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The significance of methionine cycle enzymes in plant virus infections

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Both biotic and abiotic stresses cause changes in the activities of plant methionine cycle (MTC) enzymes. These changes contribute to the ability of the plant to manage stress. On the other hand, viruses utilize MTC enzymes to promote infection. Here, we review the growing but still limited knowledge of the interactions between plant viral proteins and MTC enzymes. Virus-induced changes in *S*-adenosyl methionine synthetase and *S*-adenosyl homocysteine hydrolase activities debilitate transcriptional and post-transcriptional RNA silencing and affect antiviral defense reactions connected to ethylene and polyamine biosynthesis pathways. Viral perturbations of host methionine homeostasis couple *trans*-sulfuration and gluthathione biosynthesis pathways to MTC functions. Large multiprotein complexes, which contain viral proteins and MTC enzymes, may represent metabolons assembled for specific viral functions or host defense responses. Proper understanding of the MTC-associated metabolic and regulatory interactions will reveal those with potential to create virus resistance in plants.

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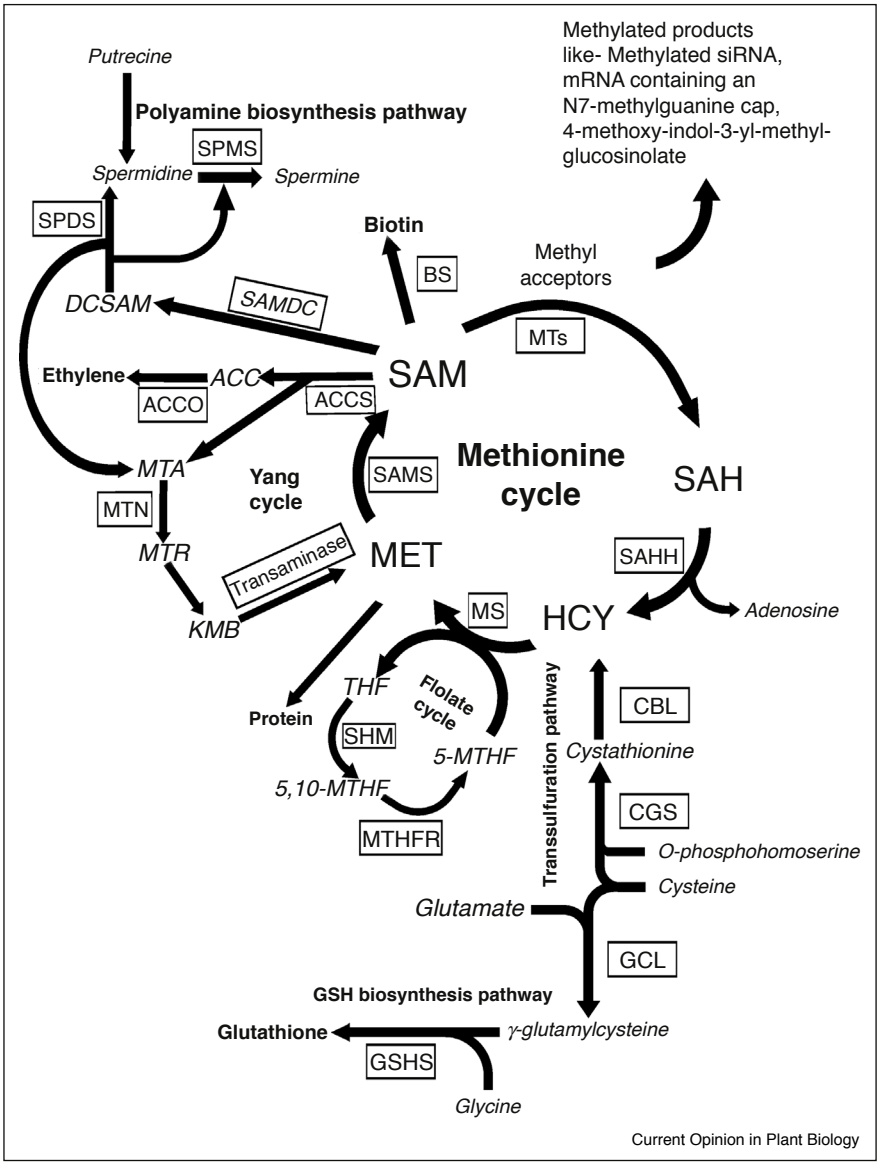
Introduction

Plant virus diseases cause a global threat to agricultural production and food security. While research-based knowledge forms a foundation for planning management strategies for established viral diseases, many challenges persist. Furthermore, a substantial portion of detrimental emerging and re-emerging plant diseases are due to viruses [1]. As intracellular parasites, viruses are obliged to exploit the resources of the host cell to reproduce. The host and viral factors together genetically determine plant virus replication and translation within the host cells and their movement throughout the host plant. Pro-viral compatible host–virus interactions support whereas incompatible interactions and those activating defense responses limit these functions essential for establishing an infection. Furthermore, interactions of viral proteins with the host reprogram several processes associated with primary metabolism [2]. Among the primary metabolic pathways affected by plant virus infection is the host methionine cycle (MTC), also known as activated methionine cycle.

With its approximately 450 known virus species [3], the family *Geminiviridae* forms the largest plant virus family. Geminiviruses cause significant crop losses in various plant species especially in tropical and subtropical countries. Potyviruses form another widespread plant virus group infecting most cultivated plants worldwide. Differently from geminiviruses, which have a single-stranded DNA genome, the genome of potyviruses is a single-stranded RNA molecule of positive polarity, which makes this group the largest group of plant RNA viruses (reviewed in Ref. [4]). Recent examples arising from studies on, for example, geminivirus and potyvirus reveal that viral proteins are able to interact directly with MTC enzymes to promote infection [5••,6••]. Considering the important impact of these two large virus groups and plant viruses in general to food production, evaluation of various strategies for generating resistant crops is crucial. Manipulation of the interactions between MTC enzymes and viral proteins is one possible strategy to be scrutinized.

Attempts to gain virus resistance by modifying the activities of MTC enzymes were initialized already at 1990s [7]. Although it was found that SAMS silencing by transgenic expression of antisense RNA results in a broad virus resistance, which is especially effective toward capped plant viruses, this approach is not applicable in resistance breeding because it results in distorted plant growth. Therefore, more subtle ways to modulate MTC functions and the *trans*-methylation reactions are required to mitigate the losses caused by viral diseases in plants. Figure 1 introduces MTC and the pathways converging to and diverging from it with the key enzymes and metabolites indicated. Understanding the cross talk between MTC, its associated pathways and virus infection will provide tools to devise strategies against the adverse effects on growth and crop yield caused by the infections. We propose that identification of multiprotein complexes formed within stress-exposed plants to target specific

Figure 1



MTC and its associated pathways. This figure demonstrates major pathways converging to and diverging from MTC. Metabolite flow directions are presented by arrows, while the major pathways involved therein are marked in bold. Key intermediates from the individual pathways are in italics and the enzymes involved in their bioconversion are presented in adjacent boxes. ACC, 1-aminocyclopropane-1-carboxylic acid; ACCO, ACC oxidase; ACCS, ACC synthase; BS, biotin synthase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; DCSAM, decarboxylated *S*-adenosyl methionine; GCL, glutamate cysteine ligase; GSH, glutathione; GSHS, glutathione synthetase; HCY, homocysteine; KMB, 2-keto-4-methylthiobutyric acid; MAM,Methylthioalkylmalate synthase; MET, methionine; MS, methionine synthase; MTA, 5′-methylthioadenosine; MTs, methyltransferases; 5-MTHF, 5-methyltetrahydrofolate; 5,10-MTHF, 5,10-Methylenetetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; MTN, 5-methylthioadenosine nucleosidase; MTR, 5-methylthioribose; SAH, *S*-adenosyl-homocysteine; SAHH, *S*-adenosyl-homocysteine hydrolase; SAM, *S*-adenosyl methionine; SAMDC, *S*-adenosylmethionine decarboxylase; SAMS, *S*-adenosyl methionine synthetase; SHM, serine hydroxymethyltransferase; SPDS, spermidine synthase; SPMS, spermine synthase; THF, tetrahydrofolate.

trans-methylation reactions represents an important research area for future investigations. Such studies will be instrumental for understanding the metabolic and regulatory MTC-associated interactions and discovering their potential targets for engineering virus resistance.

Regulation of antiviral RNA silencing by SAMS and SAHH activities

Methylation of macromolecules and small molecules regulates a wide variety of cellular processes linked to primary metabolism, gene expression and protein functions in

plants (reviewed in Ref. [8•]). The *trans*-methylation reactions are catalyzed by cellular methyl transferases, which utilize *S*-adenosyl methionine (SAM), a product of MTC, as the methyl group donor. *S*-adenosyl-L-methionine synthase (SAMS) catalyzes SAM production from adenosine triphosphate and L-methionine. After ATP, SAM is the second most used enzymatic co-factor in the cell (reviewed in Ref. [9]) revealing the abundant nature of *trans*-methylation reactions. One well-known example of SAM-dependent methyl transfer reaction is mRNA capping. Plant and animal viruses having 5′ capped genomic RNAs encode their own SAM-dependent capping enzymes to ensure their efficient replication (reviewed in Ref. [10]). Methyl group transfer from SAM to the target molecules produces *S*-adenosyl-homocysteine (SAH), a universal methyl transferase inhibitor [11], as a byproduct. *S*-adenosyl-L-homocysteine hydrolase (SAHH) converts SAH to adenosine and L-homocysteine (HCY). Methionine synthase (MS), the third essential enzyme of MTC, completes the cycle by catalysing the conversion of HCY back to methionine.

DNA cytosine methylation is a modification that can affect plant gene expression and development. 5-methyl cytosine (5-mC), formed in a reaction catalyzed by cytosine methyl transferase, is also the epigenetic marker for transcriptional RNA silencing (TGS) (reviewed in Ref. [12•]). *Arabidopsis* plants carrying mutations in the genes of either cytosine methyltransferase or adenosine kinase, an enzyme having an important role in efficient production of SAM, are hypersensitive to two distinct genera of geminiviruses, begamoviruses, and curtoviruses [13], suggesting that plants may use chromatin methylation as a defense against DNA viruses. Beet severe curly top virus (BSCTV) C2 protein interacts with *S*-adenosyl-methionine decarboxylase 1 (SAMDC1) [14•]. SAMDC1 catalyses the conversion of SAM to decarboxylated *S*-adenosyl-methionine (dcSAM), which likely acts as a competing methyltransferase inhibitor against SAM. Both SAMDC1 overexpression and the presence of BSCTV C2 reduce 5-mC methylation of the viral DNA consequently promoting infection. The authors showed that SAMDC1 is stabilized in the presence of BSCTV C2 protein and propose that viral DNA methylation gets inhibited due to the disturbed SAM/dcSAM ratio [14•]. C4 protein of cotton leaf curl multan virus (CLCuMuV; family *Geminiviridae*; genus *Begomovirus*) suppresses both TGS and post-transcriptional gene silencing (PTGS). CLCuMuV C4 interacts with SAMS of *Nicotiana benthamiana* and reduces its enzymatic activity [6••]. The study demonstrates directly that disturbance of the proper functioning of SAMS by its interaction with C4 enhances accumulation of CLCuMuV DNA, reduces SAMS-dependent DNA methylation, and inhibits TGS and PTGS. Interestingly, C4 protein with a mutation at its putative ATPase activity site is not able to suppress SAMS activity. On this basis, the authors propose that ATP hydrolysis by C4 may block the ATP-dependent SAM production by SAMS.

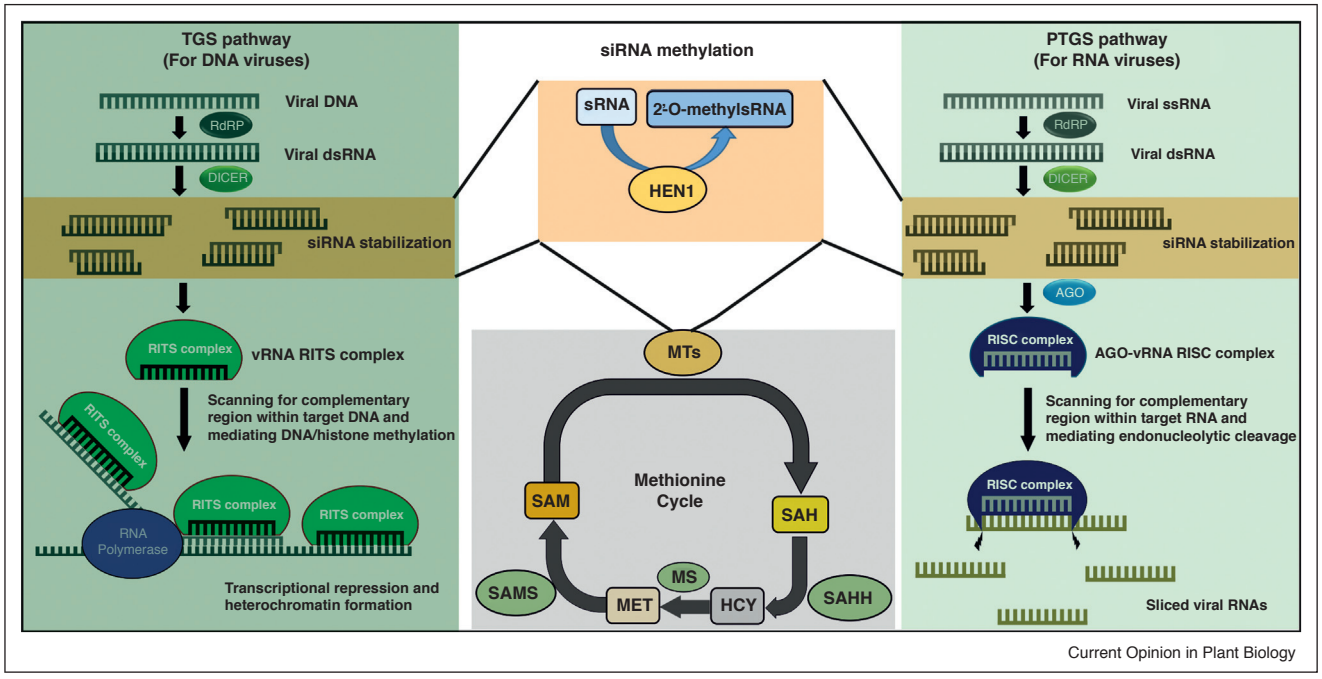
Antiviral RNA silencing against potyvirus infection is suppressed by the potyviral helper component-proteinase (HCPro). HCPro's interaction with SAMS and SAHH has been proposed as a possible mechanism enabling RNA silencing suppression [5••,15•]. Potato virus A (PVA; genus *Potyvirus*) infection reduces SAMS activity in an HCPro-dependent manner [5••]. The authors hypothesized that this inhibition may contribute to the HCPro-mediated suppression of RNA silencing, since Hua enhancer 1 (HEN1), the methyl transferase responsible for stabilization of siRNAs via 2′-0-methylation of their 3′ ribose [16,17], requires a constant supply of SAM. An RNASeq analysis of transcript levels in plum pox virus (PPV; genus *Potyvirus*)-susceptible or resistant apricot revealed that SAMS2 expression is higher in the resistant genotype [18], supporting the importance of SAMS in antiviral defense against potyviruses. HEN1 activity is potentially further downregulated by HCPro interaction with SAHH, which may lead to accumulation of the HEN1 inhibitor SAH. In support, downregulation of SAHH activity leads to reduced RDR6-dependent accumulation of secondary siRNAs and to a similar suppression of local silencing than achieved in the presence of the silencing suppressor proteins of tomato chlorosis virus (genus *Crinivirus*; family *Closteroviridae*) and potato virus Y (genus *Potyvirus*; [15•]). A model, which proposes that disturbed methylation and subsequent destabilization of sRNAs essential for RNA silencing may explain reduced TGS and PTGS during viral infections, is presented in Figure 2.

Altogether, these studies imply that smooth running of the MTC ensures the undisturbed SAM supply. Resulting active chromatin and siRNA methylation provides plants protection against geminivirus and potyvirus, respectively. It would be worthwhile to investigate how MTC functions could be sustained to support optimal antiviral RNA silencing during an attempted virus invasion.

Virus-induced changes in ethylene and polyamine biosynthesis and MTC enzymes

MTC is closely associated with several other metabolic pathways (Figure 1). Concomitant variation in the transcript levels of SAMS, SAHH and SAMDC indicates major perturbations in sulphur metabolism and polyamine (PA) biosynthesis during viral infection [19,20]. Conversion of HCY into methionine in a reaction catalyzed by MS forms a crucial control point between the flow of metabolites to methionine biosynthesis and its diverging biosynthetic pathways, including those of ethylene, PA and glycinebetaine [21,22]. Compatible interaction between citrus leprosis virus, a (+)RNA virus of genus *Cilevirus*, and citrus plant leads to repression of MS gene [20], while systemic infection by soybean mosaic virus (genus *Potyvirus*) first upregulates and subsequently downregulates the MS transcript level in soybean [19]. In spite of the transcriptional

Figure 2



siRNA methylation connects MTC to transcriptional and post-transcriptional RNA silencing pathways. DICER-like endoribonucleases (DCLs) cleave double-stranded intermediates of viral RNA into 21–24 nucleotides long siRNAs before subsequent 3′ end methylation catalysed by methyl transferase HEN1. The MTC provides SAM substrate to HEN1. The 3′ end methylation stabilizes siRNAs by protecting them from polyuridylation and degradation and is therefore, an important step before loading of the stabilized siRNAs onto either RNA-induced transcriptional silencing (RITS) complex for subsequent DNA/histone methylation and transcriptional repression or RNA-induced silencing complex (RISC) for endonucleolytic cleavage of the target RNA. This is essential for the success of both TGS and PTGS. The MTC is additionally responsible for removing SAH, which is the byproduct of siRNA methylation and a feedback inhibitor of methyltransferases including HEN1. Taken together, controlled operation of the MTC is essential for RNA silencing and successful antiviral defense.

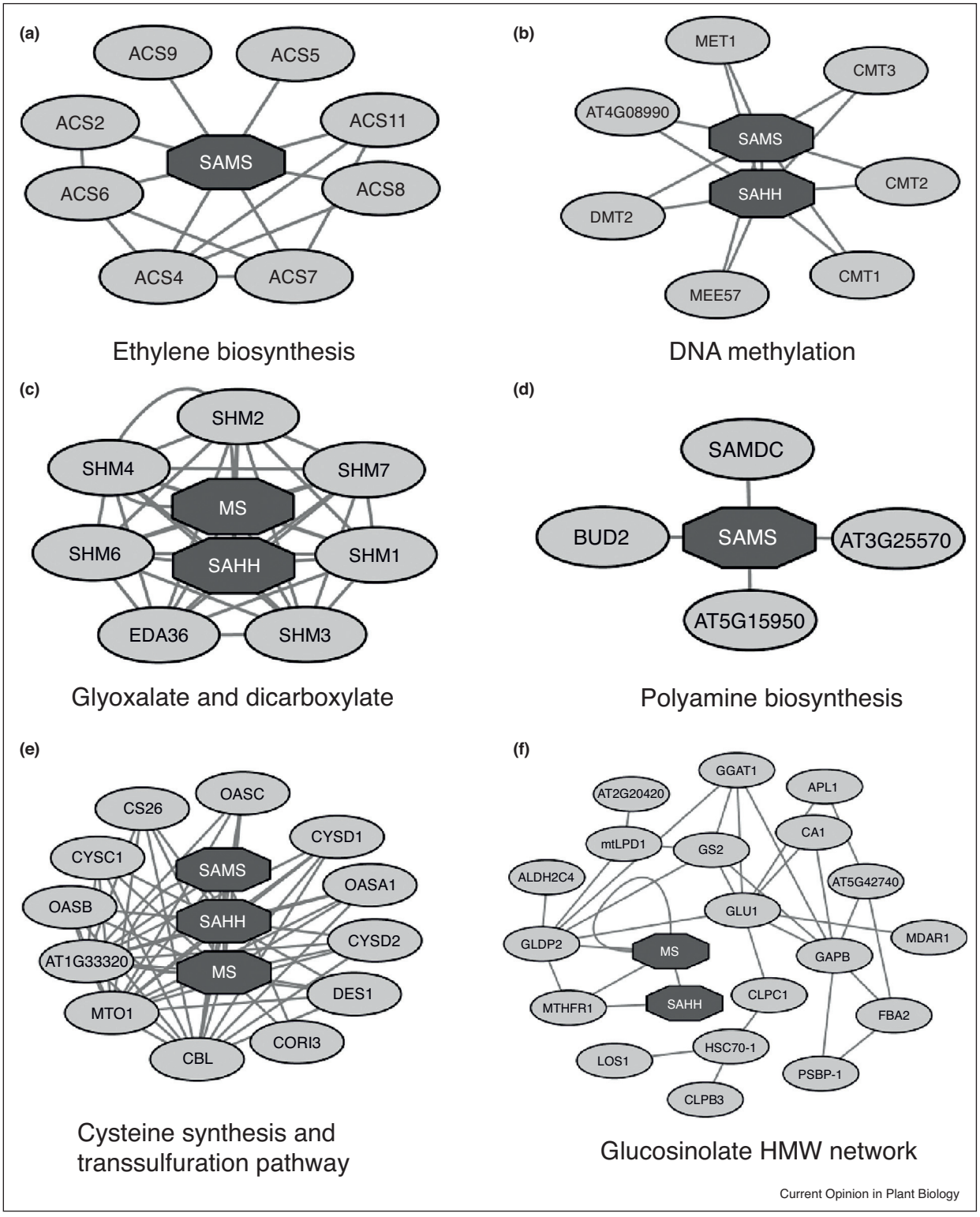
regulation of the MS gene, no examples of direct involvement of MS in virus infection were found.

In addition to serving as a methyl donor SAM plays important roles as a signal molecule. Stress factors and viral proteins influencing SAMS activity can, therefore, manipulate SAM-dependent signaling pathways leading to alterations in stress tolerance and susceptibility of the plant to virus infection (Figure 1). Expression of *Solanum brevidence* SAMS in transgenic *Arabidopsis* plants enhances expression of ethylene biosynthesis genes as well as stress-responsive and wound-responsive genes [23]. Similarly, rice dwarf virus (RDV; genus *Phytoreovirus*, family *Reoviridae*) infection induces the ethylene biosynthesis genes in rice leading to increased ethylene production [24••]. Further studies on the role of ethylene in RDV infection revealed that both the virus and overexpression of RDV protein Prn11 stimulates SAMS activity [24••]. Under the circumstances of SAMS-activated ethylene signalling, plants become more susceptible to RDV infection [24••]. It remains to be studied, whether other SAM-dependent pathways are involved in RDV infection as well.

SAMS expression is upregulated in leaves and roots of *Beta vulgaris* L. during salt stress [25]. Ectopic expression of *Beta vulgaris* L. SAMS in *Arabidopsis* in turn leads to enhanced salt and H_2O_2 tolerance [25]. Similarly, ectopic expression of *Solanum lycopersicum* SAMS in transgenic tomato plants increases tolerance to alkali stress and lowers oxidative stress [26]. In both cases, the reason for the enhanced salinity–alkalinity tolerance is increased PA biosynthesis initiated from its precursor SAM, which alleviates accumulation of reactive oxygen species *in planta* [27]. PA biosynthetic pathway genes are also upregulated upon cucumber mosaic virus (CMV)-elicited hypersensitive reaction in *Arabidopsis* [28]. Out of the PAs, spermidine is the one that specifically induces CMV resistance. However, this study did not reveal whether interference of the virus with the MTC enzyme activities preceded the increase in the PA biosynthesis.

Changes in the MTC enzyme activities affect also bacterial infections. Knock down of SAHH1 and MS1 decreases resistance to *Ralstonia solanacearum* [29].

Figure 3



Interaction maps of *Arabidopsis* MTC enzymes with proteins belonging to MTC-linked pathways. **(a–e)** compiles the primary interactors of the *Arabidopsis thaliana* MTC enzymes with components from pathways involved in viral infections. (a) ethylene biosynthesis, (b) DNA methylation, (c) glyoxalate and dicarboxylate, (d) polyamine biosynthesis and (e) cysteine synthesis and *trans*-sulfuration pathway. **(f)** presents the interaction network of MTC enzymes associated with glucosinolate metabolism. Interaction data were retrieved from STRING database network [54] and visualization of the networks was carried out using Cytoscape [53]. SAMS, SAHH and MS herein includes all variants of the enzymes in

However, SAHH appears to have a reverse role in *Pseudomonas syringae* infection, as increased resistance toward infection results from co-silencing of all three SAHH genes of tomato [30]. Both the viral and bacterial examples here indicate that an alteration in MTC function suppressing the infection by one pathogen may be beneficial for another pathogen. This diversity of responses needs to be kept in mind when considering crop plant improvements by modulation of MTC-related enzyme activities.

Convergence between the methionine cycle, *trans*-sulfuration pathways and virus infection

Cytoplasmic MS is primarily responsible for recovery of methionine via the route of MTC, whereas chloroplastic MS is responsible for *de novo* methionine synthesis via *trans*-sulfuration pathway. Cystathionine γ-synthase, cystathionine β-lyase and MS are the major enzymes sequentially contributing to *de novo* methionine biosynthesis, SAM being the central feedback regulator of the process. The *trans*-sulfuration pathway and MTC share a common metabolite, HCY, which contributes to methionine synthesis in both cases. Interestingly, upregulation of the proteins involved in *de novo* methionine biosynthesis is associated with oxidative stress responses in plants. Increased expression of cystathionine γ-synthase in tobacco and *Arabidopsis* seeds leads to increased flux of HCY toward methionine synthesis with a concomitant reduction in the level of its precursor cysteine and cysteine-derived metabolite glutathione (GSH) [31,32•]. The variation in GSH level is more dependent on the flux of cysteine toward GSH/methionine biosynthesis pathways, than the transcript levels of the genes involved therein [32•]. GSH is a major antioxidant *in planta* (reviewed in Ref. [33]), and its reduction induces oxidative stress [31,32•]. In line with this, transgenic tobaccos with a constitutively higher level of methionine were shown to be more sensitive to oxidative stress [34]. Furthermore, *Phytophthora infestans* infection in potato induces oxidative stress and increased HCY accumulation with simultaneous upregulation of SAHH and cystathionine β-lyase gene [35], again indicating coupling between MTC, *trans*-sulfuration pathway and the oxidative stress response.

Mixed infections by potyvirus and potexvirus cause remarkable oxidative stress and severe symptoms in the affected plants [36]. Imbalance in the flow of metabolites through the MTC, *trans*-sulfuration and GSH biosynthesis pathways has recently been proposed to induce the oxidative stress during mixed potexvirus and potyvirus infection [37•]. It was found that both SAHH silencing and expression of potyviral HCPro in potexvirus-infected background causes a significant reduction in the cellular GSH concentration. Inhibition of SAHH by HCPro together with a still to be identified potexvirus component likely reduces the conversion of SAH to HCY, which may lead to low recovery of methionine via the MTC. The authors propose that *de novo* methionine synthesis is upregulated at the expense of GSH biosynthesis in order to maintain the methionine homeostasis [37•]. However, further studies are required to verify the connection between reduced SAHH activity, low GSH and oxidative stress during synergistic potyvirus and potexvirus infection.

PPV causes sharka, which is a disease causing significant problems in stony fruit tree orchards. PPV infection induces oxidative stress in susceptible peach plants. Interestingly, an artificial cysteine precursor, L-2-oxo-4-thiazolidine-carboxylic acid (OTC), was shown to reduce PPV symptoms, stimulate plant growth and alleviate virus-induced oxidative stress in peach [38••]. This study revealed that OTC-treatment is able to prevent PPV infection-induced changes in the cellular glutathione levels. While more investigations are required to understand the mechanism how OTC functions in PPV-infected plants, this example shows that the use of environmentally safe compounds, like OTC, has potential to support healthy growth of virus- infected plants.

(Figure 3 Legend Continued) *Arabidopsis*. SAMS (AT1G02500, AT2G36880, AT3G17390, AT4G01850); SAHH (AT3G23810, AT4G13940), MS (AT3G03780, AT5G17920, AT5G20980). ACS, 1-aminocyclopropane-1-carboxylate synthase; ALDH2C4, Aldehyde dehydrogenase family 2; APL1, Glucose-1-phosphate adenylyltransferase large subunit 1; AT3G25570, Adenosylmethionine decarboxylase family protein; AT5G15950, Adenosylmethionine decarboxylase family protein; AT4G08990, DNA (cytosine-5-)-methyltransferase family protein; AT1G33320, Pyridoxal phosphate (PLP)-dependent transferases superfamily protein; AT5G42740, Glucose-6-phosphate isomerase; BUD2, Adenosylmethionine decarboxylase family protein; CA1, Beta carbonic anhydrase 1; CBL, Cystathionine beta-lyase; CLPC1, Chaperone protein ClpC1; CLPB3, Chaperone protein ClpB3; CMT, DNA (cytosine-5)-methyltransferase (chromomethylase); CORI3, Tyrosine transaminase family protein; CS26, Probable *S*-sulfocysteine synthase; CYSC1, Bifunctional L-3-cyanoalanine synthase/cysteine synthase C1; CYSD1, Bifunctional L-3-cyanoalanine synthase/cysteine synthase D1; CYSD2, Bifunctional L-3-cyanoalanine synthase/cysteine synthase D2; DES1, Bifunctional cystathionine gamma-lyase/cysteine synthase; DMT2, DNA (cytosine-5)-methyltransferase 4; EDA36, Pyridoxal phosphate (PLP)-dependent transferases superfamily protein; FBA2, Fructose-bisphosphate aldolase 2; GAPB, Chloroplast localized glyceraldehyde-3-phosphate dehydrogenase; GGAT1, Glutamate–glyoxylate aminotransferase 1; GLDP2, Glycine dehydrogenase (decarboxylating) 2; GLU1, Ferredoxin-dependent glutamate synthase 1; GS2, Glutamine synthetase; HSC70-1, Probable mediator of RNA polymerase II transcription subunit 37e; LOS1, Ribosomal protein S5/Elongation factor G/III/V family protein; MDAR1, Monodehydroascorbate reductase 1; MEE57, DNA (cytosine-5-)-methyltransferase family protein; MET1, DNA (cytosine-5)-methyltransferase 1; mtLPD1, Dihydrolipoyl dehydrogenase 1; MTO1, Pyridoxal phosphate (PLP)-dependent transferases superfamily protein; MTHFR1, Methylenetetrahydrofolate reductase 1; PSBP-1, Oxygen-evolving enhancer protein 2-1; OASA1, Encodes a cytosolic isoform of cytosolic *O*-acetylserine(thiol)lyase; OASB, Cysteine synthase; OASC, *O*-acetylserine (thiol) lyase isoform C; SAMDC, *S*-adenosylmethionine decarboxylase proenzyme 1; SHM, Serine hydroxymethyltransferase.

Future perspectives – identification and functional characterization of large multiprotein complexes containing viral proteins and MTC enzymes

Regarding the wide range of cellular processes regulated by the activities of MTC enzymes and SAM-dependent *trans*-methylation reactions, an important question about the mechanisms, which ensure specific responses via the distinct pathways, arises. Metabolons are supramolecular enzyme complexes dynamically regulating substrate channelling and metabolic pathway flux [39,40,41••]. The network of MTC enzymes and enzymes of the associated pathways (Figure 1) advocate the need to study possible existence of metabolons containing MTC enzymes. The wide range of SAMS, SAHH and MS interactors in *Arabidopsis* presented in Figure 3 could potentiate specific assemblies between the MTC enzymes, methyl transferases and methylation targets functionally connected to specific stress-responsive pathways. Protein phosphatase 2A regulatory subunit B′γ (PP2A-B′γ) modulates light-dependent oxidative stress responses [42,43] and formation of 4-methoxy-indol-3-yl-methyl glucosinolate (4MO-I3M; [44••]) in *Arabidopsis*. PP2A-B′γ interacts physically with SAHH1 and cobalamin-dependent MS (CIMS1) and modulates the abundance of SAHH1 and CIMS1-containing multi-enzyme complexes [44••]. The authors propose that these oligomeric protein complexes are needed to connect MTC with specific metabolic pathways. Interactions between SAHH1 and indole glucosinolate methyl transferase isoforms 1 and 4 (IGMT1 and IGMT4), which catalyze 4MO-I3M formation in *Arabidopsis* leaves, were also demonstrated in Rahikainen *et al.* [44••]. Of interest is that 4MO-13M is a defense molecule against the green peach aphid (*Myzus persicae*; [45]), which is an efficient vector for plant virus transmission. Such findings may open up possibilities to design novel resistance solutions against plant viral diseases.

Interestingly, at least one variant of each MTC enzyme associates with potyviral multiprotein complexes studied so far. SAMS (AT2G36880) and SAHH (AT4G13940) are present in the HCPro-proteome [5••]; SAMS (AT1G02500) in viral replication complex proteome [46]; SAHH (AT4G13940, AT3G23810) in the VPg-NIa proteome [47] and MS (AT5G17920) in PVA riboproteome [48]. Also, several of the primary interactors of MTC cycle enzymes can be found in potyviral proteomes. For example, 1-aminocyclopropane-1-carboxylate synthase 8 of ethylene biosynthesis pathway exists in potyviral riboproteome [48] and serine hydroxymethyltransferase 1 of glyoxalate and dicarboxylate metabolic pathway is present in the potyviral VPg-NIa interactome [47]. Glyoxalate and dicarboxylate metabolism is another MTC related pathway reported to be significantly altered during several viral infections [49–51]. Interestingly, glycolate oxidase (GOX) is reported to co-localize physically with P8 protein of the rice dwarf phytoreovirus and aid its replication [52]. In similar lines, PVA replication complex proteome contains several glyoxalate and dicarboxylate metabolism components including GOX [46]. The potyviral HCPro forms high molecular weight complexes containing SAMS, viral RNA helicase protein called CI and VPg [5••]. In accordance with these findings, it seems possible that SAMS and SAHH are sequestered in complexes with HCPro and other viral proteins to support virus-specific functions during the infection. Further studies on the potyvirus-linked functions within these assemblies will probably reveal novel insights into the versatility of MTC enzyme functions in plant virus infection. We propose that purification and analysis of the stress-specific large enzyme complexes is an important future direction. Detailed interactomic, proteomic, and metabolomic studies with a focus on the MTC enzymes and viral factors will be instrumental for understanding the metabolic and regulatory connections with a potential to create virus tolerance or resistance in plants.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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