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Generation of transgenic wheat (*Triticum aestivum* L.) accumulating heterologous endo-xylanase or ferulic acid esterase in the endosperm

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SUMMARY

Endo-xylanase (from *Bacillus subtilis*) or ferulic acid esterase (from *Aspergillus niger*) were expressed in wheat under the control of the endosperm specific *1DX5* glutenin promoter. Constructs both with and without the endoplasmic reticulum retention signal KDEL were used. Transgenic plants were recovered in all four cases but no qualitative differences could be observed whether KDEL was added or not. Endo-xylanase activity in transgenic grains was increased between two and three fold relative to wild type. The grains were shriveled and had a 25-33% decrease in mass. Extensive analysis of the cell walls showed a 10-15% increase in arabinose to xylose ratio, a 50% increase in the proportion of water extractable arabinoxylan, and a shift in the MW of the water extractable arabinoxylan from being mainly larger than 85 kD to being between 2 kD and 85 kD. Ferulic acid esterase expressing grains were also shriveled and the seed weight was decreased by 20-50%. No ferulic acid esterase activity could be detected in wild type grains whereas ferulic acid esterase activity was detected in transgenic lines. The grain cell walls had 15-40% increase in water unextractable arabinoxylan and a decrease in monomeric ferulic acid between 13 and 34%. In all the plants the observed changes are consistent with a plant response that serves to minimize the effect of the heterologously expressed enzymes by increasing arabinoxylan biosynthesis and cross-linking.

Abbreviations: FA, ferulic acid; FAE, ferulic acid esterase; AX, arabinoxylan; WES, water extractable solids; WUS, water unextractable solids; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan; CWM, cell wall material; HMW, high molecular weight material; LMW, low molecular weight material; DX, glutenin promoter controlled xylanase expression; DFK, glutenin promoter controlled xylanase expression with C-terminal KDEL; DF, glutenin promoter controlled ferulic acid esterase expression; DFK, glutenin promoter controlled ferulic acid esterase expression with C-terminal KDEL

INTRODUCTION

Arabinoxylan (AX) is the major non-starch polysaccharide in wheat (*Tritium aestivum* L.) cell walls. In wheat cell walls AX consists mainly of a β-1,4 linked D-xylose backbone with single α-1,2 or α-1,3 linked L-arabinofuranosyl substitutions. Ferulic acid (FA) or coumaric acid can furthermore be esterlinked to the O-5 of arabinose. The FA esters have been shown to crosslink to other FA esters or lignin (Bunzel et al. 2001; Grabber et al., 2004). The overall structure of water-unextractable arabinoxylan (WU-AX) and water-extractable arabinoxylan (WE-AX) has been proposed to mainly vary in the degree of arabinose substitution with WE-AX having the highest degree of substitution (Izydorczyk and Biliaderis 1995).

The properties of the AX influences the baking qualities of the flour, e.g. water binding, rheology and viscosity. Generally WU-AX has a negative influence on the baking properties of wheat flour opposed to the beneficial properties of high molecular weight (HMW) WE-AX (Courtin et al., 1999). In order to convert some of the WU-AX to HMW WE-AX, addition of endo-xylanase is widely used in bread production. Excellent results are obtained with endo-xylanases that specifically degrade WU-AX into HMW WE-AX. The bacterial xylanase used in this study is specific for WU-AX and is used commercially in baking applications. Further degradation to low molecular weight (LMW) WE-AX is not desirable as this type of polysaccharide lacks the beneficial properties of HMW WE-AX (Courtin et al., 1999). For a review on the properties of the flour constituent in the baking process see Goesaert et al. (2005).

The properties of AX also influence the feed quality of the grains. Increased viscosity in the guts of monogastric animals with following reduced uptake of minerals is observed with increased content of HMW WE-AX. WU-AX is not digested and passes intact through the digestive tract. Degradation of both WE-AX and WU-AX to LMW AX or free sugars is beneficial as both mineral and calorie uptake is increased and therefore endo-xylanase is also used in the feed industry to improve feed quality (Twomey et al., 2003).

In the production of second generation biofuels from plant-based lignocellulosic biomass, cellulases and hemicellulases are applied to generate glucose and other sugars that can be fermented into fuels. Accessory enzymes could be anticipated to facilitate increased glucose yield by decreasing cross-linking of cellulose microfibrils and general cell wall loosening. For degradation of grass based lignocellulosic material addition of endo-xylanase and/or ferulic acid esterase (FAE) are of course of great interest. Both endo-xylanase and FAE have been mixed with commercially available cellulases and indeed increased glucose yield could be observed (Tabka et al., 2006, Berlin et al., 2006). However, rather than exogenous application it would be highly relevant to generate designated energy crops that express and store cell wall degrading enzymes during plant development. Hereby, the need for exogenous addition of enzymes might be obviated. Likewise, it might be envisaged that the cell wall structure and composition could be altered *in planta* by heterologous expression of enzymes facilitating increased glucose yields when processed for bioethanol production.

In the present study we have expressed a bacterial endo-xylanase or a fungal FAE in wheat. In the first series of experiments the expression was designed to be ubiquitous using the strong constitutive maize ubiquitin promoter (Christensen et al. 1992). In *Festuca arundinacea* FAE has been expressed successfully using the maize ubiquitin promoter, with no major alterations in plant morphology (Buanafina et al., 2008). Generally, lines with ubiquitous expression of FAE harbored lower levels of FAE activity than when expression was controlled with more targeted senescence or heat shock inducible promoters suggesting that high-level FAE expression may have a toxic effect, particularly at the tissue culture stage (Buanafina et al., 2008). For each enzyme we introduced two constructs, one with and one without the KDEL endoplasmic reticulum retention sequence. The rationale for the use of the KDEL retention signal was to keep the enzymes away from the apoplast as expression of cell wall degrading enzymes have previously been shown to be facilitated by withholding in the endomembrane system (Skjøt et al., 2002). A few transgenic lines were obtained but these showed severe growth retardation and sterility (results not shown). We therefore focused on endosperm specific expression to assess if such *in planta* expression could provide insight into the composition of the endosperm cell wall as well as improve the functional properties of the wheat grain for baking and as animal feed. Patel et al. (2000) have shown that endo-xylanase can be expressed without detrimental effect on growth and development in the barley endosperm and expression of endo-xylanases in rice has also been accomplished (Kimura et al., 2003). However, while the barley endosperm cell walls contain only 30% AX with mixed linkage b-glucan comprising the predominant cell wall polymer, the wheat endosperm has much less b-glucan with 70% of the cell wall consisting of AX. Endosperm expression of endo-xylanase and FAE might therefore have more drastic effects in wheat.

RESULTS

Generation of wheat with heterologous expression of endo-xylanase or ferulic acid esterase

Chimaeric bacterial endo-xylanase and fungal FAE genes under the control of the endosperm-specific 1DX5 promoter from the wheat high-molecular-weight-glutenin gene were introduced into wheat immature embryos by particle bombardment. For both of the two genes a variant containing the endoplasmic reticulum retention signal KDEL was generated. The bialaphos resistance gene (*bar*) was used as a selectable marker. According to PCR analysis 31 primary transformants were generated and for most of these T_0 plants the presence of one or more copies of the inserted endo-xylanase or FAE gene was confirmed by Southern blotting (Figure 1). However, only seven of these lines produced transgenic offspring and the kernels were shriveled and with a lower mass than wild type kernels (Figure 2 and Table 1).

Table 1. Grain weight of the transgenic lines and corresponding WT sister lines.

Line	1000 grain mass (g) WT line	1000 grain mass (g) transgenic line
DX	43.6	32.2
DXK	44.4	29.7
DF	35.1	17.7
DFK	33.3	25.7

The remaining primary transformants were either sterile or produced only non-transgenic T_1 seeds. Five of the seven stably transformed lines were transformed with the endo-xylanase gene (DX) and three of these lines had the KDEL extension (DXK). One of the two DX lines was partially sterile and no homozygous offspring was generated. The other DX line had normal fertility and homozygous offspring was selected. The fertile DX line and one DXK line were selected for further analysis.
The two remaining lines had been transformed with the FAE gene, one with (DFK) and one without KDEL (DF). Both lines were used for further analysis.

For each transgenic line a non-transgenic sister line from the T_2 generation was chosen as control, designated as WT, but derived from the same callus as the transgenic line. This was essential in order to ensure the same genetic background in the transformants and the controls since the used Bobwhite variety is not an entirely inbreed variety (Warburton et al., 2002).

For all the selected lines, fertile homozygous offspring was selected in T3 or T4. In general, the transformed lines and the sister lines had the same phenotype as WT, except for the shriveled kernels and lower grain mass compared to the non-transgenic sister lines (Figure 2, Table 1).

Endo-xylanase and FAE activity

Endo-xylanase activity was measured in mature seeds of the DX and DXK lines, and FAE activity was measured in the DF and DFK lines. The DX and DXK lines had two to three fold increase in endo-xylanase activity compared to the non-transgenic sister lines from 0.03 U / mg protein (Table 2). FAE activity of approximately 0.02 U / mg protein could be detected in the DF and DFK lines whereas no endogenous activity could be observed in WT (Table 2). No significant difference in protein content per grain could be observed in either of the transgenic lines when compared to corresponding WT (t-test, $p \geq 0.1$, n=5, results not shown).

Table 2. Xylanase or ferulic acid esterase activity in transgenic lines. The values shown are mean ± SD (n=3). Significant difference between WT and transgenic lines are indicated (t-test; *, $p < 0.05$; *** $p < 0.001$).

Line	Endo-xylanase activity (U / mg protein)		Ferulic acid esterase activity (U / mg protein)	
	WT	transgenic	WT	transgenic
DX	0.031 ± 0.003	0.067 ± 0.017 *		
DXK	0.027 ± 0.003	0.072 ± 0.016 ***		
DF			0	0.017 ± 0.003 ***
DFK			0	0.020 ± 0.003 ***

Cell wall analysis

The sugar composition analysis of the non-cellulosic part of the cell wall material (CWM) revealed large amounts of arabinose and xylose (total around 80 mol%) and lesser amounts of glucose (around 15 %). This is indicative for the commenilid cell wall of wheat with AX and mixed linkage β-glucan as the major constituents. Small amounts of galactose (5 mol%) were also present and could originate from glycan structures on cell wall proteins. Fucose, rhamnose, galacturonic acid and glucuronic acid were only detected at trace levels in the analysis of CWM (Data not shown). After fractionation (see below) fucose, rhamnose, galacturonic acid and glucuronic acid were detected in small quantities in the water extractable solids (WES) fraction constituting a total of approximately 10 % of (w/w) of non cellulosic sugars(Data not shown). Mannose has been reported to be present in minor amounts in wheat grains in the form of glucomannan (Bacic and Stone 1981; Mares and Stone 1973). However, the high xylose to mannose (w/w) ratio resulted in a poor resolution of the xylose/mannose peaks and therefore mannose was not quantified.

In all transformants, an 8 to 20 % increase in AX content was observed in the CWM, irrespective of whether they expressed the xylanase or the FAE. To investigate how the expression of xylanase or FAE affected the solubility of AX, the CWM was fractionated into Water Unextractable Solids (WUS) and Water Extractable Solids (WES), and the monosaccharide composition determined. Between 15 % and 25 % of the non-cellulosic polysaccharides were water extractable. The increased AX observed in CWM was ascribed to an increase of about 70% in the amount of AX in WES in the case of DX and DXK and to an increase of about 27% in the amount of AX in WUS in the case of DF and DFK. Increases in the molar arabinose to xylose ratio between 11 and 18% were observed in DX and DXK, respectively. After fractionation into WUS and WES these changes could be ascribed to 18 and 35 % increase in Ara/Xyl ratio in WUS (Table 3). In contrast, a 33 and 40 % decrease in Ara/Xyl ratio of WES were observed in DF and DFK, respectively (Table 3). In DF and DFK changes alike to DX and DXK were observed but numerically smaller, with changes in WUS between 6 and 12 % and changes in WES between 17 and 25 %.

The WES was subjected to preparative size exclusion chromatography with refractive index detection (Figure 3). In the WES of DX and DXK extensive changes in size distribution, with a clear shift from high (>150 kD) to lower molecular weight (<80 kD) fraction, could be seen. The changes observed in DF and DFK were more subtle though a slight shift from fractions with a molecular weight ranging from 25 to 80 kD to larger masses was observed. To verify that the changes in the refractive index chromatogram were due to changes in the carbohydrate amount and/or composition, the sugar composition of the collected fractions was analyzed (Results not shown). Good agreement between refractive index detection and sugar composition analysis was observed.

In order to quantify any possible changes in FA content due to the increased endo-xylanase or FAE activity, FA was extracted and quantified. Approximately 2 µg FA / mg CWM corresponding to 20 µg FA / mg AX were extracted. The most abundant ferulate dimer (diFA) was 8-*O*-4' linked (approximately 50 % (w/w) of total diFA) with smaller amounts of 8,5' and 5,5' linked diferulic acid (Figure 4). Small amounts of cis-FA (included in monomeric FA) and coumaric acid were also detected (not shown).

In DX and DXK no differences were observed in the total FA or mFA content, but a 20 % increase in DiFA was observed indicating an increased cross linking of AX. In DF and DFK the mFA content was decreased 30 % and 10 %, respectively. The decrease in mFA content was not followed by a similar decrease in diFA so an increased dimerization ratio was observed (Figure 4).

DISCUSSION

Transgenic wheat expressing a bacterial endo-xylanase and a fungal ferulic acid esterase

In the present study we have generated wheat plants that express endo-xylanase or ferulic acid esterase in the endosperm. The *Aspergillus* esterase is the only FAE that has been described previously. Many xylanases are known, but we specifically chose a *Bacillus* xylanase, which is known for its superior performance in baking applications. The 1Dx5 promoter was used to drive endosperm specific expression, and to ensure targeting to the secretory pathway, the sequence encoding the barley a-amylase signal peptide (Rogers and Milliman, 1983) was fused to the 5'-end of the two genes. In addition we also introduced two constructs where the sequences of the endoplasmic reticulum retention signal KDEL was fused to the C-terminal. A two to three fold higher endo-xylanase activity was observed in the lines transformed with the endo-xylanase gene compared to non-transgenic sister lines. Likewise we found significant ferulic acid esterase activity in the lines transformed with the fungal gene while there was no enzyme activity in the control materials. There were no differences between the lines harboring the KDEL sequence and the line without this sequence.

Our initial attempts aiming at generating lines with a constitutive expression of the two enzymes largely failed since the few transformants obtained suffered from poor growth and development as well as sterility. Buanafina et al. (2008) successfully obtained transgenic *F. arundinacea* lines expressing FAE under control of constitutive promoters, but they observed that it was more difficult than when using more specific or inducible promoters. When using the endosperm specific 1Dx5 promoter a large number of lines were identified and their transgenic nature confirmed by PCR, Southern blotting and enzyme assays. However, it was apparent that the presence of these two heterologous activities drastically affected reproduction as most seeds in the T1 generation turned out to lack the gene. This may result from selection during formation of the female or male gametes or at fertilization and early development where only the non-transgenic gametes were viable and functional. Furthermore, the few transgenic T1 lines identified produced T2 seeds that were shriveled and with a low grain weight.

The activities observed for the heterologous endo-xylanase were considerably lower than the 10-fold increase reported for endosperm specific expression of an endo-xylanase in barley using the hordein D promoter, hordein D being a homologue of the high molecular weight glutelins (Patel et al., 2000, Kimura et al., 2003). Interestingly, the barley grains expressing endo-xylanase exhibited a lower fertility, and the authors speculated if this could be due to the transformation event and not due to the endo-xylanase activity. However, the effect on fertility that we observed in wheat was clearly linked to the active xylanase activity. A likely explanation that can account for the more adverse effect of expressing xylanase in wheat than in barley is that AX constitutes a much higher proportion of the wheat cell wall while in barley the predominant wall polymer is mixed linkage b-glucan. Another explanation that should be considered is the presence of endo-xylanase inhibitors. Two classes of endo-xylanase inhibitors are known from wheat grains, TAXI exclusively inhibits bacterial and fungal family GH11 endo-xylanases (reviewed in Gebruers et al., 2004), while the second type of inhibitor, endo-xylanase-inhibiting protein (XIP) (McLauchlan et al., 1999), inhibits both family GH10 and family GH11 fungal endo-xylanases but not bacterial endo-xylanases (Flatman et al., 2002). This would indicate that only TAXI inhibitors would affect the endo-xylanase activity originating from the expressed *Bacillus* endo-xylanase. The content of TAXI-like inhibitors in barley is around tenfold lower than in wheat and no TAXI inhibitors have to the authors knowledge been reported in rice which might explain the higher activities reported in the endo-xylanase expressing barley (Goesaert et al., 2004). The observed two to three fold increase in endo-xylanase activity might be significantly higher if the inhibitory effect of TAXI inhibitors were subtracted. TAXI insensitive versions of the xylanase used in this study have been generated (Sorensen and Sibbesen, 2006) and future work could include expression of a TAXI insensitive xylanase in order to circumvent the possible inhibition of the expressed xylanase. On the other hand, a higher xylanase activity would likely lead to even stronger negative effects on grain yield.

DF and DFK transformants with evident FAE activity were also found. No endogenous FAE activity could be detected in the WT. To our knowledge this is the first study aiming at expressing ferulic acid esterase in the endosperm of a cereal. However, the effects of FAE expression has been analyzed in leaves of the two fodder grasses *Lolium* and *Festuca* leading to reduced levels of mFA and diFA in the cell wall and increased in vitro dry matter digestibility (Buanafina et al. 2006, 2008). The present study has documented that the expression of a heterologous ferulic acid esterase has a similar effect on grain morphology and reproduction as that observed for the heterologous endo-xylanase. The reason for this similarity could be that both endo-xylanase and FAE decrease the degree of cross

linking in the cell wall. In the case of endo-xylanase the AX backbone is digested into smaller polymers hence decreasing the cross linking. In FAE expressing plants the cross linking of AX via inter- or intrachain FA dimerization is lowered. The constraints on reproduction as well as the effects on the grain may accordingly indicate that the intactness of the cell wall is very important leading to severe pleiotropic effects if compromised.

In the evaluation of the effects of heterologous expression, the intracellular localization of the two enzymes is of paramount importance. Recent immunolocalization studies have thus documented that although the heterologous proteins are targeted to the apoplast via the fusion of signal sequences to the N-terminal, the proteins end up in the protein storage vacuoles of the endosperm (Arcalis et al. 2004, Brinch-Pedersen et al. 2006). It thus appears that the endosperm provides an environment for protein trafficking that overrules the normal secretion mechanisms to the apoplast and instead favors deposition in protein storage vacuoles. In the present study we used the same promoter and α-amylase signal sequence as used in the study by Brinch-Pedersen et al. (2006). Endosperm dominant protein trafficking mechanisms may also explain why we failed to find any differences between plants transformed with the KDEL constructs and those without.

Other approaches for expression of cell wall degrading enzymes include targeting the enzymes to apoplast (Skjøt et al., 2002; Obro et al., 2009), Golgi (Skjøt et al., 2002; Obro et al., 2009), cytoplasm (Patel et al., 2000, Kimura et al., 2003), vacuole (Buanafina et al., 2006, 2008) and endoplasmic reticulum (this study). No consistent correlation between the cell location where the enzymes are expressed and the observed severity of possible phenotypes are observed. In potato the expression of fungal arabinanase led to a severe phenotype when targeted to the apoplast whereas Golgi retention abolished this severe phenotype (Skjøt et al., 2002). In *Arabidopsis* the apoplastic targeting of the same fungal arabinanase did not lead to a more severe phenotype than with Golgi retention (Obro et al., 2009).

Cell wall sugar composition, ferulic acid content and composition in control plants

The non-cellulosic sugar composition with ≈ 75 % AX and ≈ 20 % glucan (w/w) is typical of a wheat grain cell wall. The Ara/Xyl ratio of approximately 50% is also in line with previously reported analyses of wheat grains (Basic and Stone 1981, Maes et al. 2005, Mandalari et al., 2005). Approximately 2 µg FA / mg CWM was extracted which is comparable to the 2-4 µg FA / mg bran and approximately 2 µg FA / mg grain reported in other studies (Rybka et al., 1993, Adom et al., 2005). Likewise,
the composition of the FA with mostly mFA and 8-*O*-4' as the main diFA followed by smaller amounts of 5,5' and 8,5' diFA is in agreement with available data (Mandalari et al. 2005).

When the endo-xylanase or FAE were expressed it resulted in clear changes in the cell wall composition of the wheat plants. In DX and DXK an increase in WES non-cellulosic sugar content was observed which could be ascribed to an increase in AX content in the WES fraction (Table 3). The structure and composition of the AX in WES and WUS were changed, as indicated by the changes in Ara / Xyl ratio. The increase in Ara / Xyl ratio in WUS, the corresponding decrease in Ara / Xyl ratio in WES and the change in size distribution towards polymers of lower molecular mass all indicate that the expressed endo-xylanase in the transformants changed the sugar composition and structure of AX in the WUS by digesting the unsubstituted areas of the AX. The released AX fragments were thereby shifted from the WUS to WES fraction. Surprisingly, the digestion of the unsubstituted AX in the WUS fraction did not lead to a decreased WUS AX content, indicating that the plants were compensating for the change in AX structure. Apparently, the plants increased the deposition of AX and increased the cross-linking, thereby efficiently preventing any change in the amount of AX in WUS.

In DF and DFK an increase in the non-cellulosic carbohydrate content of the CWM could be observed. This increase correlated with a significant increase in the AX content of the CWM. After fractionation of the CWM into WUS and WES fractions the increased non-cellulosic carbohydrate content was ascribed to increased content of WUS AX. The Ara / Xyl ratio in CWM did not change in DF and DFK lines as opposed to DX and DXK. However, after fractionation of CWM into WUS and WES it was clear that the Ara / Xyl ratio changed in the fractions, with an increase in Ara / Xyl ratio in WUS. Unlike, the DX and DXK lines, the changes in AX in the DF and DFK lines is not a redistribution of the AX between WES and WUS but a more complex change with deposition of extra AX, possibly as a compensatory mean. The increased deposition of AX into the WUS fraction is somewhat unexpected as a decrease in the mFA could be assumed to lead to decreased diFA content and thereby an increased solubility of the AX. However, mFA content is decreased in both DF and DFK, whereas the diFA content was not consistently changed in DF and DFK. Thus, the plants appear to react to the decreased FA content by increasing the ratio of diFA to total FA and by increasing AX

deposition and AX molecular mass. Thereby, the plants prevent the change to a more soluble AX that was a priori predicted in the DF and DFK lines.

Several aspects in the presented work points towards compensatory regulation of the cell wall biosynthesis and modification. In both endo-xylanase and FAE expressing plants an up-regulation of xylan deposition was observed. Whether the extra deposited xylan is of the same or has a different structure in either endo-xylanase or FAE expressing plants cannot be determined unambiguously based on this study. It could be argued that in both cases the extra deposited xylan is part of the WUS fraction. In the endo-xylanase plants the WUS fraction is unchanged even with the presence of the extra endo-xylanase activity that is expected to increase the water solubility of the xylan. In the FAE expressing plants the xylan content in WUS is increased directly. Since it has previously been hypothesized that xylan is deposited as a highly substituted polymer and then later *in muro* the arabinose substitutions are partially removed (Gibeaut and Carpita, 1991), the extra xylan deposition observed in this study is not only a regulation of xylan biosynthesis but also a regulation of modifying enzymes.

In all the transgenic plants there was an increase in the ratio of diFA to total FA, suggesting a compensatory effect in response to the effects of the expressed enzymes.

The responses in the transgenic plants can all be understood as a reaction that prevents the wall changes that would be a priori predicted to result from the action of the heterologoulsy expressed enzymes. Apparently, the wheat plants strongly react to any attempt to increase solubility of wall AX by increasing AX deposition and cross-linking. A major unanswered question in cell wall biology is how plants are able to sense the integrity of the wall and react to even relatively small changes in wall properties. Receptor-like kinases have been proposed to be involved in the cell wall to cell cross talk (Hématy et al., 2007; Humphrey et al. 2007, Hematy and Hofte 2008). Physical connections that link the cell wall to the membrane and 'sense' cell wall integrity by its physio-mechanical state have also been suggested (Gillmora et al., 2005). The changes in response to xylanase could also be due to oligosaccharide signals rather than to changes in the AX itself, similarly to what was observed with arabinanase in potato, where adverse effects were only observed when the enzyme was active in the apoplast and were unrelated to the amount of arabinan (Skjot et al., 2002).

That strong compensatory effects were observed in all four transformants have implications for future research into modifying the grass cell wall. Currently, large efforts are put into research that could lead to increased yields in biofuel production. Many alterations of the grass cell wall have been proposed that would increase second generation biofuel production. Some of these suggestions are related to changing the xylan content or changing xylan-lignin interactions. The present study suggests that successful generation of crops with improved polysaccharide composition may require not only changes to single genes, but changes to signal transduction mechanisms as well.

EXPERIMENTAL PROCEDURES

Construction of plasmids

A total of four constructs containing the coding region from either the *Xyn*A endo-xylanase gene from *Bacillus subtilis* (GenBank accession NC_000964.2) or the *Fae* ferulic acid esterase gene from *Aspergillus niger*,(de Vries et al. 1997, GenBank accession Y09330.2) were generated. The recombinant genes were under control of the endosperm-specific promoter fragment from the wheat high-molecular-weight-glutenin *1DX5* gene (Halford et al., 1989). To ensure targeting via the secretory pathway the sequence encoding the barley a-amylase signal peptide (Rogers and Milliman, 1983) was used to replace the endogenous bacterial & fungal signal peptides of the *Xyn*A and *Fae* genes, respectively. The constructs were made with or without the sequence encoding the signal KDEL for retention in the endoplasmic reticulum(Munro and Pelham, 1987).

A plasmid containing the endo-xylanase gene was provided by Danisco A/S. The coding region was PCR amplified with the forward primer XynA-F1 (5'-*AA*CTGCAGGCTAGCACAGACTACTGGCAAAATTGG-3') introducing a 5´ *Pst*I site (underlined) and the reverse primer XynAR2 (5'-*CGG*GGTACCTCACCACACTGTTGCGTTAGAGCTTCCGCTGCTTTGATATCC-3') introducing a 3´ *Kpn*I site (underlined). A KDEL sequence was amplified using the forward primer XynA-F1 and the reverse primer XynAR1 (5'-*CGG*GGTACCTCA**TAGCTCGTCCTT**CCACACTGTTGCGTTAGAGCTTCCGCTGCTTTGATATCC-3') which added the KDEL sequence (bold). The two amplified fragments were cloned into the pCR-Blunt-II-TOPO (Invitrogen). To adapt the endo-xylanase coding region for expression in wheat, rare codons, mostly in the third wobble base position, were modified by PCR based single nucleotide mutagenesis. A possible cryptic intron was changed by a single base mutation at the potential splicing

site without changing the corresponding amino acid. Altogether 12 nucleotides were substituted in the coding region of the endo-xylanase gene. The mutagenesis was performed according to Xu et al. (1996). The upper half of the endo-xylanase coding region was amplified with the forward primer XynA-F1 and the reverse primer XynAMR1 (5'-CCCATC**G**CTTTTTACAGT**C**CCTTT**G**TACGTTCCAGT**T**GG**C**CT**G**TAAGTAC- 3'). Bold indicates nucleotide changes compared to the native sequence. The lower half of the endo-xylanase coding region including the KDEL sequence was amplified with the forward primer XynAMF1 (5'-GGTACATATGACATATA**C**ACAACTACACG**G**TATAACGCACC**A**TCCATTGA-3') and the reverse primer (minus KDEL version) XynAMR3 (5'-*CGG*GGTACCTCACCACACTGTTACGTTAGA**G**CTTCC**G**CT**G**CTTTGATATCC -3'). The KDEL coding sequence was amplified with XynAMF1 and XynAMR2 (5'-*CGG*GGTACCTCA**TAGCTCGTCCTT**CCACACTGTTACGTTAGA**G**CTTCC**G**CT**G**CTTTGATA TCC-3'). The upper and lower fragments of the coding region were directionally cloned in pCR-Blunt-II-TOPO. The modified endo-xylanase sequence was inserted as a *Pst*I-*Kpn*I fragment into *Pst*I-*Kpn*I-digested p1DX5signal peptidePhyN (Brinch-Pedersen et al. 2003), replacing the signal peptide and Phy sequences and yielding p1DX5XynN (minus KDEL) and p1DX5XynKN (plus KDEL). Finally the α-amylase signal peptide, isolated as a *Pst*I-*Pst*I fragment from p1DX5signal peptidePhyN was ligated into p1DX5XynN and p1DX5XynKN yielding p1DX5signal peptideXynN and p1DX5signal peptideXynKN, respectively.

Mycelium of an *Aspergillus niger* transformant NW154::pIM3207.7, which overproduces ferulic acid esterase (de Vries et al. 1997), was used to clone the FaeA coding sequence. The *FaeA* gene within the fungal chromosome contained an intron, and the two exons were PCR amplified separately. Codon optimization and generation of restriction sites for further cloning were performed during the amplification. Exon 1 was amplified with the forward primer FAEAE1F (5'-*AA*CTGCAGGCCTC**T**AC**T**CAAGG**A**ATCTC**T**GAAGACCTCTACAAT) and the reverse primer FAEAE1B (5'-*CATCGACGCTCCCAGA*CTATGTCC**A**GTCACGGTAAGCGCATAGTCCGG. Bold indicates nucleotide changes compared to the native sequence. The second exon was amplified with the forward primer FAEAE2F (5'-*ACCGTGACAGGACATAG*TCTGGGAGCGTCGATGGCAGCACTCACT) and the reverse primer FAEAE2B0 (5'-GGGGTACCTTACCAAGTGCAAGCTCCGCTAGTCATCCCAAAATAAGT) (minus KDEL version) or FAEAE2BK (5'-GGGGTACCTTAGAGCTCGTCCTTCCAAGTGCAAGCTCCGCTAGTCATCCCAAAATAAGT) (KDEL version). Primer sequence in italics represents overlap used for joining the two exons via PCR. The fragments were cloned in pCRÒII-TOPO vector (Invitrogen). The remaining cloning steps yielding p1DX5signal peptideFaeN and p1DX5signal peptideFaeKN was performed as described already for p1DX5signal peptideXynN and p1DX5signal peptideXynKN.

In order to facilitate large-scale preparation of linearized DNA for transformation experiments, the *BAR* and the *FaeA* genes were mutagenized to remove internal restriction sites for *Pvu*II. For changing a single base within the internal *Pvu*II sites we used the method by Stratagene, QuikChange Site-Directed Mutagenesis. The entire transformation cassette could then be excised using *Pvu*II as the only restriction enzyme.

Wheat transformation

*Pvu*II-fragments containing the four different expression cassettes were introduced into immature embryos of wheat cultivar Bob White using the biolistic procedure previously described by Brinch-Pedersen et al., (2000). Homozygous lines and corresponding WT sister lines were identified in T2. Thus, for each transformant genotype, a WT plant to be used as control originated from the same callus as the transformant, but did not contain the transgene. The T2 plants were propagated in pots. The T3 plants were propagated in plots containing 139 plants in the greenhouse. The grains from these T3 plants were harvested and used for analysis. The seed batch for each genotype thus consisted of seeds from 139 different T3 plants.

Identification of transgenic plants by PCR and Southern blot

Genomic DNA was isolated by FastDNA® Kit (Qbiogene). PCR amplification for detection of the expression cassettes with endo-xylanase was achieved by using the primers a-signal peptide-F2 (5'-ACAAACATTTGTCCCTCTCCCTCTTCC-3') and Xyn 503-R (5'-AGCCCAATTACTGCCCAGATTCATTCC-3'). The upper strand primer corresponds to the bases 8 to 34 of the sequence encoding the signal peptide. The lower primer anneals at position 547 to 573 within the chimeric ORF. Thus the amplified fragment was 566 bp long.

The primers a-signal peptide-F (5'-TGGCGAACAAACATTTGTCCC-3') and FAE-R (5'-CTCGCAATCGTTGCATTGAGG-3') were used for detection of the expression cassettes with ferulic acid esterase. The upper strand primer corresponds to the bases 2 to 22 of the sequence encoding the signal peptide. The lower primer anneals to position 343 to 363 within the chimeric ORF. Thus the amplified fragment was 362 bp long.
Southern blotting was done as described in Brinch-Pedersen et al. (2000) with some modifications. The genomic DNA (10 µg) was digested using EcoRV, which cuts once in *XynA* and *FaeA*. Blots for *XynA* identification were hybridised with a 509 bp PstI – KpnI fragment of *XynA* and the fragment used in the *FaeA* blots were a 480 bp EcoRV- NcoI fragment of the FaeA gene.

Protein extraction

Twenty five grains were shattered with a hammer and transferred to appropriate tubes. Three different samples of 25 grains were extracted for each genotype. The whole meal flour was then further ground in 7.5 ml of extraction buffer (100 mM MES pH 6.0; 2 mM EDTA; 1.4% ascorbic acid; 1 mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride)(Calbiochem, CA, USA); 1 µg/ml leupeptin (Sigma-Aldrich)) using an Ultra Turrax homogenizer for 2 times 20 sec at 24000 rpm. The sample was centrifuged for 10 min at 12000 g at 4 ˚C. The supernatant was decanted and protein content was determined using Bradford reagent (Bradford, 1976). The supernatant was kept at -20 ˚C for further analysis.

Enzyme activity assays

Endo-xylanase activity was measured using azo-xylan derived from oat spelt (Megazyme). 50 µl of supernatant from the protein extraction was incubated with 100 µl 150 mM MES pH 6.0 and 150 µl of azo-xylan prepared as described by Megazyme (http://secure.megazyme.com/downloads/en/data/S-AXYO.pdf). Assays were incubated for 18 h at 37 ˚C and terminated by addition of 1 ml of 96 % ethanol followed by centrifugation for 5 min at 10000 g. The absorbance of the supernatant at 590 nm was then measured. In order to correlate the measured absorbance with the number of reducing ends, so a specific activity in enzyme units (U) (µmol product/min) could be calculated, a standard curve was made which correlated the number of reducing ends (Lever 1972) with the absorbance at 590 nm. In the assays for the standard curve endo-xylanase from *B. subtilis* (Danisco A/S) was used.

Ferulic acid esterase (FAE) activity was measured using treated ferulated xylan (P-WAXYI) (Megazyme) as substrate. The substrate for the assays was prepared by adding 250 mg P-WAXYI to 22.5 ml water under stirring and further stirring for 5 min. 2.5 ml 1 M NaOH was added and stirring continued for additionally 2 min. Sodium acetate to a final concentration of 50 mM was added and the pH adjusted to 5.2 using 1 M HCl. After addition of 50 mU of *B. subtilis* endo-xylanase it was incubated overnight at 30 ˚C. The substrate preparation was centrifuged for 20 min at 20000 rpm in a SS34 rotor and the supernatant was decanted and 300 µl aliquots were dried under vacuum. One aliquot was then used as substrate for one assay. The assay was performed by incubating 10 µl of supernatant from the protein extraction, the substrate aliquot dissolved in 100 µl water, 20 µl of 0.5 M MES pH and 70 µl water for 15 min at 37 ˚C. The assay was stopped by addition of 15 µl of glacial acetic acid. Released ferulic acid (FA) was extracted by adding 215 µl ethyl acetate, vortexing, centrifuging for 1.5 min at 13000 g and transferring the top ethyl phase to a new tube. The extraction procedure was repeated trice to ensure near total extraction of released FA. The ethyl acetate with extracted FA was dried under vacuum and re-dissolved in 1 ml of methanol and the absorbance at 320 nm was measured and compared to a standard curve of absorbance using pure FA (Sigma-Aldrich). One unit of FAE activity was defined as µmol released FA / min.

Cell wall material preparation

Wheat grains (triplicate samples of 2.5 g for each genotype) were smashed with a hammer and transferred to appropriate tubes. The whole meal was further homogenized for 2 min at 24000 rpm in 15 ml of 50 mM MOPS, pH 7.0. The sample was boiled for 10 min, cooled to 85 ˚C and 200 U of heat stable α-amylase (Megazyme) was added and incubated for 30 min. In the next step, 20 ml of 200 mM sodium acetate pH 4.5 was added followed by 20 U of amyloglucosidase (Megazyme) and then incubated for 30 min at 50 ˚C. Subsequently, 105 ml of 96 % ethanol was added and the whole meal flour was shaken vigorously followed by centrifugation at 10000 rpm (Sorvall, SLA 1500). The supernatant was discarded and the pellet was dried under vacuum. The dried pellet was wetted in 1 ml ethanol immediately followed by addition of 2 ml of dimethyl sulfoxide, vortexed and boiled for 5 min followed by amylase and amyloglucosidase treatment as described above. The pellet was then homogenized further in a mortar. The resulting sample was termed “cell wall material” (CWM).

Determination of ferulic acid content

Ferulic acid and other hydroxycinnamates were extracted and quantified as described in Obel et al. (2002). In brief CWM was incubated with 2 M NaOH and incubated overnight. After acidification by glacial acetic acid the hydroxycinnamates was solvent extracted into ethyl acetate. The ethyl acetate was evaporated away and the hydroxycinnamates redissolved in methanol and analyzed by HPLC.

Noncellulosic carbohydrate monosaccharide composition analysis

Approximately 5 mg of CWM was hydrolyzed in 1 ml of 2 M trifluoroacetic acid for 1 h at 120 °C. Trifluoroacetic acid was removed by drying under vacuum. Monosaccharide composition was subsequently determined by high performance anion exchange chromatography with pulsed amperiometric detection (HPAEC-PAD) of hydrolyzed material using a PA20 column (Dionex, CA, USA) as described previously (Obro et al., 2004). Monosaccharide standards included L-Fuc, L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl, D-GalUA and D-GlcUA (all from Sigma). For verification of the response factors a standard calibration was performed before analysis of each batch of samples. The AX content in CWM was estimated as the sum of arabinose and xylose content.

Cell wall material fractionation and mass distribution analysis

Cell wall material was fractionated into a water soluble (WES) and a water insoluble (WUS) fraction by incubating approximately 30 mg of CWM in 1 ml extraction buffer (50 mM phosphate pH 7.0; 0.02 % sodium azide) overnight with shaking (1400 rpm) at 65 ˚C for 24 h. The CWM was centrifuged at 13000 g for 15 min and the supernatant was collected. The pellet was re-extracted with the same procedure, and the supernatants combined. The combined supernatant and pellet, containing WES and WUS respectively, were dried under vacuum. Monosaccharide composition analysis as described above was performed on WUS and WES and a mass distribution of WES was performed using size exclusion chromatography. Size exclusion chromatography was carried out on a Superose 12 column (1 X 30 cm, Amersham Pharmacia, Sweden) equilibrated in 0.05 M ammonium formate. Sample was applied to the column and eluted with 0.05 M ammonium formate at a flow rate of 24 ml/h. 0.8 ml fractions were collected. The eluent was monitored using a refractive index detector (model 131, Gilson, USA). The sugar composition analysis of the collected fractions was analyzed using trifluoroacetic acid followed by high performance anion exchange chromatography with pulsed amperiometric detection as described above.

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Table 3. Sugar content and analysis of CWM, WUS and WES. The values shown are mean ± SD (n=3). Significant difference between WT and transgenic lines are indicated in bold (2-way ANOVA, $p < 0.05$).

	Non-cellulosic carbohydrate (µg/mg CWM)			Arabinoxylan (µg/mg CWM)			Molar Ara / Xyl ratio (%)		
Line	CWM	WUS	WES	CWM	WUS	WES	CWM	WUS	WES
DX WT	166±14	138±12	**32±3**	131±11	113±10	**21±2**	**52±2**	**50±3**	**52±1**
DX transg.	161±12	128±19	**44±5**	136±17	110±17	**32±3**	**58±1**	**60±3**	**35±0**
DXK WT	151±11	118±17	**23±8**	116±7	95±13	**14±5**	**47±1**	**46±1**	**53±2**
DXK transg.	161±4	117±18	**34±3**	132±3	98±15	**25±2**	**63±1**	**71±1**	**32±0**
DF WT	165±5	**132±4**	**33±6**	**125±3**	**107±3**	18±0	51±1	**50±1**	**55±1**
DF transg.	174±3	**147±5**	**27±3**	**142±3**	**122±4**	20±2	52±0	**53±1**	**46±2**
DFK WT	137±4	**102±7**	**36±4**	**100±3**	**80±5**	19±2	51±1	**49±1**	**59±1**
DFK transg.	165±27	**139±20**	**26±9**	**130±21**	**112±16**	19±7	54±0	**56±1**	**44±2**

Figure Legends

Figure 1. Southern blot analysis of transgenic wheat lines. Lines that were tested positive by PCR were further analyzed by Southern blotting using probes for the XynA or FaeA genes. The lines DX-1, DXK-3, DF-2 and DFK-4 contained the genes of interest and were fertile. These lines were used for the further experiments.

Figure 2. Severe phenotype of the grains was observed in the transgenic lines. The top row shows the WT sister lines corresponding to the transgenic lines (bottom row). A) DX,;B) DXK ; C) DF; D) DFK.

Figure 3 Size exclusion chromatography of WES. The amount of sugar in the eluate was detected by refractive index and confirmed by sugar composition analysis (not shown). Size exclusion chromatography chromatograms for transgenic lines (broken lines) and corresponding sister WT lines (solid lines) are shown. A) DX and corresponding WT; B) DXK and corresponding WT; C) DF and corresponding WT; D) DFK and corresponding WT. A clear shift towards lower molecular mass was observed in DX and DXK. In contrast, only a small change towards higher molecular mass was observed in DF and DFK. Elution of molecular mass markers (dextrans in kD) are indicated at the top.

Figure 4. Content of ferulic acid monomer and dimers in CWM from transgenic and WT lines. The AX content is measured as the sum of arabinose and xylose. Significant differences between transgenic lines and the corresponding WT line are indicated with asterisk (t-test, n=3, $p < 0,05$).

Figure1

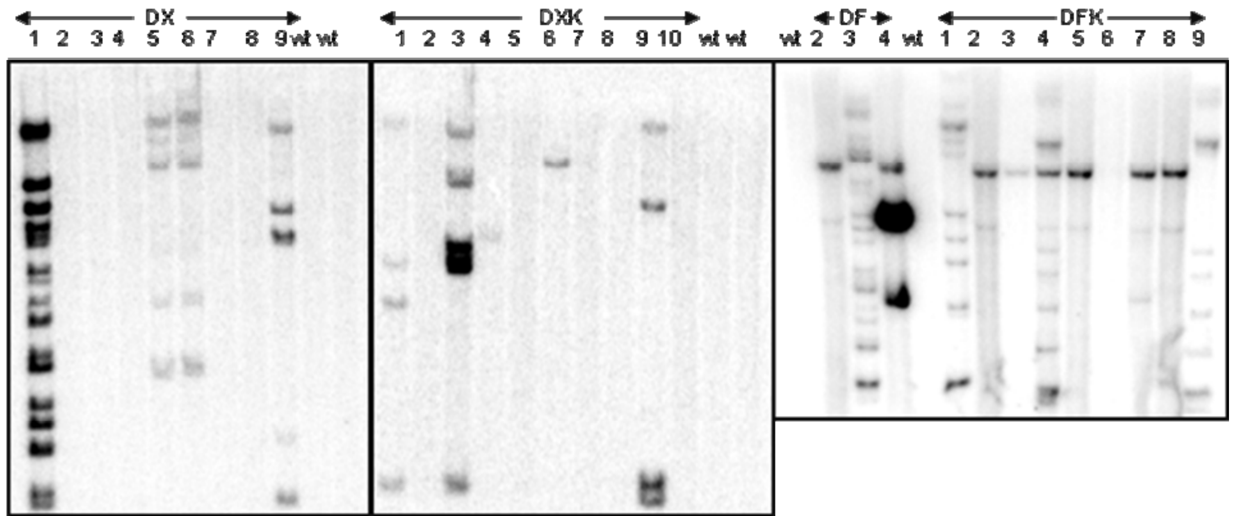


Figure 2

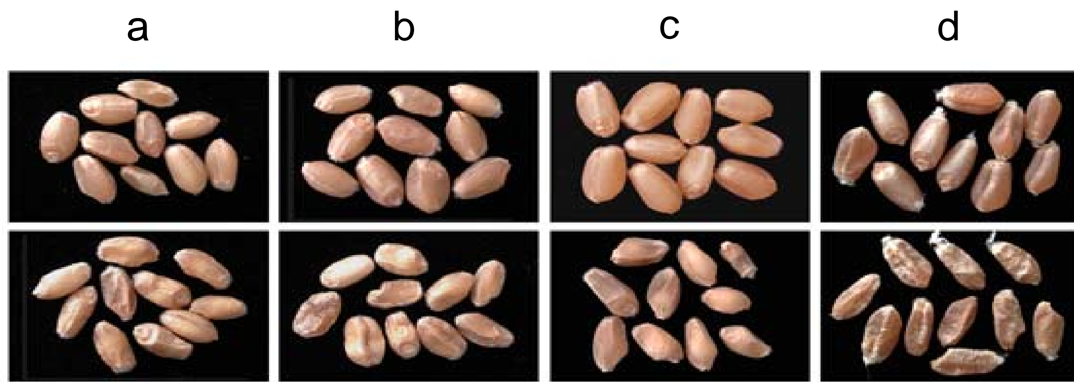
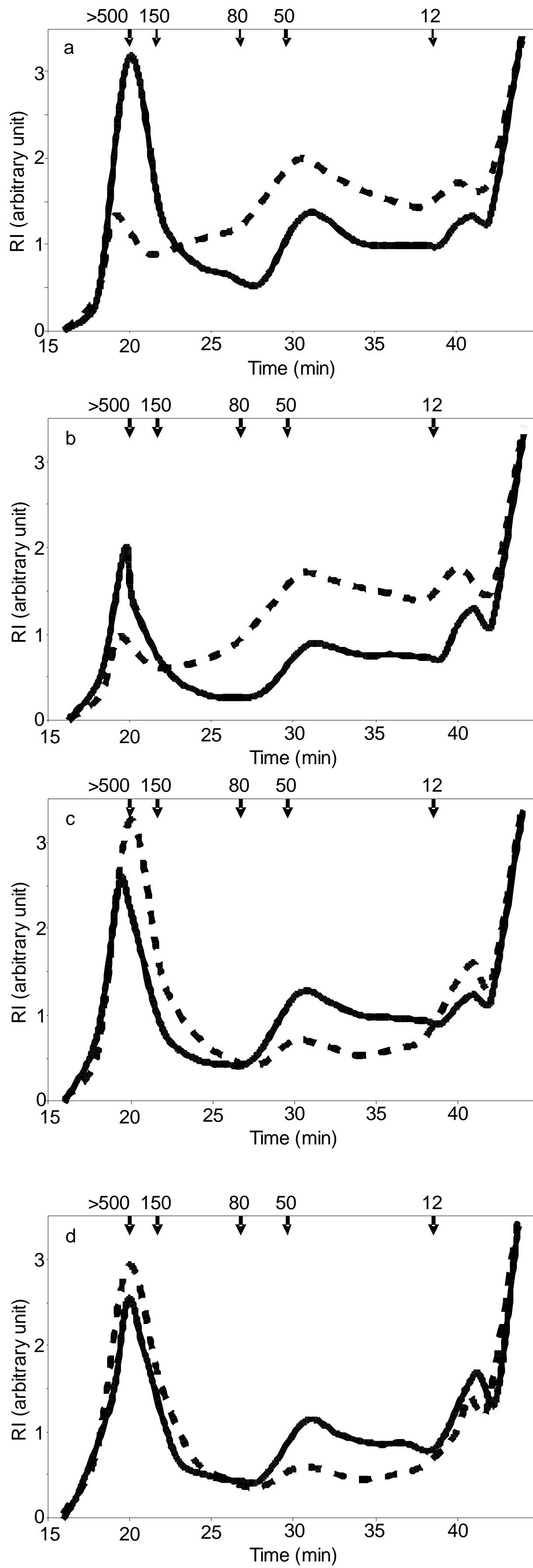
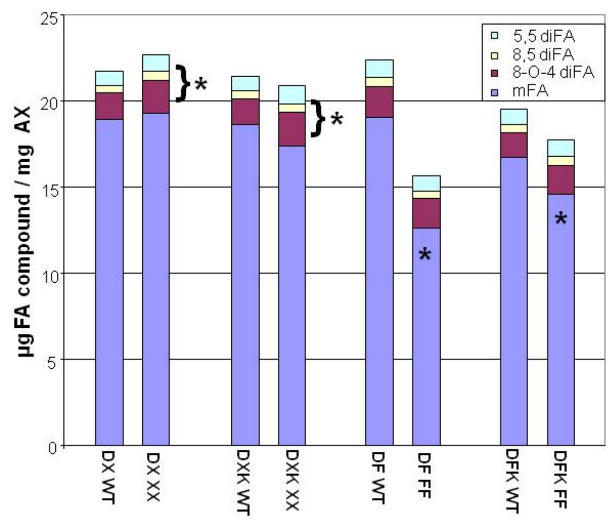


Figure 3
Figure 4




5,5 diFA
8,5 diFA
8-O-4 diFA
mFA
µg FA compound / mg AX
25
20
15
10
5
0
DX WT
DX XX
DXK WT
DXK XX
DF WT
DF FF
DFK WT
DFK FF
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