Front. Agric. China 2007, 1(2): 188–192
DOI 10.1007/s11703-007-0032-9

RESEARCH ARTICLE

SUN Feifei, HOU Xilin, LI Ying, CUI Xiumin

Molecular cloning and characterization of nitrate reductase gene cDNA from non-heading Chinese cabbage

© Higher Education Press and Springer-Verlag 2007

Abstract Four non-heading Chinese cabbage (*Brassica campestris* ssp. c*hinensis* Makino) cultivars, *Suzhouqing*, *Xuekeqing*, *Huangxinwu*, *Aijiaohuang*, were planted to investigate the activity of nitrate reductase (NRA) in leaves. After being induced by KNO_3 at 50 mmol/L for different hours, the NRAs of the four cultivars were determined *in vivo*. The results showed that the NRAs changing trends of these four cultivars were similar. The highest NRAs in leaves reached their maximum at the 4th, 4th, 6th, and the 6th hour of induction, respectively. According to these results, the level of NR mRNA in plants could be enhanced by nitrate inducement. Then, the total RNA was isolated from the leaves of *Suzhouqing* that was induced by KNO_3 at 50 mmol/L for four hours, and two fragments of NR cDNA were obtained through RT-PCR using specific primers. The products of PCR were cloned and sequenced. They are 1 125 and 438 base pairs, which were named nr_{1125} and nr_{438}, encoding 374 and 135 amino acids, respectively. Finally, nr_{1125} was accepted and released by GenBank (accession number DQ001901).

Keywords non-heading Chinese cabbage (*Brassica campestris* ssp. *chinensis* Makino), nitrate reductase, PCR, clone

1 Introduction

Leafy vegetables, especially those nitrogen-relying plants, are rich in nitrate, and are the main nitrate source for people (Chen et al., 2000; Shen et al., 1982; Vogtmann et al., 1984; Santamaria and Elia, 1997). The accumulation of nitrate in plants depends on several environmental factors, including light intensity (Gaudreau et al., 1995), temperature, nitrogen availability (Gunes et al., 1995; Poulsen et al., 1995) and water supply (Goodman, 1979). Nitrate is not toxic, but when reduced to nitrite during digestion in the human body, the ion can oxidize haemoglobin to methaemoglobin, causing methaemoglobinemia (National Research Council/National Academy of Science, 1981). In addition, nitrate is the precursor of carcinogenic nitrosamine (Walker, 1990; Dich et al., 1996). The acceptable daily intake of nitrate is 220 mg for a 60 kg individual (Eenink et al., 1984).

Therefore, many experts pay attention to the ways to reduce nitrate content in vegetables, like proper fertilization, improving environmental conditions, determination of the correct harvesting time, appropriate storage, pre-eating treatments and processing, etc. (Liu et al., 2003). However, there may be a limit as to how much these methods can reduce the leaf nitrate content in production due to many internal and external factors (Xiong et al., 2004).

However, when absorbed by plants, nitrate must be reduced to NO_2^- in the cytoplasm of root and mesophyll cells, then NO_2^- is carried into the plastid rapidly and reduced to NH_4^+ by nitrite reductase, and finally, it is used to synthesize amino acid. Nitrate reductase catalyzes the reduction of nitrate to nitrite and is considered to be a limiting and also a key factor in plant growth and development. Thus, an increase in the amount and activity of nitrate reductase leads to a corresponding increase in the potential for nitrate reduction and confers a greater capacity for general amino-acid synthesis, protein synthesis or total nitrogen assimilation (Hirel et al., 2001). Furthermore, more active nitrate reductase is good for increasing the yield of vegetables and the utilizing ratio of fertilizers under the same fertilizing conditions.

Thereby, an alternative approach, involving the transfer of a *nia* gene (Vincentz and Caboche, 1991) into target plants, has been shown to be effective in elevating nitrate reductase activity and, consequently, reducing nitrate content following insertion and expression in NR-deficient mutants of *Nicotiana plumbaginifolia* (Vaucheret et al., 1990; Dorbe et al., 1992). In this investigation, we successfully cloned two fragments of nitrate reductase gene from non-heading Chinese cabbage, which is the basis for assembling a full-length cDNA to gene transformation.

Translated from *Journal of Nanjing Agricultural University*, 2006, 29(2): 15–19 [译自: 南京农业大学学报]

SUN Feifei, HOU Xilin (✉), LI Ying, CUI Xiumin
State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China
E-mail: hxl@njau.edu.cn

2 Materials and methods

2.1 Plant materials and growth condition

Seeds of four non-heading Chinese cabbage (*Brassica campestris* ssp. c*hinensis* Makino) cultivars of *Suzhouqing*, *Xuekeqing*, *Aijiaohuang* and *Huangxinwu* were surface-sterilized with 1% NaClO for 10 min, rinsed with deionized water, then soaked in deionized water for four hours, and germinated in sterilized moist filter paper in the dark at 25°C. At the very beginning of germination, seeds were placed in pots containing vermiculite and pearlite (ratio = 1:2), and watered with deionized water once everyday, and then replaced with 1/2 Hoagland nutrient solution after cotyledon unfolding.

2.2 Treatment and sampling

The seedlings with four euphyllas were used for analysis of nitrate reductase activity. When leaves were fully expanded, the roots of the plants were induced for 0, 2, 4, 6, 8, 10 and 12 hours in KNO_3 at 50 mmol/L respectively, and then sampled. The fully expanded leaves of *Suzhouqing* induced for four hours were used for RNA extraction.

2.3 Determination of activity of nitrate reductase (NRA) *in vivo*

Fresh leaves without the midrib were cut into 5 mm × 5 mm slices. The samples (0.50 g) were placed in 10 mL of incubation medium, 0.1 mol potassium phosphate buffer (pH 7.5) containing 0.1 mol KNO_3 with 1% (v/v) propanol. Prior to determination, the buffer solution was purged with N_2 (gas) for 30 min to remove dissolved oxygen. Then the samples were vacuum-infiltrated (two times), and the reaction flasks were purged with N_2 (gas) for 5 min, then sealed with rubber stoppers and incubated in a water bath at 30°C for 30 min. After incubation, the samples were added 1 mL of 30% trichloroacetic acid to stop the enzyme reaction. The nitrite released to the medium was measured by the method of Chen et al. (1980). The amount of nitrite (NO_2^{-1}) formed during the reaction was measured with a spectrophotometer at 540 nm, and NRA was expressed in mmol · ($g^{-1} \cdot h^{-1}$).

2.4 RNA extraction and RT-PCR

Total RNA was isolated from younger leaves of *Suzhouqing* induced for four hours with 50 mmol KNO_3 according to the instruction of a Simply P Total RNA Extraction Kit (Bioflux, China). RNA quality was determined by 1% agarose gel electrophoresis. One mg of total RNA was used to reverse-transcribe the first strand cDNA using TaKaRa RNA PCR Kit (AMV) Ver.2.1 (TaKaRa, Japan). Fifty ng of the cDNA was added to the PCR mixtures to amplify the cDNA fragment. Conserved domains were identified by alignment of the amino acid sequences of several nitrate reductase genes. According to the conserved domains, we designed two pairs of primers S1 and S2. S1sense: 5′-CCATTCCTTCTCCCC-GTCA-3′, S1antisense: 5′-CCTCCACCCTCGTTACCT-3′, anticipate product size was 1 125 bp; S2sense: 5′-AGAGTTC-TACCTTCTCTTG-3′. S2antisense: 5′-GCAGTTGTTCG TCATCCC-3′, anticipate product size was 428 bp. These two products would have an overlap of about 217 bp. The 20 μL PCR mixtures were set up as follows: 10 × PCR Buffer 2.0 μL, cDNA 1 μL, 200 μmol/L dNTPs 1.6 μL, 25 mmol/L $MgCl_2$ 1.6 μL, 0.5 μmol/L each specific primer 1 μL, ddH_2O 11.6 μL, 5U/μL LA-*Taq* DNA polymerase (TaKaRa, Japan) 0.2 μL. Amplification profile used was 94°C 3 min, 30 cycles of 94°C for 30 sec, 1 min at 57°C and 1 min at 72°C and a final extension of 10 min at 72°C. The products were resolved in 1.0% agarose gel.

2.5 Products purification and gene cloning

The RT-PCR products were gel-purified by Agarose Gel DNA Purification Kit Ver 2.0 (TaKaRa, Japan) and subsequently cloned into the pGEM-T Easy vector (Promega), then transformed into *E. coli* JM109 competent cells (TaKaRa, Japan). After being cultured for one hour, the cell suspension was placed on LB medium with ampicillin (100 mg/L), IPTG and X-gal, and then cultured over night. White colonies were selected to detect the recombinant plasmids by PCR. Positive clones were sequenced at the Gene Company, Shanghai, China. The sequences were analyzed using CLUSTALX, DNAMAN, BLAST, ORF FINDER and DNASTAR programs.

3 Results

3.1 Effect of nitrate inducing time on non-heading Chinese cabbage NRA

Inducing time with nitrate had a significant effect on non-heading Chinese cabbage NRA (Fig. 1). During inducing, NRAs of the four cultivars showed similar changing trends, with an obvious single bell-like curve. NRAs of *Suzhouqing* and *Xuekeqing* reached the maximum after being induced for four hours, and for *Huangxinwu* and *Aijiaohuang*, the optimum inducing time was six hours, then, NRAs quickly decreased as time increased. Two hours later, the NRAs of *Suzhouqing*, *Xuekeqing* and *Aijiaohuang* decreased to the beginning level, but *Huangxinwu* lasted for four hours. The concrete results are shown in Fig. 1. According to the above results, the level of nitrate reductase mRNA could be enhanced by nitrate inducement.

3.2 RNA extract from *Suzhouqing*

Under natural conditions, NR constitutively expressed at a low level, however, NR mRNA could rapidly accumulate in

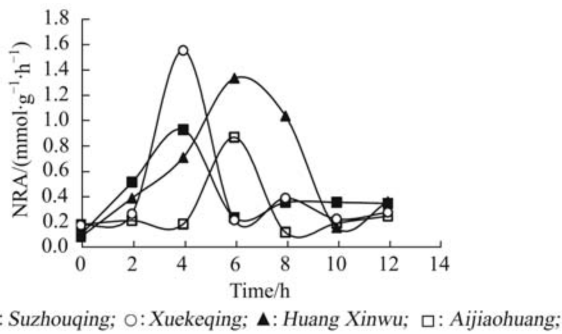

Fig. 1 Influence of inducing time on non-heading Chinese cabbage NRA in leaf

the cytoplasm after nitrate inducement. Therefore, we selected *Suzhouqing* leaves induced for four hours for total RNA extracting. There were two clear bands (28s and 18s rRNA) and a faint one (5s rRNA) on 1% agarose gel (Fig. 2), indicating that the RNA could be used for later experiment.

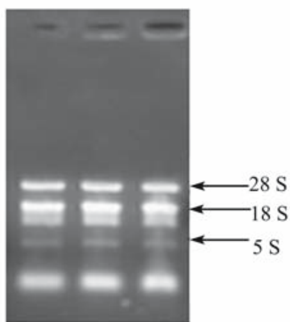

Fig. 2 Electrophoresis of total RNA from *Suzhouqing* (three replicates)

3.3 Cloning of two NR gene fragments

Two gene fragments were amplified using specific primers (S1 and S2) from the cDNA, which were reversely transcripted from total RNA of *Suzhouqing* (Fig. 3). They were 1 125 and 438 base pairs after sequence analysis, and named nr_{1125} (GenBank: DQ001901) and nr_{438}, respectively. Subsequently, the RT-PCR products were cloned into the pGEM-T Easy vector, and then transformed into *E. coli* JM109 competent cells. After being cultured overnight, white spots were selected to detect recombinant plasmids (Fig. 4). The result showed that recombinant plasmids PCR products were the same size as cDNA PCR products, indicating that target fragments had been cloned successfully.

3.4 Sequence analysis of two NR gene fragments

Positive clones were sequenced in both directions, and two cDNA fragments were obtained. One was 1 125 base pairs encoding 374 amino acids, the other was 438 base pairs encoding 135 amino acids. They were named $nr_{1\,125}$ (GenBank: DQ001901) and nr_{438}, respectively (Figs 5 and 6). The results of BLAST analysis showed that the nucleotide sequence of $nr_{1\,125}$ was 99%, 97% and 93%, similar to *Brassica napus*, *Arabidopsis thaliana* and *Brassica rapa*, respectively. The homology to other species was also very high (data not given). The nucleotide sequence of nr_{438} was 99%, 89% and 82% identical to *B. napus*, *A. thaliana* and *B. rapa*, respectively. The homology to other species was all above 82% (data not given). Finally, we submitted $nr_{1\,125}$ to the Genebank, and obtained the accession number DQ001901.

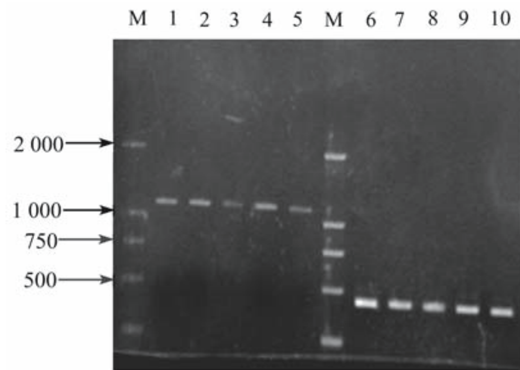

Fig. 3 Electrophoresis analysis of RT-PCR product (M: DL2000 marker; 1, 2, 3, 4, 5: RT-PCR product of primer S 1; 6, 7, 8, 9, 10: RT-PCR product of primer S2)

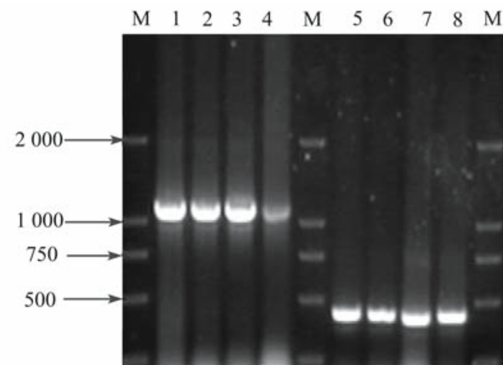

Fig. 4 PCR analysis of the recombinant plasmids (M: DL2000 marker; 1, 2, 3, 4: PCR product of recombinant plasmids of primer S1; 5, 6, 7, 8: PCR product of recombinant plasmids primer S 2)

4 Discussion

Vegetable NRAs distinctly differ among different genotypes, and the genotypes of high NRA have a high efficient nitrate inducement system. To select and breed the varieties with high NRA and low nitrate content is a new direction for improvement of vegetable quality. Earlier studies showed that the introduction of the NR recombination gene *nia* transferred into the genome of lettuce led to a remarkable decrease in nitrate content in leaves (Curis et al., 1999). Expression of chimeric gene of tobacco NR ligased to CaMV35S promotor

```
   1 ccattccttc tccccgtcac caaaacaaga ccgtatccgt tccaaccgaa aagatcgttg
  61 tcagagaaac caaaaacgac gtcgtggaac acagttatga ctccagcgac gacgaggacg
 121 agagccacaa ccgtaacgtg tcatactaca aggagatgat acgcaaatcc aacagcgacg
 181 tagaaccgtc gatcctagac tcgcgagacg aatccacggc tgacaactgg atccaccgta
 241 actcctctat ggtgcgtctc acgggaaaac accccttcaa cgtcgagcctcctctccctc
 301 gcctcatgca ccacggcttc atcacccccg tccctctcca ctacgtccgc aaccacggca
 361 ccgtcccaaa ggccgactgg tcggactgga ccgttgagat caccggcctc gtcaagcgtc
 421 cggcgaagtt caccatggag cagcttatct ccgagttccc tagccgcgag tttccggtca
 481 ctctcgtctg cgccggtaac cgccggaaag aacagaacat ggtgaagcag acgataggtt
 541 tcaactgggg ctccgccgga gtatccacct ccctatggaa aggtgttcct ctcagtgaga
 601 tcctccgtcg atgcgggata tacagtagga gaggcggcgc gctcaacgtc tgcttcgaag
 661 gagcggagga tcttcccgga ggcggcgggt cgatgtacgg aacgagtatt aagaaagaga
 721 tggcgatgga tcctgcgaga gacatcatat tagcgtacat gcagaacagc gagcttctca
 781 cgccggatca cgggtttccg gttcggatca tcgtacccgg tttcatcggt ggtcgaatgg
 841 ttaaatggtt gaaacgtatc atcgtcacgc ctcaagaatc cgacagttgc tatcattaca
 901 aggacaatag agttctacct tctcttgtcg atgctgaact ggcaaatgca gaagcatggt
 961 ggtacaagcc ggaatatata atcaacgagc ttaatataaa ctcggtgata accacacccg
1021 gtcaccaaga gattttgcct attaatgcat ttaccactca aaagccgtac acgttaaaag
1081 gctatgctta ctctggagga gggaagaagg taacgagggt ggagg

   1 IPSPRHQNKTVSVPTEKIVVRETKNDVVEHSYDSSDDEDESHNRNVSYYKEMIRKSNSDV
  61 EPSILDSRDESTADNWIHRNSSMVRLTGKHPFNVEPPLPRLMHHGFITPVPLHYVRNHGT
 121 VPKADWSDWTVEITGLVKRPAKFTMEQLISEFPSREFPVTLVCAGNRRKEQNMVKQTIGF
 181 NWGSAGVSTSLWKGVPLSEILRRCGIYSRRGGALNVCFEGAEDLPGGGGSMYGTSIKKEM
 241 AMDPARDIILAYMQNSELLTPDHGFPVRIIVPGFIGGRMVKWLKRIIVTPQESDSCYHYK
 301 DNRVLPSLVDAELANAEAWWYKPEYIINELNINSVITTPGHQEILPINAFTTQKPYTLKG
 361 YAYSGGGKKVTRVE
```

Fig. 5 Nucleotide sequences (up) and deduced amino acid sequences (down) of nr_{1125} from non-heading Chinese cabbage (The shaded region was the primer position; the underlined part was superposed filed with nr_{438})

```
  1 agagttctac cttctcttgt cgatgctgaa ctggcaaatg cagaagcatg gtggtacaag
 61 ccggaatata taatcaacga gcttaatata aactcggtga taaccacacc cggtcaccaa
121 gagattttgc ctattaatgc atttaccact caaaagccgt acacgttaaa aggctatgct
181 tactctggag gagggaagaa ggtaacgagg gtggaggtga ctctagacgg aggagagacg
241 tggagcgtgt gtgagcttga ccaccaagag aagccaaaca agtatggcaa gttctggtgc
301 tggtccttct ggtcgcttga cgttgaggtt cttgatctgc ttagtgctaa agaggtagct
361 gttcgagcct gggacgagtc tttgaacacc cagcctgaaa aactcatctg gaacgtgatg
421   gggatgacga acaactgc
```

Fig. 6 Nucleotide sequences of nr_{1125} from non-heading Chinese cabbage (The shaded region was the primer position; the underlined part was superposed filed with nr_{1125})

in potato could depress 95% nitrate content in potato tubes (Djennane et al., 2002).

NR is a substrate inducement enzyme, so expression of NR gene can be stimulated by nitrate. In our study, before obtaining NR cDNA fragments by RT-PCR, we investigated the effect of nitrate inducement on NRA in order to evaluate NR-mRNA transcriptional discriminations. We selected *Suzhouqing* leaves induced with nitrate after four hours to extract total RNA, although its NRA was not the highest (Fig. 1). We cloned the target gene fragments by RT-PCR. It indicated that the transcriptional discrimination of this NR-mRNA was up to the requirement of PCR. However, *Xuekeqing* and *Huangxinwu* were higher in NRA than *Suzhouqing*, and the NRA of *Aijiaohuang* was a little different from that of *Suzhouqing*. In addition, we designed primers within the conservative region, so we presumed that we could obtain the same result in the other three varieties with the same primers and method. This presumption needs to be tested.

RT-PCR is a simple and quick method to clone target genes, but it requires high specific primers. In this study, we designed two pairs of primers whose products would have an overlapped region of 271 base pairs. The sequencing result showed that these two fragments indeed had a 271 base pairs overlapped region, which indicated that RT-PCR is an efficient method for amplifying target genes of less than 2 000 base pairs. Our study greatly supports the feasibility of cloning of segmented target genes.

Acknowledgments This research was financially supported by Hi-Tech Research and Development Program of China (No. 2003AA207120)

References

Chen X P, Zou C Q, Liu Y P, Zhang F S (2000). The nitrate content difference and the reason among four spinach varieties. Plant Nutrition and Fertilizer Science, 6(1): 30–34 (in Chinese)

Shen M Z, Zhai B J, Dong H R, Li J G (1982). Studies on nitrate accumulation in vegetable crops I. Evaluation of nitrate and nitrite

in different vegetables. Acta Horticulture Sinica, 9(4): 41–48 (in Chinese)

Vogtmann H, Temperli A T, Kunsch U, Eichenberger M, Ott P (1984). Accumulation of nitrates in leafy vegetables grown under contrasting agricultural systems. Biol Agr Hortic, 2: 51–68

Santamaria P, Elia A (1997). Producing nitrate-free endive heads: effect of nitrogen form on growth, yield, and ion composition of endive. J Am Soc Hortic Sci, 122(1): 140–145

Gaudreau L, Charbonneau J, Vezina L P, Gosselin A (1995). Effects of photoperiod and photosynthetic photon flux on nitrate content and nitrate reductase activity in greenhouse grown lettuce. J Plant Nutr, 18: 437–453

Gunes A, Kirkby E A, Aktas M (1995). Influence of partial replacement of nitrate by amino acid nitrogen or urea in the nutrient medium on nitrate accumulation in NFT grown winter lettuce. J Plant Nutr, 17: 1929–1938

Poulsen N, Johansen A S, Sørensen J N (1995). Influence of growth conditions on the value of crisphead lettuce. 4. Quality changes during storage. Plant Foods Hum Nutr, 47: 157–162

Goodman P J (1979). Genetic control of inorganic nitrogen assimilation of crop plants. In: Hewitt E J, Cutting C V, eds. Nitrogen Assimilation of Plants. New York: Academic Press, 1–708

Walker R (1990). Nitrate, nitrite and N-nitroso compound: a review of the occurrence in food and diet and the toxicological implications. Food Add Cont, 7: 717–768

Dich J, Jrvinen R, Knekt P, Penttila P L (1996). Dietary intakes of nitrate, nitrite and NDMA in the finish mobile clinic health examination survey. Food Add Cont, 13: 514–552

Eenink A H, Blom-Zandstra M, Hollman P C H, Aarts P, Groenwold R (1984). Research on reduction of nitrate content in lettuce via breeding. In: Proc Eucarpia Meet Leafy Vegetables. INRA ed. Versailles, France, 100–109

Liu X R, Liu J L, Ren J Q (2003). Study on influence factors on nitrate accumulation in vegetables and its control measures. Soil and Fertilizers, (4): 3–6 (in Chinese)

Xiong G H, Lin X Y, Zhang Y S, Zhen SH J, Zhou G D (2004). Effect of fertilization on nitrate accumulation. Chinese Journal of Soil Science, 35(2): 217–221 (in Chinese)

Hirel B, Bertin P, Quillere I, Boundoncle W, Attagnant C, Dellay C, Gouy A, Cadiou S, Rettailliau C, Falque M, Callais A (2001). Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. Plant Physiology, 125: 1258–1270

Vincentz M, Caboche M (1991). Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia*. EMBO J, 10: 1027–1035

Vaucheret H, Chabaud M, Kronenberger J, Caboche M (1990). Functional complementation of tobacco and *Nicotiana plumbaginifolia* nitrate reductase deficient mutants by transformation with the wild-type allele of the tobacco structural genes. Mol Gen Genet, 220: 468–474

Dorbe M F, Caboche M, Daniel-Vedele F (1992). The tomato *Nia* gene complements a *Nicotiana plumbaginifolia* nitrate reductase-deficient mutant and is properly regulated in heterologous transgenic plants. Plant Mol Biol, 18: 363–375

Chen W, Zhang D Y (1980). Extraction, measurement and purification of nitrate reductase in plant tissues. Plant Physiology Communication, 16(4): 45–49 (in Chinese)

Curis I S, Power J B, Laat A M M, Caboche M, Davey M R (1999). Expression of a chimeric nitrate reductase gene in transgenic lettuce reduces nitrate in leaves. Plant Cell Reports, 18(11): 889–896

Djennane S, Chauvin J E, Quilere I, Meyer C, Chupeau Y (2002). Introduction and expression of deregulated tobacco nitrate reductase gene in potato lead to highly reduced nitrate levels in transgenic tubers. Transgenic Res, 11(2): 175–184