Final Report

2020-EB-00XX

Quantitative proteomics of potato leaves treated with XXXX

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This is a sample report, results were generated for illustrative purposes only.

Contents

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Abbreviations | Glossary

ABA	Abscisic acid
SDS–PAGE	Electrophoresis method that allows protein separation by mass
LC MS/MS	Liquid chromatography–mass spectrometry
FDR	False Discovery Rate
ANOVA	Analysis of Variance statistical test
BIN	MapMan term describe biological pathways and concepts
GO	Gene Ontology

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I. Summary

In order to get a better understanding of the mechanism of action of the test compound XXXX, we investigated the proteome dynamics of *Solanum tuberosum*, after foliar application of XXXX, followed by inoculation with pathogen. A total of 3697 proteins were identified by Mass Spectrometry. Annotation, functional category classification, and enrichment tests highlighted significant coordination and enrichment in small heat shock proteins and an increase in endochitinases. These results suggest that XXXX is capable of inducing a response in plants against biotic and heat/osmotic stress, characterized by protein modification and protein homeostasis, which may or may not be related to ABA and chloroplast regulation. These results, which complement other independent observations, should provide further insight into the molecular mechanism of XXXX action.

Aim of the Experiment

To measure and compare the changes in protein profiles and abundance of potato (*Solanum tuberosum*) after foliar application of XXXX.

Traceability

The experiment was performed at the Sponsor's laboratory. Samples were collected by Sponsor and sent for processing at Elysia Bioscience. Remaining samples after analysis may be kept in storage for 4 weeks after approval of the final report. Data (raw and processed) is returned to the Sponsor; copy of all the data remains stored at Elysia Bioscience unless otherwise instructed by the Sponsor.

Test samples: Foliar tissue, 7-week-old plants, *Solanum tuberosum*, specific treatment unknown to Elysia Bioscience.

Reference test item: Substance, extract, specific characteristics unknown to Elysia Bioscience.

Observations/Notes: Test was performed open labelled.

Storage conditions at Elysia Bioscience: Samples were processed at reception. Protein extraction stored at -20°C.

Date of analysis: Analyses were carried out 15 June to 10 July 2019.

Identification of test samples

After receipt at Elysia Bioscience, test samples were stored at -20°C according to the requirements of the experiment and the nature of the samples. Test samples batched-delivered were noted in an identification sheet (**Annexe 1, separate file**). Samples were not be further analysed outside of this study (cf. Terms and Conditions).

Handling of test samples

According to the sponsor's specifications, remaining test samples are to be stored at the end of the experiments. The sponsor remains responsible for test samples (cf. Terms and Conditions).

II. Materials and Methods

Sample Preparation and LC-MS/MS Analysis

Protein was extracted using the Pierce Plant Total Protein Extraction Kit (Thermo Fisher) according to manufacturer's recommendations. The protein concentration was measured by Bradford (Roti Nanoquant), mixed with Laemmli buffer and heated to 37°C for 30 min. The samples were separated by SDS–PAGE and digested with trypsin overnight. The peptides generated were acidified and separated using an LTQ Orbitrap Fusion LUMOS (Thermo Fisher). The mass spectra were queried using Proteome Discoverer (version 2.4). The resulting MS/MS data were queried against the *Solanum tuberosum* reference proteome UP0000049XX (34647 entries). Search parameters were as follows: monoisotopic mass; trypsin as cleavage enzyme; two max missed cleavages, carbamidomethylation of cysteine as fixed modifications; and N-

terminal acetylation and oxidation of methionine as variable modifications. Results were filtered based on a false discovery rate (FDR) ≤0.01. The Background Based ANOVA was used to test differentially expressed proteins. The test correctly assumes that most protein abundances usually do not change in response to any one stimulus. This method automatically determines the range of protein ratios that are essentially constant between conditions and then tests each protein ratio against the median and variance derived from this background population. Proteome Discoverer 2.4 Protein quantitation values were exported for further analysis in Excel. Results are presented in **Annexe 2**.

Bioinformatics and Statistical Analysis

Proteins of *p*-values <0.05 by Student t-test and a fold-change of >1.50 or <0.50 in expression between groups were considered significant. MapMan 3.0.0 was used for pathway analysis (Thimm et al., 2004). Proteins fold-change values were transformed into Log2 fold-change, and their means were calculated. The non-redundant proteins were classified into MapMan BINs and their annotated functions were visualized using MapMan by searching against reference proteome UP000004994 mapping. Differentially expressed proteins were classified by their biological processes using the publicly available gene ontology (GO) database provided by the Gene Ontology Consortium (Ashburner et al., 2000). Enriched GO terms were identified with Fisher's exact test and FDR <1%. Visualization of these pathways and enrichment analysis was performed using REVIGO (Supek et al., 2011). Interactome analysis was performed using Cytoscape (Shannon et al., 2003) combined with STRING (Jensen et al., 2009). Known interactions among differentially-expressed proteins in treated leaves were used to draw the protein interaction network.

III. Results

Overview of Protein Expression in potato leaves treated with XXXX

To shed light on the mechanisms responsible for the biological activity XXXX in plant protection during the early stages of disease, we performed a comparative proteome survey on leaves of glasshouse-grown potato var. name treated with XXXX and inoculated an unspecified pathogen. The treated leaves were compared with potato treated with water and inoculated with the same pathogen. Three biological replicates of control and treated leaves were collected at the same time. Proteomic analysis returned a total of 38707 high-quality peptides corresponding to 3697 identifiable and quantifiable proteins in the samples (**Annexe 2**). Of the 3697 proteins, 202 were found to be differentially expressed proteins in a pairwise comparison between XXXX treated and control leaves. Among the 202 differentially expressed proteins, 156 had clearly referenced functional annotations.

Pathway analyses

Proteins identified were first analysed by organization into relevant functional pathways. This is displayed onto diagrams, to enable quick and easy observation of the similarities and differences between the treatments. Diagrams (**Figure 1**) show the log2 fold-change in protein abundance in XXXX versus Control for the 2567 annotated proteins. A detail of the analysis, related to biotic stress and pathogen attack, is shown in **Figure 2**. Of note, the analysis is limited to proteins annotated or known to belong to a given pathway. Unannotated proteins may have a known function but not be associated to any pathway (also referred to as “not assigned”). In the case of the present study, a total of 1130 detected potato proteins were unannotated and therefore could not be included in the analyses.

The overview of metabolism (**Figure 1**) shows an overall good coverage of all cell pathways. Overall, we noticed an **upregulation of protein folding and homeostasis (Figure 3 A, B), and a downregulation of redox regulation (Figure 4 A, B) in response to XXXX (Not shown in this Sample Report).** Additionally, a mixed regulation of proteins related to secondary metabolite synthesis was observed **(Figure 2, not shown).**

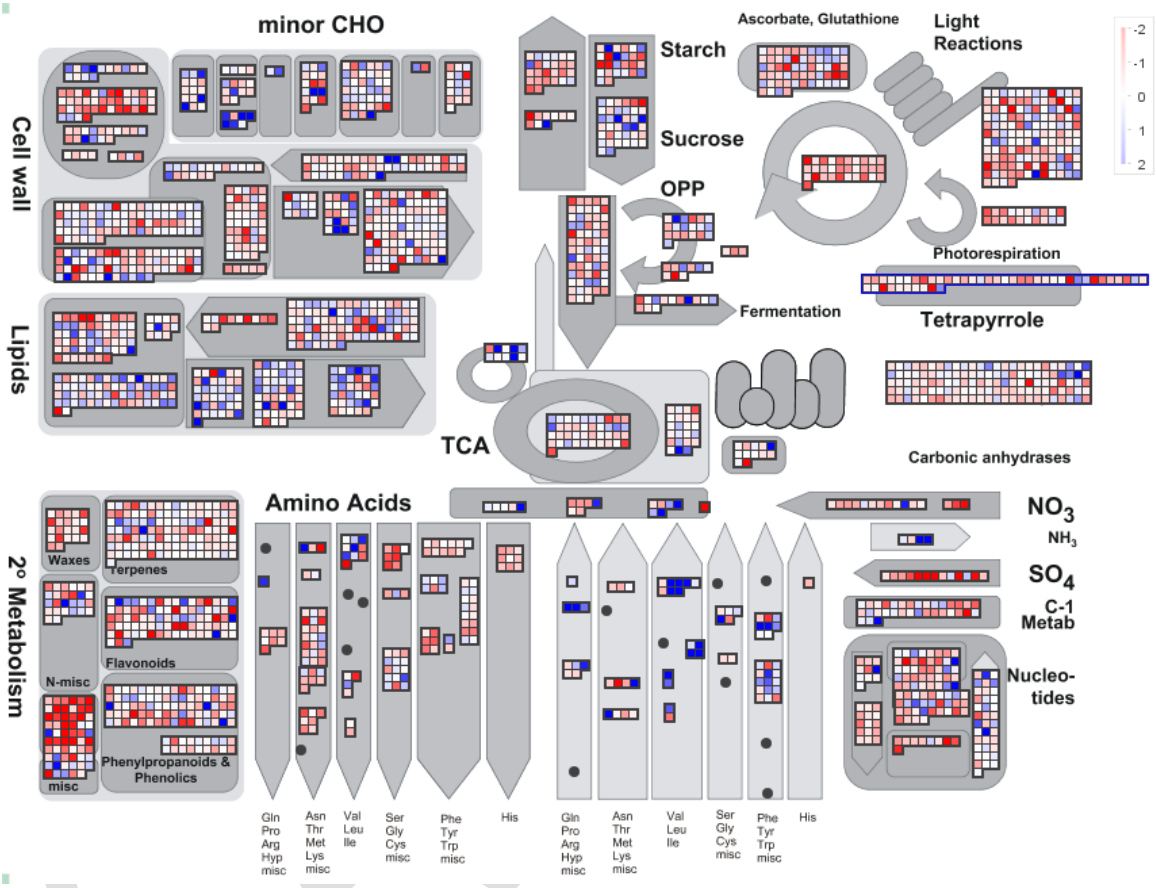

Figure 1. Sample Metabolism overview and functional annotation of the identified proteins (MapMan). Increased and decreased protein abundance is shown under a blue and red colour scheme, respectively. Differential proteins are shown under a stringent cut-off, colour saturates at 2 log2 fold-change and -2 log2 fold-change. Proteins unchanged from control values appear as white. All proteins quantified were used for this mapping.

Gene Ontology Classification of Proteins modified by XXXX

Gene ontology (GO) classification of proteins was used to reveal the **biological processes** associated to the proteins and pathways potentially modified by XXXX. Biological processes (also called "GO terms") designate specific objectives that the plant achieves. As such, a biological process is not equivalent to a pathway. Instead, a biological process is often accomplished by particular sets of proteins, or a specific combination of pathways. Association to a biological process is useful to define and narrow-down interesting proteins and pathways by their relationship to a final process or event. Proteins associated with pathways modified by XXXX were retrieved (listed in **Annexe 3**) and analysed for related biological process using REVIGO. The GO analysis is shown in **Figure 5**. The summary table is presented **Annexe 4.** GO analysis suggest that **the pathways and proteins modified by XXXX are implicated in responses to hydrogen peroxide, responses to abiotic stimuli, and responses to antibiotic or chemical. These proteins could also be involved in chaperone-mediated protein folding and protein homeostasis**.

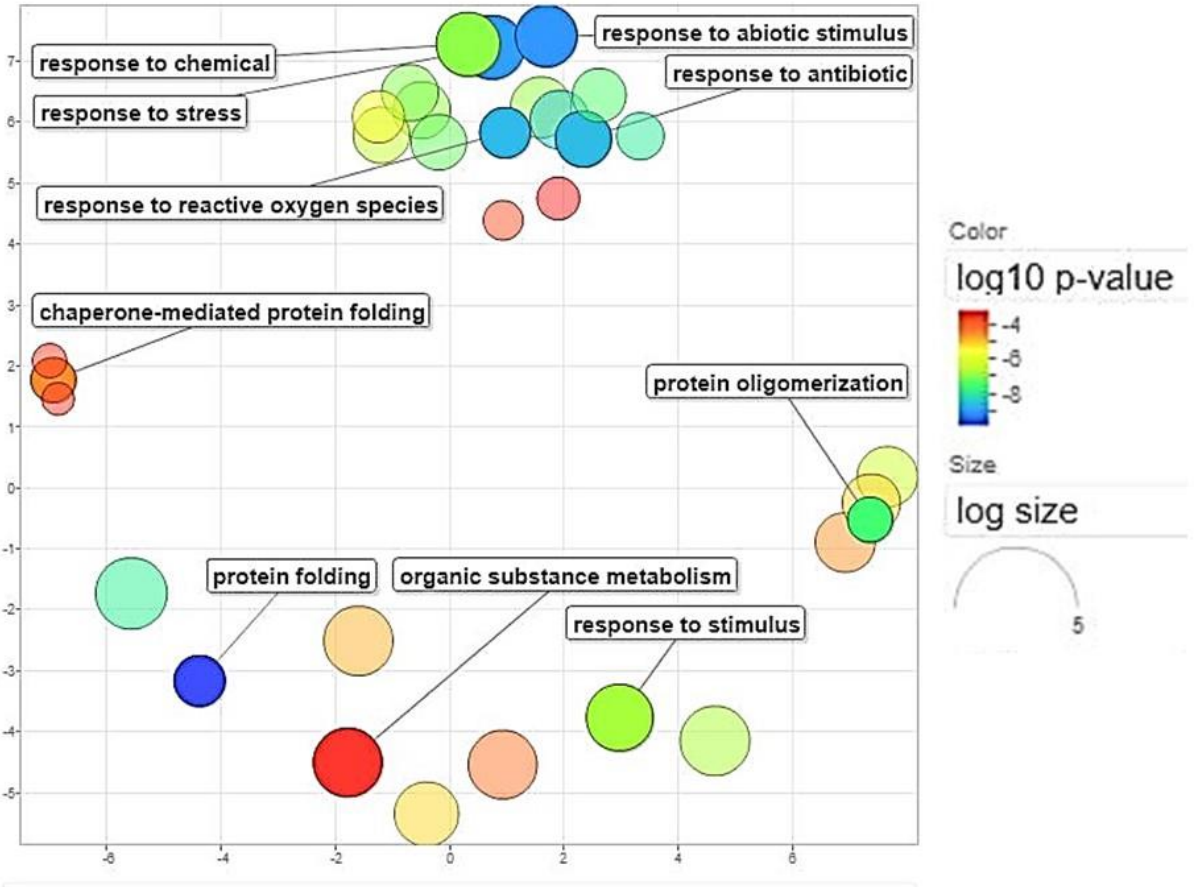

Figure 5. Sample GO analysis of biological processes visualised using REVIGO. Diagram of significantly associated GO terms by clustering the GO terms based on semantic similarity. Bubble colour indicates the *p*-value (blue lower p-value than red), only GO terms with $p < 0.05$ are displayed. Bubble size indicates the frequency of the GO term shared by proteins, as several GO terms can be associated to a single protein.

Protein–Protein Interactions

Cytoscape was used to map the protein-protein interactions among the proteins associated with modified pathways by XXXX. Biological networks help to visualize the data in one plot to help identify patterns and relationships between proteins from diverse pathways. We analysed the known interactions of proteins using the following criteria: 1) All proteins with a significant difference in abundance (*p*-value < 0.05), and/or 2) Proteins associated with modified pathways identified in treated samples. Results are shown in **Figure 6**. The summary **Table 1** is also presented in detail in **Annexe 5**.

Table 1. Sample List of differentially-expressed proteins and interacting proteins identified by proteomic analyses. Proteins with the most prominent change from control are highlighted.

Uniprot Entry	Description	Ratio (TR) / (CTRL)	p-Value: (TR) / (CTRL)
O48616	MAP kinase kinase (MAP kinase kinase 2)	0.00	7.6E-16
Q2MI42	Protein TIC 214 (AtTIC214)	0.28	9.7E-07
P07179	RuBisCO small subunit 2A (LESS 5)	0.57	1.2E-06
P17340	Plastocyanin, chloroplastic	0.62	3.2E-04
Q40163	Photosystem II 10 kDa polypeptide, chloroplastic	0.63	4.4E-04
O80432	Mitochondrial small heat shock protein	2.67	4.5E-13
Q05539	Acidic 26 kDa endochitinase	3.61	7.6E-16
Q05538	Basic 30 kDa endochitinase	5.86	7.6E-16
Q95661	Small heat shock protein, chloroplastic	11.91	7.6E-16
Q6UJX4	Molecular chaperone Hsp90-1	1.15	9.4E-01
K4CXQ6	UBC13-2 (Ubc13-type ubiquitin-conjugating enzyme 2)	1.22	9.5E-01
Q9SMC4	Oligosaccharyl transferase subunit DAD1 (DAD-1)	1.38	8.7E-01
K4BB06	Proteasome subunit beta (EC 3.4.25.1)	1.86	1.5E-02
O24030	Proteasome subunit alpha type-7	0.89	6.8E-01
P27065	Ribulose bisphosphate carboxylase large chain	0.97	8.7E-01
Q96489	Class II small heat shock protein Le-HSP17.6	1.02	9.9E-01
D3TI69	Beta-hexosaminidase	1.05	9.9E-01
Q84MI6	Mitogen-activated protein kinase (EC 2.7.11.24)	1.06	9.8E-01
Q9M5A8	Chaperonin 21	1.08	9.8E-01
Q8GUQ5	Brassinosteroid LRR receptor kinase (tBRI1)	1.12	9.5E-01

Prominent among the upregulated proteins in the network are Mitochondrial small heat shock protein (**O80432**, fold-change = 2.67), Chloroplastic small heat shock protein (**Q95661**, fold-change = 11.91), Acidic 26 kDa endochitinase (**Q05539**, fold-change = 3.61), and Basic 30 kDa endochitinase (**Q05538**, fold-change = 5.86). In this study, heat shock proteins appear to be associated molecular chaperone regulation.

It has been shown that heat shock proteins (Dangi et al., 2018) and endochitinases (Girhepuje and Shinde, 2011) may inhibit the growth of phytopathogenic fungi. In addition, Endochitinases may be induced by abiotic stress. For instance, these proteins interact with Class II small heat shock protein HSP17 (**Q96489**), which is also involved in responses to heat and reactive oxygen species. Heat shock proteins and abiotic stress are also important for oxidative and energy metabolism.

The Acidic 26 kDa endochitinase (**Q05539)** interacts with beta-hexosaminidase, which is also a chitinolytic enzyme. The Chloroplastic small heat shock protein (**Q95661**) interacts with chaperone HSP90 (**Q6UJX4**), which is involved in protein folding and homeostasis (Sangster and Queitsch, 2005).

Among the downregulated proteins are MAP kinase kinase MEK1 (**O48616**), which was not detected in XXXX treated leaves despite relatively abundant levels in control leaves. MEK1 is involved in mitosis and the control of cell division (Colanzi et al., 2000). It is possible that XXXX has an impact on plant growth.

Other downregulated proteins included the chloroplast proteins TIC 214 (**Q2MI42**, fold-change = 0.28), RuBisCO small subunit 2A (**P07179**, fold-change = 0.57), Plastocyanin (**P17340**, fold-change = 0.62), and Photosystem II 10 kDa polypeptide (**Q40163**, fold-change = 0.63) involved in plant photosynthesis and cellular metabolism. It could be that downregulation of these proteins act to counteract the increase in heat shock protein activity and, alternatively, to balance out tetrapyrrole regulation between light reactions and stress protection.

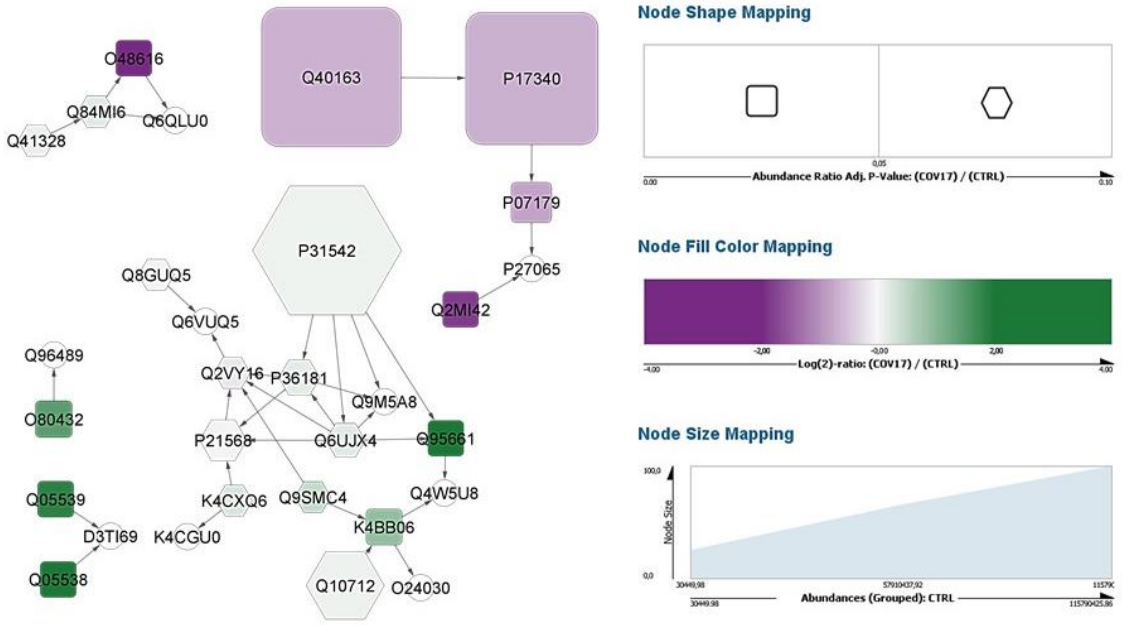

Figure 6. Sample protein-protein interaction networks. Proteins are represented with colour nodes (upregulated in green and downregulated in magenta, white denotes no change). Differentially expressed proteins in XXXX vs. Control are shown by a rectangle, the size on the node denotes the relative abundance of the protein. The direction of the interactions is shown by arrows. Circles denote interacting proteins that were not detected in the proteomic analysis.

IV. Conclusions et recommendations

Based on the analyses shown above, application of XXXX increases small heat-shock proteins and endochitinases. These proteins may interact with chaperones involved in protein homeostasis, in the regulation of heat and ROS responses, and plant pathogen defense. These results suggest that XXXX is capable of inducing a response in plants against biotic and heat/osmotic stress. XXXX also decreases MEK1 and chloroplast related proteins, possibly linked to a balance between growth and stress responses.

Figure 6 shows a summary of the associated keywords associated to the effects of XXXX on potato leaves. The results presented in this study can be monitored by Western Blot or RT-qPCR to verify and validate overall levels of the proteins identified as markers of the action of XXXX.

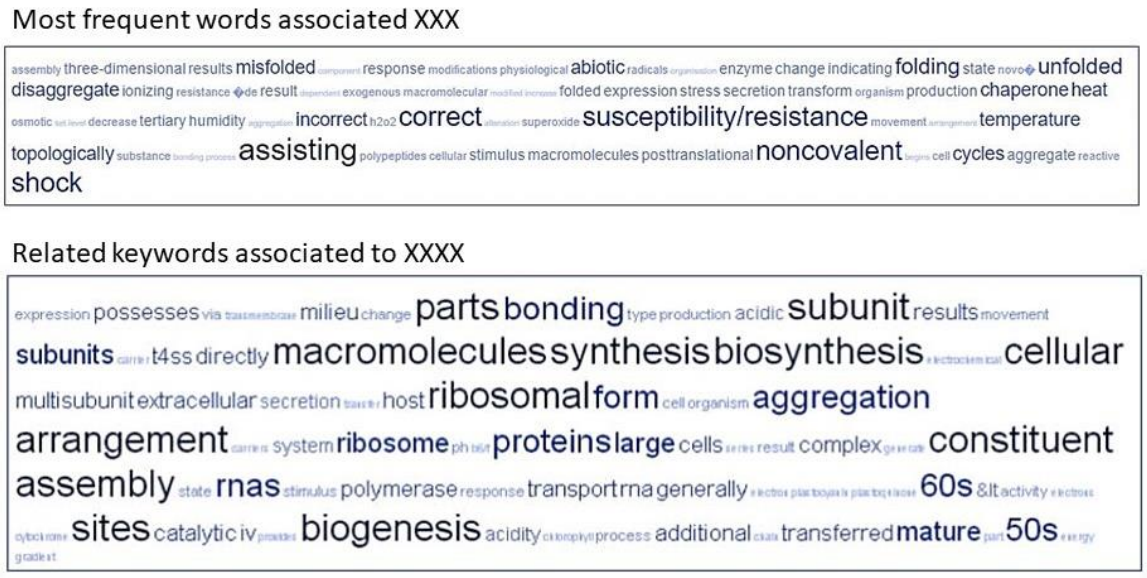

Figure 6. Tag Cloud. Summary of keywords associated with the effects of XXXX. All displayed words are overrepresented in the descriptions of the GO terms, with larger and darker letters signifying stronger overrepresentation.

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Annexes

Annexes 1 to 5 can be found in accompanying files.

Provided are the full texts of selected relevant publications.