ABSTRACT

Transgenic flax as a source of fiber with improved properties. Manipulation of the cell wall polymers metabolism in flax fiber.

Flax (*Linum usitatissimum* L.) is the most commonly cultivated fibrous plant in our climate zone. Flax fiber has high water absorption, mechanical strength and it contains a variety of the antioxidant compounds. It is composed of four main polymers: cellulose, hemicellulose, pectin, lignin, and a number of secondary metabolites, proteins, waxes and inorganic compounds. What determines the properties of flax is precisely its composition. Lignin provides strength and hydrophobicity to the cell wall, also creates a mechanical barrier to pathogens, however, it causes substantial rigidity and complicates the use of flax fiber as a raw material in various industries. Pectin is involved in the cell adhesion and plant response to pathogen attack, whereas during extracting the fiber from flax straw (retting), pectin is degraded by microorganisms, which determines retting duration and fiber quality. Difficulties associated with growing flax and obtaining fiber (susceptibility to infection, the risk of crop loss or deterioration of fiber quality when retting is performed incorrectly) and some of its characteristics (stiffness, lignification) cause permanent loss of interest in the cultivation of flax.

Aiming to obtain new types of flax with improved properties, plants with reduced lignin or pectin level were generated. The purpose of all modification was to improve the quality of flax fiber and to diversify of its applications by manipulating the amount/composition of the cell wall polymers. A number of the literature data and preliminary laboratory tests have confirmed that the cinnamyl alcohol dehydrogenase (CAD) is the key enzyme in the lignin biosynthesis pathway and lowering its activity is a good tool to reduce the level of lignin in the cell wall. The transgenic flax plants with repressed *CAD* gene were not only characterized by decreased lignin level, but also by changed cell wall composition, and consequently altered mechanical properties. In contrast, the polysaccharide component of flax cell wall was changed by two separate modifications based on the same principle - expression of exogenous fungal enzyme degrading pectin. Using the molecular biology tools, the cDNA encoding for polygalacturonase I (PGI) from *Aspergillus aculeatus* or cDNA encoding for rhamnogalacturonate lyase A (RHA) from *A. aculeatus* was introduced into the flax genome. The available data indicates that these enzymes effectively degrade pectin and are effective tools to manipulate the amount of pectin. The resulting plants were characterized by a reduction in the content of pectin and shortened retting duration in the laboratory tests. The obtained data showed the effectiveness of the modifications, which allows expecting that the composition of flax fiber would also change and would result in improved properties of flax fiber. Therefore, the main aim of this study was to evaluate the effect of the modifications and, consequently, reduction of the pectin or lignin level on the composition, structure and properties of the cell wall of the flax fiber. Furthermore, it was examined how the introduced modification affected the expression of genes involved in the metabolism of the individual cell wall polymers.

The first stage of the study was to analyze the expression of selected genes of the cell wall metabolism and genes involved in the pathogenesis in *in vitro* cultured plants. In particular, the expression of selected genes encoding the synthesis and/or degradation and rearrangement of cellulose, lignin, pectin and hemicelluloses was studied, as well as the expression of the pathogenesis-related genes and the cell wall polymers biosynthesis regulatory genes. Realizing this

task, the sinapyl alcohol dehydrogenase (SAD) gene was identified in the flax genome. Its phylogenetic analysis revealed that flax SAD belongs to the Class II of the plant alcohol dehydrogenases' family. In addition, it was observed that SAD is not necessary to maintain the proper level of lignin in flax. Moreover, several genes involved in the biosynthesis of lignin (*HCT*, *C3H*, *C4H*), metabolism of sucrose (*SUS*, *SPS*, *SPP*) and regulators of the cell wall synthesis (*WAK*, *THE*, *FEI*, *PERK1*, *PERK2*, *COBRA1*, *COBRA2*) were identified and isolated from flax genome.

It was observed, that *CAD* gene repression affected the expression of number of genes involved in the cell wall metabolism. However, primarily, it neither caused the down-regulation of any gene from the lignin biosynthesis pathway, nor it was compensated by an increase in the *SAD* expression. In addition, various changes were observed in the expression of genes associated with the methylation of pectin. An increase in the mRNA level of genes responsible for the degradation of pectin and hemicelluloses (*PL*, *PTL*, *XYLa*, *GS*, *MS*, *GLS*) was also noticed. Moreover, the reduction in the expression *β-1,3-glucanase* gene was observed. Although the molecular mechanism linking the lower amount of lignin and changes in the expression of pathogenesis-related genes remains unclear pectin methylesterases were pointed out as a possible connection.

The introduction of exogenous polygalacturonase into flax caused an increase in the expression of endogenous *polygalacturonase* and *pectic lyase*. The observed changes in the expression of synthesis and degradation genes compensate for each other, which indicate that the decrease in the total pectin amount is an effect of the introduced modification. Among the other analyzed genes the attention shall be given to the increased expression of *pectin methylesterase 1* and *β-1,3-gucanase*, genes involved in lignin (*PAL*, *C4H*, *4CL*) and cellulose (*CesA3*, *CesA4*, *CesA5*) biosynthesis, and also genes involved in sucrose metabolism (*SPP* i *SPS*) and hemicellulose degradation (*XYN*, *GS*).

Although RHA7 plants also have reduced pectin level, similarly as PGI11 plants, the introduced enzyme has different activity, which caused different that in PGI11 expression of genes involved in the metabolism of the cell wall polymers. In plants form RHA7 line, the expression of *pectic lyase* was reduced, which leads to the conclusion that the reduction in the total pectin level is caused exclusively by the introduced modification. Moreover, various changes in the expression of genes responsible for pectin demethylation were observed. It was also revealed, that the expression of several genes involved in lignin (*C4H*, *CCR*) and cellulose (*CesA2*, *CesA4*) biosynthesis, and hemicellulose degradation or rearrangement (*GMT*, *XXT*, *XYLa*, *GS*, *GLS*) has decreased. In contrast, the increase in the level of *β-1,3-gucanase* mRNA was observed.

The second stage of the study involved the cultivation of transgenic lines in the field at laboratory scale in order to obtain the fiber and the phenotypic analysis of transgenic plants growth *in vivo*. CAD27 flax plants were visually indistinguishable from the control in the field trial. Careful analysis showed that they were characterized by the delayed and/or reduced lignification of the fiber elementary cells and by the changes in the anatomical structure of the stem (larger diameter of fiber elementary cells and thinner thickness of the fiber bundle). There were no differences in the phenotypic parameters (height, weight of seeds, and number of seeds in the capsule); however, lowered crop yield was observed (both seeds and fibers). The verification of the resistance to the *Fusarium* infection showed that plants form CAD27 line were more susceptible to *F. oxysporum* infection, but showed greater resistance to the attack by *F. culmorum*. For CAD27 straw, no apparent shortening of retting duration was observed, although it was unified and effective from the very first day of retting. For both lines with the reduced amount of pectin (PGI11 and RHA7) *in vivo* retting

tests confirmed the results obtained *in vitro*, the significant reduction in the retting duration and its greater efficiency. Moreover, in the field trial, there were no changes in the phenotypic characteristics and yielding of straw and fiber. However, a reduction in the seed yield for PGI11 plants and increase for RHA7 plant was observed. Furthermore, these two lines were characterized by an increased resistance to the infection by both *F. oxysporum*, and *F. culmorum* strains. These results indicate that it is possible to obtain a normal phenotype of plants with reduced content of pectin/lignin and their field growth, which does not agriculturally differ from the control plants.

The third stage of the study was to analyze the composition and structure of fiber from three transgenic flax lines, namely CAD27, PGI11 and RHA7. The fiber form pants with repressed *CAD* gene was characterized by reduced lignin content, increased content of cellulose and hemicelluloses, with unchanged pectin content. The analysis of the phenylpropanoid pathway metabolites showed accumulation of vanillin and vitexin, which resulted in increased antioxidant potential of the extract from the fibers. Furthermore, the accumulation of chlorophyll B and sterols: campesterol, stigmasterol and β-sitosterol was shown. In PGI11 fiber the decline in total pectin content was observed and it was associated with the decrease in the content of sugars from water soluble fraction (mainly the decrease in mannose content), with a slightly higher content of uronic acids. The measured reduction in hemicellulose content was due to the decrease in the content of sugars (glucose and partially ribose), although partially it was compensated by an increase in the uronic acid, which however, has changed from galacturonic to glucuronic acid. The analyses of the other components of the cell wall showed no significant changes in the lignin content, while there was an increase in the cellulose amount. The quantities of all identified phenylpropanoid compounds associated with the cell wall were decreased, except syringic aldehyde, whose amount increased in relation to the control. In contrast, there was an increase of chlorophyll B level, as well as a significant accumulation of sterols (campesterol, stigmasterol and β-sitosterol). The fiber from plants with introduced RHA gene was characterized by a marked reduction in the content of pectin and hemicelluloses. The decrease of the total pectin content was associated with a reduction in the concentration of sugars (mainly mannose) in the water soluble fraction of pectin, at the same time uronic acid content slightly increased. In contrast, the reduction of the amount of hemicellulose is associated mainly with a significant decrease in the content of uronic acids, while the overall level of sugars did not change significantly. The analyses of the other cell wall polymers in RHA7 fibers revealed a slight increase in the concentration of cellulose and no change in the content of lignin. In contrast to PGI11 fibers, the RHA7 fibers showed significant accumulation of the phenylpropanoid compounds. An increase in the level of lutein, chlorophyll b, CBD and sterols was also observed

Infra-red spectroscopic analysis of transgenic fibers showed changes in the chemical interaction and spatial organization of the cell wall polymers. The fiber from CAD27, PGI11 and RHA7 lines was characterized by the lower number of hydroxyl groups involved in the hydrogen bonds and by the reduced crystallinity index (the strongest for RHA7 fibers). The shortening of microfibrils length and rearrangement of pyranoid rings was also observed. The strength of the measured changes follows the tendency $I_{NIKE} < I_{CAD2\,7} < I_{PGI11} < I_{RHA7}$.

Owing to the disclosed changes in the amount of modified polymers and overall cell wall composition throughout the last stage of the study the impact of modifications and changes caused by it on the properties of flax fiber was assess. Three potential applications of flax fiber were taken into account:

one in the textile industry (linen yarn/fabric) and two biomedical: as a dressing material or as a drugs carrier and stabilizer. For this purpose the three aspects of the obtained fibers were analyzed: mechanical properties, the impact of the extract from fibers containing phenylpropanoid compounds on the growth of human pathogenic bacteria, and the ability to bind drugs. Mechanical tests of the low-lignin fiber showed an increase in the tensile strength and stiffness. The phenylpropanoid extract from the CAD27 fiber suppressed the growth of Gram negative bacteria *E. coli* and visibly retarded the growth of the *P. aeruginosa* strain. Moreover, the binding experiment showed high ruitn binding capacity from the fiber from CAD27 line, far greater than the control fiber. The research determining the properties of the two types of the low-pectin fibers (PGI11 i RHA7) showed similar characteristic for both lines. The significant increase in the tensile strength and binding capacity was observed. The fiber from the PGI11 line better than RHA7 bound rutin, whereas the fiber RHA7 had a much better tensile strength than PGI11 fiber. Moreover, the phenylpropanoid extract from the PGI11 fiber had the slightest impact on bacterial growth, however, it exclusively delayed the growth of *E. hirae*. In contrast, the extract from the RHA7 fiber had the best inhibitory effect on the growth of *S. aureus* and *P. aeruginosa*.

In summary, in the case of the CAD27 fibers, although despite the reduction of the lignin amount the increase in the flexibility was not obtained, the low-lignin fiber has improved tensile strength and other tested properties. Reducing the amount of pectin (PGI11 lines and RHA7) resulted in a shorter and more efficient retting, and it has a positive impact on the composition and properties of flax fiber. The comparison of the studied transgenic plant lines proved that the most effective modification was the introduction to the plant genome gene encoding for RHA. The plants and fiber from RHA7 line were characterized by increased seed yield and resistance against infections, while changes in the cell wall of flax fiber correlated with the significant improvement in the fiber properties.

In this study it was showed that the cell wall is a dynamic and interactive structure, where changes in the amount of one component alter the amount and spatial organization of other components. This implies alteration in a genetic network related to the cell wall. The changes in the cell wall structure (induced, caused by environmental or developmental factors), and therefore its mechanical properties are the signal transduced to genes, which stimulates the compensatory mechanism to maintain the correct functionality of the cell wall. This, in turn, leads to the remodeling of the cell wall structure. Moreover, the signal coming from the cell wall alters also the expression of other genes, eg pathogen-related genes, which correlates with the changes in the susceptibility to the pathogen infections. It was showed that reducing lignin or pectin level in the cell wall was compensated by the increased cellulose level and changes in its spatial organization, as cellulose is the main component responsible for the strength.