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STUDY ON DETECTION OF SPECIFIC COLOR GENE INTRODUCED INTO CARNATION

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Abstract

The objectives of this study is to design primer pairs which will amplify a part of transgene (flavonoid3' and 5' –hydroxylase) and discriminate between non-GM carnation variety and GM carnation variety with DNA markers. The sequence of the transgene was unknown. Then it had to be searched on Patent and Utility Model Gazette Database on the web site of Japan Patent Office and on the DNA Database Gene Bank (NCBI) to design primer pairs. The sequences of candidate primer pairs were designed automatically by OLIGO 6.0 (Molecular Biology Insights, USA) with information of desired length of amplified products. As a result, two primer pairs were prepared. PCR conditions, especially for annealing temperature, concentration of $MgCl_2$ and number of cycles, were optimized. Three individuals of non-GM variety and GM variety had been discriminated with GM variety specific bands amplified by two primer pairs respectively. A process to detect certain transgene by DNA markers is very short, then combination of this method with conventional DUS test will (have a possibility to) improve efficiency of present DUS test.

I. Introduction

1. National Center for Seeds and Seedlings (NCSS) in Japan conducts DUS (Distinctness, uniformity and Stability) test to provide the data for examination to clarify whether the candidate varieties are new or not. During DUS testing, candidate varieties are cultivated on farms or in greenhouse and compared with similar existing varieties for morphological characteristics (color, shapes, size, etc.) and physiological (resistance to disease and pests, etc.) characteristics.

2. In general, phenotypic characteristics are likely to be influenced by environmental conditions and it makes field examination difficult. But DNA markers are independent of environmental conditions. Moreover DUS test based on the phenotype takes long period to judge whether candidate varieties are new or not. In contrast, DNA markers can discriminate among plant variety very quickly. So combining traditional methods with DNA markers has a possibility to make the current DUS test more efficient and precise.

3. Research section of NCSS has been studying the methods of discrimination plant varieties on the basis of DNA markers.

4. Genetic engineering techniques of biotechnology are rapidly progressing to enable the creation of transgenetic plants (genetically modified organisms: GMO) with external genes from other organisms like bacteria for characters as resistance to diseases and insects.

5. During recent years, genetically modified plants are being developed in some kind of major crops such as soybean, maize, cotton and potato.

6. Genetically modified carnation was applied for registration and NCSS had conducted DUS test in FY 2002, Research section of NCSS has tried to detect the gene introduced into GM carnation.

7. Objective of this study is to make a manual for the detection of a part of specific gene introduced into plant which is applied for registration. Especially, we focused on the case with no previous knowledge of DNA sequence of transgene beforehand.

8. Also at present BMT meeting have been discussing to consider model for the use of biochemical and molecular techniques in DUS testing. So we have tried the case study for option 1 related to gene specific makers that are introduced by genetic modification. In this study, the genetically modified carnation developed by using the purple color gene of petunia origin was used.

II. Materials and methods

a) Design of primer pairs for amplifying a part of gene introduced into carnation

9. GM carnation has introduced the gene of the flavonoid 3' and 5' – hydroxylase activity. The DNA sequence information of transgene was needed to design sequences of primer pair which would amplify a part of gene by PCR. To get the sequence information of the gene, firstly we searched for the DNA sequence information of the gene on Patent and Utility Model Gazette DB on the web site of Japan Patent Office. Secondly, same gene was searched also on the DNA Database GeneBank (National Center for Biotechnology Information). Candidate primer pairs sequences were designed automatically by OLIGO 6.0 (Molecular Biology Insights, USA) after inputting desired length of .amplified products.

b) DNA extraction

10. Materials used for in this study were original variety (three plants) and GM carnation derived from original variety(three plants). Main phenotypic difference was flower color. Flower color of Non-GM plants is cream and GM is violet.
Genomic DNA was extracted from frozen leaves of greenhouse-grown three individuals of non-GM variety and three GM variety according to the CTAB procedure.

c) PCR conditions and detection of amplified products

11. Three factors (annealing temperature, $MgCl_2$ and number of cycles) were examined how they would affect amplification of bands:
PCR-cycling conditions consist of an initial denaturation and activation for AmplitaqGold of 95°C for 10min followed by 30 or 40 cycles of 60s at 94℃, 60s at 60℃ or 65℃ and 60s at 72℃. A final extension step of 7min at 72℃ ended the cycle. Concentrations of $MgCl_2$ examined were 1.5mM or 2.5mM. PCR-cycling was carried out by GeneAmp PCR System 9700(PE-Applied Biosystems, USA)
Amplified products were loaded on 1.8% Metaphor agarose gel with running condition 80min at 15V/cm at constant 15℃ and then stained with Ethidium Bromide solution. After staining, gel was photographed under UV condition.

III. Results and Discussion

a) Design of primer pairs

12. Two primer pairs (with different size of amplified product) were selected from many candidate primer pairs which Primer analysis software 'OLIGO 6.0' designed. One of them would amplify product of 561bp (named Carn-S) and the other would amplify product of 1104bp (named Carn-L). Figure 1 showed sequences of two primer pairs

Carn-S Forward 5' GGTTGTGGTGGCGGAGAT 3'.
Reverse 5' CAAATTGCTCGGAGGTAAGG 3'
Carn-L Forward 5'ATGGCAGTTGCTTCTACCC 3'
Reverse 5' TGCACAAATTCTTCGTCCAG 3'

Fig. 1 Sequences of primer pairs

b) PCR conditions and band detection

13. Low annealing temperature (at 60℃), 2.5mM $MgCl_2$ and 40 cycles conditions tended to amplify non-specific bands. 65℃, 30 cycles and 1.5mM $MgCl_2$ condition could amplify only specific bands with two primer pairs respectively (Fig.2)

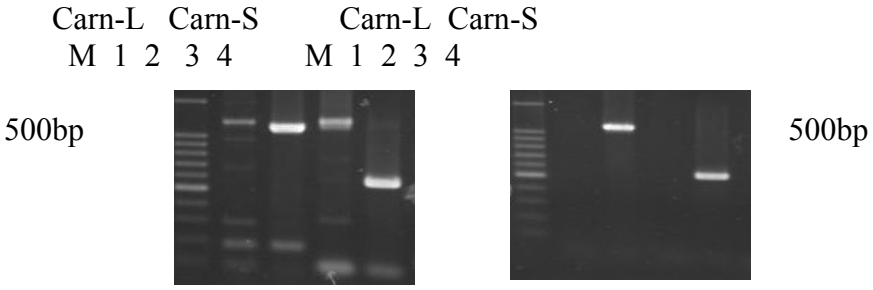

Fig.2 Amplification of specific bands and non - specific bands
Lane 1and 3: non-GM Lane 2 and 4 : GM
Left: 60℃,2.5mM $MgCl_2$ and 40cycles non-specific bands were visible in all lanes
Right: 65℃,1.5mM $MgCl_2$ and 30cycles
Only specific band was visible
M: size marker 100bp ladder

PCR-cycling conditions adopted finally consist of an initial denaturation and activation for AmplitaqGold of 95℃ for 10min followed by 30 cycles of 60s at 94℃, 60s at 65℃ and 60s at 72℃. A final extension step of 7 min at 72℃ ended the cycle .

c) Morphological difference between GM and Non-GM carnation

Both carnation's characteristic show the different type of ground color only as table 1. And the other characteristics are apparently quite same except for anthocyanin coloration of anthers.

Table 1 Flower color of Non-GM and GM carnation

Varieties	Origin	Ground Color* (states)
Applicant Variety	Genetic modified Carnation	JHS color chart;8605(vivid purple)
Original Variety	*Cream Cinderella*	2902(yellowish white)

Foot note; One (ground color) out of 92 characteristics items for dianthus plant
* means important characteristic

GM carnation Non-GM

Upper: GM carnation Down; Non-GM

Fig.3 Flowering stage at field trial Fig.4 Flower color of GM and Non-GM

d) Discrimination between non-GM and GM carnation

14. Genomic DNA extracted from three individuals of non-GM variety and three GM variety each had been tried to amplify respectively. Specific bands could be amplified from only genomic DNA extracted from GM variety with both primer pairs (Fig.5).

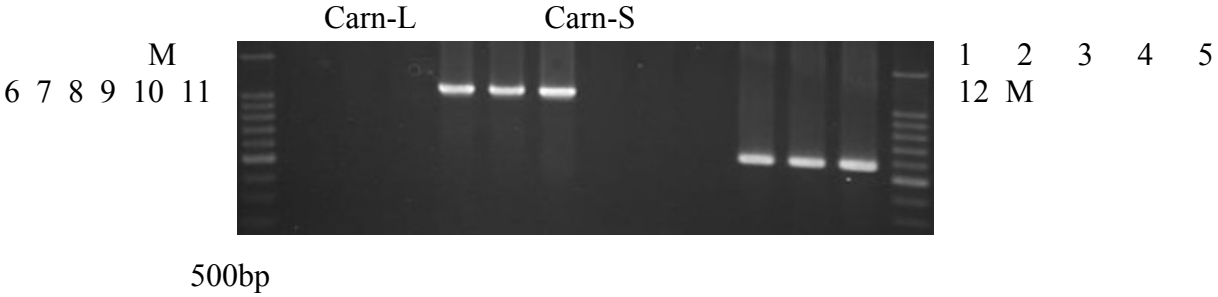

Fig.5 Amplified products from genomic DNA of GM plants
Lane 1, 2, 3, 7, 8, 9: non GM no band
Lane 4, 5, 6, 10, 11, 12: GM specific band
M: size marker,100bp ladder

15. Important issue of this study is how do we get the DNA sequence information of transgene. As described above, if we can get the DNA sequence information of transgene , it is comparatively easy to design primer pair and to discriminate between non-GM and GM plants. Method of comparing phenotypic characteristics is most reliable under conventional DUS test, but it takes longer period to check the expression of a specific characteristics which is directly linked to transgene than to check presence or absence of the transgene by DNA markers.

References

1. Japanese Patent Office document FY1994 NO.6-500239: “DNA sequence that code enzyme of flavonoid 3’ and 5’-hydroxylase activity and its usage”. Seeds and Seedlings Division, MAFF, Japan (1995) Test Guidelines for Dianthus.

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