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AtAzg1 and AtAzg2 comprise a novel family of purine transporters in Arabidopsis

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ABSTRACT

In plants, nucleobase biochemistry is highly compartmented relying upon a well-regulated and selective membrane transport system. In Arabidopsis two proteins, AtAzg1 and AtAzg2, show substantial amino acid sequence similarity to the adenine–guanine–hypoxanthine transporter AzgA of *Aspergillus nidulans*. Analysis of single and double mutant lines harboring T-DNA insertion alleles *AtAzg1-1* and *AtAzg2-1* reveal a marked resistance to growth in the presence of 8-azaadenine and 8-azaguanine but not to other toxic nucleobase analogues. Conversely, yeast strains expressing *AtAzg1* and *AtAzg2* gain heightened sensitivity to growth on 8-azaadenine and 8-azaguanine. Radio-labeled purine uptake experiments in yeast and *in planta* confirm the function of AtAzg1 and AtAzg2 as plant adenine–guanine transporters.

1. Introduction

Nucleobases play a pivotal role in plant metabolism, contributing to DNA and RNA metabolism, and the biochemistry of carbohydrates, glycoprotein, phopsolipids and secondary metabolites such as cytokinins, and caffeine [1]. A hallmark of nucleobase biochemistry is the high degree of compartmentation, necessitating extensive intra- and inter-cellular transport as illustrated in ureide biochemistry [2].

The Arabidopsis genome contains six different families that encode putative nucleobase transporters. Nucleobase-ascorbate transporters (NATs) are ubiquitous and transport oxidized purines, xanthine, hypoxanthine and uric acid, uracil, or ascorbate [3–6]. None of the 12 identified Arabidopsis NAT loci are yet functionally characterized. Equilibrative nucleoside transporters (ENT) found in plants, protozoans and mammals, act primarily as nucleoside transporters but can also transport nucleobases adenine, guanine, xanthine and hypoxanthine or cytokinin [7–13]. Eight identified ENT loci are present in the Arabidopsis genome. The Arabidopsis genome contains purine permease (PUP) and ureide permease (UPS) gene families that are common only among plant species. Of the 21 Arabidopsis PUPs several are known to transport adenine, cytosine or secondary compounds such as cytokinins and caffeine [14,15]. Two of the five transporters in the Arabidopsis UPS family transport uracil, allantoin and the purines xanthine and hypoxanthine [16,17]. Locus At5g03555 encodes a protein with significant amino acid similarity to FUR4, a uracil transporter of *Saccharomyces cerevisiae* – belonging to the purine-related transporter or nucleobase:cation symporter 1 family [18]. No data concerning the function of this Arabidopsis transporter is yet published. Another Arabidopsis nucleobase transporter family consists of AtAzg1 (locus At3g10960) and AtAzg2 (locus At5g50300) encoding proteins with significant similarity to the AzgA adenine–guanine–hypoxanthine transporter of *Aspergillus nidulans* [19].

Using Arabidopsis lines harboring homozygous T-DNA insertion alleles for *AtAzg1* and *AtAzg2*, we performed toxic purine analogue growth studies as well as *in planta* [^{3}H]nucleobase uptake experiments. In addition, yeast strains expressing *AtAzg1* and *AtAzg2* were grown in the presence of toxic purine analogues and assayed for the ability to transport [^{3}H]nucleobases. Our results show that AtAzg1 and AtAzg2 transport adenine and guanine and are the first report linking adenine and guanine transport with specific genes in plants.

2. Materials and methods

2.1. Arabidopsis growth conditions and genetics

Arabidopsis lines SAIL114-E03 and SALK020651 were obtained from Arabidopsis Biological Resource Center (Columbus, OH) [20,21]. Seeds were grown for 12–14 days at 20 °C under constant

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Table 1
Oligonucleotide primers used in this study.

Oligonucleotide name	DNA sequence (5′–3′)
At3g10960A	GATCCCATTCCTCTTAACCAGA
At3g10960B	TGCAATCACTTGTCCTTTCATC
At3g10960RSA	CCGCTCGAGATGGAGCAACAGCAACAACAACAACT
At3g10960RSB	ATAAGAATGCGGCCGCCTAAACGGTAGTATCAATCTCAC
At5g50300A	ATTTGTTTTCTTTGCAATTTGAT
At5g50300B	ACCAGTTTTGTGACATCGGTTA
SAILLB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
SALKLBb1	GCGTGGACCGCTTGCTGCAACT
UNI51REV	TGGCAACTAGAAGGCACAGAC
U22685A	CCGCTCGAGATGGGAAGAGAAGACGTTA

light on agar-solidified Minimal Arabidopsis Medium (MAM) [22] containing 30 μM 8-azaguanine (8-azg). Light was supplied by a combination of Cool White fluorescent and incandescent lamps at a quantum flux rate of 130 μmol m^{-2} s^{-1} (400–700 nm). Seedlings exhibiting resistance were transferred to potting mix BM-2 (Berger, Saint-Modeste, Québec, Canada) and grown at 20 °C under same light conditions. DNA extraction was done using DNeasy Plant Mini Kit (Qiagen, Valencia, CA) or QuickExtract (Epicentre, Madison, WI). Individual plants were genotyped by multiplex PCR using two gene-specific primers [At3g10960A & B or At5g50300A & B] and one T-DNA specific primer [SAILLB3 or SALKLBb1, respectively] (Table 1). Homozygous T-DNA insertion mutants in each locus were identified then crossed to produce a double homozygous insertion mutant (double mutant) whose genotype was confirmed by multiplex PCR as mentioned above.

2.2. Construction of yeast expression plasmids

The coding regions derived from loci At3g10960 and At5g50300 were cloned into yeast expression vector pRG399 (vector pRS424 [23] with the *PMA1* promoter [24]). Oligonucleotides At3g10960RSA and At3g10960RSB were used to amplify a DNA fragment from cDNA clone U17958 [GenBank # BT010556] and oligonucleotides U22685A and UNI51REV were used to amplify a DNA fragment from cDNA clone U22658 [BT006441]. Resulting DNA fragments were cloned into pRG399 producing plasmids pNS418 and pRH162, respectively.

2.3. Yeast cultures and transformation

S. cerevisiae strains INVSC [MATα *his3-Δ1, leu2, trp1-289, ura3-52*] (Invitrogen, Carlsbad, CA) and RG191 [Mat a, *fcy2 Δ:: kanMX4*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*] (Research Genetics, Stanford, CA) [25] were grown in YPD or on Synthetic Complete medium (SC) [26] at 30 °C. Yeast transformation was by the lithium acetate method [27]. Sensitivity to toxic nucleobase analogues was assayed by adding filter-sterilized stock solutions to the growth media.

2.4. Arabidopsis radionucleobase uptake

Eleven-day-old seedlings were transferred from agar-solidified MAM onto agar-solidified MAM supplemented with [8-^{3}H]guanine (3.7 kBq ml^{-1}) or [2,8-^{3}H]adenine (3.7 kBq ml^{-1}) (Moravek Biochemicals, Brea, CA). The plants were grown for 2 days in conditions of constant light at 20 °C, then removed and weighed. Three 75 mg samples of selected genotype tissue were homogenized in 0.5 ml of 20 mM Tris–HCl, pH 8. After homogenization, samples were centrifuged at 11 750×*g* for 1 min. For each sample, the radioactivity in 0.1 ml of extract was determined by mixing with 3 ml of EcoLume (MP Biochemicals, Solon, OH) and measured by using a scintillation counter.

2.5. Radionucleobase uptake by yeast expressing AtAzg1 and AtAzg2

Freshly grown yeast were concentrated to OD_{600} = 6 and incubated for 0 and 2.5 min with 0.5 μM [8-^{3}H]guanine or [2,8-^{3}H]adenine in 100 mM citrate buffer (pH 3.5) with 1% glucose. Fifty microliter aliquots were added to 4 ml of ice-cold water and filtered through a 0.45 μm Metricel membrane filter (Gelman Sciences, Ann Arbor, MI). Filters were then washed with 8 ml of water and radioactivity was measured by scintillation counter. Statistical analysis used an independent paired *t*-test. Significance was measured at $P = 0.1$(*) and at $P = 0.05$(**).

3. Results and discussion

3.1. Arabidopsis genome encodes proteins similar to A. nidulans adenine–guanine transporter AzgA

The Arabidopsis genome contains two genes that encode for proteins showing substantial amino acid similarity to the *A. nidulans* guanine–adenine transporter, AzgA [19]. Amino acid alignments of AtAzg1 and AtAzg2 show a 36.5%/56.3% and 38.5%/65.8% amino acid identity/similarity to AzgA, respectively. Comparison of the two Arabidopsis proteins reveals levels of sequence similarity at 48.3%/77.6% aa identity/similarity, however, these values suggest that AtAzg1 and AtAzg2 are not the result of a very recent gene duplication (Fig. 1A). Secondary structure predictions strongly suggest that AtAzg1 and AtAzg2 are integral membrane proteins supporting similarly placed transmembrane spanning domains (Fig. 1A). AtAzg1 is predicted to have eight transmembrane spanning domains when analyzed by TMHMMv2.0 or 10 transmembrane spanning domains when analyzed by SVMtm, Split4.0 or MINNOU protein secondary prediction programs, while AtAzg2 always has a strong prediction for ten transmembrane spanning domains [28–30]. AtAzg1 contains two larger hydrophilic regions while AtAzg2 contains a predicted chloroplast targeting sequence (Fig. 1A).

Genes encoding AzgA-like proteins are found throughout the plant kingdom and present as three independent clades as shown in a phylogram including representative members of Plantae (Fig. 1B). Two clades consist of dicotyledonous species *Arabidopsis thaliana* and *Vitis vinifera* and the monocotyledon *Oryza sativa* contain AtAzg1 and AtAzg2 paralogues, while the non-vascular moss *Physcomitrella patens* contains only one AtAzg1-like paralogue. In contrast, a separate clade containing two AzgA-like proteins from the green alga *Chlamydomonas reinhardii* indicates an earlier divergence of AzgA-like genes. Similar AzgA-like proteins are encoded in diatom and cyanobacterial genomes (data not shown). Although AzgA-like proteins are related to NATs they lack a motif essential for NAT substrate specificity and binding [19,31]. Together, the sequence similarities and structural predictions support the view that AtAzg1 and AtAzg2 are adenine–guanine transporters in Arabidopsis.

3.2. T-DNA insertion mutations in AtAzg1 and AtAzg2 confer resistance to some toxic purine analogues in Arabidopsis seedlings

Gene expression compiled from publically available databases (e-FP Browser, Genevestigator Gene Atlas and Chronologer found at The Arabidopsis Information Resource Web site) reveals that on a developmental view that AtAzg1 and AtAzg2 have similar levels of expression in seedlings and young plants. AtAzg1 has higher levels of gene expression in mature leaves, inflorescences and developing flowers compared to AtAgz2, while AtAzg2 is more highly expressed in developing endosperm and seed stages 4–7 [32].

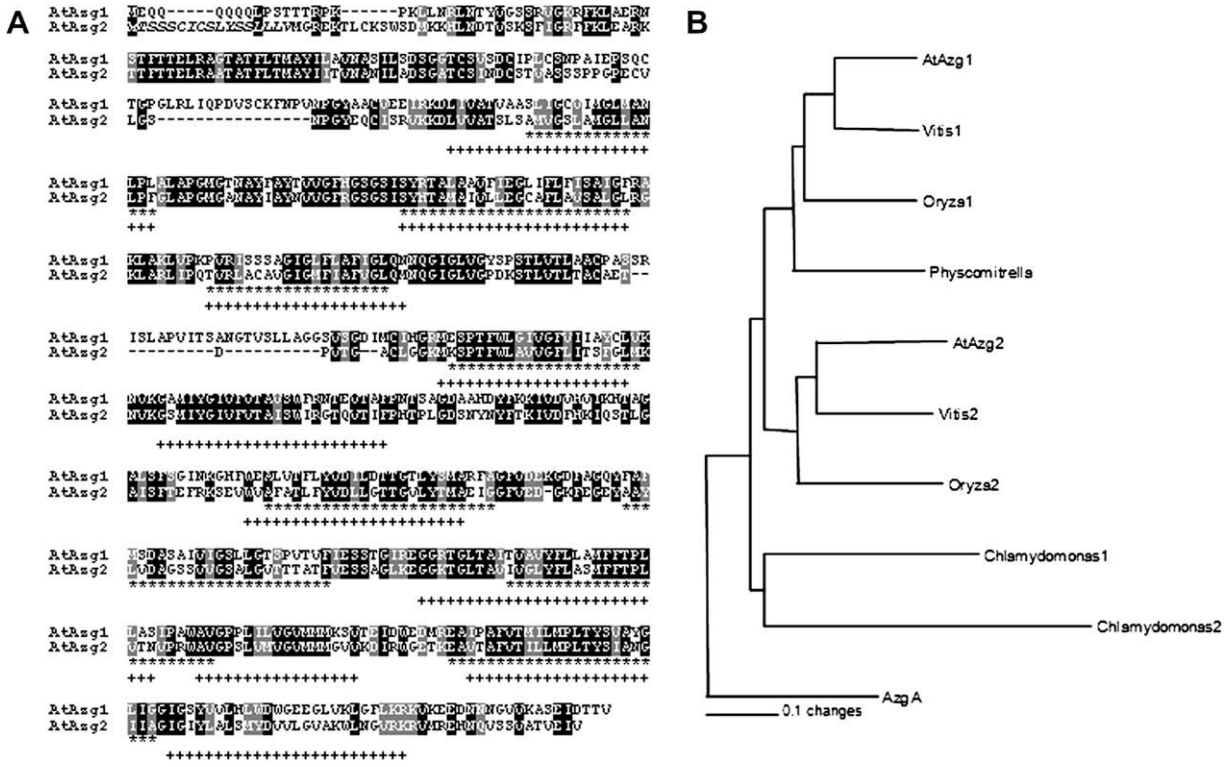

Fig. 1. Amino acid sequence similarity and phylogenetic relationships of AtAzg1 and AtAzg2. (A) Alignment of AtAzg1 and AtAzg2 amino acid sequences by Clustal W [35]. Black boxes represent amino acid sequence identity while grey boxes identify similar amino acids. Bold italic font on AtAzg2 denotes a probable chloroplast transit sequence [36]. Location of transmembrane spanning domains for AtAzg1 (stars*) and AtAzg2 (pluses +) predicted by TMHMM v 2.0 [37]. (B) Phylogenic relationship of AzgA-like proteins from *Arabdiopsis thaliana* GenBank #AAQ65179.1 (AtAzg1), BAB09401.1 (AtAzg2); *Vitis vinifera* CAN76560.1 (Vitis1), CAO64167.1 (Vitis2); *Oryza sativa* EAY97568.1 (Oryza1), EAZ18210.1 (Oryza2); *Physcomitrella patens* EDQ66119.1 (Physcomitrella); *Chlamydomonas reinhardtii* EDP08303.1 (Chlamydomonas1), XP001698486.1 (Chlamydomonas2) and *Aspergillus nidulans* Q07307.3 (AzgA). The tree represents an unrooted maximum parsimony [38].

AtAzg1 and AtAzg2 are expressed in germinating seedlings, young roots and leaves [32]. Seedlings offer an informative developmental stage to access the effect of toxic nucleobase analogues on growth. Homozygous mutant lines carrying allele AtAzg1-1 (SAIL_114_E03) or allele AtAzg2-1 (SALK 020651) exhibited strong resistance to 8-azg (Fig. 2). Double mutant plants show increased

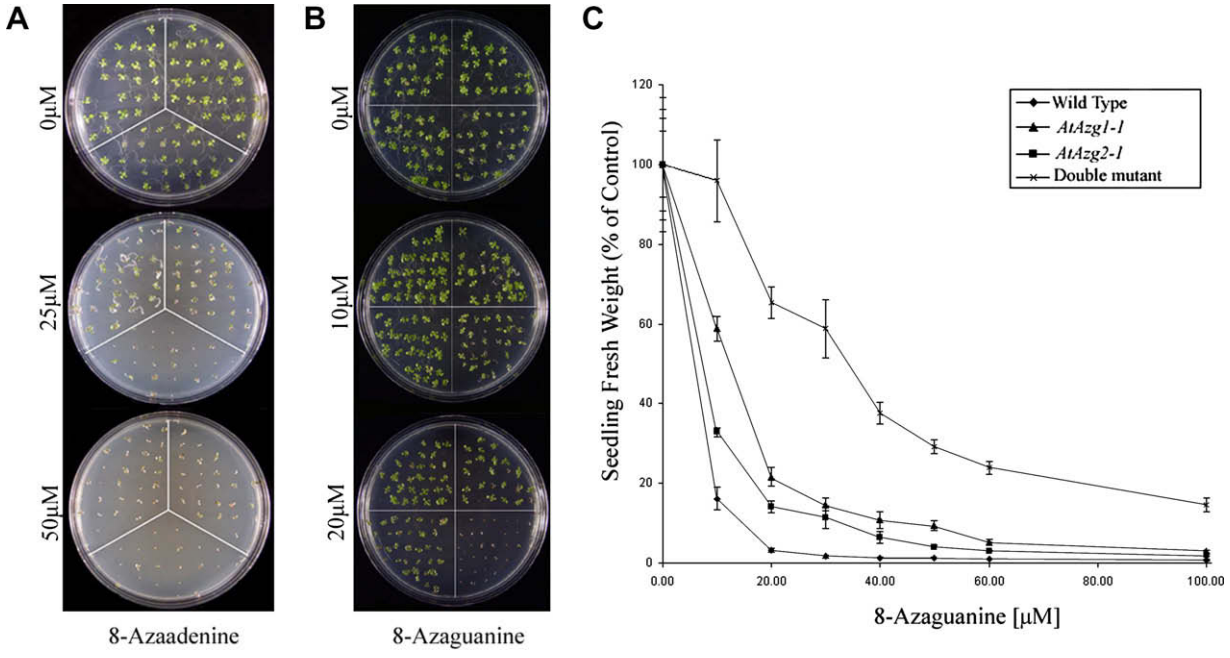

Fig. 2. Growth of *AtAzg* mutants on toxic purine analogues. (A) *AtAzg1-1* (top left) and *AtAzg2-1* (top right), and wild-type (bottom) on 8-azaadenine. (B) *AtAzg1-1* (top right), *AtAzg2-1* (bottom left), and double mutant (top left) and wild-type (bottom right) on 8-azgaguanine. (C) Effect of a concentration series of 8-azg on the fresh weight of *AtAzg1-1*, *AtAzg2-1*, double mutant, and the wild-type. Values shown are the mean of three independent experiments. Error bars indicate the standard error of the mean.

Table 2
Resistance of mutants *AtAzg1-1* and *AtAzg1-2* to toxic purine vs. pyrimidine analogues.

Toxic analog	*AtAzg1-1*	*AtAzg2-1*
8-Azaadenine	++	+
8-Azaguanine	+++	++
2-Bromodeoxyuridine	–	–
2-Fluorocytosine	–	–
2-Fluorodeoxyuridine	–	–
5-Fluoroorotic acid	–	–
5-Fluorouracil	–	+

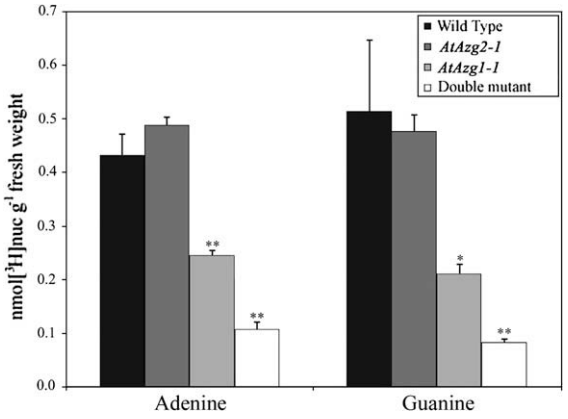

Fig. 3. Uptake of [^{3}H]-adenine and -guanine by 11-day-old seedlings. The code for the bars is as follows: wild-type, black; *AtAzg2-1*, dark grey; *AtAzg1-1*, light grey; and double mutant, white. Values shown are the mean of three independent experiments. Error bars indicate the standard error of the mean. Statistical analysis used an independent paired *t*-test. Significance was measured at $P = 0.1$(*) and at $P = 0.05$(**).

resistance when grown on 8-azg over either single mutant (Fig. 2B and C). *AtAzg1-1* mutant plants exhibited a stronger resistance phenotype than did the *AtAzg2-1* mutant plants (Fig. 2C). Based on IC_{50} values, *AtAzg1-1* is 3-fold more resistant to 8-azg (IC_{50} = 12.5 μM) while *AtAzg2-1* is nearly 2-fold more resistant to 8-azg (IC_{50} = 7.5 μM) when compared to wild-type (IC_{50} = 4 μM) (Fig. 2C). The double mutant exhibited a synergistic effect with an IC_{50} value of 34.5 μM for 8-azg. This represents an 8.5-fold resistance compared to wild-type (Fig. 2C). The stronger resistance to 8aza and 8azg observed with *AtAzg1-1* vs. *AtAzg2-1* plants can be explained in terms of gene expression levels. Plants were exposed to 8-aza and 8-azg for 12 days during which time *AtAzg1* is expressed 2.5–3 times higher than *AtAzg2* (Genevestigator Gene Chronologer). The higher level of resistance reflects the greater depletion of adenine/guanine transporters in *AtAzg1-1* plants. When *AtAzg1-1* mutants were grown on 5-fluorouracil no resistance was observed, however, a small level of resistance was observed with the *AtAzg2-1* mutants. This observation suggests that the substrate specificity of AtAzg2-1 differs slightly from that of AtAzg1-1. No resistance was observed to any other compound at concentrations lethal to wild-type (Table 2).

3.3. AtAzg1-1 but not AtAzg2-1 seedlings are deficient in the uptake of [^{3}H]-adenine and -guanine

AtAzg2-1 seedlings did not show a significant decrease in uptake for either [^{3}H]-adenine or -guanine ($P = 0.3631$ and $P = 0.8144$, respectively) when compared to the wild-type (Fig. 3). However, *AtAzg1-1* seedlings showed a statistically-significant reduction in the uptake of [^{3}H]-adenine (43%) and [^{3}H]-guanine (59%) (Fig. 3). Double mutant seedlings showed a highly statistically-significant reduction in the uptake of [^{3}H]-adenine (75%) and [^{3}H]-guanine (84%) when compared to wild-type (Fig. 3).

Several interesting results emerge from the data. First, in both the growth studies and in the [^{3}H]-purine uptake studies the *AtA-*

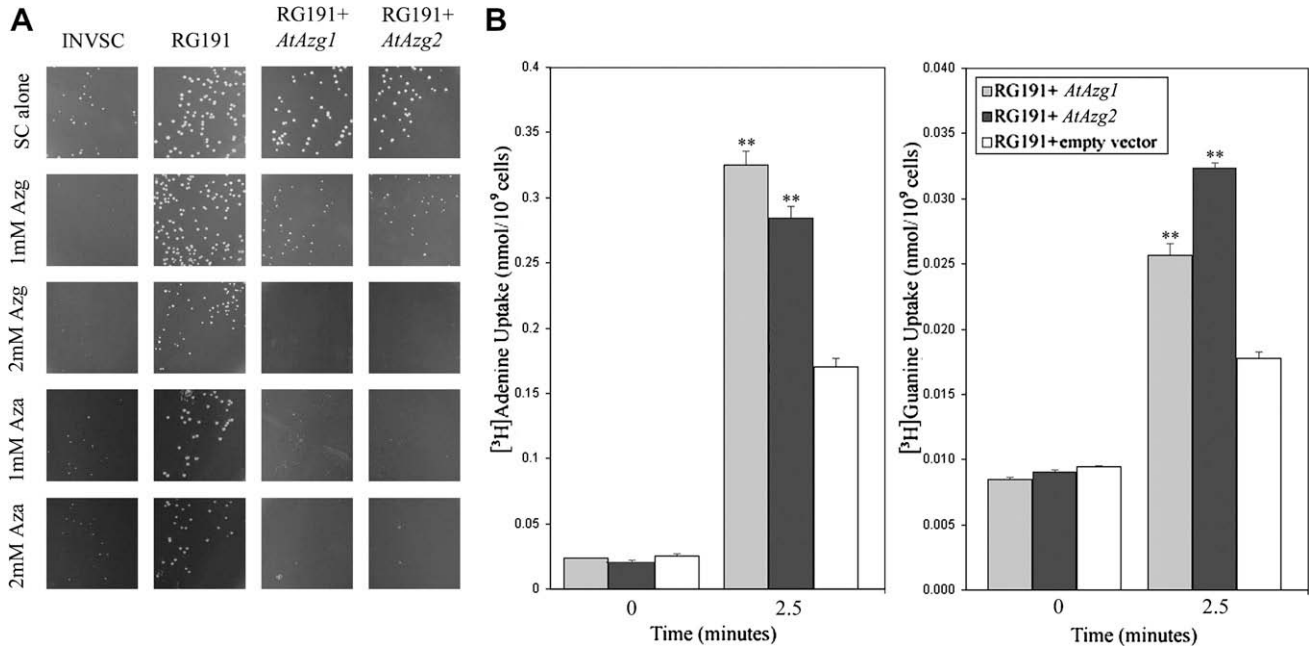

Fig. 4. Growth of *S. cerevisiae* cells expressing *AtAzg1* and *AtAzg2* on toxic purine analogues and uptake of [^{3}H]-adenine and -guanine. (A) Wild-type (INVSC), *fcy2* mutant (RG191), RG191 expressing *AtAzg1* (RG191 + *AtAzg1*) and RG191 expressing *AtAzg2* (RG191 + *AtAzg2*) grown on SC medium supplemented with 0, 1 or 2 mM 8-azaguanine or 8-azaadaneine. (B) RG191 + *AtAzg1* (light grey), +*AtAzg2* (dark grey) or empty (white) was incubated with 0.5 μM of either [^{3}H]-guanine or -adenine in citrate buffer (pH 3.5). Aliquots were taken at 0 and 2.5 min and filtered. Radioactivity is expressed as nmol of labeled nucleobase per 10^9 cells. Values shown are the mean of at least three independent experiments. Error bars indicate the standard error of the mean. Statistical analysis used an independent paired *t*-test. Significance was measured at $P = 0.1$ (*) and at $P = 0.05$(**).

zg1-1 plants are more deficient in adenine or guanine transport than *AtAzg2-1* plants. Second, plants deficient for both *AtAzg* loci are far more affected in purine transport than either single mutant or the additive deficiency of both. Third, *AtAzg2-1* plants show some resistance to growth on 8-azg and 8-aza but no statistical difference in the uptake of [^{3}H]-adenine or [^{3}H]-guanine compared to wild-type. These results can be explained in part by considering different subcellular locations of AtAzg1 and AtAzg2. If AtAzg1 is located in the plasma membrane and represents the major route for adenine and guanine entry, then a loss of this protein will result in toxic analogue resistance and reduction in uptake of adenine and guanine. If AtAzg2 is located in the plastid membranes – as suggested by the predicted chloroplast targeting motif in AtAzg2 (Fig. 1A) – then labeled purines can still enter the cell but toxic purine analogues are inefficiently catabolized due to restricted entry into the plastid. When compared to wild-type, little difference in radiolabel uptake by *AtAzg2-1* would be evident, yet resistance to toxic analogues would be observed. This was the case.

3.4. Heterologous experiments in yeast reveal that AtAzg1 and AtAzg2 act as adenine–guanine transporters

Two separate sets of experiments used *S. cerevisiae* strains to test whether AtAzg1 and AtAzg2 act as transporters. The first experiment determined if AtAzg1 and AtAzg2 could influence the growth of yeast on toxic purine analogues. The second experiment measured the ability of yeast containing *AtAzg1* or *AtAzg2* to take up [^{3}H]-adenine or -guanine. *S. cerevisiae* do not have AzgA-like transporters, but do contain a transporter named Fcy2 that transports adenine, guanine and cytosine [33]. Fcy2 belongs to the Nucleobase:Cation Symporter-1 family. Yeast strains deficient in Fcy2 display heightened resistance to growth on 5-fluorocytosine (5-FC), 8-aza and 8-azg [34]. The addition of functional adenine–guanine transporters to *fcy2* strains would increase the sensitivity to growth on 8-aza and 8-azg, but not 5FC. The coding regions of *AtAzg1* and *AtAzg2* were cloned into yeast expression vectors, introduced into an *fcy2* deficient strain, RG191, and the resulting transformants challenged for growth on varying concentrations of the three toxic nucleobase analogues. While untransformed control RG191 was unaffected by growth on 1–2 mM 8-aza or 8-azg, wild-type yeast (INVS) as well as *AtAzg1*- and *AtAzg2*-containing RG191 strains showed limited or no growth (Fig. 4A). No difference in growth on 20 and 40 mM 5FC was observed for RG191 or transformants, yet was lethal for wild-type strains (data not shown). This data reflects the nucleobase specificity observed in the mutant Arabidopsis growth experiments mentioned above, namely resistance to adenine and guanine but not cytosine analogues. Further, these data reflect the transport specificity observed for *Aspergillus* AzgA but not the yeast FCY2.

If AtAzg1 and AtAzg2 act as adenine–guanine transporters then the presence of these transporters in yeast should enhance the uptake of radio-labeled adenine and guanine. This is indeed the case as yeast strains transformed with *AtAzg1* or *AtAzg2* showed a highly significant increase in uptake of [^{3}H]-adenine, 91% and 67%, and [^{3}H]-guanine, 45% and 82%, over control yeast, respectively (Fig. 4B). The ability of each of AtAzg1 & 2 to transport adenine and guanine in yeast cells further supports their role as plant adenine–guanine transporters. The data presented in this paper clearly define AtAzg1 and AtAzg2 as AzgA-like adenine–guanine transporters in plants.

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