Université de Picardie Jules Verne
Ecole Doctorale Sciences, Technologie, Santé (ED 585)

Intitulé du sujet de la thèse / Title of thesis subject : **Functional regulation of pLAnt polygalacturonases through REdox status. (FLARE)**	
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Description of the PhD subject / Description of the PhD subject:

The plant primary cell wall is a complex structure, mainly composed of proteins and polysaccharides that play a central role in controlling plant shape and development. Over the recent years, cell wall pectins have been reported as major actors regulating the mechanical properties of the cell wall that can mediate changes in growth (Levesque-Tremblay et al., 2015; Sénéchal et al., 2014). Pectins are complex polysaccharides rich in galacturonic acids and comprise four main domains: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II) and xylogalacturonan (XG) (Caffall and Mohnen, 2009). One key feature of HG, a homopolymer of beta-1,4 linked-D-galacturonic acid units, is its degree of methylesterification and acetylesterification. The degree of polymerization (DP) of HG is subsequently temporally and spatially modulated *in muro* by pectin-degrading enzymes such as PolyGalacturonases (Endo-PGs, EC 3.2.1.15 and Exo-PGs, EC 3.2.1.67) that finally generate oligogalacturonides (OGs) and result in cell wall loosening. Among PGs, QUARTET2 (QRT2), ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1 and 2 (ADPG1 and ADPG2) were shown to play a role in organ dehiscence, while QRT3 was involved in the degradation of HG in pollen tetrad (Ogawa et al., 2009; Rhee et al., 2003). This shows that PGs play a central role in plant reproductive development. In parallel, PGX1 (POLYGALACTURONASE INVOLVED IN EXPANSION 1) was described to be involved in dark-grown hypocotyl elongation (Xiao et al., 2014). More recently, PGX2 was shown to play a role in cell expansion and to influence secondary cell wall formation (Xiao et al., 2017), and PGX3 was presented as a major actor in stomata formation and dynamics (Rui et al., 2017).

In plants, proteins secreted in apoplast through the secretory pathway, including PGs, need to be oxidised to reach their stable and active forms. The regulation of PG activity through their redox status would require changes in cellular redox conditions, referring to the balance between oxidized and reduced forms of molecules in the cell. Polygalacturonases often contain redox-active cysteine residues in their structure, and the thiol groups in these cysteines can undergo reversible oxidation-reduction reactions, influencing the enzyme's conformation and activity. The first two structures of Arabidopsis PGs, PGLR and ADPG2 have 15 and 13 cysteine residues respectively, twelve of which are present in both enzymes and can form disulphide bridges. These disulphide bridges contribute to the enzyme's stability as they are positioned at the N- and C-terminal ends and protect the hydrophobic core. These structural data led us to explore the possible contribution of redox regulation of disulphide bridges in PG structure and how this could contribute to plant development.

Our hypothesis is that the high number of cysteines and disulphide bonds among plant polygalacturonases play an important role in the regulation of enzymes' conformation and activity. This would mean that these cysteines can undergo, through interactions with putative cell wall-localized thioredoxins (TRXs), glutaredoxins (GRXs) and gamma-interferon-inducible lysosomal thiol reductase (GILT), reversible oxidation-reduction reactions in specific cell wall microenvironments, at acidic pH.

FLARE is a collaborative project between two research groups; BioEcoAgro from University of Picardie Jules Verne (UPJV) and Nara Institute of Science and Technology (**NAIST, Japan**) with highly complementary expertise ranging from protein production and enzymes' biochemistry (UPJV), structural biology (UPJV), proteomics and in planta studies (NAIST). This is determinant to understand how plant cell wall oxidoreductases could modulate polygalacturonase activity.

FLARE has been divided in 3 scientific work packages.

WP1. Sampling the oxidoreductase diversity in plant cell wall

In this WP, we will identify and characterize Arabidopsis cell-wall related oxidoreductases.

Arabidopsis GILT, TRX and GRX oxidoreductase sequences will be retrieved from Uniport and NCBI databases, aligned and compared. UPJV will perform phylogeny and structural modelling to provide proteins classification. Sequence and structural superimposition with known protein will lead to uncovering active site specificities. Based on the uncovered sequences, NAIST will perform the proteome analysis of plant cell walls (roots and leaves). Cell wall proteins account for approximately 10% of the plant cell wall composition but previous analyses mainly targeted structural proteins. In contrast while some GILT, TRX and GRX proteins contain signal peptides, secretions signal for the apoplast, little is known about their presence and relative abundance. Sequence/structure and proteomic analyses will allow identification of the most prominent candidates for further analyses.
To assess the activity of GILT, TRX and GRX that are present in the cell wall the proteins will be produced in *Pichia pastoris*, purified and biochemically characterized. The need for co-factors will be assessed. This will drastically increase the number of characterized oxidoreductases whose role in the regulation of PG activity will be determined.
In a second approach, characterized oxidoreductases will be crystallized and submitted to experimental 3D structure determination using synchrotron facilities. Up to date no structures from the GILT family are present in PDB.

WP2. Redox control of endo-polygalacturonase activity

Considering the high number of cysteins present in the structures of plant PG, that can potentially engage disulphide bridges, this WP will explore this potential novel, and unique, way of regulating PG activity.

We will used site-directed mutagenesis of specific cysteines in ADPG2 and PGLR to formally demonstrate their roles in the control of PG activity (preliminary results available). For this purpose, mutated versions of the proteins will be produced in *Pichia pastoris*, purified and biochemically characterized. To determine that, albeit the mutations, the fold is conserved, proteins will be subjected to circular dichroism as well as to protein crystallization and crystal X-ray diffraction.
The processivity PGs cysteine mutants will be further determined using LC-MS/MS oligo profiling approach to finely map the diversity of hydrolysis products from either commercially available substrates or cell walls from Arabidopsis. In parallel, kinetic parameters, including Kon/Koff/Km/Vmax/Kd for each of the enzyme will be determined.
The direct interaction of wild-type and mutated forms of ADPG2 and PGLR with oxidoreductases produced in WP1 will be determined using MicroScale Thermophoresis (MST). Proteins that are in interaction show a different signal which will allow calculating their interaction strength. Moreover, the effects of interaction on protein integrity and folding will be determined using Differential Scanning Fluorimetry (DSF) analysis and Circular Dichroism (CD). To relate the redox-mediated changes in PG activity to potential differences in the dynamical behavior of the proteins in complex with substrates, MD simulations will be ran using either wild-type or mutated versions of the proteins.

WP3. From in-vitro to in vivo: roles of cell wall oxidoreductase in the control of plant development

From bioinformatic, proteomics and biochemistry results gathered in WP1-WP2, WP3 aims at understanding the role of selected TRX, GRX and GILT on plant development. This WP will highlight the contribution of oxidoreductases in regulating the fine-tuning of pectins and the consequent effects on cell wall structure, cell wall mechanics and plant development.

We will determine the subcellular localization of selected TRX, GRX and GILT and their interaction with PG in vivo at the cell wall. For this purpose, we will express proteins under the control of the CaMV35S promoter with oxidoreductases being fused to monomeric red fluorescent protein (mRFP) while PG being fused to enhanced green fluorescent protein (EGFP). Following transient transformation of *N. benthamiana* leaves or stable transformation of *Arabidopsis thaliana*, we will determine the localization of proteins using confocal microscopy. In parallel, Fluorescence cross-correlation spectroscopy (FCCS) will be used to determine the oxidoreductase-PG interaction in vivo.
The influence of the oxidoreductase on the plant cell wall structure will be assessed by generating overexpressing or mutant lines for the selected GRX, TRX and GILT. Following their genotyping, plants will be characterized for defects in plant development (root and hypocotyl length, plant size…) as well as changes in cell wall structure. Cell walls from the different lines will be characterized using analytical chemistry and well as immunocytochemistry (specific antibodies for pectic domains). At NAIST, the use of Atomic force Microscopy (AFM) will relate the changes in cell wall chemistry to the changes in cell wall mechanics. The effects of the changes in expression of oxidoreductases on PG activity will be determined using spectrophotometric assay as well as profiling of endogenous OG. Using overexpressing a knock-out lines for oxidoreductases and thanks to NAIST, we will determine the oxidation states of proteins at the cell wall. Using a thiol labelling reagent like isotope coded affinity tag (ICAT) and mass spectrometry for quantification. The ICAT method reveals cysteines that can be oxidised. The ICAT reagents consist of isotopically labelled linker containing light or heavy isotopes and a biotin group which allows affinity purification of proteins. Cell wall extracts from a control and from Arabidopsis mutants/overexpressing lines will be used and following digestion of proteins by trypsin, peptides will be analysed by mass spectrometry to determine which cysteines are susceptible to oxidation and form disulphides bonds. This approach will help identifying the specific cysteine in proteins that may be involved in redox regulation.

Mots clés / Key-words (5) : Oxidoreductase, Polygalacturonases, Regulation, Reduction, Interaction

Profil du candidat / Profile of the candidate :

We are looking for an enthusiast candidate with a master 2 in plant biology or biochemistry or related fields. The PhD student will be trained by staff in scientific approaches as diverse as molecular biology (nucleic acid extractions, PCR, qRT-PCR, molecular cloning, microbiology, plant transformation), structural biology, biochemistry (protein extraction, enzymology), analytical chemistry (immunolabeling, chromatography, proteomics, mass spectrometry) and confocal microscopy.

FLARE is a collaborative project between UPJV (France) and NAIST (Japan), and the PhD student is expected to travel for some training period in the laboratory of Prof Taku Demura (Nara, Japan).

Composition du dossier de candidature / To candidate

1- CV + lettre de motivation/ CV + Motivation letter
2- Relevés de notes des 3 dernières années d'études dont l'année de L3 ou équivalent / Grades for the last three years, including BSc
3- Diplômes de la Licence & du Master ou équivalents / BSc and Master diploma
   Les étudiants n'ayant pas encore validé leur M2 devront fournir / For those who do not have yet the Master:
   - *Relevé de notes du premier semestre du M2 / Grades from 1st semester of Master 2*
   - *Lettre d'appréciation du responsable de stage / Recommendation letter of person in charge of training period during the Master.*
4- Lettre d'appréciation du responsable de CV la formation précisant le classement du candidat et l'effectif de la promotion / Recommandation letter from the person in charge of formation indicating grade and ranking.
5- Lettres de recommandation / Recommandation letter

Please send the documents as a single pdf file to: jerome.pelloux@u-picardie.fr and josip.safran@u-picardie.fr before the 20th May 2024.