FUNCTIONAL IDENTIFICATION AND CHARACTERIZATION OF AN

ARABIDOPSIS THALIANA GENE INVOLVED IN VITAMIN B_6 BIOSYNTHESIS

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FUNCTIONAL IDENTIFICATION AND CHARACTERIZATION OF AN *ARABIDOPSIS THALIANA* GENE INVOLVED IN VITAMIN B_6 BIOSYNTHESIS

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Charmaine LaShawn Porter, daughter of Claude Porter and Charles and Shari Brown, was born July 30, 1981 in Baton Rouge, Louisiana. She graduated with honors from Baton Rouge Magnet High in 1999. From there she attended Tuskegee University in Tuskegee, Alabama for 1 1/2 years and then transferred to Auburn University in January, 2001. She graduated cum laude from Auburn University with a Bachelor of Science degree in Microbiology, May 2003. Immediately after graduation, she entered graduate school at Auburn University in the Department of Biological Science, in May 2003, to pursue her Master of Science degree in Molecular Biology.

THESIS ABSTRACT

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Vitamin B_6 (pyridoxine) is a biological coenzyme seemingly involved in environmental stress tolerance and an important vitamin in human nutrition produced in plants as well as fungi and bacteria. A series of studies from these different organisms revealed that *SNZ1/SNO1* homologues are the genes involved in the production of pyridoxine. We decided to functionally define *SNO1* and *SNZ1* orthologs and/or paralogs in the *Arabidopsis thaliana* genome by using transgenic yeast expressing putative *SNZ1* or *SNO1* homologs to suppress the growth defects of yeast Δ*snz*1 and Δ*sno*1. Using a blast search we were able to find *Arabidopsis* homologs of yeast *SNZ1/SNO1*. Moreover, we have obtained knockout deletion mutants of *SNZ1* and *SNO1* from *S. cerevisiae* and were able to confirm that the *SNZ1* and *SNO1* deletions did not grow as efficiently as wild type in media lacking pyridoxine. In addition, we were able to clone the putative *A. thaliana SNZ1* into a high expression yeast shuttle vector (p426 GPD). Our results indicate that the *AtSNZ1* does complement phenotypic expression of the *SNZ1* knockout in yeast.

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LITERATURE REVIEW

Vitamins are indispensable dietary compounds that are required by many organisms. Most unicellular organisms and plants are autotrophic for the production of vitamins. However, as heterotrophs, humans are not able to synthesize vitamins and therefore we must obtain vitamins from other sources, such as fruits and vegetables. Vitamins are essential, in that, they are the substances that we need in order to grow and develop properly. Each group of vitamins has a specific role to play in our development. For instance, vitamin D in milk helps our bones become stronger, vitamin A in carrots aids night vision, vitamin C in oranges assists the body in healing and the B vitamers, which are found in green leafy vegetables, help the body make protein and energy.

Table 1. Description of the various types of vitamins

Vitamin	Function
A	For vision, eyes, clear skin, healthy bones, hair, teeth
A	(Beta carotene) Antioxidant which turns into vitamin A when the body needs it
D	Calcium and phosphorous metabolism needed for strong bones and teeth
E	Prevents oxidation of proteins, fats, and vitamin a, protects red blood cells
K	Helps the blood clot properly
C	An antioxidant that helps create the immune system
B-1	(Thiamine) Functioning of nervous system, appetite and energy
B-2	(Riboflavin) For the skin, eyes, and energy
B-3	(Niacin) For the skin, the nervous system and mental performance
B-5	(Panthothenic Acid) develops acetylcholine
B-6	Helps metabolize protein and fat, needed for the hemoglobin formation
Biotin	Metabolize amino acids and stimulate hair growth
Folic Acid	Red blood cell formation, metabolizes fat, amino acids, and protein
Choline	Prevents fat accumulation in the liver
Inositol	Involved in calcium mobilization

One member of the eight soluble B vitamers, vitamin B_6, has become of great interest to the scientific community in recent years. This attention is due to the fact that vitamin B_6 has been implicated in a variety of biological processes throughout nature. Vitamin B_6 is a combined term for, pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL) and their phosphorylated derivatives pyridoxine-5'-phosphate (PNP), pyridoxamine-5'-phosphate (PMP) and pyridoxal-5'-phosphate (PLP) (Dakshinamurti, 1990). These six vitamers are all B_6 -vitamers but pyridoxine is the vitamer that is commonly referred interchangeably as vitamin B_6.

Vitamin B_6, principally in the form of the coenzyme pyridoxal 5'-phosphate (PLP), is involved in a wide range of biochemical reactions, including the metabolism of amino acids and glycogen; the synthesis of nucleic acids, hemoglobin, sphingomyelin and other sphingolipids; and the synthesis of the neurotransmitters serotonin, dopamine, norepinephrine and gamma-aminobutyric acid (GABA) (Dakshinamurti, 1990). Current research has suggested that vitamin B_6 is involved in, but not limited, to the growth of cells, maintenance in the immune systems, and the balancing of hormonal changes in women (Wyatt et al., 1999). Relative to other vitamins, only a minuscule amount of information is known about the biosynthesis of vitamin B_6.

The first biochemical pathway for pyridoxine biosynthesis was elucidated in *E. coli* (Laber et al., 1999). This pathway was based on biochemical evidence that indicated that in *E. coli*, the active form of vitamin B_6, pyridoxal 5' phosphate (PLP) was synthesized de novo by a pathway involving the condensation of the amino acid 4-(phosphohydroxy)-L- threonine and the sugar 1-deoxy-D-xylulose-5-phosphate (Laber et al., 1999). The genes *pdx*A and *pdx*J code for enzymes that have been implicated in the

intramolecular condensation reaction between the two substrates to yield PNP. PNP is then oxidized by the gene product of PdxH to form the active coenzyme PLP (Zhao et al., 1995).

It has also been shown that *Rhizobium meliloti* uses the same precursors, 1-deoxy-d-xylulose and 4-hydroxy-L-threonine, as *E. coli,* in order to synthesize pyridoxine (Tazoe et al., 2000). The original work with *E. coli* involving pyridoxine auxotrophic mutants (Osami et al., 1999) has been followed by extensive bioinformatics analyses that have shown that most bacteria and archea contain this pathway. However, this predominantly bacterial pathway has never been demonstrated in Eurkaya and some Archea.

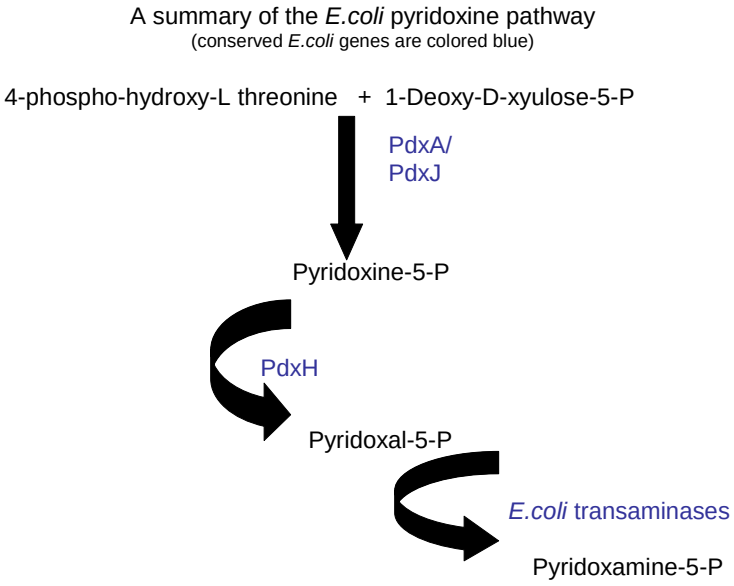

Figure 1. The proposed pyridoxine synthesis pathway in *E.coli*
Precursors to the de novo pathway of pyridoxine (vitamin B_6) involves the condensation of the amino acid, 4-phospho-hydroxy-L threonine and sugar, 1-Deoxy-D-xyulose-5-P which are then catalyzed by the enzymes PdxA and PdxJ to form pyridoxine-5-P (PNP).

The biosynthesis of pyridoxine has evolved via two different pathways during the evolution of eubacteria (Ehrenshaft et al., 1999). Some bacteria utilize the previously defined *E. coli* pathway and other organisms (primarily Archea and Eukarya) use an, at the present, a poorly defined Eukarya pathway. Radiolabling experiments support the notion of two mutually conserved pathways for pyridoxine synthesis. However, the point at which the divergence occurred between prokaryotes and eukaryotes is not well understood. We do know from feeding studies using ^{15}N-labeled substrates that glutamic acid is donor of the amide group in bacterial pyridoxine biosynthesis (Tanaka et al., 2000). Contrarily, in the yeast *Saccharomyces cerevisiae* as well in three additional eukaryotes, ^{15}N –labeling revealed that the nitrogen atom of pyridoxine arises from the amide group of glutamine and not glutamic acid (Tanaka et al., 2000).

Additional evidence indicated a divergence of pathways when ^{13}C labeling provided evidence that the C_5 carbon skeleton of vitamin B_6 in yeast originates from a glucose-derived pentulose or pentose intermediate (Gupta et al., 2001). On the other hand, in *E. coli*, the C_5 chain of pyridoxine originates by the condensation of pyruvic acid with glyceraldehyde 3- phosphate which leads to the production of one of the major building blocks of pyridoxine in *E. coli*, 1-deoxy-D-xylulose 5- phosphate (Gupta et al., 2001). Although the *E. coli* pathway of pyridoxine formation has been extensively studied and understood, this isotopic labeling has given us a start point in understanding the mechanism of eukaryotic pyridoxine biosynthesis.

More information on the genetics of pyridoxine synthesis became available when bioinformatics analysis using the BLAST search utility at NCBI revealed that

microorganisms either contain homologues to *E. coli pdxA/pdxJ* or homologues to a gene called *SOR1*; but does not contain copies to both types of genes (Ehrenshaft et al., 1999). *SOR1* is the important gene that became the catalyst in understanding the genetic and phenotypic relationship of pyridoxine in eukaryotes.

SOR1 (singlet oxygen resistance) is a gene first identified in the filamentous fungal pathogen of plants, *Cercospora nicotianae,* with linkage to resistance against compounds that produced singlet oxygen (Ehrenshaft et al., 1999). *Cercospora* produces a potent toxin called cercosporin as part of its mechanism of plant pathogenesis. Cercosporin is a photosensitizer that is activated by light and produces singlet oxygen ($O_2^{singlet}$). This singlet oxygen can cause damage to critical cellular components, such as membranes, proteins and DNA which often leads to cellular death. Organisms have developed methods to protect themselves against reactive oxygen species (ROS) that are reduced in form, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$). Naturally, because these ROS are by products of cellular metabolism, they have received a tremendous amount of attention in the literature. Conversely, most organisms are unable to tolerate the non-radical singlet oxygen ($O_2^{singlet}$) and there are only a few biological defenses that have been identified for scavenging ($O_2^{singlet}$) (Daub 1998).

Because *Cercospora* is unique in using a ($O_2^{singlet}$) –generating toxin as part of its mechanism of pathogenesis, it must have mechanisms to protect itself from toxicity of the ($O_2^{singlet}$). The mechanism that *Cercospora* has evolved utilizes the gene product of *SOR1* gene to detoxify ($O_2^{singlet}$). Furthermore, *SOR1* has another role other than the conferring resistance to ROS in *C. nicotianae* (Ehrenshaft et al., 1999). Studies have revealed that

SOR1 is involved in a de novo pathway of vitamin B_6 synthesis. Experiments involving *C. nicotianae sor1* mutants demonstrated that such mutants were unable to grow in the absence of pyridoxine but pyridoxine prototrophy was restored by a *SOR1* transformation (Ehrenshaft et al., 1999). In addition, because of the studies with the *Cercospora SOR1* gene, pyridoxine was implicated in the quenching ($O_2^{singlet}$) through a mechanism that consumed pyridoxine inside the cells (Ehrenshaft et al., 1999). Actually, the authors were able to show that pyridoxine quenches ($O_2^{singlet}$) at a rate comparable to two of the most highly efficient antioxidants, vitamins C and E. This was the first demonstrations that showed pyridoxine had antioxidant properties and was capable of quenching or protecting against ($O_2^{singlet}$). Thus, the reports with *Cercospora nicotianae* on the *SOR1* gene was the beginning of characterizing a novel biochemical function for pyridoxine in eukaryotes.

The fact that studies with the *SOR1* gene have indicated that pyridoxine aids *Cercospora* in resisting ($O_2^{singlet}$) production is consistent with results that have been published in other systems. The antioxidant properties of vitamin B_6 (pyridoxine) were examined in *Schizosaccharomyces pombe* by treating cells with menadione, which is a generator of superoxide (Chumnantana et al., 2004). It was shown that pyridoxal-5'-phosphate, pyridoxamine-5'-phosphate and pyridoxamine demonstrated higher antioxidant activity than vitamin C. Moreover, numerous studies in humans have indicated that a pyridoxine deficiency occurs in both type 1 and type 2 diabetic patients (Ellis et al., 1991) and a study using red blood cells (RBC) showed that pyridoxine could inhibit ($O_2^{singlet}$) and radical production, which helps prevent lipid peroxidation, protein glycosylation and lowers hyperglycemia in diabetic patients (Jain and Lim, 2000). Thus,

it seems that pyridoxine has the dual function of supplying the coenzyme PLP to many enzymatic reactions along with providing resistance against active oxygen species.

As a result of widespread genomic sequencing, it is now understood that *SOR*1 gene of unknown biochemical function, is a highly conserved gene throughout the three domains of life (Stolz and Vielreicher, 2003). Sequence alignments revealed that this gene exhibits approximately 60% homology throughout the archea, eubacteria and eukaryotes kingdoms. All of the organisms in these kingdoms have evolved a different name for the gene involved in vitamin B_6 production. A study in *Neurospora crassa* was able to effectively indicate that *PDX1* is the *SORI* homologue through genomic analysis of the fungi's genome; they were also able to demonstrate that mutations in the *PDX1* gene conserved regions caused the pyridoxine mutant phenotype that has been documented in the literature (Bean et al., 2001). In *Aspergillus nidulans*, *pyro A,* is the name used for the *SOR1* homologue. Interestingly, mutations of the *pyroA,* cause increased sensitivity to the (O_2 ^{singlet}) generated by the photosensitizer, methylene blue (Osmani et al., 1999). However, the *pyroA* auxotrophic mutants were able to grow in the methylene blue at media concentrations of pyridoxine of 0.5 ug/ml in the media. Thus, comparable to the studies cited above in other fungi, this data indicates that in *A. nidulans, pyroA* is required for pyridoxine biosynthesis and for resistance to (O_2 ^{singlet}).

Since several independent experiments provided data to show that *SOR*1 and its homologues are involved in pyridoxine biosynthesis, *SOR*1 and its homologues are now commonly referred to as *PDX* (pyridoxine requiring) gene, as a measure to give a consensus name in the literature among the homologues. Some organisms have more than one copy of *PDX1* gene. In fungus, *Neurospora crassa* contains two copies of the *PDX1*

homologue, i.e. *pdx1/pdx2,* which are in close physical proximity to each other (Bean et al., 2001). Additionally, other organisms, such as *Bacillus subtilis* and *Haemophilus influenzae,* also encode this second gene that is in close proximity to its *PDX1* homologue (Ehrenshaft and Daub, 2001). Moreover, in the yeast *Saccharomyces cerevisiae*, the analysis of the *PDX* genes becomes more complicated, in that yeast have three unlinked *PDX1* homologues called *SNZ1* (Snooze), *SNZ2* and *SNZ3* respectively. In addition there are three *PDX2* homologues that are adjacent to the *PDX1* homologues and have been named *SNO* (Snz proximal ORF).

Paralogous gene families, such as the *SNZ/SNO* family, comprise approximately 40% of the yeast genome (Blandin et al., 2000). Sometimes these gene redundancies involve genes with exact functional redundancy although there are many cases in which the paralogous genes have undergone evolutionary divergence where the genes are only partially functionally redundant or not functionally redundant at all (Rodriguez-Navarro et al., 2002). This divergence is what is believed to have happened to the *SNZ* gene family in yeast. *SNZ1* "snooze" (YMR096c) was originally identified when it was shown that the SNZ1p protein was expressed during the stationary phase of the yeast life cycle (Braun et al., 1996). Additional homologues, *SNZ2* (YNL333w) and SNZ3 (YFL059w) were confirmed after publication of the entire yeast genome. *SNZ2* and *SNZ3* encode proteins that exhibit approximately 99% homology with each other and approximately 80% identity to the *SNZ1* amino acid sequence (Padilla et al., 1998). Furthermore, analysis of yeast genomic sequences has led to the discovery of another conserved gene family located on the same chromosome immediately adjacent to each *SNZ* gene that is called *SNO* (SNZ-proximal reading frame) (Figure 2). Comparably, *SNO2* (YNL334c)

and SNO3 (YFL069c) encode for proteins that are nearly 100% identical to each other and approximately 72% identical to the *SNO1* (YMR095c) protein (Braun et al., 1996).

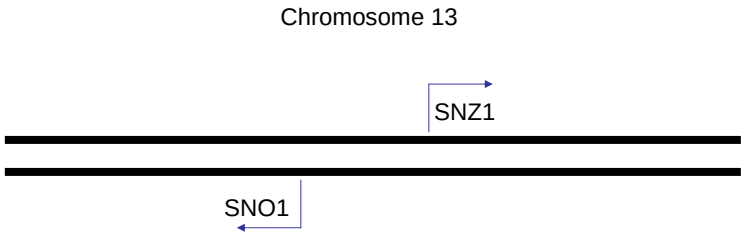

Figure 2. Direction of the Open Reading Frames for *SNZ1* and *SNO1* On Chromosome 13 in *Saccharomyces cerevisiae*. In yeast, the paralogous members of the SNZ and SNO family, i.e. *SNZ1* and *SNO1*, are located adjacently on the same chromosome and are transcribed in divergent directions.

Now, a conundrum exists in the literature about *SNZ* homologues. Bioinformatics studies have shown *SNZ* and its homologues to be one of the most highly conserved proteins present within bacteria, fungi and higher plants but these proteins have not had a previously defined function. Usually a high degree of conservation throughout evolutionary time signifies the importance of a protein in nature. This is not the case for the *SNZ/SNO* family in yeast. In fact, single null deletions of each gene results in viable cells. Ironically, the expression of these genes increases during stationary phase which is a time when most genes decrease in expression (Braun et al., 1996). This observation

along with evolutionary conservation has led to the hypothesis that the *SNZ/SNO* gene families are involved in the universal nature of the growth response.

As mention previously, the three different studies from *Aspergillus nidulans*, *Neurospora crassa*, and *Cercospora nicotiniae* have revealed that the *SNZ/SNO* homologous genes (*PDX1/2*, *PyroA*, and *SOR*1) are related to the biosynthesis of pyridoxal (vitamin B_6). To support the hypothesis that the yeast *SNZ/SNO* genes have an analogous phenotypic expression to their homologues involving pyridoxine biosynthesis, it was demonstrated that *SNZ1* and *SNO1* mutants were sensitive to methylene blue, a producer of singlet oxygen (Padilla et al., 1998). Surprisingly, the other mutant alleles *snz1, 2, 3*Δ*3*, *sno1, 2, 2*Δ*3*, *snz2, 3*Δ*3*, *sno2, 3*Δ*3,* were not sensitive to growth on methylene blue (Padilla et al., 1998). Furthermore, only *SNZ1* and *SNO1* are required for growth in media lacking pyridoxine, but *SNZ2*, *SNZ3*, *SNO2* or *SNO3* are not required for growth. Copies 2 and 3 of *SNZ/SNO* are activated by vitamin B_1 (thiamin) biosynthesis, and thus may play a role in the synthesis of that vitamin (Rodriguez-Navarro et al., 2002). In addition, yeast 2-hybird analysis indicated that there was a strong interaction between Snz1p and Sno1p (Padilla et al., 1998). Thus, it can be concluded that only the *SNZ1* and *SNO1* copies are responsible for the metabolism of pyridoxine in yeast, while the other copies are involved in some other growth response. The conserved aspects of the *SNZ/SNO* homologues in nature has led to the quest of understanding the manner in which *SNZ1/SNO1* participates in the pyridoxine biosynthetic pathway. Moreover, yeast has become the model system in which the eukaryotic pyridoxine pathway is being studied and understood.

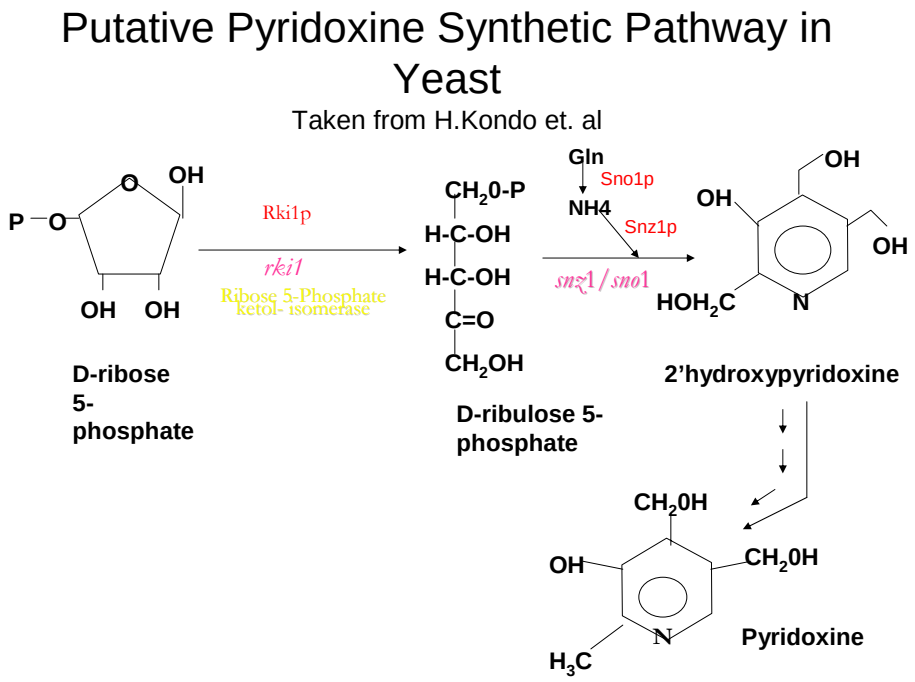

Figure 3. Proposed pyridoxine pathway in *Saccaharomyces cerevisiae*
The pyridoxine pathway in yeast begins with D-ribose 5'-phosphate by which ribose 5'-phosphate ketol-isomerase (Rki1p) catalyzes the compound into D-ribulose 5'-phosphate. The Snz1p and the Sno1p complex release ammonia from glutamine, where the nitrogen is incorporated into the ring, leading to the formation of 2'-hydroxypyridoxine.

Currently, data strongly suggest that the Snz1p and Sno1p complex functions as a glutaminase to supply ammonia for the ring nitrogen of pyridoxine in yeast (Dong et al., 2004). The Snz1p and Sno1p complex is believed to participate in a glutamine amidotransferase reaction with Sno1p serving as a glutaminase (Dong et al., 2004). Recently, it has been shown through a cross complementation experiment using the *E. coli pdxJ* with *Cercospora SOR1* (*Pdx1/SNZ1*) gene, that *PDX1* is the responsible for pyridoxine ring closure reaction (Wetzel et al., 2004). Since this finding, a putative pathway of the synthetic pathway of pyridoxine in yeast has been proposed in the literature (Kondo et al, 2004). The synthesis starts with D-ribose 5-phosphate being

converted to D- ribulose 5-phosphate by a ribose 5-phosphate ketol-isomerase, ultimately providing the C_5 carbon skeleton of the vitamin (Kondo et al., 2004). From this point, the Sno1p and Snz1p releases the ammonia from glutamine and the nitrogen atom is incorporated in the ring that formed by the condensation of D-ribulose 5-phosphate with an unidentified substrate forming, 2-hydroxypyridoxine, the starting biosynthetic precursor of vitamin B_6 (Zeidler et al., 2001).

There is a commonality among both the prokaryotic and eukaryotic pathway in which these organisms have a resourceful salvage pathway that converts the three natural free forms of vitamin B_6, pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), to the major operating coenzyme, pyridoxal-5'- phosphate (PLP). This salvage pathway involves the use of two enzymes, pyridoxal kinase and pyridoxine-phosphate oxidase. Pyridoxal kinase catalyzes the phosphorylation of the 5-hydroxymethyl group of all three vitamins while pyridoxal –phosphate oxidase catalyzes the oxidation of the alcohol of pyridoxine (PNP) and the amine of pyridoxamine (PMP) to an aldehyde liberating ammonia in the latter case (McCormick and Chen, 1998). In addition, there are phosphatases that are able to convert the phosphates of the vitamins to back to the free form of the vitamin.

Pyridoxine involving genes, such as *SNZ* and *SNO*, are not found in the genome of higher eukaryotes (animals), with the exception of the marine sponge *Suberites domuncula* (Seack et al., 2001). Sponges (Urmetazoa) were one of the first organisms to diverge from the common ancestor of all Metazoa (Seack et al., 2001). Since animal lineages have obviously lost the *SNZ* and *SNO* genes, it is possible loss of *SNZ* and *SNO* in animals occurred at the Poriferal (sponges) lineage (Tanaka et al., 2004). Interestingly,

it has been reported that there is mammalian pyridoxal kinase and oxidase activity. A study indicated that the mammalian pyridoxal oxidase is developmentally regulated in the liver and brain. Moreover, it was shown that there is an absence of pyridoxal oxidase in certain tumors (Ngo et al., 1998). It was hypothesized that the mammalian pyridoxal kinases and oxidases help to facilitate entry of vitamin B_6 into the cell (McCormick and Chen, 1998). This observation is appealing in that humans can not synthesize vitamin B_6 and must uptake the vitamin nutritionally from other sources, such as plants, fungi or bacteria. From these experiments, it is apparent that vitamin B_6 plays major roles in mammals that are not yet fully identified. As we are constantly looking for means to improve the longevity of humans, logically, it would be appropriate to study how humans obtain and metabolize their vitamin B_6 source, which is plants, and to examine how the source of vitamin B_6 could be manipulated in plants.

Pyridoxine 5'β-D-glucoside (PNG), is a natural occurring glycosylated derivative of vitamin B_6, which is found in most fruits, vegetables, and cereal grains comprising approximately 75% of the total amount of vitamin B_6 found in plants (Gregory,1998). Unfortunately, there is little information known about pyridoxine biosynthesis in plants. Bioinformatic searching of the genomes of different plants species has shown that there are several genes that have been identified which show a high degree of homology to *PDX1*. However, none of these genes have a function identified, and none of the genes have been extensively characterized. One study, attempted to characterize the *PDX1* orthologue in the plant *Phaseolus vulgaris*. The results indicated that the *pvPDX1* (putative *PDX I*) gene of *P. vulgaris*, was able to partially reverse pyridoxine auxotrophy in null mutants of *C. nicotianae* (Graham et al., 2004). This is the first data to suggest

that plants also use the eukaryotic divergent pathway of pyridoxine biosynthesis. However, further work is needed in order to elucidate the pyridoxine biosynthetic pathway of plants.

Since the genome has already been sequenced, *Arabidopsis thaliana* is a useful model system in order to learn more information about the intrinsic factors of vitamin B_6. Also, *Arabidopsis* plants are easily grown in the laboratory, making the plant an appealing system for studying the *SNZ1/ SNO1* (*Pdx1/Pdx2*) genes functional characteristics in plants. In, *Arabidopsis* mutants, it was shown that the *SOS4* (salt overly sensitive) gene encodes a pyridoxine, pyridoxal or pyridoxamine (PN/PL/PM) kinase homolog that functions in the biosynthesis of PLP (Shi et al., 2001). In addition, a blast search revealed that *Arabidopsis* genome contains at least three homologs to *SORI/ PDXI/ SNZ1* that may putatively function as the protein involved in pyridoxine biosynthesis with the gene accession numbers as follows: At2g38240, At3g16050, and At5g01410. In addition, At5g60540 and At4g26900 represent putative *SNO1* (*Pdx2*) homologs. Detailed knowledge of vitamin synthesis in plants might lead to better nutritional quality in foods which would indirectly enhance the life of humans. With this perspective in mind, current studies were initiated in an attempt to elucidate the pyridoxine biosynthesis pathway in plants.

INTRODUCTION

Eukaryotic and certain prokaryotic organisms have a gene family homologous to the *SOR*1 gene of *Cercospora nicotianae* (yeast *SNZ*) that expresses a highly conserved protein which exhibits at least 60% homology in Archea, bacteria, and eukaryotes. Despite this conservation, only a minuscule amount of information is known about its function. However, it has been shown in *Saccharomyces cerevisiae* (Baker's yeast) that *SNZ* synthesis increases dramatically during stationary phase (Braun et. al., 1996). *SNZ* is a member of a paralogous gene family consisting of three members, *SNZ1*, *SNZ2* and *SNZ3*. In addition, each of these genes is found adjacent to members of another conserved family named *SNO* (SNZ-proximal reading frame). Studies show that both *SNZ1* and *SNO1* are involved in pyridoxine (pyridoxal) biosynthesis, which is the first step of vitamin B_6 production. Vitamin B_6 is an essential nutrient that provides the coenzyme pyridoxal-5-phosphate (PLP) to most biological systems. Furthermore, it has been documented that PLP plays a role in protective responses to stress in certain organisms.

Using bioinformatics analysis, we have found that the *Arabidopsis thaliana* genome contains homologues to the *SNZ1* and *SNO1* genes found in yeast and other fungi. We have used PCR to obtain cDNA clones of the coding sequence of these *Arabidopsis* genes, and we have obtained single gene deletions of the *Saccharomyces cerevisiae SNZ1* and *SNO1* genes. These mutants are unable to grow as efficient, in comparison to the WT strain, when placed in media lacking pyridoxine/pyridoxal phosphate. We have expressed an *A.thaliana* homologue of yeast *SNZ1* in these yeast deletion mutants and

demonstrated functional complementation of the phenotype of the *SNZ1* growth and biochemical phenotypes.

MATERIALS AND METHODS

Strains and Media

The following strains *S. cerevisiae* were used in this study: BY4742 (Matα hisΔ1leu2Δ2lysΔ2ura3Δ3), BY4742::ΔSNZ1 (Matα hisΔ1leu2Δ2lysΔ2ura3Δ3) and BY4742::ΔSNO1 (MatαhisΔ1leu2Δ2lysΔ2ura3Δ3).

The following media was used for the cultivation of yeast in this study: YPD (1% yeast extract, 2% peptone, 2% glucose), SC (Synthetic Complete) (0.67% yeast nitrogen base without amino acids, 2% glucose, and supplemented with auxotrophic requirements which included histidine, leucine, lysine, and uracil), SC-Ura (same as SC except lacking uracil supplementation); Minimal Medium (vitamin B_6 deficient media) composed of the same components as SC media except that vitamin B_6 was specifically excluded from the yeast nitrogen base mixture used; and methylene blue medium (where YPD was supplemented with 37 μg of methylene blue per ml and subjected to a light source. All solid media used for Petri Plates contained 2% agar.

Strain Cultivation

All strains grown on plates were incubated at 30°C for 2-3 days unless otherwise indicated. In experiments, where liquid culture was conducted, the yeast cells were shaken at 250 rpm at 30°C and samples taken from the culture were assayed for growth at time points shown in the various figures and the text.

Yeast Knock Out Strains

Both the Δ*SNZ1* and Δ*SNO1* single deletion knockouts were purchased from the American Type Culture Collection (ATCC). The knock out strains were constructed based on a PCR-based gene deletion strategy which was use to generate a start-to-stop deletion of the open reading frame of *SNZ1* and *SNO1*. The deletion module consisted of a KanMX4 cassette that was constructed with the addition of the 45 base pairs immediately upstream of the ATG start codon and the 45 base pairs of sequence immediately downstream of the stop codon. During the deletion process, both the *SNZ1* and *SNO1* genes were replaced with a KanMX4 module via homologous recombination. The KanMX4 module was uniquely tagged with two 20mer sequences which were detected via hybridization to a high density oligonucleotide array.

Yeast Knock Out Strain Verification

For verification of specific deletions, specific primers used for polymerase chain reaction verification were designed between lengths of 17 and 28 bases and a melting temperature (T_M) of around 65°C. Primers A and D were designed to be homologous to the region which is about 200 bases upstream or downstream of the open reading frame. Primers B and C were designed to bind to regions within the open reading frame and yield a PCR product of 300-1000 bases when used with the A or D primers. The KanB and KanC primers were picked from regions within the KanMX4 cassette. Specific primer combinations for both SNO1, SNZ1, and KanB and KanC are shown in Table 2.

Table 2. List of *SNZ1* and *SNZ1* specific verification primer combinations

Primer Name	Primer Sequence
SNO1-A	GCGTCCTCCTATTTATATCGAAAAT
SNO1-B	AAACTACCTTTTCCGGATTATGAAC
SNO1-C	AATTAGAAAACGAAAGTGCCCTAGT
SNO1-D	CACACAAGAATAAAAGAGCTTGACA
SNZ1-A	TTTATGGCACTAGTTGGAATAGCTC
SNZ1-B	GCCTTTATCTCCTCTGTAATTCTCC
SNZ1-C	GGAGAATTACAGAGGAGATAAAGGC
SNZ1-D	ATATTTGACCCTGAGGTACTGTTGA
KanB	TAATGCCGAGGAGGGACGTC
KanC	TGATTTTGATGACGACGAGCGTAAT

All strains were verified using a standard 25ul PCR reaction which included template DNA obtained from the appropriate strain to be tested, 2.5mM dNTPs, 50mM $MgCl_2$, 10X PCR buffer, Taq polymerase (Qiagen) and primers consisting of the combinations A and B, C and D, A and D, A and KanB, KanC and D. KanB and KanC primers were used in order to verify that the *SNZ1* and *SNO1* genes were correctly knocked out and replaced by a kanamycin resistance cassette. A successful deletion yielded PCR products using primers A and KanB, and KanC and D, but did not yield PCR products using primers A and B or C and D. The A-D primers yield a product regardless if the strain was WT or a deletion, but the size of this product could vary between wild type and mutant strains depending on the length of the gene in question.

Arabidopsis cDNA

Arabidopsis SNZ1 and *SNO1* homologs were prepared by PCR using an Arabidopsis cDNA, purchased from the Ohio State stock center, as template. Primers for the 5' and 3' ends of the putative *AtSNZ1* and *AtSNO1* were designed that contain restriction sites

on their 5' ends (AtSNZ1-Xho1 (5'-primer) and EcoRI (3'-primer); AtSNO1-XhoI (5'-primer) and SpeI (3'-primer)). The amplified PCR product were cloned into pGEM T-Easy and sequenced to verify the PCR product. The cloned pGEM T-Easy vector was then cut with appropriate restriction enzymes, and inserts were then cut out, separated from plasmid DNA using agarose gel electrophoresis, cleaned from the gel, and directionally cloned into the p426 GPD shuttle vector. This construct was grown in *E. coli*, and subsequently transformed into appropriate yeast strains for phenotypic evaluation.

E. coli Transformation

E. coli transformations involving the pGEM T-Easy plasmid and yeast shuttle vector (p426 GPD) were performed by using standard heat shock protocol. First, 50ng of plasmid DNA was mixed in a 1.5ml microfuge tube with 50ul of *E. coli* DH5α competent cells and incubated on ice for 30 minutes. Then the tubes containing the mixture of plasmid and competent cells was placed into a water bath at 42^{o}C for 45 seconds. The tubes were then put back on ice for 2 minutes to reduce the damage to the *E. coli* cells. Following the previous step, 500ul of LB (minus antibiotic) was added to the tubes which were then incubated for 1hr at 37^{o}C. After the 1 hr incubation period, 100ul of the culture was spread on LB plates containing ampicillin and incubated overnight.

Yeast Transformation

Transformation of yeast deletions with the putative *Arabidopsis SNZ1* gene was accomplished by the lithium acetate method (Gietz, 1994).

Sequencing

Sequencing was done by Auburn University Genomic and Sequencing Lab using appropriate primers for each sequenced construct.

Serial Dilutions

The yeast strains were grown in the appropriate media for about 1-3 days until the cells reached a density of 2.0 at OD_{600}. These cells were then diluted to approximately a 1.0 OD_{600} and then aliquoted to five 1:10 serial dilutions and plated on $100mm^2$ plates.

Growth Curve

WT and mutants were inoculated into test tubes containing minimal media at a starting OD of 0.02. To minimize error, three replicates of each growth curve were made and the standard error of each time point was calculated using the statistical package in Microsoft XP. The OD was taken every two hours for 48 hrs. The control followed the procedure above but used synthetic complete media instead of minimal media.

RESULTS

Verification of the *Saccharomyces cerevisiae snz*1 and *sno*1 Deletion Mutants

Strain deletion was performed by using a PCR deletion strategy that allowed the *SNZ*1 and *SNO*1 genes to be replaced by a KanMX4 module which breaks down the antibiotic, kanamycin and geneticin (a kanamycin analog more toxic to yeast). The yeast knockouts were initially screened based on the fact that the deletion containing the KanMX4 module would confer resisitance to geneticin. A successful deletion results in the replacement of the sites where the B and C primers land in the wild type SNO or SNZ genes with sites where the KanB and KanC primers would land in the KanMX4 module. The A and D primers which are outside the reading frame of SNO and SNZ will remain. The wild type and knockout strains were verified by using the A/D, A/KanB, A/B, KanC/D, and C/D primers in a PCR reaction. For the knockout strains only the A/D, A/KanB and KanC/D PCR products were obtain which indicated that the gene was knocked out (Figure 4b and 4c). WT did not produce any PCR product with the KanB or KanC primers but did with the B and C primers (Figure 4a). Table 3 shows the expected size of the PCR products for each gene and primer combination. The appropriate size of the PCR products obtained confirmed that we had appropriate yeast deletion strains containing the *sno*1 deletion and the *snz*1 deletion.

Table 3. Expected Sizes of PCR product for verification by using the designated deletion primers

Strain	A-D	A-KanB	KanC-D	A-B_WT	C-D_WT
WT	1450 bp	_____	_____	805 bp	666 bp
ΔSNZ1	2140 bp	592 bp	890 bp	_____	_____
ΔSNO1	2182 bp	655 bp	869 bp	_____	_____

The Phenotype of Yeast *sno*1 and *snz*1 Deletion Mutants

Yeast cells are potentially a model system for understanding the eukaryotic pathway for pyridoxine biosynthesis. It has been described that the yeast *SNZ*1 and *SNO*1 are required for growth in media lacking vitamin B_6 (Rodriguez- Navarro et al., 2002). However this phenotype is difficult to demonstrate. Thus, in order to use yeast as a model system to functionally characterize the *Arabidopsis SNZ*1 and *SNO*1 homologues, it was necessary to verify that yeast *sno*1 and *snz*1 mutants cannot grow on media lacking pyridoxine. Yeast deletion mutants and wild type cells were cultivated overnight in liquid SC media without pyridoxine and subsequently plated on Petri plates on the same SC minimal media minus pyridoxine. The cells were washed 2X to remove traces of pyridoxine pre-cultured media and diluted to an OD_{600} of 0.2. The cells were then plated in a set of serial dilutions of this titer, and the results of this experiment are shown in Figure 4a.

From this experiment, it was apparent that both *sno*1 and *snz*1 deletion mutants were not able to grow as efficiently as wild type on minimal media lacking supplied pyridoxine. The wild type had growth even at the last 10^{-4} dilution. There was no growth of the Δ*snz*1 after the 10^{-2} dilution while Δ*sno*1 growth was lost at the 10^{-3} dilution. These results confirm that the Δ*snz*1 and Δ*sno*1 knockout are sensitive to the lack of pyridoxine and more interestingly, that Δ*snz*1 has a slightly more drastic phenotype than the Δ*sno*1 on minimal media (Fig. 5a).

When WT and the 2 deletion mutant strains were plated on SC medium at the same serial dilutions as described, both of the mutants as well as the WT grew comparably on SC medium containing pyridoxine (Fig. 5b). This demonstrates that the growth defect in the mutants observed on minimal medium lacking pyridoxine is only related to the absence of pyridoxine in the medium.

To further substantiate the phenotype of Δ*snz*1 and Δ*sno*1, quantitative measurements of the growth of Δ*snz*1 and Δ*sno*1 in liquid media with and without pyridoxine were made. The growth curve was carried out for 48 hrs in minimal media without pyridoxine. Our results show that after 48 hrs there was a significant difference of growth between WT and the Δ*snz*1 and Δ*sno*1 mutant (Fig. 6). There was a slight difference of growth between WT and the *SNO1* mutant. This growth curve analysis is consistent with the observation that mutants are not able to grow as efficiently as WT on media lacking pyridoxine. This phenotype further substantiates that these genes do play a role in the synthesis of pyridoxine.

The original discovery of the *SOR*1 gene has led to the subsequent finding that the pyridoxine and its metabolites have antioxidant properties. Methylene blue, under visible

light, produces toxic singlet oxygen which can be damaging to the cellular components, leading to apoptosis. Padilla et al. reported that out of all the three *SNZ/SNO* homologues of yeast, only *SNZ1* and *SNO1* were sensitive to methylene blue in the light (Padilla et al., 1998). To further analyze the defects of the yeast Δ*snz*1 and Δ*sno*1 deletions, we subjected the cells to methylene blue-induced singlet oxygen stress. Figure 6 indicates, that the Δ*snz*1 and Δ*sno*1 deletion mutants are more sensitive to methylene blue than wild type. Again, we were able to show Δ*snz*1 mutant was more sensitive than Δ*sno*1 (Figure 7).

Heterologous Expression of the Arabidopsis putative *SNZ1* gene

Now that the phenotypic characteristics of yeast *SNZ1* and *SNO1* genes and their deletion mutants have been determined, it is possible to use these mutant strains to examine the phenotype of the strains expressing the homologs of SNO and SNZ from the plant *Arabidopsis thaliana*. This will permit the identification of functional homologs of these genes from this plant. To begin these experiments a bioinformatic analysis was done using the WU-BLAST2 search utility at The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/). The results of these BLAST searches are given in Table 4. Note that there are 3 genes in the *Arabidopsis* genome that code for proteins that show greater than 60% similarity to the yeast SNZ1p amino acid sequence. The alignment of these sequences with the sequence of yeast SNZ1p is shown in Figure 8. Note that while there is significant divergence of the *Arabidopsis* and yeast sequences at both the amino-terminal and carboxy-terminal regions, there is substantial identity of the sequences in the

middle regions of the protein. There is only one gene that codes for a protein with greater than 50% similarity to the yeast SNO1p amino acid sequence.

Table 4. Results of BLAST searches using the yeast SNO1p and SNZ1p amino acid sequences to detect homologs of those genes in the genome of *Arabidopsis thaliana*.

Gene	**(Bit score) E-value**	**Length**	**Identity**	**Similarity**
***Arabidopsis* homologs to Yeast *SNZ*1 (NP_013814)**				
At5g01410	68418.m00054 stress-responsive protein, putative similar to ethylene-inducible protein HEVER [Hevea brasiliensis] SWISS-PROT:Q39963			
	(851) 3.9e-86	310 aa	57% (165/284)	74% (212/285)
At2g38230 (NP_181358.1)	68415.m04695 stress-responsive protein, putative similar to ethylene-inducible protein HEVER [Hevea brasiliensis] SWISS-PROT:Q39963; contains Pfam domain, PF01680: SOR/SNZ family			
	(830) 6.5e-84	310 aa	58% (162/276)	75% (209/276)
At3g16050 (NP_188226.1)	68416.m02029 stress-responsive protein, putative similar to ethylene-inducible protein HEVER [Hevea brasiliensis] SWISS-PROT:Q39963; contains Pfam domain, PF01680: SOR/SNZ family			
	(632) 6.2e-63	315 aa	43% (121/276)	64% (183/276)
***Arabidopsis* homologs to Yeast *SNO*1 (NP_013813)**				
	(238) 3.5e-21	256 aa	38% (66/172)	54% (93/172)

Having found sequences for genes from *Arabidopsis* that are putatively functional homologs of both yeast SNZ1 and SNO1, clones of these genes were obtained. One of the putative *Arabidopsis SNZ1* genes was functionally characterized and used for the functional characterization in the yeast system. It was demonstrated that the *Arabidopsis* gene functionally complements the yeast Δ*sno*1 deletion mutant. Our focus was on the *SNZ1* because our previous research has provided strong evidence that *SNZ1* has the stronger phenotype and *Arabidopsis* contains multiple genes that are putatively SNZ-homologs. We were able to engineer the At5g01410, putative *Arabidopsis SNZ1* gene, into a p426 GPD, which is a multicopy expression vector with a strong yeast promoter.

This plasmid was then transformed into its perspective knockout mutant. The presence of the glyceraldehyde 3 –phosphate dehydrogenase (GPD) promoter in the vector, allowed for the plant gene to be constitutively expressed in yeast. Our results indicate that the *AtSNZ1* gene functionally complements the yeast *SNZ1* knockout phenotype in minimal media. When we plated WT, Δ*SNZ1* and the Δ*SNZ1: AtSNZ1* on minimal media, it was clear that cells complemented with the *AtSNZ1* grew comparably to WT (Fig 9), while the mutant showed inhibition of growth at an earlier dilution. This is the first direct experimental evidence that indicates that the putative *AtSNZ1* gene is actually involved in pyridoxine biosynthesis.

DISCUSSION

Vitamin B_6 and its intermediates are important coenzymes in many enzymatic reactions. The implication of vitamin B_6 importance in many aspects of nature has led scientists to research the synthesis of the vitamin. Bioinformatics studies have revealed that most organisms use one of the following two pyridoxine biosynthetic pathways that have been discovered to date, i.e. the *E. coli* pathway or eukaryotic pathway. In addition, ^{15}N –labeling has shown that glutamine was incorporated into pyridoxine ring of yeast unlike *E. coli* which uses glutamate, confirming that two different pathways exist (Tazuya et al., 1995). The *E. coli* pathway has been well studied, and how pyridoxine is formed in this bacterium is well understood. However, more research needs to be conducted in order to grasp the full picture of pyridoxine biosynthesis in eukaryotes. We do know that the eukaryotic pathway contains a conserved *PDX*1 (*SOR*I/*SNZ*1) gene family that has been proven to be important for vitamin B_6 production.

SOR 1, originally known for protecting the fungus *Cercospora nicotianae* from singlet oxygen production, mutants were unable to grow unless they were supplied with pyridoxine. It was the study of *SOR 1* gene in *Cercospora* that has uncovered a novel role of dual function in which pyridoxine was involved in antioxidant properties as well as pyridoxine biosynthesis. Genomic sequencing has exposed the fact that *SOR 1*is a gene that has homologues in many organisms and is distributed widely in nature, but the function of this gene has not been extensively characterized. We now know that *SOR*I/*PDX*1 genes are orthologues to a larger family of genes that are commonly referred to as *SNZ* genes.

SNZ (Snooze) gene family was found in the yeast, *Saccharomyces cerevisiae,* and its members consist of three unlinked *PDX1* homologues, *SNZ1*, *SNZ2* and *SNZ3*. In addition, there is an *SNO* (Snz- proximal reading frame) family with each member adjacent to the open reading frame of one *SNZ* gene and transcribes in opposite directions (Fig.2). This family consists of three homologues to the *PDX2* gene, *SNO1*, *SNO2*, and *SNO3*. As mentioned previously, little is known about the function of *SNZ/SNO,* but a few reports have given some insight to the function of the gene families. It has been shown that both *SNZ1/SNO1* increases in synthesis during stationary phase (Braun et al., 1996). Furthermore, it was shown that only the *SNZ1/SNO1* homologues were sensitive to methylene blue (Padilla et al., 1998). Lastly, functional analysis showed that both *SNZ1* and *SNO1* were required for growth in media lacking vitamin B_6, and *SNZ2*/*SNO2*, *SNZ3*/*SNO3* genes pairs seemed to be involved in vitamin B_1 (thiamin) biosynthesis (Rodriguez-Navarro et al., 2002).

Our results have confirmed that both the *SNZ1* and *SNO1* in yeast are involved in the vitamin B_6 biosynthesis. We were able to go back and repeat some of the experiments in the literature that were done on *SNZ1* and *SNO1* mutants. Our experiment with vitamin B_6 less media (minimal media) clearly indicated that both *SNZ1* and *SNO1* were needed for growth in media lacking pyridoxine. We became more convinced of this phenotype when we quantitatively assessed the growth curve between WT and the mutants in minimal media; where *SNZ1* and *SNO1* grew less than the WT strain. Additionally, we were able to show that *SNZ1* and *SNO1* are sensitive to growth on methylene blue plates. Thus, it is very likely that pyridoxine functions as an antioxidant against singlet oxygen production in yeast. In all of our experiments, it was clear that

SNZ1 mutant had a more pronounced phenotype that *SNO1*. There is strong evidence that suggest that *SNO1* is a glutaminase possibly supplying the nitrogen atom for the pyridoxine ring when it is in a complex with *SNZ1* (Dong et al., 2004). However, it is not clearly defined as to what role *SNZ1* plays in the formation of pyridoxine but we believe that this stronger phenotype may be due to the fact that *SNZ1* may function in one of the early steps in the formation of the pyridoxine ring. More research needs to be done in order to characterize the function of *SNZ1*.

Our goal in this investigation was to characterize the function of the putative *Arabidopsis thaliana SNZ1* and *SNO1* genes. Since *SNZ1* is the gene that is more important in pyridoxine biosynthesis we decided to focus on the just the *AtSNZ1* gene. Our complementation of the *AtSNZ*1 gene on minimal media strongly suggests that the *AtSNZ1* may play a role in the formation of pyridoxine as its homologous counterparts. We attempted to demonstrate that the *AtSNZ1* construct complemented the methylene blue sensitive phenotype of the yeast Δ*snz*1 mutant, but were unable to demonstrate this (data not shown). This may be the result of the fact that the *AtSNZ1* gene does not fully complement the yeast mutant phenotype. This could be related to expression levels in yeast, or possible targeting sequences that wrongly target some or all of the AtSNZ protein. Additionally, in our hands the methylene blue phenotype is not very strong, and thus it was not clear that there is actually a strong methylene blue phenotype in the mutants.

In literature, there have not been many reports involving the production of vitamin B_6 in plants. This is surprising since humans obtain most of our dietary vitamin B_6 source from plants. It has been shown that vitamin B_6 has potential to the aid humans

in diseases such as eczema and psoriasis. The study of the vitamin B_6 genes in *Arabidopsis* could be the first step in characterizing the vitamin B_6 genes throughout the plant kingdom. Thus, identifying genes in pyridoxal metabolism may possibly help obtain a greater yield and nutritional quality in plants. Therefore, a goal in our lab is to designed more experiments in order to decipher the exact function of the *AtSNZ1* genes in *Arabidopsis thaliana*.

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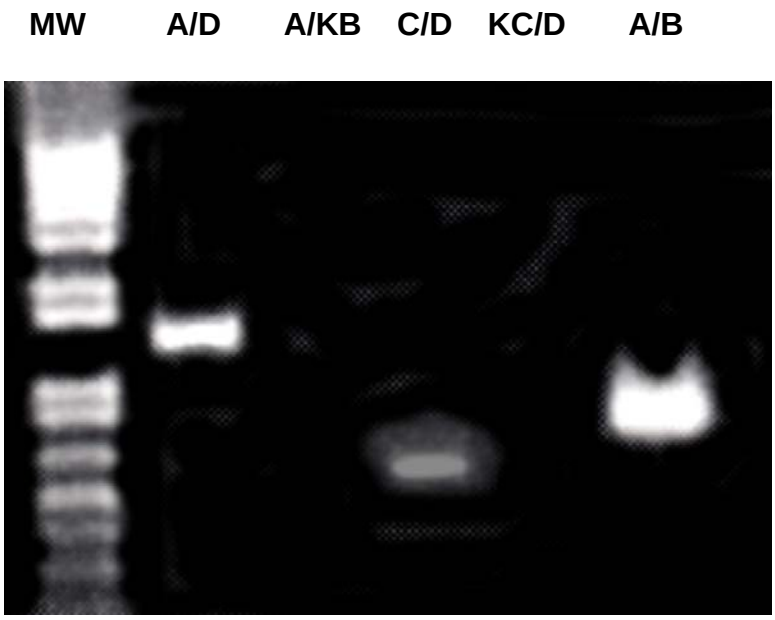


Figure 4a. WT verification PCR products obtained with *Saccharomyces cerevisiae* strain BY4742 (WT) the molecular weight markers (MW) are shown along with the products of reactions using the primer pairs A/B, A/KB, AB, KC/D and CD. Note that only products were obtained with the WT primers.

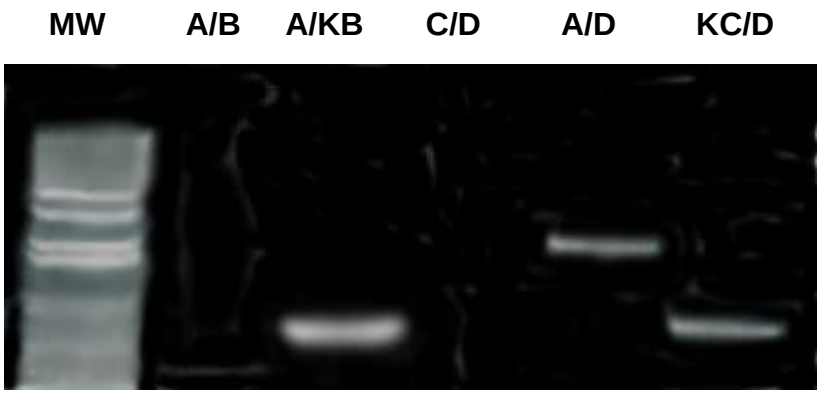


Figure 4b. SNZ1 mutant verification PCR products obtained with *Saccharomyces cerevisiae* strain BY4742 (Δ*snz*1) the molecular weight markers (MW) are shown along with the products of reactions using the primer pairs A/B, A/KB, AB, KC/D and CD. Note that besides the A/D primer pair, no products were obtained with the WT primers.

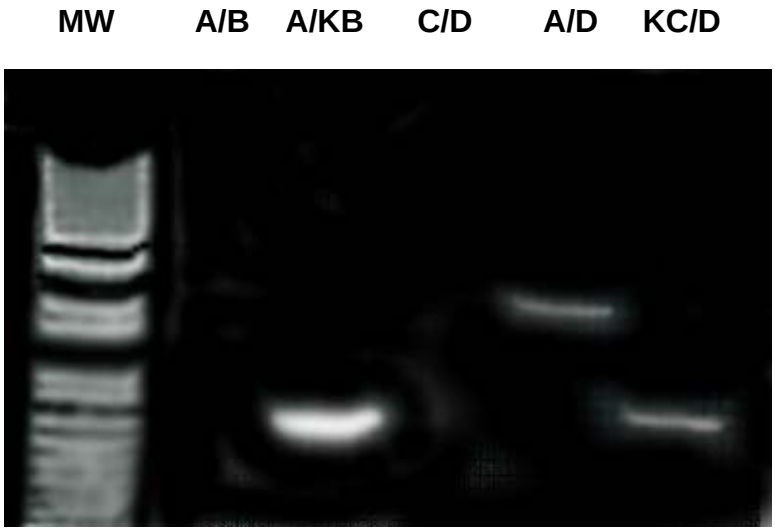


Figure 4c. SNO1 mutant verification PCR products obtained with *Saccharomyces cerevisiae* strain BY4742 (Δ*sno*1) the molecular weight markers (MW) are shown along with the products of reactions using the primer pairs A/B, A/KB, AB, KC/D and CD. Note that besides the A/D primer pair, no products were obtained with the WT primers.

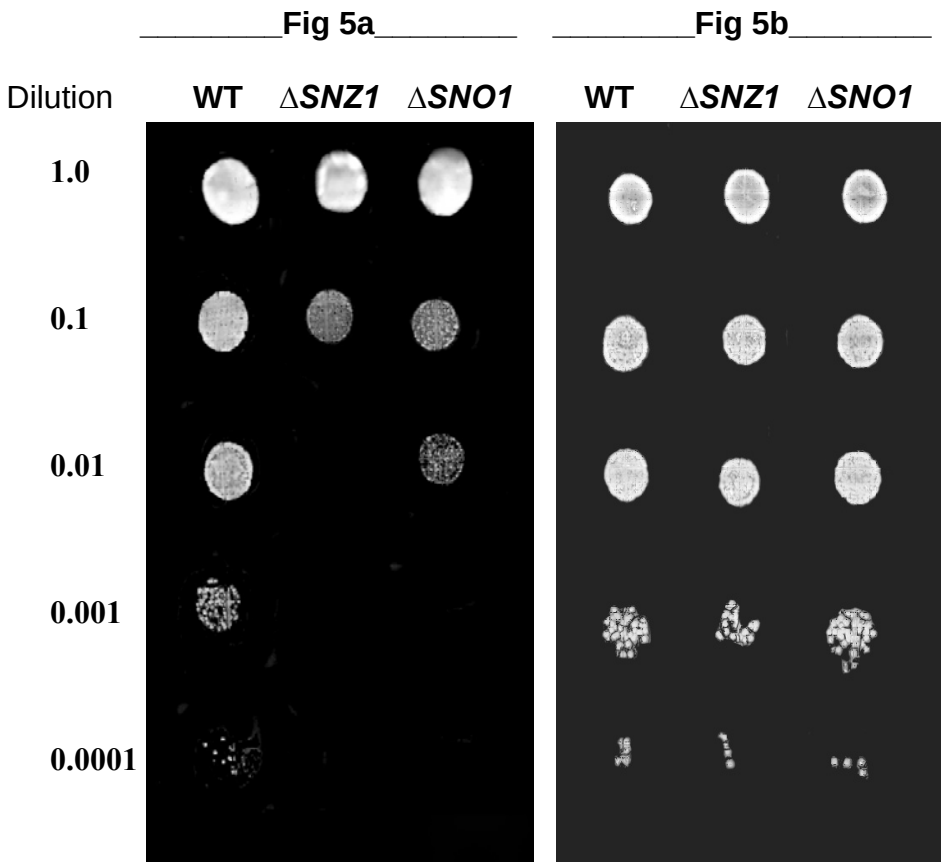


Figure 5a & b. Minimal Media Phenotypic Experiments The growth of wild type (BY4742), BY4742:: Δ*SNZ*1*,* and BY4742:: ΔSNO1 was determined by plating a culture of the appropriate yeast strain on Petri plates in either minimal medium (Figure 4a, see methods) or SC medium (Figure 4b, see methods) at varying dilutions (as indicated). The reduced ability of the 2 deletion mutant to grow on media lacking pyridoxine is shown in Figure 4A, while the lack of a growth defect on media containing pyridoxine is shown in Figure 4b.

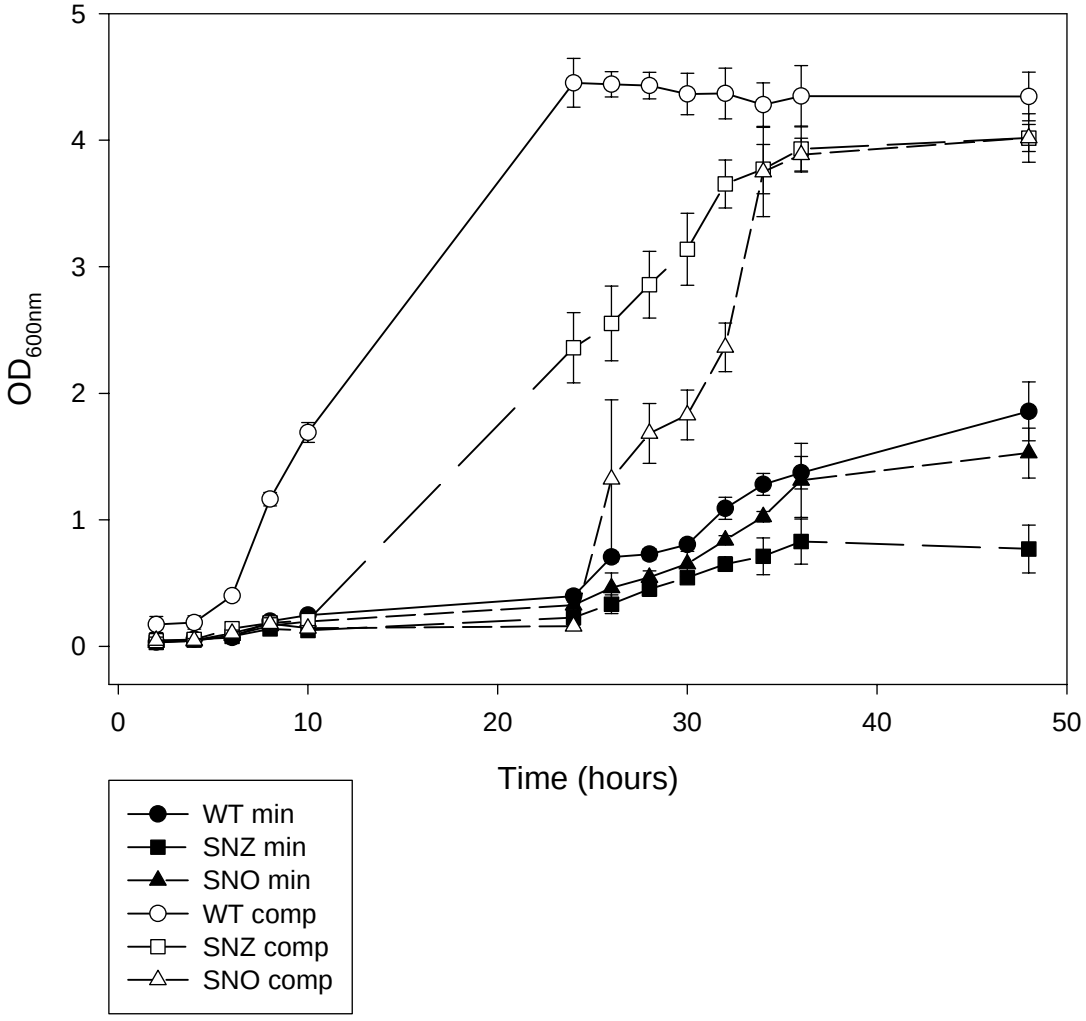


Figure 6. 48hr Growth Curve Analysis Forty eight hour growth curves of WT (BY4742) (diamond), BY4742 Δ*SNZ1* (square), and BY4742 Δ*SNO1* (triangle) on minimal (closed symbols) and synthetic complete media (open symbols). Growth of 3 replicate cultures of each treatment were estimated by determining the optical density of the culture at 600 nm at the time points indicated in the figure. Error bars shown represent the standard error of 3 replicate measurements at each time point.

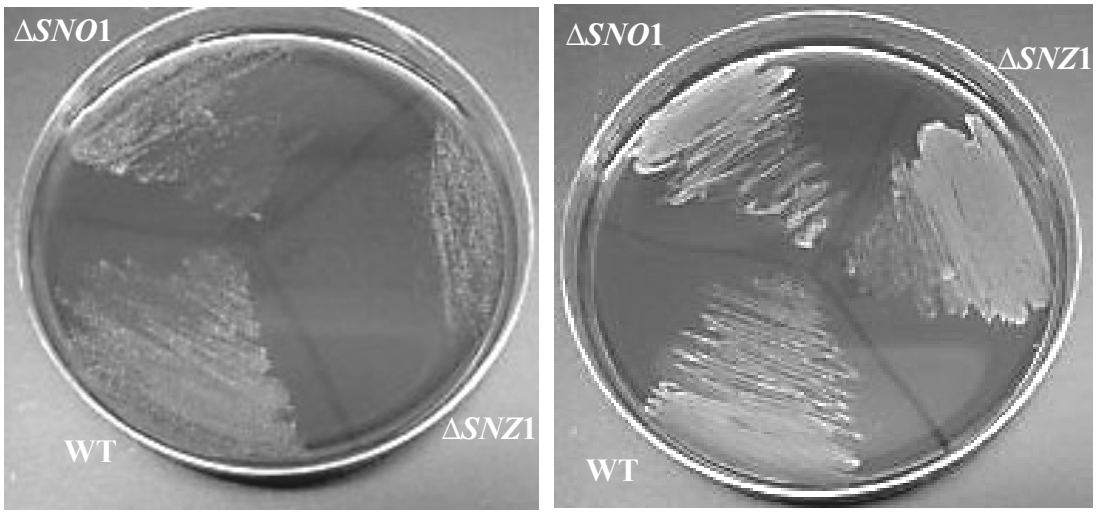


Figure 7. Methylene Blue Experiments WT, Δ*SNZ*1*, and* Δ*SNO*1 streaked on methylene blue plates. Incubation of the plate in the light leads (left plate) to the production of singlet oxygen which reduces the growth of the *snz*1 deletion mutant, but not the *sno*1 deletion mutant compared to wild type. The plate on the right was incubated in the dark, where no singlet oxygen is produced.

```
CLUSTAL W (1.82) multiple sequence alignment


At5g01410       -----MEG--TGVVAVYGNGAITEAK-KSPFSVKVGLAQMLRGGVIMDVVNAEQARIAEE 52
At2g38230       -----MAG--TGVVAVYGEGAMTETKQKSPFSVKVGLAQMLRGGVIMDVVNAEQARIAEE 53
At3g16050       MADQAMTDQDQGAVTLYSGTAITDAKKNHPFSVKVGLAQVLRGGAIVEVSSVNQAKLAES 60
SNZ1p           -----MTG-------------------EDFKIKSGLAQMLKGGVIMDVVTPEQAKIAEK 35
                     * .                      *.:* ****:*:**.*::* . :**::**.

At5g01410       AGACAVMALERVPADIRAQGGVARMSDPQMIKEIKQAVTIPVMAKARIGHFVEAQILEAI 112
At2g38230       AGACAVMALERVPADIRAQGGVARMSDPEMIKEIKNAVTIPVMAKARIGHFVEAQILEAI 113
At3g16050       AGACSVIVSD----PVRSRGGVRRMPDPVLIKEVKRAVSVPVMARARVGHFVEAQILESL 116
SNZ1p           SGACAVMALESIPADMRKSGKVCRMSDPKMIKDIMNSVSIPVMAKVRIGHFVEAQIIEAL 95
                :***:*:. :     :*  * * **.** :**:: .:*::****:.*:********:*::

At5g01410       GIDYIDESEVLTLADEDHHINKHNFRIPFVCGCRNLGEALRRIREGAAMIRTKG-EAGTG 171
At2g38230       GVDYVDESEVLTLADEDNHINKHNFKIPFVCGCRNLGEALRRIREGAAMIRTKG-EAGTG 172
At3g16050       AVDYIDESEIISVADDDHFINKHNFRSPFICGCRDTGEALRRIREGAAMIRIQGDLTATG 176
SNZ1p           EVDYIDESEVLTPADWTHHIEKDKFKVPFVCGAKDLGEALRRINEGAAMIRTKG-EAGTG 154
                 :**:****::: **  :.*:*.:*: **:**.:: *******.******* :*  :.**

At5g01410       NIIEAVRHVRSVNGDIRVLRNMD-DDEVFTFAKKLAAPYDLVMQTKQLGRLPVVQFAAGG 230
At2g38230       NVVEAVRHVRSVNGAIRLLRSMD-DDEVFTYAKKIAAPYDLVVQTKELGRLPVVQFAAGG 231
At3g16050       NIAETVKNVRSLMGEVRVLNNMD-DDEVFTFAKKISAPYDLVAQTKQMGRVPVVQFASGG 235
SNZ1p           DVSEAVKHIRRITEEIKACQQLKSEDDIAKVAEEMRVPVSLLKDVLEKGKLPVVNFAAGG 214
                :: *:*:::* :   ::  ..:. :*:: . *::: .* .*: :. : *::***:**:**

At5g01410       VATPADAALMMQLGCDGVFVGSGIFKSGDPARRARAIVQAVTHYSDPEMLVEVSCGLGEA 290
At2g38230       VATPADAALMMQLGCDGVFVGSGVFKSGDPVKRAKAIVQAVTNYRDAAVLAEVSCGLGEA 291
At3g16050       ITTPADAALMMQLGCDGVFVGSEVFDGPDPFKKLRSIVQAVQHYNDPHVLAEMSSGLENA 295
SNZ1p           VATPADAALLMQLGCDGVFVGSGIFKSSNPVRLATAVVEATTHFDNPSKLLEVSSDLGEL 274
                ::*******:************ :*.. :* :   ::*:*. :: :.  * *:*..* :

At5g01410       MVGINLNDEKVERFANRSE---- 309
At2g38230       MVGLNLDD-KVERFASRSE---- 309
At3g16050       MESLNVRGDRIQDFGQGSV---- 314
SNZ1p           MGGVSIESISHASNGVRLSEIGW 297
                * .:.: .      .
```

Figure 8. Sequence Alignments The amino acid sequences for yeast SNZ1p and the proteins coded for by Arabidopsis genes At5g01410, At2g38230, and At3g60650 were obtained from the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov/) and used to conduct an alignment using the CLUSTAL-W program online at EMBL (http://www.ebi.ac.uk/clustalw/) the alignment is shown above. An (*) under a column indicates an exact match of sequence, while (:) or (.) show stronger and weaker similarity of amino acids.

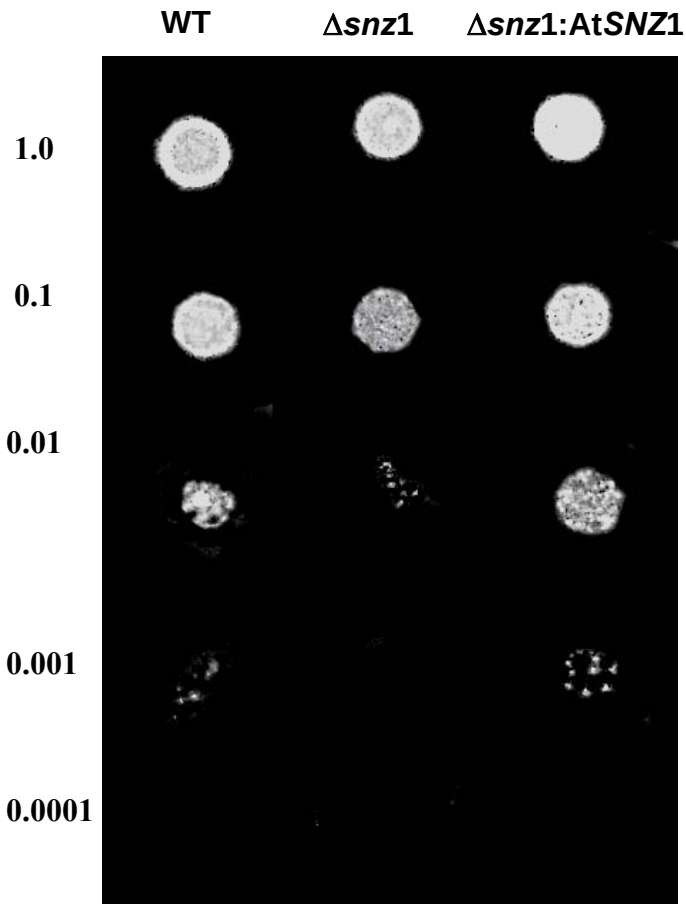
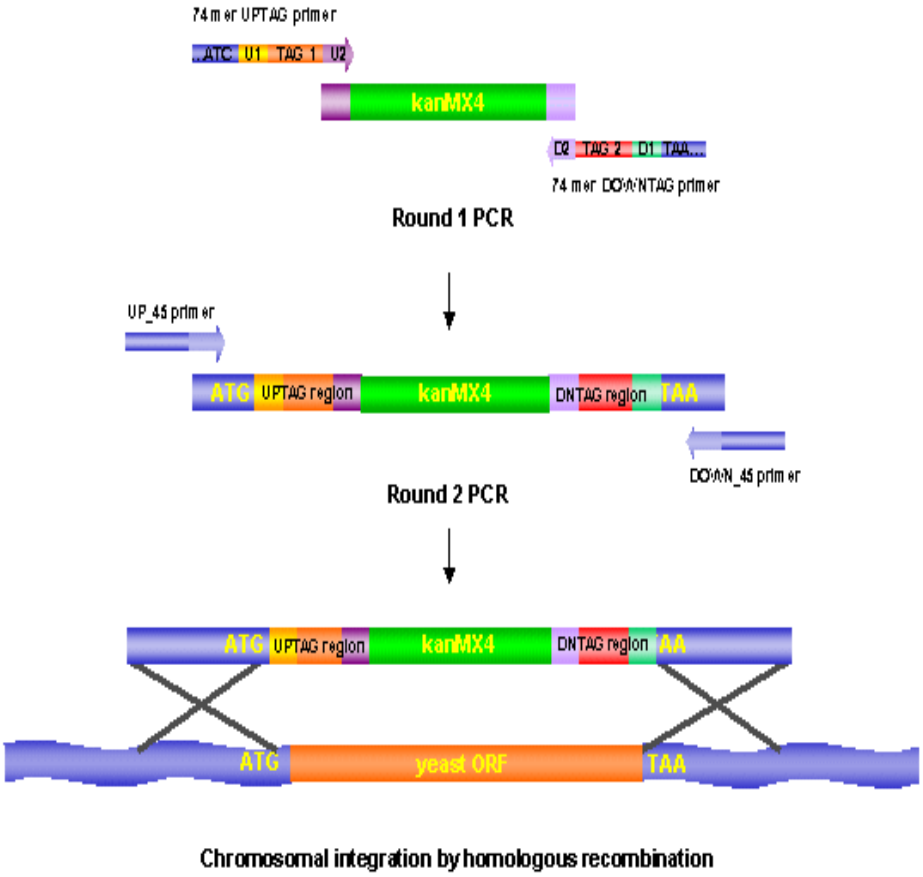


Figure 9. ***Arabidopsis SNZ1*** **complementation** Growth of Wild type (WT), the *snz*1 deletion mutant (Δ*snz*1), and the *snz*1 deletion mutant containing the putative *Arabidopsis SNZ*1 homolog transformed into the mutant on p426 shuttle vector driven by the yeast GPD promoter (Δ*snz*1: At*SNZ*1).

APPENDIX



Chromosomal integration by homologous recombination

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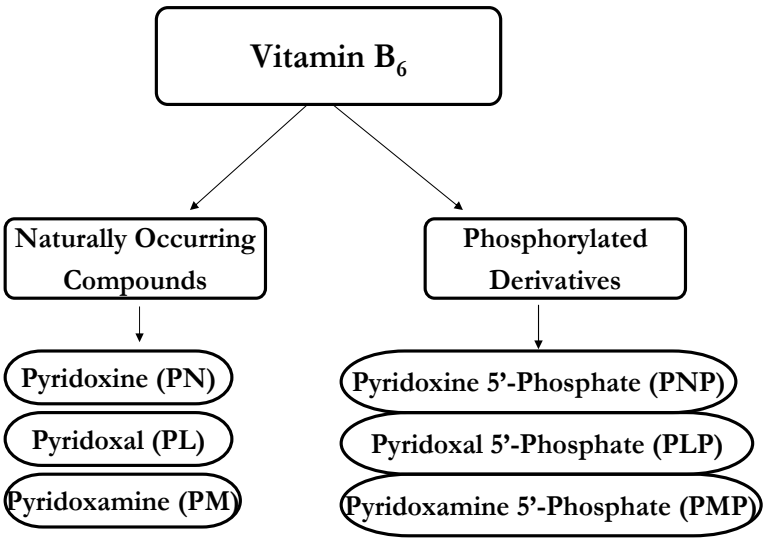
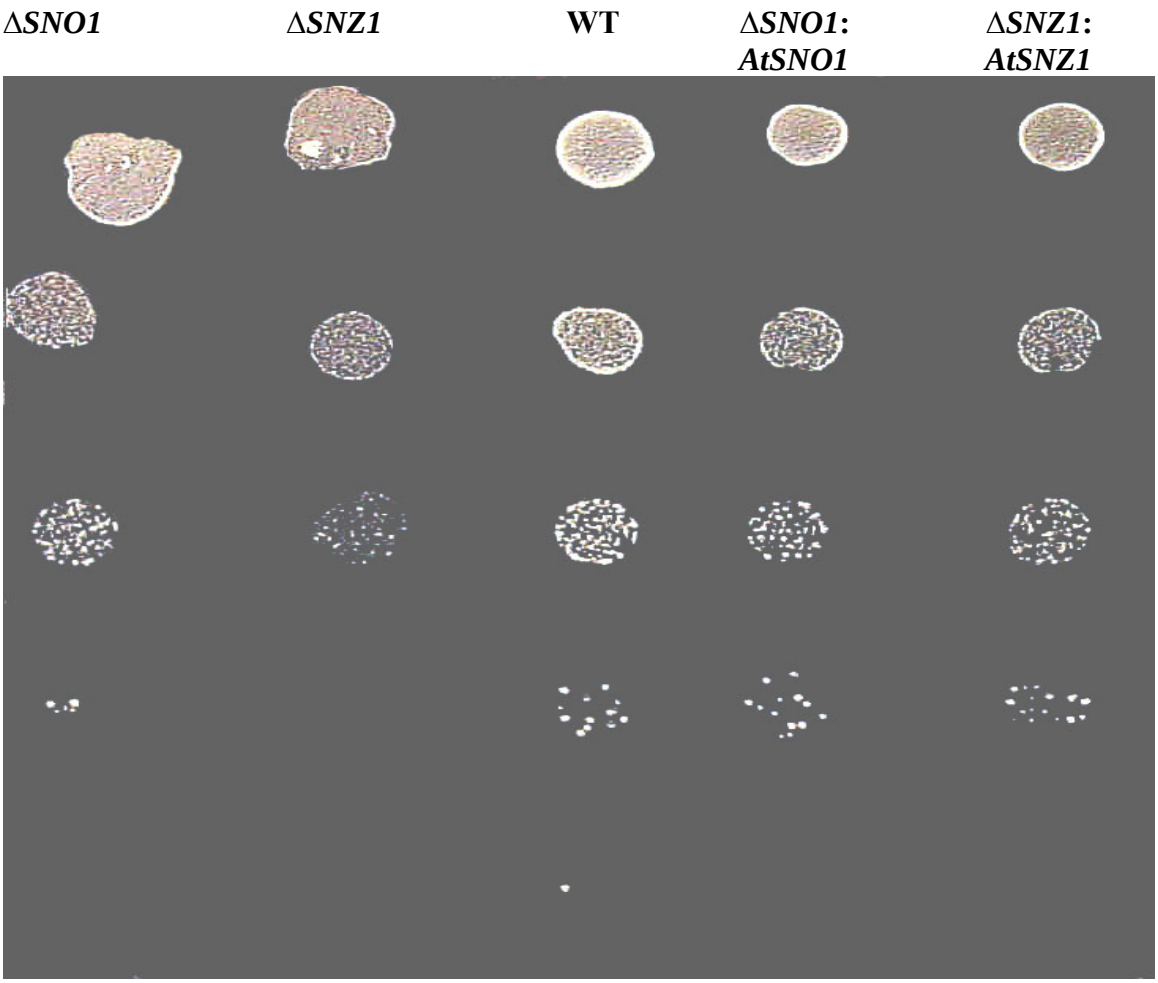
Appendix I. Deletion Strategy



Appendix II. Flowchart of the six B_6 vitamers

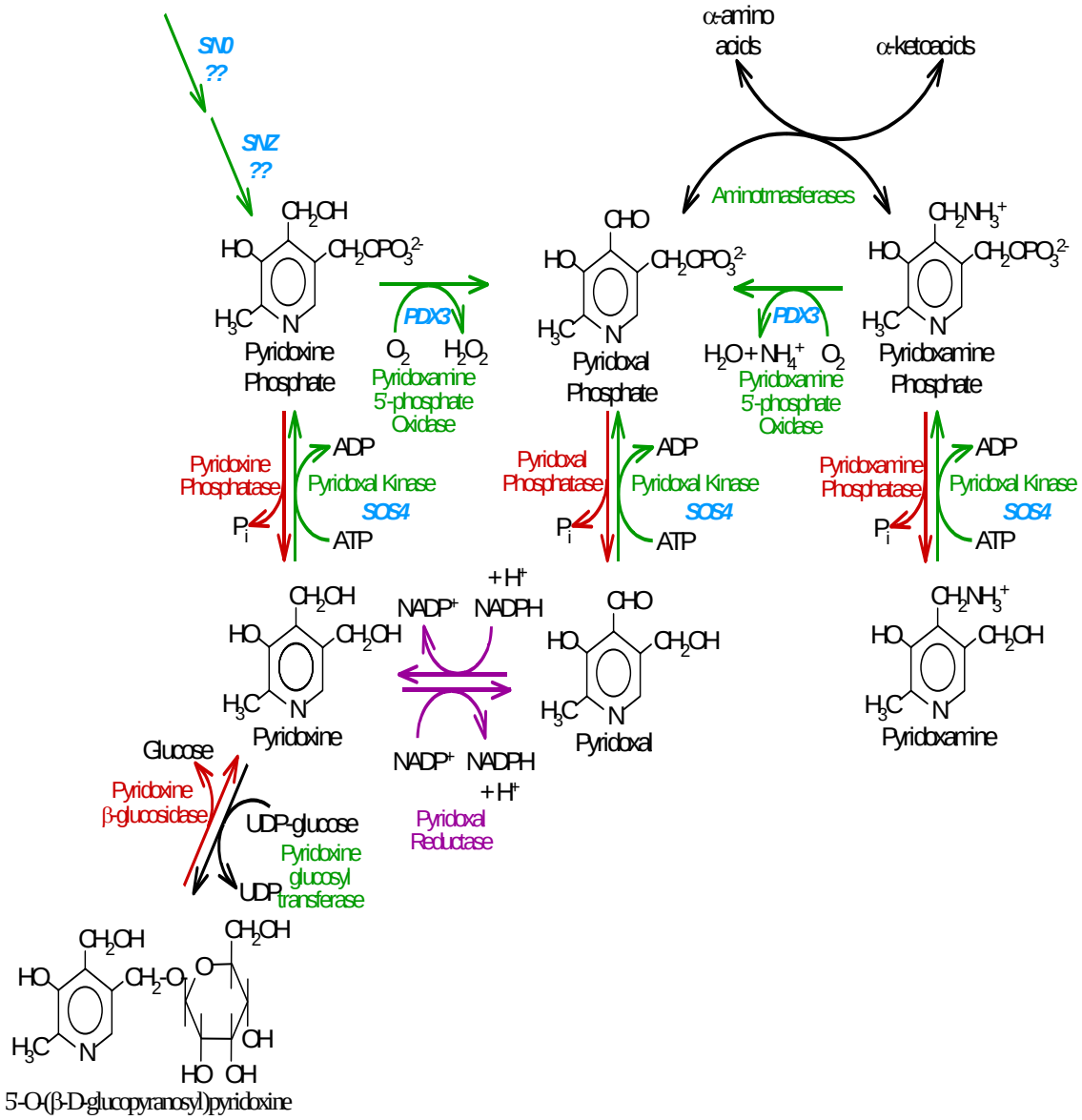
Studies	Pdx (Pyridoxine Requiring) Name	Experiments	Involved in Pyridoxine Biosynthesis
Cercospora nicotianae	*Sor* 1	Auxotrophic mutants; antioxidant properties	Yes
Aspergillus nidulans	*Pyro* A	Auxotrophic mutants; methylene blue	Yes
Neurospora crassa	*Pdx*1/ *Pdx*2	Genomic analysis; Auxotrophic mutants	Yes
Saccharomyces cerevisiae	*SNZ* 1, 2, 3 *SNO* 1,2, 3	Auxotrophic mutants; Methylene blue	Yes (copy 1)
Arabidopsis thaliana	Putative *SNZ*1; *SNO*1	N/A	?

Appendix III. Summary of Eukaryotic Pyridoxine Studies



Appendix 1V. *AtSNZ1* complementation

Pyridoxal Metabolism in Plants



Appendix V. Proposed pathway for the biosynthesis of pyridoxine in plants