Wioleta Wojtasik

THE EVALUATION OF THE SIGNIFICANCE OF CELL WALL POLYMERS IN FLAX RESPONSE TO FUSARIOSIS

ABSTRACT

Having been priced for centuries as a source of valuable raw materials, flax (*Linum usitatissimum*) thanks to its multipurpose applications, nowadays, is perceived as a non-waste plant. Residues after pressing the oil (the seedcake) and extracting the fiber (the shives) are both unappreciated materials containing important compounds which have antioxidant, antibacterial, antifungal and anticancer properties. The factors that limit flax growth and development are connected with the climate conditions, however, the pathogen infections, especially with fungi from genus *Fusarium* are responsible for the biggest losses in flax crop yield. The most dangerous flax pathogens are: *Fusarium oxysporum* whose infection leads to *Fusarium*-derived leaves wilting, and *Fusarium culmorum* which causes *Fusarium* rot base of the shoots. Both types of *Fusarium* reduce the crop yield and the quality of raw materials derived from flax.

The cell wall is the first, mechanical barrier against pathogen infection. The pathogens' attack starts when they secrete enzymes degrading the host's cell wall. At the first stage, by decomposing pectin with pectinases, the cell wall structure loosens and other cell wall components are exposed to degradation by fungal celullases and hemicellulases. As a result of polygalacturonases, belonging tofungal pectinases, action oligogalactouronides (OG) are released. OGs are short pectin fragments, which acting as the elicitors, activate the plant defense mechanisms. Through the signal pathways OGs induce the expression of genes involved in the pathogenesis and other genes related to the metabolic and systemic response.

The main aim of the thesis was to evaluate the role of the cell wall polymers in the flax plant response to the infection with *Fusarium oxysporum* and *Fusarium culmorum*. For this purpose, the flax seedlings were incubated with fungal strains from genus *Fusarium*, and theplant tissue was collected duringthe progress of the infection (in 6th, 12nd, 24th, 36th and 48th hour after the infection beginning). The next step was to investigate changes in the expression of genes involved in the cell wall polymers metabolism using real time PCR technique. These analyzes have to be preceded by the identification and verification of the exact, flax mRNA sequences of tested genes. Due to the lack in the database of the flax mRNA sequence of the majority of investigated genes as well as the lack of flax genome the cDNA library was used to isolate particular genes using degenerate primers. These primers were designed for the most homologous mRNA fragments from other plants. Successfully, a few dozens of partial sequences of PR genes and genes involved inthe cell wall polymer metabolismwere identified, and later they were confirmed after the publication of the flax genome sequence. Particular attention was paid to pecitn, constituting the first target of pathogenic enzymes.Other polymers like cellulose, hemicellulose and lignin were also investigated to fully understand the defense mechanism of plants. Additionally, polyamines were taken into consideration, in particular those linked to the cell wall, as it is known from the literature that they are involved in the plant response to the biotic stress. In order to accurately determine the molecular background of successive stages of plant-pathogen interactions in flax and to correlate them with the changes in cell wall polymers, genes related to pathogenesis (β-1,3-glucanase and chitinase) were examined.

The analysis of the expression of genes involved in the metabolism of the cell wall polymers in flax after infection with *F. oxysporum* and *F. culmorum* allowed selecting two groups of genes which differently responded to the infection. The first group was represented by genes whose expression was strongly affected by the infection; five to ten fold increase was observed. These genes belonged to the systemic response mechanisms, as despite strong stimulation by the infection, they answered later (at least 12 hours after incubation with pathogens), and the highest increase in the expression was in 48^{th} hour.

After *F.culmorum* infection in flax, the genes that showed the highest expression level were genes involved in lignin synthesis (phenylalanine ammonia-lyase; PAL, glycosyltransferase; GT, sinapyl alcohol dehydrogenase; SAD, cinnamyl alcohol dehydrogenase; CAD and hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; HCT), polyamine synthesis (arginine decarboxylase; ADC and ornithine decarboxylase; ODC) and chitinase gene. Similar results were observed after infection with *F. culmorum*, where the first group was represented by the genes involved in lignin synthesis (phenylalanine ammonia-lyase; PAL and glycosyltransferase; GT) and genes related to pathogenesis (β-1,3-glucanase and chitinase). To the second group belong genes which are induced only in slight amount. They participate in a metabolic response whose activation is dependant on elicitors (oligosaccharides) releasement as a result of cell wall degradation by the fungal enzymes. The gene expression in this group varies, form slight increase in the transcript amount (up to 5 times) to measurable decrease, and often the changes in the gene expression are non-systemic. The first subgroup, after infection with both fungi strains, gather genes from lignin biosynthesis, and also β-glycosidase (GLS) gene and β-1,3-glucanase 2. Additionally, after *Fusarium culmorum* infection increase in the expression of cellulose 2 and β-1,3-glucanase 1 was noticed. The second subgroup is represented by genes with the reduced expression. Here belongs genes from pectin metabolism (galacturonosyltransferase 1; GAUT1, galacturonosyltransferase 7; GAUT7, rhamnogalacturonan xylosyltransferase; RGXT, pectin methyltransferase; PMT, pectin methylesterase 3; PME3, polygalacturonase; PG and pectin lyase; PaL), hemicellulose metabolism (glucomannan 4-β-mannosyltransferase; GMT, galactomannan galactosyltransferase; GGT, xyloglucan xylosyltransferases; XXT, xylanase; XYN, endo--1,4-xylanase; XYLb and α-galactosidase; GS) and cellulose synthesis (cellulose synthases: CLS1, CSL2 and CSL4). The third and the last subgroup gather remaining genes from pectin, hemicellulose and cellulose metabolism, whose expression changes independently. There are speculations that genes weekly responding to the pathogen infection are only transcription noises and they do not participate in the plant's defense mechanisms.

The next step was to verify how the expression of genes involved in the polymers metabolism correlate with the polymers amount. Using spectrophotometric methods the levels of pectin, lignin, cellulose and hemicelluloses were determined, whereas UPLC was used to analyze polyamine amount. Obtained results showed that the concentration of particular polymers did not change significantly, and the only differences were noticed after 48 hours of incubation with pathogens. However, resulting from the analysis of particular pectin and hemicelluloses fractions changes in the arrangement of the cell wall were observed. These results were confirmed using infra-red spectroscopy, which additionally revealed some changes in the structure of cellulose. The most significant changes were observed for polyamine fraction which is connected to the cell wall. In particular, spermidyne was highly accumulated. The results from this part of the thesis suggest that the role of the cell wall polymers in the plant response to the fusariose is manifested through the changes in their genes' expression rather than changes in the polymers amount.

Next part of the thesis was to evaluate the importance of the cell wall polymers in the plant's response to the pathogenic infection through the analysis of the transgenic flax with the

overexpression of the β-1,3-glucanase (flax type B). Due to the high response of genes involved in the flax pathogenesis induced *Fusarium* infection, it was advisable to evaluate if the induced increased activity of PR genes (here β-1,3-glucanase and chitinase) has a direct impact on the activation of genes form cell wall polymers metabolism and whether there are different that through PR genes pathways that induce such changes. For this purpose, the expression of these genes together with their metabolites content in a transgenic flax type B (lines B10, B11 and B14) were determined. The most significant changes in the level of genes expression were observed for B14, in which the genes had demonstrated induction of both systemic and metabolic response.

Despite smaller changes in the gene expression in flax type B than in the flax after infection with *F. oxysporum* and *F. culmorum*, some similarities as well as some differencies were observed. The analysis of the polymer amounts revealed changes in the rearrangement of the cell wall despite almost unchanged quantity of polymers. In addition, the flax type B showed increased content of polyamines associated with the cell wall, despite the lack of measurable differences in their total amounts. The results obtained in this section showed that the increase in expression of β-1,3-glucanase affects the genes expression of the cell wall polymers. However, due to the differences in the levels of genes expression and different patterns of the rearrangement of polymers in flax infected with pathogenic *F. oxysporum* and *F. culmorum* strains and in the transgenic flax type B, it might be concluded that during the pathogen infection, genes involved in the metabolism of the cell wall polymers are activated not only by the pathways dependent on PR genes, but also by other signal routes.

The last part of the thesis has partially application nature. Its purpose was to assess the quality of the fibers obtained from transgenic flax-type B. B14 line was chosen for field trial and fiber analysis because this line was characterized by the best productivity due to the largest seed production compared with lines B10 and B11.

Flax overexpressing potato β-1,3-glucanase was characterized by increased expression of endogenous β-1,3-glucanase and chitinase and an increased resistance to *Fusarium culmorum* and *Fusarium oxysporum*. Mechanical and biochemical analysis of flax had to answer the question whether the changes caused by the introduction of exogenous β-1,3-glucanase gene into flax genome had an influence on the crop yield and affected fibers and shives composition. Shives are by-product remaining after the separation of fibers from flax straw.

The fourth generation of transgenic flax from B-type was grown on an experimental plot. After reaching the maturity state, the plants were harvested, seeds were separated from the straw, which was then subjected to a dew retting. The next step was to mechanically extract the fibers from retted flax straw and carry out experiments determining the mechanical properties of fibers. Furthermore, in obtained flax fibers and shives from B14 line exact composition of the particular cell wall polymers: pectin, hemicellulose, cellulose, lignin also callose and attached to these components phenolic compounds were checked. Due to the possibility of using flax products as raw materials having high antioxidant capacity, the antioxidant potential of the extracts isolated from whole raw products (fibers, shives) was estimated as well as from individual cell wall fraction to determine the exact source of these constituents.

Flax fibers derived from flax overexpressing β-1,3-glucanase were characterized by the modified composition of the cell wall polymers. The levels of the following polysaccharides: cellulose, hemicellulose and pectin were higher, while the lignin content was reduced. Also decline in callose was observed, the substrate for β-1,3-glucanase, which could suggest that callose released from

glucose molecules are consumed in the production of other polysaccharides. The increase in the monosaccharides in hemicellulose fraction translates to the accumulation of phenolic compounds in this fraction, and further raised antioxidant potential. To sum up, fibers obtained from transgenic flax B14 had higher antioxidant potential in comparison with the control fibers, indicating the possibility of their use as biomedical feedstock. Furthermore, for the first time it was shown that β-1,3-glucanase affects the metabolism of cell wall polymers, and its overproduction in flax leads to the improved fiber quality.

Analysis of flax shives revealed changes in the composition of the cell wall polymers: an increase in content of pectin, hemicellulose and lignin, and decrease in callolse level. Moreover, by-products derived from the transgenic flax B14 were characterized by the changed structure of cell wall polymers and reduced level of phenolic compounds associated with the wall, and thus lower antioxidant potential of pectin and hemicellulose fractions. However, despite the reduced antioxidant capacity as compared to the control, shives as unexploited by-products are a valuable source of phenolic compounds, which are accumulated in much higher amount in the shives than in the flax fibers. In conclusion, from the transgenic flax lines B14 better quality fiber was obteinned but shives had slightly worse biochemical parameters, however, still being a rich source of the antioxidant compounds.