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# Plant Metabolomics: A Characterisation of Plant Responses to Abiotic Stresses

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## 1. Introduction

As with all organisms, plants thrive within a range of environmental conditions that are optimal for their growth and development. They must, however, respond and adapt to conditions that deviate from the optimal, such as low/high temperature, dehydration, high salinity, oxidative stress, heavy metals and nutrient deficiency; these deviations are often responsible for losses in productivity and for spatial (geographical) and temporal (growing season) limitations in the cultivation of crops.

Although plants and animals share some responsive mechanisms to unfavourable environmental conditions, plants, as sessile organisms, have developed highly sophisticated and efficient strategies of response.

Because of the great interest for both basic and applied research, many scientific endeavours have long addressed the understanding of the mechanisms underlying the stress response and the identification of the specific genes/metabolites that are responsible for tolerance phenotypes. In recent years, the "omics" approaches have allowed high-throughput analyses of the changes that are induced by environmental stresses, confirming data previously obtained with targeted analysis and extending the scope of investigation.

It is noteworthy that the metabolomic changes that have been observed in plants subjected to stress conditions depend on different causes; therefore, they have different significance and are expected to differently correlate with tolerance/sensitivity phenotypes. Namely, changes in the metabolome composition due to adverse environmental conditions may depend on i) the stability and catalytic activity of enzymes involved in the production/degradation of specific metabolites, ii) the production of abnormal compounds (or abnormal concentrations of normal compounds) as a result of cell damage, iii) the adjustment of concentration of some metabolites to restore homeostasis and normal metabolic fluxes and iv) the synthesis and/or accumulation of compounds involved in mediating tolerance mechanisms.

The main goal of studying metabolic changes during stress responses is to identify metabolites belonging to the (iii) and (iv) groups that are responsible for stress tolerance.

Upon exposure to osmotic stress as a result of low temperature, drought and high salinity, plants accumulate a range of osmolytes with the primary function of turgor maintenance.

The solutes accumulated vary among species and include sugars (i.e., sucrose, glucose, fructose and trehalose), polyols, betaines and amino acids, such as proline (Shulaev et al., 2008; Smirnoff, 1998). Many compounds are known to play a role as osmoprotectants, acting as low molecular weight chaperones, stabilising the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging the reactive oxygen species (ROS). Examples of these molecules are glycine betaine, proline and mannitol (Chen & Murata, 2008; Szabados & Savourè, 2010). Other compounds act as chelating agents (sequestering toxic metals and ions), redesigners of lipids (optimising the structure and fluidity in membranes), energy sources and/or signalling molecules (Alcázar et al., 2010; Valluru & Van den Ende, 2008).

Although the involvement in tolerance phenotypes for some metabolites is inferred on the basis of their increase under stress and of their physico-chemical and biological properties, it may be very difficult to assign a specific function.

For some compounds, such as proline and glycine betaine, the exogenous application of the molecule or the enhancement of their biosynthesis through the ectopic expression of a rate-limiting gene has resulted in a stress tolerance improvement (Chen & Murata, 2008; Kishor et al., 1995; Quan et al., 2004; Szabados & Savourè, 2010). Moreover, another transgenic approach has included the overexpression of transcription factors involved in stress-specific gene regulation, such as DREB or MYB factors, in particular, those regulating the synthesis of osmoprotectants (Gosal et al., 2009). However, even if genetic engineering offers a good tool for a substantial improvement in a desired trait within a short time, it must be considered that most of the transgenic lines obtained thus far have not been field-tested (Ashraf, 2010).

The possibility of monitoring a complete set of metabolites could largely improve the understanding of the adaptation mechanisms. This systematic study defined "metabolomics" is intended to provide an integrated view of the functional status of an organism, significantly contributing to the study of stress biology in plants. Depending on the question addressed, specific approaches or their combination can be used in metabolomic investigations: metabolic fingerprinting, metabolic profiling and targeted analysis. A variety of analytical techniques, such as GC-MS, LC-DAD-MS, FT-IR and NMR, are successfully employed for metabolic fingerprinting and profiling, whereas targeted analysis is performed using both the above-mentioned techniques (integrated with the use of spiking experiments or *in vivo* labelling) and the more traditional biochemical analyses. The huge volumes of data generated by these approaches require advanced multivariate statistical analysis (supervised or unsupervised) to increase the knowledge base. Moreover, in the last years, metabolomics data handling has been improved because of the development of publicly available bioinformatic tools and databases.

Here, we review the recent progress in this field, highlighting the advantages and limitations of the above-mentioned approaches and techniques. We will focus on metabolite changes induced by abiotic stresses and discuss the meaning of specific and non-specific responses to different stresses. Moreover, a comparison of metabolite profiling among species and/or cultivars differing in their stress tolerance, as well as the metabolic content of wild type plants versus mutants or transgenics, will be reported to highlight qualitative and/or quantitative differences correlating with the phenotypes.

The differences in the metabolite content can also represent good predictors for stress tolerance phenotypes both in screening of varieties and in plant breeding programs.

Finally, we will discuss the potentiality of the global analysis of data obtained with different "omics" approaches, such as an integrated metabolome, transcriptome and proteome analysis, as a valuable strategy to attain a holistic view of mechanisms sustaining stress tolerance in plants.

## 2. Analytical techniques

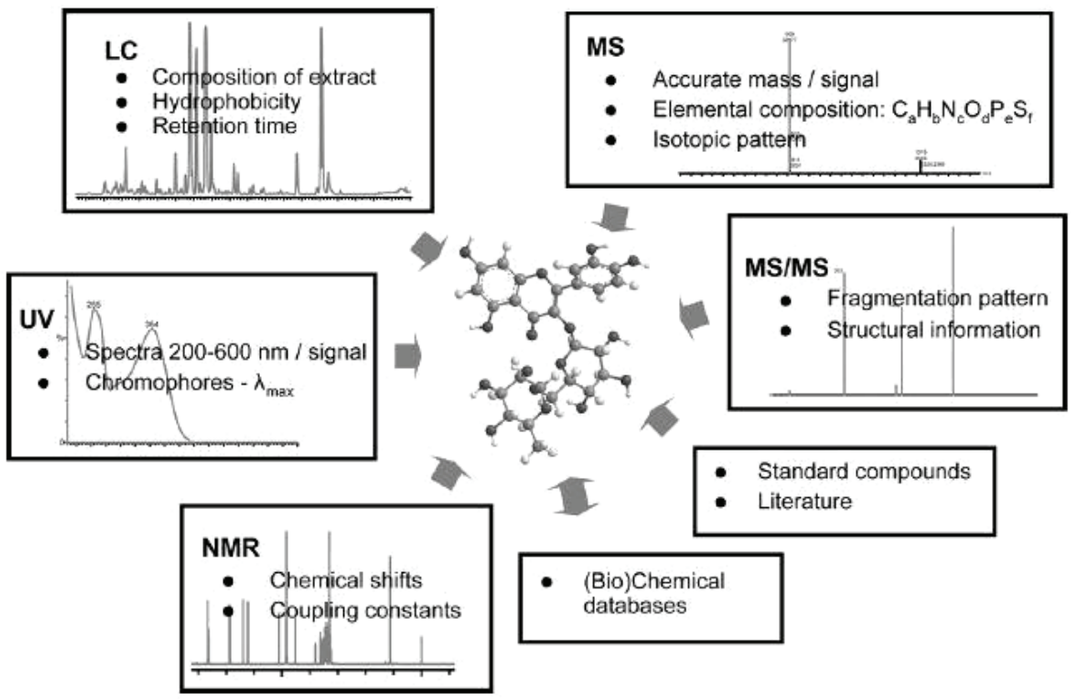
It has been estimated that hundreds of thousands of different metabolites are present in plants, with various chemical structures and, for many of them, with well-established bioactivities (Verpoorte, 1998). The analysis, chemical characterisation and quantification of these metabolites usually involves a multidisciplinary approach, based on different analytical techniques.

Metabolomics studies have been applied to different fields (Krastanov, 2010), ranging from environmental science, food science, human safety and plant biology (Bundy et al., 2008; Cevallos-Cevallos et al., 2009; Fukushima et al., 2009).

The aim of the metabolomic approach is to identify a much larger possible number of metabolites to better understand the biological system under investigation. Recently, (Dettmer et al., 2007) several terms have been used for metabolomics related definitions, such as metabolic profiling, metabolic fingerprinting and metabolic footprinting. These three approaches are fully integrated into the metabolomic investigations.

Metabolite identification is a real challenge, where many factors play a relevant role, including the analytical tool used, the sample preparation, the bio-computational tool for the data mining and the quality of the acquired data. Sample preparation is the most underestimated problematic part of the metabolomics analysis; a wide chemical diversity of compounds is present with a very high range of concentrations that could be present simultaneously. Appropriate extraction procedures need to be evaluated to obtain the maximum number of chemical components within the same sample. In this respect, the chemical classes of compounds could require specific separation processes involving solvents with different polarity. Furthermore, especially when NMR analysis is performed, the presence of buffered solutions (to control small shifts of NMR signals due to different pH values) or deuterated solvents is required. Detailed sample preparation procedures can be found in a recent review (Schripsema, 2010). Extraction procedures can be followed by chromatographic techniques, including TLC, HPLC, UPLC, HILIC (Hydrophilic Interaction LIquid Chromatography) (Bajad & Shulaev, 2011) and GC to eliminate possible contaminants and to obtain selected fractions. The commonly accepted analytical platforms to investigate plant metabolome are MS- and NMR-based systems, and, even more frequently, these two approaches have been combined to address the identification of metabolites in complex extracts (Moco et al., 2007). In Scheme 1, a pictorial diagram of different platforms used in metabolite identification is represented. MS-based approaches are often limited by separation and derivatisation protocols, as well as the detection capability, which usually allows single metabolite detection and quantification. Furthermore, the physico-chemical properties of metabolites (e.g., volatibility, low ionisability, lack of chromophores) could limit the determination; in such cases, only limited metabolic profiling can be performed. Other techniques, such as NMR and all of its technical modifications, do not require any derivatisation and limited (liquid state) or no extraction procedures of the sample (solid state), thus, allowing for the identification and quantification of different kinds of metabolites from the same sample in the shortest time.

This technique is partially limited by the relatively low sensibility when compared to MS spectrometry (the detection limit in the sub-microgram region at 14.1 T). As a matter of fact, with the advent of new ultra-high field magnets (1 GHz is now commercially available) and cooled probes, NMR methods have experienced a dramatic increase in sensibility and, thus, have become a valid alternative to mass spectrometry, reaching almost the same sensitivity (ppb). Moreover, the advent of NMR microprobes, with active volume as low as 1.5 μL, have provided new possibilities for analysing molecules in very low volumes, increasing concentration of the analyte without compromising the Signal/Noise ratio.




Scheme 1. Example of possible analytical technologies and databases that can be used to identify rutin. (Reproduced with permission from Moco et al., 2007).

The development of the so-called "hyphenated techniques" take advantage of the separation and detection processes performed continuously in a single step; these techniques range from the basic LC-MS and GC-MS approaches recently reviewed by T'Kindt et al. (2009). It has been demonstrated that combination of high-end analytical technologies facilitates the structure elucidation process of small mass molecules present in minute quantities in valuable samples. In this respect, several applications have been recently developed, such as LC-SPE-NMR or the more efficient LC-SPE-NMR-MS set up which overcomes the limitation of direct coupling between LC and NMR (Schlotterbeck & Ceccarelli, 2009; Yang et al., 2009; Van Beek et al., 2009). The instrumental setup of these techniques implies the physical joint of chromatographic-based instruments to spectroscopic detection-based instrumentations, making these systems not easily affordable to standard research laboratories. Nevertheless, improvements have been obtained either with the introduction of reproducible strategies for metabolite identifications or with the exchange of identifications among laboratories. The introduction of the concept of Mass Spectral Tag (MST), defined as ensemble of properties

(molecular mass to charge ratio, chromatographic retention index and the induced mass fragmentation pattern) (Kopka, 2006), enhanced the GC-MS data exchange and then the identification of compounds. The term "hyphenated techniques", first introduced by Hirschfeld et al. (1980), has experienced a progressive evolution among differently combined techniques selected to tackle challenging problems. The theoretical application of multiple hyphenation steps, usually called "hyphernation" (e.g., LC-DAD UV-NMR/MS-MS method), is technically very difficult and not applicable due to the high cost. These approaches have been reviewed and discussed previously (Wilson & Brinkman, 2003).

### 2.1 Hyphenated chromatographic techniques

Chromatographic techniques, usually adopted to select chemically equivalent compounds, are essentially based on two different phases: liquid and gas. With recent technological improvements (e.g., long narrow bore capillary columns, capillary columns), the liquid phase techniques can reach sensitivity magnitudes on the order of nano-g. In the gas phase, sensitivity is largely affected by the ionisation source conditions because ion production is the basic requirement for this type of analysis. The detection limit can be as low as a few ng/L.

Based on the different mobile phases adopted in the chromatographic techniques, an arsenal of different methods has grown during recent years, and all of them took the advantage of the combination of techniques, due to the fact that both quantification and identification are important in metabolomic research. Techniques, such as HPLC-MS, GC-MS, CE-UV and HPTLC, or multi-combined techniques, such as LC-MS-NMR, GC-IT-MS-MS and LC-MS-MDF, are adopted almost routinely in current plants metabolomic studies (Abou-Donia et al., 2007; Gotti et al., 2006; Llop et al., 2010). Recently, Berkov et al. (2011) developed and validated a new GC-MS method for the rapid determination of galanthamine in *Leucojum aestivum*, a study that also focused on the determination of the origin of the plant. This method, with the aid of Principal Component Analysis (PCA), was rather informative (metabolomic based), providing information not only on the galanthamine content but also on alkaloid profiles; these data could be successfully correlated with the plant species, plant organs and the geographical origin of the plant.

### 2.2 Hyphenated spectroscopic techniques

The possible combination of a high performance chromatographic technique with a high characterisation technique is probably one of the best possible approaches for the quantification and characterisation of metabolite composition. As a matter of fact, hyphenated LC-MS, HPLC-MS, GC-MS and HILIC-LC-MS have been largely adopted in plant metabolite profiling (Allwood et al., 2009; Allwood & Goodacre, 2010; Cubbon et al., 2010) for their performance for selectivity and sensitivity in targeted analysis, enabling the detection of very low abundant and/or volatile compounds. In contrast, NMR hyphenated techniques take the advantage of a non-targeted analysis performed in a quantitative fashion to detect most of the highly abundant primary and secondary metabolites, with relevant structural information. A comparison between MS and NMR techniques have been reviewed by Krishnan et al. (2005). More recently Dai et al. (2010a, 2010b) successfully applied and combined NMR and LC-DAD-MS analysis to investigate the metabolic variations of three cultivars of *Salvia milthiorrhiza* Bunge (SMB), as well as changes induced by water depletion. The combination of these two analytical techniques has allowed the

detection of both the primary and secondary metabolites content. In particular, the authors have found that the metabolome of SMB is dominated by 28 primary metabolites (sugars, amino acids and carboxylic acids) and 4 secondary metabolites (polyphenols) (Figs 1 and 2).

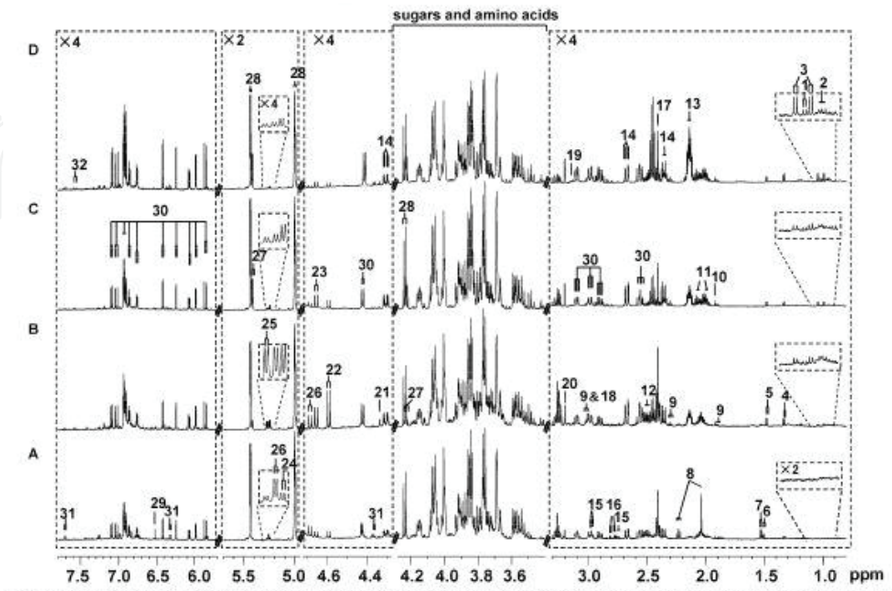



Fig. 1. $^{1}$H NMR spectra (600 MHz) of *Salvia milthiorrhiza* Bunge extracts from four different geographical origins: A) Zhongjiang, Sichuan B) Wuhan, Hubei C) Anding, Hebei D) Nanyang, Henan (Reproduced with permission from Dai et al., 2010a).

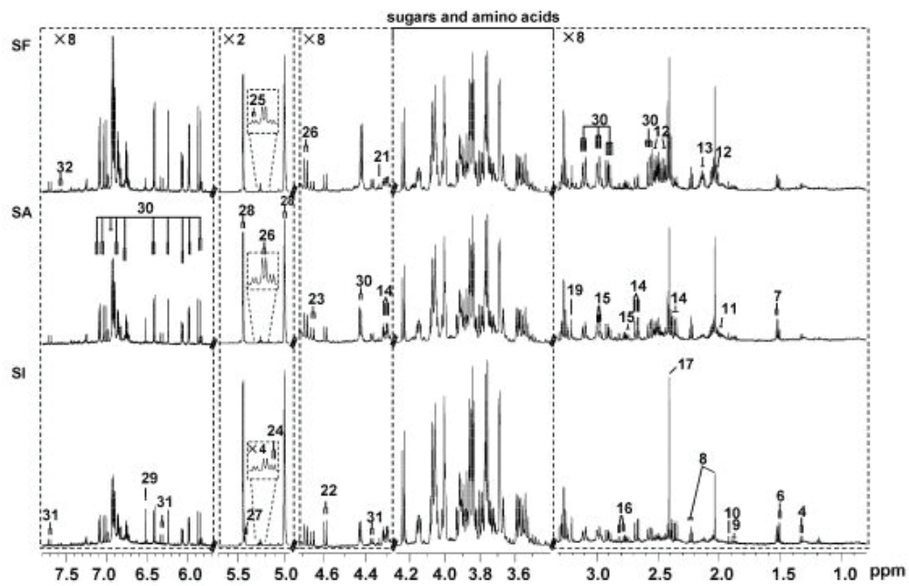



Fig. 2. $^{1}$H NMR spectra (600 MHz) of three different cultivars from *Salvia milthiorrhiza* Bunge: SF) Folium SA) Sativa SI) Silcestris (Reproduced with permission from Dai et al., 2010a).

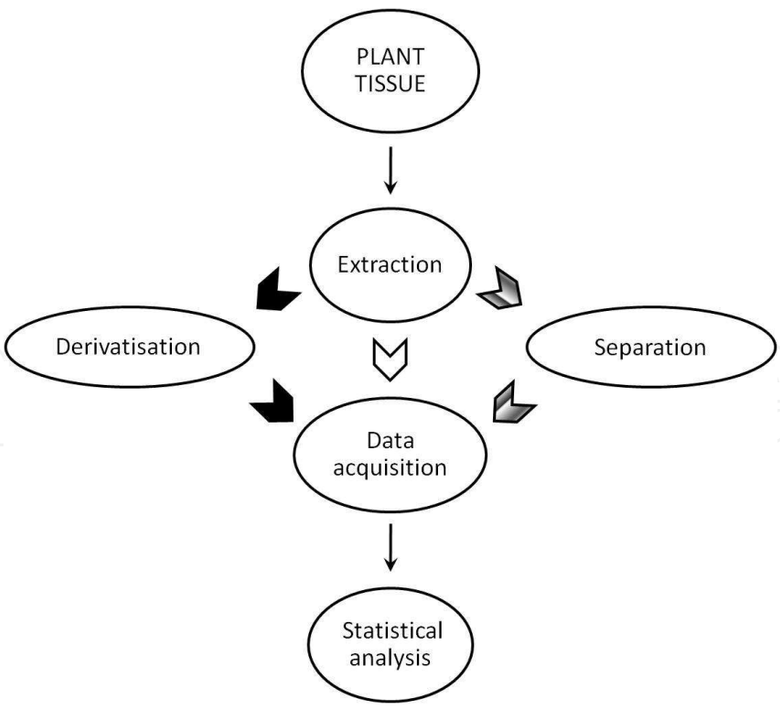
The systematic analysis of the metabolite composition of these three cultivars of SMB, grown in four geographical areas, allowed the assessment of differences among ecotypes and growing-location effects for the same cultivars.

## 3. Data treatment

### 3.1 Chemometrics

The increased specificity and sensitivity of the analytical tools has offered the feasibility of obtaining a wide range of information with a single experiment. This technological breakthrough has allowed large dataset collections, enabling the possibility to evaluate similarities/differences among samples not possible in earlier studies. This approach, known under the general term ''metabolomics,'' properly refers to the collection of small molecules that can be found in a cell, organ, or organism. The metabolomic approach can be created by the following two different schools of thought: i) the chemometric approach, in which the chemical compounds are not identified, but their spectral patterns are statistically analysed to identify relevant spectral features that could differentiate samples and ii) the targeted or comprehensive profiling approach, in which the aim is firstly to identify and quantify most of the chemical compounds and secondly to perform statistical methods to identify relevant biomarkers. In Scheme 2, a pictorial representation of the flowchart used for metabolite identification is represented.

The term "chemometrics" is largely accepted today as a general statistical approach coupled to analytical techniques. The statistical approaches could be represented into two different classes: monovariate statistical analysis and multivariate statistical analysis.




Scheme 2. Schematic representation of the processes in metabolite analysis. Filled, dashed and empty arrows indicate the routes for GC–MS, chromatographic (LC/HPLC/CE) and NMR based metabolomics, respectively.

### 3.1.1 Monovariate statistical analysis

Significant amounts of data are obtained by measuring many variables on an ensemble of chemical samples or by recording many signals from an industrial process to track its behaviour. A data collection task, whether in science, business, or engineering, typically involves many measurements made on several samples. The unavoidable data variability has traditionally been analysed using one or two variables at a time. However, to discover the relationships among all of the samples and variables efficiently, all of the data must be processed simultaneously. Chemometrics is intended to extract information in multivariate chemical data, using the tools of statistics and mathematics. It is typically used for three primary purposes: to explore the patterns of similarities in the data, to track the properties of materials on a continuous basis, and to prepare and use multivariate classification models. In general, the algorithms applied have demonstrated significant capacity in analysing and modelling a wide variety of data types for an even more diverse set of applications. Different mathematical methods can be used to explore experimental data, based on the different possible targets; this phase provides information about statistical parameters of each variable and correlations among variables, reducing the data dimensionality. Among the possible systems, the analysis of variance, ANOVA (Miller & Miller, 1993), is used to select the variables that are most significant in the sample differentiation. This univariate statistical technique is used for testing the null hypothesis when two or more samples are drawn from the same population; high values of the F-test suggest that the null hypothesis can be discarded. This technique is no longer used for large data sets (especially in the case of spectroscopic data). The extension of ANOVA is called ''multivariate data analysis'' (MANOVA), and it is used whenever more than one correlated variable is concerned and they cannot be simply combined. MANOVA selects discriminant variables with high indices of reliability.

### 3.1.2 Multivariate statistical analysis

Unlike monovariate methods, where only one variable is considered, in multivariate statistical analysis, correlations among more variables are concerned. Multivariate data analysis is frequently used to address the following aspects: i) data overview ii) classification and or discrimination among groups of observations and iii) regression modelling between two blocks of data (X and Y). These applications reflect the main stages of multivariate analysis. One of the aims of this technique is to reduce the system dimensionality. Among the so-called ''compression techniques'', PCA (Geladi & Kowalski, 1986; Jackson, 1991) is widely used and recognised as the main ''unsupervised'' technique for the primary analysis of data. This method finds linear combinations of the variables in the original data, called PCs, which are orthogonally related and describe the major trends in the data. When the minimum meaningful number of PCs has been found, by means of loadings and score matrices, the original data matrix can be rebuilt. Inspection of the loadings gives indications on how the PCs are obtained from the original variables and how much the variable has in common with that PC. Scores show how the observations are clustered together on the basis of their variables.

Another compression technique, member of the so-called ''classification methods'', is the cluster analysis (CA) (Romesburg, 1984), that is applied to evaluate similarities and clusters among samples. This approach based on ''similarities'' or ''classification'' methods can also be split into hierarchical or non-hierarchical approaches. Commonly,

two types of clustering are used: K-Mean and Tree Clustering, named TCA. These classification methods are without *a priori* hypotheses in finding meaningful groups, and the result is often used for further statistical analysis. Dendrograms are usually adopted as graphical representation tools to visualise the data clustering. The same representation could be used to visualise the results of Hierarchical Clustering Analysis (HCA), included in the so called "segmentation methods", which group samples in dataset by their similarities according to their distances. These distances can be measured by different methods: Euclidean, Manhattan distances or correlations.

Another clustering method is the K-mean, which uses a fixed number (K) of groups. In this case, a metric distance should be defined to govern the clustering but the way of making groups is different. Several other clustering algorithms exist, essentially consisting of small theme variations, but none of them is best for a specific problem. As a matter of fact, these clustering methods are no longer used because more sophisticated and precise grouping methods have been developed.

Discriminant analysis (DA), also called ''discriminant function analysis'', performs sample classification with *a priori* hypothesis. This hypothesis could be based on a previously determined TCA or other CA protocols. The natural extension of DA is the "multiple discriminant analysis" (MDA), which sometimes is named ''discriminant factor analysis'' or "canonical discriminant analysis" (CDA). Among these type of analyses, "linear discriminant analysis" (LDA) has been largely used to enforce differences among samples classes.

Another classification method is called "quadratic discriminant analysis" (QDA) (Frank & Friedman, 1989) and consists of an extension of LDA. Another method, named "regularised discriminant analysis" (RDA), works better with various class distribution and in the case of high-dimensional data, is a compromise between LDA and QDA. More recently, "independent component analysis" (ICA) has been developed for the analysis of signals from complex mixtures (Comon, 1994). In this approach, the coefficients of the linear expansion of the data vectors must be mutually independent; this requires higher order statistics in determining the ICA expansion and some non-linearities must also be used in the learning phase, thus, resulting in a more meaningful data representation with respect to PCA. "Generalised discriminant analysis" (GDA) (McLachland, 1992) is used to determine whether a given classification of samples into a group is appropriate. Therefore, each sample is assigned to a group and a model is searched and computed to maximise the classification.

The general aim is to find out a mathematical model with high predictive capacity for a variable obtained from known values derived from the ensemble of independent variables; these types of protocols are called ''regression methods.'' The simplest model describes the Y variable that is linearly dependent on the X variable; this casual dependence is a linear regression. Science often involves controllable variables (factor or predictor variables) to explain, to regulate, or to predict the behaviour of other variables (response variables). When factors are few, not significantly redundant (collinear), and show a correct relationship to the responses, the multiple regression can be the proper means to turn data into information. When spectroscopic data are considered, factors (variables) can be hundreds and highly collinear; the responses are components that need to be predicted for future samples. In these cases, Partial Least Squares Projections to Latent Structures (PLS) (Wold et al., 1984) is used to create multivariate calibration models with predictive capacity. In principle, multiple linear regression can be used with a large number of factors. However, if this number is bigger than the number of observations, the model will fail to predict a new data set because of problems with overfitting. In such cases, there can be only a few

underlying or latent factors that account for most of the variation in the response. The origin of PLS acronym can be explained by considering the general idea of PLS, which is to extract these latent factors accounting for the largest manifest factor variation possible, while optimally modelling the response. In PLS, factors are used to predict responses in the population, which is achieved indirectly by extracting the latent factors, T and U, from factors and responses, respectively. The extracted factors, T (X scores), are used to predict the Y scores, U, and then to build predictions for the responses. In PLS, the X and Y scores are chosen so that the relationship between successive scores is as strong as possible. Currently, several linear and nonlinear multivariate classification methods exist: the choice implies the evaluation of discriminatory power against the ability to interpret the meaning of class differences. In this respect, Soft Independent Modelling of Class Analogy (SIMCA) (Wold, 1976) is a well-established method for multivariate classification; disjoint PCA is used for fitting each class and it is largely used, even though it does not give easily accessible class difference information, thus, hampering the quality of interpretation of the classification model. PLS discriminant analysis (PLS-DA) has largely been used for explaining differences among overall class properties that become progressively more complicated with an increasing number of classes. The relatively new orthogonal PLS-DA (OPLS-DA) (Bylesjö et al., 2006) approach has been demonstrated to be the most revealing of the generated models. OPLS-DA was obtained as an extension of the PLS method featuring an orthogonal signal correction (OSC) filter (Trygg & Wold, 2002). In other words, compared to PLS-DA, OPLS-DA effectively separates predictive from non-predictive (orthogonal) loadings variation, which is particularly enforced when a two-class model is concerned.

## 4. Abiotic stresses

During recent decades, most studies investigating the complex cascade of events occurring in plants upon exposure to abiotic stresses have been mainly focused on the gene expression level. Through the application of transcriptomics (in addition to forward and reverse genetics) hundred of genes have been linked with environmental stress responses and regulatory networks of gene expression have been delineated.

Relatively less is known about changes at the metabolomic level, but in the last few years the global metabolite analysis of plant stress response is representing a very rapidly expanding research field.

Here we will review a selection of recent publications, describing results obtained both in the model plant, *Arabidopsis thaliana*, and in several crop species, focusing on the following main abiotic stresses: low- and high-temperature, drought, high salinity and oxidative stress.

### 4.1 Temperature stress

Temperature is one of the most crucial environmental factors determining plant growth and development. Plants are subjected to continuous diurnal and seasonal temperature fluctuations, with consequences depending on whether these deviations from the optimal values remain within a natural temperature range for each species or whether extremes of this range are reached. However, the temperature range of survival in some species can be extended through "cold acclimation" or "acquired thermotolerance", the adaptive processes whereby plants increase in chilling/freezing or in heat tolerance in response to a prior low non-freezing or high temperature exposure, respectively. These highly complex inducible

mechanisms are accomplished by extensive reprogramming of the plant transcriptome, proteome and metabolome.

### 4.1.1 Low-temperature stress response in Arabidopsis

Metabolic changes in response to low temperatures (chilling and freezing) have been extensively characterised in the model plant, *A. thaliana*.

In one of the first studies aimed to explore (on a large scale) the Arabidopsis metabolome variations occurring in response to low temperature, Cook et al. (2004) compared the stress-induced global changes in two Arabidopsis ecotypes, differing for their acclimation ability (Ws-2: high acclimation and Cvi-1: low acclimation). They reported that, out of 434 metabolites monitored in cold-acclimated plants, 75% and 62% increased their amount in Ws-2 and in Cvi-1, respectively; most of the changes (91%) observed in the Cvi-1 plants significantly overlapped with those occurring in Ws-2 plants. Moreover, 114 metabolites showed a fivefold or higher increase in Ws-2, compared to only 47 metabolites in Cvi-1. Altogether, these findings suggested that the ability to acclimate may depend on and correlate with the magnitude of cold-induced global changes in the metabolome (Cook et al., 2004). However, Hannah et al. (2006), through a comparison of nine different Arabidopsis accessions, found that the extent of cold-driven metabolic responses did not simply correlate with the cold-acclimation capacity.

Several of the cold-induced metabolites, such as sugars, proline and polyamines, identified through global analysis have been previously reported to accumulate under cold stress in Arabidopsis and other species in studies utilising targeted analysis (Guy et al., 2008). However, the power of metabolite profiling with respect to targeted analysis consists of the identification of changes in the amount of metabolites not yet known to be involved in the process being considered (i.e., cold acclimation).

To elucidate the role of the CBF pathway in the reconfiguration of the low-temperature metabolome, Cook et al. (2004) also compared the metabolic profiles of Ws-2 wild-type (wt) plants and transgenic lines ectopically expressing the transcription factor CBF3. In non-acclimated transgenic lines, they found an increase in almost 80% of the metabolites that were cold-induced in wt (90% if metabolites induced more than fivefold were considered), thus, confirming a prominent role of the CBF pathway in the cold response.

More recently, Maruyama et al. (2009) reported analogous studies, with results that were only partially in agreement with those of Cook et al. (2004). Namely, Maruyama and co-workers compared the metabolite profile of wt Arabidopsis plants grown under control or stress conditions (cold and drought) and transgenic plants ectopically expressing DREB1A/CBF3 or an active form of DREB2A. The overexpression of DREB1A/CBF3 has been previously shown to confer a higher tolerance to both freezing and dehydration, whereas the overexpression of DREB2A significantly improved the tolerance to dehydration but not to freezing in transgenic plants.

Maruyama and co-workers found that DREB1A/CBF3 ectopic expression resulted in an increased amount of 37 metabolites, 33 of which also accumulated in cold-treated wt plants. Because cold induced the accumulation of 155 metabolites in these experimental conditions, the CBF pathway appeared to be involved in the increase of only 21% of the cold-induced metabolites (in comparison to 80%, as reported by Cook et al., 2004). In DREB2A transgenic plants (that are dehydration, but not freezing, tolerant), the level of 28 metabolites increased, 17 of which were also positively affected by DREB1A/CBF3.

Seventeen metabolites (including myo-inositol, galactinol, raffinose, sucrose and 13 unknowns) were found to increase both in cold-exposed wt plants and DREB1A/CBF3-overexpressing plants but not in DREB2A-overexpressing plants. Thus, these compounds were considered as possible candidates that play an active role in the cold response. However, the level of the 4 known and 7 of the 13 unknown metabolites also significantly increased in dehydration-exposed wt plants.

Interestingly, gene expression data obtained by a microarray analysis of the different lines and growth conditions were found to be in agreement with the metabolic data; in particular, the expression levels of genes involved in carbohydrate metabolism positively correlated with the accumulation of specific sugars and sugar alcohols under stress conditions (Maruyama et al., 2009).

Vannini et al. (2004) analysed the low temperature stress response of wt and transgenic Arabidopsis plants ectopically expressing *Osmyb4*, a rice gene involved in cold responses. The non-acclimated *OsMyb4*-transgenic plants exhibited a degree of tolerance to both chilling and freezing comparable to that developed by wt plants after cold acclimation. Subsequently, through both targeted and profiling analyses, Mattana et al. (2005a) compared the changes in the metabolic content of wt and *OsMyb4*-transgenic plants during a ten-day-long cold experiment. They correlated the better tolerance of transgenic plants to a higher content of several metabolites (proline, sucrose, glucose, fructose, glycine betaine, alanine and sinapoyl malate) that were present in the transgenics prior to the stress treatment and that may prepare plants to face the stress. Moreover, during cold treatment, the degree of tolerance of the transformed plants further increased; the amount of the above-mentioned metabolites raised in both wt and transgenic plants, but it was always higher in the transgenic lines than in wt during the time course of the experiment (Mattana et al., 2005a). Furthermore, the increased metabolic contents in transformed plants were consistent with the global changes observed in the mRNA population (Mattana et al., 2005a; Vannini et al., 2004, 2006).

Kaplan et al. (2004), in a time course experiment, found that the amount of 311 out of the 497 low-$M_r$ polar compounds that were detected was affected by cold exposure. The authors showed that changes in the metabolite contents were evenly distributed across all of the temporal stages of the cold-response: early, intermediate and late (corresponding to 1-4 hours, 12-24 hours and 48-96 hours, respectively), with either sustained or transient increase or decrease. These results indicated that acclimation is a long-term dynamic process.

This idea was strengthened by the finding (Gray & Heath, 2005) that the metabolic profile of Arabidopsis leaves that were shifted to low temperature was constantly changing (at least up to 49 days, the maximum cold-treatment period evaluated by the authors), whereas leaves that had developed at low temperature exhibited a stable metabolite composition.

The profile of the shifted leaves became more distinct from the profile of the untreated ones the longer the shifted leaves stayed at low temperature. However, the profile remained different from the profile of leaves that had developed at 4 °C. Therefore, the authors suggested the existence of two distinct metabolic networks in response to cold stress: one that is environmentally modulated and another that is developmentally modulated at low temperature. The authors hypothesised that the same might be true also in response to any other environmental stimulus.

Although many quantitative and qualitative differences among the results reported by several authors do exist, one of the aspects that is consistent in all of the studies on cold acclimation is the crucial role of carbohydrate metabolism.

Its prominent function in the cold response was confirmed by the comparative metabolite analysis conducted by Hannah et al. (2006). As we have already mentioned, these authors analysed nine different Arabidopsis accessions, originating from Scandinavia to the Cape Verde Islands and differing in their freezing tolerance, combining transcript and metabolite profiling. They showed that the global changes of transcripts, but not of metabolites, correlated with the cold acclimation ability. However, the accumulation of individual metabolites, including several carbohydrates (i.e., glucose, fructose, sucrose and raffinose), correlated significantly with freezing tolerance. Although the important role of soluble sugars and particularly of raffinose has been highlighted by many studies, it has been demonstrated that raffinose accumulation alone is neither necessary nor sufficient for cold acclimation (Zuther et al., 2004).

An important role for carbohydrate has also been reported in relation to heterosis for freezing tolerance (Korn et al., 2008, 2010; Rohde et al., 2004). A significant heterosis effect on leaf-freezing tolerance was first observed by Rohde et al. (2004) in the $F_1$ progeny resulting from reciprocal crosses between the accessions Columbia-0 (Col) and C24, where Col plants are more freezing-tolerant than C24 plants, in both non-acclimated and acclimated conditions. In this case, among the soluble sugars measured, only raffinose showed a strong correlation with the leaf-freezing tolerance.

Korn et al. (2008) extended this study to the analysis of 24 $F_1$ hybrid lines, generated by reciprocal crosses of either Col or C24 accessions with six other parental accessions, widely differing in freezing tolerance. The degree of heterosis for freezing tolerance depended on the analysed cross (with C24 showing a better combining ability than Col) and was genetically unrelated to the heterosis for biomass production. Through a targeted analysis, they found that freezing tolerance in acclimated and non-acclimated plants correlated with the content of sugars (glucose, fructose, sucrose and raffinose), flavonols and proline. Very interestingly, heterosis for freezing tolerance correlated with heterosis for flavonols and sugars accumulation, whereas the proline content exhibited no correlation with heterotic effects in freezing tolerance (Korn et al., 2008).

More recently, the same research group (Korn et al., 2010) used global metabolic profiling to discover metabolite combinations able to predict freezing tolerance and its heterosis. They identified several compatible solutes as crucial predictors for both phenotypes, in particular, metabolites belonging to the raffinose biosynthetic pathway and other yet unidentified compounds, in addition to some TCA cycle intermediates that specifically contributed only to predict the heterotic phenotype.

The approach used by Korn et al. (2010), aimed to identify groups of metabolites, instead of individual metabolites, that together possess a predictive potential, seems to be well suited to analyse a redundant cellular protection system, such as that represented by compatible solutes, where single compounds might act non-specifically and substitute for each other with compensatory mechanisms (Panikulangara et al., 2004; Zuther et al., 2004).

#### 4.1.2 High-temperature stress response in Arabidopsis

In comparison with the numerous works conducted on the metabolic changes induced by low temperature, only a few non-targeted metabolomic studies have been carried out on plants subjected to heat stress.

Kaplan et al. (2004; see section 4.1.1) performed a global metabolite profiling analysis by GC-MS to identify similarities and differences in temporal metabolite responses associated with

the induction of acquired thermotolerance in response to heat shock (HS) and acquired freezing tolerance in response to cold shock (CS).

One of the most prominent differences between the plant responses to high and low temperature was represented by the temporal dynamics of the induced metabolic changes. Indeed, whereas during cold exposure, changes were evenly distributed along the time course of the treatment, most of the heat-induced metabolic alterations occurred within the first 30 minutes (Gray & Heath, 2005; Kaplan et al., 2004).

Out of the 497 low-$M_r$ polar compounds detected, 143 were affected by HS; among them, several pyruvate- and oxaloacetate-derived amino acids, fumarate and malate (oxaloacetate precursors), some amine-containing metabolites (β-alanine, GABA and putrescine) and several carbohydrates (including maltose, sucrose, raffinose, galactinol and myo-inositol) were found to be coordinately increased (Kaplan et al., 2004).

Because 311 metabolites or mass spectral tags were altered in response to CS, it appeared that cold shock influenced metabolism more profoundly than heat shock. Moreover, a very large proportion of the HS metabolite response was shared with the CS response (with only a very small fraction being HS specific); in contrast, the majority of metabolites (60%) that were responsive to cold shock were CS-specific.

Ninety-three metabolites showed a common response between the two thermal stresses; among these, pyruvate- and oxaloacetate-derived amino acids, polyamines and several carbohydrates (including fructose, sucrose, myo-inositol-phosphate, galactinol and raffinose) were increased in their content under both HS and CS. Several of these molecules are known to be either compatible solutes or precursors for secondary metabolites with properties of protection against pathogens.

Concerning the role of raffinose in the heat stress response, Panikulangara et al. (2004) determined the content of this sugar in Arabidopsis wt plants, in transgenic plants overexpressing the major heat-shock transcription factor HSF3 and in two *Galactinol synthase1* (*GolS1*) T-DNA knockout mutants. Galactinol synthase is a key enzyme in the biosynthetic pathway of the raffinose family oligosaccharides (RFOs): raffinose, stachyose and verbascose. The expression of *GolS1* was heat-inducible in wt plants, constitutively up-regulated in HSF3-overexpressing transgenic plants and almost completely inhibited in the T-DNA insertion mutants. Wild-type plants showed a basal level of raffinose under non-stress conditions, that was increased following heat stress; in transgenic lines, a constitutive strong accumulation was observed that was further induced after heat stress; on the contrary, only a basal level without any increase after heat stress was detectable in the knockout mutants. Surprisingly, when the heat tolerance phenotype was investigated, no differences between the wt and knockout mutant lines were detected.

These results, together with those obtained by Zuther et al. (2004; see section 4.1.1.) about a lack of difference in the cold tolerance phenotype among Arabidopsis lines accumulating very different levels of raffinose, led to the unexpected conclusion that altering the raffinose content (using T-DNA inactivated or transgenic plants) did not affect the stress temperature tolerance phenotype.

One possible explanation is the presence of feedback and/or compensative mechanisms, whereby alterations of raffinose levels are accompanied by changes in the amount of other sugars/metabolites important in temperature stress tolerance. This hypothesis was supported by the increased amount of galactinol under cold acclimation found in the raffinose synthase knockout mutant (Zuther et al., 2004).

An increased level of the signalling molecule, salicylic acid (SA), was also observed by Kaplan et al. (2004) during both heat and cold stresses, although with different time courses. This finding suggested that SA, already known to play a key role in systemic acquired resistance to pathogens, could also function as an early signalling molecule in temperature stress responses. Actually, comparing wt and mutant plants accumulating different SA amounts, a correlation between pre-stress levels of endogenous SA and both basal and acquired thermotolerance was demonstrated (Clarke et al., 2004; Larkindale et al., 2005). Clarke and co-workers also have suggested an involvement of jasmonates in conferring basal thermotolerance to Arabidopsis plants.

### 4.1.3 Temperature stress response in crop species

Two rice genotypes, contrasting in chilling tolerance, were investigated for their response to cold, as well as to salt and osmotic stress (Morsy et al., 2007). Targeted metabolite analysis revealed that the two genotypes responded differently to the different stresses. Unexpectedly, the chilling-tolerant (CT) genotype was found to be more sensitive to drought and especially to salt stress than the chilling-sensitive (CS) one.

Differences in stress tolerance matched with, and may depend on, differences in metabolite accumulation between the two cultivars: indeed, under cold stress, CT accumulated galactose and raffinose, whereas these sugars decreased in CS. Conversely, CS specifically accumulated higher levels of osmoprotectants, such as mannitol and threalose under salt and drought conditions, respectively. The endogenous content of oxidative products and the activities of some antioxidative enzymes were also measured, leading the authors to hypothesise on the presence of a more efficient ROS scavenging metabolism in CT genotype during chilling stress (Morsy et al., 2007).

In addition to the studies in transgenic Arabidopsis mentioned above, Mattana et al. (2005b) performed similar studies on stress tolerance response in wt and *Osmyb4*-expressing transgenic plants in several species, using a constitutive (pCaMV35S) or a stress-inducible (pCOR15a) promoter.

The authors reported that the increase in Myb4-driven cold tolerance in maize, apple and Osteospermum well correlated with the increased concentration of sugars and proline. The transgenic maize plants grown under control conditions did not show any difference in metabolite concentration with respect to the wild-type, as the *Osmyb4* gene expression in maize was under the pCOR15a stress-inducible promoter. However, a 6-day cold-treatment increased the sugar (fructose, sucrose and glucose) and proline contents in both wt and transgenic maize plants, with the concentration being significantly higher for all of them in the *Osmyb4*-transgenic plants.

The use of the pCaMV35S constitutive promoter to drive *Osmyb4* expression in Osteospermum and apple transgenic plants resulted in increased sugars and proline concentration prior to the stress exposure (Mattana et al., 2005b).

In a further investigation, the authors reported the first metabolic profile of the Osteospermum species under control and stress growth conditions, thus, confirming and extending the previous observations on Myb4-driven cold tolerance and metabolic changes (Laura et al., 2010). Namely, in the PCA score plot, Osteospermum samples distributed according to genotype and treatments: control and 2-day cold-treated plants (wt and transgenics) clustered together, whereas 10-day cold-treated and freezing-treated plants separated into different regions based on genotype. The samples separation correlated with different amounts of sucrose (higher in transgenic plants), inuline, glucose, fructose and

amino acids (higher in wt) that accumulated under stress conditions (Laura et al., 2010). Determination of proline content and proline/amino acids ratio confirmed previous reported results on a higher concentration in *Osmyb4*-transgenic plants and highlighted that differences between accumulation in the two genotypes was boosted during the cold stress treatment (Laura et al., 2010).

In transgenic apple, in agreement with the previously described overlap between cold and heat stress responses, *Osmyb4* was found to ameliorate the tolerance to both cold and heat stress (Mattana et al., 2005b).

In a more detailed analysis, the authors confirmed that the observed tolerance in transgenic apple plants may be driven by the higher content of sugars (glucose, sucrose and fructose) and proline present in plants grown under control conditions. In particular, the cold treatment amplified the differences of metabolites accumulation between wt and transgenics. Moreover, the *Osmyb4*-overexpressing plants showed an improved tolerance phenotype towards drought stress (Pasquali et al., 2008).

A common characteristic displayed by all of the Myb4-transgenic plants under cold treatment was the increase in the proline content, whereas wt plants showed an increase in free amino acids (Laura et al., 2010; Mattana et al., 2005a; Pasquali et al., 2008). This result underlined the importance of proline accumulation during the stress, in agreement with the several roles proposed for this amino acid during abiotic stress (Verbruggen & Hermans, 2008).

To identify the metabolites associated with differential heat tolerance in two perennial grass species, Du et al. (2010) performed a metabolite profile analysis of C4 warm-season bermudagrass (a tolerant species) and C3 cool-season Kentucky bluegrass (a susceptible species), grown under optimum temperature conditions or subjected to short-term and long-term heat stress. All of measured physiological parameters confirmed that bermudagrass exhibited better heat tolerance than Kentucky bluegrass. The metabolic profile analysis revealed differences in the accumulation of many metabolites, depending both on species and growth conditions. In particular, bermudagrass accumulated a higher content of most of the metabolites identified (including organic acids, amino acids, sugars and sugar alcohols) in comparison to Kentucky bluegrass, especially following long-term heat stress.

### 4.2 Drought stress

In addition to extreme temperatures, drought is one of the major constraints for plant productivity worldwide. Timing and severity of water deficit may vary a lot, ranging from long drought seasons (when the water supply by rain is lower than the demand) to short periods without rain at all.

Whether exposed to mild or severe drought conditions, plants exhibit a range of specific responses, aimed to reduce water loss and/or to optimise water uptake. Among the earliest responses, reduction in vegetative growth, stomatal closure and a decrease in the rate of photosynthesis are observed. Osmotic adjustment, that is the active accumulation of solutes in response to drought, resulting in reduced osmotic potential and contributing to maintain cell turgor, represents an important adaptation mechanism to water deficit in several plants.

Drought triggers the production of the phytohormone abscisic acid (ABA) and the occurrence of both ABA-dependent and ABA-independent pathways involved in plant drought response has been well described (Yamaguchi-Shinozaki & Shinozaki, 2006). Many

drought-inducible genes have been identified so far in many species and the complex gene networks are becoming to be elucidated. More recently, the global metabolic changes induced by water deficit have also been addressed by several authors.

### 4.2.1 Drought stress response in Arabidopsis

The global transcriptional and metabolic changes induced in Arabidopsis by drought and their dependence on ABA-mediated or not mediated pathways have been investigated in an integrated analysis, comparing wt and a T-DNA-tagged NCED3 knockout mutant (*nc3-2*, impaired in the dehydration-inducible ABA biosynthesis) under control and water deficit conditions (Urano et al., 2009). The metabolic analysis indicated that drought strongly affected the metabolic profile, influencing the level of 82 metabolites in the wt (61 increased and 21 decreased) and 78 metabolites in the mutant (46 increased and 32 decreased). The authors classified the changes in the profiles of metabolites into categories based both on the timing (early, middle and late phase) and the trend of variations throughout the time-course experiment (transient or stable).

Comparison of the wt and mutant metabolic profiles highlighted that, among a total of 64 dehydration-increased metabolites, 16 were regulated by ABA-dependent pathways, including some amino acids, ethanolamine, glucose and fructose, 35 were regulated by ABA-independent pathways, such as raffinose and galactinol, metabolites belonging to TCA cycle and GABA shunt, and 13 were regulated by both ABA-dependent and ABA-independent pathways, including proline, agmatine, methionine, lysine, saccharopine and phenylalanine.

The metabolic analysis performed by the authors revealed that most of the drought-induced amino acids showed global correlation with each other, whereas the sugar groups did not show any correlation with the amino acid groups, thus, indicating a response to drought stress through completely different metabolic networks.

The authors also reported the integrated analysis of drought-induced transcriptome and metabolome changes.

The relationship between temperature and drought responses has been widely documented (Yamaguchi-Shinozaki & Shinozaki, 2006); mutations affecting the tolerance to both stresses, as well as transgenic plants with increased tolerance to both stresses, have been described (Bouchabke-Coussa et al., 2008; Kasuga et al., 1999; Mattana et al., 2005a).

An emblematic example of the overlap between the temperature and drought stress responses is represented by the *eskimo1* (*esk1*) mutant phenotype. Although *esk1* was originally isolated as a freezing-tolerant mutant in the absence of cold acclimation, able to constitutively accumulate high amounts of proline (Xin & Browse, 1998), genes regulated by the ESK1 protein showed a larger overlap with genes regulated by osmotic, salt and ABA treatment than with genes regulated by cold acclimation or belonging to the CBF/DREB pathway (Xin et al., 2007).

Lugan et al. (2009), comparing global metabolic profiles of wt and *esk1* plants grown under control and three different stress conditions (cold, salinity or dehydration), found that the mutant constitutively mimicked the phenotypic traits of wt abiotic stress response, in terms of development, osmotic status and metabolic profile. Despite some discrepancies, the changes at the metabolomic level were consistent with changes observed in the transcriptome, previously described by Xin and co-workers (2007).

A more detailed comparison between mutant and stressed wt metabolomes indicated that *esk1* was closer to drought-stressed than cold-acclimated wt plants. Indeed, the mutant

accumulated melibiose, raffinose, galactinol, proline, galactose, fructose and GABA (all associated with the three considered stress conditions), but not other metabolites, such as glutamine, trehalose and sucrose, involved in the wt cold response. Therefore, the authors suggested that the freezing tolerance exhibited by the *esk1* mutant was a side effect of a constitutive acclimation to dehydration. Based on transcriptome analysis, a major role of ESK1 in the plant response to water shortage and in the whole-plant water economy was also suggested by Bouchabke-Coussa et al. (2008).

The improved tolerance to multiple stresses as a consequence of the altered expression, in mutant or transgenic lines, of a gene normally activated in response to a specific stress, depends on the overlap at the molecular level of the response to different stresses. The rationale of this overlap is evident considering the two aspects of abiotic stress effects on plant cells: i) some primary cellular damages may be shared by several stresses, as for example the water deficiency caused not only by drought, but also by salinity, freezing and hypoxia (damaged roots being unable to transport water to the aerial parts) and ii) different primary effects may induce a common secondary stress (and, therefore, common secondary damages), such as oxidative stress, derived from the ROS production following the impaired photosynthetic ability in most of the suboptimal growth conditions (for a review on the specific and unspecific responses to cold and drought stress, see Beck et al., 2007).

In Arabidopsis plants subjected to cold or drought treatment, Mattana et al. (2005a) observed similar metabolic changes, such as an increase of proline, sugars and amino acids, although with a different time-course accumulation between the two stress conditions. In the same paper, the authors also reported the increased tolerance to both stresses of plants ectopically expressing the rice gene *Osmyb4*. Under stress conditions, the *Osmyb4* action seemed to amplify the changes in metabolites observed in wt, maintaining the difference in the timing of accumulation between the two stresses.

We have already cited the studies performed by Maruyama and co-workers (2009) on wt and Arabidopsis transgenic plants overexpressing DREB1A/CBF3 or DREB2A, the former conferring tolerance both to freezing and dehydration and the latter only to dehydration in transgenic plants. Indeed, in agreement with these tolerance phenotypes, on the basis of the metabolic profile data (in particular, the accumulation of arginosuccinate, fumarate, malate and several unknown metabolites), DREB2A transgenic plants clustered with drought-treated, but not with cold-treated wt plants.

We have also discussed that DREB1A/CBF3 transgenic plants and cold acclimated wt plants shared the accumulation of 17 metabolites (see section 4.1.1.), which were not affected in DREB2A transgenic plants and, therefore, were presumed to be involved in freezing tolerance. In contrast, as both transgenic lines showed strong drought tolerance, it was proposed that this phenotype did not depend on metabolites shared by DREB2A transgenic plants and drought-treated plants, but on those metabolites whose accumulation was increased in both transgenic lines (Maruyama et al., 2009). The comparison of global transcriptomes performed by the authors led to analogous conclusions.

Because of the possible simultaneous occurrence in nature of drought and high temperature conditions, Rizhsky et al. (2004) performed an analysis of the molecular and metabolic response of Arabidopsis plants to these stresses, considered either individually or in combination. The metabolic profile of plants subjected to both stresses was more similar to that of drought-treated plants than to that of heat-treated plants, with accumulation of high levels of sugars, such as sucrose, maltose and gulose. However, double stress-treated plants also accumulated sugars that are specific of heat-treated plants (i.e., fucose and melibiose)

and did not accumulate other metabolites typical of the drought response, the most remarkable example being proline. The authors suggested that proline might be toxic during a combination of drought and high temperature stress. A parallel transcriptome profiling of the same samples highlighted a similar preferential overlapping of the double-stressed plants with the drought-stressed ones.

### 4.2.2 Drought stress response in crop species

Many wild species are more tolerant to unfavourable environmental conditions than their relative cultivated crops, suggesting that crossing between wild species and elite cultivars could lead to an improvement of stress adaptation in modern crops.

On the basis of this observation, Semel et al. (2007) compared the metabolic profile of tomato fruit pericarp from irrigated and non-irrigated field grown plants, belonging either to the cultivated tomato, *Solanum lycopersicum* (cv. M82), or to its interspecific hybrid with *Solanum pennellii*. A total of 72 identified metabolites were detected, and the variance due to the genotype and the environment was evaluated.

Under irrigated field conditions, the metabolite composition of the elite cultivar and the F1 hybrid strongly differed, with a significantly higher content of several amino acids in M82 and a higher level of the majority of fatty and organic acids in the F1 hybrid. The two genotypes were also quite distinct with regard to the contents of sugars, sugar phosphates, sugar alcohols and other metabolites, with most of them (i.e., fructose, glucose, maltose, sucrose, trehalose and myo-inositol) being present at higher levels in the F1 hybrid and only a few of them (putrescine and fructose-6-phosphate) more abundant in M82 (Semel et al., 2007).

In the cultivated tomato, M82, the stress strongly affected the content of many metabolites, with large increases in several amino acids (including proline, β-alanine, GABA, glutamate and glycine), fatty and organic acids (including TCA cycle intermediates), as well as sugars and sugar derivatives. Whereas a change in the content and/or a role in response to water stress had largely been documented for some of these solutes (e.g., proline and some sugars), the increase of other compounds was considered more intriguing, such as for branched amino acids, TCA cycle intermediates or gentiobiose. It is noteworthy that a signalling role during tomato fruit development had been proposed for this latter molecule.

On the contrary, the F1 hybrid metabolic profile was not significantly affected by the experimental growth conditions, possibly because of the "constitutive" elevated concentration of many of the metabolites known to be involved in drought stress response.

The statistical analysis performed on the entire dataset supported the analytical results. Indeed, PCA clearly discriminated M82 from the F1 hybrid as well as irrigated from non-irrigated M82, but was unable to discriminate irrigated from non-irrigated F1 samples. Similarly, Hierarchical Clustering Analysis (HCA) revealed a strong influence of the genotype and a lower influence of the environment on the metabolic profiles (Semel et al., 2007).

Vannini et al. (2007) analysed the drought-tolerant phenotype and targeted metabolite accumulation in wt and transgenic tomato plants overexpressing the rice *Osmyb4* gene either under a constitutive (pCaMV35S) or a stress-inducible (pCOR15a) promoter. They found that the ameliorated tolerance of the transgenic lines was associated with a higher accumulation of sugars (sucrose, fructose and glucose) and proline (measured as percentage of total amino acid content).

Following drought treatment, the content of these molecules increased in both wt and transgenic plants, with the levels observed in transgenics always higher than those observed

in wt. The Myb4-constitutively expressing plants accumulated a higher content of free sugars even under control conditions. As expected, because of the use of a stress-inducible promoter, no significant difference in the concentration of any analysed metabolite was observed between wt and pCOR15a-Myb4 plants under control conditions. However, under water deficit conditions, the concentration of these compounds was found to be significantly higher in pCOR15a-Myb4 transgenic lines than in wt and to be comparable to that found in pCaMV35S-Myb4 transgenic plants (Vannini et al., 2007).

Dai et al. (2010b) have performed a systematic characterisation of the metabolic changes induced by water depletion in the roots of the medicinal plant, *Salvia miltiorrhiza* Bunge, comparing the results obtained using two metabolite analysis techniques ($^1$H-NMR and LC-DAD-MS) and four extraction methods based on different solvents (see also section 2.2). As phytomedicines are usually either air-dried or sun-dried for the purposes of transportation, storage or pharmacological requirements, the effect of these two different drying processes on the metabolite composition was also investigated and compared with a freeze-drying process, taken as the control.

$^1$H-NMR analysis revealed the presence of both primary and secondary metabolites, whereas LC-DAD-MS detected 44 secondary metabolites, among which 5 polyphenolic acids, genipin, umbelliferone and tormentic acid had not been previously described in this plant.

Both approaches revealed distinct metabolite profiles of the extracts obtained from the different drying treatments and the PCA score plots generated from both data series could discriminate the samples depending on the drying process.

However, the two approaches detected different metabolic changes following the two drying processes. Among the primary metabolites detected by $^1$H-NMR, an increase in proline, alanine and succinate accompanied by a decrease in *n*-butanol and lactate was observed in both the air-dried and sun-dried samples; in contrast, an increase in the content of sucrose and glutamine was observed only in air-dried roots, whereas an increase in leucine, melibiose and raffinose was found only in sun-dried roots. Different effects of air- and sun-drying processes were also highlighted on secondary metabolism by the LC-DAD-MS method, with air-drying enhancing the biosynthesis of oligomeric caffeic acids and tanshinones and sun-drying promoting the biosynthesis of tanshinones but inhibiting that of polyphenolic acids. The differences in metabolite content variations between the two sample groups was suggested to be attributable to the different drying speed of the two methods and to the concomitant occurrence of light and thermal stresses in sun-, but not in air-dried roots (Dai et al., 2010b). This study has the merit of showing the effectiveness of the combination of two different analytical approaches ($^1$H-NMR and LC-DAD-MS) and of highlighting the importance to carefully consider and optimise the extraction method when metabolomic analyses are performed.

To assess the association of osmotic adjustment (OA) with drought tolerance, seed yield and specific metabolites accumulation, a recent study was conducted on three different castor (*Ricinus communis* L.) hybrids and their respective parents, grown under irrigated and non-irrigated field conditions (Babita et al., 2010). The authors reported that genotypes with a greater OA also had higher leaf Relative Water Content (RWC) and maintained higher leaf water potential under water deficit; moreover, a positive relationship existed between OA and total seed yield under drought stress conditions. The high-OA genotypes accumulated significantly higher amounts of proline, total soluble sugars, total free amino acids and potassium, with sugars representing the major contributors to OA (Babita et al., 2010).

Three cotton near-isogenic lines (NILs), obtained via marker-assisted selection from the elite cultivars of the two species *Gossypium barbadense* (GB) cv. F-177 and *Gossypium hirsutum* (GH) cv. Siv'on, have been characterised for their metabolic and mineral compositions and compared to their parental genotypes (Levi et al., 2011). Plants were field-grown under well-watered and water-limited conditions and comparative analysis was performed between i) GB and GH genotypes ii) the two water regimes and iii) each NIL and its recipient parent.
The HCA, based on either 27 metabolites or 5 minerals, clearly distinguished between GB and GH genotypes. Within each species, in most but not all of the cases, the irrigation treatments had a more pronounced effect on the clustering than the genotypes. Comparisons between plants grown under well-watered and water-limited conditions for each genotype showed different trends in the various solutes. On the basis of the previously reported improved drought tolerance of the NILs versus their recipient parents, the authors focused their attention on those metabolites whose amount increased under stress in one or more of the NILs. In particular, an increase in aspartic acid, citric acid, malic acid, threonic acid, alanine, glycerol and myo-inositol among metabolites as well as in potassium, magnesium and calcium among minerals was suggested to contribute to the ameliorated adaptation to drought of these NILs (Levi et al., 2011).

### 4.2.3 Resurrection plants

Whereas the term "drought tolerance" refers to the ability of plants to survive a moderate dehydration (down to ~ 0.3 g $H_2O$ $g^{-1}$ dry weight), the capacity to tolerate further dehydration (down to an absolute water content of 0.1 g $H_2O$ $g^{-1}$ dry weight) is referred to as "desiccation tolerance" (Moore et al., 2009). This term also includes the ability of plants to rehydrate successfully and to regain normal metabolism and growth within several hours of rewatering.
Although desiccation is part of the normal developmental program of seeds in most higher plants, only a few species possess desiccation-tolerant vegetative tissues. These include the individual members of different angiosperm families and are termed "resurrection plants" (Moore et al., 2009).
Such species have been extensively studied in attempts to identify the mechanisms associated with their remarkable tolerance and with the aim of using the obtained knowledge to improve drought tolerance in economically important crop species.
Many different approaches have been employed in these studies, focusing on molecular, biochemical, metabolic, ultrastructural and physiological aspects of such a complex trait (Moore et al., 2009).
Among the plethora of data obtained, the identification of several upstream-acting genes, such as those encoding transcription factors and small regulatory RNA molecules, are of particular interest (Moore et al., 2009).
With regard to metabolites involved in desiccation tolerance, the importance of antioxidants, such as phenolic acids and polyphenols (galloylquinic acid) has been highlighted. Namely, a correlation between the galloylquinic acid content/composition and the maximum desiccation period that different populations of *Myrothamnus flabellifolius* can survive has been reported. These molecules have been suggested to act as a "reservoir", able to determine the length of the desiccation period that a plant can suffer before its viability is irreversibly compromised (Moore et al., 2005).
As in other species, in resurrection plants dehydration leads to an increase in the content of proline and of soluble carbohydrates (i.e., sucrose, trehalose, raffinose and glucose).

Moreover, the localisation of glucose and sucrose in plant tissues was reported in accordance with their possible function as cellular protectants during water stress (Martinelli, 2008).
Despite all of the results achieved in elucidating single aspects of the desiccation tolerance phenomenon, from gene regulation to metabolic adjustment or macromolecular stability, the secrets of resurrection plants still remain to be discovered. A holistic comprehension of how the identified individual factors interact spatially and temporally and the identification of (if it exists) the master switch is still lacking. Consistently, the ectopic expression of *Craterostigma plantagineum* transcription factors-encoding genes in Arabidopsis, tobacco and desiccation-sensitive callus tissue from *C. plantagineum* itself has led to inconsistent results: either improved drought tolerance or no effects on the phenotype, or even unexpected side effects, such as ABA insensitivity (Moore et al., 2009).

### 4.3 Salt stress

The increased salinisation of arable land, due to both natural processes and agricultural practises, is expected to have a dramatic negative impact on soil fertility in the next decades, resulting in a high percentage of land loss by the middle of the century. Most of the economically important crop species are very sensitive to high salt concentration in the soil.
High salinity engenders both hyper-osmotic stress (caused by the reduction of water availability due to the reduced water potential) and hyper-ionic stress (caused by the toxic effects of the accumulated ions). Plants are thus subjected to dehydration, ion toxicity, nutritional deficiencies and oxidative stress, with the main negative effects being the disruption of ionic equilibrium, the inhibition of cell division and expansion, and the reduction in photosynthesis and growth. Plant acclimation responses include ion exclusion and tissue tolerance, osmotic adjustment and several molecular and biochemical changes, with both conserved and divergent metabolic responses among different species (D.H. Sanchez et al., 2008).

#### 4.3.1 Salt stress response in Arabidopsis

Kim et al. (2007) have reported a detailed analysis of metabolic changes occurring during a time-course experiment (up to 72 hours) on salt-stressed Arabidopsis cell cultures. PCA and Batch Learning Self-Organising Mapping analysis (BL-SOM) revealed a coordinated induction of several pathways at different time points. Namely, short-term responses included the induction of the methylation cycle (for the supply of methyl groups), of the phenylpropanoid pathway (for lignin production) and of glycine betaine biosynthesis, whereas long-term response was characterised by the co-induction of glycolysis and sucrose metabolism and the co-reduction of the methylation cycle. In particular, metabolites that transiently increased in the short-term period included S-adenosyl-L-methionine (SAM), ethanolamine, cysteine and aromatic amino acids. Twenty-four hours after salt treatment, a decrease in SAM and the aromatic amino acid content and an increase in glycerol, inositol and S-adenosyl-L-homocysteine (SAH) were observed. As a consequence, the methylation index SAM/SAH increased as a short-term response to salt stress and constantly decreased after 12 hours of salt stress. Finally, long-term stressed cells abundantly accumulated sucrose and lactate (Kim et al., 2007).
The metabolic response to high salinity stress was also addressed in a time-course experiment by Kempa et al. (2008). These authors investigated the ABA involvement in the

complex re-adjustment of carbohydrate metabolism during salt stress, by exploring the temporal dynamics of the Arabidopsis metabolome in response to high soil salinity (up to 5 days) or to ABA treatment (up to 3 days). Comparison of the salt- and ABA-induced metabolic changes in an Independent Component Analysis (ICA) revealed both common and distinct metabolic responses, indicating the existence of ABA-dependent and ABA-independent pathways. Notably, both high salt and ABA treatments led to depletion of starch and increase in maltose levels, suggesting a role of this hormone in triggering stress-induced starch mobilisation.

The authors also addressed the question of whether a correlation exists between changes in specific metabolite levels and changes in the expression levels of genes encoding the corresponding metabolic enzymes and found such a correlation in several, but not all, of the pathways examined.

As plant hormones play a crucial role in responses to various environmental stresses, studies on the effects of hormone treatments on intracellular metabolites have also to be mentioned here. One of such studies was performed by Okamoto et al. (2009), who investigated by NMR the metabolic profiling of Arabidopsis T87 cultured cells following various hormone treatments (ABA, salicylic acid [SA], auxin and brassinosteroid). Moreover, as ABA and SA are known to mediate abiotic and biotic stress responses and to act antagonistically each other, the authors also monitored the dynamic metabolic changes in cells treated with ABA and SA simultaneously or successively for different time periods. Based on their data, the authors suggested that ABA and SA do not have simple antagonistic effects but that they cross-talk at the metabolite levels in a much more complex manner.

The single and combinatorial effect of salinity stress and elevated $CO_2$, two environmental conditions that are expected individually to affect plant growth in opposite directions, has also been investigated (Kanani et al., 2010). The authors found that, while the transcriptional responses to the salinity and to the combined stresses were very similar, this was not the case for the metabolic responses, thus, representing an example of "inconsistency" between these two levels of plant response. In particular, the combination of the two perturbations had a milder effect on the metabolic physiology than the salinity stress alone. This suggested a beneficial role of elevated $CO_2$ on salt-stressed plants at the metabolic level, at least within the experimental timeframe (30 hours), probably due to the provided additional resources in the presence of elevated $CO_2$ concentration.

### 4.3.2 Salt stress response: *Thellungiella* vs. Arabidopsis

*Thellungiella halophila* (also known as *T. salsuginea*), a *Brassicaceae* species closely related to Arabidopsis, displays "extremophile" characteristics represented by a remarkable tolerance to a variety of abiotic stresses, namely high salinity, water-deficit and freezing. Studies have taken advantage of the high nucleotide sequence identity between *Thellungiella* and Arabidopsis, utilising tools developed for the model species to investigate the transcriptome of the halophyte species.

Gong and co-workers (2005) investigated the salinity stress adaptation competence of *Thellungiella*. To identify pathways relevant for the stress adaptation phenotype of *Thellungiella*, they compared the transcript and metabolite profiles of the two species, grown under both optimal and salt-stressed conditions. In addition to stress responses shared by the two species, three *Thellungiella*-specific response categories were defined: i) additional

pathways that are stress-activated in *Thellungiella* but not activated in Arabidopsis, ii) genes with a significantly higher pre-stress intensity in *Thellungiella* and iii) novel stress-relevant genes whose homologs are not stress-responsive in Arabidopsis.

At the metabolic level, changes in Arabidopsis plants subjected to 150 mM NaCl for 24 hours were mainly represented by an increase, with respect to control plants, of proline, sucrose and an unknown compound (putative complex sugar). Drastic differences distinguished the two species, the most relevant being a higher amount in *Thellungiella* of sugars and sugar alcohols, both under control and salinity growth conditions. Under salt stress, *Thellungiella* also accumulated higher levels of proline, glutamic acid, malic acid, succinic acid, whereas in both control and stress conditions, Arabidopsis showed a higher accumulation of fumaric acid and mannitol.

Metabolome data, together with transcriptome results, have pointed towards the presence of a stress anticipatory strategy in *Thellungiella* as responsible for its "extremophile" characteristics.

More recently, Arabidopsis and *Thellungiella* responses to salinity and osmotic stress have been compared with an analogous approach by Lugan et al. (2010).

The authors found that, apart from a few differences in raffinose and secondary metabolites, salt stress affected the same metabolic pathways in the two species, the main differences being quantitative. *Thellungiella* had a higher concentration of many stress-related metabolites than Arabidopsis, independent of the growth conditions. It also contained less water and showed a higher ability to lose water following stress, without any detrimental effect, which could contribute to maintaining a water potential gradient between the soil and plants in water-limiting conditions.

PCA analysis sharply separated the samples, both depending on the species and on the environmental conditions, the genetic background being the main contributor to the metabolome variations. The species-dependent differences appeared to relate partially to the stress anticipatory strategy that has been hypothesised for *Thellungiella*; indeed, 42 of the 58 metabolites that were more abundant, and 19 of the 34 metabolites that were less abundant, in *Thellungiella* under the control growth conditions, were found to increase and decrease, respectively, in Arabidopsis under stress treatment. Therefore, the Arabidopsis metabolic response to salt seemed to, at least partially, mimic the constitutive status of *Thellungiella*.

A very original contribution to the metabolomic analysis approach that was provided by this study is represented by the idea of considering the metabolome of each species as a single "virtual molecule", the physicochemical properties of which are the weighted averages of the properties of the individual metabolites.

Therefore, based on this idea, the significant differences between the two species can be summarised as follows: i) under both standard and stressed conditions, the *Thellungiella* metabolome was more soluble, polar, massive and reduced than the Arabidopsis metabolome; ii) osmotic and salinity stresses changed the metabolome biophysical properties in a different way, depending on the stress and on the species; iii) both stresses induced more dramatic changes in Arabidopsis than in *Thellungiella*; iv) in Arabidopsis, salt affected the metabolome biophysical properties more than osmotic treatment and v) in *Thellungiella*, water stress induced more dramatic changes than salt stress (Lugan et al., 2010).

### 4.3.3 Salt stress response in crop species

One of the first applications of metabolomic analysis to the plant response to salt stress was reported by Johnson et al. (2003) on tomato fruits. Extracts from two varieties

differing in their salt tolerance were analysed using FT-IR spectroscopy coupled with chemometric techniques.

Whereas the unsupervised method, PCA, was not able to discriminate between the control and salt-treated fruits for either variety, the supervised method, Discriminant Function Analysis (DFA), classified the untreated and salt-treated samples of both varieties. The application of Genetic Algorithms (GAs) enabled the identification of key regions within the FT-IR spectra important for this discrimination, corresponding to nitrile-containing compounds and amino radicals.

Analyses of both gene expression and metabolite profiles were performed to elucidate the mechanisms responsible for the ability of a salt-tolerant tree species, *Populus euphratica*, grown in one of its natural habitats, a saline semi-arid area (the Ein Advat valley, located in the Negev desert in Israel) to acclimate to high salinity (Brosché et al., 2005). Leaf samples were collected from trees grown in four experimental sites in the valley, represented by three distinct areas that are characterised by a different degree of soil salinity, in addition to a non-saline well-irrigated area used as a control.

The accumulation of 22 selected metabolites in the leaves was examined by GC-MS. Trees growing in the most saline area, which accumulated more Na+, displayed a significantly higher concentration of the amino acids, β-alanine, valine and proline, whereas changes in stress-responsive carbohydrates and organic acids were of relatively limited extent, when compared to what is observed in Arabidopsis, and were statistically significant only for glycerol, glyceric acid and myo-inositol.

An interesting comparison between water deficit and salt stress has been described by Cramer et al. (2007). These authors monitored the early and late changes in the transcript and metabolite profiles induced in the vegetative tissues of grapevines (*Vitis vinifera*, cv. Cabernet Sauvignon) by long-term (16 days) water deficit and salinity stresses. Both stresses were gradually applied to the plants to better mimic field conditions. Moreover, the uniqueness of the experimental design was represented by the imposition of equivalent water potentials over time in the two stress treatments, thus, allowing the discrimination of the osmotic effects from the ion toxicity effects.

As expected, the relative abundance of several metabolites was altered by both stress conditions; however, at equivalent water potentials, water deficit had a more severe effect than salinity. Namely, among the key compounds involved in energy metabolism and osmotic adjustment, malate, proline and glucose were significantly higher in drought-treated than in salt-treated plants; moreover, only drought caused an increase in citrate and tartrate. With regard to inorganic molecules, a higher accumulation of sulphate, chloride and phosphate was observed under salinity than under drought stress. These differences in metabolite accumulation between the two growth conditions were correlated to differences observed in the transcript levels of genes involved in energy metabolism and nitrogen assimilation. Altogether, the data reported by Cramer et al. (2007) suggested a greater need for osmotic adjustment, ROS detoxification and photoinhibition amelioration in drought-treated than in salt-treated plants.

Another example of a multiple stress comparison is represented by the afore-mentioned results by Morsy and co-workers (2007; see section 4.1.3). These authors characterised two rice genotypes that contrasted in chilling tolerance for their response to water-deficit and high salinity stresses and found that, unexpectedly, the chilling-tolerant (CT) genotype was more sensitive than the chilling-sensitive (CS) one to both of the stresses. The high

accumulation of specific osmoprotectants, such as trehalose (under drought conditions) and mannitol (under salt conditions), observed in CS relative to CT, might account for its higher tolerance under these stresses.

Two rice cultivars (Arborio and Nipponbare) have been characterised by $^{1}$H-NMR analysis for their metabolic profiles under either osmotic or salt-stress conditions in *in vitro* experiments by Fumagalli et al. (2009). Nipponbare was found to be more tolerant to both stresses than Arborio, as shown by the percentage of inhibition on shoot and root growth. For both genotypes, PCA score plots clustered the samples into three distinct groups, depending on the growth conditions: untreated, osmotic treated (PEG 20%) and salt treated (NaCl 150 mM) seedlings. In comparison to control growth conditions, shoots of both cultivars accumulated a higher amount of glucose, glutamine and glutamate under both stress conditions; under salt stress, an increase in the content of sucrose, threonine, valine and lactate was also induced.

Although the two rice cultivars showed the same trend in metabolic changes during stress, they significantly differed in the relative amount of some metabolites, namely in the sucrose/glucose ratio and in the glutamate/total amino acids and glutamine/total amino acids ratios. These results suggested that both sugar and glutamine-glutamate metabolism were differentially regulated in the two cultivars in response to abiotic stresses.

More recently, Widodo et al. (2009) conducted an analysis of the metabolic responses to salinity stress in barley, a species of particular interest for metabolomic studies among cereals, as it is characterised by a higher $Na^+$ tissue tolerance (i.e., the capacity of accumulation of high concentrations of $Na^+$ in leaves) in comparison to rice and wheat.

Two barley cultivars differing in their salt tolerance, Sahara (more tolerant) and Clipper (more susceptible), were compared for their metabolic profiles under normal or saline conditions in a time-course experiment (24 hours, 3 and 5 weeks). The PCA of the leaf metabolites separated the samples belonging to the two cultivars grown in any conditions, the distance increasing with the time of the experiment for both control and treated samples. In both cultivars, a clear separation between short-term (24-h) and long-term responses (3 and 5 weeks) was also evident. Indeed, after 24 hours of salt treatment, only a few changes in metabolite concentrations were detected, whereas after long-term exposure (3 and 5 weeks) a greater number of metabolic changes and a larger magnitude of these changes were observed in both cultivars.

The authors suggested that, with the exception of proline, the observed accumulation of several amino acids in Clipper leaves after long-term salt exposure might correlate, as reported for other species, with slower growth and/or leaf necrosis, thus, being an indicator of general stress and cell damage rather than part of an adaptive response to salinity.

On the contrary, the specific accumulation in Sahara leaves of organic acids (including TCA cycle intermediates), sugars, polyols and other compounds, already known to be involved in cellular protection, may actually have a functional role in establishing the salt-tolerant phenotype of the cultivar (Widodo et al., 2009).

An interesting aspect of stress response is represented by the observation that mycorrhizal plants exposed to osmotic constraints generally perform better than nonmycorrhizal plants. Most of the knowledge on the improved stress protection comes from plants interacting with arbuscular mycorrhizas (AMs), whereas relatively little information is available on molecular and physiological mechanisms underlying the enhancement of stress tolerance in host plants by ectomycorrhizas (EMs).

Luo et al. (2009) have investigated the transcriptional and metabolic profiles in EM and non-EM roots of gray poplar (*Populus x canescens*) under control or excess-salinity conditions. The mycelia of the fungus *Paxillus involutus* were used for mycorrhizal inoculation. Unstressed EM roots accumulated osmolytes, such as soluble carbohydrates, sugar alcohols and free amino acids, at a higher extent than non-EM roots. Moreover, sugars of both major and minor pathways were more abundant in EM than non-EM roots also under stress conditions. Conversely, there were no significant differences in the amino acid content between stressed non-EM roots and both unstressed and stressed EM-roots. In agreement with the metabolic data, a microarray analysis indicated a constitutive activation of stress-related genes in control EM-roots, that are activated by salt stress in non-EM roots. Altogether, the data of Luo et al. (2009) indicated a stronger induction of defence pathways and metabolites in EM roots than in non-EM roots exposed to excess salinity, suggesting that the fungus *P. involutus* was able to prime the poplar plants for increased stress tolerance.

### 4.4 Oxidative stress response

A common consequence of most abiotic stresses is an increased production of reactive oxygen species (ROS), which are highly toxic and cause damage to proteins, lipids, carbohydrates, chlorophyll and DNA, thus, resulting in oxidative stress (Gill & Tuteja, 2010). ROS are mainly by-products of processes, such as photosynthetic or respiratory electron transport. Under normal growth conditions, there is an equilibrium between the production and the scavenging of ROS, but abiotic stress factors may disturb this equilibrium, leading to a sudden increase in intracellular levels of ROS. Most of the studies on this topic have been performed on ROS-scavenging enzymatic antioxidants, which represent the initial defence mechanism, whereas fewer studies have been reported about the direct consequences of oxidative stress on the plant metabolome.

Baxter et al. (2007), using the redox-active quinone menadione (MD), induced oxidative stress in Arabidopsis cell suspension cultures and characterised the dynamics of metabolic responses by following changes in metabolite abundance and in $^{13}C$-labeling kinetics. A total of 23 metabolites out of the 50 analysed were significantly affected (16 decreasing and 7 increasing). The integrated evaluation of such metabolic changes (an increase in hexose and triose phosphates, gluconate, ribose, a decrease in malate and some amino acids) indicated a dramatic inhibition of the TCA cycle and a diversion of carbon into the oxidative pentose phosphate pathway (OPPP). The decrease of ascorbate (one of the principal cell antioxidant molecules), concomitant with the accumulation of threonate (an ascorbate breakdown product), indicated a prolonged severe oxidative stress with a failure to recycle the oxidised ascorbate entirely (Baxter et al., 2007).

Analogous studies performed on Arabidopsis roots from hydroponically-grown plants highlighted similar metabolic changes in short-term menadione responses (30 minutes), whereas after longer oxidative stress (2 and 6 hours), changes observed in Arabidopsis roots and cultured cells clearly differed (Lehmann et al., 2009). In menadione-treated roots, among 56 identified polar metabolites, 33 were significantly affected within the first 30 minutes, and 39 were altered in at least two time points. The early changes, analogous to the observations in cell cultures, consisted of a decrease in the TCA cycle metabolites and associated amino acids and an increase in the OPPP intermediates Ribose 5-P and Ribulose 5-P and some glycolytic intermediates. As the time course proceeded, the amount of many

metabolites (i.e., TCA cycle intermediates and some amino acids) returned to normal values and further increased in the roots, in contrast to the response of cultured cells, in which most metabolites remained depressed throughout the time course. A major difference in the response of cells in culture and roots was in glycolysis: whereas in cultured cells a sustained increase in hexose 6-phosphates and a transient increase in 3-PGA were observed, in roots a significant decrease in hexose 6-phosphates and a linear increase in pyruvate were found. Moreover, the following variations in metabolites that can prevent oxidative damage were reported in menadione-treated roots: an increased abundance of proline (with a concomitant decrease in its precursor, glutamate), changes in polyamine metabolism with a decrease in putrescine, and accumulation of some methionine-derived aliphatic glucosinolates (Baxter et al., 2007; Lehmann et al., 2009).

An enhanced tolerance to menadione-induced oxidative stress was displayed by rice cultured cells overexpressing the Arabidopsis *Bax Inhibitor-1* (*AtBI-1*) gene (Ishikawa et al., 2010). Bax Inhibitor-1 is an endoplasmic reticulum membrane protein, acting as a cell death suppression factor, that is widely conserved in animals and higher plants.

Using Capillary Electrophoresis–Mass Spectrometry (CE-MS), the authors investigated the metabolic responses to cell death-inducible oxidative stress. The control rice cells showed a shift in carbon flow from the central pathway to the OPPP, probably due to an increased requirement for NADPH as reducing power, in agreement with data obtained in roots and cultured cells of Arabidopsis (Baxter et al., 2007; Lehmann et al., 2009). However, despite the depression of carbon metabolism in the central pathway, a marked accumulation of most amino acids derived from PEP, pyruvate and oxaloacetate was found in MD-treated rice cells. This observation was inconsistent with results obtained in MD-treated Arabidopsis, in which decreased levels of several amino acids correlated with decreases in their precursors (Baxter et al., 2007; Lehmann et al., 2009). *AtBI-1* overexpression did not produce any significant effect on primary metabolism in non-stressed cultured cells. However, clear differences between *AtBI*-1 overexpressing and control cells were found following a 24 h exposure to stress (but not at earlier time points), mainly in some metabolic pathways, i.e., glycolysis, amino acids of the glutamate and aspartate families, and components of redox and energy metabolism. These results suggested that tolerance to oxidative stress conferred by the AtBI-1 factor was due to a higher capacity of metabolic acclimation, with a recovery of metabolite composition that was depleted during the early response.

Oxidative stress and programmed cell death may be induced both in natural and cultivated plants, including forest trees, by ozone ($O_3$) exposure. In the past few years the increase in tropospheric ozone concentration has become one of the most serious environmental stress factors, that negatively affect plant growth, development and productivity. Ozone is a photochemically generated air pollutant, that can enter the intercellular space of leaves through the stomata, react with water and spontaneously generate ROS. Depending on the severity of the stress ($O_3$ concentration and length of exposure) and on the susceptibility of the plants (varying with age and genotypes), damage symptoms may range from visible chlorosis and necrosis in the leaves to inhibition of photosynthesis and reduced yield. The ozone effects have been studied with two main approaches, by exposing plants either to a high-dose of $O_3$ for a short period (acute ozone exposure) or to a weaker dose for a longer period (chronic ozone exposure), which represents a more realistic stress condition. The plant responses to this atmospheric pollutant have been recently investigated through "omics" tools in different

species, such as Arabidopsis, rice and birch (Cho et al., 2008; D'Haese et al., 2006; Kontunen-Soppela et al., 2007; Li et al., 2006; Ludwikow & Sadowski, 2008).

Cho et al. (2008) performed a systematic analysis of rice seedling molecular responses, using parallel transcriptomics, proteomics and metabolomics approaches, thus, providing a global view of signalling and metabolic pathways involved in rice response to $O_3$ exposure. CE-MS based metabolomic profiling revealed an increase in the content of several amino acids, GABA, glutathione and sakuranetin, a main rice secondary metabolite. The integration of the outputs from all these different approaches allowed the authors to indicate glutamate, GABA and glutamate dehydrogenase as possible biomarkers for $O_3$ damages in rice.

A long-term ozone exposure experiment was conducted in realistic open field conditions by Kontunen-Soppela et al. (2007), to compare $O_3$-induced leaf metabolome changes in two genotypes of white birch (*Betula pendula* Roth) differing in their ozone sensitivity. Among 339 low molecular weight metabolites, ozone enrichment led to increased concentrations of phenolic compounds (such as chlorogenic acid and quercetin glycosides) and lipophilic compounds related to leaf cuticular wax formation. On the contrary, decreases in concentrations of many carbohydrates and chlorophyll-related compounds were induced by elevated ozone.

### 4.5 Plant stress response and circadian clock

A very interesting aspect that has been more recently addressed is the interaction between the endogenous circadian clock and the transcriptional and metabolic reprogramming that occurs during the plant stress responses.

A role for the circadian clock in cold stress responses has been demonstrated (Nakamichi et al., 2009) and a large overlap between cold- and circadian-regulated genes has been observed (Bieniawska et al., 2008). These authors reported that diurnal- and circadian-regulated genes were responsible for the majority of the substantial variation observed between different experiments carried out to characterise the cold-responsive transcriptome in Arabidopsis. That is, genes identified as cold-responsive were dependent on the time of day the experiment was performed and a control at normal temperature did not correct for this effect, contrary to what is currently assumed.

Espinoza et al., (2010) have investigated the role of diurnal and/or circadian regulation in metabolic cold-induced responses, performing an integrated analysis of both transcripts and metabolites. Their findings also underlined the importance of understanding cold acclimation in the correct day-night context. Furthermore, they observed that a mutant with a disruption in the circadian clock was more sensitive to freezing and impaired in its cold acclimation capacity. This finding was in agreement with data reported in *Populus* on a reduced ability to cold acclimate of transgenic lines where the expression of some clock-component homologs genes had been down-regulated by RNA interference (Ibáñez et al., 2010). On the contrary, an Arabidopsis triple mutant for other clock-component genes has been reported to have an increased freezing tolerance, associated with a higher accumulation of the compatible solutes, proline and raffinose (Nakamichi et al., 2009). The reason for these contrasting phenotypes of different clock mutants remains to be elucidated.

It must be mentioned that in Arabidopsis, the time-of-day has also been shown to influence the transcriptome alterations following drought exposure (Wilkins et al., 2010).

The circadian clock seems to function as a central coordinator of plant metabolism, to maintain homeostasis by determining the levels of both primary and secondary metabolites

and also to allow plants to anticipate future environmental stresses, such as drought at midday and cold at midnight. In addition, a feedback mechanism is brought about by metabolic and stress cues on the central oscillator itself (A. Sanchez et al., 2011).
It is noteworthy that a functional link between the circadian clock and plant immunity has also been reported very recently (W. Wang et al., 2011), with a remarkable and intriguing example of a plant tuning its immune response against a pathogen. Arabidopsis defence genes involved in the response to an oomycete pathogen were found to be under the control of a central circadian regulator (the *cca1* gene), thus, allowing plants to anticipate infection at dawn (when the pathogen disperses the spores) through a maximal expression of the relevant genes at the time of day when attack is most likely (W. Wang et al., 2011).
Thus, the circadian clock and the response to both abiotic and biotic stresses appear to be firmly interconnected in plants. Furthermore, the integration of the circadian clock with the stress signalling pathways might have played a crucial role in the development of plant adaptation to their environments during evolution.

## 5. Integration of "omics" results

The availability of high-density microarray and next generation sequencing technologies has opened the route to carry out whole genome transcriptome (WGT) analyses in a high-throughput manner.
Likewise, high throughput LC-MS approaches enable the rapid identification of large sets of proteins and of their post-translational modifications (Huang & Xu, 2008).
In fact, plant acclimation to abiotic stress conditions is associated with profound changes in proteome composition. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to unravel the possible relationships between protein abundance and plant stress acclimation (Kosovà et al., 2011)
Post-translational modifications (PTMs) are also involved in the regulation of a wide range of cellular responses to abiotic stress stimuli and greatly affect protein structure, activity and stability. Several hundred PTMs have been described in the literature and the advent of high-throughput quantitative proteomics technologies has allowed the systematic identification of the PTMs (phosphorylation, S-nitrosylation, ubiquitylation, SUMOylation, glycosylation) and the determination of their functional relevance in the context of regulation and response to abiotic stress (Ytterberg & Jensen, 2010).
These global analyses were in numerous cases coupled to metabolomic approaches (see above), reinforcing the tight link between changes in specific transcriptional patterns of candidate gene and/or specific proteomic patterns to the production of metabolites (both primary and secondary).
The WGT technologies enable to precisely pinpoint the classes of genes under transcriptional control (down/up-regulation) and to define not only responses at the gene level but also at that of "network" (Yamaguchi-Shinozaki et al., 2006; Swindell et al., 2007).
In fact, as described for the metabolomic approaches, the characterisation of the whole transcriptome enables researchers to identify the whole cascades of target genes from the transcriptional factors to the effector genes (whose expressions is dependent upon that of specific transcriptional factors).
The transcriptome analyses allow to define co-regulatory pathways that often underlie the concerted up/down-regulation of large sets of genes involved in the same regulatory

and/or biosynthetic pathways (Krouk et al., 2010). This is of high relevance to define the genetic components of specific or common plant responses to abiotic stress conditions.
The advent of high-throughput sequencing technologies has markedly accelerated the generation of whole transcriptome data and the capability of capturing changes in expression also of rare transcripts. It is now possible to globally define transcription start sites, polyadenylation signals, alternative splice sites and generate quantitative data on gene transcript accumulation in single tissues or cell types (L. Wang et al., 2010). These deep-sequencing technologies (also called Next Generation Sequencing Technologies) are thus paving the way for global genome transcriptomics and will undoubtedly lead to novel insights into plant abiotic stress responses. However, several challenges exist to making this technology broadly accessible to the plant research community, including the current need for a computationally intensive analysis of large data sets.

## 6. Conclusion

Metabolome analysis has become an invaluable tool to study plant metabolic changes that occur in response to abiotic stresses. This approach has already enabled to identify a large number of metabolites whose accumulation is affected by exposure to stress conditions. However, despite the many progresses that have been achieved in this field, much work is still required to identify novel metabolites and pathways not yet linked to stress response and tolerance and to decipher the extensive coordination and interaction among the various metabolic pathways.
To better understand the role of stress-associated metabolites in abiotic stress response, it has to be taken into account that metabolites not only have functional roles in stress tolerance but also act as signalling molecules. In most of the studies, the production, increase or depletion of metabolites are mainly regarded as the final, downstream response of the plant cell to the external stimuli. However, the question should be addressed whether the changes in metabolic networks that are observed are driven by alterations in gene expression, or whether the transcriptome changes are responding to a specific metabolic perturbation. In addition to hormones or other canonical mediators, such as sucrose and glucose, many other small molecules may play a crucial role in signalling pathways; it seems likely that only a subset of the metabolites with a mediator function in the regulation of transcription in response to stresses has been identified so far.
To this purpose, it is essential to consider the temporal dynamics of the response, through an integration of the "omics" data obtained at different time points during stress exposure. But even this "snapshots"-based approach, consisting in the comparison among different samples taken at different time points, has been recently considered as a rather static approach and its usefulness for obtaining a comprehensive and global information on stress-induced molecular changes has been questioned. More dynamic approaches, such as fluxomics (Wiechert et al., 2007), aimed to follow the flux of metabolites through pathways, are being currently developed and might reveal to be much more informative.
To elucidate the function of a single compound, it is also important to be aware that compensatory mechanisms are commonplace and that a change in the content of a single metabolite may have no effects on the phenotype, because of compensative modulation of other components of the same family of compounds.
The original approach proposed by Lugan et al. (2010), considering the metabolome as a single "virtual molecule" whose physicochemical properties are the weighted averages of

the properties of individual metabolites, also appears to be quite promising both to investigate the global metabolic strategies of a species to maintain cell function under stress and to evaluate differences among species.

In addition, further efforts to make stress treatment conditions more relevant to plant growth outside of the lab are required. Because plants are often subjected to a combination of multiple adverse conditions rather than to individual stresses (a common example being represented by the simultaneous occurrence of heat and drought in the field), tolerance to multiple abiotic stresses is an important breeding target in crops. Studies performed comparing single or combined stresses have already demonstrated that metabolic responses may be quite different and these results have to be considered in identifying strategies to improve stress tolerance, either by breeding or by transgenics approaches.

Moreover, as relatively little is known about the molecular mechanisms that underlie the acclimation of plants at a long-term realistic exposure to specific stressors, the focus has to move from how plants survive "acute" (sudden and short-term) stress conditions to how plants respond to "chronic" (long-term), sub-optimal growing conditions.

Another aspect to be considered is that, besides classical stress factors, plants also have to cope with emerging stressors (such as tropospheric ozone and anthropogenic stressors), which were not previously met by species during evolutionary times.

The recent findings on a firm interconnection between the plant circadian clock and the response to both abiotic and biotic stresses also emphasize the importance of having a diurnal perspective when plant stress responses are characterized and of investigating stress response in the correct day-night context.

Another major challenge is the elucidation of epigenetic regulation mechanisms, including changes in nucleosome distribution, histone modification, DNA methylation, and non–protein-coding RNAs (npcRNAs), which also play important roles in abiotic stress gene networks (Urano et al., 2010).

The integration of the -omics approaches, that have markedly increased our understanding of global plant systems in response to stress conditions, is likely to enable researchers to reconstruct the whole cascade of cellular events leading to rapid responses and adaptation to the various abiotic stress stimuli.

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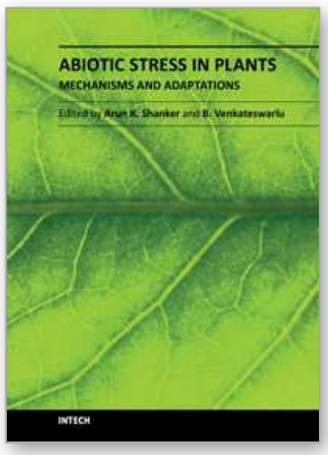


**Abiotic Stress in Plants - Mechanisms and Adaptations**

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World population is growing at an alarming rate and is anticipated to reach about six billion by the end of year 2050. On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for this are water shortages, depleting soil fertility and mainly various abiotic stresses. The fast pace at which developments and novel findings that are recently taking place in the cutting edge areas of molecular biology and basic genetics, have reinforced and augmented the efficiency of science outputs in dealing with plant abiotic stresses. In depth understanding of the stresses and their effects on plants is of paramount importance to evolve effective strategies to counter them. This book is broadly dived into sections on the stresses, their mechanisms and tolerance, genetics and adaptation, and focuses on the mechanic aspects in addition to touching some adaptation features. The chief objective of the book hence is to deliver state of the art information for comprehending the nature of abiotic stress in plants. We attempted here to present a judicious mixture of outlooks in order to interest workers in all areas of plant sciences.

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