Inducible DNA Promoters for Use in Apple

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Abstract

A chemically inducible and a light inducible DNA promoter are being developed and evaluated for their ability to regulate gene expression in transgenic apple. The estradiol-induced XVE gene expression system was cloned into a binary vector (pBinPlusARS) compatible for use in Agrobacterium-mediated transformation of apple. To evaluate the estradiol-inducible binary vectors, a GUS marker gene was cloned into pBinPlusARS.XVE and pBinPlusARS.XVE.Gateway. When evaluated in tobacco under non-inducing conditions, GUS expression from the GUS containing XVE constructs was indistinguishable from that of an empty vector (no GUS coding region) negative control. However, in the presence of estradiol, GUS expression from the GUS containing XVE constructs was greater than that from a 35S promoter positive control. To evaluate the activity of the light inducible promoter of the peach chlorophyll a/b binding (CAB) protein and XVE in transgenic apple, binary vectors were constructed to allow comparison of test promoter activity with 35S activity.

INTRODUCTION

Genomic research and genetic engineering have tremendous potential to enhance crop performance, improve food quality and increase farm profits. However, significant social and scientific constraints remain before transgenic technologies can become a reality in the orchard. Among these are consumer concerns regarding the safety of transgenics and the impact this may have on marketing.

The research reported here was part of a United States - Israel Binational Agricultural Research and Development Fund (BARD) project to regulate expression of site-specific DNA recombinases in apple in order to eliminate kanamycin-resistance transgenes (nptII) from transgenic apple. Site-specific recombination has previously been shown to function in plants and can be used to mediate gene excision in plants (Dale and Ow, 1990; Onouchi et al., 1991; Lyznik et al., 1993). Excision of the nptII transgene results from the interaction between a recombinase protein and its DNA recognition sequence placed at each end of the targeted gene (Gleave et al., 1999; McCormac et al., 1999; Luo et al., 2000; Sugita et al., 2000). Because most perennial fruit crops have a long generation time and are asexually propagated, we aimed to develop non-sexual methods for gene excision through the controlled expression of recombinase protein from inducible promoters. The goal of the research reported here was to evaluate inducible promoters for their ability to control gene expression in apple. The promoters evaluated included a light inducible promoter (chlorophyll a/b binding protein of peach) and a chemically inducible promoter (estradiol-induced XVE) (Zou et al., 2000).

The estradiol-induced XVE gene expression system is regulated by a chimeric transcription factor (activator) containing the DNA-binding domain of LexA from E. coli, the herpes simplex virus VP-16 activation domain and the regulatory region of the human estrogen receptor. In the presence of estradiol this transcription factor activates expression
from its target DNA promoter containing multiple copies of the LexA operator fused upstream to a minimal CaMV 35S promoter (Zou et al., 2000).

MATERIALS AND METHODS

The chimeric XVE transcription factor and its corresponding inducible OLexA-46 promoter were cloned from pER8 (Zou et al., 2000) into the pBinPlusARS binary vector which is compatible for use in Agrobacterium-mediated transformation of apple (Fig. 1). A 2.144 kb DNA fragment containing XVE was PCR amplified from pER8 using primers XVE.F.KpnI and XVE.R.KpnI that included KpnI sites (Table 1). The resulting PCR fragment was cloned into the KpnI site of pUCAP to yield pUCAP.XVE. pER8 was digested with EcoRI and BspEII, and the ca. 1.325 kb OlexA-46/MC5/T3A region of pER8 (3419-4744 bp) was cloned into the EcoRI/XmaI site of pBinPlusARS to yield pBinPlusARS.OLEX. The KpnI fragment of pUCAP.XVE containing the coding region of the chimeric XVE transcription factor was then cloned into the KpnI site of pBinPlusARS.Olex to yield pBinPlusARS.XVE (Fig. 1). An intron-containing GUS marker gene (Vancanneyt et al., 1990) was then cloned into pBinPlusARS.XVE by conventional PCR cloning and via the pCR2.1-TOPO (Invitrogen) vector intermediate. For pBinPlusARS.XVE.GUS-int.PCR, the GUS-int coding region was PCR amplified from pCAPGUS35S using primers F2.XhoI and R6.SpeI (Table 1), digested with XhoI and SpeI, and ligated into pBinPlusARS.XVE. For pBinPlusARS.XVE.GUS-int.TOPOinframe and pBinPlusARS.XVE.GUS-int.TOPOoutframe, the GUS-int coding region was PCR amplified from pCAPGUS35S using primers Topoinframe.F2 and Topoinframe.R2, and Topooutframe.F4 and Topooutframe.R5 (Table 1), respectively, and cloned into pCR2.1-TOPO (Invitrogen); the resulting plasmid was digested with XhoI and SpeI, the GUS-int containing-fragment was gel purified and ligated into pBinPlusARS.XVE.

pBinPlusARS.XVE was also converted to a GATEWAY destination vector by inserting a GATEWAY cassette into the multiple cloning site downstream of the OLexA-46 promoter (Fig. 1). pBinPlusARS.XVE was digested with SpeI and treated with Pfu DNA polymerase to create blunt ends. The Gateway Vector Conversion System Reading Frame Cassette A was inserted into pBinPlusARS.XVE by blunt end ligation to yield pBinPlusARS.XVE.gateway. The GUS-int coding region was PCR amplified from pCAPGUS35S using primers F2directional and Topoinframe.R2 (Table 1), cloned into pENTR/D-TOPO (Invitrogen), and then recombined into pBinPlusARS.XVE.gateway using LR Clonase (Invitrogen) to yield pBinPlusARS.XVE.gateway.GUS-int.

All constructs were confirmed by DNA sequencing.

A promoter-evaluation (test) vector was constructed to allow evaluation of promoter activity in transgenic plants relative to a standard 35S promoter (Fig. 2). The erGFP6 INT green fluorescent protein gene, specifically modified for expression in transgenic plants (Mankin and Thompson, 2001), was cloned into the HindIII site of pCAMBIA2301 to yield pCAMBIAgfp94. The 35S promoter (control), the chlorophyll a/b binding protein promoter of peach, and estradiol-inducible XVE promoter were cloned into pCAMBIAgfp94 (Fig. 2). Test vectors were transformed into apple and the promoters are currently being evaluated in apple.

RESULTS AND DISCUSSION

A GUS marker gene was cloned into pBinPlusARS.XVE using different technologies including PCR cloning, GATEWAY (Invitrogen) recombination, and cloning from a TOPO cloning (Invitrogen) vector where the insert was first cloned in 2 different reading frames (the native AUG translation initiation codon in-frame and out-of-frame with an upstream AUG in the 5' leader). When evaluated in tobacco under non-inducing conditions, GUS expression from GUS-containing XVE transgenics was indistinguishable from that of empty pBinPlusARS vector transgenic (negative control). However, in the presence of 1 µM estradiol, transgenics containing the PCR and GATEWAY derived vectors had significantly higher levels of GUS expression than transgenics containing...
either of the TOPO cloned vectors or the negative control. The TOPO cloned vectors contained an upstream AUG carried over from the TOPO-cloning intermediate (pCR2.1-TOPO) that is in a favorable context to initiate translation in eukaryotes (Fig. 2). No expression was detected from the transgenics containing the out-of-frame TOPO cloned vector under inducing conditions. This was most likely due to the initiation of translation from the AUG in the 5′ leader upstream of the valid translation start codon (Kozak, 2002). Surprisingly, a lower level of expression was also observed from the transgenics containing the in-frame TOPO cloned vector under inducing conditions. This may have been due to a negative effect of the additional amino-acids on GUS expression or a repression of translation caused by the two proximal 5′ AUGs.

To evaluate the activity of peach chlorophyll a/b binding protein gene-promoter and XVE in transgenic apple, binary vectors were constructed to allow comparison of test promoter activity with 35S activity (Fig. 3). Using pCAMBIA2301, vectors were constructed with test promoters driving expression of green fluorescent protein (ERGFP6<sup>GUS</sup>) and 35S driving expression of GUS (Fig. 3). Test vectors were transformed into apple and the promoters are currently being evaluated in apple.

**ACKNOWLEDGEMENTS**

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**Literature Cited**


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**Tables**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>F2directional</td>
<td>5'-CACCATGGTCGGTCTCTGTAGAAAC-3'</td>
</tr>
<tr>
<td>F2.XhoI</td>
<td>5'-GCCTGCCTCGAGACCATGGTCGGTCTCTGTAGAAA-3'</td>
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<td>R6.SpeI</td>
<td>5'-CATAGAACTAGTTCCCGAGGTGTGATCTG-3'</td>
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<td>Topoinframe.R2</td>
<td>5'-GTGCCACGGAGAGTGGTTGATTT-3'</td>
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<tr>
<td>Topooutframe.F4</td>
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<tr>
<td>XVE.R.KpnI</td>
<td>5'-ATCAGATTGTGGTACCCCGCTTCACTGTTTC-3'</td>
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Fig. 1. The binary plasmid vector pBinPlusARS.XVE for estradiol-inducible gene expression in apple. PG10-90 = synthetic promoter controlling expression of XVE (Ishige et al., 1999), LexA = DNA-binding domain of LexA (X), VP16 = transcription activation domain of VP-16 (V), hER = regulatory region of human estrogen receptor, Te9 = rbcS E9 poly(A) sequence, OLexA-46 = XVE target promoter containing multiple copies of the LexA operator fused upstream to a minimal CaMV 35S promoter, MCS = multiple cloning site, T3A = rbscS3A poly(A) sequence, LB = T-DNA left border, RB = T-DNA right border.

Fig. 2. The DNA 5’ leader sequences upstream of the AUG translation start for the GUS coding region in A) pBinPlusARS.XVE.GUS-int.PCR, B) pBinPlusARS.XVE.GUS-int.TOPOinframe and C) pBinPlusARS.XVE.GUS-int.TOPOoutframe. The binary vectors constructed via a TOPO cloning intermediate contain a translation initiation AUG in a favorable eukaryotic context [a purine (G or A) in the -3 position and a G in the +4 position] upstream of the valid AUG start. The illustration of all sequences starts at the pBinPlusARS.XVE cloning site downstream of the inducible OLexA-46 promoter.
Fig. 3. pCAMBIAgfp94 is a promoter-evaluation (test) vector constructed to allow comparison of gfp expression in transgenic plants from test promoters under inducing and non-inducing conditions relative to GUS expression from a standard 35S promoter. pCAMBIAcab19gfp94 contains the light-inducible chlorophyll a/b binding protein promoter of peach controlling expression of the erGFP\textsuperscript{INT} gene (GFP).