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Linkage Map of a Gene Controlling Zero Tannins (*zt-1*) in Faba Bean (*Vicia faba* L.) with SSR and ISSR Markers

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Abstract: Faba bean (*Vicia faba* L.), a partially allogamous species, is rich in protein. Condensed tannins limit the use of faba beans as food and feed. Two recessive genes, *zt-1* and *zt-2*, control the zero tannin content in faba bean and promote a white flower phenotype. To determine the inheritance and develop a linkage map for the *zt-1* gene in the faba bean germplasm M3290, F_2 and F_3 progenies were derived from the purple flower and high tannin content genotypes Qinghai12 and *zt-1* line M3290, respectively. Genetic analysis verified a single recessive gene for zero tannin content and flower colour. In total, 596 SSR markers and 100 ISSR markers were used to test the polymorphisms between the parents and bulks for the contrasting flower colour via Bulked Segregant Analysis (BSA). Subsequently, six SSR markers and seven ISSR markers were used to genotype the entire 413 F_2 population. Linkage analysis showed that the *zt-1* gene was closely linked to the SSR markers SSR84 and M78, with genetic distances of 2.9 and 5.8 cM, respectively. The two flanked SSR markers were used to test 34 faba bean genotypes with different flower colours. The closely linked SSR marker SSR84 predicted the *zt-1* genotypes with absolute accuracy. The results from the marker-assisted selection (MAS) from this study could provide a solid foundation for further faba bean breeding programmes.

Keywords: faba bean; *zt-1*; linkage map; SSR; ISSR

1. Introduction

Faba bean (*Vicia faba* L.), one of the most important temperate food crops, is widely grown for human consumption in China, Ethiopia, Egypt and the Andean States of South America and for livestock feed in Europe and Australia [1]. To date, the average faba bean cultivation area is close to 2.5 million hectares annually, which ranks fourth among cool-season crops [2]. It has been demonstrated that growing faba bean is the most effective strategy for managing soil fertility through crop rotation, which contributes to sustainable agriculture [3].

Faba bean seeds together with other relative beans, have high nutritional values as they are excellent sources of protein, carbohydrates, minerals and fibre [4]. Nevertheless, faba bean also suffer from both biotic and abiotic factors that constrain their productivity and digestibility. Previous studies have demonstrated that condensed tannins are responsible for low-protein seeds and may

decrease feed consumption due to their astringent taste [5]. Condensed tannins from faba bean may also decrease the efficiency of food utilization [6,7]. Although several methods, such as cooking and autoclaving, have been used to remove condensed tannins, these processes may also promote other changes in the seed compounds. Meanwhile, a significant problem in tannin removal is the high cost [8]. Compared to traditional methods, growing cultivars with low-tannin and zero-tannin content are the most effective, economic and environment-friendly strategy.

A previous study first reported the absence of tannins in the white flowers of faba bean varieties; this served an important role in the in vitro digestibility of nutrients in monogastric animals [9]. According to Picard [9], there are two inherited recessive genes, *zt-1* and *zt-2*, that control the zero-tannin characteristic in faba bean and promote a white flower characteristic in the plant. Genetic studies also discovered that the genes in faba bean that control white-flowered plants actually block anthocyanin synthesis [10,11]. Breeders usually use crosses between intergeneric and interspecific plants to improve their characteristics. However, faba bean genotype hybrids carrying different zero tannin genes generally give rise to segregating progenies. Therefore, identifying varieties with zero tannins will be helpful for both choosing appropriate crosses for breeders [12] and representing a reservoir of genes for tannin-free plants. To date, great progress has been made in developing faba beans with zero tannins. Several markers have been mapped to the *zt-1* region. The number of markers is still limited, and more markers are needed to fill the gaps for more efficient marker-assisted selection, further fine mapping and map-based cloning of the gene.

Faba bean, a partially allogamous and genetically isolated plant, tolerates no exchange of genes with any other species, including its close relative *Vicia narbonensis* [13]. The perception is that genetic mapping and marker-assisted selection (MAS) in faba bean faces enormous challenges because of its huge genome size (13,000 Mb) [14,15], even though faba bean is diploid and has fewer chromosomes ($2n = 2x = 12$) than other species in the genus *Vicia* L. [16].

Various molecular markers have been widely used in faba bean, especially in genetic diversity and relationships among germplasm collections. For example, amplified fragment length polymorphism (AFLP) markers were used to assess the genetic diversity in 22 recent faba bean elite cultivars [17]. Zong et al. [18] subsequently analysed winter and spring [19] faba bean accessions worldwide using AFLP markers. Linkage maps of the gene controlling zero tannin in faba bean with SCAR markers developed from linked RAPD markers has been published [20]. Compared with other molecular markers, simple sequence repeats (SSR) markers are based on the amplification of sequence repetitions. It is a simple and repeatable method that can produce abundant polymorphic fragments. Therefore, SSR markers have the advantage of being a valuable tool for constructing genetic linkage maps and marker-assisted trait selection in faba bean breeding efforts.

As mentioned above, Picard [9] and Bond [10] first reported that the seed coat of all white flowered varieties of faba bean was free of tannins. The faba bean germplasm M3290, which is originally from the Mediterranean region, is a tannin-free cultivar with white flowers and the *zt-1* gene [9]. The variety was collected from the International Center for Agricultural Research in the Dry Areas (ICARDA) Syria [1]. It was then developed by the Qinghai Academy of Agricultural Science and has been widely used in faba bean breeding programmes in China in the past few years (unpublished data).

The objective of this study was to (1) construct a linkage map of the temporary named gene *zt-1* the controls the zero-tannin trait in the M3290 variety and (2) identify closely linked markers that could be useful for marker-assisted selection (MAS) in faba bean and further cloning of the gene.

2. Materials and Methods

2.1. Plant Materials

The Qinghai12 variety has coloured flowers (purple) and high tannin content, while the genotype *zt-1* M3290 [1] produces white flowers and has a tannin-free seed coat. M3290 was used as the male parent and Qinghai12 was used as the female parent to develop the population lines. An F_2 population

with 413 plants and the derived $F_{2:3}$ families with 8–10 plants each that were derived from the cross between the tannin-free cultivar, M3290, and a condensed tannin line, Qinghai12, were used for mapping the tannin-free gene *zt-1*. The parents and populations used in this study were grown in the fields of the Qinghai Agriculture and Forestry Academy. A total of 413 F_2 progenies were grown during the 2015 growing season on the experimental farm and all F_3 lines with ten or twenty plants each were grown in the next spring in 2016. All the parents and progenies were carefully characterized for their phenotypes by their colour in the field and tested for tannin content in the laboratory during the flowering period. The colours of the offspring were classified as two types, e.g., "white" and "purple", those with the same flower colour as M3290 were scored as "white" and the remainder with the same flower colour as Qinghai12 were scored as "purple".

A representative collection of 34 elite faba bean accessions from the major faba bean production regions in China, including ten main spring varieties from Qinghai province, four spring varieties from Gansu province, four Yunnan germplasms, six winter varieties from Sichuan province, seven Jiangsu winter cultivars and three Zhejiang winter cultivars, were used to validate the molecular markers identified to be linked to the *zt-1* gene. Two main types in China, the spring and winter faba bean, were both selected in this study to compare different ecotypes of faba bean germplasm. All the representative faba bean samples from different areas were grown on the experimental farm at the Qinghai Agriculture and Forestry Academy.

2.2. Tannin Measurement

To enhance the phenotype accuracy and to confirm the tannin content in the genotypes with different colours, the tannin content was determined in the parents and F_2 individuals with different flower colours. The Folin-Donis (F-D) method was used to measure the tannin content with a few modifications [21].

2.3. DNA Extraction

After measuring the condensed tannin content, the newly expanded faba bean leaves were used to extract genomic DNA. Genomic DNA was extracted using the DS (Sodium Lauroylsarcosine) protocol [22,23]. Tannin-free and condensed tannin bulks were established from 20 free (white flower) and 20 condensed (purple flower) tannin content F_2 plants, respectively. Bulked segregant analysis (BSA) [24] was used to identify whether the markers were linked to the gene controlling the zero-tannin characteristic.

2.4. Marker Analysis

A total of 596 pairs of SSR primers were screened between the two parents and bulks. Among them, 128 SSR markers were referred to in Ma et al. [25], 236 SSR markers were selected from a linkage map developed by El-Rodeny et al. [26], and the remaining SSR markers (unpublished data) were kindly provided by the Institute of Crop Science, Chinese Academy of Agricultural Sciences (ICP, CAAS). The ISSR markers used in this study were according to Zietkiewicz et al. [27]. All the primers used in this study were synthesized by Shanghai Sangon Biological Engineering Technology and Services Company Ltd., Shanghai, China.

SSR reactions were performed in a 20 µL reaction volume containing 1 unit of *Taq* DNA polymerase (TaKaRa), 2 µL of 10× buffer (50 mmol KCl (TaKaRa), 10 mmol Tris-HCl (TaKaRa, pH 8.3), and 1.5 mmol $MgCl_2$ (TaKaRa), 200 µmol of each dNTP (Roche, Basel, Switzerland), 6 pmol of each primer and 50–100 ng of template DNA. The PCR conditions were as follows: denaturation at 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 50–61 °C (depending on primers) for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. ISSR-PCR amplifications were performed in 25 µL reaction volumes with 80 ng of genomic template DNA, 2 µL of 10 mM Tris–HCl, 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM of each dNTP, 120 nM of each primer, and 1 U of Taq DNA polymerase. ISSR-PCR reactions were performed with the following conditions: denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s,

annealing at optimal temperature for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min.

PCR reactions were performed in a PTC200 Peltier Thermal Cycler. PCR products were then mixed with 4 µL of the formamide loading buffer (98% formamide, 10 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol, pH 8.0, Shanghai Sangon) and heated at 94 °C for 5 min. The PCR products were separated on 6% denaturing polyacrylamide gels, 8% non-denaturing polyacrylamide gels or 1.5% agarose gels. Each 5–7 µL sample was loaded and then resolved using the silver staining method as described by Bassam et al. [28] or ethidium bromide and then photographed.

2.5. Statistical Analysis and Genetic Mapping

The Chi-square tests (χ^2) were used to determine the theoretical expectation based on the assumption of a single Mendelian gene controlling *zt-1*. Recombination fractions were converted to centiMorgans (cM) and the genetic distances of closely linked markers were calculated with software JOINMAP version 4.0 using the Kosambi mapping function [29]. A LOD score of 3.0 was used as a threshold for grouping and a maximum recombination fraction of 0.5 were employed as linkage criteria to establish the linkage group [30].

3. Results

3.1. Phenotypic and Genetic Analyses

In the flower testing in the field, M3290 had white flowers, whereas Qinghai12 had purple flowers. In the F_2 population, there were 95 white flowers and 318 purple flowers in the flower test, which fits a 1:3 ratio ($\chi^2_{1:3} = 0.879$, $p = 0.348$), consistent with the *zt-1* gene behaving as a single recessive gene in this population. Simultaneously, the tannin content also segregated in a 1:3 ratio after measuring the F_2 population, as the 95 plants with white flowers were all tannin-free (0 mg/mL), and the 318 plants with purple flowers presented tannin content ranging from 0.1 to 0.4 mg/mL. The histogram for tannin content was drawn to see the distribution of the trait (Figure 1). When the flower colours of F_3 families were tested during the same period in the next year, the segregation of these families conformed to a 1:2:1 ratio ($\chi^2_{1:2:1} = 1.59$, $p = 0.451$) as expected for a single gene (Table 1).

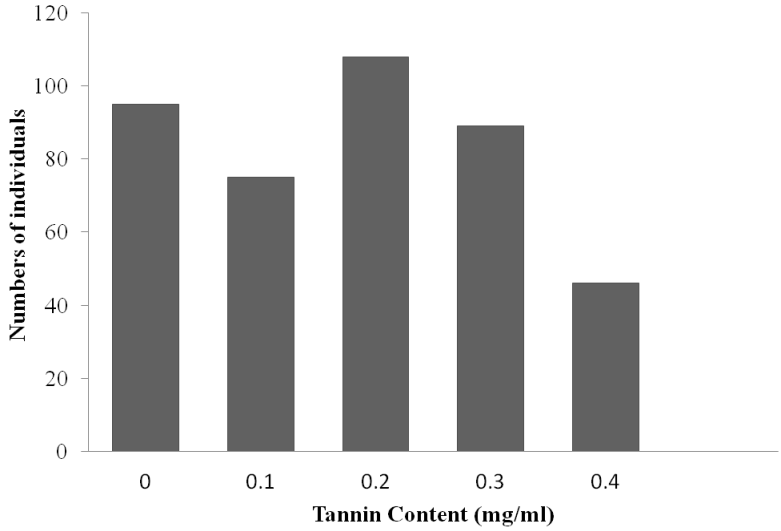

Figure 1. Frequency distributions of tannin content in 413 plants of F_2 population.

Table 1. Segregation for flower colours in the M3290/Qinghai12 F_1, F_2 and $F_{2:3}$ progenies.

CrossProgeny	Observed Number of Plants or Lines			Expected Ratio	χ^2	*P*
	W	Seg	P			
M3290	15	0	0			
Qinghai12	0	0	15			
F_1	15	-	-	1:0		
F_2	95	-	318	1:3	0.879	0.348
F_3	95	205	113	1:2:1	1.59	0.451

W, white flowers; P, purple flowers; Seg, segregation.

3.2. Identification of the SSR and ISSR Markers

Of the tested SSR primers, fifteen SSR markers, were polymorphic and contrasted between the purple and white flower bulks as well as the two parents (Figure 2). The selected polymorphic SSR markers were all co-dominant and could only be tested by 6% denaturing polyacrylamide gels. For example, the association between the SSR marker SSR84 in part of the F_2 population segregating for the *zt-1* gene is shown in Figure 2. The results shown in Figure 2 indicated that there were five genotypes with white flowers which showed the 900 bp bands with M3290, five genotypes with purple flowers which showed the same bands as Qinghai12 (1100 bp) and the other ten genotypes which showed heterozygous bands. For the tested 100 ISSR markers, seven markers, namely ISSR7, ISSR9, ISSR10, ISSR12, ISSR25, ISSR36 and ISSR48, were identified as polymorphic between the parents and bulks in this study (Figure S1). Among the seven ISSR markers, ISSR12 and ISSR25 were co-dominant and the other five were dominant (Figure 3).

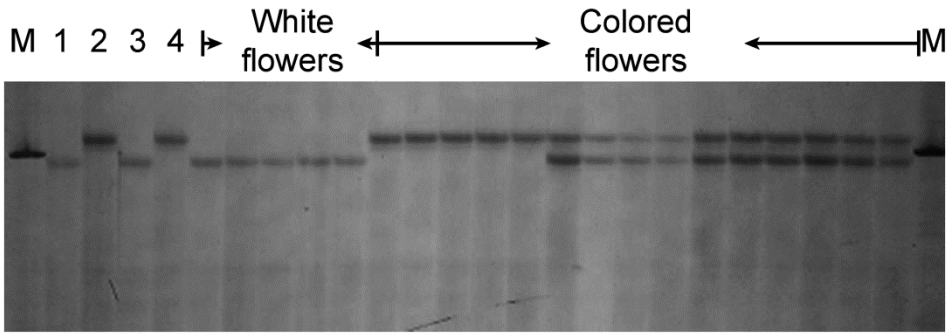

Figure 2. PCR amplification results of the SSR marker SSR 84 in part of the F_2 population segregating for *zt-1*. 1, M3290 (900 bp); 2, Qinghai12 (1100 bp); 3, white flower bulk (900 bp); 4, purple flower bulk (1100 bp); M, Marker (100 bp). The F_2 population includes 5 white flower genotypes (zz, 900 bp), 5 purple flower genotypes (ZZ, 1100 bp) and 10 heterozygous genotypes (Zz, 1100 bp); This is a composite picture of several different gel picture.

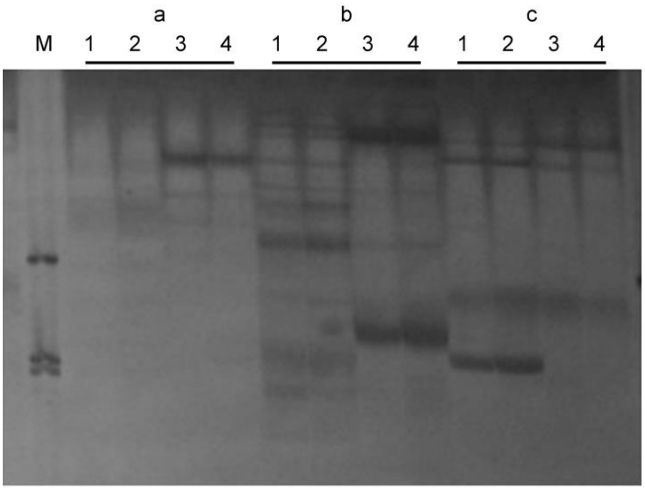

Figure 3. Polymorphism analysis of the partial ISSR markers in the parents and bulks. 1, M3290; 2, white flower bulk; 3, Qinghai12; 4, colored flower bulk. M, Marker (100 bp). a, ISSR7; b, ISSR12; c, ISSR36.

3.3. Mapping the SSR and ISSR Markers

The selected fifteen SSR markers and seven ISSR markers were then used to genotype the 413 F_2 plants to construct the linkage map. After testing the 413 plants in the F_2 population, it was evident that six SSR markers, M78, ssi85H, M233, SSR84, M81 and M38 (Table 2) and three ISSR markers were linked to *zt-1* (Table 2). The results of the *zt-1* linkage estimates with the nine polymorphic markers based on the phenotype and genotype data are shown in Figure 4.

Table 2. Molecular markers mapped at or close to the *zt-1* locus.

Name	Marker Type	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Annealing Temperature (°C)
SSR84	SSR	TCTGAAAACGAGTTCAGTGGA	CTGGTGCCGAACTAACCAGT	52
M38	SSR	GCTACTGGAGGAGGCTTTCA	GCCTTCTACACAACGGCTTC	53
M78	SSR	GTCAAATCGAGTGGCGAAAG	TTGGGATATGGAAGTAGCTTCAG	52
M81	SSR	CCTCATGCCATTCCTCTGAT	TTCCGCGTGGTAAATTCTATG	55
M233	SSR	CATCCCAACAATATACCGGC	CTGGGGTACCACCGTAACTC	51
ssi85H	SSR	AACAACTACGTAATGCCAGAC	ACATGAGGGGCCAAGTAT	52
ISSR7	ISSR	AGA GAG AGA GAG AGA GT		53
ISSR9	ISSR	AGA GAG AGA GAG AGA GG		50
ISSR10	ISSR	GAG AGA GAG AGA GAG AT		51

The selected nine markers were mapped within a genetic interval of 20.6 cM flanking *zt-1* (Figure 4). The flanking markers, *SSR84* and *M78*, were closely linked with the *zt-1* gene with genetic distances of 2.9 cM and 6.2 cM, respectively (Figure 4).

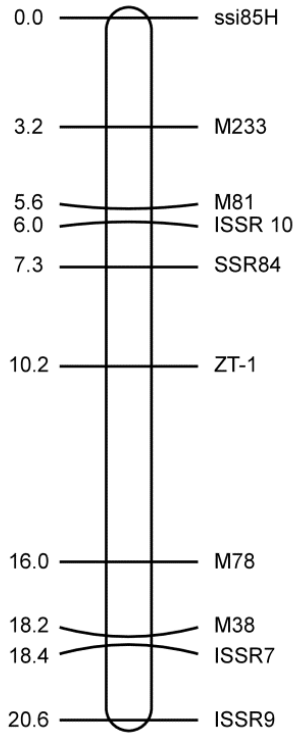

Figure 4. Linkage map of the zero tannins *zt-1* gene flanked by six SSR and three ISSR markers. The locus name and corresponding locations are indicated on the right side and the genetic distances between them are indicated on the left side.

3.4. Closely Linked Markers for the Marker-Assisted Selection of zt-1

The two flanked markers (Figure 4), SSR84 and M78, with genetic distances of 2.9 cM and 6.2 cM, respectively, were first used to identify representative varieties from different areas of China to assess their potential use in the MAS. The results showed that when SSR84 was used for MAS, only M3290 and *zt-1*-carrying genotypes (white flowers) produced the expected band of 900 bp and the genotypes with purple flowers (without the *zt-1* gene) produced 1100 bp bands. Nevertheless, the results of molecular detection with M78 was not helpful in the selection of the *zt-1* gene in faba bean breeding programs (Table 3).

Table 3. The 34 Chinese faba bean genotypes used for validation of the closely linked markers.

No.	Variety	Province	Flower [a]	SSR84 [b]		M78 [b]	
				900 bp	1100 bp	400 bp	420 bp
1	Qinghai11	Qinghai	Purple	- [c]	+ [c]	-	+
2	Qinghai12	Qinghai	Purple	-	+	-	+
3	Qinghai13	Qinghai	Purple	-	+	-	+
4	Qingcan14	Qinghai	Purple	-	+	-	+
5	Qingcan15	Qinghai	Purple	-	+	-	+
6	M3290	Qinghai	White	+	-	+	-
7	TF26	Qinghai	White	+	-	+	-
8	TF29	Qinghai	White	+	-	-	+
9	TF34	Qinghai	White	+	-	-	+
10	2005-00	Qinghai	White	+	-	-	+
11	Lincan6	Gansu	Purple	-	+	-	+
12	Lincan7	Gansu	Purple	-	+	-	+
13	Lincan8	Gansu	Purple	-	+	-	+

Table 3. *Cont.*

No.	Variety	Province	Flower [a]	SSR84 [b]		M78 [b]	
				900 bp	1100 bp	400 bp	420 bp
14	Yangyandou	Gansu	Purple	-	+	-	+
15	Yundou7	Yunnan	Purple	-	+	-	+
16	Yundou8	Yunnan	Purple	-	+	-	+
17	Yundou9	Yunnan	Purple	-	+	-	+
18	Touxinlv	Yunnan	Purple	-	+	-	+
19	Dahudou	Sichuan	Purple	-	+	-	+
20	Xiaohudou	Sichuan	Purple	-	+	-	+
21	Honghudou	Sichuan	Purple	-	+	-	+
22	Chenghu9	Sichuan	Purple	-	+	-	+
23	Chenghu10	Sichuan	Purple	-	+	-	+
24	Chenghu11	Sichuan	Purple	-	+	-	+
25	Tongcanxian7	Jiangsu	Purple	-	+	-	+
26	Tongcanxian8	Jiangsu	Purple	-	+	-	+
27	Qidongbaipi	Jiangsu	Purple	-	+	-	+
28	Haimendabaipi	Jiangsu	Purple	-	+	-	+
29	Tongcan5	Jiangsu	Purple	-	+	-	+
30	Nantongsanbai	Jiangsu	Purple	-	+	-	+
31	DAqingpi	Jiangsu	Purple	-	+	-	+
32	Lvpidou	Zhejiang	Purple	-	+	-	+
33	Luohandou	Zhejiang	Purple	-	+	-	+
34	Xiaoqingdou	Zhejiang	Purple	-	+	-	+

[a] Flower colour. Purple: genotype with tannin content and without the *zt-1* gene.; white: genotype with zero tannin content carrying the *zt-1* gene. [b] Closely linked markers. [c] '+' and '-' indicate the presence and absence of the specific alleles of the SSR markers, respectively.

The other different PCR fragments amplified with other linked markers also could not distinguish lines with white flowers from coloured varieties. Therefore, it was verified that only SSR84 is helpful for selecting the *zt-1* gene in faba bean programmes for tannin content in this study.

4. Discussion

4.1. Zt-1 Gene in Faba Bean Variety M3290

In this study, we identified markers to the single recessive gene *zt-1* in the faba bean variety M3290 and mapped it with six SSR markers and three ISSR markers. We also tested the elite faba bean germplasms with the closely linked markers and the results provided a sound basis for further MAS in faba bean.

The absence of tannin content in faba bean is determined by two recessive genes, *zt-1* and *zt-2*. In this study, *zt-1* was inherited as a single recessive gene in the M3290/Qinghai12 population. This result is in accordance with that of Gutierrez et al. [20], who used a segregated F_2 population derived from Vf6 and a *zt-1* line. The segregation for both the flower colour and tannin content fit the expected 1:3 and 1:2:1 ratios, respectively, which is consistent with a single recessive gene that controls zero tannin content in faba bean. The linkage map of Vf6 × *zt-1* F_2 populations showed that the *zt-1* gene was flanked with two SCAR markers with genetic distances of 3.6 cM ($OPAF20_{776}$) and 9.7 cM ($SCC5_{551}$).

4.2. SSR and ISSR Markers

A total of 596 SSR markers and 100 ISSR markers were used to screen the polymorphisms between parents as well as bulks in this study. The SSR markers were firstly randomly selected from each linkage group (LG) according to Ma et al. [25] and El-Rodeny et al. [26]. Also subsequently, unpublished SSR markers (including EST sequences) were kindly provided by the Institute of Crop Science,

Chinese Academy of Agricultural Sciences (ICP, CAAS). Finally, ISSR markers were downloaded according to Zietkiewicz et al. [27] and used to test the polymorphisms between parents and bulks. The polymorphism selection results indicated that six SSR markers and seven ISSR markers showed clear and repeatable bands between parents and bulks. The linkage analysis showed that the six SSR markers were all linked with the *zt-1* gene after genotyping the 413 F_2 plants, but only three ISSR markers indicated linkage correlation with the *zt-1* gene. The polymorphism tests verified the application of SSR markers in faba bean, and also provided us with a sound basis for further fine mapping of the *zt-1* gene. Nine markers, including six SSR markers and three ISSR markers were mapped at *zt-1* locus in this study. The flanking markers, SSR84 and M78, were closely linked with the *zt-1* gene with genetic distances of 2.9 cM and 6.2 cM, respectively. Although several markers have been mapped to the *zt-1* region, the number of the markers is still limited, and more are needed for more efficient marker-assisted selection, fine mapping and map-based cloning of the *zt-1* gene.

Compared to previous studies, a linkage map of the *zt-1* region with nine markers, including six SSR markers and three ISSR markers were constructed with a F_2 population.

4.3. Closely Linked Markers and Their Application in MAS

Molecular markers closely linked to the target gene are considered important tools for MAS in plant breeding programmes [31]. However, faba bean possess a large genome size and limited molecular markers. Therefore, MAS progress in faba bean breeding faces enormous challenges [1,32]. In this regard, it is urgent to develop more valuable and closely linked markers for faba bean breeding.

Previous studies allowed the prediction of the *zt-1* genotypes with a 95% accuracy [20]. In this study, two flanking markers were used to test their suitability in MAS, and the closely linked marker SSR84 was verified to be a powerful tool (100% accuracy of the selection of the *zt-1* gene) for further faba bean breeding. Owing to the recessive nature of the faba bean flower and tannin content traits, crops are often segregated by crosses that cause devaluation a few years after being grown for commercial production [33]. We present a convenient marker in this study that is closely linked with the *zt-1* gene and might resolve this problem.

4.4. Faba Bean Breeding Program of Variety M3290

White flowers and zero tannin content are controlled by a single recessive gene. These and many other good agronomic traits make M3290 a desirable donor for faba bean breeding programmes. In fact, M3290 was used in breeding programmes a few years ago in China. The faba bean variety 'TF26' and some other lines were developed with M3290 by the Qinghai Academy of Agriculture and Forestry Sciences; these varieties showed pure white flowers in fields and zero tannin contents. However, the limited genetic background and linked molecular markers for the *zt-1* gene still hampers the use of the gene in breeding programs. The demonstration of the *zt-1* gene in the germplasm M3290 and the closely linked markers identified in this study should accelerate its application in breeding programmes and SSR84 closely linked with the *zt-1* gene could exactly distinguish flowers with different genotypes.

5. Conclusions

F_2 and F_3 progenies derived from M3290 and Qinghai12 were used for phenotypic and genetic analyses, and the results indicated that the *zt-1* gene in this population behaved as a single recessive gene. Selected SSR markers and ISSR markers were used to genotype the entire 413 F_2 population, and linkage analysis showed that the *zt-1* gene was closely linked to the SSR markers SSR84 and M78, with genetic distances of 2.9 and 5.8 cM, respectively. SSR marker SSR84 could predict the *zt-1* genotypes in faba bean breeding.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/6/80/s1, Figure S1: PCR amplification results of the ISSR marker ISSR10 in part of the F_2 population segregating for *zt-1*.

Author Contributions: X.Z., W.H., Y.L. conceived and designed the experiments; Q.Y. and W.S. performed the experiments; P.L. and Y.T. analyzed the data; W.H. contributed reagents/materials/analysis tools; X.Z. and W.H. wrote the paper.

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Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Duc, G.; Bao, S.; Baumc, M.; Redden, B.; Sadiki, M. Diversity maintenance and use of *Vicia faba* L. genetic resources. *Field Crop. Res.* **2010**, *115*, 270–278. [CrossRef]
2. FAO. United Nations. 2016. Available online: http://faostat3.fao.org/download/Q/QC/E (accessed on 10 October 2016).
3. Stoddard, F.L.; Hovinen, S.; Kontturi, M.; Lindström, K.; Nykänen, A. Legumes in Finnish agriculture: history, present status and future prospects. *Agric. Food Sci.* **2009**, *18*, 191–205. [CrossRef]
4. Friedman, M. Nutritional value of proteins from different food sources. A review. *J. Agric. Food Chem.* **1996**, *44*, 6–29. [CrossRef]
5. Wiseman, J.; Cole, D.J.A. European legumes in diets for non-ruminants. In *Recent Advances in Animal Nutrition*; Haresign, W., Cole, D.J.A., Eds.; Butterworths: London, UK, 1988; pp. 13–37.
6. Martin-Tanguy, H.; Guillaume, J.; Kossa, A. Condensed tannins in horse bean seeds: Chemical structure and apparent effects on poultry. *J. Sci. Food Agric.* **1977**, *28*, 757–765. [CrossRef]
7. Cansfield, P.E.; Marquardt, R.R.; Campbell, L.D. Condensed proanthocyanidins of faba beans. *J. Sci. Food Agric.* **1980**, *31*, 802–812. [CrossRef] [PubMed]
8. Vander Poel, A.F.B.; Dellaert, L.M.W.; Van Norel, A.; Helsper, J.P.F.G. The digestibility in piglets of faba bean (*Vicia faba* L.) as affected by breeding towards the absence of condensed tannins. *Br. J. Nutr.* **1992**, *68*, 793–800. [CrossRef]
9. Picard, J. Apercu sur l'héréditédu caractére absence de tannins dans les graines de féverole (*Vicia faba* L.). *Ann. Amelior. Plant.* **1976**, *26*, 101–106.
10. Bond, D.A. In vitro digestibility of the testa in tannin-free field beans (*Vicia faba* L.). *J. Agric. Sci. Camb.* **1976**, *86*, 561–566. [CrossRef]
11. Cabrera, A.; Martin, A. Genetics of tannin content and its relationship with flower and testa colours in *Vicia faba* L. *J. Agric. Sci. Camb.* **1989**, *113*, 93–98. [CrossRef]
12. Crofton, G.R.A.; Bond, D.A.; Duc, G. Potential seed multiplication problems arising from the existence of two genes for the absence of tannin in *Vicia faba* L. *Plant Var. Seeds* **2000**, *13*, 131–139.
13. Alghamdi, S.S.; Migdadi, H.M.; Ammar, M.H.; Paull, J.G.; Siddique, K.H.M. Faba bean genomics: Current status and future prospects. *Euphytica* **2012**, *186*, 609–624. [CrossRef]
14. Bennett, M.D.; Smith, J.B. Nuclear DNA amounts in angiosperms. *Proc. R. Soc. Lond. B Biol. Sci.* **1982**, *216*, 179–199. [CrossRef]
15. Johnston, J.S.; Bennett, M.D.; Rayburn, A.L.; Galbraith, D.W.; Price, H.J. Reference standards for determination of DNA content of plant nuclei. *Am. J. Bot.* **1999**, *86*, 609–613. [CrossRef] [PubMed]
16. Raina, S.N.; Ogihara, Y. Ribosomal DNA repeat unit polymorphism in 49 *Vicia* species. *Theor. Appl. Genet.* **1995**, *90*, 477–486. [CrossRef] [PubMed]
17. Zeid, M.; Schon, C.C.; Lin, W. Genetic diversity in recent elite faba bean lines using AFLP markers. *Theor. Appl. Genet.* **2003**, *107*, 1304–1314. [CrossRef] [PubMed]
18. Zong, X.; Liu, X.; Guan, J.; Wang, S.; Liu, Q.; Paull, J.G.; Redden, R. Molecular variation among Chinese and global winter faba bean germplasm. *Theor. Appl. Genet.* **2009**, *118*, 971–978. [CrossRef] [PubMed]
19. Zong, X.; Ren, J.; Guan, J.; Wang, S.; Liu, Q.; Paull, J.G.; Redden, R. Molecular variation among Chinese and global germplasm in spring faba bean areas. *Plant Breed.* **2010**, *129*, 508–513. [CrossRef]
20. Gutierrez, N.; Avila, C.; Rodriguez-Suarez, C.; Moreno, M.; Torres, A. Development of SCAR markers linked to a gene controlling absence of tannins in faba bean. *Mol. Breed.* **2007**, *19*, 305–314. [CrossRef]
21. Hagerman, A.E.; Butler, L.G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809–812. [CrossRef]

22. Song, W.N.; Langridge, P. Identification and mapping polymorphism in cereals based on polymerase chain reaction. *Theor. Appl. Genet.* **1991**, *82*, 209–213.
23. Song, W.N.; Henry, R. Polymorphisms in the a-amy1 gene of wild and cultivated barley revealed by the polymerase chain reaction. *Theor. Appl. Genet.* **1994**, *89*, 509–512.
24. Michelmore, R.W.; Paran, I.; Kesseli, R.V. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9828–9832. [CrossRef] [PubMed]
25. Ma, Y.; Yang, T.; Guan, S.; Wang, H.; Wang, X.; Zong, X. Development and characterization of 21 EST-derived microsatellite markers in *Vicia faba* (faba bean). *Am. J. Bot.* **2011**, *98*, 22–24. [CrossRef] [PubMed]
26. El-Rodeny, W.; Kimura, M.; Hirakawa, H.; Sabah, A.; Shirasawa, K.; Sato, S. Development of EST-SSR markers and construction of a linkage map in faba bean (*Vicia faba*). *Breed. Sci.* **2014**, *64*, 252–263. [CrossRef] [PubMed]
27. Zietkiewicz, E.; Rafalski, A.; Labuda, D. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **1994**, *20*, 176–183. [CrossRef] [PubMed]
28. Bassam, B.J.; Anolles, G.C.; Gresshoff, P.M. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* **1991**, *196*, 80–83. [CrossRef]
29. Van Ooijen, J.W. *JoinMap4, Software for the Calculation of Genetic Linkage Maps in Experimental Populations*; Kyazma BV: Wageningen, The Netherlands, 2006.
30. Kosambi, D.D. The estimation of map distances from recombination values. *Ann. Eugen.* **1944**, *12*, 172–175. [CrossRef]
31. Kelly, J.D. Use of random amplified polymorphic DNA markers in breeding for major gene resistance to plant pathogens. *HortScience* **1995**, *30*, 461–465.
32. Terzopoulos, P.J.; Bebeli, P.J. Genetic diversity analysis of Mediterranean faba bean (*Vicia faba* L.) with ISSR markers. *Field Crop. Res.* **2008**, *108*, 39–44. [CrossRef]
33. Link, W.; Ederer, W.; Metz, P.; Buiel, H.; Melchinger, A.E. Genotypic and environmental variation for degree of cross-fertilization in faba bean. *Crop Sci.* **1994**, *34*, 960–964. [CrossRef]