Scientia Horticulturae 172 (2014) 47–53

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

A dexamethasone-inducible gene expression system is active in Citrus plants

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ARTICLE INFO

Article history:
Received 11 November 2013
Received in revised form 22 January 2014
Accepted 16 February 2014
Available online 28 April 2014

Keywords:
Citrus
Transgenic
Inducible
LhGR
Dexamethasone
β-Glucuronidase expression

ABSTRACT

Inducible gene expression systems, in which the level or timing of activity of a gene of interest can be controlled exogenously, are an effective means to assess gene function. However, such systems have not been widely employed in crop plants. Here we show that the glucocorticoid receptor (GR)-based inducible gene expression system functions in Citrange (*Citrus sinensis* × *C. trifoliata*) plants. We generated transgenic Citrange plants containing a two component 35S::*LhGR*/pOp6::*β-glucuronidase* (GUS) system (Wielopolska et al. (2005) Plant Biotechnol. J. 3, 583), in which the synthetic transcription factor, LhGR, is glucocorticoid-inducible and can activate the pOp6 promoter driving expression of the *β-glucuronidase* (GUS) reporter gene. We describe a method for inducing *LhGR* activity using the synthetic glucocorticoid dexamethasone (DEX) in transgenic Citrange. With the advent of transgenic approaches to engineer new traits in Citrus, control of transgene activity will be essential to realize the full potential of such manipulations. The method we describe here is likely to be of general use in many Citrus cultivars to temporally control the activity of introduced transgenes.

1. Introduction

Although traditional breeding has been the main driving force to create novel Citrus varieties with desirable traits, genetic transformation is a promising option to tackle the new challenges facing the industry (Pons et al., 2012). Commonly, the generation of transgenic Citrus has relied on the use of constitutive promoters to drive the expression of target genes throughout the plant (Gong and Liu, 2013). However, constitutive expression from the cauliflower mosaic virus (CaMV) 35S or nopaline synthase (Nos) promoters (Benyon et al., 2013; Pena et al., 2001) is not always suitable if continued expression of the gene of interest is deleterious to plant viability or fertility. In some cases it is preferable to use tissue- or developmentally-specific promoters to refine spatial or temporal patterns of gene expression. For example, phloem specific promoters are actively being employed in Citrus in an effort to find strategies to counteract the effect of the phloem-limited *Candidatus Liberibacter* bacteria causing citrus greening (Benyon et al., 2013; Dutt et al., 2012; Singer et al., 2011).

An alternative approach to control when a transgene is active is to use an inducible system that relies on the addition of an exogenous chemical inducer to activate gene expression or function (Borghi, 2010; Corrado and Karali, 2009; Moore et al., 2006). One such system utilizes the sequence of the ligand-binding domain of the glucocorticoid receptor (GR), which is fused to the gene of interest, usually encoding a transcription factor (Lloyd et al., 1994; Sablowski and Meyerowitz, 1998; Simon et al., 1996). In the absence of the steroid-ligand dexamethasone (DEX), the chimeric protein remains in the cytoplasm where it is sequestered by HSP90 protein complex, and cannot enter the nucleus (Picard, 1993). Upon addition of DEX, the chimeric protein is freed from its interaction with HSP90 and can localize to the nucleus and exert its function. In addition to being inducible, transgene activity can be tissue specific or ubiquitous, depending on the promoter used to drive the chimeric transgene.

The development of a two-component GR-based system, consisting of an inducible transcription factor driver (LhGR), whose activity is only inducible in presence of DEX, and a target gene of interest driven by a synthetic *pOp6* responsive promoter, has enhanced the versatility of this approach (Craft et al., 2005; Moore et al., 1998; Samalova et al., 2005). The expression of the target gene of interest can only be induced in response to DEX when both constructs are present in the same cell. This two-component system can lead to very specific and robust expression of the

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http://dx.doi.org/10.1016/j.scienta.2014.02.041
0304-4238/

target gene of interest (Craft et al., 2005; Samalova et al., 2005). The pOpOff2 version of this system, in which the two cassettes are resident on the same plasmid, allows for integration of both components via a single transformation event (Wielopolska et al., 2005). In the pOpOff2 vector, *LhGR* expression is under the control of the constitutive *35S* promoter, and the bidirectional *pOp6* promoter drives expression of the *β-glucuronidase* reporter gene and of a hair-pin RNAi cassette. It is only in the presence of the inducer, that LhGR can bind to *pOp6* and activate transcription of both target genes.

In the present study we focused on the inducibility of the reporter *β-glucuronidase* gene as a proof of concept that a DEX-inducible system using the pOpOff2 vector is functional in transgenic Citrange plants. We defined parameters for successful inducible *β-glucuronidase* reporter gene expression and demonstrated that, despite a low level of basal expression, following treatment, gene expression was induced up to 25 fold in transgenic plants.

2. Materials and methods

2.1. Generation of pY141

The pOpOff2 plasmid, containing the 35S::*LhGR* and *pOp6::β-glucuronidase*/*hair-pin RNAi* cassettes, was obtained from Dr Chris Helliwell, CSIRO (Wielopolska et al., 2005). A fragment of *Citrange carrizo* (*Citrus sinensis* × *C. trifoliata*) *WUSCHEL (WUS)* cDNA (NCBI accession no. KJ136020) was cloned into the hair-pin RNAi cassette. For this, total RNA from young vegetative shoot tips of Citrange was extracted using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA). Contaminant DNA was removed with TURBO DNA-free (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Specific RNA concentrations were determined using the Qubit RNA broad range kit and a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA). cDNA was synthesized from 400 ng of total RNA using SuperScript® III (Invitrogen) and oligodT (Invitrogen, Carlsbad, CA). The fragment of Citrange *WUS* cDNA was amplified using the following primers: opr8 (CACCAGAGACCAGCTGCTGCTA) and opr9 (CTGAAGAAGAGGTGCTGCTACCATC), and was subcloned in a pENTR™/SD/D-TOPO (Invitrogen) backbone. Using the Gateway LR Clonase system (Invitrogen, Carlsbad, CA) this fragment was subcloned into the pOpOff2 vector (a gift of Chris Helliwell, CSIRO) to generate the pY141 vector. In this study one transgenic line (no. 24, pY102 vector) was used as a negative control and contains a cDNA fragment subcloned in the RNAi vector pK7GWIWG2 (II) (Karimi et al., 2002) and will be described elsewhere (Rossignol, Orbović and Irish, in prep.). Control *35S::β-glucuronidase* transgenic Citrange plants were generated previously (Orbović and Grosser, 2006).

2.2. Generation of transgenic Citrange carrying T-DNA from pY141

Citrange transformation was carried out at the Citrus Research and Education Center at Lake Alfred, Florida and all transgenic lines were maintained at the Center throughout the duration of this study. Transgenic lines were generated as follows. pY141 was introduced into the EHA105 Agrobacterium strain and transformation of Citrange hypocotyl explants and selection of the transgenics were carried out using established protocols (Orbović and Grosser, 2006). Regenerated shoots from explants co-incubated with Agrobacterium were obtained after selection on kanamycin media. A PCR screen to confirm the presence of the transgene was done by making a punch in a small leaf (0.5 mm in diameter) when the shoots were less than one cm tall. This piece of tissue was used as direct source of template for PCR. Primers used correspond to the Citrange *WUS* specific primer opr9 (CACCAGAGACCAGCTGCTGCTA) and opr75 (GAGCTACACATGCTCAGG), which anneals in the terminator region. PCR conditions were as follows: denaturation step at 98 °C for 2 min; 40 cycles of 3-step amplification: 98 °C for 5 s—57 °C for 5 s—72 °C for 20 s; and additional extension at 72 °C for 4 min. These conditions were optimized for the use of "Phire" hot start II DNA polymerase (New England Biolabs, Ipswich, MA) and reactions were set up following the manufacturer's recommendations. Shoots that were PCR positive for the T-DNA were grown and subsequently micro-grafted on 4 week-old, etiolated Citrange rootstock (Orbović and Grosser, 2006). Newly grafted shoots were grown in vitro for an additional four weeks before they were transferred to 5 × 5 × 5 cm cube pots filled with Oasis foam rootcubes (Smithers-Oasis, Kent, OH). To establish the young grafts, these were kept on the light bench under 24 h light (55 μmol/m^2/s) under a dome to preserve humidity. After 3 months, grafted plants were transferred to soil in 15 × 30 cm (diameter × height) pots and kept in the greenhouse. Genotyping of established grafted transgenic plants was carried as follows: a single leaf was ground in liquid nitrogen and the powder was used to extract the DNA with a DNeasy plant kit (Qiagen, Valencia, CA). PCR reactions were performed using NEB Taq polymerase following the manufacturer's recommendation (New England Biolabs, Ipswich, MA), with 200 nM of primers opr20 (GATGACGCACAATCCCACTATC) and opr55 (TTATTTTTGATGAAACAGAAGCTTTTTGATATTTCC) to amplify the 35S::*LhGR* component and opr46 (CACCATGTTACGTCCTGTAGAAACCCCA) and opr47 (TCATTGTTTGCCTCCCTG) primers to amplify the *β-glucuronidase* gene Sequences were amplified as follows: one step of denaturation at 95 °C for 5 min followed by a three-step amplification: 20 s of denaturation at 95 °C, 1 min of annealing at 55 °C, and 1 min (*LhGR*) or 2 min (*β-glucuronidase*) extension at 68 °C, with a total of 35 cycles. A final extension step at 68 °C for 5 min was performed.

2.3. Induction method and treatments

A 20 mM stock solution of dexamethasone (D1756; Sigma-Aldrich, St. Louis, MO) was prepared in 96% ethanol and stored at −20 °C. Plants were watered twice a week with a freshly prepared solution containing a 1:1000 dilution of the dexamethasone stock solution (DEX, 20 μM dexamethasone in 0.1% ethanol) or with a solution of 0.1% ethanol only for mock control plants (MOCK). 80 mL of either solution was applied to 15 × 30 cm (diameter × height) pots and 25 mL of solution was used to water the 5 × 5 × 5 cm cube pots (for plants nos. 37–40 in experiment 3). In all cases, care was taken so that the pots were dry before the following watering to ensure drenching with fresh solution each time.

Induction experiments were performed at three different times for different lengths of time (Table 1): experiment 1 (50 days, total of 14 applications), experiment 2 (20 days—6 applications), and experiment 3 (14 days—4 applications). In experiment 3, a time course was performed (Fig. 4a). The first time point corresponded to two weeks before the plants started to receive treatment (time point before induction, tbi). The plants were then watered 4 times across two weeks with either DEX or MOCK solution. Then, four days after the last application, leaf samples were taken (t0), and the plants were watered one last time with DEX or MOCK solution. Leaf samples were taken 2 h (t2), 4 h (t4) and 24 h (t24) after this last watering.

2.4. Assessment of LhGR and β-glucuronidase expression levels

Total RNA extraction from leaf tissue, and cDNA synthesis on 400 ng of total RNA, were performed as described above. Real time quantitative RT-PCR was performed using a CFX system (Biorad,