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Poster Abstract – E.01

IN SEARCH OF A SELECTABLE MARKER GENE FROM *MEDICAGO SATIVA*

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GSA-AT, Gabaculine resistance, Alfalfa

Selectable marker genes (SMG) for plant genetic engineering are mostly derived from bacteria, and very few derive from plants; however, plant SMG could be perceived as safer for human health and the environment.

In plants, the enzyme glutamate 1-semialdehyde aminotransferase (*GSA-AT*) catalyses the conversion of glutamate-1-semialdehyde into aminolaevulinic acid, a step in the synthesis of tetrapyrrole compounds including chlorophyll. This enzyme is irreversibly inhibited by gabaculine (3-amino-2,3-dihydrobenzoic acid). In a gabaculine resistant mutant strain of *Synechococcus elongatus,* a gene encoding a mutated GSA-AT was isolated, that has been successfully used as a selectable marker gene in genetic transformation of tobacco Gough et al. 2001) and *Medicago sativa* (Rosellini et al., unpublished). The mutant bacterial protein differs form the wild type four a 3-aminoacid deletion close to the ammino terminus and a substitution in the catalytic domain.

We found that plant GSA-AT have high similarity (more than 70% at the aminoacid level) with this bacterial enzyme. Therefore, we decided to test the possibility to obtain a gabaculine resistant plant GSA-AT that could be used as selectable marker gene.

To this purpose, we took advantage of a published *Medicago truncatula* tentative consensus sequence (TC93991) highly homologous to GSA-AT, and designed primers to amplify the coding sequence using *Medicago sativa* and *Medicago truncatula* cDNA as templates. Single PCR products of the expected size were obtained, cloned and sequenced. Sequence analysis revealed that the cDNAs from the two species indeed encode GSA-AT, and have about 98% identity at both the nucleotide and aminoacid level.

Multiple alignment was performed to investigate the conservation of the active domains of the protein and the phylogenetic realationships among GSA-AT from plants.

We hypothesize that a single aminoacid substitution in the highly conserved catalytic domain could be sufficient for gabaculine resistance.

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Poster Abstract – E.02

EXCISION OF A SELECTABLE MARKER IN TRANSGENIC WHEAT (*TRITICUM AESTIVUM*) USING A CHEMICALLY REGULATED Cre/loxP SYSTEM

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Marker-free transformation, wheat, Cre/loxP, β-estradiol induction

Selectable marker genes are required to ensure the selection of transgenic plants during plant transformation. However, once transformation is accomplished, the presence of a marker gene in transgenic plants becomes undesirable, particularly as most of the selectable marker genes currently used confer resistance to antibiotics and herbicides.

The removal of selectable marker genes will not only lead to the elimination of potential environmental and health-related risks as well as technical barriers, but also increase the consumer acceptance of GM crops and their products.

Several strategies have been employed to remove selectable markers from transgenic plants.

Recently, chemical-inducible Cre/loxP DNA recombination systems (CLX) have emerged and seem to provide a highly reliable method to generate marker-free transgenic *Arabidopsis* and rice plants in a single step transformation.

Wheat is a recalcitrant species and, in comparison with rice and maize, progress in this species has been slower. A robust method for the transformation of wheat has only recently been developed. Furthermore, in wheat, selection can be carried out only after the regeneration phase, otherwise no plants will regenerate. For this reason, it is not possible to obtain transgenic marker-free wheat plants by single step transformation - chemical induction needs to be carried out on the T1 generation after T0 selection.

We have transformed wheat plants with pX6-GFP, which contains the CLX system. Transgenic plants were grown in order to obtain seeds and the immature embryos were then collected and subcultured on callus induction medium for 10 days. The calli were then transferred onto fresh medium containing β-estradiol for 20 days.

Excision of the marker gene was monitored through the detection of GFP fluorescence.

Fluorescent calli were transferred onto regeneration medium in order to obtain marker-free plants.

These plants will be molecularly analysed in order to verify the excision and the efficiency of this chemically-inducible system.

Proceedings of the 50th Italian Society of Agricultural Genetics Annual Congress
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Poster Abstract – E.03

CO-TRANSFORMATION FOR MARKER-FREE GENETICALLY ENGINEERED ALFALFA

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gabaculine, GSA-AT, Medicago sativa, transgenic plants

Among the arguments against Genetically Modified Plants (GMPs) is the fact that they contain, linked to a useful gene, an antibiotic or herbicide resistance gene serving as selectable marker (SMG) to facilitate the process of *in vitro* selection of transgenic cells. The development of techniques to avoid the use of these SMGs could improve public perception of plant genetic engineering.

In co-transformation, the useful gene and the SMG are introduced together, but using separate vectors, so that they are integrated in different genomic sites. Progeny plants carrying only the useful gene can then be obtained thanks to genetic segregation.

A mutant *Synechococcus* gene encoding glutamate 1-semialdehyde aminotransferase was used as the SMG for alfalfa genetic transformation in this experiment. This gene has been shown to confer resistance to gabaculine (3-amino-2,3-dihydrobenzoic acid) in tobacco (Gough et al. 2001) and performs very well in alfalfa (unpubished data from our lab). The conventional SMG *Npt*II played the part of the useful gene in this experiment. Both genes were placed under the control of the Dual CaMV35S promoter and the *Nos* polyadenylation sequence.

Alfalfa leaf explants were co-cultivated with a mixture of two *Agrobacterium* (strain LBA4404) cultures, each harbouring a pPZP201BK binary vector containing either the *hem*L or the *Npt*II cassette. Regeneration was performed on gabaculine-containing media, and each mature somatic embryo was divided into two parts and transferred onto regeneration medium containing either gabaculine or kanamycin in order to check for the expression of *hem*L and *Npt*II.

In a first experiment, 10 kanamycin resistant events were obtained in 107 gabaculine resistant events. So far, 5 of these 10 events have been tested by PCR for the presence of the *hem*L and *Npt*II genes, and three were positive for both.

In a second experiment we have 5 kanamycin resistance events in 83 gabaculine resistant events.

The relatively low efficiency of co-transformation that we obtained may be due to the presence of homologous control regions in the two constructs, involving silencing of the non selected gene (*Npt*II) during the first regeneration cycle on gabaculine. To check this hypothesis the experiment will be repeated using different control sequences for the two genes. In any case, our results indicate that an acceptable number of co-transformed plants can be obtained without much effort. The number of copies and independence of the two genes is being assessed by Southern blot experiments. Three plants containing both genes were crossed to a non transgenic, unrelated pollen parent and the segregation of the two genes will be assessed in the progeny.

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Poster Abstract – E.04

MARKER GENE REMOVAL AND ALTERNATIVE MARKER GENES FOR AN IMPROVED GENE TRANSFER IN GRAPE

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Vitis, marker gene, gene transfer, self excision, antibiotic resistance

Improvement of grape molecular breeding was attempted by exploiting two strategies based respectively on the marker gene excision and the use of marker genes alternative to antibiotic resistance.

To exploit the self-excision, embryogenic calli of *V. vinifera* (cvs. Chardonnay, Brachetto), and the rootstock 110 Richter were co-cultured with *Agrobacterium tumefaciens* carrying the chemically-inducible site-specific *cre/loxP* pX6 vector with the genes for the Green Fluorescent Protein (GFP) and for the neomicin phosphotransferase (NPTII). In this construct, kindly provided by prof. Nam-Hai Chua (The Rockefeller University, New York), the expression of the *cre* recombinase is regulated by the 17-β-estradiol (Zuo *et al.*, 2001, *Nature Biotechnol.* 19 :157-161) and should result in the NPTII gene excision and the consequent GFP gene expression. Besides, the construct was also tested on *Nicotiana benthamiana* as model system. Putatively transgenic cultures were selected on kanamycin, and individual somatic embryos were isolated and converted into plantlets. Preliminary molecular assays showed the transfer of the GFP gene into the plant genome, and observations at the fluorescence stereomicroscope proved its expression, as the result of the successful induction of the marker gene self-excision mechanism.

To test the efficiency of the constructs based on marker genes alternative to antibiotic resistance genes, we verified the possibility to make cells able to metabolize specific carbon sources, such as mannose as substitute to sucrose (Posytech, Sygenta licence). This strategy has proved successful with various plants, however gave variable results in grapes (Reed *et al.*, 2001, In Vitro Cellular & Devel. Biolol. Plant, 37:127-132; Kieffer *et al.*, 2004, Vitis 43:35-39). The effect of the selective medium on the callus growth ability and morphogenic potential was verified. Embryogenic calli of *V. vinifera* cv. Brachetto were grown and monthly subcultured on different formulations of the same medium containing mannose, sucrose or free of the carbohydrate source, respectively. Calli cultured on the substrate containing mannose or sucrose showed a similar growth rate. As expected, no growth was observed in calli placed on the medium without carbohydrate source, which progressively turned brown and died.

At this point of the work, the removal of the marker gene seems to be the most promising between this two strategies compared for grape gene transfer. First of all, tissue culture conditions related to the PMI gene still require a labor-intensive optimization in the view of accurately selecting transgenic- from non-transgenic cells. On the contrary, the presence of the NPTII gene in the pX6 vector allows an effective selection among cell lines. Moreover, the elimination of the

marker gene after induction with 17-β-estradiol allows to take advantage of an effective selection method, and also to perform the actual elimination from the grape cells of the non-desired genetic material that were inserted during the gene transfer process.

Authors wish to tank Ivana Gribaudo (CNR, Unità Grugliasco) for providing us with the somatic embryos cultures. This research is supported by the Trento Autonomous Province (Project *EcoGenEtic.Com*).

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Poster Abstract – E.05

TRANSGENIC POPLAR AGAINST LEPIDOPTERA: AN ITALY-CHINA PROJECT FOR BIOSAFETY AND CONSERVATION

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resistance gene, resistance management, Populus, Cry IAb, refuge area

The area under genetically engineered plants producing *Bacillus thuringiensis* (Bt) toxins is steadily increasing. This increase has magnified the risk of resistance alleles being selected in natural populations of target insect pests. The speed at which this selection is likely to occur depends on the genetic characteristics of Bt resistance but also on the resistance management plans. Poplar plants expressing CryIAb against several species of Lepidoptera were selected. Initially they were growth in alternate rows (one row of wild type and one row of transgenic plants), however, considering several parameters, we suggest a different model for plantation strategy of GM plants and constitution of refuge areas. This approach was developed in the framework of the project "Sustainable research and development in biotechnology applied to the protection of the environment, in collaboration with the Popular Republic of China" Sponsored by the Italian Ministry of Environment, to implement and improve a resistant management plan according to the different species of pests and to the Chinese environment and regulations. A similar approach could be adopted also in Italy, in the case of a future liberalisation of Bt poplar transgenic cultures. Moreover the helpfulness of male-sterile /Bt poplars in the resistance management and on the environmental impact is discussed. In fact the release of commercial Bt poplar trees was made possible by an early Chinese regulatory system for transgenic cultures, somehow looser than the North American and European one. The Italian- Chinese project aims at the development of guidelines that could answer to European biosafety requests as well as from the new rules introduced in China aimed at the best preservation of this culture very important for the Asian Country.

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Poster Abstract – E.06

A TRANSGENIC WHEAT LINE WITHOUT ANTIBIOTIC AND HERBICIDE MARKER GENES

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pmi, phosphomannose isomerase, marker genes, HMW glutenin subunit, clean gene

Genetic engineering of monocots is usually carried out by direct methods such as particle bombardment or protoplast transformation and involves the use of selective agents and marker genes to favour the multiplication of the initially transformed cell. Antibiotics or herbicides are used as selective agents. However, both may have some undesirable effects. It has been argued that there might be a risk, that the antibiotic-resistance gene could be transferred from plants to pathogenic bacteria while herbicide resistance might be transferred to weeds. Moreover the routine production of transgenic plants involves transformation with foreign DNA carried on plasmids, and causes the integration of vector backbone sequences into the genome along with the transgenes.

Several strategies have been developed to avoid the use of antibiotic and/or herbicide marker genes in plant transformation. Among them there are the elimination of marker genes by recombinases or the use of alternative marker genes.

In the present work durum wheat transformation was carried out with minimal gene cassettes, which were linear DNA fragments lacking vector sequences excised from the plasmids. Two transformation experiments were carried out using as target genes two wheat sequences encoding the Dx5 and Dy10 HMW glutenin subunits and *phosphomannose isomerase (pmi)* gene as the selectable marker. Phosphomannose isomerase catalyzes the reversible interconversion of mannose-6-phosphate and fructose-6-phosphate, allowing plant cells to utilize mannose as a carbon source and to grow and differentiate on media containing mannose. PCR assay of T_1 generation identified fifteen transgenic plants of which the line TC-52 was subsequently investigated. A stable integration and expression of Dy10 HMW glutenin subunit gene was confirmed by protein gel analyses of T_1, T_2 and T_3 generations. In order to identify transgenic lines free of marker genes, segregation analysis of *Dy10* and *pmi* genes was conducted in 150 T_3 progenies. The absence of co-segregation of the two genes suggested the localization on two different chromosomes and allowed the identification of transgenic lines completely marker free.

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Poster Abstract – E.07

VERIFICATION OF THE PRINCIPLE OF SUBSTANTIAL EQUIVALENCE IN A TRANSGENIC BREAD WHEAT LINE OVER-EXPRESSING A LOW-MOLECULAR-WEIGHT GLUTENIN SUBUNIT BY MEANS OF A PROTEOMIC ANALYSIS OF ENDOSPERM PROTEINS

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transgenic wheat, substantial equivalence, glutenin, proteome, allergenic proteins

Recent efforts to increase the quantity of specific wheat gluten proteins, which are directly correlated with the quality of end-use products, have focused on the introduction of additional gene copies by means of genetic engineering. With the aim of improving the quality traits of wheat-derived end products, we have produced and characterized a transgenic bread wheat line over-expressing a LMW (low-molecular-weight) glutenin subunit.

In order to define the consequences of transgene(s) insertion/expression and the effects of genetic transformation on the global abundances of the various classes of endosperm proteins, we have carried out an extensive, comparative proteomic profiling between the seeds of the transgenic line with their corresponding non-transformed counterpart. The investigation of the consequences of this transgenic event on the global endosperm protein expression is also of particular interest in the context of defining the "substantial equivalence" of this particular transgenic wheat line, since major concerns of genetic modification regard the potential of unintended side effects, which could originate depending on transgene integration site(s), spatial and temporal expression and/or from its putative interactions within several pathways of plant metabolism.

Proteomic analyses showed that, during the seed development, several classes of endosperm proteins were significantly differentially regulated. As a result of the high over-expression, and subsequent accumulation, of the transgenic LMW glutenin subunit, HMW (high-molecular-weight) glutenins and all sub-classes of gliadins were heavily down-regulated during seed filling in the transgenic genotype. Similarly, we also had evidences that CM-like proteins, a class including several components involved in food allergy to wheat, are less abundant in the transgenic genotype with respect to the wild-type. We also investigated the relative abundances, in developing endosperms, of the metabolic protein fraction, which showed only minor differences between the transgenic and the wild-type genotype. Protein identification of differentially expressed spots is currently underway, using a combination of N-terminal sequencing and MS.

In all protein classes, the differential expression detected by proteomic analyses, both in mature and developing seeds, had also been suggested by the corresponding transcript abundances assessed by comparative microarray experiments (Scossa et al., SIGA 2005).

In our opinion, this particular transgenic bread wheat line does not seem to contradict the principle of substantial equivalence, at least for that regarding the development of novel allergenic polypeptides in the endosperm, although showing significant transcriptional and proteomic

differences. Such differences, in fact, do not involve polypeptides implicated in differential allergenic properties with respect to its comparator. In conclusion, the global magnitude of the differential expression observed in this transgenic bread wheat line seems not greater than the natural variability usually observed in the cultivars obtained by traditional breeding methods.

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Poster Abstract – E.08

A METABOLOMIC STUDY OF TRANGENIC MAIZE (*ZEA MAYS*) SEEDS REVEALED VARIATIONS IN OSMOLITES AND BRANCHED AMINO ACIDS

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GMO, metabolomics, multivariate analysis, NMR, Zea mays

Aim of research was to investigate metabolic variations in the grains associated to genetic modifications of *Zea Mays*, using metabonomic techniques. Concerning this point, non-targeted characteristic of the technique is useful to identify metabolites peculiar to the genetic modification and initially undefined. The obtained results showed that the genetic modification, introducing *Cry1Ab* gene expression, induces metabolic variations involving primary nitrogen pathway. Concernig methodological aspects, the experimental protocol used has been applied for the first time in this field. It consists of a combination of Partial Least Squares-Discriminant Analysis and Principal Component Analysis (PCA). The most important metabolites for discrimination were selected and the metabolic correlations linking them are identified. PCA on selected signals confirms metabolic variations, highlighting important details about the changes induced on the metabolic network by the presence of Bt transgene in the maize genome.

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Poster Abstract – E.09

COMBINING POLYPLOIDY AND PARTHENOCARPY TO ENSURE GENE CONTAINMENT AND PROMOTE THE COEXISTENCE OF CONVENTIONAL AND GM CROPS

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gene containment, polyploidy, Solanum lycopersicum, tomato

In a tomato production field, near-sterile variants appear spontaneously as diploid (21%), triploid (68%), tetraploid (5%) or aneuploid (6%) plants. The first artificial triploid (3x) was obtained by Jörgensen in 1928 by crossing 4x with 2x strains; with the same procedure few others were produced later on. Triploid plants showed features that were intermediate between diploids and tetraploids and exhibited a pollen and ovule fertility that was reduced to 5 and 15% of the diploid, respectively. Seed set was also extremely low (1-2% of the diploid), even when the triploid was hand-pollinated with 2x pollen. Triploid plants were therefore practically unfruitful, due to the high gametic and zygotic sterility. Differently, tetraploid plants showed a satisfying fruit set, but fruits were highly variable in size due to the low and variable number of seeds.

Recently, in our lab, the first fruitful tomato triploid plants were synthesized by crossing 4x with 2x somaclones. The synthesis of 3x plants was achieved in both a wild-type (cv Chico III genetic background) and a near-isogenic line carrying the parthenocarpy allele *parthenocarpic fruit* (*pat*). In addition to parthenocarpy, the *pat* mutation shows reduced pollen production and ovule viability. In this work, data about pollen and seed fertility of the two near-isogenic (*pat* and wild-type) series of 2x, 3x and 4x plants are reported. The triploid parthenocarpic plants produced a reduced amount of pollen with very low viability; however, they resulted very fruitful although the fruits were completely seedless. Therefore, in addition to preventing gene flow by pollen, parthenocarpic triploids avoid the contamination of soil with plenty of seeds as it happens in open field during mechanical harvest of conventional processing tomato cultivars. Taken together, such properties make 3x parthenocarpic plants a truly innovative breeding material, suitable to permit the coexistence of transgenic and conventional tomato cultivations and crop rotation.

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Poster Abstract – E.10

GENETIC TRANSFORMATION OF ELITE *P.* X *CANADENSIS* CLONES WITH A MORPHOLOGICAL MARKER GENE.

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Populus x *canadensis, Neva, genetic trasformation, Agrobacterium tumefaciens, ipt gene*

Interspecific hybrid poplars (*P. deltoides x P. nigra*) usually known as *P.x canadensis* are intensively cultivated worldwide for very interesting commercial wood properties. Despite their importance, relatively few research works have reported successful results *in vitro* culture and genetic engineering to *P x canadensis* hybrid clones (Confalonieri et al., 2003). Actually it is very difficult to establish and manipulate *in vitro* culture tissues of most of these clones for their high recalcitrance (Busov et al., 2005). In this work we show the results *in vitro* manipulation of clones *P .x canadensis*, selected by C.R.A. - Poplar Research Institute of Casale Monferrato and largely used in intensive poplar cultivations around the world. We tested a number of regeneration protocols using different media and phytormone combinations until we obtained a single regeneration protocol with an efficiency close to 100%. Afterwards we have conducted some genetic transformation experiments employing disarmed vectors carrying either reporter or selective marker genes (*uidA, bar, nptII*). We also employed a monitoring vector (MAT-type) carrying a morphological marker gene (*ipt*) in our transformation experiments. PCR analysis to assess the presence of the *nptII* gene were performed. We obtained positive results only with MAT-vector system. Moreover we selected putative transgenic lines through observation of anomalous phenotypes (*ipt*-type) and through GUS histochemical assays. We observed 6,7 % and 8,9 % of *ipt*-phenotypes in Neva and I-214 clones, respectively. Molecular analysis and genetic transformation experiments with other *P. x canadensis* clones are currently under way to confirm our preliminary results. Considering the commercial importance of hybrid *P. x canadensis*, these results are very interesting to obtain transgenic plants without antibiotic resistance genes, for a possible use in the open field cultivation, in compliance to the current law on OGM.

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Poster Abstract – E.11

COMPARISON OF FIVE REAL-TIME PCR VALIDATED METHODS FOR SPECIFIC IDENTIFICATION AND QUANTIFICATION OF *ZEA MAYS*

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maize, real-time PCR, endogenous genes

According to the European Regulations (Reg 1829/2003; Reg 1830/2003), to obtain the authorization to place on the market a Genetically Modified Organism (GMO) for feed and/or food use, the applicant must submit a dossier containing detailed information about several aspects such as description of the GM trait, risk analysis studies and other relevant issues. One of the most important tools to be provided by the applicant to the enforcement system is a suitable method for detection identification and quantification of the transformation event.

The more widespread techniques for GMO detection and quantification, are based on PCR and real time PCR, for qualitative and quantitative analysis respectively. The GM percentage for a single food/feed ingredient is calculated by the ratio of the GM vs. the food/feed ingredient DNA content. This ratio is obtained by comparing the quantitative results of two different real time PCR: GM event-specific one, and species-specific one. The CRL (Community Reference Laboratory for GM food and feed) has to evaluate and validate the method provided by the applicant on the bases of defined guidelines. Since different applicants generally submit different species-specific real time PCR methods for the same species, a number of *Zea mays* species-specific real time PCR has been validated by the CRL so far.

In order to harmonize methods among the enforcement laboratories involved in GMO detection, we compared five validated real time PCR methods for maize quantification whose PCR targets are *adh* (alcohol dehydrogenase) gene for three of them, and *hmg* (high mobility group) gene for the other two.

Template DNA extracted from seven GM maize Certified Reference Material (CRM) and a pool DNA template made by mixing an identical portion of each extracted DNA were tested under repeatability conditions (same operator using the same equipment within short intervals of time) , at several dilution levels, according to the five real time PCR methods. Slopes and R^2 coefficients obtained from the adopted regression model and other validation parameters were compared, within the same method and among all the five methods, by analysing their distribution at statistical level.

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Poster Abstract – E.12

QUALITATIVE AND QUANTITATIVE PCR ANALYSIS OF DIFFERENTLY PROCESSED ROUNDUP READY SOYBEAN

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roundup soybean, agri-food supply chain, qualitative PCR, real time PCR

Eight types of processed Roundup soybean materials, namely, seeds, cracked seeds, expander, crude flour, proteic flour, crude oil, degummed oil and lecithin, all derived from different steps of an industrially production food supply chain, were examined by qualitative and quantitative PCR. Specific combinations of primers differentiated sequences from the whole insert. The amplification of marker PCR fragments long not more than 500 base pairs was successful both in raw material (seeds) and in partially (cracked seeds) and highly (flours, crude and degummed oils and fluid lecithin) processed materials. Real time quantification was also applied as a screening assay in all processed foods. Results obtained show that the developed screening, gene-specific and construct-specific markers can be used in HACCP protocols for the control and traceability of GMO contamination of food and feed products containing soybean, according to the current EU regulations for the labelling and traceability of novel foods.

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Poster Abstract – E.13

FATE OF FEED-INGESTED PLANT DNA IN MONOGASTRICS AND RUMINANTS

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horizontal gene transfer, plant DNA survival, rabbit, water buffalo

The effect of the digestion process in the gastro-intestinal tract (GIT) of animal models on the fate and integrity of plant DNA has been widely evaluated since DNA availability and integrity is a key factor for hypothetical gene transfer of recombinant DNA from GM-crop-derived feeds to animal and human gut microflora and it is therefore one aspect of risk assessment for novel food and feed.

Using a real-time PCR approach to track plant DNA in animal samples, we have evaluated the persistence of feed-ingested DNA in blood and tissues of buffaloes and rabbits raised with conventional feeding and slaughtered under routine conditions (Tudisco et al., in press).

The results obtained demonstrate that fragmented plant DNA can be recovered from several tissues and from GIT of water buffalo, with maximum level of persistence for kidney. In rabbits the presence of fragmented DNA sequences from low and high copy number plant genes has been detected in blood, muscular tissues, organs and GIT.

In conclusion, our data can add some information on the fate of feed plant DNA in farm animals to widen the case histories available.

Tudisco R, Infascelli F, Cutrignelli MI, Bovera F, Morcia C, Faccioli P, Terzi V. Fate of feed plant DNA monitored in water buffalo (*Bubalus bubalis*) and rabbit (*Oryctolagus cuniculus*). Livestock Production Science, in press.