PhD School on Agriculture, Environment and Bioenergy

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(XXXIX cycle, 20123-25)

Project draft

1. Field of interest

07/E – Agricultural chemistry, agricultural genetics and soil science – AGR/13 - Agricultural chemistry

2. Project title

Study of the protein–protein interactions as component of plant responses to nitrogen availability.

3. Tutor

Bhakti Prinsi

Associated Professor in Agricultural Chemistry at the Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy - University of Milan. Her research activity is aimed at studying topics of plant physiology and biochemistry relevant to crop quality and production: physiological and molecular responses to mineral nutrition and abiotic stresses; fruit ripening in woody plants; relations between primary and secondary metabolism in plants; characterization and biological activities of seed storage proteins in legume and cereal crops. These research activities have enabled her to gain a good expertise in the fields of plant proteomics and metabolomics based on mass spectrometry approaches.

Author of 45 "peer-reviewed" articles. *h-index (Scopus): 20; total citations (Scopus): 1405.*

Prof. Luca Espen

Full Professor in Agricultural Chemistry at the University of Milan. His recent research activity can be divided in the following topics: a) biochemical and physiological aspects of seed germination; b) biochemical and physiological processes of the fruit ripening of orchard tree species; c) plant mineral nutrition; d) biochemical and physiological aspects involved in the abiotic stress responses.

Author of 79 "peer-reviewed" articles. *h-index (Scopus): 28; total citations (Scopus): 2142.*

4. Relevance of the topic and state of the art:

Nitrogen (N) fertilization in agricultural systems is necessary to sustain crop yields, but it has huge economic, social and environmental costs. Plant N use efficiency is very low, therefore the selection of new strategies to manage N inputs is needed to improve the sustainability of agricultural production [1]. Maize (*Zea mays* L.), among more important cereals, is a model organism for the study of plant physiology, biochemistry and genetics [2]. Nitrate is the main form of inorganic N used by crops. Its availability affects growth, morphology, and metabolism of plants and, ultimately, grain production and fruit quality [3].

In the past, proteomics was successfully applied to gain new knowledge on crop metabolic adaptations to different N inputs, highlighting the involvement of different post-translational modifications and enzyme isoforms [8,9,10,11]. The study of protein complexes is an expanding area of research in plant biology. Indeed, there is increasing evidence that enzymes functionality and regulative proteins are influenced by protein-protein interactions and formation of large macromolecule complexes, which cluster cooperating enzymes. The formation of protein complexes can affect catalytic activities, substrate specificity and subcellular localization, representing a further level of control in the plant responses [12]. Interestingly, it is now well documented that proteins involved in root nitrate uptake and plant water balance work as oligomeric complexes [13, 14].

The aim of the project is to unravel the changes in abundance and composition of protein complexes in response to N availability. Based on the proteomics results produced by previous studies, the attention will be paid to the analysis of protein-protein interactions that could play a central role in the N supply responses.

5. Layout of the project (draft)

The aim of the project is to study the changes in the protein-protein interactions occurring in the adaptation to different N availabilities in model crops, such as maize (*Zea mays* L.). The proteomic evaluations will be combined with physiological and biochemical analyses and will be focalized on root organ.

Materials & Methods:

Plant materials and nutritional treatments: the project will make use of maize seedlings as the main experimental model. Moreover, to broaden knowledge and find common or specific responses, the use of other crop species as well as different phenological stage could be evaluated. In details, commercial lines of maize will be grown in hydroponic systems. During vegetative growth, the effects of different N nutritional treatments on plant growth and plant metabolism will be tested. The nutritional treatments will be based on the reintroduction of nitrogen (*i.e.* nitrate) after a starved period to analyze the typical induction phase, characterized by the activation of root transport systems as well as nitrogen assimilation pathway.

Evaluation of physiological effects: key physiological parameters related to sensing and utilization of N availability, such as nitrate uptake capacity, *in vivo* biochemical test, such as the ability to acidify the rhizosphere, and biomass accumulation will be evaluated by mean of phenotyping tools and biochemical assays.

Evaluation of biochemical responses: western-blot and enzymatic analyses will be performed in order to evaluate the abundance and activities of key enzymes involved in N acquisition (*i.e.* Nitrate Reductase, Glutamine Synthetase) in roots. In order to describe metabolic adaptations, the levels of key metabolites, such as sugars and amino acids, will be evaluated by colorimetric and/or mass spectrometry analysis.
Characterization of protein complexes in the roots: The first step of the research activity will be devoted to optimizing the technical protocols to isolate protein complexes of interest. For this purpose, the native electrophoresis analyses will be performed combining different approaches [Blue native (BN) PAGE, 2D-BN/SDS-PAGE] to achieve the best resolution and reliability. The detection of the protein complexes of interest will be obtained using specific antibodies in Western blot analyses. Finally, LC-ESI-MS/MS analyses will be used to complete protein identification and quantitation and investigate changes in composition and stoichiometry of the protein complexes. Bioinformatic analyses will be used to perform mass spectrum interpretation and quantitation, utilizing the large set of public genetic information, such as the maize reference proteome available in UniProt (https://www.uniprot.org/taxonomy/4577 - 62236 entries). Proteomic data interpretation will be implemented by using statistical and informatic tools, such as KEGG (https://www.genome.jp/kegg/pathway.html) and STRING (https://string-db.org/cgi/input?sessionId=bT98mcTOiy7a&input_page_show_search=on), among others.
Finally,

Validation of the results: the most interesting changes will be validated by alternative approaches, such as immunoblotting, using commercial and/or novel antibodies or size-exclusion chromatography (SEC) coupled with LC-ESI-MS/MS [15].

5.1. Schedule and major steps (3 years):

First year:
The activities of the first year will be mainly devoted to study nitrogen nutrition and to optimize proteomic protocols.
- Study of the scientific literature related to plant N nutrition and proteomics.
- Definition of the nutritional treatments (N inputs, timings) useful to define the nitrate uptake/induction phase in maize and, eventually, in other model crops.
- Optimization of the protocols to analyze protein complexes in maize roots.

Second year:
The activities of the second year will be aimed at studying changes in protein-protein interaction in maize roots.
- Insights on plant physiological and metabolic responses to the experimental conditions defined in the previous steps.
- Large-scale analysis of protein complexes in roots of plants exposed to the experimental conditions defined in the previous steps.

Third year:

Interpretation and validation of the results

- Interpretation and comparison of the proteomics results in relation to the plant physiological and metabolic responses to different N inputs.
- Validation of the results by means of complementary techniques, bioinformatic tools, and targeted analyses.

The training and dissemination activities (schools, congresses, scientific publications) and research periods abroad will be planned during the three years of the PhD school.

6. Available funds (source and amount)

The project will be funded with a grant of 20.000,00 € (PNNR Projects).

7. Literature:

1. Xu G, Fan X, Miller AJ. Plant nitrogen assimilation and use efficiency. *Ann Rev Plant Biol* 2012, 63:153-82. DOI: 10.1146/annurev-arplant-042811-105532.

2. Strable J, Scanlon MJ. Maize (*Zea mays*) a model organism for basic and applied research in plant biology. *Cold Spring Harb Protocols* 2009. DOI: 10.1101/pdb.emo132.

3. Andrews M, Raven JA, Lea PJ. Do plants need nitrate? The mechanisms by which nitrogen form affects plants. *Ann Appl Biol* 2013, 163:174–199. DOI: 10.1111/aab.12045.

4 Paungfoo-Lonhienne C, Visser J, Lonhienne TG, Schmidt S. Past, present and future of organic nutrients. *Plant and Soil* 2012, *359*(1): 1-18. DOI: 10.1007/s11104-012-1357-6.

5. Forsum O, Svennerstam H, Ganeteg U, Näsholm T. Capacities and constraints of amino acid utilization in Arabidopsis. *New Phytologist* 2008, *179*(4):1058-1069. DOI: 10.1111/j.1469-8137.2008.02546.x.

6. Forde BG. Glutamate signaling in roots. *J Exp Bot* 2014, 65:779-787. DOI:10.1093/jxb/ert335.

7. du Jardin P. Plant biostimulants: definition, concept, main categories and regulation. *Sci Hortic* 2015, 196: 3-14. DOI: 10.1016/j.scienta.2015.09.021.

8. Pii Y, Alessandrini M, Dall'Osto L, Guardini K, Prinsi B, Espen L, Zamboni A, Varanini Z. Time-resolved investigation of molecular components involved in the induction of NO_3^- High Affinity Transport System in maize roots. *Front Plant Sci* 2016, 7: 1657. DOI: 10.3389/fpls.2016.01657.

9. Prinsi B, Negri AS, Pesaresi P, Cocucci M, Espen L. Evaluation of protein pattern changes in roots and leaves of Zea mays plants in response to nitrate availability by two-dimensional gel electrophoresis analysis. *BMC Plant Biol* 2009, 9:113. DOI: 10.1186/1471-2229-9-113.

10. Prinsi B, Espen L. Mineral nitrogen sources differently affect root glutamine synthetase isoforms and amino acid balance among organs in maize. *BMC Plant Biol* 2015, 15:96. DOI: 10.1186/s12870-015-0482-9.

11. Prinsi B, Espen L. Time-course of metabolic and proteomic responses to different nitrate/ammonium availabilities in roots and leaves of maize. *Int J Mol Sci* 2018, 19(8) 2202. DOI: 10.3390/ijms19082202.

12. Aryal, U. K.; Ding, Z.; Hedrick, V.; Sobreira, T. J. P.; Kihara, D.; Sherman, L. A. Analysis of Protein Complexes in the Unicellular Cyanobacterium Cyanothece ATCC 51142. *J Proteome Res* 2018, *17* (11), 3628–3643. doi.org/10.1021/acs.jproteome.8b00170.

13. Jacquot, A.; Li, Z.; Gojon, A.; Schulze, W.; Lejay, L. Post-Translational Regulation of Nitrogen Transporters in Plants and Microorganisms. *Journal of Experimental Botany* 2017, *68* (10), 2567–2580. doi.org/10.1093/jxb/erx073.

14. Muratore, C.; Espen, L.; Prinsi, B. Nitrogen Uptake in Plants: The Plasma Membrane Root Transport Systems from a Physiological and Proteomic Perspective. Plants 2021, 10 (4), 681. doi.org/10.3390/plants10040681.

15. Senkler, J.; Senkler, M.; Eubel, H.; Hildebrandt, T.; Lengwenus, C.; Schertl, P.; Schwarzländer, M.; Wagner, S.; Wittig, I.; Braun, H.-P. The Mitochondrial Complexome of Arabidopsis Thaliana. The Plant Journal 2017, 89 (6), 1079–1092. https://doi.org/10.1111/tpj.13448.