Molecular farming: plants as a production platform for high value proteins

Gent, 14-16 September 2011

Program and abstracts
Sponsors

Organizing committee Ghent:

Prof. Dr. Ann Depicker (VIB-UGent)
Dr. Bart Van Droogenbroeck (ILVO)
Dr. Sylvie De Buck (VIB-UGent)
Drs. Kirsten De Wilde (VIB-UGent)
Drs. Vikram Virdi (VIB-UGent)
Program COST meeting  
14 – 16 September 2011, Gent, Belgium

Wednesday, 14 September 2011 (FSVM building, VIB)

14.00 – 15.00  Registration – coffee

Chairman: Ann Depicker

15.00 – 15.30  Ann Depicker (VIB, UGent): Welcome
15.30 – 16.00  Alain Goossens (VIB, UGent, Belgium) : Production of novel bioactive molecules by combinatorial biosynthesis in plant cells
16.00 – 16.30  Wout Boerjan (VIB, UGent, Belgium) : Bio-energy from poplar

16.30 – 17.30  Guided tour in the Schell/Fiers/VanMontagu VIB building
17.30 – 18.00  Marc De Loose (ILVO, Belgium) : Coexistence field trial
18.00 – 18.30  Bart Van Droogenbroeck (ILVO, Belgium) : Field trial with transgenic potatoes
18.30 – 19.30  Walk to “Kasteel van Zwijnaarde”
19.30 – 22.30  Reception and Welcome Dinner in “Kasteel van Zwijnaarde”

Thursday, 15 September 2011 (Hotel Poortackere Monasterium)

08.30 – 09.00  Registration

Chairman: Dirk Bosch

09.00 – 09.15  Introduction: Ann Depicker and Kirs i-Marja Oksman
09.15 – 10.00  Nico Callewaert (VIB, UGent, Belgium): Which glycans in which expression system for which therapeutic application?
10.00 – 10.15  Herta Steinkellner (Vienna, Austria): Plant glycoengineering: an advantage over mammalian cell based systems?
10.15 – 10.30  Bieke Nagels (UGent, Belgium): Production of human erythropoietin with multi-antennary N-glycan structures in Nicotiana benthamiana plants
10.30 – 10.45  Alexandra Castilho (Vienna, Austria): In planta sialylation of recombinant proteins

10.45 – 11.15  Coffee break and poster viewing

11.15 – 12.00  Peter Casteels (Ablynx, Belgium): Manufacture of Nanobodies: from Pipeline into the Clinic
12.00 – 12.15  Sylvie De Buck (VIB, UGent, Belgium): Production of VHH and VHH-Fc antibodies in Arabidopsis thaliana seeds
12.15 – 12.30  Vikram Virdi (VIB, UGent, Belgium): Passive immunization of piglets against post weaning diarrhoea via anti-ETEC antibodies produced in seeds

12.30 – 13.45  Lunch in Monasterium hotel
13.45 – 14.45  Guided STSM poster tour
Chairman: Stefan Schilberg

14.45 – 15.30 Anni Van Broeckhoven (Crea Bio Support, Ghent, Belgium): GMP Processing and purification of recombinant proteins

15.30 – 15.45 Geert Angenon (VUB, Belgium): Production in plant seeds of an oral veterinary vaccine against avian influenza

15.45 – 16.05 Siva Reddi (New Delhi, India): Tobacco chloroplast transformation for over production of antibodies and cellulolytic enzymes

16.05 – 16.30 Coffee break and poster viewing

16.30 – 16.45 Kristiina Makinen (Helsinki, Finland): Methods to enhance protein expression from Potato virus A gene vector in plants

16.45 – 17.00 George Lomonosoff (Norwich, UK): Production of virus-like particles for therapeutic applications

17.00 – 17.15 Anders Kvarnheden (Uppsala, Sweden): Begomovirus-associated DNA-satellites and their potential as expression vectors in plants

17.15 – 17.30 Catherine Navarre (UCLouvain, Belgium): Expression of different antibody isotypes in suspension cell cultures

17.30 - 17.45 Henrik Brinch-Pedersen (Slagelse, Denmark): A cereal platform for the production of phytases

17.45- 18.00 Udo Conrad (Gatersleben): Production of very large spider silk proteins by posttranslational fusions in vivo.

18.30 – 20.00 Guided tour in Gent
20.15 Dinner in “De Foyer”

Friday, 16 September 2011 (Hotel Monasterium)

Chairman: Paul Christou

09.00 – 09.45 Jan Desomer (Bayer BioScience N.V., Belgium): From "green juice" to "galactosylated plantibodies": IP landscape of molecular farming

09.45 – 10.00 Harry Thangaraj (United Kingdom): Intellectual Property and molecular farming

10.00 – 10.15 Stefan Schillberg (Aachen, Germany): Recent progress of the EU FP7 CoMoFarm project

10.15 – 10.30 Dirk Bosch: Molecular farming database feedback and update on target products (WP3)

10.30 – 11.00 Coffee break and poster viewing

11.00 – 11.45 Marc Zabeau (Tech Transfer, UGent): Tech transfer from academics

11.45 – 12.00 Stefan Schilberg and Einar Mantyla: update on WP2 -plant production platforms and downstream processing

12.00 – 12.15 Paul Christou and Bart van Droogenbroeck: Update on WP1 -the social economic position of molecular farming

12.15 – 12.45 Discussion – chaired by the WP leaders

12.45 – 13.00 Concluding remarks: Kirsi-Marja Oksman

13.00 - Lunch in Monasterium hotel

14.00 – 17.00 Management committee meeting chaired by Kirsi-Marja Oksman
Like many towns in the Middle Ages, Ghent was enclosed by several canals. These were meant to protect the town from the dangers that threatened its inhabitants. Near the gate ("poort" in Dutch) leading into town, there was a field ("akter" in Dutch): the Poortackere.

In this field a complex for beguines was built, which was also called Poortackere by the people of Ghent. In one of the walls of the building there is an 18th century stone which reveals that the beguine house was founded in 1278. Not long after this, a chapel and a churchyard were added. Poortackere mainly housed older and sick beguines.

When during the French revolution all cloisters, churches, and beguin complexes were seized by the town councils, the council of Ghent confiscated Poortackere. In 1863 the site was sold to the Count Joseph De Hemptinne, a notorious Maecenas of the Neogothic Movement. With his permission, a congregation was housed here.

At first there were plans to restore the old buildings, but in the end a new, neogothic cloister was built. Most buildings of Poortackere date from that period. A new chapel, two inside gardens, and an orphanage became part of the complex.

After World War II, the site was successively an orphanage, a house for young women, and a student residence. In 1998 there were still 6 nuns living here, but due to the extravagant maintenance costs the buildings were sold.

Thanks to private initiative, the site has been transformed into a hotel, guest rooms, seminar centre and a restaurant. As a result, everybody can now enjoy its architecture and its unique atmosphere.
Dinner 14 September 2011

Already since the eleventh century, there is a castle situated on the territory of the current Castle Zwijnaarde. In 1526, the Castle of Zwijnaarde, the residence of the abbot of St. Peter’s Abbey in Ghent, was the scene of a human tragedy. Isabella, the half sister of Emperor Charles V and the Queen of Norway, Sweden and Denmark, died there at the age of 25, as she was exhausted by five successive pregnancies. The history of the Castle in Zwijnaarde is as dramatic as that of Isabella. The impressive medieval castle was destroyed during the Calvinist government in Ghent between 1577 and 1584. In the late seventeenth century, under abbot Joachim Schaeyck, the castle was rebuilt. In 1797, the family della Faille d’Huyse bought the castle from the French government, and rebuilt it around 1836 into a neoclassical style. However, in the first world ware, the castle was destroyed again. But again, the family della Faille d’Huyse re-draw the castle and now, the castle has a more neorococostyle. Since many years (more than 25 years), the family does not life in the castle anymore, and it became a restaurant.

Dinner 15 September 2011

The Foyer is situated on the first floor of the Royal Dutch Theatre. In the 19th century, this building, with an impressive balcony, was designed by architect Edmond de Vigne. The authentic interior with beautiful wood panelling and beautiful ceiling was fully restored. On the balcony, you have a beautiful view over “Het Sint-Baafs plein”. Also the theatre company NTGent is located in this building. NTGent increasingly promotes itself as ‘a playhouse’, a house of players, and deriving pleasure from acting is regarded as key.
Abstracts

Oral presentations
Global warming, environmental disasters, and increasing oil prices have catalyzed a worldwide trend to use plant biomass as a renewable source for liquid biofuels and bio-based materials. Plant biomass can be processed into bio-ethanol by enzymatic depolymerization of the cell wall polysaccharides into simple sugars, followed by fermentation. However, the presence of lignin in the cell wall is an important recalcitrance factor. One approach to overcome this hurdle is to engineer lignin amount or alter its composition to make lignin more susceptible to chemical degradation. Down-regulation of cinnamoyl-CoA reductase (CCR) in poplar results in reduced lignin content in greenhouse conditions. Field trials are necessary to evaluate whether the results obtained in the greenhouse can be extrapolated to field conditions. After a long regulatory Calvary, field trials have been initiated under short rotation coppice culture to evaluate their potential as raw material for bioethanol production. The regulatory procedure and the latest scientific results from these trials will be presented.
Production of human erythropoietin with multi-antennary N-glycan structures in *Nicotiana benthamiana* plants

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Until now pharmaceutical proteins were mainly produced in bacteria, yeasts or mammalian cells. Plant systems can offer advantages for cost effective and flexible production of biopharmaceuticals. For this, the magnICON® expression system was developed which enables high level production of biotherapeutics in leaves of *Nicotiana benthamiana* plants. This system allows generating protein levels of 500 mg monoclonal antibodies per kg fresh weight within 7-9 days.

Although protein synthesis is conserved between plants and humans, there are differences in the N-glycosylation pathway. Since these differences can influence the safety and efficacy of plant made pharmaceuticals (PMPs) the plant N-glycosylation pathway needs humanization. Although many of the differences have already been addressed, little attention has been given to the structural differences. Our research is aimed at introducing multi-antennary glycan structures in *N. benthamiana* by expressing the responsible enzymes GnT-IV and GnT-V. In addition, this humanization step will be combined with the removal of xylose and fucose (XylT/FucT RNAi plants). For the production of multi-antennary N-glycans the non-host glycosyltransferases, GnT-IV and –V, were fused to the plant specific localization signals of XylT and FucT. Both wild type and XylT/FucT RNAi plants were used for transformation. The plants with the highest relative amount of multi-antennary N-glycan structures were further used to transiently express a therapeutically relevant protein, such as EPO, with the magnICON system.

The presented research shows that it is possible to introduce humanized, multi-antennary N-glycan structures in plants and combine this modification with the removal of xylose and fucose residues. Furthermore, it was shown that a recombinant protein expressed in these plants carried the humanized N-glycan structures. This opens the road to further expand the PMP production platform by introducing galactose and sialic acid residues and create fully humanized plants.
Production of VHH and VHH-Fc antibodies in *Arabidopsis thaliana* seeds

*Sylvie De Buck*1,2, Jonah Nolf1,2, Thomas De Meyer1,2, Kirsten De Wilde1,2, Els Van Lerberge1,2, Bart Van Droogenbroeck3 and Ann Depicker1,2

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Among the many plant-based production systems that have been developed for molecular farming, seeds have the particular advantage that they can accumulate high levels of recombinant proteins in a small volume and stable environment. Using the regulatory sequences of the seed storage protein genes arceline 5-I and -phaseolin of *Phaseolus* spp., the seed-specific accumulation of a model single chain Fv (scFv) recombinant antibody fragment boosted to exceptionally high levels, reaching no less than 36% of total soluble protein in seeds of transgenic homozygous *Arabidopsis thaliana* plants, which is equivalent to approximately 7% of seed weight (De Jaeger et al., 2002). Also high accumulation levels of bivalent scFv-Fc antibodies and full antibodies, corresponding to approximately 1% to 5% of the *Arabidopsis* seed weight, were achieved using the same expression cassette in *Arabidopsis* seeds (Van Droogenbroeck et al., 2007; Loos et al., 2011).

Nowadays, there is much interest in the production and use of nanobodies®. A nanobody is the smallest antigen-binding domain, and is part of the heavy-chain antibodies, produced by camelids (Harmsen and De Haard, 2007).

Our aim was to evaluate the accumulation of monovalent nanobodies® (VHH) in plant seeds. Nanobodies have many applications, but for some, bivalent antibodies perform much better. Fusion of the Fc chain to the VHH domain results in a VHH-Fc protein and due to the Fc oligomerization based on the disulfide bridges in the hinge region, a stable bivalent dimeric complex is formed. This bivalence results in a strengthened antigen—antibody interaction, because avidity superimposes on the affinity.

Therefore, in the current research, we will answer several questions: (1) What are the accumulation levels of a nanobody in seeds?, (2) What are the accumulation levels of a bivalent VHH-Fc antibody in seeds, and (3) Does the type of signal sequence peptide (plant versus camel) has an influence on the accumulation levels of both VHH and VHH-Fc? Results will be presented and discussed.

References


Passive immunization of piglets against post weaning diarrhoea via anti-ETEC antibodies produced in seeds

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Plants can be used to produce a plethora of recombinant therapeutic proteins at fraction of a cost as compared to conventional production platforms. Plant seeds in particular are natural protein producing and storage organs, which enable production of abundant recombinant protein in a stable confined environment. The seed based expression system is particularly interesting for oral passive immunisation against veterinarian diseases, such as enterotoxigenic Escherichia coli (ETEC) causing post weaning diarrhoea. We therefore investigated the production of anti-ETEC antibodies in seeds of the model plant Arabidopsis thaliana, to later transfer the technology to a grain crop that can be used in piglet starter feed and produced in sufficient quantities.

In the pilot phase, we focused on the F4 bearing ETEC (F4⁺ETEC) strains that are most predominantly isolated from diseased piglets. For successful seed based anti-F4⁺ETEC passive immunotherapy it is imperative that the antibodies are produced in abundant amounts and that they survive gut transit. We hence aimed at designing a robust antibody by fusing the antigen binding domains of camelid antibodies (VHH) with the crystallising fragment (Fc) of porcine IgG3. The anti-F4-ETEC VHHs were panned from a lymphocytic library derived from a llama immunised with purified adhesion molecule FaeG ac, present on F4 fimbriae. These VHH’s were grafted to the Fc, then inserted into a seed specific expression cassette and transformed in A. thaliana. Each antibody expressed to its own particular accumulation level, the highest being 3% of seed weight.

These highly expressed anti-F4⁺ETEC antibodies were correctly folded in planta and recognised the FaeG adhesion molecule in an enzyme-linked immunosorbent assay. The disulphide bond within the hinge enabled production of dimeric antibodies that agglutinate the ETEC bacteria and inhibited ETEC attachment to gut villous enterocytes in an in vitro assay.

These antibodies are thus good candidates to evaluate their functionality in a piglet feed trial.
Tobacco chloroplast transformation for over production of antibodies and cellulolytic enzymes

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Experiences gained from all over the world over the last one decade showed that molecular farming based on plant based expression systems have several advantages over the conventional microbial expression systems for the large scale production of economically valuable proteins and enzymes at very low cost. Plant biomass can be produced in large quantities by simply covering large acreage and easy to scale up, reducing the initial capital investments. In the present study a number of genes coding for cell wall degrading enzymes useful in biofuel industry were expressed in the chloroplasts of tobacco, a non-food and non-feed plant, considered to be more suitable for the molecular farming. In addition, genes coding for monoclonal antibodies raised against specific milk antigens were also expressed as FD, ScFv fragments either alone or as fusion proteins with a view to purify them from the plant biomass. Molecular analysis showed the stable site-specific integration of transgenes into the plastid genome and efficient transcription of introduced genes in the chloroplasts. Results based on the biochemical and proteomic analysis of expressed proteins/enzymes will be presented and discussed.
A method to enhance protein expression from Potato virus A gene vector in plants

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*Potato virus A* (PVA), a potyvirus with a (+)ssRNA genome, can be used as a gene vector for transient expression of heterologous proteins in its host plants. An infection assay, which allows quantitation of viral gene expression with *Renilla* luciferase activity, was developed (Eskelin et al., 2010 J.Virol. Methods, 164, 101-110). This assay is sensitive enough to quantitate changes in translation, replication and movement capacity of PVA at the onset of infection and was utilized when we showed that the viral protein VPg promotes viral RNA (vRNA) stability and translation (Eskelin et al., 2011. J.Virol. 85, 9210–9221). A host protein was identified as a component of a viral protein complex. Co-expression of the host protein with non-replicating vRNA caused a 4-fold increase in Rluc amount, whereas vRNA was stabilized 10-fold. Co-expression of VPg alone resulted in a 10-fold increase in viral RNA level, and Rluc accumulated 20-fold. When the host protein and VPg were co-expressed, vRNA accumulation was increased 20-fold, and Rluc 45-fold. Hence, the host protein and VPg showed an additive effect on vRNA accumulation and a similar translational efficiency as vRNA during co-expression of VPg only, which suggests that VPg promotes the translation of the host protein stabilized vRNA. We propose that with the aid of these enhancer proteins it is possible to increase substantially the yield of any target protein from PVA gene vector.
Production of virus-like particles for therapeutic applications


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Though Cowpea mosaic virus (CPMV) capsid-based technologies have enjoyed considerable successes, the range of applications has been restricted by the need to propagate the virus by the infection of plants. This means that viral particles must be fully competent for movement from cell-to-cell, placing limitations on sequences that can be successfully expressed on the particle surface. Furthermore, the particles must retain their ability to package the genomic RNA, as this is essential for the virus to move between cells. As a result, the majority of particles isolated from plants contain either RNA-1 or RNA-2 meaning that preparations remain infectious, raising biosafety issues. A further disadvantage is that, being full of RNA, the particles cannot be loaded with heterologous material. The CPMV-HT expression system [1,2] has provided a means of producing CPMV capsids in plants without the need for infection [3], thereby overcoming these concerns and limitations.

By co-expressing the VP60 coat protein precursor and the 24K proteinase, empty (devoid of RNA) virus-like particles (eVLPs) of can now be readily obtained from agro-infiltrated plant tissue. The ability to produce such eVLPs greatly extends the range of applications of CPMV-based bio- and nano-technologies. For example, we have shown that CPMV eVLPs can encapsulate, within the protein capsid, cobalt or iron oxide by environmentally benign processes [4]. Furthermore, the external surface remains amenable to chemical modification. Thus the development of CPMV as a targeted delivery agent of therapeutics is now becoming a reality.

References
Begomovirus-associated DNA-satellites and their potential as expression vectors in plants

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Plant-infecting viruses are used as molecular tools for specific down-regulation of gene expression in plants as well as for heterologous expression of proteins. Begomoviruses are common in tropical and subtropical regions around the world and they have a genome of circular single-stranded (ss) DNA. Begomoviruses from the Old World are often associated with DNA-satellites. Betasatellites have been shown to be necessary for the development of wild type disease symptoms in natural hosts. Betasatellites depend on their helper begomovirus to support replication, systemic infection, and whitefly vector-mediated transmission. Except for a conserved nonanucleotide sequence, TAATATTAC, betasatellites do not share any significant sequence identity with begomoviruses. Betasatellites have a single gene, which encodes the protein βC1. This protein has been shown to assist in begomovirus accumulation by suppressing host-mediated silencing of viral transcripts, and possibly to facilitate viral movement in the plant.

Some begomovirus-betasatellite complexes have also been found to associate with another type of ssDNA satellite, termed alphasatellite. Alphasatellites encode a replication-associated protein (Rep) and they are capable of autonomous replication. However, alphasatellites require a helper begomovirus for systemic infection of the host plant and for whitefly vector-mediated transmission, suggesting that they are encapsidated by the begomoviral coat protein. Initially, there was no apparent role for alphasatellites in the aetiology of begomoviral diseases, but recent evidence suggests they may be involved in modulating symptom severity, indicating that they may affect the RNA silencing either directly and/or indirectly.

We are characterising different begomovirus-satellite complexes, which infect dicot plants, such as cotton, okra and tomato, in Africa and Asia. These complexes are important pathogens on crops, but also provide possibilities as expression vectors in plants. The presentation will give an overview of the system and our research on begomoviruses and their satellites.
Production of very large spider silk proteins by posttranslational fusions in vivo.

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Highly repetitive technical proteins as spider silk proteins are difficult to produce as large multimers because of genetical instabilities. Here, posttranslational fusion could help to overcome this problem and to achieve spider silk proteins of native size. Large multimers of the spider silk protein FLAG have been produced in transiently and stably transformed tobacco plants by intein-based posttranslational fusions. Multimers from dimers to dekamers (360000 Dalton) and more have been isolated. The specificity of the intein-based fusion has been demonstrated by mutations in the intein coding sequence.
Recent progress of the EU FP7 CoMoFarm project

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The CoMoFarm project aims to develop high-yielding production systems based on plants, plant tissues and plant cells for the large-scale production of pharmaceutical and industrial proteins. The concept behind the project is to develop ways to standardize the growth and behavior of plants (and plant tissues and cells) to achieve a consistent yield and product quality.

The project involves the comparative analysis of four different plant-based systems: hydroponic plants, root cultures, cultivated moss and suspension cells. A number of different species are also compared. The best performers have been used for strain and process optimization. In parallel we developed automated systems for plant monitoring and maintenance, ultimately leading to a full production platform including downstream processing. Platform and process development will be carried out with due consideration for the emerging regulatory landscape relevant to plant-derived pharmaceutical products.

The CoMoFarm project is the first to look at plant-based production systems holistically, with a view to optimizing the entire production train from cell to pure protein, specifically focusing on pharmaceutical industry standards. The project will involve many innovative elements including in-process monitoring and the automated control of environmental parameters to optimize product yield, quality and homogeneity. Novel downstream processing technologies and enhanced bioreactor and hydroponic facility designs will be used to keep plants and plant cells in peak health.

Ultimately, the results from the CoMoFarm project will help to reduce the costs involved in the production of pharmaceutical and industrial proteins and to ensure that pharmaceuticals from plants are produced to the highest possible standards.
Abstracts

Poster presentations
Challenging the moss bioreactor: Production of biologically active recombinant human factor H

Juliana Parsons¹,², Annette Büttner-Mainik¹, Hanna Jérôme¹, Andrea Hartmann³, Stephanie Lamer⁴, Ralf Reski¹,², Andreas Schaaf¹, †, Andreas Schlosser⁴,⁵, Peter F. Zipfel³, and Eva L. Decker¹,²

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The complex glycoprotein Factor H (FH) is a key regulator from the innate immune system. Deficiency of FH is associated with various human diseases such as severe retinal and rare kidney disorders. Although administration of FH constitutes a promising therapeutic, FH is not available as a medication. Physcomitrella patens was already shown to be an interesting alternative platform for the production of biopharmaceuticals. Yet, the expression of FH is a novel challenge for the moss bioreactor. FH is a 155 kDa single-chain protein, with 8 N-glycosylation sites and 20 repetitive globular domains, which need 40 internal disulphide bonds to be built up. The structure of FH is essential for its biological activity. In fact, the molecular mass of FH is similar to that of IgG antibodies, which have already been successfully expressed in P. patens, but its tertiary conformation differs completely, what makes the production of this protein an ambitious assessment for our expression system. Here, we present the production of FH in P. patens. Moss-produced FH displays a biological activity comparable to the control protein purified from human plasma. The correct processing of the signal peptide and integrity of the protein was verified via peptide mapping by mass spectrometry. Despite the big size and conformation of the protein, rFH was secreted into the culture medium (1).

References


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Influence of recombinant antibody expression on the *Arabidopsis* seed transcriptome

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Among the many plant-based production systems that are being tested for molecular farming, seeds are very attractive as they provide a stable environment in which the accumulating recombinant proteins can be stored. Using the regulatory sequences of the seed storage protein genes arcelin 5-I and β-phaseolin of *Phaseolus vulgaris*, the seed-specific accumulation of a model single chain Fv (scFv) recombinant antibody fragment boosted to exceptionally high levels, reaching 7\% of seed weight of transgenic homozygous *Arabidopsis thaliana* plants (De Jaeger \textit{et al}, 2002). Also high accumulation levels of more complex scFv-Fc antibodies (Van Droogenbroeck \textit{et al}, 2007) and full length antibodies (Loos \textit{et al}, 2011), corresponding up to 2\% and 0.3\% of seed weight respectively, were achieved using the same expression cassette in *Arabidopsis* seeds.

To determine whether these extreme high production levels of recombinant antibody are influencing the endogenous proteome and in that way change the cellular homeostasis of the seed, we studied the transcriptomic status of several antibody expressing lines. Two VHH-Fc producing lines accumulating up to 2\% and 1\% of seed weight respectively, and a transgenic line accumulating a scFv-Fc antibody up to 1.6\% of seed weight, were selected for that purpose. RNA was isolated from developing transgenic seeds at 13 days post anthesis (dpa). In comparison to the wild type Col-0, we could identify differentially expressed genes of which several were up- and only a few down-regulated. By means of a qPCR approach, the microarray analyses for up- and down-regulation of particular genes were verified. Up-regulated genes are related to protein folding, glycosylation/modification, translocation, vesicle transport and protein degradation, suggesting that the transgenic seeds are in a state of cellular stress, called the ‘unfolded protein response’ (UPR; Urade, 2007). We hypothesize that overloading the endoplasmic reticulum with recombinant antibody results in disturbance of the normal cellular homeostasis. In line with these findings, abundant seed proteins localized aberrantly (Van Droogenbroeck \textit{et al}, 2007; Loos \textit{et al}, 2011).

References
Development and production of VHH and VHH-Fc antibodies as functional tools for plant research

Thomas De Meyer1,2, Sylvie De Buck1,2, Serge Muyldermans3 and Ann Depicker1,2

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Antibodies and antibody derived fragments are excellent tools for the detection and purification of proteins. However, only a few antibodies targeted against plant proteins are currently available on the market. Researchers therefore are forced to fuse their protein of interest to one of the many different protein tags (GFP, His, ...). This step is not only time consuming, the protein tag might also interfere with the function of the protein. We are currently developing an approach that will meet this demand for high quality antibodies against *Arabidopsis thaliana* specific proteins.

Conventional antibodies (150kDa) are composed of two light and two heavy chains, dimerized via disulfide bridges. Due to their large size and complex nature, researchers progressed towards the development of smaller antibody fragments that fully retain their antigen-binding ability: Fab (50kDa) and scFv (25kDa). However, these antibody derivatives still require the assembly of two variable domains to bind their respective antigens. Camelids on the other hand produce heavy-chain antibodies (HCAb) which are devoid of light chains (Harmsen & De Haard, 2007). Their N-terminal domain, called a Nanobody® or VHH, represents the smallest antigen-binding domain (15kDa).

As a proof-of-concept, we immunized a dromedary with a crude *A. thaliana* seed extract. After isolating the peripheral blood lymphocytes, we were able to construct a VHH phage library of $6 \times 10^8$ independent transformants. We believe that such a library provides a good basis for the production of VHHs against plant specific proteins on a large scale and in an efficient way.

To evaluate the usefulness of the VHHs in plant research, we randomly selected 18 VHHs which showed strong binding in ELISA. These VHHs could easily be produced in *E. coli* at high levels: 1 to 9 mg per liter culture. Subsequently, the respective antigens for each VHH were identified by affinity purification of an *Arabidopsis* seed extract with purified VHH. Apparently, the subset of 18 VHHs was strongly biased towards the most abundant seed storage proteins: 2S albumin and 12S globulin.

In order to further increase their sensitivity in protein assays, the VHHs will be fused to an Fc-fragment to allow for dimerization. These VHH-Fc antibodies will be produced stably in *A. thaliana* seeds and transiently in *N. benthamiana* leaves. To expand the usability of the antibodies, both the VHH and VHH-Fc formats will also be tested in Western blot and immunolocalisation assays.

References
Production of recombinant proteins by *Arabidopsis lyrata subsp. kamchatka*, a polyploid and perennial plant

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Commercially interesting proteins, such as therapeutic proteins, vaccines and industrial enzymes can be produced using genetically modified plants. Seeds are especially attractive as a production platform: These compact organs offer a stable environment in which recombinant proteins can be stored for many years. A powerful expression technology, developed at UGent, has already proven its strength in *Arabidopsis thaliana* (*Brassicaceae*). In this work, related *Brassicaceae* species are evaluated to evaluate their potential as a seed-based production platform. Small *Brassicaceae* can be grown in greenhouses for production of proteins with a high added value, which have to be very pure, but are only needed in relatively small amounts.

*Arabidopsis lyrata* subsp. *kamchatka* is a natural allotetraploid, derived from a cross between *A. arenosa* en *A. halleri*. Compared to *A. thaliana*, *A. lyrata* subsp. *kamchatka* is bigger, has a longer life cycle, and its seeds are also bigger. Previous experiments showed that *A. lyrata* subsp. *kamchatka* produces a similar amount of seed per surface and per time as *A. thaliana* (accession Col-0), but with a higher protein content (unpublished results). To be useful as a production platform, susceptibility to genetic transformation of the targeted production host is a prerequisite. *A. lyrata* subsp. *kamchatka* plants were co-transformed by floral dip with two different *Agrobacterium* strains: One containing a DsRed expression cassette allowing simple visual selection of transformed seeds, the other one containing an expression cassette for a complex bivalent antibody format as an example of a commercially interesting protein. Both proteins are expressed using a different seed specific promoter.

Transformation efficiency with the DsRed T-DNA was 0.18%. Twenty-two percent of the primary transformants carrying the DsRed cassette were cotransformed with the T-DNA containing the antibody expression cassette. Antibody accumulation levels in seeds of *A. kamchatka* cotransformants varied between 0.66 and 1.34 µg intact antibody per mg seed. However, high degradation levels between 72% and 93% were detected.

The levels of intact bivalent antibody are rather low compared to scFv accumulation levels obtained in *A. thaliana* (up to 73 µg/mg, De Jaeger et al., 2002). Therefore, successful expression of recombinant proteins can only be evaluated on a case-by-case basis for any production platform (Van Droogenbroeck et al., 2007).

References


GoldenBraid: a simple and standardized system for the assembly of recyclable multigene constructs in Molecular Farming

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Multigene engineering in plants has the potential to generate unprecedented Molecular Farming products. Unfortunately most DNA assembly methodologies currently used by plant genetic engineers are poorly adapted to multigene assembly. Here we present GoldenBraid (GB) a simple standardized system for multigene engineering in plants. GB is based in the previously described Golden Gate cloning strategy [1], an efficient multipartite assembly method based in type IIIS restriction enzymes. The GB system consists in a set of four destination plasmids (pDGBs) designed to incorporate multipartite assemblies made of standard DNA parts and to combine them binarily to build increasingly complex multigene constructs. GB special cloning design consists in a double loop ("braid") of iterative cloning that facilitates the reusability of the constructs and ensures the indefinite growth of multigene structures, while maintaining the simplicity of the cloning toolkit.

New versions of GB are currently being developed. Among other improvements, we made it compatible with MoClo, another multigene cloning strategy appeared in 2011 [2], so pieces built following a common standard can be used for both strategies.

We propose the use of GB as an assembly standard for Molecular Farming and Plant Synthetic Biology. Fast GB-engineering of genetic devices for antibody-production, identity preservation, male sterility, etc, will be presented. In addition, two alternative multigenic constructs made of five recyclable devices each, and comprising a total of 19 basic parts will be also described.

References
Functional recombinant antibody against milk specific antigen, beta-lactoglobulin, produced in barley (Hordeum vulgare, L.) cell cultures and grains

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The most common form of allergy, immunoglobulin E mediated hypersensitivity, affects more than 25 % of the world’s population. The hypersensitivity can be determined as an exaggerated and inappropriate immune response to an allergen. Cow’s milk allergy is a very complex disorder. It is most prevalent in early childhood, with reported incidences between 2-6%, and decreases toward adulthood to occurrence of 0.1–0.5%. One of the major allergens in cow’s milk is \(\beta\)-lactoglobulin (BLG). Extensive research in plant molecular farming has shown that a wide range of monoclonal antibodies and other pharmaceutically important proteins can be efficiently expressed in plants. The general eukaryotic protein synthesis pathway is very well conserved between plants and animals so plants can fold and assemble very complex proteins. The overall aim of the project was to develop plant-based production systems for \(\beta\)-lactoglobulin specific antibodies for diagnostic and milk processing purposes.

For the production of a recombinant \(\beta\)-lactoglobulin specific IgE antibody, a barley codon usage optimized scFv sequence of the antibody (D1 scFv) was placed either under constitutive maize ubiquitin or seed-specific \(\beta\)-glutelin promoter. ER targeting signal was included and the role of ER retention sequence HDEL in obtained accumulation levels was studied. Keeping in mind the downstream process to purify the recombinant proteins, a His-tag was inserted at the C-terminal part of the protein. The Agrobacterium-mediated transformation, selection and regeneration of infected barley embryos (\textit{H. vulgare} L. cv. Golden Promise) followed the procedure described in Eskelin et al. (2009). The best transgenic barley cell lines produced on the average 0.96 ± 0.22 mg D1 scFv / kg cells (FW) when screened by ELISA over growth period of 21 days. In case of barley grains, 108 transgenic barley plants representing 35 different clones were generated. The best productivity was obtained with the seed-specific promoter and the ER retention signal. The D1 scFv accumulation was followed and stable to T1 generation (T2 grains). A pilot scale extraction was performed for transgenic grains and the crude extract was purified by a two-step chromatographic purification protocol including IMAC and size exclusion chromatography. The chromatographic purification of D1 scFv resulted in a yield of 0.47 mg purified protein with high purity corresponding to a yield of 9.5 mg purified D1 scFv / kg of transgenic barley seeds. The binding properties of the purified D1 scFv were further characterised by ELISA and immunoprecipitation assay. The ELISA revealed that 29 % of the purified protein was functional corresponding to a yield of 2.7 mg purified fully functional D1 scFv / kg of transgenic seeds. In the immunoprecipitation assay the D1 scFv fragment recognised the native BLG with the molecular weight of 18 kD in the milk sample without heat treatment and no binding was analysed with the heat-treated milk sample as expected.

References
Optimization of plant-based recombinant production systems for expression of the human complement factor 5a and interleukin 6 for therapeutic purposes

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Emerging challenges of modern medicine are autoimmune diseases, which spread rapidly in our aging society. Among them, the sepsis is one of the most severe one with a mortality up to 70%. Conventional treatments fail, due to the increasing occurrence of antibiotic-resistant bacteria, being the elicitor of the hyperinflammatory response. Consequently, novel immunoregulatory therapies are required. Both human complement factor 5a (C5a) and the cytokine interleukin-6 (IL6) are pleiotropic proteins, which are associated with the fatal outcome of sepsis. Since clinical therapies that targeting both have been promising, efficient production of a functionally active C5a and IL6 in high amounts is requested.

Since post-translational modifications of C5a and IL6 are essential for the production of therapeutics, both human proteins were expressed in a eukaryotic host: N. tabacum. Low expression levels of transgenes that are stable integrated in the nuclear-genome, are one of the main hindrances that limit the full realization of the economic potential of plant made pharmaceuticals. In order to overcome this obstacle, C5a and IL6 were transformed in the cultivar ‘Geudertheimer’ with an extraordinary high biomass yield. Both antigens were targeted to the apoplasm, retended in the ER or translocated to the vacuole. In case of C5a the highest accumulation level was detected in the vacuole, whereas IL6 was predominantly enriched in the ER. The expression levels on basis of the total soluble protein (TSP) were equal in leave tissue and seeds of tobacco. However, the yield per fresh weight differed significantly. This is owed the high TSP content of seeds, exceeding with up to 40% of fresh weight that of leaves, which is normally below 1%.

By conventional breeding of T₂ progenies, the transgene expression was increased as well.
**Exploiting mutant P19, a virus-encoded suppressor of silencing, to enhance protein expression in transgenic plants**

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Foreign gene expression in plants is often hampered by the onset of RNA silencing that negatively affects target gene expression. Plant virus-encoded suppressors of RNA silencing, such as P19 from the *Tomato bushy stunt virus*, are useful tools for counteracting silencing and enhancing expression. While P19 successfully enhances transient expression in plants following agro-infiltration, plants transgenic for P19 are not viable. This is probably because P19 interferes with endogenous gene silencing associated with growth and development.

We hypothesized that a previously characterized mutant of P19 (P19/R43W), typified by reduced symptomatic effects while maintaining the ability to sequester short-interfering RNAs (siRNAs), could be used to suppress RNA silencing without the concomitant developmental effects. We have found that P19/R43W is able to enhance expression levels of the fluorescent marker, GFP, in transient assays about seven-fold. This is half the level of expression achieved in presence of wt P19, which confirms that P19/R43W is not as strong as wt P19. Furthermore, we have been able to regenerate phenotypically normal, fertile *Nicotiana benthamiana* plants from tissue co-transformed with P19/R43W and GFP. By contrast, as previously found, tissue transformed with wild type P19 did not develop viable plants. Results show that in the presence of P19/R43W, GFP expression levels of about 0.1 g/kg of fresh weight tissue can be achieved in T1 transgenic plants. In addition, we have shown that plants homozygous for P19/R43W are also developmentally normal.

To extend the use of P19/R43W to high-level expression of heterologous proteins in plants, genes encoding the heavy and light chains of the human anti-HIV antibody, 2G12 were expressed in presence of P19/R43W. P19/R43W was seen to enhance transient expression of 2G12 as expected. Transgenic plants expressing 2G12 and P19/R43W have been regenerated from transformed *Nicotiana benthamiana* tissue and exhibit a sustained level of 2G12 expression. The P19/R43W system is currently being used to create transgenic plants expressing *cowpea mosaic virus* virus-like particles for use in bio-nanotechnology. We propose that the use of P19/R43W can mitigate the effects of transgene silencing and hence, enhance expression of valuable proteins in transgenic plants.

Reference

Triggering secretion of the human antibody M12 in tobacco hairy roots

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Our aim is to develop high-yielding production systems based on plants, plant tissues and plant cells for the large-scale production of pharmaceutical and industrial proteins. The concept behind the EU-funded CoMoFarm project (www.comofarm.org) is to develop ways to standardize the growth and behaviour of plants (and plant tissues and cells) to achieve a consistent yield and product quality. Diagnostic and therapeutic antibodies are an important target in plant molecular farming. The antibody M12 was constructed by fusion of the constant domain of human IgG, to the variable domains of the scFv M12, which were earlier selected from a naïve human phage display library directly on a breast carcinoma cell line. M12 binds to vitronectin that has been speculated to be involved in hemostasis and tumor malignancy. Our hypothesis is that by using the targeting properties of M12 antibody delivery of cytotoxic agents or radioisotopes to solid tumors can be conducted.

Hairy roots offer an attractive option for industrial scale production of valuable proteins by having commonly more stable production pattern than those of undifferentiated cells. Hairy roots were initiated by infecting transgenic Nicotiana tabacum cv Petite Havana SR1 plants expressing M12 antibody with a wild type Agrobacterium rhizogenes strain LBA9402/12. In order to trigger secretion of the target antibody to culture medium several factors including phytohormones, signaling and stabilizing agents and macronutrients were studied by using statistical experimental design (Modde, Umetrics). It was observed that NAA and additional KNO₃ increased the M12 accumulation in dose-dependent manner and application of PVP clearly stabilized the protein in culture medium, leading 1.7 to 2.5 -fold M12 levels in PVP added samples.

Reference
Human interleukin 2 (hIL-2) is a pharmaceutically important cytokine which activates diverse cells of the immune system, including helper T cells, B cells, macrophages, natural killer cells, and lymphokine-activated killer precursor. Recombinant hIL-2 is available as a commercial preparation Proleukin® (aldesleukin) mainly used in immunotherapy of metastatic melanoma and kidney cancer. This cytokine has been also tested in HIV infection treatment and therapy of colorectal, gastric, liver and many other cancers. Its application as adjuvant for many animals and human vaccines has also been reported. The production of large quantities of this cytokine in *Escherichia coli* and baculovirus-infected *Trichoplusia ni* insects cells is too expensive. Therefore, adaptation of plant-based expression systems seems to be a good idea. Our studies were focused not only on the production of recombinant hIL-2 but also on improving its stability especially in the alimentary canal when considering its potential oral application. For these purposes hIL-2 was produced as a fusion protein with two low molecular weight proteinase inhibitors CMTI and SPI2s. CMTI strongly inhibits trypsin and a number of medically important serine proteinases. SPI2 inhibits fungal and bacterial serine proteinases. We used SPI2 mutant which should also possess weak activity against trypsin. Three groups of transgenic tobacco plants were obtained and analyzed by Western blot. The recombinant proteins level in the leaves was estimated by ELISA and the biological activity of plant-produced hIL-2 (alone or in a fusion with SPI2 or CMTI) as well as both proteinase inhibitors were confirmed.

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Expression and purification of a mutated form of human GAD65 from transgenic tobacco leaves.

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Type 1 insulin-dependent diabetes (T1D) is caused by the autoimmune destruction of insulin-secreting beta cells, leading to a life-long insulin deficiency (Gepts, 1965). The young age of affected patients, the need for insulin therapy and the high prevalence of late-onset complications make T1D a major health problem.

The 65 kDa isoform of glutamic acid decarboxylase (GAD65), present on pancreatic islet beta cells, is one of the major autoantigens implicated in the development of human T1D (Baekkeskov et al., 1982) and it has been recently demonstrated in a Phase II Clinical Trial that two injections of the molecule can give protection to T1D. The final therapeutic aim for T1D is primary prevention because of the difficulty in identifying people at risk of T1D within the population. Vaccination studies and subsequent vaccination treatment of a large number of people would require a huge amount of purified protein, but the current production platforms are too expensive and unable to provide enough GAD65 to meet global demand.

GAD65 has previously been expressed in Nicotiana tabacum plants but yields were disappointing (maximum 0.25% of total soluble protein, TSP) (Porceddu et al., 2009; Ma S. et al., 2004; Wang et al., 2008; Avesani et al., 2003). In order to improve the recombinant protein expression level, we expressed a mutated form of the molecule with no catalytic activity (hGAD65mut), hypothesising that the enzymatic activity might interfere with its accumulation.

The mutated form of the molecule we used was previously described (Hampe et al., 2001) and it was characterised by the substitution of the amino acid residue responsible for cofactor binding in the catalytic site. We showed that GAD65mut accumulates to higher levels in transgenic plants (2.2% TSP) than GAD65 (Avesani et al., 2010), suggesting that the catalytic properties of GAD65 could contribute to its poor yields.

A 1% total soluble proteins (TSP) yield of a recombinant protein in transgenic plant is considered to be the minimum required to make the extraction of a plant-derived pharmaceutical protein economically viable. Given that we obtained GAD65mut maximum yield of about 2.2% TSP, the highest-expressing GAD65mut plants allowed us to begin the set up of all steps and parameters for the purification process of the recombinant protein from plant tissue and the evaluation of plant platform capacity to meet global demand of GAD65 protein for vaccination studies and treatment, in comparison to other expression platforms.

Within the Cost Action FA804 a Short Term Scientific Mission was held in the Fraunhofer Institute, in collaboration with Dr Schillberg’s team in order to plan a purification strategy for hGAD65mut from tobacco plants. The results obtained during the set up of the extraction and the first steps of the purification protocol are discussed.
Molecular characterization of antibodies expressed in plants

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Before the STSM applicant’s arrival two antibody framework constructs were designed, each of them in six variants. Those constructs were transferred to a T-DNA destination vector using the Gateway cloning system. After confirmation of correct sequences of constructs, Arabidopsis var. Colombia (Col O) plants were transformed using floral-dip method by Agrobacterium tumefaciens containing the appropriate constructs. Transformed plants were selected on medium with kanamycin, and the presence of the transgene was confirmed by PCR using specific primers and DNA extracted from seedlings. The T2 seedstocks of selected primary transformed plants were harvested individually.

The aim of the applicant’s STSM was to learn various methods subsequently used for molecular characterization of antibodies permanently expressed in A. thaliana seeds. It included especially the isolation of seed proteins, detection of target recombinant proteins and testing of their functionality. The relative amount of expressed antibodies and their functionality was established by functional ELISA of seed extracted proteins in samples of the same total soluble proteins concentration to divide them into high-, middle-, low- and non- recombinant antibodies expressing lines. According to the functional ELISA screening 5 lines with high- and 2 - 3 lines of middle-expression of recombinant proteins were chosen and the relative amount of antibodies was checked by SDS-PAGE/Coomassie Brilliant Blue R-250 or Western blot analysis. In order to obtain homozygous plants it is imperative to know the ones bearing transgene at a single locus. Plants with highest antibody expression were classified into single locus and multiple loci integration via segregation analysis.

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Dynamic trafficking of wheat γ-gliadin and of its structural domains in tobacco cells, studied with fluorescent protein fusions

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Prolamins, the main storage proteins of wheat seeds, are synthesized and retained in the endoplasmic reticulum (ER) of the endosperm cells, where they accumulate in protein bodies (PBs) and are then exported to the storage vacuole. The mechanisms leading to these events are unresolved. To investigate this unconventional trafficking pathway we fused wheat γ-gliadin and its isolated repeated N-terminal and cysteine-rich C-terminal domains to fluorescent proteins and expressed them in tobacco leaf epidermal cells. Our results indicated that γ-gliadin and both isolated domains were able to be retained and accumulated as protein body-like structures (PBLS) in the ER, suggesting that tandem repeats are not the only sequence involved in γ-gliadin ER retention and PBLS formation. We also report on the high actin-dependent mobility of γ-gliadin PBLS, and demonstrate that most of them do not colocalize with Golgi body or prevacuolar compartment markers. Both γ-gliadin domains are found in the same PBLS when coexpressed, which is most likely due to their ability to interact with each other as indicated by the yeast two-hybrid and FRET-FLIM experiments. Moreover, when stably expressed in BY-2 cells, green fluorescent protein (GFP) fusions to γ-gliadin and its isolated domains were retained in the ER for several days before being exported to the vacuole in a Golgi-dependent manner, and degraded, leading to the release of the GFP “core”. Taken together, our results show that tobacco cells are a convenient model to study the atypical wheat prolamin trafficking with fluorescent protein fusions.
Genetic engineering of barley – methods and applications

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Barley belongs to the most important crops worldwide. For this species, numerous genomics tools and resources such as specific cDNA libraries, EST databases, molecular markers as well as physical and genetic maps have been developed. As a result, many DNA-sequences are available for which a detailed functional analysis is desirable. Therefore, a powerful cereal transformation platform based on the use of Agrobacterium tumefaciens has been established in our laboratory. Either immature embryos or isolated microspores stimulated to undergo embryogenic development have been routinely used as gene transfer targets. The employment of these methods have resulted in the transformation of various spring and winter type cultivars of barley. Functional gene analyses and biotechnological approaches further require cell-specific promoters. In this respect, we are facing the general problem that most promoters from dicotyledons are not useful in monocotyledonous plants. The recent identification and utilization of several monocot-compatible promoters have resulted in the establishment of a number of valuable expression systems for barley, including those with specificities for the leaf epidermis, the endosperm or the egg cell. Examples are presented for the production of recombinant proteins in barley grains.
Expression analysis of sppA gene encoding bacteriocin sakacin P

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Protein expression in E.coli enables to obtain large amounts of the desired protein that can further be used for the antigen and vaccine production, as well as molecular immunology or structural, biochemical and cell biology studies. Sakacin P is a 61 amino acid residue long class Ila bacteriocin. The gene cluster of sakacin P comprises six consecutive genes transcribed in the same direction with the genes sppA and spiA encoding the sakacin P preprotein and the putative immunity protein, respectively.

In this study, the structural bacteriocin gene (achieved after oligonucleotides extension) was amplified by PCR with the specific primers for the sppA gene and the resultant product was cloned into pEAQ-HT or pM81-FSC2-POW vectors. The pEAQ-HT-sppA construct was transformed into Agrobacterium tumefaciens LBA4404 containing the disarmed Ti plasmid and bacterial suspensions were infiltrated into Nicotiana benthamiana for the sakacin P expression. Using this system, sakacin P is expected to be produced at levels equal to or higher than those obtained within the wild strains.
Expression of recombinant human lactoferrin in transgenic alfalfa plants

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Human lactoferrin (hLF), a valuable milk glycoprotein, known to have a lot of physiological functions such as antimicrobial, anti-inflammatory and immunomodulatory activities, antivirus and anti-cancer effects, is of increasing interest to molecular farming.

A suitable candidate for development of a new and efficient system for recombinant hLF production for clinical application is alfalfa (Medicago sativa L.) due to its favorable agronomic characteristics, lack of toxic compounds and the ease of in vitro manipulation.

In order to produce hLF in alfalfa plants a construct, containing hLF cDNA under the control of 35S CaMV promoter, was engineered. As selectable marker was used bar - gene, which expression in plant cells confers tolerance to L - phosphinotrocin (ppt). Plants from a highly embryogenic alfalfa clone from the Bulgarian cultivar Obnova 10 were transformed using Agrobacterium tumefaciens - mediated leaf disc method. Transgenic alfalfa plants were established from ppt - resistant calli via indirect somatic embryogenesis. The presence of hLF cDNA into the genome of the selected regenerants was confirmed by PCR analysis. RT-PCR and western blot assays showed expression of hLF in leaf tissue. Studies on antibacterial effect of the recombinant glycoprotein were conducted and resistance of the transgenic alfalfa plants to some bacterial pathogens was demonstrated.

The obtained results suggest that expression of hLF in alfalfa plants could be beneficial not only for producing recombinant protein for clinical application but also for crop quality improvement.
Molecular farming of selected viral antigenes for vaccination in *Arabidopsis* seeds

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Besides the conventional production platforms such as animal and insect cell cultures, yeast and bacteria, the use of transgenic plants is a promising alternative production system for high value recombinant proteins. Plants offer general advantages in terms of production scale and economy, product safety, and ease of storage and distribution. Recently, plants have been used as recombinant biofactories to express a number of proteins including pharmaceuticals and potential vaccines. Levels of expression are critical and vary greatly depending on the protein expressed, the plant species used for expression, and the expression construct design. Seeds have the useful advantage of accumulating proteins in a relatively small volume and stable environment.

Using the regulatory sequences of the seed storage protein genes *arceline 5-I* and *β-phaseolin* of *Phaseolus vulgaris*, a single-chain variable fragment (scFv) accumulated to exceptionally high levels as high as 36.5% of total soluble protein (TSP) in *Arabidopsis* seeds which is equivalent to approximately 7% of seed weight (De Jaeger et al., 2002). Also high accumulation levels of more complex scFv-Fc antibodies, corresponding to 2% of seed weight, were achieved using the same expression cassette in *Arabidopsis* seeds (Van Droogenbroeck et al., 2007; Loos et al., 2011).

Working towards the production of safe and effective vaccines for a number of animal pathogens, our research occupies seed specific molecular farming to generate a set of antigenic proteins and variants thereof. To this goal, selected viral protein formats from the Porcine Circovirus type 2 (PCV2) and the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) were expressed in *Arabidopsis thaliana* seeds. Transformants were screened and the proteins were analyzed by SDS-page analysis, Western blotting and ELISA. The obtained results, insights and further perspectives will be discussed.

References


Expression of chimeric *Chlamydia trachomatis* MOMP protein antigen in *Arabidopsis thaliana* and *Daucus carota*

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Urogenital chlamydial infection, caused by *Chlamydia trachomatis*, is the main sexually transmitted infection in Sweden. Despite active programmes for detection and case finding, nearly 37 000 cases were reported in 2010. Serovar E strains are considered to cause approximately 40-50% of these cases. A vaccine would be highly valuable in order to control the epidemic.

The major outer membrane protein (MOMP) of *Chlamydia trachomatis* is a highly antigenic and hydrophobic transmembrane protein. Our attempts to express the full-length protein in a soluble form in transgenic plants failed. A chimeric gene construct of *Chlamydia trachomatis* serovar E MOMP was designed in order to increase solubility of the MOMP protein but with retained antigenicity. The construct was based on known T and B cell epitopes located in the variable segment (VS) 2 and 4 loops of MOMP.

The designed construct was successfully expressed in *Arabidopsis thaliana*, and in *Daucus carota*. A chimeric MOMP expressed in and purified from *E. coli* was used as antigen for production of antibodies in rabbits. The anti-chimeric MOMP antibodies recognized the corresponding protein in the transgenic plants, as well as in inactivated *C. trachomatis* elementary bodies. Transgenic Arabidopsis and carrots were characterized for the number of MOMP chimeric genetic inserts and for protein expression. Stable integration of the transgene and the corresponding protein expression were demonstrated in Arabidopsis plants over at least six generations. Transgenic carrots showed a high level of expression of the chimeric MOMP– up to 3% of TSP.
Our group is involved in the development of new vaccines by exploiting plants both as biofactories (for safe and low-cost formulations) and as a source of innovative immuno-stimulating sequences/molecules with less clinical use constraints (i.e. auto-immune reactions/pre-existing T-cell immunity to the carrier) of some immune response modifiers currently tested in experimental DNA/protein-based immunization.

We are particularly focused on the Human Papilloma Virus (HPV)-derived cancers. In fact, the two commercially available prophylactic vaccines (based on L1/VLPs, virus-like particles), despite their recognized efficacy, have significant shortcomings: they are expensive and might be an insufficient tool to tackle cervical cancer worldwide. Second generation, low-cost prophylactic vaccines against high immunogenic, cross-reacting HPV antigens as well as alternative expression systems (DNA-, plant-based) may provide ways of overcoming these problems. Moreover, the development of therapeutic vaccination is necessary for the treatment of established HPV infections and related pre- and invasive oncologic disease of the lower genital tract, in co-operation with currently used surgical and radio/chemo-therapies. Such treatment would help the cure of high-risk HPV-associated pathologies such as 50% of tonsillar carcinomas, 40% of penis cancers and 25% of Head/Neck tumours. As a consequence, a therapeutic treatment wouldn’t need to involve the whole women population (like a prophylactic intervention), but would be restricted to already infected women/men, having remarkably lower health care costs with respect to the prophylactic vaccination currently undertaken.

A therapeutic vaccine against cervical cancer should trigger effector T-cell trafficking, overcome local immuno-suppression and generate acute inflammation at the tumour site. These requirements can be fulfilled improving the poor immunogenicity of the target antigen, in particular of the HPV E7 oncoprotein (a tumor-specific antigen, responsible for the onset and maintenance of the malignant status.).

In 2002 we demonstrated that a plant extract of *Nicotiana benthamiana*, obtained after the ectopic expression of the E7 from HPV type 16 (HPV16), induced a cell-mediated immune response in vaccinated mice able to protect from tumour challenge, notably even without adjuvant. Thereafter, it was demonstrated that the plant extract induced maturation of human dendritic cells and primed a HPV16 E7-specific cytotoxic activity. In parallel, we also produced a purified plant-derived E7 fusion protein by agroinfiltration, through an advanced plant viral expression vector, that was able to inhibit tumour development in mice and exhibited a dramatic therapeutic effect in presence of fully established tumours. This experimental vaccine is currently being tested in prime-boost heterologous (DNA/protein) experiments performed on a newly developed orthotopic mouse model for HPV-related Head/Neck tumors. Preliminary experiments indicate that the combination of DNA and plant-derived fusion-protein vaccine induce size reduction of mouth implanted experimental tumours. The characterization of the purified E7-fusion protein is under study with the aim to start a Phase I clinical trial. More recently, we started exploring the production of these vaccines in contained systems (i.e. roots and microalgae) and, in parallel, we are developing chimeric vaccines using mutant plant-derived sequences/genes (like the ribosome inactivating proteins) in genetic fusions with tumour-specific antigens, to enhance immunogenicity and efficacy of cancer vaccines.
A comparative study of the elicitation effects of coronatine and methyl jasmonate on taxane production in transgenic cell cultures of *Taxus media*

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The biotechnological production of the anticancer compound taxol and other taxanes used in the semisynthesis of its derivatives is a commercial reality for several pharmaceutical companies, but the low productivity of *Taxus* sp. cellular cultures requires the use of elicitors to increase taxane accumulation. We have recently developed a study with a cell line of *Taxus media* carrying the TXS transgene of *Taxus baccata* (Expósito et al. 2010) in which we compare the effects of two elicitors, methyl jasmonate (MeJA) and coronatine (COR), on growth, the taxane profile and production, and expression level of the genes that encode the enzymes TXS, DBAT, BAPT and DBTNBT, all involved in taxol biosynthesis. The results demonstrate that, over a period of 24 days, elicitation with COR (1 \(\mu\)M) is less harmful for *T. media* cells than with MeJA (100 \(\mu\)M). Although both elicitors effectively increased the taxane yield, there were variations in the production profile. As previously reported, high taxane productivity depends on the addition of elicitors, even when the cells overexpress the TXS transgene of *T. baccata*. Maximum levels of taxol were obtained in COR-elicted cultures, whereas the addition of MeJA significantly increased the contents of the precursor baccatin III. In addition, COR promoted the maximum excretion of taxanes to the culture medium compared to the control cells and those treated with MeJA.

Regarding the transcript accumulation of the studied genes, it was observed that the induction of *TXS, DBAT, BAPT* and *DBTNBT* was similar in the presence of 100 \(\mu\)M MeJA and 1 \(\mu\)M COR. In general, it is possible to conclude that both elicitors activate the expression of genes encoding enzymes involved in the initial phase of taxane biosynthesis as well as subsequent steps, and that the selective induction of key genes by each system of elicitation is responsible for the overall production of taxanes obtained.
Social Leverage of Intellectual Property in Molecular Farming

Harry Thangaraj and Rodrigo Corredor
Saint Georges University of London-ATP project

While several patents protecting pharmaceutical products are near to their expiration period, the race for innovative products able to deliver solutions to health problems in developed and developing countries is silently supported by years of basic research undertaken by public sector research institutions (PSRI) and universities. In this scenario, molecular farming is probably one of the most promising fields of research with potential to deliver solutions to health problems at a global scale.

Providing the right options to the economic and social concerns in an environment of extreme technological change have become a challenge for, both public and private entities dealing with biomedical R&D. According to the OECD, in recent years a surge on the implementation of collaborative and integrator business models as a response to the challenges imposed by the increase of disruptive and radical innovations in the biotechnological sector is challenging the capacity of PSRI to play an active role in the R&D process.

In the specific case of COST Action FA0804, the retrospective analysis of the activities framed in this research consortium has allow to depict a map of some relevant issues regarding the intellectual property management, namely the incidence of the use of proprietary technologies in the availability of economic and scientific resources required by member institutions. It has also allow for a better understanding of the effects of implementation of collaborative models such as consortia in relation with the aim to play a more active role in commercialization of end products and research tools obtained in the course of public fund research.

Accordingly, we focus our analysis on the identification of a set of measures that must be taken inside the consortium to improve interaction with transfer of technology offices charged to define IP strategies. From this perspective, introduction of mechanisms such as internal peer review inside consortia may help to define pro-market strategies that enable disclosure of some relevant research outcomes without eliminate the incentives from the private sector.

We assume that an improvement in the management of IP in the context of collaborative agreements may have a social impact in the context of molecular farming. The introduction of so-called humanitarian use exemption in licensing agreements with private sector is recognized as a potential vehicle for introduction of competition in local markets. However, access to technologies involved in molecular farming is also dependent on the absorptive capacity of developing countries, which is very limited due to the knowledge and capital-intensive nature of biotechnological research. Therefore to make molecular farming an attractive and affordable technology for both, developed and developing countries, further collaborative research must be based in other forms of collaborative agreements such as Patent Pools, Publics Private Partnerships or Public Domain initiatives which allows for a more comprehensive and framed negotiation of the diseases object of research and ensure the availability of the research results for targeted populations in developing countries. Although, market and welfare effects of implementation of those collaborative business models remain ambiguous.
Plants have emerged as an alternative platform for the production of recombinant proteins, known as Molecular Farming. In order to cope with uncertainties in organizing Molecular Farming, a conceptual framework based on cost accounting is built to evaluate the impact of conversion from existing enterprises. The case of conversion from a traditional greenhouse cropping like lettuce, tomatoes, cutting flowers, to a Molecular Farming cropping is studied. This is illustrated for the exploitation of *Arabidopsis thaliana* as host plant for recombinant protein production, and starting from a representative Flemish greenhouse setting. As Molecular Farming has to compete with two distinct decision levels (the traditional greenhouse cropping and the standard recombinant protein production platforms), the framework is based on opportunity cost comparison.

The conceptual framework and cost accounting model that is proposed can be used to locate the competition frontier for different product-platform combinations. Calculations of other case studies can be made by entering different values for recombinant protein prices, production costs and production volumes generated by a variety a possible Molecular Farming crops. Thus, despite case specificity, the framework can be generalized to different categories of proteins, plant-based systems, scales of production and cultivation infrastructures. The model can be used as a decision support tool used in farm-specific conditions. The strength of the framework is the graphical visualization of sensitivity analyses, clarifying various uncertainties that characterize the decision making process.
EU legitimizes GM crop exclusion zones

To the Editor:
On July 13, 2010, the European Commission (EC) officially proposed to give member states the freedom to veto the cultivation of genetically modified (GM) crops on their own territory without having to provide any scientific evidence relating to new risks. The objective of the legislation is ostensibly to make individual member states responsible for their own policy on GM crops, and therefore to speed up pending authorizations by removing the ability of those member states to veto approval throughout the European Union by avoiding a qualified majority (Fig. 1). However, we argue that the opt-out will have exactly the opposite effect to that intended, allowing the creation of arbitrary GM-free zones in Europe that will cause untold damage to the EU economy and its global scientific standing.

The removal of any need for scientific justification in decisions concerning GM crops effectively serves to legalize the currently illegal practice in which individual member states arbitrarily declare GM-free zones within their borders, or ban GM crops altogether. The only GM crops currently grown in Europe are the pest-resistant maize variety MON810 and the Amflora potato variety engineered to produce modified starch. Both are banned in Austria, Hungary and Luxembourg, and the MON810 event is also banned in France, Greece and Germany (Table 1). Poland is currently drawing up legislation to ban all GM seeds, and other member states are considering similar proposals. Although these existing and proposed bans are technically in breach of EU regulations, at least those member states implementing a ban have to make some sort of effort to justify their decision on scientific grounds, even if the evidence used in such cases is dubious (the ‘safeguard clause’). When the legal amendment enters into force, member states will be free to restrict or prohibit the cultivation of all or particular GM crops within their territory, including crops that have already been approved for cultivation under Directive 2001/18/EC and Regulation EC 1829/2003, and will be able to do so without explanation. This places the future of GM agriculture in Europe at the whim of politicians who may feel compelled to act in response to the media or activist propaganda.

EU policy on agriculture has evolved over the past 50 years as the continent has moved from the position of a net importer struggling to feed its population at the end of a devastating war, to today’s near trade parity with the rest of the world. Even so, the EU is still a net importer of agricultural raw materials and 55% of imports come from ten countries, with Brazil, the United States and Argentina ranking in the top three positions. The same three countries also happen to be the world’s largest adopters of GM technology, with the United States planting 64 million hectares of transgenic crops in 2009 and both Brazil and Argentina planting just over 21 million hectares. Paradoxically, although the proposed amendment will allow member states to adopt measures against the cultivation of GM crops, they will not be allowed to adopt measures prohibiting the import or marketing in the European Union of authorized GM products from elsewhere, which means that EU markets are likely to be flooded with imported GM products that could just as easily be homegrown. However, the import of GM products is also heavily regulated, as is particularly apparent in the EU’s treatment of imported maize and soybean from the United States and elsewhere, which has a substantial knock-on effect on animal agriculture. In this context, the European Union is deficient in feed protein and is ultimately dependent on soybean meal imports. However, imports have declined considerably (from $2.8 billion in 1997 to $1.9 billion in 2008), predominantly because of the complex and onerous process for approving imported GM products, which is administered at the member state level after the European Food Safety Authority (Parma, Italy) has issued opinions declaring that products are safe. The United States Department of Agriculture states that GM events take on average 15 months to approve in the

Figure 1  The previous voting habits of the EU ministers on matters concerning GM crops. Europe divides into two roughly equal camps (pro-GM in green and anti-GM in red) based on votes cast on 19 GM proposals between 2003 and 2005. In 2005, the pro-GM camp had a slight advantage because several of the most populous member states were pro-GM. More recently, there has been a slip towards the anti-GM stance with Germany and France now adopting an anti-GM position, although they are more likely to abstain on proposals rather than actively oppose. This is reflected in the voting for the approval of the Amflora GM potato on February 18, 2008. (Data from Friends of the Earth; symbols represent votes for, against and abstentions; not all countries voted on every proposal; the authors thank Michael Green for assistance in the preparation of this figure.)
1.2 million metric tons of CO₂ saved from 35 million metric tons of pesticides, over tillage). In 2008, these savings amounted to the need for spraying, and by eliminating use and also fuel consumption by reducing agriculture (that is, by reducing pesticide environmental footprint of conventional that biotech can be used to limit the GM adoption rates have also recognized and other countries with substantial presence from surrounding farms. will be fined accordingly, and risk litigation from surrounding farms.

The United States, Brazil, Argentina and other countries with substantial GM adoption rates have also recognized that biotech can be used to limit the environmental footprint of conventional agriculture (that is, by reducing pesticide use and also fuel consumption by reducing the need for spraying, and by eliminating tillage). In 2008, these savings amounted to 35 million metric tons of pesticides, over 1.2 million metric tons of CO₂ saved from the use of fossil fuels and an additional 13.2 million metric tons of CO₂ sequestered into the soil through the implementation of no-tillage policies. If the EU continues obstructing the deployment of GM crops in Europe, it will force farmers to use environmentally hazardous, expensive and unsustainable agricultural practices, spend unnecessary resources on fossil fuels and agrochemicals, while at the same time letting the same products be imported from the United States and South America, further tipping the balance of trade in the wrong direction.

One of the reasons for the low take-up of GM crops in Europe is low consumer demand and public trust in the technology compared with conventional or organic crops. Mistrust of GM crops by the European public is hard to rationalize, given that >70% of processed foods consumed by humans in the United States and Canada contain GM ingredients, a similar proportion of white maize in Africa is transgenic and several GM products are consumed by humans in China, all with no reported ill effects after 10 years. These countries also export GM seeds to other markets (including the European Union) with no reported incidents.

The failure of the EU to support the adoption of GM crops is symptomatic of the largely unsuccessful Lisbon Agenda, which set out in 2000 to change the EU into a highly competitive knowledge-based economy with innovation, economic vitality, social and environmental renewal and sustainability as its core values. GM crops are an innovation supported by many EU research organizations and they have already proven successful in other countries in terms of economic growth, environmental sustainability and competition. Yet every possible obstacle has been erected in the European Union to prevent this beneficial form of agriculture from being adopted, leading to economic stagnation, trade disputes and the continued destruction of the environment through chemical use and intensive tillage.

One additional impact of the EU’s policy on GM agriculture is to discourage homegrown research in agbiotech and drive researchers overseas where the value chain can be realized in terms of released GM crops. Within the European Union, researchers working on transgenic plants know that the best they can expect for their products is greenhouse cultivation, and that despite their benefits, transgenic plants are unlikely to be deployed in any setting where they could perform a useful function. Here the EU policy on GM crops is attacking its own foundations as a competitive bioeconomy because with one hand the EC offers funding for innovative biotech research and values (or even requires) the

### Table 1 National bans currently implemented under the ‘safeguard clause’

<table>
<thead>
<tr>
<th>Country</th>
<th>Event</th>
<th>Date</th>
<th>Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>BT176 maize</td>
<td>1997</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>MON810 maize</td>
<td>1999</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>T25 maize</td>
<td>2000</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>GT73 rapeseed</td>
<td>2007</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>MON863 maize</td>
<td>2008</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>Ms8 rapeseed</td>
<td>2008</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>Rf3 rapeseed</td>
<td>2008</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>Ms8/Rf3 rapeseed hybrid</td>
<td>2008</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>EH92-527-1 potato</td>
<td>2010</td>
<td>Import</td>
</tr>
<tr>
<td>France</td>
<td>Topas 19/2 rapeseed</td>
<td>1998</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>MS1/R1 rapeseed hybrid</td>
<td>1998</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>MON810 maize</td>
<td>2008</td>
<td>Cultivation</td>
</tr>
<tr>
<td>Germany</td>
<td>BT176 maize</td>
<td>2000</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>MON810 maize</td>
<td>2009</td>
<td>Cultivation</td>
</tr>
<tr>
<td>Greece</td>
<td>BT176 maize</td>
<td>1997</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>T25 maize</td>
<td>1997</td>
<td>Import</td>
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<tr>
<td></td>
<td>Topas 19/2 rapeseed</td>
<td>1998</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>MS1/R1 rapeseed hybrid</td>
<td>1998</td>
<td>Import</td>
</tr>
<tr>
<td></td>
<td>MON810 maize</td>
<td>2001</td>
<td>Cultivation</td>
</tr>
<tr>
<td>Luxembourg</td>
<td>BT176 maize</td>
<td>1997</td>
<td>Cultivation</td>
</tr>
<tr>
<td></td>
<td>MON810 maize</td>
<td>2009</td>
<td>Cultivation</td>
</tr>
<tr>
<td>Hungary</td>
<td>MON810 maize</td>
<td>2005</td>
<td>Cultivation</td>
</tr>
</tbody>
</table>

*These bans were amended in 2008.*
The new EC recommendations relating to the approval of GM crops in Europe aim to combine the EU science-based authorization system with freedom for member states to decide on their own cultivation policies. We firmly believe that these concepts are irreconcilable because the freedom of member states to ban GM crops is granted with no conditions, allowing decisions to be made based on irrational criteria (‘any grounds’) and essentially rendering the whole process of risk assessment obsolete. The new recommendation on co-existence recognizes that member states may adopt measures to avoid the unintended presence of GM material in other products below the labeling threshold of 0.9%, and such measures may include the restriction or outright banning of GM agriculture within their borders. Such measures should be proportionate to the objective, but obviously they are not, given the arbitrary and excessive isolation distances that are enforced without any scientific data to show that such distances are necessary. The recommendations apply to cultivated GM crops but not to imported GM products, even if the two represent identical events. This is clearly an illogical position to adopt. Any safety concerns about commercialized GM products should apply equally to cultivated and imported material, and the artificial distinction only serves to highlight the double standard that is being employed in this context. The EC states that “…ensuring a safety assessment following the highest scientific standards and a reinforcement of the monitoring function were and remain priorities for the Commission as concerns GMO cultivation…” while at the same time stating that no grounds are required at all for member states to limit or ban GM agriculture. This clearly is not a high scientific standard by any stretch of the imagination. The proposed legislation does nothing to smooth the authorization process for GM crops in Europe but does provide activists and the media with fuel for their anti-GM propaganda and will no doubt cause massive confusion among the public. The EC proposal also contrasts with one of the EU’s key goals (that is, the creation of a free market economy without border controls) because it imposes arbitrary segregation with respect to GM and non-GM agriculture. Although the authorization system is still science-based, the EC has rendered this system toothless by giving member states the means to ignore or overturn regulatory guidance and allow the implementation of policies that have no rational basis by people who have no accountability.

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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