Genomic Approaches to Unveil the Physiological Pathways Activated in *Arabidopsis* Treated with Plant-Derived Raw Extracts

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Keywords: biostimulants, microarrays, ABA

Abstract

DNA microarrays can be used to obtain a fingerprint of the transcriptional status of the plant or cell under a given condition and may be useful for characterising which genes respond, either by induction or repression, to novel stimuli or specific treatments. An in-depth bioinformatical analysis of all the data produced by microarrays can further highlight the metabolic or functional pathways most affected by the treatment. This approach has been used to investigate the effects induced by the treatment of different plant-derived raw materials, provided by Valagro SpA, on *Arabidopsis* seedlings. A clear example is represented by treatment with a raw plant-derived protein extract (VAL-P01). In this case the treatment induced genes related to ABA and osmotic stress treatment. We therefore demonstrated that VAL-P01 was able to mimic in planta the same pattern of responses linked to ABA treatment or osmotic stress, making the plant stronger against possible further stresses. Another plant extract, VAL-P02, was shown to be significantly altering the transcription of senescence genes, making it an ideal candidate adjuvant for the prolonged shelf-life of vegetal products.

INTRODUCTION

Natural products in agriculture can limit the use of environmentally harmful chemical inputs used for increasing crop yield and promote plant development, growth and nutrients uptake. Recently, a class of natural products for agricultural applications is attracting the interest of the market and the research community: the biostimulants. As defined in Khan et al. (2009), biostimulants correspond to "materials, other than fertilizers, that promote plant growth when they are applied in small quantities". A more recent definition better explains the characteristics of these products: "plant biostimulants are substances and materials, with the exception of nutrients and pesticide, which when applied to plants, seeds or growing substrate in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress response" (Du Jardin, 2012). According to the above reported definition, the application of biostimulants has a positive impact on plant nutrition and plant growth, while at the same time providing anti-stress effects (Richardson et al., 2004). Biostimulant formulas are proprietary but most of them contain similar components: plant hormone-like compounds, amino acids, humic acids, manure and/or sea kelp extracts (Maini, 2006; Vinković et al., 2007; Mora et al., 2010).

Several reports highlight the beneficial physiological effects induced by the crop treatment with biostimulants, but the molecular mechanisms behind these effects are still unknown (Vernieri et al., 2006). Gene expression determines the plant's phenotype, physiology and response to the environment. Therefore, analysis of gene expression can provide clues about regulatory mechanisms, biochemical pathways and broader cellular functions that are affected by biostimulants. Nowadays, DNA microarrays represent a high-throughput technology to rapidly and quantitatively measure the parallel expression

of thousands of genes (Aharoni and Vorst, 2002). Transcript profiling provided by microarray datasets can generate a picture of cellular functions under a given experimental condition (Schena et al., 1995; Tan et al., 2009).

Here, we present a microarray-based gene expression study aimed at elucidating the molecular mechanisms ignited by several crude plant extracts, acting as biostimulants. We measure the effect of these compounds on a well-studied model plant, *Arabidopsis thaliana* (Lamesch et al., 2012), by using the Affymetrix ATH1 GeneChip microarray, which is able to detect the expression of more than two-thirds (21,377 out of 27,416) of the *Arabidopsis thaliana* genes (Giorgi et al., 2013). A subsequent bioinformatical analysis is applied on the results to investigate which are the physiological pathways most affected by the biostimulants.

MATERIAL AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used in this study. Arabidopsis seedlings plates have been prepared as described in Banti et al. (2010). The samples have been harvested 4, 12 and 24 hours after the treatments with the biostimulants. For the treatments we used eight plant crude extract (VAL-P01 to VAL-P08) with different natural origin. Including a two-replicates control plants panel, this sums up to 30 samples, plus two plant controls harvested before the treatment. For drought stress experiments, Arabidopsis adult plants were grown in a climatic cell, with controlled conditions (T=23°C, 14-/10-h photoperiod at 150 mmol photons m^{-2} s^{-1}) and stress was induced by stopping watering for 10 days.

RNA Isolation, cRNA Synthesis, and Hybridization to Affymetrix GeneChips

Total RNA was extracted from the seedling samples, using the Ambion RNAqueous extraction kit (Ambion). RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix Arabidopsis ATH1 GeneChip arrays (Affymetrix, Santa Clara, CA), according to the manufacturer's protocol. In brief, 10 µg of total RNA was used in a reverse transcription reaction (Ambion MessageAmp kit) to generate first-strand cDNA. After second-strand synthesis, double-strand cDNA was used in an in vitro transcription reaction to generate biotinylated cRNA. After purification and fragmentation, biotinylated cRNA was used for hybridization. This generated 32 raw probe intensity numerical matrices, stored as cell intensity (CEL) files.

Microarray Data Analysis

Raw microarray signal intensities for each of the 32 measured samples were loaded as CEL files into the R environment (Ihaka and Gentleman, 1996) via the *affy* package (Gautier et al., 2004). The probe signals were background-corrected following the RMA procedure (Wu et al., 2004), then all samples were quantile-normalized and the probes were finally summarized into unlogged single gene values via the median polish procedure (Giorgi et al., 2010) using the standard probeset-to-gene map (CDF) provided by Affymetrix. All data passed all default quality tests assessed by the Robin pipeline for Affymetrix microarrays (Lohse et al., 2010).

Genes were flagged as "differentially expressed" in each treated vs. control contrast if (i) change is significant ($P \leq 0.05$) as defined by the PUMA pipeline with default parameters (Pearson et al., 2009) (ii) second, the absolute $\log_2$ fold change must be higher than 2 ($\log_2$FC, logarithm of the ratio between the treated signal divided by the control signal $\log_2 FC \geq 2$ ("induced") $\log_2 FC \leq 2$ ("repressed").

UPGMA-like hierarchical clustering of the samples was performed using the R package *pvclust* (Suzuki and Shimodaira, 2006), applying the average method after calculating the distance matrix between samples as 1-PCC (Pearson Correlation Coefficient). 100 bootstrap values were generated and reported in each node of the tree as

BP (bootstrap probability).

Ontological assignment of each gene to a particular molecular pathway or biological function was done using the Mapman annotation (Usadel et al., 2005). Over-representation analyses of particular functions/pathways within lists of genes were performed on the MEFISTO tool (http://www.usadellab.org/cms/index.php?page =mefisto). Visual representation of transcriptional induction/repression per pathway was obtained via the Mapman software (Usadel et al., 2005). Similarities between the expression profiles generated in this study and other GeneChip-based publicly available data were investigated; this was obtained via two independent cross-experiment comparative approaches: FARO http://www.cbs.dtu.dk/services/faro/ (Manijak and Nielsen, 2011) and AtCAST v2.0.6 http://atpbsmd.yokohama-cu.ac.jp/cgi/network/ home.cgi (Sasaki et al., 2011).

RESULTS

Arabidopsis seedlings were treated with eight different biostimulants (Table 1). A time-course experiment was performed to identify responses that are time-dependent. The microarray analysis demonstrates that Arabidopsis plants respond to each treatment by changes in gene expression at all three time points analysed. To find similarities among the different treatments we performed an unsupervised hierarchical clustering of the gene expression profiles from all the samples. This yielded a tree with distinct subclusters (Fig. 1). A first distinct branch groups together all samples treated with VAL-P02; a second branch groups together all VAL-P07 and two out of three VAL-P01 treatments. The rest of the samples are grouped together with the respective time controls, indicating that, for these samples, diurnal changes have a stronger effect than the treatment itself, with respect to general transcriptional state.

We decided, therefore, to deepen our analysis on the more peculiar classes of response, specifically focusing on VAL-P01 and VAL-P02, which clustered separately (Fig. 1) and which markedly change gene expression compared to other treatments with the exception of VAL-P07 (Fig. 2), which clustered with VAL-P01. We found that VAL-P01 induced 99 genes 4 h after the treatment, 110 genes after 12 h and 85 genes after 24 h. The repressed genes were 58 after 4 h, 59 after 12 h, and 41 after 24 h from the treatment. VAL-P02 induced genes were 343 genes after 4 h, 375 genes after 12 h and 415 genes after 24 h from the treatment. The VAL-P02 down regulated genes were 115 after 4 h, 493 after 12 h, and 235 after 24 h (Fig. 2). Amongst the biostimulants used, VAL-P02 has the stronger effects in terms of transcriptional changes in comparison to the control samples (Fig. 2).

In order to obtain a global picture of the effects of the treatments on biological processes, all transcriptional changes were analysed using the MapMan software (Fig. 3). This analysis highlighted the most interesting biological processes affected by VAL-P01 and VAL-P02.

Significantly induced Mapman classes of genes (i.e., over-represented biological pathways or functions) are indicated for VAL-P01 in Table 2 and for VAL-P02 in Table 3 at three time points after treatment. VAL-P01 exerted a clear effect on ABA signalling and influenced anthocyanin synthesis. *At2g47770* (TSPO-related) was the most induced one and it is mainly expressed in dry seeds, after osmotic and salt stress or by the application of abscisic acid (ABA) (Guillaumot et al., 2009). *At5g59220* (*SAG113*) belongs to the group of ABA signalling genes associated to water loss during leaf senescence (Zhang et al., 2012). ABA responsive elements-binding factors are highly induced by the treatment with VAL-P01, namely *At4g34000* (*ABF3*), *At3g19290* (*ABF4*), *At3g56850* (*AREB3*), *At3g19290* (*AREB2*), all considered as key transcriptional regulators of ABA pathway (Choi et al., 2005; Finkelstein et al., 2005). Other ABA signalling genes are also present in the VAL-P01-induced list: *At4g26080* (*ABI1*), *At5g57050* (*ABI2*), *At3g24650* (*ABI3*) and *At3g08550* (*ABI8*) (Merlot et al., 2001; Brady et al., 2003; Brocard-Gifford et al., 2004). Another stress gene, whose expression is enhanced by VAL-P01is *At5g15960* (*KIN1*), is a well-known responder to cold- and drought-stress

(Izawa et al., 1993).

VAL-P01 also induces several anthocyanin pathway genes, principally *At1g56650* (*PAP1*, *Production of Anthocyanin Pigment 1*), a key transcription factor for anthocyanins biosynthesis (Tohge et al., 2005) and *At5g42800* (*DFR*, *DihydroFlavonol 4-Reductase*) (Harborne and Williams, 2000; Winkel-Shirley, 2001). Other flavonoid synthesis pathways are induced by VAL-P01, for example we found two chalcone/stilbene synthases (*At4g00040* and *At1g02050*) and flavonol synthases *At5g63580* (*FLS2*) and *At5g63590* (*FLS3*). Furthermore, the nucleotide sugar metabolism, responsible for synthesizing, for example, pectin precursors, is deeply affected by the VAL-P01 biostimulant (specifically, four UDP-glycosyl transferases are significantly induced: *At1g01390*, *At5g49690*, *At2g18560* and *At5g65550*).

Concerning the genes responding to VAL-P02 we noticed the strong induction of genes belonging the *DIN* family (Dark INducible genes): *At3g60140* (*DIN2*), *At3g49620* (*DIN11*), *At4g35770* (*DIN1*), *At3g13450* (*DIN4*), *At3g47340* (*DIN6*), *At3g06850* (*DIN3*), *At1g67070* (*DIN9*), *At5g20250* (*DIN10*) and *At3g06850* (*DIN3*). *DIN* genes are typically induced under dark condition and sugar starvation (Trethewey and Rees, 1994), but also accumulate during natural leaf senescence (Fujiki et al., 2000; Fujiki et al., 2008).

We further proceeded investigating how the transcriptional responses to biostimulants VAL-P01 and VAL-P02 could mimic classical plant treatments available in Arabidopsis microarray database with the AtCAST pipeline (Sasaki et al., 2011). The AtCAST results were confirmed by the FARO server, whose purpose is also to find expression pattern similarities across microarray experiments (Manijak and Nielsen, 2011). The experiments that were found to be significantly similar to our treatments are listed in Table 4, connected in a network view displayed in Figure 4, showing genes are transcriptionally responding in a similar way. The pattern of gene expression for VAL-P02 correlates with that of both abiotic stress (particularly osmotic) and biotic stress (such as that induced by the bacterial extracts HrpZ or flagellin, or by living pathogens). The pattern of gene expression for VAL-P01 is strictly related with the microarray datasets from experiments related to ABA treatments, plus various abiotic stresses at least partly involving ABA signalling pathways.

In order to validate the hints given by the general pathway analysis for VAL-P01, and in particular the prediction that ABA-related stress tolerance mechanisms are highly activated by this biostimulant, we performed a tolerance drought stress. Arabidopsis adult plants were deprived of water for 10 days and, remarkably, plants that were previously treated with VAL-P01 could better tolerate the stress (Fig. 5). These results demonstrate that VAL-P01 is able to mimic the ABA induced-effects, not only at the transcriptional, but also at the physiological level.

DISCUSSION

In this work we demonstrated that microarrays constitute a powerful tool to characterize the effect of natural, raw substances predicting their possible use as biostimulants. The analyses of our microarray data and the further functional analyses, using the MapMan and AtCAST softwares, allowed us to link gene expression changes induced by the treatments with physiological processes. Our results not only validate the proposed method, but also provide new data on not-yet characterized raw materials that can be used to rapidly develop new biostimulants.

The results obtained in the drought-tolerance experiments are in agreement with the microarray data highlighting an effect of VAL-P01 on ABA and abiotic-stress related genes. Abscisic acid activates genes associated with different processes in plant such as storage proteins, dormancy, germination, the arrest of embryonic development, leaves senescence and the closure of stomata (Sreenivasulu et al., 2006, 2010). Different stress conditions such as low temperatures, drought, salinity, heat, wounding, desiccation, drought, cold and light result in increased levels of ABA, which is considered as a plant stress hormone (Hauser et al., 2011). Several studies have indeed demonstrated a pivotal role for ABA in the modulation, at the gene level, of adaptive responses for plants in

adverse environmental conditions (Cutler et al., 2010; Fujita et al., 2011). ABA is produced under drought and low temperature stresses and increased ABA content in leaves was observed during hardening, cold acclimation and salt stress in several crops such as winter wheat, potatoes, and alfalfa (Lalk and Dörffling, 1985; Luo et al., 1992). A number of genes have been described that respond to drought and low temperature stress at the transcription level (Thomashow, 1998), and it was demonstrated that their regulation is related to ABA as a mediator in triggering plant responses to adverse environmental stimuli (Zeevaart and Creelman, 1988). For cold stress acclimation the key role of ABA production is also well documented (Yamaguchi-Shinozaki and Shinozaki, 2005), and the role of ABA in drought tolerance has been extensively studied in Arabidopsis (Bray, 1997). Our results show that VAL-P01 activates genes related to ABA response and also induces tolerance to drought stress further support the link between ABA and drought stress. VAL-P01 can represent an ingredient to develop a new biostimulant to protect plants against a series of stresses that are linked to ABA as a signalling molecule. We observed that VAL-P02 induces a specific class of genes, which are involved in the senescence process in plant, namely the *DIN* genes. *DIN* transcripts also accumulate during natural leaf senescence (Fujiki et al., 2008), and their expression in darkness seems to be triggered, at least in part, by sugar starvation, which often prevails in plants within several hours after darkness (Trethewey and Rees, 1994). This observation sustains the idea that the VAL-P02 might affect the senescence process in treated plants. VAL-P02 could therefore be used to prolong the shelf-life of horticultural products, but further experimental evidence is required to support this proposal.

ACKNOWLEDGEMENTS

The present work was possible thanks to the support by Valagro SpA.

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Tables

Table 1. List and generic composition of biostimulant products experimented in the current study.

Code of raw material	Composition	
VAL-P01	10%	total amino acids
	6-10%	betaines
VAL-P02	2.8%	crude proteins
	14.5%	ashes
	47%	total carbohydrates
	72%	organic matter
VAL-P03	44%	total organic C
	75%	organic matter
	47.6%	humic acids
	12.1%	fulvic acids
VAL-P04	8%	crude proteins
	40-55%	ashes
	9%	total amino acids
	6%	sugar alcohols
	35-45%	organic matter
VAL-P05	6%	crude proteins
	40-55%	ashes
	3-4%	organic acids
	4-6%	total amino acids
	4%	sugar alcohols
	1%	betaines
VAL-P06	40%	total organic C
	60%	organic matter
	48%	wood derived polymer complex
VAL-P07	2-4%	crude proteins
	12%	ashes
	46-48%	total carbohydrates
	30-40%	disaccharides
	50-60%	organic matter
VAL-P08	25%	crude proteins
	8.5%	ashes
	11-12%	total amino acids
	4-5%	free amino acids
	13%	organic acids
	30-35%	organic matter

Table 2. List of the most induced functional groups affected by the treatment with VAL-P01, at the three different time points. The mainly affected pathways were ABA signalling and flavonoids/anthocyanins synthesis (MEFISTO test, Fisher's Exact Test with Bonferroni correction, P-value<0.05).

Induced gene group	Over-representation corrected P-value
VAL-P01 4 hours (99 genes)	
Hormone metabolism - abscisic acid	3.2319E-04
Secondary metabolism	1.5126E-03
Secondary metabolism - flavonoids	3.1329E-03
Hormone metabolism - abscisic acid.induced-regulated-responsive-activated	7.2410E-03
Secondary metabolism - flavonoids.anthocyanins	1.3072E-02
Development - late embryogenesis abundant	1.9013E-02
Stress	2.0278E-02
Miscellaneous - protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.1421E-02
Hormone metabolism	2.8193E-02
Not assigned - unknown genes	4.7777E-02
VAL-P01 12 hours (110 genes)	
Miscellaneous	1.4423E-07
Miscellaneous - protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.9000E-06
Hydroxyproline rich proteins	2.5290E-05
Miscellaneous - cytochrome P450	4.2145E-04
Not assigned - unknown genes	1.7033E-03
Hormone metabolism - abscisic acid.induced-regulated-responsive-activated	8.2087E-03
Hormone metabolism - abscisic acid	1.0089E-02
Stress	1.1941E-02
VAL-P01 24 hours (55 genes)	
Hormone metabolism - abscisic acid	6.5378E-04
Hormone metabolism - abscisic acid.induced-regulated-responsive-activated	1.0266E-03
Not assigned - unknown genes	1.3140E-02
Hormone metabolism	1.4001E-02
Lipid metabolism - lipid transfer proteins	4.9165E-02

Table 3. List of the most induced functional groups affected by the treatment with VAL-P02, at the three different time points. The mainly affected pathways were stress signalling (MEFISTO test, Fisher's Exact Test with Bonferroni correction, P-value<0.05).

Induced gene group	Over-representation corrected P-value
VAL-P03 4 hours (343 genes)	
Miscellaneous - glutathione S transferases	5.4767E-12
Stress	1.4080E-06
Stress - biotic	4.8357E-04
Redox - glutaredoxins	1.6900E-03
Miscellaneous - nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	2.1198E-03
Protein metabolism	9.0506E-03
Miscellaneous - cytochrome P450	9.2628E-03
Amino acid metabolism - degradation - branched-chain group	2.0613E-02
Amino acid metabolism - degradation	2.1886E-02
Development	4.0141E-02
Lipid metabolism - lipid degradation.lipases	4.3466E-02
VAL-P02 12 hours (375 genes)	
Amino acid metabolism - degradation	1.5639E-10
Amino acid metabolism - degradation - branched-chain group	1.1643E-06
Miscellaneous - cytochrome P450	1.2009E-03
Development	2.7210E-03
Redox - glutaredoxins	3.3228E-03
Amino acid metabolism - degradation - branched-chain group.leucine	4.2222E-03
Minor CHO metabolism.trehalose.potential TPS/TPP	3.5632E-02
Secondary metabolism - flavonoids - anthocyanins	1.1868E-02
VAL-P02 24 hours (415 genes)	
Hormone metabolism	2.5360E-08
Protein metabolism	5.4674E-08
Not assigned - unknown genes	2.2613E-06
Miscellaneous - glutathione S transferases	6.9239E-06
RNA - regulation of transcription- WRKY domain transcription factor family	3.3093E-05
Miscellaneous - cytochrome P450	7.5490E-04
DNA metabolism	2.0105E-03
Protein metabolism - degradation - ubiquitin	8.5949E-03
Miscellaneous - nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	9.1785E-03
Development	1.6316E-02
Stress - biotic	2.8210E-02

Table 4. List of the 10 experimental conditions most correlated to VAL-P01 or VAL-P02, sorted by absolute AtCAST-derived Spearman Correlation coefficient. The transcriptional similarities were assessed upon gene expression changes (all treatments vs. all controls) detected by the Arabidopsis microarray. Negative correlation values signify an opposite behaviour; positive correlation values signify a similar behaviour. Details are available on the AtCAST publication (Sasaki et al., 2011).

Experiment	Genotype	Treatment/tissue	Spearman correlation
		VAL-P01	
ABA 1 h	Col-0	10 µM ABA/seedling	0.86
ABA 3 h	Col-0	10 µM ABA/seedling	0.80
ARR22-ox (t-zeatin 3 h)	ARR22-ox	20 µM t-zeatin/seedling	-0.70
Drought stress 24 h (shoot)	Col-0	drought (15 min dry air then further incubation in closed vessel)/shoots	0.68
Prohexadione 12 h	Col-0	10 µM prohexadione/seedling	0.67
ABA 0.5 h	Col-0	10 µM ABA/seedling	0.65
Norflurazon	Col-0	5 µM Norflurazon/seedling	-0.65
Osmotic stress 24 h (root)	Col-0	osmotic stress (300 mM mannitol)/roots	0.63
Drought stress 24 h (root)	Col-0	drought (15 min dry air then further incubation in closed vessel)/roots	0.61
UV stress 24 h (shoot)	Col-0	UV-B stress (15 min 1.815 min 1.8 W/m^2 Philips TL40W/12; thereafter recovery/shoots	-0.60
		VAL-P02	
$AgNO_3$ 3 h	Col-0	10 µM $AgNO_3$/seedling	0.84
Phytoprostane A1 4 h	Col-2	75 µM phytoprostane A1/seedling	0.74
HairpinZ 4 h (leaf)	Col-0	infiltrated with 10 µM HrpZ/leaves	0.74
Phaseolicola 2 h (leaf)	Col-0	infiltrated with 108 cfu/ml *P. syringae* pv. *phaseolicola* harvested after 2 h/leaves	0.73
P. infestans 6 h (leaf)	Col-0	*P. infestans* 6 h (leaf)/leaves	0.71
OPDA 4 h	Col-2	75 µM OPDA/seedling	0.70
Flagellin 4 h (leaf)	Col-0	infiltrated with 1 µM Flg22/leaves	0.70
Osmotic stress 24 h (root)	Col-0	osmotic stress (300 mM mannitol)/roots	0.68
Salt stress 24 h (root)	Col-0	salt stress (150 mM NaCl)/roots	0.64
ARR22-ox (t-zeatin 3 h)	ARR22-ox	20 µM t-zeatin/seedling	0.55

Figures

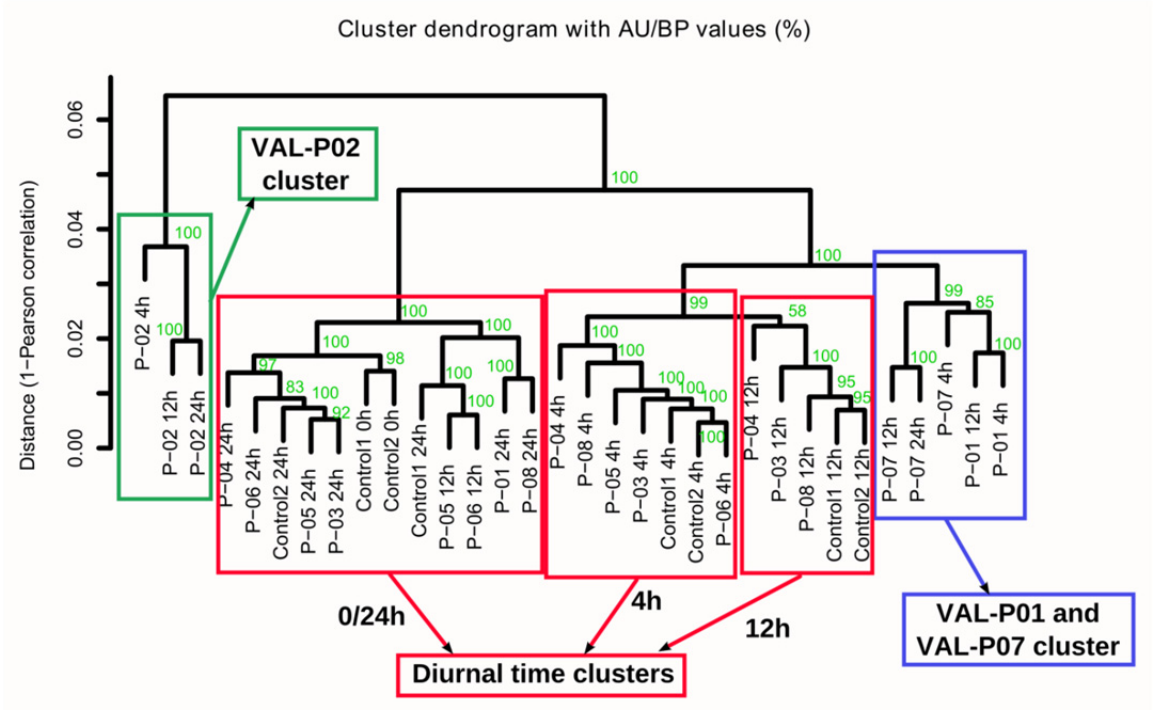

Fig. 1. UPGMA-like hierarchical clustering of the control and biostimulant-treated samples generated in this work. 100 bootstrap values were generated and reported in each node of the tree as BP (Bootstrap Probability). The tree was generated using the *pvclust* R package.

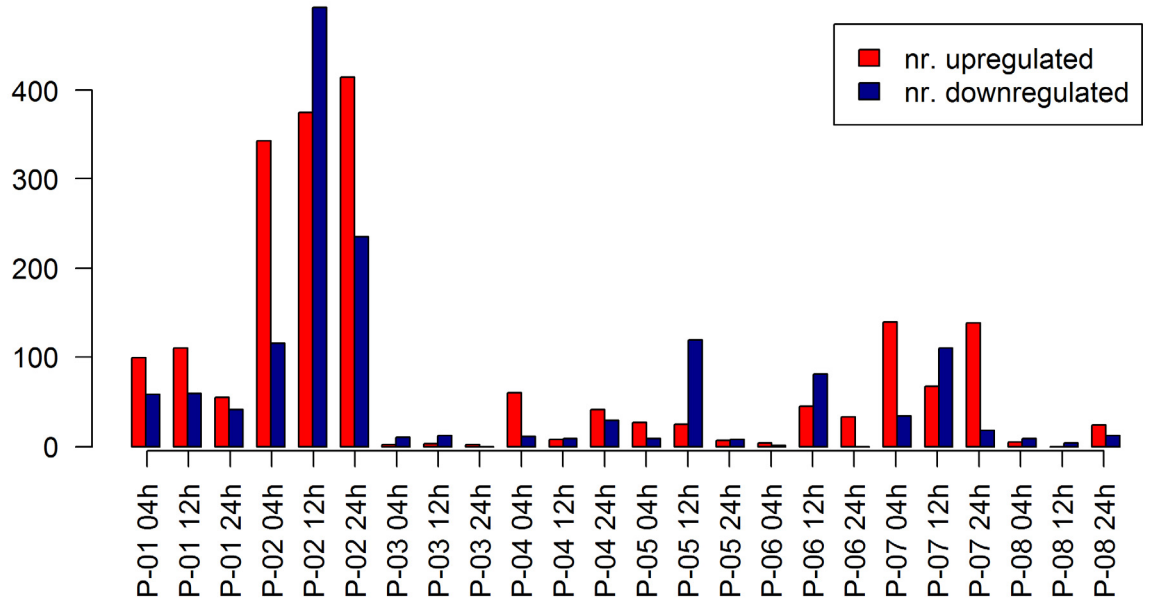

Fig. 2. Barplots indicating the number of significantly induced (red) and repressed (blue) genes for each treatment and each time point compared to control (untreated) plants.

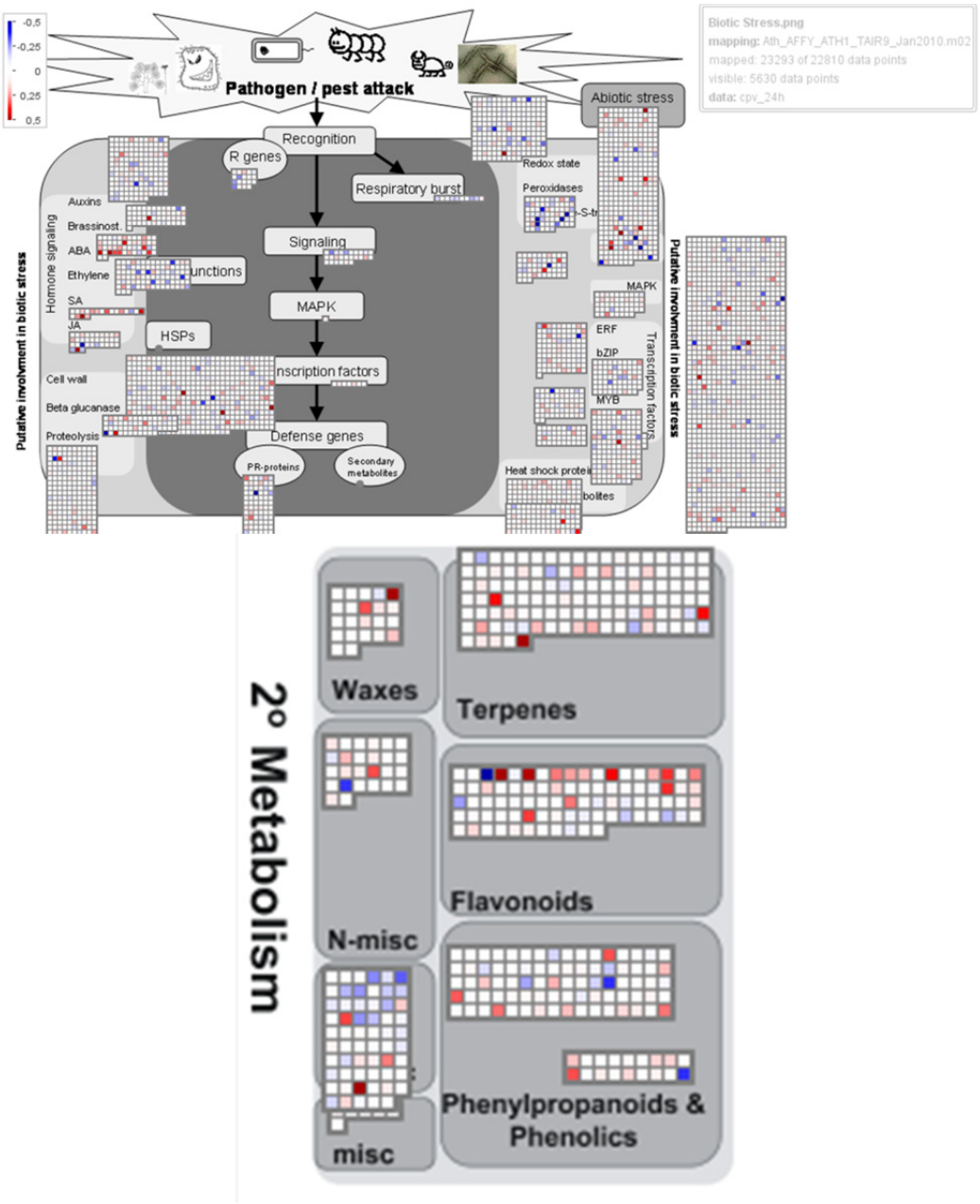

Fig. 3. Microarray data of VAL-P01 and VAL-P02 (averaged changes in transcript level from two biological replicates) were analysed using the MapMan software. The output of the software is shown, only for VAL-P01, with the genes involved in each metabolic step represented by a small square. A blue square (and blue shades) indicates a gene whose transcript level decreased following the treatment. A red square (and red shades) indicates a gene whose transcript level increased following the treatment.

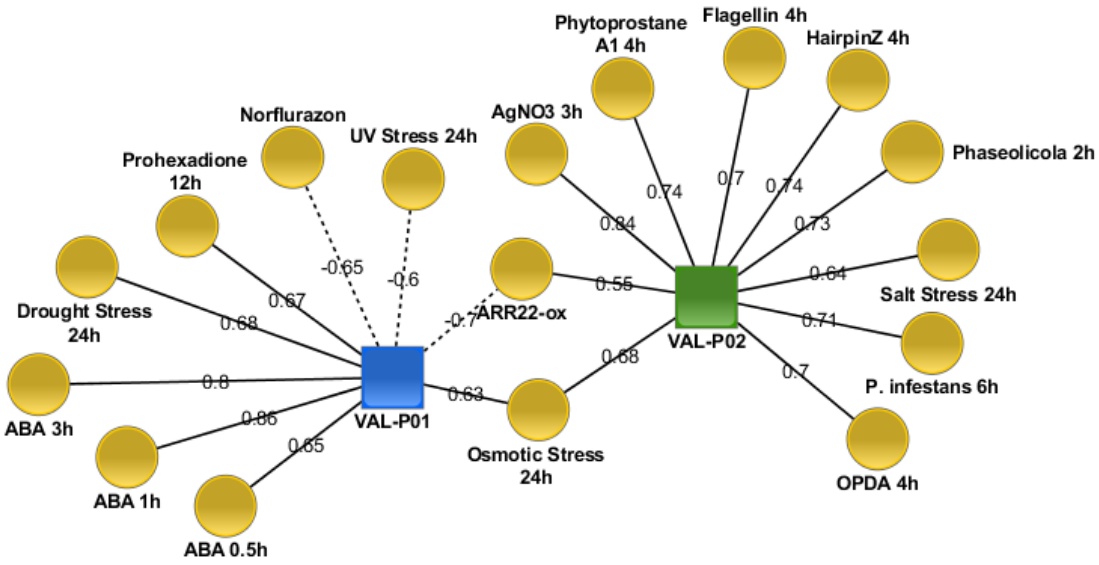

Fig. 4. AtCAST-inferred cytoscape network representations of the 10 public experiments most correlated to VAL-P01 and VAL-P02. Dashed lines represent negative correlations. Identical treatments on different tissues are merged into unique network nodes.

A

B

Fig. 5. Effect of drought stress on *Arabidopsis* plants deprived of water for 10 days. The plants that were previously treated with VAL-P01 (B) display a much better tolerance in comparison with the control plants (A).