Ancient coding sequences underpin the spatial patterning of gene expression in C_4 leaves

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Abstract

Photosynthesis is compromised in most plants because an enzymatic side-reaction fixes O_2 instead of CO_2. The energetic cost of oxygenation led to the evolution of C_4 photosynthesis. In almost all C_4 leaves compartmentation of photosynthesis between cells reduces oxygenation and so increases photosynthetic efficiency. Here we report that spatial expression of most C_4 genes is controlled by intragenic *cis*-elements rather than promoter sequence. Two DNA motifs that co-operatively specify the patterning of genes required for C_4 photosynthesis are identified. They are conserved in plants and algae that use the ancestral C_3 pathway. As these motifs are located in exons they represent duons determining both gene expression and amino acid sequence. Our findings provide functional evidence for the importance of transcription factors recognising coding sequence as previously defined by genome-wide binding studies. Furthermore, they indicate that C_4 evolution is based on ancient DNA motifs found in exonic sequence.

Introduction

Photosynthesis allows atmospheric CO_2 to be fixed into organic molecules and therefore forms the basis of life on the planet. When plants moved onto land they inherited the photosynthetic system first developed by bacteria, in which the enzyme Ribulose Bisphosphate Carboxylase Oxygenase (RuBisCO) generates the three-carbon compound phosphoglyceric acid (PGA) (Anbar et al., 2007). As PGA contains three carbon atoms, this form of photosynthesis is known as the C_3 pathway. However, a side-reaction of RuBisCO fixes O_2 rather than CO_2, and this generates the toxic compound phosphoglycolate. Although plants use the photorespiratory pathway to remove phosphoglycolate, it is energetically expensive and some carbon is lost in the process (Bauwe et al., 2010). Around 30 million years ago, some plants evolved a photosynthetic system in which CO_2 is concentrated around RuBisCO such that oxygenation is minimised, and so photosynthetic efficiency increases by around 50% (Hatch and Slack 1966; Sage et al., 1999). These species now represent the most productive vegetation on the planet (Sage et al., 2004; Ray et al., 2012), and because they initially generate a C_4 acid in the photosynthetic process, are known as C_4 plants.

The mechanism by which CO_2 supply to RuBisCO is increased in C_4 species depends on the spatial separation of photosynthetic reactions. Initial production of C_4 acids takes place in one compartment, and then their re-release to concentrate CO_2 occurs in another. Although in some species this can take place within a single cell (Edwards et al., 2004), in the majority of C_4 plants, evolution has co-opted the existing compartmentation afforded by multi-cellularity to separate these carboxylation and decarboxylation reactions (Hatch and Slack 1966). The separation of photosynthesis between cells requires the co-ordinated regulation of numerous enzymes and transporters. For example, in mesophyll (M) cells, enzymes such as carbonic anhydrase, phospho*enol*pyruvate carboxylase and malate dehydrogenase allow the production of C_4 acids from HCO_3^-, whereas in the bundle sheath (BS), high activities of C_4 acid decarboxylases and RuBisCO allow efficient entry of carbon into the Calvin-Benson cycle (Furbank, 2011). The importance of each enzyme accumulating in the correct cell-type is considered critical for the efficiency of the pathway, and so the spatial patterning of photosynthesis gene expression in C_4

55 plants has received significant attention. As with most other eukaryotic systems, although post-
56 transcriptional and translational regulation are acknowledged (Hibberd and Covshoff 2010; Kajala
57 et al., 2011; Williams et al., 2016; Fankhauser and Aubry, 2016), most analysis has focussed on
58 the importance of promoters in regulating gene expression in M or BS cells (Sheen, 1991; Viret et
59 al., 1994; Taniguchi et al., 2000; Nomura et al., 2000; Kaush et al., 2001; Gowik et al., 2004;
60 Akyildiz et al., 2007). Previously, we found that expression of multiple *NAD-dependent MALIC*
61 *ENZYME (NAD-ME)* genes in the BS of C_4 *Gynandropsis gynandra* was dependent on sequences
62 not found in promoter elements upstream of these genes, but rather in exonic sequence within the
63 gene (Brown et al 2011). Whilst the exact *cis*-elements were not defined, orthologous genes from
64 the C_4 model *Arabidopsis thaliana* also contained the regulatory DNA necessary for preferential
65 expression in BS cells of the C_4 leaf. Overall, these data implied that evolution has repeatedly
66 made use of pre-existing regulatory DNA found within genic sequence to pattern gene expression
67 in C_4 leaves. However, the specific sequences responsible for preferential expression in the BS
68 were not defined, and so it was not clear if exactly the same *cis*-elements were used by each gene.
69 Furthermore, without a definition of the motifs specifying expression in the BS contained within
70 these *NAD-ME* genes, it was not possible to identify if they control expression of additional genes,
71 nor to understand if these same elements are used in other species. To address this, we first
72 identified the sequence motifs responsible for patterning these *NAD-ME* genes, and in doing so,
73 show that they act as duons impacting both on patterns of gene expression as well as amino acid
74 sequence of the encoded protein. We used these sequence motifs to predict and validate other
75 genes that are also controlled by these elements, and also to investigate the likely origin of such
76 regulation within land plants. We report both widespread use of, and ancient origins for, two *cis*-
77 elements that act within coding sequence co-operatively to generate BS expression in C_4 leaves.
78 We place these findings in the context of genome-wide studies reporting the widespread binding of
79 transcription factors to genic sequence rather than promoter elements, as well as the polyphyletic
80 evolution of the complex C_4 pathway.

Results

Two motifs within coding sequence act co-operatively to generate BS specificity

To better understand the mechanism responsible for generating preferential gene expression in BS cells analysis was focussed on a region of 240 nucleotides within the coding region of *GgNAD-ME1*. Although previous work showed this fragment confers BS accumulation of GUS in *G. gynandra* (Brown et al., 2011) it was unclear if this was due to recognition of the DNA or RNA sequence. To test if regulation was lost when a complementary mRNA was produced, an antisense construct for this 240 nucleotide sequence (Figure 1A) was placed under control of the constitutive CaMV35S promoter, and fused to *uidA* encoding the β-glucoronidase (GUS) reporter. Whereas the CaMV35S promoter alone lead to similar accumulation of GUS in M and BS cells (Figure 1B), microprojectile bombardment of the antisense construct maintained preferential accumulation in the BS (Figure 1B) indicating that DNA sequence is recognised by *trans*-acting factors and therefore that preferential accumulation in BS cells is regulated during transcription.

To identify the specific nucleotides responsible for BS expression within this 240bp fragment a deletion series was generated by removing fragments from either the 5' or 3' end of *GgNAD-ME1* (Figure 1A). Deletion of 24, and 78 nucleotides from the 3' end did not affect preferential accumulation in BS cells (Figure 1A-B), but removal of 90 nucleotides resulted in loss of cell specificity (Figure 1A-C). Similarly, deletion of the first 63 nucleotides from the 5' end did not abolish preferential accumulation of GUS in BS cells, but removing 78 nucleotides did (Figure 1A-C). A fragment incorporating bases 64 to 162 was sufficient to retain cell preferential accumulation in the BS both after microprojectile bombardment (Figure 1B), and after production of stable transformants (Figure 1C, S2). We conclude that one region composed of the nucleotides TTGGGTGAA (64 to 79 downstream of the translational start codon) and a second region made up of nucleotides GATCCTTG (141 to 162 nucleotides downstream of the translational start codon) are necessary for preferential accumulation of *GgNAD-ME1* in BS cells of C_4 *G. gynandropsis*. These two regions will hereafter be referred to as Bundle Sheath Motif 1a (BSM1a) and Bundle Sheath Motif Ib (BSM1b), and they are separated by 75 nucleotides.

108 To test if the sequence separating BSM1a and BSM1b is required for preferential expression in
109 BS cells, it was replaced with exogenous sequence lacking homology to the native region of
110 *GgNADME1* (Figure 2A). The fragment that contained BSM1a and BSM1b separated by this
111 exogenous sequence led to preferential accumulation of GUS in BS cells (Figure 2A, 2B). Although
112 the exact sequence separating BSM1a and BSM1b does not impact on their function, the distance
113 separating them could play an important role. The length of the spacer was therefore modified, and
114 this indicated that BSM1a and BSM1b do not generate preferential accumulation in the BS cells
115 when fused together directly, or when separated by 999 base pairs (Figure S3). However, when
116 the intervening sequence was between 21 and 550 base pairs preferential accumulation in BS
117 cells occurred (Figure S3). Site-directed mutagenesis of each motif showed that the first two
118 nucleotides of BSM1a had no impact on preferential accumulation of GUS in the BS, but that
119 substitution of the guanine at position 3 and thymine at position 6 abolished BS accumulation of
120 GUS (Figure 2B). Similarly, three and five base pair substitutions in BSM1b resulted in a decrease
121 of cell specificity (Figure 2B). Based on these results we propose that within the coding region of
122 *NAD-ME1,* two separate sequences separated by a spacer are necessary and sufficient to
123 generate strong expression in BS cells.
124

125 **BSM1a and 1b specify the spatial patterning of additional genes**

126 Although thousands of genes are differentially expressed between M and BS cells of C_4 plants,
127 to our knowledge no DNA motifs that determine the patterning of more than one gene in BS cells
128 have been identified. To test whether BSM1a and BSM1b operate more widely to generate
129 preferential expression in BS cells, the coding sequences of other genes were scanned using
130 FIMO (Grant et al., 2011). Sequences similar to BSM1a and BSM1b in genes annotated
131 mitochondrial *MALATE DEHYDROGENASE* (*mMDH*) and *GLYCOLATE OXIDASE 1* (*GOX1*) were
132 identified. In both cases, fragments from *mMDH* and *GOX* containing the two motifs were sufficient
133 to drive BS accumulation of GUS in *G. gynandra* (Figure 3A), and when they were deleted
134 preferential accumulation in BS cells was lost (Figure 3A). The identification of BSM1a and BSM1b

135 in these additional genes allowed consensus sequences to be defined (Figure 3B). These data
136 imply that the DNA sequences defined by BSM1a and BSM1b form the basis of a regulon that
137 operates through conserved *cis*-elements located in the exons of multiple genes to generate
138 preferential expression in BS cells of C_4 leaves. Altogether these results suggest multiple gene
139 families involved in C_4 photosynthesis and photorespiration have been recruited into the BS
140 (Figure 3C) using a regulatory network based on these two motifs.
141

142 **BSM1a and BSM1b are ancient and conserved within land plants**

143 Using the sequences that define BSM1a and BSM1b (Figure 3B), and the minimum and
144 maximum distance that can separate them, *NAD-ME* genes from *G. gynandra* and the closely
145 related C_3 species *A. thaliana* were assessed. Similar sequences close to the predicted
146 translational start site of *GgNAD-ME2, AtNAD-ME1* and *AtNAD-ME2* were identified (Figure 4A).
147 BSM1a is located in the predicted mitochondrial transit peptide and its position varies relative to
148 the translational start site. It is noteworthy that compared with *AtNAD-ME2* and *CgNAD-ME1 & 2*,
149 BSM1a is found on the opposite DNA strand in *AtNAD-ME1*, further supporting the notion that BS
150 preferential expression is mediated by a transcription-based mechanism. BSM1b is located in the
151 mature processed protein and its position appears invariant (Figure 4A). When either of these
152 motifs was removed from *GgNAD-ME2, AtNAD-ME* or *AtNAD-ME2* preferential accumulation in BS
153 cells was lost (Figure 4B). These data indicate that the consensus sequences defined by BSM1a
154 and BSM1b from these eight genes (Figure 4C) are necessary and sufficient to generate BS
155 expression in the C_4 leaf.

156 As BSM1a and BSM1b are present in *NAD-ME* genes of C_3 *A. thaliana*, we next investigated
157 the extent to which these sequences are conserved across 1135 wild inbred *A. thaliana* accessions
158 with genome sequence available. Single nucleotide polymorphism (SNP) data were retrieved
159 (1001 Genomes Consortium, 2016), and analysis showed an unexpectedly high level of
160 conservation with no SNPs detected within either BSM1a or BSM1b (Figure 5A&B). This high level
161 of conservation is consistent with both Motifs acting as "duons" in C_3 *A. thaliana* as well as C_4 *G.*

162 *gynandra*. To investigate whether these motifs are also found more widely in *NAD-ME* genes
163 across the land plant phylogeny, *NAD-ME* gene sequences were retrieved from 44 species in
164 Phytozome (v10.1, www.phytozome.org) and analysed for the presence of BSM1a and BSM1b
165 (Figure S4). All dicotyledons contained at least one *NAD-ME* gene carrying the sequences that
166 define BSM1a and BSM1b (Figure 5C). In the monocotyledons, BSM1a was completely conserved
167 in rice, *Brachypodium* and *Panicum*. Although BSM1b showed one nucleotide substitution in all
168 monocotyledenous genomes available it appears more ancient as it is conserved in spikemoss and
169 moss (Figure 5C, S4). The hypothesis that BSM1b is more ancient is supported by the finding that
170 a version with one nucleotide substitution was also found in the chlorophyte algae *C. reinhardtii*
171 (Figure S4). It was also noticeable that both BSM1a and BSM1b are highly conserved in *GOX1*
172 and *MDH* genes in land plants, and that BSM1b appears more ancient as it is found in all *GOX1*
173 genes from all land plants and even in the chlorophyte algae. It is possible that BSM1a found in
174 land plants is derived from the *MDH* genes of the algae, as it is observed in *C. subellipsoidea* and
175 *M. pusilla,* two members of the chlorophyta. Comparing the sequence of BSM1a and BSM1b in
176 *NAD-ME, MDH* and *GOX1* indicates that BSM1b is less variant, but in both cases, their
177 conservation implies an ancient role across the plant kingdom that likely is derived from the algal
178 ancestor.
179
180 **Widespread use of genic DNA for spatial patterning of gene expression**
181 Although genome-wide analysis of transcription factor binding sites indicates a significant
182 amount of binding occurs within genes, to our knowledge, there is little functional knowledge
183 confirming the importance of such sites. Having functionally defined BSM1a and BSM1b as being
184 important for patterning gene expression in the C_4 leaf, the extent to which other genes important
185 for the C_4 pathway are regulated by sequences within the gene rather than the promoter was
186 investigated. Based on *de novo* transcriptome assemblies, transcribed regions of twelve genes
187 recruited into C_4 photosynthesis in *G. gynandra* were cloned, with 3' and 5' ends verified using
188 3'RACE and genome walking (Supplementary Files I and 2). The gene sequences were placed

189 under control of the constitutive CaMV35S promoter, and fused to *uidA* encoding the β-
190 glucoronidase (GUS) reporter. Combined with publically available datasets for *PPDK* and *CA*
191 (Kajala et al 2011, Williams et al., 2016) this approach showed that preferential expression of
192 genes encoding C_4 proteins in either M or BS cells is commonly driven by elements within their
193 coding sequences (Figure 6). *CA2, CA4, PPCk1* and *PPDK* gene sequence without their
194 endogenous promoters all led to preferential accumulation of GUS in M cells, whereas *MDH1,*
195 *NAD-ME1* and *NAD-ME2* caused preferential accumulation of GUS in the BS. *Rubisco Activase*
196 *(RCA)* coding sequence drove a small but significant increase in the number of BS accumulating
197 GUS (Figure 6A) (p-value <0.05, CI 95%). These data indicate that regulatory elements within
198 genic sequence impact on cell preferential expression in the majority of genes recruited into the
199 core C_4 pathway.

200 In some cases, cell-preferential expression of C_4 genes has evolved from regulatory elements
201 found in ancestral C_3 species (Kajala, et al., 2011; Brown et al., 2011, Williams et al., 2013). To
202 investigate the extent to which genic sequence from ancestral C_3 species contain regulatory
203 elements sufficient for expression in either M or BS cells, orthologues to each of the C_4 genes were
204 cloned from *A. thaliana,* placed under the same reporter system and tested by microprojectile
205 bombardment. This showed that with the exception of *AtPPCk1*, the orthologous genes from C_3 *A.*
206 *thaliana* contained regulatory elements in coding sequence that can specify spatial patterning of
207 gene expression in the C_4 leaf of *G. gynandra* (Figure 6). Overall, these data indicate that spatial
208 patterning of gene expression in the C_4 leaf is largely derived from regulatory elements present in
209 coding sequences of genes found in the ancestral C_3 state. In the case of those defined at the
210 nucleotide level in *NAD-ME1, NAD-ME2, MDH* and *GOX1,* these elements appear highly
211 conserved and therefore ancient within land plants.

212 **Discussion**

213 The data presented here, combined with previous reports (Brown et al. 2011; Kajala et al. 2011;
214 Williams et al. 2016) portray an overview of the contribution that untranslated regions (UTRs) and
215 coding sequences make to the generation of cell-specific gene expression in leaves of C_4 *G.*
216 *gynandra*. Eight of the eleven core C_4 cycle genes possess regulatory elements in their transcript
217 sequences that are sufficient for preferential accumulation in either M or BS cells of the C_4 leaf.
218 These data strongly imply that, in addition to promoters being involved in generating cell-specificity
219 in C_4 leaves (Gowik et al., 2004; Sheen, 1999), coding sequences and UTRs play a widespread
220 role in the preferential accumulation of C_4 transcripts to either M or BS cells. It remains to be seen
221 whether this high degree of regulation from genic sequence is a common phenomenon in C_4
222 leaves of species other than *G. gynandropsis*, or whether it is critical for the spatial control of gene
223 expression in other tissues and other species. However, as genome-wide studies of transcription
224 factor recognition sites in organisms as diverse as *A. thaliana* and human cells (Stergachis et al.,
225 2013; Sullivan et al., 2014) have reported significant binding occurs in genic sequence, we
226 anticipate many more examples of spatial regulation of gene expression being associated with *cis*-
227 elements outside of promoter sequences.

228 The accumulation of *GgNADME1*, *GgNADME2, mMDH* and *GOX1* transcripts in BS cells is
229 dependent on the co-operative function of two *cis*-elements that are separated by a spacer
230 sequence, all of which are located in the first exon of these genes. These motifs are conserved
231 both in their sequences, but also in the number of nucleotides that separates them. In orthologous
232 *NAD-ME* genes from C_3 *A. thaliana,* which diverged from the Cleomaceae ~38 million years ago
233 (Schranz and Mitchell-Olds, 2006; Couvreur et al., 2010), although these motifs are present, they
234 are not sufficient to generate cell preferential expression in the C_3 leaf (Brown et al. 2011). This
235 finding indicates that for *NAD-ME* genes to be preferentially expressed in BS cells of C_4 plants, a
236 change in the behaviour of one or more *trans*-factors was a fundamental event. At least in *G.*
237 *gynandropsis*, evolution appears to have repeatedly made use of *cis*-elements that exist in genes
238 of C_3 species that are orthologous to those recruited into C_4 photosynthesis (Brown et al., 2011;

239 Kajala et al., 2012; Williams, Burgess et al., 2016). The alteration in *trans*-factors such that they
240 recognise ancestral elements in *cis* in the M or BS therefore appears to be an important and
241 common mechanism associated with evolution of the highly complex C_4 system.

242 BSM1a and BSM1b, which we defined first in the *GgNAD-ME1* gene, are also present and
243 operational in the *GgNAD-ME2*, *GgMDH1* and *GgGOX1* genes. If, as seems likely, these motifs
244 are recognised by the same *trans*-factors to generate preferential expression of all of these genes
245 in the C_4 BS*,* this finding also identifies a mini-regulon that during evolution could have recruited at
246 least four genes simultaneously into specialised roles in the BS. By combining Flux Balance
247 Analysis constrained by a model of carbon fixation, it has previously been proposed that
248 upregulation and preferential expression of multiple genes of the C_4 cycle would be required to
249 balance nitrogen metabolism between M and BS cells (Mallmann et al., 2014). The presence of
250 BSM1a and BSM1b in at least four genes from *G. gynandropsis* provides a mechanism that may
251 have facilitated this patterning of multiple genes during the evolution of C_4 photosynthesis.

252 The dual role of exons in protein coding as well as the regulation of gene expression has
253 received significant attention in vertebrates (Lang et al., 2005; Nguyen et al., 2007; Goren et al.,
254 2006; Tumpel et al., 2008; Dong et al., 2010, Stergachis et al., 2013). Although, 11% of
255 transcription factor binding sites are located in exonic sequence in *A. thaliana* (Sullivan et al.,
256 2014), to our knowledge, the identification of BSM1a and BSM1b represents the first functional
257 evidence for *cis*-elements in plant exons. The fact that these motifs are present in C_3 *A. thaliana*,
258 and in fact, also found in the genomes of many land plants and some chlorophyte algae, indicates
259 that these duons play ancient and conserved roles in photosynthetic organisms. The role of such
260 regulatory elements within coding sequences has previously been proposed to be associated with
261 constraints on both protein coding function and codon bias. For example, mutation to these *cis*-
262 elements could be deleterious to both the correct function of the protein, but also to codon usage
263 and so translational efficiency (Robinson et al., 1984; Tuller et al., 2010; Nakahigashi et al., 2014).
264 If this is the case, BSM1a and BSM1b could be highly conserved across deep phylogeny because
265 of strong positive selection pressure on these elements due to impact on translation, and this

266 conservation is then co-opted to also regulate transcription during the evolution of C_4
267 photosynthesis to generate cell-specific gene expression. Establishing the role of BSM1a and
268 BSM1b in C_3 plants would provide insight into the extent to which their role has altered during the
269 transition from C_3 to C_4 photosynthesis.

270 Duons under strong selection pressure may represent a rich resource of *cis*-elements upon
271 which the C_4 pathway has evolved. Although C_4 photosynthesis is a complex trait that requires
272 multiple changes to gene expression, the repeated recurrence of C_4 species across multiple plant
273 lineages suggests that a relatively low number of changes may be required to acquire the C_4
274 syndrome (Sinha & Kellogg, 1996; Hibberd et al., 2008; Westhoff & Gowik, 2010). A single C_4
275 master switch has been proposed (Westhoff & Gowik, 2010) but despite multiple comparative
276 transcriptomic studies (Brautigam et al., 2011; Aubry et al., 2014; Kulahoglu et al., 2014), there is
277 as yet no evidence for it. Given the repeated and highly convergent evolution of the C_4 pathway, as
278 well as evidence that separate lineages can arrive at the C_4 state via different routes (Williams et
279 al., 2013), it appears more plausible that C_4 photosynthesis made use of a number of gene sub-
280 networks. This is now supported by a number of findings. First, just as core photosynthesis genes
281 encoding the light harvesting complexes and Calvin-Benson-Bassham cycle are regulated by light,
282 the vast majority of genes that encode proteins of the C_4 cycle in C_3 *A. thaliana* are also regulated
283 by light signalling, yet, during the evolution of C_4 photosynthesis there was a significant gain of
284 responsiveness to chloroplast signalling (Burgess et al., 2016). Second, it has been suggested that
285 evolution of the C_4 pathway is associated with the recruitment of developmental motifs into leaves
286 that in C_3 species operate in roots (Kulahoglu et al., 2014). Lastly, the identification of the *cis*-
287 element MEM2 (Williams, Burgess et al., 2016), which controls preferential expression of multiple
288 genes in C_4 M cells, and now BSM1a and BSM1b in four different genes that are strongly
289 expressed in BS cells, indicates that that C_4 evolution has made use of small-scale recruitment of
290 gene sub-networks in both cell-types.

291 **Methods**

292 **Growth of plant material and production of reporter constructs**

293 Sterile *G. gynandra* seed was sown directly from intact pods and germinated on moist filter
294 papers in the dark at 30°C for 24 h. Seedlings were then transferred to Murashige and Skoog (MS)
295 medium with 1% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) and grown for a further 13 days in a
296 growth room at 22°C and 200 μmol m^{-2} s^{-1} photon flux density (PFD) with a photoperiod of 16 h
297 light.

298 *G. gynandropsis* mRNA sequences were predicted from a *de novo* assembled transcriptome
299 and UTRs were verified by 3' RACE (Supplementary File I) and genome walking (Supplementary
300 File 2). *A. thaliana* cDNA sequences were extracted from Phytozome v10.1. Reporter constructs
301 were generated by ligation of the fragment of interest with a modified reporter cassette containing
302 the Cauliflower Mosaic Virus 35S promoter (pCaMV35S), 13 bp of its 5'UTR, the *uidA* gene
303 (encoding GUS), and the *nosT* terminator sequence (Brown et al., 2011). Vectors were assembled
304 in this cassette using Gibson assembly (Gibson et al., 2009) (Supplementary Table I). Site-directed
305 mutagenesis was performed using the Quickchange method.

306

307 **Microprojectile bombardment and production of stable transformants**

308 350 ng M-17 tungsten particles (1.1-μm diameter; Bio-Rad) were washed with 100% (v/v)
309 ethanol and resuspended in ultrapure water. 1.5 μg of plasmid DNA was mixed with the tungsten
310 particles while vortexing at slow speed. After addition of the DNA, 50 μL 2.5 M calcium chloride
311 (Fisher Scientific) and 10 μL 100 mM spermidine (Sigma-Aldrich) were added to the particle
312 suspension to facilitate binding of DNA to the particles. The tungsten-DNA suspension was
313 incubated for 10 min on ice, with frequent agitation to prevent pelleting. Particles were then
314 washed and resuspended in 100 μL 100% (v/v) ethanol. 10 μL aliquots of tungsten-DNA were
315 transferred to plastic macrocarriers (Bio-Rad) and allowed to dry for 3 minutes at room
316 temperature. Three macrocarriers were used for each transformation. Following bombardment with
317 a Bio-Rad PDS-1000/He particle delivery system, seedlings were placed upright in a sealed Petri

318 dish, with the base of their stems immersed in 0.5x MS medium and incubated in a growth room at
319 22°C and 150 µmol m^{-2} s^{-1} PFD with a photoperiod of 16 h light for 48 h, prior to GUS staining.
320 Stable plant transformation was performed by introducing constructs into *G.gynandra* via
321 *Agrobacterium tumefaciens* LBA4404 as described previously (Newell et al., 2009). Plant tissue,
322 after bombardment or stable transformation, was GUS stained (0.1 M Na_2HPO_4 pH7.0, 0.5 mM K
323 ferricyanide, 0.5 mM K ferrocyanide, 0.06% v/v Triton X-100, 10 mM Na_2EDTA pH8.0, 1mM X-
324 gluc) at 37°C for 6-16 h and then fixed in a 3:1 solution of ethanol to acetic acid at room
325 temperature for 30 min. Chlorophyll was cleared with 70% (v/v) ethanol and tissue treated with 5%
326 (w/v) NaOH at 37°C for 2 h. M and BS cells containing GUS were identified and counted using
327 phase-contrast microscopy. At least 50 cells were counted per construct in each experiment, and
328 for each construct, three independent experiments were conducted (Supplementary Table II).
329

330 ***cis*-Element prediction and localization**

331 *De novo* motif prediction was performed using the Multiple Em for Motif Elucidation (MEME)
332 suite v.4.8.1 with the following parameters: *meme sequences.fa -dna -oc. -nostatus -time 18000 -*
333 *maxsize 60000 -mod oops -nmotifs 3 -minw 7 -maxw 9 -revcomp*. To scan for motif instances
334 across various datasets FIMO was used with the following parameters: *fimo --oc . --verbosity 1 --*
335 *thresh 0.1 motifs.meme sequences.fa*. Only hits located within the first 550 bp, allowing a spacing
336 between the motifs of 35 to 550 bp were accepted.

337 **Figure legends**

338 **Figure S1: Transformation of *G. gynandra* M and BS cells by microprojectile bombardment.**
339 Leaves of *G. gynandra* arranged concentrically prior to bombardment (**A**). Representative GUS
340 stained *G. gynandra* leaf transformed with *pCaMV35s:GgNAD-ME1(25-240bp)::gfp/uidA::nosT* (**B**).
341 Mesophyll cells (**C**) and Bundle Sheath cells (D, black arrows) stained with GUS after
342 bombardment. Scale bars represent 100 µm.

343

344 **Figure S2: Pixel intensity of stable transgenic lines.** Pixel intensities across regions of the leaf
345 containing mesophyll and bundle sheath. Data are derived from GUS stained leaves from three
346 independent transgenic lines. Data are presented as histograms for whole datasets and dots that
347 represent single measurements. At least 20 measurements were made per transgenic line.

348

349 **Figure S3: Topological requirements for BSM1a and BSM1b function.** Summary of the
350 constructs used in this experiment. BSM1a and BSM1b were separated by 0, 21, 240, 347, 413,
351 550 and 999 base pairs derived from the gene encoding Green Fluorescent Protein (GFP) **(A)**.
352 Percentage of cells containing GUS after microprojectile bombardment of *G. gynandra* leaves.
353 Bars represent the percentage of stained cells in Bundle Sheath (BS - blue) and mesophyll (M -
354 grey) cells. Error bars denote the standard error of the mean. * represents statistically significant
355 differences with P-values <0.05 and CI = 95% determined by a one-tailed t test.

356

357 **Figure S4: Location of BSM1a and BSM1b across the land plant phylogeny.** BSM1a and
358 BSM1b in *NAD-ME, mMDH* and *GOX1* coding sequences retrieved from 44 species in Phytozome
359 (v10.1). Green dots represent identical versions of the motifs while yellow and orange dots denote
360 alternative versions with one or two substitutions respectively.

361 **Figure 1: Two regions within the coding sequence of *GgNAD-ME1* are necessary for**
362 **preferential gene expression in the bundle sheath.** An antisense construct, as well as a
363 deletion series from the 5' and 3' ends of *GgNAD-ME1*$_{(1\text{-}240\ bp)}$ coding sequence were translationally
364 fused to the *uidA* reporter under the control of the CaMV 35S promoter **(A)**. Percentage of cells
365 containing GUS after microprojectile bombardment of *G. gynandra* leaves. Bars represent the
366 percentage of stained cells in BS (blue) and M (grey) cells, error bars denote the standard error. *
367 represents statistically significant differences with P-values <0.05 and CI = 95% determined by a
368 one-tailed t test (**B**). GUS in *G. gynandra* transformants containing uidA fused to 1-240, 1-141, 79-
369 240 and 64-162 bp from the translation starting site of *GgNAD-ME1* **(C).** Scale bars, 100 µm.
370
371 **Figure 2: Two *cis*-elements that are sufficient for preferential accumulation of GUS in the**
372 **bundle sheath.** Non-mutated and mutated versions of BSM1a and BSM1b flanked by 75
373 nucleotides derived from GFP were translationally fused to *uidA* encoding GUS and placed under
374 control of the CaMV35S promoter (**A**). The percentage of cells containing GUS after microprojectile
375 bombardment of *G. gynandra* leaves (**B**). Error bars denote the standard error. * represents
376 statistically significant differences with P-values <0.05 and CI = 95% determined by a one-tailed t
377 test.
378
379 **Figure 3: BSM1a and BSM1b drive the expression of additional genes in C_4 and**
380 **photorespiration pathways.** Sequences similar to BSM1a and BSM1b were predicted to be
381 present in coding sequences of *mMDH* and *GOX1* genes of *G. gynandra*. Deleting the motifs
382 resulted in the loss of preferential accumulation of GUS in the BS (**A**). A consensus sequence for
383 both motifs was defined based on *NAD-ME1, mMDH* and *GOX1* versions of the motifs (**B**). BSM1a
384 and BSM1b coordinate BS gene expression of multiple gene families (highlighted in red) relevant
385 to C_4 photosynthesis and photorespiration (**C**). Error bars denote the standard error. * represents
386 statistically significant differences with P-values <0.05 and CI = 95% determined by a one-tailed t
387 test.

388

389 **Figure 4: Functional versions of BSM1a and BSM1b are present in additional *NAD-MEs.***
390 BSM1a and BSM1b are found in *GgNAD-ME2* and in orthologs of *GgNAD-ME1&2* from the C_3
391 species *A. thaliana* (**A**). Translational fusions carrying these fragments confer BS preferential
392 expression in *G. gynandra* leaves. When BSM1a or BSM1b were removed this pattern of GUS was
393 lost (**B**). A consensus sequence generated from all versions of BSM1a and BSM1b tested
394 experimentally (**C**). Error bars denote the standard error. * represents statistically significant
395 differences with P-values <0.05 and CI = 95% determined by a one-tailed t test.

396

397 **Figure 5: BSM1a and BSM1b are highly conserved in land plants.** Single nucleotide
398 polymorphisms (SNP) in *AtNAD-ME1* (**A**) and *AtNAD-ME2* (**B**) genes from 1135 wild inbred *A.*
399 *thaliana* accessions. On the left, the position of BSM1a and BSM1b are highlighted by dashed blue
400 lines, UTRs, exons and introns are denoted by black, grey and white bars respectively on the X-
401 axis. To the right an expanded area representing exon 1, intron 1 and exon 2 is shown, with
402 BSM1a and BSM1b marked within the blue dashed lines. For both genes, no SNP were detected
403 in either motif. The presence of each motifs was investigated in gene sequences of *NAD-ME1,*
404 *mMDH* and *GOX1* retrieved from 44 species in Phytozome (v10.1). Each Pie-chart shows the
405 percentage of motif instances that were identical (green), or had 1 base pair (yellow), 2 base pair
406 (orange) substitutions or no similarity (white) detected.

407

408 **Figure 6: Pre-existing intragenic regulatory sequences play a major role controlling C_4**
409 **photosynthesis genes.** Coding sequences encoding for core proteins of the C_4 pathway from *G.*
410 *gynandra* together with orthologs from *A. thaliana* were translationally fused to *uidA* and placed
411 under control of the CaMV35S promoter. After introduction into *G. gynandra* leaves by
412 microprojectile bombardment mesophyll preferential expression of *CA2, CA4, PPDK* and *PPCk*,
413 together with Bundle Sheath preferential expression of *mMDH, NAD-ME1* and *NAD-ME2* were
414 observed (**A**). With the exception of *PPCk* these regulatory elements are conserved in orthogues

415 from *A. thaliana* (**B**). The contribution of intragenic sequences controlling gene regulation of the C_4
416 pathway is summarized in (**C**), *CA2, CA4, PPDK* and *PPCk* (blue) and *mMDH, NAD-ME1&2* (red)
417 denote genes where intragenic sequences control cell preferential gene expression. Error bars
418 denote the standard error. * represents statistically significant differences with P-values <0.05 and
419 CI = 95% determined by a one-tailed t test.
420
421 **Supplementary File 1:** FASTA sequences from Rapid Amplification of cDNA ends used to verify
422 3' UTR sequences of C_4 genes from *G. gynandra*.
423
424 **Supplementary File 2:** FASTA sequences from Genome Walking experiments used to verify 5'
425 ends of C_4 gene sequences from *G. gynandra.*
426
427 **Supplementary Table I:** Primer sequences used in generation of constructs, 3' RACE
428 experiments and Genome Walking.
429
430 **Supplementary Table II:** Total cell counts for the microprojectile bombardment experiments.
431
432 **Competing Interests**
433 The authors have no competing interests.

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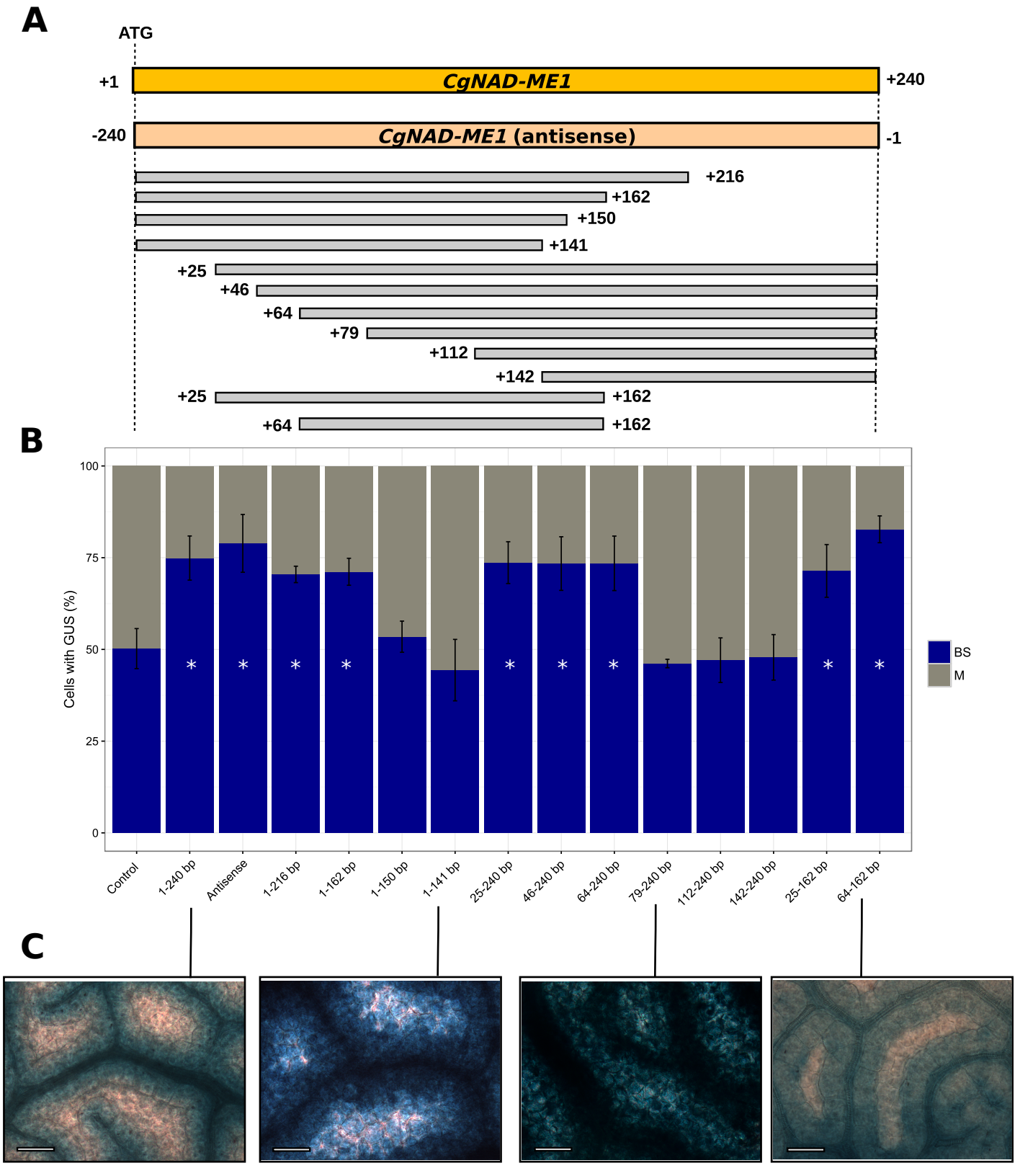
A
ATG
+1
CgNAD-ME1
+240
-240
CgNAD-ME1 (antisense)
-1
+216
+162
+150
+141
+25
+46
+64
+79
+112
+142
+25
+162
+64
+162
B
Cells with GUS (%)
100
75
50
25
0
BS
M
Control
1-240 bp
Antisense
1-216 bp
1-162 bp
1-150 bp
1-141 bp
25-240 bp
46-240 bp
64-240 bp
79-240 bp
112-240 bp
142-240 bp
25-162 bp
64-162 bp
C

Figure 1

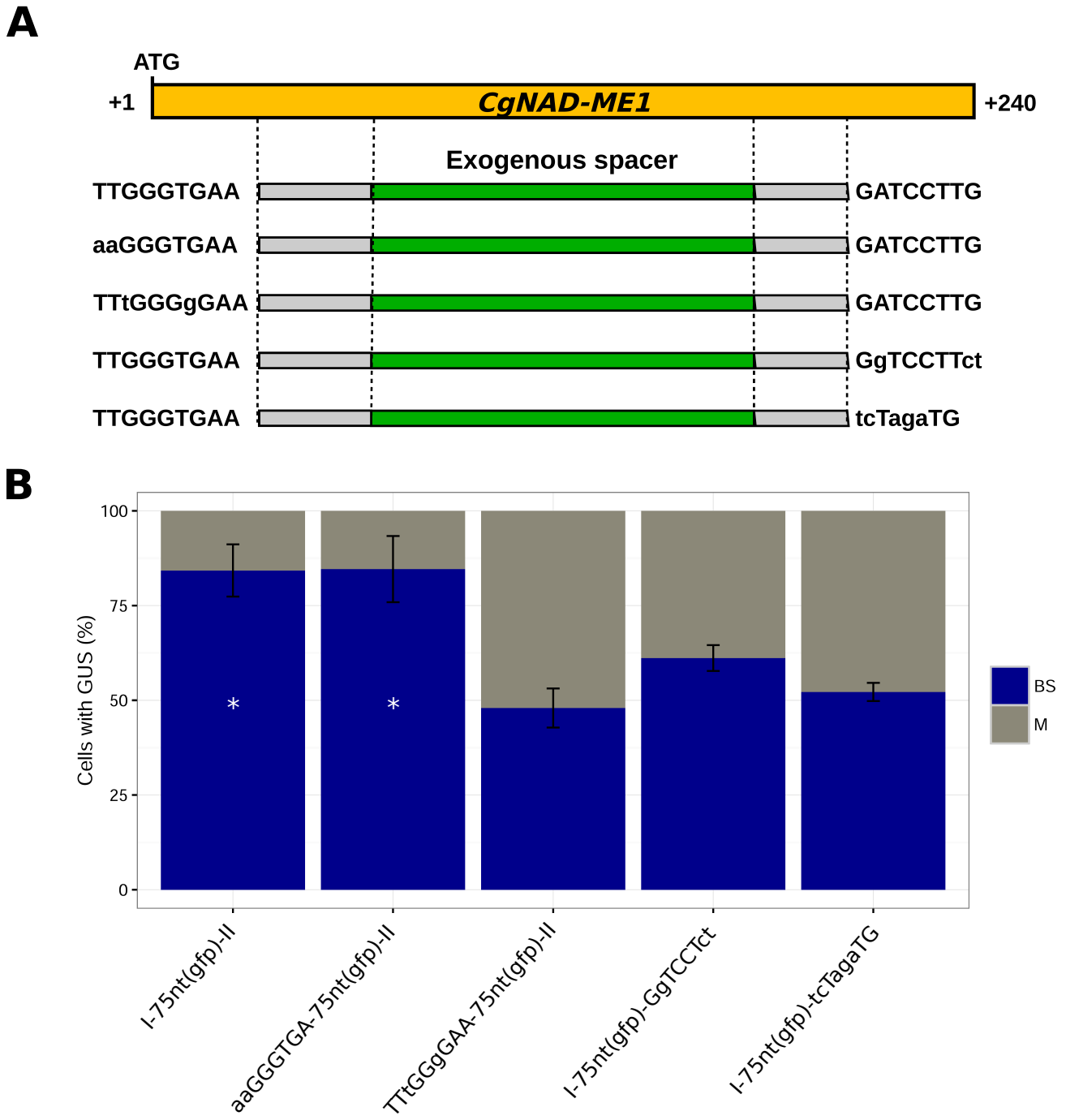
A
ATG
+1
CgNAD-ME1
+240
Exogenous spacer
TTGGGTGAA
GATCCTTG
aaGGGTGAA
GATCCTTG
TTtGGGgGAA
GATCCTTG
TTGGGTGAA
GgTCCTTct
TTGGGTGAA
tcTagaTG
B
Cells with GUS (%)
100
75
50
25
0
BS
M
I-75nt(gfp)-II
aaGGGTGA-75nt(gfp)-II
TTtGGGgGAA-75nt(gfp)-II
I-75nt(gfp)-GgTCCTct
I-75nt(gfp)-tcTagaTG

Figure 2

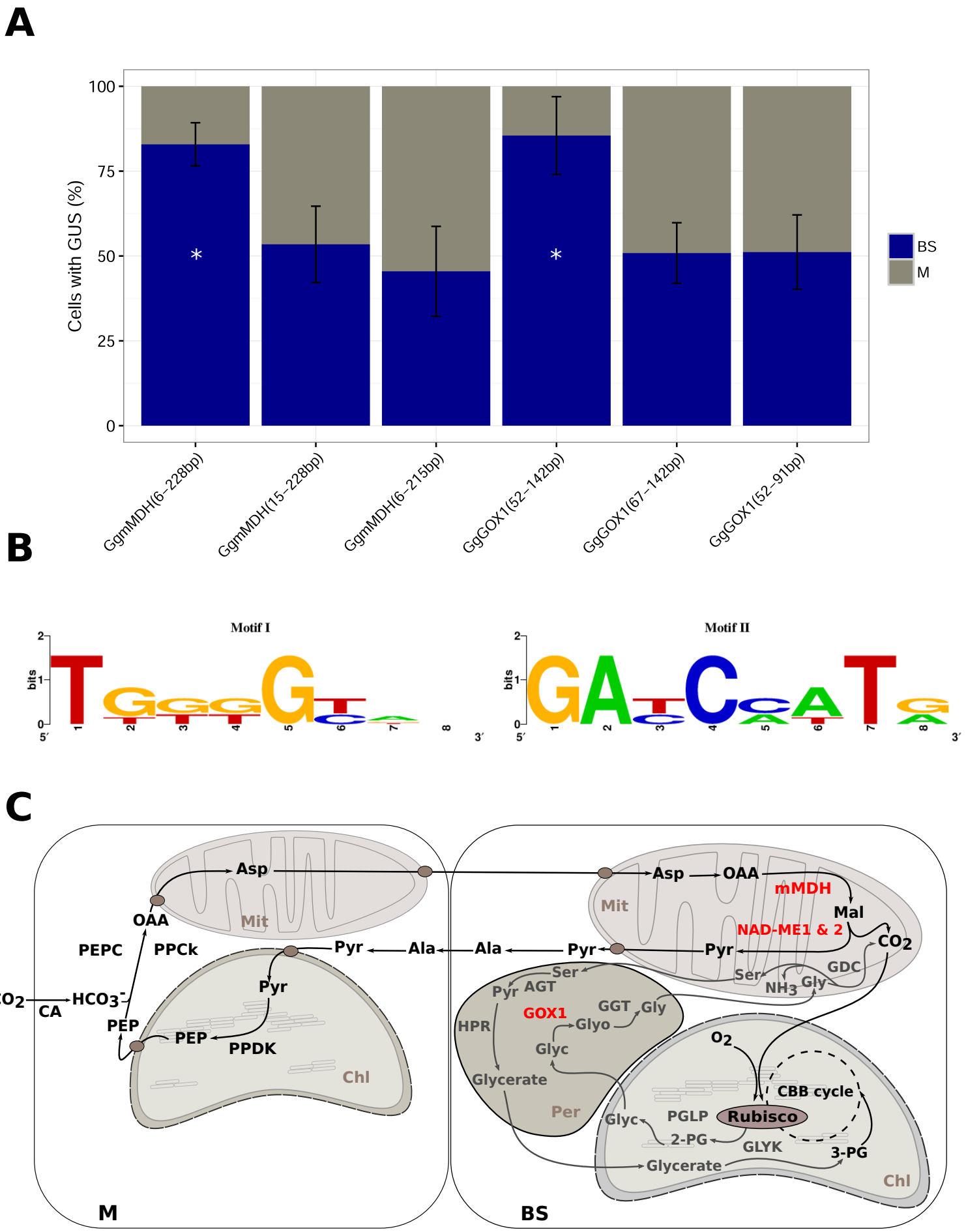
A
Cells with GUS (%)
100
75
50
25
0
BS
M
GgmMDH(6–228bp)
GgmMDH(15–228bp)
GgmMDH(6–215bp)
GgGOX1(52–142bp)
GgGOX1(67–142bp)
GgGOX1(52–91bp)
B
Motif I
Motif II
bits
5′
3′
C
Asp
OAA
Mit
PEPC
PPCk
Pyr
Ala
CO2
HCO3-
CA
PEP
PPDK
Chl
M
mMDH
Mal
NAD-ME1 & 2
GDC
Ser
AGT
GOX1
GGT
Gly
Glyo
Glyc
HPR
Glycerate
Per
O2
CBB cycle
Rubisco
PGLP
2-PG
GLYK
3-PG
BS

Figure 3

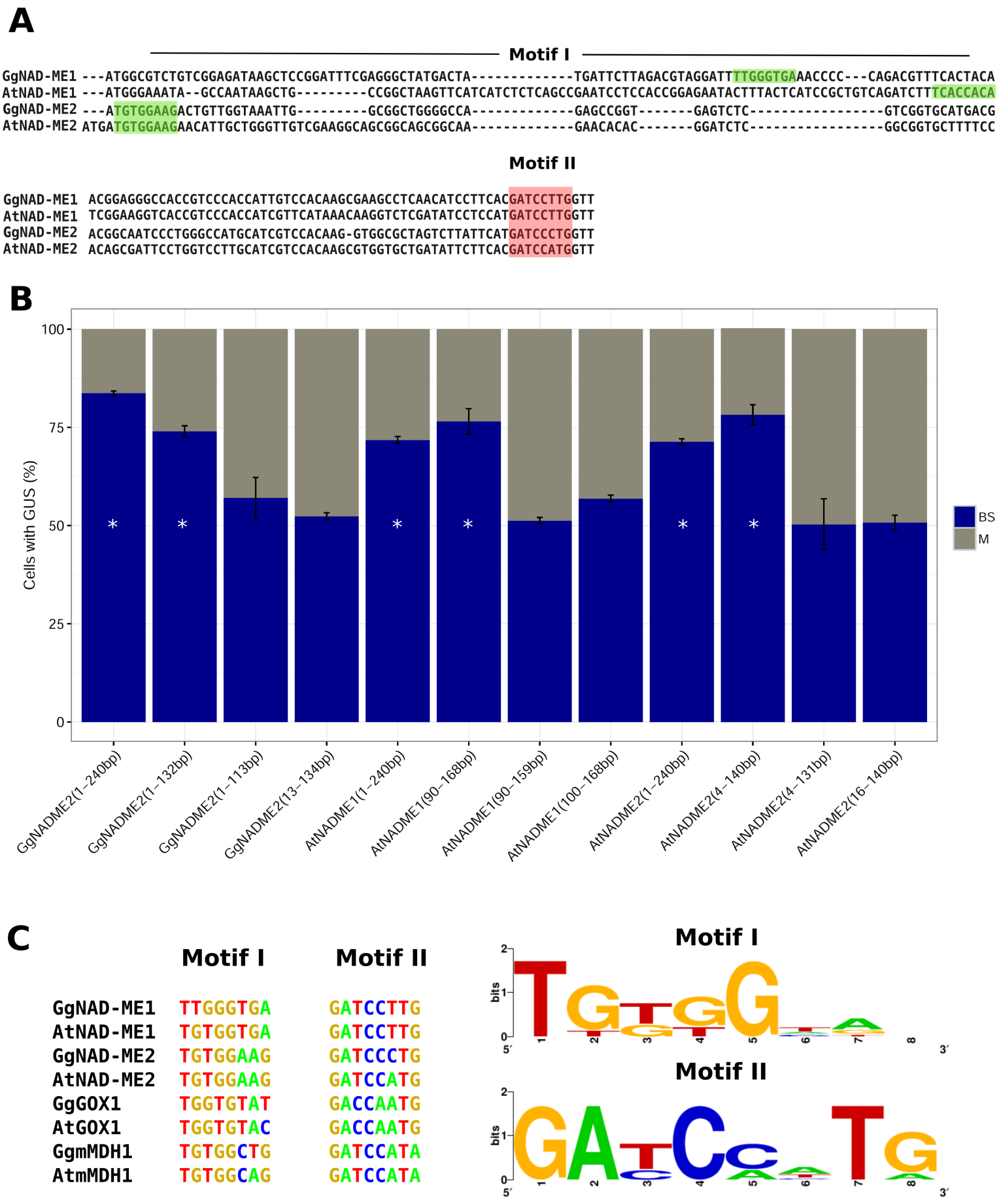
A
Motif I
GgNAD-ME1 ---ATGGCGTCTGTCGGAGATAAGCTCCGGATTTCGAGGGCTATGACTA----------TGATTCTTAGACGTAGGATTTTGGGTGAAACCCC---CAGACGTTTCACTACA
AtNAD-ME1 ---ATGGGAAATA--GCCAATAAGCTG---------CCGGCTAAGTTCATCATCTCTCAGCCGAATCCTCCACCGGAGAATACTTTACTCATCCGCTGTCAGATCTTTCACCACA
GgNAD-ME2 ---ATGTGGAAGACTGTTGGTAAATTG---------GCGGCTGGGGCCA----------GAGCCGGT-------GAGTCTC----------GTCGGTGCATGACG
AtNAD-ME2 ATGATGTGGAAGAACATTGCTGGGTTGTCGAAGGCAGCGGCAGCGGCAA----------GAACACAC-------GGATCTC----------GGCGGTGCTTTTCC
Motif II
GgNAD-ME1 ACGGAGGGCCACCGTCCCACCATTGTCCACAAGCGAAGCCTCAACATCCTTCACGATCCTTGGTT
AtNAD-ME1 TCGGAAGGTCACCGTCCCACCATCGTTCATAAACAAGGTCTCGATATCCTCCATGATCCTTGGTT
GgNAD-ME2 ACGGCAATCCCTGGGCCATGCATCGTCCACAAG-GTGGCGCTAGTCTTATTCATGATCCCTGGTT
AtNAD-ME2 ACAGCGATTCCTGGTCCTTGCATCGTCCACAAGCGTGGTGCTGATATTCTTCACGATCCATGGTT
B
Cells with GUS (%)
100
75
50
25
0
BS
M
GgNADME2(1–240bp)
GgNADME2(1–132bp)
GgNADME2(1–113bp)
GgNADME2(13–134bp)
AtNADME1(1–240bp)
AtNADME1(90–168bp)
AtNADME1(90–159bp)
AtNADME1(100–168bp)
AtNADME2(1–240bp)
AtNADME2(4–140bp)
AtNADME2(4–131bp)
AtNADME2(16–140bp)
C
Motif I
Motif II
GgNAD-ME1 TTGGGTGA GATCCTTG
AtNAD-ME1 TGTGGTGA GATCCTTG
GgNAD-ME2 TGTGGAAG GATCCCTG
AtNAD-ME2 TGTGGAAG GATCCATG
GgGOX1 TGGTGTAT GACCAATG
AtGOX1 TGGTGTAC GACCAATG
GgmMDH1 TGTGGCTG GATCCATA
AtmMDH1 TGTGGCAG GATCCATA
Motif I
bits
Motif II
bits

Figure 4

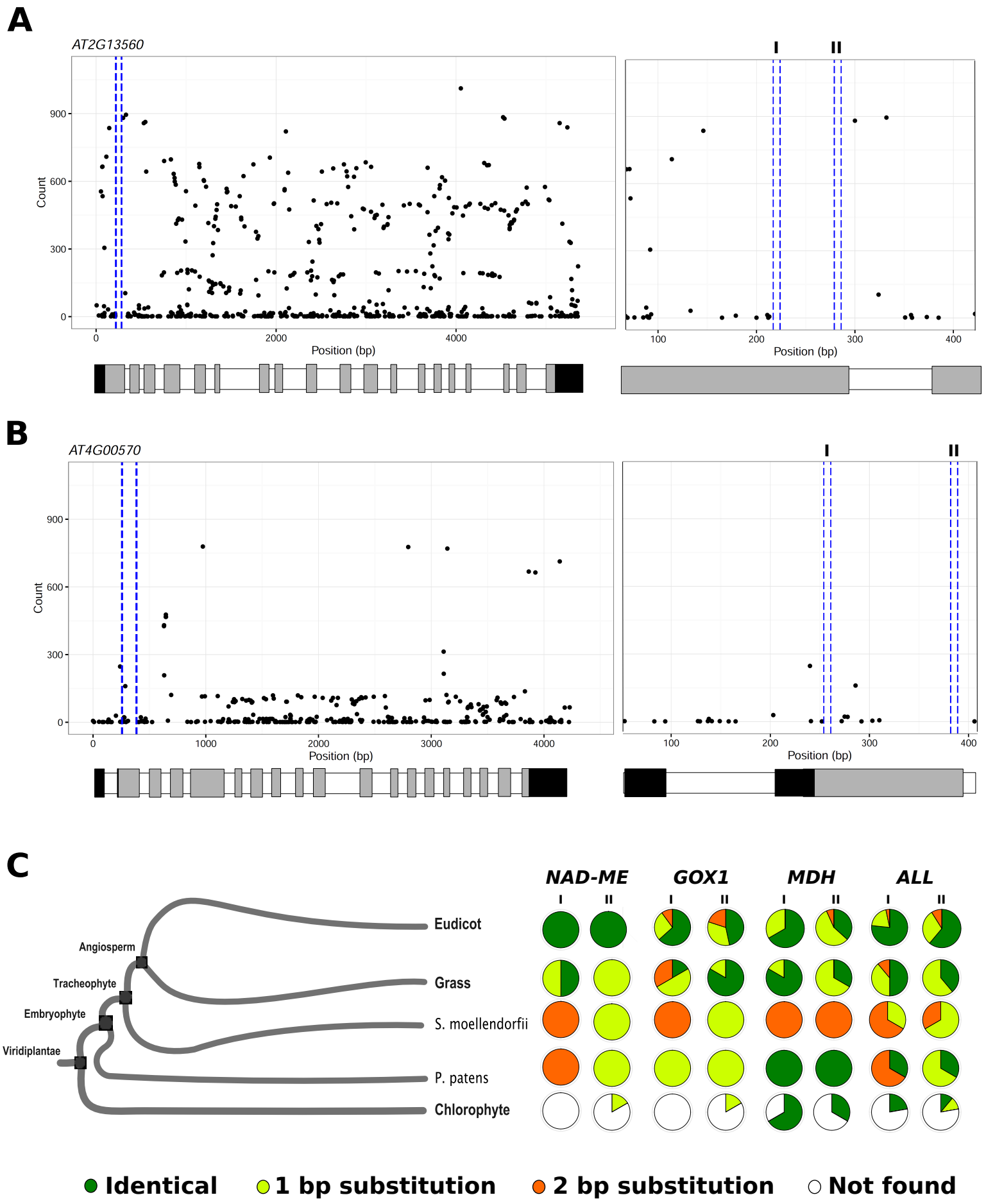
A
AT2G13560
Count
Position (bp)
I
II
B
AT4G00570
C
Angiosperm
Tracheophyte
Embryophyte
Viridiplantae
Eudicot
Grass
S. moellendorfii
P. patens
Chlorophyte
NAD-ME
GOX1
MDH
ALL
Identical
1 bp substitution
2 bp substitution
Not found

Figure 5

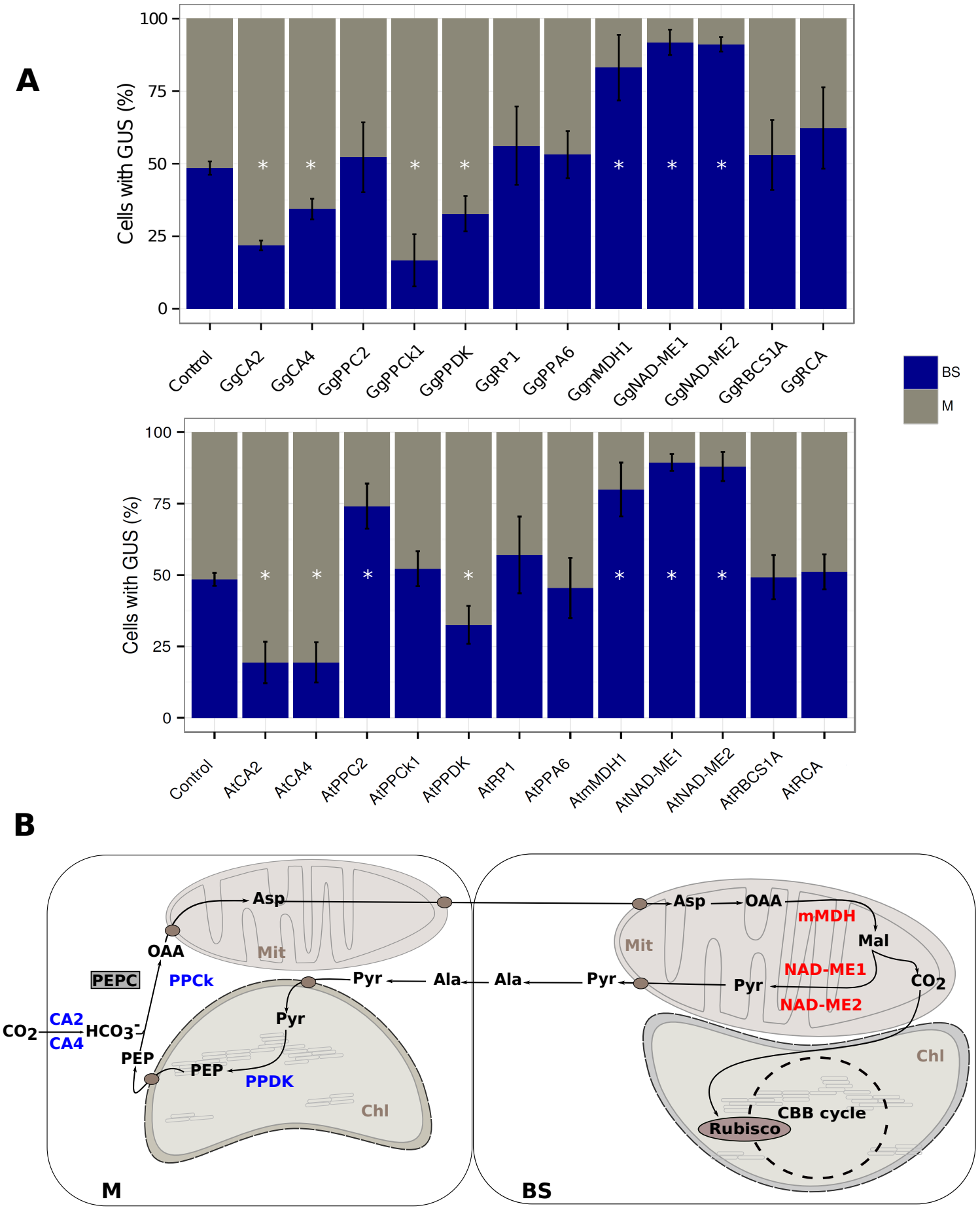
A
Cells with GUS (%)
100
75
50
25
0
Control
GgCA2
GgCA4
GgPPC2
GgPPCk1
GgPPDK
GgRP1
GgPPA6
GgmMDH1
GgNAD-ME1
GgNAD-ME2
GgRBCS1A
GgRCA
BS
M
AtCA2
AtCA4
AtPPC2
AtPPCk1
AtPPDK
AtRP1
AtPPA6
AtmMDH1
AtNAD-ME1
AtNAD-ME2
AtRBCS1A
AtRCA
B
Asp
OAA
Mit
PEPC
PPCk
Pyr
Ala
CO2
CA2
CA4
HCO3-
PEP
PPDK
Chl
mMDH
Mal
NAD-ME1
NAD-ME2
CBB cycle
Rubisco
M
BS

Figure 6