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 or an inducer of non-plant origin are desirable tools for both basic plant research as well as advanced biotechnological utilizations. Although, the technology has been widely exploited in model plant species, optimal solution is missing for the major monocotyledonous crop species – rice. Here, we characterise the inducible properties of the pOp6/LhGR adapted for rice as fast, sensitive, tight, with high levels of induction that remain stable over several generations. Most importantly, the system does not cause negative growth defects *in vitro* or in soil grown plants. We describe various methods of application and optimization of induction by finding out that another steroid, triamcinolone acetonide, is more potent inducer then dexamethasone in rice. 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 for regulated 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; regulated expression at different stages of plant development and for specific duration; conditional genetic complementation, co-suppression and overexpression studies. However, to adapt existing inducible systems developed in model plants to other species including monocots and crops *e.g*. rice, it is not an easy task.

The systems typically contain two transcription units. Whereas 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) systems, 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 details (Moore *et al*., 2006) and potential applications in plant biotechnology reviewed (Corrado and Karali, 2009) previously.

Rice is the world`s staple diet crop and there have been several attempts to develop an inducible system for gene manipulation in this important monocotyledonous species. The XVE system was tested in rice (Sreekala *et al*., 2005; Okuzaki *et al*., 2011) but its use remains very limited due to the lack of systemic movement of the estradiol inducer (Hirose *et al*., 2012, Chen *et al*., 2017). Other two steroid-inducible systems tested, 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).

Therefore, easier and more common strategy exploited in rice so far 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). For various other crops promoters induced 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) were adopted. 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. The 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 was developed that confers resistance to geminiviruses in model plants *Arabidopsis* and tobacco (Ji

54 *et al*., 2018). Only recently, the technology was integrated with the estradiol-inducible XVE-based
55 cell-type-specific system (Siligato *et al*., 2016; Zuo *et al*., 2000) to create an inducible genome editing
56 (IGE) system in *Arabidopsis* that enables efficient generation of target gene knockouts in desired cell
57 types and developmental stage (Wang *et al*., 2020).

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

69 This report describes the functionality of pOp6/LhGR system in rice, its stability over several
70 generations, time course and dose response characteristics, optimization of induction by testing
71 various steroids as inducers and finally methods of systemic and localised applications without
72 causing any detrimental effects in rice even after prolonged induction.

73

74 **RESULTS**

75 **The pOp6/LhGR system is functional in rice**

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

86 To adopt the pOp6/LhGR system for rice, firstly we choose a binary pVec8-overexpression vector
87 (Kim and Dolan, 2016) in which we placed the LhGR2 activator sequence that incorporates the
88 *Arabidopsis* codon-optimized *GAL4* sequence (Rutherford *et al*., 2005) under the control of a maize
89 ubiquitin promoter that contains an intron (*pZmUbi*). The inclusion of an intron is well known to
90 greatly increase expression efficiency in monocots, but similar effects have been reported in dicots
91 and other eukaryotes (Rose, 2004). Secondly, we checked in the literature (Mitsuhara *et al.*, 1996;
92 Segal *et al*., 2003) that none of the sequences of the pOpIn2 bidirectional reporter cassette

93 (Samalova *et al*., 2019) including the *lacOp*, minimal promoters and tobacco mosaic virus (*TMV*)
94 omega (Ω) translation enhancers have been reported to be toxic or non-functional in monocots and
95 cloned it into the activator construct to create pVecLhGR2 as depicted in Fig. 1 and Supp. Fig. 1. For
96 simplicity of testing the regulated expression of the pOp6/LhGR in rice, we used the *uidA* (encoding
97 β-glucuronidase; GUS) and the yellow fluorescent protein (*YFP*) as the genes of interest.

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

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

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

112 We grew 166 putative primary transformants (T0) into maturity and tested the first generation of
113 seedlings (T1) for resistance to hygromycin by germinating them on ½ MS medium supplemented
114 with the antibiotics (Table 1). Only 33 lines germinated and grew, suggesting that only 20 % were
115 real transformants, out of which 8 lines (25 %) showed positive GUS staining after induction with
116 Dex. Five most strongly inducible lines were grown to the next generation (T2) and these lines
117 retained HYG-resistance and showed positive GUS staining upon Dex induction. Two lines (65 and
118 121) were tested further and proved stable inducible GUS expression in the subsequent (T3)
119 generation (Fig. 2A).

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

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

127 To characterise the induction property of the pOp6/LhGR system in rice we performed time course
128 and dose response experiments. To increase the efficiency of induction in shoots, we induced the
129 newly developed leaves of app. 2-week old seedlings in open air condition by paining the leaves with
130 a Dex solution supplemented with 0.1% (v/v) Tween-20. Significant increase in fluorometrically
131 determined GUS activity was detected 12h after induction with 10 μM Dex in two independent

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

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

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

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

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

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

167 We expanded the range of possible steroid inducers readily available and tested them in a 30 μM
168 water solution supplemented with 0.1% Tween-20. We painted leaves of 5-week-old plants (Fig. 4C,
169 lines 65A top and 121C bottom) with steroids: betamethasone (Bet), fludrocortisone acetate (Flu),
170 prednisone (Pre) or prednisolone (Plo) and Dex and performed histochemical GUS staining 24h later.
171 None of the new glucocorticoids reached GUS activity levels comparable to Dex in both transgenic

172 lines tested and the GUS staining was also much weaker than that of the constitutive *pZmUbi*
173 promotor activity.

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

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

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

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

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

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

201 The final experiment that we conducted was to observe whether there are any undesirable effects
202 due to long term induction of the pOp6/LhGR system in rice. We watered 4-week old plants with
203 either 30 μM Dex or 30 μM TA or a control solution (DMSO) at 2-day intervals for a week (Fig. 6, left)
204 or two weeks and let to recover for further 6 weeks (Fig. 6, right). The images suggest that the plants
205 grew and developed normally compared to WT plants treated with the control solution. However,
206 for the long-term induction through soil, DMSO should be avoided and ethanol should be used as a
207 solvent for the inducers to prevent accumulation of DMSO in the soil over several weeks of watering
208 (Moore *et al*., 2006; Samalova *et al*., 2019).

209

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

307 The major limitation and possible reasons for low levels of target gene induction in rice leaves using
308 the XVE system is inefficient estradiol uptake from hydroponics. Interestingly, cut leaf segments
309 placed in liquid MS media with 10 μM estradiol showed detectable GFP fluorescence in whole leaf
310 segments within 48 h (Okuzaki *et al*., 2011) and similar activation was confirmed by PCR in another
311 study (Hirose *et al*., 2012). Also spraying of leaves with the inducer proved as possible method of
312 induction (Hirose *et al*., 2012). The limiting factor therefore seems to be the estradiol uptake that is
313 depending on the roots. The inducer might have been trapped by XVE expressed in roots or diffused
314 and attenuated in leaves. Interestingly, activation by soaking transgenic seeds (Chen *et al*., 2017)
315 with estradiol solution induced highly efficient site-specific recombination in germinating embryos,
316 resulting in high-level expression of target gene or RNAi cassette in intact rice 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). Very unexpected were the findings of Vlad and colleagues
320 (2019) that the pOp6/rcoLhGR transgenic plants manifested severe developmental perturbations
321 when grown on concentrations > 0.1 μM Dex. The direct cause of these growth defects is not known,
322 but the authors suggested that the rice genome contains sequences with high similarity to the *lacOp*
323 sequence, suggesting non-specific activation of endogenous genes by Dex induction. Although, the
324 pOp promoter was not activated by endogenous factors in maize (Moore *et al*., 2006; Segal *et al*.,
325 2003) it is possible that the rice-codon optimization rendered the system too efficient. The off-target
326 effects can be minimized by quenching with IPTG that acts as an effective antagonist for the LacI
327 DNA binding domain in LhGR (Craft *et al.*, 2005).

328 Also, the GVG system caused growth defects in rice (Owerkverk *et al*., 2001) and other species (Kang
329 *et al*., 1999; Andersen *et al*., 2003; Amirsadeghi *et al.,* 2009). It is not clear why GVG generates these
330 effects as each domain of the molecule have been used successfully in other expression systems,
331 however, one possibility is that GVG requires higher expression levels to achieve full promoter

activation, resulting perhaps in squelching or non-specific binding to *CCG(N11)CGG* sequences in the genome (Moore *et al*., 2006). Apparently, the GVG level was not limiting for target-gene activation (Aoyama and Chua, 1997), therefore, selection of plants with a mild GVG phenotype in combination with optimized induction conditions was recommended for the system applications. Although, the XVE system had no effect on growth, all tested lines including controls grown in hydroponics presented a different root pattern with oestradiol treatment so the system does not appear to be suitable for root morphology studies (Okuzaki *et al*., 2011).

EXPERIMENTAL PROCEDURES

Construct preparation

To prepare the pVecLhGR2 construct (Fig. 1A, Fig. S1), firstly, the LhGR2 and the octopine synthase terminator (Tocs) sequences were amplified by polymerase chain reaction (PCR) using vector pOpIn2 (Samalova *et al*., 2019) as the template. The two PCR fragments, LhGR2 (forward primer P1: 5'-AAAAAGGTACCATGGCTAGTGAAGCTCGAAAAACAAAG-3'; reverse primer P2: 5'-GCATATCTCATTAAAGCAGGACTCTAGTTCACTCCTTCTTAGGGTTAGGTGGAGTATC-3') and Tocs (forward primer P3: 5'-TACTCCACCTAACCCTAAGAAGGAGTGAACTAGAGTCCTGCTTTAATGAGATATGC-3'; reverse primer P4: 5'- AAAAAGGTACCCTAAGGCGCGCCGTTGTCG CAAAATTCGCCCTGGACCC -3') were joined together by overlapping PCR with primers P1 and P4 and cloned into the unique *Kpn*I site of the binary rice overexpression vector pVec8-*Kpn*IOX (Kim and Dolan, 2006). Secondly, an *Asc*I fragment containing the bidirectional pOp6 operator array together with *TMV* Ω translation enhancer sequences driving expression of YFP and GUS (including 35S terminator sequence) reporters of the pOpIn2YFP vector (generated by M. Kalde, I. Moore lab) was cloned into a unique *Asc*I site created in the previous cloning step. The correct orientation of the fragment as well as the LhGR2 in the final pVecLhGR2 vector was confirmed by sequencing.

Plant material and growth conditions

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

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

Dexamethasone (Dex) was prepared as a 30 mM stock solution in dimethyl sulfoxide (DMSO) and stored at -20 ^{o}C. Similarly, we prepared stock solutions of betamethasone (Bet), deoxycorticosterone (Doc), fludrocortisone acetate (Flu), prednisone (Pre), prednisolone (Plo) and triamcinolone acetonide (TA), all purchased from Sigma-Aldrich. For each treatment, either a glucocorticoid (induction) or the equivalent volume of DMSO (control) were added to obtain desired concentration.

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

375 For induction of soil grown plants, 4-week-old rice plants were either watered with 50 ml of 30 μM
376 Dex or 30 μM TA or newly developed leaves (3rd leaf) were painted on both sides with 10 μM Dex or
377 10 μM TA solution supplemented with 0.1% Tween-20. The treatments were repeated at 2-day
378 intervals for one or two weeks.

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

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

387 **Detection of YFP fluorescence**

388 The YFP signal was detected using a Zeiss LSM 510 Meta confocal laser-scanning microscope with
389 514-nm excitation from an Argon laser and a BP535-590IR emission filter.

390

391 **ACKNOWLEDGEMENT**

392 I would like to dedicate this publication to my colleague and friend Ian Moore, and his beloved
393 family, without whom science is much less fun. I want to thank to Peng Wang (Chinese Academy of
394 Sciences, Shanghai, China) for the control pZmUbi::GUS line and the rice transformation protocol; to
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396 Oxford) for the pOpIn2YFP vector.

397

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TABLES

Table 1: Number of putative transformants and generated transgenic lines tested for hygromycin (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

FIGURES AND FIGURE LEGENDS

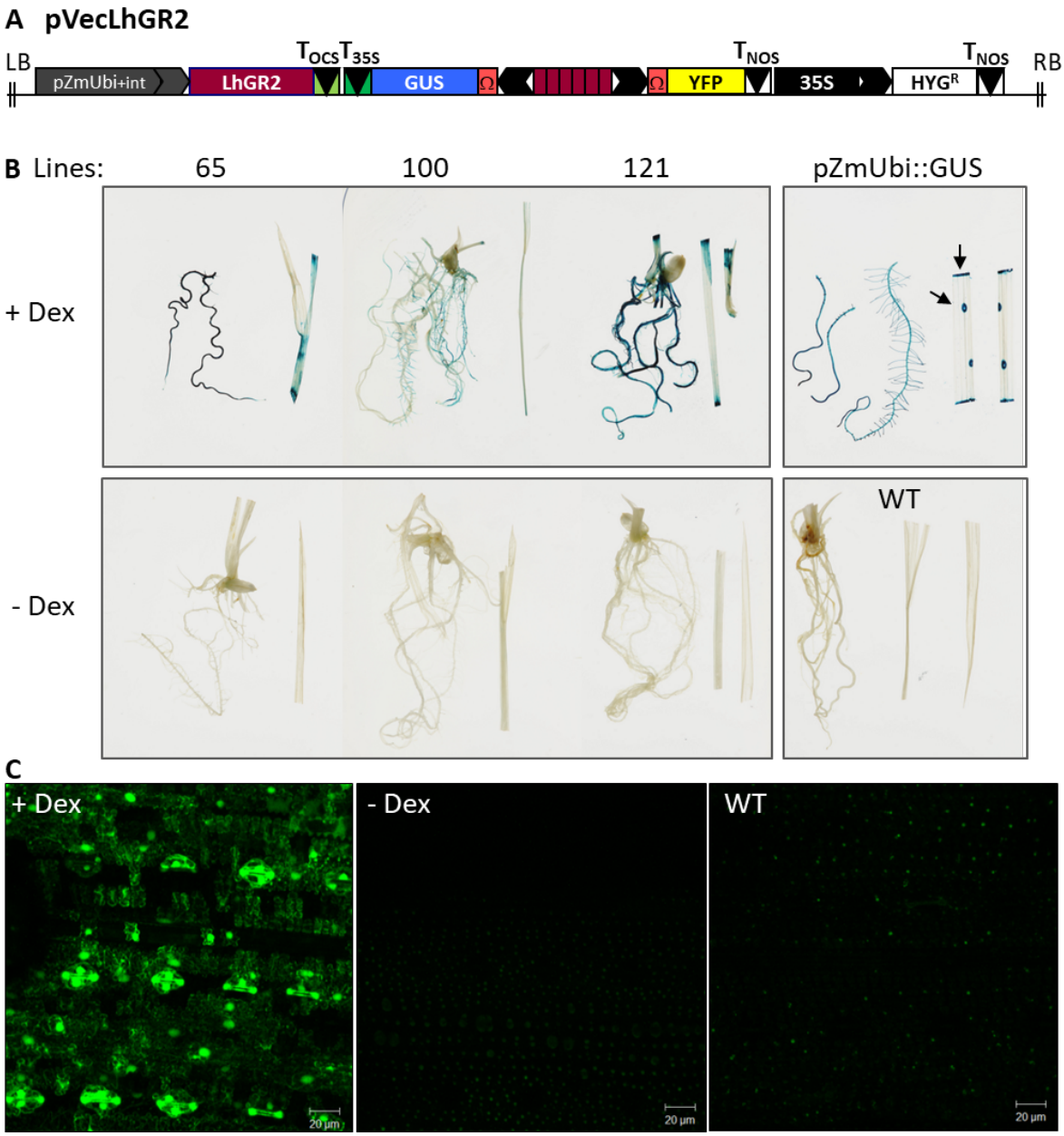
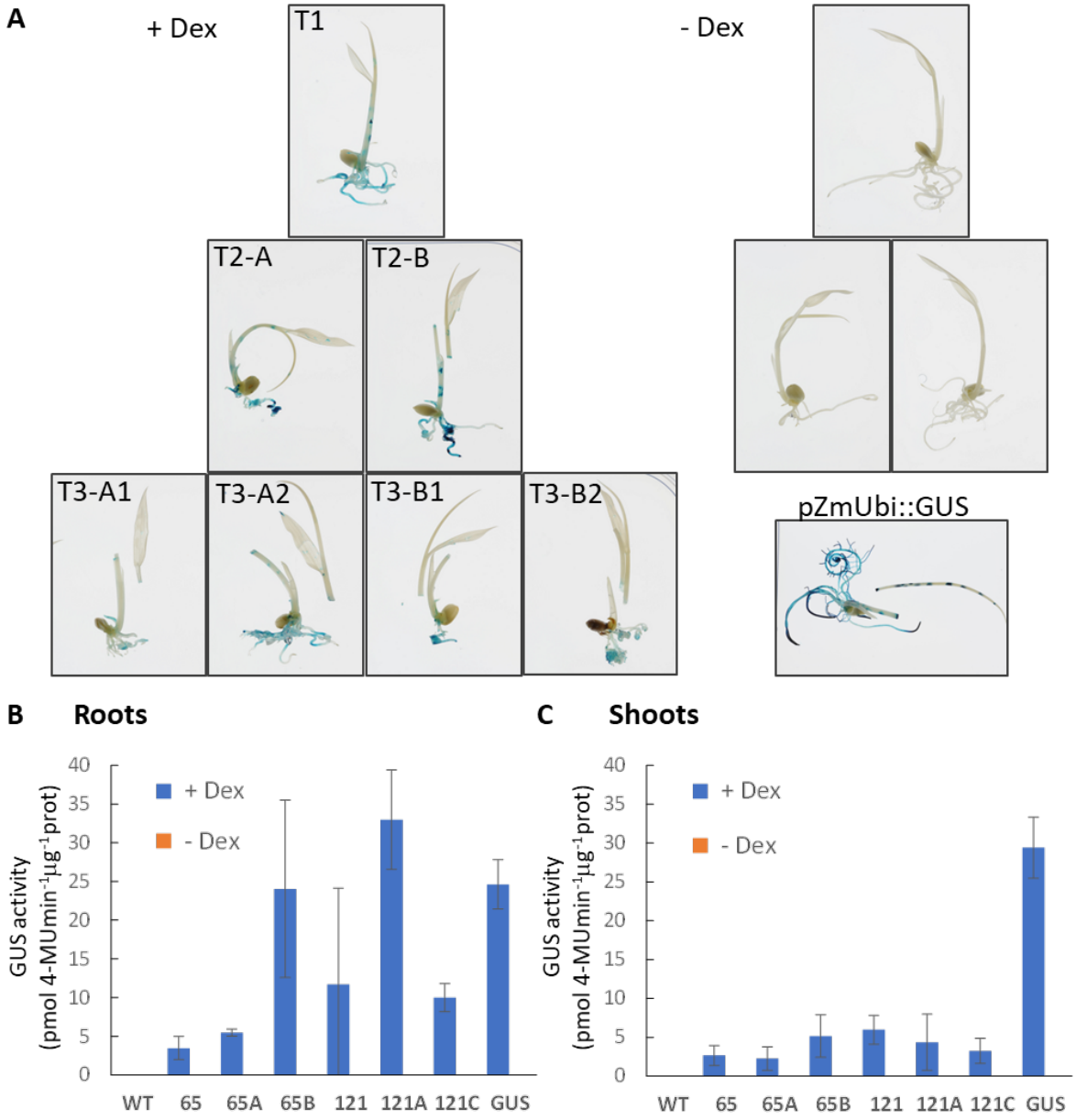


Figure 1: The pOp6/LhGR system in rice: a proof of principle

(A) A schematic representation of the pVecLhGR2 construct that contains the *Arabidopsis* codon-optimized *GAL4* sequence (Rutherford *et al.,* 2005) of the transcriptional activator LhGR2 driven by a *pZmUbi* promoter (containing an intron), a bidirectional pOp6 promotor version with two *TMV* Ω translation enhancers driving two reporter genes - *uidA* (encoding β-glucuronidase; GUS) and a yellow fluorescence protein (*YFP*), and a pCaMV35S::HYG selectable marker cassette conferring hygromycin resistance. The construct was assembled as detailed in Supplementary Figure S1. **(B)** Twelve-day-old rice seedlings of three independent transgenic lines (number 65, 100 and 121) histochemically stained for GUS activity that was induced by seedling transfer onto ½ MS plates containing 30 µM Dex or control DMSO (-Dex) for 6 days. Seedlings of pZmUbi::GUS and wild-type (WT) were included as controls. The arrows point to an example of damaged cells/ cuts where the GUS staining substrate (5-bromo-4-chloro-3-indolyl glucuronide; X-Gluc) penetrated inside the cells.

587 **(C)** Leaves of 4-week-old plant (line 121) were painted with 30 µM Dex or control solution (-Dex) and
588 imaged using a confocal laser scanning microscope 96 h later to detect YFP fluorescence. WT was
589 included as a control for autofluorescence. The scale bar is 2 µm.



590

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

592 **(A)** Ten-day-old rice seedlings (line 65) of generation T1, T2 (A and B) and T3 (A1, A2 and B1, B2)
593 histochemically stained for GUS activity induced by germinating and growing the seedlings on ½ MS
594 plates containing 30 µM Dex or control DMSO (-Dex) for 10 days. Seedlings of pZmUbi::GUS were
595 included as a control for the GUS staining. **(B, C)** GUS activity determined fluorometrically in roots (B)
596 and shoots (C) of 7-day-old rice seedlings (lines 65 and 121, T1 and T2) germinated and grown on ½
597 MS plates containing 30 µM Dex or control DMSO (-Dex) for 7 days. Seedlings of WT and
598 pZmUbi::GUS were included as controls. The error bars represent SD.

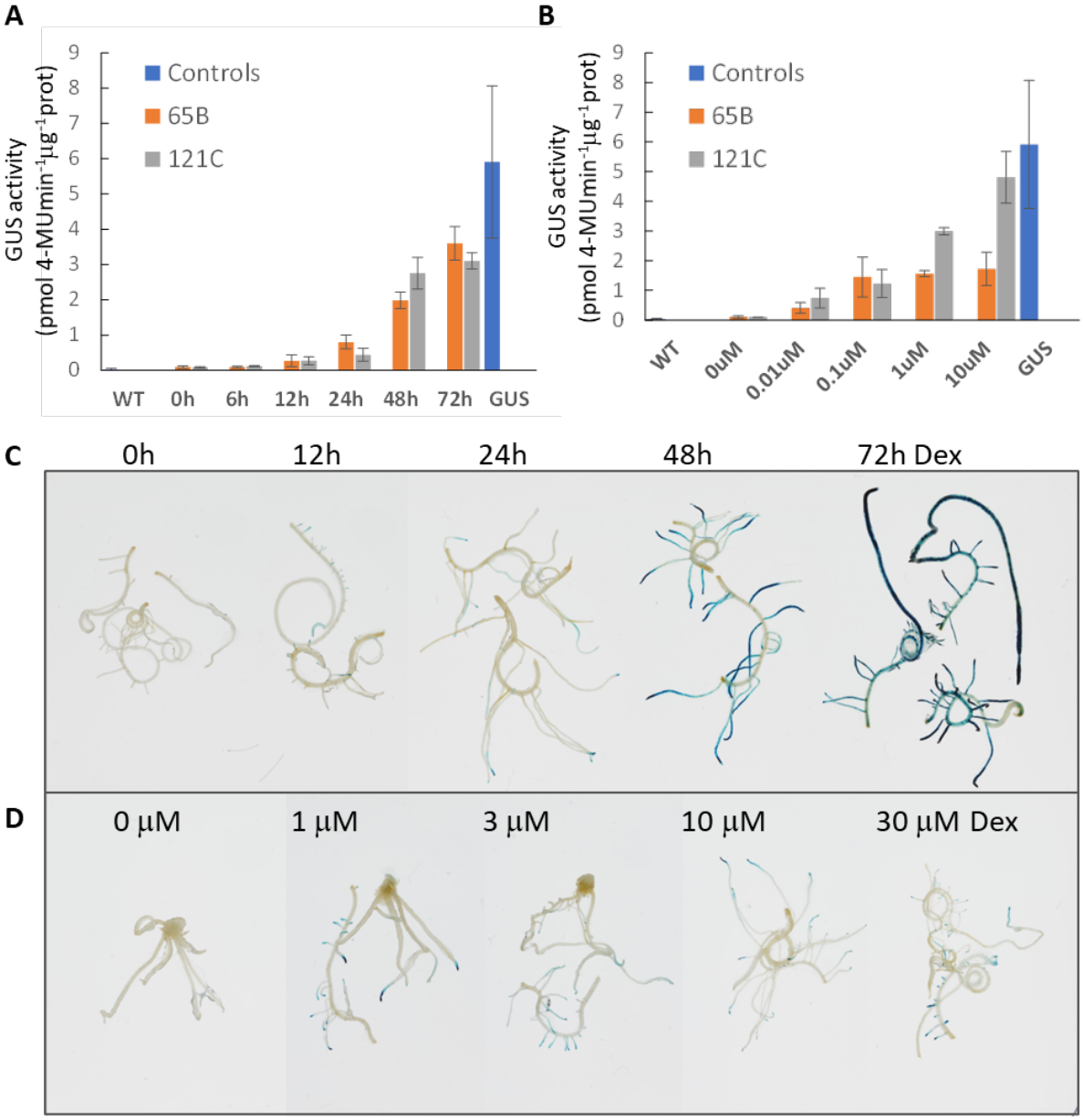


Figure 3: Time course accumulation of induced GUS activity and dose-response of the pOp6/LhGR system in rice

GUS activity determined fluorometrically in 7-day-old rice seedlings (lines 65B and 121C) germinated on ½ MS plates (vertically placed in the incubator) and then stood up in 15 ml Falcon tubes with 2 ml water for further 5 days. The newly developed leaves were induced by painting with water supplemented with 0.1% Tween-20 and **(A)** 10 µM Dex for 0, 6, 12, 24, 48 and 72h or **(B)** with 0, 0.01, 0.1, 1 and 10 µM Dex for 48h. Seedlings of WT and pZmUbi::GUS were included as controls. The error bars represent SD. **(C)** Roots of 10-day-old seedlings (lines 65A, 121C) were induced in liquid ½ MS with 30 µM Dex for 0, 12, 24. 48 and 72h, then histochemically stained for GUS activity. Representative images are shown. **(D)** Roots of 10-day-old seedlings (lines 65A, 121C) were induced in liquid ½ MS for 24h with 0, 1, 3, 10 and 30 µM Dex, then histochemically stained for GUS activity. Representative images are shown.

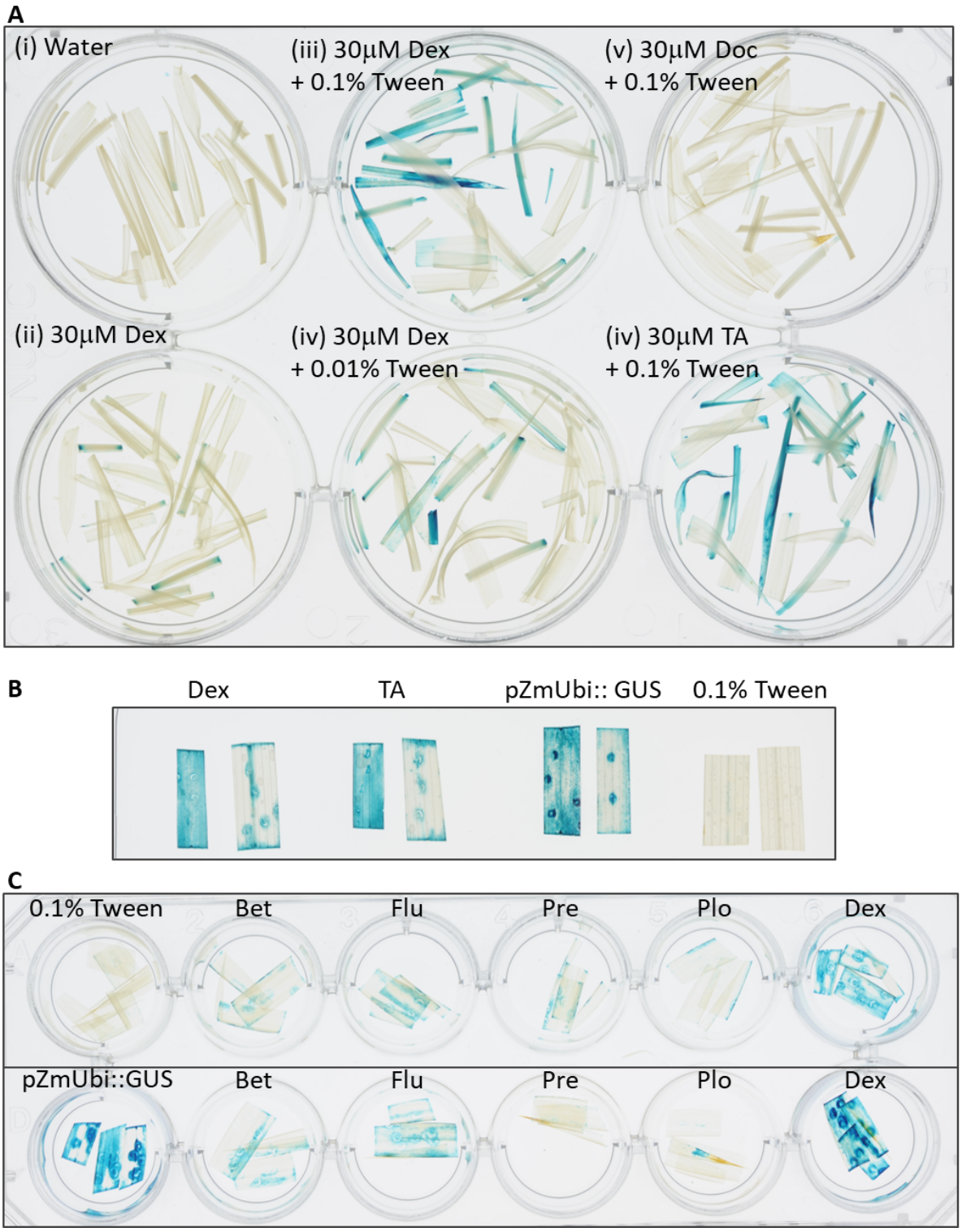


Figure 4: Optimization of the pOp6/LhGR induction in rice by testing different steroid inducers

(A) Shoots of 10-day-old seedlings (lines 65A, 121C) were induced in water supplemented with either 0.01% or 0.1% Tween-20 and 30 µM steroid inducer Dex, deoxycorticosterone (Doc) or triamcinolone acetonide (TA) for 24 h, then histochemically stained for GUS activity. **(B)** Leaf cuttings of the same leaf of 6-week-old plants (lines 65A, 121C) were induced in water supplemented with

618 0.1% Tween-20 and 10 μM Dex or 10 μM TA for 24h, then histochemically stained for GUS activity.
619 Leaves of pZmUbi::GUS were included as controls. Representative images are shown. **(C)** Leaves of 5-
620 week-old plants (lines 65A top, 121C bottom) were induced by painting with water supplemented
621 with 0.1% Tween-20 and 30 μM steroid inducer: Dex, betamethasone (Bet), fludrocortisone acetate
622 (Flu), prednisone (Pre) or prednisolone (Plo) for 24 h, then histochemically stained for GUS activity.

623

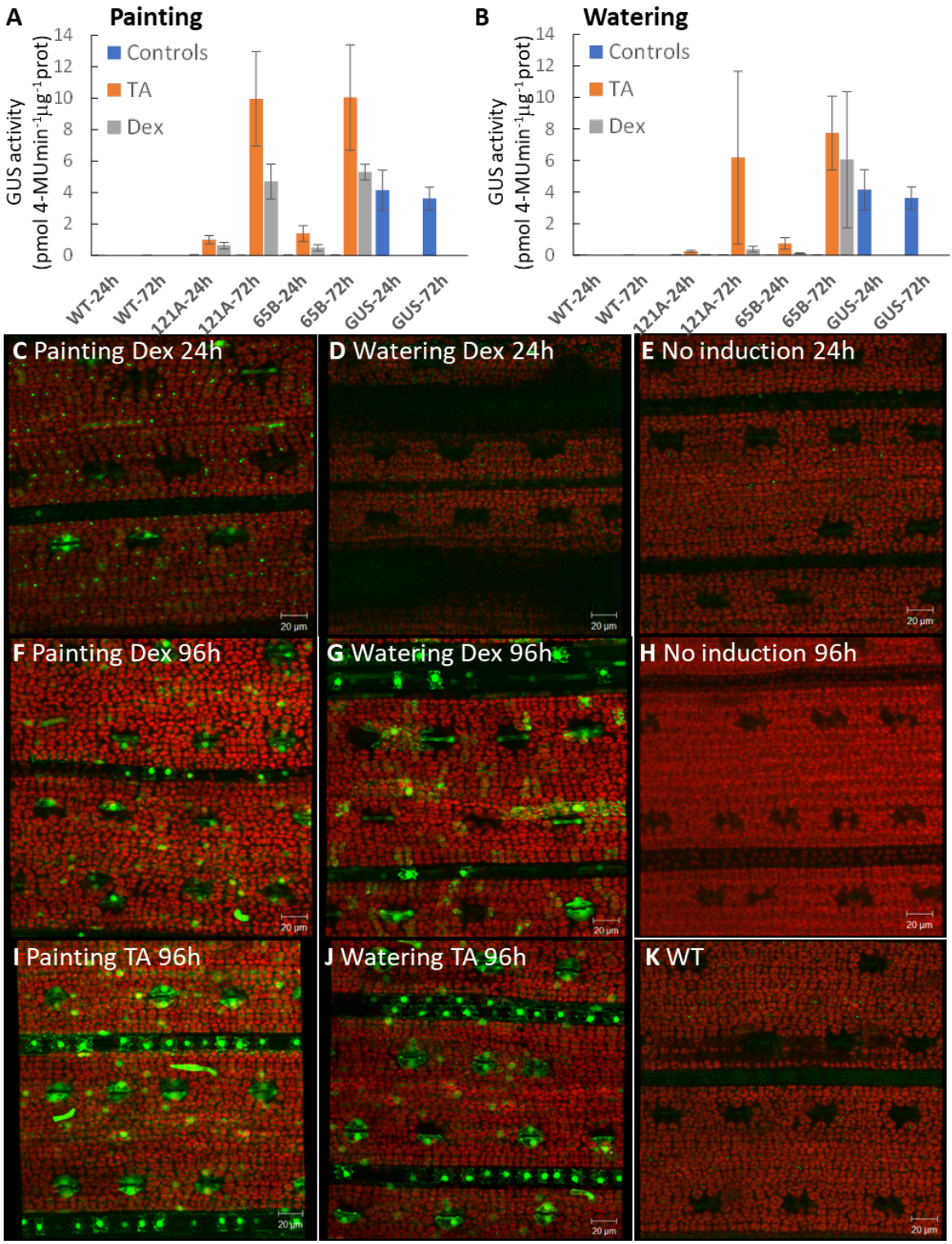


Figure 5: Expression characteristics of the pOp6/LhGR system induced in soil-grown plants by painting and watering

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

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

636

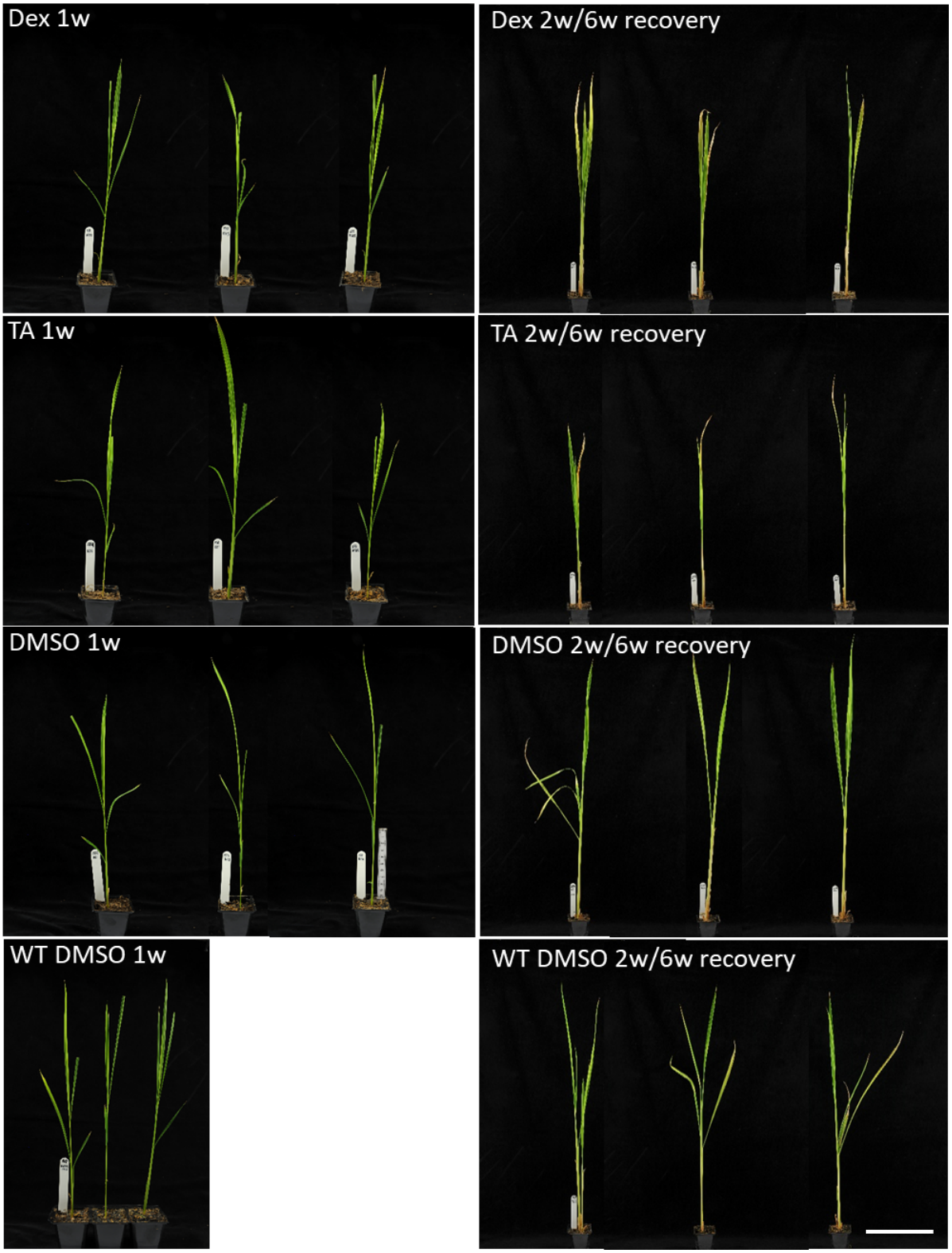


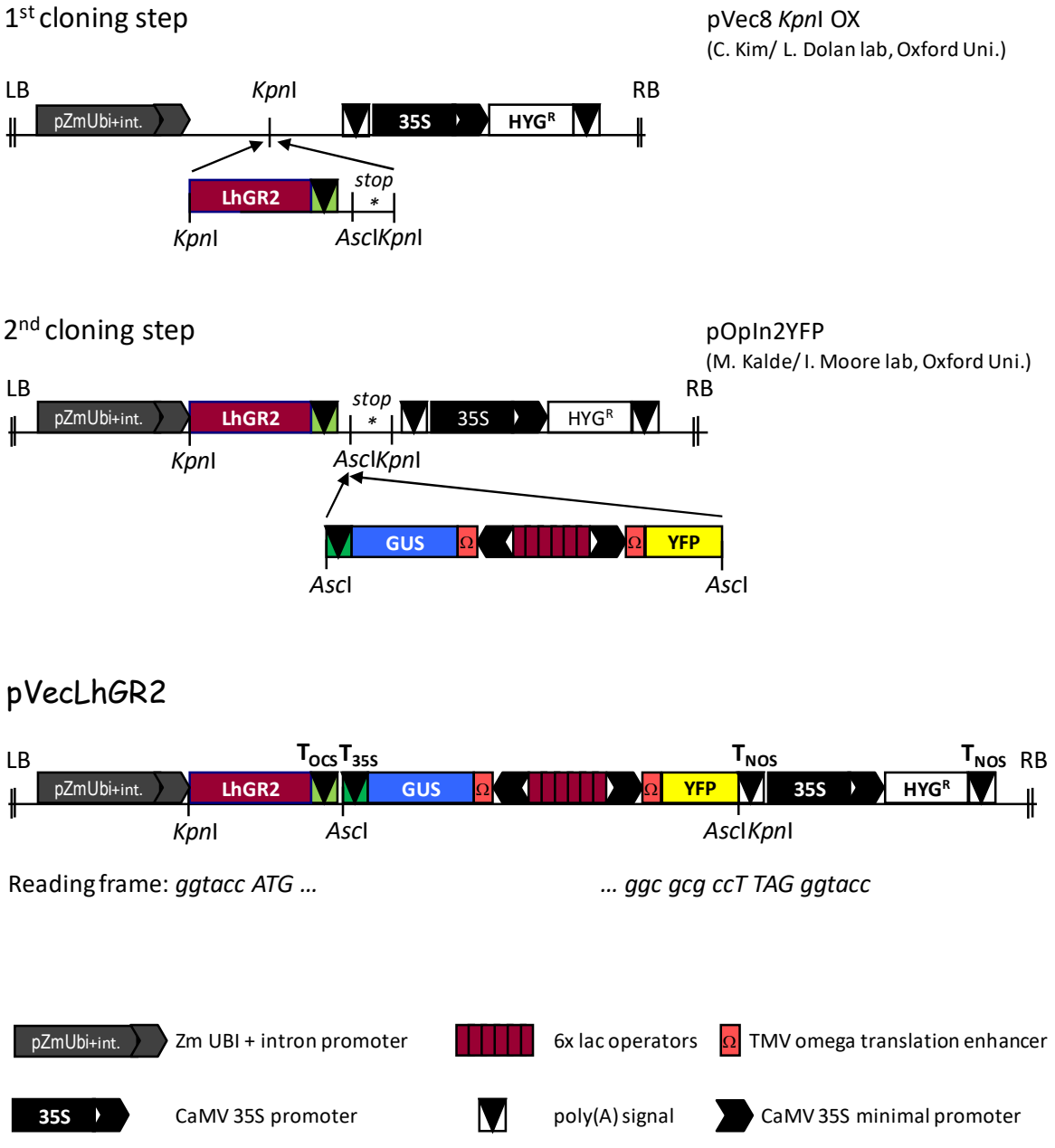
Figure 6: Long-term effect of the pOp6/LhGR system induction on mature rice plants

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

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

643

644 **SUPPLEMENTARY FIGURES**



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