

## **Expression of a glucomannan mannosyltransferase gene (*GMMT*) from *Aloe vera* is induced by water deficit and abscisic acid**

**Running Title: Expression of *GMMT* in *Aloe vera* plants under water stress**

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1 **Highlight**

2 *GMMT* (a possible *CSLA9*) from Aloe vera is upregulated during water stress. Aloe  
3 vera *GMMT* expression is also induced by exogenous application of the plant  
4 stress hormone abscisic acid (ABA) in non-water-stressed plants.

5

## 6 **Summary**

7 In *Aloe barbadensis* Miller (Aloe vera), a xerophytic crassulacean acid metabolism  
8 (CAM) plant, the main polysaccharide of the gel present in the leaves is an  
9 acetylated glucomannan named acemannan. This polysaccharide is responsible  
10 for the plant succulence, helping it to retain water. In this study we determined  
11 using polysaccharide analysis by carbohydrate gel electrophoresis (PACE) that the  
12 acemannan is a glucomannan without galactose side branches. We also  
13 investigated the expression of the gene responsible for acemannan backbone  
14 synthesis, encoding a glucomannan mannosyltransferase (GMMT). It was found  
15 by *in silico* analyses that the *GMMT* gene belongs to the cellulose synthase like A  
16 type-9 (*CSLA9*) subfamily. Using RT-qPCR it was found that the expression of  
17 *GMMT* increased in Aloe vera plants subjected to water stress. This expression  
18 correlates with an increase of endogenous ABA levels, suggesting that the gene  
19 expression could be regulated by ABA. To corroborate this hypothesis, exogenous  
20 ABA was applied to non-water-stressed plants, increasing the expression of *GMMT*  
21 significantly 48 h after ABA treatment.

22

23 Key words: ABA, Acemannan, *CSLA9*, *GMMT*, PACE, RT-qPCR, water stress.

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25

## 26 **Introduction**

27 *Aloe barbadensis* Miller (Aloe vera) is a monocotyledon of the Order Asparagales  
28 originally from Africa and the Arabian peninsula. It is a xerophytic plant that is  
29 capable of tolerating water deficit (Silva et., 2010). Aloe vera has succulent leaves  
30 (which can store water due to the presence of polysaccharides) and has a CAM  
31 photosynthetic metabolism that allows greater water use efficiency, reducing the  
32 evapotranspiration rate during the day (Nobel, 1997; Sushruta et al., 2013). The  
33 leaves have an outer green cortex which is photosynthetically active, while the  
34 inner gel contains mainly an acetylated glucomannan known as acemannan  
35 (Hamman, 2008). The gel also has in lower amounts other soluble  
36 polysaccharides, sugars, anthroquinones, amino acids, vitamins and proteins  
37 (Chow et al., 2005; Chun-hui et al., 2007).

38 Acemannan is the most abundant polysaccharide of the gel present in the leaf  
39 parenchyma cells (Ni et al., 2004), apparently not being a component of the cell  
40 walls, even though glucomannans are considered to be hemicellulose-type  
41 polysaccharides. This molecule has several medicinal properties, such as  
42 acceleration of wound healing, antioxidant and anti-inflammatory effects, prebiotic  
43 properties (Quezada et al., 2017) with retardation of colon cancer development,  
44 reduction of blood pressure, reduction of blood glucose (glycemia) and  
45 improvement of lipid profile in diabetic patients (Sanchez Machado et al, 2017;  
46 Minjares-Fuentes et al., 2017).

47 Acemannan contributes to plant tolerance to the lack of water as a compatible  
48 solute. It also contributes to the succulence of the plant, because the parenchyma

49 where this polysaccharide is synthesized accumulates water, making the plant  
50 tolerant to water stress. This polysaccharide has a main backbone of mannose and  
51 glucose linked by  $\beta$ -(1→4) linkages and has been shown to increase its amount  
52 under water restrictions (Silva et al., 2010). There is a controversy regarding the  
53 existence of galactose side chains in the polysaccharide. Recently Minjares-  
54 Fuentes et al. (2017) stated that the polysaccharide has a low frequency of  
55 galactose side chains, while Campestrini et al., (2013) using NMR found that the  
56 polysaccharide contains only partially acetylated mannose and glucose without  
57 side chains of galactose.

58 For this reason, in this study we wanted to define whether acemannan is a  
59 glucomannan or a galactoglucomannan and to analyze the expression of the gene  
60 that encodes the enzyme glucomannan mannosyltransferase (GMMT, EC:  
61 2.4.1.32) in Aloe vera plants subjected to water deficit. This enzyme catalyzes the  
62  $\beta$ -(1→4) linkage that incorporates glucose or mannose to a mannan or  
63 glucomannan backbone (Liepman and Cavalier, 2012). The GMMT enzyme  
64 belongs to the cellulose synthase-like (CSL) protein family and the cellulose  
65 synthase-like A (CSLA) subfamily. The glucomannan 4-beta-mannosyltransferase  
66 enzyme from Aloe vera could be any CSLA (Liepman and Wilkerson, 2005;  
67 Liepman et al., 2007) that synthesizes glucomannan from nucleotide sugars (GDP-  
68 mannose and GDP-glucose) by generating a  $\beta$ -(1→4) glycosidic bond between C1  
69 and C4 of the hexoses (mannose and/or glucose). The glucomannan backbone is  
70 later acetylated in C2, C3 or C6 of the mannose units by the action of other  
71 enzymes (Simões et al., 2012). Figure 1 gives the structure of acemannan.

72 This polysaccharide as a cell wall component in other plant species is synthesized  
73 in the Golgi lumen and later transported to form part of the plant cell wall (Maeda et  
74 al.,2000; Lerouxel et al., 2006; Goubet et al., 2009; Temple et al., 2016).

75 Tolerance to drought in Aloe vera has been previously studied in detail by our  
76 group (Silva et al., 2010, Delatorre-Herrera et al., 2010, Ramírez et al., 2012.  
77 Huerta et al. 2013, Salinas et al, 2016). Since acemannan increases under  
78 conditions of lack of water and abscisic acid (ABA) is the main hormone that  
79 regulates the response to water content of plants, the purpose of this study was to  
80 analyze the expression of the *GMMT* gene under water stress and to determine if  
81 its expression is regulated by ABA.

## 82 **Material and Methods**

83 Adult *Aloe barbadensis* Miller (Aloe vera) plants were used for the experiments and  
84 analyses. These were brought from a private plantation near Los Choros in the  
85 Coquimbo Region, Chile and transferred to pots in the laboratory greenhouse.  
86 Each pot contained a mixture of sand and topsoil in the proportion 2: 1,  
87 respectively. All pots were irrigated with 300 mL of water or 100% field capacity  
88 (FC) once a week for two months to match the water conditions among all plants.  
89 The plants were maintained at 25 °C and a photoperiod of 16 h light and 8 h dark.  
90 After two months the plants were divided into four groups. These consisted of: T1,  
91 the control group, which maintained irrigation with 100% FC; T2, watered with 75%  
92 FC (225 mL); T3, watered with 50% FC (150 mL) and T4, watered with 25% FC

93 (80 mL). The irrigations were applied once a week for a total of 13 weeks. Each  
94 group consisted of 5 different plants.

#### 95 **Preparation of plant tissue**

96 Leaves of Aloe vera were cut and immediately frozen in liquid nitrogen and stored  
97 at -80 °C. For RNA extraction the leaves were maintained frozen to avoid the  
98 activation of RNAses. Subsequently, the leaf cortex was macerated avoiding  
99 contamination with the leaf gel due to the presence of sugars and polysaccharides  
100 which interfere with RNA purification.

#### 101 **Polysaccharide analysis by carbohydrate gel electrophoresis (PACE).**

102 The PACE analyses of glucomannan polysaccharide from Aloe vera was  
103 performed according to Goubet et al. (2002) with modifications. For this, 50 µg of  
104 glucomannan obtained from the gel as described by Quezada et al. (2017), was  
105 initially deacetylated with 20 µL of 4M NaOH for 1 h at room temperature. Next it  
106 was neutralized with 1N HCl, adding water to a final volume of 500 µL. After  
107 neutralization the glucomannan was digested in 1M ammonium acetate buffer pH  
108 6.0 with 4 µL of 1mg/mL mannanase 26A from *Cellvibrio japonicus* (Goubet et al.,  
109 2009) and/or 0.8 µL α-galactosidase (Megazyme) overnight at room temperature  
110 under agitation. After digestion, the enzymes were inactivated by heating the  
111 samples at 100 °C for 10 min in a heating block. Following inactivation, the  
112 samples were dried in a centrifugal vacuum evaporator at 60 °C for at least 4 h.  
113 The dried samples were derivatized with 8-aminonaphthalene-1,3,6-trisulfonic acid  
114 (ANTS) and 2-picoline–borane (2-PB) to be visualized under UV light according to

115 Pidatala et al. (2017). The electrophoresis was run in 24 cm glass plates, 0.75 mm  
116 spacer and wells of width 0.25 cm for 30 min at 200V, followed by 1 h 45 min at  
117 1000V at 10 °C. The oligosaccharides and sugars were visualized in a Syngene G-  
118 box under UV light. For this analysis we used standards of glucose, mannose and  
119 galactose and locus bean gum galactomannan (from seeds of *Seratonia siliqua*)  
120 purchased from Sigma-Aldrich Corp. (MO, USA) and Konjac glucomannan from  
121 Megazyme (Chicago, USA).

## 122 **RNA extraction**

123 The CTAB method was used for RNA extraction (Meisel et al., 2005) with some  
124 modifications. The RNA was purified with an RNA purification kit, described below.  
125 For this, 1.5 g of frozen Aloe vera powder was added to 20 mL of CTAB extraction  
126 buffer (2% w/v CTAB, 2% w/v PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M  
127 NaCl, 0.05% spermidine) containing 400  $\mu$ L  $\beta$ -mercaptoethanol, previously heated  
128 to 65 °C in a water bath. The tissue was mixed well in the buffer to be  
129 homogenized, avoiding the formation of crusts. The samples were incubated at 65  
130 °C for 15 min, shaking slowly every 5 min. Subsequently, 20 mL of chloroform:  
131 isoamyl alcohol (24:1) was added. Then the mixture was homogenized, using a  
132 vortex at maximum speed. The sample was then centrifuged at 9000 g for 40 min  
133 at 4 °C and the supernatant (yellow color) was transferred to a 50 mL falcon tube  
134 and placed on ice. A volume of chloroform: isoamyl alcohol (24:1) equivalent to  
135 that of the supernatant was added to the pellet and vortexed again, then  
136 centrifuged at 9000 g for 40 min at 4 °C. The supernatant was saved and  
137 combined with the supernatant of the first centrifugation. To precipitate the RNA,



138 10 M LiCl was added in a volume equivalent to one-fourth volume of the total  
139 supernatant obtained, gently shaken and incubated overnight at 4 °C.

140 The extracted RNA was purified using a column of the E.Z.N.A Total RNA Kit I  
141 (Omega Biotec, Norcross, GA, USA), using the protocol recommended by the  
142 manufacturer. The RNA was quantified in a NanoDrop™ spectrophotometer at 260  
143 nm. The 260/280 and 260/230 ratios were determined to evaluate the presence of  
144 proteins or phenolic compounds. The integrity of the RNA was verified by  
145 electrophoresis in a 1.5% agarose gel made up with DEPC water, MOPS and 37%  
146 formaldehyde. The electrophoresis was run at 70 V for 50 min and the gel was  
147 stained with ethidium bromide (EtBr). The gel was photographed under the UV light  
148 in a transilluminator with a photographic camera (Syngene, model MultiGenious,  
149 Synoptic Ltd., UK).

## 150 **Primer design**

151 Because the nucleotide sequence of the gene encoding the GMMT enzyme of Aloe  
152 vera is unknown, the primers were designed from three ortholog nucleotide  
153 sequences of *CSLA1* genes from *Setaria italica* (XM\_004951549), *Oryza*  
154 *brachyantha* (XM\_006646934) and *Brachypodium distachyon* (XM\_003571071), all  
155 monocotyledons. The access codes for these genes were obtained from the  
156 National Center for Biotechnology Information (NCBI).

157 The primers selected were:

158 GMMT-F1: 5'- GTCCAGATCCCCATGTTCAACGAG -3'

159 GMMT-R1: 5'- CCAACAGAATTGAGAAGGGTGAT -3'

160 These primers amplify a sequence of 935 bp. The *actin* gene was amplified and  
161 used as a control gene.

### 162 **RT-PCR analysis**

163 The extracted RNA was treated with DNase I (Thermo Scientific) to eliminate  
164 contaminant gDNA. For this, 1.5  $\mu$ L of the 10X enzyme buffer plus 5  $\mu$ L DNase I  
165 and 0.5  $\mu$ L RiboLock RNase Inhibitor (ThermoScientific) (40U/ $\mu$ L), were added to 5  
166  $\mu$ g RNA, completing 15  $\mu$ L with H<sub>2</sub>O DEPC treated, and incubated at 37 °C for 1 h  
167 in the thermocycler. The enzyme was inactivated with 1 $\mu$ L 50 mM EDTA and  
168 incubated at 65 °C for 15 min followed by 75 °C for 5 min. To quantify the RNA  
169 after the DNA digestion was performed by spectrophotometry in a NanoDrop™ at  
170 260 nm. The quality of the RNA was verified by gel electrophoresis.

171 The RNA free of gDNA was used to generate cDNA. Two  $\mu$ g of DNA-free RNA was  
172 mixed with 1  $\mu$ L oligo (dT) primer (0.5  $\mu$ g/ $\mu$ L) (Promega) and incubated at 70 °C for  
173 5 min in the thermocycler. After incubation, the tubes were kept on ice for 5 min.  
174 Twenty  $\mu$ L of the ImProm-II reverse transcription mix (Promega Corporation,  
175 Madison, WI, USA) was incorporated in each tube. The thermocycler program  
176 used was 25 °C for 5 min, 42 °C for 60 min and 70 °C for 15 min. The product was  
177 kept at -20 °C.

178 A PCR analysis was performed with the final cDNA using the designed primers for  
179 the amplification of the partial *GMMT* sequence. The GoTaq Green Master Mix  
180 (Promega Corporation, Madison, WI, USA) was used for the PCR, which contains

181 a Taq DNA polymerase. The thermocycler program was: a pre-incubation cycle at  
182 95 °C for 2 min, 35 cycles of amplification with 30 s of dissociation at 95 °C, 40 s of  
183 hybridization at 51.8 °C, 56 s of extension at 72 °C, a final extension cycle of 5 min  
184 at 72 °C and standby cycle at 10 °C until the tubes were extracted.

185 To determine the size of the PCR product an electrophoresis was run in a 1.5%  
186 agarose gel for 58 min at 85 V. In the gel 5 µL of the PCR product was loaded and  
187 the gel was stained with EtBr and photographed with the Syngene camera  
188 equipment. The expected fragment of 935 bp was obtained.

#### 189 **Restriction analysis of the amplified PCR fragment**

190 Using the *Setaria italica GMMT* gene sequence and *in silico* digestion, the selected  
191 enzyme was Avall. For this enzyme the expected fragments were three because  
192 Avall cuts the *GMMT* sequence in two sites. These fragments are of 550, 244 and  
193 141 bp. *In vitro* digestion was carried out with Avall fast digest of Thermo Scientific  
194 (USA) according to the manufacturer's instructions. Agar gel electrophoresis  
195 confirmed the presence of these three fragments.

#### 196 **Sequence determination of the cDNA *GMMT* fragment of Aloe vera**

197 The PCR product of the *GMMT* gene from Aloe vera was sequenced by Macrogen  
198 (Rockville, MD, USA). The fragment obtained from Aloe vera was 938 bp instead of  
199 the expected 935 bp.

200

201

## 202 **Phylogenetic analysis of the Aloe vera *GMMT* sequence**

203 Phylogenetic trees were generated using nucleotide and amino acid sequences.

204 This was done by using the free online program Phylogeny.fr ([www.phylogeny.fr](http://www.phylogeny.fr)).

205 The nucleotide phylogenetic tree was built with the "One Click" mode, which

206 consists of the following programs: MUSCLE for multiple sequence alignment,

207 Gblocks for automatic alignment curation, PhyML for tree building and TreeDyn for

208 tree drawing (Dereeper et al., 2008). The *GMMT* sequence from Aloe vera was

209 analyzed along with several orthologous sequences of *CSLA* and *CSLC* from other

210 monocotyledon species: *Hordeum vulgare* (*CSLC1*: GQ386981.1; *CSLC2*:

211 GQ386982.1; *CSLC3*: GQ386983.1; *CSLC4*: GQ386984.1), *Oryza brachyantha*

212 (*CSLC3*: XM\_006659911.1; *CSLA1*: XM\_006646934.2; *CSLA9*:

213 XM\_006656206.2), *Setaria itálica* (*CSLA1*: XM\_004951549.3; *CSLA9*:

214 XM\_004965770), *Zea mays* (*CSLA9*: XM\_008661585.2), *Brachypodium*

215 *distachyon* (*CSLA9*: XM\_003560517.3; *CSLA1*: XM\_003571071.3); *Phoenix*

216 *dactylifera* (*CSLA9*: XM\_008806163.2); *Ananas comosus* (*CSLA9*:

217 XM\_020251756.1); *Asparagus officinalis* (*CSLA9*: XM\_020391479.1) and *Elaeis*

218 *guineensis* (*CSLA9*: XM\_010913922.2).

219 The Aloe vera nucleotide sequence was translated *in silico* using the online

220 bioinformatics tool ExPASy translate ([web.expasy.org/translate](http://web.expasy.org/translate)). We built a second

221 phylogenetic tree using the amino acid sequence obtained from *in silico* translation

222 of Aloe vera *GMMT* and partial *CSLA* amino acid sequences from other monocot

223 plants. The tree topology was generated by using the "One Click" mode (Dereeper

224 et al., 2008) which constructed a tree by a maximum-likelihood method.  
225 Additionally a third phylogenetic tree was generated using the same matrix but with  
226 the TNT program ([www.phylogeny.fr](http://www.phylogeny.fr)) and utilizing a mPAM250 amino acid  
227 substitution stepmatrix. Previously the sequences were aligned by ClustalW  
228 (Thompson et al., 1994). This third tree employed a parsimony method for tree  
229 building.

230 In both latter trees the amino acid sequences of monocots were from: *Ananas*  
231 *comosus* (CSLA9: XM\_020251756.1), *Asparagus officinalis* (CSLA9:  
232 XM\_020391479.1), *Brachypodium distachyon* (CSLA1: XP\_003571119.1, and  
233 CSLA9: XP\_003560565), *Elaeis guineensis* (CSLA9: XM\_010913922.2), *Oryza*  
234 *brachyantha* (CSLA1: XP\_006646997.2; and CSLA9: XP\_006656269), *Phoenix*  
235 *dactylifera* (CSLA9: XP\_008804385), *Setaria italica* (CSLA1: XP\_004951606.1 and  
236 CSLA9: XP\_004965827), and *Zea mays* (CSLA9: XP\_008659807.1). Both trees  
237 also included all the known CSLA amino acid sequences from *Oryza sativa*  
238 (CSLA1: XP\_015625335.1; CSLA2: AAK98678.1; CSLA3: BAD37274.1; CSLA4:  
239 AAL84294.1; CSLA5: AAL82530.1; CSLA7: ABG34297.1 and CSLA9:  
240 AAL25128.1) and *Dendrobium officinale* (CSLA1: AIW60927.1; CSLA2:  
241 KM980200.1; CSLA3: KP003920.1; CSLA4: KM980201.1; CSLA5: KM980202.1;  
242 CSLA6: KF195561.1; CSLA7: KP205040.1 and CSLA8: KP205041.1). While *Oryza*  
243 *sativa* belongs to the Poales, *Dendrobium officinale* belongs to Asparagales.

244 To determine node support for our tree (TNT generated) a bootstrap 1000 analysis  
245 was performed ([www.phylogeny.fr](http://www.phylogeny.fr)) using all the monocots CSLA amino acid  
246 sequences previously described.

247 For multiple alignments additional amino acid sequences from the *CSLAC* gene of  
248 two monocots were considered: *Hordeum vulgare* (CSLC1: ACV31212.1, CSLC2:  
249 ACV31213.1, CSLC3: ACV31214.1 and CSLC4: ACV31215.1) and *Oryza*  
250 *brachyantha* (CSLC3: XP\_006659974.1).

### 251 **Quantitative RT-PCR analysis (RT-qPCR)**

252 qPCR primers were designed using the previously sequenced *GMMT*  
253 fragment from Aloe vera, using the online program Primer3  
254 (<http://primer3.ut.ee>) (Untergasser et al., 2012; Koressaar and Remm,  
255 2007). The primers generated were:

256 qGMMT-F1: 5'-GCTATCGTGGCCGTC CG-3' with a melting temperature of 60 °C.

257 qGMMT-R1: 5'-CTTTGCTCGACCACCTCTGG-3' with a melting temperature of 60  
258 °C.

259 These primers amplified a fragment of 107 bp. Primers were tested using  
260 the cDNA from Aloe vera plants subjected to different water treatments.  
261 The sizes of the PCR products were verified by electrophoresis in agarose  
262 gel.

### 263 **Determination of primer efficiencies**

264 Serial dilutions from 100 ng/μL cDNA (1/10 1/100, 1/1,000, 1/10,000,  
265 1/100,000, 1/1,000,000, 1/10,000,000), were produced using DNase-free  
266 water. RT-qPCR assays were performed with these cDNA dilutions in a  
267 Stratagene Mx3000P™ thermocycler (Agilent Technologies, Waldbronn,

268 Germany) with the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix  
269 from Agilent Technologies. The reaction mixture had a final volume of 20  
270  $\mu\text{L}$ , containing 10  $\mu\text{L}$  Brilliant III 2x Master Mix, 8  $\mu\text{L}$  nuclease-free water,  
271 0.5  $\mu\text{L}$  forward primer, 0.5  $\mu\text{L}$  reverse primer and 1  $\mu\text{L}$  of each dilution,  
272 according to the manufacturer's instructions.

273 The efficiency (E) of each primer pair was obtained from the equation:

274  $E = (10^{(-1/\text{slope})} - 1) \times 100\%$  (Radonić et al. 2004)

275 The qPCR program was: initial denaturation at 95 °C for 3 min, 40  
276 amplification cycles at 95 °C for 15 s followed by 60 °C for 15 s,  
277 denaturation at 95 °C for 1 min, final elongation at 60 °C for 30 s and a  
278 final denaturation at 95 °C for 30 s.

279 **RT-qPCR analyses of Aloe vera plants subjected to water stress and**  
280 **exogenous ABA treatment**

281 Three different plants were used per water treatment for water stressed  
282 plants, taking one leaf per plant. For the exogenous ABA experiments, 7  
283 plants of the T1 water treatment (100% FC) were utilized. From these,  
284 three plants were sprayed and irrigated with 100 mL of the control solution:  
285 10% v/v ethanol, 0.1% v/v Tween-20 and distilled water. Four plants were  
286 sprayed with 50 mL of 10  $\mu\text{M}$  ABA (PhytoTechnology Laboratories, KS,  
287 USA) dissolved in the control solution plus an additional 50 mL of the  
288 same ABA solution was applied directly to the pot (Chu et al., 1990). Both  
289 control (100 mL/ plant) and ABA (100 mL/ plant) solutions were applied

290 once to the plants during the dark photoperiod. This was done to allow  
291 better permeation of the respective solutions via the stomatal aperture that  
292 occurs in CAM plants. One leaf from each plant was collected prior to any  
293 treatment (0 h control). Another leaf was also collected from each plant at  
294 12 h, 24 h, 48 h, 60 h, 1 week and 2 weeks after ABA or control  
295 treatments.

296 The reaction mix used for the RT-qPCR analyses contained 1  $\mu$ L cDNA  
297 (10 ng/ $\mu$ L) from each treatment plus the components described above.

#### 298 **ABA and ABA derivatives quantification by HPLC-ESI-MS/MS**

299 The quantifications were performed from the same leaves used in the RT-  
300 qPCR analyses of plants subjected to the 4 water treatments. For this, the  
301 green cortex was used to extract the ABA and ABA derivatives. The cortex  
302 was ground into a fine powder and kept at -80 °C.

303 ABA and its derivatives were extracted according to Ordaz-Ortiz et al.  
304 (2015) with some modifications. One hundred and fifty mg of fresh ground  
305 tissue was extracted with 5 mL of methanol/water/formic acid (75:20:5  
306 v/v/v). The resulting solution was shaken using a vortex for 15 min at 4 °C.  
307 The resulting suspension was centrifuged at 1.500 x g for 45 min at 4 °C.  
308 The supernatant was recovered and mixed with deuterated internal  
309 standards (5 ng/mL final concentration of each standard). The mixture was  
310 filtered through 0.45  $\mu$ m PVDF filters, and 20  $\mu$ L of the filtered mix was  
311 injected into a 1200s Agilent liquid chromatograph coupled to a 6410



312 Agilent electrospray ionization triplequad tandem mass spectrometer (ESI-  
313 MS/MS). Chromatographic separation was performed according to Pan et  
314 al. (2010), using a C18 column (150x4.6mm, 5µm Intersil-ODS-3, GL-  
315 Sciences); the mobile phases were 0.1% formic acid in water (A) and 0.1%  
316 formic acid in methanol (B), with 0.3 mL min<sup>-1</sup> flow rate at room  
317 temperature. The elution was done using linear steps; 30% B from 0 to 2  
318 min, 2 to 20 min increasing to 100% B, 20 to 22 min with 100% B and 22  
319 to 25 min with 30% B. The MS/MS detection was performed using the  
320 multiple reactions monitoring mode (MRM) at -4500 V, 25 psi nebulization  
321 pressure and 10 L min<sup>-1</sup> nitrogen flow. Deuterated standards were  
322 purchased from Olchemim and Canada National Research Council of  
323 Canada-Plant Biotechnology Institute (Ordaz-Ortiz et al., 2015). ABA  
324 standard was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO,  
325 USA).

### 326 **Statistical analyses**

327 One way ANOVA and Tukey's post-hoc test were performed to determine  
328 the significant differences among the results obtained from each  
329 quantitative analysis.

### 330 **Results**

331 Two leaf anatomical parameters were measured to demonstrate that Aloe vera  
332 plants were subjected to water deficit, total leaf fresh weight and leaf thickness in  
333 plants subjected to four water treatments. In Figure 2 (A and B) the leaf fresh

334 weight and thickness decrease with increasing water deficit. The greatest variation  
335 of both parameters was in T4 plants.

### 336 **PACE analyses of Aloe vera glucomannan**

337 Polysaccharide analysis of carbohydrate gel electrophoresis was  
338 performed to determine if the acemannan from Aloe vera gel was a  
339 glucomannan or a galactoglucomannan. Figure 3 shows the results of  
340 these analyses. The presence of glucomannan is clearly seen by the  
341 production of oligosaccharides that migrate with the pure mannan  
342 standards, and also the glucomanno-oligosaccharides from Konjac  
343 glucomannan.

344 The results shown in Figure 3C demonstrated that galactose is not  
345 released from GMT<sub>1</sub> and GMT<sub>4</sub> after digestion of the acemannan with  $\alpha$ -  
346 galactosidase. Galactose was only detected after digestion of the locus  
347 bean galactomannan with this enzyme. From these results we can  
348 conclude that the acemannan from Aloe vera is a glucomannan without  
349 galactose branches.

### 350 **Amplification of the cDNA of *GMMT***

351 To identify the *GMMT* gene, we used primers based on *CsIA* genes as described in  
352 materials and methods. Using the selected primers a single fragment of cDNA of  
353 approximately 935 bp was obtained from total mRNA extracted from Aloe vera  
354 leaves, as shown in Figure 4.

355 To corroborate that the fragment of 935 bp was from the *GMMT* gene, this cDNA  
356 was subjected to a restriction enzyme digestion. The online program Restriction

357 Mapper was used for this. Avall was selected by preliminary *in silico* analysis using  
358 the sequence of *CSLA1* from *Setaria italica*. This endonuclease cuts in two  
359 restriction sites in this sequence, generating three fragments of 550, 244 and 141  
360 bp. The digestion of the Aloe *GMMT* cDNA indeed resulted in three fragments of  
361 approximately the expected sizes, as seen in Figure 5.

### 362 **Sequence analysis of the isolated *GMMT* fragment**

363 The expected size of the amplified fragment was 935 bp, in which the  
364 forward and reverse primer sequences were included with some minor  
365 changes in their sequences. In the reverse primer sequence there were  
366 three extra nucleotides, which gave rise to a fragment of 938 bp. The three  
367 extra nucleotides might be due to an artifact caused by the DNA  
368 polymerase (Taq polymerase). Figure 6 shows the nucleotide sequence  
369 obtained for the amplified Aloe vera *GMMT* fragment.

370 With this sequence a BLAST analysis was done using the NCBI program. Table I  
371 shows this BLAST.

372 This BLAST showed between 79% and 84% identity with the species shown. The  
373 E value was 0.0 in all cases, indicating a high certainty that the Aloe vera  
374 nucleotide sequence encodes a glucomannan mannosyl synthase CSLA9-like  
375 enzyme. The nucleotide sequence from Aloe vera was 84% identical to the cDNA  
376 of a glucomannan 4-beta-mannosyltransferase 9-like of *Asparagus officinalis* and  
377 *Phoenix dactylifera*. Aloe vera shared 82% identity with the Asparagales  
378 *Dendrobium officinale* and *Dendrobium catenatum*, both encoding a 4-beta-

379 mannosyltransferase 9-like. The sequence also has 79% identity with  
380 *Brachypodium distachyon* mannan synthase 9-like.

### 381 ***In silico* analyses of the translated *GMMT* sequence**

382 It was found that the deduced amino acid sequence contains two aspartic residues  
383 (D), which form the sequence DXD (where X is a variable amino acid residue)  
384 identified as the binding site to divalent cations and sugar nucleotides present in  
385 the glycosyl transferase enzymes.

386 A second conserved amino acid region was also found constituted by glutamine, a  
387 variable residue (X), histidine, arginine and tryptophan (QXHRW). The variable  
388 residue was found to be glutamine (Q) in Aloe vera, resulting in the conserved  
389 region QQHRW (Table 3). This region is different from the CESA enzymes which  
390 synthesize plant cellulose. The sequence of CESA has a second residue of valine  
391 (V) instead of glutamine.

392 With this analysis we were able to build a phylogenetic tree, shown in Figure 7.  
393 This tree was constructed using the nucleotide sequence and it shows that the  
394 Aloe vera gene sequence is in the *CSLA* cluster, apparently in the subgroup of  
395 *CSLA9* next to *Asparagus officinalis*. Both are Asparagales and among other  
396 monocotyledon species. The closest group to *CSLA9* is the *CSLA1* cluster, while  
397 the sequences for *CLSC*, which encodes the enzyme xyloglucan synthase, were  
398 grouped further apart from the *CLSA1* and *CLSA9* encoding the glucomannan  
399 synthases (Figure 7).

400 The bootstrap 1000 analysis performed with all the monocot amino acid  
401 sequences and generated with One Click Mode confirmed that the Aloe

402 vera sequence is grouped with the CSLA9 and in the Asparagales group  
403 closer to *Asparagus officinalis* and *Dendrobium officinale*. The amino acid  
404 phylogenetic tree also indicates that Aloe vera sequence is near to CSLA1,  
405 Figure 8. Results not shown suggest that the amino acid sequence from  
406 Aloe vera is further away from CESA (cellulose synthase) and from CSLC  
407 (xyloglucan synthase). Figure 8 shows the results from both phylogenetic  
408 trees generated with the amino acid sequences.

#### 409 **RT-qPCR analysis of Aloe vera *GMMT***

410 The primers chosen for the qPCR analyses amplified a fragment of 107 bp  
411 for the *GMMT* of Aloe vera. Figure 9 shows the RT-qPCR product for  
412 *GMMT* and *ACTIN*. The *ACTIN* fragment was 116 bp.

413 Using the designed primers, it was found that *GMMT* expression in Aloe vera  
414 increased a significant amount in the plant leaves of T3 water treatment (50% FC)  
415 compared to the control group T1 and the most severe water restriction treatment  
416 T4. Between T2 (75% FC) and T3 plants there was no significant difference in  
417 *GMMT* expression, Figure 10.

#### 418 **Quantification of ABA and its metabolites in water-stressed Aloe vera plants.**

419 ABA and ABA metabolites were quantified to study correlation between the  
420 expression of *GMMT* and the endogenous concentration of this hormone and its  
421 derivatives in plants subjected to water treatments. The results of these  
422 quantifications are shown in Table 4.

423 The table shows that ABA and its metabolites, PA, DPA and ABA-GE, increased  
424 significantly under water restriction. There was a 15.5-fold increase of ABA in T4  
425 plants compared to the control group T1. For ABA derivatives, PA increased 41.5  
426 times and DPA 10.6 times in T4 plants with respect to the control group. The  
427 conjugated form of ABA (ABA-GE) increased 7.9 times in the most severe water-  
428 stressed group of plants compared to T1.

#### 429 ***GMMT* expression under exogenous ABA treatment**

430 To determine if ABA is involved in *GMMT* expression, plants of Aloe vera from the  
431 control group (T1) were subjected to a single dose of 10  $\mu$ M of ABA. Figure 11  
432 shows the expression of this gene before and after the hormone treatment.

433  
434 The results show that the expression gradually increased 4 times after 48 h of ABA  
435 treatment. *GMMT* expression decreased at 60 h, returning to the basal level and  
436 gradually increasing again at 1 and 2 weeks after the initial ABA application. The  
437 control group did not show any significant variation in the expression of *GMMT* at  
438 any time interval.

439

#### 440 **Discussion**

441 The CAM metabolism of Aloe vera optimizes water use efficiency and allows the  
442 plant to tolerate water deficit. However, Aloe vera shows morphological changes in  
443 its leaves in plants subjected to the most severe water deficit (25% FC, T4). Fresh  
444 weight decreases gradually in plants with increasing water restrictions;  
445  $T1 \square T2 \square T3 \approx T4$ . The thickness also decreases gradually with increasing water  
446 deficit;  $T1 \square T2 \square T3 \square T4$ . Water stress also causes changes in leaf pigmentation.

447 The leaves of T4 plants have a more purple color compared to the green of well-  
448 watered plants.

449 The changes in fresh weight and leaf thickness are probably due to a decrease in  
450 water content of leaves, specifically of the water stored in the leaf gel made of  
451 acemannan polysaccharide (Silva et al. 2010). This polysaccharide is the main  
452 molecule that retains water in the mesophyll of the leaf. Since the length of leaves  
453 is affected by water stress, it is very likely that the photosynthetic area is also  
454 reduced. Silva et al. (2014) found that the photosynthetic cells decrease in length  
455 and mesophyll thickness is reduced with water stress in Aloe vera leaves, therefore  
456 the amount of gel is also reduced. We can conclude that Aloe vera plants can  
457 suffer water stress in spite of being tolerant to drought.

458 Acemannan from Aloe vera has been described in the literature as a  
459 galactoglucomannan (Minjares-Fuentes et al., 2017) and/or as a glucomannan  
460 without galactose branches (Campestrini et al. 2013). In this study, using PACE  
461 analysis we show that Aloe vera acemannan lacks detectable galactose branches,  
462 since digestion with  $\alpha$ -galactosidase did not release any galactose and is only  
463 composed of glucomannan oligosaccharides similar to those described for Konjac  
464 glucomannan (Goubet et al. 2002). Although, it might be possible that the  $\alpha$ -  
465 galactosidase we used was unable to hydrolyze the glycosidic bond to release  
466 galactose from the glucomannan backbone. This enzyme, however can release  
467 galactose from glucomannan from other plants. A previous GC-MS analysis of the  
468 alditol acetates sugar derivatives performed by our group indicated negligible  
469 (0.05%) galactose. This amount of galactose might be too low for the  $\alpha$ -

470 galactosidase to release galactose or, probably galactose is a contaminant from  
471 cell walls. The polysaccharide is made up of 87% mannose and 13% glucose,  
472 indicating that it is a glucomannan (Quezada et al. 2017). Although Minjares-  
473 Fuentes et al. (2017) performed a methylation analysis to determine the glycosidic  
474 linkages of the polysaccharide, a reliable analysis, it is unknown if the Aloe vera  
475 cultivar that they used was the same as ours. Other variables that can account for  
476 the presence or lack of galactose could be the age of the plants, the environmental  
477 conditions of the plant cultivation and/or the plant tissue from which the gel is  
478 extracted from. This may explain the absence of galactose side branches in our  
479 samples.

480 Since water stress seems to affect gel production (Silva et al. 2010) in Aloe vera  
481 and the main compound of the gel is acemannan, it was important to determine the  
482 expression level of the gene encoding the *GMMT* enzyme. This is the main  
483 enzyme that synthesizes the glucomannan backbone, by transferring mannose and  
484 glucose to the growing polysaccharide using GDP-mannose and GDP-glucose  
485 (Liepman and Cavalier, 2012). With the designed primers and our experimental  
486 conditions, we were able to amplify a single fragment of 938 bp of the *GMMT* gene.  
487 Even though the fragment was of the expected length and a restriction enzyme  
488 analysis gave the expected fragment pattern, it was necessary to sequence the  
489 amplified cDNA segment, since the Aloe vera genome has not been sequenced.

490 *GMMT* belongs to a complex family of genes encoding the cellulose synthase like  
491 (CSL) enzymes which are glycosyltransferases. Since acemannan is a  
492 glucomannan, the gene encoding the corresponding enzyme has to be any *CSLA*



493 (Gille et al. 2011; Liepman and Cavalier 2012). Searching the databases for  
494 sequences of these genes, we were able to identify the *CSLA* gene subfamily to  
495 which the Aloe vera sequence most likely belongs.

496 It is important to point out that the amino acid sequence from the *in silico*  
497 translation corroborates that the conserved region “DXD” encodes the enzyme  
498 binding site to sugar nucleotides. The amino acid sequence also shows another  
499 conserved region of 5 amino acids “QQHRW”, characteristic of the enzyme active  
500 site of the CSL subfamily (Liepman and Cavalier, 2012). The second glutamine  
501 (Q), and histidine (H) of this sequence are variable, while the first glutamine  
502 residue from this region gives the specificity of the CSL enzymes that synthesize  
503 hemicelluloses, *CSLA* (Saxena and Brown, 1997, Saxena and Brown 2000,  
504 Liepman and Cavalier 2012). This region is different from the cellulose synthase A  
505 (*CESA*) which synthesizes only cellulose. *CESA* is known to contain a valine (V)  
506 and a lysine (L) in this conserved region, resulting in the sequence QVLRW  
507 (Saxena and Brown, 1997; Saxena and Brown, 2000).

508 Even though both types of enzymes (CSL and *CESA*) belong to the  
509 glycosyltransferase superfamily, our results provide enough evidence to indicate  
510 that the 938 bp nucleotide sequence from Aloe vera encodes for part of a *CSLA*  
511 and not for a *CESA* gene. The phylogenetic tree constructed with the nucleotide  
512 sequence confirmed that the isolated fragment is from a *CSLA* gene. The  
513 sequence is specifically in the subgroup of *CSLA9* next to *Asparagus officinalis*.  
514 Both the sequence from Aloe vera and from *A. officinalis* are in this *CSLA9* cluster  
515 among other monocotyledon species and near the *CSLA1* gene cluster. Since Aloe

516 vera belongs to the Order Asparagales, the proximity of the Aloe vera gene to that  
517 of *Asparagus officinalis* is expected. The phylogenetic tree also indicates that the  
518 Aloe vera *GMMT* is further from the *CSLC* genes which encode for xylan  
519 synthases (Liepman and Cavalier, 2012). In conclusion, the sequence from Aloe  
520 vera is most likely part of the *CSLA9* genes.

521 To confirm these findings, we built a cladogram with the in vitro translated amino  
522 acid and other monocot sequences which were considered in this analysis, like  
523 most of the *CSLA* from *Oryza sativa* and *Dendrobium officinale*. The Aloe vera  
524 sequence is closest to the *Asparagus officinalis* *CSLA9* and near to the *CSLA6*  
525 from *Dendrobium officinale*. He et al., (2015) found that the *CSLA6* of *Dendrobium*  
526 *officinale* has high homology with the *CSLA9* of *Arabidopsis thaliana*. Therefore,  
527 the bootstrap analysis confirmed that the Aloe vera *GMMT* gene sequence belongs  
528 to a *CSLA9*.

529 With our results we cannot exclude the existence of more than one gene and/or  
530 more than one isoform of this enzyme present in Aloe vera. But, even though the  
531 primers we designed were from the most conserved region of the *GMMT* genes,  
532 only one sequence was amplified in this research. On the other hand, since the  
533 total mRNA extracted was from leaves of plants of the same age, this might explain  
534 why we amplified a single fragment. By using other plant tissues of Aloe vera or  
535 leaves from different ages, other *CSLA* genes might have been expressed.

536 The expression of Aloe vera *GMMT* increased 4.5 times under water stress,  
537 between T1 and T3 (50% FC). This increment was found to be gradual from the

538 control plants to T3 plants and to decrease later in T4 plants. Therefore, *GMMT*  
539 expression in Aloe vera responds to water stress, suggesting that the synthesis of  
540 this polysaccharide increases under water deficit. These results make sense, since  
541 it is the molecule that helps to retain water, confirming previous results from our  
542 group in which a mild water deficit increases gel production (Silva et al., 2010).  
543 Similar results were found by Huerta et al. (2013) in Aloe vera water-stressed  
544 plants where the maximum expression of *HSP70* gene was in T3 plants.

545 A previous study by He et al. (2015) reported that the expression of *CSLA* genes  
546 related to the biosynthesis of glucomannan was up-regulated with polyethylene  
547 glycol (PEG) and salt stress treatments in *Dendrobium officinale*, a plant of the  
548 Order Asparagales. This suggests that these genes have a role in abiotic stress  
549 responses.

550 The water deficit in T4 was probably too severe for the plant to respond with  
551 greater *GMMT* expression. This could be due to the fact that T4 plants were not  
552 acclimated to a less severe water stress (such as conditions of T2 and T3 for short  
553 periods of time) to withstand 25% FC. The study of Huerta et al. (2013)  
554 demonstrated that the expression of *HSP* genes is greater when Aloe vera plants  
555 are previously subjected to temperature and water acclimation treatments.

556 Since the *CSLA* gene seems to be expressed under water deficit, our group  
557 considered it important to study if this greater expression of *AvCSLA9* is under the  
558 control of abscisic acid. For this, the endogenous concentration of ABA was  
559 determined in plants of these four water treatments. Indeed, ABA concentration

560 increases gradually and significantly with increasing water deficit. The ABA  
561 concentration was highest in T4, 15.5-fold the concentration of control plants, while  
562 in T3 plants ABA was 4.5-fold the concentration of control group. This increment is  
563 probably sufficient to induce greater expression of Aloe vera *GMMT* (*CSLA9*).  
564 There are no reports so far on ABA regulation of the *CSLA* gene subfamily.  
565 However, there is a recent publication on the upregulation by ABA a xyloglucan  
566 galactosyl transferase encoded by a *CSLC* in *Sorghum bicolor* (Rai et al., 2016).

567 Transcription factors involved in the up-regulation of *CSLA9* in *Arabidopsis thaliana*  
568 have been recently described, such as MYB46, ANAC041 and bZIP1 (Kim et al.,  
569 2014). However, it is still unknown if these transcription factors are ABA-induced.

570 Exogenous ABA application to control plants not subjected to water deficit induced  
571 the expression of the *GMMT* of Aloe vera, suggesting that the expression of this  
572 gene appears to be regulated by ABA. Our results are probably the first finding that  
573 *CSLA9* of a CAM plant might be ABA-regulated.

574 In many seeds the testa cell wall is synthesized during seed development. During  
575 this time the mother plant injects ABA to the seed that keeps it dormant  
576 (Buckeridge, 2010). It is unknown if this ABA injection induces synthesis of the  
577 galactomannans present in the testa of these seeds. But it is known that the cell  
578 wall softens and remodels when seed starts germination and this remodeling  
579 occurs when the level of ABA decreases in the seeds (Bento et al., 2013). In any  
580 case, these hemicelluloses of the testa trap water when the seed initiates  
581 germination and help with seed hydration. These galactomannans are similar in

582 their physiological role to the Aloe vera acemannan as a polysaccharide which  
583 helps to retain water in the leaf tissue. It would be interesting if further research can  
584 confirm that part of this gene family is ABA-regulated. Additionally, further studies  
585 in Aloe vera plants are needed to identify possible ABRE sequences in the  
586 promoter regions of this gene. The analyses of the promoter region of Aloe vera  
587 *CSLA9* may elucidate in the future the transcription factors that can bind to it.

588 The conclusions of this study are:

- 589 1. The acemannan from Aloe vera is a glucomannan without galactose  
590 branches.
- 591 2. The *GMMT* enzyme in Aloe vera is encoded by a gene that belongs to the  
592 *CSLA9* subfamily.
- 593 3. *GMMT* expression increases in Aloe vera plants under water deficit.
- 594 4. ABA appears to be involved in the control of the expression of *GMMT* of  
595 Aloe vera plants subjected to water deficit.

596

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**Table 1. BLAST analysis of the nucleotide sequence of the possible *GMMT* fragment of *Aloe vera*.** The table shows the Cover percentage, E value and Identity between the *Aloe vera* *GMMT* sequence and those of several plant species. In parenthesis is the respective NCBI accession identifier.

Description	Cover	E value	Identity
PREDICTED: <i>Asparagus officinalis</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_020391479.1)	100%	0.0	84%
PREDICTED: <i>Phoenix dactylifera</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_008806163.2)	100%	0.0	84%
PREDICTED: <i>Elaeis guineensis</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_010913922.2)	99%	0.0	84%
PREDICTED: <i>Ananas comosus</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_020251756.1)	97%	0.0	84%
PREDICTED: <i>Musa acuminata</i> subsp. <i>malaccensis</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_009422847.2)	98%	0.0	82%
<i>Dendrobium officinale</i> glucomannan 4-beta-mannosyltransferase 9 mRNA, complete cds, (KF195561.1)	100%	0.0	82%
PREDICTED: <i>Dendrobium catenatum</i> glucomannan 4-beta-mannosyltransferase 9-like, mRNA, (XM_020817384.1)	100%	0.0	82%
PREDICTED: <i>Setaria italica</i> probable mannan synthase 9, mRNA, (XM_004965770.3)	100%	0.0	80%
PREDICTED: <i>Brachypodium distachyon</i> probable mannan synthase 9, transcript variant X1, mRNA, (XM_003560517.3)	100%	0.0	79%

**Table 2. Multiple alignment of a partial region from the translated fragment of CSLA (GMMT) from Aloe vera.** The alignment was performed with several cellulose synthase A-like and C-like from different monocotyledon species. Within the solid box are the aspartic residues of the DXD domain characteristic of the glycosyltransferases that allow the interaction with sugar nucleotides.

Species	Enzyme	Sequence	Final Residue
<i>Setaria italica</i>	CSLA1	VAIFDADFQDPDFL	192
<i>Brachypodium distachyon</i>	CSLA1	VVIFDADFQDPDFL	189
<i>Oryza brachyantha</i>	CSLA1	VAIFDADFQDPDFL	140
<i>Phoenix dactylifera</i>	CSLA9	VAIFDADFQPEPDFL	203
<i>Setaria italica</i>	CSLA9	VAIFDADFQPEPDFL	203
<i>Zea mays</i>	CSLA9	VAIFDADFQPEPDFL	195
<i>Oryza brachyantha</i>	CSLA9	VAIFDADFQDPDFL	186
<i>Brachypodium distachyon</i>	CSLA9	VAIFDADFQPDADFL	154
<i>Elaeis guineensis</i>	CSLA9	VAIFDADFQPEPDFL	198
<i>Ananas comosus</i>	CSLA9	VAIFDADFQPEPDFL	198
<i>Asparagus officinalis</i>	CSLA9	VVIFDADFQPEHDFL	200
<i>Aloe vera</i>	CSLA9	VVIFDADFQPEPDFL	100
<i>Hordeum vulgare</i>	CSLC2	VAIFDADFQPNPDFL	169
<i>Hordeum vulgare</i>	CSLC1	VVIFDADFQPQADFL	338
<i>Hordeum vulgare</i>	CSLC4	VVIFDADFQPQEDFL	167
<i>Hordeum vulgare</i>	CSLC3	VAIFDADFQPNPDFL	208
<i>Oryza brachyantha</i>	CSLC3	VAIFDADFQPNPDFL	250
		*.*****:***	

**Table 3. Multiple alignment of the active site of the translated fragment of CSLA (GMMT).** The alignment was performed with several cellulose synthase A-like and C-like from different monocotyledon species. Within the dashed line is the conserved aspartic acid (D) residue from the active site in the proteins that have a glycosyltransferase-like 2 region (GT2). In the solid box is the conserved region QQHRW, while in the dotted line is the second conserved glutamine (Q) that gives specificity to CSL enzymes.

Species	Enzyme	Sequence	Final Residue	Sequence	Final Residue
<i>Setaria italica</i>	CSLA1	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAIRA	283	KAFRF <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	323
<i>Brachypodium distachyon</i>	CSLA1	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAIRA	280	KAFRF <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	320
<i>Oryza brachyantha</i>	CSLA1	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAIRA	283	KAFRF <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	278
<i>Phoenix dactylifera</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	301	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	341
<i>Setaria italica</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	301	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	341
<i>Zea mays</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	293	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	333
<i>Oryza brachyantha</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	284	KAFRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GRANL	324
<i>Brachypodium distachyon</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	252	KAFRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	292
<i>Elaeis guineensis</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	296	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	336
<i>Ananas comosus</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	296	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	336
<i>Asparagus officinalis</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	297	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	337
<i>Aloe vera</i>	CSLA9	GGWKDRTTVE <span style="border: 1px dashed red;">D</span> MDLAVRA	197	KAYRY <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">SC</span> GPANL	237
<i>Hordeum vulgare</i>	CSLC2	GGWMERTTVE <span style="border: 1px dashed red;">D</span> MDIAVRA	267	EAYRK <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">HS</span> GPMQL	307
<i>Hordeum vulgare</i>	CSLC1	GGWMERTTVE <span style="border: 1px dashed red;">D</span> MDIAVRA	436	EAYRK <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">HS</span> GPMQL	476
<i>Hordeum vulgare</i>	CSLC4	GGWMERTTVE <span style="border: 1px dashed red;">D</span> MDIAVRA	266	EAYRK <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">HS</span> GPMQL	306
<i>Hordeum vulgare</i>	CSLC3	GGWMERTTVE <span style="border: 1px dashed red;">D</span> MDISVRA	306	QAYRK <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">HS</span> GPMQL	346
<i>Oryza brachyantha</i>	CSLC3	GGWMERTTVE <span style="border: 1px dashed red;">D</span> MDIAVRA	341	QAYRK <span style="border: 1px solid blue;">Q</span> QHRW <span style="border: 1px dotted red;">HS</span> GPMQL	381
		*** :*****: : **		:*:* ***** .* :*	

**Table 4. Quantification of ABA and its derivatives in Aloe vera plants subjected to water deficit.** ABA, phaseic acid (PA), dihydrophaseic acid (DPA) and ABA-glucose ester (ABA-GE) were quantified by HPLC-ESI-MS/MS as described in Material and Methods. The hormones were extracted from 3 different plants for each water treatment (N=3). ABA quantifications are given in nmol, while PA, DPA and ABA-GE are given in pmol. Each value is given with its SD. Different letters within the same row indicate significant differences among water treatments (one way ANOVA,  $P < 0.05$  and Tukey's post-hoc test).

ABA and ABA derivatives	Water Treatment (% FC)			
	T1 (100 %)	T2 (75 %)	T3 (50 %)	T4 (25 %)
ABA (nmole/g DW)	1.26 ± 0.18 a	4.08 ± 0.27 b	5.70 ± 0.27 c	19.56 ± 4.19 d
PA (pmole/g DW)	3.18 ± 0.44 a	23.84 ± 0.85 b	45.68 ± 5.77 c	132.98 ± 16.35 d
DPA (pmole/g DW)	9.45 ± 0.75 a	27.61 ± 2.12 b	59.40 ± 8.15 c	100.04 ± 5.29 d
ABA-GE (pmole/g DW)	100.08 ± 16.85 a	289.49 ± 34.20 b	504.46 ± 40.82 c	851.77 ± 107.7 d

**Figure 1. Acemannan structure.** The structure corresponds to an acetylated glucomannan. Mannosyl (Man) and glucosyl (Glc) residues are linked by  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkages. The acetyl groups are encircled by dashed lines.

**Figure 2. Leaf anatomical parameters of plants subjected to different water treatments.** A. shows the fresh weight of the whole leaves. B. shows the average thickness of whole leaves. Different letters indicate a significant difference between treatments by one way ANOVA ( $p < 0.05$ ) and Tukey's post-hoc test.

**Figure 3. PACE analyses of acemannan from Aloe vera leaf gel.** **A.** Sugar standards (Std), stained with ANTS, from left to right are: galactose (Gal), oligosaccharide ladder of mannose (M), glucose (Glc). M<sub>1</sub>: mannose, M<sub>2</sub>: disaccharide, M<sub>3</sub>: trisaccharide, M<sub>4</sub>: tetrasaccharide, M<sub>5</sub>: pentasaccharide, M<sub>6</sub> hexasaccharide, all mannan standards. **B.** Different mannan polysaccharides digested with 26A mannanase (26A) and/or  $\alpha$ -galactosidase ( $\alpha$ -Gal). K: Konjac glucomannan. GMT<sub>1</sub>: glucomannan from Aloe vera of T1 treatment. GMT<sub>4</sub>: glucomannan from Aloe vera of T4 treatment. LB: Locust bean gum (galactomannan). Numbers indicate oligosaccharides from glucomannan from Aloe vera double digestion with the 26A and  $\alpha$ -Gal enzymes. 1: disaccharide of glucosyl mannose, 2: disaccharide of mannosyl mannose, 3: trisaccharide of mannosyl glucosyl mannose, 4: glucosyl glucosyl mannose, 5: glucosyl mannosyl mannose. Beyond number 5, there are other larger oligosaccharides of glucomannan. **C.** Corresponds to different mannans digested with  $\alpha$ -Gal enzyme. Enz: only  $\alpha$ -Gal enzyme. +: with enzyme digestion. -: without enzyme digestion.

**Figure 4. Gel electrophoresis of *GMMT* cDNA amplicons from *Aloe vera* plants.** **A** and **B** are the *GMMT* fragments, amplified using the designed primers mentioned in Material and Methods. The gel shows a clear single band of approximately 935 bp. **M**, molecular markers (Thermo Scientific GeneRuler 100 bp Plus DNA ladder).

**Figure 5. Restriction analysis of the *GMMT* cDNA of *Aloe vera* using *Avall*.** Lane **A**, shows the cDNA without the restriction enzyme. Lanes **B** and **C** show the fragments obtained from two different cDNA samples after digestion with *Avall*. **M**, molecular marker (Thermo Scientific GeneRuler 100 bp Plus DNA ladder). The top left arrow indicates the undigested cDNA. The left dotted arrows indicate cDNA fragments after *Avall* digestion.

**Figure 6. Sequence of the amplified fragment of *Aloe vera GMMT*.** The nucleotides in red indicate the forward (*GMMT-F1*) and reverse primer sequences (*GMMT-R1*). The three extra nucleotides in the reverse primer sequence are underlined.

**Figure 7. Phylogenetic tree of *CSLA* and *CSLC* nucleotide sequences from different monocotyledon species.** Within the box is shown *Aloe vera* (*Av*) *GMMT* fragment (a possible *CSLA9*), which has a high identity with the sequences of *CSLA9* from other monocotyledons. *Hv*: *Hordeum vulgare*; *Ob*: *Oryza brachyantha*; *Si*: *Setaria italica*; *Bd*: *Brachypodium distachyon*; *Ac*: *Ananas comosus*; *Ao*: *Asparagus officinalis*; *Eg*: *Elaeis guineensis*; *Pd*: *Phoenix dactylifera*; *Zm*: *Zea mays*. The numbers indicate the percent of Maximum-likelihood between clades (the percent for branch support), results only greater than 70% are shown in the tree. The bar length indicates the number of substitutions per site.

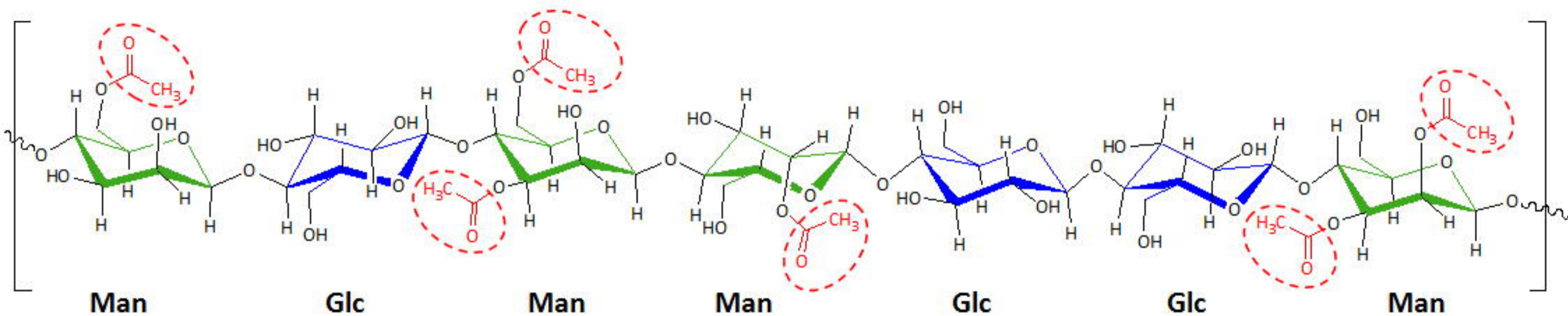


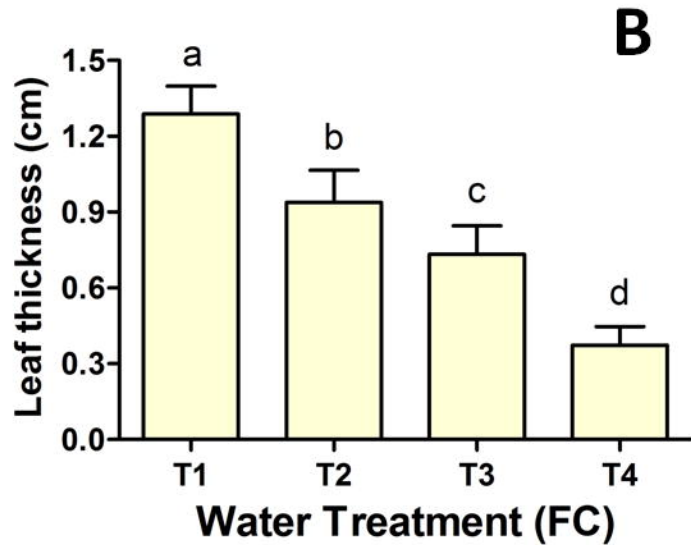
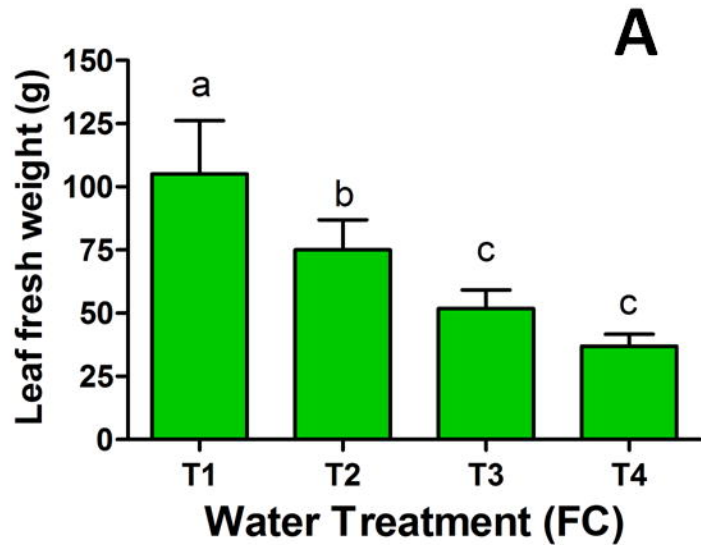
**Figure 8. Phylogenetic tree of CSLA amino acid sequences from monocotyledon species.** All the sequences are CSLA enzymes exclusively from monocotyledons. The Aloe vera (Av) fragment is highlighted within the box and is clustered with other CSLA9 enzymes. Since the resulting topology was similar between both amino acid sequence trees generated, only the maximum-likelihood method tree is shown. See Materials and Methods for details for both trees. The numbers in front of each node indicates the statistical node support; on left side is the maximum-likelihood branch support and on the right side are the corresponding bootstrap values considering 1000 replicates were used. Only results greater than 70% for each node are shown, bootstrap values lower than 70% are represented by (-). The bar length indicates the number of substitutions per site. Ac: *Ananas comosus*; Ao: *Asparagus officinalis*; Bd: *Brachypodium distachyon*; Do: *Dendrobium officinale*; Eg: *Elaeis guineensis*; Ob: *Oryza brachyantha*; Os: *Oryza sativa*; Pd: *Phoenix dactylifera*; Si: *Setaria italica*; Zm: *Zea mays*.

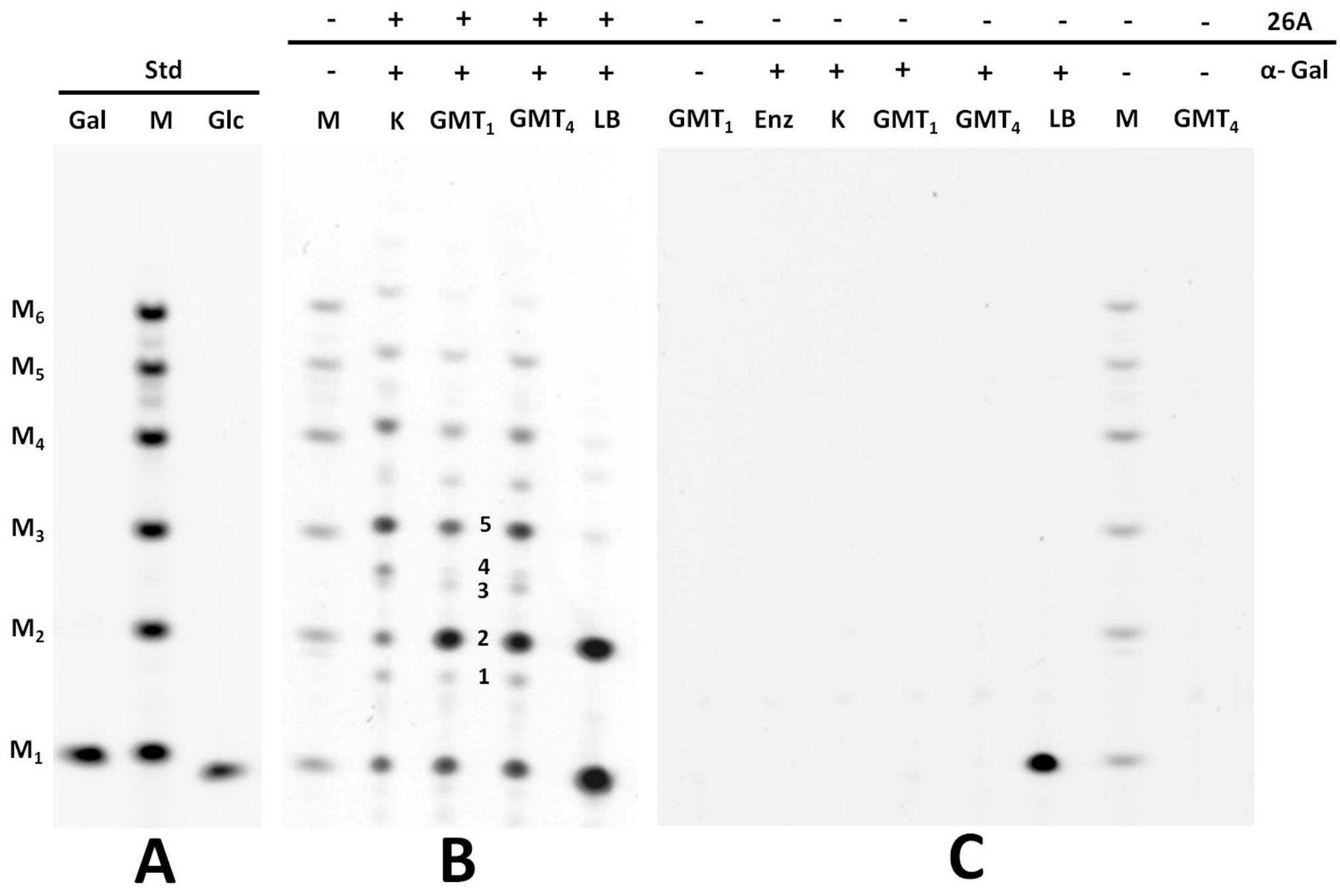
**Figure 9. RT-qPCR products for *GMMT* and *ACTIN* genes from Aloe vera.** I, in lanes A and B are replicates of the 107 bp *GMMT* fragment. In lanes C and D are also replicates of the *ACTIN* gene of 116 bp, used as control a housekeeping gene. II, lanes E and F contain the respective negative controls (no template control). M are molecular markers (Thermo Scientific GeneRuler Low Range DNA Ladder).

**Figure 10. *GMMT* expression levels in Aloe vera plants subjected to 4 water treatments.** Adult plants were subjected for 13 weeks to weekly irrigation as described in Materials and Methods. Treatment 1 was used as a calibrator and the *ACTIN* expression levels as a normalizer. Three biological replicates and one technical replicate were used. Different letters denote significant differences determined by one way ANOVA ( $p < 0.05$ ) and Tukey's post-hoc test.

**Figure 11. *GMMT* expression in Aloe vera plants treated with exogenous ABA.** The gene expression was determined at different time intervals, 0, 12, 24, 48 and 60 h, 1 and 2 weeks after ABA treatment. 0 h is the control group before ABA treatment. The dotted line indicates the basal expression level of *GMMT*. Four different plants were used for the assay. Each bar is the average of three technical replicates for each time interval. Error bars indicate SD. Different letters denote significant differences between time intervals by one way ANOVA ( $P < 0.05$ ) and Tukey's post-hoc test.



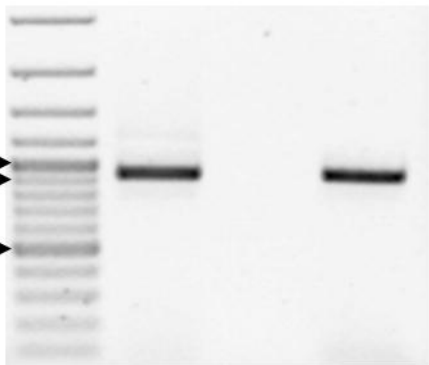


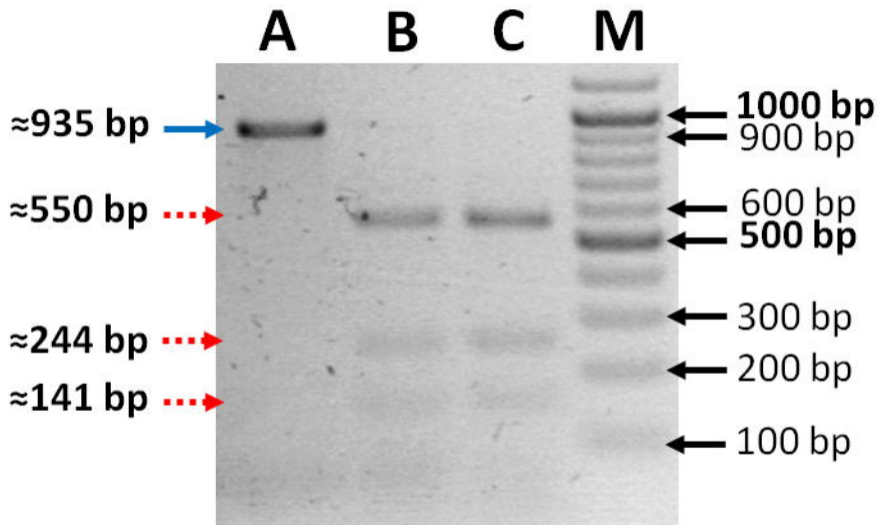


**M****A****B**

**1000 bp** →  
**900 bp** →

**500 bp** →





5'-**GTCCAGATTCCCATGTTCAACGAG**AAAGAGGTGTATCAGCTGTCAATTGGAGCTGCATGCGGGGCTATCGT  
GGCCGTCCGATCGGATCATCATCCAAGTGCTTGATGATTCCAAGTATCCTATCATTAAAGGATATGGTGGAGGT  
GGAGTGCCAGAGGTGGTCGAGCAAAGGGATAAACATAAAGTACGAGATTAGGGACAACAGGAACGGATAC  
AAAGCCGGCGCATTGAAAGAAGGGATGAAGCACAGCTACGTGAAGCACTGCGACTACGTCGTCATCTTCGAC  
GCCGACTTCCAGCCGGAGCCCGACTTCCTCTGGCGTTCAGTTCCTTCTACTTCACAACCCTGAGGTCGGACTC  
GTCCAGGCTCGCTGGAAGTTCGTAAACTCAGATGAGTGCCTAATGACAAGGATGCAAGAGATGTCCCTCGATT  
ACCACTTCACTGTGGAACAGGAGGTCGGCTCCGCTACCTACGCATTCTTCGGTTTCAACGGAACTGCTGGAGTA  
TGCGCGGATCGCGGCTCTTAATGAAGCGGGAGGGTGGAAAGGATCGGACCACGGTGGAGGACATGGACTTGGC  
TGTCGAGCAAGCCTCAAAGGGTGGAAAGTTTGTCTTCTCGGAGACCTAAAGGTTAAGAATGAACTACCGAGC  
ACTCTAAAGGCATACCGATATCAGCAGCATAGATGGTCTTGCGGACCAGCAAACCTTGTTTCAGGAAAATGGTGA  
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AAGATTGTGGCTCACATTGTGACCTTTGTATTCTACTGCGTTGTGATCCCCACAACCGTGTTGGTTCCTGAAGTT  
GTAATACCAAAGTGGGGAGCAGTTTACATTCCCTCCATC**ATTCACCCTTCTTCAATTCTGGTTGG** - 3'



