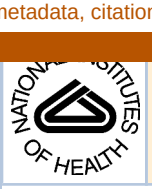

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Regulation of ACS protein stability by cytokinin and brassinosteroid

Maureen Hansen, **Hyun Sook Chae**[†], and **Joseph J. Kieber**[*]

Department of Biology, University of North Carolina, Chapel Hill, NC 27599−3280, USA

Summary

A major question in plant biology is how phytohormone pathways interact. Here, we explore the mechanism by which cytokinins and brassinosteroids affect ethylene biosynthesis. Ethylene biosynthesis is regulated in response to a wide variety of endogenous and exogenous signals, including the levels of other phytohormones. Cytokinins act by increasing the stability of a subset of ACC synthases, which catalyze the generally rate-limiting step in ethylene biosynthesis. The induction of ethylene by cytokinin requires the canonical cytokinin two-component response pathway, including histidine kinases, histidine phosphotransfer proteins and response regulators. The cytokinin-induced myc–ACS5 stabilization occurs rapidly (<60 min), consistent with a primary output of this two-component signaling pathway. We examined the mechanism by which another phytohormone, brassinosteroid, elevates ethylene biosynthesis in etiolated seedlings. Similar to cytokinin, brassinosteroid acts post-transcriptionally by increasing the stability of ACS5 protein, and its effects on ACS5 were additive with those of cytokinin. These data suggest that ACS is regulated by phytohormones through regulatory inputs that probably act together to continuously adjust ethylene biosynthesis in various tissues and in response to various environmental conditions.

Keywords

brassinosteroid; ethylene; protein stability; ACC synthase

Introduction

Ethylene is a gaseous plant hormone that is involved in many growth and developmental processes, including germination, abscission, senescence, plant defense and fruit ripening (Abeles *et al*., 1992). Ethylene is derived from the amino acid methionine, and is produced in three steps: conversion of methionine to Ado-met by *S*-adenyl methionine synthetase, conversion of Ado-met to ACC by ACC synthase (ACS), and conversion of ACC to ethylene by ACC oxidase (ACO) (Yang and Hoffman, 1984). The conversion of Ado-met to ACC by ACS is the first committed step and is generally the rate-limiting step in this pathway (Adams and Yang, 1979; Yang and Hoffman, 1984). The ACS family in Arabidopsis consists of nine proteins, of which eight have ACS activity as homodimers (Tsuchisaka and Theologis,

*For correspondence (fax +1 919 962 1625; e-mail jkieber@unc.edu)..

[†]Present address: Syngenta Biotechnology Inc., Research Triangle Park, NC 27709, USA.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The half-life of the myc–ACS5 protein is increased in response to BR.

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2004a; Yamagami *et al*., 2003). These proteins form homodimers and heterodimers with distinct enzyme kinetics (Tsuchisaka and Theologis, 2004a). The protein sequence of the catalytic core is highly conserved, but the C-terminal region of ACS proteins is divergent and the proteins may be sub-divided into three groups on that basis: type 1 ACS proteins have the longest C-terminus with a single putative calcium-dependent protein kinase (CDPK) phosphorylation site and three mitogen-activated protein kinase (MAPK) phosphorylation sites, type 2 ACS proteins have an intermediate length C-terminus containing a single putative CDPK phosphorylation site, and type 3 ACS proteins have a very short C-terminus and no predicted kinase phosphorylation sites (Chae and Kieber, 2005; Liu and Zhang, 2004; Sebastià *et al*., 2004; Tatsuki and Mori, 2001; Yoshida *et al*., 2005).

The *ACS* genes are transcriptionally regulated both developmentally and in response to environmental stimuli (Tsuchisaka and Theologis, 2004b; Wang *et al*., 2005; Yamagami *et al*., 2003). Recent evidence suggests that an important component of the regulation of ACS and ethylene biosynthesis occurs post-transcriptionally by stabilization of the ACS proteins (Argueso *et al*., 2007; Chae and Kieber, 2005; Chae *et al*., 2003; Liu and Zhang, 2004; Tatsuki and Mori, 2001; Wang *et al*., 2004). Phosphorylation of the MAPK sites in type 1 ACSs increases the stability of the protein and thus increases ethylene production (Joo *et al*., 2008; Liu and Zhang, 2004). Mutations in the C-terminal region of type 2 ACSs (*eto2* and *eto3* in ACS5 and ACS9, respectively) increase the stability of the protein and increase ethylene production in the dark (Chae *et al*., 2003; Joo *et al*., 2008; Vogel *et al*., 1998b). The type 2 ACSs are degraded by the 26S proteasome, a process mediated by an E3 ligase in a C-terminus-dependent manner (Wang *et al*., 2004; Yoshida *et al*., 2005, 2006). ETO1 is an adaptor BTB-TPR (broad-complex, tramtrack, bric-a-brac/tetratricopeptide repeat) protein that is a component of this E3 ligase complex, and *eto1* hypomorphic mutations result in the over-production of ethylene (Gingerich *et al*., 2005; Guzman and Ecker, 1990; Pintard *et al*., 2004; Wang *et al*., 2004; Weber *et al*., 2004). Loss-of-function mutations in RUB (related-to-ubiquitin) components of the E3-mediated protein degradation pathway, the *rub1 rub2* double mutant, the *rce1* (RUB1 conjugating enzyme) and *ecr1* (E_1-conjugating enzyme-related 1) mutants, display triple responses in the absence of exogenous ethylene, and some phenotypes in these mutants are dependent on the ethylene perception pathway, indicating that additional components of the E3 ligase pathway are involved in regulating ethylene biosynthesis (Bostick *et al*., 2004; Larsen and Cancel, 2004; Woodward *et al*., 2007).

Several hormones are known to elevate ethylene biosynthesis, including auxins, cytokinins and brassinosteroids (Arteca and Arteca, 2008; Woeste *et al*., 1999a; Yi *et al*., 1999). Auxin treatment results in an increase in the level of several *ACS* transcripts, while cytokinin has been shown to increase ACS5 protein stability (Abel *et al*., 1995; Chae *et al*., 2003; Liang *et al*., 1992; Tsuchisaka and Theologis, 2004b; Vogel *et al*., 1998a; Wang *et al*., 2004; Woeste *et al*., 1999a,b; Yamagami *et al*., 2003). Brassinosteroids have been shown to increase *VrACS7* transcripts in mung bean, although the mechanism by which brassinosteroids increase ethylene synthesis in Arabidopsis has not been studied.

The cytokinin-signaling pathway has been elucidated, and involves four groups of proteins that show partial functional redundancy: Arabidopsis histidine kinases (AHK), Arabidopsis histidine phosphotransfer proteins (AHP), type B Arabidopsis response regulators (ARRs) and type A ARRs (Kakimoto, 2003; To and Kieber, 2008). The three cytokinin receptor AHKs bind cytokinin and transmit the signal to the AHPs via phosphorylation (To and Kieber, 2008). The AHPs then phosphorylate the type B and type A ARRs, which act as positive and negative elements, respectively, in cytokinin signaling. Cytokinin response factors (CRFs) are downstream transcription factors that are transcriptionally up-regulated in response to cytokinin (Rashotte *et al*., 2003, 2006).

Brassinosteroid (BR) is a plant hormone that is implicated in germination, stem elongation, leaf growth, fertility and response to disease and stress (reviewed by Belkhadir *et al*., 2006; Fujioka and Yokota, 2003; Li and Jin, 2007). Exogenous brassinolide increases ethylene synthesis in etiolated Arabidopsis seedlings, in inflorescence stems in conjuction with auxin, and in mung bean hypocotyls with auxin or cytokinin (Arteca and Arteca, 2008; Woeste *et al*., 1999a; Yi *et al*., 1999). BR is perceived by the BRI1 receptor, a leucine-rich receptor-like kinase (LRR-RLK) that can dimerize with and phosphorylate BAK1 (Belkhadir *et al*., 2006; Fujioka and Yokota, 2003; Li and Jin, 2007). There are two other BRI1-like genes (*BRLs*) in Arabidopsis that can also act as BR receptors (Cano-Delgado *et al*., 2004; Li, 2003; Zhou *et al*., 2004). BRI1 transmits the signal to inactivate BIN2 (BR-insensitive 2), a GSK3-like kinase that is a negative regulator of the BR pathway. BIN2 phosphorylates the transcription factors BES1 (BRI1-EMS-suppressor 1) and BZR1 (brassinazole-resistant 1), which promotes their degradation, and inhibits the DNA-binding activity of BES1.

Here, we investigate the mechanism by which cytokinin and BR increase ethylene biosynthesis. We examine the impact of cytokinin-signaling mutants on ethylene biosynthesis and the kinetics of cytokinin-mediated ACS protein stability, and investigate points of intersection between cytokinin, BR and ethylene biosynthesis.

Results

Cytokinin and BR both increase ethylene biosynthesis, but BR does not induce a full triple response

We examined whether elevated ethylene in response to cytokinin and BR resulted in a triple response, the morphology that dark-grown Arabidopsis seedlings display in the presence of ethylene. Application of cytokinin increases ethylene biosynthesis and does induce a triple response (Figure 1) (Vogel *et al*., 1998a). However, the increase in ethylene production in response to BR does not result in seedlings displaying complete triple-response morphology. In particular, although growth on BR does result in a shortened and thickened hypocotyl, the exaggerated curvature of the apical hook is absent, and the lengths of the root and hypocotyl are not reduced as much as in ACC or benzyladenine (BA)-treated seedlings. Furthermore, the hypocotyl displays irregular spiral growth (Figure 1). Therefore, in addition to increasing ethylene production, BR has other effects on seedling morphology and prevents a complete triple response. Growth in the presence of either BA or BR results in some shortening of the hypocotyl in the strong ethylene-insensitive mutant *ein2−5*, and, in the case of BR, the *ein2−5* seedlings display an irregular spiral-hypocotyl phenotype (Figure 1). These data indicate that both cytokinin and BR have effects on etiolated seedling hypocotyl growth, independent of ethylene. Measurement of ethylene levels confirmed that exogenous cytokinin and BR increase ethylene production in etiolated seedlings (Figure 2a) (Vogel *et al*., 1998a;Woeste *et al*., 1999a).

Cytokinin perception pathway mutants show decreased cytokinin-induced ethylene biosynthesis

We examined whether cytokinin induction of ethylene biosynthesis required the canonical two-component phospho-relay response pathway, and whether there is specificity for this response within the gene families that encode the proteins involved in each step of the pathway. To this end, loss-of-function cytokinin-signaling mutants for each step of the signaling pathway were examined for ethylene production in response to cytokinin.

We first examined the cytokinin-receptor double mutants *ahk2,3, ahk2,4* and *ahk3,4*. In the absence of cytokinin, *ahk* double mutants produce amounts of ethylene comparable to those produced by wild-type seedlings (Figure 2b). *ahk2,4* and *ahk3,4* mutants show reduced

cytokinin-mediated induction of ethylene biosynthesis, indicating that these receptors are required for this process. The double mutants with loss of function of *ahk4* have lower ethylene responses to cytokinin, indicating that AHK4 is probably the greatest primary contributor to ethylene biosynthesis in response to cytokinin. We were unable to measure ethylene biosynthesis in the *ahk2,3,4* triple mutant as it is sterile.

The AHPs function redundantly in various cytokinin responses, including hypocotyl elongation in the dark, which is known to be partially mediated by ethylene (Cary *et al*., 1995; Hutchison *et al*., 2006; Vogel *et al*., 1998b). To test whether the AHPs are involved in cytokinin up-regulation of ethylene biosynthesis, we examined the *ahp1,2,3* and *ahp1,2,3,4* mutants. In the absence of exogenous cytokinin, both multiple loss-of-function mutants produced less ethylene than the wild-type (Figure 2c). This probably reflects the effect of endogenous cytokinin in regulating ethylene biosynthesis in wild-type seedlings, as these mutant displays a stronger reduced cytokinin function phenotype than any of the double receptor mutants (Hutchison *et al*., 2006). In response to cytokinin, the *ahp1,2,3* and *ahp1,2,3,4* mutants displayed greatly reduced induction of ethylene, indicating that AHPs are necessary for transduction of the signal for cytokinin induction of ethylene biosynthesis.

We also examined disruption of the type A *ARR* genes, which encode negative regulators of cytokinin signaling (To *et al*., 2004). Ethylene production from wild-type and *arr3,4,5,6,8,9* mutant seedlings was measured in response to increasing concentrations of cytokinin, ranging from 10 nM to 50 μM. The *arr3,4,5,6,8,9* mutant seedlings produced an elevated level of ethylene in the absence of exogenous cytokinin, probably due to a heightened response to endogenous cytokinin (Figure 2e). This multiple type A *ARR* mutant also produced more ethylene than the wild-type in response to all levels of cytokinin tested (Figure 2e), which is consistent with the hypersensitivity of this mutant in other response assays (To *et al*., 2004).

The type B ARRs are transcription factors that belong to a subgroup previously shown to be involved in cytokinin signaling (Mason *et al*., 2004). Of the 11 type B ARRs, we tested the *arr1*, *arr2*, *arr10* and *arr12* single mutants as well as the quadruple *arr 1,2,10,12* mutant for their impact on ethylene biosynthesis (Mason *et al*., 2005; Rashotte *et al*., 2006). Consistent with the response in other assays, the *arr1,2,10,12* mutant showed a marked decrease in the elevation of ethylene in response to cytokinin (Figure 2d). Neither the basal or cytokinin-induced levels of ethylene biosynthesis were affected in any of the single mutants tested, except *arr1*, which was similar to the *arr1,2,10,12* quadruple mutant. This suggests that ARR1 is the primary type B ARR mediating this response.

The final pathway components that we tested were the CRFs, of which there are six in Arabidopsis. Two triple mutants *crf1,2,5* and *crf2,3,6* have previously been shown to alter cytokinin-regulated transcription (Rashotte *et al*., 2006). The triple mutants showed no changes in ethylene biosynthesis, indicating that the CRFs are not required for ethylene induction by cytokinin.

BR increases the half-life of ACS5 protein

To determine whether BR increases ethylene biosynthesis via changes in ACS5 stability, we examined the half-life of ACS5 protein in a dexamethasone (Dex)-inducible myc-tagged system (Chae *et al*., 2003). Seedlings were grown on a low level of Dex to induce near-endogenous levels of ACS5 protein, and were then treated with either 0.1 μM BR or a carrier control. Cycloheximide was then applied to inhibit *de novo* protein synthesis, and the turnover of the myc–ACS5 fusion protein was examined by Western blotting (Figure 3a). ACS5 protein has a half-life of 15−30 min (Chae *et al*., 2003; Spanu *et al*., 1994). Similar to cytokinin, BR treatment caused a marked decrease in myc–ACS5 protein degradation. Although there is a band at 60 min in the control that is more intense than neighboring bands, this is most likely

the result of variations in the low level of protein induced in at this concentration of Dex; several independent blots support this conclusion (e.g. Figure S1). These results indicate that BR elevates ethylene biosynthesis at least partly through an increase in the stability of the ACS5 protein, although BR may also regulate ethylene through changes in transcription. Indeed, examination of public microarray data suggests a 3.7-fold induction of *ACS5* in response to brassinolide in Arabidopsis (Zimmermann *et al*., 2004).

Kinetics of cytokinin stabilization of ACS5 protein

We have shown that cytokinin induction of ethylene biosynthesis is mediated via the components of the known cytokinin-signaling pathway, and previous studies have indicated that this occurs through stabilization of ACS5 protein (Chae *et al*., 2003). We examined the kinetics of cytokinin-induced stabilization of the ACS5 protein in 4-day-old etiolated seedlings (Figure 3c). In the control treatment, there is a transient increase in protein at 1 h, probably due to increased stress on the seedling when moved to a liquid environment. Cytokinin-treated seedlings clearly show further increased levels of steady-state ACS5 after 1 h, and this elevated level persists until at least 26 h after cytokinin treatment. This time frame is consistent with an early transcriptional response to cytokinin or a direct output of the signaling pathway.

Cytokinin and BR stabilize multiple type 2 ACSs

ACS proteins fall into three classes, based primarily on the presence or absence of regulatory phosphorylation sites in the C-terminal domain. We have shown that cytokinin and BR both up-regulate ethylene biosynthesis, at least in part through stabilization of ACS5 protein, a type 2 ACS. We examined whether the steady-state levels of ACS9, the type 2 ACS most closely related to ACS5, were elevated in response to BA and brassinolide (Figure 4). Similar to ACS5, ACS9 protein levels increase in response to both BA and brassinolide, suggesting that the effect of cytokinin and BR is not specific for ACS5, but rather may be a general feature of type 2 ACSs. The effect of BA and brassinolide on protein stability was additive for both ACS5 and ACS9. This is consistent with measurements of ethylene biosynthesis in which the effects of BA and cytokinin are additive (Figure 2a) (Woeste *et al*., 1999a). This suggests that the two hormones act through distinct targets on these ACS proteins.

The C-terminal domain of ACS5 and ACS9 is important in regulation of the stability of the proteins (Chae *et al*., 2003; Wang *et al*., 2004; Yoshida *et al*., 2006). We examined whether mutations in these C-termini affect the response to cytokinin and BR. Both ACS5^{eto2} and ACS9^{eto3} are still stabilized by the hormones, indicating that these C-terminal mutations do not disrupt the mechanism for this stabilization. It is possible that these mutations do not completely abrogate the C-terminal regulatory function.

Discussion

Ethylene biosynthesis is highly regulated by multiple exogenous and endogenous inputs. A primary point of regulation is control of the level and activity of the ACS enzymes. This key enzyme is encoded by a multigene family that is subject to multiple layers of control that act in concert to precisely mediate the level of ethylene produced by a cell in a given situation. The first layer of control is regulation of transcription of the various *ACS* genes. A large number of regulatory inputs, including brassinosteroid, auxin, wounding, anoxia and developmental signals such as ripening and floral senescence, act at least in part by regulating the transcription of distinct subsets of ACS genes (Argueso *et al*., 2007). A second level of control relates to the diverse enzymatic properties of the large number of homo- and heterodimers that potentially can form in different cells from the various ACS isoforms (Tsuchisaka and Theologis, 2004a). This can alter the amount of ethylene produced from the varying levels of *S*-adenyl methionine substrate that may be present at any given time in a cell. Here, we explored a third

regulatory input into ethylene biosynthesis, regulation of the stability of ACS protein in response to other hormonal cues.

BR increases ethylene biosynthesis and does so by stabilizing ACS protein

BR treatment results in an increase in ethylene biosynthesis (Woeste *et al*., 1999a), and this ethylene probably plays an important role in the many effects of BR on plant growth and development. This is clearly shown by the growth of etiolated seedlings in the presence of BR (Figure 1), for which there are ethylene-dependent and ethylene-independent effects. For example, BR has been shown to promote cell elongation in hypocotyls in the light; if dark-grown seedlings respond similarly to BR, then this effect would be counteracted by the action of the elevated ethylene; thus the elongation of the hypocotyl in the presence of BR is the summation of these two counteracting forces (De Grauwe *et al*., 2005; Fujioka and Yokota, 2003). In addition, BR causes spiral growth of the hypocotyl that is independent of ethylene. Finally, BR promotes opening of the apical hook while ethylene causes an exaggeration in its curvature.

We showed that ACS5 protein was stabilized in response to BR, suggesting that, like cytokinin, BR increases ethylene synthesis by post-transcriptional regulation of ACS5. However, our results also indicate an effect of BR on *ACS* transcription. Public transcriptome data indicate that both *ACS5* and *ACS6* transcripts are slightly elevated (approximately 3.5-fold) in response to brassinolide, and studies in mung bean indicate that *VrACS7* is regulated transcriptionally by BR (Yi *et al*., 1999; Zimmermann *et al*., 2004). Similarly, in etiolated seedlings, BR treatment results in increase of ACS5 transcript levels (M.H. and J.J.K., unpublished results). Thus, similar to cytokinin, BR has an effect on the transcription of *ACS* genes, but also acts by increasing ACS protein stability.

The turnover of the three classes of ACS proteins appears to be regulated through distinct mechanisms. For example, a pathogen-activated MAP kinase has been shown to phosphorylate specifically type 1 ACSs (Joo *et al*., 2008; Liu and Zhang, 2004). The ETO1 protein is involved in turnover specifically of type 2 ACS proteins (Wang *et al*., 2004; Yoshida *et al*., 2005, 2006), and cytokinin appears to act by increasing the stability of type 2 ACS proteins (Chae *et al*., 2003). Here we show that BR also increases the stability of two type 2 ACS proteins. Genetic and molecular analyses have demonstrated a role for the C-terminus of both type 1 and type 2 ACS proteins in targeting the respective proteins for rapid degradation. A simple model suggests that BR and cytokinin act by inhibiting this C-terminus-dependent targeting. However, surprisingly, both hormones still increase the stability of the *eto2* and *eto3* versions of ACS proteins. Thus, the *eto2* and *eto3* mutations reduce, but do not eliminate interaction with ETO1, and the *eto3* mutant produces a normal BR-induced ethylene response (Woeste *et al*., 1999b; Christians *et al*., 2008).

Cytokinin-induced ACS stabilization requires type B ARR transcription factors

We determined that the cytokinin signal that results in the stabilization of ACS5 passes through the canonical cytokinin two-component pathway. We also found that the type B response regulator ARR1 is necessary for the cytokinin-induced ethylene response. As type B ARRs mediate transcriptional responses to cytokinin, this suggests that the action of cytokinin in stabilizing ACS5 acts through altered transcription of a regulatory input. As the alteration of ACS5 protein stability occurs fairly rapidly in response to cytokinin, it is likely that this regulatory input is a primary target of cytokinin-activated ARR1. These data indicate that this input is the primary type B ARR that mediates the response to cytokinin in the stabilization of ACS5. Furthermore, basal ethylene biosynthesis is altered in several of the cytokinin-signaling mutants, suggesting that endogenous cytokinin is important in regulating the level of ethylene biosynthesis.

A model summarizing the above data is shown in Figure 5. Type 2 ACSs are ubiquitinated by E3 ligase containing an ETO1 component, and targeted to and degraded by the 26S proteasome. This targeting is reduced by mutations in the C-terminus of the ACS protein, and by cytokinin or brassinosteroid stabilization. Cytokinin stabilization occurs through the cytokinin-signaling pathway: AHK dimers perceive the signal and phosphorylate AHPs, which in turn phosphorylate type A ARRs and type B ARR1. The former negatively regulate both the cytokinin-signaling pathway and ethylene biosynthesis, while the latter is necessary for ethylene production.

Conclusions

ACS enzymes are encoded by a moderately large gene family, which is regulated by multiple distinct transcriptional and post-transcriptional inputs. This allows exquisite control of ethylene biosynthesis in plants in various developmental and environmental contexts. Here we highlight two such regulatory inputs, cytokinin and BR. The data suggest that both hormones act to stabilize type 2 ACS proteins. The precise nature of how cytokinin and BR stabilize ACS protein awaits further study.

Experimental procedures

Plant materials and growth conditions

Unless stated otherwise, all wild-type controls were Col-0. The mutants used were *ahk2−7*, *ahk3−3*, *cre1−12* (*ahk4* allele), arr1−3, 2−2, 10−2, 12−1, ahp2,3,5, arr3,4,5,6,8,9, crf1,2,5, crf2,3,6 and *ein2−5*, and various combinations thereof (Mason *et al*., 2005; Rashotte *et al*., 2006) (Hutchison *et al*., 2006; To *et al*., 2004). The ransgenic lines used were Dex-inducible myc-tagged ACS5, ACS5^{eto2} (Chae *et al*., 2003), ACS9 and ACS9^{eto3}. The coding region of ACS9 was amplified from cDNA of wild-type and eto3, fused to a 6 × myc cassette, and then cloned into the binary GVG vector pTA7002 (Aoyama and Chua, 1997). Col plants were transformed with the plasmids by the floral dip method (Clough and Bent, 1998), and transformants were selected on MS medium containing hygromycin. T_2 seedlings for lines that expressed the myc-tagged proteins at low levels in an inducible manner were grown on MS medium containing 10 nM Dex screen. Seeds were sterilized and plated on 1 × MS/1% sucrose/0.4% agar medium. The plates were incubated at 4°C for 5 days in the dark and then incubated in the light for 4−6 h at room temperature. Plates were then moved to a 22°C dark chamber for 4 days. All cytokinin treatments comprised 5 μM benzyladenine from a 5 mM stock in 100% DMSO, all BR treatments comprised 100 nM brassinolide from 100 μM stock in 80% ethanol, all ACC treatments comprised 5 μM ACC from 25 mM stock in 100% DMSO, and all controls comprised appropriate carrier added to MS. Dex treatment for the myc–ACS5 line was with 15 nM alone or 8 nM with BR, 30 nM for ACS9, 10 nM for ACS5^{eto2} and 40 nM Dex for ACS9^{eto3}.

Gas chromatography measurements

Seeds were sterilized using chlorine gas sterilization (100 ml bleach + 3 ml HCl in a 6 l closed chamber for approximately 4 h), and 40−80 seeds in 0.4% agar were aliquoted into 3 ml 1 × MS/1% sucrose medium in 22 ml gas chromatography vials. Vials were cold-treated for 5 days at 4°C in the dark, then incubated in the light for 4−6 h at room temperature, and then incubated in the 22°C dark for 4 days. For cytokinin mutant experiments, vials were capped at day 3; all others were capped when moved to 22°C. After growth at 22°C, the accumulated ethylene was measured by gas chromatography as described previously (Vogel *et al*., 1998b). All genotypes and treatments are represented by at least three vials each.

Protein analysis

Proteins were extracted from 4-day-old etiolated seedlings in two volumes of 6 × SDS buffer, and ground with a mini-pestle. Extracts were incubated at 95°C for 3 min and at room temperature for 5 min, and then centrifuged for 3 min at room temperature at 16 000 ***g*** in a microcentrifuge (Eppendorf, http://www.eppendorf.com). Western analysis was performed essentially as described previously (Chae *et al*., 2003).

Half-life experiment

Seedlings were grown on 15 nM Dex or 100 nM brassinolide plus 8 nM Dex in 1 × MS/1% sucrose/0.4% agar under the standard growth conditions for etiolated seedlings described above. Four-day-old etiolated seedlings were washed twice for 7 min in liquid 1 × MS and then moved to individual tubes containing 200 μM cycloheximide in 1 × MS liquid. A subset of seedlings was then extracted for the time 0 control, or samples were incubated for various times before proteins were extracted.

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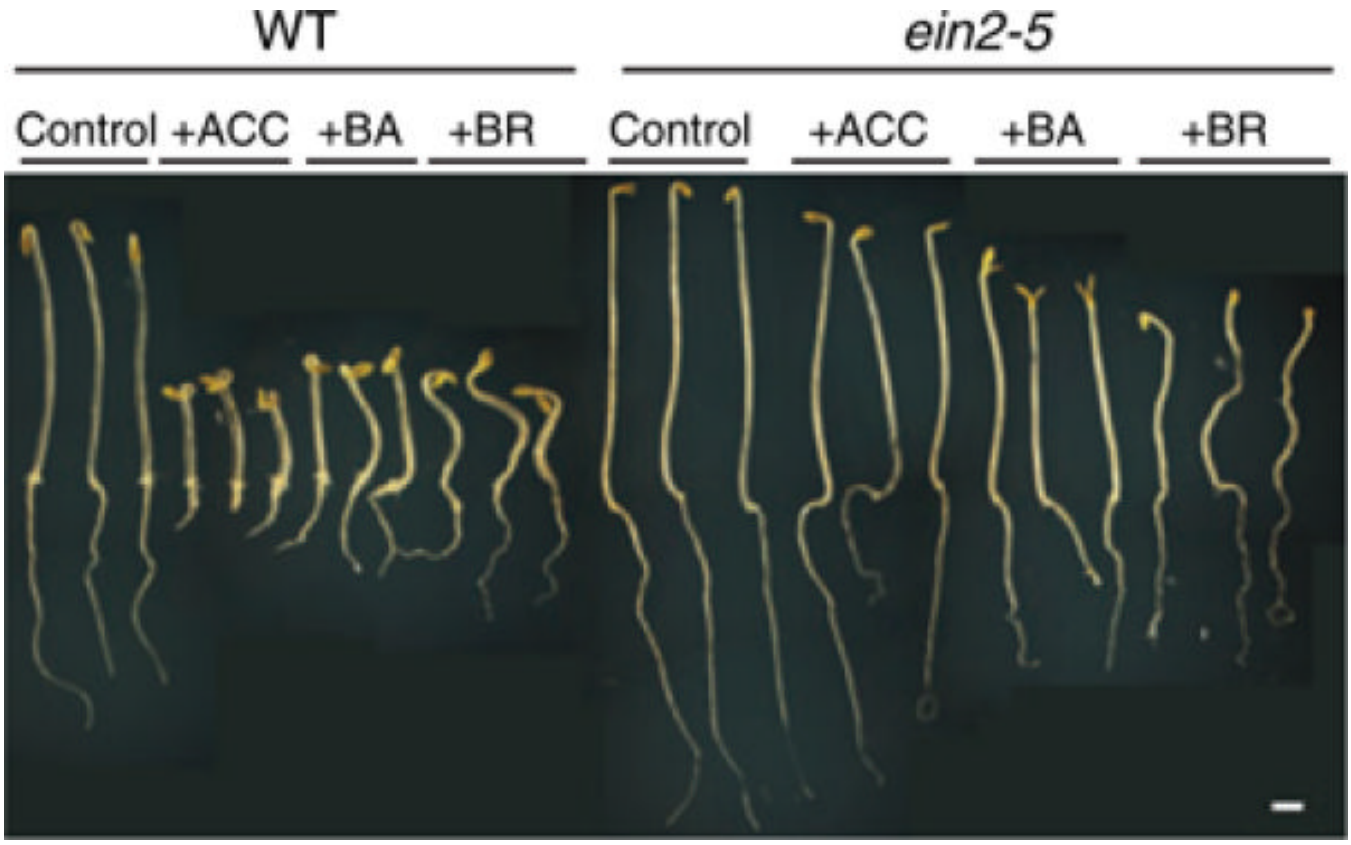

Figure 1. Morphology of four-day-old etiolated seedlings

Wild-type and *ein2−5* seedlings were grown on MS with control carrier, 1 μM ACC, 5 μM BA or 0.1 μM BR. Three representative seedlings of each genotype and treatment are shown. Seedlings treated with BR were flattened to keep the entire seedling in focus. Scale bar = 1 mm.

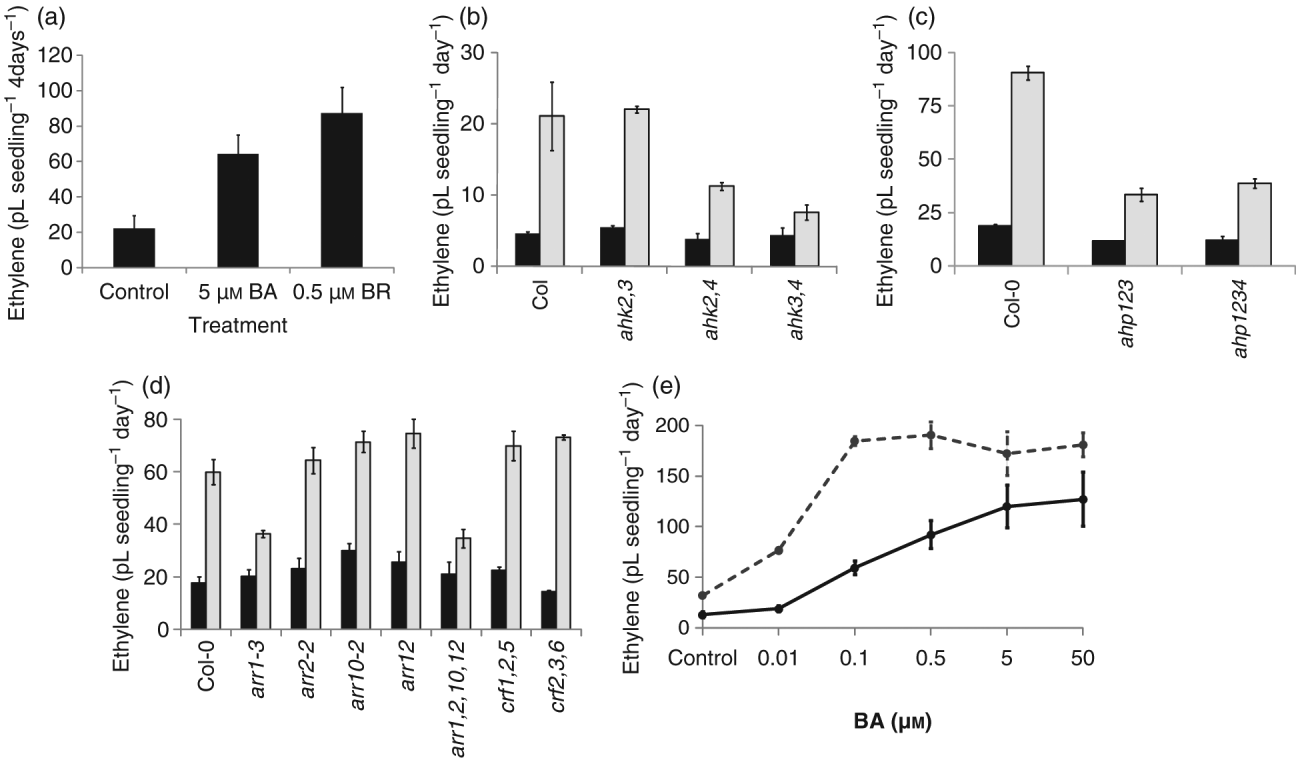

Figure 2. Ethylene production by four-day-old etiolated seedlings in response to BA and BR

(a) Ethylene produced over 4 days in wild-type seedlings treated with carrier control, 5 μM BA or 0.5 μM BR.

(b) Ethylene produced by *ahk* receptor mutants in carrier control (black bars) or 5 μM BA (shaded bars).

(c) Ethylene produced by *ahp1,2,3* and *ahp1,2,3,4* mutants in carrier control (black bars) or 5 μM BA (shaded bars).

(d) Ethylene produced by type B *arr* or *crf* mutants in response to carrier (black bars) or 5 μM BA (shaded bars).

(e) Ethylene produced by wild-type (black circles and solid line) and the type A multiple mutant *arr3,4,5,6,8,9* (gray circles and dotted line) for a range of BA treatments.

For (a), ethylene was accumulated over 4 days; for (b)–(e), ethylene accumulation from day 3 to day 4 was determined. Error bars represent the standard error.

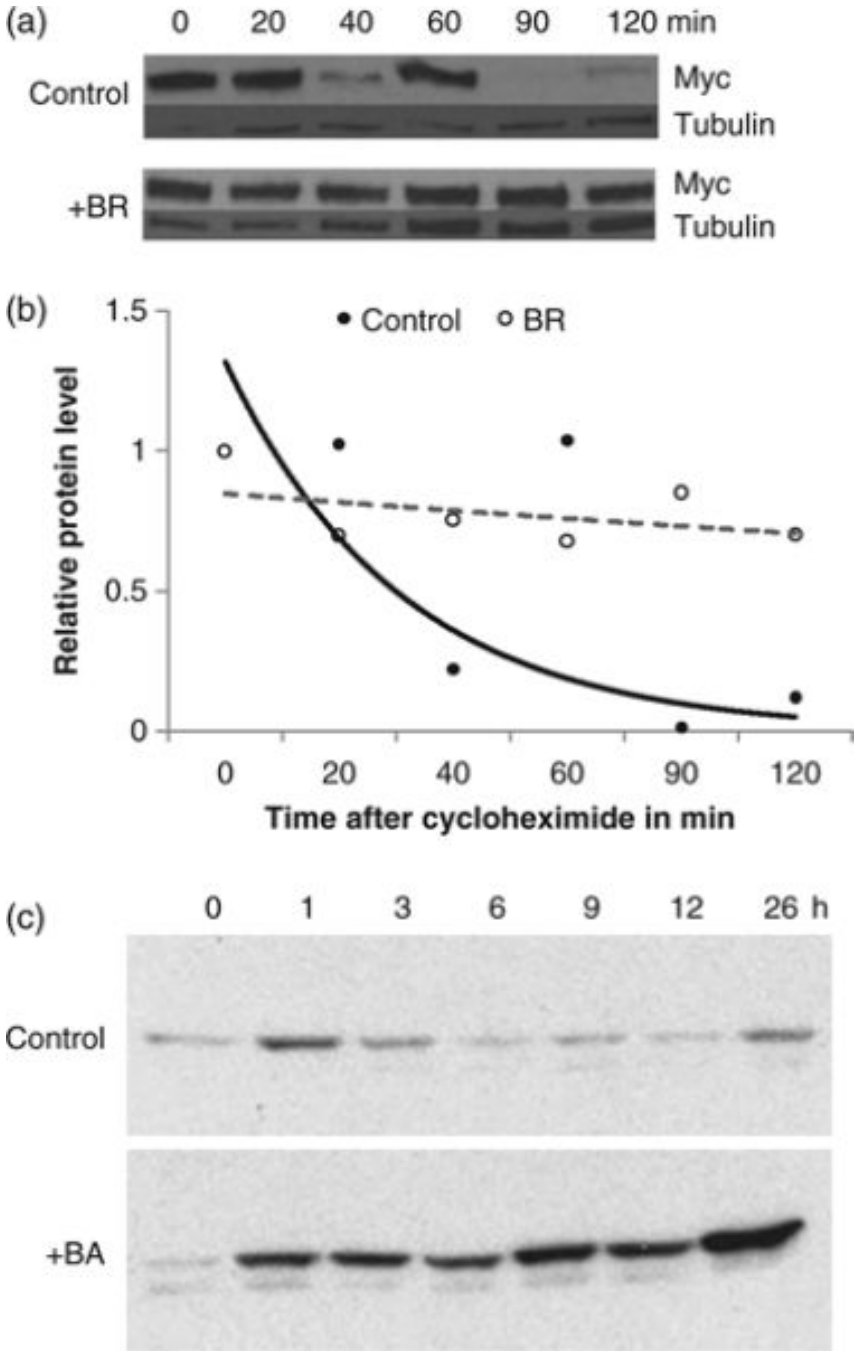

Figure 3. myc–ACS5 protein levels are altered in response to BR and BA

(a) Western blot analysis of protein extracts from 4-day-old etiolated seedlings grown on either carrier control or 0.1 μM BR and treated with cycloheximide for the time indicated. The blots were stripped and then probed with an anti-β-tubulin antibody as a control. This image is representative of five blots performed.

(b) Quantification of the protein level in (a). Open circles represent BR treatment; closed circles represent treatment with the carrier control. Each band in (a) was quantified, normalized to its relevant tubulin control, and plotted as a percentage of the time 0 control. The lines show the best fit using an exponential equation: solid line, carrier treatment; dashed line, BR treatment.

(c) Steady-state protein level of myc–ACS5 at various times after addition of 5 μM BA or a carrier control.

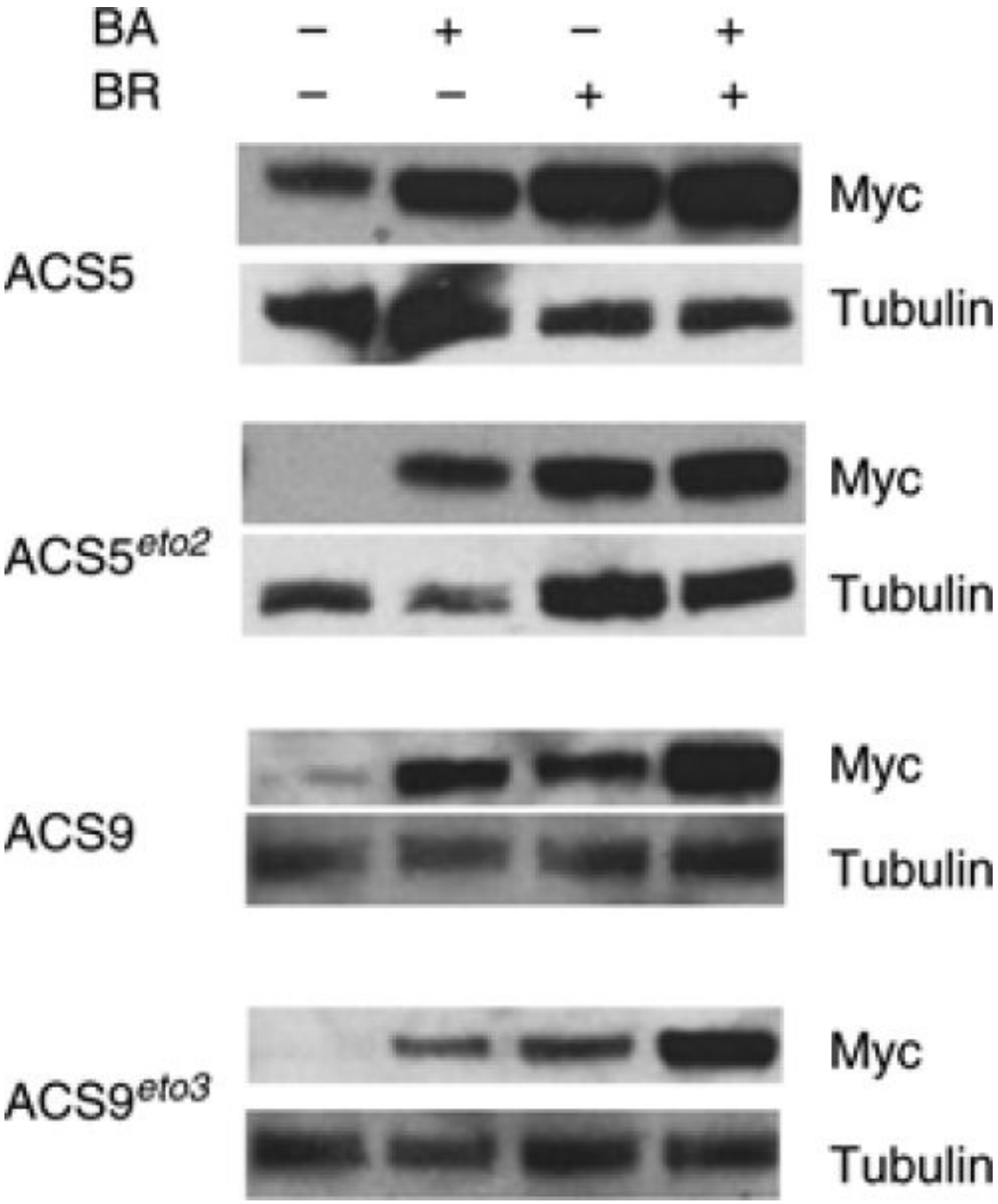

Figure 4. Steady-state levels of myc-tagged ACS proteins in response to BA and/or BR

Four-day-old etiolated seedlings harboring a Dex-inducible promoter expressing myc fusion proteins myc–ACS5, myc–ACS5^{eto2}, myc–ACS9 or myc–ACS9^{eto3} were grown on carrier control, 5 μM BA, 0.1 μM BR, or 5 μM BA plus 0.1 μM BR as indicated. A representative blot from three replicates is shown for each experiment.

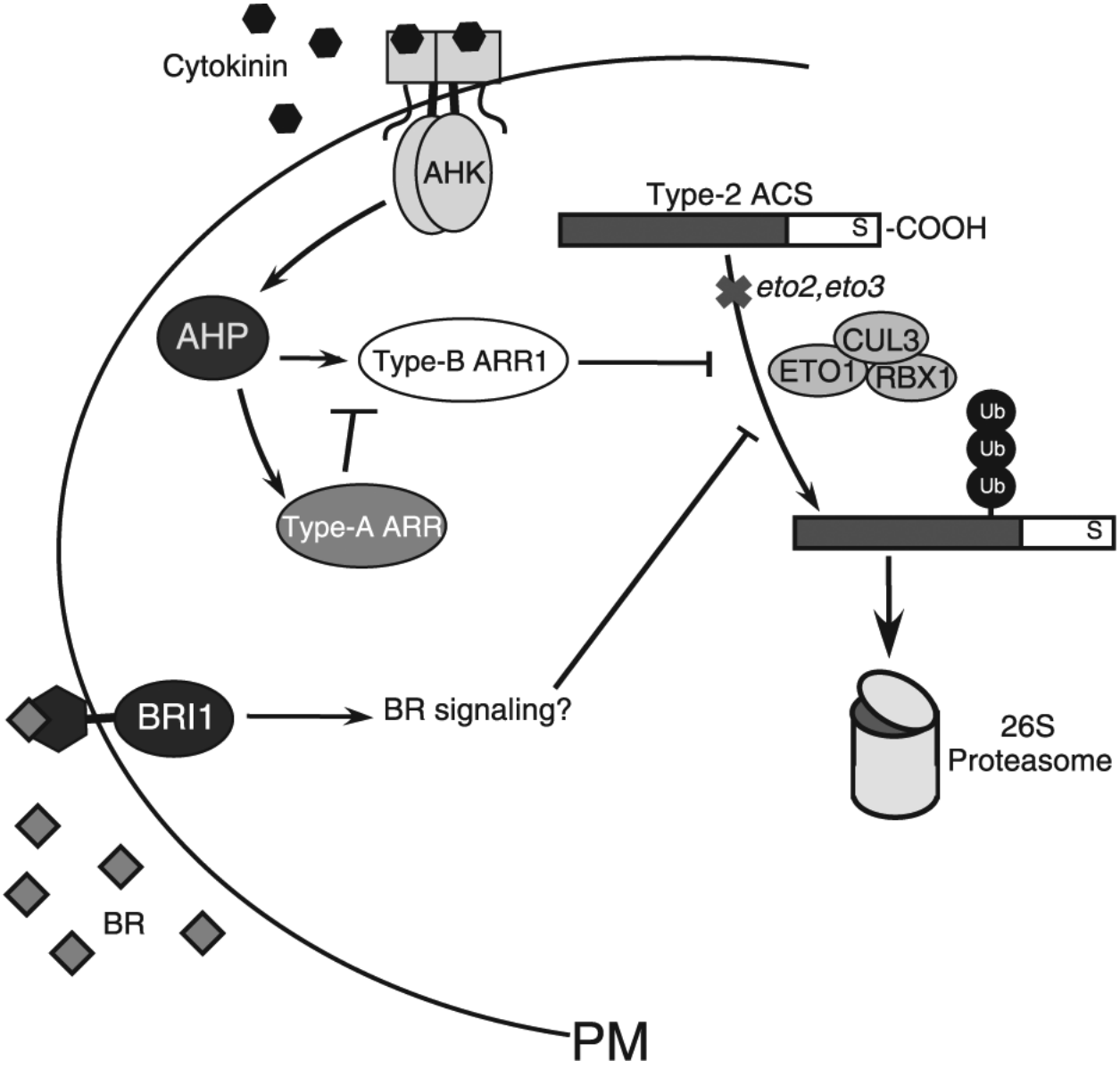

Figure 5. Model of regulation of type 2 ACSs by BR and cytokinin

Cytokinin binds to AHK receptors, which auto-phosphorylate and subsequently transmit a phosphoryl group to the AHPs. The AHPs can then phosphorylate type B or type A ARRs. The type B ARR1, and perhaps other untested type B ARRs, act to prevent degradation of type 2 ACS, in a non-C-terminus-dependent manner. The type A ARRs negatively regulate ethylene biosynthesis, and also cytokinin signaling. The blocking arrow denotes negative feedback on cytokinin signaling, but not at a particular point in the cytokinin-signaling pathway. BR blocks degradation of type 2 ACS, presumably through receptor BRI1. PM denotes the plasma membrane; S in the ACS protein represents Ser461, a putative CDPK phosphorylation site.