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

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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. \( S. \) bicolor) 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-fer tile; S, male-sterile; TPA, transcript processing activity.

Hybrid 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
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 F₁ 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 Mmnt1, which confers enhanced transcript processing 5’ to the mitochondrial gene atp4 (orf25); IS1112C is Mmnt1, while A3Tx398 is mmnt1 (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 r4 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 × sudangrass F₁ 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 F₁ 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 amylase 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 fertility 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 S₁ (male-sterile) lines from parents with A3 CMS restoration capability were identified from each population and designated NP28S₁-1, NP28S₁-2, NP35S₁-1, and NP35S₁-2.

To capture and characterize this new source of restoration, these S₁ lines were grown and utilized for bulk pollinations of A3Tx398 at Gainesville, FL. Five fertile F₁ populations were grown and self-pollinated, and 193 F₂ panicles were scored for fertile-sterile plants. Pollen staining of 80 plants from the F₂ families revealed numerous individuals with >95% staining, and one such individual was selected for advance. Among individuals in this F₂ 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 F₁ population designated S04-28. A3Tx398 was pollinated with 14M18, generating a BC₁F₁ population; among these plants, an individual designated 24-65 was selected and used to pollinate A3Tx398, generating the BC₂F₁, S04-29. Individual 24-65 was also self-pollinated, generating the BC₂F₂ population S04-30. Two individuals from the BC₂F₁, 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 BC₂F₂ population S05-35. A backcross to A3Tx398 generated the BC₃F₁ population S05-36. The second individual from the BC₂F₁, S04-30, S04-30-200 was self-pollinated to generate the BC₃F₂ population S05-37. A backcross to A3Tx398 generated the BC₄F₁ population S05-38.

The BC₃F₁ population S05-37 was examined for pollen staining, and 16 individuals were self-pollinated to generate BC₃F₂ 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 BC₄F₁ populations, and self-pollinated to generate two BC₄F₂ lines.

**Development of 14M21 Progeny**

The 14M21 individual was self-pollinated to generate a F₁ population. A3Tx398 was pollinated with 14M21, generating the BC₁F₁ 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 BC₁F₂ 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 BC₂F₁ 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 pHCl04, 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 F₁ A3Tx398/IS1112C is included. Line designations and identifications are described in the text.

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<th>70</th>
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<th>30</th>
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<td>30</td>
<td>29:1</td>
<td>64</td>
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<td>16</td>
<td>10</td>
<td>4</td>
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<td>1</td>
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<td>2</td>
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<td>A3Tx398/IS1112C</td>
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and carries sequences of atp9 (Tang et al., 1996b). Membranes were also probed the clone pH1T160 to detect activity of the single dominant gene Mut1, 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 F₁’s, representing 6 to 8 plants from each of the six crosses of A3Tx398 with NP28S₁-1, NP28S₁-2, NP35S₁-1, and NP35S₁-2, revealed no seed set in 26 panicles and 5- to 100% seed set in the remaining 16 panicles. Therefore individuals within the four S₁ sudangrass lines transmitted fertility restoration capability, which was observed in F₁’s from five of the six original crosses to A3Tx398. F₂ families from each of the five fertile F₁’s were scored for fertile:sterile plants. The five F₂ 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 F₂’s resulting from pollination of A3Tx398 with IS1112C (Tang et al., 1996a, 1998; Pring et al., 1999). Examinations of 80 plants from these F₂ 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 F₄ population designated S04-28 segregated 61F:31S plants (Table 1), consistent with an F₃ 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 F₁’s resulting from pollination of A3Tx398 with IS1112C (Table 1). These heterozygous $Rf^3rf^3Rf^4rf^4$ F₁’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 F₁ 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 F₁ population that is assumed to genetically homogeneous. This variability in seed set was similar to that observed within the F₄ 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 F₄ S04-28 population indicated that the segregation ratio fit 3:1 and 9:7 ratios (Table 2).

Pollination of A3Tx398 with 14M18 generated a BC₁F₃ 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 Yates' correction (Yates, 1934) is used. Ratio of fertile: sterile plants is F:S.

<table>
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<th>Ratio tested</th>
<th>F:S</th>
<th>3:1</th>
<th>9:7</th>
<th>13:3</th>
<th>15:1</th>
<th>63:1</th>
<th>1:1</th>
<th>1:3</th>
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<td>3.78</td>
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<td>F1(S01-30)</td>
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<td>BC1F1(S03-25)</td>
<td>62:0</td>
<td>**</td>
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<tr>
<td>BC1F1(S04-26)</td>
<td>5:26</td>
<td>**</td>
<td>1.30</td>
<td>0.37</td>
<td>**</td>
<td>**</td>
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<tr>
<td>BC1F1(S04-27)</td>
<td>2:38</td>
<td>**</td>
<td>2.06</td>
<td>**</td>
<td>**</td>
<td>**</td>
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*Significant at the 0.05 probability level.
**Significant at the 0.01 probability level.

The BC1F1 S05-37 population (derived from a second BC1F1 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 BC1F1 S05-38 population segregated 8F:56S, with a seed set pattern similar to other backcross BC1F1's examined (Table 1). These segregation data fit only a 1:7 ratio (Table 2). Forty-six BC1F1 S05-38 individuals were examined, and only 5 had at least 50% stained pollen. Thus, each of three BC1F1 progenies had a lower percentage stained pollen percentage than the BC1F1.

Seed set among 14M18 progenies exhibited a trend toward higher percentage seed set among fertile members of the F2 and F3 populations (S04-30, 61%; S05-35, 67%; S05-37, 79%), similar to the parental population (S04-28, 68%), than observed for the backcross BC1F1 populations (S04-29, 38%; S05-36, 29%; S05-38, 44%) (Table 1).

To develop lines homozygous for the putative restorers we advanced the BC1F1 population S05-37 by selection and self-pollination. Among 16 BC1F1 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 BC1F1 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 BC1F1'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 F1'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 BC1F1'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 F1 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 BC1F1 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 BC1F1 developed from 14M18.

Subsequent backcrosses, however, provided variable results. Among the BC1F1 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\(_{1}\), 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\(_{2}\), 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 R\(_{3}\) restoring allele (Tang et al., 1996b, 1998; Pring et al., 1999). Members of the original F\(_{1}\) population resulting from pollination of A3Tx398 with the sudangrass lines NP285-1, NP285-2, NP255-1, and NP355-2, and bulk seedlings of the F\(_{1}\)'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\(_{1}\) 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\(_{1}\), S05-37 (Fig. 1A, F), three individuals from the BC\(_{2}\), S05-38 (Fig. 1B, C, G), and one individual from the BC\(_{2}\), S05-36, each exhibited abundant 1110-, 870-, and 810-nt whole-length orf107 transcripts, while the 380-nt transcript results from processing. The 650-nt transcript is derived from atp9.

Phenotypic Expression of Male Fertility Restoration Conferred by Sudangrass

Male-sterile sorghum carrying IS1112C cytoplasm is associated with exsertion of turgid anthers filled with noniodine-staining pollen with a diameter about 90% that of pollen from normal, fertile plants (Tang et al., 1998). Heterozygous F\(_{1}\)’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 exserted anthers that carried mixtures of stained and unstained pollen. In segregating progeny from a self-pollinated S05-37 individual, a male-fertile plant scored for a male-fertile BC\(_{1}\) 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.
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 occurring backcross, F2, and F3 populations derived from sudangrass (taxonomically the same subspecies) crosses. Decreases in pollen stainability were particularly evident in A3Tx398 pollinated with certain of the developed lines. These data are in marked contrast to gametophytic restoration systems of the alternative 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 D1 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 F2 population S04–28 fit a one gene (3:1) and a two-gene complementary (9:7) sporophytic model, while the BC1F2 S04–30 and the BC1F2 S05–35 populations fit only the latter, two-gene model. The BC1F2 S04–37, derived from a different F2 individual than was used for the BC1F2 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 F2 population, the derived BC1F2 S04–30, and the BC1F2 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 BC1F2 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 et al., 1996b, 1998; Pring 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.

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subset of the alleles needed to achieve full fertility. Perhaps
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ment would then produce BC2F1 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. BC1F2 plants derived by self-pollination of fully
fertile BC1F1 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 BC1F1 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 BC1F1 progeny were therefore expected to
inhibit a full set of major and minor restoring alleles.

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 BC1F1 populations.


