Planta (2005) 222: 957–967
DOI 10.1007/s00425-005-0037-7

ORIGINAL ARTICLE

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Modification of plant architecture through the expression of GA 2-oxidase under the control of an estrogen inducible promoter in *Arabidopsis thaliana* L

Received: 18 March 2005 / Accepted: 29 April 2005 / Published online: 4 November 2005

Abstract The gibberellin (GA) 2-oxidase (PcGA2ox1) from bean catalyses the 2β-hydroxylation of some precursor and bioactive GAs resulting in their inactivation. We have expressed *PcGA2ox1* under the control of the estrogen receptor-based chemical-inducible system, XVE, to modify plant architecture and assess whether transgene expression is localised. Applications of estradiol to the shoot apical region of inducible *PcGA2ox1* overexpressors exhibited delays in both bolting (maximum of 46 days) and times to anthesis (maximum of 62 days) compared to wildtype (36 and 41 days, respectively), without altering leaf area. Individual treated leaves showed signs of epinasty and became dark green; such estradiol-treated regions maintained these 'green-islands' well beyond the onset of leaf senescence. Northern blots revealed that the *PcGA2ox1* transcript could be detected within 1 h of treatment. The level of *PcGA2ox1* transcript appeared to peak 3–5 h after estradiol application in both high and semi expressors. Quantitative Reverse Transcription (QRT)-PCR data showed that GA down-regulated genes *AtGA3ox1*, *AtGA20ox1* and *SCARECROW-LIKE3* (*SCL3*) were up-regulated and the GA up-regulated genes *AtGA2ox1* and *AtExp1* were down-regulated in estradiol-treated leaves of inducible *PcGA2ox1* overexpressors; neighbouring non-treated leaves showing no significant changes. Further molecular analyses revealed that expression of the transgene was confined to estradiol-treated leaves only. Expression profiles of GA down- and up-regulated genes in inducer-treated overexpressors appeared to be synchronised with changes in leaf phenotype. These observations suggest that *PcGA2ox1* under the control of the XVE system can be used effectively to alter plant architecture in Arabidopsis by localised 2β-hydroxylation of GAs at estradiol-treated sites.

Keywords Chemical-inducible system · Estrogen receptor · GA 2-oxidase · Gene expression · Gibberellin

Abbreviations CaMV: Cauliflower mosaic virus · GA: Gibberellin · GUS: β-Glucuronidase · DIG: Digoxigenin · PcGA2ox1: *Phaseolus coccineus* L. GA 2-oxidase1 · QRT-PCR: Quantitative reverse transcription-PCR · *SCL3*: *SCARECROW-LIKE3*

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Introduction

GAs are plant hormones that are involved in the regulation of growth and development in higher plants. Hence, due to their importance throughout the life cycle of plants, the biosynthesis of GAs has been intensively studied. The biologically active GAs, are produced from *trans*-geranylgeranyl diphosphate (GGPP) by the action of cyclases, cytochrome P450 monooxygenases and 2-oxoglutarate-dependent dioxygenases (2 ODDs) (Hedden and Phillips 2000). The dioxygenases catalyse the final steps of the GA pathway, the GA 20-oxidase (GA20ox) oxidises C-20 and the GA 3β-hydroxylase (GA3ox) introduces a 3β-hydroxyl group. Another dioxygenase which inserts a 2β-hydroxy group, called the GA 2-oxidase (GA2ox), results in the inactivation of GAs. Indeed, the GA 2-oxidase also oxidises the precursors of bioactive GAs, such as GA_{20} and GA_9. This suggests that GA 2-oxidase plays a key role in determining or regulating the amounts of active GAs in plants.

Several GA 2-oxidase cDNAs have been cloned, initially from runner bean (*Phaseolus coccineus* L.) by functional screening (*PcGA2ox1*) and three from *Arabidopsis thaliana* L. using the elucidated sequence (Thomas et al. 1999). In garden pea (*Pisum sativum* L.),

two GA 2-oxidase cDNAs have been cloned (Lester et al. 1999; Martin et al. 1999). Studies in rice (*Oryza sativa* L.) showed that ectopic expression of the cloned GA 2-oxidase (*OsGA2ox1*) cDNA from rice produced transformants with shortened stems and impaired reproductive organs (Sakamoto et al. 2001). Overexpression of a pea GA 2-oxidase2 cDNA in transgenic *Arabidopsis thaliana* L. plants caused seed abortion and reduced pollen tube growth which confirmed that GAs play a vital role in seed development and normal pollen tube growth (Singh et al. 2002). Indeed, the ectopic expression of *OsGA2ox1* under the control of the promoter of a GA biosynthesis gene, *OsGA3ox2* (*D18*) produced semi-dwarf rice plants with normal flowering and grain development suggests that modulation of GA catabolism and bioactive GA content in a site-specific manner can offer a potential improvement in cereal crops (Sakamoto et al. 2003). These previously cloned GA 2-oxidases use C_{19}-GAs as their substrates. Recently, through activation-tagging, a further two 2β-hydroxylases (*AtGA2ox7* and *AtGA2ox8*) which hydroxylate C_{20}-GA precursors were cloned in *Arabidopsis thaliana* L. (Schomburg et al. 2003). This study confirmed that through overexpression in tobacco or in Arabidopsis, the levels of active GAs are reduced and dwarf plants are produced. Overall, these studies confirm that GA 2-oxidases play a major role in plant growth and development and their overexpression can lead to GA deficient phenotypes.

Constitutive promoters such as the cauliflower mosaic virus (CaMV) 35S promoter are not useful for the transcription of genes where over- or under-expression can lead to deleterious effects on the plant. Although the application of tissue-specific promoters can circumvent the problem, to some degree, the use of a chemical-inducible system offers a more flexible control on gene expression due to the requirement of an inducer for its function. Here, we investigate the value of expressing the GA 2-oxidase (*PcGA2ox1*) from bean in Arabidopsis under the control of the estrogen receptor-based inducible system (Zuo et al. 2000) to see whether plant architecture can be modified and how GA precursors are subsequently modified.

Materials and methods

Construction of pER8 vectors

The selectable marker CaMV 35S promoter-*bar*-CaMV 35S terminator was cut out of pGreen-Cassette using *Eco*RV and ligated into *Pvu*II site within the T-DNA of pER8 to allow transformed plants to be screened using the herbicide Basta® and used throughout all constructs. This plasmid was used as an empty vector control in transformation studies (designated as CaMV 35S-*bar*-CaMV 35S transformants). The reporter *gus*A gene was cloned in-frame to the estrogen-induced promoter using the GATEWAY Cloning Technology Kit. An *attB* flanked PCR product of *PcGA2ox1* was synthesised using the primers: 5′-AAAAAAGCAGGCTTCATAGT AACAATCGACAAC-3′ and 5′−AGAAAGCTGGG TAATTTGAAGGGTGTGTTTGG-3′ in a Thermal Cycler (Perkin Elmer) set at 94°C (2 min), and 10 cycles of 94°C (15 s), 60°C (30 s) and 72°C (60 s). The *attB-PcGA2ox1* expression clone was finalised following 5 cycles of 94°C (15 s), 50°C (30 s) and 72°C (3 min) and then 20 cycles of 94°C (15 s), 60°C (30 s) and 72°C (3 min) in a Thermal Cycler. The first recombination into pDONR201 to create the entry clone and a second recombination using a destination vector were according to manufacturer's instructions. All modified pER8 vectors were sequenced to confirm gene of interest and induced promoter were in-frame. Binary vectors were transferred into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) by electroporation.

Production and screening of transgenic plants

Seeds of *Arabidopsis thaliana* L. ecotype Columbia (Lehle Seeds, TX, USA) were cold-treated in water for 4 days at 4°C in the dark then germinated and grown in a peat and vermiculite medium contained in 10 cm diameter pots (5 plants/pot) in a growth chamber (18 h photoperiod, 70 μmol m^{-2} s^{-1}, 24 ± 2°C). At the onset of flowering, plants were dipped into a suspension of *Agrobacterium*, 5% (w/v) sucrose and 0.05% (v/v) Silwet L-77 (OSi Specialities, Inc., Danbury, CT, USA) (Clough and Bent 1998) before returning to the growth chamber for seed production. Prior to sowing, seeds where stored in an incubator (30°C, 40% relative humidity) for 6 weeks to improve ripening. Following cold-treatment, seeds where sown in trays containing a soil mix soaked in 0.05% (v/v) Basta®. After germination, seedlings were sprayed every 2–3 days with 0.05% Basta and only transformed plants remained green after 9 days from sowing. Transformed plants were raised as described until homozygous lines were produced and used in subsequent studies.

GUS histochemical studies

In order to determine whether the estrogen-induced promoter could activate the *gus*A gene where estradiol, an estrogen analogue (Nacali, Japan) was applied, pER8-*gus*A lines were selected for investigation. Three-week-old plants were raised in soil and leaves from lower (third leaf), middle (sixth leaf) and upper (eighth leaf) regions and apical shoot were treated with a single droplet (approx. 10 μl) of 17-β-estradiol (0, 0.4, 4, 40 and 100 μM) in the presence or absence of a surfactant Tween 20 (Sigma) or Tergitol (Sigma) at 0.02%. Five hours after treatment, individual leaves and apical shoots were excised and treated for GUS activity (Curtis et al. 2000a) prior to being examined using a stereomicroscope.

Table 1 Primers and TaqMan® probes used for QRT-PCR

Gene (AGI code)	Forward primer	Reverse primer	Probe
AtGA2ox1 (At1g78440)	CTTCGCTGGACCTTCATTGAC	ACAACCTCTCGTCCTCATTGTCT	CAGAGAATCGCTCCGTTGACATGTTTGA
AtGA3ox1 (At1g15550)	TCCGAAGGTTTCACCATCACT	TCGCAGTAGTTGAGGTGATGTTG	TCGCCTCTCAACGATTTCCGTAAACTTTG
AtGA20ox1 (At4g25420)	GCCTGTAAGAAGCACGGTTTCT	CTCGTGTATTCATGAGCGTCTGA	CAATCACGGCATCAGCGAGGAGCTTAT
AtExp1 (Atg69530)	AACGCACACGCCACATTCTAC	AAAGGCATAGGTGTTCGTGGAG	GTGGTGGTGATGCTTCCGGCACAATGG
SCL3 (At1g50420)	TCATGGTGGTCACTGAGCAAG	TGCTGCGTAGGTGTAAAGTGATTC	CTCAGACCACAACGGCTCCACA
PcGA2ox1 (At132438)	CATGGTTGTTCTGTCTCAGCC	TGGACATTGCTAATGGTTTCAGAA	GCATTGAACCAGTGGTTTTC

Northern blotting

Northern analyses were performed to determine the time-scale for the activation of the estrogen-induced promoter to express transgenes in relation to the application of estradiol. Three-week-old transformed plants grown in soil were sprayed with 2 μM 17-β-estradiol containing 0.02% Tween 20 and then the tops of the plants were excised at 0, 1, 3, 5, 7 and 9 h intervals and frozen immediately in liquid nitrogen. Total RNA was extracted from frozen tissue using an RNA isolation kit (Tri Reagent, MRC, Cincinnati, OH, USA), which was modified according to Chomczynski and Mackey (1995) to allow the removal of polysaccharides. Fifteen micrograms of RNA was fractionated on 1.2% (w/v) agarose gels, blotted onto positively charged nylon membranes and probed with a digoxigenin (DIG)-labelled *PcGA2ox1* fragment (for *PcGA2ox1* transformants) or a *gus*A gene fragment (*gus*A transformants). Both probes were produced from their respective binary vectors by PCR. A pair of primers was used for the amplification of *PcGA2ox1* (Primer 1, 5′-CCATGGT TGTTCTGTCTCAGC-3′; Primer 2, 5′-AATCAGCAG CAGATTTCTGG-3′) using 30 cycles of 94°C (30 s), 53°C (30 s) and 72°C (75 s) in a PTC-100 Thermal Cycler (MJ Research, Inc., Watertown, Mass., USA). The DIG-labelled *gus*A fragment was prepared as described (Curtis et al. 2000a). Membranes were stripped and reprobed with a DIG-labelled 18S rRNA fragment from *Saccharomyces cerevisisae* to confirm loading efficiency of RNA.

Phenotypes of transformed plants

For in vitro studies, surface-sterilised cold-treated seeds (20 seeds/9 cm Petri dish) were sown on agar-solidified (0.8%) medium containing half-strength Murashige and Skoog (MS 1962) salts and vitamins, 1% (w/v) sucrose and pH 5.8 (designated as GT medium) supplemented with or without 17-β-estradiol (0, 0.4, 4, 40, 100 μM). Cultures were maintained in growth cabinets set at 24 ± 2°C with a 18 h photoperiod and light intensity of 70 μmol m^{-2} s^{-1}. Plants were assessed in terms of plant size, leaf senescence and time to bolting. To determine whether GA_4 could rescue the dwarf character of the 2ox overexpressing line 2oxH2, seeds where sown on GT medium supplemented with or without GA_4 (1 μM) in the presence of 17-β-estradiol (0.4 μM) and the phenotypes of the seedlings were subsequently recorded. Cultures were grown under the same conditions as for the phenotypic assessment study.

To study the effects of time of 17-β-estradiol application (0, 0.4, 4, 40 and 100 μM) on time of bolting (approx. 3 mm of stem was visible) and anthesis (time when the first flower opened) transformed plants where grown under the same conditions as for in vitro cultures and a 10 μl droplet was applied to the rosette or the inducer was used during seed imbibition (10 plants/

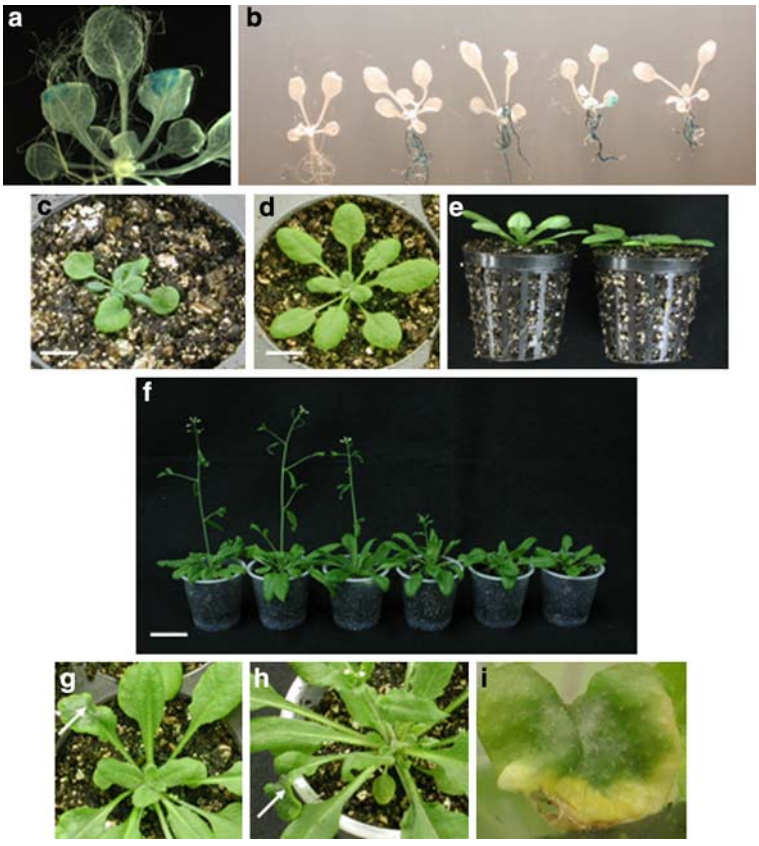

Fig. 1 The effects of estradiol on transgene activity and phenotype of XVE transformants. **a** Localised GUS activity in leaves treated with 40 μM of 17-β-estradiol. **b** Intense GUS activity in roots of the line GUS2 cultured on medium containing 17-β-estradiol. Plants were cultured on medium containing 0, 0.4, 4, 40 and 100 μM of 17-β-estradiol (from left to right). GUS activity in lower leaves of the 40 μM estradiol-treated plant was due to contact with the medium. **c** A 2oxH2 transformant treated with 100 μM 17-β-estradiol at the apical shoot (21 days post-sowing) exhibiting dwarfism, dark green leaves and prostrate leaves (*bar* = 1 cm). **d** For comparison, a non-treated 2ox-H2 plant of the same age (*bar* = 0.8 cm). **e** Application of 100 μM of 17-β-estradiol to the apical shoot of 2oxH transformants resulted in leaves to become prostrate (left, untreated; right, treatment after 2 d). **f** Forty-two days after the application of 100 μM 17-β-estradiol (applied 21 days post-sowing), both the 2oxH1 and 2oxH2 lines developed rosettes of similar size compared to wildtype and GUS2 line. From left to right: wildtype treated and without estradiol, GUS2 line treated and non-treated and 2oxH1 and 2oxH2 treated with estradiol (*bar* = 4 cm). **g** The application of 40 μM of 17-β-estradiol to individual leaves (see *arrow*) of line 2oxH2 resulted in dark 'green islands' and leaf surface distortion. **h** At anthesis, the treated leaf (see *arrow*) remains dark green and curves towards the soil as opposed to a more erect-habit of non-treated leaves. **i** Treated leaf retains its 'dark green island' where estradiol was applied but the surrounding leaf undergoes senescence

treatment, 3 replicates/treatment). In a separate study, an exogenous application of GA_4 (2 μM) to the apical shoot of 20-day-old rosette plants was applied to the 2ox overexpressor 2oxH2 to see whether a reversal of dwarfing could be observed.

Quantitative reverse transcription (QRT)-PCR

To investigate whether the estrogen-induced promoter linked to *PcGA2ox1* functions exclusively in leaves treated with 17-β-estradiol (40 μM) and not function in neighbouring leaves (seventh or eighth true leaves), RNA was extracted from both treated and neighbouring non-treated leaves. Total RNA was extracted from leaves using the RNAqueous RNA Isolation Kit with Plant RNA Isolation Aid (Ambion, Austin, TX, USA). The first-strand cDNA was produced from 2 μg of DNaseI-treated total RNA using the SuperScript First-Strand Synthesis for QRT-PCR (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Transcript levels were quantified using a sequence detector system (model 7700; Applied Biosystems, Foster City, CA, USA) utilising specific primers (Table 1) with a 18S rRNA as internal standard (Yamauchi et al. 2004).

Gibberellin analysis

Three-week-old plants grown in soil were liberally sprayed with 40 μM 17-β-estradiol with 0.02% Tween 20. Two days after treatment, the tops of treated plants were harvested and frozen immediately in liquid nitrogen. Approximately 15 g (f.wt.) of plant material was used from each transgenic line for each GA

Table 2 The effects of 17-β-estradiol application on times to bolting and anthesis[a]

Line/estradiol μM	Time of estradiol application (d)			
	0 (Imbibed seeds)		21	
	Bolting	Anthesis	Bolting	Anthesis
2oxH1/0	37.6 ± 3.1	42.1 ± 2.9	37.1 ± 1.4	41.7 ± 1.8
2oxH1/0.4	39.9 ± 4.6	43.8 ± 3.3	37.6 ± 2.2	42.5 ± 3.0
2oxH1/4	40.5 ± 2.4	49.1 ± 3.1[b]	39.3 ± 3.6	45.1 ± 2.9[b]
2oxH1/40	42.7 ± 1.9[b]	50.7 ± 3.9[b]	42.0 ± 3.2[b]	49.4 ± 3.4[b]
2oxH1/100	43.4 ± 3.3[b]	51.8 ± 3.6[b]	43.1 ± 4.0[b]	51.7 ± 4.4[b]
2oxH2/0	35.4 ± 4.1	40.1 ± 3.2	36.9 ± 3.0	42.0 ± 2.6
2oxH2/0.4	41.1 ± 3.8[b]	47.7 ± 3.1[b]	41.3 ± 2.6[b]	46.6 ± 3.3[b]
2oxH2/4	42.2 ± 6.4	52.0 ± 3.6[b]	41.5 ± 3.8	46.9 ± 3.8[b]
2oxH2/40	43.0 ± 2.8[b]	55.7 ± 4.3[b]	42.0 ± 2.3[b]	47.4 ± 3.0[b]
2oxH2/100	45.7 ± 3.2[b]	61.5 ± 4.5[b]	43.0 ± 2.0[b]	49.0 ± 3.7[b]
GUS2/0	37.0 ± 3.6	41.8 ± 3.3	37.5 ± 1.3	42.8 ± 2.0
GUS2/0.4	34.8 ± 3.7	39.7 ± 3.9	36.3 ± 3.3	41.6 ± 3.9
GUS2/4	38.1 ± 3.7	42.8 ± 3.4	37.2 ± 1.9	42.4 ± 2.4
GUS2/40	39.7 ± 3.1	44.1 ± 3.4	36.8 ± 1.8	41.6 ± 2.1
GUS2/100	38.8 ± 3.5	42.8 ± 3.4	35.0 ± 1.2	39.6 ± 1.9
Wildtype/0	36.3 ± 3.3	40.5 ± 3.8	36.5 ± 1.5	41.0 ± 1.6
Wildtype/0.4	38.8 ± 3.4	42.5 ± 2.8	37.6 ± 1.8	42.1 ± 2.2
Wildtype/4	37.4 ± 3.7	41.6 ± 3.1	38.2 ± 1.9	42.5 ± 2.3
Wildtype/40	37.9 ± 2.5	42.6 ± 2.5	37.6 ± 3.8	41.6 ± 3.3
Wildtype/100	38.0 ± 3.6	42.4 ± 3.5	35.6 ± 1.8	39.9 ± 2.1

[a]Data are presented as mean ± standard deviation of 15 plants/treatment; 3 replicates/treatment. [b] Differences between replicate means of transformant and respective wildtype treatments are significantly greater than zero at the 0.05 level of significance

measurement. GAs were quantified using ^{2}H-labelled internal standards as described previously (Gawronska et al. 1995). GA analyses were performed twice using different batches of plants grown in a growth chamber as described earlier.

Statistical analysis

Differences in the times to bolting and anthesis between homozygous transgenic lines and their respective wildtype treatments were assessed by the paired sample Students *t*-test (Bishop 1971). Each replicate mean was subtracted from their respective wildtype treatment and the difference of the three mean values together was assessed to determine whether the overall difference was significantly greater than zero.

Results

GUS activity in high expressing pER8-*gus*A transformants

To test whether the estrogen-induced promoter will function only at sites where 17-β-estradiol was applied, GUS activity in an overexpressing pER8-*gus*A line (GUS2) was studied both in vivo and under culture conditions. Twenty-one-day-old plants (rosette-stage of growth) raised on compost were treated either on individual leaves or apical shoot with a single droplet containing 17-β-estradiol with surfactant (Tween 20 or Tergitol). Both surfactant treatments produced very similar responses in terms of area size of blue colouration in leaf and apical shoot following GUS histochemical staining. The higher the concentration of estradiol applied to the plant, the greater the size of blue stain was produced. A concentration of 0.4 μM

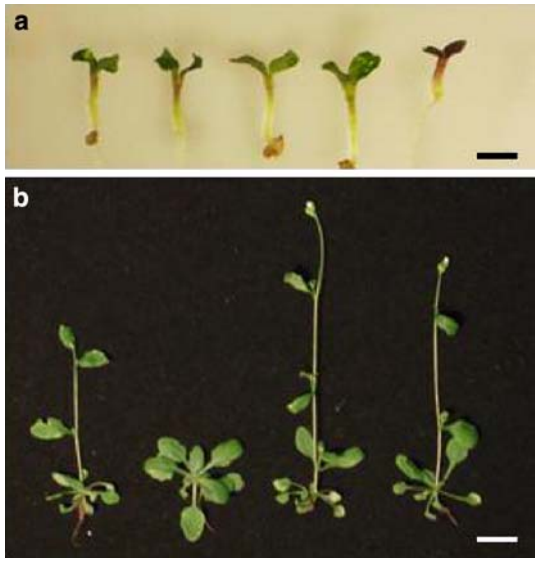

Fig. 2 The effects of GA_4 on reversing the dwarf habit of 2ox overexpressor 2oxH2. **a** Seven-day-old seedling of CaMV 35S-*bar*-CaMV 35S cultured on medium without GA_4 and 17-β-estradiol (control), 2oxH2 seedling without estradiol and GA_4, 2oxH2 seedling in the presence of 1 μM GA_4 but no estradiol, 2oxH2 seedling cultured with 17-β-estradiol (0.4 μM) and 1 μM GA_4 and 2oxH2 seedling with 1 μM GA_4 but without estradiol (left to right; *bar* = 0.5 cm). **b** Thirty-two-day-old plants of the 2oxH2 line and wildtype treated with (time 0) or without 100 μM 17-β-estradiol and with or without 2 μM GA_4 (applied at apical shoot at 20 days from sowing). A 2oxH2 plant treated with GA_4 and estradiol, 2oxH2 plant treated with estradiol only, 2oxH2 plant treated with GA_4 but no estradiol and a non-treated 2oxH2 plant (left to right; *bar* = 2 cm)

of 17-β-estradiol was sufficient to elicit GUS activity in treated leaves. In all treatments, GUS activity could not be detected outside the area where estradiol was applied (Fig. 1a). Transformants treated with estradiol without surfactant and also without the inducer failed to reveal GUS activity.

In a separate study, GUS activity was also studied in young plantlets which were raised from seed in culture on agar-based medium containing estradiol. Three-week-old plants were carefully removed from culture and treated for GUS activity. Roots of the high expressing line exhibited an intense blue colouration over a wide range of inducer concentrations (Fig. 1b). However, both leaves and the apical shoot which were not in contact with the medium, failed to show GUS activity. In the case of cotyledons and lower leaves in contact with the surface of the agar-medium, revealed GUS activity mainly in the vascular tissue (Fig. 1b). Plants grown in a culture medium containing 17-β-estradiol up to 100 μM exhibited a phenotype comparable to wildtype and transformants cultured in the absence of estradiol (data not shown).

Phenotypes of pER8-2ox transformants raised in culture

Following the confirmation that GUS activity could only be detected at sites where 17-β-estradiol was applied, 10 homozygous pER8-2ox lines where sown on medium containing estradiol for phenotypic analysis. The time of germination (radicle emergence) was consistent in all lines tested (6 days). However, after 8 days, two pER8-2ox lines exhibited both severe dwarfism and darker green leaves (designated as 2oxH1 and 2oxH2) compared to other pER8-2ox lines, positive control transformants (pER8-*gus*A and CaMV 35S-*bar*-CaMV 35S) and wildtype. These severely dwarfed 2ox lines exhibited dwarfism at 0.4 μM 17-β-estradiol and severe dwarfism at 4, 40 and 100 μM 17-β-estradiol. Such dwarf lines exhibited slower growth compared to untreated lines and produced smaller, dark green cotyledons with anthocyanin pigmentation in their smaller hypocotyls (approx. half the length of untreated 2oxH lines). At the time when true leaves emerge (approx. 3 mm in length) from the inducible *PcGA2ox1* overexpressors in the presence of 40 μM 17-β-estradiol, the untreated lines had formed 7–8 true leaves. One 2ox line exhibited slight dwarfism only when cultured at 40 and 100 μM of 17-β-estradiol after 13 days from sowing (designated as 2oxS1). Another 2ox line exhibited no dwarfism but produced darker green leaves when cultured on medium containing 40 and 100 μM of 17-β-estradiol (2oxS2). All other pER8-2ox transformants (2oxL1-6) exhibited no phenotypic differences in culture compared to positive control transformants and wildtype plants. A supplementation of 1 μM of GA_4 in the culture was sufficient to reverse the dwarfing effect of 0.4 μM of 17-β-estradiol on germinating seeds of the 2oxH2 line (Fig. 2a). Seedlings of 2oxH2 grown on medium containing GA_4 with estradiol developed longer hypocotyls and larger cotyledons (similar to untreated seedlings) compared to seedlings treated with estradiol alone which exhibited dwarfism with increased anthocyanin pigmentation.

Effects of estradiol on bolting and flowering times

To assess the effects of 17-β-estradiol on bolting and anthesis, the chemical inducer (0, 0.4, 4, 40 and 100 μM) was applied to the apical shoot of rosette plants at different times of plant development (21-, 24- and 31-day-old plants), a two treatment application at 24 and 31 days post-sowing and also during the vernalisation of seeds (time 0). The most effective treatments of estradiol on bolting and anthesis were the single applications at 0 and 21 days (Table 2). The 2oxH1 and 2oxH2 lines exhibited significant delayed bolting and anthesis times compared to both wildtype and GUS transformants, when 17-β-estradiol was applied at 40 and 100 μM. Line 2oxH2 showed the greatest delay in bolting (45 days) and anthesis (62 days) times when treated with the highest concentration of estradiol at 100 μM during vernalisation. Seeds treated with either 40 or 100 μM 17-β-estradiol resulted in dwarf plants with dark green leaves. Rosette plants treated at 21-day-old, showed that in approximately 24 h after estradiol application (treatments 40 and 100 μM only), both 2ox dwarf lines exhibited dark green leaves where the estradiol was applied and such leaves became prostrate (Fig. 1c) compared to a non-treated plant (Fig. 1d). Such plants appeared dwarf in terms of a reduced rosette diameter compared to wildtype plants (11 days after treatment: 2oxH2, 40 μM 3.3 ± 0.7 cm, 100 μM 2.8 ± 0.7 cm; wildtype, 40 μM 6.0 ± 0.4 cm, 100 μM 6.0 ± 0.6 cm) but later, the newly formed leaves formed rosettes of size similar to wildtype plants (42 days after treatment: 2oxH2, 40 μM 10.3 ± 1.4 cm, 100 μM 9.6 ± 1.1 cm; wildtype, 40 μM 9.3 ± 0.8 cm, 100 μM 9.7 ± 0.8 cm; Fig. 1e). The untreated 2oxH1 and 2oxH2 lines developed similarly to wildtype plants (Table 2) with comparable rosette diameters (data not shown). The application of estradiol as a single application (24-day-old plants) or as a double application (24 and 31 days after sowing) onto the 2oxH transformants also delayed bolting and anthesis times compared to wildtype and GUS transformants but were less effective than earlier treated plants (data not shown). Transformants treated with estradiol at 31 days post-sowing bolted and flowered at a similar time compared to wildtype and control transformants (data not shown). However, these 2oxH lines formed 'dark green islands' where estradiol was applied and developed prostrate leaves 2 days after treatment compared to the upright leaves of non-treated plants (Fig. 1f).

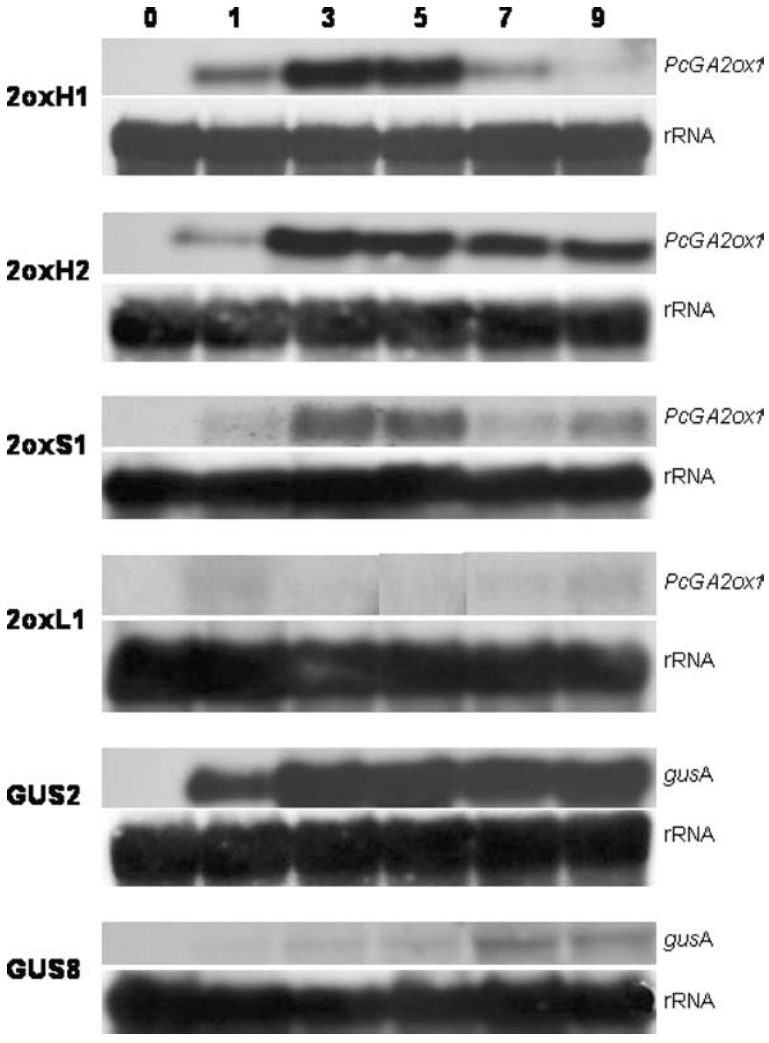

Fig. 3 Effect of 17-β-estradiol on the expression of a transgene linked to the estrogen receptor-based chemical-inducible system. Twenty-one day-old plants were sprayed with 2 μM of estradiol and then harvested at 0, 1, 3, 5, 7 and 9 h after treatment for RNA extraction. Total RNA (15 μg) was subjected to RNA gel-blot analysis with either *PcGA2ox1* or *gus*A gene DIG-labelled fragments as probes. Loading efficiency was tested using a rRNA probe

Rosette plants (20-day-old, previously subjected to 100 μM of 17-β-estradiol at time 0) of the 2oxH2 line were treated with 10 μl of GA_4 (2 μM) at the apical shoot to see whether dwarfism could be reversed. Twelve days after GA_4 treatment, plants of the 2oxH2 line developed a prominent bolt almost equal to untreated plants (Fig. 2b). This observation suggests that the delay in anthesis times in the 2oxH2 line can be reversed by a single application of GA_4.

The two 2oxS lines (2oxS1 and 2oxS2) also exhibited delayed bolting and anthesis times compared to controls when estradiol was applied at time 0 at 40 and 100 μM (S1 and S2 at 40 μM: bolting 40.4 ± 2.1 days and 42.4 ± 3.3 days, respectively; 100 μM, 41.1 ± 3.1 days and 44.3 ± 3.2 days, respectively; anthesis, at 40 μM, 44.9 ± 1.7 days and 47.6 ± 3.6 days, respectively; 100 μM, 46.1 ± 3.4 days and 47.6 ± 3.6 days, respectively). The six 2oxL lines only exhibited a delay in bolting and anthesis times when estradiol was applied during vernlization at 100 μM. With the exception of the 2oxH lines, none of the other 2ox transformants exhibited changes in leaf colour and plant stature when estradiol was applied at the highest concentration of 100 μM.

Expression profiles of estradiol-induced genes

In order to understand the effect of estradiol on the expression of a transgene linked to the chemical-induced promoter, total RNA was extracted from estradiol-treated pER8-2ox and pER8-*gus*A transformed plants over time and assessed by Northern blotting (Fig. 3). In all cases, the transcribed gene could be detected in transformants within 1 h of treatment with estradiol. The blots of the dwarf 2oxH transformants gave a stronger signal when probed with the *PcGA2ox1* DIG-labelled probe compared with the 2oxS transformants but their profile of expression appeared similar with a peak between 3–5 h after the application of estradiol. Northern blots of the 2oxH2 line revealed a strong signal for *Pc2ox1* expression up to 48 h after estradiol treatment (data not shown). The high GUS expressing line GUS2, revealed a strong signal of GUS expression throughout all harvested samples but the weak GUS expressor GUS8 showed a gradual increasing weak signal of expression through time. All 6 blots of the 2oxL lines revealed a very weak signal when probed with *PcGA2ox1*. There was no signal of *PcGA2ox1* expres-

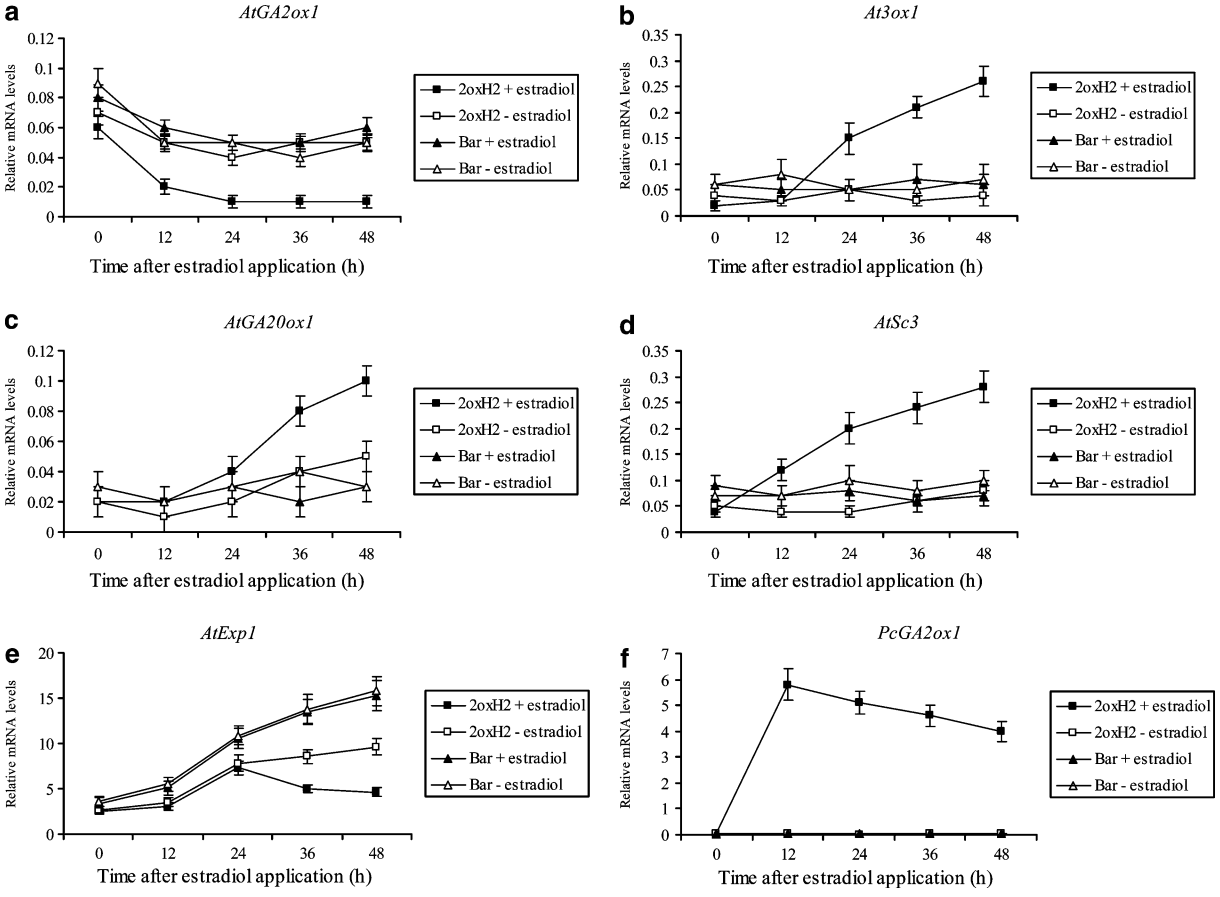

Fig. 4 Effects of 17-β-estradiol on the localised expression of GA-regulated genes. Individual leaves (seventh or eighth leaf) from the 2ox overexpressor 2oxH2 and plants carrying CaMV 35S-*bar*-CaMV 35S (plant control) were treated with 40 μM of estradiol, whilst its neighbouring leaf was not treated (control). Treated and non-treated leaves (5–6 leaves/treatment) were excised over a range of times (0–48 h) after estradiol treatment and RNA extracted. QRT-PCR was performed using first-strand cDNA as template on a sequence detector system. Transcript levels of GA up-regulated genes *AtGA2ox1* (**a**) and *AtExp1* (**e**), along with the GA down-regulated genes *AtGA3ox1* (**b**), *AtGA20ox1* (**c**) and *SCL3* (**d**) are given over a time course performed twice using two batches of rosette-grown plants. Expression of the transgene *PcGA2ox1* (**f**) is also given to provide evidence that induction by estradiol is confined to treated leaves. Data is presented as mean ± s.e.m. of two replicate experiments

sion in non-treated plants of 2ox transformants, suggesting that there was no evidence of leakage in the expression of the transgene.

Changes in GA-regulated gene expression through the application of estradiol

In an attempt to see whether overexpression of *PcGA2ox1* at localised sites can change the levels of bioactive GAs which leads to differences in the level of expression of GA-responsive genes, the 2ox dwarf line 2oxH2 was treated with 17-β-estradiol in rosette plants. In this study, the seventh or eighth leaf was treated with estradiol and its neighbouring seventh or eighth leaf was left untreated so that the effect of leaf age would be limited; a transformed line carrying CaMV 35S-*bar*-CaMV 35S without a transgene linked to the XVE system was also treated the same way and used as a control. Both lines were exposed to estradiol over a time course

Table 3 Concentration (ng g^{-1} fresh weight) of GAs in rosette leaves of XVE transformants, treated with or without 40 μMs of 17-β-estradiol. Endogenous GAs were quantified by GCMS using internal standards. Data are presented as the means of two replicate experiments

Plant/estradiol	GA_{12}	GA_{24}	GA_9	GA_{51}	GA_4	GA_{34}
GUS2/+	3.95	5.58	0.08	0.28	0.35	0.52
2oxL1/+	3.30	5.08	0.07	0.31	0.61	0.30
2oxH2/+	1.80	0.88	0.03	2.23	0.24	0.09
GUS2/−	3.13	5.44	0.06	0.30	0.51	0.50
2oxL1/−	3.88	4.89	0.06	0.26	0.46	0.45
2oxH2/−	3.39	4.89	0.06	0.22	0.44	0.48

of 48 h at 12 h intervals; total RNA was extracted from these individual leaves and the expression of GA regulatory genes was assessed by QRT-PCR (Figs. 4a–e). The expression of the endogenous *AtGA2ox1* gene (Thomas et al. 1999) decreased rapidly soon after the application of estradiol in the 2oxH2 line, whilst untreated leaves and control plants showed little change in expression levels. The GA down-regulated genes *AtGA3ox1* (Chiang et al. 1995), *AtGA20ox1* (Phillips et al. 1995) and *SCARECROW-LIKE3* (*SCL3*, Ogawa et al. 2003) were all up-regulated in leaves treated with estradiol in the 2oxH2 line but non-treated leaves showed no change throughout the 48 h time course experiment. However, the GA up-regulated gene *AtExp1* (Ogawa et al. 2003), declined in expression after 24 h in the 2oxH2 line when treated with estradiol with non-treated leaves revealing a steady increase in expression. These results suggest that the expression of *PcGA2ox1* under the control of the estrogen-induced promoter was localised and that individual treated leaves were becoming deficient in active GA.

To determine whether transgene expression was confined to estradiol-treated leaves only, both treated and adjacent untreated leaves were monitored for *PcGA2ox1* transcripts by QRT-PCR (Fig. 4f). Expression of *PcGA2ox1* in treated leaves revealed a peak 12 h after estradiol application, followed by a gradual decline at 48 h after treatment. However, in the adjacent non-treated leaves, transgene expression could barely be detected and was similar to both treated and non-treated leaves of CaMV 35S-*bar*-CaMV 35S control plants.

To understand the phenotypic effects of individual leaves being treated with estradiol in the 2oxH2 line, some treated plants were left untouched along with control plants and their phenotypes were assessed. Between 24 and 36 h after treatment, sites of leaves treated with estradiol exhibited 'dark green islands' and leaf distortion in only the 2oxH2 line (Fig. 1g). At anthesis, these treated leaves showed further distortion and curved towards the soil, whilst untreated leaves remained erect in habit (Fig. 1h). Later observations revealed that at the time when the treated leaf was undergoing senescence, the 'dark green islands' remained up to the time of seed-shed (Fig. 1i).

Evidence for 2β-hydroxylation in 2ox dwarf plants in the presence of estradiol

To confirm the 2β-hydroxylation function of PcGA2ox1 in transformed plants, the dwarf 2ox line 2oxH2, plants were sprayed with 40 μM of 17-β-estradiol and the whole rosette was harvested for GA analysis (Table 3). As a control, untreated plants were also analysed along with treated and untreated plants of 2oxL1 and GUS2. Two days after estradiol treatment, only the 2oxH2 line exhibited both dark green leaves and prostrate leaves but no dwarfism, the other lines failed to show any phenotypic changes in the presence or absence of estradiol. GA analysis revealed that the treated 2oxH2 line clearly demonstrated the 2β-hydroxylation of GA_9 (0.03 ng g^{-1}f wt) to GA_{51} (2.23 ng g^{-1}) compared with non-treated plants (GA_9, 0.06 ng g^{-1} ; GA_{51}, 0.22 ng g^{-1}). The other transformed lines (treated or not) showed little variation in GA_9 (0.06–.008 ng g^{-1}) and GA_{51} (0.22–0.31 ng g^{-1}). Despite the level of GA_4 being reduced in estradiol-treated 2oxH2 line (0.24 ng g^{-1}) compared to untreated (0.44 ng g^{-1}), it could not be explained by its 2β-hydroxylation to GA_{34} (2oxH2 treated with estradiol, 0.09 ng g^{-1} ; non-treated, 0.48 ng g^{-1}). The decrease in the levels of GA_{34} and GA_{24} in the estradiol-treated 2oxH2 line and the presence of GA_4 in all samples are discussed (see Discussion).

Discussion

We have demonstrated that the estrogen-induced promoter for controlling the expression of *PcGA2ox1* can be used effectively in the modification of plant architecture in the model plant, *Arabidopsis thaliana* L. A previous study using the estradiol-activated XVE system linked to the green fluorescent protein (GFP) reporter gene demonstrated the system to be highly inducible, tightly controlled, specifically activate only the target gene and have no undesirable effects on the host (Zuo et al. 2000). Our initial studies revealed that when the XVE system was linked to the *gus*A gene, GUS activity was highly inducible (only 0.4 μM of 17-β-estradiol was sufficient to activate GUS expression), transgene activity occurred only where the inducer was applied and the transformants were phenotypically similar to wildtype plants. However, the use of a detergent (Tween 20 or Tergitol) with estradiol was essential for the XVE system to be highly inducible as treatments without detergent could not induce GUS activity even at a concentration of 100 μM.

Alteration of plant phenotype by site-specific activity of PcGA2ox1

Following the confirmation that the XVE system can be used to express a transgene under tight regulation, studies were performed to test whether the system could be used to express the bean GA 2-oxidase (*PcGA2ox1*) to specifically alter plant architecture at a fixed localised region. Previous studies investigating the modification of plant stature have been achieved through mutations in GA biosynthetic genes (Martin et al. 1997), reducing GA production by expressing antisense constructs (Coles et al. 1999), by overexpression of a pumpkin GA20 oxidase (Curtis et al. 2000b; Niki et al. 2001) and by enhancing GA catabolism (Sakamoto et al. 2001). However, although the heights of these plants were much reduced compared to wildtype, the surface areas of leaves were also reduced in size or development of reproductive organs was impaired. Here, by using

estradiol during seed imbibition, we initially delayed plant development as shown by a delay in bolting times (especially in the 2oxH overexpressors treated with 40 or 100 μM 17-β-estradiol) compared to untreated controls and wildtype plants. However, in later development of the 2oxH lines, the effect of *PcGA2ox1* expression appeared to be more directly related to stem elongation as leaf area was unaffected and thus reducing the chance of photosynthetic efficiency being reduced. In addition, all *PcGA2ox1* transformants, including the 2oxH over-expressors, developed normal reproductive organs and produced viable seed at yields equivalent to wildtype. Our results show that the effect of estradiol on both times to bolting and anthesis depends on the time of application, concentration of inducer and the transformant used for investigation. The earliest application of estradiol was the most effective treatment (at seed imbibition) when applied at the highest concentration of 100 μM. Two transformed lines which exhibited severe dwarfism when grown in culture in the presence of estradiol (designated 2oxH1 and 2oxH2) were the most effective in showing a delay in these two processes. In this case, the transformed lines of 2oxH1 and 2oxH2 exhibited a delay in bolting by 7 and 10 days compared to wildtype and time to anthesis was delayed by 11 and 21 days, respectively. Interestingly, the dwarf growth habit exhibited by the 2oxH2 line could be reversed by an exogenous application of GA_4 in both culture (germinating seeds) and also to the apical shoot to produce plants with similar anthesis times compared to untreated 2oxH2 plants. In a similar study, GAs which are not substrates of GA 2-oxidase, such GA_3 (at 2 μM) could also reverse dwarfism in rosette plants of 2oxH2 line by promoting stem elongation compared to GA_1 (2 μM) which is degraded by the enzyme (data not shown). The other 2ox transformed lines also revealed a delay in bolting and anthesis times compared to wildtype and positive control transformants but to a reduced level. Interestingly, applying estradiol at either at seed imbibition or 21 days after sowing, caused the 2oxH1 and 2oxH2 lines to produce smaller rosette plants but at the time of bolting these plants developed new leaves to form rosettes similar to wildtype and untreated plants.

Molecular studies of site-specific expression of *PcGA2ox1*

Studies on the expression of *PcGA2ox1* using the XVE system confirmed that the activity of the transgene was localised as supported by molecular analysis and phenotypic observations. Northern blots showed that the transcript was detectable within 1 h after estradiol application and that the pattern of expression for the 2ox high expressors (2oxH1 and 2oxH2) and semi-expressor (2oxS1) peaked between 3–5 h but the *gus*A gene control lines (GUS2 and GUS8) revealed an increase in transcript levels up to 9 h after inducer application. This time-dependent reduction in the expression of *PcGA2ox1* under the XVE system has previously been reported in the expression of GFP (Zuo et al. 2000) but this occurred 14–21 days after exposure to estradiol. Due to an unknown post-transcriptional mechanism (Fire 1999), the abundance of *PcGA2ox1* transcript may have been regulated early, although the comparison between GFP and *PcGA2ox1* transcripts should be made carefully in an identical experimental condition in order to confirm this observation. Our QRT-PCR data further confirmed that the production of GA is tightly regulated and that such regulation can be localised using the XVE system. Applying estradiol to individual leaves resulted in the expression of the GA down-regulated genes *AtGA3ox1*, *AtGA20ox1* and *SCL3* to increase 12–48 h after the application of the inducer, but the GA up-regulated gene *AtExp1* declined in expression after 24 h compared to neighbouring non-treated leaves. In addition, expression of the GA up-regulated gene *AtGA2ox1* decreased rapidly soon after the application of inducer relative to non-treated leaves (control). The lack of transgene expression in untreated adjacent leaves further supports that induction by estradiol is solely confined to treated leaves. Due to the localised reduction in bioactive GA in treated leaves by 2β-hydroxylation such leaves became dark green within 24–48 h. The reduction in expression of *AtExp1* after 24 h appeared to coincide with the distortion of leaves at sites where estradiol was applied and the epinastic behaviour of leaves which were observed 24–48 h after treatment. This reduction in the levels of active GAs at estradiol-treated sites may have disrupted normal cell division and cell elongation, which require GA (Sauter and Kende 1992; Ogawa et al. 2003), and thus causing treated leaves to exhibit irregular growth.

Activity of PcGA2ox1 causes 2β-hydroxylation of precursor and bioactive GAs

Our GA analysis data further confirmed the catabolic activity of PcGA2ox1 as the 2ox overexpressing line 2oxH2 contained ten-fold more GA_{51} after a 2-day exposure to 40 μM of 17-β-estradiol compared to non-treated controls which indicates the possible 2β-hydroxylation of GA_9 as mentioned previously (Sakamoto et al. 2001). However, although the level of GA_4 was marginally reduced, it could not be explained by its 2β-hydroxylation to GA_{34} (decreased by five-fold). We think the decrease in GA_{34} in the estradiol-treated 2oxH2 line to be a result of a reduced flux through GA_9 to GA_4 and subsequently to GA_{34}, due to the increased conversion of GA_9 into GA_{51}. The reduction in the level of GA_{24} in treated 2ox overexpressing lines maybe a result of the upregulation of GA 20-oxidase activity, which coincides with the increase in *AtGA20x1* transcript levels through feedback regulation, presumably due to reduced GA_4 synthesis (Fig. 4). Previous studies have indicated that the homeostasis of active GA levels is maintained in part through negative regulation of GA

biosynthesis genes, including those encoding GA 20-oxidases (Phillips et al. 1995). Additionally, 2 days after estradiol-treatment, the 2oxH2 line failed to show marked changes in dwarfism and so the metabolic change occurred drastically (i.e. increase in GA_{51}), but the GA_4 level was maintained due to a homeostatic mechanism. Finally, the very weak expression of *PcGA2ox1* in the 2oxL1 line (Fig. 3) was insufficient to cause the 2β-hydroxylation of precursor and bioactive GAs and thus their GA profile (Table 3) and their phenotypes in response to estradiol application were comparable to wildtype.

This study has provided evidence that expression of *PcGA2ox1* under the control of the XVE system can be effective in modifying plant architecture by the 2β-hydroxylation of bioactive GAs in site-specific regions. Although the release of estrogen or its analogue estradiol to the environment may cause ecological problems, the use of an inducible system for the 'switching-on' of a GA catabolic gene has the potential to improve existing crops by, for example, delaying bolting and flowering times (Curtis et al. 2002). The availability of other inducible promoter systems such as those induced by ethanol (Caddick et al. 1998) and other inducers (Gatz and Lenk 1998) could potentially avoid the use of hazardous chemicals being released to the environment and thus allow agronomically-useful plants being produced in a more safer way. This system not only offers an alternative approach for modifying plant stature but also the ability to induce highly localised reductions in GA levels which may provide a new insight in GA action.

Acknowledgements We would like to thank N.-H. Chua and A.W. Mellon (The Rockefeller University, New York, USA) for providing pER8 and P. Hedden (Rothamsted Research, Harpenden, Herts, UK) for cDNA of *PcGA2ox1*. This work was supported by a RIKEN Fellow Award (to I.S.C.).

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