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ORIGINAL ARTICLE

Down-regulation of *S*-adenosyl-L-homocysteine hydrolase reveals a role of cytokinin in promoting transmethylation reactions

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Abstract *S*-adenosyl-L-homocysteine hydrolase (SAHH) is a key enzyme for maintenance of cellular transmethylation potential. Although a cytokinin-binding activity had been hypothesized for SAHH, the relation between cytokinin and transmethylation reactions has not been elucidated. Here we show that, of the two *Arabidopsis thaliana SAHH* genes, *AtSAHH1* has a much higher expression level than *AtSAHH2*. A T-DNA insertion mutant of *AtSAHH1* (*sahh1-1*) and the RNA interference (RNAi) plants (*dsAtSAHH2*) accumulated a higher level of cytokinins, exhibited phenotypic changes similar to those of cytokinin-overproducers, and their global DNA methylation status was reduced. On the other hand, cytokinins positively regulate the transmethylation pathway genes, including *AtSAHH1*, *AtADK1* (for adenosine kinase), and this regulation involves the cytokinin activity. Furthermore, expression of three cytosine DNA methyltransferase genes examined was inducible by cytokinin treatment. Unlike adenine and adenosine which are SAHH inhibitors, the adenine-type cytokinins have no effect on SAHH activity at protein level. Changing of endogenous cytokinin levels by transgene expression resulted in alterations of DNA methylation status in the *sahh1-1* background, suggesting that cytokinins promote DNA methylation, at least under transmethylation stringent conditions. These data demonstrate that the phytohormone cytokinin plays a role in promoting transmethylation reactions, including DNA methylation.

Keywords *Arabidopsis* · Cytokinin · DNA methylation · *S*-Adenosyl-L-homocysteine hydrolase

Abbreviations

ADA	Adenosine deaminase
ADK1	Adenosine kinase 1
6-BA	6-Benzylaminopurine
CKX	Cytokinin oxidase
DHPA	Dihydroxypropyladenine
DPU	Diphenylurea
dsRNA	Double-strand RNA
GFP	Green fluorescent protein
GUS	*β*-Glucuronidase
HOG1	Homology-dependent gene silencing 1
HPLC	High performance liquid chromatography
iPA	Isopentenyl adenosine
IPT	Adenosine phosphate-isopentenyltransferase
KT	Kinetin
MgPMT	Mg protoporphyrin methyltransferase
MTase	Methyltransferases
PVPP	Polyvinylpolypyrrolidone
RGP	GTP-binding protein
RNAi	RNA interference
RR	Ruthenium red
SAHH	*S*-adenosyl-L-homocysteine hydrolase
SAM	*S*-adenosyl-L-methionine
SAH	*S*-adenosyl-L-homocysteine

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Introduction

Transmethylation reactions are commonly involved in modifications of almost all the classes of metabolites in plants, animals and microorganisms. Recently, increasing evidences show that DNA and histone transmethylation plays an important role in regulating gene expression and epigenetics (Kinoshita et al. 2004; Jullien et al. 2006; Mull et al. 2006), such as transcriptional and post-transcriptional gene silencing (Morel et al. 2000; Jones et al. 2001). In many cases, mutations in methylation-related genes release the inactivation of genes, suggesting a role of DNA methylation in suppressing gene expression (Elmayan et al. 1998; Morel et al. 2000; Rocha et al. 2005).

Methionine has two major fates: incorporation into proteins and conversion into *S*-adenosyl-L-methionine (SAM). SAM plays a pivotal role as methyl donor in many aspects of biological and biochemical events (Chiang et al. 1996). A large portion of SAM is used as methyl donor, and after the methyl group is transferred to acceptors, such as phospholipids, proteins, DNA and RNA, *S*-adenosyl-L-homocysteine (SAH) is formed. SAH is a product inhibitor for most of the transmethylation reactions, and the ratio of SAM/SAH is referred to as methylation index for elevated levels of SAH inhibit activities of almost all methyltransferases (Chiang 1998). *S*-adenosyl-L-homocysteine hydrolase (SAHH) catalyzes the conversion of SAH into adenosine and L-homocysteine, releasing the feedback inhibition by SAH and promoting SAM-dependent transmethylation. SAHH is therefore a key enzyme in maintenance of the methylation potential in cells (De Clercq et al. 1989; Miller et al. 1994; Tanaka et al. 1997).

Although the hydrolysis of SAH into adenosine and L-homocysteine catalyzed by SAHH is reversible, under normal conditions, the removal of adenosine facilitates the hydrolysis reaction. Adenosine removal is mainly accomplished by adenosine deaminase (ADA) in mammalians and adenosine kinase (ADK) in plants, respectively (Migchielsen et al. 1995; Moffatt et al. 2002). Antisense inhibition of *ADK* gene expression in *Arabidopsis* resulted in abnormal phenotypes, including rounded and wavy leaves, bushy stature, and hypomethylesterified pectin (Moffatt et al. 2002).

The biological function of SAHH in human and mouse has been elucidated by numerous studies using inhibitors and genetic mutants. In vivo and in vitro studies manifested that SAHH activity can be inhibited by adenine and adenosine and their derivates (Chiang 1998). Neplanocin A is a carbocyclic adenosine analog; when applied to rat pituitary cells it caused a dose-dependent inactivation of SAHH and an increase of intracellular SAH level; after treatment for 4–5 days, methylated cytosine was reduced by 50% (Wolfson et al. 1986). The inhibitive effect was also observed for 3-deazaneplanocin A, another analog of adenosine (Miller et al. 1994).

For plants, analysis of *Arabidopsis homology-dependent gene silencing1* (*HOG1*) mutants revealed that *HOG1* gene encodes SAHH, and *HOG1* mutations caused DNA demethylation in the whole genome, resulting in relief of transcriptional gene silencing (Rocha et al. 2005). A more recent investigation showed that a partial loss-of-function missense mutation of the *Arabidopsis SAHH1* gene caused loss of cytosine methylation specifically in non-CG contexts (Mull et al. 2006). Tobacco callus cultures treated with dihydroxypropyladenine (DHPA) preferentially inhibited DNA methylation at CNG and non-symmetrical motifs (Kovarik et al. 1994; Koukalova et al. 2002). Reduced *SAHH* gene expression in tobacco plants due to antisense inhibition resulted in inhibition of viral replication (Masuta et al. 1995) and DNA hypomethylation (Tanaka et al. 1997).

Natural cytokinins are adenine derivatives (Electronic supplementary material Fig. S1) that regulate numerous aspects of plant growth and development, including apical and floral meristem development, stem growth and branching, leaf senescence, light signal transduction, and stress tolerance. Many components involved in cytokinin biosynthesis and signaling pathways have been isolated (Banno et al. 2001; Hwang and Sheen 2001; Inoue et al. 2001; Nishimura et al. 2004; To et al. 2004). In an early investigation, SAHH was found to be present in a 130-kDa cytokinin-binding protein complex isolated from tobacco leaves, thus the enzyme was proposed to be a cytokinin-binding protein (Mitsui et al. 1993). Furthermore, increased levels of bioactive cytokinins and related phenotypic changes were reported for antisense *SAHH* tobacco plants (Masuta et al. 1995). An investigation of maize proteins, however, did not prove the cytokinin-binding potential of SAHH, as the SAHH and cytokinin-binding activities were found in separate chromatographic fractions (Romanov and Dietrich 1995). Despite these discrepant but intriguing findings, up to date evidence for a cross-talk between SAM-dependent transmethylation pathway and cytokinin action is lacking. By analysis of a T-DNA mutant and transgenic RNAi plants, we demonstrate that down-regulation of *SAHH* affects the expression of cytokinin pathway genes, and cytokinin positively regulates the transmethylation cycle and DNA methylation.

Materials and methods

Material

Arabidopsis thaliana (ecotype Col-0) plants were grown at 22°C with a 16-h photoperiod. A T-DNA insertion mutant

of *AtSAHH1* (SALK_068487) was obtained from ABRC (Ohio State University, Columbus, OH, USA). Chemicals were purchased from Sigma (St. Louis, MO, USA).

Plant transformation and analysis

The upstream fragments of *AtSAHH1* and *AtSAHH2*, approximately 2.2 kb, were cloned into pBI121 vector to form *proSAHH1::GUS* and *proSAHH2::GUS*, respectively. A cotton *RDL1* promoter (Wang et al. 2004) was subcloned into the pCAMBIA1300 vector and a 550-bp inverted repeat fragment of the second exon of *AtSAHH2* linked by *RTM1* intron was then inserted to form *proRDL1::dsAtSAHH2* vector. The 35S promoter was also introduced to pCAMBIA1300 vector and the genomic coding sequence of *AtSAHH1* and *AtSAHH2* genes were in-frame fused to the *enhanced green florescence protein* gene to form *pro35S::GFP-SAHH1 and pro35S::GFP-SAHH2*, respectively. The *AtSAHH1* promoter was used to form *proSAHH1::GFP-SAHH1*. A 2-kb promoter sequence of *SAG12* (At5g45890) and the *IPT* cDNA from *Agrobacterium tumefaciens* were inserted into pCAMBIA1301 to generate *proSAG12::IPT*. A cDNA fragment of *CKX2* (At2g19500) was placed behind the 35S promoter to form *pro35S::AtCKX2*. Primers used in this investigation are listed in Electronic supplementary Table S1.

Binary vectors were introduced into *A. tumefaciens* strain GV3101, followed by transformation of *Arabidopsis* by floral dipping (Clough and Bent 1998). Transgenic plants were selected on 1/2 MS medium containing 50 mg/l of kanamycin or hygromycin. For liquid culture of *Arabidopsis* seedlings, seeds were germinated and grown on MS medium for 5 days, followed by transferring the seedlings to 1/2 MS liquid medium for another 3–5 days before treatments.

For GUS histochemical analysis (Jefferson et al. 1987), six independent transgenic lines for each of the constructs were selected. Chlorophyll content was measured according to a published protocol (Nakatani and Baliga 1985). For GFP fluorescence of onion epidermal cells, images were obtained with an LSM510 laser scanning confocal microscope (Zeiss, Jena, Germany), with argon laser excitation at 488 nm and a 505- to 550-nm emission filter set; cell plasmolysis was performed with 20% (w/v) sucrose for 10 min. Staining of seed coat with Ruthenium red (RR) was performed as described (Moffatt et al. 2002).

DNA isolation and analysis

DNA was isolated as described (Katterman and Shattuck 1983). For southern-blot analysis the DNA was digested with restriction enzyme (5 U enzyme/1 μg DNA) overnight, followed by another 6-h digestion with newly added enzyme (4 U enzyme/1 μg DNA) to ensure complete digestion. The DNA samples, 10 μg each lane, were separated by electrophoresis on 1.2% agarose gel and transferred to a Hybond-N^+ filter membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Fragments of *At5S rDNA* and 180-bp centrometric repeat were ^{32}P-labeled with a Random Primer DNA Labeling kit (TaKaRa, Dalian, China). Membranes were hybridized and washed according to the manufacturer's manual.

RNA isolation and gene expression analysis

RNA was isolated by using TRIzol reagent (Invitrogen). The total RNAs, 15 μg per lane, were separated on agarose (1.2%) or urea polyacrylamide (15%) gel and transferred to a Hybond-N+ filter membrane. For probes the gene-specific 3′-untranslated regions (UTRs) of *AtSAHH1* and *AtADK1* were used and labeled as described for southern.

For RT-PCR, total RNAs of 1 μg were reverse transcribed in a 20-μl reaction using the RNA PCR (AMV) kit (TaKaRa). Quantitative analysis of gene expression was performed by using TaKaRa Real-Time PCR Core Kit and EvaGreen™ (Biotium, Shanghai, China) as the dye. A ubiquitin gene (At4g05320) was used as the internal control (ESM Table S1).

Assay of SAHH activity

The coding region sequence of *AtSAHH1* was in-frame fused to the pET28b vector and transformed into *Escherichia coli* strain BL21 (DE3). The N-terminal 6× His-tag fused SAHH proteins were purified by a Ni-affinity column (Qiagen, Valencia, CA, USA). SAHH enzyme activity was assayed following published methods (Aiyar and Hershfield 1985; Wolfson et al. 1986) with slight modifications. The purified recombinant proteins, 2 and 10 μg, were used to assay the synthesis and hydrolysis activities, respectively. For synthesis direction, the protein was added to a 50 μM potassium phosphate buffer, pH 7.2, containing 4 mM of homocysteine 1 mM of EDTA, in a total volum of 500-μl. Adenosine was then added to a final concentration of 0.2 mM and the mixture was incubated at 37°C for 5 min. After termination by adding 25 μl of 5 N $HClO_4$, and placing on ice water for 5 min, the insoluble material was removed by centrifugation. The synthesis activity was determined by measuring the formation of *S*-adenosyl-L-homocysteine (SAH). The hydrolysis activities were determined by incubation with *S*-adenosylhomocysteine (0.2 mM) in the presence of excess ADA, followed by measuring the amount of inosine formed in the reaction solution. For inhibition assay, adenine, 2iP or 6-BA (at a given concentration) was included in the assay, or pre-incubated in the reaction mixture, respectively, for 10 min before adding substrate. Supernatant was analyzed by HPLC (Agilent

1100 series) using a C18 reversed-phase column which was eluted with 1 M sodium acetate, pH 4.5, containing 5% methanol at an elution rate of 1 ml/min. The amount of SAH and inosine was monitored at 260 nm by measuring the peak area. Assays were repeated for four to six times and the data were analyzed by Student's *t* test.

Quantitative analysis of metabolites

Seedlings were grown vertically on plates for 10 days, roots were collected and frozen in liquid nitrogen. Cytokinin extraction and purification were performed as previously described (Zhang et al. 2001). Briefly, fresh prepared roots were homogenized to slurry over ice and extracted with pre-cold 80% methanol at 4°C overnight. The supernatant was collected (3,000 g, 10 min), and the pellet was re-extracted with 80% methanol. The two supernatants were combined and dried in vacuum and resolved in NH_4Ac (0.1 M, pH 9.0). After passing through a polyvinylpolypyrrolidone (PVPP), a DEAE sephadex A-25 and a C18 Sep-pak column, the eluted solution was dried and dissolved in H_2O and subjected to reverse-phase C18 HPLC (Agilent 1100). For SAM and SAH analysis, rosette leaves of ~1-month-old plants were extracted as described (Rocha et al. 2005). The final supernatant fractions were analyzed by HPLC and the UV absorbance was monitored at 254 nm. A detailed protocol is available upon request. Analysis was repeated with at least three different samples, calculations were verified using purchased standards.

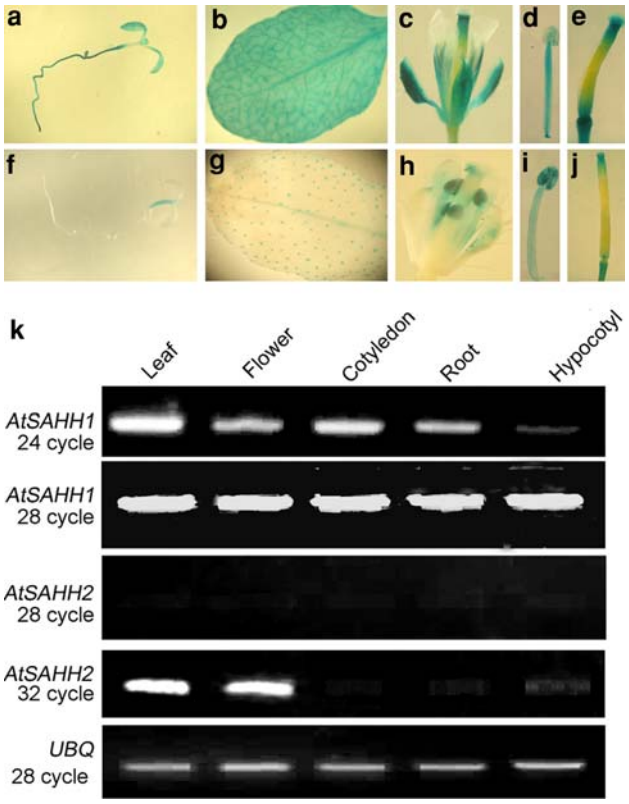

Fig. 1 Expression patterns of *AtSAHH1* and *AtSAHH2*. **a**–**j** GUS staining of the *proSAHH1::GUS* (**a**–**e**) and the *proSAHH2::GUS* (**f**–**j**) seedling (**a**, **f**), leaf (**b**, **g**), flower (**c**, **h**), anther (**d**, **i**), and young silique (**e**, **j**). **k** RT-PCR analysis of *AtSAHH1* and *AtSAHH2* in 9-day-old seedlings (cotyledon, root, and hypocotyl) and in ~5-week-old plants (leaf and flower)

Results

Expression patterns of *AtSAHH1* and *AtSAHH2* in *Arabidopsis*

The *A. thaliana* genome contains two genes coding for SAHH, namely *AtSAHH1* (At4g13940) and *AtSAHH2* (At3g23810), and they share 86% identity at the nucleotide level and are 95% identical at the amino acid level. After expression in *E. coli*, recombinant proteins of both AtSAHH1 and AtSAHH2 exhibited the expected activity of hydrolyzing SAH (data not shown).

To examine spatial patterns of expression, promoters (about 2 kb upstream to the translational start site) of *AtSAHH1* and *AtSAHH2* were fused to a *β*-glucuronidase (*GUS*) reporter gene, respectively. GUS staining of the 8-day-old seedlings showed that *proSAHH1::GUS* was expressed in the cotyledon and root (including root tip), but the expression in hypocotyls was weak; whereas *proSAHH2::GUS* expression was restricted to hypocotyl (Fig. 1a, f). In leaves, *proSAHH1::GUS* was widely expressed with strong GUS-staining in both primary and secondary veins; interestingly, the GUS activity was confined to trichomes of the *proSAHH2::GUS* leaves (Fig. 1b, g). In flowers, *proSAHH1::GUS* was expressed in sepal, stigma, filament, and the basal part of silique (Fig. 1c–e), whereas *proSAHH2::GUS* was mainly expressed in anthers (Fig. 1h–j). The promoter activities of the two *SAHH* genes are largely non-overlapping, and *proAtSAHH1* has a much wider pattern of activity than *proAtSAHH2*.

Because gene expressions can be regulated by sequences outside the promoter region, the two *SAHH* genes were further analyzed by RT-PCR, which confirmed that the transcript level of *AtSAHH1* was much higher than that of *AtSAHH2* in all the organs examined, including leaves, flowers, cotyledons, hypocotyl and root, though the level was relatively low in hypocotyl (Fig. 1k). While *AtSAHH1* transcripts were clearly detected by 24 cycles of PCR amplification, those of *AtSAHH2* were undetectable after 28 cycles. At higher cycles (32), *AtSAHH2* expression became evident in leaves and flowers, weak or undetectable in hypocotyl, cotyledons and root (Fig. 1k). Consistently, the gene chip data from NASC (Nottingham Arabidopsis Stock Centre) showed that *AtSAHH1* mRNA is approximately

five times more abundant than *AtSAHH2* (see also Rocha et al. 2005). In combination, these data suggest that AtSAHH1 accounts for most of the SAHH activities in *Arabidopsis* plants.

To determine the subcellular distribution of SAHH proteins, a *GFP* (green fluorescent protein) gene was in-frame fused to the 5′-end of *AtSAHH1* and *AtSAHH2* open reading frames, respectively. When the 35S promoter was used, both constructs were able to complement a T-DNA insertion mutant of *AtSAHH1* (*sahh1-1*, see below), indicating that the fusion proteins were functional (data not shown). After transient expression in onion epidermis cells, most of the GFP signal appeared in the cytoplasm, supporting previous reports that SAHH protein is present in cytosol (Wasternack et al. 1985). However, weak signal in nucleus could not be excluded (Electronic supplementary material Fig. S2). The identical subcellular localization and catalytic activities indicate that both proteins are functionally identical.

Abnormal phenotypes of *sahh1-1* mutant and *AtSAHH2*-RNAi plants

A T-DNA mutant (SALK_068487), named *sahh1-1*, contained an insertion at 82-bp upstream to the *AtSAHH1* translation starting codon. The heterozygous plants appeared normal, but the homozygous plants exhibited pleiotropic phenotypic changes. In continuous light condition, length of the *sahh1-1* root was less than half of the WT root (Fig. 2a). When grown at 16-h photoperiod for four weeks, the *sahh1-1* leaves were dark green (Fig. 2b) with increased levels of both chlorophyll a and chlorophyll b in comparison with the WT leaves (Electronic supplementary material Fig. S3). Furthermore, flowering was delayed by about 10 days, and leaf senescence was also delayed (data not shown).

In contrast with the *hog1-4* and *hog1-5* mutants which were zygotic embryo lethal (Rocha et al. 2005), the *sahh1-1*

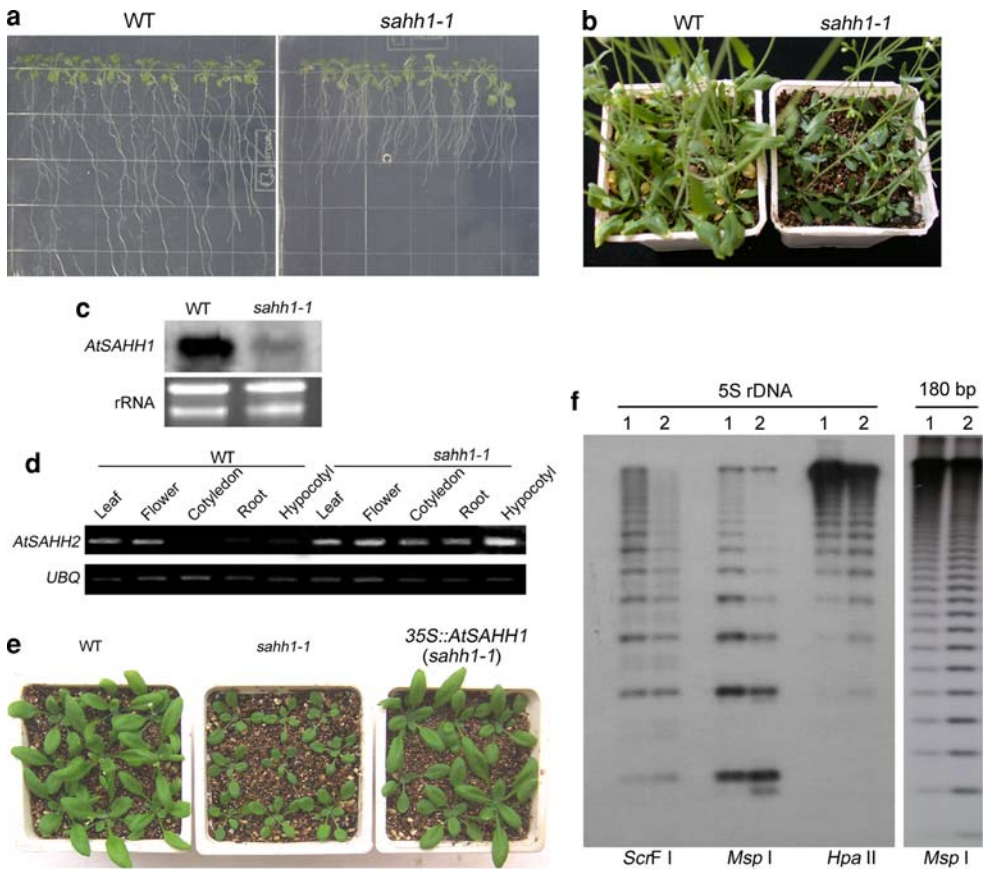

Fig. 2 **a**–**f** Abnormal phenotypes and DNA methylation status of *sahh1-1* plants. **a** Roots of the *sahh1-1* and the WT seedlings grown on MS medium in the greenhouse with continuous light for 8 days. Growth (elongation) of *sahh1-1* roots was reduced. **b** Comparison of *sahh1-1* and WT plants grown in soil in the greenhouse for 4 weeks with 16-h photoperiod. The *sahh1-1* leaves were dark green. **c** Northern blot showing the transcript level of *AtSAHH1* in leaves of the WT and *sahh1-1* plants. The transcript level in *sahh1-1* plants was greatly reduced. A 248-bp fragment of *AtSAHH1* at the 3′UTR was used as probe. **d** RT-PCR analysis of *AtSAHH2* transcripts in *sahh1-1* plants (see also Fig. 1k). PCR was performed by 32 cycles. **e** Plants of WT, *sahh1-1* and *pro35S::AtSAHH1 sahh1-1*, showing complementation of *sahh1-1* by *pro35S::AtSAHH1*. **f** Southern blots showing global DNA methylation status of WT (*lane 1*) and *sahh1-1* mutant (*lane 2*) plants. Genomic DNA (5 μg) was digested with methylaton-sensitive enzymes (*Scr*FI, *Msp*I and *Hpa*II) and probed against an At5S rDNA or a 180-bp centrometric fragment, respectively

plants were completely fertile. Northern blot showed that *AtSAHH1* expression in *sahh1-1* was greatly reduced, but not abolished (Fig. 2c). As shown in Fig. 1, *AtSAHH1* has a much higher level of expression than *AtSAHH2*. To see if *AtSAHH2* expression was changed due to *AtSAHH1* mutation, RT-PCR was performed on the *sahh1-1* mutant. We found that *AtSAHH2* transcript level was increased in cotyledons, hypocotyl and root, but largely unchanged in leaves and flowers. Furthermore, the level of *AtSAHH2* expression was still low, as high cycles of PCR were required for the detection (Fig. 2d). Thus a slight elevation of *AtSAHH2* expression was unlikely able to compensate the reduced expression of *AtSAHH1* in the *sahh1-1* mutant.

Analysis of SAM and SAH by high performance liquid chromatography (HPLC) showed that the SAM/SAH ratio was decreased in *sahh1* leaves (12.06 ± 0.8 for *sahh1* vs. 14.04 ± 1.2 for WT), consistent with a previous report that *hog1* mutant had a lower SAM/SAH ratio (20.44 for *C-insert-hog1-1* homozygous vs. 25.38 for WT) (Rocha et al. 2005). Introduction of *AtSAHH1* genomic DNA, placed behind the CaMV 35S promoter, resulted in completely normal plants (Fig. 2e), confirming that the abnormal phenotypes of *sahh1-1* plants were caused by reduced expression of *AtSAHH1*.

It was reported that point mutations in *AtSAHH1/HOG1* gene caused global DNA hypomethylation (Rocha et al. 2005). To see if reduced *AtSAHH1* expression due to T-DNA insertion exerts a similar effect, DNA methylation status of *sahh1-1* plants was examined. Using 5S rDNA as a probe, we observed DNA hypomethylation in *sahh1-1* plants after digestion with methylation-sensitive enzymes of *Scr*FI, *Msp*I and *Hpa*II, respectively. Hypomethylation was also observed when the *Msp*I-digested *sahh1-1* DNA was probed against a repeated 180-bp centrometric fragment (Fig. 2f).

To reveal functional redundancy between *AtSAHH1* and *AtSAHH2*, we generated RNA interference (RNAi) lines expressing an inverted repeat derived from a 550-bp fragment of the second exon of *AtSAHH2*, which likely also targets *AtSAHH1* due to a high DNA sequence identity. Of the 21 double-strand RNA (dsRNA) producing lines (*dsAtSAHH2*) obtained, twelve exhibited a bushy phenotype with shorter and much branched stem, dark green and wrinkly leaves, and the rosette leaves were slightly bigger (Fig. 3a). Although the *dsAtSAHH2* and *sahh1-1* plants were similar in many aspects, such as the dark green leaves, the *sahh1-1* mutant plants did not show the bushy phenotype (Fig. 2b). Six T1 plants of each line with moderate to severe phenotypic changes were retained for further analysis. In many cases, introduction of double-stranded RNA molecules into different types of organisms generates 21- to 25-nt fragments which are associated with the degradation of homologous transcripts in vivo (Hammond et al. 2001). By denaturing polyacrylamide gel electrophoresis, we

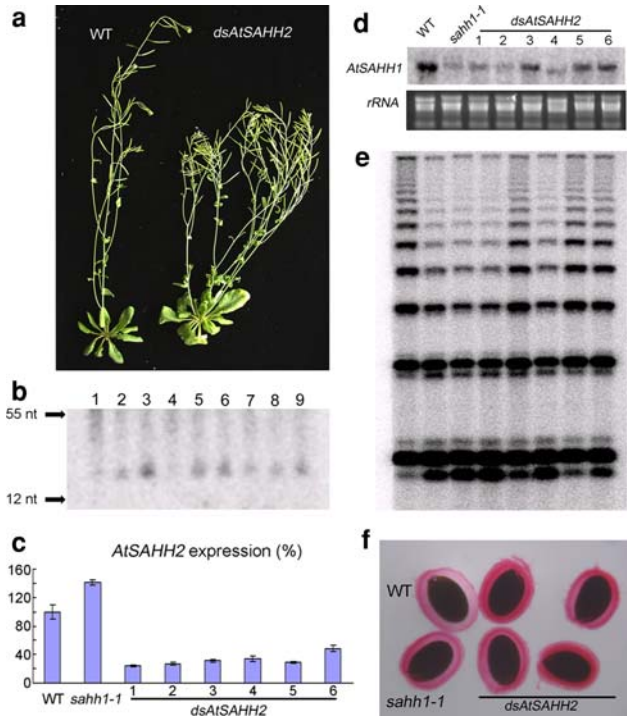

Fig. 3 Analysis of RNAi (*dsAtSAHH2*) plants. **a** Phenotype of the WT and *dsAtSAHH2* plants grown in soil in the greenhouse for 4 weeks. Note the bushy architecture (shorter and much branched stem and increased rosette leaves) of *dsAtSAHH2* plants. **b** Polyacrylamide gel separation of RNAs from different *dsAtSAHH2* lines, showing small RNAs, approximately 21–25 nt, specific to *AtSAHH2*. The 550-bp fragment of the second exon of *AtSAHH2* was used as probe. *Lines (2, 3, 4, 6, 7, 8)* showing differential accumulation of small RNAs were chosen for subsequent analysis (and designated *1, 2, 3, 4, 5, 6* in **c**, **d**, **e**). **c** Real-time RT-PCR analysis of *AtSAHH2* expression in leaves of the 3-week-old plants of the WT, *sahh1-1* and *dsAtSAHH2* lines. Expression of *AtSAHH2* in WT was set as 100%. *Error bars* indicate standard error. **d** RNA-gel blot showing transcript levels of *AtSAHH1* in leaves of the 3-week-old of the WT, *sahh1-1* and *dsAtSAHH2* lines. The specific probe derived from the 3′ UTR of *AtSAHH1* was used to determine the transcript level. **e** Gel blot of *Msp*I digested genomic DNA prepared from the WT, *sahh1-1* and *dsAtSAHH2* lines, after hybridization with the probe of At5S rDNA. Note that the global DNA hypomethylation was correlated with At*SAHH1* gene repression. **f** Ruthenium red (*RR*) staining of seed coat of the WT, *sahh1-1* and *dsAtSAHH2* lines. Note the more intensive RR staining of *sahh1-1* and *dsAtSAHH2* seeds

detected the 21- to 25-nt RNA fragments (Fig. 3b). Due to the low abundance of *AtSAHH2* transcripts in plants, we conducted real-time RT-PCR which showed that *AtSAHH2* expression was reduced by two- to fourfolds (Fig. 3c). Northern blot with a gene-specific 3′-end probe showed that *AtSAHH1* transcript abundance was also decreased in the RNAi lines, comparable to the reduction in the *sahh1-1* mutant (Fig. 3d). As expected, global DNA methylation levels of these transgenic plants with abnormal phenotypes were reduced to a different degree, which was correlated to the decrease of the *SAHH* transcript abundance (Fig. 3d, e). To test whether SAM-dependent methylation was generally

affected in *SAHH*-repressed lines, we also compared the pectin methylation levels in the seed coat of *sahh1-1* and *dsAtSAHH2* lines. When immersed in an aqueous solution of Ruthenium red, both the *sahh1-1* and *dsAtSAHH2* seeds showed more intensively pink-stained capsule than the WT seeds (Fig. 3f), an indication of reduced methylesterified pectin due to down-regulated *SAHH*.

Elevated cytokinin content and response in *SAHH*-deficient plants

Such phenotypes with dark green leaves, delayed flowering, and much branched stems observed on either the *sahh1-1* mutant or the transgenic *dsSAHH2* plants, are reminiscent of cytokinin overproducers (Ori et al. 1999; Catterou et al. 2002; Chang et al. 2003). It was reported that tobacco plants expressing an antisense RNA for *SAHH* contained an excess level of cytokinins (Masuta et al. 1995). To see if *sahh1-1* plants also have an elevated level of cytokinins, we quantitatively analyzed zeatin and isopentenyl adenosine (iPA), two biologically active cytokinins, in roots. As shown in Fig. 4a, the zeatin level was about threefold higher in *sahh1-1* roots than in the WT, and the iPA level was also substantially increased in the mutant. These results confirm that knockdown of *SAHH* gene expression in *Arabidopsis* induces cytokinin accumulation.

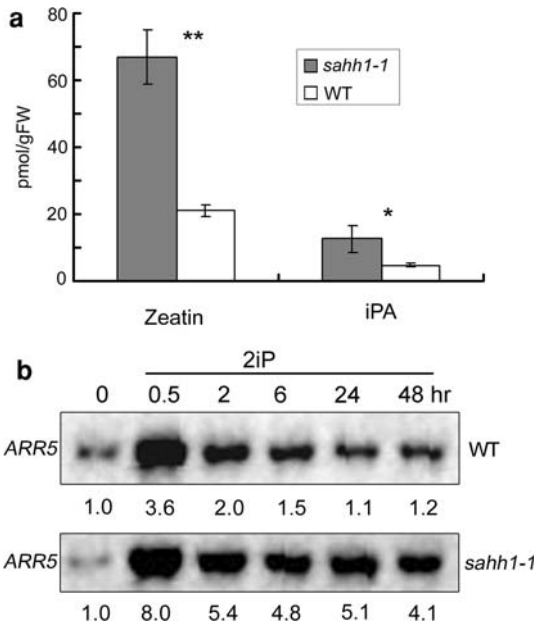

Fig. 4 Analysis of cytokinin contents and *ARR5* expression. **a** Quantitative analysis of cytokinins in roots. Levels of zeatin and isopentenyl adenosine (*iPA*) in 2-week-old roots of the WT and *sahh1-1* seedlings were determined by HPLC. *Asterisks* indicate the statistical difference (* $P < 0.05$; ** $P < 0.01$) by Student's *t* test. **b** Northern blot showing changes of transcription levels of *ARR5* in the WT and *sahh1-1* seedlings in response to the cytokinin 2-isopentenyladenine (2iP, 20 μM). RNA loading was normalized with 25S rRNA

We next performed a root growth assay to examine the sensitivity of *sahh1-1* seedlings to exogenous cytokinins. We found that, after treatment with 2-isopentenyladenine (2iP, 2 μM) for eight days, the WT root growth (elongation) was inhibited by ~60%; when the *sahh-1* roots were assayed, the inhibition rate was increased to ~76% (data not shown). In order to further compare the consequences of *SAHH* repression and the cytokinin overproduction, we generated transgenic pants expressing a bacterial gene encoding adenosine phosphate–isopentenyltransferase (IPT), a key enzyme of cytokinin biosynthesis, under the promoter of a senescence-associated gene *SAG12* (Gan and Amasino 1995). The *proSAG12:IPT* roots also exhibited a higher inhibition rate (~70%) of elongation than the WT in response to 2iP-treatment (data not shown), consistent with previous reports that plants are more sensitive to exogenous cytokinins when endogenous cytokinin levels are increased (Catterou et al. 2002; Yang et al. 2003). Furthermore, the transgenic *proSAG12::IPT* plants were similar to *sahh-1* plants in their dark green leaves and higher contents of leaf chlorophylls (ESM Fig. S3).

Examination of cytokinin response gene revealed that, when the WT seedlings were treated with 2iP, expression level of *ARR5*, a type-A cytokinin response regulator gene, was increasing in 30 min after treatment, and then declined (Fig. 4b). *ARR5* expression level in *sahh1-1* seedlings was at first similarly increased after 2iP treatment, but the elevated level was maintained, rather than quickly declined as in the WT seedlings (Fig. 4b), indicating a prolonged response of *sahh1-1* seedlings to the cytokinin. These results suggest a possibility that cytokinin turnover was affected in the *sahh1-1* mutant relative to the wild type; or alternatively, the cytokinin pathway activation was retained longer when *SAHH* gene was repressed.

Cytokinin induces *SAHH* and *ADK* gene expression

Having demonstrated that down-regulation of *SAHH* promotes cytokinin accumulation, we investigated the effect of cytokinin on transmethylation pathways. We treated the suspension cultured *Arabidopsis* seedlings with different types of cytokinins. The adenine-type cytokinin 2iP was able to induce *AtSAHH1* expression (Fig. 5), the *AtSAHH1* mRNA level increased at around 6 h after treatment. We found that *AtSAHH1* gene expression was also responsive to other purine derivative cytokinins, such as kinetin (KT), 6-benzylaminopurine (6-BA) and zeatin (Fig. 5a). Furthermore, cytokinin was also able to induce the expression of the ADK gene *AtADK1* (At3g09820; Fig. 5a).

To see if the purine structure of cytokinin molecules contributed to *SAHH* gene induction, we tested a synthetic cytokinin, diphenylurea (DPU), which is structurally different from purine derivatives (ESM Fig. S1) but biologically

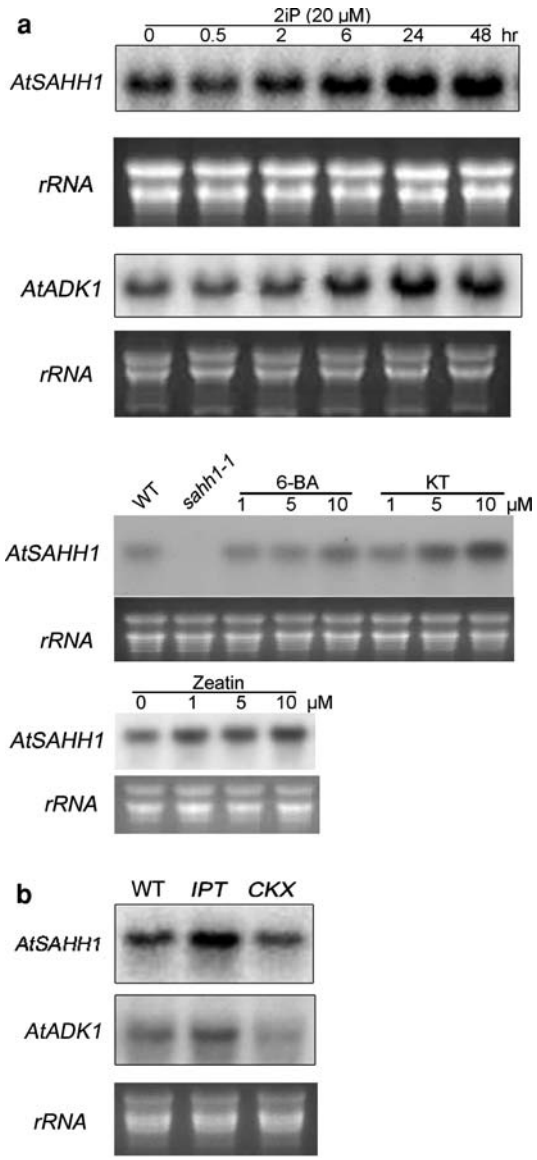

Fig. 5 Induction of *AtSAHH1* and *AtADK1* expression by cytokinins. **a** Transcript levels of *AtSAHH1* and *AtADK1* in suspension cultured seedlings treated with 20 μM 2-isopentenyladenine (*2iP*), or with different concentrations of 6-benzylaminopurine (*6-BA*), kinetin (*KT*) and zeatin, respectively, for 24 h. **b** Transcript levels of *AtADK1* and *AtSAHH1* in leaves of 4-week-old WT plants transformed with *proSAG12::IPT* and *pro35S::CKX2*, respectively

active in assay, though the activity is comparatively low. We found that DPU at 20 μM induced *AtSAHH1* expression (Electronic supplementary material Fig. S4). On the other hand, adenine (a purine) and adenosine (a purine derivative), both lacking cytokinin properties, showed no effect on *AtSAHH1* expression at this concentration (ESM Fig. S4). These data demonstrate that cytokinin-induction of *AtSAHH1* expression requires the cytokinin activity, whereas the purine structure that may bind to SAHH proteins is unlikely involved in the *SAHH* induction pathway.

To further elucidate the effects of endogenous cytokinins on transmethylation pathway, we generated plants overexpressing a cytokinin degradation gene, *CKX2*, which encodes cytokinin oxidase that inactivates cytokinins by removing the isoprenoid side chain from the molecule (Werner et al. 2003). We found that leaf surface area and petiole length of the *pro35S::CKX2* plants, grown in soil in greenhouse for four weeks, were reduced; in addition, root growth of *pro35S::CKX2* seedlings was less sensitive to cytokinin-treatment (data not shown). These data were indicative of a decreased content of cytokinin in *pro35S::CKX2* plants. The *proSAG12::IPT* plants, in which cytokinin biosynthesis was elevated, were also analyzed. In the WT background, *AtSAHH1* gene expression was reduced in *pro35S::CKX2* plants but increased in *proSAG12::IPT* plants; therefore, *AtSAHH1* expression level correlates with the endogenous cytokinin level. Furthermore, expression of *AtADK1* showed a similar change of transcript level in these transgenic plants, though the overall expression level was lower (Fig. 5b). These data uncover a role of cytokinin in regulating the transmethylation cycle at the gene expression level.

Cytokinin has no effect on SAHH enzyme activity

Since both natural cytokinins and SAHH substrates are adenine derivatives, an interesting question is that if cytokinin would affect SAHH catalytic activities. An earlier and preliminary assay of SAHH activity with a fraction of maize soluble proteins showed no influence of cytokinins on the enzyme (Romanov and Dietrich 1995). To clearly elucidate this, we performed a detailed assay with purified SAHH recombinant proteins. First, we examined the activity of catalyzing SAH synthesis by AtSAHH1 in the presence of cytokinin. Adenine was chosen as a positive control as its interaction with and inhibition of SAHH have been well elucidated (Hershfield and Krodich 1978). HPLC showed that adenine at 50 μM inhibited SAH formation by ~45%; at 500 μM the inhibition was close to 80%. When AtSAHH1 was pre-incubated with adenine for 10 min, the inhibition was more evident: at the same concentrations the inhibitions were 56 and 90%, respectively. By contrast, when 2iP or 6-BA, both are adenine-type cytokinins, were added to the reaction, neither affected the formation of SAH, even at a concentration as high as 1 mM (Fig. 6). Pre-incubation of the two cytokinins exerted no effect on SAHH activity either (data not shown). Because SAHH catalyzes reversible hydrolysis of SAH, we also conducted the assay in the hydrolytic direction. Again the SAH hydrolysis was hypersensitive to adenine, but not to 2iP and 6-BA (data not shown). Similar results were obtained for recombinant proteins of AtSAHH2 (data not shown). The enzyme assay data presented here support that, in vitro, cytokinins have no effect on SAHH activity.

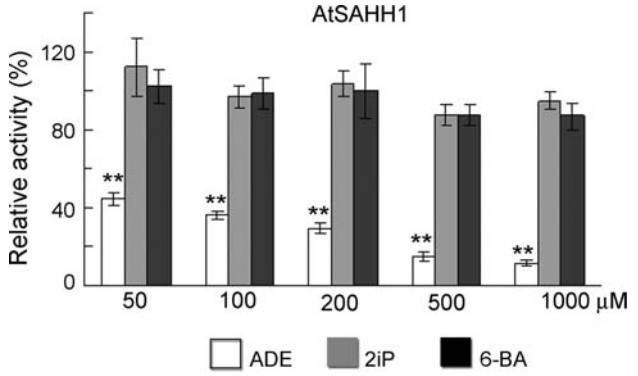

Fig. 6 Effects of adenine and adenine-type cytokinins on SAHH activity. The effects of adenine (*ADE*), 2iP and 6-BA on enzyme activities of recombinant AtSAHH1 proteins were assayed in SAH synthesis direction. The activity without adding adenine-type compounds was set as 100%. *Error bars* indicate standard error. *Asterisks* indicate the statistical difference ($P < 0.01$) by Student's *t* test

Cytokinin promotes DNA methylation

Although cytokinins do not affect SAHH activity in vitro, they stimulate expression of *AtSAHH1* and *AtADK1* genes, both encoding enzymes of the methyl recycling pathway. This suggests a role of cytokinin in up-regulating cellular methylation potential and, consequently, DNA methylation.

Analysis of the DNA methylation status in *pro35S::CKX2* transformants revealed that, in *sahh1-1* background, global DNA methylation was reduced comparing to the untransformed plants; in WT background, however, expression of *pro35S::CKX2* did not result in an obvious change of DNA methylation status (Fig. 7a). We then examined the effect of elevated cytokinin levels on DNA methylation by using *proSAG12::IPT* plants. Again, in WT background the DNA methylation status remained largely unchanged, but in *sahh1-1* background DNA methylation level was increased (Fig. 7a).

Cytosine methylation is fulfilled by specific DNA methyltransferases. We asked if cytokinin affects the expression of cytosine methyltransferase genes. The DNA methyltransferase *MET1* modifies the cytosine methylation status of the CG sites (Finnegan et al. 1996). Real-time RT-PCR showed that expression level of *MET1* in seedlings was increased by 2.6- and 2.8-fold after 2iP-treatment for 24 and 48 h, respectively (Fig. 7b). Expression of two other cytosine methyltransferase genes, *CMT3* and *DRM2*, was also substantially increased in response to cytokinin treatment (Fig. 7b). Both *CMT3* and *DRM2* are responsible for de novo methylation at the asymmetric and CpNpG sites (Cao et al. 2003).

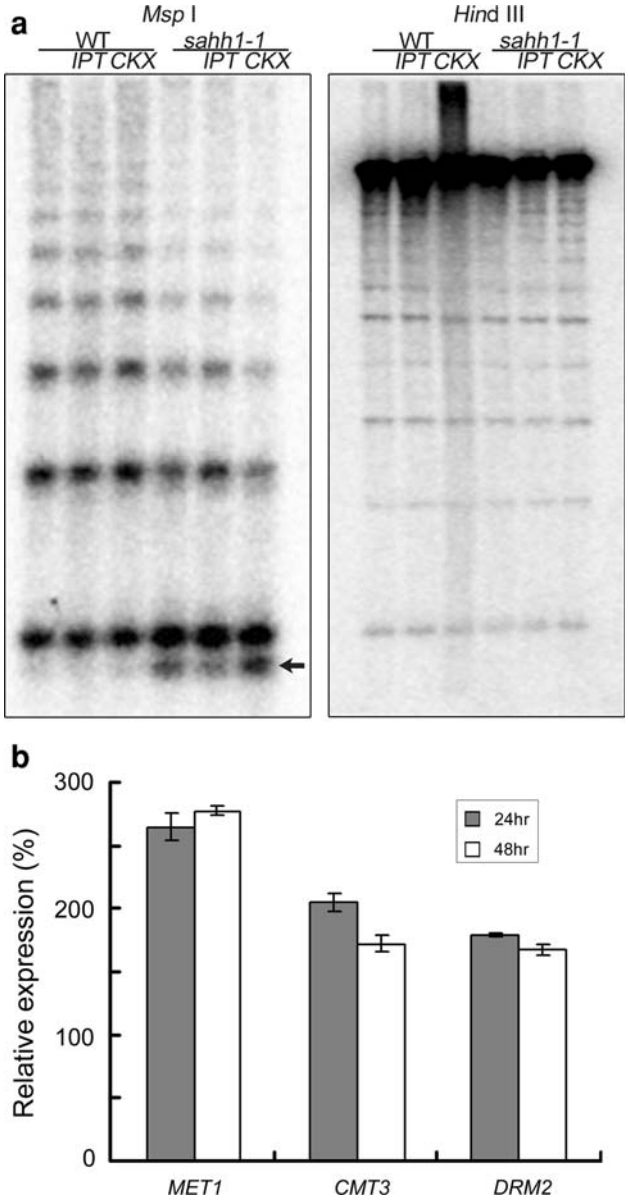

Fig. 7 Effects of cytokinin on DNA methylation. **a** DNA methylation status of plants (Col-0 and *sahh1-1*) transformed with *proSAG12::IPT* (*IPT*) or *pro35S::CKX2* (*CKX*). Genomic DNA from the 4-week-old plants was digested with the methylation-sensitive enzyme *Msp*I and probed against 5S rDNA. Note the increased and reduced DNA methylation levels in *sahh1-1* plants expressing *proSAG12::IPT* (*IPT*) and *pro35S::CKX2* (*CKX*), respectively. *Arrow* notes the differential hybridization signal. As control, the samples were also digested with the methylation-resistant *Hin*dIII and probed against 5s rDNA. **b** Real-time RT-PCR analysis of cytosine DNA methyltransferase genes. Seedlings were treated with 20 µM 2iP for 24 and 48 h, respectively; for each gene, expression level in untreated seedlings was set as 100%. Mean values of triplicate experiments ±SE

Discussion

Phytohormones are important regulators in modulating plant growth and metabolism. It was reported that tobacco SAHH binds to cytokinins, and a decreased amount of SAHH proteins possibly releases the cytokinin and results in increased cytokinin content (Mitsui et al. 1993; Masuta et al. 1995). Our analysis of *Arabidopsis* showed that

endogenous levels of cytokinins (zeatin and iPA) were indeed increased in the *sahh1-1* mutant. However, antisense inhibition of not only *SAHH*, but also *ADK* and *MET1* expressions were associated with similar morphological features, such as dark green leaves and bushy stature (Finnegan et al. 1996; Tanaka et al. 1997; Moffatt et al. 2002). Our data support that suppression of *SAHH* gene expression, and more likely the reduced methylation potential due to perturbation of the SAM-dependent transmethylation cycle, result in enhanced cytokinin content and prolonged pathway activation, and this at least partly accounts for such phenotypic changes as higher chlorophyll content, delayed flowering, retarded root growth and bushy stature. Chlorophyll synthesis requires a methylation step catalyzed by Mg protoporphyrin methyltransferase (MgPMT), a SAM-dependent methyltransferase (Alawady and Grimm 2005). However, chlorophyll contents were increased, rather than decreased, in *sahh1-1* and *dsAtSAHH2* plants. Analysis of barley seedlings showed that cytokinins promote the MgPMT activity (Yaronskaya et al. 2006). SAM is one of the central metabolites in plant metabolism in that it not only donates methyl group for modifications of various metabolites, but also serves as substrate for ethylene and polyamine biosynthesis (Roje 2006). Therefore, suppression of SAM-dependent methyl recycle may also affect these pathways, causing abnormality in plant growth and development.

Unlike adenosine and adenine, cytokinins have no inhibitive effect on SAHH enzyme activity, at least in vitro, suggesting that adding an isopentenyl side chain to the N^6 position of adenine or adenosine abolishes their effect on SAHH. Thus the possibility that cytokinin turnover may affect SAHH activity in vivo could not be excluded. On the other hand, cytokinin positively regulates the expression of methyl recycling pathway genes, such as those coding for SAHH and ADK, promoting transmethylation reactions. Although a firm conclusion awaits further investigation, data presented here suggest a possible feedback-loop of regulation between cytokinin and the transmethylation cycle: reduced transmethylation potential in plant cells leads to an increased level of cytokinin, which in turn stimulates the transmethylation pathway genes, resulting in the maintenance of cellular transmethylation reactions.

Interestingly, cytokinins also stimulate gene expression of the enzymes directly involved in cytosine methylation. The induction of *AtSAHH1*, *AtADK1* and cytosine DNA methyltransferase genes by cytokinin is consistent with a recent report that methyl recycling activities are coordinately regulated (Pereira et al. 2007). Cytosine DNA methyltransferases (MTase) transfer the methyl group from SAM to double-strand DNA. *Arabidopsis* plants with deficiency in MTase show a genome level demethylation of DNA and developmental abnormality (Ronemus et al. 1996; Bartee and Bender 2001; Xiao et al. 2006). DNA methylation level was found higher in the adult than in the juvenile and juvenile-like meristems; and at the same time, a wide-spread and stronger signal of zeatin was detected in adult meristems by immunolocalization (Bitonti et al. 2002). In this investigation, we provide evidence showing that cytokinin plays a role in promoting plant DNA methylation. In *sahh1-1* plants, in which the cellular transmethylation potential was reduced, manipulating endogenous cytokinin levels by transgene expression induced a measurable effect on global DNA methylation status: DNA methylation status increased with the level of cytokinin. Furthermore, the induction of cytosine DNA methyltransferase genes by cytokinin suggests that this promoting role is general. However, similar alterations of cytokinin levels did not result in marked changes of DNA methylation in WT *Arabidopsis* plants. A plausible explanation is that DAN hypermethylation status of WT plants is prevalent, further increase of DNA methylation cannot be achieved just by elevated levels of DNA methyltransferases. Another possibility is that SAHH activity was affected due to a reduction of SAM/SAH ratio in *sahh1-1* plants, thus an elevation of SAHH level has a more profound effect on DNA methylation in the *sahh1-1* than in the WT plants. Furthermore, not only SAHH but also ADK, which removes SAH, are inducible by cytokinins, thus the phytohormone provides a condition for elevating the transmethylation potential; in the WT *Arabidopsis* plants, on the other hand, the *AtSAHH1* expression is high and there can an abundant supply of the methyl group. Recent genome-wide analyses revealed that over one-third of *Arabidopsis* genes are methylated (Zhang et al. 2006; Zilberman et al. 2007). It would be interesting to examine the effect of cytokinins on methylation status of specific genes in relation to various nutrient and other environmental conditions. In summary, the phytohormone cytokinin participates in regulating global DNA methylation, particularly under transmethylation stringent conditions. This provides a new insight into the role of phytohormones in plant metabolism and epigenetics.

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