1. PHD PROJECT DESCRIPTION (4000 characters max., including the aims and work plan)

Project title:

Post-translational regulation of auxin-conjugating enzymes in plant tissues

Project goals

The aim of the project is to verify the hypothesis that the activity of enzymes synthesizing auxin conjugates is regulated at the post-translational level by covalent modifications and/or by interactions of enzyme with low-molecular-weight ligands (amino acids, other phytohormones and other signaling compounds).

1.1. Outline

Auxins are a group of low molecular weight chemical compounds, derivatives of aromatic amino acids - tryptophan (e.g. indole-3-acetic acid (IAA) and phenylalanine. As phytohormones, auxins control almost all physiological processes of plants, so it is necessary to precisely regulate their concentration during growth and development and during the response to environmental factors. One of the mechanisms that maintains the appropriate concentration of auxin is conjugation - a covalent modification of the hormone consisting in the enzymatic attachment of a sugar, a cyclic alcohol, an amino acid or a protein. Conjugation of auxin with sugar is an example of an ester conjugate. The vast majority of the auxin pool occurs in the plant as the bound form (conjugates), and only a small share exhibits the biologically active form (free auxin). There is well documented that the synthesis of auxin conjugates is regulated by transcription of genes encoding the IAA-glucose synthase (IAGLU) and IAA-amino acid amidosynthetases (GH3s). Also, little is known about the regulation of the functions of GH3 family proteins by protein-protein interactions. So far, only two proteins are known that interact with the GH3 family polypeptide (JAR1/GH3.11) and regulate its activity. Amidosynthetases ,which belong to the GH3 family, catalyze the synthesis of auxin linkages with amino acids (e.g. IAA-aspartate) in a two-step reaction using ATP to form acyladenylate (IAA-AMP) intermediate. The mechanism of this reaction is relatively well understood. On the other hand, ther is some evidence suggesting that there must be some other regulatory mechanisms, possibly at the level of the enzymatic protein. This is a completely novel approach to examining the control of the concentration of active phytohormone. So far, no such research has been undertaken in relation to hormone conjugation. The expected result will be the description of a new mechanism that regulates the level of a plant hormone.

In this project, having the genes encoding IAA-glucose synthase (IAGLU) from maize and IAA-aspartate synthetase (PsGH3) from pea, amino acid residues that constitute potential sites of post-translational modifications will be identified. The possibility of covalent

protein modification will be verified using immunochemical methods (immunoprecipitation, Western blot). Then, using site-specific mutagenesis, changes will be introduced within the sequences coding for the amino acids subject to modification. In order to verify the thesis that post-translational modifications of auxin-conjugating enzymes modulate their activity, kinetic analyzes of the purified enzymes will be performed (substrate specificity, determination of K_m, k_{cat}, V_{max} parameters). Moreover, proteins can be also subject to allosteric regulation through non-covalent interactions with low-molecular-weight ligands. The aim of the research will be to examine whether catecholamines (dopamine, adrenaline, noradrenaline) and indole amines (melatonin, serotonin, tryptamine), due to their structural similarity with auxins, can influence the activity of enzymes synthesizing conjugates.

1.2. Work plan

1. Analysis of the amino acid sequences of the PsGH3 protein in order to search for motifs susceptible to phosphorylation and possibly other post-translational modifications (glutathionylation, carbonylation, nitrosylation, ubiquitylation) 2. Production of primers generating mutated motifs subject to post-translational modification in the recombinant PsGH3 protein. 3. Production and purification of modified forms of PsGH3. 4. Immunochemical analyzes of post-translational modifications of PsGH3. 5. Analyzes of the enzymatic activity of mutant and native forms of the PsGH3 protein. 6. Testing the effect of different concentrations of selected catecholamines and indole amines on the enzymatic activity and kinetic parameters (Km, Vmax/Km, kcat) of the PsGH3 protein. 7. Search for proteins interacting with PsGH3 in plant extracts - protein isolation using the pull down method, 2-DE electrophoretic separation (IEF/SDS-PAGE), protein identification using the

LC-MS/MS method (in cooperation with Prof. Jorg Fettke, University of Potsdam).

1.3. **Literature** *(max. 10 listed, as a suggestion for a PhD candidate)*

Wojtaczka P, Ciarkowska A, Starzyńska E, Ostrowski M (2022) The GH3 amidosynthetases family and their role in metabolic crosstalk modulation of plant signaling compounds. Phytochemistry DOI:10.1016/j.phytochem.2021.113039

Ciarkowska A, Ostrowski M, Kozakiewicz A (2021) Biochemical characterization of recombinant UDPG-dependent IAA glucosyltransferase from maize (Zea mays) International Journal of Molecular Sciences DOI:10.3390/ijms22073355

Ostrowski M, Mierek-Adamska A, Porowińska D, Goc A, Jakubowska A (2016) Cloning and biochemical characterization of indole-3-acetic-amino acid synthetase PsGH3 from pea. Plant Physiology and Biochemistry doi: 10.1016/j.plaphy.2016.05.031

Cohen jD, Strader LC (2024) An auxin research Odyssey: 1989-2023. Plant Cell doi: 10.1093/plcell/koae054.

Xu G, Zhang Y, Li M, Jiao X, Zhou L, Ming Z. (2021) Crystal structure of the acyl acid amido synthetase GH3-8 from Oryza sativa. Biochemical and Biophysical Research Communications doi: 10.1016/j.bbrc.2020.11.098.

Mateo-Bonmati E, Casanova-Saez R, Simura J, Ljung K (2021) Broadening the roles of UDP-glycosyltransferases in auxin homeostasis and plant development. New Phytologist doi: 10.1111/nph.17633.

Solanki M, Shukla LI (2023) Recent advances in auxin biosynthesis and homeostasis. 3Biotech https://doi.org/10.1007/s13205-023-03709-6

1.4. Required initial knowledge and skills of the PhD candidate

Knowledge of the structure, function, isolation and characterization of proteins, especially enzymes, skills in laboratory work (preparation of solutions, spectrophotometric analysis, preparation of samples for chromatographic and electrophoretic analyses). Knowledge of mutagenesis, bacterial expression systems, production of recombinant proteins. Ability to perform kinetic characterization of enzymes and interpret the results. Knowledge about phytohormones, in particular the metabolism, functions and mechanisms of action of auxins. Knowledge of the literature on the characteristics and metabolism of auxin

conjugates.

1.5. Expected development of the PhD candidate's knowledge and skills

Ability to critically analyze the results of own research and compare them with the results of other authors. Improving methods of purification and biochemical and molecular characterization of recombinant enzymes. Ability to plan and perform research in the field of identifying post-translational modifications of proteins and protein-protein interactions. Searching for research methods to verify the hypotheses. Ability to edit a manuscript containing research results (original paper) and a critical review of the literature (review paper). Ability to prepare an application for research funding from external sources (NCN).