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1 # Epigenetic regulation of sulphur homeostasis in plants

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9 **Running title**: Epigenetic regulation of sulphur homeostasis

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11 **Highlight statement:** We summarize and discuss recent findings on the epigenetic
12 regulation of sulphur homeostasis and response to sulphur deficiency in plants, including
13 DNA methylation, histone modifications and noncoding RNA mediated gene silencing.

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Abstract

Plants have evolved sophisticated mechanisms for adaptation to fluctuating availability of nutrients in soil. Such mechanisms are of importance for plants to maintain homeostasis of nutrient elements for their development and growth. The molecular mechanisms controlling the homeostasis of nutrient elements at the genetic level have been gradually revealed, including the identification of regulatory factors and transporters responding to nutrient stresses. Recent studies have suggested that such responses are not only controlled by genetic regulation but also by epigenetic regulation. In this review, we present recent studies on the involvement of DNA methylation, histone modifications and noncoding RNA mediated gene silencing in the regulation of sulphur homeostasis and response to sulphur deficiency. We also discuss the potential effect of sulphur containing metabolites such as *S*-adenosylmethionine (SAM) on the maintenance of DNA and histone methylation.

Keywords: sulphur, epigenetics, DNA methylation, histone modifications, non-coding RNAs, miRNAs, *S*-adenosylmethionine

46 **Introduction**

47 As one of the essential macronutrients, sulphur (S) plays a pivotal role in plant growth and
48 development. Plants take up S from the rhizosphere in the form of inorganic sulphate. In
49 *Arabidopsis thaliana* (Arabidopsis), this process is mainly driven by two root-specific
50 high-affinity sulphate transporters, SULTR1;1 and SULTR1;2 (Rouached *et al.*, 2008;
51 Takahashi *et al.*, 2000; Yoshimoto *et al.*, 2002). After being transported into root cells,
52 sulphate is either transported into the plastids by SULTR3 sulphate transporters (Cao *et al*.,
53 2013; Chen *et al*., 2019), where it is assimilated into organic sulphur compounds, or
54 transported into the vacuoles for storage. In the sulphur assimilation pathway, sulphate is
55 first activated by ATP sulfurylase (ATPS) to adenosine 5′-phosphosulfate (APS), which is
56 either reduced to sulphite in the primary assimilation pathway or phosphorylated to form
57 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Takahashi *et al.*, 2011). These two
58 reactions are catalyzed by APS reductase (APR) and APS kinase (APK), respectively.
59 PAPS provides an activated sulphate for many sulfation reactions, while sulphite is further
60 reduced to sulphide by sulphite reductase (SiR). Sulphide is condensed with *O*-acetylserine
61 (OAS) by *O*-acetylserine (thiol) lyase (OAS-TL) to form the S-containing amino acid
62 cysteine (Cys). Cys can be used directly for protein biosynthesis or serves as a precursor
63 for the biosynthesis of methionine (Met) and glutathione (GSH). These two molecules can
64 be used for biosynthesis of many sulphur containing derivatives such as glucosinolates and
65 phytochelatins, which are important for plants to alleviate biotic stress and detoxify heavy
66 metals, respectively (Cobbett, 2000; Halkier and Gershenzon, 2006).

67 The transporters responsible for sulphate uptake, and enzymes involved in the S
68 assimilation pathway have been well-characterized (Leustek *et al.*, 2000; Takahashi *et al.*,
69 2011). The regulation of S homeostasis at the genetic level in plants has also been gradually
70 revealed. Such regulation includes the modulation of sulphate acquisition and distribution,
71 S assimilation and the biosynthesis and recycling of sulphur containing compounds at both
72 the transcriptional and posttranscriptional levels. In term of the regulation of sulphate
73 uptake and distribution, several *trans*-acting factors and *cis*-elements have been identified.
74 The most important regulatory factor identified so far is the transcription factor SLIM1
75 (SULFUR LIMITATION 1). SLIM1 regulates the expression of *SULTR1;1* and *SULTR1;2*

76 to activate sulphate uptake in roots, and *SULTR4;2* to release sulphate from vacuoles
77 (Maruyama-Nakashita *et al.*, 2006). Several *cis*-elements responsive to S deficiency have
78 been identified, including the sulphur-responsive element (SURE) in the promoter of
79 *SULTR1;1* (Maruyama-Nakashita *et al.*, 2005), a SURE-like element in the promoter of
80 the wheat *Sulfur deficiency-induced-1* (*sdi-1*) gene (Howarth *et al.*, 2009), the UPE-box in
81 tobacco *UP9C* gene (Wawrzynska *et al.*, 2010), and SURE21A and SURE21B in the 3'-
82 untranslated region of *SULTR2;1* (Maruyama-Nakashita *et al.*, 2015). It appears that
83 SLIM1 does not target directly to the SURE element in the promoter of *SULTR1;1* and
84 *SULTR1;2* though it regulates the expression of these two gens. Rather, SLIM1 forms a
85 homodimer and binds to the UPE-box, which also exists in the promoters of sulphur
86 deficiency induced genes in Arabidopsis, such *LSU*, *APR* and *SULTR2;1* (Wawrzynska *et*
87 *al.*, 2010; Wawrzynska and Sirko, 2016).

88 Similar to the complex regulation of sulphate uptake and distribution, sulphate assimilation
89 is also tightly controlled, being highly regulated by the demand for reduced sulphur, in a
90 regulatory system known as the 'demand-driven' regulatory pathway (Davidian and
91 Kopriva, 2010; Lappartient and Touraine, 1996; Lappartient *et al.*, 1999). However, the
92 molecular mechanisms underlying the regulation of sulphate assimilation remain largely
93 unclear. SLIM1 is likely involved in regulating the expression of *ATPS4* and *SERAT3;1* as
94 these two genes are downregulated in the *slim1* mutant (Maruyama-Nakashita *et al.*, 2006).
95 The transcriptional factor LONG HYPOCOTYL5 (HY5) has been shown to regulate the
96 expression of *APR1* and *APR2* in Arabidopsis by directly targeting the promoters of these
97 two genes (Lee *et al.*, 2011). However, HY5 seems to not regulate the expression of *APR3*,
98 suggesting multiple genetic pathways for the regulation of the reduction of APS. The
99 regulation of the biosynthesis of sulphur containing secondary metabolites such as
100 glucosinolates is much more complex. Many transcription factors, including at least eight
101 MYBs, six MYC-bHLHs, two WRKYs, and a DNA-binding-with-one-finger (DOF)
102 transcription factor OBP2, have been shown to be involved in this process (Frerigmann,
103 2016). Recently, two repressors controlling glucosinolate biosynthesis, *sulfur deficiency*
104 *induced 1* (*SDI1*) and *SDI2* have been identified in Arabidopsis (Aarabi *et al.*, 2016). Under
105 sulphur limited conditions the nuclear localized SDI1 interacts with MYB28, a major
106 transcription factor that promotes glucosinolate biosynthesis, to suppress the biosynthesis

107 of glucosinolates and prioritize sulphate utilisation for primary metabolites (Aarabi *et al.*,
108 2016). The catabolic recycling of organic S compounds such as glucosinolates and GSH is
109 essential for plants to adapt to sulphur limiting conditions. Glucosinolates are thought to
110 function as a sulphur storage pool in plants in the Brassicaceae as their levels fluctuate
111 according to the environmental sulphur status (Falk *et al.*, 2007; Maruyama-Nakashita,
112 2017; Maruyama-Nakashita *et al.*, 2006). Although the catabolic enzymes of
113 glucosinolates and GSH have been identified and well characterized (Bachhawat and
114 Yadav, 2018; Kumar *et al.*, 2012; Kumar *et al.*, 2015; Ohkama-Ohtsu *et al.*, 2008; Paulose
115 *et al.*, 2013; Wittstock and Burow, 2010), the genetic regulation of the breakdown of these
116 compounds is largely unknown. Except SLIM1 which functions as a central transcriptional
117 regulator in the degradation of glucosinolates under sulphur limited conditions
118 (Maruyama-Nakashita *et al.*, 2006), other transcription factors and corresponding targeting
119 *cis*-elements involved in the degradation of glucosinolates and GSH remain to be identified.

120 It is well recognized that the regulation of S homeostasis is under complex genetic control.
121 Emerging evidence suggests that epigenetic regulation of gene expression plays an
122 important role in the adaptive response to S deficiency and the maintenance of S
123 homeostasis (Huang *et al.*, 2016). Epigenetic changes refer to heritable genetic changes
124 resulting from modification of a chromosome without alteration of the DNA sequence
125 (Berger *et al.*, 2009). Epigenetic regulation of gene expression in response to biotic and
126 abiotic stresses, and adaptation to environmental cues, has been gradually revealed (Alonso
127 *et al.*, 2019; Chinnusamy and Zhu, 2009; Lamke and Baurle, 2017; Sahu *et al.*, 2013; Secco
128 *et al.*, 2017). Epigenetic regulation mainly occurs at three levels; DNA methylation, histone
129 modifications, and noncoding RNA regulation. Perhaps the most direct link between S
130 homeostasis and DNA and histone methylation is the fact that S-adenosylmethionine
131 (SAM), a major methyl donor required for many transmethylation reactions, is a sulphur
132 containing compound. In this review, we discuss what is currently known about the
133 regulation of S homeostasis at these three epigenetic levels.

134

135 **DNA methylation**

136 DNA methylation is one of the most well studied epigenetic modifications, playing an
137 important role in the regulation of gene expression, transposon silencing, and imprinting.
138 DNA methylation generally refers to the transfer of a methyl group from SAM to the 5'
139 position of cytosine to form 5-methylcytosine. In plants, DNA methylation occurs in three
140 different sequence contexts CG, CHG and CHH (where H is A, C or T). A specific DNA
141 methylation state in a given genomic region is determined by the dynamic regulation of *de*
142 *novo* DNA methylation, maintenance of DNA methylation and DNA demethylation (Law
143 and Jacobsen, 2010; Zhang *et al.*, 2018). In plants, *de novo* DNA methylation is mediated
144 by the RNA-directed DNA methylation (RdDM) pathway, which requires DNA
145 methyltransferase DOMAINS REARRANGED METHYLASE 2 (DRM2), and many
146 other proteins. The maintenance of DNA methylation during DNA replication depends on
147 the cytosine sequence context, and different DNA methyltransferases are involved. The
148 methylation of symmetric CG is maintained by METHYLTRANSFERASE 1 (MET1), and
149 CHG is maintained by DNA methyltransferase CHROMOMETHYLASE 2 (CMT2) and
150 CMT3, whilst the asymmetric CHH is maintained by DRM2 at RdDM target regions or
151 CMT2 at histone H1-containing heterochromatin. DNA demethylation can be divided into
152 passive and active demethylation, with the former refering to the failure of maintenance of
153 methylation during DNA replication. Such passive DNA demethylation can be due to the
154 shortage of the methyl donor, or loss of function of DNA methyltransferase. Active DNA
155 demethylation is mediated by a base excision repair pathway which requires different
156 bifunctional 5-methylcytosine DNA glycosylases, including REPRESSOR OF
157 SILENCING 1 (ROS1), TRANSCRIPTIONAL ACTIVATOR DEMETER (DME),
158 DEMETER-LIKE PROTEIN 2 (DML2) and DML3. A detailed description of *de novo*
159 DNA methylation, maintenance of methylation, and DNA demethylation, can be found in
160 recent reviews (Law and Jacobsen, 2010; Zhang *et al.*, 2018).

161 Nutrient stresses, such as phosphate starvation (Secco *et al.*, 2015; Yong-Villalobos *et al.*,
162 2015) and zinc deficiency (Chen *et al.*, 2018), have been shown to change the global DNA
163 methylation at the whole genome level. Using whole genome bisulphite sequencing (BS-
164 Seq), changes in DNA methylation, at base level resolution throughout the genome, have
165 been revealed in rice (Secco *et al.*, 2015) and Arabidopsis (Yong-Villalobos *et al.*, 2015).
166 Under phosphate starvation conditions widespread changes in DNA methylation were

167 observed in the rice genome, and such changes mainly derive from the hypermethylation
168 of transposable elements in the vicinity of phosphate starvation inducible genes (Secco *et*
169 *al.*, 2015). Similarly, extensive remodelling of global DNA methylation also occurs in
170 Arabidopsis plants, with some of this DNA methylation remodelling being correlated with
171 changes in the expression of phosphate starvation inducible genes (Yong-Villalobos *et al.*,
172 2015). Although a limited number of changes in DNA methylation were reported in
173 Arabidopsis under phosphate starvation (Secco *et al.*, 2015), this may be due to different
174 treatment conditions and/or different approaches in the identification of differentially
175 methylated regions (Secco *et al.*, 2017). Zinc deficiency also triggers genome-wide
176 differential DNA methylation, with prominent changes in transposable elements (Chen *et*
177 *al.*, 2018). Depletion of the macronutrient nitrogen alters locus-specific DNA methylation
178 patterns, although the changes on genome-wide DNA methylation are currently unknown
179 due to the limitation of the technique used (Kou *et al.*, 2011).

180 Sulphate deficiency is assumed to affect genome-wide DNA methylation in plants because
181 the universal methyl donor for DNA methylation SAM is synthesized from Cys, the first
182 organic sulphur compound in the primary sulphate assimilation pathway. The biosynthesis
183 of SAM can be initiated by the condensation of Cys and *O*-phosphohomoserine (OPH) to
184 form cystathionine (Cyst), which is further converted to homocysteine (Hcy) by
185 cystathionine γ-synthase (CGS) and cystathionine β-lyase (CBL), respectively (Fig. 1)
186 (Hesse and Hoefgen, 2003). Methionine synthase (MS) subsequently converts Hcy to Met
187 using the methyl group from 5-methyltetrahydrofolate (5-CH3-THF), and ultimately Met
188 is converted to SAM catalysed by SAM synthetase (SAMS). The biosynthesis of SAM is
189 tightly controlled and the concentration of SAM is affected by the availability of sulphate.
190 Under sulphate deficient condition, SAM concentration decreases (Nikiforova *et al.*, 2005).
191 Recently, using BS-seq to investigate genome-wide changes in DNA methylation in
192 response to sulphur deficiency, we observed that cytosine methylation levels in all three
193 sequence contexts CG, CHG and CHH decreased in both roots and shoots under sulphate
194 depletion conditions (Fig. 2A). This might be due to a shortage of the methyl donor SAM
195 which potentially lead to enhanced passive DNA demethylation (Zhang *et al.*, 2018).
196 Interestingly, DNA methylation levels tend to increase under phosphate starvation (Fig. 2B)

197 (Yong-Villalobos *et al.*, 2015), suggesting distinct mechanisms in the modulation of
198 genome-wide DNA methylation under different nutrient stresses.

199 During DNA methylation, the methyl group of SAM is transferred to cytosine by a specific
200 DNA methyltransferase, and results in the production of a molecule of *S*-
201 adenosylhomocysteine (SAH). SAH is a strong inhibitor of all known SAM-dependent
202 methyltransferases and is thus rapidly hydrolyzed into Hcy and adenosine by *S*-
203 adenosylhomocysteine hydrolase (SAHH) (Hoffman *et al.*, 1979; Moffatt and Weretilnyk,
204 2001). This reaction is reversible, and the equilibrium is largely driven towards SAH
205 hydrolysis by the rapid removal of Hcy and adenosine. The by-product adenosine is
206 phosphorylated to adenosine monophosphate (AMP) by adenosine kinase (ADK) (Moffatt
207 *et al.*, 2002). Hcy can be re-methylated to Met for biosynthesis of SAM to complete the
208 SAM cycle (Fig. 1). The SAM cycle, as well as the SMM (*S*-methylmethionine) cycle, are
209 two Met recycling systems essential for sustaining the high demand of Met for SAM-
210 dependent transmethylation reactions and also for maintaining the optimized ratio of SAM
211 to SAH (Sauter *et al.*, 2013). The SAM to SAH ratio is generally termed the 'methylation
212 potential' and can be used as a metabolic indicator for the methylation status in cells. The
213 alteration of the SAM to SAH ratio usually leads to changes in global methylation patterns.
214 Partial loss-of-function of *SAHH1* (also known as *HOMOLOGY-DEPENDENT GENE*
215 *SILENCING1*, *HOG1*) leads to increased SAH levels and a decreased SAM to SAH ratio
216 resulting in DNA hypomethylation in Arabidopsis (Ouyang *et al.*, 2012; Rocha *et al.*, 2005).
217 A subset of genes is up-regulated in the hypomethylated *hog1* mutant, which shows a
218 dramatic growth defect (Jordan *et al.*, 2007; Rocha *et al.*, 2005). Reduction of ADK activity
219 in Arabidopsis also increases SAH levels and reduces DNA methylation (Moffatt *et al.*,
220 2002). Both SAHH1 and ADK1 are targeted to the nucleus, and form a complex with a
221 methyltransferase CMT (Lee *et al.*, 2012). Such a protein complex may facilitate the rapid
222 removal of SAH and adenosine to avoid the inhibition of methyltransfereases by SAH.

223 The impairment of SAM biosynthesis itself could also lead to global DNA methylation
224 changes. Mutation of *SAMS3* (also called *METHIONINE ADENOSYLTRANSFERASE 4*,
225 *MAT4*) reduces whole-genome DNA methylation mostly in the CHG and CHH sequence
226 contexts (Meng *et al.*, 2018). The null mutant of *SAMS3* is lethal, and the weak allele
227 mutants accumulate extremely high levels of Met and SAH, and lower levels of SAM (Goto

228 *et al.*, 2002; Meng *et al.*, 2018). Four isoforms of SAMS in Arabidopsis interact with each
229 other and may form homo- and/or hetero-polymers to fulfill the biosynthesis of SAM
230 (Meng *et al.*, 2018). A similar genome-wide DNA hypomethylation was also observed for
231 the other three *SAMS* mutants in Arabidopsis (Meng *et al.*, 2018). Knockdown of three
232 *SAMS* genes in rice by RNA interference reduces DNA methylation at several flowering
233 related genes, and lead to a late-flowering phenotype (Li *et al.*, 2011). Although the effect
234 of the Met and Hcy biosynthesis defect on DNA methylation is largely unexplored in plants,
235 it is assumed that the perturbation of Met and Hcy levels may change SAM levels, and thus
236 modulate the DNA methylation pattern. Indeed, increased plasma Hcy is associated with
237 the elevation of plasma SAH levels, and results in DNA hypomethylation in human (Castro
238 *et al.*, 2003; Yi *et al.*, 2000). This might be due to the fact that high levels of Hcy suppress
239 the expression of *SAHH* and thus elevates the level of SAH (Jiang *et al.*, 2007a; Jiang *et*
240 *al.*, 2007b), which inhibits the activity of most of the SAM-dependent methyltransferases
241 (Hoffman *et al.*, 1979)., Such lines of evidences have suggested that interruption of the
242 SAM cycle alters the genome-wide DNA methylation. However, it is still unclear how
243 global DNA methylation is affected by sulphate assimilation or which step in the
244 assimilation pathway plays the key roles in epigenetic regulation.

245 The one-carbon metabolism pathway plays an important role in epigenetic modifications
246 including DNA methylation. This is because the one-carbon unit carrier 5-methyl
247 tetrahydrofolate (5-CH_3-THF) provides the methyl group for the biosynthesis of Met (Fig.
248 1). 5-CH_3-THF is converted from 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) by
249 methylenetetrahydrofolate reductase (MTHFR) in a NADH-dependent manner (Roje *et al.*,
250 1999). Although the impact of MTHFR on epigenetic modifications is unclear, mutation
251 of MTHFR in maize has been shown to reduce lignin levels which is likely due to a shortage
252 of the methyl donor SAM (Tang *et al.*, 2014). In fact, defects in several steps of folate
253 biosynthesis or turnover have been shown to affect SAM levels and thus change genome-
254 wide DNA methylation (Fig. 1). Suppression of folate biosynthesis by treatment with
255 sulfamethazine, which is a structural analog and competitor of the folate synthesis
256 precursor *p*-aminobenzoic acid (*p*ABA), decreases folate pool size and SAM level, and
257 thus causes a reduction in DNA methylation (Zhang *et al.*, 2012). Inhibition of
258 dihydrofolate reductase (DHFR), which catalyses the conversion of DHF to THF, by

259 methotrexate also decreases the level of SAM, and is thought to lead to genome-wide DNA
260 hypomethylation (Loizeau *et al.*, 2008). The interruption of folate turnover also changes
261 the methylation potential, and alters global DNA methylation. Mutation in the cytoplasmic
262 bifunctional methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate
263 cyclohydrolase (MTHFD1), which is required for the turnover of 5,10-CH_2-THF to THF,
264 causes a strong genome-wide decrease in DNA methylation (Groth *et al.*, 2016). The
265 *mthfd1* mutant accumulates a higher level of Hcy due to impaired folate metabolism, and
266 the increased Hcy level leads to decreased SAHH activity and accumulation of SAH, which
267 competitively inhibits SAM-dependent DNA methylation. Even though both SAM and
268 SAH are increased, the stronger increase in SAH levels leads to an overall decrease in
269 methylation potential, resulting in DNA hypomethylation. Folate polyglutamylation, which
270 is carried out by folylpolyglutamate synthetase (FPGS), is essential for folate affinity,
271 stability and subcellular compartmentation (Hanson and Gregory, 2011; Matherly and
272 Goldman, 2003; Shane, 1989). Folate-dependent enzymes prefer polyglutamylated folates
273 to the monoglutamyl form (Shane, 1989). Mutation of *FPGS1* in Arabidopsis dramatically
274 reduces DNA methylation, and releases chromatin silencing at a genome-wide scale (Zhou
275 *et al.*, 2013). Similar to the *mthfd1* mutant, the Hcy level also significantly increases in the
276 *fpgs1* mutant, following elevation of the SAH level, and the reduction of the methylation
277 potential.

278 We recently identified a high S Arabidopsis mutant and identified the casual gene as *MORE*
279 *SULPHUR ACCUMULATION1* (*MSA1*) (Huang *et al.*, 2016). MSA1 was previously
280 annotated as serine hydroxymethyltransferase 7 (SHM7). Although MSA1 is catalytically
281 inactive *in vitro* and might require other co-factors to facilitate activity, SHM family
282 proteins are believed to catalyse the reversible conversion of serine and THF to glycine and
283 5,-10-CH2-THF (Schirch and Szebenyi, 2005). Mutation of *MSA1* leads to a reduction of
284 cytosine methylation levels in roots and increased levels in shoots, which may be due to
285 lower levels of SAM in roots but slightly increased SAM levels in shoots (Huang *et al.*,
286 2016). Interestingly, a large number of differentially methylated genes (DMGs) were found
287 between the mutant and wild-type (Huang et al., 2016), even though the detailed
288 mechanism underlying the opposite effect of *msa1* on genome-wide DNA methylation
289 between roots and shoots is unclear. Several S-deficiency responsive genes and genes

290 involved in glucosinolate and anthocyanin metabolisms are differentially methylated in
291 *msa1*, including *SULTR1;1*, *SULTR1;2*, *APR3* and *ATPS4*. Methylation in the promoter
292 region of a gene usually inhibits its expression (Zilberman *et al.*, 2007). In Huang et al.,
293 (2016) we found that a 258-bp genomic region 118-bp upstream of the sulphur responsive
294 element (SURE) in the promoter of *SULTR1;1*, which is essential for the S deficiency
295 response (Maruyama-Nakashita *et al.*, 2005), is hyper-methylated under S sufficient
296 condition but is hypo-methylated under S deficiency (Huang *et al.*, 2016). This is correlated
297 with the low expression level of *SULTR1;1* under S sufficient condition and its strong
298 induction by S deficiency. In the *msa1-1* mutant, the upstream region of SURE in the
299 promoter of *SULTR1;1* is hypo-methylated and is associated with the elevation of its
300 expression level and the increase of S levels in shoots (Huang *et al.*, 2016). Similar hypo-
301 and hypermethylations in the vicinity of *cis*-acting elements, such as MBS, P1BS and W-
302 box, in the promoter of phosphate–responsive genes have also been shown to correlate with
303 increased or decreased expression of phosphate responsive genes (Yong-Villalobos *et al.*,
304 2016). Therefore, dynamic DNA methylation particularly in the gene promoter region may
305 represent an important mechanism in regulation of the expression of nutrient deficiency
306 responsive genes.

307 Promoter DNA methylation could repress transcription in two ways (Domcke *et al.*, 2015).
308 First, methylation in the promoter could inhibit the binding of transcriptional activators
309 thus hindering the activation of gene expression. Second, DNA methylation in the promoter
310 could present an epigenetic mark that recruits the binding of transcriptional repressors to
311 the promoter, thus repressing gene expression. Therefore, for nutrient deficiency induced
312 genes such as *SULTR1;1*, DNA methylation in the promoter would inhibit the binding of a
313 transcriptional activator (Fig. 3A) or promote the binding of a transcriptional repressor (Fig.
314 3B), thus keep gene expression at a low level under nutrient sufficient conditions. However,
315 under nutrient deficient condition, the cytosines in the promoter would be demethylated,
316 allowing binding of a transcriptional activator, or releases a transcriptional repressor,
317 leading to the activation gene expression (Fig. 3).

318

319 **Histone modifications**

320 Histones are the protein components of nucleosomes and fundamental units of chromatin.
321 Canonical histones include, histone 2A (H2A), H2B, H3 and H4. A typical nucleosome
322 contains an octameric protein complex consisting of two of these four core histones which
323 are wrapped with 147 base pairs of DNA (Kouzarides, 2007). Histone modifications refer
324 to posttranslational covalent modifications on the amino-terminal tails of these core
325 histones, including methylation, acetylation, phosphorylation, ubiquitination, and many
326 other less investigated modifications (Bannister and Kouzarides, 2011; Kouzarides, 2007;
327 Liu *et al.*, 2010). Such modifications are carried out by specific modifying enzymes ('the
328 writers') to establish different histone marks, which can be recognized and translated by
329 regulatory proteins (the readers/effectors) to trigger downstream signaling events. In
330 certain cases, these histone marks can be removed by particular enzymes ('the erasers')
331 (Liu *et al.*, 2010). Histone modifications alter the accessibility of DNA to the
332 transcriptional machinery, and influence gene expression. In general, histone acetylation
333 and phosphorylation are associated with transcriptional activation, whereas the effect of
334 histone methylation on gene expression is more complicated (Berger, 2007). Histone
335 methylation occurs on lysine and arginine residues at different amino acid positions of H3
336 and H4, in which lysine can undergo mono-, di- or tri-methylation while arginine may be
337 mono-, or di-methylated symmetrically or asymmetrically. Among these diverse histone
338 methylations, methylations on histone H3 lysine-4 (H3K4) and H3K36 are typically
339 associated with active gene transcription, whereas methylation on H3K9 and H3K27
340 generally leads to gene repression (Bannister and Kouzarides, 2011; Liu *et al.*, 2010; Xiao
341 *et al.*, 2016). Dynamic histone modifications maintained by various 'writers' and 'erasers'
342 play critical roles in regulation of gene expression during development, and responding to
343 environmental stimuli including nutrient stresses.

344 Several studies have demonstrated the involvement of histone modifications in modulating
345 the expression of nutrient responsive genes. For example, at the gene body of the
346 Arabidopsis nitrate transporter gene *NRT2.1*, the level of tri-methylation of lysine 27 on
347 histone H3 (H3K27me3) is much higher at high N supply compared to the low N supply,
348 whereas the levels of H3K4me3 and H3K36me3 showed an opposite response (Widiez *et*
349 *al.*, 2011). As mentioned above, H3K27me3 is associated with gene repression while
350 H3K4me3 and H3K36me3 leads to gene activation. Therefore, the deposition of

351 H3K27me3 on the *NRT2.1* locus mediated by HNI9/AtIWS1 is essential for feedback
352 repression of *NRT2.1* by high N supply. The involvement of H3K4me3 in regulation of
353 gene expression under phosphate deficiency was also reported. The H3K4me3 mark can
354 be recognized and bound by a plant homeodomain (PHD)-containing putative transcription
355 factor AL6 which acts as a histone mark reader (Lee *et al.*, 2009). Under phosphate
356 deficient condition, the H3K4s at the promoter-proximal nucleosomes of the MYB
357 transcriptional factor gene *ETC1* are likely tri-methylated. AL6 then binds to the H3K4me3
358 at the *ETC1* locus through its PHD domain and activates the expression of *ETC1*, which
359 might further regulate downstream gene expression and promote root hair elongation
360 during phosphate deficiency (Chandrika *et al.*, 2013a; Chandrika *et al.*, 2013b). Not only
361 methylation on histone 3 is involved in the nutrient stress response, the symmetric
362 dimethylation on histone 4 arginine-3 (H4R3sme2) was also reported to be involved in
363 regulation of Fe homeostasis. Global H4R3sme2 level increase under excess Fe but
364 decrease in the absence of sufficient Fe supply, which requires the Shk1 binding protein 1
365 (SKB1/AtPRMT5), a histone modification 'writer' catalyzing the symmetric dimethylation
366 of histone H4R3 (Fan *et al.*, 2014). SKB1 targets the chromatin of the Ib subgroup bHLH
367 genes (*AtbHLH38*, *AtbHLH39*, *AtbHLH100* and *AtbHLH101*) to regulate their transcription
368 by deposition of H4R3sme2. Although SKB1 does not response to Fe status, the association
369 of SKB1 to the chromatin of Ib subgroup *bHLH* genes and the H4R3sme2 levels on these
370 loci decrease under limited Fe supply, and thus enhance the expression of these genes in
371 order to enhance Fe uptake (Fan *et al.*, 2014). Besides histone methylation, histone
372 acetylation might also regulate expression under phosphate starvation. Knockdown of a
373 histone deacetylase HDA19, which acts as a histone acetylation 'eraser', alters the
374 expression of a subset of genes involved in the phosphate starvation response (Chen *et al.*,
375 2015).

376 Although there is no direct evidence to support histone modifications involvement in
377 regulation of sulphur homeostasis, histone methylations and acetylations are found in many
378 genes involved in sulphate uptake and assimilation in Arabidopsis (Table 1), including
379 H3K27me3 (Zhang *et al.*, 2007), H3K4me3 and H3K36me3 (Luo *et al.*, 2013), H3K23ac
380 and H4K16ac (Lu *et al.*, 2015), and H3K9ac (Zhou *et al.*, 2010). Therefore, it can be
381 assumed that histone modification may also play a role in maintaining sulphur homeostasis.

382 In fact, the interruption of the SAM cycle, which leads to abnormal SAM to SAH ratio,
383 affects histone methylation (Fig. 1). Mutations of *FPGS1*, *MTHFD1* and *SAMS3*, which all
384 lead to lower SAM to SAH ratios, not only reduce global DNA methylation but also
385 decrease H3K9me2 levels (Groth *et al.*, 2016; Meng *et al.*, 2018; Zhou *et al.*, 2013).
386 Furthermore, elevation of SAH has been shown to decrease the methylation of histone H3
387 at the arginine 8 (H3R8me2a) site in brain of hyperhomocysteinemic rats (Esse *et al.*, 2013),
388 and methylation of H4R3me2a in the liver of cystathionine β-synthase-deficient mice (Esse
389 *et al.*, 2014).

390

391 **Noncoding RNA regulation**

392 Noncoding RNAs (ncRNAs) refer to functional RNA transcripts that do not code for
393 proteins. ncRNAs comprise different groups of transcripts, including the ribosomal RNAs,
394 transfer RNAs, and regulatory ncRNAs that play critical roles in transcriptional and post-
395 transcriptional regulation in eukaryotes. According to their length, ncRNAs can be divided
396 into small ncRNAs (sRNAs), and long ncRNAs (lncRNAs). The micro RNAs (miRNAs),
397 and small interfering RNAs (siRNAs), are two main groups of small regulatory RNAs with
398 different biogenesis processes and functions (Axtell, 2013). ncRNAs that are longer than
399 200 nucleotides are generally considered as lncRNAs (Kapranov *et al.*, 2007). Many
400 lncRNAs function as regulators of gene expression during development and responses to
401 environmental stimuli (Kim and Sung, 2012), though very recent studies suggest some
402 individual lncRNAs may not function as previously thought (Goudarzi *et al.*, 2019). An
403 example of lncRNA responding to nutritional stress is *INDUCED BY PHOSPHATE*
404 *STARVATIONA* (*IPS1*), which prevents the cleavage of *PHO2* by miRNA399 through a
405 target mimicry mechanism (Bari *et al.*, 2006). lncRNAs responsive to sulphur deprivation
406 have been identified in microalgae *Chlamydomonas reinhardtii* (Li *et al.*, 2016). However,
407 lncRNAs are largely unexplored in plants, and their function in regulation of S homeostasis
408 is still unknown though some of lncRNAs are conserved among species (Li *et al.*, 2016).
409 Similarly, the involvement of siRNAs in the response to S deficiency and maintenance of
410 S homeostasis is less studied in plants. Here, we focus on the functions of miRNAs in
411 regulation of gene expression in the maintenance of S homeostasis.

412 miRNAs are major post-transcriptional regulators of gene expression through guiding the
413 degradation of target mRNAs and/or inhibiting the translation of target genes (Axtell, 2013;
414 Jones-Rhoades et al., 2006). More than three hundred miRNAs have been identified in
415 Arabidopsis by computational and experimental approaches, including those responding to
416 nutrient deprivation (Kozomara and Griffiths-Jones, 2011). Among these miRNAs, the
417 expression of 32 miRNAs was found to be down- or up-regulated under S deficient
418 condition, accounting for approximately 10% of the total miRNAs identified in
419 Arabidopsis so far (Liang *et al.*, 2015). miR395 is one of the most well investigated
420 miRNAs in response to S deficiency, and plays a central role in sulphate assimilation and
421 allocation. miR395 was first identified by a computational approach, and was confirmed
422 experimentally to be highly induced by sulphur starvation (Jones-Rhoades and Bartel,
423 2004). Such induction requires redox signalling as the S deprivation induction of miR395
424 is compromised in the GSH biosynthesis mutant *cad2* and the thioredoxin reductase double
425 mutant *ntra ntrb*, which are defective in glutaredoxin- and thioredoxin-dependent redox
426 signaling, respectively (Jagadeeswaran *et al.*, 2014). Furthermore, external
427 supplementation of GSH suppresses the induction of miR395 by S deprivation.

428 miR395 was predicted to target three ATP sulfurylase genes (*ATPS1*, *ATPS3* and *ATPS4*),
429 and a low-affinity sulphate transporter *SULTR2;1* in Arabidopsis (Jones-Rhoades and
430 Bartel, 2004). The cleavage of these four target genes by miR395 was validated
431 experimentally in different tissues (Allen *et al.*, 2005; Jagadeeswaran *et al.*, 2014; Jones-
432 Rhoades and Bartel, 2004; Kawashima *et al.*, 2009). Overexpression of the *MIR395* gene
433 strongly suppresses the accumulation of transcripts of these four genes and increases the
434 sulphate level in shoots. Furthermore, knockout of *ATPS1* and *SULTR2;1*, and knockdown
435 of *ATPS4*, simultaneously phenocopies the high sulphate level of miR395-over-expressing
436 plants, supporting the notion that miR395 targets to *ATPS1*, *ATPS4* and *SULTR2;1* (Liang
437 *et al.*, 2010). Although the cleavage of target genes by miR395 is clear, the transcript levels
438 of the four target genes are not always negatively correlated with the level of miR395.
439 miR395 is strongly induced by sulphate starvation in both roots and shoots, whereas the
440 transcript levels of the four target genes show distinct responses to sulphate deficiency in
441 roots and shoots. *ATPS4* shows a canonical regulation by miR395 as its expression
442 decreases in both roots and shoots following the induction of miR395 by sulphate

443 starvation (Jagadeeswaran *et al.*, 2014; Liang *et al.*, 2010). Target mimics of miR395 also
444 leads to over-accumulation of *ATPS4* transcripts under both sulphate sufficient and
445 deficient conditions (Kawashima *et al.*, 2011). The transcript levels of *ATPS1*, *ATPS3* and
446 *SULTR2;1* in shoots decrease in response to sulphate deficiency as expected
447 (Jagadeeswaran *et al.*, 2014). However, in roots under sulphate deficient condition, *ATPS1*
448 and *ATPS3* maintain consistent expression levels (Jagadeeswaran *et al.*, 2014), or are
449 slightly induced, depending on the period of sulphate deficiency (Kawashima *et al.*, 2011;
450 Liang *et al.*, 2010). *SULTR2;1* is consistently strongly induced by sulphate deficiency in
451 roots, even though miR395 is also induced (Jagadeeswaran *et al.*, 2014; Kawashima *et al.*,
452 2011; Liang *et al.*, 2010). The positive correlation between miR395 and *SULTR2;1*
453 expression in roots is due to their non-overlapping expression pattern in the root vascular
454 tissues. *SULTR2;1* is specifically expressed in the xylem parenchyma and pericycle cells,
455 whereas the expression of miR395 is restricted in phloem companion cells, which leaves
456 the target mRNA of *SULTR2;1* intact (Kawashima *et al.*, 2009).

457 There are four *ATPS* genes in the Arabidopsis genome. *ATPS1*, *3* and *4* encode the plastid-
458 localized isoforms, whereas *ATPS2* dually encodes plastidic and cytosolic isoforms
459 (Hatzfeld *et al.*, 2000; Rotte and Leustek, 2000; Bohrer *et al.*, 2015). The plastidic isoforms
460 function in the initial activation of sulphate for assimilation into cysteine, while the
461 cytosolic ATPS2 is involved in sulphation reaction for biosynthesis of glucosinolates
462 (Hatzfeld *et al.*, 2000). Interestingly, miR395 only targets plastidic isoform genes, but not
463 the cytosolic *ATPS2*, indicating that miR395 specifically regulates sulphate assimilation in
464 plastids, but not in the cytosol. Therefore, miR395 plays an important role in sulphate
465 assimilation and root-to-shoot translocation of sulphate by regulating mRNA levels of ATP
466 sulfurylase genes and *SULTR2;1*. Such regulation seems to be conserved among different
467 species, such as rice (Guddeti *et al.*, 2005; Jagadeeswaran *et al.*, 2014; Yuan *et al.*, 2016)
468 and *Brassica napus* (Huang *et al.*, 2010). miR395 is also induced in response to heavy
469 metals such as arsenic (As) and copper (Cu), and is suppressed by nitrogen and carbon
470 deficiency, suggesting broad functioning of miR395 in the regulation of gene expression
471 in response to nutrient stresses (Jagadeeswaran *et al.*, 2014; Liang *et al.*, 2015).
472 Interestingly, under phosphate limiting conditions miR399 is involved in the regulation of
473 phosphate uptake and translocation through the targeting of *PHO2* to maintain phosphate

474 homeostasis (Chiou *et al.*, 2006; Fujii *et al.*, 2005), further highlighting the importance of
475 miRNAs in regulation of adaptation in response to nutrient deficiency.

476

477 **Conclusions and future perspectives**

478 Emerging evidence is starting to indicate the important roles of epigenetic regulation in
479 controlling responses to nutrient stresses, and the maintenance of nutrient homeostasis in
480 plants. miRNAs mediated gene silencing which is well-established to participate in the
481 regulation of sulphate uptake and assimilation, whereas the examples of the involvement
482 of DNA methylation and histone modifications in regulation of S homeostasis are still
483 limited. Given that the universal methyl group donor SAM is derived from sulphate in
484 plants, the reduction of SAM levels either due to impairment of its biosynthesis, or the
485 interruption of folate metabolism, all leads to alterations in genome-wide DNA methylation,
486 and in some cases also changes in histone methylation (Fig. 1). Therefore, a tight link
487 between sulphur metabolism and DNA and histone methylation appears to exist in plants.
488 Indeed, mutation of *MSA1*/*SHM7* leads to a reduction of SAM levels and alters global DNA
489 methylation, including the methylation level of several S homeostasis related genes, which
490 triggers S deficiency response and enhances sulphate uptake and assimilation in the *msa1-*
491 *1* mutant (Huang *et al.*, 2016). Such enhancement of sulphate uptake and assimilation may
492 be a feedback response to the reduction of SAM levels observed in this mutant. It is
493 therefore necessary to detect whether a similar S deficiency response occurs in those folate
494 metabolism related mutants with alteration of DNA and histone methylation due to the
495 shortage of SAM. Several enzymes involved in SAM biosynthesis or metabolism have
496 isoforms localized to the nucleus, including SAMS1/2/3 (Mao *et al.*, 2015; Meng *et al.*,
497 2018) and MSA1 (Huang *et al.*, 2016) for SAM biosynthesis, and SAHH1 and ADK1 for
498 recycling SAM (Lee *et al.*, 2012). It is therefore likely that SAM is synthesized in the nuclei
499 to locally sustain the methyl group for DNA and histone methylation (Huang et al., 2016).
500 The perturbation of such a nuclear SAM pool may then trigger S deficiency responses
501 through unknown signalling pathways.

502 Several studies have demonstrated that dynamic DNA methylation at *cis*-elements in
503 promoter regions may influence the expression of nutrient responsive genes such as

504 *SULTR1;1* (Huang *et al.*, 2016) and several phosphate starvation responsive genes (Yong-
505 Villalobos *et al.*, 2016; Yong-Villalobos *et al.*, 2015). Such a relationship between gene
506 expression and DNA methylation is largely based on their correlation, which might not
507 necessarily reflect causality. With the development of epigenome editing tools that enable
508 the specific methylation or demethylation of targeted cytosine residues in the promoter of
509 the genes of interest (Gallego-Bartolome *et al.*, 2018; Gallego-Bartolome *et al.*, 2019), it
510 is now possible to reliably establish the causality of DNA methylation status and
511 transcriptional activity. Furthermore, most studies usually assess DNA methylation in
512 whole roots and shoots or even in whole plants, which may mask functionally important
513 heterogeneity among different cell types. Unique patterns of DNA methylation in specific
514 cell types, or in a single cell, have been revealed (Kawakatsu *et al.*, 2016; Li *et al.*, 2019).
515 It is thus necessary to determine cell-type specific or even single cell DNA methylation
516 profiles to link DNA methylation and gene expression. The application of single cell
517 methylome analysis techniques and precise epigenome editing tools will enable functional
518 analyses of DNA methylation in gene expression, and allow the direct demonstration of its
519 role in response to nutrient stresses.

520

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Figure legend

Fig. 1. The interconnection of sulphate assimilation, folate metabolism and the SAM cycle with the DNA and histone methylation. The sulphate uptake and assimilation pathway, the biosynthesis and turnover of folate and the SAM cycle were shown in the background in light green, light blue and orange, respectively. Interruption of pathways with enzymes highlighted in blue and red alters genome-wide DNA methylation, and mutation of

enzymes in red change histone methylation. Abbreviations for enzymes: ADK, adenosine kinase; APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; DHFR, DHF reductase; DHFS, DHF synthase; DHPS, DHP synthase; γ-ECS, γ-glutamylcysteine synthetase; FPGS, folylpolyglutamate synthase; GSHS, glutathione synthetase; MS, methionine synthase; MTHFD1, bifunctional methylene THF dehydrogenase/methenyl THF cyclohydrolase; OAS-TL, OAS(thiol)lyase; SAHH, SAH hydrolase; SAMMT, SAM-dependent methyltransferase; SAMS, SAM synthetase; SAT, serine acetyltransferase; SHM, serine hydroxymethyltransferase; SiR, sulphite reductase; SOT, sulfotransferase; SULTR, sulphate transporter; SYN, 10-formyl THF synthetase. Abbreviations for compounds: Ado, adenosine; AMP, adenosine monophosphate; APS, adenosine 5'-phosphosulfate; Cys, cysteine; Cyst, cystathionine; DHF, dihydrofolate; DHP, dihydropteroate; Glun, polyglutamate; Hcy, homocysteine; Met, methionine; OAS, O-acetylserine; pABA, UDP-glucose-p-aminobenzoate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Ser, serine; THF, tetrahydrofolate; γ-GluCys, γ-glutamylcysteine.

Fig. 2. Whole genome methylation levels of *Arabidopsis* under sulphate and phosphate starvation conditions. (**A**) Methylation levels at all cytosines in the genome (Total C) and the CG, CHG and CHH sequence context under +S and -S conditions. Methylation level was determined by whole genome bisulfite sequencing (BS-Seq) of the shoots and roots of plants grown on MGRL agar media with 1.5 mM sulphate (+S) or without added sulphate (-S) for two weeks. (**B**) Methylation levels at all cytosines in the genome (Total C) and the CG, CHG and CHH sequence context under +Pi and -Pi conditions. Data were derived from Yong-Villalobos et al. (2015) and recalculated based on the raw data. Plants were grown hydroponically with 1 mM phosphate for 7 days and then transferred to hydroponic media containing 1 mM (+Pi) or 5 μM phosphate (-Pi) to be grown subsequently for 16 days. Methylation level was determined by BS-Seq of the shoots and roots, respectively.

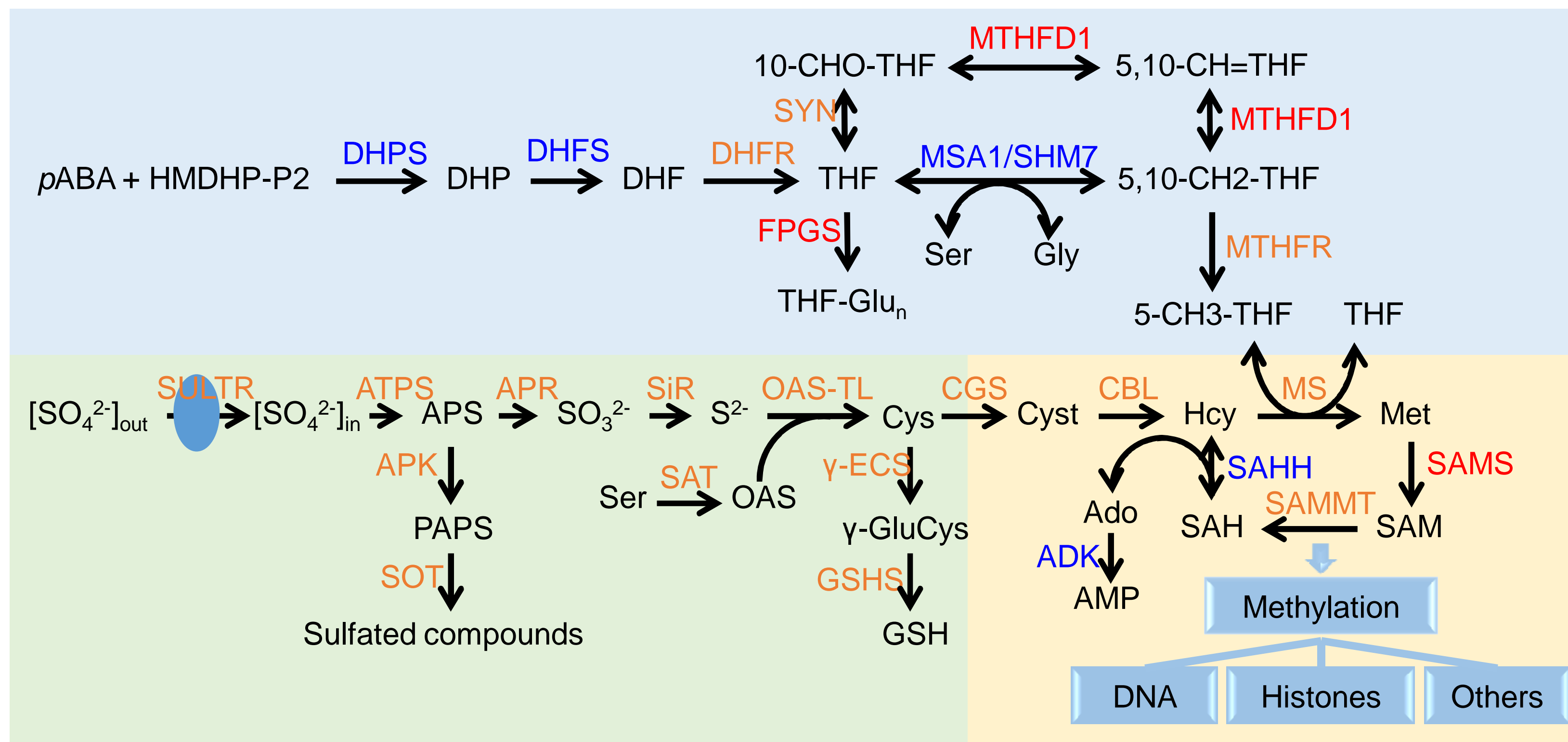
Fig. 3. A potential model of dynamic DNA methylation in regulation of gene expression. The nutrient deficiency responsive genes are methylated at the *cis*-element in the promoter under sufficient nutrient condition. The methylation may prevent the binding of the transcriptional activator (TA) [upper panel in (A)] or recruit the transcriptional repressor

(TR) [upper panel in (A)] and thus inhibits gene expression. However, under nutrient deficient condition, the hypo-methylated promoter allows the binding of transcriptional activator to promote the transcription.

Table 1. Histone modifications of genes involved in sulphate uptake and assimilation.

Gene ID	Gene symbol	Histone modifications					
		H3K27me3 [a]	**H3K4me3 [b]**	**H3K36me3 [b]**	**H3K23ac [c]**	**H4K16ac [c]**	**H3K9ac [d]**
Sulphate transporter							
At4g08620	*SULTR1;1*						
At1g78000	*SULTR1;2*						
At1g22150	*SULTR1;3*						
At5g10180	*SULTR2;1*						
At1g77990	*SULTR2;2*						
At3g51895	*SULTR3;1*						
At4g02700	*SULTR3;2*						
At1g23090	*SULTR3;3*						
At3g15990	*SULTR3;4*						
At5g19600	*SULTR3;5*						
At5g13550	*SULTR4;1*						
At3g12520	*SULTR4;2*						
ATP sulfurylase							
At3g22890	*ATPS1*						
At1g19920	*ATPS2*						
At4g14680	*ATPS3*						
At5g43780	*ATPS4*						
APS reductase							
At4g04610	*APR1*						
At1g62180	*APR2*						
At4g21990	*APR3*						
APS kinase							
At2g14750	*APK1*						
At4g39940	*APK2*						
At3g03900	*APK3*						
At5g67520	*APK4*						
Sulfite reductase							
At5g04590	*SiR*						
Serine acetyltransferase							
At5g56760	*SERAT1;1*						
At1g55920	*SERAT2;1*						
At3g13110	*SERAT2;2*						
At2g17640	*SERAT3;1*						
At4g35640	*SERAT3;2*						
O-acetylserine (thiol)lyase							
At4g14880	*OASTL-A1*						
At3g59760	*OASTL-C*						
At2g43750	*OASTL-B*						
At3g22460	*OASTL-A2*						
Cysteine synthase							
At3g03630	*CS26*						
At3g04940	*CYSD1*						
At3g61440	*CYSC1*						
At5g28030	*CYSD2*						

Whole genome analysis of histone modifications was carried out by using chromatin immunoprecipitation (ChIP) coupled with high-density whole genome tiling microarrays (ChIP-chip), or ChIP coupled with high throughput sequencing (ChIP-seq). Genes involved in sulphate uptake and assimilation were extracted and shown in Table 1. Cells in grey background mean the presence of histone modifications. Data from: [a] Zhang *et al*., 2007; [b] Luo *et al*., 2013; [c] Lu *et al*., 2015; [d] Zhou *et al*., 2010.



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Abbreviations for enzymes: ADK, adenosine kinase; APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; CBL, cystathionine β-lyase; CGS, cystathionine γ-synthase; DHFR, DHF reductase; DHFS, DHF synthase; DHPS, DHP synthase; γ-ECS, γ-glutamylcysteine synthetase; FPGS, folylpolyglutamate synthase; GSHS, glutathione synthetase; MS, methionine synthase; MTHFD1, bifunctional methylene THF dehydrogenase/methenyl THF cyclohydrolase; OAS-TL, OAS(thiol)lyase; SAHH, SAH hydrolase; SAMMT, SAM-dependent methyltransferase; SAMS, SAM synthetase; SAT, serine acetyltransferase; SHM, serine hydroxymethyltransferase; SiR, sulphite reductase; SOT, sulfotransferase; SULTR, sulfate transporter; SYN, 10-formyl THF synthetase.

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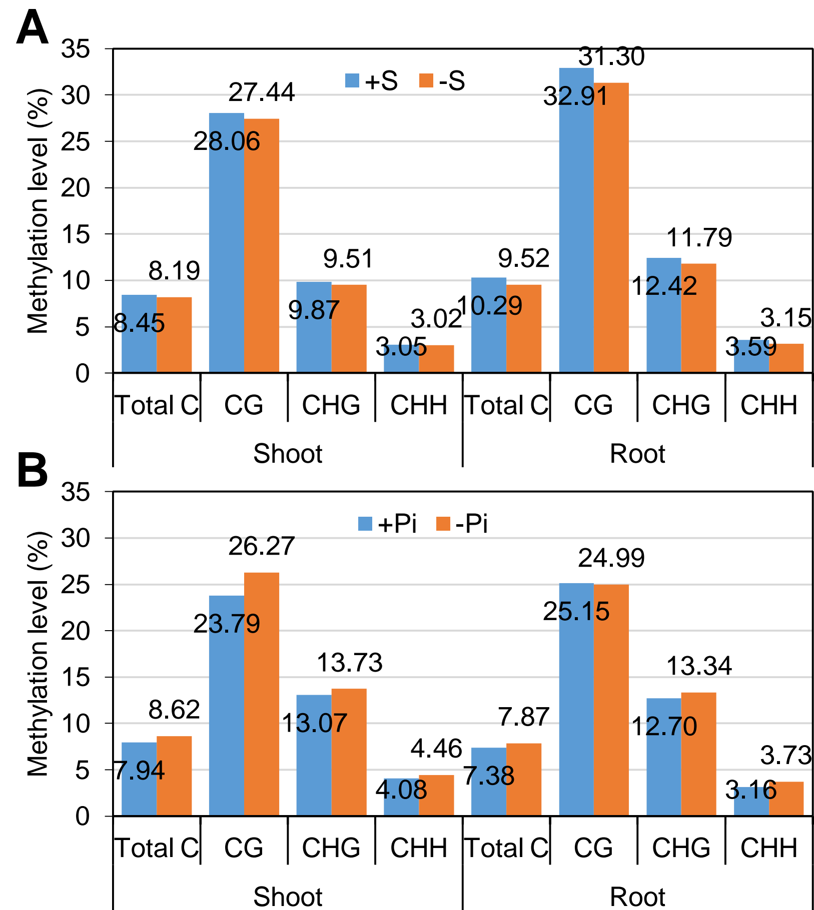

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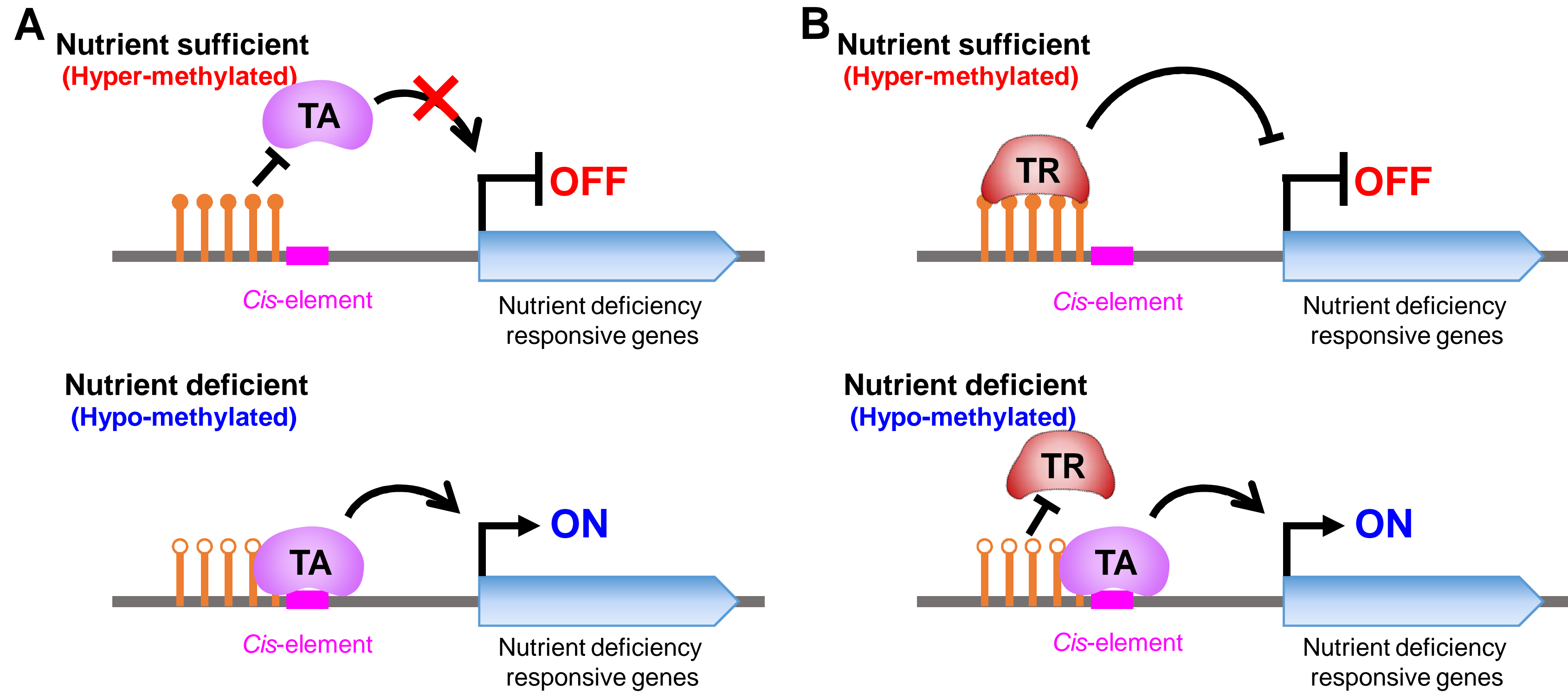

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