Inheritance of *dml* and *ti* genes in Soybean

Mi Kyung Sung¹, Min Hwan Kim¹, Hyung Jin Seo¹, and Jong Il Chung^{1*}

¹Department of Agronomy, Research Institute of Life Science, Gyeongsang National University, JinJu, Korea, 660-701

ABSTRACT *dml* mutant newly identified displays necrotic spots with chlorotic halo despite the absence of any detectable pathogens in soybean. Kunitz trypsin inhibitor (KTI) protein of mature soybean seed is a main anti-nutritional factor and is controlled by *Ti* allele in soybean seed. The objective of this research was to determine the linkage or independent assortment between *dml* allele and *ti* allele. F₂ population was made by crossing between “Gaechuck2ho” (*DlmDlmtiti*) and T363 (*dldmlTiTi*) parents. The normal leaf or disease lesion mimic leaf trait of parents and F₂ individual plants was recorded at R2 and R3 growth stage from field. The random seeds from individual F₂ plants harvested were analysed electrophoretically to determine the presence of the KTI protein. The disease lesion mimic trait was matched to 3 : 1 ratio at the 0.01 levels of probability. The absence of the KTI protein was controlled by a single recessive gene. In F₂ generation, the ratios of 102 *Ti_Dlm_* : 21 *Ti_dldml* : 24 *titiDlm_* : 4 *titidldml* were observed. Segregation ratio gave a good fit to the 9 : 3 : 3 : 1 ratio based on F₂ generation at the 0.01 levels of probability. The data confirmed that *dml* allele controlling disease lesion mimic trait was inherited independently with the *ti* allele controlling absence of KTI protein in soybean.

Keywords *dml*, *ti*, Independent assortment, Linkage, Kunitz trypsin inhibitor, Soybean

INTRODUCTION

A new phenotypic traits are useful in genetic and breeding studies. By gamma-irradiated treatment, disease lesion mimic mutant have been identified in soybean [*Glycine max* (L.) Merr.,] leaf. Mutant displays necrotic spots with chlorotic halo despite the absence of any detectable pathogens. The mutant was behaved as a single recessive allele, designated *dml* (Chung et al. 1998). The *dml* allele controlling disease lesion mimic trait is useful in basic research aimed at better understanding disease hypersensitive response and programmed cell death in soybean. Kim et al. (2005) reported that *dml* mutant was light-dependent and observed that the mutant showed a defect in starch degradation of chloroplasts during diurnal fluctuations. The position of *dml* allele on linkage group were not reported. However, inheritance between *dml* trait and a few morphological traits was identified. The *dml* allele was inherited independently with the *PI* allele (controlling glabrous trait), the *y9* allele (controlling

chlorophyll- deficient leaf trait), the *f* allele (controlling fasciation stem trait) and the *lf2* allele (controlling seven-leaflet trait) in soybean (Jeong et al. 2007).

Soybean protein has been widely used in the food industry due to excellent nutritional quality and health benefit. But, the several antinutritional factors present in raw mature soybean seeds. Different trypsin inhibitors have been reported to be present in soybeans (Birk 1961, Frattali and Steiner 1968, Kunitz 1945), but much of the soybean trypsin inhibitor activity is thought to be due to Kunitz trypsin inhibitor (Rackis et al. 1962). Kunitz trypsin inhibitor (KTI) protein of mature soybean seed is a main anti-nutritional factor in soybean seed. KTI protein is a small and non-glycosylated protein containing 181 amino acid residues with 21.5 kDa. KTI protein was first isolated and crystallized from soybean seeds by Kunitz (1945). The Kunitz trypsin inhibitor protein strongly inhibits trypsin, thus reducing food intake by diminishing digestion and absorption. In USDA germplasm collection, two soybean accessions (PI157440 and PI196168) have been identified

Received Mar 2, 2013; Revised Mar 3, 2013; Accepted Mar 13, 2013; Published March 31, 2013

*Corresponding author Jong Il Chung, jongil@gnu.ac.kr, Tel: +82-55-772-1872, Fax: +82-55-772-1879

which lacks KTI protein (Orf and Hymowitz 1979). Five electrophoretic forms of KTI have been discovered. The genetic control of four forms, Tia, Tib, Tic, and Tid, has been reported as a codominant multiple allelic series at a single locus (Sing et al. 1969; Hymowitz and Hadley 1972; Orf and Hymowitz 1979). Orf and Hymowitz (1979) also found out that the fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated as ti. The Ti locus has been located on linkage group 9 in the classical linkage map of soybean (Hildebrand et al. 1980; Kiang 1987), which was integrated in linkage group A2 (chromosome 8) of the USDA/Iowa State University soybean molecular linkage map (Cregan et al. 1999). The genetic removal of the KTI protein will improve the nutritional value of soybean. Oliveira et al. (2007) observed that total isoflavone contents in KTI free soybean seed were higher than those in the normal soybean seed. Relationship between leaf trait and seed compositional trait is useful in genetic and breeding research of soybean. The objective of this research was to determine the linkage or independent assortment between dlm allele and ti allele.

MATERIALS AND METHODS

Genetic materials

Two parents were used to develop F₂ populations. “Gaechuck2ho” parent has *titi* genotype (KTI protein absent in mature seed) and normal leaf trait (*DlmDlm* genotype). T363 parent has the *dlm* allele that controls disease lesion mimic trait in leaves (*dlmdlm* genotype) and has KTI protein in mature seed (*TiTi* genotype). The seeds of “Gaechuck2ho” and T363 parents were planted to cross in a greenhouse. The crosses of “Gaechuck2ho” (*DlmDlmtiti*) × T363 (*dlmdlmTiTi*) were made and F₁ seeds were obtained. F₁ seeds obtained were planted in a greenhouse. F₁ hybridity was confirmed on morphological traits. F₂ seeds from F₁ plant confirmed were harvested. The seeds of parent and F₂ were planted in the field in Jun 2011. The normal leaf or disease lesion mimic leaf trait of parents and F₂ individual plants was recorded at R2 and R3 growth stage from field.

Determination of Kunitz trypsin inhibitor protein

Crude protein from parent seed and random seed from each F₂ plant harvested was extracted to determine the presence (+) or absence (-) of KTI protein electrophoretically. Seed coats were removed and were ground. The samples were incubated for 30 min (at room temperature) in 1 ml Tris-HCl, pH 8.0, containing 1.56% v/v β-mercaptoethanol. After centrifugation, 50 μl of the supernatant was added to an equivalent amount of 5X sample buffer [10% w/v sodium dodecyl sulfate (SDS), 50% v/v glycerol, 1.96% v/v β-mercaptoethanol, 1M Tris-HCl, pH 6.8]. The samples were boiled at 97° for 5 min and then centrifuged. Two microliters of the supernatant were loaded on a 12% acrylamide SDS polyacrylamide gel electrophoresis (SDS-PAGE) medium gels in Owl Separation Systems Inc (Model : P9DS, Portsmouth, NH USA). Electrophoresis was performed at 120 V for 7 hrs. Gels were stained overnight in an aqueous solution of 0.25 g coomassie blue R250, 10% acetic acid, and 45% methanol. The gels were then destained with destaining solution (5% acetic acid, 14% methanol) for several hours. A Wide-Range SDS-PAGE molecular mass standard (Sigma Marker™, Product Code: M4038, St. Louis MO USA) containing 21.5 kDa (for KTI protein) was used to aid recognition of samples lacking KTI protein. Chi-square analysis was used to test the goodness-of-fit of observed ratios with expected ratios for independent assortment or linkage.

RESULTS AND DISCUSSION

Leaf trait of F₁ plant obtained from the cross of “Gaechuck2ho” and T363 parents was normal. A necrotic spot on the leaf of F₁ plant was not observed. A total of 157 F₂ plants were grown in field. Disease lesion mimic trait was segregated in F₂ population (Fig. 1). 126 F₂ plants showed the normal leaf and 25 F₂ plants showed disease lesion mimic trait (Table 1).

Chi-square value of 5.74 was higher than critical point of 3.84 at the 0.05 levels of probability and the value was lower than 6.635 point of 0.01 levels of probability. The disease lesion mimic trait was matched to 3 : 1 ratio at the 0.01 levels of probability. Previous studies observed that

Fig. 1. Segregation of normal and disease lesion mimic trait in F_2 plant generation obtained from the cross of “Gaechuck2ho” (*titiD1mD1m*) and T363 (*TiTiD1md1m*). N: normal leaf type, S: necrotic spots with chlorotic halo leaf type.

disease lesion mimic trait in soybean leaf was inherited as a single recessive gene at the 0.05 levels of probability (Chung et al. 1998; Jeong et al. 2007). Deviation from the 3 : 1 ratio at the 0.05 levels of probability might be due to the small population size and death of a few plants with disease lesion mimic trait by bad field condition. All mature F_1 seeds obtained produced Kunitz trypsin inhibitor (KTI) protein, indicating that the presence of KTI protein was dominant to the absence of it. A part of the SDS-PAGE pattern for KTI protein that appeared in the parents and F_2 plants is shown in Figure 2.

Bands for KTI protein were segregated in F_2 generation. The segregation of F_2 generation for presence and absence

Fig. 2. Segregation of the Kunitz trypsin inhibitor (KTI) protein in the parents and F_2 plants. P1:T363, P2: “Gaechuck2ho”. +, -; presence and absence of KTI protein, respectively.

of KTI protein is summarized in Table 2.

Among 151 F_2 plants harvested, 123 F_2 plants showed KTI protein and 28 F_2 plants did not show KTI protein. The segregation ratio of the presence or absence of the KTI protein in the F_2 plants well fitted to an expected 3:1 ratio ($\chi^2=3.35$, $P=0.066$). This result shows that the absence of the KTI protein is controlled by a single recessive gene. Previous studies have shown that KTI protein is inherited as a single recessive allele (Orf and Hymowitz, 1979; Kim et al. 2006; Eun et al. 2012).

The Segregation between *d1m* allele and *ti* allele was observed (Table 3). The F_2 plant ratios of 102 normal leaf type, KTI protein present: 24 normal leaf type, KTI protein absent: 21 disease lesion mimic type, KTI protein present : 4 disease lesion mimic type, KTI protein absent were observed.

Table 1. Inheritance of *d1m* allele in segregated F_2 plant population obtained from the cross of “Gaechuck2ho” (*titiD1mD1m*) and T363 (*TiTiD1md1m*).

No. of F_2 plant	Leaf trait		Expected ratio	χ^2	P
	Normal (<i>D1m</i> _)	Necrotic (<i>d1md1m</i>)			
Observed	126	25	3 : 1	5.74	0.016
Expected	113.25	37.75			

Table 2. Segregation of the presence or absence of the Kunitz trypsin inhibitor (KTI) protein in F_2 generation from the cross between “Gaechuck2ho” and T363.

KTI Protein	No. of F_2 plant		χ^2 value (3:1)	P
	Observed	Expected		
+	123	113.25	3.35	0.066
-	28	37.75		

+, -: presence(+) or absence(-) of KTI protein.

Table 3. Segregation of *Ti* (*ti*) and *Dlm* (*dmlm*) alleles in F₂ generations obtained from the cross between “Gaechuck2ho” (*DlmDlmtiti*) and T363 (*dmlmdlTiTi*) parents.

Genotype	F ₂ generation		Expected ratio	χ^2	P
	Observed	Expected			
<i>Ti</i> _ <i>Dlm</i> _	102	84.94	9	9.106	0.027
<i>Ti</i> _ <i>dmlmdl</i>	21	28.31	3		
<i>titiDlm</i> _	24	28.31	3		
<i>titi dmlmdl</i>	4	9.44	1		

Segregation ratio gave a good fit to the 9 : 3 : 3 : 1 ratio based on F₂ generation at the 0.01 levels of probability (Chi-square value = 9.106, $P=0.027$). Chi-square value of 9.106 was higher than critical point of 7.815 at the 0.05 levels of probability and the value was lower than 11.345 point of 0.01 levels of probability. Generally, the plant with both disease lesion mimic type and KTI protein absent type (*titidlmdl* genotype) is very weak in growth. By the bad field condition, a few F₂ plant with *titidlmdl* genotype was died in early growth stage. By this reason, deviation from the 9 : 3 : 3 : 1 ratio at the 0.05 levels of probability was observed. However, at the 0.01 levels of probability, the data confirmed that *dml* allele controlling disease lesion mimic trait was inherited independently with the *ti* allele controlling absence of KTI protein in soybean. So far, the position of *Dlm* locus on classical linkage group or molecular linkage map has not been identified. The *dml* allele was inherited independently with the *PI* allele, the *y9* allele, the *f* allele and the *lf2* allele (Jeong et al. 2007). From these results, *dml* allele would not be located on classical linkage group 9 (molecular linkage group A2, chromosome 8) in soybean. These results will be useful in genetic and breeding research of soybean.

CONCLUSION

To determine the genetic relationship between *dml* allele controlling disease lesion mimic trait and *ti* allele controlling the presence or absence of Kunitz trypsin inhibitor (KTI) protein in mature seed, F₂ population was developed from the cross of “Gaechuck2ho” (*DlmDlmtiti*) and T363 (*dmlmdlTiTi*) parents. The leaf trait of parents and F₂ individual plants was recorded at R2 and R3 growth stage from field and the

random seeds from individual F₂ plants harvested was analysed to determine the presence of the KTI protein. A total of 157 F₂ plants were grown in field. 126 F₂ plants showed the normal leaf and 25 F₂ plants showed disease lesion mimic trait. The disease lesion mimic trait was matched to 3 : 1 ratio at the 0.01 levels of probability. Among 151 F₂ plants harvested, 123 F₂ plants showed KTI protein and 28 F₂ plants did not show KTI protein. The segregation ratio of the presence or absence of the KTI protein in the F₂ generation was well fitted to an expected 3:1 ratio ($\chi^2=3.35$, $P=0.066$). This result shows that the absence of the KTI protein is controlled by a single recessive gene. The ratios of 102 *Ti*_ *Dlm*_ : 21 *Ti*_ *dmlmdl* : 24 *titiDlm*_ : 4 *titidlmdl* were observed. Segregation ratio gave a good fit to the 9 : 3 : 3 : 1 ratio based on F₂ generation at the 0.01 levels of probability (Chi-square value = 9.106, $P=0.027$). The data confirmed that *dml* allele controlling disease lesion mimic trait was inherited independently with the *ti* allele controlling absence of KTI protein in soybean.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (grant number: 2010-0002513)

REFERENCES

- Birk Y. 1961. Purification and some properties of a highly active of trypsin and chymotrypsin from soybeans. *Biochem. Acta.* 54:378-381.

- Chung J, Staswick PE, Graef GL, Wysong DS and Specht JE. 1998. Inheritance of a disease lesion mimic mutant in soybean. *J Hered.* 89:363-365.
- Cregan PB, Jarvik T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, Kaya N, VanToai TT, Lohnes DG, Chung JI and Specht JE. 1999. An integrated genetic linkage map of the soybean. *Crop Sci.* 39:1464-1490.
- Fraternali V and Steiner RF. 1968. Soybean inhibitor. I. Separation of some properties of three inhibitors from commercial crude trypsin inhibitors. *Biochem.* 7:521-530.
- Hymowitz T and Hadley HH. 1972. Inheritance of a trypsin variant in seed protein of soybeans. *Crop Sci.* 12:197-198.
- Hildebrand DF, Orf JH and Hymowitz T. 1980. Inheritance of an acid phosphatase and its linkage with the Kunitz trypsin inhibitor seed protein of soybeans. *Crop Sci.* 20:83-85.
- Jeong WH, Nam KC, Park MS, Lee KJ, Kim MS and Chung JI. 2007. Independent inheritance of *d1m* allele with *lf2* and *P1* alleles in soybean (*Glycine max* L.). *Korean J. Breed Sci.* 39(2):232-235.
- Jeong WH, Nam KC, Park MS, Lee KJ, Kim MS and Chung JI. 2007. Inheritance of *d1m* gene with *f* and *y9* alleles in soybean (*Glycine max* L.). *Korean J. Breed. Sci.* 39(4):473-477.
- Kunitz M. 1945. Crystallization of a soybean trypsin inhibitor from soybean. *Science.* 101:668-669.
- Kiang YT. 1987. Mapping three protein loci on a soybean chromosome. *Crop Sci.* 27:44-46.
- Kim HK, Kim YJ, Paek KH, Chung JI and Kim JK. 2005. The phenotype of the soybean disease-lesion mimic (*d1m*) mutant is light-dependent and associated with chloroplast function. *Plant Pathol. J.* 21(4):395-401.
- Kim MS, Park MJ, Jeong WH, Nam KC and Chung JI. 2006. SSR marker tightly linked to the *Ti* locus in [soybean *Glycine max* (L.) Merr.]. *Euphytica* 152:361-366.
- Oliveira MIP, Nilton D, Piovesan ICJ, Everaldo GB, Maurilio A and Moreira LO. 2007. Protein, oil, and isoflavone contents in lipoxygenase- and kunitz trypsin inhibitor-deficient soybean seeds. *Chromatographia.* 66:521-527.
- Orf JH and Hymowitz T. 1979. Inheritance of the absence of the trypsin inhibitor in seed protein of soybeans. *Crop Sci.* 19:107-109.
- Rackis JJ, Sasame HA, Mann RR, Anderson RL and Smith AK. 1962. Soybean trypsin inhibitors: Isolation, purification and properties. *Biochem. Biophys.* 98:471-478.
- Singh L, Wilson CM and Hadley HH. 1969. Genetic soybean trypsin inhibitors separated by disc electrophoresis. *Crop Sci.* 9:489-491.
- Eun HH, Sung MK, Baek WJ, Shim SI, Kim MC and Chung JI. 2012. Inheritance of Kunitz trypsin inhibitor and P34 protein in soybean seed. *Korean J. Crop Sci.* 57(1):78-82.