SUMMARY
Laccases (LAC) are multi-copper containing oxydases distributed in plants, fungi, bacteria and insects that catalyses the oxidation of a wide range of different compounds. Well characterized in fungi for their role in lignin-degradation, a little is know on their role in plants.
Three laccases found in Arabidopsis thaliana have been characterized, and are involved in lignin polymerization in the stem (LAC4 and LAC17) and flavonoids oxidation in the seed coat (LAC15). Specific sub cellular localization of the proteins and specific substrate oxidation in these tissues ask the question of a specificity of the enzymes for their substrate. Enzymes specificity will also be assayed using a fungal laccase from Trametes versicolor, to check for a plant / fungi specificity.
In vivo and in vitro analysis of these four laccases will be performed to understand the factors that could lead the proteins activity either for polymerization or degradation of lignin.

INTRODUCTION
Plant cell wall is surrounded by a lignocellulosic cell wall constituted of polysaccharides, cellulose 45 % and hemicelluloses 30 % and of lignin, a phenolic polymer accounting for 20 % of the biomass. Lignins impart strength to cell walls, facilitate water transport, and impede the degradation of cell wall polysaccharides.

Lignins are hetero-polymers constituted of three monolignols, p-coumaryl alcool, sinapyl alcool and coniferyl alcool that once incorporate in the cell wall will be called H, G ans S
respectively\(^1\). Monolignols are oxidized in the cell wall by two types of enzymes peroxidases and laccases. However, these enzymes being part of multigenic families (73 and 17 respectively identified in *Arabidopsis thaliana*) it is challenging to identify the one specifically involved in lignin biosynthesis.

In *Arabidopsis thaliana* 8 of the 17 laccases are mostly expressed in the stem. Studies on mutants in which the genes expression is highly reduced or totally knockout, showed that the absence of two laccases LAC4 and LAC17, causes a reduction of total lignin content in stems. For these mutants, lignin content is reduced by 8-14 % compared to a wild type plant and the double mutant *lac4g lac17* has even much lower lignin levels, 40 % less than the control\(^2\).

Analyses of gene expression localize LAC4 in the fibers and vascular bundles, and LAC17 in fibers only. This tissue specific gene expression, as well as the presence of the G and S monolignols in the fibers and of S only in the vascular bundles, asks the question of substrate specificity for the enzymes.

The opposite activity of structurally close laccases, i.e. LAC4, LAC17, and *Trametes versicolor*, on lignin, also asks the question of specific chemical mechanisms for the oxidation of monolignols and/or lignin by plant or fungi enzymes.

Purification of heterologously produced proteins will allow in vitro experiments on model compounds by checking the ability of an enzyme to oxidize the monolignols. It will enable us to understand the specific features leading the oxidative process to either polymerization of monolignols or degradation of lignin.

The results of these in vitro experiments will be associated to in vivo complementation experiments of *Arabidopsis* mutants by LAC4, LAC17 and LAC15 genes and lignin analysis.

**RESULTS**

**Mutant plants complementation: in vivo analysis of proteins**

Mutants lacking one or two of the laccases (simple or double mutant) will be complemented by introducing one of the laccase gene to see if the wild type phenotype can be recovered.

The results obtained so far for the double mutant (*lac4 lac17*) complementation with each of the 3 laccases, did not give much information on specificity and enzymatic properties of the proteins. Simple mutant complementation is in course, and might give more information on lignin specificity.
Expression and purification of the proteins

For the in vitro experiments, we transiently over expressed the plant laccases genes in tobacco leaves, to obtain a high-amount of proteins for purification, structural analysis and in vitro experiments. So far, several strategies have been investigated with different tags in order to test diverse purification methods to produce active enzymes: tags FLAG and GFP\(^3\) for affinity chromatography and hydrophobin for liquid-liquid extraction\(^4\).

An alternative strategy has also been developed using seeds of mutants Arabidopsis thaliana as an enzymatic tool. Indeed LAC15 is the only laccase expressed in seeds and LAC15 deficient mutant can be complemented by LAC4 or LAC17 genes and produce these enzymes in the seeds, themselves being used in in vitro experiments without protein purification.

Substrate disappearance kinetics: in vitro analysis of proteins

Substrates and enzymes are stirred together in a buffered solution at pH 6 for plant laccases or 4.2 for Trametes laccase. Disappearance of the substrate is monitored through HPLC quantification and the products of the reactions are analyzed through LC/MS and, if possible, quantified by LC analysis. For the moment 4 substrates have been tested (see below) and 2 enzymes LAC15 (expressed in seeds) and Trametes laccase. Control experiments have been performed to check substrate stability in the conditions of the reaction.

In our preliminary studies, we found no differences between the qualitative results of the oxidation of monomers by both laccases. Indeed, we observed the formation of several oligomers up to a polymerization degree (PD) of 8, structure of which has not been further investigated. On the other hand, while also producing oligomers, the oxidation of the β-5 dimer by both enzymes, leads to the formation of the G monomer and degradation products such as vanillin and valinic acid. These results show that both plant and fungal laccases can either polymerize phenolic compounds or induce the degradation and/or the recondensation of oligomers in in vitro experiments.
CONCLUSIONS

We were able to have the first comparative study on plant/fungi behavior on model compound. This was possible with the use of a commercial fungal enzyme and *Arabidopsis thaliana* seeds expressing the laccase 15.

*In vivo* plants and fungi are known to have opposite activities. Fungi degrade lignins when plants polymerize it. But *in vitro* both plants and fungi laccases exhibit the same enzymatic activities. From a single monolignol or from a dimer, a whole range of compounds can be recovered, going from the monomer to the 8 units species. Experiments will be repeated with the laccases 4 and 17 as well indicating if there is differences for the plant proteins.

This absence of difference *in vitro* needs to be further combined with *in vivo* analysis of mutant plants complemented with the different enzymes. This results prompt us to undergo further *in vitro* studies in order to determine the factors that will selectively drive the oxidative process to either oligomerization or degradation. This will enable us to understand the polymerization in planta as well as the degradation of lignin by fungi, and to finally propose some new catalytic methodology for lignocellulosic biomass deconstruction for second generation biofuel production.

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