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Research article

Nitrate metabolism in tobacco leaves overexpressing Arabidopsis nitrite reductase

Susie Davenport*, Pascaline Le Lay, Juan Pablo Sanchez-Tamburrrino

British American Tobacco, R&D Cambridge, 210 The Science Park, Cambridge, CB4 0WA, UK

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ABSTRACT

Primary nitrogen assimilation in plants includes the reduction of nitrite to ammonium in the chloroplasts by the enzyme nitrite reductase (NiR EC:1.7.7.1) or in the plastids of non-photosynthetic organs. Here we report on a study overexpressing the *Arabidopsis thaliana NiR* (*AtNiR*) gene in tobacco plants under the control of a constitutive promoter (CERV – Carnation Etched Ring Virus). The aim was to overexpress AtNiR in an attempt to alter the level of residual nitrite in the leaf which can act as precursor to the formation of nitrosamines. The impact of increasing the activity of AtNiR produced an increase in leaf protein and a stay-green phenotype in the primary transformed AtNiR population. Investigation of the T_1 homozygous population demonstrated elevated nitrate reductase (NR) activity, reductions in leaf nitrite and nitrate and the amino acids proline, glutamine and glutamate. Chlorophyll content of the transgenic lines was increased, as evidenced by the stay-green phenotype. This reveals the importance of NiR in primary nitrogen assimilation and how modification of this key enzyme affects both the nitrogen and carbon metabolism of tobacco plants.

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1. Introduction

Nitrogen (N) assimilation remains a widely studied pathway in plants because of its fundamental importance for growth and development. Nitrate is a major source of this crucial plant nutrient and its availability is a rate-limiting factor in the growth and development of many plant species (Masclaux-Daubresse et al., 2010; Hirel et al., 2011; Krouk et al., 2011; Andrews et al., 2013). Therefore the transport, assimilation and recycling of nitrogen is a highly complex and regulated process, as it is the mineral nutrient that is required in the greatest abundance by the plant (Lea and Azevedo, 2006; Dechorgnat et al., 2011; Hirel et al., 2011; Wang et al., 2012).

The first two enzymes involved in nitrate assimilation are nitrate reductase (NR) and nitrite reductase (NiR). NiR requires reduced ferredoxin to reduce nitrite to ammonia, which is subsequently assimilated via glutamine synthetase (GS) and glutamate synthase (GOGAT) (Andrews et al., 2013). There is recent evidence that NiR, GS and GOGAT are present in enzyme complexes within the chloroplast (Kimata-Ariga and Hase, 2014). NR has been extensively studied, with significant research carried out on the regulation of the NR enzyme protein in tobacco, potato, lettuce, *Arabidopsis thaliana* and early vascular plants (for example; Curtis et al., 1999; Djennane et al., 2002; Heidari et al., 2011; Lambeck et al., 2012; Konishi and Yanagisawa, 2013; Nemie-Feyissa et al., 2013). NR is also able to synthesise NO from nitrite in tobacco (Lu et al., 2014). Genes encoding *NR* have been cloned and used in both overexpression (Ferrario-Méry et al., 1998; Djennane et al., 2004; Lea et al., 2006; Zhao et al., 2013) and down regulation studies (Stitt and Feil, 1999; Kruse et al., 2002; Lillo et al., 2004). These studies have resulted in an increased understanding of post-translational regulation of NR, which has probably evolved to avoid the potential problems associated with nitrite accumulation (Lillo et al., 2004; Lea et al., 2006; Heidari et al., 2011).

Nitrogen assimilation is not a process in isolation, as it needs to be tightly linked to carbon (C) metabolism and photosynthesis (Vincentz et al., 1993; Tobin and Bowsher, 2005; Lillo, 2008). NR and NiR are reliant on photosynthetic activity and in particular NiR, due its localisation in the chloroplast, underpins the strong relationship between C and N metabolism. When nitrogen is in short supply, the photosynthetic apparatus in the leaf chloroplast breaks down and there is a reduction in both NR and NiR activity.

* Corresponding author.
E-mail address: susie_davenport@bat.com (S. Davenport).

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Remobilisation and recycling processes then take over in order to supply the newly developing sink leaves with metabolites (Okumoto and Pilot, 2011; Masclaux-Daubresse and Chardon, 2011; Guiboileau et al., 2012, 2013). Visual markers for remobilisation, include the loss of chlorophyll and protein and increases in glutamate dehydrogenase (GDH) activity in the older source leaves. These markers were identified in the study of Masclaux et al. (2000), who investigated the transition of metabolites from young sink leaves to older source leaves in tobacco.

As previously mentioned many studies on nitrogen metabolism have focused on NR, in contrast here we have investigated the role of NiR in nitrogen assimilation in tobacco. Previous studies have shown the detrimental effect of silencing NiR (Vaucheret et al., 1998; Morot-Gaudry-Talarmain et al., 2002; Morikawa et al., 2003, 2004) resulting in the accumulation of toxic levels of nitrite in the cell. However, in a recent study by Kyaing et al. (2012), the overexpression of a *nii2* gene in tobacco led to an increase in the activity of NiR. The study further demonstrated that the overexpression of NiR decreased the concentrations of nitrate and to a lesser extent nitrite in the leaves as well as increasing NR activity. Kyaing et al. (2012) concluded that the increase in NiR activity had a clear effect on the nitrate content of the plant.

Here we describe the overexpression of the Arabidopsis *NiR* gene in tobacco and the subsequent effects on NiR activity, key metabolites and enzymes involved in both nitrogen assimilation and recycling. The results demonstrate that NiR does in fact play a central role in nitrogen metabolism, not only being involved in the detoxification of nitrite but also in the regulation of NR activity and senescence. Throughout the older leaves are referred to as source leaves and the younger leaves as sink leaves.

2. Materials and methods

2.1. Plant material and treatments

Wild type (WT) tobacco (*Nicotiana tabacum*. K326) plants, and transgenic *NtAtNiR* plants derived from the WT were used in the experiments described. Seeds were germinated in a propagation mix compost (Levingtons F1) in a glasshouse at 22 °C under high pressure 400 W sodium lamps at 16 h light/8 h dark photoperiod. After four weeks, WT and homozygous transgenic tobacco (*NtAtNiR* T_1) plants were transplanted into clay pebbles (Hydro-Leka Clay Pebbles; Gro Well Hydroponics, Warwick, UK). Four week old WT tobacco and transgenic plants were watered with N free Hoagland's solution (Matt et al., 2001) containing added 1 mM or 10 mM potassium nitrate. The 90 × 3 l pots were stood in 17.5 cm saucers, which were topped up each day with the appropriate Hoagland's solution. The plants were allowed to grow for a further eight weeks, until the first signs of flower buds had appeared. The effects of the different nitrate concentrations on plant growth were not noticeable until four weeks into the treatments, when the plants grown with 1 mM nitrate began to show signs of N limitation, e.g. they had yellowing leaves and were visibly shorter.

The feeding trial was carried out using a randomised block design in order to assess any significant differences between the lines, following statistical analysis of the results by ANOVA. Three blocks were set out, each block contained treatments of 1 mM and 10 mM nitrate. Each block contained three homozygous *NtAtNiR* individuals of four different transformed lines and WT controls subjected to the two nitrate treatments to give a total of 30 plants in each block. This design was repeated with each block having the plants and treatments randomised within it.

Leaf samples were taken from the older source leaves (leaves 3 and 4 from the base of the plant) and the younger sink leaves (leaves 14 and 15 from the base) after eight weeks of growth on the nitrate nutrient solutions in clay pebbles.

2.2. Plasmid construction and plant transformation

Genomic DNA was extracted from WT *A. thaliana* leaves using a QIAGEN DNeasy Plant DNA extraction kit (#69106) (QIAGEN Ltd., Crawley, UK). The full length *AtNiR* gene (AK221199 – http://www.ncbi.nlm.nih.gov/nuccore/AK221199) was amplified by a polymerase chain reaction (PCR) with specific primers 5′-ATCGAGCTCGGATCCATGACTTCTTTCTCTCTCAG-3′ (forward) and 5′-GATGAGCTCGGATCCTACCTCAATCTTCATTCTC-3′ (reverse). The PCR product was digested with *Bam*H1 and *Kpn*1 and inserted into the binary vector pBinplus (pBNP; van Engelen et al., 1995). It contained the constitutive Carnation Etched Ring Virus (CERV: Hull et al., 1986) promoter and a nopaline synthase (NOST) terminator sequence. A luciferase control line was also constructed in the same manner (LUC). The recombinant plasmids were transformed into *Agrobacterium tumefaciens* LBA4-404 by the electroporation method. Tobacco leaf disks were surface sterilized, infected with the *A. tumefaciens* strain containing the recombinant plasmid, and then cultured for 2 d. The leaf disks were incubated on MS medium containing 3 mg l^{-1} 6-benzylaminopurine and 0.2 mg l^{-1} 1-naphthaleneacetic acid to induce shoots. Within 4–6 weeks, the regenerated shoots were transformed to a root-inducing medium. Both media contained kanamycin (50 mg l^{-1}) and cefotaxime sodium (250 mg l^{-1}).

2.3. Absolute quantitation method for copy number

Quantitation of the number of transgenes inserted was detected by the absolute quantitation method, based on using a quantitative amount of DNA and relating the PCR signal to a standard curve in order to extrapolate the copy number (BIO-RAD technical note 2697). A virtual calibrator was also employed, which is a known transgenic line but of unknown copy number, to compare to other transgenic lines.

Genomic DNA was digested with *Ssp*I (1 μg genomic DNA, 5 μl *Ssp*I buffer, 10 units *Ssp*I endonuclease (Promega), SDW to 50 μl) by incubating at 37 °C for at least 2 h. The enzyme was inactivated at 70 °C for 10 min. Standard curves were generated using serial dilutions of plasmid DNA (pBNP*AtNiR*) and genomic DNA of one transgenic line (virtual calibrator) to compare standard curve efficiencies. The primer pair At1300F (5′-TGCTGATGACGTTCTTCC-3′) and At1440R (5′-TGCAAGAAGCATGTAC-3′) were used in the PCR reaction (0.25 pM forward primer, 0.25 pM reverse primer, 12.5 μl SYBR-green mix (Bio-Rad laboratories, UK), 1 μl diluted DNA, SDW to 25 μl). The PCR conditions were; 1 cycle at 95 °C for 3 min followed by 45 cycles of 95 °C at 30 s and 55 °C for 30 s. A melt curve was generated by 80 cycles at 30 s intervals, increasing the temperature from 55 °C to 95 °C in 0.5 °C increments. All reactions were performed in 4 replicates and the data collected by the i-cycler iQ™ software (Bio-Rad laboratories, UK). For each transgenic sample, 250 ng of genomic DNA was analysed and compared with the standard curves. This procedure makes the assumption that one of the transgenics will only contain one copy of the transgene and the highest threshold cycle (C_t) values will be the lines with the lowest copy number. C_t values were compared and differences of 1 C_t represented a two fold difference in target DNA. Controls containing no DNA and non-transgenic genomic DNA were also included.

2.4. Comparative C_t copy number method

This method allows the analysis of unknown amounts of DNA

by comparing the C_t value of a transgene to that of an endogenous gene (German et al., 2003; Weng et al., 2004). The number of copies of the endogenous gene (or reference gene) per genome remains constant, whereas the number of copies of a transgene (or target gene) can vary, therefore the ratio of target to reference gene will indicate the copy number of the transgene. For the C_t calculation to be valid, the efficiency of the target amplification and the efficiency of the reference amplification must be taken into account, therefore standard curves have to be produced for both the target and reference gene. The reference gene used was nitrate reductase (*NR*), with the primer pair NIAF (5′-GGTCTTTCAAGCCTCGGTCTG-3′) and NIAR (5′-GGAAGG-GAATTCGTTAACCAA-3′), and the target gene was *AtNiR*, with the primer pair At1300F and At1440R. Genomic DNA was extracted but not quantified and each sample was amplified twice using both sets of primers with the same volume of DNA added. The PCR reaction (0.25 pM forward primer, 0.25 pM reverse primer, 12.5 µl SYBR-green mix (Bio-Rad laboratories, UK), 1 µl DNA, SDW to 25 µl) was 1 cycle at 95 °C for 3 min followed by 45 cycles of 95 °C for 30 s and 55 °C for 30 s. A melt curve was generated by 80 cycles at 30 s intervals of increasing temperature from 55 °C to 95 °C in 0.5 °C increments. This melt curve was used to identify that the correct amplicons had been generated. The equation from Weng et al. (2004) was used to calculate copy number;

$$[(C_{t\ \mathrm{tar}} - \mathrm{I_{tar}})/\mathrm{S_{tar}}] - \left[\left(C_{t\ \mathrm{ref}} - \mathrm{I_{ref}}\right)\middle/\mathrm{S_{ref}}\right]$$

$$\mathrm{X_0}/\mathrm{R_0} = 2$$

where:

$C_{t\ \mathrm{tar}}$ or $C_{t\ \mathrm{ref}} = C_t$ value for target and reference genes.
$\mathrm{I_{tar}}$ or $\mathrm{I_{ref}}$ = Intercept of relative standard curves.
$\mathrm{S_{tar}}$ or $\mathrm{S_{ref}}$ = Slope of relative standard curves.
2 is the dilution factor for the standard curves.
$\mathrm{X_0}$ = initial amount of target molecule.
$\mathrm{R_0}$ = initial amount of reference molecule.

2.5. Western blot analysis

Total soluble leaf protein in the leaves of WT and transgenic lines was extracted by grinding approximately 100 mg of leaf material with 100 µl of Overcoat buffer (100 mM Tris pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, 10% glycerol, 10 mM β-mercaptoethanol). Proteins were separated by running denaturing SDS-PAGE gels as described by Laemmli (1970), using a NuPAGE mini gel system (Invitrogen™, Paisley, UK).

The separated proteins were transferred to a nitrocellulose membrane (0.45 µm pore size; Invitrogen™, Paisley, UK) using a SureLock™ mini-cell (Invitrogen™, Paisley, UK) system.

Specific antibodies for the detection of *A. thaliana* NiR (AtNiR) polypeptides were raised in rabbits using two synthetic peptides KSMEELDSEKSSKD and LKVTEEVERLVSVP (14-mers) corresponding to sequences of the NiR protein (Covalab Ltd., Cambridge, UK). These primary antibodies were prepared (1:1000 dilution) in TBS-Tween (20 mM Tris pH 7.6, 0.8% NaCl, 0.1% Tween20). The membrane was added to the antibody solution and left to shake at room temperature for at least 1 h before being washed three times with TBS-Tween. The secondary antibody (anti-rabbit, horseradish peroxidase linked antigen; Amersham Biosciences, Bucks, UK) was added at 1:4000 dilution in TBS-Tween and incubated for 2 h with shaking. Membranes were washed as above. Pre-stained SeeBlue® Plus2 Protein Standard molecular markers were run for each gel.

Antibody binding was detected using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Bucks, UK) and then exposed to film (Kodak BioMax Film, Anachem, Luton, UK). Bands usually appeared after 5 min exposure.

2.6. Determination of enzyme activities

Leaf samples were collected between 9 and 10 am in the morning and kept on ice, prior to extraction.

NiR activity in leaf extracts was measured by following the disappearance of nitrite, determined by the action of acidic sulphanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride, as described by Takahashi et al. (2001). Maximal unphosphorylated NR activity in leaf extracts was determined by following the appearance of nitrite as described above and by Ferrario-Méry et al. (1998). Glutamate dehydrogenase (GDH) activity in leaf extracts was determined in the aminating direction by following the oxidation of NADH in the presence of 2-oxoglutarate and ammonia as described by Turano et al. (1996).

The protein concentration in the leaf extracts was measured according to Bradford (1976).

2.7. Analysis of soluble amino acids

To further investigate the impact of the overexpressed AtNiR enzyme on nitrogen metabolism, the concentrations of five soluble amino acids were determined in the leaves. Glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp) and asparagine (Asn) were selected as they are normally the first four amino acids to be synthesized following the assimilation of ammonium ions (Forde and Lea, 2007; Lea et al., 2007), whilst proline (Pro) may be synthesised directly from Glu, particularly under stress conditions (Szabados and Savouré, 2010).

The EZ:faast™ kit (Phenomenex®, Macclesfield, Cheshire, UK) was used to extract and derive the soluble amino acids present in the leaf samples. Quantification was then carried out by liquid chromatography–mass spectrometry (LC/MS: Perkin Elmer Series 200, Applied Biosystems, Warrington, Cheshire, UK). Amino acids were derivatised with chloroformate reagent that derivatises both the amino and carboxyl groups of the amino acids and separated on a EZ:faast™ AAA-MS column with the mobile phase components (10 mM ammonium formate in water: 10 mM ammonium formate in methanol 1:2, v/v). The EZ:faast™ kit provided amino acid standards and the procedure was calibrated by running 10, 50 and 100 µl of the amino acid standards through the column prior to the samples.

2.8. Analysis of nitrate and nitrite concentrations in leaf tissue

In order to determine the nitrate concentration of the leaves, the nitration of salicylic acid by leaf extracts was followed under highly acidic conditions. The 5-nitrosalicylic acid chromophore formed was determined at 410 nm, as described by Cataldo et al. (1975) in a method that is not sensitive to interference from chloride, nitrite or ammonium ions.

The nitrite content of the leaves was determined as part of either the NR assay or the NiR assay, as described previously. Both assays measured the amount of nitrite in the extract at time zero and this value was calculated from the nitrite calibration curve generated during each assay (Takahashi et al., 2001).

2.9. Chlorophyll measurements

A hand-held CCM-200 Chlorophyll Content Meter (Opti-Sciences, Inc., Hudson, NH, USA) was used for the non-destructive

determination of chlorophyll content of intact leaf samples, by taking absorbance measurements at 653 nm and 931 nm (Grsic and Cavelek, 2008).

2.10. Statistical analysis

The statistical significance of the differences in the measured parameters between the WT and transgenic plants was tested by the analysis of variance (ANOVA) test using mini-tab software. The values shown in the figures are the mean values ± SE. Mean values are significantly different at $P < 0.05$, as indicated by asterisks (*) in the figures.

3. Results

3.1. AtNiR gene expression

Initially 16 tobacco plants were confirmed as transformed with the *AtNiR* gene by a polymerase chain reaction (PCR) using primers specific to *AtNiR*. Leaf extracts of individual T_0 *NtAtNiR* plants were then analysed by western blotting, using the custom made antisera to the AtNiR protein (Fig. 1A). This demonstrated that 12 of the *NtAtNiR* transgenic plants were synthesising the AtNiR protein, although in varying amounts. No cross-reacting AtNiR protein was detected in control leaf extracts of the LUC overexpressing and WT tobacco, thus confirming the specificity of the antisera.

Following copy number analysis, four of the twelve T_0 *NtAtNiR* expressing lines (3, 14, 20 and 22) were confirmed as possessing a single copy of the *AtNiR* gene and therefore heterozygous (Fig. 1B). Visual analysis of the single copy lines at the flowering stage (16 weeks), revealed a stay-green phenotype present on the lower senescing leaves (Fig. 2). NiR activity measured in leaves at position 12 was higher in the *NtAtNiR* T_0 transgenic plants, when compared to the 3 LUC and 3 wild-type controls (Fig. 3A), a difference that was statistically significant. The total soluble protein content of the leaves of these plants was also determined (Fig. 3B) and there was a slight increase in protein in the *NtAtNiR* transgenic leaves, as compared to the WT and LUC controls, but this increase was not statistically significant.

For each of the 4 single copy T_0 *NtAtNiR* plants, T_1 seeds were harvested and plants were grown up. Homozygous individuals were identified by copy number analysis (Fig. 4A and B), in order to select plants for nitrate treatment in the glasshouse study. Data for the T_1 progeny of plant 14, shows a ratio of 10 homozygous: 17 heterozygous: 9 null. As a double check a set of 24 T_1 seeds from line 14 were also germinated and tested by western blot analysis for the AtNiR protein in leaf extracts (Fig. 4C). Six of the plants (3, 7, 10, 12, 13 and 22) clearly contained no cross reacting AtNiR protein, whilst the leaves of the other plants contained a variable amount of AtNiR protein. This indicates a ratio of 18:6 homozygous plus heterozygous: null, which is exactly what would be expected for a classical Mendelian segregation.

3.2. Enzyme activities in the leaves of T_1 NtAtNiR transgenic lines grown under two differing nitrate regimes

Individual homozygous T_1 (selected from the original T_0 *NtAtNiR* plants 3,14 20 and 22) and WT plants were separated into three blocks with each block randomised to include three plants of each

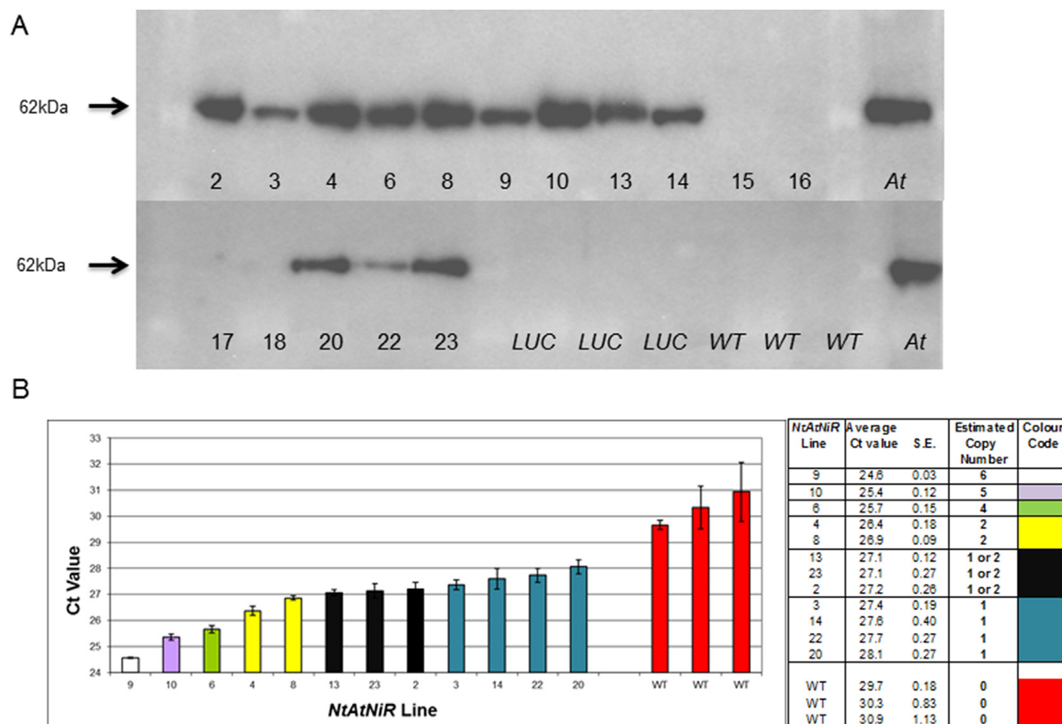

Fig. 1. (A) Western blot analysis of AtNiR protein in leaf extracts of sixteen different T_0 tobacco plants numbered 2–23, transformed with the *AtNiR* gene. Three wild type (WT) and three luciferase (*LUC*) transgenic plant leaf extracts are shown as negative controls with leaf extracts of *A. thaliana* (*At*) as positive controls. The AtNiR antibodies used were raised against synthetic peptides as described in the Materials and methods. (B) Copy number analysis of *NtAtNiR* transformants of tobacco, using the absolute quantification method. The threshold cycle (C_t) values of leaf extracts of the T_0 tobacco plants were measured using SYBR-green in a BioRAD i-cycler. Three measurements were taken from each individual plant to give a mean value for copy number for each line. Three WT plants are shown as controls.

line grown on either 1 or 10 mM potassium nitrate in Hoagland's solution for 8 weeks.

When the plants were grown on 1 mM nitrate, NiR activity in the WT source leaves was very low, whilst in the transgenic lines, activity was up to 65 fold higher. Although in the sink leaves of the WT, NiR activity was considerably higher than in the source leaves, NiR activity in the sink leaves of all the transgenic lines was still even higher (Fig. 5A). For plants grown on 10 mM nitrate, NiR activity was elevated in both the source and sink leaves of the WT, but again NiR activity was higher in the transgenic lines, particularly in the sink leaves and the *NtAtNiR* 3 source leaf (Fig. 6A).

NR activity in both leaves of the WT plants grown on 1 mM nitrate was almost undetectable, whilst low activity compared to NiR, was measurable in three of the transgenic source leaves. NR activity in the sink leaves of all of the transgenic lines was considerably elevated compared to the source leaves (Fig. 5B). Growth on 10 mM nitrate stimulated NR activity in the WT, particularly in the source leaves, to such an extent that the activities in the WT and transgenic leaves were very similar. However in the sink leaves, NR activity was 3–4 fold higher than the WT activity in three of the transgenic lines (Fig. 6B).

The highest GDH aminating activity was detected in the source leaves of the WT plants grown on low 1 mM nitrate, which was twice that of the source leaves of any of the transgenic lines. However, lower GDH activity was detected in the WT sink leaves, which was similar to those of the transgenic leaves (Fig. 5C). GDH activity was again higher in the source leaves compared to the sink leaves of the WT grown on high nitrate, but there was little difference between the activities of GDH in the leaves of the WT and the transgenic lines (Fig. 6C).

Fig. 2. Tobacco T_0 plants (lines *NtAtNiR* 3 and 20) overexpressing *A. thaliana* nitrite reductase, Negative control tobacco T_0 plants (lines *LUC* 1 and 2) overexpressing luciferase and wild type tobacco (WT 1 and 2). Plants were grown in a glasshouse for 16 weeks to flowering, as described in Materials and methods with the equivalent of 10 mM nitrate.

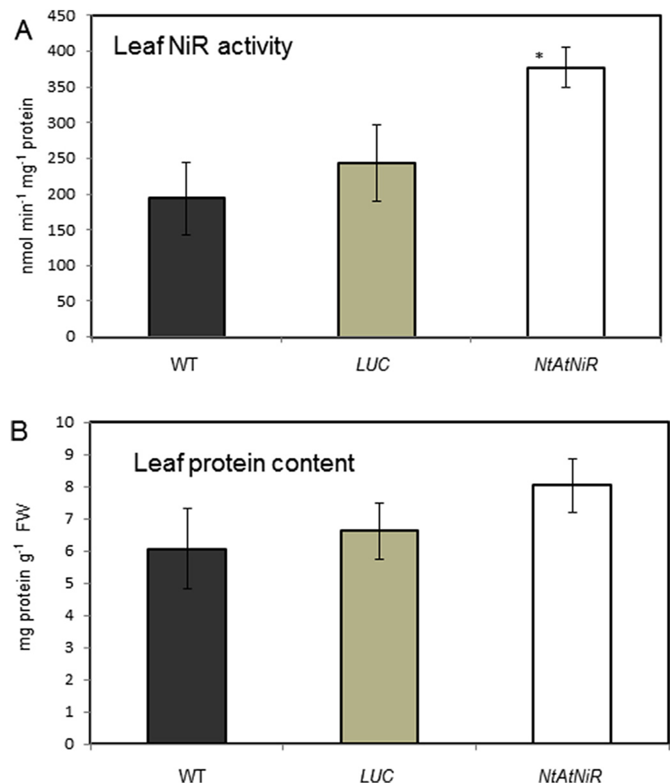

Fig. 3. Nitrite reductase activity and protein content of leaves of WT and four different T_0 lines of tobacco transformed with the *AtNiR* gene. (A) Nitrite reductase activity of leaf 12 of 16 week old plants. (B) Leaf protein content of leaf 12 of 16 week old plants. Results are presented as mean values of four *NtAtNiR* plants (T_0 line 3, 14, 20 and 22); *LUC* and WT are the means values of three plants and the bars indicate SE. Asterisks represent statistical difference compared to WT plants under the same conditions (ANOVA one-way test: $^*P < 0.05$).

Total soluble protein was measured in all plants (Figs. 5D and 6D). There was no clear trend, apart from a slight increase in total soluble protein in the source leaves of the *NtAtNiR* plants grown on 1 mM nitrate, which was not statistically significant (Fig. 5D).

3.3. Metabolite concentrations in the leaves of T_1 NtAtNiR transgenic lines grown under two differing nitrate regimes

To evaluate further the effect of the overexpression of NiR, the concentrations of nitrite and nitrate in the source and sink leaves of the WT and the T_1 transgenic lines were determined. Overall apart from one line, the WT and *NtAtNiR* transformed plants contained similar concentrations of nitrite in the leaves of the 1 mM nitrate grown plants (Fig. 7A), whilst there were significantly lower nitrate concentrations in the sink leaves of three of the transformed lines, when compared to the WT (Fig. 7B). Following growth on 10 mM nitrate the nitrite concentration was decreased to a slight extent, in both the source and sink leaves of the four transgenic lines, although this difference was not significant (Fig. 8A). There was a high level of variability in the nitrate concentrations measured in both leaf types of the transgenic lines (Fig. 8B), but overall there was an indication of lower nitrate, particularly in the source leaves.

Analysis of the leaf chlorophyll content again indicated that

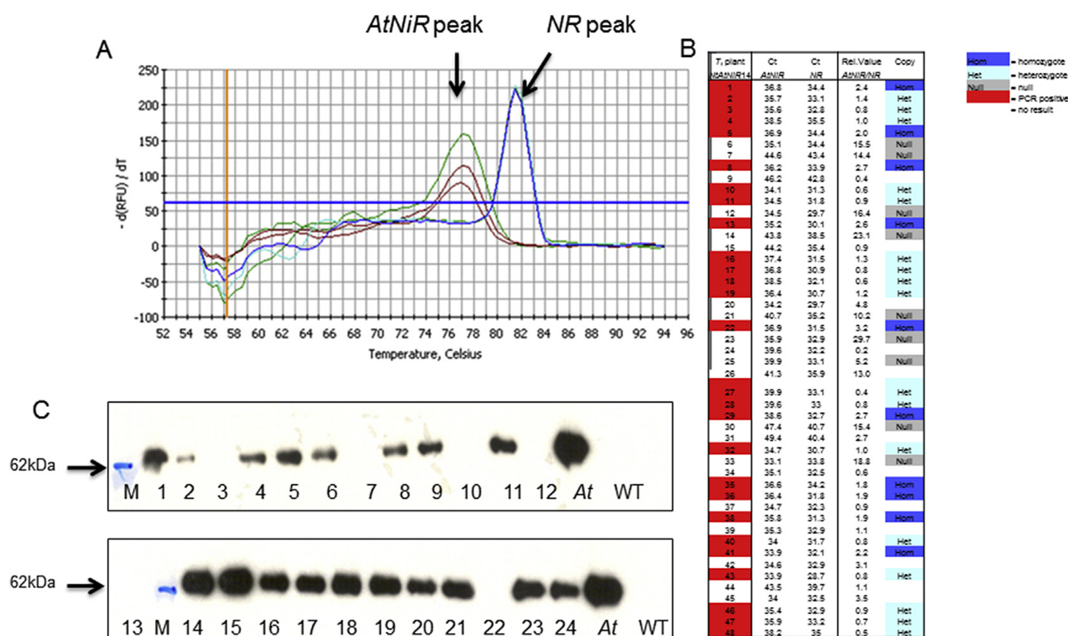

Fig. 4. (A) Melt curve peaks of *NR* and *AtNiR* isolated from the leaves of three individual *NtAtNiR* T_1 tobacco plants, progeny of parent line 14. The comparative C_t copy number method compares the melt curve peak of the inserted *AtNiR* gene to that of an endogenous *NR* gene to give a comparative C_t value and ratio of transgene number to that of the endogenous gene. The *AtNiR* peak is at 77 °C and *NR* is at 81.5 °C. (B) Calculated results for 48 T_1 progeny of the *NtAtNiR* line 14. In total, 96 plants were sown and analysed in order to select the desired number of homozygous individuals for the two nitrate treatments. (C) Western blot analysis of AtNiR protein in leaf extracts of twenty-four T_1 *NtAtNiR* tobacco plants, progeny of line 14, using specific antibodies raised against synthetic polypeptides of AtNiR (see Materials and Methods). M denotes the molecular marker at 62 kDa for bovine serum albumin. Leaf extracts of the WT are show as negative controls (WT) with leaf extracts of *A. thaliana* (*At*) as positive controls. Western blot analysis was carried out on the progeny of all four of the lines judged to be single copies from Fig. 1B and showed similar results.

there was variation in the amounts determined in the leaves of the individual transgenic lines. However, there was a trend showing that both the sink and source transgenic leaves contained slightly more chlorophyll than those of the WT grown on both 1 mM and 10 mM nitrate (Figs. 7C and 8C).

The five key soluble amino acids were analysed in the source and sink leaves of plants grown on 1 mM nitrate, but the concentrations were lower and more variable (Fig. 9A–E) than determined on those grown on 10 mM nitrate. The plants grown on 10 mM nitrate had concentrations of Gln, Glu and Pro that were clearly depressed in the source leaves of the transgenic lines compared to the WT (Fig. 10A, B and E), although the results were not statistically significant. In contrast, the concentrations of Asp and Asn were more variable and tended to be higher in the transgenic lines (Fig. 10C and D).

The next generation (T_2) of the *NtAtNiR* lines (3, 14, 20 and 22) were grown in compost with a 10 mM nitrate supply. The stay-green phenotype in the source leaves of the transgenic plants was clearly visible after twelve weeks of growth (Fig. 11) and the chlorophyll measurement data also reflect the phenotype, even though these were not statistically significantly different from the WT.

4. Discussion

The first attempt to construct plants with increased NiR activity was carried out by Crété et al. (1997), who transformed tobacco with a tobacco *Nii* gene and obtained a maximum of a 63% increase in leaf NiR activity in plants that had no obvious phenotype. Takahashi et al. (2001) constructed Arabidopsis plants containing a spinach *NiR* gene with a maximum of an 82% increase in NiR activity and concluded that NiR was an important enzyme in the control of nitrate assimilation in plants, far more than nitrate reductase and glutamine synthetase. More recently Kyaing et al. (2012) constructed tobacco plants with an overexpressed tobacco *nii2 gene*, which had a 30% increase in NiR activity, but again did not display a phenotype distinguishable from the WT plants. In contrast, when the Arabidopsis gene (*AtNiR*), encoding NiR was overexpressed in tobacco in this study, the older leaves of the transgenic *NtAtNiR* plants sustained a green appearance and appeared to senesce more slowly over a longer period of time, when compared to the WT (Figs. 2, 7C, 8C and 11).

The transformation of tobacco lines with *AtNiR* resulted in a statistically significant increase in NiR activity of up to 65 fold in the source leaves and 4 fold in the sink leaves, when the plants were grown on 1 mM nitrate (Fig. 5A). In plants grown on 10 mM nitrate, the increase in NiR activity in the transgenic lines was similar in the source and sink leaves and ranged between 2 and 7 fold compared to the WT (Fig. 6A). These data clearly suggest that not only is the *AtNiR* transgene transcribed in the tobacco plants, but that it is expressed to form a protein, which is transferred to the chloroplast where the transit peptide is cleaved and the protein is able to efficiently catalyse the NiR reaction. The increase in NiR activity in the leaves of the *NtAtNiR* transgenic lines was far greater when compared to the WT, than previously determined in the three studies discussed above.

The NR activity determined was very much dependent on the concentration of nitrate applied, as following treatment with 1 mM nitrate it was not possible to measure NR activity in the WT, whilst considerable activity was determined in the majority of the transgenic lines (Fig. 5B). However, in the plants grown on 10 mM nitrate there was little difference between the NR activity in the source leaves of the WT and the transgenic lines, but in the sink leaves of the transgenic plants, NR activity was 3–4 fold higher than the WT (Fig. 6B). In the *nii2* overexpressing tobacco plants constructed by

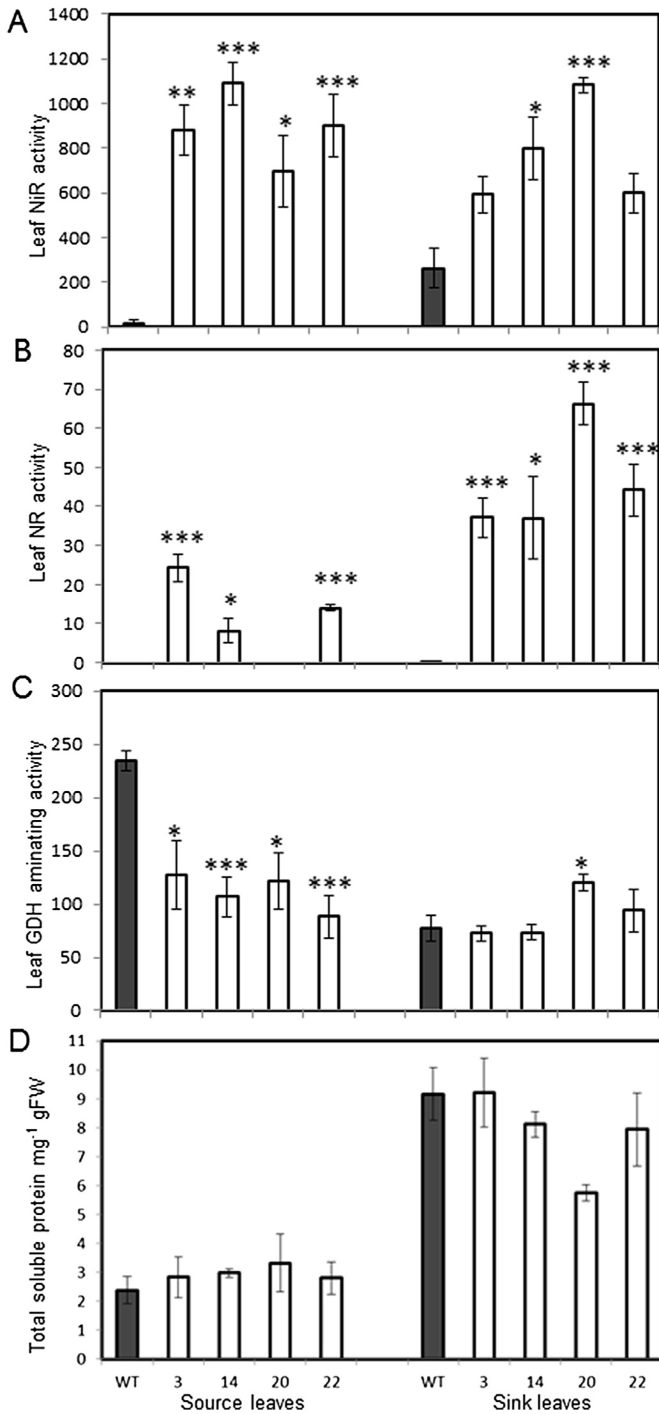

Fig. 5. Enzyme activities in the leaves of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 1 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. (A) Nitrite reductase (NiR) activity in source leaves and sink leaves. (B) Nitrate reductase (NR) activity in source leaves and sink leaves. (C) Glutamate dehydrogenase (GDH) aminating activity in source leaves and sink leaves. (D) Total soluble protein in source leave and sink leaves. Results are means of three independent biological repeats. Asterisks represent statistical difference compared to WT plants under the same conditions (ANOVA one-way test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). All enzyme activities are expressed as nmol min^{-1} mg^{-1} protein.

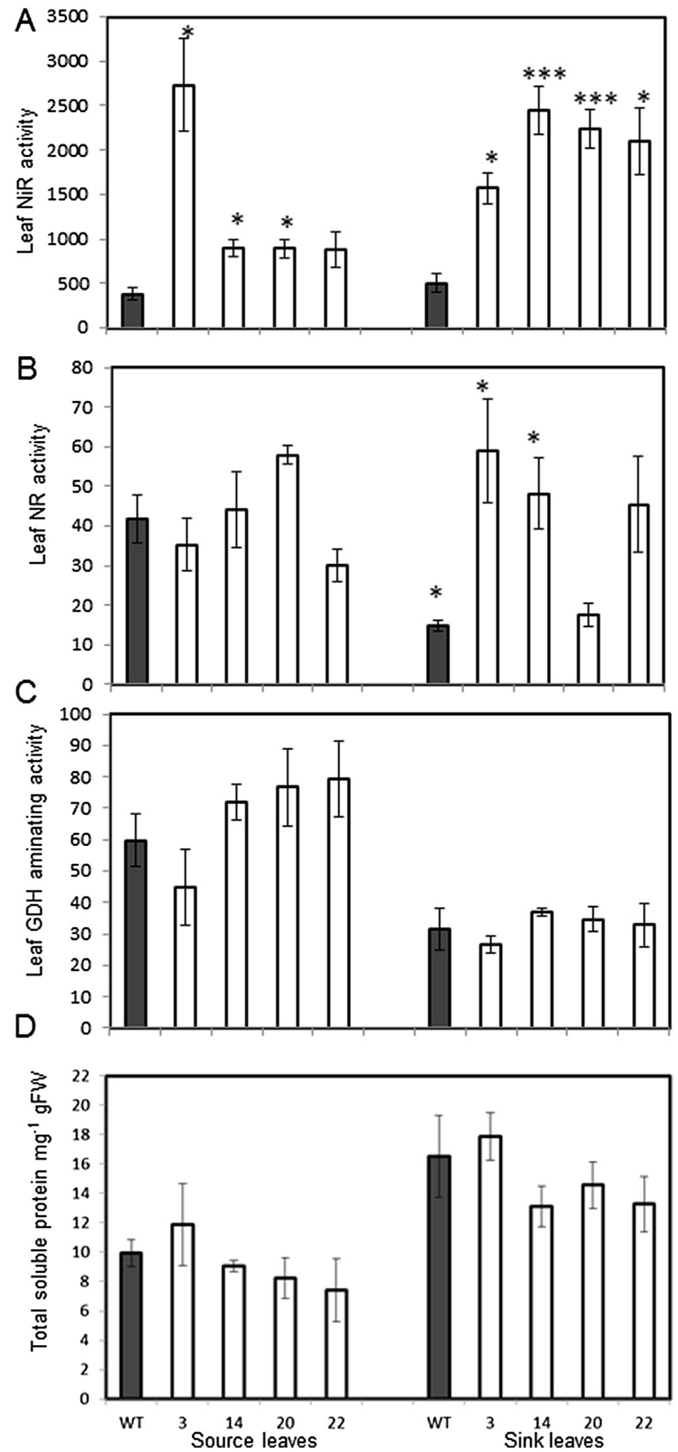

Fig. 6. Enzyme activities in the leaves of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 10 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. (A) Nitrite reductase (NiR) activity in source leaves and sink leaves. (B) Nitrate reductase (NR) activity in source leaves and sink leaves. (C) Glutamate dehydrogenase (GDH) aminating activity in source leaves and sink leaves. (D) Total soluble protein in source leave and sink leaves. Results are means of three independent biological repeats. Asterisks represent statistical difference compared to WT plants under the same conditions (ANOVA one-way test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). All enzyme activities are expressed as nmol min^{-1} mg^{-1} protein.

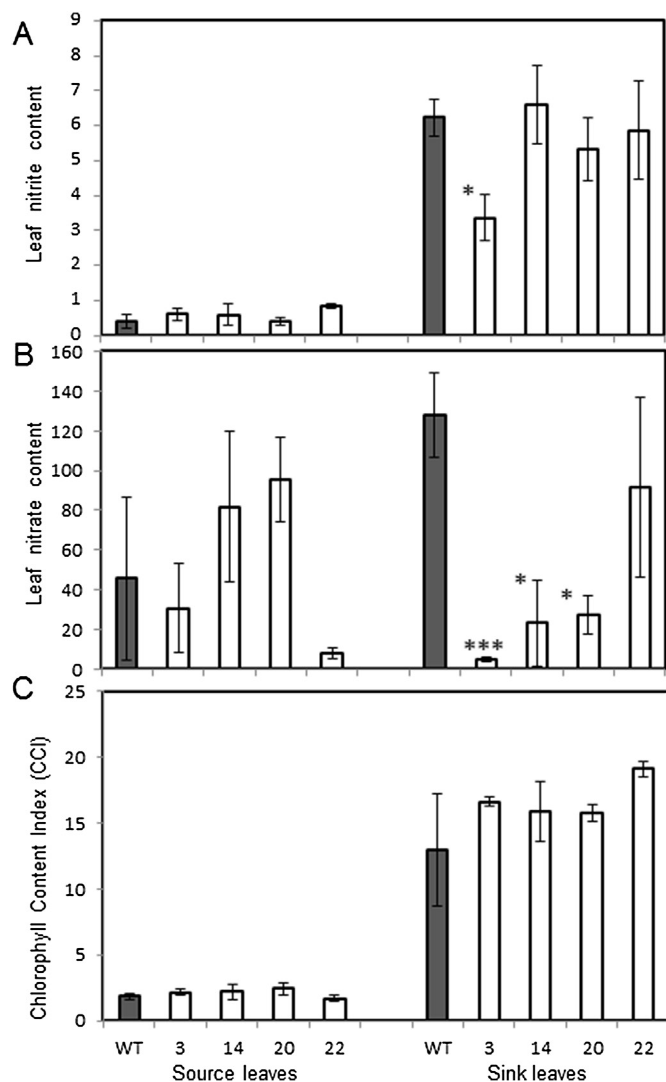

Fig. 7. Leaf nitrogen analysis of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 1 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. (A) Nitrite content (nmol g^{-1} FW) of source leaves and sink leaves. (B) Nitrate content (μmol g^{-1} FW) of source leaves and sink leaves. (C) Chlorophyll Content Index of source leaves and sink leaves. Results are means of three independent biological repeats. Asterisks represent statistical difference compared to WT plants under the same conditions (ANOVA one-way test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

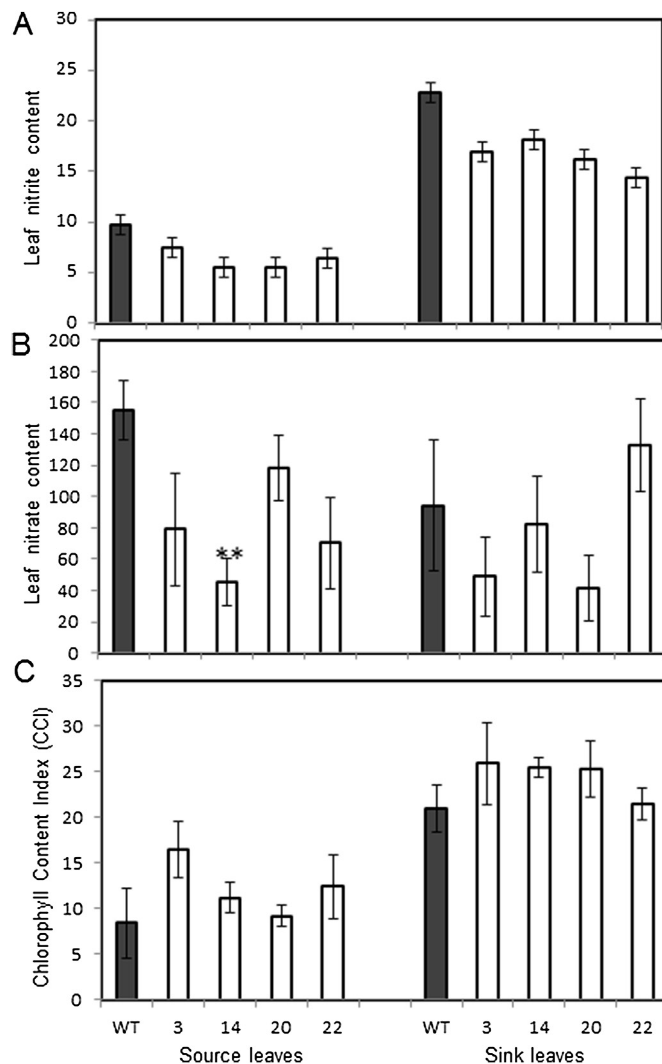

Fig. 8. Leaf nitrogen analysis of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 10 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. (A) Nitrite content (nmol g^{-1} FW) of source leaves and sink leaves. (B) Nitrate content (μmol g^{-1} FW) of source leaves and sink leaves. (C) Chlorophyll Content Index of source leaves and sink leaves. Results are means of three independent biological repeats. Asterisks represent statistical difference compared to WT plants under the same conditions (ANOVA one-way test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

Kyaing et al. (2012) grown in 20 mM nitrate, there was an increase in NiR activity of 30% and a 2-fold increase in NR activity, but the leaf age and type were not given. The varying, but overall stimulatory effect of overexpressing NiR on NR activity, is consistent with the complex mechanisms that have been shown to regulate NR activity in higher plants. These include the inductive action of light, sugars, nitrate and nitrite at the level of transcription (Konishi and Yanagisawa, 2013; Reda, 2013; Krapp et al., 2014) and post-translational regulation by light/dark dependent reversible phosphorylation and subsequent binding of inhibitory 14-3-3 proteins. The latter action is able to operate in a matter of minutes (Lillo, 2008; Heidari et al., 2011; Lambeck et al., 2012; Nemie-Feyissa et al., 2013).

Leaf senescence is characterized by a transition from nutrient assimilation to nutrient remobilization and an increase in glutamate dehydrogenase gene expression and activity has been proposed as a marker for senescence (Pageau et al., 2006; Marchi et al., 2013). Glutamate dehydrogenase (GDH) catalyses the reversible conversion of 2-oxoglutarate and ammonia to glutamate and was originally thought to play a role in ammonia assimilation. However there is now considerable evidence that GDH only operates in the direction of the deamination of glutamate during periods of protein catabolism (Fontaine et al., 2012).

The older source leaves of the WT tobacco lines contained higher GDH activity than younger sink leaves, in addition GDH activities were higher in plants grown on 1 mM compared to 10 mM nitrate (Figs. 5C and 6C). These observations are consistent

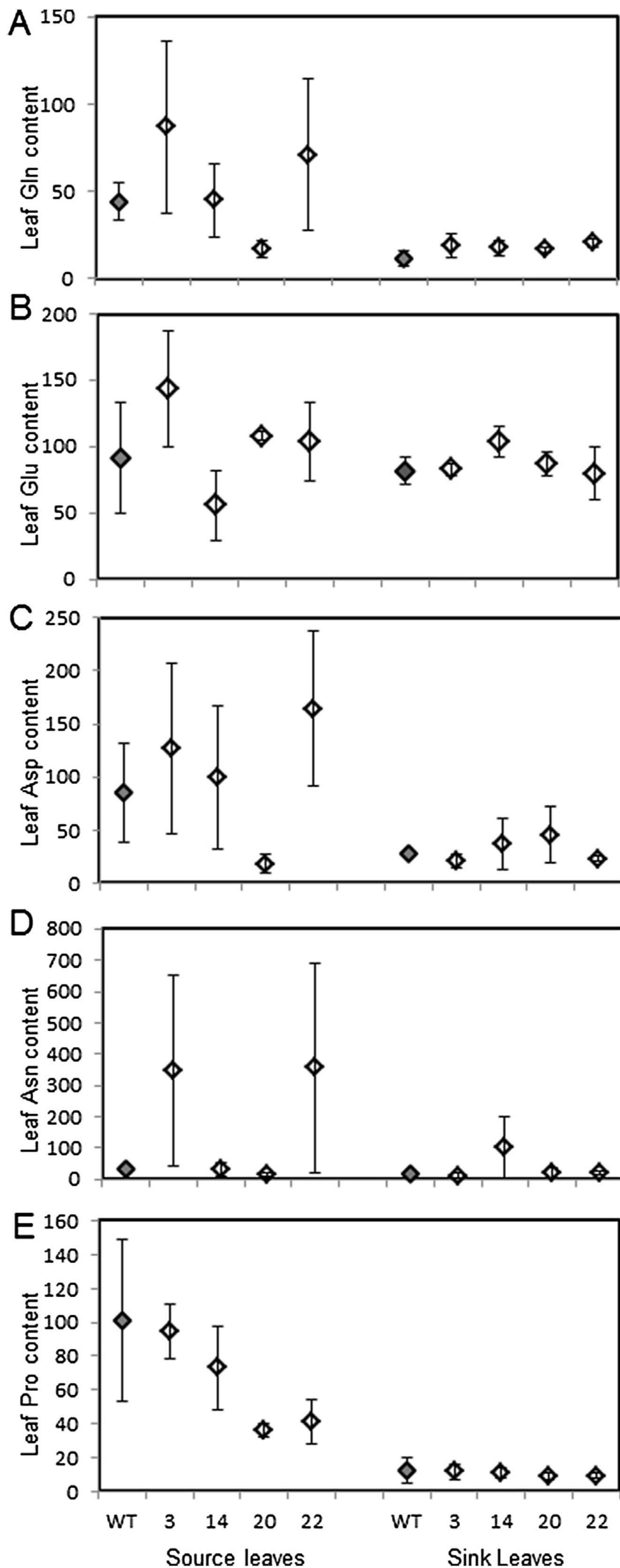

Fig. 9. Soluble amino acid content of leaves of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 1 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. Amino acid content (nmol g^{-1} FW) of source leaves and sink leaves. (A) Glutamine (Gln) content of source leaves and sink leaves. (B) Glutamate (Glu) content of source leaves and sink leaves. (C) Aspartate (Asp) content of source leaves and sink leaves. (D) Asparagine (Asn) content of source leaves and sink leaves. (E) Proline (Pro) content of source leaves and sink leaves.

with the increase in GDH protein determined in WT and autophagy-defective mutants of Arabidopsis during the senescence of plants grown under 2 mM nitrate rather than 10 mM nitrate (Guiboileau et al., 2013). The investigations in Arabidopsis demonstrated that autophagy resulted in protein break down and amino acid recycling and that the remobilised nitrogen is transported to the seed, especially when the plants were grown on limited nitrogen (Guiboileau et al., 2012; Avila-Ospina et al., 2014). In the studies reported here, the highest GDH activity was detected in the older source leaves of the WT tobacco grown on the low 1 mM nitrate concentration, whilst the GDH activity was 50% reduced in the same leaves of the *NtAtNiR* plants (Fig. 5C). The source leaves of the transgenic lines grown on low 1 mM nitrate also appeared to have more protein in comparison to the WT lines, although again the data was not significant (Fig. 5D). These results suggest that in the older leaves, grown on low nitrate, the senescence process may have progressed at a lower rate in the *NtAtNiR* transgenic plants when compared to the WT.

Most investigations have concentrated on the overexpression of genes encoding NR, as the enzyme was originally considered to be the rate limiting step in growth and nitrogen assimilation (Quilleré et al., 1994; Camacho-Cristóbal et al., 2002; Wang et al., 2004; Jonassen et al., 2008; Zhao et al., 2013). In our study we have shown there is positive influence of increased *NiR* expression on NR activity. Both NR and NiR activities were increased in the *NtAtNiR* transgenic plants, and this is supported in part by the lower concentrations of nitrate and nitrite determined in the sink leaves of some of the transgenic lines following growth on 1 mM nitrate (Fig. 7A and B). Although there was a trend for lower concentrations of nitrate and nitrite in the leaves of the transgenic plants grown in 10 mM nitrate, the differences were small compared to the WT and apart from nitrate in the source leaves of one line, were not significant (8A and B). Overall the chlorophyll content of the sink and source leaves of all the transgenic lines was higher than the WT (Figs. 7C and 8C), confirming the photographs shown in Figs. 2 and 11, and suggesting that there could have been increased nitrate assimilation in the transgenic lines.

Analysis of the soluble amino acid content of the leaves of plants grown on 1 mM nitrate produced variable data and it was difficult to draw clear conclusions (Fig. 9A–E). The Gln content of the source and sink leaves of all of the plants grown on 10 mM nitrate was considerably higher than plants grown on 1 mM nitrate, whilst the Gln in the *NtAtNiR* leaves was lower than the WT, although the difference was not significant (Fig. 10A). There was a comparable trend in the glutamate (Glu) content of the 10 mM nitrate grown leaves (Fig. 10B), although unlike Gln, the concentration of Glu in the leaves of the plants grown in 1 mM nitrate, was similar to those grown in 10 mM nitrate, further evidence that the Glu concentration is subject to strict regulation (Fritz et al., 2006; Forde and Lea, 2007).

Aspartate (Asp) and asparagine (Asn) are two amino acids produced downstream from Glu and each of these amino acids showed a trend of an increase in the transformed lines irrespective of leaf age, particularly in the 10 mM nitrate grown plants, although the results were variable. The concentrations of Asp and Asn were also higher in plants grown on 10 mM nitrate, when compared to 1 mM

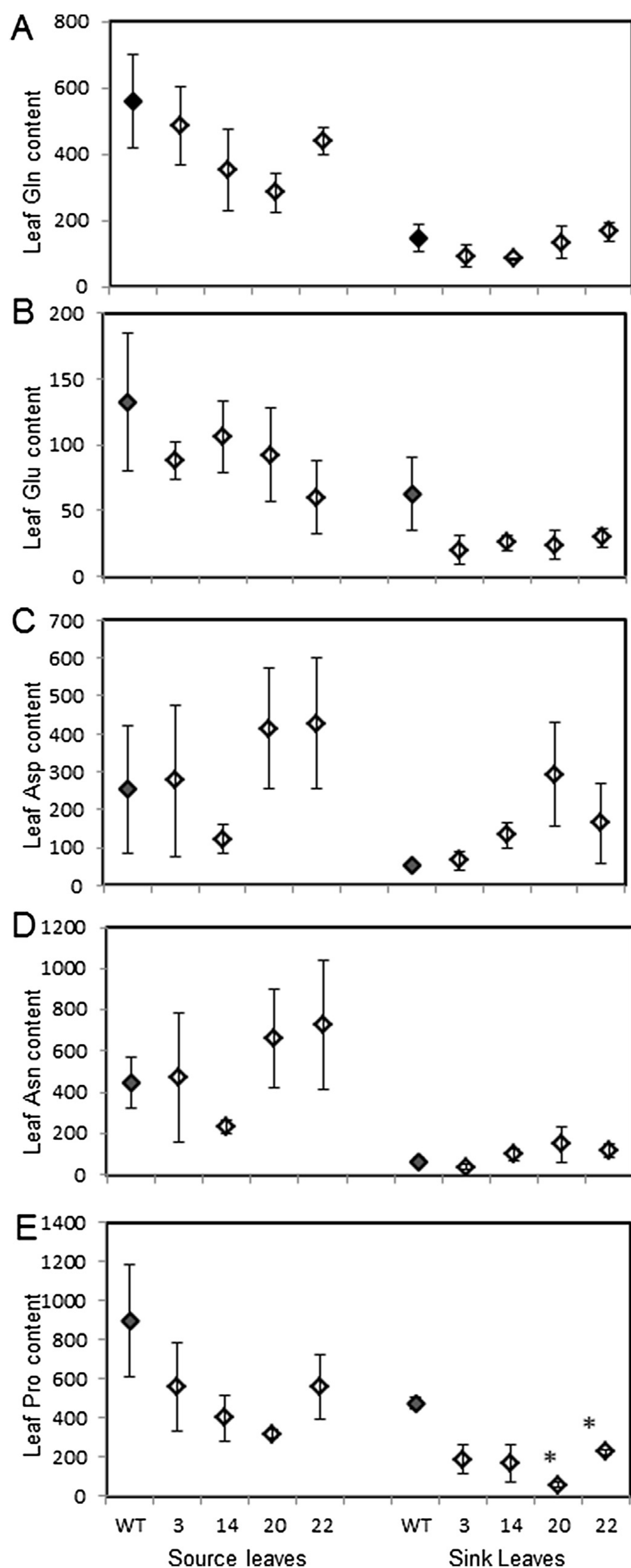

Fig. 10. Soluble amino acid content of leaves of WT tobacco (■) and four T_1 homozygous transgenic lines (□) overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Plants were grown on Hoagland's solution containing 10 mM potassium nitrate. Samples were taken from leaves 3–4 (source leaves) and leaves 14–15 (sink leaves). Three plants from each line were grown for 8 weeks in a glasshouse. Amino acid content (nmol g^{-1} FW) of source leaves and sink leaves. (A) Glutamine (Gln) content of source leaves and sink leaves. (B) Glutamate (Glu) content of source leaves and sink leaves. (C) Aspartate (Asp) content of source leaves and sink leaves. (D) Asparagine (Asn) content of source leaves and sink leaves. (E) Proline (Pro) content of source leaves and sink leaves.

nitrate (Figs. 9C, D and 10C, D).

There was almost a 40 fold higher concentration of Pro in the sink leaves of 10 mM nitrate grown WT plants, when compared to 1 mM nitrate grown plants, whilst the difference in the source leaves was almost 10 fold. However, there was overall a lower concentration of Pro in the *NtAtNiR* plants when compared to the WT plants, irrespective of the leaf age and nitrate concentration (Figs. 9E and 10E). Pro, which may be synthesised directly from Glu or via ornithine, has been shown to accumulate following exposure to drought, high salinity, high light and UV irradiation, heavy metals, oxidative stress and in response to biotic stresses (Hare et al., 1999; Szabados and Savouré, 2010; Ben Rejeb et al., 2014). Following the relief of the stress, Pro is oxidised through proline dehydrogenase, with the subsequent production of ATP in the mitochondria (Servet et al., 2012; Schertl and Braun, 2014). The data presented here would suggest that the transgenic *NtAtNiR* plants have been less exposed to stress than the WT, although the relationship with nitrite metabolism is not clear.

Although not always statistically significant, the data presented in this paper suggests that increased expression and activity of NiR in the leaves of tobacco delays the breakdown of protein and chlorophyll during the senescence period. This would allow the chloroplasts to prolong photosynthetic activity through the production of ATP and reduced ferredoxin required not only for the assimilation of CO_2, but also for the reduction of nitrite by NiR and the GS/GOGAT pathway. The properties of the *NtAtNiR* transgenic lines are similar to some of the "functional stay-green" mutants and transgenic lines, which have recently been reviewed by Gregersen et al. (2013) and Thomas and Ougham (2014), in which the entire senescence syndrome is delayed or slowed down, or both. In transgenic tobacco with an enhanced content of cytokinin, there was a considerable delay in the senescence of the lower leaves, which resulted in a 40% increase in biomass and 52% increase in seed yield (Gan and Amasino, 1995).

Therefore the relationship between NiR activity and the stay-green phenotype may lie in the dependence of NiR on photosynthetically reduced ferredoxin as an electron acceptor. Likewise downstream metabolites Asp and Asn appear to show an increase which implies an increased flux through Fd-GOGAT, an enzyme also dependant on photosynthetically reduced ferredoxin and may be fuelled by the increased chlorophyl content. As a hypothesis, potentially these two strong sinks for reduced ferredoxin maintain chloroplast function and therefore delay the onset of chloroplast dismantling and senescence leading to the stay-green phenotype. Further studies looking at the relationship of reduced ferredoxin levels to chloroplast dismantling may add another insight into the molecular events that trigger the onset of senescence.

This study highlights the importance of NiR in the nitrate assimilation pathway. The enzyme has received relatively little attention in transgenic studies in the past. The research supports the suggestion that NiR may be an important controller of nitrate assimilation (Takahashi et al., 2001). Further studies with the transgenic *NtAtNiR* tobacco plants will hopefully reveal the effects that increased NiR activity has on other key enzymes and metabolites in the N and C assimilation pathways.

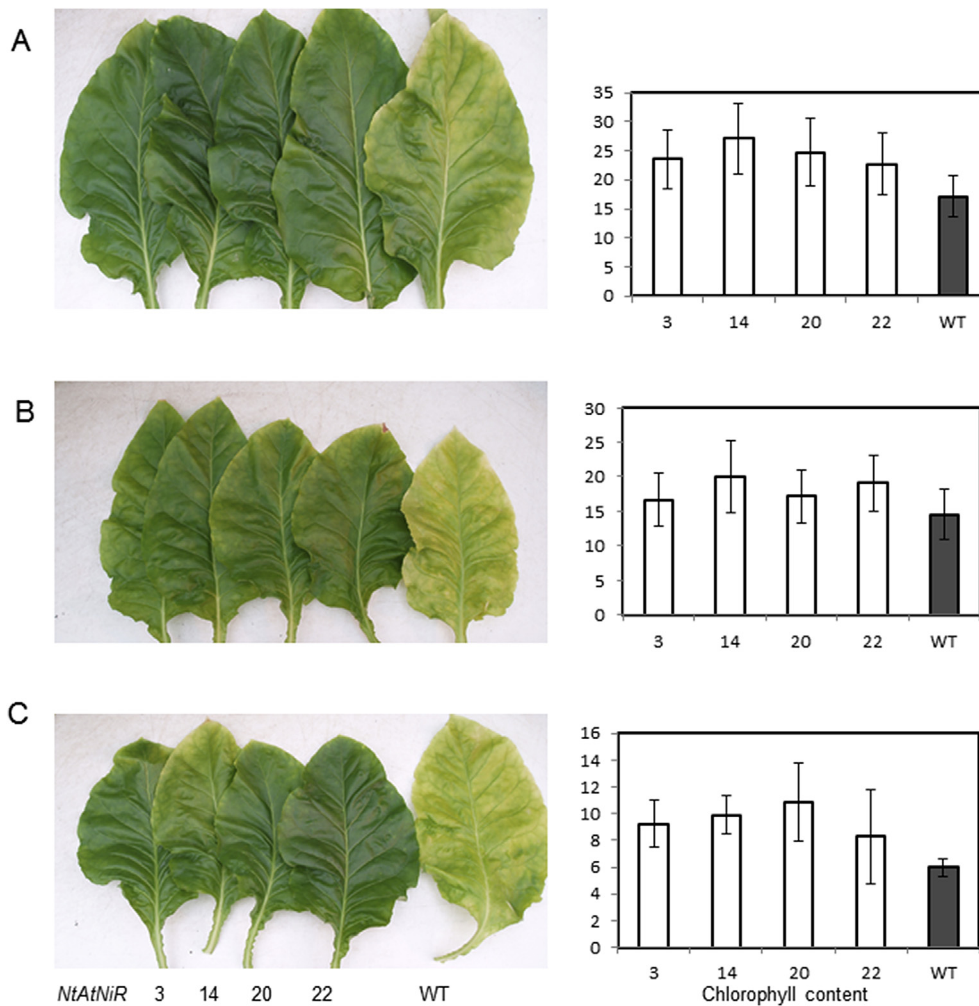

Fig. 11. Leaf phenotype and chlorophyll content of WT tobacco (■) and four T_1 (□) homozygous transgenic lines of overexpressing *A. thaliana* nitrite reductase (*NtAtNiR*). Leaves were sampled after 12 weeks of growth on Hoagland's solution containing 10 mM potassium nitrate. (A) Leaf 7 from the base. (B) Leaf 6 from the base. (C) Leaf 5 from the base. Chlorophyll was determined using a chlorophyll content metre providing units as chlorophyll content index (CCI).

Author contribution

S.D. produced all the clones and transformed plants and performed molecular and biochemical characterization and wrote the article. P.L.L. contributed to the biochemical characterisation under different growth conditions and the design of the experiments. J.P.S-T. contributed in design and discussion of the study.

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