Synergistic action of histone acetyltransferase GCN5 and receptor CLAVATA1 negatively affects ethylene responses in Arabidopsis thaliana

Stylianos Poulios and Konstantinos E. Vlachonasios*
Department of Botany, School of Biology, Faculty of Science, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

* To whom correspondence should be addressed. Email: kvlachon@bio.auth.gr

Received 1 September 2015; Revised 21 October 2015; Accepted 4 November 2015

Editor: Richard Napier, University of Warwick

Abstract

GENERAL CONTROL NON-REPRESSIBLE 5 (GCN5) is a histone acetyltransferase (HAT) and the catalytic subunit of several multicomponent HAT complexes that acetylate lysine residues of histone H3. Mutants in AtGCN5 display pleiotropic developmental defects including aberrant meristem function. Shoot apical meristem (SAM) maintenance is regulated by CLAVATA1 (CLV1), a receptor kinase that controls the size of the shoot and floral meristems. Upon activation through CLV3 binding, CLV1 signals to the transcription factor WUSCHEL (WUS), restricting WUS expression and thus the meristem size. We hypothesized that GCN5 and CLV signaling act synergistically to inhibit ethylene responses in Arabidopsis. This genetic and molecular interaction was mediated by ETHYLENE INSENSITIVE 3/EIN3-LIKE1 (EIN3/EIL1) transcription factors. Our data suggest that signals from the CLV transduction pathway reach the GCN5-containing complexes in the nucleus and alter the histone acetylation status of ethylene-responsive genes, thus translating the CLV information to transcriptional activity and uncovering a link between histone acetylation and SAM maintenance in the complex mode of ethylene signaling.

Key words: Arabidopsis, auxin, CLV1, EIN3, ethylene, GCN5, histone acetylation, triple response.

Introduction

Ethylene is a gaseous plant hormone that affects several developmental processes and responses to environmental cues, including seed germination, seedling morphology, fruit ripening, leaf senescence, sex expression, biotic defense, and abiotic tolerance (Bleecker and Kende, 2000). Based on genetic and biochemical studies in Arabidopsis thaliana, ethylene perception at the endoplasmic reticulum (ER) membrane is translated through signaling mechanisms and complex regulatory networks to transcriptional regulation in the nucleus (Guo and Ecker, 2004; Zhao and Guo, 2011). In plants, ethylene is perceived by a group of ER membrane-located receptor proteins, ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ETHYLENE RESPONSE SENSOR2 (ERS2), and ETHYLENE-INSENSITIVE4 (EIN4), that are similar in sequence and structure to bacterial two-component
histidine kinases (Hua et al., 1998; Stepanova and Alonso, 2009). In the absence of ethylene, the ethylene receptors interact with a member of the Raf kinase family, CONSTITUTIVE ETHYLENE RESPONSE 1 (CTR1) (Guo and Ecker, 2004), which acts as negative regulator of the ethylene signaling pathway (Kieber et al., 1993). The physical interaction of ethylene receptors with CTR1 keeps downstream signaling components EIN2 and EIN3 inactive (Chao et al., 1997; Solano et al., 1998; Alonso et al., 1999). In the absence of ethylene, the EIN3-Binding F-box protein 1 (EBF1) and EBF2 target the transcription factors EIN3/EIN3-LIKE1 (EIL1) for degradation through the 26S proteasome-mediated pathway (Guo and Ecker, 2003; Potuschak et al., 2003; Binder et al., 2007). In the presence of ethylene, EIN3 and EIL1 accumulate in the nucleus and are necessary and sufficient for the induction of most ethylene-response genes (Guo and Ecker, 2004; Stepanova and Alonso, 2009; Chang et al., 2013). EIN3/EIL1 binds as a homodimer immediately to the 5′ upstream region of the ETHYLENE RESPONSE FACTOR 1 (ERF1) gene (Solano et al., 1998). This motif is also present in other early ethylene-response genes, such as GLUTATHIONE S TRANSFERASE 1 (GST1) (Solano et al., 1998). The transcriptional factor ERF1 then activates several secondary ethylene-inducible genes that contain the GCC box in their promoter (Solano et al., 1998). During the first day of ethylene treatment, four distinct waves of transcription are observed, suggesting that several layers of transcription control are present (Chang et al., 2013).

Acetylation of the N-terminal tails of histones was one of the first chromatin modifications to be characterized and is generally correlated with increased accessibility and transcription of the associated DNA (Verdone et al., 2005). The identification of the transcriptional regulator GCN5 as a histone acetyltransferase (HAT) (Brownell et al., 1996) gave rise to characterization of enzymes and their regulatory partners, which form large multiprotein complexes that function to alter chromatin states (Grant et al., 1997). GCN5 physically associates with ADA2 in several transcriptional coactivator complexes that are particularly well characterized in the yeast Saccharomyces cerevisiae (Grant et al., 1997). Similar to yeast and mammals, Arabidopsis GCN5 has been shown to acetylate H3 in vitro (Stockinger et al., 2001; Mao et al., 2006), and global H3 acetylation is reduced in gcn5 mutant plants (Benhamed et al., 2008). Specifically, H3K14 and H3K27 acetylation is reduced at defined loci in gcn5 mutants (Benhamed et al., 2006). Also, Arabidopsis GCN5 interacts in vitro with the two homologs ADA2a and ADA2b (Stockinger et al., 2001; Mao et al., 2006). Arabidopsis ADA2b enhances the HAT activity of GCN5 (Mao et al., 2006). Expression of 5% of a set of 8200 genes tested was changed in gcn5 and ada2b mutant leaves (Vlachonasios et al., 2003). As a result, gcn5 mutants exhibit pleiotropic developmental defects, including dwarfism, loss of apical dominance, aberrant meristem function in the root and shoot, leaf and flower development, short petals and stamens, flower infertility, and plant responses to abiotic stress (Bertrand et al., 2003; Vlachonasios et al., 2003; Benhamed et al., 2006; Long et al., 2006; Cohen et al., 2009; Hark et al., 2009; Kim et al., 2009; Kornet and Scheres, 2009; Pavangadkar et al., 2010; Kaldis et al., 2011). Moreover, AtGCN5 protein is associated with 40% of the promoters in the Arabidopsis genome (Benhamed et al., 2008).

The aerial parts of the plant are generated by shoot apical meristem (SAM) function. Shoot stem cells are harbored in the central zone at the tip of the meristem, while organ initiation takes place in the peripheral zone of the meristem (Sablowski, 2011). SAM maintenance is regulated by CLAVATA3 (CLV3), an arabinosylated tridecapeptide (Ohyama et al., 2009), which is produced by shoot stem cells and diffuses to underlying cells to inhibit the gene expression of the transcription factor WUSCHEL (WUS) via a negative-feedback loop that regulates the size of the meristem (Brand et al., 2000; Schoof et al., 2000). CLV3 is perceived by two different receptors, CLV1, a putative receptor kinase, that controls the size of the shoot and floral meristems (Clark et al., 1993, 1997) and CLV2/ CORYNE subunits (Muller et al., 2008; Bleckmann et al., 2010).

Mutations in GCN5 resulted in overproliferation of young buds, the development of abnormal structures around the inflorescence meristem, and upregulation of several meristem regulatory genes including WUS (Bertrand et al., 2003; Cohen et al., 2009). Therefore, GCN5-dependent gene expression programs or developmental regulation may be repressive for WUS expression (Servet et al., 2010). In this report, we hypothesized that GCN5 and CLV1 act together to regulate SAM function. To test this, we used genetic and molecular approaches to generate and characterize clv gcn5 double mutants. Surprisingly, the clv1-1 gcn5-1 mutant exhibited constitutive ethylene responses suggesting that, in the absence of ethylene, CLV1 and GCN5 could act in synergy as negative regulators of ethylene responses.

Materials and methods

Plant materials and growth

The gcn5-1, ada2a-2, and ada2b-1 mutants in Wassilewskija-2 (Ws) ecotype have been previously described (Vlachonasios et al., 2003; Hark et al., 2009). The clv1-1 and clv3-2 mutants in the Landsberg erecta (Ler) ecotype and the eil3-3, ein3-1, cr1-1, eto2-1, and hsl1-1 mutants in the Columbia-0 (Col-0) ecotype were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Plants were grown at 20–22 °C with 100–150 μmol m^2 s^-1 cool-white fluorescent lamps under long-day conditions (16h light/8 h dark). Soil-grown plants were irrigated twice weekly with half-strength Hoagland’s nutrient solution.

Triple-response assays

Seeds were surface sterilized and cold treated at 4 °C for 3–4 d in the dark. For plating, seeds were sown on Gamborg B5 medium (Duchefa) supplemented with 1% sucrose (Duchefa). The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Sigma-Aldrich) was added to the medium at final concentrations of 0.1, 1, and 10 μM. The ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) (Sigma-Aldrich) and the ethylene perception inhibitor AgNO3 (Sigma-Aldrich) were added to the medium at a final concentration of 10 μM. Seedlings were grown vertically at 20–22 °C in
the dark for 4 d, and then hypocotyl length, root length, and apical hook angle were measured using ImageJ software (Abramoff et al., 2004).

Genetic analysis
The clv-1 gcn5-1 double mutant was created using pollen from gcn5-1 to fertilize clv-1-4. The resulting F1 generation was self-fertilized and the segregating F2 population was genotyped using PCR-based methods (ExTaq™, TakaRa; KAPA2G™ Fast PCR, Kapa Biosystems) with the primers listed in Supplementary Table S1 at JXB online, to confirm the double mutants. The double mutants were backcrossed to either the Ws-2 or Ler background for at least four generations. The triple-response phenotype was observed in both ecotype backgrounds. The clv-1 mutation was tracked using specific primers to amplify a region of the gene and then the PCR product was digested with Cac8I (New England Biolabs). The triple mutant ein3-1 clv-1-1 gcn5-1 was created using pollen from clv-1-1 gcn5-1/+ flowers to fertilize ein3-1 mutants. The F1 generation was genotyped to identify the triple heterozygous plants that were left to self-fertilize and produce F2. In the F2 population, triple mutants were identified by PCR-based genotyping. The ein3-1 mutation was tracked by PCR with specific primers and digestion with HaeIII (TaKaRa). The triple mutant was backcrossed for at least four generations to the Col-0 background. The eil3-3 ein3-1 clv-1-1 gcn5-1 quadruple mutant was created using pollen from ein3-1 clv-1-1 gcn5-1/+ to fertilize eil3-3 mutants. The F1 generation was genotyped to identify quadruple heterozygotes, which were left to self-fertilize to produce F2. The quadruple mutant was found in the F3 generation after identifying eil3-3/ein3-1 clv-1-1 gcn5-1/+ double heterozygotes and leaving them to self-fertilize to produce F3. The eil3-3 mutation was tracked by PCR with specific primers. The clv3-2 gcn5-1 double mutant was created using clv3-2 pollen to fertilize gcn5-5. The F1 double heterozygotes were left to self-fertilize and the F2 population was genotyped for the gcn5-1 mutation to identify the double mutants. The clv3-2 mutation was tracked phenotypically. The clv3-2 gcn5-1 double mutant was backcrossed to the Ler background for at least four generations. The clv-1 ada2a-1 double mutant was created using pollen from clv-1-1 to fertilize heterozygous ada2a-1 plants. The F1 generation was genotyped for both mutations using PCR with specific primers to track ada2a-1 and the double heterozygotes were self-fertilized. The F2 generation was genotyped as above to identify double mutants. The clv-1 ada2a-2 ada2a-1 double mutant was created by using pollen from clv-1-1 ada2a-1/+ to fertilize ada2a-2. The triple mutant was backcrossed to the Ws-2 background for at least four generations. The ada2a-2 mutation was tracked by PCR with specific primers. Finally, the hsl1-1 clv-1-1 gcn5-1 triple mutant was created by using clv-1-1 gcn5-1/+ pollen to fertilize hsl1-1. The triple mutant was backcrossed for at least four generations to the Col-0 background. The hsl1-1 mutation was tracked by PCR with specific primers and digestion with EclI (New England Biolabs).

Gene expression analysis
Plants were grown on Gamborg B5 medium supplemented with 1% sucrose for 7 d and then transferred on Gamborg B5 medium without sucrose for another 7 d. The seedlings were then transferred to Petri dishes covered with wet paper for 24 h. Six to eight seedlings were treated with 1 ml of 5% ethylene/air mix for 1, 3, and 6 h, and then flash frozen in liquid nitrogen. For gene expression analysis in adult plants, 30-d-old soil-grown plants were treated with 100 μM ACC solution. The solution was sprayed on to the plant rosettes until they were saturated. Rosette leaves were collected before treatment or after 4 h and flash frozen in liquid nitrogen. RNA extraction was performed using a Nucleospin® RNA Plant kit (Macherey-Nagel). Reverse transcription was carried out using 0.5 μg of total RNA with the Promega Reverse Transcription System (Promega) or the PrimeScript™ 1st strand cDNA Synthesis kit (Takara) in three independent biological repeats. Quantitative PCR (qPCR) analysis was performed using a KAPA™ SYBR® Green Fast qPCR kit and the ABI StepOne™ platform using gene-specific primers (Supplementary Table S1). Gene-specific primers for the PDF2 ORF were used as controls. The Cq values obtained from the ethylene-induced genes were normalized to the values obtained from PDF2. The values were expressed as PDF2-normalized levels of the target genes. Student’s t-test was used to compare the expression of the target genes between wild-type and mutant plants to determine whether the difference was significant at P<0.05 or P<0.01.

Chromatin immunoprecipitation
Chromatin immunoprecipitation assays were performed according to Kaldis et al. (2011) with minor modifications. Fifteen-day-old Ws, clv-1-1, and gcn5-1 plants and clv-1-1 gcn5-1 mutants were used. Approximately 300 mg of plant tissue was used for each sample. Seedlings were fixed in 1% formaldehyde for 15 min in a vacuum and neutralized with 0.125 M glycine in a vacuum for an additional 5 min. After washing twice with cold, sterilized PBS solution, the tissue was ground in liquid nitrogen to a fine powder. The isolated nuclei were sheared to an average length of 500 bp by sonication five times for 10 s each. Sonicated chromatin was diluted ten times and 1 ml was used for each immunoprecipitation. Antibody against acetylated histone H3 was used (Upstate). The immunoprecipitated samples were incubated with protein A–agarose beads (Roche) for 60 min. Elution of chromatin fragments attached to the beads was done at 65 °C with 1% SDS, 0.1 M NaHCO3. Proteins were detached from the chromatin by reverse cross-linking with 200 mM NaCl at 65 °C overnight. This was followed by proteinase K (Sigma-Aldrich) treatment. The DNA was isolated using a commercially available PCR clean-up kit (Macherey-Nagel). Immunoprecipitated DNA was diluted in water and analyzed with qPCR using specific primers (Supplementary Table S1). Real-time PCR was carried out in reactions using buffer containing KAPA SYBR® FAST qPCR Master Mix ABI Prism® (Kapa Biosystems). Input samples were used in 10-fold serial dilutions to construct the standard curve. The percentage input values obtained for the target regions of ERF1, EBF2, and GST1 were normalized and presented as fold change relative to wild type. The promoter region of RBCS1A was used as a positive control (Benhamed et al., 2006). The At2G19390 gene was used as a negative control, indicating no change in acetylation status between the mutants. The means and standard error of three biological replicates were calculated.

Confocal microscopy
Seeds for the DR5rev:GFP transgenic lines were obtained from NASC. The DR5rev:GFP transgene was introgressed into gcn5-1, clv-1-1, and clv-1-1 gcn5-1 mutants. Pollen from clv-1-1 gcn5-1/+ plants was used to fertilize the DR5rev:GFP pistil. The F1 generation was left to self-fertilize and plants from the F2 generation were genotyped by PCR using green fluorescent protein (GFP)-specific primers (Supplementary Table S1). A Zeiss AxioObserver.Z1 confocal scanning microscope equipped with an LSM 780 confocal laser scanning system was used to detect the GFP fluorescence. ZEN 2011 software was used to analyze the results according to the manufacturer’s instructions.

Results
The clv-1-1 gcn5-1 double mutant displays constitutive ethylene responses
In Arabidopsis, GCN5 is required for a plethora of developmental processes such as leaf development, apical dominance, root meristem activity, inflorescence and floral
meristem function, and flower fertility (Bertrand et al., 2003; Vlachonasios et al., 2003; Cohen et al., 2009; Kornet and Scheres, 2009; Servet et al., 2010). gcn5 mutants exhibit enlarged inflorescence meristems and elevated WUS expression (Bertrand et al., 2003; Cohen et al., 2009). The SAM is regulated primarily through a negative-feedback loop involving WUS and CLV (Brand et al., 2000; Schoof et al., 2000). In order to investigate whether GCN5 controls SAM size through the CLV/WUS pathway, we constructed a double mutant of gcn5-1 and clv-1, an intermediate dominant-negative allele (Dievart et al., 2003). If GCN5 acts in the CLV/WUS pathway, we expected to observe similar phenotypes of the double mutant as the clv-1 mutant. Surprisingly, the clv-1 gcn5-1 double mutant displayed a synthetic phenotype characterized by dwarfism, a compact rosette, epinastic leaves, delayed transition to reproductive growth, and floral sterility (Fig. 1A and data not shown). Moreover, when grown in the dark, the clv-1 gcn5-1 mutant displayed a strong triple-response phenotype characterized by a short hypocotyl, short root, and exaggerated apical hook (Fig. 1B and Supplementary Fig. S1 at JXB online). The progeny of the heterozygous clv-1 gcn5-1/+ plants showed segregation of the triple-response phenotype at a ratio of approximately 1:3 (Supplementary Table S2 at JXB online), indicating that the triple-response phenotype is caused by a recessive loss-of-function mutation. These phenotypes were reminiscent of constitutive ethylene responses (Guo and Ecker, 2004), suggesting a synergistic action of CLV1 and GCN5 that leads to negative regulation of ethylene responses.

We next measured the hypocotyl length of 4-d-old etiolated seedlings grown in the presence of the ethylene biosynthesis precursor ACC, to monitor the effects of exogenous ethylene on clv-1 and gcn5-1 as well as on the clv-1 gcn5-1 double mutant. The wild-type plants and the clv-1 and gcn5-1 mutants responded to ACC treatment by displaying short hypocotyls and exhibiting the triple response (Fig. 1C). At low concentrations of ACC, the clv-1 gcn5-1 mutant showed a slight but statistically significant reduction in hypocotyl length (Fig. 1C), indicating that the double mutant was still responsive to ethylene.

The constitutive ethylene-response phenotypes observed in the clv-1 gcn5-1 mutant could result from either ethylene overproduction or activation of the ethylene signaling pathway. To test these hypotheses, the single and double mutants as well as the wild-type seedlings were treated with either ethylene biosynthesis inhibitor AVG or the ethylene perception inhibitor AgNO3. In the presence of either inhibitor, at a concentration that reverses the ethylene overproducer eto2 phenotype (Vogel...
et al., 1998), the clv1-1 gcn5-1 seedlings continued to display the triple response (Fig. 1D, E and Supplementary Fig. S2 at JXB online), indicating that the phenotypes of clv1-1 gcn5-1 plants did not arise as a result of increased ethylene production or by increased sensitivity toward ethylene. This result suggests that CLV1 and GCN5 act below the level of ethylene biosynthesis and perception by activating downstream components of the ethylene signaling pathway.

Genetic interaction of the clv1-1 gcn5-1 double mutant with ethylene signaling mutants

The ethylene signaling pathway has largely been elucidated by genetic screens designed to identify mutations in genes that cause ethylene insensitivity or the constitutive ethylene response (Chao et al., 1997; Guo and Ecker, 2003, 2004; Qiao et al., 2009; Stepanova and Alonso, 2009). Analysis of double, triple, or higher-order mutants of those genes gave rise to the current model for the perception and signal transduction of ethylene (Stepanova and Alonso, 2009; Zhao and Guo, 2011). For example, the ctrl-1 mutant displays constitutive ethylene responses (Kieber et al., 1993), whereas the ein3-1 mutant is partially insensitive to ethylene and does not exhibit the triple response (Chao et al., 1997). To position the action of both CLV1 and GCN5 in the classical ethylene signaling pathway, we constructed the ein3-1 clv1-1 and ein3-1 gcn5-1 double mutants as well as the ein3-1 clv1-1 gcn5-1 triple mutant. As expected the ein3-1 clv1-1 double mutant had characteristics of ein3-1, indicating that CLV1 acts in an independent pathway upstream of EIN3 (Fig. 2A and Supplementary Fig. S1). The ein3-1 gcn5-1 double mutant displayed characteristic of gcn5-1, suggesting that GCN5 acts downstream of EIN3 in an independent pathway (Fig. 2A and Supplementary Fig. S1). However, the ein3-1 clv1-1 gcn5-1 triple mutant showed a triple-response-like phenotype, similar to ctrl-1 (Fig. 2A and Supplementary Fig. S1). These genetic results suggested that CLV1/GCN5 synergistic action is either downstream from or parallel to EIN3 in the ethylene signaling pathway.

Fig. 2. Genetic analysis of double, triple, and quadruple mutants for the triple response in the absence of ethylene, in 4-d-old etiolated seedlings. (A) Genetic interaction of EIN3/EIL1 transcription factor genes with CLV1 and GCN5. All multiple mutants containing ein3-1 or eil1-3 are in the Col-0 background, while clv1-1 gcn5-1 is in theWs-2 background. (B) Genetic interaction of the HLS1 gene with CLV1 and GCN5. Double and triple hls1-1 clv1-1 gcn5-1 mutants are in the Col-0 background. (C) Synergistic action of ADA2a and ADA2b with CLV1 on the negative regulation of the ethylene response. The clv1-1 ada2a-2, clv1-1 ada2b-1, and clv1-1 ada2a-2 ada2b-1 multiple mutants are in theWs-2 background. (D) CLV3 regulation of ethylene responses through GCN5 function. The clv3-2 gcn5-1 double mutant is in Ler background.
Interestingly, the hypocotyl and root were longer in the \textit{ein3-1 clv1-1 gcn5-1} triple mutant than in the \textit{clv1-1 gcn5-1} double mutant, suggesting that both hypocotyl and root elongation are controlled at least partially by EIN3 (Supplementary Fig. S1). Since EIN3 acts with its close homolog EIL1, and EIL1 overexpression can compensate for EIN3 loss of function (Chao \textit{et al.}, 1997), we generated the \textit{eil1-3 ein3-1 clv1-1 gcn5-1} quadruple mutant to examine whether CLV1 and GCN5 act through EIL1 to affect ethylene responses. The quadruple mutant shows no sign of the triple response, mainly by the absence of an apical hook (Fig. 2A and Supplementary Fig. S1), suggesting that the action of EIN3 and EIL1 is necessary and sufficient for CLV1 and GCN5 signals to affect ethylene responses. However, the slightly shorter hypocotyl length observed in the quadruple mutant in comparison with \textit{eil1-3} could arise from the \textit{gcn5-1} mutation (Supplementary Fig. S1A). One of the late components of ethylene signaling is the putative acetyltransferase HOOKLESS1 (HLS1). HLS1 is induced by ethylene and acts to modulate the function of auxin-response factors in the formation of the apical hook structure (Lehman \textit{et al.}, 1996; Li \textit{et al.}, 2004). To position the synergistic action of CLV1/GCN5 in the late components of ethylene signaling, we constructed the \textit{hls1-1 clv1-1 gcn5-1} triple mutant. The \textit{hls1-1 clv1-1 gcn5-1} mutant is characterized by short and thick hypocotyls, lack of the \textit{clv1-1 gcn5-1} triple mutant resembled the \textit{gcn5-1} phenotype more than the \textit{ada2b-1} phenotype (Supplementary Fig. S6). In Arabidopsis, GCN5 is known to affect genome function by acting independently with ADA2a and ADA2b (Hark \textit{et al.}, 2009). Here, our data suggest that CLV1 signaling requires both ADA2a- and ADA2b-dependent GCN5-containing complexes to affect ethylene responses.

**CLAVATA signaling acts synergistically with GCN5 to affect ethylene responses**

CLV1 is a transmembrane receptor kinase expressed in the rib meristematic cells (Clark \textit{et al.}, 1997). Its ligand, the extracellular peptide CLV3, is produced in the central zone of the SAM (Fletcher \textit{et al.}, 1999). When CLV3 binds to CLV1, the transcription of \textit{WUS} is reduced (Mayer \textit{et al.}, 1998; Brand \textit{et al.}, 2000). Loss of CLV3 activity causes enlargement of the SAM. This is achieved by increasing cell division or reducing cell differentiation, or both (Reddy and Meyerowitz, 2005). To examine whether CLV3 is also involved in regulation of ethylene responses through GCN5 function, we generated the \textit{clv3-2 gcn5-1} double mutant. Consistent with our-response phenotype (Fig. 2D and Supplementary Fig. S7 at JXB online), suggesting that CLV3 acts as a negative signal by binding to the CLV1 receptor to regulate ethylene signaling through GCN5 action. In the vegetative stage, the \textit{clv3-2 gcn5-1} double mutant had some characteristics of the \textit{gcn5-1} mutant (Supplementary Fig. S6).

**CLV1 and GCN5 negative regulation of ethylene-induced gene expression is mediated by EIN3**

To elucidate further the role of \textit{CLV1} and GCN5 in ethylene signaling, the expression of ethylene-inducible genes in \textit{clv1-1 gcn5-1} double mutant plants was compared with that in single mutants and wild-type seedlings. Specifically, after genetic selection of the double mutants and homozygous \textit{gcn5-1} mutants, 15-d-old seedlings were exposed to ethylene gas for several hours and the expression of genes participating in ethylene biosynthesis (Bleecker and Kende, 2000), perception (Hua \textit{et al.}, 1998), signal transduction, and responses (Chao
GCN5 and CLV1 negatively affect ethylene responses

et al., 1997; Solano et al., 1998) was monitored. In the absence of ethylene, the transcription activator ERF1 (Solano et al., 1998) was expressed more than 10-fold higher in the clv1-1 gcn5-1 double mutant than in wild-type and clv1-1 and gcn5-1 single mutants (Fig. 3A). After exposure to ethylene gas, ERF1 expression increased rapidly in the wild-type and clv1-1 and gcn5-1 mutants (Fig. 3D). Notably, upon ethylene treatment, the induction of ERF1 expression in the clv1-1 mutant was lower than in wild-type plants (Fig. 3D). Interestingly, the level of ERF1 expression was slightly increased upon ethylene treatment in clv1-1 gcn5-1 (Fig. 3D), suggesting that the double mutant still responded to ethylene. Similar to the ERF1 expression pattern, in the absence of ethylene, the expression of EBF2 (Fig. 3B) was up-regulated in the double mutant. After exposure to ethylene gas, EBF2 expression was rapidly increased in the wild-type and clv1-1 and gcn5-1 mutants (Fig. 3E) and only slightly increased in clv1-1 gcn5-1 (Fig. 3E). Likewise, GST1 was also overexpressed in clv1-1 gcn5-1 and in clv1-1 but not in gcn5-1 mutants (Fig. 3C), suggesting that CLV1 is a negative regulator of GST1. Upon ethylene treatment, the expression of GST1 in the double mutant was up-regulated 2- or 3-fold more than in the wild-type and single-mutant plants (Fig. 3F), indicating that the synergistic action of CLV1 and GCN5 enhances the negative regulation of GST1 expression in the presence of ethylene. Moreover, one of the ethylene receptor, ERS1, was also up-regulated in the double mutant as well as in the gcn5-1 mutant in comparison with the wild-type and clv1-1 seedlings (Supplementary Fig. S8A at JXB online), suggesting that ERS1 is negatively regulated by GCN5 action. Interestingly, in the absence of ethylene, the negative regulator of ethylene signaling CTR1 (Kieber et al., 1993) and ACO4, a member of the ACC oxidase gene family (Bleecker and Kende, 2000) were also overexpressed in clv1-1 gcn5-1 (Supplementary Fig. S8B, C), suggesting that CTR1 and ACO4 are negatively affected by the synergistic action of CLV1 and GCN5. Furthermore, in the double mutant, CTR1 and ACO4 expression were also induced by ethylene treatment, but this induction was not statistically significant in contrast to what was observed in the wild-type and single mutants (Supplementary Fig. S8B, C). In contrast to the expression pattern observed in the double mutants, the ada2b-1 mutant displayed a similar expression pattern of ethylene-regulated genes to the pattern observed in wild-type plants (Supplementary Fig. S9 at JXB online). Overall, these results demonstrated that not only does simultaneous inactivation of CLV1 and GCN5 activate the ethylene signaling pathway but also that the ethylene signaling pathway is not saturated, suggesting that CLV1 and GCN5 could act together as negative regulators of ethylene responses in the absence of ethylene.

It is known that ERF1 and EBF2 are direct targets of the EIN3/EIL1 transcription factors (Solano et al., 1998; Konishi...
CLV1 and GCN5 affect the acetylation status of H3 in ethylene-inducible genes

To examine whether the observed changes in the expression of ethylene-inducible genes in the clv1-1 gcn5-1 double mutant resulted from changes in the acetylation status of histone associated with target gene promoters, we performed chromatin immunoprecipitation experiments using an antibody for histone H3 acetylated at lysines 9 and 14 (H3K9/14), followed by qPCR in 15-d-old seedlings. For the ERF1 gene, the gcn5-1 mutant showed a statistically significant reduction in H3K9/14 acetylation in all three regions examined, suggesting that GCN5 targets the ERF1 locus (Fig. 5A). The clv1-1 mutant showed no difference from the wild type suggesting that CLAVATA signaling does not affect H3 acetylation. Interestingly, the clv1-1 gcn5-1 mutant showed increased H3 acetylation in comparison with the gcn5-1 single mutant at levels similar to those of the wild type, especially in the core promoter region of the gene, indicating that, despite the loss of GCN5, the levels of H3 acetylation remained high (Fig. 5A). A similar pattern was observed for the EBF2 gene; for the two regions examined, gcn5-1 mutant plants show reduced H3 acetylation in both regions and the clv1-1 gcn5-1 mutant had acetylation levels similar to those of the wild type or even higher (Fig. 5B). In the GST1 gene, in the ORF region examined, gcn5-1 had reduced acetylation, which was restored in the double mutant (Fig. 5C). These data indicated that CLV1 and GCN5 affect the acetylation of H3K9/14 in ethylene-inducible genes in an antagonistic way. The possible repressive effect of CLV1 in H3K9/14 acetylation is masked when GCN5 is active, but with the loss of CLV1 action, in the double clv1-1 gcn5-1 mutant, histone H3 acetylation is de-repressed. Another possibility is that CLV signaling could suppress GCN5 action on H3 acetylation.

CLV1 and GCN5 affect WUS expression in the absence of ethylene

A constitutive triple response, such as that observed in the clv1-1 gcn5-1 double mutant, is mostly caused by defects in cell elongation (Ecker, 1995). We considered how CLV genes acting in the SAM could influence the whole seedling in a gcn5 background. Mutations in GCN5 showed overproliferation of young buds, development of abnormal structures around the inflorescence meristem, and upregulation of several meristem regulatory genes including WUS (Bertrand...
et al., 2003; Cohen et al., 2009). CLV signaling is known to inhibit the expression of transcription factor WUS via a negative-feedback loop that regulates the size of the meristem (Brand et al., 2000; Schoof et al., 2000). To address whether WUS mRNA levels are regulated simultaneously by CLV and GCN5, we monitored WUS expression in 15-d-old clv1-1 gcn5-1 seedlings. Indeed, the level of WUS mRNA was increased in the double mutant to similar levels as observed in clv1-1 mutants (Supplementary Fig. S8D), suggesting that WUS expression is not necessary to alter ethylene responses. Moreover, the level of WUS expression in gcn5-1 mutants was similar to that of wild-type plants, indicating that WUS is not regulated by GCN5 alone at this developmental stage.

CLV1 and GCN5 affect HAT gene expression

The restored H3 acetylation in the ERF1 and EBF2 loci observed in the clv1-1 gen5-1 mutant (Fig. 5A, B) could arise from the activity of other HATs in the region. To test this hypothesis, we examined the expression levels of all known A. thaliana HATs in wild-type, clv1-1, gen5-1, and clv1-1 gen5-1 young seedlings. Interestingly, we found that the expression levels of HAC1, HAC12, HAG2, and IDM1 (Earley et al., 2007; Han et al., 2007; Qian et al., 2012; Li et al., 2014) were decreased in the clv1-1 gen5-1 mutants (Fig. 6), suggesting that CLV1 and GCN5 interaction affect negatively the expression levels of these HAT genes. The expression of the other HAT genes was unaffected in the double mutant with one exception: the expression of At1g77540, an uncharacterized H3/H4 acetyltransferase, was slightly, but not statistically significantly, increased. Therefore, the restored H3 acetylation was not a result of elevated expression of HATs but could be mediated by the remaining activity of other HATs recruited at the ERF1 and EBF2 loci.

HAT activity is reversed by histone acetylases (HDACs), and so the increase in H3K9/14 acetylation found in the double-mutant clv1-1 gen5-1 could also arise from reduced histone deacetylation activity. HDACs are known to be involved in ethylene signaling (Zhou et al., 2005). To test whether the synergistic action of CLV1 and GCN5 leads to regulation of HDAC genes, we monitored the expression of HDA19 and HDA6. We observed that HDA19 expression was not affected in the clv1-1 gcn5-1 double mutant (Supplementary Fig. S8E). However, the expression of HDA6 was slightly but significantly reduced in the double mutant (Supplementary Fig. S8F). These results indicated that synergistic action of CLV1 and GCN5 could have a modest effect on HDA6 expression in the absence of ethylene.

CLV1 and GCN5 do not affect EIN3 transcription

Since EIN3 is a central regulator of ethylene responses, its expression level could be activated by the synergistic action of
CLV1 and GCN5. Therefore, EIN3 gene expression was monitored in wild-type and clv1-1 gcn5-1 double mutant plants. EIN3 gene expression was not affected by the loss of CVL1 and GCN5 action (Supplementary Fig. S8G), suggesting that EIN3 is probably stabilized in the nucleus and recruited to the promoters of ERF1 and EBF2.

CLV1 and GCN5 affect auxin-related gene expression in the absence of ethylene

Histone acetylation is implicated in auxin signaling (Weiste and Droge-Laser, 2014). Synergistic effects of auxin and ethylene have been well defined in the regulation of hypocotyl elongation (Ruzicka et al., 2007), apical hook formation (Li et al., 2004), and root growth (Rahman et al., 2001), suggesting that these two signaling pathways also interact at the molecular level. In Arabidopsis, gcn5 and ada2b mutants exhibit several abnormal auxin-related growth phenotypes (Vlachonasios et al., 2003; Kornet and Scheres, 2009). Moreover, GCN5 was found in a mutant suppressor screen for the co-repressor TPL (Long et al., 2006), and ADA2B was shown to be required for histone acetylation in response to auxin (Sieberer et al., 2003; Anzola et al., 2010). In order to check the effect of GCN5 and CLV1 signaling on auxin signaling, we used the reporter line DR5:GFP in the background of gcn5-1, clv1-1, and clv1-1 gcn5-1 mutants. The DR5 promoter can quantify the level of indole-3-acetic acid (IAA) responses (Ulabasov et al., 1997; Friml et al., 2003). As shown in Fig. 7A, DR5:GFP was weakly expressed in the cotyledons of wild-type and clv1-1 dark-grown seedlings. In gcn5-1, the DR5:GFP signal was observed in the convex side of the apical hook, indicating that auxin signaling is impaired by GCN5. However, a strong DR5:GFP signal was observed on the convex side of the apical hook and on the stele as well as on the cotyledon veins of dark-grown clv1-1 gcn5-1 double-mutant seedlings, suggesting that auxin signaling is up-regulated. In the presence of ACC, the DR5:GFP signal was weakly accumulated in the dark-grown seedlings in all genotypes tested (Supplementary Fig. S10 at JXB online), suggesting that ethylene affects auxin responses in dark-grown seedlings. We next monitored the expression of the auxin-related gene IAA3, a negative regulator of auxin signaling (Tian et al., 2003) in 15-d-old seedlings. In gcn5-1, IAA3 expression was reduced in comparison with the wild-type and clv1-1 mutant plants (Fig. 7B). In the double clv1-1 gcn5-1 mutant plants, IAA3 expression was further reduced, suggesting that the clv1-1 mutant enhances the gcn5-1 effect on IAA3 expression (Fig. 7B). Overall, these results suggested that auxin is induced in the clv1-1 gcn5-1 double

Fig. 6. Gene expression of HATs of A. thaliana in wild-type, clv1-1, gcn5-1, and clv1-1 gcn5-1 plants. Gene expression analysis was performed by RT-qPCR using specific primers. Error bars represent standard error. Asterisks indicate differences from the wild type with statistical significance at *P<0.05 and **P<0.01 (t-test).
mutant in several developmental stages including dark-groin and young light-growing seedlings.

Discussion

Previous physiological and molecular genetic analyses have revealed extensive interactions between ethylene and other signals (Stepanova and Alonso, 2009; Zhao and Guo, 2011). Taken together, our data suggest that, in the absence of ethylene, another level of regulation exists for the ethylene signaling pathway that incorporates developmental signals from the CLV pathway and the HAT GCN5 (Fig. 8). CLV3 stimulates CLV1 action by providing information about the meristem and its developmental potential, since the shoot meristem gives rise to all above-ground tissues of the plant. Signals from the CLV transduction pathway reach the GCN5-containing complexes in the nucleus through an unknown mechanism and possibly alter the histone acetylation status of specific genes, thus translating the CLV information into transcriptional activity. These target genes either negatively affect ethylene-induced gene expression or act as co-repressors of ethylene responses. Alternatively, the loss of synergistic action of CLV1/GCN5 leads to auxin accumulation that stabilizes EIN3/EIL1 in the absence of ethylene and as a result activates ethylene responses.

It remains a question as to how CLV signaling acting in the SAM could influence the whole seedling in a gcn5 background resulting in ethylene responses. One possibility could be that GCN5 causes global changes in the histone acetylation status that is initiated in the SAM and these changes are then maintained in differentiated cells. In Arabidopsis, GCN5 is associated with a large number of promoters possibly affecting their histone acetylation pattern (Benhamed et al., 2008).

Our results suggest that, in the absence of ethylene, GCN5 is required for the acetylation status of the ethylene-inducible genes, suggesting that GCN5-containing complexes could occupy the promoter of these genes. This association leads to a 'permissive state' of chromatin so that the genes are activated with the appropriate stimulus. In gcn5 mutants, the reduced H3 acetylation observed is a reflection of GCN5-dependent...
H3 acetylation. GCN5-containing complexes acting as transcriptional co-activators could also recruit EIN3/EIL1 in the promoters of ethylene-response genes. It was reported that an Arabidopsis homolog of TFIID-interacting transcription factor TAF12b/Enhanced Ethylene Response 4 (EER4), a putative member of the GCN5-containing complex in yeast (Grant et al., 1997), interacts and co-localizes with EIN3 in the nucleus, suggesting that EER4 could recruit EIN3 and/or other transcription factors to induce downstream gene transcription (Robles et al., 2007).

In contrast, clv1-1 mutants do not change histone H3 acetylation status in the same genes (Fig. 5). Simultaneous loss of CLV signaling and GCN5 action results in the activation of ethylene responses in whole-plant vegetative developmental stages, as exhibited by the triple-response phenotype of the clv1-Ig cn5-1 double mutant and the activation of ethylene-related gene expression such as ERF1 and EBF2 in young and adult rosette stages. Moreover, H3 acetylation is restored, presumably by a GCN5-independent mechanism, resulting in increased accessibility to ethylene-inducible genes. Recently, it was reported that members of the CBP/p300 HAT (HAC) family are involved in the ethylene signaling pathway in Arabidopsis (Li et al., 2014) as negative regulators. Our data suggest that the synergistic action of CLV signaling and GCN5 is required for HAC1 expression. However, the mechanism by which HAC1 and HAC5 modulate ERF1 gene expression and affect the acetylation status in the absence of ethylene is unknown.

The synergistic CLV signaling and GCN5 action, in the absence of ethylene, depends on EIN3/EIL1 activity, since the ERF1 and EBF2 induction seen in clv1-I gcn5-1 is absent in the ein3-I clv1-I gcn5-1 triple mutant and the triple response is eliminated in the eil1-3 ein3-I clv1-I gcn5-1 quadruple mutant. The increased accessibility to ethylene-inducible genes allows EIN3/EIL1 to activate the genes and manifest ethylene-response phenotypes. It was suggested that the committed chromatin structure of ethylene-responsive genes before induction by ethylene stimuli is important for the transcription activity (Hu et al., 2011). Therefore, the synergistic action of CLV signaling and the activity of GCN5 could maintain the basal chromatin structure by limiting the accessibility of transcription factors EIN3/EIL1 to immediate targets.

Histone acetylation, and especially the transcriptional adaptor protein ADA2b (known also as PROPORZ), has been shown to modulate auxin-responsive gene expression (Anzola et al., 2010). Moreover, basic leucine zipper transcription factors interact with ADA2b and recruit GCN5-containing complexes to specific auxin-responsive genes (Weiste and Droge-Laser, 2014). Furthermore, GCN5 has been found in a mutant suppressor screen for the co-repressor TOPLESS (Long et al., 2006), which is thought to interact with HDACs as well as with IAA/AUX co-repressors to target promoters of auxin-responsive genes leading to transcriptionally inactive chromatin (Szemenyei et al., 2008). In gcn5-1 and ada2b-I mutants, auxin-responsive genes were either up-regulated, such as GH3.3, or down-regulated, such as IAA3 and IAA7 (Vlachonasios et al., 2003; Weiste and Droge-Laser, 2014), suggesting that auxin signaling was affected. Recently, it was shown that auxin stabilizes EIN3 nuclear accumulation in an EBF1/EBF2-dependent manner, indicating a positive-feedback loop between auxin biosynthesis and ethylene signaling (He et al., 2011). Our results fit well with this model, suggesting that CLV signaling and GCN5 act synergistically to limit auxin levels and as a result repress ethylene responses (Fig. 8). In clv1-I gcn5-1 plants, auxin is induced and thus EIN3 protein is stabilized, leading to activation of ethylene responses.

In summary, translation of positional signals in the SAM, affected by CLV signaling, into developmental cues involves changes in histone acetylation machinery, and synergistically could orchestrate auxin effects on cell proliferation leading to ethylene responses. The interm components of such genetic interactions, as well as their biochemical interactions, are still unknown; however, this study has uncovered a link between SAM maintenance and histone acetylation in the complex mode of ethylene signaling.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used in this study.

Table S2. Segregation analysis of F2 and F3 for the multiple mutants used in this study.

Fig. S1. Genetic interaction of EIN3/EIL1 transcription factor genes with CLV1 and GCN5.

Fig. S2. clv1-I gcn5-1 double mutants do not respond to ethylene biosynthesis and perception inhibitors.

Fig. S3. Genetic interaction of HLS1 gene with CLV1 and GCN5.

Fig. S4. ada2b-I mutant is hypersensitive to ethylene.

Fig. S5. Synergistic action of ADA2a and ADA2b with CLV1.

Fig. S6. Three-week-old mutant and wild-type plants used in this study.

Fig. S7. Genetic interaction between CLV3 and GCN5.

Fig. S8. Gene expression analysis of ERS1, CTR1, ACO4, WUS, HDA19, HDA6, and EIN3.

Fig. S9. Ethylene-induced gene expression in ada2b-I mutants.

Fig. S10. The effect of ethylene on DR5:GFP expression in wild-type, gcn5-I, clv1-I, and clv1-I gcn5-I double mutant.

Acknowledgements

We would like to thank Professor Evangelos Sfakiotakis from the Department of Agriculture, Aristotle University of Thessaloniki (AUTH), for the ethylene gas gift and Professor Emmanuel Panteris from Department of Biology, AUTH, for his help with the confocal microscopy. We also thank Professors Steve Triezenberg (Van Andel Institute, USA) and Amy Hark (Muhlenberg College, Allentown, USA) for stimulating discussion and critical reading of this manuscript. Financial support by the MSc Graduate Program Studies ‘Applied Genetics and Biotechnology’ from School of Biology, AUTH, is gratefully acknowledged. Stylianos Poulios is a scholar of the Alexander S. Onassis Public Benefit Foundation.

References


The putative Arabidopsis transcriptional adaptor protein, mediates auxin and gene expression in Arabidopsis. Molecular Plant
AtGCN5/HAG1 is a versatile regulator of developmental and inducible servet C, Conde e Silva N, Zhou D-X.

Histone dynamics and roles of histone acetyltransferases during cold-induced gene regulation in Arabidopsis. Plant Molecular Biology

Pavangadkar K, Thomashow MF, Trienzenberg SJ. 2010. Histone dynamics and roles of histone acetyltransferases during cold-induced gene regulation in Arabidopsis. Plant Molecular Biology


