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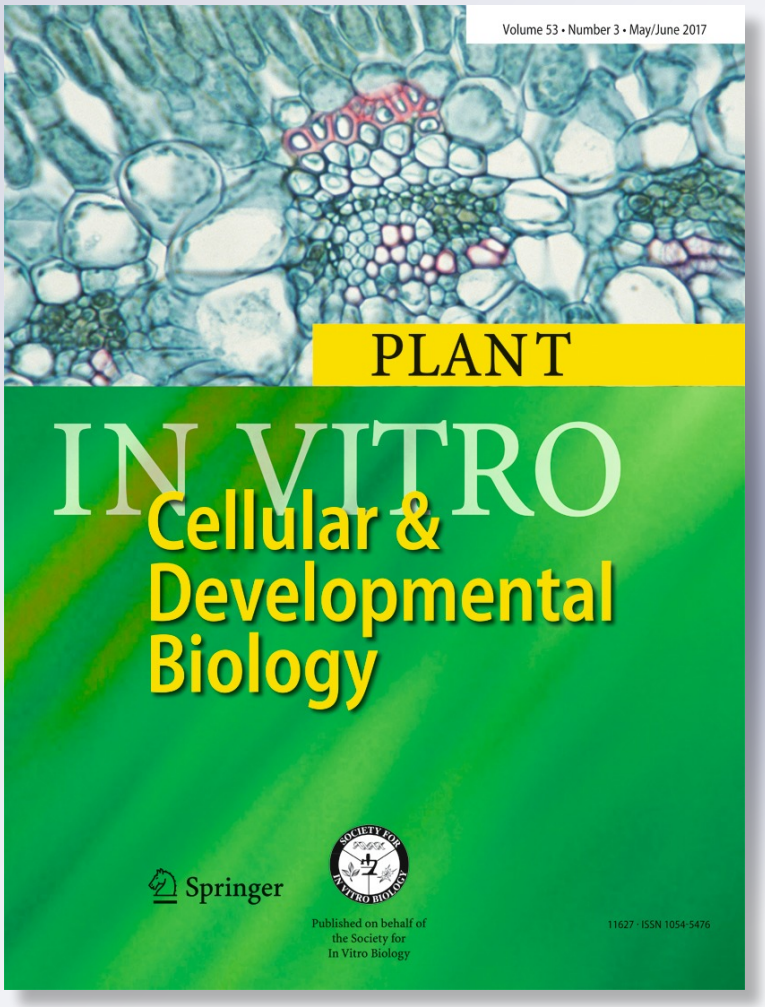

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DEVELOPMENTAL BIOLOGY

Loss of developmental pluripotency occurs in two stages during leaf aging in *Arabidopsis thaliana*

Manish N. Raizada[1] · Travis L. Goron[1] · Ovik Bannerjee[1] · May Quach Mason[1] · Michael Pautler[1] · Jan Brazolot[1] · Adam D. Morris[1] · Arani Kajenthira[1] · Stephen J. Dinka[1] · Natalie DiMeo[1]

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Abstract Aging in plants and animals can result in the loss of cellular pluripotency and the inability to regenerate multiple organs from differentiated, somatic tissues. Detached cotyledons from *Arabidopsis thaliana* (L.) Heynh. seedlings can proliferate to form callus as well as root and shoot organs in the presence of the phytohormones auxin and cytokinin or their synthetic analogs. In this study, detached cotyledons from the ecotype Landsberg *erecta* (Ler-0) demonstrated two separate developmental intervals of pluripotency loss. During a 48- to 60-h interval, occurring approximately 4 to 6 d after germination (DAG), detached cotyledons from Ler-0 could proliferate new cells (callus) but lost shoot regeneration competency, which the ecotype Nossen-0 (No-0) retained. In older cotyledons, the pluripotency loss was more severe, as detached cotyledons failed to proliferate callus. In the first interval, Ler-0 cotyledons lost the ability to respond to treatment with a high concentration of synthetic auxin, a level previously hypothesized to promote dedifferentiation. However, both young and old Ler-0 cotyledons were found to induce WUSCHEL::NLS-GUS, a marker for undifferentiated shoot apical meristem cells. The late-stage interval positively correlated to the timing of slowed growth in cotyledons still attached to intact seedlings. Also, cytokinin overexpression could rescue (or delay) this late-stage callus proliferation failure, but not the early-stage decline in shoot regeneration ability. Combined, the results demonstrate that loss of pluripotency *in vitro* occurs in distinct temporal stages and involves auxin and/or cytokinin signaling. Furthermore, there is genetic variation for this loss of pluripotency within *Arabidopsis*.

Keywords Arabidopsis · Pluripotency · Shoot regeneration · Senescence · Aging

✉ Manish N. Raizada
raizada@uoguelph.ca

[1] Department of Plant Agriculture, University of Guelph, Guelph, ON N1G 2W1, Canada

Introduction

Understanding the relationship between plant tissue aging and pluriopotency remains of great interest (Gurtner *et al.* 2007) and affects the improvement of vegetative propagation of agricultural and horticultural crops (Preece 2003; Ikeuchi *et al.* 2016). In general, younger somatic cells are more readily stimulated to dedifferentiate into pluripotent stem cells than are those from older tissue (Steeves and Sussex 1989; Conboy and Rando 2005; Chen *et al.* 2014; Rasmussen *et al.* 2015; Quach Mason *et al.* 2017). Data from mice suggests the maintenance of a youthful, non-committed state associated with pluripotency involves epigenetic DNA modifications and specific transcription factors that prevent cell differentiation while permitting proliferation (Boyer *et al.* 2006; Surani *et al.* 2007). The connection between aging and pluripotency remains poorly understood, but the loss of pluripotency has been observed to accelerate in humans cells cultured *in vitro* (Rubin 2002).

Many plant cells remain pluripotent throughout development, a characteristic that has formed the basis of several *in vitro* regeneration studies (Steeves and Sussex 1989). Two classes of phytohormones, auxins and cytokinins, synergistically promote organ regeneration (Christianson and Warnick 1983; Su *et al.* 2011; Pulianmackal *et al.* 2014). Both have been directly implicated in age-dependent changes in cell pluripotency (Rasmussen *et al.* 2015; Zhang *et al.* 2015; Chen *et al.* 2014), but exact roles are unknown. The

mechanisms by which these and other plant hormones act can be studied *in vitro* with synthetic plant growth regulators (PGRs). During normal development, auxins, which are often supplemented during some *in vitro* studies with synthetic auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) promote cell expansion and tissue patterning as unidirectional morphogens (Bhalerao and Bennett 2003). Various types of cytokinins, *e.g.*, 6-furfuryladenine (kinetin) promote cell division by directly up-regulating cyclins (Riou-Khamlichi *et al.* 1999) and shoot meristem-specifying genes (Rupp *et al.* 1999; Schaller *et al.* 2014). The combined effect can be an inhibition of programmed organ death, or senescence (Gan and Amasino 1995).

The transgene *35S-IPT161* confers overexpression of isopentenyl transferase, the rate-limiting enzyme of cytokinin biosynthesis (Gan and Amasino 1995). Overexpression of this gene can delay senescence in *Arabidopsis thaliana* (Gan and Amasino 1995) and may serve as a useful tool to examine the relationship between aging, cytokinin, and regeneration.

While both classes of PGRs discussed above are individually important for organogenesis, their respective ratio is also critical to the nature of development. A high auxin to cytokinin ratio promotes *de novo* root organ development from differentiated somatic cells. In contrast, a high cytokinin to auxin ratio promotes shoot organogenesis and provides the rationale behind the components of shoot induction media (SIM). An intermediate cytokinin to auxin ratio promotes unorganized cell division, leading to a tumor-like callus referred to as a neoplasm (Christianson and Warnick 1983). Numerous reports suggest that the PGRs in callus induction media (CIM) promote dedifferentiation and increase shoot and root regeneration in multiple species (Christianson and Warnick 1983, 1985; Hicks 1994; Sugiyama 1999; Che *et al.* 2002; Che *et al.* 2006). A few days of high auxin/low cytokinin exposure (CIM) are thought to make detached tissues "competent" for regeneration.

Availability of well-characterized mutants and cell-identity reporters makes *Arabidopsis* an excellent model for studying molecular mechanisms of stem cell regeneration and age-dependent loss of pluripotency (Banno *et al.* 2001; Che *et al.* 2002; Ohtani and Sugiyama 2005; Che *et al.* 2006; DeCook *et al.* 2006; Konishi and Sugiyama 2006; Xu *et al.* 2006; Zhang *et al.* 2015). During normal development, shoots and leaves are initiated from a population of organized shoot apical meristem (SAM) cells. These meristematic cells express a suite of critical regulators, including the homeodomain-containing transcription factor WUSCHEL (WUS) (Mayer *et al.* 1998) and the short signaling peptide CLAVATA3 (CLV3) (Fletcher *et al.* 1999). WUS and CLV3 interact to maintain stem cells in an undifferentiated, controlled state of proliferation (Schoof *et al.* 2000). The presence and ratio of PGRs are thought to influence these and other meristem regulators (Shani *et al.* 2006; Gordon *et al.* 2009; Cheng *et al.* 2010; Somssich *et al.* 2016). In *Arabidopsis*, overexpression of *WUS* in combination with auxin was shown to induce ectopic adventitious shoot formation from intact transgenic roots (Gallois *et al.* 2004). WUS (and therefore undifferentiated SAM) can be visualized directly *in planta* with the transgenic marker WUS::NLS-GUS.

In the present study, the regenerative potential of the embryonically derived cotyledon (seed leaf) was systematically analyzed throughout the aging process. The cotyledon is an ideal model system because its age can be easily measured as days after seed germination (DAG). Tissue is available within a few days following germination, allowing for rapid experiments with reduced time needed to test environmental influences, and less variation caused by competing organs (*e.g.*, shading). This research builds upon the previous observation that shoot regeneration potential in *Arabidopsis* ecotype C24 decreased 3.5-fold when donor cotyledons reached a threshold age of 6 DAG (Zhao *et al.* 2002).

In this report, it is demonstrated that during cotyledon development of another ecotype, Landsberg *erecta* (Ler-0), experiences two temporal intervals when pluripotency is lost. Along with tracking callus growth rate, these intervals were examined in a cytokinin-overexpressing transgenic line (carrying *35S-IPT161*), and *via* post-detachment expression of the shoot apical meristem (SAM) marker WUS::NLS-GUS.

Materials and methods

Plant material and growth Single-seed descended, wild-type *A. thaliana* ecotypes Landsberg *erecta* (Ler-0, WT-4) and Nossen-0 (WT-9) were used (Lehle Seeds, Round Rock, TX). Where noted, an alternative Ler-0 stock (CS20) was obtained from the ABRC Stock Centre (Ohio State University, Columbus, OH). Wild-type C24 (CS906) and *35S-IPT161* (C24 inbred, CS117) were also from ABRC. Columbia-0 (Col-0) transgenic seed stock WUSCHEL::NLS-GUS was courtesy of T. Laux (University of Freiburg, Freiburg, Germany); the reporter was introgressed for four generations into Ler-0 (Lehle).

Only seeds <2 yr. old were utilized in this study. Surface sterilization was performed in 15% (*v/v*) Clorox® (Oakland, CA) bleach (5.25% NaClO) for 30 s followed by a sterile water rinse, immersion in 70% (*v/v*) ethanol for 30 s, and then five final rinses in sterile water. Seeds were immersed in sterile water and cold-treated in the dark at ~4°C for 2–7 d, depending on the ecotype, to promote uniform germination. Seeds were then suspended in 0.1% (*v/v*) agar (BD Biosciences, Franklin Lakes, NJ) to facilitate pipet-mediated, even-placement on germination medium in 100 × 25 mm Petri dishes. The germination medium was composed of 2.2 g L^{-1} MS + B5 (M404, PhytoTechnology Laboratories, Overland

Park, KS), 0.97 g L^{-1} MES (M825, PhytoTechnology Laboratories), 10 g L^{-1} sucrose (1% [*w/v*] final concentration), pH 5.7 adjusted with KOH, and 3.0 g L^{-1} Phytagel™ (P8169, Sigma-Aldrich®, St. Louis, MO). All reagents were added prior to autoclaving at 121°C for 35 min. Seeds were individually positioned into a grid pattern with a Pasteur pipet (26 evenly spaced spots surrounding a 100-mm-diameter circle) to ensure homogenous micro-growth environments (*e.g.* nutrient accessibility). Plates were sealed with Micropore™ tape (1530-1, 3M Company, Maplewood, MN) during growth. Germination was conducted indoors without any sources of natural sunlight at 25°C under 24-h continuous light (50–80 μmol m^{-2} s^{-1}, F72T12CW/VHO cool white fluorescent lamps, Osram Sylvania Inc., Wilmington, MA) for 6–7 d.

Cotyledon regeneration assay and analysis Cotyledon detachment occurred at 6 d after germination (DAG) unless otherwise noted. The injury procedure involved detaching cotyledons (excluding the petiole) with fine forceps. Large, small, or chlorotic cotyledons were not selected. Explants were positioned with the abaxial surface in contact with induction medium when possible, but a pilot experiment suggested this was unimportant. Detached tissues were placed onto either high cytokinin/low auxin, 4.4 μM 6-(γ,γ-Dimethylallylamino) purine (2-ip) and 0.5 μM NAA, Shoot Induction Medium (SIM) (default) or high auxin/low cytokinin, 0.1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D, Callus Induction Medium (CIM) (with a CIM pretreatment if noted) in Petri dishes as described above. SIM (Zhao *et al.* 2002) consisted of 20 g L^{-1}glucose (2% [*w/v*] final concentration), 0.5 g L^{-1}MES, regular strength Gamborg's B5 with vitamins (G5893, Sigma-Aldrich®) and 3.0 g L^{-1} Phytagel™ (pH 5.8 by KOH addition). All PGRs were added after media were autoclaved at 121°C for 35 min and cooled to 55°C. Basal medium was prepared using the same recipe as SIM, but lacking PGRs.

The default regeneration consisted of 14 d on SIM followed by a one-time transfer to fresh SIM for the remaining 3 wk. to keep the nutrient and PGR content high. The CIM pretreatment was for 4–5 d, followed by SIM for 10 d, and then a final transfer onto fresh SIM. Again, plates were sealed with Micropore tape. Plates containing PGRs were kept at 4°C until use; plates older than 1 mo were discarded. Post-detachment regeneration conditions were 24 h of low-light (cool white fluorescent light ~20 μmol m^{-2} s^{-1}) in a 23°C growth chamber (Conviron, Winnipeg, Canada) set to 50% relative humidity, where tissues were kept until scoring. Tissues with precocious regeneration 1 wk post-injury were noted and/or removed as these were assumed to contain residual SAM cells. Continuous randomization of plates was performed during regeneration to buffer against local light or air-flow effects. All work was conducted in a laboratory without natural light, and tissues were never exposed to sunlight during transportation. When possible, growth bulbs more than 1 yr old were replaced to reduce experimental variation in tissue culture.

Regeneration was scored 5 wk post-detachment, unless otherwise noted. Regeneration was scored for number of explants with at least one shoot (defined as a minimum of two cotyledon leaves), total number of shoots on each plate, and number of explants with at least one root. In many cases, the mass of the regenerating explants (dissected for organ type) was also recorded. The number of explants and replicates was reported separately for each experiment. To measure cotyledon lengths, detached cotyledons were placed onto a clear Petri dish, with a ruler below, prior to transfer onto regeneration medium.

GUS staining and scoring Ler-0 WUSCHEL::NLS-GUS cotyledons of different ages were detached and placed first on CIM for 5 d and then on SIM for 2 d. After this 7-d period, cotyledons were GUS-stained using a protocol adapted from Jefferson *et al.* (1987). Specifically, tissues were permeabilized with 90% (*v/v*) acetone pre-chilled for 20 min at −20°C, rinsed with 0.1 M $NaPO_4$ (pH 7.2) for 10 min at 23°C, and incubated under 24-h light (20 μmol m^{-2} s^{-1}) in GUS buffer [50 mM $NaPO_4$ (pH 7.0), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10 mM Na_2EDTA (pH 7.0)] containing 1 mg mL^{-1} 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronic acid (X-Gluc) substrate (B7300, Biosynth AG, Staad, Switzerland) dissolved in 1% (*v/v*) *N*,*N*-dimethylformamide (DMF) (D4551, Sigma-Aldrich®) at 37°C for 3–4 h. After GUS staining, cotyledons were rinsed once with 23°C fixing solution (70% [*v/v*] ethanol, 5% [*v/v*] acetic acid) before being fully submerged in fixing solution and stored at 4°C. Fixing solution was replaced with 70% (*v/v*) ethanol 2–3 d after staining to clear the tissues, with the ethanol replaced as necessary to permit complete clearing. Except if otherwise noted, reagents were from Sigma-Aldrich®. Tissues were scored using a light microscope (Stemi™ DV4, Carl Zeiss Inc., Oberkochen, Germany) with ×20 magnification after they were sufficiently cleared (~10–14 d after GUS staining).

Ploidy analysis Nuclei were isolated by adapting the protocol of Arumuganathan and Earle (1991). Cotyledons from approximately 25 *Arabidopsis* plantlets were excised and placed in 65 × 15 mm Petri dishes (on wet ice) containing 1 mL of Solution A (15 mM HEPES, 1 mM EDTA, 80 mM KCl, 30 mM NaCl, 300 mM sucrose, 0.2% (*v/v*) Triton™ X-100, 0.5 mM spermine, and 1% [*w/v*] PVP). The pH was adjusted to 7.5 with NaOH (Bino *et al.* 1992). Cotyledon tissues were then chopped using a new, one-sided razor blade for 1.5 min and subsequently passed through a 30-μm Nitex® nylon mesh filter (03–30/18, Sefar, Heiden, Switzerland) into a 1.5-mL microcentrifuge tube. Each tube was centrifuged at 16,100×*g* for 4 s and the supernatant was discarded. The pellet was resuspended in 300 μL Solution B (3 mL Solution A plus

7.5 μL of 10 mg mL^{-1} RNAse). Samples were incubated at 37°C for 15 min after the addition of 10 μL of 1 mg mL^{-1} propidium iodide. All reagents were from Sigma-Aldrich® unless otherwise noted.

A Coulter EPICS Elite ESP flow cytometer (Beckman-Coulter, Fullerton, CA) equipped with a 15-mW argon laser (emission = 488 nm) was used for flow cytometric measurements. Samples were analyzed using a 100-μm flow cell tip with a sheath pressure of 82.7 kPa. Forward light and 90° side scatter signals were collected using 488-nm dichroic and band pass filters positioned in front of the photomultiplier tube. Both peak and integrated red fluorescence signals were measured with a 640-nm dichroic filter, followed by a 610-nm band pass filter. Analysis was conducted with Coulter's Elite Software (version 4.01). Analytical gates were used to minimize debris signals. A total of 3000 nuclei were analyzed per sample.

Statistical analysis All analyses were performed in GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). Where the data exhibited normality, as identified with Bartlett's test, means were compared with Dunnett's or Tukey's multiple means comparisons ($P < 0.05$) as indicated in the figure legends. Dunn's multiple means comparisons ($P < 0.05$) were used where data exhibited non-normality. Šidák's multiple comparisons ($P < 0.05$) were applied to natural log-transformed data where indicated.

Results

Ler-0 cotyledons lost shoot regeneration potential 4–6 d after germination When younger and older cotyledons (4 to 14 DAG) were detached and first placed on high auxin/low cytokinin-containing CIM for 4 d, and then high cytokinin/low auxin-containing SIM for 30 d, differences in shoot organ regeneration were observed. The shoot regeneration from 4 DAG Ler-0 cotyledons was 5-fold higher than from 6 DAG cotyledons, as scored 5 wk after injury (Fig. 1*a*–*g*). The mass of the regenerating shoot and callus tissues was also 4-fold higher in the youngest cotyledons (Fig. 1*e*). There was no significant decrease in shoot regeneration in No-0 cotyledons (Fig. 1*b*–*g*), as all ages displayed a regeneration frequency of >90%. The results (Fig. 1*a*) suggested that a 48-h developmental time window (between 4 and 6 DAG) may be critical for Ler-0 shoot regeneration potential.

Callus proliferation potential declined in older cotyledons In addition to the effects on shoot regeneration shown for young (<6 DAG) Ler-0 cotyledons, a second, dramatic phenotype associated with older cotyledons was observed. All Ler-0 cotyledons detached at 4 or 8 DAG formed significant callus due to cell proliferation (Fig. 2*a*), whereas up to 25% of older cotyledons (14 DAG) failed to undergo any visible signs of post-detachment proliferation or expansion (Fig. 2*b*), even 5 wk following exposure to regeneration plant growth regulators (PGRs). These cotyledons had all been cultured on CIM for 4 d, followed by 30 d on SIM, and were part of the same experiment shown in Fig. 1.

Older Ler-0 cotyledons expressed WUSCHEL::NLS-GUS In the cotyledon age experiments (Fig. 1), detached cotyledons were pretreated with CIM (high auxin) for 4–5 d, a presumptive dedifferentiation treatment, followed by 4 wk of SIM (high cytokinin/low auxin). Simultaneously, the regeneration assay was repeated without the CIM pretreatment (Fig. 3*a*–*d*). In comparison to the non-pretreated tissues (Fig. 3*b*), 4-d-old Ler-0 cotyledons responded positively to the CIM dedifferentiation treatment, whereas cotyledons at least 48 h older did not (Fig. 3*a*). In contrast, Nossen-0 cotyledons regenerated shoots at >90% frequency with and without the CIM pretreatment (Fig. 3*c*–*d*).

To test the hypothesis that older Ler-0 cotyledons lose competency to dedifferentiate, post-detachment expression of the shoot apical meristem marker WUS::NLS-GUS was examined in young and old Ler-0 cotyledons pretreated with CIM. WUS::NLS-GUS was equally induced after detachment and exposure to CIM PGRs in both young (4 DAG) and old (12 DAG) cotyledons (Fig. 3*e*–*i*). In intact plants, GUS expression was only localized to the SAM, with no expression found in non-detached cotyledons (Fig. 3*e*; data not shown).

Since the WUS transgene was introgressed from a different ecotype (Col-0) for 4 generations, the stock was not purely Ler-0. Therefore, as a control, the transgene-carrying Ler-0 stock was assayed for the age-dependent decline in shoot regeneration observed in wild-type Ler-0. Detached cotyledons were exposed to the shoot regeneration protocol (5 d of CIM, 4 wk of SIM). Whereas young cotyledons (detached 4 DAG) regenerated shoots at 34% frequency, older cotyledons (12 DAG) displayed 0% shoot regeneration. Interestingly, 32% of cotyledons detached 8 DAG did regenerate shoots (two replicates, $n = 25$ cotyledons per replicate, data not shown), while less than 10% frequency was observed in the non-hybrid Ler-0. Therefore, the decline in shoot regeneration during aging of the WUS::NLS-GUS stock was similar but delayed as compared to the wild-type Ler-0.

Cotyledon growth potential and pluripotency showed a positive relationship in ecotype Ler-0 For a subset of plants (from Fig. 1), cotyledon length was measured as an indicator of growth (and age) after detachment and before placement onto regeneration medium. In ecotype Ler-0, cotyledons substantially grew between day 4 and day 8. From 4 to 6 DAG, cotyledons grew by 260%, while cotyledons measured on 6 DAG grew only 44% more (Fig. 4*a*). Thus, when detached cotyledons showed maximum ability to regenerate shoots, at 4 DAG, they also showed their greatest growth potential while still on the

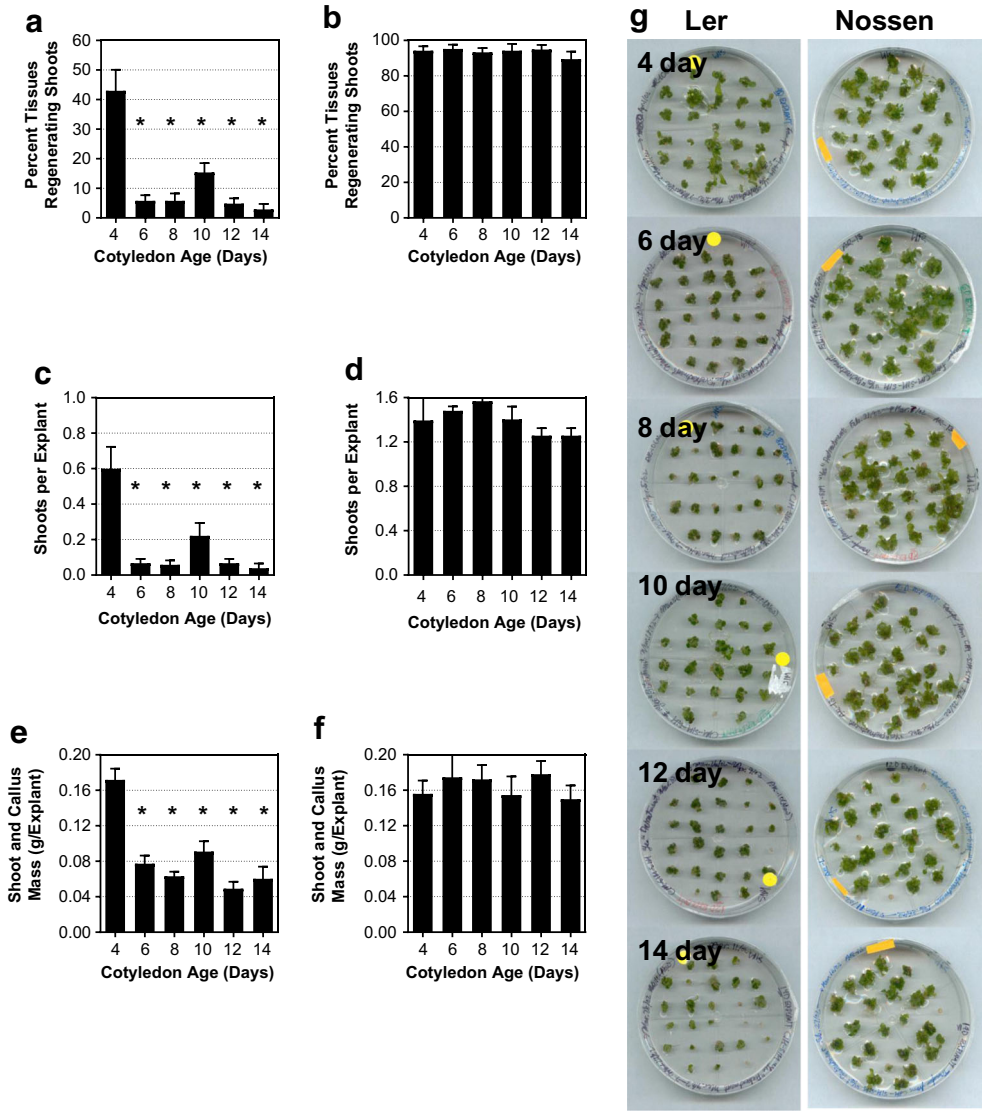

Figure 1. Loss of totipotency in cotyledons related to age at the time of detachment in two ecotypes: Shoot regeneration ability loss at a younger age. Uniformly-sized cotyledons from ecotypes Landsberg *erecta*, Ler-0 (*a, c, e*) and Nossen-0, No-0 (*b, d, f*) were detached at different d after germination (DAG) and then pretreated with high auxin/low cytokinin (callus-inducing medium, CIM) for 5 d prior to placement on high cytokinin/low auxin (shoot induction medium, SIM). Histograms showing the mean of four replicates for the number of cotyledons that regenerated shoots 5 wk after being detached in (*a*) Ler-0 and (*b*) No-0 were calculated as a percentage of the total number of explants subjected to treatment; the average number of regenerated shoots per explant for (*c*) Ler-0 and (*d*) No-0; and the average fresh weight of dissected, regenerated callus and shoot tissue for (*e*) Ler-0 and (*f*) No-0. Each replicate was one petri plate containing 26 cotyledons; the total experiment was $n = 2786$ cotyledons; +/− standard deviation (SD). *Asterisks* above separate cotyledon ages indicate significant difference ($P < 0.05$) compared to the cotyledons detached at 4 DAG, as identified by Dunnett's multiple comparisons. (*g*) Corresponding pictures of one representative plate for each treatment are shown.

plant. Furthermore, by the age at which callus failure was first observed (10–14 DAG), non-detached cotyledons had stopped growing (Fig. 4*a*). These results suggested that cotyledon growth potential and pluripotency are positively correlated in ecotype Ler-0. In contrast, 12–14 d No-0 cotyledons remained highly competent to regenerate shoots, despite having lost growth potential (Fig. 4*b*).

Cytokinin overexpression improved shoot regeneration and callus induction in older cotyledons The effect on regeneration and callus induction of the transgene *35S-IPT161*, which causes increased cytokinin biosynthesis, inhibits leaf senescence and increases leaf longevity (Gan and Amasino 1995; Lara *et al.* 2004; Kim *et al.* 2006) was investigated in detached cotyledons. This transgene is present in ecotype C24, which also showed a decline in regeneration due to cotyledon age. The transgene increased the frequency of shoot regeneration in ecotype C24 above that in wild type (Fig. 4*c*), improving shoot regeneration in transgenic cotyledons detached 8 DAG to a level approaching younger (4

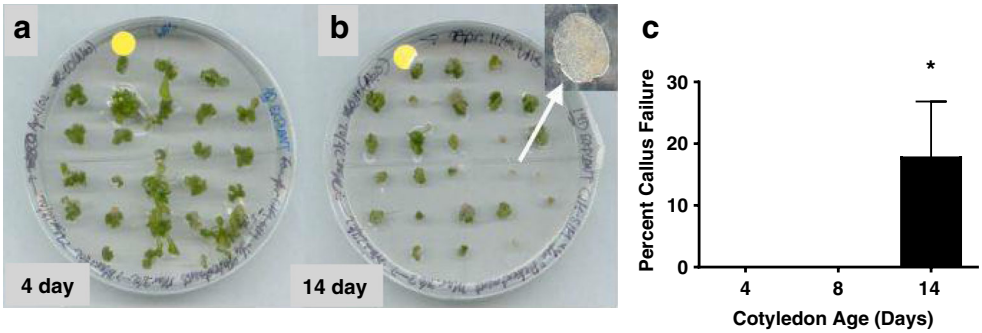

Figure 2. Loss of totipotency in cotyledons related to age at the time of detachment in two ecotypes: Cell proliferation ability loss at an older age in Ler-0 cotyledons. (*a*) Regeneration response of 4-d-old detached Landsberg *erecta* (Ler-0) cotyledons (Lehle Seeds) and (*b*) 14-d-old Ler-0 cotyledons, 4 wk after detachment. (*b*) Shows that a subset of cotyledons failed to induce visible post-injury mitosis (magnified in inset image). (*c*) Callus failure of Ler-0 cotyledons from an additional source (ABRC, CS20) was quantified. Cotyledons were pretreated with CIM for 4 d followed by 30 d on SIM. Tissues were exposed to cool white fluorescent light (20–30 μmol m^{-2} s^{-1}). Shown are the means of four replicates (n = 26 per replicate) +/− SD. The *asterisk* above the separate cotyledon ages indicates significant difference ($P < 0.05$) with the other two age treatments, as identified with Dunn's multiple comparison.

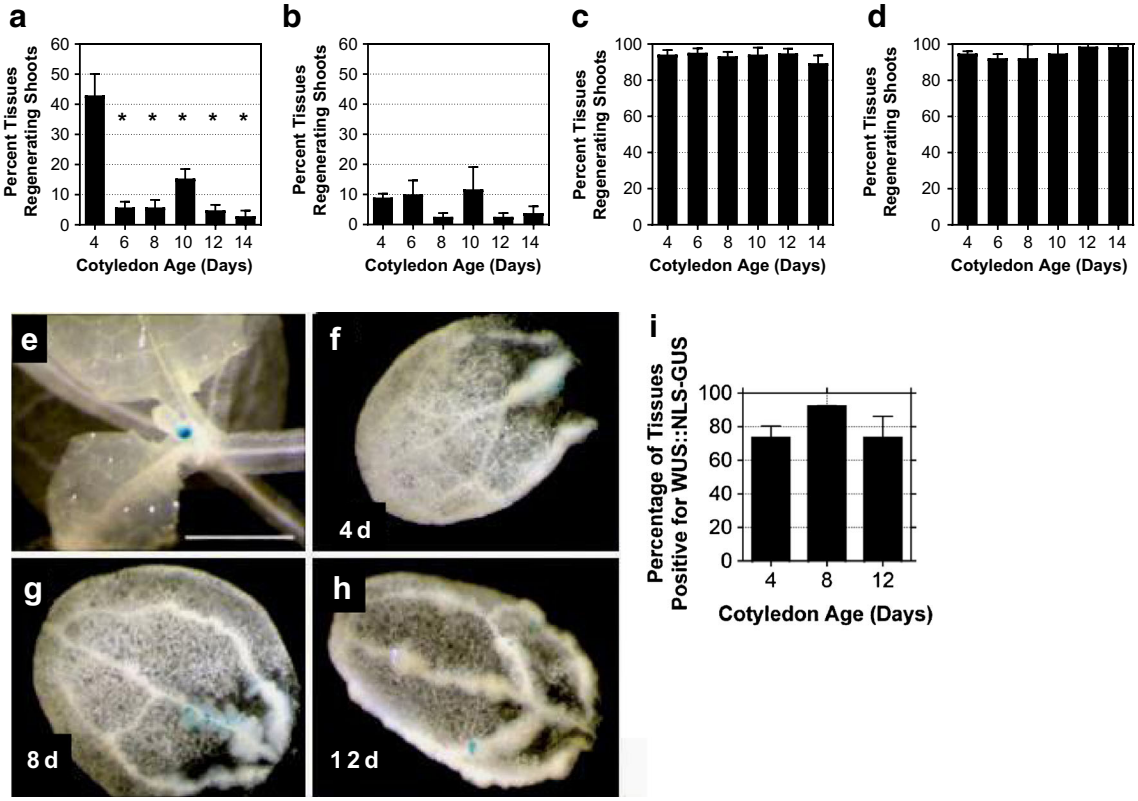

Figure 3. Shoot regeneration response of detached Ler-0 cotyledons to a presumptive dedifferentiation treatment (high auxin/low cytokinin, CIM) and expression of the shoot meristem reporter, WUSCHEL::NLS-GUS. (*a–d*) Shoot regeneration in response to plant growth regulators. Following detachment, Ler-0 and No-0 cotyledons were either first pretreated with CIM for 5 d followed by 4 wk of shoot induction media (SIM; high cytokinin/low auxin) or SIM alone. (*a*) Ler-0, CIM + SIM. (*b*) Ler-0, SIM alone. (*c*) Nossen-0, CIM + SIM. (*d*) Nossen-0, SIM alone. (*e–i*) Post-detachment expression of the shoot apical meristem marker WUS::NLS-GUS in Ler-0 cotyledons pretreated with CIM (5 d) and then SIM (2 d). WUS::NLS-GUS expression in (*e*) intact Ler-0 seedlings, (*f*) cotyledon detached 4 DAG, (*g*) cotyledon detached 8 DAG, and (*h*) cotyledon detached 12 DAG. (*i*) The percentage of Ler-0 cotyledons (detached at different DAG) expressing WUS::NLS-GUS 7 d after detachment was quantified. Non-detached cotyledons did not express WUS::NLS-GUS (data not shown). In panels, (*a–d*), *histograms* represent the mean of four replicates (n = 26 per replicate) +/− SD. (*a*, *c*) are reproduced from Fig. 1; the experiments in (*b*) and (*d*) were performed simultaneously with those in (*a*) and (*c*). Total experiment n = 2496 cotyledons. *Histograms* represent the mean of three replicates (each replicate n = 26 cotyledons) +/− SD. *Asterisks* above separate cotyledon ages indicate significant difference ($P < 0.05$) with the cotyledon detached 4 DAG, as identified with Dunnet's multiple comparisons, or with Dunn's multiple comparisons where non-normality was identified with Bartlett's test.

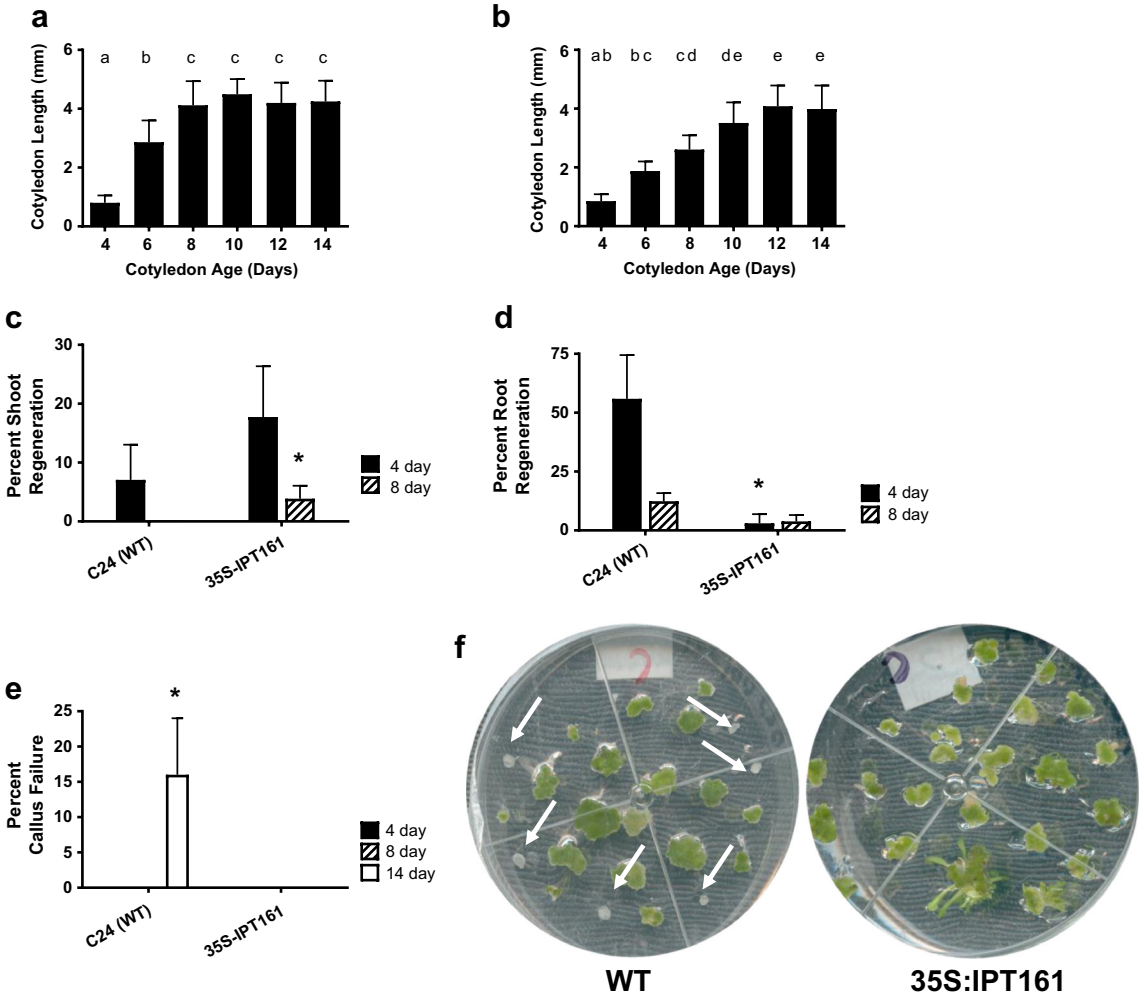

Figure 4. Cotyledon growth and the effects of a senescence-delaying transgene on regeneration. Growth rates for (*a*) Ler-0 (Lehle Seeds) and (*b*) No-0 cotyledons. The base-to-tip lengths of the cotyledons used in Figs. 1 and 2 were measured before being placed onto regeneration media ($n = 20$). *Different letters* above separate cotyledon ages indicate significant difference ($P < 0.05$) as identified with Tukey's multiple comparisons or Dunn's multiple comparisons where non-normality was indicated by Bartlett's test. (*c–f*) The effect of delayed leaf senescence on regeneration using the cytokinin-overproducing transgene *35S-IPT161*. Shown are the effects of the transgene on (*c*) shoot regeneration, (*d*) root regeneration, and (*e–f*) callus failure following cotyledon detachment in ecotype C24. (*f*) A representative picture shows cell proliferation failure (noted by *arrows*) in wild-type C24 explants (*left plate*), but no failures in the cytokinin-overexpressor (*right plate*). For (*c–f*), detached cotyledons were treated with 5 d of CIM medium, followed by 30 d of SIM medium. Each *histogram* represents the mean of four replicates ($n = 26$ per replicate) +/− SD. In (*c–e*), *asterisks* indicate significant difference ($P < 0.05$) between C24 and the cytokinin-overexpressing line for individual ages as identified with Šidák's multiple comparisons of the log-transformed data.

DAG) wild-type cotyledons. However, the shoot regeneration ability of the cytokinin-overexpressing line did decline as the cotyledons aged, with a ~5-fold decrease in shoot regeneration as the tissue aged from 4 to 8 DAG. Therefore, as a control to ensure that the cytokinin-overexpressing construct remained functional in older cotyledons, root regeneration in young and old cotyledons was measured (predicted to remain diminished in the presence of a higher cytokinin to auxin ratio). Root regeneration was almost non-existent in both young and old transgenic cotyledons (Fig. 4*d*), demonstrating that the transgene was still inducing high cytokinin levels even as the cotyledons aged.

The effect of *35S-IPT161* on callus failure was also tested. The non-transgenic C24 control displayed significant failure to induce callus in cotyledons detached 14 DAG (Fig. 4*e–f*). All transgenic cotyledons harvested 14 DAG induced callus (Fig. 4*e–f*). Combined, these results suggested that cytokinin over-expression could fully rescue the late stage (callus failure) and partially rescue the early stage (shoot regeneration) age-related pluripotency decline.

Discussion

Pluripotency in the cotyledons of *A. thaliana* ecotype Ler-0 was lost in two distinct temporal stages Cotyledons from Ler-0, but not No-0, progressively lost shoot regeneration ability during a critical 48–60 h interval early in development (4 to 6

DAG; Fig. 1*a–e*), earlier than previously observed in ecotype C24 (Zhao *et al.* 2002). The results (Fig. 1*a*) suggested that a 48-h developmental time window (between 4 and 6 DAG) was critical for Ler-0 shoot regeneration potential. Cotyledon growth potential in intact seedlings and regeneration post-detachment were observed to be positively related in ecotype Ler-0, but not No-0 (Fig. 4*a, b*). It was concluded that age caused Ler-0 cotyledons to experience a progressive loss in pluripotency during a 48–60 h interval in early cotyledon development, whereas during this same interval, pluripotency in No-0 was age-independent. Later in development, a subset of older Ler-0 cotyledons failed to develop callus (Fig. 2), the first step in the regenerative process (Christianson and Warnick 1983). Loss of post-detachment cell proliferation potential may be a general phenomenon of older cotyledons.

The role of WUSCHEL and auxin in age-related loss of pluripotency Young (4 DAG) Ler-0 cotyledons regenerated after exposure to a high auxin treatment (CIM) thought to promote dedifferentiation, while cotyledons that were 48 h older did not. This result suggested that Ler-0 cotyledons lose competency to respond to a high auxin/low cytokinin plant growth regulator (PGR) treatment during aging (Fig. 3*a*, *b*). However, WUSCHEL::NLS-GUS (a marker for cells associated with an undifferentiated state in the shoot apical meristem) showed expression in both young and old Ler-0 cotyledons (Fig. 3*e–i*), suggesting that the old-age block in shoot development occurs downstream or parallel to WUSCHEL. In No-0, both young and old cotyledons were largely insensitive to the CIM (high auxin) pretreatment (Fig. 3*c, d*).

The results also suggested that age-dependent declines in shoot pluripotency may be caused in part by a reduction in response to auxin (CIM pretreatment) by Ler-0 cotyledons as they age (Fig. 3*a, b*). Evidence that auxin is a central organizer of new embryo and organ formation includes the observation that tissue-localized auxin concentrations peak during early embryogenesis (Ribnicky *et al.* 2002; Friml *et al.* 2003; Jenik and Barton 2005). Because auxin is transported unidirectionally *via* asymmetric distribution of PIN protein efflux carriers and can form quantitative gradients, auxin is thought to be a morphogen of tissue and organ polarity (Benkova *et al.* 2003; Bhalerao and Bennett 2003; Pan *et al.* 2015). Interestingly, ectopic WUS expression can redirect root cells to two different fates: shoot organogenesis (leaves) or somatic embryogenesis, depending on whether auxin is present (Gallois *et al.* 2004). Exposure to high levels of auxin (CIM) has been discussed as making plant cells more competent to regenerate (Christianson and Warnick 1983), perhaps by stimulating dedifferentiation (Banno *et al.* 2001; Che *et al.* 2006). Use of the polar auxin transport inhibitor, TIBA, has shown that there is a critical time interval when auxin is required to gain this competence (Christianson and Warnick 1984).

The data presented here indicate that aging can reduce the ability of a tissue to regenerate in response to auxin. The age-dependent decline in Ler-0 shoot regeneration may not be due to a decline in dedifferentiation, but rather a post-WUSCHEL block in shoot apical meristem or shoot formation. Alternatively, there may be a high auxin-induced dedifferentiation pathway acting in parallel to, or after WUSCHEL. For example, auxin has been linked to increased activity of AINTEGUMENTA (ANT) and other transcription factors (Yamaguchi *et al.* 2016), which promote pluripotency into old age and enhance the persistence of cell division (Mizukami and Fischer 2000; Nole-Wilson *et al.* 2005; Bandupriya *et al.* 2014; Horstman *et al.* 2015).

Neither leaf senescence nor cell ploidy are critical for shoot regeneration ability Shoot regeneration potential may have declined rapidly in Ler-0 because of organ senescence (Chandlee 2001). Here, the lengths of cotyledons were measured as an indication of growth, age, and senescence (Mizukami and Fischer 2000) prior to placement on regeneration medium. Growth of Ler-0 cotyledons continued until 8 DAG (Fig. 4*a*), indicating that the cotyledons were not yet senescent at the time of the lowest shoot regeneration rate (6 DAG, Fig. 1*a*). The growth of No-0 cotyledons was delayed and more gradual (Fig. 4*b*) than Ler-0. When growth potential was lost, No-0 cotyledons continued to regenerate shoots, suggesting that no general statements can be made about the relationship between the growth potential of cotyledons and their regenerative ability. In Ler-0, there was a correlation between callus failure and growth cessation leading to the hypothesis that these two traits may be functionally linked in that ecotype (Fig. 2; Fig. 4*a*). In future studies, additional indicators of cotyledon senescence, including reactive oxygen species (ROS) accumulation in growing cotyledons or signs of programmed cell death (PCD), may be measured.

Another possibility is that older cells may not regenerate or form callus because they might be terminally differentiated. Cell ploidy is a marker of cell differentiation (Caro *et al.* 2012). Differentiating leaf cells endo-reduplicate, resulting in increased ploidy. Therefore, in a preliminary experiment (Fig. 5), the frequency of diploid cells was measured as a marker for meristematic potential, compared to polyploid cells as a marker for differentiation. Although older cotyledons increased in ploidy, contrary to expectation, the diploid fraction remained higher in Ler-0 cotyledons than in No-0 during the critical 4–7 DAG interval (Fig. 5*a*). Furthermore, both ecotypes, not only Ler-0, experienced a dramatic increase in polyploidy from 4–7 DAG (only peak ploidy at 16N is shown, Fig. 5*b*). These results suggest that ploidy may have little bearing on the regeneration potential of *Arabidopsis* cotyledons.

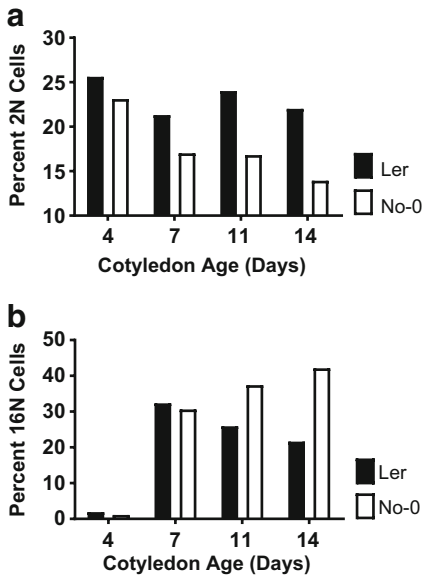

Figure 5. Cotyledon ploidy and differentiation. Preliminary measurements of ploidy as a marker for organ differentiation during the aging of Ler-0 (Lehle) and No-0 cotyledons ($n = 26$). Shown are percentages of cells that were (*a*) diploid (2N) and (*b*) 16N. 16N was the peak polyploidy observed and thus represents a marker for terminal cell differentiation as opposed to potentially younger mitotic cells (2N).

The role of cytokinin in regeneration potential The cytokinin biosynthesis transgene *35S-IPT161* increased overall shoot regeneration in ecotype C24, perhaps the result of an increased cytokinin to auxin ratio (Christianson and Warnick 1983). However, a dramatic (5-fold) decrease in regeneration ability was still observed in the overexpression line between 4 to 8 DAG (Fig. 4*c*), indicating that cytokinin cannot bypass the age-dependent loss of shoot regeneration ability. Transgenic cotyledons experienced no callus failure even at 14 DAG, a dramatic result compared to the non-transgenic control (Fig. 4*e, f*), indicating that cytokinin can bypass (or delay) the late-stage loss of pluripotency. The presence of cytokinin might have upregulated cell division (Riou-Khamlichi *et al.* 1999; Schaller *et al.* 2014), and/or shoot meristem-specifying genes directly (Rupp *et al.* 1999; Schaller *et al.* 2014). There is evidence that cytokinin signaling proteins are upregulated during early regeneration events in *Arabidopsis* (Che *et al.* 2002; Mahonen *et al.* 2006; Besnard *et al.* 2014).

Conclusions

The results demonstrated that there was variation among *Arabidopsis* ecotypes for the loss of pluripotency of detached cotyledons. This loss of pluripotency occurred at two distinct temporal stages in the cotyledons of ecotype Ler-0. In the early interval, Ler-0 cotyledons lost the ability to respond to a high synthetic auxin treatment previously hypothesized to promote dedifferentiation as a first step for shoot regeneration. At the later stage, cytokinin overexpression could rescue (or delay) callus failure. Cotyledon senescence, ploidy, or competency to express WUSCHEL were not found to be determinants in the age-dependent loss of pluripotency. Unlocking the mystery of age-dependent loss of regeneration could potentially improve *in vitro* propagation of plants.

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References

Arumuganathan K, Earle E (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9:229–233

Bandupriya HDD, Gibbings JG, Dunwell JM (2014) Overexpression of coconut *AINTEGUMENTA-like* gene, *CnANT*, promotes *in vitro* regeneration of transgenic *Arabidopsis*. Plant Cell Tissue Organ Cult 116:67–79

Banno H, Ikeda Y, Niu QW, Chua NH (2001) Overexpression of Arabidopsis ESR1 induces initiation of shoot regeneration. Plant Cell 13:2609–2618

Benkova E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591–602

Besnard F, Refahi Y, Morin V, Marteaux B, Brunoud G, Chambrier P, Rozier F, Mirabet V, Legrand J, Laine S, Thevenon E, Farcot E, Cellier C, Das P, Bishopp A, Dumas R, Parcy F, Helariutta Y, Boudaoud A, Godin C, Traas J, Guedon Y, Vernoux T (2014) Cytokinin signaling inhibitory fields provide robustness to phyllotaxis. Nature 505:417–424

Bhalerao RP, Bennett MJ (2003) The case for morphogens in plants. Nat Cell Biol5:939–943

Bino RJ, Devries JN, Kraak HL, Vanpijlen JG (1992) Flow cytometric determination of nuclear replication stages in tomato seeds during priming and germination. Ann Bot 69:231–236

Boyer LA, Mathur D, Jaenisch R (2006) Molecular control of pluripotency. Curr Opin Genet Dev 16:455–462

Caro E, Desvoyes B, Gutierrez C (2012) GTL1 keeps cell growth and nuclear ploidy under control. EMBO J 31:4483–4485

Chandlee JM (2001) Current molecular understanding of the genetically programmed process of leaf senescence. Physiol Plant 113:1–8

Che P, Gingerich DJ, Lall S, Howell SH (2002) Global and hormone-induced gene expression changes during shoot development in Arabidopsis. Plant Cell 14:2771–2785

Che P, Lall S, Nettleton D, Howell SH (2006) Gene expression programs during shoot, root, and callus development in Arabidopsis tissue culture. Plant Physiol 141:620–637

Chen X, Qu Y, Sheng L, Liu J, Huang H, Xu L (2014) A simple method suitable to study *de novo* root organogenesis. Front Plant Sci 5:208

Cheng ZJ, Zhu SS, Gao XQ, Zhang XS (2010) Cytokinin and auxin regulates *WUS* induction and inflorescence regeneration *in vitro* in *Arabidopsis*. Plant Cell Rep 29:927–933

Christianson ML, Warnick DA (1983) Competence and determination in the process of *in vitro* shoot organogenisis. Dev Biol 95:288–293

Christianson ML, Warnick DA (1984) Phenocritical times in the process of *in vitro* shoot organogenesis. Dev Biol 101:382–390

Christianson ML, Warnick DA (1985) Temporal requirement for phytohormone balance in the control of organogenesis *in vitro*. Dev Biol 112:494–497

Conboy IM, Rando TA (2005) Aging, stem cells and tissue regeneration—lessons from muscle. Cell Cycle 4:407–410

DeCook R, Lall S, Nettleton D, Howell SH (2006) Genetic regulation of gene expression during shoot development in Arabidopsis. Genetics 172:1155–1164

Fletcher LC, Brand U, Running MP, Simon R, Meyerowitz EM (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science 283:1911–1914

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jurgens G (2003) Efflux-dependant auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426:147–153

Gallois JL, Nora FR, Mizukami Y, Sablowski R (2004) WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem. Genes Dev 18:375–380

Gan SS, Amasino RM (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. Science 270:1986–1988

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. Proc Natl Acad Sci U S A 106:16529–16534

Gurtner GC, Callaghan MJ, Longaker MT (2007) Progress and potential for regenerative medicine. Annu Rev Med 58:299–312

Hicks GS (1994) Shoot induction and organogenesis *in vitro*—a developmental perspective. In Vitro Cell Dev Biol - Plant 30P:10–15

Horstman A, Fukuoka H, Muino JM, Nitsch L, Guo C, Passarinho P, Sanchez-Perez G, Immink R, Angenent G, Boutilier K (2015) AIL and HDG proteins act antagonistically to control cell proliferation. Development 142:454–464

Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: cellular origins and molecular mechanisms. Development 143: 1442–1451

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jenik PD, Barton MK (2005) Surge and destroy: the role of auxin in plant embryogenesis. Development 132:3577–3585

Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, Lee IC, Sheen J, Nam HG, Hwang I (2006) Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis. Proc Natl Acad Sci U S A 103:814–819

Konishi M, Sugiyama M (2006) A novel plant-specific family gene, ROOT PRIMORDIUM DEFECTIVE 1, is required for the maintenance of active cell proliferation. Plant Physiol 140:591–602

Lara MEB, Garcia MCG, Fatima T, Ehness R, Lee TK, Proels R, Tanner W, Roitsch T (2004) Extracellular invertase is an essential component of cytokinin-mediated delay of senescence. Plant Cell 16: 1276–1287

Mahonen AP, Bishopp A, Higuchi M, Nieminen KM, Kinoshita K, Tormakangas K et al (2006) Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development. Science 311: 94–98

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell 95:805–815

Mizukami Y, Fischer RL (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. PNAS 97:942–947

Nole-Wilson S, Tranby TL, Krizek BA (2005) *AINTEGUMENTA*-like (*AIL*) genes are expressed in young tissues and may specify meristematic or division-competent states. Plant Mol Biol 57:613–628

Ohtani M, Sugiyama M (2005) Involvement of SRD2-mediated activation of snRNA transcription in the control of cell proliferation competence in Arabidopsis. Plant J 43:479–490

Pan X, Chen J, Yang Z (2015) Auxin regulation of cell polarity in plants. Curr Opin Plant Biol 28:144–153

Preece JE (2003) A century of progress with vegetative plant propagation. Hortscience 38:1015–1025

Pulianmackal AJ, Kareem AVK, Durgaprasad K, Trivedi ZB, Prasad K (2014) Competence and regulatory interactions during regeneration in plants. Front Plant Sci 5:142

Quach Mason M, Goron TL, Arnold ED, Dickinson P, Kajenthira A, Dinka SJ, Oro R, Raizada MN (2017) Pyramid screening: combining three genetic screens into one efficient screen for shoot regeneration mutants in *Arabidopsis thaliana*. J Plant Growth Regul. doi: 10.1007/s00344-016-9651-9

Rasmussen A, Hosseini SA, Hajirezaei MR, Druege U, Geelen D (2015) Adventitious rooting declines with the vegetative to reproductive switch and involves a changed auxin homeostasis. J Exp Bot 66: 1437–1452

Ribnicky DM, Cohen JD, Hu WS, Cooke TJ (2002) An auxin surge following fertilization in carrots: a mechanism for regulating plant totipotency. Planta 214:505–509

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science 283:1541–1544

Rubin H (2002) The disparity between human cell senescence *in vitro* and lifelong replication *in vivo*. Nature Biotechnol 20:675–681

Rupp HM, Frank M, Werner T, Strnad M, Schmulling T (1999) Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. Plant J 18:557–563

Schaller GE, Street IH, Kieber JJ (2014) Cytokinin and the cell cycle. Curr Opin Plant Biol 21:7–15

Schoof H, Lenhard M, Haecker A, Mayer KFX, Jurgens G, Laux T (2000) The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100:635–644

Shani E, Yanai O, Ori N (2006) The role of hormones in shoot apical meristem function. Curr Opin Plant Biol 9:484–489

Somssich M, Je BI, Simon R, Jackson D (2016) CLAVATA-WUSCHEL signaling in the shoot meristem. Development 143:3238–3248

Steeves TA, Sussex IM (1989) Patterns in plant development, 2nd edn. Cambridge University Press, New York

Su YH, Liu YB, Zhang XS (2011) Auxin-cytokinin interaction regulates meristem development. Mol Plant 4:616–625

Sugiyama M (1999) Organogenesis *in vitro*. Curr Opin Plant Biol 2:61–64

Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. Cell 128:747–762

Xu J, Hofhuis H, Heidstra R, Sauer M, Friml J, Scheres B (2006) A molecular framework for plant regeneration. Science 311:385–388

Yamaguchi N, Jeong CW, Nole-Wilson S, Krizek BA, Wagner D (2016) AINTEGUMENTA and AINTEGUMENTA-LIKE6/PLETHORA3 induce LEAFY expression in response to auxin to promote the onset of flower formation in Arabidopsis. Plant Physiol 170:283–293

Zhang TQ, Lian H, Tang H, Dolezal K, Zhou CM, Yu S, Chen JH, Chen Q, Liu H, Ljung K, Wang JW (2015) An intrinsic microRNA timer regulates progressive decline in shoot regenerative capacity in plants. Plant Cell 27:349–360

Zhao QH, Fisher R, Auer C (2002) Developmental phases and STM expression during Arabidopsis shoot organogenesis. Plant Growth Regul 37:223–231