Mapping of QTLs controlling root shape and red pigmentation in radish, *Raphanus sativus* L.

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Genetic factors controlling root shape and red pigmentation on the surface of the hypocotyl (upper part of root) were investigated using a molecular linkage map based on an F₂ population derived from a cross between two radish cultivars, 'Huang-he hong-wan' and 'Utsugi-gensuke'. One hundred and ninety-eight segregating markers (169 AFLPs, 28 *Brassica* SSRs and one SLG-CAPS) were located on 14 linkage groups (LGs) at a LOD threshold value of 5.0. The mapping position of *Brassica* SSRs revealed that frequent genomic rearrangements occurred between the *Brassica* and *Raphanus* genomes. Three quantitative trait loci (QTLs) for root shape with LOD values of 2.42, 3.22 and 2.88 were identified on LGs 3, 8 and 9, respectively. These three QTLs accounted for 42.4% of the phenotypic variance when joined together. Analysis of QTL(s) for root diameter revealed the presence of two QTLs on LG4 and LG8. The QTL on LG8 was considered to control root thickening and to affect the root shape. For hypocotyl pigmentation, a QTL which exerted a large genetic effect with a LOD value of 9.58, was recognized on LG 11 and accounted for 43.8% of the phenotypic variance.

Key Words: *Raphanus sativus*, linkage map, QTLs, root shape, red pigmentation.

Introduction

During the last decade, a number of molecular markers linked to important agronomic traits were developed in many plants (Peleman and van der Voort 2003). Marker-assisted selection (MAS) using these molecular markers has accelerated the development of new cultivars and the introduction of desirable traits in breeding programs.

However, many agriculturally important traits are not controlled by a single locus and can involve more than two loci. These loci that underlie a particular trait are designated as quantitative trait loci (QTLs). Genetic linkage map, which is based on molecular markers, is a powerful tool for crop breeding, as it provides a framework for mapping loci that control important traits and enables to gain information about QTLs to be clarified (Paran and Zamir 2003). In addition, comparative mapping analysis of related genomes has been conducted using simple sequence repeat (SSR) and restriction fragment length polymorphism (RFLP) markers to detect the conservation and order of genes, referred to as 'synteny' (Choi *et al.* 2004, Moore *et al.* 1995, Suwabe *et al.* 2006, Tanksley *et al.* 1992). In cruciferous plants, a large number

of reports have dealt with the development of molecular markers linked to important traits and the construction of molecular linkage maps (Ajisaka *et al.* 2001, Hirai *et al.* 2004, Kole *et al.* 1996, Marwede *et al.* 2005, Mongkolporn *et al.* 2003, Suwabe *et al.* 2004b, Tanhuanpää *et al.* 1996).

Radish, *Raphanus sativus* L. (2n=2x=18), is one of the most important vegetables in East Asia, along with other cruciferous crop plants (Kitamura 1958, Kaneko *et al.* 2007). Its hypertrophied root with hypocotyl is mainly used in cooking, as a pickled vegetable, and in salads. Wide morphological variations have been observed in the roots of local and commercial radish cultivars. These traits seem to be controlled by QTLs. Some molecular markers linked to important agronomic traits have also been developed in radish. *S*-alleles, which control self-incompatibility, were identified by using the cleaved amplified polymorphic sequence (CAPS) of the *S*-locus-specific glycoprotein (SLG) gene (Niikura and Matsuura 1998). Furthermore, Murayama *et al.* (2003) screened an amplified fragment length polymorphism (AFLP) marker linked to a fertility restorer (*Rf*) gene for Ogura cytoplasmic male sterility for conversion into a sequence-tagged site (STS) marker. However, these studies were conducted on traits controlled by single dominant genes. To our knowledge, markers for more complex genetic traits have not been studied because whole genome mapping and subsequent QTL analysis would be required. Although

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Bett and Lydiate (2003) reported a linkage map of *Raphanus* using *Brassica* RFLP markers, no information about agronomic traits was available from this map.

In a previous study, we constructed a molecular linkage map for an F_1 population of radish cultivars based on AFLP, *B. rapa* genome-derived SSR and SLG markers by adopting a pseudo-test-cross strategy (Tsuro *et al.* 2005). Although this study provided information about the framework of mapping loci in the *R. sativus* genome, most agronomic traits were not detectable due to the lack of segregation in the F_1 population. The objective of the present study was to estimate the locations of QTLs controlling the root shape and surface pigmentation of the root and leaf veins in the newly constructed F_2 linkage map, based on the above-mentioned framework markers of the F_1 population.

Materials and Methods

Plant materials

Two radish cultivars, 'Huang-he hong-wan (Kouga-benimaru)' (Sakata Seed Co., Yokohama, Japan) and 'Utsugi-gensuke' (Ishikawa Agricultural Research Center, Kanazawa, Japan) were used in the present study. 'Huang-he hong-wan' is a Chinese radish cultivar showing a globular root with red skin. 'Utsugi-gensuke' is a Japanese radish with a long white root. Both parental plants used in the present study were the same as those used in the previous study (Tsuro *et al.* 2005). In each parent, homozygosity was increased by three successive selfings (S_3). Since outcrossed crucifer plants generally display a high heterology, the S_3 plants may still retain some heterozygous loci. However, we consider that such residual heterozygosity did not cause a problem in the present study because the F_2 population was derived from a single F_1 plant, in which, for each locus, only one of the alleles should have been transmitted from each parent. One hundred and four F_2 seeds were germinated on commercial soil ('Ryujo-baido'; Zen-noh Agricultural Co-operatives, Tokyo, Japan) in Jiffy pots placed in a greenhouse in September 2004. After the development of three or four leaves, the seedlings were transferred to the field, planted at 80 cm intervals in a row 100 cm wide, and grown under natural conditions. Compound fertilizer (N:P:K = 15:15:15) at the rate of 180 g m^{-2} was applied as basal dressing. All the plants were harvested at 95 days after seeding, in mid-December 2004.

The root axis of radish is composed of two anatomically distinct parts. The upper part originates from the hypocotyl, namely the stem, and lateral roots do not occur in this part of the root. The lower part consists of true root tissue, and lateral roots are found in this part of the root. These two parts of the radish root become thicker, with the formation of a succulent tissue, which is used as a vegetable for eating. Hereafter, these two parts will be referred to as the 'root' in the present paper. However, since pigmentation on the surface of the two parts of the radish root can differ, the 'root' will be referred to as 'hypocotyl' and 'lower root', respectively. As the roots of the F_2 plants showed wide variations

from slender to hypertrophic like in 'turnip', the ratio of the root length to the diameter at the level of the thickest part of the root was used as an index of root shape. Since the red pigmentation level on the surface of the hypocotyl also showed wide variations in the F_2 population, we used a visual five-color score, namely 0 (white) to 4 (deep red) to indicate increasing pigmentation grade. In addition, the pigmentation of the leaf veins and the surface pigmentation of the lower root were evaluated as red or white, respectively.

DNA polymorphism analysis

Total DNA was extracted from young leaves of the two parental and 104 F_2 plants grown in a greenhouse using the CTAB method (Murray and Thompson 1980). AFLP, *Brassica*-SSR, and self-incompatibility locus (*S*-locus) markers were used for the polymorphism analysis. AFLP analysis was conducted according to the method of Vos *et al.* (1995), with some modifications, as reported previously (Tsuro *et al.* 2005). Analysis of the SSR polymorphism was carried out using the 'BRMS' primer pairs developed for *B. rapa* (Suwabe *et al.* 2002, 2004a). Nineteen SSR loci had already been placed on the genetic linkage maps of *B. rapa* and *R. sativus* (Suwabe *et al.* 2006, Tsuro *et al.* 2005), and eight additional markers are indicated in Table 1. The SSR markers were detected as previously reported (Tsuro *et al.* 2005). The fragments amplified from BRMS-268 and BRMS-302 were separated by electrophoresis on an 8% polyacrylamide gel. Polymorphism analysis of SLG was conducted as described previously (Tsuro *et al.* 2005).

Linkage analysis and QTL detection

The marker data were scored according to the definition of JoinMap3.0 (Van Ooijen and Voorrips 2001). The F_2 segregation population was analyzed as an 'F2' population type. The Kosambi mapping function was used for linkage analysis (Kosambi 1944).

For QTL analysis, QTLs for all traits were identified using the Kruskal–Wallis and interval mapping procedure of MapQTL4.0 (Van Ooijen *et al.* 2002). Significance thresholds for interval mapping were determined using the permutation test of MapQTL ($P < 0.05$, 2500 permutations). Although interval mapping is widely used, we also applied the Kruskal–Wallis method because in some cases, the residuals obtained with interval mapping were not distributed normally, which might have induced incorrect results (Zini *et al.* 2005). For the interval mapping approach, five neighboring intervals and a step size of 2 cM were used. A LOD score of 2.0 was adopted to indicate the presence of a QTL.

Results and Discussion

Linkage mapping and genome rearrangement

In the AFLP analysis, 280 polymorphic markers were detected using 38 primer pairs and scored as dominant markers. Among the 26 *Brassica*-SSR primer pairs examined, 16 pairs amplified single polymorphic bands, segregating in a

Table 1. BRMS markers located on the linkage map of *R. sativus*

BRMS No.	Primer sequence (5'–3')	Expected length in <i>B. rapa</i> (bp) ^a	Detected length in <i>R. sativus</i> (bp)	
			'Huang-he hong wan'	'Utsugi-gensuke'
042	GGATCAGTTATCTGCACCACAA TCGGAATTGGATAAGAATTCAA	122	101	99
089	GACTTTCCCGATTTGCTTTTCCAG GGAGGGTTCGGGTCTTACAAGTTTG	233	243	245
203	GAATCAATGGTCCAGATTTCTTTTA ATCTCTCCCTCTTGTCTCATCT	279	328	334
258	ATCTGATAACTGAGTGTCTATTGTGC CTCTGCTTCTTCATCGGAAAAAT	221	193	ND ^b
268	GAGTAGTACCAGGAAGGGTGACCTC ACGCTTAACTGCGGAGTTCTGAT	508	ND ^b	Ca. 100 ^c
270	ACCCAAGTACGTGATGTGAACCTTG GAAACAGAAGAGCAGGAGGAGGA	228	ND ^b	194
302	CAAGTCGAAGAAGACATCTCCAAAC TAAGCTTCTTCTCGAGCTTTTGGTT	331	Ca. 600 ^c	Ca. 580 ^c
313	TCATGGTCAACCGAAGTAATTAAG ACCGCAATCTCTCCGATAAACTCTA	187	188	182

^a SSRs were extracted from the genomic library of a doubled-haploid line of *B. rapa* 'G004' (Suwabe *et al.* 2002, 2004).

^b Not detected and treated as 'null'.

^c Approximate size of band separated by electrophoresis on 8% polyacrylamide gel.

dominant (3:1) mode, whereas eight pairs generated codominant polymorphic bands (1:2:1). All these primer pairs detected single loci. Two other primer pairs, BRMS-303 and BRMS-330, detected two independent loci with two discrete polymorphic bands, respectively. One hundred and ninety-eight segregating markers (169 AFLP, 28 *Brassica*-SSR and one SLG-CAPS markers) that were used for linkage mapping, generated 14 linkage groups (LGs) at a LOD threshold value of 5.0 (Fig. 1). The total length of the map was 667.6 cM, and the length of the LGs ranged from 8.0 (LG14) to 122.2 cM (LG8). The number of markers per LG ranged from 2 (LG7, LG13 and LG14) to 33 (LG1), and the average interval between the markers was 3.4 cM. As 111 AFLP markers did not join any linkage group, they remained unmapped. The number of LGs did not match the chromosome number ($n=9$) of this species. The present map was slightly shorter than reported based on a cross between *R. sativus* and *R. raphanistrum* (Bett and Lydiate 2003). A portion of the genome may have remained unmapped in the present map, although the *R. sativus* and *R. raphanistrum* maps were constructed with Mapmaker, which tends to generate larger maps than those generated with JoinMap (Bradeen *et al.* 2001).

In a previous study, we constructed a linkage map of *R. sativus* based on an F_1 population derived from the same parentage as that in the present analysis (Tsuro *et al.* 2005). Although the map length in the present study nearly corresponded to that in our previous study (657.8 cM), only seven BRMS loci (BRMS-051, 057-2, 129, 287-1, 303-1, 303-2 and 324) were detected in both populations. The other 12 loci, detected in the F_1 map, were homozygous, and were not mapped in the F_2 map. In contrast, 21 BRMS markers, which did not show any segregation in the F_1 population, were

newly located in the present F_2 population map. Based on the locations of the SSR and SLG markers, LG1, LG3, LG4 and LG11 in the present F_2 map could correspond to LG3, LG5, LG1 and LG2 in the previous F_1 map (Tsuro *et al.* 2005), respectively. The correspondence of LG8 in the F_2 map with an LG in the F_1 map remained unclear because BRMS-057-2 and BRMS-303-1 were located on LG8 in the present map and on LG3 and LG5 in the previous F_1 map, respectively. In another linkage map based on an F_2 population derived from the same parental plants as those in the F_1 map, LG1, LG3 and LG8 in the F_1 map fully corresponded to three independent linkage groups of the other map, based on the location of SSR markers (data not shown). These results suggested that mapping of BRMS-057-2 at the bottom of LG3 in the previous F_1 map may have been an artifact due to the lack of connecting markers. LG5 in the previous F_1 map exhibited two marker clusters, and may have separated when additional markers were used.

In comparative mapping of the SSRs between *B. rapa* (Suwabe *et al.* 2006) and *R. sativus* (present study), frequent rearrangements might have occurred in the genomes. Thus, each SSR located on the same LG (LG1, LG3, LG4 and LG8) was derived from different *Brassica* LGs (BLG), respectively. These results corresponded to our previous findings (Tsuro *et al.* 2005). In cruciferous plants, extensive genomic rearrangements were described based on comparative mapping (see Schranz *et al.* 2006 for a review). Based on these and our results, the radish genome may have also undergone frequent rearrangements in the evolutionary process, as in other Crucifers. More detailed genomic rearrangements between *Brassica* and *Raphanus* genomes could be elucidated by the mapping of a larger number of common markers.

Fig. 1. Linkage map of *R. sativus* based on an F_2 population derived from a cross between ‘Huang-he hong-wan’ and ‘Utsugi-gensuke’ using AFLP, *Brassica*-SSR and SLG-CAPS markers. SSR and SLG markers are indicated in bold letters. The number identifying each linkage group is shown at the top. The location of the SSR markers on the *B. rapa* map (Suwabe *et al.* 2006) is indicated on the left side in each group (e.g. BLG5). SSRs denoted by ‘ND’ were not located on the *B. rapa* map.

Root shape

The value of the root shape index of S_3 ‘Utsugi-gensuke’, which has a long root, was 3.59 ± 0.21 , whereas that of S_3 ‘Huang-he hong-wan’, which has a globular root, was 1.09 ± 0.07 . The value of the root shape index of the F_2 individuals showed a continuous normal distribution (Fig. 2A), suggesting that the root shape is controlled by multiple genes. Three QTLs were detected on LG3, LG8 and LG9, respectively. These QTLs exhibited LOD values between 2.42 and 3.22 (Fig. 3A, arrows) and accounted for 42.4% of the phenotypic variance when joined together (Table 2). These results suggested that the root shape trait is controlled by many QTLs with small effects in addition to the above three QTLs. When the QTL for root length and that for root diameter were separately analyzed, two QTLs controlling the root diameter were detected on LG4 and LG8 (Table 2 and Fig. 3B, arrows). The alleles of ‘Huang-he hong-wan’ induced an increase of the diameter at both loci, in spite of the small difference in the root diameter of the parents, namely 7.5 cm for ‘Utsugi-gensuke’ and 9.6 cm for ‘Huang-he hong-wan’. As expected, since the QTLs for root shape and root diameter were located at the same position on LG8, (Table 2), it was assumed that the QTL on LG8 controlled primarily root thickening and, as a result, affected the root

shape. However, the QTL for root length was not detected in the present study, although wide variations in the root length were observed in the parental lines, namely 26.9 cm for ‘Utsugi-gensuke’ and 10.3 cm for ‘Huang-he hong-wan’, and the segregating F_2 population. The length of the root may thus be controlled by a number of genes exerting small effects in this population. The present study did not exclude the presence of a QTL for root length with a large effect in Japanese radish cultivars, because the root of the present parent, ‘Utsugi-gensuke’ was shorter than that of most of the Japanese radish cultivars. Although, the root shape of radish cultivars is known to vary under several environmental conditions, e.g. arcuation and divarication (Iwata *et al.* 1998), the environmental effect was not as large as to induce a change from long type root to spherical one. We used here a parentage with two distinct phenotypes, namely long root and spherical root. At least in the parental lines, duplicated plants showed similar phenotypes. However, since we used here an F_2 population, and evaluated the phenotype of the corresponding genotype based on only one plant, it may be difficult to analyze the environmental effects. Additional data on environmental effects might be obtained by using a recombinant inbred population.

Fig. 2. Frequency distribution of root shape index (A) and red pigmentation of the hypocotyl (B) in an F_2 population derived from a cross between ‘Huang-he hong-wan’ and ‘Utsugi-gensuke’. (A) The score for the parental lines was 1.09 ± 0.07 for the S_3 of ‘Huang-he hong-wan’ ($n = 5$) and 3.59 ± 0.21 for the S_3 of ‘Utsugi-gensuke’ ($n = 5$). (B) Pigmentation grade is scored from 0 (white) to 4 (deep red). Scores in the parental lines were 0 for the S_3 of ‘Utsugi-gensuke’ ($n = 5$) and 4 for the S_3 of ‘Huang-he hong-wan’ ($n = 5$).

Pigmentation level of root surface and leaf veins

In all the F_1 plants, the surface of the hypocotyls showed a deep red pigmentation, while the surface of the lower root was white. The F_1 plants displayed reddish leaf veins with a gradation from deep red at the base of the vein

to green at the tip of the vein. The hypocotyls in 85 out of 104 F_2 plants showed various levels of red pigmentation, while in the remaining plants, red pigmentation was not observed. The level of pigmentation in the F_2 population displayed a continuous right-biased distribution (Fig. 2B), suggesting that one locus exerted a large effect on pigmentation. Two large peaks in LOD scores were detected on LG11 at positions 29.1 cM and 56.1 cM in interval mapping (Fig. 3C), with only one at 29.1 cM, being statistically significant, based on the Kruskal–Wallis test. This QTL exhibited a LOD value of 9.58 and accounted for 43.8% of the phenotypic variance (Table 2). Genotypic effects on pigmentation were not observed by using the marker closest to the peak at 56.1 cM (ACG/CTC1; data not shown). Since in the present map, only two markers were located in the QTL region at 56.1 cM (Fig. 1), this QTL region requires further analysis.

In the parent, ‘Huang-he hong-wan’, extensive pigmentation of the leaf veins was observed unlike in the other parent, ‘Utsugi-gensuke’. We also examined the segregation of the pigmentation for the two traits: in 92 out of 104 F_2 plants, a reddish color of the surface of the leaf veins was observed, while in only six of 104 F_2 plants, a red pigmentation of the lower root surface occurred at a very low level. However, the number of loci controlling the pigmentation could not be determined by statistical analysis.

Aoba (1966, 1968) reported the presence of an F_2 segregation of anthocyanin pigmentation for the petiole, hypocotyl and lower root, in several combinations of pigmented or non-pigmented turnips (*B. rapa*). Nevertheless, the precise number of loci for the pigmentation of the petiole and lower root could not be determined, except for that of the hypocotyl. Based on these and our results, the pigmentation of the leaf veins and lower root of radish may be controlled by complex systems. More detailed analysis on a large scale enable to elucidate the genetic systems involved.

Here, we constructed an F_2 genetic linkage map of *R. sativus*, and obtained QTLs for root shape and hypocotyl pigmentation, in addition to some intergenomic information between *Raphanus* and *Brassica*. This information might be more useful for *Raphanus* breeding if the conversion of the present markers to sequence-specific ones could be achieved for saturation of the present map. Further molecular

Table 2. Putative QTLs detected for root shape, thickening and red pigmentation of the hypocotyl in the F_2 population of ‘Huang-he hong wan’ and ‘Utsugi-gensuke’

Trait	Linkage group	Marker interval ^a	QTL position (cM)	LOD score	Additive effect ^c	Phenotypic variance accounted for (%)
Root shape (length/width)	3	BRMS-303-2–AAC/CTG6	39.7	2.42	–0.24	11.0
	8	AAG/CTA12–ACT/CTT8	86.0	3.22	–0.33	19.3
	9	ACA/CTG5–ACC/CTA6	45.4	2.88	0.25	12.1
Thickening	4	ACA/CTT5 ^b	77.6	3.03	0.71	13.1
	8	AAG/CTA12–ACT/CTT8	86.0	4.51	0.95	23.8
Red pigmentation	11	SLG–AAG/CAG10	29.1	9.58	1.37	43.8

^a QTL peak position, detected by interval mapping and Kruskal–Wallis analysis (1%), between two markers.

^b Terminal side of LG4.

^c Additive effect of the ‘Huang-he hong wan’ allele.

Fig. 3. QTL LOD plots for root shape (A), root thickening (B) and pigmentation of the surface of the hypocotyl (C) in *R. sativus*. The LOD threshold of 2.0 is indicated with a dashed line. Thick line on the horizontal axis indicates statistical significance at the 1% level based on Kruskal–Wallis analysis. Arrow denotes the peak position of a LOD score in the region, supported by both interval mapping and Kruskal–Wallis analyses.

mapping, including SSR development, is currently being conducted, which could enable to elucidate the genetic factors controlling agronomical traits in *R. sativus*.

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