International Journal of Agricultural Technology 2018 Vol. 14(7): 2261-2270
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

Fine mapping of quantitive trait loci for seed-related traits in yardlong bean

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Yoshida, A. K. and Tomooka, N. (2018). Fine mapping of quantitive trait loci for seed-related traits in yardlong bean. International Journal of Agricultural Technology 14(7): 2261-2270.

Abstract Yardlong bean is an important legume of Southeast and East Asia. It is believed to have been domesticated from vegetable (pod) cowpea. Among domestication-related traits, seed size is a distinctly trait that distinguish yardlong bean from its wild ancestor which has resulted in an approximately three-fold increase in seed length. Previously, we identified major QTLs for seed-related traits on linkage group 7, which were located on pleiotropic quantitative loci. Seed-related traits are highly complex quantitative traits that are controlled by multiple quantitative loci (QTLs) with a major and several minor effects and are influenced by multiple genetic and environmental factors. Thus, it is challenging to identify the major genes for controlling seed-related traits in yardlong bean. As the basis for fine mapping, a set of near isogenic lines (NILs) was developed from the cross between yardlong bean (JP81610) and wild cowpea (JP89083) population based on three generations of backcrossing and three generations of selfing. We have been able to narrow down the location of the genes underlying seed-related traits from 4.3 Mbp to 1.65 Mbp region. The locus was associated with transgressive variation for seed-and pod-related traits in this population. The phenotype was difficult to evaluate due to the influence of pod-related traits (pod length, pod width and pod softness) affected to seed size variation, underscoring the value of using multiple approaches to phenotyping, including extreme sampling and NILs group-mean comparisons. The fact that the QTLs controlling pod-related traits have also been detected on this target region, in which the genes for seed-related traits were associated, suggest that this region may generally not randomly distributed across the genome.

Keywords: yardlong bean, QTL, fine mapping

Introduction

Yardlong bean [*Vigna unguiculata* (L.) Walp. cv-gr. *sesquipedalis*] is one of important legume of Southeast and East Asia. It is believed to have been domesticated from vegetable (pod) cowpea which is characterized by its very long pods with seeds usually 8-12 mm long. Yardlong bean and cowpea differ phenotypically as a result of domestication process such as changes in plant architecture, gigantism in the consumed plant organs, reduced seed dispersal

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and loss of seed dormancy. Among domestication-related traits, seed size is a distinctly trait that distinguish yardlong bean from its wild ancestor which has resulted in an approximately three-fold increase in seed length.

The genetics of domestication-related traits of yardlong bean have been reported by Kongjaimun *et al.* (2012). 153 QTLs for 21 traits were identified using BC_1F_1 and F_2 populations from a cross between inter-subspecies; yardlong bean and wild cowpea. Most traits related to seed, pod, stem, and leaf were controlled by between one and eleven QTLs. QTLs for these traits show co-location on several narrow genomic regions on almost all linkage group, but especially on linkage group 7 that major QTLs for sizes of seed, pod, stem and leaf were principally located. Pleiotropy or close linkage of genes for the traits is suggested in these chromosome regions. Moreover, QTLs for seed-related traits have been reported in a limited number of legume crops including azuki bean (Kaga *et al.* 2008), rice bean (Isemura *et al.* (2010), mungbean (Isemura *et al.* 2012) and soybean (Zhang *et al.* 2004). Among these legumes, *BIG SEEDS1* (*BS1*) gene plays a key role in the control of increasing soybean seed size, weight and amino acid content and down-regulation of soybean *BS1* orthologous also resulted in similar phenotypes as the Medicago *bs1* mutants, supporting a conserved role of *BS1* in the control of organ size in legumes (Ge *et al.* 2016). Recently, Naito *et al.* (2017) reported *MOG* gene that produced *multiple-organ-gigantism* (*mog*) mutant, a recessive mutant of blackgram that produced bigger leaves, more biomass and larger seed but less number of seeds.

As seed-related traits are highly complex quantitative traits that are controlled by multiple QTLs with a major and several minor effects and are influenced by multiple genetic and environmental factors. Thus, it is challenging to identify the major genes for controlling seed-related traits in yardlong bean. This study was undertaken to refine the position of seed-related traits, mapped by Kongjaimun *et al.* (2012) to an interval 12.3 cM and to developed a set of near-isogenic lines (NILs) that would provide the foundation for isolation of the gene underlying these QTLs. We aimed to use the NILs to characterized the magnitude and behaviour of the wild cowpea-derived allele in a yardlong bean (domesticated type) background.

Materials and methods

Fine mapping development

The previous report from Kongjaimun *et al.* (2012) stated that the domestication-related QTLs detected in BC_1F_1 and F_2 populations. Kongjaimun *et al.* (2012) showed that the major QTLs for seed-related traits; seed length,

seed width and spacing between seeds in pod except for seed thickness were found on linkage group 7. In this study, BC_1F_1 population [(yardlong bean accession JP81610 x wild cowpea accession JP89083) x JP81610] of Kongjaimun *et al.* (2012) was used to develop fine mapping. Each backcross generation, flanking marker of the target region; cp07863 and cp00806 were used as marker-assisted selection and phenotypic data of seed-related traits were recorded. Finally, two BC_3F_1 plants (No.5-3 and No.5-18) were selected based on heterozygous genotype within the target region and as many other regions fixed allele of JP81610 as possible to produced BC_3F_2 population. Seed-related QTLs were re-evaluated in a segregation population of 1358 BC_3F_2 plants and confirmed that they were located in this target region. However, the phenotypic data of BC_3F_2 population was disturbed by virus infection, BC_3F_3 population was used to phenotypic confirmation for obtaining the accurate result. 16 BC_3F_3 seeds from each 96 BC_3F_2 lines or a total of 1536 BC_3F_3 seeds, were screened to reveal either recombinant or homozygous. Finally, selected 160 BC_3F_3 seeds with genotype covering the recombination point within the target region were evaluated for phenotype comparison (Fig. 1).

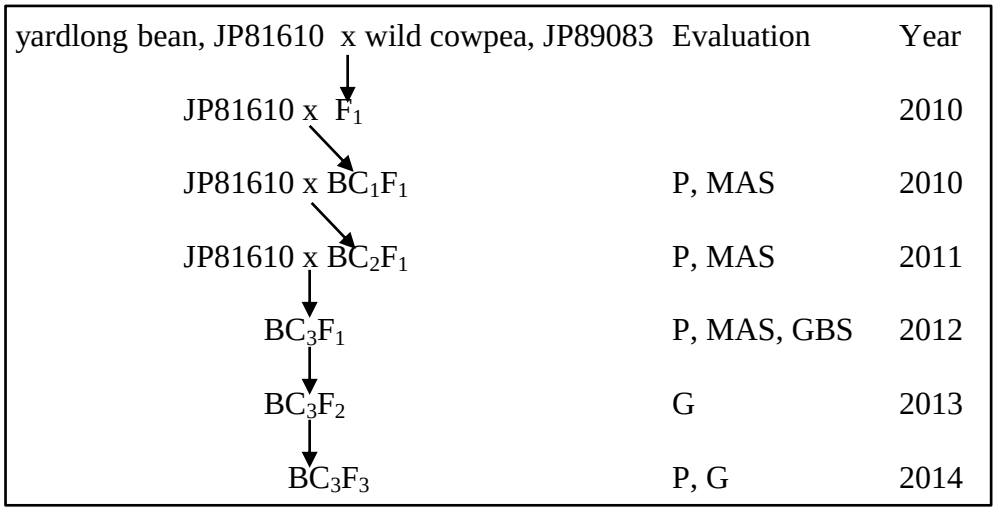

Figure 1. Procedures of NIL development and fine mapping. P, phenotyping; MAS, marker-assisted selection; GBS, genetic background selection; G, genotyping

Phenotypic evaluation

Seed phenotyping of 160 recombinant BC_3F_3 plants and 160 homozygous BC_3F_3 plants (control) were evaluated. 5 seed-related traits namely

seed length (SDL), seed width (SDW), seed thickness (SDT), spacing between seeds (PDSBS) and seed area (SDA), were average of ten seeds (Table 1).

Table 1. seed-related traits examined in BC_3F_3 populations of the cross between yardlong bean and wild cowpea

Trait	Trait abbreviation	Evaluation method
Seed length	SDL	Maximum distance from top to bottom of the seed use 10 seeds (mm)
Seed width	SDW	Maximum distance from hilum to its opposite side use 10 seed (mm)
Seed thickness	SDT	Maximum distance between both sides of the hilum use 10 seeds (mm)
Spacing between seeds in pod	PDSBS	Spacing between seeds is calculated by formula: [(PDL*10)-(SDL*SDNPPD)/SDNPPD]
Seed area	SDA	Seed area is calculated by formula: (SDL*SDW*SDT)

DNA extraction

Total genomic DNA was extracted from cotyledon of each 1356 BC_3F_3 seed (Fig. 2) using CTAB method (Lodhi *et al.* 1994). DNA concentration was estimated and adjusted to 50 ng uL^{-1} for simple sequence repeat analyse by Nanodrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific)

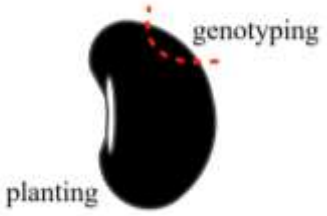

Figure 2. Each BC_3F_3 seed was extracted for genotyping and was planted for phenotyping

Molecular analysis and fine mapping strategies

The required density of molecular markers in the target region was achieved by using previous published SSRs of Kongjaimun *et al.* (2017) reported that the QTL of pod length, was located between molecular markers cp07863 and cp00806 on linkage group 7, with a marker interval of 12.3 cM where seed-related traits were also located. Kongjaimun *et al.* (2017) was successfully added 25 newly developed SSRs to this region. The marker interval of 8 recombination point within the target region (cp05517, VAGN2061, VAGN2007, VAGN1925, VAGN1912, VAGN1829, CEDG111,

VAGN0018 and VAGN0005) were used for genotyping of 1356 BC_3F_3 seeds to identified the recombinant seeds, then the recombinant seeds in each recombination region were planted to reveal the phenotypic segregation. PCR mixture in a total volume of 5 µL, containing 1.0 µL of template DNA, 2.5 µL of 2 × QIAGEN Multiplex PCR Master Mix, 1.0 µL of Q-solution, 0.2 µL of 20 pmol primers mix, and 0.2 µL of Taq. The 5'-end of the reverse primer was fluorescent labeled with one of the f following fluorescent dyes, Fam, Hex, or NED (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems). The PCR thermal cycling was programmed as follows: 95 ℃ for 15 min followed by 40 cycles of 94 ℃ for 30 s, 55 ℃ for 60 s, 72 ℃ for 60 s and final extension 72 ℃ for 10 min. After amplification, 1 µL of PCR product was mixed with 10 µL of Hi-Di formamide and 0.125 µL of 500 LIZ size standards (Applied Biosystems) and run on an ABI Prism 3100 or 3130xl Genetic Analyzer (Applied Biosystems). Using GENEMAPPER ver. 3.0 software (Applied Biosystems), alleles with three different colors in a multiplex PCR product were separated into respective loci and their sizes were determined.

Data analysis

Phenotypic means of two group (recombinant plants and homozygous plants) in each recombinant region were compared using T-test by R program version 3.1.2 (R Development Core Team, 2013).

Results

Fine mapping population used in this study derived from a cross between yardlobg bean accession JP81610 and wild cowpea accession JP89083. JP81610 had larger seed (11.9x3.7x6.2 mm) whereas JP89083 has smaller seed (3.9x2.0x2.7 mm). The heritabily for seed length, seed width and seed thickness were 92.5%, 81.0% and 65.7%, respectively. Seed size is remarkable domesticated trait of yardlong bean which has resulted in an approximately three-fold increase in seed length. Spacing between seeds in pod is about 26.6 mm for JP81610 and 1.9 mm for JP89083 with heritabily of 87.8%. It suggests that seed-related traits are highly inherited.

Kongjaimun *et al*. (2012) showed that the major QTLs of seed-related traits; SDL, SDW and PDSBS except for SDT were found on linkage group 7 with high phenotypic variance explained (PVE) and LOD score (Table 2). In this study, we focused on the target region of seed-related QTLs between cp07863 and cp00806 with the marker interval of 12.3 cm or approximately 4.3 Mbp by comparing to reference genome (azukibean; http://viggs.dna.affrc.go.jp).

Table 2. QTLs detected for seed-related traits in previous report of Kongjaimun *et al.* (2012)

Trait	QTL name	BC_1F_1				F_2			
		LOD	P	Loci	PVE (%)	LOD	P	Loci	PVE (%)
SDL	*Sdl7.1*+	13.6	0.0009	23.4	13.5	41.8	0.0009	46.3	26.5
SDW	*Sdw7.1*+	22.9	0.0009	21.6	20.9	5.3	0.0009	63.4	18.5
SDT	*Sdt7.1*+	8.5	0.0009	39.4	7.5	14.2	0.0009	43.0	15.2
PDSBS	*Pdsbs7.1*+	30.4	0.0009	34.2	25	6.2	0.0009	9.0	5.1

Table 3. Phenotype comparison between recombinant and homozygous plants within the region of interest

Recombinant region	Homozygous	Recombinant	T-test	*P*
	SDL			
VAGN2061-cp05517	11.22	9.72	1.86	0.1507
VAGN2007-VAGN2061	10.40	11.21	1.99	0.1203
VAGN1925-VAGN2007	12.04	11.34	1.50	0.2498
VAGN1912-VAGN1925	10.62	11.10	0.77	0.5131
VAGN1829-VAGN1912	9.41	10.66	3.29*	0.0176
CEDG111-VAGN1829	12.25	11.37	4.41*	0.0190
VAGN0018-CEDG111	12.13	11.07	5.17**	0.0050
VAGN0005-VAGN0018	11.69	10.69	2.33	0.0897
	SDW			
VAGN2061-cp05517	5.71	5.09	1.94	0.1159
VAGN2007-VAGN2061	5.36	5.67	5.81**	0.0052
VAGN1925-VAGN2007	6.06	5.45	3.83*	0.0304
VAGN1912-VAGN1925	5.33	5.81	2.36	0.1049
VAGN1829-VAGN1912	5.24	5.85	4.66**	0.0055
CEDG111-VAGN1829	6.04	5.59	3.50*	0.0201
VAGN0018-CEDG111	6.39	6.02	2.89*	0.0334
VAGN0005-VAGN0018	5.88	5.76	1.22	0.3243
	SDT			
VAGN2061-cp05517	3.96	3.65	0.90	0.4083
VAGN2007-VAGN2061	3.80	3.73	0.25	0.8356
VAGN1925-VAGN2007	3.65	3.81	0.84	0.4497
VAGN1912-VAGN1925	3.88	3.89	0.02	0.9861
VAGN1829-VAGN1912	3.88	3.95	0.53	0.6299
CEDG111-VAGN1829	3.99	3.83	0.59	0.5855
VAGN0018-CEDG111	4.05	3.87	1.35	0.2271
VAGN0005-VAGN0018	4.08	3.98	0.33	0.7565

* significant at 5% level, ** significant at 1% level

Table 3 (continued).

Recombinant region	Homozygous	Recombinant	T-test	*P*
	PDSBS			
VAGN2061-cp05517	20.00	12.20	1.58	0.2310
VAGN2007-VAGN2061	11.45	16.35	4.65*	0.0119
VAGN1925-VAGN2007	23.13	15.53	3.48	0.0516
VAGN1912-VAGN1925	11.30	17.10	6.39**	0.0084
VAGN1829-VAGN1912	11.78	16.48	7.75**	0.0006
CEDG111-VAGN1829	24.20	14.98	1.68	0.1895
VAGN0018-CEDG111	25.45	18.33	3.56*	0.0175
VAGN0005-VAGN0018	21.13	17.87	1.01	0.4063
	SDA			
VAGN2061-cp05517	253.63	187.08	1.80	0.1606
VAGN2007-VAGN2061	211.30	235.70	1.99	0.2690
VAGN1925-VAGN2007	266.25	236.73	1.20	0.3282
VAGN1912-VAGN1925	219.58	253.70	0.84	0.4824
VAGN1829-VAGN1912	192.43	246.15	3.34*	0.0270
CEDG111-VAGN1829	295.15	243.25	2.42	0.0821
VAGN0018-CEDG111	313.83	257.58	4.55**	0.0039
VAGN0005-VAGN0018	281.40	246.03	1.19	0.2985

* significant at 5% level, ** significant at 1% level

To finely map the seed-related loci, we conducted the population of three generations of backcrossing and three generations of selfing (BC_3F_3). Of the 1538 BC_3F_2 plants, 337 showed heterozygous allele in the target region (24.82% were recombinant lines). In addition, we screened 96 recombinant BC_3F_3 line (16 seeds per line) with 9 markers, cp05517, VAGN2061, VAGN2007, VAGN1925, VAGN1912, VAGN1829, CEDG111, VAGN0018 and VAGN0005, covered 8 recombination point to classified into two goup; recombinant and homozygous seeds. Finally, 160 plants for each recombinant and homozygous were evaluted for phenotypic comparison. Phenotypic comparison between recombinant plants and homozygous plants within the region of interest showed that seed length gene was located between recombination region of VAGN1912 and VAGN0018 with the marker interval of about 1.65 Mbp by revealing the significant difference of seed length mean between recombinant and homozygous plants (Table 3). Gene of seed width was probably located on between VAGN2061 and VAGN1925, and VAGN1912 and VAGN0018. Gene controlling spacing between seeds per pod was found between VAGN2007-VAGN2061, VAGN1925-VAGN1829 and

VAGN0018-CEDG111. Seed area gene was found between VAGN1912 and VAGN0018, same location as seed length gene. Gene of seed thickness was not appeared in this study due to only minor QTL was detected on this linkage group which was difficult to detect for fine mapping (Table 3). As seed length, seed width, spacing between seed were clustered on the pleiotropic region in which they were probably linked, phenotyping their character were rather difficult due to other traits influenced. However , the result revealed that a locus for these traits were found between VAGN1829 and VAGN0018 with a limited region of 1.65 Mbp (Fig.3). Thus , it suggested that gene controlling seed-related traits were principally existed.

Discussion

In previous study of Kongjaimun *et al*. (2012), multiple QTLs were identified throughout the genome of yardlong bean. Especially, on chromosome 7 QTLs with the largest phenotypic contribution for seed-related traits were identified. The seed-related traits are remarkable domesticated trait of yardlong bean, have resulted in an approximately three-fold increase in seed length. Seed-related traits are shown by co-location of the QTLs on narrow region of linakge group 7, where pod-related traits such as pod length, pod width, pod dehiscence were also located. Clustering of QTLs is due to either close linkage or pleiotropy or both. Pleiotropic effects on various organs have been reported in several crops such as common bean (Koinange *et al.* 1996), maize (Doebley *et al.* 1995), tomato (Downie *et al.* 2003), rice bean (Isemura *et al.* 2007, 2010; Kaga *et al.* 2008) and sunflower (Bachlava *et al.* 2010). As seed size is one of domestication-related trait, QTLs controlling these traits are generally not randomly distributed across the genome (Gepts, 2004). QTLs controlling these traits may be related to cultivation magnetism and should be considered under the protract transition paradigm of crop demoestication (Allaby, 2010). In this study, gene controlling seed-related traits (*sdl7.1+*, *sdw7.1+*, *sda7.1+* and *pdsbs7.1+*) were found in the limited region of about 1.65 Mbp between VAGN1829 and VAGN0018. However, another gene of seed width (*sdw7.2+*), spacing between seeds in pod (*pdsbs7.2+*, *pdsbs7.3+*) and seed area (*sda7.2+*) were probably located on near expected region (Fig. 3).

Unlike other legumes studies, we focused on gene controlling seed-related traits in yardlong bean mainly located on linkage grouop 7 following previous reported of Kongjaimun *et al.* (2012). Ge *et al.* (2016) found *BIG SEEDS1* (*BS1*) gene plays a conserved role in the control seed size and weight in the model legume *Medicago truncatula* and the grain legume soybean (*Glycine max*). we BLASTed two *BS1* orthologus namely *GmBS1* (*Glyma10g38970*) and *GmBS2* (*Glyma20g28840*) to azuki bean genome

database (http://viggs.dna.affrc.go.jp), the reference genome for yardlong bean, demonstrated that this gene was detected on chromosome 8. Also, Naito *et al.* (2017) identified *multiple-organ-gigantism* (MOG) locus produced bigger leaves, more biomass and larger seed but less number of seeds located on chromosome 8. Comparing seed size QTLs in *Vigna* crops, large effect of QTLs was found in various chromosome (Isemura *et al.* (2010, 2012)); Kaga *et al.* (2008); Zhang *et al.* 2004). Unfortunately, we did not investigate gene controlling seed-related trait in chromosome 8 and another due to only small effect of QTLs was found by Kongjaimun *et al.* (2012).

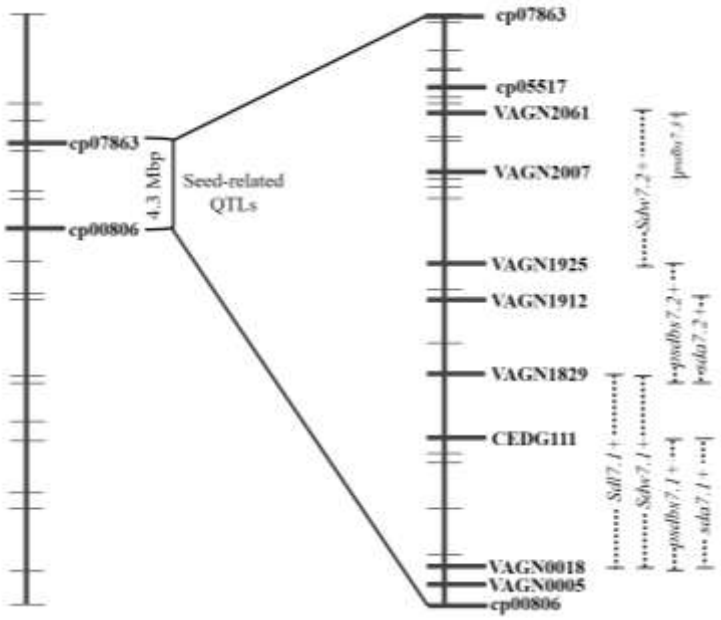

Figure 3. Fine mapping for seed-related traits on linkage group 7

Here we narrowed down the target region of gene controlling seed-related traits on linkage group 7 to obtain the candidate region for gene sequencing that will be useful for legume breeding program with improving commercial value and yield.

Acknowledgement

This study was funded by the Japan Socity for the Promotion of Science (JSPS).

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(Received: 15 September 2018, accepted: 5 November 2018)