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The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants

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Abstract The pEAQ vectors are a series of plasmids designed to allow easy and quick production of recombinant proteins in plants. Their main feature is the use of the Cowpea Mosaic Virus *hypertranslational* "CPMV-*HT*" expression system, which provides high yields of recombinant protein through extremely high translational efficiency without the need for viral replication. Since their creation, the pEAQ vectors have been used to produce a wide variety of proteins in plants. Viral proteins and Virus-Like Particles (VLPs) have been of particular interest, but other types of proteins including active enzymes have also been expressed. While the pEAQ vectors have mostly been used in a transient expression context, through agroinfiltration of leaves, they have also been shown to be suitable for the production of stably transformed lines of both cell cultures and whole plants. This paper looks back on the genesis of the pEAQ vectors and reviews their use so far.

Keywords pEAQ · CPMV-*HT* · Virus-Like Particles · Recombinant protein

Introduction

The use of plants as expression systems for the production of recombinant proteins has emerged as an attractive alternative to bacterial, yeast and animal cell production systems. Producing proteins in plants potentially has advantages in terms of cost and also safety as plant pathogens do not infect mammals including humans, thereby reducing contamination risks when pharmaceutical proteins are produced (Kusnadi et al. 1997; Lico et al. 2008). Moreover, certain plants such as *Nicotiana benthamiana* can be grown in high density and still produce large amounts of biomass in a matter of weeks. There are essentially two approaches to producing heterologous proteins in plants—stable transformation (either nuclear or plastid) and transient expression. Stable transformation involves the production of true-breeding lines of genetically transformed plants. This approach has advantages in terms of reproducibility and potential large-scale production; however it is often very time-consuming and is unsuitable for the rapid screening of a wide variety of different constructs (Kusnadi et al. 1997). Transient expression in plants allows for production of high titres of recombinant proteins in a matter of days in what is essentially a batch process (Pogue et al. 2002). This can be achieved by using either modified plant viruses, or with *Agrobacterium tumefaciens* carrying an expression plasmid. In the latter case, the intercellular space within the plant leaves is flooded with a suspension of bacteria in a process known as agro-infiltration (Bechtold and Pelletier 1998). Many transient expression systems are based on RNA plant viruses, such as Potato virus X (PVX), Tobacco mosaic virus (TMV) and Cowpea mosaic virus (CPMV), which rely on their rapid replication and ability to spread throughout the plant to achieve high levels of protein expression (Cañizares et al. 2005; Giritch et al. 2006; Lindbo 2007). However, the use of replicating RNA viruses has disadvantages in terms of the size and complexity of the proteins which can be expressed, potential problems of genetic drift and concerns regarding biocontainment (Scholthof et al. 1996). To address these issues, a non-replicating system based on Cowpea mosaic virus, the CPMV-*HT* expression system, was developed and subsequently refined to give the pEAQ series of transient expression vectors. These have proven

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highly effective at producing high titres of a variety of recombinant proteins in plants in a matter of days without biocontainment or genetic drift concerns.

The pEAQ vectors

The pEAQ vectors have their origin in attempts to develop CPMV as a virus-based expression system. CPMV is a bipartite virus in the family *Comoviridae*, with a genome consisting of separately-encapsidated RNA-1 (6.0 kb) and RNA-2 (3.5 kb) molecules, each of which has a single long open reading frame encoding a polyprotein. RNA-1 encodes the viral replication machinery and the 24K protease, which is responsible for processing the polyproteins encoded by both RNAs at a variety of specific sites. This activity by 24K includes releasing itself from the RNA-1 polyprotein (Goldbach and Wellink 1996). RNA-2, which is entirely dependent on RNA-1 for its replication, encodes both viral coat proteins and the viral movement protein. It has been shown that most of the RNA-2 polyprotein can be deleted and replaced with a foreign sequence without abolishing the ability of the molecule to be replicated by RNA-1 (Cañizares et al. 2006; Rohll et al. 1993), leading to the development of replication-competent deleted RNA-2 (delRNA-2) constructs. These had advantages over the previous vectors based on full-length RNA-2 molecules both in terms of biocontainment and the size of insert tolerated. However, because of their inability to spread from cell-to-cell and the absence of the small (S) coat protein, which acts as the native suppressor of gene silencing (Liu et al. 2004), these delRNA-2 constructs had to be introduced into leaves by agro-infiltration (Liu and Lomonossoff 2002) in the presence of RNA-1 and a heterologous suppressor (Cañizares et al. 2006). The delRNA-2 approach was successfully used to express a number of proteins (Sainsbury et al. 2009a). However, to preserve the ability of delRNA-2 constructs to be replicated by RNA-1, it was essential to retain the sequence of the first 512 nucleotides at the 5′ end of RNA-2, including two in-frame AUGs at positions 161 and 512, as well as the entire RNA-2 3′UTR (Rohll et al. 1993). This required the precise in-frame positioning of the heterologous sequence between AUG 512 and the 3′ UTR which made insertion of heterologous sequences into delRNA-2 constructs a cumbersome two-step process (Sainsbury et al. 2009a).

During these studies, it was noticed that co-inoculation of delRNA-2 constructs with the powerful P19 suppressor of gene silencing from Tomato bushy stunt virus stabilised the mRNA transcribed from the incoming T-DNA to such an extent as to make replication by RNA-1 unnecessary to achieve high levels of protein expression. Through studies originally intended to simplify cloning, it was found that removal of AUG 161 caused a 10-fold increase in yield of an inserted GFP gene through increased translational efficiency of the mRNA transcribed from the T-DNA (Sainsbury and Lomonossoff 2008). A further enhancement was obtained when an additional out-of-frame upstream AUG at 115 was also removed, despite the fact that removing AUG 115 alone decreases translation compared to wild-type. These unexpected results indicate that AUG 161 inhibits translation initiation at AUG 512. This may be explained by the need to promote replication of RNA-2, a process which requires the activity of RNA-dependent RNA polymerase on a region of an RNA relatively unoccupied by ribosomes. This could also explain the observed effect of removing AUG 115, which initiates an open reading frame that extends beyond AUG 161: AUG 115 might allow ribosomes to bypass AUG 161 and re-initiate at AUG 512 (Sainsbury and Lomonossoff 2008). Such regulatory activity provided by upstream open reading frames is known to occur in other eukaryotic systems (Meijer and Thomas 2002).

The new expression system, based on a deleted version of CPMV RNA-2 with a mutated 5′UTR, was shown to enhance expression of GFP, DsRed, the Hepatitis B virus core antigen HBcAg, and human anti-HIV antibody 2G12 (Sainsbury and Lomonossoff 2008; Sainsbury et al. 2010b). Because high level expression is based on enhanced translation rather than replication, the system was termed CPMV-*hypertrans* or CPMV-*HT*. This early *HT* expression system, which is the direct precursor to the pEAQ vector series, facilitated *in planta* production of Influenza virus VLPs. Influenza hemagglutinin (HA) is highly immunogenic and, although it is highly variable, it is a good target for narrow host-range VLP vaccines. Influenza HA was produced in *N. benthamiana* using CPMV-*HT* and this protein budded from plant-cell membranes to form vesicular VLPs (D'Aoust et al. 2009). In fact, CPMV-*HT* was determined to provide higher yields of VLPs than the alfalfa plastocyanin gene-based expression system which was previously used. For this reason, CPMV-*HT* was chosen for industrial-scale production of HA VLPs which are currently undergoing clinical trials in a commercial context (D'Aoust et al. 2010).

The pEAQ series of transient expression vectors (Sainsbury et al. 2009b) are designed to utilise the CPMV-*HT* effect to achieve high levels of expression while at the same time facilitate the insertion of heterologous sequences between the modified (*HT*) 5′ UTR and the 3′ UTR. They are small binary vectors carrying genes essential for replication of the plasmid in *E. coli* and *A. tumefaciens* on the backbone, along with a T-DNA transfer region for DNA transfer to the plant genome. The 5.2 kilobase backbone of all pEAQ plasmids carries the *oriV* and *colE1* origins of replication, the *trfA* gene required for plasmid replication, and the *nptIII*

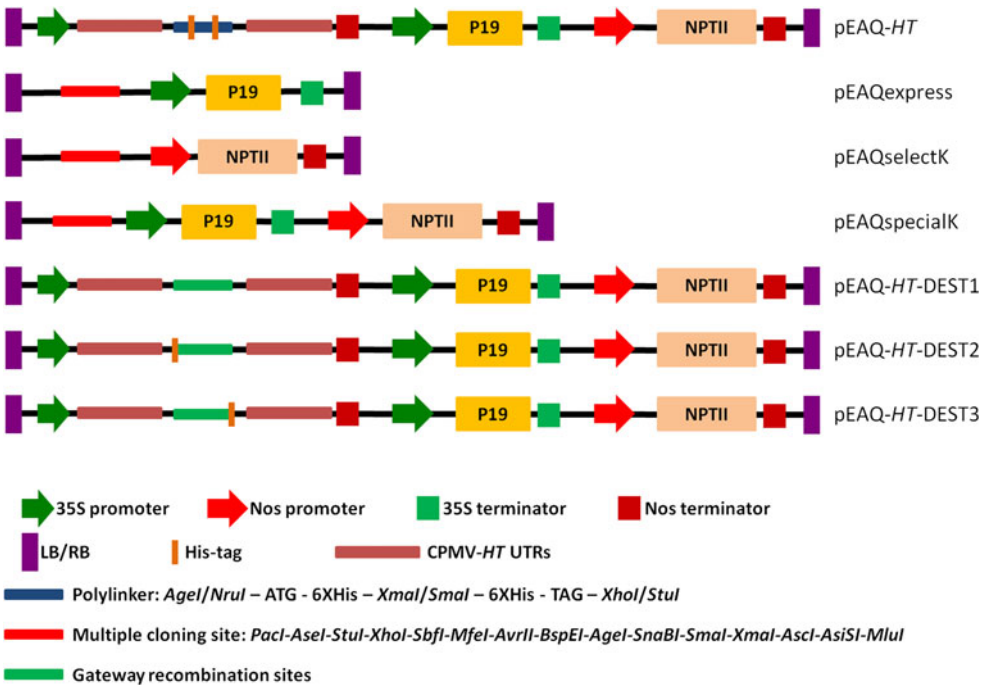

Fig. 1 The pEAQ vectors. The T-DNA of the different pEAQ vectors can contain the CPMV-*HT* expression cassette, the P19 suppressor of gene silencing, and the NPTII kanamycin resistance gene. They can be compatible with restriction enzyme or Gateway cloning and allow for the addition of an N- or C-terminal hexahistidine tag. The vector backbones are identical

prokaryotic neomycin phosphotransferase gene which confers resistance to kanamycin. The T-DNA region varies between pEAQ plasmids to allow for maximum flexibility in cloning and expression. This flexibility includes the presence or absence of the P19 suppressor of gene silencing and NPTII eukaryotic kanamycin resistance gene (Fig. 1). All pEAQ vector sequences are published and annotated in the GenBank nucleotide database. To the best of the authors' knowledge, neither the transient expression of recombinant proteins using pEAQ, nor the activity of such recombinant proteins, has ever been adversely affected by P19 or NPTII. The design of the pEAQ vectors is modular, allowing the insertion of multiple CPMV-*HT* cassettes on the same T-DNA. This is important to maximise the efficiency with which multiple proteins can be expressed within the same cell (Montague et al. 2011). Once a gene or genes of interest have been inserted into the appropriate pEAQ vector, the DNA is transformed into *A. tumefaciens* prior to inoculation into *Nicotiana benthamiana*. The *Agrobacterium* strain of choice for transfer of pEAQ vectors to plants has usually been LBA4404 (Sainsbury et al. 2009b), although strains C58C1, EHA105 and the cysteine auxotroph C58::pEHA105/Cys32 have also been used with success (Larsen and Curtis 2012; Sun et al. 2011). Inoculation of bacterial suspensions can either be accomplished using a syringe (small scale experiments) or by vacuum infiltration when larger amounts of material are required. The ease of use of these vectors allows the recombinant protein to be harvested within 2–3 weeks of DNA sequence identification, including the 5–9 days normally required between agro-inoculation and harvest (Fig. 2).

Production of VLPs and other viral proteins

The need to develop a method for the rapid production of candidate vaccines and vaccine carriers in plants led to the pEAQ vectors being adopted as a major expression platform for the European Union FP7 Project "Plant-Produced Vaccines" (PLAPROVA). As part of this consortium, proteins from a wide range of enveloped and non-enveloped viruses, including Hepatitis B virus (HBV), papillomaviruses and Porcine respiratory and reproductive syndrome virus (PRRSV) were expressed in plants using the pEAQ vectors. An overview of the results of this project can be found in Thuenemann et al. (2013) and only selected details are given here.

One of the first successes in terms of using pEAQ to produce a viral capsid was the core antigen of Hepatitis B virus (HBcAg), which readily forms immunogenic stable core-like particles (CLPs, Fig. 3a) in *N. benthamiana* and reached yields of 200–500 mg per kg of fresh weight tissue (FWT) (Thuenemann et al. 2013). These HBcAg particles are currently undergoing immunogenicity and uptake studies to demonstrate their use as vaccine candidates. Another success for plant-produced VLPs is the L1 major coat protein of Human papillomavirus type 8 (HPV-8), which was expressed in plants using the pEAQ vectors. It was shown to form higher order structures including VLPs, yielding up to 240 mg/kg FWT provided that the C-terminal 22 amino acids were removed, as this peptide appears to be a nuclear localisation signal (Matić et al. 2012). These HPV-8 L1 VLPs are the first ever produced in plants, and are potential vaccine candidates (immunogenicity trials are underway).

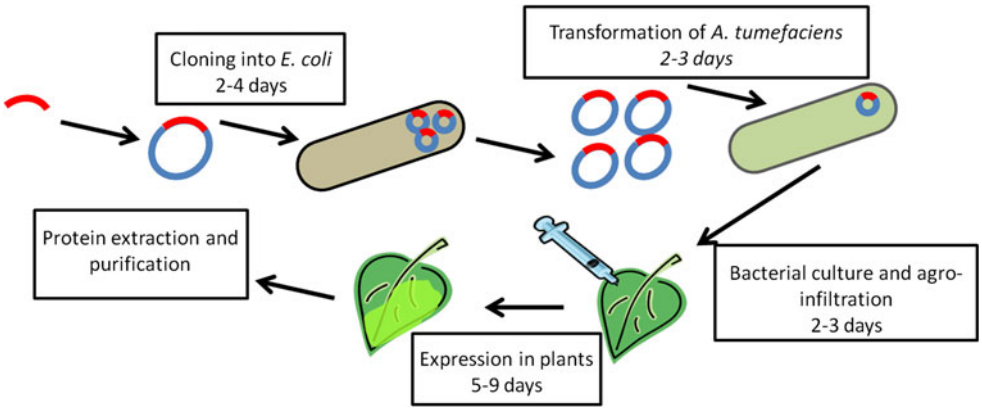

Fig. 2 The pEAQ pipeline. Using pEAQ vectors allows for cloning and expression of the protein of interest to take place within 2–3 weeks

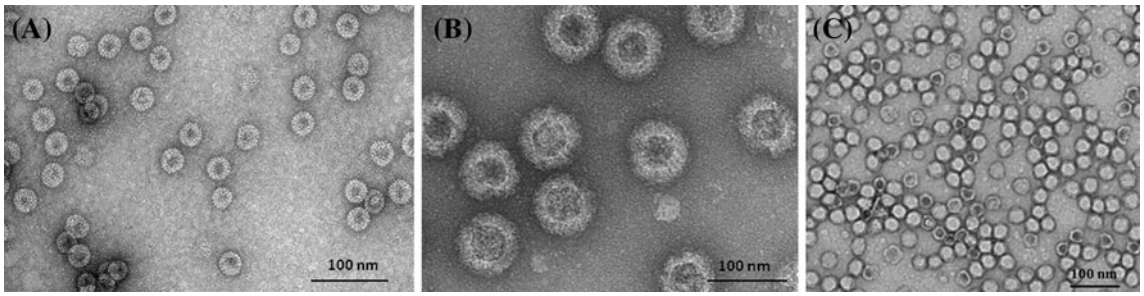

Fig. 3 Examples of plant-produced CLPs and VLPs. **a** HBV HBcAg CLPs; **b** BTV VPs 3,7,2 and 5 VLPs; **c** CPMV processed VP60 (L and S) eVLPs. All particles produced in *N. benthamiana* and visualised by staining with 2 % uranyl-acetate on a FEI Tecnai 20 TEM

Interestingly, this study compared production of HPV-8 VLPs in plants using pBIN19, pEAQ-*HT* and a TMV-based system from Icon genetics. It was found that while pBIN19 did not yield detectable levels of L1 (even with co-infiltration of a P19-bearing construct), recombinant HPV-8 VLPs were produced with the pEAQ and TMV systems, although yields were 15 times higher with pEAQ. This contrasts with a comparable experiment, published alongside the first, which assayed production of GFP with these different vectors. That experiment demonstrated that the TMV-based system gave better expression of GFP than pEAQ. The authors hypothesise that the difference may be due to the size of the transgene being expressed: because the TMV-based system requires RNA replication and cell-to-cell movement, the probability of genetic instability increases with insert size. This issue is obviously resolved by the use of an entirely non-replicating system such as pEAQ-*HT*. Using a similar approach, the L1 major coat protein of Bovine papillomavirus type 1 (BPV-1) was produced in *N. benthamiana* using pEAQ-*HT*, with accumulation in the tissue reaching 224 mg/kg FWT, allowing a recovered yield of 183 mg/kg FWT (Love et al. 2012). The L1 protein was shown to self-assemble into VLPs which elicited a strong specific immune response in rabbits. This is the first report of a BPV vaccine candidate produced in plants.

The most complex VLP successfully produced to date with pEAQ is the Bluetongue virus (BTV) VLP. In this virus, the VP3 and VP7 proteins form core-like particles (CLPs), and the VP2 and VP5 proteins use this core to assemble on its surface to produce VLPs which can stimulate a neutralising antibody response in sheep (Thuenemann et al. 2013). Expression in plants of either VP3 and VP7, or all four viral coat proteins was compared using different expression systems including pEAQ-*HT*. The sequences used were plant codon-optimised sequences from BTV serotype 8, and the pEAQ vector was determined to be most suitable for the production of VP3/7 CLPs as well as VP3/7/2/5 VLPs (Fig. 3b). The optimised production of BTV VLPs using pEAQ relied on modulating the expression of each of the four proteins in order to arrive at the correct expression ratio for VLPs to form efficiently. The details of how this was achieved are in preparation (Thuenemann, E.C., Meyers, A., Rybicki, E., Heath, J. and Lomonossoff, G.P., in preparation).

In addition to the production of animal virus VLPs, it has also been shown that the pEAQ-*HT* system is able to direct the production of CPMV empty (RNA-free) virus-like particles (eVLPs) in plants (Fig. 3c). This required the expression of two proteins, the VP60 coat protein precursor and the RNA-1—encoded 24K protease to achieve processing of VP60 to the mature L and S proteins; these were initially introduced on two separate pEAQ constructs (Saunders et al. 2009). The co-infiltration of these two constructs yielded assembled CPMV eVLPs which are structurally identical to

wild-type CPMV particles apart from the absence of RNA. The yield of recovered eVLPs from this initial, two construct approach was 0.1–0.2 g/kg FWT. However, two to five -fold higher yields of CPMV eVLPs have since been achieved by placing both the VP60 and 24K constructs on the same T-DNA (Montague et al. 2011). This development opened the door for a plethora of biotechnological applications. Empty particles can potentially be loaded with molecular cargo, and the non-replicating expression system in principle allows modifications to be made to the CPMV coat proteins for display or targeting of the particles (Montague et al. 2011). A study to this effect was carried out which demonstrated efficient internal and external mineralisation of the CPMV eVLPs with cobalt (Sainsbury et al. 2011). The key to both types of mineralisation is the 24 amino acids at the C-terminus of the small (S) coat protein, which occlude the pore located on the fivefold axis of the CPMV particles. This peptide is essential for capsid assembly but can be cleaved post-assembly to allow internal mineralisation. Alternatively, it can be replaced with a hexahistidine tag to allow external mineralisation. The work carried out over the years on using CPMV as a biotechnological tool has been reviewed (Sainsbury et al. 2010a). Recent studies have also demonstrated that it is possible to produce wild-type as well as modified Turnip crinkle virus (TCV) VLPs in *N. benthamiana* using pEAQ-*HT*, although in this case the VLPs encapsidate host RNA (Saunders K., personal communication).

A similar approach to that which was used to produce eVLPs of CPMV was applied in an attempt to produce Foot and mouth disease virus (FMDV) VLPs in plants. This involved the co-expression of the FMDV P1 protein precursor (containing VP0, VP3 and VP1 capsid proteins) modified to contain a CPMV 24K protease recognition site instead of the native FMDV 3C protease cleavage site, since the 3C protease is toxic in plants. When co-expressed with a CPMV 24K-containing construct, the individual capsid proteins were identified, suggesting that they had been processed from the FMDV P1 polyprotein by the CPMV 24K protease. However assembled VLPs were not observed, indicating that plant cells are probably not suitable for either assembly or stability of FMDV particles (Thuenemann et al. 2013). This indicates the kind of limitations that future work on recombinant protein production in plants may face.

The successes obtained with the production of VLPs from non-enveloped viruses contrast with the difficulties encountered when trying to produce VLPs from the enveloped Porcine respiratory and reproductive syndrome virus (PRRSV) in *N. benthamiana* with pEAQ. Full length GP5 and M envelope proteins, which both confer partial protective immunity, could not be expressed, and severe necrosis was rapidly apparent on the leaves. This is thought to be due to the fact that these are transmembrane proteins: upon removal of the transmembrane domain of GP5, necrosis was alleviated and enough protein was purified for immunogenicity and protection studies to be carried out (Lenzi et al. unpublished). It seems probable that the problems encountered with the expression of the envelope proteins of PRRSV will be generic for all envelope proteins with a trans-membrane domain.

To deal with the challenge posed by enveloped viruses, numerous groups have adapted easily-produced CLPs and VLPs to display surface epitopes of Influenza virus. The M2 transmembrane protein of Influenza virus would make a broadly protective vaccine due to its low variability; and its outer domain, the M2e epitope, is likely to be most useful in this regard. Because HBcAg is so easily produced in plants (see above), it was chosen as a potential epitope carrier. The M2e peptide was fused either to the N-terminus or, in a separate approach, to the immunodominant e1 loop of HBcAg, and this yielded about 15–50 mg/kg of recombinant VLPs (Thuenemann et al. 2013). Another study demonstrated that it was possible to use the Human papillomavirus type 16 (HPV) L1 protein as a carrier for M2e and produce the chimeric VLPs in plants using pEAQ (Matić et al. 2011). The L1 protein of HPV-16 was modified to substitute the helix 4 (h4) region with the Influenza A M2e antigen, and this construct was successfully expressed in *N. benthamiana*. The resulting particle yield reached up to 120 mg/kg FWT, and these VLPs were recognised by both anti-HPV and anti-influenza monoclonal antibodies. While M2e has low variability, the Influenza virus surface protein hemagglutinin (HA) is easy to produce and is a good candidate for strain-specific vaccines. One group which was not associated with the PLAPROVA project managed to use pEAQ to transiently express avian Influenza A H7N7 HA to a yield of 200 mg/kg FWT. While this HA did not form VLPs, it did cause hemagglutination of chicken erythrocytes, and this process was blocked by hyperimmune rabbit antiserum raised against H7 subtype virus (Kanagarajan et al. 2012b).

Production of enzymes

The pEAQ expression system has been used to produce high titres of an active human enzyme, human gastric Lipase (hGL), through transient expression in *N. benthamiana*. This enzyme, responsible for degradation of lipids in the human stomach, has uses in in vitro digestion models as well as a potential medical use for patients suffering from pancreatic insufficiency. Previous efforts to produce recombinant hGL, in insect cells or yeast, have suffered from very low yields and poor recovery (Canaan et al. 1998; Crabbe et al. 1996). By using pEAQ, hGL has been produced at levels of about 0.5 g/kg FWT, representing a

maximum yield of enzymatic activity of 193 U/g FWT (Vardakou et al. 2012). This corresponds to an activity of about 310 U/mg of protein, which is about three-fold lower than native human- produced hGL. The reasons for this discrepancy are unknown as of yet, but it is possible that the purification process could be further optimised to better preserve enzymatic activity. The recombinant hGL from plants shares important characteristics with its native human-produced version: it is stable at 40 °C as well as at low pH, it exhibits higher affinity for short-chain lipids over long-chain lipids, and crucially, it appears to be resistant to digestion by pepsin, the protease present in the stomach. This resistance is thought to be caused by the glycosylation of the protein, and although the glycosylation profile is predicted to be different for plant-produced hGL from native hGL in terms of the glycans used, this does not appear to compromise resistance to digestion by pepsin. While hGL was produced in plants with a view to use the enzyme as a reagent or even a medical therapeutic post-purification, pEAQ has also been used to produce enzymes for fundamental research. The expression in *N. benthamiana* of hexahistidine-tagged recombinant OsChia4a, a rice chitinase, allowed research to be carried out on this enzyme's anti-fungal activity as well as on the regulation of the gene coding for this enzyme by the plant hormone jasmonic acid (Miyamoto et al. 2012). While this is the only currently published example of the pEAQ system being used to study gene function, it is currently being deployed for many such studies worldwide.

It has also been shown that the pEAQ vectors are well suited to the manipulation of entire synthetic pathways through the co-expression of multiple enzymes. The coding sequences for two sesquiterpene synthases from *Artemisia annua*, amorpha-3,11-diene synthase (ADS) and *epi*-cedrol synthase (ECS), were cloned into pEAQ and expressed in *N. benthamiana* to yields of 90 and 96 mg/kg FWT respectively (Kanagarajan et al. 2012a). Moreover, these two enzymes converted the product farnesyl diphosphate into (respectively) amorpha-4,11-diene and *epi*-cedrol, which are intermediates in the biosynthetic pathway leading to the production of artemisinin, an important antimalarial drug. This indicates that pEAQ allowed relatively high-level expression of these enzymes and that these function *in planta* with appropriate product specificity. Two enzymes from the monocot crop species *Avena sativa* (oat) have also been expressed in order to study their mode of action (Sainsbury et al. 2012). The oxidosqualene cyclase (OSC) and the cytochrome P450 (CYP450) enzymes were expressed both separately and on the same T-DNA in pEAQ vectors, and both were found to be active *in planta.* The product β-Amyrin was produced by OSC, and when both enzymes were co-expressed, a novel compound was produced which is currently under investigation. This indicates that the pEAQ vectors can be used not only to produce large quantities of a particular enzyme, but also to study their modes of action alone or with other enzymes involved in the same biosynthetic pathway, as well as potentially creating novel compounds.

Production of proteins in cell suspension cultures

The pEAQ vectors have also been used to overexpress proteins of interest in cell cultures. recombinant human serum albumin (HSA) was produced in a transgenic tobacco Bright Yellow-2 (BY-2) cell suspension system (Sun et al. 2011), and yields of this protein reached 22.1 mg/l of growth medium (or 0.7 % total soluble protein). While this may appear to be a relatively low yield, the authors argue that it is 20–40 times higher than yields of other recombinant proteins obtained with this cell line, indicating that the pEAQ vectors may be a way to improve productivity of BY-2 cell suspension cultures, thus potentially opening doors for the production of pharmaceutical proteins in this system. A separate study actually compared different virus-based expression systems for the production of β-glucuronidase (GUS) in *N. benthamiana* cell suspension cultures and hairy root cultures, and compared both systems with agroinoculation of leaves (Larsen and Curtis 2012). The expression systems that were compared were two replicating systems, based on Potato virus X (PVX) and Tobacco rattle virus (TRV); and two non-replicating systems, pEAQ-*HT* and a Tobacco etch virus (TEV)-based vector, pGPTVK-GI. In cell suspension cultures, pEAQ allowed the production of about 3 mg/l of GUS, which corresponds to 0.51 % TSP and is not significantly greater than the amount of GUS produced by the TEV-based control. In agroinfiltrated leaves however, pEAQ allowed the production of GUS at up to 40 % TSP, which was far higher than the control. In hairy root cultures, the pEAQ vectors yielded GUS at 0.006 % TSP compared to 0.076 % TSP for the replicating pTRV2 vector. The conclusion of this study was that although pEAQ (along with the other vectors) are compatible with cell suspension cultures and hairy root cultures, they are far better suited to transient expression through agroinfiltration of leaves. The authors speculate that this may be due to leaves providing a better environment for efficient bacterial attachment and transfer of T-DNA.

Use of pEAQ vectors in transgenic plants

Though the pEAQ system could be used to produce transgenic suspension cultures (Sun et al. 2011), it proved impossible to regenerate lines of transgenic *N. benthamiana*

with constructs expressing wild-type P19 as this interferes with plant regeneration (Saxena et al. 2011). This was addressed for the last stage in the creation of the pEAQ vector series: the development of a version of the P19 suppressor of gene silencing (P19/R43W) that is not developmentally toxic to plants and therefore compatible with the production of stable transgenics (Saxena et al. 2011). Through a point mutation in the P19 gene that substitutes arginine 43 with tryptophan, P19 ceases to prevent regeneration of fully-grown *N. benthamiana* plants from callus after transformation with *Agrobacterium.* It was found that plants transiently expressing P19/R43W along with GFP produce half as much recombinant GFP as plants transiently expressing wild-type P19 and GFP, but this reduced yield still represents a sevenfold increase compared to transient expression of GFP without any suppressor of gene silencing. Because the difference between wild-type P19 and P19/R43W is a single nucleotide substitution, the pEAQ-*HT* vector can be used to rapidly test a construct transiently, and if yield is considered adequate, a straightforward site-directed mutagenesis reaction on the same plasmid can yield a vector which can itself be tested transiently, but which is also ready for the production of transgenics. This is advantageous as the production of transgenics is far more time-consuming than transient expression. This development has allowed pEAQ to be used for the production of high-yielding, fertile, stable, homozygous transgenic lines that constitutively produce GFP (Saxena et al. 2011) and human gastric lipase (Peyret, H; Thuenemann, E.C.; Eldridge, T; Saxena, P; Lomonossoff, G.P., unpublished).

Conclusion

In less than five years since initial publication, the pEAQ series of vectors and the CPMV-*HT* system on which they are based have proven to be viable alternatives to replicating expression systems for the expression of heterologous proteins in plants. The dramatic increase in translational output provided by the CPMV-*HT* mutation in the CPMV RNA-2 5′ UTR eliminates the need for replication in order to achieve high yields of recombinant protein. The creation of a suite of user-friendly expression vectors which include selection markers as well as a suppressor of gene silencing has allowed this expression system to be used by a wide range of research groups in many different fields. While the production of viral proteins, and notably fully-assembled stable VLPs have doubtlessly been the primary use of the pEAQ vectors so far, they have also been used to produce recombinant enzymes and other proteins in transient, transgenic, cell culture and even hairy root systems. In the future, we hope that pEAQ vectors will prove their worth by becoming part of the production system for commercially available recombinant proteins, notably plant-produced vaccines. We also expect that there will be the opportunity to test the usefulness of the pEAQ vectors in plants other than *Nicotiana*: as a research tool with *Arabidopsis*, but also perhaps with algae and monocotyledonous plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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