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Genetic Transformation of *Triticeae* Cereals for Molecular Farming

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1. Introduction

The *in planta* production of recombinant proteins is a newly emerging area. The use of transgenic crops enjoys several comparative advantages over established heterologous protein production systems based on bacteria, yeasts, mammalian or plant cells, particularly in terms of cost and practicality. Thanks to the development of effective transformation protocols, the generation of recombinant vaccines, antibodies and enzymes in the grains of the *Triticeae* cereals has become a feasible proposition in recent years. A further advantage of *in planta* synthesized recombinant proteins over bacterial and yeast-derived ones relates to post-translational modifications, in particular glycosylation. Since the majority of pharmaceutically active proteins are glycoproteins, their synthesis in bacteria and yeast is not possible. Therefore most of these proteins are currently synthesized in mammalian cell cultures. Since such cultures need complex (and therefore expensive) media, they also bear the risks of contamination by human pathogens. At present, about a dozen plant-derived pharmaceuticals are in the clinical phase of testing. Beside that a secretory IgA targeting tooth decay (CaroRx™-from Planet Biotechnology Inc, Ma et al., 1998, 2005) and a human intrinsic factor targeted as a dietary supplement to alleviate vitamin B-12 deficiency (Cobento Biotech AS) are already approved for human use (Faye & Gomord, 2010). A number of field trials are currently underway to investigate and validate additional products (Dunwell, 2009; APHIS, 2011).

The *Triticeae* family includes the major temperate crop species barley and wheat, which have been intensively bred over many decades to become well adapted to a wide range of growing environments. Although the major end-use of the temperate cereal grain is for food and feed, a significant focus of certain improvement programmes is aimed at the bioenergy market. Barley is seen as a more suitable host than wheat for transgenic applications because it is more easily transformed. An important advantage of barley and wheat in the context of biosafety is that they are largely self-pollinating, and so have been accorded G.R.A.S. (generally recognized as safe) status by the European regulatory agency EFSA. The infrastructure associated with cereal grain production, harvest and post-harvest storage is well established, and production volume is readily scalable by simply adjusting acreage. A number of transgene expression systems are available, some designed to restrict expression to the grain, but others allowing ubiquitous expression (for review, see Hensel et al., 2011).

The purification of heterologous products can be a costly process, although in some situations this step is not needed; a good example is provided by the feeding to poultry of

transgenic pea expressing an scFv antibody directed against the *Eimeria* parasite (Zimmermann et al., 2009). In dicotyledonous species such as *Nicotiana benthamiana*, pharmaceutical proteins have been produced primarily using virus-based magnICON system (ICON Genetics, Germany) in combination with agroinfiltration of the leaf: this approach has been exploited by Bayer Innovation GmbH to develop a patient-specific tumour-vaccine against non-Hodgkin's lymphoma (NHL) which is at present in the clinical phase of testing (Bayer Innovation GmbH, Germany). So far, however, this technology has not been usable in *Triticeae* species. At present either transient expression based on particle bombardment or virus vectors, or via stable expression by integration within the nuclear genome or the plastome, using *Agrobacterium*-mediated transformation or particle bombardment are used, respectively.

To date, the main focus of pharma transgenic research in the *Triticeae* cereals has been concerned with the expression of human growth factors in the barley endosperm (Maltagen Forschung GmbH, Germany; ORF Genetics, Iceland; Ventria Bioscience, CO). The transgenic production of antigens, vaccines and antibodies must abide by GMP (Good Manufacturer Practice), which is intended to guarantee the quality and uniformity of the approved product. The major challenge that this creates is to provide a uniform product given that transgene expression and the accumulation of the transgene product can be affected by uncontrollable variation in a field environment. Although it is possible to grow material in a closely controlled environment, such as in a sophisticated glasshouse, this forfeits much of the advantage of plant production systems, as such facilities are expensive to operate, and thus are only appropriate for high value products which require only small production volumes.

Uniform planting material is a necessity, and one means of obtaining this in the cereals is to generate doubled haploid plants from immature pollen. In barley, Kumlehn et al. (2006) were able by using *Agrobacterium*-mediated gene transfer into embryogenic pollen cultures to produce haploid primary transformants, which were subsequently treated with colchicine to diploidize the material, thereby avoiding segregation of the transgene in later progeny. This immediate fixation of the transgene is particularly attractive in terms of time-saving in winter varieties of wheat and barley.

This review aims to summarize the current state of the art regarding strategies, targets and future challenges in order to achieve high expression levels of *Triticeae* species-based recombinant proteins.

2. The generation of transgenic plants

The progress achieved over the past 20 years towards *Triticeae* cereal transformation has been reviewed recently by Kumlehn & Hensel (2009). The various approaches differ from one another with respect to the means employed to transfer the alien DNA, and/or in the choice of recipient host tissue. Methods include the use of PEG to transfer the DNA into isolated protoplasts, the exploitation of a virus as a vector, the biolistic introduction of DNA-coated particles and *Agrobacterium*-mediated gene transfer. The two latter methods will be described here in some detail, since they have been used intensively in the temperate cereals. Most transformation events involve the integration with nuclear DNA, but transplastomic *Triticeae* plants have also been reported (Cui et al., 2011). The commonest target tissue has been immature embryos, although isolated ovules have also shown some potential (Holme et al., 2008), and embryogenic barley pollen has distinct advantages

(Kumlehn et al., 2006). In wheat, Chauhan et al. (2010) have demonstrated that *Agrobacterium*-mediated gene transfer is also feasible for anther-culture derived haploid embryos.

2.1 Biolistic gene transfer

The biolistic technique involves the bombardment of the recipient tissue with gold or tungsten particles coated with the transgene DNA. It has been widely used to achieve transient expression, particularly where the purpose has been to assess the functionality of gene candidates, the effectiveness of RNAi constructs or the activity of promoter/reporter fusions (Onate et al., 1999; Rubio-Somoza et al., 2006). The major advantage of the technique is that it can rapidly characterize a large number of sequences (Ihlow et al., 2008). Most biolistic protocols seek to effect transfer into either leaf epidermal cells (Douchkov et al., 2005) or into the scutellar tissue of an immature embryo (Knudsen & Müller, 1991). The first stable transgenic wheat plants generated by this means involved the introduction of a gene determining herbicide resistance into embryogenic callus (Vasil et al., 1992). Thereafter, the method was improved and applied successfully to barley (Wan & Lemaux, 1994), cereal rye (Castillo et al., 1994), triticale (Zimny et al., 1995) and macaroni wheat (Bommineni et al., 1997).

2.2 *Agrobacterium*-mediated gene transfer

Although *Agrobacterium*-mediated gene transfer is based on a natural process, the *Triticeae* cereals were not originally considered as being amenable to the technique, as they are not infected by *Agrobacterium* spp. in nature. After the first reports of its successful use to transform wheat (Cheng et al., 1997) and barley (Tingay et al., 1997), the range of transformable species was extended to cereal rye (Popelka & Altpeter, 2003) and triticale (Hensel et al., 2009; Nadolska-Orczyk et al., 2005). However, transformation efficiency remains still variable and rather genotype dependent. In barley, the most readily transformed cultivar is 'Golden Promise', which allows an average of >10 independent transformation events per immature embryo (Bartlett et al., 2008; Hensel et al., 2009; Murray et al., 2004); other cultivars, while being amenable to transformation, show a lower level of efficiency (Hensel et al., 2008; Murray et al., 2004). One suggested means of overcoming this genotype dependency was to replace immature embryos with isolated ovules as the recipient tissue. Holme and colleagues (2008) showed that genotypes with a poor regeneration capacity can be transformed by this method, although the efficiency was lower but not statistically different from that of 'Golden Promise'. Kumlehn et al. (2006) preferred to target embryogenic cultures of pollen as the target plant tissue for transformation in barley.

3. Expression systems

A comprehensive summary of the expression systems developed to date has been given by Hensel et al. (2011). In the context of the cereal grain, a prime target has been to exploit the regulatory system responsible for the expression of the endosperm storage proteins, which represent a major proportion of the protein synthesized within the grain. A particularly frequently exploited sequence for barley is the *HORDEIN D* promoter, and for wheat the various *GLIADIN* and *GLUTENIN* promoters. Vickers et al. (2006) suggested that even

higher levels of transgene expression in barley and wheat endosperms could be obtained by using the oat *GLOBULIN 1* promoter. But till now there is no published study using this expression system. One strategy to maximize transgene expression involves the directed targeting to a particular cellular compartment, by attaching a signal peptide to the 5'- or 3'- terminus of the transgene; a second approach exploits promoter sequences that are only active during a distinct developmental stage or within a specific tissue. Further possibilities involve the use of viral transcriptional enhancer elements or the suppression of the recipient's endogenous protein degradation machinery. When transgenes encoding either antibodies or vaccines have been expressed in both tobacco and maize, only weak accumulation of the recombinant protein occurred in the cytosol, but targeting to the endoplasmic reticulum (ER) by attaching a H/KDEL tag led to a dramatically improved level of heterologous product accumulation (Schillberg et al., 1999; Streatfield et al., 2003). Unfortunately, the choice of the (a) signal peptide remains somewhat empirical, and to a large extent varies from one recombinant protein to other. Where glycosylation is required, targeting to the ER is essential, but nevertheless it remains necessary to evaluate the glycosylation pattern, since this property can itself be polymorphic (Floss et al., 2009).

4. Targets

Three major groups of products have been targeted to date for molecular farming. The first two consist of human or animal antigens and antibodies, which have applications in disease diagnosis, prophylaxis and recovery. The third, which has reached a more advanced stage thanks to a lesser regulatory load, is a range of technical enzymes. The first plant-made protein to be marketed was chicken avidin, produced in maize by ProdiGene (Hood et al., 1997). A number of companies have been active in making recombinant proteins in *Triticeae* plants - these include Ventria Bioscience, ORF Genetics and Maltagen Forschung GmbH. The full set of published outcomes in this area has been summarized in Table 1, and each is described in more detail below.

Promoter, specificity	Coding sequence	Effect	Species	References
Vaccines, Antigenes				
Barley *TRYPSIN INHIBITOR* (*TI*), endosperm	Enterotoxigenic *Escherichia coli FIMBRIAL ADHESIN FaeG* F4 (K88)	Edible vaccine for pigs partialy effective against ETEC-induced diarrhea	Barley	Joensuu et al., 2006
Antibodies				
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	ScFvT84.66	Antibody against carcinoembryonic antigen (CEA), tumor-associated diagnostic reagent	Wheat	Stoeger et al., 2000
Wheat High-molecular-weight *GLUTENIN 1Bx17* (*HMW 1Bx17*), endosperm	Synthetic anti glycophorin scFv-HIV epitope fusion	HIV diagnostic reagent	Barley	Schuenmann et al., 2002

Promoter, specificity	Coding sequence	Effect	Species	References
Human Proteins and Growth Factors				
Barley *a-AMYLASE*, aleurone	*ANTITHROMBIN III*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Barley *HORDEIN D* (*HOR-D*), endosperm				
Barley *a-AMYLASE*, aleurone	*a1-ANTITRYPSIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Barley *a-AMYLASE*, aleurone	*SERUM ALBUMIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	*COLLAGEN Ia*	Molecular farming of pharmaceutical proteins	Barley	Ritala et al., 2008; Eskelin et al., 2009
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				
Barley *HORDEIN D* (*HOR-D*), endosperm	*FLT3-LIGAND*	Molecular farming of pharmaceutical proteins	Barley	Erlendsson et al., 2010
Barley *HORDEIN D* (*HOR-D*), endosperm	*LACTOFERRIN*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous				Kamenarova et al., 2007
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				
Barley *a-AMYLASE*, aleurone	*LYSOZYME*	Molecular farming of pharmaceutical proteins	Barley	Stahl et al., 2002
Rice *GLUTENIN B1* (*GLUB-1*), endosperm				Huang et al., 2006
Wheat High-molecular-weight *GLUTENIN 1Bx17* (*HMW 1Bx17*), endosperm			Wheat	Huang et al., 2010
Barley *HORDEIN D* (*HOR-D*), endosperm	*ISOkine™, DERMOkine™*	Molecular farming of pharmaceutical proteins	Barley	ORF Genetics
Technical Enzymes and Recombinant Proteins				
Wheat High-molecular-weight *GLUTENIN 1-D1* (*HMW GLU-1 D1*), endosperm	An-*FERULIC ACID ESTERASE*	Molecular farming of second generation biofuels	Wheat	Harholt et al., 2010

Promoter, specificity	Coding sequence	Effect	Species	References
Barley *HORDEIN-D* (*HOR-D*), endosperm	Heat stable *(1,3-1,4)-β-GLUCANASE*	Grains containing thermostable 1,3-1,4-β-glucanase for better malting	Barley	Horvath et al., 2000
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	*Vitreoscilla HAEMOGLOBIN* (*VHb*)	Grains with altered oxygen availability	Barley	Wilhelmson et al., 2007
Wheat Low-molecular-weight *GLUTENIN G1D1* (LMWG1D1), endosperm	Ps-*LEGUMIN A*	Grains with altered protein composition	Wheat	Stoeger et al., 2001
Cauliflower Mosaic Virus 35S (*35S*), ubiquitous	Hv-*LIPOXYGENASE2* (*LOX2*)	Plants with modified oxylipin signature	Barley	Sharma et al., 2006
Maize *UBIQUITIN-1* (*UBI-1*), ubiquitous	Heat-stable An-*PHYTASE*	Grains with improved digestibility for non-ruminant animal feed	Wheat	Brinch-Pederson et al., 2000
Barley *HORDEIN D* (*HOR-D*), endosperm	Td-*THAUMATIN*	Grains containing a natural sweetener for brewing industry	Barley	Stahl et al., 2009
Wheat High-molecular-weight *GLUTENIN 1-D1* (*HMW GLU-1 D1*), endosperm	Bs-*ENDO-XYLANASE*	Grains with improved baking quality	Wheat	Harholt et al., 2010

Table 1. Bio-pharmaceuticals and technical enzymes expressed in *Triticeae* species.

4.1 Vaccines and antigens

Epidemics of the major infectious human diseases are becoming rare in the developed world thanks to the widespread use of vaccination. In less developed countries, the high cost of vaccine and a poorer level of social infrastructure exposes the population to such diseases. The production of a cheap prophylactic product, such as a plant-made vaccine, would make a material contribution to development. The ideal expression system for producing such vaccines needs to be readily transformable, inherently safe and economical, and therapeutically effective (Fischer and Schillberg, 2004). Current systems capable of producing antigens and antibodies in transgenic plants have recently been described (Daniell et al., 2009; Floss et al., 2009; Joensuu et al., 2008). While vaccines can be administered either orally or by injection, the former method is preferably from an organizational point of view and the use of grains (or other plant parts) is particularly attractive for the vaccination of domesticated animals. A disadvantage of the oral delivery route is the relatively large quantity of antigen required (Streatfield & Howard, 2003). The only published report which describes the use of *Triticeae* plants as a vehicle for producing/expressing antigens is concerned with the control of infection of enterotoxigenic *E. coli* in pigs, chickens and cows (Joensuu et al., 2006). Here, the major subunit of the F4 fimbriae (FaeG) protein was expressed in barley grains, where it comprised up to 1% of total soluble protein. The recombinant protein was able to evoke F4 fimbria-specific antibodies in mice. In a second approach, a company (Novoplant, Germany) expressed a gene responsible for

the production of an FaeG-specific antibody in transgenic pea, and were able to demonstrate a level of antibody expression in the seed of up to 1-2 g scFv/kg.

4.2 Antibodies

Following the first discovery of immunity-conferring substances in the blood (Behring & Kitasato, 1890), antibodies have been exploited in the fight against several diseases. Most antibodies are large Y-shaped proteins that include an antigen-binding site formed by the two variable segments of their heavy and light chain. The five major classes of antibody (IgA, IgD, IgE, IgG and IgM) are recognized by their conserved region structure and their immunological function (Woof & Burton, 2004). Hiatt et al. (1989) pioneered the expression of immunoglobulin chains in tobacco, since then, various portions of these chains have been expressed heterologously, including single chain molecules (scFvs), Fab fragments, small immune proteins (SIPs), IgGs and chimeric secretory IgAs (for a review, see De Muynck et al., 2010). The commonest plant host to date has been tobacco, with only a small number of examples among the *Triticeae* species. In wheat, the earliest success was achieved with the single chain Fv antibody ScFvT84.66, active against carcinoembryonic antigen (CEA), a well characterized tumour-associated marker (Stoeger et al., 2000). The production level was around 1 µg antibody/g grain, which compared unfavourably with what was possible at the time in rice. Storage of the dry grain at room temperature produced no discernible alteration in the antibody's biological activity, demonstrating the attractiveness of the *in planta* transgene expression of therapeutic molecules. A second example concerned a diagnostic antibody for HIV (Schuenmann et al., 2002), where an anti-glycophorin single-chain antibody was fused to an HIV epitope and expressed in tobacco leaves and stems, in potato tubers and in barley grains. In each case, the production level of the fusion protein was adequate, allowing the *in planta* method to replace the more conventional one based on bacterial and murine cells. The yield of heterologous protein in the barley grain reached as much as 150 µg/g, suggesting that transgenic barley could represent a highly suitable means of producing this particular antibody. The rather strict regulatory framework associated with GM plants in Europe has meant that no other example of *in planta* vaccine or antigen production in *Triticeae* has been published in the last ten years.

4.3 Human proteins and growth factors

The earliest published account of the use of cereal grain to express human genes concerned the five proteins antithrombin III, α1-antitrypsin, lysozyme, serum albumin and lactoferrin (Stahl et al., 2002). Here, the concern was not the quantity or quality of the recombinant proteins, but rather the detection of the T-DNA integration sites in the barley genome. However, these targets remain in the portfolio of Maltagen Forschung GmbH, whose website provides detailed information concerning the company's interest in these genes (Maltagen, Germany). Similar products are also offered by ORF Genetics, which exploits an endosperm-specific expression system. They produce a number of hormones and cytokinines like endothelial monocyte activating polypeptide-2 (EMAP2), various fibroblast growth factors, interferons and interleukins. A recent product from this company was human FLT3-ligand, with the gene under the control of the barley *HORDEIN D* promoter (Erlendsson et al., 2010). Ritala et al. (2008) were able to express a codon-optimized version of *COLLAGEN Ia* in barley endosperm-derived suspension cells, and showed that the recombinant protein was equivalent to a version produced in *Pichia pastoris* yeast. The gene

was driven by the maize *UBIQUITIN-1* promoter and the resulting protein yield was rather low (2-9 µg/l). However, the yield was improved by substituting the endosperm-specific rice *GLUTENIN B1* promoter and expressing the construct in the barley grain. The collagen content in the transgenic grain reached ~45 mg/kg dry weight in the best-performing transgenic derivatives. By way of comparison, the heterologous protein content of grain carrying the transgene driven by the same *UBIQUITIN-1* promoter was just ~13 mg/kg (Eskelin et al., 2009). This level was calculated to be sufficient to produce some 5 t of product were ~10% of Finland's barley production to be used for this purpose. Since the annual demand of the pharmaceutical sector is for at least ten times this amount, there is clearly a need to improve the efficiency to compete with existing production systems.

4.4 Technical enzymes and recombinant proteins

Here, the focus was on transgenes whose products are designed to either improve the technical quality of wheat (baking) or barley (brewing), to alter feed quality, or to improve biofuel properties. The earliest report of this sort of manipulation dates back about a decade, when Horvath et al. (2000) described the heterologous expression of a gene encoding a heat-stable *(1,3-1,4)-β-GLUCANASE*, designed to improve the digestibility of barley-based feed pellets used as chicken feed. The chicken gut is unable to break down complex glycans, and this failure can lead to the formation of excessive viscosity in the intestine. In commercial practice, this problem is commonly resolved by the addition to the diet of purified (1,3-1,4)-β-glucanase extracted from *Bacillus amyloliquefaciens*. A fully active and heat non-labile enzyme is present in the transgenic barley grain, which therefore represents an improvement in the nutritional value of the feed containing it. In a related approach, Brinch-Pederson et al. (2000) expressed in the wheat grain a heat-stable *PHYTASE* driven by the *UBIQUITIN-1* promoter in an attempt to encourage the release of phosphate, iron and zinc from the feed. Note that up to 85% of the phosphate present in the cereal grains is bound to phytic acid (Lott, 1984), which is deposited in the grain as phytin, a mixed salt containing potassium, magnesium, iron, calcium and zinc (Raboy, 1990). In the dry grain (as well as in the digestive tract of non-ruminant animals), no phytase activity is detectable (Lantzsch et al., 1992; Usayran & Balnave, 1995), so chicken diets are commonly supplemented by *Aspergillus niger* derived phytase (Nelson et al., 1968, 1971). The presence of the transgenic wheat increased grain phytase activity by a factor of four (from 0.7 to 3 kFTU/kg), whereas even an increase of 10% would have been sufficient to significantly improve the quality of wheat-based feed.

Barley malt and wheat flour are common ingredients of processed food and beverages, so the improvement of their technical quality is of commercial interest. The protein thaumatin is a low-calorie sweetener and flavour modifier (Gibbs et al., 1996; Green, 1999), initially isolated from the West African katemfe fruit (*Thaumatococcus daniellii* Bennett). It is heat stable up to 70°C and is 2,000-3,000 times sweeter than sugar. It has been produced heterologously in bacteria, yeast and various dicotyledonous plants, with an *in planta* yield reaching 1 g/kg leaf in tobacco (Icon Genetics). It has also been successfully synthesized in the barley grain, yielding 2-3 g/kg on a dry matter basis (Stahl et al., 2009).

The germinating seed frequently suffers from oxygen deficiency (Bewley & Black, 1994). This presents a problem during the malting process, and is not readily counteracted by continuous aeration (Wilhelmson et al., 2006). The hypoxia inhibits the *de novo* production of

starch-hydrolyzing enzymes (Guglielminetti et al., 1995), but the heterologous expression of *Vitreoscilla HAEMOGLOBIN* (VHb) in the barley grain reduces the level of hypoxia, and thus increases the availability of starch-hydrolysing enzymes during malting (Wilhelmson et al., 2007). However, the constitutive expression of *VHb* did not improve the germination rate of barley.

Several studies have highlighted the role of oxylipins in the regulation of environmentally induced or developmental-specific processes (Weber, 2002). Oxylipins are a product of the lipoxygenase pathway. When barley *LIPOXYGENASE2* was over-expressed as a means of determining the effect of altering the oxylipin status, Sharma et al. (2006) were able to show that they act as regulators, possibly by enhancing the level of endogenous jasmonic acid.

The baking property of wheat flour is influenced largely by the quantity and quality of the endosperm storage proteins, but arabinoxylan, the major non-starch polysaccharide present in the flour, also has some influence. When Harholt et al. (2010) created transgenic wheat plants expressing an *A. niger* gene responsible for the synthesis of ferulic acid esterase, the resulting grains were shrivelled and their test weight was reduced by up to 50 per cent. The increased ferulic acid esterase activity in the transgenic grain produced a higher than wild type level of water non-extractable arabinoxylan in the cell wall, but the effect of this alteration on the baking property of the flour has yet to be determined. The same authors performed similar experiments using a *B. subtilis ENDO-XYLANASE* gene, the product of which is used as an additive in some commercial baked wheat products. Just as for the ferulic acid esterase grain, the transgenic grains were shrivelled and of smaller test weight than the wild type. In the cell walls of these transgenic materials, the arabinose to xylose ratio was increased by 10-15%, and the proportion of water-extractable arabinoxylan was increased by 50%; the molecular weight range of this water-extractable arabinoxylan was reduced from >85 kDa to 2-85 kDa. There may be some potential for this transgene in the use of wheat as a bioenergy crop.

The major classes of endosperm storage proteins in the *Triticeae* species grain are the albumins, the globulins and particularly the prolamins. Transgenic wheat expressing a pea *LEGUMIN A* gene under the control of an endosperm-specific promoter were studied by Stoeger et al. (2001) to determine whether this globulin protein would be correctly processed and form the hexameric structure which it adopts in the pea seed. An unexpected result was that the legumin was condensed within endosperm inclusion bodies, and eventually formed crystals. This led the authors to suggest this transgenic material as a suitable means of producing large quantities of pure 11S globulin protein.

5. Protein modifications

Several modifications occur during the processing of proteins; these include cleavage of signal peptides after entry into the ER, formation of disulphide bonds in the lumen of the rough ER, phosphorylation by protein kinases, and the attachment of sugar side chains (glycosylation) initiated in the ER but occurring primarily in the Golgi apparatus. These modifications can be an important determinant of a protein's stability and activity.

5.1 Disulfid bridges

The conformation of a protein is sequence-dependent. One of the primary determinants of folding is the formation of a disulphide bridge between pairs of thiol groups. Most prolamins contain a number of cysteine residues capable of forming such disulphide bonds.

The retention of a phaseolin γ-zein fusion protein in the ER of tobacco protoplasts was shown to be dependent on disulphide bonding (Pompa & Vitale, 2006). Prolamins are synthesized in the ER of the wheat and barley endosperm, and are then transported to protein storage vacuoles (PSVs) in a process thought to involve both Golgi-dependent and independent pathways (Galili et al., 1993; Levanony et al., 1992; Rechinger et al., 1993). Autophagy and the *de novo* formation of PSVs has also been reported to mediate the transport of prolamins to the PSVs in wheat (Levanony et al., 1992), but the molecular and cellular mechanisms underlying these routes remain unknown.

5.2 Glycosylation

More than 50% of eukaryotic proteins are glycosylated (Apweiler et al., 1999), with the sugar linked either to an asparagine (*N*-glycosylation) or to a serine or threonine (*O*-glycosylation) residue. The synthetic pathway of N-glycans is conserved among animals, plants and fungi (for a review, see Kukuruzinska & Lennon, 1998). The majority of mammalian N-glycans are terminated by Neu5Ac and other sialic acids linked to terminal β1,4- or β1,3-Gal residues. These negatively charged sugars affect the biological activity and half-life of many therapeutic glycoproteins (Erbayraktar et al., 2003; Schauer, 2000; Varki, 2007). The synthesis of complex N-glycans takes place in various compartments of the plant cell and has been recently reviewed in the context of therapeutic protein production by Gomord et al. (2010). Retention in the ER prevents the addition of xylose and fucose residues to a recombinant antibody (Sriraman et al., 2004) that limites its applications to some human antibodies or antigenes. In tobacco, the pattern of glycosylation depends on whether the antibody is expressed in the leaf or in the seed, a phenomenon explained by proposing that the transport pathways from the ER to the protein storage vacuole differ in these organs (Floss et al., 2009), as suggested by Vitale and Hinz (2005). In monocotyledonous species, as in dicotyledonous ones, leaves (Fitchette et al., 1999; Wilson et al., 1998) and roots (Mega, 2004; Wilson et al., 2001) produce both high-Mannose-type N-glycans and complex N-glycans containing β1,2-xylose, α1,3-fucose and terminal GlcNAc or Lea antennae. A similar structural glycoprotein diversity has also been described for the fruits of both monocotyledonous (Leonard et al., 2004) and dicotyledonous (Wilson et al., 2001) species. The N-glycosylation patterns of seed glycoproteins differ significantly between monocotyledonous and dicotyledonous species. In the former, there is a little, if any presence of terminal Lea antennae (Bardor et al., 2003; Leonard et al., 2004), whereas this structural element is common in the seed of buckwheat, walnut, hazelnut, peanut, pea and mung bean (Wilson et al., 2001).

6. Concluding remarks

This review has set out to summarize the information in the public domain regarding the use of *Triticeae* species for the heterologous production of valuable products. A number of plant species have been suggested as vehicles for molecular farming, but relatively little attention has been paid to this important group of crop species, perhaps because they have been regarded as rather difficult to transform and/or because expression systems are less developed than in more commonly used plants such as tobacco.

A number of challenges remain before plant-made pharmeceuticals (PMPs) can reach the market. A major one is the expense and low efficiency of target purification. The attachment of fungal hydrophobins, elastin-like polypeptides (ELPs) or the use of a domain of the maize

storage protein zein as a purification tag represents promising strategies. The principle behind these purification tagging approaches can be based on either a temperature dependent change in solubility (ELP) termed inverse transition cycling (Meyer & Chilkoti, 1999), on a change in hydrophobicity in the case of the hydrophobins (Linder et al., 2001), or on the assembly of the proteins into so-called protein bodies by the use of γ-zein (Coleman et al., 1996; Geli et al., 1994). Although inverse transition cycling has been used to purify cytokines (Lin et al., 2006), antibodies (Floss et al., 2009; Joensuu et al., 2009) and spider silk proteins (Scheller et al., 2004) from transgenic plants, no application has yet been reported in *Triticeae* species. The same applies also for hydrophobins. Recently Joensuu et al. (2010) showed that the transient expression of a hydrophobin-GFP fusion transgene increased the accumulation in the leaves of *N. benthamiana* and eased the purification of the product. The γ-zein protein induces the formation of ER-derived protein bodies (PBs) in the seed and some vegetative tissues in dicotyledonous transformants in the absence of other zein subunits (Coleman et al., 1996; Geli et al., 1994). This observation has been exploited in the development of the Zera® expression system by ERA Biotech (Barcelona, Spain), which is effective in a number of plant species (Ludevid Mugica et al., 2007, 2009; Saito et al., 2009; Torrent et al., 2009a, 2009b). A rather different system has been pioneered by ORF Genetics, in which a carbohydrate-binding domain is used to purify the target protein (Mantyla & Orvar, 2007).

A more inexpensive approach is possible where the whole seed (or grain) is a component of feed, since in this case no purification is necessary. Nevertheless it remains important that the PMP is stable under ambient temperature conditions for several weeks. The stability of an antibody in the wheat grain was already demonstrated a decade ago (Stoeger et al., 2001). Where the PMP is heat stable, then heat treatment during feed processing is possible (Horvath et al., 2000). Achieving an adequate level of expression is essential, one approach would be to lower the amount of endogenous storage proteins competing with the transgene. Such a strategy has been followed by ORF Genetics by the down regulation of an transcription factor (*Hv-HoxB4*) which specifically affects the expression of the barley *HorB* and *HorC* genes (Orvar, 2005).

Public acceptance of GM products and a straightforward means of their detection require the availability of clear markers. In barley it is possible to use testa colour for this purpose by conventionally transferring an exotic testa colour into a readily transformable cultivar, which then becomes suitable for the production of PMPs (Orvar, 2006). With the imminent acquisition of the genomic sequences of barley and wheat, it can be expected that the key genes for the synthesis and processing underlying the pattern of glycosylation of *Triticeae* proteins will soon be known. Progress towards establishing plants as a vehicle for the production of PMPs is likely to accelerate in the coming years.

7. References

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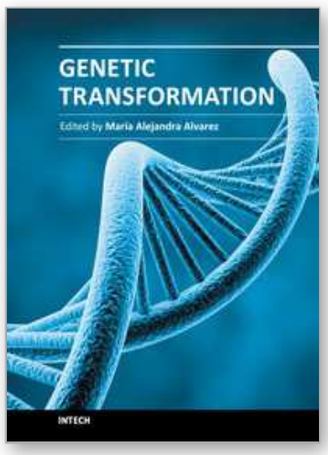

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Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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