

Marker-assisted pyramiding of γ-tocopherol methyltransferase and glutamate formiminotransferase genes for development of biofortified sweet corn hybrids

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Micronutrients, including vitamins, minerals, and other bioactive compounds, have tremendous impacts on human health. Much progress has been made in improving micronutrient content of inbred lines in various crops through biofortified breeding. However, biofortified breeding still fall short for rapid generation of high-yielding hybrids rich in multiple micronutrients. Here, we bred multi-biofortified sweet corn hybrids efficiently through marker-assisted selection. Screening by molecular marker for vitamin E and folic acid, we obtained 15 inbred lines carrying favorable alleles (6 for vitamin E, 9 for folic acid, and 3 for both). Together with the genetic diversity analysis, multiple biofortified corn hybrids were developed through crossing.

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Abstract

- Micronutrients, including vitamins, minerals, and other bioactive compounds, have tremendous 27 impacts on human health. Much progress has been made in improving micronutrient content of 28 inbred lines in various crops through biofortified breeding. However, biofortified breeding still fall 29 short for rapid generation of high-yielding hybrids rich in multiple micronutrients. Here, we bred 30 multi-biofortified sweet corn hybrids efficiently through marker-assisted selection. Screening by 31 molecular marker for vitamin E and folic acid, we obtained 15 inbred lines carrying favorable 32 33 alleles (6 for vitamin E, 9 for folic acid, and 3 for both). Together with the genetic diversity analysis, multiple biofortified corn hybrids were developed through crossing. 34
- 35 Key words: Biofortification, Hybrids, Molecular Breeding, Sweet Corn

Introduction

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- Micronutrients (vitamins and minerals) are essential to people's health (Farré et al. 2014). At present, billions of people (mainly in developing countries) still suffer from "hidden hunger" due
- 39 to insufficient intake of micronutrients (Muthayya et al. 2013). In the past 20 years,
- 40 biofortification, enhancement of the levels of micronutrients in food crops through agricultural
- 41 technologies, has been used as an important strategy to produce "healthier food" (Garg et al. 2018;
- 42 Saltzman et al. 2017).
- Sweet corn (Zea mays L. var. saccharata), a type of maize with high level of sugar, is an
- 44 invaluable source of protein, calories, essential fatty acids, vitamins, and minerals for human



45 nutrition (Wu et al. 2020). However, the varied contents of micronutrients present in the different sweet corn varieties were significant. The wide variability for micronutrients content in sweet corn 46 unveils the great prospect of developing biofortified sweet corn varieties. Many quantitative trait 47 loci (QTL) associated with micronutrients content have been identified (Baseggio et al. 2019; 48 Baseggio et al. 2020; Diepenbrock et al. 2017; Hershberger et al. 2021; Lone et al. 2021; Simic et 49 50 al. 2012; Wu et al. 2022). For example, ZmVTE4, encoding γ -tocopherol methyltransferase, is capable of catalyzing γ -tocopherol to α -tocopherol, the major constituent of vitamin 51 E, shows the highest vitamin E activity (Burton & Ingold 1981; Kamal-Eldin & Appelqvist 1996). 52 53 Two insertions in ZmVTE4 promoter region and 5' untranslated region (5' UTR) affect the level of α-tocopherol through regulating gene expression (Li et al. 2012). Molecular markers (InDel7 54 and InDel118) corresponding to the two insertions were developed to screen favorable allele. 55 ZmCTM (catalysis of 5-M-THF to MeFox) functions as a key enzyme to convert 5-methyl-56 tetrahydrofolate (5-M-THF) to a pyrazino-s-triazine derivative of 4α-hydroxy-5-methyl-57 tetrahydrofolate (MeFox) in folate metabolism. MeFox is the stable storage form of folic acid in 58 seeds (Goyer et al. 2005). The natural asparagine-to-glycine substitution caused by an A-to-G 59 single nucleotide variation in ZmCTM coding region enhances its enzymatic activity (Zhang et al. 60 61 2016). G-allele can be identified by marker SNP682. Commercial seeds of sweet corn are mostly F₁ hybrids, which are phenotypically superior 62 and with significantly higher yield compared to their parents. Traditional corn breeding based on 63 64 genetic crosses requires identifying the best parental combinations for creating elite hybrids. This process is very laborious, time-consuming, and cost ineffective. Moreover, the results are usually 65 unpredictable and not always accurate. The level of genetic diversity between two parents has been 66 67 proposed as a possible predictor of F₁ performance in crops (Yousuf et al. 2021). Accurate



characterization of the genetic background of inbred lines can be very useful in selecting inbred lines for crossing (Beckett et al. 2017). The genetic variability can be assessed using agromorphological traits, which may result in misleading estimates due to higher influence of environment on them. With the development of functional genomics and genome sequencing, marker-assisted selection has become an important approach for current crop improvement (Nie et al. 2014). Previous study established a core set of SSR molecular marker for characterizing genetic diversity of Chinese maize varieties and establishing the identity of new varieties (Wang et al. 2011).

Impressive progress has been made in biofortification of different elite crop inbred lines (Prasanna et al. 2019). There is an increasing demand for hybrid lines in practical production. Based on these requirements, we wondered whether genetic diversity together with favorable allele for vitamin E and/or folic acid could be analyzed to develop multi-biofortified sweet corns hybrids. Here, we obtained 15 inbred lines carrying favorable alleles through screening by molecular marker for vitamin E and folic acid (Li et al. 2012; Zhang et al. 2016). Together with the genetic diversity analysis (Wang et al. 2011), multiple biofortified corn hybrids were developed through crossing. This approach should greatly accelerate future biofortified breeding of sweet corn hybrids via effective selection of elite inbred lines with biofortification traits suit for optimal combination.

Materials and methods

Plant material

A set of 52 sweet corn inbred lines procured from different sources and maintained through selfing

90 were taken for the study (Supplemental Table S1). All these inbreds were planted in randomized



- 91 block design with two replications at the farmland of Zhejiang Academy of Agricultural Sciences
- 92 (Dongyang, China) during 2020 and 2021.

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- Genetic diversity analysis and allele screening
- 95 Genomic DNA was extracted using a modified CTAB extraction protocol (Clarke et al. 1989).
- 96 The core 40 SSR primers were used for genetic diversity analysis (Supplemental Table S2) (Wang
- et al. 2011). PCR amplifications were performed with a final reaction volume of 20 µL containing
- 98 30~40 ng genomic DNA. The PCR conditions were: 94 °C for 2 min, followed by 35 cycles of
- 99 denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec, and
- a last extension step at 72 °C for 10 minutes. The amplified products were resolved using 1.5%
- agarose gel or 12% PAGE (polyacrylamide gel electrophoresis) gel. Calculation of the PIC
- 102 (polymorphism information content value) was based on the results obtained from SSR using the
- following formula: PIC= $1-\Sigma fi^2$, where fi^2 is the frequency of the allele. Dendrogram were created
- using UPGMA (Unweight Pair Group Method Using Arithmetic Averages) feature of NTSYS-pc
- software Version 2.2. InDel7 and InDel118 were used for ZmVTE4 allele screening (Li et al. 2012),
- 106 SNP682 was used for ZmCTM allele screening (Table 1) (Zhang et al. 2016).

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Quantification of free α-tocopherol

- 109 The endogenous free α-tocopherol contents were determined by Wuhan Greensword Creation
- 110 Technology Co. Ltd., (http://www.greenswordcreation.com/index.html) based on UHPLC-
- 111 MS/MS analysis. In brief, sample were frozen in liquid nitrogen, ground to fine powder, and
- extracted with 1.0 mL n-hexane at -20 °C for 12 h. After centrifugation (10,000 g, 4 °C, 20 min),
- the supernatants were collected and evaporated under mild nitrogen stream at 35 °C followed by



re-dissolving in 100 μL ACN for UHPLC-MS/MS analysis (Thermo Scientific Ultimate 3000 UHPLC coupled with TSQ Quantiva).

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Quantification of free folic acid

The endogenous free folic acid contents were determined by Wuhan Greensword Creation Technology Co. Ltd., (http://www.greenswordcreation.com/index.html) based on UHPLC-MS/MS analysis. In brief, sample were frozen in liquid nitrogen, ground to fine powder, and extracted with 1.0 mL 80% methanol aqueous solution at -20 °C for 12 h. After centrifugation (10,000 g, 4 °C, 20 min), the supernatants were collected and evaporated under mild nitrogen stream at 35 °C followed by re-dissolving in 100 μL 50% ACN for UHPLC-MS/MS analysis (Thermo Scientific Ultimate 3000 UHPLC coupled with TSQ Quantiva).

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Results and discussion

To test the feasibility of the strategy, we analyzed the genetic diversity of widely used 52 sweet 127 corn inbred lines using 40 pairs of SSR core markers (Wang et al. 2011). These markers produced 128 226 alleles, an average of 5.7 alleles per marker, suggesting a high frequency of allelic variation. 129 130 The value of polymorphism information content (PIC) for each SSR locus varied between 0.27 and 0.87 with an average of 0.60. Based on the classification of PIC (PIC value < 0.25, low; 131 132 0.25 < PIC value < 0.5, intermediate; and PIC value > 0.5, high polymorphism) (Botstein et al. 133 1980), all the 40 SSR makers were found with moderate polymorphism and heterozygosity. The results suggested that these 40 SSR markers are suitable for assessing genetic diversity of sweet 134 135 corn resources.

The dendrogram was obtained from the similarity coefficient and clustering was done by



using the UPGMA algorithm with the NTSYS software program. The 52 inbred lines were divided into six distinct groups at the similarity coefficient level of 0.55 (Fig. 1). The first group accounted for 67.31% (35 inbred lines), the other groups were only for 32% (17 inbred lines). These results indicated that most of the inbred lines have similar genetic background.

Furtherly, we analyzed the favorable alleles associated with vitamin E and folic acid content in 52 sweet corn inbred lines. ZmVTE4 and ZmCTM were identified to regulate biosynthesis of free α -tocopherol and folic acid content, respectively (Fig. 2A). Molecular markers (InDel7 and InDel118) corresponding to the two insertions in *ZmVTE4* promoter region and 5' untranslated region (5' UTR) were used to screen favorable allele for free α -tocopherol. Marker SNP682 in *ZmCTM* coding region was used to characterize alleles for free folic acid. Genotypic screening showed that there was 11.54% (n=6) lines with deletion-allele in InDel7 and InDel118 loci, 17.31% (n=9) lines with G-allele in SNP682 and 5.77% (n=3) lines with both deletion-allele and G-allele (Fig. 2D). Our results revealed that most of the elite inbred lines used in breeding do not contain favorable alleles associated with vitamin E and folic acid content.

To develop hybrids with high level of vitamin E and folic acid, we chose inbred lines with micronutrients associated favorable alleles for crossing. Previous studies have suggested that the level of genetic diversity between two parents could be used as a possible predictor of F_1 performance in crops (Xiao et al. 1996). Among the inbred lines carrying favorable alleles associated with vitamin E and folic acid content, lines with different genetic distance were selected to cross as parents. F_1 progenies from lines crosses with a large genetic distance (140×225, 142×225, 140×15, and 142×15) were observed with favorable agronomic traits (ear length, number of rows per ear, grain yield per main panicle, and 1,000-grain weight) (Fig. 3A-3C, Table 2). Notably, the highest yield per plant (272.58 g) was hybrid 140×225. In contrast, F_1 progenies of



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lines from same group had poor agronomic traits (Fig. 3A-3C, Table 2). The same trend can be found for other hybridization combination.

Meanwhile, we measured free α -tocopherol and folic acid in F_1 progenies. Based on the allele analysis, we found that hybrid 140×15 and hybrid 140×225 contains α-tocopherol favorable allele (InDel7^{+/-}InDel118^{+/-} for hybrid 140×15 and InDel7^{-/-}InDel118^{-/-} for hybrid 140×225). Insertion in InDel7 and InDel118 loci affect the expression of ZmVTE4 (Li et al. 2012). Quantification of free α-tocopherol (main component of vitamin E) revealed that the concentration in hybrid 140×15 and hybrid 140×225 was lower than that in hybrid 20×15 carrying no α-tocopherol favorable allele (InDel7^{+/+}InDel118^{+/+})(Fig. 4). A similar variation pattern was observed for free folic acid in sweet corn kernel. The asparagine-to-glycine substitution caused by an A-to-G single nucleotide variation (SNP682) in maize ZmCTM enhances its enzymatic activity (Zhang et al. 2016). Homozygous G (SNP682^{G/G}) carrying hybrid 140×15 and 20×39 had significantly higher levels of free folic acid than heterozygous G/A (SNP682^{G/A}) carrying hybrid 140×15 in kernel (Fig. 4). In addition, there are differences between hybrid 140×15 and 20×39. Folates are unstable compounds, susceptible to oxidative and photo-oxidative catabolism (Blancquaert et al. 2014). Vitamin E is a potent antioxidant in plants, widely used to increase the shelf life of β -carotene in foods (Choe & Min 2009). High level of α-tocopherol in Hybrid 140×15 may enhance folate stability. Our results demonstrated the validity of the strategy and provided supporting evidence for the notion that ZmVTE4 (Li et al. 2012) and ZmCTM (Zhang et al. 2016) are key for the regulation of vitamin E and folic acid level in maize kernel.

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Conclusion

It is known that molecular marker-assisted selection is used in crop breeding (Jena & Mackill



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2008). Given that most commercial seeds are hybrids, we envisage that the strategy used here will be widely adopted to accelerate biofortification breeding of various crops. The strategy allows for biofortification in elite F₁ hybrid with much higher efficiency and accuracy. A further improvement of this strategy could be achieved by integrating morphological traits assay to characterize genetic structure of parent lines comprehensively (Mahato et al. 2018). Further, the development of new polymorphic detection technologies such as KASP (Semagn et al. 2014), and whole-genome resequencing (Jiao et al. 2012; Mace et al. 2013) would also greatly expand the utility of this strategy. The strategy described here hold great promise to future biofortification breeding.

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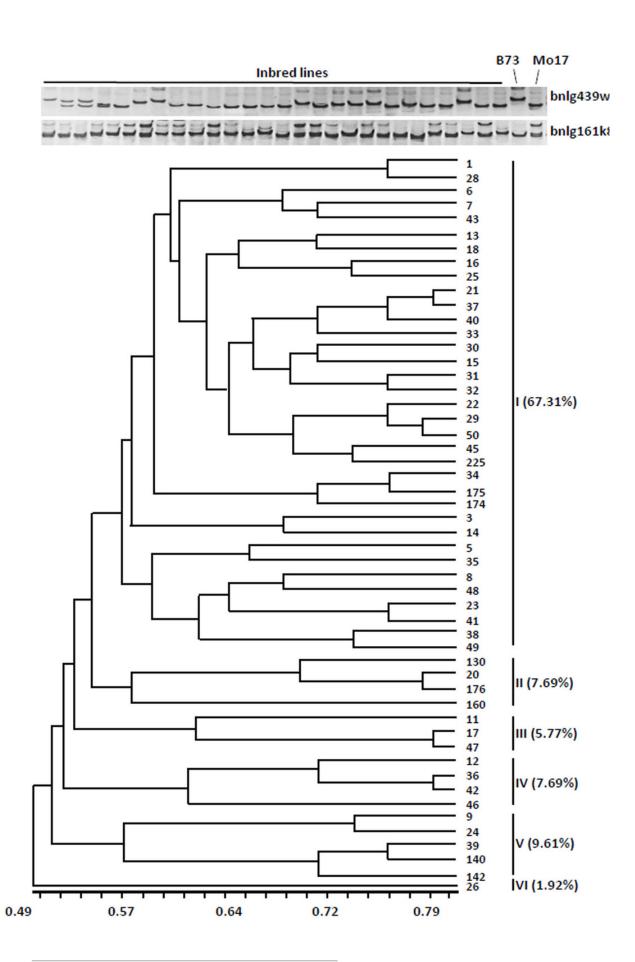


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Cluster dendrogram depicting genetic divergence among 52 inbreds based on 40 core molecular markers.

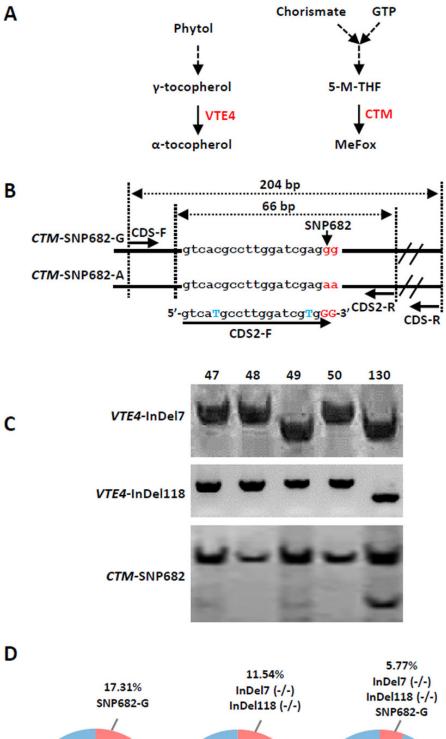
(A) Microsatellite polymorphism among sweet corn inbreds. (B) Cluster dendrogram depicting genetic divergence among 52 inbreds based on 40 core molecular markers.

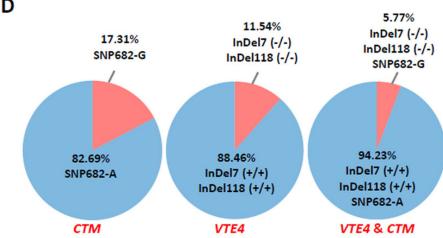




Screening of favorable alleles for vitamin E and/or folic acid in the 52 inbred lines.

(A) Scheme of α-tocopherol and folate metabolism. VTE4, γ-tocopherol methyltransferase; 5-M-THF, 5-methyl-tetrahydrofolate; MeFox, a pyrazino-s-triazine derivative of 4α-hydroxy-5-methyl-tetrahydrofolate; CTM, catalysis from 5-M-THF to MeFox. (B) Schematic illustration of SNP682 loci primer design, blue upper-case letters represent bases substituted to balance primer GC content of primer. (C) Representative pictures of allele assay at InDel7, InDel118, and SNP682 loci among 52 inbreds. InDel7 (+/+), homozygous 7-bp insertion in the 5' untranslated region (5' UTR) of *ZmVTE4*; InDel7 (-/-), homozygous 0-bp insertion in the 5' untranslated region (5' UTR) of *ZmVTE4*; InDel118 (+/+), homozygous 118-bp insertion in the promoter region of *ZmVTE4*; InDel118 (-/-), homozygous 0-bp insertion in the promoter region of *ZmVTE4*; SNP682-G, homozygous G at position 682 in the coding sequence of *ZmCTM*; SNP682-A, homozygous A at position 682 in the coding sequence of *ZmCTM*.

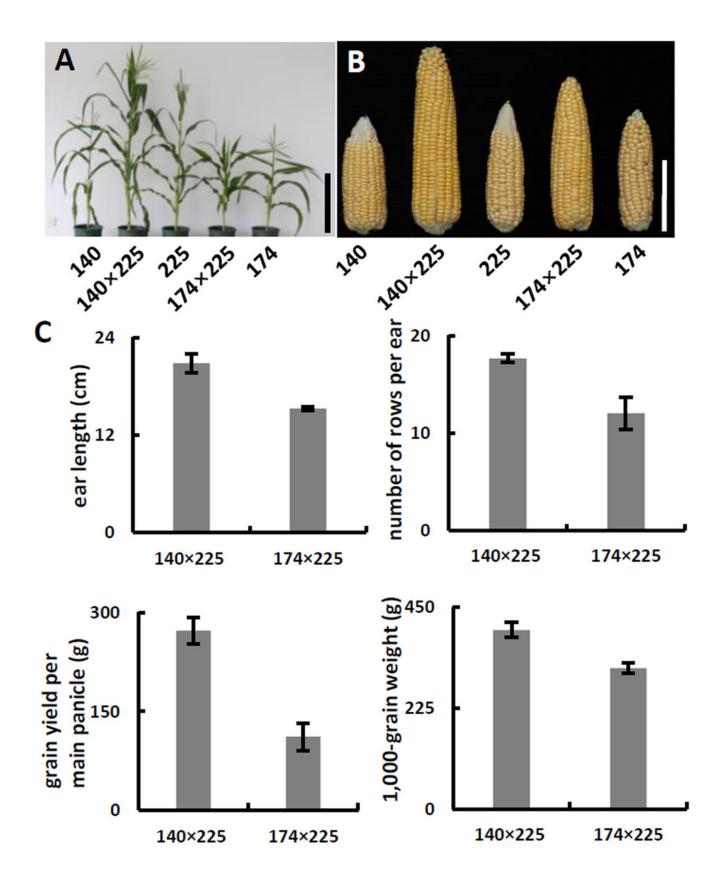






Phenotypes and agronomic traits of parental inbreds and hybrids.

(A) Plant phenotype of parental inbreds and hybrids. Bar, 30cm. (B) Phenotype of parental inbreds and hybrids on ears. Bars, 10cm. (C) Analysis of agronomic traits hybrid 140×225 and hybrid 174×225 .





Quantification of free α -tocopherol and folic acid in kernel of hybrids.



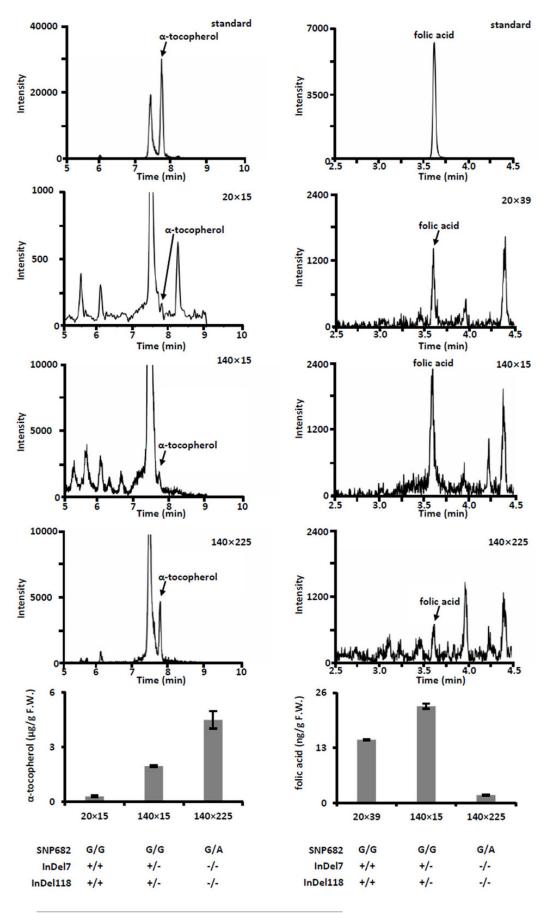




Table 1(on next page)

Primers for InDel7, InDel118, and SNP682 used in this study.

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Gene	Polymorphic site	Prime direction	Primer sequences (5'-3')	
ZmCTM	ZmCTM-CDS	Forward	TACGACGGTGGGTGTCAC	
		Reward	TGATAGGCGCTGGCATGATC	
	ZmCTM-CDS2	Forward	GTCATGCCTTGGATCGTGGG	
		Reward	ATGACGTCCTTACACAGCAC	
ZmVTE4	ZmVTE4-InDel7	Forward	TGCCGGCACCTCTACTTTAT	
		Reward	AGGACTGGGAGCAATGGAG	
ZmVTE4	ZmVTE4-InDel118	Forward	AAAGCACTTACATCATGGGAAAC	
		Reward	TTGGTGTAGCTCCGATTTGG	

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Table 2(on next page)

Characterization of agronomic traits of hybrids.

Note, different letters show significant differences among treatment combinations at 5% probability level using Duncan's multiple range test.

Hybrid F1	Growth phases (days)	Plant height (m)	Ear length (cm)	Number of rows per ear	1,00-grain weight (g)	Grain yield per main panicle (g)	Sucrose content (mg/g)
15×20	86	1.75±4.24°	16.17±0.97gh	15±2.16 ^{abc}	47.27±3.96a	203.77±25.1 ^{bcd}	172.39±17.72 ^b
15×28	90	2.38 ± 0.02^{a}	19.77±0.33bc	15±0.82abc	34.31 ± 1.85^{de}	245.79±7.49 ^{cd}	148.59±13.72bcde
20×39	86	1.65±1.25 ^{cd}	18.23 ± 0.63^{def}	16.67 ± 2.49^{ab}	48.59±2.24 ^a	231.24±36.95abc	118.53±24.05
140×15	92	2.32 ± 0.02^{ab}	20.73 ± 0.45^{ab}	15.67±0.47 ^{abc}	45.04 ± 2.85^{a}	263.29±24.72a	126.18 ± 0.97^{ef}
142×15	92	2.28 ± 0.09^{ab}	18.97±0.92 ^{cde}	17.33±1.25ab	35.22 ± 0.3^{cde}	199.14±9.95bcd	140.8 ± 7.82^{def}
140×142	89	2.36±0.06a	17.5 ± 0.82^{efg}	14±2.83abc	36.11 ± 1.06^{bcd}	158.08±13.49 ^{de}	144±15.49 ^{cde}
140×174	88	2.28 ± 0.07^{ab}	17.93 ± 0.19^{def}	14.67 ± 0.47^{abc}	33.23 ± 0.74^{de}	159.11±5.27 ^{de}	173.94±4.33 ^b
174×175	87	1.69 ± 0.03^{cd}	17.4 ± 0.38^{fg}	15.33±0.47 ^{abc}	34.31 ± 0.85^{de}	172±10.71 ^d	172.55±3.51 ^b
142×175	91	2.22±0.04b	19.37 ± 0.7^{bcd}	18.67±0.47a	33.36±1.58de	232.2±11.52 ^{abc}	157.06±4.85bcd
39×225	89	2.34±0.07a	21.77±0.39a	17±2.16ab	38.92±0.47bc	261.75±20.23a	159.05±7.84bcd
140×225	89	2.31±0.01ab	20.83 ± 1.19^{ab}	17.67±0.47ab	39.92±1.68b	272.58±20.23a	167.1±5.19bc
142×225	90	2.28 ± 0.04^{ab}	21.4±0.43a	16.67±1.7ab	36.55 ± 1.3^{bcd}	237.5 ± 41.05^{ab}	163.12±2.65 ^{bcd}
174×225	92	1.59±0.03d	15.23±0.21 ^h	12±1.63°	31.41±1.23e	111.29±20.79 ^e	205.52±3.79 ^a