Trends in Plant Science

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REVIEWS

Review

The Pivotal Role of Ethylene in Plant Growth

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Being continuously exposed to variable environmental conditions, plants produce phytohormones to react quickly and specifically to these changes. The phytohormone ethylene is produced in response to multiple stresses. While the role of ethylene in defense responses to pathogens is widely recognized, recent studies in arabidopsis and crop species highlight an emerging key role for ethylene in the regulation of organ growth and yield under abiotic stress. Molecular connections between ethylene and growth-regulatory pathways have been uncovered, and altering the expression of ethylene response factors (ERFs) provides a new strategy for targeted ethylene-response engineering. Crops with optimized ethylene responses show improved growth in the field, opening new windows for future crop improvement. This review focuses on how ethylene regulates shoot growth, with an emphasis on leaves.

Highlights

An increasing number of transcriptome studies in plants exposed to biotic or abiotic stress highlight a role for ethylene under a broad range of stresses.

The role of ethylene under stress is dual: it regulates a defense response, mostly in full-grown leaves, and a growth response in young leaves.

In young leaves, ethylene and the downstream ERFs emerge as central regulators of leaf growth inhibition, orchestrating both cell division and cell expansion.

The knowledge of ethylene-mediated growth inhibition can be successfully implemented in crops to improve plant growth and stress tolerance.

Adapting Plant Growth to the Environment: Why and How?

The sessility of plants is undoubtedly their most disadvantageous feature compared to other living organisms, and implies that their survival can be threatened by environmental perturbations. However, plants have developed fascinating mechanisms enabling rapid detection of changing conditions accompanied by highly complex molecular responses, resulting in remarkable phenotypic plasticity. During the vegetative growth stage, one tightly controlled process is plant growth. Under favorable conditions, root and shoot growth is crucial to enable continuous nutrient uptake and energy production through photosynthesis, respectively. Leaf growth, for example, is controlled by no less than six different cellular mechanisms, including precise orchestration of the switch between cell division, that drives the growth of very young leaf primordia, and cell expansion and differentiation (reviewed in [1]). By contrast, sustaining growth under unfavorable conditions could be detrimental. For example, growth under drought stress would increase the evaporative surface of the plant, rendering the plant even more susceptible. Plants thus constantly evaluate whether the environmental signals are favorable for growth or not, and redirect their resources either for growth or for stress defense.

At the physiological level, the integration of environmental signals into proper phenotypic responses is orchestrated by phytohormones. Ethylene, the smallest phytohormone with the simple C_2H_4 structure, is gaseous and therefore enables plant-to-plant communication. Since its discovery around one century ago, the multiple facets of this hormone as a signaling molecule have fascinated scientists, and this led to the unraveling of its biosynthesis and signaling (Box 1 and Figure 1), and the identification of its various functions: regulation of leaf development, senescence, fruit ripening, stimulation of germination, etc. Importantly, ethylene is produced in response to multiple environmental stresses (Figure 1), both abiotic and biotic, suggesting that it acts as a bridge between a changing environment and developmental adaptation. The abiotic stress conditions that trigger ethylene synthesis include submergence, heat, shade, exposure to heavy metals and high salt, low nutrient availability, and water deficiency [2–6].

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Box 1. Recent Advances in Ethylene Biosynthesis and Signaling

The ethylene biosynthesis pathway consists of a simple, three-step process: methionine is converted into ***S*-adenosyl methionine** (SAM; see Glossary), which is further converted by ACC-synthases (ACS) to **ACC**, the direct precursor of ethylene (Figure 1). Recycling of methylthioadenosine enables rapid ethylene biosynthesis when necessary [85]. Because the conversion from ACC to ethylene is an exothermic reaction that only requires oxygen, ethylene biosynthesis is regulated at the level of ACS enzymes, which are also under post-translational control: they can be phosphorylated before ubiquitin-mediated protein degradation by, for instance, ETO1 and CUL3 [86,87]. ACS induction and activation are responsive to environmental factors that trigger ethylene accumulation. As such, ACS genes are transcriptionally induced by drought [5] and by shade, under the control of PIF4 [58]. ACS2 and ACS6 are post-translationally activated through phosphorylation by a MAPK-phosphorylation cascade involving MKK9 and MPK3/6 [88]. ACC levels are also regulated by conjugation and release from conjugates such as malonyl- or jasmonyl-ACC [89]. The soluble ethylene precursor ACC can be taken up by the amino acid transporter **LHT1** and further transported through the plant via the xylem (Figure 1) [90].

In the destination organ, ethylene triggers a signaling cascade initiated by ethylene receptors in the ER and Golgi membrane: ERS1 (ETHYLENE RESPONSE SENSOR 1), ERS2, ETR1 (ETHYLENE RESISTANCE 1), ETR2 and EIN4 (ETHYLENE INSENSITIVE 4). These receptors are active in the absence of ethylene, and their activity can be controlled by complex formation with RTE1 (REVERSION TO ETHYLENE SENSITIVITY) and ARGOS proteins: these are positive regulators of the ethylene receptors, and thus are negative regulators of ethylene sensitivity [11,91,92]. In the absence of ethylene, active receptors subsequently bind to and thereby activate the **CTR1** protein [93]. The levels of the receptors are regulated by ethylene and CTR1: slightly increasing ethylene levels stimulate the transcription of the receptors and stabilization of CTR1, whereas higher ethylene levels push the receptor/CTR1 towards proteasome-mediated degradation [94]. CTR1 is a kinase that represses EIN2, an ER-located membrane protein. When this repression is released in the presence of ethylene, EIN2 is dephosphorylated and cleaved, releasing a C-terminal fragment that either moves to P-bodies or to the nucleus [95,96]. The downstream mode of action of the EIN2 fragment has long been a mystery, but recent studies have shown that it is involved in gene-specific regulation of translation [95,96]. The EIN2 fragment binds to the 3'-untranslated regions (3'-UTRs) of *EBF1* and *EBF2* transcripts, thereby repressing their translation. EBF1 and EBF2 are two central F-box proteins that target the primary ethylene-responsive TFs EIN3 and EIN3-LIKE 1 (EIL1) for protein degradation in the absence of ethylene [97,98]. In the presence of ethylene, EIN3 and EIL1 induce the expression of numerous secondary transcription factors (TFs), the ERFs [99]. The activity of some ERFs has been reported to be increased by phosphorylation through the MPK3/6-cascade that also regulates ethylene biosynthesis, providing dual-level regulation of the ERF-mediated response [24,100].

Ethylene: An Inhibitor of Leaf Growth

Arabidopsis (*Arabidopsis thaliana*) plants overproducing ethylene are generally dwarfed, and plant growth is reduced by exposure to ethylene [7–9]. Consequently, when the positive regulators of the ethylene signaling pathway (Box 1 and Figure 1) are mutated, plants are generally found to have larger rosettes with larger leaves in comparison to control plants. Increased growth has, for example, been observed upon mutation the endoplasmic reticulum (ER)- anchored protein EIN2 [10]. Conversely, mutants of negative regulators of ethylene signaling, such as the receptors ETR1 and ERS1 (Box 1), show a growth decrease [9]. Accordingly, overexpression of the negative regulators *ARGOS* or *ARGOS-LIKE* (*ARL*) stimulates leaf growth in arabidopsis [11,12]. Moreover, plant lines in which the ethylene sensitivity is reduced, or treatments reducing sensitivity to ethylene, cause larger leaves. For instance, plants overexpressing *NEIP2* or *TCTP*, genes encoding proteins interacting with the *Nicotiana tabacum* ethylene receptor, show decreased ethylene sensitivity but improved growth [13,14]. Similarly, *Pseudomonas frederiksbergensis*, a soil bacterium that reduces plant sensitivity to ethylene, promotes the growth of red pepper plants [15]. Finally, some rhizosphere bacteria that promote plant growth do so by expressing *ACC-DEAMINASE*, decreasing the levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in plants exposed to stress, and this has a positive effect on growth [16].

Exceptionally, ethylene has been reported to stimulate leaf growth. In the presence of very low ethylene concentrations, *Poa alpina* and *Poa compressa* show increased leaf elongation rates [17], and also the primary leaves of sunflower (*Helianthus annuus*) are enlarged [18]. However,

Glossary

S-Adenosyl methionine (SAM): a conjugated form of the amino acid methionine. It is an intermediate product of ethylene biosynthesis and the precursor of ACC.
1-Aminocyclopropane-1-carboxylic acid (ACC): the precursor of ethylene. It is stable and can be transported throughout the plant.
CDKA: A-TYPE CYCLIN-DEPENDENT KINASE, a key regulator of the cell cycle that is important at both the G1–S and G2–M phase transitions.
CTR1: CONSTITUTIVE TRIPLE RESPONSE 1. When mutated, CTR1 confers the constitutive triple response, a signature phenotype of ethylene-treated, dark-grown seedlings characterized, in comparison to wild-type etiolated seedlings, by a less-elongated, thickened hypocotyl, and a curling apical hook.
CYCLINs: key proteins controlling the different steps of the cell cycle by associating with the CYCLIN-DEPENDENT KINASES (CDKs). Their cyclic expression and subsequent protein degradation ensures unidirectional progression through the cell cycle.
DEL1 and UVI4: proteins that control the shift between the mitotic cell cycle and endoreduplication.
Endoreduplication: a variant of the plant cell cycle in which the S-phase (DNA synthesis) still takes place but not the M-phase (mitosis), resulting in doubling of the amount of DNA per cell. In many plants, endoreduplication coincides with cell expansion and differentiation.
EXPANSIN: an enzyme responsible for loosening the cell wall.
KRP1/ICK1 and SMR1: CDK-inhibitory proteins that bind to and inhibit CDKs, and thus repress cell-cycle progression.
LHT1: LYSINE HISTIDINE TRANSPORTER has been shown to also mediate ACC transport.

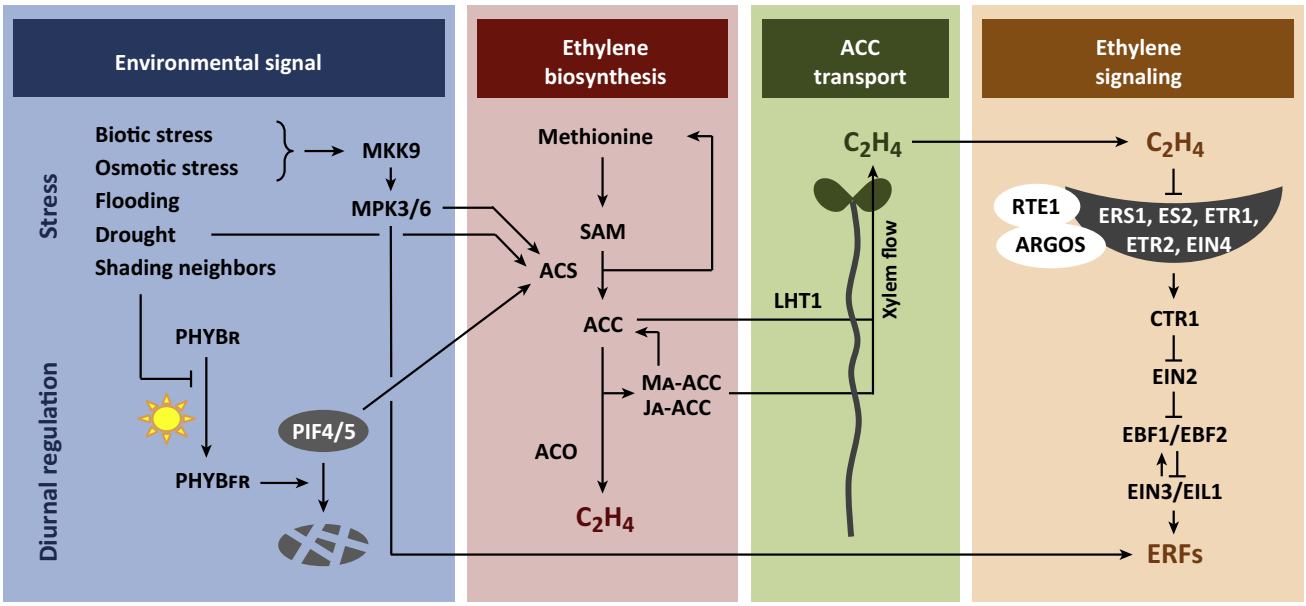

Figure 1. Overview of Ethylene Biosynthesis and Signaling Pathways in Arabidopsis and Environmental Factors That Modulate Ethylene Signaling. Ethylene is synthesized from the amino acid methionine by a three-step pathway (Box 1). Synthesis of the intermediate product 1-aminocyclopropane-1-carboxylic acid (ACC) by ACS enzymes is rate-limiting and controlled by numerous environmental conditions including biotic, osmotic, and drought stress. Ethylene biosynthesis is also diurnally regulated by the red:far-red light ratio. Light-activated PHYB (PHYBFR) binds to and degrades PIF4/5, which can no longer induce *ACS* transcription. Shading by neighboring plants also influences the PHYB–PIF4/5 pathway. The direct ethylene precursor ACC can be transported through the xylem via the LHT1 transporter or can be conjugated into malonyl-ACC (Ma-ACC) or jasmonyl-ACC (JA-ACC), which are also transported through the xylem. In the destination organ, ethylene targets ethylene receptors, and thus relieves CTR1 inhibition of EIN2. EIN2 activation triggers the stabilization of EIN3 and EIL1, primary transcription factors that further control the expression of the downstream *ERFs* (Box 1).

the opposite effect was observed as soon as ethylene levels are increased to concentrations higher than this low growth-promoting optimum. This general negative correlation between ethylene sensitivity and leaf growth has led to the classification of ethylene as a growth-repressing hormone.

Effects of Ethylene on Cell Division

In plants, where growth mainly occurs post-embryonically through well-orchestrated cell divisions, the progression through the cell cycle is tightly governed by more than 70 core cell-cycle proteins (reviewed in [19]). Controlled by endogenous cues and environmental signals, cell-cycle progression and regulation vary depending on the plant organ, and the effect of ethylene is similarly organ-dependent. For instance, during the early development of the apical hook, ethylene participates in stimulating cell divisions, although its contribution is not crucial for curving of the apical hook [20]. Moreover, ethylene and the downstream transcription factors (TFs) ERF018 and ERF109 promote cell division during vasculature development in arabidopsis stems [21]. Thus, in these specific developmental contexts, ethylene can have a positive effect on cell division.

In leaves of plants exposed to environmental stress, ethylene appears to have a negative effect on the cell cycle. When plants are exposed to less than 10 h of osmotic stress, ethylene mediates a temporary and reversible stop of the cell cycle. This is likely to occur through the inactivation of the **CDKA** by phosphorylation, possibly through the MPK3/6 pathway but independently from EIN3/EIL1 (Figure2) [2]. Moreover, at least four mechanisms in leaves link

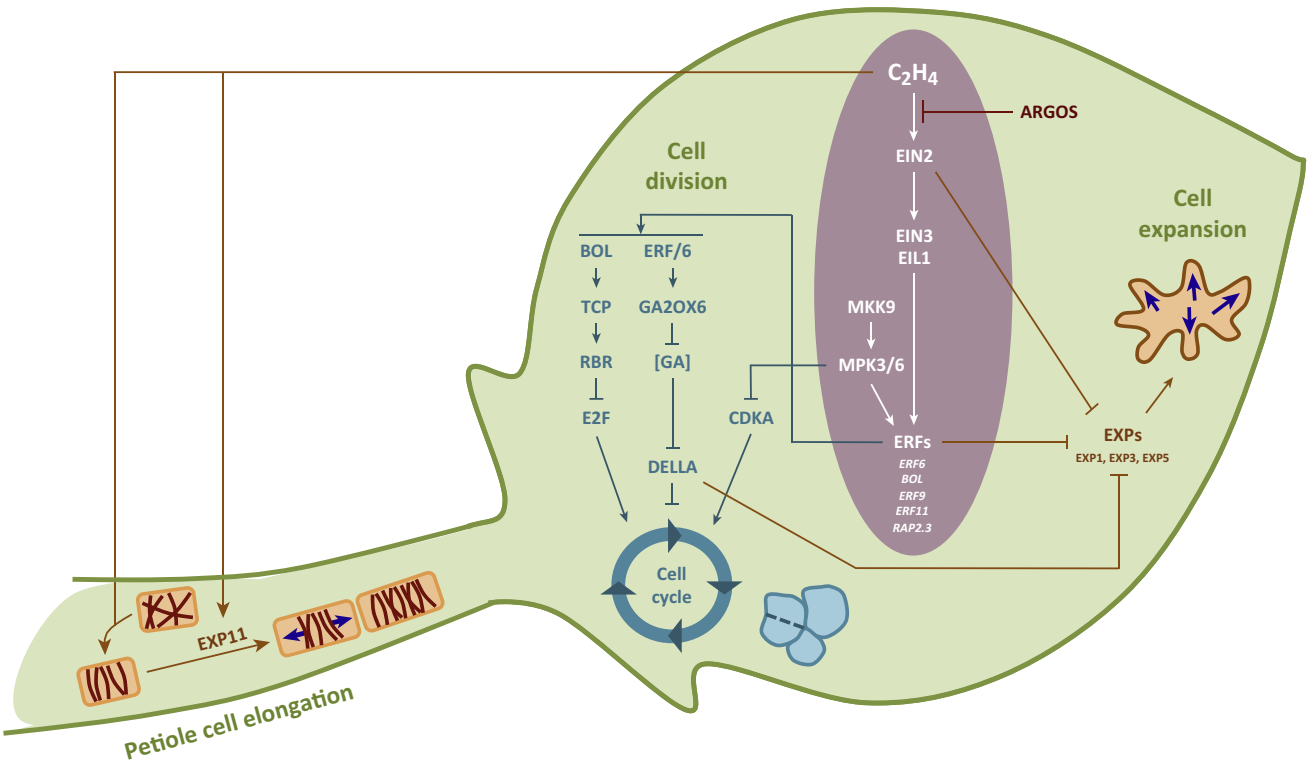

Figure 2. Molecular Pathways in Arabidopsis Leaves Connecting Ethylene to Cell Division, Cell Expansion, and Petiole Cell Elongation. In actively growing arabidopsis leaves, ethylene regulates cell division through different pathways. The MPK3/6-phosphorylation cascade regulates ethylene biosynthesis and ethylene response factors (ERFs) (simplified view, a complete scheme is given in Figure 1), and inactivates CDKA in a EIN3/EIL1-independent manner. Downstream ERFs inhibit cell division directly through *E2F* inhibition, and indirectly by inducing DELLA protein stabilization. Positive regulators of ethylene signaling, such as EIN2 or ERFs, negatively affect leaf growth by inhibiting cell expansion. Conversely, negative regulators of ethylene sensitivity, such as ARGOS and ARGOS-LIKE proteins, have a growth-stimulatory effect in leaves. Ethylene also stimulates the elongation of the abaxial petiole cells, causing hyponasty (Box 2).

ethylene to the exit of cell division and a shift to **endoreduplication** and differentiation. First, accumulation of ethylene and induction of the *BOLITA* TF (an ERF, Table 1) triggers the activation of type II *TEOSINTE BRANCHED 1/CYCLOIDEA/PCF* (*TCP*) genes (Figure 2) [22]. These TCP proteins bind to the promoter of *RETINOBLASTOMA RELATED 1* (*RBR1*), and the encoded protein phosphorylates E2Fa and thus represses the transcription of the E2F target genes, thereby inhibiting progression into the S-phase and cell division. Second, ethylene induces the expression of *ERF5* and *ERF6*, two closely related TFs, in actively growing leaves of plants exposed to stress [2,23,24]. ERF6 induces the expression of a gibberellic acid (GA)-inactivating enzyme, *GA2-OX6*, which triggers a reduction in bioactive GA levels and the accumulation of DELLA proteins (Figure 2). The DELLA proteins further repress the expression of the ***DEL1* and *UVI4*** genes, causing a premature exit from the cell cycle [25]. A third cell-cycle inhibitory mechanism relies on the downregulation of the ***CYCLIN*** genes. Overexpression of *ACS8* in poplar leaves results in downregulation of several A- and B-type *CYCLIN* genes and a *CDKA*-like gene [26]. Notably, in roots, where ethylene also represses the cell cycle, *CYCB1;1* expression is unaffected, but at the protein level CYCB1;1 was degraded in the presence of ethylene, highlighting a post-translational regulatory mechanism [27]. Finally, it should be noted that the CDK-inhibitory genes *SIAMESE* and *SIAMESE-RELATED 8* (*SMR8*) are direct targets of EIN3 in etiolated seedlings [28], and that, in roots, ethylene has been shown to induce the

expression of ***KRP1/ICK1* and *SMR1*** [27] (M.D. and P. Genschik, unpublished data). These evidences could provide a fourth mechanism of action that potentially also occurs in leaves as well. Multiple mechanisms thus connect ethylene to cell-cycle inhibition, but the precise regulatory connections have not yet been fully elucidated.

Effects of Ethylene on Cell Expansion

In a simplified view, cellular growth requires both uptake of water and the corresponding extensibility of the cell wall, thus involving two basic actions: relaxation of the cell wall, mediated by cell wall-remodeling enzymes, and the import of water, mainly through a difference in water potential and facilitated by aquaporins. Similarly as for cell division, specific examples are known where ethylene has a positive effect on cell expansion, such as in petioles or in hypocotyls grown in light (Box 2) [29,30]. Ethylene does so by directly acting on microtubule orientation and on genes of the ***EXPANSIN*** family (Box 2) [30]. In *Sagittaria pygmaea* and grape berry, ethylene induces the expression of xyloglucan endotransglycolases/hydrolases (XTHs), also stimulating cell-wall loosening and cell expansion [31,32].

However, similarly to cell division, the influence of ethylene on cell growth in leaves is almost exclusively negative. Overproduction of ethylene or overexpression of proteins of the signaling cascade results in smaller leaves because of restricted cell expansion, as illustrated by overexpression of *ACS8* [26], *EIN2* [10], *BOLITA* [22], *ERF6* [23], and multiple other ERF-encoding genes, discussed in the next section. By contrast, mutants with a reduced ethylene sensitivity have an increased leaf size resulting from enhanced cell expansion, as demonstrated in *ein2* [10] and in lines overexpressing negative regulators of ethylene signaling of the *ARGOS* family [12]. Molecularly, the connection between the proteins downstream of ethylene signaling and effectors of cell expansion is not entirely clear, but the data point toward convergence at the level of EXPANSINs. *EXP3* and *EXP5* are downregulated in plants overexpressing *EIN2* and are upregulated in *ein2* knockout plants, and expression of *EXP1* and *EXP5* is strongly repressed in dwarfed BOLITA gain-of-function plants (Figure 2) [10,22]. Alternatively, in the ERF6-mediated growth-inhibitory pathway, the inhibition of cell expansion might be regulated by DELLA proteins that are stabilized by *ERF6* overexpression. Numerous molecular mechanisms

Box 2. Hyponasty – Growth-Related and Ethylene-Mediated

In addition to growing, leaves also move up and down to optimize light capture in changing environments. This phenomenon, called hyponasty (up) and epinasty (down), has been observed in multiple plant species but is most pronounced in rosette plants such as arabidopsis. Leaves move in a diurnal way, moving upwards during daytime to reach their most vertical position at dusk [101]. Leaves also move upwards during shade avoidance, light stress, or flooding stress [102,103]. The involvement of ethylene in hyponastic leaf movement under shade or submergence has been known for a long time: *ACS* genes are induced by stress-responsive TFs (Figure 1) [57,104], and *etr1-1* tobacco mutants as well as the arabidopsis *aco5* mutants show reduced hyponastic responses [102,105]. Whether ethylene also regulates the diurnal hyponastic leaf movements under non-stress conditions is still under debate, but recent evidence points in this direction: *etr1-1*, *ein2*, and *acs2* mutants show reduced leaf-movement amplitudes throughout the day [106].

At the cellular level, hyponasty is established by elongation of the cells on the lower side of the petiole. To enable elongation, cortical microtubules (CMTs), which strengthen the cell wall and inhibit growth in their orientation, are reoriented to enable longitudinal growth. This reorientation is stimulated by ethylene, specifically in the proximal abaxial petiole cells, and coincides with ethylene-mediated transcriptional induction of *EXPANSIN11* (Figure 2) [30]. At the molecular level, this is also likely to involve alterations in brassinosteroid and auxin metabolism [104]. Recently, elongation-mediated petiole growth and the involvement of ethylene have been modeled mathematically, also highlighting a role for cell division in this process [107]. The model suggests that the extent of elongation should be greater than what was actually observed, unless the increase in cell elongation is compensated by repression of cell division in the proximal abaxial petiole cells. Experimental validation indeed showed that, in addition to stimulating cell expansion, ethylene also moderates the level of hyponasty by negatively acting on the cell cycle of petiole cells [107].

connect DELLAs to the inhibition of cell expansion (reviewed in [25]), but a notable mechanism is the DELLA-mediated degradation of the PIF4 and PIF5 proteins, which activate genes involved in cell-wall remodeling. In conclusion, at least two parallel pathways are likely to repress cell expansion upon ethylene accumulation in leaves.

Exploring ERFs as Growth-Regulating TFs

In arabidopsis, 65 ERFs have been identified and, to our knowledge, 31 have been studied at the phenotypic level. Of these, 22 show a growth phenotype when overexpressed or knocked down (Table 1), which provides multiple additional connections between ethylene and growth-regulating pathways.

Interestingly, a large number of ethylene-responsive ERFs, including ERF-1, ERF2, ERF5, ERF6, ERF8, ERF9, ERF11, ERF59, ERF98, and RAP2.6L (Table 1), have been shown to be part of a transcriptional network that regulates leaf growth inhibition upon mild osmotic stress [33]. In this network, which also contains TFs outside the ERF family, all the TFs are densely connected and regulate each other's transcription, rendering the network-mediated growth regulation particularly complex. The network is mainly composed of inhibitors of leaf growth, including ERF6, ERF8, ERF9, ERF11, and ERF98, but also contains some growth-promoting ERFs, such as ERF2, ERF59, and RAP2.6L. Most ERFs of the network are transcriptional activators that are induced quickly upon stress to activate the response, whereas two repressing ERFs, *ERF8* and *ERF9*, are induced later to avoid overactivation and enable fine-tuning of the stress response. This network is transcriptionally induced upon osmotic stress, but most likely also acts under other abiotic stress conditions such as salt or drought stress. ERF8, for example, is a strong inhibitor of cell division and leaf growth, and is an important factor in the drought stress response [5,23,33].

Several ERFs, including the core components of this growth-regulatory network, have been shown to directly or indirectly regulate the expression of GA biosynthesis or degradation enzymes in growing leaves. ERF6, ERF9, ERF11, and ERF98 regulate the transcription of *GA2-OX6*, encoding a GA degradation enzyme, upon osmotic stress, possibly resulting in growth inhibition when these *ERFs* are overexpressed [23,33]. Rice plants overexpressing *OsEATB* (an ERF) display a dwarfed phenotype because of decreased GA levels resulting from downregulation of the GA biosynthesis gene *ENT-COPALYL DIPHOSPHATE SYNTHASE 2* (*OsCPS2*), although the *GA20-OX2* gene was upregulated, and *SLR1*, encoding a DELLA protein, was downregulated [34]. Moreover, the dwarfed phenotype of the *ERF6*-overexpression line and of *dwarfy*, an *ACS8*-overexpression poplar line in which several *ERFs* are upregulated, could be rescued by increasing GA levels [23,26].

Connections between ethylene, ERFs, and the growth-regulating GA/DELLA pathway have also been established for stem growth. Ethylene accumulation under flooding conditions induces the expression of SNORKEL1 and SNORKEL2, which promote internode elongation possibly through GA [35]. These two ERFs have a contrasting function with SUB1A, another ERF induced by ethylene, that inhibits elongation upon submergence [36]. This example illustrates the diverse modes of action of different ERFs in the same environmental context. In addition, the opposite holds true: one ERF can exert different growth-related functions in different biological contexts. As such, the growth-regulatory capacities of ERF11 depend on the organ: *ERF11* overexpression induces *GA2-OX6* in leaves and hence inhibits leaf growth [33,37], whereas in the internodes it results in downregulation of *GA2-OX6* and increased elongation [38]. In the latter, ERF11 was also found to interact with the DELLA protein RGA, counteracting its growth-repressing function [38]. These observations show that ERFs are

Table 1. Overview of ERF Mutant Lines with Shoot Growth Phenotypes[a]

Gene name	Origin	Studied plant	Shoot/leaf size	Downstream of ethylene?	EIN3 target [28]	Refs
BOL/DRN-like	*A. thaliana*	*A. thaliana*	GOF: reduced	GOF: reduced ACC sensitivity	N	[22,61]
BOL/DRN-like	*A. thaliana*	*N. tabacum*	GOF: reduced	NT	N	[22]
CaPF1	*Capsicum annuum*	*Pinus virginiana*	GOF: increased	Induced by ethephon	NA	[46]
CRF6	*A. thaliana*	*A. thaliana*	GOF: increased	Induced by ACC and ethylene	N	[62]
CRF8/ERF070	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	N	[63]
DRN/ESR1	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	N	[61]
EBE	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	N	[64]
ERF-1	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	Y	[65]
ERF2	*A. thaliana*	*A. thaliana*	LOF: reduced	Induced by ACC and ethylene	Y	[33]
ERF11	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	Y	[33,37]
ERF14	*A. thaliana*	*A. thaliana*	GOF: reduced	LOF: reduced ethylene signaling	N	[66]
ERF15	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	Y	[67]
ERF4	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC	N	[68]
ERF6	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	N	[23,33]
ERF73/HRE1	*A. thaliana*	*A. thaliana*	RNAi: reduced	Induced by ACC	N	[69]
ERF8	*A. thaliana*	*A. thaliana*	GOF: reduced LOF: increased	Induced by ACC and ethylene	Y	[5,33,68]
ERF9	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	N	[33]
ERF98	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	N	[33]
HhERF2 and PeDREB2a	*Hamilodendron halodendron and Populus euphratica*	*Gossypium hirsutum* L.	Double GOF: reduced	NT	NA	[70]
HYR	*Oryza sativa*	*Oryza sativa*	GOF: increased	NT	NA	[47]
JcERF011	*Jatropha curcas* L.	*A. thaliana*	GOF: reduced	NT	NA	[71]
LEP	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	Y	[72]
NtERF3	*N. tabacum*	*A. thaliana*	GOF: reduced	NT	NA	[68]
ORA59/ERF59	*A. thaliana*	*A. thaliana*	GOF: reduced GOF: increased	Induced by ethylene	N	[33,73]
OsEATB	*Oryza sativa*	*Oryza sativa*	GOF: reduced	Repressed by ethephon	NA	[34]
OsERF1	*Oryza sativa*	*A. thaliana*	GOF: reduced	Induced by ethrel	NA	[74]
OsERF48	*Oryza sativa*	*Oryza sativa*	GOF: reduced	NT	NA	[75]
pti4	*Lycopersicon esculentum*	*A. thaliana*	GOF: reduced	Induced by ethylene	NA	[76]
PvERF001	*Panicum virgatum*	*Panicum virgatum*	GOF: increased	NT	NA	[48]
RAP2.12	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	N	[77]
RAP2.6	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC and ethylene	Y	[78]
RRTF1	*A. thaliana*	*A. thaliana*	GOF: reduced	Induced by ACC	N	[79]
SHN1/WIN1	*A. thaliana*	*A. thaliana*	GOF: reduced	NT	N	[80]
SlERF5	*Solanum lycopersicum*	*Solanum lycopersicum*	GOF: reduced	Induced by ACC	NA	[81]
SUB1A	*Oryza sativa*	*Oryza sativa*	GOF: reduced	Induced by ethylene	NA	[82]
TERF1	*Solanum lycopersicum*	*N. tabacum*	GOF: reduced	Induced by ethylene	NA	[83]
WXP2	*Medicago truncatula*	*A. thaliana*	GOF: reduced	NT	NA	[84]

[a]Abbreviations: *BOL*, *BOLITA*; *CRF*, *cytokinin response factor*; *DRN*, *DORNRÖSCHEN*; *EATB*, *ERF protein associated with tillering and panicle branching*; *EBE*, *ERF BUD ENHANCER*; *ERF*, *ethylene response factor*; *ESR1*, *ENHANCER OF SHOOT REGENERATION 1*; GOF, gain of function; *HRE1*, *hypoxia responsive ERF 1*; *LEP*, *LEAFY PETIOLE*; LOF, loss-of-function; NA, not applicable; NT, not tested; *ORA59*, *octadecanoid-responsive AP2/ERF*; *RAP*, *RELATED TO APETALA*; *RRTF1*, *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1*; *SUB1A*, *SUBMERGENCE 1A*; *WIN/SHN1*, *WAX INDUCER 1/SHINE 1*; *WXP*, *WAX PRODUCTION*.

connected with each other and with the GA signaling pathway, as has been also reported for other hormones [39], resulting in complex crosstalk that regulates shoot growth.

Ethylene and Crop Yield

Over the past decade research on the genetics of ethylene biosynthesis has successfully been translated from arabidopsis to crops, and from the laboratory to the field. For example, a *Zea mays ACS6* gene loss-of-function mutant shows slower leaf senescence and maintains photosynthesis for a longer period when exposed to drought stress [40]. In accordance, *ACS6* RNAi lines, which have reduced ethylene biosynthesis and sensitivity, show a significant increase in grain yield when exposed to drought stress in the field [41].

In addition to the targeted alteration of ethylene biosynthesis, modified expression of ethylene signaling genes appears to have promising applications for improving field crops, the most notable example being maize plants with increased expression levels of *ARGOS*. Overexpression of some genes of the ARGOS family, including *ARGOS*, *ARL*, *AtOSR1*, and *AtOSR2*, stimulates cell division, cell expansion, and thereby leaf size in arabidopsis [42,43]. ARGOS proteins interact with the ethylene receptors and negatively regulate ethylene responses [11,12]. The same ethylene insensitivity and growth advantage were observed in arabidopsis plants overexpressing the maize orthologs *ZmARGOS1* and *ZmARGOS8*. Most interestingly, CRISPR/Cas9-engineered variants of maize with increased *ZmARGOS8* expression levels show a higher grain yield under drought stress conditions, and also a mild but significant increase in plant height under well-watered conditions, albeit without grain yield advantage [44]. Conversely, inducing strong constitutive ethylene responses can be disadvantageous. For example, overexpression of *MHZ7*, the rice *EIN2* homolog, results in the field in shorter plants and reduced yield [45].

Genetic alteration of upstream members of the ethylene signaling pathway results in pleiotropic phenotypes, both desirable and undesirable, because ethylene has wide-ranging molecular functions in almost all plant organs [10,27]. To avoid these undesirable effects, more downstream players, such as the ERFs, could be more attractive candidates to target because they are more specific in terms of molecular function or expression pattern. Overexpression of specific *ERFs* has therefore successfully resulted in increased shoot biomass and yield in the field. For example, overexpression of a pepper ethylene-responsive ERF, *CaPF1*, in Virginia pine resulted in increased tolerance to a range of stresses and in enhanced shoot growth as a result of a larger number of cells [46]. In addition, the overexpression line of *HIGHER YIELD RICE* (*HYR*), a rice *ERF*, has increased shoot biomass and grain yield under normal and drought conditions [47]. Finally, increased biomass was observed in switchgrass overexpressing *PvERF001* [48]. To avoid unwanted side effects, the use of spatially or temporally regulated promoters to control *ERF* expression could also provide success in the field. For example, increased drought tolerance without growth penalty under normal conditions was obtained with rice plants overexpressing *OsERF71* specifically in the roots [49].

Because ERFs are known to have dual roles in regulating both growth and stress-tolerance mechanisms, attempts have been made to increase defense mechanisms by altering the expression of *ERF* genes. Plants with improved defense mechanisms upon stress will survive or grow better following stress, and are expected to produce more yield at the end of the season. *Sl-ERF.B.3* transcripts accumulate upon ethylene treatment, and antisense transgenic tomato plants had improved tolerance to cold stress, without growth penalty, and even a slight tendency towards enhanced height [50]. Using CRISPR/Cas9, some rice variants with deletions in *OsERF922* have been generated, and these showed enhanced resistance to

Magnaporthe oryzae, but no difference with the wild type for several yield-related traits [51]. In several cases, *ERF* overexpression resulted in increased tolerance to stress without affecting growth under normal conditions. *AP37* and *AP59* overexpression gave increased tolerance to severe drought stress and high-salinity stress in rice, without affecting growth [52]. Overexpression of *OsERF109* or *OsERF3*, that are both induced by ethylene, reduced drought tolerance without causing any growth defect under normal conditions [53,54]. However, in a series of studies, *ERF* overexpression improved stress tolerance, but with a negative effect on plant growth. This was observed for rice *OsERF1* and *OsERF48* (Table 1). This shows that, although numerous crops with altered ethylene sensitivity or *ERF* expression have successfully made it to the field, undesired effects are still unavoidable. The highly complex regulatory connections observed between growth-promoting and growth-repressing ERFs [33] might explain such undesired and unpredictable phenotypic effects. In this respect, unraveling the networks in which ERFs are involved will be crucial for understanding the growth-regulatory pathways in shoots, and will enable new advances in targeted engineering of ethylene-mediated shoot growth.

Does Ethylene Regulate Leaf Growth Dynamics?

Ethylene levels are not only increased by adverse environmental conditions but also vary in growth-favorable conditions, for example throughout a day/night cycle. Diurnal oscillations of ethylene levels have been observed in several plants species including sorghum [55], the potato subspecies Andigena [56], and arabidopsis [57]. In general, ethylene levels are low at dawn, increase during the first half of the day, peak between midday and evening, and decrease again during the evening, with the peak slightly shifting depending on the species [55–57]. Interestingly, these oscillations are maintained when plants are transferred to continuous light or dark, pointing to endogenously controlled regulation [57]. In arabidopsis, the fluctuating levels of ethylene in seedlings result from oscillating expression patterns mainly of *ACS8,* but also of *ACS5* and *ACS9* [57]. *ACS8* is most likely a target of the circadian clock because the *ACS8* promoter contains an element typically found in clock-regulated genes. Accordingly, ethylene oscillations are altered in the arabidopsis clock mutant *toc1-1* and in a *CCA1* overexpressor [57]. Moreover, the *ACS8* gene is also under the control of the dark-stabilized PIF4 and, accordingly, *phyB* (PHYTOCHROME B) mutants show higher *ACS8* transcript levels in leaves (Figure 1) [58,59]. Finally, in sorghum, the amplitude of diurnal ethylene oscillations can be influenced by light or shade treatments, as well as by the presence of PHYB [55]. These observations thus suggest that ethylene oscillations are controlled both by a clock-entrained mechanism and a light/dark-regulated response.

This tightly controlled regulation of ethylene fluctuations, even when plants are not exposed to stress conditions, supports the hypothesis that ethylene could also act under normal conditions to exert its growth-regulatory function. Indeed, leaf growth dynamics also vary according to the time of the day under control of the circadian clock [60]. The diurnal oscillations of ethylene levels and growth dynamics have both been thoroughly studied independently, but whether oscillating ethylene levels regulate these leaf growth dynamics has, surprisingly, never been investigated. Based on both the oscillation patterns and the anti-correlation between ethylene and leaf growth, largely illustrated in this review, it may be speculated that ethylene could regulate diurnal leaf growth dynamics. Arabidopsis leaf growth has a maximal rate at dawn [60], which corresponds to the moment when ethylene levels are the lowest [57]. Subsequently, until the afternoon, ethylene levels increase, and leaf growth decreases correspondingly. During the night, leaf growth rates increase, while ethylene levels remain low and invariable. This hypothetical model shows some inconsistencies, particularly regarding growth in the evening when both ethylene and growth rates are low. To further investigate this model it will be crucial to

measure ethylene levels specifically in young and actively growing leaves instead of in whole seedlings or hypocotyls as it has been done until now. Validation of this model could subsequently be obtained by detailed measurements of leaf growth dynamics in ethylene-overproducing lines or mutants. Such detailed investigations would enrich our basic knowledge of the role of ethylene in leaf growth, which is currently restricted to biotic and abiotic stress conditions.

Concluding Remarks and Future Perspectives

Over the past decade, studies have provided multiple molecular connections between ethylene and growth, cell division, and cell expansion. The effects of ethylene accumulation on cell division and cell expansion can be either positive or negative, depending on the environmental context and the organ. In leaves, the effect of ethylene on cellular processes that mediate growth is almost exclusively negative, with the exception of several ERFs, illustrated in this paper, that appear to have positive effects. Although the inhibitory effect of ethylene on shoot growth has been observed in multiple studies, including studies on field-grown crop species, the precise molecular pathways connecting ethylene to growth inhibition are far less understood. Ethylene accumulation in leaves causes rapid inhibition of cell division and cell expansion, either through DELLA-mediated mechanisms or through more direct connections with core cell-cycle or *EXPANSIN* genes, respectively. These connections, however, are still vague, and strong evidence for direct regulatory links is still missing (see Outstanding Questions). It is likely that the ERF TFs play a major role in these regulatory pathways. Identification of their direct target genes would be helpful and would improve our understanding of their sometimes contradictory roles in shoot growth. Given the emerging importance of ethylene-mediated growth inhibition of plants exposed to environmental stresses, unraveling the molecular connections with the effector genes of cell division and cell expansion would be highly valuable for engineering of crops with less-pronounced growth inhibition in adverse conditions. Although impressive results were already obtained in the field by engineering ethylene sensitivity or signaling, undesired effects are still observed, and progress can still be made in uncoupling the defense-inducing and growth-inhibitory mechanisms by targeted engineering using new genome-editing techniques.

Outstanding Questions

What are the precise molecular mechanisms underlying ethylene-mediated inhibition of the cell cycle in leaves? Are the cell-cycle genes directly targeted by EIN3/EIL1 and/or by ERFs in leaves?

Ethylene-engineered crops in the field score high success rates in terms of biomass and grain yield production, but what is the molecular origin of this advantage? Understanding the molecular reasons would enable future engineering in a more targeted manner.

Is ethylene a pure stress-responsive hormone, or does it also have a function under growth-favorable conditions, for example in controlling the diurnal dynamics of leaf growth?

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