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Breeding for dual-purpose wheat varieties using marker-trait associations for biomass yield and quality traits

Pernille L. Malik^a, Luc Janss^b, Linda K. Nielsen^c, Finn Borum^c, Henning Jørgensen^a, Birger Eriksen^c, Jan K. Schjoerring^a, Søren K. Rasmussen^{a*}

^aDepartment of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

^bDepartment of Molecular Biology and Genetics, Aarhus University, Blichers Alle 20, 8830 Tjele, Denmark

^cSejet Plant Breeding, Nørremarksvej 67, 8700 Horsens, Denmark

*Corresponding author: skr@plen.ku.dk, Phone +45 35333436

ORCID iD: 0000-0002-2852-3298 (JKS), 0000-0001-6169-2504 (SKR)

Abstract

Wheat straw is a valuable feedstock for bioethanol production, but due to the recalcitrant nature of lignocellulose, its efficient use in biorefineries is limited by its low digestibility and difficult conversion of structural carbohydrates into free sugars. A genome-wide association study (GWAS) was conducted to search for significant SNP markers that could be used in a breeding program to improve the value of wheat straw in a biorefinery setting. As part of a three-year breeding program (2015-2017), 190 winter wheat lines were phenotyped for traits that affect the yield and quality of the harvested biomass. These traits included straw yield, plant height, lodging at three growth stages, and *Septoria tritici* blotch (STB) susceptibility. Release of glucose, xylose and arabinose was determined after hydrothermal pretreatment and enzymatic hydrolysis of the straw. The lines were genotyped using 15K SNP markers and 5552 SNP markers could be used after filtering. Heritability for all traits ranged from 0.02 to 0.74. GWASs were conducted using CMLM, SUPER and FarmCPU algorithms, to analyse which algorithm could detect the highest number of marker-trait associations (MTAs). Comparable tendencies were obtained from CMLM and FarmCPU, but FarmCPU produced the most significant results. MTAs were obtained for lodging, harvest index, plant height, STB, glucose, xylose and arabinose at a significance level of $p < 9.01 \times 10^{-6}$. MTAs in chromosome 6A were observed for glucose, xylose and arabinose, and could be of importance for increasing sugar release for bioethanol production.

Keywords: GWAS, harvest index, lodging, saccharification, *Septoria tritici* blotch, winter wheat (*Triticum aestivum*)

Author contributions: P.L.M., J.K.S., B.E. and S.K.R. conceived and designed the experiments; L.K.N., F.B. and B.E. designed and carried out the field experiments, conducted phenotyping and provided SNP marker data; P.L.M. performed all the experiments; P.L.M. analysed the data with the contribution of L.J.; P.L.M. drafted and finalized the article with writing contributions from all the authors; the final article was approved by all the authors.

Key message: This study demonstrates that an active breeding nursery with rotation can be used to identify marker-trait associations for biomass yield and quality parameters that are important for biorefinery purposes.

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Introduction

Winter wheat (*Triticum aestivum* L.) breeding is crucial to obtain a sustainable and competitive wheat production and would benefit from the continual development of molecular-markers to select new varieties with improved growth characteristics. The green revolution and the discovery of reduced height (*Rht*) genes in particular have had a considerable impact on increasing grain yields while simultaneously reducing straw biomass and lodging (Gent and Kiyomoto 1997; Hedden 2003; Pearce et al. 2011; Thomas 2017). One challenge for Danish wheat breeders has been to overcome the problems of strict regulations on nitrogen fertilization in order to maintain yield with reduced fertilizer use (van Grinsven et al. 2012; Dalgaard et al. 2014), and breeding programmes have therefore focused on delivering new varieties with competitive yields at lower fertilizer application. In order to introduce greater value into their winter wheat varieties, dual-purpose varieties are of interest to breeders because they could provide food for the world's growing population along with bio-based raw materials that can replace those from fossil sources (Townsend et al. 2017). A dual-purpose wheat variety can be used for both food/feed and biorefinery purposes, thus utilizing the whole biomass and making agricultural production more sustainable.

In winter wheat, marker-trait associations (MTAs) and genetic markers have been studied intensively for grain yield (Crossa et al. 2007; Bordes et al. 2014; Ogbonnaya et al. 2017; Assanga et al. 2017), harvest index (Edae et al. 2014; Ain et al. 2015; Sukumaran et al. 2015; Qaseem et al. 2018), plant height (Liu et al. 2011; Zhang et al. 2011; Griffiths et al. 2012; Zanke et al. 2014; Bellucci et al. 2015), lodging (Verma et al. 2005; Zhang et al. 2008; Berry and Berry 2015), and STB susceptibility (Brown et al. 2015; Arraiano and Brown 2017), while few studies have been undertaken on straw biomass (Sukumaran et al. 2015; Qaseem et al. 2018) and straw quality (Bellucci et al. 2015). It is known that MTAs for grain yield are distributed throughout the genome, as also documented in a study by Crossa et al. (2007) in which only chromosome 4D showed no MTAs. Plant height and lodging are largely controlled by *Rht* genes, and allele *Rht-B1b* and *Rht-D1b* in particular, located on chromosome 4B and 4D respectively, are of great importance (Würschum et al. 2015). A reduced plant height gene has been demonstrated on chromosome 2D (*Rht8*) (Korzun et al. 1998) and *Rht* alleles on 4D (*Rht-D1c* and *Rht-D1d*) and in 4B (*Rht-B1c*, *Rht-B1d*, *Rht-B1e*, and *Rht-B1f*) have also been identified (Börner et al. 1996; Pearce et al. 2011). Another study has also shown that plant height seems to be controlled by many small and major effector genes throughout the wheat genome and that many of the SNPs have an additive effect (Zanke et al. 2014). Besides being controlled by *Rht-B1* and *Rht-D1* genes, lodging has also been

71 associated with chromosomes 1B, 1D, 2B, 2D, 3A, 6D and 7D (Verma et al. 2005; Zhang et al. 2008). The harvest
72 index has been increased by the introduction of the semi-dwarfing genes and MTAs for harvest index have been found
73 on chromosomes 1B, 1D, 3B, 3D, 5A, 6B and 7A (Edae et al. 2014; Ain et al. 2015; Sukumaran et al. 2015; Qaseem et
74 al. 2018). Identification of markers for Septoria tritici blotch (STB), a wheat disease caused by the fungus
75 *Mycosphaerella graminicola* (anamorph *Septoria tritici*) (Brading et al. 2002), is becoming increasingly important due
76 to the development of resistance to fungicides. A recent search revealed MTAs for STB on all chromosomes in the A
77 genome except for 1A, and in genome B and genome D except for 4D (Brown et al. 2015; Arraiano and Brown 2017;
78 Vagndorf et al. 2017). In addition to identifying markers for the above mentioned priority traits, it will also be relevant
79 to include markers for straw yield and quality. Wheat straw is rich in carbohydrates (cellulose, hemicellulose and ligno-
80 cellulose) and their bioconversion into ethanol (2nd generation biofuel) rather than using the starchy grains is important
81 for obtaining a sustainable biofuel production. In the present study, straw quality is defined by its ability to release
82 monosaccharides (glucose, xylose and arabinose) from straw for fermentation or conversion into biomaterials after
83 enzymatic saccharification.

84 True MTAs identified for biomass yield and quality are extremely rare, while the degree of heritability or genetic
85 variation has been analyzed in several studies. Recently, Jørgensen et al. (2018) has shown that straw and total biomass
86 yield in a panel of fourteen modern elite cultivars varies significantly. Other studies have identified MTAs on
87 chromosomes 3B, 3D, 5A, 6A and 7A (Sukumaran et al. 2015; Qaseem et al. 2018). However, when looking at
88 monosaccharide release from wheat straw after enzymatic hydrolysis, Jørgensen et al. (2018) found no genetic variation
89 between the 14 varieties. In contrast, a study by Lindedam et al. (2014) reports genetic variation in sugar release after
90 enzymatic saccharification of 20 modern and old varieties. Genetic variation has also been further detected in GWAS of
91 an historical Scandinavian winter wheat collection, where a weak MTA for glucose release was identified on
92 chromosome 1B (Bellucci et al. 2015).

93 Many of the previous studies on wheat have used breeding programmes specifically designed for the traits analyzed in
94 the MTA analysis. The use of active breeding nurseries to identify genetic markers is more challenging than using a
95 mix of modern elite lines, old varieties and landraces. The lines used in active breeding nurseries are likely to have
96 chromosomal regions that are highly inbred for desirable traits, and the segregation of alleles is not completely random.
97 This might make it more difficult to identify known QTLs for cross referencing in the association study such as reduced
98 height genes.

99 This study aimed to identify MTAs in an active wheat breeding nursery, rather than in a designed population, as this is
100 what breeding companies use on a daily basis. The study therefore intended to identify GWAS algorithms that are able
101 to capture MTAs in material from active breeding nurseries. The lines in the breeding nursery were phenotyped for a
102 large number of agronomic traits that are important in the development of dual-purpose varieties. Three different
103 GWAS algorithms (CMLM, SUPER and FarmCPU) were tested for identification of MTAs using 190 winter wheat
104 lines, grown over three years using the 15k SNP array from TraitGenetics. It addressed the biometric challenge of an
105 active breeding nursery, embracing a relatively small number of lines and with 50 % of the lines removed and replaced
106 with new lines every year. Finally, there is a discussion about the ability to identify markers for biomass yield and
107 quality and about limitations in the breeding of dual-purpose varieties.

108 *Materials and Methods*

109 *Growth conditions for plant material*

110 A collection of hexaploid winter wheat lines and varieties (*Triticum aestivum*) was grown in a field trial at Sejet Plant
111 Breeding (Horsens, Denmark) for three years from 2013 to 2016. The experimental layout of the active breeding
112 nursery was a randomized alpha block design with rotation, such that 50 new lines were introduced each year and 50
113 lines from the previous year were excluded, increasing the final number of lines grown partly over three years to 194.
114 The yearly block design included 94 lines and two check cultivars, giving 96 plots (size 8.1 m²) per block. Four blocks
115 (replicates), consisting of 12 columns and 8 rows were included in the yearly design, in which each column defined a
116 mini-block resulting in 12 mini-blocks per block. Phenotypic data were available from four blocks in 2013/14 (with the
117 exception of data for straw yield and quality traits which were only available from two blocks) and three blocks each for
118 2014/15 and 2015/16. The soil type was JB6, referring to a heavy clayey soil. The experimental site was fertilized
119 following the yearly recommendations of the Danish Agricultural Agency (lbst.dk). Plant protection against pathogens
120 followed the guidelines of The Danish Agricultural Advisory Service.

121 *Agronomic traits*

122 Traits important for yield and quality of grain and straw were obtained for all three years and included grain yield, plant
123 height, lodging, foliar susceptibility to STB (*ZymoSTB*), straw yield, and sugar release after enzymatic hydrolysis of the
124 straw.

125 Plant height was recorded as the length of the straw without the ear at full canopy height and the majority of lines in the
126 breeding nursery were semi-draws either with respect to *Rht-B1b* or *Rht-D1b*. Both plant height and STB susceptibility
127 were assessed in the field once at full height, while lodging was assessed at three different time points. The first
128 assessment of lodging coincided with ear emergence, while the timing of the middle assessment depended on the actual
129 intensity of precipitation events. The final assessment of lodging was at full maturity, just before harvest. The three time
130 points when lodging was assessed are referred to as early, medium and late-growth stage. Following standard
131 procedures by the breeders, lodging and STB susceptibility were scored on a scale from 0 to 9, with 0 characterizing
132 plots with no lodging or plots with no STB infection.

133 The straw and grain material was harvested using an experimental combine harvester providing grain (whole plot) and a
134 straw sample representative for the whole plot. The weights of the grain, the straw sample and straw from the entire plot
135 were recorded directly in the field. The straw samples were later re-measured after they had been dried (for two days at
136 60°C for straw) to calculate water loss. The grain yield of each plot was calculated assuming a water content of 15%
137 (equations for grain yield, straw yield and harvest index are provided in supplementary material eq. 1 to 4).

138 *Pretreatment, enzymatic saccharification and quantification of sugars*

139 To assess the sugar release from straw after enzymatic saccharification, straw material was cut into 4 mm fragments and
140 further milled into fine powder by ball milling using a robotic system (Labman Automation Ltd, Stokesley, North
141 Yorkshire, UK; see also Studer et al. 2010; Selig et al. 2010; Zhang et al. 2014). The pretreatment was conducted using
142 a high-throughput system with 96-well plates as described by Zhang et al. (2014). In brief, 27.0-30.0 mg of the sample
143 was pretreated in triplicates in each 96-well plate. For each plate, three control samples and 3 blanks were included. To

each well, 422 µl of 50 mM citrate buffer pH 5.0 was added and the plates were heated to 190 °C for 10 minutes. The enzymatic hydrolysis was performed in the plate using Cellic CTec2 (a mixture of aggressive cellulases, β-glucosidases and hemicellulase) with an enzyme loading of 5 FPU/g DM sample (1g enzyme to 16 g buffer), provided by Novozymes. Hydrolysis was performed at 50 °C for 72 hours during which time the samples were shaken at 450-600 rpm. The hydrolysis was terminated by heat-inactivation at 90°C for 30 minutes. The plates were centrifuged (rotor 11240, Sigma 3-18K) at 1100 g for 10 minutes and the supernatant filtered through a microtiter filter (0.45 µm GHP membrane, product no. 8084, Pall Corporation AS) mounted in a microtiter plate (product no. 82.1581, Hounisen AS). The samples were stored at 4 °C for a maximum of three days before analysis. For the analysis, 5µl of the sample was transferred to a new microtiter plate and diluted with 245 µl 50 mg/L fucose solution. Quantification of glucose, xylose and arabinose was performed using a Dionex IC5000 HPAEC-PAD under isocratic conditions (20mM NaOH) with a Dionex CarboPac™ PA1 (2 x 50mm) guard column and a Dionex CarboPac™ PA1 (2 x 250mm) analytic column.

The following equation was used to convert the results obtained by sugar analysis into milligram monosaccharide released per gram dry matter (mg/g DM):

$$Monosaccharide = \frac{C \times V \times 1000}{DM \times k} \quad (\text{eq. 5})$$

where C is the concentration of monosaccharide released (g/L), V is the total liquid volume (0.472×10^{-3} L) added to each well, DM is the quantity of DM analysed (g) and k is the hydration factor (1.111 for glucose and 1.136 for arabinose and xylose). Inclusion of the hydration factor accounted for hydration caused by depolymerisation.

The sugar release data were adjusted for plate-to-plate variation and differences in the assay performance between years using standards. The standards consisted of either a mixture of samples from the 2014 harvest included in each plate or of samples analysed in both 2015 and in 2017 (control samples). For normalization of plate-to-plate variation, a multiplication factor was calculated by the following equation:

$$f_{m,p} = \frac{\overline{X_{C,Total}}}{\overline{X_{C,Plate}}},$$

where $\overline{X_{C,Plate}}$ denotes the mean of standards for each plate and $\overline{X_{C,Total}}$ denotes the mean of standards for all plates. This enabled the creation of a multiplication factor for each plate, which was multiplied with each sugar measurement on that plate to adjust for plate-to-plate variation. Saccharification of samples harvested in 2014 was performed in 2015, while samples harvested in 2015 and 2016 were analysed together in 2017. To normalize for variation between the rounds of saccharification, mainly associated with enzyme batch performance, 10 samples from the 2014 harvest were randomly chosen and included in the saccharification performed in 2017 (control samples). The difference in mean sugar release for the 10 control samples analysed in 2015 and in 2017 was then used to adjust the saccharification conducted in 2015.

Correlations between monosaccharide release after enzymatic hydrolysis and straw yield was calculated in R using the `pairs.panels` function applying Pearson correlations.

176 *Genotyping*

177 DNA extracted from the leaf material of 194 lines were genotyped by TraitGenetics GmbH
 178 (<http://www.traitgenetics.de>) using the Illumina iScan 15K SNP array (Akhunov et al. 2009). Data were obtained for
 179 13006 SNPs and 5552 markers remained after a QC check in which markers with more than 10 % missing data, more
 180 than 5 % heterozygosity, or a minor allele frequency below 5 % were removed (also removing monomorphic markers).
 181 The final number of cultivars was reduced to 190, as four lines from year 2016 were removed, one failed to produce
 182 DNA for SNP genotyping, and the other three lines showed more than 5 % heterozygosity. SNP data were converted to
 183 numeric format using the numeric conversion option in GAPIT, with the major allele, minor allele and heterozygotes
 184 given the numbers 1, 3 and 2 respectively. These were then converted to 0, 2 and 1, which is the numeric format used in
 185 GAPIT. The missing values were then imputed using the KNN function in R (knncatimpute), which is a method that
 186 imputes the missing values based on k nearest neighbours (k = 3) (Troyanskaya et al. 2001). The numeric formatted
 187 SNP data with imputed markers were used for GWAS in R. R version 3.2.1 was used for all calculations, statistical
 188 tests and plots and Inkscape 0.92 was used for figure editing.

189 *Estimated trait means and genetic variation*

190 Phenotypic trait data were analysed for outliers and modelled through mixed linear models using the R-package
 191 ASreml-R (VSN International, Hemel Hempstead, UK). The modelling removed variations caused by environmental
 192 sources, such as block and year differences, or variations caused by technical errors during laboratory analyses such as
 193 pipetting uncertainties and plate-to-plate variation. For a given variable Y , corresponding to a trait value, the following
 194 model was applied:

$$195 \quad Y = X\beta + Zu + \epsilon \quad (\text{eq. 6})$$

196 Equation 6 consists of a vector of random effects u , a vector of fixed effects β , two design matrices X and Z with the
 197 residual ϵ denoting the random error. The design matrices associate trait observations with the appropriate fixed effects
 198 and random effects. The vector of random effects u and fixed effects β are defined in equations 7 and 8 respectively,
 199 and consists of a $p \times 1$ and $q \times 1$ vectors:

$$200 \quad u = A_i + B_{ki} + b_{mi} + R_{ji} + C_{li} + GxE_{ni} \quad (\text{eq. 7})$$

$$201 \quad u = A_i + B_{ki} + b_{mi} + R_{ji} + C_{li} + GxE_{ni} + P_o + P_p \quad (\text{eq. 7.1})$$

$$202 \quad \beta = \mu + G_j \quad (\text{eq. 8})$$

203 In equation 8, μ defines the overall mean (eq. 7 and 7.1 $\mu = 0$). The other parameters in equation 7 are the explanatory
 204 variables that defines the random effect of year variation (A_i , where $i = 2013-2015$), field variation consisting of block
 205 (B_{ki} , $k = 1-4$), mini-block (b_{mi} , $m = 1-48$), row (R_{ji} , $j = 1-48$), and column (C_{li} , $l = 1-12$), and the genotype by
 206 year/location effect ($G \times E_{ni}$, $n = 1-190$). The explanatory variable of equation 8 defines the fixed effect of the genotypic
 207 variation (G_n , $n = 1-190$). Equation 7.1 shows the random effects included for sugar release data, where plate number
 208 (P_o , $o = 1-30$) and plate position (P_p , $p = 1-96$) are also included.

Mixed linear models were used to estimate means (best linear unbiased prediction, BLUPs) such that the most accurate phenotypic estimate was used in GWAS. The best predicted trait values were used to show the distribution of the data (Fig. S1). The mixed linear model was modified for estimation of broad sense heritability by including genotypic variation as a random effect rather than a fixed effect. Broad sense heritability is defined here as the variation estimate of an individual measurement that was based on a random replicate, a random variety, and a random location in the field (Visscher et al. 2008; Leplat et al. 2016),

$$H^2 = \frac{\sigma_G^2}{\sigma_P^2} = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{GxE}^2 + \sigma_e^2} \quad (\text{eq. 9})$$

where the variance terms for phenotypic variance (σ_P^2) consisted of variance components of the explanatory variables of the random effects of genotype, genotype by environment (σ_{GxE}^2) and the residual variance (σ_e^2 , the error term). The repeatability (R^2) for cultivar-means (the heritability of variety means (Leplat et al. 2016)), as applied in Sukumaran et al. (2015) , was calculated using

$$R^2 = \frac{\sigma_G^2}{\sigma_G^2 + \frac{\sigma_{GxE}^2}{Y} + \frac{\sigma_e^2}{R}} \quad (\text{eq. 10})$$

where σ_G^2 denotes the genetic variation, σ_{GxE}^2 the genotype by year variation and σ_e^2 the residual variation. Y is the number of years and R is the number of replicates.

Genome-wide association analysis

Three different mixed linear model algorithms, all available in R, were used to run GWAS in order to identify the association between SNP markers and traits. The first and second were the mixed linear models CMLM (Compressed Mixed Linear Model; (Zhang et al. 2010)) and SUPER (Settlement of MLM Under Progressively Exclusive Relationship; (Wang et al. 2014)), supported by GAPIT 2.0 (Genome Association and Prediction Integrated Tool) (Lipka et al. 2012). The third model was FarmCPU (Fixed and random model circulating probability unification; (Liu et al. 2016)) supported by FarmCPU (now included in GAPIT 3.0). GAPIT has been widely used to identify QTLs and the newer integrated algorithm SUPER is designed to increase the power compared to CMLM (Wang et al. 2014). However, these methods suffer from accepting false positives. Normally, the kinship matrix (K-matrix) and inclusion of principal components (PCs) will control for false positives, but in return it also weakens the power such that more false negatives are obtained. FarmCPU improves the control of false positives and false negatives caused by the confounding problem between PC's or K-matrix and test markers by controlling the inflation of p-values (Liu et al. 2016).

All three methods control for false positives by fitting a Q-matrix (population structure or PCs) and a K-matrix (family relatedness) as covariates, but only SUPER and FarmCPU include pseudo quantitative trait nucleotides (pseudo QTNs) as covariates as well. A pseudo-QTN defines a SNP that is associated with the trait.

In CMLM, the K-matrix and Q-matrix are constant irrespective of the trait analysed and the K-matrix is based on groups of individuals. CMLM has three different algorithms for generating the K-matrix, VanRaden, Emma, and Loiselle (Zhang et al. 2010), which were all tested in the present study.

241 In SUPER and FarmCPU, the kinship matrix differs from trait to trait as only SNPs affecting the trait are included as
 242 pseudo QTNs for deriving the kinship matrix. The SUPER algorithm is based on the FaST-LMM-Select model
 243 (factored spectrally transformed linear mixed models) which is a mixed effect model that incorporates a map of markers
 244 when estimating pseudo QTNs (Wang et al. 2014).

245 The FarmCPU algorithm combines the models FaST-LMM-select model (testing genetic markers one at a time) and the
 246 Multi-locus Mixed model (MLMM) (testing multiple markers simultaneously) in order to remove any confounding
 247 between test markers and K and Q matrices. The FarmCPU algorithm is therefore based on a fixed effect model and a
 248 random effect model, which are run separately and repeatedly and affect one other (Liu et al. 2016).

249 Fixed effect model: $y_i = S_{i1}E_1 + \dots + S_{it}E_t + S_{ij}e_j + \epsilon_i$

250 Random effect model: $y_i = u_i + \epsilon_i$

251 In both models, y_i defines the trait value of the i^{th} wheat cultivar and ϵ_i corresponds to the residual $\sim N(0, \sigma_\epsilon^2)$. In the
 252 fixed effect model $S_{i1} \dots S_{it}$ and $E_1 \dots E_t$ gives the i^{th} and the t^{th} pseudo QTNs genotype and effect respectively, where
 253 the first defines the covariates that are zero at the beginning. S_{ij} corresponds to the genotype of the i^{th} wheat cultivar
 254 and the j^{th} SNP marker, while e_j is the effect of the j^{th} SNP marker. In the random effect model, u_i defines the total
 255 genetic effect of the i^{th} wheat cultivar, where the variance and covariance matrix is given by $G = 2K\sigma_a^2$. K is the kinship
 256 matrix created based on pseudo QTNs and σ_a^2 is the genetic variance (Liu et al. 2016).

257 The fixed effect model included pseudo-QTNs as covariates and test markers that were tested one at a time. The
 258 association between test markers and the trait was validated by the random effect model with K derived from pseudo-
 259 QTNs. The two models were run repeatedly until no more pseudo-QTNs were added to the fixed effect model. The p-
 260 value of the marker that corresponded to the pseudo-QTN was substituted by the most significant p-value of the pseudo-
 261 QTN.

262 The SUPER method, which consists of the random model, is used to optimize pseudo-QTNs, incorporating the genetic
 263 map information and test statistics from the fixed effect model. The individual's genetic effect, including variance and
 264 covariance matrix, was used as a random effect. After the evaluation of pseudo-QTNs, the pseudo-QTNs are inserted
 265 back into the fixed effect model and this continues until no more pseudo-QTNs are added to the model (Liu et al. 2016).

266 Irrespective of the GWAS algorithm applied, SNPs were considered to be significantly associated with a trait at a p-
 267 value below 9.00576×10^{-6} ($p = \frac{0.05}{no.SNPs}$), corresponding to a $-\log_{10}(p)$ above 5.05. If no markers exceeded this
 268 threshold, weak associations represented by SNP markers that had a p-value below 0.001, corresponding to a $-\log_{10}(p)$
 269 above 3, were analysed instead.

270 Results

271 Validation of genotyping

272 The filtration of the SNP markers reduced the 15k SNP array to 5552 markers. These were relatively evenly distributed
273 across the 21 chromosomes (Table 1). The SNP frequency was significantly lower on all chromosomes in the D genome
274 compared to the other two genomes, as was expected due to the later introduction of the D genome in hexaploid wheat.
275 The coverage was also lower on chromosome 4 in all genomes. For chromosomes 4B and 4D this might be due to less
276 recombination because the majority of lines in this study were semi-dwarfs (either *Rht-B1b* or *Rht-D1b*) and therefore
277 more inbred at these chromosomes. Consequently, detection of *rht* markers might be more difficult compared to a study
278 that also includes landraces.

279 **Table 1** Distribution of SNPs across the 21 wheat chromosomes

Genome\Chromosome	1	2	3	4	5	6	7	Total
A	308	336	284	161	375	372	394	2230
B	389	407	455	224	463	378	293	2609
D	141	129	86	38	108	124	85	711

280 *586 additional SNP markers were unmapped and not assigned to a chromosome

281 The SNP markers were used to generate a kinship matrix and principal components to control for family relatedness and
282 population structure, respectively. The kinship algorithm was consistently included in the mixed linear models used to
283 run GWAS. The number of principal components included in the model depended on whether they improved the QQ-
284 plot (quantile-quantile) and was highly dependent on the GWAS algorithm used (GAPIT-CMLM, GAPIT-SUPER, or
285 FarmCPU). The principal components were evenly distributed and did not indicate a strong population structure (Fig.
286 S2). Three different kinship matrices (van Raden, Emma, and Loiselle) were tested in GAPIT and all gave equally good
287 kinship matrices that did not change the effect on the QQ-plots (Fig. 1 and S3). All three K-matrices revealed that there
288 was no strong genetic resemblance between the lines, as illustrated by the bright white to orange colours in Figure 1a-c.
289 The QQ-plots followed the diagonal line and did not indicate the presence of population structure (Fig. 1d-f; results
290 shown for straw yield only, see Fig. S3 for grain yield and plant height). Based on the QQ-plot comparison (Fig.1 and
291 S3), it is apparent that the Emma and Van Raden algorithms performed equally well, while the Loiselle algorithm
292 experienced more noise indicating that Loiselle corrected less well for population structure (Fig. S3). Here the kinship
293 matrix conducted with the Emma algorithm, which also happens to be the most recent kinship algorithm in GAPIT, was
294 chosen as the kinship algorithm for all traits, but the Van Raden algorithm would have been equally good to apply. For
295 the plot of principal components and the heterozygosity of the lines see Figure S2.

296 **Fig. 1** Comparison of kinship matrices (a, b, and c) and the QQ-plots for straw yield (d, e, and f) between the three
297 algorithms van Raden, Loiselle, and Emma: a) kinship matrix and d) QQ-plot when applying the van Raden algorithm,
298 b) and e) when applying the Loiselle algorithm and c) and f) when applying the Emma algorithm. The K-matrix
299 illustrates the genetic resemblance between the 190 lines as shown by the heat map and the dendrograms (each axis).
300 The diagonal line represents inbred lines as is revealed by a dark orange colour. The darker colour, the more inbred the
301 lines. Straw yield was chosen as an example of the similarity between the QQ-plots. The QQ-plot shows the expected -
302 $\log_{10}(p)$ values plotted against the observed $-\log_{10}(p)$ values. The grey shadow resembles the 95%-confidence interval

Evaluating GWAS algorithms

Three different GWAS algorithms (GAPIT-CMLM, GAPIT-Super and FarmCPU) were used to analyse the data in order to test which algorithm provided the best QQ-plot combined with the lowest p-values. The GAPIT-Super algorithm were not able to correct properly for population structure for all traits as the p-values seemed to be inflated (above the diagonal) and the QQ-plot showed more noise (Fig. S4.b). The fitness of the lines in the QQ-plots for FarmCPU and GAPIT-CMLM were in general comparable, as they followed the diagonal well, but for most traits FarmCPU decreased the p-values compared to GAPIT-CMLM (Fig.S4.a-c with plant height as an example). There were also cases where FarmCPU failed to improve and even slightly reduced the quality of the QQ-plot compared to GAPIT-CMLM, as illustrated for straw yield and grain yield in Figure S4a-c. FarmCPU have a tendency of overcorrecting their models, with the straight line a bit below the diagonal, but the algorithm experience a minimum of noise as the line are straight (QQ-plot for plant height Fig.S4.a). Besides this FarmCPU had the ability to detect marker-trait associations with lower p-values for the majority of traits (plant height is shown as an example in Fig.S4) compared to the two other algorithms. As all algorithms failed to identify MTAs for grain yield and straw yield and the FarmCPU algorithm succeeded in identification of MTAs for the majority of the other traits, the FarmCPU algorithm was used as the GWAS algorithm for all traits.

The effect of including principal components

The advantage of including principal components (PCs) in GWAS was tested in the GAPIT software using Bayesian information criterion (BIC) values. For all traits in this study, exclusion of PCs gave the highest BIC values, therefore zero PCs were applied in GAPIT. Since the same test could not be performed in FarmCPU, all combinations of PCs were included in GWAS with the number of PCs included for each trait being based on improvements in the QQ-plot. If the QQ-plot was unchanged by including more PCs, then the model with the least number of PCs was chosen. The number of PCs included in FarmCPU therefore differed between traits and the inclusion or exclusion of PCs had a considerable effect on correcting for the population structure.

Modelling agronomic and biorefinery traits

Based on a visual inspection of the distribution of the predicted phenotypic means all traits with exception of early and intermediate lodging seemed to be normal distributed (Fig. S1), however only means of plant height, grain yield, STB and harvest index passed the Shapiro-Wilk normality test ($p > 0.001$) while the remaining traits failed to pass it (See table S1). Logarithmic transformation of data did only improve normality for straw yield, but not enough for it to become normal distributed. Early lodging had a skewed distribution due to the high number of zeros, i.e. breeding lines that did not show any lodging. Residual-plots (normal probability plots and residuals vs. fitted value plots) showed that residuals were normally distributed (data not shown). Table 2 lists the summary information (mean, range and coefficient of variance) for the phenotypic data before and after being modelled through mixed linear models to predict estimates for the phenotypic trait values for each breeding line.

Table 2 Range of agronomic and biorefinery traits

Phenotype	Mean		Range		CV [%]	
	Raw	Estimate ^a	Raw	Estimate ^a	Raw	Estimate ^a
Straw yield [t/ha]	7.7	7.5	3.6-18.6	3.7-12.1	28	15
Plant height [cm]	85	85	63-110	70-99	11	7
Grain yield [t/ha]	9.0	9.0	6.9-10.9	7.2-10.1	7	5

Harvest index [%]	55	55	31-70	43-70	11	7
Early lodging [score]	1	0	0-7	-1-3	845	554
Intermediate lodging ^b [score]	3	3	0-9	-3-11	106	62
Late lodging [score]	3	3	0-9	-3-9	124	82
STB [score]	5	5	2-7	4-6	17	13
Arabinose ^b [mg/g DM]	11.1	11.1	6.1-19.1	9.6-12.6	16	5
Xylose [mg/g DM]	129.3	130.0	61.4-201.0	112.8-151.6	13	5
Glucose [mg/g DM]	184.1	184.1	103.7-287.5	158.3-211.4	14	5

^aData obtained from predicted values using mixed linear models in ASReml.

^bData obtained from two years data compared to three years' data as for the other traits

The coefficient of variance (CV) defines the quality of the data and it was acceptable (below 30%) for the majority of traits. However, CV was generally high for lodging, which might be due to the relatively high number of cases of zero (absence of lodging), variation between years and the absence of normally distribute data.

Table 3 shows the heritability for each phenotype and the components of genetic variance, the genotype by year (G×E) variance and the stochastic and measurement error variance. The repeatability (R^2) of each trait, taking the number of locations, years and replicates into account, is also given in Table 3.

Heritability was relatively low for many traits including sugar release, early stage lodging, harvest index and straw yield, ranging between 0.02 and 0.15 (Table 3). The genotype by environment (G×E) variance ($\sigma_{G \times E}^2$), defining the genotype by year variance, was also comparable or greater than the genetic variance for most of these traits, with the exception of harvest index. The G×E variance for sugar release traits was similar to the genetic variation (σ_G^2), while the error variance (σ_e^2) had the grates effect with respect to reducing heritability. This was illustrated by the error variance being 15x, 27x, and 50x greater than the genetic variation for xylose, arabinose and glucose, respectively. The major contributors to the error variance for monosaccharide release was believed to be coursed by analytical and experimental variances, which could indicate that the low heritability was caused by experimental noise and that proper noise correction or other methods might be able to uncover more genetic variation. H^2 was based on all replicates without adjustment for the number of replicates and years, as was done for R^2 ; this difference decreased the error variance and the G×E variance and made the R^2 estimates higher. Heritability for STB susceptibility, plant height, grain yield and the later stages of lodging ranged between 0.35 and 0.74, while the repeatability for the same traits was between 0.67 and 0.94 (Table 3).

Table 3 Variance estimates for the different traits including broad sense heritability and repeatability

Phenotype	σ_G^2	$\sigma_{G \times E}^2$	σ_e^2	σ_{Total}^2	%G	%G×E	%e	H^2 ^b	R^2
Straw yield ^a	0.006	0.01	0.03	0.046	13.04	21.78	65.22	0.15	0.47
Plant height	33.65	3.62	8.00	45.27	74.33	8.00	17.67	0.74	0.94
Grain yield	9.48	10.97	4.90	25.35	37.40	43.28	19.33	0.37	0.69
Harvest index	2.59	8.87	19.9	31.36	8.26	28.28	63.46	0.08	0.33
Early lodging	0.01	0.05	0.16	0.22	4.55	22.73	72.73	0.05	0.24
Intermediate lodging	1.99	2.41	1.19	5.59	35.60	43.11	21.29	0.35	0.67
Late lodging	2.30	2.87	1.08	6.25	36.80	45.92	17.28	0.58	0.68
STB	0.27	0.09	0.17	0.53	50.94	16.98	32.08	0.51	0.84
Arabinose	0.06	0.08	1.64	1.78	3.37	4.49	92.13	0.04	0.17
Xylose	11.53	8.26	169.39	189.18	6.09	4.37	89.54	0.06	0.35
Glucose	8.81	21.56	441.91	472.28	1.87	4.57	93.57	0.02	0.14

360 ^a log transformed data

361 ^b Calculations were conducted before the variance components were rounded to two decimals

362 σ_G^2 defines the genetic variance, $\sigma_{G \times E}^2$ the genotype by year variance, σ_e^2 the error variance,

363 σ_{Total}^2 the sum of the three variance components (total variance), H^2 is broad sense heritability and R^2 is repeatability.

364 %G, %G×E and %e defines how much of the total variance the specific variance components accounts for in
365 percentage.

366 *GWAS results*

367 *Lodging and plant height*

368 The agronomic trait lodging was scored three times, viz. twice during development (early and intermediate), and once
369 just before harvest (late) to reveal if lodging during plant development and before harvest was controlled by both
370 common and different genes, with the priority given to genes for late lodging. Fig. 2 shows the Manhattan plots from
371 GWAS run by FarmCPU, revealing the SNPs and their chromosomal location that were significantly associated with
372 lodging and plant height.

373 For early lodging, significantly associated SNPs were located on chromosomes 3B, 5B, 6A, 7A, and 7D with two
374 markers on 5B being the most significant ($-\log_{10}(p)$ at 18.3 and 19.2 respectively) (Table 4). No significantly
375 associated markers were identified for intermediate stage lodging, however, there was a weak association for markers
376 on chromosomes 1B, 4B, 5B, and 7A which had a $-\log_{10}(p)$ above 3, to be specific 3.02, 3.41, 3.11 and 3.13
377 respectively (Fig. 2). MTAs were identified for late stage lodging on chromosome 1B, 2A, 3D, 4B, 5B and 6D with -
378 $\log_{10}(p)$ values between 5.1 and 8.6 (Fig. 2 and Table 4). Five MTAs were identified for plant height; one on
379 chromosomes 1B, 6B, and 7B and two on chromosome 4B (Fig. 2). Their $-\log(p)$ scores ranged from 5.3 to 6.6 (Table
380 4).

381 Four markers for early stage lodging and one marker for late stage lodging had a minor allele frequency (MAF) close to
382 the limit of 0.05, which for early stage lodging could be explained by the greater frequency of absence of lodging at this
383 stage. Beside this, the MAFs were between 0.15-0.16, 0.15-0.47, and 0.16-0.48 for early stage lodging, late stage
384 lodging and plant height respectively (Table 4).

385
386 Boxplots in Figure 3 and the SNP effect in Table 4 illustrate the SNP effect for lodging and plant height. The SNP
387 effect can be described as the difference between the two allelic versions. For early stage lodging, the SNP effect
388 corresponding to reducing early stage lodging by between 0.1 and 2.2 points (on a scale from 0-9) depended on the
389 SNP. A reduction of 2.2 points could be of great importance for minimizing early lodging. The markers identified for
390 late stage lodging could decrease lodging between 1.2 and 1.9 points, which corresponds to a reduction of mean lodging
391 by approximately 36%. The largest SNP effects were for the markers BS00009777_51 and Ra_c31466_694 on
392 chromosomes 5B and 7A respectively (2.18 and 2.19, on a scale from 0 to 9) for early lodging while marker GENE-
393 2712_130 and Ku_c18920_414 on chromosome 3D and 1B had the greatest SNP effect for late lodging (Table 4). The
394 markers on 5B and 1B were also the second most significant markers for early and late lodging respectively. The most
395 significant marker for early lodging (wsnp_Ex_c9362_15546626, 5B) only had a minor SNP effect of 0.1 while the
396 marker for late lodging (Tdurum_contig91519_224, 2A) had a more pronounced SNP effect of 1.55 (Table 4). This

could reflect the low number of lines that showed early lodging (not normally distributed data). For plant height the SNP substitution was able to decrease plant height by between 2.3 and 3.8cm and the markers on 1B and 4B were those with the greatest SNP effect and also the most significant for plant height.

No MTAs were shared between all three lodging stages as no MTAs were observed for intermediate stage of lodging. Shared MTAs for early and late lodging were in general different, except for MTAs located in a single chromosomal region on 5B (from 403.9Mb to 712.9Mb) (Table 4). If weak associations for intermediate lodging, corresponding to markers with a $-\log_{10}(p)$ -value above 3, were included then there were overlapping chromosomal regions identified between the early and intermediate stage lodging (7A) and late and intermediate stage lodging (1B and 4B). The markers identified on chromosome 7A differed, but they were located in the same region (586.2Mb and 732.8Mb for early and intermediate-stage lodging, respectively). The markers identified for both intermediate (weak associations) and late stage lodging on chromosome 1B and 4B were identical, hence they could share the QTLs. The MTA on 1B for late stage lodging was also identical to the MTA identified for plant height and the MTAs on 4B for both plant height and late lodging were in the same chromosomal location (506.1-552.8 for plant height and 552.0 for lodging) (Table 4).

Fig. 2 Manhattan plots for lodging and plant height. The significance level is set at a $-\log_{10}(p)$ of 5.05 illustrated by the solid green line. The dotted black line defines a $-\log_{10}(p)$ at 3 ($p = 0.001$) and is used as a threshold to illustrate the chromosomes where there are some weak associations

Fig. 3 Boxplot showing the SNP effect for a) early lodging, b) late lodging, c) plant height and d) harvest index. The y-axis defines the score of lodging and the x-axis defines SNP allele 1 and SNP allele 2, corresponding to absence and presence, respectively. The two boxplots visualise the estimated means of lodging for all 190 lines and how the mean changes depending on the differences in allele1 and 2

Grain yield, harvest index, and STB

The results of the association analysis for grain yield, harvest index and STB susceptibility are illustrated in Figure 4. No significant MTAs for grain yield were detected, only weak associations at a $-\log_{10}(p)$ at 3, corresponding to a p -value at 0.001, which for grain yield was on chromosome 1A, 1B, 2A, 3B, 5B, 7A, 7B, and 7D. The most promising marker (lowest p -value) was found on chromosome 7A with a $-\log_{10}(p)$ at 3.6, while the other markers had a $-\log_{10}(p)$ of between 3.0 and 3.6 (Fig. 4).

For the harvest index, two SNPs were significantly associated on chromosome 2A and 5B with $-\log_{10}(p)$ -values of around 6 and an effect size of 2.5% and 2.1% respectively, suggesting that substituting the allele increases the harvest index by approximately 2% (Table 4).

STB susceptibility also had markers associated significantly, namely those located on chromosomes 1A, 3B, 5B, 5D, 6B, and 7A with marker Ku_c22026_1410 located on chromosome 6B being the most significant (see Table 4). The $-\log_{10}(p)$ -values ranged between 5.3 and 9.1 with an allele effect of between 0.3 and 0.6 on a scale from 0 to 9.

Two markers for STB susceptibility (BS00012069_51 and BS00110130_51) had a minor allele frequency of around 0.08, which was close to the threshold. For all other significant markers the MAFs were from 0.11 to 0.42 and from

0.23 to 0.35 for STB and harvest index respectively (Table 4). None of the observed MTAs for STB and harvest index and weak associations for grain yield were identical to MTAs found for other traits.

Fig. 4 Manhattan plots for grain yield, harvest index, and STB susceptibility: The significance level is set at a $-\log_{10}(p)$ of 5.05 which is illustrated by the solid green line. The dotted black line defines a $-\log_{10}(p)$ at 3 ($p = 0.001$) and is used as a threshold to illustrate the chromosomes where there are some weak associations

Biorefinery traits: straw quality and yield

Figure 5 shows SNPs that were significantly associated with straw yield and quality traits, while the boxplots in Figure 6 illustrates the SNP effects. MTAs were observed for all types of monosaccharides ($-\log_{10}(p) > 5.05$), while straw yield only showed markers that were weakly associated ($-\log_{10}(p) > 3$). Straw yield showed too small a variation to allow marker identification, and only weak associations were found on chromosomes 1B (Ku_c18920_414) and 4B (wsnp_Ku_c12399_20037334) with $-\log_{10}(p)$ -values of 3.0 and 3.4 respectively. The marker identified on 1B was identical to that found for plant height, which seems logical since straw yield is greatly affected by plant height (Table 4). The marker wsnp_Ku_c12399_20037334 found on 4B was located near the telomere of the chromosome (1.3Mb) while the markers identified for plant height were located towards the other end of chromosome 4B (506.1-552.8Mb) (Table 4).

MTAs were identified for all monosaccharides despite of the observed low heritabilities. Release of glucose (mg/g DM) showed MTAs on chromosome 1B and 6A with a $-\log_{10}(p)$ -value of 7.1 and 5.8, respectively (Fig. 5). The effect of the SNP substitution increased the glucose release by between 4.8 and 7.3 mg/g DM corresponding to the mean glucose yield being increased by approximately 2-4%.

Four MTAs were found for xylose with a $-\log_{10}(p)$ of between 5.3 and 7.2. The markers were located on chromosomes 5A, 5B, 6A and 7D, with the last being the most significant and having the greatest SNP effect (Table 4). The effect of substituting the SNP increased the xylose release by between 3.2 and 5.6 mg/g DM, also equivalent to increasing the mean xylose yield by 2-4%.

Significant associations were observed for arabinose release (mg/g DM) on chromosomes 1A, 4A, 4B, and 6A ($-\log_{10}(p)$ at 5.1 to 7.9). Their SNP effect ranged from 0.3 to 0.7 mg/g DM and was able to increase the arabinose release by approximately 3-6% (Table 4). Arabinose release had one significant marker with a MAF of 0.05 (BS00108351_51) while all other significant markers for arabinose, xylose and glucose had MAFs of between 0.09 and 0.44 (Table 4).

There was no overlap between the MTAs for biorefinery traits and those identified for other traits, but MTAs were found on chromosome 6A for all three monosaccharides. The markers were located relatively close to one another at 597.9Mb, 524.8Mb and 429.4Mb for glucose, arabinose and xylose, respectively. Chromosome 6A therefore seemed to carry important alleles for increasing the yields of all three types of monosaccharides.

Fig. 5 Manhattan plots for straw yield and quality traits, The significance level was set at a $-\log_{10}(p)$ of 5.05, illustrated by the solid green line. The dotted black line defines a $-\log_{10}(p)$ at 3 ($p = 0.001$) and is used as a threshold to illustrate the chromosomes where there are some weak associations

465 **Fig. 6** Boxplot showing the SNP effect for a) STB, b) glucose, c) xylose and d) arabinose. The y-axis defines the score
 466 of harvest index, STB and plant height, while absence and presence defines SNP allele 1 and SNP allele 2, respectively.
 467 The estimated means of the different traits for all 190 lines were divided into groups depending on whether they had
 468 allele 1 or 2 and plotted as boxplots

469 **Table 4:** MTAs identified by GWAS

Trait	Marker type	Chr. ^a	Pos. [Mb] ^a	% identity ^b	MAF	-log ₁₀ (p)	SNP effect ^{c,d}
Early lodging	wsnp_Ex_c9362_15546626	5B	693.6	99	0.16	19.19	0.10
	BS00009777_51	5B	712.9	99	0.15	18.34	-2.18
	GENE-3659_162	6A	101.3	99	0.06	9.56	0.40
	Kukri_c5789_1029	7D	605.0	99	0.05	8.55	0.42
	wsnp_Ex_c123_244117	3B	485.4	99	0.05	7.18	0.42
	Ra_c31466_694	7A	586.2	99	0.05	6.57	-2.19
Late lodging	Tdurum_contig91519_224	2A	451.8	98	0.27	8.56	1.55
	Ku_c18920_414	1B	557.4	99	0.16	6.86	1.73
	CAP8_c3273_136	4B	552.0	99	0.47	6.61	1.20
	BS00082101_51	5B	551.7	99	0.15	5.63	-1.42
	GENE-2712_130	3D	593.2	98	0.07	5.12	1.85
	RAC875_rep_c105937_467	6D	708.9	98	0.19	5.11	-1.18
Harvest index	wsnp_BE406351A_Ta_2_3	2A	562.6	98	0.35	6.80	2.48
	Tdurum_contig52447_814	5B	456.5	99	0.23	5.42	2.06
Plant height	Ku_c18920_414	1B	557.4	99	0.16	6.59	3.83
	BS00022181_51	4B	552.8	99	0.48	6.38	-2.89
	wsnp_Ex_c4358_7854194	4B	506.1	99	0.46	5.75	-2.56
	Kukri_c338_109	6B	708.7	99	0.28	5.31	-2.89
	Kukri_c46310_84	7B	1.3	99	0.41	5.25	-2.27
STB	Ku_c22026_1410	6B	178.0	99	0.42	9.12	-0.43
	BS00012069_51	5D	561.7	99	0.08	8.23	-0.58
	Kukri_rep_c108378_52	5B	583.2	99	0.21	6.13	-0.34
	Kukri_rep_c109098_132	7A	4.8	99	0.12	5.72	0.40
	Kukri_c23958_187	3B	106.3	99	0.11	5.65	-0.47
	BS00110130_51	1A	535.0	99	0.08	5.25	0.43
Glucose	wsnp_Ex_c18384_27213023	1B	619.7	99	0.18	7.07	-7.25
	wsnp_Ex_c2236_4189774	6A	597.9	99	0.44	5.84	-4.81
Xylose	RFL_Contig3607_648	7D	634.1	99	0.09	7.20	5.57
	wsnp_Ex_c18965_27868480	6A	429.4	99	0.44	6.88	-3.58
	RAC875_c29488_56	5B	663.5	99	0.12	6.62	-4.79
	Excalibur_c53930_53	5A	416.2	98	0.30	5.31	3.17
Arabinose	BS00108351_51	1A	10.0	99	0.05	7.94	-0.74
	GENE-1756_115	4A	593.4	99	0.26	5.78	-0.30
	wsnp_CAP12_c1226_628910	6A	524.8	98	0.44	-0.25	5.35
	BS00084226_51	4B	377.8	99	0.47	-0.26	5.05

470 ^a The chromosomal position of the SNP was obtained from BLAST of the marker sequence applying the new IWGSC
 471 RefSeq v1.0 sequence available at
 472 https://urgi.versailles.inra.fr/blast_iwgsc/?dbgroup=wheat_iwgsc_refseq_v1_chromosomes&program=blastn and
 473 marker sequences at <https://triticeatoolbox.org/wheat/>.

474 ^b % identity between the SNP marker sequence and the wheat reference genome ; 99% corresponds to a perfect match
 475 with 1% being the SNP.

476 ^c The estimate for the SNP effect defines the effect of substituting the major allele with the minor allele. The lines are
 477 homozygote, hence twice the SNP effect, which corresponds to the effect the lines with one version of the SNP vs the
 478 other will have on trait value. The sign (-) indicates the better allele.

479 Grey markers define markers located in the same chromosomal region (chromosome 5B or 6A) as markers found for
 480 other traits.

481 ^d the unit of the SNP effect corresponds to the trait unit listed in table 2.

482 Bold markers define identical markers

483 *Breeding for dual-purpose wheat varieties*

484 The heritability for release of monosaccharides was small ranging from 0.02-0.06 (Table 3) and the expected effect size
 485 obtained were therefore also low (Table 4). There still seems to be variation in data when connecting the release of
 486 monosaccharides [mg/g DM] with the straw yield [t/ha] of the specific lines (Fig. 5, eq. S5), however, the release of
 487 monosaccharides were not correlated with straw yield (Table 5) meaning that an increase in sugar release phenotype
 488 will not necessarily be followed by an increase in straw yield. When converting the effect size of [mg/g DM] to [kg/ha]
 489 based on an average straw yield, the effect size obtained seemed more pronounced compared to SNP effect values of
 490 [mg/g DM] (Table 6, eq. S5). For glucose the SNP effect could correspond to an increase in sugar release between 36
 491 and 55 kg/ha, for xylose between 24 and 42 kg/ha and for arabinose between 2 and 40 kg/ha. If the effect of the alleles
 492 are additive then it corresponds to an increase in total monosaccharide release by 305.7 [kg/ha]. The correlation
 493 between the three monosaccharides was highly significant (Table 5) such that an increased release of glucose also
 494 results in a higher release of xylose and arabinose (Table 5). This could indicate that there are wheat lines with
 495 structures that more easily gets solubilized and accessible for enzymatic hydrolysis and therefor developing markers
 496 targeting this trait seems feasible.

497 Figure 5: Histograms of the frequency of sugar release in t/ha based on estimated means for sugar release and the
 498 corresponding straw yield means of each wheat line.

499 Table 5: Correlation between straw yield and the three monosaccharides

traits	Xylose	Arabinose	Straw yield
Glucose	p < 0.001 r = 0.64	p < 0.001 r = 0.35	p = 0.408 r = 0.06
Xylose		p < 0.001 r = 0.34	p = 0.042 r = 0.15
Arabinose			p = 0.535 r = 0.045

500

501 **Table 6:** The SNP effect for sugar release in kg/ha based on average straw yield

trait	SNP marker	SNP effect [mg/g DM]	SNP effect [kg/ha]
Glucose	wsnp_Ex_c18384_27213023	-7.25	54.57
	wsnp_Ex_c2236_4189774	-4.81	36.20
Xylose	RFL_Contig3607_648	5.57	41.92
	wsnp_Ex_c18965_27868480	-3.58	26.95
	RAC875_c29488_56	-4.79	36.05
	Excalibur_c53930_53	3.17	23.86
Arabinose	BS00108351_51	-0.74	5.57
	GENE-1756_115	-0.30	2.26
	wsnp_CAP12_c1226_628910	5.35	40.27
	BS00084226_51	5.05	38.01

502

503 *Discussion*

504 *MTAs for traits relevant to dual-purpose wheat*

505 The main purpose of this study was to identify MTAs for several agronomic traits, and especially for traits that impact
506 the yield and quality of mature straw biomass, in order to facilitate the breeding of dual-purpose varieties. Many of the
507 standard agronomic traits, including plant height, harvest index, lodging, and STB susceptibility, affect the yield and
508 quality of straw biomass. Lodging results in greater loss of straw biomass and the quality of the straw may deteriorate
509 due to degradation by microbes. The STB disease is also thought to reduce straw quality (Brading et al. 2002). The
510 yield of straw biomass is related to plant height and harvest index, where breeding for more straw biomass will increase
511 the height or density and decrease the harvest index. Grain yield was included in the association analysis because it will
512 always be the main breeding objective.

513 *Lodging and plant height*

514 The MTAs observed for plant height were all located in chromosomes previously associated with plant height (Liu et al.
515 2011; Zhang et al. 2011; Griffiths et al. 2012; Zanke et al. 2014) however, the specific markers were not identical even
516 though they mapped to the same chromosomal regions. The markers on chromosome 4B (RAC875_c1357_860 and
517 RFL_Contig5341_816), reported by Zanke et al. (2014), were located at approximately the same locus (609.0 and
518 645.3Mb respectively) close to the chromosomal region identified in the present study (506-553Mb) (all chromosomal
519 locations are based on IWGSC blast results). The marker RAC875_rep_c71463_98, located on 6B (699Mb; Zanke et al.
520 2014) also co-localized with the marker identified in the present study (709Mb), while the markers on 7B were located
521 in the same region of the chromosome (Ex_c101666_634 (24.1Mb) in Zanke et al. (2014) vs. Kukri_c46310_84
522 (0.1Mb) in the present study). The sequence for the SSR marker on 1B GDM0126_196 (124.9 cM) identified by Zanke
523 et al. (2014) was not available, rendering a full comparison of chromosomal locations impossible, but based on linkage
524 maps of the SSR markers, GDM0126_196 seemed to be located in the middle of the chromosome upstream of the
525 marker identified in the present study, which were located towards the telomere of chromosome 1B.. The observed
526 difference in significant marker positions could be explained by differences in the germplasm between the studies. In the
527 present study the majority of lines are semi-dwarfs (with respect to either *Rht-B1b* or *Rht-D1b*) compare to 7% and 58%
528 respectively of the winter wheat varieties used in the study Zanke et al. (2014). Their germplasm also differed further as
529 14 spring varieties also were included in the analysis.

530 Lodging was scored between once and three times during the growth season, although only if relevant due to weather
531 conditions (rain, hail and wind). Due to good weather conditions, there was no scoring of intermediate-stage lodging
532 during 2015/2016. Significantly associated SNPs were identified for early lodging and late lodging, while for
533 intermediate lodging only weak associations were found (Fig. 2 and Table 4). This indicates that the early and late
534 stages of lodging were controlled by different genes, except for genes located on chromosome 5B at around 551.7-
535 712.9Mb. Intermediate stage lodging shared some tendencies with both the early and late stages and did not show any
536 strong MTAs. It is therefore likely to be a less expressed trait, which might explain why it was harder to identify true
537 MTAs for lodging at this stage. The absence of scores for intermediate-stage lodging in the final year (2015/16) also
538 weakened the strength of the trait and may explain why it was difficult to identify true MTAs.

539 The marker identified on chromosome 1B was identical between late lodging and plant height, and the markers found
540 on 4B were at approximately the same locus 552.0-552.8Mb (Table 4). It is likely that the absence of the *Rht-B1b* allele
541 in 4B had an impact on lodging because it is negatively affected by plant height (Verma et al. 2005). The marker
542 identified on 1B for lodging was therefore likely to be a marker associated with plant height. Association between 1B
543 and plant height has been demonstrated in other studies (Liu et al. 2011; Griffiths et al. 2012; Zanke et al. 2014). Verma
544 et al. (2005) found associations between the width of cell walls in the first internode and chromosome 1B as well as a
545 strong correlation between the length of the first internode and cell wall width. It is possible that the MTA observed in
546 the present study was involved in the height and stem thickness of the first internode and therefore occurred as a
547 significant marker for both plant height and lodging. The results indicate that plant height has a greater influence on
548 lodging in the later developmental stages compared to the early growth stage (Table 4). Tendencies at intermediate
549 stage lodging confirmed this theory since the same marker was identified as having weak associations in 1B and a
550 marker at approximately the same locus in 4B.

551 There have been few GWAS studies published on wheat lodging as a trait, but more studies have been conducted on
552 association mapping/ QTL analysis. MTAs for lodging have been reported for chromosomes 3A and 3B when running
553 GWAS with 1322 winter wheat lines and 15K SNP array (Edriss et al. 2017). In the present study, only 3B was
554 identified as a MTA and only for early stage lodging. The association found on 1B, 4B, 6D, and 7D (Fig. 2) has
555 previously been documented in at least two QTL studies on winter wheat (in one or both) (Verma et al. 2005; Zhang et
556 al. 2008). A recent study that analyzed lodging-associated plant characteristics in winter wheat found markers on
557 chromosomes 3A, 5B, and 6B that were associated with the root plate width, stem strength, stem wall width and stem
558 diameter indicating that the loci found here might play a role in cell wall strength (Berry and Berry 2015). The MTAs
559 on 5B identified for early and late stage lodging have not previously been associated with lodging, but based on the
560 study by Berry and Berry (2015) they are likely to be involved in cell wall strength. Zhang et al. (2008) suggest that the
561 MTA located on 7A represents an epistatic association for lodging between 7A and 4D, such that an effect in 7A is
562 associated with the absence or presence of genes on 4D. However a direct link was not observed. To the authors'
563 knowledge, MTAs for early lodging on chromosomes 5B, 6D and 7A and for late lodging on chromosomes 2A, 3D and
564 5B have not been reported and hence are likely to be novel markers. The strongest markers for lodging are those
565 associated with chromosome 5B and these could be used in marker-assisted selection in breeding programmes to screen
566 for lodging.

567 In conclusion, only the chromosome 5B markers were identical at all stages of lodging, indicating that this region could
568 carry the same QTL due to the greater extent of linkage disequilibrium (LD) in modern wheat varieties (Hao et al. 2011;
569 Giraldo et al. 2016). Moreover the three stages of lodging were likely to be controlled by different genes. Intermediate
570 and late stage lodging seemed to be affected more by plant height, since they both mapped to the same SNP in 1B and
571 to the same location in 4B as identified for plant height.

572 *Grain yield, harvest index, and STB*

573 It was not possible to detect significant SNPs for grain yield, but the chromosomes that showed a weak association
574 with grain yield (1A, 1B, 2A, 3A, 5B, 7A, 7B and 7D; Fig. 4) were in agreement with previous studies (Bordes et al.
575 2014; Edae et al. 2014; Assanga et al. 2017). Grain yield is a highly quantitative trait that is controlled by many minor

576 and major genes and identification of new QTLs might be difficult in modern European wheat partly due to inbreeding
577 and common genetic backgrounds.

578 The significantly associated MTAs for harvest index were not previously reported (Edae et al. 2014; Ain et al. 2015;
579 Sukumaran et al. 2015) which could be due to differences in germplasm as all the above studies were based on panels of
580 spring wheat varieties either in moist, temperated, stressed or rainfed climates. Furthermore, the panel in Ain et al.
581 (2015) consisted of historical wheat cultivars from Pakistan, which are expected to have an even more diverse genetic
582 background. No studies to our knowledge have been performed in winter wheat addressing MTAs for harvest index and
583 the observed MTAs in chromosome 2A and 5B are therefore likely to be novel markers that can be tested in breeding
584 programmes to improve the harvest index.

585 The markers identified for STB have already been documented in other studies (Brown et al. 2015; Arraiano and Brown
586 2017; Vagndorf et al. 2017), with some of them located either in the same or close to the loci of known Septoria tritici
587 blotch (*Stb*) genes. The MTA in 3B was located close to the *Stb14* gene (Cowling 2006), the MTA in 5B was located
588 upstream of the *Stb1* gene (Adhikari et al. 2004), and the MTA in 7A was at approximately the same locus as the *Stb3*
589 gene (Goodwin and Thompson 2011) and the *TmStb1* gene (Brown et al. 2015).

590 Vagndorf et al. (2017) conducted GWAS with 15k SNP markers on a panel of 164 North European winter wheat
591 varieties and breeding lines grown for three years at three locations in Denmark. They observed an association in 5D
592 and 7A, with the association in 5D at approximately the same locus (560.5 Mb) as that observed in the present study.
593 The association in 7A, however, was at opposite ends of the chromosome and the present finding on chromosome 7A
594 was therefore more in agreement with the findings of Goodwin and Thompson (2011) and Brown et al. (2015).

595 The MTAs observed in 1A and 6B did not co-localize with any of the known *Stb* genes but did co-localized with the
596 QTLs (QTL2 and MQTL21) identified by Brown (2015). The observed association in chromosome 5D was in the same
597 region as a QTL observed in a later study (Arraiano and Brown 2017). Therefore, none of the identified MTAs for STB
598 are novel markers, but they may still be important for STB-resistance in breeding.

599 *Biorefinery traits: straw quality and yield*

600 Difficulties in identifying MTAs are likely to be attributable to the low heritability of straw yield (Fig. 5). Only markers
601 that were weakly associated ($p < 0.001$) with straw yield were identified on chromosome 1B and 4B (552.8Mb and
602 1.3Mb respectively), indicating that these loci could play a role in controlling straw yield. The 1B loci were identical to
603 those for plant height, corroborating the finding that plant height affects the yield of straw biomass. The association in
604 4B was not in the same chromosomal region as that for plant height (Table 4) but it might still be due to the presence or
605 absence of the *Rht-B1* gene. None of the weak associations have previously been documented (Sukumaran et al. 2015;
606 Qaseem et al. 2018). Qaseem et al. 2018 conducted an association study on bread wheat, testing for a wide number of
607 agronomic traits, including biomass, and the effects of drought and heat. For the control group, only one locus was
608 identified and localized on chromosome 5A, while MTAs on chromosome 2B were identified under heat conditions, 3B
609 under drought conditions and 2A, 5A, and 7A under combined heat and drought conditions. After conducting GWAS
610 on a population of spring wheat, Sukumaran et al. (2015) showed an association between biomass and chromosome 3D
611 and 6A at a significance level of 0.0001.

Both Sukumaran et al. (2015) and Qaseem et al. (2018) succeeded in identifying markers for biomass with a higher power ($p < 0.0001$) than that documented in the present study ($p < 0.001$). One explanation is obviously a difference in mapping populations (spring wheat vs. winter wheat) but in addition the study conducted by Qaseem et al. (2018) was a pot experiment performed in a greenhouse with a smaller influence of variation in environmental factors compared to a field experiment. However, a greenhouse environment might not reflect the environment in the field, and the MTAs identified might therefore differ between the two. A recent study also conducted on spring wheat in Kazakhstan also failed to identify any MTAs for biomass using a diverse panel of 194 lines at three locations over three years (Turuspekov et al. 2017). The number of lines was similar to that used in the present study, which could indicate that the lack of identified MTAs is due to the number of lines being too small. Biomass yield is also affected by other traits such as plant height, and controlling for this, by including plant height as a covariate in the mixed linear model in GWAS, might make it easier to detect significant MTAs. Moreover, like grain yield, straw yield is a quantitative trait possibly controlled by several small effector genes, and this may also have contributed to the difficulties in identifying significant MTAs for straw yield.

Despite the low heritability observed for straw quality traits (glucose, xylose and arabinose release), MTAs for all three monosaccharides were identified, which all had a pronounced effect when converted to kg monosaccharide per hectare. None of the observed MTAs for monosaccharide release were identical between the monosaccharide types. However, the markers in chromosome 6A were located in approximately the same chromosomal region (597.9, 429.4, and 524.8Mb for glucose, xylose and arabinose respectively). It is therefore likely that there are genes in this region that affect the cell wall structure, making the hemicellulose and cellulose more accessible for enzymes, such that sugar release is increased. The fact that all monosaccharide release yield were positively correlated also supports this finding (Table 5). Of the 108 cellulose synthase (Csl) genes identified in wheat, three are located on 6A which could directly control the structure of hemicellulose (Kaur et al. 2017). Alternatively, it could be the genes involved in cell wall composition (causing changes in lignin, hemicellulose structure or compactness) or those directly involved in cell wall bindings, such that the cell wall becomes more open and easily accessible. Further studies are needed to clarify the impact of chromosome 6A on releasing monosaccharides from straw.

The markers identified in 1B for glucose and in 4B for arabinose were not identical with those found for plant height, but the MTA identified in 1B for glucose might be located in the same chromosomal region due to large LD observed in wheat (Giraldo et al. 2016), i.e. 619.7Mb and 557.4Mb for glucose and plant height. The markers for arabinose and plant height (377.8Mb and 506.1 Mb respectively) were located further apart and are not likely to be associated with the same genes. These findings indicate that plant height might also affect the yield of released glucose (Table 4).

To the best of the authors' knowledge, only one association study on sugar release after enzymatic saccharification has been conducted to date (Bellucci et al. 2015). In that study, only weak MTAs for glucose release were identified on chromosome 1B. One of the MTAs for glucose release documented in the present study was also localized on chromosome 1B and in approximately the same location (Bellucci et al. 2015). These two studies used different saccharification methods on completely different wheat germplasm, which strongly supports the genes on chromosome 1B being involved in regulating glucose release.

648 In the present study, four MTAs were observed for xylose release and four for arabinose release, while none were
 649 identified in a study by Bellucci et al. (2015). It is likely that these chromosomes have a role in controlling the
 650 hemicellulose structure (arabinose and xylose modifications), such that they can be accessed more easily by enzymes,
 651 which in turn will contribute to increasing sugar release. More studies are needed to verify this. Both xylose and
 652 arabinose are part of the hemicellulose structure in the cell wall, and this complex structure is presumably controlled by
 653 many small effector genes, which also explains the small effect size (See Figure 6 and Table 4). In addition, the
 654 experimental protocol used for the sugar release assay may be highly important in identifying MTAs for sugar release
 655 (Oakey et al. 2013; Lindedam et al. 2014; Jørgensen et al. 2018) and further experimental optimization may increase the
 656 possibility of identifying them. Especially the noise introduced during the pretreatment, the enzymatic hydrolysis and/or
 657 the quantification of monosaccharides should be reduced, to obtain higher heritability estimates and to increase the
 658 chance of identifying MTAs with bigger effect sizes. Although the effect size seemed low, it still would correspond to
 659 and pronounced increase in sugar release in kilograms per hectare and especially if the effect were additive (Table 6).

660 *Controlling for plant height in GWAS*

661 Many of the traits analyzed by GWAS, including lodging, straw yield and glucose seemed to be affected by plant
 662 height. To verify whether plant height was indeed the factor affecting these traits, GWAS was conducted with plant
 663 height included as a covariate.

664 Some of the MTAs identified for late lodging seemed to be affected by plant height, while early-stage lodging was
 665 unaffected (MTAs in 1B and 4B; Table 4). This difference in plant height dependency between late and early lodging
 666 was partly confirmed by controlling for plant height in GWAS, since MTAs for early lodging were almost unaffected
 667 while the MTAs and weak MTAs for late and intermediate lodging, respectively, were greatly affected (Table 7). For
 668 early lodging it was only the significance level that changed, where the $-\log_{10}(p)$ was higher for 3B and 5B and lower
 669 for 6A, 7D and 7A. The MTA in 7A was actually below the significance tradeoff level corresponding to a $-\log_{10}(p)$ of
 670 5.05 (Table 7). Early lodging was therefore not dependent on plant height and its inclusion as a covariate did not
 671 improve the QQ-plot (results not shown).

672 The weak associations observed for intermediate lodging were greatly affected by plant height, since chromosomes 1B,
 673 2B and 3B were now the only chromosomes with weak associations, eliminating weak associations in 4B, 5B, and 7A.
 674 1B was still weakly associated, and even though the markers differed, two of them mapped to the same chromosomal
 675 region (502.5Mb and 548.7Mb for wsnp_Ra_c5088_9081927 and Tdurum_contig42749_3287, respectively) while the
 676 last marker BS00064032_51 was located at the start of the chromosome (144.9Mb) (Tables 4 and 5). Based on this, the
 677 association in 1B might not only be an effect of plant height.

678 The MTAs for late lodging also differed, with associations found in 6B and 5D (705.0Mb and 341.6Mb, respectively),
 679 while associations in 1B and 4B became weak associations. None of the MTAs identified for late lodging were identical
 680 with the MTAs identified without plant height as a covariate, however the weak associations identified in 1B (520.5Mb)
 681 and 4B (609.3Mb) were in the same chromosome region. Therefore plant height seemed not to be the only factor
 682 responsible for the associations in 1B and 4B. Moreover, the introduction of plant height as a covariate did not improve
 683 the QQ-plot for intermediate and late lodging, and therefore the results in Table 4 are the most reliable. Association in
 684 1B and 4B for late lodging are therefore likely to be novel markers for lodging and not just an effect of plant height.

685 When plant height was included as a covariate for glucose release, the effect of the MTAs in 1B disappeared, however
686 the other effects all disappeared as well (Table 7). The QQ-plots indicated that the GWAS model that best described
687 data was the one in which plant height was not included as a covariate, which is why the correct MTA results are those
688 given in Table 4.

689 In conclusion, controlling for plant height did not improve the QQ-plots for any of the affected traits and plant height
690 did not seem to be the major QTLs identified in 1B and 4B. The markers are therefore likely to be novel markers for the
691 traits investigated.

692 **Table 5:** Comparison of MTAs identified by GWAS with and without plant height as covariate

Trait	Standard GWAS			GWAS with plant height as covariate			
	Marker type	Chr. ^a	-log10(p)	Marker type	Chr. ^a	Pos. [Mb] ^a	-log10(p)
Early lodging	wsnp_Ex_c9362_15546626	5B	19.19	wsnp_Ex_c9362_15546626	5B	693.6	20.57
	BS00009777_51	5B	18.34	BS00009777_51	5B	712.9	19.01
	GENE-3659_162	6A	9.56	GENE-3659_162	6A	101.3	8.84
	Kukri_c5789_1029	7D	8.55	wsnp_Ex_c123_244117	3B	485.4	8.12
	wsnp_Ex_c123_244117	3B	7.18	Kukri_c5789_1029	7D	605.0	7.09
	Ra_c31466_694	7A	6.57	Ra_c31466_694	7A	586.2	4.83
Intermediate lodging^b	CAP8_c3273_136	4B	3.41	IACX1098	2B	58.3	3.36
	BS00075731_51	7A	3.13	RFL_Contig1456_842	3B	39.9	3.36
	Jagger_c505_141	5B	3.11	wsnp_Ra_c5088_9081927	1B	502.5	3.36
	Ku_c18920_414	1B	3.02	Tdurum_contig42749_3287	1B	548.7	3.36
				BS00064032_51	1B	144.9	3.36
Late lodging	Tdurum_contig91519_224	2A	8.56	BS00046264_51	6B	705.0	5.76
	Ku_c18920_414	1B	6.86	GENE-3583_319	5D	341.6	5.49
	CAP8_c3273_136	4B	6.61	wsnp_Ex_c7988_13555376	5B	316.7	4.44
	BS00082101_51	5B	5.63	BS00057153_51	4B	609.3	4.03
	GENE-2712_130	3D	5.12	BS00091666_51	6A	31.4	3.55
	RAC875_rep_c105937_467	6D	5.11	wsnp_Ra_c5088_9081927	1B	520.5	3.47
Glucose	wsnp_Ex_c18384_27213023	1B	7.07				
	wsnp_Ex_c2236_4189774	6A	5.84				

693 ^a The chromosomal position of the SNP is obtained from BLAST of the marker sequence applying the new IWGSC RefSeq v1.0 sequence available at
694 https://urgi.versailles.inra.fr/blast_iwgsc/?dbgroup=wheat_iwgsc_refseq_v1_chromosomes&program=blastn and marker sequences at
695 <https://triticeatoolbox.org/wheat/>. For exact chromosomal locations for standard GWAS results please refer to Table 4.

696 Markers in grey are identical for the two different GWAS methods.

697 ^b MTAs are based on week associations and are not true MTAs

698 *Applying GWAS on lines from an active breeding nursery*

699 Using an active breeding nursery for GWAS was highly effective at identifying MTAs, but due to limited variation in
700 more inbred traits, the genetic effect was expected to be relatively small. This was clearly also the case for grain yield
701 where no markers were detected. The lines included in the breeding programme had been bred primarily for yield, so
702 the variation should have been relatively low. However, the obtained grain yield still exhibited a relatively high genetic
703 variance (9.48) and heritability (0.37). In theory, this should increase the likelihood of detecting MTAs, but since grain
704 yield is controlled by many small QTLs, it may be difficult to detect the associated chromosomes even though
705 heritability is present.

706 Using rotation in breeding nurseries that replace 50% of the lines each year is both an advantage and a disadvantage.
707 The advantage is that it increases the number of lines included in GWAS, hence increasing its power, but the
708 disadvantage is it increases the G×E variance, making identification of the same markers in the three consecutive years
709 difficult. Despite the use of rotation and the lack of MTAs for grain yield, straw yield, and intermediate lodging MTAs
710 for all the other traits analysed, including plant height, were still obtained. It may now be common practice in breeding
711 companies to conduct GWAS in an active breeding nursery rather than using a designed population but this is not
712 widely published and here it shows indeed to be highly versatile at identifying MTAs through GWAS.

713 Using active breeding nurseries for the identification of dual-purpose varieties with a high yield of both grain and straw
714 is a challenge, as demonstrated by Townsend et al. (2017) and as confirmed in the present study with the difficulties in
715 identifying MTAs for straw and grain yield. However, it was still possible to identify MTAs for biorefinery traits in the
716 lines bred for a high grain yield, indicating that dual-purpose varieties may be indeed obtained without the introduction
717 of more distinct germplasm. Landraces that can add greater biomass variation may be desirable for breeding dual-
718 purpose varieties, but this will also introduce undesirable MTAs related to height, which will need to be removed in
719 order to develop competitive cultivars, and therefore it probably will have limited use in later stages of breeding.

720 *Challenges in identifying genetic effects*

721 The detection of a genetic effect was challenging for the majority of the traits despite the fact that MTAs were identified
722 in most cases (Tables 3 and 4). Heritability obtained by mixed linear models was considered low for early stage
723 lodging, harvest index and release of the three monosaccharides after enzymatic hydrolysis ($H^2 < 0.15$) (Table 3).
724 Repeatability, which were based on the genetic variance, the genotype by year variance and the error term (like
725 heritability) were divided with the number of repeats and number of years and were therefore considerably higher than
726 heritability (Table 3).

727 Repeatability was much higher than heritability for straw yield for which no MTAs were identified (H^2 of 0.15, Table
728 3). Other studies have experienced similar difficulties in obtaining high heritability and repeatability for straw yield
729 (Bellucci et al. 2015; Sukumaran et al. 2015). Regardless of the low heritability, Sukumaran et al. (2015) still succeeded
730 in identifying MTAs for biomass yield for spring wheat in the controlled glasshouse environment ~~and on spring wheat~~.
731 No MTAs for grain yield and straw yield were identified in the present study, which may be explained by the fact that
732 both traits are considered to be highly quantitative and that little variation existed in the analysed germplasm. However,
733 both the heritability and the repeatability for grain yield were high indicating that there indeed was genetic variation in
734 the germplasm even though it was not possible to identify MTAs. The number of varieties used in the present study

might be too low to capture the variation because grain yield is known to be controlled by many small ~~and major~~ effector genes. The G×E variance was also high for grain yield as it explained about 43% of the total variation (based on genetic, G×E and error variance components) indicating that grain yield also were greatly affected by the environment or the rotation of varieties each year. Furthermore, ~~were~~ none of the analysed GWAS algorithms for straw yield and grain yield gave a perfect fit which calls for developing other algorithms or including other co-variables that capture the population structure better to increase the chance of identifying MTAs for these traits.

Heritability was relatively low for early-stage lodging and increased at each developmental stage (Table 3), nevertheless MTAs were only identified for the early and late developmental stages (Table 4). All three stages of lodging were greatly affected by the environment as lodging is mainly initiated by rainy and windy weather conditions (Pinthus 1974). Plants that were lodging at an early stage might be standing in the final growth stage because plants have the ability to rise again. This is also apparent in the relatively high G×E components for lodging (Table 3) which are explaining between 23 and 46% of the total variation with the G×E variance increasing with the stage of lodging (early, intermediate and late). The severity of lodging at a late development stage was expected to be more dependent on plant genetics since changes in the plants physical architecture would be more prominent. The combination of plant height, root development, stem strength and seed weight was therefore likely to have a greater effect at the intermediate or late development stages compared to the early stages (Verma et al. 2005; Berry and Berry 2015). This was also confirmed by the association between the observed MTAs for late and intermediate-stage (weak associations) lodging and plant height (Table 4). MTAs for intermediate and late-stage lodging can therefore be harder to detect, even when high heritability is present, because they seem to be more complex traits that are considerably affected by interactions between weather conditions and genetics.

For almost every trait (with the exception of plant height, STB and xylose), the G×E effect was greater than the genetic effect. It was therefore harder to find associated SNPs that were fully consistent between years. The fact that 50 lines were excluded and 50 new ones were introduced each year may be a reasonable explanation for increasing the G×E variance. Using an active breeding nursery with rotation for MTA analysis therefore makes it difficult to find stable markers throughout years.

The low heritability for sugar release was primarily due to variance caused by analytical errors, as the error variance explained the majority of the variance in the model (around 90% of the total variance). The identification of genetic variance in sugar release between cultivars was therefore highly challenged by the analytical variance introduced. It was not clear which step in the chemical/enzymatic treatment was responsible for the analytical variation. Even though, variation from plate to plate and between enzyme batches might be the main contributors. To normalize data for plate-to-plate variation, control samples were included in every plate, but this was not sufficient to account completely for the experimental variance. Another study has shown that the organization of the experiment and the location of samples on the plate was important for detecting heritability (Oakey et al. 2013). By including these parameters in the mixed linear model applied, Oakey et al. (2013) succeeded in correcting for this variance and increase the heritability. In the present study, plate position and plate number were included as explanatory random variables in the mixed linear model to adjust for this variance. Despite the adjustment, heritability was still low. The plate position did have an impact, however the effect was small compared to the variance introduced by the plate number (results not shown). In future

772 studies, reducing the plate-to-plate variation might be one way of increasing the heritability for release of
773 monosaccharides after enzymatic saccharification. Despite the difficulties in obtaining high heritability for the release
774 of monosaccharides, it was still possible to obtain MTAs, where for glucose one of the MTAs was identical with a locus
775 previously identified by Bellucci et al. (2015).

776 Therefor heritability and repeatability are not always correlated with the ability to detect MTAs. Instead, heritability
777 gives information about how much of the phenotypic variation is due to genetic variation and how much of any
778 variation can be theoretically explained by an SNP effect.

779 *Conclusions*

780 This study demonstrates that the GWAS algorithm FarmCPU can be applied successfully in an active breeding nursery,
781 thereby providing identification of MTAs for a number of agronomic traits including plant height, lodging, STB
782 susceptibility, and harvest index. Significant MTAs for glucose, xylose, and arabinose release from straw by enzymatic
783 saccharification were identified, demonstrating that it is possible to breed for biorefinery-related traits.

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913

a Early lodging

b Late lodging

c Plant height

d Harvest index

a STB

b Glucose

c Xylose

d Arabinose

Supplementary material

Breeding for dual-purpose wheat varieties using marker-trait associations for biomass yield and quality traits

Theoretical and applied genetics

Pernille L. Malik^a, Luc Janss^b, Linda K. Nielsen^c, Finn Borum^c, Henning Jørgensen^a, Birger Eriksen^c, Jan K. Schjoerring^a, Søren K. Rasmussen^{a*}

^aDepartment of Plant and Environmental Sciences, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark

^bDepartment of Molecular Biology and Genetics, Aarhus University, Blichers Alle 20, 8830 Tjele, Denmark

^cSejet Plant Breeding, Nørremarksvej 67, 8700 Horsens, Denmark

*Corresponding author: skr@plen.ku.dk, Phone +45 35333436

Supplementary equations:

Equations for grain yield (eq. 1), straw yield (eq. 2 and 3) and harvest index (eq. 4) are given below.

$$Grain_{yield} \left[\frac{hkg}{ha} \right] = \frac{Grain \left[\frac{kg}{parcel} \right] * (100 - 15) * 100 \left[\frac{hkg * m^2}{kg * ha} \right]}{\frac{8.1 \left[\frac{m^2}{parcel} \right]}{100}}$$
$$Grain_{yield} \left[\frac{t}{ha} \right] = Grain_{yield} \left[\frac{hkg}{ha} \right] * 0.1 \left[\frac{t}{hkg} \right] \quad (eq. S1)$$

The straw yield in hkg/ha was obtained using the following equations:

$$Straw_{waterloss} [\%] = 100 - \left(\left(\frac{100}{straw_{wet}[g]} \right) * straw_{dry}[g] \right) [\%] \quad (eq. S2)$$

$$Straw_{yield} [hKg/ha] = \frac{straw[kg] * (100 - Straw_{waterloss} [\%]) * 100}{\frac{8.1}{100}}$$

$$Straw_{yield} [t/ha] = Straw_{yield} \left[\frac{hkg}{ha} \right] * 0.1 \left[\frac{t}{hkg} \right] \quad (eq. S3)$$

The harvest index was calculated based on the mass of the grain yield and the mass of the biological yield (sum of grain yield and straw yield) by the following equation:

$$harvest\ index\ [\%] = \frac{economic\ yield[hKg/ha]}{biological\ yield[hKg/ha]} * 100 \quad (eq. S4)$$

The conversion of sugar release from mg/g DM to t/ha was based on estimated means of sugar release and straw yield and was calculated as follows:

$$Sugar\ release\ \left[\frac{t}{ha}\right] = Straw_{yield}\left[\frac{t\ DM}{ha}\right] * sugar\ release\ \left[\frac{mg}{g\ DM}\right] * 10^{-3}\left[\frac{g\ DM}{mg}\right] \quad (eq. S5)$$

This equation was also used for converting the SNP effect for sugar release to kg/ha and here was an average of the estimated means of straw yield used.

Figure S1: The distribution of phenotypic data from estimated means. As all the histograms show are all the phenotypic traits relatively normal distributed (with exception of the three stages of lodging) and there seem to be variation between cultivars. However only grain yield, plant height, STB and harvest index passed the Shapiro-Wilk normality test ($p > 0.001$) (See Table S1). Septoria defines STB.

Table S1: Shapiro-Wilk normality test of estimated means

Trait (estimated means)	p-value (Shapiro-Wilk normality test)
Straw yield	$8.98 \times 10^{-5} *$
Plant height	0.5013
Grain yield	0.0724

Early lodging	$2.2 \times 10^{-16} *$
Intermediate lodging	$6.575 \times 10^{-4} *$
Late lodging	$1.814 \times 10^{-4} *$
STB	0.1172
Harvest index	1.807×10^{-3}
Glucose	0.6037
Xylose	0.3269
Arabinose	$4.072 \times 10^{-5} *$

* defines p-values that are below 0.001 and show which traits that are not normal distributed.

Figure S2: Population structure and heterozygosity. a) shows the heterozygosity of the 190 lines used in this study and the 6137 markers (including unknown markers) b) shows the distribution of the first 3 principal components plotted against each other.

Figure S3: Comparison of QQ-plots between the three kinship algorithms available in standard GAPIT; a) Emma, b) VanRaden, and c) Loiselle. As an example was straw yield, grain yield and plant height chosen however, equivalent results were obtained for other traits. The QQ-plots are judged based on how well the plotted values follow the diagonal (red line) and drift off toward the end, as too much noise can indicate missing correction for population structure.

Figure S4: Comparing QQ-plots between a) FarmCPU b) SUPER and c) GAPIT-CMLM. Shown are QQ-plots for grain yield and straw yield as these traits behaved differently than the other traits and for plant height as serve as an example for all other traits. The QQ-plots are judged based on how well the plotted values follow the diagonal (red line) and drift off toward the end, as too much noise can indicate missing correction for population structure.