Genomic study of Raphanus sativus L.for analysis of genes controlling glucosinolate content in roots

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論文題目	Genomic study of Raphanus sativus L.for analysis of genes controlling glucosinolate content in roots（根のグルコシノレート含量を制御する遺伝子の分析のための Raphanus　sativus　L.のゲノム研究）
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論 文 内 容 要 旨

Introduction

Radish (*Raphanus sativus* L., $2n$=2x=18), a member of the tribe Brassiceae, is one of the stably cultivated crops in Japan and is closely related to *Brassica rapa,* which is an important commercial crop all over the world. Genomic study of *R. sativus* has not progressed as much as that of *B. rapa*, but high density genetic maps of *R. sativus* have been constructed. BAC clones harboring DNA markers on the genetic maps are essential for physical map construction. BAC-end sequence analysis will contribute to the genome study of *R. sativus*.

In radish breeding, lowing the pungency caused by isothioyanates, which are degradation products of glucosinolates, is an important objective, while glucosinolates have been reported to have anti-carcinogenic effect. Although radish cultivars having high and low contents of glucosinolates have been identified, genes responsible for such difference have not been analyzed.

Chapter 1. BAC clone screening and end sequence analysis

Since a BAC library of *R. sativus* has been constructed, I screened BAC clones harboring EST-SNP markers for physical map construction based on a high-density linkage map developed in our laboratory. The library consisted of 36,864 clones stored in ninety-six 384-well plates. Insert size was 130 kb on average, ranging from 97 kb to 242 kb (**Table 1**). The total length of inserts in the BAC library was estimated to cover eight times of *R. sativus* genome. Among 904 DNA markers of SNPs and SSRs on 9 linkage groups, 706 SNP markers were employed for BAC library screening and finally BAC clones harboring 570 SNP markers, one or more clones per marker, were positively screened **(Fig. 1)**.

Both ends of 20,736 BAC clones were sequenced. After trimming sequences of a vector, *E. coli* contamination, and low quality read, I applied 34,793 (84%) BAC end sequences for further analysis. Both end sequences were obtained in 92% of the BAC clones, providing 16,008 mate pairs. The lengths of high-quality BAC end sequences ranged from 100 bp to 1,015 bp with an average length of 580 bp. All the sequences analyzed with BLAST using *Arabidopsis thaliana* chloroplast and mitochondrial genome sequences revealed 7.5% and 0.8% clones to be of chloroplast and mitochondrial DNAs, respectively **(Table 2)**.

Based on similarity searches of the repeat database, 5,661 elements accounting for 8.6% of lengths of total good quality sequences were identified to be repeat sequences. The most common repeat family was LTR-retrotransposon (68.6%) including Copia (36.9%) and Gypsy (31.7%). Another prominent class of repeat sequences was DNA transposon (17.2%), followed with Non-LTR retrotransposon (7.8%) **(Fig.2)**. In total, 2,632 SSR markers with a motif length of more than 1 nucleotide were selected from the BAC end sequences. The frequency was one SSR per 7.7 kb of the genomic sequence (13 SSRs per 100 kb). Di-nucleotide motifs were the most abundant (1,598 SSRs, 60.7%), followed by tri-nucleotide motifs (570 SSRs, 21.7%), and mono-nucleotide motifs (401 SSRs, 15.2%) **(Fig. 3)**.

After removing the repeat sequences, BlastX search of BAC end sequences were conducted using non-redundant protein database of *A. thaliana*. To obtain putative protein-coding sequences, BlastX search of BAC end sequences were also conducted using the Swissprot database of *A. thaliana*. For all BlastX searches, an E-value cutoff of 10^{-4} was used. A total of 14,200 BAC end sequences (40.8% of total good quality end sequences) were found to match at least once to non-redundant protein database of *A. thaliana*. A total of 7,486 BAC end sequences (21.5% of total good quality end sequences) were matched to sequences in Swissprot database of *A. thaliana*. BlastN search of BAC end sequences was conducted with

a cutoff of E-value $=10^{-100}$ using Unigene set of *A. thaliana*. A total of 8,827 BAC end sequences (25.3% of total good quality end sequences) were found to match to *A. thaliana* sequences.

In a previous study, the whole linkage group 8 of *R. sativus* has been found to be collinear with more than half of chromosome 1 of *A. thaliana*. Among 123 BAC clones harboring 69 SNP markers on linkage group 8 **(Fig.1)**, 97 BAC clones had both end sequences showing significant unique hits to chromosome 1 of *A. thaliana*. Eight clones had end sequences containing seven DNA markers mapped on linkage group 8. Finally, a total of 76 microsyntenic blocks anchoring 105 BAC clones, which spanned 13.6 Mb, were identified and mapped on linkage group 8 **(Fig.4)**. I am searching BAC clones having end sequences homologous with the published *B. rapa* genome sequences.

Chapter 2. QTL analysis for identification of candidate genes controlling 4-methylthio-3-butenyl glucosinolate contents in roots

Radish roots contain 4-methylthio-3-butenyl glucosinolate (4MTB-GSL) with the common name of glucoraphasatin as a characteristic common glucosinolate. An inbred line, 'TBS-2-5-3-2-(3)' ('TBS' hereafter) having low glucosinolate contents, derived from a radish F_1 hybrid cultivar 'Taibyousoubutori', and an inbred line 'AZ26H-24-6-5-(3)' ('AZ26H' hereafter) having high glucosinolate contents, derived from a pungent local cultivar 'Karamijidaikon', were used as seed and pollen parents of F_2 plants.

4MTB-GSL contents in radish roots of the F_2 plants harvested in 2010 were distributed from 5.5 to 130.6 μmol/g DW with an average of 60.7 μmol/g DW **(Fig. 5A)**. 4MTB-GSL

contents of the F_2 plants harvested in 2011 ranged from 6.6 to 103.6 μmol/g DW and the average content was 45.6 μmol/g DW (**Fig. 5B**). Frequency distribution of 4MTB-GSL contents in the F_2 population showed a continuous, bell-shaped distribution.

Analysis of multiplex PCR products amplified with 2,880 primer pairs using an Illumina sequencer determined sequences of 2,301 and 2,328 fragments of 'TBS' and 'AZ26H', respectively. The numbers of read bases of 'TBS' and 'AZ26H' were 175 Mb and 140 Mb, respectively. Comparison of sequence data of 1,777 fragments between 'TBS' and 'AZ26H' revealed 2,655 possible SNPs. Among them, SNPs with more than five repetitive reads were regarded as credible SNPs, and 1,953 SNPs in 750 DNA fragments (**Table 3**), 437 of which had been previously mapped in a linkage map, were identified.

Using 188 and 118 dot-blot-SNP markers selected by the sequence analysis of 'TBS' and 'AZ26H', 189 and 174 F_2 plants harvested in 2010 and 2011, respectively, 4MTB-GSL contents of which have been analyzed by a coworker, were genotyped. In the present study, five QTLs associated with 4MTB-GSL contents were identified in the two F_2 populations in radish roots, and three of them, i.e., GSL-QTL-1, GSL-QTL-3, and GSL-QTL-4, were repeatedly detected (**Table 4, Fig. 6**). The genotypes of BB-BB (genotype of GSL-QTL-3-genotype of GSL-QTL-4), BB-AB, and AB-BB, in which A is a 'TBS' allele and B is an'AZ26H' allele, showed significantly higher contents of glucosinolates than those of BB-AA, AA-BB, and AA-AA in the two populations (**Table 5**). In a combination of GSL-QTL-1 (positive additive effect of 'TBS' alleles) and GSL-QTL-3 (negative additive value effect of 'TBS' alleles), BB-AA (genotype of GSL-QTL-1- genotype of GSL-QTL-3), BB-BB, and BB-AB showed lower glucosinolate contents than those of AA-AB, AA-BB, and AA-AA **(Table 6).**

By comparing syntenic QTL regions of radish with *A. thaliana* and *B. rapa* genome sequences, candidates of genes controlling glucosinolate contents in the identified QTL regions were selected after adding some new markers in the QTL regions (**Table 7**). By comparing nucleotide sequences of candidate genes between 'TBS' and 'AZ26H', one SNP, one SNP, and one SCAR markers in *RsMAM3*, *RsBCAT4*, and *RsIPMDH1*, respectively, were developed for candidate gene mapping **(Fig. 7, Table 8).** *RsMAM3* and *RsBCAT4* were found to be linked to the GSL-QTL-4 and GSL-QTL-1 regions, respectively, and *RsIPMDH1* was revealed to be linked to GSL-QTL-2 **(Fig. 6, Table 8)**. Three other important genes involved in glucosinolate contents were also analyzed. *RsCYP83A1* had one insertion in an intron region in the allele of 'TBS', and *RsMAM1* had five SNPs and three indels throughout intron and exon regions. There was no sequence variation in *RsCYP79F1* between the parental lines (**Fig. 8, Table 8**).

RsMAM3 was strongly expressed in 'AZ26H' having higher 4MTB-GSL content. One nonsynonymous SNP (G-T) in the first exon changes the amino acid from leucine to phenylalanine in the coding region **(Fig. 7)**. Expression of *RsIPMDH1* was significantly higher in 'AZ26H' than that in 'TBS', implying that *RsIPMDH1* may play a co-regulation in the 4MTB-GSL biosynthesis pathway with other genes. Expression of *RsBCAT4* was also higher in 'AZ26H' than that in 'TBS' **(Fig. 9, 10)**. Although there were sequence variations in *RsCYP83A1* and *RsMAM1*, the expression levels were similar between 'TBS' and 'AZ26H'. Sequences of *RsCYP79F1* were the same between the parental lines and there was no significant difference in the expression level between them (**Table 8, Fig. 8, 9**). *RsMAM3*, *RsIPMDH1*, and *RsBCAT4* were considered to be candidate genes to regulate 4MTB-GSL content in radish, while *RsCYP83A1*, *RsMAM1*, and *RsCYP79F1* may not contribute to the difference of 4MTB-GSL contents between 'TBS' and 'AZ26H'.

Conclusion

The main objective of this study is to construct a physical map of *R. sativus*. Determination of BAC end sequences provided a fruitful resource for repeat sequence categorization, SSR detection, gene searching, and *in silico* alignment of screened BAC clones to linkage group 8. After QTL analysis of 4MTB-GSL contents, I inferred three candidate genes *RsMAM3*, *RsIPMDH1*, and *RsBCAT4* from the corresponding regions in *A. thaliana* and *B. rapa*. Nucleotide sequences and expression analysis of these genes suggested their possible contribution to the variation of 4MTB-GSL contents in radish root. BAC clones will be useful for identification of genes involved in glucosinolate contents in the QTL regions.

Table 1. Characteristics of a *R. sativus* BAC library

Cloning vector	pCC1 BAC vector
Partial digesting enzyme	*Hin*dIII
Number of clones	36,864
Number of 384-well plates	96
Missed wells	35 (0.09%)
Mean insert size	130 kb
Minimum insert size	97 kb
Maximum insert size	242 kb
Number of genome equivalents	8×

Table 2. Summary of BAC end sequences

Number of BAC clones for sequencing	20,736
Number of good-quality BAC end sequences	34,793 (84%)
Number of BAC clones with both two end sequence of good quality	16,008
Number of BAC clones with single end sequence	2,778
Total base count	20,172,078
GC content	37.1%
Minimum length	100 bp
Maximum length	1,015 bp
Mean length	580 bp
Chloroplast sequence	7.5%
Mitochondrial sequence	0.8%

Table 3. SNPs between the parental lines identified by next generation sequencing

Parental lines	Primer sets used	Total amplicons	Number of SNPs with read depth more than five	Total length with read depth more than five	Frequency	Amplicons having SNPs with read depth more than five
TBS	2,880	2,301	1,952[a]	151,839	1/77.8	729
AZ26H	2,880	2,328	2,656[a]	203,267	1/76.5	970
between lines	-	-	1,953	-	-	750

a: The sequence data of 'Aokubi' were used as references for SNP identification. Numbers of SNPs with 'Aokubi' are shown.

Table 4. QTL analysis of 4MTB-GSL contents in radish roots

QTL	Linkage group	Nearest marker	2010 F_2 population			2011 F_2 population		
			LOD	Additve[a] effect	Variance explained (%)	LOD	Additve effect	Variance explained (%)
GSL-QTL-1	LG1	RS2CL6432s	5.87	7.94	13.39	19.1	11.84	36.70
GSL-QTL-2	LG2	RS2CL6594s	5.62	-5.62	14.05	1.54[b]	-4.27[b]	3.20[b]
GSL-QTL-3	LG6	RS2CL4585s	5.19	-16.98	29.50	3.62	-10.53	16.72
GSL-QTL-4	LG9	RS2CL4290s	7.36	-8.21	13.28	4.09	-9.54	11.09
GSL-QTL-5	LG7	RS2CL3356s	1.83[b]	0.31[b]	3.31[b]	3.85	-9.19	11.30
Threshold value			4.90			3.40		

a, Additive effects of 'TBS' alleles are shown.
b, Not significant.

Table 5. Comparison of 4MTB-GSL contents between different genotypes of SNP markers in GSL-QTL-3 and GSL-QTL-4

Group number	Marker genotype		2010 F_2 population		2011 F_2 population	
	RS2CL4585s in GSL-QTL-3	RS2CL4290s in GSL-QTL-4	Number of plants	4MTB-GSL content (μmol/g DW)	Number of plants	4MTB-GSL content (μmol/g DW)
1	BB	BB	6	78.3±5.14 a	9	74.1±3.24 a
2	BB	AB	19	65.3±4.32 a	14	68.5±5.12 ab
3	AB	BB	12	59.6±3.01 a	23	59.2±3.54 ab
4	AB	AB	25	53.7±2.32 ab	46	52.5±2.84 ab
5	AA	AB	18	51.2±3.12 ab	17	42.4±3.26 bc
6	AB	AA	10	46.5±4.09 ab	14	47.5±2.53 b
7	BB	AA	5	44.3±5.07 b	13	44.9±4.72 bc
8	AA	BB	8	41.0±4.31 b	9	38.1±4.08 c
9	AA	AA	7	36.4±6.23 b	10	35.0±5.24 c

Note: Values followed by the same letter within each experiment were not significantly different at the 5% level by Tukey's multiple comparison test.

Table 6. Comparison of 4MTB-GSL contents between different genotypes of SNP markers in GSL-QTL-1 and GSL-QTL-3

Group number	Marker genotype		2010 experiment		2011 experiment	
	RS2CL6432s	RS2CL4585s	Number of plants	4MTB-GSL content(μmol/g DW)	Number of plants	4MTB-GSL content(μmol/g DW)
1	AB	BB	11	73.6±7.45 a	25	58.8±3.39 a
2	AB	AB	47	61.7±2.97 ab	42	53.7±2.08 a
3	AB	AA	17	60.2±5.14 abc	20	41.9±3.49 a
4	AA	AB	17	56.9±2.89 abcd	22	46.7±2.23 a
5	AA	BB	8	54.3±3.14 abcd	13	41.5±4.39 a
6	AA	AA	4	53.5±13.10 abcd	10	43.8±3.26 a
7	BB	AA	3	39.4±7.01 abcd	7	17.2±2.33 b
8	BB	BB	6	38.3±7.78 bd	8	41.4± 8.46 a
9	BB	AB	13	35.1±5.87 d	11	17.5±3.70 b

Note:Values followed with same letter within each experiment are not significantly different at the 5% level were determined by Tukey's multiple comparison test.

Table 7. Newly developed markers in QTL regions

Linkage group	QTL region	Marker name	Forward primer (5'-3')	Reverse primer (5'-3')	TBS-SCR27	AZ26H-SCR52	Position(cM)
LG1	GSL-QTL-1	RS2CL5813	ATGGCAACCAGCTTACCAGTCT	AACCAAGTCCAAACTTGCCATC	CTAGTGCCACCGCCGCA	CTAGTGCCGCCGCAGCA	32.9
		RS2CL3740	TGGGACAAGCTCTGGACTATCA	AACGTGAGCTTCACCAACTCAT	TTTGAAGGTGCTGGAGA	TTTGAAGGCGCTGGAGA	33.7
		RS2CL3718	CAGTTTTGAGGCAAGTTTGTGC	TCAAGTTCTGCTCAGGGGAGAT	CTTTGGATCATTGCAGC	CTTTGGATTATTGCAGC	35.5
LG2	GSL-QTL-2	RS2CL7568	ATTGCATCTCCTTCCACTCCAT	GCTTAGGCATTGCGTTTCTAGC	GAAGCCGTAGCCCACGG	GAAGCCGTTGCCCACGG	37.9
		RS2CL4817	CTCGTCTGCGCTTATGGTTATG	ACTAACGTTTGCCCCTGTCAAT	CTTAAGTACCATTTTGC	CTTAAGTATCATTTTGC	39.7
LG9	GSL-QTL-4	RS2CL5785	AGATTGTGATGTGGGCTGAGAA	TCTCGTTTAGCAACTCCACTGC	AAGGGTATCGGAAGGTT	AAGGGTATAGGAAGGTT	73.6
		RS2CL2646	AACATAACATGGGACGTTCTGC	GAAACAGGGGAGAAACAAGAGG	TTTGCAATCACCTCGTA	TTTGCAATTACCTCGTA	91.3
LG7	GSL-QTL-5	RS2CL2041	GCCCAGTCCTGTTCTTGAGATT	TAATATGGGTGGCCTCTGCTCT	GTGATCGTATTCAAAAA	GTGATCGTTTTCAAAAA	38.7
		RS2CL5401	ACATCAGAACGTGGAACAATGC	TTGAAACCGAGAAGAGCTGGAT	TTTCCCTGATGATGTGG	TTTCCCTGGTGATGTGG	29.7

Note: The oligonucleotide probes were designed as bridge probes (Shiokai et al. 2010). Sequences excluding the bridge sequence are shown. A sequence, TATATTTACATTCGCAATTAAGAGGCTTCGT designated as SCR-27, and a sequence, TATATTCCCTCCGTCAGCGGATC designated as SCR-52, were added to allele-specific sequences of TBS and AZ26H, respectively.

Table 8. Sequence analysis of glucosinolate biosynthesis genes in *R. sativus*

Gene name	Position	Length (bp)		Number of SNPs in exons	Number of indels in exons	Number of SNPs in introns	Number of indels in introns
		TBS	AZ26H				
RsMAM3	GSL-QTL-4	1620	1621	1	0	0	0
RsIPMDH1	GSL-QTL-2	1781	2285	1	0	5	3
RsBCAT4	GSL-QTL-1	1952	1963	0	0	8	7
RsCYP79F1	Not mapped	968	968	0	0	0	0
RsCYP83A1	Not mapped	1624	1623	0	0	0	1
RsMAM1	Not mapped	2512	2513	0	1	5	2

Fig. 1 Screening of BAC clones harboring EST-SNP markers on high-density linkage map. Markers with red color indicate those anchoring positive BAC clone. Markers without positive BAC clones are shown in green color. Blacks have not been used for BAC screening.

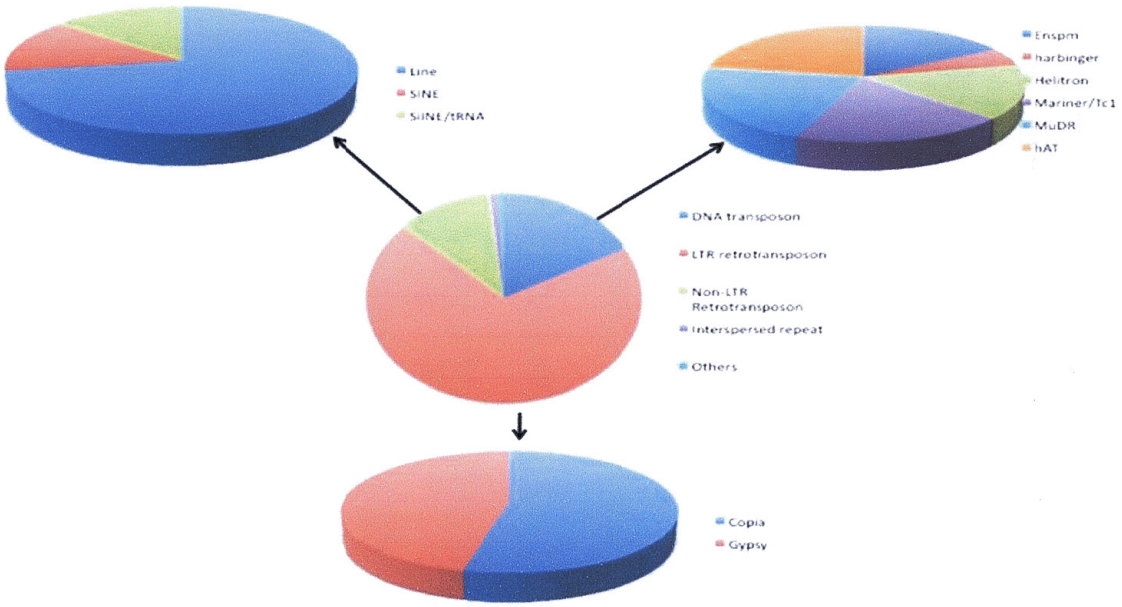

Fig.2 Classification of repeat sequences in BAC-end sequences of *R. sativus*. BAC-end sequences were searched with repeat sequences database. DNA transposons, LTR-retrotransposons, Non-LTR retrotransposons, interspersed repeats, and others were identified and classified with annotation of repeat sequences.

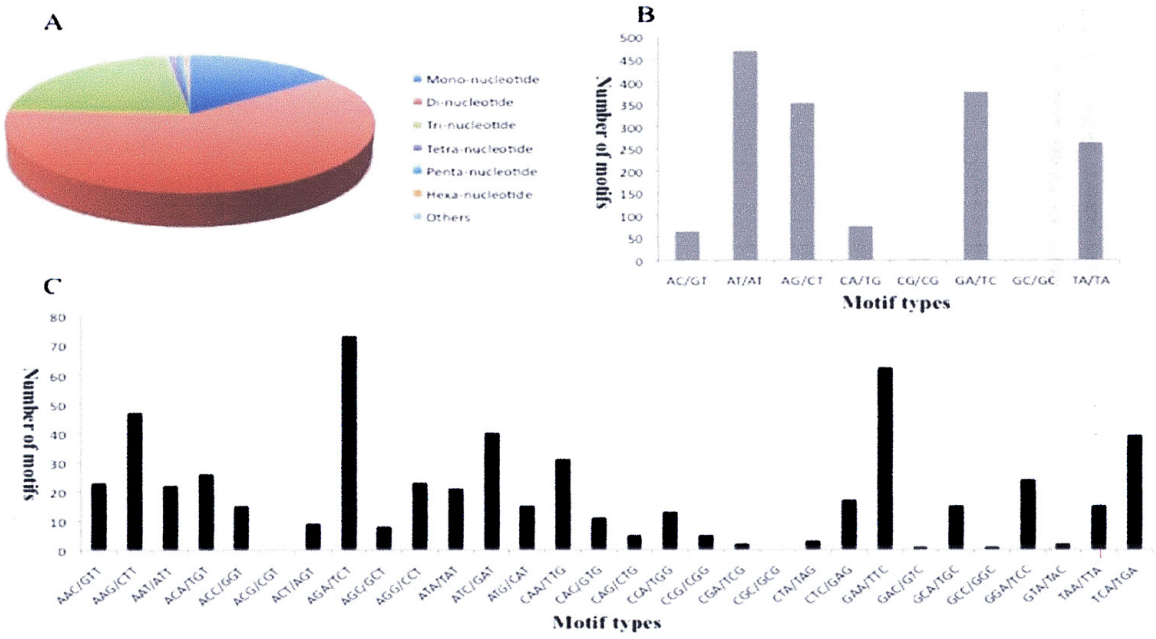

Fig. 3 Distribution of SSR motifs. A, Distribution of SSRs based on motif types. B, Distribution of SSRs by motif types in di-nucleotide repeat motifs. C, Distribution of SSRs by motif types in tri-nucleotide repeat motifs.

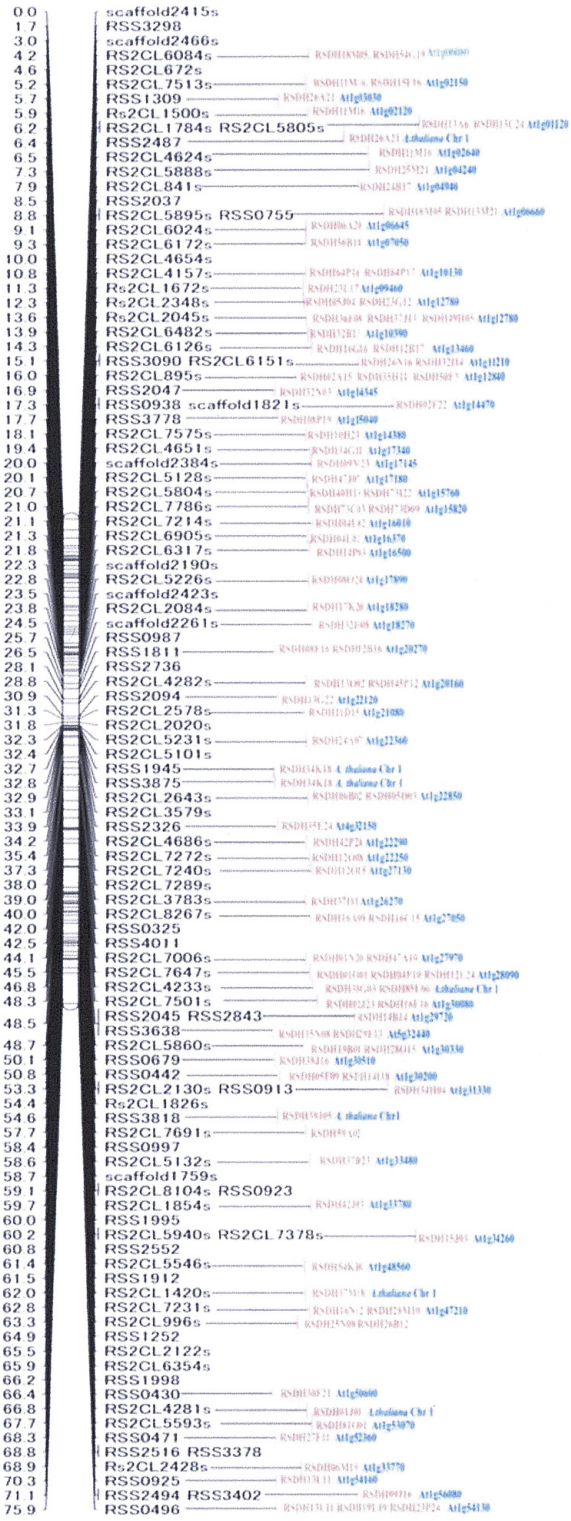

Fig. 4 *In silico* alignment of BAC clones to linkage group 8 of *R. sativus*. Microsyntenic blocks are shown with pink bars. BAC clones are shown in red, and *A. thaliana* genes in the regions syntenic to the BAC clones are shown in blue.

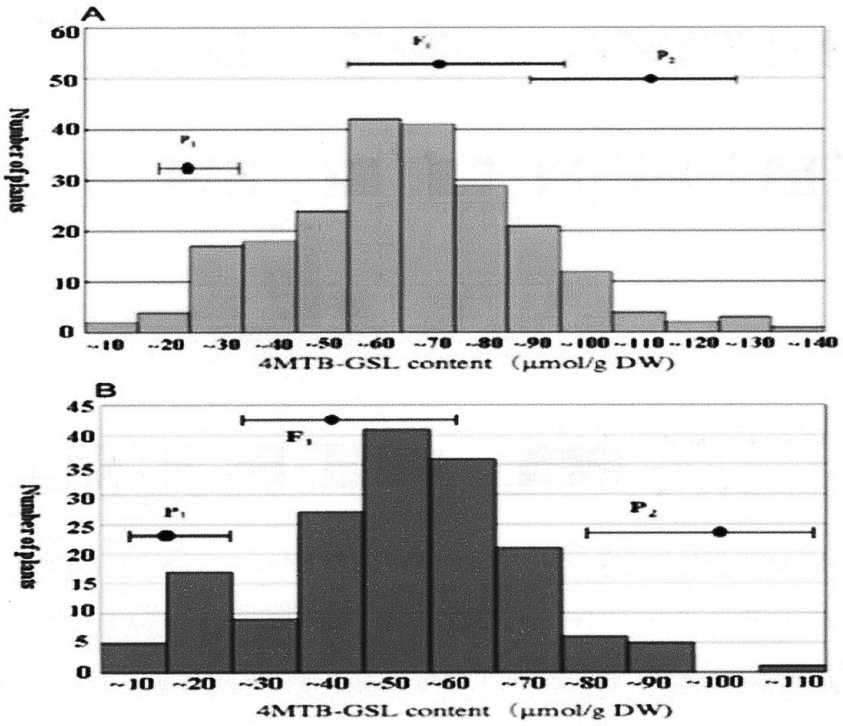

Fig. 5 Distribution of 4MTB-GSL contents in the two F_2 populations obtained by crossing 'TBS' and 'AZ26H'. (A) F_2 population grown in 2010, (B) F_2 population grown in 2011.

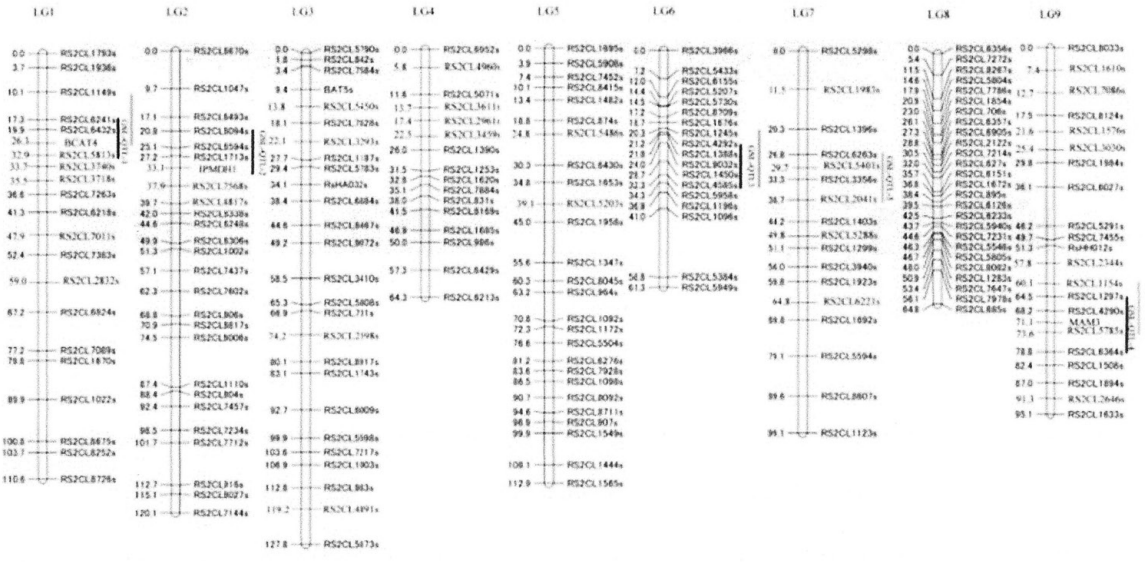

Fig. 6 Linkage map of EST-SNP markers showing polymorphism between 'TBS' and 'AZ26H'. Black bars and gray bars indicate QTL regions of 4MTB-GSL contents detected in 2010 and 2011, respectively.

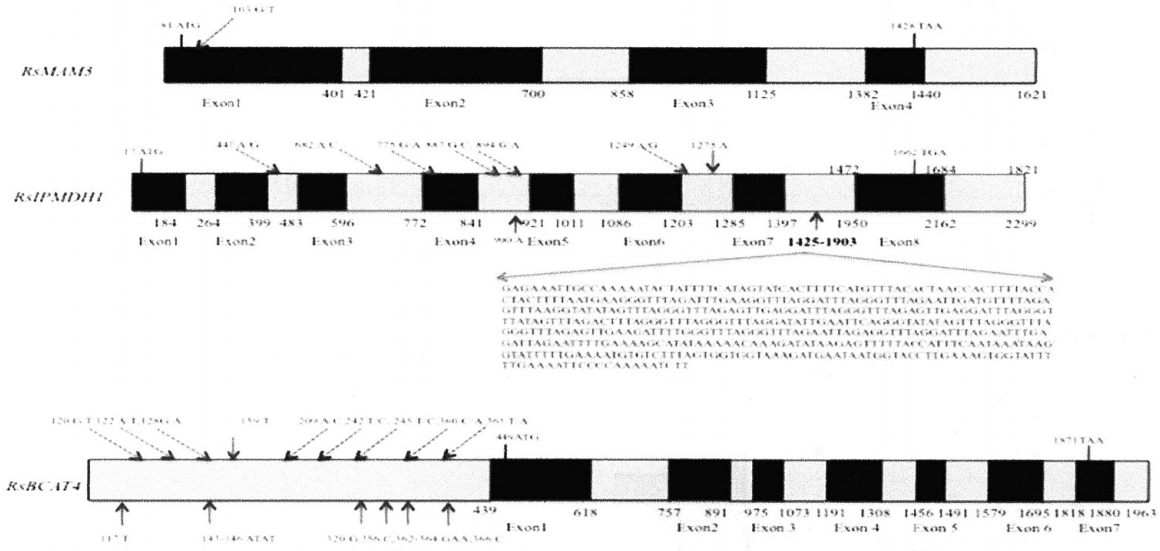

Fig. 7 Nucletide polymorphisms of *RsMAM3*, *RsIPMDH1*, and *RsBCAT4* between 'TBS' and 'AZ26H'.

The black and gray boxes indicate exons and introns, respectively. The black arrows show indels. The positions of dashed arrows indicate SNP sites and nucleotide variations ('TBS'/'AZ26H'). Insertions are shown by black arrows above the box ('TBS') and beneath the box ('AZ26H'). The numbers under the boxes indicate the start and stop sites of exons.

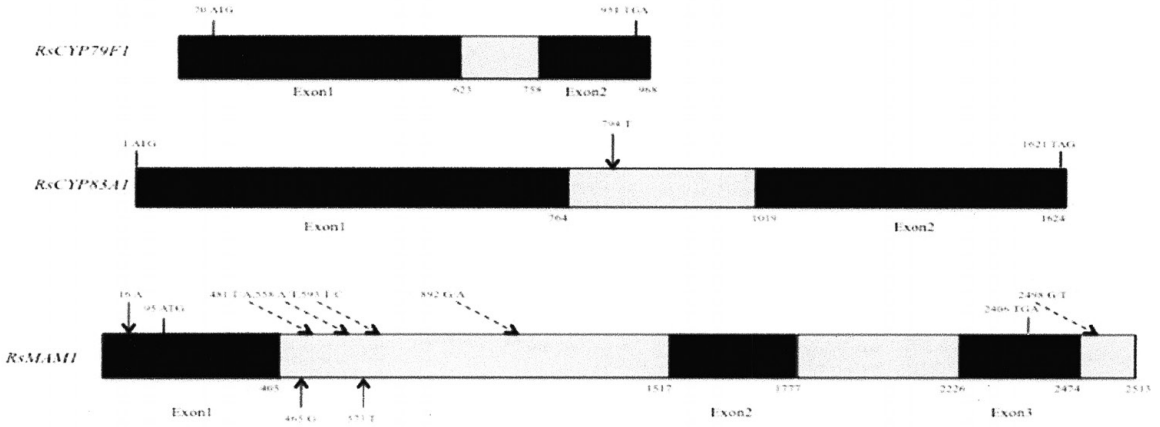

Fig. 8 Nucletide polymorphsims of *RsCYP79F1*, *RsCYP83A1*, and *RsMAM1* between 'TBS' and 'AZ26H'.

The black and gray boxes indicate exons and introns, respectively. The black arrows show indels. The positions of dashed arrows indicate SNP sites and nucleotide variations ('TBS'/'AZ26H'). Insertions are shown by black arrows above the box ('TBS') and beneath the box ('AZ26H'). The numbers under the boxes indicate the start and stop sites of exons.

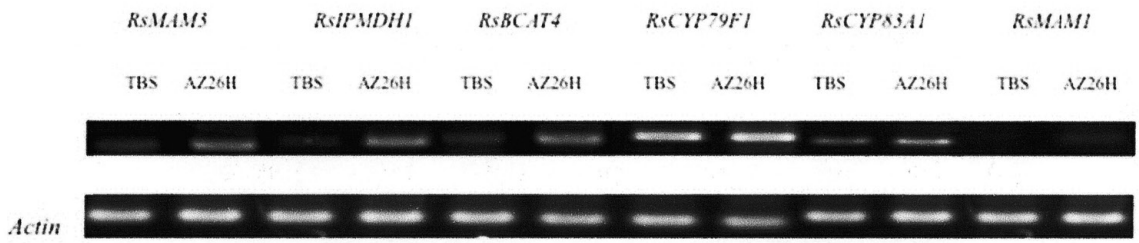

Fig. 9 RT-PCR analyis of candidate gene transcripts in 'TBS' and 'AZ26H'. RNAs were extracted from radish roots. *Actin* was used as a control to demonstrate equal RNA loading.

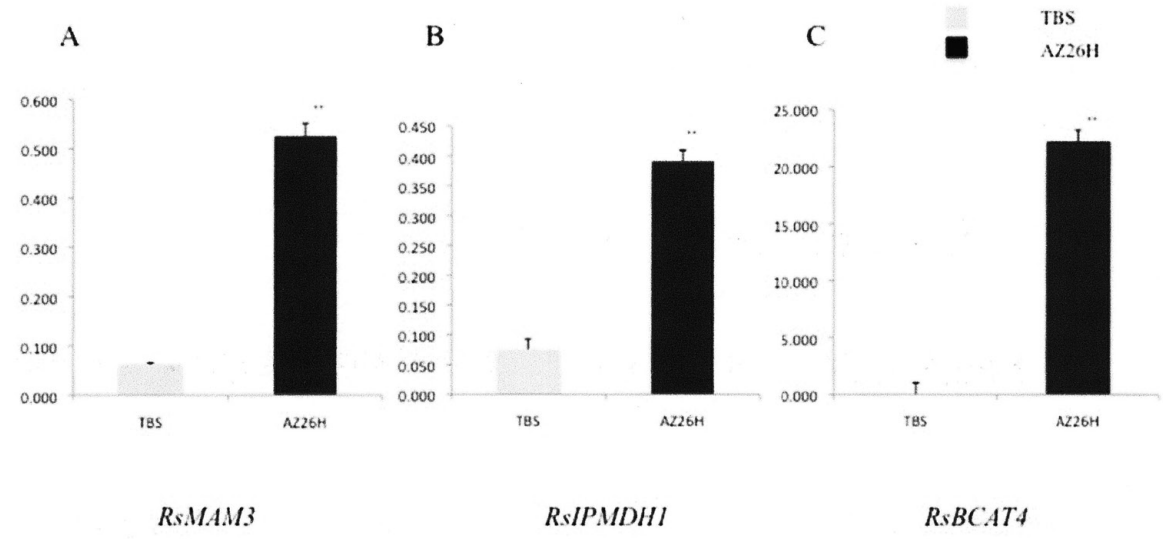

Fig. 10 Gene expression analyses of parental lines by real-time PCR. (A) *RsMAM3,* (B) *RsIPMDH1,* (C) *RsBCAT4*. Gray and black bars indicate parental lines 'TBS' and 'AZ26H'. Significant differences ($P<0.01$, LSD-t test) between the parental lines are indicated by asterisks.

論文審査結果要旨

ダイコン（*Raphanus sativus* L.）は、日本においては重要作物であるが、遺伝学的研究やゲノム研究は進んでいない。ダイコンにおいては、根の形態や辛味成分の含量が作物として重要な特性であるが、これらの特性の遺伝子は明らかとなっていない。本研究では、ダイコンの辛味を決定する4-メチルチオ-3ブテニルグルコシノレート（4MTB-GSL）の根における含量の品種間差に関わる遺伝子を見出すため、多数のDNAマーカーを作成してQTL解析を行うとともに、マッピングした遺伝子同定のための基盤となるゲノム塩基配列情報を蓄積するため、ダイコンゲノムのBACライブラリーから、連鎖地図上のDNAマーカーを含むクローンのスクリーニングと多数のクローンの両端塩基配列の解析を行った。

連鎖地図上の706のSNPマーカーを用いてBACライブラリーをスクリーニングし、570のSNPマーカーについて、それを含むBACクローンを同定した。20,736クローンの両端塩基配列を決定し、34,793の塩基配列情報を得て、塩基配列を決定した。8.6%が反復配列であり、25%がシロイヌナズナの遺伝子の塩基配列と高い相同性があることを示した。

4MTB-GSL含量が高い系統と低い系統のF_2集団を用いて4MTB-GSL含量のQTL解析を行うため、2系統のDNAをもとに2,880プライマー対で増幅したDNAを次世代シーケンサーで塩基配列分析を行い、750のDNA断片の中の1,953のSNPが信頼性が高いSNPと推定した。その情報に基づいてSNPマーカーを作成し、189個体と174個体の2集団についてSNPの遺伝子型分析と4MTB-GSL含量を測定した。188のSNPマーカーで連鎖地図を構築し、5カ所にQTLを検出した。その内3カ所は、2つのF_2集団で共通して検出され、信頼性が高いQTLと考えられた。シロイヌナズナやアブラナとのゲノムのシンテニーに基づいて、QTL領域にグルコシノレートの生合成に関わる酵素の遺伝子 *RsMAM3, RsBCAT4, RsIPMDH1* を見出した。これら遺伝子が、ダイコン染色体のQTL領域にあることを遺伝子マッピングで確認し、遺伝子発現レベルが、いずれも含量が高い系統で高いことを見出した。これらのうち、特に *RsMAM3* が4MTB-GSL含量の変異に関わっている可能性を示唆した。

以上のように本研究は、ダイコンのBACクローンの解析によりダイコンゲノムの物理地図作成に貢献するとともに、4MTB-GSL含量のQTLを見出して、4MTB-GSL含量の変異に関わる遺伝子の候補を見出したもので、その農学上の貢献は大きい。よって、審査員一同は本論文は博士（農学）の学位を授与するに値する内容であると判定した。