

CHARACTERISATION OF THE 3' INTEGRATION SITE IN MON810 YIELDGARD® MAIZE COMMERCIAL LINES

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Zea mays, MON810 event, Bt resistance, 3' insertion site analysis

The interest for the stability and biosafety of the genetically modified (GM) crops has increased in the last 20 years, together with their diffusion on the world market, causing a strong demand for the development of both analysis and detection methods. In Europe, among the mandatory labelling requirements for GM plants and derived food, the analysis of the insertion site and the characterization of the transgene flanking regions are requested. YieldGard® MON810 maize, produced by Monsanto, is one of the major GM crops. It contains the CaMV 35S promoter, the *hsp70* intron of maize, the *cryI(A)b* gene for resistance to lepidoptera, and the NOS terminator. After commercialization, Hernandez and collaborators (2003) evidenced a truncation event at the 3' end of the *cryI(A)b* gene with the complete loss of the NOS terminator. Moreover, the 3' maize genome junction region isolated did not show any homology with known sequences.

Here we describe the isolation of a larger portion of the 3' integration junction from genomic DNA of two MON810 maize lines commercially available in Spain (www.oecd.org/dataoecd/1/48/33999570.PDF) and the molecular characterisation of the insertion site in the genome of *Zea mays*. Specific primers were designed targeting the 3' integration junction in the plant amplifying a 475 bp fragment downstream of the sequence previously detected (Hernandez *et al.*, 2003). *In silico* characterization identified the 3' flanking region as a putative gene coding for the HECT E3 ubiquitin ligase (Moon *et al.*, 2004). Expression analysis of this region evidenced a read-through transcription giving rise to different RNA variants. Finally, results obtained suggested that the insertion of MON810 cassette in the maize genome caused a complex recombination event.

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VECTOR BACKBONE INTEGRATION IN GENETICALLY ENGINEERED ALFALFA

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GMO, vector backbone, Medicago sativa, Agrobacterium

Gene transfer from *Agrobacterium tumefaciens* to the plant genome is not a flawless process. Theory wants that only the sequences between LB and RB are transferred by the *Vir* machinery into the plant genome. In reality, T strand production, either from natural or artificial plasmids, shows high variability, mostly due to inefficient termination at the LB during T-strand generation, and many studies have revealed integration of Vector Backbone Sequences (VBS) in the plant genome along with the desired genes. This presence of unwanted foreign DNA in transgenic plants is one of the issues raised against genetically modified plants.

We designed a multiple PCR assay in order to evaluate the frequency of this phenomenon in transgenic alfalfa events from several independent transformation experiments with a pPZP201-derived binary vector hosting different constructs.

Our first results indicate that the percentage of events with VBS integration is 12.5 – 44 %, consistent with the literature, and appears to vary among transformation experiments. VBS close to the LB are found at a higher frequency, as expected based on the T strand production mechanism. Southern hybridizations are being carried out to confirm these results.

Strategies to obtain transgenic plants without VBS are discussed.

Cry TRANSGENIC POPLAR AND RISKS ANALYSES

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Populus alba, cry gene, transformation, transcription genomic variation

Problems, such as the impact on biodiversity and the potential escapes of the trans-genes into wild populations can be amplified in the case of long-lived forest species. Thus, studies of possible environmental impacts of transgenic forest plants (poplar) in the forest environment are important. Although a number of studies have been carried out so far considering the use of transgenesis for genomic studies, only little is known about biosafety issues of transgenic trees foreseen for breeding purposes.

As a model transgenic tree system, we have produced *Populus alba* and *P. tremula* transgenic lines carrying the *cry* gene from *Bacillus thuringiensis*. These transgenic are under screening to unravel possible pleiotropic metabolic effects in the transgenic trees following *cry* gene expression. It is also not known how the use of *cry*-transgenic poplars affects composition and activities of soil microbial communities which affect soil health. In addition to foreign DNA, proteins from *cry*-transgenic poplars can be released into the soil via plant residues and their persistence can constitute not only a selective pressure towards the target organisms, but also a possible threat for non-target ones with unpredictable consequences on the food chain sustained by these organisms.

The work is carried out on an integrated and multidisciplinary approach (physiology, soil microbiology and biochemistry, genomic, proteomic, and microscopy), involving different scientific groups with different and specific expertise, to assess the mentioned biosafety issues important for forest trees.

The screening of the transgenic plants is in course, and the evaluation of the number copy of the inserted gene as well as expression of the inserted gene is under evaluation.

GRAPE LEAF AGROINFILTRATION: A PROTOCOL FOR AGROBACTERIUM-MEDIATED TRANSIENT ASSAY OF GENE EXPRESSION

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Vitis vinifera, leaf agroinfiltration, *Agrobacterium tumefaciens*, Green Fluorescent Protein,
cell cultures

Agrobacterium-mediated transient assays for gene function can now be adopted also on *Vitis vinifera* (grape) leaves as an alternative to genetic complementation and stable plant transformation, as it is increasingly being used for a number of other plant species. We developed an efficient protocol of agroinfiltration of grape leaves for routine transient assay for gene expression. In order to optimize the technique, we analysed several grape cultivars *in vitro* propagated and different *Agrobacterium tumefaciens* strains transformed with different binary vectors harbouring GFP constructs targeted to various cell compartments. We identified grape cultivars exhibiting high efficiency of transient transformation and others with moderate efficiency of transient transformation, determined as GFP fluorescence. In our experiments, transient expression occurred in the majority of the cells within the infiltrated areas few days after agroinfiltration and, later on, in a larger portion of the leaf. The hypervirulent non-tumorigenic *Agrobacterium* strain GV3101 gave us the best results on grape without inducing defence response in the plants.

Agroinfiltrated leaves with *A. tumefaciens* harbouring GFP-construct, incubated on cell proliferation medium, generated stably transformed transgenic cell lines that could be maintained *in vitro*.

MARKER-FREE AND MARKERLESS ENGINEERING IN ALFALFA

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GMO, in vitro selection, Medicago sativa

Selectable Marker Genes (SMGs) are often linked to useful genes to efficiently obtain transgenic plants, but are not desired in transgenic crops. Techniques to avoid the presence of SMGs in transgenic plants are available, but have not yet been implemented in many cultivated species.

We are assessing the efficiency of co-transformation and markerless transformation in obtaining marker-free transgenic alfalfa. Co-transformation with two T-DNAs, each carried by a different culture of *Agrobacterium tumefaciens*, was performed. Fourteen putative co-transformed plants were regenerated from 400 leaf explants in two independent experiments. Southern analyses indicated integration of one or two copies of each T-DNA in most plants.

A Real-Time PCR protocol has been developed in order to confirm, and in the future hopefully avoid, Southern Blot hybridization experiments for copy number assessment. Our results indicate that copy number discrimination is possible even though distinguishing one- from two-copy events can be tricky.

Some plants were crossed to a non transgenic plant, and two progenies examined by PCR for transmission of both T-DNAs. Segregation of the two T-DNAs was observed in one progeny, indicating the feasibility of this marker-free approach. We are now attempting to improve co-transformation efficiency.

For markerless transformation, we are using both a SMG and a reporter gene (GUS) to estimate the percentage of transgenic events that can be obtained without selection. In a first experiment, underway, 1.7% of the somatic embryos were transgenic. Further experiments are in progress and will allow us to establish whether markerless transformation can be routinely applied to alfalfa.

REAL-TIME qPCR DETECTION OF GENETICALLY MODIFIED TOMATO USING NEW REFERENCE MATERIALS: CLONED PLASMID GENES

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Solanum lycopersicum, reference materials, plasmid, LAT52 gene, Real-Time PCR

Since new regulations for the labeling of transgenic food products came into force in the European Union, there has been a necessity to use reference materials for detection of transgenic elements in food for Real-Time quantitative PCR. Notably GM tomato was the first GMO to be obtained for commerce in USA and UK. Presently there are no certified reference materials for tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) species, so intra-laboratory evaluations developed to validate house-standards based on the use of known concentration of genomic DNA for the detection of event-specific target and endogenous gene. Considering that genomic extracted DNA quality depends on many factors, there is the necessity of an alternative: cloned plasmid DNA fragments are the best calibrators for their easy production, storage and distribution, the high stability and the universal applicability. To create new reference standards we cloned in a plasmid vector the CaMV35S promoter and the endogenous tomato gene LAT52; it was already studied that LAT52 is a single-copy gene and specific for tomato. In this work we confirm the specificity of LAT52 gene for six cultivars of tomato and the absence of sequence similarity between LAT52 gene and ten different species: wheat, maize, barley, arabidopsis, soybean, canola, tobacco, potato, egg-plant and pepper. The limit of detection on genomic DNA is 1 pg equal to one copy of LAT52 gene. The analysis in Real-Time PCR was validated verifying the possibility of using the plasmids as calibrators in the detection of transgenic tomato *tlcy-b* whose number copy of CaMV35S promoter was previously determined by Southern Blot analysis.

DEVELOPING A METHOD FOR SUGAR BEET CHLOROPLAST TRANSFORMATION

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sugar beet, plant regeneration, chloroplast, biolistic transformation

Sugar beet (*Beta vulgaris* L.) is an important industrial crop of the temperate zone. Although improvements in various sugar beet traits, such as sugar yield or disease resistance, have been achieved through conventional breeding, other traits such as herbicide resistance could be introduced in this crop by genetic engineering. Unfortunately, this species is still considered recalcitrant to genetic transformation and a routine method for regeneration of transgenic plants is lacking. Moreover, a lot of concerns could arise from the introduction of transgenic sugar beet in the field due to its well documented cross-compatibility with the wild-relative sea beet (*B. vulgaris* ssp. *maritima*). In many crop species chloroplast DNA is not transmitted through pollen. Thus the development of transplastomic sugar beet plants could avoid the risk of gene flow between the commercial transgenic sugar beet and wild-type plants or relative wild species.

We screened twenty-six *Beta vulgaris* L. varieties from Italian, German, and North American germplasm for optimal tissue culture response. Two regeneration protocols have been used. The first one is based on indirect regeneration from callus obtained from hypocotyls and cotyledons, whereas the second one is based on direct regeneration from leaf petioles. Four varieties have been identified that give high frequency regeneration (ca. 20%) from leaf petioles with the direct regeneration method. To attempt sugar beet chloroplast transformation, four vectors were constructed called pSB1, pSB2, pSB1-*bar* and pSB2-*bar*. Vector pSB1 was constructed cloning the chloroplast sugar beet genes *rrn16*, *trnV* and *rps12/7* in pBlueScript.KS plasmid. pSB2 is the same as pSB1 except for the chloroplast genes cloned in the vector that are *rbcL* and *accD*. Both the *aadA* gene, encoding spectinomycin resistance under control of the chloroplast *Prm* promoter, and the reporter *gfp* gene regulated by the chloroplast *psbA* promoter, have been inserted in the intergenic spacers of the two vectors. Other than *aadA* and *gfp* genes, plasmid pSB1-*bar* and pSB2-*bar* contain a cassette for expression of the *bar* gene. Preliminary experiments were carried out on leaf petioles using the direct regeneration method to optimize the transformation parameters. Three thousands petiole explants, derived from sterile plants grown *in vitro* have been bombarded with pSB1 or pSB2 DNA and they are currently cultured on regeneration medium containing spectinomycin 50 mg/l.

GMO TRACEABILITY IN CROPS, FOOD AND FEED

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genetically modified organisms, traceability, transgenic event, labelling, real-time PCR

In Europe, after the introduction of the Directive 2001/18/EC, Member States must adopt measures to guarantee traceability and labelling of genetically modified organisms (GMOs), in application of the precautionary principle. Traceability has the purpose of facilitating (i) withdrawal of products in case of unforeseen risks, (ii) targeted monitoring of effects, and (iii) control and verification of labelling claims. This requires the possibility of unique identification of a specific GMO event. The products containing GMOs or consisting of GMOs must be labelled according to Regulations EC 1829/2003 and 1830/2003. The threshold for labelling is set at 0.9% for food and feed containing authorised GMO events, even if the final product does not contain DNA or protein from the genetic modification. Therefore, in Europe, labelling is based on the evaluation, both qualitative and quantitative, of the presence of material of transgenic origin in ingredients and components of the food product. Recommendation 2004/787/EC declares that the concentration of GMO has to be expressed as ratio between an event-specific target and a reference target, species-specific, in terms of haploid genome. This ratio has to be established for every species and for every gene. Currently, the debate on labelling in legislation and science concerns the specificity of the analytical methods which can be used to detect GMOs under the new different conditions. In order to be highly specific, a method must have as target a unique feature of the event. Several detection methods developed before the 2004 recommendation employed construct-specific targets.

Research has been fundamental in bringing analytical methodologies to their current status of advancement. The European Commission and different other National and International institutions have financed several projects in which new strategies and approaches to GMO detection in food and feed have been tested and developed. Currently, the main project dealing with GMO detection is CO-EXTRA “GM and non-GM supply chains: their CO-Existence and TRAcability”, coordinated by INRA, France. Its main purpose is to develop comprehensive tools and methodologies and integrating them with existing ones into embedded decision-support systems aimed at enabling co-existence between GM and non-GM crops.

Within this project we have assessed the robustness of the “matrix-DNA extraction module” interaction with a predefined analytical module according to the modular procedure for the validation approach in GM food and feeds developed by Holst Jensen and Berdal. (JAOAC International, 2004, 87:927-936). The efficiency of the template producing module has been assessed using Singleplex and Duplex quantitative RT PCR. assays based on TaqMan MGB chemistry and aimed to the detection of RRS line GTS40 3-2 containing the junction region within the CTP4-EPSPS/T-nos sequence. In addition we have prepared recombinant plasmids based on pGEM-T Easy (Promega) containing specific sequences present in genetically modified plants such as RRS soybeans, maize MON810 and GA21, to be used, along with pJANUS, for creating standard curves in quantitative PCR assays.

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