

Mutagenesis and transcriptome mapping in oat and characterization of high β -glucan lines

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For Anette...

“Never give up, never surrender”

-Peter Q Taggart, Cdr., NSEA Protector

“I can’t believe it, reading and writing finally paid off!”

-Homer Simpson

Mutagenesis and transcriptome mapping in oat and characterization of high β -glucan lines

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Abstract

Oat (*Avena sativa*) is a hexaploid crop that is cultivated in Europe, North America, Russia, Australia and northern China. During recent years, oat has seen increased interest due to its potential as a functional food crop. Among beneficial health promoting bioactive molecules expressed in oat are unique antioxidants, galactolipids and high levels of globular proteins. Oat is also an excellent source of β -glucan (1-3;1-4 mixed link β -D glucan), a dietary fiber with blood glucose stabilizing and cholesterol lowering properties.

However, traditional breeding has hitherto had limited success in developing a high β -glucan oat. To improve the variation and precision of the breeding process and to provide a new tool for trait development in oat, an EMS-mutagenized TILLING-population of 2,500 oat lines was developed (**Paper I**). The mutation frequency was assayed by several different methods, including screening for mutations at the genetic level using the *AsCsIF6* and *AsPAL1* genes as a model.

Taking advantage of the TILLING-population, seeds from 1,700 lines from the TILLING population were ground and biochemically tested for β -glucan content. Several mutated lines with dramatically increased or decreased β -glucan levels were identified. This variation also validated the high mutation frequency in the mutagenized lines. The mutant line with the highest levels of β -glucan saw an increase of 52% compared to the starting variety. Six lines with increased and four with decreased β -glucan levels were finally selected and parameters like structure, molecular weight, solubility and localization of β -glucan in the seed kernel were analyzed. This showed that also the β -glucan quality varied greatly between mutants, suggesting that quantitative and qualitative differences may in some cases be linked (**Paper II**). The high β -glucan lines will now be used in various breeding applications as well as in studies to elucidate physiological effects of altered β -glucan structure on humans.

To increase and improve the genetic resources available in oat, a high coverage transcriptome map was developed using next generation sequencing from a diverse range of tissues and during seed development. In total, 190,261 contigs were obtained after assembly and annotated using BLAST, Interproscan and Gene Ontology. The quantitative nature of the data also allowed for expression analysis, creating an unparalleled view into the oat transcriptome. The data was then used to identify tissue-specific transcription factors and characterize the expression of β -glucan producing genes during seed development (**Paper III**). The oat transcriptome atlas will be useful in comparative studies between oat and other cereals and also provide an important reference in future oat-genome sequencing projects.

Finally, as a complement to the transcriptome map, an oat miRNA atlas was generated by sequencing small RNA from seed development, various tissues and during abiotic stress. These microRNAs can now be used in conjunction with the transcriptome data for a further unraveling of the complex regulatory networks in oat (**Paper IV**).

List of publications

The thesis is based on the following papers, which will be referred to in the text as Paper I-IV.

Paper I.

Chawade, A., **Sikora, P.**, Bräutigam, M., Larsson, M., Vivekanand, V., Nakash, M. A., Chen, T., Olsson, O. (2010) **Development and characterization of an oat TILLING-library and identification of mutations in lignin and β -glucan biosynthesis genes**

BMC Plant Biology, 8:304

Paper II.

Sikora, P., Tosh, S. M., Brummer, Y., Olsson, O. (2013) **Identification of high β -glucan oat lines and localization and chemical characterization of their seed kernel β -glucans**

Food Chemistry, 137:1-4

Paper III.

Sikora, P., Chawade, A., Lindlöf, A., Olsson, O. (2013) **Generation of an oat (*Avena sativa*) transcriptome and expression analysis of transcription factors and mixed-link β -glucan biosynthesis genes**

Manuscript

Paper IV.

Lindlöf, A., **Sikora, P.**, Chawade, A., Olsson, O. (2013) **Identification and Classification of MicroRNAs in Oats (*Avena sativa*) by Deep Sequencing of Small RNAs Isolated from Various Tissues and Conditions**

Manuscript

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1. Introduction

History and cultivation of oat

Oat has been actively cultivated in northern Europe since the Middle Ages, but has grown as a domesticated plant for more than 2000 years. Originating from the Fertile Crescent and areas around the Mediterranean, it originally grew as a weed among previously cultivated crops and spread northwest with the migration of agriculture during the bronze and iron ages (Suttie, Reynolds et al. 2004). Although aware of the crop, ancient Greeks believed the seed to be a diseased form of wheat and thus unfit for human consumption, usable only as fodder (Bunte, Granström et al. 2013). This observation was likely compounded by the tendency of dehulled oats to spoil due to their high lipid-content, giving them a rancid taste. The Romans, who first encountered the grain north of the Alps, referred to it as the “barbaric bread grain of the Germans”. In the colder, wetter climate of north Europe, oat flourished and quickly became a staple crop in Scandinavia, Germany, Ireland and Scotland, used both for food and feed. Cultivation in Sweden became prominent in the 17th century and increased steadily up until the late 19th century when 800 000 hectares were cultivated, and close to half of Sweden’s grain production consisted of oats (Bunte, Granström et al. 2013). With the mechanization of agriculture and the decreased reliance on horses for work and transportation, oat saw a steady decrease in cultivation during the 20th century. Today, oat is mainly grown in Canada and northern United States, northern Europe, Russia, China and Australia. The current world production (2011) is estimated to 22.5 million tons (FAOSTAT 2012).

Oat has the ability to grow in a wide range of soil types and can thrive in both low and high pH, where more sensitive cereals, such as wheat, have trouble surviving. Paired with a relatively high resistance to disease this allows cultivation on marginal land that is unsuited for other crops (Suttie, Reynolds et al. 2004). Oats have lower energy requirements compared to other cereals and a well-developed root system. These traits, combined with tolerance to pathogens affecting wheat and barley, make it an excellent rotational or mixing crop, especially with nitrogen-binding legumes such as pea or vetch, as it helps to reduce soil erosion, magnesium loss and loss of biomass (Bullock 1992).



Figure 1: Oat growing in the field in south Sweden.
Photograph courtesy of Prof. Olof Olsson

Classification and genome

Oat (*Avena*) is a member of the *Poaceae* or “true grass” family of monocots and is thus closely related to both wheat and barley, but falls into a separate subtribe, *Aveneae* (USDA 2008). The genus *Avena* contains 29 known subspecies but only a few of these are grown commercially, namely: *A.sativa* (common oat), *A.nuda* (naked oat), *A. byzantina* (red oat), *A. abyssinica* (Abyssinian oat) and *A. strigosa* (sand oat) (Bunte, Granström et al. 2013). The subspecies are divided into diploids, tetraploids and hexaploids, of which *A. sativa*, *A. nuda* and *A. byzantina* are hexaploid, *A. abyssinica* is tetraploid and *A. strigosa* is diploid (Coffman and Stanton 1977). In this thesis, *A. sativa* will be referred to as “oat”.

The genome of *A. sativa* is comprised of three subgenomes: A, C and D, each with seven chromosomes, giving a total of 21 chromosome pairs (Rajhathy 1974). The combined 1C genome size has been estimated to approximately 13.5 gb, the second largest among the cereals, the largest being wheat with an approximate size of 16 gb (Bennett and Smith 1976). As in wheat, all three genomes contribute to gene expression, complicating breeding and trait development.

The diploid progenitor of *A. sativa* has long been held to be *A. strigosa* but this assumption has come under doubt in recent years as cytological analysis has advanced considerably, disproving previous assumptions (Ladizins.G 1969, Ladizinsky 1995).

The current theory is that a recently discovered tetraploid, *A. insularis*, growing in Sicily, is the tetraploid progenitor contributing the C- and D-genomes (Ladizinsky 1998) while the diploid progenitor of the A-genome remains unknown, although some evidence suggests it may grow wild in north-west Africa (Ladizinsky 1998).

Health benefits of oats

During the last twenty years oat has gained recognition as a “healthy” cereal due to several factors. These include phenolic compounds such as tocopherols and tocotrienols (Vitamin E) that have antioxidative properties (Peterson 2001). Tocopherols and trienols are classified in four categories, alpha, beta, gamma and delta, and the Vitamin-E family thus consists of eight members (Sen, Khanna et al. 2006). In oat, α -tocotrienol is the primary constituent and α -tocopherol is the secondary constituent. These two account for between 85% and 91% of total Vitamin-E concentration although small amounts of β -tocopherol/trienol are also detectible (Peterson 1995). Gamma and delta tocopherols/trienols, on the other hand, are only present in trace amounts (Peterson 1995). Tocotrienol has displayed anti-carcinogenic (Gould, Haag et al. 1991, Rahmat, Ngah et al. 1993, Nesaretnam, Dorasamy et al. 2000), antioxidative (Kamat and Devasagayam 1995, Begum and Terao 2002), cholesterol-lowering (Qureshi, Sami et al. 2002, Iqbal, Minhajuddin et al. 2003) and neuroprotective (Anderson, Qiu et al. 2003) properties in human, rat and mouse. In addition, tocotrienol has also exhibited a greater bioavailability than tocopherols in humans (Noguchi, Hanyu et al. 2003, Yoshida, Niki et al. 2003).

Oats also contain a class of antioxidants, unique among the cultivated cereals, known as avenanthramides (Collins 1989). Avenanthramides are a group of N-cinnamoylanthranilate alkaloids derived from hydroxycinnamic and hydroxybenzoic acids with anti-proliferative, anti-inflammatory and anti-atherogenic properties and make up the majority of unbound phenolic antioxidants in the oat seed kernel (Emmons and Peterson 1999, Emmons and Peterson 2001). Like the tocotrienols/tocopherols, avenanthramides show a good bioavailability in humans (Nie, Wise et al. 2006, Nie, Wise et al. 2007) Avenanthramides have also been suggested to be a causative agent for the characteristic taste of oats (Molteberg, Solheim et al. 1996).

In addition to antioxidants, oat also contains plant sterols, known to lower cholesterol levels significantly in humans (Ostlund 2002) and like most cereals, Vitamin-B in the form of folate (Goldberg and British Nutrition Foundation. 2003).

Oat is naturally free of glutenin and gliadin, the main storage protein in oat instead being avenin, and is therefore safe for most sufferers of celiac disease (Janatuinen, Pikkarainen et al. 1995). However, as oats are often processed at the same site as wheat and barley, small amounts of gluten contamination could be present in the finished product. Several companies are now making “pure oat” products in dedicated plants to prevent cross-contamination, specifically targeting the celiac disease-segment of the market. However, the lack of gluten limits the use of oat in baking, as bread made from oat flour do not rise as well as bread made from

wheat. Oat also contains high levels of essential amino acids and the highest levels of globular proteins among the cereals (Welch 2011).

Finally, oat has large amounts of soluble dietary fibers in the form of arabinoxylans and β -glucans (1-3;1-4 mixed-link β -D glucan). On average, the oat seed kernel contains ~16% protein, 7% fat, ~10% dietary fiber (whereof 4.5% β -glucan) and ~63% starch (Asp, Mattsson et al. 1992).

The Future of oats

Heart disease and obesity-related illness is a growing problem, not only in the west but also on a global scale. For the first time in history, deaths related to cardiovascular disease overshadow deaths related to malnutrition and starvation. Humans are now eating themselves to death. To reverse this trend, and reduce pressure on the public health system, new dietary habits need to be introduced and the change needs to be supported by food products with improved health properties. Oats already contain a number of compounds with established positive health effects but these could be enhanced even further by focused breeding efforts and by employing new trait development techniques based on molecular methods. In addition, other traits can be improved further to increase the adaptation of oat as a rotational crop, strengthening the root system and thus improving soil-binding capabilities. Another area of improvement could be heightened resistance to stresses, both abiotic, such as salt and drought, as well as biotic stresses, such as diseases and fungi.

β -Glucans

β -Glucans have been linked to cholesterol lowering and blood-glucose stabilization in humans. The high levels of β -glucan in oat and barley kernels are the direct cause for health claims in the European Union, Canada and the United States (EFSA Panel on Dietetic Products and Allergies 2010, EFSA Panel on Dietetic Products 2011). According to the conclusions drawn by EFSA, there is a cause-effect relationship between β -glucan and a reduction in blood cholesterol levels provided at least 3 grams/day of β -glucans from oats, oat bran, barley, barley bran or a mixture of minimally processed β -glucans from the two cereals are consumed (EFSA Panel on Dietetic Products and Allergies 2010, EFSA Panel on Dietetic Products 2011).

β -Glucan is a linear poly-glucose fiber that is chiefly found in the cell wall of the *Poales*, but also in horsetail grass (*Equisetum arvensae*) (Sorensen, Pettolino et al. 2008), fungi (*Aspergillus fumigatus*) (Fontaine, Simenel et al. 2000), lichen (*Cetraria islandica*) (Wood, Weisz et al. 1994), algae (Popper and Fry 2003), bryophytes (Popper and Fry 2003) and the plant pathogen *Rhynchosporium secalis* (Pettolino, Sasaki et al. 2009) among others. The amount of β -glucan in the seed kernel of the *Poales* also varies drastically with oat and barley generally containing between 2 and 8%, rye 1.5-2.5% and wheat 0.5-1.5% (Marlett 1993). Corn and rice also contain β -glucan but at negligible levels (<0.5%) (Marlett 1993).

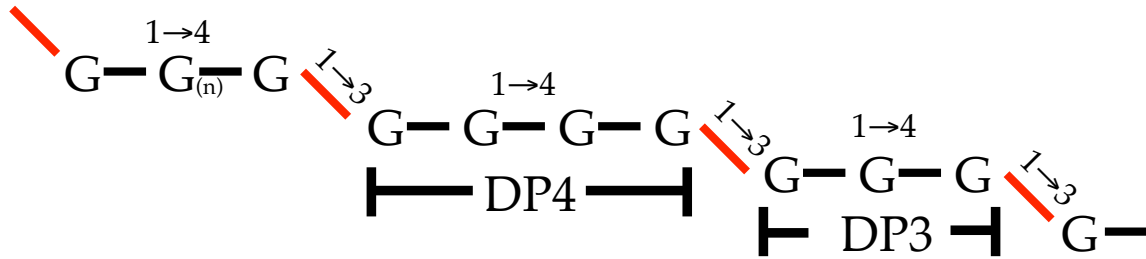


Figure 2: The “kinked chain” structure of β -glucan.

Oligomers comprised of 1-4 linked glucose molecules are in turn linked together by 1-3 bonds. The two most common oligomer lengths are trimers and tetramers. These are usually referred to by their degree of polymerization (DP), a trimer thus becomes DP3 and a tetramer becomes DP4.

Structure

The backbone of the fiber is made up of glucose molecules linked by 1-4 bonds similar to those in cellulose, but interspersed with the 1-4 bonds are single instances of 1-3 bonds, linking short oligomers of 1-4 bonded glucose molecules together in a kinked chain. The 1-3 bonds are much less rigid than their 1-4 counterparts, allowing for torsion around the length of the 1-3 bond, increasing flexibility of the fiber as a whole. This flexibility allows β -glucan fibers to retain water-solubility and associate with other β -glucan fibers forming large gel-like matrices that are pliable and porous enough to allow water to pass through the matrix.

The two main components of the fiber are trimers and tetramers (cellotriosyl and cellotetraosyl respectively), however, the ratio between the two, also known as the DP3/DP4-ratio differs markedly between different species and can be used as a key distinguishing feature (Wood, Weisz et al. 1991, Wood, Weisz et al. 1994). Oat has the lowest DP3/DP4 range among the cultivated cereals followed by barley and then wheat (Cui, Wood et al. 2000). The amount of DP3 in all three is always greater than DP4 and the DP3/DP4 ratio is thus always greater than one. In horsetail grass, the situation is reversed and DP4 is the most common oligomer (Sorensen, Pettolino et al. 2008).

In addition to trimers and tetramers, pentamers up to nonamers are detectible using HPLC (Lazaridou and Biliaderis 2007), sizes up to DP14 can be seen on high performance anion-exchange chromatography (HPAEC) systems (Lazaridou, Biliaderis et al. 2004) and DP15 and DP16 oligomers have been detected using MALDI-TOF (Sorensen, Pettolino et al. 2008). Approximately 90% of the β -glucan fiber is made up of DP3 and DP4 oligomers whereas the remaining 10% are in the range of DP5 to DP14 (Wood, Weisz et al. 1991, Tosh, Brummer et al. 2004). The long stretches of 1-4 linkages, known as cellulose-like domains, can conceivably provide a secondary interface of association between β -glucan fibers using hydrogen bonds (Izydorczyk and MacGregor 2000). There also seems to be a link between the DP3/DP4-ratio and solubility of the β -glucan fiber, it has been suggested that this may be due to the fact that an arrangement of three cellotriosyl units in a row can form a stable crystalline structure. The structure would limit flexibility, and in turn, solubility

(Tvaroska, Ogawa et al. 1983). This observation is further strengthened by the differences in gelation time between the cereals, i.e the time it takes for the solubilized fiber to go from viscous solution to a gel-like state (Lazaridou, Biliaderis et al. 2004). Oat has the longest gelation time of the three cereals, suggesting a high degree of flexibility in the fiber structure and a good ability to remain as a viscous solution, while barley has an eightfold shorter gelation time and the gelation time of wheat is sevenfold shorter than barley (Lazaridou, Biliaderis et al. 2004). The concentration of DP5+ oligomers do not differ significantly between the cereals and it is thus unlikely that this change is caused by different amounts of cellulose-like domains reducing overall solubility (Lazaridou, Biliaderis et al. 2004).

It is interesting to note that the degree of polymerization does not follow a linear curve in oat, wheat and barley. Instead, the lowest recorded levels in the DP3-9 range are those of DP7, followed by a small concentration increase of DP8 and DP9 oligomers. In addition, the concentration of DP8 is proportional to the concentration of DP4 and the concentration of DP9 is inversely proportional to the concentration of DP3 (Lazaridou, Biliaderis et al. 2004). The conservation of this relationship across all three cereals may indicate a non-trivial relationship between different length oligomers in the β -glucan fiber and needs to be explored further.

The direct amount of β -glucan is commonly measured using the McCleary-method (McCleary and Codd 1991). Soluble fiber is extracted using a mild sodium phosphate buffer in a boiling water bath followed by digestion using 1-3;1-4- β -D glucanase (lichenase). Lichenase selectively cleaves the fiber after each 1-3 bond, producing short fragments of fiber. These short oligomers can then be further digested into glucose using β -glucosidase. The increase in free glucose in the sample after the glucosidase digestion is directly proportional to the amount of β -glucan in the sample. This technique is commonly used in industry to measure β -glucan content in a wide variety of samples.

The molecular weight distribution of β -glucan varies between species but also within species (Storsley, Izydorczyk et al. 2003). However, an important factor in molecular weight determination is the extraction method used, as it is a potential source of differences. Extraction using common methods such as boiling water and sodium hydroxide can extract different populations of fiber with different solubility and can, by themselves, cause degradation of the fiber (Cui and Wood 2000). It can therefore be very complicated to get a true overview of the molecular weight distribution within a sample as each method only provides a subset of the true distribution.

The function of β -glucan in the plant cell wall

Plants can be divided by the composition of their cell walls into type-I and type-II walls. Type-I cell walls are found in dicots and non-commelinoid monocots while type-II walls are found in the commelinoids, a subsection of which is the *Poales*. In the type-I cell wall, cellulose is encased in a gel-like layer formed by pectins to provide elasticity and stability. By contrast, the type-II cell wall contains much less

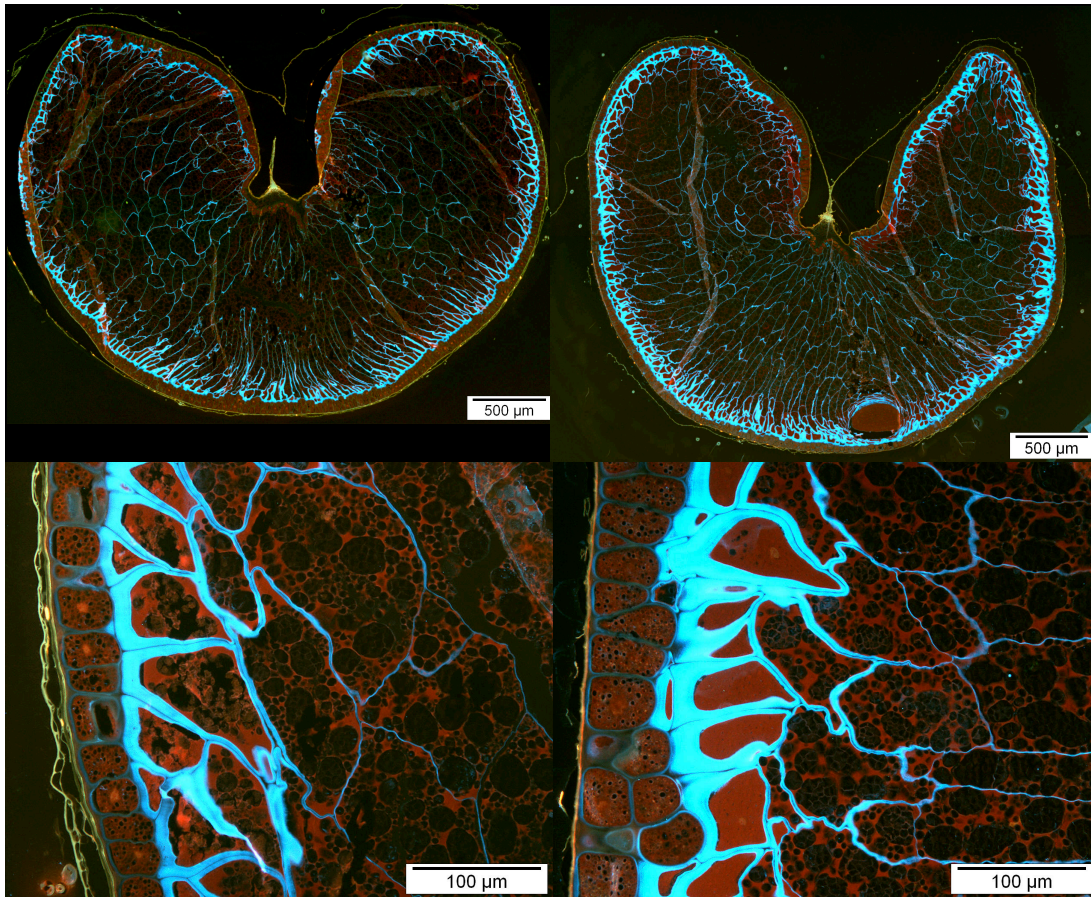


Figure 3: β -glucan in oat seed kernels visualized by calcofluor

Calcofluor-stained β -glucan appears blue (**Top Left**) Seed cross-section of the commercial cultivar *SW Belinda*. (**Top Right**) Seed cross-section of the high β -glucan mutant CT828. (**Bottom Left**) Magnified view of the aleurone layer in *SW Belinda* (**Bottom Right**) Magnified view of the aleurone layer in CT828.

pectin, and these attributes are instead provided by β -glucan together with arabinoxylans (Carpita 1996). During cell elongation arabinoxylans and β -glucans accumulate to high levels in the cell wall. β -Glucan can, by itself, constitute as much as 20% of dry cell wall mass during peak elongation (Kim, Olek et al. 2000). The high levels are rapidly turned over once the elongation has ceased, returning to pre-elongation levels.

The gel-layer formed by β -glucans in the cell wall may also act as a defensive wall, protecting the cell from invasion by fungi but also providing a potential signal system to indicate when such an attack is under way. As fungi invade plant cells it releases β -glucanases intended to digest the protective gel-layer surrounding the cell, slowly dissolving the protective layer of β -glucans and exposing the cell membrane. A recent study on rice β -glucan knock-out mutants have shown a spontaneous lesion-defense response phenotype, possibly suggesting that β -glucan fibers work as a repressor of a signal cascade aimed at programmed cell death (Vega-Sanchez, Verherbruggen et al. 2012). The same study also revealed an increase in the

brittleness of stem tissues at low cell-wall β -glucan concentrations lending some credence to the proposed function of improving pliability and elasticity of cells.

The third function is that of energy deposit in elongating cells and in the seed. The breakdown of β -glucans into glucose is relatively straightforward and involves only two enzymatic steps, allowing quick mobilization compared to the more long-winded mobilization process of starch (Fincher 2009). The rapid accumulation of β -glucan in the elongating cell may, in this light, hold a double purpose. In addition to the stability increase described above, it may also act as a quickly mobilized source of glucose. This theory seems to be corroborated by the fact that barley seedlings when moved from light to dark show increased expression of β -glucan endohydrolases and glucolases, suggesting that the plant is mobilizing glucose reserves stored in β -glucan to compensate for the decrease in photosynthetic activity (Roulin, Buchala et al. 2002). It may also be the case that plant tissues not traditionally associated with photosynthetic activity use β -glucan as a storage vector for glucose in addition to, or instead of, starch. This may also help explain the high concentration of β -glucan seen in cereal seeds, where up to 18% of the total glucose is comprised of β -glucans (Morrall and Briggs 1978), as germination occurs without the aid of photosynthesis and require sizable deposits of easily accessible energy (Burton and Fincher 2009).

Synthesis

The existence and structure of β -glucans have been known for some time, but the biochemical pathway involved in β -glucan biosynthesis has remained a mystery. It was proposed early that the site of β -glucan synthesis was located in the membranes of the Golgi apparatus; as such membranes isolated from maize coleoptiles retained the ability to synthesize β -glucan *in vitro*. This study used gel-permeation chromatography to detect β -glucan fibers and HPLC to detect β -glucan oligomers from endoglucanase-digested samples (Gibeaut and Carpita 1993). This claim has recently been restated, this time using immunocytological labeling in maize coleoptiles (Carpita and McCann 2010). However, tests carried out in barley during the cellularization of the endosperm showed no evidence of β -glucan inside the cell or in the Golgi membrane, even when high levels were detected in the cell wall (Wilson, Burton et al. 2006). In this case, β -glucan specific monoclonal antibodies were used to detect the presence of β -glucan and the authors suggested that the fiber could be heavily methyl-esterified while inside the cell, preventing antibodies from binding. After export, the fiber would then be de-esterified, exposing the epitope once again. This type of mechanism has previously been observed in pectins (Ridley, O'Neill et al. 2001).

Another theory suggests that the assembly of β -glucan fiber may actually be a two-step process where shorter fragments are synthesized within the Golgi-membrane for subsequent transport to the cell membrane (Fincher 2009). In the cell membrane short oligomers are then further assembled into long fibers as they pass through the membrane and are deposited into the cell wall. In this case, associated transport molecules could presumably block the epitope of the antibody on the monomer. This

would explain the perceived absence of β -glucan within the cell. Another issue addressed by this second hypothesis is that of the practical aspects of synthesizing large viscous fibers, with high molecular weight and gel-like properties, within the cytosol. Conceivably, there must then be advanced mechanisms in place preventing the gelation to commence before the fiber is exported through the cell membrane and into the cell wall, mechanisms that are unnecessary in the case of short oligomers.

Genetics

While a putative location of synthesis was identified early (Gibeaut and Carpita 1993), nothing was known about the genes involved in the biosynthesis; early studies proposed that some members of the cellulose synthase (*CesA*) gene family might be involved in producing mixed link β -glucans, mannans and other polysaccharides as well as cellulose (Dhugga 2001). The main candidate for β -glucan synthesis among the cellulose synthases was believed to be *CesA5* but this claim went unverified and the true β -glucan synthases remained elusive.

The starting point for a new era of cell wall genetics came with the sequencing of the rice genome and subsequent identification of a large family of genes known as the “cellulose synthase-like” genes (*Csl*) of which the *CesA* family is a subgroup. This family could be further divided into several other subclasses, namely: *CslA*, *CslB*, *CslC*, *CslD*, *CslE*, *CslF*, *CslG* and *CslH*. Recently, another subfamily has been identified in certain cereals, *CslJ* (Farrokhi, Burton et al. 2006). Of these, *CslF* and *CslH* are only present in the *Poales*, *CslB* and *CslG* are only seen in dicots (*Arabidopsis*). Despite being present in sorghum, wheat, barley and maize, no *CslJ*-genes have been identified in *Brachypodium* or rice.

The ability of some *CslF* genes to synthesize β -glucans was elegantly proven by the cloning of *CslF2* and *CslF4* from rice into *Arabidopsis*, a dicot that has a Type I cell wall that does not contain β -glucan (Burton, Wilson et al. 2006). However, mutants carrying the cloned *OsCslF2* and *OsCslF4* genes both displayed detectible levels of β -glucan in the cell walls as visualized by immunogold labeled antibodies. Despite that the 35S-constitutive promotor drove the expression of the two cloned genes, the amounts of produced β -glucan remained low. This suggests that the *F2* and *F4* genes would normally rely on one or more cofactors, possibly other *CslF*-genes, to drive β -glucan production, cofactors that were either not expressed or even present in the *Arabidopsis* genome. The gene cluster in rice was identified using QTLs (Quantitative Trait Loci) important for β -glucan production in barley, located between the *Adh8* and *Bmy2* markers on chromosome 2H, the same region in rice, located on chromosome 7, revealed no less than six *CslF* genes, tightly clustered (Burton, Wilson et al. 2006). Two more *CslF*-genes were also identified on rice chromosome 8 and 10. Several of the *CslF* genes have now been identified and mapped in many of the sequenced grass genomes, including *Brachypodium* and wheat.

In addition to *CslF2* and *CslF4*, five other genes have been functionally tested and identified as β -glucan synthases: *CslF3*, *CslF6*, *CslF8*, *CslF9* and *CslH1*. The *CslH*-family is a three-member group in rice and a single gene in barley, the activity

of which was assayed in a way identical to the early experiments where *Arabidopsis* was transformed with monocot *CsIF*-genes, now with the added step of analyzing the oligomers produced using β -glucan endohydrolase and HPAEC (High Performance Anion Exchange Chromatography) (Doblin, Pettolino et al. 2009). The gene *CsIF6* has now been conclusively linked to β -glucan production in wheat, barley and rice through a variety of experiments. Overexpression of *HvCsIF6* using both a constitutive and an endosperm specific promoter increased the levels of β -glucan in the leaf and seed respectively, although leaf overexpression seemed to be lethal in many cases (Burton, Collins et al. 2011). This could suggest that the β -glucan-producing pathway is competing with other pathways for substrate, negatively impacting viability. In this study, it was also noted that in the *HvCsIF6* overexpressor β -glucans of relatively lower DP3/DP4 ratios were produced compared to *HvCsIF4* overexpressors, hinting at different functions for different *CsIF*-genes (Burton, Collins et al. 2011). However, as there also seems to be a difference depending on which promoter was used for overexpression, there may be other factors affecting the results as well.

Sharp decreases in β -glucan production has also been observed in *CsIF6* knockouts: RNAi inhibition of *CsIF6* in wheat lead to a 42% reduction of β -glucan in the mature grain and a β -glucan lacking trait in barley (*bgl*; *beta-glucanless*) was linked to a functional mutation in *HvCsIF6* (Nemeth, Freeman et al. 2010, Taketa, Yuo et al. 2012). In addition, a rice *CsIF6*-knockout displayed a 97% reduction of β -glucan in coleoptiles and an increased activation of plant defense responses (Vega-Sanchez, Verhertbruggen et al. 2012).

The expression levels of the *CsIF* family have been extensively studied in barley tissues and during barley seed development (Burton, Jobling et al. 2008) with members of the gene family displaying remarkable differences in expression between different tissues. *HvCsIF6* is expressed in almost all tissues and during seed development but other members seem to be more specialized, being expressed in short bursts during particular stages of seed development or in a specific tissue. These include *HvCsIF3* that is predominantly found in the coleoptile and root tips, *HvCsIF9* that is found in coleoptile, root and the developing seed 8-10 days after pollination as well as *HvCsIF7* that is found in the stem and peduncle (Burton, Jobling et al. 2008). The difference in which genes are expressed in which tissues might suggest that the functional role of the fiber may differ between different tissues and that the *CsIF*-genes are controlled by tissue specific factors. The tight regulation of the *CsIF*-gene family is especially evident during seed development where at least four genes are expressed in very specific phases. This could indicate a multi-stage assembly process where genes early in the pathway are expressed first followed by subsequent members in the pathway. Using this model *HvCsIF6* would be among the last in a long chain of cellulose-like synthases involved in β -glucan synthesis. It has been established that β -glucan accumulation happens during late stages of seed development, matching the expression of *CsIF6* (Brown, Lemmon et al. 1997).

Another explanation for the tissue-specific expression could be that the permeability and thickness of the cell wall is tightly regulated. Thus, by using

different cellulose synthases, β -glucans with subtle differences in structure can be synthesized. By altering the DP3/DP4 ratios of the formed β -glucans the gelation properties of the cell wall could be altered and customized for the specific tissue or developmental stage.

Although strides have been made in elucidating the function of the β -glucan synthases there is still work to be done on fully exploring the complexities of synthase gene regulation and what roles the different members of the *CsIF* and *CsIH* gene families play in β -glucan production. More efforts could also be made to compare different species with regard to how the *CsIF*-genes are expressed and if this somehow correlates to structural differences of β -glucan fibers.

Health Benefits of β -Glucans

The health claim for oat and barley is based on the presence of high levels of β -glucan. β -Glucans have been linked to cholesterol lowering and blood glucose stabilization by lowering of the glycemic index (GI) of foodstuffs.

The postprandial glucose stabilization effect has been demonstrated using various foodstuffs in human trials including breakfast cereals, drinks, bread and granola-bars (Wood 2007). The mechanism of action for β -glucan in the gut is not known but several models have been proposed, mainly centered on the ability of β -glucan fiber to generate viscous solutions at high molecular weights (Wood, Weisz et al. 1994). A viscosity increase in the meal bolus can trap glucose and sugars within it, slowing the absorption of sugars into the blood. The slower release, in turn, leads to a slow increase in insulin levels, preventing rapid insulin spikes and decreasing the chances of insulin attenuation as seen in patients with type II diabetes (Braaten, Scott et al. 1994, Tappy, Gugolz et al. 1996). This theory is further strengthened by the fact that several studies have observed a dose-response relationship between β -glucan content and postprandial glucose load, and that acid hydrolysis of the soluble fibers has been shown to decrease the beneficial effects (Jenkins, Wolever et al. 1978, Cavallero, Empilli et al. 2002, Jenkins, Jenkins et al. 2002). Additionally, it has been suggested that β -glucans might directly interfere in starch degradation by either limiting the access of starch degrading enzymes, or the amount of water available for starch hydration (Regand, Chowdhury et al. 2011, Brennan, Derbyshire et al. 2012).

The cholesterol lowering effect of β -glucans was discovered indirectly as it had previously been established that viscous polysaccharides could lower LDL-cholesterol levels. The results seen in oat were thus attributed to β -glucans and the basis of the original health claim (Food and Drug Administration 1997). However, controversy still surrounded the claim as several studies saw little to no effect at the minimum daily intake of 3 grams of β -glucan. The focus of early studies on whole oat rather than β -glucan isolates have complicated subsequent analysis (Ripsin, Keenan et al. 1992). However, four studies have instead used purified β -glucan rather than oat or barley extracts. Of these four studies, two saw an average decrease in LDL cholesterol of 10 and 13% respectively compared to the starting value, using oat β -glucan with mildly hypercholesterolemic patients (Braaten, Scott et al. 1994, Behall, Scholfield et

al. 1997). A third study, using barley β -glucan, saw a 3.8% decrease in LDL-levels, also using mildly hypercholesterolemic patients and a fourth study saw no detectible effects on young, healthy normocholesterolemic patients using oat β -glucan (Beer, Arrigoni et al. 1995, Keogh, Cooper et al. 2003).

There are three suggested mechanisms for the cholesterol lowering effect. The first is the prevention of bile-acid reuptake. Bile acids are secreted in the small intestine, forming micelles and promoting uptake of lipids before being reabsorbed by the body in the colon. A viscous solution of dietary fiber can trap the bile acids, preventing reuptake, the bile acids instead being excreted. One of the bile-acid precursors is LDL-cholesterol and the continual loss of bile acid causes a decrease in cholesterol levels as the body converts more and more LDL-cholesterol into bile (Ellegard and Andersson 2007). Secondly, the viscosity may lead to decreased absorption of dietary cholesterol in the intestine (Theuwissen and Mensink 2007). Thirdly, β -glucans may downregulate cholesterol producing genes in the intestinal lumen and thus preventing *de novo* synthesis of cholesterol (Drozdowski, Reimer et al. 2010).

If the effect of β -glucans in lowering cholesterol is dependent on viscosity, the results may also be affected by the delivery system used. There are suggestions that solubilized β -glucans have a larger effect on cholesterol levels even at relatively low molecular weights than high molecular weight β -glucans baked into breads or cookies (Kerckhoffs, Hornstra et al. 2003). If this is indeed the case, it might explain some of the contradictions found in the studies. Another factor that likely plays a role is what other food is consumed together with the dietary fiber, as the effect would only be pronounced if sugars and lipids were consumed at the same time, driving glucose uptake and bile acid excretion respectively. More tightly controlled studies are needed in this area to conclusively and unambiguously link β -glucans to the cholesterol lowering effect seen in oat and barley.

Mutagenesis and TILLING

During the 20th century, mutagenesis has helped generate a vast amount of genetic variability and has played a significant role in plant breeding programs throughout the world. Records maintained by the joint FAO/IAEA Division in Vienna show that 2,965 crop cultivars, with one or more useful traits from induced mutations, were released worldwide over a period of 40 years. Notable examples are several wheat varieties (e.g. durum wheat used in pasta), barley including malting barley, rice, cotton, sunflower and grapefruit, resulting in an enormous positive economic impact.

The goal of mutagenesis in breeding is to cause maximal genomic variation with a minimum decrease in viability of the plant. This goal has been achieved in either one of two ways: through the use of radiation or chemical mutagens.

Among the radiation-based methods, γ -ray and fast neutron bombardment are most often employed. Of these, γ -ray bombardment is less destructive, causing point mutations and small deletions whereas fast neutron bombardment can cause translocations, chromosome losses and large deletions. Compared to chemical mutagens, both types of radiation cause major damage and severely reduce viability (Leung, Wu et al. 2001, Wu, Wu et al. 2005).

Chemical mutagens have become popular, as they are easy to use, do not require any specialized equipment and can generate a very high mutation frequency. Compared to radiological methods chemical mutagens tend to cause single base-pair changes or single nuclear polymorphisms (SNPs), rather than large deletions and chromosome translocations. The chemical mutagen EMS (Ethyl-Methanesulphonate) is today the most widely used. EMS selectively alkylates guanine bases leading to a favoring of the placement by the DNA-polymerase of a thymine residue instead of a cytosine residue opposite to the O-6-ethyl guanine during DNA replication. This results in a point mutation and the majority of the changes (70-99%) in EMS-mutated populations are therefore GC to AT basepair transitions (Till, Reynolds et al. 2004, Till, Cooper et al. 2007). In coding regions, such mutations can be silent, missense or nonsense. Outside coding regions, mutations can change critical promoter cis elements or other regulatory regions, resulting in up- or down-regulation of transcription. Aberrant splicing of mRNA, altered mRNA stability and changes in protein translation may also occur as a result of mutagenesis.

Other mutagens, such as Azide (NaN_3) and MNU (Methyl-NitrosoUrea) are also used and often combined into an Az-MNU solution. Mutagenesis using Az-MNU predominantly leads to GC to AT shifts, or vice versa. Thus, contrary to EMS the shift can happen in either direction (Till, Cooper et al. 2007).

During the last decade, the use of chemically induced mutagenesis has had a renaissance with the development of TILLING (Targeting Induced Local Lesions IN Genomes) technologies. As in conventional mutagenesis, TILLING seeds are exposed to a strong mutagenic compound, which introduce random mutations across the entire genome. However, mutagenesis is optimized to introduce as many mutations as possible in the target genome. After mutagenesis, the seeds (M1) are planted, allowed to self-fertilize and produce a new generation of seeds (M2). Typically, one seed from

each line is sown to produce an M2 population. Chromosomal DNA is isolated from every M2 plant, and is followed by a screening of the population at the DNA level.

Provided the number of mutations per genome is high enough and the size of the population is large enough, it is likely that a mutated allele of all genes in the genome exists somewhere in the population.

To determine the optimal size of a particular TILLING-population, the ploidy of the target crop should be considered. There seems to be a strong correlation between the polyploidy and the induced mutation frequency. It has been shown that as many as one mutation per every 25 kb can be introduced in a hexaploid plant without killing it or making it sterile, while the maximum mutation frequency of diploid plants such as rice and barley is much lower. Therefore, a hexaploid TILLING-population seldom needs to exceed 5,000 individual lines. Diploid populations, on the other hand, often need to be in the range of tens of thousands (Caldwell, McCallum et al. 2004, Uauy, Paraiso et al. 2009).

There are two approaches to screening a TILLING-population that can either be used separately or in combination. The first method is by using phenotypical screening techniques to detect the sought trait in the population and is especially useful when the visible number of phenotypes is high, suggesting high genetic variability. This method is however, reliant on the availability of high-throughput, low-cost and accurate methods to detect the desired phenotype. It also negates to address the issue of linking the trait to the underlying mutation in the identification step. Phenotypic screenings might be employed when the sought trait is complex, or the genetic mechanisms and pathways are partly known or completely unknown. Or to select the dominant mutation directly and thus avoid excessive crossings to combine different recessive mutations

The second method is a genetic screen, utilizing isolated chromosomal DNA from each mutated line. In this case, a gene (or genes) of interest needs to be identified beforehand. Early methods for screening TILLING-libraries relied on mismatch-cleavage techniques followed by separation on agarose or acrylamide gels. Fragments were amplified by PCR and then, through repeated heating/cooling cycles the amplified fragments from mutants were hybridized with wildtype fragments. An enzyme would then identify the characteristic bulge of the mismatched base in the mutant strand and cleave the hybridized PCR-product at that location. The result would be two shorter bands on the gel, their combined length equal to the wildtype band. Although efficient once set up, this technique requires the use of expensive specialized equipment and analysis software for high-throughput screenings (Till, Reynolds et al. 2003, Slade and Knauf 2005).

If the number of possible target genes is large, the first screening can be performed on the phenotypical level, reducing the number of candidate lines and allowing for a deeper analysis and identification of mutations in those few lines. Conversely, if the phenotype is weak or expensive to assay, lines with a mutation in the gene of interest would first be identified followed by a phenotypic assay.

Next Generation Sequencing (NGS) technologies now allows for identifying mutations at the whole genome level. Decreasing sequencing costs due to improved

technical accuracy, increased capacity and wide acceptance compared to only a few years ago has led to a large potential for NGS in TILLING. The two most commonly used NGS platforms today are the 454 GenomeSequencer FLX Ti (Roche Applied Science) and the Illumina Genome Analyzer. While the average read length for 454 is 750 basepairs, Illumina only gives 100 basepairs per read, or 2x100 in paired configuration. The total output per run, however, significantly favors the Illumina platform where up to 18 gigabases of data can be generated while a 454 run presently generates approximately 750 megabases.

NGS has been successfully applied to TILLING applications. Using 3-dimensional pooling it is possible to screen one or several genes of interest in a single FLX-454 run. Using a theoretical setup of several 96-well plates filled with DNA one PCR-reaction using uniquely barcoded primers would be performed on pooled columns and rows from all plates resulting in 20 separate PCR reactions (12 pooled columns and 8 pooled rows). In addition, DNA is pooled on a per-plate basis, with all 96 samples from each plate pooled into one. SNP-mutations in a single sample can then be correlated to the three barcodes after sequencing (column, row and plate) allowing for deconvolution of the data. Experiments suggest that as many as 12,000 samples may be analyzed simultaneously on a single 454-picotiter plate (PTP) using KeyPoint™ technology, successfully tested on a tomato TILLING-population (Rigola, van Oeveren et al. 2009). Illumina sequencing has also been adapted to high-throughput TILLING and has been used to screen bread-wheat, durum-wheat and rice populations (Tsai, Howell et al. 2011). The method, known as CAMBa (Coverage Aware Mutation-Calling using Bayesian analysis), succeeded in identifying several mutations that were missed by traditional mismatch-cleave TILLING as well as confirming already characterized mutations (Tsai, Howell et al. 2011). Compared to older mismatch-cleavage techniques NGS-TILLING does not require any specialized equipment, apart from the sequencing platform. It is also comparatively cheap and analysis tools are freely available. With the increased capacity of sequencing centers around the world prices will likely fall even further, making NGS-TILLING a competitive choice.

Once a putative trait has been identified and characterized, lines may need to be backcrossed into commercial cultivars. This is done to reduce the impact of non-trait mutations on yield and viability. Depending on the distribution of mutations in the plant, one or more backcrosses may be necessary to completely eliminate deleterious mutations. After each cross, plants have to be rescreened for the sought phenotype and self-crossed to obtain a stable phenotype. The process of backcrossing can therefore be both time consuming and complicated. To our knowledge, no TILLING-mutant has yet been the source of a new variety although work is proceeding in this area.

Transcriptomics

The transcriptome is defined as the complete set of transcripts within a cell or tissue and their relative abundance with regard to cell or tissue type, developmental stage or physiological situation. As the number and types of transcript continuously fluctuate and change, an analysis of the transcriptome can provide a unique insight into the dynamics of biological processes and networks that merely a genomic sequence is unable to supply. Before the current era of genomics, a transcriptome analysis would either be performed directly through hybridization experiments or using microarrays. Although microarray studies could provide valuable information, they were limited by current knowledge of expressed genes and availability of manufactured microarray plates for the specific organism of interest. Microarrays also lack a wide dynamic range due to background saturation and cross-linking of gene products, meaning that highly or lowly expressed genes will not stand out (Okoniewski and Miller 2006).

With the revolution in sequencing technologies during the last half decade, researchers have gained an enormously powerful tool to elucidate complex functions within living organisms. By querying the entire unfiltered transcriptome, rather than a subset of previously identified genes as in microarray experiments, new information on previously unknown genes can be elucidated. Before the era of next generation sequencing (NGS), sequence data would be obtained by EST-sequencing (expressed sequence tag). In this method, mRNA is reverse transcribed and cloned into sequencing vectors followed by Sanger sequencing. An example of this workflow, where 9,792 oat ESTs were generated can be seen in the work of Bräutigam et al. (Brautigam, Lindlof et al. 2005). Although Sanger sequencing is sensitive and robust, the throughput is very low and the price-per-base is high compared to current technologies.

As the transcriptome is only a subset of the complete genome sequence, a transcriptomic atlas can be both cheaper and easier to obtain than a full genome sequence. However, RNA-libraries from several different tissues at many different growth stages need to be sequenced in order to obtain a comprehensive view of the total transcriptome. Compared to genome sequencing this might still be preferable, as non-coding genomic sequence can be highly repetitive and thus difficult to sequence. The current generation of sequencing technologies is able to generate immense amounts of data in a comparatively short time. However, the challenge is no longer how to generate the primary sequence data but how to handle it efficiently.

New transcripts can be identified after a *De Novo* assembly of reads from the sequencing platform of choice. During the assembly process, overlaps between the reads are calculated and the reads joined together. This results in one or more contigs from each expressed transcript. Current assembly software use DeBruijn-graphs to divide each read into shorter words, each word having (n-1) overlaps with the previous and following word (Zerbino and Birney 2008, Li, Zhu et al. 2010, Gnerre, MacCallum et al. 2011). Searching the entire dataset a directed graph is constructed of all connected words, where one word connects to two other words downstream instead of one, a fork is formed and word connection continues in the two forks.

Finally the graph is collapsed into contigs and if a chain of words contains a fork, two contigs will be generated containing the consensus sequence and one fork each. The use of words rather than full reads reduces the complexity of overlap detection and, by extension, the computational intensity.

Assemblies can be further improved by the use of paired-end data. The Illumina genome analyzer uses this type of sequencing extensively. In the case of paired end reads, a stretch of cDNA is sequenced from both ends simultaneously and although the entire fragment is not sequenced, the distance between the two sequenced ends is known. Using the distance information, paired reads with one read on one contig and the other on another contig allows the two contigs to be joined into a scaffold of contigs. This technique is especially useful in genome sequencing where long stretches of repetitive sequence can be bridged by scaffolds.

After all contigs are generated, the original reads can then be mapped to contigs one by one and counted, resulting in expression data, as highly expressed genes will have comparatively more reads mapping to them. If available, finished contigs can also be mapped to the genome sequence, allowing for discovery of new splice forms, modeling of transcription factor networks and epigenetic studies of expression (Slater and Birney 2005). If an annotated genome sequence is not available, assembled contigs need to be identified using BLAST against closely related species.

After contigs are identified as coding for a certain protein the contig can be functionally annotated using Gene Ontology (GO) (Ashburner, Ball et al. 2000). GOs are divided into three subsets: Molecular Function (F), Biological Process (P) and Cellular Component (C). Each of the three subsets in turn takes the form of a tree graph with the top node being the most general definition of function or location. Additional levels denote more and more detailed information while retaining a link to the level above, and thus by extension, to the root of the tree. A high level annotation contains all information pertaining to a certain contig from the root of the tree down. This allows contigs to be assigned to large functional groups as well as unique positions inside groups. The separation of C, F and P also allows biological processes to extend over several compartments in the cell. To further illustrate the place of a specific contig in the context of cellular function, contigs are often mapped to KEGG-pathways (Kyoto Encyclopedia of Genes and Genomes). A KEGG-pathway describes the actual pathway in many metabolic processes as deduced by experimental data. Combining KEGG-pathways with time spaced or stress induced RNA-seq data can provide insight into signal cascades and varying expression within metabolic networks. The Gene Ontology information can also be used in combination with expression clustering, where genes with similar expression patterns are grouped. The combination of matching Gene Ontologies and matching expression is a common way to identify co-expressed genes.

Although immensely powerful, RNA-seq comes with its own set of problems and limitations. Current sequencing methods have an error rate in the range of 1-0.1%, and to accurately assemble data, a great deal of redundancy is required (Glenn 2011). Furthermore, mapping of expression to contigs relies on the uniqueness of short sequence reads, these can in some cases be part of conserved regions of genes

making it impossible to determine from which gene the fragment in question originates. Finally, technical aspects of library preparation and sequencing can adversely affect the result by introducing artificial bias, in addition to biological differences, necessitating pooling of several individuals, biological duplicates or verification using alternate methods such as qPCR.

With the current increase in sequencing and computer power, research now has the opportunity to shift from gene-centric to transcriptome wide studies. This allows researchers to make new inferences about hitherto unknown co-expressors and study the transcriptome as a whole, rather than as a controlled subset of total transcription as is done in microarray studies. A wealth of transcriptomic and genomic data can also be of use in marker assisted breeding and comparative genomics.

miRNA

The ability of short chains of RNA to inhibit gene expression was discovered in *C. elegans* more than two decades ago. Extensive research has now revealed miRNA to function as a regulator of post-transcriptional gene expression, translation and chromatin modification in plants and mammals, providing a separate pathway for controlling gene expression and epigenetic changes.

Most miRNA genes are transcribed by RNA polymerase II and the promoter regions are similar to protein encoding genes, including a TATA-box and other basic cis-elements (Meng, Huang et al. 2009). After transcription, the primary miRNA (pri-miRNA) forms into an imperfect stem-loop that is recognized by DICER-like proteins in plants and sequentially cleaved into precursor-miRNA (pre-miRNA) and finally into a mature 21-25 nucleotide long miRNA-duplex (Voinnet 2009). After the two miRNA strands are dissociated they are mostly incorporated into Argonaute associated miRNA induced silencing complexes (Ago-RISCs). The miRNA template then guides RISCs to those mRNAs that have a highly complementary sequence, where it subsequently binds to the 3' untranslated region (3'-UTR). Once bound, the complex blocks translation initiation by interfering with ribosome recruitment as well as ribosome elongation (Olsen and Ambros 1999, Humphreys, Westman et al. 2005). The exact mechanism behind how the RISC-complex elicits the two functions remains unclear, although it may include recruitment of secondary proteins. Furthermore, the bound complex then mediates deadenylation of the 3' end of the mRNA followed by decapping and degradation (Giraldez, Mishima et al. 2006, Wu, Fan et al. 2006). Although seemingly straightforward, there are several other factors that affect the efficiency of the silencing. Among other things, the level of sequence complementarity as well as the target abundance has an impact of the silencing as RISCs with low complementarity sequences catalyzing fewer rounds of cleavage before being broken down (Arvey, Larsson et al. 2010). The primary mechanism of action also differs between plants and animals, where translational inhibition is more common in animals and degradation is more common in plants.

The majority of miRNA targets in plants are transcription factors. Consequently, miRNA expression can form complex interlaced networks of

regulation and feedback loops intertwined with normal gene expression. However, as miRNA act on already transcribed products they can be a way for the plant to quickly stop translation of genes in conjunction with slower transcriptional repression.

The PHR1-miR399-PHO2 pathway in *Arabidopsis* exemplifies this type of fast response. In this case, the phosphate starvation response factor (PHR1) upregulates a miRNA gene that selectively inhibits translation of the gene *pho2*. Lack of *pho2* in turn, starts a signaling cascade leading to expression of several phosphate starvation genes, quickly shifting the plant into phosphate conservation mode (Bari, Pant et al. 2006).

Despite the work put into understanding the complex connections between miRNA synthesis, inhibition and gene expression little is still known about the precise mechanisms of action. It is clear, however, that the miRNA regulatory networks may be as complicated and important as traditional gene expression to the organism.

2. Scientific aims

Overall aims

The overall aim of the thesis was to develop an oat with β -glucan levels exceeding commercial Swedish varieties and to provide insight into the transcriptomic regulation of β -glucan associated genes.

Specific aims

1. Identify key genes in the oat β -glucan biosynthetic pathway in oat and characterize their expression
2. Modify biosynthesis of β -glucans in oat using mutagenesis
3. Identify novel oat phenotypes and characterize their β -glucan structure
4. Establish tools and data for further development of oat genomics and characterization of bioactive compounds

3. Current Study

Paper I

Development and characterization of an oat TILLING-library and identification of mutations in lignin and β -glucan biosynthesis genes

In this work we developed a mutagenized TILLING (Targeting Induced Local Lesions in Genomes) population in the commercial Swedish oat cultivar *SW Belinda*.

To optimize the population size and mutation density several different EMS-concentrations were tested and a kill-curve established. The optimal concentration of 0.9% (v/v) was used in the final library construction. Approximately 9,000 seeds were mutagenized and sown in greenhouses, seeds from the surviving 2,880 M1-plants were then harvested and a single seed resown. This resulted in 2,600 M2 plants out of which 2,550 produced M3 seeds. Several phenotypes were visible during the growth of the M2 generation, these included chlorosis (2.3%), semi-dwarves (1.3%), giants (0.6%), semi-dwarves (0.6%) and high tiller as well as early/late flowering and late senescence. In total, approximately 5% of the population had visually detectible phenotypes.

Genomic DNA was extracted from the leaves of each of the 2,600 M2 plants using the CTAB method. The mutation frequency was then established using RAPD-PCR, Sanger sequencing and MALDI-TOF analysis. The RAPD-PCR analysis suggested a mutation frequency of 1/20 kb, Sanger sequencing of *AsCsIF6* and *AsPAL1* indicated a frequency of 1/33 kb and MALDI-TOF analysis of the same genes reported a frequency of 1/38 kb. The MALDI-TOF analysis and Sanger sequencing of *AsPAL1* and *AsCsIF6* also identified six SNP mutations in *AsPAL1* and ten mutations in *AsCsIF6*. Of these, all but two mutations in *AsPAL1* were silent or tolerated according to their PSSM score.

To assay the population on the phenotypic level, 1,824 M3 seeds were stained using a phloroglucinol-HCL assay. This identified 17 mutants with a higher or lower lignin content in the husk compared to the commercial cultivar *SW Belinda*. The identified lines were then confirmed using a quantitative acetyl bromide method (Iiyama and Wallis 1988).

The sequences for *AsPAL1* and *AsCsIF6* were submitted to genbank (accession GQ373155 and GQ379900 respectively) and the mutagenized population will be submitted to Nordgen when enough seeds from propagation are available.

Discussion

The aim of this project was to develop a mutagenized library with a high mutation frequency, statistically ensuring a mutation in each expressed gene. This population can now be used to screen for mutations both at the phenotypic and genetic level. The identification of several *CsIF6* mutants even in the comparatively small screen suggests many interesting phenotypes exist within the entire population. The large number of visible phenotypes also indicates a high mutation frequency.

This population will now form the basis for identifying new β -glucan phenotypes in oat through forward and reverse genetics.

Paper II

Identification of high β -glucan oat lines and localization and chemical characterization of their seed kernel β -glucans

In this study we scaled down and optimized the McCleary mixed link β -glucan quantification method (McCleary and Codd 1991) and screened a previously developed TILLING library (**Paper I**) in the cultivar *SW Belinda* for changes in β -glucan accumulation in the seed kernel. In total, 1,700 lines were individually screened and several lines showing either high or low β -glucan levels were identified. The highest β -glucan content was found in the line CT828 (7.54%) and the lowest in CT837 (1.85%). Additionally, three commercial varieties were included in the analysis, namely *SW Belinda* (4.95%), *SW Betania* (4.84%) and *SW Kerstin* (4.24%). The top six high β -glucan mutants and four low β -glucan mutants were selected for further analysis.

β -Glucan was then stained with calcoflour and localized in the seed kernel using fluorescent microscopy. Several of the high β -glucan lines showed a significant accumulation of β -glucan in the aleurone layer. The highest accumulation was found in CT98 (7.13%) while in CT828 the β -glucans were more evenly distributed with a relatively higher expression in the starchy endosperm. In the low β -glucan mutants CT837 and CT2472 we saw significantly lower amounts of β -glucan in the starchy endosperm as well as in the bran. In addition, the three highest and two lowest β -glucan mutants were also characterized using longitudinal cross sections and a similar distribution of the β -glucans was seen as in the cross sections.

In addition to localization, the structure of the β -glucan fiber in the different mutants was characterized. The molecular weight and polydispersity was determined in extractions using sodium hydroxide and water. The two low mutants CT837 and CT2472 contained significantly lower molecular weight β -glucans when extracted using sodium hydroxide. In addition, CT837 also had significantly lower molecular weights when β -glucans were extracted using water. The lowest molecular weights were found in CT2472 when extracted using sodium hydroxide and CT1293 when extracted with water. The DP3/DP4 ratio was measured in all samples using lichenase digestion followed by HPLC separation. The highest DP3/DP4 ratio was measured in CT828 (2.72). All mutants had an equal or higher DP3/DP4 ratio compared to the commercial starting cultivar *SW Belinda* (2.39). Finally, the solubility of β -glucans

extracted using sodium hydroxide and water were measured using FIA (Flow Injection Analysis). Several mutants displayed altered solubility in both sodium hydroxide and water. The most significant change was seen in the low β -glucan mutant CT1293 that had a 4% reduction of solubility in fibers extracted using sodium hydroxide and a 13% reduction in solubility in fibers extracted in hot water. Several mutants also exhibited an increased solubility; CT98 showed a 4% increase in solubility in sodium hydroxide extractions and a 6% increase in water extractions. The highest solubility was found in the high β -glucan mutant CT970 where an increase of 8% was found in water-extracted samples only.

Discussion

In this paper we demonstrate that the increased variation induced in mutagenized populations opens up the possibility to identify new phenotypes in commercial cultivars. By screening 1,700 lines we identified a mutated line, which showed a 52% increase of β -glucan in the seed kernel compared to the original non-mutated *SW Belinda* cultivar. The fact that mutagenesis does not fall under GMO-regulation means that any new variety derived from this population can be commercialized without legal constraints. Furthermore, we highlight the structural differences found both within existing commercial cultivars and mutagenized lines. The fact that concentration, DP3/DP4 ratio, molecular weight and solubility all vary between mutants and commercial cultivars suggest that the critical mutation in each case is pleiotropic and affects both β -glucan level and structure.

The identified high and low β -glucan lines will now be further characterized using transcriptomics and direct sequencing in order to more specifically link the β -glucan phenotype to a genetic event, and elucidating how gene function and structural changes come together. In addition, β -glucans with altered structure can also be evaluated for their bioactive properties, once sufficient material has been obtained, allowing comparison of different β -glucan structures against the same genetic and biochemical background. This can be helpful in separating the β -glucan effect from an overall oat effect on cholesterol lowering and blood glucose stabilization.

Paper III

Generation of an oat (*Avena sativa*) transcriptome and expression analysis of transcription factors and mixed-link β -glucan biosynthesis genes

In this work we generate a high coverage transcriptome atlas from root, stem, leaf, crown, and seed development. Seed development covered 15 time points from the first emergence of the ear to 20 days post-completed ear emergence. In total, 190,261 contigs were obtained after sequence assembly, the average length was 649 basepairs and the N50-value (defined as the contig length where half the assembly is in longer contigs) was 858 basepairs.

The complete dataset was annotated using BLAST and gene ontology, 93,328 contigs had a significant hit in a BLAST-database and 71,921 of those had one or more associated Gene Ontologies. The top blast hit species was *Brachypodium distachyon* followed by *Hordeum vulgare*. All contigs without relevant BLAST-hits were *de novo* annotated using reciprocal BLAST against contigs with an existing BLAST-hit, TMHMM to detect putative transmembrane domains and SignalP to detect eukaryotic signal peptide domains. In addition, contigs were translated in all six frames and the longest open reading frame (ORF) recorded. Of the 96,933 contigs without BLAST-hits 9,746 contigs had a reciprocal BLAST-hit, 43,330 had an ORF longer than 60 amino acids, 24,932 contained a putative signal peptide domain and 16,618 contained a predicted transmembrane helix.

Gene Ontology annotated sequences were then analyzed according to Molecular Function, Cellular Component and Biological Process. The top Molecular Function annotations were Nucleotide Binding (39% of contigs) and kinase activity (18% of contigs). The top Biological processes were Response to Stress (10% of contigs), Catabolic process (10% of contigs) and cellular protein modification (9% of contigs). In total 48% of contigs were localized in either the mitochondrion or chloroplast. The dataset was further investigated by looking at annotations in contigs that were uniquely expressed in crown, leaf or seed. The unique leaf annotations contained a large portion of “cellular protein modification”-annotations (20%) and transport proteins (14%). The crown also contained large numbers of unique “cellular protein modification” (15%) annotations but also response to stress (11%). The crown also contained the largest diversity of unique annotations. The seed mostly contained “catabolic process”-annotations (12%) but also annotations related to cell growth (3%) and others. The annotated dataset was then used to investigate transcription factor expression, genes associated in avenanthramide synthesis and genes involved in β -glucan synthesis.

In total we identified 175 tissue specific transcription factors, 60 expressed in crown and leaf, 41 expressed during ear emergence, 12 during flowering, 24 during early seed development and 28 during late seed development. Four avenanthramide producing genes were identified and their expression characterized across crown, leaf and seed. Finally seven β -glucan synthase genes were identified in the dataset.

A phylogenetic analysis of the identified oat β -glucan compared to wheat and

barley β -glucan synthases revealed a previously uncharacterized *CsIF* gene tentatively named *CsIF15*. Aside from *CsIF15*, *CsIF3*, 4, 6, 8, 9 and *CsIH1* were also identified. *AsCsIF6*, *AsCsIF8* and *AsCsIF9* all showed close relation to their barley counterparts while *AsCsIF3*, *AsCsIF4*, *AsCsIF9* and *AsCsIH1* showed less conservation. Expression analysis of the β -glucan producing genes in seed, leaf and crown revealed high expression of several genes in the crown tissue while neither *AsCsIF4* or *AsCsIF9* were expressed in the leaf. All *CsIF* and *CsIH* genes were expressed during seed development.

In addition to characterizing expression across tissues, expression of all β -glucan synthase genes was also mapped over seven time points during seed development. This revealed that *AsCsIF4*, 6, 8 and *H1* all see heightened expression during later stages of seed development when much of the β -glucan accumulation in the seed kernel is thought to occur. These results were then compared to previously published material in barley. Comparisons showed that barley, contrary to oat only sees increased expression of *CsIF6* during later stages of seed development while expression of *CsIF4* and *CsIF8* remained low.

Discussion

This paper lays the groundwork for further genomic and transcriptomic research in oat. The diverse tissues queried and the large volume of reads makes it likely that a majority of the total oat transcriptome is now characterized. We identify several uncharacterized contigs that still contain protein like domains such as signal peptides, transmembrane helices and long open reading frames. These could be labeled as “unique oat proteins”.

We also demonstrate the versatility of the transcriptome expression data by identifying several tissue specific transcription factors, avenanthramide producing genes and β -glucan producing genes. The seed development time points also allow us to map expression of β -glucan genes during seed development. There are several interesting aspects of β -glucan synthase expression in this work, one of which is the very high expression values seen in crown. It has previously been argued that β -glucan might serve as an energy deposit in addition to its traditional role in providing stability to the cell wall and this might be the case in the crown, where little to no photosynthesis takes place. It is also possible that β -glucan is actively synthesized in the crown and then somehow exported into the stem as it elongates during growth. Further studies are needed in this area to fully explore the role of β -glucan in the crown.

The expression of β -glucan genes during seed development also hint at some interesting differences when comparing oat and barley. The differences in β -glucan structure between cereals are still unexplained. However, the increased expression of two additional β -glucan producing genes in addition to *CsIF6* in oat suggest that several different synthases act concurrently, either in separate pathways or in the same pathways producing different types of short oligomer precursors. As absolute levels of β -glucan in the seed kernel are similar in oat and barley, the expression of multiple β -glucan synthases apparently does not change β -glucan levels significantly.

This data can now be used to provide a stable baseline when characterizing the previously developed β -glucan TILLING-mutants (**Paper I**, **Paper II**). The dataset obtained in this paper will also be very useful to the oat community and plant researchers in general, providing data for comparative expression studies.

Paper IV

Identification and Classification of MicroRNAs in Oats (*Avena sativa*) by Deep Sequencing of Small RNAs Isolated from Various Tissues and Conditions

In this paper we extend the transcriptomic data obtained in **Paper III** by deep sequencing of small RNAs in several unique tissues as well as during seed development and cold and drought stress. In total 330,445,824 reads were obtained from unstressed crown, leaf, stem, root and seed development in addition to cold stressed leaf and crown as well as from drought stressed leaf tissue were obtained by Illumina sequencing. Seed development was further divided into four distinct developmental stages: Pre-flowering, Flowering, Early Seed Development, and Late Seed Development. After cleanup, removal of non-miRNA reads and removal of tags with an expression <1 TPM (transcript per million) a total of 63,219 plausible oat miRNAs remained in all sequenced libraries. A majority of these (36,698) were found in the Flowering seed development time point.

The 63,219 oat miRNAs were then further characterized by length, identification of isoMIRs and matches against known miRNAs through the miRBase-database. The length characterization revealed a bias towards 24 nt miRNAs in stem and during seed development while all other libraries aside from root showed a bias towards 21-22 nt miRNAs. The distribution in root was highly uniform across all lengths. IsoMIRs are miRNAs varying only by a few basepairs in length or composition. By assaying the number of miRNAs with length variants and the number of miRNAs with high sequence similarity a network could be inferred, connecting isoMIRs into groups. A total of 44.4% of all oat miRNAs had an isoMIR in the form of a sequence heterogeneity (38.5%), a length isoform (38.2%), or both (23.2%). Finally, annotation was done by matching the oat miRNAs to known miRNAs using the miRBase database. This resulted in confirmed 793 hits, although the majority of the dataset had no hits.

The oat miRNA population was then used in conjunction with the transcriptome atlas developed in **Paper III** to predict target genes for miRNA regulation. In total, 99.3% of all miRNA had a plausible target in the oat transcriptome atlas and 83.9% of all contigs with a significant BLAST-hit in the transcriptome atlas had at least one potential miRNA regulator. The large number of contigs with a potential miRNA regulator may in this case be due to the relaxed threshold values used in the analysis.

Discussion

Here we extend the transcriptomic atlas of oat by adding data to another regulatory layer, that of miRNA. The large number of length and sequence heterogeneities found in the oat miRNA data could suggest that identical miRNA genes exist in the different genomes, only differing slightly. The low number of confirmed oat miRNAs in miRBase also suggest that, like in previous studies, plant miRNAs have not yet been mined deeply enough and that many real miRNAs remain uncharacterized. It is interesting to see that the stem and seed development miRNAs seem to share similar length distributions. This suggests that miRNAs are partly shared between these tissues through the plasmodesmata, although more studies are of course needed to confirm this assumption however. The availability of miRNA data will be a valuable tool in future studies of tissue specific expression during development and under the influence of stress in oat.

4. Conclusions

In this work we developed a mutagenized TILLING-population in oat. We then screened the population for mutants with high or low β -glucan content. During the screenings we discovered mutants with up to a 52% increase and a 267% decrease compared to the β -glucan content in the starting, non-mutated variety. The specific β -glucan mutant lines were further characterized by analyzing the structure and distribution of the β -glucan fiber in the seed kernel as well as the solubility and molecular weight revealing differences in DP3/DP4 ratio, solubility, distribution and molecular weight.

To further our understanding of the oat genome, a high coverage transcriptome map of oat was developed, significantly increasing the amount of genetic data available to researchers. In addition, the quantitative aspects of the data will allow for comparison of expression and identification of novel gene pathways on oat. The transcriptomic data was then further extended by miRNA sequencing and expression analysis.

Major results from this work

- A mutagenized TILLING population was produced. This population has proved to have a high variation and can now be used for genetic and phenotypic screenings for many new traits.
- High and low β -glucan mutants, including those with altered β -glucan structure were identified and characterized. These can now be used as breeding material to develop novel high β -glucan cultivars. They can also be used to elucidate the influence of β -glucan structure on the cholesterol lowering and blood glucose stabilizing effects seen in humans. Finally, identified mutants can be used to study the transcriptomic alterations causing the high or low β -glucan content and the altered β -glucan structure.
- A high coverage transcriptome atlas of oat was developed and annotated. This atlas will be an invaluable tool for further research into gene regulation and novel trait development. A comprehensive transcriptome dataset will also be of great use in a future oat genome-sequencing project.
- The transcriptome atlas was further extended by sequencing and characterization of oat miRNA and their expression across a variety of tissues. These results can be used in conjunction with the transcriptomic atlas to further understanding of the regulatory networks governing gene expression in oat.

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