

Shortening the Breeding Cycle of Sorghum, a Model Crop for Research

Govinda Rizal, Shanta Karki, Michael Alcasid, Florencia Montecillo, Kelvin Acebron, Nikki Larazo, Richard Garcia, Inez Hortense Slamet-Loedin, and William Paul Quick*

ABSTRACT

Sorghum [*Sorghum bicolor* (L.) Moench] is a model C4 cereal for both basic and applied research. It has most of the traits of a model plant species: large embryos that are easy to rescue, moderate genome size of about 760 Mb, several unique traits not found in other species, plenty of seeds, and many important agronomic as well as commercial uses. However, it takes a long time to complete its breeding cycle. Other problems encountered during the research on sorghum breeding were early desiccation of embryos from mutants and wide hybridization, and the high-yielding cultivars and plants grown in controlled environments are usually unicum, which limits their use in crossing to obtain both selfed and crossed seeds. The objective of this research was to find ways to obtain cross- and self-pollinated seeds from the same plant, conserve the vital embryos, and most important, shorten the breeding cycle. Two methods are reported here. The first method was to produce crossed as well as selfed seeds on the same panicle of the usually uni-culm plant. The second method was to carry out embryo rescue to save vital embryos as well as shorten the breeding cycle from the regular 17 to 11 wk. By these two methods, the breeding cycle of sorghum was made comparable or even shorter than that of other model crops, which would allow the development of breeding materials much faster.

G. Rizal, S. Karki, M. Alcasid, F. Montecillo, K. Acebron, N. Larazo, R. Garcia, and W.P. Quick, C4 Rice Center, International Rice Res. Institute, Los Banos, Laguna, DAPO Box 7777, Metro-Manila, Philippines; I.H. Slamet-Loedin, Plant Breeding, Genetics and Biotechnology Division, International Rice Research Institute, Los Banos, Laguna, DAPO Box 7777, Metro-Manila, Philippines; W.P. Quick, Dep. of Animal and Plant Sciences, Univ. of Sheffield, Sheffield, UK. G. Rizal, and S. Karki contributed equally. Received 19 July 2013. *Corresponding author (w.p.quick@irri.org).

Abbreviations: MS, Murashige and Skoog; PP, polymorphic parents; YCS, Yoshida culture solution.

SORGHUM [*Sorghum bicolor* (L.) Moench] is the first C4 crop and second cereal after rice (*Oryza sativa* L.) to have the whole genome sequenced and made publicly available. It has a moderate genome size of 760 Mb with minimum gene duplication (Patterson et al., 2009). It is a representative species for genomic study of the world's economically important C4 species with large genomes like maize (*Zea mays* L.) (Gaut and Doebley, 1997), sugarcane (*Saccharum officinarum*) (Ming et al., 1998), and switchgrass (*Panicum virgatum* L.) (Sharma et al., 2012). It evolved in high-temperature and drought-prone areas of Central Africa (Kimber et al., 2013); thus, it possesses inherent abilities to survive and excel in extreme temperatures (Peacock, 1982) and under drought conditions (Kharrazi and Rad, 2011) more effectively than other cereals like maize. It is a reservoir of genes for tolerance to stresses like salt (Younis et al., 2007), aluminum (Caniato et al., 2007), drought (Sasaki and Antonio, 2009), and heat (Peacock, 1982). It has a high photosynthetic efficiency and, hence, high productivity. It also harbors genes for higher biomass (Habyarimana et al., 2002) and other yield-related traits (Staggenborg et al., 2008).

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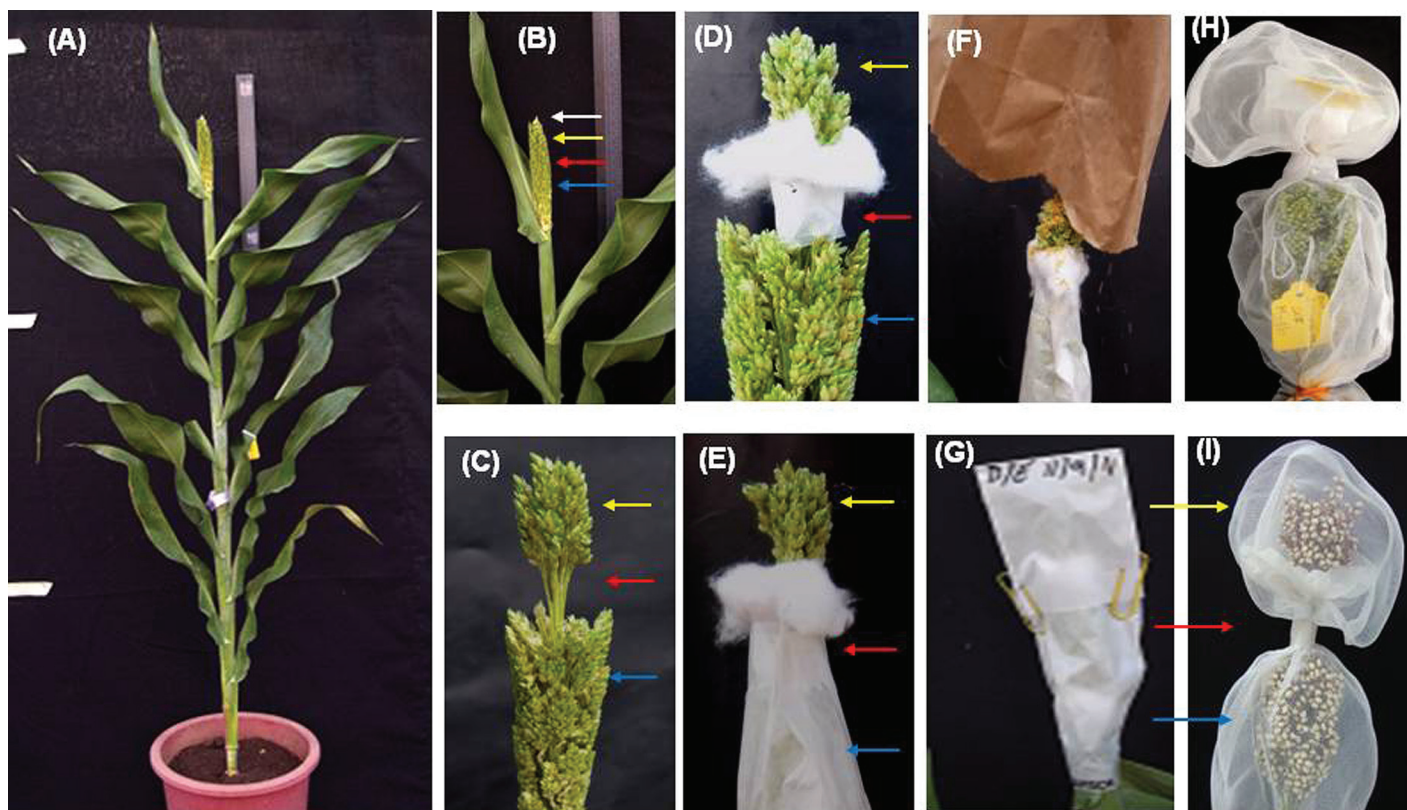


Figure 1. Strategy to produce both hybrid and inbred seeds in one panicle of unicum sorghum plant. (A) A unicum sorghum plant. (B) A panicle was divided into four parts: the tip (P1) shown by white arrow, the second part (P2) shown by yellow arrow, the third part (P3) shown by red arrow, and the last part (P4) shown by blue arrow. (C) Part P1 was trimmed off, the spikelets from P3 were clipped off, and the spikelets in P2 and P4 were retained. (D) The P3 region was covered with fine cotton. This part provides the neck to attach a bag to enclose the P4. (E) P4 was bagged for self-pollination of the spikelets, and the spikelets in the P2 were hand-emasculated. (F) The emasculated spikelets of P2 were sprayed with pollens from selected male parent. (G) The cross-pollinated P2 was separately bagged and kept undisturbed for seed set. (H) After 2 wk of pollination, pollinator bags were replaced with mesh bags. (I) The bag was tied at the neck to avoid mixing of seeds from each part. When the panicles matured, the selfed (P4) seeds were harvested first, and then the crossed (P2) seeds were harvested. The figures are not to scale. The diameter of the pot at its brim was 25 cm, to be considered for comparison of the plants.

Sorghum ranks as the fifth-most important cereal crop in the world (FAO: www.fao.org/docrep/W1808E/w1808e01.htm, accessed 4 Dec. 2013). It is a staple food of people living in the semiarid parts of Africa and India (US Congress, 1988; Deb et al., 2005). It is a natural, gluten-free substitute of wheat to those people suffering from celiac sprue disease, which causes allergy to wheat gluten (Schober et al., 2005). It is grown at a commercial scale for animal feed and biofuel production in the United States, China, and many other countries (Sorghum Handbook, US grain council: www.grains.org/images/stories/technical_publications/Sorghum_Handbook.pdf, accessed 22 Nov. 2013). It provides large amount of lignocellulosic biomass for the production of biofuels on a sustainable basis, so it is proposed as a model crop for the genetic study of biomass and quality in bioenergy grasses (Carpita and McCann, 2008).

Sorghum has several traits that make it an excellent model for the study of monocotyledons, cereals, C4 species, and stress tolerance. These traits include ease to cultivate, large seeds and embryos, ease for crossing (it is self-pollinated and cross-compatible), and grows in a wide

range of soils and environments. Medium to tall plant sizes make the study of phenotypes easier, including the study of leaf veins (Rizal et al., 2012).

In spite of being one of the major crops of the world and an excellent model for research, the use of sorghum in scientific research lags far behind other cereals like maize and rice (Bowers et al., 2003). Moreover, the outcomes of sorghum research have not been used widely in crop improvement compared with other model crops (Rensink and Buell, 2004). This lag in adopting sorghum as a model species is due mainly to its long life cycle. Researchers prefer species with short life cycles, which allow them to test many generations in a short time. For example, *Arabidopsis* requires 8 wk to complete its life cycle from seed to seed, whereas sorghum takes up to 17 wk. Although sorghum produces many large seeds per panicle, the widely cultivated varieties, including the first sequenced sorghum BTx623 and plants grown in controlled conditions, are usually unicum (Fig. 1A). Being uni-culmed limits choice in crossing. Unlike maize, which is also uni-culm but has multiple cobs in different internodes, sorghum produces one

panicle per culm. In breeding plants with single culm, it is not possible to get both inbred and hybrid seeds from a panicle. The problem was even more obvious in mutation breeding, where a large population was required for screening purposes. To produce both hybrids and inbreds, it is necessary to maintain genetic purity and prevent off-types (seeds from unintended pollination). Often the F_1 seeds from hand-emasculated spikelets showed desiccation before maturity. Such seeds needed an immediate rescue.

The objective of this research was to discover ways to obtain self-pollinated as well as cross-pollinated seeds from a single panicle, to shorten the breeding cycle, and optimize methods to rescue vital embryos from mutants and from distant hybridization. Two methods that were developed and optimized to solve the problem and make sorghum a model species for research and breeding are reported here.

MATERIALS AND METHODS

Plant Materials and Their Cultivation

Seeds of *Sorghum bicolor* accession BTx623 that was first sequenced were obtained from two sources and named as BTx623 (R), BTx623 (Z) after the names of the providers. BTx623 (R) was mutagenized with 0.28% ethyl methanesulfonate (EMS), and the mutants were named beginning with R.28. BTx623 (Z) was mutagenized with γ rays of 100, 200, 300, 400, 500, and 600 Gy, and their names begin with M100 to M600, respectively. Seeds of sorghum cultivars BTx623 (R) and BTx623 (Z) as wild types, IS 18551, IS 40653, and *S. propinquum* were used as polymorphic parents (PP) in crosses, and several generations of mutant lines obtained from mutation of BTx623 were grown in field conditions and inside greenhouses in Los Banos, Philippines. The plants were cultivated all year round. Los Banos receives rainfall almost throughout the year. In cases of no precipitation and when grown inside greenhouses, the plants were irrigated using sprinkler irrigation. Plants were grown at a rate of one plant per 8-L-volume pot.

Segregation of a Panicle into Different Parts

Panicles that emerged 3 to 5 cm out of the leaf sheath were selected. The flag leaf sheath was gently removed using sharp scissors and without injuring the stems (Fig. 1A). The panicle head was divided into four parts, designated as P1 to P4 (Fig. 1B). The spikelets in the tip (P1) were trimmed off (Fig. 1C). Spikelets in the second part (P2) were used for emasculation and crossing (Fig. 1E). For emasculation, P2 was left with approximately 100 sessile spikelets. Some sessile spikelets that formed clusters and all the pedicellate spikelets were removed. The spikelets from 2 to 5 cm in P3 region were removed to separate the P2 from P4 (Fig. 1C). Sterilized cotton was used to cover the P3 to provide a soft neck to tie the lower end of the glassine bag covering the P2 and upper end of the bag covering the P4 (Fig. 1D, Fig. 1E, and Fig. 1G). The lowest part (P4) was bagged using waterproof glassine bags to produce inbred seeds (Fig. 1E).

Emasculation and Crossings

Part P2 was emasculated and cross-pollinated to produce hybrid seeds (Fig. 1F). A manual technique of emasculation was adopted.

The spikelets were opened by gently pushing aside glume and palea with the tip of a bamboo splinter designed locally. Three anthers were held from behind and pressed downward without affecting the female part. The fragile filaments broke and the anthers fell off. Care was taken to emasculate all the spikelets by removing all three stamens from each spikelet. Emasculation was done in the afternoon and the pollination performed during the subsequent two or three mornings. Before the first pollination, the emasculated flowers were checked for any unemasculated spikelets. If there were some spikelets with anthers still intact, they were discarded or judiciously removed before pollination.

Several combinations of crossings were performed. Mutants at M_2 generations were crossed with their respective wild types or with other mutant lines of M_2 to M_6 generation. The backcrossed lines were crossed at BC_1F_2 generations. The mutant and backcrossed lines were also crossed with polymorphic parents (Table 1 and Table 2). The immature seeds from the crosses and selfed seeds from the mother plants were rescued to test their germination in medium.

Explant Sterilization for Embryo Rescue

Fertilized spikelets of sorghum, 8 to 12 d after pollination, were harvested for immature embryos (Fig. 2A). The spikelets were washed thoroughly under running tap water and surface-sterilized with 70% ethanol for 1 min. Then, they were placed in 50% sodium hypochlorite with one drop of Tween 20 (Sigma Aldrich), sterilized for 30 min by continuous rotation, and rinsed with sterile distilled water five times or until the water became clear (Fig. 2B). Immature embryos were aseptically removed from the spikelets using forceps and a sharp blade under a dissecting microscope and placed in solid MS (Murashige and Skoog, 1962) medium (Fig. 2D and 2E). The embryo germination medium contained MS basal mineral nutrients and MS vitamins with 3% sucrose. The pH of the medium was adjusted to 5.8, and 2 g/L Gelrite (Sigma) was used as the gelling agent. For rapid germination of the immature embryos without any callus induction, the embryos were placed with the scutellum side touching the medium (Fig. 2F). Up to five immature embryos were placed in a petri dish. Then they were placed in 30°C under continuous light, and the germination and growth conditions were checked regularly (Fig. 2G and Fig. 2H). One week later, the seedlings were transferred to Yoshida culture solution (YCS) (Yoshida et al., 1972) and left to grow for one more week (Fig. 2I). The 2-wk-old seedlings with good root and shoot systems were transplanted into pots containing sterilized soil and fertilized with 10 g of slow releasing 14-14-14 N-P-K fertilizer (Fig. 2J).

RESULTS

Segregated Crossing Strategy to Obtain Hybrid and Inbred Seeds on a Single Panicle

The number of seeds obtained from a cross depended on the type of parent, size of panicle, and the growing condition of the plants. BTx623 had a large panicle, easy-to-emasculate spikelets, and large and easily separable embryos. A sorghum landrace *S. propinquum* had grassy panicles and were difficult to emasculate and to obtain crossed seeds. Fewer seeds were obtained from the crosses, including either

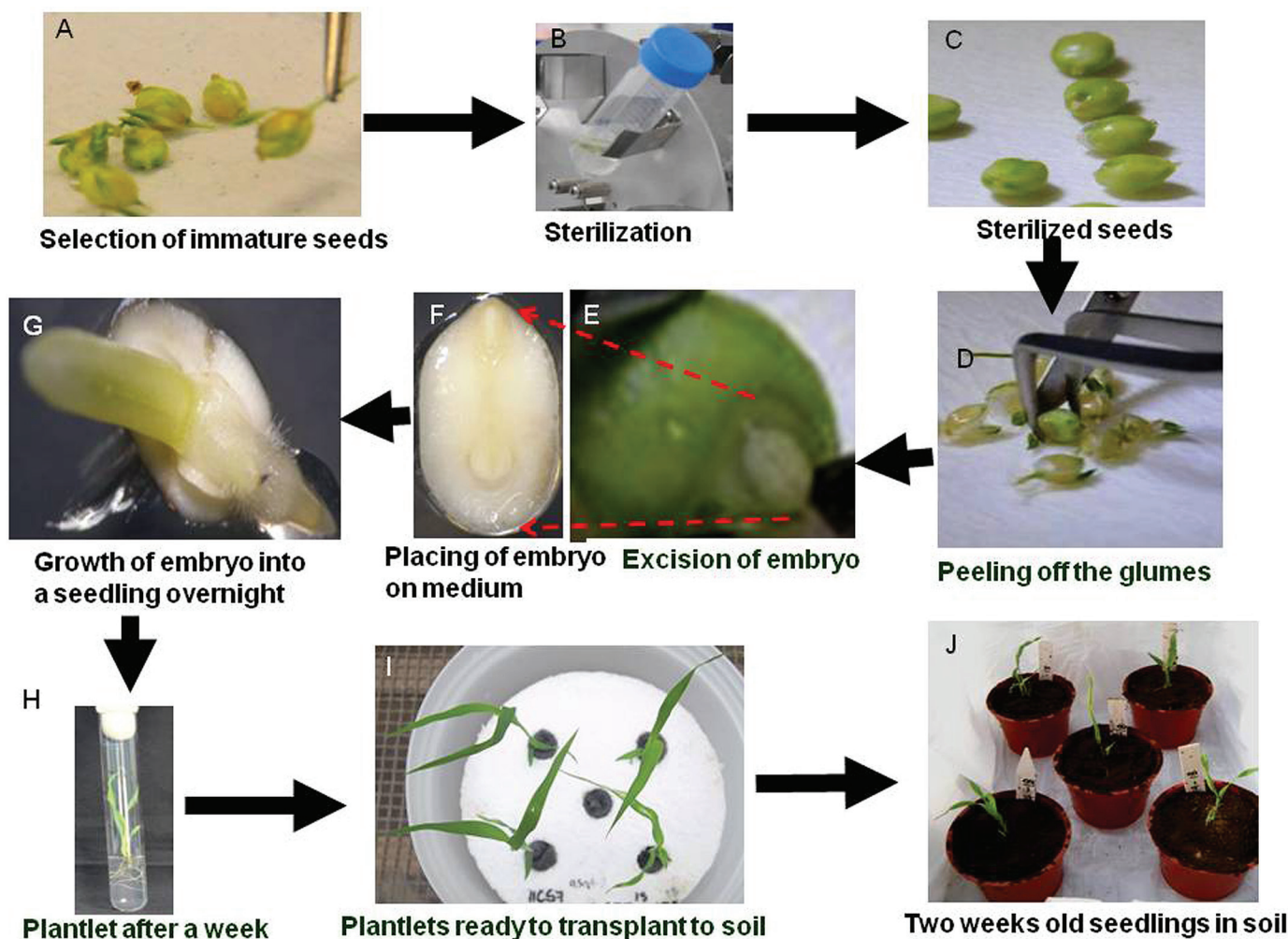


Figure 2. An outline of the embryo rescue process of sorghum. (A) Ten- to 14-d-old embryos were harvested from the panicle. (B) The embryos were sterilized using 50% sodium hypochlorite solution and washed with autoclaved water. (C) The glumes and palea were removed using forceps. (D) All the surrounding covers were removed, leaving only the succulent grain. (E) Embryos were mined from the immature seeds using sterilized forceps and scalpel, inside a clean bench to ensure aseptic condition. (F) Freshly excised embryo was grown in Murashige and Skoog (MS) medium. (G) The embryo grew and differentiated into plumule and radical overnight, in less than 12 h. (H) The embryo may be grown in a petri dish or test tube containing MS medium. (I) Seven-day-old seedlings from MS media were transferred to Yoshida culture solution (YCS), where they grew for next 7 d, before they were transplanted into soil. (J) Seedlings transferred from YCS were grown in individual pots.

one or both parents from the mutant population, because the mutants had smaller panicles. On average, 100 sessile spikelets were left in P2 after emasculation. With this strategy, as many as 109 F_1 and up to 1000 self-pollinated seeds were obtained from a single panicle (Table 1). This number of seeds is enough for genetic analysis and to advance to the next generation. To obtain more crossed seeds, more spikelets can be selected in P2 for emasculation. In some cases, less than 10 F_1 seeds and less than 50 selfed seeds were obtained, which was due to the very small size of panicles and/or low seed-filling rates. In this way, the need to wait for a generation was eliminated. Both selfed and cross-pollinated seeds were used for advancing the generation and for further genetic studies.

Embryo Culture Rescued Valuable Plant Materials

Seeds from most mutants as well as the crossed seeds showed premature desiccation (Fig. 3A and 3B), which could lead to embryo degeneration as the embryo could not be supported by the endosperm that was drying. Growing immature embryos in medium eliminated the exposure to desiccation. The embryos began to develop into roots and shoots within 12 h of germination in media (Fig. 2G and 3C). There were no phenotypic differences between the seedlings from the rescued immature embryos and normally matured seeds (Fig. 4). Embryos less than 10 d old were fragile to handle and susceptible to damage. It was found that immature embryos isolated 10 to 12 d after pollination were the youngest embryos that gave the best results with respect to ease in embryo

Table 1. Summary of crosses made to produce cross- and self-pollinated seeds from a single panicle of sorghum [*Sorghum bicolor* (L.) Moench]. The generations and names of both parents used in the crossing and the number of crossed (F_1) and selfed seeds obtained from the female parent are given for each cross. Mutant (M) populations were obtained from mutation of the elite cultivar BTx623 and advanced to subsequent mutant generations (M_2 to M_6). Sorghum land-race *S. propinquum* and other cultivars such as IS 18551, IS 3620, and IS 40653 were used as polymorphic parents (PP). Two accessions of BTx623 (BTx623 [R] and BTx623 [Z]) were used as wild types (WT). SN, serial number.

SN	Parents used in crossing		F_1 seeds	Selfed seeds	SN	Parents used in crossing		F_1 seeds	Selfed seeds
	Female	Male				Female	Male		
1	M300-18561-02 (M_2)	BTx623 (Z) (WT)	109	177	51	R.28-00413-19 (M_2)	BTx623 (R) (WT)	14	35
2	M300-20208-21 (M_2)	BTx623 (Z) (WT)	99	54	52	R.28-00082-08 (M_2)	BTx623 (R) (WT)	10	24
3	M500-2186-04 (M_2)	BTx623 (Z) (WT)	91	16	53	M400-0715-01-03 (M_3)	M500-0944-19 (M_2)	83	702
4	M300-15204-17 (M_2)	BTx623 (Z) (WT)	90	185	54	M400-0715-01-11 (M_3)	R.28-10989-23-14 (M_3)	68	886
5	M500-2083-17 (M_2)	BTx623 (Z) (WT)	84	101	55	M400-0715-01-16 (M_3)	M300-20208-08-26 (M_3)	3	965
6	M500-2408-12 (M_2)	BTx623 (Z) (WT)	82	171	56	R.28-16899-01-22 (M_3)	M400-0715-01-04 (M_3)	1	19
7	M500-2311-01 (M_2)	BTx623 (Z) (WT)	80	152	57	M400-0715-01-02 (M_3)	R.28-01648-22-11-20 (M_4)	42	889
8	M500-2307-05 (M_2)	BTx623 (Z) (WT)	78	323	58	M400-0715-01-28 (M_3)	R.28-01648-22-11-20 (M_4)	3	409
9	M500-2076-22 (M_2)	BTx623 (Z) (WT)	77	190	59	M400-0715-01-28 (M_3)	IS 40653 (PP)	34	409
10	M300-19695-01 (M_2)	BTx623 (Z) (WT)	76	272	60	R.28-07554-26-9 (M_3)	IS 3620 (PP)	21	79
11	M300-15920-03 (M_2)	BTx623 (Z) (WT)	74	296	61	R.28-10709-10-31 (M_3)	IS 40653 (PP)	18	56
12	M500-2582-07 (M_2)	BTx623 (Z) (WT)	72	228	62	M400-0715-01-04 (M_3)	BTx623 (Z) (WT)	100	656
13	M500-2186-01 (M_2)	BTx623 (Z) (WT)	69	193	63	M300-20208-21-09 (M_3)	BTx623 (Z) (WT)	82	71
14	M500-2305-11 (M_2)	BTx623 (Z) (WT)	69	54	64	M300-20208-8-23 (M_3)	BTx623 (Z) (WT)	78	343
15	M300-17590-15 (M_2)	BTx623 (Z) (WT)	61	41	65	M500-2734-15-10 (M_3)	BTx623 (Z) (WT)	65	207
16	M300-14032-34 (M_2)	BTx623 (Z) (WT)	48	325	66	M300-20208-8-08 (M_3)	BTx623 (Z) (WT)	62	397
17	M300-20208-18 (M_2)	BTx623 (Z) (WT)	45	147	67	M300-20208-8-03 (M_3)	BTx623 (Z) (WT)	58	459
18	M500-2186-17 (M_2)	BTx623 (Z) (WT)	36	76	68	M300-20208-8-19 (M_3)	BTx623 (Z) (WT)	56	351
19	M500-2328-16 (M_2)	BTx623 (Z) (WT)	35	125	69	M400-0715-01-15 (M_3)	BTx623 (Z) (WT)	50	706
20	M400-7754-03 (M_2)	BTx623 (Z) (WT)	31	60	70	M400-0715-01-16 (M_3)	BTx623 (R) (WT)	48	965
21	M500-2308-23 (M_2)	BTx623 (Z) (WT)	30	193	71	M300-20208-8-29 (M_3)	BTx623 (Z) (WT)	41	152
22	M500-2036-24 (M_2)	BTx623 (Z) (WT)	28	141	72	R.28-3521-11-16 (M_3)	BTx623 (R) (WT)	55	64
23	M500-2036-23 (M_2)	BTx623 (Z) (WT)	27	19	73	R.28-00082-08 (M_3)	BTx623 (R) (WT)	46	38
24	M300-15204-17 (M_2)	BTx623 (Z) (WT)	22	185	74	R.28-3521-11-22 (M_3)	BTx623 (R) (WT)	44	24
25	M400-7754-03 (M_2)	BTx623 (Z) (WT)	21	60	75	R.28-10989-23-16 (M_3)	BTx623 (R) (WT)	25	133
26	M500-2076-22 (M_2)	BTx623 (Z) (WT)	21	190	76	R.28-08219-10-16 (M_3)	BTx623 (R) (WT)	20	54
27	M500-2308-23 (M_2)	BTx623 (Z) (WT)	20	193	77	R.28-08219-07-16 (M_3)	BTx623 (R) (WT)	16	38
28	M500-2311-01 (M_2)	BTx623 (Z) (WT)	18	152	78	R.28-16899-01-22 (M_3)	BTx623 (R) (WT)	14	19
29	M500-2328-16 (M_2)	BTx623 (Z) (WT)	18	125	79	R.28-3521-11-25 (M_3)	BTx623 (R) (WT)	9	10
30	M500-2307-05 (M_2)	BTx623 (Z) (WT)	13	323	80	R.28-10989-23-25 (M_3)	BTx623 (R) (WT)	7	20
31	M300-4301-07 (M_2)	BTx623 (Z) (WT)	12	155	81	R.28-08219-07-11 (M_3)	BTx623 (R) (WT)	4	5
32	M500-2582-07 (M_2)	BTx623 (Z) (WT)	9	228	82	R.28-08219-10-24-14 (M_4)	BTx623 (R) (WT)	9	8
33	M300-18561-02 (M_2)	BTx623 (Z) (WT)	8	177	83	R.28-01648-22-01-03 (M_4)	BTx623 (R) (WT)	6	197
34	M500-2408-12 (M_2)	BTx623 (Z) (WT)	8	171	84	R.28-08219-10-24-30 (M_4)	BTx623 (R) (WT)	5	60
35	M500-2036-24 (M_2)	BTx623 (Z) (WT)	6	141	85	M300-20208-21-16-07-04 (M_5)	IS 3620 (PP)	62	726
36	M300-22632-38 (M_2)	BTx623 (Z) (WT)	5	96	86	M300-20208-18-01-06-06 (M_5)	IS 3620 (PP)	49	303
37	M500-2186-01 (M_2)	BTx623 (Z) (WT)	5	193	87	M300-20208-18-01-06-07 (M_5)	IS 3620 (PP)	34	133
38	M300-15920-03 (M_2)	BTx623 (Z) (WT)	1	296	88	M300-20208-18-01-06-09 (M_5)	IS 3620 (PP)	28	231
39	R.28-08235-10 (M_2)	BTx623 (R) (WT)	97	163	89	M300-20208-21-16-07-03 (M_5)	BTx623 (Z) (WT)	71	276
40	R.28-12066-21 (M_2)	BTx623 (R) (WT)	96	103	90	M400-6349-17-03-05-10-09 (M_6)	M300-20208-18-01-06-06 (M_5)	20	32
41	R.28-16899-01 (M_2)	BTx623 (R) (WT)	80	182	91	1CS106-09-129 (BC $_1$ F $_2$)	1CS171-08 (BC $_1$ F $_1$)	12	56
42	R.28-10989-23 (M_2)	BTx623 (R) (WT)	66	65	92	1CS92-04-261 (BC $_1$ F $_2$)	R.28-0709-10-09 (M_3)	11	464
43	R.28-12092-01 (M_2)	BTx623 (R) (WT)	65	294					
44	R.28-07101-19 (M_2)	BTx623 (R) (WT)	60	231					
45	R.28-03277-13 (M_2)	BTx623 (R) (WT)	53	155					
46	R.28-11182-15 (M_2)	BTx623 (R) (WT)	53	41					
47	R.28-10613-08 (M_2)	BTx623 (R) (WT)	49	90					
48	R.28-10709-10 (M_2)	BTx623 (R) (WT)	35	6					
49	R.28-12880-15 (M_2)	BTx623 (R) (WT)	23	35					
50	R.28-13039-36 (M_2)	BTx623 (R) (WT)	23	2					

(cont'd)

Table 1. Continued.

SN	Parents used in crossing		F ₁ seeds	Selfed seeds
	Female	Male		
93	1CS106-09-001 (BC ₁ F ₂)	R.28-01648-22-11-18 (M ₄)	9	270
94	1CS117-05-03 (BC ₁ F ₂)	IS 40653 (PP)	30	572
95	1CS117-05-105 (BC ₁ F ₂)	IS 40653 (PP)	24	6
96	1CS106-09-101 (BC ₁ F ₂)	IS 40653 (PP)	22	175
97	1CS117-05-20 (BC ₁ F ₂)	<i>S. propinquum</i> (PP)	20	335
98	1CS117-05-102 (BC ₁ F ₂)	IS 18551 (PP)	15	443
99	1CS106-09-279 (BC ₁ F ₂)	IS 40653 (PP)	10	19
100	1CS57-02-245 (BC ₁ F ₂)	IS 40653 (PP)	10	7
101	1CS57-02-245 (BC ₁ F ₂)	IS 40653 (PP)	10	7
102	1CS57-02-89 (BC ₁ F ₂)	IS 40653 (PP)	9	11
103	1CS57-02-89 (BC ₁ F ₂)	IS 40653 (PP)	9	11
104	1CS117-05-194 (BC ₁ F ₂)	<i>S. propinquum</i> (PP)	7	54
105	1CS57-02-153 (BC ₁ F ₂)	IS 18551 (PP)	6	10
106	1CS106-09-108 (BC ₁ F ₂)	IS 3620 (PP)	5	113
107	1CS57-02-89 (BC ₁ F ₂)	IS 18551 (PP)	5	11
108	1CS92-02-151 (BC ₁ F ₂)	IS 40653 (PP)	3	162
109	1CS216-07-280-08 (BC ₁ F ₃)	IS 3620 (PP)	12	110

rescue, the rate of germination, and plant development. The germination rates of selfed seeds rescued to hasten the generation were 100% for all lines except one line with BC₁F₂ seeds with 90% germination. The germination rate of crossed seeds rescued to avoid premature desiccation was 100% in 33 of 44 lines. One rescued line had 90%, seven lines had 80%, and two lines had 60% germination. The lowest germination was 40% in one line that came from a cross between two mutant parents (Table 2). The plantlets that were kept in YCS 1 wk after their germination in MS medium developed better shoots and roots and, hence, survived better than plantlets that were directly transplanted to soil immediately after removal from the solid MS medium (Fig. 2J). When plantlets were directly transplanted to soil, the survival rate was 80%, while the survival was 100% when they were transplanted to soil after acclimatization in YCS for a week (Table 2).

Embryo Rescue Shortened Breeding Cycle in Sorghum

To shorten the time of obtaining F₂ plants for analysis, 5 to 10 F₂ immature embryos from each cross were germinated on MS medium 10 to 12 d after pollination (Fig. 4). The F₂ immature embryos gave a 100% germination rate (Table 2). Therefore, immature embryos can be grown in culture medium instead of waiting for the seeds to mature so that genetic analysis can be done earlier. While the unharvested seeds were maturing in the panicle, the seedlings from immature seeds germinated in MS medium were about 1 month old already (Fig. 4). The mature seeds need to be fumigated and dormancy broken, which takes 2 to 3 wk more. Similarly, rescuing F₁ immature seeds hastened the production of F₁ plants.

In most cases, all F₁ immature seeds produced healthy plants, thereby producing F₂ seeds in less than 3 mo (Fig. 4). The direct germination of immature seeds also significantly reduced the time required for the next generation of seed production. Normally it takes 5 to 6 wk more for seeds to mature, harvest, and give postharvest treatments before they can be germinated to produce plants. The embryo rescue method saved a minimum of 6 wk per generation, which adds up to several months in a continuous cycle of breeding. The combination of these two methods significantly shortened the life cycle of sorghum for research. The methods developed in this study are of immense use to the breeders and geneticists who need to cross sorghum and/or want advanced generations in a short time.

DISCUSSION

All plant models for genetic studies have some limitations. In *Arabidopsis*, small seed size and wide variation within seeds add to the limitations in their genetic studies (Herridge et al., 2011). Similarly, rice requires quite a lot of water for cultivation. Although sorghum is a model plant for research (Patterson et al., 2009), the long life cycle restricts its wide usage. Therefore, in this study effort was undertaken to shorten the life cycle of sorghum to make it a preferred model crop.

There is wide variation within the sorghum species (Zheng et al., 2011). The landraces usually have multiple tillers, but the widely cultivated elite cultivars and most mutants had single culm. Lafarge et al. (2002) reported that modern sorghum cultivars grown in field conditions produced from zero to four fertile tillers. Although sorghum plants grown in the field with wide spacing produce a few tillers, those grown in pots and/or greenhouses are usually single culmed. Until now, there has been no mechanism to predict fertile tiller at early stage or to enhance fertility in tillers. Breeders have had to depend on the main tiller for their crossing work. There is wide variation in the architecture of inflorescence, which is associated with grain yield (Hmon et al., 2013). A sorghum plant has an erect stem with a panicle on the top (Fig. 1A). The panicle has spikelets in pairs; the sessile spikelets are hermaphrodite and fertile, whereas the pedicellate spikelets are sterile. Flowering occurs in the morning up to midday, and the anthesis starts from the tip and proceeds in a downward direction along the panicle. Therefore, sorghum is easy to cross-breed compared with other C₄ model species such as *Setaria viridis*. Different methods of emasculation of sorghum flowers are available, but each has its own pros and cons. Warm water treatment of flowers at 50°C for 5 to 10 min is a rapid method. In 5 to 10 min, multiple panicles can be treated, depending on the size of the water container. The warm water treatment of sorghum panicles was not effective in completely sterilizing the anthers. Unlike the emasculation of rice, the vacuum suction method is not effective for sorghum

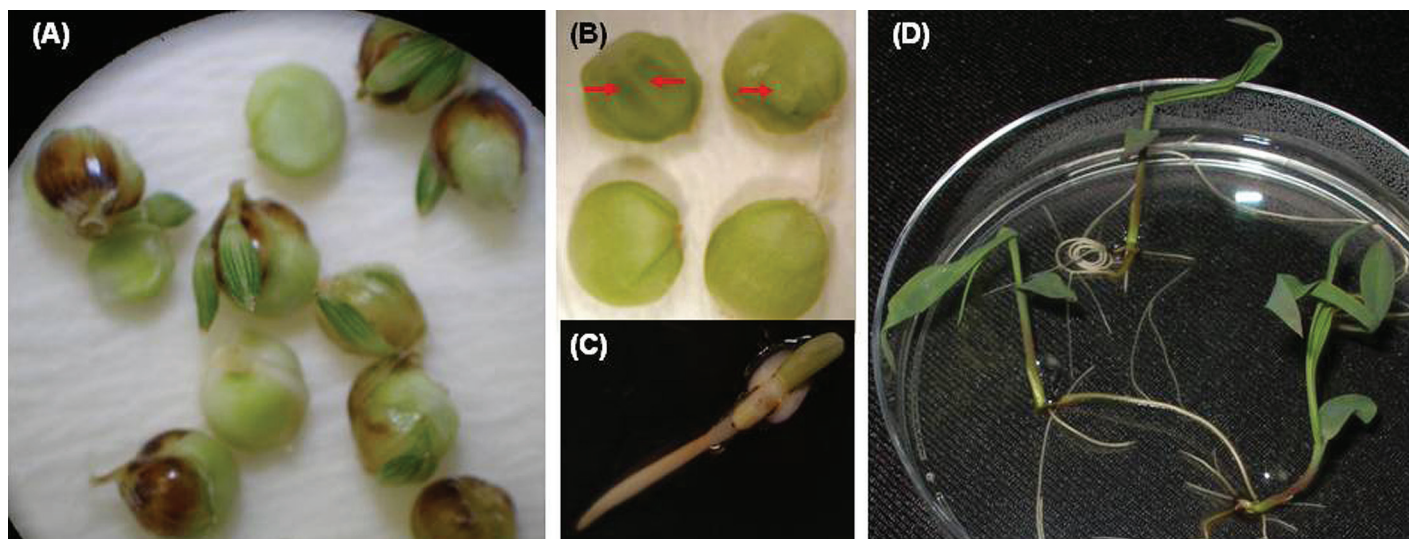


Figure 3. Rescue of embryos from distant hybridization. (A) Immature seeds from hybridization were harvested to rescue them from early desiccation. (B) Ten-day-old immature seeds show signs of desiccation; they formed wrinkles (upper images shown with red arrows). (C) One-night-old seedling from the rescued embryo. (D) Seven-day-old seedlings from the distant hybridization.

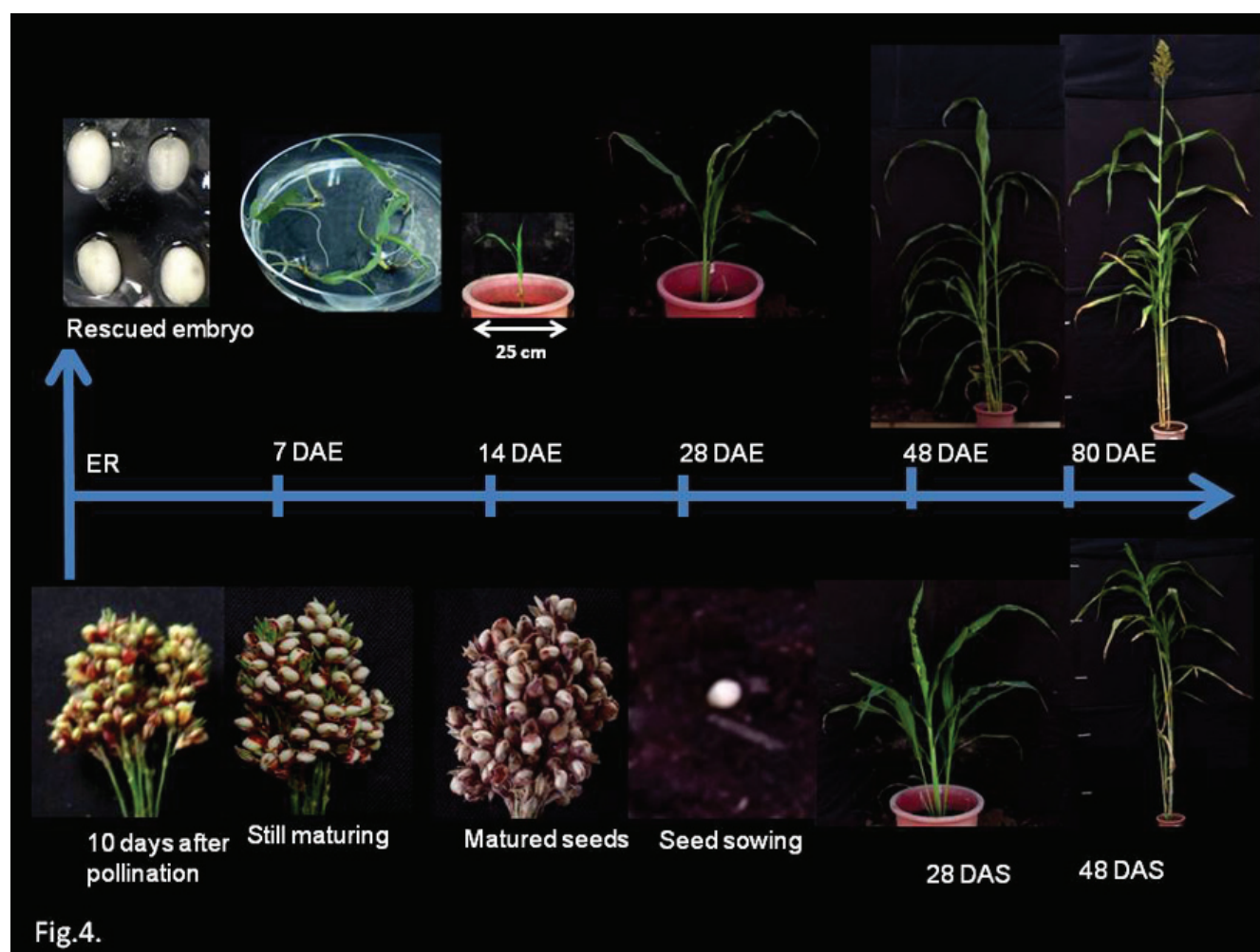


Figure 4. Shortening the breeding cycle of sorghum for research. Crossed seeds from the upper part of the panicle (Fig. 11, P2) were harvested 10 to 12 d after pollination for embryo rescue (upper row), and the remaining seeds were left to mature normally in the panicle (lower row). Embryos were grown in Murashige and Skoog (MS) medium for 1 wk and then in Yoshida culture solution for 1 wk. Then they were transplanted into the soil. All this time the seeds in the panicle passed through normal maturity. When the seeds in the panicle were matured and ready to harvest, the seedlings from immature embryo were 4-wk old already. The embryo rescue technique fastened the breeding cycle. ER, embryo rescue; DAE, days after embryo rescue from immature seeds; DAS, days after sowing of mature seeds.

Table 2. Details of the plant materials used in embryo rescue. Total number of immature seeds harvested for embryo rescue and the germination percentage of the rescued embryos are summarized. The embryos were rescued from immature seeds 10 to 12 d after pollination. SN, serial number; WT, wild type; PP, polymorphic parents.

SN	Parents	Generation	No. of immature seeds used	Germi- nation rate (%)	SN	Plants used in crossing	Female	Male	No. of immature seeds used	Germi- nation rate (%)
A. Selfed immature seeds germinated on Murashige and Skoog (MS) medium to shorten the life cycle					B. Immature F₁ seeds rescued to protect from early desiccation					
1	BTx623 (R)	WT	5	100	1	R.28-16899-1-22 × M400 0715-01-04	M ₃	M ₃	5	100
2	BTx623 (Z)	WT	5	100	2	M400-0715-1-28 × IS 40653	M ₃	PP	5	100
3	IS 18551	PP	5	100	3	M400-0715-01-28 × R.28-01648-22	M ₃	M2	5	100
4	IS 40653	PP	5	100	4	M400-0715-01-26 × 1CS92-04-261	M ₃	BC ₁ F ₂	5	100
5	<i>S. propinquum</i>	PP	5	100	5	M400-0715-01-15 × BTx623 (Z)	M ₃	WT	5	100
6	1CS106-09-108	BC ₁ F ₂	5	100	6	M400-0715-01-15 × BTx623 (Z)	M ₃	WT	5	100
7	1CS106-09-279	BC ₁ F ₂	5	100	7	M400-0715-01-11 × R.28-10989-23-14	M ₃	M ₃	5	100
8	1CS117-05-03	BC ₁ F ₂	5	100	8	M400-0715-01-04 × BTx623 (Z)	M ₃	WT	5	100
9	1CS117-05-102	BC ₁ F ₂	5	100	9	M400-0715-01-03 × M500-0944-19	M ₃	M ₂	5	100
10	1CS117-05-105	BC ₁ F ₂	5	100	10	M400-0715-01-02 × R.28-01648-22-11-20	M ₃	M ₄	5	100
11	1CS117-05-194	BC ₁ F ₂	5	100	11	R.28-08219-10-24-20 × BTx623 (R)	M ₄	WT	5	100
12	1CS117-05-20	BC ₁ F ₂	5	100	12	1CS106-09-127 × 1CS171-07	BC ₁ F ₂	BC ₁ F ₁	5	100
13	1CS117-05-70	BC ₁ F ₂	5	100	13	1CS106-09-01 × R.28 01648-22-11-18	BC ₁ F ₂	M4	5	100
14	1CS117-05-92	BC ₁ F ₂	5	100	14	1CS106-09-261 × R.28 01648-22-11-30	BC ₁ F ₂	M ₄	5	100
15	1CS117-05-92	BC ₁ F ₂	5	100	15	1CS106-09-108 × IS 3620	BC ₁ F ₂	PP	5	100
16	1CS253-4-85-02	BC ₁ F ₂	10	100	16	1CS106-09-279 × IS40653	BC ₁ F ₂	PP	5	100
17	1CS253-4-85-08	BC ₁ F ₂	11	100	17	1CS117-05-03 × IS 40653	BC ₁ F ₂	PP	5	100
18	1CS254-10-78-02	BC ₁ F ₂	11	100	18	1CS117-05-102 × IS 18551	BC ₁ F ₂	PP	5	100
19	1CS252-02-100-06	BC ₁ F ₂	10	90	19	1CS117-05-105 × IS 40653	BC ₁ F ₂	PP	5	100

because of the large size of the anthers. It required much higher pressure, which consumed more energy and generated loud noise. The high suction pressure also affected the style of the female. Hand emasculatation, though more time demanding than other methods, is the most effective method of emasculatation for sorghum flowers. This technique was reliable, highly efficient, and convenient enough to pollinate a portion of the panicle. Sorghum has three large stamens on fragile filaments that were easy to break without damaging the stigma during manual emasculatation. Anthesis along the panicle was not uniform. With segregation for emasculatation, the P2 and P4 clusters were left with spikelets with uniform anthesis time. Now it is possible to get both self- and cross-pollinated seeds from the same panicle in the same season. This will reduce the time as well as prevent genetic contamination. In addition, by removing the nonproductive pedicellate spikelets, the developing seeds received more nutrients.

Embryos from distant hybridizations and from crosses involving mutant parent(s) often do not mature normally. Such embryos were immediately rescued and were given intensive care to prevent desiccation and death. Although sorghum is one of the most recalcitrant plants for tissue culture and plant regeneration (Grootboom et al., 2010), direct germination of immature embryos was possible. The embryos produced vigorous shoots and roots

in 2 wk. The plants from immature embryos have been successfully established in soil. Their phenotypes were normal and grew well (Fig. 4). Both the plants from rescued embryos and the normally matured direct seeded plants had similar amounts of seed production. Therefore, this method produced healthy plantlets from immature embryos. This protocol has been optimized using culture medium first described by Inagaki and Mujeeb-Kazi, (1995). It has proven efficient under different cases, thereby offering an opportunity to shorten the breeding cycle. This step reduced the time from seed maturity to

(cont'd)

Table 2. Continued.

SN	Plants used in crossing	Female	Male	No. of immature seeds used	Germination rate
20	1CS117-05-194 × <i>S. propinquum</i>	BC ₁ F ₂	PP	5	100
21	1CS117-05-20 × <i>S. propinquum</i>	BC ₁ F ₂	PP	5	100
22	1CS117-05-70 × IS 18551	BC ₁ F ₂	PP	5	100
23	1CS57-02-251-05 × IS 40653	BC ₁ F ₂	PP	5	100
24	1CS92-04-131 × <i>S. propinquum</i>	BC ₁ F ₂	PP	5	100
25	1CS92-04-162 × IS 3620	BC ₁ F ₂	PP	5	100
26	1CS92-04-287 × <i>S. propinquum</i>	BC ₁ F ₂	PP	1	100
27	1CS92-04-67 × IS 40653	BC ₁ F ₂	PP	5	100
28	1CS117-05-92 × BTx623 (R)	BC ₁ F ₂	WT	5	100
29	1CS117-05-92 × BTx623 (R)	BC ₁ F ₂	WT	5	100
30	1CS92-01-151 × BTx623 (R)	BC ₁ F ₂	WT	5	100
31	1CS92-02-152 × BTx623 (R)	BC ₁ F ₂	WT	5	100
32	1CS92-04-106 × Rooney	BC ₁ F ₂	WT	5	100
33	1CS92-04-124 × BTx623 (R)	BC ₁ F ₂	WT	5	100
34	1CS92-04-60 × BTx623 (R)	BC ₁ F ₂	WT	10	90
35	1CS92-04-261 × R.28-10709-10-09	BC ₁ F ₂	M ₃	5	80
36	1CS106-09-127 × IS 18551	BC ₁ F ₂	PP	5	80
37	1CS92-04-202 × <i>S. propinquum</i>	BC ₁ F ₂	PP	5	80
38	1CS92-04-287 × <i>S. propinquum</i>	BC ₁ F ₂	PP	10	80
39	1CS92-04-67 × IS 40653	BC ₁ F ₂	PP	5	80
40	1CS92-04-106 × BTx623 (R)	BC ₁ F ₂	WT	5	80
41	1CS92-04-167 × BTx623 (R)	BC ₁ F ₂	WT	5	80
42	1CS92-04-207 × IS 40653	BC ₁ F ₂	PP	5	60
43	1CS92-04-287 × <i>S. propinquum</i>	BC ₁ F ₂	PP	5	60
44	M400-0715-01-16 × M300-20208-08-26	M ₃	M ₃	5	40

harvest to germination by 6 wk. The embryo rescue in each generation will greatly shorten the time required to obtain homozygous lines.

A model species should also be easily transformable to test gene functions. For sorghum, highly efficient transformation using the microprojectile bombardment system and *Agrobacterium*-mediated transformation are available (Liu and Godwin, 2012; Zhao et al., 2000). These methods, along with methods to shorten the breeding cycle and availability of whole genome sequences, make *S. bicolor* an efficient model crop for research.

CONCLUSION

Sorghum bicolor can be an excellent model system to study C4 photosynthesis, food crops, biofuel crops, and a representative of large genomes like sugarcane and switchgrass because it is easy to produce different types of mapping populations and breeding materials. The techniques developed in this study to obtain both selfed and crossed seeds from a plant in the same season and with high-germination efficiency of the immature embryos shortened the breeding cycle. Because of its big plant size and wide leaves, it is suitable for physiological measurements, leaf anatomical analysis, and growth and developmental studies. Use of techniques developed in this study and comparative genomic approaches to sorghum research opens opportunities for discovering genetic factors related to C4 photosynthesis and valuable agronomic traits.

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