Analyzing quantitative trait loci of agronomic traits for plant architecture and yield in adzuki bean (*Vigna angularis*).

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KACHAPILA Modester Takondwa

Doctoral Program in Animal Science and Agriculture

Graduate School of Animal and Veterinary Science and Agriculture

Obihiro University of Agriculture and Veterinary Medicine

アズキ（*Vigna angularis*）の草型および収量性に関与する農業形質の量的形質遺伝子座の解析

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帯広畜産大学大学院畜産学研究科

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カチャピラ・モデスター・タコンドワ

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Adzuki bean production

Adzuki bean (*Vigna angularis* (Ohwi &Ohashi)) is a leguminous crop belonging to the *Fabaceae* family. It is a self-pollinating plant and diploid in nature having the chromosome number 2n=2x=22 [1] [2]. Adzuki bean is a culturally important crop in over 30 East Asian countries, particularly Japan, China, Korea, Nepal and Bhutan [3,4]**.** There is a growing popularity for adzuki bean in other countries including Canada and United States, however, there is dire need for varieties suitable for mechanized farm operations [5]. In Japan, adzuki bean is the second important legume after soybean [6–8]. Adzuki bean is normally small-seeded but some large-seeded varieties known as 'Dainagon' exist as well. The small-seeded types on average weighs 13 to 15 g per 100 seeds while the Dainagon type on average weighs 18 to 20 g per 100 seeds [9]. Adzuki bean is an exceptional source of starch and protein, with a protein content threefold higher than cereals and recommendable health food [2]. Hence, it is a regular ingredient in East Asian cuisine for both sweet and spicy dishes. Adzuki beans from Hokkaido are well known and sought after in the Japanese confectionery industry due to the quality of bean for jam making characteristic [10]. However, compared to other crops, research work on adzuki bean genomic breeding and improved production systems is still limited [1,2]. Therefore, there is need to develop varieties with high yield, good quality [11]and suitability for efficient production systems to sustain productivity.

On average, Hokkaido produces 49,800 out of 53,4000 tons of adzuki bean produced annually in Japan, representing around 93% of the domestic production (Figure 1.1) [12,13]. However, of late, there has been a decreasing production trend due to reduced farming families but increased land area coupled with high labor demand but limited mechanization for critical farm operations like weeding and harvesting [12]. For instance, about 20% adzuki bean production decline has been observed in Hokkaido over the past 10 years from around 23,800 hectares to 19,000 hectares (Figure 1.1) [10,13]. It is therefore important to improve the mechanization systems for sustainable adzuki bean production in Hokkaido.

1.2 Plant architecture in adzuki bean

The sustainable agricultural productivity to generate sufficient food stocks for the globally continuously increasing population remain a big challenge [14]. The prime goal in breeding programs therefore is to improve crop productivity and plant architecture is one of the agronomic traits of significance to high productivity [15,16]. Plant traits that are associated with architecture such as resistance to lodging and mechanization ability are determinants for productivity in most crops [14,17]. Therefore, genetic improvements in plant height-related traits is necessary for the breeding of genotypes with ideal architecture [14,18]. The genomics of plant architecture are well-known in other legumes such as soybean [14,19,20]. Although some studies have been reported on plant architecture traits in adzuki bean [7,21–24], still more work is needed to fully explore the genetic control mechanisms for effective application in adzuki bean breeding programs.

Quantitative traits such as plant height, are complex and vary significantly in phenotypes based on the genetic control mechanisms and other external factors for example the growing environment [14]. Genotype analysis provides useful information for advanced methodologies such as molecular breeding for desirable traits including ideal plant architecture. Notable progress has been reported on genomic crop improvements. For instance; in cereal crops shorter stem lengths have shown to enhance yields and improve resistance to lodging, light capture and nutrient use [14,25,26]. In mung bean (*Vigna radiata), VrDet1* gene was identified as a functional ortholog of *DT1* in soybean, *PvTFL1y* in common bean and *TFL1* in Arabidopsis, that control stem elongation by regulating levels in the shoot apical meristem [27]. Interestingly, the gene for an indeterminate stem growth habit *stdet5.9.1* was mapped in mung bean, and considered to be associated with a QTL responsible for loss of twinning habit in adzuki bean [28]. Cumulatively, plant height in legumes is influenced by stem growth habit (determinacy), which varies according to the genetic control mechanism. Regardless of, the similarity observed in the control of stem growth habit among mung bean and adzuki bean [28], variations exist in the in the phenotypic expressions

of architectural traits in legumes. In this view, studies to isolate genes responsible for stem elongation and other architectural traits are required to advance breeding approaches in adzuki beans.

One desirable component of plant architecture is epicotyl length, which is the lower part of the stem from the cotyledon to the primary node. Long epicotyl traits in adzuki bean are crucial in improving fitness for mechanized work. However, excessively long stems are not desirable due to plant lodging susceptibility and tillage incapability that reduces the production efficiency and productivity. Previously, Mori et al. and Kachapila et al., reported important QTLs, loci interactions, parental allele effects and potential candidate genes for epicotyl in recombinant inbred lines developed from cultivars [23,24]. However, the studies could not fully conclude on the responsible genes for epicotyl length in the identified target regions. Thus, signifying the need for more detailed analysis using the information generated in the previous studies on epicotyl length, to fully dissect the control mechanisms of epicotyl length. Besides, epicotyl length, other factors including plant height, stem internode length and node number equally contribute to plant architecture. An understanding of all corresponding factors and trait combinations is hence needed.

1.3 Yield traits in adzuki beans

Hokkaido produces a bulk of adzuki bean in Japan, therefore sustainable production in this region is important. Lately, there has been an increase in the average farm holding size per farmer, because of a reduction in the number of farmers. Indicatively farming households reduced from 2,848,166 to 2,155,082 over a 10-year period from 2005 to 2015, on the other hand, the population of farmers owning a minimum 5.0 ha farmland registered and increase from 87,724 to 90,992 [29]. A contribution factor to the changes in the farm holding sizes is the observed increment in the number of aged farmers in the range of 56 to 60 years [29]. Therefore, the aged farmer population, labour scarcity and laborious weeding and harvesting farm operations, call for urgent action on the development of improved plant materials, labor-saving and efficient technologies in adzuki bean production.

The cold weather is another challenge affecting yield levels in Hokkaido region. Particularly due to the short frost-free period. Adzuki bean growing period is 100 to 140 days from end May to mid-October [12]Normally, frost starts by mid-October [9,12] , however, around this time most late maturing adzuki beans are still in field and in such cases, the yield and grain quality of adzuki beans is affected. Therefore, to minimize the effect of frost of adzuki bean, breeding of varieties with early maturity is paramount. In view of this, Tokachi agricultural experiment station (TAES) has been working on adzuki bean improvement including breeding for early maturity and efficient production systems. Recent developments include a medium maturity variety 'Kitaroman' and early maturity 'Chihayahime'. However, 'Kitaroman' variety show poor soil-borne disease resistance and lower yields. The variety 'Chihayahime' is relatively stable in yield level and has better disease resistance [30], however, the genomic mechanism controlling the earliness trait is not fully exploited. Apart from the earliness, disease resistance and high yield traits, higher leaf senescence at maturity is desirable. Green leaves and stems with higher moisture content at maturity stage entangle during the mechanized harvesting process, hence, reducing the pace as well staining the pods and grains resulting into low quality beans. Therefore, further understanding of underlying factors for the respective yield-related traits is necessary to achieve optimal yield in adzuki bean production.

1.4 QTL mapping

QTL mapping and other developments in genome sequencing tools give opportunities for advanced analysis of genetic factors that regulate agronomic traits and application of the generated information into breeding programs of interest. Adzuki bean is a crop of economic significance, and there is growing need for genetic improvements to address current production challenges. While model plants including *Arabidopsis thaliana* and other key legumes such as soybean are adequately studied, studies in adzuki bean are limited. As such, the estimated genome size in adzuki bean is small around 500 Mb [1,6,11]. Nonetheless mapping populations that has been useful in adzuki bean genome analysis have been developed. Initially, a backcross population from (*V. nepalensis x V. angularis*) was used to construct a genetic map

consisting 11 linkage groups, with single sequence repeats (SSR), amplified fragment length polymorphisms (AFLPs) and restriction fragment length polymorphisms (RFLPs) [31]. Discovery for next generation sequencing technologies has enabled high speed whole-genome studies and development of several other sequencing tools. The Vigna Genome Server (ViGGS) (http:// http://viggs.dna.affrc.go.jp/) was developed as an access to genomic information using 'Shumari' variety sequencing [32]. Henceforth, studies in adzuki bean have based on the develop sequences as a reference genome [2,6–8,21,23,24].

The advances in next generation sequencing tremendously enabled development of mapping tools such as QTL-seq [33]. Despite the improved QTL mapping accuracy in techniques like QTL-seq, the genetic architecture for causal interactions on QTLs are not sufficiently detectable. Hence, the need to employ other techniques that enhance the understanding of casual inferences for target traits, such as *qtlnet* as proposed by [34,35]. The *QTLNetwork* or *qtlnet* is a methodological software that goes beyond simply QTL mapping by enabling visualization of the web of underlying interactions for complex traits in experimental populations [35]. So far, the QTL networking methodology has been successfully applied in studies for rice [36] , rye [37] , and *Brassica rapa* [38].

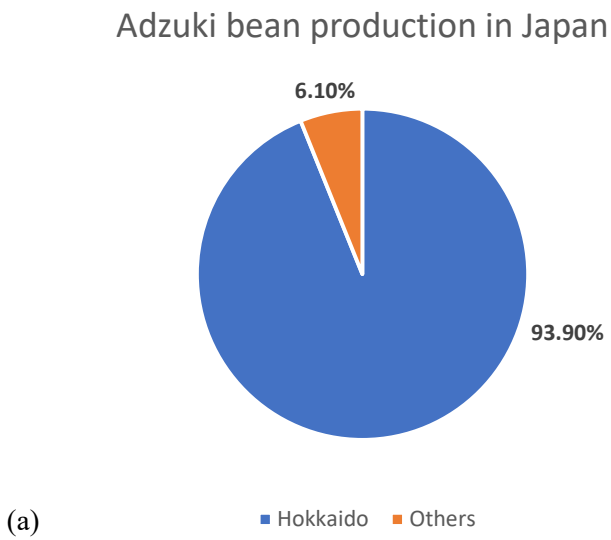
1.5 Study purpose

The overall objective of this study was to analyze the quantitative trait loci of agronomic traits for improved plant architecture and yield in adzuki beans (*Vigna angularis*). The specific objectives of this study were therefore:

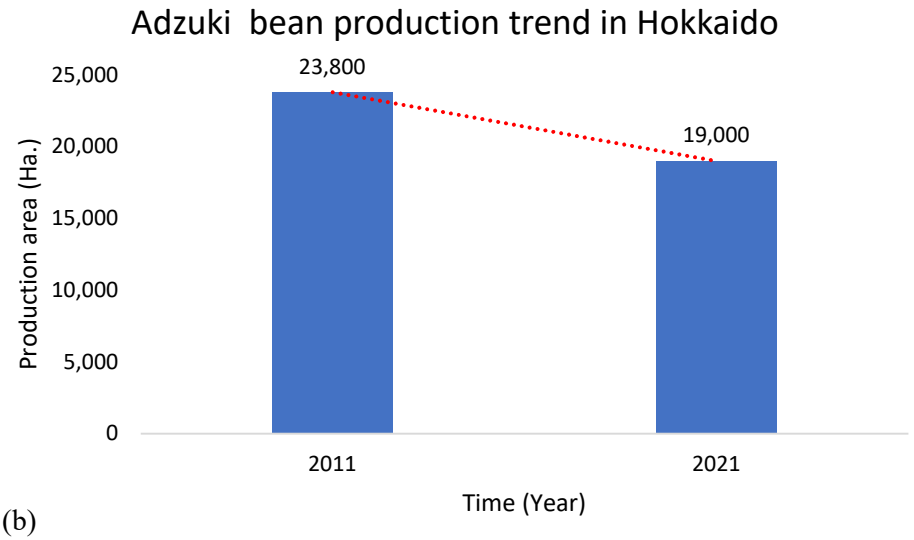
(i) To clarify the genetic mechanisms associated with epicotyl length for improved mechanization efficiency in adzuki beans.

(ii) To clarify the genetic mechanisms for plant height-related traits for the development of adzuki beans with ideal plant architecture.

(iii) To clarify the genetic mechanisms for maturity and yield-related traits in adzuki beans.



(a)



(b)

Figure 1-1 (a) Adzuki bean production status in Hokkaido; (b) Decreasing adzuki bean production trend in Hokkaido

Source: (a) National Agriculture and Food Resource organization, (b) Hokkaido Agricultural Administration Department.

CHAPTER 2: FINE-MAPPING OF EPICOTYL LENGTH TRAIT IN ADZUKI BEAN (*Vigna angularis*).

2.1 Introduction

Epicotyl length (ECL) in legume crops is an important agronomic characteristic due to the trait's effect on plant height. ECL influences productivity through plant density, competition for light and suitability for mechanization [39] . In adzuki bean (*Vigna angularis* [Willd.] Ohwi and Ohashi), ECL traits strongly influence the efficiency of cultivation with large machinery [12]. Adzuki bean seedlings are very small, hence, tilling and weeding with large machinery poses a risk of burying the seedlings in the soil [12]. Plants with long epicotyls, however, are less likely to be covered by soil or damaged by mechanical tillage or weeding. Long epicotyls are also advantageous at the pod harvesting stage. During mechanical harvesting, pods close to the soil surface are damaged by the cutting blade, resulting in harvest loss, which potentially reduces yield in adzuki bean [23] . Plants with long ECL have the capability to minimize harvest losses because they have few pods close to the soil surface. Current cultivars, however, have short epicotyls that are less than 5 cm long [23]. Thus, weeding at the seedling stage and harvesting at pod maturity are labor-intensive stages in the cultivation of adzuki bean, because the operations have not been efficiently mechanized. One of the main breeding goals is for improving adzuki bean productivity is to develop long ECL genotypes, that allow for enhanced mechanization efficiency [12,23]. Studies on the genetic mechanisms controlling ECL are needed to support that breeding goal.

Quantitative trait loci (QTLs) associated with ECL have been mapped in adzuki bean. The morphological and physiological traits were explored to understand the genetics related to the domestication of adzuki bean [7,21]. ECL QTLs were detected on linkage groups 2 and 9 in a study using an adzuki bean landrace accession (JP81481) and closely-related wild species (*V. nepalensis*) accession (JP107881) [21]**.** Two QTLs associated with ECL were also detected on linkage groups 1 and 9 in a population derived from a cross between wild adzuki bean accession JP110658 and adzuki bean cultivar Kyoto Dainagon [7]**.** The adzuki bean cultivar (*V. angularis*) show longer epicotyls than the wild species (*V. nipponensis*) due to ECL

increasing effect from the cultivated parent allele [7,21].Thus, the domestication led to increased ECL in cultivars. However, ECL in common cultivars is still not ideal for efficient mechanization.

In our prior research, ECL was evaluated in an F_2 population derived from a cross between breeding line Tokei1121 (with longer epicotyls) and cultivar Erimo167 (with ordinary length epicotyls). The results showed that ECL is controlled by multiple genes. Four QTLs associated with differences in ECL in cultivated germplasm were detected on chromosomes 2 (*qECL2*), 4 (*qECL4*), 7 (*qECL7*) and 10 (*qECL10*). The results of segregation pattern analysis using residual heterozygous lines (RHLs) at *qECL10* showed that a single recessive gene derived from Tokei1121 contributed to the longer ECL phenotype. This gene was mapped to a ~255 kb region near the end of chromosome 10 [23]. The chromosomal loci for the ECL QTLs detected in studies using wild species differed from those detected in studies using cultivated germplasm. This suggests that genetic variations for ECL among cultivars is governed by different QTL than those that contribute to variations in ECL between cultivars and wild species. However, the inadequate marker-saturation of the linkage map in [23] was a limiting factor in the identification of QTLs associated with ECL in that study. Hence, there is a need for more in-depth analysis of the genomic control mechanisms for ECL in cultivated adzuki bean.

In this study, a breeding line with longer epicotyls, Toiku161, and a cultivar with ordinary length epicotyls, Chihayahime, were re-sequenced to take advantage of the accurate and rapid QTL detection. Then, using a population of recombinant inbred lines (RILs) derived from a cross betweenToiku161 and Chihayahime, QTLs associated with ECL were identified by QTL-seq and linkage mapping. Finally, the QTL target region on chromosome 7 was narrowed down using substitution mapping.

2.2 Materials and methods

2.2.1 Plant materials and phenotypic evaluation in RILs

For QTL-seq analysis, an F_7 population with 155 individuals was developed by crossing 'Toiku161' (female parent) with 'Chihayahime' (male parent). 'Toiku161' is a breeding line with long epicotyls, and 'Chihayahime' is an early maturing cultivar released in 2016 that lacks the long epicotyl trait (Figure 2.1). Both parental materials were developed by the breeding program at the Tokachi Agricultural Experimental Station (TAES), Hokkaido Research Organization, Japan.

The F_7 population and the parental lines were grown in greenhouses maintained at 22 °C during the day and 15 °C at night and a daylength of 16 h. This experiment was conducted from mid-November to mid-December 2017. Because of the short natural day-length (9 to 10 h) at this time, supplemental lighting was provided from 6 to 9-am and from 3 to 10-pm using high intensity LED lighting units (PFQ-300P/DT: Nippon Medical and Chemical Instruments, Osaka, Japan). The photosynthetic photon flux density of supplemental lighting was 1500 μmol m^{-2} s^{-1}. A paper pot (Nippon Beet Sugar Manufacturing, Tokyo, Japan) with 4.7 cm × 4.7 cm × 5.0 cm depth was used to grow each plant. The paper pot was placed in a plastic tray (60.5 cm × 30.7 cm × 3.0 cm depth) and filled with potting soil (Hokusan, Hokkaido, Japan) containing chemical fertilizer (N 340 mg L^{-1}, P 1350 mg L^{-1}, K 220 mg L^{-1}, pH 6.2). A single seed was planted in the center of each pot at 1.0 cm depth. Twelve plants were grown per line. An adequate amount of water was supplied when the soil surface became dry. The plant trays were rotated every 3 days to minimize positional effects in the phytotron. ECL of the F_7 population and the parental lines was measured at 35 days after planting when epicotyl elongation has ceased. ECL was measured from the ground surface to the primary leaf node on the main stem of the plant in 0.1 cm increments. Mean ECL was calculated from the ECL values of each of the 155 individuals in the F_7 population and parental line.

2.2.2 Construction of sequencing libraries and sequencing

From the 155 individuals in the F_7 population, 20 individual lines with extreme phenotypes were selected based on ECL phenotyping data in F_7 population. The ten (10) lines with lowest mean ECL (over 10% of the total population) were selected for the shorter epicotyl length (SECL) group and the 10 lines with the longest mean ECL were selected for the longer epicotyl length (LECL) group. DNA extracted from these two groups were used to construct the DNA bulking groups following the QTL-seq method described by [6]. Genomic DNA was extracted from the 20 selected lines and parental lines using the DNeasy® Plant Mini Kit (QIAGEN) according to the manufacturers' protocol. The DNA concentrations were measured with QubitTM dsDNA BR Assay Kits (Life Technologies, Carlsbad, CA, USA) with a Qubit fluorometer 2.0 (Invitrogen, Grand Island, NY, USA), according to the manufacturers' directions. The DNA concentration of each sample was adjusted to a final concentration of 20 ng μL^{-1}. Equal amounts of DNA from the 10 RILs in the SECL-group and the 10 RILs in the LECL-group were mixed to produce a bulk DNA sample for the SECL-group and the LECL-group, respectively.

Each bulk DNA sample and DNA from the parental lines was fragmented using Covaris S220 conditions that resulted in a fragment length of 300 bp. Libraries were prepared according to the KAPA Hyper Plus Kit manual (Kapa Biosystems, Wilmington, MA, USA) using adapters from the FastGene Adapter Kit (FastGene). To obtain li-braries of the desired DNA size, DNA was fractionated using Labchip XT (PerkinElmer, Waltham, MA, USA) and the concentration of the prepared libraries was measured with the Qubit dsDNA Assay Kit (Life technologies). The quality of the prepared libraries was then checked using Fragment Analyzer and the dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Ankeny, IA, USA). The prepared libraries were sequenced by paired-end sequencing at 150 bp on a Hiseq X Ten (Illumina, San Diego, CA, USA), with a data output of around 30 Gb per sample.

The QTL-seq pipeline (https://genome-e.ibrc.or.jp/resource/mutmap, accessed on 5 April 2018) was used to estimate the candidate QTL regions following the protocol de-scribed by [33]. Firstly, quality control of short reads was performed to exclude reads with low quality values using the FASTX-Toolkit

(http://hannonlab.cshl.edu/fastx_toolkit/download.html, accessed on 5 April 2018). To develop a 'Chihayahime reference sequence', the sequence reads of 'Chihayahime' were aligned to V. angularis v1.0 reference genome (https://viggs.dna.affrc.go.jp/download, accessed on 5 April 2018) using BWA software ver. 0.5.9-r16 (https://sourceforge.net/projects/bio-bwa/files, accessed on 5 April 2018) and SNPs were detected using SAM tools [40]. A synthetic 'Chihayahime reference sequence' was con-structed by replacing the detected SNPs with DNA sequence from the V. angularis v1.0 reference genome. In order to increase the accuracy, for subsequent analyses, additional SNPs were detected by re-aligning the short reads from the 'Chihayahime' parent to the constructed 'Chihayahime reference sequence'. The short reads containing SNPs detected at this stage were not used in subsequent analyses because they were considered to contain false-positive SNPs. SNP-index and Δ(SNP-index) were calculated to identify genomic regions for ECL, following [33,41,42]. SNP-index was referred to as to the proportion of reads with SNPs different from the reference parent (Chihayahime) sequence. To calculate SNP-index values for the SECL-group and LECL-group samples, sequence reads were first aligned to the 'Chihayahime reference sequence' using BWA software. High-quality SNPs were identified by mapping the short reads of both SECL and LECL groups with an average read depth of ≥10, using SAMtools [40] and based on the proto-col described by [33]. The SNP-index was calculated for each bulked sample using the formula: SNP-index (at a position) = count of alternate base/count of reads aligned. Differences in SNP-index values (Δ(SNP-index)) were calculated by subtracting the SNP-index of the SECL-group from the SNP-index of the LECL-group for each identified SNP across the genome at 2 Mb intervals using a 50 Kb sliding window.

2.2.3 Phenotypic trait evaluation in F_2 population

An F_2 population with 96 individuals was used to validate the position of QTLs detected by QTL-seq. These plants were obtained by self-pollinating F_1 seed derived from a cross between 'Chihayahime' (female parent) and 'Toiku161' (male parent).

The F_2 population and 4 plants of each parent were grown in the experimental field of TAES in 2018. Single seeds per planting station were planted at 60 cm ridge spacing and 20 cm within ridge plant spacing with 3 to 4 cm planting depth. ECL was measured 35 days after planting, at a time when the epicotyls were fully extended.

2.2.4 Genome-wide detection of SNPs and InDels, and marker analysis

SNP and InDel position between Toiku161 and Chihayahime were detected using GATK ver. 3.8 software (https://doi.org/10.1101/gr.107524.110) [43]. To remove adapter sequences and low-quality reads, the raw data were cleaned using Trimmomatic ver. 0.38 (https://doi.org/10.1093/bioinformatics/btu170) [44]. After trimming, only sequences with pair-end reads were aligned to the *V. angularis* v1.0 reference genome (https://viggs.dna.affrc.go.jp/download) using BWA ver. 0.7.17 (https://doi.org/10.1093/bioinformatics/btp324) [45] and SAMtools ver. 1.9 (https://doi.org/10.1093/bioinformatics/btp352) [40]. Duplicate reads sequenced multiple times were deleted using picard ver. 2.18.23 (http://broadinstitute.github.io/picard). Realignment of short reads close to insertion and deletion regions was done using the GATK. The SNPs and InDels identified were filtered by a mapping quality of ≥30 and minimum read depth of 10. Sequence variants between Toiku161 and Chihayahime were estimated from SNP/InDel information detected between Toiku161 and the reference genome, and between Chihayahime and the reference genome, respectively. InDel markers were developed based on SNP/InDel info5mation between Toiku161 and Chihayahime. A total of 34 InDel markers on the QTL region of chromosome 2, 7, 10, and 11 were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Table S2.1).

The PCR reaction solution comprised 2.9 µL sterile water, 0.05 µL each 100 pmol Forward and Reverse primers, 5 µL GoTaq Master Mix (Promega) and 2 µL DNA, making a total mixture of 10 µL. The PCR reaction conditions included an initial heat denaturation at 95°C for 2 minutes, 35 cycles denaturation at 95°C for 15 seconds, annealing at 55 to 60°C for 15 seconds and extension at 72°C for 30 seconds; and

a final extension step at 72°C for 2 minutes. The PCR products were subjected to 1xTAE buffer electrophoresis at 200V for 30 minutes and analyzed on 4% agarose gel. The gels were stained with ethidium bromide for 15 minutes and the PCR products were visualized by UV radiation.

2.2.5 Validation of QTLs in the F_2 population using linkage mapping

A linkage map for the F_2 population was constructed using JoinMap® ver.6 [46], based on the genotypes of 96 individuals in the population. Genetic distances (cM) were calculated using the Kosambi map function [47].

QTL analysis was performed using MapQTL® ver. 6 [48]. A permutation test was done using 1000 permutations at 5% LOD value significance, adopted as threshold level. Interval Mapping (IM) analysis was conducted. If any LOD peak exceeded the threshold, the closest marker to the peak was set as the cofactors and Multiple QTL Mapping (MQM) analysis using MapQTL® ver. 6 software. The procedure was repeated until no new peaks with values greater than the set LOD 3.0 threshold appeared.

2.2.6 Interaction assessment between major QTLs

To analyze interaction between two QTLs (*qECL7.1* on chromosome 7 and *qECL10.1* on chromosome 10), four F_2 plants (Plant ID: #236, #223, #206, and #286) were selected using DNA markers flanking *qECL7.1* and *qECL10.1*. Four DNA markers were used, namely TC13 and TC15 for *qECL7.1*, and TC24 and TC26 for *qECL10.1,* respectively (Table S2.1.). Both #236 and #223 possessed heterozygous allele at *qECL7.1* region, while #236 had homozygous for Chihayahime allele and #223 had homozygous for Toiku161 allele at *qECL10.1* region. On the contrary, both #206 and #286 possessed heterozygous allele at *qECL10.1* region, while #206 had homozygous for Chihayahime allele and #286 had homozygous for Toiku161 allele at *qECL7.1*. These four F_2 plants were self-pollinated and generated four F_3 residual heterozygous lines (RHLs).

The four F_3 RHLs were grown in a growth chamber (LPH-411SP: Nippon Medical & Chemical Instruments, Osaka, Japan). Seeds were sown in 5 cm × 5 cm × 5.5 cm depth planting trays filled with culture soil at a sowing depth of 2 cm. The temperature condition was set at daytime 22°C (16 h) and nighttime 15°C (8 h). The environment of photosynthetic photon flux density was 360 μmol m^{-2} s^{-1}. On the sowing day, the plants were irrigated and the tray was covered with a plastic sheet to retain moisture and the plastic sheet was removed on the third day. Supplementary irrigation was applied as needed during the plant's growing period. The plant trays were rotated daily to minimize positional effects in the chamber growing conditions. The ECL survey was done on day 21 after sowing, due to adequate lighting that promoted rapid growth of the plant materials,

2.2.7 Fine mapping of *qECL7.1*

A total of 6 DNA markers consisting of InDel, CAPs, dCAPs, and SNP markers were designed using Primer3Plus (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) to find different recombination points in the *qECL7.1* region (Table S2.2.). Self-pollinating plant #223 and genotyping inbred progeny over several generations generated five recombinant lines with different recombination sites. These included three F_5 (#223_81_75, #223_112_347, #223_160_46) and two F_6 (#223_95_178_193, #223_91_134_15) generations. In addition, two F_5 lines (#223_105_7 with Chihayahime sequence and #223_105_1 with Toiku161 sequence) that were homozygous for large unrecombined regions within the *qECL7.1* region, were chosen as isogenic controls.

The five recombinant lines and two lines with parental sequence were grown in a growth chamber as described above. Ten seeds resulting from self-pollination of each line were planted and the ECL was measured 21 days after planting. The mean ECLs of the five recombinant lines were compared using Dunnett's test. The chromosomal region containing *qECL7.1* was refined by comparing phenotypes and genotypes among the recombinant and nonrecombinant control plants.

Sequence information (Vangularis_V1) and inferred gene information (Gene structure and function information) downloaded from VIGGS (https://viggs.dna.affrc.go.jp/download) were imported into the Integrative Genomics Viewer (IGV: https://software.broadinstitute.org.software/igv/download), to identify predicted genes within the *qECL7.1* region.

2.2.8 Statistical analysis

To determine statistically significant differences, the mean ECL of the recombinant lines and parent lines used in the fine-mapping analysis were compared against control line #223_105_7 using Dunnett's test at $p < 0.01$ in R software version 3.6.2 [49].

2.3 Results

2.3.1 QTL-seq analysis for epicotyl length trait in F_7 population

The frequency distribution of the ECL trait showed a continuous variation in the 155 F_7 mapping population (Figure. 2.2). The ECL in the female parent Toiku161 was significantly higher at 12.1 cm than male parent 'Chihayahime' at 6.5 cm, indicating that 'Toiku161' had longer epicotyls than 'Chihayahime'. The F_7 population showed differences in ECL ranging from 3.2 to 14.1 cm with a mean length of 7.7 cm.

Based on the phenotypic data of the F_7 population, 10 plants with the longest epicotyls and 10 plants with the shortest epicotyls were selected to prepare the long epicotyl length (LECL) and short epicotyl length (SECL) DNA pools used in the QTL-seq analysis. The epicotyl lengths of lines in the LECL pool ranged from 12.0 cm to 14.1 cm while lines in the SECL pool had ECLs that ranged from 3.2 cm to 4.4 cm. Mean epicotyl lengths were of 12.9 ± 0.8 cm and 3.9 ± 0.4 cm for the long and short groups, respectively (Figure 2.2.).

A Manhattan plot of Δ(SNP-index) for the LECL and SECL pools was done for chromosomes 1 to 11 at 95% and 99% confidence intervals (Figure S2.1.). Values of Δ(SNP-index) that exceeded the QTL threshold at the 99% statistical level were identified at four locations, on Chromosomes 2, 7, 10 and 11 (Figure 2.3.). The QTLs were named *qECL2.1, qECL7.1, qECL10.1* and *qECL11.1* (*quantitative trait locus for EPICOTYL LENGTH 2.1, 7.1, 10.1 and 11.1*). At *qECL7.1*, one peak was detected above the QTL threshold between 6.50 and 10.65 Mb and at *qECL10.1* multiple peaks were detected between 19.75 to 28.85 Mb with the largest peak between 26.55 to 28.85 Mb (Table 2 1). No distinct peaks could be detected at *qECL2.1* and *qECL11.1*. All detected QTLs had a positive Δ(SNP-index) value indicating the presence of a QTL where the 'Toiku161' genotype contributes to long epicotyls.

2.3.2 Validation of major ECL QTLs in F_2 population

Multiple QTL mapping of an F_2 population was used to validate the candidate genomic regions detected by QTL-seq. The F_2 population showed a continuous distribution in ECL (Figure S2.2.). A linkage map was constructed using 34 InDel markers (Figure 2.4.). On chromosome 7, *qECL7.1* had a peak position at 51.1 cM that was associated with marker TC13 at 9,479,153 Mb physical position, flanked by markers TC12 and TC15. On Chromosome 10, *qECL10.1* had a peak position at 88.6 cM associated with marker TC26 at 28,568,685 Mb physical position, flanked by markers TC24 and TC26 (Figure 2 4., Table 2.2., Table S2.1.). The peak for *qECL7.1* had 6.7 LOD value and 21.2 % of phenotypic variance explained, while *qECL10.1* had 4.4 LOD value and 13.4 % of phenotypic variance explained. The additive effect of *qECL7.1* (-0.8 cm) was slightly higher than that of *qECL10.1* (-0.6 cm). At both loci, the Toiku161 sequence contributed to longer epicotyl lengths. The physical positions of these two QTLs corresponded to the genomic regions for the *qECL7.1* and *qECL10.1* detected by the QTL-seq method (Table S2.1.). The data from the F_2 mapping population failed to confirm ECL QTLs on chromosome 2 and chromosome 11 but validated the finding of major QTLs *qECL7.1* on chromosome 7 and *qECL10.1* chromosome 10.

2.3.3 Interaction between *qECL7.1* and *qECL10.1*

Four F_2 plants (#236, #223, #206 and #286) and F_3 populations derived from each plant were used to analyze the interaction between *qECL7.1* and *qECL10.1*. The distribution of ECL for each of the four F_3 RHL is shown in (Figure 2.5.).

A mono-modal distribution was observed in the ECLs of F_3 populations #236 ranging from 2.8 – 4.0 cm (mean 3.3 ± 0.3 cm) and #206 ranging from 2.5 – 4.3 cm (mean 3.3 ± 0.4 cm). A bimodal distribution was observed in the ECLs of RHL populations #223 ranging from 2.3 cm – 8.9 cm (mean 4.7 ± 2.0 cm) and #286 ranging from 2.7 cm – 9.7 cm (mean 5.0 ± 2.0 cm). Populations with a bimodal distribution could be divided into two groups. The 126 RHLs from #223 included 95 plants with short epicotyls (ECL ≤ 5.9 cm) and 31 plants with long epicotyls (ECL ≥ 6 cm). The 81 individuals from #286 included 61 plants with short epicotyls (ECL ≤ 5 cm) and 20 plants with long epicotyls (ECL ≥ 6 cm). This segregation pattern fits a monogenic 3:1 ratio, (χ^2= 0.10, p = 0.76), indicating that recessive genes at *qECL7.1* and *qECL10.1* from Toiku161 may contribute to the long epicotyl phenotype.

Plants in the F_3 population derived from plant #223 that were classified as 'Toiku161'-homozygous at *qECL10.1* had significantly longer mean epicotyl length (6.5 ± 0.5 cm) than those classified as 'Chihayahime' (2.6 ± 0.2 cm) homozygous or heterozygous plants. In the #286 population, plants classified as 'Toiku161'-homozygous at *qECL7.1* showed significantly longer mean epicotyl length (mean 6.6 ± 1.1 cm) than plants classified as 'Chihayahime' (2.8 ± 0.4 cm) homozygous or heterozygous plants. The 'Toiku161' effect for increasing ECL at *qECL10.1* was likewise observed in the Toiku161 homozygous class at *qECL7.1*, but not in the classes that were heterozygous and homozygous for the 'Chihayahime' sequence at *qECL7.1.* Hence, the results indicate that there is an interaction between the two loci for epicotyl length on chromosomes 7 and 10.

2.3.4 Fine mapping of epicotyl length trait QTL *qECL7.1* on chromosome 7

The genetic position for *qECL7.1* on chromosome 7 was fine-mapped using substitution lines developed by self-pollinating plant #223. Based on the phenotypic analysis, #223_112_347, which was classified as 'Toiku161' homozygous (7.3 ± 0.8 cm) at marker TC64 to TC101, showed significantly longer epicotyls than that of Chihayahime. Line #223_81_75, which was homozygous for 'Toiku161' at marker TC64 to TC102, likewise showed significantly longer epicotyls (7.0 ± 0.7 cm) than 'Chihayahime.' Mean ECLs for #223_112_347 and #223_81_75 did not differ from that of the Toiku161 control line (6.9 ± 0.7 cm) (Table 2.3.). Plants classified as 'Chihayahime'-like showed ordinary ECLs and mean ECL for #223_160_46 (3.9 ± 0.6 cm), #223_95_178_193 (4.0 ± 0.4); and #223_91_134_15 (4.0 ± 0.5 cm) was not different from that of the 'Chihayahime' control line (3.4 ± 0.5 cm) (Table 2.3.). Thus, the phenotypic classification appeared to be linked with genotype classes that were homozygous for the 'Toiku161' allele and for the 'Chihayahime' allele. Detailed mapping of the ECL trait on chromosome 7 showed that *qECL7.1* was linked to markers TC99 and TC102. The candidate genomic region of *qECL7.1* covers 418 kb between TC99 at 10,211,134 bp and TC102 at 10,628,880 bp physical position (Table 2.3.). According to gene prediction models in VIGGS (https://viggs.dna.affrc.go.jp), 35 putative genes (Table 2.4.) are contained within the 418 kb region of *qECL7.1.* Annotation for these genes include 3 non-protein coding genes, 7 hypothetical proteins, 18 uncharacterized proteins and 7 genes with putative functions.

2.4 Discussion

ECL is a key agronomic trait of adzuki bean. An important goal of adzuki bean breeding programs is the development of cultivars with increased ECL, within the range 6 to 8 cm [23]. ECL is a complex trait in legumes, that is controlled by one or multiple genes and QTLs [41,50]. An in-depth analysis of genetic mechanisms underlying complex traits such as ECL is therefore necessary for successful identification and fine-mapping of genomic regions contributing to crop improvement.

In this study, four QTLs for ECL were detected by QTL-seq on chromosomes 2 (*qECL2.1*), 7 (*qECL7.1*), 10 (*qECL10.1*) and 11 (*qECL11.1*) in an F_7 population developed from parental linesToiku161 and Chihayahime. Linkage mapping in F_2 population detected one QTL on chromosome 7 (*qECL7.1*) and one QTL on chromosome 10 (*qECL10.1*). Since both *qECL7.1* and *qECL10.1* were identified at the same chromosomal region using two different analysis approaches, it is likely that these two are consistent QTLs. In the present study, *qECL10.1* had a peak position associated with marker TC89 at 28,568,685 bp. Previously, *qECL10* was mapped to a 28,630,875 bp region at the terminal end of chromosome 10 using a RHL population developed from a cross between breeding line Tokei1121 (long epicotyls) and cultivar Erimo167 (ordinary length epicotyls) [23]. The data suggest that *qECL10* and *qECL10.1* are within the same region. Substitution mapping narrowed the chromosomal region of *qECL7.1* to a 418 kb region flanked by DNA markers TC99_10,211,134 bp and TC102_10,628,880 bp on chromosome 7. The expected physical position of *qECL7* detected by [23], using an F_2 population derived from Tokei1121 and Erimo167 was in 28.0 Mbp region. Alleles from the long epicotyl parents (Toiku161 and Tokei1121) at both loci increased ECL. The loci of *qECL7.1* and *qECL7* are about 18 Mbp apart, suggesting that they may be different QTLs. However, the study could not confirm this result due to the narrow chromosome 7. Thus, further studies are needed to determine if two different ECL-genes are present on chromosome 7, or if they are the same.

This study used substitution mapping to narrow down *qECL7.1* to the 418 kb region described above [24]. Annotation analysis using the VIGGS database (https://viggs.dna. affrc.go.jp, accessed on 29 October 2020) predicted 35 genes in the *qECL7.1* region. However, because the molecular function of *qECL7.1* is unknown, it was difficult to precisely identify candidate genes predicted in the *qECL7.1* region that contribute to ECL. Annotation of variants between parent lines revealed a mutation in the 5' untranslated regions (5' UTR) of *Vigan 07G1141000.01* that is similar in sequence to the kinesin-like protein found in soybean. Kinesins are adenosine triphosphate (ATP)-dependent biological motor proteins,

that function to transport molecules along microtubules, segregate chromosomes, and elongate cell wall [51]. No mutations were detected in the protein-coding region of this gene and the insertions observed in the 5'UTR region had many repeated sequences. Therefore, the present study could not confirm that *Vigan 07G1141000.01* is the gene responsible for *qECL7.1*. The mutations in 'Chihayahime' and 'Toiku161' may have altered the expression of *Vigan 07G1141000.01*, suppressed the transportation of essential cell wall molecules and reduced the cell elongation in epicotyls. In addition, there could be undetected mutations in the *qECL7.1* region that may affect the function and expression of genes in the candidate region. Therefore, further studies are needed to test whether differences exist in the transcription and translation of 'Chihayahime' and 'Toiku161' *Vigan 07G1141000.01*.

Transgressive segregation was observed in the F_2 population. Two QTLs, *qECL7.1* and *qECL10.1*, were identified in this study, and only the allele derived from 'Toiku161' showed an effect of increasing ECL. Beneficial alleles were not identified in the ordinary ECL parent 'Chihayahime'. The identified QTLs, *qECL7.1* and *qECL10.1*, explained 21% and 13% of the phenotypic variation for ECL in the adzuki bean mapping population, respectively. The constructed linkage maps covered approximately 92% of the whole adzuki bean genome, although there were large gaps between DNA markers in some regions. Therefore, there may be additional unidentified loci in the mapping population that contribute to ECL.

This study applied the QTL-seq method to take advantage of high-throughput genome sequencing and bulked segregation analysis (BSA). Based on a 2 Mb and 50 kb sliding window, four QTL regions *qECL2.1, qECL7.1, qECL10.1* and *qECL11.1* were identified. The QTL-seq methodology has proved useful in accurate determination of QTL regions, as it mainly requires whole genome resequencing of distinct DNA bulks rather than individual genotyping of an entire population [52,53]. Furthermore, the availability of a draft genome assembly facilitates the QTL sequencing process since initial marker development is not necessarily required. QTL-seq has been applied to legume crops including; soybean (*Glycine max*) plant height [41]; chickpea (*Cicer arietinum*) 100 seed weight [54]; pod number [55]; flowering time [56]; aschochyta blight resistance [42]; pigeon pea (*Cajanus cajan*) flowering time and leaf

shape [57]; ground nut *(Arachis hypogaea*) fresh seed dormancy; and leaf spot resistance [58]. For adzuki bean, many putative genes are contained within each identified QTL with approximately 329 genes in the *qECL7.1* region and 690 genes in the *qECL10.1* region. This study used ten individual lines per DNA bulk, which may not capture the full extent of variation and recombinants in the mapping population. Further refinement of the *qECL7.1* region is recommended using a larger population having more recombinants between DNA markers that co-segregate with the genomic region. This may allow for identification of candidate genes responsible for the ECL trait, and genomic regions with high utility for marker-assisted selection [57].

Mapping and isolation of QTLs promotes efficiency in crop breeding through marker assisted selection and enhances understanding of molecular mechanisms associated with traits of interest [33,59]. To facilitate the use of study findings in breeding programs, it was important to understand the interaction between detected ECL QTLs. An assessment of F_3 lines from plant #223 and plant #286 in this study, indicated that both *qECL7.1* and *qECL10.1* had the 'Toiku161' effect for increasing epicotyl length, indicating the presence of an epistatic interaction between the two loci [59]. In adzuki bean, epistatic reactions are critical genetic factors for ECL and specific pathways including *qECL10* and *qECL7* underline the genetic control mechanism for ECL[23]. In soybean, 11 epistatic loci and nine candidate genes associated to internode number were identified, and 10 epistatic loci and 10 epistatic candidate genes associated to plant height were identified [60]. In addition, significant additive effects were detected in 11 QTLs for plant height among which six QTLs had additive by environment interaction effects. Of six digenic epistatic QTL pairs, four QTLs had additive effects for plant height [20]. The significant epistatic interaction detected in soybean derived additive effects from both parent lines. In the present study, epistatic interactions between *qECL7.1* and *qECL10.1* mainly derived additive effects from the 'Toiku161' parent allele. Detailed genomic analysis, using near isogenic lines (NILs) of the QTLs, for example, will be helpful for a comprehensive understanding of these interactions.

The Identification of *qECL7.1* and *qECL10.1* and the interaction between these loci will contribute to marker-assisted gene pyramiding and the generation of adzuki bean genotypes with superior ECL traits, in the range of 6 to 8 cm as reported by [23]. The new genotypes will improve adzuki bean suitability for efficient mechanization at critical stages such as weeding at the seedling stage and harvesting at pod. This study illustrated the development of a co-dominant InDel marker, TC64_9,479,153 nearest to *qECL7.1*, and co-dominant marker TC89_28,568,685 nearest to *qECL10.1* and these may be useful in marker-assisted selection for adzuki bean long epicotyl genotypes.

ECL is one of several important plant architecture traits that affect mechanization efficiency in adzuki bean production. An understanding of how overall plant architecture effects suitability for mechanization and productivity is important for the successful breeding of ideal genotypes. A study to clarify the genetic control mechanism for other plant architecture-related traits and the interactions among the traits using RILs is currently in progress.

2.5 Summary

This study found two major QTLs for epicotyl length in adzuki bean, *qECL7.1* and *qECL10.1* on chromosome 7 and 10 using QTL-seq method, that was confirmed by InDel marker-based QTL mapping in an F_2 population. *qECL7.1* on chromosome 7 was fine-mapped to a 418 kb region flanked by markers TC99_10,211,134 bp and TC102_10,628,880 bp by substitution mapping. Gene annotation analysis, predicted 35 candidate genes on chromosome 7 in the target region. One candidate gene *Vigan 07G1141000.01*, contains a mutation that might contribute to ECL variation in adzuki bean cultivars, however, this study could not conclusively identify the candidate genes for ECL. Therefore, further fine-mapping of *qECL7.1* are recommended to precisely identify associated candidate gene functions, and facilitate marker-based selection of ideal genotypes for epicotyl length in adzuki bean breeding.

Table 2-1: Putative QTLs for ECL among 155 RILs crossed between 'Toiku161' with 'Chihayahime'

Chromosome	Physical interval (Mb)	Δ(SNP-index)[1] range
2	25.30-25.60	0.64-0.67
2	25.75-26.15	0.64-0.66
2	27.10-27.25	0.64-0.65
2	27.55-27.85	0.64-0.65
2	28.10-29.05	0.64-0.68
2	31.65-32.75	0.63-0.70
7	6.50-10.65	0.65-0.83
10	19.75-21.75	0.66-0.76
10	22.75-24.35	0.66-0.73
10	26.55-28.85	0.66-0.80
11	6.30-7.45	0.65-0.75
11	7.80-11.10	0.65-0.73

[1] Single nucleotide polymorphism index

Table 2-2: QTL peaks on chromosome 7 and 10 for epicotyl length in the 96 F_2 population

Chromosome	LOD[1] peak position (cM)	Nearest marker	Marker interval	LOD[1]	AE[2] (cm)	DE[3] (cm)	PVE[4] (%)
7	51.1	TC64	TC63-TC66	6.7	-0.8	-0.1	21.2
10	88.6	TC89	TC87-TC89	4.4	-0.6	-0.5	13.4

[1] Logarithm of odds. [2] Additive effect; positive values indicate positive effect on ECL derived from T161, while negative values indicate negative effect on ECL derived from Chihayahime. [3] Dominance effect. [4] Percentage of the total phenotypic variation explained by each QTL.

Table 2-3: Genotypes of 9 DNA markers at the *qECL7.1* region on chromosome 7 and ECL in the recombinants

Line	Genotype of marker in RHLs									Epicotyl length (cm)				
	TC64_9,479,153	TC65_9,984,711	TC98_10,131,117	TC_99_10,211,13	TC100_10,305,60	TC101_10,308,09	TC102_10,628,88	TC103_11,772,49	TC66_13,081,534	Mean		SD		Estimated Genotype of *qECL7.1*
Chihayahime	A	A	A	A	A	A	A	A	A	3.2	±	0.5		CH
Toiku161	B	B	B	B	B	B	B	B	B	6.9	±	0.7	*	T161
#223_105_7	A	A	A	A	A	A	A	A	A	3.4	±	0.5	---	CH(Control)
#223_105_1	B	B	B	B	B	B	B	B	B	6.9	±	0.7	*	T161(control)
#223_81_75	B	B	B	B	B	B	B	A	A	7.0	±	0.7	*	T161
#223_112_347	B	B	B	B	B	B	A	A	A	7.3	±	0.8	*	T161
#223_160_46	B	B	B	B	A	A	A	A	A	3.9	±	0.6		CH
#223_95_178_193	B	B	B	A	A	A	A	A	A	4.0	±	0.4		CH
#223_91_134_15	A	A	A	A	A	A	A	B	B	4.0	±	0.5		CH

Plants were homozygous for the Toiku161 (black boxes) and Chihayahime (white boxes) sequences at 9 DNA markers. Asterisks indicate difference in ECL between genotypes using Dunnett's test with a significance level of $p < .0.01$

Table 2-4: Predicted genes within *qECL7.1* region from TC99_10.2M to TC102_10.6M

Gene	Position (bp)	Length (bp)	Description
Vigan.07G111100.01	Chr07:10217067...10225123 (+ strand)	8056	Biological Process: cell redox homeostasis (GO:0045454)
Vigan.07G111200.01	Chr07:10225971...10227563 (- strand)	1592	Similar to early nodulin-like protein 2-like. [XP_006592879.1, Glycine max]
Vigan.07G111300.01	Chr07:10245110...10253581 (+ strand)	8471	Biological Process: regulation of Rab GTPase activity (GO:0032313)
Vigan.07G111400.01	Chr07:10259315...10262198 (+ strand)	2883	cellulose microfibril organization
Vigan.07G111500.01	Chr07:10267247...10267599 (+ strand)	352	Similar to Integrase core domain containing protein. [I6ZTT9, Phaseolus vulgaris]
Vigan.07G111600.01	Chr07:10270113...10274630 (- strand)	4517	Mitochondrial outer membrane translocase complex, subunit Tom22, plant (IPR017411)
Vigan.07G111700.01	Chr07:10274131...10274487 (+ strand)	356	Hypothetical protein.
Vigan.07G111800.01	Chr07:10278476...10286164 (- strand)	7688	DDRGK domain containing protein (IPR019153)
Vigan.07G111900.01	Chr07:10287789...10289985 (- strand)	2196	Hypothetical protein.
Vigan.07G112000.01	Chr07:10291534...10291999 (- strand)	465	Hypothetical protein.
Vigan.07G112100.01	Chr07:10292828...10293475 (- strand)	647	Hypothetical protein.
Vigan.07G112300.01	Chr07:10297927...10298963 (+ strand)	1036	Hypothetical protein.
Vigan.07G112400.01	Chr07:10311827...10312393 (+ strand)	566	Hypothetical protein.
Vigan.07G112500.01	Chr07:10349998...10352995 (+ strand)	2997	Biological Process: oxidation-reduction process (GO:0055114)
Vigan.07G112600.01	Chr07:10363325...10365026 (- strand)	1,701	Hypothetical protein.
Vigan.07G112700.01	Chr07:10444164...10448247 (+ strand)	4083	Biological Process: oxidation-reduction process (GO:0055114)
Vigan.07G112800.01	Chr07:10463548...10464907 (+ strand)	1359	Similar to Uncharacterized protein. [D8WJ57, Glycine max]
Vigan.07G112900.01	Chr07:10472813...10481791 (+ strand)	8978	Similar to Uncharacterized protein. [K7LW88, Glycine max]
Vigan.07G113000.01	Chr07:10482576...10483609 (- strand)	1033	Similar to Potassium/sodium hyperpolarization. [XP_007021055.1, Theobroma cacao]
Vigan.07G113100.01	Chr07:10495792...10500041 (+ strand)	4249	WD40/YVTN repeat-like-containing domain (IPR015943)
Vigan.07G113200.01	Chr07:10501026...10509455 (- strand)	8426	UHRF1-binding protein 1-like (IPR026728)
Vigan.07G113300.01	Chr07:10505128...10506155 (+ strand)	1027	Non-protein coding gene.
Vigan.07G113400.01	Chr07:10537568...10544061 (+ strand)	6493	WD40/YVTN repeat-like-containing domain (IPR015943)
Vigan.07G113500.01	Chr07:10558674...10564005 (+ strand)	5331	Glycosyl transferase, family 8 (IPR002495)
Vigan.07G113600.01	Chr07:10562715...10563808 (- strand)	1093	Domain of unknown function DUF1995 (IPR018962)
Vigan.07G113700.01	Chr07:10564235...10566639 (- strand)	2404	Myc-type, basic helix-loop-helix (bHLH) domain (IPR011598)
Vigan.07G113800.01	Chr07:10578898...10580621 (- strand)	1723	Biological Process: cell redox homeostasis (GO:0045454)
Vigan.07G113900.01	Chr07:10584354...10585861 (+ strand)	1507	Cellular Component: photosystem II oxygen evolving complex (GO:0009654)
Vigan.07G114000.01	Chr07:10584354...10585924 (- strand)	1570	Non-protein coding gene.
Vigan.07G114100.01	Chr07:10586027...10592438 (- strand)	6411	Molecular Function: microtubule motor activity (GO:0003777)
Vigan.07G114200.01	Chr07:10597826...10599550 (- strand)	1724	HSP20-like chaperone (IPR008978)
Vigan.07G114300.01	Chr07:10599645...10600897 (- strand)	1252	Non-protein coding gene.
Vigan.07G114400.01	Chr07:10602299...10606380 (+ strand)	4081	Cellular Component: actin filament (GO:0005884)
Vigan.07G114500.01	Chr07:10617379...10618239 (- strand)	860	Molecular Function: DNA binding (GO:0003677)
Vigan.07G114600.01	Chr07:10625237...10626614 (- strand)	1377	Molecular Function: DNA binding (GO:0003677)

Table S 2. 1: InDel markers used to detect QTLs in F_2 population

Marker name	Chromosome	Physical position (bp)	Genetic position (cM)	Primer sequence (5'→3')		Product size (bp)		An. Tm. (°C)
				Forward	Reverse	Chihayahime	Toiku161	
TC16	2	215,480	0.0	ACTCCCTACACCATTGTTCCCT	AAACTCAACGGATGTAGCACGT	193	157	55
TC17	2	3,968,366	20.8	AATACGCAGTCCCCGTAACCAA	GAGGCACGGATGTAGCGTTTTC	263	274	55
TC18	2	25,084,309	61.4	GAGGTATTAGACGACTCGGCCC	AGTGCAAAAATAGGACCCAACTGA	290	308	55
TC19	2	29,668,229	67.1	TCTCCAAACCAGAGAGCGACC	ACACCTCTGTTGTTGCGTTGTC	245	266	55
TC20	2	37,069,131	79.9	GGATGTTACAGGATGGCAAGCC	GATACTGCTTTTAGACCGGCGC	225	197	55
TC21	2	45,030,121	115.1	ACGATCTTGCAGTTAAACTAAACAGA	ACCCAAAACATTGTAAAGACACTT	213	230	55
TC58	7	160,123	0.0	AGATCAGCCACAATGCCGAATA	TGCAATGGCATGGTGCAATTAA	285	261	55
TC59	7	2,746,363	17.7	GGGAAGGGTATCTCAATGGACC	ACGAAATATGAACGGTATGTTTTGT	217	187	55
TC60	7	5,411,094	35.2	TTGAAGAGTGAAGTGAGCACAC	TGACCAAGTTTGTAGGTTACACA	298	274	55
TC61	7	6,586,919	42.7	TGAAACTTGGATCCATGAAAATGT	TCCCGCGCATAGTCTGTTTT	188	200	55
TC62	7	7,843,631	45.3	TCGCATGAGCAAATCACGAAGC	TCGTTTGGCAAGTTCCTACTAACCT	292	254	55
TC63	7	8,549,506	47.6	TGGGGGCGTGAAATTTAAAATGA	ACAACACTGCAAATCATTAACAAATT	200	188	55
TC64	7	9,479,153	51.1	AGAGGTGGAGGGAGAAGATGGG	TTCGATTCGCTTTTTGGGCCAA	284	305	55
TC65	7	9,984,711	51.5	CCGGTTCTTTAGGGAGCTTAGA	CGCGATCACCGGAATGAAAT	356	400	55
TC66	7	13,081,534	56.8	TGATGCAGCAAGTCGAGTTCT	ACAGGATAGGATGAGACAATTCACA	153	142	55
TC67	7	25,162,481	67.4	TGGTGTGCGAGATTGTATCCGT	AAGAGCAAGCATCCCAAACAGC	197	191	60
TC80	10	51,418	0.0	AGTGGGCTTTTTAGAGTGGGCT	AAAAAGTTGTGGCGGTAGTGGC	265	283	55
TC81	10	4,926,505	25.1	AACTCGGCAACCCAATTAACCG	GATTTCTCCAAGCGCCCTCAAA	202	217	55
TC82	10	9,163,805	36.9	ATATCGTTGACAGAGCGGAGGG	CACCAGGATGACCCTAATCCGT	203	191	55
TC83	10	10,312,821	39.6	CGGAAGGTTCTTGTGGGAAGGA	TGACGCTCTATTTTGTGCAATCGA	313	273	50
TC84	10	15,288,117	43.8	GGTGTGTTTGGGGTTGAAGATG	AGGAGCATAAGGTTTTGGGAGA	302	370	55
TC85	10	23,091,471	58.6	TGTTAAAGTCAAAACTAACGAAAACAG	TGCATGGTGTTGAAATGTGATCT	239	259	55
TC86	10	26,324,877	76.4	GCATTGAAGGAATCCACGTGCA	TTCTTGTCGCTCCTCCATCTCG	127	111	60
TC87[1)]	10	27,485,959	86.8	AAACCAGAATGAGCGCACGAAG	AGGGGTATGAGTGAAGAATGGTGG	194	178	55
TC88	10	28,128,765	87.2	ACGTCACTTGTTGCATAGGAACA	GGCTTTTAAGTGTAGAGGGGTCG	161	152	60
TC89[1)]	10	28,568,685	88.6	AATTTGGAGCTTGCTGTTCCCG	TGGTCACCGAAGAAGAAGGTTCA	317	285	60
TC90	11	654,400	0.0	GCAGTTCAATTTTCATTGCCAGGC	GGGGGAGTATGGTTGATGCTCA	311	299	55
TC91	11	1,197,408	5.3	TCCTCTGGGGTAGCCATTTTCA	TTTGATCCGCTACAGCACTTGC	193	184	55
TC92	11	1,718,463	8.8	TCACAATGGAGAAAAAGTGTTGTCC	CTCCAAAGCTGCAACCAGAATG	210	200	55
TC93	11	4,894,003	13.3	TCCCAAAGGCCAAAAGTGAGA	TCACCTTCTTGACATTGGAGAGA	225	207	55
TC94	11	10,002,825	21.9	AGGAGTGAGCAAGGAGAAATGA	TGGTCAAAGCGTCGAGTTTCT	235	221	55
TC95	11	15,242,614	26.0	CTCGGGCAAAATTGAAGGCTCA	GCTTCCACCTCCAAATCTCCCT	236	257	55
TC96	11	34,902,417	55.5	AGAGTTTGGCTGCATCACATG	TCAACCAATAAGCTTGGATCAGT	165	175	55
TC97	11	38,028,974	83.7	GAAGAATCCAGGCCCTTGCT	CGAGATGCGGCGGTATATGT	344	300	55

Table S 2. 2: DNA markers used to narrow down *qECL7.1*

Marker name	Chromosome	Physical position (bp)	Primer sequence（5'→3'）		Marker type	Product size (bp)		An. Tm. (°C)
			Forward	Reverse		Chihayahime	Toiku161	
TC98	7	10,131,117	CAACTGCCATGACAACTGCC	ATTCAGTTAGGCCCTCACCG	CAPs/DdeI	90, 180, 390	90, 180, 115, 280	55
TC99	7	10,211,134	AAACTCCCTCAAGTGAACAAAACG	CCCCGTTACTTTTAAACCACGTTT	SNP	794	794	64
TC100	7	10,305,602	ATGTGGTTACTACGTCATGTTTTGG	ACGTGAATTAGCACTAGTACAACCA	InDel	951	950	64
TC101	7	10,308,093	AGGGTCTTGACATTATCACCAACA	AACGCCCATCTTTTTCTCTTTCTG	SNP	785	785	60
TC102	7	10,628,880	TAAAGGTCAATAACGGGAGGGG	AGAAAAATGTTTGTTGCGGCAA	SNP	750	750	60
TC103	7	11,772,495	GGGCAAAACAATAATGGGGAGG	GTTTCTTTTAGAATGTCTAAGTGG	dCAPs/TaqI	184	160, 24	50

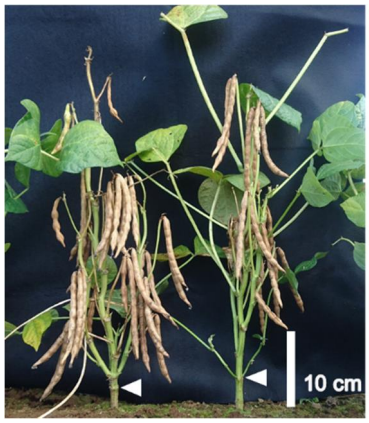


Figure 2-1: Phenotypic variation between parent lines "Toiku161' and 'Chihayahime' at maturity stage

Note: Phenotypic variation for ECL between parent lines Chihayahime (left) and T161 (right) varieties at harvesting stage. The white line represents 10 cm scale bar. The White arrowheads indicate position of primary node, which is the endpoint of epicotyl in each parent line.

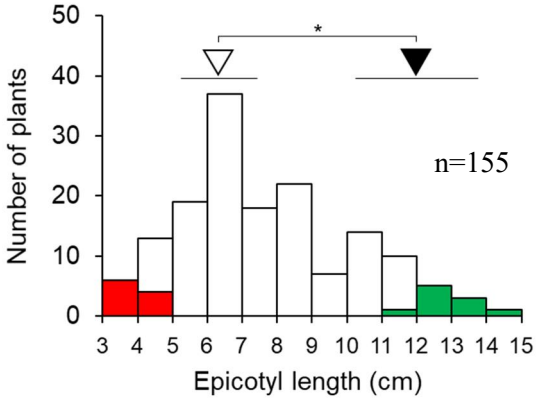


Figure 2-2: Phenotypic variation of ECL in F_7 population

Note: Frequency distribution of ECL measured in 155 RILs of an F_7 population. Arrowheads and horizontal lines indicate the mean values and standard deviation for parent lines Chihayahime (*white arrowhead*) and T161 (*black arrowhead*) at $p<0.05$ significance. Individua RILs used for the short and long epicotyl length pools are highlighted in red and green, respectively.

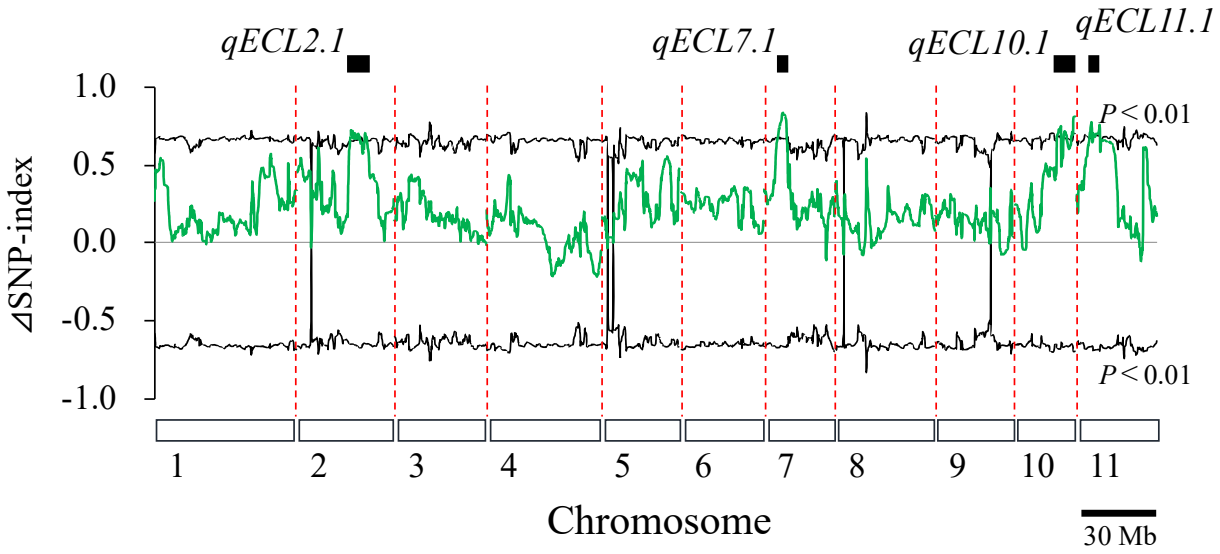


Figure 2-3: Manhattan plot for QTL-seq analysis of pooled F_7 population from a cross between T161 and Chihayahime.

Note: Manhattan plot for QTL-seq analysis in F_7 population from a cross between T161 and Chihayahime on four chromosomes with putative QTLs. The SNP-index was calculated based on 2 Mb intervals with a 50 kb sliding window analysis (green line), at a Statistical confidence interval (p <0.01).

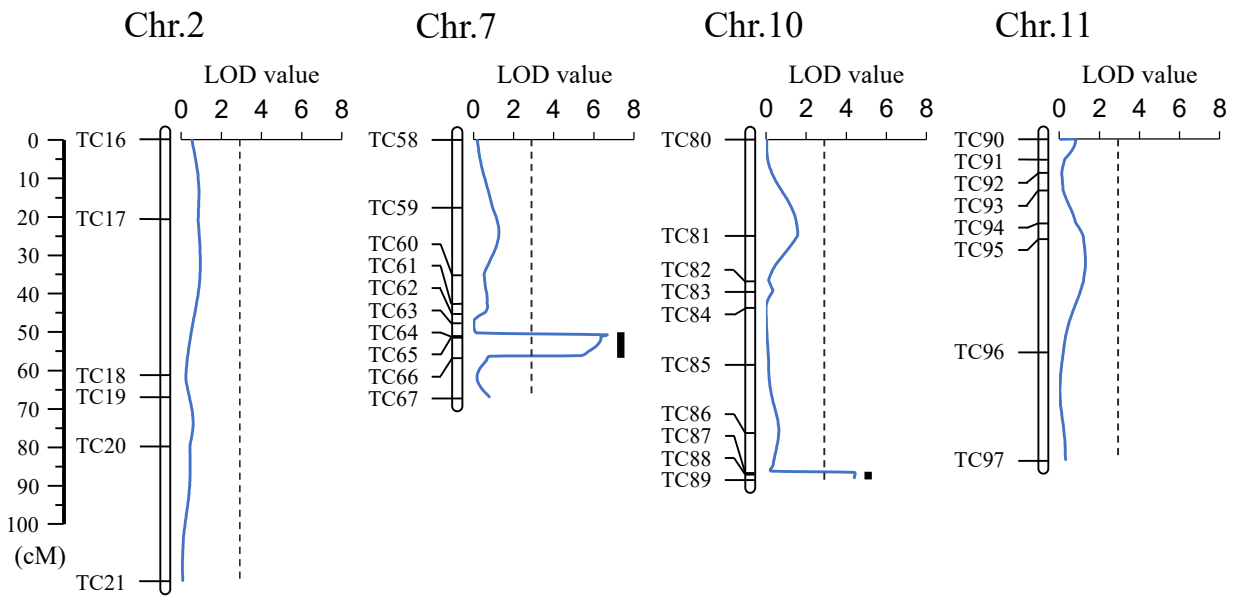


Figure 2-4: Multiple QTL mapping for ECL in F_2 Population

Note: Linkage map constructed for an F_2 population with 96 individuals using the indicated 97 InDel markers. Horizontal axis represent marker positions and vertical axis represents LOD score (blue). *Black rectangles* represent the two LOD support factor for candidate QTL regions on chromosome 7 and 10.

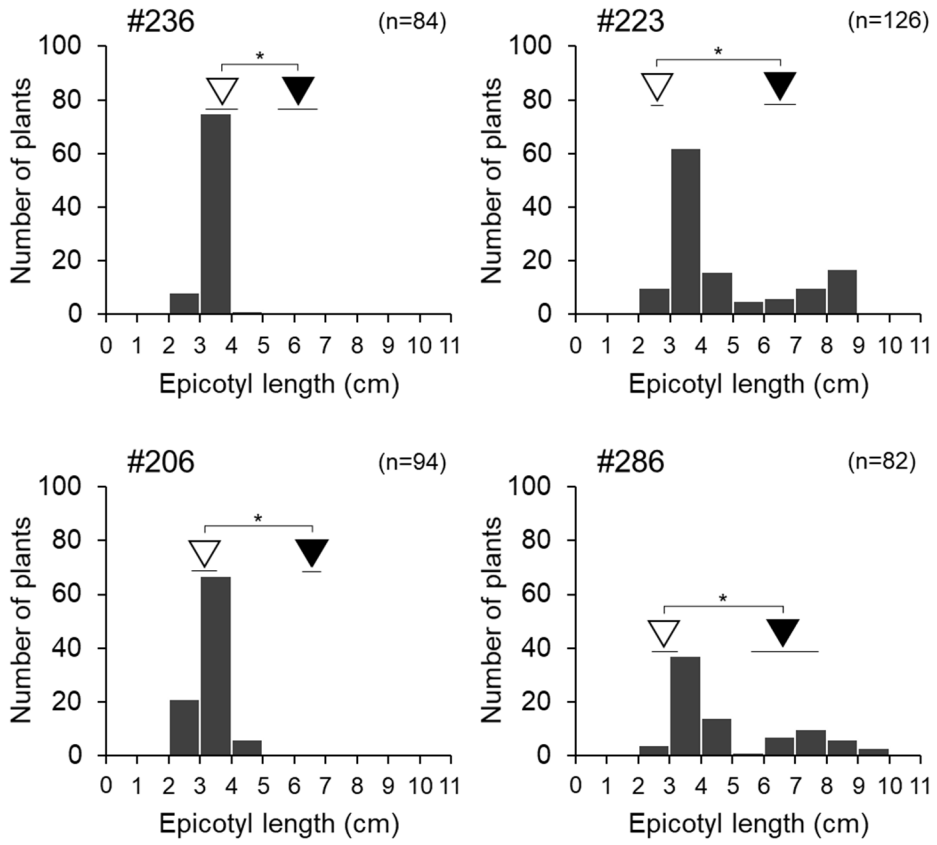


Figure 2-5: Segregation patterns F_3 RHLs on *qECL7.1* and *qECL10.1* target region

Note: ECL of F_3 RHLs derived from F_2 individuals #236, #223, #206 and #286. Arrowheads and horizontal lines indicate the mean values and range of values within one standard deviation of the mean for progenitor lines Chihayahime (white arrowhead) and Toiku161 (black arrowhead). * indicate statistical confidence at $p < 0.01$.

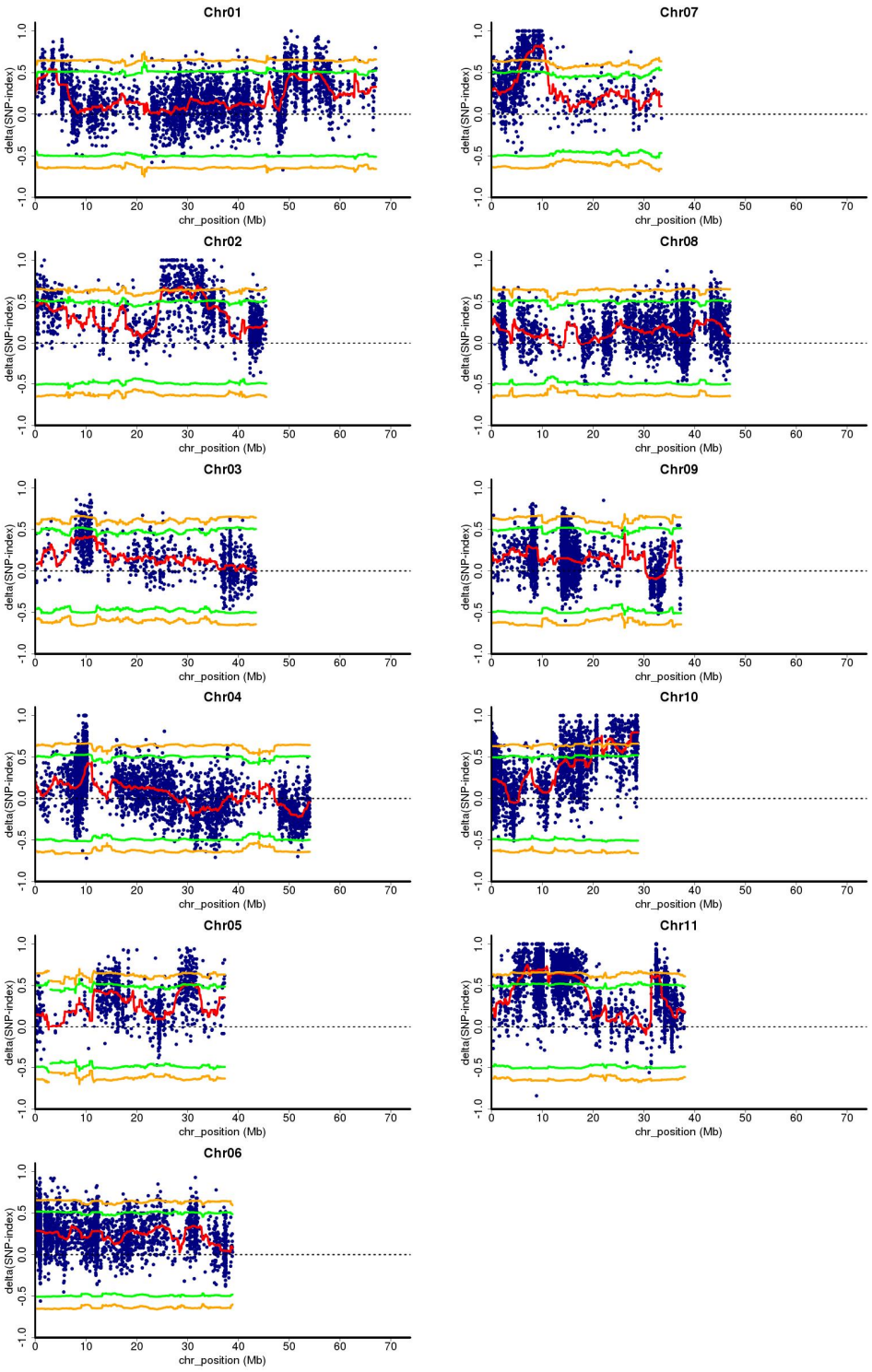


Figure S 2. 1: Manhattan plot for QTL-seq analysis of pooled F_7 population on adzuki bean chromosome 1 to 11.

Note: Combined QTL seq analysis to map ECL locus. Figure shows plots of the short and long epicotyl bulks on chromosome 1 to 11 and Δ(SNP-index) plot with confidence intervals under the null hypothesis of no QTLs (green, $p<0.05$; orange, $p<0.01$)

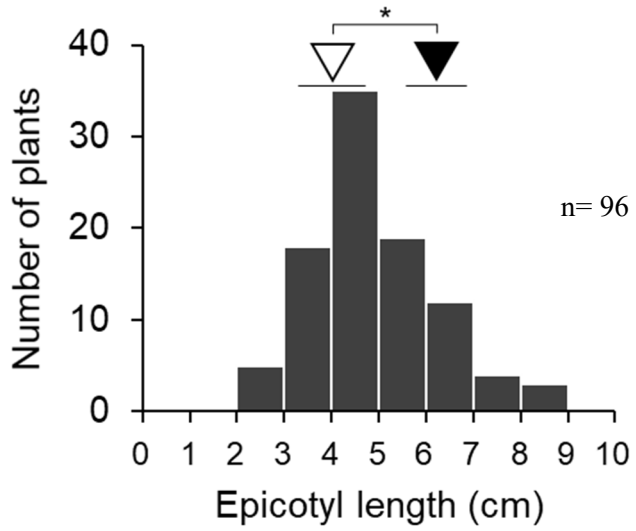


Figure S 2. 2: Phenotypic variation of ECL in F_2 population

Note: Frequency distribution of ECL in an F_2 population and parent lines T161 (*black arrow*) and Chihayahime (*white arrow*). Arrowheads represent parental means and vertical lines represent standard deviation at $p<0.05$ significance.

CHAPTER 3: QTL ANALYSIS FOR PLANT HEIGHT-RELATED TRAITS IN ADZUKI BEAN (*Vigna angularis*)

3.1 Introduction

Plant height, which affects production and lodging, is mostly determined by the number and length of internodes in legumes [15]. It is a complex trait associated with many genes, particularly genes involved in the biosynthesis and signaling of proteins for plant growth [61] **.** Plant height is one of the key criterions used by plant breeders in the selection for suitable cultivars, as an indicative basis for above ground biomass, flowering, mechanization capacity and crop sensitivity to lodging [18,62].

Plant architecture QTLs in adzuki bean have been studied before. The modification of plant architectural trait components in some legume crops including the mapping of genomic regions that control plant height traits for productivity improvements, have contributed to significant improvements in yield levels [63] **.** In a population derived from wild species *V. nepalensis* and a landrace, stem length QTLs were identified on linkage groups (LG) 1 and 2, and the cultivated parent allele effect increased internode length in the lower part of the stem but shortened internode length in the upper part of the stem [21]. Three epicotyl length (ECL) QTLs identified on linkage groups 1 and 2 possessed alleles from the cultivated parent that increased ECL. In the study, co-localization was observed between ECL QTLs and lower internode length. In a population developed from a wild adzuki bean accession and cultivar 'Kyoto Dainagon', QTLs for stem length *Stl3.9.1* and *Stl3.10.1*; internode lengths at late stem-growth stage (sixth to tenth) QTLs Stl*7-10i3.9.1 and Stl5-10i3.9.1* were mapped at a similar position [7]. The QTLs for the first to fifth internode lengths, categorized as early to middle-stage stem growth was mainly detected on LG 1, LG 2, LG 4b and LG 9 [7], with the cultivated parent alleles increasing internode length as similarly observed in [21]. A close association was also observed among QTLs for the first to third internode lengths and ECL [7,21]. In a population developed from a cultivar Ass001 and wild accession CWA108, 26 agronomic QTLs were mapped including *VaST1-10I9*, a stem internode length trait for the first to tenth internodes on LG 9 [22]. Epicotyl length QTLs have also been analyzed using a population developed from cultivars 'Tokei1121'

and ‘Erimo167’ [23]. The study identified four QTLs namely *qECL2, qECL4, qECL7* and *qECL10* on LG 2, LG 4, LG 7 and LG 10 respectively, with large effect QTLs on LG 10 and LG 7 bearing ‘T1121’allele for increased ECL.

Previously, most of the QTL analysis studies based on wild species, therefore necessary to conduct more detailed studies using study populations developed from crosses between cultivars to facilitate applicability into adzuki bean breeding programs. In this view, [24] used QTL-seq methodology in RIL population developed from a breeding line ‘Toiki161’ and cultivar ‘Chihayahime’ that identified four QTLs for epicotyl length trait on chromosomes 2,7,10 and 11 and confirmed two QTLs *qECL7.1* and *qECL10.1* on chromosomes 7 and 10 using InDel-based mapping. The *qECL7.1* was narrowed down to 418 kb region containing 35 predicted genes, based on substitution mapping using InDel, CAPS, dCAPS and SNP markers.

However, ECL is just one of the key traits contributing to overall variation in plant height-related traits. This necessitated further studies to clarify genetic mechanisms for traits with potential contribution to the development of genotypes with ideal architecture in adzuki bean production. Therefore, this study component analyzed QTLs for plant height (PH) and its related traits namely; epicotyl length (ECL), stem internode length of below node seven (STIL7), stem internode length above node 8 (STIL8), total number of nodes on the main stem (NN), flowering date (FLD) and maturity date (MAD) in order to understand genetic control mechanisms and interactions for plant height related traits.

3.2 Materials and Methods

3.2.1 Mapping population

The $F_{9:10}$ populations used in this study was derived from a cross between cultivars ‘Toiku161’ (T161) and ‘Chihayahime’ both developed by TAES, Hokkaido research organization. ‘T161’ has long epicotyl and was the female in the cross while ‘Chihayahime’ has normal epicotyl and was the male in the

cross (Figure 3.1)**.** The recombinant inbred lines (RIL) population consisted of 113 lines generated by self-pollination.

3.2.2 Field experiment and trait measurement

The RILs and parent lines were grown at TAES from May to October in 2020, 2021, 2022 and 2023. Three seeds that were later thinned to one seed per station were planted at 20 cm plant spacing and 60 cm ridge spacing. Each RIL had 8 plants from which 6 representational plants were selected for trait evaluation. A total of 13 plants in 2020, 8 plants in 2021 and 6 plants in 2022 and 2023 were planted for the parent lines. Main traits recorded included plant height measured from the soil surface to the top node of the main stem; epicotyl length measured from the cotyledon node to the primary node; node number which was recorded as the total number of nodes on the main stem; stem internode length below the seventh node which was recorded as the average total length of stem internode 1 to 7, stem internode length beyond the eighth node which was recorded as the average total length of nodes beyond eighth node; flowering date which was recorded when each line had at least one flower ; and maturity which recorded when 80% of the lines had reached mature pod color (Table 3.1). Data analysis for the traits was done using the mean value of each RIL and parent line over a four-year period.

3.2.3 Statistical analysis

Statistical analysis of the phenotypic variation and principal component analysis (PCA) were analyzed using R scripts (https://cran.r-project.org). Distribution charts were created using the *PerformanceAnalytics* package and correlation plots were created using the *corrplot* package [64]. Principal component analysis was conducted using *FactomineR* package [65]. Descriptive statistics for PH, ECL, NN, STIL7, STIL8, FLD and MAD were analyzed in excel and t-test was used to detect significant

differences of the evaluated traits among the parent lines and RILs. Two-way ANOVA was used to analyze significant interactions between the detected major QTLs.

3.2.4 DNA extraction

DNA was extracted from 113 individual F_9 and F_{10} plants and 6 plants of each parent line using CTAB DNA extraction method. Samples from young and fully opened trifoliate leaves were collected and freeze- dried. 400 µL EDTA was added to each sample then ground in multi-species grinding machine (TissueLysserZ Qiagen Cat. No 85200: Retch; QIAGEN). Leaf samples were centrifuged at 1,500rpm for 10 mins at 22^{o}C then incubated at room temperature for 60 minutes. After incubation, 100 µL isopropanol was added to 100 µL sample, mixed on ice then cooled overnight at 4^{o}C. Afterwards, samples were centrifuged again at 15,000 rpm for 20 mins and the supernatant was decanted. 100 µL of 70% ethanol was added, mixed gently by vortex, centrifuged at 4^{o}C and 15,000 rpm for 2 minutes. Later on, the supernatant was decanted again and put on a towel to dry to evaporate remaining Et-OH. DNA was dissolved in 200 µL of 1/10 TE buffer, vortexed, flushed in mini centrifuge for 30seconds at 2,500 rpm and allowed to allowed to stand overnight at room temperature and diluted at 1/100 for subsequent experiments.

3.2.5 Marker analysis

The PCR amplification was done in a 10 µL reaction volume containing 2.9µL of sterile water, 0.05 µL each of 100pmol Forward and Reverse primers, 5µL of Go-Tag Master Mix (Promega) and 2 µL of DNA. The PCR program was as follows: initial heat denaturation at 94^{o}C for 2 mins, 35 cycles of 94^{o}C for 15 s and extension at 72^{o}C for 30 s. The annealing temperature ranged from 45 – 60^{o}C depending on the primer pair sequences. The PCR products were analyzed on a 4% agarose gel electrophoresis in 1xTAE buffer at a constant voltage of 200V for 30 min. After electrophoresis, the gels were stained with ethidium

bromide for 15-20 mins and exposed to UV light to visualize the PCR products. Only markers that showed polymorphism between parents were used for the RILs genotyping.

DNA markers namely insertion and deletion (InDel), cleaved amplified polymorphic sequences (CAPS) and derived CAPS (dCAPS), were developed from re-sequence data of 'T161' and 'Chihayahime'. Mutations detected between 'T161' and 'Chihayahime' were classified as single nucleotide polymorphisms (SNPs) and insertions and deletions (InDel). InDel genotypes of 10bp or more were extracted on agarose gels, and those within 2.5Mbp on either side of the identified sequences were used in the development of InDel Markers. Primers 1000 bp on either side of the target InDel were sequenced using Vigna Genome server (https://viggs.dna.affrc.go.jp) and Primer3plus (https://primer3plus.com) was used to design the primers. DNA ladder was used as the basis for estimating fragment sizes.

3.2.6 Linkage map construction

A linkage map was constructed from the genotypes of the F_9 and $_{F10}$ populations using JoinMap software 4.1 [46]. Chi-square test was used in calculating marker segregation ratios whereby markers with distorted segregation (p<0.01) were excluded and the remaining 108 markers were used for the genetic map construction. Logarithm of odds (LOD) score was used to determine the likelihood for presence of a linkage between selected marker(s) and a QTL. Marker loci with a significant likelihood ratio greater than LOD 1.0 was used create linkage groups (LGs) for the genetic map and recombination frequencies were translated to genetic distances using Kosambi's mapping function [47].

3.2.7 QTL analysis

QTLs were determined by Interval Mapping (IM) and Multiple QTL Model (MQM) using MapQTL software version 6.0 [48]. Firstly, a permutation test was run to get genome-wide LOD thresholds

for each trait, at $p<0.05$ significance level based on 1000 times permutations [66]. Secondly, significant markers associated with QTLs (LOD ≥2.7) were detected by IM analysis. The significant markers were then used as cofactors in the MQM analysis and markers with the highest LOD values at each associated QTL were considered optimal. Genetic information including marker position, percentage of phenotypic variation explained, additive effect direction and dominance effect of each QTL were obtained from the MapQTL analysis output. Naming of the identified QTLs was based on the nomenclature suggested in [67] by denoting 'q' representing QTL, followed by the respective trait abbreviation then the chromosome number in Italic font.

3.2.8 Interaction assessment among detected plant height and stem length QTLs

Two major QTLs were identified for plant height trait. The RILs were divided into four groups based on the genotypes of two DNA markers (AZ02_4.0M and AZ04_50.7M) that were closely associated to the two QTLs (*qPH2* and *qPH4*). The RILs were classified into 'T161' homozygous, heterozygous and 'Chihayahime' homozygous. The '*aov*' function in R statistical software and JASP (version 17.3.0) was used for analyzing the QTL interactions.

3.3 Results

3.3.1 Constructed genetic linkage map

The genetic map was constructed with a total of 108 DNA markers, that generated 11 linkage groups (Figure 3.4). The number of linkage groups generated in this map matched the basic number of chromosomes in adzuki bean $2n = 2x = 22$. In total 95.6% of the polymorphic InDel, CAPS and dCAPS markers were mapped into the genome. The linkage map covers an average genetic distance of 963.6cM and represents about 94.7% of the adzuki bean genome. The average distance between adjacent markers was 9.0cM. There were some gaps on the linkage groups but no gap was more than 40.8cM (Table 3.2**).**

3.3.2 Phenotypic variation in plant height traits among the parent lines and RILS

Variations were observed in the seven traits based on the mean, standard deviation and range for parent lines and RILs evaluated in the four years from 2020 to 2023 (Table 3.3). Plant height and node number were among the traits with largest ranges within the RILs. The means of the RILs generally fell within the parent line means in all the evaluated traits except PH for RILs that was lower than parental mean in 2021 and 2022. Some RILs had trait means exceeding that of the parent lines.

PH was almost the same between the parent lines except in 2020 and 2023 'T161' was significantly taller than 'Chihayahime'. NN was almost the same between parent lines but in 2022 'Chihayahime' was significantly higher than 'T161'. ECL significantly differed between the parent lines in all years. STIL7 was longer in 'T161' than 'Chihayahime' while STIL8 was higher in 'Chihayahime' than 'T161' (Table 3.3). All the traits analyzed showed a relatively normal distribution among the RILs in the study population (Figure S3.1). These results therefore suggest that all the 7 traits are controlled by multiple genes.

3.3.3 Correlation analysis for plant height traits

To analyze the genetic associations among the plant height related traits, a correlation analysis was conducted for the 113 RILs. Most combinations showed positive correlation for the plant height-related traits over the three years period (Figure 3.2, Figure S3.1). Strong positive relationship was observed between plant height and average stem internode length above 8^{th} node (r >0.80); epicotyl length and average stem internode length (r >0.80); plant height and flowering date (r >0.60); and plant height and maturity date (r >0.40). however, strong negative relationship was observed between node number and epicotyl length (r >-0.80); node number and average stem internode length below 7^{th} node (r >-0.60); weak positive relationship was observed between node number and average stem internode length above 8^{th} node in 2020 and 2021(r >0.20), while a negative relationship was observed in the traits in 2022 (r >-0.40). These results indicate the possibility that some genetic factors for traits related to plant height are likely controlled

by different QTLs. Based on the correlation coefficient matrix, principal component association were analyzed.

3.3.4 Principal component analysis

To minimize the redundancy of the raw data and to effectively identify the distribution structure of phenotypic variation among plant height-related traits, principal component analysis was conducted for seven traits using 3-year data. The first three principal components were selected based on the principle that eigenvalues are equal to or greater than 1. Calculations were made for the trait loading scores and contribution to total variation. The first three principal components were closely related to plant height traits, contributing to 85.1% of the total variation in 2020, 84.1% in 2021, 85.3% in 2022 and 77.7% in 2023 (Table 3.4).

The principal component analysis shows differences among the phenotypic traits. Two-dimensional analysis explains 39.9% and 31.5% in 2020, 40.6% and 30.7% in 2021, 39.6%, and 33.6% in 2022 and 33.2% and 30% in 2023. The PCA biplot illustrates distinct clusters of the plant height traits (Figure 3.3). Biplot loadings that clustered together indicate strong positive relationships within the cluster, while biplot loadings that appearing on opposite ends indicate strong negative relationships among the traits. The plant height-related traits clustered into three groups. Plant height, stem internode length above the 8th node, flowering and maturity constituted major components with high biplot loading scores for principal component 1 in all three years although flowering trait registered low biplot loading score in 2022. Epicotyl length and stem internode length below the 7th node clustered together and constituted major components based on biplot loading scores for principal component 2 although the stem internode length below the 7th node trait that registered lower biplot loading score in 2022 (Table 3.4; Figure 3.3). The biplot loadings for the number of node traits were separated from the rest and positioned opposite the clustered loadings for

epicotyl length and stem internode length below the 7th node, indicating a negative relationship between the traits.

3.3.5 QTLs detected for plant height traits in 'Toiku161' and 'Chihayahime' RILs

The QTL analysis of the four years data for the 7 traits PH, ECL, NN, STIL7, STIL8, FLD and MAD, detected a total of 39 QTLs (Table3.5.) covering 9 regions on the 11 chromosomes of the constructed map (Figure 3.5.). One to four QTLs were detected for each trait per year at significance level (p<0.05). Several QTLs were detected for PH, ECL, STIL and NN, which were located on chromosomes 1, 2,3,4,7,8 and 10. The QTL analysis results for each trait are presented in Table 3.5 and Figure 3.5. Four QTLs for PH were detected on chromosomes 2, 4 and 8. A large effect and stable QTLs were detected on chromosomes 2 and 4 explaining 12.3 to 21.9% and 14.2 to 8.3% phenotypic variation (PVE), respectively. The 'Chihayahime' type alleles on chromosome 4 increased PH while 'T161' alleles increased the PH chromosomes 2 and 8. QTLs for PH were observed within the same region on chromosome 2 as *qPH2.2* on marker AZ02_5.3M overlapped *qPH2.1* on marker AZ02_4.0M. Four QTLs were detected for ECL on chromosomes 1, 2, 7 and 10. Two large effect QTLs were detected on chromosomes 7 and10 explaining 22.8 to 41.3% and 11 to 14.3% PVE respectively. On all four QTLs 'T161' allele effect increased the ECL. Four QTLs were identified for NN on chromosomes 1, 4 and 7. On all QTLs 'Chihayahime' type alleles increased the NN. Two node number QTLs were detected on chromosome 4. The QTLs *qNN4.1* and *qNN4.2* were observed to be different since there was no overlap based on 2 LOD factor coverage. Accordingly, stem growth at different plant development stages thus early, middle to late stages is controlled by different QTLs [7,21]. In this study, QTLs for STIL7 (vegetative phase) were detected on chromosomes 2, 3, 4, 7 and 8. A large effect QTL for STL7 was found on chromosome 7 (34 to 52% PVE). Alleles from the 'T161' parent increased the stem internode length below node 7 on all chromosomes except on chromosome 3 and 4. Stable QTLs in three to four years for STIL8 (reproductive phase) were detected on chromosome 2, 4, and 10. Alleles from 'T161' parent increased the stem internode length above node 8 on chromosome 2,

while alleles from 'Chihayahime' parent increased the stem internode length above node 8 on chromosomes 4 and 10. QTLs for FLD were detected on chromosomes 2, 4, 7, 8, 9 and 10. A stable QTL was detected on chromosomes 2 explaining about 14.8 to 33.9% PVE, respectively and had the 'Chihayahime' allele effect for reducing the flowering time. Two FLD QTLs on chromosome 2 and chromosome 4 were associated with different markers but located within the same region based on a two LOD factor. QTLs for MAD were detected on chromosomes 1, 2, and 4. Two peaks with opposite effects were detected for MAD on chromosome 4, likely could be different QTLs.

3.3.6 Co-localization of detected plant height QTLs on the linkage groups

Important QTLs and clusters for plant height traits were found on chromosomes 2, 4, 7 and 10, detailed as follows. On chromosome 7, QTLs for ECL (*qECL7*), STIL7 (*qSTIL7.7*) and NN (*qNN7.1*) were located on the same marker position at AZ07_10.0M. Alleles for 'T161' had an increasing effect for *qECL7* and *qSTIL7.7* while 'Chihayahime' alleles had an increasing effect for *qNN7.* QTLs for epicotyl length *qECL1* and *qECL7* were consistently located close to the traits node number (*qNN1*) and stem internode length (*qSTIL7*), respectively. On chromosome 2, plant height QTL (*qPH2*) was co-located with upper stem internode length (*qSTIL8.2)* between markers AZ02_4.0M and AZ02_5.3M, and 'T161' allele effect increased the trait values. On chromosome 4, in the same region near marker AZ04_50.7M, QTLs for plant height (*qPH4*), upper internode length (*qSTIL8.4*) and total number of nodes (*qNN4.2*) were detected, and alleles from the parent 'Chihayahime' increased the trait values. On chromosome 2, QTLs for STIL7 (*qSTIL7.2*) and ECL (*qECL2*) was located within the same region between markers AZ02_2.6M to AZ02_4.0M and notably, on all QTLs, alleles from the parent 'T161' increased the trait values. The co-localization indicates the possible linkage of the traits.

3.3.7 QTL interaction for plant height-related traits

Two-way analysis of variance was conducted for plant height trait QTLs detected *qPH2* on chromosome 2 and *qPH4* on chromosome 4 (Table 3.6 – 3.9). There was no significant interaction between the QTLs *qPH2* and *qPH4* except in 2020 at $p = 0.04$ significance level. However, significant differences were observed only in independent factors thus *qPH2* ($p = 0.003$) and *qPH4* to ($p = 0.001$) in 2021, *qPH2* ($p = 0.001$) and *qPH4* ($p = 0.001$) in 2022, and *qPH2* ($p = 0.002$) and *qPH4* ($p = 0.000$) in 2023.

Further to the interaction analysis, the combination effect for plant height trait was analyzed to determine potential allelic combinations for desirable plant height in adzuki bean. Transgressive segregation was observed in the study population (Figure S3.1). Consequently, the 113 RILs were grouped into four classes based on the different alleles for nearest markers to the plant height QTL on chromosomes 2 and 4 (Table 3-10). The combinations for the 'Chihayahime' type allele (CH), at both *qPH2* on chromosome 2 and *qPH4* on chromosome 4 resulted into a moderate plant height ranging from 45.4 to 71.8 cm over the four years. Similarly, the combination of 'T161'type allele (T161), at both loci on *qPH2* and *qPH4* resulted into a moderate plant height ranging from 49.4 to 75.7 cm over the four years, respectively. On the other hand, the combination of 'Chihayahime' type allele on *qPH2* and 'T161' type allele on *qPH4* (CH – T161), resulted into the shortest plant height ranging from 38.4 to 62.9 cm over the four years. The combination for the 'T161' type allele on *qPH2* and 'Chihayahime' type allele on *qPH4* (T161 – CH), resulted into the tallest plant height ranging from 58.5 to 96.1 cm over the four years, respectively. The transgressive segregation observed in the study population could be of potential use in determination of combined allelic effects that would yield desirable traits, particularly plant height for effective mechanization and minimized plant lodging in adzuki beans.

3.4 Discussion

3.4.1 Comparison of detected and previously reported QTLs on plant height traits

Quantitative trait loci (QTL) analysis plays an important role in clarifying the genomics influencing particular phenotypic expression, and such information is potentially useful in Marker Assisted Selection (MAS) for crop improvement. Therefore, genetic mechanisms contributing to seven key plant architecture traits namely plant height, epicotyl length, lower stem internode length, upper stem internode length, total number of nodes, flowering, and maturity, were examined in this study.

Mori et al., [23] analyzed epicotyl length QTLs *qECL2, qECL4, qECL7* and *qECL10,* and narrowed down *qECL10* to 25.76 Mbp region. The QTL *qECL7* detected on physical position 10.0M in this study in contrast with 28.0M, could be due to the narrow linkage group 7 comprising only four markers within the marker region AZ07_26.5M5 and AZ07_30.0M5 in Mori et al., [23]. Therefore, to confirm the *qECL7* comparative position, in our previous work [24], a RIL population developed from long epicotyl breeding line 'T161' and ordinary epicotyl variety 'Chihayahime' was re-sequenced. An InDel based mapping validated QTLs identified by QTL-seq analysis. An important loci *qECL7.1* was fine-mapped to a 418 kb region flanked by DNA markers TC99_10,211,134 bp and TC102_10,628,880 bp using substitution mapping. The epicotyl length QTL *qECL1* detected in this study at 55.0M on linkage group 1, was different based on physical positions from the previously described *qECL2.1.1* and *qECL2.1.2* [21] and *qECL3.1.1* [7] both detected at position 67.1M on linkage group 1.

Isemura et al., [21], analyzed QTLs for the sixth internode *St6i2.2.1* and *St6i2.2.2* and seventh internode *St7i2.2.1* and *St7i2.2.2* On linkage group 2, at 45.5M physical position that was different from lower internode length below 7 QTL (*qSTIL7.2*) detected on 2.6M in this study. On Linkage group 4, Isemura et al., [21] detected QTL *St7i1.4.1* on physical position 54.1M observed to be within the same region with *qSTIL7.4* on physical position 52.0M. On linkage group 7, Kaga et al., [7], detected sixth internode QTL *St6i3.7.1*; while Isemura et al., [21], detected QTLs for sixth internode *St6i2.7.1*, seventh

internode *St7i1.7.1,* and *St7i1.7.2* on physical position 33.5M for all QTLs, that was different from *qSTIL7.7* all detected on position 10.0M in this study. On linkage group 8, Kaga et al., [7], detected *St4i3.8.1* on physical position 47.0M that was different from *qSTIL7.8* detected on 17.9M in this study. On linkage group 10, Kaga et al., [7], detected *St8i3.10.1* on physical position 28.9M that differed to *qSTIL8.10* detected on position 17.3M in this study.

Therefore, the plant height and total node number QTLs reported in this study are considered different since no previous reports were found based on the researchers' literature search. On the other hand, the stem internode length QTLs except *qSTIL7.4* reported in this study are considered different from previous reports as either have not been described before or are located on different positions to previous reports on similar linkage groups in the adzuki bean genome.

3.4.2 Relationship between detected QTLs

Plant height is an accumulation of epicotyl length (ECL) and stem internode length (STIL), whereby STIL is also dependent on total number of nodes (NN). A high correlation and close linkage between the traits plant height, epicotyl length, stem internode length and total number is thus expected. Results in this study however showed strong correlation between plant height and upper internode length; epicotyl length and lower stem internode length. A negative relationship was detected in traits such as total number of nodes with stem internode length below node seven; and epicotyl length with total number of nodes. This result relates to Yang et al., [14] study on QTL contribution to plant height traits in soybean using principal component analysis. It was observed that traits associated with plant height namely shoot height, stem node number and average internode length, grouped together confirming a high correlation among the traits [14].

In this study, no QTL for total node number was detected on chromosome 2, while a QTL for epicotyl length was detected but not stable and with minor effect explaining only 7.7% phenotypic variation.

However, stable QTLs in the two-year study period for plant height (*qPH2*) and stem internode length above node seven (*qSTIL8.2)* were detected in the same region. Therefore, independence was observed among the traits for plant height, total number of nodes and epicotyl length on chromosome 2. On the other hand, an association between plant height and upper internode length was noted on the same chromosome 2. In the same region of chromosome 4, a relationship existed between QTLs for plant height (*qPH4*), total node number (*qNN4.2*) and upper internode length (*qSTIL8.4*), (Table 3.5, Figure 3.5). The results therefore suggest possible contribution of node number on upper stem and plant height, and further indicate the independence of plant height from epicotyl length QTLs in this study population. This result therefore indicates the possibility for modifying overall plant height in adzuki bean by separately controlling epicotyl length and stem internode length on the upper part of the plant.

On chromosome 10, QTL for internode length above node number 7 (*qSTIL8.10*) was stable in three years, but not linked to plant height, since no plant height QTL was detected on chromosome 10. On the same chromosome 10, stable QTL for epicotyl length (*qECL10*) was on different positions with the loci for upper stem node *(qSTIL8.10)* and neither related to plant height. This suggest that on this genetic region the detected QTLs mainly affect stem elongation but not height.

Clustering of QTLs was mainly observed on chromosome 7, as well as, 2, 4, and 8 (Figure 3.5). Clustering of the QTLs maybe attributed to pleiotropy or close linkage of QTLs, as single mutations can have an effect on two or more phenotypic traits [7,21,68]. However, further studies are recommended to better understand the effect of pleiotropy or close linkage in clustering of QTLs in the studied adzuki bean population.

3.4.3 Significance of detected QTLs in breeding for ideal plant architecture

Plant architecture traits in adzuki been have been reported especially on the domestication of adzuki bean [7,21]. Potential agronomic traits for the improvement of growth habit, yield and seed coat

pigmentation have also been reported [6,69]. Interestingly, the previous studies clearly distinguish the lower, middle and upper stems length QTLs [7,21]. This is a useful analysis for understanding the control mechanisms in accordance with plant development stages and the associated genomic functions influencing the transition between plant development phases. In our experiments, it was observed that by the time adzuki bean plant develops the 7th node, the first flowering starts developing on the plants as well (unpublished data). In this study, principal component analysis showed clustering of epicotyl length and stem internodes below 7th node as well as plant height, stem internodes above 8th node, flowering and maturity traits, therefore confirming the linkages between lower stem internodes with vegetative development and upper stem internodes with reproductive development. Previously, a classification was done for stem internode lengths from 1 to 5 as lower to middle growth stages, and internode length 6 to 10 as late growth stage [7]. Therefore, to reduce dimensionality of the data and improve accuracy in the analysis, the stem length traits in this study were grouped as stem internode length below the 7th node and stem internode length above the 8th node.

Several adzuki bean varieties have been developed for production in Hokkaido. These include; 'Chihayahime' released in 2016 that has ordinary epicotyls, higher number of total nodes, disease resistance and higher yield. 'Erimoshozu' released in 2017 a cultivar with comparatively higher disease resistance, better yield but shorter epicotyls. 'Toiku161' which is a promising breeding line in terms of improved epicotyl length but has less total number of pods, comparatively low disease resistance and lower yield. Recently, TAES developed breeding line Toiku number 180 'Kitairoha' which has ideal plant architecture attributes including long epicotyls, raised pod position 10 cm above the ground surface, resistance to soil-borne diseases, good processing ability, that makes the breeding line ideal for combine harvesting and yield losses reduction [10]. However, to fully elucidate the genomic control mechanisms and facilitate effective achievement of the breeding goal for improved architecture, more adzuki bean lines with ideal architecture are required.

A major aim of this study was to analyze QTLs for plant height traits and assess the significance in the development of adzuki bean cultivars with improved plant architecture. According to reports, epicotyl length QTLs *qECL7.1* and *qECL10.1*; as well as *qECL7* and *qECL10* are considered stable [23,24] . The identified QTLs on chromosome 7 and 10 are presumably of great significance in clarifying genomic control mechanisms for improved epicotyl length genotypes. In this study, QTLs for epicotyl length and lower stem internode length were co-localized, hence, difficult to separate. This study findings show that internode length of the upper nodes and the total number of nodes on the main stem are the main factors influencing plant height as evidenced by the co-localization of detected QTLs. The results, suggest that plant height and epicotyl length are likely regulated by different genes. Furthermore, the results present a possibility for separation of upper part (above node 8) and the lower part (below node 7) enabling development of plants with a longer lower part but shorter upper part by using a combination of QTLs on chromosomes 2 and 4 to control overall plant height.

In soybean, the manipulation of plant architecture genes that control stem elongation, flowering and maturity time to balance the development of pod bearing nodes with lodging resistance is suggested as one of the approaches for improving productive potential [19]. Plant height related trait QTLs for plant height *qPH4*, flowering *qFLD4* and maturity *qMAD4* were detected in this study on chromosome 4. A total of 12 orthologues were identified including *Dt1, VaFT1, VaFT2* and *VaFT5* on chromosome 4 in population obtained from crossing 'Chihayahime' cultivar and 'Toiku161' breeding line, based on the Phylogenic tree and gene expression analysis for adzuki bean orthologues of soybean flowering-related genes [70]. The *Dt1* and *VaFT5* genes were observed to be within the locality of the plant height related QTLs identified in this study on chromosome 4.

Plant architecture is a fundamental agronomic characteristic that is dependent on indeterminate and determinate meristems development [71]. Comprehensive studies identified the *Dt1* and *Dt2* genes as control agents for determinacy and indeterminacy of stem growth habits in soybean [19,71,72]. The *Dt1* gene is functionally maintained in Arabidopsis Terminal flower1 (*TFL1)* gene that affects stem termination

[19,72]**.** Genes associated with reproductive traits have been identified as well including Arabidopsis flowering *Locus T* [73].

The expression of *Dt1* in the shoot apical meristem prevents the transition from vegetative to reproductive phase and promotes indeterminate growth [74]. The genes regulating stem elongation affects diversity in phenotypes of soybean plant architecture [19]. The indeterminate stem elongation soybean plant varieties tend to have a longer growing period and late maturity with increased number of pods in comparison with determinate varieties [75]. The *Dt1* could also be involved in the determinate and indeterminate elongation in adzuki bean. The identified orthologues or other genes [70] close to the plant height and yield related loci maybe responsible for the phenotypic variation in number of nodes, flowering time and maturity time of the RILs evaluated in this study. However, comparison gene sequences between the 'Toiku161' and 'Chihayahime' indicated sequence variations in the intron region but not in the exon region. Although the *VaFT5* and *Dt1* genes were observed in the vicinity of plant height and yield traits loci in the study population, the roles on plant height traits, flowering, maturity and yield remains unclear in adzuki bean. Therefore, in-depth research is required to clarify the *Dt1* and *VaFT5* genes influence in the reproductive functions and stem elongation contribution to the phenotypic variations of plant height and yield traits in adzuki bean, to facilitate the development of desirable plant architecture trait combinations.

3.4.4 Ideal plant height in adzuki bean

Traditional breeding processes are laborious and time demanding, whereas advanced technologies such as marker- assisted selection (MAS) are being effectively applied in many crops enabling rapid selection of preferable traits thereby facilitating breeding work for ideal plant types including plant height for optimal yields [14]. Furthermore, it is suggested that higher plants, larger node numbers, stronger stems, and longer growth periods are ideal genotypes for optimal plant architecture in soybean [19]. Currently, the priority in adzuki bean breeding efforts for ideal architecture include the development of genotypes with

longer epicotyls for improved mechanization and lodging resistance to minimize crop yield losses. This study sought to analyze and understand the usefulness of plant height related traits in regard to breeding for ideal genotypes of adzuki beans for optimal productivity.

Generally, plant height is a combination of epicotyl length and average internode length plus node number. The mean epicotyl length among the RILs evaluated in this study ranged from 5 to 7 cm. This is in-line with the findings from Mori et al., [23], that an epicotyl length within 5 to 8 cm is desirable in adzuki beans, as it allows optimal pod position and height at least above 10cm from the field surface and minimizes pod damage during combine harvesting. Combine harvesting improves efficiency and minimizes the labor shortage which is among the challenges for adzuki bean production in Hokkaido. The results from this study showed that ideal plant height can be attained by; a combination of either (1) long epicotyl length on chromosome 7 and short upper internode length on chromosome 4 using alleles derived from 'T161' parent line, or (2) short epicotyl length on chromosome 7 and long stem internode length on chromosome 4 using alleles derived from 'Chihayahime' parent line**.** However, such combinations may yield undesirably tall or short plants with potential lodging susceptibility and combine harvesting inefficiency. In soybean the optimal plant height at present for commercial cultivars ranges between 70 to 90 cm, with compromised yield levels observed for taller or shorter crop stands [14]. In adzuki bean, observation so far indicate that ideal plant height for optimal yield approximately ranges from 60 to 80 cm (TAES personal communication). The combination effect analysis of plant height trait from this study, showed that sole combination of either the 'Chihayahime' type alleles or 'T161' type alleles on both *qPH2* (chromosome 2) and *qPH4* (chromosome 4) yielded a moderate plant height across the four-year study period. Therefore, an ideal plant height within the proposed 60 to 80 cm range in adzuki bean, could be attainable by utilizing a sole allelic combination of 'Chihayahime' or 'T161' type alleles on both *qPH2* and *qPH4* to control the length of the upper plant part.

However, observations and data collected so far lacks sufficient scoring on lodging susceptibility, as lodging is one of crucial factors affecting productivity. In this study, only one QTL for lodging was

detected in the year 2022. Therefore, further research work is needed for verification of the current observations, because in some incidences within the 60 to 80cm plant stands, high lodging frequencies occur due to various factors including heavy weights of pods in varieties with high total pod numbers.

3.5 Summary

A better understanding of the key traits such a plant architecture is essential in modern crop breeding programs for improved crop productivity. This study identified a total of 15 QTLs in 2020, 12 QTLs in 2021, 12 QTLs in 2022 and 11 QTLs in 2023 for plant height related traits, and further confirmed the different genetic control mechanisms between plant height and epicotyl length traits. An epistatic interaction was detected between plant height QTLs on *qPH2* and *qPH4* in a single year. The information generated in this study will be useful towards molecular breeding efforts in developing cultivars with useful plant architecture traits such as long epicotyls for improved mechanization while controlling poor traits like excessive long plants to avoid lodging in adzuki beans. Earlier studies, fine-mapped epicotyl length trait QTLs *qECL7.1* and *qECL10* and identified candidate genes within the target regions. However, more detailed studies are needed on fine mapping and associated candidate genes for the detected other plant architecture traits QTLs such as plant height, stem internode length and number of nodes in order to further clarify the genetic basis in plant architecture for effective adzuki bean breeding programs implementation. This work focused much on plant height related traits, however to clearly understand the significance of plant architecture on yield, an in-depth analysis of yield related traits is necessary.

Table 3-1: Plant height traits evaluation

Trait	Abbreviation	Evaluation criteria
Plant height	PH	Distance from the base to the tip of plant at maturity
Epicotyl length	ECL	Length from the cotyledon to primary leaf
Node number	NN	Total number of nodes on the main stem
Stem internode length below 7th node	STIL7	Total length of nodes below 7th node
Stem internode length above 8th node	STIL8	Total length of nodes above 8th node
Flowering	FLD	Number of days from planting to 1st flowering
Maturity	MAD	Number of days from planting to 80% pod maturity

Table 3-2: Description of the 11 linkage groups in the constructed genetic map

Linkage group	Length (cM)	Marker number	Marker distance (cM)	Max. gap (cM)	Map coverage (%)
LG1	137.1	14	9.8	20.8	88.5
LG2	81.4	8	10.2	21.1	99.6
LG3	70.6	9	7.8	15.4	91.0
LG4	99.8	13	7.7	13.3	99.6
LG5	88.3	9	9.8	35.8	99.5
LG6	89.3	9	9.9	18.1	98.8
LG7	80.0	9	8.9	19.7	82.1
LG8	94.2	10	9.4	15.5	98.3
LG9	49.0	8	6.1	14.0	87.6
LG10	83.7	11	7.6	19.1	98.4
LG11	90.2	8	11.3	40.8	98.2
Total	**963.6**	**108**	**9.0**		**94.7**

Table 3-3: Descriptive statistics for plant height related traits in parent lines

			Parent line			RILs[a]					
Trait		year	T161	Chihayahime	Difference	Min	Max	Mean	CV(%)	Kurtosis	Skewness
PH	(cm)	2020	61.1±3.0	47.4±3.1	13.7***	31.9	119.5	51.7±12.1	21.2	7.1	1.7
		2021	52.8±1.0	52.8±3.3	0.0^{ns}	32	87.0	47.5±8.4	17.7	3.3	1.1
		2022	75.1±4.2	70.6±2.4	4.5^{ns}	39.8	126.4	67.8±16.9	24.9	1.4	1.0
		2023	74.2±4.1	67.8±3.7	6.4*	46.6	116	70.2±11.2	16.0	2.2	0.9
ECL	(cm)	2020	10.4±0.5	5.2±0.5	5.2***	2.8	12.2	6.9±2.0	29.0	0.1	0.6
		2021	8.2±1.2	4.3±0.8	3.9***	2.8	10.1	5.2±1.6	30.8	0.9	1.2
		2022	7.1±0.5	3.2±0.2	3.9***	2.6	9.3	4.9±1.3	26.5	1.1	1.3
		2023	6.6±0.6	3.2±0.2	3.4***	2.6	8.2	4.4±1.1	25.0	1.2	1.2
NN	(no. per plant)	2020	11.6±0.3	12.5±0.5	0.9^{ns}	9.0	15.5	11.9±1.1	9.2	1.1	0.4
		2021	13.6±0.8	14.4±0.5	0.8^{ns}	10.5	16.2	13.2±1.1	8.3	0.6	0.2
		2022	12.3±0.6	14.2±1.3	1.9**	9.8	16.8	13.0±1.3	10.0	0.1	0.0
		2023	14.3±1.1	14.4±0.7	0.1^{ns}	10.2	18.8	14.8±1.4	9.5	0.9	-0.3
STIL7	(cm)	2020	29.8±4.1	18.8±8.6	11**	13.3	60.0	21.9±6.3	28.6	10.9	2.3
		2021	24.3±1.2	15.7±12.6	8.6**	10.7	37.3	19.0±4.7	24.6	1.4	1.0
		2022	37.1±1.8	23.0±0.7	14.1***	16.6	63.9	27.8±7.8	28.1	4.6	1.7
		2023	23.5.±9.6	17.9±7.6	5.6***	11.5	32.5	19.2±3.7	19.1	2.1	1.1
STIL8	(cm)	2020	20.0±2.8	25.0±3.0	5.0**	4.7	47.0	20.6±8.5	41.2	0.7	0.9
		2021	24.0±3.6	37.3±3.4	13.3**	11.3	49.6	30.2±7.9	26.1	-0.2	0.3
		2022	34.2±3.2	51.1±2.7	16.9***	12.0	90.7	40.4±15.2	37.6	0.8	0.9
		2023	41.8±6.8	45.6±6.0	3.8^{ns}	20.7	128.3	45.5±13.3	29.3	12.6	2.4

[a:] Recombinant inbred lines, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant

Table 3-4: Variable loading scores for plant height related traits and variation proportion

	2020			2021			2022			2023		
	PC1	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**	**PC1**	**PC2**	**PC3**
Eigen value	2.8	2.2	1.0	2.8	2.1	0.9	2.8	2.4	0.8	2.3	2.1	1.0
Variance (%)	39.9	31.5	13.7	40.6	30.7	12.9	39.6	33.6	12.1	33.2	30.0	14.5
Cumulative variance (%)	39.9	71.4	85.1	40.6	71.3	84.1	39.6	73.2	85.3	33.2	65.2	77.7
PH	0.8	0.5	-0.2	0.6	0.7	-0.2	0.5	0.8	-0.3	0.6	0.7	-0.1
ECL	-0.1	0.9	0.0	-0.5	0.8	0.2	-0.5	0.7	0.0	0.8	-0.2	0.1
NN	0.6	-0.6	-0.3	0.9	-0.2	-0.2	0.6	0.3	0.6	-0.7	0.7	0.0
STIL7	0.3	0.9	-0.1	-0.2	0.9	0.0	0.7	0.2	0.4	0.9	-0.1	0.0
STIL8	0.9	-0.1	-0.4	0.9	0.2	-0.3	0.8	-0.5	-0.2	0.2	0.8	-0.2
FLD	0.6	0.0	0.7	0.6	0.2	0.4	-0.3	0.9	0.0	0.7	0.7	0.2
MAD	0.7	-0.1	0.5	0.6	-0.1	0.7	0.8	0.4	-0.4	0.1	0.1	1.0

Note: Eigen vectors of the first three dimensions of variance for each trait identified in the principal component analysis (PCA) of phenotypic variation and correlation

Table 3-5: Detected Plant-height related traits QTLs in T161 x Chihayahime RILs population

Trait name	QTL[a]	year	LG[b]	Nearest Marker	Peak position	LOD[c]	T161[d]	CH[e]	Add[f]	PVE %[g]
Plant height	*qPH2.1*	2020	2	AZ02_4.0M	20.9	5.37	55.2	43.6	5.8	14.7
	qPH4		4	AZ04_50.7M	71.2	9.80	41.2	56.0	-7.4	30.2
	qPH8		8	AZ08_11.9M	46.1	3.94	53.2	44.1	4.6	10.4
	qPH2.2	2021	2	AZ02_5.3M	22.9	4.00	54.2	46.6	3.8	12.3
	qPH4		4	AZ04_50.7M	70.2	9.01	44.8	56.0	-5.6	30.1
	qPH2.1	2022	2	AZ02_4.0M	20.9	4.95	82.0	65.9	8.0	13.5
	qPH4		4	AZ04_50.7M	70.2	12.17	60.5	85.3	-12.4	38.2
	qPH2.2	2023	2	AZ02_5.3M	22.9	7.07	81.1	67.6	6.8	21.9
	qPH4		4	AZ04_50.7M	69.2	3.76	66.7	76.3	-4.8	14.2
Epicotyl length	*qECL1*	2020	1	AZ01_55.0M	106.7	4.88	7.6	6.1	7.4	9.9
	qECL7		7	AZ07_10.0M	48.8	9.86	7.8	5.9	9.7	22.9
	qECL10		10	AZ10_27.5M	79.5	6.68	7.6	6.1	7.8	14.3
	qECL7	2021	7	AZ07_10.0M	47.8	15.55	6.4	4.2	10.7	41.3
	qECL10		10	AZ10_28.5M	83.7	5.05	5.8	4.8	5.2	11
	qECL2	2022	2	AZ02_4.0M	14.0	4.48	5.5	4.6	0.5	7.7
	qECL7		7	AZ07_10.0M	48.8	14.69	5.9	4.2	0.9	33.2
	qECL10		10	AZ10_28.5M	83.5	7.57	5.7	4.5	0.6	14.8
	qECL7	2023	7	AZ07_10.0M	49.5	7.95	5.0	3.9	0.5	22.5
	qECL10		10	AZ10_26.3M	77.1	9.63	5.0	3.9	0.5	24.6
Node number	*qNN4.1*	2020	4	AZ04_54.0M	99.8	2.92	11.7	12.6	-0.4	11.2
	qNN7		7	AZ07_10M	48.8	5.05	11.7	12.6	-0.5	16.5
	qNN1	2021	1	AZ01_55.0M	112.5	2.94	13.1	13.7	-0.2	6.6
	qNN4.2		4	AZ04_50.7M	72.3	7.67	12.9	13.9	-0.5	19
	qNN7		7	AZ07_10.0M	49.5	6.97	12.9	13.8	-0.4	17
	qNN11		11	AZ11_0.6M	0.0	3.74	13.7	13.1	0.3	8.5
	qNN4.2	2022	4	AZ04_50.7M	72.3	3.97	12.7	13.6	-0.5	11.5
	qNN7		7	AZ07_10.0M	47.8	8.05	12.4	13.8	-0.7	25.2
	qNN4	2023	4	AZ04_50.7M	72.3	2.9	14.5	15.3	-0.4	7.6
	qNN7		7	AZ07_10.0M	49.5	6.76	14.1	15.5	-0.7	24.1
Total Node length ≤7	*qSTIL7.2*	2020	2	AZ02_2.6M	12.0	5.48	259.9	210.9	24.5	11.5
	qSTIL7.4		4	AZ04_52.1M	77.3	4.89	211.7	258.8	-23.6	10.1
	qSTIL7.7		7	AZ07_10.0M	48.8	13.76	273.2	195.6	38.8	34.8
	qSTIL7.8		8	AZ08_17.9M	49.1	3.22	253.5	216.4	18.5	6.4
	qSTIL7.7	2021	7	AZ07_10.0M	48.8	18.28	226.8	156.7	35.0	52.5
	qSTIL7.2	2022	2	AZ02_2.6M	13.0	3.74	314.1	262.5	25.7	8.4
	qSTIL7.3		3	AZ03_9.4M	23.1	3.23	259.7	307.9	-24.1	7.4
	qSTIL7.7		7	AZ07_10.0M	48.8	12.79	332.1	228.8	51.7	40.6

Trait name	QTL[a]	year	LG[b]	Nearest Marker	Peak position	LOD[c]	T161[d]	CH[e]	Add[f]	PVE %[g]
	qSTIL7.7	2023	7	AZ07_10.0M	47.8	11.68	216.7	168.7	24.0	37.9
Total Node length ≥8	*qSTIL8.2*	2020	2	AZ02_5.3M	23.9	3.26	256.1	193.8	31.2	8.6
	qSTIL8.4		4	AZ04_50.7M	72.2	8.08	178.0	272.1	-47.1	23.5
	qSTIL8.10		10	AZ10_17.3M	48.6	4.03	196.3	253.7	-28.7	10.8
	qSTIL8.2	2021	2	AZ02_5.3M	23.9	7.42	363.1	282.7	40.2	16.5
	qSTIL8.4		4	AZ04_50.7M	72.2	10.22	278.7	367.4	-44.4	24.2
	qSTIL8.10		10	AZ10_17.3M	47.9	8.52	285.9	359.5	-36.8	19.3
	qSTIL8.2	2022	2	AZ02_5.3M	22.9	3.56	499.3	393.3	53.0	7.3
	qSTIL8.4		4	AZ04_50.7M	70.2	16.23	327.4	567.6	-120.1	42.3
	qSTIL8.10		10	AZ10_17.3M	48.6	3.61	403.9	489.3	-42.7	7.5
	qSTIL8.2	2023	2	AZ02_2.6M	14.0	3.58	537.1	430.2	53.3	11.9
	qSTIL8.4		4	AZ04_48.3M	67.2	4.89	422.3	545.5	-61.6	16.9
Flowering date	*qFLD2*	*2020*	2	AZ02_5.3M	22.9	4.44	65.0	63.0	0.9	14.8
	qFLD10		10	AZ10_0.05M	0.0	2.96	64.6	63.3	0.7	9.6
	qFLD2.2	*2021*	2	AZ02_4.0M	19.9	11.9	62.8	59.2	1.8	33.9
	qFLD4		4	AZ04_48.3M	69.2	6.73	59.7	62.2	-1.3	17.2
	qFLD8		8	AZ08_12M	40.1	3.46	61.6	60.0	0.8	8.8
	qFLD2	*2022*	2	AZ02_4.0M	19.9	14.2	63.0	59.3	1.9	32.2
	qFLD4.2		4	AZ04_50.7M	70.2	9.52	59.6	62.4	-1.4	20.5
	qFLD7		7	AZ07_10M	49.5	2.90	60.4	61.6	0.6	5.6
	qFLD9		9	AZ09_15.1M	28.0	3.12	61.7	60.3	0.7	6.0
	n.d	*2023*	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Maturity date	*qMAD4*	*2020*	4	AZ04_35.7M	50.1	3.37	111.7	110.0	0.8	12.8
	qMAD4	*2021*	4	AZ04_35.7M	50.1	3.22	126.9	125.3	1.6	12.3
	qMAD1	*2022*	1	AZ01_5.2M	20.0	3.66	110.9	109.7	1.2	9.8
	qMAD4.2		4	AZ04_50.7M	72.3	8.17	108.3	110.1	-1.8	20.0
	qMAD2	*2023*	2	AZ02_4.0M	20.9	5.42	103.5	100.8	1.3	15.5
	qMAD4		4	AZ04_50.7M	71.2	8.14	100.4	103.5	-1.5	25.3

[a] QTL: Quantitative trait loci; [b] LG: Linkage group; [c] LOD: Logarithm of odds; [d] T161: T161 type allele; [e] CH: Chihayahime type allele; [f] Add: Additive effect; [g] PVE%: Phenotypic variation explained in percentage, n.d: not determined.

Table 3-6: Interaction of plant height QTLs 2020

Cases	Sum of Squares	df	Mean Square	F	p
qPH2	2163.56	1	2163.56	23.103	< .001
qPH4	3770.58	1	3770.58	40.263	< .001
qPH2 ✻ *qPH4*	400.705	1	400.705	4.279	0.041
Residuals	10020.4	107	93.648		

Table 3-7: Interaction of plant height QTLs 2021

Cases	Sum of Squares	df	Mean Square	F	p
qPH2	486.651	1	486.651	9.128	0.003
qPH4	1373.75	1	1373.75	25.766	< .001
qPH2 ✻ *qPH4*	33.935	1	33.935	0.636	0.427
Residuals	5704.81	107	53.316		

Table 3-8: Interaction of plant height QTLs 2022

Cases	Sum of Squares	df	Mean Square	F	p
qPH2	3061.17	1	3061.17	18.095	< .001
qPH4	8387.43	1	8387.43	49.58	< .001
qPH2 ✻ *qPH4*	439.121	1	439.121	2.596	0.11
Residuals	18101	107	169.168		

Table 3-9: Interaction of plant height QTLs 2023

Cases	Sum of Squares	df	Mean Square	F	P
qPH2	867	1	867.4	9.936	0.00211**
qPH4	2925	1	2924,5	33.499	7.33e08***
qPH2 ✻ *qPH4*	186	1	186.0	2.131	0.14731
Residuals	9254	107	87.3		

Table 3-10: Combination effect analysis for plant height trait

qPH2	*qPH4*	2020	2021	2022	2023
		Mean PH	Mean PH	Mean PH	Mean PH
CH	CH	45.4±9.13^{b}	47.1±7.61^{bc}	66.0±13.2^{c}	71.8±11.4^{b}
CH	T161	38.4±7.9^{c}	42.7±6.54^{c}	56.9±10.4^{d}	62.9±7.17^{c}
T161	CH	67.2±24.0^{a}	58.5±16.2^{a}	96.1±18.5^{a}	90.1±18.4^{a}
T161	T161	49.4±8.94^{b}	51.1±5.89^{ab}	75.7±14.5^{b}	73.2±7.76^{b}
CH		47.4±3.1	52.8±3.3	70.6±2.4	67.8±3.7
T161		61.1±3.0	52.8±1.0	75.1±4.2	74.2±4.1
Significance		***	ns	ns	*

Figure 3-1 Plant architecture differences in parent lines T161(left) and Chihayahime (right) at harvesting stage.

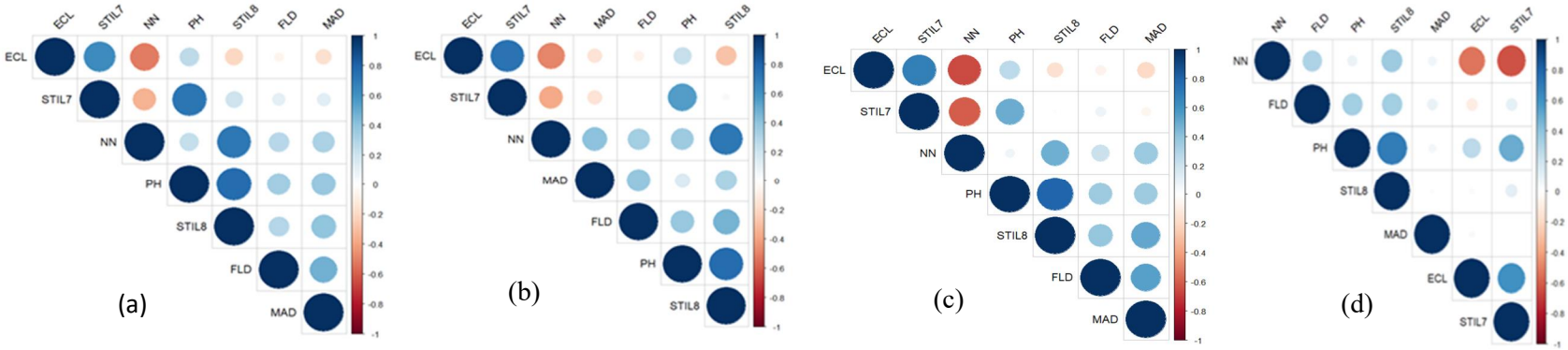


Figure 3-2: Correlation analysis for plant height traits in (a), (b), (c) and (d) for years 2020, 2021, 2022 and 2023, respectively

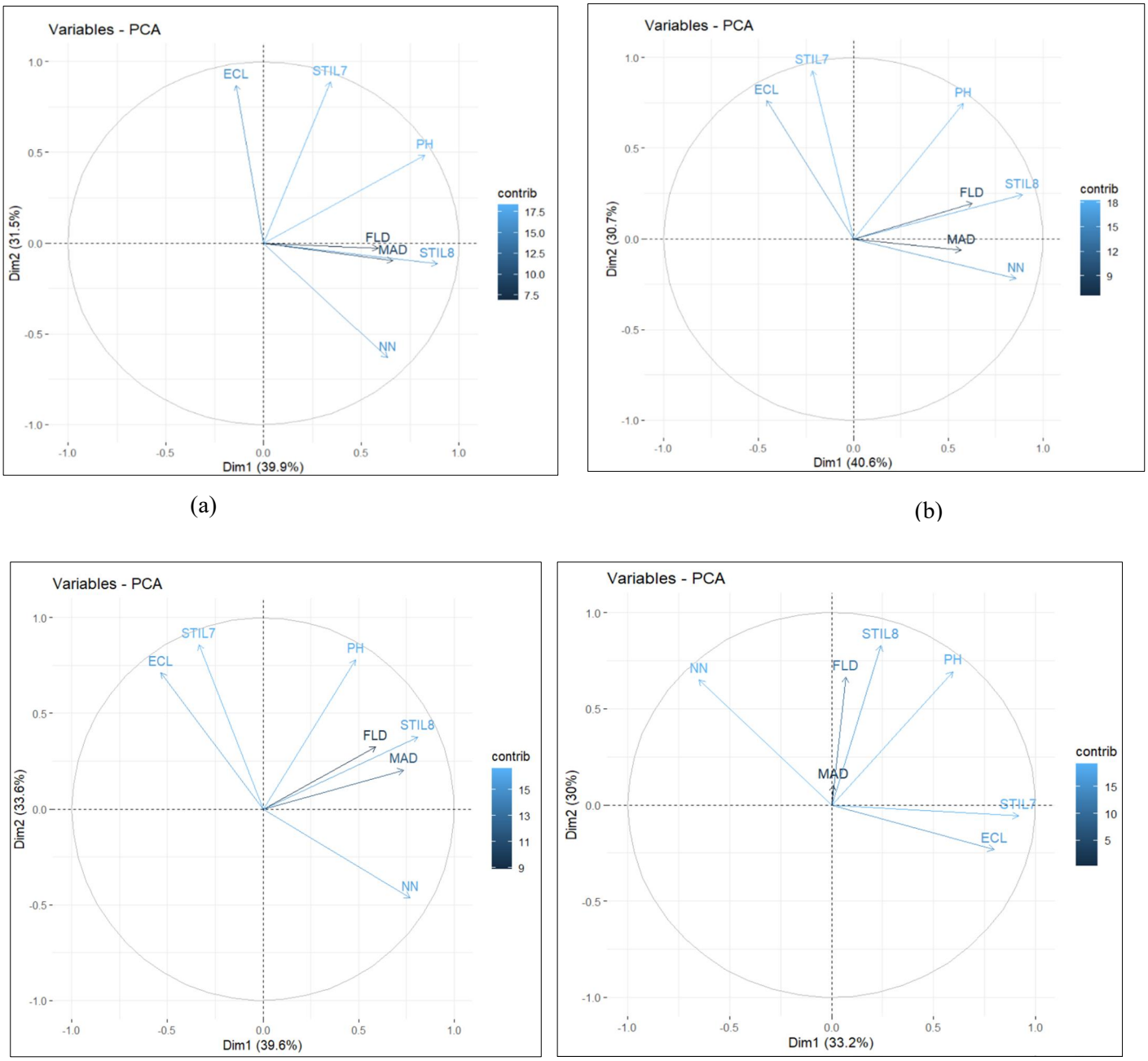


Figure 3-3: Principal component analysis for plant height traits

Note: Principal component analysis of phenotypic variation and contribution of plant height traits in (a), (b), (c) and (d) for the years 2020, 2021, 2022 and 2023, respectively. The two-dimensional correlation structure among 113 RILs. Horizontal axis represents Dimension 1 and the vertical axis represents Dimension 2. Dimension 1 explains 39.9% in 2020, 40.6 % (2021), 39.6% (2022) and 33.2% (2023), and Dimension 2 explains 31.5% (2020), 30.7 % (2021), 33.6% (2022) and 30% (2023). Arrows indicate the strength of relationship between traits. The colour shade in the bar indicate trait contribution to phenotype

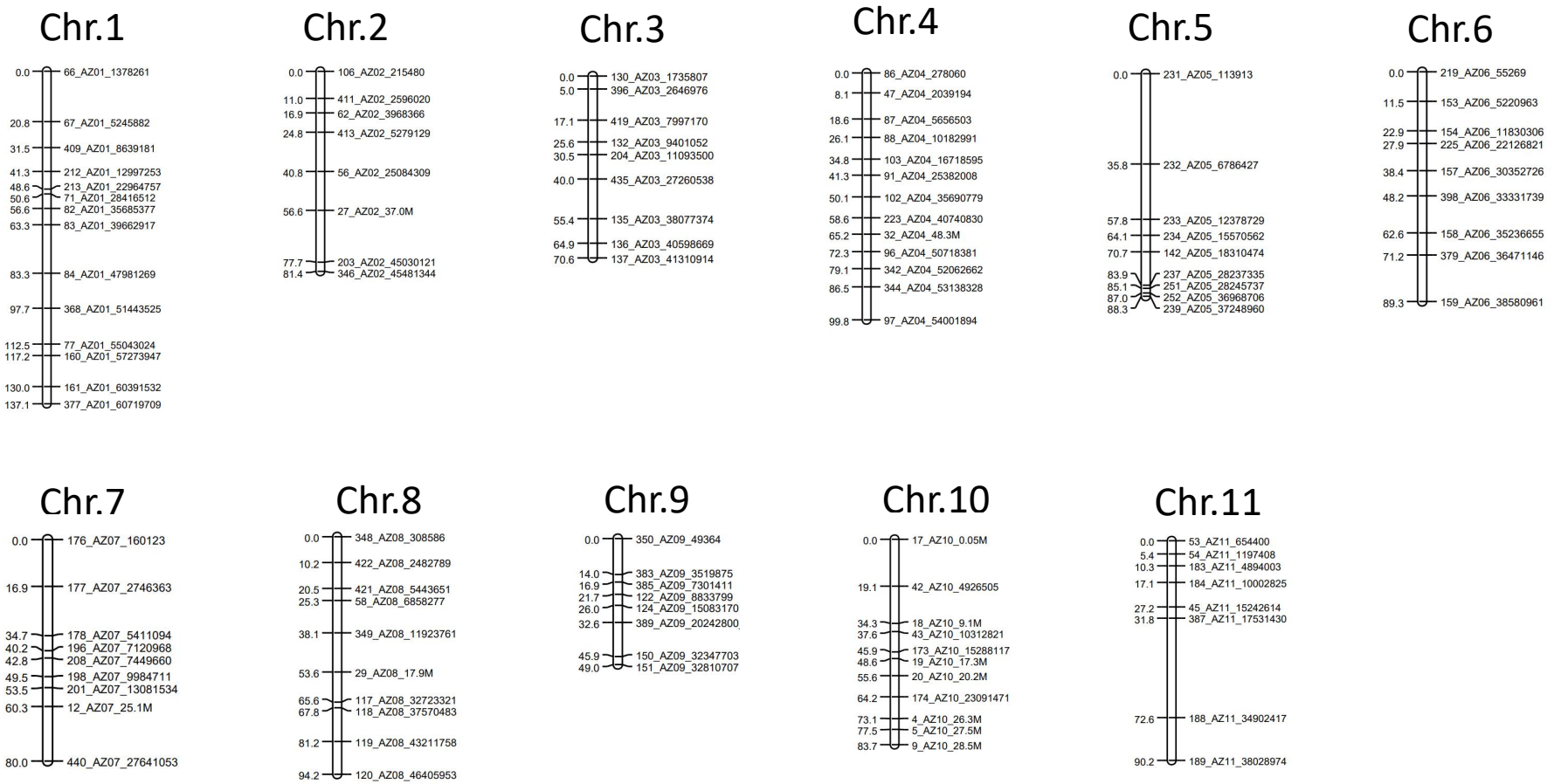


Figure 3-4: Adzuki bean linkage map constructed at LOD1.0 in JoinMap software

Note: Adzuki bean linkage map constructed with 108 DNA markers in 113 RILs developed from ‘T161’ x ‘Chihayahime’ cross.

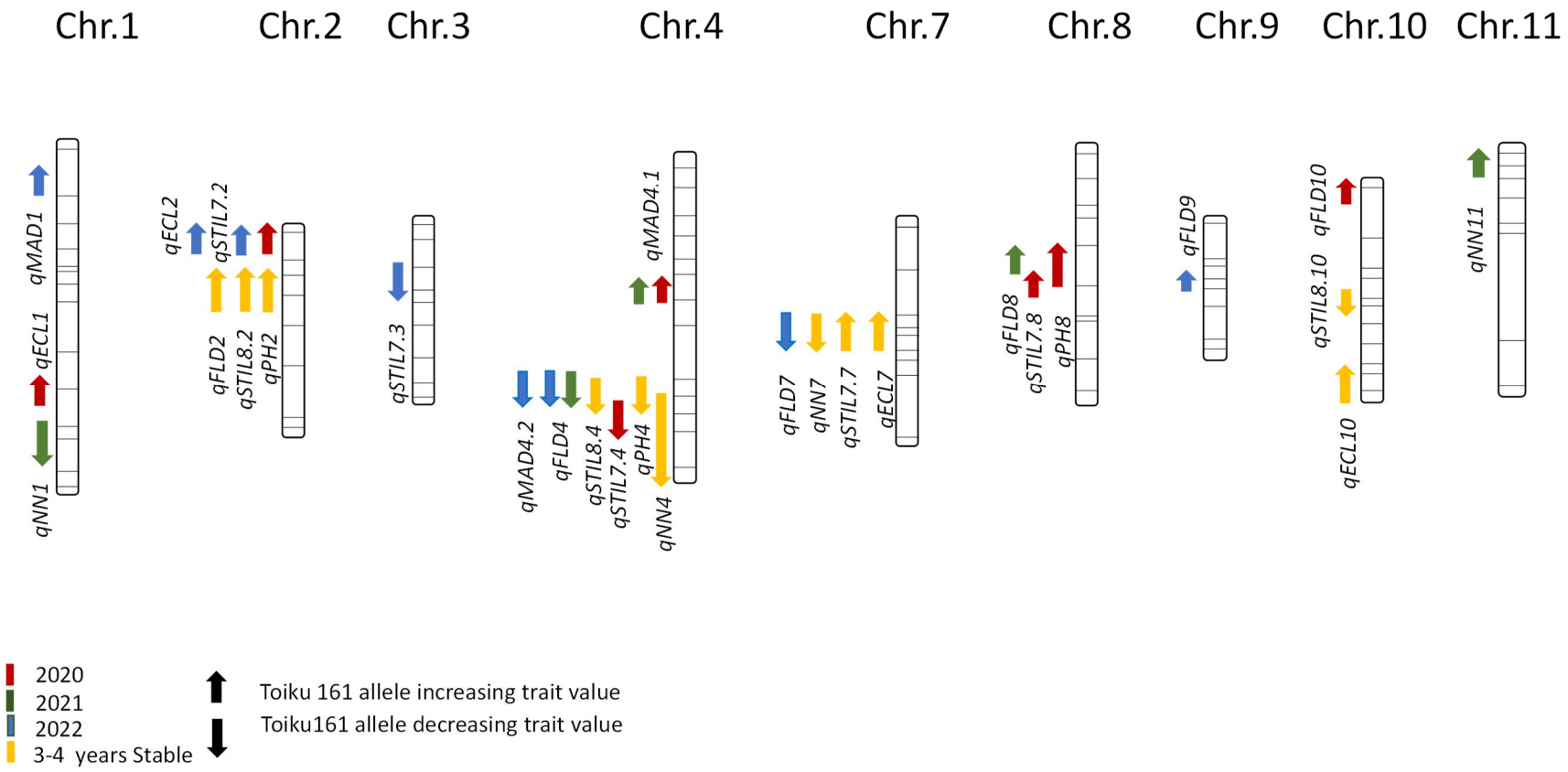
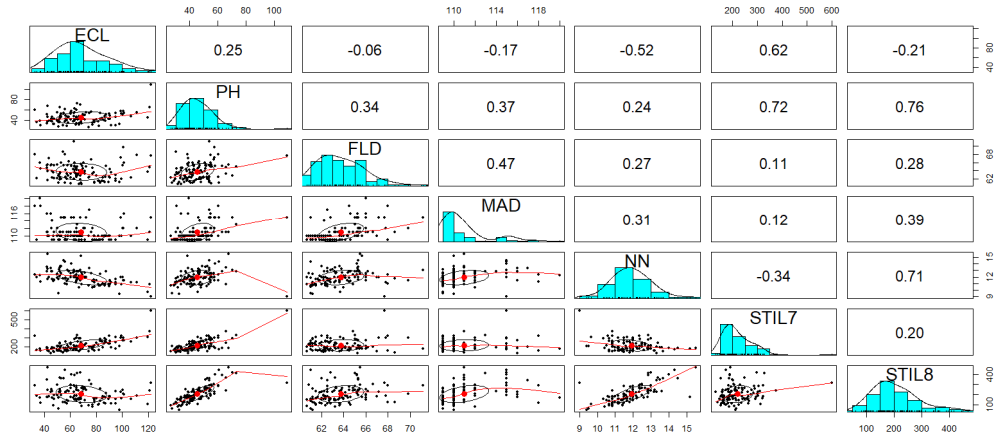


Figure 3-5:QTL mapping for plant height traits on constructed linkage map

Note: Mapping for the detected plant-height related trait QTLs in 113 RILs developed from 'T161' x 'Chihayahime' cross. Red colour indicates QTLs detected in 2020, green colour indicates QTLs detected in 2021, blue colour indicates QTLs detected in 2022 and yellow colour indicates Stable QTLS in the three to four (2020 – 2023) year period. Black upward arrow indicates an increasing effect from Toiku161 allele, and black downward arrow indicates a decreasing effect from Toiku161 allele.

(a)



(b)

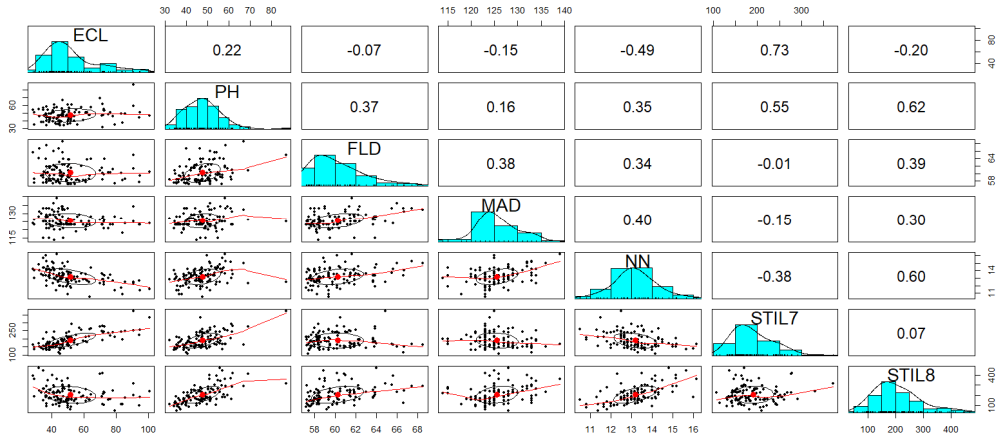


Figure S 3. 1: Phenotypic variation of plant height traits (a) and (b)

(c)

(d)

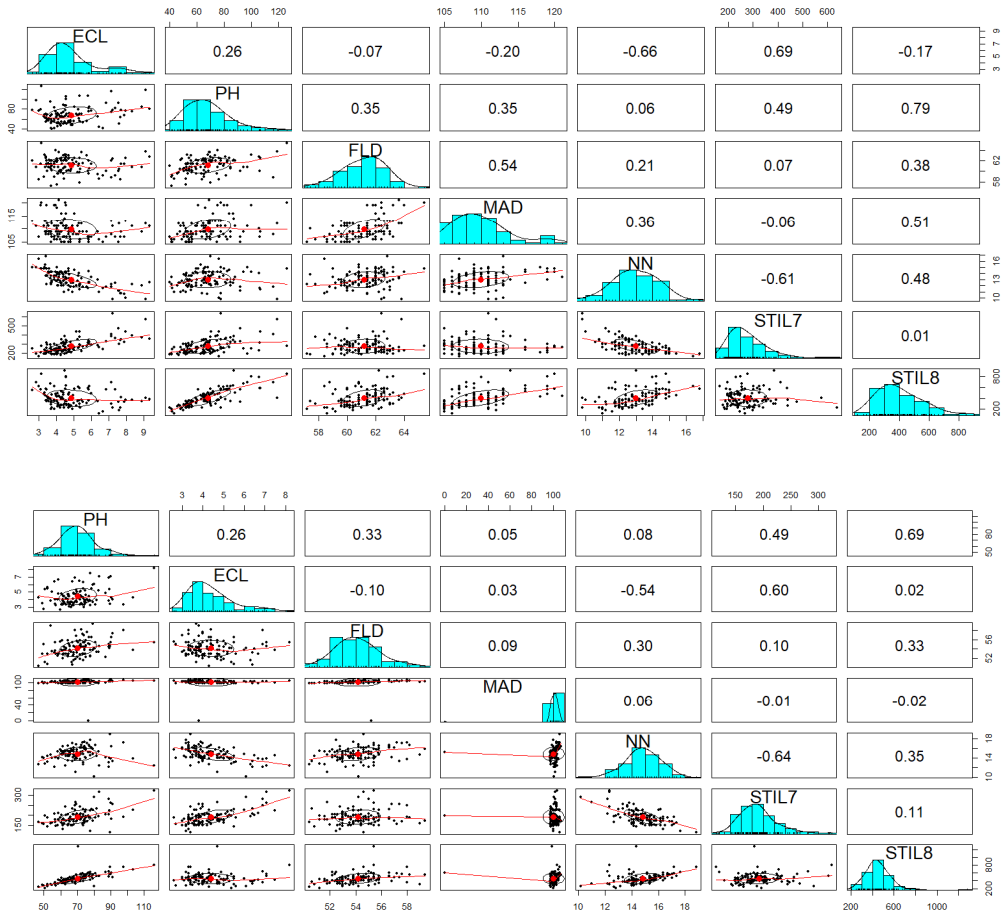


Figure S 3. 2: Phenotypic variation of plant height traits (c) and (d)

Note: Phenotypic distribution of PH, ECL, NN, STIL7, STIL8, FLD and MAD. Pairwise trait correlations across 113 RILs in 2020 (a), 2021 (b), 2022 (c) and 2023 (d). The RILs differed phenotypically and displayed high relationships between PH with STIL8, and ECL with STIL7 across the years. Positive and negative numbers indicate the Pearson correlation coefficient (*r*).

CHAPTER 4: QTL ANALYSIS FOR YIELD-RELATED TRAITS IN ADZUKI BEAN (*Vigna angularis*)

4.1 Introduction

For some decades now yield has been a focus area in crop breeding programs to ensure achievement of productivity goals. Yield is a complex trait influenced by several other factors including plant type, lodging susceptibility and production systems efficiency [16]. Previously, Kachapila et al. (Unpublished data), analyzed the phenotypic variation and genomic control mechanism for plant height related traits in adzuki bean. Studies in soybean suggest that plant architecture traits such as; internode characteristics, pod number at the stem apical area, raceme length and lodging susceptibility influence yield levels [19]. Lodging affects the effectiveness for harvesting using combine machines and causes bad quality grains [9], thereby reducing overall yield productivity levels. The flowering pattern of a plant and the timing of transition from vegetative to reproductive phase also determines the plant growth habit thus either discreet flowering (determinate) or non-discreet flowering (Indeterminate) plants [61]. It is in this context that this study component was designed to understand the genomic background of yield-related traits and their association to the identified plant height traits, for improved plant architecture and yield-related traits in adzuki beans programs.

Climatic conditions are very important considerations in agricultural production. Hokkaido the largest producer of adzuki bean in Japan, has very cold winter and cool summer unlike other regions of Japan. Specifically, Tokachi district which is a hub for adzuki bean production in Hokkaido, has a short frost-free period about 134 days with the last frost on average by May 20 and the early frost by early October [9]. The coincidence of early frost occurrence and maturity time for medium to late adzuki bean varieties affects the grain quality and reduces yield levels. As such prevention of frost damage is a focus area for sustainable adzuki bean production. On the other hand, temperature is a critical factor influencing the plant development and yield in adzuki beans. Extremely cooler summer causes delay in plant growth and maturity, pod and seed development in turn decreasing the seed yield [9,12]. Therefore, flowering and maturity time of adzuki bean needs critical consideration to avoid frost damage on late maturing plants. An early maturing and medium yielding cultivar ‘Chihayahime’ developed in Hokkaido by TAES, however the genetic mechanism controlling maturity and yield remains unclear which affects the effective improvement for early maturity and high yielding traits. Therefore, an understanding of the genetic mechanisms controlling maturity and yield levels to sustain Hokkaido region adzuki bean productivity is necessary.

To date, a number of studies have analyzed QTLs for flowering, maturity time and yield related traits. Using a population from a cross between wild species from Nepal *(V. nepalensis)* and landrace from

Tokushima prefecture, QTLs were identified for flowering on Linkage group 4, 100 seed weight on linkage groups 1, 2, 5 and 9 [21]. In a study that using a population from a cross between wild adzuki bean accession from Yamanashi prefecture and cultivar 'Kyoto Dainagon', identified QTLs for flowering date and maturity on Linkage groups 2, 3 and 4; total pod number on linkage groups 4 and 9; 100 seed weight on linkage groups 1, 2, 5, 6, 9, 10 and 11; seed weight on linkage groups 4, 6, 9 and 10; seed number per pod on linkage groups 5, 6, 7, 10 and 11 [7]. Another study used a population crossed between 'Shumari' a cultivar from Hokkaido prefecture and landrace Acc2265, and found *FD1* QTL on linkage group 2 [8]. A study based on a population crossed from wild adzuki bean accession (*Vigna nipponensis*: Yesheng10) from Dandong, China and adzuki bean cultivar (Jihong9218) widely grown in northern China, found flowering time QTLs on linkage groups 3 and 5 [2]. In addition, using a population crossed between adzuki bean cultivar 'Ass001' and wild adzuki bean accession 'CWA108', QTLs for flowering were detected on chromosome 4, 7 and 10; maturity on chromosome 4; seed number per pod on linkage groups 3 and 4; and 100 seed weight on linkage groups 1, 2 and 3 [22].

However, most of the studies based on crosses between cultivated and wild species. Therefore, it is not clearly known whether the identified QTLs equally affect maturity and yield in cultivated varieties such as 'Chihayahime'. This study evaluated the phenotypic variations and correlation of yield-related traits and as well as analyzed quantitative trait loci contributing to genotypic variations in yield traits.

Agronomic traits such as yield are typically controlled by multiple genetic loci and interactions between genetic loci including environmental factors. As such, QTL detection alone may not fully explore the phenotype and genotype associations including the underlying network traits with significant contributing to the genotypic and phenotypic trait expressions. Significant correlations were observed between flowering and maturity with some plant height related traits (Kachapila et al., Unpublished data). Therefore, in this study a QTL network analysis using the QTL-directed dependency graph (QDG) was performed to understand the direct and indirect relationships among the traits of interest for improved plant architecture and yield in adzuki bean.

4.2 Materials and Methods

4.2.1 Mapping population and field experiments

The RIL populations used in this study was derived from a cross between cultivars 'Toiku161' (T161) and 'Chihayahime' which are both varieties developed by TAES, Hokkaido research station. T161 is a medium maturity variety and was the female in the cross while 'Chihayahime' was the male in the cross

and is an early maturity variety, which attains maturity about a week early than existing medium maturity varieties in Hokkaido region. The study population consisted of 113 RILs generated by self-pollination.

The RILs and parent lines were grown at Tokachi Experimental Research Station from May to October in 2020 to 2023 (Figure 4.1.). Three seeds that were later thinned to one seed per station were planted at 20cm between planting stations and 60cm ridge spacing. Each RIL had 8 plants from which 6 representational plants were selected for trait evaluation. A total of 13 plants in 2020, 8 plants in 2021 and 6 plants in 2022 and 2023 were planted for the parent lines.

The main traits that were evaluated in this study included flowering date (FLD), maturity date (MAD), reproductive period (RP), total number of pods (PDTN), Lodging (LOD), Leaf senescence (LS), number of grains per pod (NGP), 100 seed weight (100GW) and total grain weight (GW) (Table 4.1**)**. Data analysis for the traits was done using the average value of each RIL and parent line over a four-year period.

4.2.2 DNA extraction

DNA was extracted from 113 recombinant inbred lines and 6 parent lines using CTAB DNA extraction method. Samples were collected from young and fully opened trifoliate leaves, then freeze- dried and ground in multi-species grinding machine (TissueLysser Qiagen Cat. No 85200: Retch; QIAGEN).

4.2.3 Marker development

The DNA markers, insertion and deletion (InDel), cleaved amplified polymorphic sequences (CAPS) and derived CAPS (dCAPS), were developed from re-sequence data of ‘T161’ and ‘Chihayahime’. Polymorphic markers between parents were used for genotyping of the RILs.

4.2.4 Genetic map construction

A linkage map was constructed from the genotypes of the RIL populations using JoinMap software 4.1 [46]. Chi-square test was used in calculating marker segregation ratios, markers with distorted

segregation (p<0.01) were excluded and the remaining 108 markers were used for constructing the genetic map. Logarithm of odds (LOD) score was used to determine the likelihood for presence of a linkage between selected marker(s) and a QTL. Marker loci with a significant likelihood ratio greater than LOD 1.0 was used create linkage groups for the genetic map and recombination frequencies were translated to genetic distances using the Kosambi mapping function [47]. The linkage map previously constructed and used for analyzing plant height traits by Kachapila et al (unpublished) was used in the analysis for yield-related traits.

4.2.5 QTL analysis

QTLs were determined by Interval Mapping (IM) and Multiple QTL Model (MQM) using MapQTL software version 6.0 [48]. A permutation test was run to get genome-wide LOD thresholds for each trait, at p<0.05 significance level based on 1000 times permutation score [66]. Significant markers associated with QTLs at a determined LOD threshold level for each trait were detected by IM analysis. The significant markers were then used as cofactors in the MQM analysis and markers with the highest LOD values at each associated QTL were considered optimal. Genetic information including marker position, percentage of phenotypic variation explained, additive effect direction and dominance effect of each QTL were obtained from the MapQTL analysis output. Naming of the identified QTLs was based on the nomenclature suggested by [67], using ‘q’ followed by the respective trait abbreviation then the chromosome number in Italic font.

4.2.6 QTL network analysis

To determine the causal relationship between detected QTLs for plant height and yield traits, QTL network analysis was performed using the ‘*qdg*’ function of *qtlnet* package in R statistical software

described by [34], http://www.stat.wisc.edu/~yandell/sysgen. Prior QTL information for the *qtlnet* analysis input was run in *R/qtl* package based on the *find.marker* and *makeqtl* functions [76].

4.3 Results

4.3.1 Phenotypic variation

The trait means, standard deviation of the mean, coefficient of variation, skewness and kurtosis of the parental and RIL populations (Table 4.2). A stable and earlier FLD and MAD was observed in 'Chihayahime' than 'T161'over the four-year study period. However, mean RP for 'Chihayahime' was 3 days longer than 'T161' in 2020 ($p<0.001$) unlike in the other years. The mean PDTN for 'Chihayahime was higher than 'T161' across the four years. The total grain weight (GW) was almost the same in 'Chihayahime' and 'T161' but differenced were observed in yield components such as 100GW.

Frequency distribution of all yield-related traits in the RILs showed continuous and relatively normal distribution except for LS and LOD (Figure S4.1). These results suggested that most of the yield-related traits are controlled by multiple genes. The means of all the RILs were within the parental line means except for PDTN in 2020 and GW in 2021, suggesting possible presence of transgressive segregation.

4.3.2 Phenotypic trait correlation

To analyze the genetic associations among the yield-related traits, a correlation analysis was conducted for the 113 RILs. Most combinations showed correlation for the yield-related traits over the four years period (Figure 4.2, Figure S4.1). Positive and significant correlations were observed between the traits; including: FLD*MAD ($r>0.6$); MAD*RP ($r>0.8$); PDTN*LOD ($r>0.6$); PDTN*GW ($r>0.8$); GW*MAD* RP ($r>0.4$-0.6); and GW*100GW ($r>0.4$ -0.6). However, negative relationship was observed in some traits including: - LS *FLD, MAD, RP, 100GW ($r>-0.4$), and FLD*RP ($r>-0.2$ to 0.2) in the study

period. Therefore, indicating possibility that some genetic factors for yield-related are traits are controlled by different QTLs.

4.3.3 Detection of QTLs for yield-related traits

The QTL analysis of the four years data for yield-related traits, detected a total of 36 QTLs covering 9 regions of the 11 linkage groups on the previously constructed genetic map (Kachapila et al., unpublished). The details of the detected genetic loci are as described below (Table 4.3, Figure 4.3). Six QTLs for FLD were detected on chromosomes 2, 4, 7, 8, 9 and 10. A stable QTL *qFLD2* was detected on chromosome 2 (4.4 – 14.2 LOD value, and 14.8– 33.9% PVE). Four QTLs *qFLD2, qFLD8, qFLD9* and *qFLD10* promoted FLD when RILs possessed the 'Chihayahime' type allele whereas the QTLs, *qFLD4 and qFLD7*, reduced flowering time when RILs possessed the 'T161' type allele. QTLs for MAD (*qMAD1, qMAD2, qMAD4, qMAD7* and *qMAD10*) were detected on chromosomes 1, 2, 4, 7 and 10 that accelerated maturity date when RILs possessed 'Chihayahime' type alleles. On Chromosome 4, two peaks were detected for MAD at AZ04_35M and AZ04_50.7M and the QTLs at the two loci had opposite effects. Five QTLs for reproductive period were detected (*qRP1, qRP2, qRP4, qRP5* and *qRP7*) on chromosomes 1, 2, 4, 5 and 7. On chromosome 4 *qRP4* two peaks were detected at similar marker positions to MAD and the 'Chihayahime' type allele reduced the reproductive period. Two QTLs for PDTN (*qPDTN4* and *qPDTN11*) were detected on chromosomes 4 and 11 an increased pod number when RILs possessed 'Chihayahime' type allele. The QTL *qPDTN4* was stable over three years with a major effect (LOD 3.5 – 5.2 and 13.3 – 19.1% PVE). NGP QTLs were detected on chromosomes 1, 2 and 4. On chromosome 1, *NGP1* was stable over two years increased number of grains with the 'Chihayahime' allele effect. Four 100GW QTLs were detected on chromosomes 1, 5, 7 and 9. The QTLs *q100GW1* and *q100GW5* were stable over two years period and had opposite effects with the 'T161' type allele increasing the trait value on chromosome 1, while the 'Chihayahime' type allele increased the trait value on chromosome 5, respectively. Three QTLS for GW were detected on chromosomes 2, 4 and 7. None of the QTLs were stable over the four years period,

however, *qGW4* had a major effect (LOD7.7 and 26.3% PVE) with Chihayahime allele effect for increasing total grain weight. Four QTLS were detected for LS on chromosomes 1, 3, 7 and 10. The QTL *qLS7* was stable over two years period and the 'T161' allele effect promoted leaf senescence in 2020 while the 'Chihayahime' allele effect promoted leaf senescence in 2022. Only one QTL was detected for LOD on chromosome 1 and the 'T161' allele effect increased the lodging susceptibility

4.3.4 Co-existence of yield-related traits QTLs on linkage groups

Important QTLs and clusters for yield-related traits were found on chromosomes 1, 2, 4, and 7 with details as follows. On chromosome 1, *qRP1*, *qLS1* and *q100GW1* formed a cluster on a region flanked by the markers AZ01_55M and AZ01_57.3M. The QTLs *qMAD1* and *qLOD1* close to marker AZ01_5.2M were also co-located on chromosome 1. On chromosome 2, a cluster of *qFLD2* and *qRP2* and *qGW2* was detected flanked by markers AZ02_4.0M and AZ02_5.3M. On chromosome 4, a cluster of *qFLD4, qPDTN4* and *qRP4.2* was detected on the region flanked by markers AZ04_48.3M and AZ04_50.7M*,* while *qMAD4* and *qRP4.1* were co-located on the same marker AZ04_35.7M. On chromosome 7, *qFLD7*, *qRP7*, *q100GW7*, *qGW7* and *qLS7* formed a cluster on a region flanked by the markers AZ07_10M and AZ07_25.1M. The presence of clusters therefore signals co-existence of QTLs controlling the detected associations of yield-related traits.

4.3.5 QTL interactions

In order to assess the interaction between detected QTLs, a two-way ANOVA was done for the QTLs MAD (*qMAD1* and *qMAD4*), FLD (*qFLD2* and *qFLD4*) and 100GW (*q100GW1* and *q100GW5*) (Table 4.4 – 4.11). For flowering time had significant interaction only in 2021 (p<0.019) and not in the other years (p = 0.687in 2020, p = 959 in 2022). For maturity time, no significant interaction was noted over the three-year evaluation period (p<0.250, p<0,822 and p<0.483). Between the 100 grain weight QTLs

significant interaction was observed in 2021 (p<0.011) and not in 2022 (p<0.067). This ANOVA results suggest that FLD, MAD and 100GW QTLs have an independent additive effect on traits for yield and maturity in adzuki bean.

4.3.6 QTL networks

This study evaluated causal inferences of QTL networks for 11 agronomic traits using QDG method. Genetic interactions were observed between two or more QTLs and traits. The analysis findings showed a direct graph (DG) for plant architecture and yield built with either traits or QTLs presented as nodes and corresponding interactions presented as edges (Figure 4.4). The strength of the identified edges was estimated using LOD scores. A total of 16 QTLs with an impact on 11 plant architecture and yield traits were initially identified. Further association analysis indicated 3 discrete networks, related to stem elongation and productivity (yield); seed weight and seed quantity.

Stem length and yield was the major node comprising of plant height (PH), upper stem length (STIL8), node number, (NN), epicotyl length (ECL), lower stem length (STIL7), flowering (FLD), maturity (MAD), pod number (PDTN) and total grain weight (GW) traits. The other 2 nodes comprised one trait each thus 100 seed weight (100GW) in seed weight and grain number per pod (NGP) in seed quantity, respectively. Notably, the network outline shows that most traits are closely associated particularly stem elongation traits (PH, STIL8, NN, ECL and STIL7) and yield traits (FLD, MAD, PDTN and GW). However, 2 subgroups for NGP and 100GW are disjointed from the rest. Surprisingly, contrary to our expectation the loci for the two seed weight traits 100GW and GW were not connected. The PH and ECL traits could be considered as accelerator/facilitator nodes, based on the understanding that both nodes had direct and indirect interactions with traits in other subgroupings of the QTL network.

According to Feng et al.,[77], an interaction of four or more loci or traits is defined as a hub. The study findings indicate that the QTL node 198_AZ07_10M and 96_AZ04_50.7M are likely important hubs

for plant architecture and yield traits since the two QTLs were connected with at least four edges. The QTL node 198_AZ07_10M had direct effects on ECL, STIL7, NN which indirectly affected PH. The QTL node 96_AZ04_50.7M had direct effects on the traits PH, GW, MAD and MAD that had indirect effects on FLD. QTL node 32_AZ04_48.4M directly affected STIL8 trait that had indirect effect on PH and NN traits. QTL node 62_AZ04_4.0M showed direct effect on FLD and ECL traits and indirectly affected STIL7 that had edges towards PH and NN.

The QTL network findings revealed pleiotropic QTLs that had an effect on more than a single trait such as 198_AZ07_10M,96_AZ04_50.7M, 62_AZ02_4.0M. All yield traits except FLD, as well as two plant height traits ECL and STIL8 were observed to be controlled by more than one QTL indicating polygenic effect nature

4.4 Discussion

4.4.1 Comparative position analysis of detected QTLs with previous reports

To determine the uniqueness and usefulness of the QTLs found in this study, a comparative analysis based on physical positions was done on QTLs detected in this study from previous reports by [7,11,21] The results showed that QTLs found in this study differed from previous reports except three QTLs:-*qFLD4* detected on AZ04_48.3M in this study and *Fld2.4.1*; *Fld3.4a.1* detected on 48.7M [7,21]; *q100GW1* on 57.3M compared to 55.2 to 57.6M [7,21]; *q100GW5* on 12.4 to 15.6M compared to 11.3M [7]. Therefore, all QTLs except *qFLD4*, *q100GW1* and *q100GW5* are considered unique since there has been no similar reports previously or exist on different positions with the reported QTLs.

4.4.2 Distribution on QTLs for yield related traits

Yield is a complicated trait that is affected by many QTLs and factors [78]. The mapping of major effect QTLs and relationship between yield traits is therefore important for the understanding of underlying control mechanisms and application for improving crop productivity. Important QTLs were identified in this for the 6 to 9 yield-related traits over a three-year study period. As expected, a high correlation was observed between flowering and maturity traits in line with most reports on the similar genetic basis for flowering and maturity. High correlation was also observed between maturity and reproductive period; pod number and lodging; maturity and grain weight; and between grain weight traits. The high correlations observed therefore suggests that the traits are likely governed by the same QTLs. The results also showed negative correlations and weak correlations between some traits particularly leaf senescence and flowering, maturity and reproductive period traits as well as between flowering and reproductive period. There was a high variation of the degree of leaf fall in the RILs with some lines having the poorest and highest leaf fall (0-5 scores), which may have contributed to the low correlations. Additionally, since yield traits are affected by several factors including environmental factors, the temperature variations over the 3-year trial period may have contributed to variations in the flowering and reproductive periods.

A collective localization of yield trait QTLs was observed in the study particularly on linkage groups 1, 2, 4 and 7. The results showed co-localization for major effect QTLs including; -flowering, pod number and grain weight within 48.3 – 50.7M on chromosome 4; and the alleles from 'Chihayahime' promoted pod number and grain weight. Flowering, reproductive period, 100 grain weight, total grain weight and leaf senescence were within 10 – 25.1M on chromosome 7 and the 'Chihayahime' allelic effect promoted grain weight traits. Regardless of the negative correlation, leaf senescence and 100 grain weight as well as reproductive period traits were co-located within the physical distance 55 – 57.3M on chromosome 1. Flowering and reproductive period were also co-located within 4.0 – 5.3M on chromosome 2. Suggesting the traits are possibly other factors besides the genetic mechanisms influence the traits. The co-localization of QTLs reflects presence of more polymorphic markers [78], possibly a similar scenario

applied for chromosomes 1, 2, 4 and 7 in this study population. The information on the frequency and distribution of QTL regions, generated in the study, maybe useful as potential basis for further research and genetic effect combinations in adzuki bean breeding.

4.4.3 Different control mechanism for flowering and maturity

A correlation analysis was performed to ascertain the relationship between flowering and maturity times in the RILs. Despite the presence of a significant positive correlation *(r*≥0.6) between flowering and maturity times, there was a wide variation in the traits and transgressive segregation was observed in the RILs since some individual lines had earlier or later flowering and maturing times.

This study detected important QTLs for flowering on chromosome 2, flowering and maturity on chromosome 4. In Soybean flowering and maturity has been reported to be controlled by same genes, for instance *E* loci that regulates flowering and maturity [79]. Previously, the co-localization of flowering and maturity QTLs in adzuki bean was also reported [2,7]. Unlike the previous reports, in this study the QTLs *qFLD4* and *qMAD4* was mapped on different locations (48.3 - 50.7 Mb and 35.7 Mb respectively) of chromosome 4. The findings in this study therefore suggest that flowering and maturity time is likely to be controlled by different genetic loci or genes in this population. This presents an opportunity for the independently controlling flowering and maturity in adzuki bean breeding programs.

4.4.4 QTL network

The mechanisms for plant growth are normally associated with architecture and yield [38]. For sustainable agricultural production, it is important to analyze the underlying genetic interactions that regulate plant growth and its potential effects on phenotypic expression of useful traits. Although QTL mapping helps in uncovering the genetic mechanisms of particular loci, an understanding of the causal

genetic interactions amongst the loci is more meaningful. This study analyzed QTL networks to clarify the genotype and phenotype interactions for plant height traits that could be used in the selection of ideal plant architecture and yield traits in adzuki bean. QTL data from RILs developed from 'Toiku161' and 'Chihayahime' parental lines was used in the QTL network analysis.

In total 16 QTLs with a genotypic impact on 11 phenotypic traits related to plant height and yield were found. The identified QTLs formed a causal network of phenotypes that differentiated the direct and indirect QTL networks contribution to plant height and yield traits in the adzuki bean population. The QDG approach used in this study effectively identified the QTL network outline illustrating the direct and indirect effects on associated plant height and yield traits in adzuki bean.

The findings in this study presented 3 isolated networks existing in the study population, that mainly covered the stem elongation and yield; seed weight and seed quantity traits for related to plant height and yield in adzuki bean. Based on the literature search, no studies have been reported on QTL networks in adzuki bean. However, some studies have been done in other crops, including mapping for causal genes and genetic interactions for plant height, heading date and grain yield in F_2 rice population [77], and gene expression network reconstruction for eQTL analysis for genomic architecture and plant development in *Brassica rapa* RILs [38,77]. Complex genetic interactions between QTLs were identified and suggested that arrangement of the genetic interactions may be helpful towards precise predictions of useful phenotypic expressions in rice breeding [77]. It is considered that plant height could be used as an estimation for yield and the growing period of plants significantly determines the final plant height unlike the growth rate [38]. In this study, pleiotropic QTLs and polygenic trait interactions were detected, possibly suggesting that multiple traits within the network are controlled by similar genetic loci in adzuki bean. These findings could help in the understanding of plant height and yield relationships that maybe useful in the determination of potential interactions for development of adzuki bean genotypes with ideal plant architecture and yield.

The presence of hub QTLs in the plant height and yield traits network indicate a possible pleiotropic gene regulation, and most of the associated traits were polygenic in this study. It is considered that most

network studies rarely find polygenic trait regulation as compared to basic QTL studies [38]. There was a point of departure in this study findings from Baker et al., [38], as a number of traits were found to be polygenic in nature. However, most QTLs in network analysis are usually of relatively large effect, hence, some small effect QTLs may not be detectable contributing to the polygenic gene expression [38,77].

Two prominent QTL nodes in the network were 198_AZ07_10M and 96_AZO4_50.7M. These nodes had edges towards at least four traits each, out of the total 11 traits. Given the high clustering on the nodes, indirect connections also existed in QTL node interactions. The indirect connections included PH trait on 198_AZ04_10M and FLD trait on 96_AZ04_50.7M. A notable indirect effect also existed for PH via the route 32_AZ04_48.3M to STIL8 to PH. The study findings confirm pleiotropic effect for plant height and yield traits based on the existence of extensive direct and indirect effects detected in the QTL network. In a study of causal phenotypic networks for egg traits in poultry [80], defined a collider node as one having both direct and indirect trait effects from different subgroups connecting to the node. In this study, PH was considered a collider trait that connected the subgroups for yield-related traits and plant height-related traits. On the node 96_AZ04_50.7M, the 'Chihayahime' type allele was favourable for improved PDTN, GW and Maturity explaining a maximum PVE of 17.1%, 26.3% and 12.8% respectively. On the node 198_AZ07_10M, the 'T161' was favourable for increasing ECL and STIL7 explaining a maximum PVE of 41.3% and 52.5%, respectively. The information presented here is of significant use in molecular assisted breeding for improved mechanization efficiency and yield levels by utilizing the alleles conferring desirable traits for plant height and yield on Chromosomes 4 and 7.

4.5 Summary

In this study, 36 QTLs for yield related traits were detected and it is envisaged that analysis of the identified genomic regions will be of great interest for in breeding for improved maturity and other associated yield traits using molecular markers in adzuki beans. An independent control mechanism was detected for flowering and maturity time traits. This is therefore, an opportunity for effective separation of the two traits on chromosome 4 by fixing the flowering time but improving the maturity time to minimize adverse cold weather effect and at the same time promoting pod number and grain weight to improve productivity in adzuki bean. Lodging and Leaf senescence QTLS identified on chromosome 1 and 7 respectively are important for improving lodging susceptibility and degree of leaf fall by utilizing the beneficial alleles from 'Chihayahime' parent line. There was no significant interaction between QTLs for flowering and maturity, denoting existence of an independent additive effect. Two QTL hubs AZ04_50.7M and AZ07_10M were identified that are considered to be useful in mapping the underlying factors for the regulation of plant height and yield traits, therefore could be ideal for breeding adzuki bean genotypes with improved architecture and productivity.

Table 4-1: Yield-related traits evaluation

Trait	Abbreviation	Evaluation method
Flowering date	FLD	Number of days from planting to 1st flowering
Maturity date	MAD	Number of days from planting to 80% pod maturity
Reproductive period	RP	Difference between maturity date and flowering date
Pod number	PDTN	Total number of harvested pods per plant
Lodging	LOD	Degree of plant fall on a 5- point scale
Leaf Senescence	LS	Degree of leaf fall on a 5- point scale
100 grain weight	100GW	Weight of 100 seeds
Grain weight	GW	Total weight of harvested seeds
Grain number	NGP	Number of grains per pod

Table 4-2: Descriptive statistics for yield related traits

Trait			Parent line			RILs[a]					
		Year	T161	Chihayahime	Difference	Min	Max	Average	CV(%)	Kurtosis	Skewness
FLD	(days)	2020	67.8±0.3	62.5±0.2	5.3***	60.6	71.2	63.7±2.1	3.3	0.9	0.9
		2021	62.0±1.3	58.1±0.4	3.9***	57.1	68.5	60.2±2.5	4.2	0.8	1.1
		2022	62.6±0.6	61.2±1.6	1.4*	57.1	65.4	61.2±1.5	2.5	0.1	-0.3
		2023	55.8±1.1	53.36±1.1	2.5**	50.4	59.4	54.1±1.7	3.1	0.5	0.5
MAD	(days)	2020	111.4±1.2	109.2±0.4	2.2***	109.0	120.0	111±2.3	2.1	2.5	1.7
		2021	128.2±1.1	121.8±0.8	6.4***	114.0	139.0	125.6±4.5	3.6	0.6	0.3
		2022	111.4±0.9	108.2±1.1	3.2***	105.0	121.0	109.9±3.8	3.5	0.9	1.1
		2023	102.8±2.0	100.8±0.8	2*	97.0	107.0	101.2±2.5	2.5	-0.8	0.2
RP	(days)	2020	45.2±2.2	48.2±1.3	3.0***	42.0	61.5	48.2±2.8	5.8	3.3	1.1
		2021	67.0±1.8	65.9±1.8	1.1^{ns}	53.2	75.0	66.1±3.8	5.7	1.2	-0.3
		2022	49.1±1.9	47.6±1.1	1.5^{ns}	43.8	53.4	48.3±2.4	5.0	-0.8	0.1
		2023	47.0±2.7	47.4±1.6	0.4^{ns}	42.0	52.8	47.0±2.2	4.7	-0.2	0.03
PDTN	(no.per plant)	2020	30.1±5.4	32.2±3.8	2.1^{ns}	19	56	34.5±7.2	20.9	0.1	0.5
		2021	43.9±2.7	53.0±2.7	9.1***	26.0	77.7	51.6±9.9	19.2	0.0	0.5
		2022	46.3±7.1	58.7±8.6	12.4*	31.6	78.6	51.7±7.8	15.1	0.6	0.2
		2023	34.4±6.1	42.1±5.3	7.7^{ns}	24.6	57.5	40.8±6.5	15.9	-01	0.3
LOD	(score of lodged plants)	2020	0.3±0.4	0.2±0.3	0.1^{ns}	0.0	2.0	0.2±0.4	200	5.4	2.1
		2021	1.6±0.5	0.1±0.2	1.5***	0.0	3.0	0.5±0.8	160	1.7	1.6
		2022	2.8±0.4	1.5±0.7	1.3***	0.0	3.0	1.3±1.1	84.6	-1.3	0.3
LS	(score of leaf fall)	2020	4.4±0.6	3.4±0.5	1***	1.0	4.0	2.1±0.7	33.3	0.1	0.4
		2021	2.4±0.5	5.0±0	2.6***	2.0	5.0	3.3±1.1	33.3	-1.3	0.1
		2022	3.0±0.0	1.6±0.5	1.4***	1.0	4.0	2.0±1.0	50.0	-1.0	0.5
100GW	(g)	2021	18.1±0.6	16.1±0.4	2.0***	13.4	21.0	16.4±1.6	9.8	0.1	0.6
		2022	15.1±0.6	13.1±0.5	2.0***	0.0	18.5	14.0±1.9	13.6	22.3	-3.0
		2023	13.0±0.4	13.1±0.6	0.1^{ns}	10.4	18.1	13.1±1.1	8.4	2.2	0.6
GW	(g)	2021	36.5±5.4	33.8±6.9	2.7^{ns}	18.1	64.2	41.2±9.7	23.5	-0.2	0.2
		2022	35.2±3.6	37.1±3.9	1.9^{ns}	18.0	62.1	34.9±7.7	22.1	0.7	0.4
		2023	24.0±4.0	30.3±4.0	6.3	19.2	37.3	30.0±3.6	12.0	-0.1	-0.2
NGP	(no.per pod)	2022	5.8±0.5	6.0±0.3	0.2^{ns}	4.6	7.6	5.9±0.6	10.2	-0.01	0.33
		2023	5.6±0.3	5.7±0.3	0.1^{ns}	4.4	7.4	5.9±0.6	10.2	-0.3	-0.01

[a:] Recombinant inbred lines, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant

Table 4-3: Detected yield-related traits QTLs in T161 x Chihayahime RILs population

Trait name	QTL[a]	year	Chr[b]	Nearest Marker	Peak position	LOD[c]	T161[d]	CH[e]	Add[f]	PVE%[g]
Flowering date	*qFLD2*	2020	2	AZ02_5.3M	22.9	4.44	65.0	63.0	0.9	14.8
	qFLD10		10	AZ10_0.05M	0.0	2.96	64.6	63.3	0.7	9.6
	qFLD2.2	2021	2	AZ02_4.0M	19.9	11.88	62.8	59.2	1.8	33.9
	qFLD4		4	AZ04_48.3M	69.2	6.73	59.7	62.2	-1.3	17.2
	qFLD8		8	AZ08_12M	40.1	3.46	61.6	60.0	0.8	8.8
	qFLD2	2022	2	AZ02_4.0M	19.9	14.23	63.0	59.3	1.9	32.2
	qFLD4.2		4	AZ04_50.7M	70.2	9.52	59.6	62.4	-1.4	20.5
	qFLD7		7	AZ07_10.0M	49.5	2.90	60.4	61.6	0.6	5.6
	qFLD9		9	AZ09_15.1M	28.0	3.12	61.7	60.3	0.7	6.0
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Maturity date	*qMAD4*	2020	4	AZ04_35.7M	50.1	3.37	111.7	110.0	0.8	12.8
	qMAD4	2021	4	AZ04_35.7M	50.1	3.22	126.9	125.3	1.6	12.3
	qMAD1	2022	1	AZ01_5.2M	20.0	3.66	110.9	109.7	1.2	9.8
	qMAD4.2		4	AZ04_50.7M	72.3	8.17	108.3	110.1	-1.8	20.0
	qMAD2	2023	2	AZ02_4.0M	20.9	5.42	103.5	100.8	1.3	15.5
	qMAD4		4	AZ04_50.7M	71.2	8.14	100.4	103.5	-1.5	25.3
Reproductive period	*qRP4*	2020	4	AZ04_35.7M	50.1	2.71	49.0	47.1	0.9	10.5
	qRP5		5	AZ05_37.0M	90.0	2.97	49.1	47.1	1.0	10.3
	qRP2	2021	2	AZ02_5.3M	21.9	6.34	62.8	67.2	-2.1	21.8
	qRP4.2		4	AZ04_48.3M	62.6	3.53	66.5	63.2	1.7	11.5
	qRP1	2022	1	AZ01_55.0M	109.7	3.01	48.7	47.2	0.8	7.6
	qRP4		4	AZ04_35.7M	52.1	2.94	48.8	47.2	0.7	8.4
	qRP7		7	AZ07_25.1M	58.5	3.42	47.2	48.9	-0.9	9.9
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Pod number	*qPDTN4*	2021	4	AZ04_50.7M	72.3	3.51	48.9	56.5	-0.4	13.3
	qPDTN4	2022	4	AZ04_50.7M	75.3	4.60	49.2	56.7	-3.7	17.1
	qPDTN11		11	AZ11_10M	17.1	3.59	48.3	52.9	0.8	13.6
	qPDTN4	2023	4	AZ04_50.7M	74.3	5.20	38.6	45.1	-3.2	19.1
Grain number per pod	*qNGP1*	2022	1	AZ01_55.0M	108.7	3.28	5.8	6.3	-0.2	10.9
	qNGP2		2	AZ02_5.3M	28.8	3.40	6.3	5.8	0.3	11.4
	qNGP1	2023	1	AZ01_55.0M	108.7	6.36	5.6	6.2	-0.3	19.1
	qNGP4		4	AZ04_53.1M	88.5	2.85	6.1	5.7	0.2	8.1
100 grain weight	*q100GWg1*	2021	1	AZ01_57.3M	121.2	5.39	17.3	15.5	0.9	18.1
	q100GWg5		5	AZ05_12.4M	59.8	4.10	15.7	17.0	-0.6	13.5
	q100GWg1	2022	1	AZ01_57.3M	116.5	9.74	14.6	13.1	0.8	23.2
	q100GWg5.2		5	AZ05_15.6M	61.8	5.39	13.3	14.4	-0.6	11.7
	q100GWg7		7	AZ07_13.1M	55.5	3.71	13.4	14.3	-0.5	7.8

Trait name	QTL[a]	year	Chr[b]	Nearest Marker	Peak position	LOD[c]	T161[d]	CH[e]	Add[f]	PVE%[g]
	q100GWg9		9	AZ09_32.8	49.0	4.91	14.4	13.4	0.5	10.7
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Grain weight	*qGWg2*	2021	2	AZ02_5.3M	23.9	3.80	48.6	40.3	4.2	11.9
	qGWg4		4	AZ04_50.7M	72.3	7.72	38.8	50.1	-5.6	26.3
	qGWg7	2022	7	AZ07_25.1M	59.5	3.30	188.1	223.2	-17.6	12.6
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Leaf senesence	*qLS3*	2020	3	AZ03_38M	51.0	3.90	1.9	2.5	-0.3	13.0
	qLS7		7	AZ07_25.1M	59.5	4.19	2.5	1.9	0.3	14.2
	qLS10	2021	10	AZ10_23.1M	67.2	5.31	2.8	3.8	-0.5	19.4
	qLS1	2022	1	AZ01_55M	113.5	4.01	2.2	1.5	0.4	12.0
	qLS7		7	AZ07_25.1M	59.5	3.03	1.6	2.3	-0.4	10.4
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Lodging	*qLOD1*	2022	1	AZ01_5.2M	15.0	3.34	1.8	0.9	0.5	12.7
	n.d	2023	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

[a] QTL: Quantitative trait loci; [b] LG: Linkage group; [c] LOD: Logarithm of odds; [d] T161: T161 type allele; [e] CH: Chihayahime type allele; [f] Add: Additive effect; [g] PVE%: Phenotypic variation explained in percentage, n.d: not determined.

Table 4-4: Interaction for flowering QTLs in 2020

Cases	Sum of Squares	df	Mean Square	F	p
qFLD2	52.812	1	52.812	14.613	< .001
qFLD4	13.424	1	13.424	3.714	0.057
qFLD2 ✻ *qFLD4*	0.589	1	0.589	0.163	0.687
Residuals	386.702	107	3.614		

Table 4-5: Interaction for flowering QTLs in 2021

Cases	Sum of Squares	df	Mean Square	F	p
qFLD2	198.230	1	198.230	52.390	< .001
qFLD4	97.866	1	97.866	25.865	< .001
qFLD2 ✻ *qFLD4*	21.457	1	21.457	5.671	0.019
Residuals	404.857	107	3.784		

Table 4-6: Interaction for flowering QTLs in 2022

Cases	Sum of Squares	df	Mean Square	F	p
qFLD2	34.143	1	34.143	23.425	< .001
qFLD4	25.109	1	25.109	17.227	< .001
qFLD2 ✻ *qFLD4*	0.004	1	0.004	0.003	0.959
Residuals	155.958	107	1.458		

Table 4-7: Interaction for maturity QTLs in 2020

Cases	Sum of Squares	df	Mean Square	F	p
qMAD1	24.425	1	24.425	5.394	0.022
qMAD4	78.339	1	78.339	17.300	< .001
qMAD1 ✻ *qMAD4*	6.067	1	6.067	1.340	0.250
Residuals	484.519	107	4.528		

Table 4-8: Interaction for maturity QTLs in 2021

Cases	Sum of Squares	df	Mean Square	F	p
qMAD1	22.142	1	22.142	1.309	0.255
qMAD4	251.902	1	251.902	14.896	< 0.001
qMAD1 ✻ *qMAD4*	0.863	1	0.863	0.051	0.822
Residuals	1809.393	107	16.910		

Table 4-9: Interaction for flowering QTLs in 2022

Cases	Sum of Squares	df	Mean Square	F	p
qMAD1	148.917	1	148.917	14.118	< 0.001
qMAD4	248.101	1	248.101	23.520	<0.001
qMAD1 ✻ *qMAD4*	5.228	1	5.228	0.496	0.483
Residuals	1128.669	107	10.548		

Table 4-10: Interaction for 100 grain weight QTLs in 2021

Cases	Sum of Squares	df	Mean Square	F	p
q100GW1	38.64	1	38.64	21.004	<0.001
q100GW5	34.57	1	34.57	18.792	<0.001
q100GW1 ✻ *q100GW5*	12.27	1	12.27	6.671	0.011
Residuals	189.46	103	1.84		

Table 4-11: Interaction for 100 grain weight QTLs in 2022

Cases	Sum of Squares	df	Mean Square	F	p
q100GW1	61.4	1	61.39	19.562	<0.001
q100GW5	20.0	1	20.03	6.383	0.013
q100GW1 ✻ *q100GW5*	10.8	1	10.79	3.437	0.067
Residuals	323.2	103	3.14		

(a)

(b)

Figure 4-1 (a) and (b): Parent lines ‘Toiku161 and ‘Chihayahime’ at flowering and maturity stages growing in the field at Tokachi agricultural research station

Note: Parent lines at flowering stage (95 days after planting) and maturity stage (135 days after planting), in the field at Tokachi agricultural research station. On the left side is ‘Toiku161’ exhibiting late flowering and maturity, on the right side is ‘Chihayahime’ exhibiting earlier flowering and maturity.

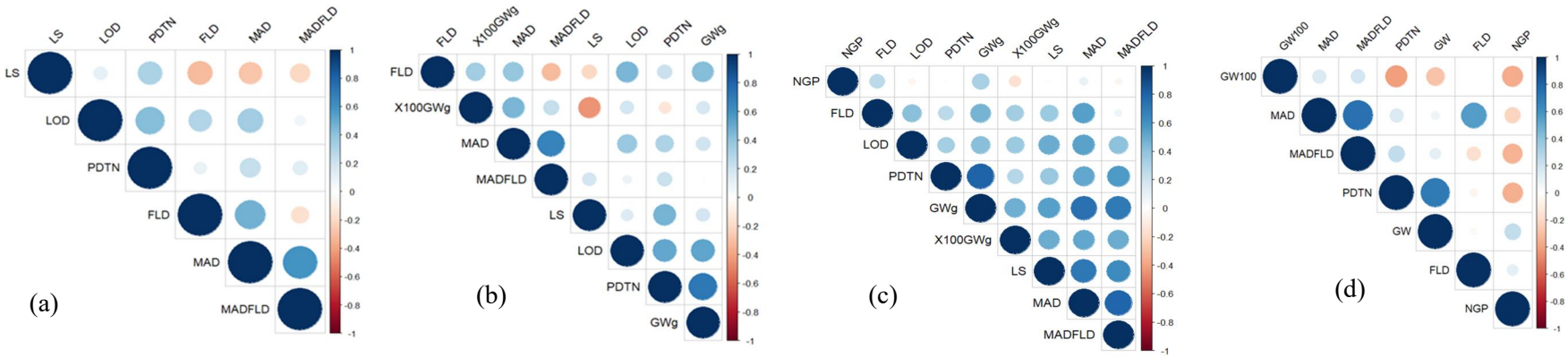


Figure 4-2: Correlation analysis for yield -related traits in (a), (b), (c) and (d) for the years 2020, 2021, 2022 and 2023, respectively

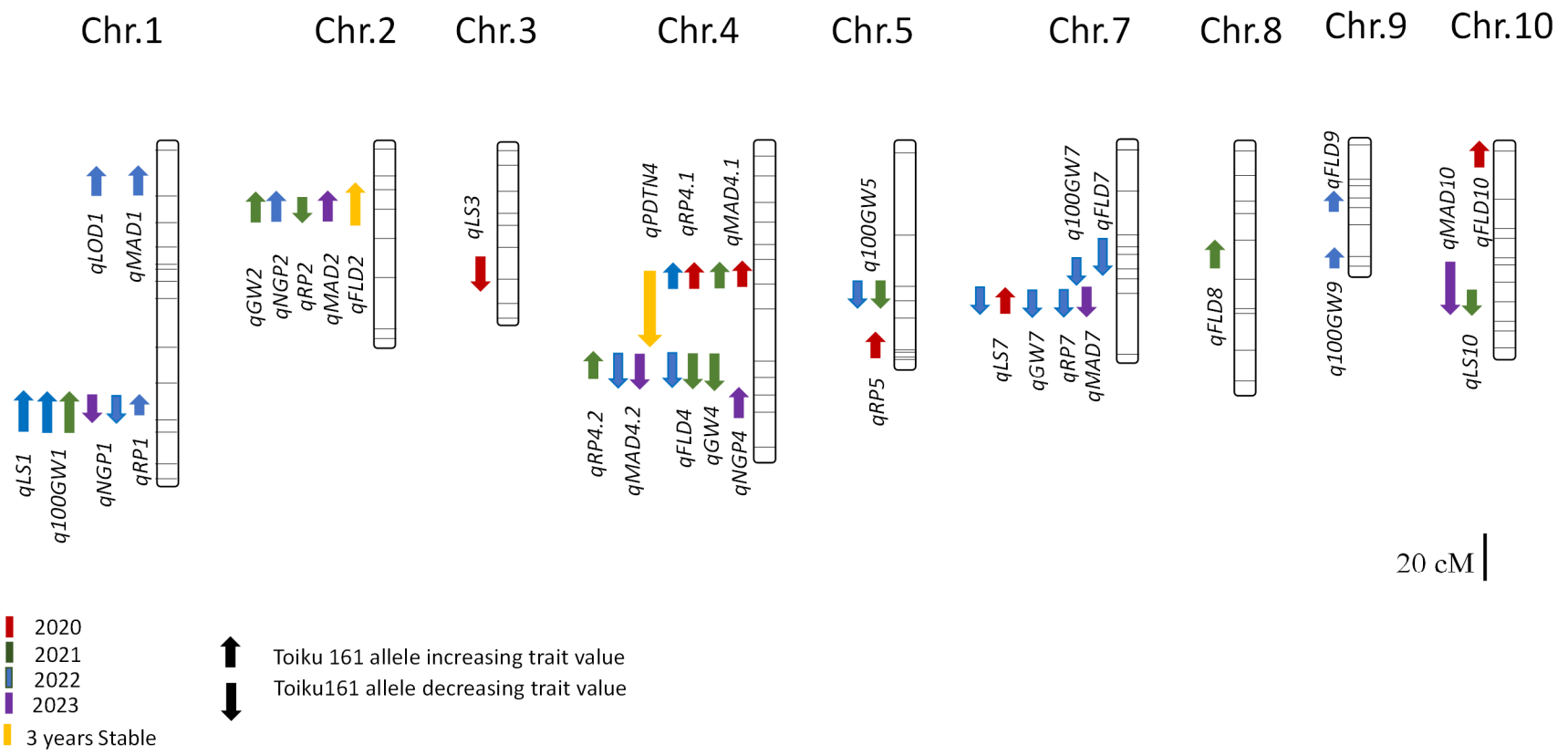


Figure 4-3: QTL mapping for yield-related traits on the constructed linkage map

Note: Mapping for the detected yield-related trait QTLs in 113 RILs developed from 'T161' x 'Chihayahime' cross. Red colour indicates QTLs detected in 2020, green colour indicates QTLs detected in 2021, blue colour indicates QTLs detected in 2022, purple colour indicates QTLs detected in 2023 and yellow colour indicates Stable QTLS in the three-year period. Black upward arrow indicates an increasing effect from Toiku161 allele, and black downward arrow indicates a decreasing effect from Toiku161 allele.

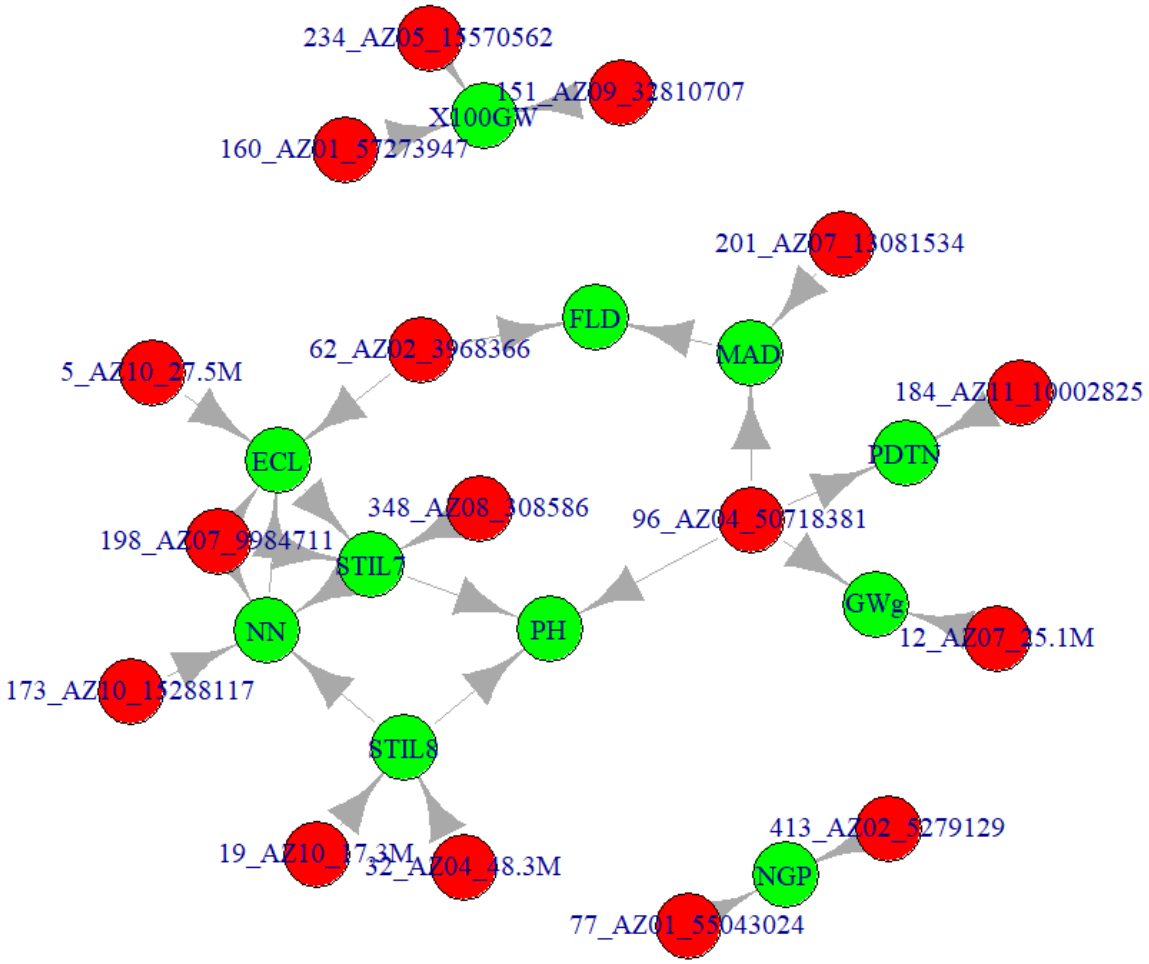
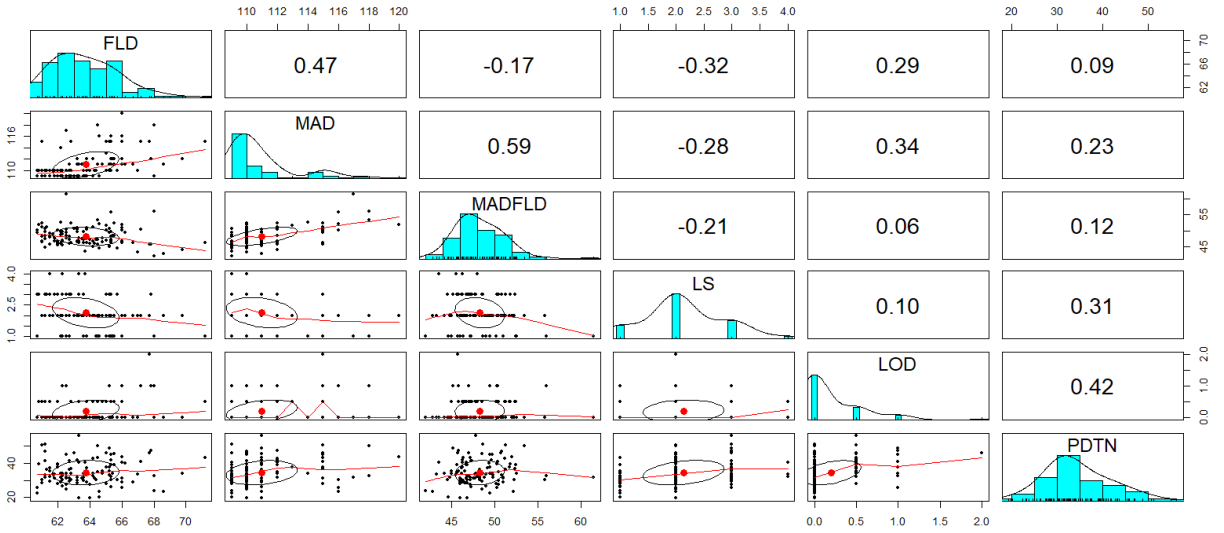


Figure 4-4: QTL network analysis for plant height and yield traits

Note: QTL network analysis for plant height and yield traits. QTL nodes are shown in red and traits in green, respectively.

(a)



(b)

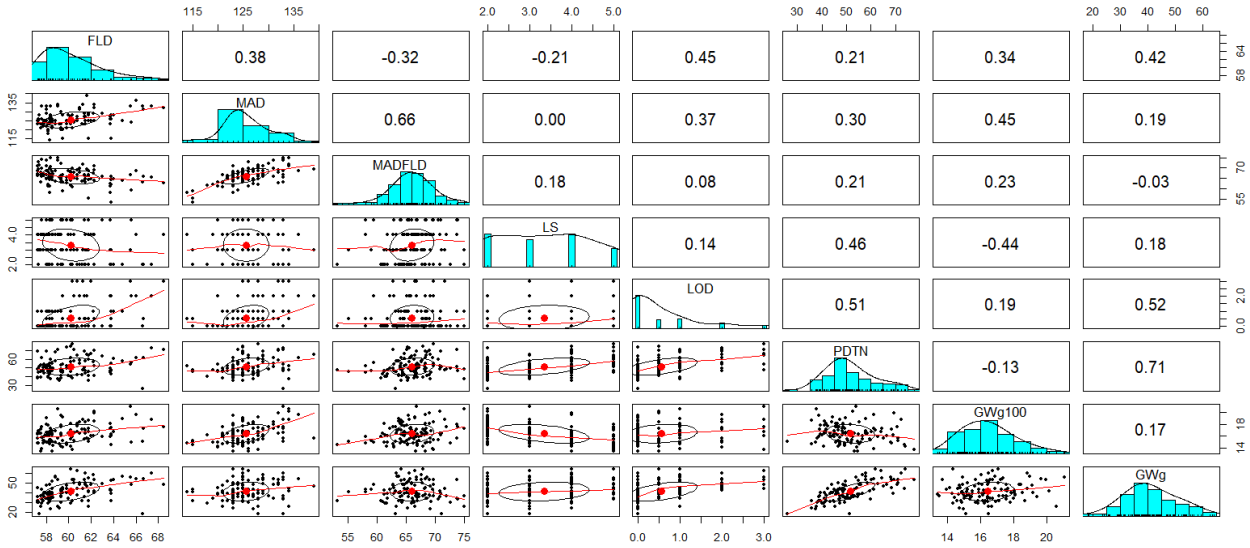
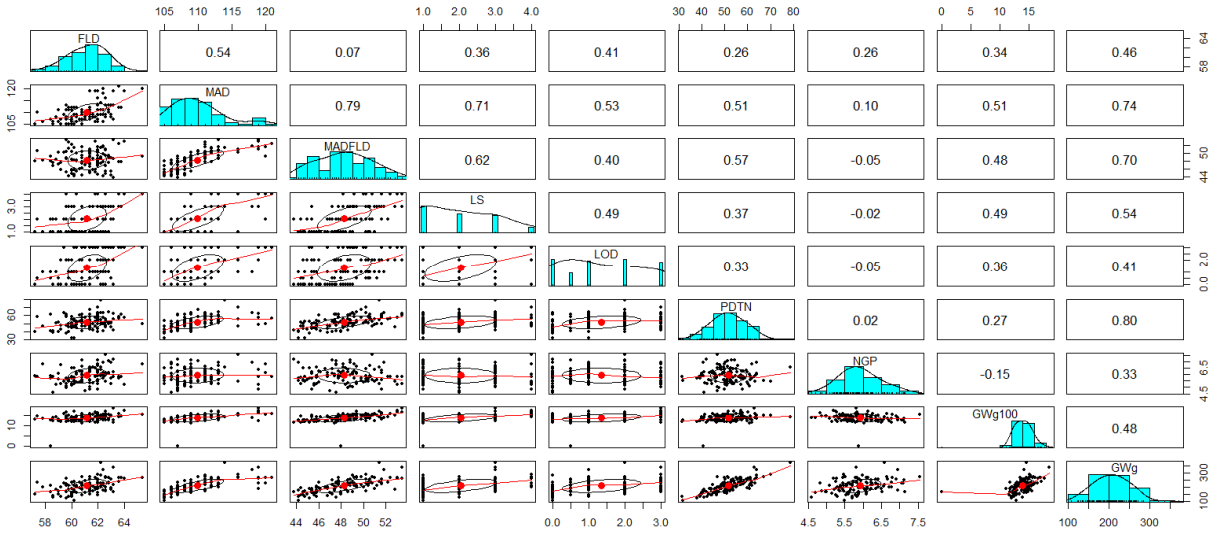


Figure S 4. 1: Phenotypic variation of yield-related traits (a) and (b)

(c)



(d)

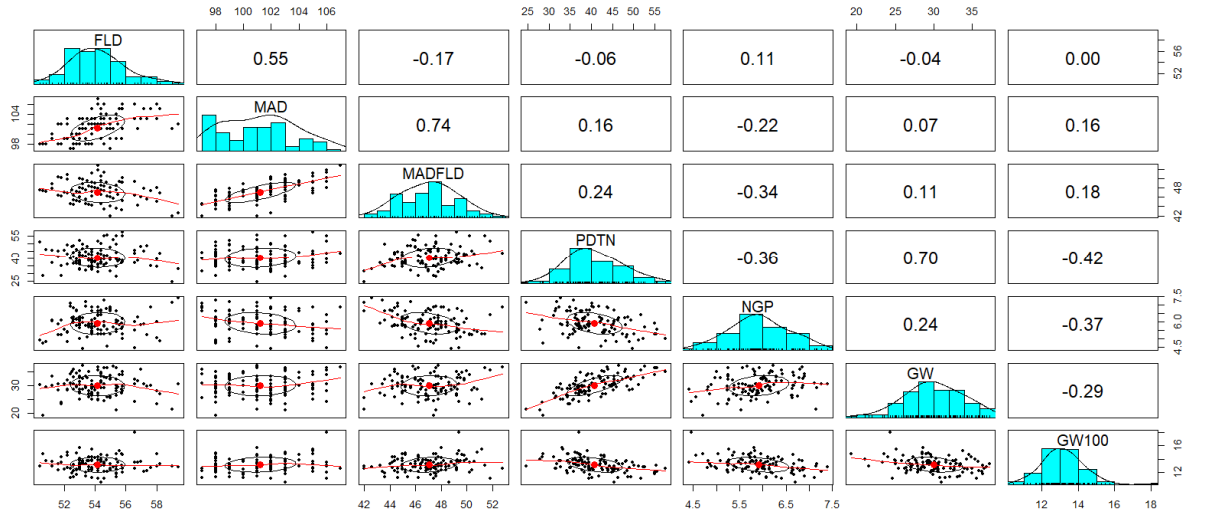


Figure S 4. 2: Phenotypic variation of yield-related traits (c) and (d)

Note: Phenotypic distribution of FLD, MAD, MADFLD (RP), LS, LOD, PDTN, NGP, 100GW and GW. Pairwise trait correlations across 113 RILs 2020 (a), 2021 (b), 2022 (c), and 2023 (d), respectively. The RILs differed phenotypically and displayed high relationships between FLD and MAD, GW and PDTN, MAD, MADFLD (RP) across the years. Positive and negative numbers indicate the Pearson correlation coefficient (*r*).

CHAPTER 5: GENERAL DISCUSSION

5.1 Relationship between plant height and yield-related traits

Generally, plant height traits have significant correlations with yield-related traits such as flowering and maturity [6]. The relationships between key agronomic traits such as architecture and yield, contribute to adaptability and productivity in soybean [60]. Although co-existence of plant height and yield -related traits is well-known, in some cases QTL mapping indicate that genetic loci or genes responsible for the architectural and yield traits also co-exist besides the trait correlation [78]. In this study, the QTLs for flowering and maturity were correlated but on different chromosomes or positions of the same chromosome. Most studies report high correlation and co-existence of flowering and maturity [7]. In adzuki bean and [81]**.** In soybean. On the other hand, [82] used a population crossed between cultivated *G. Max* and wild specie *G. soja*, and reported a non-pleiotropic locus for flowering and maturity in soybean. The authors suggested that it is possible to genetically separate non-pleiotropic effect in flowering and maturity traits. This is useful information for understanding the study finding, A similar however it is necessary to explore further if similar control mechanisms exist in adzuki bean. Hopefully, the trait correlations and clusters (co-existence) identified herein will be of significance in marker-assisted breeding for yield and architecture traits improvement.

This study found no co-existence between plant height and maturity on chromosomes 2, 4 and 8, indicating that the traits maybe controlled by different genetic loci and associated genes. However, co-existence was found between plant height, node number, pod number and total grain weight on chromosome 4 with alleles from ‘Chihayahime’ parent line increasing the trait values, suggesting control of the traits by the same genetic mechanism.

The genetic loci and genes associated with flowering time and stem growth habit mostly possess pleotropic effects on other traits [61]. It was necessary to explore the relationship of yield and plant architecture traits, which was done by QTL network analysis using a QDG method in this study. The

network analysis identified 16 QTL nodes with an effect on 11 plant height and yield traits. Further, the results discovered two QTL hubs with pleiotropic effect and polygenic traits. In a nutshell, the QDG analysis in this study, will be helpful in the comprehensive dissection of the genetic architecture on plant height and yield traits therefore contributing to the possible identification of causal relationships underlying genetic loci for plant height and yield that may facilitate the development of adzuki bean genotypes with ideal plant architecture and yield.

5.2 Significance of detected QTLs in breeding

In Hokkaido, adzuki bean in the past was commonly harvested in a two-stage process by using a cutting then pick-up machine [12]. Therefore, most cultivars and breeding lines in Hokkaido have short epicotyls and low hanging pods below 10 cm, constituting a plant architecture that is not well- adapted to more efficient systems such as the combine harvesting that reduces harvestability and yield. On average, pod shattering causes 13% yield loss in dry adzuki beans with 8% of the loss caused by ineffective combine harvesting [83]. However, the cut and pick-up system is laborious and time consuming. To facilitate breeding of improved cultivars, hybridization of the long epicotyl materials such as 'Toiku161' or 'Kitairoha' possessing high-yielding and disease-resistant attributes is necessary. Additionally, effective and efficient breeding for ideal plant height requires the clear understanding of mode of inheritance of the associated traits. Previously, epicotyl length QTLs have been identified but not much details to ascertain if epicotyl length acts independently or epistatic effect exists in the control of plant height traits. The findings from this study therefore helped clarify the relationship between epicotyl length and other plant height traits, particularly the association of ECL and lower stem internode loci but de-linkage with upper stem loci that could be useful in breeding for ideal plant height.

Plant height trait is based on the stem internode number and length, and is among the critical traits due to for its effect on yield and lodging. In soybean, the short plant heights reduce lodging susceptibility however presents a trade-off for pod numbers given the fewer number on nodes [61]. Leaf senescence at

pod maturity stage, is desirable to facilitate harvesting of adzuki bean using machinery. In soybean, high moisture levels in the plants during harvesting, affects the effectiveness of harvesting with combine machines as the stems and leaves cut from the plant during the harvesting process piles up in the machine [12]. This study detected leaf senescence QTLs on chromosome 3 and 10 with a high degree of leaf fall effect from 'Chihayahime' alleles. However, the two QTLs were detected only once each, therefore need for detailed analysis to check the reproducibility of the QTLs.

The phenotypic expression of architectural traits confer probable benefits and combination effects of such advantageous traits are ideotypes sought after by plant breeders [61]. Therefore, results found in this study are likely an initial step towards the breeding goal for optimizing plant architecture and yield in adzuki bean. The major effect QTLs and association discovered in this study can be focus area coupled with aggregation of the preferable trait combinations for respective crop improvements.

5.3 Marker-assisted selection

In genetics, mapping of genetic loci and associated genes to determine the genomic positions, effect and functions of respective genes, is very important for molecular marker-assisted breeding [22] Unfortunately, there is a limited number of studies on QTL mapping and candidate gene analysis in adzuki bean. Therefore, the information generated in this study will contribute toward the knowledge body on adzuki bean genetic mechanisms to facilitate the use of molecular marker-assisted breeding for improving key agronomic traits and development of ideal plant types and architecture.

Marker-assisted breeding (MAS) entails the use of DNA (molecular) markers that exist in close proximity with traceable genetic loci and associated genes, for application in the breeding for traits that are complex, difficult and tedious to evaluate [15]. Conventional breeding approach has been applied in breeding programs for some time and yielded significant improvements in breeding programs, however the approach is tedious, takes long time and requires evaluation of large population to generate desirable frequency of allelic combinations [59]. Therefore, advanced breeding strategies such as MAS are

considered as more precise, fast and cost-effective breeding programs [59]. The level of polymorphism in DNA markers determines the effectiveness of MAS [15] .In this study, QTL clusters were found on chromosomes 1, 2, 4, and 7. As explained by Swamy et al., [78], the presence of QTL clusters may reflect high-level polymorphism on the chromosomes. Therefore, results from this study are likely valuable in high efficiency MAS using co-dominant markers for QTLs *qFLD4, qPDTN4*, *qGW4* and *qMAD4* to generate a desirable combination of the traits. Polygenic traits and pleiotropic gene effects presence in a mapping population may complicate the gene introgression process and establish the effect on the introgressed loci [59]. Nonetheless, MAS has been successfully applied in the selection of complex traits and introgression into target genetic backgrounds for trait improvement [59]. Therefore, the polygenic traits (NGP, GW, 100GW, PDTN, MAD, STIL8, NN, ECL and STIL7), and the pleiotropic effects (198_AZ10_10M and 96_AZ04_50.7M) identified through QDG approach in QTL analysis in this study could be selected for desirable phenotypic expression using MAS.

In a nutshell, based on the research findings in this study, MAS could be applicable to effectively aggregate beneficial alleles in the selection of ideal phenotypes for plant height and yield-related traits possessing epicotyl length between 6 to 8 cm, early maturity (less than 109 days) and yield (100 seed weight over 18g). The manipulation of QTLs or gene pyramiding on potential loci identified in this study particularly, chromosomes 2, 4 and 7 for plant height trait and yield traits, will be of potential value in addressing current adzuki bean production challenges especially, yield levels, frost prevention by improved maturity lines, labour demand through improved suitability for machine use at peak production times.

ABSTRACT

The Tokachi region of Hokkaido is the primary adzuki bean (*Vigna angularis*) producer in Japan. However, sustainable production is affected by limitations in genetic knowledge related to plant architecture and yield, and machine harvesting efficiency. This study aimed to gain insights into the genetic mechanisms underlying key traits for enhanced adzuki bean production. The primary objectives encompassed: (i) elucidating genetic control of epicotyl length to improve mechanization efficiency; (ii) identifying genetic loci associated with plant height traits for ideal plant architecture; and (iii) clarifying genetic mechanisms underlying maturity and yield-related traits in adzuki bean. Mapping populations were generated using the breeding line 'Toiku161' and the cultivar 'Chihayahime' for subsequent quantitative trait loci (QTL) analyses.

Chapter 2 focused on the genetic control of epicotyl length (ECL) by analyzing recombinant inbred lines using QTL-seq. Four significant QTLs on chromosomes 2, 7, 10, and 11 were identified that contribute to increased ECL. Two of these QTLs, *qECL7.1* and *qECL10.1*, were validated using insertion/deletion (InDel) markers. The chromosomal location of *qECL7.1* was fine-mapped to a 418 kb region using a substitution mapping method with InDel, cleaved amplified polymorphic sequence (CAPS), derived cleaved amplified polymorphic sequence (dCAPS), and single nucleotide polymorphism (SNP) molecular markers. This region harbors 35 candidate genes possibly influencing ECL. The identified genetic loci offer valuable insights for breeding adzuki bean genotypes with enhanced epicotyl length, a critical trait for mechanical weed control and harvesting efficiency.

Chapter 3 mapped the genetic loci affecting plant height (PH) and its related traits, including ECL, stem internode length below node 7 (STIL7), stem internode length above node 8 (STIL8), and the total number of nodes (NN). Over 39 QTLs were related to these traits. Some QTLs showed stability by being detected consistently across the three years, including *qPH2* and *qSTIL8.2* on chromosome 2, *qPH4* and *qSTIL8.4* on chromosome 4, and *qSTIL7.7* and *qECL7.1* on chromosome 7. Remarkably, on chromosomes

2 and 4, a QTL affecting STIL8, an upper stem trait, co-localized with a QTL influencing PH. Likewise, on chromosome 7, a QTL affecting STIL7, a lower stem trait, co-localized with a QTL influencing ECL. This suggests the prospect of controlling the upper and lower stem parts independently to achieve enhanced plant height without compromising overall plant architectural integrity.

Chapter 4 analyzed genetic mechanisms for maturity and yield traits, including flowering (FLD), maturity (MAD), reproductive period (RP), total pod number (PDTN), Grain weight (GW), and number of grains per pod (NGP). A total of 36 QTLs were detected and a stable QTL for flowering *qFLD2* was found on chromosome 2 across the three-year study period. Flowering and maturity traits were generally located on either different chromosome or chromosomal positions. This suggests independent genetic regulation of flowering and maturity times, presenting prospects for developing early-maturity varieties to minimize yield losses by frost. The study findings showed co-localization between plant architecture (PH, STIL, FLD, MAD) and yield (PDTN, GW) traits, especially on chromosomes 2 and 4. The QTL network further evaluated causal relationships between plant height and yield traits, revealing 16 QTLs with direct and indirect effects on 11 traits. These QTL-to-trait interactions were distinctively grouped into 3: stem length and yield; seed weight, and seed quantity. Two important QTL hubs were identified within chromosomes 4 and 7. The identified pleiotropic and polygenic effects in the QTL-to-trait interactions provide useful insights into the understanding of regulatory mechanisms governing plant height and yield traits.

In conclusion, the QTL, candidate genes, co-dominant markers, QTL-to-trait interactions, and QTL hubs identified in this study are invaluable aspects in the molecular-assisted breeding for ideal key traits including plant height, epicotyl length, maturity, and yield. This understanding is crucial to the development of genotypes with improved mechanized compatibility and increased yields thereby contributing to sustainable production of adzuki bean in Tokachi. Further studies are, however, recommended particularly on fine-mapping of remaining key traits and candidate gene identification for more impactful application in adzuki bean breeding programs.

概要

北海道の十勝地域は，日本におけるアズキの主要な生産地であり，今後も持続的にアズキを生産することが求められている．しかしながら現状において，アズキの草型や収量性に関する遺伝的な知見は少なく，効率的な栽培に関わる機械作業適性をもつ植物体の育成が困難な状況である．本研究では，アズキの効率的な生産を可能とする農業形質の遺伝的メカニズムの知見を得るために，(i) 機械作業効率向上に重要な上胚軸長の遺伝的制御を明らかにすること，(ii) 理想的な草型を得るために草丈および草型関連形質の遺伝子座を特定すること，(iii) アズキの収量関連形質の遺伝的メカニズムを明らかにすることを目的とした．そこで，育種系統である「十育 161 号」と栽培品種の「ちはやひめ」を交雑して得られた後代系統をマッピング集として用い，量的形質遺伝子座 (QTL) 解析を実施した．

第 2 章では，上胚軸長（ECL）の遺伝的制御を明らかにするために，組換え自殖系統を供試して QTL-seq 解析を行った．その結果，第 2，第 7，第 10 および第 11 番染色体上に上胚軸長の増加に寄与する 4 つの QTL が座乗することを確認した．挿入・欠失（InDel）マーカーで作製した連鎖地図を用いてこれら QTL を再検証したところ，2 つの QTL（*qECL7.1* と *qECL10.1*）のみが大きな効果をもつことが明らかとなった．第 7 染色体に検出した *qECL7.1* の座乗領域を絞り込むために，新たな DNA マーカーを開発し置換マッピングを行った結果，418 kb の領域（35 の推定遺伝子）に *qECL7. 1 をファインマッピングした．これらの結果は*，アズキの機械除草や機械収穫にとって重要な特性である上胚軸長の効率的な選抜に役立つと考えられる．

第 3 章では，草丈（PH）および草丈に影響を与える ECL，下位節の節間長（STIL7），上位節の節間長（SIL8）および主茎の総節数（NN）について QTL 解析を行い，計 39 の QTL を検出した．これら QTL のうち，第 2 染色体の *qPH2* と *qSTIL8.2*，第 4 染色体の *qPH4* と *qSTIL8.4*，そして第 7 染色体の *qSTIL7.7* と *qECL7.1* は，同一領域に 3 年間を通じて安定的に座乗した．以

上の結果から，草丈の構成要素である胚軸長・下位節の節間長と上位節の節間長は異なる遺伝メカニズムを有しており，独立して草丈を制御していることを明らかにした．

第 4 章では，開花期（FLD），成熟期（MAD），登熟期間（RP），総莢数（PDTN），100 粒重（GW）および一莢内粒数（NGP）の QTL 解析により，これらの特性に関連する計 30 の QTL を検出した．第 2 染色体上に特定した *qFLD2* は，3 ヵ年安定して検出された．また，FLD と MAD の QTL が異なる遺伝子座として検出されたため，開花期と成熟期を独立的に制御できると考えられた．また，第 2，第 4 染色体上に検出した草型関連形質と収量関連形質は同一領域に共局在していた．草型関連形質と収量関連形質との間の因果関係を調査するために QTL ネットワーク解析を行った．その結果，QTL と形質間の相互作用において，第 4 染色体と第 7 染色体の 2 つの QTL が草丈と収量性を調節するうえで重要であることが明らかとなった．

この研究の成果から得られた各種 QTL，候補遺伝子，QTL に連鎖する DNA マーカー，QTL と形質間の相互作用に関する情報は，マーカー支援選抜を通して理想的な特性（上胚軸長，草丈，開花期と成熟期，収量）をもつ品種の早期開発につながると考えられる．また，省力的なアズキ栽培に必要な機械作業適性の向上や収量性の増加に寄与し，持続可能なアズキの生産を可能とするだろう．今後の研究において，重要形質のファインマッピングと候補遺伝子の特定を進めることにより，アズキの育種プログラムにおいてより効果的な成果が期待される．

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