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# Fertility Restoration of the Sorghum A3 Male-Sterile Cytoplasm through a Sporophytic Mechanism Derived from Sudangrass

Hoang V. Tang, Jeffrey F. Pedersen, Christine D. Chase, and Daryl R. Pring\*

## ABSTRACT

Fertility restoration of sorghum [*Sorghum bicolor* (L.) Moench] lines carrying the IS1112C (A3 group) male-sterile cytoplasm has been documented as a two-gene gametophytic mechanism involving complementary action of restoring alleles designated *Rf3* and *Rf4*, as derived from IS1112C. Fertility restoration capability has also been reported from sudangrass (*S. bicolor* subsp. *drummondii*) populations. We describe characteristics of a fertility restoration system derived from sudangrass, in which male-sterile individuals were observed at high frequency in backcross and  $F_2$ - $F_3$  segregating populations. Segregation analyses were consistent with a sporophytic restoration system involving two complementary genes. Pollen iodine staining in fertile progeny indicated that the restorers were not efficacious, and fertility was decreased in progeny of backcrosses. Silencing of restoring alleles through paramutation might be operative in these examples. Sudangrass-derived fertility restoration did not involve enhanced transcript processing of the chimeric mitochondrial open reading frame *orf107*. Thus male sterility induced by the A3 cytoplasm can be restored through different mechanisms.

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**Abbreviations:** CMS, cytoplasmic-nuclear male sterility; F, male-fertile; S, male-sterile; TPA, transcript processing activity.

**H**YBRID GRAIN sorghum [*Sorghum bicolor* (L.) Moench] production in the USA is essentially dependent on one source of cytoplasmic-nuclear male sterility (CMS), the milo or A1 source (Pring et al., 1995; Schertz et al., 1997). Seven male-sterile cytoplasm groups have been defined based on differential fertility restoration requirements (Xu et al., 1995; Schertz et al., 1997). Among these groups is sterility conferred by cytoplasm of the line IS1112C (type member, A3 group). This source of CMS has not been commercially utilized for hybrid seed production because of the low frequency of restorer genes among sorghum lines and lines developed through the Sorghum Conversion Program (Worstell et al., 1984; Bosques-Vega et al., 1989; Torres-Cardona et al., 1990; Schertz et al., 1997; Kuhlman et al., 2006). Genetic analyses of fertility restoration in the A3 source of CMS lead to the determination that restoration conferred by the male-fertile source of the cytoplasm, IS1112C, was through a gametophytic mechanism requiring complementary action of two restoring alleles designated *Rf3* and *Rf4* (Tang et al., 1996b, 1998; Pring et al., 1999). In gametophytic restoration, the haploid pollen must carry restoring alleles to function. Only restoring

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alleles are transmitted through the paternal parent, and all resulting progeny are male fertile.

The mitochondrial chimeric open reading frame *orf107* is associated with CMS conferred by IS1112C (Tang et al., 1996b, 1998). In male-fertile  $F_1$  populations of A3Tx398/IS1112C and A3Tx7000/IS1112C, an enhanced transcript processing activity (TPA) is evident, cleaving about 75% of *orf107* transcripts within the open reading frame, reducing the abundance of full-length transcripts available for possible translation (Tang et al., 1996a, 1998). Segregation analyses indicated that enhanced TPA was required for fertility restoration, leading to the conclusion that enhanced TPA was tightly linked to, or represented action of, a restoring allele, which was assigned to *Rf3* (Tang et al., 1998; Pring et al., 1999). Enhanced *orf107* TPA is linked to the single dominant gene *Mmt1*, which confers enhanced transcript processing 5' to the mitochondrial gene *atp4* (*orf25*); IS1112C is *Mmt1*, while A3Tx398 is *mmt1* (Tang et al., 1996b, 1998). Designation of the restoring alleles as dominant is tentative because restoration occurs in haploid tissues. No mechanism has been established for *Rf4* in restoration, but the *rf4* locus has been mapped and assigned to linkage group E, or chromosome 7 (Wen et al., 2002; Kim et al., 2005).

Fertility restoration capability for the A3 male-sterile cytoplasm has also been identified in some populations of sudangrass (*S. bicolor* subsp. *drummondii*) (Pedersen and Toy, 1997), wherein 14% seed set was observed in A3Tx398  $\times$  sudangrass  $F_1$  hybrids. Additionally, fertility restoration of A3 male-sterile cytoplasm was observed in a small number of lines (4 of 1007) derived from open-pollinated progeny of A3Tx398 and A3KS57  $F_1$  hybrids produced in near proximity to shattercane (*S. bicolor* subsp. *drummondii*) (Pedersen et al., 2003).

We report here the recovery and characterization of fertility restoration capability for the A3 male-sterile cytoplasm resulting from bulk pollinations with the NP28 and NP35 sudangrass populations. The molecular and genetic features of sudangrass-derived restoration are distinct from those of the two-gene gametophytic restoration mechanism conferred by IS1112C.

## MATERIALS AND METHODS

All data reported here were obtained from plants grown in field plots near Gainesville, FL, during the period 1997–2006. Panicles to be used for crosses were bagged on emergence. Plants to be used for backcrossing to A3Tx398 and for self-pollination were selected after pollen iodine staining. Since starch amylose deposition in pollen grains is initiated about 72 h before anthesis (Pring and Tang, 2004), pollen was collected and stained with iodine about 24 h before anther exertion. Seed set under pollinating bags was visually estimated at maturity.

## Generation of Self and Backcross Populations

Sudangrass individuals from the populations NP28 (Gorz et al., 1990a) and NP35 (Gorz et al., 1990b) that restored fertil-

ity to A3 CMS were identified by crossing them to A3Tx398 or A3Tx430 and observing subsequent seed set at Lincoln, NE. These individuals were simultaneously self-pollinated by covering panicles with pollinating bags before anthesis. Two  $S_1$  (male-sterile) lines from parents with A3 CMS restoration capability were identified from each population and designated NP28 $S_1$ -1, NP28 $S_1$ -2, NP35 $S_1$ -1, and NP35 $S_1$ -2.

To capture and characterize this new source of restoration, these  $S_1$  lines were grown and utilized for bulk pollinations of A3Tx398 at Gainesville, FL. Five fertile  $F_1$  populations were grown and self-pollinated, and 193  $F_2$  panicles were scored for fertile:sterile plants. Pollen staining of 80 plants from the  $F_2$  families revealed numerous individuals with >95% staining, and one such individual was selected for advance. Among individuals in this  $F_3$  population, two plants with 95% stained pollen were self-pollinated and crossed to A3Tx398. These plants were designated 14M18 and 14M21.

## Development of 14M18 Progeny

The 14M18 individual was self-pollinated, generating the  $F_4$  population designated S04-28. A3Tx398 was pollinated with 14M18, generating a  $BC_1F_1$  population; among these plants, an individual designated 24-65 was selected and used to pollinate A3Tx398, generating the  $BC_2F_1$  S04-29. Individual 24-65 was also self-pollinated, generating the  $BC_1F_2$  population S04-30. Two individuals from the  $BC_1F_2$  S04-30 population with greater than 80% pollen staining were selected for self-pollination and backcrossing to A3Tx398. One of those individuals, S04-30-69, was self-pollinated to generate the  $BC_1F_3$  population S05-35. A backcross to A3Tx398 generated the  $BC_2F_1$  population S05-36. The second individual from the  $BC_1F_2$  S04-30, S04-30-200 was self-pollinated to generate the  $BC_1F_3$  population S05-37. A backcross to A3Tx398 generated the  $BC_2F_1$  population S05-38.

The  $BC_1F_3$  population S05-37 was examined for pollen staining, and 16 individuals were self-pollinated to generate  $BC_1F_4$  families. These families were grown, and 8 to 10 individuals in each family were examined for pollen staining and seed set. Two individuals from one family, each with 100% pollen staining, were backcrossed to A3Tx398, generating two  $BC_2F_1$  populations, and self-pollinated to generate two  $BC_1F_5$  lines.

## Development of 14M21 Progeny

The 14M21 individual was self-pollinated to generate a  $F_4$  population. A3Tx398 was pollinated with 14M21, generating the  $BC_1F_1$  population S03-25. Among the S03-25 population, an individual, designated 25-69 was identified, with 80% pollen staining and 30% seed set. A3Tx398 was pollinated with 25-69, generating the  $BC_2F_1$  population S04-26. A second backcross was made with another individual, designated 25-72, which had 80% pollen staining and 90% seed set. This cross generated the  $BC_2F_1$  population S04-27.

## Transcript Analyses

Plants near anthesis were examined for iodine pollen staining. Leaf RNA from fertile plants was prepared as described (Tang et al., 1998), and RNA from the equivalent of 2.5 g fresh weight was electrophoresed in agarose gels, blotted to membranes, and hybridized as described (Tang et al., 1996a). The membranes were probed with the *orf107* clone pHC104, which spans *orf107*

**Table 1. Percentage seed set among lines derived from pollination of A3Tx398 with the individuals 14M18 and 14M21, and derived progenies. Values are numbers of plants with estimated percentage seed set. For comparison, the  $F_1$  A3Tx398/IS1112C is included. Line designations and identifications are described in the text.**

	Percentage seed set													Total	F:S	Mean seed set
	100	90	80	70	60	50	40	30	20	10	5	1	0			
M18 lines																
F <sub>4</sub> (S04-28)		22	17	4	1	4	3	1	2	1	4	2	31	92	61:31	67
BC <sub>2</sub> F <sub>1</sub> (S04-29)			1	1	2	1	0	3			1	2	48	59	11:48	38
BC <sub>1</sub> F <sub>2</sub> (S04-30)	2	18	10	2	1	6	1	1	4	3	1	5	34	88	54:34	61
BC <sub>1</sub> F <sub>3</sub> (S05-35)	1	16	9	2	1	2	2	3	3	2	1		29	71	42:29	67
BC <sub>2</sub> F <sub>1</sub> (S05-36)				2		1		1	2			3	47	56	9:47	29
BC <sub>1</sub> F <sub>3</sub> (S05-37)	5	26	9	4	1	2	1	1	1	1		1	20	72	52:20	79
BC <sub>2</sub> F <sub>1</sub> (S05-38)			2	1		1		2		1		1	56	64	8:56	44
BC <sub>2</sub> F <sub>1</sub> (S06-17)		1	8	2		3		1					0	15	15:0	70
BC <sub>2</sub> F <sub>1</sub> (S06-19)		4	6	1	1	2							0	14	14:0	71
M21 Lines																
F <sub>4</sub> (S01-30)	10	6	7			8		2				3	3	39	36:3	71
F <sub>4</sub> (S04-25)		5	10	2	2	4	2		2	1		1	1	30	29:1	64
BC <sub>1</sub> F <sub>1</sub> (S03-25)	17	16	10	4	6	6		1	2					62	62:0	83
BC <sub>2</sub> F <sub>1</sub> (S04-26)			1			1			1			2	26	31	5:26	30
BC <sub>2</sub> F <sub>1</sub> (S04-27)												2	38	40	2:38	1
A3Tx398/IS1112C																
F <sub>1</sub> (S00-13)	[>90; 37]		44	36	19	15	13	13	9	8	[<10; 10]		1	205	204:1	61

and carries sequences of *atp9* (Tang et al., 1996b). Membranes were also probed the clone pHT160 to detect activity of the single dominant gene *Mmt1*, which is tightly linked or allelic to enhanced *orf107* TPA, and confers enhanced TPA 5' to the mitochondrial gene *atp4* (*orf25*) (Tang et al., 1996a, 1998).

## RESULTS

### Evaluation of Progenies Derived from Self-Pollinations

Scoring of seed set in 42  $F_1$ 's, representing 6 to 8 plants from each of the six crosses of A3Tx398 with NP28S<sub>1</sub>-1, NP28S<sub>1</sub>-2, NP35S<sub>1</sub>-1, and NP35S<sub>1</sub>-2, revealed no seed set in 26 panicles and 5- to 100% seed set in the remaining 16 panicles. Therefore individuals within the four S<sub>1</sub> sudangrass lines transmitted fertility restoration capability, which was observed in  $F_1$ 's from five of the six original crosses to A3Tx398.  $F_2$  families from each of the five fertile  $F_1$ 's were scored for fertile:sterile plants. The five  $F_2$  families segregated 36F (fertile):19S (sterile), 27F:10S, 31F:9S, 20F:6S, and 22F:13S plants. The high frequency of male-sterile plants observed, 22 to 37%, is consistent with a sporophytic restoration mechanism, and not a gametophytic mechanism, wherein male-sterile plants are extremely rare among  $F_2$ 's resulting from pollination of A3Tx398 with IS1112C (Tang et al., 1996a, 1998; Pring et al., 1999). Examinations of 80 plants from these  $F_2$  families revealed 66 with stained pollen, consistent with seed set data. Of the 66 plants, 41 exhibited 50 to 100% stained pollen, indicating that efficacy of the restoration mechanism was such that not all pollen grains were apparently viable.

### Evaluation of 14M18 Progeny

The 14M18  $F_4$  population designated S04-28 segregated 61F:31S plants (Table 1), consistent with an  $F_3$  parent heterozygous for fertility restoration alleles. The mean seed set was 67%, skewed toward a high percentage seed set, but seven individuals were scored at less than 10% seed set. We evaluated these data in comparison to fertility restoration conferred by IS1112C by scoring 205  $F_1$ 's resulting from pollination of A3Tx398 with IS1112C (Table 1). These heterozygous *Rf3rf3Rf4rf4*  $F_1$ 's are predicted to shed 25% viable pollen (Tang et al., 1998; Pring et al., 1999) and exhibit approximately 50% seed set (Worstell et al., 1984). We observed 61% mean seed set in this  $F_1$  population, which included 10 plants with less than 10% seed set, and one sterile plant. Thus, highly variable percentage seed set was observed within an  $F_1$  population that is assumed to genetically homogeneous. This variability in seed set was similar to that observed within the  $F_4$  S04-28. Consequently we included individuals with very low seed set resulting from sudangrass-derived restoration in the "fertile" category. Chi-square analysis of the  $F_4$  S04-28 population indicated that the segregation ratio fit 3:1 and 9:7 ratios (Table 2).

Pollination of A3Tx398 with 14M18 generated a  $BC_1F_1$  population. Among 19 of 27 fertile plants examined from this population, 10 had 70 to 90% stained pollen while 9 had less than 60% staining. Thus, the population included many individuals with pollen staining clearly exceeding the expected 25% stained pollen associated with the gametophytic restoration mechanism conferred



**Table 2. Analyses of segregation of male-fertile and male-sterile A3 cytoplasm sorghum backcross and selfed lines resulting from pollination with sudangrass populations. Line designations are described in the text. The 13:3 value of S05-37 (3.85) is significant only if Yate's correction (Yates, 1934) is used. Ratio of fertile:sterile plants is F:S.**

	F:S	Ratio tested							
		3:1	9:7	13:3	15:1	63:1	1:1	1:3	1:7
M18 Lines									
F <sub>4</sub> (S04-28)	61:31	3.71	3.78	**	**	**			
BC <sub>2</sub> F <sub>1</sub> (S04-29)	11:48						**	1.27	2.04
BC <sub>1</sub> F <sub>2</sub> (S04-30)	54:34	**	0.94	**	**	**			
BC <sub>1</sub> F <sub>3</sub> (S05-35)	42:29	**	0.24	**	**	**			
BC <sub>2</sub> F <sub>1</sub> (S05-36)	9:47						**	2.38	0.65
BC <sub>1</sub> F <sub>3</sub> (S05-37)	52:20	0.30	**	*(3.85)	**	**			
BC <sub>2</sub> F <sub>1</sub> (S05-38)	8:56						**	*	0.00
BC <sub>2</sub> F <sub>1</sub> (S06-17)	15:0								
BC <sub>2</sub> F <sub>1</sub> (S06-19)	14:0								
M21 Lines									
F <sub>4</sub> (S01-30)	36:3	*	**	3.13	0.14	**	**		
F <sub>4</sub> (S04-25)	29:1	**	**	*	0.44	0.65			
BC <sub>1</sub> F <sub>1</sub> (S03-25)	62:0								
BC <sub>2</sub> F <sub>1</sub> (S04-26)	5:26						**	1.30	0.37
BC <sub>2</sub> F <sub>1</sub> (S04-27)	2:38						**	**	2.06

\*Significant at the 0.05 probability level.

\*\*Significant at the 0.01 probability level.

by IS1112C (Tang et al., 1998; Tang and Pring, 2003). The population segregated for fertile and sterile plants, but we were unable to obtain an accurate ratio because of extensive tillering. Fertile plants in this population had a mean seed set of 77%. Analysis of the BC<sub>2</sub>F<sub>1</sub> S04-29 population indicated 11F:48S plants, with a highly variable percentage seed set among the fertile plants, ranging from 1 to 80% (Table 1). Chi-square analysis indicates that the data fit 1:3 and 1:7 ratios (Table 2). Examinations of 14 plants revealed a low percentage of stained pollen, with only 1 plant carrying 50% stained pollen, in contrast to the BC<sub>1</sub>F<sub>1</sub>. The BC<sub>1</sub>F<sub>2</sub> S04-30 population segregated 54F:34S, with seed set among the fertile plants skewed toward high percentages (Table 1). Chi-square data indicates that the data fit a 9:7 ratio (Table 2). The BC<sub>1</sub>F<sub>3</sub> S05-35 population segregated 42F:29S, with seed set distribution among the fertile plants similar to that of the parental BC<sub>1</sub>F<sub>2</sub> S04-30 (Table 1). Chi-square analyses fit a 9:7 ratio (Table 2), as did the data from the parental BC<sub>1</sub>F<sub>2</sub> S04-30. The BC<sub>2</sub>F<sub>1</sub> S05-36 population segregated 9F:47S (Table 2), which fit 1:3 and 1:7 ratios, paralleling data derived from the BC<sub>2</sub>F<sub>1</sub> S04-29. Among the nine fertile BC<sub>2</sub>F<sub>1</sub> S05-36 plants, percentage seed set was 1 to 70%, with a distribution similar to that of the BC<sub>2</sub>F<sub>1</sub> S04-29. Thirty-six BC<sub>2</sub>F<sub>1</sub> S05-36 individuals were examined for pollen staining, and only two showed at least 50% stained pollen, similar to data from the BC<sub>2</sub>F<sub>1</sub> S04-29 population.

The BC<sub>1</sub>F<sub>3</sub> S05-37 population (derived from a second BC<sub>1</sub>F<sub>2</sub> S04-30 individual) segregated 52F:20S with a 79% percentage seed set skewed toward high percentages (Table 1). The segregation data fit a 3:1 and a 13:3 (two-gene) model, the latter only if Yates (1934) correction was utilized (Table 2). The corresponding A3Tx398 backcross BC<sub>2</sub>F<sub>1</sub> S05-38 population segregated 8F:56S, with a seed set pattern similar to other backcross F<sub>1</sub>'s examined (Table 1). These segregation data fit only a 1:7 ratio (Table 2). Forty-six BC<sub>2</sub>F<sub>1</sub> S05-38 individuals were examined, and only 5 had at least 50% stained pollen. Thus, each of three BC<sub>2</sub>F<sub>1</sub> progenies had a lower percentage stained pollen percentage than the BC<sub>1</sub>F<sub>1</sub>.

Seed set among 14M18 progenies exhibited a trend toward higher percentage seed set among fertile members of the F<sub>2</sub> and F<sub>3</sub> populations (S04-30, 61%; S05-35, 67%; S05-37, 79%), similar to the parental population (S04-28, 68%), than observed for the backcross BC<sub>2</sub>F<sub>1</sub> populations (S04-29, 38%; S05-36, 29%; S05-38, 44%) (Table 1).

To develop lines homozygous for the putative restorers we advanced the BC<sub>1</sub>F<sub>3</sub> population S05-37 by selection and self-pollination. Among 16 BC<sub>1</sub>F<sub>4</sub> families examined, only 1 family showed no segregation for sterile plants. Two members of this family were self-pollinated, and each of 25 and 26 progeny from the two BC<sub>1</sub>F<sub>5</sub> lines showed 100% pollen staining, indicating that the parental plants were homozygous for the restoring alleles. The two parental plants were backcrossed to A3Tx398, generating BC<sub>2</sub>F<sub>1</sub>'s S06-17 and S06-19. Pollen staining of S06-17 showed 5 plants with 50% staining and 11 plants with 75% staining, while S06-19 showed 3 plants with 25% staining, 11 plants with 50% staining, and 2 plants with 75% staining. Staining patterns of these F<sub>1</sub>'s, specifically members of the populations with greater than 50% pollen staining, do not resemble characteristics of gametophytic restoration conferred by IS1112C (Tang et al., 1998; Tang and Pring, 2003). Seed set in the two BC<sub>2</sub>F<sub>1</sub>'s was 70 and 71% (Table 1), with no male-sterile plants.

## Evaluation of 14M21 Progeny

Evaluations of 14M21 progeny were more limited because variability among the progenies examined did not allow consistent interpretations in terms of the characteristics of fertility restoration. The F<sub>4</sub> was evaluated in two separate years, and progeny segregated 36F:3S and 29F:1S, respectively, with highly variable seed set averaging 72 and 64% (Table 1). Segregation ratios fit 13:3 and 15:1 ratios for the first year data, and 15:1 and 63:1 ratios for the second-year data (Table 2). Sixty-two plants were evaluated in the BC<sub>1</sub>F<sub>1</sub> S03-25 population; all were fertile with an average of 83% seed set (Table 1). Sixty-one were examined for pollen staining, and 49 had 50 to 100% stained pollen, similar to staining data from the BC<sub>1</sub>F<sub>1</sub> developed from 14M18.

Subsequent backcrosses, however, provided variable results. Among the BC<sub>1</sub>F<sub>1</sub> S03-25 progeny, the individual 25-69 had 80% pollen staining and 30% seed set.

When A3Tx398 was pollinated with 25-69 to generate the BC<sub>2</sub>F<sub>1</sub> S04-26, the resultant population segregated 5F:26S, and the five fertile plants had only 30% seed set (Table 1). Chi-square analyses indicate that these data fit 1:3 and 1:7 models (Table 2). A second backcross was made with individual 25-72, which had 80% pollen staining and 90% seed set, to generate the BC<sub>2</sub>F<sub>1</sub> S04-27, which segregated 2F:38S, fitting a 1:7 ratio, and the two fertile plants had an estimated 1% seed set (Table 2), rendering these of questionable value. Progeny from these two backcrosses thus had low seed set percentages, paralleling observations of backcrosses with the 14M18 populations.

## Transcript Analyses

We examined individuals and populations derived from the sudangrass pollinations for enhanced *orf107* TPA, thought to be tightly linked to, or to represent action of the *Rf3* restoring allele (Tang et al., 1996b, 1998; Pring et al., 1999). Members of the original F<sub>1</sub> population resulting from pollination of A3Tx398 with the sudangrass lines NP28S<sub>1</sub>-1, NP28S<sub>1</sub>-2, NP25S<sub>1</sub>-1, and NP35S<sub>1</sub>-2, and bulk seedlings of the F<sub>2</sub>'s, were scored for enhanced *orf107* TPA and for activity of *Mmt1*. None of the lines exhibited enhanced *orf107* TPA (not shown), indicating that the restoration mechanism did not involve altered *orf107* transcripts. Some of the F<sub>1</sub> individuals exhibited *Mmt1*, indicating that the sudangrass populations were heterogenous for the presence of *Mmt1* (data not shown).

Six fertile members of advanced progenies derived from 14M18, selected by scoring for percentage stained pollen before leaf RNA isolation, were individually examined for enhanced *orf107* TPA. Two individuals from the BC<sub>1</sub>F<sub>1</sub> S05-37 (Fig. 1A, F), three individuals from the BC<sub>2</sub>F<sub>1</sub> S05-38 (Fig. 1B, C, G), and one individual from the BC<sub>2</sub>F<sub>1</sub> S05-36, each exhibited abundant 1110-, 870-, and 810-nt whole-length (Tang et al., 1996a) *orf107* transcripts, along with a trace of the processed 380-nt transcript resulting from basal level processing. None of the individuals showed evidence of enhanced TPA resulting in the accumulation of the 380-nt product, such as shown for a male-fertile BC<sub>15</sub>F<sub>2</sub> derived from A3Tx398/IS1112C (Pring and Tang, 2004; Pring et al., 2006)(Fig. 1D).

Six fertile members of the 14M18-derived populations were also examined for action of *Mmt1*, linked to enhanced *orf107* TPA in IS1112C (Tang et al., 1996a, 1998), which conditions enhanced cleavage of a 1044-nt *atp4* transcript, generating a 832-nt transcript. A male-fertile *Mmt1* BC<sub>11</sub>F<sub>3</sub> resulting from pollination of A3Tx398 with IS1112C (Pring and Tang, 2004; Pring et al., 2006) exhibited the 1044-nt transcript and abundant levels of the processed 832-nt transcript (Fig. 2A), while A3Tx398 (*mmt1*) displayed only a trace of the processed transcript (Fig. 2B). Three individuals from the BC<sub>1</sub>F<sub>3</sub> S05-37 (Fig. 2C, F, G), two individuals from the BC<sub>2</sub>F<sub>1</sub> S05-38 (Fig.

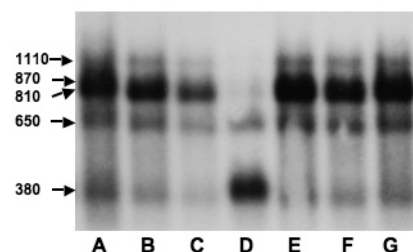


Figure 1. Transcript analyses for enhanced mitochondrial *orf107* transcript processing. Total leaf RNAs were prepared, and RNA from 2.5 g tissue was blotted to membranes and probed with the *orf107* clone pH104, which spans *orf107* and carries sequences of *atp9* (Tang et al., 1996b). A. S05-37, #38; B. S05-38, #40; C. S05-38, #49; D. a male-fertile BC<sub>15</sub>F<sub>2</sub> resulting from pollination of A3Tx398 with IS1112C; E. S05-36, #176; F. S05-37, #67; and G. S05-38, #48. The 1110-, 870-, and 810-nt transcripts represent whole-length *orf107* transcripts, while the 380-nt transcript results from processing. The 650-nt transcript is derived from *atp9*.

2D, H), and one individual from the BC<sub>2</sub>F<sub>1</sub> S05-36 (Fig. 2E), each displayed abundant levels of the 832-nt processed transcript, indicating that all plants were *Mmt1*. Although the sudangrass-derived fertility restoration is associated with the presence of *Mmt1*, linked to enhanced *orf107* TPA in IS1112C, the latter is not expressed in these lines, and thus we conclude that the mechanism of fertility restoration in A3Tx398 derived from the sudangrass populations does not involve enhanced *orf107* TPA.

## Phenotypic Expression of Male Fertility Restoration Conferred by Sudangrass

Male-sterile sorghum carrying IS1112C cytoplasm is associated with exertion of turgid anthers filled with non-iodine-staining pollen with a diameter about 90% that of pollen from normal, fertile plants (Tang et al., 1998). Heterozygous F<sub>1</sub>'s exhibit a mixture of iodine-stained and unstained pollen grains. We observed a similar phenotypic expression of CMS in lines segregating for fertility/sterility in the sudangrass-derived progeny, wherein many plants exerted anthers that carried mixtures of stained and unstained pollen. In segregating progeny from a self-pollinated S05-37 individual, a male-fertile plant scored

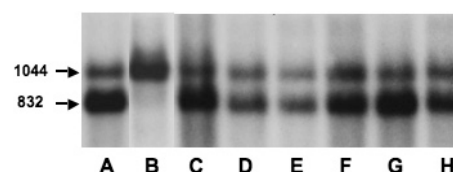


Figure 2. Transcript analyses for action of *Mmt1*. Total leaf RNAs were prepared, and RNA from 2.5 g tissue was blotted to membranes and probed with the *atp4* clone pHT160 (Tang et al., 1996a). A. Male-fertile BC<sub>11</sub>F<sub>3</sub> resulting from pollination of A3Tx398 with IS1112C; B. A3Tx398; C. S05-37, #30; D. S05-38, #40; E. S05-36, #167; F. S05-37, #167; G. S05-37, #67; and H. S05-38, #48. The population of 1044-nt transcripts is partially cleaved in the presence of *Mmt1*, generating the 832-nt transcript.

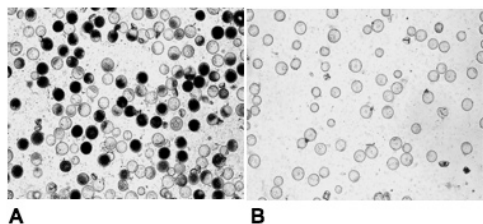


Figure 3. Iodine staining of pollen from self-pollinated progeny of the BC<sub>1</sub>F<sub>3</sub> S05-37. (A) Male-fertile plant, (B) male-sterile segregant. Note similarity in size of stained and unstained pollen in (A), and low frequency of collapsed unstained pollen in (A) and (B).

as 50% stained pollen (Fig. 3A) included turgid, near-full-sized nonstaining pollen, and a male-sterile plant (Fig. 3B) was characterized by turgid, nonstaining pollen.

## DISCUSSION

The recovery of sudangrass-derived fertility restoration capability for the A3 male-sterile sorghum cytoplasm, first observed by Pedersen and Toy (1997), and further developed here, identifies possible alternative approaches to developing the A3 cytoplasm for utilization in hybrid development. In addition to the low frequency of restorer genes in sorghum germplasm, the low percentage seed set in heterozygous F<sub>1</sub>'s restored to fertility with IS112C, 50 to 61% (Worstell et al., 1984; this work), represents a barrier in utilization of the A3 male-sterile cytoplasm for seed production. Alternatively, even low levels of viable pollen may increase risk of unwanted gene flow in a pollination control system based on A3 CMS as described by Pedersen et al. (2003). If fertility restoration can be conferred by sudangrass or shattercane, gene flow could potentially occur via fertile progeny from cultivated sorghum × uncontrolled sudangrass or shattercane (taxonomically the same subspecies) crosses.

The appearance of male-sterile individuals in segregating backcross, F<sub>2</sub>, and F<sub>3</sub> populations derived from sudangrass indicates that the mechanism of restoration is sporophytic, wherein the diploid genotype determines pollen viability. This possibility was first apparent in the occurrence of up to 37% male-sterile individuals in F<sub>2</sub> families derived from pollination of A3Tx398, with the bulked sudangrass lines, and observed in progenies derived from 14M18 and 14M21. These data are in marked contrast to gametophytic restoration conferred by IS112C (Tang et al., 1996b, 1998; Pring et al., 1999), wherein male-sterile individuals occur at very low frequency in backcrosses and F<sub>2</sub>'s. Compilation of data from A3Tx398/IS112C F<sub>2</sub>'s grown in Gainesville, FL, College Station, TX, and Mayaguez, Puerto Rico, 1990–1995, identified only four male-sterile plants among 430 plants examined (unpublished data, 2006). Thus it is clear the mechanism of sudangrass-derived fertility restoration, at least as evaluated in A3Tx398, is distinct from the previously described restoration conferred by IS112C. Addi-

tionally, sudangrass-derived restoration does not involve enhanced *orf107* TPA, although we have also identified one sorghum line, SC215, that carries a restorer that complements the *Rf4* allele but does not confer enhanced *orf107* TPA (Kuhlman et al., 2006).

Four other CMS systems (maize, *Zea mays* L.; common bean, *Phaseolus vulgaris* L.; rice, *Oryza sativa* L.; and cotton, *Gossypium hirsutum* L.) exhibit multiple, independent mechanisms of fertility restoration. Of these, cotton offers a precedent for gametophytic and sporophytic fertility restoration mechanisms for a male-sterile cytoplasm of higher plants, as developed for the D<sub>8</sub> cytoplasm, wherein the *Rf1* gene restores fertility through a sporophytic mechanism, and *Rf2*, through a gametophytic mechanism (Zhang and Stewart, 2001; Feng et al., 2005). At the molecular level, the alternative maize (Wen et al., 2003), common bean (He et al., 1995; Sarria et al., 1998), and rice (Wang et al., 2006) restoration systems restrict expression of mitochondrial CMS loci by different molecular mechanisms. These mechanisms include changes in mitochondrial DNA organization, transcript accumulation, transcript processing, and protein stability. Fertility restoration mechanisms therefore reveal the complex events of plant mitochondrial gene expression regulated by nuclear restorer alleles. The molecular mechanism of fertility restoration by sudangrass appears to be posttranscriptional. Further investigation into this mechanism will provide additional insights into the causal mechanism of A3 CMS.

Estimates of the genetic complexity of restoration derived from the 14M18 sudangrass derivative were not entirely consistent. Segregation patterns of the original F<sub>4</sub> population S04-28 fit a one gene (3:1) and a two-gene complementary (9:7) sporophytic model, while the BC<sub>1</sub>F<sub>2</sub> S04-30 and the BC<sub>1</sub>F<sub>3</sub> S05-35 populations fit only the latter, two-gene model. The BC<sub>1</sub>F<sub>3</sub> S04-37, derived from a different F<sub>2</sub> individual than was used for the BC<sub>1</sub>F<sub>3</sub> S05-35, was consistent with a sporophytic one-gene 3:1 pattern, and a two-gene 13:3 pattern if the Yates (Yates, 1934) correction was utilized. Therefore the original 14M18 F<sub>4</sub> population, the derived BC<sub>1</sub>F<sub>2</sub> S04-30, and the BC<sub>1</sub>F<sub>3</sub> S05-35 share a 9:7 two-gene complementary action model, and the two backcrosses S04-29 and S05-36 segregated as one or two genes.

Confounding these conclusions are observations that the sudangrass-derived restorers may not be efficacious, indicated by less than 100% stained pollen in progeny of A3Tx398 pollinated with certain of the developed lines. Decreases in pollen stainability were particularly evident in backcross generations. Three BC<sub>2</sub>F<sub>1</sub> populations derived from 14M18 exhibited 50% or less stained pollen, which mimics aspects of gametophytic restoration patterns for the A3 male-sterile cytoplasm, wherein 50 and 25% stainability are observed, dependent on the requirement of one or two restorer genes, respectively (Tang et al., 1998; Tang



and Pring, 2003). We were able to reproduce the phenomenon of less than 100% stained pollen in backcross progeny with the two  $BC_1F_4$  lines apparently homozygous for the required restoring alleles. The  $BC_2F_1$  progeny lines S06-17 and S06-19 displayed a range of 25 to 75% pollen staining, similar to observations with 14M18 as a pollen parent. Associated with this decrease in pollen stainability is reduced seed set among 14M18-derived backcross populations compared to  $BC_1F_2$  and  $BC_1F_3$  generations. Specifically, the three  $BC_2F_1$  populations derived from 14M18 displayed lower percentage stained pollen and reduced mean seed set compared to a  $BC_1F_1$  and three  $BC_1F_2$  and  $BC_1F_3$  populations. This phenomenon, decreased seed set associated with backcross generations compared to self-pollinated generations, has also been observed for rice carrying the Chinsurah boro II male-sterile cytoplasm (Sano et al., 1992). These factors may have contributed to the high frequency of male-sterile plants in the three  $BC_2F_1$  populations. These effects were not manifested in the  $BC_2F_1$  progeny lines S06-17 and S06-19, derived from the apparent homozygous parent, wherein seed set was comparable to that of  $BC_1F_2$  and  $BC_1F_3$  populations, and no male-sterile plants were observed.

The partial pollen staining and low seed sets observed in populations segregating for restoring alleles derived from sudangrass could result from a requirement for restoring alleles at minor-effect loci that sort independently from the major-effect restorer genes, such that full fertility is recovered only when all major- and minor-effect restoring alleles are present. Fully fertile  $BC_1F_1$  plants might be heterozygous for restoring alleles at each of the required major and minor restoration loci. Segregation and independent assortment would then produce  $BC_2F_1$  plants inheriting only a subset of the alleles needed to achieve full fertility. Perhaps the absence of all minor-effect restoring alleles accounts for the excess of male-sterile progeny in advanced backcross families.  $BC_1F_2$  plants derived by self-pollination of fully fertile  $BC_1F_1$  plants have a higher probability of inheriting a restoring allele at each of the major- and minor-effect loci, and segregants homozygous for restoring alleles at all loci might be recovered. Fully fertile  $BC_1F_5$  plants derived from S05-37 were probably homozygous for restoring alleles at most, if not all, of the major and minor restorer loci. The S06-17 and S06-19  $BC_2F_1$  progeny were therefore expected to inherit a full set of major and minor restoring alleles. The absence of male-sterile plants in these populations is consistent with fertility restoration conditioned by major and minor gene effects, although the highly variable pollen staining among the progeny would require invoking heterozygosity of the  $BC_1F_5$  parents.

Alternatively, silencing of a restoring allele following fertilization of an egg carrying a nonrestoring allele could account for reduced pollen stainability and the recovery of excess male-sterile progeny in  $BCF_1$  populations.

Silencing of an active allele following association with an inactive allele is a feature of paramutation, first investigated in maize (Brink et al., 1968). Paramutation is an epigenetic phenomenon, and the rates of gene silencing are variable and allele specific, with penetrance ranging from 10 to 100% (Chandler and Stam, 2004). In the present context, the frequency of male-fertile plants in  $BCF_1$  populations would depend largely on the efficiency with which a pollen-transmitted restoring allele is silenced by the female-contributed nonrestoring allele. Silencing that occurs during plant development, before gamete formation, might result in sectorized anthers that produced both stained and unstained pollen, thereby accounting for the variable frequency of pollen staining observed among  $BCF_1$  plants, particularly for the S06-17 and S06-19  $BC_1F_2$  populations. The effects of silencing would be less apparent in  $F_2$  or  $F_3$  populations because a significant fraction of the female gametes producing these populations also carry restoring alleles.

Documentation of a novel fertility restoration mechanism for the A3 male-sterile cytoplasm raises the possibility of development of a system useful in exploiting this source of CMS. Instability of restorer function, as defined in the Tx398 background, and particularly in backcrosses, represents a potential barrier to utilization. Other lines, however, may not carry paramutagenic alleles, as defined by Chandler and Stam (2004), and thus not exhibit instability in fertility restoration. Development of markers linked to the restorer loci will facilitate introgression of the loci into other agronomic lines to further evaluate the utility of this system.

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