Exploring a new detergent-inducible promoter active in higher plants and its potential biotechnological application

Gretel M. Hunzicker [1], Elmar W. Weiler, and I. Kubigsteltig

Pflanzenphysiologie, Ruhr-Universit?t Bochum, Universit?tsstr. 150, D-44780 Bochum, Germany.
[1] Email grechenm@yahoo.com.ar

Abstract

For the biotechnological use of higher plants, it is necessary to ensure the formation of the desired recombinant product in the right organ at the right time, and in the right cellular location, in large quantities and without exerting toxic effects in the cell. For many purposes, it may be desirable to exert exogenous control over the production of recombinant proteins using chemically controllable promoters. In our group, the promoters of 12-oxophytodienoic acid reductases (OPR1 and OPR2) have been isolated in order to investigate jasmonic acid biosynthesis. Although OPR1 is not the isoform that is important for in vivo jasmonate production, the OPR1-gene has a very sensitive promoter which can be activated by mechanical and chemical inducers (Biesgen and Weiler, 1999; Sanders et al 2000; Schaller et al 2000; Stintzi et al 2000; He and Gan, 2001). One of those chemical inducers is the non-ionic detergent Tween 20. A number of deletions of the *OPR1*-promoter have been designed and tested for their properties and range of applications, using reporter gene analysis (?-glucuronidase) to assay for promoter strengths and characteristics by Northern Blot and GUS fluorometric assays. In order to know which genes are induced by the application of Tween 20, microarray analysis has been done. Promoter functionality will be tested in a range of agricultural plants. The aim of the project is to advance our understanding of application-oriented promoter-design in the biotechnological use of higher plants.

Media summary

Detergent inducible promoters have not been described before. The *OPR1*-promoter is induced after the application of the detergent Tween 20. Biotechnological aspects will be investigated.

Key words

Inducible promoter, detergent, Tween 20, OPR, promoter-GUS-fusion.

Introduction

Many advantages exist for the use of plants in biotechnology over other organisms like animals, mammalian cell cultures, bacteria or yeast. Transgenic plants have some remarkable features that make them particularly well suited for cost-effective bioproduction of proteins for pharmaceutical uses (Cramer et al. 1999). These include low production cost, low capital cost, low risk of carrying human pathogens or endotoxins and relatively high protein yield. Additional advantages are excellent scalability, eukaryotic post translational modifications, relatively fast gene-to-protein time and high biomass production (Raskin et al. 2002). For the biotechnological use of higher plants, it is necessary to be very careful in the selection of a particular plant species. It is important to consider how readily it can be manipulated to produce a stable transgenic line, the tissue and subcellular compartment best suited for stable expression of the heterologus protein and the availability of methods for the initial processing of the plant material. The product should be formed in large quantities, it should be easy to purify in fully functional form and should remain intact during storage time (Cramer et al. 1999; Twyman R.M et al. 2003; Fischer et al 2004). Choice of promoter, which mediates the timing, tissue-specificity, and level of transgene expression, is a key to transgene product yield and recovery strategies (Chinn and Comai 1996). The expression level of the promoter should be close to zero in the absence of an inducer. Its is important that the chemical used is highly specific for the target promoter. It should neither influence the expression of other genes nor affect other cell functions. Uptake is another important issue: once it is applied, either by foliar spray or root drenching, the chemical should enter every cell (Gatz and Lenk 1998). Chemicals that

are used to regulate transgene expression include the antibiotic tetracycline, the steroids dexamethasone and estradiol, copper, ethanol, the inducer of pathogen-related proteins, benzothiadiazol, herbicide safeners, and the insecticide methoxyfenozide (Padidan, 2003). Until now nothing has been published about detergents as chemical inducers. Moreover, they are widely used in experimental assays without knowing how the plant is responding in their presence. The OPR1 gene is located on chromosome I, it has been cloned as a 7079-bp-long genomic fragment from *Arabidopsis thaliana*. Its specific function is not well known but its gene activity is related to the senescence stage (Biesgen and Weiler, 1999; He and Gang, 2001). The *OPR1*-promoter is induced by Tween 20 (C_{12}-sorbitan-E_{20};Polysorbate 20) detergent (Biesgen, 1999). This detergent is a very cheap and biodegradable detergent that can be easily diluted in water and applied by foliar spraying or root drenching. It has been used as emulsifying agent for the preparation of stable oil-water emulsions, in pre-extractions of membranes to remove peripheral proteins, and for lysing mammalian cells. The specificity of gene expression depends on cis-elements present in the promoter and enhancer regions and their interaction with specific transcription factors (Zhou, 1999). In the present work, we try to identify the detergent inducible cis–element present in the *OPR1*-promoter, fuse it with a minimal promoter to produce a chemically inducible chimeric promoter able to function in crops.

Methods

Growth conditions and plant treatment

Arabidopsis thaliana (L.), strain Columbia (Col-0) was grown on soil in a greenhouse with an 8 h photoperiod for 3-4 weeks. Seeds were sterilized 2 times in 70 % ethanol for 1 minute, once in 5 % (v/v) sodiumhypochloride, rinsed with 3 changes of sterile distilled water for 10 minutes, and stored during 72 h in 0.3 % agar at 4 ?C. Plants grown under sterile condition were sown on MS medium (Murashige and Skoog 1962) supplemented with or without the appropriate herbicide and vitamins, with 1 % (w/v) sucrose and 0.8 % agar in a growth chamber with 16 h photoperiod (24 ?C at day/20 ?C at night). The growing time was 22 days. Heterozygous transgenic plants with the *OPR1*-promoter 5?-deletions (pCBi67 –2498 bp, pCBi68 –2170 bp, pCBi69 –2079 bp, pCBi70 –1661 bp, pCBi71 –1103 bp, pCBi72 –713 bp, pCBi73 –227 bp, pCBi74 –47 bp) fused whit the *uidA*-gene and the *bar*-gene were provided by C. Biesgen (Ruhr-Universit?t Bochum). New deletions of the *OPR1*-promoter were performed by PCR with the following primers: S71OPR1 5?-AAG CTT ATC TCC AAC ACA TTG TCACA-3?; A72OPR1 5?-TCTA GAT GTT TAT GTT TAT GTT ATG AGC CCA GT-3?and A73OPR1 5?-TCT AGA GTT CCT TCC TAT CAT CTA CAGT-3?. The PCR products were cloned into the multiple cloning vector pGEM-T (Promega Corp, Madison, WI). Afterwards, the PCR products were fused to the T-DNA vector pCBI74 (Biesgen, 1999), used as a minimal promoter which contains the TATA box, the 5?-unstranslated region, and the first 114 bp of the *OPR1*-promoter. As a selectable marker this construct has the *bar*-gene and as a reporter gene, the *uidA*-gene. GUS assays in transgenic plants grown under sterile conditions were performed histochemically with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) as the substrate and quantitatively with 4-methylumbelliferyl-β-D glucuronide (MUG), as described by Jefferson (1987).

Northern blot experiments

Plant RNA was isolated according to Barkan (1989). The harvested plant material was 1 g and for each sample, 15 ?g RNA was size-fractionated on denaturating formaldehyde agarose gels 1.8 % (w/v) and blotted onto nylon membranes. Ethidium bromide was used for staining the labeled nucleic acid probes, and RNA blot analysis was done following standard procedures (Sambrook et al. 1989). The probes used were *OPR1*-cDNA. It is important to note that due to high sequence similarity, it is not possible to distinguish between *OPR1* and *OPR2* genes.

Results and Discussion

The OPR1-promoter is induced after detergent spraying

In order to corroborate results from Biesgen (1999) and to detect whether the detergent induction takes place, *Arabidopsis thaliana* wild type plants were sprayed with 0.2 % Tween detergent (fig.1 C, D). The

induction caused by the detergent is maximal after 2 h and disappears completely between 4 h and 6 h after the treatment. To confirm that the detergent is the only responsible factor for the induction of the *OPR1*-promoter in the spraying procedure, controls were sprayed with water (fig.1 A) and air (fig.1 B). No *OPR1*-induction detected after water or air spraying during three hours. There were no adverse effects seen in the plant after detergent application, and treated plants were able to generate viable seeds (data no shown).

Promoter activity

Homozygous transgenic lines containing the above deletions were tested in order to define the putative cis-element location. Control plants were sprayed with water, and the samples with 0.2 % Tween detergent, (fig. 2 A). The 67 (-2498 bp), 68 (-2170 bp), 69 (-2079 bp), 70 (-1661 bp) transgenic plants lines (Figure 2 A) show a very high background, and the induction of the promoter caused by detergent is weak. This can be explained by the presence of one unique enhancer (DNA sequence that inc reases the expression of genes in its vicinity) which is active also in the control situation.

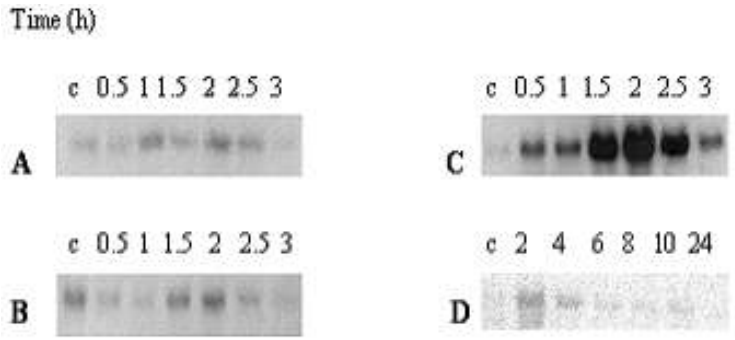

Figure 1. RNA blot analysis of total RNA (15 µg per lane) isolated from *Arabidopsis thaliana* wild type (Col-0). Plant controls (c) were taken before the induction. The samples were harvest every 0.5 h in A) air, B) distilled water, C) 0.2% (w/v) Tween 20 detergent, after spraying in order to detect whether the detergent induction takes place. To detect when the detergent induction disappears, D) 0.2 % (w/v) Tween 20 detergent was sprayed and the samples were collected every 2 h and at 24 h. The probes used were labeled *OPR1*-cDNA. Note that the analysis does not discriminate between *OPR1* and *OPR2* due to high sequence similarity between them.

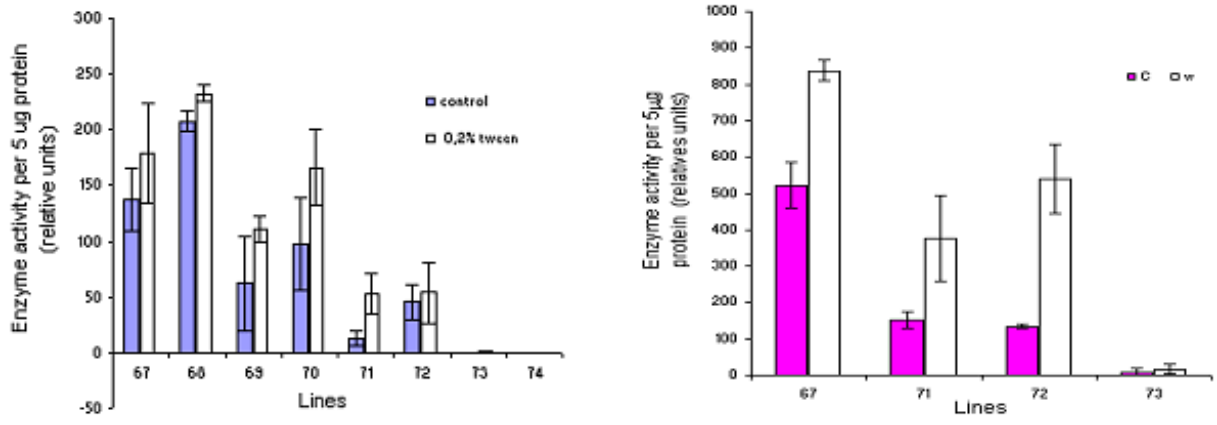

Figure 2. GUS activities in leaves of homozygous plants (22-days old, grown in sterile condition) containing 5?-deletions (pCBi67 –2498 bp, pCBi68 –2170 bp, pCBi69 –2079 bp, pCBi70 –1661 bp, pCBi71 –1103 bp, pCBi72 –713 bp, pCBi73 –227 bp, pCBi74 -47 bp). A) GUS activities per hour after treatment with detergent (0.2 % Tween 20) or control solution (water); B) GUS activities per

hour after wounding with a hemostat. The samples were harvested 2.5 hours after the treatment. The activity shown is the mean of A) 2 independent experiments with 2 independent samples each B) 3 independent experiments with 3 independent samples each. GUS activity was determined fluorometrically using 4-MUG as substrate. Error bars indicate the standard error of the mean.

The line 71 (-1103 bp) has the lowest background and in this line, the inducibility (difference between control and induction amounts) is the highest found in all the lines. Therefore, we suspect that in the 71 line, there might be a repressor protein which is released after the detergent application. This would fit with the results from line 72 (-713 bp), where we have nearly comparable promoter activity in the control and in the induced state. Therefore, new deletions were created in the OPR1-promoter between lines 71 (-1103 bp) and 72 (-713 bp) and fused to a minimal promoter. These plants will be tested in the near future (data not shown).Wounding assays were performed with the lines 67 (-2498 bp), 71 (-1103 bp), 72 (-713 bp) and 73 (-227 bp) (fig. 2, B) to know whether there is a wound-responsible cis-element located in the same DNA-region as the detergent element. The lines 67, 71 and 72 are wound-inducible. It is possible that both effects (wounding and detergent) are mediated by the same cis-element. In this case, the detergent is causing a mechanical damage which activates a wounding response. The 73 line is not induced, it shows nearly no GUS-activity.

References

Barkan A (1989). Tissue-dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. Plant Cell. 1, 437-445.

Biesgen (1999). Charakterisierung der Expression und Funktion von 12-Oxophytodiens?ure-Reduktasen. Dissertation, Bochum Germany, 102 p.

Biesgen C, Weiler EW (1999). Structure and regulation of *OPR1* and *OPR2*, two closely related genes encoding 12-oxophytodienoic acid-10,11-reductases form *Arabidopsis thaliana.* Planta. 208, 155-165.

Chinn AM, Comai L (1996). Gene transcription. In: Owen MRL, Pen J (eds) Transgenic plants: a production system for industrial and pharmaceutical proteins. Wiley, Chichester, 27-48 p.

Cramer CL, Boothe JG, Oishi KK (1999).Transgenic Plants for therapeutic Proteins: linking upstream and downstream strategies. Curr. Top. Microbiol. Immunol. 240, 95-118.

Fischer R, Stoger E, Schillberg S, Christou P, Twyman MR (2004). Plant-based production of biopharmaceuticals. Curr. Opin. Plant Biol. 7, 152-158.

Gatz C, Lenk I (1998). Promoters that respond to chemical inducers. Trends in Plants Science. 3, 352-358.

He Y, Gan S (2001). Identical promoter elements are involved in regulation of the *OPR1* gene by senescence and jasmonic acid in *Arabidopsis.* Plant Mol. Biol. 47, 595-605.

Jefferson. RA (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. 5, 387-405.

Ma JKC (2003).The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. 4, 794-805.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Padidam M (2003). Chemically regulated gene expression in plants. Curr. Opin. Plant Biol. 6, 169-177.

Raskin L, Ribnicky DM, Komarnytsky S, Llic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O?Neal JM, Cornwell T, Pastor I, Fridlender B (2002). Plants and human health in the twenty-first century. TIBTECH. 20, 522-531.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York.

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB (2000). Plant Cell. 12, 1041-1061.

Schaller F, Biesgen C, Mussig C, Altmann T, Weiler EW (2000). *Planta* 210, 979-984

Stintzi A, Browse J (2000). *Proc.* Natl. Acad. Sci. *USA* 97, 10625-10630.

Twyman, Stoger E, Schillberg S, Christou P, Fischer R (2003). Molecular farming in plants: host systems and expression technology. TIBTECH. 21, 570-578.

Zou DX (1999). Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. Trends in Plant Science. 4, 210-214.