# Diurnal down-regulation of ethylene biosynthesis mediates biomass heterosis

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**Heterosis is widely applied in agriculture; however, the underlying molecular mechanisms for superior performance are not well understood. Ethylene biosynthesis and signaling genes are shown to be down-regulated in *Arabidopsis* interspecific hybrids. Ethylene is a plant hormone that promotes fruit ripening and maturation but inhibits hypocotyl elongation. Here we report that application of exogenous ethylene could eliminate biomass vigor in *Arabidopsis thaliana* F1 hybrids, suggesting a negative role of ethylene in heterosis. Ethylene biosynthesis is mediated by the rate-limiting enzyme, 1-aminocyclopropane-1-carboxylate synthase (ACS). Down-regulation of *ACS* genes led to the decrease of ethylene production, which was associated with the high-vigor F1 hybrids, but not with the low-vigor ones. At the mechanistic level, expression of *ACS* genes was down-regulated diurnally and indirectly by *Circadian Clock Associated 1* (*CCA1*) during the day and directly by *Phyotochrome-Interacting Factor 5* (*PIF5*) at night. Consistent with the negative role of ethylene in plant growth, biomass vigor was higher in the *acs* mutants than in wild-type plants, while increasing endogenous ethylene production in the hybridizing parents reduced growth vigor in the hybrids. Thus, integrating circadian rhythms and light signaling into ethylene production is another regulatory module of complex biological networks, leading to biomass heterosis in plants.**

hybrid vigor | ACS | circadian clock | PIF | epigenetics

Heterosis or hybrid vigor describes a phenomenon of superior growth and fitness in the F1 hybrid offspring relative to the parents (1). Although several genetic models, including dominance and overdominance, are used to explain heterosis, the molecular basis remains poorly understood (1–3). In tomato, a single-locus overdominance is shown to affect fruit yield, probably through changes in floral architecture (4). In *Arabidopsis* interspecific and intraspecific hybrids, altered circadian rhythms increased photosynthesis and starch metabolism (5, 6) and decreased stress responses (5, 7–9), leading to biomass heterosis (2, 10). Mechanisms for these changes could be related to epigenetic perturbations of key regulatory genes in the F1 hybrids (2, 11–14). In addition, basal expression levels of stress-responsive genes can be used as genetic distance indicators between the parents to predict heterosis levels in the F1 hybrids (5). These data suggest that either increasing carbon metabolism and/or decreasing energy cost for defense can promote heterosis (15). Consistent with the notion, many genes involved in the ethylene biosynthesis and signaling pathway including those encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS*) and ethylene response factor (*ERF*) are down-regulated in the *Arabidopsis* allotetraploids that are formed between *Arabidopsis thaliana* and *Arabidopsis arenosa* (16). However, the mechanism for ethylene in biomass heterosis is largely unknown.

Ethylene is a plant hormone that regulates growth and developmental processes; disruption of ethylene biosynthesis or signal transduction affects growth and development of roots, hypocotyls, and seeds (17). Ethylene biosynthesis begins with the conversion of methionine to S-AdoMet (SAM) by S-AdoMet synthetase. SAM is then converted to ACC by ACS, which is a rate-limiting step (17). Finally, ACC is oxidized to ethylene by ACC oxidase (ACO). The *Arabidopsis* genome contains 12 annotated *ACS* genes, 8 of which (*ACS2*, *ACS4–9*, and *ACS11*) show functional activities (17). Ethylene production is circadian regulated by CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and TIME OF CAB EXPRESSION 1 (TOC1) in *A. thaliana* (18), but the biological significance of circadian-mediated ethylene production remains unknown.

Here we show that applying excessive exogenous ethylene could eliminate biomass heterosis, suggesting negative effects of ethylene on heterosis. Consistently, *ACS* genes were transcriptionally down-regulated in *Arabidopsis* hybrids, and consequently ethylene production was reduced in the hybrids. In contrast to previous findings (19), all *ACS* genes tested were subject to diurnal but not circadian expression. *ACS* genes were indirectly regulated by *CCA1* during the day and directly by PHYTOCHROME-INTERACTING FACTOR 5 (*PIF5*) at night, both of which were also down-regulated in the hybrids. Consistent with the down-regulation of ethylene production and increased growth vigor in the hybrids, disruption of ethylene biosynthesis or

## Significance

**Heterosis is a fundamental biological phenomenon and important to crop and animal production. However, molecular mechanisms for heterosis remain elusive. Ethylene promotes fruit ripening but inhibits vegetative growth. Here we report that diurnal down-regulation of ethylene biosynthesis genes in *Arabidopsis thaliana* F1 hybrids corresponds to the decrease of ethylene production and biomass heterosis. Expression of *ACS* genes is diurnally and indirectly regulated by CCA1 during the day and directly regulated by PIF5 at night. Disruption of ethylene biosynthesis or signaling transduction leads to higher biomass, while application of exogenous ethylene reduced growth vigor, further validating the negative roles of ethylene in biomass heterosis. Thus, ethylene can integrate circadian rhythms and light signaling to regulate growth vigor in plant hybrids.**

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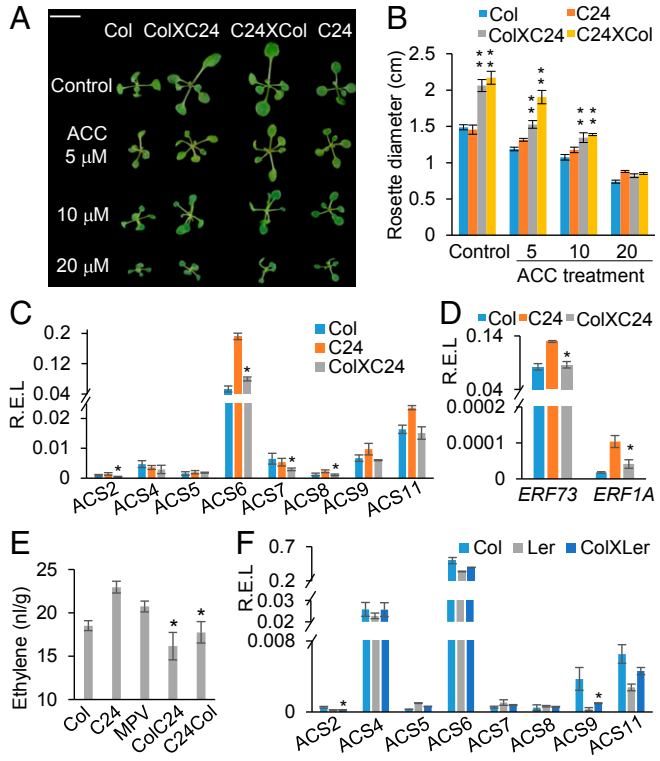


**Fig. 1.** Ethylene biosynthesis is down-regulated in *Arabidopsis* hybrids. (*A*) Representative images of seedlings of Col-0, C24, reciprocal F1 hybrids (ColXC24 and C24XCol) with or without ACC treatments. (Scale bar, 1 cm.) (*B*) Quantitative analysis of rosette diameter in *A*. Double asterisks indicate statistical significance levels at $P < 0.01$ compared with the midparent value (MPV). (*C*) Relative expression levels (R.E.L.) of *ACS* genes at ZT0 in Col-0, C24, and F1 hybrid (ColXC24). Asterisks indicate down-regulation in the hybrids at the statistically significant level ($P < 0.05$) relative to MPV. (*D*) R.E.L. of *ERF1A* and *ERF73* as the materials used in *C*. (*E*) Ethylene production in F1 and F1r hybrids and their parents. (*F*) R.E.L. of *ACS* genes in hybrid (ColXL*er*).

signaling transduction resulted in higher growth traits, including rosette areas and biomass. Increasing endogenous ethylene production could reduce biomass heterosis in the hybrids, further validating a critical role of ethylene in heterosis.

## Results

**Ethylene Production Is Down-Regulated in *A. thaliana* Hybrids.** A previous study has found an overall down-regulation of ethylene biosynthetic and signaling pathway genes in *Arabidopsis* interspecific hybrids or allotetraploids (16), suggesting a potential role for ethylene in hybrid vigor. To determine the relationship between ethylene and heterosis, we applied exogenous ACC, the precursor of ethylene, to the growth media to test the effect of excessive ethylene on growth vigor in F1 hybrids and their parents. In the control, the better parent heterosis (BPH) value was 39–45% in the hybrids, which was reduced in a manner depending on ACC concentrations (Fig. 1 *A* and *B*). As the ACC concentration increased from 5 μM to 10 μM, BPH values gradually decreased from 16–44% to 14–15%. When the ACC concentration increased to 20 μM, the heterosis was nearly eliminated (Fig. 1 *A* and *B*). These data indicate that ethylene has a negative effect on heterosis.

This negative effect could be reversed by repressing *ACS* genes in *A. thaliana* intraspecific hybrids, as in the *Arabidopsis* interspecific hybrids (16). Indeed, among eight *ACS* genes, *ACS2* and *ACS6–8* were significantly down-regulated below the midparent value (MPV) in the F1 intraspecific hybrid (ColXC24) (Fig. 1*C*). To further validate down-regulation of *ACS* genes in heterosis, we examined *ACS* expression changes in several other F1 hybrids produced from different *A. thaliana* ecotypes, including Col-0 (United States), C24 (Portugal), Ws (Russia), L*er* (Germany), and Est-1 (Russia), which displayed high biomass heterosis (5). All eight *ACS* genes tested (*ACS2*, *ACS4–9*, and *ACS11*) were down-regulated below the MPV in all or most hybrid combinations (*SI Appendix*, Fig. S1). Moreover, some examined ethylene-responsive genes, including *ERF1A* and *ERF73*, were also down-regulated below the MPV in the high-vigor hybrids (Fig. 1*D* and *SI Appendix*, Fig. S2). As a result, ethylene production was significantly reduced in the high-vigor F1 hybrids (Fig. 1*E*). These data indicate a role for repressing *ACS* genes in heterosis. Consistent with this notion, in the F1 hybrid (ColXL*er*) with low-level or no biomass heterosis, most *ACS* genes, except for *ACS2* and *ACS9*, were expressed at similar levels to the MPV (Fig. 1*F*).

Since ethylene is a negative regulator for plant vegetative growth (17), down-regulation of *ACS* expression and ethylene production in the hybrids could increase growth vigor. Indeed, all single *acs* mutants and the double mutants with *erf*, and the pentuple mutant *acs24569* showed higher shoot length than the wild type (Col-0) (Fig. 2*A*). The double mutants *acs6 erf1a* and *acs6 erf73* grew larger than the single *acs* mutants (Fig. 2*A*). Biomass traits, including rosette size and dry weight, were significantly increased in the double mutants *acs6 erf1a* and *acs6 erf73* and in the pentuple mutant *acs24569* (Fig. 2 *B* and *C*). These data provide independent genetic evidence that suppression of individual *ACS* and *ERF* genes in the hybrids could increase biomass heterosis as in the diploid mutants.

**Expression of *ACS* Genes Is Subject to Diurnal but Not Circadian Regulation.** As ethylene production is diurnally regulated in both short-day (12/12 h of light/dark cycle) and long-day (16/8 h of

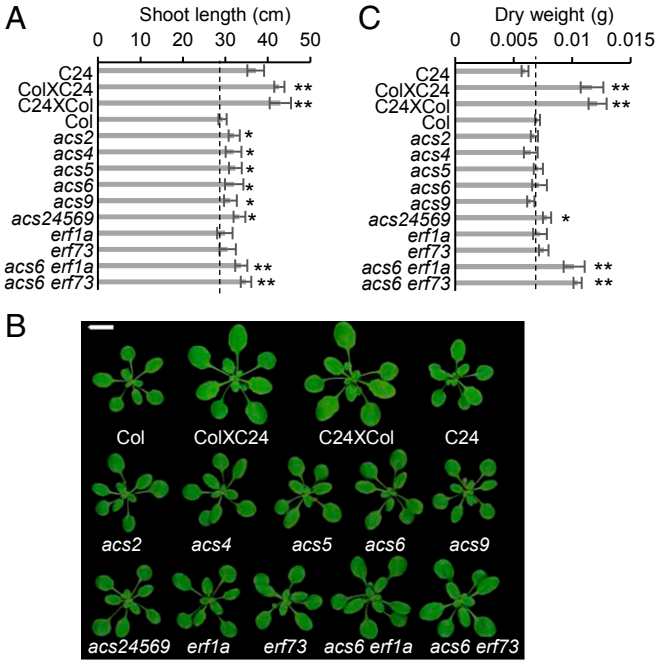


**Fig. 2.** Growth vigor is reduced in *ACS* gene mutants. (*A–C*) Comparison of Col-0, C24, F1 (ColXC24), and reciprocal F1 (C24XCol) hybrids, *acs* and *erf* single mutants, *acs* pentuple mutant (*acs24569*), and *acs erf* double mutants (*acs6 erf1a* and *acs6 erf73*), showing shoot length in mature plants (*A*), rosette size (*B*), and dry weight (*C*) in plants at 18 d after sowing. Asterisks indicate statistically significant levels (hybrid vs. MPV or mutant vs. wild type). Vertical dashed lines indicate phenotypic values of the wild type (Col-0). $*P < 0.05$, $**P < 0.01$. (Scale bar, 1 cm.)

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light/dark cycle) conditions (18), we further examined expression of *ACS* genes in a diurnal cycle. All *ACS* genes, except for *ACS4*, were down-regulated in the *A. thaliana* F1 (ColXC24) and F1 reciprocal (F1r, C24XCol) hybrids in both day and night, especially at zeitgeber time 0 (ZT0 = 6 AM, dawn, or beginning of the light cycle), ZT4, and ZT16 (Fig. 3*A* and *SI Appendix*, Fig. S3).

Given the role of circadian rhythms in ethylene production (18), we proposed that the circadian clock regulates expression of *ACS* genes, in addition to photosynthetic, metabolic, and stress-responsive genes in the hybrids and allotetraploids (5, 6). To test this, we examined *ACS* gene expression subjected to diurnal (24 h, 16 h light/8 h dark) and circadian conditions (48 h, constant light) in the wild-type (Ws) and *cca1 lhy* mutant (Ws background), respectively. Under the diurnal condition, all *ACS* genes showed expression peaks at ZT4 in the wild type, and they were significantly down-regulated in the *cca1 lhy* mutant compared with the wild type during the day, particularly during the peak expression time at ZT4 (Fig. 3*B* and *SI Appendix*, Fig. S4*A*). However, under the constant light condition, all *ACS* genes were expressed at lower levels and did not show rhythmic peaks in the wild type, while *ACS* expression levels were similar between the *cca1 lhy* mutant and the wild type (Fig. 3*B* and *SI Appendix*, Fig. S4*A*). The data suggest that *ACS* expression is subject to diurnal but not circadian regulation. This notion was supported by the analysis of chromatin immunoprecipitation (ChIP) quantitative PCR (qPCR). Using antibodies against CCA1, ChIP-qPCR results showed that CCA1 was significantly enriched in the *TOC1* promoter (as a positive control, which was normalized to *UBQ10*), but not in the promoters of four *ACS* genes (*ACS2* and *ACS6–8*) (Fig. 3*C* and *SI Appendix*, Fig. S4*C*), although each has at least one CCA1-binding site (CBS) in its promoter sequence (*SI Appendix*, Fig. S4*B*). These data suggest an indirect role of CCA1 in altering *ACS* expression.

***ACS* Genes Are Directly Regulated by PIF5 at Night.** *CCA1* was repressed in the hybrids during the day (*SI Appendix*, Fig. S5), and *ACS* genes were also down-regulated in the *cca1 lhy* mutant during the day but not at night (Fig. 3 *B* and *C* and *SI Appendix*, Fig. S4*A*), indicating that down-regulation of *CCA1* in the hybrids contributes to the down-regulation of *ACS* genes during the day. However, *ACS* genes were down-regulated both during the day and at night in the hybrids (Fig. 3*A* and *SI Appendix*, Fig. S3). This suggests that other genes are responsible for *ACS* down-regulation at night in the hybrids. The other factors could be PIF4 and PIF5 because they affect ethylene levels, *ACS* expression, and hypocotyl elongation in darkness (19–21). PIF4 and PIF5 normally activate *ACS* expression at night and inhibit hypocotyl elongation. Consistent with this notion, *PIF4* and *PIF5* were down-regulated at night (ZT0 and ZT18, 16 h light/8 h dark) but not during the day (ZT12) in the F1 hybrids derived from different combinations of *A. thaliana* ecotypes (Fig. 4*A* and *SI Appendix*, Fig. S6), which could reduce *ACS* expression in the hybrids. Indeed, in *PIF5* overexpression (PIF5-OE) plants, all *ACS* genes were significantly up-regulated, whereas in the *pif1 pif3 pif4 pif5* (*pif1345*) mutant, several *ACS* genes (*ACS2*, *ACS5*, and *ACS9*) were significantly down-regulated at night (ZT0), but not during the day (Fig. 4*B* and *SI Appendix*, Fig. S7). These data suggest a role for PIFs in *ACS* and ethylene regulation at night in the hybrids as in the diploids (19).

The nightly expression change of *ACS* genes is dependent on PIFs. Three *ACS* genes (*ACS2*, *ACS7*, and *ACS9*) contain at least one PIF-binding motif G-box within 2-kb promoter sequences (*SI Appendix*, Fig. S4*B*). In the *Nicotiana benthamiana* transient expression experiment, overexpression of *PIF5* could elevate expressions of *ACS2*, *ACS7*, and *ACS9* under the control of native promoters; this activation was abolished when the G-box in the promoters was mutated (Fig. 4 *C* and *D* and *SI Appendix*, Fig. S8). ChIP-qPCR analysis also showed that PIF5 bound to G-box in vivo in the promoters of *ACS2*, *ACS7*, and *ACS9* (Fig. 4*E*). A previous study also observed an enrichment of PIF4 in promoters of *ACS5* and *ACS7–9* (22). Together, these results suggest that reduced expression of *PIF4* and *PIF5* in hybrids led to down-regulation of *ACS* genes at night to promote hypocotyl elongation and growth vigor.

**Excessive Endogenous Ethylene Could Eliminate Heterosis in F1 Hybrids.** Higher growth traits resulting from disrupting ethylene biosynthesis or signaling transduction prompted us to test whether increasing endogenous ethylene production could inhibit heterosis. As constitutively expressing $ACS6^{DDD}$, a gain of function for *ACS6*, results in constitutive ethylene production (23), we tested the effect of $ACS6^{DDD}$ overexpression on heterosis in the hybrids. Transgenic plants overexpressing $ACS6^{DDD}$ in Col (Col_*ACS6-OX*) and in C24 (C24_*ACS6-OX*) were used to generate reciprocal F1 hybrids (ColXC24_ *ACS6-OX* and C24XCol_ *ACS6-OX*), which all showed more ethylene production (11- to 15-fold) than the wild type (Fig. 1*E* and *SI Appendix*, Fig. S9*A*). Biomass traits, including cotyledon area, rosette size, and dry weight in the hybrids ColXC24_*ACS6*-OX

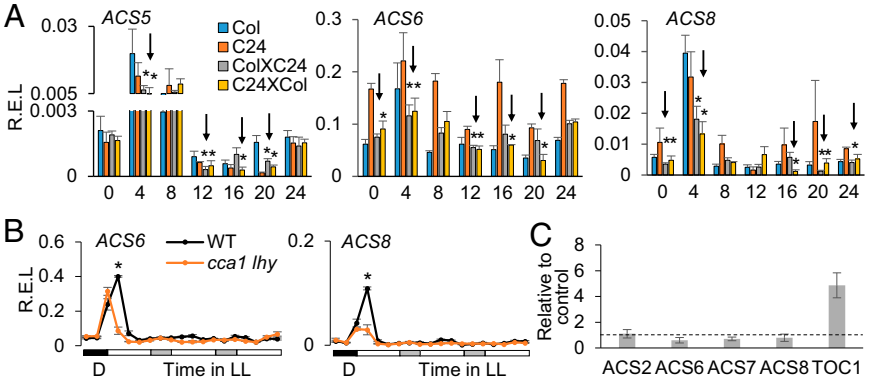


**Fig. 3.** *ACS* genes are regulated diurnally and indirectly by *CCA1* during the day. (*A*) Relative expression levels (R.E.L.) of *ACS* genes every 4 h in a 24-h period (ZT0 = dawn) in the Col-0, C24, and reciprocal F1 hybrids (ColXC24 and C24XCol). Asterisks indicate down-regulation (arrows) in the hybrids at the statistically significant level ($P < 0.05$) relative to MPV. *ACT7* was used as an internal control. (*B*) R.E.L. of *ACS6* and *ACS8* in the wild type (Ws) and *cca1 lhy* double mutant under a diurnal cycle (24 h) followed by constant light (LL) (48 h) conditions. Black, white, and gray boxes indicate dark, light, and subjective night, respectively. The asterisk indicates the statistically significant level ($P < 0.05$). (*C*) ChIP-qPCR showed the fold enrichment (*y* axis) of CCA1-binding fractions in the promoters of *ACS2* and *ACS6–8*. The *TOC1* promoter containing evening elements was used as the positive control. *UBQ10* was used as a nonbinding control for normalization. *$P < 0.05$.

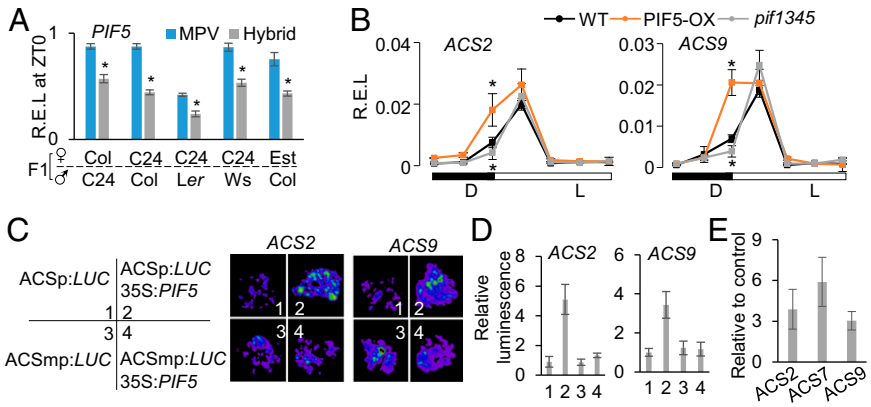


**Fig. 4.** *ACS* genes are regulated indirectly by *PIF5* at night. (*A*) R.E.L. of *PIF5* at ZT0 in F1 hybrids between different combinations of ecotypes. Asterisks indicate down-regulation in the hybrids at the statistically significant level ($P < 0.05$) relative to MPV. (*B*) R.E.L. of *ACS2* and *ACS9* in Col-0 (WT), *PIF5-OX*, and *pif1345* mutant lines in diurnal conditions. Asterisks *Above* and *Below* the graphs, respectively, indicate up-regulation in PIF5-OX and down-regulation in *pif1345* at the statistically significant level ($P < 0.05$) relative to WT. (*C*) Transient luciferase expression assays indicate that PIF5 directly activated expression of *ACS2* and *ACS9*. The infiltrated constructs are ACS promoter fused with luciferase (*ACSp:LUC*, 1), *ACSp:LUC* cotransfected with *35S:PIF5* (2), ACS mutated promoter:luciferase (*ACSmp:LUC*, 3), and *ACSmp:LUC* with *35S:PIF5* (4). Luciferase activity images of *N. benthamiana* leaves are shown for *ACS2* and *ACS9*. (*D*) Relative luminescence intensity (*y* axis) for each comparison in *C*. (*E*) ChIP-qPCR showed fold enrichment (*y* axis) of PIF5 in the promoters of *ACS2*, *ACS7*, and *ACS9*. Coding region of *PP2A* was used as a nonbinding control for normalization.

and C24XCol_*ACS6*-OX were significantly lower than the hybrids between the wild-type parents (Figs. 2 and 5 *A*–*D*). The BPH value of cotyledon area was 47–85% in the hybrids of wild-type parents (Fig. 5*A*). However, in the F1 hybrids of *ACS6*$^{DDD}$-overexpression parents, no heterosis was observed in the cotyledon area (Fig. 5*B*) or in the rosette size and dry weight (Fig. 5 *C* and *D*). These results indicate a negative effect of the constitutive ethylene production on heterosis during plant growth and development.

## Discussion

Our data collectively support a model for CCA1-mediated ethylene emission during the day and PIF-mediated *ACS* expression at night

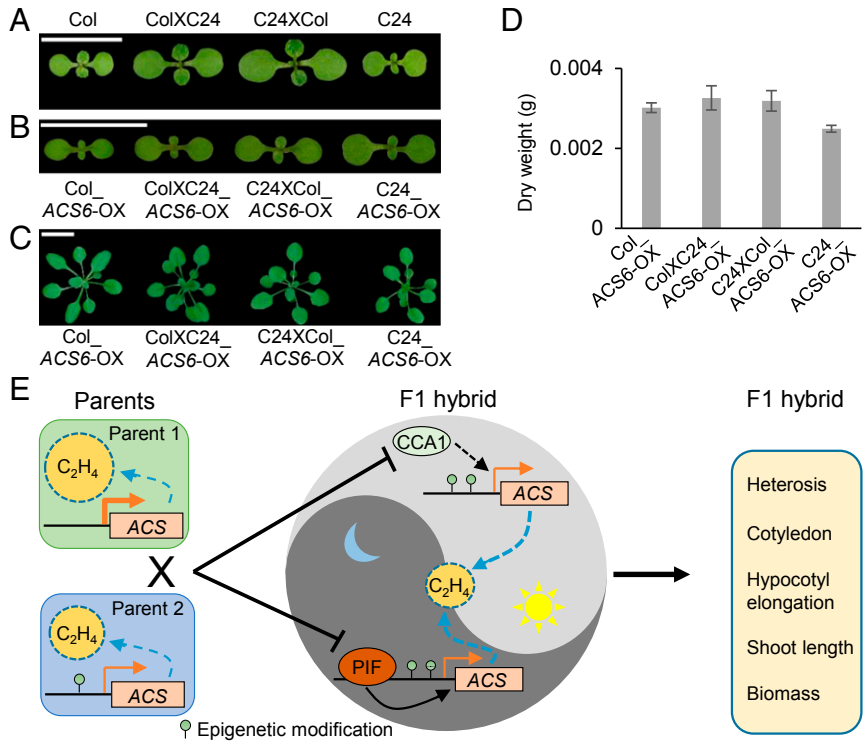


**Fig. 5.** Production of excessive endogenous ethylene inhibits heterosis. (*A* and *B*) Representative cotyledon images of F1 reciprocal hybrids of the wild type (*A*) and *ACS6*$^{DDD}$-overexpression (*B*) parents. (Scale bars, 1 cm.) (*C* and *D*) Representative rosette-size images (*C*) and quantified dry weight (*D*) of Col_*ACS6-OX*, C24_*ACS6-OX*, and their F1 (ColXC24_*ACS6-OX*) and reciprocal F1 (C24XCol_*ACS6-OX*) hybrids at 18 d after sowing. (Scale bar, 1 cm.) (*E*) Model showing the role of ethylene in heterosis. Different ethylene levels between the parents result in the reduced level of ethylene production in the hybrids. The down-regulation of ethylene production is associated with down-regulation of *ACS* genes, which is mediated indirectly by CCA1 during the day and directly by PIF at night. Decrease in ethylene biosynthesis (yellow circle) promotes superior performance of growth and biomass traits (cotyledon area, hypocotyl elongation, and shoot length) in the hybrids, leading to heterosis.

to promote growth vigor in hybrids (Fig. 5*E*). In *A. thaliana* F1 hybrids and *Arabidopsis* allotetraploids, circadian clock genes such as *CCA1* and *LHY* are repressed by DNA methylation and histone modifications during the day (6, 14). As a result, repressing *CCA1* during the day decreases ethylene production by indirectly inhibiting *ACS* expression in the hybrids. At night, *PIF* genes, which regulate skotomorphogenesis (etiolation) (19), were repressed in hybrids, and repressing *PIFs* could decrease ethylene production through direct regulation of *ACS* genes. As PIF proteins are degraded under light (24), and CCA1 cannot directly bind promoters of *ACS* genes, other circadian clock regulators such as ELF3 could also regulate *ACS* expression during the day and night (25). Taken together, our data indicate that decrease of ethylene production leads to superior growth traits in the hybrids.

Current studies have revealed an emerging complexity of biomass heterosis under the control of regulatory networks involving circadian rhythms, which mediate photomorphogenesis, PIF signaling, skotomorphogenesis, ethylene production, and stress responses. Altered circadian rhythms in the hybrids could not only gate the timing of photosynthesis and starch biosynthesis and degradation, which stimulates carbon metabolism and photomorphogenesis and growth phenotypes (2, 6), but also gate the timing of stress responses, which balances the tradeoff between defense and growth (5). Ethylene emission is also regulated by diurnal rhythms (18). In the hybrids, ethylene biosynthesis and signaling pathways are repressed through integration with circadian rhythms and PIFs to promote growth vigor in F1 hybrids, which reveals another regulatory module of complex networks that lead to heterosis.

In *Arabidopsis*, more than 7,000 genes display diurnal expression patterns (26). As a result, circadian-regulated genes could exhibit opposite trends of expression changes during day and night in the hybrids or allotetraploids (5, 6). A recent study reported up-regulation of *PIF4* in the F1 hybrid (C24XL*er*) during the day (ZT7) (27). In our study, *PIF4* and *PIF5* were repressed at night (ZT18) in the F1 hybrids produced from several different *A. thaliana* ecotypes, and their expression changes during the day were not obvious in the hybrids relative to the parents (Fig. 4*A* and *SI Appendix*, Fig. S6). This discrepancy could result from different hybrids and time points tested in two studies. It is notable that PIF proteins are rapidly degraded by light-activated phytochromes, and they are more potent at night to regulate hypocotyl growth (24). Consistent with this notion, expression of *ACS* genes is significantly up-regulated in the *PIF5*-OX plants and down-regulated in *pif1345* mutant at night but not during the day (Fig. 4*B* and *SI Appendix*, Fig. S7). We also noticed an expression increase of several *ACS* genes (*ACS4–7* and *ACS9*) during the day in the *pif1345* mutant and PIF5-OX lines as in the wild-type plants (Fig. 4*B* and *SI Appendix*, Fig. S7), suggesting that *ACS* genes are required for ethylene production and related biological activities independent of *PIFs* during the day. Indeed, constantly overexpressing or repressing *PIFs* affected overall growth as observed in the PIF5_OX lines and *pif1345* mutant, which showed severe and moderate reductions of vegetative growth, respectively (*SI Appendix*, Fig. S9 *B* and *C*). In the *pif1345* mutant, the positive effect of reducing *PIFs* at night on hypocotyl growth could be counterbalanced and uncoupled by the increased ethylene activities during the day. We predict that altered expression of *PIFs* in the hybrids at night is likely more biologically relevant to growth vigor, while their roles during the day remain to be investigated.

Ethylene is also known as a stress-related hormone (28), and many ecotypes used for the study have different basal expression levels of stress-responsive genes (5). C24 is more stress tolerant, especially to biotic stresses than Col-0 (29), and consequently more ethylene is produced in C24 than in Col-0, which is less sensitive to the ethylene treatment (Fig. 1 *A*, *B*, and *E*). Natural variation of ethylene production and sensitivity suggests that plants can adapt to local environments by adjusting ethylene biosynthetic and signaling pathways. This adaptation could be related to altered expression of *ACS* genes through epigenetic modifications, as observed for some stress-responsive genes (5). Notably, many stress-responsive genes are also repressed in the F1 hybrids to promote growth vigor (5, 9). Thus, optimizing diurnal and circadian expression of ethylene and stress-responsive genes under stress and nonstress conditions provides a better resource allocation for promoting growth vigor as well as environmental adaption in the hybrids as in the parents.

## Materials and Methods

**Plant Materials and Growth Conditions.** *A. thaliana* ecotypes including Col-0, C24, Ws, L*er*, and Est-1 (CS22629) were used to generate F1 hybrids by hand pollination. The *cca1 lhy* (CS9380), *pif1345* (CS66049), *acs2* (CS16564), *acs4* (CS16566), *acs5* (CS16568), *acs6* (CS16569), *acs9* (CS16571), *acs24569* (CS16644), *erf1a* (SALK_036267), and *erf73* (SALK_012913) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The PIF5-OX line (35S:PIF5-myc) was obtained from a previous report (30). Transgenic plants that overexpressed $ACS6^{DDD}$ in Col (Col_*ACS6-OX*) were generously provided by Shuqun Zhang, University of Missouri, Columbia, MO. Transgenic plants that overexpressed $ACS6^{DDD}$ in C24 (C24_*ACS6-OX*) were generated by *Agrobacterium tumefaciens*-mediated transformation. For diurnal conditions, plants were grown under the light/dark (L/D) cycle of 16 h/L at 22 °C and 8 h/D at 20 °C. For circadian conditions, plants were grown under the constant light of 16 h/L at 22 °C and 8 h/L at 20 °C. Plant transformation was performed in 4- to 5-wk-old flowering plants following the floral dipping method (31).

**Plasmid Constructs.** The *N. benthamiana* transient expression assay employed luciferase reporter constructs, each containing 2-kb sequence upstream from the ATG codon of *ACS2* and *ACS6–9* that was cloned into pFAMIR-LUC vector (5). For 35S-driven overexpression constructs, each contained coding sequence of *PIF5* or *CCA1* that was cloned into pF35SE vector (5). For generating $ACS6^{DDD}$-overexpression transgenic lines in C24 backgrounds, coding sequences of $ACS6^{DDD}$ were amplified from Col_$ACS6^{DDD}$-*OX* and cloned into pF35SE vector. Each construct was cloned into *Agrobacterium* strain GV3101 for plant transformation (31) or *N. benthamiana* transient expression assay (32). Primer sequences for individual inserts are listed in *SI Appendix*, Table S1.

**RNA Extraction and qRT-PCR.** Total RNA was isolated from aerial rosette tissues of ∼18-d-old seedlings using Plant RNA Reagent (Invitrogen). After digestion by RNase-Free DNase (Promega), total RNA (1 μg) was used to produce first-strand cDNA with the Omniscript RT Kit (Qiagen). The cDNA was used as the template for qRT-PCR using FastStart Universal SYBR Green Master (Roche). The reaction was run on the LightCycler 96 System (Roche). The relative expression level was quantified using internal control *ACT7* (*At5g09810*) (6). Three biological replicates were performed for each sample and three technical replicates were used for each biological replicate in qRT-PCR experiments. The MPV was calculated as the mean of parental expression value for each replicate. One-way ANOVA was used to determine the significance in each comparison. The primer sequences are listed in *SI Appendix*, Table S1.

**Ethylene Measurement by Gas Chromatography.** For each genotype, 12 seeds were germinated in 40 mL in each gas chromatography (GC) vial containing 30 mL of Murashige and Skoog agar. These vials were sealed with permeable tape. After 10 d, the vials were capped and the plants were incubated for another 36 h in capped GC vials in a growth chamber. The ethylene level was measured using GC-2014 (Shimadzu). Ethylene production was normalized to fresh weight of seedlings with five biological replicates.

***N. benthamiana* Transient Expression Assay.** The transient expression assays were performed in leaves of *N. benthamiana* as previously described (32). The *A. tumefaciens* strain GV3101 containing the luciferase reporter construct or 35S-driven overexpression construct was incubated in Luria–Bertani medium and finally resuspended in infiltration buffer (10 mM Mes, 0.2 mM acetosyringone, 10 mM $MgCl_2$) to an ultimate concentration of $OD_{600}$ = 1.0. Equal amounts of different combined bacterial suspensions were infiltrated into the young leaves of the 5-wk-old plants using a needleless syringe. After infiltration, the plants were grown first in the dark for 12 h and then with a cycle of 16 h/8 h (light/dark) for 48 h at 24 °C before imaging. The leaves were sprayed with 100 μM luciferin (Promega) and placed in the dark for

5 min. Luciferase activity was observed using NightOWL LB 983 in Vivo Imaging System (Berthold). Each experiment was performed using three independent biological replicates.

**ChIP-qPCR.** ChIP was performed as previously described (33). Briefly, 2 g of rosette leaves from 2-wk-old Col-0 or 35S:PIF5-myc seedlings were used as starting materials for ChIP experiments. Antibodies against CCA1 (Abiocode) were used to immunoprecipitate the protein–DNA complex. For PIF5, antibodies against MYC (Cell Signaling) were used for immunoprecipitation. The isolated chromatin without antibodies was used as input control. *UBQ10* and *PP2A* were used as internal controls for normalization. Primers used for ChIP-qPCR are listed in *SI Appendix*, Table S1.

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