10-2017

Brassinosteroid and gibberellin control of seedling traits in maize (Zea mays L.)

Songlin Hu
Iowa State University

Darlene l. Sanchez
Iowa State University

Cuiling Wang
Henan University of Science and Technology

Alexander E. Lipka
University of Illinois at Urbana-Champaign

Yanhai Yin
Iowa State University

See next page for additional authors

Brassinosteroid and gibberellin control of seedling traits in maize (Zea mays L.)

Abstract
In this study, we established two doubled haploid (DH) libraries with a total of 207 DH lines. We applied BR and GA inhibitors to all DH lines at seedling stage and measured seedling BR and GA inhibitor responses. Moreover, we evaluated field traits for each DH line (untreated). We conducted genome-wide association studies (GWAS) with 62,049 genome wide SNPs to explore the genetic control of seedling traits by BR and GA. In addition, we correlate seedling stage hormone inhibitor response with field traits. Large variation for BR and GA inhibitor response and field traits was observed across these DH lines. Seedling stage BR and GA inhibitor response was significantly correlate with yield and flowering time. Using three different GWAS approaches to balance false positive/negatives, multiple SNPs were discovered to be significantly associated with BR/GA inhibitor responses with some localized within gene models. SNPs from gene model *GRMZM2G013391* were associated with GA inhibitor response across all three GWAS models. This gene is expressed in roots and shoots and was shown to regulate GA signaling. These results show that BRs and GAs have a great impact for controlling seedling growth. Gene models from GWAS results could be targets for seeding traits improvement.

Keywords
Zea amys, Brassinosteroid, Gibberellins, Seedling traits, Field traits

Disciplines
Agronomy and Crop Sciences

Comments
This article is published as Hu, Songlin, Darlene L. Sanchez, Cuiling Wang, Alexander E. Lipka, Yanhai Yin, Candice AC Gardner, and Thomas Lübberstedt. "Brassinosteroid and gibberellin control of seedling traits in maize (Zea mays L.)." Plant Science 263 (2017): 132-141. 10.1016/j.plantsci.2017.07.011. Posted with permission.

Authors
Songlin Hu, Darlene l. Sanchez, Cuiling Wang, Alexander E. Lipka, Yanhai Yin, Candice A.C. Gardner, and Thomas Lubberstedt

Plant Science 263 (2017) 132–141

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Brassinosteroid and gibberellin control of seedling traits in maize (*Zea mays* L.)

Songlin Hu[a,*], Darlene L. Sanchez[a], Cuiling Wang[b], Alexander E. Lipka[c], Yanhai Yin[d], Candice A.C. Gardner[a,e], Thomas Lübberstedt[a]

[a] Department of Agronomy, Iowa State University, 100 Osborn Drive, Ames, IA 50011, USA
[b] Department of Agronomy, Henan University of Science and Technology, 263 Kaiyuan Avenue, Luoyang, Henan 471023, China
[c] Department of Crop Sciences, University of Illinois, Champaign, IL 61801, USA
[d] Department of Genetics, Development and Cell Biology, Iowa State University, 100 Osborn Drive, Ames, IA 50011, USA
[e] U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS), 100 Osborn Drive, Ames, IA 50011, USA

ARTICLE INFO

Keywords:
Zea mays
Brassinosteroid
Gibberellins
Seedling traits
Field traits

ABSTRACT

In this study, we established two doubled haploid (DH) libraries with a total of 207 DH lines. We applied BR and GA inhibitors to all DH lines at seedling stage and measured seedling BR and GA inhibitor responses. Moreover, we evaluated field traits for each DH line (untreated). We conducted genome-wide association studies (GWAS) with 62,049 genome wide SNPs to explore the genetic control of seedling traits by BR and GA. In addition, we correlate seedling stage hormone inhibitor response with field traits. Large variation for BR and GA inhibitor response and field traits was observed across these DH lines. Seedling stage BR and GA inhibitor response was significantly correlate with yield and flowering time. Using three different GWAS approaches to balance false positive/negatives, multiple SNPs were discovered to be significantly associated with BR/GA inhibitor responses with some localized within gene models. SNPs from gene model *GRMZM2G013391* were associated with GA inhibitor response across all three GWAS models. This gene is expressed in roots and shoots and was shown to regulate GA signaling. These results show that BRs and GAs have a great impact for controlling seedling growth. Gene models from GWAS results could be targets for seeding traits improvement.

1. Introduction

Brassinosteroids (BRs) and gibberellins (GAs) are two groups of plant hormones that control various plant developmental processes. Aberrations occurring in BR/GA biosynthesis or signaling can greatly alter plant stature and change plant responses to environmental and developmental cues. BRs are steroid hormones similar to those found in animals [1]. They regulate important traits such as germination, cell elongation, root development, fertility, flowering time, and plant architecture traits such as leaf angle and plant height [2–4]. In addition, BRs play positive roles in resistance to both biotic and abiotic stresses such as drought, salt, heat, cold, oxidative stress and pathogen attacks [5]. BR biosynthesis and signaling pathways have been well established in the model species *Arabidopsis thaliana* [6–10], and in rice (*Oryza sativa*) [11,12]. BR pathway components have shown a great potential to serve as targets for genetic engineering for crop improvement. For example, seed-specific over-expression of *AtDWF4* in Arabidopsis can enhance cold tolerance, which was attributed to up-regulation of cold-response gene *COR15A* [13]. Loss-of-function of *OsGSK* improved rice tolerance to cold, heat, salt, and drought stress [14]. In contrast, role and pathway information of BRs in maize is still limited [15,16].

GAs are a large group of cyclic diterpene compounds [17]. They are involved in seed germination and vegetative growth including elongation of stems, roots, and the expansion of leaves, development of flowers, fruit set, and the control of fertilization [18–23]. GA biosynthesis and signaling pathways have been extensively studied in Arabidopsis, rice, maize and other crops [11,23–26]. Mutations in GA pathway genes played a vital role in the green revolution such as the *semidwarf1* (*sd1*) gene (GA biosynthesis pathway), which encodes a GA20oxidase (*OsGA20ox2*) (Sasaki et al. [21]) and reduced height-1 (*RHT-1*) (GA signaling pathway) of wheat (*Triticum aestivum* L.) [27]. With the knowledge of BR and GA control of traits and stress tolerance, crop improvement can greatly benefit in three ways 1) genetic engineering of BR and GA biosynthesis and/or signaling pathways can be employed to improve stress tolerance, and perhaps biomass and yield of agricultural crops [28]; 2) molecular strengthening [29] treatments can be developed to optimize trait expression temporally; 3) functional markers [30] can be developed for BR/GA pathway genes for marker-

* Corresponding author.
E-mail address: hsonglin1@gmail.com (S. Hu).

http://dx.doi.org/10.1016/j.plantsci.2017.07.011
Received 8 May 2017; Received in revised form 9 July 2017; Accepted 11 July 2017
Available online 19 July 2017

aided selection.

Establishment of seedlings is a key factor for uniform, high-yielding field stands and, consequently, stable yields [31]. Several traits were used to measure seedling vigor in maize, including seed germination rate, seedling shoot length, root length, seedling dry weight, root dry weight, and these traits have been extensively used for correlation studies with adult plant traits in different maize genotypes [32–35]. Early maize seedling vigor greatly enhances crop establishment, especially in stressful environments such as under low temperatures in central Europe and northern Mediterranean areas [36]. In low precipitation areas, where the soil moisture in the upper layer is quite limited and seeds are exposed to frequent dehydration events, deep sowing ensures adequate seed-zone moisture before germination and thereby enhances seedling establishment [37,38]. However, when sown deep, an elongated and vigorous mesocotyl is needed to push the shoots to the surface. It was reported that deep-seeding tolerance was significantly associated with mesocotyl elongation [39,40]. BRs and GAs control maize seedling architecture traits such as mesocotyl length, seedling, and root length [41]. In addition, they are associated with seedling stress tolerance such as chilling tolerance and salinity stress [42–44]. In this study, we used the BR and GA inhibitors Propiconazole (Pcz) and Uniconazole (Ucz) to explore the BR and GA levels in different maize genotypes due to their easy accessibility, high stability and low costs [41,45], and investigated the control of seedling traits by BR and GA from physiological and genetic perspectives. Our main objectives were to i) study phenotypic variation of BR and GA inhibitor responses of four seedling traits within two diverse maize association panel, ii) use Genome-wide association studies (GWAS) to identify SNPs throughout the genome associated with BR and GA inhibitor responses, iii) investigate the correlation between seedling stage hormone level with field traits of plant height, flowering time and yield.

2. Materials and methods

2.1. Plant materials

A set of 207 BGEM (BC_1 derived doubled haploid (DH) lines – with expected 25% donor and 75% recurrent parent genome composition) were used in this study, which were obtained from the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, Iowa. Donor parents are landraces included in the Germplasm Enhancement of Maize (GEM) project. Recurrent parents are two expired PVP (Plant Variety Protection) lines PHB47 and PHZ51, which are from the two maize heterotic groups Stiff Stalk and Non Stiff Stalk in corn belt area of United States, respectively. The methods used to create the BGEM lines were described in a previous study [46]. Briefly, various landrace accessions were used as donor parents, and PHB47/PHZ51served as recurrent parents for one generation of backcrossing. BC_1 individuals were subsequently used to produce DH lines. There was no intentional selection during BGEM line development. BGEM lines are intended as a resource for biological research and potential discovery of unique alleles and traits. In this study, 76 BGEM lines are in PHZ51 and 131 BGEM lines in PHB47 background (DHZ51 and DHB47 subset, respectively). The pedigree, DH code, and donor accession information of the 207 BGEM lines is listed in Table S1.

2.2. Phenotyping

A paper roll cultivation method was employed as described in a previous study [47] to germinate seed. All BGEM lines were treated with three independent treatments of 80 μM BR inhibitor propiconazole (Pcz), 80 μM GA inhibitor uniconazole (Ucz) and water as mock treatment. 80 μM was picked as it was the highest concentration that produced stable and clear results, tolerated by seedlings without negative impact on their phenotype. Each treatment was applied in three independent experiments completed June 8, 2015, June 19, 2015, and June 29, 2015 using a completely randomized design (CRD). For each treatment, 18 seeds from each BGEM line were used with 6 seeds assigned to each experiment. Seed was germinated in three steps: 1) seed was soaked 24 h for absorption of sufficient treatment solution; 2) seed was transferred into paper rolls containing the respective soaking solution (water, Pcz, or Ucz); 3) paper rolls were placed in covered buckets in a growth chamber without light at 25 °C. After eight days, seedlings were removed from the growth chamber and different traits were measured. Each paper roll with six seedlings was considered as an experimental unit.

Four seedling traits – mesocotyl length, shoot length, primary root length, and total dry weight were manually evaluated for the three treatments (BR inhibitor, GA inhibitor, and water). Mesocotyl length (cm) was measured with a ruler from the root-shoot transition zone to the first node of the seedling. Shoot length (cm) was measured with a ruler from the root-shoot transition zone to the tip of the seedling. Primary root length (cm) was measured with a ruler from the root-shoot transition zone to the tip of the primary root. After these three measurements, seedlings were dried for 48 h at 55 °C [48], to determine the seedling dry weight (g). Hormone inhibitor response was calculated as the ratios of the measurements taken under BR (or GA) inhibitor and mock treatment [49]. Except for seedling traits, field traits plant height (PHT), Growing Degree Units (GDUs) to anthesis, GDUs to silking, anthesis silking interval (ASI) and yield (tons per hectare) were measured under regular field conditions (nutrition, irrigation etc.,). PHT was evaluated at Agronomy Farm (Boone, IA) across two years 2014–2015 using a randomized complete block design with two replications (50 individuals per plot – with two rows). Two weeks after tasseling, a representative plant in the center of each plot was selected and PHT was measured from the ground to the top of the tassel. Yield and flowering time (anthesis, silking, ASI) were evaluated across three environments (with one replication within each environment) at Agronomy Farm2014, Agronomy Farm 2015, and the ISU research farm (2015, Nashua IA) following a completely randomized design. In each replication, 50 individuals from each BGEM line were grown per plot. At maturity, ears were machine-harvested and yield was measured on a plot basis at 15.5% moisture content and subsequently converted to tons per hectare. Days to anthesis and silking were counted from date of sowing to the day, when 50% of the plants in a plot had tassels with anthers exerted from the glumes and ears with emerged silks, respectively. These were converted to GDUs: $\text{GDUs} = \frac{(Tmax + Tmin)}{2} - 10$, where Tmax is maximum daily temperature and is set equal to 30 °C when temperatures exceeded 30 °C. Tmin is the minimum daily temperature and is set equal to 10 °C when temperatures fall below 10 °C. ASI was calculated as the difference in GDUs between anthesis and silking.

2.3. Phenotypic data analysis

From each experimental unit (each roll): four seedlings out of six were sampled to eliminate poorly germinating seedlings, and means were taken per roll. The additive model for analysis of variance of seedling traits, flowering time and yield was: $y_{ij} = \mu + E_i + G_j + e_{ij}$ [48], where y_{ij} represents the observation from the ij^{th} experimental unit, μ is the overall mean, E_i is the experiment, and G_j is the genotype, and e_{ij} is the error. Best linear unbiased prediction (BLUP) was calculated by fitting genotype and experiment as random effects in SAS 9.2 (SAS Institute, 2008). The additive model for analysis of variance of PHT was: $y_{ijk} = \mu + E_i + R_j(E_i) + G_k + (G \times E)_{jk} + e_{ijk}$, where y_{ijk} represents the observation from the ijk^{th} experimental unit, μ is overall mean, E_i is the environment, $R_j(E_i)$ is the replication nested within each environment, G_k is the genotype, and $(G \times E)_{jk}$ is the interaction between genotype and environment, e_{jik} is the error. The BLUPs were calculated by fitting genotype, environment and replication as random effects in SAS 9.2 (SAS Institute, 2008). Heritability for all traits was calculated based on a plot basis [50]. For each evaluated trait, variance

Table 1
Trait designations and descriptions.

Trait name	Trait description
BRM	Ratio[a] of mesocotyl length with BR inhibitor treatment to water treatment
GAM	Ratio of mesocotyl length with GA inhibitor treatment to water treatment
BRS	Ratio of shoot length with BR inhibitor treatment to water treatment
GAS	Ratio of shoot length with GA inhibitor treatment to water treatment
BRR	Ratio of primary root length with BR inhibitor treatment to water treatment
GAR	Ratio of primary root length with GA inhibitor treatment to water treatment
BRW	Ratio of seedling dry weight with BR inhibitor treatment to water treatment
GAW	Ratio of seedling dry weight with GA inhibitor treatment to water treatment

[a] Ratio value close to 1 means that the trait is more tolerant to BR or GA inhibitor.

component estimates were obtained from a mixed linear model fitted across all environments in SAS PROC MIXED. Variance components (σ_g^2, $\sigma_{g\times e}^2$, σ_e^2) were estimated according where σ_g^2, $\sigma_{g\times e}^2$, σ_e^2 corresponds to genotypic variance, genotype by environmental interaction variance, and error variance, respectively. Entry mean-based heritability (h^2) was calculated from variance component estimates as $h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{g\times e}^2/n + \sigma_e^2/rn}$ where r is the number of replications within each location, and n is the number of locations [50]. Pearson's correlation coefficients were calculated between the BLUPs of each phenotype, and graphs were obtained using ggplot2 in R [51].

2.4. Marker data

The diversity panel was genotyped with a genotyping-by-sequencing (GBS) protocol [52] by Cornell University from Buckler lab. Removal of monomorphic and low-quality SNPs, as well as those with minor allele frequencies below 2.5% and missing data rate > 25%, generated a data set with 247,775 SNPs. For SNPs located at the same position (with genetic distance equal to 0 cM) in the genetic map, only one randomly selected SNP per map position was retained, which reduced the final number of SNPs to 62,049. For missing SNP markers, the LD k-nearest neighbor algorithm (LD KNNi imputation) was used for imputation using TASSEL 5.0 [53]. We observed many short recurrent parent segments interspersed with exotic donor genotypes, leading to more than 1000 recombinations across the genome per line. Because we only conducted one generation of backcrossing, we would expect recombination frequency to be much lower. Thus, we corrected for monomorphic markers within large donor segments based on Bayes theorem, with underlying assumption that the short recurrent parent segments are monomorphic markers instead of due to double recombinations. These short recurrent parent segments were corrected or kept as original genotype based on the results from the monomorphic marker correction approach. After correction the donor genome composition was closer to the expected 25% compared to original marker data, and the average recombination rate substantially reduced. The corrected dataset was used for genome-wide association studies.

2.5. Population structure, linkage disequilibrium, and genome-wide association analyses

Population structure was estimated using the genome-wide 62,049 SNPs with principal component analysis (PCA). PCA was calculated with R package Genome Association and Prediction Integrated Tool-R package (GAPIT) [54]. The most probable number of subpopulations was picked by plotting the number of PCAs (X-axis) against the variance explained by PCA numbers (Y-axis). The best number of subpopulations was selected, when the decrease of variance reached a plateau (no more variance can be explained by adding more PCs) [54]. The software program TASSEL 5.0 [53] was used to calculate linkage disequilibrium (LD) among SNP markers.

BLUPs of trait values for BR/GA inhibitor response of mesocotyl length, shoot length and primary root length were used for GWAS with 62,049 SNPs. GWAS analysis for field traits is summarized by Sanchez et al. (2017 in preparation) with the same plant materials and markers, thus will not be discussed in this manuscript. Seedling weight was not included due to low heritability (< 0.3).To balance false positives and false negatives, three association analysis methods were applied in this study: 1) General Linear Model (GLM) + PCA, with covariate PCA from GAPIT was included as fixed effects to account for population structure, 2) mixed linear model (MLM) [55] with population structure (PCA) and kinship included as covariates, and 3) Fixed and random model Circulating Probability Unification (FarmCPU) with kinship and population structure (PCA) as covariates, but with additional algorithms solving the confounding problems between testing markers and covariates [56]. The software program TASSEL 5.0 [53] was used to conduct GWAS with GLM + PCA method. GAPIT [54] was applied to conduct MLM. The R package FarmCPU was used to conduct GWAS with FarmCPU model. The statistical program simpleM implemented in R was used to account for multiple testing [57]. The threshold level was based on the effective number of independent tests m (Meff_G) and m was used in a similar way as the Bonferroni correction [48]. To obtain Meff_G for SNP data, a correlation matrix for all markers needs to be constructed and corresponding eigenvalues for each SNP locus calculated. A composite LD (CLD) correlation is calculated directly from SNP genotypes. Once this SNP matrix is created, the effective number of independent tests is calculated. In this study, with $\alpha = 0.05$ as family-wise error rate, the threshold for significant trait-marker associations was set as 2.55×10^{-6} (multiple testing threshold level).

3. Results

3.1. Hormone inhibitor responses of BGEM lines

Designations of measured BR and GA inhibitor trait responses and trait descriptions are summarized in Table 1. All traits have shown considerable variation and diversity for BR and GA inhibitor responses within both DHB47 and DHZ51. The standard deviation for BRR and GAR (See Table 1) varied the most with ratio values of 0.14 and 0.11 within DHZ51, and 0.13 and 0.1 within DHB47. This corresponds to previous studies that root traits varied the most for seedling traits [48]. All trait BR and GA inhibitor responses maximum, minimum, mean, median and standard deviations are listed in Table 2. Specifically, averages of BRM, BRS, BRR, GAM, GAS, and GAR ranged from 0.2 to 0.8 (ratio value close to 1 means that the genotype is more tolerant to either BR or GA inhibitor) across the entire panel. For these traits, the BR and GA inhibitor responses were below 1.0, indicating that the mesocotyl, shoot, and primary root lengths were inhibited by hormone inhibitor application. Moreover, GA inhibitor Ucz showed a stronger effect compared to BR inhibitor, as the values for GAM, GAS, and GAR were 0.21, 0.38, and 0.71 in DHZ51, lower than those for BRM, BRS, and BRR, which were 0.42, 0.57, and 0.84, respectively. Similar results were observed in DHB47 (Table 2). Means for BRM and GAM were lowest with 0.3–0.4 for BRM, and 0.1–0.2 for GAM, compared to other traits with BRR, BRS, BRW ranging from 0.5–1 and GAR, GAS, and

Table 2
Summary statistics for both hormone inhibitor response and field traits.

Library: DHZ51							
Trait	Min	Max	Mean	Median	PHZ51	SD	H^2
BRM	0.18	0.81	0.42	0.4	0.46	0.12	0.71
BRS	0.39	0.88	0.57	0.55	0.54	0.11	0.52
BRW	0.87	1.5	1.12	1.11	1.11	0.12	0.01
BRR	0.54	1.18	0.84	0.82	0.94	0.14	0.19
GAM	0.02	0.56	0.21	0.21	0.28	0.09	0.87
GAS	0.2	0.79	0.38	0.38	0.4	0.09	0.68
GAR	0.48	1.07	0.71	0.71	0.73	0.11	0.38
GAW	0.75	1.39	1.04	1.04	0.96	0.11	0.05
Anthesis (GDUs)	723.9	881.8	786.8	784.1	766.3	28.8	0.65
Silking (GDUs)	745.2	927.8	813.2	811.1	783.7	40.7	0.70
ASI (GDUs)	−11.8	104.7	26.2	19.7	17	22.8	0.53
PHT(cm)	177.2	268.3	222.6	222.2	225	20	0.85
Yield (tons/hectare)	0.9	4.6	2.6	2.5	3.9	0.9	0.59

Library: DHB47							
Trait	Min	Max	Mean	Median	PHB47	SD	H^2
BRM	0.12	0.63	0.31	0.31	0.28	0.1	0.8
BRS	0.31	0.73	0.5	0.49	0.47	0.09	0.5
BRR	0.47	1.12	0.75	0.75	0.58	0.13	0.39
BRW	0.86	1.64	1.13	1.12	1.11	0.13	0.03
GAM	0.04	0.32	0.12	0.1	0.09	0.06	0.85
GAS	0.15	0.53	0.29	0.28	0.27	0.07	0.68
GAR	0.38	1.06	0.64	0.62	0.57	0.1	0.39
GAW	0.79	1.3	1.03	1.02	1	0.1	0.01
Anthesis (GDUs)	664.6	864.8	778.9	780.1	746.1	32.7	0.73
Silking (GDUs)	691.1	867	787.4	784.7	746.1	32.9	0.65
ASI (GDUs)	−24.7	45.8	9.2	8.8	0.2	15.1	0.39
PHT(cm)	173.2	279	225.4	225	230.8	21.5	0.88
Yield	1.1	5.3	3.2	3.1	5.3	0.9	0.62

GDUs, Growing Degree Units (°C); SD, standard deviation; H^2, heritability; ASI, anthesis silking interval; PHT, plant height. BRM, BRS, BRR, BRW, GAM, GAS, GAR, GAW: see Table 1 for explanation.

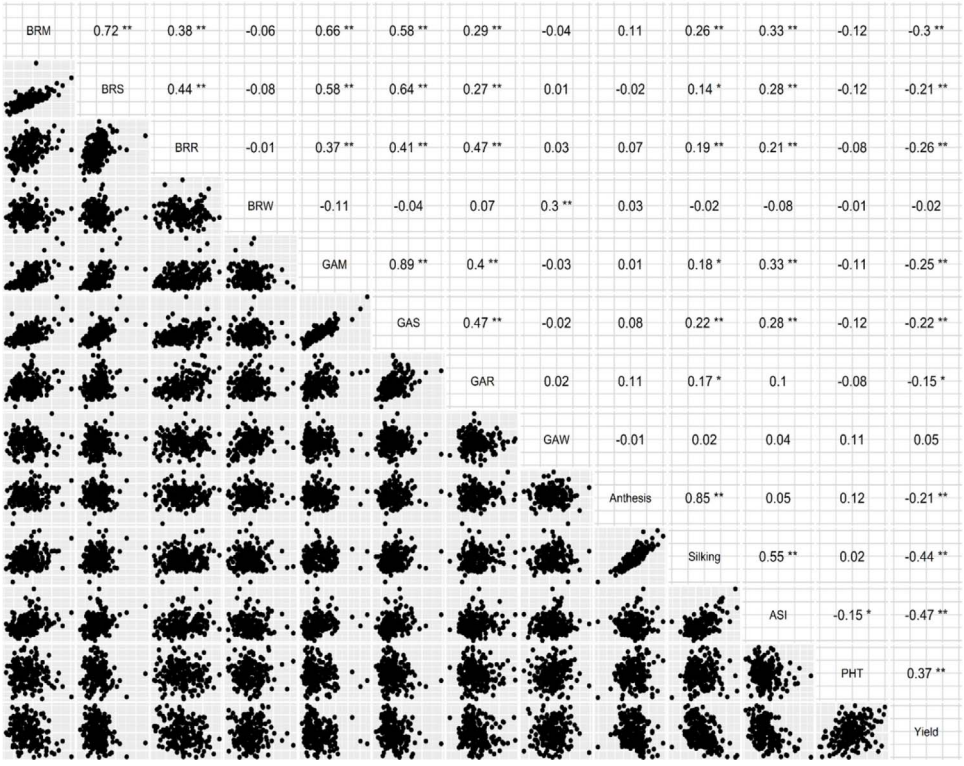

Fig. 1. Pearson correlations between all 13 traits collected. * and ** represent significance levels at α = 0.01 and 0.001, respectively. BRM, BRS, BRR, BRW, GAM, GAS, GAR, and GAW: see Table 1 for explanation; ASI, anthesis silking interval; PHT, plant height.

GAW ranging from 0.3–1. This indicates that mesocotyl length was more sensitive in response to BR and GA inhibitors compared to shoot length, primary root length, and seedling dry weight. We noticed that the median of trait values in DHB47 and DHZ51 was close to their recurrent parent (PHB47 and PHZ51) performance (Table 2), expected as these are derived from backcrosses without any selection. For example, the median of BRS in DHB47 was 0.49, close to BRS for PHB47 (0.47). The median of BRS in DHZ51 was 0.55, close to BRS (0.54) for PHZ51. This indicates that around half of the BGEM lines were more tolerant to BR and GA inhibitors compared to their recurrent parents. BRM and GAM showed the highest heritabilities ($H^2 \sim 0.8$), compared to seedling length ($H^2 \sim 0.55$), root length ($H^2 \sim 0.3$), and dry weight ($H^2 \sim 0.1$). BRM, BRS, BRR, GAM, GAS, and GAR were significantly and positively correlated ($P = 0.001$) with each other (Fig. 1), suggesting that higher levels of BR and GA increase elongation of mesocotyl length, shoot length, and primary root length. However, BRW and GAW were not significantly correlated with any other traits, although significantly correlated with each other ($P = 0.001$).

3.2. Field performance of BGEM lines

Large variation was observed for field traits (Table 2). Standard deviation of Growing Degree Units (GDU) for anthesis and silking was similar with 28.8 °C and 40.7 °C in DHZ51, and 32.7 °C and 32.9 °C in DHB47. For ASI (GDUs), standard deviation in DHB47 was 15.1 °C, smaller than 22.8 °C in DHZ51. For PHT (cm) and yield (t/ha), the standard deviation within DHB47 was 20 cm, 0.9 t/ha, almost the same as in DHZ51 which was 21.5 cm and 0.9 t/ha, respectively. When BGEM lines were compared to their recurrent parents, the medians of field trait values in DHB47 and DHZ51 were close to PHB47 and PHZ51 (Table 2). For example, the median value of PHT in DHB47 and DHZ51 were 225 and 222.2 cm, and for PHB47 and PHZ51 230.8 and 225 cm, respectively. However, for ASI (GDUs) of DHB47, the median value was 8.8 °C, while PHB47 was 0.2 °C. Moreover, PHB47 and PHZ51 performed better for grain yield than most of the BGEM lines (PHB47 had the maximum value for yield within DHB47). PHT showed the highest value of $H^2 \sim 0.9$, followed by flowering time (anthesis and silking) with $H^2 \sim 0.7$, grain yield with $H^2 \sim 0.6$, and ASI with $H^2 \sim 0.4$ in DHB47 and $H^2 \sim 0.5$ in DHZ51, respectively. The closest correlation was observed between GDUs to anthesis and silking, with r = 0.85. Both traits were significantly and negatively correlated with grain yield ($\alpha = 0.001$). ASI was negatively correlated with grain yield and PHT, and there was a positive correlation between grain yield and PHT ($\alpha = 0.001$).

3.3. Correlation between hormone inhibitor responses and field traits

Since BR and GA control both seedling development and field traits, we calculated the correlations between seedling stage hormone inhibitor responses and field traits. There was no significant correlation ($\alpha = 0.01$) between field traits PHT, GDUs for anthesis, and seedling BR and GA inhibitor responses (Fig. 1). However, field traits grain yield, GDUs to silking, and ASI were significantly ($\alpha = 0.01$) correlated with seedling BR and GA inhibitor responses. Specifically, grain yield was significantly ($\alpha = 0.001$) and negatively correlated with BRM, BRS, BRR, GAM, GAS, and GAR. The highest correlation was between grain yield and BRM with a negative correlation of 0.3. GDUs for silking was positively and significantly ($\alpha = 0.01$) correlated with BRM, BRS, BRR, GAM, GAS, and GAR. The highest correlation was between GDUs for silking and BRM with a positive correlation of 0.26. ASI was significantly ($\alpha = 0.001$) and positively correlated with BRM, BRS, BRR, GAM, and GAS. The highest correlation was observed between BRM, GAM, and ASI, with a positive correlation of 0.33. In summary, seedling stage hormone inhibitor responses were correlated with field traits grain yield, ASI and GGD for silking, and BRM, GAM are with the highest correlations.

3.4. Population structure

Consistent with the two recurrent parents PHB47 and PHZ51 (donor accessions were crossed with either PHB47 or PHZ51 to produce DH lines), we found two sub-populations, used for joint analysis of DHB47 and DHZ51. One subpopulation comprised 60% BGEM lines used for GWAS including PHB47, which include mostly BGEM lines from DHB47

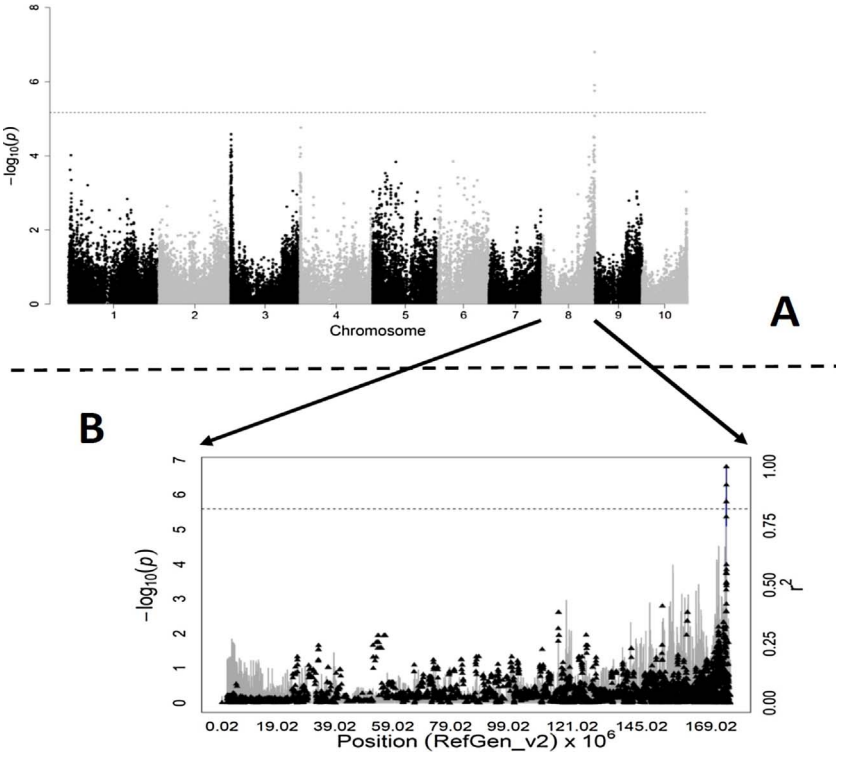

Fig. 2. GWAS for GAM in maize.
(A) GWAS results from the mixed linear model (MLM) of GAM. GAM represents for GA control of mescocotyl length and calculated as ratio of mesocotyl length under GA inhibitor treatment to the length under water treatment. X-axis represents the ten chromosomes and y-axis represents for the −log10P-values. The horizontal line represents for the threshold for multiple testing corrections calculated from SimpleM (See Experimental procedures). (B) Scatter plot of association results from MLM analysis of GAM and LD estimates (r2) across Chromosome 8 where SNPs were found to be significantly associated with GAM. −log10P-values (left, y-axis) are from GWAS results and r2 values (right, y-axis) represent the LD between all SNPs from Chromosome 8 to the top marker which locates within GRMZM2G01339. The grey vertical lines are −log10 P-values for SNPs. Triangles are the r2 values of each SNP relative to the peak SNP (indicated in red) at 174,376,713 bp. The black horizontal dashed line indicated the threshold from SimpleM.

group, and nine BGEM lines BGEM-0005-N, BGEM-0085-N, BGEM-0107N, BGEM-0121N, BGEM-0129N, BGEM-0215N, BGEM-0227N, BGEM-0248N, BGEM-0260N from DHZ51 group. The other subpopulation comprised 40% BGEM lines including PHZ51, including mostly BGEM lines from DHZ51 group, with nine BGEM lines BGEM-0007-S, BGEM-0052S, BGEM-0078-S, BGEM-0094-S, BGEM-0165-S, BGEM-0166-S, BGEM-0175-S, BGEM-0220-S, BGEM-0266-S from the DHB47 group. These BGEM lines, which were grouped with a different recurrent parent, had on average around 50% donor genome proportion, larger than the average of donor genome proportion for the whole BGEM collection which was 18.3%.

3.5. Genome-wide association study

Three SNP markers on Chromosome 8, *S8_174376713* ($P = 1.57 \times 10^{-7}$), *S8_174338368* ($P = 1.24 \times 10^{-6}$), and *S8_174376891* ($P = 1.75 \times 10^{-6}$), were significantly associated with GAM ($H^2 \sim 0.85$) using MLM: *S8_174376713* and *S8_174376891* were from the same gene *GRMZM2G013391* and *S8_174338368* is located in an intergenic region 40 kb upstream of its transcription start site (Fig. 2A). No SNPs were identified for the other traits except for GAM. Linkage disequilibrium (LD) between *S8_174338368* and *GRMZM2G013391* was $r^2 = 0.85$. Tissue specific expression of *GRMZM2G013391* has been determined using high density NimbleGen Microarrays based on B73. It was shown that *GRMZM2G013391* was expressed with absolute expression level of 422 in shoots and 1899.04 in primary roots at seedling stage [58]. Thus, this gene is involved in metabolic processes for seedling growth. The homologs of *GRMZM2G013391* in model species Arabidopsis and rice are zinc-finger type transcription factors, designated as WRKY. Interestingly, it was shown that a rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway [59]. We searched for GA candidate genes near *GRMZM2G013391* to test the hypothesis that the signal was caused by strong LD between GA candidate genes and *GRMZM2G013391*. The closest GA candidate gene *ZmGA2ox1* is 500 kb away from *GRMZM2G013391*. However, the r^2 value between a SNP derived from this GA candidate gene and the two SNPs (significantly associated with GAM) from *GRMZM2G013391* was below 0.1 (Fig. 2B).

With the FarmCPU model, 19 significant SNPs were found using the same threshold of $P = 2.55 \times 10^{-6}$ (multiple testing threshold calculated from SimpleM: See Experimental procedures) for GAM, GAS, BRM, and BRS. For root traits GAR and BRR (H^2 ranged from 0.2–0.4), no significant markers were detected using the FarmCPU model. Eleven candidate genes were predicted based on the 19 SNPs and their surrounding regions of 2 kb up- and downstream of markers (Table 3). Among them, four genes *GRMZM2G024657*, *GRMZM2G091919*, *GRMZM2G177050*, *GRMZM2G114911* were associated with BR inhibitor response (BRM and BRS), and seven genes *GRMZM2G013391*, *GRMZM2G022258, GRMZM2G001169*, *GRMZM2G148229*, *GRMZM5G891656*, *GRMZM2G412085, GRMZM2G132663* were associated with GA inhibitor responses (GAM and GAS*)*. All 11 genes are expressed at seedling shoots based on B73 [58], with gene *GRMZM2G022258* associated with the highest expression value of 9118.35, which means that these genes were associated with the metabolic process for seedling development. Its orthologue in Arabidopsis is exportin protein that mediates the nuclear export of proteins, rRNA, snRNA, and some mRNA [60]. Orthologues for the other genes in Arabidopsis and rice are WRKY transcription factor, cysteine proteinases, kinase, and binding proteins, among others (Table S2). None of these genes were co-localized with any BR or GA candidate genes. We noticed that the most significant marker associated with GAM is exactly the same detected both by FarmCPU (SNP *S8_174376713* in Fig. 2) and MLM, with $P = 3.22 \times 10^{-11}$ for FarmCPU.

Considering that the use of a MLM and FarmCPU could generate false negative results as both kinship and population structure are included to control false positive results, we identified the most significant associations with GLM + PCA method, using the same threshold as MLM and FarmCPU of $P = 2.55 \times 10^{-6}$, resulting in 134 significant markers for all traits (Table S3). More significant markers were detected, and some markers overlapped with MLM and FarmCPU results. In particular, the top three markers from GLM + PCA are exactly the same as the three significant markers with MLM method (for GAM), with *P*-values 3.23×10^{-13}, 3.45×10^{-13}, 4.32×10^{-12} for *S8_174376891*, *S8_174376713*, and *S8_174338368,* respectively. In total, 24 markers were significantly associated with GAM and GAS on Chromosome 8, clustered within a 1Mb region up- and downstream of gene *GRMZM2G013391*. As a result, gene *GRMZM2G013391* is significantly associated with GAM across all three methods of MLM, FarmCPU, and GLM and always associated with the lowest *P*-values.

Except for associated markers from gene *GRMZM2G013391,* four other markers were identified consistent across FarmCPU and GLM + PCA methods with two associated with BR inhibitor response and another two with GA inhibitor response. These four markers were from four gene models. Specifically, markers from *GRMZM2G024657* and *GRMZM2G114911* were associated with BRM and BRS, respectively. *GRMZM2G024657* codes for a cysteine proteinase. In common bean a

Table 3
Significant SNPs from FarmCPU.

Trait	SNP	Chr	Position	*P*-value	Gene	Absolute expression value in seedling
BRM	*S5_3538677*	5	3538677	3.29E-08	*GRMZM2G024657*	69.88
BRM	*S7_143003800*	7	143003800	3.03E-07	*GRMZM2G091919*	102.82
BRM	*S7_172879110*	7	172879110	2.50E-06	*GRMZM2G177050*	163.72
BRS	*S4_154986604*	4	154986604	1.06E-06	*GRMZM2G114911*	2173.76
BRS	*S3_184357485*	3	184357485	1.41E-06	Intergenic	
BRS	*S9_76294986*	9	76294986	2.44E-06	Intergenic	
GAM	*S8_174376713*	8	174376713	3.22E-11	*GRMZM2G013391*	422
GAM	*S5_749312*	5	749312	2.63E-09	*GRMZM2G022258*	9118.35
GAM	*S4_2511802*	4	2511802	5.05E-09	*GRMZM2G001169*	46.65
GAM	*S7_170538823*	7	170538823	4.37E-08	*GRMZM2G148229*	8265.48
GAM	*S8_4409026*	8	4409026	7.41E-08	Intergenic	
GAM	*S9_152757586*	9	152757586	1.25E-06	*GRMZM5G891656*	256.55
GAS	*S8_174377648*	8	174377648	6.44E-12	*GRMZM2G013391*	422
GAS	*S3_2813553*	3	2813553	1.85E-11	*GRMZM2G412085*	423.37
GAS	*S8_119054591*	8	119054591	1.93E-08	*GRMZM2G132663*	1704.02
GAS	*S4_2511802*	4	2511802	2.14E-08	*GRMZM2G001169*	46.65
GAS	*S3_3123403*	3	3123403	1.34E-07	Intergenic	
GAS	*S1_292671200*	1	292671200	2.96E-07	Intergenic	
GAS	*S10_140978407*	10	140978407	4.33E-07	Intergenic	

Trait abbreviations see Table 1 for explanation. Chr: Chromosome.

cysteine proteinase is regulated by BR for germination and seedling elongation [61]. Markers from *GRMZM2G001169* and *GRMZM5G891656* were associated with GAM, however, they did not overlap with predicted GA candidate genes. *GRMZM2G001169* is a gene with unknown function, and *GRMZM5G891656* is an oxidoreductase family protein.

4. Discussion

4.1. BR and GA control of seedling traits

Plant hormones are small molecules that regulate many aspects regarding to plant growth and development, as well as responses to changing environmental conditions. By modifying the production, distribution or signal transduction of plant hormones, plants are able to regulate and coordinate both growth and/or stress tolerance to promote survival or escape from environmental stress [62]. In this study, we focused on BR and GA control of seedling traits, as BR and GA promote seedling vigor and growth [63–67] and they are positively correlated to seedling stress tolerance of drought, salinity, cold/heat, and heavy metals in different plant species such as maize, sorghum, Arabidopsis and rice [43,62,68,69]. For example, exogenous application of BRs increased the cold tolerance of maize seedlings [70], and increased endogenous levels of GAs were effective in protecting maize seedlings from drought stress [71]. As a high quality seedling establishment can lead to uniform field stands and stable yields, we investigated the BR and GA activities at seedling stage across 207 BGEM lines. On average, an expected 25% (with observed 18.3%) tropical germplasm was introgressed into PHB47 and PHZ51, representing the two maize heterotic groups Stiff Stalk and Non Stiff Stalk in the corn belt area of United States. We measured the hormone inhibitor responses of these 207 BGEM lines, and an extensive amount of phenotypic variation was found for all traits measured (Table 2). For example, BGEM-0191-N was 28 times more tolerant to GA inhibitor compared to BGEM-0215N with regard to GAM, and BGEM-0269-S was six times more tolerant to BR inhibitor compared to BGEM-0079-S. Moreover, without selection, the donor segments have either increased or decreased effects for hormone inhibitor response – around half BGEM lines are more tolerant/not tolerant to BR and GA inhibitors for each trait measured compared to recurrent parents. In conclusion, there is substantial variation for BR and GA control of seedling traits.

For both DHB47 and DHZ51, shoot length, mesocotyl length, and primary root length were reduced with the application of BR and GA inhibitors compared to water treatment. Ratio values of BRM, BRS, GAM and GAS were below 1.0 for all but one of the 207 BGEM lines (BGEM-0123-N with BRS equal to 1.1). Primary root length was less sensitive to BR/GA inhibitor compared with shoot and mesocotyl length, as on average BRR and GAR was larger than BRM, BRS, GAM and GAS. We noticed that on average, BRW and GAW was close to 1 (Table 2). The dry weight of the tissue of most BGEM lines was not affected with the application of BR and GA inhibitors, although the length of both shoot and primary root length was substantially reduced. One explanation of this result is that BR and GA regulate cell length instead of cell number and weight at seedling stage, and we observed that the seedlings were fatter and shorter with hormone inhibitors compared to water treatment. However, it was noted that BRW and GAW had very low estimated heritabilities in both the DHB47 and DHZ51 libraries.

Heritability values ranged from 0.01 to 0.87. BRM and GAM showed the highest heritability (> 0.8) across both DHB47 and DHZ51, followed by BRS and GAS (0.5–0.7). By keeping all conditions equal, BRM and GAM was the most stable and repeatable trait for reflecting BR and GA inhibitor response across different maize genotypes. BRR and GAR had a heritability close to 0.35, this corresponds to previous studies with similar ranges of heritability for root traits both under controlled environmental and field conditions [48,72,73]. For BRW and GAW, the heritability was extremely low and less than 0.1. This may because biomass traits were not measured as accurately as length traits, or seedling dry weight was very sensitive to environmental conditions.

4.2. BR and GA inhibitor response prediction for field traits

Seedling traits are easy to measure in controlled environments and have been used for comparison with adult plant traits. Because root traits are difficult and laborious to measure at the adult stage, measurements of seedling root architectural traits were compared with field root traits; interestingly some root traits have been shown to be positively correlated with yield [35,48]. Instead of using seedling traits *per se*, we calculated the correlation between seedling stage BR/GA inhibitor response and field traits. Because BRs and GAs regulate both seedling growth and field traits such as plant height, sex determination, and yield in maize [4,16,74], our hypothesis is that genotypes with elevated early BR and/or GA activities are more tolerant to BR and GA inhibitors, and will grow taller and may produce higher yields. If this hypothesis is correct, we can use seedling stage BR and GA inhibitor response to predict field traits for inbred lines. We found that BRM, BRS, BRR, GAM, GAS were positively ($\alpha = 0.01$) correlated with GDUs to silking and ASI, and were negatively correlated with yield. Reduced yield may result from a longer ASI, as grain yield and its component, ears per plant, showed a dependence on ASI and were negatively correlated [75], as the asynchrony of male and female flowering can cause reduced pollination rate. Although there was a positive correlation between PHT and yield, seedling stage BR and GA inhibitor response was not significantly correlated with field PHT for BGEM lines. This result may have arisen because the BR and GA genes functioning at seedling stage and the developmental process to form PHT are different sets of genes in an inbred background.

4.3. Genome-wide association study

Genome-wide association studies identified markers within or near candidate genes affecting field traits, seedling traits, stress tolerance, or nutritional quality in maize [48,49,76–79]. In this study, we used three statistical models (GLM + PCA, MLM, FarmCPU) to conduct genome-wide association studies for BR and GA inhibitor response, and found in total 3, 19, and 134 significant SNPs with MLM, FarmCPU, and GLM + PCA methods, respectively. As noted in other studies, MLM created type II errors [80] and GLM method can create type I errors. Of interest, we applied a recently developed method FarmCPU, which included both population structure and kinship matrix in the GWAS model like for the MLM, but used additional algorithms to address confounding problems between testing markers and covariates [56]. As a result, it is less stringent compared to the MLM method, but more stringent than the GLM + PCA model, and markers not detected by MLM, but significant using FarmCPU, are putatively in LD with causal variants masked by kinship or between subpopulations. However, significant markers from MLM are consistent with GLM + PCA and FarmCPU methods. Using all three methods in conjunction is preferable to balance the potentially false positives and false negatives for gene trait associations.

Across all three association models, SNPs from *GRMZM2G013391* were consistently associated with GAM and always had the lowest *P*-values. Although there is a GA candidate gene close to (within 0.5 Mb) *GRMZM2G013391*, the LD between these two genes is below 0.1. Moreover, *GRMZM2G013391* is expressed throughout seedling development with absolute expression level of 422 in B73 shoots [58], which means that this gene is involved in the metabolic process for seedling development. It needs to be noted that expression is only based on B73, and variation in transcriptome profiles between multiple inbred lines has been reported [81]. The gene model of *GRMZM2G013391* codes for WRKY transcription factors predicted from MaizeGDB [82]. Plant WRKY gene family represents an ancient and complex class of zinc-

finger transcription factors (TFs) that are involved in the regulation of various physiological processes and many plant pathways such as development and senescence, and in plant response to many biotic and abiotic stresses [83,84]. In rice, it was shown that a *WRKY* gene encodes a transcriptional repressor of the GA signaling pathway in aleurone cells: OsWRKY71 blocks GA signaling by functionally interfering with GA-inducible transcriptional activator OsGAMYB and exogenous GA treatment decreases the steady-state mRNA level of OsWRKY71 and destabilizes the GFP:OsWRKY71 fusion protein [59]. Moreover, synergistic interaction of ABA-inducible WRKY genes regulates GAMYB-mediated GA signaling in aleurone cells, thereby establishing a novel mechanism for ABA and GA signaling cross-talk [85]. If the function of *GRMZM2G013391* is similar as WRKY transcription factors in rice to regulate GA signaling, this candidate gene could be a vital player in regulating GA levels in maize seedlings. In maize, WRKY transcription factors were found to be associated with chilling tolerance of maize seedlings, with a GWAS analysis of chilling tolerance indices (ratio of measurements taken under chilling stress and control conditions) [49]. Because WRKY genes are associated with both GA activities and chilling tolerance, it is possible that WRKY genes are regulating chilling tolerance through increasing endogenous GA levels. It was shown that endogenous GA levels were positively associated with chilling stress tolerance: with external GA_3 treatment, chilling tolerance was increased with decreased electrolyte leakage and malondialdehyde content, increased proline content, and improved antioxidant enzyme activities. Moreover, treatment with GA inhibitors exacerbated chilling injury [86]. This may explain that in our study, we detected WRKY genes with GA inhibitor response of seedlings, and WRKY genes were also associated with seedling chilling tolerance in previous studies.

Except for markers from gene model *GRMZM2G013391,* markers were found to be significantly associated with BRM, BRS, GAM, and GAS from other ten gene models with FarmCPU method, and they are all expressed throughout seedling development at different expression levels in B73 (Table 3). Based on the known functions of the homologs of these candidate genes in Arabidopsis and rice, these candidate genes are related to proteinases, protein kinase, transferase, binding proteins, and SNARE (Soluble NSF Attachment Protein Receptor) proteins (Table S2). Some genes with enzyme activity functions were found to be regulated by BR and GA in different species. For example, *GRMZM2G024657* is predicted to code for cysteine proteinases and associated with BRM in this study. It was shown that BRs and GAs are involved in the expression of cysteine proteinases in cotyledons of common beans for germination. BRs and GAs regulate the synthesis of cysteine proteinases to degrade storage proteins in cotyledons of legume plants, to provide enough nutrition for the germination and growing process [61]. In addition, *GRMZM2G091919* is predicted to code for glycosyl transferase and also associated with BRM. Glycosyl transferase is one of the most important modification reactions towards plant secondary metabolites, and plays a key role in maintaining cell homeostasis [87]. It was shown that glycosyl transferase regulates BR activities. When UDP-glycosyltransferase 73C5 (UGT73C5) was ectopically overexpressed in Arabidopsis, the transgenic plants displayed characteristic BR-deficient dwarf phenotypes, and this dwarfism was reverted to wild-type morphology by exogenous application of *epi*-BL [88]. The genes associated with BR and GA inhibitor responses in this study may represent BR/GA pathway components, or they regulate BR/GA activities, or they are regulated by BR/GA. They may play key roles for the BR and GA control of seedling traits such as mesocotyl length, shoot length and primary root length. Several loci with small effects were likely missed in our study due to limited population size [79]. However, the genes identified by GWAS in this study are in various cases consistent with findings of earlier studies of large effect loci, which could be used as direct targets of marker-assisted selection [49].

Authors' contributions

SH and TL conceived the study, designed the experiments, discussed the results and finalized the manuscript; DLS collected field data for BGEM lines and helped with genotype analyses; CW helped with plant hormone related experiments; AEL helped with statistical analysis. YY established protocol for measuring BR/GA inhibitor response. CG provided BGEM seeds and field support. TL, AEL, YY, CG edited the manuscript; all authors read and approved the final manuscript.

Acknowledgements

We are grateful to China Scholarship Council for Songlin Hu and Cuiling Wang's funding. In addition, authors would also like to thank USDA's National Institute of Food and Agriculture (Project Numbers: IOW04314, IOW01018), and Hatch Multistate Project NC-007, as well as the Plant Sciences Institute, RF Baker Center for Plant Breeding and K.J. Frey Chair in Agronomy at Iowa State University for funding or supporting this work. There is no conflict of interest for this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.07.011.

References

[1] S. Fujioka, T. Yokota, Biosynthesis and metabolism of brassinosteroids, Annu. Rev. Plant Biol. 54 (2003) 137.
[2] L. Taiz, E. Zeiger, Plant Physiology, 5th ed., Sinauer Assoc, Sunderland, 2010.
[3] S.D. Clouse, Molecular genetic studies confirm the role of brassinosteroids in plant growth and development, Plant J. 10 (1996) 1–8.
[4] C. Vriet, E. Russinova, C. Reuzeau, Boosting crop yields with plant steroids, Plant Cell 24 (2012) 842–857.
[5] P. Krishna, Brassinosteroid-mediated stress responses, J. Plant Growth Regul. 22 (2003) 289–297.
[6] S.D. Clouse, Brassinosteroid signal transduction: from receptor kinase activation to transcriptional networks regulating plant development, Plant Cell 23 (2011) 1219–1230.
[7] J. Hao, Y. Yin, S.-Z. Fei, Brassinosteroid signaling network: implications on yield and stress tolerance, Plant Cell Rep. 32 (2013) 1017–1030.
[8] J. Jiang, C. Zhang, X. Wang, Ligand perception, activation, and early signaling of plant steroid receptor brassinosteroid insensitive 1, J. Integr. Plant Biol. 55 (2013) 1198–1211.
[9] B. Zhao, J. Li, Regulation of brassinosteroid biosynthesis and inactivation, J. Integr. Plant Biol. 54 (2012) 746–759.
[10] J.-Y. Zhu, J. Sae-Seaw, Z.-Y. Wang, Brassinosteroid signalling, Development 140 (2013) 1615–1620.
[11] H. Tong, C. Chu, Brassinosteroid signaling and application in rice, J. Genet. Genomics 39 (2012) 3–9.
[12] G.J. Bishop, C. Koncz, Brassinosteroids and plant steroid hormone signaling, Plant Cell 14 (2002) S97–S110.
[13] U.K. Divi, P. Krishna, Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance, New Biotechnol. 26 (2009) 131–136.
[14] S. Koh, S.-C. Lee, M.-K. Kim, J.H. Koh, S. Lee, G. An, S. Choe, S.-R. Kim, T-DNA tagged knockout mutation of rice OsGSK1 an orthologue of Arabidopsis BIN2, with enhanced tolerance to various abiotic stresses, Plant Mol. Biol. 65 (2007) 453–466.
[15] I. Makarevitch, A. Thompson, G.J. Muehlbauer, N.M. Springer, Brd1 gene in maize encodes a brassinosteroid C-6 oxidase, PLoS One 7 (2012) e30798.
[16] T. Hartwig, G.S. Chuck, S. Fujioka, A. Klempien, R. Weizbauer, D.P.V. Potluri, S. Choe, G.S. Johal, B. Schulz, Brassinosteroid control of sex determination in maize, Proc. Natl. Acad. Sci. 108 (2011) 19814–19819.
[17] T. Sakamoto, K. Miura, H. Itoh, T. Tatsumi, M. Ueguchi-Tanaka, K. Ishiyama, M. Kobayashi, G.K. Agrawal, S. Takeda, K. Abe, An overview of gibberellin metabolism enzyme genes and their related mutants in rice, Plant Physiol. 134 (2004) 1642–1653.
[18] S.L. Dellaporta, A. Calderon-Urrea, The sex determination process in maize, Science 266 (1994) 1501.
[19] M.P. Lange, T. Lange, Gibberellin biosynthesis and the regulation of plant development, Plant Biol. 8 (2006) 281–290.
[20] T. Oikawa, M. Koshioka, K. Kojima, H. Yoshida, M. Kawata, A role of OsGA20ox1 encoding an isoform of gibberellin 20-oxidase, for regulation of plant stature in rice, Plant Mol. Biol. 55 (2004) 687–700.

[21] A. Sasaki, M. Ashikari, M. Ueguchi-Tanaka, H. Itoh, A. Nishimura, D. Swapan, K. Ishiyama, T. Saito, M. Kobayashi, G.S. Khush, Green revolution: a mutant gibberellin-synthesis gene in rice, Nature 416 (2002) 701–702.
[22] Y. Zhang, Y. Zhu, Y. Peng, D. Yan, Q. Li, J. Wang, L. Wang, Z. He, Gibberellin homeostasis and plant height control by EUI and a role for gibberellin in root gravity responses in rice, Cell Res. 18 (2008) 412–421.
[23] C.M. Fleet, T.-P. Sun, A DELLAcate balance: the role of gibberellin in plant morphogenesis, Curr. Opin. Plant Biol. 8 (2005) 77–85.
[24] M. Ueguchi-Tanaka, M. Ashikari, M. Nakajima, H. Itoh, E. Katoh, M. Kobayashi, T.-Y. Chow, C.H. Yue-ie, H. Kitano, I. Yamaguchi, GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin, NatuZre 437 (2005) 693–698.
[25] M. Ueguchi-Tanaka, M. Nakajima, E. Katoh, H. Ohmiya, K. Asano, S. Saji, X. Hongyu, M. Ashikari, H. Kitano, I. Yamaguchi, Molecular interactions of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin, Plant Cell 19 (2007) 2140–2155.
[26] M. de Lucas, J.-M. Davière, M. Rodríguez-Falcón, M. Pontin, J.M. Iglesias-Pedraz, S. Lorrain, C. Fankhauser, M.A. Blázquez, E. Titarenko, S. Prat, A molecular framework for light and gibberellin control of cell elongation, Nature 451 (2008) 480–484.
[27] J. Peng, D.E. Richards, N.M. Hartley, G.P. Murphy, K.M. Devos, J.E. Flintham, J. Beales, L.J. Fish, A.J. Worland, F. Pelica, 'Green revolution'genes encode mutant gibberellin response modulators, Nature 400 (1999) 256–261.
[28] S.P. Choudhary, J.-Q. Yu, K. Yamaguchi-Shinozaki, K. Shinozaki, L.-S.P. Tran, Benefits of brassinosteroid crosstalk, Trends Plant Sci. 17 (10) (2012) 594–605.
[29] S. Hu, T. Lübberstedt, Getting the 'MOST'out of crop improvement, Trends Plant Sci. 20 (6) (2015) 372–379.
[30] J.R. Andersen, T. Lübberstedt, Functional markers in plants, Trends Plant Sci. 8 (2003) 554–560.
[31] A. Amram, A. Fadida-Myers, G. Golan, K. Nashef, R. Ben-David, Z. Peleg, Effect of GA-sensitivity on wheat early vigor and yield components under deep sowing, Front. Plant Sci. 6 (2015).
[32] M.J. Asghar, I.A. Khan, Variability for seedling vigour in two maize (Zea mays L.) populations I: means, variance components and heritabilities, Pak. J. Biol. Sci. 8 (2005) 839–843.
[33] F. Cervantes-Ortiz, A. Carballo-Carballo, D. Bergvinson, J. Crossa, M. Mendoza-Elos, E. Moreno-Martinez, Seedling Vigor Inheritance and Its Relationship to Adult Plant Traits in Inbred Tropical Maize Lines, (2007).
[34] R. Molatudi, I. Mariga, The effect of maize seed size and depth of planting on seedling emergence and seedling vigour, J. Appl. Sci. Res. 5 (2009) 2234–2237.
[35] A.H. Abdel-Ghani, B. Kumar, J. Reyes-Matamoros, P.J. Gonzalez-Portilla, C. Jansen, J.P. San Martin, M. Lee, T. Lübberstedt, Genotypic variation and relationships between seedling and adult plant traits in maize (Zea mays L.) inbred lines grown under contrasting nitrogen levels, Euphytica 189 (2013) 123–133.
[36] A. Hund, Y. Fracheboud, A. Soldati, E. Frascaroli, S. Salvi, P. Stamp, QTL controlling root and shoot traits of maize seedlings under cold stress, Theor. Appl. Genet. 109 (2004) 618–629.
[37] R.A. Richards, G.J. Rebetzke, M. Watt, A.T. Condon, W. Spielmeyer, R. Dolferus, Breeding for improved water productivity in temperate cereals: phenotyping, quantitative trait loci, markers and the selection environment, Funct. Plant Biol. 37 (2010) 85–97.
[38] H. Zhang, P. Ma, Z. Zhao, G. Zhao, B. Tian, J. Wang, G. Wang, Mapping QTL controlling maize deep-seeding tolerance-related traits and confirmation of a major QTL for mesocotyl length, Theor. Appl. Genet. 124 (2012) 223–232.
[39] A.F. Troyer, The location of genes governing long first internode of corn, Genetics 145 (1997) 1149–1154.
[40] G. Zhao, P. Ma, J. Wang, G. Wang, Identification of deep-seeding tolerance in different maize inbred lines and their physiological response to deep-seeding condition, J. Maize Sci. 17 (2009) 9–13.
[41] T. Hartwig, C. Corvalan, N.B. Best, J.S. Budka, J.Y. Zhu, S. Choe, B. Schulz, Propiconazole is a specific and accessible brassinosteroid (BR) biosynthesis inhibitor for arabidopsis and maize, PLoS One 7 (2012).
[42] A. Strigens, N.M. Freitag, X. Gilbert, C. Grieder, C. Riedelsheimer, T.A. Schrag, R. Messmer, A.E. Melchinger, Association mapping for chilling tolerance in elite flint and dent maize inbred lines evaluated in growth chamber and field experiments, Plant Cell Environ. 36 (2013) 1871–1887.
[43] S. Anuradha, S.S.R. Rao, Effect of brassinosteroids on salinity stress induced inhibition of seed germination and seedling growth of rice (Oryza sativa L.), Plant Growth Regul. 33 (2001) 151–153.
[44] X. Li, H. Jiang, F. Liu, J. Cai, T. Dai, W. Cao, D. Jiang, Induction of chilling tolerance in wheat during germination by pre-soaking seed with nitric oxide and gibberellin, Plant Growth Regul. 71 (2013) 31–40.
[45] W. Rademacher, Growth retardants: effects on gibberellin biosynthesis and other metabolic pathways, Annu. Rev. Plant Biol. 51 (2000) 501–531.
[46] E.A. Brenner, M. Blanco, C. Gardner, T. Lubberstedt, Genotypic and phenotypic characterization of isogenic doubled haploid exotic introgression lines in maize, Mol. Breed. 30 (2012) 1001–1016.
[47] B. Kumar, A.H. Abdel-Ghani, J. Reyes-Matamoros, F. Hochholdinger, T. Lübberstedt, Genotypic variation for root architecture traits in seedlings of maize (Zea mays L.) inbred lines, Plant Breed. 131 (2012) 465–478.
[48] J. Pace, C. Gardner, C. Romay, B. Ganapathysubramanian, T. Lübberstedt, Genome-wide association analysis of seedling root development in maize (Zea mays L.), BMC Genomics 16 (2015) 1.
[49] J. Huang, J. Zhang, W. Li, W. Hu, L. Duan, Y. Feng, F. Qiu, B. Yue, Genome-wide association analysis of ten chilling tolerance indices at the germination and seedling stages in maize, J. Integr. Plant Biol. 55 (2013) 735–744.
[50] J.B. Holland, W.E. Nyquist, C.T. Cervantes-Martínez, Estimating and interpreting heritability for plant breeding: an update, Plant Breed. Rev. 22 (2003) 9–112.
[51] H. Wickham, Ggplot2: Elegant Graphics for Data Analysis, Springer Science & Business Media, 2009.
[52] R.J. Elshire, J.C. Glaubitz, Q. Sun, J.A. Poland, K. Kawamoto, E.S. Buckler, S.E. Mitchell, A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species, PLoS One 6 (2011) e19379.
[53] P.J. Bradbury, Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, E.S. Buckler, TASSEL: software for association mapping of complex traits in diverse samples, Bioinformatics 23 (2007) 2633–2635.
[54] A.E. Lipka, F. Tian, Q. Wang, J. Peiffer, M. Li, P.J. Bradbury, M.A. Gore, E.S. Buckler, Z. Zhang, GAPIT: genome association and prediction integrated tool, Bioinformatics 28 (2012) 2397–2399.
[55] J. Yu, G. Pressoir, W.H. Briggs, I.V. Bi, M. Yamasaki, J.F. Doebley, M.D. Mcmullen, B.S. Gaut, D.M. Nielsen, J.B. Holland, A unified mixed-model method for association mapping that accounts for multiple levels of relatedness, Nat. Genet. 38 (2006) 203–208.
[56] X. Liu, M. Huang, B. Fan, E.S. Buckler, Z. Zhang, Iterative usage of fixed and random effect models for powerful and efficient genome-wide association studies, PLoS Genet. 12 (2016) e1005767.
[57] R.C. Johnson, G.W. Nelson, J.L. Troyer, J.A. Lautenberger, B.D. Kessing, C.A. Winkler, S.J. O'Brien, Accounting for multiple comparisons in a genome-wide association study (GWAS), BMC Genomics 11 (2010) 1.
[58] R.S. Sekhon, H. Lin, K.L. Childs, C.N. Hansey, C.R. Buell, N. de Leon, S.M. Kaeppler, Genome-wide atlas of transcription during maize development, Plant J. 66 (2011) 553–563.
[59] Z.-L. Zhang, Z. Xie, X. Zou, J. Casaretto, T.-H.D. Ho, Q.J. Shen, A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells, Plant Physiol. 134 (2004) 1500–1513.
[60] C.M. Andorf, C.J. Lawrence, L.C. Harper, M.L. Schaeffer, D.A. Campbell, T.Z. Sen, The Locus Lookup tool at MaizeGDB: identification of genomic regions in maize by integrating sequence information with physical and genetic maps, Bioinformatics 26 (2010) 434–436.
[61] D. Yamauchi, Hormonal regulation of the expression of cysteine proteinase genes in germinated cotyledons of common bean seeds, Plant Biotechnol. 24 (2007) 231–235.
[62] E.H. Colebrook, S.G. Thomas, A.L. Phillips, P. Hedden, The role of gibberellin signalling in plant responses to abiotic stress, J. Exp. Biol. 217 (2014) 67–75.
[63] M. Ashraf, N. Akram, R. Arteca, M. Foolad, The physiological, biochemical and molecular roles of brassinosteroids and salicylic acid in plant processes and salt tolerance, Crit. Rev. Plant Sci. 29 (2010) 162–190.
[64] F. Özdemir, M. Bor, T. Demiral, İ. Türkan, Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (Oryza sativa L.) under salinity stress, Plant Growth Regul. 42 (2004) 203–211.
[65] S.D. Clouse, Integration of light and brassinosteroid signals in etiolated seedling growth, Trends Plant Sci. 6 (2001) 443–445.
[66] Y. Fuchs, M. Lieberman, Effects of kinetin, IAA, and gibberellin on ethylene production, and their interactions in growth of seedlings, Plant Physiol. 43 (1968) 2029–2036.
[67] M.M. Evans, R.S. Poethig, Gibberellins promote vegetative phase change and reproductive maturity in maize, Plant Physiol. 108 (1995) 475–487.
[68] B.V. Vardhini, S.S.R. Rao, Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum, Plant Growth Regul. 41 (2003) 25–31.
[69] S. Kagale, U.K. Divi, J.E. Krochko, W.A. Keller, P. Krishna, Brassinosteroid confers tolerance in Arabidopsis thaliana and Brassica napus to a range of abiotic stresses, Planta 225 (2007) 353–364.
[70] I. Singh, U. Kumar, S. Singh, C. Gupta, M. Singh, S. Kushwaha, Physiological and biochemical effect of 24-epibrassinoslide on cold tolerance in maize seedlings, Physiol. Mol. Biol. Plants 18 (2012) 229–236.
[71] C. Wang, A. Yang, H. Yin, J. Zhang, Influence of water stress on endogenous hormone contents and cell damage of maize seedlings, J. Integr. Plant Biol. 50 (2008) 427.
[72] A.M. Krill, M. Kirst, L.V. Kochian, E.S. Buckler, O.A. Hoekenga, Association and linkage analysis of aluminum tolerance genes in maize, PLoS One 5 (2010) e9958.
[73] H. Cai, F. Chen, G. Mi, F. Zhang, H.P. Maurer, W. Liu, J.C. Reif, L. Yuan, Mapping QTLs for root system architecture of maize (Zea mays L.) in the field at different developmental stages, Theor. Appl. Genet. 125 (2012) 1313–1324.
[74] N.B. Best, T. Hartwig, J. Budka, S. Fujioka, G.G.S. Johal, B. Schulz, B.P. Dilkes, nana plant2 encodes a maize ortholog of the Arabidopsis brassinosteroid biosynthesis protein Dwarf1, identifying developmental interactions between brassinosteroids and gibberellins, Plant Physiol. 171 (4) (2016) 2633–2647 (00399.02016).
[75] G. Edmeades, J. Bolanos, A. Elings, J.-M. Ribaut, M. Bänziger, M. Westgate, The role and regulation of the anthesis-silking interval in maize, Physiol. Model. Kernel Set Maize (2000) 43–73.
[76] A.E. Lipka, M.A. Gore, M. Magallanes-Lundback, A. Mesberg, H. Lin, T. Tiede, C. Chen, C.R. Buell, E.S. Buckler, T. Rocheford, Genome-wide association study and pathway-level analysis of tocochromanol levels in maize grain, G3: Genes| Genomes| Genetics 3 (2013) 1287–1299.
[77] J. Weng, C. Xie, Z. Hao, J. Wang, C. Liu, M. Li, D. Zhang, L. Bai, S. Zhang, X. Li, Genome-wide association study identifies candidate genes that affect plant height in Chinese elite maize (Zea mays L.) inbred lines, PLoS One 6 (2011) e29229.
[78] E.S. Buckler, J.B. Holland, P.J. Bradbury, C.B. Acharya, P.J. Brown, C. Browne, E. Ersoz, S. Flint-Garcia, A. Garcia, J.C. Glaubitz, M.M. Goodman, C. Harjes, K. Guill, D.E. Kroon, S. Larsson, N.K. Lepak, H.H. Li, S.E. Mitchell, G. Pressoir, J.A. Peiffer, M.O. Rosas, T.R. Rocheford, M.C. Romay, S. Romero, S. Salvo,

H.S. Villeda, H.S. da Silva, Q. Sun, F. Tian, N. Upadyayula, D. Ware, H. Yates, J.M. Yu, Z.W. Zhang, S. Kresovich, M.D. McMullen, The genetic architecture of maize flowering time, Science 325 (2009) 714–718.
[79] J. Yan, M. Warburton, J. Crouch, Association mapping for enhancing maize (L.) genetic improvement, Crop Sci. 51 (2011) 433–449.
[80] Y. Xue, M.L. Warburton, M. Sawkins, X. Zhang, T. Setter, Y. Xu, P. Grudloyma, J. Gethi, J.-M. Ribaut, W. Li, Genome-wide association analysis for nine agronomic traits in maize under well-watered and water-stressed conditions, Theor. Appl. Genet. 126 (2013) 2587–2596.
[81] C.N. Hansey, B. Vaillancourt, R.S. Sekhon, N. De Leon, S.M. Kaeppler, C.R. Buell, Maize (Zea mays L.) genome diversity as revealed by RNA-sequencing, PLoS One 7 (2012) e33071.
[82] M.K. Monaco, T.Z. Sen, P.D. Dharmawardhana, L. Ren, M. Schaeffer, S. Naithani, V. Amarasinghe, J. Thomason, L. Harper, J. Gardiner, Maize metabolic network construction and transcriptome analysis, Plant Genome 6 (2013).
[83] L. Wang, W. Zhu, L. Fang, X. Sun, L. Su, Z. Liang, N. Wang, J.P. Londo, S. Li, H. Xin, Genome-wide identification of WRKY family genes and their response to cold stress in Vitis vinifera, BMC Plant Biol. 14 (2014) 1.
[84] L. Zhang, L. Gu, P. Ringler, S. Smith, P.J. Rushton, Q.J. Shen, Three WRKY transcription factors additively repress abscisic acid and gibberellin signaling in aleurone cells, Plant Sci. 236 (2015) 214–222.
[85] Z. Xie, Z.L. Zhang, X. Zou, G. Yang, S. Komatsu, Q.J. Shen, Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells, Plant J. 46 (2006) 231–242.
[86] Y. Ding, J. Sheng, S. Li, Y. Nie, J. Zhao, Z. Zhu, Z. Wang, X. Tang, The role of gibberellins in the mitigation of chilling injury in cherry tomato (Solanum lycopersicum L.) fruit, Postharvest Biol. Technol. 101 (2015) 88–95.
[87] J. Wang, B. Hou, Glycosyltransferases: key players involved in the modification of plant secondary metabolites, Front. Biol. China 4 (2009) 39–46.
[88] B. Poppenberger, S. Fujioka, K. Soeno, G.L. George, F.E. Vaistij, S. Hiranuma, H. Seto, S. Takatsuto, G. Adam, S. Yoshida, The UGT73C5 of Arabidopsis thaliana glucosylates brassinosteroids, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 15253–15258.