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Growth Regulators and the Control of Nucleotide Sugar Flux

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A small number of plant growth regulators are involved in the control of cell expansion. Despite knowledge of some of their signal transduction cascades, surprisingly little is known of how basic cell expansion–related processes, such as cell wall biosynthesis, are affected during growth. The Arabidopsis (*Arabidopsis thaliana*) mutant *root hair defective1* (*rhd1*) lacks a functional UDP-glucose 4-epimerase gene, *UGE4*, which is involved in channeling UDP-D-galactose (UDP-D-Gal) into cell wall polymers. Here, we use *rhd1* as a genetic model to analyze the physiological and genetic controls of nucleotide sugar flux. We find that ethylene specifically suppresses all visible aspects of the *rhd1* phenotype. The ethylene-triggered suppression of *rhd1* is negatively regulated by *CONSTITUTIVE TRIPLE RESPONSE1* and requires the function of the wild-type genes *ETHYLENE INSENSITIVE2* (*EIN2*), *EIN4*, *AUXIN-RESISTENT1*, and *ETHYLENE-INSENSITIVE ROOT1* but does not depend on the activity of wild-type *ETHYLENE RECEPTOR1* or *EIN3* genes, highlighting the nonlinearity of ethylene signal transduction. Ethylene does not induce the expression of alternative *UGE* genes but, instead, suppresses the expression of two isoforms, *UGE1* and *UGE3*, in a tissue-specific manner. Ethylene restores the biosynthesis of galactose-containing xyloglucan and arabinosylated galactan cell wall polymers in *rhd1* back to wild-type levels. However, the dependence on *UGE4* of pectic (1→4)-β-D-galactan and glucuronosyl-modified AGP biosynthesis is exacerbated. Our data suggest that ethylene and auxin together participate in the flux control of UDP-D-Gal into cell wall polymers and that the genetic control of this process is qualitatively distinct from previously described responses to ethylene.

INTRODUCTION

The root of Arabidopsis (*Arabidopsis thaliana*) offers a robust and amenable model to analyze biochemical processes in a well-defined developmental genetic context. Its simple morphology features single cell layers of epidermis, cortex, endodermis, and vasculature, which divide, expand, and differentiate in a spatially well-defined and predictable fashion (Dolan et al., 1993). Mutations in the UDP-glucose 4-epimerase gene *UGE4* of Arabidopsis, which is identical to *ROOT HAIR DEFECTIVE1* (*RHD1*), cause dramatic bulging of root epidermal cells (Schiefelbein and Somerville, 1990; Baskin et al., 1992) because of a deficiency of D-galactose in specific cell wall polymers (Seifert et al., 2002). The observation that *UGE4* is essential for the biosynthesis of galactosylated xyloglucan (XG) in both epidermal and cortical cells and that a type II arabinosylated (1→6)-β-D-galactan (AG II) is absent from the epidermis of *rhd1*, gave rise to the hypothesis that the flux of UDP-D-galactose (UDP-D-Gal) into particular cell wall polymers involves the formation of complexes between cytosolic UGE isoforms (Reiter and Vanzin, 2001) and Golgi-localized galactosyltransferases (Seifert et al., 2002). The recent demonstration that a β-D-glucuronosylated epitope on arabinogalactan proteins (AGPs), recognized by the monoclonal antibody (mAb) LM2 (Smallwood et al., 1996; Yates et al., 1996), is deficient only in trichoblasts of *rhd1* roots but is present in all other cell types (Andeme-Onzighi et al., 2002) suggests that the flux of UDP-D-Gal into different types of AGPs is compartmentalized at the level of UGE isoforms and is regulated in a cell type–specific manner. On a developmental level, the correlation of epidermal bulging with AGP deficiency in the *rhd1* root and the fact that the *rhd1* mutant can be phenocopied by treatment of wild-type roots with the AGP binding β-glucosyl Yariv reagent suggest a role for AGP in the control of cell expansion (Willats and Knox, 1996; Andeme-Onzighi et al., 2002; Seifert et al., 2002). To better understand the role of AGP and other galactosylated cell wall polymers in root development and the role of *UGE4* in the channeling of UDP-D-Gal into the cell wall, we searched for genetic and physiological modifiers of the *rhd1* mutant phenotype. A suppressor of *rhd1* is expected to compensate for the loss of UDP-D-Gal either at the level of its generation or of its channeling into cell wall polymers. Alternatively, reversion of the *rhd1* phenotype may involve a UGE-independent compensation of the mechanical inadequacies of galactose-deficient cell wall polymers responsible for the root epidermal bulging phenotype in *rhd1* mutants. The genetic approach we have taken is to characterize epistatic relationships between *rhd1* and previously characterized regulatory pathways that operate in the same cell types in which the *rhd1* phenotype is expressed. We find that ethylene specifically suppresses the *rhd1* phenotype and modulates the channeling of UDP-D-Gal into cell wall polymers.

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RESULTS

Ethylene Specifically Suppresses the *rhd1* Phenotype

We attempted to modify the *rhd1* phenotype by treatment with growth regulators and by the construction of double mutants.

Exposure of wild-type and *rhd1* seedlings to ethylene, or its precursor 1-aminocyclopropane-1-carboxylic acid (ACC), results in a reduction of root elongation and an increase in hair length and density (Figure 1A). Treatment of *rhd1* with abscisic acid, the artificial auxin 2,4-D (Figure 1B), methyl jasmonate, salicylic acid, and gibberellic acid fails to suppress root epidermal bulging (data not shown). Various mutants that display a short root phenotype, such as *cpd* (Szekeres et al., 1996), *prl1* (Nemeth et al., 1998), *det3* (Schumacher et al., 1999), *alf1* (Celenza et al., 1995) (Figure 1C), *sab* (Aeschbacher et al., 1995), *cob* (Benfey et al., 1993), *rsw1*, *rsw2* (Baskin et al., 1992), and *prc1* (Fagard et al., 2000), showed additional root epidermal bulging when brought into the *rhd1* mutant background (data not shown). Ethylene does not abolish the swelling of epidermal cells in mutants, such as *cob* (Benfey et al., 1993) and *prc1* (Fagard et al., 2000), that display a root epidermal bulging phenotype similar to *rhd1* (Figure 1D). We conclude that ethylene specifically suppresses the root epidermal bulging phenotype of *rhd1* mutants.

Genetic Analysis of Ethylene-Triggered Suppression of *rhd1*

The transduction of the ethylene signal has been genetically dissected previously using the triple response of dark-grown seedlings exposed to ethylene (Roman et al., 1995; Alonso et al., 2003). To test if the triple response and ethylene-triggered suppression of *rhd1* are controlled by the same set of genes, we generated double mutants between *rhd1* and known ethylene response mutants.

CONSTITUTIVE TRIPLE RESPONSE1 (*CTR1*) is a negative regulator of all known ethylene responses (Bleecker and Kende, 2000) acting upstream of a mitogen-activated protein kinase cascade (Ouaked et al., 2003), and *ctr1* mutants display a constitutive ethylene response. In *ctr1 rhd1* double mutants, the *rhd1* phenotype is fully suppressed (data not shown), suggesting control of ethylene-triggered suppression of *rhd1* by the *CTR1* mitogen-activated protein kinase pathway. *ETHYLENE-INSENSITIVE2* (*EIN2*) acts downstream of *CTR1* (Bleecker and Kende, 2000) as a positive regulator of ethylene action, and *ein2* loss-of-function mutants are insensitive to ethylene. *ein2 rhd1* double mutants display strong root epidermal bulging and wrinkled roots both on control medium (data not shown) and in the presence of ACC (Figure 2). The loss of many cortical and epidermal cells early in elongation growth might cause root wrinkling. Our results indicate that *EIN2* is required for the ethylene-triggered suppression of *rhd1*. In the absence of externally applied ethylene, *EIN2* might somehow mask the full effect of the *rhd1* mutation, explaining the exacerbated phenotype of the *ein2 rhd1* double mutants. *EIN4* is a member of a family of five putative ethylene receptors (Hua and Meyerowitz, 1998), and the dominant allele *ein4-1* causes ethylene insensitivity. In the *ein4 rhd1* double mutant, ethylene-triggered suppression of *rhd1* is abolished (Figure 2). *ETHYLENE-RECEPTOR1* (*ETR1*) is another putative ethylene receptor (Chang et al., 1993; Hua and Meyerowitz, 1998), and the dominant *etr1* mutation is strongly ethylene insensitive (Roman et al., 1995). Nevertheless, ethylene-triggered suppression of

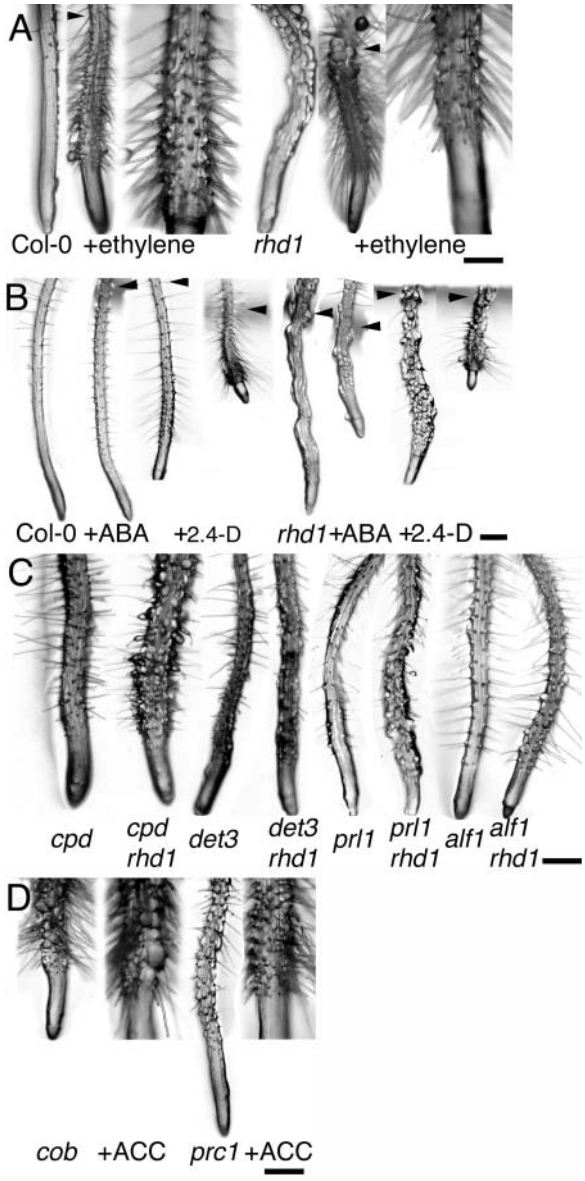

Figure 1. Specific Suppression of *rhd1* by Ethylene.

(A) From left to right, Columbia-0 (Col-0) and *rhd1* grown in air and Col-0 and *rhd1* in the presence of 10 ppm ethylene. Arrowheads indicate the position of the root tip at the start of the treatment.

(B) Growth regulators that inhibit root elongation but do not suppress root epidermal bulging. From left to right, Col-0 control, 100 μM abscisic acid (ABA), 10 nM 2,4-D, 50 nM 2,4-D, *rhd1* control, 100 μM ABA, 10 nM 2,4-D, and 50 nM 2,4-D.

(C) Additive phenotypes between various short root mutants and *rhd1*.

(D) Transfer to 5 μM ACC does not suppress root epidermal bulging in *cob* and *prc1*.

Bars in **(B)** and **(C)** = 250 μm; for higher magnification, bars in **(A)** and **(D)** = 125 μm.

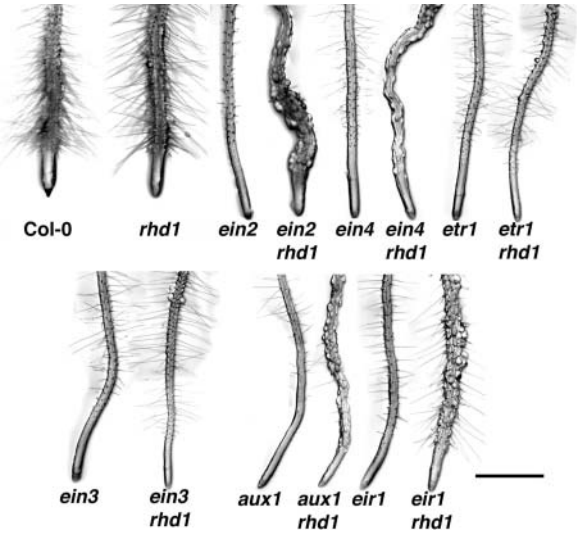

Figure 2. Genetic Dissection of Ethylene-Triggered Suppression of *rhd1*.

Effect of ethylene on the *rhd1* phenotype in various ethylene-resistant mutant backgrounds 2 d after transfer to 5 μM ACC. Bar = 1 mm.

rhd1 took place in the *etr1 rhd1* double mutant (Figure 2). *EIN3* is a positive transcriptional regulator of the ethylene response (Solano et al., 1998). However, as with *etr1 rhd1* double mutants, ethylene efficiently suppresses the *rhd1* phenotype in *ein3 rhd1* double mutants (Figure 2). Mutations in the putative auxin influx carrier *AUXIN-RESISTANT1* (*AUX1*) (Bennett et al., 1996) and the putative auxin efflux carrier *ETHYLENE-INSENSITIVE ROOT1* (*EIR1*) (Luschnig et al., 1998) both confer ethylene insensitivity on the root (Roman et al., 1995). Both *eir1 rhd1* and *aux1 rhd1* double mutants show a strong *rhd1* phenotype in the presence of ACC (Figure 2).

In summary, we find that the ethylene-triggered suppression of *rhd1* is mediated by *EIN4*, *CTR1*, and *EIN2*. By contrast, this novel ethylene response does not involve *ETR1* and *EIN3*. The requirement for intact polar auxin transport generated by *AUX1* and *EIR1* for this response is a new example of the interaction between ethylene and auxin signaling in root development (Swarup et al., 2002).

Ethylene Suppresses the Expression of *UGE1* and *UGE3*

One mechanism to explain the ethylene-triggered suppression of *rhd1* could be the transcriptional induction of alternative *UGE* genes. To address this possibility, we compared, by semi-quantitative reverse transcription (RT)-PCR, the mRNA levels of the five Arabidopsis *UGE* genes (Reiter and Vanzin, 2001; Seifert et al., 2002) in roots of seedlings grown in the absence or presence of 5 μM ACC. We find that none of the *UGE* genes are upregulated but that *UGE1* and *UGE3* are slightly suppressed by ACC (Figure 3A). We then analyzed the spatial expression pattern of the five *UGE* gene family members in *rhd1* plants using transcriptional fusions of genomic regions flanking the five UGE genes and β-glucuronidase (GUS). In untreated controls (C in Figure 3B), *UGE1:GUS* is strongly expressed in all cells of *rhd1* roots, whereas the other four *UGE:GUS* constructs are expressed in a more tissue-specific manner. *UGE2:GUS* is strongly expressed in the root cap (Figure 3B) and appeared in the stele in the mature zone of the root (data not shown). *UGE3:GUS* expression is detected in the columella cells of the root cap and in stele tissue in the differentiation and hair zones (Figure 3B). *UGE4:GUS* is strongly expressed in the stele, the endodermis, and the cortex of the elongation, differentiation, and hair zones and weakly expressed in the epidermis in the division and elongation zones (Figure 3B). *UGE5:GUS* expression is not detected in root tips under these conditions (data not shown). After treatment with ACC, the expression of *UGE2:GUS* and *UGE4:GUS* remained unchanged (A in Figure 3B). However, *UGE1:GUS* expression in the division zone and *UGE3:GUS* expression in the stele is visibly reduced. This is consistent with the slightly suppressed RT-PCR signals for *UGE1* and *UGE3* in ACC-treated roots versus untreated wild-type roots.

Taken together, our data indicate that ethylene does not induce expression of *UGE* genes in roots but rather partially suppresses the expression of *UGE1* and *UGE3* in a tissue-specific manner. We conclude ethylene-triggered suppression of

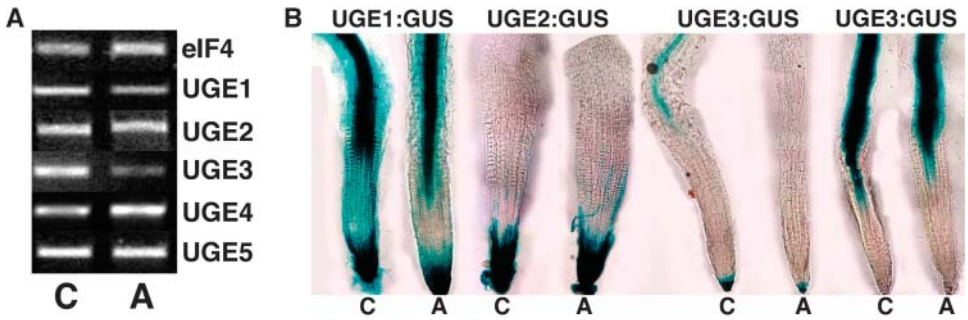

Figure 3. Effect of ACC on the Expression of the Five Arabidopsis *UGE* Genes.

(A) RT-PCR on RNA isolated from roots grown in the absence (lane C) or presence (lane A) of 5 μM ACC.
(B) Root tips of *rhd1* plants transformed with *UGE* promotor:GUS:*UGE* 3′ UTR fusions grown in the absence (C) or in the presence (A) of 5 μM ACC.

rhd1 cannot be explained by increased transcription of alternative *UGE* genes.

Modulation of Cell Wall Defects in the *ctr1 rhd1* Background

To investigate the impact of ethylene on the role of *UGE4* for UDP-D-Gal channeling, we immunolabeled sections of roots of wild-type and *rhd1* mutants (Figure 4A) and of *ctr1* mutants and *ctr1 rhd1* double mutants (Figure 4B) using six mAbs that recognize different cell wall matrix carbohydrate structures that directly or indirectly require the presence of galactosyl residues. The mAb CCRC-M7, which recognizes an arabinosylated (1→6)-β-D-galactan (Steffan et al., 1995) found in AG II, uniformly labeled all cell walls in the wild type (Figure 4A) (Freshour et al., 1996; Seifert et al., 2002). The epitope, however, is totally absent from the *rhd1* root epidermis, including root hairs (Figure 4A) (Seifert et al., 2002). However, CCRC-M7 labels every cell type in *ctr1* single mutants and *ctr rhd1* double mutants, including epidermal cells and root hairs. The mAb CCRC-M1 binds to (1→2)-α-L-fucosyl (1→2)-β-D-galactosyl side chains of XG (Puhlmann et al., 1994) in all cell types of both the wild type (Figure 4A) (Freshour et al., 1996; Seifert et al., 2002) and the *ctr1* single mutant (Figure 4B). Whereas CCRC-M1 labeling is absent in the epidermis, cortex, and endodermis of *rhd1* single mutants (Figure 4A) (Seifert et al., 2002), ubiquitous immunodecoration was restored in the *ctr1 rhd1* double mutant (Figure 4B). The mAb LM2 that recognizes a β-D-glucuronosyl residue on AGP (Yates et al., 1996) binds to the walls of all cell types in the wild type and in *ctr1* mutants (Figures 4A and 4B). In agreement with a previous report on the *rhd1-2* mutant also named *reb1-1* (Andeme-Onzighi et al., 2002), we observed that in the *rhd1-1* allele, labeling with LM2 disappeared only from trichoblast cells but remained strong in all other cell types (Figure 4A). In the *ctr1 rhd1* double mutant, however, LM2 signal was generally absent apart from weak labeling in the division zone (Figure 4B). The mAb LM5 that recognizes (1→4)-β-D-galactan side chains of pectic rhamnogalacturonan I (Jones et al., 1997) labeled stele endodermis, cortex cells, and non-hair cells in the elongation zone of wild-type roots. In the *rhd1* single mutant, the epidermis was unlabeled, but all underlying tissue layers were heavily labeled (Figure 4A). LM5 label was low in the division zone of the wild type and absent from the *rhd1* mutant in all cell types except the root cap (data not shown). In *ctr1* mutants, LM5 profusely labeled every cell in the cell division, elongation, and differentiation zones. However, the *ctr1 rhd1* double mutant was only faintly labeled, and the epidermis and cortex in the transition between cell division and cell elongation zone was completely unlabeled (Figure 4B). The mAb JIM14 binds to an unknown epitope of AGPs and is expressed in all cell types of wild-type roots and *ctr1* single mutants. As for CCRC-M7, JIM14 failed to bind to the epidermis of *rhd1* single mutants but labeled every cell in the *ctr1 rhd1* double mutants (Figure 4B). The mAb JIM13 binds to

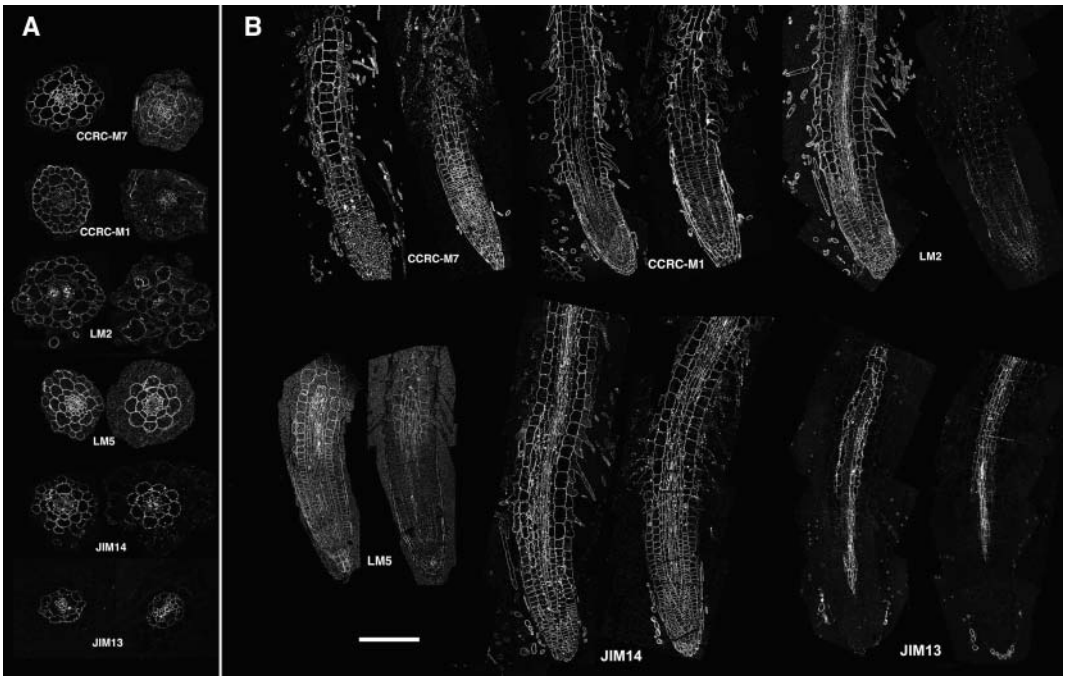

Figure 4. Distribution of Galactose-Dependent Cell Wall Epitopes.

CCRC-M7 recognizes an arabinosylated (1→6)-β-D-galactan (Steffan et al., 1995) found in AG II; CCRC-M1 binds to (1→2)-α-L-fucosyl (1→2)-β-D-galactosyl side chains of XG (Puhlmann et al., 1994); LM2 recognizes a β-D-glucuronosyl residue on AGP (Yates et al., 1996); LM5 recognizes (1→4)-β-D-galactan side chains of pectic rhamnogalacturonan I (Jones et al., 1997); and JIM14 and JIM13 bind AGPs (Yates et al., 1996). Transverse sections through elongation zone of wild-type and *rhd1* single mutants **(A)** and longitudinal sections of the *ctr1* single mutant and *ctr1 rhd1* double mutant **(B)**. Bar = 100 μm.

a complex epitope on AGP (Yates et al., 1996), and labeling in Arabidopsis roots is initially restricted to metaxylem cells and later extends to the endodermis and some pericycle cells (Dolan et al., 1995). The same pattern was observed in *rhd1* and *ctr1* single mutants and *ctr1 rhd1* double mutants (Figure 4B).

Taken together, these data suggest that ethylene dramatically alters the role of *UGE4* in the supply of UDP-D-Gal for the biosynthesis of cell wall polysaccharides (Figure 5). Under ethylene-free conditions, *UGE4* is essential for the supply of UDP-D-Gal to synthesize arabinosylated (1→6)-β-D-galactan and pectic (1→4)-β-D-galactan in the epidermis and for fucogalactosylated XG in the epidermis, cortex, and endodermis. The dependence of the biosynthesis of AGP-bound LM2 epitope on UDP-D-Gal supplied by *UGE4*, on the other hand, is narrowly restricted to trichoblasts. When ethylene signaling is constitutively active, the synthesis of arabinosylated (1→6)-β-D-galactan and fucogalactosylated XG becomes totally independent of *UGE4*. Pectic (1→4)-β-D-galactan and the formation of AGPs bearing the LM2 epitope, on the other hand, become far more dependent on *UGE4* function than under ethylene-free conditions.

DISCUSSION

Novel Role of Ethylene in the Coordination of Nucleotide Sugar Metabolism

Ethylene exerts a large variety of physiological effects during development and stress (reviewed in Wang et al., 2002). In this report, we find that ethylene specifically suppresses the aberrations caused by mutations in the *UGE* gene *RHD1/UGE4* (Schiefelbein and Somerville, 1990; Baskin et al., 1992; Seifert et al., 2002). We argue that this effect reflects a novel physiological role for ethylene in the control of nucleotide sugar flux. Using a variety of growth regulators and genetic treatments, we show that ethylene specifically suppresses the *rhd1* mutant phenotype. Ethylene-triggered suppression of *rhd1* is not an unspecific or indirect effect of the inhibition of root growth by ethylene. Other growth regulators or mutant backgrounds that inhibit root growth do not suppress *rhd1* but rather act in an additive manner. This is in line with previous studies that found additive interactions between *rhd1* and mutants affected in root hair formation (Schiefelbein and Somerville, 1990; Grierson et al., 1997; Parker et al., 2000; Baumberger et al., 2001). Other root morphology mutants, such as *cob* (Benfey et al., 1993) and *prc1* (Fagard et al., 2000), are not rescued by ethylene, and in some cases, such as *sab*, the radial swelling phenotype is even enhanced by ethylene (Aeschbacher et al., 1995). Moreover, we genetically separated the effects of ethylene on root growth and on the *rhd1* phenotype by demonstrating that the suppression of *rhd1* does not depend on the inhibition of root growth in the *ein3 rhd1* and *etr1 rhd1* double mutants. At the histological level, we show that the formation of galactose-containing XG and AG II is rescued in the *ctr1 rhd1* double mutant, demonstrating that ethylene modulates the supply of UDP-D-Gal. The *rhd1* mutant phenotype is caused by a defective flux of UDP-D-Gal into cell surface arabinogalactan-proteins and cell wall polymers. Ethylene-triggered suppression of *rhd1* might reflect a specific role of this growth regulator in stress responses that affect nucleotide sugar metabolism. It was shown previously that externally applied galactose, which is toxic because of profound imbalances in nucleotide sugar metabolism (Dormann and Benning, 1998), induces the production of ethylene in *Phaseolus aureus* (mung bean) hypocotyls (Colclasure and Yopp, 1976). Consistent with the possibility that ethylene is a stress signal for defective nucleotide sugar metabolism, the exaggerated *rhd1* phenotype in the *ein2 rhd1* double mutant indicates that wild-type *EIN2* plays a role that counteracts the deleterious effects of the *rhd1* mutation. This action could be part of a feedback loop involving ethylene. Interestingly, an ethylene- and jasmonate-mediated feedback mechanism acts in mutants defective in cellulose synthase (Ellis et al., 2002; Pilling and Hofte, 2003).

Ethylene Signal Transduction for *rhd1* Suppression Reveals a Novel Genetic Topology

Using known mutants in ethylene response genes to genetically dissect the ethylene-triggered suppression of *rhd1*, we established that this novel response depends on the basic signaling components that have been isolated using the triple response of dark-grown seedlings such as *EIN4*, *CTR1*, and *EIN2*. However, the overlap between the role of ethylene in the control of the triple response and nucleotide sugar flux was not complete, revealing a separation between different ethylene responses on various

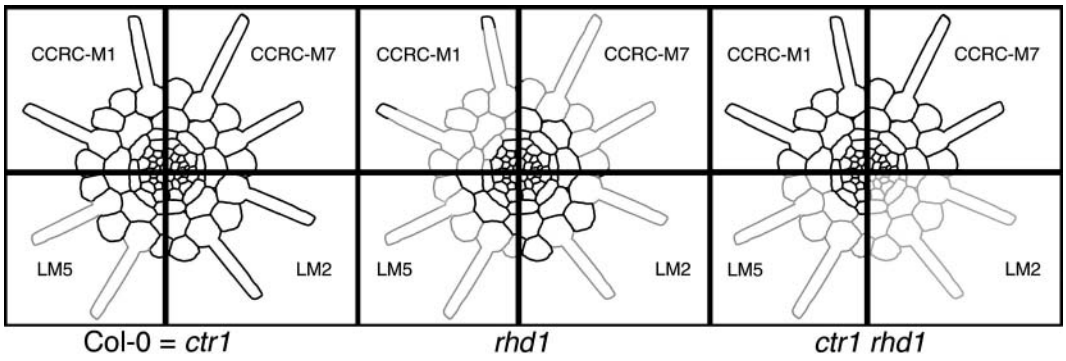

Figure 5. Schematic Overview of Epitope Distribution in Plants in Relation to Genotype at the *RHD1/UGE4* and *CTR1* Loci.

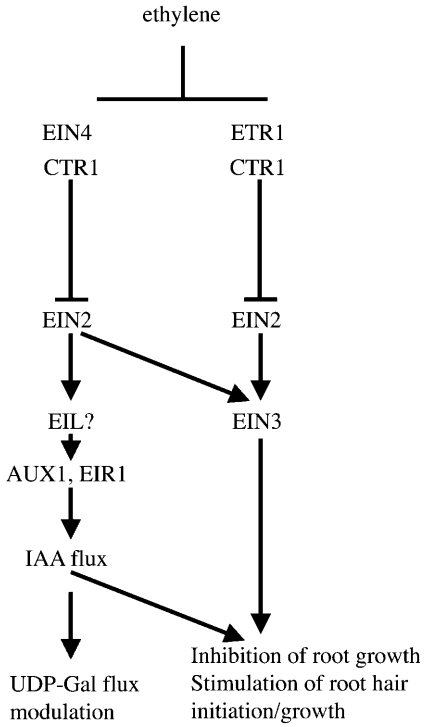

Figure 6. Genetic Pathways Involved in Ethylene-Triggered Suppression of *rhd1* in Relation to Other Root-Specific Growth Responses to Ethylene.

Question marks indicate unclear genetic relations.

genetic levels (Figure 6). The genetic topology of ethylene signal transduction is usually modeled as a linear series of events involving the sensing of ethylene by five functionally redundant ethylene receptors, the inhibitory action of the protein kinase CTR1, and the putative membrane protein EIN2 that in turn triggers nuclear events involving the transcriptional activator EIN3 and structurally related *EIN3*-like (*EIL*) genes that, together with ethylene response factors, control the transcription of ethylene-regulated genes (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Wang et al., 2002). The observation that ethylene suppresses *rhd1* in the *etr1-1* mutant background, but not in the *ein4-1* background, is a striking example of the nonequivalence of individual putative ethylene receptors and might reflect a separation of signal transduction pathways of *ETR1* and *EIN4*, respectively (Figure 6). It was shown previously that the ethylene receptor subfamily I that comprises *ETR1* and *ERS1* has a unique function that cannot be functionally complemented by group II, which includes *EIN4*, *ETR2*, and *ERS2* (Wang et al., 2003). Conversely, *EIN4* and other group II members might play a role that is not covered by family I members. We find that *EIN3*, like *ETR1*, is not involved in the ethylene-triggered suppression of *rhd1*. However, besides *EIN3*, there exist six *EIL* genes in Arabidopsis, and it will be interesting to see if ethylene-triggered suppression of *rhd1* requires *EIL1*, which recently has been found to be allelic with the *WEAKLY ETHYLENE INSENSITIVE5* gene (Alonso et al., 2003), or other *EIL* genes. Apart from the involvement of general ethylene response genes in ethylene-triggered suppression of *rhd1*, we found that the putative auxin influx permease AUX1 (Bennett et al., 1996) and the putative auxin efflux carrier EIR1 (Luschnig et al., 1998) are required for the ethylene-triggered suppression of *rhd1* (Figure 6). In this respect, it is surprising that neither artificial auxins such as 2,4-D (Figure 1C), naphthylacetic acid, nor the auxin-overproducing mutant *alf1* (Celenza et al., 1995) were found to suppress *rhd1*. This suggests that precise homeostasis of auxin is required as part of the ethylene-induced events that suppress *rhd1*. From the hypothetical genetic pathway drawn for the ethylene-triggered suppression of *rhd1* (Figure 6), we predict the existence of novel ethylene response loci that could be isolated from an *rhd1* modifier screen. Such loci might be specifically required for the growth regulator–controlled modulation of nucleotide sugar flux and therefore could not have been identified in previous ethylene and auxin response mutant screens.

Possible Mechanism of Ethylene-Triggered Suppression of *rhd1*

Consistent with the idea that ethylene-triggered suppression of *rhd1* involves a modulation of nucleotide sugar flux, we found that some galactose-containing cell wall polymers were restored in the *ctr1 rhd1* double mutant compared with the *rhd1* single mutant. Given the high degree of genetic redundancy of the *UGE* gene family in Arabidopsis, the simplest mechanism to suppress the effect of *UGE4* deficiency would be the induction of an alternative *UGE*. However, our data indicate that *UGE* transcript levels are not increased by ethylene. Restoration of the normal pattern of CCRC-M1 and CCRC-M7 labeling in *ctr1 rhd1* compared with *rhd1* single mutants is more likely to be the consequence of a redirection of UDP-D-Gal flux rather than increased UDP-D-Gal synthesis. Consistent with this concept, we found that some cell wall polymers, such as pectic galactan labeled with LM5 and the AGP species labeled with LM2, became more stringently dependent on the function of *UGE4* in the *ctr1 rhd1* background compared with the *rhd1* single mutant. Because *UGE1:GUS* expression and LM5 immunolabeling on the one hand and *UGE3:GUS* expression and LM2 immunolabeling on the other were reduced in approximately overlapping tissue areas, this could indicate roles for *UGE1* in the formation of pectic galactan and for *UGE3* in the formation of AGP species bearing the LM2 epitope in cooperation with *UGE4*. We have hypothesized previously that UDP-D-Gal is channeled into various cell wall polymers by a physical interaction among cytosolic UGE, Golgi-localized UDP-D-Gal transporters, and polymer-specific galactosyl transferases (Seifert et al., 2002). This also would explain the differential action of ethylene on different cell wall polymers in a *UGE4*-deficient mutant background. Ethylene might increase the flux of UDP-D-Gal generated from alternative UGE isoforms such as UGE1, UGE2, and UGE3 for the biosynthesis of galactosylated XG and AG II and thereby suppress the deficiencies of cell walls and plasma membrane that are characteristic of the *rhd1* mutant phenotype. This might involve the induction of adaptor proteins and/or post-translational modifications of UGE isoforms, creating new

channeling complexes. Although this model still lacks direct support, further genetic dissection of the *rhd1* phenotype and its modifiers as well as the identification of molecular interactors of UGE isoforms could provide mechanistic insight.

METHODS

Plants and Growth Conditions

Arabidopsis wild-type (Columbia-0) and mutant seeds were sterilized and germinated as described previously (Seifert et al., 2002). We generated double mutants by pollinating *rhd1* plants with mutant pollen and selfing the resulting F1 plants. In most cases, the isolation of double mutants segregating in the F2 generation was performed based on the additive nature of the phenotype. Except for the infertile *rhd1 cpd* and *rhd1 sab* double mutants, all observations were performed in homozygous F3 families. To confirm the genotype of ethylene-insensitive *rhd1* double mutants, we either tested the hypocotyl elongation of dark-grown seedlings (*ein2*, *etr1*, *ein3*, and *ein4*) or root gravitropism (*aux1* and *eir1*), which are both normal in *rhd1* single mutants. Because *ctr1 rhd1* double mutants cannot be phenotypically identified in the F2 generation, they were selected from *rhd1 rhd1 CTR1 ctr1* offspring, and observations were performed on homozygous *ctr1 rhd1* F4 families.

Immunohistochemistry

Reflection microscopy of silver-enhanced, immunogold-labeled, resin-embedded tissue sections and electron microscopy were performed as described previously (Bush and McCann, 1999). All treatments of the wild type and mutant, including image acquisition and processing, were performed in parallel.

Expression Analysis

Semiquantitative RT-PCR of UGE isoforms was performed as described previously (Seifert et al., 2002). To produce *UGE* promoter:GUS:*UGE* 3′ UTR fusions, we amplified ~2 kb of the genomic 5′ region adjacent to the respective *UGE* start codon and 1 kb downstream of the respective UGE stop codon of the five known Arabidopsis UGE genes, introducing additional restriction sites for cloning. The products were cloned into pGEM-T (Promega, Madison, WI). The inserts of sequenced recombinant clones were introduced into a derivative of binary vector pGREEN0179 (Hellens et al., 2000) that contained the bacterial *GUS* gene (Jefferson et al., 1987). The resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and was transformed into *rhd1* plants by floral dip (Clough and Bent, 1998). Transformants were selected on plates containing hygromycin (40 μg mL^{-1}). GUS expression was detected in homozygous T3 plants as described previously (Jefferson et al., 1987).

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