Metabolic studies in plant organs: don’t forget dilution by growth

Michel Génard1*, Valentina Baldazzi1 and Yves Gibon2

1 UPR 1115 Plantes et Systèmes de Culture Horticoles, Institut National de la Recherche Agronomique, Avignon, France
2 UMR1332 Biologie du Fruit et Pathologie, Institut National de la Recherche Agronomique, Villenave d’Ornon, France

*Correspondence: michel.genard@avignon.inra.fr

INTRODUCTION

The recent development of high-density technologies has enabled a dramatic extension of the exploration of living organisms. Such exploration typically consists in the parallel measurement of large numbers of compounds, at different developmental stages or following the application of contrasted environmental stimuli. Correlation analysis or more sophisticated statistical approaches are then used to identify clusters of co-varying compounds with the aim of reconstructing the underlying regulatory network governing system responses. Such approaches are now routinely used to identify predictive biomarkers (Steinfath et al., 2010; Riedelsheimer et al., 2012), including candidate genes (Carreno-Quintero et al., 2012).

Originally developed for unicellular organisms, these approaches are now applied to plants for the analysis of transcripts, proteins, metabolites (Gibon et al., 2004; Saito et al., 2008; Stitt et al., 2010; Liberman et al., 2012) and more recently enzymes activities (Gibon et al., 2004; Saito et al., 2008; Moreno-Risueno et al., 2010).

Most studies involve homogenization of specific tissues or even whole organs (e.g., fruit pericarp, leaf), without considering the subcellular localization of the measured compounds. Plant cells distinguish from other cells in possessing a large central vacuole, which size may vary dramatically between tissues, genotypes (species, cultivars) and developmental stages. Whereas young cells have small vacuoles, mature cells have large vacuoles that can encompass more than 95% of the cell volume. Here, we will demonstrate that without taking into account the volumes of the cell compartments, the analyses of the dynamics of the compounds and the subsequent compound-compound correlation analyses might be biased, especially when the functional significance of the study is bound to concentrations, as it is the case for enzymes and metabolites. Possible correction strategies are discussed, with special emphasis to their pertinence and applicability to specific questions.

RESULTS

DILUTION BY GROWTH STRONGLY AFFECTS METABOLIC CONCENTRATIONS

Let us consider for the sake of simplicity a cytosolic metabolite M. In many studies the metabolite concentration is expressed per volume (or, equivalently, per gram of fresh mass) of the whole tissue, as

\[ [M] = M/V \]

where \( V \) is the volume of the measured part of the tissue and \( M \) the metabolite quantity in this tissue. The metabolite being cytosolic, its concentration, to be physically meaningful, should be calculated as

\[ [M] = M/V_c \]

where \( V_c \) is the volume of cytosol in the tissue.

It is clear that \([M]\) underestimates \([M_c]\) because \( V_t \) is often much bigger than \( V_c \) due to the presence of other subcellular compartments (e.g., vacuole and plastids). More importantly, the ratio \( [M_c]/[M] = V_t/V_c \), may not be constant among genotypes or during the development of organs where the ratio \( V_t/V_c \) strongly increases as the cell vacuole enlarges. This may lead to an erroneous interpretation of changes in metabolite concentration when comparing different experiments. Let’s make an example. Assuming that \( V_c \) is constant and \( V_t \) increases with time, Figure 1A shows that depending on metabolite dynamics, the measured metabolite concentration \([M]\) can be strongly different from the real cytosolic concentration, both quantitatively and qualitatively. In the extreme case, a decrease of \([M]\) can be measured instead of an increase when the growth of the volume is faster than the metabolite accumulation in the cytosol (Figure 1A, blue line). This means that the seasonal variations of \([M]\) during development of organs may not have any biological significance, reflecting the evolution of cell growth rather than a specific regulatory strategy.

Note that the effect of dilution depends on the specific features of both the growth curve and metabolite time course, so that it is difficult to correctly estimate the changes in \([M_c]\) in absence of a good knowledge of the subcellular compartmentalization and its evolution in terms of relative volume occupancy within the cell. Of course the effect of dilution by growth is expected to be limited, at least in the last developmental stages, as long as vacuolar compounds are considered but, as shown before, it can become dramatic when compounds are localized in smaller organelles or in the cytosol.

METABOLIC CORRELATION ANALYSIS ARE SEVERELY BIASED BY DILUTION

The above considerations naturally extend to the analysis of correlations among concentrations of compounds, in a system-level perspective.
As an example, let’s now consider 50 cytosolic metabolites, with linear or non-linear seasonal variation (Figure 1B). As shown in Figure 1B the pattern of correlation between the different metabolites can change dramatically due to dilution by growth. Indeed, most correlation values are altered and 47% of the metabolites display an opposite correlation when considering the total tissue in place of the cytosol (Figure 1C).

As a consequence, metabolites that could be meaningfully clustered together according to their cytosolic variations are now split into separate groups whereas non-correlated metabolites are incorrectly merged into a common cluster by dilution effect (Figures 1D,E). Overall, the effect of volume expansion is to increase positive correlation among cytosolic metabolites, damping variations at late developmental stages.

**DISCUSSION AND PROSPECTS**

Plant development is a dynamic process that involves a complex series of molecular and biochemical events associated to volume expansion. Understanding its regulation is one of the great challenges of modern biology and has been the goal for many omics studies over the last years.

It is striking that in many studies fresh weight is used as the reference to express concentrations of biomolecules, irrespective of their subcellular localization. We show here that considering subcellular compartments is of primary importance for the correct interpretation of experimental results, especially when dealing with a system level perspective. Note that in the example we used, the organ volume was assumed to change with time but our conclusions are valuable for any other factor inducing organ volume variation, such as carbon or water stress, temperature, or genotypic traits. As a consequence, the bias induced by growth can potentially affect not only studies on dynamic profiling (Lombardo et al., 2011; Osorio et al., 2011, 2012) but also comparison among genotypes or the investigation of system response to stress (Cross et al., 2006; Sulpice et al., 2010; Osorio et al., 2012). For instance, Steinhauser et al. (2010)
found that most of tomato enzyme activities decrease during fruit development and interpreted this as being at least in part due to vacuole expansion (in their study, the fruit volume varied from almost 0 to 60 cm³).

In the last few years a number of studies have pointed out subcellular compartmentalization as a key feature of plant cell biology and as an unavoidable requirement for accurate experimental measurements (Sun et al., 2004; Kruger et al., 2007; Fernie and Stitt, 2012) and models (Grafahrend-Belau et al., 2004; Kruger et al., 2007; Fernie and Roberts, 2003), so that nuclear size could also be used as a proxy for the cytoplasmic compartment. However, methods for routine measurement of nuclear size have to be developed.

In absence of molecular measurements, dry mass, can be used as a normalization factor in situations where the water content and thus fresh mass can undergo strong variations (e.g., under water stress). If in addition to water, the content in storage compounds (starch, sucrose, amino acids, etc.) is expected to change, the above strategy can be iterated by expressing the concentration with respect to the structural dry mass (SDM) of the tissue, as:

\[ [X] = \left( \frac{DM}{FM} \right) \left( \frac{SDM}{DM} \right) \frac{M_x}{SDM} \text{ with } SDM = DM - STO \]

where STO is storage compounds content of the tissue. In this case, the first component describes the dilution due to water, the second describes the dilution due to the accumulation of storage carbohydrate and the third describes the concentration of \( X \), independently of the dilution processes.

Another standardization used in water stress studies consists in expressing data on fresh mass at full turgor (Hummel et al., 2010). This method provides the possibility to evaluate the contribution of accumulated metabolites to osmotic adjustment. It nevertheless involves an additional and work intensive experimental step, which restricts its use to small scale experiments.

In the case of studies on leaf tissues, normalization with respect to leaf surface (Krapp et al., 1993) is generally advantageous from a practical point of view (collection of leaf discs of known surface) and perhaps also conceptually. In particular, various studies indicate that under stresses or genetic manipulations affecting photosynthesis, photosynthetic activity appears less sensitive when expressed on a mass-basis than on an area-basis, the latter being the one that correlates best with relative growth rate (Poorter et al., 2009). Chlorophyll content has also been used for leaves (Holaday et al., 1992) but its stability has to be checked.

Another way to circumvent the problem bound to subcellular volumes is to express data in a “semi-quantitative” way, i.e., not an absolute amount per unit of biomass but a relative amount. In the simplest case a signal obtained for a given analyte is divided by the signal obtained by another analyte, either internal, or external to the system under study. In the case of mass spectrometry-based metabolomics (Katajamaa and Oresic, 2007), reference samples are often used and metabolite levels are expressed as “fold-changes.” Their use assumes that variations in the relative sizes of compartments do not affect the output. However, only a fraction of the metabolites are solely located within one cell compartment, and their distributions between compartments may vary. The use of isotope-labeled biological standards does actually not solve this problem. Metabolomics also use single or multiple “internal” (added before the extraction) or “external” standards (added after the extraction). Metabolites are then expressed relative to one or more standards, but subcellular compartmentation is again critical. In contrast to metabolomics, transcriptomics (Irizarry et al., 2003; Bullard et al., 2010) and proteomics (Clough et al., 2012) usually involve normalization procedures where the whole population of analytes of a sample, an experiment or a series of experiments represents the reference, thus reducing the bias induced by changes in compartments/organelles volumes. Although such normalization probably is the most suitable for correlation analyses, it is considered as less robust for metabolomics (Katajamaa and Oresic, 2007). It is worth mentioning that “semi-quantitative” data are suitable for studying fold-changes or for looking for correlations between variables, but not necessary for building mechanistic models (Rohwer, 2012). Indeed, whereas a range of integrative approaches can cope with alternatives to fresh weight to express amounts and/or activities of biomolecules, in others, such as mechanistic modeling, it will be crucial to evaluate the concentrations and thus the volume of the compartments where the enzymes or the metabolites are present. In that perspective, a challenging issue for the histologists moving to systems biology would be to find new techniques allowing quick measurement of compartment volumes and to feed existing databases on organelles dynamics (Mano et al., 2008, 2011).

**ACKNOWLEDGMENTS**

This work has been funded by the Eranet Erasysbio+ FRIM project and by INRA.
REFERENCES

Bullard, J. H., Purdom, E., Hansen, K. D., and Dudoit, S. (2010). Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinform. 11:94. doi: 10.1186/1471-2105-11-94

Carreno-Quintero, N., Acharjee, A., Maliepaard, C., Bachem, C. W. B., Mumm, R., Bouwmeester, H., et al. (2012). Untargeted metabolic quantitative trait loci analyses reveal a relationship between primary metabolism and potato tuber quality. Plant Physiol. 158, 1306–1318. doi: 10.1104/pp.111.188441

Clough, T., Thaminy, S., Ragg, S., Aebersold, R., and Vitk, O. (2012). Statistical protein quantification and significance analysis in label-free LC-MS experiments with complex designs. RMC Bioinform. 13:S6. doi: 10.1186/1471-2105-13-S16-S6

Cross, J. M., Von Korff, M., Altmann, T., Bartzetko, L., Sulpice, R., Gibon, Y., et al. (2006). Variation of enzyme activities and metabolite levels in 24 Arabidopsis accessions growing in carbon-limited conditions. Plant Physiol. 142, 1574–1588. doi: 10.1104/pp.106.086629

Fernie, A. R., and Sitt, M. (2012). On the discordance in measuring exposure of plants to low temperature. In Arabidopsis rosettes and Junker, B. (2009). Flux balance analysis of Arabidopsis thaliana leaf metabolism. PLoS ONE 6:e17806. doi: 10.1371/journal.pone.0017806

Krueger, S., Steinhauer, D., Willmitzer, L., and Giavalisco, P. (2012). High-resolution plant metabolomics: from mass spectral features to metabolites and from whole-cell analysis to subcellular metabolite distributions. Plant J. 70, 39–50. doi: 10.1111/j.1365-313X.2012.04902.x

Li, Z., Palmer, W. M., Martin, A. P., Wang, R., Rainsford, F., Jin, Y., et al. (2012). High invertase activity in tomato reproductive organs correlates with enhanced sucrose import into, and heat tolerance of, young fruit. J. Exp. Bot. 63, 1155–1166. doi: 10.1093/jxb/err329

Liberman, I. M., Sozzani, R., and Benfey, P. N. (2012). Integrative systems biology: an attempt to describe a simple weed. Curr. Opin. Plant Biol. 15, 162–167. doi: 10.1016/j.pbi.2012.01.004

Lombardo, V., Osorio, S., Borsani, J., Lauxmann, M., Bustamante, C., Budde, O. C., et al. (2011). Metabolic profiling during peach fruit development and ripening reveals the metabolic networks that underpin each developmental stage. Plant Physiol. 157, 1696–1710. doi: 10.1104/pp.111.186064

Mano, S., Miwa, T., Nishikawa, S., Mimura, T., and Nishimura, M. (2008). The plant organelles database (PODB): a collection of visualized plant organelles and protocols for plant organelle research. Nucleic Acids Res. 36, D929–D937. doi: 10.1093/nar/gkm789

Mano, S., Miwa, T., Nishikawa, S., Mimura, T., and Nishimura, M. (2011). The Plant Organelles Database 2 (PODB2): an updated resource containing movie data of plant organelle dynamics. Plant Cell Physiol. 52, 244–253. doi: 10.1093/pcp/pcr184

Moreno-Risueno, M. A., Busch, W., and Benfey, P. N. (2010). Omics meet networks - using systems approaches to infer regulatory networks in plants. Curr. Opin. Plant Biol. 13, 126–131. doi: 10.1016/j.copb.2009.11.005

Oikawa, A., Matsuda, F., Kikuyama, M., Mimura, T., and Saito, K. (2011). Metabolomics of a single vacuole reveals metabolic dynamism in an alga Chama australis. Plant Physiol. 157, 544–551. doi: 10.1104/pp.111.183772

Okumoto, S., Jones, A., and Frommer, W. B. (2012). Quantitative imaging with fluorescent biosensors. Annu. Rev. Plant Biol. 63, 663–706. doi: 10.1146/annurev-arplant-042110-103745

Osorio, S., Alba, R., Damasceno, C. M. B., Lopez-Casado, G., Lohse, M., Zanor, M. I., et al. (2011). Systems biology of tomato fruit development: combined transcript, protein, and metabolite analysis of tomato transcription factor (nor, rin) and ethylene receptor (Nr) mutants reveals novel regulatory interactions. Plant Physiol. 157, 405–425. doi: 10.1104/pp.111.175463

Osorio, S., Alba, R., Nikoloski, Z., Kochevenko, A., Fernie, A. R., and Giovannoni, J. I. (2012). Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behaviour. Plant Physiol. 159, 1713–1729. doi: 10.1104/pp.112.199711

Poorter, H., Niinemets, U., Poorter, L., Wright, I. J., and Villar, R. (2009). Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytol. 182, 565–588. doi: 10.1111/j.1469-8137.2009.02830.x

Riedelsheimer, C., Czedik-Eysenberg, A., Grieder, C., Liseck, J., Technow, F., Sulpice, R., et al. (2012). Genomic and metabolic prediction of complex heterotic traits in hybrid maize. Nat. Genet. 44, 217–220. doi: 10.1038/ng.1013

Rohwer, J. M. (2012). Kinetic modelling of plant metabolic pathways. J. Exp. Bot. 63, 2275–2292. doi: 10.1093/jxb/ers080

Saito, K., Hirai, M. Y., and Yonekura-Sakakibara, K. (2008). Decoding genes with coexpression networks and metabolomics - majority report by precogs. Trends Plant Sci. 13, 36–43. doi: 10.1016/j.tplants.2007.10.006

Sternath, M., Sternath, N., Peters, R., Schauer, N., Groth, D., Hummel, J., et al. (2010). Discovering plant metabolic biomarkers for phenotype prediction using an untargeted approach. Plant Biotechnol. J. 8, 900–911. doi: 10.1111/j.1467-7652.2010.00516.x

Steinhauer, M.-C., Steinhauer, D., Koehl, K., Carrari, F., Gibon, Y., Fernie, A. R., et al. (2010). Enzyme activity profiles during fruit development in tomato cultivars and Solanum pennellii. Plant Physiol. 153, 80–98. doi: 10.1104/pp.110.154336

Stitt, M., Sulpice, R., and Keurentjes, J. (2010). Metabolic networks: how to identify key components in the regulation of metabolism and growth. Plant Physiol. 152, 428–444. doi: 10.1104/pp.110.150821

Sugimoto-Shirasu, K., and Roberts, K. (2003). Big it up: endoreduplication and cell-size control in plants. Curr. Opin. Plant Biol. 6, 544–553. doi: 10.1016/j.pbi.2003.09.016

Sulpice, R., Trenkamp, S., Steinhath, M., Usadel, B., Gibon, Y., Wittucka-Wall, H., et al. (2010). Network analysis of enzyme activities and metabolite levels and their relationship to biomass in a large panel of Arabidopsis accessions. Plant Cell 22, 2872–2893. doi: 10.1105/tpc.110.076653
Sun, Q., Emanuelsson, O., and Van Wijk, K. J. (2004). Analysis of curated and predicted plastid subproteomes of Arabidopsis. Subcellular compartmentalization leads to distinctive proteome properties. Plant Physiol. 135, 723–734. doi: 10.1104/pp.104.040717

Tohge, T., Ramos, M. S., Nunes-Nesi, A., Mutwil, M., Giavalisco, P., Steinhauser, D., et al. (2011). Toward the storage metabolome: profiling the barley vacuole. Plant Physiol. 157, 1469–1482. doi: 10.1104/pp.111.185710

Received: 17 December 2013; accepted: 23 February 2014; published online: 11 March 2014.

Citation: Génard M, Baldazzi V and Gibon Y (2014) Metabolic studies in plant organs: don't forget dilution by growth. Front. Plant Sci. 5:85. doi: 10.3389/fpls.2014.00085

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.