The Use of Legume Seed for Expression and Storage of High Value Proteins

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Abstract

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There is an ever growing need for the use of recombinant proteins both in medicine and industry; however their widespread use is limited by the lack of production capacity. Transgenic plants offer the possibility to produce and deliver recombinant proteins on a large scale with low production costs and with minimal purification or enrichment requirements. Among crop plants, legumes have great potential as a protein production platform because of their naturally high protein content, nutritional value, independence of N-nutrition, pollen containment, available processing technology, storage stability etc. There have been several proof-of-principle attempts to produce large amounts of recombinant protein in seed of both soybean and pea, however, our knowledge of processes regulating the foreign protein production and deposition is still limited.

Keywords: pea; pharming; protein bodies; soybean; vaccine

With advances in biology and medicine the number of well-characterized proteins with significant research, clinical or industrial potential is rapidly increasing. Together with the increasing number of various recombinant proteins such as enzymes, vaccines or antibodies, the demanded production scale is also growing. So, while the global demand for proteins important in research might be in milligrams or grams, other clinical or industrial proteins might be demanded in kilograms or even tons. It is clear that an improvement of the current expression systems is needed to satisfy growing needs for recombinant proteins in the near future.

It has been recognized recently that plants offer some significant advantages over the more conventional expression systems such as microbial, yeast, insects or mammalian cells (Ma *et al.* 2005). These advantages include the proper protein folding of eukaryotic proteins, ability to assemble large multimeric complexes and to express extremely large proteins, the rapid and simple scalability and economy of scale, the absence of human pathogens, availability of processing methods which are well known in the food industry and a potential for direct oral administration of raw or partially purified plant material. It has also been recognized that plant-specific glycosylation might pose problems with rapid degradation and/or allergenicity of recombinant product in some applications.

Seed expression

Most of the current effort in pharmaceutical protein expression in plants is concentrated on the use of green leaf biomass. Expression in leaves is easily obtained both with conventional constitutive promoters or using chloroplast transformation (Daniell *et al.* 2005). The expression in green tissue has also advantages for transient expression systems such as viral vectors (Fitchen *et al.* 1995; Marillonet *et al.* 2005; Peyret & Lomonossoff 2013) which combine high expression levels (about 40-50% of total soluble protein, TSP) with unparalleled speed and scalability (a few weeks from vector design to purified protein in scale relevant for clinical applications, Bendandi *et al.* 2010). However, leaves mostly consist of water and fibre while their protein content is low (1–3%). Recombinant proteins are susceptible to degradation by endogenous proteases and modification by phenolic compounds present in leaves. Large volumes of green plant tissue have to be processed *e.g.* frozen and lyophilised immediately after harvest.

There are significant advantages in directing the expression of the recombinant protein into the plant seed. Plant seeds are low in water and thus offer high stability and natural microencapsulation. Seeds of different plant species have evolved a diversity of different storage tissues and organs which are evolutionarily specialized to store energy in many different forms such as polysaccharides, oils, proteins as well as other compounds such as phytate, antioxidants and a variety of secondary metabolites. During long periods of dormancy, major storage products remain substantially intact. The different storage products are deposited in various plant species in a variety of tissues or organs such as the embryo, cotyledons or scutellum, endosperm and aleurone. This natural ability of seed to store proteins and other products essentially intact for prolonged periods makes seed an excellent organ for recombinant protein expression. Low water content and high protein density also simplify the logistics of storage. The protein processing facility can thus be separated from the plant growth facility both in space and time, i.e. the seed can be grown, harvested and stored long before the actual need for the processing of final product arises.

Downstream processing is usually inversely proportional to the initial recombinant protein content of raw material, thus a high accumulation of proteins in seed can significantly reduce the processing costs. Another indirect advantage of seeds might be in biosafety, since sufficient amounts of recombinant proteins can be grown under controlled greenhouse conditions, provided that high accumulation per individual seed is achieved.

Specific advantages of legume seed

Among all seed-based expression platforms, seed of legume crops stands out because of its exceptionally high protein content. Unlike cereals and other plants which store energy in the seed primarily in the form of starch or oil, legumes conserve energy in the form of proteins, which is facilitated by their independence of exogenous N supply. In some legumes *e.g.* in soybean, the seed protein content can be as high as 45% of its dry mass, which is about four times more protein than in cereal grain.

Moreover, some legumes (*e.g.* soybean) offer an additional advantage for the production of pharmaceuticals – practically no cross pollination, either under greenhouse or field conditions. The self-fertilisation is not only advantageous for growing multiple transgenic events within the same greenhouse compartment, but also it is considered as an additional biosafety containment, preventing the spread of biologically active transgenes such as oral vaccines to non-transgenic food crops.

The excellent long-term stability of foreign proteins in legume seed has been demonstrated by Oakes *et al.* (2009), who showed that the levels of adhesin fanC expressed and stored in soybean seed were unchanged upon 4 years of storage at ambient temperature or upon mail shipment from USA to India and back. Similar observations were made with B-subunit of heat labile enterotoxin (LTB) in soybean, where the vaccine was stable for 7 years (Moravec, unpublished observations).

The total amount of recombinant protein expressed in legume seed is often underestimated when compared to other established seed-based expression systems such as maize or rice. The reason is that the amount of recombinant protein is most commonly reported as a percentage of total soluble protein (TSP) and not as a percentage of the seed dry weight. The cereal seed typically contains on average 8–12% of total protein. Since most cereal seed storage proteins are water insoluble prolamins and glutelins, the actual base for TSP calculation to which recombinant protein expression is compared represents only about 2–3% of the seed dry weight. So if, for example, protein is expressed to 1% of TSP in both soybean and maize, this would translate to 4 mg of protein per 1 g of soybean seed (at 40% soluble protein content) while 1% TSP equals only to approximately 0.25 mg per 1 g of maize seed (at 2.5% of soluble protein content). In our hands, when grown in the greenhouse, soybean can yield about 30 g of seeds per plant at a density of 5 plants per square meter (recommended field densities are about 30 plants per square meter), which translates to 150 g of seed and 1.5 g of recombinant protein (at 2.5% of soluble protein content) per square meter. In the greenhouse, two or three harvests per year are possible; a 100 m^2 greenhouse can thus yield 450 g of recombinant protein per annum, with a potential to increase the seed yield per plant, plant density and most importantly the concentration of recombinant protein in the seed, as was demonstrated by Schmidt & Herman (2008), who achieved about 8% TSP with reporter gene GFP (Table 1).

The use of legumes for recombinant protein production has been hindered so far mostly because of the difficult transformation protocols and relatively low transformation rate. Whereas the first reports on successful transformation of legumes appeared many years ago, *e.g.* transformation of soybean was reported

by Hinchee *et al.* (1988), of pea by Puonti-Kaerlas *et al.* (1990) and of common bean by Russell *et al.* (1993) (biolistic), the recalcitrance of some legumes towards *in vitro* regeneration was and still is a major bottleneck in transgenic plant production. After many years of stepwise improvements, today multiple protocols exist using both *Agrobacterium*-mediated DNA transfer (Švábová *et al.* 2005; Paz *et al.* 2006) or direct biolistic methods (Rech *et al.* 2008) that provide relatively high transformation efficiency in some legumee crops. However, transformation of some important and potentially relevant legume crops for recombinant protein expression is still impossible *e.g.* lupin.

It is also important to note that most legume seeds contain anti-nutritional factors such as lectins, protease inhibitors and in some cases potent allergens (Helm *et al.* 1997), which might interfere with some applications, particularly when the oral administration of unprocessed seed material is intended. On the other hand, the protease inhibitors might be beneficial for the recombinant protein stability during processing and/or prolonged release of orally delivered antigens. For oral applications of seed material, known allergens might also be removed as part of the transformation process (Herman *et al.* 2003).

Expression cassette design

The choice of the right regulatory sequences, namely the promoter, has a decisive impact on the final yield of recombinant protein. Some of the early attempts to express recombinant proteins in legumes used strong constitutive promoters such as CaMV35S to drive its expression. Russell *et al.* (2005) used 35S promoter to express hGH (human growth hormone) in soybean. Only one of the four tested plants was shown to be expressing detectable levels of hGH in the dry seed, at a level of 0.0008% total soluble protein (TSP). The 35S promoter was also used to express adhesin fanC in soybean (Piller *et al.* 2005) or cholera toxin B fused to VP60 in pea (Mikschofsky *et al.* 2009). And while strong expression levels of about 0.7% TSP in pea or 0.5% TSP in soybean have been reported in the leaf tissue, much lower levels of antigen (0.004% of TSP) accumulated in the seed. However, even such a low dose of antigen was sufficient to achieve effective immunization (Mikschofsky *et al.* 2009; Mikschofsky & Broer 2012).

It is currently preferred to use a strong seed specific promoter *e.g.* storage protein promoter such as glycinin G1 promoter (Meinke *et al.* 1981), which was used to achieve levels in the order of 2.5–3% in soybean (Ding *et al.* 2006; Moravec *et al.* 2007), the promoter of the alpha subunit of beta-conglycinin yielding about 2.9 % of TSP of human growth hormone (Cunha *et al.* 2011a). Similarly, the seed specific promoter USP (unknown seed protein) was used to produce scFv antibody that accumulated about 2% TSP in pea seed (Saalbach *et al.* 2001). However, the use of strong seed specific promoters does not always ensure the high seed accumulation as reported by Perrin *et al.* (2000), who used the legumin A promoter to drive the expression of scFV antibody resulting in the expression of only about 0.0036% TSP in pea seed.

Besides strong constitutive or seed specific promoter, promoters inducible by chemical signal have also been tested in soybean seed (Semenyuk *et al.* 2010), where the methoxyfenoside inducible gene switch has been successfully used to drive the expression of reporter protein GFP.

While the results obtained with the glycinin, conglycinin or USP promoters seem promising, a comprehensive promoter strength analysis is missing, mostly due to difficulties with legume transformation and the need for generating a large number of independent lines bearing the same coding sequence.

Choice of cellular compartment

Another important factor to consider is the subcellular compartment used for protein accumulation. It has often been shown in plants that retention of proteins in the endoplasmic reticulum (ER), using the H/KDEL terminal peptide, enhances recombinant protein accumulation. Abnormal accumulation of proteins within the ER is a principal cause of several serious human diseases. Mutations in cargo proteins are mainly responsible for these disorders (Rutishauser & Spiess 2002). As a result of mutations, the proteins fail both to traffic and to be disposed of by the ER protein quality control mechanism (Kleizen & Braakman 2004), resulting in large aggregates within this compartment that are toxic to cellular functions in the long term. Conversely, the ER compartment of plant cells is known to tolerate unusually high accumulation of proteins without compromising plant development and reproduction. ER synthesis is highly desirable for the production of active foreign proteins that require chaperone assistance, oligomer formation, disulphide bond formation and/or cotranslational glycosylation (Pompa & Vitale 2006). Also, proteins residing within the ER acquire the basic N-linked glycans which are identical in plants and

animals. However, upon further transport into the Golgi apparatus and PSVs, or to the apoplast, foreign proteins might acquire the complex plant type N-linked glycans, which are markedly different from those of mammals (Petruccelli *et al.* 2006). Such a modification is often undesirable.

It has been shown in soybean that foreign proteins can be retained within the ER compartment and if sufficient expression levels are obtained, can be sequestered into ER-derived protein bodies (PBs, Moravec *et al.* 2007). While these protein bodies are known as storage organelles in cereals, so far their formation in legumes was observed only in soybean (Kinney *et al.* 2001; Moravec *et al.* 2007; Schmidt & Herman 2008). These electron-dense protein accretions are inert storage compartments which are stable also during the seed desiccation phase when the rest of the ER undergoes degradation. Targeting the accumulation of foreign proteins to seed PBs naturally microencapsulates the foreign proteins to provide protection against degradation both within the seed during seed maturation (Moravec *et al.* 2007) and in the gastrointestinal tract, which might be useful for the potential production of orally available biopharmaceuticals. Deposition in PBs may also enhance potential mucosal immune responses. From all the above-mentioned arguments it is clear that the deposition of foreign protein product into ER derived protein bodies is both highly desirable and achievable, however the mechanism of their formation is still unclear. It has been suggested that ER body-forming peptides from storage proteins of cereals might be used to induce the protein body formation in legume seed, however this has not been tested so far.

Protein storage vacuoles (PSVs) are another compartment which can potentially accumulate foreign proteins. PSVs are different from regular lytic vacuoles present in most plant cells. PSVs are ER-derived cisternae specialized to store large amounts of seed storage proteins. However, while the PSVs can stably accumulate the intrinsic storage proteins, early attempts to produce recombinant proteins targeted to PSVs (Pueyo *et al.* 1995; Coleman *et al.* 1996) were unsuccessful since the proteins turned out to be posttranslationally unstable. Although high levels of foreign proteins can often be synthesized, subsequent degradation during seed desiccation left little of this protein remaining in dormant seeds. In contrast, some recent reports demonstrate successful high-level expression and accumulation of several proteins including human growth hormone (hGH, 2.9% TSP), human coagulation factor IX (hFIX; 0.23% TSP), mammary cancer marker scFvDIR83D4 (0.93% TSP) and other proteins in PSVs, see e.g. Cunha *et al.* (2010, 2011a, b). The major difference between PSVs and ER-derived protein bodies would thus be the different glycosylation pattern. It is clear that the localization of storage products at the cellular level is a major determinant of accumulation levels of the product as well as its stability during prolonged periods of dormancy.

Storage protein downregulation

Seeds have evolved to store precise amounts of proteins and other reserve compounds at maximum density within storage cells, leaving little cellular space to add additional products resulting from transgene expression. Within its developmental programme, the seed exhibits a limited degree of storage substance plasticity. Other than variability caused by changes in nutrient availability and environmental effects, the relative distribution of various storage compounds is primarily genetically determined.

To make the best use of the protein synthesis capacity of legume seed, it is desirable to redirect a significant part of the protein synthesis capacity from the production of intrinsic seed proteins to the synthesis of foreign protein(s). It was shown previously that soybean seed lacking one or both major seed storage proteins germinates normally and develops into a normal plant (Kinney *et al.* 2001; Takahashi *et al.* 2003), while total seed protein content is similar to that of wild-type plants. This observation is explained by the overall increase in the levels of other seed proteins compensating for the absence of either 11S or 7S seed storage proteins or both. When the transgenic soybean line expressing GFP was crossed with a line lacking 11S beta-conglycinin expression, the resulting hybrid accumulated 4 times as much GFP (about 8% of TSP) as the parental plant (Schmidt & Herman 2008). Interestingly, even though the recombinant protein is fused to the ER retention signal KDEL, it does not share the fate of the ER compartment, *i.e.* its degradation during seed desiccation, but is secreted into membrane-bound electron-dense vesicles–protein bodies. Proteins deposited in these bodies do not show any signs of degradation even after 7 years of storage (Moravec, unpublished observations). Quite surprisingly, the accumulation of proglycinin in these ER bodies which was observed in the parental delta11S line was restored to normal trafficking to the protein storage vacuole and processing to mature glycinin. This may suggest that there are protein or chaperone cofactor preferences to accrete one protein

Table 1. Milestones in the use of legume seed as a platform for the expression of recombinant proteins

Legume	Expressed protein	Promoter used	Reported yield	Cellular compartment	Note	Reference
Glycine max	*E.coli* adhesin fanC	CaMV35S	0.5%TSP	not reported	high expression both in leaves and in seed, useful as a fodder vaccination	Piller *at al.* (2005)
	Basic fibroblast growth factor	glycinin Gy1, CaMV 35S	2.3% TSP (Gy1) or 0.056% TSP(35S)	not reported		Ding *et al.* (2006)
	E.coli heat labile enterotoxin B (LT-B)	glycinin Gy1	2.4% TSP	ER-derived bodies	oral application protects mice from challenge with native toxin	Moravec *et al.* (2007)
	GFP	glycinin Gy1	8% TSP	ER-derived bodies	fourfold enhancement of GFP expression upon introgression into line with suppressed β-conglycinin expression	Schmidt & Herman (2008)
	Human proinsulin	γ-kafirin		protein storage vacuole	protein stable for at least 7 years at room temperature	Cunha *et al.* (2010)
	GFP	Ecdysone inducible promoter (VGE)	0.1% TSP upon induction	ER-derived bodies	the protein expression was induced by application of methoxyfenozide (synthetic ecdysone analogue); there was no expression in noninuced plants nor in other tissues than seed	Semenyuk *et al.* (2010)
	human coagulation factor IX	β-conglycinin α' subunit	0.23% TSP	protein storage vacuole	enzymatic activity retained after 6 years of storage	Cunha *et al.* (2011b)
	Human growth hormone	β-conglycinin α' subunit	2.9% TSP	protein storage vacuole		Cunha *et al.* (2011a)
Pisum sativum	scFv antibody	legumin A	9 μg per 1 g of seeds	ER		Perrin *et al.* (2000)
	scFv antibody	USP (unknown seed protein	2% TSP	ER	high level and stable expression up to R3 generation.	Saalbach *et al.* (2001)
	Rabbit haemorrhagic disease virus VP60: choleratoxin B subunit	CaMV35S	up to 0.7% TSP	ER	rabbits immunized with pea-derived CTB:VP60 showed anti-VP60-specific antibodies, similar to commercial vaccine -immunized rabbits, and survived RHDV challenge	Mikschofsky *et al.* (2009)

TSP – total soluble protein; ER – endoplasmic reticulum

in favour of another with the result that accreting one protein, GFP, impedes the accretion of another protein, proglycinin (Schmidt & Herman 2008). Contrary to these findings, when the GFP-producing line was crossed with the line lacking both 7S and 11S major storage proteins, the reporter protein did not participate in proteome rebalancing, *i.e.* it was expressed at a similar level like in the parental line (2% of TSP, Schmidt *et al.* 2011). Aproteomic analysis of the seed showed that the selection of upregulated intrinsic proteins is selective. Although the attempt to increase the foreign protein expression above 8% was not successful, the understanding of the processes underlying the proteome rebalancing could unlock the potential to obtain high yields of recombinant proteins in soybean and possibly other legumes as well.

Downstream processing

Downstream processing and/or purification of expressed recombinant protein from the seed are clearly among the most important and difficult tasks when using seed as a production platform. The various options and purification strategies should be considered prior to plant transformation, at the time when the expression cassette is being designed. It is clear that the final protein purity and purification strategy are greatly dependent on the nature of produced protein and its intended application.

The most advantageous case is when the intended route of protein administration to either human or animal subjects is oral or topical. In this case no or minimal processing is necessary. It has been shown by our work (Moravec *et al.* 2007) that protective immune responses can be achieved in experimental animals by feeding them with raw soybean extract. More recently, protective passive immunisation has been observed by feeding piglets with seed-expressed sIgA (Virdi *et al.* 2013). Optionally, other formulations well known in the food and feed processing industry, *e.g.* formulations based on soymilk, tofu, protein extracts, extruded meal, etc., can be used.

When the application demands the use of pure protein, then purification could be simplified by a known method – *e.g.* genetic fusion with peptide simplifying the purification process such as elastin-like protein (ELP, Floss *et al.* 2010). ELPs are proteins which change their solubility as a function of temperature and/or salt concentration, making purification very fast and efficient. They were also shown to improve the yields of expressed protein (Conrad *et al.* 2011). Another promising option is the oleosin fusion technology (Nykiforuk *et al.* 2006) which, however, has not yet been reported to produce recombinant protein in legumes.

Still in some applications the genetic fusion with purification tag is not possible. In these cases, the purification protocol should take the advantage of our extensive knowledge of the legume seed composition. Dry legume seed typically contains 20–40% of protein, 10–20% of oil, 20–50% of starch and is usually very low in polyphenols, which is a clear advantage over leaf biomass. Most of the protein content is seed storage proteins that can be classified into two major classes: salt-soluble globulins and water-soluble albumins (Osborne & Campbell 1898). The globulin fractions – 11S legumin and 7S vicilins and convicilin are predominant storage proteins that account for about 50–75% of the total seed protein depending on variety (Tzitzikas *et al.* 2006). These storage proteins would be major contaminants during the purification process. An exemple of a protocol to purify recombinant beta glucuronidase from soybean seed was described by Robic *et al.* (2010). Briefly, the seed is ground to a fine powder to improve contact with solvents. Then, optionally, the oils are extracted with organic solvent. Some experimentation is usually necessary during the extraction of aqueous phase to find optimal pH and ionic strength which will enrich the extract with the recombinant protein while depleting it from storage proteins. Then, the aqueous phase is typically subjected to various chromatography steps until the desired enrichment and purity are achieved.

CONCLUSIONS

Transgenic plants offer the possibility of producing and storing high value recombinant proteins on an extremely large scale with low production costs and with minimal purification requirements. The potential exists for direct formulation of oral vaccines in animal feed and/or human consumables. The risks associated with human pathogen contamination and needle-based delivery could thus be avoided. Vaccines made in crop plants, when administered orally, can elicit both systemic and mucosal immunity that is protective. In addition, due to current high production costs, some therapies put significant pressure on or are inaccessible for healthcare systems, especially in poor countries. It is thus possible that with significant cost reduction of large-scale recombinant protein production even new, currently unforeseen applications will emerge. Seed, and especially legume seed, is a very efficient expression platform which has the unparalleled capacity to store large amounts of biologically active protein for extended periods of time without

detectable reduction of its activity. Even though the building blocks have been established, the use of the state of the art tools of omics, synthetic biology and metabolic engineering is needed to expand our knowledge of protein expression, trafficking and deposition in legume seed and to establish it as an economically competitive production platform.

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References

Bendandi M., Marillonnet S., Kandzia R., Thieme F., Nickstadt A., Herz S., Frode R., Inoges S., Lopez-Diaz De Cerio A., Soria E., Villanueva H., Vancanneyt G., Mccormick A., Tuse D., Lenz J., Butler-Ransohoff J.E., Klimyuk V., Gleba Y. (2010): Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. Annals of Oncology, **21**: 2420–2427.

Coleman C.E., Herman E.M., Takasaki K., Larkins B.A. (1996): The maize gamma-zein sequesters alpha-zein and stabilizes its accumulation in protein bodies of transgenic tobacco endosperm. Plant Cell, **8**: 2335–2345.

Conrad U., Plagmann I., Malchow S., Sack M., Floss D.M., Kruglov A.A., Nedospasov S.A., Rose-John S., Scheller J. (2011): ELPylated anti-human TNF therapeutic single-domain antibodies for prevention of lethal septic shock. Plant Biotechnology Journal, **9**: 22–31.

Cunha N.B., Araujo A.C.G., Leite A., Murad A.M., Vianna G.R., Rech E.L. (2010): Correct targeting of proinsulin in protein storage vacuoles of transgenic soybean seeds. Genetics and Molecular Research, **9**: 1163–1170.

Cunha N.B., Murad A.M., Cipriano T.M., Araujo A.C.G., Aragao F.J.L., Leite A., Vianna G.R., Mcphee T.R., Souza G., Waters M.J., Rech E.L. (2011a): Expression of functional recombinant human growth hormone in transgenic soybean seeds. Transgenic Research, **20**: 811–826.

Cunha N.B., Murad A.M., Ramos G.L., Maranhao A.Q., Brigido M.M., Araujo A.C.G., Lacorte C., Aragao F.J.L., Covas D.T., Fontes A.M., Souza G., Vianna G.R., Rech E.L. (2011b): Accumulation of functional recombinant human coagulation factor IX in transgenic soybean seeds. Transgenic Research, **20**: 841–855.

Daniell H., Chebolu S., Kumar S., Singleton M., Falconer R. (2005): Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine, **23**: 1779–1783.

Ding S.H., Huang L.Y., Wang Y.D., Sun H.C., Xiang Z.H. (2006): High-level expression of basic fibroblast growth factor in transgenic soybean seeds and characterization of its biological activity. Biotechnology Letters, **28**: 869–875.

Fitchen J., Beachy R.N., Hein M.B. (1995): Plant virus expressing hybrid coat protein with added murine epitope elicits autoantibody response. Vaccine, **13**: 1051–1057.

Floss D.M., Schallau K., Rose-John S., Conrad U., Scheller J. (2010): Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. Trends in Biotechnology, **28**: 37–45.

Helm R.M., Cockrell G., Stanley J.S., Sampson H.A., Herman E.M., Burks A.W., Bannon G.A. (1997): IgE-binding epitopes on P34, also identified as Gly m Bd 30K, a major soybean allergen. Journal of Allergy and Clinical Immunology, **99**: 571–571.

Herman E.M., Helm R.M., Jung R., Kinney A.J. (2003): Genetic modification removes an immunodominant allergen from soybean. Plant Physiology, **132**: 36–43.

Hinchee M.A.W., Connorward D.V., Newell C.A., Mcdonnell R.E., Sato S.J., Gasser C.S., Fischhoff D.A., Re D.B., Fraley R.T., Horsch R.B. (1988): Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. Bio-Technology, **6**: 915–921.

Kinney A.J., Jung R., Herman E.M. (2001): Cosuppression of the alpha subunits of beta-conglycinin in transgenic soybean seeds induces the formation of endoplasmic reticulum-derived protein bodies. Plant Cell, **13**: 1165–1178.

Kleizen B., Braakman I. (2004): Protein folding and quality control in the endoplasmic reticulum. Current Opinion in Cell Biology, **16**: 343–349.

Ma J.K., Chikwamba R., Sparrow P., Fischer R., Mahoney R., Twyman R.M. (2005): Plant-derived pharmaceuticals – the road forward. Trends in Plant Science, **10**: 580–585.

Marillonnet S., Thoeringer C., Kandzia R., Klimyuk V., Gleba Y. (2005): Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. Nature Biotechnology, **23**: 718–723.

Meinke D.W., Chen J., Beachy R.N. (1981): Expression of storage-protein genes during soybean seed development. Planta, **153**: 130–139.

Mikschofsky H., Broer I. (2012): Feasibility of *Pisum sativum* as an expression system for pharmaceuticals. Transgenic Research, **21**: 715–724.

Mikschofsky H., Schirrmeier H., Keil G.M., Lange B., Polowick P.L., Keller W., Broer I. (2009): Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus. Plant Biotechnology Journal, **7**: 537–549.

Moravec T., Schmidt M.A., Herman E.M., Woodford-Thomas T. (2007): Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine, **25**: 1647–1657.

Nykiforuk C.L., Boothe J.G., Murray E.W., Keon R.G., Goren H.J., Markley N.A., Moloney M.M. (2006): Transgenic expression and recovery of biologically active

recombinant human insulin from *Arabidopsis thaliana* seeds. Plant Biotechnology Journal, **4**: 77–85.

Oakes J.L., Bost K.L., Piller K.J. (2009): Stability of a soybean seed-derived vaccine antigen following long-term storage, processing and transport in the absence of a cold chain. Journal of the Science of Food and Agriculture, **89**: 2191–2199.

Osborne T.B., Campbell G.F. (1898): Proteids of the pea. Journal of American Chemical Society, **20**: 348–362.

Paz M.M., Martinez J.C., Kalvig A.B., Fonger T.M., Wang K. (2006): Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. Plant Cell Reports, **25**: 206–213.

Perrin Y., Vaquero C., Gerrard I., Sack M., Drossard J., Stoger E., Christou P., Fischer R. (2000): Transgenic pea seeds as bioreactors for the production of a single-chain Fv fragment (scFV) antibody used in cancer diagnosis and therapy. Molecular Breeding, **6**: 345–352.

Petruccelli S., Otegui M.S., Lareu F., Tran Dinh O., Fitchette A.C., Circosta A., Rumbo M., Bardor M., Carcamo R., Gomord V., Beachy R.N. (2006): A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and sorted to protein storage vacuoles in seeds. Plant Biotechnology Journal, **4**: 511–527.

Peyret H., Lomonossoff G.P. (2013): The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants. Plant Molecular Biology, **83**: 51–58.

Piller K.J., Clemente T.E., Jun S.M., Petty C.C., Sato S., Pascual D.W., Bost K.L. (2005): Expression and immunogenicity of an *Escherichia coli* K99 fimbriae subunit antigen in soybean. Planta, **222**: 6–18.

Pompa A., Vitale A. (2006): Retention of a bean phaseolin/maize gamma-zein fusion in the endoplasmic reticulum depends on disulfide bond formation. Plant Cell, **18**: 2608–2621.

Pueyo J.J., Chrispeels M.J., Herman E.M. (1995): Degradation of transport-competent destabilized phaseolin with a signal for retention in the endoplasmic reticulum occurs in the vacuole. Planta, **196**: 586–596.

Puonti-Kaerlas J., Eriksson T., Engström P. (1990): Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. Theoretical and Applied Genetics, **80**: 246–252.

Rech E.L., Vianna G.R., Aragao F.J.L. (2008): High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nature Protocols, **3**: 410–418.

Robic G., Farinas C.S., Rech E.L., Miranda E.A. (2010): Transgenic soybean seed as protein expression system: aqueous extraction of recombinant beta-glucuronidase. Applied Biochemistry and Biotechnology, **160**: 1157–1167.

Russell D.A., Spatola L.A., Dian T., Paradkar V.M., Dufield D.R., Carroll J.A., Schlittler M.R. (2005): Host limits to accurate human growth hormone production in multiple plant systems. Biotechnology and Bioengeneering, **89**: 775–782.

Russell D.R., Wallace K.M., Bathe J.H., Martinell B.J., Mccabe D.E. (1993): Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle-acceleration. Plant Cell Reports, **12**: 165–169.

Rutishauser J., Spiess M. (2002): Endoplasmic reticulum storage diseases. Swiss Medical Weekly, **132**: 211–222.

Saalbach I., Giersberg M., Conrad U. (2001): High-level expression of a single-chain Fv fragment (scFv) antibody in transgenic pea seeds. Journal of Plant Physiology, **158**: 529–533.

Semenyuk E.G., Schmidt M.A., Beachy R.N., Moravec T., Woodford-Thomas T. (2010): Adaptation of an ecdysone-based genetic switch for transgene expression in soybean seeds. Transgenic Research, **19**: 987–999.

Schmidt M.A., Herman E.M. (2008): Proteome rebalancing in soybean seeds can be exploited to enhance foreign protein accumulation. Plant Biotechnology Journal, **6**: 832–842.

Schmidt M.A., Barbazuk W.B., Sandford M., May G., Song Z.H., Zhou W.X., Nikolau B.J., Herman E.M. (2011): Silencing of soybean seed storage proteins results in a rebalanced protein composition preserving seed protein content without major collateral changes in the metabolome and transcriptome. Plant Physiology, **156**: 330–345.

Švábová L., Smýkal P., Griga M., Ondřej V. (2005): *Agrobacterium*-mediated transformation of *Pisum sativum in vitro* and *in vivo*. Biologia Plantarum, **49**: 361–370.

Takahashi M., Uematsu Y., Kashiwaba K., Yagasaki K., Hajika M., Matsunaga R., Komatsu K., Ishimoto M. (2003): Accumulation of high levels of free amino acids in soybean seeds through integration of mutations conferring seed protein deficiency. Planta, **217**: 577–586.

Tzitzikas E.N., Vincken J.P., De Groot J., Gruppen H., Visser R.G.F. (2006): Genetic variation in pea seed globulin composition. Journal of Agricultural and Food Chemistry, **54**: 425–433.

Virdi V., Coddens A., De Buck S., Millet S., Goddeeris B.M., Cox E., De Greve H., Depicker A. (2013): Orally fed seeds producing designer IgAs protect weaned piglets against enterotoxigenic *Escherichia coli* infection. Proceedings of the National Academy of Sciences of USA, **110**:11809–11814.

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