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ASSESSMENT OF GENETIC VARIATION AND PRESENCE OF NITRATE REDUCTASE GENE (*NR*) IN DIFFERENT LETTUCE GENOTYPES USING PCR-BASED MARKERS

[42]

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ABSTRACT

Thirteen lettuce genotypes belonging to four lettuce types (Butterhead, Cos/Romaine, Crisphead, and Stem lettuce) were investigated using RAPD fingerprints as genetic marker to assess genetic diversity. Also, the presence of nitrate reductase gene (NR) in lettuce genotypes was investigated by PCR assays. From 30, random primers tested, only 13 were reproducible, giving 85 bands. The genetic similarity was estimated based on the pair-wise comparison of amplification products. The thirteen lettuce genotypes showed variation at the DNA level. The UPGMA cluster analysis separated the 13 lettuce genotypes into two distinct groups. Moreover, gene-specific primer pairs for amplification of nitrate reductase revealed the presence of nitrate reductase gene in Ambassador, Bath, Merveille des Quatre, Romain de Benicardo, Colona and Chinese stem genotypes only. The work presented in this paper illustrated that sensitive specific PCR assays represent a valuable and a new tool for screening lettuce breeding material for low nitrate content.

Key Words: Lettuce, Nitrate, Genotypes, PCR, Marker.

INTRODUCTION

Some species of vegetables, such as beet (*Beta vulgaris* L.) celery (*Apium graveolens* L. var. *dulce* (Mill.) Pers.), lettuce (*Lactucas sativa* L.), and spinach (*Spinacia oleracea* L.), can accumulate more than 2500 mg nitrate per kg fresh weight of leaf tissue (**Blom-Zandstra, 1989**). High amounts of nitrate in edible plant parts may adversely affect both human and animal health due to the reduction of nitrate to nitrite, which is a toxicant and a precursor of other harmful, and possibly lethal, compounds (**Lee, 1970; Hodgson & Levi, 1997 and Al-Redhaiman, 2000**).

Nitrate reductase (NR, EC 1.6.6.1) is the first enzyme of the nitrate assimilation pathway in higher plants. It reduces the major plant nitrogen source, NO^-_3, into NO^-_2, which is then further reduced to NH_3 by NO^-_2 reductase (**Redinbaugh & Campbell, 1991; Pelsy & Caboche, 1992 and Crawford, 1995**) .While variation in nitrate accumulation in lettuce plants can be caused by environmental conditions (especially light intensity) and

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by nitrogen fertilizer source, a body of literature also reports significant genetic effects (**Reinink *et al* 1987; Reinink, 1992 and Belligno *et al* 1996**).

Information on the genetic diversity in breeding materials is essential for the optimal design of plant breeding program for low nitrate content (**Van Hintum, 2003**). In horticultural crops, RFLP (restriction fragment length polymorphisms) markers have been used successfully to identify cultivars of a wide variety of species (**Hubbard *et al* 1992; Parent & Page, 1992; Vosman *et al* 1992 and Bowers *et al* 1993**). Recently, a modification of the automated polymerase chain reaction (PCR) has created another class of molecular markers, i.e. random amplified polymorphic DNAs (RAPD) (**Williams *et al* 1990**). Compared to RFLPs, the RAPD procedure is a fast and sensitive method that avoids using radioactive isotopes and is well suited for studies of many samples (**Thormann and Desborn, 1992**). Therefore, RAPD analysis can be used to identify many useful polymorphisms quickly and efficiently, and as such, it has tremendous potential for use in cultivar identification (**Lu *et al* 1996**). The objectives of this study were to (1) estimate the genetic relationship between lettuce genotypes based on RAPD markers and (2) investigate the presence of nitrate reductase gene (NR) in lettuce genotypes for low nitrate content by PCR analysis.

MATERIAL AND METHODS

Plant material and DNA extraction

Thirteen lettuce genotypes belonging to the following types: Butterhead (Ambassador, Augusta, Bath, Kennedy, Lilian, and Merveille Des Quatre Saisons genotypes), Cos/Romaine (Little Gem, Lobjoit's Green, and Romain de Benicardo genotypes), Crisphead (Saladin and Colona genotypes), and Stem lettuce (Chinese stem and New Chicken genotypes) were chosen to form as broad a genetic base as possible.

The extraction of genomic DNA was done following the modified CTAB procedure (**Hoisington *et al* 1994**). The concentration of extracted genomic DNA was adjusted to 10 ng/µl. Lettuce genotypes were duplicated after DNA extraction, and reduplicated in consecutive steps of RAPD analysis, to test reliability and reproducibility of the RAPD protocol.

Specific pcr amplification for the *NR* gene

Specific primer pairs for amplification of nitrate reductase gene were as follows: forward primer, 5'-GGTAGGCGATTGGCTAACATTG TCTGC-3' reverse primer 5'-GAGACAC-CAACAGTCTTTCCTCTGCG-3' (**Sherameti *et al* 2002**). Amplification was carried out in 25 µL reaction volumes, containing 1X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each dNTPs (Pharmacia Biotech, Germany), 25 pmol primer, and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for 1 cycle of 30s at 94°C; and 40 cycles of 1 min at 94°C, 1 min at 63°C, and 1 min at 72°C; followed by 5 min at 72°C. An aliquot of

10 µl from each reaction product was resolved by electrophoresis on 1.5% agarose gel in 1X TBE buffer, stained with ethidium bromide, and visualized with UV light.

RAPD amplification

Thirty RAPD analysis was conducted in a thermal cycler (Thermolyne Amplitron) using 13/30 random primers obtained from Operon Technology, USA. The conditions reported by **Williams *et al* (1990)** for creating RAPD markers by PCR were optimized for use with lettuce template DNA. PCR products were visualized along with a DNA marker on 2% agarose gel with 1X TBE buffer and detected by staining with ethidium bromide. Gels were photographed on Polaroid films under UV light.

Statistical analysis

The bands were scored manually as present (1) or absent (0), and transferred to a binary matrix. Only distinct and major bands were analyzed using NTSYSpc **(Rohlf 1993)**. Genetic similarity (GS) between the two genotypes i and j was calculated according to the formula of **Jaccard (1908)**, using the SIMQUAL module of the NTSYSpc.

$$GSij = Nij / (Nij + Ni + Nj)$$

Where: Ni is the number of detected bands in the genotype i and not in genotype j, Nj is the number of detected bands in the genotype j and not in genotype i , and Nij is the number of bands common to genotype i and j .

The generated similarity matrices were further analyzed using the UPGMA (unweighted pair group method using arithmetic averages, **Sneath and Sokal, (1973)** clustering method in the SAHN module of the NTSYSpc . Dendrograms were created using the TREE module.

RESULTS AND DISCUSSION

Extent of polymorphism revealed in lettuce by the RAPD technique

Thirty primers of arbitrary nucleotide sequence were used to amplify DNA segments from 13 lettuce genotypes. For each primer evaluated, a multiple bands profile or fingerprint was produced comprising from one to five major bands plus a varying number of minor bands (Fig. 1). With most primers, the overall signal strength was good although some ambiguities arose in the scoring of minor bands. Overall, the complexity of the band profiles was similar to those obtained with other plant species **(Williams *et al* 1990)** confirming previous observations that the number of bands in RAPD profiles is independent of genome complexity **(Rafalski *et al.*, 1991)**. Of the 30 primers tested, 13 primers were selected for further analysis based on the intensity, size, and number of amplified products (85 bands). A considerable degree of polymorphism was detected with all 13 primers (Table 1). Certain amplified bands appeared to be common to several genotypes while others were present in some genotypes but absent in others (Fig. 1).

Cluster analysis of the genetic distance values was conducted to generate dendrograms indicating relationships between lettuce genotypes. UPGMA

Table 1. The 13 Selected Operon primers and the number of amplified products and polymorphic fragments.

Primers	Sequence 5` to 3`	Amplified products	Polymorphic fragments
OP-A04	*AATCGGGCTG*	7	6
OP-A05	AGGGGTCTTG	3	1
OP-A06	GGTCCCTGAC	6	6
OP-A07	GAAACGGGTG	6	4
OP-A08	GTGACGTAGG	8	7
OP-A09	GGGTAACGCC	4	1
OP-A11	CAATCGCCGT	11	7
OP-A12	TCGGCGATAG	7	5
OP-A14	TCTGTGCTGG	6	4
OP-A15	TTCCGAACCC	4	3
OP-A16	AGCCAGCGAA	9	7
OP-A17	GACCGCTTGT	9	6
OP-A18	AGGTGACCGT	5	3

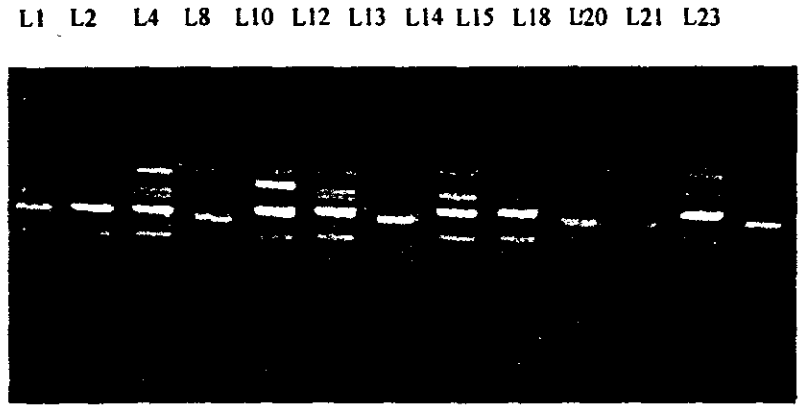

Fig. 1. Polymorphism revealed using primer OP-A06 to amplify genomic DNA purified from 13 lettuce genotypes: (*left to right*) Ambassador (L1), Augusta (L2), Bath (L4), Chinese stem (L8), Colona (L10), Kennedy (L12), Lilian (L13), Little Gem (L14), Lobjoit's Green (L15), Merveille (L18), Romain (L20), Saladin (L21) and New Chicken (L23).

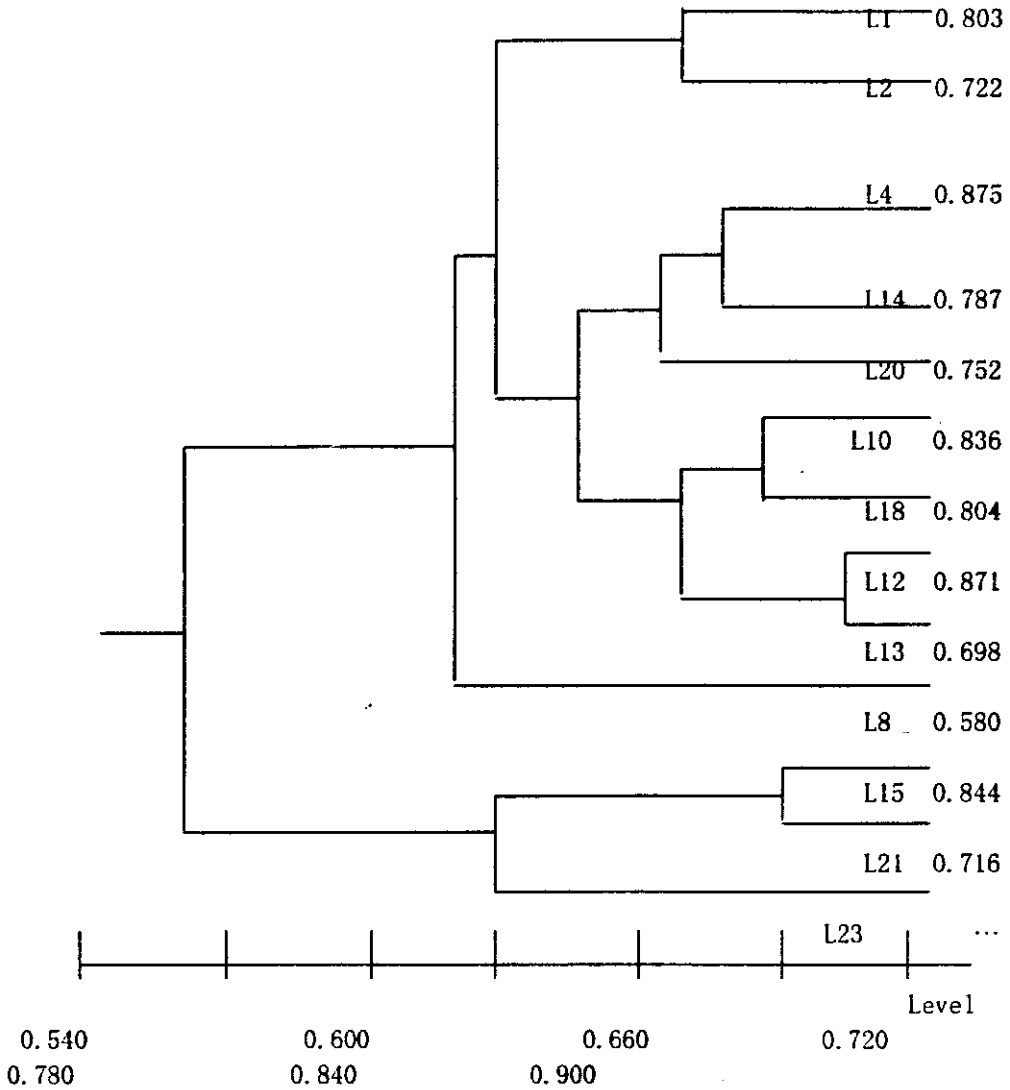

Fig. 2. Dendrogram constructed from similarity coefficients and showing the clustering of lettuce genotypes.

analysis of 13 lettuce genotypes based on 85 RAPD markers differentiated two major groups (Fig. 2). The first group contained the Butterhead type (Ambassador and Augusta, Bath, Merveille des Quatre Saisons, Kennedy and Lilian genotypes), Romaine type (little Gem and Romaine de Benicardo genotypes), Crisphead type (Colona genotype), and Stem lettuce type (Chinese stem genotype) . The second group contained Cos/Romaine type (Lobjoit's Green genotype), Crisphead type (Saladin genotype), and Stem lettuce type (New Chicken genotype). Regardless of the basis for their distinct genetic patterns; these two groups should be intercrossed in further breeding programs for low nitrate content in lettuce.

Nitrate reductase gene (NR) of lettuce genotypes

The nitrate reductase gene was amplified from Butterhead type (Ambassador, Bath and Merveille des Quatre Saisons genotypes), Cos/ Romaine type (Romain de Benicardo genotype), Crisphead type (Colona genotype), and Stem lettuce type (Chinese stem genotype). The amplification of the NR gene of these genotypes yielded one fragment of approximately 800bp long. On the other hand, the NR gene was not amplified in PCR product from other genotypes. **Escobar-Gutierrez *et al* (2002)** found that nitrate concentration showed not only great variability between lettuce cultivars in general, but also between the main lettuce types and between cultivars within the Butterhead type . Therefore, the low nitrate content in lettuce may be due to nitrate reductase genes. **Sherameti *et al* (2002)** demonstrated that the total nitrate reductase activity is regulated comparably to the expression of the nitrate reductase genes. Moreover, **Curtis *et al* (1999)** concluded that the presence of the nitrate reductase gene (NR) in transgenic lettuce was confirmed by nitrate reductase enzymatic assay, a reduction in the nitrate content of leaves and by Southern hybridization.

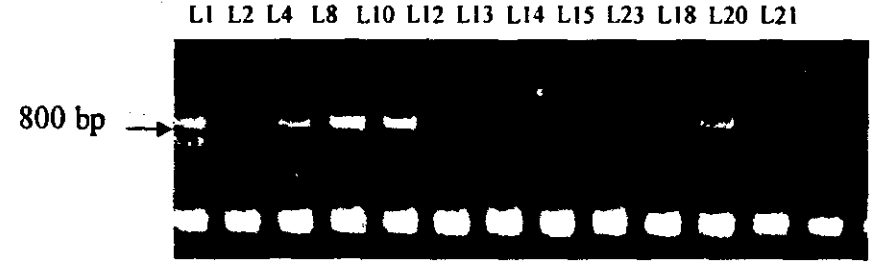

Fig. 3. Agarose gel of amplified nitrate reductase gene from 13 lettuce genotypes: (*left to right*) Ambassador (L1), Augusta (L2), Bath (L4), Chinese stem (L8), Colona (L10), Kennedy (L12), Lilian (L13), Little Gem (L14), Lobjoit's Green (L15), Merveille (L18), Romain (L20), Saladin (L21), and New Chicken (L23).

The work presented in this paper illustrated that sensitive specific PCR assays represent a valuable and a new tool for screening of lettuce breeding material for low nitrate content which will be a major objective in lettuce breeding program to limit nitrate concentration in salad crops. That could be a great importance since the high nitrate concentration can be toxic and may cause illness or even death to humans (**Al-Redhaiman, 2000**).

REFERENCES

Al-Redhaiman, K.N. (2000). Nitrate accumulation in plants and hazards to man and livestock health: *A Review. J. King Saud Univ., 12:143-156.*

Belligno, A.; G. Fisichella; M. Tropea; G. Sambuco and G. Muratore. (1996). Effects of different nitrogenous fertilizers on nitrate content of lettuce plants. 1. Comparison of a new slow-release fertilizer with traditional fertilizers. *Agrochimica 40: 85-93.*

Blom-Zandstra, M. (1989). Nitrate accumulation in vegetables and its relationship to quality. *Ann. Appl. Biol. 115: 553-561.*

Bowers, J. E.; E. B. Bandman and C. P. Meredith. (1993). DNA fingerprint characterization of some wine grape cultivars. *Amer. J. Enol. Viticult. 44:266-274.*

Crawford, N.M. (1995). Nitrate: nutrient and signal for plant growth. *Plant Cell 7: 859-868.*

Curtis, I.S.; J.B. Power; A.M.M. de Laat; M. Caboche, and M.R. Davey. (1999). Expression of a chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. *Plant Cell Reports 18: 889-896.*

Escobar-Gutierrez, A.J.; I.G. Burns, A. Lee and R.N. Edmondson (2002). Screening lettuce cultivars for low nitrate content during summer and winter production. *Journal of Horticultural Science & Biotechnology. 77: 232-237.*

Hodgson, E. and P.E. Levi (1997). *Modern Toxicology, 2 nd Ed.* pp 22-35 Appleton and Lange,Connecticut..

Hoisington, D.A.; M.M. Khairallah and D. Gonzales-de-Leon. (1994). *Laboratory. Protocols. pp. 1-25, CIMMYT Applied Molecular Genetics Laboratory,* Mexico, DF..

Hubbard, M.; J. Kelly; S. Rajapakse; A. Abbott and R. Ballard. (1992). Restriction fragment length polymorphism in rose and their use for cultivar identification. *Hort Science 27: 172-173.*

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaudoise Sci. Nat. 44: 223-270.*

Lee, D.H.K. (1970). Nitrate, Nitrites and Methemoglobinemia. *Environ. Res. 3: 484-511.*

Lu, Z.; G.L. Reighard and W.V. Baird. (1996). Identification of Peach Rootstock cultivars by RAPD markers. *Hort. Science 31: 127-129.*

Parent, J. and D. Page. (1992). Identification of raspberry cultivars by nonradioactive DNA fingerprinting. *Hort. Science 27: 1108-1110.*

Pelsy, F. and M. Caboche. (1992). Molecular genetics of nitrate reductase in higher plants. *Adv. Genet. 30: 1- 40.*

Rafalaski, J.A.; S.V. Tingey and J.G.K. Williams. (1991). RAPD markers a new technology for genetic mapping and plant breeding. *Agric. Biotech. News Info. 3: 645-648.*

Redinbaugh, M.G. and W.H. Campbell. 1991. Higher plant responses to environmental nitrate. ***Physiol. Plant, 82: 640-650.***

Reinink, K. (1992). Genetics of nitrate content in lettuce. 2.Components of variance. ***Euphytica 60: 61-74.***

Reinink, K.; R. Groenwold and A. Bootsma (1987). Genotypical differences in nitrate content in *Lactuca sativa* L. and related species and correlation with dry matter content. ***Euphytica 36: 8-11.***

Rohlf, F.J. (1993). NTSYS pc Numerical Taxonomy and Multivariate Analysis System, Version 1.8 user guide. ***Applied Biostatistics Inc O' New York pp. 6-25.***

Sherameti, I.; S.K. Sopory; A. Trebicka; T. Pfannschmidt and R. Delmuller (2002). Photosynthetic electron transport determines nitrate reductase gene expression and activity in higher plants. ***J. Bio. Chem., 277: 46594-46600.***

Sneath, P.H.A. and R.R. Sokal. (1973). ***Numerical Taxonomy.*** The principles and practice of numerical classification. ***pp. 10-45.*** W.H. Freeman and Company, San Francisco, U.S.A.

Thormann, C.E. and T.C. Desborn. (1992). Use of RAPD and RFLP markers for germplasm evaluation. In: ***Applications of RAPD Technology to Plant Breeding, pp. 9-11.*** Joint Plant Breeding Symp. Ser. Crop Sci. Amer., Amer Soc. Hort. Sci., and Amer. Genet. Assn., Minneapolis, Nov. 1992.

Van Hintum, T. (2003). Molecular characterisation of a lettuce Germplasm Collection. ***Eucarpia Leafy Vegetables 10: 99-104.***

Vosman, B.; P. Arens; W. Rus-Korteka as and M.J.M. Smulders (1992). Identification of highly polymorphic DNA regions in tomato. ***Theor. Appl. Genet. 85: 239-244.***

Williams, J.G.K.; A.R. Kubelik; K.J. Livak; J.A. Rafalski, and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. ***Nucleic Acids Res. 18: 6531-6535.***

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تحديد الاختلافات الوراثية والكشف عن نشاط جين النترات في التراكيب الوراثية المختلفة للخس باستخدام الدلائل الجزيئية بواسطة الـ PCR

[٤٢]

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درست الاختلافات الوراثية لثلاثة عشر تركيب وراثي من الخس ينتمون إلى أربعة طرز هي (طرز الخس ذات الأوراق الدهنية، طرز الرومين، طرز ذات الأوراق الخشنة وطرز الخس الساقي) باستخدام الدلائل الجزيئية بطريقة الـ RAPD. وتم أيضا الكشف عن نشاط الجين الخاص بالنترات في سلالات الخس بواسطة تحليل الـ PCR. واختير ١٣ بادئا جزيئي من ثلاثين بادئا جزيئي وأعطوا ٨٥ حزمة. وقورنت درجة التشابه الوراثي بين سلالات الخس، حيث أظهرت اختلافات على مستوى الحامض النووي DNA بين الثلاثة عشر سلالة خس. وقسمت سلالات الخس باستخدام تحليل UPGMA إلى مجموعتين كبيرتين. وكشف عن النترات في سلالات الخس حيث وجد الجين في سلالات Ambassador, Bath, Merveille des Quatre, Romain de Benicardo, Colona and Chinese stem باستخدام دليلين جزيئين متخصصين، وقد بين العمل في هذا البحث على قيمة حساسية طرق الـ PCR كوسيلة جديدة في حصر التراكيب الوراثية للخس لخفض محتواها من النترات.

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