Identification and molecular characterization of the nicotianamine synthase gene family in bread wheat

Julien Bonneau¹, Ute Baumann², Jesse Beasley¹, Yuan Li² and Alexander A. T. Johnson¹,*

¹School of BioSciences, The University of Melbourne, Melbourne, Vic., Australia
²Australian Centre for Plant Functional Genomics, The University of Adelaide, Adelaide, SA, Australia

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*Correspondence (Tel: +61 3 8344 3969; fax: +61 3 9347 5460; email johnsa@unimelb.edu.au)

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Keywords: phytosiderophore, iron, Triticum aestivum, gene expression, nutrient and metal transport, ion transport.

Summary
Nicotianamine (NA) is a non-protein amino acid involved in fundamental aspects of metal uptake, transport and homeostasis in all plants and constitutes the biosynthetic precursor of mugineic acid family phytosiderophores (MAs) in graminaceous plant species. Nicotianamine synthase (NAS) genes, which encode enzymes that synthesize NA from S-adenosyl-L-methionine (SAM), are differentially regulated by iron (Fe) status in most plant species and plant genomes have been found to contain anywhere from 1 to 9 NAS genes. This study describes the identification of 21 NAS genes in the hexaploid bread wheat (Triticum aestivum L.) genome and their phylogenetic classification into two distinct clades. The TaNAS genes are highly expressed during germination, seedling growth and reproductive development. Fourteen of the clade I NAS genes were up-regulated in root tissues under conditions of Fe deficiency. Protein sequence analyses revealed the presence of endocytosis motifs in all of the wheat NAS proteins as well as chloroplast, mitochondrial and secretory transit peptide signals in four proteins. These results greatly expand our knowledge of NAS gene families in graminaceous plant species as well as the genetics underlying Fe nutrition in bread wheat.

Introduction
Nicotianamine (NA) is a non-protein amino acid found in all higher plants (Noma and Noguchi, 1976; Noma et al., 1971). Nicotianamine was originally described as the ‘normalizing factor’ for tomato (Lycopersicon esculentum L) mutant chloronerva (Budesinsky et al., 1980). The chloronerva plants displayed retarded growth and signs of iron (Fe) deficiency despite containing high concentrations of Fe and other heavy metals in vegetative tissues, symptoms which were reversed upon exogenous application of NA. These findings, combined with three-dimensional studies of NA structure, indicated that NA was crucial for in planta chelation, transport and homeostasis of Fe and other heavy metals (Budesinsky et al., 1980; Scholz et al., 1985). Later studies confirmed important roles for NA in short- and long-distance transport of ferrous Fe (Fe²⁺) and other divalent metal cations such as zinc (Zn²⁺), manganese (Mn²⁺), copper (Cu²⁺) and nickel (Ni²⁺) as well as ferric iron (Fe³⁺) (Scholz et al., 1985; Stephan and Scholz, 1993; von Wiren et al., 1999).

Synthesis of NA occurs via trimerization of S-adenosyl-L-methionine (SAM) in a process catalysed by nicotianamine synthase (NAS) enzymes (Higuchi et al., 1994). Nicotianamine is further converted to mugineic acid family phytosiderophores (MAs), such as 2'-deoxymugineic acid (DMA), specifically in graminaceous plant species that utilize Strategy II Fe uptake (Marschner and Romheld, 1994; Marschner et al., 1986). Strategy II Fe uptake has been characterized in a number of graminaceous crops including rice (Oryza sativa L.), barley (Hordeum vulgare L.), maize (Zea mays L.) and wheat (Triticum aestivum L.) and involves the release of MAs into the rhizosphere, particularly under plant Fe deficiency, for chelation of Fe³⁺ and other essential transition metals (Marschner et al., 1986). Following chelation of Fe³⁺ with DMA or other MAs, the MAs-Fe³⁺ complexes are reabsorbed through yellow stripe-like (YSL) transporters at the root surface (Curie et al., 2001). The release of MAs facilitates the growth of Strategy II plant species on calcareous soils where high pH impedes the Fe reductive approach utilized by Strategy I plants (Guerrinot and Yi, 1994; Romheld et al., 1982; Scholz et al., 1992).

Studies of the NAS genes and proteins of rice, barley and maize have revealed significant differences in NAS gene number and expression level as well as NAS protein structure, length and function across the three species. Three NAS genes are present in the rice genome (hereafter referred to as OsNAS genes), consisting of the highly homologous OsNAS1 and OsNAS2 genes...
on chromosome Os3 and the OsNAS3 gene on chromosome Os7 (Inoue et al., 2003). The presence of 9-10 NAS genes in the maize genome (hereafter referred to as ZmNAS genes) located on chromosomes 1, 7 and 9 represents a gene family three times larger than that of rice (Mizuno et al., 2003; Zhou et al., 2013a, b). The ZmNAS proteins vary between 327 and 601 amino acid (aa) in length and phylogenetic comparison to the NAS proteins of rice (three proteins), barley (nine proteins), Arabidopsis thaliana (three proteins) and Solanum lycopersicum (one protein) identified two clades or classes of NAS proteins within the Gramineae; these are referred to as clade I and II NAS proteins in this paper (Inoue et al., 2003; Mizuno et al., 2003; Perovic et al., 2007; Zhou et al., 2013a, b). The pairs of clade I NAS genes in maize are believed to result from chromosomal block duplication (Wei et al., 2007). Such duplication of NAS genes in rice and barley has also been described (Higuchi et al., 2001; Perovic et al., 2007).

Expression of the clade I NAS genes in rice and maize is root specific, induced ubiquitously in root and shoot tissues under Fe deficiency and decreased under conditions of Fe excess; these findings suggest that clade I NAS genes function primarily in the regulation of NA and MAS biosynthesis, Fe uptake and long-distance Fe translocation. Uniquely to maize, a pair of expressed clade I NAS genes (ZmNAS2;1,2,2) encodes NAS proteins approximately twice as long (601 aa) as the clade I NAS proteins found in rice and barley; however, enzymatic activity of these maize NAS proteins has not been detected (Mizuno et al., 2003; Zhou et al., 2013b). Expression of the clade II NAS genes in rice and maize is shoot specific, decreased and/or relocated to root tissues under Fe deficiency and induced ubiquitously in root and shoot under conditions of Fe excess. These expression patterns suggest that clade II NAS proteins do not contribute to MAS biosynthesis under Fe deficiency and are instead primarily involved in NA biosynthesis for Fe loading of vascular tissues and maintenance of cellular Fe homeostasis.

Evidence suggests that clade I and II NAS proteins are targeted to various compartments within the plant cell; a recent bioinformatics study of the ZmNAS protein sequences revealed signal peptides localizing ZmNAS1 (clade II) and ZmNAS6;1 (clade I) to the mitochondria and chloroplasts, respectively (Zhou et al., 2013a). In rice, the OsNAS2 (clade I) protein contains two motifs (YXXΦ and LL) involved in vesicular transport and OsNAS2 enzyme activity was found to be localized to rice root vesicles under Fe deficiency (Nozoye et al., 2014).

The presence of 9-10 NAS genes in the barley genome (hereafter referred to as HvNAS genes) represents a gene family similar in size to that of maize (Herbik et al., 1999; Higuchi et al., 1999a,b, 2001; Mizuno et al., 2003; Perovic et al., 2007). The HvNAS genes have been classified into three synteny-based groups through comparison with the rice genome (Perovic et al., 2007). Group 1 consists of the HvNAS2, HvNAS3 and NASH01a genes and is located on barley chromosome 4H. The NASH01a gene is orthologous and syntenic to OsNAS1 and OsNAS2 on rice chromosome Os3. Group 2 contains the NASH02 gene which is orthologous and syntenic to OsNAS3 on rice chromosome Os7. Group 3 consists of five HvNAS genes that are nonsyntenic with rice. Four of the group 3 genes are located on chromosome 6H (HvNAS1, HvNAS4, HvNAS5, NASH01b) while HvNAS5 is located on chromosome 2HS; these HvNAS genes are considered unique to the barley genome and are thought to have arisen from ectopic HvNAS gene duplication events (Perovic et al., 2007). In contrast to rice and maize, all HvNAS genes investigated to date show Fe deficiency inducible expression specifically in root tissues with no expression under Fe sufficiency nor in shoot tissues under any Fe status (Higuchi et al., 2001). The HvNAS proteins vary between 267 and 340 aa in length and little is known about their cellular localization.

While the NAS gene families of rice, maize and barley have been described in publications for over 10 years, there is only one publication to date (Pearce et al., 2014) identifying two genes belonging to the NAS gene family of bread wheat (Triticum aestivum), hereafter referred to as the TaNAS gene family. The paucity of information about TaNAS genes is surprising in the light of recent publications identifying NA and/or DMA as the predominant chelators of Fe in wheat white flour (Eagling et al., 2014a,b). In this paper, we describe the identification and characterization of a large TaNAS gene family in bread wheat using cv. Chinese Spring—the reference genome of the International Wheat Genome Sequence Consortium—and high-yielding Australian cv. Gladius as our plant materials.

**Results**

**Identification of 21 TaNAS genes located on multiple bread wheat chromosomes and subgenomes**

We identified a total of 21 unique TaNAS genes and determined the genomic location of 19 of these genes on chromosome groups 2, 3, 4, 5 and 6 (Figure 1, Table 1). Several sets of homologous TaNAS genes were identified on chromosome groups 2, 4 and 6 (Figure 1, Table S5). Chromosome group 2 contains three homologous TaNAS genes located on the A, B and D subgenomes (TaNAS9-A/TaNAS9-B/TaNAS9-D) and two pairs of homologous TaNAS genes located only on the A and B subgenomes (TaNAS3-A/TaNAS3-B and TaNAS1-A/TaNAS1-B). Chromosome group 4 contains three homologous TaNAS genes located on the A, B and D subgenomes (TaNAS6-A/TaNAS6-B/ TaNAS6-D). The homologous TaNAS4-D and TaNAS4-A genes on chromosome groups 4 and 5 were likely separated by the known 4AL-5AL wheat chromosome translocation (Hernandez et al., 2012). Chromosome group 6 contains three homologous TaNAS genes located on the A and D subgenomes (TaNAS2-A/ TaNAS2-D1/TaNAS2-D2) and the two 6DS genes share 98.5% genomic sequence identity. Chromosome group 6 also contains an additional three homologous TaNAS genes located on the A and D subgenomes (TaNAS7-A1/TaNAS7-A2/TaNAS7-D) and the 6AS and 6DL genes share 96.2% genomic sequence identity. We identified one TaNAS gene on chromosome group 3 without any homologues (TaNAS3-B).

The genomic location of two TaNAS genes (TaNAS4-U and TaNAS8-U) could not be determined based on currently available wheat genome sequence. The genomic sequence of TaNAS4-U shares over 95% nucleic acid identity with the TaNAS4-A and TaNAS4-D genes and most likely represents the homologues gene located on subgenome B. The TaNAS4-U gene could only be amplified from cv. Gladius DNA, suggesting that the gene is present in only one of the investigated wheat cultivars or has significant polymorphisms between the two wheat cultivars. The partial (168 bp) sequence obtained for the TaNAS8-U gene shares 94.6% nucleic acid identity with the two homologous TaNAS3-A and TaNAS3-B genes located on wheat chromosome group 2 and likely represents the homologues gene TaNAS3-D located on subgenome D. Overall, the 20 full-length TaNAS genes identified in this study share medium to high identity in terms of nucleic acid sequence (67.8%–98.5%) as well as medium to high similarity in protein sequence
The TaNAS genes separate into two distinct clades and show high similarity to HvNAS genes

Our phylogenetic analyses identified two distinct clades of TaNAS genes that share most similarity with barley HvNAS genes (Figure 3). Clade I contains 17 of the TaNAS genes as well as the previously described clade I NAS genes of rice, maize and barley; clade I further separates into three subgroups with bootstrap proportions of <75%. Phylogenetic analyses revealed that the TaNAS and HvNAS genes within each clade I subgroup are most closely related with bootstrap proportions of 99.6%–100%. Clade I subgroup 1 contains two TaNAS genes from wheat chromosome groups 2A and 2B (TaNAS1-A and TaNAS1-B) and the orthologous barley gene HvNAS5-1/2 located on chromosome 2HS. Clade I subgroup 2 contains three TaNAS genes from wheat chromosome groups 6A and 6D (TaNAS2-A, TaNAS2-D1 and TaNAS2-D2) and the orthologous barley gene HvNAS1 located on chromosome 6HS. Clade I subgroup 3 is the largest of the subgroups and comprises 12 TaNAS genes located on various wheat chromosome groups and six orthologous HvNAS genes. Clade II is a much smaller NAS gene clade with no subgroups and contains three homeologous TaNAS genes located on wheat chromosome group 2 (TaNAS9-A/TaNAS9-B/TaNAS9-D) as well as the previously described clade II NAS genes of rice, maize and barley.

The TaNAS proteins show variation in the length of N- and C-terminal regions and are targeted to several subcellular locations

Alignment of the protein sequences encoded by the 20 full-length TaNAS genes showed that TaNAS proteins vary in length from 287 to 385 amino acids and that several regions of the proteins are highly conserved (Figure 2). Two of the proteins have uncharacteristically long N-terminal regions that contain either mitochondrial targeting or chloroplast transit peptides (TaNAS5-B and TaNAS6-D), two others have uncharacteristically short C-terminal regions (TaNAS1-A and TaNAS1-B), while one protein has both an uncharacteristically long N-terminal containing a chloroplast transit peptide and a short C-terminal region (TaNAS6-A). The TaNAS2-A protein has an N-terminal secretory pathway signal peptide (Table S4). All of the TaNAS proteins contain YXXΦ (Φ = amino acids with bulky hydrophobic residues) and di-leucine (LL) motifs in the N-terminal region that have previously been described as characteristic features of NAS superfamily proteins in plants and indicate that they are localized to cytoplasmic vesicles (Figure 2).

Clade I and II TaNAS genes are expressed during germination, seeding growth and reproductive development

Eighteen of the 21 TaNAS genes were expressed in young root tissues, seedling leaf and/or developing reproductive organs of cv.
Table 1 Identification of 21 TaNAS genes in bread wheat. Each TaNAS gene was assigned a unique name (first column) based on homoeologous grouping and subgenome A, B or D (U indicates unknown). An additional number was added to the gene name when more than one NAS gene belonging to the same homoeologous group was located on the same chromosome. The third and fourth columns provide the IWGSC contig (https://urgi.versailles.inra.fr/blast/blast.php) on which the gene was identified and the MIPS database gene annotation (http://pgsb.helmholtz-muenchen.de/plant/index.jsp) for each of the TaNAS genes. The sequences of PCR primers used to amplify each gene from genomic DNA, as well as the expected PCR product sizes (bp), are provided in columns 5–7.

| Gene name | Chromosome | IWGSC contig | MIPS database gene annotation | Forward primer sequence | Reverse primer sequence | PCR product length |
|-----------|------------|---------------|-------------------------------|------------------------|------------------------|--------------------|
| TaNAS1-A  | 2AS        | ctg5260464    | Traes_2AS_1F8B6F760.1         | TCAAGCATCAGCTCATCATTAC | AGGCTCCCTTTTCTTCTTA   | 973                |
| TaNAS1-B  | 2BS        | ctg5186953    | Traes_2BS_D50EDE8A4.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS2-A  | 6AS        | ctcg4374229   | Traes_6AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS2-U  | 6AL        | ctcg4418337   | Traes_6AL_89FA588F1.1         | CAAGCTCGAGAGAGACAT     | TTGGGACGTCGAGAGAGCG   | 1006               |
| TaNAS3-B  | 2AS        | ctcg223571    | Traes_2AS_A948C71A6.1         | TCAAGCATCAGCTCATCATTAC | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS3-A  | 2AS        | ctcg4374229   | Traes_2AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS4-A  | 2AS        | ctcg4590953   | Traes_2AS_D50EDE8A4.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS4-U  | 2AS        | ctcg4590953   | Traes_2AS_D50EDE8A4.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS5-A  | 2AS        | ctcg4590953   | Traes_2AS_D50EDE8A4.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS5-B  | 2AS        | ctcg4418337   | Traes_2AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS5-U  | 2AS        | ctcg4418337   | Traes_2AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS6-A  | 4AS        | ctcg6090953   | Traes_4AS_34072BFC9.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS6-B  | 4AS        | ctcg6090953   | Traes_4AS_34072BFC9.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS6-U  | 4AS        | ctcg6090953   | Traes_4AS_34072BFC9.1         | GAGTATCAGCTCATCATTAC   | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS7-A  | 6AL        | ctcg4374229   | Traes_6AL_89FA588F1.1         | CAAGCTCGAGAGAGACAT     | TTGGGACGTCGAGAGAGCG   | 1006               |
| TaNAS7-B  | 6AL        | ctcg4374229   | Traes_6AL_89FA588F1.1         | CAAGCTCGAGAGAGACAT     | TTGGGACGTCGAGAGAGCG   | 1006               |
| TaNAS7-U  | 6AL        | ctcg4374229   | Traes_6AL_89FA588F1.1         | CAAGCTCGAGAGAGACAT     | TTGGGACGTCGAGAGAGCG   | 1006               |
| TaNAS8-A  | 6AS        | ctcg4374229   | Traes_6AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS8-B  | 6AS        | ctcg4374229   | Traes_6AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS8-U  | 6AS        | ctcg4374229   | Traes_6AS_15A8F3BEE1.1        | AATCGGAGGCGTACATCATTAC | TGGTGAGAGACTGGAAGCG   | 973                |
| TaNAS9-A  | 2AS        | ctcg4374229   | Traes_2AS_1F8B6F760.1         | TCAAGCATCAGCTCATCATTAC | AGGCTCCCTTTTCTTCTTA   | 973                |
| TaNAS9-B  | 2BS        | ctcg4374229   | Traes_2BS_A948C71A6.1         | TCAAGCATCAGCTCATCATTAC | ATGTCGGGGAATCATCAC    | 1006               |
| TaNAS9-D  | 2DS        | ctcg4374229   | Traes_2DS_2BS_A948C71A6.1     | TCAAGCATCAGCTCATCATTAC | ATGTCGGGGAATCATCAC    | 1006               |

Chinese Spring (Figure 4). In most cases, homoeologous TaNAS genes displayed similar expression profiles; however, in some instances specific homoeologous genes were either more tissue specific or higher in expression relative to the corresponding homoeologues. The homoeologous TaNAS3-A/TaNAS3-B genes as well as the TaNAS7-A1, TaNAS7-D and TaNAS2-D2 genes were most highly expressed in the embryonic radicle 2 days after sowing (DAS) and seedling root 10–12 DAS (Figures 4a–e). The TaNAS2-A gene (Figure 4f), a homoeologous gene to TaNAS2-D2, was not only highly expressed in young root tissues but also in seedling leaf and reproductive organs with highest expression in the carpogenic tissue 3–5 days after pollination (DAP). The TaNAS7-A2 gene, a homoeologous gene to TaNAS7-D, displayed highly anther-specific expression with approximately 100-fold higher expression in anther tissue relative to all other organs (Figure 4g). The homoeologous TaNAS4-D/TaNAS4-A genes and the homoeologous clade II TaNAS9-A/TaNAS9-B/TaNAS9-D genes were all most highly expressed in leaf tissues (Figure 4h–i). Expression of the subgenome A TaNAS9-A gene was 10-fold higher than the corresponding subgenome B and D homoeologues (TaNAS9-B and TaNAS9-D). The TaNAS5-B gene as well as the homoeologous TaNAS6-A/TaNAS6-B/TaNAS6-D genes had generally low expression levels with most expression detected in the carpogenic tissue 3–5 DAP (Figure 4j–p). Expression of the subgenome D TaNAS6-D gene was threefold higher than the corresponding subgenome A and B homoeologues (TaNAS6-A and TaNAS6-B). The TaNAS1-A and TaNAS8-U genes had the most ubiquitous expression profiles of the TaNAS genes with expression detected in most tissue types and developmental stages (Figure 4q–r). No expression of the TaNAS4-D, TaNAS2-D1 and TaNAS4-U genes was detected in cv. Chinese Spring.

Clade I TaNAS genes are expressed in root tissues and up-regulated under iron deficiency during vegetative growth

The analysis of TaNAS gene expression in the root and shoot of 4-week-old cv. Gladius plants found that nearly all of the clade I TaNAS genes were expressed in root tissues of the vegetatively growing bread wheat plants under conditions of Fe deficiency and Fe deficiency (Figure 5). Under Fe-sufficient growth conditions (control treatment), 14 of the clade I TaNAS genes were expressed at low levels in root tissues until day 5 of the treatment period and then moderately expressed from days 5–7 (dashed lines in Figure 5a–n). Increased levels of TaNAS gene expression towards the end of the control treatment period may reflect increased demands for NA- and/or DMA-mediated Fe transport as
Figure 2 Amino acid sequence alignment of the NAS proteins from wheat (TaNAS), rice (OsNAS) and barley (HvNAS) identifies conserved regions and motifs. Blue shading corresponds to 100% conservation of amino acids between species. The YXXΦ and LL motifs of the NAS superfamily protein are shaded in green and outlined in red (Y = tyrosine; X = any amino acid residue; Φ = amino acids with bulky hydrophobic residues; LL = di-leucine).

Figure 3 Phylogenetic relationship of the wheat, rice, barley and maize NAS genes. An unrooted tree of NAS gene coding sequences from wheat (TaNAS), rice (OsNAS), barley (HvNAS) and maize (ZmNAS) identifies the clade I and II NAS genes of these species. Clade I separates into three subgroups comprising only wheat and barley genes. Black nodes (●) represent weak bootstrap values (<75%). The scale bar corresponds to branch length and longer branches correspond to greater numbers of nucleic acid polymorphisms along the sequence.
Relative expression of 18 TaNAS genes in 12 different tissues and developmental stages of bread wheat cv. Chinese Spring. Relative expression of TaNAS genes is provided for the following: (1) embryo 1 DAS; (2) coleoptile 2 DAS; (3) radicle 2 DAS; (4) seedling root 10-12 DAS; (5) seedling crown 10–12 DAS; (6) seedling leaf 10–12 DAS; (7) immature inflorescence; (8) bracts; (9) pistil; (10) anthers; (11) caryopsis 3-5 DAP; (12) embryo 22 DAP. Units on the y-axis indicate normalized mRNA copies per µg of total RNA. Error bars indicate SEM of three technical replicates derived from one bulked biological replicate.
Figure 5 Relative expression of 17 TaNAS genes in root tissues of bread wheat cv. Gladius under iron-sufficient/deficient conditions. Gene expression in plants grown under Fe-sufficient (dashed line) or Fe-deficient (solid line) conditions is presented at four time points of the 7 day treatment: day 0 (1), day 1 (2), day 5 (3) and day 7 (4). Units on the y-axis indicate normalized mRNA copies per µg of total RNA. Error bars indicate SEM of three biological replicates for time points 1, 3 and 4 and two biological replicates for time point 2. Asterisks indicate statistically significant differences for the effect of condition (+Fe and –Fe) at each time point (one-way ANOVA, Tukey’s test, *P value ≤0.05; **P value ≤0.01; ***P value ≤0.001).
wheat plants begin to rapidly grow and tiller at the 4-week growth stage. Under Fe-deficient growth conditions, expression of the 14 clade I TaNAS genes was significantly up-regulated in root tissues with approximately 15-fold higher expression at day 5 and threefold higher expression at day 7 of the treatment relative to controls (solid lines in Figure 5a-n). Three additional TaNAS genes, including the clade II TaNAS9-A and TaNAS9-D genes, were differentially expressed under Fe deficiency in the root; however, their expression levels were significantly lower than that of the previously mentioned 14 genes and no clear expression patterns were identified (Figure 5c-o).

Only seven TaNAS genes, including the three homeologous clade II TaNAS genes located on wheat chromosome group 2 (TaNAS9-A/TaNAS9-B/TaNAS9-D), were expressed at low levels in cv. Gladius shoot tissues (Figure S3). The average level of TaNAS gene expression under both Fe-sufficient and Fe-deficient growth conditions in shoots was 30-fold lower than that of roots and bordered on the detection limit for qRT-PCR. Unlike the expression patterns observed in roots, Fe-deficient growth conditions tended to decrease the already low TaNAS gene shoot expression levels. No expression of the TaNAS1-B, TaNAS2-D1 and TaNAS2-D2 genes was detected in cv. Gladius.

**Discussion**

The 21 TaNAS genes located on the A, B and D subgenomes of bread wheat represent the largest plant NAS gene family identified to date. Most members of this large gene family are transcriptionally active at multiple stages of bread wheat growth and development as well as under conditions of Fe deficiency. The results obtained from our molecular characterization of the TaNAS gene family allow several conclusions to be made about the origin of TaNAS genes as well as their function within the wheat plant.

**Many clade I and II TaNAS genes share a common origin with HvNAS genes**

Our study identified two distinct clades of TaNAS genes, a result that is consistent with previous studies of maize, barley and rice NAS genes (Perovic et al., 2007; Zhou et al., 2013b). The separation into the two clades is supported by high bootstrap values and branch lengths. Seventeen of the 20 full-length TaNAS genes belong to clade I, and in our phylogenetic analyses, these genes clustered as three subgroups comprising only wheat and barley NAS genes. This suggests that each *Triticaceae* subgroup is derived from distinct ancestors.

Clade I subgroups 1 and 2 contain two groups of homeologous TaNAS genes—the TaNAS1-A/TaNAS1-B genes located on chromosome group 2 and the TaNAS2-A/TaNAS2-D1/TaNAS2-D2 genes located on chromosome group 6—which are syntenic with the duplicated HvNAS5-1/2 and HvNAS1 genes located on barley chromosomes 2 and 6, respectively (Perovic et al., 2007). It was originally hypothesized that a barley-specific NAS gene duplication event gave rise to the HvNAS5-1 and HvNAS1 genes; however, our results indicate that this gene duplication event likely occurred in a diploid wheat–barley ancestor.

Clade I subgroup 3 contains 12 TaNAS and 6 HvNAS genes and our results suggest that there is macro-synteny of NAS genes between wheat and barley in this subgroup. In addition, our results indicate that intra- and interchromosomal NAS gene duplication events gave rise to this large subgroup of NAS genes in both wheat and barley. The homeologous TaNAS4-D/TaNAS4-A genes located on wheat chromosome groups 4 and 5 and the homeologous TaNAS7-A2/TaNAS7-D genes located on wheat chromosome group 6 are orthologous and syntenic to the HvNAS2/HvNAS3 and HvNAS4/HvNAS7 intrachromosomal gene duplication pairs located on barley chromosomes 4HL and 6HL, respectively (Perovic et al., 2007). The homeologous TaNAS6-A/TaNAS6-B/TaNAS6-D genes located on wheat chromosome group 4 are orthologous and syntenic to the NASHORTa gene located on barley chromosome 4HL. The putatively homeologous TaNAS3-A/TaNAS3-B/TaNAS3-U genes are not orthologous nor syntenic to any of the barley NAS genes, indicating that wheat-specific NAS gene duplication events most likely occurred in a diploid wheat ancestor to give rise to these genes. Similar wheat-specific gene duplication events and/or chromosomal translocations are also likely responsible for origin of the TaNAS7-A1, TaNAS2-D1, TaNAS2-D2, TaNAS4-U and TaNAS5-B genes. Interestingly, the TaNAS4-U and TaNAS5-B genes of clade I subgroup 3 contain significant differences between cvs. Gladius and Chinese Spring, suggesting that some genes within this subgroup are evolving rapidly. The TaNAS4-U gene may be specific to cv. Gladius or have significant polymorphisms in cv. Chinese Spring as it could not be amplified from genomic DNA nor cDNA of cv. Chinese Spring. The TaNAS5-B gene, which has no homeologues on the A and D subgenomes, is functional in cv. Gladius yet contains a SNP (G→C) in the cv. Chinese Spring coding sequence that creates a premature stop codon (Figure S4).

Clade II contains the homeologous TaNAS9-A/TaNAS9-B/TaNAS9-D genes that are orthologous and syntenic to the barley NASHOR2 gene, orthologous to the rice OsNAS3 gene and orthologous to the maize ZmNAS3, ZmNAS4 and ZmNAS5 genes (Figure 3). In contrast to the clade I TaNAS genes, there is no evidence for gene duplication, translocation or deletion in the origin of the clade II TaNAS genes.

The TaNAS gene expression profiles indicate key roles for nicotianamine in bread wheat germination, seedling growth, reproductive development and iron deficiency response

The finding that several TaNAS genes are expressed during seed germination and seedling growth (namely in the embryonic radicle, seedling root and seedling shoot) indicates a requirement for NA biosynthesis at early stages of bread wheat growth and development. The rice OsNAS genes are also expressed at early stages of rice seed germination (Nozoye et al., 2007). We can hypothesize that NA functions as an important chelator of divalent metal cations such as Fe²⁺ and Zn²⁺ during wheat germination and seedling growth and helps to redistribute these metals at the tissue/cellular level and maintain metal homeostasis. Nicotianamine is also the biosynthetic precursor to mugineic acid family phytosiderophores such as DMA that wheat plants secrete from root tissues in order to absorb rhizospheric Fe³⁺ and it is likely that a significant proportion of wheat seedling root NA is diverted towards DMA biosynthesis. In general, the clade I TaNAS genes appear to play a major role in root-specific NA production at the germination and seedling stages while the clade II TaNAS genes contribute more towards shoot-specific NA production at these stages. Similar expression trends have been observed for the clade I and II NAS genes of rice and maize (Inoue et al., 2003; Zhou et al., 2013b).

Expression of several TaNAS genes in tissues of the bread wheat inflorescence, particularly the anther, and in developing grain indicates that NA is essential for bread wheat reproduction.

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and complements the results of biochemical studies that have identified NA and/or DMA as predominant chelators of Fe in bread wheat white flour (Eagling et al., 2014a,b; Walker and Waters, 2011). Anthers are known to be major sink tissues for Fe in Arabidopsis and high expression of several TaNAS genes in the wheat anther, in particular the anther-specific TaNAS7-A2 gene, suggests that wheat anthers are major Fe sinks and that NA is required to chelate Fe and protect against oxidative damage in this reproductive organ (Chu et al., 2010; Roschzttardtz et al., 2013; Takahashi et al., 2003). The finding that several TaNAS genes are expressed in the carpel 3-5 DAP and the embryo 22 DAP likely reflects a requirement for NA to mobilize Fe and other divalent metals to the developing embryo. Expression of the rice OsNAS1 and OsNAS2 genes has also been detected in developing rice seed and the embryo (Dash et al., 2012; Fujita et al., 2010; Jain et al., 2007).

Nearly all of the clade I TaNAS genes were expressed at low to moderate levels in the roots of 4-week-old cv. Gladius plants grown under Fe sufficiency. These low levels of root-specific TaNAS expression presumably provide sufficient NA to enable DMA biosynthesis as well as root to shoot translocation of Fe under sufficient conditions. Expression of the clade I TaNAS genes was significantly up-regulated in root tissues under Fe deficiency and this result is consistent with the Fe deficiency response of barley where all of the HvNAS genes are significantly up-regulated in root tissues in response to Fe deficiency (Higuchi et al., 2001). Increased expression of the clade I TaNAS genes in root tissues should result in greater quantities of NA (and DMA) to increase Fe uptake from the rhizosphere as well as Fe mobilization within the plant. The Fe deficiency response of barley differs somewhat from that of barley in that the rice clade I OsNAS1 and OsNAS2 genes are up-regulated in both root and leaf tissues under Fe deficiency (Inoue et al., 2003). The lack of significant TaNAS gene expression in the shoot of vegetatively growing wheat plants under Fe deficiency indicates that the wheat Fe deficiency response is more similar to that of barley than rice and involves root-specific induction of clade I TaNAS genes.

Our finding that, in some cases, TaNAS genes located on one subgenome were more highly expressed than corresponding homeologous genes on other subgenomes is a common feature of gene expression in hexaploid bread wheat. Genetic studies have found that approximately 55% of bread wheat genes in the three subgenomes display 'unbalanced' expression where at least one homeologous gene dominates. Silencing or loss of homeologous genes also frequently occurs in the bread wheat genome (Borg et al., 2012; Leach et al., 2014; Nussbaumer et al., 2015).

The TaNAS proteins localize to cytoplasmic vesicles, chloroplasts and mitochondria within bread wheat cells and show variation in the length of N- and C-terminal regions

The 20 full-length TaNAS proteins identified in this study contain di-leucine (LL) and YXXΦ endocytosis motifs in the N-terminal region as previously found in rice and barley proteins which suggests that the TaNAS proteins are enzymatically active and localized to cytoplasmic vesicles where NA biosynthesis is likely to take place (Figure 2, Geldner and Robatzek, 2008; Robert et al., 2005; Zuo et al., 2000). Rice OsNAS proteins also contain these motifs and substitution mutations in either motif of the OsNAS2 protein disrupt MA vesicle formation and movement in rice root cells (Nozoye et al., 2014).

Three of the clade I TaNAS proteins have uncharacteristically long N-terminal regions that were determined by in silico analyses to contain peptides for mitochondrial targeting (TaNAS5-B) or chloroplast transit (TaNAS6-A and TaNAS6-D). We identified an EST corresponding to the long N-terminal region of the TaNAS6-D gene (GenBank identifier CA637385), indicating that these long N-terminal regions are transcribed and that specific clade I TaNAS proteins are targeted to mitochondria and chloroplasts to facilitate NA biosynthesis and maintenance of Fe homeostasis within these organelles. Furthermore, the presence of a secretory signal peptide in the N-terminal region of the TaNAS2-A protein suggests that certain TaNAS proteins may be secreted from the cell to enable NA biosynthesis in other parts of the plant. Similar subcellular targeting of NAS proteins has been suggested in maize, where four of the nine ZmNAS proteins contain peptides for secretion or targeting to chloroplasts and mitochondria (Zhou et al., 2013a). The function of the short C-terminal regions of the TaNAS1-A and TaNAS1-B proteins is currently unknown; however, a similar short C-terminal region is present in the orthologous HvNAS5-1 protein of barley (Figure 2).

Applications towards plant breeding

The 21 TaNAS genes described in this study represent a valuable resource for plant breeders working to improve the growth, yield and nutritional value of bread wheat, a major food staple that provides 20% of the calories and protein consumed by humans worldwide. Iron toxicity often occurs when wheat plants are cultivated under waterlogged conditions, while Fe deficiency can occur in plants grown on calcareous soil. These abiotic stresses frequently lead to early leaf senescence and negatively impact on grain yield and quality. Altered expression of specific TaNAS genes could either increase or suppress the Fe deficiency response and thereby facilitate the development of bread wheat cultivars adapted to Fe-rich or Fe-poor growth conditions. Furthermore, increased activity of TaNAS genes with expression within the developing grain could lead to the production of more nutritious wheat varieties containing enhanced concentrations of bioavailable grain Fe.

Experimental procedures

Bread wheat cv. Gladius germination and hydroponic growth conditions

Seeds of cv. Gladius were germinated on moist filter paper for 5 days. Seedlings were transferred to 20-L tubs (30 seedlings per tub) containing hydroponic growth solution as described in Genc et al. (2007) and grown in a University of Melbourne glasshouse (Parkville, Vic., Australia) for 4 weeks over January 2014 with average daily temperatures of 24.6 °C and 58.8% humidity. Hydroponic solutions were replaced every 2 days and pH was maintained between 6.5 and 7. Iron deficiency treatments commenced when plants were 3 weeks old; one tub of seedlings was grown for seven additional days in hydroponic solution lacking Fe, whereas a control tub of seedlings was grown for seven additional days in hydroponic solution with Fe. Three plants were individually harvested from the Fe-deficient and control tubs at four time points—days 0 (experiment start), 1, 5 and 7—and the root and shoot tissues were snap-frozen in liquid nitrogen. Prior to harvest, shoot weight and length and leaf SPAD value (Chlorophyll Meter SPAD-502Plus, Konica Minolta, Europe) were recorded for each plant. Plants grown under Fe deficiency developed leaf Fe deficiency symptoms as indicated by
significantly reduced chlorophyll content of the fourth fully expanded leaf on day 5 (P < 0.05) and the fifth fully expanded leaf on day 7 (P < 0.001) of the treatment (Figure S1).

Identification and naming of the TaNAS genes

The HvNAS coding sequences (Table S1) were used as queries in BLAST searches against the 41 genomic databases of the International Wheat Genome Sequencing Consortium (IWGSC, 2014—http://www.wheatgenome.org) to identify sequences encoding putative TaNAS genes. Matching sequences from the IWGSC portal were annotated using the TriAnnot v1.4 (http://wheat.urgi.versailles.inra.fr) and FGENESH (http://www.softberry.com/) pipelines (Solovyev et al., 2006). In cases where a full gene sequence was not available from IWGSC, we further searched the Triticum aestivum cv. Chinese Spring 5x genome database (http://www.cerealsdb.uk.net). Gene sequences were merged and assembled using Geneious Pro 5.6.6 (Biomatters, http://www.geneious.com/). Each TaNAS gene was assigned a unique name based on homoeologous grouping and subgenome and followed the recommended rules for gene symbolization in wheat (http://wheat.pw.usda.gov/ggpages/wycg/98/intro.htm, Table 1). Gene names TaNAS1-A and TaNAS9-A/TaNAS9-B correspond to the TaNAS1 and TaNAS9 genes, respectively, described in Pearce et al. (2014). Homoeologous TaNAS genes were identified on the basis of their chromosome group, arm location and percentage of nucleic acid identity with a 94% identity threshold level for homoeologous genes. The classification of homoeologous genes also took into account the known 4AL-5AL wheat chromosome translocation (Hernandez et al., 2012) and the pericentric inversion of wheat chromosome 4AS-4AL (Liu et al., 1992).

Gene prediction models including predicted CDS and protein sequence were obtained for each of the identified TaNAS genes using FGENESH software (http://www.softberry.com). CDS alignments (CLUSTALW) and protein alignments based on the degree of amino acid conservation (CLUSTALW—cost matrix BLOSUM62, threshold 1) were performed in Geneious Pro 5.6.6. The TaNAS protein sequences were analysed for the presence of target peptides using the recommended protocol for TargetP 1.1 as described by Emanuelsson et al. (2007). Validation of the TaNAS genes was obtained by BLASTN searches to the 19 TaNAS coding sequences to the recently released TGACv1 genome assembly (http://pre.plants.ensembl.org/Triticum_aestivum/info/ index), all of which returned a single TGAC scaffold with identity equal to or higher than 99%.

Gene sequencing

Subgenome-specific PCR primers for each TaNAS gene were designed using the respective IWGSC contig sequences and Primer3 software (Table 1; Untergasser et al., 2012); the primer pairs were used to amplify fragments from cvs. Gladius and Chinese Spring genomic DNA. The subgenome specificity of each primer pair was verified using cv. Chinese Spring nulli-tetrasomic DNA (Sears, 1954). PCR amplification cycles consisted of 1 cycle = 1 min 95 °C; 35 cycles = 20 s 95 °C, 30 s 61–62 °C, 1 min 72 °C; 1 cycle = 5 min 72 °C. PCRs were performed in a final volume of 30 μl according to manufacturer instructions for MyTaq™ HS DNA polymerase (Bioline, Boston, MA, USA). Amplification products were sequenced by Sanger sequencing at the Australian Genome Research Facility Ltd (http://www.agr-f.org.au/). Most primer pairs amplified expected PCR products from both cvs. Gladius and Chinese Spring except for those amplifying the TaNAS3-A, TaNAS1-A, TaNAS7-A2, TaNAS4-D and TaNAS4-U genes; in these cases, the primer pairs amplified the PCR products from cv. Chinese Spring only. In the case of the TaNAS4-U gene, the primer pairs amplified a PCR product from cv. Gladius only.

Table 2 Quantitative reverse transcription PCR (qRT-PCR) analysis of the 21 TaNAS genes. The table provides gene name, forward and reverse primer sequences, PCR product length (bp) and the qRT-PCR efficiency

| Gene name | Forward primer sequence (5'-3') | Reverse primer sequence (5'-3') | PCR product length | qRT-PCR efficiency |
|-----------|---------------------------------|---------------------------------|-------------------|-------------------|
| TaNAS1-A  | AAGTCGACGCCTCCTGTTACC          | GCTCAGTTGATGATGTTGCTCA          | 230               | 0.98              |
| TaNAS1-B  | AAGTCGACGCCTCCTCAAG            | GCTCAGTTGATGATGTTGCTGC          | 230               | –                 |
| TaNAS2-A  | GTGCGTACGACGTGCTTCTTCC         | GCTCCTTGTGGCAGCTCA              | 388               | 0.92              |
| TaNAS2-D1 | CTTCCTTGGCCAGCTCA              | CACCCCAAGGTGAGGTC               | 189               | –                 |
| TaNAS2-D2 | CTTCCTTGGCCAGCTCA              | CACCCCAAGGTGAGGTC               | 189               | 0.98              |
| TaNAS3-A  | GGTGGTGTACGACGCGGTCAA          | TGTGACCCTCCCACTCGA              | 221               | 0.97              |
| TaNAS3-B  | GGTGGTGTACGACGCGGTCAA          | CACCCCAAGGTGAGGTC               | 391               | 0.97              |
| TaNAS4-A  | CACCCCAAGGTGAGGTC              | ACAGTTGTTGCGAGGTC               | 198               | 1.03              |
| TaNAS4-U  | AATAGCGCTCTTGCCCCAGGA          | ATCCACCTCCCTGCCGAGCA            | 187               | 0.97              |
| TaNAS4-D  | ACAGTTGTTGCGAGGTC              | ATCCACCTCCCTGCCGAGCA            | 197               | 1.01              |
| TaNAS5-B  | GGTGGTGTACGACGCGGTCAA          | TGAGTTGTTGCGAGGTC               | 130               | 0.96              |
| TaNAS5-A  | CTGCGGCTCGGCTGCCCA             | GGTGCGGCTCGGCTGCCCA             | 330               | 0.97              |
| TaNAS6-B  | CAGCTGAGGCTCGCTCA              | AGGTAGCGCTCGCGCTCA              | 283               | 0.95              |
| TaNAS6-D  | GTCGGACGCTCGCTCA              | ATGGCTAGCGCTCGCGCTCA            | 203               | 0.92              |
| TaNAS7-A1 | GTGCGGACGCTCGCTCA              | AAGACTGCTCTCTCTCTCCAGTTCA       | 473               | 0.97              |
| TaNAS7-A2 | TGCCCTCGTCCCTCGCCCA            | AGTAGCTGCTCGCTCGCCCA            | 174               | 0.98              |
| TaNAS7-D  | TGGGCACGCTCGCTCA              | CATGCGGCTCGCTCGCCCA             | 258               | 0.88              |
| TaNAS8-U  | GTACCCCAAGGTGAGGTC             | GAGTTGCTAGCGCTCGCA              | 168               | 0.9              |
| TaNAS9-A  | GGGTGCTGACGACGCGGTCAA          | GGGTGCTGACGACGCGGTCAA           | 154               | 1.07              |
| TaNAS9-B  | CAGCTGAGGCTCGCTCA              | AGGTAGCGCTCGCTCGCTCA            | 265               | 0.92              |
| TaNAS9-D  | GTCGGACGCTCGCTCA              | ATGGCTAGCGCTCGCGCTCA            | 287               | 0.92              |

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Phylogenetic analysis

Phylogenetic analysis of the 20 full-length TaNAS coding sequences was performed in Geneious Pro 5.6.6 using the Geneious plugin PhyML (Guindon et al., 2010) and a Kimura 2-parameter substitution model with bootstrap value of 1000. Published coding sequences of 3 rice OsNAS (OsNAS1, LOC_Os03 g19427.1; OsNAS2, LOC_Os03 g19420.1; OsNAS3, LOC_Os07 g48989.1), 10 barley HvNAS (Table S1) and nine maize ZmNAS (Table S2) genes were included in the phylogenetic analysis.

Quantitative reverse transcription PCR (qRT-PCR) analysis of TaNAS genes

Preparation of a wheat cv. Chinese Spring cDNA library representing a range of tissues and developmental stages has been described previously, where each cDNA was generated from pooled RNA representing 7–10 individual plants (Schreiber et al., 2009). We prepared a cv. Gladius cDNA library; the snap-frozen shoot and root tissues harvested from individual 4-week-old hydroponically grown plants were pulverized and total RNA was extracted from 90 to 100 mg of ground plant material using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer instructions with the following modifications: 250 μL of a 0.8 M sodium citrate and 1.2 M sodium chloride solution was added at the precipitation step and two final washes were performed. Approximately 2 μg of RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA) in 20 μL reactions to remove genomic DNA contamination and oligo-dt-primed reverse transcription was performed following manufacturer instructions (Tetro cDNA synthesis Kit, Bioline). Reactions were performed in a final volume of 20 μL and then diluted 1 : 10. The cDNA samples (cv. Gladius) were verified as genomic DNA free through lack of amplification in PCRs using primer sets specific to the noncoding microsatellite Xcfd43 as well as a noncoding region of the TaWIN1 gene (Table S3).

Subgenomic-specific qRT-PCR primer pairs for each TaNAS gene were designed using the same methodology as described under ‘Gene Sequencing’ (Table 2). The specificity of each qRT-PCR primer pair was determined by melt curve analysis and sequencing. For each primer pair, primer efficiency was calculated using the formula $10^{(1 – \text{ct}_{	ext{std}})/10}$, where m corresponds to the slope of the standard curve generated using triplicate ten-fold serial dilutions (10⁻¹–10²) of purified template for each primer pair (Table 2). The qRT-PCR analysis of TaNAS gene expression was performed using the cDNA libraries of cvs. Chinese Spring and Gladius according to the protocols described in Vandesonompe et al. (2002). The geometric mean of the three housekeeping genes TaCyclophilin, TaGAPDH and TaEFA was used as a normalization factor to normalize the expression of the TaNAS genes as described in Schreiber et al. (2009). A one-way ANOVA followed by a Tukey’s test was performed within days (Days 0, 1, 5 and 7) and between treatments (+Fe and -Fe) to identify significant differences in the cv. Gladius qRT-PCR experiments. Normalization of cv. Gladius qRT-PCR gene expression was performed independently for the root and shoot tissues (Figure S2).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Morphology of hydroponically grown bread wheat cv. Gladius plants.

Figure S2. Expression profiles of the three control genes prior to normalization—TaCyclophilin (black), TaGAPDH (grey) and TaEFa (white) in bread wheat cv. Gladius (a) shoot and (b) root tissues.

Figure S3. Relative expression of seven TaNAS genes in shoot tissues of bread wheat cv. Gladius under Fe sufficient/deficient conditions.

Figure S4. Bread wheat cv. Chinese Spring contains a premature stop codon in the TaNAS5-B coding sequence.

Table S1. Barley HvNAS genes used in phylogenetic analyses.

Table S2. The maize ZmNAS genes used in phylogenetic analyses.

Table S3. Sequence of PCR primers used to screen for genomic DNA contamination in cv. Gladius cDNA samples.

Table S4. Prediction of subcellular localization peptides for TaNAS proteins.

Table S5. Percentage nucleic acid identity between the 20 full length TaNAS genes.
Author/s: 
Bonneau, J; Baumann, U; Beasley, J; Li, Y; Johnson, AAT

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