Linkage and regional association analysis reveal two new tightly-linked major-QTLs for pod number and seed number per pod in rapeseed (*Brassica napus L.*)

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To facilitate the pseudochromosomes assembly and gene cloning in rapeseed, we developed a reference genetic population/map (named BnaZNF2) from two sequenced cultivars, Zhongshuang11 and No.73290, those exhibit significant differences in many traits, particularly yield components. The BnaZNF2 genetic map exhibited perfect collinearity with the physical map of *B. napus*, indicating its high quality. Comparative mapping revealed several genomic rearrangements between *B. napus* and *B. rapa* or *B. oleracea*. A total of eight and 16 QTLs were identified for pod number and seed number per pod, respectively, and of which three and five QTLs are identical to previously identified ones, whereas the other five and 11 are novel. Two new major QTL respectively for pod number and seed number per pod, *qPN.A06-1* and *qSN.A06-1* (*R² = 22.8% and 32.1%*), were colocalised with opposite effects, and only *qPN.A06-1* was confirmed and narrowed by regional association analysis to 180 kb including only 33 annotated genes. Conditional QTL analysis and subsequent NILs test indicated that tight linkage, rather than pleiotropy, was the genetic causation of their colocalisation. Our study demonstrates potential of this reference genetic population/map for precise QTL mapping and as a base for positional gene cloning in rapeseed.

Rapeseed, *Brassica napus* L. (AACC genome, *2n = 38*), is mainly cultivated for the production of edible oil and is the second largest oil crop globally, after soybean. Rapeseed originated from recent (~0.01 million years) allopolyploidy between the ancestors of two diploid species, *B. rapa* (AA genome, *2n = 20*) and *B. oleracea* (CC genome, *2n = 18*)1. Yield is the most important target for the genetic improvement of most crops, including rapeseed. Yield is also the most complex trait in crops. Yield is multiply determined by its component traits2, each of which is quantitatively inherited and controlled by many quantitative trait loci (QTLs). In addition, negative correlations are usually observed among yield components, which has traditionally been considered the genetic trade-off among competitive demands, i.e., antagonistic pleiotropy3. Among the three yield components of rapeseed, pod number is most highly correlated with seed yield, followed by seed number per pod, while the correlation of seed weight with yield is relatively low2, suggesting that the pod number and seed number per pod are the major contributors to the yield in rapeseed.

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Nearly ten linkage QTL mapping studies and one association analysis study involving pod number and/or seed number per pod in rapeseed have been reported, but the genetic and molecular bases of both traits remain ambiguous. First, the linkage maps used in these studies involved markers with unknown sequence information, and the genomic sequence of rapeseed was unknown, preventing the integration of these QTLs in the reference physical map and the identification of the underlying genes. Second, the genomic sequences of the parental cultivars used in these studies were unknown, complicating the development of molecular markers and the screening of candidate genes. More importantly, only a few major QTLs for pod number and/or seed number per pod have been identified, and none have been fine-mapped or further cloned.

Recent innovations in genome sequencing technology and bioinformatics have enabled the sequencing of European winter type rapeseed cultivar Darmor-bzh and Chinese semi-winter type cultivar Zhongshuang11 (http://www.brassica.info/resource/sequencing.php) by our own and several other institutes. Approximately a dozen linkage maps have been developed using different genetic populations of rapeseed (http://www.brassica.info/resource/maps/published-data.php); however, the number of sequence-based markers in these linkage maps was insufficient, and none of these genetic populations were derived from Zhongshuang11. To accurately anchor and orientate the 2730 sequence scaffolds of Zhongshuang11 (http://www.ncbi.nlm.nih.gov/genome/genomes/203), a skeleton linkage map of sequence-based markers must be constructed using a reference population derived from itself. In addition, tens of rapeseed cultivars have also been resequenced (http://www.brassica.info/resource/sequencing.php). These sequenced cultivars may represent a unique genetic resource for the development of reference populations for genetic study in rapeseed.

Among the sequenced cultivars, Zhongshuang11 (de novo sequencing) and No.73290 (resequencing) exhibit significant differences in many traits related to agronomy, plant/root architecture and yield (particularly pod number and seed number per pod), suggesting that they are ideal parents for reference population construction and genetic study in rapeseed. The main objectives of the current study were the following: (1) construction of a skeleton linkage map with sequence-based polymerase chain reaction (PCR) markers using the reference population derived from Zhongshuang11 and No.73290; (2) validation of the quality of reference skeleton linkage map by comparative mapping with the physical maps of *B. napus* and *B. rapa/B. oleracea*; (3) mapping and comparison of QTLs for pod number and seed number per pod using the reference skeleton linkage map; (4) validation and fine mapping of the major QTLs by regional association analysis; and (5) dissection of the genetic basis of the colocalised QTLs for pod number and seed number per pod by conditional QTL analysis.

**Results**

**Construction of a skeleton linkage map using the BnaZNF population.** Of the 9351 screened primer pairs, i.e. 6504 simple sequence repeat (SSR), 2592 single nucleotide polymorphism (SNP), 179 sequence tagged site (STS) and 76 Insertion/Deletion (InDel), 961 (10.3%) primer pairs displayed clear polymorphism between the two parental cultivars Zhongshuang11 and No.73290, producing 1038 sequence-based PCR markers in the BnaZNF population (Supplementary Table S1).

All 1038 markers were subjected to Joinmap 4.0 software for linkage analysis, which resulted in a linkage map of 19 linkage groups and 803 markers (710 SSR, 48 SNP, 31 InDel and 14 STS) (Fig. 1; Table 1). Of these, 499 SSR markers (prefixed by “BoSF”, “BrSF” and “BnSF”), 31 SNP (prefixed by “ns”) markers and all 31 InDel markers (prefixed by “BnID”) were newly developed and mapped (Supplementary Table S2). The 19 linkage groups were successfully assigned to the 19 chromosomes of the A (A01–A10) and C (C01–C09) genomes, respectively. The length of the 19 linkage groups ranged from 32.1 (C07) to 148.7 (C03) cM, with a sum and mean of 1763.2 and 92.8 cM, respectively. It should be noted that A05, A08, A10, C07 and C09 were typically shorter than the other 14 linkage groups. The marker density of the 19 linkage groups ranged from 4.01 (C07) to 1.45 (A09) cM, with an average of 2.19 cM.

Only 40 (5.0% of the total) markers exhibited significant (P ≤ 0.001) segregation distortion, of which 5 and 35 skewed to Zhongshuang11 and No.73290, respectively (Table 1). Interestingly, the distorted markers tended to be concentrated on/near the two ends of the linkage groups. The distorted markers on A06 bottom, A09 top and C02 bottom skewed to Zhongshuang11, whereas those on A01 top, A04 bottom and A09 bottom skewed to No.73290 (Supplementary Table S3).

**Validation of the quality of BnaZNF linkage map by comparative mapping with the physical maps of *B. napus* and *B. rapa/B. oleracea*.** The availability of the pseudochromosomes of *B. napus*, *B. rapa* and *B. oleracea* facilitated the comparison of genetic and physical maps in *Brassica*. Comparative mapping between the BnaZNF linkage map and the physical maps of *B. napus* and *B. rapa/B. oleracea* was based on the markers aligned to their pseudochromosomes, and the results are displayed using dot-plots (Fig. 2).

A total of 26, 17 and 733 markers were successfully aligned to the pseudochromosomes of *B. napus*, *B. rapa/B. oleracea* and both, respectively, while the remaining 27 failed (Supplementary Table S4). Nearly all markers mapped on each of the 19 linkage groups were collinear with the corresponding pseudochromosomes of *B. napus*. This collinearity was also supported by the high correlation between the genetic and physical positions of the aligned markers, with coefficients ranging from 0.884 (C05) to 0.997 (A03) for the different chromosomes (mean = 0.963) (Table 2). These results strongly indicate the high quality...
of the current BnaZNF2 linkage map. High collinearity was also observed between most linkage groups and the corresponding pseudochromosomes of *B. rapa*/*B. oleracea*, with the exception of C05 and C06. The discontinuous collinearity observed for C05 and C06 was also supported by the intermediate correlation between the genetic and physical positions of the aligned markers, with $r = 0.586$ and 0.640, respectively. A detailed comparison of the physical positions of these aligned markers revealed several obvious genomic rearrangements between the pseudochromosomes (including C05 and C06) of *B. napus* and *B. rapa*/*B. oleracea*: translocation in one location in A01, A02 and C01 and in two locations in C05 and C06; inversion in one location in C02 and C06; and reshuffling in A05 and C04 (Fig. 2; Supplementary Table S4).

**Phenotypic variation and correlation of pod number and seed number per pod in the parents and linkage population.** The two parents (Zhongshuang11 and No.73290) differed significantly in pod number and seed number per pod in all four investigated environments (Table 3). The pod number of No.73290 was approximately 50% greater than that of Zhongshuang11, while the seed number per pod of Zhongshuang11 was nearly twice that of No.73290. Interestingly, the pod number of branch
inflorescence was nearly twice that of main inflorescence in both the parents and populations. A normal or near-normal distribution was observed in the populations for both traits in all four investigated environments (Fig. 3), indicating a quantitative inheritance suitable for QTL identification. In addition, the populations exhibited transgressive segregation of both traits but with a small degree (Table 3), indicating that the favourable alleles were mainly distributed on one of the two parents.

The pod number of the whole plant was significantly positively correlated with those of the main and branch inflorescence with moderate and high coefficients, respectively (Table 4), suggesting that the pod number of the whole plant is mainly determined by that of the branch followed by the main inflorescence. The pod numbers of the main and branch inflorescence were also significantly positively correlated with moderate coefficient, suggesting that the genetic determination of the pod number of the main and branch inflorescence differs. The seed number per pod of the main and branch inflorescence were significantly positively correlated with high coefficient, indicating similar genetic control. As expected, the pod number and seed number per pod were significantly negatively correlated for the main and branch inflorescence as well as the whole plant, suggesting competition among sinks for assimilates. However, these correlation coefficients were all small, suggesting that the final seed yield of rapeseed (*B. napus* L.) could be improved by increasing its component traits, such as pod number and/or seed number per pod.

Analysis of variance (ANOVA) revealed that genotype, environment and the genotype × environment interaction all have significant effects on both the pod number and seed number per pod of the main and branch inflorescence as well as whole plant (Supplementary Table S5). The broad-sense heritability of the pod number was moderate as follows: main inflorescence (0.65) > whole plant (0.49) > branch inflorescence (0.45) (Table 4). Whereas, the broad-sense heritability of the seed number per pod was equally high for the main (0.83) and branch (0.82) inflorescence as well as whole plant (0.83).

**Linkage mapping and integration of the QTLs for pod number and seed number per pod.** A total of 69 QTLs (51 and 18 at significant and suggestive levels, respectively) were detected for pod number and seed number per pod in five experiments (Supplementary Table S6A). After deleting seven non-reproducible suggestive QTLs, 16 and 46 QTLs were identified for pod number and seed number per pod, respectively, which were distributed on seven (A01, A03, A05, A06, A09, C02 and C06) and nine (A01-A03, A05-07, A09, C02 and C06) of the 19 linkage groups (Supplementary Table S6B). Where the

| Linkage group | Markers number | Genetic distance (cM) | Recombination frequency* | Marker density* | Coverage ratio (%)* | Number of marker skewed to Zhongshuang11 | No.73290 | Total |
|---------------|----------------|----------------------|--------------------------|----------------|-------------------|----------------------------------------|----------|-------|
| A01           | 49             | 128.8                | 5.6                      | 2.63           | 98.2              | 0                                      | 5        | 5     |
| A02           | 54             | 82.9                 | 3.7                      | 1.54           | 91.0              | 0                                      | 0        | 0     |
| A03           | 72             | 129.1                | 5.3                      | 1.79           | 81.7              | 0                                      | 0        | 0     |
| A04           | 33             | 87.1                 | 4.8                      | 2.64           | 94.4              | 0                                      | 2        | 2     |
| A05           | 33             | 55.6                 | 4.0                      | 1.68           | 61.1              | 0                                      | 0        | 0     |
| A06           | 67             | 113.3                | 4.7                      | 1.69           | 99.3              | 1                                      | 0        | 1     |
| A07           | 27             | 96.8                 | 4.2                      | 3.59           | 96.7              | 0                                      | 0        | 0     |
| A08           | 14             | 32.6                 | 12.5                     | 2.33           | 13.9              | 0                                      | 0        | 0     |
| A09           | 87             | 125.9                | 4.2                      | 1.45           | 88.5              | 2                                      | 28       | 30    |
| A10           | 18             | 48.5                 | 6.9                      | 2.55           | 40.6              | 0                                      | 0        | 0     |
| C01           | 34             | 102.4                | 2.7                      | 3.01           | 99.5              | 1                                      | 0        | 1     |
| C02           | 38             | 113.7                | 2.4                      | 2.99           | 96.7              | 1                                      | 0        | 1     |
| C03           | 75             | 148.7                | 2.6                      | 1.98           | 95.4              | 0                                      | 0        | 0     |
| C04           | 67             | 123.4                | 2.6                      | 1.84           | 97.4              | 0                                      | 0        | 0     |
| C05           | 26             | 67.3                 | 2.3                      | 2.59           | 68.2              | 0                                      | 0        | 0     |
| C06           | 31             | 109.7                | 3.3                      | 3.43           | 89.4              | 0                                      | 0        | 0     |
| C07           | 8              | 32.1                 | 3.3                      | 4.01           | 21.5              | 0                                      | 0        | 0     |
| C08           | 42             | 115.6                | 3.7                      | 2.75           | 81.6              | 0                                      | 0        | 0     |
| C09           | 28             | 49.7                 | 1.4                      | 1.78           | 72.7              | 0                                      | 0        | 0     |
| Total         | 803            | 1763.2               | 3.4                      | 2.19           | 80.8              | 5                                      | 35       | 40    |

Table 1. Summary statistics of the BnaZNF2 linkage map. *which is calculated as the genetic distance (cM) divided by physical distance (Mb). † which is calculated as genetic distance (cM) divided by markers number. ‡ which is calculated as the covered physical distance (Mb) of linkage group divided by the length (Mb) of the corresponding pseudochromosome.
confidence intervals of these identified QTLs in different experiments overlapped, they were integrated into a single consensus QTL for each trait (Supplementary Table S6C). Eight and 16 consensus QTLs were obtained for pod number and seed number per pod, respectively, and five and 13 of these were reproducible (Table 5).

Interestingly, the numbers of identified QTLs and consensus QTLs for seed number per pod were both much larger than those for pod number, consistent with the much higher heritability of seed number per pod compared to pod number (Table 4). In addition, the Zhongshuang11 alleles in the majority

Figure 2. BnaZNF2 genetic map versus physical maps of *Brassica napus* and *B. rapa/B. oleracea*. The genetic and physical distances of the mapped markers are presented on the horizontal and vertical axes, respectively. The scatter dots indicate the genetic positions of the mapped markers on the linkage groups of BnaZNF2 genetic map and their physical positions on the pseudochromosomes (A01–A10; C01–C09) of *B. napus* (black) and *B. rapa/B. oleracea* (grey).
of both the identified and consensus QTLs decreased the pod number but increased the seed number per pod (Table 5; Supplementary Table S6C), consistent with the higher seed number per pod and lower pod number of Zhongshuang11 compared to No.73290 (Table 3). The consensus QTLs for pod number and seed number per pod explained 2.3–22.8% and 2.7–32.1% of the phenotypic variance, respectively. The additive/dominant effect of the consensus QTLs for pod number and seed number per pod ranged from −9.65/−7.48 to 7.29/9.17 and from −1.73/−1.05 to 1.97/1.59, respectively. Two consensus QTLs on the A06 linkage group (qPN.A06-1 and qSN.A06-1) were repeatedly detected in four and all five experiments, respectively, and displayed a large effect ($R^2$ = 22.8% and 32.1%); thus, they can be treated as major QTLs (Fig. 4). Interestingly, the confidence intervals of three pairs of consensus QTLs for pod number and seed number per pod (qPN.A05-1/qSN.A05-1, qPN.A06-1/qSN.A06-1 and qPN.C02-1/qSN.C02-1) overlapped well (Table 5). Of these, only qPN.A06-1 and qSN.A06-1 exhibited an opposite additive/dominant effect in the same experiments, explaining the moderate genetically negative correlation between pod number and seed number per pod (Table 4).

Physical map-based comparison of currently and previously detected QTLs for pod number and seed number per pod in rapeseed. The availability of the pseudochromosomes of B. napus also enabled the physical map-based comparison of currently and previously detected QTLs for pod number and seed number per pod in rapeseed. The corresponding genomic intervals of these QTLs were determined by BLAST/e-PCR analysis using the associated markers (within confidence intervals) with available probe/primer sequences against the physical map of B. napus.

The genomic regions of the majority (∼80%) of these QTL could be determined, of which approximately a quarter overlapped (Table 6; Supplementary Table S7). In detail, qPN.A03-1, qPN.A03-2, qPN.C06-1, qSN.A09-1, qSN.C06-1 and qSN.C06-2 identified in the current study corresponded to Sil/dm2_N3, qPN.A3-2, PNNP-C6, qSN.A1-1, qSN-LP1-A9a/qSN-OP2-A9, qSN.C2-1, qSN.C6-4 and qSN.C6-1, respectively, identified in the previous studies. In addition, PNLP-A3 and qPN-LP2-A3b, PNNP-A5 and qPN.A5-2, qPN.A6-1 and PNLP-A6, Sil/dm2_N7 and qPN.A7-1, NPP1_LG4 and qPN.A7-2, qPN-LP1-A9 and Sil/dm2_N9, qPN-LP3-C3a and qPN.C3-1, qSNA1 and qSN.A1-7, qSN-OP3-A2/qSN-OP2-A9a/qSN-LP3-A2 and SNLP-A2, qSN.A3-1 and SNLP-A3, qSN.A5-1 and qPN-OP1-A5, S/Sil_N5 and qSN.A5-2, qSN.A9-1 and SNNP-A9, qSN-OP1-C1 and S/Sil_N11.

Table 2. Correlation coefficients between the genetic and physical positions of the aligned markers for each of the 19 linkage groups/pseudochromosomes of B. napus and B. rapa/B. oleracea.
qSN-OP3-C6b/qSN-LP1-C6 and qSNC6, qSS.N19 and qSN.C9-3, which were identified in previous studies\(^2,4,5,9,11,12\), were also determined to be identical.

**Fine mapping of the major QTLs for pod number and seed number per pod by regional association analysis.** To further fine-map the two colocalised major QTLs for pod number and seed number per pod, regional association analysis was conducted. The corresponding genomic region of the target major QTL was identified by the alignment of the primer-pairs sequences of the molecular markers within the QTL interval and reference genomic sequences of *B. napus*\(^13\) and *B. rapa*\(^16\). A total of 103 putative single-locus SSR markers (Supplementary Table S8) within the corresponding ~1.7 Mb genomic region of *B. rapa*\(^18\) were synthesised and used to screen polymorphism among several mini-core collection of the association population\(^15\). Of these, 35 SSR markers that amplified only one main band and exhibited obvious polymorphism were selected for further regional association analysis.

A large range of phenotypic variation was observed for both pod number (32–106) and seed number per pod (7.6–28.0) among the 576 inbred lines of the association population. Based on population structure and family relatedness, regional association analysis was conducted using mixed linear

### Table 3. Phenotypic variation of pod number and seed number per pod for the two parents and populations in four investigated environments.

| Environments | Materials       | Parents | PNm | Pnb | PNw | SNPPm | SNPPb | SNPPw |
|--------------|----------------|---------|-----|-----|-----|-------|-------|-------|
| W09          | Parents        | Zhongshuang11 | 76  | /   | /   | 24.1  | /     | /     |
|              |                | No.73290   | 124 | /   | /   | 13.7  | /     | /     |
|              |                | F,       | 51  | /   | /   | 12.4  | /     | /     |
|              |                | Min      | 149 | /   | /   | 29.2  | /     | /     |
|              |                | Mean     | 100 | /   | /   | 20.8  | /     | /     |
|              | F,3            | Zhongshuang11 | 58  | 82  | 140 | 16.3  | 17.1  | 17.1  |
|              |                | No.73290   | 89  | 148 | 237 | 7.0   | 8.1   | 7.8   |
|              |                | F,9       | 48  | 30  | 83  | 5.3   | 6.4   | 6.3   |
|              |                | Min      | 97  | 183 | 277 | 18.7  | 18.3  | 18.2  |
|              |                | Mean     | 76  | 86  | 159 | 13.6  | 13.8  | 13.8  |
| W10          | Parents        | Zhongshuang11 | 65  | 126 | 191 | 21.0  | 19.5  | 20.3  |
|              |                | No.73290   | 94  | 173 | 267 | 11.4  | 10.3  | 10.2  |
|              |                | F,3      | 56  | 81  | 136 | 11.4  | 9.2   | 10.1  |
|              |                | Max      | 104 | 344 | 397 | 22.3  | 20.0  | 20.3  |
|              |                | Mean     | 76  | 165 | 233 | 17.4  | 15.1  | 15.9  |
| W11          | Parents        | Zhongshuang11 | 45  | 149 | 194 | 22.9  | 21.2  | 21.2  |
|              |                | No.73290   | 76  | 235 | 311 | 12.3  | 10.8  | 11.2  |
|              |                | F,3      | 26  | 80  | 119 | 10.9  | 10.0  | 9.7   |
|              |                | Max      | 78  | 395 | 456 | 24.8  | 23.5  | 23.8  |
|              |                | Mean     | 57  | 197 | 247 | 18.6  | 17.2  | 17.4  |
| X11          | Parents        | Zhongshuang11 | 61  | 119 | 175 | 21.1  | 19.3  | 19.5  |
|              |                | No.73290   | 96  | 185 | 272 | 11.1  | 9.7   | 9.7   |
|              |                | F,3      | 37  | 110 | 153 | 9.5   | 10.6  | 9.9   |
|              |                | Max      | 85  | 284 | 344 | 24.5  | 23.9  | 25.2  |
|              |                | Mean     | 60  | 176 | 232 | 17.9  | 16.4  | 16.8  |
| Total        | Parents        | Zhongshuang11 | 52  | 79  | 134 | 10.6  | 8.2   | 9.2   |
|              |                | No.73290   | 99  | 236 | 315 | 21.8  | 20.4  | 20.1  |
|              |                | Mean     | 75  | 153 | 216 | 17.0  | 15.4  | 15.8  |

Table 3. Phenotypic variation of pod number and seed number per pod for the two parents and populations in four investigated environments. PNm/PNb/PNw and SNPPm/SNPPb/SNPPw are the abbreviations of pod number and seed number per pod, respectively, for the main inflorescence/branch inflorescence/whole plant.
mode (MLM) by TASSEL3.0 software. Interestingly, a significant (P < 0.001) association signal was only observed for pod number and not for seed number per pod (Fig. 5). Of the six SSR markers associated with pod number, BrSF46–177 displayed the strongest association signal (P = 2.2E-08 and R² = 8.7%); this signal was very close (13 kb) to the peak marker (BrSF46–167) of qPN.A06-1 identified by linkage analysis (Fig. 1; Table 5). Therefore, the confidence interval of qPN.A06-1 was defined by genomic region between BrSF46–136 and BrSF46–238, which corresponds to 180 kb (from 22,607 to 22,787 kb) including

Figure 3. Distribution of pod number and seed number per pod in the BnaZNF2 and corresponding F2:3 and F2:4 populations planted in four environments. The horizontal axis represents the trait value of pod number and seed number per pod for the main inflorescence, branch inflorescence and whole plant. The vertical axis represents the number of individuals/families within the population. The different experiments (environment and population combination) are represented by different colours as indicated in the legend. W09F2, W10F2:3, W11F2:3, X11F2:3 and X11F2:4 were the codes of the five experiments which have been detailedly described in the first section of Methods.

|          | PNm | PNb | PNw | SNPPm | SNPPb | SNPPw |
|----------|-----|-----|-----|-------|-------|-------|
| PNm      |     |     |     |       |       |       |
| PNb      | 0.49*** |     |     |       |       |       |
| PNw      | 0.61*** | 0.96*** |     |       |       |       |
| SNPPm    | −0.41*** | −0.31*** | −0.34*** |       |       |       |
| SNPPb    | −0.27*** | −0.22*** | −0.23*** | 0.87*** |       |       |
| SNPPw    | −0.34*** | −0.28*** | −0.31*** | 0.94*** | 0.97*** |       |
| h²       | 0.65 | 0.45 | 0.49 | 0.83 | 0.82 | 0.83 |

Table 4. Genetic correlation and broad-sense heritability of pod number and seed number per pod.
* , ** and *** represent the significant levels of P = 0.05, 0.01 and 0.001, respectively. PNm/PNb/PNw and SNPPm/SNPPb/SNPPw are the abbreviations of pod number and seed number per pod, respectively, for the main inflorescence/branch inflorescence/whole plant.
| Consensus QTL | Peak position | Confidence interval | Additive effect | Dominant effect | LOD value | R² (%) | Experiments |
|----------------|---------------|---------------------|----------------|----------------|------------|--------|-------------|
| qPN.A01-1       | 104.4         | 94.3–105.8          | −4.90          | 2.52           | 3.0        | 12.5   | W10F2:3m/X11F2:3m |
| qPN.A03-1       | 61.5          | 57.5–61.9           | −9.65          | 7.90           | 3.6        | 3.0    | W11F2:3w |
| qPN.A03-2       | 81.6          | 64.6–98             | −1.73          | −6.23          | 3.8        | 3.4    | W11F2:3m |
| qPN.A05-1       | 10.8          | 4–13.8              | −4.67          | 9.17           | 3.4        | 12.6   | W09F2m |
| qPN.A06-1       | 86.3          | 82.6–93.0           | −6.04          | −2.49          | 8.1        | 22.8   | W10F2:3m/W11F2:3m/X11F2:3m/11F2:4m |
| qPN.A09-1       | 44.0          | 42.5–54.4           | 4.05           | −7.20          | 2.7        | 2.3    | X11F2:3bw |
| qPN.C02-1       | 8.2           | 3.2–21.5            | −5.14          | 2.91           | 4.3        | 18.5   | W10F2:3m/W10F2:3m/X11F2:3m |
| qPN.C06-1       | 51.2          | 49.1–53.0           | 7.29           | −7.48          | 4.4        | 15.0   | W10F2:3m/W11F2:3m |
| qSN.A01-1       | 17.3          | 12.4–23.2           | −1.01          | −1.05          | 4.2        | 8.8    | X11F2:3b |
| qSN.A01-2       | 113.9         | 106.6–117.7         | −0.89          | 1.09           | 3.7        | 2.7    | X11F2:4bmw |
| qSN.A02-1       | 6.1           | 0.4–17.6            | 0.36           | 0.76           | 4.7        | 5.3    | W10F2:3bw |
| qSN.A02-2       | 43.1          | 36.2–45.9           | 1.11           | −0.64          | 5.8        | 7.0    | X11F2:4bmw |
| qSN.A02–3       | 81.6          | 79.7–82.9           | 0.32           | 0.06           | 4.2        | 4.3    | X11F2:4mw |
| qSN.A03-1       | 14.1          | 12–20.1             | −1.73          | 1.33           | 4.3        | 6.3    | X11F2:3m |
| qSN.A03–2       | 118.0         | 116.0–123.7         | −0.82          | 1.59           | 3.1        | 4.3    | X11F2:3bw |
| qSN.A05-1       | 9.5           | 4.1–13.7            | −1.03          | 1.47           | 4.1        | 4.7    | X11F2:4bmw |
| qSN.A06-1       | 88.9          | 85.0–97.6           | 1.97           | 0.59           | 11.0       | 32.1   | W09F2m/W10F2:3bmw/W11F2:3bmw/X11F2:3bmw/X11F2:4bmw |
| qSN.A07-1       | 6.7           | 3.7–15.9            | 0.75           | 0.53           | 5.9        | 6.9    | W11F2:3bw |
| qSN.A07-2       | 54.4          | 35.8–61.1           | 0.86           | 0.62           | 7.5        | 15.8   | W10F2:3bw/W11F2:3b/X11F2:4b |
| qSN.A07–3       | 75.7          | 69.6–78.8           | 0.48           | 0.52           | 3.9        | 3.8    | W10F2:3m/W11F2:3m |
| qSN.A09-1       | 68.7          | 68.5–82.2           | 0.59           | 0.15           | 3.6        | 4.9    | W11F2:3bw |
| qSN.C02-1       | 5.8           | 2–18.1              | −0.71          | −0.13          | 3.8        | 6.7    | W11F2:3b |
| qSN.C06-1       | 53.4          | 47.8–58.2           | 0.72           | 0.39           | 3.7        | 4.7    | W11F2:3bw |
| qSN.C06-2       | 16.6          | 82.9–97.7           | 1.03           | −1.02          | 4.6        | 8.6    | W10F2:3bw |

Table 5. Identified and consensus QTL for pod number and seed number per pod.

Figure 4. QTL scanning curves for the A06 linkage group for pod number and seed number per pod in five experiments. The horizontal and vertical axes, respectively, represent the genetic distance and LOD value. The lines and curves, respectively, indicate the threshold and true LOD values. The different traits and experiments are represented using different colours and (line/curve) styles, respectively, as indicated in the legend. PNm/PNb/PNw and SNPNPm/SNPNPb/SNPNPw are the abbreviations of pod number and seed number per pod, respectively, for the main inflorescence/branch inflorescence/whole plant. W09F2, W10F2:3, W11F2:3, X11F2:3, and X11F2:4 were the codes of the five experiments.
Table 6. The common QTLs identified in the current and previous studies for pod number and seed number per pod.

| Trait name | QTL name | Linkage group | Marker interval | Genomic region (Mb) | Reference |
|------------|----------|---------------|-----------------|---------------------|-----------|
| Pod number |          |               |                 |                     |           |
| PNLP-A3    | A03      | BoGMS0230/BnGMS257 | 5.1–6.1         | Ding et al.2        |
| qPN-LP2-A3b| A03      | MR123/BRMS0543  | 5.1–7.4         | Shi et al.4         |
| Sil/dmr-N3 | A03      | E32M51_283E/MR12 | 10.8–13.2       | Radoev et al.3      |
| qPN-A03-1  | A03      | BrS0295/CNU098  | 11.4–13.5       | Current study       |
| qPN-A03-2  | A03      | FITO285/BoSF2828 | 14.2–15.4       | Current study       |
| qPN-A3-2   | A03      | SA28/CNU276     | 14.0–15.2       | Shi et al.2         |
| PNLP-A5    | A05      | BnPHT1/BoGMS1565c | 3.3–4.0         | Ding et al.9        |
| qPN-A5-2   | A05      | E1HM31–130/CNU344 | 3.6–4.6         | Shi et al.2         |
| qPN-A6-1   | A06      | IGF1075d/nia3b   | 7.3–11.1        | Shi et al.2         |
| PNLP-A6    | A06      | H001J23/BoGMS531 | 8.5–12.5        | Ding et al.7        |
| Sil/dmr-N7 | A07      | CB10439/MR153b  | 10.5–13.5       | Radoev et al.3      |
| qPN-A7-1   | A07      | E1HM34–220/35RXTRAP10-4 | 11.0–12.9 | Shi et al.2         |
| qPN-A7-2   | A07      | nia6b063/sr7223 | 14.1–14.4       | Shi et al.2         |
| qPN-LP1-A9 | A09      | HBr075/H091P21-4 | 32.5–32.8       | Shi et al.3         |
| qPN-LP3-C3a| C03      | BRMS106/pX141aE | 3.9–6.7         | Shi et al.4         |
| qPN-C3-1   | C03      | STS01/pX141bE   | /–6.0           | Shi et al.2         |
| qPN-C06-1  | C06      | BoSF1254/BoSF2507 | 17.9–18.8   | Current study        |
| PNLP-C6    | C06      | BnGMS118/MR133.1 | 18.1–18.9       | Ding et al.7        |
| qSN-A01-1  | A01      | BoSF2770/nia6b063 | 0.5–1.5        | Current study        |
| qSN-A1-1   | A01      | nia6b71/CNU142  | 0.7–1.5         | Shi et al.2         |
| qSN-A1-7   | A01      | E10HM34-230/EST165 | /–8.7–/       | Shi et al.2         |
| qSNA1      | A01      | Na14G06/CB10819 | 8.2–8.9         | Qi et al.11         |
| qSN-OP3-A21/qSN-OP1-A2a/qSN-LP3-A2 | A02 | xR6293a/BnSIZ1-A2 | 3.0–4.7 | Shi et al.4         |
| SNLP-A2    | A02      | Na14H11/B049H114 | 4.2–4.5         | Ding et al.9        |
| qSN-A3-1   | A03      | CNU210/S13M08-1-157 | 15.3–15.7  | Shi et al.2         |
| SNLP-A3    | A03      | CNU270/He131    | 15.4–16.2       | Ding et al.5        |
| qSN-A5-1   | A05      | CNU257/BRS063   | 3.2–3.3         | Shi et al.2         |
| qPN-OP1-A5 | A05      | BnPHT1-A5/nia12335b | 3.2–4.4        | Shi et al.4         |
| S/Sil_N5   | A05      | MD21/MR113     | 8.0–12.4        | Radoev et al.3      |
| qSN-A5-2   | A05      | CNU206/pW161-2b | 11.2–12.8       | Shi et al.2         |
| qSN-A9-1   | A09      | P04M21-130/IGF5222b | 1.0–1.8       | Shi et al.2         |
| SNLP-A9    | A09      | HBr199c/HBr097c | 1.2–1.8         | Ding et al.7        |
| qSN-OP1-C1 | C01      | HR-Sp2-18/BRMS175 | 36.5–38.8     | Shi et al.4         |
| S/Sil_N11  | C01      | CB10536/CB10357b | 35.3–37.7       | Radoev et al.3      |
| qSN-C02-1  | C02      | BoSF1425/CNU461 | 0.8–2.2         | Current study        |
| qSN-C2-1   | C02      | pW119/nia12095  | 1.4–1.7         | Shi et al.2         |
| qSN-C6-4   | C06      | HUA64-2/Na12E01a | 16.9–18.1      | Shi et al.2         |
| qSN-C6-1   | C06      | BoSF1254/BoSF1126 | 17.8–19.0   | Current study        |
| qSN-C6-2   | C06      | pW134/AP1a      | 29.6–32.0       | Shi et al.2         |
| qSN-C6-3   | C06      | BoSF1951/CNU331a | 30.6–34.2       | Current study        |
| qSN-OP3-C6b/qSN-LP1-C6 | C06 | BnPHT1-C6/pA28 | 34.4–36.0 | Shi et al.4         |
| qSNC6      | C06      | CNU182/CNU352   | 34.8–35.9       | Qi et al.11         |
| qSS.N19    | C09      | EA13MC03-150/Na10D07 | 39.4–/       | Zhang et al.12      |
| qSN-C9-3   | C09      | IGF5193b/CB10288 | 39.0–40.0      | Shi et al.2         |

Table 6. The common QTLs identified in the current and previous studies for pod number and seed number per pod.
only 33 annotated genes in the reference genome of *B. napus* (Table 7). Because the extent of linkage disequilibrium (LD) decay for the target QTL region was estimated to be 193 kb (Fig. 6), it was impossible to further fine-map it using the current association population. In addition, the \(-\log(P)\) values of the association of the six SSR markers with pod number and seed number per pod were not accordant (Fig. 5). These results also suggested that the colocalisation of \(qPN.A06-1\) and \(qSN.A06-1\) was not likely caused by pleiotropy.

**Dissection of the genetic basis of the colocalised QTLs for pod number and seed number per pod by conditional QTL analysis.** To determine the genetic basis (pleiotropy vs. tight-linkage) of the colocalisation of \(qPN.A06-1\) and \(qSN.A06-1\) in the same experiments, conditional QTL analysis was performed in both the linkage and association populations (Table 8). Regardless of whether pod number was conditioned by seed number per pod or seed number per pod was conditioned by pod number, the LOD values and \(R^2\) of the two major QTLs, as well as the \(-\log(P)\) values and \(R^2\) of the six associated markers, exhibited only a small variation. These results suggest that tight linkage rather than pleiotropy is more likely the genetic basis of the colocalisation of \(qPN.A06-1\) and \(qSN.A06-1\).

**Discussion**

In the current study, an F2 population from two sequenced rapeseed cultivars was developed. Among the reported genetic populations in rapeseed\(^{13-19,24}\), this is the first for which parental genomic information is available, which will greatly facilitate subsequent studies. In addition, Zhongshuang11 and No.73290 exhibit large differences in many traits related to agronomy, plant/root architecture and yield\(^{15}\). More importantly, many QTLs with relatively large effects have been identified in this population, including for pod length, seed weight\(^{15}\), pod number, seed number per pod (Table 5) and flowering time (Shi et al., unpublished data). Due to the heterozygosity of the current BnaZNF\(_2\) population, the corresponding recombinant inbred lines population (named BnaZNRIL) was also developed in our lab. All major QTLs identified from the BnaZNF\(_2\) population have been fine-mapped in our laboratory, and the functional validation of the candidate genes is in progress. Therefore, the BnaZNF\(_2\)/BnaZNRIL population is an ideal reference population for QTL mapping and map-based gene cloning in rapeseed.

To facilitate pseudochromosome assembly of Zhongshuang11, a skeleton linkage map of 803 sequence-based PCR markers was constructed using the BnaZNF\(_2\) population (Table 1). A total of 561 markers (499 SSR, 31 SNP and all 31 InDel) on the BnaZNF\(_2\) linkage map were developed in this study (Supplementary Table S2), increasing the number of sequence-based PCR markers in rapeseed\(^{14}\). To our knowledge, this is the first rapeseed linkage map comprising only sequence-based PCR markers.
In addition, the current linkage map mainly (88.4%) comprises SSR markers and has more SSR markers (710) than other published linkage maps of *B. napus* (http://www.brassica.info/resource/maps/published-data.php). These favourable characteristics will facilitate the easy transfer of these markers to other genetic populations and the comparison/integration of different linkage maps in *B. napus* as well as in other *Brassica* species25,26.

The existence of short linkage groups is commonly observed in published linkage maps of *B. napus*5,9,12,27–30, but the detailed causation (insufficient number of markers, poor polymorphism of parents and low frequency of recombination) has not been investigated. Although a total of 9351 primer-pairs were used to screen the polymorphic markers for map construction (Supplementary Table S1), five short linkage groups were also identified in the current BnaZNF 2 linkage map (Table 1). Of these, four linkage groups (A08, A10, C07 and C09; A05 was the exception) were nearly the same short length in a *B. napus* gene code | *A. thaliana* gene model | Gene name | Annotation
---|---|---|---
GSBRNA2T00001820001 | AT2G02370.1 | / | SNARE associated Golgi protein family
GSBRNA2T00001830001 | AT2G02360.1 | PP2-B10 | Phloem protein 2-B10
GSBRNA2T00001840001 | AT2G02550.1 | PP2-B2 | Phloem protein 2-B2
GSBRNA2T00001860001 | AT2G02230.1 | PP2-B1 | Phloem protein 2-B1
GSBRNA2T00001880001 | AT2G02230.1 | PP2-B1 | Phloem protein 2-B1
GSBRNA2T00001890001 | / | / | /
GSBRNA2T00001811001 | AT2G02230.1 | PP2-B1 | Phloem protein 2-B1
GSBRNA2T00001812001 | AT2G02230.1 | PP2-B1 | Phloem protein 2-B1
GSBRNA2T00001813001 | / | / | /
GSBRNA2T00001814001 | AT2G02170.1 | / | Remorin family protein
GSBRNA2T00001815001 | AT3G49520.1 | / | F-box and associated interaction domains-containing protein
GSBRNA2T00001816001 | AT1G25055.1 | / | F-box family protein
GSBRNA2T00001817001 | AT2G02150.1 | EMB2794 | Tetrameric repeat (TPR)-like superfamily protein
GSBRNA2T00001818001 | AT2G02150.1 | EMB2794 | Tetrameric repeat (TPR)-like superfamily protein
GSBRNA2T00001820001 | AT2G02880.1 | IDD4 | Indeterminate(ID)-domain 4
GSBRNA2T00001821001 | AT2G02130.1 | LCR68, PDF2.3 | Predicted to encode a PR (pathogenesis-related) protein
GSBRNA2T00001822001 | AT2G02880.1 | IDD4 | Indeterminate(ID)-domain 4
GSBRNA2T00001824001 | AT2G02070.1 | IDD5 | Indeterminate(ID)-domain 5
GSBRNA2T00001825001 | AT2G02061.1 | / | Nucleotide-diphospho-sugar transferase family protein
GSBRNA2T00001826001 | AT2G0260.1 | / | Homeo-domain-like superfamily protein
GSBRNA2T00001828001 | AT2G02050.1 | / | NADH-ubiquinone oxidoreductase B18 subunit
GSBRNA2T00001829001 | AT2G02040.1 | NTR1, PTR2, PTR2-B | Encodes a di- and tri-peptide transporter
GSBRNA2T00001830001 | AT2G01940.1 | IDD15, SGR5 | A putative transcription factor in gravity-perceptive cells
GSBRNA2T00001832001 | AT2G01950.1 | BRL2, VH1 | Encodes a LRR receptor kinase and associated with provascular/procambial cells
GSBRNA2T00001835001 | / | / | /
GSBRNA2T00001836001 | AT2G01913.1 | / | Unknown protein
GSBRNA2T00001837001 | AT2G01910.1 | MAP65-6 | Microtubule associated protein (MAP65/ASE1) family protein
GSBRNA2T00001838001 | AT2G01905.1 | CYC18 | Cyclin-dependent protein kinase regulator activity
GSBRNA2T00001839001 | AT2G01880.1 | PAP7 | Serine/threonine phosphatase
GSBRNA2T00001840001 | AT2G01870.1 | / | Unknown protein
GSBRNA2T00001841001 | AT2G01870.1 | / | Unknown protein
GSBRNA2T00001842001 | AT2G01860.1 | EMB975 | Embryo defective 975
GSBRNA2T00001843001 | AT2G01850.1 | EXGT-A3, XTH27 | Homology to xyloglucan endotransglycosylases/hydrolases (XTHs)

Table 7. List of 33 annotated genes within the fine-mapped genomic region of qPN.A06-1.
high-density genetic map constructed using the BnaZNRIL population and the Brassica 60K Illumina Infinium SNP array (Shi et al. unpublished data); therefore, they were unlikely to be caused by an insufficient number of markers. Because its genomic coverage ratio was not low, the short length of the C09 linkage group was obviously due to its low recombination frequency among all 19 linkage groups. For the A08, A10 and C07 linkage groups, their short length is likely due to the low polymorphism between parents because their genomic coverage ratios were very low, while the recombination frequencies were not low. In fact, the A08 and A10 linkage groups are also short in more than half of the published linkage maps of B. napus\textsuperscript{5,9,12,27–29,31}. Thus, a lack of genetic diversity is likely a common characteristic of the

Table 8. Conditional analysis on the target major QTL region in both linkage and association population.

| Experiment code | LOD/R² (%) | −log(P)/R² (%) |
|-----------------|------------|---------------|
|                 | PNm | PNm/SNPPm | SNPPm | SNPPI/PNm |
|                 | PNm | PNm/SNPPm | SNPPm | SNPPI/PNm |
| Linkage population |   |           |       |           |           |
| W09F₂         | NS | NS | 10.0/25.6 | 11.9/23.3 |
| W10F₂-3       | 9.9/23.9 | 10.5/19.7 | 10.4/28.1 | 11.4/27.8 |
| W11F₂-1       | 6.9/20.5 | 6.2/18.1 | 13.4/34.6 | 8.2/24.2 |
| X11F₂-1       | 7.7/24.0 | 7.6/19.8 | 21.8/44.7 | 18.9/48.2 |
| X11F₂-1       | 8.0/22.8 | 9.7/20.2 | 11.3/43.6 | 12.9/47.6 |
| Association population |   |           |       |           |           |
| BrSF46-136    | 4.3/4.6 | 4.1/4.2 | NS | NS |
| BrSF46-161    | 7.4/6.8 | 6.5/5.8 | NS | NS |
| BrSF46-167    | 6.8/8.5 | 5.4/6.8 | NS | NS |
| BrSF46-177    | 7.7/8.7 | 6.6/7.4 | NS | NS |
| BrSF46-186    | 4.4/4.5 | 4.8/4.3 | NS | NS |
| BrSF46-238    | 3.7/4.1 | 3.0/3.6 | NS | NS |

Figure 6. Trend of LD decay with increase in physical distance in the target major QTL region. The horizontal and vertical axes, respectively, represent the physical distance and the R² of LD. The scatter plots show the mean R² of the LD for each window. The dotted line indicates the background level of LD. The curve on the figure shows the trendline of the regression between physical distance and R² of LD. The regression equation and its determination coefficient are also shown on the figure.
two chromosomes in rapeseed, which may indicate a new direction for breaking the genetic bottleneck in rapeseed cultivars.

Interestingly, most of the distorted markers tended to cluster on both ends of the linkage groups and skew to the same parent (Supplementary Table S3). This clustering is not likely due to chance and suggests the presence of a segregation distortion region (SDR) in rapeseed, which can be explained by factors such as the selection of gametophytes and sporophytes, homeologous recombination and residual heterozygosity in parents.

The major part of the constructed BnaZNF linkage map and physical map of B. napus were collinear (Fig. 2; Table 2), suggesting its high quality and suitability for rapeseed genomic studies such as pseudochromosome assembly and comparative mapping. The inconsistency between the genetic and physical distance of a few tightly-linked markers (Supplementary Table S4) may be due to genotyping errors, misanchored scaffolds or distorted markers. This is the first comparative study of the genetic and physical map of rapeseed. The BnaZNF, skeleton linkage map-based comparison of the physical maps of B. napus, B. rapa or B. oleracea revealed several genomic rearrangements (including inversion, translocation and reshuffling) between these species. These rearrangements should occur very recently, after the formation of B. napus from the progenitors of the two diploid species B. rapa and B. oleracea. This finding is consistent with the reported genomic rearrangements in the resynthesised Brassica polyplids, including B. napus.

In the reported linkage6,7,11 and association10 mapping studies of pod number and/or seed number per pod, more than 70 and 80 QTLs have been detected for pod number and seed number per pod, respectively, distributed on 16 (excluding A04, C04 and C07) and 17 (excluding C04 and C07) of the 19 linkage groups (Supplementary Table S7). Most of these QTLs exhibited a moderate effect, and only two on A02 (qPN.A02) and C02 (qSN.A02) for pod number, three on A01 (qSN.A01), C03 (qSS.C03) and C09 (qSS.C09) for seed number per pod, and one on C01 (qSN11a) for both traits could be considered major QTLs6,11. In the current study, 20 and 16 QTLs were identified for pod number and seed number per pod, respectively, which were distributed on seven (A01, A03, A05, A06, A09, C02 and C06) and nine (A01-A03, A05-07, A09, C02 and C06) of the 19 linkage groups (Table 5). One and two thirds of them, respectively, exhibited a relatively large ($R^2 > 10\%$) or moderate ($2 < R^2 < 10\%$) effect, and only two each for pod number and seed number per pod should be considered major QTLs. The recent completion of sequencing and assembling of rapeseed genome enabled the first physical map-based comparative QTL analysis in the current study, which undoubtedly increased the accuracy of comparison. Only three QTLs for pod number and five QTLs for seed number per pod identified in the current study had been reported previously (Table 6), whereas the remaining five and 11 are novel (Supplementary Table S7). In addition, seven pairs of QTLs for pod number and nine pairs of QTLs for seed number per pod, those had been reported in the previous studies6,7,11,12, are also identical. The aforementioned 24 QTLs common to the current and previous studies should be potential targets for marker-assisted selection to improve yield in rapeseed.

More importantly, the major QTLs identified across these studies will be important targets of map-based gene cloning for elucidating the molecular basis of yield in rapeseed. These results demonstrate that both pod number and seed number per pod are controlled by a large number of loci that are distributed on nearly all of the 19 linkage groups and mostly exhibit moderate effects, which strongly suggests the complexity of the genetic architecture of both traits. This complexity is understandable because the pod number per plant and seed number per pod are affected by many biological/developmental processes, including inflorescence/flower/ovule differentiation, fertilization and pod/seed development, of which many genes40–41.

Several major QTLs for pod number and/or seed number per pod had been identified in previous QTL mapping studies6,7,11,12, but none have been fine-mapped and further cloned. The traditional/classical fine mapping strategy is based on screening of recombinant individuals from a large-scale segregation population of near-isogenic lines (NILs) developed from several rounds of successive backcrossing and/or self-crossing, which is time-consuming and labour intensive. To directly fine-map the two colocalised major QTL (qPN.A06-1 and qSN.A06-1) identified in the current study, a regional association analysis was proposed by using the high resolution of the natural population. Using this strategy, qPN.A06-1 was successfully narrowed to a much smaller (approximately 1/7) interval of 180 kb including only 33 annotated genes (Fig. 5). This result suggests that regional association analysis is an effective and highly efficient strategy for direct fine mapping of target major QTLs identified by preliminary linkage analysis. According to the detailed annotation of the 33 genes (Table 7), a homeodomain-like superfamily protein gene GSBRNA2T00001826001 was likely to be the candidate43, because the two parents Zhongshuang11 and No.73290 showed significant difference in flowering time and flower organ number. To further confirm the candidate gene(s), comparative sequencing of the 180 kb genomic region between the two parents is in progress. However, qSN.A06-1 was not validated by regional association analysis using the same association population and markers. This failure may have occurred because the genetic variation responsible for the major QTL of seed number per pod is rare in our association population or the expression of this major QTL depends on the genetic background. These results suggest that the use of regional association analysis for fine mapping of the target major QTL depends on the type of variants (rare or common) and/or genetic background. These results also suggest that the fine mapping of qSN.A06-1 have to use the traditional/classical NIL strategy.
Resource limitation force an organism to allocate energy to processes in a competitive manner. In seed plants, the major trade-off between the number of fruits per inflorescence/plant and the number of seeds per fruit, which was traditionally explained by antagonistic pleiotropy, can be quantitated/ reflected by the negative coefficients of genetic correlation. For rapeseed, the negative correlation between pod number and seed number per pod has been commonly observed, including in the present study, and is consistent with the opposite additive-effect direction of most of the colocalised QTLs for both traits. However, the genetic basis of these colocalised QTLs with the opposite effect for both traits has not previously been investigated and remains unknown. The existence of two colocalised major QTL with opposite direction for pod number and seed number per pod in the current study provided an ideal example to test the hypothesis of antagonistic pleiotropy. The genetic basis of their colocalisation was then investigated by further regional association analysis (Fig. 5) followed by conditional QTL analysis (Table 8). The results of both analyses suggested that their colocalisation was caused by tight-linkage rather than pleiotropy, which was also confirmed by the subsequent measurement of both traits on the NILs that were recombinant at the target major QTL region (Shi et al. unpublished data). This is the first study that provides solid evidences to clearly demonstrate a negative correlation between yield component traits in rapeseed as well as other crops. The existence of two tightly linked major QTLs for pod number and seed number per pod also indicates the retention of a large linkage drag during the breeding of the elite rapeseed cultivar Zhongshuang11. Therefore, the strategy described in this paper will be effective in increasing the pod number of Zhongshuang11 without decreasing the seed number per pod via the introgression of qPN.A6 through backcross and marker-assisted selection.

Methods
Population construction, field experiment and trait measurement. The reference genetic population (named BnaZNF2) used for the linkage analysis included 184 F2 individuals derived from Zhongshuang11 and No.73290. The natural population used for the association analysis was described in our previous study and comprises 576 global inbred lines, including the two parents of the linkage population.

The F2 individuals were planted in Wuhan from Oct. 2008 to May 2009 (code W09F2). The F2 families were planted in Wuhan from Oct. 2009 to May 2010 (code W10F2) and Oct. 2010 to May 2011 (code W11F2) as well as in Xining from April to Aug. 2011 (code X11F2). The F2 families were planted in Xining from April to Aug. 2011 (code X11F2). The association population was planted in Wuhan from Oct. 2011 to May 2012 (code W12AP). The planting of the F2, F3 and association populations followed a randomised complete block design with two to three replications. Each block contained two rows, with 33.3 cm between rows and 16.7 cm between individual plants. The seeds were sown by hand, and the field management followed standard agricultural practice. In each block, 10 representative individuals from the middle of each row were harvested by hand at maturity.

Pod number was measured as the number of effective pods on the main inflorescence, branch inflorescence and whole plant, respectively (PNm, PNb and PNw). Seed number per pod was calculated as the number of well-filled seeds on the main inflorescence, branch inflorescence and whole plant, respectively, divided by the corresponding pod number (SNPPm, SNPPb and SNPPw).

Molecular marker analysis and linkage map construction. Three groups of molecular markers were first used to screen polymorphism between the two parents of the linkage population, and the single-locus markers were then used to screen the mini-core collection of the association population. The first group, mainly consisting of SSR and STS markers, was downloaded from the public database of Brassica (http://www.brassica.info/resource/markers.php; http://brassica.nbi.ac.uk/IMSORB/). The second group, consisting of SSR markers (prefixed “BoSF”, “BrSF” and “BnSF”), was developed by our lab from the sequence scaffolds of B. rapa, B. oleracea and B. napus. The third group consisted of SNP (prefixed “snap” and “ns”) and InDel (prefixed “BnID”) markers and was developed in our lab from the re-sequencing of several B. oleracea cultivars and No.73290. For markers detected at more than one polymorphic locus, a lowercase alphabetic letter was added to distinguish the different loci. PCR, electrophoresis and silver staining were performed as previously described.

The genetic linkage map was constructed using the software JoinMap 4.0 (http://www.kyazma.nl/index.php/mc.JoinMap) using a threshold for goodness-of-fit of ≤ 5, a recombination frequency of < 0.4 and minimum logarithm of odds (LOD) score of 2.0. All genetic distances were expressed in centimorgans (cM) as derived by the Kosambi function. Double-crossover events were examined, and the original scores were rechecked for potential scoring errors.

Linkage groups were assigned to the corresponding chromosomes (A01–A10; C01–C09) based on the common markers in the reported linkage maps of B. napus (http://www.brassica.info/CropStore/maps.php)5,20,22,49–52.

QTL mapping and integration. QTL mapping was conducted using the composite interval mapping (CIM) procedure incorporated in WinQTLCart v2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). The walk speed, number of control markers, window size and regression method were set to 1 cm, 5, 10 cm and forward regression, respectively. The default minimum distance between QTLs (5 cM) and minimum LOD from top to valley (1) were used to define a QTL peak in an experiment.
experiment-wise LOD threshold was determined by permutation analysis with 1000 repetitions. The LOD score corresponding to P = 0.05 (3.0–4.6) was used to identify significant QTLs. To avoid missing QTLs with a relatively small effect, a lower LOD score, corresponding to P = 0.10 (2.6–4.1), was adopted for the presence of suggestive QTLs. Both significant QTLs and reproducible suggestive QTLs were admitted and named as identified QTLs.

Because QTLs detected in different experiments and mapped to the same region of a chromosome might be several estimates of the position of a single QTL, integration of these reproducible QTLs was conducted.

**Linkage disequilibrium and regional association analysis.** Linkage disequilibrium (LD) was estimated as the correlation coefficient $R^2$ between all pairs of markers using the standalone software TASSEL v3.0. Rare alleles with a frequency of <0.05 were treated as missing data. The background level of LD was defined as the 95% quantile of the $R^2$ value among unlinked markers. The LD decay was calculated using the linked markers as previously described.

The structure of the association population was inferred using the software STRUCTURE v2.3.4. The iteration number and K-value (the putative number of genetic groups) were set to 3 and from 1 to 10, respectively. Both the length of the burnin period and number of MCMC (Markov Chain Monte Carlo) replications after burnin were set to 10000. The most likely K-value was determined by posterior probability [LnP(D)] and an ad hoc statistic $\Delta k$ based on the rate of change in LnP(D) between successive $k$.

The relative kinship of the 576 inbred lines of the association population was calculated using SPAGeDi v1.4 software. All negative kinship values between individuals were set to zero.

The regional association analysis was conducted using single-locus SSR markers within the confidence interval of the target major QTL in the association population of 576 inbred lines. Based on the population structure and relative kinship (Q and K matrix), the calculation was performed with a mixed linear model (MLM) incorporated into the TASSEL v3.0 software. The threshold of the significant marker-trait association was set to $P \leq 0.001$.

**Conditional QTL analysis.** To determine the genetic basis of the colocalisation of QTLs for different traits, conditional QTL analysis was performed using the conditional phenotypic values. The conditional phenotypic values $y(T1\mid T2)$ were obtained by the mixed model approach for the conditional analysis structure and relative kinship (Q and K matrix), the calculation was performed with a mixed linear model (MLM) incorporated into the TASSEL v3.0 software. The threshold of the significant marker-trait association was set to $P \leq 0.001$.

**Statistical analysis.** Broad-sense heritability was calculated as $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/n + \sigma_n^2/nr)$, where $\sigma_g^2$, $\sigma_e^2$ and $\sigma_n^2$ are the variances of genotype, genotype by environment and error, respectively, while $n$ and $r$ are the number of environments and replicates, respectively. The genetic correlation was calculated as $r_{Gx} = \text{cov}_{Gx}/(\sigma_{Gx}^2 \times \sigma_{Gy}^2)^{1/2}$, where $\text{cov}_{Gx}$ is the genotypic covariance and $\sigma_{Gx}^2$ and $\sigma_{Gy}^2$ are the variances of the pairwise traits. The significance of each genetic correlation was determined using a t-test of the correlation coefficients. The components of variance/covariance and the coefficients of correlation were estimated using the PROC GLM or CORR procedure, respectively, incorporated into SAS software version 8.1. The Excel statistical functions CHISQ.TEST and T.TEST were used to obtain the significance level ($P_{\text{test}}$ and $P_{\text{t-test}}$) of the degree-of-fit and differences, respectively.

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**Author Contributions**

J.S. and H.W. designed the study. J.S. and J.Z. performed the marker analysis in the F2 population. J.S. and Y.Y. performed the marker analysis in the association population. J.S. and J.Y. performed the regional association analysis of the target major QTL region. S.H. and R.L. performed the SSR/InDel marker development. X.W. and G.L. performed the field experiments and traits investigation. J.S. analysed all the data and wrote the manuscript. All authors have read and approved the final manuscript.

**Additional Information**

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