5*, which does not require CIM preincubation for SIM upregulation, would not. It is known that several A-type ARR genes are upregulated by the action of B-type ARRs (Hwang and Sheen 2001; Sakai et al. 2001), and we have shown in other studies that *ARR5* is a direct transcriptional target of *ARR2*, a major B-type ARR (P. Che, unpublished observations). We have observed that *ARR15* is also directly targeted by *ARR2*, however, we speculate that its potential for upregulation might be offset by the action of some repressive mechanism that can be inactivated by CIM preincubation. We have investigated whether repression relief would be prevented by treatment with the proteasome inhibitor MG132, and so far we have observed that it is not (P. Che, unpublished observations).

ARR15 upregulation could be useful as a developmental marker for green callus formation. *ARR15* is expressed in the pericycle (Kiba et al. 2002) and other vascular bundle tissues (P. Che, unpublished observations). Competence to form green callus and to upregulate *ARR15* expression are both acquired only after one day preincubation on CIM and both are unaffected by the action of cell cycle inhibitor treatment during CIM preincubation. The requirements for *WUS* expression correlate well with those for shoot formation. For upregulation on SIM, *WUS* requires three days preincubation on CIM—similar to the requirements for shoot formation. *WUS* expression may be key to shoot regeneration because expression of the gene is required for specifying the stem cell niche in the shoot meristem (Baurle and Laux 2005; Laux et al. 1996; Mayer et al. 1998). Gallois et al. (2002) demonstrated that ectopic *WUS* and *STM* expression promotes shoot development. The subsequent interplay between *WUS* and the A-type ARR genes in shoot regeneration is probably very interesting, since the A-type ARRs are negative regulators of shoot development and *WUS* is a repressor of A-type ARR genes, such as *ARR5* and *-15* (Leibfried et al. 2005).

Fundamental to understanding the role of CIM preincubation is the question why a two-step process is required for shoot regeneration. The answer may relate to the counteracting effects of cytokinin and auxin on LRP formation. Li et al. (2006) reported that cytokinin blocks the formative

divisions in LRP formation and that increasing concentrations of auxin were not able overcome the cytokinin effects. If cytokinin blocks the formative steps in shoot primordia formation, then that would justify sequential treatment with the two hormones. Justification for the two-step process may also relate to cell cycle events. Menges et al. (2002) reported that nearly 500 genes in *Arabidopsis* show significant fluctuation in expression in the cell cycle, and the CIM requiring genes in our study might be differentially inducible at different cell cycle stages. It is assumed that most of the pericycle cells from which shoots arise are arrested in the G1 phase of the cell cycle (Beeckman et al. 2001). Preincubation on an auxin-rich CIM medium could be the only treatment able to overcome the arrest of pericycle cells at the G1–S boundary.

The requirement for DNA synthesis or overcoming G1–S arrest during CIM preincubation does, indeed, distinguish various developmental events. *ARR15* upregulation and green callus formation do not require DNA synthesis but shoot formation does. A possible interpretation of this finding is that cells in G1 are incapable of responding to signals required for shoot formation, however, G1 cells are capable of responding to signals required for *ARR15* upregulation and green callus formation.

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