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A multiway approach to analyze metabonomic data: a study of maize seeds development

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Abstract

The aim of this research was to show that the application of multiway partial least square-discriminant analysis to nuclear magnetic resonance spectra is a valuable tool to analyze metabonomic data of transgenic maize. We evaluated the effects, on the development of seeds, of the introduction of the antisense-mediated downregulation and overexpression of the *Rpd3* gene (*ZmRpd3*) in the genome of a maize inbred line, we identified the metabolites involved in the differentiation between classes of samples, directly integrating the evolution of each metabolic perturbation over time in the model. Major differences were found at the beginning of development, confirming the results obtained by transcript analysis: *ZmRpd3* transcripts and proteins accumulate during the initial stage of development, suggesting a role for this gene in cell cycle control.

Keywords: Metabonomics; Maize; NMR; N-way methods; N-PLS

Metabonomics is a well-established technique to measure metabolite levels and to follow their systematic and temporal changes due to external perturbations, such as genetic modifications [1,2], environmental stressors [3], diet [4], lifestyle [5], and pharmaceuticals [6,7], both beneficial and adverse, in whole organisms.

Many different global analytical tools have been employed to obtain metabolic profiles of the systems, including nuclear magnetic resonance (NMR)[1] and mass spectrometry, which are the two main complementary techniques used in this field. In particular, NMR spectra provide a "metabolic fingerprint" of the system, revealing crucial details of the biological machinery without any preconstituted hypothesis (i.e., by a non-targeted approach).

Application of multivariate analysis is an important step to extract the metabolic information embedded in the spectra. Usually, two-way chemometrics methods, such as principal components analysis (PCA) and partial least square (PLS), are used [8]. In particular, there is a lot of attention to PLS-discriminant analysis (PLS-DA) applications in metabonomics studies because it allows a more focused evaluation and analysis of the data that includes additional knowledge of the samples, such as genotype, dose, and treatment. However, it is well known that this method requires rigorous validation both for the classification and for the selection of important markers. It has been shown that single cross validation is insufficient because score plots always show an overfitted situation [9].

Furthermore, it is very common to obtain time series data that are naturally arranged as a three-way data set, where the first dimension represents the treatment, the second dimension represents the metabolite concentrations, and the third dimension represents the time. Two structural models, either to rearrange the multiway data to fit existing two-way algorithms or to generalize two-way algorithms to the multiway case, have been investigated to determine which is most suitable for multiway data with regard to model fit, parsimony, and interpretation. Some comparisons have shown that whereas the former approach generally gives a better or comparable fit, the latter may give

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[1] *Abbreviations used:* NMR, nuclear magnetic resonance; PLS, partial least square; DA, discriminant analysis; N-PLS, multiway PLS; PCA, principal components analysis; DAP, days after pollination; 2D, two-dimensional.

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better interpretation through the use of a simpler structure employing fewer model parameters [10]. Moreover, interpretation becomes difficult for the rearranged data because the primary, secondary, etc., variable modes are not modeled explicitly but are mixed up during the unfolding step.

For this reason, in recent years, multiway methods are gaining attention [11,12] in the metabolomic research community. Multiway analysis is the extension of the traditional multivariate analysis to arrays with more than two ways, where data are characterized by several sets of variables that are measured in a cross fashion. In this case, data are arranged not in matrices, as in standard data sets, but in cubes: a typical example comes from fluorescence measures, where emission spectra can be measured at several excitation wavelengths for several samples [13].

The multiway partial least squares (N-PLS) is the regression method for the analysis of higher-order arrays [14]. As in the traditional two-way PLS, it searches for a compromise between better fitting (i.e., less error in describing the array of the independent variables) and better prediction (i.e., less error in evaluating the response space). N-PLS has been applied successfully in many areas, ranging from the analysis of food characteristics by fluorescence and gas chromatography [15,16] to the simultaneous determination of ions by electrochemical sensors [17]. Recently, the use of N-PLS to the quantification of lipoprotein fractions obtained by 2D diffusion-edited NMR spectra has been evaluated [18].

In this case, this kind of data treatment seems the "natural" choice. In fact, the study aims to reveal discriminant dynamical details in the growth of seeds belonging to different genotypes.

The method was applied considering as response block a "dummy matrix," which records sample membership, obtaining a model based on the discriminating characteristics among the groups analogous to the PLS-DA approach traditionally used in metabonomics. The advantage of a multiway analysis with respect to the two-way multivariate technique is to obtain not a better fit but rather more adequate, robust, and interpretable models [13]. In fact, the constructed model integrates the information contained in the entire structure of the multiway array, without any loss due to the unfolding procedure.

In the field of metabonomics, the characterization of metabolic changes in time is a fundamental aspect of elucidating the biochemical response of an organism to an external perturbation, so a multiway approach can be very useful. In fact, the analysis of growth profiles distills the peculiar characteristics allowing discrimination; i.e., it is not the change of a specific metabolite but the dynamic variation of the whole system in time that enters directly in the discrimination among the groups.

As processes develop through time, the metabolic responses also exhibit dynamic variation, and monitoring these changes results in characteristic patterns for each type of perturbation. Principal component trajectories have been constructed from NMR data to investigate the changing multivariate biochemical profile during development of a toxic lesion [19]. However, this kind of analysis, although effective for trajectory analysis, is not suitable for the simultaneous comparison of several parallel systems, and thus the use of alternate multiway tools for optimally extracting metabolic trajectory and biomarker information have been investigated [11,12].

The aim of this paper is to show that the application of multiway PLS-DA to NMR spectra is a valuable tool to evaluate metabonomic data. In particular, this study evaluates the effects, on the development of seeds, of the introduction of the antisense-mediated downregulation and overexpression of *Rpd3* gene (*ZmRpd3*) [20] in the genome of a maize inbred line. This gene encodes histone deacetylase, one of the enzymes responsible for maintaining the steady state balance of histone acetylation [21], a posttranslational modification of histones that plays a key role in modulating dynamic changes in chromatin structure and gene activity [22].

Materials and methods

Plant material

Seed samples of the inbred line B73 (control) and their transgenic versions containing a modified *ZmRpd3-101* maize gene [23] were used (four repeats per class). In particular, the two modified genotypes contained an antisense-mediated downregulated (AS-33) and an overexpressed (OE-3) *Rpd3* gene. For further details about the preparation of the samples, see Varotto et al. [20].

Plants of the inbred line B73 and its transgenic versions were grown under greenhouse conditions at 25:18 °C (day:night) with a 16:8 (light:dark) h cycle. At flowering, plants were self-pollinated. Identification of the developmental stages of maize seeds was made according to indications from the Iowa State University of Science and Technology [24]. Ears were harvested at 8, 13, 18, and 23 days after pollination (DAP) and stored in sealed plastic bags at −80 °C. Furthermore, ears were harvested after physiological maturity, dried at 30 °C, and stored in sealed plastic bags at 4 °C. For each genotype, four seed samples derived from the central portion of an ear was used for metabolomic analyses.

NMR sample preparation

For each sample a single maize seed was weighed and then frozen in a stainless steel mortar by liquid N_2 before being pulverized to a fine powder with a pestle chilled in liquid N_2 and maintained in liquid N_2 bath during the pulverization procedure.

Three milliliters of methanol/chloroform mixture (2:1) was added to the powder. The powder was stirred and 1 ml of chloroform and 1.2 ml of water were added (Bligh–Dyer method modified in our laboratory [25,26]). The sample was stored at 4 °C for 1 h and then centrifuged

at 10,000*g* for 20 min at 4 °C. The resulting upper hydroalcoholic and lower chloroformic phases were separated. The extraction procedure was performed twice on the pellet to obtain a quantitative extraction. After the second extraction, the two hydroalcoholic phases obtained were recollected, dried under N_2 flux, and stored at −80 °C prior to analysis.

This extraction procedure has a good performance compared to other procedures, as recently shown [27].

NMR data collection

For the NMR spectra, the dried sample was dissolved in 1 ml of 0.5 mM TSP (sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid) solution in D_2O phosphate-buffered saline (pH 7.4) to avoid chemical-shift changes due to pH variation [28]. The dissolved extracts were transferred to a 5-mm NMR tube.

NMR spectra were recorded on a Bruker (Bruker GmbH, Rheinstetten, Germany) DRX 500 spectrometer, operating at ^1H frequency of 500.13 MHz. Single-pulse spectra were acquired using a solvent presaturation pulse sequence to suppress residual water resonances. Spectra were obtained at $T = 300$ K; 256 scans were acquired, with data collected into 64 k data points and a spectral width of 12 ppm using a 20-s delay for a full relaxation condition. Prior to Fourier transformation, an exponential multiplication was performed using a line broadening equal to 0.09 Hz.

The spectra were phased and baseline corrected using ACD/Spec Manager 7.00 software (Advanced Chemistry Development Inc., Toronto, Canada), and they were referenced to TSP for chemical shift (0.00 ppm) and quantization of the signals [28].

NMR data preprocessing treatment

The ^1H spectra were reduced to 499 discrete chemical shift regions by digitization to produce a matrix of sequentially integrated regions of 0.02 ppm in width between −0.5 and 9.5 ppm, using ACD software: column 1 corresponds to the bucket −0.5 to −0.48 ppm. Use of bucketing means that many metabolites are represented more than once in the data matrix. This can lead directly to identification of peaks from the same molecule and hence aid molecule identification, in addition to the identification of substances whose concentrations are interdependent or under some common regulatory mechanism [29]. In this case, a fixed width was chosen to explore potentialities of an unsupervized first step in the analysis. The spectra were normalized to the TSP signal, set at 10. The region between 4.6 and 4.9 ppm was removed to eliminate baseline effects due to presaturation of the water signal.

Multiway analysis

The N-PLS regression model is an extension of the two-way PLS to higher orders [14], as PARAFAC is an extension of PCA. Using a bilinear, PCA-like, model, the two-way PLS model decomposes data (the X block) in such a way that each score successively has the property of maximum covariance with the unexplained part of the dependent variables (the Y block) [18]. The N-PLS model does exactly the same but using a trilinear, PARAFAC-like, model instead.

As a result of the N-PLS model, the X array is decomposed in terms of a scores matrix (T) relative to sample mode and two loadings matrices (W_J, W_K), relative to NMR and time mode, respectively, while the Y matrix (a dummy matrix in this case) is decomposed in terms of a score matrix (U) relative to the first mode and a loadings matrix Q_M.

The expression relating the two decomposition models of X and Y is

$$U = TB + E_U,$$

where B represents the regression coefficients and E_U is the error matrix.

The bucketed and normalized NMR data were imported in Matlab v.7.0 (Mathworks, Natick, MA) equipped with the N-way Toolbox for Matlab version 3.0 (obtained from R. Bro at http://www.models.kvl.dk/source/[30]).

Data were arranged in a three-way data cube with individual seeds as mode 1, bucketed NMR spectra as mode 2, and time as mode 3, giving a data cube of dimensions $12 \times 484 \times 5$.

Fig. 1 shows a schematic representation of the chosen strategy. Prior to analysis, the data were mean centered and scaled at unitary variance across the sample mode. This allows us to remove offsets and to treat equally all levels of variations of the metabolites, such as minimizing the effects of the high concentration of sucrose.

Y is a dummy matrix with three columns, each representing a genotype, containing 1 for the samples belonging to the group and zeros for all the others.

The number of significant latent variables has been chosen by leave one out cross validation, using the routine included in the N-way Toolbox. In particular, the parameter Root Mean Square Error of Prediction (RMSEP) has been calculated, increasing the number of latent variables and determining its minimum [16]. Furthermore, class prediction has been calculated, considering half of the seeds as

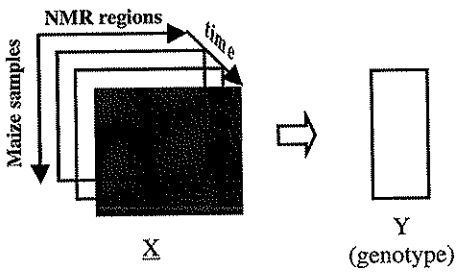

Fig. 1. Scheme of the three-way approach.

training set and the other half as test set. All seeds were correctly assigned to their genotype.

Results and discussion

To test the efficacy of the multiway PLS-DA approach to the evaluation of metabonomic studies, a total of 60 spectra (4 spectra per each genotype for five time points) were acquired and processed.

Examples of typical ^{1}H NMR spectra of the hydroalcoholic extracts of ripe samples, belonging to control, antisense, and overexpressing seeds are shown in Fig. 2. The assignment of the peaks was based on the literature [2,31,32] and a combination of 2D experiments. The spectra contained a number of assignable amino acids, organic acids, and sugars and confirmed previous findings for this kind of plant [32].

N-PLS-DA regression was developed between the array containing the bucketed NMR spectra and a "dummy matrix" containing information about samples groups. As a result, two factors were found to be significant, explaining 46.33% of X variance and 76.33% of Y variance. The total explained variation of X for this model is relatively low because many regions of the spectra contained only noise, and the autoscaling of the corresponding variables contributes to increasing the random variance, which is impossible to model. However, 76% of Y variance can be related to this variation in X. Extracting too many components means that noise is imported in the construction of the model and that the true factors are described by correlated variables.

With regard to class prediction, all of the seeds were correctly assigned to their genotype, using two latent variables.

Fig. 3, shows the score plot. It is clearly evident that it is possible to separate the three genotypes. In particular, the first factor discriminates between OE-3 seeds, characterized by positive values of the scores, and those of the other two genotypes, with negative values. Furthermore, the second factor discriminates between the control seeds, with positive values and the AS-33 with negative values. The third genotype is spread along the axis, indicating that it is not characterized by this factor.

Such a discrimination among the three groups is obtained taking into account the entire growth pattern of the three genotypes. This is the main advantage of this kind of approach: the three-way data structure is maintained and actively used in the analysis. Unlike the bivariate analysis methods, here the time mode is not mixed with the sample mode; therefore the evolution of the metabolic perturbation over time enters directly in the discrimination among the groups.

In fact, when a traditional PLS approach is applied to the data set, discriminating characteristics due to the genetic modification are mixed with those corresponding to the development of the seeds. By N-PLS-DA, instead, the discrimination among the three genotypes is modeled considering the entire growth pattern, and random or less general changes in metabolite levels, which do not have discriminating time profiles, are considered noise, making simpler the interpretation of the obtained model.

Fig. 4 shows the plot of the loadings corresponding to the NMR mode. A positive value in the loadings plot implies a positive correlation with the scores in the first latent variable. Thus, all variables with positive values are positively correlated with the samples with positive scores, whereas the variables with negative values are correlated with the samples with negative scores, making it possible to identify the metabolites that discriminate between the three genotypes. For the first latent variable, positive values are obtained for NMR descriptors corresponding to all resonances of sucrose.

Sucrose plays a central role in regulating cellular metabolism and physiology in plants, functioning as the major transported sugar from photosynthetic tissues to sink organs and as a signaling molecule [33,34]. Because cell division in plants is responsive to energy availability, the presence of sucrose or its metabolites is a likely regulator of cell cycle progression. Control mechanisms that interface between the presence of sugar and the cell cycle are therefore probable, as previously reported for yeast [35–37].

In addition, it is possible to determine that, for the second latent variable, Ala and Gln have positive loading values, whereas guanine, sucrose, and α and β glucose have negative loading values. It is worth noting that guanine is present in all the genotypes from 08 DAP to 18 DAP.

In Fig. 5, the loadings corresponding to the time mode are plotted: they show the discriminant trajectories among the three genotypes. From this plot, it is evident that there are differences in the growth pattern of the three genotypes. Moreover, combining these results with the scores shown in Fig. 3, it is possible to assert that the first latent variable discriminates OE-3 growth pattern with respect to the other two variables, whereas the second latent variable discriminates control growth pattern with respect to that of the other genotypes. In particular, major differences are present at the beginning of development, confirming the results obtained by transcript analysis [20]: *ZmRpd3* transcripts and proteins accumulate during the initial stage of development, suggesting a role of this gene in cell cycle control.

Differences in the growth "dynamics" of the three genotypes underline the primary role of Rpd3 function in the control of cell cycle progression and in the expression of anabolic genes. This has emerged through transcriptional profiling of the yeast *Rpd3* mutant and by an extensive analysis of Rpd3 binding and deacetylation activities throughout the complete genome [38–40].

It was reported that the ZmRpd3 protein is able to interact with the maize retinoblastoma-related protein ZmRBR1, a key regulator of the G1/S transition [41], and with the maize retinoblastoma-associated protein ZmRbAp1, a histone-binding protein believed to be involved in nucleosome assembly [42,20].

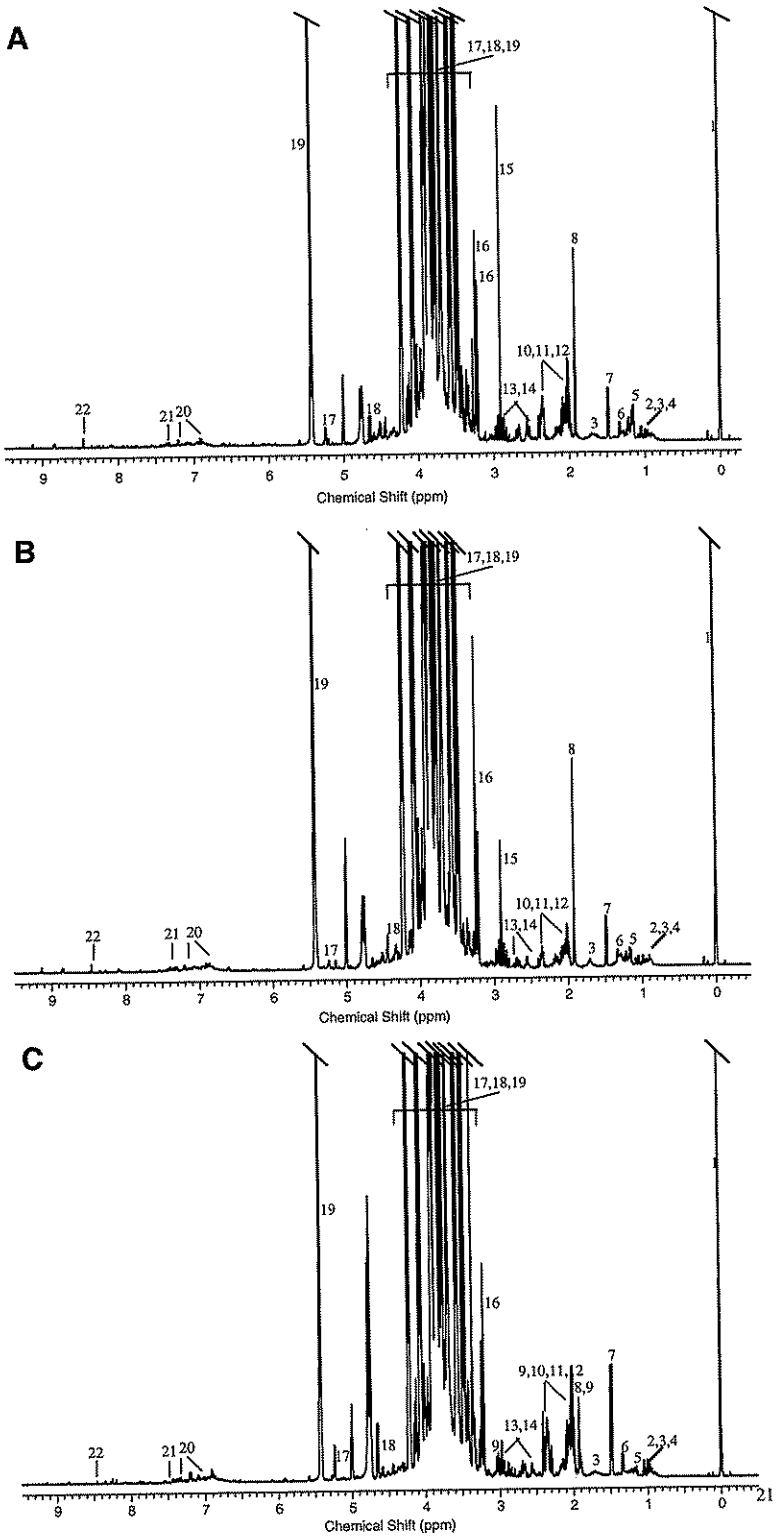

Fig. 2. Example of 500-MHz ^{1}H NMR spectra for extracts of ripe maize seeds. (A) Control sample; (B) seed containing the antisense-mediated downregulated *Rpd3* gene (AS-33); (C) seed containing the overexpressed *Rpd3* gene (OE-3). Key: 1, TSP; 2, isoleucine; 3, leucine; 4, valine; 5, 3-hydroxybutyrate; 6, threonine; 7, alanine; 8, acetate; 9, GABA; 10, proline; 11, glutamate; 12, glutamine; 13, aspartate; 14, asparagine; 15, trimethylamine; 16, choline; 17, α-glucose; 18, β-glucose; 19, sucrose; 20, tyrosine; 21, phenylalanine; 22, formate.

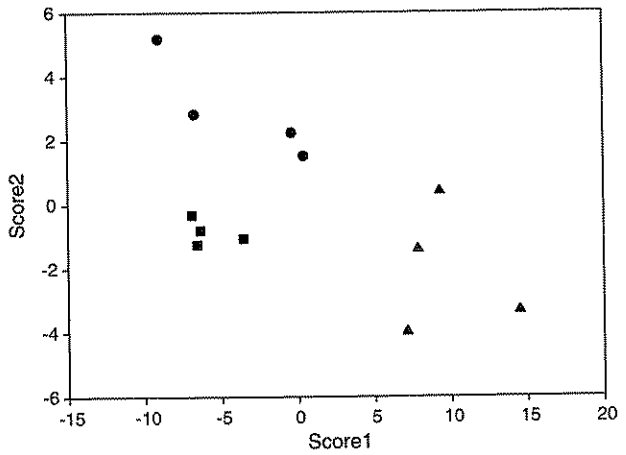

Fig. 3. Score plot obtained for N-PLS-DA. The control samples are represented as circles, AS-33 (antisense-mediated downregulation of *Rpd3* genes) as squares, OE-3 (overexpression of *Rpd3* genes) as triangles.

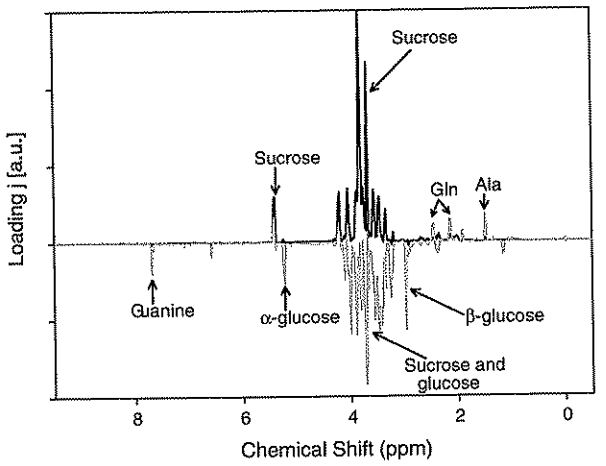

Fig. 4. Plot of the loadings corresponding to the NMR mode, with the assignment of the main signals. The first latent variable is represented in black and the second in gray.

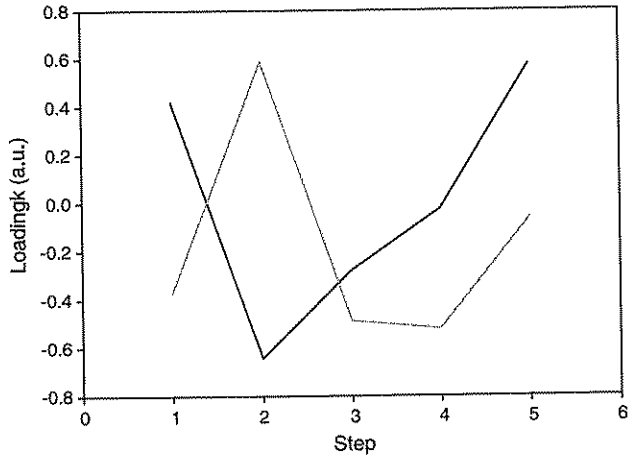

Fig. 5. Plot of the loading along the time dimension. The first latent variable is represented in black and the second in gray.

As underlined in the application of the Tucker model to toxicological studies [12], no constraints are imposed on time profiles, and, thus, the analysis can handle perturbations with any kind of time evolution, including reversible and oscillating behavior. Furthermore, as in the Tucker model, different rates in the response of the system can be considered simultaneously. This is one of the major drawbacks with batch modeling, where all study objects must have similar metabolic and response rates, without the possibility of considering slow and fast responders in the same model [11].

Conclusions

This work demonstrates that multiway PLS is a powerful tool to analyze metabonomic data because the developing of perturbation over time enters directly in the discrimination among the groups. It allows both a classification of the samples based on their characteristics and a way of going in deep in the metabolic changes associated with the perturbation. In particular, major differences are present at the beginning of development confirming the results obtained by transcript analysis: *ZmRpd3* transcripts and proteins accumulate during the initial stage of development, suggesting a role of this gene in cell cycle control.

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