Selection of the best comparator for the risk assessment of GM plants – conventional counterpart *vs*. negative segregant.

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Introduction

In risk assessment of GM plants and derived food its important to identify the **similarities** and **differences between GM plants and its comparators**. Therefore, selecting the right comparators must be one of the top priorities.
The unintended differences may:
a) Be dependent on the **transgene expression**;
b) Occur as a consequence of **epigenetic changes**, **host DNA disruption** or DNA **sequence rearrangements** promoted by transgene insertion;
c) Be due to the ***in vitro* culture procedures** necessary for the transformation process.
The **question** is which control would allow us to better evaluate the potential **unintended differences arising from a) and b)**, discounting the potential effect of *in vitro* culture procedures; since they are non-controversial and largely used in conventional breeding.
Aiming to answer the previous question we used the following

Materials

Three different rice lines (*Oriza sativa L. ssp. Japonica cv Nipponbare*):

- A **control** conventional counterpart.
- An *Agrobacterium* transformed transgenic line (**Transgenic 1**).
- A negative segregant (**Neg. segregant 2**) - homozygous negative progeny - from a different transgenic line.

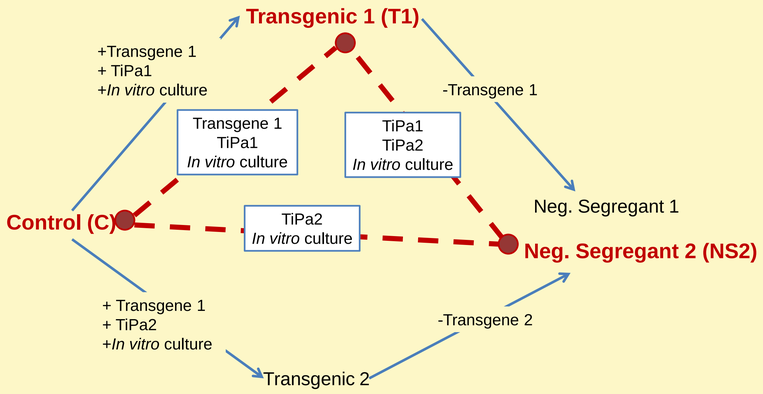

Fig. 1 – TiPa1 – Transgene 1 insertion promoted alterations; TiPa2 – Transgene 2 insertion promoted alterations

Methods

Protein Extraction and RuBisCO deplection aiming to enrich the samples in low abundant proteins

Refraction-2D ™ Gel Electrophoresis –
All samples were run in duplicate with both GDye 200 and GDye300 and an internal standard was used in every gel to assist in gel alignments and normalization of spot volume

Identification of differential regulated spots with DIGE Enabled Samespots Software

MS/MS Analysis

Results

From the all spots identified in the Refraction-2D™ Gels, **81 were selected as differentially regulated spots** (fold difference ≥ 1.5, Anova P value <0.05, FDR adjusted P<0.05 between at least two of the three tested groups (C, NS2 and T1)) to continue to MS analysis.

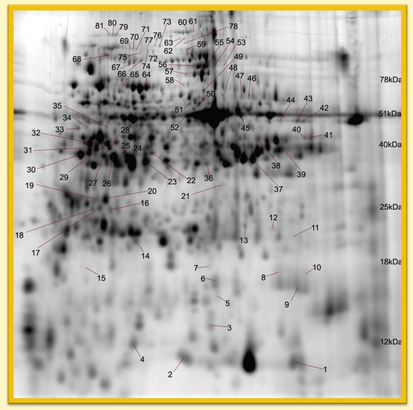

Fig. 2 – Example of Refraction-2D™ Gel – Sample: Internal Standard.

Although the 81 chosen spots were distributed between different categories (as seen below) , **all spots showed the same profile in T1 and NS2 *vs.* C**, with **only one spot being statistically different between T1 and NS2**.
60,4% of the chosen spots were statistically **different in both T1 and NS2 *vs.* C** and also had a **fold difference ≥ 1,5**.

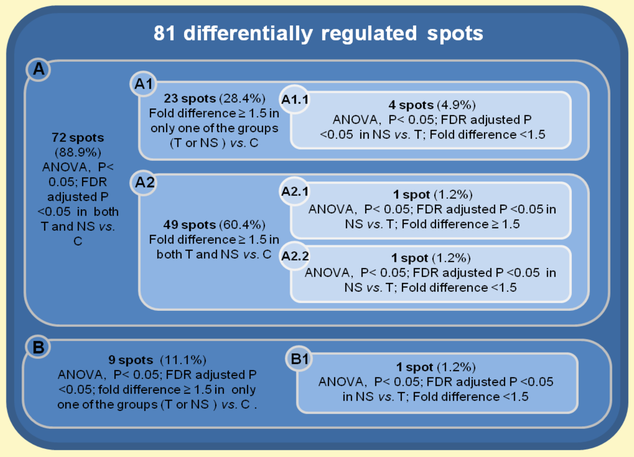

Fig. 3 – Categorization of the 81 differentially regulated spots.

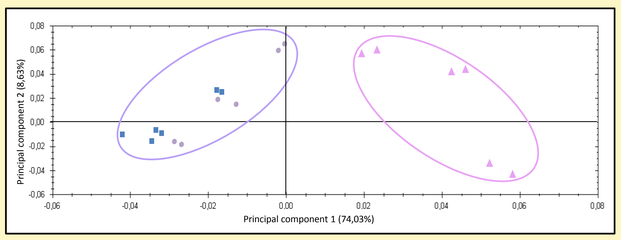

Fig. 4 - PCA analysis using 81 selected spots. Triangle – Control line; Circle - Negative segregant 2 line; Square – Transgenic 1 line.

T1 and NS2 lines group together and apart from C.
The only **feature in common between T1 and NS2** is the fact that they both suffered ***in vitro* culture** procedures.
The results obtained indicate that in this study, different transgene insertion promoted alterations and the presence/absence of transgene were factors with less impact on the rice proteome than the *in vitro* culture.

36 proteins identified by MS corresponding to 55 spots
Some examples

	Spot nº	Protein identification
	4 (A1)	gi|125546229 – Blast: gi|108711793 Actin-depolymerizing factor 3 [O s] (I=99%; P= 100%; G=0%)
	9 (A1)	gi|115461585 – Blast: gi|226491656 peptidyl-prolyl cis-trans isomerase [Z m] (I=85%, P=90%, G=2%)
	21 (A2)	gi|146386456 chain A, Crystal Structure Of Class I Chitinase [Os]
	48 (A2)	gi|313575769 chaperonin protein [O s]
	...	...

Table 1 – **Blue**: Down-regulated protein. **Red**: Up-regulated protein. A1 and A2 group in Fig. 3

The majority of these proteins have functions that can be associated with stress responses or belong to metabolic pathways that can be activated under stress. Interestingly, *in vitro* culture was already reported as a stressful activity.

One of the most evident alteration in T1 and NS2 lines was the decline of photosynthetic efficiency and the enhancement of photorespiration and glyoxylate cycle

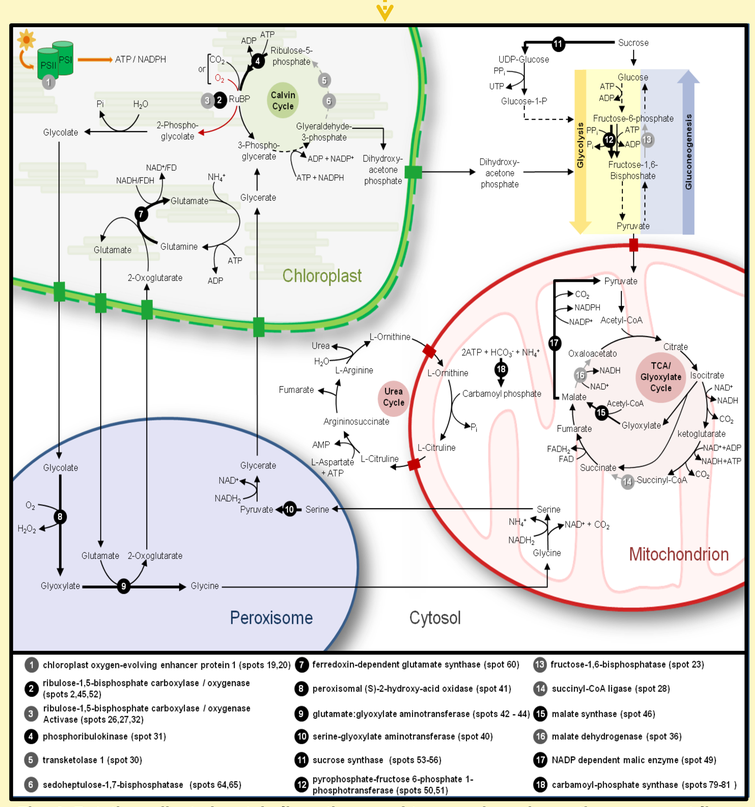

Fig. 4. Putative adjusted metabolic pathways of transgenic and negative segregant lines. Black and grey (circles and arrows) indicate up-regulation or down regulation of the correspondent protein, respectively. TCA – Tricarboxilic acid. PSI and PSII – Photosystem I and photosystem II . PPi – Pyrophosphate. RuBP- Ribulose 1,5-Bisphosphate.

Conclusions

The results obtained indicate that, ***in vitro* culture, and eventually the stress caused by this process**, was the **major factor influencing the differences** between Control and Transgenic lines. The Negative segregant analyses was essential for corroborating these findings.
This work highlights the importance of continuous revision and upgrade of the guidance criteria to be followed for the selection of suitable comparators in GMO risk assessment.

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