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Sexual compatibility of transgenic soybean and different wild soybean populations

HU Yu-qi[1*], SHENG Ze-wen[1*], LIU Jin-yue[1], LIU Qi[1], QIANG Sheng[1], SONG Xiao-ling[1], LIU Biao[2]

[1] *Weed Research Laboratory, College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, P.R.China*
[2] *Nanjing Institute of Environmental Sciences, Ministry of Ecology and Environment, Nanjing 210042, P.R.China*

Abstract

The introduction of genetically modified (GM) soybean into farming systems raises great concern that transgenes from GM soybean may flow to endemic wild soybean *via* pollen. This may increase the weediness of transgenic soybean by increasing the fitness of hybrids under certain conditions and threaten the genetic diversity of wild soybean populations. Although pollen-mediated gene flow between GM crops and wild relatives is dependent on many factors, the sexual compatibility (SC) determined by their genetic backgrounds is the conclusive factor. The considerable genetic variation among wild soybean may cause compatibility differences between different wild and cultivated soybeans. Thus, evaluation of the SC between transgenic soybean and different wild soybeans is essential for assessing the environmental consequences of cultivated soybean-wild soybean transgene flow. The podding and seed sets were assessed after artificial hybridization using transgenic glyphosate-resistant soybean as the paternal parent and 18 wild soybean populations as the maternal parents. Then, the average number of filled seeds produced in 200 flowers (AFS) was calculated for wild soybean under natural self-pollination as well as under artificial crossing with transgenic soybean. Finally, the index of cross-SC was calculated (ICSC) (ratio of the AFS of wild soybean artificially crossed with transgenic soybean and the AFS of naturally self-pollinated wild soybean). The results demonstrated that after self-pollination and crossing with transgenic soybean, the average podding rates of 18 wild soybean populations ranged in 96.50–99.50% and 4.92–18.03%, and the average filled seed number per pod varied from 1.70 to 2.69 and 0.20 to 0.48, respectively. The results showed that approximately 89% of wild soybeans displayed medium or higher than medium SC with transgenic soybean (ICSC>1.0%). This implied the high possibility of gene flow *via* pollen from transgenic soybean to wild soybean.

Keywords: transgenic soybean, wild soybean, sexual compatibility, artificial hybridization, gene flow

1. Introduction

Biotechnology has been the most quickly adopted crop technology in the world. Twenty-six countries planted 191.7 million hectares of genetically modified (GM) crops in 2018, a number which has increased 113-fold since 1996. GM soybeans, which were planted on 95.9 million hectares, reached their highest level of adoption worldwide, covering 50% of the global biotech crop area (James 2018).

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HU Yu-qi, E-mail: 2017116028@njau.edu.cn; SHENG Ze-wen, 2018116085@njau.edu.cn; Correspondence SONG Xiao-ling, Tel: +86-25-84395117, E-mail: sxl@njau.edu.cn; LIU Biao, Tel/Fax: +86-25-85287064, E-mail: 85287064@163.com
* These authors contributed equally to this study.

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Herbicide resistance, including mono trait resistance and stacked trait resistance wherein insect resistance and herbicide resistance are combined, has consistently been used as a dominant trait in soybeans; in 2017, soybeans with herbicide resistance accounted for 77% of the global biotech soybean area (James 2019). Currently, GM soybean has not been permitted for commercial release, but biotechnology has been applied to develop herbicide-resistant, high-yield, high-quality and stress-tolerant varieties in China (Chen *et al*. 2012; Liu M *et al*. 2012; Song *et al*. 2013; Zhang *et al*. 2013; Xu *et al*. 2017). Soybean production is not self-sufficient in the country, although the Chinese government has made efforts to revitalize the industry. Eighty percent of soybeans consumed since 2012 have been imported, reaching 88.04 million tons in 2018 (http://data.stats.gov.cn), and most of these are herbicide-resistant soybeans. On 21 January 2020, Ministry of Agriculture and Rural Affairs, China issued a security certificate for the production and field planting in the south of China for the glyphosate-resistant transgenic soybean SHZD32-01, developed by Shanghai Jiaotong University, representing further progress towards the commercialization of transgenic soybean in China. It is highly possible that these soybeans will be cultivated in China in the near future.

The introduction of GM soybean into farming systems raises great concern that transgenes *via* pollen from GM soybean may flow to endemic wild soybean (Kuroda *et al*. 2006; Wang *et al*. 2010; Yoshimura *et al*. 2011; Goto *et al*. 2017). The gene flow from GM soybean to wild soybean is the major avenue for transgenic soybean to become a weed if the fitness of hybrids between transgenic soybean and wild soybean increases compared to that of their wild soybean parents under certain conditions. On the other hand, gene flow from GM soybean might threaten the genetic diversity of wild soybean populations (Wang *et al*. 2010; Yoshimura *et al*. 2011).

Wild soybean (*Glycine soja* Sieb. et Zucc.) (G genome, 2n=40) is classified within the same genus as cultivated soybean (*Glycine max* (L.) Merr.) and is an ancestor of soybean and an important part of soybean genetic resources, and also has important value for research on the origin and evolution of soybean (Li X H *et al*. 2010; Li Y H *et al*. 2010; Stupar 2010; Akpertey *et al*. 2014). Wild soybeans are distributed in all soybean growing areas in China, from the north (53°N) to the south (24°N) and from the east (134°E) to the west (97°E) (Dong *et al*. 2001). The area of overlap between soybean cultivation areas and wild soybeans habitats is gradually increasing with increases in the soybean planting area (Wang and Li 2012a).

Cultivated and wild soybeans have no crossing barrier and produce highly fertile F_1 hybrids by artificial crossing, and *G. soja* and *G. max* hybrids can survive in semi-natural conditions for at least 3 yr without intervention (Oka 1983). Additionally, introgression between cultivated and wild soybeans in China has been reported (Wang *et al*. 2010; Wang and Li 2011a, b). The outcrossing rate in wild soybean varied in 2.4–19.0% (Kiang *et al*. 1992) and 9.3–19% (Fujita *et al*. 1997; Ohara and Shimamoto 2002) using allozymes and 0–37.4% (Kuroda *et al*. 2006) applying nSSRs. The mean outcrossing rate reached 8.1%, with extremely high values from 21.2 to 66.4% based on nuclear and chloroplast microsatellite variations (He *et al*. 2012).

The frequency of natural hybridization through pollen flow from cultivated soybean to a single wild soybean has been measured quantitatively, and the mean natural outcrossing rate was 0.73% (five hybrids from 686 progeny), ranging from 0 to 5.89% when *G. soja* and *G. max* were alternately planted at a distance of 50 cm under their simultaneous flowering and adequate pollinators (Nakayama and Yamaguchi 2002). However, in adjacent cultures spaced 5 cm apart, only one glyphosate-tolerant seedling was detected among 32 502 seedlings from the 51 wild soybean plants examined (Mizuguti *et al*. 2009). Furthermore, a 2-yr hybridization experiment was conducted to understand the factors that affect natural hybridization between GM and wild soybean. The experiment found that hybridization frequencies ranged from 0 to 0.097% (25 hybrids out of 25 741 cultivated adjacent to wild soybean). Differences in flowering phenology, isolation distance and the presence of buffer plants accounted for half of the variation in the hybridization frequency found in this study (Mizuguti *et al*. 2010). In China, Chen *et al*. (2006) reported that one glyphosate-resistant hybrid among 12 728 plants of wild soybean was found 10 m from the pollen donor in an experiment utilizing a concentric circle sampling scheme. Recently, the outcrossing rate from transgenic soybean to five wild soybeans was 0.06–0.19% in alternating cultivations (Liu *et al*. 2020). However, Liu *et al*. (2008) and Liu J *et al*. (2012) did not find gene flow from glyphosate-resistant transgenic soybean to wild soybean. It is widely recognized that pollen-mediated gene flow between GM crops and their wild relatives is dependent on many factors, including compatibility, the degree of flower synchronization, sympatry, and circumstance (such as wind force and wind direction). However, compatibility, which is determined by genetic background, is the conclusive factor (Dale 1992; Légère 2005; FitzJohn *et al*. 2007; Song *et al*. 2009; Zuo *et al*. 2011). Wild soybean has been shown to exhibit genetic differentiation in the process of adapting to different growth environments over a long time (Kiang *et al*. 1992; Wen *et al*. 2009; Li X H *et al*. 2010; Li Y H *et al*. 2010; He *et al*. 2012; Wang and Li 2012b). Estimations of the genetic variability of *G. soja* have been made by many researchers (Choi *et al*. 1999; Dong *et al*. 2001; Ohara and

Shimimoto 2002; Li X H *et al*. 2010; Li Y H *et al*. 2010; Wang and Li 2011a). The considerable genetic variation among wild soybean may cause compatibility differences between different wild and cultivated soybeans.

Although studies have shown that GM soybeans can hybridize with wild soybeans, they still do not fully reflect the potential ecological risks of cultivating GM soybeans in China due to the few populations of wild soybeans studied (Chen *et al*. 2006; Liu *et al*. 2020). The current study used a glyphosate-resistant (GR) soybean (T14R 1251-70) as the male parent and 18 wild soybean populations of different origins as the female parents for artificial hybridization. The podding and seed set rates were counted, finally index of cross sexual compatiblity was calculated to comprehensively evaluate the compatibility differences between GR soybeans and different populations of wild soybeans. The results provide a theoretical basis for assessing gene flow from herbicide-resistant soybean to wild soybean.

2. Materials and methods

2.1. Materials

Glyphosate-resistant soybean (T14R 1251-70) and receptor soybean (NJR44-1) were provided by the National Soybean Improvement Center of Nanjing Agricultural University. T14R 1251-70 contains one single-copy *cp4-epsps*. It was obtained by Agrobacterium-mediated co-transformation of the receptor soybean NJR44-1.

A total of 18 wild soybean populations were collected from 12 provinces, including Heilongjiang, Jilin, Liaoning, Hebei, Shangdong, Gansu, Henan, Jiangsu, Anhui, Zhejiang, Jiangxi, and Hunan, and two autonomous regions, Inner Mongolia and Guangxi Zhuang, in China (Table 1).

2.2. Methods

Planting methods Experiments were conducted in a greenhouse at the Pailou Experimental Farm (32°02′N, 118°37′E), Nanjing Agricultural University, China, from 2016 to 2017. Experimental pots were exposed to natural light and photoperiods, and the temperature fluctuated from 30–38°C during the experiment.

Single seeds of each wild soybean population and transgenic soybean were sown at 1 cm depth in individual pots (23 cm diameter and 25 cm height) previously filled with a clay loam of medium fertility from the experimental field. Eight pots were considered an experimental unit, and there were four replicates of each population of wild soybean. The 160 transgenic soybean seeds were sown four times (40 seeds each time) at 10-day intervals to guarantee flowering synchronization with the different wild soybeans.

Selection of flower buds of wild soybean for emasculation and transgenic soybean flowers for pollination The anthers of wild soybean dehisce before anthesis. Emasculation is necessary to prevent self-pollination of wild soybean, and thus, for hybridization purposes, the flowers of wild soybean must be emasculated before anthesis. Therefore, the feasibility and reliability of the method of artificially emasculating wild soybean flowers were assessed using population HLJHRB-1. The flower buds were picked at 05:00–06:00 in the morning, and their morphological characteristics and the development of the stigmas and anthers were observed under a dissecting microscope (Olympus SZX7, Carl Zeiss, Germany) and recorded. Flower buds in which the stigma was developed

Table 1 Information of wild soybeans (*Glycine soja*) used in the experiments

Number	Population number	Collecting site	Latitude and Longitude
1	HLJHRB-1	Harbin City, Heilongjiang Province	46°06′34′′N, 127°21′43′′E
2	HLJHRB-2	Harbin City, Heilongjiang Province	46°04′44′′N, 127°23′02′′E
3	JLBC-1	Baicheng City, Jilin Province	45°31′23′′N, 124°17′19′′E
4	JLBC-2	Baicheng City, Jilin Province	45°31′20′′N, 124°19′57′′E
5	LNTL	Tieling City, Liaoning Province	42°17′28′′N, 123°51′47′′E
6	LNSY	Shenyang City, Liaoning Province	41°32′41′′N, 123°27′29′′E
7	IMBT	Baotou City, Inner Mongolia Autonomous Region	40°37′37′′N, 109°54′14′′E
8	HBBD	Baoding City, Hebei Province	38°51′08′′N, 115°43′16′′E
9	SDDY	Dongying City, Shandong Province	37°25′10′′N, 118°46′55′′E
10	HBHD	Handan City, Hebei Province	36°38′59′′N, 114°36′35′′E
11	GSBY	Baiying City, Gansu Province	36°32′58′′N, 104°08′38′′E
12	HNSQ	Shangqiu City, Henan Province	34°22′21′′N, 115°40′18′′E
13	JSCZ	Changzhou City, Jiangsu Province	31°37′13′′N, 119°29′53′′E
14	AHAQ	Anqing City, Anhui Province	31°07′26′′N, 116°59′59′′E
15	ZJHN	Haining City, Zhejiang Province	30°28′08′′N, 120°29′42′′E
16	JXYT	Yingtan City, Jiangxi Province	28°16′29′′N, 117°01′24′′E
17	HNCZ	Chenzhou City, Hunan Province	25°44′34′′N, 112°57′52′′E
18	GXGL	Guilin City, Guangxi Zhuang Autonomous Region	25°04′06′′N, 110°18′41′′E

completely (the day before anther dehiscence) but the anthers did not dehisce were chosen for emasculation and pollination. The morphological characteristics of flower buds at this stage were recorded. After establishing the criteria for choosing these flower bud, 50 optimal flower buds in the middle and upper sections of the five wild soybean plants were emasculated artificially at 05:00–06:00 in the morning and were not pollinated to verify the reliability of the method of emasculating wild soybean flowers artificially. The details were as follows according to Lu and Shi (2009).

The optimal flower buds selected for emasculation were held between the thumb and forefinger. The peduncles holding the flowers were weak and tender and were thus handled with care. First, sharply pointed forceps were used to gently remove the upper part of the sepals. Then, carefully holding the middle of the flower bud, the entire corolla with the anthers was removed with the forceps by grasping the middle of the corolla and breaking them it from the flower to complete the emasculation. Then, the emasculated flower bud was wrapped with the fresh leaves around it to protect the exposed style and tagged. After 10 days, the emasculated flower bud was observed to develop into a pod. The developed pod number was recorded.

At 07:00–08:00, transgenic soybean flower buds with corollas that had not been fully opened but had dehiscent anthers were selected as pollen donors (Appnedix A). To determine the pollen viability of transgenic soybeans used as pollen donors, pollens were collected from these flower buds, and the *in vitro* pollen germination rate from 20 to 100 min was tested according to the method described by Liu and Liu (2018). At least 500 pollens from five flower buds on each of the five plants were used as one replicate, and a total of four replicates were conducted each time over the 2 yr. Finally, the *in vitro* pollen germination rate was calculated as follows: Pollen germination rate=(Pollen germinated/Pollen observed)×100

Self-pollination of wild soybean Wild soybean is autogamous except for occasional limited outcrossing. Because its flower bud is very small, 2–3 mm, it is difficult to perform emasculation. Therefore, for self-pollination, appropriate flower buds were selected and tagged. Surplus flower buds around the chosen flower buds were removed. At maturity, data were recorded for the number of flowers, developed pods, seeds and filled seeds in pods. The podding and seed set rates were calculated. Podding rate (%)=Pods/The selected appropriate flower buds×100; Seed set rate=Filled seeds/Seeds in a single pod

For most populations, 50 appropriate flower buds were selected and observed. For the five populations (JSCZ, AHAQ, ZJHN, HBHD, and LNTL), 100 appropriate flower buds were selected and observed. For seed set, numbers of seeds and filled seeds of 40 developed pods were recorded. The experiment was replicated four times.

Hybridization of wild soybean crossed with transgenic soybean The optimal flower buds of wild soybean were selected and emasculated according to the methods established. Hybridizations were carried out by four people with similar crossing experience from 2016 to 2017. On the same day, at 07:00–08:00, transgenic soybean flower buds with corollas that had not been fully opened but with dehiscent anthers were selected and picked up (Appendix A). Then, all petals were removed to expose the anthers. The anthers were gently rubbed on the stigma of the emasculated wild soybean flowers (stigmas were rubbed twice with anthers of two transgenic flowers) to ensure that the donor pollens were scattered on the stigmas of the wild soybean buds completely. After pollination, the pollinated flower buds were wrapped with fresh leaves around it and tagged. For each wild soybean population, 37–180 flower buds were emasculated and crossed with transgenic pollen. The experiment was replicated four times. All crosses were made in a similar period of the same year during the self-pollination period of wild soybean. At 60 days after crossing, data were recorded for the developed pods, seeds and filled seeds in pods. The podding and seed set rates were calculated: The podding rate (%)=Developed pods/Crossed flower buds×100; Seed set rate (%)=Filled seeds/Seeds in a single pod×100

Assessment of sexual compatibility of wild soybean crossed with transgenic soybean First, the number of average filled seeds produced in 200 pollinated flowers (AFS) was calculated for naturally self-pollinated wild soybean as well as for that artificially crossed with transgenic soybean. AFS=200×Podding rate×Average filled seed number per pod

Index of cross sexual compatibility (ICSC)=AFS of wild soybean by artificially crossed with transgenic soybean/AFS by natural self-pollination. ICSC was used to evaluate cross-sexual compatibility (SC). Greater ICSC means higher SC between wild soybean and transgenic soybean. The five grades were divided to assess cross-SC (Table 2).

2.3. Data and statistical analysis

The podding and seed set rates were calculated using Microsoft Office Excel 2007. All data analysis was performed using SPSS (SPSS 22.0) statistical software. One-way ANOVA was performed using Duncan's multiple range test to compare the *in vitro* pollen germination rates of transgenic soybean from 20 to 100 min at the same planting time, podding rate, number of seeds per pod, number of filled seeds per pod, and seed set of wild soybean under natural

Table 2 The grade for assessing cross sexual compatibility between wild soybean and transgenic soybean

Grade	Index for cross sexual compatibility	Level of cross sexual compatibility
1	ICSC>3.0%	Extremely high
2	3.0%≥ICSC>2.0%	High
3	2.0%≥ICSC>1.0%	Medium
4	1.0%≥ICSC>0.5%	Low
5	0.5%≥ICSC	Extremely low

self-pollination or that artificially crossed with transgenic soybean between different wild soybean populations.

3. Results

3.1. Selection of flower buds of wild soybean for emasculation and transgenic soybean flowers for pollination

The wild soybean flower is typical of those in the subfamily Papilionoideae, i.e., the flowers exhibit a polypetalous, irregular corolla. Out of the five petals, the odd fifth posterior petal is the outermost, forming the standard; the two lateral petals, the wings and the two anterior petals, are connate, forming a boat-shaped keel. Both the androecium and gynoecium are enclosed inside the keel tube (Appendix B).

The morphological characteristics of HLJHRB-1 wild soybean flower buds were recorded, and photos were taken as follows (Appendix C):

Stage 1: The flower bud is tightly wrapped with an indehiscent calyx, and the corolla is not visible. Inside, stigma and anther appear transparent and small.

Stage 2: The top of the calyx is slightly dehiscent, and the white or light purple corolla is seen indistinctly. Inside, the stigma and anther are larger than those in stage 1.

Stage 3: The top of the calyx is dehiscent, and the purple corolla can be seen clearly. The stigma and anther appear larger than those at stage 2, but anther dehiscence has not occurred.

Stage 4: The calyx lobes are prominent, and the corolla has stretched from the top of the calyx. Inside, the dehiscence of anthers can be seen clearly.

The time from stages 1 to 2 was 2 d, and the time from stages 2 to 3 and from stages 3 to 4 was approximately 1 d each. In stage 4, anther dehiscence occurred. Since stage 3 occurs just before stage 4, the stigma should be receptive during stage 3. The flower buds of wild soybean at stage 3 were considered optimal flower bud for emasculation and pollination.

Fifty optimal flower buds from five wild soybean plants were emasculated. After 10 d, no pods developed from these emasculated flower buds. Therefore, the emasculation techniques used in this study for wild soybean are feasible and reliable.

The pollen germination rates of transgenic soybean were similar at four planting times in two years *in vitro* experiments from 20 to 100 min, which were approximately 87–94% (Fig. 1). These results confirmed that the transgenic soybeans used as pollen donors four times in 2 yr had high viability.

3.2. The podding rate of wild soybean after self-pollination and crossing with glyphosate-resistant transgenic soybean

The average podding rates of 18 wild soybean populations after self-pollination were very high and quite similar, ranging from 96.50 to 99.50%, in which those of JLBC-2, GSBY and GXGL were the lowest, and that of LNSY was the highest. However, when crossed with transgenic soybean, the podding rates varied among the different wild soybeans, ranging from 4.92 to 18.03%, among which that of HBBD was the lowest, and that of HNCZ was the highest.

The podding rate of wild soybean crossed with transgenic soybean differed greatly from that of self-pollinated wild soybean and was significantly lower than that of the respective self-pollinated wild soybeans by 80.47–94.08%; HNCZ had the smallest difference and HBBD had the largest difference (Table 3).

3.3. The seed set rate of wild soybean after self-pollination and pollination with transgenic soybean

After self-pollination, the each eight populations of wild soybean produced 1–3 and 1–4 filled seeds in each pod, and most pods contained 2–3 filled seeds, except for those of SDDY and AHAQ, in which 7.5 and 0.63% of pods did not contain filled seeds. The proportion of pods containing one filled seed was 0.63–15.63%, except for those of SDDY, which reached 31.25% of pods containing one filled seed. The proportions of pods containing two, three and four filled seeds were 33.75–70.00%, 16.25–62.50% and 0–6.88%, respectively (Appendix D).

When crossed with transgenic soybean, wild soybean produced fewer filled seeds. A total of 55.95–80.28% of the pods of wild soybean did not contain filled seeds, and 18.31–40.85% of pods contained one filled seed. Most wild soybeans did not have pods containing two filled seeds, and only the four populations, LNTL, HBHD, IMBT, and HNSQ, produced two filled seeds in 1.27–3.57% pods (Appendix D).

With the self-pollination of the 18 wild soybean populations, the average seed number per pod and the average filled seed number per pod varied from 2.56 to 3.21 and from 1.70 to 2.69, respectively. After crossing with

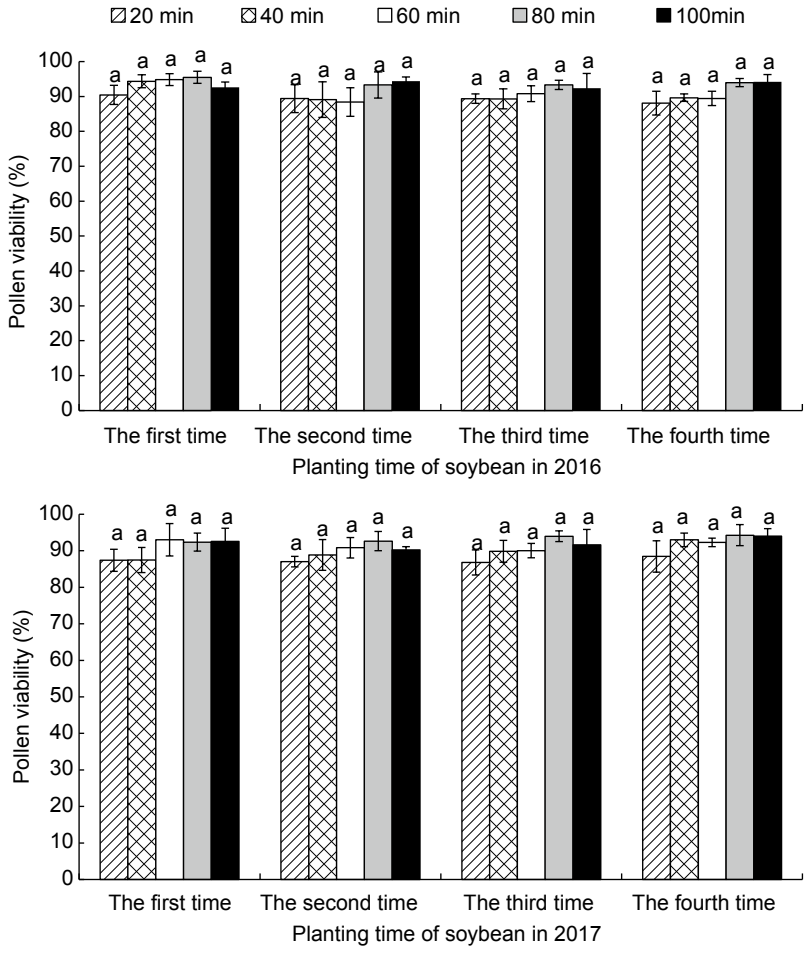

Fig. 1 Pollen germination rate of transgenic soybean *in vitro* from 20 to 100 min in four planting times in 2 yr. The same lowercase showed no significant difference *in vitro* 20 to 100 min in the same planting time. Bars mean SE (*n*=4).

transgenic soybeans, the average seed number per pod and the average filled seed number per pod in wild soybean decreased to 1.30–2.07 and 0.20–0.48, respectively. The average seed set in the different wild soybean populations shifted from 63.85–90.31% after self-pollination to 8.88–28.02% after crossed with transgenic soybean, showing a reduction of 50.66–78.14% with artificial crossing (Table 4).

3.4. Assessment of sexual compatibility of wild soybean crossed with transgenic soybean

The ICSC of HNCZ was 3.2%, showing extremely high cross-SC. Then ICSCs of JLBC-1, HBHD, ZJHN, IMBT, HNSQ, and JXYT ranged from 2.08–2.88%, showing high cross-SC. Nine populations, HLJHRB-1, HLJHRB-2, JLBC-2, LNSY, SDDY, JSCZ, AHAQ, GSBY, and GXGL, whose ICSC varied from 1.13 to 1.71%, demonstrated medium cross-compatibility. LNTL and HBBD, with ICSCs of 0.99 and 0.46%, showed low and extremely low cross-SC, respectively (Table 5).

4. Discussion

4.1. Importance of sexual compatibility

The introgression of transgenes of genetically modified crops may change the persistence or multiplication of wild/weedy relatives in the environment. Because of the possession of modified novel, beneficial transgenes may enhance the fitness associated with the competitiveness and invasiveness of wild/weedy relatives under selection pressure (Stewart *et al*. 2003; Lu and Yang 2009; Hokanson *et al*. 2015; Wang *et al*. 2016; Kim *et al*. 2019). Moreover, hybridization allows other crop alleles to spread to wild/weedy populations (Snow *et al*. 2010). On occasion, introgression of crop alleles leads to the evolution of increased weediness (Mercer *et al*. 2007; Uwimana *et al*. 2012). Ultimately, gene flow from crop taxa may have a

Table 3 The podding rate of different wild soybeans after self-pollination and crossed with glyphosate-resistant transgenic soybean[1)]

Population	Pod number/Flower number		Podding rate (%)		Podding rate difference (%)
	Self-pollination	Crossed	Self-pollination	Crossed	
HLJHRB-1	198/200	76/525	99.00±0.58 ab	14.47±0.60 bcd	84.53
HLJHRB-2	195/200	62/420	97.50±0.96 abc	14.76±0.61 bc	82.74
JLBC-1	197/200	123/685	98.50±0.96 abc	17.97±1.06 a	80.53
JLBC-2	193/200	43/328	96.50±0.50 c	13.13±0.63 cde	83.37
LNTL	393/400	71/722	98.25±0.25 abc	9.84±0.44 fghi	88.41
LNSY	199/200	50/565	99.50±0.50 a	8.85±0.17 hi	90.65
IMBT	194/200	64/550	97.00±0.58 bc	11.62±0.55 efg	85.38
HBBD	198/200	13/265	99.00±1.00 ab	4.92±0.42 j	94.08
SDDY	197/200	14/180	98.50±0.96 abc	7.78±0.64 i	90.72
HBHD	394/400	84/665	98.50±0.29 abc	12.62±0.71 de	85.88
GSBY	193/200	47/400	96.50±0.50 c	11.75±0.48 ef	84.75
HNSQ	198/200	81/500	99.00±0.58 ab	16.20±0.38 ab	82.80
JSCZ	397/400	25/242	99.25±0.25 ab	10.32±0.71 fgh	88.93
AHAQ	391/400	19/202	97.75±0.48 abc	9.41±0.96 hi	88.34
ZJHN	396/400	28/216	99.00±0.41 ab	12.96±0.76 cde	86.04
JXYT	198/200	71/500	99.00±1.00 ab	14.20±0.68 bcd	84.80
HNCZ	197/200	53/294	98.50±0.96 abc	18.03±0.63 a	80.47
GXGL	193/200	24/250	96.50±0.96 c	9.65±0.83 ghi	86.85

[1)] Crossed means crossed by the transgenic soybean.
Values are mean±SE (*n*=4).

Table 4 The seed set rate of different wild soybeans after self-pollination and crossed with glyphosate-resistant transgenic soybean[1)]

Population	Number of seeds per pod		Number of filled seeds per pod		Seed set (%)	
	Self-pollination	Crossed	Self-pollination	Crossed	Self-pollination	Crossed
HLJHRB-1	2.91±0.03 cd	1.54±0.04 bcdef	2.59±0.03 bc	0.20±0.02 f	89.48±0.43 ab	11.34±0.53 fg
HLJHRB-2	2.76±0.02 hij	1.73±0.01 bc	2.46±0.01 e	0.27±0.01 cdef	90.31±0.73 a	13.18±0.64 efg
JLBC-1	2.81±0.02 fgh	1.35±0.02 ef	2.48±0.04 de	0.30±0.02 bcdef	88.65±1.12 ab	16.88±1.24 def
JLBC-2	2.90±0.02 cde	2.07±0.06 a	2.54±0.01 cd	0.32±0.02 bcde	87.81±0.31 abc	13.02±1.31 efg
LNTL	3.03±0.04 b	1.58±0.02 bcde	2.13±0.03 jk	0.21±0.02 ef	70.68±0.88 j	8.88±1.05 fg
LNSY	2.96±0.01 c	1.64±0.02 bcd	2.63±0.01 b	0.38±0.05 abc	89.17±0.17 ab	17.95±1.93 cdef
IMBT	2.65±0.00 l	1.70±0.01 bc	2.13±0.01 jk	0.37±0.01 abc	81.46±0.65 fg	19.68±1.02 bcde
HBBD	2.86±0.01 def	1.77±0.08 b	2.48±0.01 de	0.23±0.08 def	86.67±0.51 bcd	13.19±5.24 efg
SDDY	2.70±0.02 jkl	1.71±0.17 bc	1.70±0.04 l	0.29±0.02 bcdef	63.85±1.92 k	13.19±1.20 efg
HBHD	2.77±0.01 ghi	1.73±0.03 bc	2.21±0.01 hi	0.48±0.03 a	80.52±0.10 g	24.62±1.85 abc
GSBY	2.86±0.02 def	1.36±0.01 ef	2.54±0.02 cd	0.34±0.02 bcd	89.48±0.57 ab	21.49±3.17 abcd
HNSQ	2.56±0.02 m	1.40±0.02 def	2.10±0.02 jk	0.37±0.01 abc	83.85±0.79 ef	21.72±1.12 abcd
JSCZ	3.21±0.02 a	1.52±0.19 bcdef	2.69±0.01 a	0.40±0.06 abc	84.69±0.50 de	26.01±1.03 ab
AHAQ	2.82±0.01 fgh	1.50±0.09 cdef	2.16±0.01 ij	0.38±0.07 abc	77.50±0.57 h	19.17±3.63 bcde
ZJHN	2.80±0.03 fgh	1.55±0.10 bcdef	2.34±0.01 g	0.40±0.05 abc	84.84±0.69 de	22.74±2.08 abcd
JXYT	2.73±0.01 ijk	1.30±0.01 f	2.40±0.02 f	0.41±0.01 ab	88.54±1.10 ab	28.02±1.62 a
HNCZ	2.84±0.02 efg	1.49±0.03 cdef	2.06±0.03 k	0.36±0.03 abc	73.85±1.72 i	18.30±1.36 cdef
GXGL	2.68±0.01 kl	1.52±0.10 bcdef	2.26±0.01 h	0.34±0.02 bcd	85.73±0.43 cde	18.99±2.32 cde

[1)] Crossed means crossed by the transgenic soybean.
Values are mean±SE (*n*=4).

substantial impact on the evolution of wild populations (Ellstrand *et al*. 1999, 2013).

The initial stage of introgression is the formation of a hybrid (Chapman and Burke 2007; Mizuguti *et al*. 2010). Estimating hybridization frequencies between GM crops and their relatives is the first step towards evaluating the introgression of a transgene. For these reasons, understanding the factors that affect hybridization between GM crops and their wild relatives is important and useful for the development of regulations in countries where wild relatives of GM crops are distributed.

Pollen-mediated transgene flow is driven by many factors. Among these factors, some degree of reproductive compatibility between flowering times of geographically overlapping species is the most important factor controlling the natural hybridization between species (Dale 1992; Ellstrand *et al*. 1999; Chapman and Burke 2007; Song *et al*. 2009; Zuo *et al*. 2011).

Table 5 The index of cross sexual compatibility, sexual compatibility grade and sexual compatibility level of different wild soybean crossed with transgenic soybean[1)]

Number	Population	AFS of crossed	AFS of self-pollinated	ICSC (%)	Sexual compatibility grade	Sexual compatibility level
1	HLJHRB-1	5.79	512.82	1.13	3	Medium
2	HLJHRB-2	7.97	479.70	1.66	3	Medium
3	JLBC-1	10.78	488.56	2.21	2	High
4	JLBC-2	8.40	490.22	1.71	3	Medium
5	LNTL	4.13	418.55	0.99	4	Low
6	LNSY	6.73	523.37	1.29	3	Medium
7	IMBT	8.60	413.22	2.08	2	High
8	HBBD	2.26	491.04	0.46	5	Extremely low
9	SDDY	4.51	334.90	1.35	3	Medium
10	HBHD	12.12	435.37	2.78	2	High
11	GSBY	7.99	490.22	1.63	3	Medium
12	HNSQ	11.99	415.80	2.88	2	High
13	JSCZ	8.26	533.97	1.55	3	Medium
14	AHAQ	7.15	422.28	1.69	3	Medium
15	ZJHN	10.37	463.32	2.24	2	High
16	JXYT	11.64	475.20	2.45	2	High
17	HNCZ	12.98	405.82	3.20	1	Extremely high
18	GXGL	6.56	436.18	1.50	3	Medium

[1)] AFS, the average filled seeds produced in 200 pollinated flowers; crossed, crossed with the transgenic soybean; ICSC, index of cross sexual compatibility.

Generally, the highest outcrossing rates from transgenic soybean to wild soybean were much lower than those from cultivated soybean. The highest outcrossing rates from transgenic soybean to cultivated soybean were found to be 0.19% by Yoshimura *et al*. (2006), 0.52% by Abud *et al*. (2007), 0.934% by Huang *et al*. (2014), 0.049% (Kim *et al*. 2019), and 0.93% by Liu *et al*. (2020). Direct evidence for outcrossing from transgenic soybean to wild soybean has been reported in Japan (Nakayama and Yamaguchi 2002; Mizuguti *et al*. 2009, 2010) and in China (Chen *et al*. 2006; Liu *et al*. 2008, 2020; Liu J *et al*. 2012). Of these reports, the highest outcrossing rates were 0.73, 0.19 and 0.097%, respectively. Two reports had extremely low outcrossing rates of 0.0031 and 0.0078%, and two reports did not find gene flow. These reports have one thing in common; only one wild soybean was used in these experiments except for one study by Liu *et al*. (2020). The results obtained by using limited wild soybeans are not sufficient to predict the potential possibility of outcrossing from transgenic soybean to wild soybean.

Both the horizontal and vertical distribution of annual wild soybean in China is extensive, and this contributes to the high genetic diversity of wild soybeans (Dong *et al*. 2001). Wild soybean possesses a higher level of genetic diversity than cultivated soybean (Dong *et al*. 2001; Wen *et al*. 2009), and significant variance does exist among subpopulations in Northeast China, the Huang-Huai-Hai Valley and Southern China (Wen *et al*. 2009).

Such plentiful genetic diversity of annual wild soybeans in China makes it difficult to select wild soybeans for assessing gene flow from GM soybean to wild soybean. Direct measurements of gene flow are generally difficult because such experiments require a large field area. On the other hand, in addition to innate factors, such as reproductive compatibility, sympatry, flowering synchrony, and the acreages of the pollen sources and recipient populations, the outcrossing rate is also influenced by environmental factors, such as weather and environmental conditions (Chen *et al*. 2006). This can result in findings that cannot be compared.

It is plausible that differences in the genetic background of wild soybean populations impact their compatibility with cultivated soybean. The evidence of interspecific reproductive compatibility derived from experimental crosses is an important component of risk assessment and a useful first step, especially where data from field observations are unavailable (Armstrong *et al*. 2005). Reports of experimental hybridization (manual hand pollination) between crops and their relatives are a useful source of information, as they allow measurement of the degree of reproductive compatibility between species (FitzJohn *et al*. 2007).

If the extent of reproductive compatibility between transgenic soybean and wild soybean was known in advance, wild soybean that had higher reproductive compatibility with transgenic soybean could be selected for further field assessment. The results of gene flow obtained using species with the highest reproductive compatibility under natural conditions will provide the most persuasive and useful information for assessing the possibility of gene

flow from transgenic soybean to wild soybean. Reproductive compatibility data represent an early 'tier' of risk assessment. The more detailed assessment of rates of spontaneous hybridization forms a later tier of assessment and needs to be done for populations with high reproductive compatibility (Song *et al*. 2009).

4.2. Index of cross sexual compatibility

In our paper, the SC of 18 wild soybean populations, as pollen recipients, and glyphosate-resistant soybean, as the pollen donor, was confirmed by evaluation of the podding and seed set rates. This demonstrated that the podding and seed set rates in both self- and cross-pollinated wild soybean varied among different genotypes of wild soybean. If only seed set after hybridization was used as an index for assessing SC, the difference in the seed set between self-pollinated and hybridized wild soybeans as well as the differences in the podding rate with these two methods, would be ignored. To avoid this problem, the index of cross sexual compatibility (ICSC) was proposed.

The ICSC indicates the average filled seeds produced in 200 flowers after hybridization by natural self-pollination. A greater ICSC means higher SC between wild soybean and transgenic soybean. Based on the ICSC values, SC was dependent on the genotype of the wild soybeans. Each population showed extremely high and extremely low SC with transgenic soybean, accounting for 5.56%. Six and nine populations showed high and medium SC, accounting for 33.33 and 50%, respectively. Only one population showed low SC. The results demonstrated that approximately 89% of wild soybean plants displayed medium or higher SC. This implied a high possibility of gene flow *via* pollen from transgenic soybean to wild soybean.

According to ecological regions where soybean grows in China suggested by Wang and Gai (2002), seven wild soybeans belong to the northern single cropping, spring planting eco-region (I), of which one (LNTL) and two (JLBC-1 and IMBT) populations showed low and high SC, respectively, and the other four (HLJHRB-1, HLJHRB-2, JLBC-2, and LNSY) showed medium SC. Five wild soybeans belong to the Huang-Huai-Hai double cropping, spring and summer planting eco-region (II), of which two each showed high (HBHD and HNSQ) and medium (SDDY and GSBY) SC, and one (HBBD) had the lowest SC. Two (JSCZ and AHAQ) and one wild soybean (ZJHN) from the middle and lower Changjiang Valley double cropping, spring and summer planting eco-regions (III) had medium and high SC, respectively. Three wild soybeans (HNCZ, JXYT and GXGL) from the Central South multiple cropping, spring, summer, and autumn planting eco-region (IV) demonstrated extremely high, high and medium values, respectively. There were no wild soybeans collected from the southwest plateau double cropping, spring and summer planting eco-region (V), and South China tropical multiple cropping, all season planting eco-region (VI). Comparatively, these results implied that wild soybeans from central south multiple cropping, spring, summer, and autumn planting ecoregions had high SC with transgenic soybeans.

4.3. Podding rate

The pod rate after crossing (the proportion of crossed buds developing into mature pods) ranged from 4.92 to 18.03%, depending upon the wild soybean populations. More than 80% of crossed flower buds were shed within several days after hybridization of wild soybean compared to those under self-pollination. It is well known that flower shedding is common in wild soybean, and varies with different varieties (Saitoh *et al*. 2004). The reason for the shedding of crossed buds much more than those of self-pollinated buds should be attributed, to some extent, to the cryptic structural differences between wild and cultivated soybean. In addition, the injury incurred in the emasculation and pollination processes should not be ignored, although the researchers were fully trained and practiced. The pod setting rate reported by Guan *et al*. (2015) was 9 and 6% for two different wild soybeans and one transgenic soybean, respectively. These results are identical to those of our research.

4.4 Seed set

The seed set rates of different self-pollinated wild soybeans varied from 63.85–90.31%, illustrating a genotype effect on seed setting. The seed set rates of the wild soybeans crossed with transgenic soybean, which provided similar highly viable pollens, varied from 8.88–28.02%, illustrating a maternal genotype effect on seed setting. These results indicate that there are differences in reproductive compatibility between the transgenic soybean lines and 18 wild soybean populations, and the potential for gene flow varies with the genotype of wild soybean in some instances. Similar conclusions have been reported in other studies (Chen *et al*. 2004; Sun *et al*. 2015; Tang *et al*. 2018).

Comparing seed set rates, it was found that the seed set rates of wild soybean crossed with transgenic soybean were 50.66–78.14% lower than those of self-pollinated wild soybean. However, 1–6 filled seeds per 100 pollinated flowers and 2–4 filled seeds per 100 developed pods were produced after crossing with transgenic soybean. This indicates the existence of potential transgene flow from the herbicide-resistant transgenic soybean to the 18 wild soybean populations. If transgenic soybean is released,

strategies should be taken to prevent gene flow from cultivated to wild soybeans, which may produce F_1 hybrids, the first step of introgression.

In our research, each pod developed after hybridization produced an average of less than one filled seed. Guan *et al*. (2015) found 13 and eight filled seeds in nine and six developed pods after hybridization, respectively. The filled seed number was greater than that in our research. This may be due to the use of different pollen donors and recipients between studies.

Sexual reproduction in angiosperms includes a series of stages: germination of pollen on the stigma, penetration of the stigma by the pollen tube carrying two sperm cells, downward growth towards the ovary to the ovule, fertilization of the egg cell and central cell (double fertilization) with the pollen, and development of the embryo and endosperm (Edlund *et al*. 2004; Hao *et al*. 2016). In our research, pods developed from 18 wild soybean populations after hybridization produced seeds and filled seeds. It is inferred that the pollen tubes of transgenic soybean penetrated the stigmas, entered the styles into the ovules of all experimental wild soybeans, and completed double fertilization. The results verified the SC between wild soybean and cultivated soybean.

After double fertilization, soybean seed development can be divided into three major seed development phases of seed set, seed growth and seed maturation (Weber *et al*. 2005; Ruan *et al*. 2012; Du *et al*. 2017). Seed set refers to the development of the zygote to the “heart stage”, in which endosperm cells develop and surround the embryo in the seed. The seed set stage determines seed yield potential through the establishment of the number of seeds. The seed growth stage features cell expansion and synthesis of storage products in the newly formed endosperm. At this stage, the cotyledons have developed, and primary leaf primordia are visible; the endosperm occupies approximately 1/2 of the seed volume. The seed maturation stage is characterized by cotyledons that have reached their final size, and the endosperm is completely assimilated. At this stage, the seed has obtained mature size, and seed weight begins to decrease due to desiccation. The seed growth and early seed maturation stages determine the final seed size and weight through the accumulation of storage products.

In our research, the majority of developed pods contained 1–2 seeds after hybridization and fewer than 1–3 seeds after self-pollination of maternal wild soybean plants. Meanwhile, 55.95–80.28% of the developed pods did not produce filled seeds after hybridization, only one filled seed was produced in the developed pods, which was much less than that produced with the self-pollination of wild soybean. Based on this information, it is inferred that seed abortion between wild soybean and cultivated soybean may occur at the seed set stage, as well as the seed growth and early seed maturation stages. Although several studies related to the development of the embryo and endosperm of soybean and wild soybean have been reported (Zhang *et al*. 1990; Shen *et al*. 1991; Chamberlin *et al*. 1993; Ma *et al*. 2004; Wang *et al*. 2008), the reason for seed abortion, especially embryo abnormalities, after crossing of wild soybean and cultivated soybean has not been clarified. It was suggested that the abortive embryo mechanism after crossing should be determined first based on the identification of key molecules that mediate patterning of the embryo of the model plant *Arabidopsis thaliana* (ten Hove *et al*. 2015; Jeong *et al*. 2016; Palovaara *et al*. 2016). Transgenic soybean, which has lower compatibility with wild soybean, especially at the seed set stage, should be cultivated with the help of new molecular technology to prevent gene flow from transgenic soybean to wild soybean.

5. Conclusion

The SC of transgenic glyphosate-resistant soybean (as the paternal parent) and 18 wild soybean populations (as maternal parents) were assessed. The results showed that two to four filled seeds per 100 developed pods could be produced. This indicates the high possibility of gene flow *via* pollen from transgenic soybean to wild soybean. Moreover, the podding and seed set rates of wild soybean after crossing differed due to the different genetic backgrounds of the wild maternal parents. This implied that the frequency of gene flow from transgenic glyphosate-resistant soybean to different wild soybean populations varies owing to their different reproductive compatibilities. Therefore, the compatibility between transgenic soybean and wild relatives must be assessed to scientifically evaluate the potential risk of gene flow before transgenic soybeans are commercially released.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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