pPE1000: A Versatile Vector for the Expression of Epitope-Tagged Foreign Proteins in Transgenic Plants

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Epitope tagging can enable the immunological surveillance of foreign proteins expressed in transgenic plants, thus avoiding the need to raise antibodies specific to each protein. In the course of our research, we have expressed a number of genes encoding proteins rich in sulfur-containing amino acids, in transgenic tobacco and in white clover. We discovered that there were problems associated with monitoring the levels of some of these foreign proteins in transgenic plants. For example, the pea seed storage protein PA1 (2) is a poor immunogen, and the antibodies raised against this protein are low in titer and avidity. This results in unreliable immunological detection and quantification of the protein in transgenic plants. Our findings also confirm other published results indicating that the expression of foreign monocot or dicot genes encoding seed proteins, in the leaves of transgenic plants (such as tobacco, petunia, lucerne and clover), often result in low levels of foreign protein accumulation (6). This increases the requirement for a superior antigenic monitoring system in order to enhance the probability of detecting low levels of the foreign protein.

To overcome these difficulties, we have devised a specialized cassette-vector allowing the in-frame N- or C-terminal fusion of a polymerase chain reaction (PCR)-amplified mature protein coding sequence from any gene to a sequence encoding an epitope of the influenza hemagglutanin α-chain. This plant expression cassette, pPE1000, was constructed using the pGEM-9Zf derivative pART7 (4) as the parent vector and includes a duplicated CaMV 35S promoter (Cabb B-JI isolate) from the vector PJIT166 (a gift from P. Mullineaux, John Innes Science Centre) and the 3′ transcription terminator from the nopaline synthase (*NOS*) gene subcloned from the vector pCaGUS (a gift from V. Walbot, Department of Biological Science, Stanford University). An 87-bp oligonucleotide 5′-GCGAAGCTTGAATTCACCATGTATCCATACGATTGTTCCAGATTATGCTTGGTGGTCCAGCAAGCTGCAATGG-G,-3′ was synthesized on the Model 391 DNA Synthesizer (PE Applied Biosystems, Foster City, CA, USA), amplified by PCR and cloned as an *Nco*I-*Eco*RI fragment into the

2X35S/NOS 3′ cassette.

The expression cassette-vector is shown in Figure 1 and includes a Kozac's positive translation consensus start codon and 5′ and 3′ polylinkers flanking an open reading frame (ORF) encoding the minimal HA1 epitope YPYDVPDYA (3). This nine-amino-acid epitope tag has proven sufficient for N- and C-terminal tagging of recombinant proteins in mammalian systems (8). Restriction sites flanking the 2X35S/EPITOPE/NOS 3′ cassette allow excision with either *Not*I for subcloning into the binary vector pART27 (4) or *Kpn*I for subcloning into the binary vector pRD400 (1). There are also unique *Sac*I and *Xho*I sites for either directional subcloning or insertion of other plant transgenes before subcloning.

Any gene sequence cloned beyond the *Sma*I site of pPE1000 also allows the possibility of excision with *Sma*I-*Eco*RI for subcloning into the *Xmn*I-*Eco*RI sites of the maltose-binding-protein fusion vector pMAL system (New England Biolabs, Beverly, MA, USA). This has permitted us to over-express the recombinant epitope-tagged PA1 (2) and the maize zein and rice oryzin proteins in *E. coli* (unpublished).

To demonstrate the effectiveness and function of pPE1000, the β-glucuronidase reporter gene (*gusA*) was cloned into the vector to give three variants: pPE117B (*gus* alone), pPE120 (*epi-gus*) and pPE121 (*gus-epi*). The 3 cassettes were subsequently sub-cloned into the *Kpn*I site of pRD400 and introduced into the tobacco cultivar W38 by *Agrobacterium*-mediated transformation (7). Kanamycin-resistant plants were regenerated, and several transgenic plants from each construct were investigated for the expression of the *GUS* transgene.

Histochemical staining using X-GLUC identified the activity and tissue localization of the β-glucuronidase enzyme in all 3 groups of plants, and the GUS activity was detectable in both root and leaf tissue at varying levels, indicating that the *gus* gene was functioning alone as well as with the epitope fused to the N or C terminus of the gene. Seeds collected from a number of the primary transformants with high GUS expression levels also showed GUS activity in leaf and root tissue

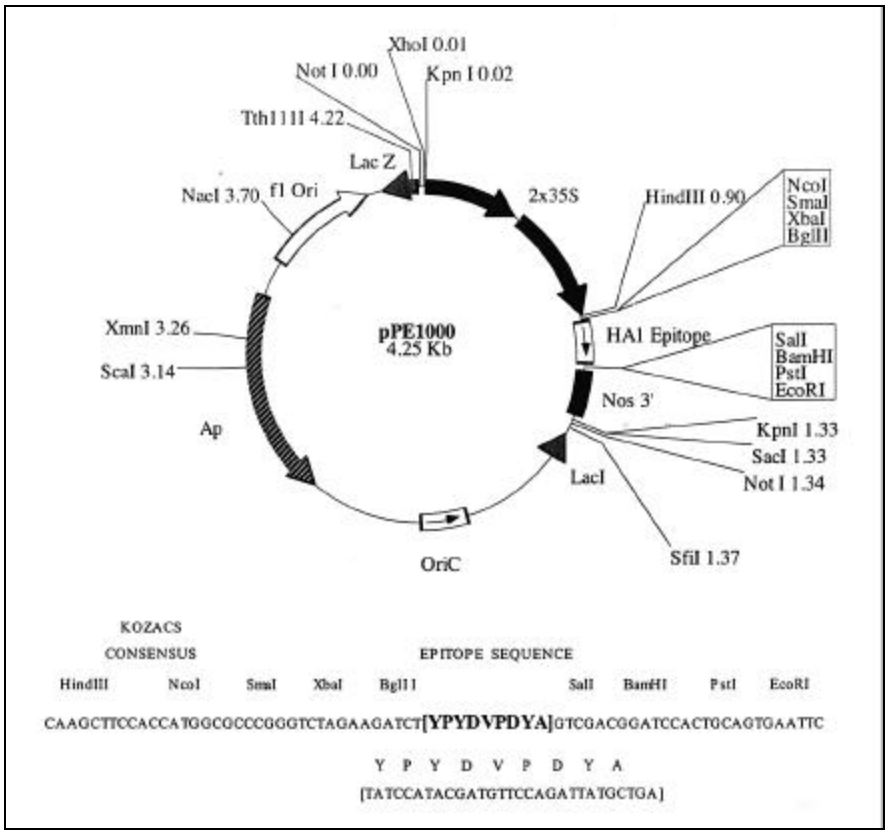

Figure 1. Restriction map of plasmid pPE1000.

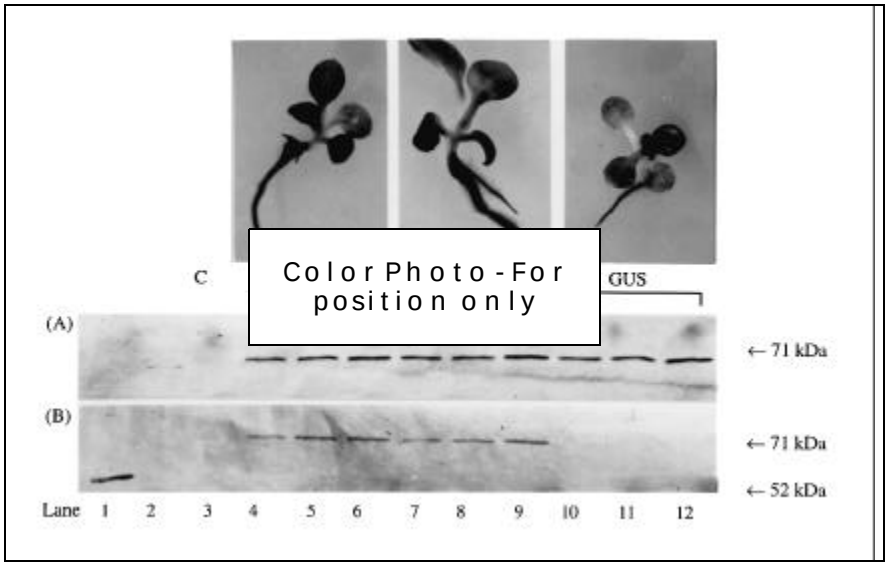

Figure 2. The histochemical localization of GUS activity and the immunological detection of transgene fusion proteins in GUS, GUSEPI and EPIGUS transgenic tobacco plants and leaf cell extracts transformed with the constructs pPE117B, pPE120 or pPE121. (A) Western blotting using β-glucuronidase antiserum. GUS was detected in all 3 series in 3 different high expressors (lanes 4–12) and also control GUS protein (lane 2). No cross-reaction was detected in control tobacco (lane 3) or with malE: EPIPA1 containing the epitope tag (lane 1). (B) Western blotting using HA1 monoclonal antibody. HA1 protein was detected only in 2 series corresponding to pPE120 (GUSEPI) and pPE121 (EPIGUS) but not in plants containing pPE117B (GUS only) or the control plant (lane 3). There was no cross-reaction with control GUS protein supplied with the GUS antiserum (lane 2), but there was a reaction with malE:EPIPA1 protein (lane 1). (C) Histochemical localization of Gus activity in F2 transgenic tobacco plants expressing the Gus fusion proteins shown below.

upon germination (Figure 2).

The presence of the GUS and epitope fusion proteins in transgenic plants was then investigated by Western blot analysis. Total soluble protein was extracted from the leaves of 3 high expressors in each group and then electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (2). A 71-kDa protein was identified by Western blotting using β-glucuronidase-specific antibodies in protein extracts from the GUS, EPIGUS and GUSEPI transgenic plants (Figure 2A). Western blotting of a duplicate gel using HA1-specific monoclonal antibodies (BAbCO, Richmond, CA, USA) also identified a 71-kDa protein in extracts from the GUSEPI and EPIGUS transgenic plants, confirming that these proteins also contained the non-apeptide epitope sequence (Figure 2B).

We have already used pPE1000 to N-terminally tag a PCR fragment comprising the mature proprotein coding region of the pea albumin 1 gene (*PA1*), resulting in increased Western blot sensitivity and enabling determination of recombinant protein levels to below 0.001% total cell protein (2). Furthermore, the S-rich proteins rice oryzin (10 kDa), maize zein (10 kDa) and a 3.2-kDa synthetic pumpkin trypsin inhibitor I (5) have all been cloned into this cassette vector, resulting in the non-apeptide epitope tag fused to the N or C terminus of these genes (unpublished data). This has allowed facile detection of all three foreign proteins in transgenic plants, by the use of a single antibody, streamlining the analysis of a large number of plants.

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