Physiological and genetic analysis of CO$_2$-induced breakdown of self-incompatibility in *Brassica rapa*

Xintian Lao$^{1}$, Keita Suwabe$^{2}$, Satoshi Niikura$^{3}$, Mitsuru Kakita$^{1}$, Megumi Iwano$^{1}$ and Seiji Takayama$^{1,*}$

$^{1}$ Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan
$^{2}$ Graduate School of Bioresources, Mie University, Tsu 514-8507, Japan
$^{3}$ Tohoku Seed Co. Ltd, Utsunomiya 321-3232, Japan

* To whom correspondence should be addressed. E-mail: takayama@bs.naist.jp

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Abstract

Self-incompatibility (SI) of the Brassicaceae family can be overcome by CO$_2$ gas treatment. This method has been used for decades as an effective means to obtain a large amount of inbred seeds which can then be used for F$_1$ hybrid seed production; however, the molecular mechanism by which CO$_2$ alters the SI pathway has not been elucidated. In this study, to obtain new insights into the mechanism of CO$_2$-induced SI breakdown, the focus was on two inbred lines of *Brassica rapa* (syn. *campestris*) with different CO$_2$ sensitivity. Physiological examination using X-ray microanalysis suggested that SI breakdown in the CO$_2$-sensitive line was accompanied by a significant accumulation of calcium at the pollen–stigma interface. Pre-treatment of pollen or pistil with CO$_2$ gas before pollination showed no effect on the SI reaction, suggesting that some physiological process after pollination is necessary for SI to be overcome. Genetic analyses using F$_1$ progeny of a CO$_2$-sensitive×CO$_2$-insensitive cross suggested that CO$_2$ sensitivity is a semi-dominant trait in these lines. Analysis of F$_2$ progeny suggested that CO$_2$ sensitivity could be a quantitative trait, which is controlled by more than one gene. Quantitative trait locus (QTL) analyses identified two major loci, *BrSIO1* and *BrSIO2*, which work additively in overcoming SI during CO$_2$ treatment. No QTL was detected at the loci previously shown to affect SI stability, suggesting that CO$_2$ sensitivity is determined by novel genes. The QTL data presented here should be useful for determining the responsible genes, and for the marker-assisted selection of desirable parental lines with stable but CO$_2$-sensitive SI in F$_1$ hybrid breeding.

Key words: *Brassica rapa*, calcium, CO$_2$, F$_1$, hybrid, QTL, self-incompatibility.

Introduction

Self-incompatibility (SI) is a widespread genetic system in many flowering plants which serves to prevent self-fertilization and maintain genetic diversity. It is based on self/non-self pollen–pistil recognition interactions followed by inhibition of self-pollen hydration, germination, or pollen tube growth. In the Brassicaceae, SI is sporophytically controlled by a multiallelic locus termed the S locus (Bateman, 1955). Male and female determinants have been identified as SP11/SCR (S-locus protein 11/S-locus cysteine-rich) (Schopfer et al., 1999; Takayama et al., 2000) and SRK (S receptor kinase) (Takasaki et al., 2000), respectively. When a compatible pollen grain lands on the stigma, it swells and a pollen tube is allowed to grow, whereas when self-pollen attaches to the stigma, SP11/SCR binds specifically to the extracellular domain of SRK of the same S-haplotype (Takayama et al., 2001), which triggers an SI signalling pathway to reject self-pollen. Another stigmatically expressed gene located at the S locus, S locus glycoprotein (SLG) (Nasrallah et al., 1987; Takayama et al., 1987), has been shown to enhance the recognition process between self-pollen and stigma (Takasaki et al., 2000).
et al., 2000); however, this function of SLG remains controversial (Silva et al., 2001). A recent study suggested that the plants in the Brassicaceae genus Leavenworthia use paralogous SRK and SP11/SCR genes, Lal2 (Leavenworthia alabamica SRK-related 2) and SCR1 (SCR-like), for self/non-self recognition in SI, but the function of their orthologues in other Brassicaceae genera also remains unknown (Chanthra et al., 2013).

Many studies focusing on the downstream components involved in this type of SI signalling pathway have been performed and, thus far, two components have been identified as positive effectors. ARC1 (arm repeat containing 1) was identified by a yeast two-hybrid screen using the kinase domain of SRK as the bait (Gu et al., 1998; Stone et al., 1999). ARC1 is a U-box protein with E3 ubiquitin ligase activity (Stone et al., 2003), and has been shown to interact with Exo70A1, a putative component of the exocyst complex required for compatible pollination (Samuel et al., 2009). MLPK (M-locus protein kinase) was identified by positional cloning as the gene responsible for the self-compatible mutation of Brassica rapa var. Yellow sarson (Murase et al., 2004). MLPK is a membrane-anchored cytoplasmic protein kinase and interacts directly with SRK to transduce SI signalling (Kakita et al., 2007). However, the importance of these two components in the Brassicaceae SI mechanism remains controversial (Kitashiba et al., 2011; Indriolo et al., 2012).

In the Brassicaceae, it has been known that SI can be overcome under some physiological and environmental conditions such as plant age (Ockendon, 1978; Horisaki and Niikura, 2008), stigmatic chemical treatments (e.g. ether, KOH, and NaCl) (Tatebe, 1968; Tao and Yong, 1986; Monterio and Gabelman, 1988), and high temperature (Matsubara, 1980; Okazaki and Hinata, 1987). CO₂ gas (3–5%) treatment (Nakanishi et al., 1969) is the most effective way to overcome SI. Today, most cultivated lines of crucifer vegetables, such as cabbage, broccoli, Chinese cabbage, and radish are F₁ hybrids whose seeds are produced by mix-planting two self-incompatible inbred parental lines. In this economical F₁ hybrid breeding system, CO₂ gas treatment has been used to suppress SI and allow self-fertilization, thereby providing large-scale seed propagation of parental lines. This method has been used all over the world for many years, but the molecular mechanism leading to SI breakdown by CO₂ gas treatment is entirely unknown.

Previous studies suggested that not all lines respond equally to CO₂, and there are variations in SI response to CO₂ (CO₂ sensitivity) in the Brassicaceae (Nakanishi and Hinata, 1973; Niikura and Matsuura, 2000). Preliminary genetic analysis using lines with different CO₂ sensitivity in radish (Raphanus sativus) suggested that high CO₂ sensitivity was controlled by a recessive gene independent of the S-locus (Niikura and Matsuura, 2000). In another genetic analysis using CO₂-sensitive and CO₂-insensitive lines of Chinese cabbage (B. rapa), high CO₂ sensitivity was suggested to be controlled by a dominant gene (Hyun et al., 2007); however, no responsible genes have been identified from these studies so far.

In this study, new inbred lines of B. rapa with different CO₂ sensitivity, a CO₂-sensitive line (HA-11621) and a CO₂-insensitive line (HA-11623), were selected and analysed. X-ray microanalysis suggested that SI breakdown in the CO₂-sensitive line was accompanied by significant calcium accumulation at the pollen–stigma interface. Independent pre-treatment of pollen or pistil with CO₂ gas before pollination showed no effect on the SI reaction, suggesting that some physiological process that occurs after pollination is necessary for SI to be overcome. Genetic analyses using F₁ and F₂ progeny of a CO₂-sensitive×CO₂-insensitive cross suggested that CO₂ sensitivity is a semi-dominant and quantitative trait. Furthermore, quantitative trait locus (QTL) analyses identified two major responsible loci, BrSIO1 and BrSIO2, which function additively in overcoming SI during CO₂ treatment.

**Materials and methods**

**Plant materials**

Two inbred lines of B. rapa (2n=20), a CO₂-sensitive line (HA-11621) and a CO₂-insensitive line (HA-11623), were established at Tohoku Seed Co., Ltd, and grown in the greenhouse with 16 h light and 8 h dark conditions at 20 °C. Both lines show stable SI under normal (open-air) condition but have different CO₂ sensitivity; SI in HA-11621 breaks down following treatment with 4.5% CO₂ whereas SI in HA-11623 is unaffected. HA-11621 and HA-11623 are reciprocally compatible, and their F₁ progeny were obtained under normal conditions. Buds (1–2 d before flowering) from a randomly chosen F₁ were used for F₂ production. Young petals and stamens were removed from the bud, and the immature pistil was pollinated with pollen grains from mature flowers of the same plant (bud pollination). Pollinated pistil was then covered with a paper bag for 3 d and seeds from the pistil were harvested. More than 20 pistils were pollinated, and harvested seeds were used as the F₂ population. A total of 110 F₂ plants were used for phenotypic and genetic analysis.

**Cryo-scanning electron microscopy and energy-dispersive X-ray analysis**

Flowers were self- or cross-pollinated and incubated for 1.5 h with or without 4.5% CO₂ gas. These pollinated and non-pollinated pistils were submerged in liquid nitrogen slush and frozen under vacuum. While under vacuum, the sample was transferred to the microscope cryo stage (ALTO 1000, Gatan), and then the stage temperature was increased to –95 °C to remove frost that had settled on top of the specimen as a result of condensation. When all surface frost had been removed by sublimation, as verified by electron microscopy, the temperature was reduced to –140 °C. Imaging was performed using an ETD (Everhart–Thornley detector) by Quant 250 scanning electron microscopy (FEI). The chamber pressure was 30 Pa and the accelerating voltage was 15 kV. EDX (energy-dispersive X-ray spectroscopy) analysis of the element assay was performed on selected papilla cells using INCA X-ray analysis software (Oxford Instruments, www.oxinst.com/Pages/home.aspx, last accessed 14 December 2013), with the detector’s processing time set at 2. X-ray data were collected with 4.5 nA probe current for 2 min. Each 2–3 pistils were used in one experiment and three individual experimental sets were performed.

**Evaluation of reaction level of SI to CO₂ (RLSICO₂)**

Three to five flowers were cut at the peduncle and stood on a 1% (w/v) solid agar plate. Flowers were self-pollinated, placed into a CO₂ incubator, and treated with 4.5% CO₂ for 4 h at 23 °C. After 1 d at room temperature, pistils were fixed in ethanol/acetic acid (3:1) overnight, softened in 1 N NaOH at 60 °C for 2 h, then stained with 0.01% (w/v) decolorized aniline blue in 2% K₃PO₄ for 6 h. Pollen tube behaviour was observed under a fluorescence microscope (Axioskop 2, Zeiss). CO₂ sensitivity was measured using the RLSICO₂ index.
RLSICO$_2$ was classified into five categories, based on the number of pollen tubes penetrating into the stigma: 1, 0 pollen tubes; 2, 1–5 pollen tubes; 3, 6–15 pollen tubes; 4, 16–30 pollen tubes; and 5, >30 pollen tubes. Three replicates were performed on each plant on different days. Non-CO$_2$-treated self-pollinated flowers were used as controls. In all cases, no pollen tubes penetrated into the control stigmas.

### Genotyping of S-haplotypes

S-haplotypes of *B. rapa* were identified using primers PS5 (5′-ATGAAAAAGGCTAAGAAAGAACCTA-3′) and PS15 (5′-CCG TGTTTTATTATAGGAGAAAGCT-3′) (Nishio et al., 1996) to amplify a fragment of the *SLG* gene. PCR-RFLP (restriction fragment length polymorphism) was used to distinguish the two *S*-haplotypes based on differential digest with the restriction enzyme *KpnI* (TaKaRa, Japan). Digested DNA was electrophoresed on a 1.5% agarose gel.

### Molecular markers and detection of DNA polymorphism

To screen for markers that show polymorphism between *B. rapa* lines, primers specific for simple sequence repeat (SSR) markers from different sources [UK, prefixes Ra, Na, Ol, and ENA (Lowe et al., 2004; http://brassica.bbsrc.ac.uk); Japan, prefixes BRMS, KB, and EST (Suwabe et al., 2002, 2004, 2006; http://vegmarks.nivot.africgo.jp, NVTS); China, prefix sau, um (Ge et al., 2011); and Korea, prefix AMCP (Ramcherry et al., 2011)] were used. SSR, RFLP, and insertion/deletion (Indel) markers (prefixes XT and Bra) were also designed based on the *Brassica* database (BRAD) (http://brassicadb.org/brad/), last accessed 14 December 2013 (Supplementary Table S1 available at JXB online).

Total genomic DNA was extracted from young leaves of two parental lines and F$_1$ progeny using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). DNA polymorphism analysis with SSRs was carried out using PCR with fluorescent dyes, performed according to Suwabe et al. (2008) with some modifications. The M13 (–21) universal primer sequence (18bp) was fused to the 5′ end of the original forward primer, and the M13 (–21) universal primer was labelled with 6-FAM, NED, VIC, or PET fluorescent dye (Applied Biosystems, CA, USA). PCRs were performed in a 10 μl reaction volume containing 10 ng of template DNA, 4.7 μM of labelled M13 (–21) universal primer and reverse primer, 0.3 μM of forward primer, 1× PCR buffer, 1× dNTP, 1× MgCl$_2$, and 0.5 U of Taq (TOYOBO, Japan). Conditions for PCR were as follows: initial denaturation was carried out at 94 °C for 3 min followed by 37 cycles at 94 °C for 30 s, 55 °C (slope of 0.5 °C s$^{-1}$) for 30 s, 72 °C (slope of 0.5 °C s$^{-1}$) for 30 s, and a final extension at 72 °C for 4 min. A 1 μl aliquot of 50-fold diluted PCR product was added to 8.9 μl of Hi-Di™ Formamide and 0.1 μl of GeneScan™ 600 LIZ™ Size Standard (Applied Biosystems, USA) and applied to an ABI 3730 DNA Analyzer (Applied Biosystems). Data were analysed using ABI GeneMapper® software.

For polymorphism analysis with RFLP and InDel markers, PCR was carried out in a 10 μl reaction volume with 5 pmol of forward and reverse primers instead of fluorescent dyes. For RFLP markers, amplified fragments were digested using restriction enzymes for 1 h. Fragments of digested DNA were separated on a 2–4% agarose gel.

### Linkage map construction and QTL analysis

A genetic map was constructed using JoinMap® version 4 (Van Ooijen, 2006) utilizing the double pseudo-testcross strategy with a log$_{10}$ of odds (LOD) threshold of 6.0 for linkage group identification. The best marker order was calculated with the regression mapping algorithm, and marker order was retained from the first round only. Map distance units in centiMorgans (cM) were converted from recombination frequencies using the Kosambi mapping function (Kosambi, 1944). Interval mapping (IM) was performed to identify putative QTLs using the established linkage map and the observed phenotypic traits. This method was run using MapQTL® version 6 (Van Ooijen, 2009). With this software, a $P < 0.05$ LOD score significance threshold was calculated by creating a group-wide distribution of the data based on a 1000 permutation test. LOD peaks were used to estimate the position of QTLs on the map.

### Statistical analysis

Box plots were prepared by Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd, Japan) to compare the phenotypic difference, as this plot type gives a good sense of environmental data distribution (Upton and Cook, 1996). Kruskal–Wallis analysis of variance (ANOVA) by ranks was used between paired comparisons of markers to examine marker association.

### Results

#### Phenotypic analysis of *B. rapa* lines in response to CO$_2$ treatment

Two inbred lines of *B. rapa* with different CO$_2$ sensitivity, a CO$_2$-sensitive line (HA-11621) and a CO$_2$-insensitive line (HA-11623), were used in this study. Flowers were self-pollinated by hand pollination and incubated in a CO$_2$ incubator (4.5% CO$_2$) for 4 h. Both lines were self-incompatible under normal conditions (control) (Fig. 1A, B), whereas they showed significantly different responses to CO$_2$ gas treatment (Fig. 1C, D). Specifically, in the CO$_2$-sensitive line, many pollen tubes were seen to penetrate into papilla cells after CO$_2$ treatment. This pollination test confirmed that the CO$_2$-sensitive line had high sensitivity to CO$_2$ while the CO$_2$-insensitive line hardly responded to 4.5% CO$_2$. Cross-pollination was performed as a positive control (Fig. 1E, F).

#### Physiological changes in papilla cells after CO$_2$ treatment

Previous work using X-ray microanalysis has revealed the accumulation of calcium at the stigmatic surface following compatible cross-pollination in *Brassica oleracea* (Ellem and Dickinson, 1999). X-ray mapping strongly indicated that a high concentration of calcium was localized at the points where the pollen grain made contact with the surface of the stigmatic papilla cell. The calcium accumulation was also observed in *B. rapa*, especially in compatible pollination (Iwano et al., 1999). To examine the physiological effect of CO$_2$ on pollination reactions, this calcium accumulation was examined by cryo-scanning electron microscopy fitted with an X-ray microanalysis system. When the CO$_2$-sensitive line was cross-pollinated with the CO$_2$-insensitive line, pollen grain hydrated and the pollen tube penetrated into the papilla cell, but when CO$_2$-sensitive and CO$_2$-insensitive lines were self-pollinated under normal conditions (without CO$_2$ treatment), few pollen grains hydrated, and no pollen tube germination was observed in either line (Fig. 2A, upper panel). After CO$_2$ treatment, the cross-pollinated pollen grains did not show a significant difference, neither did the self-pollinated CO$_2$-insensitive line. However, the self-pollinated CO$_2$-sensitive line showed obvious changes: pollen hydration and germination were observed under CO$_2$ treatment (Fig. 2A, lower panel).
Fig. 1. Phenotype of inbred Brassica rapa lines used in this study. (A, B) Pollen tube behaviour after self-pollination of CO$_2$-sensitive (HA-11621) and CO$_2$-insensitive (HA-11623) lines under normal conditions (without CO$_2$ treatment). No penetrated or elongated pollen tubes are observed in either line. (C, D) Pollen tube behaviour after self-pollination of CO$_2$-sensitive and CO$_2$-insensitive lines under 4.5% CO$_2$ gas treatment. Pollen tubes could penetrate into the stigma and elongate through the style only in the CO$_2$-sensitive line. (E, F) Cross-pollination as a positive control; the arrow shows pollen tubes which have penetrated. Pt, pollen tubes. Bar=1000 μm.

The efficiency of CO$_2$ treatment

A previous study suggested that the effect of CO$_2$ on SI breakdown depends on the timing of treatment (Nakanishi and Hinata, 1973). In the CO$_2$-sensitive line, when self-pollinated flowers were immediately treated with 4.5% CO$_2$ for 4h, SI could be overcome, and typically >10 pollen tubes penetrated into the stigma (Fig. 3A). When CO$_2$ treatment started 3h after self-pollination, SI could still be overcome (Fig. 3B). However, when CO$_2$ treatment started 6h after self-pollination, the number of penetrating pollen tubes was decreased (Fig. 3C). These results indicate that self-pollination inhibition in SI is biostatic, as previously suggested (Sarker et al., 1988), and can be reversed at least at 3h after pollination. However, at 6h after pollination, SI inhibition enters an irreversible phase that cannot be overcome by CO$_2$ treatment.

Next, in order to narrow down the stage of SI affected by CO$_2$ treatment, experiments with high CO$_2$-pre-treated pollen or pistil from the CO$_2$-sensitive line were performed (Fig. 4). SI could not be overcome by pre-treatment of either tissue, and no pollen tube penetration could be observed even when both pollen and pistil were treated separately prior to pollination (Fig. 4). SI could be overcome only when the self-pollinated pistil was treated with high CO$_2$. These results suggest that some post-pollination physiological process is affected by high CO$_2$, in the process of SI breakdown.

S-allele characterization and phenotype of CO$_2$ sensitivity in $F_1$ and $F_2$

The $S$-haplotypes of the two parental inbred lines were first determined by amplifying their SLG genes (Nishio et al., 1996). The sequence data suggested that the $S$-haplotypes of the CO$_2$-sensitive and CO$_2$-insensitive lines were $S_{55}S_{55}$ and $S_{46}S_{46}$, respectively. To dissect genetically the gene(s) that determines sensitivity to CO$_2$ treatment, six $F_1$ plants
Fig. 2. Electron micrographs and X-ray microanalysis of B. rapa. (A) Cryo-scanning electron micrographs of pollinated papilla cells were taken 1.5 h after pollination. Representative examples of the cross-pollinated (left column) and self-pollinated (middle column) CO₂-sensitive line, and the self-pollinated (right column) CO₂-insensitive line are shown. Without CO₂ treatment (upper panels), only cross-pollen is accepted, and self-pollen grains maintain a spheroid shape without swelling in both lines. With 4.5% CO₂ gas treatment (lower panels), pollen grains swell and germinate in the CO₂-sensitive line (lower middle) but not in the CO₂-insensitive line (lower right). Bar=25 μm. (B) Representative examples of energy-dispersive X-ray spectra of non-pollinated and pollinated papilla cell surfaces. Scanning positions for X-ray analyses are indicated by asterisks in (A). The emissions of Al-Kα, P-Kα, S-Kα, K-Kα, and Ca-Kα were detected at the papilla cell surfaces, and the intensity of Ca emission was increased after cross-pollination (left column). The increase of Ca emission was also observed after self-pollination with 4.5% CO₂ gas treatment in the CO₂-sensitive line (middle column) but not in the CO₂-insensitive line (right column). These spectrum patterns are reproducible in three individual experiment sets. The emission of Al-Kα is mostly derived from the stub that held the samples.
(S₄₆S₅₅) were produced by crossing CO₂-sensitive and CO₂-insensitive lines. These F₁ plants exhibited an intermediate CO₂ sensitivity phenotype where self-pollen tubes penetrated into the stigma under high CO₂ treatment but there were fewer penetrating pollen tubes than observed in a self-pollination of the CO₂-sensitive parent. An F₂ population of 110 individuals derived from a bud-pollinated F₁ plant was made and used for further genetic analyses of the CO₂ sensitivity. F₂ individuals were genotyped using PCR-RFLP to distinguish SLG alleles (Supplementary Fig. S1 at JXB online). S₅₅- and S₄₆-haplotypes were segregated in the F₂ population according to Mendelian transmission (Supplementary Table S2). Pollen tube behaviour after CO₂ treatment varied among individuals and, in order to quantify the strength of CO₂ sensitivity, the modified RLSICO₂ (reaction level of SI to CO₂) index was employed (Niikura and Matsuura, 2000), which calculates CO₂ sensitivity based on the number of penetrating pollen tubes after self-pollination under high CO₂ conditions (see the Materials and methods). The RLSICO₂ of 110 F₂ individuals is presented in Supplementary Fig. S2, and the summarized box-plot data are shown in Fig. 5, together with the RLSICO₂ of F₁ and the parental inbred lines. F₁ had an RLSICO₂ score intermediate to the two parental lines, suggesting that the high CO₂ sensitivity is a semi-dominant (incompletely dominant) trait in these inbred lines. Furthermore, the RLSICO₂ of F₂ individuals was continuously distributed and did not follow a simple one-locus biallelic Mendelian distribution (Supplementary Fig. S2). These results suggest that CO₂ sensitivity in the inbred lines used here could be a quantitative trait which is controlled by more than one gene.

Relationship between S-alleles and CO₂ sensitivity
To investigate whether CO₂ sensitivity is related to S-haplotypes, the 110 F₂ individuals were grouped into three
genotypes \((S_{45}S_{55}, S_{46}S_{46}, \text{and } S_{46}S_{55})\). The RLSICO\(_2\) of each group is shown in Fig. 5. In the three F\(_2\) groups, RLSICO\(_2\) scores were distributed from 1 to 5, and interquartile ranges overlapped, indicating that CO\(_2\) sensitivity is not linked to the S-locus in these two lines.

**Reproductive tissue controlling CO\(_2\) sensitivity**

From the F\(_2\) population, two \(S_{46}S_{46}\) homozygotes with different RLSICO\(_2\) were selected: F\(_{2}\)-16, a CO\(_2\)-insensitive line (RLSICO\(_2\)=1 ± 0.0); and F\(_{2}\)-26, a CO\(_2\)-sensitive line (RLSICO\(_2\)=4.15 ± 0.53). These two lines were used to examine the reproductive tissue controlling CO\(_2\) sensitivity. Reciprocal crosses were performed between these two SI lines with or without high CO\(_2\) gas treatment. Because all crosses under normal conditions (without CO\(_2\) treatment) were incompatible, only data from crosses performed in the high CO\(_2\) condition are shown in Fig. 6. The cross between CO\(_2\)-sensitive F\(_2\)-26 pistil and CO\(_2\)-insensitive F\(_2\)-16 pollen was CO\(_2\) sensitive, showing many penetrating pollen tubes under high CO\(_2\). On the other hand, the cross between CO\(_2\)-insensitive F\(_2\)-16 pistil and CO\(_2\)-sensitive F\(_2\)-26 pollen was CO\(_2\) insensitive, showing no penetrating pollen tubes even under high CO\(_2\). These results suggest that CO\(_2\) sensitivity is controlled by genes expressed in the female tissue (pistil).

**Marker analysis and construction of a linkage map**

In order to map QTLs that determine CO\(_2\) sensitivity, a linkage map was constructed for this F\(_2\) population. A total of 911 different genetic markers were examined in the two parental lines. To clarify the relationship between previously identified SI-related genes and CO\(_2\) sensitivity, SLG (an S-locus marker), MLPK, and ARCl were also selected. Though a very low level of polymorphism (14.7%) was detected for all types of markers, 123 polymorphic markers were selected, which include 113 SSRs, five RFLPs, and five InDel markers. These 123 markers were used for linkage mapping, and generated 10 linkage groups (A01–A10) at a LOD threshold value of 6.0 (Fig. 7). The total length of the map was 947.5 cM, and the length of the linkage groups ranged from 63.2 cM (A10) to 168.4 cM (A03). The distance between markers varied from 0 to 29.3 cM, with an average interval of 7.7 cM. SLG, MLPK, and ARCl were mapped to A07, A03, and A04, respectively, which is consistent with previous reports (Ajisaka et al., 2001; Hatakeyama et al., 2010).

**QTL analysis and association of markers with high CO\(_2\) sensitivity**

Using the constructed linkage map, QTLs responsible for high CO\(_2\) sensitivity were analysed. Three QTLs were identified on linkage groups A03 and A05 based on a LOD threshold of 3.40 (1000 permutation test, \(P < 0.05\)) (Fig. 8, Table 1). These QTLs are tentatively named Brassica rapa SI Overcome (BrSIO) 1–3, and these results further supported the prediction that CO\(_2\) sensitivity of SI is controlled by a polygenic system. BrSIO1 on A05 and BrSIO2 on A03 are two major QTLs that explained 19.3% and 19.0% of phenotypic variation, respectively. BrSIO3, located near BrSIO2, accounted for 14.5% of the variance (Table 1).

**Fig. 5.** Box plots of CO\(_2\) sensitivity phenotypes. Data show the distribution of RLSICO\(_2\) with 25th, 50th, and 75th percentiles (horizontal bars), interquartile ranges (columns), and 1.5 interquartile ranges (error bars) of RLSICO\(_2\) from six CO\(_2\)-insensitive F\(_2\) individuals, six F\(_1\) individuals (\(S_{46}S_{46}\)), and 110 F\(_2\) individuals (22 \(S_{46}S_{46}\), 64 \(S_{46}S_{55}\), and 24 \(S_{55}S_{55}\)). a indicates a significant difference \((P < 0.01)\) between CO\(_2\)-sensitive and CO\(_2\)-insensitive lines.

**Fig. 6.** Reciprocal crosses with CO\(_2\) treatment between two \(S_{46}\) homozygous individuals from the F\(_2\) population with different RLSICO\(_2\). (A) CO\(_2\)-sensitive F\(_2\) self-pollination. (B) CO\(_2\)-insensitive F\(_2\) self-pollination. (C) A CO\(_2\)-sensitive F\(_2\) pistil pollinated with pollen from a CO\(_2\)-insensitive F\(_2\). (D) A CO\(_2\)-insensitive F\(_2\) pistil pollinated with pollen from a CO\(_2\)-sensitive F\(_2\). Pt, pollen tubes. Bar=1000 μm. \(n=3\).
To examine the significance of these QTLs, F₂ progeny were classified into groups based on the genotypes of the linkage markers nearest these three newly identified loci, and the relationship of the loci to RLSICO₂ in individual plants was analysed using Kruskal–Wallis ANOVA by ranks (Table 2). Alleles from CO₂-sensitive (HA-11621) and CO₂-insensitive (HA-11623) lines are presented as S and I, respectively. Almost all classifications using the closest linkage markers showed a higher RLSICO₂ index in the SS group with significance at P < 0.01, except marker BRMS-114, which showed significance at P < 0.05.

Marker association was further examined with combinations of BrSIO₁ and BrSIO₂ since BrSIO₃ is a minor QTL closely linked to BrSIO₂, making it difficult to identify it as an independent QTL. F₂ progeny were classified into nine groups based on the genotypes of their closest linkage markers (Table 3). According to this classification, for example, the two above analysed S₄₆S₄₆ homozygous lines with different RLSICO₂, F₄₋₁₆ (CO₂-insensitive line) and F₂₋₂₆ (CO₂-sensitive line), were classified into group 8 and group 1, respectively. When groups with the same BrSIO₁ genotype were compared, the BrSIO₂ SS group showed a higher RLSICO₂ index compared with the BrSIO₂ II group. Likewise, when the groups with the same BrSIO₂ genotype were compared, the BrSIO₁ SS group showed a higher RLSICO₂ compared with the BrSIO₁ II group. Although the numbers of F₂ individuals in each group were rather low, significance (P < 0.05) was detected between groups 1 and 6, 2 and 6, and 2 and 8. These data suggest that BrSIO₁ and BrSIO₂ work additively in overcoming SI during CO₂ treatment in the CO₂-sensitive (HA-11621) line. No QTL was detected at genes known to affect SI stability (MLPK, ARC1, or the S-locus), indicating that CO₂ sensitivity is determined by novel genes in the experimental lines used here.

Associated gene prediction by in silico comparative mapping

Using the B. rapa genome sequence (Cheng et al., 2011), BrSIO₁ could be mapped to a 569 kb region flanked by InDel marker XT05-004 and SSR marker BRMS-034, and BrSIO₂ to a 1469 kb region flanked by SSR markers BRMS-042-2 and KBrH110117R. These two regions include 121 and 280 genes annotated in the Brassica database (BRAD), respectively (Supplementary Tables S3, S4 at JXB online). Comparison of the A. thaliana genome with the Brassicaceae genome (reviewed by Schranz et al., 2006) suggests that BrSIO₁ has synteny on A. thaliana chromosome 2 and BrSIO₂ has synteny on both chromosomes 3 and 4. It is assumed that these two QTLs do not have the same genetic origin and could be
CO₂-induced breakdown of Brassica self-incompatibility

**Fig. 8.** QTL analysis results. The solid line indicates the LOD score and the dotted line indicates the QTL threshold (LOD=3.4) determined using a 1000 permutation test (*P* < 0.05). The x-axis represents each linkage group (cM) and the y-axis indicates the QTL score. Two QTLs (*BrSIO2* and *3*) are detected in A03 and one in A05 (*BrSIO1*). Arrows show loci involved in SI stability reported by Hatakeyama et al. (2010).
two independent regions controlling high CO$_2$ sensitivity. Based on reciprocal cross results, the CO$_2$ sensitivity trait may be controlled by genes expressed in the female organ (Fig. 8). A total of 121 and 280 annotated genes in BrSIO1 and BrSIO2 have 103 and 243 homologues in A. thaliana, respectively, and 54 and 141 of these genes are expressed in A. thaliana pis (microarray data of carpel at stage 12, http://affymetrix.arabidopsis.info/narrays/search.pl?f1=1&s1=ATGE_37, last accessed 14 December 2013, Supplementary Tables S3, S4). Genes involved in related biological processes are often expressed cooperatively and their co-expression information is important for understanding biological systems (Eisen et al., 1998). ATTED-II (http://atted.jp/, last accessed 14 December 2013) is a gene co-expression database useful for identifying the potential partners working in the same biological processes (Obayashi et al., 2007). Co-expression analysis was performed using ATTED-II with these 195 genes and it was found that MAP kinase 6 (At2g43790 in BrSIO1) and ethylene overproducer 1 (At13g8511070 in BrSIO2) showed the strongest co-expression and calmodulin-like 41 (At3g50770 in BrSIO2) has weak co-expression with cytochrome c oxidase 10 (At2g44520 in BrSIO1) and beta glucosidase 28 (At2g44460 in BrSIO1). In addition to these co-expressed genes, these two regions encode highly homologous family member proteins, for example matrixin proteins (At2g45040 in BrSIO1 and At4g16640 in BrSIO2) and senescence-associated proteins (At2g44670 in BrSIO1 and At4g17670 in BrSIO2). All these can be candidate responsible genes, although the biological functions of these genes are mostly unknown.

**Discussion**

It has been >40 years since Nakanishi et al. (1969) first reported that SI could be overcome by CO$_2$ and Nakanishi and Hinata (1973) demonstrated the applicability of this technique to commercial use. Nowadays, seed companies have adopted this method to obtain inbred parental seeds of crucifer vegetables for large-scale commercial F$_1$ hybrid seed production. However, there is still very limited understanding of the mechanism by which SI is overcome.

Lee et al. (2001) showed a shrunken and distorted papilla cell surface in the CO$_2$-sensitive B. rapa cv. Hirasuka, and suggested that these structural changes could cause SI to be overcome. The cryo-scanning electron microscopy data reported here did not show any structural changes in CO$_2$-sensitive or CO$_2$-insensitive lines (Fig. 2A). Additionally, pretreatment of non-pollinated pistils with high CO$_2$ gas did not cause SI breakdown (Fig. 4). Therefore, a completely different SI breakdown mechanism must be present, at least in

### Table 1. Summary of CO$_2$ sensitivity QTLs

<table>
<thead>
<tr>
<th>QTL</th>
<th>LG</th>
<th>Closest marker</th>
<th>QTL peak (cM)$^a$</th>
<th>LOD</th>
<th>$R^2$</th>
<th>Additive effect$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrSIO1</td>
<td>A05</td>
<td>XT05-004</td>
<td>83.50</td>
<td>5.17</td>
<td>19.30</td>
<td>0.72</td>
</tr>
<tr>
<td>BrSIO2</td>
<td>A03</td>
<td>BRMS-042-2</td>
<td>60.87</td>
<td>4.46</td>
<td>19.00</td>
<td>0.69</td>
</tr>
<tr>
<td>BrSIO3</td>
<td>A03</td>
<td>KBrH110117R</td>
<td>41.25</td>
<td>3.76</td>
<td>14.50</td>
<td>0.65</td>
</tr>
</tbody>
</table>

$^a$ QTL peak position, detected by interval mapping, between two markers.
$^b$ Amount of phenotypic variation explained by the QTL.
$^c$ Additive effect of the CO$_2$-sensitive HA-11621 allele on RLSICO$_2$.

### Table 2. Statistical analysis of QTL effect

<table>
<thead>
<tr>
<th>QTL</th>
<th>BrSIO1</th>
<th>BrSIO2</th>
<th>BrSIO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker$^e$</td>
<td>XT05-004</td>
<td>BRMS-042-2</td>
<td>BRMS-042-2</td>
</tr>
<tr>
<td>SS</td>
<td>3.12 (27$^b$)</td>
<td>3.17 (24)</td>
<td>3.24 (20)</td>
</tr>
<tr>
<td>SI</td>
<td>2.34 (53)</td>
<td>2.36 (51)</td>
<td>2.42 (62)</td>
</tr>
<tr>
<td>II</td>
<td>2.02 (26)</td>
<td>1.93 (25$^c$)</td>
<td>2.06 (33)</td>
</tr>
</tbody>
</table>

$^a$ S, CO$_2$-sensitive HA-11621 allele; I, CO$_2$-insensitive HA-11623 allele.
$^b$ Individuals whose genotype was unidentified are excluded.
$^c$ Kruskal–Wallis analysis comparing phenotype between genotype groups with individuals in the same groups.
$^d$ Significance level: **P <0.01; *P <0.05.

### Table 3. QTL association for CO$_2$ sensitivity

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Marker$^f$</th>
<th>XT05-004</th>
<th>BRMS-042-2</th>
<th>No. of individuals$^g$</th>
<th>Mean RLSICO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SS</td>
<td>SS</td>
<td>5</td>
<td>3.86</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SS</td>
<td>SI</td>
<td>13</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>SS</td>
<td>II</td>
<td>5</td>
<td>2.40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SI</td>
<td>SS</td>
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<td>3.12</td>
<td></td>
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<tr>
<td>9</td>
<td>II</td>
<td>II</td>
<td>3</td>
<td>1.71</td>
<td></td>
</tr>
</tbody>
</table>

$^e$ S, CO$_2$-sensitive HA-11621 allele; I, CO$_2$-insensitive HA-11623 allele.
$^f$ Individuals whose genotype was unidentified are excluded.
$^g$ Significance level, *P <0.05.

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the CO₂-sensitive line. In contrast, massive Ca accumulation was observed at the pollen–stigma interface specifically in CO₂-sensitive plants under high CO₂ conditions (Fig. 2B). Brewbaker and Kwack (1963) were the first to describe the need for a high concentration of Ca²⁺ for pollen germination and pollen tube growth. The high concentration of Ca²⁺ could be needed for activating pectinase to loosen the papilla cell wall, allowing the pollen tube to penetrate (Black and Charlwood, 1995), or for keeping the pollen tube cell wall rigid enough not to burst (Hepler and Winship, 2010). Although causal relationships remain unclear, the data suggest that CO₂ treatment induces a certain compatible reaction leading to Ca²⁺ accumulation at the pollen–stigma interface.

To date, several genetic studies have been performed to understand the mechanism of SI breakdown for breeding purposes. Niikura and Matsuura (2002) reported that in Japanese radish high CO₂ sensitivity is controlled by a recessive gene that governs the construction and/or metabolism of the stigma, which reacts to CO₂ without any changes in gene expression. In contrast, Hyun et al. (2007) reported a dominant, S-haplotype-linked high CO₂ sensitivity phenotype in B. rapa. In contrast to these previous reports, F₁ plants had an intermediate CO₂ sensitivity and the F₂ population had a continuous frequency distribution of RLSICO₂ in the present study (Fig. 6). These results suggest that in the lines used for this study, CO₂ sensitivity is a quantitative trait which is controlled by more than one gene.

Genetic linkage maps based upon frequency of recombination in segregating populations are fundamental and powerful tools for associating phenotypic trait-specific genetic regions. Linkage mapping can be used to understand the biological basis of complex traits and to dissect genetic determinants underlying the expression of agronomically important breeding traits (Paran and Zamir, 2003). Using QTL analysis, two major QTLs for high CO₂ sensitivity were successfully identified (Fig. 8). BrSIO1 and BrSIO2 had similar LOD scores and explained similar amounts of phenotypic variation (19.3% and 19%), and these could be two major factors controlling high CO₂ sensitivity. Very recently, five QTLs associated with stability of SI in B. rapa have been identified. Two of them co-localized with SLG (A07) and MLPK (A03) and the other three were on A02, A06, and A10 (Hatakeyama et al., 2010). CO₂ sensitivity did not link with the S-locus in the present study (Fig. 5) and none of the other reported loci co-localized with QTLs detected here (Fig. 8), indicating that CO₂ sensitivity of the lines in this study is determined by novel genes different from those known to affect SI stability. Genes in BrSIO1 and BrSIO2 regions have 103 and 243 homologues in A. thaliana, respectively, and 54 and 141 of these genes are expressed in A. thaliana pistil. In silico comparative analyses identified several co-expressing genes and highly homologous genes encoded in these two regions. All these can be candidate responsible genes; however, to identify the genes responsible for high CO₂ sensitivity in the QTL regions in B. rapa more accurately, it would be necessary to narrow down the regions by developing near-isogenic lines (NILs).

To maintain F₁ seed quality, inbred lines with strong but CO₂-sensitive SI are ideal for F₁ hybrid breeding, and it is very important to understand the genetic relationships between SI-related genes and CO₂ sensitivity phenotypes. These results could be useful for the marker-assisted selection of parental lines with both stable SI and high CO₂ sensitivity.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. S-haplotype analysis of F₂ plants by PCR-RFLP.

Figure S2. RLSICO₂ in CO₂-sensitive and CO₂-insensitive lines, and F₁ and F₂ progeny based on the number of penetrating pollen tubes after self-pollination under high CO₂ conditions.

Table S1. Genetic markers and their primers used for linkage analysis.

Table S2. S-haplotype segregation in the F₂ population.

Table S3. Annotated genes and Arabidopsis homologues in BrSIO1.

Table S4. Annotated genes and Arabidopsis homologues in BrSIO2.

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