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RESEARCH

Characterization of a Multiseeded (*msd1*) Mutant of Sorghum for Increasing Grain Yield

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ABSTRACT

Sorghum [*Sorghum bicolor* (L.) Moench] has branched panicles with primary, secondary, and tertiary branches. These inflorescence branches are composed of a terminal triplet spikelet, consisting of one sessile (directly attached to the branch) and two pedicellate (spikelets attached to the branch through a short pedicel), followed by one or more spikelet pairs (one sessile and one pedicellate). In BTx623, and most sorghum lines, only the sessile spikelets can develop into seeds and the pedicellate spikelets, occasionally developing anthers, eventually abort. Here, we report the isolation and characterization of a stable multiseeded (*msd1*) mutant of sorghum (in BTx623 background, referred to as wild-type [WT]) in which both sessile and pedicellate spikelets are fertile. In addition, this mutant displayed increased length and total number of primary and secondary inflorescence branches. Genetic analysis showed that this multiseeded mutation is a recessive trait. Pedicellate spikelets in *msd1* mutants exhibited complete flower with functional ovary and anthers, of which 75 to 95% can develop into viable seeds. The individual *msd1* seeds are smaller than WT; however, the results indicated that this reduction in seed weight can be compensated by the increase in seed number. The total seed weight per panicle in *msd1* mutants was increased by 30 to 40% as compared to the WT. Further experiments are needed to demonstrate whether the *msd1* mutation can increase grain yield under field conditions.

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Abbreviation: WT, wild-type.

Seed number per panicle is a major determinant of grain yield in sorghum [*Sorghum bicolor* (L.) Moench] and other cereal crops (Saeed et al., 1986; Duggan et al., 2000; Richards, 2000; Ashikari et al., 2005; Reynolds et al., 2009; Sreenivasulu and Schnurbusch, 2012). Increased seed number and seed size, which is directly related to improved grain yield, is a common goal during domestication of cereal crops, resulting in inadvertent selection of a limited number of germplasm with these needed traits (Zohary et al., 2012). The number of seeds per panicle is proposed to be directly related to developmental mechanisms that control inflorescence architecture and panicle size (Bommert et al., 2005; Sreenivasulu and Schnurbusch, 2012).

In sorghum, the inflorescence, also known as panicle, consists of a main rachis on which many primary branches develop. Secondary branches and sometimes tertiary branches are developed from the primary branch (Brown et al., 2006). The main inflorescence, primary, secondary, and tertiary branches all end with a terminal spike, which consists of one sessile (directly attached to primary, secondary, and tertiary branches) fertile spikelet (floret) and two sterile pedicellate (attached to the branches by a short pedicel) spikelets (Walters and Keil, 1988). Below the terminal

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spike, one or more spikes can develop, and these adjacent spikes usually consist of one sessile and one pedicellate spikelets. In BTx623 and most other sorghum accessions, only the sessile spikelets of the terminal or adjacent spikes form complete flowers and develop into seeds, whereas pedicellate spikelets are sterile (Casady and Miller, 1970). In a few sorghum cultivars, the pedicellate spikelets are enlarged and were observed to occasionally develop anthers but completely lack female (ovary, style, and stigma) organs (unpublished observation, 2013).

In addition to its primary role of introducing variability, mutagenesis by radiation or chemical mutagens is an effective approach to elucidate morphogenesis, metabolism, and signal transduction pathways in most organisms, including higher plants (Bentley et al., 2000; Henikoff et al., 2004; Amsterdam and Hopkins, 2006). Mutation has long been used in sorghum breeding and was instrumental in isolating novel phenotypes (Sree Ramulu, 1970a, 1970b; Quinby and Karper, 1942; Gaul, 1964). A number of beneficial mutations, including dwarfing, early flowering, high protein digestibility, high lysine, and others, have been widely used in sorghum breeding (Singh and Axtell, 1973; Quinby, 1975; Ejeta and Axtell, 1985; Oria et al., 2000). Collection and preservation of these mutants was performed by the late Dr. Keith Schertz, a former sorghum geneticist with USDA–ARS. This collection displays a wide range of phenotypic variation, providing a vital resource for sorghum genomic studies. It has been released as a collection of genetic stocks through Germplasm Resources Information Network (Xin et al. [2013]; www.ars-grin.gov, accessed 2 Jan. 2014).

The completion of the genome sequence of the key public inbred line, BTx623, makes it possible to study gene function on a genome-wide scale, and to compare gene function in sorghum with other plants (Paterson, 2008; Paterson et al., 2009). To support the functional genomic studies, we have established an Annotated Individually pedigreed Mutated Sorghum (AIMS) library (Xin et al., 2008, 2009). The mutant library consists of 6144 pedigreed M_4 seed pools developed by single-seed descent from individual mutagenized seeds (M_1) to M_3 generation. This mutant library contains many biologically and agronomically interesting mutants, such as (but not limited to) brown midrib (*bmr*) mutants that produce biomass (stover) with improved digestibility and ethanol yield and erect leaf (*erl*) mutants that have the potential to improve canopy radiation capture and hence biomass yield (Xin et al., 2009; Saballos et al., 2012; Sattler et al., 2012).

Recently, we isolated and characterized a novel class of multiseeded sorghum mutants, designated as *msd*. In this class of mutants, all spikelets, sessile and pedicellate, set viable seeds, and produce greater number of panicle branches. We report here the genetic and agronomic characterization of one group of this *msd* class, *msd1*, the first of a series of multiseeded mutants, and discuss its potential in improving grain yield in sorghum.

MATERIALS AND METHODS

BTx623, a key public inbred line (Miller, 1977), was purified by single-seed descent for six generations before use in chemical mutagenesis as described previously (Xin et al., 2008). Batches of 100 g of dry seed (~3300 seeds) of purified BTx623 were soaked with agitation (16 h at 50 revolutions min^{-1} on rotary shaker) in 200 mL of tap water containing ethyl methane sulfonate (Sigma Aldrich, St. Louis, MO) concentrations ranging from 0.1 to 0.3% (v/v). The treated seeds were thoroughly washed in about 400 mL of tap water for 5 h at ambient temperature, changing the wash water every 30 min. The mutagenized seeds were air-dried and subsequently used for planting.

The air-dried seeds were planted at a density of 120,000 seeds ha^{-1}. Before anthesis, each panicle was bagged with a 400-weight rain-proof paper pollination bag (Lawson Bags, Northfield, IL) to prevent cross pollination. After bagging, each bag was injected with 5 mL of chlorpyrifos (Dow AgroSciences, Indianapolis, IN) at 0.5 mL L^{-1} to control corn earworms (*Helicoverpa zea*) that might hatch within the bag and destroy the seeds. Sorghum panicles were harvested manually and threshed individually. Each fertile panicle was planted as an M_2 head row. Three panicles from each M_2 head row were bagged before anthesis, and only one fertile panicle was used to produce the M_3 seeds. It should be noted that substantial number of mutant lines displayed diminished seed production during the M_3 generation. Thus, 10 panicles were bagged for each M_3 head row and pooled as M_4 seeds.

The multiseeded phenotype was identified by observing initially for bulky and large heads, and later by systematic and careful inspection of panicles from each of the 6144 M_3 plots from the beginning of grain filling to physiological maturity or harvesting. Any panicle with terminal spikes that developed into three seeds, instead of one seed and two aborted spikelets, was selected and confirmed in the next generation. Confirmation of the multiseeded phenotype was performed in the next generation by planting the selected lines in replicated plots and then carefully monitoring the development of seeds in spikelets in the panicle from grain fill to physiological maturity.

Backcrossing and Genetic Analysis

The confirmed mutants were backcrossed to the wild-type (WT) BTx623 by hand-emasculation in the field and also in a standard polyhouse to maximize opportunities for crossing. The first mutant used in successful backcross was designated as multiseeded1 (*msd1*). BC_1F_1 seeds from WT × *msd1* were self-pollinated to produce BC_1F_2 generation. Seeds from BC_1F_2 generation were planted as row plots in the field in summer 2011 and carefully scored for the trait at maturity and confirmed as BC_1F_3 family rows in summer 2012. Each BC_1F_3 progeny was represented by 20 to 30 individual plants in a plot. Five panicles from each plot were bagged at anthesis and were carefully evaluated after full seed set for presence or absence of the *msd* trait. Analysis of segregation for goodness of fit to 3:1 ratio as recessive trait was performed using chi-square test.

Morphology of *msd1* Inflorescence

For detailed characterization of inflorescence, two to three plants of 24 BC_1F_3 *msd1* and 24 WT families were grown in plastic pots of 20 cm by 25 cm height by depth in the polyhouse. Plants were fertilized with Osmocote (Scotts Co., Marysville, OH) and irrigated through automatic controlled emitters. At approximately midanthesis, young panicles from *msd1* and WT plant samples were obtained for microscopic examination and morphological characterization. Samples of spikelets were dissected to facilitate observation of floral structures, including anthers and ovules in the terminal and adjacent spikes along the length of the panicle. Histological observations were performed using a LEICA MZ6 microscope fitted with a DFC420 camera (Leica Microsystems [Schweiz] AG, Heerburg, Switzerland).

Characterization of Panicle and Agronomic Traits

For characterization of panicle and agronomic traits, 10 confirmed *msd1* BC_1F_3 families and 10 WT families were utilized for measurement of traits of interest. Measurements of different panicle traits were conducted based on studies of sorghum inflorescence by Brown et al. (2006). Briefly, the length of the panicle was measured from the node of first branch attachment to the tip of the panicle. Primary branches were then removed individually from the main rachis and the total number per panicle was determined. The length of 12 primary branches sampled from the bottom, middle, and tip portions of the panicle were measured. Subsequently, secondary branches from each primary branch were removed and counted. The total number of spikelets per panicle was counted after carefully dissecting each panicle. Each spikelet was scored as filled (developed seeds) or unfilled (sterile chaffy structures). Percent seed set was calculated as the ratio of filled spikelets to total number of spikelets in panicle. Seed weight was evaluated as total seed weight of cleaned seeds (no glumes) and as 100-seed weight.

To determine if the *msd1* mutation affects germination, 25 seeds each from the same 10 families of BC_1F_3 *msd1* and WT discussed earlier were subjected to germination test according to AOSA protocol for sorghum (AOSA, 1999).

Statistical Analysis

All data in this report are means from three plants per line. Statistical difference was evaluated using Student's *t* test, to achieve statistical comparison between mutant and WT. Segregation ratio was tested for goodness of fit to a 3:1 ratio using chi-square test.

RESULTS AND DISCUSSION

From 6144 independently mutated M_3 lines, >20 multiseeded mutants were selected over the past 2 yr based on careful visual inspection of spikelet development and size of panicle.

Inflorescence Structure of *msd1* Mutant

A comparison of the morphological features of terminal spikelets from WT and *msd1* mutant is shown in Fig. 1. The distinct differences between the WT and *msd1* are illustrated in Fig. 1A and B, which show that only the sessile spikelets are enlarged and had developed in WT, while in *msd1*, both sessile and pedicellate spikelets are enlarged and exhibited signs of development at an early stage of flowering. In Fig. 1 (C vs. D), the sterile pedicellate spikelets of WT are shown separate from the sessile spikelet (Fig. 1C), while the development of the ovary in each of the pedicellate and sessile spikelets of *msd1* mutant is shown using a dissected intact terminal spike (Fig. 1D). The resulting fully developed monoseeded WT and three-seeded *msd1* mutant spikelet are shown in Fig. 1E and F, respectively.

The characteristic features of a flower branch from WT and multiseeded panicles are shown in Fig. 2. In Fig. 2A and B, close-up photos of the abaxial (outside) and adaxial (inside) views of an inflorescence branch indicate that *msd1* had conspicuously larger and filled pedicellate spikelets compared to flat and undeveloped WT pedicellate spikelets. At grain fill, detailed examination of a branch showed that in WT, the pedicellate spikelets are sterile and shrunken and could only be seen from the adaxial view of an inflorescence branch, and were barely visible from the abaxial view (Fig. 2A and B). In the *msd1* mutant, the pedicellate spikelets were enlarged and could be seen clearly from both abaxial and adaxial views (Fig. 2A and B).

In Fig. 3A, the dried pedicellate spikelets are easily observed as shrunken and dried chaffy structures in WT. In this primary branch only three seeds developed, arising from three sessile spikelets (Fig. 3A). In contrast, a bulkier primary branch from *msd1* is shown in Fig. 3B. All spikelets set seed in *msd1*, producing seven seeds in this primary branch instead of three seeds. A schematic representation of the trait is shown in Fig. 3C and D.

A review of literature indicated earlier reports documenting the occurrence of rare sorghum germplasm with both sessile and pedicellate spikelets developing into complete flowers and setting seeds (Ayyangar et al., 1935; Karper and Stephens, 1936), which was referred to as "fertile pedicelled spikelets of sorghum" (compared to conventional mono-seeded phenotype). A report by Casady and Miller (1970) documented the production of probably similar recessive "hermaphroditea pedicelled spikelets" (fertile pedicelled spikelet) from a Chinese germplasm "PI 92260" at a rate of ~72%, which could be similar to multiseeded phenotype, although no photographs were provided. We obtained the germplasm, PI 92260 (possible fertile pedicelled germplasm), from the USDA–ARS Germplasm Resources Information Network (www.ars-grin.gov) sorghum germplasm repository, and a grow-out of the material in the greenhouse did not produce any plants with the hermaphrodite pedicelled spikelets. It is possible that this rare multiseeded germplasm is not well characterized and could have been overlooked during curation. Additionally, a database search of the sorghum

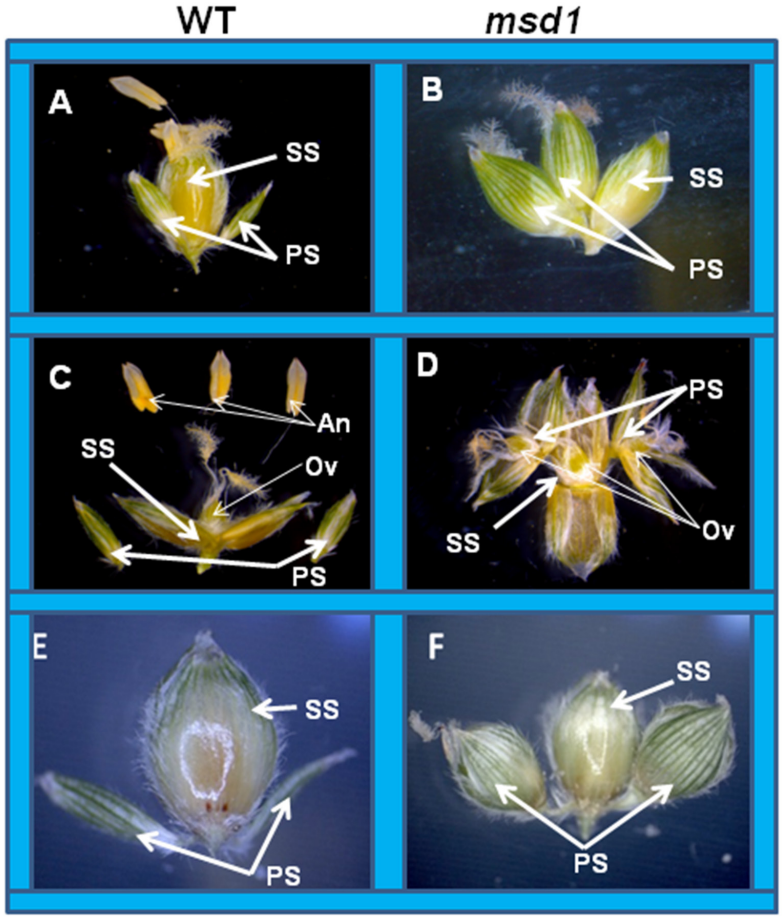

Figure 1. Inflorescence features of sorghum wild-type (WT) and multiseeded1 (*msd1*) terminal spikelets. In (A) WT BTx623 (*MSD* = mono-seeded), the sessile spikelet (SS) is enlarged and the two pedicellate spikelets (PS) are small and shrunken. (B) In *msd1* mutant, both SS and PS are enlarged. (C) In WT, only SS produces complete flower composed of three anthers (An) and an ovary (Ov), while PS are sterile. (D) In *msd1* mutant, both SS and PS developed functional ovary (Ov) and each three anthers. (E, F) Only SS develops into seed in WT, while in *msd1*, both SS and PS develop into seeds, producing three seeds.

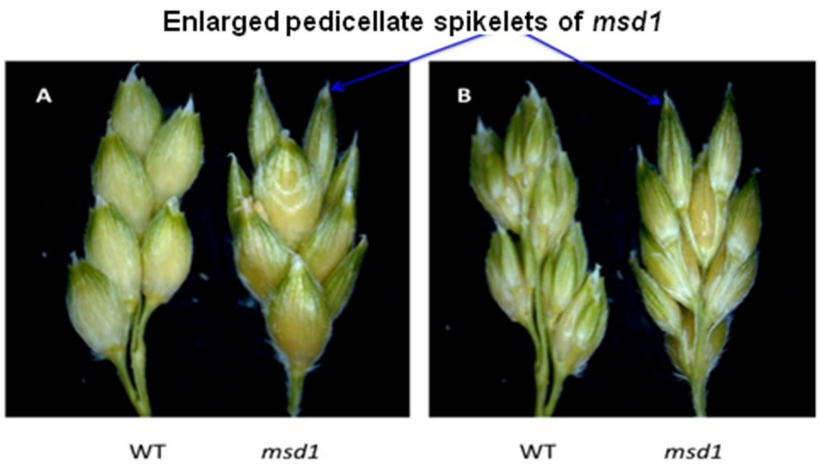

Figure 2. Phenotype of sorghum wild-type (WT) (MSD) and multiseeded1 (*msd1*) inflorescence branch. In (A), the abaxial view of a primary branch from WT (left) and *msd1* (right) panicles is shown. (B) The adaxial view of the same inflorescence branch from WT (left) and *msd1* (right) panicles. The arrows point to the visible enlargement of the pedicellate spikelets in *msd1*.

germplasm collection at the International Crops Research Institute for Semi-Arid Tropics (ICRISAT, www.icrisat.org/crop-sorghum-genebank.htm, accessed 2 Jan. 2014) indicated no record of multiseeded sorghum germplasm.

The induction of development of fertile pedicellate spikelets found in multiseeded mutant of sorghum reported here could be analogous to the phenomenon of the evolution of six-rowed barley (*Hordeum vulgare* L. ssp.

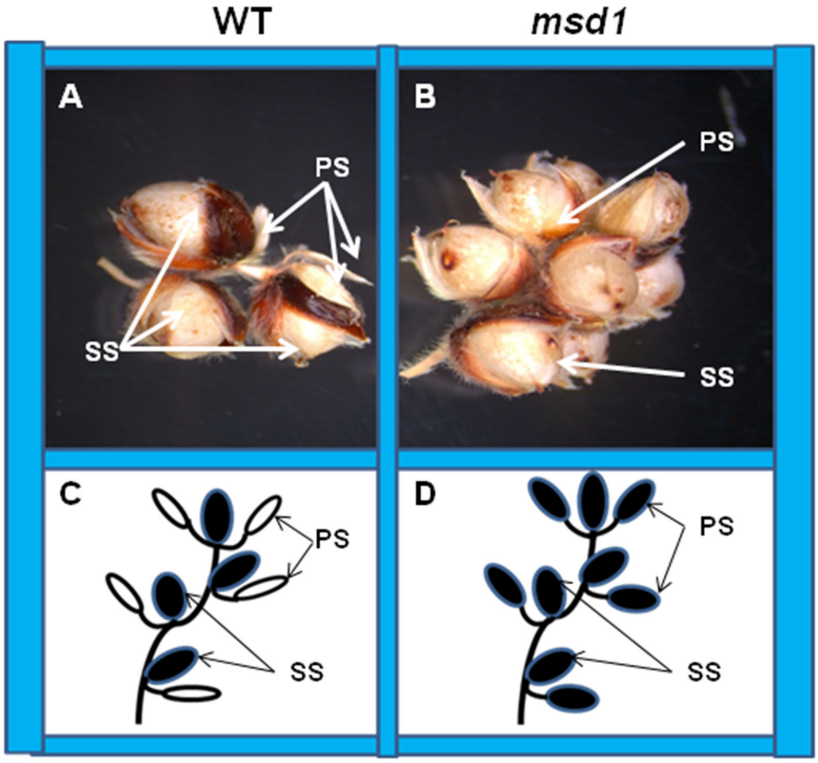

Figure 3. Seed set and schematic representation of wild-type (WT) and multiseeded1 (*msd1*) sorghum. (A) Close-up photograph of an inflorescence branch of WT panicle with arrows pointing to sessile spikelets (SS) that produced or set seeds, while sterile pedicellate spikelets (PS) produced adherent shrunken and dried chaffy structures. (B) In *msd1*, a close-up photograph of an inflorescence branch showed all spikelets (SS and PS) set seeds. (C, D) The schematic representations of the morphology of primary branch shown in A and B are provided. In WT, dried shrunken PS that did not set seed are shown as unfilled ovals, while in *msd1*, all spikelets (SS and PS) are shown as filled ovals to represent seed set.

vulgare, a cereal crop under the Triticeae tribe of Poaceae) from two-rowed germplasm. Barley spike is composed of three spikelets (one central and two lateral spikelets) parallel to that of sorghum (Kellogg, 2007). In modern six-rowed barley all three spikelets are fully fertile and produce viable seeds, while in two-rowed barley cultivars only the central spikelet developed into seeds (Komatsuda et al., 2007). Phylogenetic analysis demonstrated that six-rowed phenotype of barley originated through independent mutations of the *Vrs1*, the major gene controlling the trait (Komatsuda et al., 2007; Ramsay et al., 2011).

msd1 Is a Monogenic Recessive Mutation

To determine the genetic nature of the multiseeded mutation, and specifically *msd1*, the mutant was crossed to the WT, BTx623. All F_1 plants showed conventional sterile pedicellate spikelets and panicle morphology similar to BTx623, indicating that the mutation is recessive. Among the 99 F_2 plants, 23 were *msd1* mutants and 76 were WTs (Table 1). This ratio is consistent with a single recessive Mendelian trait. This result confirmed earlier hypothesis that this type of mutation is recessive and was designated as *msd1/msd1* formally (*msd1* in short form).

We hypothesize that *msd1* phenotype could have resulted from mutation of a putative nuclear gene that serves as a switch for the development of pedicellate spikelets in wild-type sorghum. This hypothesis is related to the finding reported from barley, wherein the gene called *Vrs1* controlled the development of six-rowed phenotype (analogous phenotype of the sorghum multiseeded mutant) [Komatsuda et al., 2007; Ramsay et al., 2011]). The *Vrs1* gene, a homeodomain-leucine zipper 1-class homeobox gene, was found to harbor 48 independent mutations that correlated with allelism test for the six-rowed barley phenotype (Komatsuda et al., 2007). Barley *Vrs1* was proposed to act as master switch that controls the growth of lateral spikelets by suppressing their development (Komatsuda et al., 2007; Ramsay et al., 2011; Koppolu et al., 2013). These studies indicate that mutation in critical portions of the

Table 1. Segregation for multiseeded trait in backcross1 F_2 (BC_1F_2) generation of wild-type (WT) (BTx623) × multiseeded1 (*msd1*) sorghum. Chi-square analysis was performed to determine goodness of fit to expected 3:1 ratio.

Phenotype	Observed	Expected	Chi square	*P* value
WT = mono/single-seeded (*MSD*)	76	74	0.05	
Multiseeded (*msd*)	23	25	0.16	
Total	99	99	0.21 ns	*P* = 0.41

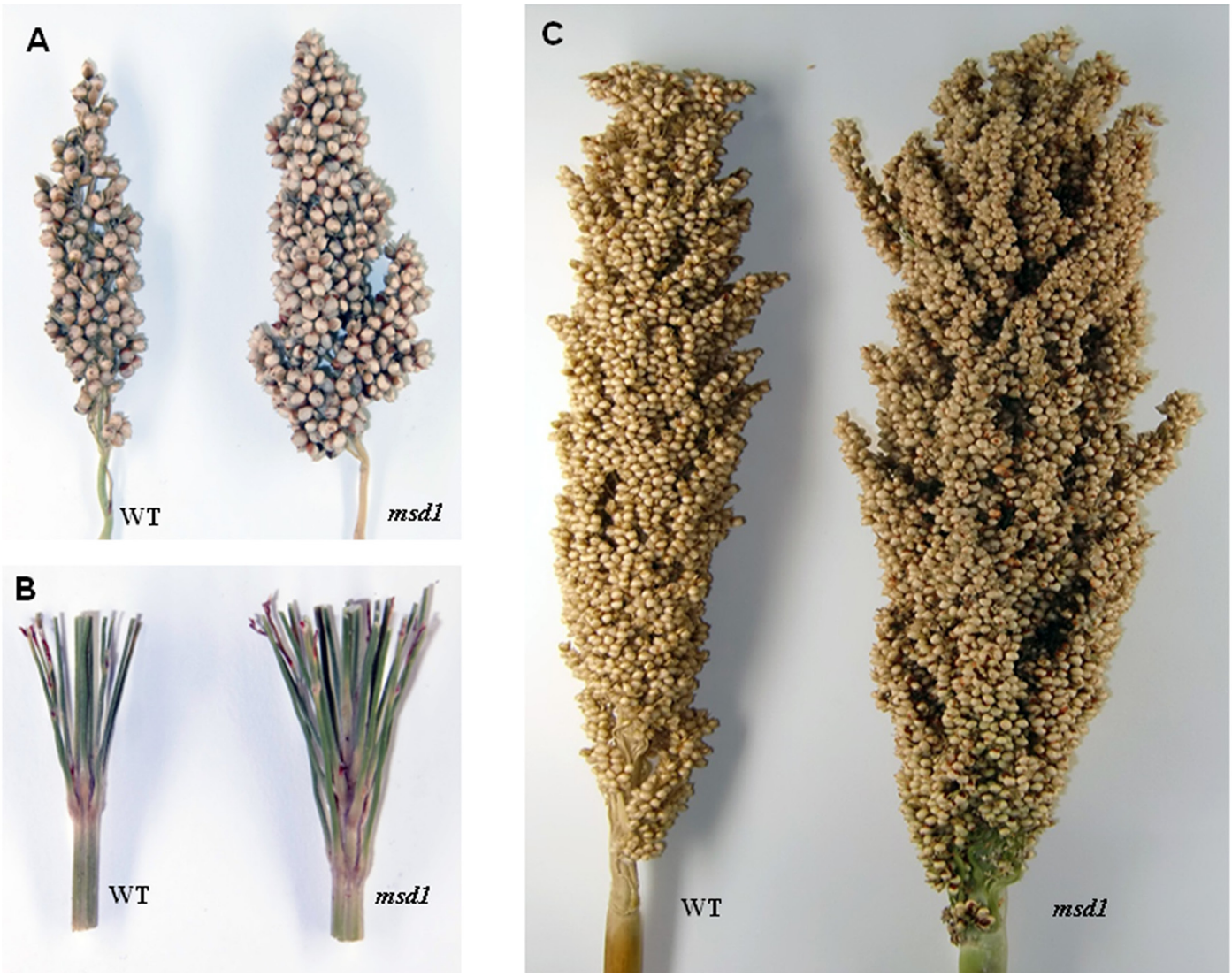

Figure 4. Phenotypic traits of panicles of wild-type (WT) and multiseeded1 (*msd1*) sorghum. (A) Photos showing the comparison of overall morphology of primary branch for WT and *msd1*. (B) Photos showing the difference in primary branch number in WT and *msd1*. (C) Photographs of mature panicles of WT and *msd1*; *msd1* panicle is heftier and bulkier than WT.

barley *Vrs1* gene released the suppression of development of lateral spikelet organs, which in turn permits growth of these floral organs alongside that of central spikelet, resulting in the six-rowed phenotype (Komatsuda et al., 2007). With the multiseeded sorghum mutant, parallel morphological events resulting in the development of pedicellate spikelet could have arisen from the mutation of the important sites of the yet to be discovered regulatory gene. One evidence that could support this hypothesis will be the existence of alleles or multiple forms of the *msd1*. Recently, results from complementation studies indicate that there are at least five independent alleles of *msd1* (data not presented). Further studies are ongoing to clone the gene for *Msd1* and determine the independent mutations that give rise to various allelic forms of the gene.

Panicle Traits and Agronomic Characteristics of *msd1* Mutant

We performed a detailed characterization of the important agronomic features of *msd1* compared to WT (Fig. 4 and Tables 2, 3, and 4). In addition to the altered fate of the pedicellate spikelets, the *msd1* mutant also displayed distinct changes in general panicle morphology and agronomic features. These include increased number of primary branches and the length of primary and secondary branches (Fig. 4B and Table 2). The differences in primary branching between WT and *msd1* are depicted in Fig. 4B. Consequently, the *msd1* panicle was bulkier and longer than the wild-type BTx623 (Fig. 4C).

Due to the distinct changes in inflorescence morphology and the fate of the pedicellate spikelets observed, the number of seeds produced per primary inflorescence branch was almost 3× compared to wild-type BTx623 (Table 4). However, the increased seed numbers came with a cost of

Table 2. Comparison of wild-type (WT) and BC_1F_2 multiseeded1 (*msd1*) sorghum mean phenotypic values for inflorescence traits. Student's *t* test was used to declare significance of difference between mean values from WT and *msd1*. Values in parentheses are standard error of the mean.

Inflorescence/ spikelet trait	WT	*msd1*	*P* value	*msd1* expressed as % of WT
				%
No. of seeds per primary branch	57.17(6.55)	189.68(10.13)	<0.0001**	332
No. of primary branches panicle^{-1}	46.12(1.55)	51.80(1.16)	0.006**	113
Length of primary branches (cm)	4.76(0.34)	6.24(1.18)	0.005**	131

** Significant at 0.01 probability level.

Table 3. Comparison of wild-type (WT) and BC_1F_2 multiseeded1 (*msd1*) sorghum mean phenotypic values for panicle traits. Student's *t* test was used to declare significance of difference between mean values from WT and *msd1*. Values in parentheses are standard error of the mean.

Trait	WT	*msd1*	*P* value	*msd1* expressed as % of WT
				%
Panicle length (cm)	30.87(0.55)	32.48(0.32)	0.02*	105
Total no. of seeds panicle^{-1}	2688(312.3)	9794(530)	<0.0001**	364
Panicle exsertion (cm)	3.00(0.72)	2.58(0.54)	0.64ns	86

* Significant at 0.05 probability level.

** Significant at 0.01 probability level.

reduced seed size (Table 4), with *msd1* showing a mean value of 1.72 g 100 seeds^{-1} while WT showed 3.11 g 100 seeds^{-1}. Notwithstanding, the total seed number on a per-panicle basis seemed to compensate for reduced seed size, resulting in an increase in total seed weight per panicle. Thus, the seed production based on weight per panicle increased in *msd1* mutant plants (Table 4). Notably, *msd1* did not show significant difference with WT based on plant height, maturity, and germination rate (Table 4). These results suggest that mutation does not affect a number of agronomic traits of sorghum and there were minimum background mutations in the *msd1* mutant line described here.

Based on the results, *msd1* mutant showed potential as a resource for increasing grain yield as a parent to use for crosses with large-seeded germplasm. Large-seeded sorghum lines have been discovered and are publicly available (Dweikat et al., 2005; Tuinstra et al., 2001). Hybridization of *msd1* with the large-seeded sorghum lines could potentially improve the seed size, and in combination with more than doubling of seed number could lead to increase yield production in sorghum. However, further studies on evaluation of the effects of *msd1* mutation on grain yield per se are needed. To date an assessment of the value of this mutant in applied breeding through cooperative research with public and private breeding programs is ongoing.

Table 4. Comparison of wild-type (WT) and BC_1F_2 multiseeded1 (*msd1*) sorghum mean phenotypic values for agronomic traits, including seed germination rate. Student's *t* test was used to declare significance of difference between mean values from WT and *msd1*. Values in parentheses are standard error of the mean.

Agronomic and yield traits	WT	*msd1*	*P* value	msd as % of WT
				%
% seed set	34.6(0.93)	82.2(2.8)	<0.001**	247
Weight of 100 seeds (g)	3.114(0.05)	1.72(0.27)	<0.001**	48
Total seed weight panicle^{-1} (g)	77.88(8.42)	105.16(7.36)	0.04*	137
Plant height	110.10(1.53)	112.18(1.68)	0.37ns	101.9
Maturity (d)	61.62 (0.42)	61.24(0.53)	0.56ns	99
Germination rate (%)	85.72(1.84)	84.26(1.28)	0.72ns	98

* Significant at 0.05 probability level.

** Significant at 0.01 probability level.

The mechanism by which the development of the pedicellate spikelets is suppressed in sorghum is not clear. Identification of the genes defined by the *msd* mutation will be necessary to elucidate the mechanism. In addition, with gene cloning and identification, perfect molecular markers can be developed that could assist in the introgression of the *msd* trait into elite breeding lines of sorghum. The use of DNA markers for the actual gene will facilitate faster development of hybrids and assessment of the effects of the mutation on yield potential. In addition, it is possible that genetic and molecular information on *msd1* could be utilized to manipulate seed number and possibly yield in other warm-season cereal crops such as corn (*Zea mays* L.) and rice (*Oryza sativa* L.).

In summary, we report here the isolation and characterization of a novel *msd1* mutant of sorghum. This mutant exhibits pedicellate spikelets that develop into seeds, stably increasing seed number to 3× that of WT and further displays increased length of panicle branches and overall larger panicle size. *msd1* also showed increased total seed weight per panicle as compared to WT under limited test. Research on application of this novel class of sorghum mutants for practical breeding for increased grain yield is in progress.

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