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1 Evaluation of synthetic promoters in 2 *Physcomitrella patens* 3

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14

15 **Highlights**

- 16 • *Cis*-elements can be randomly assembled to construct short plant promoters
- 17 • Synthetic promoters have higher mRNA expression level than the endogenous *PpAct7*
- 18 • Synthetic promoters have similar protein expression levels to *AtUBQ10* promoter

19

20

21 **Abstract**

22 Securing a molecular toolbox including diverse promoters is essential for genome engineering.
23 However, native promoters have limitations such as the available number or the length of the
24 promoter. In this work, three short synthetic promoters were characterized by using the yellow
25 fluorescent protein Venus. All of the tested promoters were active and showed mRNA activity higher
26 than housekeeping gene *PpAct7*, and similar protein expression level to *AtUBQ10* promoter. This
27 study shows that few *cis*-elements are enough to establish a strong promoter for continuous
28 expression of genes in plants. Along with this study, enhance the number of available promoters to
29 be used in *P. patens*. It also demonstrate the potential to construct multiple non-native promoters on
30 demand, which would aid to resolve the bottleneck issue of multiple pathway expression in *P. patens*
31 and other plants.

32

33 **Keywords:** *Physcomitrella patens*; Venus; Ubiquitin promoter; Actin promoter; Synthetic Biology;
34 synthetic promoters

35 **Introduction**

36 Genes in eukaryotes consist of three major regions, the promoter, the coding strand and the
37 terminator. The promoter controls the gene expression by regulating the binding of transcription
38 factors to recruit RNA polymerase (Latchman 1997). Thus, the synthesis of mRNA is directly
39 correlated with the promoter activity that may lead to the production of protein. The promoter is
40 situated thousands of base pairs (bp) upstream from the transcription start site (TSS), to about 30 bp
41 downstream from the TSS (Porto *et al.* 2014). Promoters come in different type of switches, some
42 are constitutive, some react to specific stimuli, and some are inducible and maintain a strict on/off
43 style switch.

44 Plants are known to have larger promoter sequences than those found in fungi and prokaryotes. Plant
45 promoters typically range from 500 bp to over 2,000 bp (Liu and Stewart 2016). As the possibilities
46 in synthetic biology advances, it is often necessary to introduce multiple genes and promoters to
47 achieve the desired traits. However, endogenous plant promoters are often of limited use in plant
48 synthetic biology as multiple copies of the same promoter can trigger homology-dependent gene
49 silencing (Halpin 2005). Therefore, characterizing multiple promoters from heterologous species has
50 become important for fine-tuning of multiple genes.

51 *Physcomitrella patens* is a plant model system that has been used extensively to study plant evolution,
52 physiology, and development (Vesty *et al.* 2016). The full genome is sequenced (Rensing *et al.* 2007)
53 and development growth media and transformation methods are well described (Bach *et al.* 2014).
54 Its ability to perform efficient homologues recombination, that now can be explained with RecQ
55 helicase function (Wiedemann *et al.* 2018), is unique among plants enabling *in vivo* assembly of
56 multiple DNA fragments followed by targeted genome integration by homologues recombination
57 (King *et al.* 2016). Collectively, such distinct features make *P. patens* attractive as an industrial

58 production platform for small natural products, which requires integration of numerous genes (Zhan
59 *et al.* 2014; Ikram *et al.* 2015; Pan *et al.* 2015; Sabovljević *et al.* 2016; Khairul Ikram *et al.* 2017).

60 Monocot housekeeping gene promoters and the 35S promoter from Cauliflower mosaic virus (CaMV)
61 have shown a high-level of gene expression in *P. patens* (Horstmann *et al.* 2004; Saidi *et al.* 2005).
62 However, the limited number of available promoters are a bottleneck in introducing complex
63 pathways into *P. patens* and typically long plant promoters are difficult to handle in multiple gene
64 integrations. Therefore, developing strong and short synthetic promoters has emerged as a major
65 interest to provide a solution.

66 Synthetic promoters are relatively short (300-500 bp) and can be generated in countless number with
67 similar strength or function, and could improve the genome stability (Roberts 2011). *Cis*-regulatory
68 elements (CREs) of native promoters are non-conserved among genes that are similarly expressed.
69 Thus, the synthetic promoters can be reasonably constructed to give high gene expression with a
70 smaller size (Liu and Stewart 2016). The strength of synthetic promoters depends on the selection,
71 spacing of CREs and the copy number. Using bioinformatic algorithms, novel CREs could be
72 discovered, by comparing the upstream sequence of differentially regulated genes. These CREs could
73 be put together rationally to design new synthetic promoters (Roberts 2011). Furthermore, the
74 strength of the synthetic promoter could be enhanced by proper spacing and increasing the CRE copy
75 number (Liu and Stewart 2016).

76 Here, we have tested three synthetic promoters in *P. patens*, developed using an automated high-
77 throughput screening method. The promoters were built using computational analysis of large
78 transcriptomic functional data set to identify *cis*-elements, which form the building blocks of
79 synthetic promoter libraries. All three synthetic promoters had higher mRNA expression than the
80 housekeeping gene *PpAct7* and showed similar protein expression pattern to *AtUBQ10* promoter.

81

82 **Materials and Methods**

83 *Promoter construction*

84 The synthetic promoter library was constructed at Synpromics using random assembly techniques of
85 *cis*-elements through expression data analysis of *Zea mays*. Genes showing strong expression strength
86 above *Ubiquitin1* transcripts were labeled constitutive. Using transcription factor binding site
87 database TRANSFAC, *cis*-elements of the constitutively expressed genes were identified from the
88 1,500bp upstream and 500bp downstream of the transcription start site. Subsequently, Synpromics
89 Ltd Syn-score algorithm was applied to the identified regions to rank the *cis*-elements (Roberts *et al.*
90 2017). Further, a synthetic promoter library was constructed by, random assembly of the chosen *cis*-
91 elements (300bp-800 bp), attached upstream to CaMV 35S minimal promoter (position -46 to +89).
92 Later, functional promoters were identified by the expression analysis of the *Luciferase* gene.

93

94 *Growth media*

95 *P. patens* (Gransden ecotype, International Moss Stock Center #40001) was grown on solid and liquid
96 PhyB media (Bach *et al.* 2014) under sterile conditions, with continuous 20–50 W/m² light intensity
97 at 23°C.

98

99 *DNA preparation and transformation protocol*

100 DNA fragments for transformation were prepared in blocks as below. First block, a 2.7 kb region
101 with 108 5' neutral locus, G418 selection marker with CaMV 35S promoter/ CaMV poly(A) signal
102 was amplified from the pRH004 plasmid. Second block, the synthetic promoter sequences developed
103 by Synpromics, and the *Arabidopsis Ubiquitin10* (*AtUBQ10*) promoter was amplified with 20~22 nt
104 overhangs homologous to block one and three. Block 3, with the Venus fluorescent protein, OCS
105 terminator and the 108 locus homologous recombination flanking region was amplified from pRH004

106 plasmid (Figure 1A). Purified 1.5 pmol of each DNA block (Figure 1B) was transfected into the
107 isolated moss protoplasts during transformation process and selected for positive colonies according
108 to previously published methods (King *et al.* 2016; Khairul Ikram *et al.* 2017).

109

110 *Arabidopsis Ubiquitin10 promoter*

111 *Arabidopsis UBQ10* promoter with a length of 634 bp of (Grefen *et al.* 2010) was cloned from
112 *Arabidopsis* genomic DNA using primer set; UBQ10 F 5'-GTCGACGAGTCAGTAATAAACGG-
113 3' and UBQ10 R 5'-CTGTTAATCAGAAAACTCAGATTAATC-3'. For moss transformation, 22-
114 nt overhangs that are identical to the next fragments (block one and three) were attached to both ends
115 by second PCR using overhang primers.

116

117 *Detection of Venus fluorescence*

118 Venus fluorescence was detected on protonemal cells grown for seven days in PhyB liquid media. *P.*
119 *patens* protonema cells were visualized and photographed using a confocal laser-scanning
120 microscope. Z-stacks were performed on each line using the 488nm laser line and YFP emission
121 filter. Z-stacks were put together using the Zeiss software built-in maximum projection function.
122 Fluorescent level of each promoter lines was calculated from digital Images using the software
123 ImageJ (<https://imagej.nih.gov/ij/>). A previously published method on fluorescent cell analysis was
124 used to calculate the corrected total cell fluorescence (CTCF) levels (Burgess *et al.* 2010; McCloy *et*
125 *al.* 2014).

126 For each cell, measurements were taken for the cell area, integrated density and mean grey value.

127 Final corrected total cell fluorescence (CTCF) was calculated using the following formula.

128 $CTCF = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background reading})$

129

130 *RNA extraction and qPCR*

131 Total RNA was extracted from the appropriate lines (7 days after blending), using Spectrum™ Plant
132 Total RNA Kit (Sigma, STRN250). To synthesize cDNA, 1 µg of extracted total RNA was reverse
133 transcribed by iScript cDNA synthesis kit (Bio-rad, 1708891), followed by PCR amplification of the
134 following transcripts, *PpAct7* and *Venus*. PCR reactions were carried out using (Qiagen kit name)
135 and (Bio-rad machine name), by denaturation at 95°C 5 min, 40 cycles with 95°C for 10 sec and 60°C
136 for 10 sec, and melting curve analysis to check the specificity. Relative *Venus* gene expression from
137 each promoter line was analyzed by $Exp_{Venus} = 2^{\Delta C_t[\text{promoter}]}$, $\Delta C_t[\text{promoter}] = C_t[\text{Actin}] - C_t[\text{Venus}]$.

138

139 **Results and discussion**

140 *Assembly of cis-elements to construct constitutive plant promoters*

141 To generate synthetic promoters, functional *cis*-elements should be collected since *cis*-elements will
142 form the building blocks of synthetic promoters. We used automated high-throughput screening
143 method. In this method, computational analysis of large transcriptomic functional data sets of *Zea*
144 *mays* was used to identify *cis*-elements from constitutively expressed genes. We ranked collected *cis*-
145 elements by applying Syn-score algorithm and randomly assembled selected *cis*-elements to generate
146 promoter library (Roberts et al, 2017). This technique has an advantage since elements are selected
147 based on the requirements for the synthetic promoters (e.g., inducible, constitutive and tissue- or
148 developmental stage-specific), which is a more focused approach than using completely random
149 elements. The promoter candidates consist of randomly assembled *cis*-elements of varying lengths
150 up to 30bp. Therefore, the promoter length and the position of *cis*-elements vary (Figure 1C). It has
151 been shown that the position of *cis*-elements relative to each other markedly influences promoter
152 strength (Rushton *et al.* 2002), which was shown in the transcription data of the synthetic promoters
153 we tested (Figure 2).

154 The synthetic promoters can be used for gene stacking in *P. patens*, as they do not resemble each
155 other on the sequence level and contain small *cis*-elements rather than large promoter fragments,
156 which greatly reduces the risk of homology-induced gene silencing. As it is an automated system, it
157 is reproducible, robust and faster than a manual approach. Thus, multiple functional promoters in *P.*
158 *patens* could be constructed on demand.

159

160 *Promoter activity analysis of synthetic promoters*

161 To analyze the activity of synthetic promoters, we used fluorescence protein Venus. The coding
162 sequence of *Venus* was placed downstream of each promoter including well-known constitutive

163 *AtUBQ10* promoter, and all constructs were stably transfected to *P. patens* 108 neutral locus (Bach
164 *et al.* 2014). We performed qPCR to compare the promoter activity. The mRNA expression level of
165 *Venus* was calculated in relative to the endogenous housekeeping gene *Actin7* (*PpAct7*). Actin is an
166 essential component of the plant cytoskeleton and is known to be a ubiquitous protein that is
167 constitutively expressed in eukaryotes (Meagher *et al.* 1999). It is also shown that *PpAct7* is
168 preferable to be used as a housekeeping gene (Le Bail *et al.* 2013) and perform dual functions as a
169 control and a housekeeping gene in the data analysis of this experiment. Expression of human VEGF
170 protein using the 5' promoter region of the *PpAct7* depicted an eight-fold increase in the production
171 of the VEGF protein compared to the constitutive CaMV 35S promoter (Weise *et al.* 2006). This
172 suggests *PpAct7* is highly expressed compared to the 35S promoter that was quantified previously
173 (Horstmann *et al.* 2004). Thus, the mRNA expression of *PpAct7* can be compared to the *Venus*
174 transcript levels driven by the synthetic promoters. All three synthetic promoters showed higher
175 expression than *PpAct7* (Figure 2). Synthetic promoter *I2-10* yielded the highest mRNA expression
176 level at 304.51 ± 4.21 fold relative to *PpAct7* followed by 45.91 ± 0.88 , 124.93 ± 3.4 , 258.38 ± 0.09
177 for *I2-48*, *I2-79*, and *AtUBQ10*, respectively. The *I2-10* promoter was 2.43X and 6.63X expressed
178 than *I2-79* and *I2-48*. This high level of expression can be attributed to the presence of G-box in *I2-*
179 *10* (Figure 1A). The G-box is a regulatory element in plant promoters, playing an essential role in
180 plant promoter responsiveness to light, stress, and hormones (Menkens *et al.* 1995). Some of the G-
181 box motifs have been shown to aid high-level constitutive protein expression in some plant species
182 (Ishige *et al.* 1999).

183

184 *Protein expression analysis of synthetic promoters in P. patens protonema cells*

185 After mRNA expression analysis, we wanted to identify if the protein expression would show similar
186 pattern, because protein level does not always correspond to that of mRNA. The Venus protein

187 expression level was calculated for each synthetic promotor line. This was performed in 14 days old
188 *P. patens* protonema cells (Figure 3). Micrographs of *I2-10*, *I2-48*, *I2-79*, and *AtUBQ10* promoter
189 lines were processed to measure the fluorescence intensity of Venus via ImageJ.

190 All three synthetic promoters showed a similar level of Venus fluorescent protein expression (Figure
191 3). Compared to the medium-strength of the *AtUBQ10* promoter (Grefen *et al.* 2010), the synthetic
192 promoters, *I2-10*, *I2-48* and *I2-79* displayed 1.6X, 1.5X and 1.5X decrease in protein expression,
193 respectively. All synthetic promoters share the identical sequence -123 bp from ATG, thus we
194 excluded the possibility of ribosome entry for the discrepancy in expression of mRNA and protein
195 level, and assume structure-related factor made the difference. These synthetic promoter candidates
196 were derived from a screen conducted in *Zea Mays*, where Synpromics has shown that they mediate
197 two- four-fold higher protein expression (firefly luciferase) compared to the ubiquitin-1 promoter. As
198 one would expect we have seen that the promoters show maximum activity in the organism in which
199 they were screened. The fact that promoters developed for another plant species show such high
200 activity in *P. patens*, bodes well for the further development of promoters for use in this chassis
201 organism.

202 The short length of 634 bp makes the *AtUBQ10* promoter an ideal control. However, the *AtUBQ10*
203 promoter consisted of the first 5' intron expanding 304 bps, while synthetic promoters contained
204 none. Deletion of the first 5' intron in *AtUBQ10* has shown to result in 3-fold lower protein activity
205 than the first intron intact (Norris *et al.* 1993). Thus, adding an either synthetic or natural first 5'
206 intron section to the synthetic promoters would likely lead even higher expression of the following
207 coding sequences.

208

209 In conclusion, we have tested the activity of three synthetic promoters in *P. patens*. All three
210 promoters showed high expression of mRNA compared to the *PpAct7* and similar protein activity to

211 the medium-strength *AtUBQ10* promoter. Previously, published works have revealed that the addition
212 of the first 5' intron increase the stability of the mRNA and yield several folds of higher protein
213 activity. Thus, adding a 5' intron to the end of synthetic promoters would likely increase the strength
214 of the promoters. Further, range of promoters mediating a range of different expression levels is
215 essential in building genetic circuitry in synthetic biology applications, such that synthetic promoters
216 can control the correct stoichiometry of different component proteins of the circuit at the
217 transcriptional level.

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- 308

310 **Figure text**

311 **Figure 1:** A) vector map of linearized pRH004 vector.

312 B) Three PCR fragments (blocks) were constructed to transform the promoters to the *P. patens* 108
313 neutral loci. Block 1 was amplified from the pRH004 vector with the 108 neutral locus and resistance
314 to Geneticin driven by CaMV 35S promoter. Block 2 depicts, PCR amplified *AtUBQ10*, *I2-10*, *I2-48*
315 and *I2-79* promoter sequences. Followed by the final block 3, containing the Venus fluorescent
316 protein with the OCS terminator sequence and the 108 loci.

317 C) Schematic of randomly assembled cis regulatory elements to construct the synthetic promoters.

318

319 **Figure 2:** Overview of promoter strength based on *Venus* expression, using *PpAct7* promoter as
320 background measurement.

321

322 **Figure 3:** Confocal images of Venus expression, driven by synthetic promoters and *AtUBQ10*
323 promoter. All lines are grown and imaged in identical conditions. B) Representative, promoter
324 strengths of *I2-10* (n=54), *I2-48* (n=41), *I2-79* (n=48) and *AtUBQ10* (n= 32) lines. Promoter strength
325 was calculated by measurement of the fluorescence intensity for each cell by the use of confocal
326 micrographs and ImageJ software. Scale bar =0.01 mm

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