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root uv-b sensitive Mutants Are Suppressed by Specific Mutations in ASPARTATE AMINOTRANSFERASE2 and by Exogenous Vitamin B6

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ABSTRACT Vitamin B6 (vitB6) serves as an essential cofactor for more than 140 enzymes. Pyridoxal 5′-phosphate (PLP), active cofactor form of vitB6, can be photolytically destroyed by trace amounts of ultraviolet-B (UV-B). How sun-exposed organisms cope with PLP photosensitivity and modulate vitB6 homeostasis is currently unknown. We previously reported on two *Arabidopsis* mutants, *rus1* and *rus2*, that are hypersensitive to trace amounts of UV-B light. We performed mutagenesis screens for second-site suppressors of the *rus* mutant phenotype and identified mutations in the *ASPARTATE AMINOTRANSFERASE2* (*ASP2*) gene. *ASP2* encodes for cytosolic aspartate aminotransferase (AAT), a PLP-dependent enzyme that plays a key role in carbon and nitrogen metabolism. Genetic analyses have shown that specific amino acid substitutions in ASP2 override the phenotypes of *rus1* and *rus2* single mutants as well as *rus1 rus2* double mutant. These substitutions, all shown to reside at specific positions in the PLP-binding pocket, resulted in no PLP binding. Additional *asp2* mutants that abolish AAT enzymatic activity, but which alter amino acids outside of the PLP-binding pocket, fail to suppress the *rus* phenotype. Furthermore, exogenously adding vitB6 in growth media can rescue both *rus1* and *rus2*. Our data suggest that AAT plays a role in vitB6 homeostasis in *Arabidopsis*.

Key words: Vitamin B6; pyridoxal-phosphate; aspartate aminotransferase; ultraviolet light; photo protection.

INTRODUCTION

Sun-exposed organisms inevitably encounter some amount of ultraviolet-B (UV-B; 280–320-nm) radiation. High fluence (HF) UV-B light can harmfully react with numerous biological molecules including DNA, RNA, lipids, and proteins to give rise to various photoproducts that, if not properly repaired or replaced, can lead to the death of the organism (McKenzie et al., 2003; Caldwell et al., 2007). On the other hand, low fluence (LF) UV-B can provide beneficial effects including serving as developmental signals in plants and aiding vitamin D synthesis in humans (Ulm and Nagy, 2005; DeLuca, 2009). How cells detect DNA photoproducts such as cyclobutane pyrimidine dimers (CPDs) and initiate nucleotide excisions repair (NER) processes have been well studied (Wittschieben et al., 2005; Scrima et al., 2008). Furthermore, the molecular and biochemical mechanisms of how cells, upon exposure to UV-B, adopt survival strategies by mounting stress responses and building up UV-absorbing compounds have also been extensively analyzed (Morrison and Long, 1958; Schaltenbrand et al., 1987; Suesslin and Frohnmeyer, 2003; Marrot et al., 2005). The amount of UV-B that an organism is exposed to can vary greatly, depending on the position of the sun in the sky and the atmospheric conditions. The full range of UV-B effects on plants and on many of the UV-B-sensitive molecules is currently not completely understood.

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We previously identified in two homologous genes, *ROOT UV-B SENSITIVE1* (*RUS1*) and *ROOT UV-B SENSITIVE2* (*RUS2*), mutations that cause a severe developmental arrest after germination (Tong et al., 2008; Leasure et al., 2009). The *rus1* and *rus2* phenotypes are greatly alleviated when light irradiance incident upon the roots is reduced (Tong et al., 2008; Leasure et al., 2009). Under our conditions, wavelengths in the UV-B range had the strongest effect on the mutant phenotype. *RUS2* was also recently identified as the gene mutated in *weak auxin response1* (*wxr1*), suggesting a role for auxin in the *rus* phenotype (Ge et al., 2010). *wxr1* had reduced polar auxin transport from the shoot to root tissues, and reduced PIN auxin transporters in the root (Ge et al., 2010). It remains to be seen whether reduced auxin transport is the root cause of the *rus* phenotype or whether it is the means by which some pathway affects the arrest to development after germination.

The RUS proteins all contain a conserved Domain of Unknown Function 647 (DUF647) and exist in all plant, most animal, and some fungal genomes that are sequenced to date. *Arabidopsis*, rice (*Oryza sativa*), and moss (*Physcomitrella patens*) all contain six RUS proteins, and the majority of currently sequenced animal genomes contain but a single *RUS* gene, which sequence-based comparisons suggest to be the ortholog of RUS3 in plants (Leasure et al., 2009). The *rus1* and *rus2* mutations genetically interact, as *rus1* and *rus2* plants have virtually identical phenotypes, and the *rus1 rus2* double mutant phenotype is no more severe than either of the single mutants (Leasure et al., 2009). Additionally, the RUS1 and RUS2 proteins interacted in a yeast-two-hybrid analysis, suggesting that they physically interact with each other *in vivo*.

One biologically important molecule that is extremely UV-B-sensitive is the B vitamin pyridoxal-5′-phosphate (PLP; active enzymatic form of vitamin B6; Morrison and Long, 1958). PLP is irreversibly destroyed by UV-B light, even under illumination by low-ultraviolet fluorescent lamps (Schaltenbrand et al., 1987). PLP is used as a cofactor for a large number of essential enzymes that catalyze more than 140 distinct enzymatic reactions and belong to five of the six defined large enzyme classes (Percudani and Peracchi, 2003). Studies have suggested that vitamin B6 (vitB6) vitamers are also potent antioxidants that can effectively quench reactive oxygen species (Bilski et al., 2000; Denslow et al., 2007; Mooney et al., 2009). The maintenance of PLP homeostasis is thus critically important to cellular well-being. Chronic exposure to ambient LF UV-B can be problematic to cells because of the loss of PLP to photolysis and the potential accumulation of PLP photoproducts. How cells detect PLP photolysis and maintain overall vitB6 homeostasis is currently not known.

A major class of enzymes in all organisms is the aminotransferases. Aminotransferase enzymes depend on PLP as a required cofactor and catalyze the amination of a keto acid, using an amino acid as an amino-group donor. In plants, the amino acids aspartate, asparagine, glutamate, and glutamine are typically the most abundant, with asparagine and glutamine usually used as ammonia carriers in the xylem and phloem (Lam et al., 1994). The enzyme Aspartate Aminotransferase (AAT; E.C. 2.6.1.1) is centrally positioned in the conversion of asparagine to glutamine at the middle step where aspartate is converted to glutamate, or *vice versa*. AAT enzymes are highly conserved in sequence identity and similarity between disparate species. Mutations in the *ASPARTATE AMINOTRANSFERASE2* (*ASP2*) gene of *Arabidopsis* were previously identified in mutant plants that lacked the AAT2 enzymatic activity in native gel assays (Schultz et al., 1998). Loss-of-function mutations had only minimal phenotypic consequences for the plant as a whole, suggesting that redundancy or metabolic flexibility exists for the AAT function in *Arabidopsis* (Schultz et al., 1998; Miesak and Coruzzi, 2002). Here, we report that specific *asp2* mutations in *Arabidopsis* can partially suppress the *rus* mutant phenotype. Additionally, the exogenous supplementation of vitB6 vitamers can also partially suppress the *rus* phenotype. Our results support a role for RUS1, RUS2, and ASP2 in vitB6 photoprotection and homeostasis.

RESULTS

A Screen for *rus* Mutant Suppressors Yields Mutations in *ASPARTATE AMINOTRANSFERASE2* (*ASP2*)

To better understand the RUS UV-B response pathway in *Arabidopsis*, we performed three separate suppressor screens using ethyl methane sulfonate (EMS) mutagenesis on *rus1-1*, *rus1-2*, and *rus2-1* seeds. Suppressors were defined as plants having elongated roots and increased leaf production, as compared to *rus* mutants (Figure 1A). We initially identified over 75 suppressor lines from our three independent screens, which mapped to five loci (see Supplemental Figure 1). One recessive suppressor in the *rus1-2* background was mapped using traditional map-based cloning methods (Tong et al., 2008). Broadly spaced markers placed this mutation on chromosome five and additional fine mapping and sequencing revealed a missense mutation in the *ASPARTATE AMINOTRANSFERASE2* (*ASP2*; *At5g19550*) gene (see Supplemental Figure 2). The *ASP2* gene encodes for a cytosolically targeted Aspartate Aminotransferase (AAT) enzyme (Schultz and Coruzzi, 1995; Schultz et al., 1998; Wilkie and Warren, 1998).

Three additional *rus* suppressors were mapped to the *ASP2* locus. Sequencing revealed missense mutations in all of them in the *ASP2* gene in the region that encodes for the active site of the enzyme (Figure 2A). At least one *asp2* mutation that suppresses the *rus* phenotype was found in each of our three screens. We named these mutations *asp2-11* through *asp2-14*, as mutant alleles of *ASP2* have previously been reported (Schultz et al., 1998). *asp2-12* is the allele that we used initially to map with. *asp2-11*, *asp2-12*, and *asp2-13* are recessive suppressors and *asp2-14* behaves as a dominant suppressor based on the segregation of suppression after backcrossing to wild-type (WT) (see Supplemental Figure 2). One striking phenotype for both *rus1*

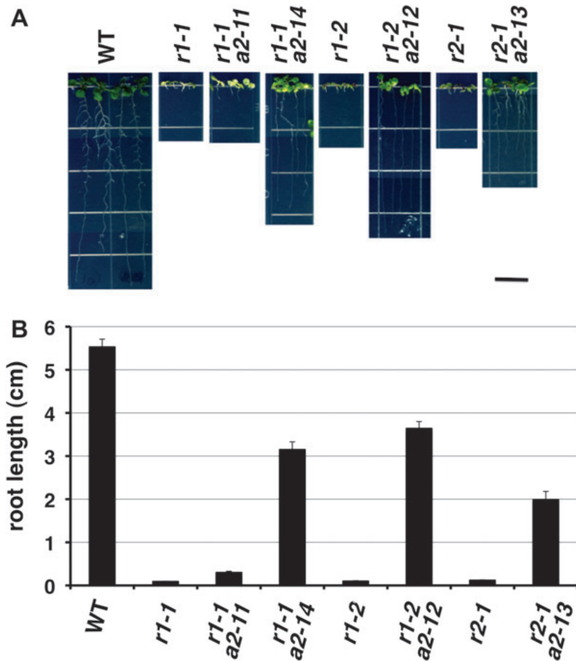

Figure 1. Identification of *rus* Suppressors.

(A) Phenotypes of 10-day-old *rus* and *rus* suppressor seedlings. WT, wild-type; *r1*, *rus1*; *r2*, *rus2*; *a2*, *asp2*. Four suppressor alleles (*asp2-11*, *asp2-12*, *asp2-13*, and *asp2-14*) that have specific mutations in the ASP2 gene have been identified. Bar = 1 cm.

(B) Root-length measurements of 10-day-old seedlings for WT, *rus* mutants (*r1-1*, *r1-2*, and *r2-1*), and the four suppressors (specific *asp2* mutations in either *rus1* or *rus2* background) (n = 9 or 10). Error bar = standard error.

and *rus2* mutants is their dramatically reduced root length in light (Tong et al., 2008; Leasure et al., 2009). As previously documented, the root lengths for *rus1-1*, *rus1-2*, and *rus2-1* are less than 4% of that for the WT (Figure 1B; Tong et al., 2008). The stunted root growth in these mutants was significantly recovered by specific asp2 mutations (Figure 1B).

We transformed *rus1-2 asp2-12* plants with constructs that express either the WT *Arabidopsis ASP2* cDNA or the orthologous Human *Glutamic-oxaloacetic Transaminase1* (*GOT1*; cytosolic AAT isoform) cDNA under the Cauliflower Mosaic Virus (CaMV) 35S promoter. Individual transformant lines for both of these constructs showed either a partial or a complete return of the *rus1-2* phenotype (Figure 2B). This result combined with the fact that we isolated four mutations in the same gene led us to conclude that the *asp2* mutations recovered in our screen are in fact responsible for the suppression of the *rus* phenotype.

asp2-12 Suppresses *rus1-2*, *rus2-1*, and the Double *rus1-2 rus2-1* Mutant

The moderately strong recessive allele *asp2-12* was recovered in the screen for *rus1-2* suppressors. We previously reported that the phenotype of *rus1-2 rus2-1* double mutants is indistinguishable from the phenotype of either single mutant alone (Leasure et al., 2009). We hypothesized that *asp2-12* can suppress the phenotypes of *rus2-1* and *rus1-2 rus2-1* double mutants. To test this, we crossed the *rus1-2 asp2-12* plants with *rus2-1*. In the F^2, we recovered homozygous *rus2-1 asp2-12* double mutants and *rus1-2 rus2-1 asp2-12* triple mutants. *asp2-12* was able to similarly suppress both the *rus2-1* phenotype and the *rus1-2 rus2-1* phenotype, supporting our hypothesis (Figure 2C and 2D). We also recovered *asp2-12* single mutants from this cross, which were indistinguishable from WT plants under our conditions (Figure 2C and 2D). This result supports our previous model whereby the loss of RUS1, RUS2, or both RUS1 and RUS2 eliminates the function of the RUS1/RUS2 complex completely.

asp2 Suppressors Lack Enzymatic Activity

All of the *asp2* suppressor mutations that we recovered in our screen create single amino acid changes in conserved residues near the active site of the ASP2 protein (Figure 2A). Therefore, we hypothesized that these mutations affect the enzymatic activity of the protein. To test the enzymatic activity of our suppressors, we utilized a native gel assay for AAT activity, which uses the dye Fast Blue BB that turns blue in the presence of the AAT reaction product oxaloacetate. This assay produces three bands on a non-denaturing acrylamide gel representing the AAT1, AAT2, and AAT3 activities, which correspond to the ASP1, ASP2, and ASP5 proteins in *Arabidopsis*, respectively (Miesak and Coruzzi, 2002). Analysis of 7-day-old plant protein extracts showed no AAT2 enzymatic activity in any of our suppressor mutants (Figure 3A). As a control, we used seedlings homozygous for the previously identified *asp2-2* allele, which was shown to lack enzymatic function (Miesak and Coruzzi, 2002) (Figure 3A). None of our asp2 suppressor mutants was able to convert aspartate to glutamate at a detectable level in this assay.

The activity of the AAT1 (encoded by *ASP1*; mitochondrial isoform) enzyme is similar in position to AAT2 on the gel, which appears faintly in all of the mutants that we tested (Figure 3A). To be certain that our mutant asp2 proteins were completely inactive, we performed a native gel activity assay on proteins purified in an *E. coli*-based Maltose Binding Protein (MBP)-fusion protein expression system. In addition to the WT ASP2 and our four suppressor mutant versions (asp2-11 through asp2-14), we purified asp2-2 and an asp2(K251A) mutant in which the pyridoxal-5′-phosphate (PLP) cofactor binding site lysine is replaced by alanine. In this experiment, only the WT ASP2 protein showed enzymatic activity (Figure 3B). We next tested whether or not the mutant forms of the asp2 protein migrated on a native gel in the same manner as the WT ASP2. Interestingly, Coomassie staining of a native gel showed that only the WT ASP2 and the asp2-K251A proteins migrate to the location of the enzymatic activity (Figure 3B). Slower migrating bands were observed, but never associated with enzymatic activity. Coomassie staining of the proteins run on SDS–PAGE showed that all were present at comparable levels (Figure 3B). From these data, we conclude

Figure 2. Specific Amino Acid Substitutions in ASP2 Suppress *rus* Phenotype.

(A) Amino acid sequence from 247–296 of the ASP2 protein with the position and nature of amino acid changes shown for each of the suppressor alleles. Arrow indicates the lysine residue that binds to pyridoxal-5′-phosphate (PLP). Human cytosolic AAT (HsGOT1) and *Arabidopsis* ASP1, ASP3, and ASP5 are shown for sequence comparison.

(B) Complementation of *asp2* suppression alleles by WT *Arabidopsis ASP2* and Human *GOT1*. Phenotypes of *rus1-2 asp2-12* (*r1-2 a2-12*) plants expressing either the *Arabidopsis ASP2* cDNA (*ASP2*) or the human cytosolic *AAT* cDNA (*Hs-cGOT1*) under the Cauliflower Mosaic Virus 35S promoter (*35S*) are shown. Bar = 0.5 cm.

(C) Suppression of *rus* single and double mutants. Phenotypes of *asp2-12* (*a2-12*) suppressor mutation either singly or in combination with *rus2-1* (*r2-1*) or *rus1-2 rus2-1* (*r1-2 r2-1*) are shown. Bar = 0.25 cm.

(D) Root-length measurements of 10-day-old seedlings shown in (C). Root lengths of single (*a2-12*), double (*r2-1 a2-12*; *r1-2 r2-1*), and triple (*r1-2 r2-1 a2-12*) mutant seedlings (n = 9 or 10) were measured. Error bar = standard error.

that our mutant asp2 suppressor proteins lack enzymatic activity and also fail to form a complex with similar electrophoretic properties as WT ASP2.

Loss of AAT2 Activity Is Not Sufficient to Suppress the *rus* Phenotype

We developed an antibody against purified ASP2 protein to use in biochemical analyses. To test the specificity of this antibody, we performed Western blotting analysis on WT samples and a null T-DNA insertion allele *asp2-15* obtained from the SALK collection (Alonso et al., 2003). This mutant lacks a WT product for *ASP2* by RT–PCR analysis (Figure 4A). Our antibody detected a protein of the predicted size (~42 kDa) for ASP2 in the WT Col sample (Figure 4A). We did not detect this protein in the *asp2-15* sample, confirming that this allele lacks ASP2 protein and that our antibody specifically recognizes ASP2 (Figure 4A). Publically available microarray data from Genevestigator suggest that *ASP2* is more highly expressed during early germination than at later stages in development (see Supplemental Figure 3) (Hruz et al., 2008). Using our ASP antibody, we performed a Western blot on 2, 3, 5, and 7-day-old WT seedling protein extracts (see Supplemental Figure 4). Our antibody detected much more protein in the 2 and 3-day-old samples than in the 5 or 7-day-old samples, confirming the expression data from Genevestigator (see Supplemental Figures 3 and 4).

All of the *asp2* suppressor mutants lack detectable enzymatic activity. Therefore, we hypothesized the lack of AAT2 activity leads to *rus* suppression. To test this hypothesis, we crossed the *rus1-2* mutants with the *asp2-2* loss-of-function allele and the *asp2-15* T-DNA null allele. The asp2-2 protein lacks AAT2 activity in the native gel assay and *asp2-15* plants lack ASP2 protein altogether (Figures 3B and 4A). Interestingly, plants homozygous for *rus1-2* and homozygous for *asp2-2* or *asp2-15* do not show suppression of the *rus* phenotype (Figure 4C). We conclude from these data that *rus* suppression is not due to the loss of AAT2 activity and that there must be an additional function of the asp2 suppressors that is responsible for *rus* suppression.

The *rus* Mutant Phenotypes Are Partially Suppressed by vitB6

Under standard Murashige and Skoog (MS) medium growth conditions (Murashige and Skoog, 1962), *rus1* and *rus2* mutant plants have near WT levels of root growth when grown in the dark (Tong et al., 2008; Leasure et al., 2009). When we grew *rus* (*rus1-2*, *rus2-1*, and *rus1-2 rus2-1*) seeds in the dark on MS medium without adding a B vitamin supplement, the roots of the *rus* seedlings failed to elongate (Figure 5A). This result led us to hypothesize that one or more of the B vitamins in the supplement are required for the suppression of the *rus* phenotype in

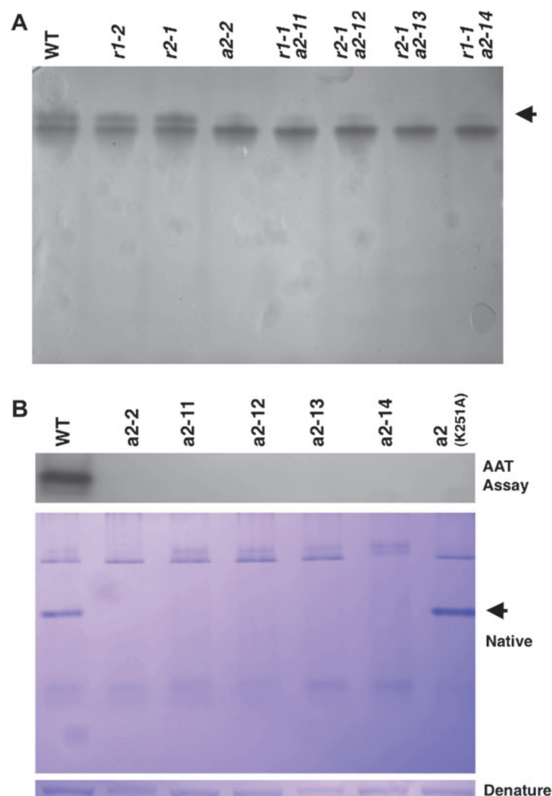

Figure 3. asp2 Suppressors Lack Both Enzymatic Activity and Protein Dimerization.

(A) AAT activity assay on 7-day-old plant extracts. The upper band, indicated by an arrow, represents the AAT2 (ASP2 protein) activity and the lower band represents the AAT3 (ASP5 protein) activity. A faint band representing the AAT1 (ASP1 protein) activity, at the same position as AAT2, can be seen in some samples. WT, wild-type; *r1*, *rus1*; *r2*, *rus2*; *a2*, *asp2*.

(B) Top row shows AAT activity assay on 2 μg total protein of WT ASP2 and various mutant asp2 proteins purified from an *E. coli* protein expression system. Only the region of gel where activity was observed is shown. Middle row shows Coomassie staining of identically run gels showing the region where enzymatic activity is seen for the WT. Bottom row shows SDS–PAGE stained with Coomassie showing the fusion protein at comparable levels in all samples.

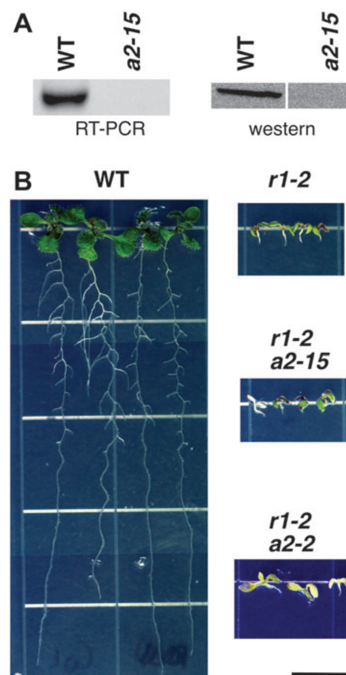

Figure 4. Loss of AAT2 in *asp2-15* and *asp2-2* Is Not Sufficient to Suppress the *rus* Phenotype.

(A) RT–PCR (RT–PCR) and Western blot (Western) analysis of wild-type (WT) and *asp2-15* T-DNA allele (*a2-15*) confirm the knockout mutation in *asp2-15*. Total RNA and total protein extracts were isolated from 2-day-old seedlings. An ASP2-specific antibody was used for the Western.

(B) Phenotypes of 10-day-old wild-type (WT), *rus1-2* (*r1-2*), *rus1-2 asp2-15* (*r1-2 a2-15*), and 14-day-old *rus1-2 asp2-2* (*r1-2 a2-2*) plants.

the dark. To test this, we grew plants on various MS medium plates with only a single B vitamin: nicotinic acid, pyridoxine HCl (PN; vitB6), thiamine, or myo-inositol. When grown in the dark, only *rus1-2* plants grown in the presence of PN showed root elongation (Figure 5A). Additionally, a mixture of all four vitamins suppressed the *rus1-2* phenotype to the same degree as PN alone, suggesting that PN is the molecule required for *rus1-2* root growth in the dark.

We next hypothesized that higher amounts of exogenous PN could suppress the *rus* mutants in the light. To test this, we grew WT Col, *rus1-2*, *rus2-1*, and *rus1-2 rus2-1* plants on plates with 0, 1, 2, 5, 10, 25, 50, or 100 μg ml^{-1} PN (the standard concentration for PN is 1 μg ml^{-1}). After 9 d of growth, root lengths were measured for each genotype (Figure 5B). Root growth increased linearly between 0 and 10 μg ml^{-1} PN, but then leveled off and began to decline slightly after 25 μg ml^{-1} PN. Wild-type Col seedlings are also slightly affected negatively by this amount of PN (Figure 5B). This result shows that high levels of PN can partially suppress the *rus* phenotype under standard light conditions.

VitB6 exists in six different forms (vitamers): pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and the three phosphorylated forms of each (PNP, PLP, and PMP, respectively). Pyridoxal-5′-phosphate (PLP) is the form of the vitamin used as a cofactor for over 140 enzymes, one of which is AAT2 (ASP2). We hypothesized that all non-phosphorylated B6 vitamers would be able to suppress the *rus1-2* phenotype. To test this, we grew *rus1-2* mutants on MS plates in the light with 10 μg ml^{-1} of PN, PL, or PM. PN, PL, and PM all suppressed the *rus1-2* phenotype to some degree, although PN had the most suppression. These results show that any of the non-phosphorylated B6 vitamers can suppress the *rus1-2* phenotype.

To test the ability of the cofactor form of vitB6 to suppress the *rus1-2* phenotype, we grew plants on MS plates in the light with 10 μg ml^{-1} PLP. We did not observe suppression of the *rus1-2* phenotype in this experiment. However, PLP is known to be unstable in the light, even under standard florescence lighting (Morrison and Long, 1958). MS medium plates with

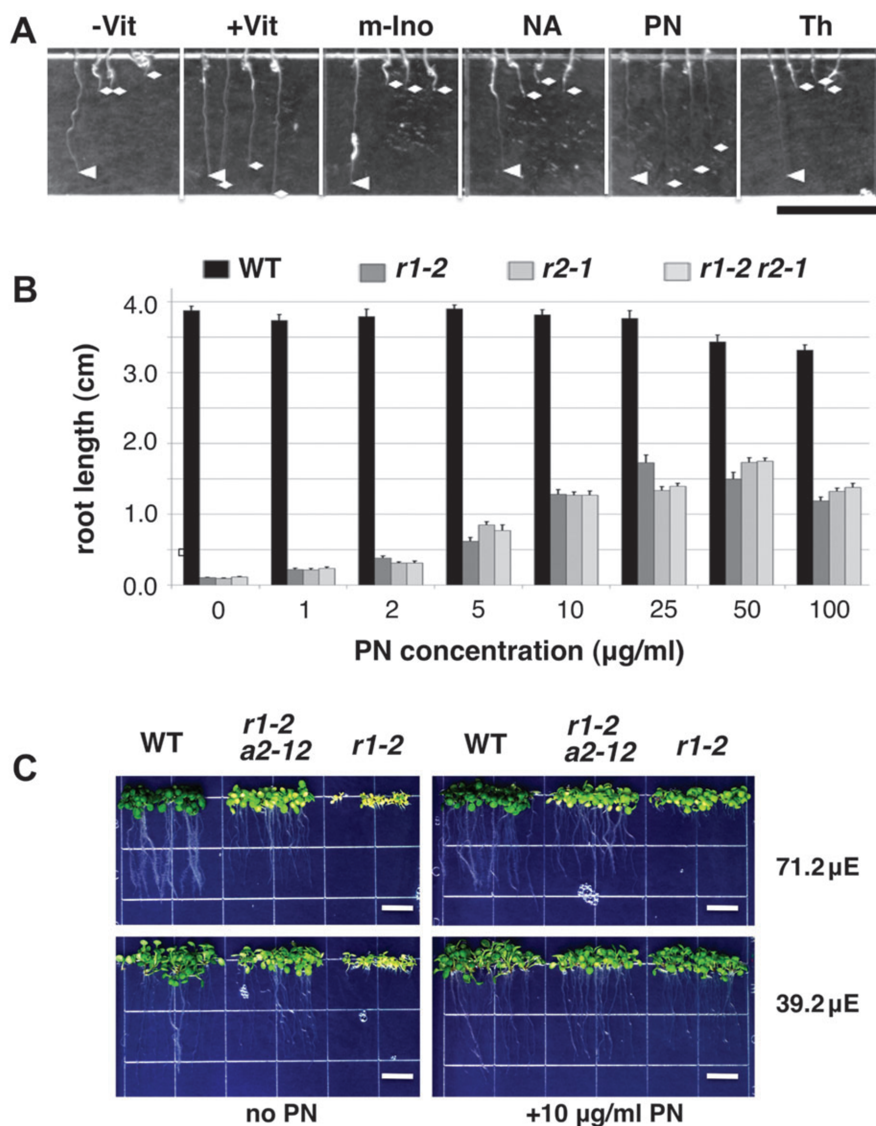

Figure 5. Effects of Light and PN on the *rus* Phenotype.

(A) Roots of dark-grown seedlings with various B vitamin supplements. For each vitamin treatment, four seedling roots (from left to right, WT, *rus1-2*, *rus2-1*, and *rus1-2 rus2-1*) are shown. The root tip position (a triangle for WT and a diamond for the rest) are marked. m-Ino, myo-Inositol (100 μg ml^{-1}); NA, Nicotinic Acid (1 μg ml^{-1}); PN, Pyridoxine (1 μg ml^{-1}); Th, Thiamine (10 μg ml^{-1}).

(B) Graph of the root lengths of 9-day-old wild-type (WT), *rus1-2* (*r1-2*), *rus2-1* (*r2-1*), and *rus1-2 rus2-1* (*r1-2 r2-1*) plants grown with increasing concentrations of pyridoxine (PN).

(C) 10-day-old wild-type (WT), *rus1-2 asp2-12* (*r1-2 a2-12*), and *rus1-2* (*r1-2*) seedlings grown under various light and PN treatments. The *rus1-2* phenotype is highly suppressed when supplemented with 10 μg ml^{-1} PN under the one stop light filter (55% normal light intensity). μE, μmol photons m^{-2} s^{-1}.

10 μg ml^{-1} PLP initially have a noticeably yellow tint due to the absorption spectrum of PLP. Within a day of being exposed to standard growth conditions, these plates become colorless. Indeed PLP is irreversibly converted to a photoproduct when exposed to UV light for 5 min (see Supplemental Figure 5). Thus, the lack of suppression of *rus1-2* by PLP could well be due to the degradation of the PLP in the medium. To circumvent this problem, we grew *rus1-2* plants in the dark on MS plates with 1 μg ml^{-1} PLP. Indeed, in this experiment, we observed that 1 μg ml^{-1} PLP can suppress *rus1-2* at levels comparable to the suppression seen with 1 μg ml^{-1} PN. This result suggests that *rus* mutants are suppressed by supplementation with the active B6 vitamer PLP, and that suppression is not limited to the non-phosphorylated B6 vitamers.

Different alleles of asp2 suppressors respond differently to exogenously added PN. *rus1-1 asp2-11* seedlings show significantly more suppression of the *rus* phenotype in the presence of 10 μg ml^{-1} PN, as compared to *rus1-1 asp2-11* seedlings

grown without PN. However, *rus1-2 asp2-12* seedlings display no obvious growth differences with or without supplemental PN (see Supplemental Figure 6). We interpret these results to mean that the *asp2* suppressor mutants act in a similar manner to exogenous vitB6 supplementation. The asp2-12 suppressor likely represents the strongest suppression of the *rus* phenotype that is possible through this pathway, so that additional PN supplementation has no additional effect on the phenotype. asp2-11 is only partially suppressive, and more PN continues to suppress the *rus* phenotype.

We analyzed B6 vitamer levels in five different genotypes (WT, *rus1*, *rus2*, *rus1* RUS1–GFP, and *rus1 asp2-14*) (see Supplemental Figure 7). We were able to measure levels of the vitamers PN, PL, PM, PLP, and PMP, but PNP was below our detection sensitivity (0.01 pmol mg^{-1} fresh tissue). Our measurements showed that PMP and PLP were reduced by about 20% in the *rus1* and *rus2* mutants, as compared to the WT (see Supplemental Figure 7). PMP levels were restored to normal in the asp2 suppressor (*rus1-1 asp2-14* double mutant), although PLP levels remained low (see Supplemental Figure 7). asp2 suppressor (*rus1-1 asp2-14* double mutant) showed twice as much PM as in seedlings of other genotypes. It is interesting to note that the levels of total B6 vitamers in the *rus1* and *rus2* mutants are only slightly lower than that in WT (see Supplemental Figure 7).

Combinatorial Effects of Light and PN on the *rus* Phenotype

Our data reveal a role for both light and vitB6 on the *rus* mutant phenotype. In order to determine the combinatorial effects of these two variables, we grew WT, *rus1-2*, and *rus1-2 asp2-12* plants under full or partial light level conditions, with or without a 10 $\mu g\ ml^{-1}$ supplement of PN. To achieve a reduction in light quantity, we utilized a one-stop neutral density filter that uniformly across wavelengths reduces light levels to 55% of normal growth light conditions (Tong et al., 2008). With 10 $\mu g\ ml^{-1}$ PN supplementation, *rus1-2* plants had increased root growth under all light conditions, and exhibited near WT root growth under the reduced light condition (Figure 5C). *rus1-2 asp2-12* plants do not appear to be significantly affected by PN supplementation (Figure 5C). These results reveal that both light intensity and PN supplementation are important for *rus1-2* suppression. Additionally, the lack of any strong effect of PN supplementation on *rus1-2 asp2-12* plants supports a role for the *asp2* suppressor mutations in the vitB6 aspect of *rus* suppression.

asp2 Suppressor Mutants Change Amino Acids Near the Cofactor Binding Site

AAT enzymes have been studied for decades and detailed crystallographic and functional details have been worked out for the structure of the protein (Yang and Metzler, 1979; Ziak et al., 1993). We utilized the Cn3D helper application (National Center for Biotechnology Information, NCBI) to identify the positions of our asp2 suppressor mutant amino acid changes based on the pig cytosolic AAT structure (Rhee et al., 1997). All of our suppressor mutants affect amino acids that are predicted to physically reside near the PLP molecule in the native AAT enzyme (Figure 6A). asp2-14 (R259H) carries a substitution in a 100% conserved Arginine residue that was shown to be critically important in forming the phosphate binding cup via two specific hydrogen bonds (see Supplemental Figure 7). The amino acid change in the asp2-2 protein, which fails to suppress the *rus* phenotype, does not appear to reside near to the PLP molecule in the protein structure (Figure 6A). These data show a correlation between the

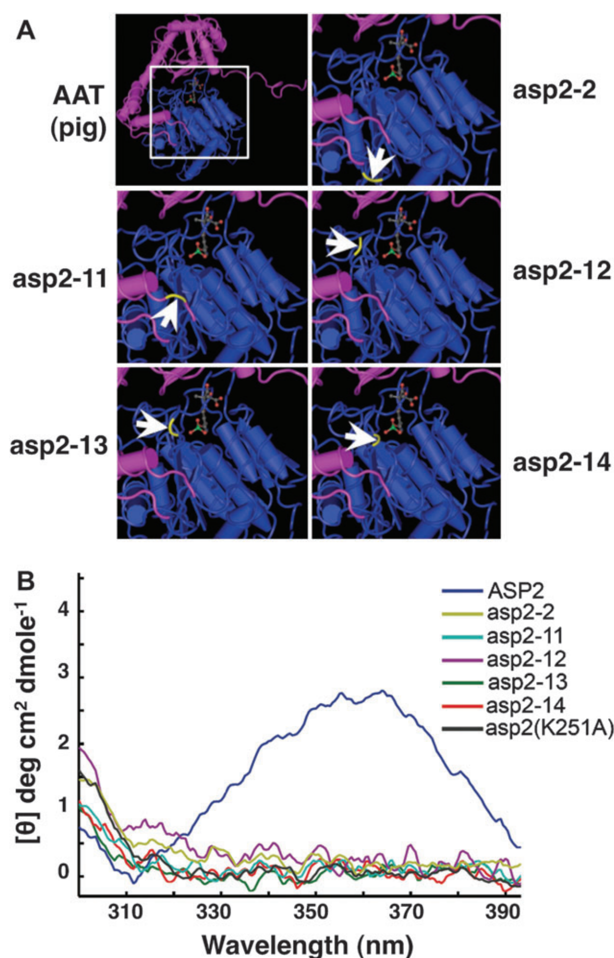

Figure 6. Amino Acid Substitutions in *asp2* Suppressors Are Located in the PLP Binding Pocket.

(A) The site of *asp2* suppressor mutation amino acid changes mapped onto the crystal structure model of the swine cytosolic AAT enzyme. The PLP cofactor is visible as a ball-and-stick structure in the center of the protein. Each mutant protein version is represented on a close-up of the swine AAT enzyme, as illustrated by the white box. Arrows indicate the positions for the mutant residues highlighted in yellow.

(B) Only the WT ASP2 has a CD-spectrum indicative of a having covalently bound PLP cofactor. Near UV circular dichroic (CD) spectrum data for WT ASP2 and various mutant proteins are shown.

position of the altered amino acid and suppression of the *rus* phenotype.

Circular-Dichroism Spectroscopy Suggests that *asp2* Suppressor Mutants Lack PLP Cofactor

In order to determine the effect of our various mutations on the overall ASP2 secondary structure and protein folding, we compared the far-UV Circular-Dichroic (CD) Spectrum (200–300 nm) of the WT ASP2 enzyme to those of the asp2 mutant proteins. There were no significant differences in the CD spectra between the WT and mutant enzymes in the region of 200–250 nm, suggesting that these mutations do not cause large changes in secondary structure. In order to assess the microenvironment of the PLP cofactor, we examined the near-UV CD of the WT and mutant enzymes (Figure 6B). The WT enzyme showed a single-band positive CD signal centered at 360 nm, characteristic of the family of aspartate aminotransferase enzymes with a de-protonated aldimine Schiff base linkage (Yano et al., 1992; Kim et al., 1994). All mutants lacked any CD signal from 300–400 nm, suggesting that the PLP cofactor is not bound to these mutants as in the native enzyme. These results show that, although the secondary structures of the asp2 mutant proteins appears similar to WT, none of the mutant asp2 proteins is able to form its proper association with the PLP cofactor.

DISCUSSION

We have isolated four unique *asp2* mutations that act as second-site suppressors of the *rus* phenotypes. These mutations create single amino acid changes in the ASP2 protein near the lysine to which the pyridoxal-5′-phosphate (PLP) cofactor normally binds (Figure 2A). Our analyses of these proteins indicate that they lack enzymatic function (Figure 3A and 3B), fail to covalently bind to their cofactor molecule PLP (Figure 6B), and fail to form a WT dimer complex (Figure 3B). Lack of dimerization in the mutants is likely a result of K251 being free of the PLP cofactor, as CD spectrum analysis suggested that the mutant monomers are folded properly. Additionally, the K251A mutant that lacks the PLP-binding lysine was able to dimerize without having a PLP molecule, again suggesting that it is the cofactor-free lysine in the active site that prevents dimerization. We have shown that the various vitamer forms of vitB6 can suppress the *rus* phenotype in a manner similar to the *asp2* suppressor mutants (Figure 5A and 5B). Reducing the intensity of light exposure can further suppress vitB6-supplemented *rus1-2* mutants (Figure 5C).

AATs are a highly conserved family of proteins present throughout all domains of life and catalyze the transamination of aspartate and α-ketoglutarate to glutamate and oxaloacetate, and the reverse reaction. AATs function as homodimers with two functionally equivalent active sites, with each protein contributing to the active site of its binding partner (Wilkie et al., 1996). The PLP cofactor forms a covalent Schiff base with the ϵ-amino group of a lysine residue in each active site (Malashkevich et al., 1995). It is clear that the chemical conditions created by the PLP-binding site pocket allow for the formation of the Schiff base attachment. If this were not true, PLP would be prone to binding with any lysine it could find, rather than at specific lysines in specific proteins.

In our experiments, WT ASP2 protein had a near-UV CD spectra single positive dichroic band centered at 360 nm, which is consistent with previously described spectra for aspartate aminotransferases from *Bacillus* sp. (Figure 6B) (Yano et al., 1992; Kim et al., 1994). The near-UV has been shown to be sensitive to both the local microenvironment and the ligation chemistry of the protein and the PLP adduct, with many different binding motifs giving rise to different CD signals (Kim et al., 1994). The CD signal is also characteristic of aspartate aminotransferases, rather than other families of enzymes that use the PLP coenzyme, such as glutamate decarboxylase (Tramonti et al., 1998) and plant phosphorylase (Shimomura et al., 1980). The positive CD signal corresponding to the absorption band at 360 nm indicates that pyroxidal-5′-phosphate is covalently attached via a deprotonated internal aldimine Schiff base linkage, as opposed to the protonated form that has a positive CD centered at 430 nm (37–39). In the various mutant asp2 proteins, the lack of any clear CD signal between 300 and 500 nm provides strong evidence that the PLP co-factor is not covalently bound (Yang and Metzler, 1979; Futaki et al., 1990). However, lack of cofactor binding does not seem to be sufficient for *rus* suppression, as the asp2-2 protein fails to suppress *rus* phenotypes despite also lacking the cofactor. All of our four *asp* suppressor mutations create single amino acid changes near the active site of the enzyme at positions that are highly conserved (Figure 2A). Based on the tertiary structure of the orthologous pig cytosolic AAT enzyme, these amino acids reside very close to the position of the PLP in the folded protein (Figure 6B). Indeed, *asp2-12* alters the alanine residue directly preceding the binding-site lysine to a valine residue. *asp2-13* replaces a negatively charged glutamate residue with a positively charged lysine. Interestingly, the dominant *asp2-14* mutation alters an arginine residue thought to be involved in stabilizing the negative charges of the phosphate group of PLP (Wilkie et al., 1996). Of the four suppressors we identified, the weakest suppressor, *asp2-11*, creates an amino acid change furthest from the PLP-binding site. The amino acid changed in the non-suppressive *asp2-2* allele appears to reside completely outside of the PLP-binding site.

Although the suppressor mutations all lead to a loss of AAT2 enzymatic function, this does not account for the suppression of the *rus* phenotype. Logically, many mutations (e.g. *asp2-2*, *asp2-15*, any non-sense mutation) in the *ASP2* gene could be expected to destroy the enzymatic function of the protein, but we only recovered specific missense mutations near the active site of the enzyme in our screen. Our biochemical results suggest that the asp2 suppressor proteins are identical to each other regarding the lack of both a covalently bound PLP cofactor and enzymatic activity. Despite their observed similarities,

the asp2 suppressor proteins have varying levels of *rus* suppression. Therefore, we are left to conclude that the asp2 suppressor proteins are performing some function in the plant in addition to transamination. This additional function may or may not be present in the WT ASP2 protein as well.

rus mutants recover most of their root growth when light intensity is decreased, but only in the presence of at least some exogenous vitB6 (Figure 5C) (Tong et al., 2008; Leasure et al., 2009). The addition of higher-than-standard amounts of PN results in the partial recovery of *rus* mutants in full light conditions (Figure 5). This recovery ends at around 25× normal PN concentrations (25 μg ml^{-1}), possibly due to other limiting factors (e.g. PN transporter levels, PN toxicity). Wild-type roots also show a slight reduction in root growth in the presence of 25 μg ml^{-1} PN, suggesting that some negative effects of high PN concentrations may limit the recovery of root growth in *rus* mutants. The combination of reduced light exposure and excessive PN supplementation nearly completely suppresses the *rus1-2* phenotype (Figure 5). *rus1-2 asp2-12* plants are not remarkably affected by PN supplementation. These data suggest that PN supplementation in the growth media mimics the effects of the *asp2* suppressor mutations.

The total amount of the measured vitB6 vitamers in the *rus* mutants is slightly lower than that in the WT and the suppressor (Supplemental Figure 7). When individual vitamers are compared, our results suggested that both PLP and PMP in *rus* mutants were reduced by about 20% when compared to those in the WT. The reduced PMP was restored in both the suppressor (*rus1-2 asp2-14* double mutant) and the complementation plant (*rus1-1* RUS1–GFP transgenic line). The reduced PLP in *rus* mutants, however, was restored only in the complementation transgenic line, not in the suppressor. In addition, levels of PM are significantly higher in the *rus* mutants, the complementation line, and the suppressor, when compared to that in the WT. It is also interesting to note that the suppressor displayed a more than 40% higher PM level than that in the WT. Since various B6 vitamers are known to be interconvertible and the overall vitB6 levels seem not significantly lower in the *rus* mutants, it is reasonable to postulate that the *rus* mutants are unlikely to be vitB6-deficient. The observed differences among the mentioned B6 vitamers, however, could suggest that individual B6 vitamers may play a direct or indirect role in contributing to the *rus* phenotypes. For example, PMP is the product of half transamination in the reaction catalyzed by ASP2, and is normally bound tightly by multiple non-covalent interactions in the B6 binding pocket in the enzyme's active site. It is possible that specific mutations in ASP2 B6 binding pocket allow PMP release at half reaction and thus result in elevated PMP levels. It remains to be tested whether and how levels of PMP (or other specific B6 vitamers) can serve as signals for development in the light. The suppressive effects of asp2 proteins with amino acid changes near the PLP-binding site implicates ASP2 in the perception of its cofactor.

Our results suggest a second role for the ASP2 protein in addition to, although probably related to, its role in metabolism. It is possible that the ASP2 protein is able to assess the status of the PLP levels in the cell and promote or repress development accordingly. RUS1 and RUS2 proteins maybe normally act to amplify the PLP signal that ASP2 sees. In this scenario, *rus* mutant plants perceive that they have low levels of PLP, when in fact they do not. The asp2 suppressors, to varying degrees, signal that there is adequate vitB6. The WT ASP2 can interfere with the signal coming from the recessive suppressors, as evidenced by the return of the *rus* phenotype in *rus1-2 asp2-12* plants transformed with *35S::cASP2* or *35S::HsGOT1*. Finally, excessive application of vitB6 to *rus* mutants increases the level of vitB6 to levels that are perceived to be satisfactory. It is likely that PLP-damaging light wavelengths increase the requirement of the cell for vitB6, as PLP is a light labile molecule. Our observation that *rus* suppression requires high levels of exogenous vitB6 application in the light supports this idea.

VitB6 is a fascinating and incredibly important molecule for all forms of life. In addition to its long-recognized role in metabolism, vitB6 has recently been implicated in plant development (Shi and Zhu, 2002; Chen and Xiong, 2005; Wagner et al., 2006), salt tolerance (Shi et al., 2002), and protection from oxidative stresses (Titiz et al., 2006; Havaux et al., 2009; Leuendorf et al., 2010). A rare form of epilepsy in humans is caused by a build-up of a compound that interferes with vitB6 action, and is treated by high doses of pyridoxine (Struys and Jakobs, 2007). PLP could also work as a UV-B receptor that photochemically responds to environmental ultraviolet light to elicit cellular responses. A better understanding of the homeostasis and metabolism of vitB6 in *Arabidopsis* will have a broad and relevant impact on many aspects of biology.

METHODS

Plant Growth and Imaging

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 plants were grown as described before (Hou et al., 2005). For Petri dish-grown seedlings, surface-sterilized seeds were cold-treated at 4°C and plated on MS growth medium (Murashige and Skoog, 1962) with 1% sucrose in square plates (100 × 1 003 × 15 mm; Fisher Scientific) and placed in a growth chamber (Percival Model CU36L5) with a 16-h/8-h light/dark cycle at a constant temperature (23°C). White growth light was provided by cold fluorescence light tubes (Philips F17T8/TL741 for CU36L5 model, Philips F32T8/TL741 for AR-66L model) and maintained at 100 μmol m^{-2} s^{-1}. Plants were grown with or without vitamin supplements as indicated in the text. The Lee brand filter ND #209 (0.3, 1 stop) was used for reducing light in some experiments. Plants were imaged with a RT Slider camera using Spot software (Diagnostic Instruments, Inc.). Some high-resolution plant images were created by piecing together two or three overlapping images using Photoshop software (Adobe Systems, Inc.). Root-length measurements were performed using the ImageJ software (http://rsbweb.nih.gov/ij/).

Mapping of *asp2* Mutants

A mapping population for mapping *asp2-12* was generated by crossing *asp2-12*, which is in the Columbia (Col) ecotype, with Landsberg *erecta* (L*er*). F^1 plants were allowed to self-fertilize and the F^2 seed collected for mapping. Linkage to genetic markers placed the mutation on a small region of chromosome five and sequencing of candidate genes revealed a mutation in the *ASP2* coding sequence. Sequencing of the four suppressor mutants revealed the following changes: *asp2-11* P292L (codon CCU to CUU) *asp2-12* A250V (codon GCC to GUC); *asp2-13* E258K (codon GAG to AAG); *asp2-14* R259H (codon CGU to CAU).

ASP2 Overexpression Constructs

cDNA for *Arabidopsis ASP2* was created by reverse transcriptase reaction using primers for the start and stop of translation. Human *GOT1* cDNA was purchased from Invitrogen. cDNAs were cloned into the pBI-1.4T binary vector behind the Cauliflower Mosaic Virus 35S promoter. *Agrobacterium*-mediated transformation was utilized to create transgenic *Arabidopsis* containing either of the transgenes.

AAT Enzyme Assay

AAT enzymatic assays were performed as described by Schultz et al. (1998). For whole-plant extracts, seedlings were collected and weighed. Seedlings were ground in AspAT extraction buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 0.1% Triton-X) in a volume of buffer two times the weight of the tissue. For whole-plant extracts, equal amounts of sample (by fresh weight) were loaded onto non-denaturing poly-acrylamide gels. For purified proteins, 2 μg of protein was loaded for each sample and run on non-denaturing gels. Gels were incubated for 5 min with AAT activity buffer (2.2 mM α-ketoglutaric acid, 8.6 mM L-aspartic acid, 0.5% PVP-40, 1.7 mM EDTA, 100 mM dibasic sodium phosphate) and then incubated with AAT Activity buffer with 0.1% Fast Blue BB for up to 30 min in the dark (or until staining appeared saturated). Gels were imaged with a Kodak Imagestation without the use of any light filter.

Protein Purification

For enzymatic assays and CD spectrum analysis, ASP2 protein variants were purified by pMAL Protein Fusion and Purification System (New England Biolabs Inc.). *ASP2* cDNAs were cloned in frame into the pMAL-c2x expression vector. Mutant *asp2* cDNA forms were created via site-directed mutagenesis of the *ASP2* cDNA using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All cloning and site-directed mutagenesis was confirmed by sequencing.

Western Blotting

Proteins were extracted in 2X extraction buffer (100 mM Tris, pH 6.8, 100 mM Dithiothreitol (DTT), 8% SDS, 0.1% bromphenol blue, 20% glycerol), boiled for 5 min and run on SDS–PAGE. Protein concentrations were controlled by extracting equal fresh weight per volume of total extract across samples in each experiment. Equal loading on SDS–PAGE gels was confirmed by Coomassie staining of simultaneously run identically loaded gels and/or Ponceau staining of blots. Proteins were transferred onto Protran BA 83 nitrocellulose membrane (Whatman) overnight at low current (~7 mA). Blots were blocked for at least 2 h in TBST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 5% (w/v) milk. 7 μl of primary antibody was added to the blot in 10 ml of TBST with 2.5% (w/v) milk for 2 h. Blots were washed three times for 10 min each in TBST and then blocked again in TBST with 10% (w/v) milk for at least 2 h. 5 μl of HRP-conjugated goat anti-rabbit secondary antibody was added to the blot in 10 ml of TBST with 5% (w/v) milk for at least 1 h. Blots were washed three times for 10 min each in TBST. Detection was performed using the Pierce ECL Western Blotting Detection System (Thermo Scientific Inc.) and a 4000R Image Station (Kodak) CCD camera system for 20 min of exposure.

Circular Dichroism Spectroscopy

Far-UV (200–300 nm) and near-UV (250–400 nm) circular dichroism (CD) measurements were taken with a J-810 spectropolarimeter (Jasco Inc.) in a 1-mm (far-UV) or a 10-mm (near-UV) quartz cuvette (Hellma, USA). Temperature was maintained at 23°C with a circulating water bath (Model F-25, Julabo). CD spectra were collected with an integration time of 0.5 s for far-UV and 1.0 s for near-UV.

Extraction and Quantification of B6 Vitamers

Extraction of vitB6 vitamers was performed according to Gonzalez et al. (2007). The obtained TCA extracts were neutralized by adding 7% v/v of 5 M NaOH. B6 vitamers were then separated by HPLC using a Cadenza CD-C18 column, 4.6 × 250 mm I.D., 3 μm (Imtakt, Japan), and measured with a Waters 2475 fluorescence detector. Excitation and emission wavelengths were 300 and 400 nm, respectively. PM, PL, and PN were determined after an isocratic separation in a mobile phase containing 0.1 M potassium dihydrogen phosphate and 0.1 M sodium perchlorate, pH 3.0, as described by Kimura et al. (1996). PLP and PMP were determined after an isocratic separation in the above mobile phase to which 0.5 g L^{-1} of the derivatizing agent sodium bisulfite was added.

To enzymatically synthesize PMP and PNP standards, pyridoxal kinase was expressed in *E. coli* cells and purified using affinity chromatography. *Arabidopsis* SOS4 cDNA encoding the pyridoxal kinase was cloned into pDEST15 (Invitrogen, CA). Overnight cultures were induced with IPTG (Sigma-Aldrich, MO) for 4 h before recombinant GST:SOS4 was affinity purified via glutathione agarose resin (Sigma-Aldrich, MO) using standard procedures. The purified enzyme (5 μl of the resin-bound enzyme) was incubated at 37°C overnight with gentle agitation in 50 mM potassium phosphate buffer, pH 7.0, containing 0.08 mM PM or PN, 1 mM ATP, and

100 μM zinc acetate. PLP standard was obtained commercially (Sigma-Aldrich, MO).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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