WALNUT ROOTSTOCK TRANSFORMATION AND REGENERATION FROM VEGETATIVE TISSUE

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ABSTRACT

The primary goal of this proposal is to develop novel regeneration technologies needed to engineer walnut rootstocks for pest and disease resistance. The productivity of the walnut industry of California is dependent the availability of superior rootstocks to combat soil disease and pest problems. Individual selections displaying resistance to either *Phytophthora* crown or root rots or to root lesion nematode, *Pratylenchus vulnus*, have been identified and can be clonally propagated but these selections are susceptible to crown gall. Rootstocks with multiple resistances are clearly desirable. Walnut transformation depends on the use of somatic embryos. Somatic embryos are readily obtained from immature, and therefore uncharacterized, seed but not from vegetative or other mature tissues. This precludes inserting any genes into a rootstock exhibiting another superior trait if the trait was identified in a seedling or mature tree. Walnuts have a long generation time and the most promising rootstocks, hybrids of English walnut x black walnut, are sterile or nearly sterile, factors that severely impede developing multiple resistances by breeding. The recent discoveries of genes that stimulate regeneration provide a unique avenue to develop cultivar independent transformation for walnut. Developing the capability to insert genes into elite walnut rootstock will provide a significant tool for improving walnut trees for the California walnut industry.

OBJECTIVES

1. To test the efficacy of the XVE inducer system in walnut somatic embryos and track expression at various stages of the regenerated plant.
2. To determine the capability the *ESR1* and *PGA6* genes, under the control of a constitutive promoter and the XVE inducible system, to stimulate the production of transgenic somatic embryos or organogenic shoots for a range of rootstock germplasm.
3. To determine the regeneration capability of vegetative tissue collected from *in vitro* shoots established from embryos transformed with the *ESR1* and *PGA6* genes and determine if this capability to regenerate is maintained as the tree ages.
4. To determine the regeneration capability of vegetative tissues collected from *in vitro* shoots established from greenhouse and field grown elite trees when transformed with the *ESR1* and *PGA6* genes under the control of the XVE inducer system.

PROCEDURES

Establishing *Agrobacterium* cultures for transformation: Forty 40 µl of the glycerol stock of *Agrobacterium* strain EHA 105 containing the binary vector pER10-GFP, pDU03-PGA6 or pDU03-ERS1 (see below) was used to inoculate 20 ml of MGL medium (pH 7.0) containing 10 mg/l rifampicin, 5mg/l tetracycline and 20 mg/l spectinomycin sulfate, and incubated overnight

at 28°C at 250 rpms. Five ml of the overnight growth was removed and transferred to 15 ml of TY medium (pH 5.5) containing 10 mg/l rifampicin, 5mg/l tetracycline and 20 mg/l spectinomycin sulfate, 200uM acetosyringone. The culture was incubated overnight at 28°C at 250 rpms. The following morning a dilution was made by adding 1.5 ml of the overnight culture to 20 ml of TY medium (pH 5.5), containing 200 µM acetosyringone to an O.D at 600 nm of 0.075 to 0.20.

1. Efficacy of the XVE inducer system in walnut somatic embryos.

Construction of binary vectors pER10-GFP: We have constructed the binary vectors pER10-GFP in order to test the utility of the XVE system in walnut. The base vector pER10 was obtained from Dr. Nam-Hai Chua (Rockefeller, NY). This particular binary vector is identical to pER8 described in their earlier publication (Zuo *et al*., 2000), except that the selectable marker in pER10 is kanamycin resistance whereas in pER8 it is hygromycin resistance. We have introduced the GFP coding sequences into the MCS (multiple cloning site) region of pER10 downstream from the chemically inducible *cis* acting sequences (LexA) creating the binary vector pER10-GFP ((figure 1). This binary has been introduced into disarmed strains of *Agrobacterium tumefaciens* EHA105 to create a functional plant transformation system.

Plant transformation with binary vector pER10-GFP

a. Tobacco: *Nicotiana tabacum* c.v. SR1 leaf pieces were inoculated with *Agrobacterium* containing the plasmid pER10-GFP and cultured on MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose, 80 mg/l adenine sulfate, 2.0 mg/l kinetin, 2.0 mg/l IAA, 8g/l TC agar, pH 5.8 (MSKI) plus 200 µM acetosyringone,. Cultures were incubated at 23°C in the dark for 48 to 72 hours. Leaves were then transferred to MSKI medium containing 400 mg/l carbenicillin, and 250 mg/l kanamycin sulfate. Cultures were maintained at 26°C and a 16 hour photoperiod. Tissue was subcultured to fresh medium every 21 days. Kanamycin resistant shoots were harvested and transferred to fresh medium and multiple shoots were produced. Clones from transgenic shoots were transferred to fresh medium with or without 5 µM 17-β-estradiol. After 24 hours, leaf tissue was harvested and observed for fluorescence under UV light using a Leica Stereomicroscope and GFP filter.

b. Walnut: Somatic embryos of Chandler and Paradox were obtained from Charles Leslie (Department of Pomology, UC Davis) and subcutlured bi-weekly on agar solidified DKW basal salts containing 20 g/L sucrose, MS vitamins and 1 ml/liter PPM and solidified with 8g/l TC agar (Phtyotechnology Inc.). For transformation, actively growing somatic embryos were harvested and placed in a 100 x 15 mm petri dish with 20 ml of the dilute *Agrobacterium* culture containing the binary vector pER10-GFP and allowed to soak for 5-10 minutes. Embryos were removed and plated onto DKW basal medium containing 20 g/L sucrose, MS vitamins and 200 µM acetosyringone (WCM). Cultures were incubated at 23°C in the dark for 48 to 72 hours. Embryos were then transferred to DKW basal medium containing 20 g/L sucrose, MS vitamins, 500 mg/l cefotaxime, 100 mg/l kanamycin sulfate (WIM) with or without 5 µM 17-β-estradiol. Cultures were maintained in the dark at 26°C. Cultures were subcultured onto fresh medium every 7-14 days. Sixty days post transformation, embryos transformed with pER10-GFP were observed under UV light using a Leica Stereomicroscope and GFP filter. GFP expressing

embryos were harvested and half were transferred to WIM minus 5 µM 17-β-estradiol and half to WIM plus 5 µM, 17-β-estradiol and cultured for further observation. Embryos with the highest GFP expression were converted into shoots.

2. Capability the *ESR1* and *PGA6* gene, under the control of a constitutive promoter and the XVE inducible system, to stimulate the production of transgenic somatic embryos or organogenic shoots for a range of rootstock germplasm.

Construction of binary vector pDU03-PGA6 and pDU03-ERS1: The coding region containing PGA6 was place downstream of the CaMV35S promoter and introduced into pDU99.2215 Similarly ESR1 was also placed downstream of the constitutive CaMV35S promoter. The CaMV35S-PGA6 and the CaMV35S-ESR1 cassette was introduced into pDU99.2215.

Construction of the binary vectors pER10-PGA6 and pER10-ERS1 : Shown in figure 2 are the two binary vectors that have been constructed to investigate the effect of the regeneration genes under the control of a constitutive promoter. These vectors have been obtained and are essentially the same as have been described in the literature. The binary vector pER10-PGA6 was described by Zuo et al., (2002) and should trigger a vegetative to embryo transition when stimulated with 17-β-estradiol. The second binary vector, pER10-ESRI, should trigger shoot formation.

Plant transformation with binary vectors pDU03-PGA6, pDU03-ERS1,pER10-PGA6 and pER10-ERS1:We inoculated into *in vitro* generated walnut stems, roots, leaves and embryos with vectors pDU03-PGA6 and pDU03-ERS1. Tissue was inoculated and maintained as described above.

3. Regeneration capability of vegetative tissue collected from *in vitro* shoots established from embryos transformed with the *ESR1* and *PGA6* genes.

Transgenic embryos expressing the PGA6 or ERS 1 gene were regenerated into plants. Shoot cultures were micropropagated on gelrite solidified DKW basal medium with 20 mg/L sucrose with 1.0 mg.L BAP and 0.1 mg/L IBA. These plants were used to excise various tissues, which were plated on media containing 17-β-estradiol in order to turn on expression of the regeneration genes. (Tables 1 and 4).

Table 1. List of transgenic lines and cytokinin combinations tested with and without the addition of estradiol

Line Number	PCR GOI	PCR nptii	1.0 BA	1.0 TDZ	1.0 CCPU	1.0 2ip	1.0 Kinetin	1.0 Zeatin	1.0 2,4 D/ 0.2 BA
093006-045	positive	positive	x	x	x	x	x	x	x
031053-048	positive	positive	x	x	x	x	x	x	x
093053-019	nt	negative	x	x	x	x	x	x	x
031053-038	negative	positive	x	x	x	x	x	x	x
031160-010	positive		x	x		x		x	x
031160-011	positive		x	x	x	x	x	x	x
031160-022	positive		x	x	x	x	x	x	x
031160-026	positive		x	x	x	x	x	x	x

Table 2. List of transgenic lines and auxin combinations tested with and without the addition of estradiol

Line Number	PCR GOI	PCR nptii	1.0 2,4-D	1.0 2,4,-5T	1.0 FA	1.0 Pic	1.0 NAA	1.0 IBA	1.0 IAA
093006-045	positive	positive	x	x	x	x	x	x	x
031053-048	positive	positive	x	x	x	x	x	x	x
093053-019	nt	negative	x	x	x	x	x	x	x
031053-038	negative	positive	x	x	x	x	x	x	x
									x
031160-010	positive		x	x	x	x	x	x	x
031160-011	positive		x	x	x	x	x	x	x
031160-022	positive		x	x	x	x	x	x	x
031160-026	positive		x	x	x	x	x	x	x

4. Determine the regeneration capability of vegetative tissues collected from *in vitro* shoots established from greenhouse and field grown elite trees when transformed with the *ESR1* and *PGA6* genes under the control of the XVE inducer system.

Once whole walnut plants have been regenerated from somatic embryos transformed with the inducible PGA6 or ERS1 genes in the absence of 17-β-estradiol, we will test various tissue sources for there ability to regenerate once the expression of the regeneration genes is induced through the addition of 17-β-estradiol to the media.

RESULTS AND CONCLUSIONS

Efficacy of the XVE inducer system in walnut somatic embryos

Tobacco: Leaves harvested from cloned shoots of tobacco plants transformed with the plasmid pER10-GFP and cultured on MS medium supplemented with 30 g/l sucrose, 80 mg/l adenine sulfate, 2.0 mg/l kinetin, 2.0 mg/liter IAA, 8g/l TC agar and 5 micromolar 17-β-estradiol, pH 5.8 exhibited GFP expression whereas leaves harvested from cloned shoots grown on media lacking 5 micromolar 17-β-estradiol failed to express GFP (see figure 3).

Walnut: Fourteen weeks post-transformation, kanamycin resistant embryos transformed with pER10-GFP were observed under UV light using a Leica Stereomicroscope and GFP filter. GFP expression was clearly visible in numerous embryos grown on media with 17-β-estradiol while fluorescence was absent in non-transgenic control, embryos or transgenic embryos grown in the absence of GFP (see Figure 4). When GFP expressing embryos were harvested and placed on MS medium containing 5 micromolar 17-β-estradiol they continued to express GFP. However, when GFP expressing embryos were transferred to medium lacking 5 micromolar 17-β-estradiol the expression of GFP dissipated within 7 days. Twenty four hours after returning these non-fluorescing embryos to medium containing 17-β-estradiol, GFP expression was again induced (Figure 5). These results demonstrate that the 17-β-estradiol induction system can be used to turn on and off genes in walnut, which will be critical to controlling the expression of the regeneration genes. We have regenerated whole plants from these embryos and observed GFP expression in regenerated tissue when grown in the presence of 17-β-estradiol. This demonstrates that the XVE system will function at the whole plant level (Figure 6)

Capability the *ESR1* and *PGA6* genes, under the control of a constitutive promoter to stimulate the production of transgenic somatic embryos or organogenic shoots for a range of rootstock germplasm.

Previous work that we have done using another embryo inducing transcription factor (LEC2) in tobacco, tomato, alfalfa and lettuce demonstrated that the response generated by these regeneration genes can be greatly effected by the tissue source and the presence or absence of exogenous hormones particularly 2,4-D. Based on these results we transformed various tissue explants from Paradox walnut on medium that lacked 2,4-D in both the co-culture and induction medium, or media which contained 2,4-D either in the co-culture or induction medium or in both the co-culture and embryo induction medium. Inoculated and non-inoculated somatic embryos, stem and root tissues exposed to 2,4-D in the induction medium or in both the co-cultivation and

induction media developed brown gelatinous callus approximately 60 days after co-cultivation whereas tissues not exposed to 2,4-D produce nodular or friable callus. Numerous sectors of yellow pro-embryonic callus developed on the gelatinous brown callus generated from somatic embryos inoculated with pER10-PGA6 and grown in the presence of 5 micromolar 17-β-estradiol. However the development of this proembryogenic callus does not seem to be the result of the expression of the PGA6 gene or a 2,4-D effect since non-inoculated control tissue yielded a similar callus phenotype.

Capability the *ESR1* and *PGA6* genes, under the XVE inducible system, to stimulate the production of transgenic somatic embryos or organogenic shoots for a range of rootstock germplasm.

Table 3. PCR results on walnut shoots regenerated from Chandler somatic embryos transformed with XVE:ERS-1 and regenerated on 200 mg/L kanamycin sulfate.

ESR1 Walnut pDU03.5101			
	Clone	Cultivar	PCR/ESR1
	Construct 3086		
1	031160-4	Chandler	-
2	031160-10	Chandler	+
3	031160-11	Chandler	+
4	031160-19	Chandler	+
5	031160-22	Chandler	+
6	031160-26	Chandler	+
7	031160-04-2	Chandler	+
8	031161-40	Chandler	+
9	031161-40-2	Chandler	-
10	031160-9	Chandler	+

Table 4. PCR results on walnut shoots regenerated from Chandler somatic embryos transformed with XVE:PGA6 and regenerated on 200 mg/L kanamycin sulfate.

pGA6 Walnut pER10-PGA6			
	Clone	Cultivar	PCR/PGA6
1	Control	Chandler	nd
2	093010-24	Chandler	-
3	093006-28	Chandler	-
4	093006-18	Chandler	nd
5	093006-43	Chandler	-
6	093006-45	Chandler	+
7	093006-58	Chandler	nd
8	093006-61	Chandler	nd

9	031053-7	Chandler	nd
10	031053-8	Chandler	-
11	031053-18	Chandler	nd
12	031053-19	Chandler	nd
13	031053-27	Chandler	+
14	031053-38	Chandler	-
15	031053-34	Chandler	-
16	031053-45	Chandler	nd
17	031053-48	Chandler	+
18	031053-61	Chandler	nd
19	031053-64	Chandler	nd
20	031053-12	Chandler	nd

We have generated a number of transgenic lines for each of the regeneration genes and the presence of the gene has been verified using PCR for a portion of the coding region of *PGA6* or *ESR1* (Table 3 and 4)

Clones of transformed shoots have been established and maintained *in vitro* using nodal sections and were used as a source of tissue for subsequent experiments using the procedures established by Leslie and MacGranahan (1992). We will continue to micropropagate the regenerated shoots *in vitro* in order to maintain enough material for regeneration studies on media containing estradiol.

We are evaluating tissue morphology and regeneration potential for lines containing the *ESR1* or *PGA6* transgenes versus the response elicited from control tissue containing only GFP. This will allow us to determine the influence of *ESR1* or *PGA6* expression on regeneration in the presence of various hormones. We include tissue from control plants containing only GFP in all hormone treatments experiments and monitored for GFP induction in the presence of estradiol to ensure that estradiol was causing gene induction. Vegetative tissues (stems and leaves and petioles) were harvested from the T_0 transgenic walnut plants and plated onto DKW basal plant culture media with supplemental auxin and/or cytokinin treatments (Figure 7). Each hormone treatment included explants plated on media containing or lacking the inducer estradiol. The regeneration genes inserted into these plants will not be expressed in tissue plated on media without estradiol. Therefore, any response we observe will be due only to the hormone tested. In contrast, the regeneration genes inserted into these plants will be expressed in tissue plated on media with estradiol. Therefore, the response we observe in these treatments will be due to both the presence of the hormone and the expression of the regeneration genes.

A summary of our observations for the various hormone treatments to date is explained below. Results were taken after 6 weeks (three subcultures at 2 week intervals).

Summary results from hormone study:

Cytokinins:

BAP-Stimulated multiple shoot development from preformed lateral buds on stem sections, necrotic and green non-differentiated callus developing on leaves and petioles. However, there is no difference between induced and non-induced or GFP control

TDZ- Stimulated fast growing green, nodular non-differentiated callus. However, there is no difference between induced and non-induced or GFP control

CCPU-Stimulated fast growing green, nodular non-differentiated callus. However, there is no difference between induced and non-induced or GFP control

2ip- Stimulated stem elongations with non-differentiated callus along the length of the stem. Non-differentiated callus developing from petioles. However, there is no difference between induced and non-induced or GFP control

Kinetin- Stimulating the development of the most interesting callus of the cytokinins tested. The callus is green, nodular and slow growing callus especially on the petioles. However, there is no difference between induced and non-induced or GFP control

Zeatin- Stimulated elongation of the stem sections . Green nodular callus developing on the stems and leaf explants. However, there is no difference between induced and non-induced or GFP control

Auxins:

2,4-D- We observed more callus development on explants cultured without estradiol and on GFP control tissue then on tissue cultured with estradiol. Some lines exhibited more nodular callus in the presence of estradiol. However, none of the callus appeared embryogenic

2,4,5-T- Explants developed beige non-differentiated callus with no significant difference between induced and non-induced or GFP control

4FA-4 FA stimulated green callus that resembled cytokinin-induced callus. These was no significant difference between induced and non-induced or GFP control

Picloram-Explants developed nodular green callus, but to date none has developed shoots or embryos. We observed no significant difference between induced and non-induced or GFP control.

NAA- In the absence of estradiol or in GFP controls NAA stimulated root formation from leaf and stem tissue. With estradiol, root development was inhibited or reduced.

2,4-D + BAP- This hormone combination caused the formation of rapidly growing non-friable callus. No significant difference between induced and non-induced or GFP control

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Figure 1. The GFP coding sequences introduced into the MCS (multiple cloning site) region of pER10 downstream from the chemically inducible *cis* acting sequences (LexA) creating the binary vectors pER10-GFP

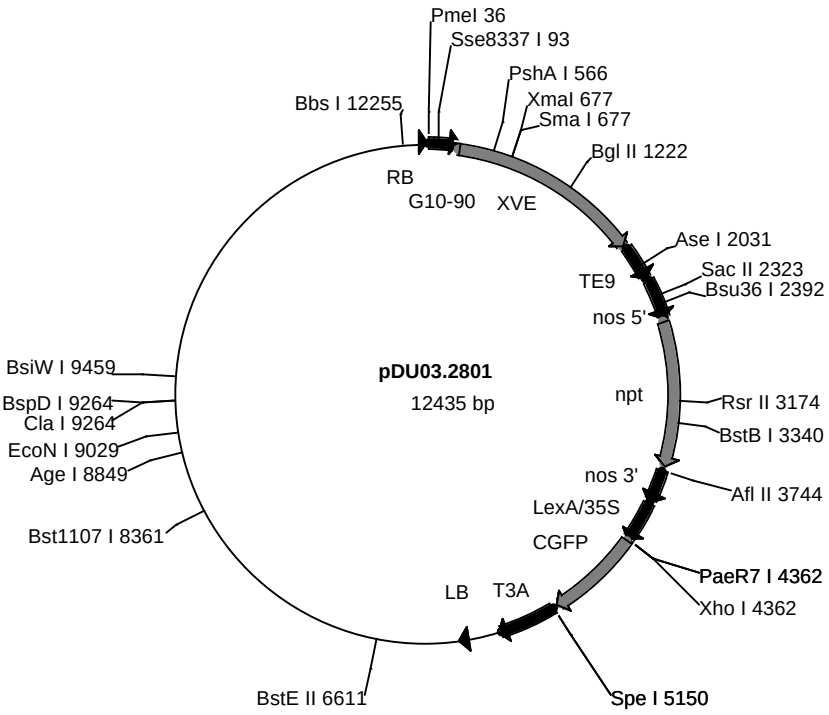
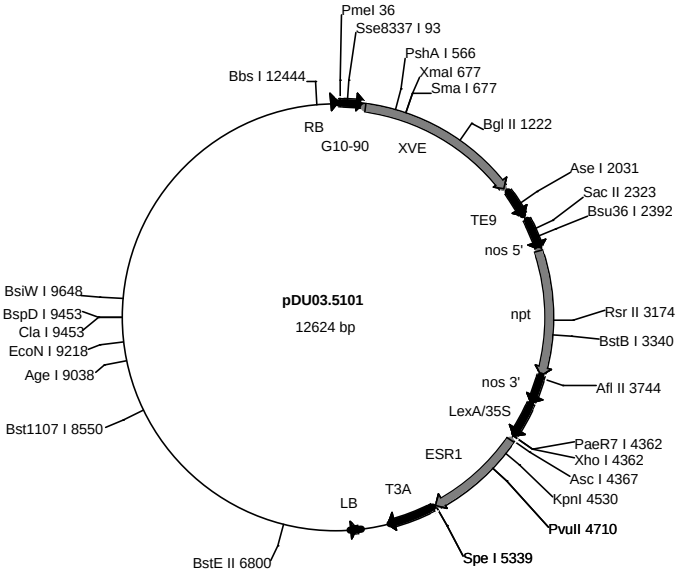


Figure 2. The PGA6 (left) or ESR-1 (right) coding sequences introduced into the MCS (multiple cloning site) region of pER10 downstream from the chemically inducible *cis* acting sequences (LexA) creating the binary vectors pER10-PGA6



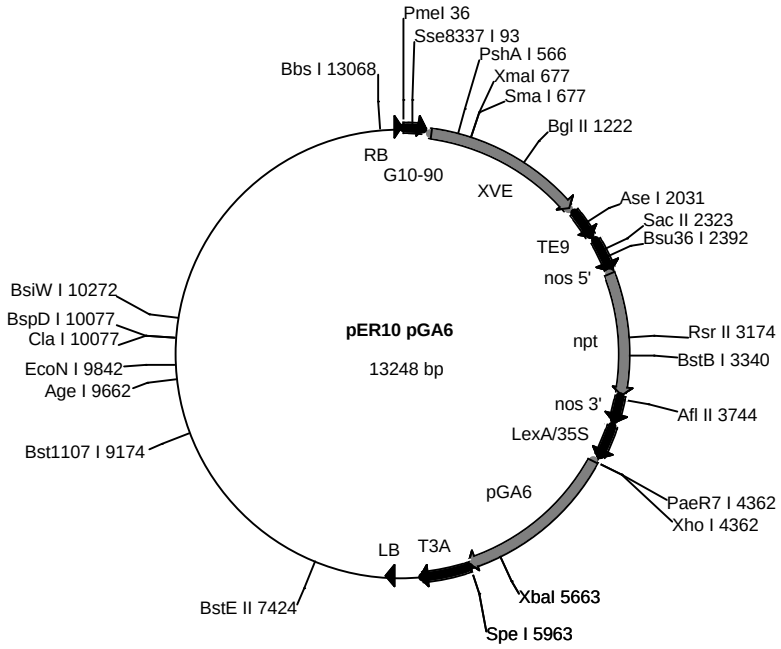


Figure 3. 17-β-estradiol induced GFP expression in transgenic tobacco leaves cultured on MS minimal organics medium supplemented with 500 mg/l cefotaxime, 100 mg/l kanamycin sulfate and 5 micromolar estradiol (right) or without 17-β-estradiol (left).

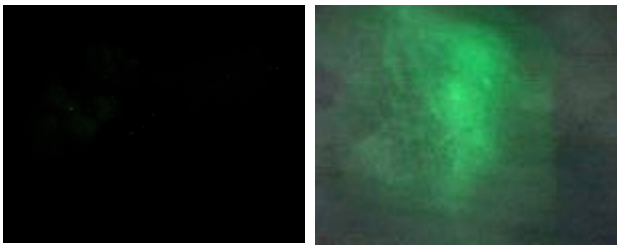


Figure 4. 17-β-estradiol induced GFP expression in transgenic walnut somatic embryos cultured on DKW minimal organics medium supplemented with 500 mg/l cefotaxime, 100 mg/l kanamycin sulfate and 5 micromolar 17-β-estradiol (bright field image to left of Fluorescent image)

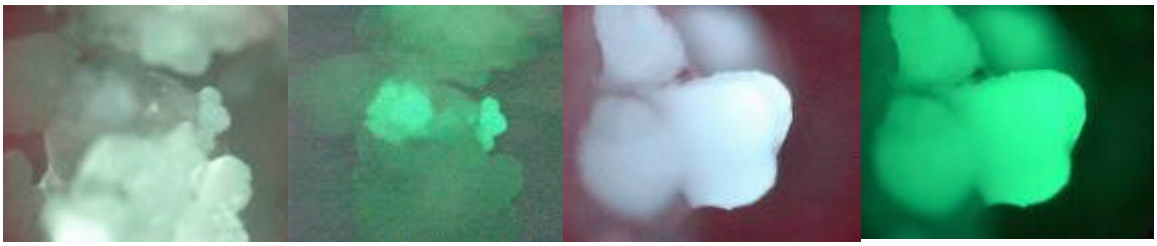


Figure 5. 17-β-estradiol induced GFP expression and subsequent loss of expression in transgenic walnut somatic embryos cultured on DKW minimal organics medium supplemented with 500 mg/l cefotaxime, 100 mg/l kanamycin sulfate with 5 micromolar estradiol and then transferred to the same media with (top row) or without (bottom row) 5 micromolar 17-β-estradiol at 24h, 72 hrs and 7 days, and then 24 hours after subculturing tissue onto fresh media containing 17-β-estradiol (far right top and bottom)

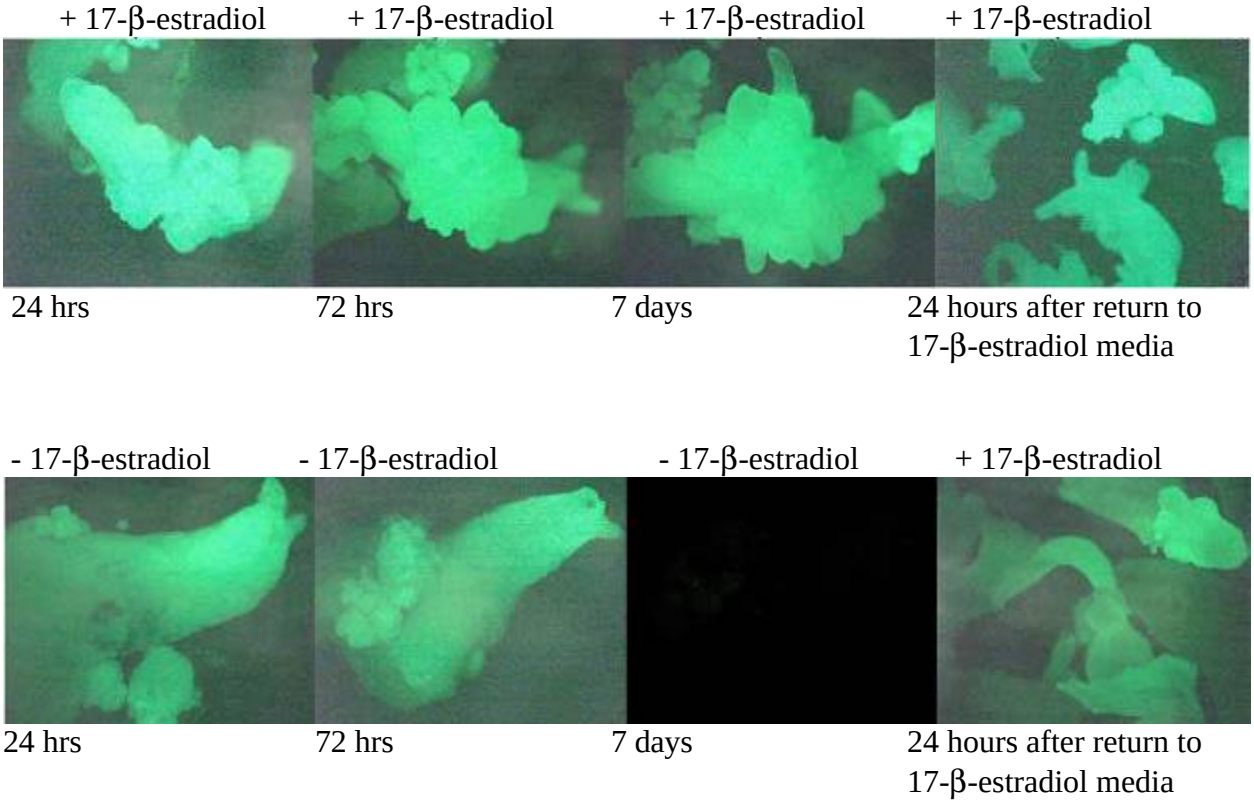


Figure 6. A transgenic walnut shoot regenerated from a transgenic somatic embryo containing the XVE:GFP construct as seen under white light (left). Estradiol induced GFP expression in stem tissue from a regenerated somatic embryo transformed with the XVE:GFP construct (right).

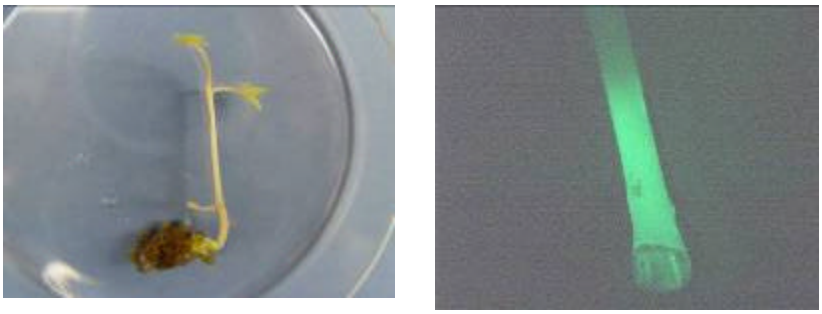


Figure 7. Tissue explants from cloned walnut plants transformed with PGA-6, ESR-1 or GFP under the control of the estradiol inducible promoter