

# Development of a complete set of monosomic alien addition lines between *Brassica napus* and *Isatis indigotica* (Chinese woad)

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## Abstract

**Key message** A complete set of monosomic alien addition lines of *Brassica napus* with one of the seven chromosomes of *Isatis indigotica* and the recombinant mitochondria was developed and characterized.

**Abstract** Monosomic alien addition lines (MAALs) are valuable for elucidating the genome structure and transferring the useful genes and traits in plant breeding. *Isatis indigotica* (Chinese woad,  $2n = 14$ , II) in *Isatideae* tribe of *Brassicaceae* family has been widely cultivated as a medicinal and dye plant in China. Herein, the intertribal somatic hybrid ( $2n = 52$ , AACCCII) between *B. napus* cv. Huashuang 3 ( $2n = 38$ , AACCC) and *I. indigotica* produced previously was backcrossed recurrently to parental *B. napus*, and 32 MAAL plants were isolated. Based on their phenotype, 5S and 45S rDNA loci and chromosome-specific SSR markers, these MAALs were classified into seven groups corresponding to potential seven types of MAALs carrying one of the seven *I. indigotica* chromosomes. One of the MAALs could be distinguishable by expressing the

brown anthers of *I. indigotica*, other two hosted the chromosome with 5S or 45S rDNA locus, but the remaining four were identifiable by SSR markers. The simultaneous detection of the same SSR marker and gene locus in different MAALs revealed the paralogs on the chromosomes involved. The recombinant mitochondrial genome in MAALs was likely related with their male sterility with carpelody stamens, while the MAAL with normal brown anthers probably carried the restoring gene for the male sterility. The complete set of MAALs should be useful for exploiting the *I. indigotica* genome and for promoting the introgression of valuable genes to *B. napus*.

**Keywords** *Brassica napus* · *Isatis indigotica* · Monosomic alien addition line · Genomic in situ hybridization (GISH) · Simple sequence repeat (SSR)

## Introduction

Monosomic alien addition lines (MAALs) are plants with an alien chromosome from one donor species added to the genome of another recipient species. A complete set of MAALs which dissects the donor genome can be used for chromosomal assignment of genes and markers (Geleta et al. 2012), constructing physical maps of specific chromosomes (Kynast et al. 2004), examining alien genes expression (Cho et al. 2006) and studying syntenic correspondence among different species (Tan et al. 2005). Furthermore, MAALs provide a novel platform for studying the chromosome homoeology among different genomes and alien chromosome behavior in recipient genetic background (Wang et al. 2005; Heneen et al. 2012; McArthur et al. 2012). In crop breeding, the prime motive for producing alien addition lines is to introgress genes of

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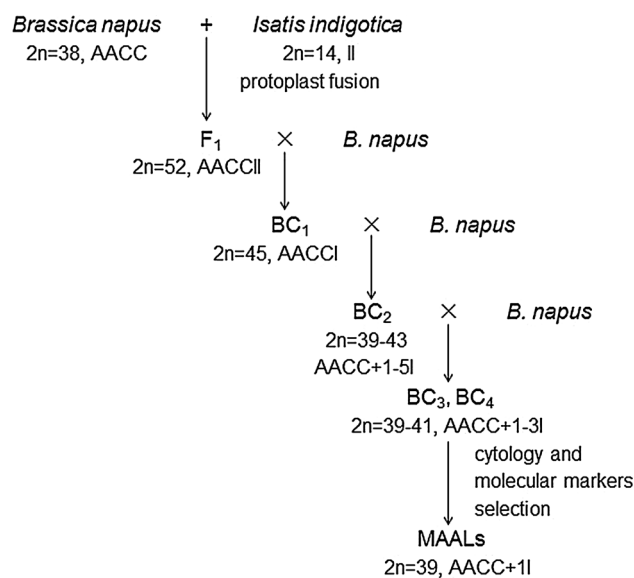
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interest from wild relatives into cultivated crops, and the introgressions with alien chromosome segments and target traits can be induced by homoeologous recombination, radiation or the gametocidal effect (Klindworth et al. 2012).

The common method for developing MAALs was recurrent backcrossing of the natural or synthesized amphidiploids to one parental species, and then addition lines were isolated from the resulting aneuploid progenies. MAALs can be identified among aneuploidy plants by morphological traits, chromosome-specific cytological and molecular markers, biochemical markers, and the method of molecular cytogenetics. In the family *Brassicaceae*, the genomes of the three cultivated *Brassica* diploids and the other related species have been dissected by the development of MAALs through interspecific, or intergeneric and even intertribal sexual and somatic hybridizations (see review by Prakash et al. 2009). These MAALs have enhanced the location of the useful traits and genes on particular chromosomes, such as disease resistance, important agronomic traits and fertility restoring gene for CMS (Chen et al. 1992; Cheng et al. 1994; Peterka et al. 2004; Wang et al. 2003; Wei et al. 2010; Heneen et al. 2012; Ding et al. 2013), and the investigation of chromosome homoeology (Geleta et al. 2012). However, the establishment of the whole set of MAALs for *Brassica* species and relatives is difficult, because of the small size of the chromosomes and lack of morphological and cytological landmarks (Budahn et al. 2008; Geleta et al. 2012; Heneen et al. 2012).

*Isatis indigotica* Fort. (Chinese woad) which belongs to the *Isatideae* tribe of the *Brassicaceae* family (Al-Shehbaz et al. 2006) has been widely cultivated as a medicinal and dye plant from ancient times in China, but now it is commonly used for medicinal purpose. Its root (*Radix isatidis*) is now the second most consumed source of plant medicine, after radix ginseng, but ginseng is mainly used as tonic. The products from *Radix isatidis* are usually prescribed for the indications of eruptive epidemic diseases caused by the bacteria and viruses (influenza, viral pneumonia, mumps, and hepatitis), to remove heat and eliminate toxin, to reduce heat in blood, and to soothe the sore throat. The medicine is also considered to have immune regulatory and even antitumor effects (Liu et al. 2000; Chung et al. 2011; Du et al. 2013). Some studies showed that this plant had resistance to tobacco mosaic virus (TMV) (Wang and Wang 1988), the fungus *Sclerotinia sclerotiorum* causing stem-rot in rapeseed (Zhao et al. 1994), and even to diamondback moth (*Plutella xylostella*) (Tang et al. 2010). However, the functional components of this plant with great consumption are still unclear, which largely suffers from the scarce information for its genetics and genome. In our previous study aiming



**Fig. 1** Crossing scheme for the development of MAALs

to produce new germplasm for the genetic improvement of *Brassica* crops and to dissect the genome of *I. indigotica*, the intertribal somatic hybrids between *Brassica napus* L. ( $2n = 4x = 38$ , genomes AACC) and *I. indigotica* ( $2n = 2x = 14$ , II) were obtained by protoplast fusion and characterized for their phenotype and cytology (Du et al. 2009). Importantly, the hybrids had the expected sum of chromosomes from the two parents ( $2n = 6x = 52$ , AACCII) and gave rise to backcross progenies (BC<sub>1</sub>,  $2n = 5x = 45$ , AACCI) after pollination by *B. napus*. In this study to continue the genetic analysis of the hybrid progenies, the complete set of *B. napus* monosomic additions carrying one of the seven different *I. indigotica* chromosomes ( $2n = 39$ , AACC + 1I<sub>1-7</sub>) is established by characterizing backcrossing progeny plants for their phenotype, cytological and molecular markers. This set of MAALs is pivotal for elucidating the genome structure of this important medicinal plant and for the introduction of useful genes and traits into *Brassica* crop. Specifically one new cytoplasmic male sterile (CMS) line with carpelody stamens is selected for the hybrid breeding of *B. napus*.

## Materials and methods

### Plant materials

The intertribal somatic hybrid plant (As1) ( $2n = 6x = 52$ , AACCII) between *Brassica napus* L. cv. Huashuang 3 ( $2n = 4x = 38$ , AACC) and *Isatis indigotica* Fort. ( $2n = 2x = 14$ , II) was previously produced by the fusion

of mesophyll protoplasts, and its BC<sub>1</sub> plants after pollinated by Huashuang 3 had the expected chromosome complement ( $2n = 5x = 45$ , AACCI) (Du et al. 2009). Herein, these BC<sub>1</sub> plants with the *Isatis* genome in haploidy state were again backcrossed to Huashuang 3 to produce BC<sub>2</sub> plants which were derived from the embryos rescued on MS medium (Murashige and Skoog 1962). These BC<sub>2</sub> plants with one to several chromosomes from *I. indigotica* were pollinated by Huashuang 3 to produce the BC<sub>3</sub> population in which monosomic addition plants with individual chromosome of *I. indigotica* were identified (Fig. 1). The plants with  $2n = 39$  were maintained by culturing their axillary buds from main stems or branches on MS medium for further study.

#### Cytology and pollen viability analysis

To determine the chromosome numbers of backcross progenies, the ovaries from young flower buds were collected in the morning. After pre-treatment with 2 mM 8-hydroxyquinoline solution for 3 h at room temperature, the ovaries were fixed in Carnoy's solution (3:1 ethanol:glacial acetic acid, v/v) overnight, and stored in 70 % ethanol at  $-20\text{ }^{\circ}\text{C}$  until use. Cytogenetic observations were carried out as described by Li et al. (1995). Pollen fertility was determined as the percentage of pollen grains stained with 1 % acetocarmine.

#### Probe labeling and GISH/FISH analysis

Total genomic DNA was extracted and purified from young leaves according to Dellaporta et al. (1983). Genomic DNA of *I. indigotica* was labeled with Bio-11-dUTP (Fermentas) or digoxigenin-11-dUTP (Roche) by nick translation. The 5S rDNA and 45S rDNA isolated from tomato provided by Prof. Lijia Li, College of Biology, Wuhan University, China, were labeled with Bio-11-dUTP and digoxigenin-11-dUTP, respectively, using nick translation method. The genomic DNA of *B. napus* was sheared by boiling for 15 min to produce DNA fragments of 100–500 bp and used as blocks. Chromosome preparations for GISH were made according to the protocol (Ge et al. 2009). The ovaries were digested in an enzyme mixture containing 4 % cellulose Onozuka RS (Yakult, Japan), 2 % pectinase (Merck, Germany) and 2 % pectinase (Sigma-Aldrich, USA) for about 60 min at  $37\text{ }^{\circ}\text{C}$ . In situ hybridization was performed following the procedures of Cui et al. (2012). Photographs were taken using a computer-assisted fluorescence microscope (Axio Scope A1, Zeiss, Germany) with a CCD camera. Images were processed by Adobe Photoshop CS5 to adjust contrast and brightness.

#### SSR marker analysis

Primer sequences for *I. indigotica* SSR markers were kindly provided by Prof. Luqi Huang from State Key Laboratory of Dao-di Herbs, National Resource Center of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China. Of 3054 SSRs, 192 primer pairs were randomly synthesized (Table S1). These markers included 97 dinucleotide, 83 trinucleotide and 12 tetranucleotide repeats. All these 192 SSR markers were evaluated for successful PCR amplification and polymorphism by testing the genomic DNA of rapeseed and woad. PCR amplifications were performed in a volume of 10  $\mu\text{l}$  containing 20 ng genomic DNA,  $1 \times$  *Taq* buffer, 2 mM  $\text{MgCl}_2$ , 0.2 U *Taq* DNA polymerase (Fermentas), 2 mM dNTPs (Fermentas), 5  $\mu\text{M}$  forward and reverse primer. PCR reaction program was the following:  $94\text{ }^{\circ}\text{C}$  for 5 min; 10 cycles with  $94\text{ }^{\circ}\text{C}$  for 30 s,  $60\text{ }^{\circ}\text{C}$  for 30 s,  $72\text{ }^{\circ}\text{C}$  for 45 s, with a  $0.7\text{ }^{\circ}\text{C}$  decrease in annealing temperature at each cycle; 30 cycles with  $94\text{ }^{\circ}\text{C}$  for 30 s,  $55\text{ }^{\circ}\text{C}$  for 30 s,  $72\text{ }^{\circ}\text{C}$  for 45 s, and a final extension at  $72\text{ }^{\circ}\text{C}$  for 10 min. Amplification products were separated on 6 % denaturing polyacrylamide gels. After electrophoresis, the gels were stained as previously described (Peterka et al. 2004) and photographs taken.

#### *I. indigotica* gene markers

Sequences of seven *I. indigotica* genes were downloaded from GenBank. These genes included *IiCCoAOMT* (GenBank: DQ115904), *IiCDPK* (GenBank: DQ482580), *IiCOMT* (GenBank: DQ115905), *IiLTK* (GenBank: DQ468350), *IiMYB* (GenBank: DQ468346), *IiPAL* (GenBank: DQ468345) and *IiSDD1* (GenBank: DQ407741). PCR primers were designed by Oligo 7 software (Table S2). Reactions (10  $\mu\text{l}$ ) contained  $1 \times$  *Taq* buffer, 2 mM  $\text{MgCl}_2$ , 5 mM dNTPs, 5  $\mu\text{M}$  forward and reverse primer, 1 U *Taq* DNA polymerase and 50 ng genomic DNA. DNA fragments were amplified after a 5-min denaturation at  $94\text{ }^{\circ}\text{C}$  for 30 cycles ( $94\text{ }^{\circ}\text{C}$  for 45 s,  $57\text{ }^{\circ}\text{C}$  for 30 s,  $72\text{ }^{\circ}\text{C}$  for 90 s), and a 10-min extension step at  $72\text{ }^{\circ}\text{C}$ . PCR products were separated on 0.8 % agarose gels.

#### Mitochondrial DNA analysis

To determine the mitochondrial genome, 21 pair primers of mitochondrial genes were designed based on the rapeseed mtDNA sequence (accession number: AP006444). Primer sequences are listed in Table S3. PCR was performed in a 10- $\mu\text{l}$  reaction mixture containing 100 ng total plant DNA,  $1 \times$  *Taq* buffer, 2 mM  $\text{MgCl}_2$ , 1 U *Taq* DNA polymerase, 5 mM dNTPs, 5  $\mu\text{M}$  forward and reverse primer. PCR amplification was carried out with an initial denaturation

**Table 1** The somatic chromosome numbers of the BC<sub>2</sub>, BC<sub>3</sub> and BC<sub>4</sub> plants

	Number of plants	Chromosome numbers								
		<38	38	39	40	41	42	43	44	45
BC <sub>2</sub>	22	1	1	2	7	4	5	2	–	–
BC <sub>3</sub>	113	2	68	25	12	5	1	–	–	–
BC <sub>4</sub>	130	2	89	31	7	1	–	–	–	–

step at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 2 min, and a final 10-min extension at 72 °C.

## Results

### Development of MAALs

After BC<sub>1</sub> plants ( $2n = 5x = 45$ , AACCI) derived from the intertribal somatic hybrids between *B. napus* cv. Huashuang 3 and *I. indigotica* were backcrossed again to Huashuang 3 as pollen parent, 22 BC<sub>2</sub> plants were successfully obtained with the rescue of immature embryos on MS medium (Fig. 1). They had  $2n = 38$ –43, besides one with  $2n = 21$ , only one plant had  $2n = 38$  origination from *B. napus*, and two were monosomic additions ( $2n = 39$ ), while the remaining 19 plants still kept 2–5 additional woad chromosomes (Table 1). Because of the poor male fertility of these BC<sub>2</sub> plants, those with  $2n > 38$  were pollinated by Huashuang 3 to obtain BC<sub>3</sub> progenies, and 113 plants were selected by their phenotype for the determination of chromosome numbers. Out of 113 BC<sub>3</sub> plants, 25 ones had  $2n = 39$  and 18 had  $2n = 40$ –42, while most (68) had  $2n = 38$ , and 2 had  $2n < 38$ . Then, 16 plants with  $2n = 39$  were successfully maintained clonally on MS medium by culturing their axillary buds available. In the similar way, 31 BC<sub>4</sub> plants with  $2n = 39$  were identified and 25 were cloned on MS medium (Table 1). Totally, 41 plants presumably with  $2n = 39$  were successfully cloned to produce enough plants for further investigations. By GISH analysis of these 41 plants, 32 were confirmed to be monosomic additions with 38 chromosomes from *B. napus* and one from *I. indigotica* (Fig. 2b), but the others were actually euploid *B. napus* ( $2n = 38$ ). In other side, several plants with  $2n < 38$  were found but no further study was made. One plant ( $2n = 20$  with one alien chromosome) appeared, which likely derived from the unfertilized egg. Two plants ( $2n = 40$ ) had one pair of alien woad chromosomes related to the chromosome d of *I. indigotica* (below).

The challenging task next was to classify these 32 monosomic additions into seven potential groups which

likely hosted one of the seven different chromosomes from *I. indigotica*, according to their phenotype, chromosome-specific cytological and DNA markers (Figs. 2, 3, 4, 5; Table 2). The seven types of MAALs (designated as Ma–Mg) included 2, 2, 3, 6, 5, 13, 1 plants, respectively, Mf included much more plants (13), and Mg corresponded to only one plant (Table 2).

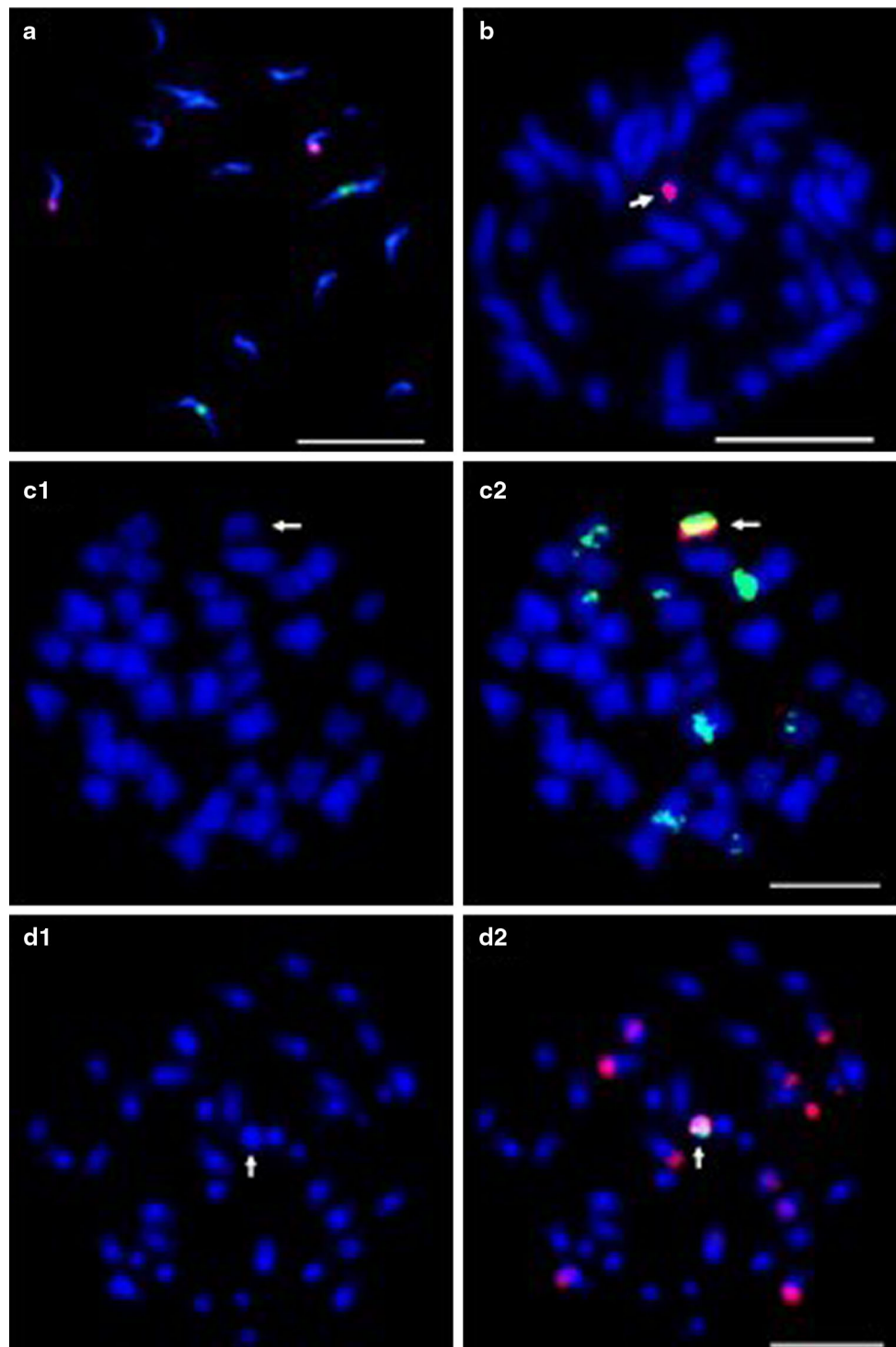
### Cytological markers of MAALs

The chromosomes of *I. indigotica* had the small and similar size, and their size was even smaller than those of *B. napus*. Fluorescence in situ hybridization (FISH) analysis with the labeled 45S rDNA and 5S rDNA as probes identified a single locus of 5S rDNA and 45S rDNA separated on different chromosomes in *I. indigotica* (Fig. 2a). The locus of 5S rDNA was located near centromeres on the long arms of two chromosomes, and the locus of 45S rDNA was at the terminal parts of the short arms of other two chromosomes, which provided the chromosomal markers for the identification of two MAALs. Consequently, Mf and Mg were found to carry the alien chromosome with 5S rDNA and 45S rDNA loci, respectively (Fig. 2c, d; Table 2). Other MAALs were undistinguishable cytologically.

### SSR markers of MAALs

Among 192 SSR markers from *I. indigotica*, 144 pairs produced the genome specific fragments, 47 pairs showed polymorphism between two parental species and one pair (ssr18) had no polymorphism (data not shown). With these woad-specific polymorphic markers to classify MAALs, most markers segregated for their presence or absence (Fig. 3), but some primers lost bands in backcross progenies or had no polymorphism between additions. According to their distribution in MAALs, these markers were divided into seven groups (a–g, Fig. 3), which was in agreement with the chromosome number of woad genome ( $n = 7$ ). The number of markers in group c (19) was fewer than the other groups (23–32) (Table 2). Markers in each group were specific to one of the seven woad chromosomes (Table 2).

Five SSR markers specific to more than one chromosome of *I. indigotica* were also identified (Table 3). The number of duplicated SSR markers which were shared by more than one chromosome was 2, 0, 4, 3, 2, 2 and 2 for the chromosomes a–g, respectively. So no such markers appeared on chromosome b. The markers 9b, 61b and 149 were detected in two chromosomes, whereas 9a, 59b and 90 in three chromosomes. Chromosome a shared no markers with b, e and f. Chromosome c shared no markers with b. Chromosome d shared no markers with b and e.



**Fig. 2** GISH and FISH analysis of MAALs. *Blue color* is from the DAPI staining of chromosomes. **a** One root-tip cell of *I. indigotica* probed with the labeled 5S rDNA (*green*) and 45S rDNA (*red*). **b** An ovary cell ( $2n = 39$ ) of one MAAL line with one labeled chromosome from *I. indigotica* (*red*). **c1, c2** One ovary cell (**c1**) of Mf with one chromosome from *I. indigotica* (*red*) which carries 5S rDNA

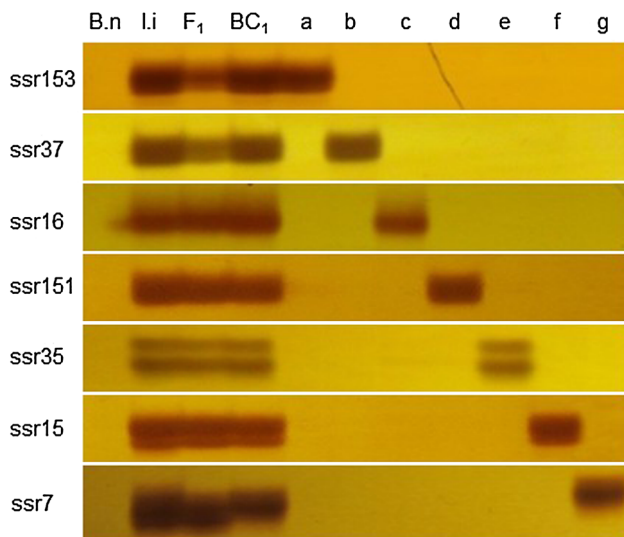
locus (*green*) (**c2**). Several chromosomes from *B. napus* also carry 5S rDNA loci. **d1, d2** One ovary cell (**d1**) of Mg with an alien chromosome (*green*) which hosts 45S rDNA (*red*) (**d2**). The *red* signal from 45S rDNA also appears on some chromosomes from *B. napus*. *Arrows* indicate alien chromosomes. *Bars* 10  $\mu\text{m}$  (color figure online)

Chromosome e only shared markers with c. Chromosome f shared markers with c, d and g. Chromosome g shared markers with c, d and f. Chromosome c shared two markers with e, and similarly d and f shared two markers,

suggesting a significant level of homology between c and e and between d and f.

#### Phenotype of MAALs

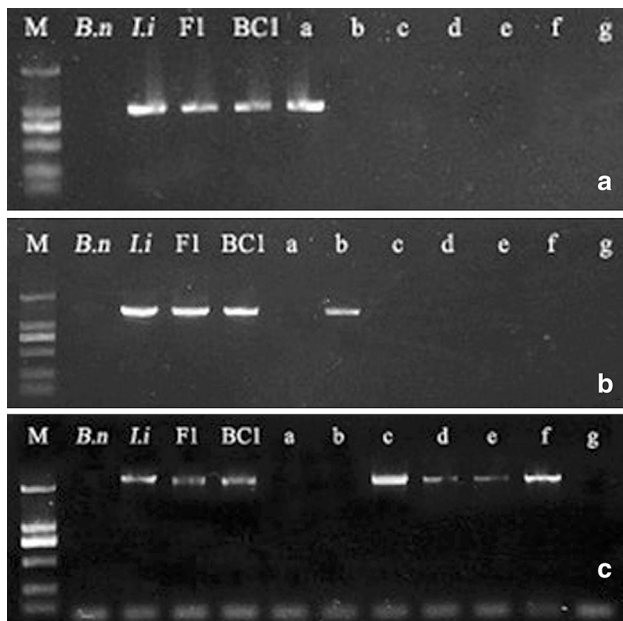
The young plants of MAALs showed a morphology biased to *B. napus* with some variations, but expressed some traits of the woad origin, mainly the darker green and thicker leaves. The margin of leaves was less serrated than *B. napus*, probably because the leaves of *I. indigotica* had no serrations. They grew slowly and also flowered later than *B. napus*. The different plants of the same MAAL line showed nearly the same phenotype. The line Ma showed weaker growth than other additions. As to flower organs, only the line Me had normal flowers with the brown anthers as *I. indigotica* did (Fig. 4c), and could be distinguished from the others by this trait. But other lines showed the disturbed stamen development and wrinkled petals (Fig. 4e1, f1). At the beginning of flowering, the stamens were weakly developed and some fused together (Fig. 4e1, e2), whereas the flowers of Mb did not open (Fig. 4d). The tetradynamous stamens developed into carpelloid structures and the two shorter stamens were only filaments without anthers (Fig. 4f1, f2). These feminized stamens contained stigmatoid structures at their tips and two from same side usually



**Fig. 3** SSR profile generated by MAALs-specific SSR markers. *B.n* represents for *B. napus*, *I.i* for *I. indigotica*, *F<sub>1</sub>* for the somatic hybrid, *BC<sub>1</sub>* for the backcross progeny of the hybrid with *B. napus* parent, *a–g* for MAALs. These codes are the same in Figs. 5, 6



**Fig. 4** Flowers of MAALs. **a** *B. napus*, **b** *I. indigotica*, **c** Me, **d** Mb, **e** and **f** Md, **e2**, **f2** and **g** flowers of MAALs with sepals and petals removed, showing abnormal anthers. Bars 1 cm, except **b** (bar 0.5 cm)



**Fig. 5** Location of the *I. indigotica* genes on different chromosomes in MAALs by PCR analysis. **a** The *liMYB* locates on chromosome a. **b** *liSDDR1* on chromosome b. **c** *liPAL* on chromosome c, d, e, f. M DL2000

fused together. Sometimes, the stamens deviated to petal-like organs in few flowers (Fig. 4g). In general, these lines gave low seed sets by self-pollination, but much

**Table 3** SSR markers specific to more than one chromosome of *I. indigotica*

Locus	Chromosome						
	a	b	c	d	e	f	g
9a	–	–	+	+	–	+	–
9b	–	–	+	–	+	–	–
59b	–	–	–	+	–	+	+
61b	–	–	+	–	+	–	–
90	+	–	+	–	–	–	+
149	+	–	–	+	–	–	–

Markers 9, 59 and 61 have two loci a and b. Markers 59a and 61a are specific to chromosome f. See Table 2

+ Marker present, – marker absent

higher seeding rates after pollination by *B. napus*, except Ma with fewer seeds in each pod (Table 4).

Location of *I. indigotica* genes in MAALs

PCR results showed that the markers *liMYB* and *liCDPK* were specific to Ma (Fig. 5a; Table 2), *liSDDR1* was related to Mb (Fig. 5b). The *liPAL* was amplified in four MAALs (Mc, Md, Me and Mf), respectively, showing that the gene had four homologous copies in *I. indigotica* (Fig. 5c; Table 2). *liCOMT* was lost in BC<sub>1</sub> plants and all

**Table 2** Cytological and molecular markers of MAALs

MAALs	Number of plants	SSR markers			Gene markers	rDNA
		Woad-specific SSR markers	Polymorphism markers	Total number		
Ma	2	1, 2, 10, 22, 54, 55, 70, 73, 77, 82, 87, 93, 140, 142, 153, 154, 159, 167, 180, 185a, 188, 192	19, 36, 57, 95, 114, 117, 125, 171, 187	31	<i>liCDPK</i> , <i>liMYB</i>	–
Mb	2	20, 37, 38, 72, 80, 110, 113, 123, 128, 130, 136, 137, 148, 156, 170, 175, 177, 179	21, 25, 45, 50, 69, 84, 91, 126, 141, 172, 176	29	<i>liSDDR1</i>	–
Mc	3	5, 6, 8, 12a, 14, 16, 51, 83, 85, 88, 99, 103, 107, 112, 129, 139, 152, 186b	169	19	<i>liPAL</i>	–
Md	6	13, 17, 26, 27, 34, 43, 49, 52, 62, 64, 65, 78, 92, 101, 104, 116, 144, 145, 151, 168, 173, 181, 184, 185b, 186c	11, 32, 98, 131, 158, 166, 190	31	<i>liPAL</i>	–
Me	5	4, 12b, 30, 35, 40, 46, 47, 63b, 76, 79, 96, 102, 108, 111, 132, 138, 146, 161, 174, 186d, 191	39, 75, 134, 147	25	<i>liPAL</i>	–
Mf	13	3, 15, 24, 29, 53, 56, 58, 59a, 60, 61a, 63a, 66, 67, 68, 89, 97, 100, 105, 109, 115, 118, 119, 127, 143, 150, 155, 162, 186a	41, 74, 157, 189	32	<i>liPAL</i>	5S rDNA
Mg	1	7, 23, 28, 31, 33, 44, 81, 94, 106, 122, 124, 135, 164, 165, 182	42, 48, 71, 120, 133, 163, 178, 183	23	–	45S rDNA

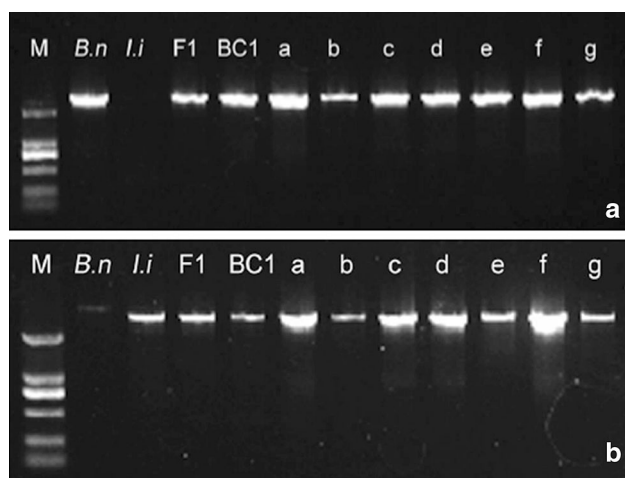
Markers 12, 59, 61, 63 and 185 have two loci a, b; 186 has four loci a, b, c, d. Marker 59b is specific to chromosome d, f and g. Marker 61b is specific to chromosome c and e. See Table 3

**Table 4** Seed sets of MAALs

Line	Thousand seed weight (g)	Seeds per pod <sup>a</sup>	Pod length <sup>a</sup> (cm)	Pod-setting rate <sup>a</sup> (%)
<i>B. napus</i>	4.13	19.8	7.74	100
Ma	4.25	1.8	5.00	76.92
Mb	4.59	5.2	3.72	100
Mc	3.11	16.6	5.44	100
Md	4.66	13.2	6.58	100
Me	4.86	16.0	5.76	100
Mf	3.89	19.8	5.90	100
Mg	–	–	–	–

–, Data lost, for siliques shattered before investigation

<sup>a</sup> Ten siliques per plant were analyzed



**Fig. 6** MtDNA analysis using primers of mitochondrial genes. The profiles are from *cox2-2* (a) and *rps3* (b). M DL2000

MAALS, probably because of a deletion of the genomic region containing this gene in the haploidy woad genome of the respective BC<sub>1</sub> plant (AACCI) used for backcrossing with *B. napus*. The marker *IiLTK* and *IiCCoAoMT* did not show polymorphism between *I. indigotica* and *B. napus*.

#### Recombinant mitochondrial genome in MAALs

Using a primer pair of *cox2-2*, one fragment of approximately 2,300 bp specific to *B. napus* was detected in F<sub>1</sub>, BC<sub>1</sub> and MAALs (Fig. 6a). The *cox1* in F<sub>1</sub> and backcross progenies was the same as *B. napus*, but the *orf 261* specific to *B. napus* was lost in F<sub>1</sub> and backcross progenies. As to *rps3*, a larger and weaker band was amplified in *B. napus* by the primer pair designed based on the rapeseed mtDNA sequence, but the smaller and brighter bands in F<sub>1</sub>, BC<sub>1</sub> and

all MAALs which were the same as the one in *I. indigotica* (Fig. 6b). This suggested not only the sequence differences at this locus between *B. napus* and *I. indigotica*, but also the genetic recombination between the loci of these two species. No polymorphisms between *B. napus* and *I. indigotica* were detected for other 17 mitochondrial genes. These results revealed that the hybrid maintained the recombinant mitochondrial genome of two parents and was maternally transmitted to MAALs.

#### Discussion

As the intertribal sexual crosses between *B. napus* and *I. indigotica* only produced the unclassical hybrids with the *I. indigotica* chromosomes largely eliminated (Tu et al. 2010), their somatic hybridizations were made and the hybrids with the sum of the parental chromosome complements and particularly the partial fertility were successfully obtained (Du et al. 2009), which makes it feasible to dissect the genome of the important medicinal plant by developing the complete set of MAALs in this study. Using the characteristics in phenotype, chromosomes and DNA sequences specific for *I. indigotica*, the large number of monosomic addition plants derived from the backcrossing progenies of several generations was put into seven groups hosting one of the seven *I. indigotica* chromosomes in the background of *B. napus* (Table 2).

The distinct phenotypic traits from the donor parent are most easily recognizable for the identification of MAALs, as shown by the intergeneric or even intertribal additions of *B. napus* with individual chromosomes from *Orychophragmus violaceus* (Ding et al. 2013). But *I. indigotica* has few phenotypic characters which are expressed and specific for particular MAALs, except for the brown color of anthers, though it belongs to different tribe. Cytologically, the rDNA loci (5S and 18S-5.8S-25S rDNA) and their chromosomal localization have been shown to be suitable for the karyotypic characterization of species with small and similarly sized chromosomes, and thus for the recognition of certain MAALs carrying such chromosomes (Table 2; Fig. 2c, d). The 5S rDNA loci are usually localized within pericentromeric heterochromatic regions in *Brassica* species (Lim et al. 2005; Koo et al. 2011) and *I. indigotica* (this study), and most 45S rDNA occupied terminal positions in *Brassicaceae* species (Ali et al. 2005), but some adjacent to terminal positions (Yang and Li 2011; this study), or fewer pericentromeric regions (Mandáková and Lysak 2008). Interestingly, the 45S rDNA loci on three *O. violaceus* chromosomes were active and gave differential amounts of rRNA transcripts in the *B. napus* background, while they were completely dominant over those of *B. napus* in the hybrids of these two species (Ge et al.



2009). However, the expression of rRNA genes from *I. indigotica* was not detected in the somatic hybrids with *B. napus* (data not shown), and also most likely not in the addition line Mg (Table 2). This also indicated the variable expression dominance of 45S rDNA in the interspecific hybrids and allopolyploids.

A complete series of MAALs enables markers and genes to be allocated to donor chromosomes on the basis of the presence/absence of markers and genes on the chromosomes added to the recipient genome (Cho et al. 2006). In this study, 184 SSR markers and 4 genes were allocated to the seven chromosomes of *I. indigotica* (Table 2). On the other hand, these unique materials could confirm interchromosomal duplicate loci. For example, PAL was encoded by a multi-gene family with four members in *Arabidopsis* (Raes et al. 2003). In *I. indigotica*, *IiPAL* had at least four homologous copies located on chromosomes c, d, e and f, respectively (Fig. 5c). As no genetic map has now been available for woad, these SSR markers would facilitate the construction of its genetic mapping. The production of radiation hybrid lines from monosomic additions can provide valuable tools for the physical mapping of alien chromosomes (Kynast et al. 2004).

The two tribes *Isatideae* and *Brassicaceae* which were put in one of the three lineages for *Brassicaceae* (Beilstein et al. 2006) descended from a common ancestral karyotype, proto-Calepineae karyotype (PCK,  $n = 7$ ) (Mandáková and Lysak 2008), which shared two homologous ancestral segments. SSR markers and *IiPAL* showed different homeology within *I. indigotica* chromosomes (Table 3; Fig. 5c), and the higher degree of homeology existed between chromosomes c and e, d and f, but lower or no homeology between chromosome b and others.

The mitochondrial genome analysis showed that the recombination of parental mtDNA which occurred in the somatic hybrid was maintained in the MAALs derived, which might be partially responsible for the male sterility shown as the carpeloid stamens in these MAALs and even the euploid *B. napus* progeny with such cytoplasm (Fig. 6), besides other factors affecting the development of floral organs. The homeotic conversion of stamens with carpeloid structures was also presented by monosomic rapeseed (*B. napus*)-radish additions with *Raphanus* cytoplasm (Budahn et al. 2008) and *Brassica napus* lines with rearranged *Arabidopsis* mtDNA (Leino et al. 2003). This is probably associated with the reshuffling mitochondrial dysfunction and nuclear–mitochondrial interactions that affect the development of floral organs. The expression of genes responsible for stamen formation depended on proper mitochondrial function and correct nuclear–mitochondrial interaction (Teixeira et al. 2005; Carlsson et al. 2007). The male sterile phenotype was present in all but

one addition lines and their euploid offspring. The normal development of stamens in the MAAL (Me) indicated that the alien chromosome carried the gene(s) for fertility restoration which could be introgressed into *B. napus* to establish new cytoplasmic male sterility system for hybrid production.

Despite the usage of large amount with an excellent safety record as a traditional Chinese medicine plant, the effective ingredients of *I. indigotica* against viruses and bacteria are still elusive. MAALs obtained here provide a feasible strategy to identify and estimate overall bioactive compounds in each one of the MAALs by integrated analysis of the metabolome and transcriptome. Preliminary result indicates that an addition plant contained chromosome d and e of *I. indigotica* has obvious antiviral activity (unpublished data). These MAALs are greatly useful for understanding the biosynthesis pathway of plant secondary metabolites and the related key genes. The addition lines also could serve to detect the genes for plant resistances to *S. sclerotiorum* and TMV.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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