Transcriptome Analysis of Age-Related Gain of Callus-Forming Capacity in Arabidopsis Hypocotyls

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Callus-forming capacity is enhanced with hypocotyl maturity in Arabidopsis. However, the genetic regulation of age-related gain in capacity for callus formation is unclear. We used a gene expression microarray assay to characterize the underlying mechanisms during callus formation in young and mature hypocotyl explants of Arabidopsis. As expected, genes involved in photosynthesis and cell wall thickening showed altered expression during hypocotyl maturation. In addition, genes involved in cytokinin perception were enriched in mature hypocotyl tissues. Phytohormone-induced callus formation in hypocotyl explants was accompanied by increased expression of genes mainly related to the cell cycle, histones and epigenetics. The induction level of these genes was higher in mature hypocotyl explants than young explants during callus formation. We identified a number of genes, including those with unknown function, potentially involved in age-related gain in callus formation. Our results provide insight into the effect of hypocotyl age on callus formation. Altered cytokinin signaling components, cell cycle regulation and epigenetics may work in concert to lead to gain of callus-forming capacity in hypocotyls with age.

Keywords: Age • Callus formation • Hypocotyls • Microarray.

Abbreviations: ARC, age-related change; CDK, cyclin-dependent kinase; CIM, callus-inducing medium; CYC, cyclin; FDR, false discovery rate; GO, gene ontology; GUS, β-glucuronidase; PCA, principal component analysis; RT–PCR, reverse transcription–PCR; VIM1, variant in methylation 1.

The microarray data described in this study have been deposited in the Gene Expression Omnibus (accession No. GSE27508).

Introduction

Plants go through developmental stages, or phases, during their life cycle. Three major phases have been described: young vegetative, mature vegetative and reproductive (Poethig 1990). Characteristics that appear early during development are referred to as young and those that appear late are referred to as mature. In Arabidopsis, the morphological and physiological traits of the successive organs vary with age (Robbelen 1957, Martinez Zapater et al. 1995, Gendreau et al. 1997, Chaffey et al. 2002, Kus et al. 2002). The morphological changes are easy to recognize in leaves and hypocotyls. The first two young leaves of Arabidopsis have rounded lamina with smooth margins and long petioles, whereas leaves in mature stages have oval lamina with serrated margins and short petioles (Robbelen 1957, Martinez Zapater et al. 1995). In addition, the secondary xylem of the hypocotyl develops in two phases: an early phase (young), in which only vessel elements develop, and a later stage (mature), with both vessel elements and fibers (Gendreau et al. 1997, Chaffey et al. 2002).

Age-related changes (ARCs) in plants occur as a result of the differential regulation of developmental processes. ARCs are involved in diverse cellular processes, including morphological processes, physiological processes, metabolism and responses to environmental cues (Bond 2000, Kus et al. 2002). An example of a process that depends on ARCs in Arabidopsis is age-related pathogen resistance (Kus et al. 2002). Furthermore, ARCs are involved in photosynthetic properties and ethylene-induced leaf senescence in Arabidopsis (Bond 2000, Jing et al. 2005). ARCs are involved in callus-forming capacity in Sequoia sempervirens and Sequoia obvallata (Huang et al. 1992, Jing et al. 2005). Callus-forming capacity is better in mature explants or cuttings of these plants than in young explants. However, little information is available on the role of ARCs in callus-forming capacity and the underlying mechanisms.

Callus development starts with a cell fate regression, whereby plant tissues regain the competency for cell division and organ regeneration (Skoog and Miller 1957, Hicks 1994). Callus consists of unspecialized cells, and growth and metabolic conditions can be controlled exogenously. Callus formation may be associated with reprogramming of gene expression and cellular metabolism (Konishi and Sugiyama 2003, Che et al. 2006). Callus cells are often used for investigating...
biochemical properties of plant functions and propagating clones by in vitro tissue culture and production of useful metabolites (Pasqua et al. 2003, Paek et al. 2005). In Arabidopsis, in vitro procedures for the regeneration of shoots or adventitious roots from hypocotyl explants are well established and typically involve callus formation (Konishi and Sugiyama 2003, Che et al. 2006). All these events can be induced under the control of exogenous phytohormones (Skoog and Miller 1957). Hypocotyl explants are pre-cultured on auxin-rich callus-inducing medium (CIM) to trigger callus formation. The callus-forming capacity varies considerably with the callus induction conditions and the genetic and epigenetic nature of explants (Che et al. 2006, Pischke et al. 2006, Krizova et al. 2009). The characteristics of explants, such as cell types and age, also modulate callus formation ability (Saxena et al. 1997, Dhar and Joshi 2005).

Phytohormones such as auxin and cytokinin are crucial signaling molecules that control cell proliferation and affect cell identities during in vitro tissue culture (Cary et al. 2001, Nordstrom et al. 2004, Rieffer et al. 2006, Teale et al. 2006, Sugimoto et al. 2010). The balance between auxin and cytokinin controls callus formation and organ regeneration (Skoog and Miller 1957, Cary et al. 2001). Exposing callus cultures to high levels of cytokinin supports shoot formation, whereas a high concentration of auxin promotes rooting. In Arabidopsis, genomic gene expression and proteomic profiling have identified groups of components that are molecular signatures of the different developmental processes during callus formation and organ regeneration (Che et al. 2006, Yin et al. 2007). Among the endogenous developmental signals, cytokinin has a particularly profound effect on cell division and longevity of plant organs. The auxin–cytokinin interaction regulates the formation and maintenance of meristems (Lopez-Juez et al. 2008), so their cross-talk is important to control developmental processes during organ regeneration in vitro and meristem formation for establishing the whole plant body.

The culture of various tissues with hormones in vitro induces callus formation by stimulating cell growth, DNA replication and cell division (Menges et al. 2005, Pischke et al. 2006). Hormonal signaling networks can influence cell division parameters and establish functional links between the regulatory mechanisms of the cell cycle and genes involved in callus formation (Pischke et al. 2006). Cyclin-dependent protein kinases (CDKs) and their regulatory cyclin subunits (CYCs) play a central role in cell cycle control (Dewitte and Murray 2003, Inze and De Veylder 2006). CDKA;1 expression reflects the state of competence for cell division (Hermel et al. 1993) whereas CYCB1;1 expression is linked to cell division (Colon Carmona et al. 1999). Phytohormone-stimulated callus formation and cell cycle reactivation in Arabidopsis hypocotyls are associated with the capacity to express CDKA;1 and CYCB1;1 (Huang et al. 2003, Ohnari and Sugiyama 2005). Furthermore, Kip-related proteins (KIPs) regulate both DNA synthesis and mitosis during the cell cycle by binding and inhibiting CDKs with their conserved CDK-binding domain (De Veylder et al. 2001, Verkest et al. 2005). Thus, modulation of the expression of cell cycle-regulated genes during callus formation determines the competence and activity for cell division.

Epigenetic mechanisms control chromatin structures and functions, including DNA methylation and histone modifications (Vaissière et al. 2008). These epigenetic changes are involved in cellular differentiation in many developmental aspects and also regulate the cellular plasticity and reprogramming necessary for callus formation (Pischke et al. 2006, Dennis and Peacock 2007, Berdasco et al. 2008, Tanurdzic et al. 2008, Ay et al. 2009, Rodriguez-Lopez et al. 2010). In Arabidopsis, both callus and cell suspensions possess a unique epigenetic signature with subsets of genes whose expression is controlled by promoter hypomethylation and hypermethylation (Berdasco et al. 2008, Tanurdzic et al. 2008). Epigenetic modifications may be involved in habituated callus cultures (Pischke et al. 2006). In addition to reprogramming gene expression and cell cycle re-entry, epigenetic perturbations may be associated with callus formation.

The hypocotyl of Arabidopsis young seedlings is the embryonic stem that forms the connection between the two cotyledons and the seedling root. Most, if not all, cells of the hypocotyl are developed in the embryo. Therefore, cells of hypocotyl tissues are mostly differentiated post-germination and are perpetuated throughout the entire life cycle (Vandenbussche et al. 2005). The morphological simplicity of the hypocotyl makes it an excellent model system to study the effects of age on callus-forming capacity (Gendreau et al. 1997).

Here, we established an in vitro experimental system to study the role of ARCs in callus-forming capacity in Arabidopsis hypocotyl explants. The regions that formed callus in young and mature explants were characterized. We used transcriptome-based analysis to explore genome-wide expression changes underlying the mechanism of ARCs in callus formation of young and mature explants. We investigated the genes responsible for hormone homeostasis, cell cycle regulation and epigenetic modifications during ARCs in callus formation and probed the potential roles of these mechanisms in gain of callus-forming capacity by explant age. These studies lay the basis for further molecular dissection of genes that regulate ARCs in callus formation of Arabidopsis.

Results

Callus-forming capacity is greater in mature than in young Arabidopsis hypocotyl explants

To investigate the effect of hypocotyl age on callus formation ability, we used an in vitro tissue culture system with Arabidopsis (Col-0) hypocotyls from plants at different growth stages: 1 week (7–10 d old), 3 weeks (21–24 d old), 5 weeks (35–38 d old) and 7 weeks (49–52 d old). Early germinating seedlings (1 week: young) had emerged above the culture medium and had a pair of cotyledons. The young seedlings then passed through a vegetative phase to production of true
leaves and entered the mature phase (5 weeks: mature). Hypocotyl segments 5–7 mm long were excised from plants of different ages (1–7 weeks), then were transferred to a CIM containing auxin for 0, 10 and 20 d to induce callus formation.

Callus formation was greater in mature (5 weeks) than young hypocotyl (1 week) explants with 20 d auxin treatment, especially at 10 d after CIM incubation (Fig. 1), and the fresh weight of mature hypocotyl-derived calli was significantly greater in mature than in young explants (Supplementary Fig. S1).

Anatomy of hypocotyl-derived callus formation from young and mature Arabidopsis

To characterize the regions that formed callus in young and mature explants, we examined cross-sections of hypocotyl explants. Hypocotyl tissue derived from young (1-week-old) and mature (5-week-old) plants was defined as Y1 and M0, respectively (Fig. 2), and those cultured on CIM for 2 d were defined as Y2 and M2, respectively. Before CIM culture, mature hypocotyls had more cells in the stele than did young hypocotyls (Fig. 2). After CIM culture, young and mature tissue showed many cytoplasm-rich cells with visible nuclei in the stele, which were not present before culture. Thus, active cell division was induced within 2 d of CIM culture to produce cytoplasm-rich cells in both young and mature hypocotyl explants. From the distribution of cytoplasm-rich cells, CIM culture induced cell division at least in the pericycle (the outermost layer of the stele) of both young and mature hypocotyl explants. The cytoplasm-rich cells were also present in the inner stele cells in CIM-treated mature hypocotyls.

Microarray assay of genes enriched in young and mature Arabidopsis hypocotyl tissues

To better understand the molecular basis of changes in the callus-forming capacity of Arabidopsis hypocotyls at different ages, we used transcriptome analysis by microarray assay of hypocotyl tissues from young and mature plants (Fig. 3). A stringent threshold was expression change >2-fold and false detection rate (FDR) <0.05 for determining significant differences. We found 2,787 genes with >2-fold differential mRNA expression between young and mature hypocotyl tissues immediately before callus induction (Supplementary Table S1). Clustering the differentially expressed transcripts produced young and mature hypocotyl-enriched gene sets (1,303 and 1,484, respectively).

To evaluate the potential functions of genes enriched in young and mature hypocotyl tissues, we assigned gene ontology (GO) categories of biological processes to the two gene sets (Supplementary Table S2), and then compared the GOs by semantic similarity-based scatterplots with use of REVIGO (Fig. 4). GO analysis of young hypocotyl-enriched genes revealed that most were involved in generating precursor metabolites and energy, photosynthesis and response to auxin (Fig. 4A). Other categories were unidimensional cell growth, macromolecular complex assembly and cell wall loosening (Supplementary Table S3). GO analysis of mature hypocotyl-enriched genes revealed diverse functional categories (Supplementary Table S2). The predominant functional themes were secondary metabolic processes, ion transport and lignin metabolic processes (Supplementary Table S3). In addition, transcripts were involved in response to toxins and oxidative stress, as well as cell wall thickening (Fig. 4B). We then analyzed various hormone signaling pathways (Supplementary Tables S4, S5). The expression of most auxin-related genes was down-regulated in mature hypocotyls (Fig. 5A), but components involved in cytokinin perception and signaling were up-regulated (Fig. 5B). Therefore, the predominance of the inferred biological roles reflects the molecular basis of the growth status for young and mature hypocotyl tissues.

Transcriptome analysis of age-related changes in callus-forming capacity

To gain insight into the molecular mechanisms underlying the age-related changes in callus formation in Arabidopsis, we used microarray assay to analyze transcriptional programs during phytohormone-induced callus formation in young and mature hypocotyl explants. Callus formation is induced after auxin treatment for 1 d (Huang et al. 2003), so we extracted total RNA from young and mature hypocotyl explants cultured on CIM for 1 d. We compared DNA microarray data from treated and untreated hypocotyl tissues by principal component analysis (PCA) to identify similarities or differences in overall gene expression between the samples. PCA is a mathematical technique used to summarize and visualize the features of the variance in complete microarray data prior to clustering of a set of arbitrarily selected genes (Yeung and Ruzzo 2001, Ringnér, 2009). PCA is used to project high-dimensional, global expression data onto the three principal components. The closer two points are in the plot, the more similar the samples are in terms
of their global gene expression profile (Fig. 3B). The first and third principal components (x- and z-axes) separated auxin-treated hypocotyl tissues from untreated samples (Fig. 3). The second principal component (y-axis) showed a further separation between young and mature hypocotyls. For each tissue type, the three biological replicate samples clustered closely together, which suggested that they were a reliable representation of the transcript profile of their respective tissues. These results illustrated strong homogeneity in the pattern of gene expression for each tissue type.

We found 5,379 and 5,167 genes with differential expression after CIM treatment for young and mature hypocotyl explants, respectively (Supplementary Tables S6, S7). To search further for genes whose expression mirrored age-related changes in callus formation, we compared the expression profiles of genes with changed expression and clustered their functions by GO analysis. A significant proportion of genes with expression modulated by age-related gain of cell division functioned in cell cycle progression and DNA replication, and many other genes with changed expression were involved in chromatin assembly (Table 1).

Expression of cell division-related genes during age-related callus formation

As expected, comparison of treated and untreated young and mature hypocotyl explants revealed significant up-regulation of genes involved in cell division-related processes, including those encoding CYCs, CDKs and histones (Supplementary Fig. S2A). In general, induction of CYC and CDK genes was relatively greater for mature (M1 vs. M0) than young (Y1 vs. Y0) explants, and genes encoding histones were induced to a higher level in mature than in young explants (Supplementary Fig. S2B). The fold induction of cell division-related genes was >3.5 for mature explants (M1 vs. M0) but 1.4–3.0 for young explants (Y1 vs. Y0). Detailed information is given in Supplementary Tables S8 and S9.

Verification of gene expression patterns from microarray data

Transcriptional regulation revealed by microarray data was confirmed by reverse transcription–PCR (RT–PCR) analysis of mRNA expression. We selected 10 genes related to cell cycle machinery for RT–PCR and detected the accumulation of all 10 CYC genes during phytohormone-stimulated callus formation, but the kinetics and levels of expression varied (Supplementary Fig. S3). Most A- and B-type CYC transcripts accumulated to a higher level in mature than in young explants cultured on CIM for 1 and 2 d. The expression of D-type CYC genes was mostly elevated in both young and mature explants cultured on CIM for 1 d (Supplementary Fig. S3). The RT–PCR results were verified by three replications, and all showed nearly identical gene expression patterns (Supplementary Fig. S3). The induction of A- and B-type CYC genes was mostly attributed to callus formation, as was seen with a temperature-sensitive callus-forming mutant, srd2 (Ohtani and Sugiyama 2005). The mRNA expression of CYCA and CYCB was greater at a permissive (22°C) than at a restrictive induction temperature (28°C) with CIM culture (Supplementary Fig. S4). Hence, the expression profiles

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**Fig. 2** Cellular details of hypocotyl-derived callus formation in young (1-week-old) and mature (5-week-old) Arabidopsis hypocotyl explants. Hypocotyl tissues from young (Y0) and mature (M0) plants and young and mature plants cultured on CIM for 2 d (Y2 and M2, respectively) were stained with toluidine blue O. The lower row shows magnification of the stele. Red arrows indicate cytoplasm-rich cells with visible nuclei at the pericycle layer and inner stele region in Y2 and M2. Stars denote cells at the endodermis. Cp, cortex parenchyma; En, endodermis; Ep, epidermis; St, stele.
of A- and B-type CYC genes showed good agreement between the RT–PCR and microarray results. These results confirmed the overall reliability of the microarray expression data.

Next, we examined the expression of CYCB1;1 in young and mature hypocotyl explants cultured on CIM by using transgenic Arabidopsis plants harboring a fusion reporter construct (CYCB1;1-GUS) linking the CYCB1;1 promoter region and partial coding sequence to a β-glucuronidase (GUS) reporter gene (Colon Carmona et al. 1999). CYCB1;1-GUS exhibited distinct spatial and temporal expression patterns during the early stages of callus formation in young and mature explants. GUS expression was induced much later in young than in mature explants. Only
trace GUS expression began to be detected in young hypocotyl explants after 48 h of CIM incubation (Fig. 6). Therefore, the increase in mitotic activity was more rapid in mature than in young explants. The rapid increase in mitotic activity in mature explants agreed with the enhanced potential for callus formation. The rapid increase in mitotic activity was more rapid in mature than in young explants. The rapid increase in mitotic activity in mature explants after 48 h of CIM incubation (Fig. 6). Therefore, the increase in mitotic activity was more rapid in mature than in young explants. The rapid increase in mitotic activity in mature explants agreed with the enhanced potential for callus formation.

Identification of genes potentially involved in age-related gain of callus-forming capacity

Genes of a similar expression pattern may share a similar function. Clustering gene expression profiles can be used for tentative assignment of functional annotation (Eisen et al. 1998). To identify the genes potentially involved in age-related gain in capacity for callus formation, we used microarray assay to search for a gene cluster with expression patterns similar to that of cell division-related genes (Supplementary Figs. S2, S3). According to the induction intensity of the cell division-related genes, we used the gene-filtering criteria of 1.4- to 3-fold change in young explants (Y1 vs. Y0) and minimum 3.5-fold change in mature explants (M1 vs. M0) with CIM incubation. The pre-processing and filtering steps revealed genes that might be involved in age-related gain of callus formation. We grouped 294 putative genes (positive factors) by GO analysis. The largest class of genes was those involved in cell division and DNA replication, which suggested that age-related gain of callus-forming capacity was accompanied by the induction of cell division-related genes (Fig. 7A). Cell division-related GO terms were significantly enriched among differentially expressed genes (Fig. 7A). These identified genes (positive factors) were further classified into regulatory genes, encoding proteins responsible for signal transduction, transcriptional regulation and epigenetics; and functional genes, involved in DNA replication, cytokinesis and cell wall modification (Supplementary Table S10). Detailed information on these regulatory and functional genes is provided in Supplementary Table S11. We found 15 putative regulatory genes associated with age-related gain of callus-forming capacity (Supplementary Table S10). This group contains genes belonging to transcription factors such as MYB3R4 (AT5G11510) and HMGB6 (AT5G23420). The regulatory group included VIM1 (AT1G57820) and VIM6 (AT4G08590), encoding proteins responsible for epigenetic modification (Supplementary Table S10).

We identified genes with unannotated biological processes and unknown functions as potential candidates involved in age-related gain of callus-forming capacity (Supplementary Table S11). To facilitate the interpretation of genes with unknown functions, we retrieved tissue-specific expression patterns for these genes from the Arabidopsis eFP browser. A large proportion of the genes with unknown functions (21 of 50) were expressed predominantly in tissues with active cell division, such as shoot apex (Supplementary Fig. S5). Moreover, GeneMANIA analysis revealed that these genes with unknown functions were co-expressed with those related to cell cycle progression, epigenetics and signal transduction (Supplementary Table S10). Thus, these unknown genes may play roles in cell cycle progression, epigenetics and signal transduction, which are potentially involved in age-related gain of callus-forming capacity.

In addition to screening putative positive regulatory factors, we screened negative factors potentially involved in age-related gain of callus formation by the same threshold criteria used for the positive factors but with decreased gene expression. The negative factors were selected by a 0.3- to 0.7-fold change in young explants and maximum 0.28-fold change in mature explants with CIM treatment. We identified 176 probe sets as...
putative negative factors of age-related gain of callus formation (Supplementary Table S11). The genes encoding the negative factors were involved in response to abiotic and biotic stimuli, ion transport and peptide transport (Fig. 7B). GO analysis revealed that they were predominantly involved in responses to stresses such as cold and water deprivation. Therefore, stress-related genes were down-regulated in age-related gain of callus formation in Arabidopsis hypocotyl explants.

### Discussion

ARCs have been described in perennial woody plants and in annual herbaceous species such as Arabidopsis (Huang et al. 1992, Jing et al. 2005). Accumulating evidence has suggested that ARCs occur in diverse biological processes, including biomass production, disease resistance, hormone homeostasis and organogenesis (Hamann 1998, Kus et al. 2002, Van Der Graaff et al. 2006). For example, loss of ability to regenerate roots by stem cuttings or explants in vitro routinely occurs during maturation in various woody plant species (Hamann 1998, Kumar Jha et al. 2002). Our Arabidopsis hypocotyl explants showed an age-related decrease in ability to form adventitious roots (Supplementary Fig. S6). Thus, the decrease in adventitious root-forming potential is characteristic of the maturation phase in both woody species and Arabidopsis (Busov et al. 2004). ARCs have been demonstrated in callus formation (Huang et al. 1992, Dhar and Joshi 2005), so we wondered whether age-related gain of callus-forming capacity also existed in an annual herbaceous plant such as Arabidopsis. We found callus-forming capacity significantly better in mature Arabidopsis hypocotyl explants than in young explants (Fig. 1). Age-related gain in callus-forming capacity in Arabidopsis hypocotyl explants was consistent with that observed in S. sempervirens (Huang et al. 1992), which implies a similarity in the underlying molecular regulation.

ARCs in Arabidopsis hypocotyls have been well investigated in terms of morphological features (Chaffey et al. 2002), but little is known about the molecular characterization during hypocotyl maturation. We used genome-wide transcriptome
The biological pathways enriched in our young or mature hypocotyls were in good agreement with those observed for physiological and morphological traits (Chaffey et al. 2002). In mature hypocotyl tissues, the cork cambium is well developed, and the vascular cambium could be identified by periclinal cortical cell divisions (Chaffey et al. 2002). Our pathway analysis revealed higher relative expression of cell cycle-related genes in young than in mature hypocotyls (Supplementary Table S1). Cell cycle activation in young hypocotyl tissues may be an early developmental program, leading to formation of vascular cambium during hypocotyl maturation.

The location of cells that form callus in response to auxin has been reported in mature hypocotyl explants of loblolly pine (Pinus taeda) (Greenwood et al. 2001). In mature hypocotyl explants, cambium cells exhibit radial swelling after auxin treatment and became similar in appearance to parenchyma cells (Greenwood et al. 2001). Thereafter, mitotic activity spreads centrifugally out into the cortex. Dividing callus cells are evident near the former cambium and become widespread in cortical regions. In young hypocotyls of Arabidopsis plants (1-week-old), the calli generally originate in the pericycle opposite the protoxylem poles (Atta et al. 2009). We found callus formation initiated mainly from the pericycle cell layers in both young and mature Arabidopsis hypocotyl explants (Fig. 2). In mature hypocotyls, not only pericycle cells but also inner stele cells might be induced to resume cell division upon CIM culture. Mature hypocotyls contain proliferating cambium mass in the stele (Chaffey et al. 2002), which probably contributed to the difference in gene expression profiles.

Most senescence-associated genes (SAGs) are expressed during natural senescence and are progressively up-regulated over time during Arabidopsis development (Buchanan-Wollaston 1997, Guo et al. 2004). For example, the transcript level of SAG29 (AT5G13170) increases with increasing maturity (See et al. 2010), which is consistent with our findings (Supplementary Table S12). WRKY transcription factors are a large family with 74 members in Arabidopsis and are involved in several developmental processes (Rushton et al. 2010). The expression of WRKYS3 (AT4G23810) and WRKY70 (AT3G64000) genes was found to be increased with developmental age in leaf tissues (Li et al. 2004, Miao et al. 2004, Ay et al. 2009). Our microarray data revealed that these two WRKY genes were induced more strongly in mature than in young hypocotyl tissues. These data suggest similar developmental regulation of these two WRKY transcription factor genes in both leaf and hypocotyl. Metallothionein (MT) expression patterns were found to be up-regulated at the late developmental phase in leaf tissues of bean, Brassica and Arabidopsis (Buchanan-Wollaston 1994, Foley et al. 1997, Garcia-Hernandez et al. 1998). In the current microarray study, MT genes such as MT1c (AT1G07610), MT2a (AT3G09390) and MT3 (AT3G15353) were predominantly expressed in mature hypocotyls (Supplementary Table S12). SAG, WRKY and MT genes in leaf and hypocotyl tissues may be regulated with increasing plant maturity. Hence, the expression of these genes potentially involved in age-related gain of callus-forming capacity. The differentially expressed genes with expression patterns similar to cell division-related genes (positive factors, Y1 vs. Y0 > 1.4–3 and M1 vs. M0 > 3.5, or negative factors, Y1 vs. Y0 > 0.3–0.7 and M1 vs. M0 < 0.28) are plotted with different functional categories highlighted by color coding. The GO analysis was similar to that described in Fig. 4. The list of genes potentially involved in age-related gain of callus-forming capacity is provided in Supplementary Table S11.
genes may be molecular indicators of hypocotyl maturity and facilitate subsequent investigation into age-related gain of callus-forming capacity.

We probed the molecular basis of age-related gain in the capacity for callus formation by integrating two aspects of transcriptomic data from Arabidopsis hypocotyls (Fig. 3): analysis of (i) functional pathways enriched in mature vs. young hypocotyl tissues and (ii) clusters of genes sharing similar expression patterns with cell division-related probe sets during phytohormone-induced callus formation. Notably, genes related to the cytokinin perception and signaling pathway were enriched in mature hypocotyl tissues, along with increased sensitivity to cytokinin (Fig. 5). Several lines of evidence suggest that an increase in cytokinin perception may contribute to enhanced callus-forming capacity (Pischke et al. 2006). Habituated T87 cell cultures have a higher capacity for callus formation than freshly derived callus cultures with low endogenous cytokinin levels, probably because of an increased expression level of the cytokinin receptor AHK4 (AT2G01830) (Pischke et al. 2006). Explants from transgenic Arabidopsis plants overexpressing cytokinin signaling components CKI1 (AT2G47430) or CKI2 (AT5G10720) showed enhanced callus-forming capacity in a cytokinin-independent fashion (Kakimoto 1996, Hwang and Sheen 2001). We found increased gene expression of cytokinin signaling components such as AHK4, AHP1 (AT3G21510), AtPUP10 (AT4G18210) and Type-B ARR10 (AT4G31920) with increased hypocotyl maturity (Fig. 5). Thus, the ability of phytohormone pre-treatment to enhance callus formation in mature hypocotyl explants may be mediated by activation of the cytokinin signaling pathway. The naturally acquired regulation of cytokinin signaling components during hypocotyl maturation may play a vital role in age-related gain of callus-forming capacity.

Cell division-related genes such as those encoding cell cycle regulators and histone proteins play vital roles in callus formation (Menges et al. 2005, Pischke et al. 2006). As anticipated, the expression profiles of these genes we found confirmed the activation of cell division during callus formation (Supplementary Fig. S2). The induction levels of these cell division-related genes were higher in mature than in young hypocotyl explants cultured on CIM (Supplementary Fig. S2). The induction kinetics of cell cycle-related genes were also more rapid in mature than in young hypocotyl explants (Supplementary Fig. S3). For example, A- and B-type CYC expression was increased during age-related gain of callus formation (Supplementary Fig. S3). The results suggest the association of early cell cycle activation and age-related gain of callus-forming capacity.

We found the expression pattern of cell division-related genes unique (Supplementary Figs. S2, S3) and, therefore, ideal for use in identifying other genes potentially involved in age-related gain of callus formation. In addition, the induction of A- and B-type CYC genes was attributed to callus-forming capacity as demonstrated by the srd2 mutant (Supplementary Fig. S4). After applying the gene-filtering steps and statistical clustering analyses, we identified a group of probe sets to gain insight into the age-related gain in callus formation (Fig. 7). These probe sets were assigned to regulatory and functional categories on the basis of their gene annotations (Supplementary Table S10). The transcriptome of hypocotyl explants in Arabidopsis revealed a coordinated response involving both regulatory and functional genes during age-related gain of callus formation. A number of genes belonging to regulatory categories were associated with cell cycle progression and cytokinesis in Arabidopsis (Supplementary Table S10). MYB3R4 (AT5G11510) is a transcription factor for B-type CYCs and regulates cytokinesis in Arabidopsis, thus leading to cell wall formation (Ito et al. 1998, Ito 2000). Another major group of regulatory genes were those encoding S-phase-acting proteins during DNA replication, including MCM7 (AT4G02060), POLA3/POLA4 (AT5G41880) and TS02 (AT3G27060), MKK6 (AT5G56580) participates in the regulatory pathway for cytokinesis (Takahashi et al. 2010). These candidate genes related to cell cycle progression and cytokinesis may contribute to age-related gain of callus-forming capacity.

Previous studies have shown epigenetic modification involved in callus formation (Pischke et al. 2006, Tanurdzic et al. 2008). Genes encoding VIM (variant in methylation) proteins (VIM1 and VIM6) with a regulatory role in epigenetics were identified as possible candidates for conferring age-related gain in callus-forming capacity (Supplementary Table S10). VIM1 is a methylcytosine-binding protein and is implicated in the regulation of chromatin modification, transcription and the cell cycle (Liu et al. 2007, Woo et al. 2007). The vim1 vim2 vim3 triple mutant displayed abnormal morphological phenotypes, including late flowering, traits that are associated with DNA hypomethylation (Woo et al. 2008). Our microarray-based approach expanded our knowledge about VIM and revealed its role in age-related gain of callus-forming capacity. We selected VIM1 with increased induction during callus formation for functional analysis. Preliminary results showed that Arabidopsis vim1 mutants displayed altered age-related gain of callus-forming capacity as compared with the wild type (data not shown). Therefore, epigenetic modification may be a factor associated with age-related gain of callus-forming capacity.

After the gene-filtering process with the cell division-related probe sets, we identified genes with unknown functions as potential candidates involved in age-related gain of callus-forming capacity (Supplementary Table S11). Exploration of Arabidopsis expression data with the Arabidopsis eFP Browser (Winter et al. 2007) revealed that these genes were preferentially expressed in dividing tissues such as shoot apex (Supplementary Fig. S5). Bioinformatics analysis of these unknown genes with GeneMANIA (Warde-Farley et al. 2010) revealed co-expression with genes mainly related to cell cycle progression (Supplementary Table S10). Results from co-expression analysis further supported the cellular localization of the unknown genes in the dividing tissues. For example, genes encoding unknown proteins (AT3G14910, AT3G12870, AT5G48310, AT5G01910, AT4G39630 and AT3G12870) were expressed in the shoot apex and co-expressed with CYCB1;3, CDKB2;1 or CYCA1;1.
In addition, unknown genes such as AT2G27775 and AT1G69070 were co-expressed with genes related to epigenetics and signal transduction. Thus, the unknown genes may play a role in cell division and potentially contribute to age-related gain of callus-forming capacity.

In summary, we investigated the molecular mechanisms underlying age-related gain of callus-forming capacity by microarray analysis of global gene expression in Arabidopsis hypocotyl explants. Enhanced cytokinin perception, early activation of core cell cycle genes and epigenetic regulation may work in concert and play important roles in the callus-forming capacity by age. In mature hypocotyls, both pericycle cells and inner stele cells might be induced to start cell division upon CIM culture. The age-related gain of callus-forming capacity might be attributed to a difference in transcriptomic regulation in the stele. We revealed the molecular basis at the RNA level. Further investigation at the protein or metabolism level is required to elucidate age-related gain of callus-forming capacity. In addition, we identified a number of genes, including those with unknown function, with potential involvement in the callus-forming capacity. Determining their functional significance in the callus-forming capacity by age is of interest. We provide insight into both the molecular regulatory mechanisms of hypocotyl maturation and age-related gain in callus-forming capacity in Arabidopsis.

**Materials and Methods**

**Phytohormone-stimulated callus formation**

*Arabidopsis thaliana* (Col-0) seeds were vernalized for 2 d at 4°C before germination. About 500 seeds were sterilized by 10 min of incubation in 1 ml of 1% (v/v) sodium hypochlorite solution with a few drops of Tween-20. The seeds were then rinsed three times with sterilized water. Surface-sterilized seeds were sown on germination medium (Murashige–Skoog medium, MS519, Sigma) supplemented with 1.0% (w/v) sucrose, buffered to pH 5.7 with 0.05% (w/v) MES and solidified with 1.5% (w/v) agar. Seedlings were grown under a 16 h white light/8 h dark photoperiod at 25 °C with an illumination intensity of 4–6 μmol m⁻² s⁻¹ by placing the Petri dish vertically.

Phytohormone-stimulated callus formation was performed as described (Ozawa et al. 1998) with minor modifications. Hypocotyl segments (5 mm long) were excised immediately above the shoot–root transition zone from Arabidopsis plants of different ages and cultured on CIM. The CIM containing MS salt (Murashige-Skoog medium, MS519) was supplemented with 2.0% (w/v) glucose and 0.5 p.p.m. (w/v) 2,4-D, buffered to pH 5.7 with 0.05% (w/v) MES and solidified with 0.25% (w/v) gellan gum. The hypocotyl-derived explants were cultured on CIM in the dark to induce callus formation. Treating hypocotyl explants on CIM containing only 2,4-D facilitated subsequent microarray analysis and data interpretation. The experiments were repeated at least three times.

**Light microscopy**

Fixation and embedding of Arabidopsis hypocotyl tissues for light microscopy was as described (Chaffey et al. 2002, Juang et al. 2012) with some modification. Hypocotyls from young (1-week-old) and mature (5-week-old) etiolated Arabidopsis plants were cultured on CIM for 2 d. Control samples without 2,4-D treatment were also collected. Hypocotyl tissues were fixed with 2% paraformaldehyde plus 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide. Samples were dehydrated in an alcohol series, embedded in LR white resin (LR White, London Resin Company) and polymerized at 65°C. Transverse sections (800 nm) were cut at the middle region of etiolated hypocotyls by use of an ultramicrotome (Richard-Jung Ultracut S), then stained with fresh 1% toluidine blue O, followed by a quick wash in water. Sections were examined by light microscopy (Axioskop 2, Carl Zeiss), and photomicrographs were captured.

**Microarray analysis**

Total RNA was isolated from Arabidopsis hypocotyl-derived samples with use of an RNeasy Plant Mini Kit (Qiagen), then purified and concentrated by use of the RNeasy MinElute Cleanup Kit (Qiagen). We used three biological replicates grown in the same growth chamber.

Microarray assay involved use of Affymetrix microarrays (GeneChip Arabidopsis ATH1 genome array) containing 22,810 probe sets on a single chip. Labeling, hybridization and data analysis (one sample per chip) were performed according to the manufacturer’s manual. The data were analyzed by use of Microarray Suite v5.0 (MAS 5.0) with Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The raw cell intensity data files (GeneChip CEL files) were imported into GeneSpring vXI software for analysis (Agilent Technologies). Biological replicates were analyzed in triplicate. The data were normalized by use of the Robust Multichip Average (RMA) algorithm and converted to log2 scale for comparing the three biological replicates in each set of experiments. Analysis of variance (ANOVA) was used to detect genes with a statistically significant change in expression. The threshold was set to 0.05 for the P-value. The Benjamini and Hochberg algorithm calculates FDRs that are inherently corrected for multiple testing (Benjamini and Hochberg 1995). Genes were considered as significantly up- and down-regulated if the FDR value for the corresponding probe set was <0.05. To enrich for biologically relevant changes, only genes with at least a 2-fold change in expression were selected. The microarray data have been deposited in the Gene Expression Omnibus (accession No. GSE27508) (Edgar et al. 2002).

**Gene ontology and bioinformatics analysis**

To classify the differentially expressed genes by biological function, we used agriGO (http://bioinfo.cau.edu.cn/agriGO/) and
ReviGO (http://revigo.irb.hr/) (Du et al. 2010, Supek et al. 2011). ReviGO calculates the enrichment of biological functions and summarizes lists of GO terms by removing redundant terms and visualizing the remaining terms in scatterplots, interactive graphs and tag clouds. The relative expression of genes with unknown function was extracted by use of Arabidopsis eFP Browser (http://www.bar.utoronto.ca/) (Winter et al. 2007). Co-expression analysis involved use of GeneMANIA (http://www.genemania.org) (Warde-Farley et al. 2010). The PCA plots of gene dimensions were generated by use of GeneSpring vXI (Yeung and Ruzzo 2001, Ringner 2008). PCA is a method for reducing the multidimensional space down to only a few dimensions. In microarray analysis, the expression of a large number of genes is obtained for several individuals or experimental units. Each gene may be thought of as a factor, variable or component of the individuals. If only two genes were measured, a simple scatter plot of the values of each individual would show the relationship of these two factors (genes). When larger numbers of genes are measured, visualization of the resulting multidimensional space can be achieved by PCA plot.

**Semi-quantitative RT–PCR**

First-strand cDNA was synthesized from 2 μg of total RNA with 1 μl of oligo(dT) primers by use of the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The fragments of cell cycle-related genes were amplified by use of gene-specific primers. Ubiquitin was an internal control. The PCR cycling involved an initial denaturation step at 94°C for 2 min, 27–40 cycles of amplification and a final elongation step at 72°C for 5 min. PCR products were analyzed on a 2% (w/v) agarose gel. The experiments were repeated at least three times for each gene.

**Histochemical localization of GUS activity**

The transgenic Arabidopsis lines harboring the CYCB1;1-GUS construct were used for determining cell division activity (Colon Carmona et al. 1999). GUS histochemical staining was as described (Jefferson 1987). Hypocotyl-derived samples were vacuum infiltrated for 10 min with histochemical GUS buffer containing 0.5 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide in 100 mM sodium phosphate buffer (pH 7.0). Samples were kept in the same solution and incubated at 37°C for 24 h in the dark. Hypocotyl samples were washed three times with sodium phosphate buffer, then incubated in 70% ethanol for 1 week to remove Chl. GUS staining was examined under a light microscope (Optiphot, Nikon). Three independent biological replicates were used for each tissue type.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


