Fine Mapping of the Maize Cross-Incompatibility Locus *Gametophytic Factor 1* (*ga1*) Using a Homogeneous Population

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ABSTRACT

Phenotyping of a mapping population is usually the bottleneck that limits the size of the mapping population and mapping resolution. A homogeneous population mapping approach was used for mapping maize (*Zea mays* L.) *gametophytic factor 1* (*ga1*) that could completely eliminate phenotyping during the mapping process. The strong allele of maize *ga1* (*Ga1-S*), from popcorn inbred line SDGa25, showed a 100% cross-incompatibility with the majority of Chinese dent and flint maize. A homogeneous mapping population was developed by crossing an (SDGa25/Jing66) F_1 male back to an SDGa25 female. During the pollination process, pollen grains of *ga1* were completely excluded from fertilization, and a homogeneous BC_1F_1 (SDGa25//SDGa25/Jing66) population was created in which only the *Ga1-S/Ga1-S* genotype existed, making phenotyping unnecessary. A total of 2245 individuals of this population were genotyped with SD9 and SD12 markers and 20 recombinants were identified. The *Ga1-S* locus was quickly delineated to a 100 Kb region between markers dCS1 and insertion deletion (ID)7 at position 9,491,422 and 9,591,946 bp based on the *B73 RefGen_v2 sequence*. By marker-assisted selection, *Ga1-S* was introgressed into parental lines of an elite white waxy maize hybrid by six generations of backcrossing and one generation of selfing. The homozygous *Ga1-S/Ga1-S* hybrid showed full cross-incompatibility to *ga1* maize. The *Ga1-S* allele could be used as a biological reproductive barrier in reducing cross-pollination between different types of maize such as waxy and non-waxy, genetically modified (GM) and non-GM maize.

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Mᴀɪᴢᴇ (*Zea mays* L.) is a cross-pollinating crop and the level of cross-fertilization among maize plants of neighboring fields is very high due to a heavy volume of pollen produced by the maize plant and factors such as wind and insects that facilitate pollen traveling. Many field experiments have been performed to study the cross-fertilization rate of maize (Henry et al., 2003; Goggi et al., 2006; Weekes et al., 2007; Della Porta et al., 2008; Langhof et al., 2010; Kozjak et al., 2011; Gonzalez et al., 2012). It was documented that at 1 m from the pollen source, the mean cross-fertilization rate in 2003 and 2004 was 29.9 and 17.0%, respectively. At 35 m from the pollen source, the average percentage was 0.4% in both years, whereas at 100 m distance from the pollen source and beyond, the percentage of cross-fertilization

decreased to < 0.1%. However, the average cross-fertilization rate never reached 0% within 250 m from the pollen source (Goggi et al., 2006). There was evidence that cross-fertilization was detected at a distance of 650 m from the pollen source and that maize pollen could travel up to several kilometers (Henry et al., 2003; Kozjak et al., 2011).

Certain types of maize fields are required to be free of foreign pollen in practice. Specialty maize, such as sweet and waxy maize, is required to be free from foreign pollen due to the xenia effect. Seed production fields are required to be free from foreign pollen to maintain high hybrid purity. Non–GM maize fields require GM-free pollen to avoid contamination. To reduce and/or avoid cross-fertilization among adjacent maize fields, numerous strategies have been taken, including physical barriers and temporal and spatial isolation. The synchrony of flowering among maize fields contributes to a higher rate of cross-fertilization (Angevin et al., 2008). A 4 to 5 d planting shift led to a 25% reduction in the cross-fertilization rate and a 6-d shift led to a 50% reduction (Della Porta et al., 2008). Up to a 70% reduction in the cross-fertilization rate could be achieved when the planting shift was over 10 d (Kozjak et al., 2011). To minimize the adventitious presence of GM maize, isolation distances have been proposed, ranging from 15 to 800 m in the EU member states (Devos et al., 2009; Riesgo et al., 2010). To maintain genetic purity in conventional maize seed production, a minimum separation distance of 200 and 300 m were prescribed in the EU and China, respectively. However, some of the distances were difficult to manage in practice. The use of the maize cross-incompatibility gene *ga1* offers a potential alternative to effectively manage pollen flow among maize fields.

Maize gametophytic factors mediate pollen–pistil interactions and affect the success of fertilization; thus, they may play a role in restricting pollen flow among maize populations. Numerous gametophytic factors have been reported on maize chromosomes 1, 2, 3, 4, 5, 6, 7, and 9 (Nelson, 1993). The first gametophytic factor reported in maize, *ga1*, was identified by its effect on segregation distortion at the linked *sugary 1* locus in a segregating population (Mangelsdorf and Jones, 1926). There were three alleles reported at the *ga1* locus in maize: the wild-type recessive allele *ga1*, typical of cross-receptive germplasm, the strong dominant allele *Ga1-S*, characteristic of cross-incompatible inbred lines, and gametophyte factor 1-male (*Ga1-M*), having the male function but lacking the pistil barrier function of *Ga1-S* (Jimenez and Nelson, 1965; Ashman, 1981). The cross-compatible relationship among *Ga1-S*, *Ga1-M*, and *ga1* is summarized in Table 1. Several lines of evidence have indicated that gametophytic factors could be used to manage pollen flow among maize fields. First of all, the homozygous *Ga1-S/Ga1-S* genotype confers complete nonreciprocal cross-incompatibility (or unilateral cross-incompatibility) with

Table 1. Cross-compatible relationship among *Ga1-S*, *Ga1-M*, and *ga1* alleles.[†]

	Ga1-S (♂)	*Ga1-M* (♂)	*ga1* (♂)
Ga1-S (♀)	√	√	X
Ga1-M (♀)	√	√	√
ga1 (♀)	√	√	√

[†]*Ga1-S*, gametophyte factor 1-strong allele; *Ga1-M*, gametophyte factor 1-male; *ga1*, gametophyte factor-1; √, cross compatible; X, cross incompatible; ♂, male; ♀, female.

ga1 pollen (Kermicle and Evans, 2010; Zhang et al., 2012). Second, the vast majority of North American and Chinese dent and flint maize have the wild-type *ga1* recessive allele that is cross-incompatible with *Ga1-S* (Nelson 1993; Zhang et al., 2012). Third, there are other gametophytic factors including *Tcb1-S*, *Ga2-S*, and *Ga★–Yugoslavia* that showed complete cross-incompatibility with *ga* alleles, and these factors together with *Ga1-S* were cross-incompatible between each other (Vancetovic et al., 2004; Kermicle and Evans, 2005, 2010).

We and other groups have independently mapped the *Ga1-S* locus in a 2.2 Mbp interval on maize chromosome 4 based on the B73 *RefGen_v2sequence* database (Bloom and Holland, 2012; Zhang et al., 2012). We used the same mapping strategy to map the *Ga1-S* allele in which a BC_1F_1 segregating population was used. To determine the phenotypes of BC_1F_1 individuals, each individual was crossed by hand as male onto the homozygous *Ga1-S* line to test their pollen compatibility with *Ga1-S* silks. At maturity, each ear resulting from hand pollination was scored. If no seed was set, the individual lacked the *Ga1-S* allele. If the seed set was complete, the individual was then carrying the *Ga1-S* allele. This mapping strategy required tagging each BC_1F_1 segregating individual and homozygous *Ga1-S* tester individual plants and making one-to-one crosses to check the presence/absence of the *Ga1-S* allele of the BC_1F_1 segregating individuals. Furthermore, to ensure seed setting was a result of crossing, not from *Ga1-S* plant selfing, *Ga1-S* plants were completely detasseled before flowering. The mapping process was time-consuming, and occasionally *Ga1-S* plants set seeds due to incomplete detasseling, which led to incorrect phenotyping results.

In this paper, we report (i) a homogeneous population mapping approach for maize *ga1*. This approach completely eliminates phenotyping of mapping populations, which is time-consuming and often difficult to do, thus limiting mapping population size and mapping resolution; (ii) fine mapping of *Ga1-S*, the strong allele of maize *gametophytic factor 1* that showed 100% cross-incompatibility to *ga1* maize; and (iii) the introgression of *Ga1-S* into an elite waxy maize hybrid by marker-assisted selection and the possibility of using *Ga1-S* as a biological reproductive barrier for the containment of gene flows between different types of maize.
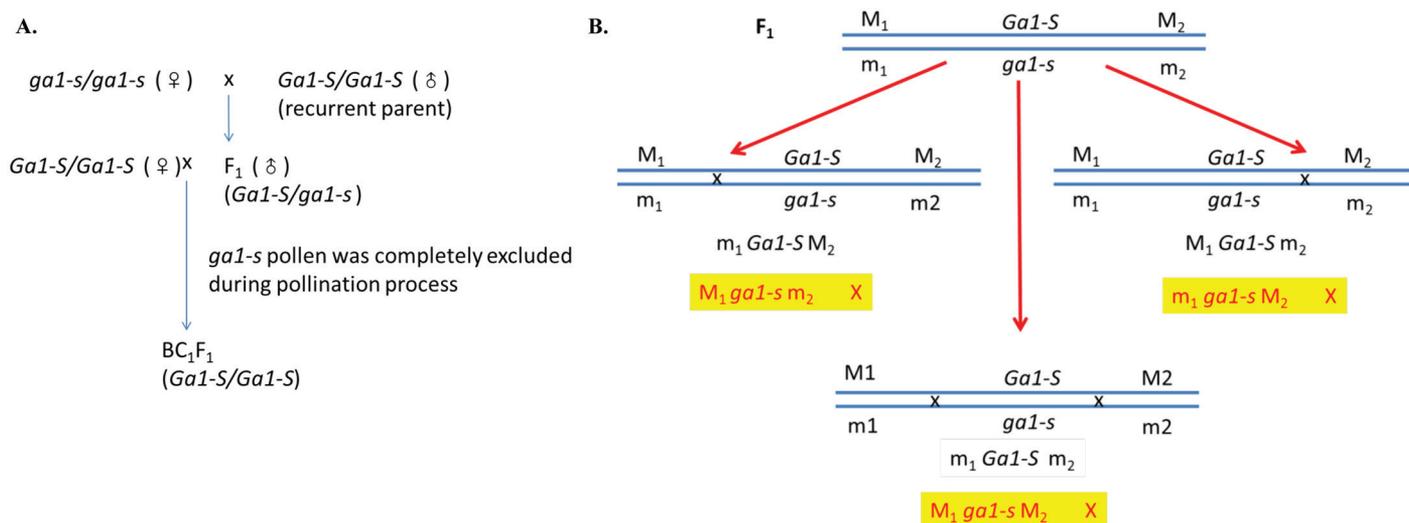
Figure 1. Diagram showing the mapping mechanism. (a) The development of homogenous BC_1F_1 mapping population. The recessive homozygous parent gametophyte factor 1-strong allele (ga1-s/ga1-s) was used as female and the dominant homozygous source parent (Ga1-S/Ga1-S) was used as male to produce F_1 (Ga1-S/ga1-s). F_1 was then used as male and the source parent as female to develop the BC_1F_1 population. The ga1-s pollen was completely excluded during pollination process and only one genotype (Ga1-S/Ga1-S) was produced. (b) Mapping mechanism. Suppose that the Ga1-S allele was initially mapped between molecular markers M_1 and M_2. If crossover occurred between M_1 and Ga1-S during meiosis of the F_1 plant, only a gamete of the m_1M_2 genotype containing the Ga1-S allele could pollinate Ga1-S silks, and recombinant genotype $M_1m_1M_2M_2$ could be identified in the BC_1F_1 population; if crossover occurred between M_2 and Ga1-S during meiosis, only a gamete of the M_1m_2 genotype containing the Ga1-S allele could pollinate Ga1-S silks, and recombinant genotype $M_1M_1M_2m_2$ could be identified in the BC_1F_1 population. If no cross over occurred between markers M_1 and M_2, only a gamete of the M_1M_2 genotype containing the Ga1-S allele could pollinate Ga1-S silks, and genotype $M_1M_1M_2M_2$ could be identified, which is the case for the majority of the BC_1F_1 population. In the scenario of double cross, only a gamete of the m_1m_2 genotype containing the Ga1-S allele could pollinate Ga1-S silks and a recombinant of $M_1m_1M_2m_2$ could exist in the BC_1F_1 population.

MATERIALS AND METHODS
Maize Materials

Popcorn inbred line SDGa25, carrying the strong allele of ga1 (Ga1-S), was provided by Dr. Baoshen Liu, Shandong Agricultural University, Taian, China. The exact pedigree of this line was unknown. This popcorn line was 100% cross–incompatible with the majority of Chinese dent and flint maize. Jing66 was a yellow dent inbred line carrying the recessive allele of ga1 and was provided by Huanong Weiye Seed Company, Beijing, China. JKN2000 was an elite white waxy maize hybrid that had been used as a national check for waxy maize variety development in China. The parental lines of JKN2000 were also provided by Huanong Weiye Seed Company. All maize materials were planted normally on the experimental farm and in the greenhouse in Beijing, and the winter nursery in Hainan province, of the Institute of Genetic and Developmental Biology (IGDB), Chinese Academy of Sciences (CAS).

Ga1-S Mapping Population Development

Since SDGa25 carries the Ga1-S allele and Jing66 carries the ga1-s allele, the F_1 SDGa25 × Jing66 was made by crossing SDGa25 as male onto Jing66 silks. The F_1 SDGa25 × Jing66 was then used as a male to pollinate SDGa25 to develop the BC_1F_1 (SDGa25//SDGa25/Jing66) homogeneous population in which all individuals had the same Ga1-S/Ga1-S genotype (Fig. 1). SDGa25 was completely detasseled before flowering.

Ga1-S Introgression into JKN2000 Parental Lines

SDGa25 was used as the Ga1-S donor, and the parental lines of JKN2000 were used as recurrent parents. JKN2000 parental lines were used as female parents for each backcross generation. During the ga1 fine mapping process, 14 closely-linked markers were developed and 5 tightly-linked markers (SD5, SD6, CS1, dCS1, and ID7) were used to select 10 individuals carrying the Ga1-S allele for further backcrossing in each generation (Table 2; Fig. 2). After six generations of backcrossing, individuals carrying the Ga1-S allele were selfed, and homozygotes of Ga1-S/Ga1-S were selected with codominant markers. The JKN2000 hybrid, homozygous at the Ga1-S locus, was evaluated for cross-incompatibility at the experimental farm in 2012. Twenty-five plants were surrounded by yellow and purple maize. To ensure flowering dates met, yellow and purple maize were planted three times at a 7-d interval. The experiment was replicated three times.

DNA Extraction

Leaf tissues of BC_1F_1 individuals were collected for DNA extraction. All individual plants were tagged and numbered. Each sample was placed in a 2.0 mL centrifuge tube and then ground to a fine powder in liquid nitrogen. A 1000 μL DNA extracting solution was preheated to 65°C (1 M NaCl, 100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, and 1% SDS) was added to each tube for incubating. After being incubated at 65°C for 30 min, each sample was then centrifuged at 12,000 rpm for 5

Table 2. Primer sequences and their types.†

Primers	Sequences	Types
ID1	F: ATCTATCGCACAAGCCCTAA	STS
	R: TTGGCCCATGTCTTCTCA	
ID2	F: CAAATTGAGCCCATTACC	STS
	R: TTCATTCTATTGCGGGTC	
ID3	F: CGATGATGAAGATGACCCTT	STS
	R: AGCCAGCGTCCACAAATA	
ID4	F: CCCCTATGATAGAAATGTAGCAC	STS
	R: CCCACTTGATGTCACCACC	
ID5	F: CTGGACAAGCACGGTAGA	STS
	R: GGTTATTTCAAGGCGGAT	
ID6	F: AACTGCAATAATACTAACCCAG	STS
	R: AATGGTTGAAACAGTGGC	
ID7	F: CATGGTATCGCCAACGACAG	STS
	R: TTCTAGCTCTGTCCTCGCGG	
ID8	F:CGGTTGTTGTTGGTTCGA	STS
	R: AAATCCTCTGGCTGTCCTG	
ID9	F: GTGCATGGCTCTGGTTAC	STS
	R: CCTTGACTACGCCTGAAA	
ID10	F: TTTAATTTCAGGCGTAGTCA	STS
	R: TCGCATCGTCCTACACTC	
CS1	F: AACTGCAATAATACTAACCCAG	CAPS
	R: AATGGTTGAAACAGTGGC	
dCS1	F: TCTGTGGAGCTTTGATAAGC	dCAPS
	R: TTATTCCTTGTTTCTGAGCC	
RS1	F: GAAAGCGATGCGGTTTAGAT	SSR
	R: TAACAACCTTCGCAAACAGC	

†ID, insertion deletion; STS, sequence-tagged site; CAPS, cleaved amplified polymorphic sequence; SSR, simple sequence repeat.

min at room temperature. A 500 µL supernatant was transferred to a fresh centrifuge tube with 200 µL 5M CH_3COOK and mixed. The sample was centrifuged at 12,000 rpm for 10 min at room temperature, and 400 µL of supernatant was transferred to a fresh centrifuge tube. A 240 µL isopropanol was added and stored at −20°C for 20 min. The sample was centrifuged at 10,000 rpm at 4°C for 5 min, and the supernatant was discarded. The pellet was washed twice with 150 µL 70% ethanol and air dried before being dissolved in 300 µL 1×TE buffer (10mM Tris-HCl pH 8.0, 0.1 mM EDTA). The DNA solution was used directly as the template for polymerase chain reaction (PCR) amplification or stored at −20°C for further analysis.

Marker Development, Genotyping, and Fine Mapping

Molecular markers on the short arm of chromosome 4 were initially screened for marker-trait association, and *Ga1-S* was previously mapped between markers SD3 and SD12 in a ~2.2 Mbp interval of the B73 reference sequence (*B73 RefGen_v2 sequence*) using four BC_1F_1 mapping populations (Zhang et al., 2012). We further delineated the locus between markers SD9 and SD12 and then switched the mapping approach to the homogeneous population approach described in this paper. The *Ga1-S* was flanked by SD9 on the telomere side and SD12 on the centromere side with genetic distances of 0.31 and 0.58 cM, respectively. For further linkage analysis, simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and In-Del markers were

developed from bacterial artificial chromosome (BAC)s in the target region. Detailed marker/primer information is provided in Table 2. A total of 2,245 individuals were used for the fine mapping and 20 recombinants were identified within the 0.97 Mbp interval. We then developed markers within this interval, and 7 recombinants on the telomere side and 13 recombinants on the centromere side were both decreased to one recombinant. The closest markers we developed were dCS1 on BAC AC208523 and ID7 on BAC AC204382 that were both 0.045 cM to the target gene. AC208523 and AC204382 were adjacent BACs. The genetic region mapped spanning the *Ga1-S* locus was estimated to be 0.089 cM in length. The above BACs were on ctg156 and the physical distance based on the B73 Ref-Gen_v2 sequence was 100,524 bp.

As a next step, PCR was performed for 35 cycles for 30 s at 95°C, 30 s at 55°C (for most primers), and 1 min at 72°C, followed by a 10 min extension at 72°C. The annealing temperature of different primers varied based on their Tm value. The 15-µL reaction mix consisted of 3 pmol of each primer, 2.5 mMol $MgCl_2$, 50 µM of each dNTP, 1 × reaction buffer, 0.75 unit of Taq DNA polymerase (Promega), and about 50 ng of template DNA. Polymerase chain reaction products were separated on a 12% polyacrylamide gel and visualized with ethidium bromide staining. The maize gene set (www.maize-equence.org) and website-based software FGENSH (www.softberry.com) was applied to predict and filter the putative genes from the mapping interval.

RESULTS
The Homogeneous Population Mapping Approach for *Ga1-S* Allele

In previous *Ga1-S* mapping studies, *Ga1-S* was mapped using BC_1F_1 (*ga1//Ga1-S/ga1*) mapping populations in which *Ga1-S/ga1* and *ga1/ga1* genotypes were segregated at a 1:1 ratio. Heterozygous *Ga1-S/ga1* and homozygous *ga1/ga1* genotypes were determined by hand-crossing them as male onto *Ga1-S/Ga1-S* silks (Zhang et al., 2012). Pollination by pollen from the *ga1/ga1* genotype produced no seeds, while pollination using pollen from *Ga1-S/ga1* genotype produced full-set seeds. This mapping method was time-consuming and limited the size of the mapping population due to the requirement of hand pollination in the field. We then switched to a different mapping approach in which a different BC_1F_1 (*Ga1-S//Ga1-S/ga1*) was used as a mapping population. The new mapping population consisted of only one genotype (*Ga1-S/Ga1-S*), and all individuals should produce full-set seeds when crossing as male onto *Ga1-S* silks, thus making phenotyping unnecessary. The homogeneous BC_1F_1 population was achieved by crossing heterozygous F_1 (*Ga1-S/ga1*) as male with the homozygous *Ga1-S/Ga1-S* parental line as female. The heterozygous F_1 (*Ga1-S/ga1*) produced two types of gametes, *Ga1-S* and *ga1*. When pollinating a *Ga1-S* homozygous plant with a mixture of *Ga1-S* and *ga1* pollens, the *ga1* pollen was completely excluded from fertilization, thus producing a BC_1F_1 population that contained only

A.

B.

	extron	Y Start Codon	♀ Stop Codon
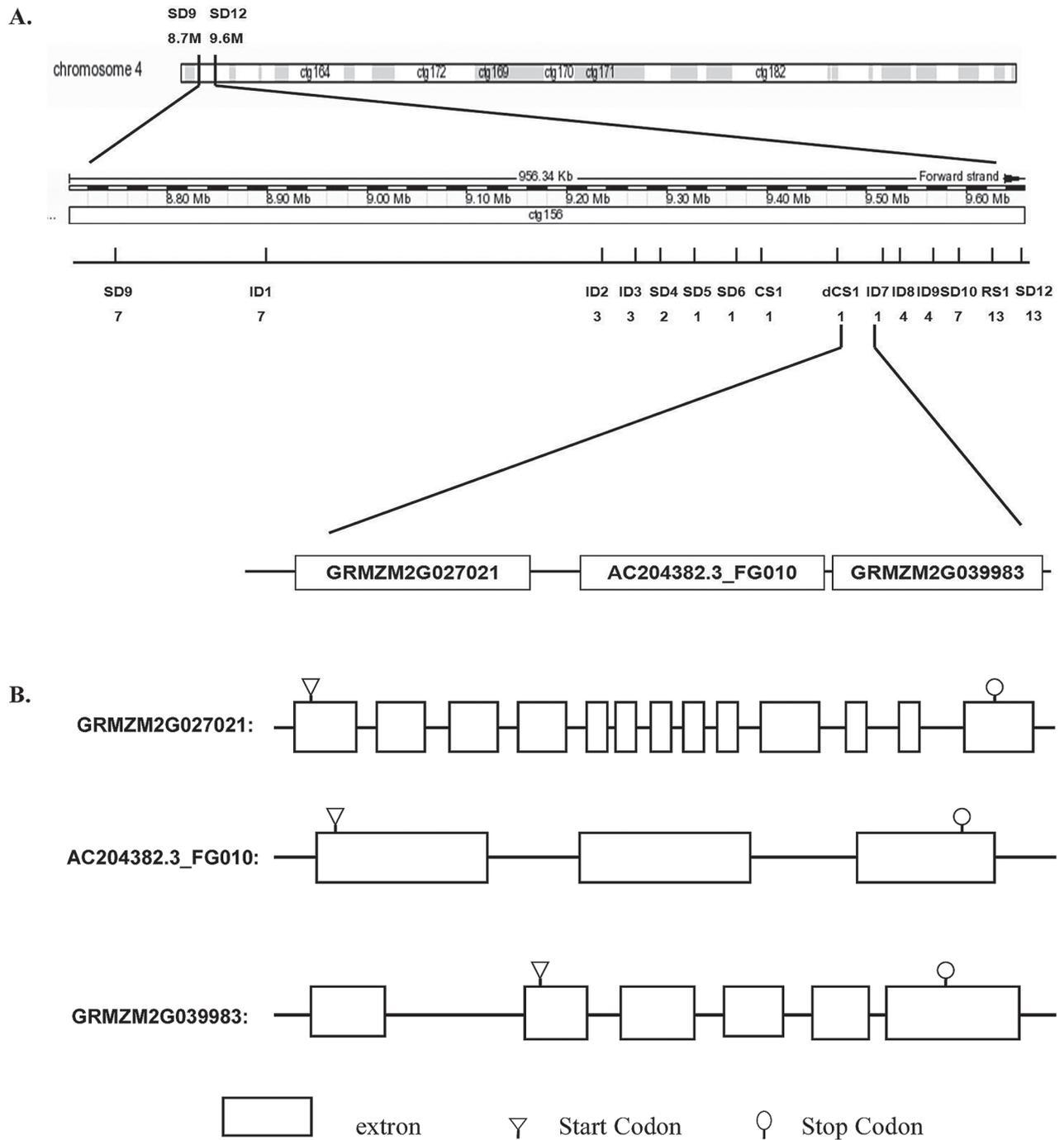
Figure 2. Fine mapping of the gametophyte factor 1-strong allele (*Ga1-S*) and candidate genes in the mapping interval. The *Ga1-S* allele was initially mapped between markers SD9 and SD12. The SDGa25//SDGa25/Jing66 BC_1F_1 population was used for fine mapping. A total of 2245 individuals were genotyped. Recombinants are indicated in the parentheses below each marker. The *Ga1-S* locus was mapped in a 100 kb region between markers dCS1 and ID7. (a) Detailed marker information is listed in Table 2. (b) Schematic gene structures of three candidate genes.

the *Ga1-S/Ga1-S* genotype (Fig. 1a). In practice, a large amount of seeds from the *Ga1-S/Ga1-S* BC_1F_1 population could be easily produced and genotyped for *Ga1-S* fine mapping. The fine mapping mechanism is illustrated in Fig. 1b. Suppose that the *Ga1-S* allele was initially mapped between molecular markers M_1 and M_2. If crossover occurred between M_1 and the *Ga1-S* during meiosis of the F_1 plant, only gametes of the m_1M_2 genotype carrying the

Ga1-S allele could pollinate *Ga1-S* silks, and the recombinant genotype $M_1m_1M_2M_2$ could be identified in the BC_1F_1 population. If crossover occurred between M_2 and *Ga1-S* during meiosis, only gametes of the M_1m_2 genotype containing the *Ga1-S* allele could pollinate *Ga1-S* silks, and the recombinant genotype $M_1M_1M_2m_2$ could be identified in the BC_1F_1 population. If no crossover occurred between markers M_1 and M_2, only the gamete of

Table 3. Candidate genes in the mapping interval region containing *ga1*.[†]

Genes ID	Location	Homologues	Conserved domains	Function
GRMZM2G027021	9485207-9494847	*Os*11:22089385–22094921	GTP-binding protein hflX	GTPase of unknown function domain containing protein, putative, expressed
AC204382.3_FG010	9583759-9584560	no		
GRMZM2G039983	9589010-9592389	*Os*11:22084084–22088761	targeting protein for Xklp2	Anisotropic growth of cells, could be involved in pollen tube growth

[†]*ga1*, gametophyte factor 1.
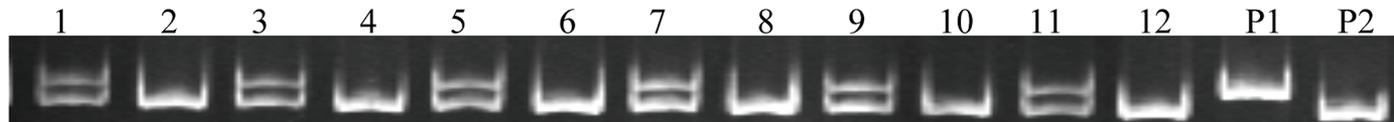
Figure 3. Banding profile showing polymorphisms identified by insertion deletion (ID)2 markers among gametophyte factor 1-strong allele (*Ga1-S*)/*Ga1-S*, *Ga1-S/ga1-s*, and *ga1/ga1* genotypes. P1, SDGa25 (*Ga1-S/Ga1-S*), the *Ga1-S* donor; P2, JKN2000 male parent (*ga1-s/ga1-s*) and recurrent parent; Lanes 1 to 12 are the BC_1F_1 segregating individuals. Lanes 1, 3, 5, 7, 9, and 11 were heterozygous individuals (*Ga1-S/ga1-s*), and lanes 2, 4, 6, 8, 10, and 12 were homozygous individuals (*ga1-s/ga1-s*).

the M_1M_2 genotype carrying the *Ga1-S* allele could pollinate *Ga1-S* silks, and the genotype $M_1M_1M_2M_2$ could be identified, which was the case for the majority of the BC_1F_1 population. In the scenario of double crossover, only gametes of the m_1m_2 genotype containing the *Ga1-S* allele could pollinate the *Ga1-S* silks, and the recombinant of $M_1m_1M_2m_2$ could exist in the BC_1F_1 population. This mapping approach completely eliminated the time-consuming phenotyping step because of the homogeneous genotype nature of the BC_1F_1 mapping population.

Fine Mapping of the *Ga1-S* Allele

We previously mapped *Ga1-S* between molecular markers SD3 and SD12 in a ~2.2 Mbp interval of the B73 reference sequence (*B73 RefGen_v2 sequence*) on chromosome 4 using four BC_1F_1 mapping populations (Zhang et al., 2012). We further delineated the locus between markers SD9 and SD12 and then switched the mapping approach to the homogeneous population approach described in this paper. We used (SDGa25 × Jing66) F_1 as male and SDGa25 as female to develop a BC_1F_1 (SDGa25//SDGa25/Jing66) population in which all individuals had the same *Ga1-S/ Ga1-S* genotype. A total of 2245 individuals were genotyped using SD9 and SD12 markers and 20 recombinants were identified within the 0.97 Mbp interval. We then developed markers within this interval, and 7 recombinants on the telomere side and 13 recombinants on the centromere side were both decreased to one recombinant. The *Ga1-S* allele was then delineated to a 100 kb-region between dCS1 and ID7 markers at positions 9,491,422 bp and 9,591,946 bp on the *B73 RefGen_v2 sequence* that contained three predicted genes (Table 3 and Fig. 2). One of the genes, GRMZM2G039983, had homology to WDL1 of *Arabidopsis*, which regulated anisotropic cell growth and perhaps could be involved in pollen tube growth (Yuen et al., 2003). Maize inbred lines shared low colinearity

(Lai et al., 2009). Thus, *Ga1-S* may not exist in the B73 genome. A BAC library was constructed from SDGa25 genomic DNA, and a single BAC covering the 100 kb region was identified and sequenced by IGDB, CAS.

Marker-Assisted *Ga1-S* Introgression into Elite Commercial *Waxy* Maize Hybrid

During the mapping process of the *Ga1-S* allele from SDGa25, tightly-linked and codominant molecular markers were developed that could easily distinguish *Ga1-S/ Ga1-S*, *Ga1-S/ga1*, and *ga1/ga1* genotypes (Fig. 3). Using SDGa25 as the *Ga1-S* allele donor and JKN2000 parental lines as recurrent parents with the assistance of five tightly-linked and codominant molecular markers (SD5, SD6, CS1, dCS1 and ID7, Table 2), JKN2000 parental lines possessing homozygous *Ga1-S* alleles were developed after six generations of backcrossing and one generation of selfing (Fig. 4). We checked the cross-incompatibility of the JKN2000 parental lines using purple maize. Twenty-five plants for each of the JKN2000 parental lines were hand-pollinated twice with pollen from purple maize, 24-h apart, and then selfed. No purple kernels were found for both male and female parental lines (data not shown). The homozygote F_1 (*Ga1-S/Ga1-S*) hybrid surrounded by yellow and purple maize was evaluated under field conditions and showed complete cross-incompatibility (Fig. 5).

DISCUSSION

Fine mapping of genes that are of agronomic importance is the first step for map-based cloning. The mapping population size is critical to mapping resolution; usually, the bigger the mapping population, the higher the mapping resolution. Phenotyping the mapping population is usually the bottleneck that limits the size of the population. A homogeneous population mapping approach could eliminate the phenotyping step. This was achieved by using a
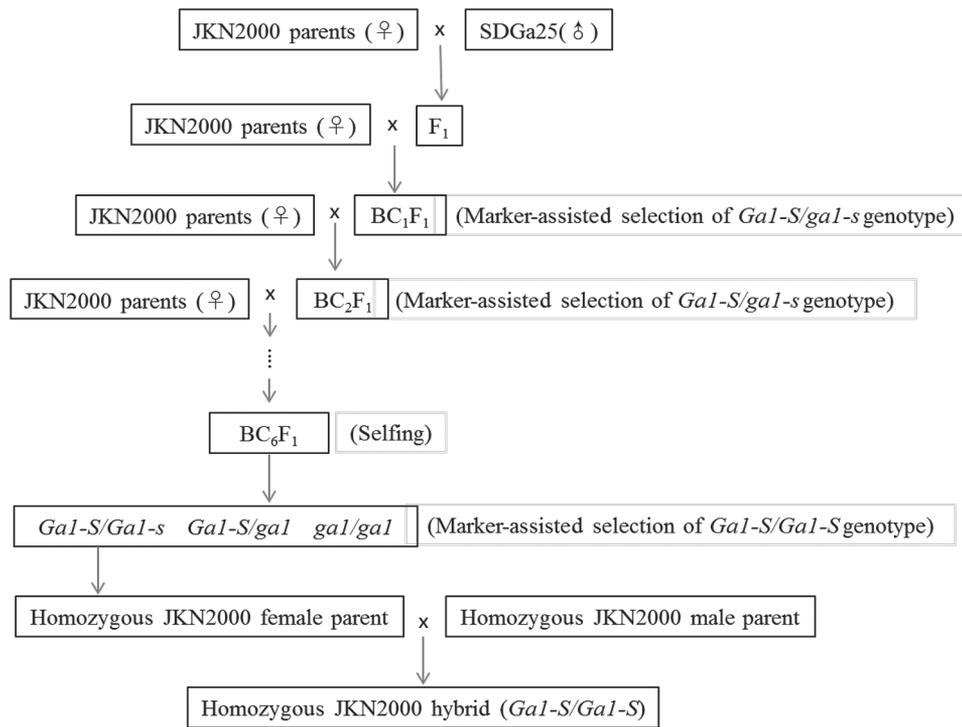
Figure 4. Diagram showing the backcross procedure utilized for introgression of gametophyte factor 1-strong allele (*Ga1-S*) into an elite waxy corn. The SDGa25 homozygous for *Ga1-S* showing complete cross-incompatibility was used as *Ga1-S* donor. The parental lines of waxy corn JKN2000 were used as recurrent parents. Individuals carrying *Ga1-S* in each backcross generation were selected with the assistance of molecular markers and were used for backcrossing. After six generation of backcross, *Ga1-S*-carrying individuals were selfed and homozygous ones were selected by codominant molecular markers. The JKN2000 homozygous at *Ga1-S* locus was created by crossing the two homozygous *Ga1-S/Ga1-S* parental lines.

A.   **B.**

Figure 5. White waxy corn JKN2000 hybrid showing difference in cross-incompatibility with yellow and purple corns (gametophyte factor 1-strong allele [*ga1-s*]/*ga1-s*). (a) JKN2000 (*ga1-s/ga1-s*). (b) JKN2000 (*Ga1-S/Ga1-S*). Both hybrids were grown in field conditions and were surrounded by yellow and purple corns.

BC_1F_1 population in which only one genotype exists. In the case of the *Ga1-S* allele, the BC_1F_1 population consists of only the *Ga1-S/Ga1-S* genotype. Using this population, the *Ga1-S* locus was quickly mapped within a 100 kb interval on chromosome 4.

We then applied this approach to map the strong alleles of *Ga2* and *Ga★*-Yugoslavia, which were completely incompatible to *ga* genotypes (Vancetovic et al., 2004; Kermicle and Evans, 2005, 2010). We also applied this approach to map the restorer gene, *Rf3*, for maize cytoplasmic male sterility S-type (CMS-S). In CMS-S maize, male sterility and fertility are determined by mitochondrial nuclear interactions in the haploid male gametophyte. The nuclear allele capable of restoring fertility to CMS-S in maize is designated as *Rf3*. All of the *Rf3* pollen is functional, whereas *rf3* pollen is nonviable (Laughnan and Gabay, 1978). The BC_1F_1 consisting of only *Rr* genotype was developed by backcrossing F_1 (*Rr*) as male to the recurrent male sterile parent. Only the *R* gametes were fertile (*r* gametes were sterile) and all BC_1F_1 individuals should then be male fertile. We grew out about 10,000 BC_1F_1 individuals in Hainan province in 2012 and 5000 in Beijing in 2013 and none of the plants were male sterile, suggesting the feasibility of this mapping method.

The homogeneous population mapping approach described in this paper is only effective for genes that are 100% excluded of one type of gametes during the pollination process. In many cases, however, this is not to be expected. Furthermore, this approach may only apply to the pollen function of *Ga1-S*. If the pollen and the pistil function could be separated by recombination (and there are naturally occurring *Ga1-M* alleles), then it would not reveal the map interval for the pistil function.

Gametophytic factor 1 is a complex locus, and there could be both a male and female factor governing cross incompatibility. The mapping approach described here was actually to map the male factor. A mapping population designed to map the female factor was constructed, and female factor mapping is under way.

Recently, there has been renewed interest in maize gametophytic factors due to cross incompatibility. This could be utilized to reduce cross fertilization in specialty maize with pollen from conventional maize and in conventional organic maize with pollen from genetically modified maize (Kermicle and Evans, 2010; Gonzalez et al., 2012; Zhang et al., 2012; Bloom and Holland 2012). Active research in breeding for cross incompatibility with *Ga1-S*, *Ga2-S*, *Ga★-Yugoslavia*, and *Tcb1* systems is underway both in China and the United States (Gonzalez et al., 2012).

During the process of mapping the *Ga1-S* allele, tightly linked codominant molecular markers were developed. These markers were able to distinguish *Ga1-S/Ga1-S*, *Ga1-S/ga1*, and *ga1/ga1* genotypes. With the assistance of these markers, in the winter nursery in the Hainan province and greenhouse facilities in Beijing, the *Ga1-S* allele was quickly introduced into the parental lines of a Chinese elite waxy maize hybrid, JKN2000, after six generations of backcrossing and one generation of selfing. Without marker assistance, individuals in the backcross segregating population carrying the *Ga1-S* allele were checked by crossing them as male onto *Ga1-S/Ga1-S* silks for seed setting evaluation. Marker-assisted selection greatly improved backcrossing efficiency by eliminating this step and by eliminating those individuals that do not carry the *Ga1-S* allele. Marker-assisted selection also reduced the time from two generations of selfing to one generation of selfing to obtain homozygous *Ga1-S/Ga1-S* individuals after the backcross process. We checked 25 ears each of the male and female parental lines with pollen from purple maize for cross-incompatibility (cross pollinated twice before selfing) and found complete cross-incompatibility to foreign pollens. The F$_1$ hybrid between these two parental lines also showed 100% cross-incompatibility to foreign pollens. The *Ga1-S* introgression did not affect much of the yield and quality of the recurrent parental lines. However, it may affect the yield of the F$_1$ hybrid since both parental lines carried the same *Ga1-S* allele from the same donor.

In 2012, 97.2 million acres of GM maize were planted in the US, representing 88% of the total acreage (www.ncga.com/home). Although biological and environmental parameters as well as technical management of coexistence measures have been taken to minimize GM-maize adventitious presence, it is still becoming increasingly difficult to ensure the safe coexistence between GM and non–GM maize. This is of specific concern to the organic maize growers and the organic industry, which needs to maintain identity-preserved non–GM maize products. The use of gametophytic factors may add one additional layer of protection for conventional maize from GM-maize contamination. Just like parental lines of GM-maize having both GM and non-GM versions, parental lines of *ga*-carrying maize also have *ga* and non-*ga* versions. As long as *ga*-carrying versions were not used for genetic transformation, GM-maize could be managed to not have gametophytic factors, making it possible for them to be utilized as biological reproductive barriers for GM and non-GM maize safe coexistence. No system intended for containment of pollen from GM-maize is 100% effective, including use of the cross incompatibility system. The combination of different strategies, however, such as the use of *Ga1-S/Ga1-S* hybrids and others may have synergistic effects in reducing cross-pollination between GM and non-GM maize to levels close to zero.

In summary, a homogeneous population mapping approach for mapping *gametophytic factor 1* was described in this paper. The approach completely eliminates the phenotyping step, which is often time-consuming, difficult to do, error-prone, and most importantly, limits the size of the mapping population and resolution. The strong allele *Ga1-S* was mapped within a 100 kb interval on chromosome 4 using this strategy. The *Ga1-S* showed 100% cross-incompatibility with the majority of dent and flint maize and could be used as a biological reproductive barrier among different types of maize such as waxy and non-waxy, GM and non-GM maize. The *Ga1-S* allele from popcorn can be quickly introgressed to conventional maize with the assistance of molecular markers.

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