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Effect of the Nitrate Reductase Genes (Nia) on the Quality of Different Lettuce Genotypes for Low Nitrate Content

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تأثير الجين المسؤول عن نشاط أنزيم اختزال النترات على جودة التراكيب الوراثية للخس من حيث خفض محتواها من النترات

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الخلاصة: درست الاختلافات الوراثية بين سلالات الخس التي تنتمي إلى أربعة مجموعات هي (مجموعة الخس ذات الأوراق الدهنية , مجموعة الرومين , مجموعة ذات الأوراق الخشنة و مجموعة الخس الساقي) بالنسبة لمحتواها من النترات وعلاقتها التي تنتمي إلى مجموعة 'Lobjoit's Green' بالجين المسؤول عن نشاط إنزيم اختزال النترات. وأظهرت النتائج أن السلالة 'Augusta and Kennedy' الرومين كانت اقل تركيز للنترات في الأوراق (4877.7) و سجل أعلى تركيز للنترات في أوراق سلالات و التي إلى 'Little Gem' التي تنتمي إلى مجموعة الخس ذات الأوراق الدهنية. و كان تركيز النترات عاليا في جذور السلالة التي إلى مجموعة الأوراق 'Augusta and Merveille Des Quatre Saisons' مجموعة الرومين و كذلك في جذور السلالتين الدهنية. و كان تركيز النيتروجين في السلالات التي تنتمي الي مجموعة الخس ذات الأوراق الدهنية و مجموعة الخس ذات الأوراق الخشنة أعلي من السلالات التي تنتمي الي مجموعتي الرومين و الخس الساقي. و أيضا كانت هناك علاقة معنوية جدا (0.82) بين تركيز النترات في الاوراق و تركيز النيتروجين. وكشف عن وجود الجين المسؤول عن نشاط إنزيم اختزال النترات في سلالات الخس حيث وجد الجين في سلالات Ambassador, Bath, Merveille des Quatre, Romain de Benicardo, Colona باستخدام دليلين جزيئين متخصصين و كانت هذه السلالات منخفضة في تركيز النترات في الأوراق مقارنة and Chinese stem بالسلالات التي تنتمي "Salddin" التي تنتمي إلى مجموعة الخس ذات الأوراق الدهنية و السلالة "Augusta and Kennedy" الي مجموعة الخس الخشن. وبناء عن هذه النتائج، يمكن استنتاج إلى أن الجين المسؤول عن نشاط إنزيم اختزال النترات يفيد في حصر التراكيب الوراثية للخس لخفض محتواها من النترات.

ABSTRACT: Genotypic variation in nitrate concentrations of different lettuce genotypes belonging to four types (Butterhead, Cos/Romaine, Crisphead, and Stem lettuce) and its relationship to nitrate reductase genes (*Nia)* for low nitrate concentrations of leaves was investigated. The results showed that the Romaine genotype ' Lobjoit's Green' exhibited the lowest leaf nitrate concentration (4877.7 mg/kg dry weight). The highest nitrate levels were recorded in the Butterhead genotypes 'Augusta and Kennedy'. Nitrate concentrations in the roots were significantly higher in the Romaine genotype ' Little Gem' and the Butterhead genotypes 'Augusta and Merveille Des Quatre Saisons' than the other lettuce genotypes. Total N concentrations were higher in the Butterhead and Crisphead genotypes than in the Romaine and Stem lettuce genotypes. A significant positive association ($r = 0.80$, $p > 0.01$) was observed between leaf NO_3^- concentrations and total N concentrations. Moreover, gene-specific primer pairs for amplification of nitrate reductase revealed the presence of the gene (*Nia*) in Butterhead type (Ambassador, Bath, Merveille des QuatreSaisons), Cos/ Romaine type (Romain de Benicardo genotype), Crisphead type (Colona genotype), and Stem lettuce type (Chinese stem genotype). These genotypes showed lower leaf nitrate concentrations than the other Butterhead genotypes "Augusta and Kennedy" and Crisphead genotype "Salddin" which did not amplify with the Nia gene. Based on these results, it is concluded that the gene-specific primer pairs for amplification of nitrate reductase gene (*Nia*) might be useful in screening lettuce breeding material for low leaf nitrate content.

Keywords: Low nitrate concentrations, *Lactuca sativa*, PCR, Saudi Arabia.

Introduction

Concerns about high nitrate levels in vegetable production have led to the introduction of limits on nitrate concentrations in some salad crops (Anon., 2001; Escobar-Gutierrez *et al.,* 2002). Among vegetable crops, lettuce, along with spinach, is routinely ranked as one of the highest accumulators of nitrate NO_3^- (Lorenz, 1978; Al-Redhaiman, 2000).

Differences in the capacity to accumulate NO_3^- among lettuce types (Crisphead, Butterhead, Romaine, Leaf) and their respective genotypes and cultivars are well documented (Reinink and Groenwold, 1988; Reinink, 1991). It has been shown that Crisphead cultivars contain higher NO_3^- than other lettuce types (Maynard *et al.,* 1976). Escobar-Gutierrez *et al*. (2002) found that nitrate concentration showed not only great variability between cultivars in general, but also between the main lettuce types and between cultivars within the Butterhead type as well.

Genotypic and species differences in NO_3^- accumulation may be related to differences in various physiological processes of nitrogen metabolism in plants, including uptake, reduction/assimilation, and translocation/partitioning (Al-Redhaiman, 1996). Cultivars can vary in efficiency of nitrogen assimilation, and accumulation of NO_3^- may be associated with a reduced capacity for NO_3^- reduction and a low nitrate reductase activity (NRA) (Goodman, 1979).

Nitrate reductase (*NR)* 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 and Campbell, 1991; Pelsy and Caboche, 1996; Crawford, 1995). *Arabidopsis thaliana* has two NR genes, *Nia1* and *Nia2* (Cheng *et al*., 1988; Wilkinson and Crawford, 1993). *Nia2* is responsible for 90 % of the total *NR* activity in developing wild-type seedlings, whereas *Nia1* accounts for the remaining 10 % (Wilkinson and Crawford, 1991; Yu *et al*., 1997). The presence of the *Nia* cDNA in transgenic lettuce was confirmed by nitrate reductase (*NR)* enzymatic assay and a reduction in the nitrate content of leaves (Curtis *et al*., 1999). The objectives of this study were to (1) study the genotypic variations in nitrate concentrations of different lettuce genotypes and (2) investigate the presence of the nitrate reductase gene (Nia) in lettuce genotypes and its relationship to low nitrate concentrations of leaves.

Materials and Methods

Seeds of eleven lettuce genotypes belonging to the types Butterhead (Ambassador, Augusta, Bath, Kennedy, and Merveille Des Quatre Saisons), Cos/ Romaine (Little Gem, Lobjoit's Green, and Romaine de Benicardo), Crisphead (Colona and Salddin), and Stem lettuce (Chinese stem) were introduced from Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK. Seeds of each genotype were germinated in a mixture of peat moss and vermiculite (1:1 v/v) at the greenhouse of the College of Agriculture and Veterinary Medicine, Al-Qassim University, Saudi Arabia.

The seedlings of each genotype were produced in mesh pots supported by the pot rims, in PVC channels approximately 14 days after germination. Plants were spaced at 19 cm within the rows. 25 liters of aerated nutrient solution were used for supplying two channels in which the nutrient solution was re-circulated. Four re-circulating hydroponics systems were used as replicates. The nutrient solution consisted of Ca $(NO_3)_2$, 0.575; KNO_3, 0.331; Mg $(NO_3)_2$.$7H_2O$, 0.219; KH_2PO_4, 0.0828 and K_2SO_4, 0.1466 (g/L). The micro nutrients were supplied to this solution as Fe-EDDHA 16; $MnSO_4.7H_2O$, 2.44; H_3BO_3, 0.68; $ZnSO_4.7H_2O$, 0.176; $CuSO_4.5H_2O$, 0.156 and (NH_4) $6MO_7O_{24}$, 0.148 (mg/L). The nutrient solution was changed weekly. The level of solution NO_3^- was monitored daily, and was replenished back to its original concentration with KNO_3 when the concentration of NO_3^- fell below one-half of its initial level. The EC of nutrient solution was about 2.5 dS/m. The pH of the solution was maintained between 5.8 and 6.2 by adjustments with nitric and phosphoric acids (3:1 v/v).

After 75 days of growth, three plants of each genotype were randomly chosen from each replicate. The samples of leaves and roots were oven dried at 70^oC then ground in a blender and stored in glass vials for NO_3^- and total N determinations. Nitrate contents of leaves and roots of each genotype were determined by nitration of salicylic acid (Cataldo *et al.,* 1975) and the results expressed as mg NO_3^-, per kg dry weight. Tissue total N was determined using a modified micro-Kjeldahl digestion procedure (Nelson and Summers, 1980).

Total genomic DNA of lettuce genotypes was extracted using the cetyltrimethyl-ammonium bromide (CTAB) method described by Hoisington *et al.* (1994). Each sample (0.5 g) of leaf tissue was transferred to a tube containing 10 ml of extraction buffer (1.5 M NaCl, 100mM Tris-HCl pH 8, 20 mM EDTA, 1% CTAB). The samples were incubated for 2 hours at 65^oC with occasional mixing. Following incubation, 5 ml of chloroform/isoamylalcohol (24:1) was added to

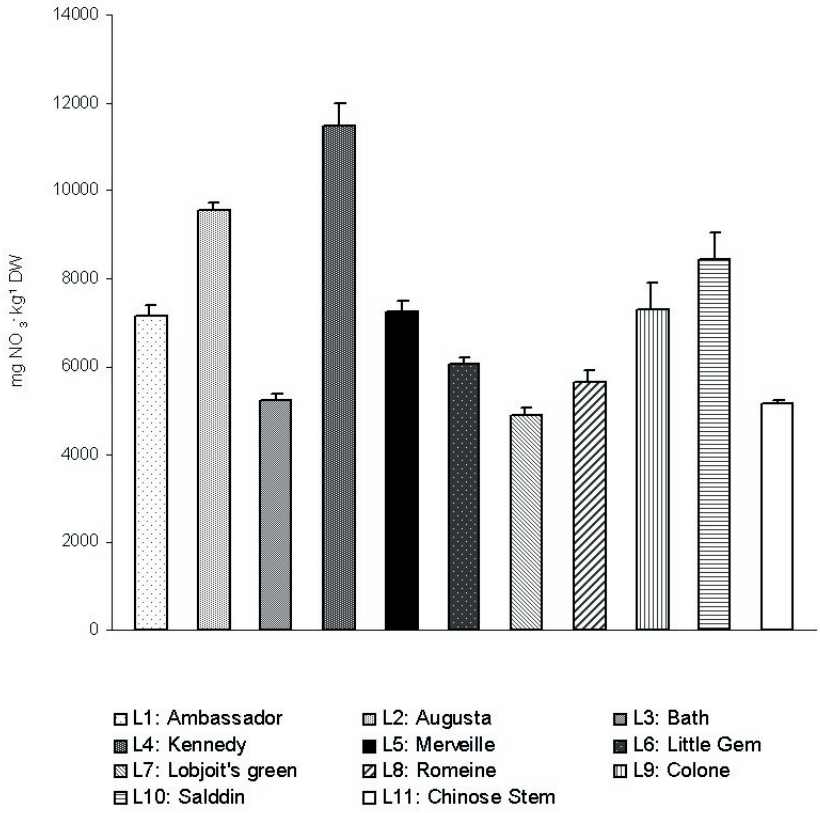

Figure 1: NO3- concentrations in leaves of eleven lettuce genotypes. Vertical bars show standard errors of four replicates.

the tubes, mixed, and centrifuged at 2600 g for 10 min. The aqueous phase was removed to a fresh tube and an equal volume of ice-cold isopropanol was added, followed by centrifugation as above to precipitate the DNA. The pellet was washed in 70% ethanol and dissolved in TE buffer (10mM Tris-HCl, pH8.0, 0.1mM EDTA).

Gene-specific primer pairs for amplification of the nitrate reductase gene (Nia) were as follows: forward primer, 5'-GGTAGGCGATTGGCTAACA TTGTCTGC-3'; and 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, 10mM Tris, pH 7.5, 1.5 mM MgCl2) 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^{o}C; and 40 cycles of 1 min at 94^{o}C, 1 min at 63^{o}C, and 1 min at 72^{o}C; followed by 5 min at 72^{o}C. An aliquot of 10 µl from each reaction product was resolved by electrophoresis on 1.5% agarose gel in 1X TAE buffer, stained with ethidium bromide, and visualized with UV light.

Data were statistically analyzed by using a randomized complete block design with four replicates through Student-Keul's Test according to Sendecor and Cochran (1980). The least significant differences were used to compare means at the 5% level. Linear association between total N of plant tissue and leaf nitrate concentrations was determined.

Results and Discussion

Leaf nitrate concentrations showed significant differences ($P < 0.05$) among lettuce genotypes (Fig. 1). The Romaine genotype 'Lobjoit's Green' exhibited the lowest leaf nitrate concentration (4877.7 mg/kg

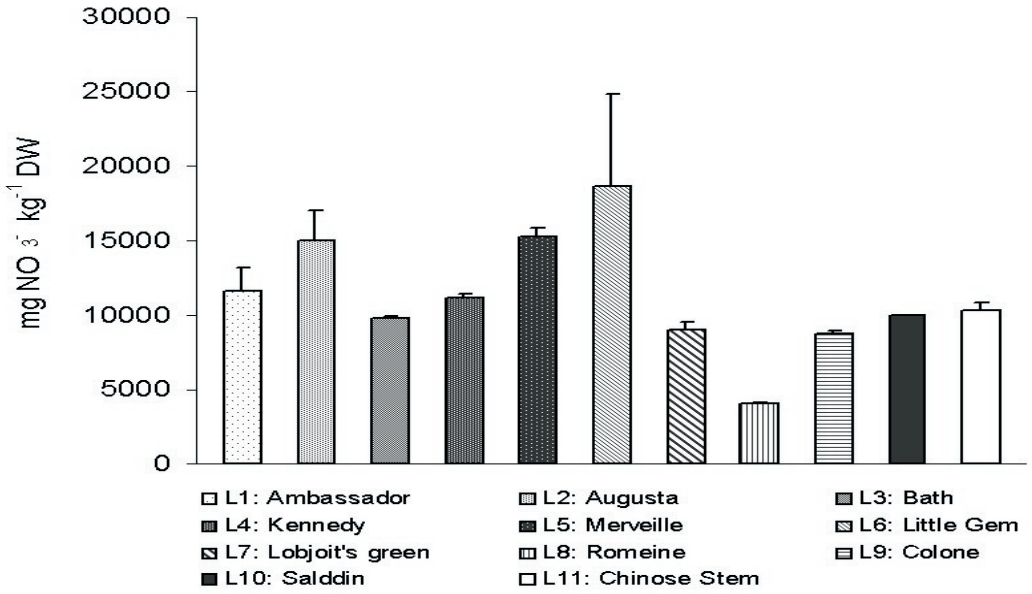

Figure 2: NO3- concentrations in roots of eleven lettuce genotypes. Vertical bars show standard errors of four replicates.

dry weight). The Stem genotype 'Chinese stem', the Buterhead genotype 'Bath', and the Romaine genotypes 'Romaine de Benicardo' and 'Little Gem' also showed lower nitrate concentrations than other genotypes. The highest nitrate levels were recorded in the Butterhead genotypes 'Augusta and Kennedy'. It was recognized that lettuce genotypes can differ in nitrate accumulation (Behr and Wiebe, 1988; Reinink, 1992; Belligno *et al.,* 1996). Regarding within Butterhead genotypes, 'Bath' showed a lower nitrate concentration than the other Butterhead genotypes. Therefore, for nitrate concentration a high variability was found, not only among genotypes in general, but also between the lettuce types, and among genotypes within the Butterhead group (Escobar-Gutierrez *et al.*, 2002).

In the roots of lettuce genotypes, the Romaine genotype 'Romaine de Benicardo' exhibited the lowest nitrate concentration (4000 mg/kg dry weight) (Fig. 2). Nitrate concentration in the roots was significantly higher in the Romaine genotype 'Little Gem' and the Butterhead genotypes 'Augusta and Merveille Des Quatre Saisons' than other lettuce genotypes. It may be noted that the Romaine genotype 'Little Gem' showed a low nitrate concentration in the leaves. This might indicate that the Romaine genotype 'Little Gem' can store a large portion of absorbed material in the roots thus translocating less NO_3^- to leaves. Al-Redhaiman (2000) reported that lettuce genotypes can accumulate NO_3- to different levels due to their differential translocation or partitioning characteristics.

Significant differences ($P < 0.05$) in total nitrogen concentrations as a percentage among lettuce genotypes were found (Fig. 3). In general, total N concentrations were higher in the Butterhead and Crisphead genotypes than in the Romaine and Stem lettuce genotypes. This confirms the findings of Al-Redhaiman (1996) that total N concentrations were higher in the Butterhead cultivars than in the Romaine type.

Correlation analysis was performed for the overall dataset to establish whether there was a significant relationship between total N concentrations of plant tissues and leaf nitrate concentrations. A significant ($p < 0.01$) positive association ($r = 0.80$, $p < 0.01$) was observed between leaf NO_3^- concentrations and total N concentrations. Genotypes such as Lobjoit's Green and Chinese stem were characterized by the lowest genotypes of leaf nitrate concentrations and total N concentrations. On the other hand, the Butterhead genotypes 'Augusta and Kennedy' were characterized by the highest genotypes of leaf nitrate concentrations and total N concentrations. A positive and significant correlation between leaf NO_3^- concentrations and total

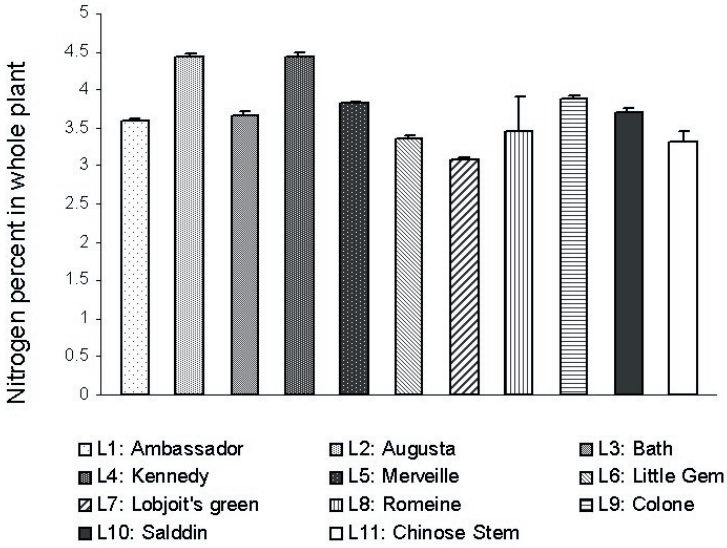

Figure 3. Nitrogen percent in plant tissues of eleven lettuce genotypes. Vertical bars show standard errors of four replicates.

N concentrations was also reported by Al-Redhaiman (1996) and Gent (2003). In general, total N in the plants results from accumulated N uptake. Therefore, variation in leaf nitrate concentrations in lettuce genotypes may be attributed to differences in nitrate uptake (Behr and Wiebe, 1992; Al-Redhaiman, 2000).

The nitrate reductase gene (*Nia*), which is responsible for the nitrate reductase activity, 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 *Nia* gene of these genotypes yielded one fragment of approximately 800bp (Fig. 4). On the other hand, the *Nia* gene was not amplified in PCR from other genotypes. It was interesting to note that the Butterhead genotypes 'Ambassador, Bath and Merveille des Quatre Saisons' had the nitrate reductase gene (Nia) and showed low leaf nitrate concentrations. However, the other Butterhead genotypes 'Augusta and Kennedy' exhibited the highest leaf nitrate concentrations and did not amplify with the *Nia* gene. Moreover, the Romaine genotype 'Romaine de Benicardo' was amplified with the *Nia* gene and showed low nitrate concentrations in the leaves and roots. Therefore, the low nitrate content in lettuce might be due to the nitrate reductase gene (*Nia*). Al-Redhaiman (1996) suggested that genotypic differences in NO_3^- accumulation in lettuce are not exclusively the result of cultivar differences in NO_3^- uptake and reduction, but may also involve other factors related to NO_3^- metabolism, such as nitrate reductase activity. The total nitrate reductase activity is regulated comparably to the expression of the nitrate reductase genes (Sharameti *et al*., 2002). Curtis *et al.* (1999) concluded that the presence of the nitrate reductase gene (*Nia*) in transgenic lettuce was confirmed by nitrate reductase enzymatic assay, a reduction in the nitrate content of leaves and by Southern hybridization.

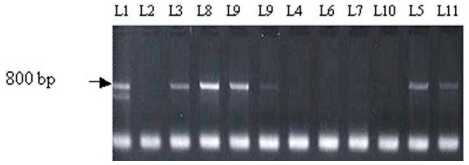

Figure 4. Agarose gel of amplified nitrate reductase gene (*Nia*) from eleven lettuce genotypes (left to right) Ambassador (L1), Augusta (L2), Bath (L3), Romaine (L8), Colona (L9), Kennedy (L4), Little Gem (L6), Lobjoit's Green (L7), Saladin (L10), Merveille (L5), and Chinese stem (L11).

The work presented in this paper illustrates that sensitive specific PCR assays represents a valuable and a new tool for screening lettuce breeding material for low nitrate content which will be a major objective in lettuce breeding programs to limit nitrate concentration in salad crops. This could be of great importance since high nitrate concentration can be toxic and may cause illness or even death in humans.

References

Al-Redhaiman, K.N. 1996. Nitrate accumulation in hydroponically-grown lettuce cultivars and relationship to nitrogen supply. *Ph.D. Dissertation*, University of Illinois, Urbana-Champagn.

Al-Redhaiman, K.N. 2000. Nitrate accumulation in plants and hazards to man and livestock health: A review. *Journal of King Saud University* 12: 143-156.

Anonymous. 2001. Commission regulation (EC) No. 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). *Official Journal of the European Communities*, L 77, 16 March 2001, 1-13.

Behr, U. and H.J. Wiebe. 1988. Relations between nitrate content and other osmotica in the cell sap of lettuce cultivars. *Gartenbauwissenschaft* 53: 206-210.

Behr, U. and H.J. Wiebe. 1992. Relation between photosynthesis and nitrate content of lettuce cultivars. *Scientia Horticulturae* 49:175-179.

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.

Cataldo, P.A., M. Harnon, L.E. Schrader and V.L. Youngs. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Community Soil Sciences and Plant Analogy* 6:71-80.

Cheng, C.L., J. Dewdney, H.G. Nam, B.G.W. Den-Boer and H.M. Goodman. 1988. A new locus (Nia1) in Arabidopsis thaliana encoding nitrate reductase. *European Molecular Biology Organization Journal* 7:3309-3314.

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 and Biotechnology* 77:232-237.

Gent, M.P.N. 2003. Solution electrical conductivity and ratio of nitrate to other nutrients affect accumulation of nitrate in hydroponic lettuce. *Horticultural Science* 38:222-227.

Goodman, P.J. 1979. Genetic control of inorganic nitrogen assimilation of crop plants. In: *Nitrogen Assimilation of Plants,* E.J. Hewitt and C.V. Cutting (Editors), 165-176. Academic Press, New York.

Hoisington, D.A., M.M. Khairallah and D. Gonzales-de-Leon. 1994. Laboratory Protocols. *CIMMYT Applied Molecular Genetics Laboratory*, Mexico, DF.

Lorenz, O.A. 1978. Potential nitrate levels in edible plant parts. In: *Nitrogen in the Environment*, D.R. Nielsen and J.G. MacDonald (Editors), 201-219, Vol 2. Acadamic Press, New York.

Maynard, D.N., A.V. Barker, P.L. Minotti and N.H. Peck. 1976. Nitrate accumulation in vegetables. *Advanced Agronomy* 28:71-118.

Nelson, D.W. and L.E. Summers. 1980. Total nitrogen analysis of soil and plant tissues. *Journal of the Association of Official Analytical Chemists* 63: 770-778.

Pelsy, F. and M. Caboche. 1992. Molecular genetics of nitrate reductase in higher plants. *Advanced Genetics* 30:1-40.

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

Reinink, K. 1991. Genotype by environment interaction for nitrate concentration in lettuce. *Plant Breeding* 107:39-49.

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

Reinink, K. and R. Groenwold. 1988. The inheritance of nitrate content in lettuce (*Lactuca sativa* L.). Euphytica 36:733-744.

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. *Journal of Biological Chemistry* 277:46594-46600.

Snedecor, G.W. and W.G. Cochran. 1980. *Statistical Methods*. Sixth Edition, Iowa State University Press, Ames, Iowa.

Wilkinson, J.Q. and N.M. Crawford. 1991. Identification of the *Arabidopsis* CHL3 gene as the nitrate reductase structural gene *Nia2*. *Plant Cell* 3:461-471.

Wilkinson, J.Q. and N.M. Crawford. 1992. Identification and characterization of a chlorate resistant mutant of *Arabidopsis* with mutations in both *Nia1* and *Nia2* nitrate reductase structural genes. *Molecular and General Genetics* 239: 289-297.

Yu, X., S. Sukumaran and L. Marton. 1998. Differential expression of the *Arabidopsis Nia1* and *Nia2* genes. *Plant Physiology* 116:1091-1096.

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