

This is a repository copy of *Breeding Dual-Purpose Maize : Grain Production and Biofuel Conversion of the Stover*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/201916/>

Version: Published Version

---

**Article:**

Gomez, Leonardo Dario [orcid.org/0000-0001-6382-9447](https://orcid.org/0000-0001-6382-9447) (2023) Breeding Dual-Purpose Maize : Grain Production and Biofuel Conversion of the Stover. *Agronomy*. 1352. ISSN 2073-4345

<https://doi.org/10.3390/agronomy13051352>

---

**Reuse**

This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:

<https://creativecommons.org/licenses/>

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

## Article

# Breeding Dual-Purpose Maize: Grain Production and Biofuel Conversion of the Stover

Noemi Gesteiro <sup>1</sup>, Ana Butrón <sup>1</sup> , Rogelio Santiago <sup>1</sup>, Leonardo D. Gomez <sup>2</sup> , Ana López-Malvar <sup>3</sup> , Lorena Álvarez-Iglesias <sup>1</sup> , Pedro Revilla <sup>1</sup>  and Rosa Ana Malvar <sup>1,\*</sup> 

<sup>1</sup> Misión Biológica de Galicia (Consejo Superior de Investigaciones Científicas), El Palacio—Salcedo, 36143 Pontevedra, Spain; ngesteiro@mbg.csic.es (N.G.); abutron@mbg.csic.es (A.B.); rsantiago@mbg.csic.es (R.S.); lalvarez@mbg.csic.es (L.Á.-I.); previlla@mbg.csic.es (P.R.)

<sup>2</sup> Center for Novel Agricultural Products (CNAP), Department of Biology, University of York, Heslington, York YO10 5DD, UK; leonardo.gomez@york.ac.uk

<sup>3</sup> Departamento Biología Vegetal & Ciencias Suelo, Facultad de Biología, Universidad Vigo, Unidad Asociada MBG-CSIC, Lagoas Marcosende, 36310 Vigo, Spain; alopezmalvar@uvigo.es

\* Correspondence: rmalvar@mbg.csic.es

**Abstract:** The improvement of maize double cropping has become increasingly important in recent years. In order to establish breeding programs for sustainable maize production, the goals of the research were (i) to understand the correlations between grain and stover yield and saccharification efficiency, and (ii) to identify QTL and metabolic pathways to design of breeding programs in a double exploitation approach. We carried out a genome-wide association study (GWAS) and a metabolic pathway analysis using a panel of highly diverse maize inbreds. As results, we have obtained that the regulation of energetic and developmental pathways have been pointed out as key pathways related to stover quantity and utilization, while no specific pathways could be identified in relation to grain yield. However, due to the moderate high heritability of yields and their positive correlation a phenotypic selection approach would be adequate for the improvement of both yields, while for saccharification efficiency improvement upcoming genomic selection models are more advisable. Overall, breeding strategies that manage the dual use of maize are viable and will contribute to a more sustainable maize crop in the near future.

**Keywords:** maize; yield; saccharification; double exploitation; GWAS; metabolic pathway



**Citation:** Gesteiro, N.; Butrón, A.; Santiago, R.; Gomez, L.D.; López-Malvar, A.; Álvarez-Iglesias, L.; Revilla, P.; Malvar, R.A. Breeding Dual-Purpose Maize: Grain Production and Biofuel Conversion of the Stover. *Agronomy* **2023**, *13*, 1352. <https://doi.org/10.3390/agronomy13051352>

Academic Editors: Shuyu Liu, Shisheng Chen, Fa Cui and Cheng Liu

Received: 13 April 2023

Revised: 8 May 2023

Accepted: 9 May 2023

Published: 11 May 2023



**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

One of the most important and widely used cereals for food, feed, industrial and energy purposes is maize. Improving the double exploitation of maize has become increasingly important in the recent years due to population increases and growing food demand [1,2]. Breeding programs have been focused on traits associated with grain yield and its related traits. Grain yield is a quantitative trait with low heritability, which may indicate several assumptions: (i) yield is determined by a large number of genes; (ii) the largest proportion of the variance is due to environment or experimental error; and/or (iii) the difference between genotypic values depends on environment ( $G \times E$  effect) [3–5].

Although a large number of quantitative trait loci (QTL) for maize grain yield have been described over the past three decades, the phenotypic variance that they explained has been very low. For example, in a mapping population of 266  $F_{2,3}$  family lines, six genomic regions associated with grain yield were mapped. One of them, located in the region between *bnlg1556* and *umc128* on chromosome 1 was identified as the major QTL, explaining between 7.1% and 16.8% of the total phenotypic variation [6]. Similarly, in a set of 253 recombinant inbred lines (RILs), Yang et al. [7] identified a single QTL for grain yield that explicated from 8.42% to 8.44% of the phenotypic variance. For grain yield per

plant, 11 genomic regions were detected in eight RIL populations explaining 2.2% to 6.7% of phenotypic variance [8].

So far, breeding efforts have been focused predominantly on improving grain yield, or silage for animal feed, with few of them specializing in exploiting stover for energy production. The production and distribution networks in maize cultivation are well-established and can supply large quantities of lignocellulosic residue with excellent logistics for exploitation [9]. Dependence on fossil biofuels has led to environmental and economic consequences of global concern; thus, lignocellulose derived from agricultural residues can represent a sustainable alternative for the production of biofuels [10].

Mapping QTL associated with lignocellulosic biomass quantity and quality is essential to choose the best breeding programs to produce biofuel [2]. While some studies described three QTL in a RIL progeny derived from the cross between WM13 and Rio for stover yield [11], others have focused on improving the quality of the lignocellulosic residue, based on three major characteristics associated with the cell wall composition: high and low concentration of cellulose and lignin, respectively, and high proportion of glucose released by enzymatic degradation [12]. Truntzler et al. [13], investigated the genetic relationship between cell wall components of the stover and saccharification efficiency (the conversion into fermentable sugars). This QTL meta-analysis included 14 studies of QTL mapping, using 11 independent populations for and cell-wall components and degradability and found 27 QTL related to saccharification. In B73 × Mo17 recombinant inbred maize population, 10 QTL were identified for sugar release after high-temperature dilute acid treatment [12] and four QTL for glucose release after a steam explosion pretreatment, what is a measure of saccharification [14]. More recently, a study performed in a MAGIC population found two significant Single Nucleotide Polymorphisms (SNPs) on chromosome 6 associated with saccharification efficiency and 16 SNPs associated with stover yield [2].

Overall, as previously mentioned, increase in both yields, grain and stover, would be a requirement to meet future food and fuel demands. Genome-wide association studies (GWAS) using panels of inbred lines presenting large diversity are an advantageous tool to identify genomic regions related to both grain and stover yield. These studies also assist the design of breeding programs for double crop exploitation [15]. Moreover, due to their being quantitative characters with a small effect for each gene, metabolic pathway analysis focusing on the action of a certain number of genes that are grouped based on their biological function could be a promising approach to complement GWAS [16]. The combination of GWAS with metabolic pathway analysis considers all genetic sequences positively associated with the trait of interest [17]. In recent studies, this approach has been utilized to investigate the genetic basis of complex traits in maize such as aflatoxin accumulation [18,19], corn earworm resistance [20], or lipid biosynthesis [21].

Aiming to advance in the identification of markers that allow to establish breeding programs for sustainable maize production, the objectives were to (i) understand the correlations between grain, stover yield and saccharification efficiency in a double exploitation approach, and (ii) identify QTL and metabolic pathways associated with these traits.

## 2. Material and Methods

### 2.1. Plant Material, Experimental Design and Yield Calculations

A subset of 836-inbred lines from the Ames association panel (North Central Regional Plant Introduction Station, Ames, Iowa, USA) [15], along with six inbred controls (A619, A632, A662, A665, PH207, EP42) were grown at Misión Biológica de Galicia in Pontevedra (42°24' N, 8°38' W, 20 m above sea level) following an augmented 17-block design in 2018 and 2019 [22]. Three hundred inbred lines well adapted to the environmental conditions in Pontevedra were selected for evaluation, in order to have a quality phenotyping. The inbred lines were divided into five precocity groups based on female flowering (date at which 50% of the plants showed visible silks) and each group was harvested at approximately 70 days post-flowering. Grain and stover yield and saccharification efficiency were recorded on

each experimental plot, each plot consisting of a single row, 2.4 m long, of 13 plants, spaced between consecutive hills in a row by 0.21 m and 0.8 m between rows.

### 2.1.1. Grain Yield

Grain yield was calculated as the weight of grain per plant and adjusted to 14% grain moisture. It was determined by the following Equation (1):

$$\text{Grain yield} \left( \frac{\text{g}}{\text{plant}} \right) = \frac{\text{Ear weight per plant (g)} \times (100 - \text{grain moisture}) \times \text{Grain weight of 5 ears (g)}}{\text{Number of plants per plot} \times 86 \times \text{Total weight of 5 ears (g)}} \quad (1)$$

where grain moisture, expressed as a percentage, was recorded using a Kett moisture meter (model PM-400) in a sample of 240 cm<sup>3</sup>. The value 86 in the equation corresponds to a constant to adjust the yield to 14% humidity.

### 2.1.2. Stover Yield

In each plot, the stover (plants without ears) of plants was harvested. The weight of fresh stover was recorded and collected to estimate the percentage of dry matter in the stover. The fresh stover was weighed, dried at 60 °C in an oven and again weighed after seven days (stover dry weight). Stover yield was calculated by the following Equation (2):

$$\text{Stover yield} \left( \frac{\text{g}}{\text{plant}} \right) = \frac{\text{weight of harvested stover (g)} \times \text{stover dry weight (g)}}{\text{Number of plants} \times \text{stover fresh weight (g)}} \quad (2)$$

### 2.1.3. Saccharification Efficiency

The stover samples, once dried (60 °C, 7 days), were ground in a Wiley mill with a 0.75 mm mesh. Saccharification efficiency determinations were carried out following the method described by Gomez et al. [23].

Ground material was weighed into 96-well plates. Each well contained 4 mg of each sample and processed using a high-throughput automated system. Samples were pretreated with 0.5 M NaOH at 90 °C for 30 min, washed four times with 500 µL sodium acetate buffer and finally subjected to enzymatic digestion (Cellic CTEC2, 7FPU/g) at 50 °C for 8 h. The amount of released sugars was assessed against a glucose standard curve using the 3-methyl-2-benzothiazolinone hydrozone method [24].

## 2.2. Statistical Analysis

### 2.2.1. Analysis of Variance and Correlation Coefficients

A combined analysis of variance was performed for the traits studied using the PROC MIXED procedure of the SAS software [25]. Best Linear Unbiased Estimators (BLUEs) for each line were calculated based on the pooled data for the 2-year analysis. Genotypes were considered as fixed effect while years and blocks were considered as random. Means comparison were carried out using the minimum significant difference (LSD) method. Traits' heritabilities were estimated following Holland et al. [26].

Correlation coefficients between grain and stover yield and saccharification efficiency were calculated using REML estimates according to a published SAS mixed model procedure [27].

### 2.2.2. Association Mapping

Inbred lines were genotyped using a genotyping-by-sequencing method (GBS) at the Institute of Biotechnology of Cornell University using version 4 of the B73 genome [22]. The genotype matrix was filtered, i.e., SNPs with more than 50% missing data and a minor allele frequency of less than 5% were omitted, as well as monomeric and multi-allelic SNPs and insertion/deletion polymorphisms (INDELs). Heterozygous genotypes were considered missing data. After filtering, 147,428 SNPs distributed across the maize genome were retained.

A genome-wide association study (GWAS) of the BLUEs across years was carried out with BLINK based on a mixed linear model using R [28]. Genotypic (G) and phenotypic (Y) data

are included in the model. BLINK eliminates the requirement of even distribution of markers in the genome and the bin method was replaced by linkage disequilibrium information [28].

An association between a trait and a SNP was considered significant when the observed  $-\log_{10}(p)$  values showed a clear derivation from the distribution of the expected  $-\log_{10}(p)$  that holds under the null hypothesis (i.e., no significant association) in quantile–quantile (QQ) plots [22,29].

### 2.2.3. Pathway Association

Significant metabolic pathways associated with grain and stover yield and saccharification efficiency were assigned using PAST tool, implemented through MaizeGDB [16,30,31]. PAST processes data in four main steps. First, GWAS output data are loaded into PAST. These data come in the form of statistics that reflect the effects of specific loci (e.g., SNPs) on a trait(s) of interest and LD data between loci. Second, the SNPs are distributed in linkage groups and each group is associated with a gene based on genomic distance between SNPs and the gene. Once SNPs are assigned to genes, attributes of SNPs, allelic effects and  $p$ -values, are transferred to genes according to the method described in Tang et al. [19]. The genes and their effects are used to find significant pathways and calculate a running enrichment score, which is plotted in a rug plot for each pathway in the fourth step [17]. In the current study, linkage groups were established based on all SNP pairs having LD exceeding an  $R^2$  of 0.8. The search window for the causative gene was set at  $\pm 1$  Kb, which was based on the distances between linked SNPs and only metabolic pathways with five or more genes were considered to reduce the bias of the small sample size.

## 3. Results

### 3.1. Analysis of Variance and Correlation Coefficients

The combined analyses of variance across years showed that differences between inbreds were significant for grain and stover yield ( $F = 2.75$ ,  $F = 2.47$ ,  $p < 0.0001$ , respectively) and for saccharification efficiency ( $F = 1.26$ ,  $p = 0.0342$ ) (Table S1). Significant genotype-by-environment interactions were observed for stover yield ( $Z = 2.93$ ,  $p = 0.0017$ ) and saccharification efficiency ( $Z = 2.07$ ,  $p = 0.0194$ ). The estimated genetic heritability for grain and stover yield was moderately high (Table 1). The genetic heritability for the estimated saccharification efficiency was not significantly different from zero (Table 1). The genotypic and phenotypic correlation coefficient between stover yield and saccharification was not significant ( $-0.599 \pm 0.317$ ,  $-0.096 \pm 0.046$ , respectively). The genotypic and phenotypic correlation coefficients between stover and grain yield were positive, moderate and significant ( $0.614 \pm 0.079$ ,  $0.547 \pm 0.035$ , respectively).

**Table 1.** Means, ranks, least significant difference (LSD) and  $h^2$  for stover yield, grain yield and saccharification efficiency (SaccEff) of the Ames association panel lines evaluated in 2018 and 2019. The  $h^2$  is considered significantly different from zero when it is two times greater than the standard deviation.

	Grain Yield (g/Plant)	Stover Yield (g/Plant)	SaccEff (nmol Glucose/mg/h)
Mean	63.21	68.04	158.43
Rank	8.83–142.98	14.14–142.52	99.05–198.04
LSD	26.13	38.28	19.04
$h^2$	$0.61 \pm 0.05$	$0.59 \pm 0.05$	$0.18 \pm 0.11$

### 3.2. Association Mapping

Following Yi et al. [22], a marker was considered significantly associated ( $\alpha = 0.05$ ) with a trait at  $p$  values less than  $1.00 \times 10^{-4}$ . We considered a region of  $+/-3$  Kb around the SNP as the SNP confidence interval following the distance at which the LD decay ( $R^2 < 0.1$ ). Two SNPs were assigned to the same QTL when their confidence intervals overlapped. We found a total of 22 significant SNPs that corresponded to 17 QTL. We

identified nine independent SNPs associated with grain yield, eight SNPs significantly associated with stover yield that were grouped into five QTL, and five SNPs grouped into three QTL, associated with saccharification efficiency (Table 2, Figure S1).

**Table 2.** Significant QTL associated with stover and grain yield and saccharification efficiency (SaccEff) in a subset of the Ames association panel lines evaluated in two years.

Trait	QTL <sup>a</sup>	Marker	Chr	Pos <sup>b</sup>	<i>p</i> Value	MAF <sup>c</sup>	Effect <sup>d</sup>
Grain yield	QTL_1_1	S1_27627320	1	27818428	$5.064 \times 10^{-5}$	0.352	8.067
	QTL_1_2	S1_29190896	1	29445156	$1.9397 \times 10^{-5}$	0.261	−9.508
	QTL_2_1	S2_4514070	2	4481454	$4.3931 \times 10^{-5}$	0.187	10.264
	QTL_2_2	S2_11756881	2	12049766	$1.4789 \times 10^{-5}$	0.250	−10.191
	QTL_3_1	S3_179251623	3	182111795	$9.3472 \times 10^{-5}$	0.109	12.663
	QTL_3_2	S3_212022847	3	215694276	$9.3124 \times 10^{-5}$	0.476	7.294
	QTL_4_1	S4_196824058	4	201140915	$9.7931 \times 10^{-5}$	0.367	−7.751
	QTL_7_1	S7_5108781	7	5367270	$4.0454 \times 10^{-5}$	0.428	7.755
	QTL_10_1	S10_148116222	10	148928081	$8.8683 \times 10^{-5}$	0.172	−11.301
Stover yield	QTL_2_1	S2_38324920	2	39888908	$4.5751 \times 10^{-5}$	0.336	−8.883
	QTL_2_1	S2_38324925	2	39888913	$4.5751 \times 10^{-5}$	0.336	8.883
	QTL_2_1	S2_38324933	2	39888921	$1.8528 \times 10^{-5}$	0.300	9.500
	QTL_2_1	S2_38324945	2	39888933	$4.657 \times 10^{-5}$	0.330	−8.757
	QTL_4_1	S4_240526011	4	246038631	$6.7154 \times 10^{-5}$	0.269	−9.633
	QTL_7_1	S7_40413622	7	41898418	$8.6845 \times 10^{-5}$	0.090	−14.817
	QTL_8_1	S8_28160359	8	29092941	$8.0953 \times 10^{-5}$	0.292	8.624
SaccEff	QTL_10_1	S10_148116222	10	148928081	$1.552 \times 10^{-5}$	0.174	−12.996
	QTL_3_1	S3_201309933	3	204497949	$3.7353 \times 10^{-5}$	0.146	5.019
	QTL_5_1	S5_178352276	5	182432287	$8.4243 \times 10^{-5}$	0.249	−3.903
	QTL_5_1	S5_178352277	5	182432288	$8.5921 \times 10^{-5}$	0.249	−3.898
	QTL_6_1	S6_150466053	6	154564447	$6.4079 \times 10^{-5}$	0.177	−4.102
	QTL_6_1	S6_150466085	6	154564479	$5.8653 \times 10^{-5}$	0.181	4.114

<sup>a</sup> The number before the underscores indicates the chromosome and the number after the underscores indicates the QTL within the chromosome. <sup>b</sup> Physical position of marker within the chromosome. <sup>c</sup> MAF: Minority allele frequency. <sup>d</sup> Additive effect calculated as half the difference between the mean of homozygotes of the second allele in alphabetical order and the mean of homozygotes of the first allele in alphabetical order.

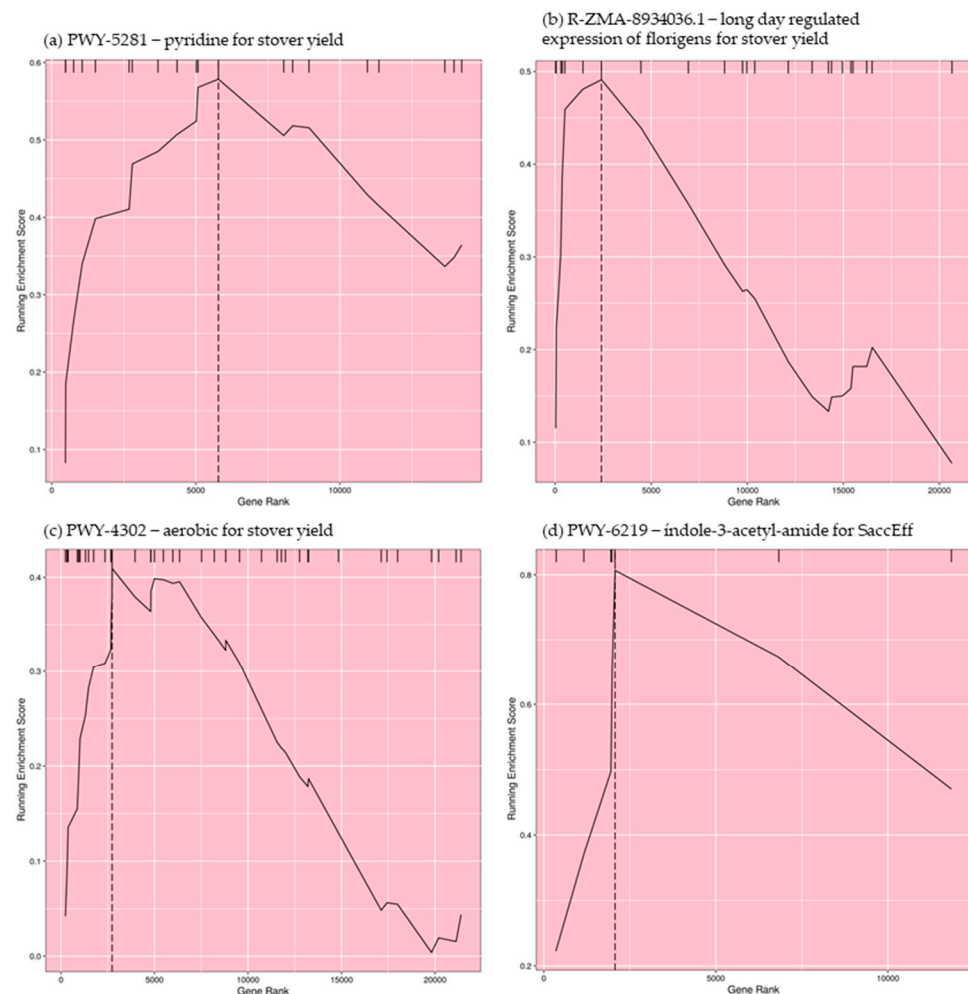
### 3.3. Pathway Association

None of the 363 pathways found with at least five genes was associated with grain yield, while aerobic, pyridine and long-day-regulated expression of florigens pathways were significantly (FDR < 0.15) enriched among genes with an effect on stover yield. On the other hand, genes of the indole-3-acetyl-amide were overrepresented (FDR < 0.15) among genes with an effect on saccharification efficiency (Table 3).

**Table 3.** Pathways affecting stover yield and saccharification efficiency (SaccEff) with enrichment scores better than  $q < 0.15$ .

Trait	Pathway ID	Pathway Name	No. of Genes	<i>p</i> Value	<i>q</i> Value
Stover yield	PWY-4302	aerobic	41	$9.16 \times 10^{-4}$	0.133
	PWY-5381	pyridine	20	$2.33 \times 10^{-4}$	0.084
	R-ZMA-8934036.1	Long-day-regulated expression of florigens	23	$1.10 \times 10^{-3}$	0.133
SaccEff	PWY-6219	indole-3-acetyl-amide	7	$3.80 \times 10^{-4}$	0.138

The plots of the pathways for stover yield and saccharification efficiency illustrate how the running enrichment score values change with genetic rank (Figure 1). The pyridine pathway (PWY-5381, Figure 1a) for stover yield had a high enrichment score (0.58) due to genes in the pathway having the highest and lowest effects on the trait and thus, increased the enrichment score value. The long-day-regulated expression of florigens pathway (R-ZMA-8934036.1, Figure 1b) for stover yield had moderate enrichment scores (0.49). This was in contrast to the aerobic pathway (PWY-4302, Figure 1c), which had a lower enrichment score (0.41) as few genes in this pathway had high effects (positive or negative) on the trait but were outnumbered by genes having low effects. For saccharification efficiency, the indole-3-acetyl-amide pathway (PWY-6219, Figure 1d) had a very high enrichment score (0.81), partly because, with the exception of one gene, the few genes assigned to this pathway were among those with the highest or lowest ranks.



**Figure 1.** Graphs of the running enrichment score calculation for (a) pyridine pathway (PWY-5381), (b) long-day-regulated expression of florigens pathway (R-ZMA-8934036.1), (c) aerobic pathway (PWY-4302) for stover yield and (d) indole-3-acetyl-amide pathway (PWY-6219) for saccharification efficiency. The X-axis shows the rank of each gene effect value; the Y-axis shows the value of the enrichment score (ES) running sum statistic as each consecutive gene effect value is processed. The x-intercept line indicates the highest point of the ES. Small hatch marks at the top of the image indicate the rank position of the effect of all genes in the pathway.

## 4. Discussion

### 4.1. Combined Analysis of Variance, Heritabilities and Correlation Coefficients

In the current study, the high heritability observed for grain and stover yield indicates that additive variance was more important than variances due to G × E interaction and

experimental error for these traits. These results agree with previous studies, where the broad-sense heritability for grain yield was very high, with values ranging from 0.77 to 0.91 [3,4,32]. However, there are no studies to contrast the heritability found here for stover yield, although there are studies on the quality and yield of corn silage around the world [33]. Unlike other crops, stover yield and grain yield were positively correlated in maize [34,35]. Based on the observed positive high genotypic and phenotypic correlation between grain yield and stover yield, these traits could be managed together. This is supported by SNP S10\_14811622, which is associated to both traits with the same sign (Table 2). This put forward that increasing stover biomass does not negatively compromise grain yield. In addition, the moderately high heritability suggests that both traits can be successfully improved using phenotypic selection, although genomic selection could also be an alternative.

Increases in biomass or stover yield have been a target for biofuel production and forage digestibility [2,12,36]. The genotypic correlation found between saccharification and stover yield was high and negative ( $r_g = -0.60$ ), although it was not significantly different from zero, probably due to the low heritability for saccharification efficiency. In this context, optimization of maize stover as a biofuel feedstock can be achieved by plant breeding to increase yield but the quality of the stover should also be taken into account. Considering the genotypic variability for saccharification efficiency and that the heritability for that trait did not differ from zero, there is hardly any variability for improving residue quality. Herein, our study represents a novel contribution for dual exploitation of the crop, since ethanolic production from lignocellulosic material does not compete with grain production.

#### 4.2. Association Mapping

For grain yield, many QTL have been previously described in the last decades. Our results show co-localization with previously detected QTL. For example, marker S1\_29190896 co-localized in bin 1.03 with previously described QTL in a RIL population [4] and in an F<sub>2:3</sub> population [6]. The QTL significantly associated with grain yield located in chromosomes 2 and 3 in this study co-localized with those found by Lima et al. [5] using a set of 256 F<sub>2:3</sub> families derived from tropical maize germplasm. The S3\_212022847 marker also co-localized at bin 3.08 with QTL identified by Wang et al. [37]. However, few consistent results have been obtained, probably due to the use of different parental materials, segregating population sizes, marker densities or environments, which could influence QTL detection.

In the current study, QTL for stover yield co-localize with QTL found in a multiparent advanced generation intercross (MAGIC) population by López-Malvar et al. [2]. As the decay linkage disequilibrium occurred at a distance greater than 100,000 bp in that MAGIC population [38,39], SNPs located far apart less than 100 kpb from those found by López-Malvar et al. [2] could be associated to the same QTL. They previously found 13 QTL significantly associated with stover yield, three of them less than 100 kpb apart from markers S2\_38324933, S7\_40413622 and S10\_148116222 associated to stover yield in the present study.

None of the markers significantly associated with saccharification efficiency in this study coincide in the same bin with those previously described for glucose yield or saccharification [12,14]. In the multiparent advanced generation intercross (MAGIC) population, López-Malvar et al. [2] identified two QTL for saccharification efficiency less than 100 kpb apart from markers associated to saccharification efficiency in the current GWAS, S6\_150466085 and S6\_150466053.

Jointly, mapping QTL associated with stover yield is an important step in optimizing selection programs to improve biofuel production. However, the correlation between stover yield and saccharification efficiency was not significant enough to support indirect selection programs to improve saccharification based on stover biomass. These results support a direct breeding strategy for saccharification, preferentially based on genomic selection. Variation in saccharification efficiency was not well explained by the significant SNPs found to recommend marker-assisted selection and the heritability is too low to recommend phenotypic selection. Genomic selection is based on trait values predicted as



the sum of the individual's genetic value at all markers distributed across the genome, and this selection strategy could lead to high correlations between the actual and predicted genetic values [40].

#### 4.3. Pathway Association

The pyridine nucleotides  $\text{NAD}^+$  and  $\text{NADP}^+$  are ubiquitous coenzymes that can have an impact on virtually all metabolic pathways in the cell [41]. Mutant studies provide evidence that changes in NAD status can alter photosynthesis and stress responses in plants, suggesting that NAD content may be a potent modulator of metabolic integration [42,43]. In this sense, the pyridine nucleotide cycle was significantly associated with maize stover yield in the current study. The regulation of dehydrogenases in mitochondria by the level of reduced and oxidized pyridine nucleotides could be a critical point of fine regulation. The plant metabolic system has features associated with remarkable metabolic plasticity of mitochondria that allow the use of energy accumulated during photosynthesis so that all anabolic and catabolic pathways are optimized and coordinated impacting yield [44].

The regulation of respiration in light also represents a phenomenon that coordinates all major pathways of metabolism during photosynthesis, including reactions of secondary metabolism. Oxidative phosphorylation produces ATP, but it is also a central sink for high-capacity electrons required by many metabolic pathways that must be coordinated and integrated in a flexible way [45]. In this context, the aerobic respiration III pathway is enriched among genes with high effect on stover yield. This alternative oxidase (AOX) pathway branches from the cytochrome pathway (aerobic respiration I) at the internal mitochondrial membrane in the ubiquinone pool and passes electrons to a single terminal oxidase. The production of reactive oxygen species (ROS) is an inevitable consequence of the mitochondrial electron transport chain (ETC), and excessive ROS production is critical for plant development. In this respect, ROS would act as a signaling compound that initiates feedback mechanisms to keep photosynthetic cell function under control [46].

Finally, the third pathway significantly impacting stover yield, long-day-regulated expression of florigens, denotes the importance of flowering time in the final biomass production. Flowering time is a very complex trait that controls the adaptation of plants to their local environment, but differences in flowering time in maize are not caused by a few large effect genes, but by the cumulative effects of numerous quantitative trait loci, each of which has only a small impact on the trait [47]. The maize genome encodes several florigen genes, i.e., floral genes expressed in the leaf vasculature that promote flowering at the shoot apex. Because flowering time is highly dependent on temperature, it is shorter for a given non-photoperiodic genotype at lower latitudes than at higher latitudes in the temperate zone. Florigens play an essential role in the genetic variability for flowering time in those lines where breeding suppressed photoperiodism [48–50].

Lastly, the only pathway significantly associated with saccharification efficiency was indole-3-acetyl-amide. Indole-3-acetic acid (IAA), a predominant form of auxin, is an important phytohormone that affects many aspects of plant development throughout the plant life cycle [51]. Stamatiou et al. [52] have observed a link between auxin transport and increased sugar release in maize. Application of a specific auxin transport inhibitor, N-1-naphthylphthalamic acid (NPA), to two different maize cultivars resulted in a significant increase in saccharification. Lines showing enhanced sugar release displayed an incompletely penetrant bishop-shaped inflorescence phenotype, which may mean that altered cell wall integrity contributes to aberrant inflorescence development. We can highlight that auxin transport may influence cytosolic sugar or starch accumulation in vegetative tissues and lead to enhanced saccharification.

## 5. Conclusions

In brief, a phenotypic selection approach using appropriate inbreds from the Ames panel will be a good starting point concerning the improvement of both yields, whereas forthcoming genomic selection models are advisable for saccharification enhancement. Overall, breeding

strategies managing dual purpose uses of maize are feasible, and will contribute in the near future to a more sustainable maize cultivation. Regulation of energy and development pathways have pointed out as key routes related to stover quantity and exploitation, whereas no specific pathways could be identified in relation to grain production.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy13051352/s1>, Table S1: BLUEs for saccharification efficiency, stover yield and grain yield; Figure S1: Manhattan and Q-Q plot for grain and stover yield and saccharification efficiency.

**Author Contributions:** Conceptualization, R.A.M., A.B. and P.R.; Methodology, N.G., L.D.G., R.S., A.L.-M. and L.Á.-I.; Software, R.A.M. and N.G.; Validation, N.G., A.B., R.S. and R.A.M.; Formal Analysis, R.A.M. and N.G.; Investigation, R.A.M., N.G., R.S. and A.B.; Resources, P.R.; Data Curation, N.G., L.Á.-I. and A.L.-M.; Writing—Original Draft Preparation, N.G.; Writing—Review and Editing, A.B., R.S., L.D.G., A.L.-M., P.R. and R.A.M.; Visualization, N.G., L.D.G., R.S., A.L.-M., L.Á.-I., R.A.M., A.B. and P.R.; Supervision, R.A.M.; Project Administration, R.A.M., A.B. and R.S.; Funding Acquisition, R.A.M., A.B., R.S. and P.R. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by subsequent coordinated projects financed by MCIU/AEI/FEDER, UE (RTI2018-096776-B-C21, RTI2018-096776-B-C22, PID2021-122196OB-C21 and PID2021-122196OB-C22) and the project funded by MCIU/AEI/FEDER, UE (AGL2016-77628-R).

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Acknowledgments:** N.G. acknowledges her FPI contract (“Contrato predoctoral para la formación de doctores”) funded by project RTI2018-096776-B-C21 (financed by MCIU/AEI/FEDER, UE). A.L.-M. acknowledges her postdoctoral scholarship to the Xunta de Galicia.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Zhou, Z.; Li, G.; Tan, S.; Li, D.; Weiß, T.M.; Wang, X.; Chen, S.; Würschum, T.; Liu, W. A QTL atlas for grain yield and its component traits in maize (*Zea mays*). *Plant Breed.* **2020**, *139*, 562–574. [[CrossRef](#)]
- López-Malvar, A.; Butron, A.; Malvar, R.A.; McQueen-Mason, S.J.; Faas, L.; Gómez, L.D.; Revilla, P.; Figueroa-Garrido, D.J.; Santiago, R. Association mapping for maize stover yield and saccharification efficiency using a multiparent advanced generation intercross (MAGIC) population. *Sci. Rep.* **2021**, *11*, 3425. [[CrossRef](#)] [[PubMed](#)]
- Flint-Garcia, S.A.; Thuillet, A.-C.; Yu, J.; Pressoir, G.; Romero, S.M.; Mitchell, S.E.; Doebley, J.; Kresovich, S.; Goodman, M.M.; Buckler, E.S. Maize association population: A high-resolution platform for quantitative trait locus dissection. *Plant J.* **2005**, *44*, 1054–1064. [[CrossRef](#)] [[PubMed](#)]
- Ma, X.Q.; Tang, J.H.; Teng, W.T.; Yan, J.B.; Meng, Y.J.; Li, J.S. Epistatic interaction is an important genetic basis of grain yield and its components in maize. *Mol. Breed.* **2007**, *20*, 41–51. [[CrossRef](#)]
- Lima, M.D.L.A.; De Souza, C.L.; Bento, D.A.V.; De Souza, A.P.; Carlini-Garcia, L.A. Mapping QTL for Grain Yield and Plant Traits in a Tropical Maize Population. *Mol. Breed.* **2006**, *17*, 227–239. [[CrossRef](#)]
- Yan, J.-B.; Tang, H.; Huang, Y.-Q.; Zheng, Y.-L.; Li, J.-S. Quantitative trait loci mapping and epistatic analysis for grain yield and yield components using molecular markers with an elite maize hybrid. *Euphytica* **2006**, *149*, 121–131. [[CrossRef](#)]
- Yang, C.; Zhang, L.; Jia, A.; Rong, T. Identification of QTL for maize grain yield and kernel-related traits. *J. Genet.* **2016**, *95*, 239–247. [[CrossRef](#)]
- Li, C.; Li, Y.; Sun, B.; Peng, B.; Liu, C.; Liu, Z.; Yang, Z.; Li, Q.; Tan, W.; Zhang, Y.; et al. Quantitative trait loci mapping for yield components and kernel-related traits in multiple connected RIL populations in maize. *Euphytica* **2013**, *193*, 303–316. [[CrossRef](#)]
- Vermerris, W.; Saballos, A.; Ejeta, G.; Mosier, N.S.; Ladisch, M.R.; Carpita, N.C. Molecular Breeding to Enhance Ethanol Production from Corn and Sorghum Stover. *Crop. Sci.* **2007**, *47*, S142. [[CrossRef](#)]
- Yuan, J.S.; Tiller, K.H.; Al-Ahmad, H.; Stewart, N.R.; Stewart, C.N. Plants to power: Bioenergy to fuel the future. *Trends Plant Sci.* **2008**, *13*, 421–429. [[CrossRef](#)]
- Barrière, Y.; Méchin, V.; Lefevre, B.; Maltese, S. QTLs for agronomic and cell wall traits in a maize RIL progeny derived from a cross between an old Minnesota13 line and a modern Iodent line. *Theor. Appl. Genet.* **2012**, *125*, 531–549. [[CrossRef](#)] [[PubMed](#)]
- Lorenzana, R.E.; Lewis, M.F.; Jung, H.-J.G.; Bernardo, R. Quantitative Trait Loci and Trait Correlations for Maize Stover Cell Wall Composition and Glucose Release for Cellulosic Ethanol. *Crop. Sci.* **2010**, *50*, 541–555. [[CrossRef](#)]
- Truntzler, M.; Barrière, Y.; Sawkins, M.C.; Lespinasse, D.; Betran, J.; Charcosset, A.; Moreau, L. Meta-analysis of QTL involved in silage quality of maize and comparison with the position of candidate genes. *Theor. Appl. Genet.* **2010**, *121*, 1465–1482. [[CrossRef](#)] [[PubMed](#)]

14. Penning, B.; Sykes, R.W.; Babcock, N.C.; Dugard, C.K.; Held, M.; Klimek, J.F.; Shreve, J.T.; Fowler, M.; Ziebell, A.; Davis, M.; et al. Genetic Determinants for Enzymatic Digestion of Lignocellulosic Biomass Are Independent of Those for Lignin Abundance in a Maize Recombinant Inbred Population. *Plant Physiol.* **2014**, *165*, 1475–1487. [CrossRef] [PubMed]
15. Romay, M.C.; Millard, M.J.; Glaubitz, J.C.; Peiffer, J.A.; Swarts, K.L.; Casstevens, T.M.; Elshire, R.J.; Acharya, C.B.; Mitchell, S.E.; Flint-Garcia, S.A.; et al. Comprehensive genotyping of the USA national maize inbred seed bank. *Genome Biol.* **2013**, *14*, R55. [CrossRef]
16. Wang, K.; Li, M.; Bucan, M. Pathway-Based Approaches for Analysis of Genomewide Association Studies. *Am. J. Hum. Genet.* **2007**, *81*, 1278–1283. [CrossRef]
17. Thrash, A.; Tang, J.D.; DeOrnellis, M.; Peterson, D.G.; Warburton, M.L. PAST: The Pathway Association Studies Tool to Infer Biological Meaning from GWAS Datasets. *Plants* **2020**, *9*, 58. [CrossRef]
18. Warburton, M.L.; Tang, J.D.; Windham, G.L.; Hawkins, L.K.; Murray, S.C.; Xu, W.; Boykin, D.; Perkins, A.; Williams, W.P. Genome-Wide Association Mapping of *Aspergillus flavus* and Aflatoxin Accumulation Resistance in Maize. *Crop. Sci.* **2015**, *55*, 1857–1867. [CrossRef]
19. Tang, J.D.; Perkins, A.; Williams, W.P.; Warburton, M.L. Using genome-wide associations to identify metabolic pathways involved in maize aflatoxin accumulation resistance. *BMC Genom.* **2015**, *16*, 673. [CrossRef]
20. Warburton, M.L.; Womack, E.D.; Tang, J.D.; Thrash, A.; Smith, J.S.; Xu, W.; Murray, S.C.; Williams, W.P. Genome-Wide Association and Metabolic Pathway Analysis of Corn Earworm Resistance in Maize. *Plant Genome* **2018**, *11*, 170069. [CrossRef]
21. Li, H.; Thrash, A.; Tang, J.D.; He, L.; Yan, J.; Warburton, M.L. Leveraging GWAS data to identify metabolic pathways and networks involved in maize lipid biosynthesis. *Plant J.* **2019**, *98*, 853–863. [CrossRef] [PubMed]
22. Yi, Q.; López-Malvar, A.; Álvarez-Iglesias, L.; Romay, M.C.; Revilla, P. Genome-Wide Association Analysis Identified Newly Natural Variation for Photosynthesis-Related Traits in a Large Maize Panel. *Agronomy* **2023**, *13*, 801. [CrossRef]
23. Gomez, L.D.; Whitehead, C.; Barakate, A.; Halpin, C.; McQueen-Mason, S.J. Automated saccharification assay for determination of digestibility in plant materials. *Biotechnol. Biofuels* **2010**, *3*, 23. [CrossRef] [PubMed]
24. Anthon, G.E.; Barrett, D.M. Determination of Reducing Sugars with 3-Methyl-2-benzothiazolinonehydrazone. *Anal. Biochem.* **2002**, *305*, 287–289. [CrossRef]
25. SAS Institute. *Base SAS 9.4 Procedures Guide*; SAS Institute: Cary, NC, USA, 2015.
26. Holland, J.B.; Nyquist, W.E.; Cervantes-Martínez, C.T. Estimating and Interpreting Heritability for Plant Breeding: An Update. *Plant Breed. Rev.* **2003**, *22*, 9–112. [CrossRef]
27. Holland, J.B. Estimating Genotypic Correlations and Their Standard Errors Using Multivariate Restricted Maximum Likelihood Estimation with SAS Proc MIXED. *Crop. Sci.* **2006**, *46*, 642–654. [CrossRef]
28. Huang, M.; Liu, X.; Zhou, Y.; Summers, R.M.; Zhang, Z. BLINK: A package for the next level of genome-wide association studies with both individuals and markers in the millions. *Gigascience* **2019**, *8*, gyy154. [CrossRef]
29. Gao, X.; Becker, L.C.; Becker, D.M.; Starmer, J.D.; Province, M.A. Avoiding the high Bonferroni penalty in genome-wide association studies. *Genet. Epidemiol.* **2010**, *34*, 100–105. [CrossRef]
30. Portwood, J.L., II; Woodhouse, M.R.; Cannon, E.K.; Gardiner, J.M.; Harper, L.C.; Schaeffer, M.L.; Walsh, J.R.; Sen, T.Z.; Cho, K.T.; Schott, D.A.; et al. MaizeGDB 2018: The maize multi-genome genetics and genomics database. *Nucleic Acids Res.* **2019**, *47*, D1146–D1154. [CrossRef]
31. Weng, L.; Macciardi, F.; Subramanian, A.; Guffanti, G.; Potkin, S.G.; Yu, Z.; Xie, X. SNP-based pathway enrichment analysis for genome-wide association studies. *BMC Bioinform.* **2011**, *12*, 99. [CrossRef]
32. Rafiq, C.M.; Rafique, M.; Hussain, A.; Altaf, M. Correlation and path analysis in maize. *J. Agric. Res.* **2010**, *48*, 35–38. Available online: [http://apply.jar.punjab.gov.pk/upload/1374660174\\_81\\_34\\_39Paper-No.4.pdf](http://apply.jar.punjab.gov.pk/upload/1374660174_81_34_39Paper-No.4.pdf) (accessed on 3 March 2023).
33. García-Chávez, I.; Meraz-Romero, E.; Castelán-Ortega, O.; Zaragoza-Esparza, J.; Osorio-Avalos, J.; Robles-Jiménez, L.E.; González-Ronquillo, M. Corn silage, a systematic review of the quality and yield in different regions around the world. *Cienc. Y Tecnol. Agropecu.* **2022**, *23*, 2547. [CrossRef]
34. Lorenz, A.J.; Gustafson, T.J.; Coors, J.G.; De Leon, N. Breeding Maize for a Bioeconomy: A Literature Survey Examining Harvest Index and Stover Yield and Their Relationship to Grain Yield. *Crop. Sci.* **2010**, *50*, 1–12. [CrossRef]
35. Mazaheri, M.; Heckwolf, M.; Vaillancourt, B.; Gage, J.L.; Burdo, B.; Heckwolf, S.; Barry, K.; Lipzen, A.; Ribeiro, C.B.; Kono, T.J.Y.; et al. Genome-wide association analysis of stalk biomass and anatomical traits in maize. *BMC Plant Biol.* **2019**, *19*, 45. [CrossRef] [PubMed]
36. Lopez-Malvar, A.; Malvar, R.A.; Butron, A.; Revilla, P.; Pereira-Crespo, S.; Santiago, R. Genetic Dissection for Maize Forage Digestibility Traits in a Multi-Parent Advanced Generation Intercross (MAGIC) Population. *Agronomy* **2021**, *11*, 104. [CrossRef]
37. Wang, H.; Zhang, X.; Yang, H.; Liu, X.; Li, H.; Yuan, L.; Li, W.; Fu, Z.; Tang, J.; Kang, D. Identification of heterotic loci associated with grain yield and its components using two CSSL test populations in maize. *Sci. Rep.* **2016**, *6*, 38205. [CrossRef]
38. Jiménez-Galindo, J.C.; Malvar, R.A.; Butrón, A.; Santiago, R.; Samayoa, L.F.; Caicedo, M.; Ordás, B. Mapping of resistance to corn borers in a MAGIC population of maize. *BMC Plant Biol.* **2019**, *19*, 431. [CrossRef]
39. Butrón, A.; Santiago, R.; Cao, A.; Samayoa, L.; Malvar, R. QTLs for Resistance to Fusarium Ear Rot in a Multiparent Advanced Generation Intercross (MAGIC) Maize Population. *Plant Dis.* **2019**, *103*, 897–904. [CrossRef]
40. Meuwissen, T.H.E.; Hayes, B.J.; Goddard, M.E. Prediction of Total Genetic Value Using Genome-Wide Dense Marker Maps. *Genetics* **2001**, *157*, 1819–1829. [CrossRef]

41. Noctor, G.; Queval, G.; Gakière, B. NAD(P) synthesis and pyridine nucleotide cycling in plants and their potential importance in stress conditions. *J. Exp. Bot.* **2006**, *57*, 1603–1620. [[CrossRef](#)]
42. Hayashi, M.; Takahashi, H.; Tamura, K.; Huang, J.; Yu, L.-H.; Kawai-Yamada, M.; Tezuka, T.; Uchimiya, H. Enhanced dihydroflavonol-4-reductase activity and NAD homeostasis leading to cell death tolerance in transgenic rice. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7020–7025. [[CrossRef](#)] [[PubMed](#)]
43. Dutilleul, C.; Driscoll, S.; Cornic, G.; De Paepe, R.; Foyer, C.; Noctor, G. Functional Mitochondrial Complex I Is Required by Tobacco Leaves for Optimal Photosynthetic Performance in Photorespiratory Conditions and during Transients. *Plant Physiol.* **2003**, *131*, 264–275. [[CrossRef](#)] [[PubMed](#)]
44. Igamberdiev, A.U.; Bykova, N.V. Mitochondria in photosynthetic cells: Coordinating redox control and energy balance. *Plant Physiol.* **2022**, *191*, 2104–2119. [[CrossRef](#)] [[PubMed](#)]
45. Møller, I.M.; Igamberdiev, A.U.; Bykova, N.V.; Finkemeier, I.; Rasmusson, A.G.; Schwarzländer, M. Matrix Redox Physiology Governs the Regulation of Plant Mitochondrial Metabolism through Posttranslational Protein Modifications. *Plant Cell* **2020**, *32*, 573–594. [[CrossRef](#)]
46. Scafaro, A.P.; Fan, Y.; Posch, B.C.; Garcia, A.; Coast, O.; Atkin, O.K. Responses of leaf respiration to heatwaves. *Plant Cell Environ.* **2021**, *44*, 2090–2101. [[CrossRef](#)]
47. Buckler, E.S.; Holland, J.B.; Bradbury, P.J.; Acharya, C.B.; Brown, P.J.; Browne, C.; Ersoz, E.; Flint-Garcia, S.; Garcia, A.; Glaubitz, J.C.; et al. The Genetic Architecture of Maize Flowering Time. *Science* **2009**, *325*, 714–718. [[CrossRef](#)]
48. Castelletti, S.; Coupel-Ledru, A.; Granato, I.; Palaffre, C.; Cabrera-Bosquet, L.; Tonelli, C.; Nicolas, S.D.; Tardieu, F.; Welcker, C.; Conti, L. Maize adaptation across temperate climates was obtained via expression of two florigen genes. *PLoS Genet.* **2020**, *16*, e1008882. [[CrossRef](#)]
49. Guo, L.; Wang, X.; Zhao, M.; Huang, C.; Li, C.; Li, D.; Yang, C.J.; York, A.M.; Xue, W.; Xu, G.; et al. Stepwise cis-Regulatory Changes in ZCN8 Contribute to Maize Flowering-Time Adaptation. *Curr. Biol.* **2018**, *28*, 3005–3015.e4. [[CrossRef](#)]
50. Stephenson, E.; Estrada, S.; Meng, X.; Ourada, J.; Muszynski, M.G.; Habben, J.E.; Danilevskaya, O.N. Over-expression of the photoperiod response regulator ZmCCT10 modifies plant architecture, flowering time and inflorescence morphology in maize. *PLoS ONE* **2019**, *14*, e0203728. [[CrossRef](#)]
51. Woodward, A. Auxin: Regulation, Action, and Interaction. *Ann. Bot.* **2005**, *95*, 707–735. [[CrossRef](#)]
52. Stamatiou, G.; Vidaurre, D.P.; Shim, I.; Tang, X.; Moeder, W.; Bonetta, D.; McCourt, P. Forward Genetic Screening for the Improved Production of Fermentable Sugars from Plant Biomass. *PLoS ONE* **2013**, *8*, e55616. [[CrossRef](#)] [[PubMed](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.