The steroid-inducible pOp6/LhGR gene expression system is fast, sensitive and does NOT cause plant growth defects in rice (*Oryza sativa*)

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Significance statement: The non-monocot codon-optimized version of the dexamethasone inducible pOp6/LhGR system does not cause severe developmental perturbations in rice plants.

Summary

Inducible systems for transgene expression activated by a chemical inducer or an inducer of non-plant origin are desirable tools for both basic plant research and biotechnology. Although, the technology has been widely exploited in model plants, it has not been optimised for use with the major monocotyledonous crop species, namely rice. We have adapted the dexamethasone-inducible pOp6/LhGR system for rice and shown that it is fast, sensitive and tightly regulated, with high levels of induction that remain stable over several generations. Most importantly, we have shown that the system does not cause negative growth defects *in vitro* or in soil grown plants. Interestingly in the process of testing, we found that another steroid, triamcinolone acetonide, is a more potent inducer in rice than dexamethasone. We present serious considerations for the construct design to avoid undesirable effects caused by the system in plants, leakiness and possible silencing, as well as simple steps how to maximize translation efficiency of a gene of interest. Finally, we compare the performance of the pOp6/LhGR system with other chemically inducible systems tested in rice in terms of the properties of an ideal inducible system.

INTRODUCTION

Chemically inducible systems that regulate gene expression are crucial tools for basic plant biology research as well as biotechnology applications. They allow analysis of gene primary effects before homeostatic mechanisms start to counteract and reveal a clear correlation between induction of a transgene and occurrence of an altered phenotype. Their applications include expression of gene products that interfere with regeneration, growth or reproduction; regulation and expression at different stages of plant development; conditional genetic complementation, co-suppression and overexpression studies. However, adaption of existing inducible systems developed in model plants to other species including monocots and important crop plants particularly rice, has not been easily achieved.

The systems typically contain two transcription units. The first unit employs a constitutive or tissue-specific promoter to express a chemical-responsive transcription factor, the second unit consists of multiple copies of the transcription factor binding site linked to a minimal plant promoter, which is used to express the target gene. The most optimal systems developed to date include dexamethasone-inducible GVG (Aoyama and Chua, 1997) and pOp6/LhGR (Craft *et al*., 2005; Samalova *et al.*, 2005), estrogen-inducible XVE (Zuo *et al*., 2000), ecdysone agonist-inducible VGE (Padidam *et al*., 2003; Koo *et al*., 2004) and ethanol-inducible *alc* systems (Caddick *et al*., 1998; Roslan *et al*., 2001; Salter *et al*., 1998). Their characteristics were compared in detail by Moore *et al*., (2006) and potential applications in plant biotechnology were reviewed by Corrado and Karali, (2009).

Several attempts have been made to develop an inducible system for gene manipulation in rice. The XVE system was tested by Sreekala *et al*., (2005) and Okuzaki *et al*., (2011) but the use remains very limited due to the lack of systemic movement of the estradiol inducer (Hirose *et al*., 2012, Chen *et al*., 2017). Two other steroid-inducible systems have been tested but the GVG and a modular gene expression system (Vlad *et al*., 2019) derived from the pOp6/LhGR system met with a mixed success in rice, as they caused severe growth and developmental defects of the plants (Ouwerkerk *et al*., 2001; Vlad *et al*., 2019).

An easier and more common strategy exploited in rice is the use of conditional promotors that can be activated by heat (Khattri *et al*., 2011; Sun *et al*., 2012; Rerksiri *et al*., 2013), pathogens (Helliwell *et al*., 2013; Quilis *et al*., 2014) or wounding (Quilis *et al*., 2014), oxidative (Woo *et al*., 2015) and other stresses (Nakashirma *et al*., 2007). Other crops promoters include induction by heat in maize (Du *et al*., 2019) and potato (Kopertekh *et al*., 2018), by light in tomato (Timerbaev and Dolgov, 2019) or, cold in barely (Eva *et al*., 2018), wheat (Meszaros *et al*., 2015) and sweet potato (Honma *et al*., 2019). Often the conditional expression is combined with the Cre-lox technology (Khattri *et a*l., 2011; Meszaros *et al*., 2015; Eva *et al*., 2018; Kopertekh *et al*., 2018; Du *et al*., 2019) or an alternative site-specific recombinase system (Woo *et al*., 2015) to generate marker-free genetically modified plants.

Adaptation of the breakthrough CRISPR/Cas9 technology to plants (Jiang *et al*., 2013) including rice (Zhang *et al*., 2014) allows genome editing and creates a powerful tool for engineering knockdown, knockout or chimeric plants. This genome editing system was combined with a heat-shock-inducible promoter to generate heritable mutations in rice (Nandy *et al*., 2019) and a virus-inducible system

54 was developed that confers resistance to Gemini viruses in model plants *Arabidopsis* and tobacco (Ji
55 *et al*., 2018). Recently, the technology was integrated with the estradiol-inducible XVE-based cell-
56 type-specific system (Siligato *et al*., 2016; Zuo *et al*., 2000) to create an inducible genome editing
57 (IGE) system in *Arabidopsis* that enables efficient generation of target gene knockouts in desired cell
58 types at different developmental stages (Wang *et al*., 2020).

59 Over the years we and others have made considerable efforts to develop the dexamethasone-
60 inducible transcription activation system, pOp6/LhGR, as a tool for the growing demands on modern
61 gene technologies. It is a widely used system for which a comprehensive library of cell-type specific
62 activator lines was created in *Arabidopsis* (Schurholz *et al*., 2018); the system was combined with
63 artificial micro-RNA (amiRNA) to knockdown multigene expression (Goth *et al*., 2012; Samalova *et*
64 *al*., 2020) and hairpin RNAi molecules to silence gene expression (Wielopolska *et al*., 2005; Liu and
65 Yoder, 2016). Apart from *Arabidopis* (Craft *et al*., 2005) and tobacco (Samalova *et al*., 2005) the
66 system was tested in various other species including citrus plant (Rossignol *et al*., 2014) and
67 *Medicago truncatula* (Liu and Yoder, 2016) and most recently in rice (Vlad *et al*., 2019). However, the
68 specific modifications made to the original version of the pOp6/LhGR system caused undesirable
69 effects in plants.

70 This report describes the functionality of the pOp6/LhGR system in rice, its stability over several
71 generations, time course and dose response characteristics; optimization of induction by various
72 steroids as inducers and methods of systemic and localised applications that do not have any
73 detrimental effects in rice even after prolonged induction.

74

75 **RESULTS**

76 **Evidence that the pOp6/LhGR system is functional in rice**

77 The pOp6/LhGR system (Craft *et al*., 2005; Samalova *et al.*, 2005) comprises of a transcription
78 activator LhGR which is a fusion between a high-affinity DNA-binding mutant of *Escherichia coli lac*
79 repressor, *lacI*^{His17}, transcription-activation-domain-II of Gal4 from *Saccharomyces cerevisiae* and the
80 ligand-binding domain (LBD) of the rat glucocorticoid receptor (GR). The pOp6 is a chimeric
81 promoter that consists of six copies of *lac* operators (*lacOp*) cloned upstream of a minimal
82 cauliflower mosaic virus (*CaMV*) 35S promoter (-50 to +8) and is apparently silent when introduced
83 into plants. The principle of the system is that in the absence of the steroid ligand, dexamethasone
84 (Dex), the transcription factor is trapped in an inactive complex via interaction of the GR LBD and
85 heat-shock protein HSP90. Upon induction with Dex, this complex is disrupted and the LhGR
86 activator binds to the pOp6 promotor and induces expression of the target gene of interest.

87 To adopt the pOp6/LhGR system for rice, first we chose a binary pVec8-overexpression vector (Kim
88 and Dolan, 2016) in which we placed the LhGR2 activator sequence that incorporates the
89 *Arabidopsis* codon-optimized *GAL4* sequence (Rutherford *et al*., 2005) under the control of a maize
90 ubiquitin promoter that contains an intron (*pZmUbi*). The inclusion of an intron is well known to
91 greatly increase expression efficiency in monocots, but similar effects have been reported in dicots
92 and other eukaryotes (Rose, 2004). Secondly, we checked in the literature (Mitsuhara *et al.*, 1996;

93 Segal *et al*., 2003) that none of the sequences of the pOpIn2 bidirectional reporter cassette
94 (Samalova *et al*., 2019) including the *lacOp*, minimal promoters and tobacco mosaic virus (*TMV*)
95 omega (Ω) translation enhancers have been reported to be toxic or non-functional in monocots and
96 cloned it into the activator construct to create pVecLhGR2 as depicted in Fig. 1 and Supp. Fig. 1. For
97 simplicity of testing the regulated expression of the pOp6/LhGR in rice, we used the *uidA* (encoding
98 β-glucuronidase; GUS) and the yellow fluorescent protein (*YFP*) as the genes of interest.

99 To create stable transgenic rice lines, we used a protocol for *Agrobacterium*-mediated
100 transformation of calli induced from seeds of *Oryza sativa spp. japonica* cultivar Kitaake as described
101 by Vlad *et al*., 2019. We generated several independent transgenic lines (T1) in which the induced
102 GUS staining was comparable to that from the constitutive promoter *pZmUbi* (Fig. 1B) and the *YFP*
103 expression (Fig. 1C) was inducible by Dex, proving that the pOp6/LhGR system is in principle
104 functional in rice. However, the transformation efficiency was relatively low and to induce higher
105 levels of expression various alternate methods were tested.

106 **Transformation efficiency and reliability of induction in subsequent generations**

107 We tested GUS activity by histochemical staining in 120 generated putative transformants that
108 included little plantlets with a piece of callus and some roots (Table 1). We induced them in liquid ½
109 MS with 30 μM Dex and stained for GUS activity 24h later. Approximately a half of them showed
110 visible (by eye) GUS staining in roots after 2h and shoots after 4h. The reaction was stopped and
111 scored at 24h with the following pattern: 30.8% root staining only, 9.2% shoots only, 12.5% stained
112 shoots, roots and callus (data not shown).

113 One hundred and sixty-six putative primary transformants (T0) were grown to maturity and tested.
114 The first generation of seedlings (T1) were tested for resistance to hygromycin by germinating them
115 on ½ MS medium supplemented with the antibiotics (Table 1). Only 33 lines germinated and grew,
116 indicating that only 20 % were real transformants, of these, 8 lines (25 %) showed positive GUS
117 staining after induction with Dex. Five of the most strongly inducible lines were grown to the next
118 generation (T2), these lines retained HYG-resistance and showed positive GUS staining upon Dex
119 induction. Two lines (65 and 121) were tested further and showed stable inducible GUS expression in
120 the subsequent (T3) generation (Fig. 2A).

121 We determined the GUS activity fluorometrically in segregating T1 and T2 progeny in roots (Fig. 2B)
122 and shoots (Fig. 2C) of 7-day-old rice seedlings germinated and grown on ½ MS plates containing 30
123 μM Dex or the same concentration of DMSO (-Dex control). Interestingly, the induced GUS activity
124 was up to 8-fold higher in roots compared to shoots and in some cases in roots it was comparable to
125 the activity from the constitutive *pZmUbi* promoter. Perhaps a low transpiration rate in Petri dishes
126 impaired the uptake and distribution of Dex into the shoots.

127 **Time course and dose response characteristics of Dex-induced GUS activity**

128 To characterise the induction property of the pOp6/LhGR system in rice we performed time course
129 and dose response experiments. To increase the efficiency of induction in shoots, we induced the
130 newly developed leaves of app. 2-week old seedlings, grown in the open air, by painting the leaves
131 with a Dex solution supplemented with 0.1% (v/v) Tween-20. Significant increase in fluorometrically,

132 determined GUS activity, was detected 12h after induction with 10 μM Dex in two independent
133 transgenic lines (65B and 121C) and this activity reached app. a half of the *pZmUbi* constitutive
134 promoter activity within 72h of induction (Fig. 3A). The GUS activity was induced in plants treated
135 with 0.01 μM Dex and while one line (65B) reached maximum levels of induction with 0.1 μM Dex,
136 the other line (121C) had increased activity with increasing Dex concentration and reached levels
137 similar to the constitutive *pZmUbi* promoter with 10 μM Dex after 48h induction (Fig. 3B).

138 To confirm the similar characteristic of the pOp6/LhGR system in roots, we induced detached roots
139 of 10-day old seedlings in liquid ½ MS media supplemented with increasing concentrations of Dex
140 and performed histochemical GUS staining after specific time durations. Visible GUS staining was
141 first detected in developing lateral roots and tips after 12h of induction and the intensity increased
142 throughout the root system up to 72h tested (Fig. 3C). The maximal GUS staining intensity was
143 detected with 1 μM Dex, the lowest concentration tested (Fig. 3D), predominantly in growing root
144 tips after 24h induction.

145 We also tested the feasibility of inducing whole seedlings (10-day old) in a liquid ½ MS medium
146 supplemented with 10 μM Dex. Histochemical GUS staining revealed the induction after 12h
147 predominantly in roots and the staining pattern did not change significantly in the 48h time-span
148 tested (data not shown).

149 **Optimization of induction by testing different steroids as inducers**

150 We tried to improve the levels of induction of the pOp6/LhGR system in rice by testing different
151 glucocorticoid derivatives (steroids) as inducers. In an attempt to reduce the surface tension at the
152 air–liquid interface that is high in rice leaves due to epicuticular waxes preventing water vapor loss,
153 we tested different concentrations of Tween-20 as the wetting agent rather than Silwet L-77 used
154 previously (Craft *et al*., 2005; Samalova *et al*., 2005). Fig. 4A (i-iv) shows clear differences in the
155 intensity of GUS staining of the 10-day-old shoots (leaves and stems) induced with a 30 μM Dex
156 solution supplemented with 0.1% Tween-20 compared to 0.01%. Almost no staining was visible
157 without the addition of the surfactant apart from damaged cells.

158 It is known that other glucocorticoid derivatives such as triamcinolone acetonide (TA) or
159 deoxycorticosterone (Doc) can be used as inducers to replace Dex (Aoyama and Chua, 1997). We
160 tested both in a 30 μM water solution supplemented with 0.1% Tween-20 (Fig. 4A v and vi) and
161 compared the GUS staining intensities. Doc induction was negligible; however, TA induction was
162 comparable if not higher than with Dex. To confirm this observation, we repeated the experiment on
163 cuttings of the same leaf of 6-week-old plants that were submerged in water supplemented with
164 0.1% Tween-20 and 10 μM Dex or 10 μM TA for 24h (Fig. 4B). Depending on the efficiency of the
165 substrate penetration, the GUS staining pattern with both inducers was comparable, but more
166 importantly, also comparable to the staining of pZmUbi::GUS line of the same age. No GUS staining
167 was detected without the inducers (Fig. 4B, 0.1% Tween-20).

168 We expanded the range of possible steroid inducers readily available and tested them in a 30 μM
169 water solution supplemented with 0.1% Tween-20. Leaves of 5-week-old plants (Fig. 4C, lines 65A
170 top and 121C bottom) were painted with the following steroids: betamethasone (Bet),
171 fludrocortisone acetate (Flu), prednisone (Pre) or prednisolone (Plo) and Dex and histochemical GUS

172 staining was carried out 24h later. None of the new glucocorticoids reached GUS activity levels
173 comparable to Dex in both the transgenic lines tested and the GUS staining was considerably weaker
174 than that of the constitutive *pZmUbi* promotor activity.

175 **Systemic and localised induction of soil-grown plants**

176 To test the feasibility of inducing gene expression at later stages of plant development, either in a
177 systemic or in a localized manner, Dex or the glucocorticoid TA were applied to soil-grown plants by
178 watering (subterranean irrigation) or painting. Local induction of expression was detected after 24 h
179 when leaves were painted with a 10 μM steroid solution supplemented with 0.1 % Tween-20 (Fig.
180 5A). The treatment was repeated and GUS activity determined 24 h and 72 h following first
181 application of the inducer. Interestingly, the TA treatment doubled the induced levels of GUS activity
182 compared to Dex, and both treatments exceeded the levels of *pZmUbi* promoter activity at 72 h post
183 induction (hpi).

184 Similarly, plants were watered with a 30 μM steroid solution and the treatment was repeated twice
185 at 0h and 24h later. The induction triggered the reporter gene expression at the whole plant level,
186 GUS activity was detectable in leaves 24 h after application of TA and 72h with both Dex and TA (Fig.
187 5B). The induced levels again exceeded the GUS activity detected in the pZmUbi::GUS line, however,
188 but only with one of the lines tested (65B). The variability in the measurements could be due to
189 segregating plant populations and a small sample size.

190 Both painting and watering experiments were repeated with a small modification of the treatment
191 done at 2-day intervals and leaves were imaged using a confocal laser scanning microscope to
192 observe induction of the second reporter gene, YFP (Fig. 5C-K). A bright YFP signal was detected 24h
193 after painting the leaves (Fig. 5C) but not watering the plants (Fig. 5D) with Dex. The signal became
194 stronger at 96 hpi with both methods of treatment (Fig. 5F and 5G) and more cells seemed to be
195 expressing YFP with the TA treatment than Dex (Fig. 5I and 5J). No signal was detected without any
196 induction (Fig. 5E and 5H) as in the WT (Fig. 5K).

197 To summarise, higher levels of GUS activity and *YFP* expression were obtained by painting the leaves
198 rather than watering the plants and using TA at equivalent concentrations of Dex, suggesting that TA
199 is a more potent inducer. Thus, whole plant and single leaf phenotypes can be assessed after
200 induction using both methods.

201 **Long-term induction of the pOp6/LhGR system has no negative effects on rice plants**

202 Final experiments were to determine whether there are any undesirable effects due to long term
203 induction of the pOp6/LhGR system in rice. Four-week old plants were watered with either 30 μM
204 Dex or 30 μM TA or a control solution (DMSO) at 2-day intervals for a week (Fig. 6, left) or two weeks
205 and let to recover for further 6 weeks (Fig. 6, right). The images suggest that the plants grew and
206 developed normally compared to WT plants treated with the control solution.

207

208

209 **DISCUSSION**

210 **A construct design: possible reasons for toxicity, leakiness and silencing**

211 In the publication of Vlad and colleagues (2019) the activator LhGR was codon optimized for use in
212 rice (*rcoLhGR*) and further "domesticated" to remove all recognition sites for the Golden Gate
213 cloning. However, severe growth defects on 10 μM Dex media were observed with all their
214 constructs tested and in all transgenic lines. As addition of isopropyl β-D-1-thiogalactopyranoside
215 (IPTG) to the growth media reduced the severity of the growth arrest phenotype, the authors
216 concluded that it was a direct consequence of LhGR activity. However, in our work, we used the
217 activator LhGR2 version that was only partially codon optimized for *Arabidopsis* to eliminate
218 premature polyadenylation events in the Gal4 domain (Rutherford *et al*., 2005) and we did not
219 observe any undesirable growth defects. Therefore, it is possible that the rice codon optimization
220 rendered the system too efficient and prone to non-specific off-target binding due to high rcoLhGR
221 protein levels in the growth inhibited lines. In this regard, to optimize efficiency of an expression
222 system it is recommended to maximize the translational efficiency of a gene of interest (Moore *et*
223 *al*., 2006). This can be achieved by taking some simple steps such as ensuring that the initiation
224 codon conforms to the consensus for efficient initiation in plants (Koziel *et al*., 1996) and including
225 translation enhancers in the 5`UTR such as the *TMV* Ω in our study.

226 Similarly, the GVG synthetic transcription factor that incorporates the glucocorticoid receptor (GR)
227 ligand binding domain was found to be detrimental when activated in rice (Owerkverk *et al*., 2001)
228 and other species including *Arabidopsis* (Kang *et al*., 1999; tobacco, Amirsadeghi *et al.,* 2009; *Lotus*
229 *japonicus*, Andersen *et al*., 2003). The growth perturbations were caused probably by the GVG
230 activator that was binding to cis-regulatory elements in the plant genome with sequence homology
231 to *GAL4* upon Dex activation (Kang *et al*., 1999). Nevertheless, the system was used successfully in a
232 number of studies in rice (Chern *et al*., 2013; Nakayama *et al*., 2013; Onodera *et a*l., 2018, Park *et al*.,
233 2012; Qu *et al*., 2009) in which the negative effects were reduced by shortening the time of Dex
234 exposure or selecting lines with low GVG expression levels and mild phenotype that could be used as
235 activator lines.

236 Moreover, when designing a construct for an inducible system, care must be taken to ensure that
237 enhancers in the promoter that drives the activator do not activate the target promoter (*e.g.* pOp6;
238 Moore *et al*., 2006) as such arrangement might result in transgene activation in the absence of
239 inducer. In the pOp6/rcoLhGR constructs (Vlad *et al*., 2019), the arrangement was not ideal, as the
240 pOp6 promoter was positioned directly next to the *pZmUbi* promoter driving the *rcoLhGR*.
241 Furthermore, two other potent promoters were used on the same T-DNA; the constitutive rice actin
242 promoter (*pOsACT*) to drive dsRed for identification of transgenic seeds, and another copy of
243 *pOsACT* or the CaMV 35S promoter (for hygromycin-resistance selectable marker) that can also
244 affect the expression of a transgene (Yoo *et al*., 2005). Curiously, the authors also used an intron in
245 the reporter gene (GUS) placed again right next to the *pOp6* promoter and revealed that the
246 presence of the intron had an enhancing effect on GUS activity levels both in the absence and
247 presence of inducer.

248 Also, it must be recognized that any transgene may be susceptible to post-transcriptional gene
249 silencing (PTGS) if transcripts accumulate to sufficient levels whether it is expressed constitutively or

250 when induced (Moore *et al*., 2006). Individual reporter genes have a gene-specific threshold of
251 mRNA abundance that will trigger PTGS (Schubert *et al*., 2004) and the probability of silencing of an
252 inducible transgene locus in increased if the locus is induced (Abranches *et al*., 2005). In an attempt
253 to minimize gene silencing, in our construct design we tried not to repeat the same sequence more
254 than once, for example we used different polyadenylation signals (namely the octopine synthase
255 terminator Tocs, nopaline synthase terminator Tnos, and T35S; Fig. 1A). However, we were not able
256 to avoid silencing as only 25 % of hygromycin-resistant transgenic lines (T1) displayed GUS activity
257 after Dex induction compared to 50 % of tested transgenic calli (T0). Others described a similar
258 decrease, for example a reporter gene (GUSPlus) was expressed in 84% of rice calli but only in 25-
259 68% of adult plants (Wu *et al*., 2003); and with the XVE system only 50 % of calli derived from
260 inducible T1 seeds showed detectable GFP signal (Okuzaki *et al*., 2011).

261 **pOp6/LhGR compared with other systems: is there an ideal inducible system for rice?**

262 The development of chemical-inducible systems for tight control of plant gene expression is a
263 challenging task. There are a number of properties that are required for an ideal system, such as low
264 basal expression level, high inducibility, specificity and dynamic range of response with respect to
265 the inducer. Also, fast response and induction by various methods is desirable. An ideal system
266 should work in several plant species and should not cause any adverse physiological effects in plants
267 by itself or its inducer. The inducer is further required to show high specificity for the transgene, high
268 efficiency at low concentrations and must not be found in target plants.

269 The pOp6/LhGR system described here **is fast**, first GUS activity was detected in painted leaves (Fig.
270 3A) and visible GUS staining in growing root tips (Fig. 3C) after 12h of induction and increased up to
271 72h tested. Similarly, with the rcoLhGR activator, 6h of induction was sufficient to induce high levels
272 of GUS activity in transgenic calli and reached maximum levels at 24-96h depending on the construct
273 (maximum levels of activity were reached after 24h in lines containing constructs with introns in the
274 reporter gene but required 4 days of induction in the intron-less versions) (Vlad *et al*., 2019). On the
275 other hand, the GVG system tested in rice (Ouwerkerk *et al*., 2001) required 4 days for the activity to
276 be detected and 2 weeks of induction for GUS activities to reach levels comparable to those
277 conferred by the strong *CaMV 35S* promoter. With the XVE system, a GFP signal was detected in rice
278 calli 48 h after induction (with 1 μM 17-β-estradiol) and reached maximum (with treatment at 25
279 μM) in 8 days. However, in calli and leaves of transgenic lines the GFP signals were weaker than
280 those in the leaves of p35S::GFP line, only in roots the signals were similar with 17-β-estradiol
281 treatment at > 10 μM (Okuzaki *et al*., 2011).

282 The pOp6/LhGR system **is very sensitive**, 0.01 μM Dex was sufficient to induce GUS activity in
283 painted leaves (Fig. 3B) and in roots of *in vitro* grown seedlings of the pOp6/rcoLhGR (Vlad *et al*.,
284 2019). Interestingly, in some lines maximum levels of induction were reached with 0.1 μM Dex,
285 while others required 10 μM Dex to reach levels similar to the constitutive *pZmUbi* promoter (Fig.
286 3B). This makes the pOp6/LhGR system ten-times more sensitive than the GVG system that required
287 minimum of 0.1 μM Dex for induction and 1-10 μM for maximum.

288 The pOp6/LhGR system **is tightly regulated and strong**, the average fold induction was app. 500 in
289 shoots of plants induced *in vitro* (Fig. 2C) or in soil by Dex, however, reached 1000-fold induction

290 when plants were treated with TA (Fig. 5). In roots the magnitude of Dex-induction reached at least
291 1000- to 6000-fold (Fig. 2B), similar to the values reported for the GVG system (Ouwerkerk *et al*.,
292 2001). Both the pOp6/LhGR as well as the pOp6/rcoLhGR systems used the same line that
293 constitutively expressed a synthetic GUS variant from *Staphylococcus* (GUSPlus) under the control of
294 the *pZmUbi* promoter as a positive control. The GUSPlus was reported to be ten-fold more active
295 than the enzyme encoded by *E. coli uidA* that was used in the inducible constructs (Broothaerts *et*
296 *al*., 2005; Jefferson *et al*., 2003), suggesting that activation of the pOp6 promoter by the activators is
297 very effective.

298 The pOp6/LhGR system **is inducible by various methods** *in vitro* and in soil-grown plants. Painting
299 the leaves with an inducer solution supplemented with 0.1% Tween-20 (to help the inducer to
300 penetrate through the protective wax layer) proved to be more effective than subterranean
301 irrigation (watering). It is worth to note that using these methods of application, TA proved to be
302 almost twice as more an efficient inducer than Dex (Fig. 5). This could be due to the nature of the
303 inducer as Dex could be more rapidly metabolized or compartmentalized than TA (Aoyama and
304 Chua, 1997). Ouwerkerk and colleagues (2001) reported that Dex was taken up efficiently by roots of
305 mature plants in hydroponics and induced GUS activity throughout the whole plant body.

306 The major limitation and possible reasons for low levels of target gene induction in rice leaves using
307 the XVE system is the inefficient uptake of estradiol from hydroponics. Interestingly, cut leaf
308 segments placed in liquid MS media with 10 μM estradiol showed detectable GFP fluorescence in
309 whole leaf segments within 48 h (Okuzaki *et al*., 2011) and similar activation was confirmed by PCR
310 in another study (Hirose *et al*., 2012). Also, spraying of leaves with the inducer was another effective
311 method of induction (Hirose *et al*., 2012). The limiting factor therefore seems to be the estradiol
312 uptake that is depending on the roots. The inducer might have been trapped by XVE expressed in
313 roots or diffused and attenuated in leaves. Interestingly, activation by soaking transgenic seeds
314 (Chen *et al*., 2017) with estradiol solution induced highly efficient site-specific recombination in
315 germinating embryos, resulting in high-level expression of target gene or RNAi cassette in intact rice
316 plants.

317 The pOp6/LhGR system **is not toxic in rice** and does not cause any undesirable negative
318 developmental growth effects even at higher concentration of Dex (up to 30 μM tested) and after
319 prolonged exposure of the plants (Fig. 6). However, for the long-term induction through soil, DMSO
320 should be avoided and ethanol should be used as a solvent for the inducers to prevent accumulation
321 of DMSO in the soil over several weeks of watering (Moore *et al*., 2006; Samalova *et al*., 2019). Very
322 unexpected were the findings of Vlad and colleagues (2019) that the pOp6/rcoLhGR transgenic
323 plants manifested severe developmental perturbations when grown on concentrations > 0.1 μM
324 Dex. The direct cause of these growth defects is not known, but the authors suggested that the rice
325 genome contains sequences with high similarity to the *lacOp* sequence, suggesting non-specific
326 activation of endogenous genes by Dex induction. Although, the pOp promoter was not activated by
327 endogenous factors in maize (Moore *et al*., 2006; Segal *et al*., 2003) it is possible that the rice-codon
328 optimization rendered the system too efficient. The off-target effects can be minimized by
329 quenching with IPTG that acts as an effective antagonist for the LacI DNA binding domain in LhGR
330 (Craft *et al*., 2005).

331 The GVG system has been shown to generate growth defects in rice (Owerkverk *et al*., 2001) and
332 other species (Kang *et al*., 1999; Andersen *et al*., 2003; Amirsadeghi *et al.,* 2009) but the reason for
333 this is not clear as each domain of the molecule has been used successfully in other expression
334 systems. However, one possibility is that GVG requires higher expression levels to achieve full
335 promoter activation, resulting perhaps in squelching or non-specific binding to *CCG(N11)CGG*
336 sequences in the genome (Moore *et al*., 2006). Apparently, the GVG level was not limiting for target-
337 gene activation (Aoyama and Chua, 1997), therefore, selection of plants with a mild GVG phenotype
338 in combination with optimized induction conditions was recommended for the system applications.
339 Although, the XVE system had no effect on growth, all tested lines including controls grown in
340 hydroponics presented a different root pattern with oestradiol treatment so the system does not
341 appear to be suitable for root morphology studies (Okuzaki *et al*., 2011).

342

343 **EXPERIMENTAL PROCEDURES**

344 **Construct preparation**

345 To prepare the pVecLhGR2 construct (Fig. 1A, Fig. S1), first, the LhGR2 and the octopine synthase
346 terminator (Tocs) sequences were amplified by polymerase chain reaction (PCR) using vector pOpIn2
347 (Samalova *et al*., 2019) as the template. The two PCR fragments, LhGR2 (forward primer P1: 5'-
348 AAAAAGGTACCATGGCTAGTGAAGCTCGAAAAACAAAG-3'; reverse primer P2: 5'-
349 GCATATCTCATTAAAGCAGGACTCTAGTTCACTCCTTCTTAGGGTTAGGTGGAGTATC-3') and Tocs (forward
350 primer P3: 5'-TACTCCACCTAACCCTAAGAAGGAGTGAACTAGAGTCCTGCTTTAATGAGATATGC-3';
351 reverse primer P4: 5'- AAAAAGGTACCCTAAGGCGCGCCGTTGTCG CAAAATTCGCCCTGGACCC -3') were
352 joined together by overlapping PCR with primers P1 and P4 and cloned into the unique *Kpn*I site of
353 the binary rice overexpression vector pVec8-*Kpn*IOX (Kim and Dolan, 2016). Secondly, an *Asc*I
354 fragment containing the bidirectional pOp6 operator array together with *TMV* Ω translation
355 enhancer sequences driving expression of YFP and GUS (including 35S terminator sequence)
356 reporters of the pOpIn2YFP vector (generated by M. Kalde, I. Moore lab) was cloned into a unique
357 *Asc*I site created in the previous cloning step. The correct orientation of the fragment as well as the
358 LhGR2 in the final pVecLhGR2 vector was confirmed by sequencing.

359 **Plant material and growth conditions**

360 To generate stable transgenic rice lines, we used *Agrobacterium*-mediated transformation of *Oryza*
361 *sativa spp. japonica* cultivar Kitaake. Calli were induced from dehulled mature seeds before co-
362 cultivation with *A. tumefaciens* strain EHA105 transformed with the pVecLhGR2 construct. Callus
363 transformation and seedling regeneration were performed at 28 ^{o}C according to a protocol modified
364 from Toki *et al*., (2006) as described by Vlad *et al*., 2019. Independent pVecLhGR2 lines were tested
365 together with positive *pZmUbi*::*GUS* (Vlad *et al*., 2019) and negative wild-type (WT) controls. Soil-
366 grown plants were cultivated in a greenhouse at 28-30 ^{o}C with a 16h/8h photoperiod.

367 **Methods of steroid induction – *in vitro*, painting and watering**

368 Dexamethasone (Dex) was prepared as a 30 mM stock solution in dimethyl sulfoxide (DMSO) and
369 stored at -20 ^{o}C. Similarly, we prepared stock solutions of betamethasone (Bet), deoxycorticosterone

370 (Doc), fludrocortisone acetate (Flu), prednisone (Pre), prednisolone (Plo) and triamcinolone
371 acetonide (TA), all purchased from Sigma-Aldrich. For each treatment, either a glucocorticoid
372 (induction) or the equivalent volume of DMSO (control) were added to obtain desired concentration.

373 For application *in vitro*, seedlings were grown on half strength Murashige and Skoog (1962) medium
374 (½ MS medium) supplemented with 15g/l sucrose and with or without agar. Dex was added to the
375 medium after autoclaving to obtain final working concentration, typically 30 μM Dex. Shoots and
376 leaf cuttings were also induced in distilled water supplemented with 0.1% Tween-20 and a
377 glucocorticoid inducer (10-30 μM).

378 For induction of soil grown plants, 4-week-old rice plants were either watered with 50 ml of 30 μM
379 Dex or 30 μM TA or newly developed leaves (3rd leaf) were painted on both sides with 10 μM Dex or
380 10 μM TA solution supplemented with 0.1% Tween-20. The treatments were repeated at 2-day
381 intervals for one or two weeks.

382 **Analysis of ß- glucuronidase (GUS) reporter activity**

383 Histochemical GUS staining and fluorometric GUS assay was performed according to Jefferson (1987)
384 at 37 ^{o}C. Extracts were prepared from ground snap-frozen samples; the protein content was
385 determined spectrophotometrically using Bio-Rad Protein Assay (Bio-Rad laboratories). The
386 fluorogenic reaction was carried out in 96 well-plates using protein extraction buffer supplemented
387 with 4-methylumbelliferyl ß-D-glucuronide (4-MUG) as a substrate. A standard curve of 4-MU was
388 used to calculate the amount of 4-MU/unit of time. Activity was calculated from three technical
389 replicates and expressed in pmoles 4-MU/min /μg protein.

390 **Detection of YFP fluorescence**

391 The YFP signal was detected using a Zeiss LSM 510 Meta confocal laser-scanning microscope with
392 514-nm excitation from an Argon laser and a BP535-590IR emission filter.

393

394

395 **ACKNOWLEDGEMENT**

396 I would like to dedicate this publication to my colleague and friend Ian Moore, and his beloved
397 family, without whom science is much less fun. I want to thank to Peng Wang (Chinese Academy of
398 Sciences, Shanghai, China) for the control pZmUbi::GUS line and the rice transformation protocol; to
399 Chul Min Kim (University of Oxford) for the pVec8-*Kpn*IOX vector and to Monika Kalde (University of
400 Oxford) for the pOpIn2YFP vector. I am grateful to Molly Dewey for her suggested word changes.

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578 **TABLES**

579

580 **Table 1: Number of putative transformants and generated transgenic lines tested for hygromycin**
581 **(HYG) resistance and induction of GUS activity in subsequent generations.**

Generation	No. plants tested	HYG resistant	GUS staining
T0	120	n.d.	63
T1	166	33	8
T2	5	5	5

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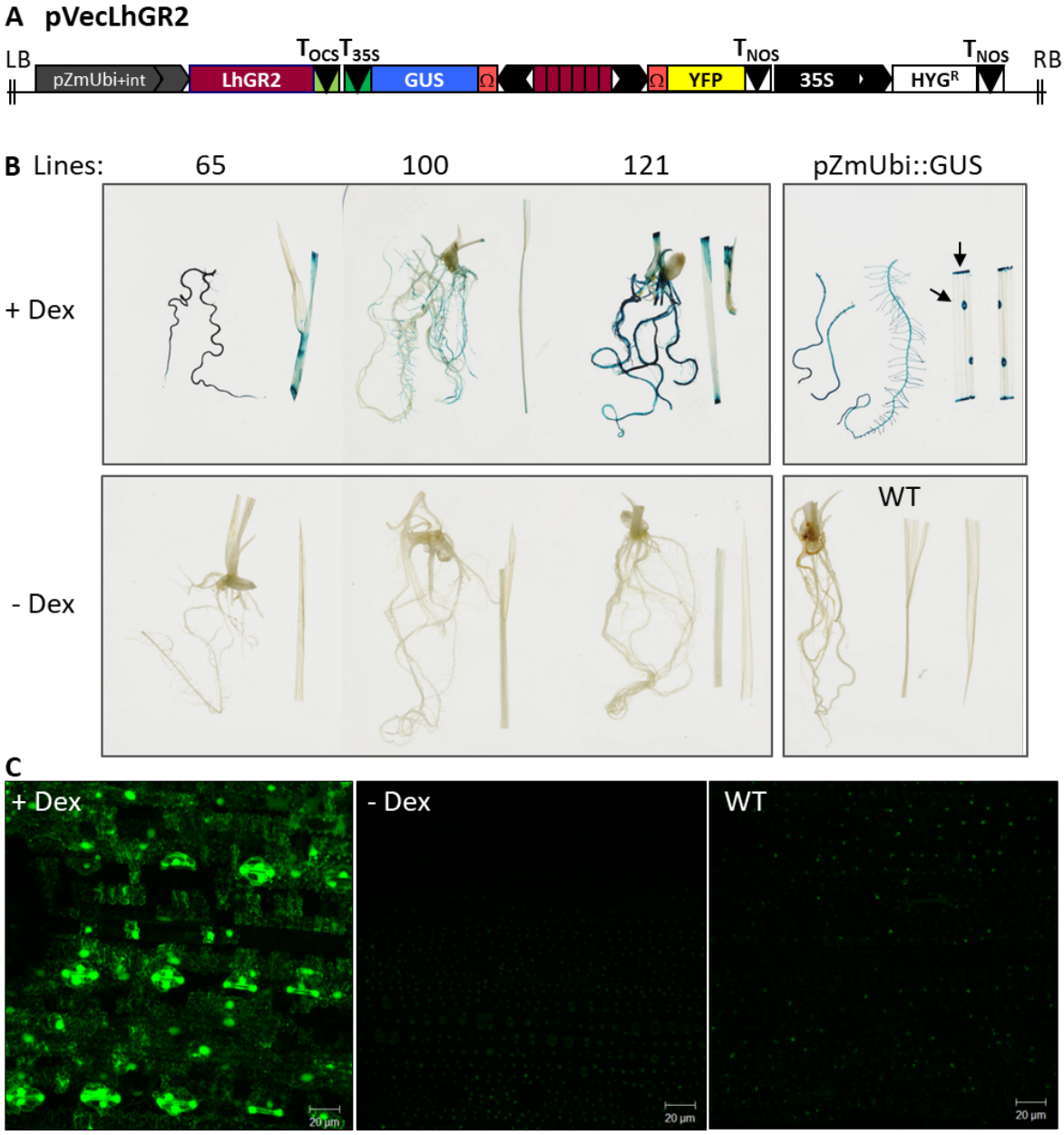
602 **FIGURES AND FIGURE LEGENDS**

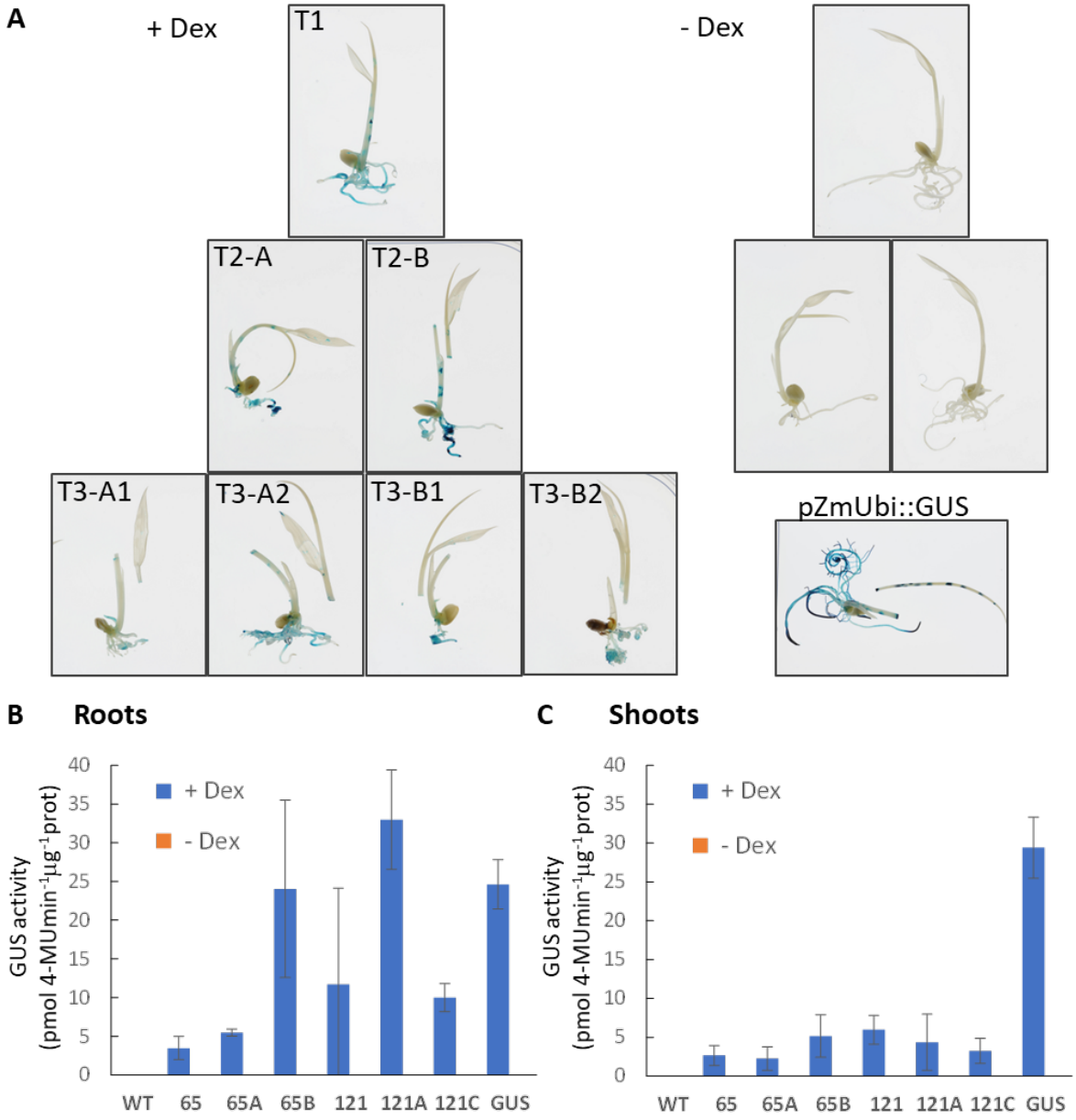


603

604 **Figure 1: The pOp6/LhGR system in rice: a proof of principle**

605 **(A)** A schematic representation of the pVecLhGR2 construct that contains the *Arabidopsis* codon-
606 optimized *GAL4* sequence (Rutherford *et al.,* 2005) of the transcriptional activator LhGR2 driven by a
607 *pZmUbi* promoter (containing an intron), a bidirectional pOp6 promotor version with two *TMV* Ω
608 translation enhancers driving two reporter genes - *uidA* (encoding β-glucuronidase; GUS) and a
609 yellow fluorescence protein (*YFP*), and a pCaMV35S::HYG selectable marker cassette conferring
610 hygromycin resistance. The construct was assembled as detailed in Supplementary Figure S1. **(B)**
611 Twelve-day-old rice seedlings of three independent transgenic lines (number 65, 100 and 121)
612 histochemically stained for GUS activity that was induced by seedling transfer onto ½ MS plates
613 containing 30 µM Dex or control DMSO (-Dex) for 6 days. Seedlings of pZmUbi::GUS and wild-type

614 (WT) were included as controls. The arrows point to an example of damaged cells/ cuts where the
615 GUS staining substrate (5-bromo-4-chloro-3-indolyl glucuronide; X-Gluc) penetrated inside the cells.
616 **(C)** Leaves of 4-week-old plant (line 121) were painted with 30 µM Dex or control solution (-Dex) and
617 imaged using a confocal laser scanning microscope 96 h later to detect YFP fluorescence. WT was
618 included as a control for autofluorescence. The scale bar is 2 µm.



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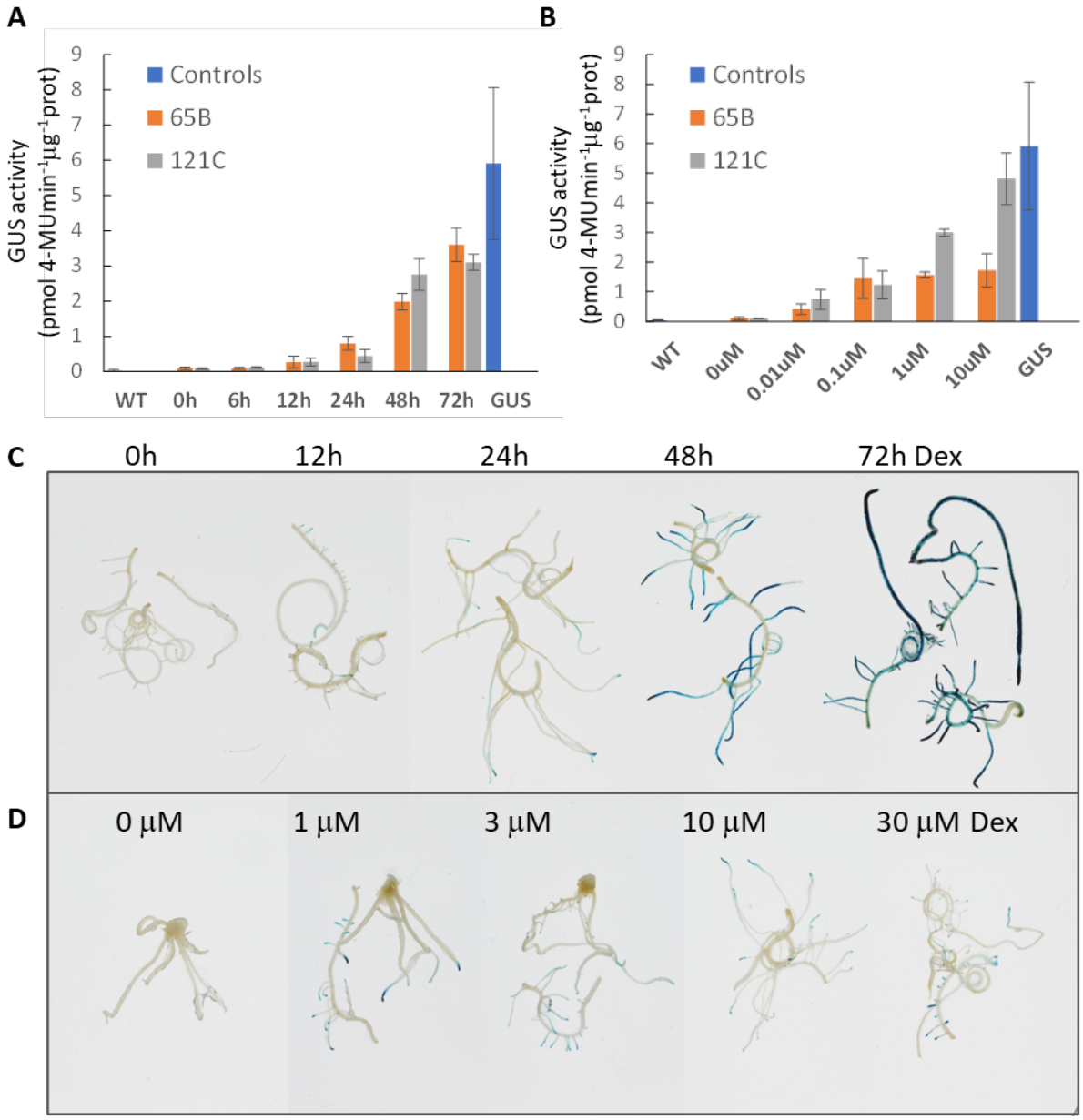
620 **Figure 2: Reliability of inducible expression in subsequent generations and GUS activity in rice**

621 **(A)** Ten-day-old rice seedlings (line 65) of generation T1, T2 (A and B) and T3 (A1, A2 and B1, B2)
622 histochemically stained for GUS activity induced by germinating and growing the seedlings on ½ MS
623 plates containing 30 µM Dex or control DMSO (-Dex) for 10 days. Seedlings of pZmUbi::GUS were
624 included as a control for the GUS staining. **(B, C)** GUS activity determined fluorometrically in roots (B)
625 and shoots (C) of 7-day-old rice seedlings (lines 65 and 121, T1 and T2) germinated and grown on ½

626 MS plates containing 30 µM Dex or control DMSO (-Dex) for 7 days. Seedlings of WT and
627 pZmUbi::GUS were included as controls. The error bars represent SD.



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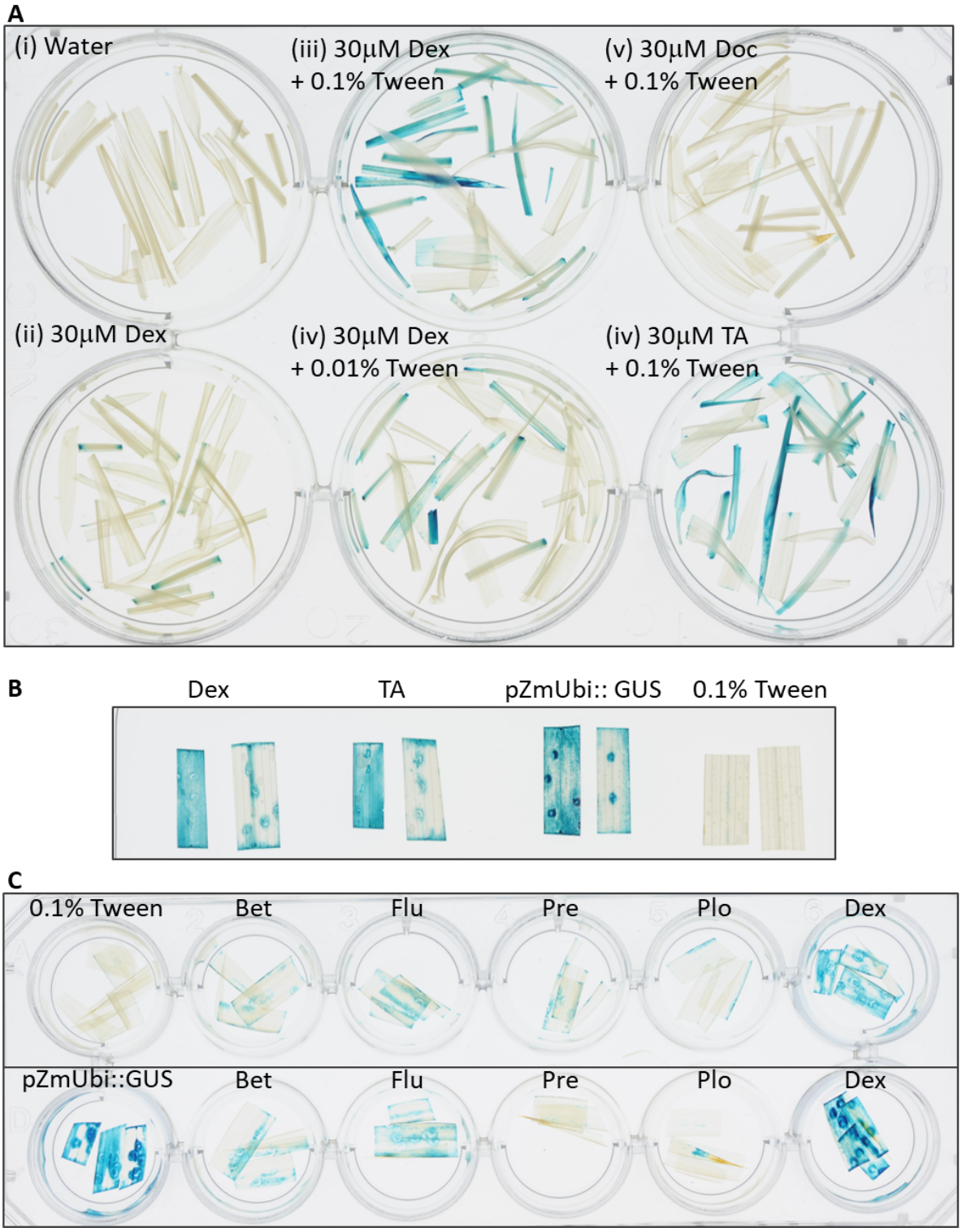
629 **Figure 3: Time course accumulation of induced GUS activity and dose-response of the pOp6/LhGR**
630 **system in rice**

631 GUS activity determined fluorometrically in 7-day-old rice seedlings (lines 65B and 121C) germinated
632 on ½ MS plates (vertically placed in the incubator) and then stood up in 15 ml Falcon tubes with 2 ml
633 water for further 5 days. The newly developed leaves were induced by painting with water
634 supplemented with 0.1% Tween-20 and **(A)** 10 µM Dex for 0, 6, 12, 24, 48 and 72h or **(B)** with 0,
635 0.01, 0.1, 1 and 10 µM Dex for 48h. Seedlings of WT and pZmUbi::GUS were included as controls.
636 The error bars represent SD. **(C)** Roots of 10-day-old seedlings (lines 65A, 121C) were induced in
637 liquid ½ MS with 30 µM Dex for 0, 12, 24. 48 and 72h, then histochemically stained for GUS activity.
638 Representative images are shown. **(D)** Roots of 10-day-old seedlings (lines 65A, 121C) were induced

639 in liquid ½ MS for 24h with 0, 1, 3, 10 and 30 µM Dex, then histochemically stained for GUS activity.
640 Representative images are shown.



641

642 **Figure 4: Optimization of the pOp6/LhGR induction in rice by testing different steroid inducers**

643 **(A)** Shoots of 10-day-old seedlings (lines 65A, 121C) were induced in water supplemented with
644 either 0.01% or 0.1% Tween-20 and 30 µM steroid inducer Dex, deoxycorticosterone (Doc) or

645 triamcinolone acetonide (TA) for 24 h, then histochemically stained for GUS activity. **(B)** Leaf cuttings
646 of the same leaf of 6-week-old plants (lines 65A, 121C) were induced in water supplemented with
647 0.1% Tween-20 and 10 μM Dex or 10 μM TA for 24h, then histochemically stained for GUS activity.
648 Leaves of pZmUbi::GUS were included as controls. Representative images are shown. **(C)** Leaves of 5-
649 week-old plants (lines 65A top, 121C bottom) were induced by painting with water supplemented
650 with 0.1% Tween-20 and 30 μM steroid inducer: Dex, betamethasone (Bet), fludrocortisone acetate
651 (Flu), prednisone (Pre) or prednisolone (Plo) for 24 h, then histochemically stained for GUS activity.

652

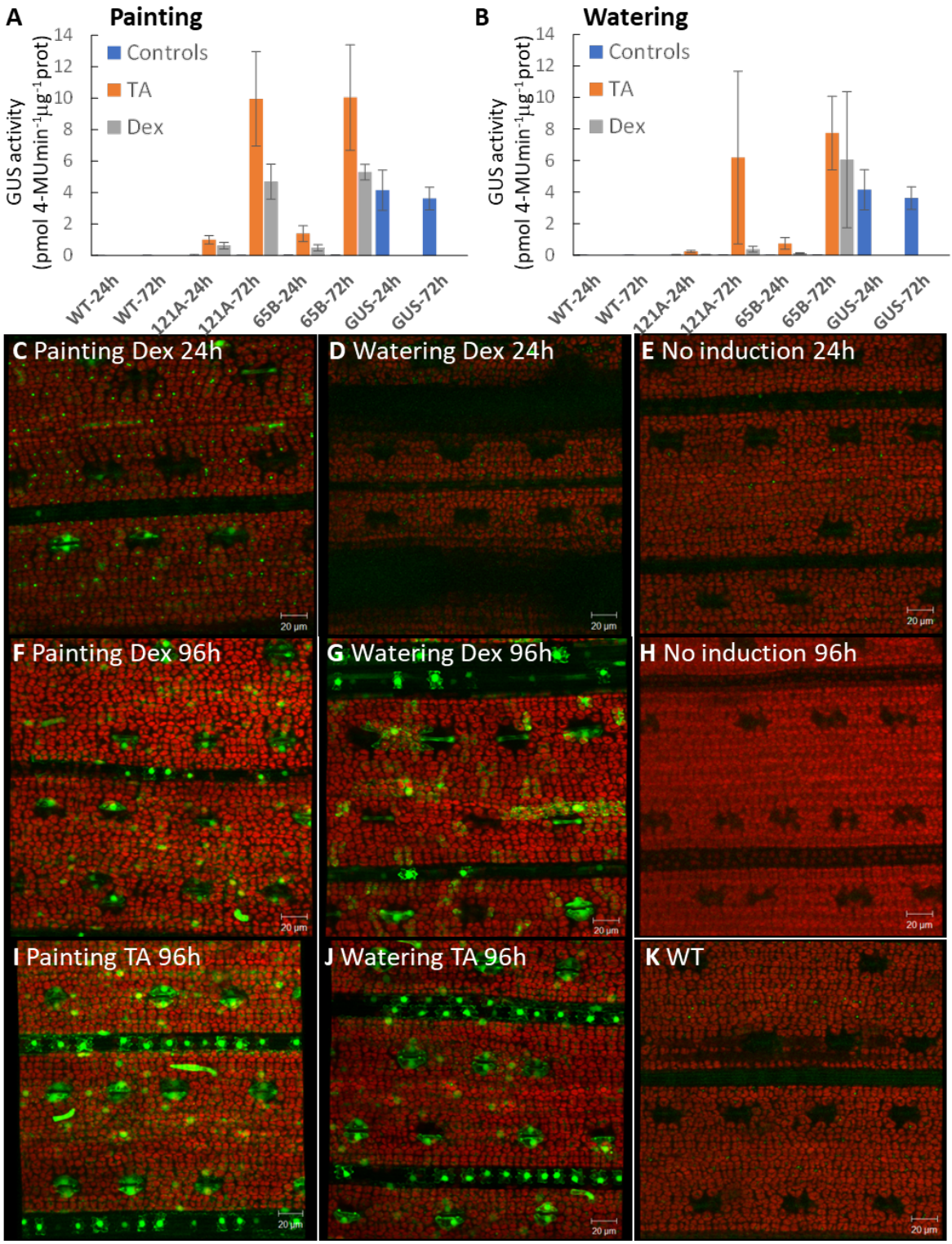


Figure 5: Expression characteristics of the pOp6/LhGR system induced in soil-grown plants by painting and watering

Four-week-old rice plants grown in soil were induced either with TA or Dex by **(A)** painting with 10 µM solution supplemented with 0.1 % Tween-20 or **(B)** watering with 30 µM solutions. The treatment was repeated twice at 0h and 24h later. Leaves were sampled and GUS activity

659 determined 24h and 72h following first application of the inducer. WT and pZmUbi::GUS plants were
660 used as controls. Three plants from segregating populations were used for each treatment. The error
661 bars represent SD. **(C-K)** Four-week-old plants (line 121C) were induced as above except the
662 treatment was repeated 48h after first induction. Leaves were imaged using a confocal laser
663 scanning microscope at 24h and 96h post-induction, YFP signal in green, chlorophyll
664 autofluorescence in red. Scale bar is 20μm.

665

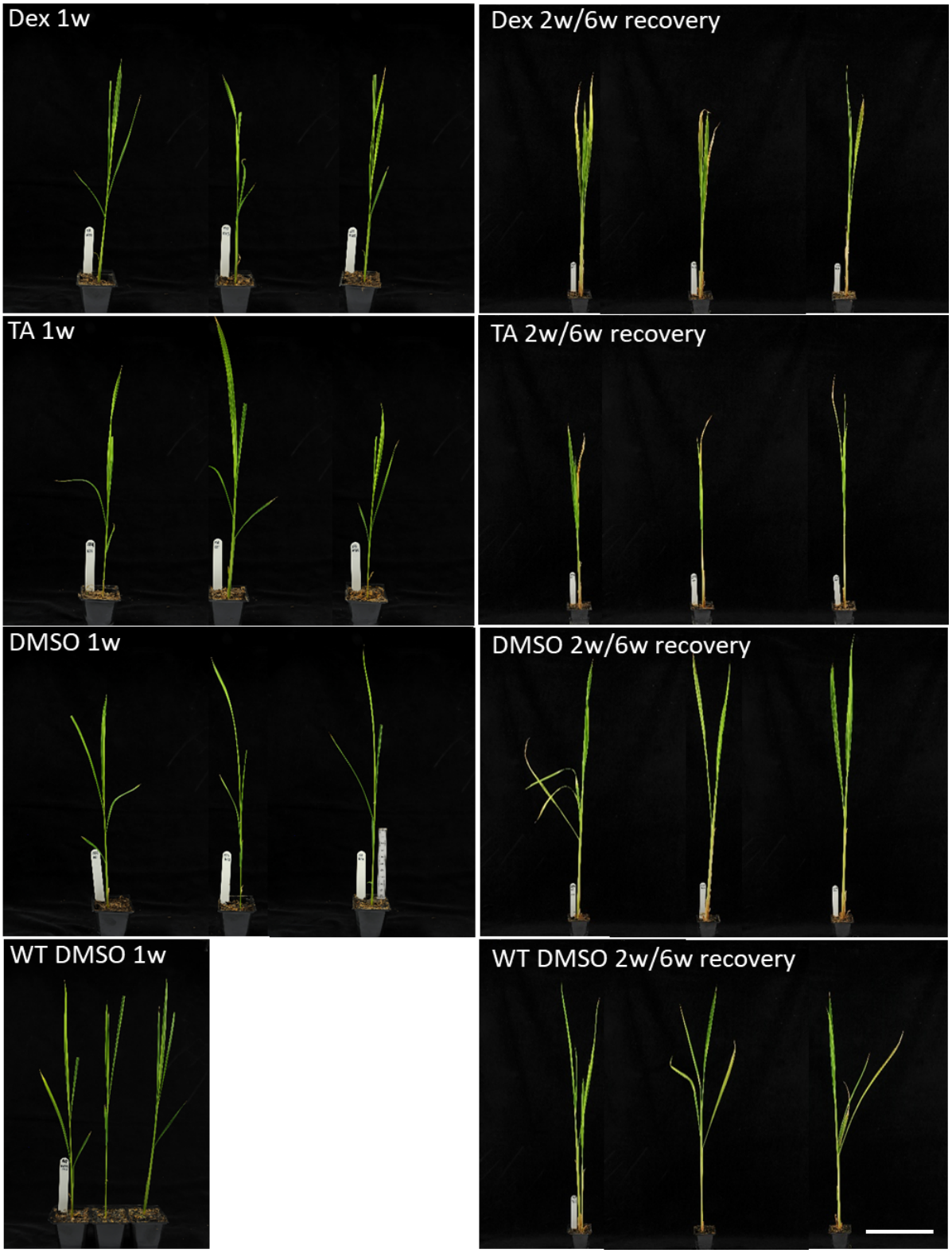


Figure 6: Long-term effect of the pOp6/LhGR system induction on mature rice plants

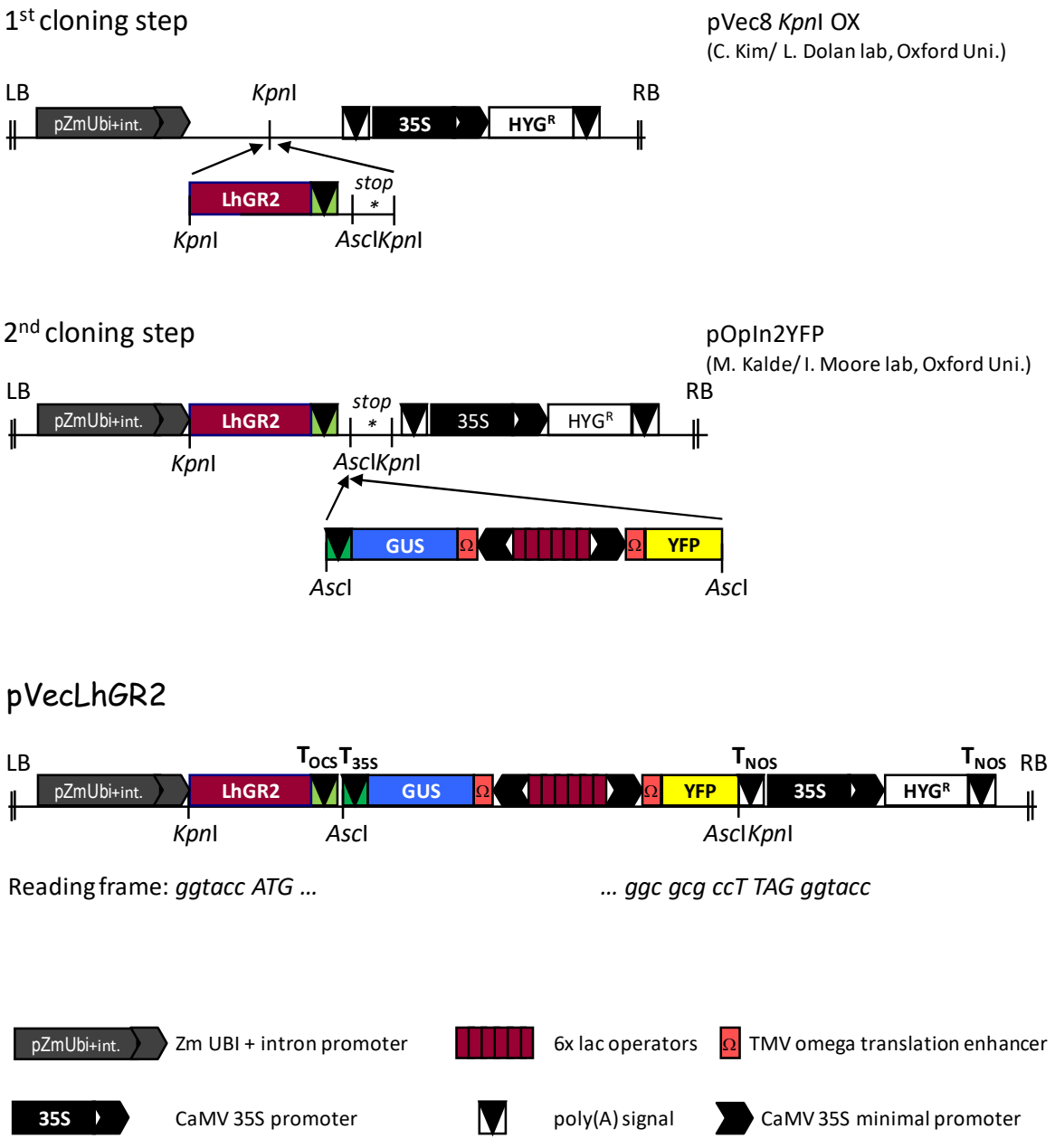
Four-week-old plants (65B and 121A) were watered with 30 µM Dex or 30 µM TA or a control solution containing DMSO (600 ml per a tray of 8 plants) at 2-day intervals for a week (left) or two

670 weeks and let to recover for further 6 weeks (right). WT plants treated with the DMSO solution were
671 used as controls. Scale bar represents 10 cm. Representative images are shown.

672

673 **SUPPLEMENTARY FIGURE**

674



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676 **Supplementary Figure S1: Cloning strategy for preparation of the pVecLhGR2 construct**