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QTL mapping for tuberous stem formation of kohlrabi (*Brassica oleracea* var. *gongylodes* L.)

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Abstract

The tuberous stem of kohlrabi is an important quantitative trait, which affects its yield and quality. Genetic command of this trait is not unveiled yet. To identify the QTLs controlling stem swelling of kohlrabi, a BC_1 population of 92 plants was developed from the cross of broccoli DH line GCP04 and kohlrabi var. Seine. A wide range of variation for tuberous stem diameter was observed among the mapping populations. We have constructed a genetic map of nine linkage groups (LGs) with different types of markers, spanning a total length of 913.5 cM with an average marker distance of 7.55 cM. Four significant QTLs for radial enlargement of kohlrabi stem, namely, *REnBo1*, *REnBo2*, *REnBo3*, and *REnBo4* were detected on C02, C03, C05, and C09, respectively, and accounted for the phenotypic variation of 59 % for the stem diameter and 55 % for the qualitative grading of tuberous stem in classes. Then, we confirmed the stability of identified QTLs using BC_1S_1 populations derived from the BC_1 plants having heterozygous alleles at the target QTL and homozygous kohlrabi alleles at the remaining QTLs. *REnBo1*and *REnBo2* using 128 plants of BC_168S_1 and 94 plants of BC_143S_1, respectively, and *REnBo3* and *REnBo4* using 152 plants of BC_157S_1 were detected at the same positions as the respective QTLs of the BC_1 population. Confirmation of QTLs in two successive generations indicates that the QTLs are persistent. The QTLs obtained in this study can be useful in marker-assisted selection of kohlrabi breeding, and to understand the genetic mechanisms of the stem swelling and storage organ development in kohlrabi and other *Brassica* species.

Keywords *Brassica oleracea* • kohlrabi • swelling • stem • QTL

Introduction

The genus *Brassica* comprises a diverse group of crops grown for vegetables, oil, condiment and forages. The species relationship of the genus *Brassica* is depicted as the triangle of U (UN 1935), in which *B. rapa* (AA, 2n = 20), *B. nigra* (BB, 2n = 16), and *B. oleracea* (CC, 2n = 18) are the three basic diploids; and *B. carinata* (BBCC, 2n = 34), *B. juncea* (AABB, 2n = 36) and *B. napus* (AACC, 2n = 38) are the three allotetraploids derived from the three progenitor diploid species. The genetic diversity and morphological variation of *Brassica* species, and their relatives, serve as model plants for biomolecular study and provide a valuable opportunity to increase our understanding of plant biology and comparative genomics (Sadowski and Kole 2012; Wang et al. 2011; Ayele et al, 2005). The *Brassica,* and closely related species, morphologically share radial enlargement in different plant organs: hypocotyl in turnip (*B. rapa*), stem in kohlrabi (*B. oleracea*) and tuber mustard (*B. juncea* var. *tumida* Tsen et Lee), root in rutabaga (*B. napus*) and radish (*Raphanus sativus*). These traits have developed independently during the domestication process of each species, but possibly arose from mutation of the same orthologous genes, providing an excellent model to study the biological functionality and morphological evolution of agronomic traits (Hovav et al. 2008; Lenser and Theiben 2013; Fuller et al. 2014; Cheng et al. 2016).

B. oleracea, one of the important species of the family *Brassicaceae*, includes many important vegetables like cabbage, cauliflower, broccoli, and kohlrabi. Kohlrabi (*B. oleracea* var. gongylodes L.) is a cold temperature tolerant cole crop, having a round to spherical shaped radially enlarged tuberous stem. The etymology of the name "Kohlrabi" derives from the combination of the German word for cabbage "Kohl", and the Swiss-German variant name for turnip "Rübe" (The Columbia Encyclopedia, 6th ed.). It is speculated that kohlrabi is derived from the marrow-stem kale that is wild cabbage and used as a fodder crop. European botanists first described kohlrabi in 1554, but descriptions of a similar vegetable referred to as "Corinthian turnip" appears in the writings of Pliny the Elder as far back as 1AD (Lim 2015). The Mediterranean and Western Europe are the primary geographic regions where kohlrabi is cultivated. Kohlrabi is an early spring or fall crop, as it does not grow well in hot weather, and is used as both food and feed. It contains high quantities of numerous functional compounds, such as the anticancer compound glucoraphanin, hydrophilic amino acids, and anti-oxidants that act to protect urolithiasis of the kidney by diuretic effect (Choi et al. 2010; Kishore 2014).

The radially enlarged stem of kohlrabi starts to develop between the 3rd and 5th nodes, followed by the initiation of meristematic activity in pith and parenchyma cells (Selman and Kulasegaram 1966). The swollen part of the stem is mainly composed of parenchyma cells, other xylem components, and pith (Havis 1940). Regulation of the rate of cambial cell division and the timing of cell expansion in cambial daughter cells is

critical for the maintenance and size of the cambium, ultimately determining radial organ size (Havis 1940). In general, this process is hormonally regulated via auxin, cytokinin and brassinosteroids; additional regulation occurs by various environmental factors such as temperature, resource availability, biotic and abiotic stresses, and physiological controls (Miyashima et al. 2013). Lan and Paterson (2001) reported that auxin is involved in cell elongation and cell division, thereby increasing the size of the stem in broccoli. Investigation into the control of cambial activation in the root tips of turnip showed that a combination of auxin, cytokinin, sucrose, and myoinositol was necessary for maximum radial growth of these excised root tips, and that the deletion of any one of these substances resulted in a reduction in cambial activity (Peterson, 2011). Root tips treated with any one of these growth regulators alone showed little cambial activation. Cytokinin signaling and its' downstream transcriptional regulation play an essential role in the radial growth of radish roots (Jang et al. 2015). The expression of *CLE* genes was altered due to auxin and cytokinin application (Gancheva et al. 2016). Overexpression of *RsCLE* genes, *CLE19* and *CLE2*, increased the number of xylem elements and *CLE41* induced the formation of additional cambium foci in *R. sativus*. Polysaccharides are the central part of the storage organs, and sucrose is the principal carbohydrate translocated to the storage organ and deposited as starch (Peter 2009). In this regard, Cheng et al. (2016) reported that energy transport related genes, such as the orthologue of Arabidopsis *SUGAR TRANSPORTER 1* (*BoSTP1*), are thought to be candidate genes for the stem swelling trait of kohlrabi.

QTLs for tuber development in turnip were reported by Lou et al. (2007), Lu et al. (2008), and Kubo et al. (2012). Genetic analyses controlling the root shape development in radish were conducted by Tsuro et al. (2008) and Zaki et al. (2010). QTLs for the stem expansion trait in *B. juncea* were reported by Li et al. (2016). Stem tuber development in kohlrabi is an important quantitative trait which affects both yield and quality. However, to date, no comprehensive investigation of stem enlargement of *B. oleracea* (kohlrabi) has been reported. QTL analysis will contribute to the elucidation of the genetic mechanism of stem enlargement in kohlrabi and understanding how tuber forming kohlrabi and other *Brassica* species were developed during the domestication process. In addition to *Brassica* species, belowground storage organs of other crops such as beets are treated as a part of the shoot (Penning de Vries et al. 1989). QTL analysis for tuber formation in kohlrabi would contribute to understanding the genetic mechanism of the development of storage organs. Therefore, a study was conducted to identify QTLs responsible for stem swelling in kohlrabi using a BC_1 population derived from a cross between kohlrabi and broccoli. Furthermore, the heritability of identified QTLs was confirmed in the offspring, BC_1S_1 populations.

Materials and Methods

Plant materials and population generation

In this study, we conducted QTL analysis for the stem swelling trait of kohlrabi using a BC_1 population and the subsequent generations (BC_1S_1). The schematic diagram of development of the studied plant population is shown in Fig. 1a. To produce BC_1, we chose two parents from *B. oleracea*: one parent broccoli and another parent from kohlrabi. The parent broccoli was a doubled haploid (GCP04) line derived from var. Green Comet (Takii Seed Co. Ltd.), which shows no tuberous stem i.e., the stem of the plant is long and cylindrical in shape (Fig. 1b). The parent kohlrabi, "Seine", (Tohoku Seed Co. Ltd., Japan) was a commercial variety, which shows a radially enlarged, spherical-round shape tuberous stem (Fig. 1c). An F_1 plant (Fig. 1d) was developed from the cross of the DH line GCP04 and Seine. Then, we produced a population of 92 accessions of BC_1 plants obtained by backcrossing an F_1 with the kohlrabi parent. We also developed an F_2 population 163 plants by self-pollination of the F_1 plant, but the low variation for segregation of the tuberous stem formation in the F_2 population was not suitable for a QTL study (Table 1). Data of the well segregating BC_1 population for stem swelling was used for linkage map construction and QTL analysis.

In order to confirm the definite position of the identified QTLs, QTL analysis was conducted in the next generation (BC_1S_1 population) derived from self-pollination of the BC_1 plants having heterozygous alleles for the target QTL and homozygous kohlrabi alleles for the other QTLs. The recombinant line number 68 and 43 of BC_1 population were selected and self-pollinated. In the subsequent generation, BC_168S_1 and BC_143S_1 were used for examination of the activity of *REnBo1* and *REnBo2*, respectively. Similarly, for examination of *REnBo3* and *REnBo4* function, the BC_157S_1 line was selected where *REnBo3* and *REnBo4* regions were heterozygous and the remaining *REnBo1* and *REnBo2* were homozygous for kohlrabi alleles. Homo- and heterozygosity of the QTL regions were determined by the markers located near each QTL region (indicated by asterisk in Fig. 2). For the QTL study, 128 plants were used in the BC_168S_1 population, 94 plants in BC_143S_1, and 152 plants in BC_157S_1 population. In addition, the presence of homozygous functional kohlrabi *BoFLC2* alleles that regulate flowering in *B. oleracea* (Okazaki et al. 2007) were confirmed during the selection of all mapping populations to ensure adequate time for vegetative growth and the development of a tuberous stem.

Seeds were sprouted in water-soaked blotting paper at 25 °C temperature and then planted in commercial soil, suitable for seed germination and seedling emergence. The seedlings were grown in 25 °C temperature with 16 hours of light and 8 hours of dark lighting conditions in a controlled room-environment for up to three weeks. Then, the seedlings were transplanted to nutrient and organic matter rich commercial soil (Honen Agri Co. Ltd., Japan) in l8 cm × 15 cm sized pots and moved to greenhouse conditions where the lowest temperature was 15°C in August-October at Niigata University, Japan. A sufficient amount of water was supplied regularly. Liquid compound fertilizer (Hyponex, N–P–K = 6–10–5) was applied at the young stage of the plants. After that, commercial organic fertilizer mixed with compound chemical fertilizer

Best-Match Vegetable (N–P–K–Mg–B = 20–8–16–1–0.15) was applied and extensive pest protection measures were taken to provide optimal conditions for growth and the swelling of the kohlrabi stem.

Collection of phenotype data

Plants were observed to have started stem swelling at the age of 26-28 days (unpublished data). Phenotype data was collected at 90 days after the sowing of seed, when the plants reached a maximum radial enlargement state of the tuberous stem. Based on the degree of stem swelling, the BC_1 and BC_1S_1 populations were then divided into four qualitative grading in classes (grade 1-4) by naked eye observation. The height and maximum diameter of the enlarged stems were also measured. The ratio of the height and the maximum diameter was determined, dividing the height by the maximum diameter of the tuberous stem, and is termed as the stem enlargement index (SEI). The calculated SEI of tuberous stem was then used to confirm the qualitative grading in classes. The height of the stem was measured from 1 cm above the root-shoot transition zone, where the stem swelling started, to the top of the apical shoot of kohlrabi. The grading of plants based on radially enlarged stems was performed as follows: grade 1, SEI > 1.80, no stem swelling (like broccoli type); grade 2, SEI 1.80 - 1.51, long-elliptical shape; grade 3, SEI 1.21 - 1.50, trophy to hypertrophy shape; and grade 4, SEI <1.21, maximum enlargement of stem (like kohlrabi type) (Supplementary figure 1).

DNA polymorphism analysis

For collection of genotype data, total genomic DNA was extracted from the young leaves of the parents, BC_1, and BC_1S_1 plants using the CTAB method (Murray and Thompson, 1980). The extracted DNA was stored at -20 °C for long-term preservation and the working DNA was kept at 4 °C temperature. In this study, SSR, InDel, and CAPS (cleaved amplified polymorphic sequence) markers were used to amplify the different positions of the genome. First, we used different public DNA markers of *Brassica* to amplify the genomic regions (Supplementary table 1). We also designed DNA markers by retrieving DNA sequences from *B. oleracea* public database EnsemblPlants (http://plants.ensembl.org/Brassica_oleracea/Info/Index) and Bolbase (http://www.ocri-genomics.org/bolbase/).

Additionally, a primer pair distinguishing between functional and non-functional *BoFLC2* was also designed. Primers designed for this study are listed in Supplementary table 2. For genotyping plants, each 10 µl polymerase chain reaction (PCR) mixture contained 1 µl DNA, 0.25 µl forward primer (30 pmol) and 0.25 µl reverse primer (30 pmol), 5 µl PCR EmeraldAmp GT PCR master mix (Takara Bio. Inc., Japan), and 3.5 µl of H_2O. The reaction mixture was incubated in a thermal cycler at 94 °C for 5 minutes (min) for denaturation, followed by 35 cycles of 94 °C for 30 seconds (s), 56 °C for 30 s, and 72 °C for 1 min for denaturation-annealing-primer extension, and finally 72 °C for 7 min. Upon completion of PCR, the samples were subjected to recommended restriction enzyme and incubated 2-5 hours for digestion in the

case of CAPS markers. Then, the samples were mixed with loading buffer and loaded onto 8% polyacrylamide gel and separated at a constant power of 250 V for 5 hours. The gel was subsequently stained using GelStar™ Nucleic Acid Gel Stain (Takara Biomedicals, Japan).

Linkage analysis and map construction

A genotyping data matrix was generated based on the band patterns observed in the BC_1 and BC_1S_1 populations. *Antmap* ver. 1.2 (Iwata, 2006) was employed to develop a linkage map. For the construction of linkage groups (LG) in the BC_1 population, we employed the "nearest neighboring locus" method and, "on recombination" criterion. The recombination threshold was 0.25, and a minimum of three markers was required to develop a group. The analysis was done via the "full course" method. The parameters for locus ordering were, "Haldane map function", "ordering LL", and "targeted groups all".

Statistical analysis and QTL mapping

QTL analysis for stem swelling of kohlrabi was carried out using the phenotypic (stem diameter and qualitative grading of tuberous stem in classes) and genotypic data of each population and their respective linkage map information using *Windows QTL Cartographer v. 2.5* (Wang et al. 2012). In order to estimate the association of each marker to a trait, composite interval mapping (CIM) (Zeng, 1994) was done using the ZMapQtl standard model 6, with a window size of 10 cM and a walking speed of 2 cM. To estimate a genome wide LOD threshold score for a QTL at 95% confidence level (P = 0.05), a 1000-permutation test was performed by shuffling the phenotypic means with the genotype (Doerge and Churchill 1996).

Results

Phenotypic segregation of the mapping population

We observed that kohlrabi had a definite pattern of stem swelling phenomenon. After germination of seed, seedlings of kohlrabi started to grow without stem swelling like other brassica plants. At the age of four weeks, they started stem swelling and reached to the maximum stem swelling at the age 85-95 days (data not shown). During the period of stem swelling, kohlrabi plants showed very low or no vertical growth of stem, thereby developed a round-spherical shaped tuberous stem. After certain growth of tuberous stem, apical shoot started to bolt if they received vernalization effect. Otherwise, kohlrabi showed no vertical growth and apical shoot meristem remained silent up to receiving vernalization effect, indicating that vernalization demarcates the period of tuberous stem formation, and consequently a definite shape. Similarly, recombinant plants having kohlrabi *BoFLC2* alleles also showed dormant apical shoot up to completion of vernalization.

Significant differences were observed among the parental and filial populations for stem swelling (Fig. 1, Table 1). Both parents used in this study displayed opposing phenotypes for the stem swelling trait.

Broccoli DH line GCP04 plants had long, cylindrical shaped stems that exhibited no stem swelling, while kohlrabi var. Seine showed rounded to spherical shaped radially enlarged tuberous stems. Our F_1 plants did not show stem swelling features, almost identical to the GCP04 parent. The highest mean diameter of swollen stem was observed in kohlrabi cv. Seine (66.59 mm), whereas the lowest mean diameter was recorded in broccoli GCP04 (27.32 mm), followed by the F_1 plants (33.91 mm). These results revealed that the stem swelling trait of kohlrabi is a partially recessive phenomenon. The mean diameter of the F_2 population (35.67 mm) did not differ significantly from the F_1 population, although the range of stem swelling in F_2 was higher than in F_1.

A wide ranged distribution of the mean diameter for stem swelling was found in the BC_1 and BC_1S_1 populations. The plants were categorized into four grades (1-4) according to the degree of stem swelling (Supplementary figure 1, 2a, and 4), showing a uniform segregation of the stem swelling trait from slim-cylindrical GCP04 type, grade 1 to round-spherical kohlrabi type, grade 4. The number of plants classified grades 1-2 was higher than that of grades 3-4. We also measured the diameter of the swollen stem. The data fit a normal continuous distribution, but was slightly skewed towards the less radially enlarged grades (Supplementary figure 2b). The distribution of stem swelling phenomenon in the BC_1 and BC_1S_1 populations revealed that kohlrabi stem swelling is a quantitative trait and is controlled by multiple genes.

Linkage map construction of BC_1S_1population

To generate a reliable linkage map of the BC_1 population, we designed CAPS, InDel and SSR primers using the *B. oleracea* genome sequence databases, Bolbase and EnsemblPlants, and assigned 54 DNA markers to the LGs derived from the BC_1 population. Sixty seven public DNA markers were also anchored to the LGs using the *AntMap* ver. 1.2 software. The generated map for kohlrabi spanned 913.5 cM, with an average marker density of 7.55 cM (Supplementary figure 3). The consensus marker order of the maps was confirmed by aligning the primer sequences to the reference genome of *B. oleracea* (http://www.ocri-genomics.org/bolbase/) and comparing the PCR amplicon size with the primer targeted region of the reference genome. The length of the longest LG was 143.7 cM on C08 followed by C05 (122.5 cM), and the shortest (63.5 cM) LG was on C04. The average distance between two adjacent markers was at a minimum (5.69 cM) for C02 and a maximum (9.56 cM) for C08. The highest number of markers (20) was anchored on C02 and C03 followed by C09 (18), and the lowest number of markers (7) was on C04 and C06. We have calculated the coverage of physical distance of public database (Bolbase) by our genetic map. Based on the physical position of the two terminal markers on each the chromosome confirmed by blast search, our map covered an average of 84.32 % of Bolbase database. The distance of the markers, and their distribution, was therefore relatively even and reliable for a QTL study over the whole genome of *B. oleracea* var. *gongylodes*.

Identification of QTL for stem swelling in BC_1 population

A mapping population of 92 BC_1 plants was developed by crossing the F_1 population with kohlrabi var. Seine. For the BC_1 plants, QTL analysis was conducted with the *Windows QTL Cartographer*, using the phenotypic data of qualitative grade of stem and stem diameter measurement. As a result, four significant QTLs were detected at the same positions of chromosomes in both measurement methods with the threshold LOD score of ≥2.6 (Fig. 2). All alleles of the QTLs contributed to stem swelling came from the kohlrabi parent. QTL1 on C02, QTL2 on C03, QTL3 on C05, and QTL4 on C09 were designated *REnBo1*, *REnBo2*, *REnBo3*, and *REnBo4*, respectively. For the grade of stem swelling, the QTLs *REnBo1*, *REnBo2*, *REnBo3*, and *REnBo4* accounted for 13 %, 16 %, 11 %, and 15 % of the total phenotypic variation, respectively, and altogether covered 55 % of the phenotypic variance (Fig. 2, Supplementary table 3). On the other hand, all four QTLs accounted for a total of 59 % of the total phenotypic variation for the stem diameter trait, where *REnBo1*, *REnBo2*, *REnBo3*, and *REnBo4* accounted for 17 %, 14 %, 13 %, and 15 % of the total phenotypic variation, respectively. The QTL *REnBo1* was detected with the maximum LOD score 6.8 at 37.3 – 62.2 cM on C02 where the leading markers were Bol015919, Bol014818, Bol031140, and BoGMS0032. This QTL was sandwiched by Bol031107 and Bol020154. *REnBo2* was detected with the maximum LOD score 6.2 at 0.00 – 23.3 cM on C03 and was bordered by DNA markers Bol035216 – Bol017491. The distinguishing markers of *REnBo2* were Bol035202, B0l035086, Bol035085, Bol035059, and Bol013022. We identified the *REnBo3* QTL with the maximum LOD score 5.2 at the 32.1 – 61.0 cM position of C05. This QTL was sandwiched by pW164 and BoSF317, and the neighboring markers were BoSF2541, pW164 and Bol026834. We found *REnBo4* on C09 with the maximum LOD score of 5.9. This QTL was anchored at 32.1 – 62.0 cM of LG9, between Bol019306 and Bol032093. The other representative markers of this QTL were Bol012694, Bol019328 and Bol012707.

Confirmation of BC_1 QTLs in BC_1S_1 generation

In order to confirm the identified BC_1 QTLs, QTL analysis was conducted in the subsequent generation (BC_1S_1 population) derived from the selected BC_1 plants, as mentioned in the Materials and Methods section. The DNA markers indicated by the asterisk in Fig. 2 were used for the determination of homo- and heterozygosity of the QTL's region. For confirmation of *REnBo1*, we chose the genotype #68 from the BC_1 population ($BC_1$68), which had heterozygous alleles for the *REnBo1* position and homozygous kohlrabi alleles at the remaining QTLs. We developed a population of 128 plants by self-pollination of $BC_1$68 plant. In the resulting population ($BC_1$68S_1), we found a good segregation of phenotypic variation for stem swelling (Fig. 3c, Supplementary figure 4). In this population, we constructed a linkage group for C02 where *REnBo1* was detected in the BC_1 QTL analysis. LGs for the other chromosomes were not constructed, but we confirmed that other QTL regions have homogeneous kohlrabi alleles in all plants of this population

275 ($BC_1 68S_1$) by using the candidate markers (asterisk marked markers in Fig. 2). We again detected QTL for
276 stem swelling on C02 with the maximum LOD score 13.6 for stem swelling grade and 21.1 for stem
277 diameter, which accounted for 24% and 67 % of the total phenotypic variation for stem swelling grade and
278 stem diameter, respectively (Fig. 3a). The position and identifying markers of *REnBo1* on C02 of the BC_1S_1
279 population were consistent with the detected position and markers of C02 in the BC_1 population. The precise
280 position of *REnBo1* was 17.3 cM to 35.6 cM, and leading markers of the QTL were Bol031187, Bol015919,
281 Bol014822, Bol031140, Bol031107, BoGMS0032 and Bol014818.
282 The genetic segregation of kohlrabi and broccoli alleles at the *REnBo1* region was associated with the
283 frequency distribution of phenotype of the $BC_1 68S_1$ population (Fig. 3c, Supplementary figure 4); plants
284 having a homozygous kohlrabi allele at the Bol031140 marker produced mostly rounded to spherically
285 shaped swollen stems (grade 3-4), whereas plants homozygous for the GCP04 allele showed little to no stem
286 swelling (grade 1-2). Plants heterozygous at the *REnBo1* region exhibited an intermediate phenotype for
287 stem swelling, although some heterozygous plants displayed a phenotype close to that of either the kohlrabi
288 or the GCP04 phenotype. The effect of environmental factors is thought to be higher in the case of absence
289 of one/more kohlrabi allele(s). Alternatively, recombination between the closest DNA marker and the
290 responsible gene might break correlation between phenotypes of plants and genotypes of DNA markers.
291
292 To confirm the perpetuity and precise position of *REnBo2*, we chose a plant from the $BC_1 43$ plant that
293 had heterozygous alleles for *REnBo2* region, but homozygous kohlrabi alleles at the other QTL positions,
294 along with homozygous *BoFLC2* alleles of kohlrabi. We then developed a $BC_1 43S_1$ population of 94 plants
295 by self-pollination. The population showed good phenotypic variation for the stem swelling trait (Fig. 3d,
296 Supplementary figure 4). *REnBo2* was also detected at the same position on C03 in QTL analysis of the
297 $BC_1 43S_1$ population, with the maximum LOD score 18.5 for the stem swelling grade and 11.2 for the stem
298 diameter, which accounted for 42 % and 51 % of the phenotypic variation, respectively (Fig. 3b).
299 Genotyping of $BC_1 43S_1$ plants by one of the nearest markers (Bol035086) of *REnBo2* showed a significant
300 association with the phenotypic variation (Fig. 3d, Supplementary figure 4).
301 In the QTL analysis of BC_1 population, *REnBo3* and *REnBo4* were detected on C05 and C09,
302 respectively. For re-examination of the effect of *REnBo3* and *REnBo4*, we utilized the $BC_1 57$ line, in which
303 these two QTL regions were heterozygous and the remaining QTLs including *BoFLC2* have homozygous
304 kohlrabi alleles. Self-pollination was done to produce BC_1S_1 seeds in $BC_1 57$ line. We developed a BC_1S_1
305 population of 152 plants and observed their stem swelling pattern. A good segregation of stem swelling in
306 the $BC_1 57S_1$ population was found (Fig. 4c, Supplementary figure 4). Likewise *REnBo1* in $BC_1 68S_1$
307 population, we also constructed LG in $BC_1 57S_1$ population only for C05 and C09, and the other QTL
308 regions were confirmed to be homozygous kohlrabi alleles using the DNA markers (asterisk marked
309 markers in Fig. 2). The QTL analysis confirmed that *REnBo3* and *REnBo4* were again detected on C05 and

C09, respectively with the threshold LOD 2.3 (Fig. 4a, 4b). The maximum LOD of *REnBo3* was 5.1 for stem swelling grade and 7.1 for stem swelling diameter, which is accounted for 11 % and 14 % of the phenotypic variation, respectively in the BC_1S_1 population. *REnBo4* was detected with a maximum LOD score 15.6 for stem swelling grade and 14.5 for stem diameter, accounting for 43% and 38% of the phenotypic variation, respectively in the BC_1S_1 population. The QTL graph of *REnBo4* in BC_1S_1 population appeared broad. The selected $BC_1$57 line contained a short heterozygous *REnBo4* region (flanked by Bol019258 and Bol012707 markers spanning 1.19 Mbp), which was sandwiched by homozygous kohlrabi chromosomal segments encompassing BoSF2564 - Bol019258 and Bol012707 - BoGMS0662. In this homozygous flanking region, it was not possible to assign polymorphic markers. Therefore, the scarce marker density in the flanking region might cause the broad QTL graph. According to the Bolbase database, the heterozygous region is flanked by Bol019258 and Bol012707 markers, spanning 1.19 Mbp which contain a total of 130 genes. A syntenic gene analysis showed that the region is about 1.22 Mbp in EnsemblPlants database.

Genotyping BC_1S_1 plants with the closest markers, BoSF2541 (*REnBo3*) and Bol012694 (*REnBo4*) showed that homozygous kohlrabi alleles at *REnBo3* and *REnBo4* mostly displayed a kohlrabi phenotype for stem swelling, likewise plants containing homozygous broccoli GCP04 alleles exhibited a broccoli phenotypes (Fig. 4c, Supplementary figure 4). Those plants having heterozygous alleles for the *REnBo3* and *REnBo4* markers tended to exhibit an intermediate phenotype for stem swelling. Additionally, we observed a significant cumulative effect for *REnBo3* and *REnBo4* for the stem swelling of kohlrabi, with a more significant contribution derived from *REnBo4*, compared to *REnBo3* (Fig. 4c, 4d).

Discussion

Linkage map development

Brassica species appear to have included a number of ecotypes that resulted in profound changes in the size, shape, and timing of development of most the plant organs. The fact that such morphological divergence has occurred among taxa that can be intercrossed to generate fertile progenies makes Brassica a fascinating subject for genetic analysis (Lan and Paterson, 2000). Construction of genetic linkage maps is the first step in genetics research, such as mapping of desirable traits, QTL analysis, and gene identification. The linkage map in this study was constructed using the 92 plants BC_1 population derived from the cross of two parents, kohlrabi and broccoli GCP04. It consisted of nine LGs corresponding to the nine chromosomes of *B. oleracea*, spanning a total length of 913.5 cM, which was consistent with the length of the linkage map (934.1 cM) used in the study of the cabbage heading trait QTL in *B. oleracea* (Lv et al. 2014), the length of linkage map (703 cM) used for development of a high-density genetic map of *B. oleracea* (Gao et al. 2007), and the integrated genetic map of 893 cM in two different F_1 haploids of *B. oleracea* (Sebastian et al. 2000).

Based on the physical position of the two terminal markers on each chromosome confirmed by blast search, our study covered an average of 84.32 % of the genome of *B. oleracea* according to the Bolbase database (http://www.ocri-genomics.org/bolbase/). The highest coverage was observed in C08 (93.7 %) followed by C03 (92.7 %) and C02 (91.1 %), and the lowest coverage was found in C06 (71.1 %). The average distance of the markers was 7.55 cM in this study indicating that the total coverage of distance, average distance of markers, and marker distribution are suitable for QTL study. We also constructed a linkage map of C02 and C03 in the $BC_1 68S_1$ and $BC_1 43S_1$ populations, respectively, and C05 and C09 in the $BC_1 57S_1$ generation to confirm the QTLs position identified within the BC_1 population. The length of these linkage maps was shorter in comparison to the respective linkage maps in the BC_1 population, because the BC_1S_1 population partially contained a fixed homozygous chromosomal segment from a parental genome, and only successful co-dominant markers were mapped to the linkage map of BC_1S_1 population.

Quantitative control of the stem swelling trait

Parents of the mapping population, kohlrabi var. Seine and broccoli GCP04 showed the highest and the lowest stem swelling, respectively (Table 1). The degree of stem swelling in F_1 plants was close to that of broccoli GCP04, revealing that the stem swelling trait of kohlrabi is expressed in a recessive manner. The range and mean of diameters of stem measured in the F_2 population were narrow, which was close to that of the F_1 population (Table 1). These results revealed that the swelling trait of kohlrabi tuberous stem is under oligogenic control with several recessive genes. Similarly, Baggett and Kean (1989) reported that the F_2 population derived from the cross of kohlrabi and broccoli showed a continuous distribution of stem diameter, which was skewed toward the broccoli type, indicating the recessive expression of the kohlrabi phenotype in the kohlrabi × broccoli F_2 population. These results suggested that F_2 population is not a suitable choice for a QTL study of stem swelling in kohlrabi, because recessive alleles are responsible for the tuberous phenotype of kohlrabi. In addition, if all of QTLs act in an additive fashion with respect to the stem swelling trait, the lack of function in one or more kohlrabi allele(s) proportionally affects the expression of the trait.

Due to the lack of suitable segregation of the stem-swelling phenotype in the F_2 population, we therefore, attempted to identify QTLs responsible for stem-swelling in kohlrabi using a BC_1 population. The BC_1 population showed ample variation for stem swelling trait, and the segregation pattern fits well to a normal continuous distribution which was suitable for a QTL study (Table 1, Supplementary figure 1, Supplementary figure 2). It can be noted that BC_1 populations have been used in previously reported genetic analyses for the fine mapping of the club root resistance QTL in Chinese cabbage (Gao et al. 2014) and the fine mapping of a major locus controlling plant height in *B. napus* (Wang et al. 2016). Lou et al. (2007) also identified QTLs for tuberous root formation using BC_1 population in turnip. Conversely, Li et al. (2016) identified QTL for stem expansion trait in the F_2 population of *B. juncea*, where two hundred F_2 plants were

used to identify a stem swelling QTL. However, the data of stem expansion in the F_2 population appeared considerably closer to that of the F_1 population, and the mean diameter had skewed to the leaf mustard parent with the cylindrical stem. Lu et al. (2008) reported 18 tuber-forming QTLs of *B. rapa* using a population of one hundred and thirteen F_2:F_3 plants, although the taproot weight was deviated from a normal distribution and skewed to the less root swelling phenotype.

In this study, we identified four significant QTLs controlling stem swelling trait of kohlrabi, which explained about 59 % of the total phenotypic variation, and those QTLs were confirmed in the successive generations. This is the first report to identify stem swelling QTLs in kohlrabi as well as in *B. oleracea*. It was revealed that stem swelling of kohlrabi is a multi-recessive trait attributed to at least four QTLs, which can be designated as a "hyper-recessive" trait. The quantitative trait found in such a BC_1 population is controlled not only by multiple genes, but also by a few individual genes that possess a sensitivity response to environmental factors. In fact, the plants with heterozygous alleles at the stem-swelling QTL region showed a wide phenotypic distribution encompassing intermediate phenotypes of GCP04 and kohlrabi (Fig. 3c, 3d). This result indicates that the stem swelling trait of kohlrabi, especially in the heterozygous genotypes, is highly affected by a complex interaction of genetic and environmental conditions. Therefore, the interactive environmental factors that contribute to the expression of radial enlargement of tuberous stem still remains to be determined.

The QTLs for turnip development have been reported by Lou et al. (2007), Lu (2008), and Kubo et al. (2010). Two QTLs controlling the size and weight of turnip in A01 and A05 were detected independently in the two trials, indicating that these two QTLs had stable effects (Kubo et al. 2010); they also detected a turnip formation QTL at the top of LG2, co-localized with *BrFLC2* locus. Similarly, Lou et al. (2007) detected a QTL for turnip formation co-localized with *BrFLC2* locus on A02 of *B. rapa*, indicating that flowering time QTL significantly affects tuber formation of turnip, because a plant that flowers early hampers tuber formation by allocating its energy to flower formation and seed development (Lou et al. 2007). To avoid the influence of the non-functional broccoli *BoFLC2* gene reported by Okazaki et al. (2007) who found the frameshift mutation in broccoli (GCP04) *BoFLC2* gene, we used the plants of BC_1 and BC_1S_1 populations with a functional *BoFLC2* gene of kohlrabi. As a result, our identified stem swelling QTLs did not show co-localization with the *BoFLC2* locus, indicating the identification of QTLs directly relevant to stem swelling. Tsuro et al. (2008) identified three QTLs for root shape index of radish, LG3, LG8, and LG9, which accounted for a total of 42.4% of the phenotypic variation. Li et al. (2016) reported four QTLs for stem weight and one QTL for stem diameter in *B. juncea*. But, we could not confirm the similarity of orthologous gene function for stem swelling in *Brassica* species and its' allies, because their studies neither used any public DNA markers, nor published their amplicon sequences. Cheng et al. (2016) reported 19 genes including *BOSTP1* that are thought to be responsible for tuber morphotype formation in kohlrabi. But we found no QTL on those regions (Supplementary figure 5).

Some of the plants of all groups of the recombinant populations (BC_1 and BC_1S_1) exhibited transgressive segregation beyond the maximum level of stem swelling of kohlrabi parents (Table 1). This result revealed that one secret QTL might contribute from broccoli GCP04, which was not accumulated in kohlrabi during domestication. Alternatively, the transgressive segregation might come from hybrid vigor appeared in the heterozygous plants of the BC_1 and BC_1S_1 populations. Transgressive segregation was also observed in anti-oxidant capacity related QTLs in *B. oleracea* (Sotelo et al. 2014), QTLs for oil content in *B. napus* (Javed et al. 2016), and QTLs for head splitting resistance in *B. oleracea* (Su et al. 2015). In this study, we could not detect any stem swelling alleles derived from GCP04, whereas we successfully identified four stem swelling alleles of kohlrabi. Additionally, we could not confirm whether this hyper-tuberization phenomenon of kohlrabi stem was the result of transgressive segregation or hybrid vigor, which needs to be investigated.

Although the QTL regions detected in this study still encompass long genomic regions, we have delimited *REnBo4* to the region of 1.2 Mbp, which contains a total of 130 genes according to the Bolbase database. Ontology of the homologous genes in *A. thaliana* showed that the delimited region contains DNA binding/transcription factors and the auxin related genes such as an auxin efflux carrier (Bol019308), auxin-responsive protein (Bol019289). These might be candidate genes, likely thereby inducing cell division in the pith and parenchyma cells and determining radial stem enlargement. Similarly, genes of the other QTL regions observed to be responsible for auxin:hydrogen symporter/transporter (PIN-Formed 4) (Bol014822), AFB2 (Bol031116), ARF (Bol026834) including some genes with unknown functions. To date, no previous QTL studies regarding the stem swelling of kohlrabi or *B. oleracea* are available to the best of our knowledge. Cheng et.al (2016) used another approach, analyzing resequencing data of a large *B. oleracea* collection to identify selective sweeps for stem swelling trait in domestication of kohlrabi. As candidate genes, they reported one gene (*BOSTP1*) for tuberous morphotype formation.

Our results provide important information into the identification of the genes responsible for the stem-swelling trait in kohlrabi as well as understanding of domestication process in kohlrabi. Different agronomic traits that have contributed to the domestication of modern crops, such as the shattering trait of rice (Li et al. 2006) and barley (Haberer and Mayer 2015), and the seed dormancy trait in rice (Sugimoto et al. 2010) were produced by single mutations of their respective genes. In contrast to some of those traits, we revealed that stem swelling in kohlrabi is attributed to multiple loci encompassing recessive alleles, indicating unique domestication process of kohlrabi. Further research is warranted to elucidate the stem swelling mechanism in kohlrabi. Identification of the responsible genes, their mode of expression, and gene interaction have yet to be revealed. Moreover, the stem swelling trait of kohlrabi is complex, and appears to be affected by environmental factors.

Author contributions MH developed BC_1 populations, designed markers, genotype and data collection of BC_1 and BC_1S_1 populations, software running and writing script. DJS provide sequence information for the design of marker and bioinformatics analysis. MA, MAUD, and MS developed BC_1 population from the parents and did partial genotype of BC_1. MH, RF, EF, and KO conceived and designed the experiment. KO contributed to the planning, provided funding, and managed the whole project. All authors contributed to the development of this manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.
Ethical standards The experiment conducted complies with the laws of Japan.

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Fig. 1 Schematic diagram of development of plant populations, and phenotypes of the parents and F_1 hybrid. **a** flow-diagram of development of plant populations (BC_1 and BC_1S_1); **b** broccoli parent (GCP04); **c** kohlrabi parent (cv. Seine); **d** F_1 hybrid. Bar = 5 cm

Fig. 2 Linkage maps of *B. oleracea* and QTLs for stem swelling trait of kohlrabi resulting from analysis of BC_1 population. Marker position is indicated in centimorgan (cM) on the left side of the linkage group, and locus of ordering were provided on the right side. The positions of QTLs were indicated by line graphs. The DNA markers for determination of homo- and heterozygosity for the QTL regions to produce the subsequent generations are indicated by the asterisks

Fig 3 QTLs and frequency distribution for stem swelling trait of kohlrabi in the BC_1S_1 populations. Linkage maps of *B. oleracea* and QTLs for stem swelling trait; **a** Confirmation of *REnBo1* in BC_168S_1 population; **b** Confirmation of *REnBo2* in BC_143S_1 population. Frequency distribution of stem swelling in different grades and the genotypes at the closet markers of each QTL; **c** BC_168S_1 population; d BC_143S_1 population. The homozygotes of GCP04, homozygotes of Seine, and heterozygotes at each marker are indicated by different colors, respectively

Fig 4 QTLs and frequency distribution for stem swelling trait of kohlrabi in the BC_157S_1 populations, which revealed segregation of the parental alleles in both of the stem-swelling QTLs, *REnBo3* and *REnBo4*. **a** confirmation of *REnBo3*; **b** confirmation of *REnBo4*; **c** frequency distribution of stem grade and the genotypes at the closet markers of each QTL. *a* and *A* represent kohlrabi allele and broccoli allele on *REnBo3* locus, respectively, and *b* and *B* represent kohlrabi allele and broccoli allele on *REnBo4* locus, respectively; **d** illustration of additive effect between *REnBo3* and *REnBo4* locus found in the BC_157S_1 population. Explanation of *A*-*a* and *B*-*b* is the same as **c**