Theor Appl Genet (2015) 128:107–118
DOI 10.1007/s00122-014-2415-7

ORIGINAL PAPER

Generation and characterization of tribenuron-methyl herbicide-resistant rapeseed (*Brasscia napus*) for hybrid seed production using chemically induced male sterility

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Received: 5 August 2014 / Accepted: 11 October 2014 / Published online: 14 December 2014

Abstract

Key message **Identification and molecular analysis of four tribenuron-methyl resistant mutants in *Brassica napus*, which would be very useful in hybrid production using a Chemically induced male sterility system.**

Abstract Chemically induced male sterility (CIMS) systems dependent on chemical hybridization agents (CHAs) like tribenuron-methyl (TBM) represent an important approach for practical utilization of heterosis in rapeseed. However, when spraying the female parents with TBM to induce male sterility the male parents must be protected with a shield to avoid injury to the stamens, which would otherwise complicate the seed production protocol and increase the cost of hybrid seed production. Here we report the first proposed application of a herbicide-resistant cultivar in hybrid production, using a CIMS system based on identifying four TBM-resistant mutants in *Brassica napus*. Genetic analysis indicated that the TBM resistance was controlled by a single dominant nuclear gene. An in vitro enzyme activity assay for acetohydroxyacid synthase (AHAS) suggested that the herbicide resistance is caused by a gain-of-function mutation in a copy of *AHAS* genes. Comparative sequencing of the mutants and wild type *BnaA.AHAS.a* coding sequences identified a C-to-T transition at either position 535 or 536 from the translation start site, which resulted in a substitution of proline with serine or leucine at position 197 according to the *Arabidopsis thaliana* protein sequence. An allele-specific dCAPS marker developed from the C536T variation co-segregated with the herbicide resistance. Transgenic *A. thaliana* plants expressing *BnaA.ahas3.a* conferred herbicide resistance, which confirmed that the P197 substitution in BnaA.AHAS.a was responsible for the herbicide resistance. Moreover, the TBM-resistant lines maintain normal male fertility under TBM treatment and can be of practical value in hybrid seed production using CIMS.

Communicated by Isobel Parkin.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2415-7) contains supplementary material, which is available to authorized users.

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Introduction

Rapeseed (*Brassica napus*) is one of the major oil crops worldwide. It is cultivated not only for edible oil, but also for industrial materials such as livestock meal, lubricants and biodiesel. The worldwide production of rapeseed increased eight-fold between 1975 and 2012, and reached 64.8 million tonnes in the 2011–2012 season (http://faostat.fao.org/). This rapid and widespread rise is due to the achievements in genetic improvement of "double low" (low erucic acid and low glucosinolate) canola quality traits, and utilization of heterosis to enhance yield.

Heterosis is defined as the superior performance of hybrids in grain yield, disease resistance and other agronomic traits relative to their parents and conventional varieties. It is a common phenomenon and has been widely used to increase yield in many crops including maize and rapeseed. A number of male sterility systems including

cytoplasmic genetic male sterility (CMS), genic male sterility (GMS), environment-sensitive genic male sterility (EGMS), genetically engineered male sterility (GEMS) and chemically induced male sterility (CIMS) have been used to produce hybrid seeds in rapeseed. At least three CMS systems have been used (Liu et al. 2005), although in the Polima and Nap systems the male sterility is somewhat sensitive to temperature fluctuations and may revert to a fertile state under low temperature, which brings the risk of false hybrids in seed production (Fan et al. 2007; McVetty 1997; Yang et al. 2006). The Ogura CMS system is environmentally stable, but it is protected by patent and only available to rapeseed breeders with a costly licence fee. In addition, effective use of CMS systems requires prior screening to match maintainer and restorer lines. This often takes a long time to achieve, and there may also be difficulties in identifying suitable maintainer or restorer lines. GMS has obvious advantages over the CMS systems. For instance, many varieties can restore the fertility, making it easy to breed an elite hybrid, and so this system has been successfully used in rapeseed hybrid seed production (Huang et al. 2007). However, all GMS systems have a common disadvantage that half of the offspring is fertile, and thus need intensive labor to remove these plants from the female parents, greatly increasing the costs of hybrid seed production (Huang et al. 2007). The EGMS system has also been successfully used in hybrid rapeseed production, although this is constrained by the potential risk of extreme temperature modification. The GEMS system has been shown to be very successful in hybrid rapeseed production, but its utilization is limited due to the issues of patent restriction and the acceptance of genetically modified (GM) food.

Compared to these other male sterility systems, CIMS has the potential to be applied to any two inbred lines as the male and female parents. This facilitates the generation of a large number of cross combinations for yield testing, which can be as simple as the single cross hybrid in maize first proposed by Shull (1908). CIMS provides a rapid, flexible and effective approach to hybrid seed production in rapeseed. Thus it has attracted considerable attention in hybrid rapeseed production, with some commercial hybrids registered in China (Guan and Stringam 1998; Yu et al. 2006). In hybrid seed production using CIMS, the female and male parents are usually grown in a ratio of 2:1 or 3:1, with two or three rows of female parent and one row of male parent. To avoid injury to the stamens of the male parents and to maintain high viability of pollen grains, the male parents must be protected with a physical barrier while spraying the chemical hybridization agent (CHA). This prevents the use of a large herbicide sprayer in hybrid seed production which would otherwise decrease the cost of hybrid seeds. Therefore there is a pressing requirement for CHA-resistant male parents to simplify the protocol, and to avoid the risk of yield reduction in hybrid seed production.

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6), the first important enzyme in the biosynthetic pathway of the branched-chain amino acids valine, leucine, and isoleucine, is the target site of several herbicidal AHAS-inhibitors. The AHAS-inhibitors mainly include five different chemical classes of sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinylthiobenzoates (PTB), and sulfonylaminocarbonyltriazolinone (SCT) (McCourt and Duggleby 2006; Powles and Yu 2010). These inhibitors can bind to the herbicide binding residues within the substrate-access channel to inhibit substrate access to the active sites of AHAS. The inhibition of AHAS activity blocks the synthesis of branched-chain amino acids and results in subsequent plant death.

Male gametocides or CHAs are usually herbicidal AHAS-inhibitors. Tribenuron-methyl (TBM), belongs to the SU family, and has been demonstrated to be an excellent male gametocide in rapeseed (Yu et al. 2006). TBM is a highly efficient systemic post-emergence herbicide and has been extensively used in controlling broad-leaf weeds. TBM has been widely used to induce male sterility since it meets the requirements of CHAs, and has very low cost, low residues and low toxicity to animals (Yu et al. 2006).

Although some herbicidal AHAS-inhibitors (mainly SU and IMI) resistant mutants have been described in *B. napus* (Hattori et al. 1995; Hu et al. 2012; Swanson et al. 1988, 1989), it is not clear if these mutants were also TBM resistant. Thus, the objective of this study was to identify novel rapeseed mutants resistant to TBM and to characterize the mechanisms of herbicide resistance and fertility performance under TBM treatment.

Materials and methods

Plant materials and field experiment

An elite oilseed rape cultivar Huashuang 5 (HS5, wild-type) was used for mutagen treatment. Two other varieties Zhongshuang 11 (ZS11) and P174 were used to develop segregation populations with herbicide-resistant mutants. Ten additional varieties, Zhongshuang2, Zhongyou9636, Qingyou331, Yunyou5, Zhongshuang9, Qinyou7, Norin24, Huyou16, Fuyou2 and Huyou15, were selected to test the allele-specific dCAPS marker. All materials were planted at the experimental station of Huazhong Agricultural University, Wuhan, China, in the winter–spring growing seasons. Approximately ten plants were grown 20 cm apart in each row, with 30 cm between rows.

Table 1 Phenotypic assessment for TBM resistance in segregation populations

Cross	Population	No. of tested plants			Ratio	χ^2	*P* value
		R	*S*	Total			
M45 × P174	F_1	18	0	18	–	–	–
	F_2	325	109	434	2.98:1	0	1
	BC_1	124	119	243	1.04:1	0.0658	0.7975
P174 × M45	F_1	21	0	21	–	–	–
M45 × ZS11	F_1	20	0	20	–	–	–
	F_2	347	122	469	2.84:1	0.2054	0.6504
	BC_1	95	98	193	0.97:1	0.0207	0.8855
ZS11 × M45	F_1	16	0	16	–	–	–

EMS mutagenesis and screening for mutants resistant to TBM herbicide

To screen for mutants resistant to TBM, about 1,000 g dry seeds of HS5 were mutagenized with freshly made 0.3 % EMS solution (W/V, Sigma) in pH 7.0 phosphate buffer for 18 h at room temperature with stirring. The mutagenized seeds (M_1 generation) were rinsed with water for 3–4 h and sown in the field. Individual plants that survived were bagged at the flowering stage and selfed seeds were harvested. M_2 seeds were then pooled and grown in the field. Seedlings at 4–6 leaf stage were sprayed twice with a dose of TBM solution lethal to wild type oilseed rape (2.5 mg l^{-1} as showed in "Results") at 7 day intervals. Plants resistant to TBM were selfed to harvest seeds for evaluation in the M_3 generation.

Herbicide treatment

To determine the lethal dose of TBM herbicide on wild-type HS5, seedlings at the 4–6 leaf stage were sprayed as above with serial concentrations of 0, 0.01, 0.025, 0.5, 2.5 and 5.0 mg l^{-1} TBM. The TBM solutions were made from commercial herbicide containing 75 % active TBM chemical (Do Pont Agricultural Chemicals Ltd, Shanghai, China). Symptoms were recorded at 20 days after second spraying of herbicide.

To induce male sterility, plants at the bolting stage, where the longest floral buds were <2–3 mm, were sprayed using 0.05 mg l^{-1} TBM, and on two subsequent occasions at seven day intervals. Male fertility was determined at flowering stage by checking the development of anthers. Pollen viability at full-bloom stage was detected by staining with 1 % acetocarmine solution (Li et al. 1995).

Inheritance analysis

One herbicide-resistant mutant, M45, was selected for further analysis. Crosses were made between M45 and susceptible varieties ZS11 and P174. Two F_2 populations were developed, one from each of the two parental combinations (M45 × P174 and M45 × ZS11; Table 1). Meanwhile, the same F_1 hybrid plants were backcrossed with their corresponding susceptible parents to obtain two BC_1 populations.

The parents, F_1 hybrids, F_2 and BC_1 progenies were grown in the field and seedlings sprayed at 2.5 mg l^{-1} (lethal dose) TBM as above described. Resistance of parents and their derived populations were scored at 20 days after the second spraying. The response phenotypes were scored with two categories: susceptible (dead) and resistant (absence of symptom or only slight injury). The segregation of each population was tested by a Chi squared (χ^2) goodness-of-fit test.

Amplification of *BnaX.AHAS* genes and sequence analysis

Genomic DNA was isolated from 250 mg young leaves and adjusted to about 50 ng μl^{-1} and used as template for PCR amplification. Two gene-specific primer pairs, BnaAHAS1-F and BnaAHAS1-R for *BnaC.AHAS.a* [corresponding to *AHAS1* described by Rutledge et al. (1991)], and BnaAHAS3-F1 and BnaAHAS3-R1 for *BnaA.AHAS.a* [corresponding to *AHAS3* described by Rutledge et al. (1991)] (Supplemental Table S1) (Hu et al. 2012) were used to amplify the open-reading frames (ORF) of *BnaC.AHAS.a* and *BnaA.AHAS.a* from M45 and the wild-type, respectively. The PCR reaction mixture (50 µl) contained 100 ng template DNA, 1 × PCR buffer, 0.2 mM each dNTP, 1.0 mM MgSO4, 0.3 µM each primer, and 1.0 U KOD-plus- (Catalog KOD-201; TOYOBO). PCR amplification was carried out with pre-denaturation at 94 °C for 3 min followed by 32 cycles at 94 °C for 15 s, 55 °C for 30 s, 68 °C for 2 min 30 s, and a final extension at 68 °C for 5 min. An additional 'A' nucleotide was added to the 3′ ends of the PCR products. The A-tailed PCR products were ligated to the pGEM®-T Easy vector (Catalog A1360; Promega) and transformed into DH5α cells. Five positive clones were selected for each PCR product and Sanger sequenced using an ABI 3500.

The coding sequences and amino acids of *A. thaliana AHAS* (*At3g48560*) were downloaded from TAIR (http://www.arabidopsis.org/). Multiple alignments of nucleotide and deduced amino acid of *AHAS* analogs and calculation of similarities between protein pairs were performed using online Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The alignment results were edited and labeled by GeneDoc software (http://www.nrbsc.org/gfx/genedoc/).

Transformation of *Arabidopsis*

To validate the function of candidate gene *bnaA.ahas3.a* for tribenuron-methyl resistance, a 2.9 kb fragment including the 2.0 kb mutated ORF and 0.9 kb downstream 3′-untranslated region was amplified from M45 with the primers BnaAHAS3-F2 and BnaAHAS3-R2 (Supplemental Table S1). PCR was performed with KOD-plus- as described above with pre-denaturation at 94 °C for 3 min followed by 32 cycles at 94 °C for 15 s, 55 °C for 30 s, 68 °C for 3 min, and a final extension at 68 °C for 5 min. Both the PCR product and the 35 s-SUNGFP vector (provided by Professor Jian Xu at Huazhong Agricultural University) were double digested by FastDigest®*Eco*RI and *Xba*I (Catalog FD0274 and FD0684; Fermentas) and ligated to construct the binary plasmid CaMV35S:bnaA.ahas3.a. After sequencing confirmation, the construct was transformed into wild-type *A. thaliana* (Col) using an *Agrobacterium tumefaciens*-mediated floral-dip method (Clough and Bent 1998). Transgenic T_1 generation was selected on Murashige and Skoog media containing 30 mg l^{-1} glufosinate ammonium (Sigma). Two positive lines in T_2 generation were selected to generate homozygous transgenic plants (T_3) for further analysis.

Enzyme extraction and activity assay

The AHAS enzyme was extracted and measured as described by (Sibony et al. 2001; Singh et al. 1988). Briefly, three grams of fresh leaf were collected from wild-type Col, transgenic *Arabidopsis*, HS5 and M45, and ground to powder in liquid nitrogen. Crude AHAS enzyme was extracted with 10 ml extraction buffer (100 mmol L^{-1} potassium phosphate buffer (pH 7.0), 1 mM sodium pyruvate,0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate,10 μM flavine adenine dinucleotide) and dissolved in 10 ml elution buffer (50 mM potassium phosphate buffer (pH 7.0), 20 mM sodium pyruvate and 0.5 mM $MgCl_2$). The extracted enzyme was detected on 8 % SDS–polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Brilliant Blue R250 (Schägger and Von Jagow 1987). A mid-range protein molecular weight marker (Catalog BM524; GeneRay) was used for size estimation of enzyme. The in vitro activity of AHAS enzymes was measured based on the amount of acetoin produced in 2.1 ml volume containing 900 μl enzyme solution, 1,000 μl reaction buffer (50 mM potassium phosphate (pH 7.0), 100 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM thiamine pyrophosphate, 10 μM flavine adenine dinucleotide) and 200 μl deionized water or TBM solutions with the same concentrations as exogenously sprayed on plants. Each assay was performed in three replications. The colored product produced by the Westerfeld reaction between acetoin and creatine and α-naphthol was observed visually and measured using absorbance at 525 nm on Spectroscopy (Westerfeld 1945). The enzyme activity was expressed as percentage of absorbance per that of positive control, in which the reaction took place with enzyme and without TBM.

Development of allele-specific dCAPS marker and polymorphism detection

The nucleotide sequence 533–537 bp from the translation start site in the wild-type allele of *BnaA.AHAS.a* in HS5 is CGA*G*G, which was mutated to CGA*A*G in M45 (the italic and underlined letter indicates the mutation). To detect the causal point mutation in *BnaA.AHAS.a*, a mismatch was introduced into the allele-specific forward primer (BnaAHAS3_dCAPS-F, Supplemental Table S1) to create an *Eco*RII recognition site (CCWGG) in the wild-type *BnaA.AHAS.a* allele (Neff et al. 2002; http://helix.wustl.edu/dcaps/). To avoid amplification interference of paralogous *BnaX.AHAS* genes, the reverse primer (BnaAHAS3_dCAPS-R, Supplemental Table S1) was located in the 5′ UTR region of *BnaA.AHAS.a* where there is significant nucleotide divergence from *BnaC.AHAS.a* and *BnaC.AHAS.b*. The PCR products amplified from both wild-type and mutant alleles are expected to be 633 bp in length. The wild-type allele was digested into fragments of 589 and 44 bp with *Eco*RII or its isoforms, while the mutant allele was undigested.

The PCR reaction mixture (20 μl) contained 75 ng template DNA, 1 × *Taq* buffer with KCl, 0.2 mM each dNTP, 2.0 mM $MgSO_4$, 0.5 μM each primer, and 5.0 U *Taq* DNA polymerase (Catalog EP0402; recombinant, Fermentas). PCR amplification was carried out as following procedure: pre-denaturation at 94 °C for 3 min followed by 40 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 5 min. 10 μl PCR product was digested for 30 min in 30 μl total volume with 1 U FastDigest® *Mva*I (Catalog FD0554; Fermentas), an isoform of EcoRII. After digestion, 10 ul of reaction were separated on 3 % agarose gel.

Identification of paralogous genes in *B. rapa* and *B. oleracea*

To study the origin of *BnaX.AHAS* genes, we sought to identify the *AHAS* multigene family in the available genomes of *B. rapa* (A genome; Wang et al. 2011) and *B. oleracea* (C genome; Liu et al. 2014), the ancestral genomes of *B. napus* (AC genome). Blastp against the *B. rapa* and *B. oleracea* genomes (Cheng et al. 2011; http://brassicadb.org/brad/) was performed to identify paralogous AHAS genes using *At3g48560* as the query gene. Firstly, paralogous genes were defined as subjects in *B. rapa* and *B. oleracea* genomes as those with an E-value $>10^{-20}$ and identity >60 %. A reversed Blastp analysis was then performed using the putative paralogous genes in *B. rapa* and *B. oleracea* as queries, and the *A. thaliana* genome ((http://www.arabidopsis.org/) as the subject. The putative genes with the best match to *At3g48560* were designated as candidate paralogous *AHAS*. The coding sequence and amino acids of *B. rapa* and *B. oleracea* gene models were downloaded from the *Brassica* database (Cheng et al. 2011; http://brassicadb.org/brad/) and *B. oleracea* Genome Database (http://www.ocri-genomics.org/bolbase/index.html), respectively. The coding and amino acid sequences of three *B. napus AHAS* genes (*BnaC.AHAS.a* (Z11524), *BnaC.AHAS.b* (Z11525) and *BnaA.AHAS.a* (Z11526)) were obtained from NCBI GenBank. Phylogenetic relationships were established using MEGA version 5.2 (Tamura et al. 2011) with Neighbor-Joining (NJ) tree based on *p*-distance model of amino acid substitutions type. A non-parametric bootstrap method was performed and the number of bootstrap replication was 1000.

Accession numbers

Amino acid sequences of AHAS enzymes used in this article can be found in GenBank with the following accession numbers: *Amaranthus retroflexus* (AAK50820), *Camelina microcarpa* (AAR07633), *Gossypium hirsutum* (CAA87083), *Descurainia Sophia* (ACB12188), *Helianthus annuus* AHAS1-AHAS3 (AAT07322, AAT07327 and AAT07329), *Glycine max* AHAS1-AHAS4 (AGL09062, AGL09059, AGL09060 and AGL09061), *Xanthium sp.* (AAA74913). Accession numbers for the amino acid sequences of AHAS in *B. napus* and *Arabidopsis thaliana* are described above.

Results

Screening of herbicide-resistant mutants

To determine the lethal concentration of TBM herbicide on rapeseed, we sprayed wild-type *B. napus* HS5 at the 4–6 leaf stage with herbicide solution containing 0, 0.01, 0.025, 0.5, 2.5 and 5.0 mg l^{-1} TBM. With the 0.01 and 0.025 mg l^{-1} TBM treatments plants showed a low level of phytotoxicity with characteristic purple leaves (Supplementary Fig. S1), and more severe damage as the concentration of TBM increased. All plants died at 20 days after spraying with 2.5 or 5.0 mg l^{-1} TBM (Supplementary Fig. S1), suggesting that the lethal dose of TBM on wild-type rapeseed might be approximately 2.5 mg l^{-1} and could be used for screening herbicide-resistant mutants in rapeseed.

Pooled M2 seeds derived from EMS-treated HS5 were grown in the field and sprayed with 2.5 mg l^{-1} TBM. Twenty putative resistant plants were obtained. Selfed seeds were obtained from individual plants and grown in the field to further validate their resistance by spraying with the same concentration of TBM solution. Eleven were confirmed to be herbicide resistant. Of these, four lines, M2, M45, M247 and M253, were confirmed to be homozygous (Fig. 1) and had a single amino acid substitution at the same position (see later description).

We selected M45 for further analysis, and grew replicate plants in the field. At the 4–6 leaf stage, seedlings were sprayed with different concentrations of TBM to test the tolerant dosage. At 20 days after spraying 0.01, 0.025 and 0.5 mg l^{-1} TBM, leaves of the wild-type HS5 plants became purple with an apparent high accumulation of anthocyanins, a typical symptom of herbicide phytotoxicity. Although growth of the wild-type HS5 plants was severely arrested when TBM was increased to 2.5 and 5.0 mg l^{-1}, the M45 plants showed no symptoms of herbicide injury at these concentrations (Fig. 2a). To determine whether M45 was also resistant to IMI, as a representative of another class of AHAS-inhibitor herbicides, we sprayed 0.5 ml l^{-1} imazapic at the 4–6 leaf stage. Leaves became purple after 20 days of spraying, indicating that the SU-resistant M45 was susceptible to IMI herbicide (Supplemental Fig. S2).

TBM herbicide resistance is controlled by a single dominant gene

To understand the inheritance of resistance in M45, crosses were made between M45 and two susceptible varieties ZS11 and P174, and F_2 and BC_1 populations were developed from these two crosses. All parents, F_1 hybrids, F_2 and BC_1 populations were grown in the field and sprayed twice with 2.5 mg l^{-1} TBM solution. F_1 plants from all reciprocal crosses were resistant to TBM (Table 1), suggesting that that the resistance was controlled by dominant nuclear gene(s) without cytoplasmic effect.

The F_2 population derived from the cross between M45 and P174 segregated in a ratio of 325 resistant plants: 109 susceptible plants. The BC_1 population segregated in a ratio of 243 resistant plants: 193 susceptible plants. A

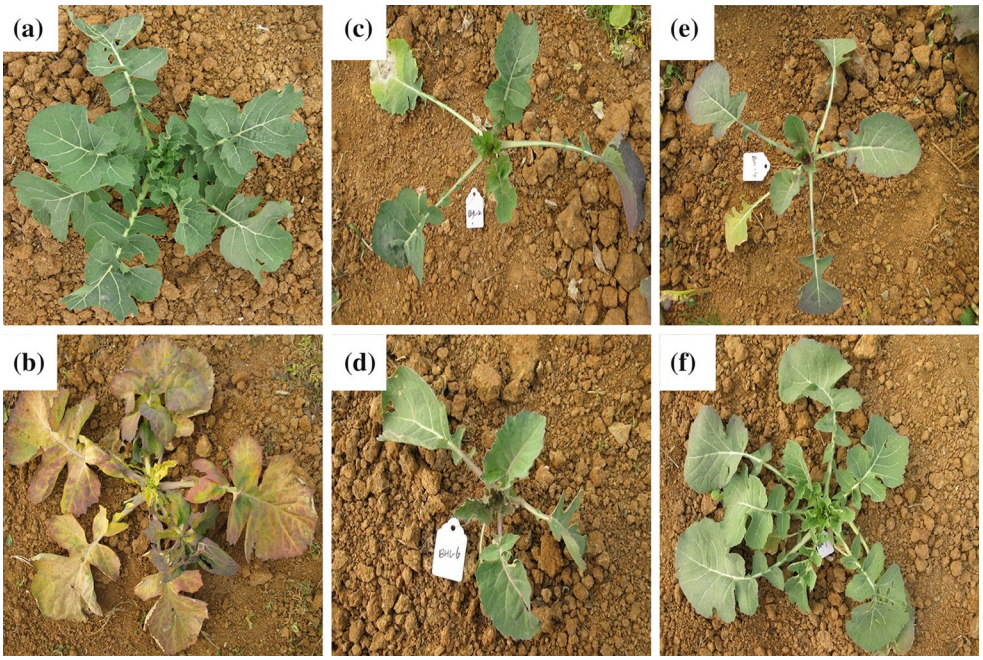


Fig. 1 Confirmation of TBM resistance. **a** untreated wild-type HS5, **b** wild-type HS5 treated by 2.5 mg l^{-1} of TBM; **c–f** Mutants M2, M45, M247 and M253 treated by 2.5 mg l^{-1} of TBM

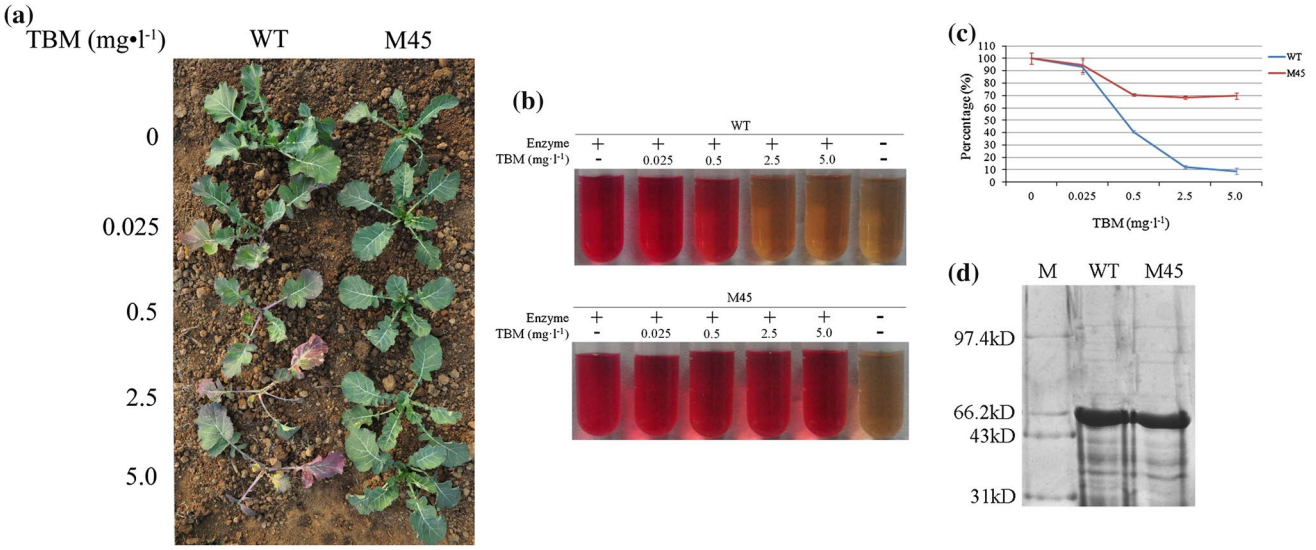
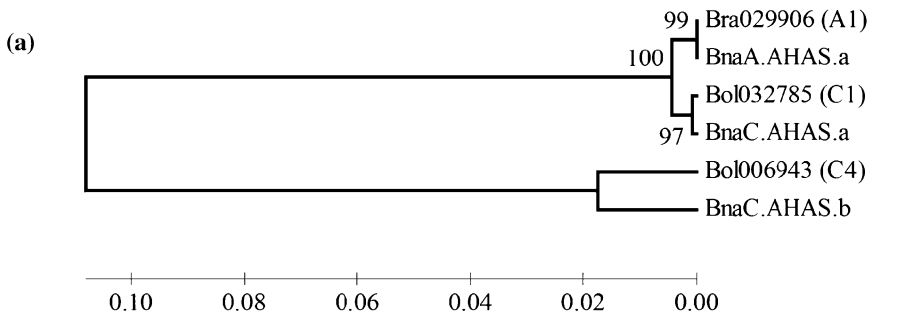


Fig. 2 Activity assay of AHAS enzymes from wild-type and mutant rapeseed plants. **a** Symptoms of wild-type HS5 and M45 plants 20 days after treatment with different concentration of TBM. **b** and **c** Colorimetric assay of AHAS enzyme from HS5 and M45 plants. The reaction with enzyme and without TBM was used as a positive control (*leftmost* tube), and reaction without enzyme and TBM as a negative control (*rightmost* tube). The *x*-axis indicates concentration of TBM added to the reaction and the *y*-axis represents relative enzyme activity (mean ± standard error, $n = 3$) compared to the positive control. **d** SDS-PAGE analysis of AHAS enzyme extracted from fresh leaves of wild-type HS5 and M45. M, protein molecular weight marker

goodness-of-fit test indicated that the segregation of resistant and susceptible plants fits the expected Mendelian ratios of 3:1 and 1:1 ($\chi^2 = 0$, $P = 1$ and $\chi^2 = 0.0658$, $P = 0.7975$; Table 1), respectively. The F_2 and BC_1 populations derived from the crosses between M45 and ZS11 gave similar results ($\chi^2 = 0.2054$, $P = 0.6504$ and $\chi^2 = 0.0207$, $P = 0.8855$; Table 1). These results indicated that the resistance was controlled by a single dominant nuclear gene.



(b)

Gene	Identity				
	Bra029906 (A1)	BnaA.AHAS.a	Bol032785 (C1)	BnaC.AHAS.a	Bol006943 (C4)
Bra029906 (A1)					
BnaA.AHAS.a	100				
Bol032785 (C1)	99.08	99.08			
BnaC.AHAS.a	99.23	99.23	99.85		
Bol006943 (C4)	77.88	77.88	77.52	77.52	
BnaC.AHAS.b	78.99	78.99	78.62	78.62	96.55

Fig. 3 Phylogenetic relationship of AHAS among *B. rapa*, *B. oleracea* and *B. napus*. The *numbers in parentheses* indicate the chromosome information of genes in *B. rapa* and *B. oleracea* genome. **a** Phylogenetic tree of AHAS paralogues based on amino acid sequence. **b** Amino acid sequence identity among AHAS paralogues

Herbicide resistance in M45 associated with AHAS enzyme activity

SU herbicides inhibit the enzyme activity of AHAS by binding to the substrate-access channel, and thus block the biosynthesis pathway of branched-chain amino acids leucine, isoleucine and alanine (Duggleby et al. 2008). To characterize whether the herbicide resistance is caused by a gain-of-function mutation in the AHAS enzyme, we measured the in vitro enzyme activity of AHAS in HS5 and M45 leaves. Proteins of AHAS enzyme extracted from both HS5 and M45 had a molecular weight (MW) of ~66 kD as determined by SDS-PAGE, which is consistent with the MW of AHAS reported in many plant species (Duggleby et al. 2008; Duggleby and Pang 2000) (Fig. 2d). The same concentrations of TBM as used for the exogenous field spraying were added to the extracted enzyme solution. The activity of AHAS was measured based on the Westerfeld reaction (Westerfeld 1945). The activities of wild-type AHAS enzyme from HS5 plants were inhibited by adding 2.5 and 5.0 mg l^{-1} TBM, and dramatically dropped to 12.1 and 8.8 % of untreated enzyme. However, the activity of the AHAS enzyme from M45 plants was only slightly inhibited by the same amount of TBM and maintained around 70 % activity of the untreated enzyme, which is sufficient to support the normal growth of rapeseed (Fig. 2b, c). These results suggested that herbicide resistance in M45 might be caused by a gain-of-function mutation in the *AHAS* genes.

Comparison of *AHAS* genes in diploid *B. rapa*, *B. oleracea* and amphidiploid *B. napus*

To identify the causal mutation in the *B. napus AHAS* genes, we first tried to identify the *AHAS* multigene family in *B. rapa* and *B. oleracea*, the two diploid ancestors of *B napus*. Reciprocal Blastp identified one *AHAS* gene (*Bra029906*) on chromosome A1 in the *B. rapa* genome, and four *AHAS* genes, *Bol032785* on C1, *Bol006943* on C4, *Bol037422* and *Bol037423* on C8, in the *B. oleracea* genome. Among these genes, *Bol037422* and *Bol037423* are two pseudogenes, and encode the N-terminal and C-terminal domain of AHAS protein, respectively, and may correspond to the two defective genes (*AHAS4* and *AHAS5*) previously reported in *B. napus* (Rutledge et al. 1991). Further analysis revealed that there is a 19.2 kb CACTA transposon inserted between these two genes, as reported previously (Zhao et al. 2013).

Three functional *AHAS* genes had originally been identified in the *B. napus* genome based on genomic library screening (Rutledge et al. 1991). We explored the relationships between the *AHAS* genes in *B. rapa*, *B. oleracea* and *B. napus* and constructed a phylogenetic tree based on amino acid sequences. The six *AHAS* genes were clearly classified into three groups, *Bra029906* and *BnaA.AHAS.a*, *Bol032785* and *BnaC.AHAS.a*, and *Bol006943* and *BnaC.AHAS.b* (Fig. 3a). Bra029906 and BnaA.AHAS.a are identical, suggesting that *BnaA.AHAS.a* originated from the A1 chromosome of *B. rapa*. BnaC.AHAS1.a and BnaC.AHAS.b have the highest identity with Bol032785 (99.85 %) and Bol006943 (96.55 %), respectively (Fig. 3b), suggesting that both *BnaC.AHAS.a* and *BnaC.AHAS.b* originated from the C1 and C4 chromosomes of *B. oleracea*. Taken together, these results indicated that there are only three functional *AHAS* genes in the *B. napus* genome, as indicated in the previous study (Rutledge et al. 1991).

Identification of the causal mutation in M45

Of the three *B. napus AHAS* genes, *BnaA.AHAS.a* and *BnaC.AHAS.a* are constitutively expressed and predicted

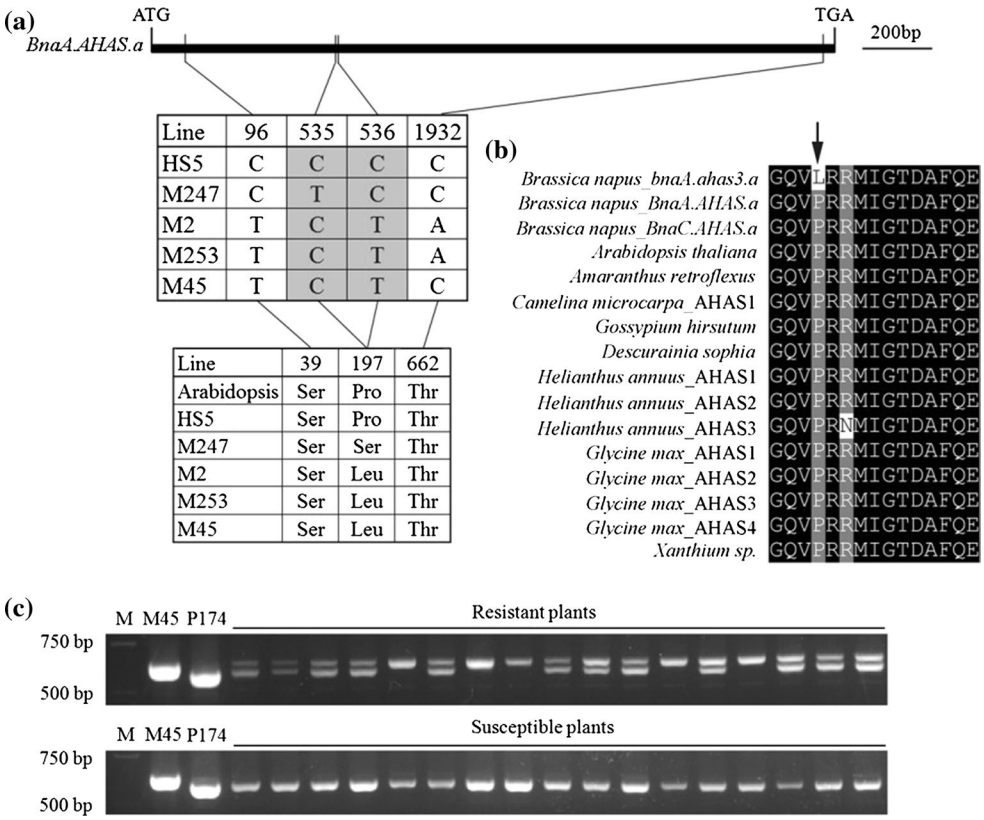


Fig. 4 Molecular characterization of mutations in *BnaA.AHAS.a*. **a** Comparisons of nucleotide and amino acid sequences between wild-type HS5 and four TBM-resistant mutants. The SNPs conferring missense mutation are highlighted by *shaded boxes*. The first nucleotide of the translation start site was numbered as 1, and the numbering of amino acids followed that of AHAS protein in *A. thaliana*. **b** Alignment of amino acid sequence surrounding the 197th amino acid of 15 AHAS proteins from different plant species. The *arrow* indicates the mutated proline at the 197th amino acid of BnaA.AHAS.a in M45. **c** Co-segregation of TBM resistance and the allele-specific dCAPS marker in F_2 plants derived from the cross between M45 and P174. M45 and P174 are the resistant and susceptible parents, respectively. M, 1 kb DNA marker (Fermentas)

to be related with herbicide resistances in *B. napus* (Ouellet et al. 1992; Rutledge et al. 1991). To identify the causal mutations induced by the EMS mutagen, the ORFs of *BnaA.AHAS.a* and *BnaC.AHAS.a* were amplified from wild-type HS5 and M45 and sequenced. Four single nucleotide polymorphisms (SNPs) at positions 197 (T197C), 1242 (A1242G), 1368 (C1368T) and 1875 (C1875A) from the translation start site were identified in the coding region of *bnaC.ahas.a* in M45 (Supplemental Fig. S3). Of these, the three SNPs at positions 1242, 1368 and 1875 represented synonymous mutations and did not alter the amino acid. Only the SNP at position 197 of *bnaC.ahas.a* gene caused a substitution of Ile with Thr in the transit peptide region at position 81 referenced to the *A. thaliana* protein sequence (Supplemental Fig. S3). The Ile at amino acid position 81 in the BnaC.AHAS.a protein is not conserved in all 24 AHAS proteins analyzed, and is not one of the common binding sites for herbicidal AHAS-inhibitors (Duggleby et al. 2008; Duggleby and Pang 2000). Comparative sequencing of four TBM sensitive rapeseed varieties (S2, M201, No. 2127 and 018A1) also revealed the C-to-T transition at this position (data not shown), suggesting that this SNP was not responsible for TBM resistance.

Two SNPs at positions 96 (C96T) and 536 (C536T) from the translation start site were identified in the coding region of *bnaA.ahas.a* in M45 (Fig. 4a). The SNP at position 96 is a synonymous mutation, while the SNP at position 536 of *bnaA.ahas.a* gene causes a substitution of proline (Pro) with leucine (Leu) at amino acid 197 with respect to the *A. thaliana* protein sequence, and so was designated P197L. Sequencing from the other three mutant lines identified the same C-to-T transition at nucleotide 536 in M2 and M253, and a C-to-T transition at the nucleotide 535 in M247, the latter causing a substitution of Pro with serine (Ser) at amino acid 197 (designated as P197S). All three mutations resulted in amino acid substitutions at the same position as in M45 (Fig. 4a). Alignment of amino acid sequences of 15 AHAS genes from nine species indicated that the Pro at position 197 is conserved in all species (Fig. 4b). Substitution of Pro at this position with other amino acids has been demonstrated to confer herbicide resistance in other species such as *A. thaliana*, yeast and redroot pigweed (Duggleby et al. 2008; Powles and Yu 2010). Taken together, these results strongly supported the hypothesis that the substitution of Pro at position 197 in BnaA.AHAS.a is the causal mutation leading to TBM resistance.

To determine by segregation analysis whether the C-to-T transition at the nucleotide 536 is directly associated with the herbicide resistance in M45, an allele-specific dCAPS marker was developed. The PCR product amplified from wild-type HS5 included the sequence CCAGG, corresponding to the recognition site of *Eco*RII (CCWGG). Digestion of the PCR product with *Eco*RII generated a 589 bp fragment, which was referred as the susceptible (S)

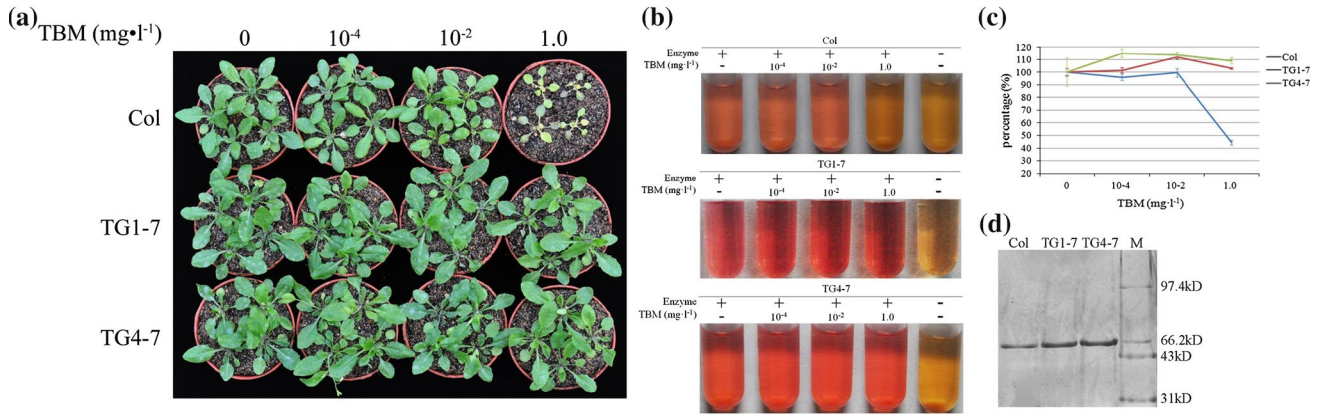


Fig. 5 TBM resistance in transgenic *Arabidopsis* plants expressing mutated *Bnaahas3*. **a** Plant symptoms of Col and transgenic lines TG1-7 and TG4-7. Plants were treated twice by TBM with interval of 1 week at stage of 4–6 leaves. **b** and **c** Colorimetric assay of AHAS enzyme in Col, TG1-7 and TG4-7. The illustration is same to that described in Fig. 2. **d** SDS-PAGE analysis of AHAS enzyme extracted from fresh leaves of Col, TG1-7 and TG4-7

allele. The corresponding site from the resistant allele (R) had the sequence CCAAG, which resulted in an uncleaved product of 633 bp (Fig. 4c). The dCAPS marker completely co-segregated with the herbicide resistance in the F_2 population, with 34 individuals derived from the cross of M45 and P174 (Fig. 4c). Furthermore, we validated the specificity of this marker in ten normal (wild-type) varieties, which are known to be susceptible to TBM, and found that all displayed the same band pattern as the susceptible parent P174 (data not shown). These results further indicated that the SNP (C536T) which caused the P197L substitution in bnaA.ahas.a is the causal mutation for herbicide resistance.

Expression of *bnaA.ahas.a* confers herbicide resistance in *A. thaliana*

To further determine that the mutant *bnaA.ahas.a* allele conferred TBM resistance, we transformed the CaMV35S:bnaA.ahas.a plasmid containing the full length ORF of *bnaA.ahas.a* gene from M45 into wild-type *Arabidopsis* (Col). Ten transgenic lines (TG1-TG10) were obtained. Two homozygous plants, TG1-7 and TG4-7, were selected from the offspring and used for resistance evaluation. The transgenic plants could tolerate up to 1.0 mg l^{-1} of TBM solution and did not show any symptoms, whilst wild-type Col plants died at this concentration (Fig. 5a).

To confirm whether the resistance in transgenic *A. thaliana* was conferred by introduction of the *bnaA.ahas.a* gene, we measured the enzyme activity of AHAS in vitro in wild-type Col, TG1-1 and TG4-7 transgenic plants by adding TBM to the enzyme extracts as described above. Enzyme was extracted from leaves of untreated Col, TG1-7 and TG4-7 plants, and a single protein with molecular weight ~ 66 kD was detected on SDS-PAGE gel (Fig. 5d). The activity of native AHAS enzyme in wild-type Col could be detected at 10^{-4} and 10^{-2} mg l^{-1} TBM (Fig. 5b, c) but was inhibited at 1.0 mg l^{-1} TBM, where activity was reduced to 40 % of untreated controls. In contrast, the activity of AHAS enzyme in transgenic plants remained almost unchanged, even at 1.0 mg l^{-1} TBM (Fig. 5b, c). Together, these results demonstrated that the mutant *bnaA.ahas.a* was able to maintain the enzyme activity of AHAS, and thus conferred resistance to TBM.

TBM does not affect the male fertility of M45

TBM has been demonstrated to be an excellent male gametocide in rapeseed (Yu et al. 2006). To check if the fertility of M45 was affected by TBM, we treated the wild-type HS5 and M45 plants with three successive sprayings of 0.05 mg l^{-1} (5 ppm) TBM at intervals of 7 days from the bolting stage. Compared to untreated plants, the TBM-treated HS5 plants displayed severe defects in stamen development. The length of filaments was significantly reduced and the development of anthers was arrested (Fig. 6a, c). However, the development of stamens in M45 plants was not affected by TBM treatment (Fig. 6a, c).

We further checked the viability of pollen grains at the full-bloom stage by acetocarmine staining. Flowers of untreated HS5 plants produced 100 % stained viable pollen grains, while the flowers of TBM-treated HS5 plants lacked any stained pollen. In contrast, the flowers of both TBM-treated and untreated M45 plants produced 100 % stained viable pollen (Fig. 6b). Taken together, these results indicated that the male fertility of M45 is insensitive to TBM, and is suitable to be widely used as the male parent in a CIMS system for hybrid rapeseed production.

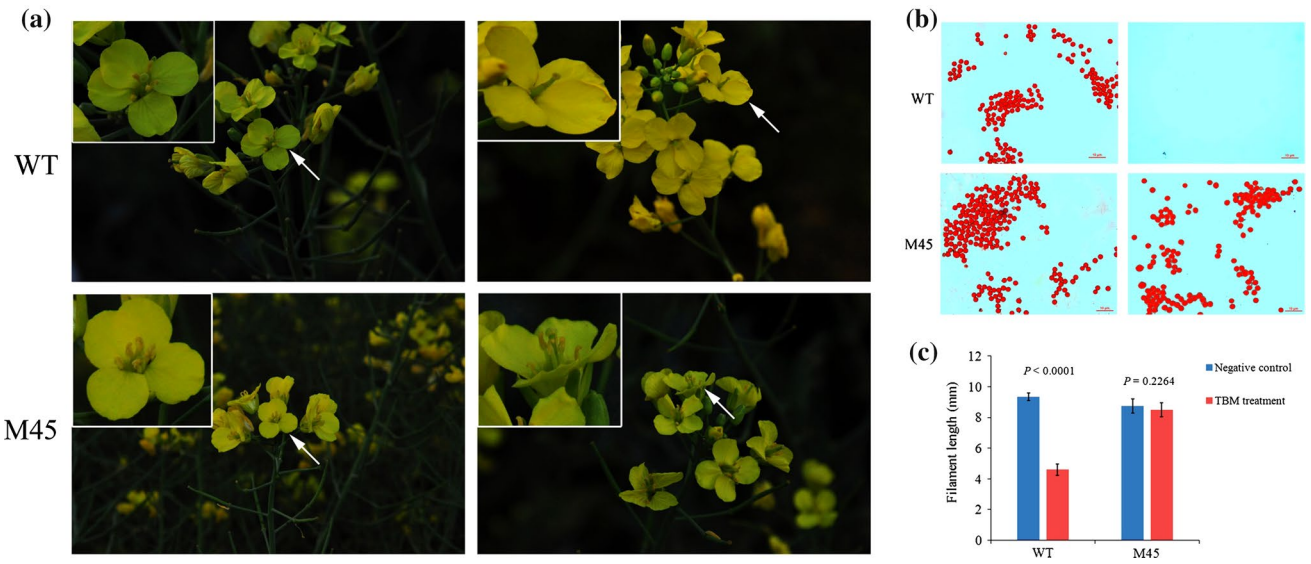


Fig. 6 Effects of TBM treatment on flower development and male fertility for wild-type and mutant plants. **a** Flowers of both wild-type and mutant plants treated with or without TBM. *Left panel* indicates flowers from both wild-type and mutant plants treated with water. *Right panel* indicates flowers from both wild-type and mutant plants treated by 5 ppm TBM for three times with an interval of 7 days. *White arrows* indicate representative flowers in each treatment. **b** Male fertility was represented by viability of pollen grains stained by acetocarmine solution. Scarlet-stained round pollen grains are fertile and lightly stained pollen grains are sterile. *Bar* 10 μm. **c** The filament length for wild-type HS5 and M45 plants treated with water and TBM treatment (mean ± standard error, $n = 10$). *P* values are calculated by one-way analysis of variance

Discussion

In this study, we identified several mutants resistant to TBM, a widely used CHA for rapeseed hybrid seed production using CIMS. We characterized the molecular mechanisms of resistance to TBM herbicide. In vitro assay of AHAS activity indicated that the resistance is due to a gain-of-function mutation in the *AHAS* genes (Fig. 2). Sequence analysis identified SNPs at nucleotides 535 and 536 from the translation start site of *BnaA.AHAS.a* in four mutants. These mutations resulted in a substitution of Pro to Ser or Leu at amino acid 197, referenced to the AHAS enzyme in *Arabidopsis* (Fig. 4a). Although several mutants resistant to the sulfonylureas or imidazolinones have been described in rapeseed (Hattori et al. 1995; Hu et al. 2012; Swanson et al. 1988, 1989), the mutations were reported at positions W574 of BnaA.AHAS.a (Hattori et al. 1995) and S653 of BnaC.AHAS.a (Hu et al. 2012). The P197L and P197S substitutions identified in this study are novel in rapeseed, although they have been reported in *Descurainia sophia*, *Arabidopsis thaliana* and *Amaranthus retroflexus* (Cui et al. 2011; Haughn et al. 1988; Sibony et al. 2001). Amino acid 197 is located at one end of the fifth α-helix (amino acid residues 197–201), which forms the entrance to the active-site access channel in the α-domain of AHAS and always contacts the aromatic ring of the sulfonylurea herbicides (Duggleby et al. 2008; McCourt et al. 2006). The proline at this position is highly conserved in all 15 AHAS genes analyzed across nine species of plants (Fig. 4b), and substitutions of this amino acid resulted in herbicide resistance in plant species including *Arabidopsis* (Haughn et al. 1988), soybean (Ghio et al. 2013), and the weeds, *Descurainia sophia* (Cui et al. 2011) and *Amaranthus retroflexus* (Sibony et al. 2001). We demonstrated that expression of the mutant *bnaA.ahas.a* conferred TBM resistance in *Arabidopsis* (Fig. 5a). Furthermore, the mutant line M45 was susceptible to IMI herbicide (Supplemental Fig. S2). However, the same mutation in redroot pigweed (*A. retroflexus*) conferred cross tolerance to both SU and IMI (Sibony et al. 2001), suggesting that the P197L substitution in *B. napus* might be different from weeds at the molecular level, resulting from different herbicide-bound AHAS structures. A significant difference between sulfonylurea-bound AHAS structures has been shown previously in plants (*A. thaliana*) and yeast (*Saccharomyces cerevisiae*) (McCourt et al. 2006). We therefore conclude that the substitution of proline at amino acid 197 has contributed to herbicide resistance in EMS-mutated rapeseed.

The CIMS system has several advantages over other MS systems in hybrid seed production and has attracted extensive attention in China, where it is likely to replace the existing male sterility systems. TBM is an effective gametocide and the main effector of CHA (Yu et al. 2006). In hybrid seed production using CIMS, complete male sterility is usually induced using as low as 5–10 ppm of TBM on the female parents, where fertile male parents are

protected with a barrier. In this study, we identified four mutants resistant to TBM by EMS mutagenesis. Spraying of TBM on wild-type HS5 for three consecutive times induced complete male sterility (Fig. 6), while the fertility of the mutant was not affected at all, demonstrating that the mutant could safely be used as a male parent in the CIMS system for hybrid seed production without intentional protection with a barrier or shield. Female wild-type HS5 plants showed no difference in yield and plant height compared with untreated controls (data not shown), and so the system is expected to increase the efficiency and security of hybrid production. To the best of our knowledge, this is the first proposed application of a herbicide-resistant cultivar in hybrid production using a CIMS system.

In addition, the mutants generated are able to tolerate up to 5.0 mg l^{-1} TBM (Fig. 2a), which is almost same as the recommended concentration for broad-leaf weed control, suggesting that the SU herbicides could be used to control broad-leaf weeds in fields of herbicide-resistant conventional and hybrid cultivars derived from M45. The Clearfield® production system is available for some IMI-tolerant crops such as rice, wheat, sunflower and rapeseed to control certain weeds with imidazolinone herbicides (Tan et al. 2005). In sunflower this system delivered complete tolerance to IMI herbicides based on two genes with additive effect including a major gene *Ahasl1-1* (*Imr1*) and an enhancer (*Imr2*) (Sala and Bulos 2012). In our study, the single mutant locus *bnaA.ahas.a* conferred resistance to TBM herbicide in M45 (Fig. 2). Stronger resistance may be expected to achieve if two AHAS genes were simultaneously mutated, which may then provide more effective weed control where higher loads-armored pernicious weeds affect rapeseed.

Author contributions HTL, JJL, BZ, LCY and CL performed the experiments. HTL and JJL analyzed the data and drafted the manuscript. JSW provided the plant materials. JW and GJK helped draft the manuscript. KDL conceived the study and helped draft the manuscript. All the authors read and approved the final manuscript.

Acknowledgments This work was supported by the National Hi-Tech R&D Program (2013AA102602), National Technology R&D Program (2012BAD49G00) and the Fundamental Research Funds for the Central Universities (Program No. 2662013YB03). GJK is a Chutian scholar, funded by Hubei province. We thank the two anonymous reviewers for their valuable suggestions.

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were performed.

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