**Trans-methylation reactions in plants: focus on the activated methyl cycle**

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**Abstract**

Trans-methylation reactions are vital in basic metabolism, epigenetic regulation, RNA metabolism, and post-translational control of protein function, and therefore fundamental in determining physiological processes in all living organisms. The plant kingdom is additionally characterized by production of secondary metabolites that undergo specific hydroxylation, oxidation and methylation reactions to obtain a wide array of different chemical structures. Increasing research efforts have started to reveal the enzymatic pathways underlying the biosynthesis of complex metabolites in plants. Further engineering of these enzymatic machineries offers significant possibilities in the development of bio-based technologies, but necessitates deep understanding of their potential metabolic and regulatory interactions. Trans-methylation reactions are tightly coupled with the so-called activated methyl cycle, an essential metabolic circuit that maintains the trans-methylation capacity in all living cells. Tight regulation of the activated methyl cycle is crucial in ensuring accurate trans-methylation reactions in different sub-cellular compartments, cell types, developmental stages and environmental conditions. This review addresses the organization and post-translational regulation of the activated methyl cycle and elaborates its critical role in determining metabolic regulation through modulation of methyl utilization in stress-exposed plants.

**Abbreviations**

4MO-I3M, 4-methoxy-indol-3-yl-methyl glucosinolate; ADK, adenosine kinase; AMC, activated methyl cycle; AMP, adenosine monophosphate; BiFC, biomolecular fluorescence complementation; BSMT1, salicylate/benzoate carboxyl methyltransferase 1; CDMS, cobalamin-dependent methionine synthase; CIMS, cobalamin-independent methionine synthase; CMT3, chromomethylase 3; CN-PAGE, clear native polyacrylamide gel electrophoresis; CPK28, calcium-dependent protein kinase 28; FER, feronia; GSNO, nitrosogluathione; H3 K9 MT SUVH4, histone 3 Lys9 methyltransferase Su(var)3-9 homologue 4; HCy, homocysteine; HOG1, homology-dependent gene silencing 1; IGMTs, indole glucosinolate methyltransferases; JMT, jasmonic acid carboxyl methyltransferase; MAT, adenosyltransferase; MeJA, methyl jasmonate; MeSA, methyl salicylate; Me-THF, methyl tetrahydrofolate; MPK, MAP protein kinase; MS3, methionine synthase 3; MT, methyltransferase; NO, nitric oxide; NPR1, nonexpressor of PR genes 1; PP2A-B'γ, phosphatase 2A regulatory subunit B'γ; PTM, post-translational modification; RBOHD, respiratory burst oxidase homologue D; ROS, reactive oxygen species; SAH, S-adenosyl homocysteine; SAHH, S-adenosyl homocysteine hydrolase; SAM, S-adenosyl-Lmethionine; SAMS or SAM, SAM-synthase; SAMCs, S-adenosyl-L-methionine carriers; TRX-H, thioredoxin H-type

## Introduction

Plants produce a plethora of natural compounds with significant health-promoting, medicinal and sensory properties potentially applicable for the benefit of mankind. Many of such plant-derived metabolites have originally evolved to defend the plant from herbivory or disease, or to attract beneficial interactors, such as pollinators. The complex chemistry of plant-derived secondary metabolites typically stems from specific functional group substitutions, such as hydroxylation, oxidation and methylation, which are catalyzed by specialised enzymes in living cells, and may be difficult to reproduce *in vitro*. Therefore, harnessing the photosynthetic production potential for industrial-scale production of value-added compounds in plants offers significant possibilities in the development of bio-based technologies.

By now, increasing research efforts have started to reveal the enzymatic pathways underlying the biosynthesis of complex metabolites in plants (Sonderby et al 2010, O'Connor 2015). Biotechnological production of these compounds will become profitable when the compound has high economical or societal value, the host plant is difficult to harvest from its natural habitat, the compound is present in very low concentrations, or its chemical synthesis is too complicated or costly. Utilization of plant-based production platforms is however currently limited by poor characterization of potential cost-efficient host plants, lack of standardized “bio-bricks” for optimal gene expression, and incomplete understanding of plant metabolic networks and their regulation. Therefore, identification of potentially profitable biosynthetic pathways and understanding their regulation represent major research opportunities in modern plant biology. On the other hand, understanding how signals arising from recognition of pathogen infection or insect infestation become translated into appropriate metabolic adjustments will aid the elucidation and fortification of plant resistance to disease and thereby mitigate losses in crop yield. Trans-methylation reactions are crucial in diverse metabolic and regulatory interactions, and the importance of mechanisms that control the utilization of methyl groups in different cellular processes is therefore evident. This review focuses on the activated methyl cycle and its essential function in the maintenance of trans-methylation potential in plant cells. Special emphasis is paid for post-translational regulation of the associated enzymes and their far-reaching effects on biotic stress resistance in plants.

## The activated methyl cycle maintains trans-methylation potential in all living cells

Trans-methylation reactions are central in biosynthetic and regulatory processes involved in primary and secondary metabolism, epigenetic regulation, RNA metabolism, and post-translational control of protein

function, and therefore vital in determining developmental programmes, physiological processes, stress tolerance and survival in all living organisms (Kim and Zilberman 2014, Kryukov et al. 2014). The vast range of trans-methylation reactions is driven by substrate specific methyltransferases (MT) that utilize S-adenosyl-Lmethionine (SAM) as a methyl donor to incorporate methyl groups into nucleophilic O, N, C or S atoms on specific target molecules, and are divided to O-MTs, N-MTs, C-MTs and S-MT, respectively (Moffatt and Weretilnyk 2001). The high specificity of the trans-methylation reactions is reflected by the high number of enzymes annotated as methyltransferases: the genome of *Arabidopsis thaliana* (hereafter Arabidopsis), for example, encodes two large protein families comprised of N-MTs and O-MTs and two smaller families comprised of S-MTs and C-MTs with 186, 124, 23 and 5 members annotated to date, respectively (http://www.uniprot.org/).

After every trans-methylation reaction, SAM-dependent methyltransferases are inhibited by the reaction by-product S-adenosyl-homocysteine (SAH). To avoid this inhibitory effect, the methyltransferases rely on the so-called activated methyl cycle (AMC; Fig. 1), which is an essential metabolic circuit that recycles the inhibitory reaction product SAH and ensures efficient regeneration of SAM, thereby re-activating the MTs and maintaining the trans-methylation capacity in all living cells. In more detail, after a trans-methylation reaction has taken place, SAH becomes rapidly hydrolyzed by S-adenosylhomocysteine hydrolase (SAHH), one of the most conserved enzymes across kingdoms (Kusakabe et al. 2015). In different plant, mammalian and bacterial species, SAHH has been found either in a dimeric, tetrameric or hexameric conformation (Shimizu et al.1984, Brzezinski et al. 2012, Kusakabe et al. 2015). Each SAHH monomer is composed of a substrate binding domain and a cofactor binding domain, where a $NAD^+$ molecule is non-covalently bound. The two domains are separated by a hinge region containing a binding site for cation which rigidifies the hinge structure (Brzezinski et al. 2012, Kusakabe et al. 2015, Manszewski et al. 2017).

The reaction catalysed by SAHH gives rise to homocysteine (HCy) and adenosine, the latter of which is converted to adenosine monophosphate (AMP) using ATP in a reaction catalyzed by adenosine kinase (ADK; Fig. 1). HCy in turn is converted back to methionine by cytosolic cobalamine-independent methionine synthase (CIMS) that utilizes methyl tetrahydrofolate (Me-THF) as a methyl donor. In contrast to mammals that possess cobalamin-dependent methionine synthase (CDMS), plants and yeast possess an evolutionarily unrelated cobalamin-independent methionine synthase (Pejchal and Ludwig 2005), while most bacteria possess both the cobalamin dependent and independent forms of the enzyme (Pejchal and Ludwig 2005). Both CDMS and CIMS utilize zinc as a cofactor. CIMS is formed by two homologous domains resulting from a gene duplication, after which the catalytic activity was lost in the N-terminal domain of the enzyme (Pejchal and Ludwig 2005). As the last step of the activated methyl cycle, SAM is

regenerated from methionine and ATP by SAM-synthase (SAMS or SAM), also called methionine adenosyltransferases (MATs). The centrality of the consecutive enzymatic reactions catalyzed by the MTs, SAHH, CIMS and SAMS/MAT can be exemplified by *in vivo* flux measurements, which have indicated that the synthesis and turnover of SAM can account for up to 80% of methionine related metabolism (Giovanelli et al. 1985). After ATP, SAM is the second most used enzyme substrate (Palmieri et al 2006), indicating that the activated methyl cycle is a major contributor in various biosynthetic and regulatory pathways in plant cells.

**The activated methyl cycle is a key contributor to DNA and RNA methylation in the nucleus**

In Arabidopsis, SAHH, CIMS and SAMS/MAT have different isoforms that, based on mutant phenotypes, appear to mediate only partially overlapping functions (Table 1): SAHH has two isoforms, CIMS has three isoforms, one of which is the chloroplastic methionine synthase 3 (MS3), and SAMS/MAT has four isoforms. Moreover, SAM-dependent trans-methylation reactions occur in multiple sub-cellular compartments, including the cytosol, nucleus and organelles. Studies utilizing Arabidopsis mutant lines available for the individual AMC components have significantly forwarded our understanding of their metabolic and regulatory interactions (Table 1). Using GFP fusions, it has been demonstrated that SAHH (Lee et al. 2012), CIMS (Ravanel et al. 2004) and SAMS/MAT isoforms (Mao et al. 2015) are all present in the cytosol as well as in the nucleus, suggesting that these cellular compartments possess a fully functional activated methyl cycle.

Protein interactions between SAHH1 and nuclear-localized methyltransferases appear to form an important layer in the regulation of transmethylation reactions. Lee et al. (2012) demonstrated that SAHH1 interacts with ADK1 and mRNA cap MT, which is required for efficient protein translation but also promotes the splicing, polyadenylation and nuclear export of mRNA. Using Bimolecular Fluorescence Complementation (BiFC) assays and structural modeling, a specific domain in the sequence of SAHH1 was shown to mediate the nuclear localisation of SAHH as well as its interactions between ADK1 and the mRNA cap MT (Lee et al. 2012). It seems plausible that the close interaction between the MTs and SAHH facilitates the immediate hydrolyses of the inhibitory reaction product HCy, and thereby maintains functionality of the MTs. Thus, the local availability of SAM and the hydrolysis of SAH *in vivo* may significantly differ from the overall methylation capacity of the entire cell.

In the nucleus, methylation of DNA and histones forms the basis for epigenetic regulation and several studies have connected the dysfunction of SAHH with hypomethylation of DNA (Rocha et al. 2005, Mull et al. 2006, Ouyang et al. 2012). Arabidopsis SAHH1 was first identified as HOMOLOGY-DEPENDENT GENE SILENCING1 (HOG1) and demonstrated to be vital in epigenetic regulation through the maintenance of trans-methylation capacity in the nucleus (Rocha et al. 2005; Table 1). Three distinct *hog1* mutants with point mutations (G386E, T414I or D444N) at the 3' end of SAHH1 displayed reduced SAH hydrolase activity with a consequent genome-wide demethylation and severe developmental defects, such as delayed germination, slow growth and reduced fertility (Rocha et al. 2005). In a *sahh1* L459F mutant carrying a point mutation at leucine 459, in contrast, DNA methylation was only specifically lost at non-CG sequences that are under the control of two distinct methyltransferases, the histone 3 Lys9 methyltransferase SU(VAR)3-9 HOMOLOGUE 4 (H3 K9 MT SUVH4) and the DNA cytosine-5-methyltransferase CHROMOMETHYLASE 3 (CMT3). Intriguingly, *sahh1* L459F mutants do not show any of the morphological phenotypes typical of *hog1*. These findings suggest that the leusine 459 in SAHH1 may be specifically needed for interaction with H3 K9 MT SUVH4 and/or CMT3, and that the joint function of these MTs does not significantly contribute to developmental processes in Arabidopsis.

Ouyang et al. (2012) analyzed *sahh1* knock-down mutants (Table 1) and showed that reduced abundance of SAHH1, and the presumable accumulation of SAH, caused DNA hypomethylation that was accompanied by extensive transcriptional up-regulation of transposable elements. This suggested that the reduced activity of SAHH released methylation-dependent DNA silencing, which acts as a defense mechanism against foreign DNA in plants. The activated methyl cycle also facilitates post-transcriptional gene silencing (PTGS), which in turn is a sequence-specific mechanism for degradation of viral RNA and provides the main defence pathway against viral infection in plants. Plant viruses may however evade such antiviral RNA silencing by targeting and manipulating the function of SAHH and SAMS (Cañizares et al. 2016). On the other hand, DNA has been found to undergo demethylation during defence activation (Pavet et al 2006, Trotta et al. 2011), likely to facilitate the expression of immunity related genes by mediating decondensation of the chromatin structure. Interestingly, the knock-down of SAHH1 affected the transcript abundance of only a few MTs, indicating that the activated methyl cycle does not have a major feedback role in the specific transcriptional regulation of MTs (Ouyang et al. 2012).

**The activated methyl cycle contributes to plant metabolism through versatile protein interactions**

Tight regulation of methyl-transfer and the regeneration of SAM through the activated methyl cycle are crucial in ensuring accurate metabolic and regulatory reactions in different cell types, developmental stages and environmental conditions. In chloroplasts, the well-known sinks for SAM-dependent methylation include DNA, RNA and the key photosynthetic enzyme Rubisco, as well as biosynthesis of photosynthesis-associated small molecules, such as chlorophyll, tocopherol and plastoquinone (Dharlingue and Camara 1985, Grimm et al. 1997, Cheng et al. 2003,). Similarly, SAM is known to be consumed in the regulation of gene expression and biosynthesis of biotin in mitochondria (Picciocchi et el. 2001). However, components of the activated methyl cycle have so far not been detected in mitochondria or plastids. Instead, these organelles rely on SAM transported from the cytosol by S-ADENOSYLMETHIONINE CARRIERs (SAMCs) that import SAM in exchange for SAH, which becomes enzymatically hydrolyzed by cytosol-resident SAHH, presumably in the vicinity of the organellar outer membrane (Bouvier et al 2006, Palmieri, et al. 2006). In Arabidopsis, SAMC1 and 2 are dually localized to chloroplastic and mitochondrial membranes (Bouvier et al. 2006, Palmieri et al. 2006). How the organellar MTs operate in isolation of the activated methyl cycle has not been revealed and remains to be established.

In rapidly growing healthy tissues, basic metabolism and various anabolic processes, such as the biosynthesis of pectins and monolignols upon the formation of cell wall, form major sinks for methyl groups (Zhong et al. 1998, Miao et al. 2011,Kim et al. 2015). Under abiotic stress conditions, SAM becomes channeled for biosynthesis of various protective substances, including complex phenolic compounds, which can protect the plant against photo-oxidative damage and have remarkable antioxidant activities in human diet. Activation of defensive measures under biotic challenges in turn consumes substantial amounts of SAM for methylation of defence compounds, such as glucosinolates, which comprise a major group of sulphur-rich chemical defences in cruciferous plants (Ranocha et al. 2001, Sonderby et al. 2010).

The activated methyl cycle is an important player also in the hormonal regulation of plant defense, since methylation of salicylic acid and jasmonic acid to methyl salicylate (MeSA) and methyl jasmonate (MeJA) has significant signaling functions in pathogen-infected plants. MeSa has been proposed to work as a systemic signal that is produced at the infection site by SALICYLATE/BENZOATE CARBOXYL METHYLTRANSFERASE 1 (BSMT1) and transduced to systemic tissues, where it is converted back to SA to induce defences in non-infected parts of the plant (Park et al 2007, Liu et al. 2010). MeJA in turn is synthetized by JASMONIC ACID CARBOXYL METHYLTRANSFERASE (JMT), whose over-expression has been shown to increase resistance to *Botrytis cinerea* infection in Arabidopsis (Seo et al.

2001). Moreover, SAM acts as a precursor for the biosynthesis of polyamines and the plant hormone ethylene, hence forming a metabolic point of cross-talk with the associated methionine salvage pathway, also known as the Yang cycle. For a more in-depth discussion on these AMC-associated metabolic circuits we refer the readers to an excellent recent review (Moffatt and Weretilnyk 2001, Sauter et al. 2013).

How the channeling of SAM to various metabolic processes in different cellular compartments is regulated remains elusive. It has been suggested that the so-called methylation index (SAM/SAH ratio) would regulate the trans-methylation reactions according to the MTs affinity to SAM and the inhibitory reaction product SAH (Cantoni 1975, Moffatt and Weretilnyk 2001). Moreover, the AMC and MTs are transcriptionally and post-translationally tightly regulated (Elmore eta l. 2012, Winterberg et al. 2014, Xu et al.2016) and more recent studies suggest an additional level of regulation through protein complex formation of AMC enzymes (Lee et al. 2012; Rahikainen et al. 2017).

The activated methyl cycle is tightly interlinked with cellular metabolism and may form different oligomeric complexes that facilitate the various methylation processes. In mammals, the active form of SAHH is a tetramer (Hu et al. 1999, Yang et al. 2003, Ishihara et al. 2010), while a crystal structure of SAHH in *Lupinus luteus* suggested that in higher plants the enzyme could be active as a dimer, whose molecular mass would correspond to 110 kDa (Brzezinski et al. 2012). In Arabidopsis leaves, however, SAHH is predominantly present in an oligomeric protein complex of an approximate molecular weight of 200 kDa, but whose subunit composition remains unresolved (Rahikainen et al. 2017). BiFC assays provided evidence indicating that SAHH1 can interact with CIMS1 and INDOLE GLUCOSINOLATE METHYLTRANSFERASEs (IGMTs) in the cytoplasm. However, SAHH1 and CIMS1 did not co-migrate in clear native (CN) gels (Rahikainen et al. 2017), suggesting that their potential *in vivo* interaction might be highly transient in nature. CIMS1 in turn could be detected in a protein complex that also contained METHYLTETRAHYDROFOLATE REDUCTASE (MTHFR), the enzyme that produces the methyl donor for CIMS, suggesting existence of an oligomeric protein complex that directly connects the activated methyl cycle with folate metabolism. Such compartmentalization within an oligomeric enzyme complex presumably ensures efficient supply of THF required for synthesis of methionine by CIMS activity (Rahikainen et al. 2017).

**Post-translational regulation of the activated methyl cycle**

Post-translational modifications (PTMs) provide efficient means for the regulation of enzyme activity, localization, turn-over and interactions with other macromolecules, and allow concomitant integration of

internal and external signals to ensure dynamic regulation of metabolic processes. As a central metabolic circuit, AMC has to promptly respond to environmental signals and changes in the cell physiology. It is therefore not surprising that different isoforms of the AMC enzymes have been identified as targets for various PTMs, including phosphorylation, nitrosylation, acetylation, thiol redox regulation and ubiquitinylation (Table 2), but only few of them have a characterized biological role. Hence, understanding whether protein-protein interactions among AMC enzymes and methyltransferases or the subcellular localization of the protein complexes is regulated by PTMs still requires further investigation.

Reversible phosphorylation by opposing actions of protein kinases and protein phosphatases is a universal mechanism of cellular regulation and the AMC components are known to undergo phosphorylation at multiple sites (Table 2). Nine phosphorylation sites, S9, S10, S20, T113, S203, T206, T207, S236 and T370, are found in peptides present in both SAHH1 and 2. Additionally, one unique phosphorylation site, T44, has been detected for SAHH1 and two unique phosphorylation sites, T214 and T219, have been observed in SAHH2. SAHH shows an interesting pattern of phosphorylation with two evolutionarily conserved phosphosites (S203 and S236) (Corrales et al. 2013) potentially forming an on/off switch in the active centre of the enzyme. In addition to these, also T206 and T207 reside close to the substrate and cofactor binding sites and may affect the enzymatic activity of the protein. Phosphorylation of distinct sites, namely S9, S10, S20, T44, T113, Y214, T219 and T370, on the surface of SAHH isoforms could mediate important regulatory functions determining e.g. protein-protein interactions and/or the subcellular localisation of the enzymes. Whether the unique sites of SAHH1 and SAHH2 phosphorylation reflect differential functional roles of the two isoforms remains to be established.

CIMS1 displays seven unique phosphorylated serine residues, S38, S43, S50, S349, S459, S707 and S759. Of these, S459, S707 and S759 reside in the catalytically active domain of the enzyme, and S707 is conserved in yeast, bacteria and Arabidopsis (Pejchal and Ludwig 2005). In contrast, CIMS2 carries only one unique phosphorylation site at S29. In addition, there are six identified phosphosites, S3, S23, S103, S408, S681 and T741, in peptides identical in CIMS1 and CIMS2, and another two, Y698 and S702, in phosphopeptides identical between CIMS1, CIMS2 and the chloroplast methionine synthase MS3. In SAMS/MAT, two phosphorylation sites, S100 and S296, are located in peptides present in SAMS1/MAT1 and SAMS2/MAT2, one phosphorylation site, S236, is found in a peptide shared by SAMS2/MAT2 and SAMS2/MAT3, and one phosphorylation site, S235, lies in a peptide conserved in all four Arabidopsis isoforms (Table 2).

The identities of the protein kinases and phosphatases responsible for reversible phosphorylation of SAHH, CIMS and/or SAMS/MAT remain largely unknown. However, SAHH1 and CIMS1 were recently shown to physically interact with a specific protein phosphatase 2A regulatory subunit B'γ (PP2A-B'γ) (Rahikainen et al. 2017). Associated with this, PP2A-B'γ was shown to modulate the abundance of SAHH1 and CIMS1 containing complexes and impact the cellular trans-methylation capacity in Arabidopsis leaves (Rahikainen et al. 2017). This is intriguing, given that the catalytic PP2A subunits are also themselves targets for regulation by reversible methylation (Wu et al. 2011). Even if the actual regulatory mechanisms and the potential PP2A-regulated phosphorylation sites remain to be established, the involvement of PP2A-B'γ in the regulation of the activated methyl cycle highlights the importance of post-translational regulation in the coordination of methyl utilization in plant metabolic networks.

Of the activated methyl cycle enzymes, SAMS/MAT is directly responsible for the supply of SAM, and the different SAMS/MAT isoforms should therefore be able to dynamically respond to the prevailing demand of SAM in different cellular compartments. Several studies have connected defects in SAMS/MAT regulation with ethylene biosynthesis or signaling (Lindermayr et al. 2006, Mao et al. 2015, Jin et al. 2017), while the trans-methylation processes that also largely depend on tightly regulated SAMS activity have drawn less attention. Despite of the central role of SAMS/MAT, evidence for its post-translational regulation and protein interactions in plants has been reported only recently (Mao et al. 2015, Jin et al. 2017).

SAMS/MAT isoforms may be targets of plasma membrane (PM) receptor signaling (Mao et al. 2015). This is an intriguing option as it would allow a fast and direct way to regulate the availability of SAM in response to developmental signals or external stimuli. SAM1/MAT1 and SAM2/MAT2 were reported to interact with the plasma membrane receptor-like kinase (RLK) FERONIA (FER), which mediates diverse functions in plant growth, hormonal signaling and immunity (Mao et al. 2015). Direct evidence of SAMS/MAT phosphorylation by FER is still missing, but the cytoplasmic FER kinase domain is a putative regulator for the two SAMS/MAT isoforms (Mao et al. 2015). Arabidopsis *fer* mutants show enhanced usage of methionine and elevated contents of SAM and ethylene as compared to wild type plants, suggesting that FER negatively impacts the SAMS/MAT isoforms. FER has a crucial role in ovule fertilization, but exerts pleiotropic effects on multiple hormonal pathways. Moreover, it has been suggested to act as a sensor for cell-wall perturbations in development and stress. However, the specific signals that act upstream of the FER-SAMS/MAT interaction, and their possible contribution to the regulation of cell metabolism, remains elusive.

In addition to possible regulation by FERONIA RLK, molecular and biochemical approaches identified SAMS/MAT proteins as targets for calcium-dependent protein kinase 28 (CPK28) mediated phosphorylation (Jin et al. 2017). CPK28 regulates early pathogen associated molecular pattern (PAMP) triggered immunity by phosphorylating BOTRYTIS-INDUCED KINASE1 (BIK1) and promoting its degradation (Monaghan et al. 2014). BIK1 is a substrate for multiple PM immune receptor complexes and its regulated turnover enables the attenuation and fine tuning of PAMP triggered signaling. Notably, the regulation of AMC poses another route of regulation of plant immunity for CPK28. In CPK28-OE plants SAMSs/MATs are degraded through the 26S proteasome pathway, while in *cpk28* mutants and MAT2/SAM2-OE plants the stabilization and consequent accumulation of SAMS/MAT resulted in formation of short hypocotyls and ectopic lignification (Jin et al. 2017). These findings underline that the maintenance of an appropriate SAM content is an important player in ethylene signaling, but also in cell wall lignification, where SAM-dependent methyltransferase reactions occur upon the biosynthesis of coniferyl and sinapyl alcohols. The potential effects of SAMS/MAT regulation by CPK28 on other aspects of plant immunity, such as biosynthesis of methylated defence compounds remain to be investigated.

In addition to post-translational regulation by phosphorylation, it seems likely that CIMS is redox regulated through its redox-active cysteine residues. In *Escherichia coli*, the cobalamin-independent methionine synthase MetE becomes inactivated under oxidative conditions and a similar effect can be attained by mutating the cysteine residue C645, which lies close to the zinc binding site (Hondorp and Matthews 2009). In Arabidopsis, CIMS is a putative target of cytoplasmic thioredoxin (TRX-H) and Yamazaki et al. (2004) suggested that the thioredoxin regulates the redox state of the two cysteines and one histidine that bind the zinc ion needed for the enzymatic CIMS activity. It seems plausible that the regulation of AMC in connected to the cell redox balance since redox signaling is one of the earliest responses to various environmental stress conditions, including pathogen infections, where the prompt metabolic adjustments and production of specific functional metabolites are needed.

Another redox-related modification regulating protein function is the reversible S-nitrosylation by nitric oxide (NO) or nitrosoglutathione (GSNO). S-nitrosylation has been reported in the regulation cell redox signaling, where classical examples include ROS scavenging enzymes (Begara-Morales et al. 2015) and the respiratory burst oxidase homologue RBOHD (Yun et al. 2011) as well as the salicylic acid responsive transcriptional regulator NONEXPRESSER OF PR GENES 1 (NPR1) (Tada et al. 2009). Therefore, it is intriguing that three ACM enzymes, SAHH, CIMS and SAMS/MAT, are also putative targets of NO signaling and have been shown to be S-nitrosylated in the presence of GSNO in Arabidopsis cell extracts (Lindermayr et al. 2005) (Table 2). Lindermayr and co-workers reported that SAMS1/MAT1, but not

SAM2/MAT2 or SAM4/MAT3, is regulated by reversible S-nitrosylation. SAMS1/MAT1 undergoes S-nitrosylation at Cys114 *in vitro* in the presence of GSNO, and this PTM inhibits its catalytic activity (Lindermayr et al. 2006). Cys114 residue is not conserved among other Arabidopsis SAMS/MAT homologues, but lies close to the catalytic site of the enzyme and in a sequence motif promoting the nitrosylation by NO (Lindermayr et al. 2006).

SAHH1 and SAHH2 also share a conserved N-terminal sequence that has been found to undergo acetylation after removal of the N-terminal methionine (Xu et al. 2017, Table 2). Similarly, CIMS1 and CIMS2 share a peptide that has been found to be N-terminally acetylated (Table 2). Moreover, a common site in CIMS1 and CIMS2 has been identified as a target for ubiquitinylation (Table 2, Saracco et al. 2009). The importance of N-terminal acetylation still remains elusive, despite the fact that more than 80% of plant proteins undergo N-terminal acetylation (Gibbs 2015). One of the latest breakthroughs in the field suggests, however, that in yeast and mammals N-terminal acetylation can target proteins for degradation via a novel branch of the N-end rule pathway (Gibbs 2015). N-terminally acetylated proteins can be targeted for degradation by the so-called N-end rule pathway, although the proteolysis ultimately depends on the exposure of the N-terminus, occurring, for example, as a consequence of protein misfolding or disruption of protein complexes (Shemorry et al. 2013). Recent studies have linked N-terminal acetylation to abiotic stress-tolerance and immunity in plants, highlighting the relevance of this PTM for plant plasticity under stress conditions and suggesting a more dynamic role for it than previously thought (Linster et al. 2015). Moreover, N-terminal acetylation has been reported to be involved in the regulation of protein-protein interactions, protein localization, folding and degradation (Aksnes et al. 2016). The localization of the N-terminus of SAHH at the surface of the protein could point to a role of N-terminal acetylation as a mediator in any of these processes.

**The role of the activated methyl cycle in defence-related glucosinolate metabolism in Arabidopsis**

A significant demand for SAM dependent methylation is formed by stress-induced biosynthesis of defense and stress metabolites. It is therefore crucial for the cell to be able to increase or decrease its methylation capacity in different cellular compartments upon environmental challenges. The methyl cycle enzymes are upregulated both transcriptionally and translationally upon plant defenses towards oomycetes, necrotrophic fungi and bacterial pathogens (Kusnierczyk et al. 2007, Elmore et al. 2012, Arasimowicz-Jelonek et al. 2013, Winterberg et al. 2014, Li et al. 2015). Accordingly, the abundance of the AMC intermediates is increased.

In different plant species, the AMC supports the biosynthesis of species-specific methoxylated secondary metabolites, which deter biotic stress agents, such as pathogens and aphids (De vos et al. 2007 and 2009, Pfalz et al. 2009). Upon tissue damage by pathogens or herbivores, glucosinolates are hydrolysed by myrosinases to thiocyanates, iso-thioyanates, and nitriles with pathogen deterring effects, which are specific according to the modifications of the basic glucosinolates structure, like methoxylation (Burow and Wittstock 2009). In species of the order *Brassicales*, the defence induced biosynthesis of methionine-derived aliphatic glucosinolates and methylation of tryptophan-derived indole glucosinolates forms a tight connection with methionine metabolism and the AMC, respectively (Hirai 2007, Gigolashvili 2007, Rahikainen et al. 2017). Interestingly, knock-out of the transcription factor MYB28, required for *de novo* synthesis of methionine and consequently of aliphatic glucosinolates, perturbs components of the AMC as well, hinting to a possible role of the AMC on the methionine pool utilized by the aliphatic glucosinolate biosynthetic pathway (Hirai 2007).

An increasing number of different glucosinolate species have been characterized from Arabidopsis ecotypes and a substantial research effort has led to identification of the enzymatic machineries involved in the biosynthesis of the core structure, oxidation, hydroxylation and methylation, as well as degradation and transport of glucosinolates (Sonderby et al. 2010, Nour-Eldin et al. 2012). Owing to such deep understanding, glucosinolate biosynthesis has become a model for studies on synthetic plant biology (Kliebenstein and Osbourn 2012). In response to biotic stress, such as infestation by green peach aphid or the necrotrophic fungal pathogen *Botrytis cinerea*, indole glucosinolates undergo trans-methylation reactions, and the resulting methylated glucosinolates, particularly 4-methoxy-indol-3-yl-methyl glucosinolate (4MO-I3M) and its degradation products are vital in deterring microbial pathogens and insect herbivores (De vos 2007 and 2009, Bednaker et al. 2009, Clay et al. 2009, Xu et al. 2016).

Transcription has been recognized as a key level of regulation in the synthesis and methylation of different glucosinolates. Since specific SAM-dependent methyltransferases undergo stress-inducible transcriptional up-regulation, the identity of methyltransferases available for interaction with the activated methyl cycle components could provide an important level of regulation (Xu et al. 2016). A recent study demonstrated that the mitogen-activated protein kinases MPK3 and MPK6 mediate transcriptional up-regulation of the stress-inducible genes encoding the cytochrome P450 CYP81F2 and IGMTs, which are responsible for formation of 4MO-I3M (Xu et al. 2016) Post-translational regulatory actions on glucosinolate biosynthesis were first reported when PP2A-B'γ was shown to physically interact with IGMTs and negatively regulate the formation of 4MO-I3M in Arabidopsis (Rahikainen et al. 2017). These findings elucidate that post-

translational regulatory mechanism can significantly impact the channeling of metabolic precursors for biosynthesis of secondary metabolites.

## Outlook

The special environmental conditions typical for Nordic regions may limit the efficiency of biomass production and crop yield. However, harsh growth conditions may also be beneficial since they enhance the accumulation of secondary metabolites, which may affect the sensory properties and improve the nutritional value of food plants. Understanding how plants modulate their metabolite contents in response to biotic factors may provide new strategies for increasing the content of value-added compounds in food plants. In long-term, assessing how external factors modulate methionine metabolism and how this contributes to the chemical defences and ultimately the formation of value-added compounds in plants will provide valuable information applicable in the quest for breeding of stress-tolerant crops for agriculture and biofuture applications.

## Author contributions

MR, SA, AT, JP and SK conceived the idea and jointly wrote the manuscript.

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**Table 1.** Genetic tools available to investigate the activated methyl cycle function in plants.

| Gene accession | Gene symbol | Mutant name | Mutation/transgene | Phenotype | References |
|---|---|---|---|---|---|
| **AT4G13940** | *AtSAHH1* | *hog1-1* | Point mutation | Genome-wide demethylation, slow growth, low fertility and reduced seed germination. | Rocha et al. 2005 |
| | | *sahh1* | T-DNA insertion | Short, hairless roots, delayed flowering and germination and slow growth. Dark green leaves and elevated cytokinin content. | Wu et al. 2009, Li et al. 2008 |
| | | *hog1-AS* | RNAi | Profuse branching, delayed flowering, increased leaf size and higher seed yield. | Godge et al. 2008 |
| | | *hog1-OE* | pro35S::SAHH1 | Early flowering with a significantly reduced plant biomass and number of leaves. | Godge et al. 2008 |
| | | *sahh1-gfp-OE* | pro35S::SAHH1-GFP | Localized to cytoplasm and nucleus | Lee et al. 2012 |
| | | *sahh1-gfp* | proSAHH1::SAHH1-GFP | Localized to cytoplasm and nucleus | Lee et al. 2012 |
| | | *gfp-sahh1* | proSAHH1::GFP-SAHH1 | Localized to cytoplasm and nucleus | Lee et al. 2012 |
| | | *sahh1-gus* | proSAHH1::GUS | Promoter active in cotyledons and root in 8-day-old plants and in primary and secondary veins in leaves. In flowers, promoter is active in sepal, stigma, filament and the basal part of the silique. | Li et al. 2008 |

| | | | | | |
|---|---|---|---|---|---|
| | | *sah1L459F* | Point mutation | Reduced DNA cytosine methylation in non-CG sequences. No morphological phenotype. | Mull et al. 2006 |
| **P68173** | *NtSAHH1* | *sahh1-AS* | RNAi | DNA hypomehylation. Wrinkled, dark green leaves with delayed senescence. Decreased apical dominance. Shorter stamen. | Tanaka et al. 1997 |
| **AT3G23810** | *AtSAHH2* | *sahh2-gfp-OE* | pro35S::SAHH2-GFP | Localized to cytoplasm and nucleus | Lee et al. 2012 |
| | | *sahh2-gfp* | proSAHH2::SAHH2-GFP | Localized to cytoplasm and nucleus | Lee et al. 2012 |
| | | *sahh2-gus* | proSAHH2::GUS | Promotor is active in hypocotyl in 8-day-old plants and in leave trichomes. In flowers, promoter is active in anthers. | Li et al. 2008 |
| | | *dssahh2* | RNAi | Bushy phenotype, short and branched stem, dark green and wrinkled leaves. Slightly bigger rosette leaves. Delayed flowering. | Li et al. 2008 |
| **AT4G39460** | *AtSAMC1* | *samt1* | T-DNA insertion | Pale-green leaves during the first weeks of growth. Severely growth-retarded phenotype. | Bouvier et al. 2006 |
| **AT1G02500** | *AtMAT1* | *mat1/sam1-1* | T-DNA insertion | No growth phenotype when grown on soil. | Jin et al. 2017, Mao et al. 2015 |
| | | *sam1-gus* | proSAM1::GUS | Promoter active in leaves, roots, root hairs and flowers. | Mao et al. 2015 |
| **AT4G01850** | *AtMAT2* | *mat2-1* | T-DNA insertion | No growth phenotype when grown on soil. Slightly insensitive to 1-aminocyclopropane- 1- | Jin et al. 2017 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | carboxylic acid (ACC) when grown on plates in dark. | |
| | | *mat2-2/sam2-1* | T-DNA insertion | No growth phenotype when grown on soil. Slightly insensitive to 1-aminocyclopropane- 1-carboxylic acid (ACC) when grown on plates in dark. | Jin et al. 2017, Mao et al. 2015 |
| | | *mat2-OE* | pro35S::MAT2-GFP | Small rosette diameter. SAM and ethylene over-producer. Short hypocotyl in etiolated seedlings. Slow primary root growth in light. Ectopic lignification in stem. | Jin et al. 2017 |
| | | *sam1-gus* | proSAM1::GUS | Promoter active in leaves, roots, root hairs and flowers. | Mao et al. 2015 |
| **AT1G02500/AT4G01850** | *AtMAT1/AtMAT2* | *sam1-1/sam2-1* | T-DNA insertion | Longer hypocotyls in dark-grown seedlings. Reduced ethylene production. | Mao et al. 2015 |
| | | *sam1-OE* | pro35S::SAM1-HA | Smaller rosette with shorter petioles and smaller leaves. Shorter hypocotyls in dark-grown seedlings. | Mao et al. 2015 |
| **AT2G36880** | *AtMAT3* | *mat3* | T-DNA insertion | Impaired pollen tube growth and reduced seed production. | Chen et al. 2016 |
| **AT3G17390** | *AtMAT4* | *mto3* | Point mutation | Methionine over-accumulation. | Goto et al. 2002 |
| **AY635050** | *SbSAMS* | *SbSAMS-OE (in A. thaliana)* | pro35S::SbSAMS | Increased levels of SAM, SAH and ethylene. Early senescence. | Kim et al. 2015 |

| | | | | | |
|---|---|---|---|---|---|
| **Z24741.1** | *SlSAMS1* | *SlSAMS1-OE (in S. lycopersicum* | pro35S:: SlSAMS1 | Higher photosynthetic capacity, better tolerance to ROS, altered polyamine metabolism and higher fruit yield under alkali stress. | Gong et al. 2014 |

**Table 2.** Known post-translational modifications of activated methyl cycle enzymes.

| Name | AGI | Site | Modification | PEPTIDE | Reference |
|---|---|---|---|---|---|
| unique peptides | | | | | |
| CIMS1 | AT5G17920 | S38 | P | STAEDLQKV(pS)ADLR | Xu et al. 2017 |
| | | S43 | P | VSADLR(pS)SIWK | Xu et al. 2017 |
| | | S50 | P | QM(pS)AAGTK | Xu et al. 2017 |
| | | S349 | | LDDEIK(pS)WLAFAAQK | Xu et al. 2017 |
| | | S459 | P | KV(pS)EEDYVK | Roitinger et al. 2015 |
| | | S707 | P | IP(pS)SEEIADR; IP(pS)SEEIADRVNK | Roitinger et al. 2015, Rahikainen et al. 2017, Xu et al. 2017 |
| | | S759 | P | LIR(pS)QLASAK | Xu et al. 2017 |
| | | N/A | S-nitrosylation | | Lindermayr et al. 2005 |
| CIMS2 | AT3G03780 | S29 | P | FALESFWDGK(pS)SADDLQK | Xu et al. 2017 |
| non-unique peptides | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| CIMS1<br>CIMS2 | AT5G17920<br>AT3G03780 | S3 | P | MA(pS)HIVGYPR | Xu et al., 2017 |
| | | S23 | P | FALE(pS)FWDGK | Xu et al. 2017 |
| | | S103 | P | GNA(pS)VPAMEMTK | Wang et al. 2013a, Roitinger et al. 2015, Xu et al. 2017 |
| | | S408 | P | AAAALKG(pS)DHR | Roitinger et al. 2015 |
| | | S681 | P | LL(pS)VFR | Xu et al. 2017 |
| | | T741 | P | KY(pT)EVKPALK | Xu et al. 2017 |
| | | A2 | Acetylation | (AcA)SHIVGYPR | Xu et al. 2017 |
| | | N/A | S-nitrosylation | | Lindermayr et al. 2005 |
| | | K406 | Ubiquitination | AAAL(UbK)GSDH | Saracco et al 2009 |
| CIMS1<br>CIMS2<br>MS3 | AT5G17920<br>AT3G03780<br>AT5G20980 | Y698 | P | YGAGIGPGV(p)YDIHSPR | Sugiyama et al. 2008, Nakagami et al. 2010, Wang et al. 2013, Umezawa et al. 2013, Xu et al. 2017 |

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|---|---|---|---|---|---|
| | | S702 | P | YGAGIGPGVYDIH(pS)PR | Sugiyama et al. 2008, Nakagami et al. 2010, 2010 , Lan et al. 2012, Umezawa et al. 2013, Wang et al. 2013a, Wang et al. 2013b, Rayapuram et al 2014, Roitinger et al. 2015, Xu et al. 2017 |
| unique peptides | | | | | |
| SAHH1 | AT4G13940 | T44 | P | (pT)EFGPSQPFKGAR | Li et al. 2014 |
| SAHH2 | AT3G23810 | Y214 | P | L(pY)QMQETGALLFPAINVN DSVTK | Xu et al. 2017 |
| | | T219 | P | LYQMQE(pT)GALLFPAINVN DSVTK | Reiland et al 2009 |
| non-unique peptides | | | | | |
| SAHH1<br>SAHH2 | AT4G13940<br>AT3G23810 | S9 | P | MALLVEKT(pS)SGR/ MALLVEKT(pS)SGREYK | Xu et al. 2017 |
| | | S10 | P | TS(pS)GREYKVK | Xu et al. 2017 |
| | | S20 | P | DM(pS)QADFGR/<br>VKDM(pS)QADFGR | Wang X. et al. 2013, Roitinger et al. 2015 |
| | | T113 | P | GE(pT)LQEYWWCTER | Li et al. 2014 |

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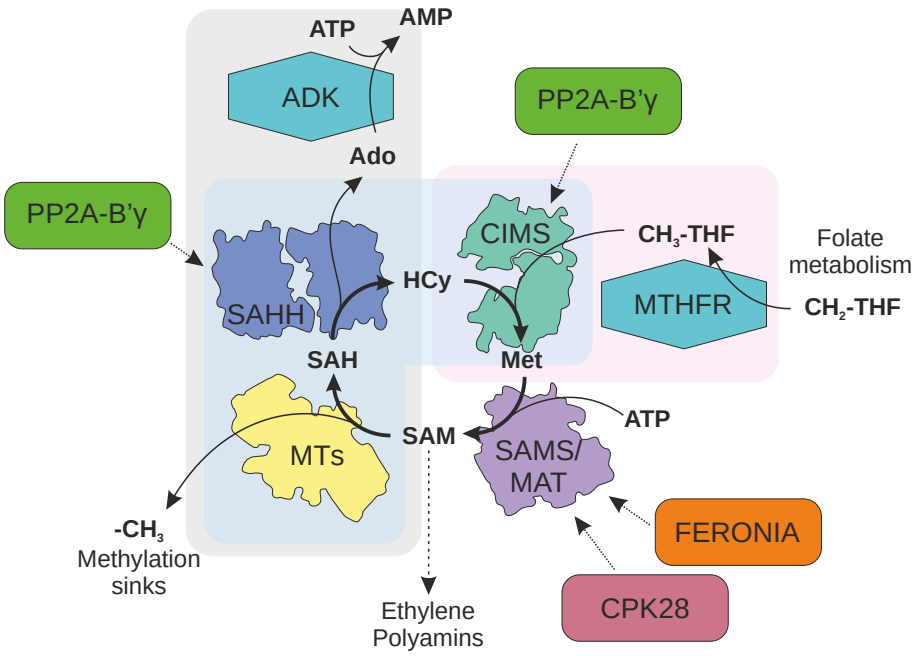
|---|---|---|---|---|---|
| | | S203 | P | LVGV(pS)EETTTGVK | Li et al. 2014, Rahikainen et al 2017 |
| | | T206 | P | LVGVSEE(pT)TTGVK | Xu et al. 2017 |
| | | T207 | P | LVGVSEET(pT)TGVK | Xu et al. 2017 |
| | | S236 | P | (pS)KFDNLYGCR | Li et al. 2014 |
| | | T370 | P | RI(pT)IKPQTDR | Roitinger et al. 2015 |
| | | | | | |
| | | C224 | S-nitrosylation | FDNLYG(sC)R | Fares et al 2011, Puyaubert J et al. 2014 |
| | | C268 | S-nitrosylation | VAVI(sC)GYGDVGK | Puyaubert J et al. 2014 |
| | | A2 | Acetylation | (AcA)LLVEKTSSGR | Xu et al. 2017 |
| | | N/A | S-nitrosylation | | Lindermayr et al. 2005 |
| unique peptides | | | | | |
| SAMS1 | | C114* | S-nitrosylation | N/A | Lindermayr et al. 2006 |
| SAMS3 | | N/A | S-nitrosylation | | Lindermayr et al. 2005 |

| | | | | | |
|---|---|---|---|---|---|
| non-unique peptides | | | | | |
| SAMS1<br>SAMS2 | AT1G02500<br>AT4G01850 | S100 | P | VLVNIEQQ(pS)PDIAQGVHG HFTK | Xu et al. 2017 |
| | | S292 | P | SGAYIVRQAAK(pS)VVANG MAR | Xu et al. 2017 |
| SAMS2<br>MAT3 | AT1G02500<br>AT2G36880 | S236 | P | YLDDKTIFHLNP(pS)GR | Xu et al. 2017 |
| SAMS1<br>SAMS2<br>SAMS3<br>MAT4 | AT1G02500<br>AT4G01850<br>AT2G36880<br>AT3G17390 | S235 | P | TIFHLNP(pS)GR | Zhang et al. 2013, Roitinger et al. 2015 |
| | | N/A | S-nitrosylation | | Lindermayr et al. 2005 |

**Figure legends**

**Figure 1.** Activated methyl cycle and its regulatory interactions. S-adenosyl-L-methionine (SAM) is used as a methyl donor in trans-methylation reactions catalyzed by methyltransferases (MTs). These reactions produce an inhibitory reaction product S-adenosyl homocysteine (SAH) that is recycled in the so called activated methyl cycle. SAH is hydrolyzed by S-adenosyl homocysteine hydrolase (SAHH) to homocysteine (HCy) and adenosine (Ado), which is further phosphorylated to adenosine monophosphate (AMP) by adenosine kinase (ADK) using adenosine triphosphate (ATP). HCy is converted to methionine (Met) by cobalamin-independent methionine synthase (CIMS) using methyltetrahydrofolate provided by methyltetrahydrofolate reductase (MTHFR). Finally, Met is recycled back to SAM by SAM-synthase (SAMS) also called methionine adenosyltransferases (MAT). The active methyl cycle enzymes form complexes and interact with each other's as well as the connected metabolic enzymes. SAHH has been found to interact with ADK and mRNA cap MT in the nucleus and with CIMS and indole glucosinolate MT in the cytoplasm, whereas CIMS forms a protein complex with MTHFR. The protein complex formation of SAHH and CIMS is regulated by a protein phosphatase 2A regulatory subunit B'γ (PP2A-B'γ). Moreover, SAMS/MAT is regulated by interactions with a receptor kinase FERONIA and a calcium-dependent protein kinase 28 (CPK28).

Figure 1.


ATP
AMP
ADK
PP2A-B'γ
Ado
PP2A-B'γ
CIMS
$CH_3$-THF
Folate metabolism
SAHH
HCy
MTHFR
$CH_2$-THF
SAH
Met
ATP
SAM
MTs
SAMS/ MAT
FERONIA
$-CH_3$ Methylation sinks
CPK28
Ethylene Polyamins