Plant natural variability may affect safety assessment data

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1. Introduction

In the bicentenary of Darwin’s birth, and the 150th anniversary of the publication of “On the Origin of Species”, it is more than convenient, and fair, to recall that Charles Robert Darwin was the first to cast doubt about “the stability of species”. In the sequence of his voyage in the Beagle, Darwin has established the basis for understanding the evolution of and, in a certain way, the diversity of life (http://darwin-online.org.uk/). Although Darwin could explain how variations and inherited characteristics contributed to his theory of evolution, he could not explain exactly how the characteristics were passed from one generation to the other. The explanation of inheritance only occurred some years later, in the next step forward in the history of genetics, and has begun with Gregor Mendel’s experiments and the subsequent discovery of DNA (De La Bedoyere, 2005). From Mendel’s era to today, much has evolved with respect to the knowledge of organisms’ adaptation and evolution. We know now that organisms change in response to the environment and that these changes continue throughout their lifetime (Fraga et al., 2005). Since plants can not move or migrate to avoid stress conditions, they are one example of organisms that have developed strong and effective survival strategies compatible with sessile life-style. These strategies comprise not only long term genomic adaptations at the population level but also short term physiological changes. Plants respond to the environment and it becomes clear that the environment to which plants must respond comprises not just soil, water and climate, but also other plants, fungi, insects and other animals, and even humans. These responses lead to plant alterations at genomic, transcriptomic, proteomic and metabolomic levels (Mahalingam et al., 2003; Schenk et al., 2000; Qureshi et al., 2007; Shulaev et al., 2008). At present we know that the coordinated patterns of gene expression in multicellular organisms involve not only genetic but also epigenetic mechanisms (Boy, 2008; Comai and Madlung, 2004). The term “epigenetics” is used to refer to heritable changes in phenotype or gene expression that occur without changes in the underlying DNA sequences. These changes may remain throughout cell divisions and may also last for multiple generations (Molinier et al., 2006). Epigenetic regulation is basically mediated by modifications in DNA methylation and chromatin structure, which plays an important role in animal and plant development throughout adult life (Probst et al., 2009). This mechanism is required to achieve stable expression or repression of genes in specific cell types, or at defined developmental stages, and can be affected by environmental factors and diet.

In recent years, a number of “omics” technologies (transcriptomics, proteomics, metabolomics, and others) have been explored to be used as complement of the current food safety analytical approaches (Batista et al., 2007; Corpillo et al., 2004; Gregersen...
These profiling techniques offer the capacity to perform broad screening for possible changes of the food product at different integration levels in a non-selective, unbiased manner. However, there are still limitations associated with the use of these new technologies that have to be addressed. One of these limitations is the interpretation of the observed differences with respect to their biological relevance and toxicological significance. It is crucial to have information on sources of natural variation that can result from crop management practices, from interactions between genotype and environment and from breeding systems (Davies, 2009).

In this study we aimed to address how important the evaluation of natural plant variability can be in a food safety assessment process.

2. Materials and methods

2.1. Plant materials

For this study, we have used a MON810 maize line (DKC6575) and the respective non-transgenic near isogenic line. Maize line MON810 was developed through a specific genetic modification to be resistant to attack by European corn borer (Ostrinia nubilalis), a major insect pest of maize in agriculture. MON810 contains a stable, genome-integrated plant expression cassette, containing the CaMV 35S promoter and hsp70 leader sequences driving the expression of a truncated version of Bacillus thuringiensis cry1Ab gene. The truncated cry1Ab gene codes for a delta-endotoxin that acts as a potent and highly specific insecticide (only insecticidal to lepidopteran insects) (http://www.gmo-compass.org). The plant materials under study were self-pollinated and grown under the same environmental conditions for four generations. At the end of the fourth generation we have analysed independently the proteome of 5 ears, each collected from one of five plants randomly selected from each line. We have also performed 2D-gel electrophoresis of the pooled five protein extracts of insect resistant (IR) maize and control lines.

2.2. Protein extraction

Five gram of seeds from each analysed ear were ground in a mill (IKA A11 basic) with liquid nitrogen and incubated with 10% (w/v) trichloroacetic acid, 60 mM DTT in cold acetone at –20 °C for 1 h. After centrifugation at 11000g for 15 min at 4 °C, pellets were incubated twice with 60 mM DTT in acetone (cooled to –20 °C) for 1 h and then centrifuged at 11,000g for 15 min at 4 °C. The pellets were vacuum dried and stored at –20 °C.

For two-dimensional gel electrophoresis, the pellets obtained were dissolved in solubilization buffer [2 M thiourea, 0.4% (v/v) Triton X-100, 7 M urea, 4% (w/v) CHAPS, 1% immobilized pH gradient (IPG) buffer 3–11]. The protein was measured according to Ramagli (1999), with albumin from chicken egg white (Sigma, Sintra, Portugal) as standard.

2.3. 2-D gel electrophoresis

Isoelectric focusing was done on 13-cm-long IPG strips (Amersham Biosciences, Carnaxide, Portugal) with a non-linear pH gradient range of 3–11 in an IPGphor instrument (Amersham Biosciences). The strips were rehydrated for 12 h at 30 V in solubilization buffer diluted in 8 M urea, 4% (w/v) CHAPS, 0.5% (v/v) IPG buffer 3–11 and 60 mM DTT to a final volume of 250 μl. After rehydration, focusing was done with the following program: 1 h at 250 V, 90 min at 500 V, 90 min at 1000 V, 1 h at 2500 V, 24 min of a linear gradient to 8000 V and 3 h at 8000 V. Prior to the second dimension, on SDS–PAGE, the IPG strips were equilibrated at room temperature for 15 min in 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and traces of bromophenol blue (equilibration buffer) plus 1% (w/v) DTT, followed by protein alkylation with 2.5% (w/v) iodoacetamide in the equilibration buffer, at the same conditions of temperature and time. SDS–PAGE, on 12.5% T, 1.4% C gels, was performed in a Hoefer SE 600 system (Amersham Biosciences). The gels were run with constant 25 mA/gel current, at 20 °C. Full Range Rainbow molecular weight markers (Amersham Biosciences) were used to calibrate protein migration.

The gels were silver stained (Blum et al., 1987).

2.4. Image analysis

For each individual tested we have run 3 two-dimensional gels. Stained gels were scanned using the ImageMaster Labscan (Amersham Biosciences) and images were aligned and analysed with the...
same spot software. For the same spot analysis we have used two replicate gels from each line (the two gels selected as best focused). In all gel analysis (Fig. 2), the third replicates were used for visual confirmation. For principal component analysis we have not considered spots with negative staining.

3. Results and discussion

Plant-to-plant variability clearly does exist and there are many variables that likely contribute to it. It is, for instance, generally accepted that when adjacent plants differ by more than two leaves (due to delayed and uneven emergence), the younger plant may not develop to its full potential (Martin et al., 2005). Delayed and uneven emergence can be caused by a variable set of factors such as variable depth of planting, wheel compaction, seed germination, location of the seed within the furrow, moisture availability, variable seed furrow closure and/or many other elements (Martin et al., 2005). So, the first fact to consider is that we will never be able to have plants in exactly the same environmental conditions. Additionally, it is important to keep in mind that the coordinated patterns of gene expression in multicellular organisms involve not only genetic but also epigenetic mechanisms; that environmental factors lead to continuous changes in an organism’s epigenetic information (Fraga et al., 2005) and that these changes can be potentially transmitted over generations (Molinier et al., 2006; Batista et al., 2008). If environmental conditions influence the stability of plant genomes and if putative changes are inherited by the progeny, then in a food safety evaluation process, we cannot forget the breeding history of the plants we are comparing. It also would be crucial, for a safety evaluation study, that the control plants are also grown for several generations under environmental conditions as close as possible to those applied to the test plants. For instance, in a recently published study (Zolla et al., 2008), an Italian group used proteomics as a complementary tool to identify unintended effects occurring in transgenic maize seeds as result of genetic modifications. This team grew the tested plants (transgenic vs.

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Fig. 2. 2-D gel electrophoresis analyses of a pooled maize control sample (I) and details of a gel region in different samples (II): individual control ears (A–E); individual IR maize ears (F–J) and pools of control and IR maize samples (showing duplicates) The data presented in this figure clearly shows that several of the differences encountered between pooled samples (control vs. IR maize) may be explained by plant variability or eventual variations in migration during electrophoresis, and thus cannot be attributed to the modification event.
non-transgenic) under different environmental conditions for five generations, and subsequently grew the 6th generation of these plants under the same environmental conditions. This team alleged that the encountered proteomic differences measured between the 5th generation of non-transgenic plants (WT5) and transgenic plants (T5) were a result of DNA manipulation plus effects induced by the different environmental conditions the plants experienced in earlier generations. Later, this team concluded that the proteomic differences found between WT6 and T6 plants could only be attributed to DNA manipulation effects, since the 6th generation of both transgenic and non-transgenic plants was grown under the same environmental conditions. This team neglected the putative effects that the different environmental conditions of the first five generations could have had on the 6th generation.

When performing a food safety evaluation between a control and an experimental line, one of two situations may apply (Fig. 1):

(a) It is possible to monitor all the events along the modification process (genetic engineering, abiotic/biotic stress, gamma irradiation...); control such processes as closely as possible and be sure that the plants being compared were always subjected to the same (or almost the same) environmental conditions (besides the specific DNA alteration to be tested): Situation A in Fig. 1. In this case, the food safety assessment process should be able to distinguish which of the detected differences are due to natural plant variability and which can be attributed to the performed intervention.

(b) Data about the exact breeding histories of the control and experimental plants are not available: Situation B, in Fig. 1. In this case there can be several degrees of knowledge about the prior history of the tested samples and the more information that is available, the fewer mistakes will be made in the food safety evaluation process. It is important in this case, to consider not only natural plant variability but also the potentially different epigenomes that the two groups of plants may have (which could result in different transcriptomes, proteomes and metabolomes) due to their distinct breeding histories.

In this study, 2D-gel electrophoresis was performed on ten different ears from ten different plants (five non-transgenic and five IR maize) obtained from progenies of plants used in a field trial. Although the modification event (introduction of Cry gene cassette) had been previously performed, the tested plants should have a similar breeding history as the control plants. To reduce (or eventually eliminate) any potential differences due to previous dissimilar environmental stimulus, we have grown these plants for four generations under the same environmental conditions and analysed ears collected at the end of the fourth generation. However, the fact that the plant material used included segregating lines from a highly heterozygous parent (a hybrid line), also implies that a strong genetic variation is expected among individuals in a progeny obtained through self-pollination. As already stated, in this study, 2D-gel electrophoresis was used to address how plant natural variability can influence an accurate food safety evaluation.

We notice, in this study (Fig. 2) that the exclusive use of data from 2D-electrophoresed pooled samples, to compare these two lines, would be insufficient for adequate safety evaluation. In Fig. 2 four different situations could be observed:

(a) Extremely variable areas were clearly detected between pools and also between individual samples. Sometimes, differences in these areas were even found between duplicates. As concluded from the different gels analysed, in these circumstances, the encountered differences could not be attributed to the performed intervention (genetic engineering in the case of IR maize).

(b) In some cases, extreme variability was detected between individual samples but not between pools. In these cases, it seems that the number of biological replicates used in the pools was sufficient to mask the natural variation among samples.

(c) Specific spots were found different between non-GM vs. GM pools but also between individual samples (GM or not). Also, in these situations, encountered differences could not be attributed to the genetic engineering event.

(d) Finally, spots were also found that were present/absent in all the control samples, and absent/present in all the test samples. These differences were obviously confirmed in the pooled gels and should be considered, and further analysed, in a food safety evaluation of these two lines.

Principal component analysis of the 24 analysed gels (five individual control samples + five IR maize samples + one control pool + one IR maize pool, all in duplicate) could differentiate 2D gels from the two maize pools (control vs. IR) but could not clearly differentiate individual control samples from individual IR maize samples (Fig. 3). These results clearly confirmed that some of the

Fig. 3. Scatter plot of the first two principal components from the principal component analysis (PCA). Pink circle: 2D-gel electrophoresis of individual control ears; Blue circles: 2D-gel electrophoresis of individual IR maize ears; Purple circles: 2D-gel electrophoresis of control pool; Yellow circles: 2D-gel electrophoresis of IR maize pool. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
differences encountered between pools could be the result of a high plant-to-plant natural variability, which emphasizes the importance of assessing natural variability. Although “omics” technologies are becoming standard tools that offer tremendous opportunities to more accurately assess the potential for any unintended effect, there are still significant challenges. One of these challenges regards the adequate management of the large quantity of complex raw data generated by these technologies in a manner such that it can be adequately analysed, scrutinized, and compared for the benefit of the scientific community (Fratamico, 2008). When using “omics” technologies, it is extremely important to ensure that all potential differences due to factors not related to the ones under study are eliminated, or at least strongly minimized.

4. Conclusions

In conclusion, for a more accurate safety evaluation of crop plants using “Omics” technologies, it might be advisable to use adequate controls, such as the exact original plant line used for the modification event. Additionally, plants with similar breeding histories, and grown in the same environmental conditions should be used. When this is not possible, the plants to be compared should be previously grown under the same environmental conditions for several generations. This could minimize potential differences due to different acclimation processes and eventually to epigenetic memory. Of course, when the mother plants are hybrids or highly heterozygous, this will also lead to genetic segregation in the progeny and further genetic differences among the individuals to analyse. Also, whenever possible, the treated and control groups should be a pool of samples made with a high number of individuals in each group, in order to minimize natural plant-to-plant variability. As an alternative, when not many individuals are available, individual analyses of the different plants should be performed to understand the role of natural plant-to-plant variability in the encountered differences.

Finally and ideally, control and test plants should be grown under a range of environmental conditions to better understand the degree of possible natural variation, and determine whether the observed differences all fall within the (wider) range of variability obtained. This should be faced as a continuous multi-laboratory task and highlights the importance of building a “database” of knowledge around natural variability in food crops.

5. Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

We thank Comissão de Fomento da Investigação em Cuidados de Saúde for funding.

References


