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Grain quality evaluations of hybrids between *Triticum aestivum* and *Sorghum bicolor* produced by pollen tube pathway method

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Abstract

Distant hybridization makes it possible to transfer the genome of one species to another, and the pollen tube pathway method has been widely used based on the hypothesis that such distant hybridization process provided the opportunity of the recombination of DNA segment. In this study, we carried out a distant hybridization between *Triticum aestivum* and *Sorghum bicolor* via pollen tube pathway method, and five new wheat lines including 9122 (*S. bicolor* 2D + *T. aestivum* L13), 9141 (*S. bicolor* 5D + *T. aestivum* L10), 9144 (*S. bicolor* 2D + *T. aestivum* G8), 9145 (*S. bicolor* 5D + *T. aestivum* G8) and 0154 (*S. bicolor* 10D + *T. aestivum* G8) were selected to determine the grain quality characteristics of these hybrids. Therefore, the properties of their flour mixogram and high molecular weight subunit of hybrids were analyzed. The variations of mixogram peak time, mixogram peak height, mixogram height in the seventh minute and sedimentation value in the five hybrids were observed, while mixogram width at the seventh minute of all the hybrids was improved. The mutation of high molecular weight subunits of glutenin happened including the composition pattern and content of HMW-GS. In hybrid 9144, the high molecular weight glutenin subunits 5+10 were presented instead of subunits 2+12 of its maternal parent G8. The results indicated that distant hybridization between *T. aestivum* and *S. bicolor* via pollen tube pathway method improved the wheat grain quality, which will provide more opportunities for the selection of new wheat cultivar with improving grain quality.

Keywords: *Triticum aestivum*, *Sorghum bicolor*, pollen tube pathway, mixograph, high molecular weight subunit, grain quality.
Abbreviations: HMW-GS- high molecular weight glutenin subunits; MHSM- mixogram height at the seventh minute; MPH- mixogram height; MPT- mixogram peak time; MWSM- mixogram width at the seventh minute.

Introduction

Wheat (*Triticum aestivum*, 2n=6X=42) is one of the most important food crops in the world (Shewry, 2009). However, in comparison with other major cereals like rice and maize, the development of high throughput wheat transformation systems has been slowed and severely affected by genotype effects on plant regeneration, low transformation efficiencies and problems with transgene inheritance and stability of expression (Sahrawat et al., 2003). Therefore, the adoption range and efficiency of specific gene(s) transformation into wheat cultivars are still limited (Jones, 2005; Wang et al., 2007). Moreover, it has been demonstrated that many important agronomic traits are controlled by multi-genes, and it is not easy to improve abiotic stress tolerance or grain quality of wheat by single gene transformation (Hu et al., 2003; Bhalla et al., 2006). Up to date, with the development of the normal hybridization breeding methods, genetic background of wheat breeding materials is becoming more monotonically than ever (Fu and Somers, 2009). Distant hybridization is commonly employed in breeding cereal crops of introgression desirable genes from wild to cultivated taxa, and much more attention has been paid on exogenous DNA of other distant species, which might be transferred into some wheat species to increase the yield and improve the quality and wide adaptation (Gandhi et al., 2006; Hegde and Waines, 2004; Trubacheeva et al., 2009; Reynolds et al., 2011). To overcome the disturbance of distant hybridization, a method for transferring foreign DNA into recipient known as the pollen-tube pathway, was first reported for cotton transformation by Zhou et al. (1983). Then, the introduction of exogenous DNA into cotton via the pollen-tube pathway with GFP as a reporter provided direct and convincing facts in cytology and molecular biology for this method (Huang et al., 1999). Since pollen tube pathway method could overcome the disturbance of distant hybridization by deliver foreign DNA into embryo sac using pollen tubes directly and the steps of plant tissue culture were avoided, so the way of gene introduction would be popularized easily. Now, being an efficient transformation technique, the pollen-tube pathway method as a new gene transfer manner has been proved effective in cotton (Chen et al., 2010), rice (Luo and Wu, 1988), soybean (Yang et al., 2011), maize (Yang et al., 2009) and wheat (Zale et al., 2009). Both of the flow of domesticated alleles into the populations of their wild relatives and the gene flow in the reverse direction (into the crop) may have important evolutionary consequences on crop taxa (Ellstrand et al., 1999). How about the gene flow among the world's most important food crops by wide cross? Although traditional wheat breeding has been improved the production and quality continually, it is limited by the gene pool required for developing varieties for stress tolerance and

grain value-added characteristics. Sorghum (*Sorghum bicolor* L.) is one of the most important food and feed crops in the arid and semi-arid regions of the world for its excellent drought and salt tolerance as well as other desired agronomic traits (Kassahun et al., 2010). On the basis of this, we have selected *Sorghum bicolor* (2n=20) varieties 2D, 5D and 10D as donor to transfer its genomic DNA into spring wheat cultivars L13, L10 and G8 via pollen tube pathway method. The genetic variations of wheat grain quality of these distant hybrids were analyzed, by which the opportunity for the selection of new wheat cultivars with improved grain qualities have been discussed.

Results

Morphological characters and agronomic traits

There are great variations of morphological characters among individual plants in earlier generations (F1-F4), including plant height, date of heading, panicle shape, panicle length, number of seeds per panicle, seed color and 1000-seed weight (Fig. 1, Table 1). All of these characters segregated to a considerable degree and were heterogeneous for some and homogeneous for others. Up to F8 generation, the segregation of characters occurred continuously, but the genetic stability of spike length and grain plumpness progressed quicker than other agronomic characters (P<0.05). Agronomic traits of distant hybrids 9122 (*S. bicolor* 2D + L13), 9141 (*S. bicolor* 5D + L10), 9144 (S. bicolor 2D + G8), 9145 (*S. bicolor* 5D + G8) and 0154 (*S. bicolor* 10D + G8) have been evaluated under various environmental conditions. The yield of hybrid 9122 was 20-22% higher than its maternal parent L13 in district test (P<0.05, data not shown).

Changes of dough mixing properties

There were great variations in the mixogram of the wheat-sorghum hybrids. Compared with its maternal parent L13, which was feebleness-muscle-powder and MPT (Mixogram Peak Time) was 1.7 min, the mixogram values of hybrid 9122 changed to be mid-muscle-powder and MPT was 2.2 min (P<0.05). However, in the case of 9144 and 9145, which were hybrids of G8 with mid-muscle-powder and MPT 2.2 min, they changed to be feebleness-muscle-powder and MPT of them were 1.0 min and 1.7 min, respectively. The Mixogram peak height (MPH) of 9141 (8.1 cm) was much higher than that of its receptor L10 (6.6 cm) (P<0.05). The mixogram height (MHSM) at seventh minute of hybrids of L10 and L13 were all higher than those of their receptors (P<0.05), but hybrids of G8 showed opposite changes. And the mixogram width at the seventh minute (MWSM) of hybrids was all improved compared to their maternal parents (P<0.05) (Table 2).

Mutations of high-molecular-weight glutenin subunits (HMW-GS)

The results of the storage protein showed that mutation of high molecular subunits of glutenin (HMW-GS) appeared in the hybrid 9144. Compared with its receptor G8, the high molecular weight subunits 5+10 were presented in 9144 instead of subunits 2+12 in G8 (Fig. 2). There were no changes on the patterns of HMW-GS in other hybrids, but the relative contents of HMW-GS were different from their recipients. Both of the patterns and content of HMW-GS affect wheat-baking quality directly.

Discussion

S. bicolor gene introgression to wheat through pollen tube pathway

The research for advantageous genes to improve the nutritional quality and increase the total yield potential of cereals and other crops has led to introgressive and distant hybridization of many cultivars with their wild relatives (Benavente et al., 2008). It has been reported that a certain part of DNA or certain individual genes may show more or less affinity, thus rendering them capable of recombination. In process of pollen tube extending, some cells of nucelli began degenerate and became pollen tube pathway through which pollen tube could enter embryo sac by nucelli (Zhou et al., 1983). The pathway was larger than pollen tube, and then between pollen tube formation and closing, heterologous DNA could enter embryo sac and integrate into the zygote cell and the forepart embryo cells (Zhou et al., 1983). According to the hypothesis above, the normal distant hybridization process had the opportunity of the hybridization of DNA segment, and a new plant transformation method was put forward, which could richen the crops genetic resources by introduction sorghum DNA into wheat. The various biological variations have been observed in hybrids of wheat (Zhang et al., 2005) and soybean (Shou et al., 2002; Yang et al., 2011) by transferring exogenous DNA via pollen tube pathway method. In this study, the progenies of *S. bicolor* and *T. aestivum* also exhibited great variations including desirable and undesirable biological traits, and such diversity did not occur only by chance and it was seen repeatedly in other combinations. The repetition of genetic variation such as albino seedling indicated that there maybe become hotspots in the combination of exogenous DNA into recipient chromosome. It has been believed that the great diversity of the morphological characteristics in the hybrid progenies is a specific of the distant hybridization of wheat and sorghum, just the same of the genetic variations reported in the hybrids of rice and sorghum (Zu et al., 1985). On the other hand, most of the progenies of such distant hybridization appeared the maternal characteristics but few had the paternal traits, such as spike shape. The results suggested that exogenous DNA had affected the gene expression of the donor through combined into its genome. Usually it costs 5-6 years to select wheat lines with genetic stability, which implies that the combination maybe only happen between some DNA fragments instead of whole genome of *S. bicolor* (Zhou et al., 1983).

Effects on grain quality of hybrids between T. aestivum and S. bicolor

Improving grain quality is an important aim for wheat breeders. To our knowledge, there have been no reports on the variations of dough stickiness in wheat hybrids via pollen tube pathway method. The Mixograph records the increase in stress as dough was mixed to its maximum resistance and the subsequent decrease in stress on over-mixing. In this study, the variations of MPT, MPH and MWSM suggested that transformation of *S. bicolor* did affect mixing properties of five hybrids (Table 2). Since dough stickiness has been attributed mainly to increased water absorption associated with higher level of soluble proteins, so the higher water absorption levels indicated higher quality wheat flour proteins (Bordes et al., 2008). Dough consistency, as measured by the height of the Mixogram, is correlated with grain hardness. And the curve peak was the highest point

Table 1. Comparisons of agronomical characteristics of five distant hybrids and their maternal parents.

Parents/Hybrids	Plant height (cm)	No. of panicle /plant	Growth duration (d)	1000-seed weight (g)
L13	84.1±1.23[a]	21±0.26[a]	97±1.26[a]	39.8±0.27[a]
9122	89.5±1.15[a]	22±0.11[a]	98±1.67[a]	42.6±0.79[b]
L10	83.9±1.46[a]	20±0.22[a]	95±1.32[a]	42.6±0.54[b]
9141	102.2±2.63[b]	20±0.19[a]	96±1.87[a]	49.6±0.76[c]
G8	107.2±2.21[b]	22±0.33[a]	96±2.52[a]	39.8±0.45[a]
9144	103.2±2.89[b]	17±0.26[b]	111±2.34[b]	34.7±0.21[d]
9145	115.5±1.68[c]	17±0.15[b]	109±1.28[b]	52.9±0.43[e]
0154	86.8±1.20[a]	19±0.18[c]	96±2.48[a]	35.2±0.69[d]

Note: The different letters on top of each value in the same column means significant difference (P<0.05).

obtained on the Mixogram and at this point the dough had optimum development, in which a well-defined peak indicated proteins that were suitable for bread making (Martinant et al., 1998). Furthermore, the time required to produce the curve peak was an indicator of gluten protein strength, and longer peak times of well-defined curves indicated stronger gluten proteins. The width of the mixogram curve and the angle of descent indicated the tolerance of the dough to over mixing, and well-defined curves with wide bands and low angles of descent indicated strong tolerance to over mixing and superior protein quality (Table 2). Some new variations of flour mixing properties observed in the hybrid progenies indicated that the introgression of exogenous *S. bicolor* DNA into wheat chromosome happened, which affected the grain quality of wheat hybrids significantly and can therefore be inherited. Wheat protein content can demand a premium price in the marketplace. High-molecular-weight glutenin subunits (HMW-GS), a family of seed storage proteins synthesized in wheat developing endosperm, are important determinants of the gluten viscoelasticity, which allow wheat dough to be processed into bread, pasta and noodles, as well as many other food products (MacRitchie, 1992; Gras et al., 2001; Shewry et al., 2003). However, protein content can be a flawed predictor and high protein content alone is not enough. The proteins must have functional properties for water uptake (absorption) and mixing strength to insure proper gluten protein development, such as HMW glutenin subunits 5+10 positively related to the baking quality (Pogna et al., 1988; Rogers et al., 1991). In this study, the basic pattern of the HMW glutenin of the hybrids was similar to that obtained from the maternal parents. However, compared with its maternal parent G8, the HMW subunits 5+10 were presented in 9144 instead of subunits 2+12 in G8, which coinciding with the sorghum but lacking in wheat (Fig. 2, Table 2). Such variations of the HMW-GS in the wheat-sorghum hybrids in this study could be explained by that the segment of *S. bicolor* DNA from the pollen tube might contain structural or regulatory genes or broken pieces, and these pieces of DNA (which might have already lost their original genetic function) could have been integrated into the ovarian genome and bringing about changes in the maternal structural genes or their mode of expression. Alternatively, a regulatory effect from DNA segment of sorghum might have stimulated a silent gene of wheat to activity. Therefore, development of reliable and reproducible systems of wheat transformation would make it possible to modify HMW-GS composition. Now, development of alternate, simple and rapid transformation protocols for development of transgenic plants, including floral dip method, pollen transformation, pistil transformation and ovary-dip transformation can overcome the constrains of *in vitro* culture, regeneration and associated problems (Eapen, 2011). Such distant DNA transformation technology is important in modern plant

Fig 1. Morphological characteristics between distant hybrids and their maternal parents. (A) plant height; (B) panicle shape and height; (C) seed color

breeding. Pollen tube pathway method is one of the means, which it is cheap, simple and relatively safe, and it is more suitable in developing country, especially. Based on these results, we concluded that above five distant hybrids selected are true hybrids of wheat and sorghum, which showed that the improvement of the wheat qualities due to introduction of exogenous DNA will provide a chance for the selection of new improved cultivars.

Materials and Methods

Plant materials

The hexaploid (2n=6x=42) wheat (*T. aestivum* L.) cultivars used in this study were Longchun No. 13 (L13), Longchun No. 10 (L10) and Ganmai No. 8 (G8), supplied by the Gansu Academy of Agricultural Science, China. The sorghum (*S. bicolor* L.) 2D, 5D and 10D, three most popular varieties planted in Gansu Province in northwestern China, were selected as donor DNA. The distant hybridization experiments via pollen-tube pathway method were carried out in the field from May to July 1991, Lanzhou, Gansu Province.

Sorghum DNA isolation

Seeds of sorghum 2D, 5D and 10D were sown in sterile soil and grown in the dark at 25°C. Two to three weeks later, about 1g etiolated seedlings were harvested. DNA extractions were done following the modified method of de Kochko and Hamon (1990). The leaves were quickly ground in liquid nitrogen in a mortar and the resulting powder was placed in a 30-ml tube. Then 15 ml extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol, pH 8.0) and 1ml 20% SDS were added. After the homogenate was incubated at 65°C for 10 min, 5 ml of 5M potassium acetate was added and placed in ice for 20 min. Materials were centrifuged at 13000 rpm for 20 min. The supernatant was decanted into a clean 30-ml tube containing 10 ml of

Table 2. Mixogram values and relative contents of HMW-GS of five wheat-sorghum hybrids and their maternal parents.

Grain quality index	G8	9144	0154	9145	L10	9141	L13	9122
mixogram value								
MPT (min)	2.2±0.05^{a}	1.0±0.02^{b}	1.2±0.05^{c}	1.7±0.12^{d}	1.7±0.09^{d}	1.5±0.12^{e}	1.7±0.06^{d}	2.2±0.16^{a}
MPH (cm)	6.9±0.12^{a}	7.2±0.34^{b}	7.8±0.33^{c}	6.2±0.25^{d}	6.6±0.18^{e}	8.1±0.36^{f}	6.6±0.27^{d}	6.7±0.34^{ad}
MHSM (m)	5.0±0.23^{a}	4.8±0.21^{a}	5.0±0.25^{a}	4.3±0.19^{b}	4.2±0.29^{b}	6.0±0.41^{c}	4.2±0.31^{b}	4.6±0.23^{a}
MWSM (cm)	0.5±0.07^{a}	0.6±0.02^{b}	0.8±0.07^{c}	0.7±0.10^{bc}	0.5±0.08^{a}	1.0±0.12^{d}	0.5±0.08^{a}	0.8±0.07^{c}
relative contents of HMW-GS								
Subunit 1	0.011±0.001^{a}	0.018±0.002^{b}	0.014±0.001^{c}	0.010±0.002^{a}	0.140±0.011^{d}	0.090±0.010^{e}	0.241±0.018^{f}	0.165±0.023^{d}
Subunit 2(5)	0.251±0.032^{a}	(0.127±0.021)^{b}	0.228±0.033^{a}	0.360±0.017^{c}	0.243±0.041^{a}	0.175±0.012^{d}	0.349±0.023^{c}	0.231±0.025^{a}
Subunit 7	0.307±0.037^{a}	0.273±0.032^{a}	0.282±0.019^{a}	0.385±0.039^{b}	0.090±0.012^{c}	0.061±0.007^{d}	0.181±0.022^{e}	0.163±0.024^{e}
Subunit 9	0.175±0.021^{a}	0.349±0.045^{b}	0.223±0.036^{c}	0.197±0.018^{a}	0.189±0.032^{a}	0.131±0.025^{d}	0.089±0.008^{e}	0.201±0.034^{ac}
Subunit 12(10)	0.256±0.034^{a}	(0.234±0.034)^{b}	0.254±0.037^{a}	0.048±0.054^{c}	0.339±0.045^{d}	0.544±0.055^{e}	0.370±0.037^{d}	0.240±0.032^{a}

Note: MPT, Mixogram Peak Time; MHSM, Mixogram Height at the Seventh Minute; MPH, Mixogram Peak Height; MWSM, Mixogram Width at the Seventh Minute; HMW-GS, High-molecular-weight glutenin subunits. The different letters on top of each value in the same line means significant difference (P<0.05)

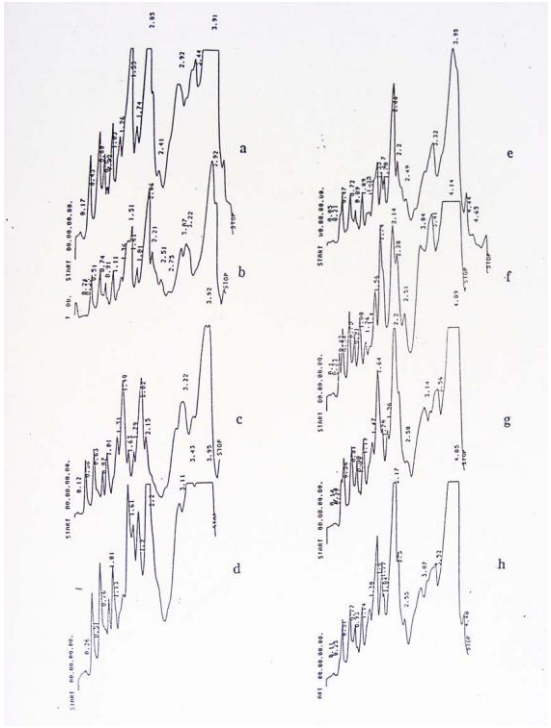

Fig 2. SDS-PAGE of HMW-GS scanning patterns of the wheat-sorghum hybrids and their maternal parents (a) L13; (b) 9122; (c) L10; (d) 9141; (e) G8; (f) 9144; (g) 9145; (h) 0154.

isopropanol. After several inversions of the tube to homogenize the contents, it was kept at -20°C for more than 30 min. A further centrifugation at 13,000 g for 15 min produced a pellet rich in nucleic acids. This pellet was air dried for 10 min and then resuspended in 700 µl of TE. Then 1 µl of RNAase (10 mg/ml) was added and the tube incubated for 1h at 37°C. 77 µl of 3M sodium acetate were added and the solution purified by adding an equal volume of phenol-chloroform (1:1). The aqueous phase was re-extracted with an equal volume of chloroform. The 0.7 volume of cold isopropanol was added to the aqueous phase, the tube inverted several times, and centrifuged for 1 min. The supernatant was decanted carefully so as not to disturb the pellet. The pellet washed with 80% (v/v) cold ethanol and centrifuged again for 30 sec. The supernatant was carefully removed, and the pellet dried in a vacuum dessicator. Then 100 µl of TE were added and the pellet left to rehydrate on ice for at least 1h or preferably overnight. The DNA quantity and purity were determined with ultraviolet spectrophotometer. The working DNA concentration was adjusted to 200 µg/ml.

Transformation of wheat through pollen tube pathway

The transformation experiments were carried out in the field from July to August 1991, Lanzhou, Gansu Province. The artificial emasculated flowers of wheat were introduced by the pollen tube pathway technique (Luo and Wu, 1988; Wędzony and Van Lammeren, 1996). The upper and basal spikelets were removed from spikes. From the remaining spikelets the central florets were dissected leaving the primary and secondary ones. These florets were emasculated by clipping anthers 2-3 d before anthesis and the spikes were bagged with manilla paper to prevent desiccation. After artificial pollination 0.5-3 h cut plumose stigma of these emasculated florets, 5-10 sorghum DNA with concentration 200 µg/ml were dropped onto the micropyle using a micropipette. All treated ears were covered with sterile paper bags and marked. At maturity stage, the seeds that developed from the treated ears (labeled as the F1 generation) were harvested. F1 seeds of distant hybridization between sorghum and wheat were planted in 1992 and F8 seeds were obtained until 1999. Five hybrid lines obtained through 8 years selection were as follows: 9122 (*S. bicolor* 2D + Wheat L13), 9141 (*S. bicolor* 5D + Wheat L10), 9144 (S. bicolor 2D + Wheat G8), 9145 (*S. bicolor* 5D + Wheat G8), 0154 (*S. bicolor* 10D + Wheat G8). After harvested in 1999, the plant height, numbers of panicle per plant, growth duration, 1000-seed weight of the five hybrids and their recipients were determined. Each treatment had 10 replicates.

Mixograph curve

Mixogram peak time, mixgram peak height and mixogram height at the seventh minute of five hybrids and their recipients were obtained using dough according to AACC (1983) method 54-40. The mixogram was conducted with 10 g of flour and 6 ml water to give optimum absorption. And the mixograms were recorded after run for 7 min, which is sufficient time for most flours to exhibit their mixing time to peak and dough breakdown. The mixograph was recorded to reflect the resistance of the dough during mixing. Each hybrid or their recipients had 10 replicates.

Seed storage protein extraction

Seed storage proteins of five wheat-sorghum hybrids and their maternal parents G8, L10 and L13 were extracted. One grain was cut into 4 pieces and placed in a tube with 1.5 ml extraction buffer. The extraction buffer was prepared by mixing stock solutions below into 100 ml volume (12.5 ml 0.5 M Tris-HC1, pH 6.7, 20 ml 10% SDS, 10 ml glycerol and 10 ml 0.002% bromophenol blue). The seed was grounded to fine powder for 3-5 min. The tubes were incubated at 100 ℃ for 3 min and then centrifuged at 6000-7000 rpm for 10 min. The glutenin was sediment by adding 125 µl ethanol (70%) into the supernatant, and then centrifuged at 10,000 rpm for 5 min and the sediment was dried and dissolved in 50 µl extraction buffer.

SDS-PAGE of high-molecular-weight glutenin subunits (HMW-GS)

The discontinuous-buffer system of SDS-PAGE used to fractionate the proteins was based on the method developed by Laemmli (1970) and modified by Payne (1981): 10% separating gel (0.375 M Tris-HC1, pH 8.9, 0.1% SDS, 10% acrylamide, 0.13% bis-acrylamide) and 3.5% stacking gel (0.12 M Tris-HC1, pH 6.7, 0.1% SDS, 3% acrylamide, 0.043% bis-acrylamide). The electrode buffer was 0.025 M Tris-glycine, pH8.3, containing 0.1% SDS. Electrophoresis was run for 16 h at 200V for 3.5% gels and 100V for 10% gels. Gels were fixed with 10% trichloroacetic acid, stained for 3 h in Coomassie Blue G250, and then destained in 7% glacial acetic acid until the bands were clear. The high molecular subunits of glutenins were analysed quantitatively by laser densitometer (LKB-bromma 2202, Sweden).

Statistical analysis

All the datasets were checked for normality prior to further analysis, and none of them were significantly different from a normal distribution, so no data transformation was required. Means of plant height, numbers of panicle per plant, growth duration, 1000-seed weight, mixogram values and relative contents of HMW-GS were calculated according to the data using SPSS (v13.0). We used LSD test to compare the mean of data. Significance level was set at 5% (0.05) for all statistics. One-way Analysis of Variances (ANOVA) was used to test the effects of pollen tube pathway method on plant height, numbers of panicle per plant, growth duration, 1000-seed weight, mixogram values and relative contents of HMW-GS with SPSS (v13.0) to show if there was significant difference between different hybrids or parents.

Conclusions

The distant hybrids between *Triticum aestivum* and *Sorghum bicolor* produced by pollen tube pathway method exhibited great genetic variations of agronomic traits such as plant height, panicle shape and seed color (Fig. 1). The mixograph of five hybrids evaluated showed that mixogram width at the seventh minute of all the hybrids was improved, which indicated the tolerance of the dough to over mixing and superior protein quality (Table 2). In hybrid 9144, the high molecular weight glutenin subunits 5+10 were presented instead of subunits 2+12 of its maternal parent G8, which coinciding with the sorghum but lacking in wheat (Fig. 2, Table 2). This study demonstrated that distant hybridization between *T. aestivum* and *S. bicolor* via pollen tube pathway method could improve the wheat grain quality, which will provide more opportunities for the selection of new wheat cultivar with improving grain quality.

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