Journal of Plant Sciences
2018; 6(4): 117-133
http://www.sciencepublishinggroup.com/j/jps
doi: 10.11648/j.jps.20180604.12
ISSN: 2331-0723 (Print); ISSN: 2331-0731 (Online)

Regulation of Phytohormone Biosynthesis Genes by Polycomb-Mediated Histone H3 Lysine 27 Trimethylation in *Arabidopsis*

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To cite this article:
Yongfeng Hu, Yan Lai, Shufang Fan, Bin Liu. Regulation of Phytohormone Biosynthesis Genes by Polycomb-Mediated Histone H3 Lysine 27 Trimethylation in *Arabidopsis*. *Journal of Plant Sciences*. Vol. 6, No. 4, 2018, pp. 117-133. doi: 10.11648/j.jps.20180604.12

Received: August 6, 2018; **Accepted**: August 21, 2018; **Published**: September 21, 2018

Abstract: Histone H3 lysine 27 trimethylation (H3K27me3) catalyzed by polycomb proteins plays a negative role in the regulation of gene expression. Several key developmental genes have been reported to be enriched with H3K27me3 and repressed by polycomb proteins. The objective of this study was to analyze the role of polycomb-mediated H3K27me3 in regulating expression of hormone biosynthesis genes in *Arabidopsis*. First of all, enrichment of H3K27me3 on hormone biosynthesis genes was investigated systematically. The results showed that genes encoding crucial enzyme tended to be enriched with H3K27me3 while the others were not. And then expression of cytokinin biosynthesis genes in *curly leaf (clf)* and *like heterochromatin protein1 (lhp1)* was detected by qPCR. Most of cytokinin biosynthesis genes expression with basal promoter activity was increased in *clf* and *lhp1*. Chromatin Immunoprecipitation (ChIP) analysis indicates that H3K27me3 levels on these genes were decreased in *clf*, suggesting repressive role of CLF and LHP1 in the regulation of these genes. To reveal the role of CLF1 and LHP1 in the dynamics of gene expression, we tested expression of various genes at different time point after IAA treatment. We found that expression of *CYP735A2* was increased in *clf1* and *lhp1* but could be repressed to the levels as in wild type plants, which indicates that CLF1 and LHP1 are not required for IAA-stimulated repression of *CYP735A2*. The induction of *gibberellin 20-oxidase1* (*AtGA20Ox1*) and *gibberellin 20-oxidase2* (*AtGA20Ox2*) by IAA application was also affected in *clf* and *lhp1*. Expression of *AtGA20Ox1* and *AtGA20Ox2* was firstly induced by IAA treatment and decreased later in wild type plants. However, decrease of *AtGA20Ox1* and *AtGA20Ox2* expression was earlier in *clf* and *lhp1* which might be caused by overproduction of gibberellins in the mutants. Thus, we propose that the role of the polycomb group proteins is to reduce expression levels of crucial enzymes to minimum, which might stop hormone production.

Keywords: *Arabidopsis*, Polycomb Protein, H3K27me3, Hormone Biosynthesis

1. Introduction

Histone H3 lysine 27 trimethylation (H3K27me3) is an epigenetic mark negatively correlating with gene transcription. The methyltransferase responsible for catalyzing H3K27me3 is E (z) protein [1]. In *Arabidopsis,* three E (z) homologs including CLF, SWINGER (SWN) and MEDEA (MEA) have conserved function to catalyze trimethylation of H3K27 in different PRC2 (polycomb repressive complex 2) -like complexes. These complexes also contain FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and EMBRYONIC FLOWER2 (EMF2) / FERTILIZATION INDEPENDENT SEED2 (FIS2) / VERNALISATION2 (VRN2). In *Arabidopsis*, PRC2 could be recruited by non-coding RNA or protein to maintain H3K27me3 levels on target genes [2-4]. Sets of cis-regulatory elements are also required for PRC2 mediated repression [5, 6]. H3K27me3 is removed by a Jumonji domain-containing protein REF6 [7]. REF6 recognizes specific DNA sequence to activate gene expression in concert with BRM, a chromatin remodeling factor [8, 9]. In mammalian and *Drosophila,* H3K27me3 serves as a binding site for the chromodomain protein POLYCOMB (PC), which

is a component of PRC1 (polycomb repressive complex 1) [10]. Interestingly, the homolog of PC in plant is not present. Instead LHP1 acts as an H3K27me3 binding protein in PRC1-like complex [11, 12]. However，it has been proposed that the genes with H3K27me3 over a 1-kb region could serve as the target for LHP1 binding, suggesting that LHP1 is not required for the regulation of all the genes enriched with H3K27me3 [12]. LHP1 may also act as a positive regulator although mechanism is not clear yet [13]. Recently it was revealed that LHP1 could interact with ATRX which is required for H3.3 deposition in *Arabidopsis* [14]. H3.3 is a histone variant distributed mainly on actively transcribed genes [15]. Thus, it is possible that LHP1 positively regulates gene expression by affecting H3.3 deposition. A novel H3K27me3 reader EBS has been identified recently [16, 17]. EBS binds H3K27me3 through BAH domain and interacts with EMF1 to mediate transcriptional repression. The EBS-EMF1 interaction is conserved in flowering plants [16, 17].

Polycomb-mediated H3K27me3 plays a crucial role in plant development. Studies in *Arabidopsis* have shown that Polycomb-group (Pc-G) proteins are essential regulators of plant developmental transitions including embryogenesis, acquisition of pluripotency, embryo-to-seedling transition, flowering time, leaf and floral organogenesis [18-23]. Large number of evidences suggest that PcG proteins negatively regulate several genes crucial for plant development such as *AGAMOUS* (*AG*), *APETALA3* (*AP3*), *SHOOT MERISTEMLESS* (*STM*), *FLOWERING LOCUS C* (*FLC*), *KNOTTED1-like homeobox* (*KNOX*), *WUSCHEL* (*WUS*), *FLOWERING LOCUS T* (*FT*), *AGL19* and *FUSCA3* (*FUS3*) [21, 23-28]. CLF and EMF2 directly repress the auxin efflux carrier gene *PIN FORMED 1* (*PIN1*) to modulate root auxin levels and control lateral root formation [29]. Recent paper revealed that PRC2 negatively regulates the expression of the auxin biosynthesis genes *YUCCA1/4* (*YUC1/4*) [30]. It has also been proved that H3K27me3 is involved in plant responses to environment [31].

Whole-genome analysis in plant reveals that H3K27me3 is mainly distributed over the transcribed region of tissue-specifically expressed genes and negatively correlates with gene expression [18, 32, 33]. Although H3K27me3 is generally considered to be negatively related to gene transcription, the function of this epigenetic mark still remains unclear. In mammalian, H3K27me3 is involved in the repression of *Hox* genes, which indicate that it might serve as a trigger to control gene activity [34]. However, it has been revealed that for Ras-induced genes changes of H3K27me3 level in the gene body are a consequence of changes in transcriptional activity. Induction of the genes by Ras signal occurs before changes of H3K27me3 level. Depletion of H3K27me3 does not affect the induction [35]. In plant, dynamics of H3K27me3 during gene activation has been analyzed at many loci which could be generally classified into two groups. For the first group genes, H3K27me3 level over the gene body is reduced after gene activation such as *FLC*, *Phytochrome A* (*phyA*), *COR15A* and *ATGOLS3* [36-38]. But the detailed investigation has shown that H3K27me3 at *FLC* is highly dependent on transcription activity, which means it could not be the cause of transcriptional repression [36]. Studies on *COR15A* and *ATGOLS3* induction by cold also show that the decrease in H3K27me3 alone is not sufficient for transcriptional reactivation [38]. Induction of the genes in the second group is not accompanied by a decrease of H3K27me3 level or LHP1 association such as *FT*, *VERNALIZATION INSENSITIVE3* (*VIN3*), *AtERF14* and *BASIC CHITINASE* (*ChiB*) [39-41]. However, mutation of PcG proteins could result in derepression or advanced induction of target genes, implying that PcG proteins repress expression levels of both group genes regardless of whether H3K27me3 is removed or not after gene activation.

Plant phytohormones are considered to be chemical messengers, which are important for developmental processes and responses to environment. They are produced in certain plant cells responding to developmental cues and environmental stimuli, and then transported to target cell to accomplish the signal transduction. The unique property of the messengers is that trace of them would generate physiological effects. Thus, their biosynthesis must be strictly controlled, as overproduction of them would take negative effect on plant development. Biosynthetic processes of all hormones contain several steps that are catalyzed by enzymes encoded by a single gene or gene family. Some enzymes are crucial for hormone biosynthesis as overproduction of these enzymes could increase the hormone levels. Therefore, the spatial and temporal specificity of hormone production may depend on the expression of enzyme-encoding genes. Taking cytokinin biosynthesis for example, *Isopentenyltransferase* (*IPT*) genes show tissue and organ-specific expression pattern to direct cytokinin production [42].

In this paper we investigated H3K27me3 enrichment over biosynthesis genes of five hormones in *Arabidopsis* and found that the genes encoding key enzymes involved in the biosynthetic processes of hormones tended to be covered with H3K27me3. We further analyzed the role of PcG proteins CLF and LHP1 in the regulation of cytokinin and gibberellin biosynthesis genes. The results demonstrate that they play some role in reducing transcription levels of these genes to restrict amounts of the enzymes, which might block hormone production in plant.

2. Material and Methods

2.1. In Silico Analysis of Histone Modification and Gene Expression

The information on H3K27me3 enrichment were obtained at EPIGARA website (http://epigara.biologie.ens.fr/cgi-bin/gbrowse/a2e/#search). Gene locus number was entered into the textbox below Landmark or region. The tracks with all H3K27me3 data deposited by different groups were ticked. Search button was clicked to get the data. The genes which were shown to be

enriched with H3K27me3 in all the data were considered to be covered by the marks. Bindings of LHP1 on the genes were analyzed by combining the data from Jacobsen lab (data were viewed at http://epigenomics.mcdb.ucla.edu/LHP1/)[12] and colot lab (gene list was provided in the paper) [11].

Gene expression data were extracted by AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp). For gene expression pattern development was selected in experiment combo box. For gene expression responding to hormones Hormones was selected in experiment combo box. Absolute values was selected in normalization combo box. Gene locus number was entered in genes texbox. Run button was clicked to get data. For gene expression pattern, we collected the data deposited by Weigel & Lohmann group [43]. For gene expression responding to hormones, we collected the data deposited by Yoshida group.

2.2. Plant Material and Exogenous IAA Treatment

The mutants used in this study are *clf-29* [44] and *lhp1* [45]. *Arabidopsis* seeds were surface-sterilized and grown in 1/2MS medium at 22°C with a 16 h light/8 h dark (long day) cycle. One μM IAA solution was added around two weeks after germination. The samples (over ten plants per sample) were harvested at indicated time points. Three biological repeats were performed.

2.3. RNA Extraction and Reverse Transcription

Total RNA was extracted from two-week old seedlings using Trizol reagent followed by the introduction from manufacture (Invitrogen). Four μg total RNA were treated first with 1 unit of DNase I (Promega) and then reverse transcribed into cDNA with a total volume of 20 μl containing 0.5 μg oligo $(dT)_{15}$, 0.75 mM dNTPs, 2.5 mM $MgCl_2$, 1 μl M-MLV reverse transcriptase (Promega). The resulting products were tested by Real-Time PCR using gene specific primers (Table A1)

2.4. Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) experiment was performed as described in [46] with slight modification. One gram of two-week old seedlings was harvested and crosslinked in 1% formaldehyde under vacuum. Nuclei were then extracted with extraction buffers. Chromatin was fragmented to 200-2000 bp by sonication and ChIP was performed using H3K27me3 (Millipore, 07-449) antibody. The precipitated and input DNAs were then analyzed by quantitative Real-time PCR with gene specific primer sets (Table A1 and Figure A1).

2.5. Real-Time PCR

Real-time PCR was performed in a total volume of 10 μL with 1.0 μl of the reverse transcription or ChIP products, 0.25 μM primers, and 5 μl SYBR on a StepOne Real-time PCR machine (Applied biosystems) according to the manufacturer's instructions. All primers were annealed at 60°C for 60 seconds and run 45 cycles. Three technical repeats were performed for each test. The ChIP enrichment was quantified by comparing the thresholdcycle (C_t) of the ChIP samples with that of the input and then normalized with the levels of control genes (*AG*): $2^{(\text{Ct of input-Ct of sample ChIP})}$ /$2^{(\text{Ct of input-Ct of control ChIP})}$. The expression of *AG* was not affected by IAA treatment (Figure A2). The expression level of target genes was normalized with that of *ACTIN*: $2^{(\text{Ct of actin- Ct of target})}$.

3. Results

3.1. H3K27me3 Enrichments on Hormone Biosynthesis Genes

The information about all the genes encoding biosynthesis enzymes of five phytohormones in *Arabidopsis* including Gibberellin (GA), Abscisic acid **(**ABA), cytokinin, ethylene and auxin were collected. For family genes, the members which have been proved to encode inactive enzymes or the enzymes not catalyzing synthesis reactions were not chosen. Pseudogene was not analyzed either. In total 62 genes were chosen for further analysis. Using locus numbers, we investigated H3K27me3 enrichments on these genes by searching the datasets obtained by different groups at epigara website. H3K27me3 coverages on ten genes were not confirmed according to the datasets, since the data from some studies showed positive results but negative results from the others. ChIP-PCR was performed to confirm whether the ten genes were covered with H3K27me3. The results showed that nine of them could be detected with low levels compared to *AG* (Figure A3). We therefore found 42 genes encoding hormone biosynthesis enzymes enriched with H3K27me3 (Table 1). Bindings of LHP1 on the genes were investigated by analyzing the data released from Jacobsen lab and Colot lab [11]. Besides, additional four genes encoding auxin biosynthesis enzymes were confirmed to be the targets of LHP1 [47]. 26 genes were found to be the targets for LHP1 bindings altogether (Table 1). To analyze the relationship between gene expression level or pattern and H3K27me3 coverage, the expression data in different tissues of these genes were extracted via AtGenExpress Visualization Tool. The obtained data were visualized and shown in Figure A4 to Figure A8. Consistent with the view proposed previously, the genes expressed in lower levels or in fewer tissues tended to be enriched with H3K27me3.

Table 1. *Collection of H3K27me3 enrichments and LHP1 binding on hormone biosynthesis genes.*

Hormone	Gene name	Locus number	H3K27me3	LHP1	Hormone	Gene name	Locus number	H3K27me3	LHP1
GA	*AtCPS*	AT4G02780	+	+	Ethylene	*ACS2*	AT1G01480	+[a]	-
	AtKS	AT1G79460	-	-		*ACS4*	AT2G22810	+	+
	AtKO	AT5G25900	-	-		*ACS5*	AT5G65800	+	+
	AtKAO1	AT1G05160	-	-		*ACS6*	AT4G11280	-	-

Hormone	Gene name	Locus number	H3K27me3	LHP1	Hormone	Gene name	Locus number	H3K27me3	LHP1
	AtKAO2	AT2G32440	-	-		*ACS7*	AT4G26200	+	+
	AtGA3ox1	AT1G15550	+[a]	-		*ACS8*	AT4G37770	+	+
	AtGA3ox2	AT1G80340	+	-		*ACS9*	AT3G49700	+	+
	AtGA3ox3	AT4G21690	+	+		*ACS11*	AT4G08040	-[a]	-
	AtGA3ox4	AT1G80330	+[a]	-		*EFE*	AT1G05010	-	-
	AtGA20ox1	AT4G25420	+[a]	-		*ACO1*	AT2G19590	-	-
	AtGA20ox2	AT5G51810	+	+		*ACO2*	AT1G62380	-	-
	AtGA20ox3	AT5G07200	+	+		*ACO3*	AT1G77330	-	-
	AtGA20ox4	AT1G60980	+	+		*ACO4*	AT1G12010	+	+
	AtGA20ox5	AT1G44090	+	-		*ACO5*	AT5G43450	-	-
	AtNCED2	AT4G18350	+	-		*TAA1*	AT1G70560	+	+
	AtNCED3	AT3G14440	-	-		*TAR1*	AT1G23320	+	-
	AtNCED6	AT3G24220	+	-		*TAR2*	AT4G24670	+[a]	-
	AtNCED9	AT1G78390	+[a]	-		*YUCCA1*	AT4G32540	+	+
	ZEP	AT5G67030	-	-		*YUCCA2*	AT4G13260	+	+
ABA	*SDR*	AT1G52340	-	-		*YUCCA3*	AT1G04610	-	-
	ABA4	AT1G67080	-	-	Auxin	*YUCCA4*	AT5G11320	+	+[b]
	AAO1	AT5G20960	-	-		*YUCCA5*	AT5G43890	+	+[b]
	AAO2	AT3G43600	-	-		*YUCCA6*	AT5G25620	+	+
	AAO3	AT2G27150	-	-		*YUCCA7*	AT2G33230	-	-
	AAO4	AT1G04580	+[a]	-		*YUCCA8*	AT4G28720	+[a]	+[b]
	AtIPT1	AT1G68460	+	+		*YUCCA9*	AT1G04180	+	+[b]
	AtIPT3	AT3G63110	+	-		*YUCCA10*	AT1G48910	+	+
	AtIPT4	AT4G24650	+	+		*YUCCA11*	AT1G21430	+[a]	-
	AtIPT5	AT5G19040	+	-					
Cytokinine	*AtIPT6*	AT1G25410	+	-					
	AtIPT7	AT3G23630	+	+					
	AtIPT8	AT3G19160	+	+					
	CYP735A1	AT5G38450	+	+					
	CYP735A2	AT1G67110	+	+					

"+" represents the genes enriched with H3K27me3 or bound by LHP1
"-" represents the genes not enriched with H3K27me3 or not bound by LHP1
"a" represents that H3K27me3 enrichments on the genes are confirmed by ChIP-PCR.
"b" represents that bindings of LHP1 on these genes are confirmed by other group [47]

3.1.1. Gibberellin

Gibberelins are biosynthesized from geranylgeranyl diphosphate (GGDP) by three classes of enzymes in plant: terpene synthases (TPSs), cytochrome P450 monooxygenases (P450s), and 2-oxoglutarate–dependent dioxygenases (2ODDs) [48] (Figure A4). These enzymes are encoded by ent-copalyl diphosphate synthase (*AtCPS*), ent-kaurene synthase (*AtKS*), ent-Kaurene oxidase (*AtKO*), entkaurenoic acid oxidase (*AtKAO*), *AtGA20ox* and *gibberellin 3-oxidase* (*AtGA3ox*) in *Arabidopsis*. It has been proposed that AtCPS might act as a gatekeeper to control the location and activity of the early stages of GA biosynthesis [49]. Regulation of *AtGA20ox* and *AtGA3ox* family genes is also important for modulating GA flux in the late stages [49]. In accordance with the functional importance of these genes we found that all the genes including single *AtCPS*, five *AtGA20oxs* and four *AtGA3oxs* were enriched with H3K27me3 (Table 1). However, ChIP-PCR results indicated that in comparison with *AG* H3K27me3 levels on *AtGA20ox1*, *AtGA3ox1* and *AtGA3ox4* were lower (Figure A3). Coincidently, except *AtGA20ox1*, *AtGA3ox1 AtGA3ox2* and *AtGA3ox4*, other genes were bound by LHP1 (Table 1), supporting the hypothesis that LHP1 binding depends on H3K27me3 levels over gene body.

3.1.2. Abscisic Acid

It is generally accepted that the main pathway for ABA biosynthesis is the indirect pathway in which ABA is derived from carotenoid [50]. The enzymes identified for catalyzing the reactions in the pathway are zeaxanthin epoxidase (ZEP), ABA4, 9-cis-epoxycarotenoid dioxygenase (NCED), short-chain dehydrogenase/reductase (SDR) and Arabidopsis aldehyde oxidase (AAO), among which NCED and AAO are encoded by family genes (Figure A5). Although in *Arabidopsis* seven *NCED* genes (*AtNCED1*, *2*, *3*, *4*, *5*, *6*, and *9*) were identified, only AtNCED2, 3, 6 and 9 had the activity to catalyze the cleavage reaction of two 9-cis-epoxycarotenoids and thus were chosen for the analysis [51]. The results showed that *AtNCED2*, *6*, *9* and *AAO4* were covered with H3K27me3 (Table 1). *AtNCED9* and *AAO4* were detected to be enriched with low levels of H3K27me3 by ChIP-PCR (Figure A3). Both biochemical and expression studies suggest that NCED is the key enzyme in ABA biosynthesis [51]. H3K27me3 enrichments on three of four *NCED* genes further proved that these genes expression

should be strictly regulated to control ABA production. *AtNCED3* without H3K27me3 enrichment might account for its higher responsiveness to abiotic stress for producing more ABA, which is important for stress tolerance [51, 52]. In addition, LHP1 association with these genes was not found, demonstrating that repression of these genes does not need LHP1 (Table 1).

3.1.3. Cytokinin

Cytokinin (CK) plays an important role in the proliferation and differentiation of plant cells. Thus, its biosynthesis must be finely controlled. In plant the key steps in the isoprenoid CK biosynthesis are *N*-prenylation of adenosine 5'-phosphates (preferable ADP or ATP) at the N^6-terminus with dimethylallyl diphosphate (DMAPP) or hydroxymethylbutenyl diphosphate (HMBDP) which is catalyzed by IPT, and conversion of CK nucleotides to the corresponding tZ-nucleotides by CYP735A [42] (Figure A6). In *Arabidopsis* seven *IPT* genes (*AtIPT1* and *AtIPT3* to *AtIPT8*) and two *CYP735A* genes (*CYP735A1*and *CYP735A2*) are identified [53, 54]. Interestingly, H3K27me3 coverage on all these genes and LHP1 association with six genes were found (Table 1). To confirm the results, we examined H3K27me3 levels on these genes by ChIP-PCR. The data showed that except *AtIPT5* with low level of H3K27me3, the other genes carried moderate H3K27me3 levels compared to *AG* (Figure 1).

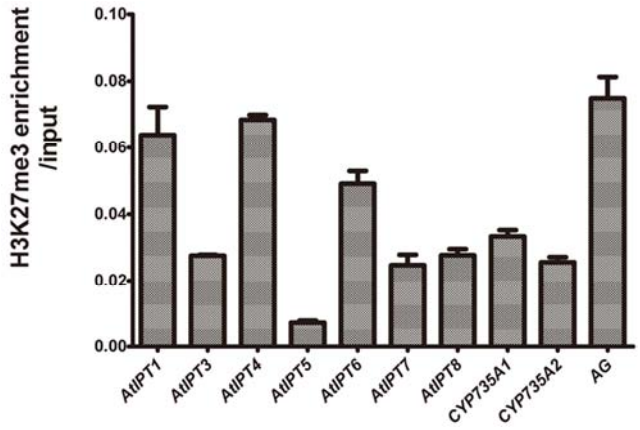


Figure 1. *H3K27me3 enrichments on cytokinin biosynthesis genes. Two-week old seedlings were harvested for chromatin immunoprecipitation using H3K27me3 antibody. Real-time PCR was performed to test H3K27me3 enrichments on seven IPT genes and two CYP735A genes. AGAMOUS (AG) was used as the positive control. Bars represent mean values +/-SD from technical repeats.*

3.1.4. Ethylene

Ethylene biosynthesis begins from conversion of S-adenosylmethionine (SAdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) [55]. ACC is subsequently oxidized by ACC oxidase (ACO) to form ethylene (Figure A7). The first step has been considered as the rate-limiting step and ACS is highly regulated by various internal and external signals [55]. Both ACS and ACO are encoded by multigene family in *Arabidopsis*. All *ACS* genes except *ACS6* and *ACS11*, and one *ACO* gene (*ACO4*) were enriched with H3K27m3 (Table 1). These genes are also bound by LHP1 except *ACS2* which carries low levels of H3K27me3 examined by ChIP-PCR (Table 1, Figure A3).

3.1.5. Auxin

Four pathways of IAA biosynthesis from Trp have been proposed in plant [56]. Recent evidence suggests that IPA pathway is the main IAA biosynthetic pathway, which consists of two steps catalyzed by TAA1 and YUCCA, respectively [57] (Figure A8). YUCCA proteins catalyze the rate-limiting step of the pathway [57]. In *Arabidopsis* three *TAA1* related genes and eleven *YUCCA* genes have been identified. Two of fourteen genes were not enriched with H3K27me3 (Table 1). LHP1 binding was found on nine genes (Table 1).

3.2. Upregulation of Cytokinin Biosynthesis Genes in clf and lhp1

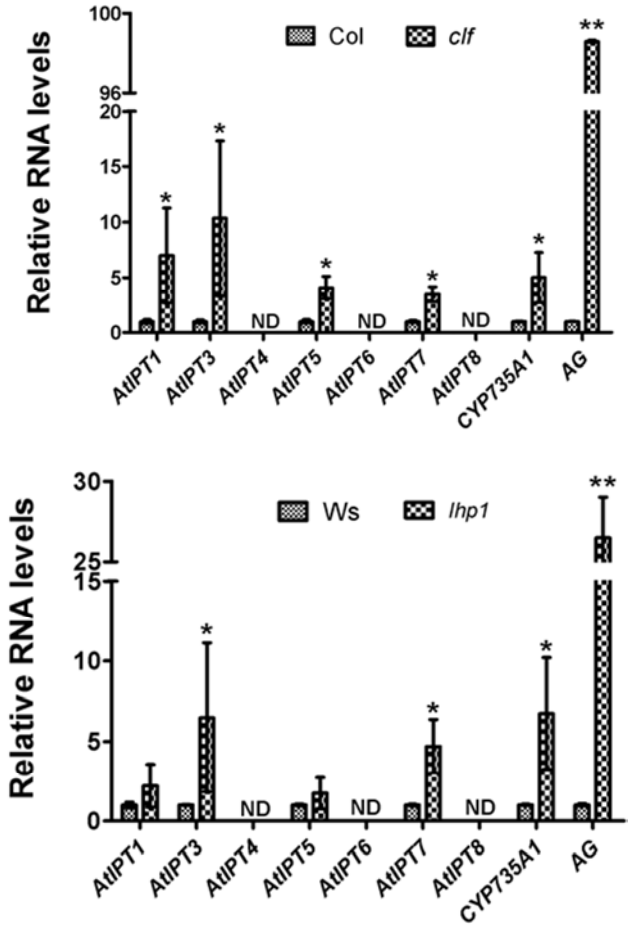


Figure 2. *Expression analysis of cytokinin biosynthesis genes in clf and lhp1. Two-week old seedlings of wild type, clf (Col) and lhp1 (Ws) plants were harvested. RT-PCR was performed to test expression of seven IPT genes and CYP735A1 in clf and lhp1 compared with corresponding wild type plants. Relative expression levels were determined by normalization with ACTIN2 transcript levels. AGAMOUS (AG) was used as the positive control. "ND" means the expression of the genes could not be detected by Real-time PCR. Bars represent mean values +/-SD from three biological repeats. Significance of expression differences between wild type and mutants (clf and lhp1) was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$.*

As mentioned above, all the genes involved in cytokinin biosynthesis were marked with H3K27me3, thus were chosen for further exploring the role of PcG proteins in their repression. Expression of these genes in *clf* and *lhp1* were tested by qRT-PCR. We found that *AtIPT3*, *AtIPT7* and *CYP735A1* were up-regulated in both *clf* and *lhp1* mutants. The expression of *AtIPT1* and *AtIPT5* was increased in *clf* but not in *lhp1* (Figure 2). *AtIPT4*, *AtIPT6* and *AtIPT8* could not be detected in wild type plants and the mutants (Figure 2).

3.3. H3K27me3 Levels Were Reduced on Part of Cytokinin Biosynthesis Genes in clf

To investigate whether repression of cytokinin biosynthesis genes by CLF is dependent on its methyltransferase activity, we compared H3K27me3 levels on these genes between wild type and *clf*. H3K27me3 enrichments on four genes were reduced in varying degrees in *clf* (Figure 3). H3K27me3 level was dramatically decreased on *CYP735A1*, implying that CLF is the main methyltransferase catalyzing H3K27 trimethylation on the gene. Reduced H3K27me3 enrichment was also observed on *AtIPT1*, *AtIPT4* and *AtIPT8* in *clf*, although expression levels of *AtIPT4* and *AtIPT8* were not altered in the mutant. This indicates that repression of the two genes may not require CLF-dependent H3K27me3. Furthermore, we noticed that expression levels of *AtIPT4* and *AtIPT8* were extremely low in the seedlings compared to the other genes (Figure 2 and Figure A6), leading us to speculate that the promoter activity of the two genes might be too low to promote transcription in the absence of CLF. In contrast, H3K27me3 levels were not altered on *AtIPT5* despite increased expression level of the gene in *clf* (Figure 2 and Figure 3), suggesting the indirect effect of CLF on the regulation of the gene.

3.4. PcG Proteins Were not Necessary for the Repression of CYP735A2 by IAA

As *CYP735A2* was repressed by IAA treatment (Figure A6), we posed the question whether PcG proteins were involved in the process of repression. Thus, we measured H3K27me3 level on the gene at different time points after IAA treatment. We found that H3K27me3 level on *CYP735A2* was increased slightly 6 hours after IAA treatment (Figure 4A). Expression of *CYP735A2* was decerased 1 hour after treatment in wild type plants, indicating that increased level of H3K27me3 was not necessary for repression of the gene (Figure 4B). Then to test whether mutation of *CLF* and *LHP1* had some effects on the repression of *CYP735A2*, we treated *clf* and *lhp1* mutants and wild type plant with IAA and harvested samples at different time points. qRT-PCR was performed to examine expression of *CYP735A2*. *CYP735A2* RNA levels were increased in both *clf* and *lhp1* before treatment but could be repressed to a level comparable to wild type shortly after treatment (Figure 4B and Figure 4C). This demonstrated that CLF and LHP1 could reduce expression levels of *CYP735A2* but were not necessary for the repression of *CYP735A2* stimulated by IAA. In *clf* mutant H3K27me3 level on *CYP735A2* was reduced slightly (Figure 4D), indicating that SWN, another H3K27 methyltransferase in *Arabidopsis*, is involved in regulating the expression of *CYP735A2* redundantly with CLF. We found that the expression of *CYP735A2* was increased 6 hours after IAA treatment to a higher level in *lhp1* in comparison with wild type plants (Figure 4C). Besides, LHP1 could bind to *CYP735A2* (Table 1). Although it is not clear why the gene was induced lately, the data led us to propose that LHP1 had a repressive role in the induction of *CYP735A2*.

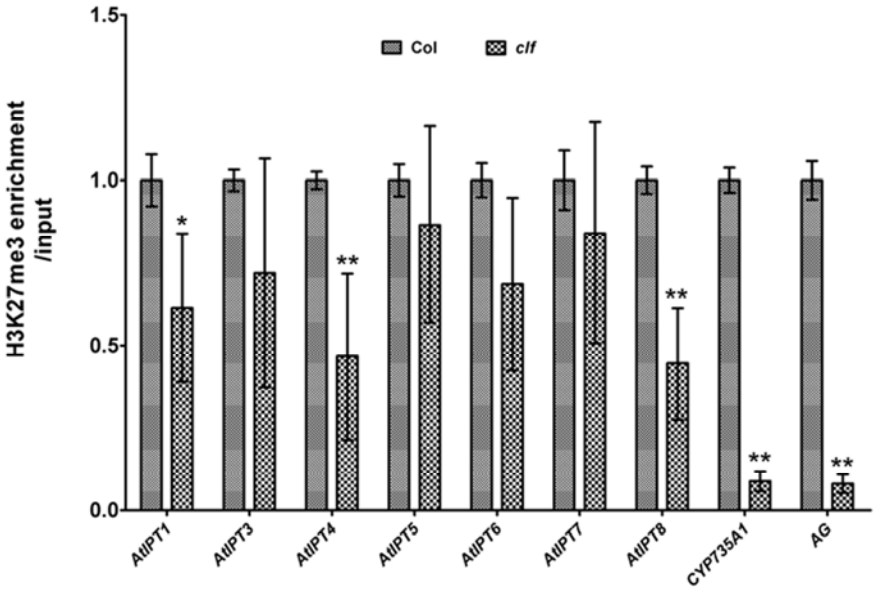


Figure 3. *H3K27me3 levels on cytokine biosynthesis genes in wild type and clf plants. Two-week old seedlings were harvested for ChIP experiment using H3K27me3 antibody. Real-time PCR was performed to compare H3K27me3 levels on seven IPT genes and CYP735A1 between wild type (Col) and clf plants. AGAMOUS (AG) was used as the positive control. Bars represent mean values +/-SD from three biological repeats. Significance of H3K27me3 difference between wild type and clf palnts was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$.*

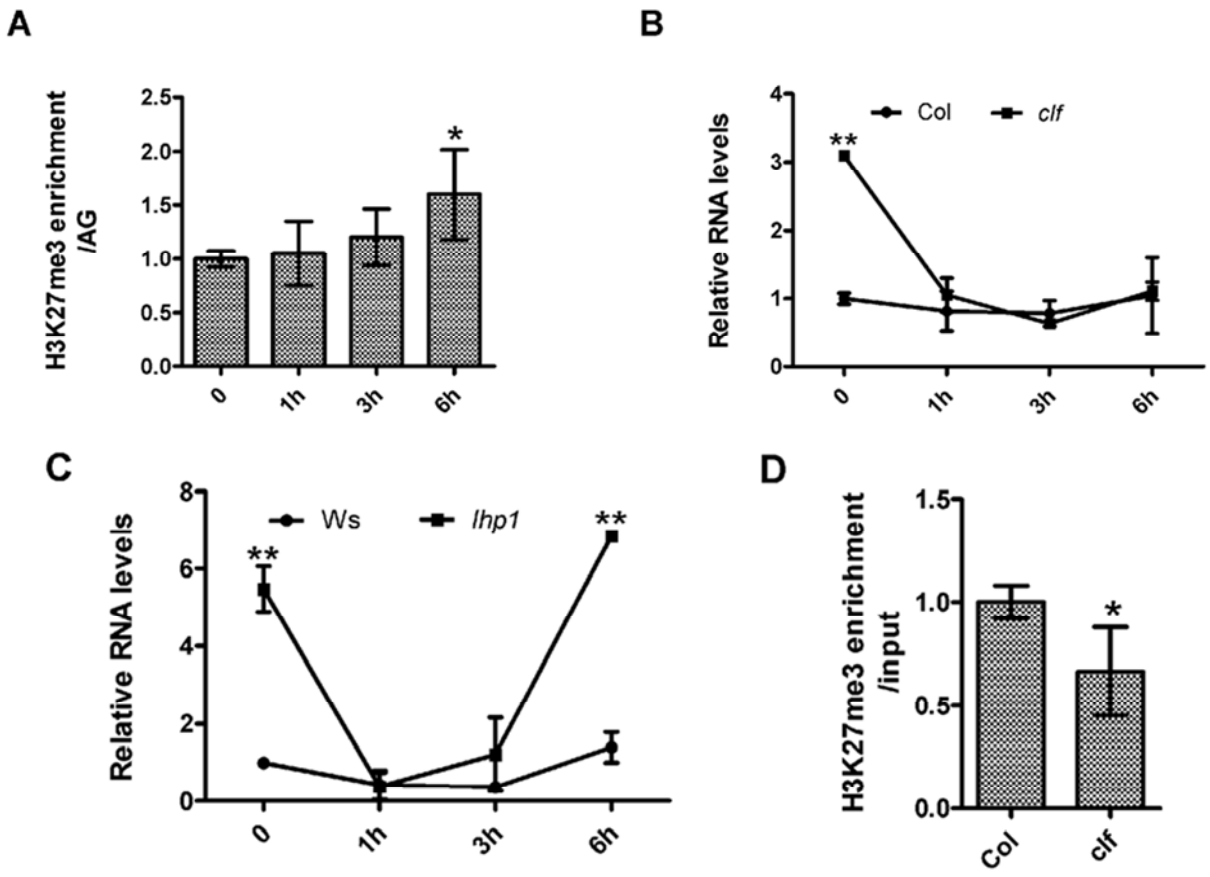
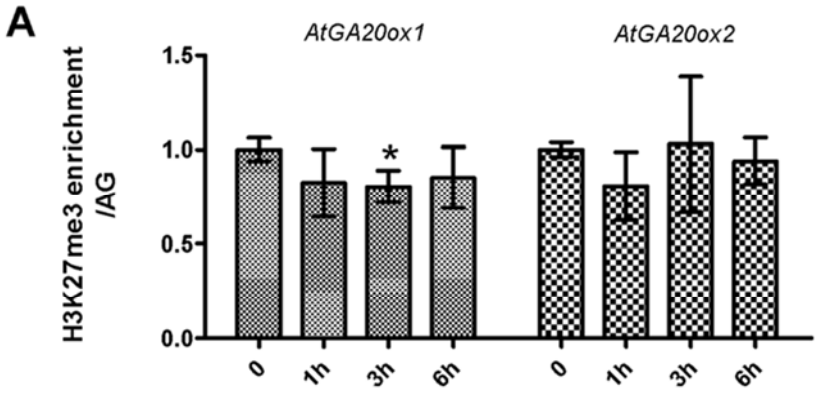


Figure 4. *CLF and LHP1 are not necessary for the repression of CYP735A2.*

(A) Analysis of H3K27me3 enrichment on *CYP735A2* after IAA treatment. Two-week old seedlings were treated with 1μM IAA and harvested at the indicated time points. ChIP experiment was performed using H3K27me3 antibody. The relative enrichments were determined by first comparing to input and then to the reference gene *AGAMOUS* (*AG*). Bars represent mean values +/-SD from three biological repeats. Significance of H3K27me3 differences between after IAA treatment (at different time points) and before treatment (0) was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (B) Repression of *CYP735A2* by IAA in wild type and *clf* plants. Two–week old seedlings were treated with 1μM IAA and harvested at the indicated time points. RT-PCR was performed to compare the repression of *CYP735A2* by IAA between wild type (Col) and *clf* plants. *ACTIN2* was used as the reference gene. Bars represent mean values +/-SD from three biological repeats. Significance of expression differences between wild type and *clf* was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (C) Repression of *CYP735A2* by IAA in wild type and *lhp1* plants. Two-week old seedlings were treated with 1μM IAA and harvested at the indicated time points. RT-PCR was performed to compare the repression of *CYP735A2* by IAA between wild type (Ws) and *lhp1* plants. *ACTIN2* was used as the reference gene. Bars represent mean values +/-SD from there biological repeats. Significance of expression differences between wild type and *lhp1* was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (D) H3K27me3 level on *CYP735A2* in wild type and *clf* plants. Two-week old seedlings of wild type (Col) and *clf* were harvested for ChIP experiment using H3K27me3 antibody. Real-time PCR was performed. Bars represent mean values +/-SD from three biolological repeats. Significance of H3K27me3 difference between wild type and *clf* palnts was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$.



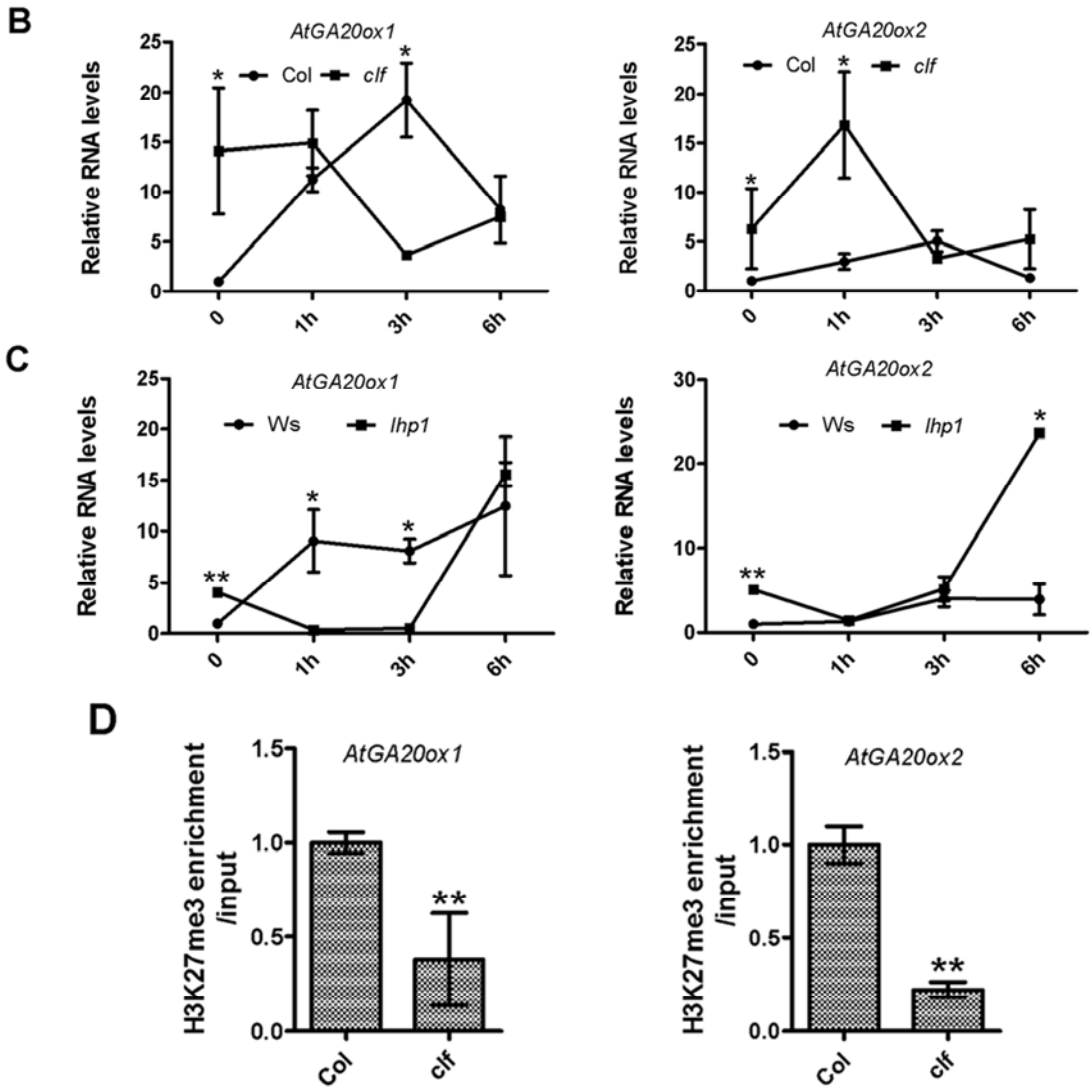


Figure 5. *Effect of PcG proteins on AtGA20Ox1 and AtGA20Ox2 induction by IAA treatment.*

(A) Analysis of H3K27me3 enrichment on *AtGA20Ox1* and *AtGA20Ox2* after IAA treatment. Two-week old seedlings were treated with 1μM IAA and harvested at the indicated time points. ChIP experiment was performed using H3K27me3 antibody. The relative enrichments were determined by first comparing to input and then to the reference gene *AGAMOUS* (*AG*). Bars represent mean values +/-SD from three biological repeats. Significance of H3K27me3 differences between after IAA treatment (at different time points) and before treatment (0) was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (B) Induction of *AtGA20Ox1* and *AtGA20Ox2* by IAA in wild type and *clf* plants. Two-week old seedlings were treated with 1μM IAA and harvested at the indicated time points. RT-PCR was performed to compare the induction of *AtGA20Ox1* and *AtGA20Ox2* by IAA between wild type (Col) and *clf* plants. *ACTIN2* was used as the reference gene. Bars represent mean values +/-SD from three biological repeats. Significance of expression differences between wild type and *clf* was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (C) Induction of *AtGA20Ox1* and *AtGA20Ox2* by IAA in wild type and *lhp1* plants. Two-week old seedlings were treated with 1μM IAA and harvested at the indicated time points. RT-PCR was performed to compare the induction of *AtGA20Ox1* and *AtGA20Ox2* by IAA between wild type (Ws) and *lhp1* plants. *ACTIN2* was used as the reference gene. Bars represent mean values +/-SD from biological repeats. Significance of expression differences between wild type and *lhp1* was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$. (D) H3K27me3 level on *AtGA20Ox1* and *AtGA20Ox2* in wild type and *clf* plants. Two-week old seedlings of wild type (Col) and *clf* were harvested for ChIP experiment using H3K27me3 antibody. Real-time PCR was performed. Bars represent mean values +/-SD from three biological repeats. Significance of H3K27me3 difference between wild type and *clf* palnts was tested by two-tailed Student's t-test, * $p<0.05$, ** $p<0.005$.

3.5. Induction of AtGA20Ox1 and AtGA20Ox2 Is Affected by PcG Proteins

To further prove the role of PcG proteins in the induction of hormone biosynthesis genes, *AtGA20Ox1* and *AtGA20Ox2*, which were induced by IAA and marked by H3K27me3, were selected for the analysis [58] (Figure A4). Firstly, we determined H3K27me3 enrichment on the two genes responding to IAA treatment. H3K27me3 level on *AtGA20ox1* was reduced 3 hours after IAA treatment, but expression of *AtGA20ox1* was notably increased 1 hour after treatment (Figure 5A and Figure 5B). RNA level of *AtGA20Ox2* was decreased slightly at first and then increased after treatment (Figure 5B). H3K27me3 on *AtGA20Ox2* was not removed in the process (Figure 5A). These data indicate that removement of H3K27me3 is not a prerequisite for the induction of the two GA biosynthesis genes.

Then we examined the induction of these genes in *clf* and *lhp1*. The qRT-PCR data indicated that by IAA treatment the expression of *AtGA20Ox1* was increased at first and then decreased lately in Columbia (Col) ecotype. We speculated that the decrease might be due to feedback inhibition by increased GA content, which was caused by increased *AtGA20Ox1* expression. It has been reported that overexpression of *AtGA20Ox1* could result in an increase of GA levels. *AtGA20Ox1* is also considered to be one of the key genes to maintain GA homeostasis, which is proved to be inhibited by GA application [48]. Our data showed that before IAA treatment *AtGA20Ox1* was de-repressed in both *clf* and *lhp1* (Figure 5B and Figure 5C). However, after IAA treatment *AtGA20Ox1* in *clf* and *lhp1* were suppressed earlier than in wild type (Figure 5B and Figure 5C). The de-repression of *AtGA20Ox1* in *clf* and *lhp1* may cause increased levels of GA, which lead to the advanced suppression of *AtGA20Ox1* in the process of IAA induction. RNA level of *AtGA20Ox2* was also elevated in *clf1* and *lhp1* before and after IAA treatment (Figure 5B and Figure 5C). The significant decrease in *lhp1* was also detected in a period of induction (Figure 5C). In addition, H3K27me3 levels on *AtGA20Ox1* and *AtGA20Ox2* were dramatically reduced in *clf* (Figure 5D). However, LHP1 bindings were observed on *AtGA20ox2* but not *AtGA20ox1* (Table 1). These results demonstrate that CLF is directly involved in the regulation of both *AtGA20Ox1* and *AtGA20Ox2* whereas LHP1 only regulates *AtGA20Ox2*.

4. Discussion

Previous studies have revealed that H3K27me3 catalyzed and recognized by PcG proteins is important for plant development. Whole-genome analysis suggests that most H3K27me3 target genes are expressed in a tissue-specific manner. Several key developmental genes enriched with H3K27me3 are repressed by PcG proteins. We analyzed H3K27me3 enrichments on hormone biosynthesis genes and found that 42 of 62 genes were covered with H3K27me3. The H3K27me3-enriched genes tended to encode key enzymes for hormone biosynthesis, demonstrating the potential importance of H3K27me3 for the control of hormone biosynthesis. Our data showed that most of the tested genes encoding cytokinin synthesis enzymes were up-regulated in the absence of PcG proteins, which uncovered the repressive role of PcG proteins in the regulation of hormone biosynthesis genes. We found that the genes with extremely low promoter activity could not be de-repressed even though H3K27me3 levels were reduced on the genes. We propose that repressive effects of H3K27me3 on gene expression may dependent on promoter activity of target genes. However, it has been revealed that LHP1 is a positive regulator of YUCCA genes that encode auxin biosynthesis enzymes and stress related genes, which contradicts our view that LHP1 serves as a repressor of gene expression [13, 47]. In *Arabidopsis* a CHD3 chromatin remodeler PICKLE, which was associated with the genes covered by H3K27me3 was initially considered to be a repressor as its homolog in animal [59-61]. But later it was found that PICKLE was required for some genes expression [62, 63]. So we could not exclude the possibility that LHP1 could either repress or promote gene expression. More evidences are required to prove the controversial argument, since existing data prefer to support our opinion that LHP1 is a negative regulator.

So far how H3K27me3 is involved in the regulation of gene expression is still obscure. The role of PcG protein remains to be explored. It has been revealed that H3K27me3 levels and/or LHP1 binding on some genes such as *ChiB*, *FT* and *VIN3* are not reduced after induction of the genes [39-41]. Analysis of H3K27me3 dynamics on *FLC* genes also shows that change of H3K27me3 enrichment on *FLC* occurs after change of gene expression [36]. These findings suggest that H3K27me3 could not play a decisive role in controlling gene expression. However, mutation of PcG proteins leads to up-regulation of all these genes, demonstrating that they may function to reduce gene expression to a lower level. We found that H3K27me3 levels on *CYP735A2* were increased after repression of gene expression by IAA. In the absence of PcG proteins *CYP735A2* was de-repressed but could be repressed to a comparable level with wild type plants by IAA treatment. This indicates that repression of *CYP735A2* by IAA does not require PcG proteins. In addition, induction of *AtGA20ox1* occurs before decrease of H3K27me3 enrichment on the gene. Increase of *AtGA20ox1* and *AtGA20ox2* expression was also detected in *clf* and *lhp1* mutants and induction curve of both genes by IAA treatment in the mutants was clearly different from wild type plants, suggesting that de-repression of the two genes in the mutants directly affect their inductions.

5. Conclusion

We speculate that the main function of PcG mediated H3K27me3 may be to restrict the expression levels of the genes encoding the crucial hormone biosynthesis enzymes, which might consequently control hormone levels. A model was proposed to explain the role of PcG proteins in controlling hormone biosynthesis genes (Figure 6). When plant needs to synthesize some hormone, a specific signal would be generated, and then transmitted into specific cells to increase the promoter activity of responding genes for enzyme production probably by transcription factors. After target genes are activated H3K27me3 might be removed, which is dependent on promoter activity. When the hormone is no longer required, the signal transduction would be terminated, and the promoter activity of the genes is decreased to the basal level. In the present of PcG proteins, the genes are enriched with H3K27m3 and transcripts of the genes are further decreased possibly to stop hormone biosynthesis completely. If PcG protein is absent, the enzyme would be produced at a low level and hormone may continue to be produced. For the genes with extremely low basal promoter activity, PcG proteins may not have the effect on them.

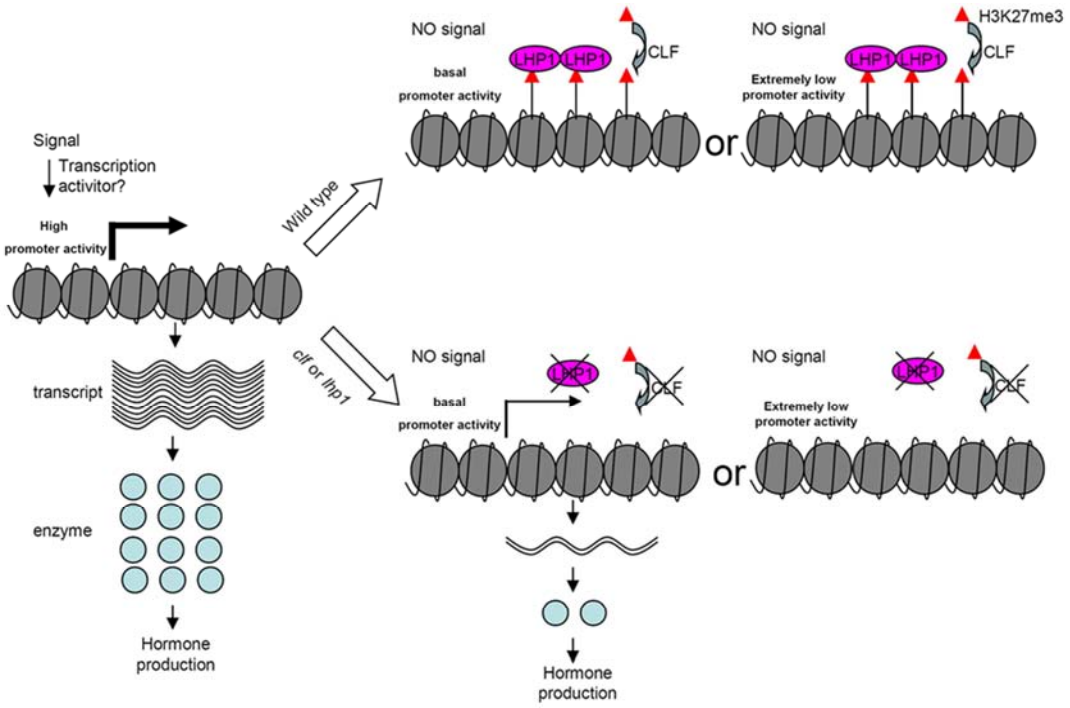


Figure 6. *Model of regulating hormone biosynthesis genes by PcG proteins. While plant development requires a hormone, a specific signal is generated and transmitted into specific cells. Finally, promoter activity of the genes encoding enzymes for the hormone production is increased probably by transcription activator. After target genes are activated H3K27me3 might be removed, which is dependent on transcription activity. When the hormone is no longer required, the signal transduction would be terminated and the promoter activity of the genes is decreased to the basal level. In the present of PcG proteins, the genes are enriched with H3K27m3 and transcripts of the genes are further decreased possibly to stop hormone biosynthesis completly. If PcG protein is absent, the enzyme would be produced in a low level, which might continue hormone biosynthesis. For the genes with extremely low basal promoter activity, PcG proteins may not have the effect on them.*

Acknowledgements

We thank Dr Dao-xiu Zhou for providing *lhp1* and *clf-29* mutants.

Appendix

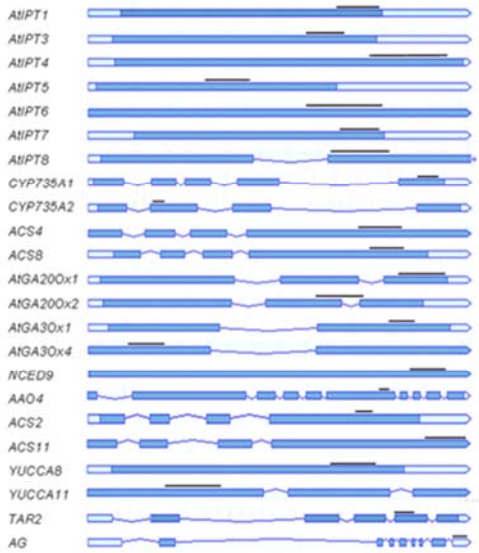


Figure A1. *Diagrams of tested genes structure. Black bars above the map indicate the amplification sites for ChIP experiment.*

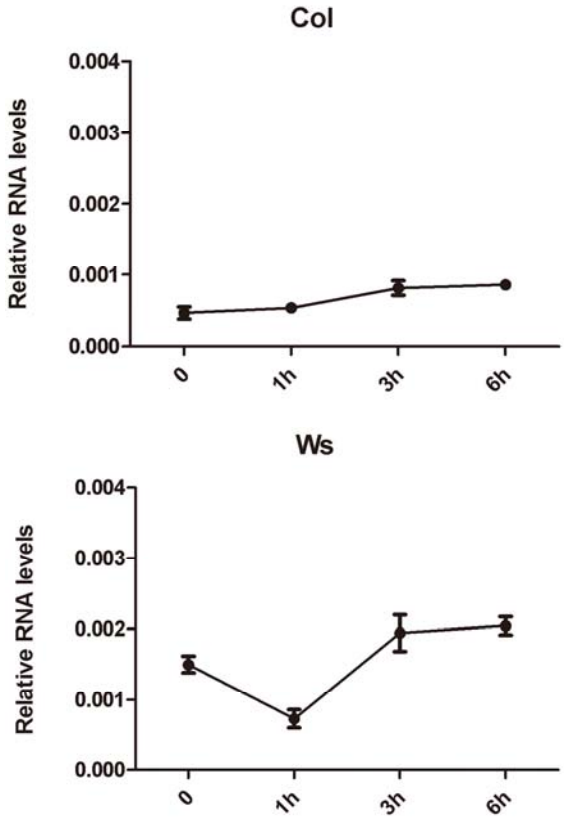


Figure A2. *Effect of IAA treatment on AG expression in Col and Ws.*

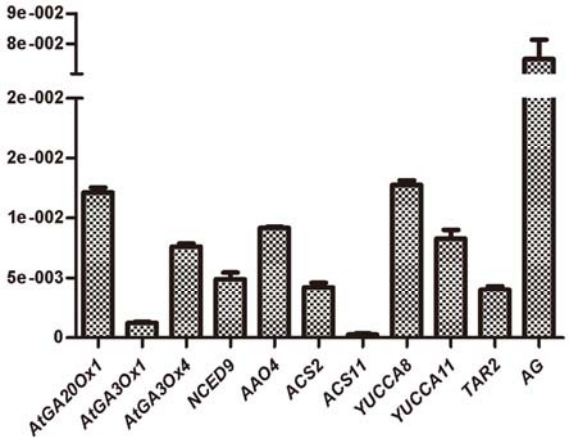


Figure A3. *H3K27me3 enrichments on ten hormone biosynthesis genes tested by ChIP-PCR. Two week-old seedlings were harvested for chromatin immunoprecipitation using H3K27me3 antibody. Real-time PCR was performed to test H3K27me3 enrichment on ten genes which are not confirmed in several datasets produced by genome-wide analysis. AGAMOUS (AG) was used as the positive control.*

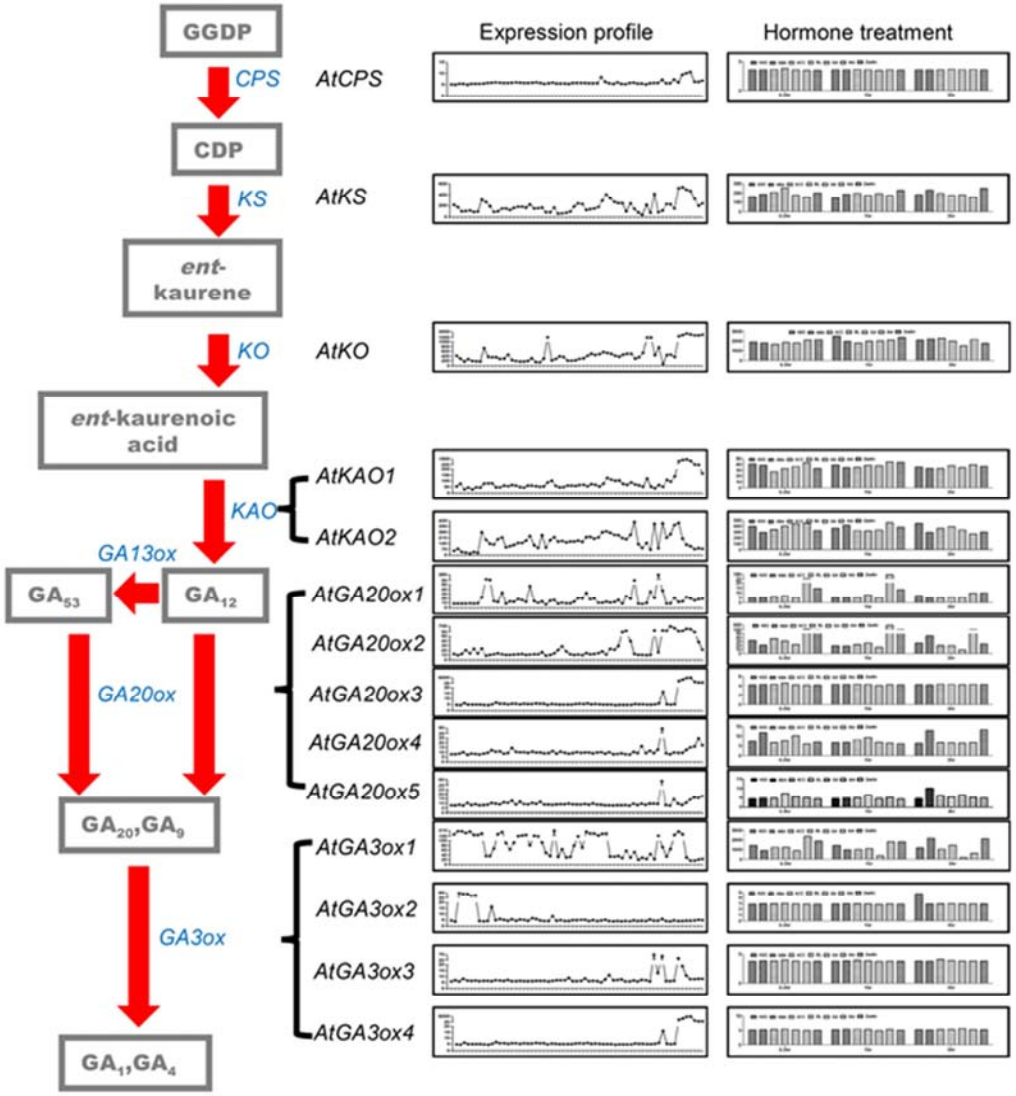


Figure A4. *Schematic diagram of gibberellin (GA) biosynthetic pathway and expression pattern of gibberellin biosynthesis genes. The data of Expression values of GA biosynthesis genes in different tissues and responding to different hormones were extracted via AtGenExpress Visualization Tool. For expression profile of the genes the tissues are as follows. 1-7: root at different stages; 8-10: stem at different stages; 11-25: leaf at different stages; 26-36: seedlings at different stages; 37-40: apex at different stages; 41-45 flowers at different stages; 46-55 floral organ at different stages; 56-63: seeds at different stages.*

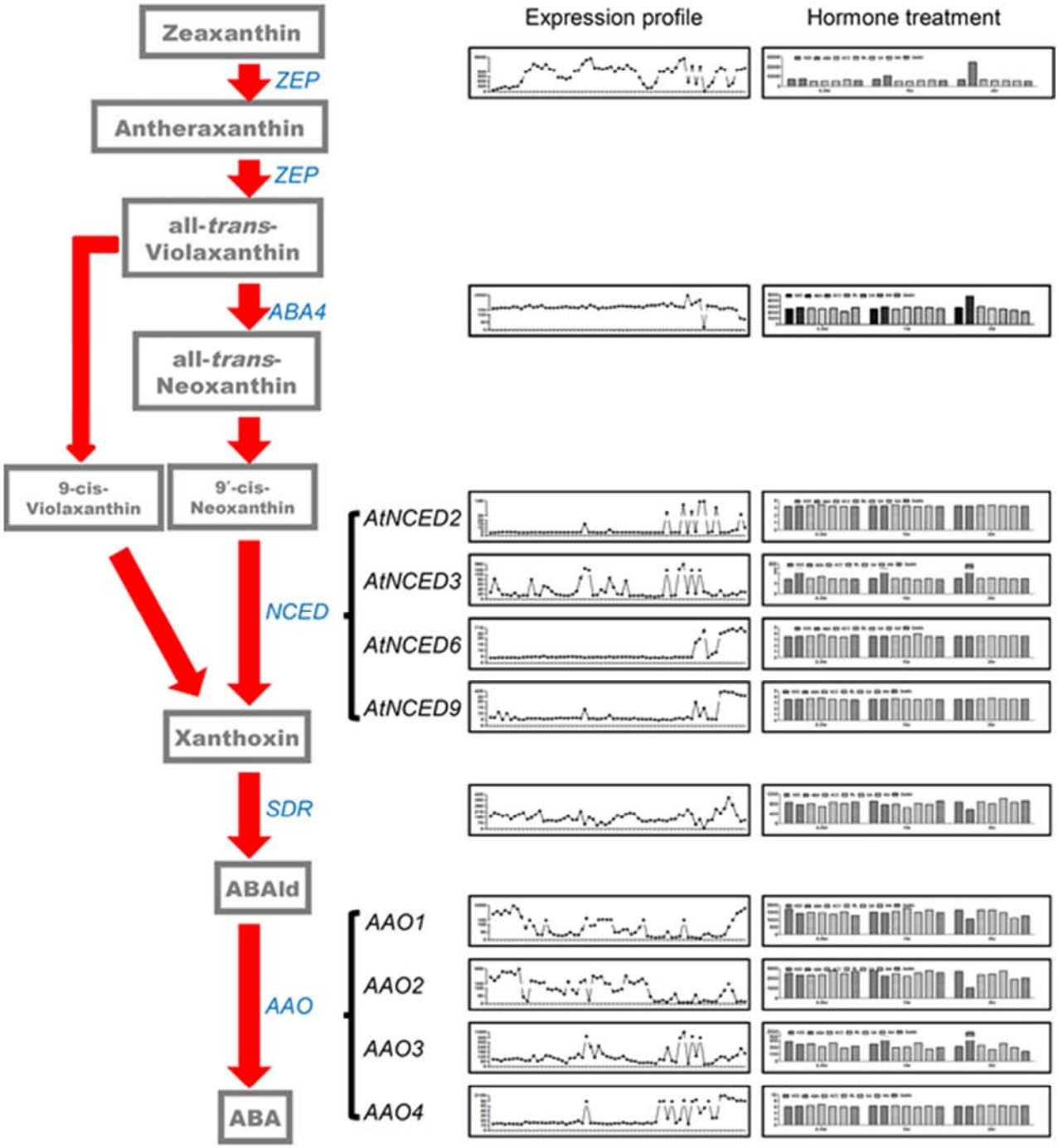


Figure A5. *Schematic diagram of abscisic acid (ABA) biosynthetic pathway and expression pattern of abscisic acid biosynthesis genes. The data of Expression values of ABA biosynthesis genes in different tissues and responding to different hormones were extracted via AtGenExpress Visualization Tool. For expression profile of the genes the tissues are as follows. 1-7: root at different stages; 8-10: stem at different stages; 11-25: leaf at different stages; 26-36: seedlings at different stages; 37-40: apex at different stages; 41-45 flowers at different stages; 46-55 floral organ at different stages; 56-63: seeds at different stages.*

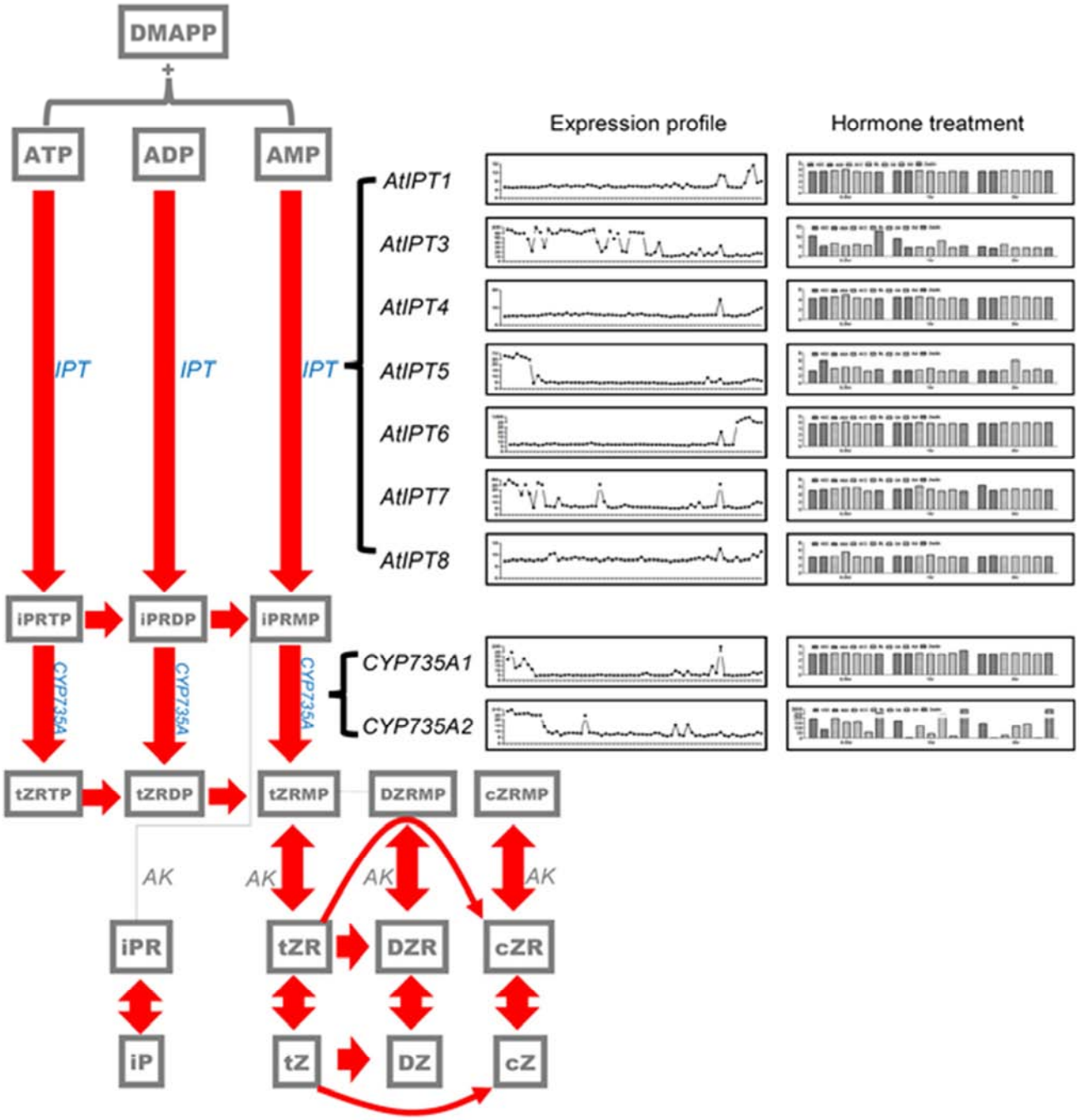


Figure A6. *Schematic diagram of cytokinin (CK) biosynthetic pathway and expression pattern of cytokinin biosynthesis genes. The data of Expression values of CK biosynthesis genes in different tissues and responding to different hormones were extracted via AtGenExpress Visualization Tool. For expression profile of the genes the tissues are as follows. 1-7: root at different stages; 8-10: stem at different stages; 11-25: leaf at different stages; 26-36: seedlings at different stages; 37-40: apex at different stages; 41-45 flowers at different stages; 46-55 floral organ at different stages; 56-63: seeds at different stages.*

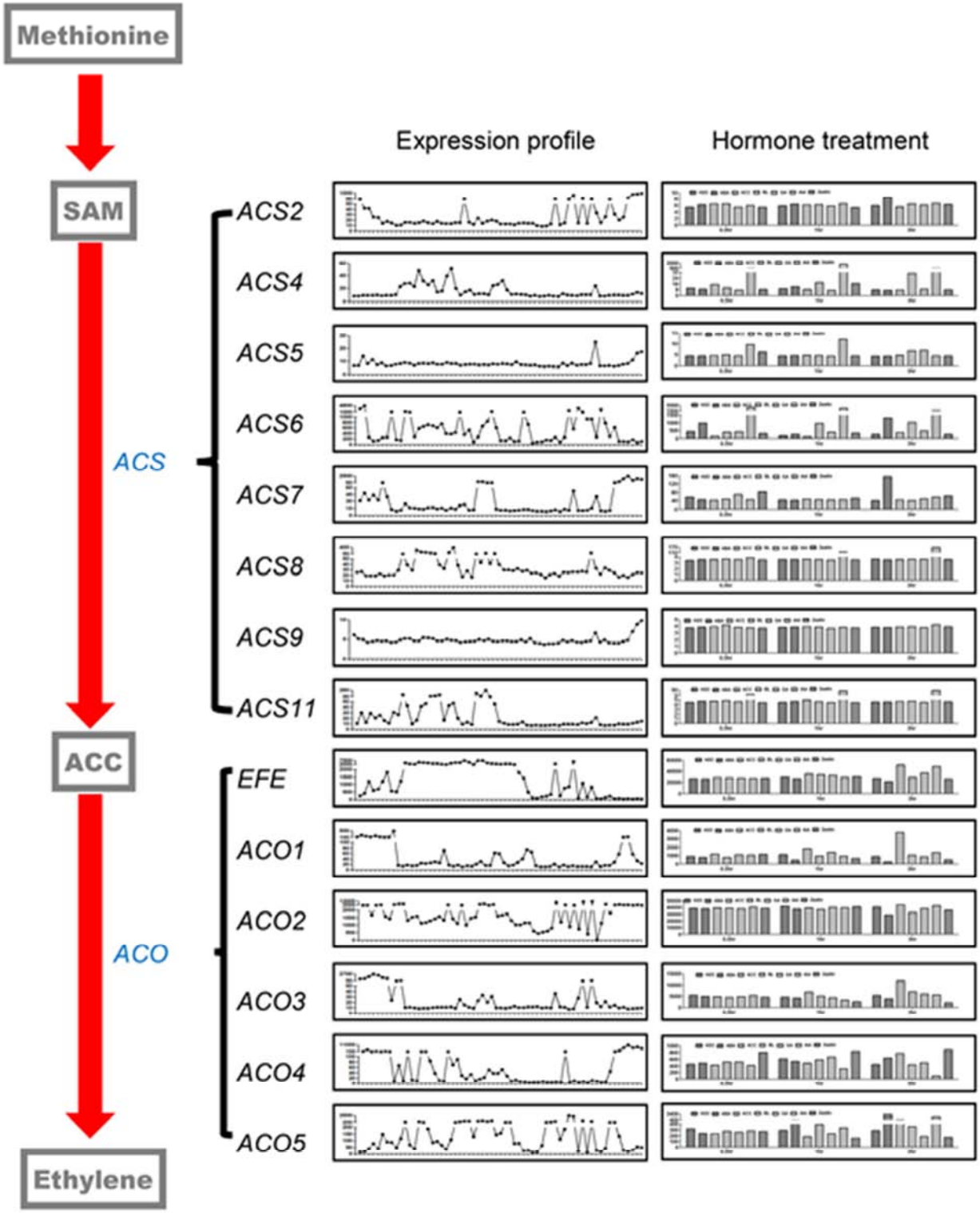


Figure A7. *Schematic diagram of Ethylene biosynthetic pathway and expression pattern of Ethylene biosynthesis genes. The data of Expression values of Ethylene biosynthesis genes in different tissues and responding to different hormones were extracted via AtGenExpress Visualization Tool. For expression profile of the genes the tissues are as follows. 1-7: root at different stages; 8-10: stem at different stages; 11-25: leaf at different stages; 26-36: seedlings at different stages; 37-40: apex at different stages; 41-45 flowers at different stages; 46-55 floral organ at different stages; 56-63: seeds at different stages.*

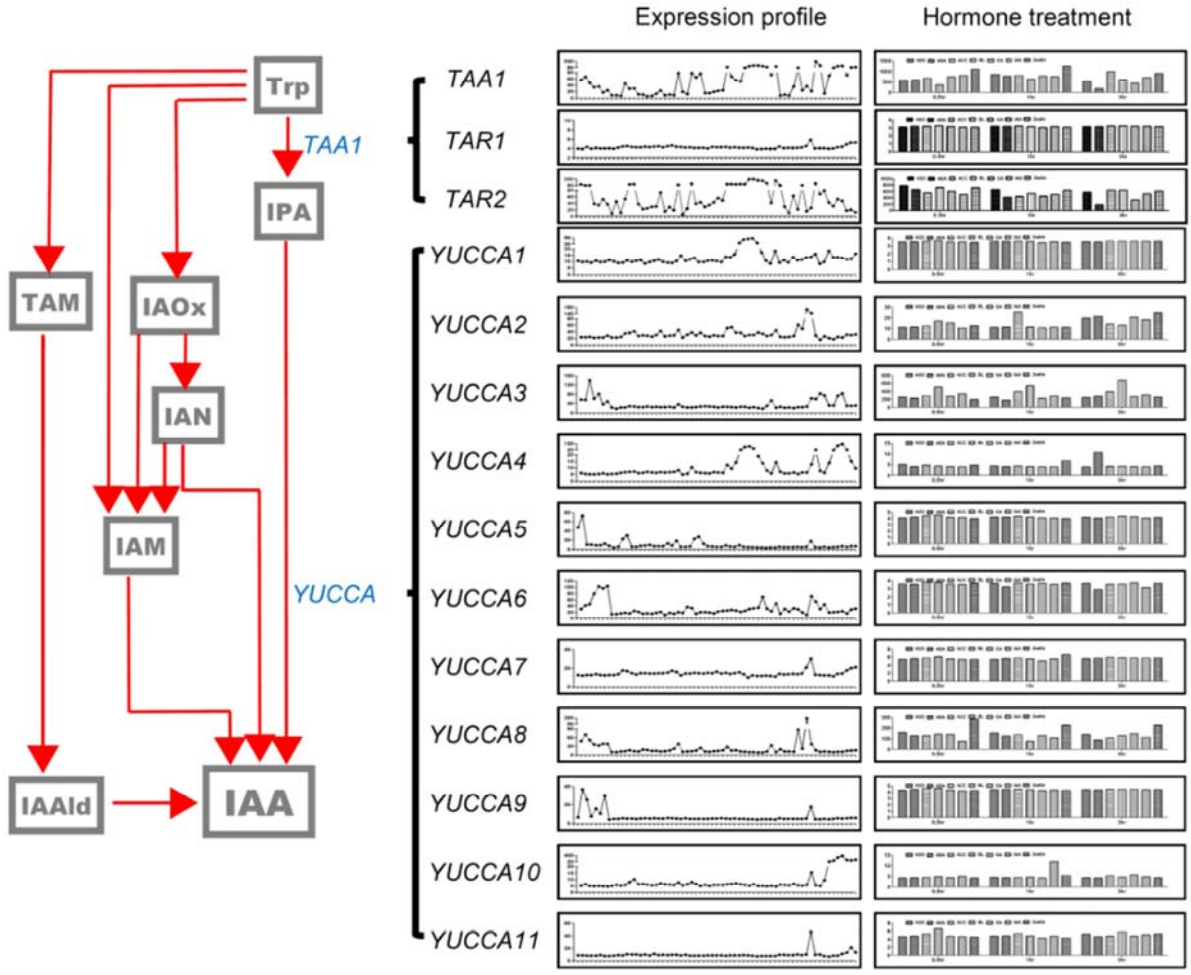


Figure A8. *Schematic diagram of auxin biosynthetic pathway and expression pattern of auxin biosynthesis genes. The data of Expression values of auxin biosynthesis genes in different tissues and responding to different hormones were extracted via AtGenExpress Visualization Tool. For expression profile of the genes the tissues are as follows. 1-7: root at different stages; 8-10: stem at different stages; 11-25: leaf at different stages; 26-36: seedlings at different stages; 37-40: apex at different stages; 41-45 flowers at different stages; 46-55 floral organ at different stages; 56-63: seeds at different stages.*

Table A1. *Primers used in this paper.*

Primer name	Nucleotide sequence（5'-3'）
AtIPT1-F	CGTACGATAAGGCGGTTGAT
AtIPT1-R	GGCTCCAACACTTGCTCTTC
AtIPT3-F	CGAGCAGTTCTTGAATGTGG
AtIPT3-R	GCCACGTTAGCATCCATCTT
AtIPT4-F	GCTTATGAGAAGGCGGTGAG
AtIPT4-R	CGGTTCCAACACTTCCTTGT
AtIPT5-F	ATTGCGTTGACTTCCGGTTA
AtIPT5-R	TCGAGCTCTGGAACTCCAAT
AtIPT6-F	AAATGATCGAGTCGGGATTG
AtIPT6-R	TCATCCCCTTCACTGTCTCC
AtIPT7-F	AACGGCTTCACAAGAAGTGG
AtIPT7-R	GTAAGATGCCGCCAAACAGT
AtIPT8-F	TTCCTTTGGGTGGATGTCTC
AtIPT8-R	CAGGCGGGTATAAGCTGAAG
CYP735A1-F	TATGGGGTAAAGACGCGAAC
CYP735A1-R	TTGGGGCATGCCTATAATTC
CYP735A2-F	ACGGGACAGAACCGAGACTA
CYP735A2-R	TGTTGTTGCAGCCATGATTT
AtGA20Ox1-F	CGAGAGCGAGAGGAAATCAC
AtGA20Ox1-R	GGTGAGCCAATCTGAAAAGG
AtGA20Ox2-F	TGCCAAACACCAGATCTCAC
AtGA20Ox2-R	GCTCTTGAATATCCCGTTCG
AtGA3Ox1-F	ACGTTGGTGACCTCTTCCAC
AtGA3Ox1-R	CCCCAAAGGAATGCTACAGA
AtGA3Ox4-F	CCTGACGTTACCACCCTCAT
AtGA3Ox4-R	CGCTTCAAGCTTCCTCTCTG
NCED9-F	GAACCGTGGCCTAAAGTGTC
NCED9-R	CGGCGTTTATGAGTTGGAGT
AAO4-F	AGGCCAACTCCAGGAAAAGT
AAO4-R	CATCACTTCCCTCGCATTTT
ACS2-F	GCTGGTTTATTTGCGTGGAT
ACS2-R	AGGAAGAGCCAGGAGACACA
ACS4-F	AATGTCAAGTTTCGGCCTTG
ACS4-R	TCGACCCAACAAAAGAGTCC
ACS8-F	CGAAGCCGAGATTGAGCTAT
ACS8-R	TCTAGTAGGCGACGGTCCAT
ACS11-F	ATCTCTCCAGGCTCATCGTG
ACS11-R	GGAAAGCTTGGAGACCCATT
YUCCA8-F	GGTTCCAGGGATCAAAAGGT
YUCCA8-R	CCGCATACAATCCTGTCCTT
YUCCA11-F	CCGCGTACTTCAAAGATGGT
YUCCA11-R	CAGCGAACTTCTCTCCGTTC
TAR2-F	TGGGGTTTCAAAGGACTCAC
TAR2-R	TCCTTTGCTGCTTGTTTCAA
AG-F	AGCTTATGCCACCACCTCAAA
AG-R	TGTTAGGTTGCAATGCCGC
ACTIN2-F	CGCTGACCGTATGAGCAAAGA
ACTIN2-R	GCAAGAATGGAACCACCGATC

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