MOLECULAR ANALYSIS OF "DE NOVO" PURINE BIOSYNTHESIS IN SOLANACEOUS SPECIES AND IN *ARABIDOPSIS THALIANA*

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1. ABSTRACT

Purine nucleotides are essential components to sustain plant growth and development. In plants they are either synthesized "*de novo*" during the process of purine biosynthesis or are recycled from purine bases and purine nucleosides throughout the salvage pathway.

Comparison between animals, microorganisms and *Arabidopsis*, the first plant species with a completely sequenced genome, shows that plants principally use the same biochemical steps to synthesize purine nucleotides and possess all the essential genes and enzymes. Here we report on the cloning and molecular analysis of the complete purine biosynthesis pathway in plants, and the in planta functional analysis of PRPP (5-phosphoribosyl-1-pyrophoshate) amidotransferase (ATase), catalyzing the first committed step of the "*de novo*" purine biosynthesis.

The cloning of the genes involved in the purine biosynthesis pathway was attained by a screening strategy with heterologous cDNA probes and by using *S. cerevisiae* mutants for complementation. Southern hybridization showed a complex genomic organization for these genes in solanaceous species and their organ- and developmental specific expression was analyzed by Northern hybridization. The specific role of ATase for plant growth and development was analyzed in transgenic tobacco plants exhibiting a reduced ATase activity and in an *Arabidopsis* T-DNA mutant (*atd2*) deficient for ATase2. The transgenic tobacco plants as well as the *Arabidopsis* mutant exhibit a specific and comparable phenotype, which is characterized by strong growth retardation and severe chlorosis in leaves. The formation of white leaves, but green cotyledons is a characteristic trait of the *Arabidopsis atd2* mutant.

2. INTRODUCTION

The vital process of nucleotide biosynthesis provides the plant with energy and direct precursors for a multitude of biochemical pathways. Therefore, purine and pyrimidine nucleotides are essential constituents for fundamental biological functions.

Purine nucleotides are of particular importance as subunits of nucleic acids, especially in dividing and elongating tissues during the process of cell division (1). In addition, purine nucleotides are direct precursors for the
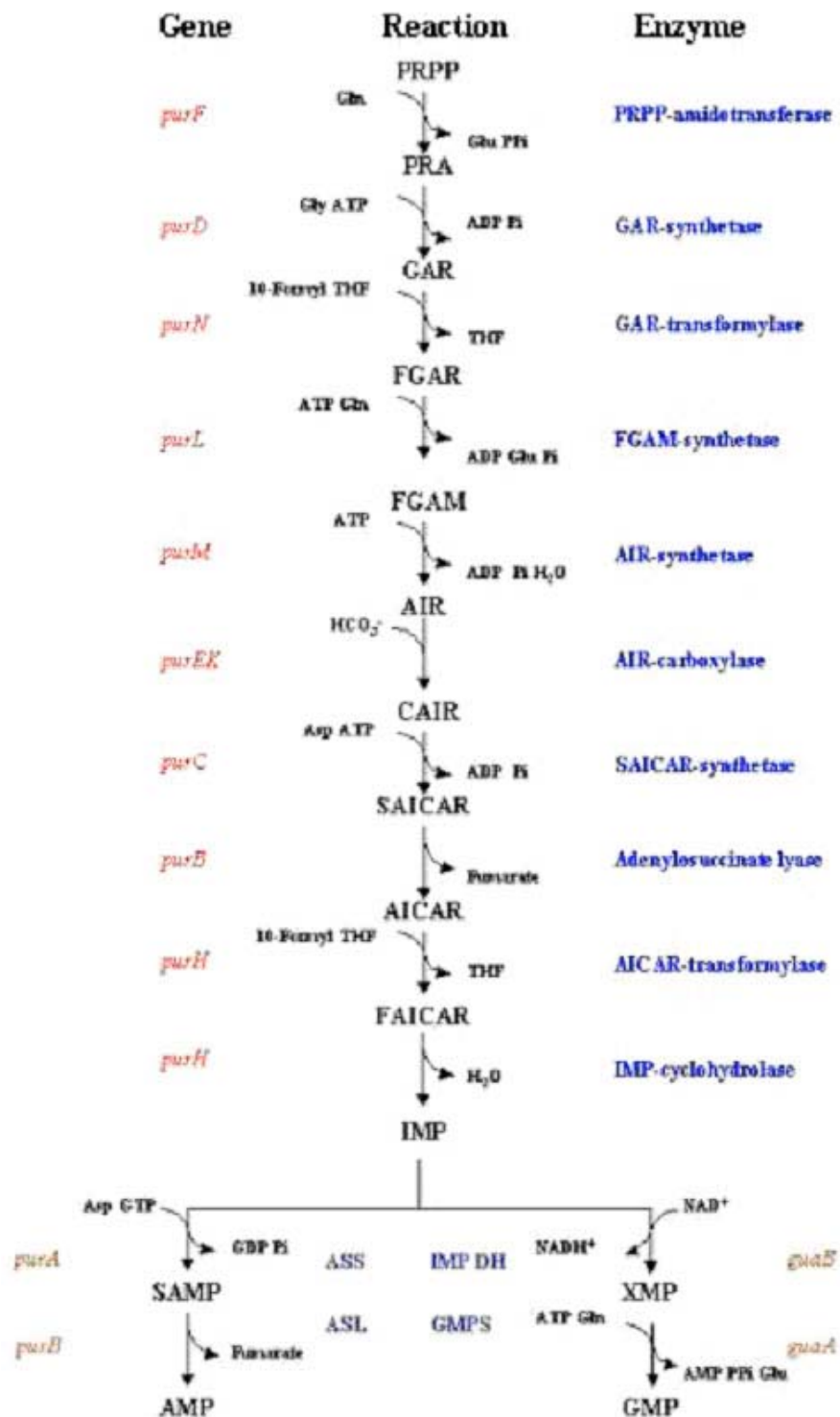
Figure 1. Schematic overview of the "*de novo*" purine biosynthetic pathway in plants. Abbreviations are: PRPP, 5-phosphoribosyl-1-pyrophosphate; Gln, glutamine; Glu, glutamate; PRA, 5-phosphoribosylamine; Gly, glycine; GAR, glycinamide ribonucleotide; THF, tetrahydrofolate; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 4-carboxy aminoimidazole ribonucleotide; Asp, aspartate; SAICAR, N-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formaminoimidazole-4-carboxamide ribonucleotide; IMP, inosine monophosphate; SAMP, adenylosuccinate; AMP, adenosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; ASS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; IMP DH, IMP dehydrogenase; GMPS, GMP synthetase

synthesis of B vitamins like thiamine, riboflavin and folates (2, 3). Purine nucleotides are furthermore directly involved in the synthesis of essential cofactors like the nicotineamide and flavine coenzymes, thereby providing constituents for the synthesis of plant hormones, act as signal molecules and, form energy rich metabolites for the synthesis of polysaccharides and secondary products (4, 5, 6). Thus, purine nucleotides are essential determinants of the primary and secondary metabolism and, consequently influence plant growth and development.

In higher plants purine nucleotides are synthesized "*de novo*" from simple molecules such as the common precursor PRPP that provides the activated ribose moiety, the amino acids glycine, glutamine, and aspartate, 10-formyl THF and carbon dioxide. Biochemical and molecular evidence indicates, that the branched pathway of the "*de novo*" purine biosynthesis comprises 14 enzymatic steps leading to synthesis of the purine monophosphates IMP, AMP and GMP (Figure 1). Based on the *Arabidopsis* genome sequence information (7), the genomic and pathway organization of purine biosynthesis in plants seems to be different from that observed for prokaryotes and other higher eukaryotes (6, 8). Prokaryotes harbor single genes coding for monofunctional proteins (except for the bifunctional ATIC), while other higher eukaryotes contain single genes encoding mono-, bi- and trifunctional proteins (8, 9). The organization of the purine biosynthesis pathway in plants is more similar to prokaryotes, with monofunctional proteins, except for the bifunctional enzymes AIR-carboxylase (AIRC) and AICAR transformylase/ IMP cyclohydolase (Figure 1). The genes involved in purine biosynthesis are organized either as single genes or in small gene families in *Arabidopsis thaliana*. The enzyme PRPP amidotransferase that catalyzes the first step in this pathway is encoded by at least three genes (*AtpurF 1*, *2* and *3*). Besides that small gene family, *AtpurEK*, *AtpurB* and *AtguaB* are each represented by two genes in the *Arabidopsis* genome (Figure 1) (6).

Despite intensive physiological, molecular and biochemical analyses of purine biosynthesis in bacteria and yeast (10, 11) and biochemical as well as physiological evidences in plants (4), very little information is available on the molecular aspects and regulation of the purine biosynthesis pathway in plants.

Genes involved in purine biosynthesis have been cloned from different plant species and experimentally verified by yeast or bacterial complementation strategies. Those genes are *purF* from *A. thaliana*, *G. max* and *V. aconitifolia* (12), *pur D* and *purN* from *G. max* and *A. thaliana* (13), *purM* and *purC* from *A. thaliana* (1, 14), *purEK* from *V. aconitifolia* (15), *purA* from *A. thaliana*, *Z. mays* and *T. aestivum* (16), *pur H* from *N. tabacum* (17) and *guaB* from *G. max* (18). The presence of all genes of the "*de novo*" purine biosynthesis could be demonstrated in the *Arabidopsis* genome and functional characterization for the majority of those genes has been performed in several species. However, sequence information and experimental evidence is still missing for *purL*, encoding FGAM

synthetase and *guaA* coding for GMP synthetase. As deduced from *Arabidopsis* and sequence data from other plant species (6, 8), proteins involved in the 10- step biosynthesis of AMP are predicted to be localized in the chloroplast. In contrast, IMPDH and GMP synthetase catalyzing the branch leading to GMP can neither be allocated to chloroplast nor to mitochondria, indicating a putative cytosolic localization for these enzymes. Biochemical and molecular analysis shows that specialized plants, like the nitrogen fixing nodulating legumes, exhibit a dual intracellular localization in chloroplasts and mitochondria for enzymes involved in IMP synthesis. Isolated chloroplast and mitochondria from *Vigna unguiculata* each showed the capability to synthesize purines "*de novo*", even if some of the involved genes are single copy encoding an enzyme that is supposedly targeted to either chloroplasts or mitochondria (19).

This complex intracellular localization of purine biosynthesis enzymes are superimposed by their simple genomic organization and indicates a multi - functional regulation of gene expression, enzyme activity, transport processes across several membranes and of pool sizes and fluxes of subcellular metabolic intermediates.

Regulation of the purine biosynthesis pathway not only occurs in microorganisms (10, 11), but also in plants through feedback inhibition of the PRPP amidotransferase. The PRPP amidotransferase from *G. max* is sensitive to feed back regulation by the products of the purine biosynthesis and the purine salvage pathway (5, 20). However, information concerning the molecular mechanisms regulating gene expression and enzyme function for the other purine biosynthesis enzymes are so far rather limiting (1, 4, 6) and neither the function of purine biosynthesis enzymes *in planta* nor viable plant mutants defective in "*de novo*" purine biosynthesis have been described.

In this study the cloning and expression of all genes involved in the complete "*de novo*" purine biosynthesis pathway in solanaceous species is presented. Furthermore, the isolation and characterization of an *Arabidopsis* T-DNA tagged mutant deficient in ATase 2, which exhibits the formation of white leaves but green cotyledons, as well as the generation and analysis of transgenic tobacco plants with reduced ATase activity are described, demonstrating here for the first time viable plants disturbed in the "*de novo* purine biosynthesis using two different strategies.

3. MATERIAL AND METHODS

3.1. Bacteria and yeast strains

Escherichia coli strain XL-1 Blue (Stratagene) was used for DNA cloning procedures. Yeast cells were cultivated at 30°C in standard yeast media (YEPD and SD with 2% glucose) and yeast manipulations were done as previously described (17). *S. cerevisiae* strain*s* SEY6210 (α leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9) and DAY4 HIS4 (α ura3-52 trp1 leu2 ser1) were used as original yeast strains during our work. Disruption of the
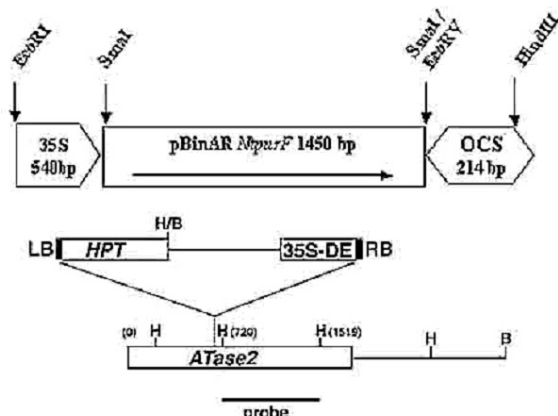
Figure 2. (a) Structure of the T-DNA construct used for gene silencing of the *NtpurF* (ATase) gene in tobacco plants. A 1450 bp SmaI/EcoRV cDNA fragment from clone AX 113070 was ligated in sense orientation in the pBinAR vector, harboring the 35 S promoter and the octopine synthase polyadenylation signal (OCS). (b) Schematic presentation of the T-DNA insert with flanking plant DNA present in the *Arabidopsis atd2* mutant. The relative positions compared to the cDNA clone for ATase2 are indicated. The T-DNA inserted 737 bp downstream of the protein translation start site. B: *Bam*HI, H: *Hin*dIII, LB and RB: left and right T-DNA border, respectively, HPT: hygromycin resistance marker and 35S-DE: CaMV 35S promoter with doubled enhancer region.

yeast *ADE16*, *ADE 17* and *ADE8* loci were done as described (17), except that the following oligonucleotides were used for *in vitro* constructed deletion of ade8::kan^r: sense oligo: 5'-GCG CAG GTG AGA GCC AAC ACA CAT CAA TAA TCT TTC CAA AAG CCA GCT GAA GCT TCG TAG GC – 3', antisense oligo: 5'- GAA TGC CAA ATA TAA AAG TAT AAA TAT GGG AAC TAT TCG CAT AGG CCA CTA GTG GAT CTG – 3'. Complementation of the respective yeast mutants was done as described (17). *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 (21) was cultivated in YEP medium (22).

3.2. Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (23). Plasmid DNA was analyzed by restriction analysis and DNA sequencing using an ALF DNA-sequencer (Pharmacia Biotech).

3.3. cDNA library screening

For isolation of cDNA's involved in purine biosynthesis a *Nicotiana tabacum* (var. Samsun NN) source leaf specific λ ZAPII (Stratagene, La Jolla, CA) cDNA library and a *Solanum tuberosum* (var. Solara) sink leaf specific λ ZAPII cDNA library were used. Library screening with ^{32}P labeled cDNA probes was performed as described (17). *In vivo* excision of the pBluescript plasmids from the λ ZAPII vector was done according to the protocols recommended by Stratagene.

3.4. Plant materials

Tobacco plants (*N. tabacum* var. Samsun NN) were grown in a greenhouse with a photoperiod of 14 h

light and 10 h dark for about 8 weeks. At that time plant material for total RNA extraction was harvested from roots, stems, source leaves (fully expanded 4th to 5th leaf), sink leaves (1st to 2nd, leaf about 5 to 10 mm in size) and closed flower buds. Petals, anthers, style/stigma and ovaries were prepared from immature flowers (emerged, but closed petals and prior to the dehiscence of anthers). Total RNA was further isolated from anthers and ovaries during flower development, followed by Northern analysis.

Arabidopsis seeds of the transgenic lines and wild-type control (both *Arabidopsis thaliana* ecotype C24) were either surface sterilised and grown in tissue culture on half strength Murashige and Skoog medium (1/2 MS10) or grown in soil as described before (24). For the genetic complementation the 35S::*ATase2* construct was introduced into the *atd2* mutant background using a leaf transformation method as described before (25). For biochemical complementation seeds of C24 wild type and *atd2* were germinated in liquid 1/2MS10 medium supplemented with 5mM inosine mono-phosphate (IMP).

3.5. Expression of sense *NtATase* in transgenic tobacco plants

The partial 1450 bp cDNA fragment from clone *NtpurF* -AX113070 was isolated as a *SmaI-EcoRV* fragment from plasmid pBluescript SK⁻(Stratagene) and ligated in sense orientation into the *SmaI* restricted pBinAR plasmid (26) between the cauliflower mosaic virus 35S promoter and the octopine synthase polyadenylation signal (Figure 2) and was introduced into tobacco by *Agrobacterium*-mediated transformation (27). After selection for kanamycin resistance, 80 primary transformants were transferred to soil and screened for reduction in *NtpurF* transcript accumulation. Four lines with reduced transcript level (AT-3, AT-25, AT-38 and AT-54) were selected and grown to seed and rescreened in the T2 generation.

3.6. Southern and northern analyses

Northern and southern blot analyses were performed as described before (17, 25), except that a probe spanning the coding region of the ubiquitin gene UBQ3 (28) was used as a loading control.

3.7. Molecular analysis of the *Arabidopsis atd2* mutant

Segregation analysis to determine the number of individually segregating T-DNA loci, Southern blot analysis to determine the actual number of T-DNA inserts, plasmid rescue and subsequent sequence analysis was performed as described before (29).

3.8. Genetic complementation of the *Arabidopsis atd2* mutant

The cDNA for ATase2 (kindly provided by Dr. T. Ito, Kyoto University) was cloned under the control of the 35S CaMV promoter, which was also used as activator tag in the pSDM1550 construct. The cDNA for ATase2 has a small 5' deletion removing the first 12 amino acids and, therefore, lacks the ATG protein translation start site. The cDNA was cloned as a translational fusion with the ATG protein translation start site of the 35S promoter. The 35S promoter was cloned as an

*Eco*RI/*Nco*I fragment of which the ssDNA overhangs were filled with klenow. This fragment was ligated to the *Eco*RI site of which the ssDNA overhangs were removed with Mungbean treatment in the pBluescript SK⁻ vector harboring the cDNA for *ATase2*. In the resulting 35S::*ATase2* translational fusion three additional nucleotides were removed as a result of the Mungbean treatment (ATG AAT GCT AAA CCC) and the ATG protein translation start was fused in frame with the cDNA clone as reported by (20).

3.9. Determination of ATase enzyme activity

Chemicals were obtained, from Sigma-Aldrich Fine Chemicals, Germany, unless stated otherwise. The enzyme assay was done according to (30). Five gram tobacco leaves were homogenized in plant extraction buffer containing in final concentrations 100 mM HEPES/KOH buffer pH 6, 8, 1 mM EDTA, 1 mM MgCl$_2$, 5 mM glutathione, 20 mM DTT and 0, 1 mM PEFABLOCK (Roth, Germany). The homogenate was filtered through four layers of Miracloth and centrifuged for 30 min at 4°C at 14 000 x g in a Sigma (3K30) centrifuge. The supernatant was desalted by using a PD-10 column (Amersham) and used for enzyme determination. ATase activity was determined by measuring the glutamate formation. A standard assay contained in final concentrations: 50 mM plant extraction buffer, 5 mM MgCl$_2$, 3 mM PRPP, 20 mM NaF, 5 mM glutamine and 100 μl plant extract. Incubation was at 30°C for 40 min. The reactions were stopped in a water bath for 3 min at 80°C, cooled on ice and centrifuged. The supernatant was used for glutamate determination using 375 μl 100 mM Tris/HCl buffer pH 8,0, 200 μl double distilled water, 75 μl 30 mM APAD (3 acetylpyridine adenine dinucleotide as the NAD analogue), 100 μl enzyme extract and one unit of glutamate DH. The formation of APADH⁺ was monitored with a Sigma photometer at 363 nm. PRPP -dependent activity was calculated by substracting the PRPP-independent activity. Control reactions were done by using all assay components except enzyme preparation.

3.10. Microscopy

Glutaraldehyde fixed material was embedded in epon, sectioned and stained as described before (31). For EM analysis, thin sections (70nm) were prepared from epon embedded material and examined using a JEOL 1010 transmission electron microscope.

4. RESULTS AND DISCUSSION

4.1. Isolation of cDNA clones encoding *N. tabacum* ATase, GARS, FGAMS, AIRC, SAICARS, ASL, ATIC, ASS, IMP-DH and GMPS

The cloning of tobacco cDNA's involved in the purine biosynthetic pathway was accomplished by screening a source leaf specific cDNA library (λZAP II, Stratagene) with heterologous cDNA probes and the results are summarized in Table 1. The probes were derived from known *Arabidopsis* cDNA's and EST clones, which either served directly as templates for PCR amplification of the respective cDNA fragments or oliconucleotides according to the sequence information were generated to amplify the probes from *Arabidopsis* cDNA. Those clones were for

NtpurF D28868 (20), for *NtpurD* X74766 (13), *NtpurL* AX 128568, *NtpurEK* N96521 (32), *NtpurC* U05599 (15), *NtpurB* I82819, *NtpurH* W43391(17, 32), *NtpurA* U49389, *NtguaB* L34684 (33) and for *NtguaA* F14426.

The isolated *NtpurF* cDNA encodes a protein of 573 aa (amino acids) and contains a 50 a N-terminal extension sequence that was predicted as a chloroplast transit peptide (34, 35), indicating targeting to the chloroplast. The deduced amino acid sequence revealed significant homologies to other plant ATase's (Table 1) and showed the typical conserved protein structures of type 1 ATases that contain propeptide sequences preceding a conserved cysteine residue and [4Fe-4S] clusters, crucial for enzyme activity (36).

ATase's catalyze the conversion of PRPP to phosphoribosylamine with glutamine as the source of the amide group. This reaction is carried out by two half reactions coupled on two separate catalytic domains. Ammonia is released at the glutaminase domain and channeled to the PRPP binding site, the phosphoribosyltransferase domain (37, 38). Both catalytic domains are conserved in the tobacco enzyme.

The isolated cDNA's of *NtpurD*, *NtpurEK*, *NtpurC*, *NtpurB NtpurH* and *NtpurA* also contain N-terminal sequences that indicate localization to the chloroplasts and furthermore show significant homologies to the respective cDNA's and proteins isolated from other plant sources (Table 1). Protein database searches (Prosite http://us.expasy.org./prosite, and NCBI conserved domain database http://www.ncbi.nlm.mih.gov/) revealed the occurrence of the individual conserved domain structures. Those were for *NtpurD* (GARS) COG0151.1, PS 00184; *NtpurEK* (AIRC) COG0026.1 and COG0041.1; *NtpurC* (SAICARS) PS01057, PS01058 and pfam01259.9; *NtpurB* (ASL) COG0015.1 and PS00163; *NtpurH* (ATIC) COG0138.1 and *NtpurA* (ASS) cd00410.1, PS01266 and PS00513. The isolated cDNA's for *NtpurL* (FGAMS) and *NtguaB* (IMP-DH) represent partial sequences that lack the 5' regions and, therefore do not allow a prediction of their subcellular localization. However, these cDNA's show significant homologies to known plant sequences (Table 1) and contain the respective conserved domain structures (*NtpurL* COG0046.1 and COG0047.1; *NtguaB* pfam 00478.9.and PS00487). Using a size fractionated *Arabidopsis* cDNA library we obtained a full length cDNA clone representing FGAM synthetase (Table 1). The isolated *AtpurL* cDNA is 4570 bp long and the sequence surrounding the deduced translation start at position 146 confirms good translation probability (39). At the 3'-untranslated region downstream of the TAA stop codon at position 4369 a typical poly (A) tail could be detected. The deduced amino acid sequence encodes for a protein of 1407 aa with a calculated molecular weight of 153,9 kDa. The sequence analysis further revealed a 53 aa N-terminal extension sequence that was predicted as a chloroplast transit peptide (35). The FGAM synthetase like the ATase and GMPS belong to the group of glutamine amidotransferases and are composed of a glutamine amide transfer or glutaminase domain and an aminator or

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Table 1. Summary of cloned genes of the purine biosynthesis pathway from *N. tabacum* (Nt), *S. tuberosum* (St) and *Arabidopsis thaliana* (At) and comparison of cDNA sequences as well as deduced amino acid sequences of different plant sources. For prediction of subcellular localization TargetP database was used. Sequence comparison was done using only coding sequences of deduced proteins and using CLUSTAL method of Lasergene software

Cloned cDNA Accession	Protein	cDNA bp	CDS bp	Deduced AS	Predicted subcellular localization TargetP	Sequence homology (% identity) nucleotide / amino acid			
						A.thaliana	*V.unguiculata* *V.aconitifolia*	*G.max*	*O.sativa*
NtpurF AX113070	ATase	1879	49-1770	573	cTP (RC1)	At2g16570 65,5 / 73,2 At4g34740 66,9 / 74,2 At4g38880 58,1 / 65,8	F071862 59,9 / 68,3	L23833 59,5 / 68,7	AP003566 44,0 / 53,4
NtpurD AY424959	GARS	1927	37-1584	515	cTP (RC2)	At1g09830 63,7 / 72,1	U308896 56,1 / 78,5	X96864 58,1 / 66,1	AK106101 51,5 / 74,9
StpurN AY424958	GART	1166	4-921	305	cTP (RC3)	At1g31220 53,5 / 54,1	U30875 58,3 / 58,2	X9686 51,7 / 58,2	-
NtpuL AX128570	FGAMS	3334	2-3055	1017	-	At1g74260 62,3 / 81,7	AY069939 66,2 /83,0	AY178840 52,4 / 60,9 AY178839 65,6 / 83,6	AP003431 60,6 / 80,1
AtpurL AX 128568	FGAMS	4570	146-4369	1407	cTP(RC5)	At1g74260 70,8 / 99,8	AY069939 66,1 / 79,8	AY178840 71,6 / 76	AP003431 63,1 / 72,4
StpurM AY429421	AIRS	1636	123-1337	404	cTP(RC2)	At3g55010 64,1 / 72,2	U30985 63,5 / 72,2	-	AC133778 60,8 / 66,8
NtpurEK AY429422	AIRC	2262	1866	621	cTP(RC2)	At2g37690 62,8 / 71,8	AF452495 66,4 / 72,6 L22584 67.4 / 71,1	-	AP003197 52,1 / 60,1
NtpurC AY429423	SAICARS	1598	47-1246	399	cTP(RC2)	At3g21110 63,5 / 68,9	AY063233 65,5 / 70,2	-	-
NtpurB AY429424	ASL	2330	37-1665	542	cTP(RC3)	At1g36280 64,3 / 67,2 At1g18440 62,4 / 65,5	AY063232 64,5 / 68,9	-	-
NtpurH AF321497	ATIC	2104	44-1882	612	cTP(RC3)	At2g35040 69,5 / 77.9	AY193836 71,5 / 78,1	-	-
NtpurA AY429425	ASS	2009	36-1553	505	cTP(RC3)	At3g57610 69,0 / 79,2	-	*L.esculentum* AF273333 86,1 / 87,1	AC097368 61,6 / 72,4
NtguaB AY433959	IMPDH	1676	1-1425	474	-	At1g79470 58,7 / 60,5 At1g16350 58.6 / 61.6	AY193837 60,2 / 61,0	AJ010201 59,7 / 61,2	AC084320 56,8 / 59,1
NtguaA AX105099	GMPS	1973	65-1681	538	-	At1g63660 67,5 / 77,9		AP003884 64,9 / 74,0	

synthetase domain (40, 41, 42). The *Arabidopsis* and tobacco FGAMS both contain the N-terminal synthetase and the C-terminal glutaminase domain.

The GMPS is implicated in the "*de novo*" synthesis of GMP and catalyzes the amination of xanthosine monophosphate to provide GMP. Until now, no plant cDNA encoding GMPS has been isolated or characterized. Our screening of a source leaf specific tobacco cDNA library resulted in the isolation of a *NtguaB* cDNA (1973 bp) with a putative start codon at position 65,

a termination codon (TAA) at position 1679 and a poly (A) tail at position 1950. The deduced amino acid sequence encodes a protein of 538 aa with a calculated molecular weight of 60,09 kDa and showed significant sequence identities to a resent released rice sequence (see Table 1) and GMPS's from microorganisms: 43,9% to *B. subtilis* NP 388517, 43,4% to *E. coli* NP417002 and 45,9% to *S. cerevisiae* NP 013944. Using TargetP prediction no targeting sequence to either chloroplasts or mitochondria could be detected. As a glutamine amidotransferase the tobacco GMPS contains a N-terminal glutaminase

domain (COG0518.1) and a C-terminal gua-GMPS synthetase domain (COG0519.1).

4.2. Isolation of cDNA clones encoding StGART and StAIRS

For the isolation of the *Solanum tuberosum StpurM* cDNA encoding AIRS we screened a potato sink leaf specific library (kindly provided by Dr. R. Zrenner, Max Planck institute of molecular plant physiology, Golm, Germany) by using a cDNA fragment homologous to the *Arabidopsis* AIRS (15) (accession number L12457) as probe. The potato cDNA clone comprises 1636 bp and contains a putative ATG start codon at position 123 and a termination codon at position 1335. Accordingly, it contains an open reading frame of 1212 bp encoding a protein of 404 aa. The deduced amino acid sequence shares high homologies to other plant AIRS (see Table 1) and contains an N-terminal extension of 13 aa that exhibits characteristic properties of a chloroplast transit peptide (35). Conserved protein structures in the potato AIRS were identified as the N-terminal ATP-binding site (pfam00586.9) and the AIRS C-terminal domain (pfam02769.9) with a so far unclear function.

In order to isolate the *Solanum tuberosum StpurN* cDNA we choose a complementation strategy. The *S. cerevisiae* strain SEY6210 was used for the generation of an *ade8* mutant (RFA8-ade8::kan^r) by gene disruption (17). This RFA8 mutant strain was used for complementation with a potato cDNA library in YEp 112. After transformation and selection against the kanamycin marker and on media without adenine, 3 positive clones could be isolated and were further analyzed. The longest isolated *StpurN* cDNA clone is 1166 bp in size and contains a putative start codon at position 4 and a termination codon at position 919 and, therefore, encodes a protein of 305 aa. The StGART protein possesses an N-terminal extension sequence that was predicted as a chloroplast transit peptide (35). With a PROSITE scan a conserved motive (PS00373) surrounding the active site of *E. coli* GART could be identified at aa position 229-252. Using the same method of gene disruption (43) we generated an ade16 / ade17 yeast double knock - out mutant (17) for complementation of the *NtpurH* cDNA (AF321497). With both the *S. cerevisiae* ade8 and ade16 / ade17 mutants we could provide evidence for the function of the potato GART and tobacco ATIC.

The survey of isolated cDNA's from solanaceous species (Table 1) and the comparison with the available *Arabidopsis* cDNA sequences (6) encoding for enzymes of the purine biosynthetic pathway, indicates that all enzymes involved in the first 10 steps leading to IMP and the branch leading to AMP are predicted to be localized in the chloroplast, whereas the enzymes in the branch leading to GMP are probably located in the cytosol.

4.3. Genomic organization of the purine biosynthetic pathway in *Nicotiana tabacum* and *Solanum tuberosum*

The genomic organization of the purine biosynthetic pathway enzymes ATase, FGAMS, ATIC, GMPS and GART was analyzed by Southern hybridization in tobacco and potato using ^{32}P labeled cDNA's (Figure 3).

The hybridization patterns clearly showed differences between the group including tobacco ATase and GMPS and a second group comprising tobacco FGAMS, ATIC and potato GART. ATase and GMPS appeared to be encoded by single copy genes, whereas the hybridization patterns of FGAMS, ATIC and GART revealed several bands indicating multiple copies for *NtpurL*, *NtpurH* and *StpurN*. Further analysis is necessary to get an overview of the genomic organization of the complete purine biosynthetic pathway and to specify if there are more genes involved in this pathway with multiple copies in solanaceous species. The probable presence of more then one isoform for the enzymes involved in purine biosynthesis could indicate the localization of these isoforms in different cell compartments.

4.4. Expression analysis by Northern hybridization with cDNA's for all steps involved in the purine biosynthetic pathway

Transcript accumulation for each step of the *de novo* purine biosynthesis was analyzed in different tobacco plant organs (Figure 4) and for selected genes in ovaries and anthers during flower development (Figure 5) and after pollination (Figure 6). Earlier expression studies, especially on *Arabidopsis purC* (1), indicated that genes involved in purine biosynthesis are primarily expressed in mitotically active tissues. However, the expression analysis in tobacco plant organs showed a different picture (Figure 4). All genes involved in the purine biosynthetic pathway exhibited high expression levels in ovaries. The genes encoding FGAMS (*purL*) and ATIC (*NtpurH*) showed a strong and constitutive expression, while the genes encoding GARS (*NtpurD*), GART (*NtpurN*) and AIRS (*NtpurM*) were also constitutively expressed, but at low levels. The predominant constitutive expression indicates a housekeeping function for those genes. In contrast, the genes encoding GMPS (*NtguaA*), IMP-DH (*NtguaB*) and SAICARS (*NtpurC*) were exclusively expressed in meristematic tissues like sink leaves (young tobacco leaves maximal 1, 5 cm in size) and floral organs. The different expression pattern for these genes indicates that transcriptional control is involved in the regulation of the purine biosynthesis. Two different expression patterns could also be observed during flower development (Figure 5). The developmental stages of tobacco flowers were defined as indicated in Figure 5. High and constitutive expression was observed in anthers and ovaries at all investigated stages of flower development for the *NtpurF* gene encoding ATase. In contrast, *NtpurH* (ATIC) and *NtguaA* (IMP-DH) mRNA accumulation increased constantly during flower development in ovaries. In anthers of young and closed flowers their expression was low, but increased in open flowers concomitantly to the development of anthers. After dehiscence of anthers and pollination the expression dropped dramatically (Figure 5). A similar expression pattern could be observed for the expression of these genes after pollination (Figure 6), whereas the expression level of *NtpurF* (ATase) remained high and constant in anthers. The expression of *NtpurH* (ATIC) and *NtguaA* (IMP-DH) increased to a maximum level one hour after pollination and declined afterwards significantly.
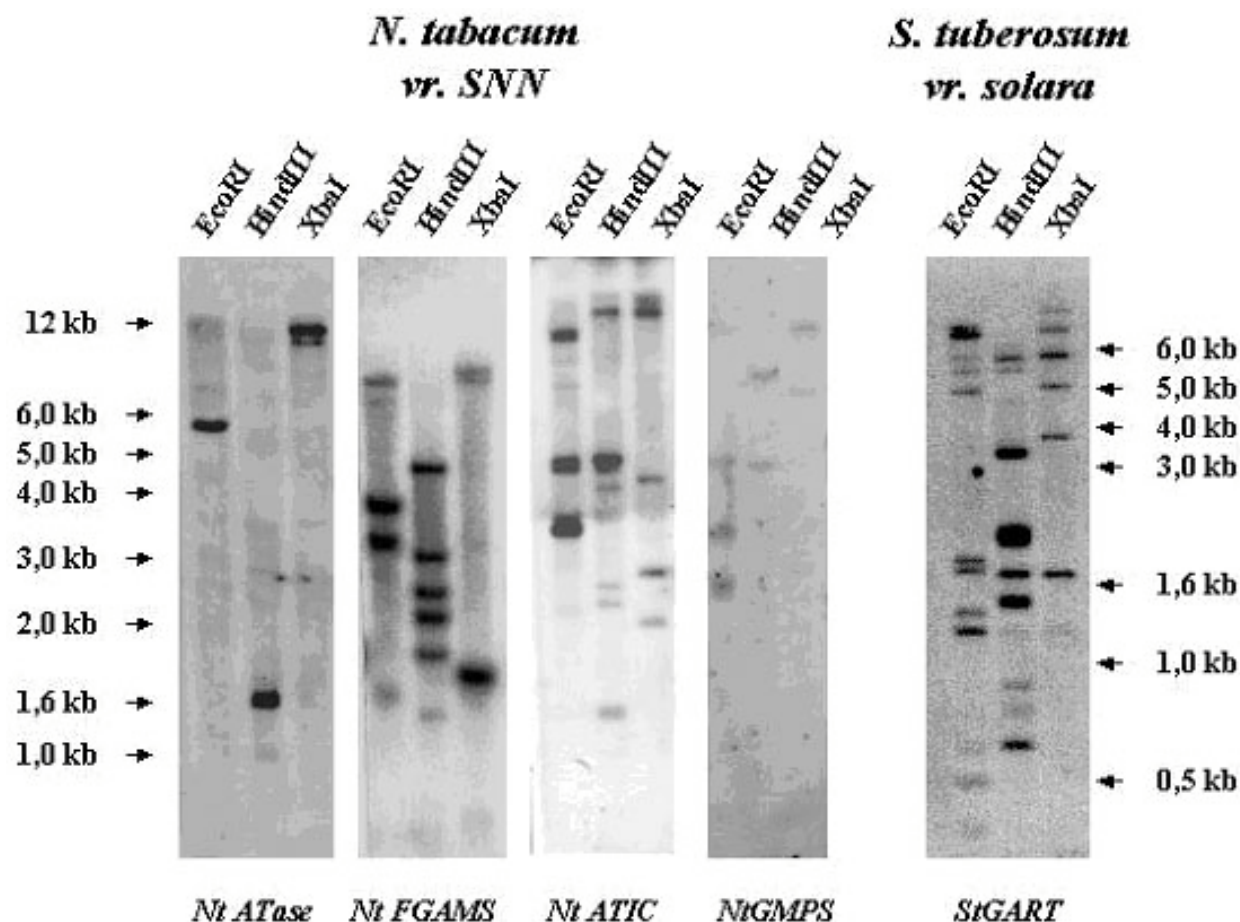
Figure 3. Genomic organization of purine biosynthesis genes in solanaceous species. Southern analyses of tobacco (*N. tabacum*) and potato (*S. tuberosum*) genomic DNA, digested with *EcoRI*, *HindIII* and *XbaI* and probed with ^{32}P labeled ATase cDNA (AX13070), FGAMS cDNA (insert from clone *NtpurL1*), ATIC cDNA (insert from clone *NtpurH5*), (17), GMPs cDNA (AX105099) and GART cDNA (AY424958). The molecular size standards (kb) are indicated for *N. tabacum* on the left and for *S. tuberosum* on the right. 20 µg of digested genomic DNA was loaded per lane. For labeling and hybridization conditions see section Material and Methods

The expression analysis demonstrated that the expression of the genes involved in purine biosynthesis is not solely confined to meristematic tissues. The constitutive expression in different non-meristematic tissues implies that purine biosynthesis has to be sustained in other processes besides nucleic acid synthesis in dividing cells. The analysis of expression during flower development further indicates a coordinated spatial and developmental control of expression for the genes involved in purine biosynthesis.

4.5. Plants with reduced function of PRPP amidotransferase (ATase)

The PRPP amidotransferase (ATase) catalyzes the first committed step of the "*de novo*" purine biosynthesis. From animal, microorganisms and plant sources it is known that this enzyme is subjected to feedback regulation and forms the rate-limiting step in the pathway (10, 11, 44, 45).

Besides the biochemical analysis of the soybean enzyme (45) and the isolation and characterization of soybean, *Vigna* and *Arabidopsis* cDNA's (12, 20), very little is known about the enzyme function in plants an its impact on the purine biosynthetic pathway.

Based on that fact, the cloning of the respective tobacco gene and sequence information available for the *Arabidopsis purF* genes (20) we generated transgenic tobacco plants with reduced activity of ATase and isolated an *Arabidopsis* T-DNA tagged mutant deficient in ATase2. The impact of reduced ATase activity on purine biosynthesis and plant growth and development was studied in these transgenic tobacco and *Arabidopsis* mutant plants.

In order to silence the tobacco *purF*(ATase) gene a 1450 bp partial cDNA from clone *NtpurF* -AX113070 was cloned in sense orientation into the pBinAR plasmid (26) between the cauliflower mosaic virus 35S promoter and the octopine synthase polyadenylation signal (Figure 2a) and was introduced into tobacco by *Agrobacterium*-mediated transformation (27). After selection for kanamycin resistance, 80 primary transformants were transferred to soil and screened for reduced *NtpurF* transcript accumulation. Four lines with reduced transcript level (AT-3, AT-25, AT-38 and AT-54) were selected and self-fertilized.
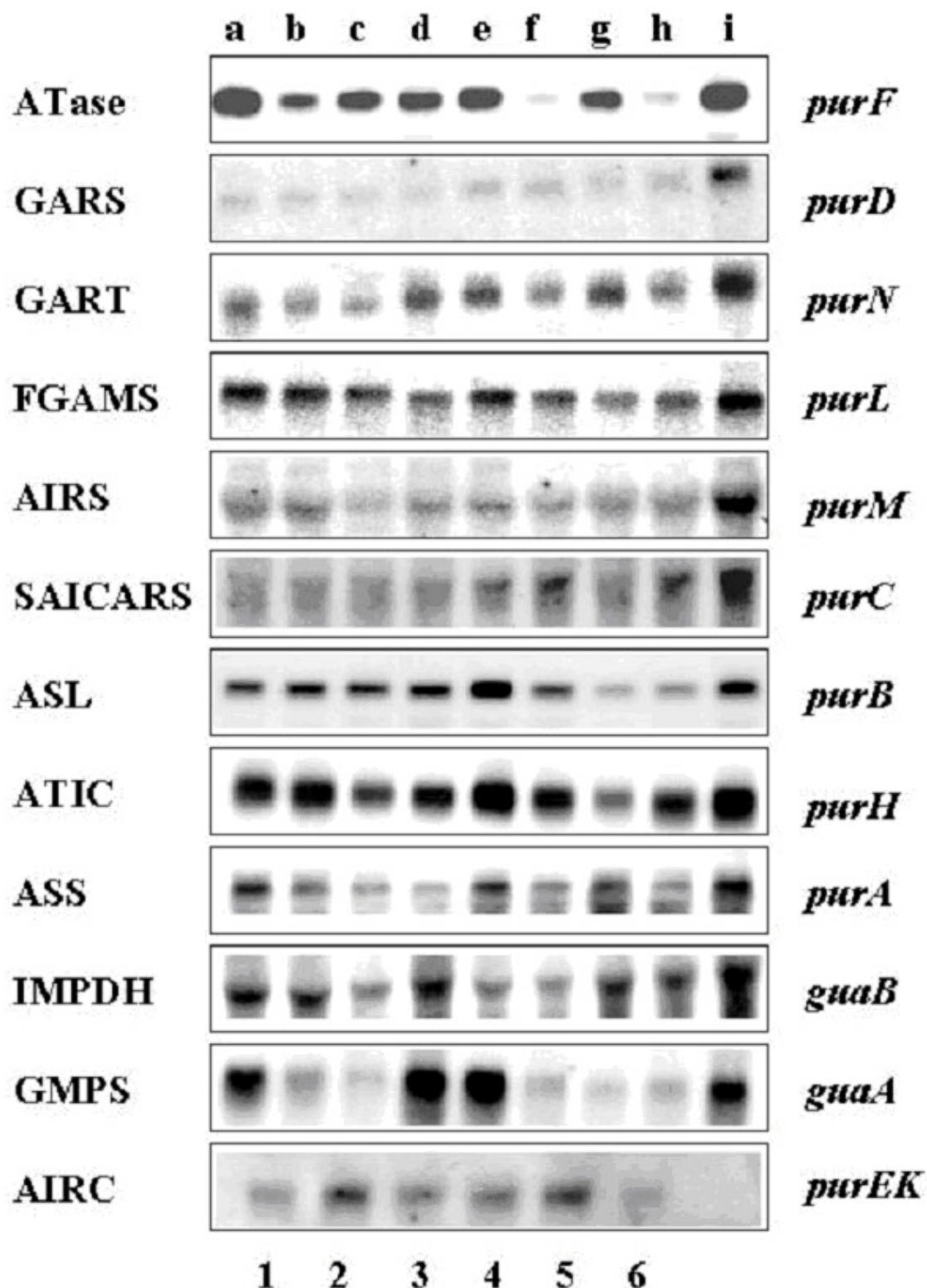
Figure 4. Expression analysis of purine biosynthesis genes in *N. tabacum*. Northern hybridization of RNA isolated from different tobacco plant organs. 20 µg of total RNA were loaded on denaturing 1,2% (w/v) agarose gels, transferred to nylon membranes and hybridized with the respective ^{32}P labeled cDNA probes (see Table. 1). a: root, b: stems, c: source leaves (3-9 cm), d: sink leaves (0,5-1,5 cm), e: closed flower buds, f: petals, g: anthers, h: stigma/style i: ovaries. Note expression of *NtpurEK* was analyzed with RNA isolated from. 1: sink leaves, 2: source leaves (2-3 cm), 3: source leaves (4-7 cm) 4: source leaves (9 cm) 5: stems and 6: roots.
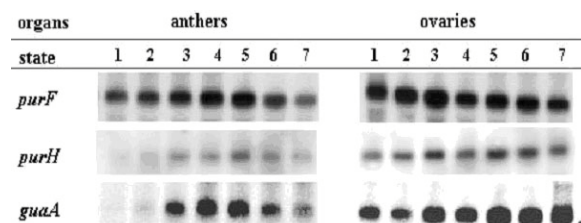
Figure 5. Expression analysis of *NtpurF* (ATase), *NtpurH* (ATIC) and *NtguaA* (IMP-DH) during flower development. Total RNA was isolated from ovaries and anthers of tobacco flowers at different states of flower development. 1: closed flower, size 0,5 cm stage -3, 2: closed flower, size 1,0 cm stage 1-2, 3: closed flower, size 1,5 cm stage3, 4: open flower, petals 0,5 cm stage 4, 5: open flower, petals 1.0 cm stage 5-6, 6: open flower, petals 2,0 cm stage 7-8 and 7: mature flower stage 12, pollinated. 20 μg RNA was loaded per lane on 1,2% denaturing agarose gels, transferred to nylon membranes and hybridized with ^{32}P labeled cDNA probes. Stages according to (49).
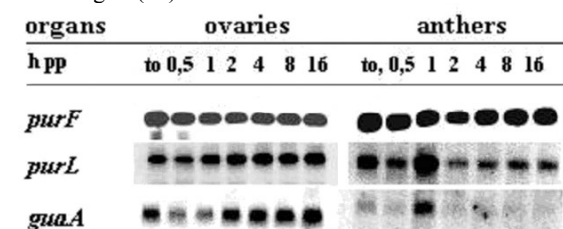

Figure 6. Expression analysis of *NtpurF* (ATase), *NtpurH* (ATIC) and *NtguaA* (IMP-DH) after pollination. RNA was isolated from ovaries and anthers at different time points: t_0: prior to pollination and 0,5, 1, 2, 4, 8, 16 h after pollination. 20 μg RNA was loaded per lane on 1,2% denaturing agarose gels, transferred to nylon membranes and hybridized with ^{32}P labeled cDNA probes.
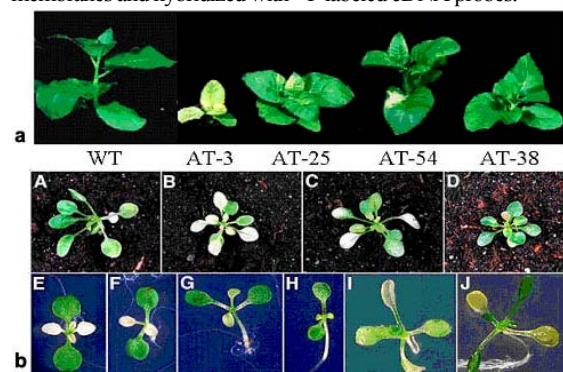

Figure 7. (a) Plant growth and visual appearance of tobacco plants transformed with the *NtpurF* silencing construct. The phenotype of the transgenic plants (AT) is characterized by strong growth retardation and the formation of chlorotic leaves (WT: wild type). (b) Development of the *Arabidopsis atd2* mutant and restoration of wild-type development by genetic and biochemical complementation. Plants were grown in tissue culture. The plants shown in A-D were transferred to soil shortly before photographs were taken. 3-week-old C24 wild type (A), *atd2* (B), 35S::*Atase2*:*atd2*/*atd2* seedling exhibiting progressive greening (C) and fully green 35S::*Atase2*:*atd2*/*atd2* seedling (D). (E-H) 1-week-old *atd2* seedlings grown under 100%, 50%, 25% and 12.5% of the standard light intensity, respectively. Note the green leaves in G and H. 2-week-old *atd2* seedling grown in liquid medium (I) or supplemented with 5 mM IMP (J).

From a pool of 550 individual transgenic *Arabidopsis* lines transformed with an activator T-DNA construct (Figure 2b) (25) a recessive mutant exhibiting the formation of white leaves was isolated and named *atd2* (*ATase deficient2*). Southern and segregation analysis showed that the *atd2* mutant harbored a single T-DNA insert. This T-DNA locus together with the plant DNA flanking the RB (right T-DNA border) was isolated by plasmid rescue and the flanking plant DNA was sequenced. The T-DNA inserted in the *AtpurF2* (At4g34740) gene encoding the *Arabidopsis* ATase2 at position 737 bp downstream of the ATG translation initiation start site of the ATase2 cDNA (20). The 3' region of the cDNA for ATase2 (kindly provided by Dr. T. Ito, Kyoto University) was used as a probe in northern blot analysis (Figure 8c). This showed that the transcript detected in wild type leaves was absent from the leaves of the *atd2* mutant. Instead an abundant smaller transcript was detected in *atd2*. This smaller transcript most likely resulted from transcription initiation in the 35S promoter used as activator tag. Such overexpression of flanking plant DNA was also observed for *let* (24) and *srh* (25) isolated from the same mutant collection. Since the T-DNA inserted in the middle of the coding region of the gene for ATase2, it is likely that this caused a knock-out of this gene and, therefore, the *atd2* mutant is ATase2 deficient resulted in an impaired purine biosynthesis in the leaves of this mutant.

The most striking observation is the similar phenotype, observed in two independent experimental approaches. Both, the transgenic tobacco plants as well as the *Arabidopsis atd2* mutants plants are characterized by strong growth retardation and strong leaf chlorosis, or the formation of white leaves but green cotyledons in the case of the *atd2* mutant (Figure 7b-B). This phenotype observed in transgenic tobacco and *Arabidopsis* mutant plants (Figure 7a) could be linked to the silencing of the *NtpurF* gene in tobacco and the disruption of the *AtpurF2* gene in *Arabidopsis* (Figure 8). Transgenic tobacco plants of the T2-generation revealed, depending of the selected line, a reduced *NtpurF* transcript level correlated with a reduced ATase activity (Figure 8a-b).

An anatomical analysis of the *Arabidopsis atd2* mutant showed that the *atd2* leaves lacked the palisade mesophyll layer (Figure 9a-B), which is normally present in dicotyledonous leaves (Figure 9a-A). Instead, an extra layer of spongy mesophyll was formed. Furthermore, *atd2* chloroplasts are defective in the proper formation of the organised thylakoid membrane structure present in wild-type chloroplasts (Figure 9b-A). In contrast, the *atd2* chloroplasts exhibited a vesiculation of membranes (Figure 9b-C). For some *atd2* chloroplasts a residual formation of thylakoid membrane structure could be observed (Figure 9b-D). Such chloroplasts were only detected in mesophyll cells located in the vicinity of vascular tissue.
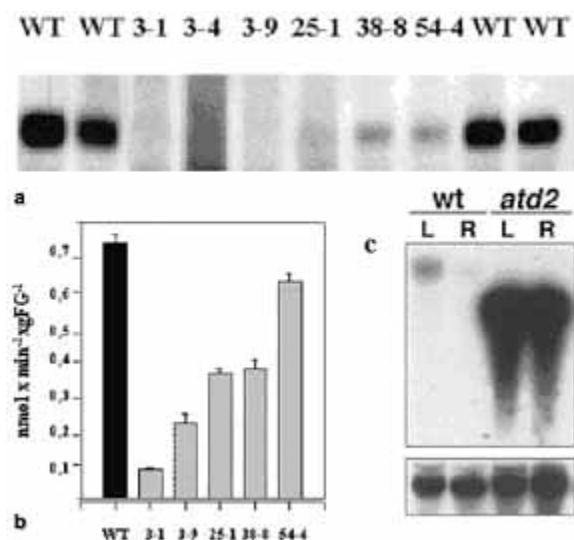
Figure 8. (a) *NtpurF* expression in WT (wild type) and transgenic tobacco plants of the T2 –generation. (b) Determination of ATase activity in WT and transgenic tobacco plants (for details see section Material and Methods). (C) Northern analysis of plants homozygous for the *atd2* mutation compared to C24 wild type. Total RNA was isolated from leaf (L) and root (R) tissue. Total RNA was isolated from *Arabidopsis* leaf (L) and root (R) and 20 μg total RNA was loaded on a denaturing 1,5% agarose gel transferred to a nylon membrane and hybridized with the 32P labeled 3' *Hin*dIII fragment of the cDNA for ATase2 (see Figure 2). The filter was stripped and rehybridized with a probe for ubiquitin (lower panel) as a loading control.

Closer examination of the leaf colour development in *atd2*, revealed that the leaves are green upon first emergence, but shortly after become white (data not shown). The growth of *atd2* under a varying range of light intensities showed that under low light conditions the leaves remained green (Figures 7b-E-H). This suggests that the defect in chloroplast development is caused by photo-oxidative damage.

To exclude the possibility that a mutation in a gene closely linked to the *atd2* locus was responsible for the phenotype observed in *atd2*, a genetic rescue construct consisting of the cDNA for ATase2 driven by the constitutive CaMV 35S promoter (35S::*ATase2* construct) was introduced into the *atd2* mutant background. This resulted in the generation of transgenic plants exhibiting the *atd2* phenotype only shortly after seed germination. However, in older seedlings the newly formed leaves progressively formed greener veins and mesophyll until the leaf became fully green (Figure 7b-c). Occasionally, fully green seedlings were observed among the progeny of such transgenic lines (Figure 7b-D). Anatomical analysis of the leaves from the 35S::*ATase2:atd2/atd2* transgenics showed that the genetic rescue restored the proper formation of the palisade mesophyll layer

(Figure 9a-C,E) and chloroplast development (data not shown).

Furthermore, we assayed whether the wild type phenotype could be restored in the *atd2* mutant by addition of the purine inosine. The purines adenine and guanine can be derived from inosine monophosphate (IMP) via a two step conversion (Figure 1). In order to avoid transport problems the seedlings were completely submerged in liquid medium. Germination and growth in the continuous presence of 5mM IMP resulted in a complete reversal of the mutant phenotype. The leaf colour was green (Figure 7b-J), the palisade mesophyll layer developed properly (Figure 9a-I) and proper thylakoid membrane structure development was restored (Figure 9b-E).

Until now, no fertile plant mutants or transgenic plants have been generated defective in purine biosynthesis. So far mutants have been isolated impaired in the purine salvage pathway, for review see (5), and in purine degradation (46). The only known plant purine (adenine) auxotroph is a cell line, which can be rescued by growth on adenine or inosine (47, 48). Both independent experimental approaches, the transgenic tobacco plants with reduced ATase activity and the *Arabidopsis atd2* knock out mutant revealed a strong impact on whole plant growth and development and indicate the central function of ATase in the plant "*de novo*" purine biosynthesis as the probable rate limiting step. Further, the dramatic effect of the *Arabidopsis atd2* mutation on chloroplast development indicates a completely new functional aspect of the purine biosynthetic pathway. Both systems combine advantages and disadvantages of gene silencing and knock out mutants, but can now be used in either way for new molecular and biochemical aspects accounting for subcellular localization and regulation of the purine biosynthetic pathway. Since the ATase enzyme is encoded by a small gene family of three *AtpurF* genes encoding ATase 1 to 3 in *Arabidopsis*, analysis of the *atd1* and *atd3* knock out mutants can specify the function of the individual ATase isoforms. This study is currently underway.
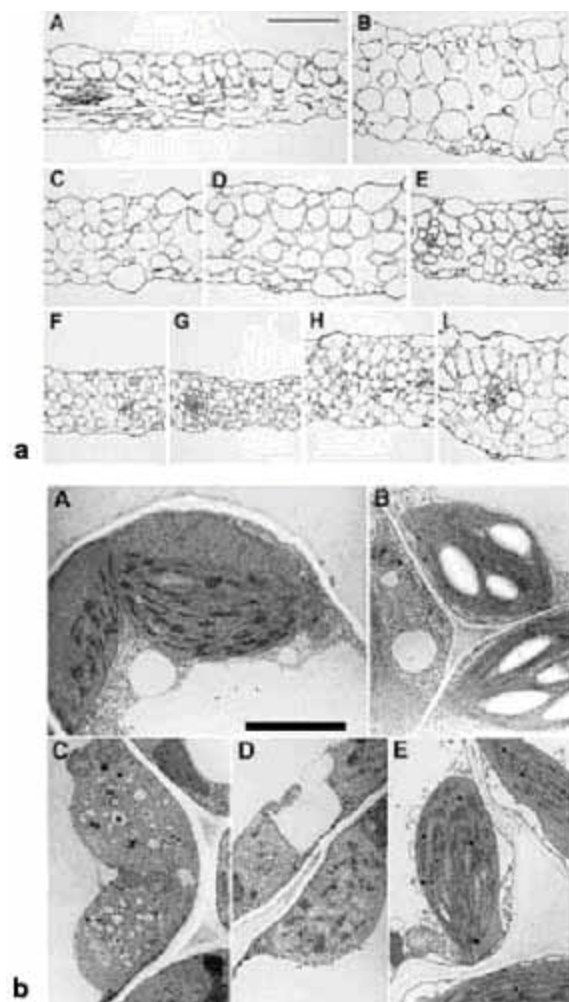
Figure 9. (a) Leaf development of the *atd2* mutant is restored by genetic complementation. Leaf anatomy of 3-week-old seedlings grown in tissue culture, (A) C24 wild type, (B) *atd2*, (C, D) leaves from 35S::*Atase2:atd2/atd2* seedlings exhibiting progressive greening and (E) leaf from fully green 35S::*Atase2:atd2/atd2* seedling. (F, G) leaves from 2-week-old wild type and *atd2*, respectively, grown in liquid culture. (H, I) leaves from 2-week-old wild type and *atd2*, respectively, grown in liquid culture supplemented with 5 mM IMP. Scale bars = 100 μm. (b) Chloroplast development of the *atd2* mutant is restored by biochemical complementation. Chloroplast structure from 2-week-old seedlings grown in liquid medium. (A) C24 wild type, (B) C24 wild type grown in the presence of 5 mM IMP, (C, D) *atd2*, note the formation of vesicles in (C) and residual thylakoid membrane structure in (D) and (E) *atd2* grown in the presence of 5 mM IMP. Scale bar:1 μm..

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