Ethylene Inhibits Cell Proliferation of the Arabidopsis Root Meristem[1][OPEN]

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The root system of plants plays a critical role in plant growth and survival, with root growth being dependent on both cell proliferation and cell elongation. Multiple phytohormones interact to control root growth, including ethylene, which is primarily known for its role in controlling root cell elongation. We find that ethylene also negatively regulates cell proliferation at the root meristem of Arabidopsis (*Arabidopsis thaliana*). Genetic analysis indicates that the inhibition of cell proliferation involves two pathways operating downstream of the ethylene receptors. The major pathway is the canonical ethylene signal transduction pathway that incorporates CONSTITUTIVE TRIPLE RESPONSE1, ETHYLENE INSENSITIVE2, and the ETHYLENE INSENSITIVE3 family of transcription factors. The secondary pathway is a phosphorelay based on genetic analysis of receptor histidine kinase activity and mutants involving the type B response regulators. Analysis of ethylene-dependent gene expression and genetic analysis supports *SHORT HYPOCOTYL2*, a repressor of auxin signaling, as one mediator of the ethylene response and furthermore, indicates that *SHORT HYPOCOTYL2* is a point of convergence for both ethylene and cytokinin in negatively regulating cell proliferation. Additional analysis indicates that ethylene signaling contributes but is not required for cytokinin to inhibit activity of the root meristem. These results identify key elements, along with points of cross talk with cytokinin and auxin, by which ethylene negatively regulates cell proliferation at the root apical meristem.

The root system of plants is responsible for the acquisition of water and soil nutrients and as such, plays a critical role in plant growth and survival (Petricka et al., 2012; Giehl et al., 2014). The architecture of the root system is developmentally plastic of necessity, responding to a changing environment by modifying such characteristics as primary root growth, lateral root density, and lateral root growth. The rate of root growth is determined through the regulation of cell division, differentiation, and elongation (Bennett and Scheres, 2010; Petricka et al., 2012; Giehl et al., 2014). Cell division occurs at the root apical meristem (RAM) comprised of a quiescent center (QC), the surrounding stem cells, and a mitotically active population of derived cells. The meristematic cells eventually cease dividing and transition from a zone of division to one of differentiation and elongation. Not surprisingly, multiple phytohormone signaling pathways interact to control root growth, including the hormone ethylene (Moubayidin et al., 2009; Petricka et al., 2012; Vanstraelen and Benková, 2012).

Ethylene plays critical roles throughout plant growth and development and regulates such diverse processes as cell expansion, senescence, and responses to biotic and abiotic stresses (Abeles et al., 1992). The ethylene signal transduction pathway has been defined through the characterization of ethylene-insensitive and constitutive ethylene response mutants of Arabidopsis (*Arabidopsis thaliana*; Chen et al., 2005; Benavente and Alonso, 2006). These signaling elements are, in order, a five-member ethylene receptor family related to the His kinases of prokaryotes comprised of ETHYLENE RESPONSE1 (ETR1), ETHYLENE RESPONSE SENSOR1 (ERS1), ETHYLENE INSENSITIVE4 (EIN4), ETR2, and ERS2; the Ser/Thr-kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1); the transmembrane protein EIN2; and the EIN3/ETHYLENE INSENSITIVE3-LIKE (EIL) family of transcription factors. This signal transduction

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pathway transduces the ethylene signal from membrane-bound receptors to the nucleus, where the EIN3/EIL transcription factors initiate the characteristic transcriptional response to ethylene (Kieber et al., 1993; Ju et al., 2012; Qiao et al., 2012; Wen et al., 2012).

According to the current model, ethylene inhibits root growth of Arabidopsis by regulating root cell elongation without impacting meristem activity (Růzicka et al., 2007; Vanstraelen and Benková, 2012). This model for ethylene inhibition of root growth is consistent with ethylene's historically delineated role in the regulation of cell expansion (Abeles et al., 1992; Kieber et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998). Ethylene inhibition of root cell elongation in Arabidopsis initiates within 20 min of exposure to exogenous ethylene (Le et al., 2001). Furthermore, the ethylene-insensitive mutants *etr1-3* and *ein2-1* exhibit increased cell elongation, and the constitutive ethylene-response mutant *ctr1-1* reduced cell elongation in the root compared with the wild type (Le et al., 2001). Cross talk with auxin is a critical component for ethylene inhibition of root cell elongation, with ethylene stimulating auxin biosynthesis in the root as well as its shootward-directed movement, the increased auxin activity in the elongation zone serving to inhibit cell elongation (Růzicka et al., 2007; Swarup et al., 2007; Strader et al., 2010).

RAM activity, however, is maintained through opposing effects of auxin and cytokinin: auxin-stimulating cell proliferation of the RAM and cytokinin-antagonizing auxin activity to reduce RAM size (Moubayidin et al., 2009; Petricka et al., 2012; Vanstraelen and Benková, 2012). Interactions of the auxin-cytokinin regulatory circuit with other plant hormones have been delineated, but to date, such interactions with ethylene in control of RAM activity have not been reported. Recent work suggests that ethylene may regulate cell proliferation in addition to its well-characterized role in controlling cell expansion. Notably, *CULLIN3* genes regulate cell proliferation at the RAM through an ethylene-dependent mechanism based on genetic analysis (Thomann et al., 2009). In addition, the inhibitory effects of osmotic stress on leaf cell proliferation are affected by ethylene mutants, and these results point to a role for ethylene signaling in cell cycle arrest (Skirycz et al., 2011). We, therefore, took advantage of the genetic and pharmacological tools available for the study of ethylene signaling to reevaluate its role in cell proliferation, focusing on the root meristem. Our results support a role for ethylene in the control of RAM size through alterations in cell proliferation. Furthermore, our results identify key elements in the signaling pathways operating downstream of ethylene as well as points of cross talk with cytokinin and auxin in control of this process.

RESULTS

Ethylene Negatively Regulates Root Meristem Size

We performed a dose-response analysis to determine the effect of ethylene on cell proliferation of the RAM. The size of the RAM was evaluated based on the number of cortex cells in a file extending from the QC to the first elongated cell (Fig. 1A), this being an established method to quantify the anticlinal divisions that occur in the RAM (Perilli and Sabatini, 2010). Seedlings were grown for 7 d in the presence of 0 to 100 μL L^{-1} exogenous ethylene (Fig. 1B). The RAM of wild-type seedlings decreased in response to all concentrations of ethylene examined, reaching 42% of its untreated size at 100 μL L^{-1} ethylene (a decrease in RAM size from 33 to 13.8 cells). Dramatically different responses were observed with the ethylene-insensitive mutant *etr1-1* and the constitutive ethylene-response mutant *ctr1-2* (Fig. 1B; Table I). The ethylene-insensitive *etr1-1* mutant was substantially less responsive to ethylene, exhibiting a larger RAM than the wild type in the absence of exogenous ethylene and decreasing to 80% of its untreated size at 100 μL L^{-1} ethylene (a decrease in RAM size from 42 to 33.5 cells), resulting in an ethylene-treated RAM that was 243% larger than that of the wild type. The constitutive ethylene-response mutant *ctr1-2* exhibited a smaller meristem than the wild type in the absence of exogenous ethylene. The *ctr1-2* mutant exhibited some responsiveness to ethylene, consistent with prior data that a CTR1-independent pathway exits for ethylene signaling (Larsen and Chang, 2001; Hall and Bleecker, 2003). Significantly, the RAM sizes of the wild type and *ctr1-2* were similar at 100 μL L^{-1} ethylene. We also performed a dose-response analysis using the ethylene biosynthetic precursor 1-aminocyclopropane-1-carboxylic acid (ACC), doing so because ACC was used in previous analysis of RAM size instead of ethylene (Růzicka et al., 2007), an approach potentially complicated by the finding that ACC has ethylene-independent effects on growth at the root tip (Xu et al., 2008; Yoon and Kieber, 2013). ACC, like ethylene, inhibited RAM activity based on a dose-response analysis (Fig. 1C).

We used the pharmacological agent 1-methylcyclopropene (1-MCP) as a complementary approach to inhibit ethylene responses (Fig. 1D). 1-MCP is a highly specific and competitive inhibitor for ethylene binding to its receptors, binding with a higher affinity to the receptors than ethylene (Hall et al., 2000; Sisler, 2006). In contrast, other pharmacological agents, such as aminoethoxyvinyl-Gly and silver, that are often used to inhibit ethylene biosynthesis and the receptors, respectively, also impact auxin activity (Strader et al., 2009; Soeno et al., 2010). As shown in Figure 1D, 1-MCP reversed the effects of exogenous ethylene on root meristem size.

We examined additional ethylene pathway mutants to gain a better understanding of how ethylene regulates RAM size. To this end, we examined ethylene-insensitive mutants of *EIN2* and the *EIN3/EIL* transcription factor family (Table I), making use of the double mutant *ein3-1 eil1-1* because of functional redundancy within the *EIN3/EIL* family (Alonso et al., 2003). Both the *ein2-50* and *ein3-1 eil1-1* mutants exhibit insensitivity for the RAM response to ethylene (Fig. 1E). We also examined several different mutants involving LOF mutations within the five-member ethylene receptor family (Table I). The receptors are functionally redundant negative regulators

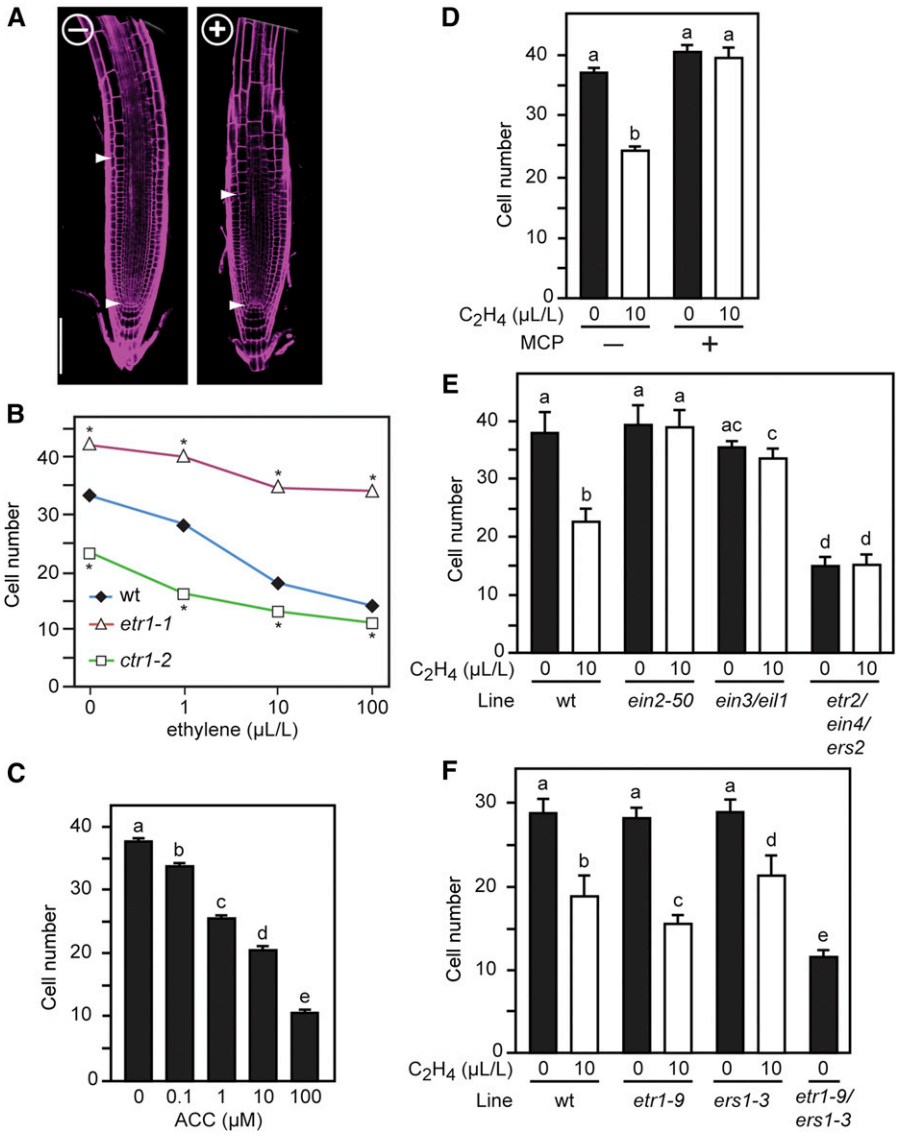

Figure 1. Ethylene negatively regulates root meristem size. Seedlings were grown on vertically oriented agar dishes for 7 d under constant illumination. RAM size was determined based on the number of cortex cells in a file extending from the QC to the first elongated cell. A, Ethylene affects RAM size based on analysis of wild-type seedlings grown in the absence (−) or presence (+) of 10 μL L^{-1} ethylene. Root cells were stained with propidium iodide for fluorescence visualization. Arrows indicate the position of QC and the end of RAM based on the position of the first elongated cell. Bar = 100 μm. B, Ethylene dose-response analysis for RAM size in the wild type (wt) compared with *etr1-1* and *ctr1-2*. Data point symbols are larger than SE, so no error bars are visible. *, Significant difference of mutant compared with the wild type ($n = 15$; $P < 0.01$). C, ACC dose-response analysis for RAM size of wild-type seedlings ($n = 15$; $P < 0.05$). D, 1-MCP inhibits effects of ethylene on RAM size. RAM size was determined by wild-type seedlings grown in the absence or presence of 10 μL L^{-1} ethylene, with the inclusion of 10 μL L^{-1} 1-MCP as indicated (+; $n = 10$; $P < 0.05$). E, Effect of additional ethylene pathway mutants on the ability of ethylene to inhibit cell proliferation at the RAM. RAM size was determined for the wild type compared with the ethylene-insensitive mutants *ein2-50* and *ein3 eil1* as well as the subfamily 2 receptor mutant *etr2 ein4 ers2*, which displays a constitutive ethylene-response phenotype ($n = 10$; $P < 0.05$). F, Effect of subfamily 1 ethylene-receptor mutants on the ability of ethylene to inhibit cell proliferation at the RAM. Wild-type seedlings (WS ecotype) were compared with single and double mutants of *etr1-9* and *ers1-3* ($n = 10$; $P < 0.05$). Error bars indicate SE. Same letters indicate no significant difference by an ANOVA applying Bonferroni correction posttest comparisons.

of the pathway for many responses, with higher order LOF mutants exhibiting constitutive ethylene-response growth phenotypes (Hua and Meyerowitz, 1998; Qu et al., 2007). The receptors fall into two subfamilies based on their features and evolutionary relationship: subfamily 1 consisting of ETR1 and ERS1 and subfamily 2 consisting of ETR2, EIN4, and ERS2 (Binder et al., 2012). The subfamily 1 LOF mutants *etr1-9* and *ers1-3* were

Table I. *Mutants used in this study*

ctr, Constitutive ethylene-response phenotype; cin, cytokinin-insensitive phenotype; ein, ethylene-insensitive phenotype; GOF, gain of function; LOF, loss of function.

Mutant	Gene(s)	Gene Product	Characteristics	Reference
etr1-1	At1g66340	Ethylene receptor	GOF; ein	Bleecker et al. (1988); Chang et al. (1993)
etr1-9	At1g66340	Ethylene receptor	LOF	Qu et al. (2007)
ers1-3	At2g40940	Ethylene receptor	LOF	Qu et al. (2007)
etr1-9 ers1-3	At1g66340; At2g40940	Ethylene receptors (subfamily 1)	LOF; ctr	Qu et al. (2007)
etr2-3 ein4-4 ers2-3	At3g23150; At3g04580; At1g04310	Ethylene receptors (subfamily 2)	LOF; ctr	Hua and Meyerowitz (1998)
tETR1-wt	At1g66340	Ethylene receptor (wild-type ETR1)	Transgene in *etr1-9 ers1-3*	Hall et al. (2012)
tETR1-H/G2	At1g66340	Ethylene receptor (kinase-inactive ETR1)	Transgene in *etr1-9 ers1-3*	Hall et al. (2012)
ctr1-2	At5g03730	Ser/Thr kinase	LOF; ctr	Kieber et al. (1993)
ein2-1 and *ein2-50*	At5g03280	Nramp-like protein	LOF; ein	Roman et al. (1995); Alonso et al. (1999); Xu et al. (2008)
ein3 eil1	At3g20770; At2g27050	Transcription factor	LOF; ein	Alonso et al. (2003)
arr1-3 and *arr1-4*	At3g16857	Type B response regulator	LOF	Mason et al. (2005)
arr10-2 arr12-1	At4g31920; At2g25180	Type B response regulator	LOF; cin	Mason et al. (2005)
arr1-3 arr10-2 arr12-1	At3g16857; At4g31920; At2g25180	Type B response regulator	LOF; cin	Mason et al. (2005)
arr2-2 arr10-2 arr12-1	At4g16110; At4g31920; At2g25180	Type B response regulator	LOF; cin	Mason et al. (2005)
shy2-24	At1g04240	AUX/IAA transcription factor	LOF	Tian and Reed (1999)
shy2-2	At1g04240	AUX/IAA transcription factor	GOF	Tian and Reed (1999)

similar to the wild type in their RAM size and response to ethylene (Fig. 1F). In contrast, the *etr1-9 ers1-3* double mutant, which was identified in a segregating *etr1-3 ers1-3*$^{-/+}$ background based on its constitutive ethylene-response phenotype (Qu et al., 2007), exhibited a substantially reduced RAM size similar to that found in the *ctr1-2* mutant (Fig. 1F). The *etr1-9 ers1-3* mutant phenotype was not characterized in the presence of ethylene, because the segregating genotypes exhibit an ethylene response indistinguishable from a constitutive ethylene-response phenotype. The subfamily 2 mutant *etr2-3 ein4-4 ers2-3* exhibited a reduced RAM size similar to what was observed with other constitutive ethylene-response mutants (Fig. 1E). Taken together, these data show that ethylene negatively regulates cell proliferation in the RAM, doing so through the canonical ethylene-response pathway that uses ethylene receptors (subfamilies 1 and 2), CTR1, EIN2, and the EIN3/EIL family of transcription factors.

Ethylene Regulates Nuclear Size during Cell Development at the Root Meristem

In roots, cells leaving the division zone exhibit an increase in cell expansion and transition from a mitotic cycle to an endocycle (Ishida et al., 2010; Takahashi et al., 2013). Endoreduplication results in an increase in DNA content and a corresponding increase in nuclear size, and therefore, the nuclear area has been used to assess the effects of cytokinin and auxin on the switch to endoreduplication at the root tip (Ishida et al., 2010; Takahashi et al., 2013). We, therefore, determined the effect of ethylene on nuclear area in cortical cells of the RAM as an independent approach to characterize the transition from cell proliferation to differentiation and expansion.

Consistent with previous reports (Ishida et al., 2010; Takahashi et al., 2013), we observed an increase in the area of 4′,6-diamidino-phenylindole (DAPI)-stained nuclei with increasing cell distance from the QC (Fig. 2). Treatment of wild-type seedlings with 10 μL L^{-1} ethylene dramatically affected the developmental profile of the nuclear area in the cortical cells (Fig. 2B). Both treated and untreated seedlings exhibited similarly sized nuclei to each other for the first 21 cells of the cortical file, but then, their nuclear areas diverged (significant nuclear size difference for cells 22 and higher; $P < 0.05$, paired Student's t test). At this point, nuclei of the ethylene-treated seedlings exhibited a rapid increase in area (characterized by a sharp change in the slope of the plot of nuclear area versus cortical cell number), coincident

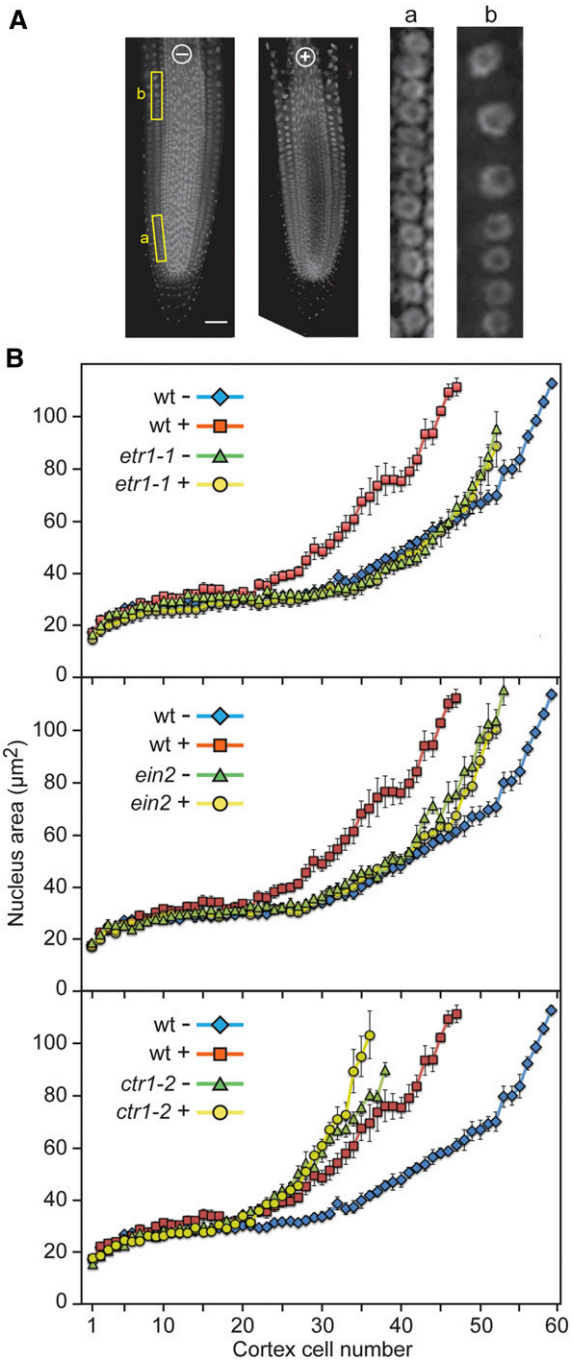

Figure 2. Effect of ethylene on nuclei size in cortical cells of the RAM. A, Nuclei visualization by DAPI staining in the root tip of a 7-d-old wild-type seedling grown in the absence (−) or presence (+) of 10 μL L^{-1} ethylene; 7-fold enlargements of the boxed areas (a and b) indicate developmental differences in nuclei area found in cortical cells. Bar = 40 μm. B, Nuclei areas of 7-d-old seedlings of the wild type (wt), *etr1-1*, *ein2-50*, and *ctr1-2* grown in the absence (−) or presence (+) of 10 μL L^{-1} ethylene (n = 10). All seedlings were grown concurrently, and data for the mutant lines are shown on separate graphs for clarity. Error bars indicate SE.

with the increased cell expansion found after transitioning from the zone of cell division. In contrast, the nuclei of the untreated seedlings exhibited a more gradual increase in area, an increased rate of change for their nuclear area not being initiated until cell 35 of the cortical file.

The ethylene-insensitive mutants *etr1-1* and *ein2-50* exhibited nuclear area developmental profiles similar to that of the untreated wild-type sample up through the transition zone, but unlike the wild type, their nuclear area developmental profiles remained substantially unchanged in response to ethylene (significant nuclear size difference compared with the wild type for cells 22 and higher of *etr1-1*; for cells 20 and higher of *ein2-50*; $P < 0.05$, paired Student's t test; Fig. 2B). The nuclear areas of *etr1-1* were smaller than those of the wild type near the transition zone (significant nuclear size difference for the data sets across RAM cell range 35–45; $P < 0.05$, Kolmogorov-Smirnov test), consistent with the expanded RAM that we observed for *etr1-1* based on cell size as a measure of the meristem (Fig. 1B). Farther away from the transition zone, the nuclei of both *etr1-1* and *ein2-50* exhibited more rapid increases in area than the wild type (Fig. 2B), consistent with the increased cell elongation predicted for such ethylene-insensitive mutants (Le et al., 2001). In contrast to the ethylene-insensitive mutants, the constitutive ethylene-response mutant *ctr1-2* exhibited a nuclear area developmental profile similar to the ethylene-treated wild-type seedling, regardless of whether *ctr1-2* was grown in the absence or presence of ethylene (Fig. 2B); the constitutive ethylene-response phenotype for *ctr1-2* can be seen in that, in the absence of ethylene, the nuclei of *ctr1-2* were significantly increased in size compared with the wild type for cells 20 and higher ($P < 0.05$, paired Student's t test). Taken together, these results based on ethylene treatment and the analysis of pathway mutants support a role for ethylene in positively regulating the transition of RAM cells to the endocycle, consistent with a role for ethylene in negatively regulating cell proliferation of the RAM.

Role of His Kinase Activity of the Ethylene Receptors in Control of Root Meristem Size

There is subfunctionalization within the ethylene receptor family, such that, although the receptors overlap in their regulation of the downstream CTR1-EIN2-EIN3/EIL1 signaling module, subsets of receptors seem to signal through alternative pathways (Shakeel et al., 2013). One potential mechanism mediating subfunctionalization is the differing enzymatic activities of subfamily 1 and 2 receptors. Subfamily 1 receptors ETR1 and ERS1 have His kinase activity, unlike the subfamily 2 receptors ETR2, ERS2, and EIN4, which lack amino acid residues critical for this enzymatic activity (Gamble et al., 1998; Moussatche and Klee, 2004). His kinase activity allows receptors to potentially participate in a multistep phosphorelay involving ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEINs (AHPs) and type B ARABIDOPSIS RESPONSE REGULATORs (ARRs) transcription factors (Schaller et al., 2008, 2011). The best characterized role for a multistep phosphorelay is in

cytokinin signaling (Kieber and Schaller, 2014), but evidence also supports a role in mediating a subset of ethylene responses (Binder et al., 2004; Hass et al., 2004; Qu and Schaller, 2004; Hall et al., 2012).

To examine the role of receptor His kinase activity in control of RAM size, we made use of lines in which wild-type (tETR1-wt) and kinase-inactive (tETR1-H/G2) versions of the receptor ETR1 were transgenically expressed in an *etr1-9 ers1-3* double-mutant background (Hall et al., 2012). Two independent transgenic lines were examined for each construct; tETR1-wt-line 1 and tETR1-H/G2-line 1 exhibit higher protein levels, whereas tETR1-wt-line 2 and tETR1-H/G2-line 2 exhibit lower protein levels, being similar to the native ETR1 protein levels (Hall et al., 2012). The RAM size was examined in response to 1 and 10 μL L^{-1} ethylene (Fig. 3A). RAM size decreased in response to ethylene in both the tETR1-wt and tETR1-H/G2 lines, indicating that His kinase activity is not required for the response. However, the ethylene sensitivity of the tETR1-H/G2 lines was significantly reduced compared with that of the tETR1-wt lines, indicating that His kinase activity may play a role in modulating the ethylene response.

If effects of receptor His kinase activity are propagated through a multistep phosphorelay, then one would predict that the ethylene regulation of RAM size would also involve the downstream type B ARRs. We tested this hypothesis by examining the ethylene control of RAM size in type B *arr* mutants (Table I), focusing on LOF mutations of *ARR1*, *ARR2*, *ARR10*, and *ARR12*, which are the principle type B ARRs expressed in the root (Mason et al., 2004; Hill et al., 2013). We initially examined the triple mutants *arr1-3 arr10-2 arr12-1* and *arr2-2 arr10-2 arr12-1* (Mason et al., 2005), examining their responsiveness to 1 and 10 μL L^{-1} ethylene (Fig. 3B). We observed reduced responsiveness of the *arr1-3 arr10-2 arr12-1* mutant, this being most pronounced at 1 μL L^{-1} ethylene; in contrast, the *arr2-2 arr10-2 arr12-1* mutant behaved similarly to the wild type. These data suggested that the *arr1* mutation might affect the RAM response to ethylene. We, therefore, examined two independent LOF alleles of *ARR1* (*arr1-3* and *arr1-4*) as well as an *arr10-2 arr12-1* double mutant, the result confirming that *arr1* mutants but not the other type B *arr* mutants examined exhibit reduced responsiveness to ethylene in the regulation of RAM size (Fig. 3C). Taken together, these results indicate that ethylene signaling through a phosphorelay involving ARR1 may contribute to the control of RAM size, although this contribution is less than that of the canonical CTR1-EIN2-EIN3/EIL1 pathway.

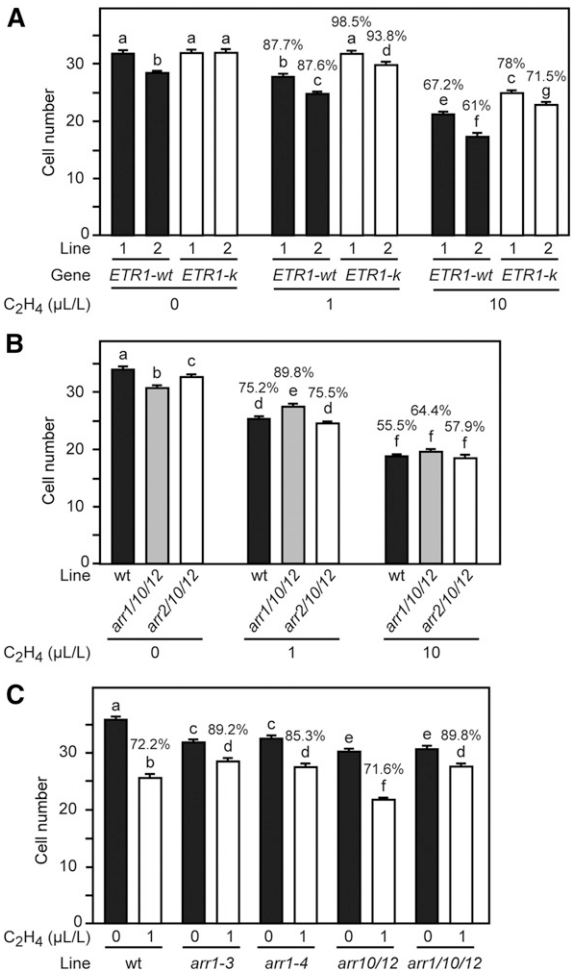

Figure 3. Role of two-component signaling in ethylene-mediated control of RAM size. A, Effect of ETR1 His kinase activity on ethylene-mediated control of RAM size. RAM size was determined for ETR1-wt and ETR1-H/G2 (ETR1-k) versions of the receptor ETR1 transgenically expressed in an *etr1-9;ers1-3* double-mutant background (Hall et al., 2012). Two independent transgenic lines were examined for each construct ($n = 15$; $P < 0.05$). B and C, Effect of type B *ARR* mutants on ethylene-mediated control of RAM size ($n = 15$; $P < 0.01$). Percentages are based on cell numbers for ethylene treatments compared with the untreated control for each line. Error bars indicate SE. Same letters indicate no significant difference by an ANOVA applying Bonferroni correction posttest comparisons. wt, Wild type.

Ethylene Facilitates Cytokinin Regulation of Root Meristem Size

Cytokinin negatively regulates cell proliferation in the RAM (Beemster and Baskin, 2000; Werner et al., 2003; Dello Ioio et al., 2008). Cytokinin also induces ethylene biosynthesis (Vogel et al., 1998a, 1998b; Chae et al., 2003; Hansen et al., 2009; Žďárská et al., 2013), raising the possibility that the inhibitory effect of cytokinin on cell proliferation might exhibit ethylene dependence. We, therefore, examined the RAM response to the cytokinin benzyladenine (BA) in the wild type, the ethylene-insensitive mutants *etr1-1* and *ein2-1*, and the constitutive ethylene-response mutant *ctr1-2* (Fig. 4). The wild-type RAM decreased in size in response to increasing BA concentration, consistent with published results (Dello Ioio et al., 2008). Both ethylene-insensitive mutants reduced the RAM responsiveness to exogenous cytokinin, with the *etr1-1* mutant exhibiting the strongest effect and affecting the cytokinin response at 0.1 and 1 μM BA and the *ein2-1* mutant affecting the cytokinin response at 0.1 μM BA. There was,

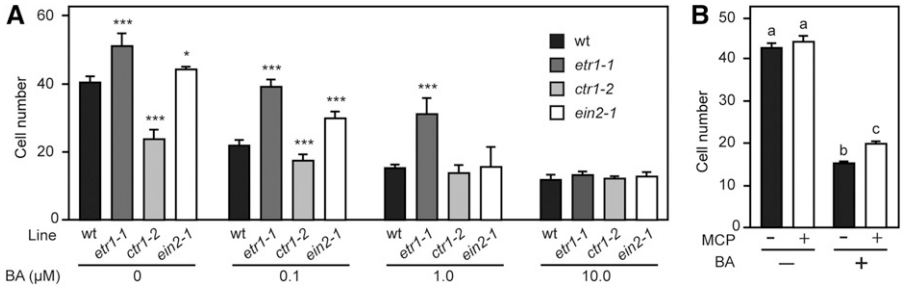

Figure 4. Role of ethylene on the cytokinin-dependent inhibition of RAM cell proliferation. A, Effect of ethylene pathway mutants. Ten-day-old seedlings were grown with the indicated concentrations of BA. Error bars indicate SE, and statistical significance was determined by performing Dunnett's multiple comparison test using the wild type (wt) as a reference group after one-way ANOVA analysis. *, $P < 0.05$; ***, $P < 0.001$. B, Effect of 1-MCP. Ten-day-old seedlings were grown in the absence (−) or presence (+) of 1 μM BA along with 10 μL L^{-1} 1-MCP as indicated. Error bars indicate SE ($n > 18$).

however, no significant difference of the RAM size between the ethylene-insensitive mutants and the wild type at 10 μM BA, indicating that an active ethylene signaling pathway was not absolutely required for the RAM response to cytokinin. The RAM of *ctr1-2* was initially substantially smaller than that of the wild type but exhibited reduced responsiveness to BA, such that it was indistinguishable from the wild type at 1 and 10 μM BA (Fig. 4A). Treatment with the inhibitor 1-MCP confirmed a role for ethylene perception in facilitating the cytokinin response (Fig. 4B). Taken together, these results indicate that the ethylene response contributes to but is not required for cytokinin effects on RAM size.

Effect of Ethylene on Molecular Reporters at the Root Meristem

To gain insight into the basis for the difference in RAM activity in response to ethylene treatment, we used the *cyclin B1;1* (*CycB1;1*):*GUS* reporter. This reporter produces a labile cyclin-GUS fusion protein that is destroyed at the end of mitosis, thereby serving as a marker for cell division (Colón-Carmona et al., 1999). In wild-type plants, GUS staining is observed in the meristematic region of the primary root, but staining is substantially reduced when plants are grown with ethylene (Fig. 5A), coincident with the reduction in RAM size. The mitotic index within the zone of cell division was also slightly reduced in response to ethylene, decreasing from 1.28 (SD = 0.10) to 0.84 (SD = 0.16). Treatment with the inhibitor 1-MCP reversed the effect of ethylene on cyclin-GUS activity, consistent with mediation through action of the ethylene receptors. Thus, the ethylene-induced reduction in RAM size of wild-type seedlings correlates with an overall reduction in cell division within this region.

To gain additional insight into the molecular changes accompanying ethylene responses at the RAM, we examined expression of selected genes in root tips. Expression was examined by quantitative reverse transcription (qRT)-PCR of the wild type grown in the absence and presence of 10 μL L^{-1} ethylene and compared with that found in the ethylene-insensitive mutants *etr1-1* and *ein2-50* as well as the constitutive ethylene-response mutant *ctr1-2* (Fig. 5B). Efficacy of the ethylene treatment was confirmed based on the expression of the known ethylene-responsive genes *ETR2* and Arabidopsis *PEROXIDASE N* (*ATP-N*; Hall et al., 2012) being elevated in response to ethylene in root tips of wild-type seedlings and exhibiting high basal expression in *ctr1-2*. Two genes were identified that exhibited a similar expression profile (i.e. elevated in response to ethylene and the *ctr1-2* mutant): *SHORT HYPOCOTYL2* (*SHY2*) and *CYCLIN-DEPENDENT KINASE INHIBITOR1/KIP-RELATED PROTEIN1* (*ICK1/KRP1*; Fig. 5B). *SHY2* encodes the auxin repressor INDOLE-3-ACETIC ACID INDUCIBLE3 (IAA3), and its induction by cytokinin has been implicated in negatively regulating cell proliferation at the RAM (Dello Ioio et al., 2008). *ICK1/KRP1* encodes a cyclin-dependent kinase inhibitor, with the *ICK/KRP* genes serving to negatively regulate cell proliferation in plants based on genetic analysis using LOF mutants (Cheng et al., 2013; Wen et al., 2013); increased levels of *ICK1/KRP1* correlate with a developmental decrease in cell cycle progression in both roots and shoots (Beemster et al., 2005). Other genes examined were largely unresponsive to ethylene. These included *CELL CYCLE SWITCH PROTEIN52A1* (*CCS52A1*), which encodes an inducer of endoreduplication implicated in mediating effects of cytokinin (Takahashi et al., 2013), and the CYC-encoding genes *CYCA2;3*, *CYCD3;1*, and *CYCB1;1* (Gutierrez, 2009). Post-transcriptional mechanisms, such as protein degradation and phosphorylation, play substantial roles in cell cycle control (e.g. degradation of CYCB1;1, such as is revealed through use of the *CYCB1;1*:*GUS* reporter), and thus, minimal changes in gene expression in response to a hormonal stimulus are not unanticipated.

SHY2 Mediates Effects of Ethylene at the Root Meristem

Ethylene-dependent induction of *SHY2* expression (Fig. 5B) suggested a potential role for this auxin repressor in mediating the inhibitory effects of ethylene on RAM

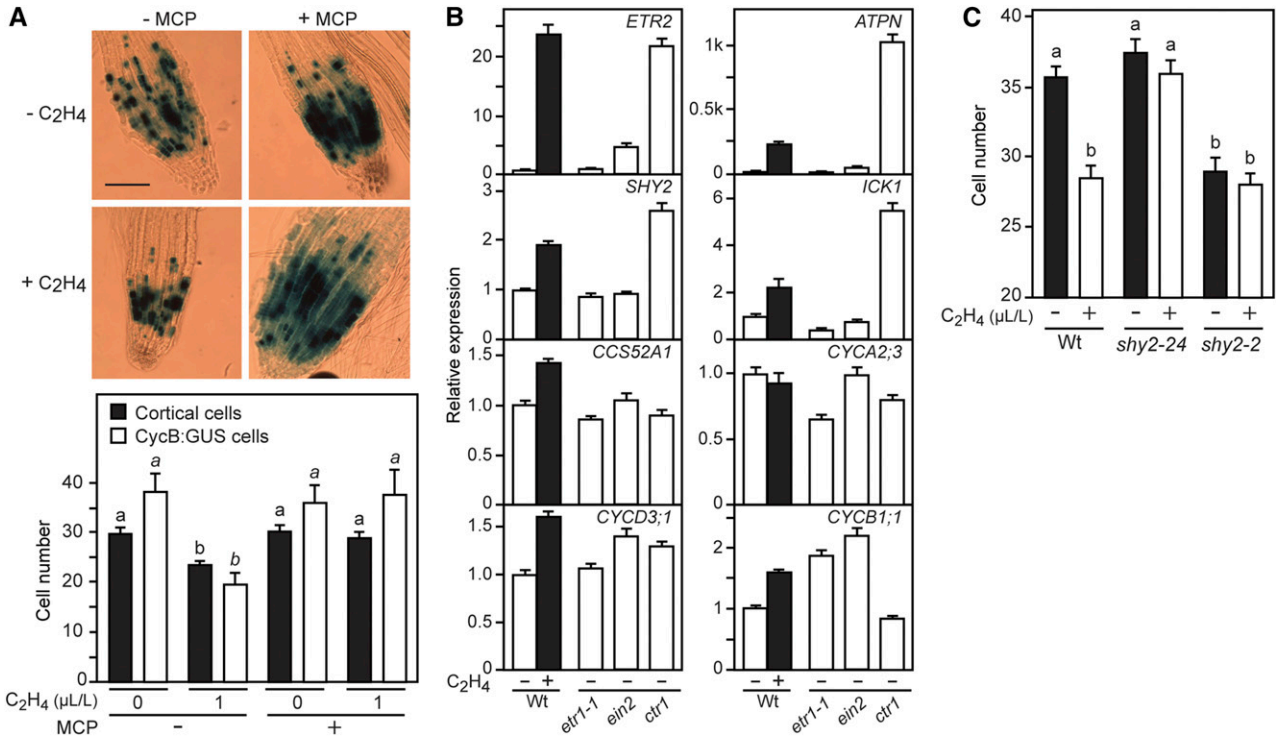

Figure 5. Molecular analysis of ethylene response at the RAM. A, Ethylene regulates cell proliferation based on a cyclin GUS reporter. Seedlings expressing the *CycB1;1:GUS* reporter were grown in the absence or presence of 1 μL L^{-1} ethylene, with the inclusion of 10 μL L^{-1} 1-MCP as indicated (+). Stained images of representative root tips are shown (upper). Bar = 100 μm. Mean number of CycB:GUS-stained cells and RAM size based on cortical cell number ($n \geq 7$; lower). Error bars indicate SE. Separate ANOVA analyses were performed for cortical cell number and CycB:GUS-stained cells ($P < 0.05$ for meristem cell numbers and $P < 0.01$ for CycB:GUS staining cells). B, Role of ethylene in regulating gene expression at the RAM. Root tips (1 mm) were isolated from wild-type (Wt) seedlings grown in the presence or absence of 10 μL L^{-1} ethylene as well as from *etr1-1*, *ein2-50*, and *ctr1-2* mutants, and gene expression was examined by qRT-PCR. Expression was normalized to a tubulin control and is presented as relative to the untreated wild-type control. *ETR2* and *ATP-N* are positive controls for ethylene-regulated gene expression. C, Effect of *SHY2* mutants on ethylene-mediated control of RAM size. Five-day-old seedlings of the wild type, the LOF *shy2-24* mutant, and the GOF *shy2-2* mutant were incubated for 12 h in the absence or presence of 10 μL L^{-1} ethylene. The RAM size was determined based on cortical cell number ($n > 18$; $P < 0.05$). Error bars indicate SE. Same letters indicate no significant difference by an ANOVA applying Bonferroni correction posttest comparisons.

cell proliferation. We, therefore, used the same genetic approach previously used to examine the role of *SHY2* in mediating the inhibitory effects of cytokinin on the RAM (Dello Ioio et al., 2008), making use of an LOF *shy2-24* mutant (premature stop codon) as well as a GOF *shy2-2* mutant (increased stability; Tian and Reed, 1999). A 12-h ethylene treatment results in a significant decrease of RAM size in wild-type roots (Fig. 5C). The RAM of the LOF *shy2-24* mutant is similar in size to the wild type but resistant to ethylene treatment, with no significant difference in RAM size being found between the ethylene-treated and -untreated *shy2-24* roots. In contrast, the GOF *shy2-2* mutant exhibits reduced RAM size in the absence of ethylene, with the ethylene treatment having no additional inhibitory effect. These results support *SHY2* as a mediator of the ethylene response on the RAM and furthermore, indicate that *SHY2* is a point of convergence for both ethylene and cytokinin in negatively regulating RAM cell proliferation.

DISCUSSION

Our results show that ethylene negatively regulates cell proliferation at the RAM based on analysis of (1) the effects of ethylene as well as its biosynthetic precursor ACC, (2) known ethylene pathway mutants that confer ethylene-insensitive as well as constitutive ethylene-response phenotypes; and (3) the inhibitor 1-MCP, a competitive inhibitor of ethylene binding to its receptors. Consistent with these cell proliferation results are those that we obtained from measuring nuclear area as an indicator of endoreduplication and analyzing a cyclin B reporter as an indicator of cell division. A model for ethylene regulation of cell proliferation at the RAM based on the genetic analysis is shown in Figure 6. Ethylene regulation of the RAM primarily functions through the canonical ethylene signaling pathway based on ethylene effects being blocked by ethylene-insensitive mutants of the ETR1 receptor, EIN2, and the EIN3/EIL

family of transcription factors. Conversely, the effects of ethylene are phenocopied in constitutive ethylene-response mutants, such as *ctr1* and the higher order receptor mutants *etr1 ers1* and *etr2 ers2 ein4*. The ethylene receptors exhibit functional overlap in control of RAM size that involves both subfamily 1 and subfamily 2 receptors, in contrast to the subfunctionalization of receptors observed for some ethylene responses (Shakeel et al., 2013).

Signaling by the ethylene receptors through an alternative two-component pathway seems to play an additional but minor role in the regulation of cell proliferation at the RAM (Fig. 6). The best-characterized role for the two-component pathway is in cytokinin signaling (Kieber and Schaller, 2014), but it is currently hypothesized that ethylene-regulated His kinase activity and signaling through a two-component system could mediate a subset of ethylene responses and/or facilitate cross talk with the cytokinin signaling pathway (Binder et al., 2012; Shakeel et al., 2013). Consistent with such possibilities are the findings that (1) plants with mutant ethylene receptors lacking His kinase activity exhibit altered ethylene growth responses (Binder et al., 2004; Qu and Schaller, 2004; Hall et al., 2012), (2) ethylene receptors interact with an AHP protein in a phospho-dependent manner (Urao et al., 2000; Scharein et al., 2008), (3) a mutant of the type B response regulator ARR2 exhibits altered ethylene and cytokinin responses (Hass et al., 2004), and (4) the type B response regulator ARR2 binds to a promoter element for the ethylene-induced gene *ETHYLENE RESPONSE FACTOR1* (Hass et al., 2004). Our findings support a role for the phosphorelay in ethylene-mediated inhibition of RAM cell proliferation based on reduced sensitivity found in kinase-deficient receptor lines as well as mutations involving the type B response regulator ARR1. In addition, of the ethylene-insensitive mutants tested, the *etr1-1* mutant exhibits the most pronounced effect on basal RAM size, consistent with the possibility that a bifurcating signaling pathway originates at the ethylene receptors. Interestingly, we did not observe functional overlap among the type B ARRs in mediating this aspect of the ethylene response, a situation that differs from that found with cytokinin-dependent inhibition of RAM cell proliferation, which involves overlapping roles for ARR1, ARR10, and ARR12 (Dello Ioio et al., 2008; Moubayidin et al., 2010; Hill et al., 2013).

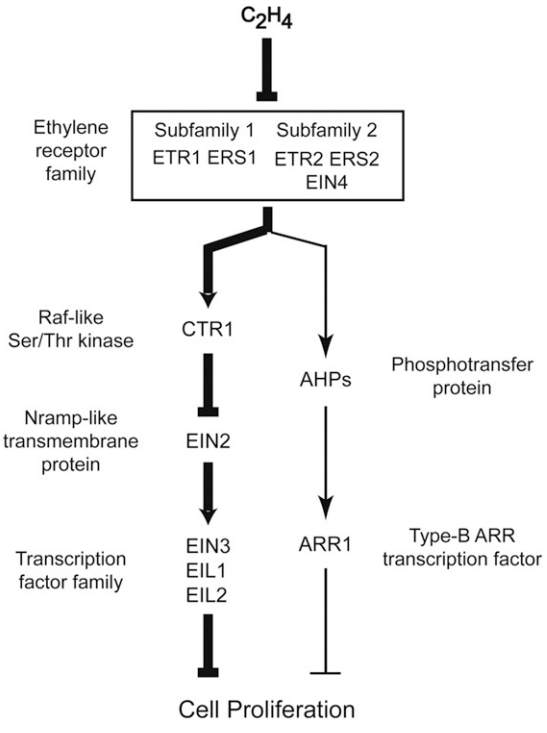

Figure 6. Model for ethylene regulation of cell proliferation at the root meristem. Ethylene negatively regulates cell proliferation through action of the ethylene receptors, which feed into two pathways. The primary regulatory pathway (thick arrows) involves CTR1, EIN2, and the EIN3/EIL transcription factor family. CTR1 is related to the Rapidly Accelerated Fibrosarcoma (Raf) protein kinases, and EIN2 is related to the Natural Resistance Associated Microphage Protein (Nramp) metal transporters. A secondary pathway (thin arrows) makes use of a two-component signaling pathway involving ARR1, a type B ARR transcription factor.

Our results are consistent with and expand on the work by Thomann et al. (2009), in which they found that *ein2-1* and *ein3-1* mutations could revert the reduced RAM size of a *cullen3* mutation, implicating the ethylene pathway in the control of RAM size. Our results differ from an earlier report, in which ethylene was proposed to negatively regulate root growth through the control of cell elongation but not cell proliferation (Růzicka et al., 2007). The effect of ethylene may have been previously missed in this case (Růzicka et al., 2007) because of (1) using the less accurate measurement of RAM length as opposed to cortical cell number and nuclear size as an indicator of RAM activity; (2) the use of the biosynthetic precursor ACC rather than ethylene itself, which is thus dependent on levels and distribution of ACC oxidase in the root; (3) differences in sensitivity for effects of ethylene on cell elongation compared with cell proliferation (the main focus of Růzicka et al. [2007] being on the analysis of cell elongation); and (4) the use of aminoethoxyvinyl-Gly and silver as pharmacological inhibitors of ethylene biosynthesis and response, respectively, which at the time, were not known to also affect auxin activity (Strader et al., 2009; Soeno et al., 2010), confounding some of the data interpretation.

Our results suggest a greater level of hormonal cross talk between ethylene and cytokinin than previously recognized. Cytokinin, like ethylene, negatively regulates cell proliferation at the RAM (Werner et al., 2003; Dello Ioio et al., 2008). Cytokinin induces the biosynthesis of ethylene, doing so through stabilization of the 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE enzymes that mediate the rate-limiting step in ethylene biosynthesis, a process that facilitates the ability of cytokinin to repress cell expansion (Vogel et al., 1998a, 1998b; Chae et al., 2003; Růžička et al.,

2009). Our results support an additional role for ethylene in the ability of cytokinin to repress cell proliferation at the RAM, contrasting with a previous report that cytokinin operates independently of ethylene in this regard (Růžička et al., 2009). The role of ethylene can be overcome as the cytokinin concentration increases, indicating that ethylene facilitates but is not absolutely required for cytokinin control of meristem cell proliferation. Ethylene, like cytokinin, also induces divisions at the QC (Ortega-Martínez et al., 2007; Zhang et al., 2013); the cytokinin response at the QC is not dependent on ethylene signaling, but it remains to be determined if ethylene may also facilitate this response at lower cytokinin concentrations.

The mechanisms used by ethylene to negatively regulate cell proliferation at the RAM overlap in part with those used by cytokinin to mediate the same effect, and this may facilitate cross talk between the pathways. In particular, we identify *ARR1* and *SHY2* as points of intersection between the ethylene and cytokinin signaling pathways in their control of RAM cell proliferation. According to the current model, cytokinin enhances the rate of cell differentiation relative to division by modulating auxin activity and transport (Moubayidin et al., 2009; Petricka et al., 2012; Vanstraelen and Benková, 2012; Schaller et al., 2014). We find that the auxin repressor *SHY2* is induced in response to ethylene, such as it also is by cytokinin (Dello Ioio et al., 2008). Consistent with this shared induction, we find that *SHY2* mediates the inhibitory effect of ethylene on the RAM in a similar manner to that found for cytokinin (Dello Ioio et al., 2008), indicating that *SHY2* is a point of convergence for both hormones in negatively regulating RAM cell proliferation. Ethylene-dependent induction of *SHY2* utilizes the canonical CTR1-dependent ethylene signaling pathway based on constitutive induction of *SHY2* in a *ctr1* mutant background; however, ethylene signaling through the two-component pathway may also play a role as ARR1 binds to the *SHY2* promoter (Dello Ioio et al., 2008). As with cytokinin, the role of *SHY2* seems to be primarily in mediating a short-term (12-h) response to ethylene, and additional factors will likely come into play for the more substantial inhibition of RAM cell proliferation observed under extended growth in the presence of these hormones.

Our results support a model in which ethylene facilitates a transition from the mitotic cycle to the endocycle at the RAM, thereby resulting in reduced cell proliferation. In particular, we observed ethylene-dependent increases in nuclear size at the RAM corresponding to the switch from cell proliferation to differentiation, with the effects on nuclear size supporting a role for ethylene in promoting endoreduplication. Such a role for ethylene at the RAM is consistent with ethylene's ability to promote endoreduplication in the hypocotyls of Arabidopsis and cucumber (*Cucumis sativus*; Gendreau et al., 1999; Dan et al., 2003). Auxin-dependent mechanisms mediate these effects of ethylene on endoreduplication at the RAM based on the auxin-repressor *SHY2* facilitating the ethylene response and auxin being a modulator of the mitotic-to-endocycle transition in roots (Ishida et al., 2010). We also find that ethylene induces expression of the cyclin-dependent kinase inhibitor *ICK1/KRP1* (Cheng et al., 2013; Wen et al., 2013), suggesting that ethylene may have more direct effects on the cell cycle. Some mechanisms used by ethylene to negatively regulate cell proliferation in the RAM may also function in the shoot, because unlike cytokinin, which positively regulates cell proliferation in the shoot, ethylene seems to negatively regulate cell proliferation in both the shoot and the root (Skirycz et al., 2011).

MATERIALS AND METHODS

Plant Materials

The wild-type and mutant lines of Arabidopsis (*Arabidopsis thaliana*) were of the Columbia-0 or Wassilewskija (WS) ecotype. The ethylene-insensitive mutants *etr1-1*, *ein2-1*, *ein2-50*, and *ein3-1 eil1-1* and the constitutive ethylene-response mutants *ctr1-2* and *etr2-3 ein4-4 ers2-3* are in the Columbia-0 background (Bleecker et al., 1988; Chang et al., 1993; Kieber et al., 1993; Roman et al., 1995; Hua and Meyerowitz, 1998; Alonso et al., 1999, 2003; Xu et al., 2008). The *etr1-9*, *ers1-3*, and *etr1-9 ers1-3* mutants are in the WS background (Qu et al., 2007). The type B response regulator mutants *arr1-3*, *arr1-4*, *arr10-2 arr12-1*, *arr1-3 arr10-2 arr12-1*, and *arr2-2 arr10-2 arr12-1* are in the Columbia-0 background (Mason et al., 2005). Generation of the tETR1-wt and tETR1-H/G2 versions of ETR1 were previously described (Hall et al., 2012). The GOF *shy2-2* and LOF *shy2-24* mutant alleles have been described (Tian and Reed, 1999). Wild-type plants containing the *CycB1;1:GUS* reporter were provided by Peter Doerner (Colón-Carmona et al., 1999).

Plant Growth Conditions

Seedlings for molecular and physiological assays were grown on Murashige and Skoog basal medium with Gamborg's vitamins and MES buffer (Research Products International), 1% (w/v) Suc, and 0.8% (w/v) phytoagar (Research Products International) as described (Argyros et al., 2008). Vertically oriented square plates were used for growth to facilitate root measurements. Seeds were surface sterilized and cold treated in the dark for 3 d at 4°C before being transferred to a growth chamber at 22°C. Seedlings were grown under continuous illumination with white light being generated by standard fluorescent bulbs augmented with 18,000-K fluorescent bulbs (Aqua-Glo; Rolf C. Hagen Corp.).

For analysis of the seedling ethylene response, plates with seedlings were transferred to 2.6-L airtight Lock&Lock Containers, with illumination in the chambers being 50 to 60 μmol m^{-2}. Ethylene was introduced through rubber septums into the growth containers using syringe and needle. Stocks of the inhibitor 1-MCP (SmartFresh Tablets; AgroFresh) were prepared in volumetric flasks sealed with rubber septums and contained a plug of saturated ammonium sulfate solution; 1-MCP was introduced into the growth containers through the rubber septums coincident with the introduction of ethylene. The ethylene biosynthetic precursor ACC was incorporated into the plate growth medium. For exogenous cytokinin treatment, BA was included in the plate growth medium, and the control contained the dimethyl sulfoxide vehicle.

Analysis of the Root Meristem

To determine the size of the root meristem, seedlings were cleared with chloral hydrate, and cells of the root cortex layer were counted in a file extending from the QC to where the cell length exceeded its width using a Nikon Eclipse 90i Optical Microscope with Nomarski Optics and a 20× objective as described (Perilli and Sabatini, 2010). Root meristem size was determined after 7 d of growth unless indicated otherwise. For fluorescence visualization, roots were stained with propidium iodide and visualized with a Nikon A1 Confocal Microscope. Histochemical analyses of GUS activity caused by the *CycB1;1:GUS* reporter were performed as described (Argyros et al., 2008).

Nuclei visualization was performed as described (Ishida et al., 2009) with some modifications. Seven-day-old seedlings were fixed in 1.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in a buffer of 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, and 0.05% (v/v) Triton X-100 (pH 7.2) for 40 min. Fixed seedling were washed with a buffer of 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, and 0.05% (v/v) Triton X-100 (pH 7.2) three times (10 min for each wash) followed by three rinses with phosphate-buffered saline (PBS) buffer. Seedlings were stained with 0.1 μg mL^{-1} DAPI (D9542; Sigma) in PBS buffer for 12 min, washed three times, and then mounted on microscope slides in PBS buffer. DAPI signals were visualized by fluorescence microscopy using a Nikon A1RSi Confocal Microscope, and the nuclei area was determined using ImageJ version 1.44 software (NIH).

For statistical analysis of cortical cell number, ANOVA tables were generated using http://www.physics.csbsju.edu/stats/anova.html, and multiple comparison tests were done using http://graphpad.com/quickcalcs/posttest1/ quick calc web tool that uses the Bonferroni correction for posttest comparisons. Duncan's multiple range test was performed among the means on the ANOVA (Duncan, 1975). For comparisons of nuclear area, either the paired Student's *t* test was performed using GraphPad QuickCalcs (http://graphpad.com/quickcalcs/) or the Kolmogorov-Smirnov test was used for comparison of two data sets (http://www.physics.csbsju.edu/stats/KS-test.html).

RNA Expression Analysis

Seeds were plated on 20-micron nylon mesh (BioDesign), and 1-mm root tips from approximately 50 seedlings were harvested into RNAlater (Life Technologies) after 7 d of growth. The RNAlater solution was removed within 24 h, and tissue was flash frozen with liquid nitrogen. RNA isolation and qRT-PCR analyses were performed as described (Argyros et al., 2008). Briefly, total RNA was isolated by using the RNeasy Plant Kit according to the manufacturer with the incorporation of a DNase Treatment (QIAGEN). First-strand complementary DNA synthesis for qRT-PCR was performed using SuperScript III with oligo(dT) primers (Invitrogen), and qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa Bio Inc.). The following primer sets were used: *SHY2* (5′-AAACAGAGCTGAGGCTGGGATTAC-3′ and 5′-AACTGGTGGCCATCCAACAATCT-3′); *ICK1* (5′-GTATCGACGGGGTACGAAGA-3′ and 5′-CCTCCCGCTACAACAACAAT-3′); *CYCB1;1* (5′-TGGTGCACTATTTGGCTGAGTTAG-3′ and 5′-CACAGTCCATGAGCTGAGTCTCA-3′); *CYCA2;3* (5′-GACCACTTCCGGACTTTATG-3′ and 5′-GAGATACACTGTGAGGTAGAGA-3′); *CYCD3;1* (5′-CTCACTGGGATTTCCTCAAC-3′ and 5′-GGTATGAAAGAGGGTCAAAGG-3′); *ATP-N* (5′-AGTGACTTAGCCGTGAACACCACA-3′ and 5′-ACGAGACCGATCAACTCCCAAACA-3′); *ETR2* (5′-AGAGAAACTCGGGTGCGATGT-3′ and 5′-TCACTGTCGTCGCCACAATC-3′); and *β-tubulin3* (*β-TUB3*) as the control for qRT-PCR with primers 5′-TGGTGGAGCCTTACAACGCTACTT-3′ and 5′-TTCACAGCAAGCTTACGGAGGTCA-3′. Average cycle threshold values were generated and analyzed by BioRad CFX384 Real-Time System and CFX Manager software version 2.1, which uses the comparative cycle threshold method (Livak and Schmittgen, 2001).

Sequence information on the genes analyzed in this study are available from the Arabidopsis Genome Initiative or GenBank/EMBL under accession numbers ARR1 (At3g16857), ARR2 (At4g16110), ARR10 (At4g31920), ARR12 (At2g25180), CTR1 (At5g03730), EIL1 (At2g27050), EIN2 (At5g03280), EIN3 (At3g20770), EIN4 (At3g04580), ERS1 (At2g40940), ERS2 (At1g04310), ETR1 (At1g66340), ETR2 (At3g23150), Per-ATP-N (At519890), SHY2 (At1g04240), ICK1/KRP1 (At2g23430), CCS52A1 (At4g22910), CYCA2;3 (At1g15570), CYCD3;1 (At4g34160), CYCB1;1 (At4g37490), and *β*-TUB3 (AT5G62700).

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