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RESEARCH PAPER

Over-expression of a tomato *N*-acetyl-L-glutamate synthase gene (*SlNAGS1*) in *Arabidopsis thaliana* results in high ornithine levels and increased tolerance in salt and drought stresses

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Abstract

A single copy of the *N*-acetyl-L-glutamate synthase gene (*SlNAGS1*) has been isolated from tomato. The deduced amino acid sequence consists of 604 amino acids and shows a high level of similarity to the predicted *Arabidopsis* NAGS1 and NAGS2 proteins. Furthermore, the N-terminus ArgB domain and the C-terminus ArgA domain found in SlNAGS1 are similar to the structural arrangements that have been reported for other predicted NAGS proteins. *SlNAGS1* was expressed at high levels in all aerial organs, and at basic levels in seeds, whereas it was not detected at all in roots. *SlNAGS1* transcript accumulation was noticed transiently in tomato fruit at the red-fruit stage. In addition, an increase of *SlNAGS1* transcripts was detected in mature green tomato fruit within the first hour of exposure to low oxygen concentrations. Transgenic *Arabidopsis* plants have been generated expressing the *SlNAGS1* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Three homozygous transgenic lines expressing the transgene (lines 1-7, 3-8, and 6-5) were evaluated further. All three transgenic lines showed a significant accumulation of ornithine in the leaves with line 3-8 exhibiting the highest concentration. The same lines demonstrated higher germination ability compared to wild-type (WT) plants when subjected to 250 mM NaCl. Similarly, mature plants of all three transgenic lines displayed a higher tolerance to salt and drought stress compared to WT plants. Under most experimental conditions, transgenic line 3-8 performed best, while the responses obtained from lines 1-7 and 6-5 depended on the applied stimulus. To our knowledge, this is the first plant *NAGS* gene to be isolated, characterized, and genetically modified.

Key words: *Arabidopsis thaliana*, arginine biosynthesis, citrulline, drought tolerance, *N*-acetyl-L-glutamate synthase, ornithine, salt stress, seed germination, transgenic plants.

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Introduction

The rapidly growing world population has made it necessary to limit the losses of crop productivity due to plant responses to environmental stress conditions, such as elevated or low temperatures, drought, salinity, poor soil nutrition, radiation, oxidative stress, and heavy metals. These abiotic factors activate an array of signalling pathways that ultimately lead to plant adaptation to stress, either through post-transcriptional or post-translational regulation (Mazzucotelli *et al.*, 2008). Alternative splicing, degradation or accumulation of stress-related transcripts define the plant response to stresses at the mRNA level, whereas protein phosphorylation and dephosphorylation, ubiquitination, and sumoylation are some of the adaptive responses at the protein level (Mazzucotelli *et al.*, 2008).

In higher plants, arginine has a high N:C ratio (4:6) and serves as a main nitrogen storage compound, where it occurs in both the protein and soluble form. L-Arginine plays a major metabolic role in seed maturation and germination, phloem and xylem transport, particularly in conifer trees, and accumulates under stress and deficiency conditions (Lea *et al.*, 2007). The conversion of glutamate to arginine involves the sequential action of a number of enzymes in nine discrete steps (Fig. 1). The first four of these steps have been distinguished as the ornithine pathway, beginning with the acetylation of glutamate into *N*-acetylglutamate (NAG) through the enzymatic activity of *N*-acetylglutamate synthase (NAGS). Subsequently, NAG is phosphorylated, reduced, and transaminated into *N*-acetylornithine (NAO). The fifth step is the production of ornithine, which is the end-product of two separate enzymatic pathways; the cyclic and the linear pathway (Slocum, 2005). In the former, ornithine and glutamate are produced after the transfer of the acetyl group from NAO to NAG through the enzymatic activity of glutamate *N*-acetyltransferase (GAT), whereas in the latter pathway, deacetylation of NAO by N^2-acetylornithine deacetylase (NAOD) yields ornithine and acetate. The linear pathway has been described in bacteria, but not in plants (Slocum, 2005). The last three steps refer to the arginine pathway, where synthesis of arginine occurs through the intermediate production of citrulline and argininosuccinate (Fig. 1).

Both ornithine and citrulline, the last intermediates in the arginine biosynthetic pathway, are non-protein amino acids. In plants, ornithine is required for the synthesis of polyamines and alkaloids (reviewed by Shargool *et al.*, 1988). Citrulline, a structural analogue of arginine, has been found as one of the major free amino acids to accumulate in the leaves of drought-tolerant watermelon plants (Kawasaki *et al.*, 2000). It is considered as a compound contributing to oxidative stress tolerance in plants subjected to severe water stress (Akashi *et al.*, 2001; Yokota *et al.*, 2002).

There is no information about the structure of the NAGS enzyme in plants. In *Escherichia coli,* however, the holoenzyme occurs either as a hexamer or as a trimer depending on the presence or lack of its ligands, arginine and NAG

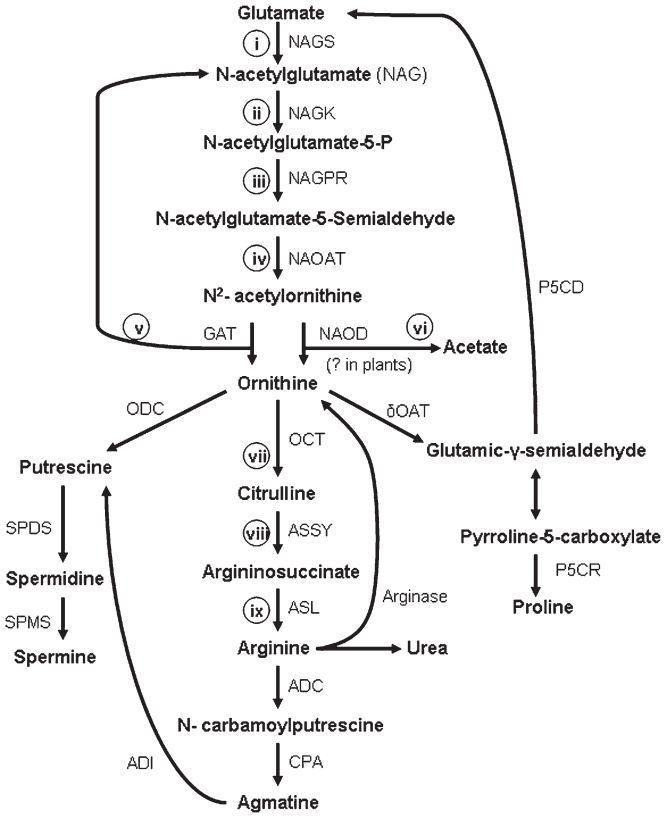

Fig. 1. The L-arginine biosynthetic pathway and its association to proline and polyamine biosynthesis. NAGS, *N*-acetylglutamate synthase; NAGK, *N*-acetylglutamate kinase; NAGPR, *N*-acetylglutamate 5-phosphate reductase; NAOAT, *N*-acetylornithine transaminase; GAT, glutamate *N*-acetyltransferase; NAOD, N^2-acetylornithine deacetylase; OCT, ornithine carbamoyltransferase; ASSY, argininosuccinate synthase; ASL, argininosuccinate lyase; δ-OAT, ornithine-δ-aminotransferase; P5CR, Δ^1 pyrroline-5-carboxylate reductase; P5CD, Δ^1 pyrroline-5-carboxylate dehydrogenase; ODC, ornithine decarboxylase; ADC, arginine decarboxylase; ADI, agmatine deiminase; CPA, *N*-carbamoylputrescine amidohydrolase; SPDS, spermidine synthase; SPMS, spermine synthase. In plants, NAGS and mainly NAGK present the possible regulation points of arginine biosynthesis since they are feedback-inhibited by arginine and alleviated by glutamate. Steps i–iv, the ornithine pathway; step v, the cyclic pathway; step vi: the linear pathway, steps vii–ix, the arginine pathway.

(Marvil and Leisinger, 1977, as reviewed in Slocum, 2005; Caldovic and Tuchman, 2003).

Two highly similar genes (At2g22910 and At4g37670) have been predicted as NAGS in the *Arabidopsis* genome. They share structural similarity to the *E. coli argA* gene, in terms of exhibiting an N-terminal *N*-acetylglutamate kinase (NAGK) domain and a C-terminal GAT domain (Slocum, 2005). Using the massively-parallel signature sequencing expression profiles for *Arabidopsis* genes involved in arginine synthesis and degradation, Slocum found that At4g37670 is more transcriptionaly active than At2g22910 (Slocum, 2005). To date, the sequences of a number of putative NAGS expressed sequence tags (ESTs) from various plant species,

such as corn, rice, soybean and tomato have been deposited in the corresponding libraries (Slocum, 2005; Qu *et al.*, 2007).

Here, the isolation and characterization of a gene (*SlNAGS1*), coding for an *N*-acetylglutamate synthase from tomato is reported. To our knowledge, *SlNAGS1* is the first *NAGS* gene isolated from plants. The tomato NAGS open reading frame (ORF) under the control of the CaMV 35S promoter was introduced into *Arabidopsis thaliana* through *Agrobacterium tumefaciens*-mediated genetic modification in order to achieve *SlNAGS1* over-expression. The resulting stable homozygous transformants exhibited increased tolerance to salt and drought stresses.

Materials and methods

Plant material and growth conditions

Tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) seeds were surface-sterilized and germinated on MS agar plates. After germination plants were transferred to pots with soil and perlite (3:1 v/v) and grown to maturity in a greenhouse. *Arabidopsis thaliana* ecotype Columbia (Col-0) was used in all experiments. Seeds were sterilized (Clough and Bent, 1998) and germinated on MS agar plus vitamins (modification 2B, Duchefa Biochemie BV, Haarlem, The Netherlands) pH 5.8 containing 2% sucrose in a growth chamber at 23 °C day temperature and 18 °C night temperature with a photocycle of 16 h light/8 h dark and photosynthetic flux density of 100 μE m^{-2} s^{-1}.

Isolation of SlNAGS *cDNA*

Initially a cDNA fragment of 252 bp was isolated by applying mRNA differential display in mature green tomato fruit subjected to 3% O_2 (data not shown). This cDNA clone was used as a probe to screen a cDNA tomato library prepared from mature green tomato tissues. As a result, a partial sequence of 1090 nucleotides corresponding to the 3′ end of NAGS was isolated, cloned into pGEM-T Easy (Promega, Madison, WI, USA) and sequenced using a Li-Cor Long Readir 4200 automated sequencer and a Sequitherm EXCELL II (Epicenter Technologies, Madison, WI, USA) kit. The full-length of the gene was isolated in two successive steps using the Marathon™ (Clontech, Palo Alto, CA, USA) and subsequently the 5' Rapid Amplification of cDNA Ends (RACE; Invitrogen, Carlsbad, CA, USA) systems. The primers used were L2.5: 5′-GAGATTGATCCATTAGAACCATTGTGACC-3′ for the Marathon™ system, and GSP1: 5′-TCAATTTGGACATGAGTT-3′ and GSP2: 5′-TGATTCCCAGGCCATGAAGGAG-3′ for the 5′ RACE system, each time following the manufacturer's instructions. The complete sequence was deposited in GenBank under the accession number FJ543466.

Expression of SlNAGS *in tomato under low oxygen stress*

Mature green tomato fruit were placed in air-tight containers and exposed to a constant gas flow of 60–100 ml min^{-1} of a gas mixture containing air, 97% N_2 and 3% O_2, 99.5% N_2 and 0.5% O_2, and 100% N_2. All treatments were performed at 22 °C. Ethylene was applied at a concentration of 10 μl L^{-1}. Samples were retrieved at 0, 1, 3, 6, 12, 24, 48, and 72 h after each treatment, the locular tissue was removed, and the pericarp was immediately frozen in liquid nitrogen and stored at –80 °C.

RNA extraction and blot analysis

Total RNA extraction from tomato was conducted according to Smith *et al.* (1986). RNA electrophoresis was run in 1% (w/v) agarose–formaldehyde gel in the presence of 20 mM phosphate buffer, pH 6.8 with 15 μg of total RNA loaded per lane. RNA was blotted onto nylon membranes (Nytran 0.45, Schleicher & Schuell Inc., Keene, USA) by capillary transfer. The probe was labelled with α-^{32}P dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden), using the Rad Prime Probe Labeling System (Invitrogen, San Diego, CA, USA). Hybridization was performed at 65 °C. Hybridized membranes were exposed to autoradiography (X-OMAT AR; Eastman Kodak Corporation, Rochester, NY, USA) for 14 d.

Vector construction and A. thaliana *transformation*

The plant transformation vector pGA643 (An *et al.*, 1988) containing the CaMV 35S promoter, an *A. tumefaciens nos* transcriptional terminator and the *npt*II kanamycin resistance gene was used for cloning the tomato *NAGS* gene in a sense orientation between the *Cla*I and *Bgl*II restriction sites. The recombinant binary plasmid was sequenced and was used to transform *A. tumefaciens* LBA 4404 (Hoekema *et al.*, 1983) by electroporation. The *A. tumefaciens* recombinant strain was used to transform *A. thaliana* plants by the floral dip method described by Clough and Bent (1998). Transformed plants were grown to maturity in a growth chamber, irrigated every 3 d with water and every 2 weeks with a commercial fertilizer (Algoflash, COMPO France SAS, Roche-lez-Beaupre, France).

Selection of transformed plants

Seeds from the transformed plants were selected on MS agar (modification 2B, Duchefa Biochemie BV, Haarlem, The Netherlands) with 2% sucrose containing 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} cefotaxime (Duchefa Biochemie BV). Resistant plants were transferred to pots with soil and perlite (3:1 v/v) and grown to maturity in a growth chamber. Seeds were harvested and sown on MS agar with 2% sucrose containing 50 μg ml^{-1} kanamycin to obtain homozygous plants. Homozygous plants were transferred to soil and were grown for seed production. Seeds from these homozygous plants were used for all further experiments.

Analyses of transgenic A. thaliana *plants*

Kanamycin-resistant plants were screened for the presence of the transgene by polymerase chain reaction (PCR) using

the tomato NAGS specific primers L2-*Cla*I (5′-ATGT-CAGCTTCACCGGCAACGC-3′) and L2-Rev1 (5′-AATGGACACAGATTAACAAGGATT-3′). The presence of the selection marker gene *npt*II was verified using the primers NPT-US1 (5′-GGTTCTCCGGCCGCTTGG-3′) and NPT-US2 (5′-TCGGGAGCGGCGATACCG-3′). DNA was isolated from leaves of homozygous transgenic and WT plants as described by Diallinas *et al.* (1997). Ten μg of DNA were digested with *Hpa*I (5 units μg^{-1} of DNA) and upon electrophoreses on a 0.7% TRIS-acetate/EDTA (TAE) agarose gel (Sambrook *et al.*, 1989), were transferred onto a positively charged nylon membrane (Schleicher & Schuell) and hybridized with a *npt*II probe and a tomato NAGS probe. Labelling was performed using the Rad Prime Probe Labeling System (Invitrogen) and α-^{32}P dCTP (Amersham). Hybridization and membrane washes were performed as described by Church and Gilbert (1984). Total RNA was extracted as described in Wadsworth *et al.* (1988). RNA was fractionated in a 1.2% formaldehyde denaturing gel in phosphate buffer as described in Sambrook *et al.* (1989). Hybridization was performed at 50 °C using a 1082 bp fragment (from the *Nde*I restriction site to the poly-adenylation site) of tomato NAGS as a probe.

Germination

For salt germination experiments, seeds were surface-sterilized as described by Clough and Bent (1998) and sown on MS plates plus 2% sucrose containing 250 mM NaCl. For these experiments, the concentration of 250 mM NaCl was selected, based on reports by Quesada *et al.* (2002). After stratification at 4 °C for 4 d, plates were transferred in a growth chamber (16 h day at 23 °C and photosynthetic flux density of 100 $\mu E\ m^{-2}\ s^{-1}$, 8 h dark at 18 °C) and monitored for germination. A set of control plates was made with no added salt. All experiments were performed in duplicate.

Drought and salt stresses

Seeds of transgenic and control plants were sown in pots of 180 cm^3 containing a mixture of soil and perlite (3:1 v/v) and after stratification were grown in a growth chamber for 4 weeks with normal watering every 3 d and watering with a commercial fertilizer (Algoflash, COMPO France SAS) every 2 weeks. After the fourth week, plants were divided into groups of eight plants each and subjected to stress treatments. One group was subjected to drought stress by withholding water, a second group was watered with a solution of 300 mM NaCl, and a control group was watered normally. The concentration of 300 mM NaCl was chosen based on the literature for salt-watering experiments (Piao *et al.*, 2001). Seven days after the beginning of the stress treatments, water-stressed plants were returned to the normal watering routine and the plants were allowed to recover.

Chlorophyll measurement

Leaves were excised from control and treated plants (subjected to salt and drought stress) and weighed 7 d after imposition of stresses. Chlorophyll extraction was performed in dimethyl sulphoxide (DMSO) as described by Richardson *et al.* (2002). Each sample consisted of six randomly selected rosette leaves from three individual plants. Absorbance (OD) was recorded at 645 nm and 663 nm, and total chlorophyll was estimated using the Arnon (1949) equation: total chlorophyll ($g\ l^{-1}$) = $0.0202OD_{645}$ + $0.00802OD_{663}$. Total chlorophyll estimated was subsequently converted to leaf chlorophyll concentration (mg chlorophyll g^{-1} fresh weight).

Arginine, ornithine, and citrulline analyses

Rosette leaves were extracted and derivatized according to Khuhawar and Rajper (2003*a*, *b*). A 20 μl sample was injected into a Symmetry C_{18} 5 mm (250×4.6 mm i.d.) column (Waters Corporation, Milford, MA, USA.) and the derivatives were eluted with methanol:water (62:38 v/v) at a flow rate of 1 ml min^{-1} using a Marathon IV HPLC Pump (Rigas Labs, Thessaloniki, Greece). Detection was performed at 330 nm by a Fasma 525 variable wavelength UV monitor (Rigas Labs, Thessaloniki, Greece). Authentic standards for arginine, ornithine, and citrulline (Sigma-Aldrich, St Louis, MO, USA) were used to identify retention times. The results are reported as μg 100 mg^{-1} fresh tissue.

Results

Isolation and characterization of a full-length cDNA for NAGS *from tomato (*SlNAGS1*)*

The initial part of the cDNA of the subsequently annotated *SlNAGS1* gene was originally identified during a differential display screen for isolating hypoxia-responsive genes from mature green tomato fruit subjected to low oxygen regimes. Later, a 1090 bp clone corresponding to the 3′ end of the gene was isolated from a cDNA library constructed from mRNA isolated from hypoxia-treated mature green tomato fruit. Finally, the full-length *SlNAGS1* cDNA clone was obtained in two successive steps via reverse-transcription PCR (RT-PCR) and RACE techniques. The full-length cDNA is 2312 nucleotides long and it contains a 218 bp 5′ untranslated region, a 1812 bp ORF, which encodes a 604 amino acids peptide, and a 263 bp 3′ untranslated region up to the polyA tail (accession number FJ543466). Further, Southern analysis revealed that there is a single copy of the gene per haploid genome (data not shown).

In silico *analysis of the* SlNAGS1

In silico analysis of the predicted amino acid sequence of the *SlNAGS1* ORF predicted that the mature protein has an estimated molecular weight of 66.4 kDa and a pI of 7.30. Moreover, a chloroplast transit peptide of 31 amino acids has been predicted to occur at the N-terminus of the predicted protein (ChloroP 1.1, Emanuelsson *et al.*, 1999). Further analysis on the primary structure of the SlNAGS1

protein predicted the occurrence of a NAG kinase-like domain from position –80 to –331aa, followed by an amino acid kinases (AAK) superfamily catalytic domain from –385 to –437aa (Fig. 2A) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer *et al.*, 2007). Close to the C-terminus of the protein, from position –443 to –591, high levels of similarity were found to the Gcn5-related *N*-acetyltransferase (GNAT) domain (Falquet *et al.*, 2002), (Fig. 2A). A BLASTP analysis (Altschul *et al.*, 1997) showed that the predicted SlNAGS1 protein exhibited 69% similarity to the *Arabidopsis* NAGS1 (at2g22910) and 66% similarity to the NAGS2 (at4g37670) proteins.

Expression of the SlNAGS1 *gene in different tissues during fruit development and under stress*

Expression levels of *SlNAGS1* were monitored in various tomato tissues, during fruit ripening and under conditions of abiotic stress. *SlNAGS1* mRNA preferentially accumulated in green tissues i.e. leaves, petioles, stems, apices, and in flowers in accordance with its chloroplast location. It was also detected at very low levels in seeds, whereas it was not detected in roots (Fig. 3A). During fruit ripening, *SlNAGS1* mRNA levels remained fairly constant with a slight peak at the red fruit stage (Fig. 3B). In order to investigate the effect of low oxygen concentration on *SlNAGS1* expression, mature green tomato fruit were exposed to different oxygen regimes (21% O_2 (air), 3% O_2, 0.5% O_2, and 0% O_2) and samples were collected at different time points after the onset of exposure. At low oxygen concentrations (0%, 0.5%) *SlNAGS1* transcript levels were high for the first 3 h of exposure, and decreased slowly and gradually thereafter (Fig. 3C). On the contrary, in 3% O_2 and in air, transcript levels after the first 3 h of exposure decreased rapidly (Fig. 3C).

Mature green tomato fruit were exposed to 10 μl L^{-1} ethylene, and samples were collected at regular time points thereafter. A peak in *SlNAGS1* mRNA accumulation was observed 3 h after exposure to ethylene with a levelling off after 24 h (Fig. 3C).

Molecular characterization of transgenic A. thaliana *plants over-expressing the* SlNAGS1 *gene*

Transgenic *A. thaliana* plants expressing the tomato *SlNAGS1* ORF under the control of the CaMV 35S promoter were generated by *Agrobacterium*-mediated transformation and seeds (T_1) of the transformed plants were selected based on their kanamycin resistance. These plants were grown to maturity and self-pollinated. T_2 seeds were subjected to another round of kanamycin selection to obtain non-segregating transgenic lines (T_3). The presence of the tomato *SlNAGS1* gene was examined by PCR using gene-specific primers (see in Materials and methods). Gene copy number

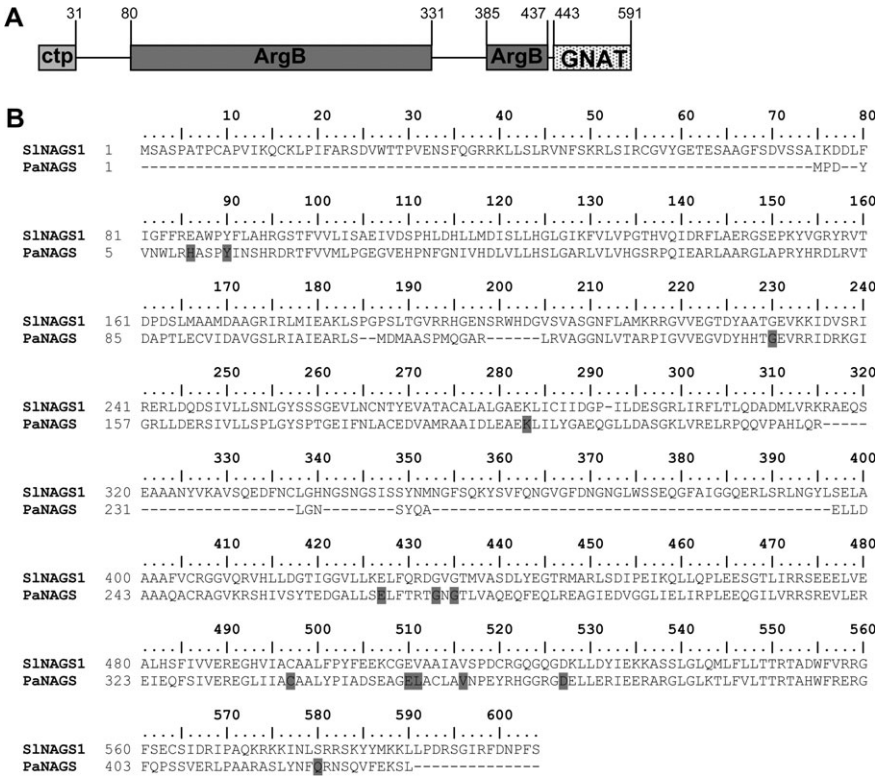

Fig. 2. Structure of the SlNAGS1 protein (A). The various sequence domains predicted by *in silico* analysis of the primary structure of the protein are indicated: light grey box, chloroplast transit peptide (as predicted by the ChloroP 1.1 software but not supported by experimental evidence); grey box, ArgB domain; grey-dotted box, GNAT domain. Amino acid positions at the beginning and end of each domain are indicated. Alignment of the tomato SlNAGS1 and the *Pseudomonas aeruginosa* (AAG08589) amino acid sequences (B). Amino acids that have been mutated in *P. aeruginosa* are grey shaded.

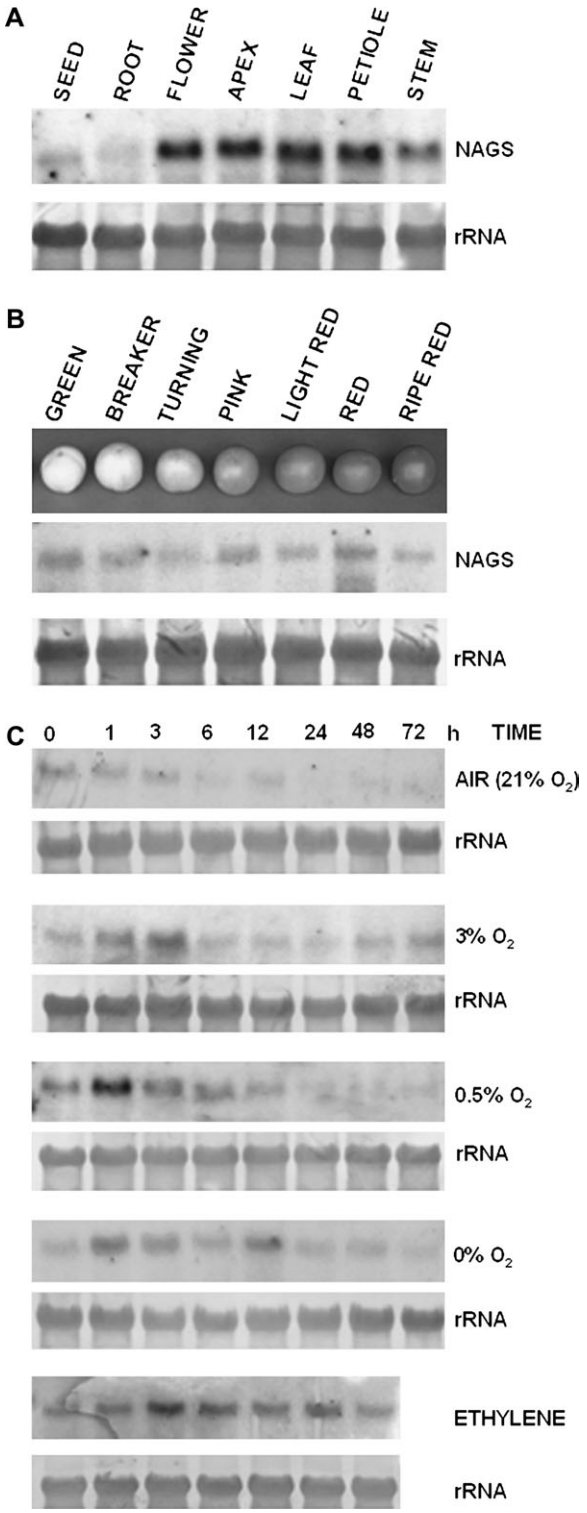

Fig. 3. Expression of NAGS in tomato vegetative tissues (A), during fruit ripening (B), and at low oxygen (air, 3%, 0.5%, 0% oxygen) and ethylene (10 ppm) conditions (C). Approximately 15 μg of total RNA were fractionated on denaturing agarose gels and after transfer to a positively charged nylon membrane was hybridized with a tomato NAGS probe labelled with ^{32}P. Equal loading of the RNA and transfer efficiency were determined by methylene blue staining. (This figure is available in colour at *JXB* online.)

was assessed in T_3 plants by Southern blot analysis and probed with both the *npt*II gene and the 3′ end of the tomato *SlNAGS1* gene. Each transgenic line showed a unique restriction pattern indicating independent transformation events (data not shown). Lines showing a single gene insertion upon hybridization with the *npt*II gene probe were further verified by probing the same blot with tomato *NAGS* (data not shown). Total RNA was isolated from fully grown rosette leaves and tomato *SlNAGS1* mRNA over-expression was confirmed in all transgenic lines but not in WT plants (Fig. 4). Three homozygous transformed lines (lines 1-7, 3-8, and 6-5) were chosen for further analysis along with the WT plants. No visible phenotypic alteration was observed between the three transgenic lines and the WT under normal growth conditions.

Accumulation of arginine, citrulline, and ornithine in the transgenic lines

The content of arginine, ornithine, and citrulline was measured in leaves of fully grown homozygous transgenic and WT plants. Ornithine levels were higher in leaves of all three transgenic lines compared with WT plants, with line 3-8 exhibiting the uppermost value, which was 9-fold higher than the controls plants (Fig. 5). Monitoring the levels of citrulline in leaves revealed that there was a 29% increase in citrulline content in transgenic line 3-8 (Fig. 5), whereas, in the other two lines, only a moderate increase (10%) was observed compared with WT levels. Concerning arginine content, transgenic line 3-8 exhibited slightly higher levels than the WT leaves, whereas the arginine content of the other two transgenic lines was lower than the WT (Fig. 5).

Stress responses of transgenic plants after exposure to salt and drought

High levels of free amino acids, including the non-protein amino acids ornithine and citrulline, have been reported to

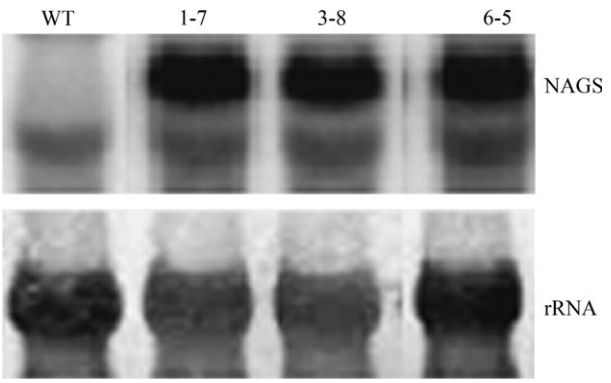

Fig. 4. Expression of tomato NAGS in selected lines of transgenic *Arabidopsis thaliana* plants and in WT plants. Total RNA isolated from leaves of fully grown homozygous plants and after fractionation and transfer to nylon membrane was hybridized with a tomato NAGS probe labelled with ^{32}P. Equal loading of the RNA and transfer efficiency were determined by methylene blue staining.

occur upon salinity stress (reviewed by Ashraf and Harris, 2004). The high ornithine levels detected in the leaves of the transgenic lines overexpressing *SlNAGS1* could contribute to increased tolerance to NaCl stress. To investigate this hypothesis, experiments were performed monitoring the seed germination ability under salinity stress by scoring radicle emergence of WT and all the transgenic lines grown on agar plates supplemented with NaCl (Fig. 6). Seeds of the transgenic line 3-8 exhibited a similar to WT germination ability on agar plates without added salt, whereas seeds of lines 1-7 and 6-5 showed reduced germination potential under these conditions (Fig. 6). Introducing high salt concentration (250 mM NaCl) in the germinating media, however, reversed this pattern; only 9% of WT seeds were able to germinate, while all three transgenic lines exhibited significantly higher germination ability compared to WT (Fig. 6).

Further, in order to determine whether the modification of the arginine biosynthetic pathway influenced the ability of whole plants to withstand osmotic stress, 4-week-old mature plants of lines 1-7, 3-8, and 6-5 were subjected to salt stress by watering them with 300 mM NaCl. Another set of plants was subjected to drought stress by withholding water. After 7 d, plants were assessed visually. Salt stressed lines 1-7, 3-8, and 6-5 exhibited clear growth retardation during the 7 d stress period as indicated by the smaller leaf size compared to the unstressed controls (Fig. 7, compare A and B). Some of the WT plants did not survive the salt treatment (Fig. 7B, lower panel). Shortage of water for 7 d caused a severe stress in the WT plants, whereas all three transgenic lines, although somewhat stressed, retained more or less their green leaf colour (Fig. 7C). Upon rewatering, the transgenic drought-stressed plants re-established their healthy appearance but not the WT plants (Fig. 7D). Moreover, leaves were collected from control and stressed plants and total chlorophyll content was measured in all plants. Control plants of all genotypes did not show any statistically significant change in comparison to the WT plants. Salt and drought stresses, however, changed the chlorophyll content of the lines tested, with all three

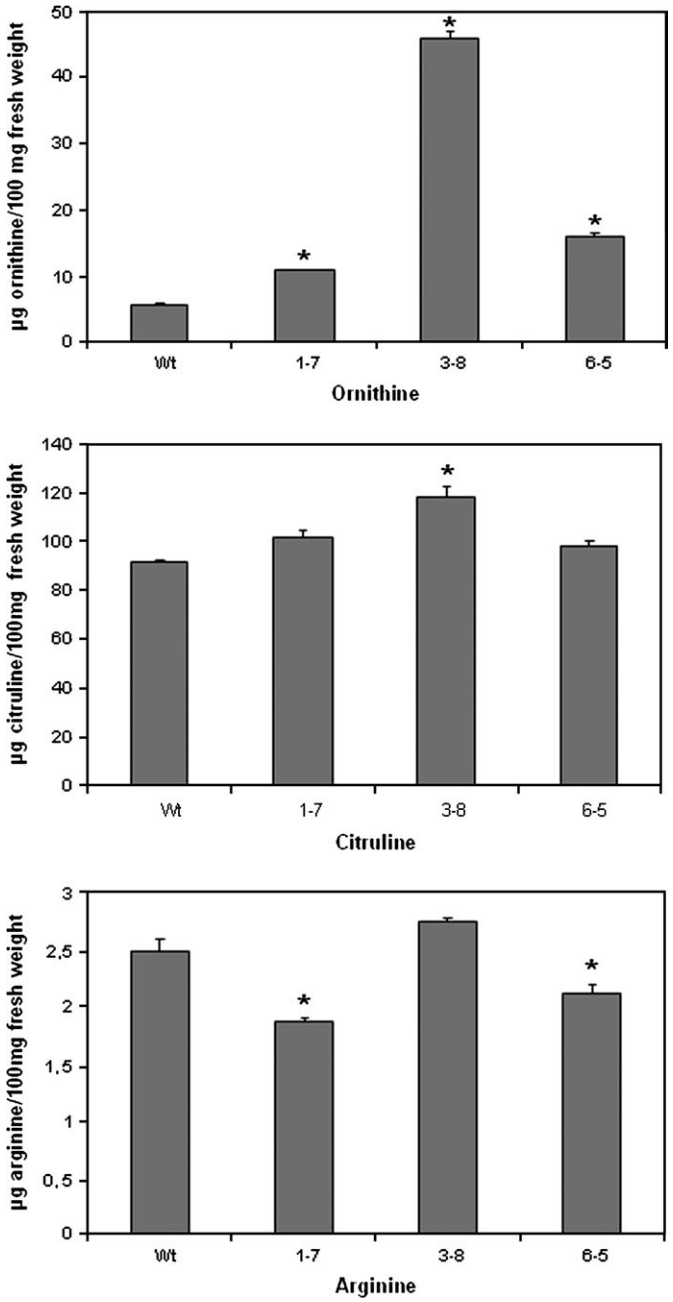

Fig. 5. Accumulation of ornithine, citrulline, and arginine in the leaves of WT and of three transformed lines. Wild-type and transgenic plants were grown to the mature rosette stage (before bolting commenced) and leaves were harvested for amino acid determinations. Data are means of three independent measurements. Error bars represent the standard error of the mean and an asterisk indicates statistically significant differences compared to WT plants.

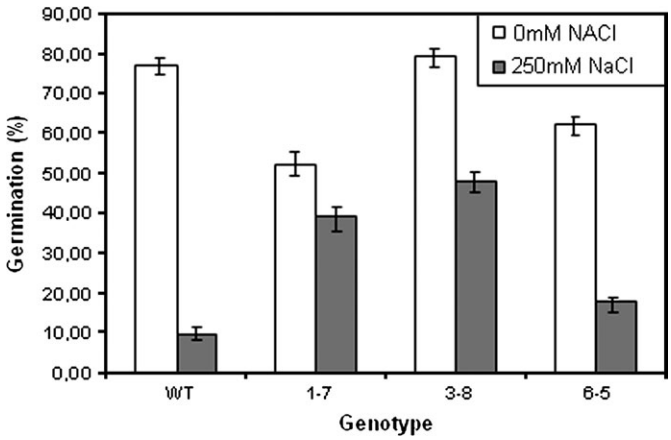

Fig. 6. Germination ability of WT and selected transgenic seeds under salt pressure. Seeds of WT and transgenic lines were germinated on MS agar medium without the addition of salt or with 250 mM NaCl supplementation. Radicle emergence was scored as positive germination. Population proportions were calculated by dividing the total number of germinated seedlings by the initial seed number plated onto each plate for each genotype. Error bars indicate the 95% confidence interval for each population proportion.

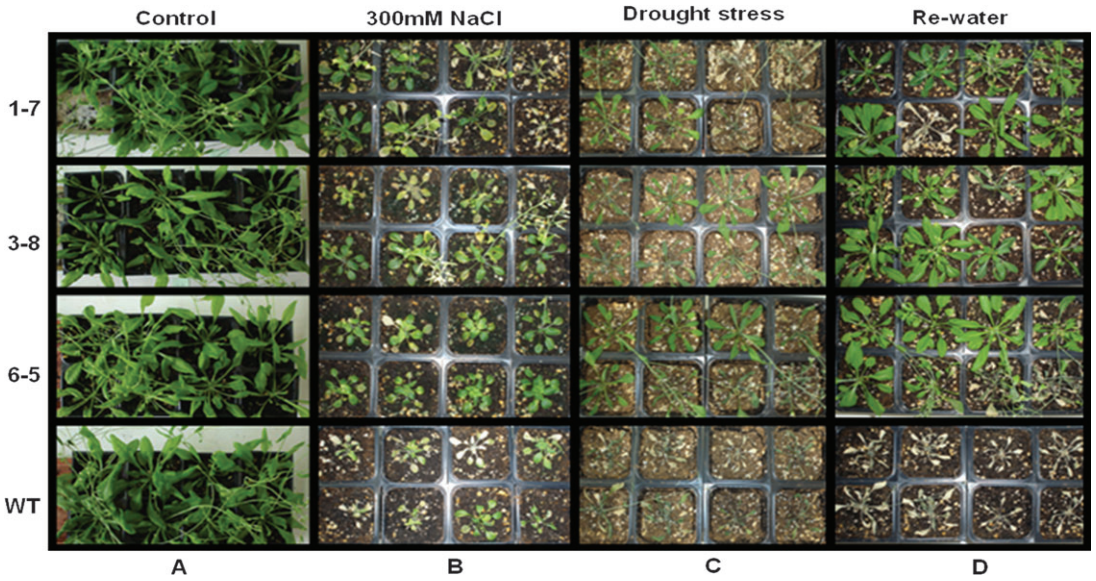

Fig. 7. Response of transgenic and control plants to salt and drought stress after 7 d. Four-week-old plants were divided into three groups; one group was watered with H_2O (A), the second group was watered with 300 mM NaCl (B), the third group was subjected to drought stress by withholding water (C). After withholding water for 7 d, the plants of this group were rewatered for 2 d (D). All plants were photographed using a digital camera.

transgenic lines accumulating larger amounts of chlorophyll than the WT plants (Fig. 8) suggesting a better tolerance of the genetically modified plants to salt and drought stresses.

Discussion

In the present study, the isolation and characterization of a [*NAGS*] gene from plants are described, as a result of an mRNA differential display approach to isolate genes responsive to hypoxia conditions in mature green tomato fruit. A full-length cDNA was obtained and found to possess high similarity to NAGS, therefore, it was designated *SlNAGS1*. It encodes for a 604 amino acid peptide that possesses the architectural organization that has been predicted to occur in other NAGS family proteins (Slocum, 2005), namely an N-terminal NAG kinase-like (ArgB) domain which is related to the bacterial ArgB domain and a C-terminal NAGS domain which corresponds to the ArgA domain (Marchler-Bauer *et al.*, 2007). The N-terminal NAG kinase-like (ArgB) found in SlNAGS shares sequence similarity to the N-terminus bacterial NAGK sequence; it is believed, however, that this similarity probably lacks the functional NAGK activity because critical amino acid positions among the plant-derived NAGS predicted proteins are not conserved (Slocum, 2005).

The ArgB and GNAT (ArgA) domains seem to be highly conserved among the plant putative NAGS proteins, including SlNAGS1 (see Supplementary Figs S1 and S2 at *JXB* online), suggesting a comparable function for these proteins among plant species. In a phylogenetic analysis, these proteins are clustered closer to the bacterial NAGSs rather than to the fungi or mammalian NAGSs (Fig. 9);

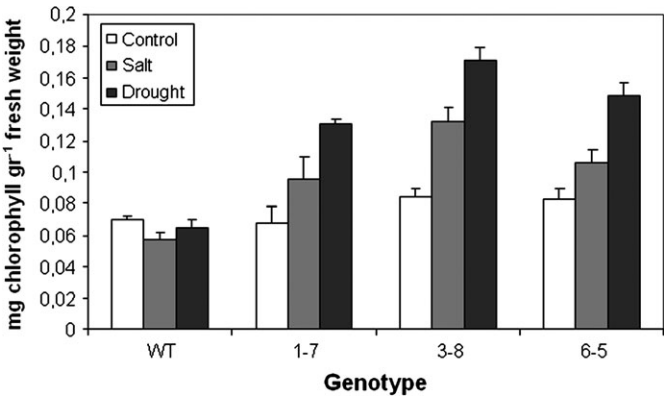

Fig. 8. Response of transgenic and control plants to salt and drought stress. Four-week-old plants were divided into three groups; one group was watered with a solution of 300 mM NaCl, another group was subjected to drought stress by withholding water, while the third group continued its normal watering routine. Six rosette leaves from three individual plants per sample were harvested for chlorophyll measurement 7 d after stress imposition. Total chlorophyll content was estimated spectrophotometrically after extraction in DMSO. Data represent means ±SE of three measurements.

nevertheless, their sequence similarity to the corresponding sequences of bacteria, fungi, and mammals is low.

In ArgB domain, predicted, predicted plant NAGS proteins contain a stretch of about 90 amino acids that has not being detected in bacteria, yeast, and fungi (Slocum, 2005). Our sequence analysis found that the N-terminus sequence of this particular stretch is highly conserved among plant-derived NAGS proteins, in contrast to its 36 amino acid C-terminus sequence that is variable in

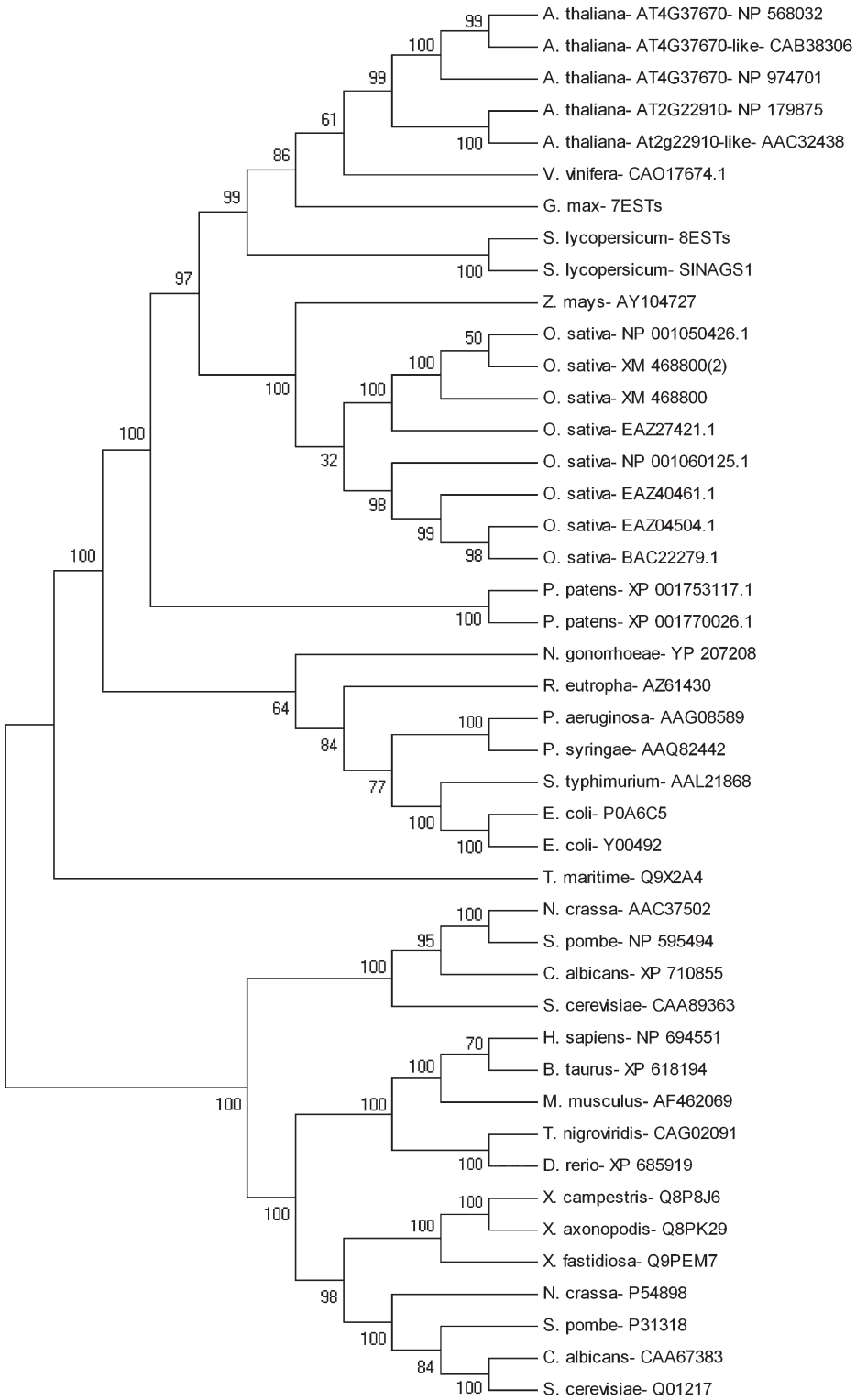

Fig. 9. Phylogenetic analysis of a total of 44 NAGS-related sequences from a variety of organisms. This analysis, which enriched and updated the previous analysis by Qu *et al.* (2007), was performed using the MEGA ver. 4.1 software (Tamura *et al.*, 2007). The phylogenetic relationship between the examined sequences was performed using the Neighbor–Joining method with p-distance correction. Bootstrap values were derived from 1000 replicate runs. Accession numbers together with the amino acid sequences used, are given in Supplementary Fig. S1 at *JXB* online. The following organisms were used in the phylogenetic analysis: *A. thaliana* (*Arabidopsis thaliana*) Arabidopsis; *B. taurus* (*Bos taurus*) cow; *C. albicans* (*Candida albicans*); *D. rerio* (*Danio rerio*) zebrafish; *E. coli* (*Escherichia coli*); *H. sapiens* (*Homo sapiens*) human; *M. musculus* (*Mus musculus*) mouse; *N. crassa* (*Neurospora crassa*); *N. gonorrhoeae* (*Neisseria gonorrhoeae*); *O. sativa* (*Oryza sativa*) rice; *P. patens* (*Physcomitrella patens*) moss; *P. syringae* (*Pseudomonas syringae*); *P. aeruginosa* (*Pseudomonas aeruginosa*); *R. eutropha* (*Ralstonia eutropha*); *S. lycopersicum* (*Solanum lycopersicum*) tomato; *S. cerevisiae* (*Saccharomyces cerevisiae*);

composition (see Supplementary Fig. S2 at *JXB* online, from position –360 to position –396).

A recent study using site-directed mutagenesis of recombinant *Pseudomonas aeruginosa* NAGS (PaNAGS) demonstrated the importance of specific positions as signature binding motifs for arginine residues, the acetyl group AcCoA or the glutamate amino group (Sancho-Vaello *et al.*, 2008). In particular, they showed that alanine substitutions of specific residues within the AAK domain resulted in complete or increased arginine inhibition, whereas mutations within the GNAT domain considerably influenced substrate kinetics (Sancho-Vaello *et al.*, 2008). Ten out of the 13 mutated sites on *P. aeruginosa* are present in the SlNAGS1 sequence with the flanking sequences to be strongly conserved (Fig. 2B). Relics of identifiable features that resemble the β11, αH, and β16 structural elements of NAGKs were also found to occur in plant NAGS amino acid sequences at positions –281 to –285, –423 to –428, and –434 to –436, respectively (Ramon-Maiques *et al.*, 2006).

The bacterial enzyme is allosterically inhibited by arginine in *E. coli* and several inhibition resistant mutants have been identified (Rajagopal *et al.*, 1998). In these mutants, specific amino acid substitutions abolish the inhibitory effect of arginine. Amino acid substitutions also exist in four positions in SlNAGS1 (positions –86, –125, –129, and –579, which correspond to positions –15, –54, –58, and –432 of the *E. coli* protein) although a different amino acid is present in the tomato protein compared to the respective site of the feedback-resistant *E. coli* protein. Similarly, other enzymes that are involved in the biosynthesis of compatible solutes in plants, for example, moth bean P5CS is allosterically inhibited by proline although less sensitive than the *E. coli* enzyme (Delauney and Verma, 1993). It is possible that, in osmotically stressed plants, this feedback inhibition of NAGS by arginine is less evident and this pathway could serve to increase the NAG pool that is directed to ornithine, polyamine, proline, citrulline, and arginine biosynthesis. Arginine inhibition of NAGS and NAGK represent key steps in the regulation of arginine biosynthesis in plants (Shargool *et al.*, 1988) whereas, in humans, NAGS is arginine activated (Sancho-Vaello *et al.*, 2008).

A chloroplast transit peptide is predicted at the N-terminus of the SlNAGS1 protein, with a putative transit peptide cleavage site after Val 31 (ChloroP 1.1 Server: Emanuelsson, *et al.*, 1999). Similarly, the other putative plant NAGS proteins also possess chloroplast transit peptides consisting of as low as 11 amino acids, to as many as 74 amino acids, with the exception of the *Arabidopsis* at2g22910 predicted NAGS protein which possesses a 4 amino acids predicted chloroplast transit peptide (ChloroP 1.1 Server - prediction results, see Supplementary Fig. S3 at *JXB* online). The prediction of the chloroplastic location of plant-derived NAGS proteins, although is generally accepted, is in contrast to 'a single report' (see review by Slocum, 2005) that detected cytoplasmic abundance of NAGS in protoplasts derived from soybean cell cultures (Jain *et al.*, 1987).

An expression analysis detected hybridizing transcripts in all green aerial tissues, namely leaves, petiole, stem, apex, and also in flowers, while the *SlNAGS1* transcripts were at extremely low levels in seeds and not detectable in roots. This expression profile is in agreement with the expected NAGS localization in plastids. In terms of fruit development and ripening, *SlNAGS1* mRNA steady-state levels accumulated in all stages tested with higher levels being in the red-stage fruit, implicating a participation in nitrogen metabolism during the late stages of ripening. It is known that glutamate accumulates in red ripe tomato fruit (Boggio *et al.*, 2000) concomitant with induced glutamate dehydrogenase (GDH) activity and transcript levels (Loulakakis *et al.*, 1994; Boggio *et al.*, 2000). GDH may be involved in ammonium detoxification *in vivo* and the replenishment of the glutamate pool, which is highly required to produce protective metabolites (citrulline, proline, phytochelatines, etc.). The above can be explained by the induction of *NAGS* during late ripening, possibly to direct glutamate to arginine (Fig. 1), and which may, subsequently, feed the urea cycle. The induction of *SlNAGS1* by ethylene further supports its involvement in tomato fruit ripening (Fig. 3). Furthermore, the early transient induction of *SlNAGS1* expression in response to low oxygen regimes indicates a possible role of this gene in a plant's adaptation to the early events of hypoxia.

To investigate the function of the *SlNAGS1* gene, transgenic *Arabidopsis* plants have been produced ectopically expressing the *SlNAGS1* gene under the transcriptional control of the CaMV 35S promoter. This modification caused elevated levels *of SlNAGS1* transcripts in transgenic lines resulting in a substantially increased concentration of ornithine. Citrulline and arginine levels were also higher compared with WT plants, however, they were noticeably lower than those of ornithine. To our knowledge, this is the first report describing elevated levels of ornithine in plants upon modification of its biosynthetic pathway. It has been suggested that ornithine accumulation can be accomplished via inhibition of any of the enzymes participating in ornithine metabolism (Patil *et al.*, 1972). For example, inhibition of ornithine carbamoyltransferase (OCT; the enzyme that catalyses the conversion of ornithine to citrulline) due to phaseolotoxin, a specific inhibitor of OCT produced by *Pseudomonas syringae* pv. *phaseolicola*, resulted in extremely high levels of ornithine in bean leaves, coupled with the formation of chlorotic lesions (Patil *et al.*, 1970).

The high ornithine levels imply that there might be an increase in the production of compounds that are derived

S. typhimurium (*Salmonella typhimurium*); *S. cerevisiae* (*Saccharomyces cerevisiae*); *S. pombe* (*Schizosaccharomyces pombe*); *T. nigroviridis* (*Tetraodon nigroviridis*) freshwater pufferfish; *T. maritime* (*Thermotoga maritime*); *X. campestris* (*Xanthomonas campestris*); *X. axonopodis* (*Xanthomonas axonopodis*); *X. fastidiosa* (*Xylella fastidiosa*); *V. vinifera* (*Vitis vinifera*) grape; *Z. mays* (*Zea mays*) corn.

from ornithine, such as polyamines, that are known to possess osmoprotective functions (see Yang *et al.*, 2007, and references therein). High levels of endogenous spermidine and spermine in rice plants due to the exogenous application of guazatine, an inhibitor of polyamine oxidase activity, prevented the loss of chlorophyll (Capell *et al.*, 2004). It is possible that the maintenance of chlorophyll in the rosette leaves of the *SlNAGS1* plants compared with the dramatic chlorophyll loss in the WT might also be due to possible high levels of polyamines.

Amino acids including arginine, alanine, serine, glycine, leucine, and valine, together with the imino acid, proline, and the non-protein amino acids, citrulline and ornithine, have been reported to build up in higher plants under salinity stress (Mansour, 2000; Ashraf and Harris, 2004). Induction of proline biosynthesis from ornithine, via δ-OAT, was evident in 12-d-old *Arabidopsis* plantlets subjected to salt stress, but not in mature (4-week-old) salt-stressed plants where the accumulation of higher levels of proline was related to the induction of the glutamate pathway (Roosens *et al.*, 1998). The improved tolerance to salt stress of *SlNAGS1* overexpressors, as judged by the increased seed germination ability and better performance of mature plants (higher chlorophyll content), can be attributed to elevated levels and/or their combination of ornithine, citrulline, and arginine, and possibly proline and polyamines. This was especially true for the 3-8 line. Thus, the genetic modification of the arginine biosynthetic pathway can improve plant tolerance to a number of stresses including salinity.

It was noticeable that our transgenic plants survived the imposed water shortage better and fully recovered upon rehydration, suggesting that, by altering the arginine biosynthetic route, the plants acquired improved properties. Recent studies showed that drought conditions lead to an excessive accumulation of citrulline in leaves of wild watermelon making them resistant to water stress (Kawasaki *et al.*, 2000). It was suggested that this contribution of citrulline could be due to its properties as a hydroxyl radical scavenger (Akashi *et al.*, 2001). In the leaves of the *SlNAGS1* transgenic plants, citrulline levels were found to be marginally higher compared to the citrulline levels of the WT plants, thus this accumulation, coupled mainly with elevated levels of ornithine and to a lower extent with arginine levels, could contribute to the water stress tolerance of *Arabidopsis SlNAGS1* overexpressors.

It is known that plant chloroplasts operate not only as the site of photosynthesis and carbon fixation, but also as the site of many metabolic biosynthetic pathways, including nitrogen metabolism such as arginine biosynthesis and the assimilation of NO_2 into an organic form and the synthesis of nucleotides and amino acids. It is interesting to note that overexpressing *SlNAGS1* prevented chlorophyll loss in osmotically and drought-treated transgenic *Arabidopsis* plants (Fig. 8), thus delaying senescence (Capell *et al.*, 1993) and contributing to stress tolerance. This might be connected to the chloroplast location of arginine biosynthesis (Heinrich *et al.*, 2004). Recently, another enzyme *N*-acetyl-glutamate kinase (NAGK) catalysing the second step in the pathway of arginine biosynthesis has been located in chloroplasts (Sugiyama *et al.*, 2004; Chen *et al.*, 2006; Mizuno *et al.*, 2007). It has been suggested that NAGK might be the major control point of arginine biosynthesis in plants because of its inhibition by arginine and its activation by NAG (Shargool *et al.*, 1988). This regulation is exhibited through the binding of PII, a 2-oxoglutarate- and amino acid-sensing protein (Lam *et al.*, 2006), which is able to alleviate the inhibition of NAGK by the end-product arginine (Sugiyama *et al.*, 2004; Chen *et al.*, 2006; Ferrario-Méry *et al.*, 2006). It will be interesting to explore the contribution of NAGS and NAGK in regulating arginine biosynthesis in NAGS overexpressors.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. 1S. Forty-four NAGS-related protein sequences from a variety of organisms were incorporated in phylogenetic analysis.

Supplementary Fig. 2S. Alignment of twenty putative NAGS-related protein sequences from various plant species.

Supplementary Fig. 3S. Prediction of chloroplastic location of putative plant-derived NAGS proteins using the ChloroP 1.1 programme (Emanuelsson *et al.*, 1999).

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