Research Paper

Characterization of tribenuron-methyl-induced male sterility in *Brassica juncea* L.

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Significant heterosis has been documented in *Brassica juncea* L. that are grown as agriculturally important oilseeds, vegetables and condiments crops. Male sterility induced by chemical hybridizing agents is an important pollination control system in hybrid crop breeding. Herein, we show that tribenuron-methyl (TBM), a sulfonylurea herbicide, is an effective male gametocide in *B. juncea* when used at a very low dosage. In the present study, foliar application of various rates of TBM induced a significant increase in pollen sterility in *B. juncea* (90.57–100%). TBM-treated plants exhibited reductions in size of floral organ and yield components; however, lower dose of TBM (0.075 g a.i. ha^{-1}) did not cause a significant reduction in seed yield per plant. Tapetum cells of TBM-treated plants were hypertrophied and degenerated earlier, and abnormal meiosis was observed at the meiotic stage. A significant decrease of acetohydroxyacid synthase (AHAS) activities was detected in buds of plants treated with 0.10 g a.i. ha^{-1} TBM, and RT-qPCR analysis showed that TBM exposure perturbed *AHAS* expression in small buds, which support that TBM induces male sterility in *B. juncea* by targeting *AHAS* expression. Our results suggest that TBM could be used as an efficient chemical hybridization agent in *B. juncea*, which has practical implications for the application of hybrid breeding in *B. juncea*.

Key Words: *Brassica juncea* L., tribenuron-methyl, AHAS, male sterility, cytological observation.

Introduction

Allopolyploid mustard (*Brassica juncea* L., 2n = 36, AABB) is an economically important crop of U's triangle model, including oilseeds, vegetables and condiments (Yang *et al.* 2016, 2017). Oilseed *B. juncea* is mainly distributed in the Indian subcontinent, northwestern China, Canada, and Australia (Yang *et al.* 2017). The global production of mustard and its oil is approximately 38–42 and 12–14 million tons, respectively (Shekhawat *et al.* 2012). Vegetable *B. juncea* is widely distributed in China and has been cultivated for 6000–7000 years. Based on their edible organs, the vegetable *B. juncea* is mainly classified into leaf-type, stem-type, root-type, and stalk-type, including 16 varieties (Chen *et al.* 1990, 1992, Yang *et al.* 1989). It is also used for condiment crops in Europe, China, and other regions (Vaughan and Hemingway 1959).

Significant heterosis has been documented in this species, including seed yield and biomass (Banga *et al.* 1984, Pradhan *et al.* 1993). To enable F$_1$ hybrid breeding, an efficient, reliable, and stable method of F$_1$ production without contamination by self-fertilized seeds from each parent is a prerequisite. To date, cytoplasmic male sterility (CMS) is the most effective way to utilize heterosis in *B. juncea* (Wan *et al.* 2018, Yamagishi and Bhat 2014). Various types of CMS have been developed by sexual or somatic hybridization with wild relatives (Chauhan *et al.* 2011, Singh and Srivastava 2006, Yamagishi and Bhat 2014). These include *Raphanus sativus* (*ogu* CMS) (Gudi *et al.* 2020, Kirti *et al.* 1995, Tian *et al.* 2014), *Moricandia arvensis* (*mori* CMS) (Bisht *et al.* 2015, Prakash *et al.* 1998), *Diplotaxis berthautii* CMS (Bhat *et al.* 2008), *Diplotaxis catholica* CMS (Pathania *et al.* 2003), '126-1' cytoplasm from a re-synthesis *B. napus* line (Sodhi *et al.* 2006), *hau* CMS (Wan *et al.* 2008, 2014, Wei *et al.* 2019), *oxa* CMS (Heng *et al.* 2018, Shi *et al.* 1991), and tuber mustard CMS (Chen *et al.* 1993). Several hybrid varieties of oilseed *B. juncea* (Chauhan *et al.* 2011) and vegetable *B. juncea* (Wan *et al.* 2018) were released in India and China. However, most of these CMS systems have not exploitable as an effective method for heterosis breeding, mainly because of the lack of full fertility restoration (Kaur *et al.* 2004). The transgenic male sterile system (*barnase/barstar* system) provides an alternative way to use heterosis in this crop (Bisht *et al.* 2004, Jagannath *et al.* 2001, 2002); however, it has transgenic safety issues. Compared with the approaches mentioned above, chemical hybridization

agents (CHAs), a class of chemical substances that can induce male sterility in plants, provide a rapid, flexible, and effective approach to the development of hybrids from a large number of parental combinations. Almost all inbred lines can be used as female parents after CHA application. Moreover, long-term use of uniform CMS in mustard heterosis breeding may cause deterioration of production features and poses a high risk for disease epidemics. Therefore, CHA is an alternative method to produce hybrids in mustard.

Indian scientists conducted pioneering studies to exploit CHAs for *B. juncea* hybrid breeding. Banga and Labana (1983) reported that the application of 0.25% ethrel twice could induce 19%–79% male sterility in *B. juncea*. However, the resultant F_1 hybrid seeds were shriveled, which affected seedling vigor and yield. Therefore, they did not suggest that this chemical is an ideal CHA for mustard hybrid breeding. Other researchers have reported that a synthetic detergent surf excel (2, 3, 4, 5, 6, and 8%), benzotriazole (0.5, 1.0, and 1.5%), and ethrel (0.2% and 0.3%) could induce 100% pollen sterility in *B. juncea* (Singh and Chauhan 2003, 2007, Singh *et al.* 2018). Acetohydroxyacid synthase (AHAS) catalyzes the first step in branched-chain amino acid biosynthesis (Umbarger 1978). AHAS activity requires three cofactors: thiamine diphosphate (ThDP), magnesium ion (Mg^{2+}), and flavin adenine dinucleotide (FAD). Recently, Chinese scientists have demonstrated that some AHAS inhibitors, such as tribenuron-methyl (TBM), amidosulfuron, monosulfuron ester sodium (MES), and SX-1 could act as effective male gametocides when applied at very low doses in *B. napus* (Cheng *et al.* 2013, 2015, Li *et al.* 2015, Ning *et al.* 2018, Yu *et al.* 2006, 2009). Furthermore, male sterility induced by SX-1 in *B. napus* was analyzed using a multi-omics integrative analysis, and the initial deficiency of protein processing in the endoplasmic reticulum and flavonoid biosynthesis were proposed as the main reasons for its effectiveness (Ning *et al.* 2018). To date, several dozen commercial hybrids in *B. napus* based on CHA-induced male sterility have been registered according to the bulletins of the Chinese National Crop Variety Approval Committee. The major ingredient of CHAs widely used in rapeseed (*B. napus*) hybrid breeding is sulfonylurea (TBM). However, the effects of TBM in *B. juncea* have not been fully elucidated. In the present study, we tested TBM as a CHA in *B. juncea*. The objectives of this study were to (1) find the suitable TBM rate to induce male sterility, (2) reveal the cytological characterization of anther abortion, and (3) determine the relationship between expression changes in *AHAS* and male sterility induced by TBM in *B. juncea*. Our results provide important scientific value in studying and utilizing CHA for heterosis in *B. juncea*.

Materials and Methods

Plant materials, filed experiments, and TBM application

In this study, two widely cultivated oilseed *B. juncea* genotypes in the Shaanxi Province, L2598 and JCYSX were used as plant materials. These materials were provided by the Rapeseed Research Center of Northwest A&F University, Yangling, Shaanxi, China. TBM, a sulfonylurea herbicide used to control broadleaf weeds in wheat fields, was used as the CHA. The TBM solutions were prepared from a commercial herbicide containing 10% of the active ingredient TBM (Hetian Chemicals, Ltd., Shenyang, China).

The field experiments were conducted at the experimental station of Northwest A&F University in Yangling (108°E, 34°15′N), Shaanxi, China, during the autumn-summer growing seasons from 2016 to 2018. The seeds were sown in an experimental field in mid-September. The plots were arranged in a randomized block design. Each plot contained four rows (2.0 m long), 10 cm between neighboring plants, and 50 cm space between rows. Each plot contained approximately 60–80 plants. To induce male sterility, plants at the bolting stage, when the longest floral buds of more than 80% of plants in a plot were <2 mm (the uninucleate stage), were sprayed with different rates of TBM solution using a hand sprayer and the adjacent plots were covered with plastic cloths. After 7 d, the plants were sprayed again with the same TBM rates. Three TBM rates (0, 0.075, and 0.10 g a.i. ha^{-1}) with a volume of 1000 L ha^{-1} were used. Each treatment was replicated three times.

Pollen viability and scanning electron microscope

The anthers were collected from at least five fresh flowers per plant and five plants per treatment. The anthers were gently squeezed using forceps to release pollen grains into an aqueous solution with 2% Alexander, and pollen viability was detected using a light microscope Olympus BX51 (Olympus Corporation, Tokyo, Japan) (Peterson and Harris 2010).

For scanning electron microscopy (SEM), pollen grains from either the male sterility or control plants of *B. juncea* line L2598 were mounted on SEM stubs and coated with gold with an MSP-IS (Hitachi, Tokyo, Japan) ion sputter coater. Their surfaces were examined with an S-3400N (Hitachi, Tokyo, Japan) scanning electron microscope with an acceleration voltage of 5.00 kV, and pollen grains were photographed (Sanders *et al.* 1999).

Specimen processing for microscopy

The anthers of *B. juncea* line L2598 were collected at the early flowering stage from the main inflorescence of control and treated plants, immediately fixed with Carnoy's liquid (ethanol:glacial acetic acid = 3:1, v/v) for 2 h, and then dehydrated using a graded ethanol series (100%, 95%, 85%, 75%, and 70%), for approximately 1–2 h each, and

stored at 4°C until use. The anthers were gently squeezed using forceps to release pollen grains into an aqueous solution with 1% acetocarmine dye, 4′,6-diamidino-2-phenylindole (DAPI) and aniline blue. Cytological observation of microspore development was performed by squashing, and bright-field photographs were taken under an Olympus BX51 microscope (Olympus, Japan) (Xiao *et al.* 2013).

Semi-thin sectioning and observation

The anthers of *B. juncea* line L2598 were dissected from flower buds of various sizes and immediately fixed by submersion in a solution composed of 4% glutaraldehyde in phosphate-buffered saline (PBS, 0.1 mol/L, pH 6.8) at 4°C until use. After rinsing with PBS (0.1 mol/L, pH 6.8) for 5, 10, 15, 20, and 30 min, the specimens were post-fixed in 1% osmium tetroxide (in 0.2 mol/L PBS, pH 6.8) at 4°C for 2 h. The samples were then washed again with the PBS buffer. The anthers were dehydrated using a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100%) for 15 min each. Next, they were allowed to be infiltrated overnight in a mixture of resin (London Resin Company, Reading, UK): alcohol of 1:1 and again twice in pure resin, 7 h and 3 h, respectively. The specimens were placed into gelatin capsules, filled with resin, and incubated at 60°C for 48 h for polymerization. The specimens were cut into 1 μm thick sections using Leica EM UC7 ultramicrotome (Leica, Nussloch, Germany), stained with 1% toluidine blue solution, finally observed on photomicroscope under bright field and photographed using an Olympus BX51 microscope (Gonzalez-Melendi *et al.* 2008).

AHAS extraction and AHAS activity determination of plants

The mustard AHAS enzyme was extracted and measured as described by Sibony *et al.* (2001) with modifications. Based on these methods, all the procedures were conducted on ice. Leaf or lateral inflorescences were collected from 5 to 10 plants in each experimental plot, and three replicates were included for each treatment. One gram of leaf or lateral inflorescence sample was ground to powder using a chilled mortar and pestle on ice. Then, 10 ml extraction buffer (100 mM potassium phosphate buffer pH 7.0, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavine adenine dinucleotide (FAD)) was added. The homogenate was centrifuged at 25000 g for 30 min at 4°C. AHAS was precipitated from the supernatant fluid with 50% saturated $(NH_4)_2SO_4$ on ice and covered for 2 h. The solution was centrifuged at 25000 g for 30 min at 4°C. The supernatant was discarded, and pellets were dissolved in 8 ml elution buffer (100 mM potassium phosphate buffer pH 7.0, 0.5 mM $MgCl_2$, and 20 mM sodium pyruvate).

The *in vitro* activity of AHAS enzymes was measured based on the amount of acetoin produced in a 2.0 ml volume containing 1 ml reaction buffer (100 mM potassium phosphate (pH 7.0), 20 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP, 10 μM FAD), and 1 ml enzyme. The assay mixture was incubated for 60 min at 37°C. The reaction was terminated by adding 200 μl of 3 M H_2SO_4, followed by incubation for 15 min at 60°C to form acetoin. Next, 1 ml of 0.5% w/v creatine and 1 ml of 5% w/v α-napthol, freshly prepared in 2.5 N NaOH, were added, and the tubes were heated for an additional 15 min at 60°C and then placed on ice. Acetoin production was measured at 530 nm wavelength. Relative AHAS activity was expressed as a percentage of the absorbance value of the corresponding control. Each assay was performed in triplicates.

Tissue sampling, primer designing, and RT-qPCR

Buds samples of *B. juncea* line L2598 were classified into three groups according to their bud length: small buds (SBs) with length <2 mm, medium buds (MBs) with 2–3 mm in length, and large buds (LBs) with lengths 4–5 mm. All samples were prepared on ice, immediately frozen in liquid nitrogen, and stored at –80°C for later use.

The primer combinations G309-forward (5′-GCTAACA GAGCTCACACTTATC-3′) and Y309-reverse (5′-TCGGA GTTCTTCTTTCTTCGTCAC-3′) were designed based on the conserved region of the coding sequence (CDS) of two functional *B. juncea AHAS* orthologs (*BjuA005211*, *BjuA040229*) (Yang *et al.* 2016). *BjuUBQ9*, a housekeeping gene, was used as a control. The primer sets G306-forward (5′-GAAGACATGTTCCATTGGCA-3′) and Y306-reverse (5′-ACACCTTAGTCCTAAAAGCCACCT-3′) for *BjuUBQ9* were described by Chandna *et al.* (2012).

Total RNA was extracted using an Ultrapure RNA Kit (KWBIO, China) according to the manufacturer's instructions. The quantity and quality of RNA samples were detected using a Nano spectrophotometer (ND-1000 Thermo Scientific), and all RNA samples were adjusted to the same concentration. First-strand cDNA was synthesized using a HiFiScript gDNA Removal cDNA Synthesis Kit (KWBIO, China) in a 20 μl reaction using oligo-dT primers according to the manufacturer's instructions. Quantitative real-time (RT) PCR was performed using the GoTaq®qPCR Master Mix Kit (Promega, USA) in Quant Studio 7 Flex to analyze the transcript levels of *B. juncea AHAS* genes. The cycling conditions were 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 60 s. A no-template control (NTC) was also included in runs for each gene. In this study, 2 ml RNase free water was used as the NTC. Each experiment was conducted in triplicates with three biological replicates for each sample. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Measurement of agronomic traits

At the full flowering stage, 15 flowers in each treatment were randomly selected. Floral morphology, including the diameter of corolla, the length and width of petal, pistil, anther, and filament length, were measured. The days from sowing to the first flower (DSF) was recorded for each plot.

At the maturity stage, 15 plants were randomly selected for each treatment. The data recorded included plant height (PH), branching height (BH), number of primary branches per plant (NB), terminal raceme length (LTR), number of siliques on the terminal raceme (NSTR), number of siliques per plant (NSP), number of seeds per silique (NS), silique length (LS), thousand-seed weight (TSW), and seed yield per plant (SYP).

Statistical analysis for significant

All statistical analyses were performed using Microsoft Excel 2010 software. Values are presented as the means and standard deviation. Variance analysis of the observed floral and agronomical traits among different treatments was conducted using the General Linear Model-Univariate program in the SPSS software (version 11.0; SPSS Inc., 2001) and a two-way ANOVA (OriginPro 2019 software), and comparison of traits was conducted by Duncan's multiple range test. To compare the *in vitro* enzyme activity of AHAS and expression of *AHAS* in male fertile and sterile plants, the means of the results were analyzed using Student's *t*-test ($*P < 0.05$; $**P < 0.01$).

Results

Gametocidal effects of TBM of different rates on B. juncea

To induce male sterility in mustard, plants were foliar sprayed with three different rates of TBM solution at the bolting stage and repeated 7 d later in 2017 and 2018. Foliar applications of TBM were effective in inducing pollen sterility in *B. juncea*. In the 2017 experiment, with the double treatments of 0.075 g a.i. ha^{-1} TBM, 90.57% and 98.33% plants exhibited induced male sterility in the lines L2598 and JCYSX, respectively (**Table 1**). The same TBM rate induced 100% male sterility in the two lines in 2018 (**Table 1**). The 0.10 g a.i. ha^{-1} TBM treatment induced male sterility in 100% of plants in both lines in both 2017 and 2018 (**Table 1**, **Fig. 1A–1C**). Pollen viability estimated

using Alexander staining showed that the pollen grains from TBM-treated plants could not be stained and had an irregular shape (**Fig. 1G–1I**). Additionally, the selfed seed-setting rate was examined. No seeds were formed in any of the treatments upon bag-selfing; however, the TBM treatment at 0.075 g a.i. ha^{-1} in 2017 resulted in 4.15% and 2.07% selfed seed setting rates for lines L2598 and JCYSX, respectively (**Table 1**, **Fig. 1D–1F**). Under open pollination, the seed set in all TBM-treated plants was normal (**Table 1**, **Fig. 1D–1F**).

Impact of TBM on floral organ morphology and main agronomic traits

The influence of TBM treatment on floral organ morphology was also investigated. These included the diameter of the corolla, the length and width of the petals, pistil, and anther, and filament length. Significant differences in the six floral organ characteristics under the three doses of TBM treatments were documented in both *B. juncea* lines (**Table 2**). The diameter of the corolla, petal length and width, pistil length, and filament length in all TBM-treated groups decreased compared to the control for both genotypes in the two consecutive years (**Table 2**). However, anther length showed no difference between treatments and controls in JCYSX in either 2017 or 2018, and nor in L2598 in 2018, although this trait was obviously reduced in L2598 in 2017 (**Table 2**).

The influence of TBM exposure was also investigated for the main agronomical traits, including PH, BH, NB, LTR, NSTR, NSP, NS, LS, TSW, and SYP. Significant effects were observed for both genotypes (**Table 3**). The trait DSF was recorded in 2018, we found that JCYSX flowered 4.67 days later than L2598, however, no obvious difference in flowering time was observed among different TBM treatments in both JCYSX and L2598 (**Table 3**). The PH of TBM-treated L2598 was lower than control plants in 2017 but increased to the same level as the control in 2018, and PH of TBM-treated JCYSX showed the same level as

Table 1. The effect of different rates of tribenuron-methyl (TBM) on the fertility of *Brassica juncea*

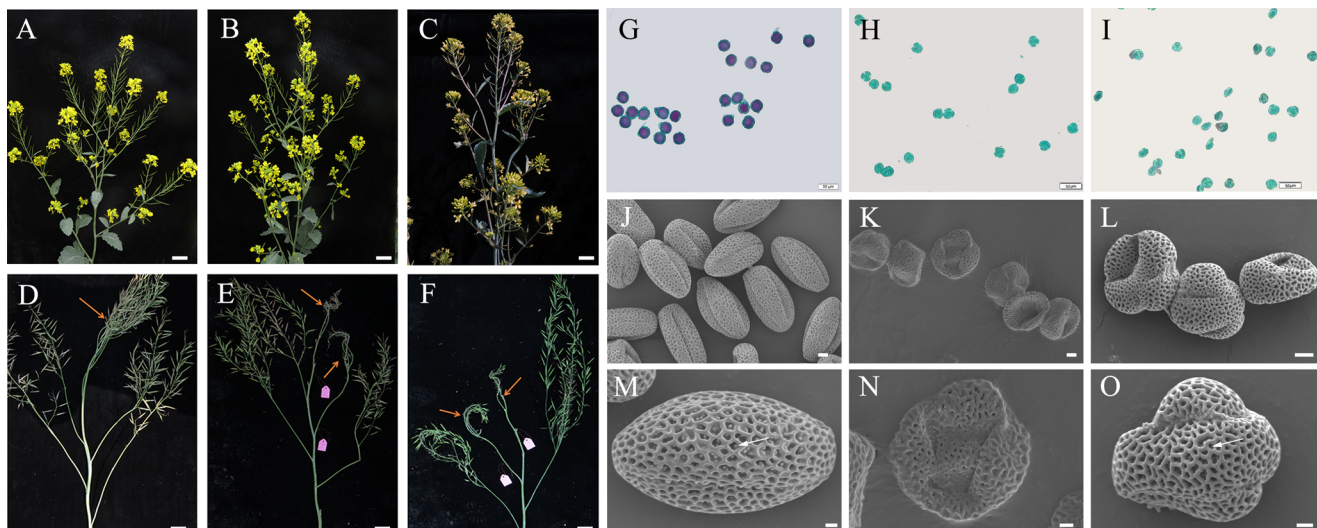| Year	Variety	TBM treatments (g a.i. ha^{-1})	Male sterile percentage (%)	Full sterile percentage (%)	Partial male sterile percentage (%)	Fertile percentage (%)	Selfed seed-setting rate of sterile plant (%)
2017	L2598	0.00	0.00	0.00	0.00	100.00	100.00
		0.075	90.57	77.36	13.21	4.43	4.15
		0.10	100.00	100.00	0.00	0.00	0.00
	JCYSX	0.00	0.00	0.00	0.00	100.00	100.00
		0.075	98.33	81.67	16.67	1.67	2.07
		0.10	100.00	96.55	3.45	0.00	0.00
2018	L2598	0.00	0.00	0.00	0.00	100.00	100.00
		0.075	100.00	100.00	0.00	0.00	0.00
		0.10	100.00	100.00	0.00	0.00	0.00
	JCYSX	0.00	0.00	0.00	0.00	100.00	100.00
		0.075	100.00	100.00	0.00	0.00	0.00
		0.10	100.00	100.00	0.00	0.00	0.00

Fig. 1. Comparing of tribenuron-methyl (TBM)-treated *Brassica juncea* line L2598 and control plants. Inflorescences (A–C), plants showing self-pollinate ability (D–F), pollen activity (G–I) and scanning electron microscopy images of pollen grains from control and TBM-treated plants. A, D, G, J, and M: control; B, E, and H: 0.075 g a.i. ha⁻¹; C, F, I, K, L, N, and O: 0.10 g a.i. ha⁻¹ TBM treatment. Arrows indicate the selfing seed set whereas other branches mean open pollination (D–F) and the granular protein substances (M and O), respectively. Bars, A–F = 2 cm, G–L = 50 μm, and M–O = 20 μm.

Table 2. The effect of tribenuron-methyl (TBM) on the floral organ morphology and the analysis of its variance

Year	Variety	TBM treatments (g a.i. ha⁻¹)	Diameter of corolla (cm)	Petal length (cm)	Petal width (cm)	Pistil length (cm)	Anther length (cm)	Filament length (cm)
2017	L2598	0.00	1.46 ± 0.05 Aa	0.66 ± 0.07 Aa	0.45 ± 0.04 Aa	0.67 ± 0.03 Aa	0.20 ± 0.01 Aa	0.50 ± 0.02 Aa
		0.075	1.23 ± 0.12 Bb	0.55 ± 0.09 Bb	0.31 ± 0.05 Bb	0.59 ± 0.05 ABb	0.15 ± 0.01 Bb	0.36 ± 0.07 Bb
		0.10	1.11 ± 0.10 Bc	0.46 ± 0.06 Cc	0.30 ± 0.05 Bb	0.56 ± 0.06 Bb	0.12 ± 0.00 Cc	0.30 ± 0.05 Bb
	JCYSX	0.00	1.44 ± 0.04 Aa	1.04 ± 0.03 Aa	0.56 ± 0.03 Aa	0.78 ± 0.04 Aa	0.13 ± 0.01 Aa	0.69 ± 0.01 Aa
		0.075	1.35 ± 0.03 Bb	0.91 ± 0.06 Bb	0.50 ± 0.02 Bb	0.65 ± 0.04 Bb	0.13 ± 0.01 Aa	0.56 ± 0.03 Bb
		0.10	1.22 ± 0.04 Cc	0.84 ± 0.04 Cc	0.47 ± 0.03 Cc	0.63 ± 0.06 Bb	0.16 ± 0.02 Aa	0.52 ± 0.03 Bc
		Source of variation						
		Variety	**	**	**	**	**	**
		Treatments	**	**	**	**	**	**
		Variety × Treatments	**	ns	**	ns	**	ns
2018	L2598	0.00	1.64 ± 0.05 Aa	1.12 ± 0.07 Aa	0.53 ± 0.03 Aa	0.96 ± 0.10 Aa	0.11 ± 0.03 Aa	0.69 ± 0.03 Aa
		0.075	1.42 ± 0.05 Bb	1.00 ± 0.07 Bb	0.48 ± 0.04 Bb	0.89 ± 0.11 Aa	0.13 ± 0.03 Aa	0.58 ± 0.04 Bb
		0.10	0.94 ± 0.03 Cc	0.81 ± 0.06 Cc	0.35 ± 0.05 Cc	0.70 ± 0.09 Bb	0.13 ± 0.03 Aa	0.49 ± 0.04 Cc
	JCYSX	0.00	1.72 ± 0.01 Aa	1.19 ± 0.03 Aa	0.56 ± 0.04 Aa	0.91 ± 0.02 Aa	0.14 ± 0.02 Ab	0.68 ± 0.02 Aa
		0.075	1.42 ± 0.01 Bb	0.98 ± 0.02 Bb	0.47 ± 0.04 Bb	0.77 ± 0.01 Bc	0.14 ± 0.02 Ab	0.59 ± 0.01 Bb
		0.10	1.34 ± 0.02 Cc	0.89 ± 0.01 Cc	0.41 ± 0.08 Bc	0.81 ± 0.01 Bb	0.17 ± 0.02 Aa	0.56 ± 0.04 Bb
		Source of variation						
		Variety	**	*	*	ns	**	ns
		Treatments	**	**	**	**	**	**
		Variety × Treatments	**	ns	**	*	ns	**

Note: Data are expressed by mean ± SD (n = 15). Comparisons among the treatment mean were made by Duncan's multiple range test. Data in the same column followed by different capital or lowercase letters indicate the significant difference at 0.01 or 0.05 level, respectively. ns, non-significant; * Significant at the $P = 0.05$ level; ** Significant at the $P = 0.01$ level.

the control in 2017 but went up to higher than control in 2018. Therefore, the ascendant trend of TBM-treated plant height from 2017 to 2018 was consistent in both genotypes, but different varieties have different responses (**Table 3**). No significant differences were observed in LTR between treated and control plants; however, a 86.43% reduction of NSTR and 32.04% reduction of SYP occurred in the

0.10 g a.i. ha⁻¹ treated L2598 plants in 2018 (**Table 3**). Compared to L2598, JCYSX was more tolerant to TBM, and there was no change in SYP at 0.05, after TBM treatment in 2018 (**Table 3**).

Table 3. The influence of tribenuron-methyl (TBM) on the agronomic traits of *Brassica juncea*

Year	Variety	TBM treatments (g a.i. ha⁻¹)	DSF (d)	PH (cm)	BH (cm)	NB (no.)	LTR (cm)	NSTR (no.)	NSP (no.)	NS (no.)	LS (cm)	TSW (g)	SYP (g)
2017	L2598	0.00	–	180.40±0.96 Aa	49.60±4.35 Aa	9.20±1.03 Aa	43.20±3.39 Aa	55.70±8.35 Aa	636.20±71.50 Bb	14.46±1.13 Aa	3.34±0.16 Aa	–	–
		0.075	–	178.70±2.61 Aa	42.10±6.57 Bb	8.70±1.64 ABa	44.60±4.09 Aa	58.70±4.69 Aa	793.80±123.30 Aa	11.35±1.42 Bb	3.01±0.25 Bb	–	–
		0.10	–	171.20±4.31 Bb	41.00±5.54 Bb	7.00±1.89 Bb	32.50±5.54 Bb	32.70±9.45 Bb	496.80±85.30 Cc	10.64±1.95 Bb	2.94±0.31 Bb	–	–
	JCYSX	0.00	–	213.30±8.25 Aa	110.08±3.25 Aa	7.70±1.70 Bb	39.10±2.58 Bb	53.60±1.56 Aa	419.60±18.88 Cc	19.46±3.76 Aa	3.33±0.11 Aa	–	–
		0.075	–	211.80±27.23 Aa	77.67±2.73 Bb	9.80±1.32 Aa	48.50±4.02 Aa	55.80±8.64 Aa	798.40±47.91 Aa	13.84±1.28 Ab	3.58±0.20 Aa	–	–
		0.10	–	198.10±24.55 Aa	78.94±3.73 Bb	7.40±1.43 Bb	28.50±4.89 Cc	54.20±6.66 Aa	524.30±40.60 Bb	13.80±3.19 Ab	3.33±0.20 Aa	–	–
	Source of variation												
	Variety		–	**	**	**	ns	*	*	**	**	–	–
	Treatments		–	ns	**	**	**	**	**	**	ns	–	–
	Variety × Treatments		–	ns	**	*	*	**	**	ns	**	–	–
2018	L2598	0.00	182.00±0.00 Aa	147.85±20.91 Aa	38.85±13.28 Ab	9.31±1.49 Aa	39.15±8.47 Aa	51.38±9.61 Aa	781.15±270.12 Aa	13.32±1.28 Aa	3.91±0.12 Aa	2.21±0.16 Aa	19.38±9.91 Aa
		0.075	183.00±1.00 Aa	135.27±21.47 Aa	48.00±13.67 Aab	6.70±2.44 Bb	35.07±8.60 Aa	6.43±12.15 Bb	598.43±257.31 ABb	12.81±1.48 Aa	4.07±0.32 Aa	2.45±0.39 Aa	10.54±6.37 Bb
		0.10	183.67±0.58 Aa	133.70±25.03 Aa	51.23±17.44 Aa	6.00±2.98 Bb	37.97±11.80 Aa	6.97±12.72 Bb	530.87±298.19 Bb	12.42±2.00 Aa	3.91±0.33 Aa	2.44±0.49 Aa	8.86±7.31 Bb
	JCYSX	0.00	186.67±0.58 Aa	186.07±7.00 Bb	45.80±21.75 Bb	9.67±1.88 Aa	39.20±7.86 Ab	53.73±9.58 Aa	503.63±59.19 Aa	17.31±1.06 Aa	4.01±0.41 Aa	2.25±0.19 Bb	17.14±11.24 Aa
		0.075	187.33±1.15 Aa	202.13±7.25 Aa	67.40±22.04 Aa	9.00±2.08 Aa	44.03±5.35 Aa	45.80±12.63 Bbc	422.87±48.85 Aa	12.81±0.92 Cc	3.45±0.31 Bb	2.12±0.12 Bb	12.04±5.82 Ab
		0.10	187.67±0.58 Aa	206.40±5.91 Aa	63.10±25.15 Aa	9.23±1.65 Aa	43.87±8.71 Aa	41.77±10.27 Bc	530.73±145.50 Aa	15.18±1.94 Bb	3.59±0.38 Bb	2.50±0.16 Aa	12.02±9.20 Ab
	Source of variation												
	Variety		**	**	**	**	**	**	*	**	**	ns	ns
	Treatments		ns	ns	**	**	ns	**	ns	**	ns	*	*
	Variety × Treatments		ns	**	ns	**	ns	**	ns	**	**	*	ns

Note: Days from sowing to the first flower (DSF; d), plant height (PH; cm), branching height (BH; cm), number of primary branches per plant (NB; no.), length of the terminal raceme (LTR; cm), number of siliques on the terminal raceme (NSTR; no.), number of siliques per plant (NSP; no.), number of seeds per silique (NS; no.), length of silique (LS; cm), thousand seed weight (TSW; g), seed yield per plant (SYP; g). Data are expressed by mean ± SD (n = 15 except n = 9 for DSF). Data in the same column followed by different capital or lowercase letters indicate the significant difference at 0.01 or 0.05 level, respectively. –, data are not collected; ns, nonsignificant; * Significant at the $P = 0.05$ level; ** Significant at the $P = 0.01$ level.

Cytological observations of anther development in TBM-induced male sterile plants

The pollen grains from 0.10 g a.i. ha^{-1} TBM-treated plants and 0 g a.i. ha^{-1} TBM-treated plants (control) for line L2598 were examined under the SEM (**Fig. 1J–1O**). The pollen grains of the control were subsphaeroidal, with three evenly distributed germinating collpates (**Fig. 1J**, **1M**). In contrast, the pollen grains of the TBM-treated plants exhibited a hollow and collapsed morphology (**Fig. 1K**, **1N**). Additionally, the tryphine of the control was filled with the granular protein substances (**Fig. 1M**), although it did not appear in the TBM-treated samples (**Fig. 1L**, **1O**).

Furthermore, anthers at different developmental stages were investigated and observed under a light microscope. As shown in **Fig. 2**, differences were observed between TBM-treated and control plants from the pollen mother cell stage to the mature pollen stage. Male sterile anthers caused by the TBM treatment did not exhibit regular meiosis as in controls (**Fig. 2C**, **2D**), and the tapetum cells developed abnormally (**Fig. 2E**, **2F**). In the uninuclear microspore stage, microspores of both male sterile anthers and those of control could be released, but aborted microspores with irregular shapes were observed (**Fig. 2G**, **2H**). Microspores and pollen grains could not be dyed as controls (**Fig. 2I–2L**). Using the 4′,6-diamidino-2-phenylindole method, we found that at the tetrad stage, the microspore mother cells completed meiosis to form tetrads, which were surrounded by the callose wall in the control. However, significant

aberrations in the male sterile anthers of the TBM-treated plants were observed (**Fig. 2O**, **2P**). Additionally, aniline blue staining analysis showed that the callose layer surrounding the tetrads in the TBM-treated samples was thicker than that in the controls (**Fig. 2U**, **2V**). Semi-thin section results indicated that at the pollen mother cell stage, no apparent cytological differences were observed between the TBM-induced male sterile anthers and controls, differentiated endothecium, middle layer, tapetum, and primary microspore mother cells were present in both anther sections (**Fig. 3A**, **3B**). However, earlier degeneration and a hypertrophied tapetum were observed during the meiotic stage (**Fig. 3C–3F**). An abnormal tapetum that detached from the inner wall (**Fig. 3D**) and with cell expansion was also observed (**Fig. 3F**) in TBM-treated plants. Serious differences were observed at the tetrad stage, mononuclear cell stage, and mature pollen stage, which did not result in tetrad, microspores, or pollen in TBM-treated plants (**Fig. 3E–3J**).

Expression of AHAS in TBM-treated and control plants

The herbicides that inhibit AHAS show little resemblance to any of the AHAS substrates and apparently act as non-competitive inhibitors, suggesting that they donot bind at the active site (Duggleby *et al.* 2008, Pang *et al.* 2002). Sulfonylurea herbicides inhibit AHAS activity by binding to the access channel, which leads to the active site and completely prevents substrate binding (Garcia *et al.* 2017,
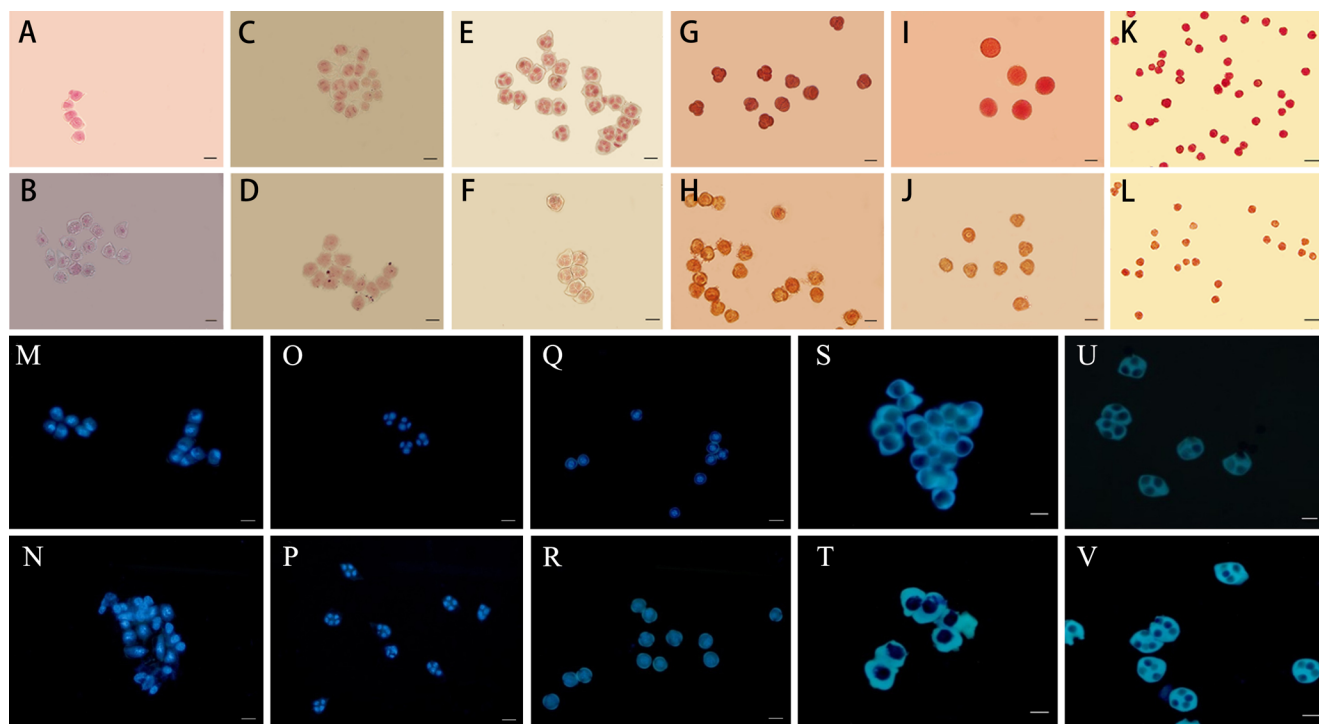

Fig. 2. Pollen development in the control and tribenuron-methyl (TBM)-treated plants of *Brassica juncea* line L2598 by acetocarmine (A–L), 4′,6-diamidino-2-phenylindole (M–R) and aniline blue (S–V) methods. A, C, E, G, I, K, M, O, Q, S, and U: control; B, D, F, H, J, L, N, P, R, T, and V: 0.10 g a.i. ha^{-1} TBM treatment; A, B, M, N, S, and T: pollen mother cell stage; C, D: meiosis stage; E, F, O, P, U, and V: tetrad stage; G, H: trilateral thickening stage; I, J, Q, and R: microspore stage; K, L: mature pollen stage. Bars, A–J, M–V = 20 μm, K and L = 50 μm.
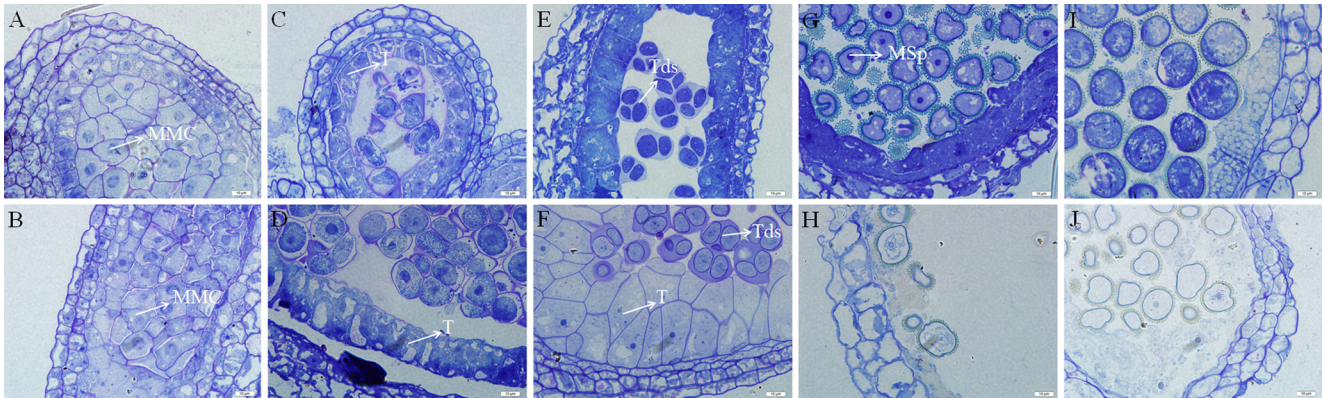
Fig. 3. Microscopic observation of anther development in *Brassica juncea* line L2598 sterile plants treated with 0.10 g a.i. ha⁻¹ of tribenuron-methyl (TBM) and its control plants. A, C, E, G, and I: control; B, D, F, H, and J: 0.10 g a.i. ha⁻¹ TBM treatment; A, B: pollen mother cell stage; C, D: meiotic cell stage; E, F: tetrad stage; G, H: mononuclear cell stage; I, J: mature pollen stage. MMC, microspore mother cells; Msp, microspores; T, tapetum; Tds, tetrads. Bars, 50 μm.
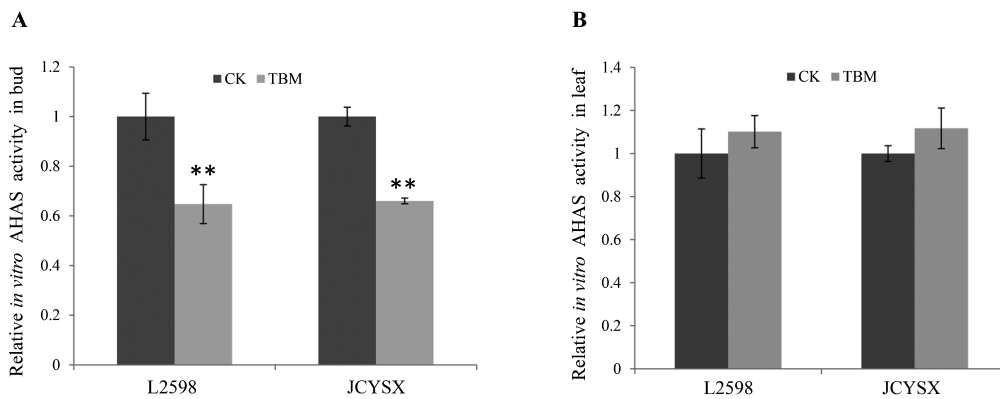

Fig. 4. *In vitro* AHAS activity in different tissues of control and 0.10 g a.i. ha⁻¹ tribenuron-methyl (TBM) treated plants. A: Bud; B: Leaf. Values are presented as the mean ± SD from three independent experiments. *In vitro* ALS activity data were compared to controls. Statistical significance was assessed using Student's *t*-test (**$P < 0.01$).

McCourt and Duggleby 2006). To determine whether male sterility was caused by inhibition of AHAS activity via transportation of the foliar-sprayed TBM, the *in vitro* enzyme activity of AHAS in the leaves and buds of TBM-treated and controls was measured (**Fig. 4**). Significant differences were detected in AHAS activities of buds in the two genotypes. The 0.10 g a.i. ha⁻¹ TBM treatment significantly decreased the AHAS activity of buds compared with control plants, 36% for L2598 and 34% for JCYSX (**Fig. 4**). However, no significant difference was observed in the AHAS activity of leaves between the control and TBM-treatment plants (**Fig. 4**). Previous studies proposed the expression of *AHAS* were not greatly affected in the TBM and amidosulfuron-treated plants though significant changes estimated in small buds were observed in *B. napus* (Lian *et al.* 2019, Liu *et al.* 2017). To verify whether TBM disturbs the expression of *AHAS* in *B. juncea*, subsequently, a more sensitive RT-qPCR analysis was conducted to measure the gene expression of *AHAS* in the buds of male fertile and sterile plants of L2598. Relative expression values were calculated after Ct normalization using *BjuUBQ9* as a

reference gene. *AHAS* expression was significantly lower in the small buds (<2 mm) of the TBM-treated plants. However, there were no differences in medium (2–3 mm) or large buds (4–5 mm) between the controls and male sterile anthers (**Fig. 5**). These together support the idea that TBM induces male sterility in *B. juncea* by targeting AHAS, and disturb the expression of *AHAS* in small buds which is partly consistent with the observations in *B. napus* (Lian *et al.* 2019, Liu *et al.* 2017, Lv *et al.* 2018).

Discussion

The development of a highly effective and inexpensive CHA without environmental or health risks is important for the utilization of heterosis in mustard (*B. juncea*). TBM has been used as an active ingredient in many AHAS-targeting herbicides (McCourt and Duggleby 2006). In recent years, it has been exploited as a potential CHA in *B. napus* at low doses (Yu *et al.* 2006, 2017). In this investigation, we determined that TBM is a very promising gametocide that can induce pollen sterility in oilseed *B. juncea*. Foliar
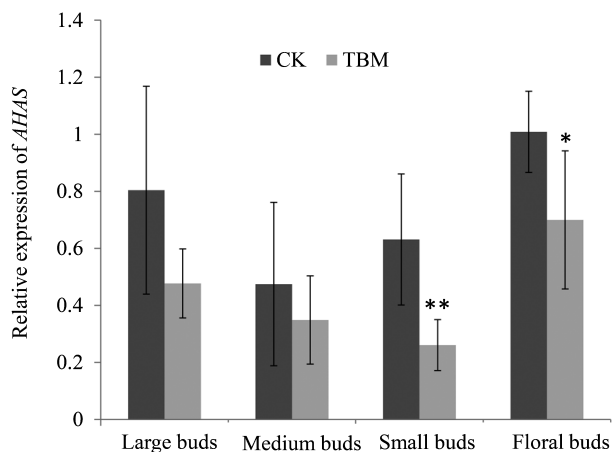

Fig. 5. Expression analyses of *AHAS* gene in the various tissues of control and 0.10 g a.i. ha⁻¹ tribenuron-methyl (TBM)-treated plants of *Brassica juncea* line L2598 through qRT-PCR. The expression results were normalized against *BjuUBQ9* as an internal control. Means ± SD are based on three biological replicates. Statistical significance was assessed by Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

applications of 0.075–0.10 g a.i. ha⁻¹ TBM at the uninucleate stage of buds were quite effective in inducing >90% male sterility in *B. juncea* in two consecutive years. The TBM treatment caused shrunken anthers and hollow and collapsed pollen; interestingly, some pollens had normal-appearing apertures without granular protein substances. Earlier degeneration, hypertrophy of the tapetum, and irregular meiosis were observed at the meiotic stage. A 36% and 34% decrease of AHAS activities was detected after 0.10 g a.i. ha⁻¹ TBM treatment in buds of L2598 and JCYSX, respectively. Moreover, *AHAS* expression was significantly lower in the small buds of TBM-treated plants than in the controls. Considering its low cost, low residues, and low toxicity to animals, our study suggests that 0.075 g a.i. ha⁻¹ TBM could be used as an efficient chemical hybridizing agent for mustard hybrid breeding.

At present, the utilization of heterosis in mustard is still in the development stage (Chauhan *et al.* 2011, Wan *et al.* 2018). Commercial mustard hybrids are currently based on *mori* and *ogu* CMS systems (Chauhan *et al.* 2011). Fertility restoration in *mori* CMS is gametophytic, where only nuclear fertility restorer (*Rf*) gene-carrying pollens are functional and 50% of F₁ plants are fertile. Thus, it is difficult to differentiate between CMS plants and fertile contaminants in seed production plots (Bhat *et al.* 2005). Issues in *ogu* CMS remain regarding the transmission of the introgressed fertility restorer gene in *B. juncea*, which results in a high frequency of male sterile plants in F₁ hybrids. Two hybrid varieties have been developed for the *Hau* CMS system in vegetable *B. juncea* (Wan *et al.* 2018). However, restorers have not been reported for oilseed *B. juncea*, and consequently, have not been applied in oilseed *B. juncea* (Wan *et al.* 2008). Fortunately, there is no maintenance and restoration relationship as required in CMS systems. CHA-

induced male sterility is physiological male sterility and has a wide range of parental selection, which greatly accelerates the search for heterotic combinations. Additionally, the CHA could be used to solve the trace pollen problem encountered by some CMS lines, which occurs in certain environments, especially those with a dramatic change in temperature. Thus, the CHA system could be an alternative way to exploit heterosis in *B. juncea* for breeders. In *B. juncea*, a few efforts have been made to identify effective and safe CHA chemicals, and without much success. Foliar applications of different concentrations of ethrel (Banga and Labana 1983), benzotrizole (Singh and Chauhan 2007), surf excel (Singh and Chauhan 2003), and EXP (Yu *et al.* 2005) have been reported to induce pollen sterility in this species. However, none of them are ideal CHAs for *B. juncea* breeding because of incomplete and unstable male sterility and serious phytotoxic effects. Yu *et al.* (2017) reported that treatment with 0.2 and 0.4 mg/L of TBM appeared to be sufficient to induce 80% to 100% male sterility in 17 tested cruciferous species, and the flowering phenology and reproductive function of the treated plants changed. In the present study, foliar applications of two doses of TBM (0.075 and 0.10 g a.i. ha⁻¹) were highly effective in inducing complete male sterility in *B. juncea* (**Table 1**); however, the suitable doses of TBM need to be tested on more genotypes. The 0.10 g a.i. ha⁻¹ TBM treatment resulted in completely male sterility, but it had a strong phytotoxic effect in plants, especially in cultivar L2598, which resulted in poor podding in the main stem, accumulation of anthocyanidins in stems, and chlorotic lesions on leaves (**Table 3**).

In the *Brassicaceae* family *Arabidopsis*, *B. napus*, and *B. juncea*, the anther developmental process is divided into the sporogenous cell stage, pollen mother cell stage, tetrad stage, uninucleate microspore stage, vacuolated microspore stage, and mature pollen stage (Li *et al.* 2016, Sanders *et al.* 1999, Singh and Chauhan 2007). During these processes, the tapetum provides nutrients for young microspores, and its degeneration initiates the pollen mitotic stage and disappears until pollen maturation. Previous studies have shown that three types of tapetal abnormalities are observed in anther development in CHAs-treated male sterile plants of *B. juncea* (Singh and Chauhan 2007). These were persistent tapetum (surf excel and benzotriazole-treated plants), formation of pseudotapetal periplasmodium, and a hypertrophied tapetum (ethrel-treated plants) (Singh and Chauhan 2007). Li *et al.* (2015) found that the plastid ultrastructure was abnormal in pollen mother cells and tapetal cells in sterile male anthers induced by CHA-MES treatment in *B. napus*. Ning *et al.* (2018) observed that tapetosomes and elaioplasts disassembled earlier in tapetal cells of CHA-SX-1 treated *B. napus* compared to those in the control. In the present study, an abnormal tapetum was also observed at the meiotic stage, and the tapetum degenerated earlier than in wild-type plants (**Figs. 2**, **3**). Here, the pollen grains that were treated with TBM in *B. juncea* showed loss of

inclusion and were shriveled compared to the control. Additionally, the pollen coat of the control was filled with some substances, whereas it did not exist after TBM treatment (**Fig. 1M**, **1O**), and a deformed callose layer surrounding the tetrad microspores was observed in TBM-treated plants, which was consistent with previous observations in SX-1 treated *B. napus* (Ning *et al.* 2018) and TBM-treated *B. napus* (Lian *et al.* 2019), respectively. Our results imply that earlier degeneration and a hypertrophied tapetum might be the main reasons for microspore abortion. In *B. napus*, the bulk cytoplasmic contents in microspores are degraded during cell death which resulting in crinkled pollen grains after TBM treatment (Zhao *et al.* 2015), and the disordered flavonoid biosynthesis directly affected by SX-1 at early stage will cause defective of other related pathways, and finally results male sterility (Ning *et al.* 2018). In our study, earlier degeneration and a hypertrophied tapetum, deformed callose layer, deletion of cytoskeleton, and incomplete deposition in the tryphine of pollen were observed in TBM-treated *B. juncea* through SEM and semi-thin sectioning. These together suggested pollen grains contained little cytoplasm and metabolic pathways related to anther and pollen wall development might cause shrunken anthers in TBM-treated plants in *B. juncea*, which require further study.

TBM blocked the access channel of the substrate leading to active sites, thereby inhibiting enzyme activity (Garcia *et al.* 2017, McCourt and Duggleby 2006). In oilseed rape *B. napus*, Zhao *et al.* (2015) suggested that TBM was absorbed by the leaves and transported to anthers through the stem, then resulted in branched-chain amino acid starvation by inhibiting AHAS activity, which ultimately triggers autophagic cell death in male sterile anthers. Lv *et al.* (2018) indicated that TBM treatments did not significantly affect AHAS activity in leaves, but significantly decreased AHAS activity in inflorescences of both *B. napus* ZS9 and a TBM-resistant mutant *K5*. This supported the hypothesis that AHAS could be the target of the AHAS-inhibiting herbicide TBM when it is used as CHA. Agreed to previous findings in *B. napus*, in the present investigation, compared with control plants, significant decrease (approximately 35%) of AHAS activities in buds, but not in leaves, was detected after the application of 0.10 g a.i. ha^{-1} TBM in *B. juncea* (**Fig. 4**), which implied that TBM was polar-transported to the flower buds, resulting in a decrease in AHAS activity in male reproductive organs and pollen abortion. Furthermore, it was reported that the expression levels of *AHAS1* and *AHAS3* were not severely affected in TBM and amidosulfuron-treated plants indicating that TBM and amidosulfuron treatment did not significantly perturb *AHAS* expression in *B. napus* (Lian *et al.* 2019, Liu *et al.* 2017). Interestingly, in above reports, significant changes in expression of *AHAS1* and *AHAS3* were estimated in small buds, though the differences were less than one-fold. In the present study, we found that the expression of *AHAS* after TBM exposure was significant reduced in small buds (stage of uninucleate), but not in medium and large buds (**Fig. 5**), which was partly consistent with previous results. We speculated that the decrease in *AHAS* gene expression in small buds is the result of the interaction between TBM and active AHAS protein, and not a direct cause. However, these interesting questions require further investigation. Together, our results suggest that TBM-induced male sterility in *B. juncea* by inhibition of AHAS activity in inflorescences via transportation of the foliar-sprayed TBM, as previously reported for *B. napus* (Lv *et al.* 2018, Zhao *et al.* 2015), although some differences exist between the species.

In conclusion, this study suggests that TBM could be used as an efficient chemical hybridizing agent in the oilseed *B. juncea*, which has practical implications for the application of hybrid breeding in *B. juncea*.

Author Contribution Statement

Shengwu Hu conceived and designed the experiments. Yuan Guo, Huhu Gao and Huaiyin Ma conducted the experiments. Yuan Guo analyzed the data. Yuan Guo wrote the manuscript. Chunlei Du, Dongsuo Zhang, Xiaoyue Wang were involved in planting and harvesting. All authors have read and approved the manuscript.

Acknowledgments

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