Plant Type Variation Induced by Grafting and the Related Genes Analysis in Pepper©

Takumi Katano, Hiroaki Miyazawa, Katsuaki Suzuki, and Yutaka Hirata
Laboratory Plant Breeding & Genetics, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509
Email: yhirata@cc.tuat.ac.jp

Investigations into the variant characters of graft-induced pepper strain "Peaton" (*Capsicum annuum* L.) have been carried out in our laboratory for many years. Although the high branching character of Peaton is an important horticultural character, the mechanism causing high branching is unknown. From previous studies, plant type such as branching pattern could be regulated by plant hormones. Specifically, the interaction of auxin and cytokinin could be a good clue to understand the mechanism of Peaton's high branching ability.

A decrease in auxin sensitivity was found by two kinds of auxin sensitivity tests. Therefore, two genes, *AXR1* and *PIN1* that relate to auxin-signal pathway, were isolated and sequenced, and G specific sequence was seen in *PIN1*. Future studies investigating the expression and sequencing of these genes would be important in understanding this phenomenon.

INTRODUCTION

The graft-variant line of pepper "Peaton" was induced by Yagishita around 50 years ago (Yagishita, 1964). Firstly, 'Yatsubusa' (Y) pepper was grafted as the scion on Spanish paprika (Sp) understock by the "mentor grafting" technique. Seeds that were derived from the fruit of the scion were sown. To increase the variation ratio, "repeat grafting" was carried out. After repeated grafting (five times), we could obtain the graft-induced variants and finally establish the stable line Peaton (G). In this study, we used a G line that has been maintained for more than 45 generations.

Graft-variant line G has wide intermediate phenotypes between Sp and G such as fruit shape, capsaicin content, sugar content, length of stoma, etc. (Yagishita, 1979). However we could observe not only such intermediate phenotypes but also G specific characters that include a high-branching character, larger number of nodes, and ability of regeneration from young cotyledon that we have not observed in Sp and Y.

Characteristics of axillary branch number and node number would be principal factors of "plant type" that construct the spatial arrangement of plant organs. A typical practical introduction of such plant types into agriculture is in the breeding of the semi-dwarf rice cultivar "IR8". In addition, we can see a deceased number of culms in the process of domestication of wild maize species by South American natives. The decease in number of culms enhances sink function of grains and enlarged the edible portion of maize. Therefore, plant type breeding has been playing an important role in breeding from ancient times to the present.

Inheritable graft-induced variation has occurred in other plants, such as eggplants and soybean (Hirata, 1979); however, such graft-induced variation is rarely

applied to agriculture breeding today. Therefore, understanding the genetic basis of G specific character that is horticulturally important would have great value in the near future. Because it will have a significant effect, we not only searched for useful genes but also the mechanism of graft-induced variation and its application in breeding science.

It is well known that plant growth regulators (PGRs) have large effects on plant morphology; especially, auxin and cytokinin in apical dominancy. Therefore, different branching patterns may be due to the difference in amount of synthesized PGR or PGR sensitivity. In fact, auxin-resistant mutant *AXR1* (auxin resistant1) of *Arabidopsis* has high branching phenotype (Lincoin, 1990).

Through molecular biological studies the teosinte branched1 gene (*TB1*) that controls number of culms was identified with QTL analysis of maize and teosinte (Doebley, 1995). Zinc finger protein *PetSPL3,* whose over-expression in plants induces a high-branching character, was found in petunia (Takatsuji, 1999). In addition, the *max* mutant in *Arabidopsis* and *rms* mutant in pea, which control branching, were investigated, and the source genes were identified. With the above research as a background, the present study was carried out to examine the hormonal involvement in the high branching character with genetic and physiological research methods.

MATERIALS AND METHODS

Plant Material. In this study, we used a G line that was purified and maintained by 47 times selfing of the graft-induced variant. In the experimental lines of Sp and Y, we also maintained their pure lines that had been used for making graft-induced variant (Figs. 1 and 2) by repeat selfing.

Investigation of Number of Axillary Branches and Nodes in Each Line. We surveyed axillary branch number and nodes in Sp, G, Y, and F_1 progenies resulting from their crosses in 2004 and 2005. Investigational time was the stage when plants stopped their vegetative and generative growth. The stem that had the largest number of branches was determined and evacuated as a main stem.

Microscopic Observation of Axillary Bud. The fourth nodes of plantlets 8 weeks after seed sowing were observed under a microscope. First, the part of the nodes was excised and embedded into an agarose gel. Agarose blocks were sliced into 60 μm widths by a micro slicer. Using these segments the growth degree of the axillary meristem was determined.

Auxin Sensitivity Test. We carried out two kinds of tests to investigate auxin sensitivity. One was the "decapitation test" and the other the "culture reaction test."

Decapitation Test. Plantlets of G and Y, 12 weeks after seed sowing, were used in the decapitation test. These plantlets were cut just under the 6th node, and a Vaseline paste containing 0, 0.1, and 0.5 $mg \cdot L^{-1}$ NAA was applied. Cut surfaces were covered with plastic caps. The number of branches was counted after 2 weeks to determine auxin response.

Culture Reaction Test. Seeds of each line were sterilized and sown on an aseptic medium in the auxin reaction test. After 1 month, cotyledonary nodes were cut around 5 mm from the upper and from the down part of nodes. These nodes were put between two kinds of media with cutting surface touching each medium (Fig. 3).

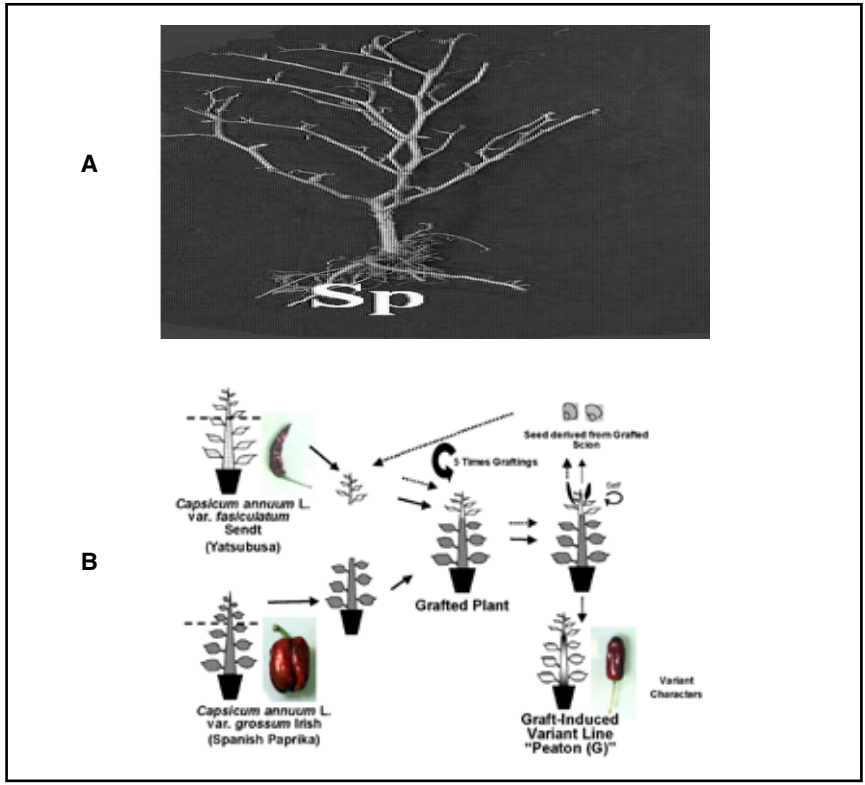

Figure 1. (A) Plant type difference of final growing stage in each line. Left to right; Sp, G, and Y. (B) Proceeding of graft-variant strain Peaton (G).

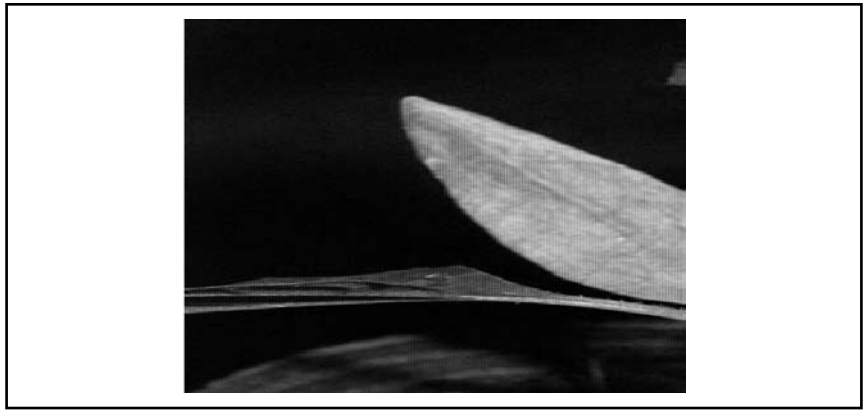

Figure 2. NAA application method in decapitation test.

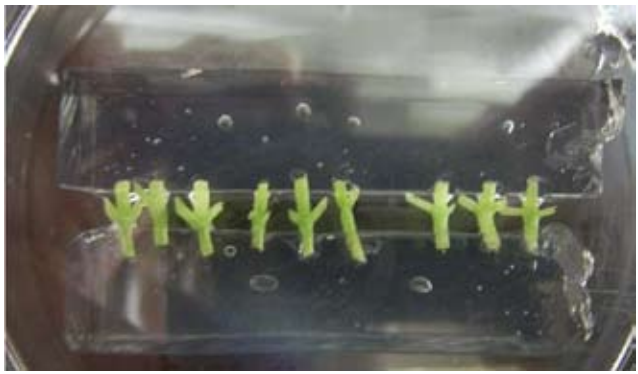

Figure 3. Auxin sensitivity test by using tissue culture. Apical medium: Murashigi and Skoog (MS) solid medium containing 0, 0.02, and 0.1 mg·L^{-1} NAA; basal medium: hormone free MS solid medium.

Solid Murashigi and Skoog (MS) medium containing 0, 0.02, 0.1, or 0.5 mg·L^{-1} of NAA were used as upper (side of shoot tips) medium. And hormone-free medium were used as lower (root side) medium.

Isolation of Auxin Signal Pathway Related Genes. We isolated from pepper putative *AXR1*, which relates to auxin signal pathway and whose mutant expresses high branching character. Two primers (forward: CCN GAY CAY TTY YTN GAY GAY, Reverse: RAA RTC NGC YTC NGC YTT NGC) that were used for amplification of *AXR1* had been designed from common sequence of *A. thaliana*, *Solanum demissum*, and *Oryza sativa*. cDNAs, which were synthesized from immature fruit, RNA of Sp, G, and Y were used as template for PCR under the following condition: initial denature at 94 °C for 5 min; setting of 35 cycles: denaturation for 30 sec at 94 °C, annealing for 30 sec at 55 °C, and extension for 1 min at 72 °C; final extension for 10 min at 72 °C. The amplified bands whose size was around 550 bp were introduced into vector and determined sequence by TA cloning and ABI prism 377 sequencer.

To isolate pepper putative *PIN1* that relate to auxin transfer, another two primers (forward: GAY YTN CAY ATG TTY GTN TGG, reverse: AAI GGN ACD ATN CCY TGN GG) were designed from common sequence of *A. thaliana*, *Momordia charantia*, and *O. sativa*. cDNAs that were synthesized from leaf RNA of Sp were used as template for PCR condition: initial denature at 94 °C for 5 min; setting of 35 cycles: denaturation for 30 sec at 94 °C, annealing for 30 sec at 50 °C, and extension for 45 sec at 72 °C; final extension for 10 min at 72 °C. Sequencing was performed according to the procedure mentioned above. More stable primers (forward: TTC AGT TGA TGA TGT CAT GTC, reverse: TTA TCC TTG TTA AGG TGG CAG) were constructed from determined sequence to amplify *PIN1* of G and Y. *PIN1* of G and Y was also determined.

Table1
The final number of nodes and branches of 'Sp', 'G' and 'Y' in 2004 and 2005

Materials	No. of nodes 2004	2005
Sp	11.76 ± 2.77 (17)	18.5 ± 1.2 (10)
G	17.71 ± 2.23 (14)	24.6 ± 1.83 (10)
Y	16.08 ± 1.26 (13)	21.0 ± 0.85 (5)
Sp × G (F_1)	14.5 ± 1.20 (8)	
Y × G (F_1)	15.21 ± 2.54 (24)	22.3 ± 0.99 (10)
Sp × Y (F_1)	13.2 ± 1.42 (15)	20.8 ± 1.78 (5)

Materials	No. of branches 2004	2005
Sp	3.56 ± 1.89 (18)	10.3 ± 0.62 (10)
G	11.67 ± 2.10 (12)	18.6 ± 1.12 (10)
Y	7.54 ± 2.07 (13)	11.4 ± 1.43 (5)
Sp × G (F_1)	2.38 ± 2.13 (8)	
Y × G (F_1)	7.17 ± 2.46 (24)	14.4 ± 0.79 (10)
Sp × Y (F_1)	2.73 ± 2.09 (15)	10.6 ± 0.92 (5)

Materials	Branching rate (No. of branches/No of nodes) 2004	2005
Sp	0.30	0.57 ± 0.04 (10)
G	0.66	0.77 ± 0.04 (10)
Y	0.47	0.55 ± 0.08 (5)
Sp × G (F_1)	0.16	
Y × G (F_1)	0.47	0.66 ± 0.05 (10)
Sp × Y (F_1)	0.21	0.53 ± 0.07 (5)

A: The number of nodes
B: The number of branches
C: The number of branches/nodes
*, **, *** and n.s.; respectively significant difference at 5%, 1%, 0.1% level and no-significant difference in T-test.

Table 1. Change of branch numbers by NAA application in decapitation test.

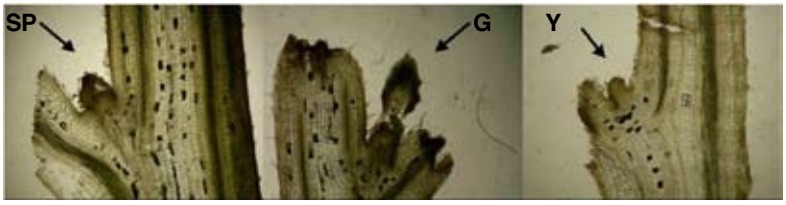

Figure 4. Microscopic observation of axillary buds. Fourth node was observed in each line.

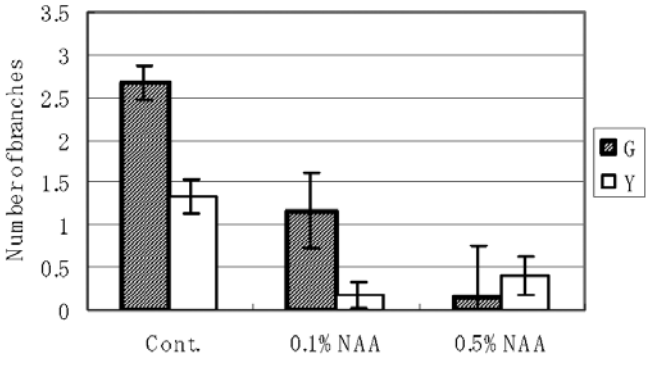

Figure 5. Change of branch numbers by NAA application in decapitation test.

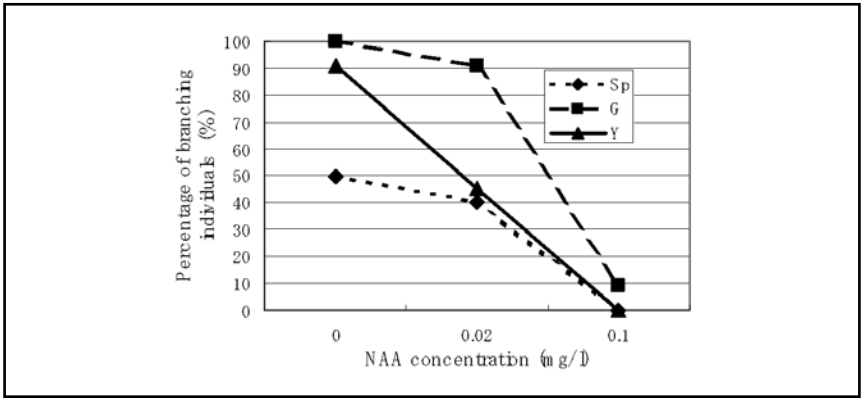

Figure 6. Change of branch formation rates by concentration of applied NAA solution in auxin sensitivity test using tissue culture. Evaluation was performed 1 week after application.

RESULTS

Number of Branches and Nodes in Each Line. Although the strengths of plant growth were different in 2004 from 2005, trends of number of branches and nodes were same in both years (Table 1 A and B). The largest number of branches and nodes occurred in G, Y was next, and Sp had the least. The F_1 progenies have similar phenotype with a pair of their parent that has less number of branches and nodes except the combination of Sp × Y in 2005.

G also had the highest average branching rate number (number of branches divided by number of nodes), Y was next, and Sp had the least number (Table1 C). From this result, high branching character in G was attributed to not only increasing nodes but also branch formation rate at the nodes was increased in G line.

Microscopic Observations of Axillary Buds. Developed axillary buds were observed among Sp, G, and Y (Fig. 4). Even further expanded leaf was seen but only in G. From this result, there was no difference in strength of axillary bud formation among Sp, G, and Y. Therefore the dormancy-breaking level or timing might be different among the lines.

Auxin Sensitivity Test.

Decapitation Test. In this control application that is performed by applying vaseline paste without auxin onto cut surface, G had 2.67 branches and Y had 1.33 branches (Fig. 5). However comparing application of 0.1% NAA and control, Y had $^1/_{10}$ (X = 0.17) branches and G had $^1/_2$ (X = 1.16) branches in 0.1% NAA application. From these results, we could estimate possibility of decreasing auxin sensitivity in G.

Culture Reaction Test. When a medium containing 0.5 $mg \cdot L^{-1}$ NAA was used as an apical medium, nodes formed calli on the cut surface and transformed their shape. In the control application (no NAA), axillary branches appeared in almost all cultured nodes of G and Y (Fig. 6). However in the application of 0.02 $mg \cdot L^{-1}$ NAA, 45% of nodes of Y formed axillary branches, 90.9% of nodes of G were formed. Consequently, we could confirm the decreasing of auxin sensitivity in G.

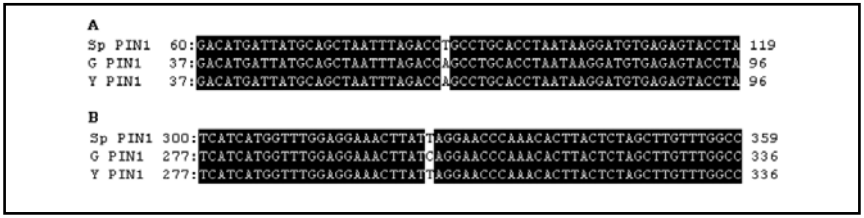

Figure 7. Partial sequence of *PIN1* derived from Sp, G, and Y.

Auxin Signal Pathway-Related Gene Isolation. In isolation of *AXR1*, separated bands around 550 bp in Sp, G, and Y were cloned and sequenced. Sequence of this gene has about 74% identity to *AXR1* of *A. thaliana*. From the determined sequence, gene specific primers were designed, and the stable band of *AXR1* from pepper was obtained. Detail of difference among Sp, G, and Y are being investigated.

In isolation of *PIN1*, separated bands around 650 bp in Sp and around 600 bp were cloned and sequenced. The 650 bp of Sp has 74% identity to auxin transport protein (*PIN1*) of *Populus tremula*. Moreover Sp-specific sequence (Fig. 7A) and G-specific (Fig. 7B) sequence were identified in their sequences.

DISCUSSION

In plant morphology, mutants of axillary branch formation are divided into three groups (Ward and Leyser, 2004). First mutant group has the changes in axillary branch formation, second has the changes in degree of breaking dormancy of axillary buds, and third has both. From the results of microscopic observation of axillary buds, Sp and Y have the same strength of axillary bud formation as G that has a high-branching character. Therefore, G is classified into second group; namely, high branching character of G is due to the difference of breaking dormancy of axillary buds.

It is generally known that dormancy of axillary bud was broken when the shoot apex is excised. Auxin that is constantly supplied to the shoot apex suppresses axillary bud outgrowth. When auxin is removed by excision of the shoot apex, suppression of outgrowth is stopped and dormancy breaking occurs. Taking this fact and G-specific decreased auxin sensitivity into consideration, it is thought that decreased auxin sensitivity may cause the high-branching character in G.

From this result, *AXR1* and *PIN1* genes that relate to auxin signal pathway was isolated by degenerated PCR. The sequence of *AXR1* has not been determined yet, however G specific sequence was determined in *PIN1* (Fig. 7). It would be a subject worth investigating whether these sequences cause the phenotypic difference.

In a previous study of auxin signal pathway, it was demonstrated that *AXR1* plays a significant role in the early stage and relates to almost all auxin signal transmission (Bennett, 2006). Therefore, not only sequence difference but also gene expression level of *AXR1* might cause the different phenotypic pattern. In addition, gene expression level of *PIN1* is also controlled by *MAX* series of genes whose mutants in *A. thaliana* express high-branching character. For that reason, expression

pattern of *PIN1* might also affect plant morphology. Therefore, it is necessary that the expression pattern of these genes in various tissues should be investigated by northern hybridization or real-time PCR.

Max1 mutant of *A. thaliana* that has a high-branching character also has rounder leaves in rosette phyllotaxis as a pleiotropic expression (Lazar and Goodman, 2006). Interestingly, G has also rounder leaves comparing with Sp and Y (Yagishita, 1979). The distribution of endogenous cytokinin in pepper leaf may affect partial expansion of leaf area (Ulvskov et al., 1992). It is well known that cytokinin and auxin have an antagonistic effect. Moreover, it is reported by Sugitani (1996) that cotyledons of G have different culture reaction. From these situations, high-branching character and changing in the ratio of width against length of leaves in G might be due to the different auxin sensitivity. At the present, we are investigating the leaf shape by Fourier-discriptor to analyze from the standpoint of genetic science (Suzuki, 2006).

This hormonal control research on graft-induced changes will be one key for the dynamic morphological change of the mechanism.

LITERATURE CITED

Bennet, T., T. Sieberer, **B. Willett**, **J. Booker**, **C. Luschnig,** and **O. Leyser.** 2006. The Arabidopsis *MAX* pathway controls shoot branching by regulating auxin transport. Current Biol. 16:553–563

Doebley, J., **A. Stec,** and **C. Gustus.** 1995. *Teosinte branched1* and the origin of maize: evidence for epistasis and the evolution dominance. Genetics. 141:333–346.

Hirata, Y. 1979. Graft-induced change in egg plant (*Solanum melongena* L.). Changes of the hypocotyl color in the grafted scions and the progenies from the scions. Jpn. Breeding 29:323–328.

Hirata, Y., and **N. Yagishita.** 1986. Graft-induced changes in soybean storage proteins. I. Appearance of the changes. Euphytica 35:395–401.

Lazar, G., and **H.M. Goodman.** 2006. *MAX1*, a regulator of the flavonoid pathway, controls vegetative axillary bud outgrowth in *Arabidopsis*. Proc. Natl. Acad. Sci. 103:472–476.

Lincoin C., J.H. Britton, and **M. Estelle.** 1990. Growth and developments of the *AXR1* mutants of *arabidopsis*. Plant Cell 2:1071–1080.

Sugitani, C. 1996. Tissue culture response in graft-induced variant strain in *Capsicum annuum* L. Tokyo univ Agri & Tech, Bachelor Thesis.

Suzuki, K. 2006. Numerical analysis of the morphogenesis mechanism in graft-induced variation in chili pepper (*Capsicum annuum* L.). 27th intl. hort. congress exhibition abstr. 228.

Takatsuji, H. 1999. Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science. Plant Molecular Biol. 39:1703–1078.

Ulvskov, P., T.H. Nielslen, P. Seiden, and **J. Marcussen.** 1992. Cytokinins and leaf development in sweet pepper (*Capsicum annuum* L.). Planta. 180:70–77.

Ward, S.P., and **O. Leyser.** 2004. Shoot branching. Current opinion in plant biol. 7:73–78.

Yagishita, N. 1964. On the induced heritable variation in *Capsicum* by means of grafting. Bull. Fac. General Educ., Tokyo univ Agri & Tech 3:1–48. (in Japanese).

Yagishita, N. 1979. Some characteristics found in the progeny from the variant of *Capsicum annuum* obtained by means of grafting. Jpn. J. Breed., 29 (Suppl. 2), 56–57. (in Japanese).