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

1.1. 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).

1.2. 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 mechanism, possibly at the level of the enzymatic protein. More recently, Liu et al. (2025) have reported that IAA-conjugating activity of AtGH3.5 is inhibited by COP1-dependent ubiquitination under different light conditions. 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 Km, kcat, Vmax 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 tryptophan- and phenylalaninie-derivative auxin precursors due to their structural similarity with active auxins, can

influence the activity of enzymes synthesizing conjugates. This study will provide insight into the signaling pathways that modulate hormone levels under different physiological conditions.

1.3. Work plan

1. Analysis of the amino acid sequences of the PsGH3 and ZmIAGLU proteins 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 and/or Zm IAGLU proteins.

3. Production and purification of modified forms of PsGH3. 5/ZmIAGLU (mutagenesis PCR, protein synthesis using bacterial expression system, purification of the recombinant protein).

4. Kinetic studies of catalytic activity of native forms and mutant of PsGH3.5/ZmIAGLUs. Testing the effect of different concentrations of tryptophan- and phenylalaninie-derived auxin precursors (e.g. L-tryptophan, L-phenylalanine, isochorismate, chorismate, antranilane, indole, indole-3-phosphate, indole-4-pyruvic acid) on the enzymatic activity and kinetic parameters (Km,

Vmax/Km, kcat) of the PsGH3/ZmIAGLU proteins.

5.Immunochemical analysis of post-translational modifications of PsGH3 and ZmIAGLU: Search for proteins interacting with PsGH3/Zm IAGLU in plant extracts - protein isolation using the pull down assay and co-immunoprecipitation, 2-DE electrophoretic separation (IEF/SDS-PAGE), protein identification using the LC-MS/MS method (in cooperation with Prof. Jorg Fettke, University of Potsdam).

1.4. **Literature** *(max. 7 listed, as a suggestion for a PhD candidate preliminary study)*

Liu Y, Xie Y, Xu D, Deng XW, Li J. (2025) Inactivation of GH3.5 by COP1-mediated K63-linked ubiquitination promotes seedling hypocotyl elongation. Nature Communications doi: 10.1038/s41467-025-58767-6

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.

1.5. 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, immunochemistry). 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.

1.6. 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).