

Phenotypic characterization of *Arabidopsis thaliana* mutants that alleviate root growth defects in a vitamin B₆ biosynthesis mutant

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Vitamin B₆ is an essential metabolite required by all living organisms and participates in numerous cellular biochemical reactions. Unlike mammals, plants can synthesize this compound *de novo*, and therefore, serve as an excellent nutritional source for the vitamin. Although the biosynthesis pathway in plants has been well understood for nearly two decades, the regulatory mechanisms that control vitamin B₆ levels remain largely unknown. Here, we describe a novel set of *Arabidopsis* mutants that display distinct phenotypic and molecular characteristics and are likely affected in either the biosynthesis or homeostasis of vitamin B₆. We propose that these new mutants will serve as valuable tools for uncovering the unknown regulatory steps governing vitamin B₆ levels in plants.

Introduction

Vitamin B₆ (further referred to as vitB₆) is one of the most versatile compounds in any living cell. It is broadly required as an enzymatic cofactor for amino acid, carbohydrate, and lipid metabolism and has been shown to function as an antioxidant [1]. It has also been implicated in plant pathogen-response related pathways as well as phytohormone homeostasis [2–7], and it provides a variety of human health benefits [8].

The vitamin comprises three chemically related isoforms which are pyridoxal (PL), pyridoxamine (PM),

and pyridoxine (PN). They differ in their 4' position with PL carrying an aldehyde group, PM an amino group, and PN a hydroxy group [1]. All three isoforms can be phosphorylated at their 5' position, but the main biologically active forms appear to be pyridoxal 5'-phosphate (PLP) and pyridoxamine-5'-phosphate (PMP) [9].

Plants represent a primary nutritional source for vitB₆ which they can *de novo* synthesize. The plant biosynthetic pathway only requires two enzymes:

Abbreviations

EMS, ethyl methanesulfonate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NPQ, nonphotochemical quenching; OPP, oxidative pentose phosphate; PDX, pyridoxine biosynthesis; PGD, 6-phosphogluconate dehydrogenase; PL, pyridoxal; PLP, pyridoxal 5'-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5'-phosphate; PN, pyridoxine; PNP, pyridoxine 5'-phosphate; PPOX, Pyridoxine/pyridoxamine 5'-Phosphate Oxidase; PRK, phosphoribulokinase; PRL, Pyridoxal Reductase 1; R5P, ribose-5-phosphate; RBSK, ribokinase; RPE, ribulose-5-phosphate-3-epimerase; RPE, ribulose-5-phosphate-3-epimerase; RPI, ribose-5-phosphate Isomerase; RPI2, Ribose-5-Phosphate Isomerase 2; *rsr4*, reduced sugar response 4; RSW10, Radial Swelling 10; RSW10, radial swelling 10; Ru5P, ribulose 5-phosphate; SOS4, salt overly sensitive 4; *sur*, suppressors of *rsr4-1*; TKL, transketolase; vitB₆, vitamin B₆.

Pyridoxine Biosynthesis Protein 1 (PDX1) and PDX2 [10]. The two PDX proteins form a large PLP synthase complex that is composed of 12 PDX1 and 12 PDX2 units that directly synthesize PLP from ribose 5'-phosphate (or ribulose 5'-phosphate), glyceraldehyde 3'-phosphate (or dihydroxyacetone phosphate), and glutamine [10–13]. PDX2 functions as a glutaminase that supplies nitrogen for PLP, and PDX1 arranges the final ring closure [10,11,14]. *Arabidopsis thaliana* has three PDX1 (PDX1.1, PDX1.2, and PDX1.3) proteins, but only a single PDX2 [15,16].

Since the vitamin is so widely involved in cell metabolism, it is not surprising that mutants impacted in vitB₆ biosynthesis show pleiotropic phenotypes. For example, null mutants in PDX2, as well as double mutants affected in PDX1.1 and PDX1.3, are disrupted in early embryo development at the early heart and globular stages, respectively, though this defect can be rescued by supplementation with PN [10,17,18]. In addition, loss-of-function mutants in just *pdx1.3* exhibit increased abiotic stress sensitivities (osmotic, salt, and oxidative stress, as well as high light and UV-B stress) [18–21]. Furthermore, an EMS (ethyl methanesulfonate)-based mutant was identified through a screen for aberrant sugar response mutants, called *reduced sugar response 4-1* (*rsr4-1*) [22]. *rsr4-1* carries a single point mutation in *PDX1.3* that changes its amino acid residue 54 from glycine (G) to serine (S) (*pdx1.3*^{G54S}) [15]. The corresponding protein is no longer able to interact with other PDX1 or PDX2 proteins to function in a PLP synthase complex, and *rsr4-1* plants show a specific reduction in PL levels with only ~37% PL left compared to the wild-type [15]. This strong reduction in PL levels correlates with reduced shoot size, delayed flowering time, reduced chlorophyll content, and most strikingly, strongly reduced primary root growth, demonstrating that vitB₆ is critical for general plant development [15]. Furthermore, the aberrant root growth in *pdx1* mutants has also been connected with ethylene signaling as well as auxin distribution and signaling [6,23].

In addition to the PDX-dependent *de novo* biosynthesis pathway, plants also possess a *salvage* pathway that relies on the concerted activities of the PDXK/SOS4 (Pyridoxal Kinase/Salt Overly Sensitive 4) pyridoxal kinase that produces PLP [24], PPOX (-Pyridoxine/pyridoxamine 5'-Phosphate Oxidase) that catalyzes the oxidation of either PNP or PMP into PLP [25], and PRL (Pyridoxal Reductase 1) that facilitates the reduction of PL to PN [26]. The salvage pathway is critical for vitB₆ homeostasis and has also been shown to impact stress tolerance in plants [1,2,24,26].

Though the biosynthetic pathways are well understood, it is still unclear how plants regulate vitB₆

homeostasis. One recent example comes from PDX1.2, which encodes for a pseudoenzyme that can assemble with PDX1.1 and PDX1.3 into a complex but lacks enzymatic activity itself [15,27,28]. PDX1.2 is thought to act as a regulatory component of the PLP synthase by impacting vitB₆ synthesis rates and stability of the PDX1 complex [12]. In addition, all PDX proteins are shown to be unstable in cell-free degradation assays, and recent work has shown that this likely involves a cathepsin B3 protein [29]. *In planta* instability has also been demonstrated for PDX1.3 and PDX2 either under starvation conditions or, specifically for PDX2, after heat shock exposure [29].

Here, we describe a set of novel mutants that suppress the short root phenotype of the *rsr4-1* mutant, which we hypothesize to be regulatory mutants for vitB₆ biosynthesis or homeostasis. The work comprises a detailed analysis of their vitB₆ content in root and shoot tissues, a general description of their phenotypic appearance and abiotic stress tolerance, and a look at the degree to which the *rsr4-1* phenotype has been normalized back to the wild-type. In addition, we generated a proteomic profile of the root and shoot for these mutants for comparison to *rsr4-1* and wild-type. The work represents a starting point to (1) better understand these different novel mutants and (2) provide working hypotheses for the suppressor phenotypes. Overall, the work represents the first step in identifying novel factors impacting vitB₆ levels in plants and novel vitB₆ regulatory pathways.

Results

Identification of novel *rsr4-1* suppressor mutants

To identify regulatory steps in vitB₆ biosynthesis, we hypothesized that the short root phenotype of *rsr4-1* is a suitable tool to identify novel mutants impacted in vitB₆ biosynthesis. The approach was to EMS-mutagenize a large pool of *rsr4-1* seeds and search for mutants that develop a long, wild-type-like root in the absence of vitB₆ supplements in the growth medium (Fig. 1A,B). We argued that these *suppressors of rsr4-1* or *sur* mutants are impacted either in regulatory steps controlling vitB₆ biosynthesis or in other aspects that allow reversion of the short-rooted phenotype in *rsr4-1* back to wild-type.

Screening of 60 pools of EMS-mutagenized seeds (~50,000 seeds) led to the identification of five mutants from five different pools (*sur10*, *sur38*, *sur50*, *sur56*, and *sur60*) with significantly elongated roots compared to *rsr4-1* (Fig. 2). However, the root length of the different *sur* mutants varies, and not all reach

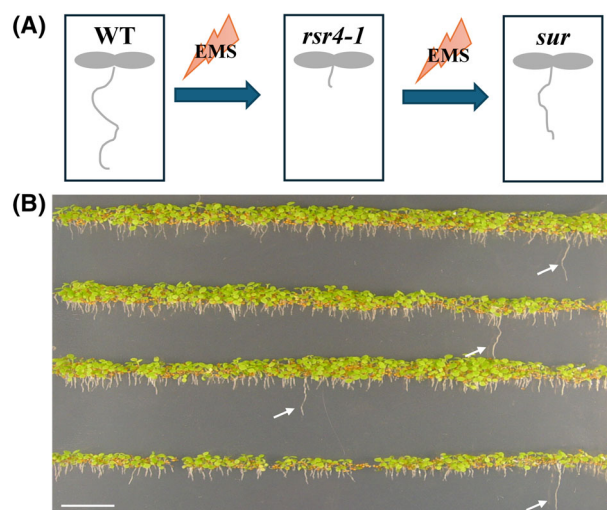


Fig. 1. Screening and identification of novel suppressor mutants of *rsr4-1*. (A) *rsr4-1* was originally identified through ethyl methanesulfonate (EMS)-mutagenesis as a *reduced sugar response* (*rsr*) mutant. Characteristically for *rsr4-1* is a short-rooted phenotype. This phenotype was used as a base to perform a second EMS mutagenesis on *rsr4-1* and to search for plants with normalized primary root growth. Identified mutants were named *suppressor of rsr4-1* (*sur*). (B) Example of an initial seed pool containing *sur* mutants (pointed out by white arrows). Scale bar represents 1 cm.

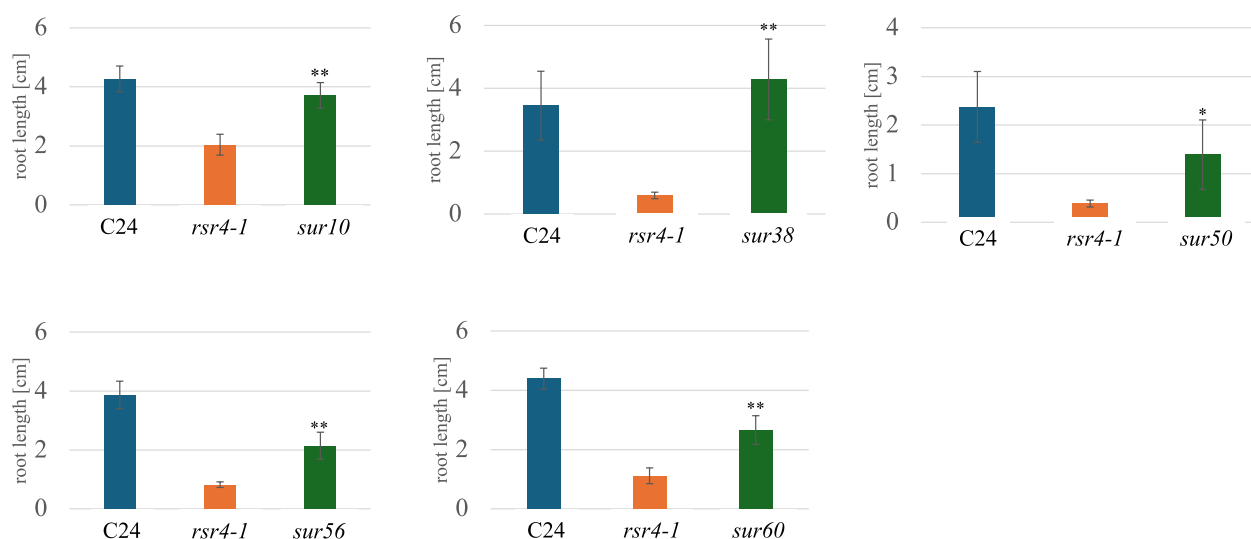


Fig. 2. Mean root length of *sur* mutants in comparison to C24 wild-type and *rsr4-1*. C24, *rsr4-1*, and *sur* mutant seedlings were grown in sterile culture for 10 days on vertical plates before primary root length was measured. All suppressor mutants had significantly longer roots than *rsr4-1*. While *sur50*, *sur56*, and *sur60* had an intermediate root length, roots from *sur10* and *sur38* were comparable to C24. Calculations are based on $n = 15$ seedlings; error bars indicate standard deviations; significance was calculated with a Student's *t*-test (*, $P < 0.05$ and **, $P < 0.01$). Significance in this graph is calculated for *rsr4-1* versus *sur* mutant.

the length of the C24 wild-type. In addition, though the root length of *rsr4-1* is always significantly shorter than C24, overall lengths can vary in the millimeter range between experiments (for example compare *sur10* and *sur38* with results gathered for *rsr4-1*; Fig. 2). This variation makes it critical to always grow

the different lines side by side to allow proper root length assessment. In general, *sur10* and *sur38* developed roots comparable to the wild-type, roots of *sur50* plants were only slightly longer than *rsr4-1*, and *sur56* and *sur60* root lengths were intermediate between *rsr4-1* and C24 (Fig. 2).

Sequencing of the *PDX1.3* genes in the different *sur* mutants showed that all five mutants still carried the G54S mutation characteristic for *rsr4-1* in *PDX1.3* [15] (Fig. S1).

Backcrossing of these mutants into *rsr4-1* showed that in the F1 generation all seedlings developed *rsr4-1*-like short roots (Fig. 3), demonstrating that in all cases the suppressor mutations are recessive in nature. Furthermore, the mutants were crossed with each other to determine allelism. As shown in Table 1, all F1 seedlings developed short roots comparable to *rsr4-1*, indicating that the five *sur* mutants are not allelic to each other and are carrying mutations in different loci.

VitB₆ measurements in shoot and root tissues

rsr4-1 was previously shown to have significantly reduced PL levels in rosette leaves of soil-grown plants compared to the C24 wild-type [15]. To determine whether the *sur* mutants exhibit normalized vitB₆ levels, we also analyzed their vitamer content. For this, 2-week-old seedlings were grown under sterile conditions to allow easy access to root tissues, which had not been included in the previous analysis.

Of the six known B₆ vitamers, PN was below the detection limit, and PM could not be quantified due to interference from a nonspecific overlapping peak. Therefore, our analysis includes PL, PLP, PMP, and PNP (Fig. 4).

The *rsr4-1* seedlings showed significantly reduced PL levels in both root (~43% reduction) and shoot (~40% reduction) tissues compared to the C24 wild-type (Fig. 4A and B). This is consistent with previously published data for rosette leaves from soil-grown plants [15]. In addition, *rsr4-1* leaves showed, on average, lower levels of PLP (~18% reduction), PMP (~19% reduction), and PNP (~18% reduction). Although these decreases were not statistically significant, they collectively contributed to an

average reduction in total vitB₆ content of about 20% compared to C24 (Fig. 4A).

Remarkably, among the five *sur* mutants, only *sur50* showed normalized levels of PL, PLP, PMP, and PNP in leaves, with average values even exceeding those of C24. This resulted in a mild increase in summed PL, PLP, PMP, and PNP of approximately 12% compared to C24 (Fig. 4A). In contrast, the remaining four *sur* mutants exhibited reduced PL levels like *rsr4-1* and showed overall vitB₆ contents closely resembling that of their parent mutant (Fig. 4A).

Interestingly, roots of all *sur* mutants—except *sur56*—showed increased PL levels that were no longer significantly different from C24, correlating with normalized root growth (Fig. 4B). Although *sur56* had significantly reduced PL levels, it tended to exhibit elevated PLP, PMP, and PNP levels, resulting in an overall ~27% increase in total vitB₆ compared to C24, and ~29% compared to *rsr4-1* (Fig. 4B). While the increase was not statistically significant, the overall higher vitB₆ content aligns with the observed normalization in root elongation.

Expression analysis for *de novo* and salvage pathway genes

Due to the observed changes in root vitB₆ levels, we analyzed the expression of the four *PDX* genes and two salvage pathway genes (*SOS4* and *PPOX*) in C24 and the respective mutant backgrounds to assess their correlation with vitB₆ content. We focused on roots because the *sur* mutants were identified through a root-based screening system, and vitB₆ changes were more pronounced in roots than in shoots (Fig. 4).

Gene expression levels were normalized to C24 (= 1) to facilitate better comparison of up- or downregulation relative to the wild-type. As shown in Fig. 5A–D, expression of *PDX1.1*, *PDX1.2*, and *PDX1.3* was largely comparable between *rsr4-1* and C24, although

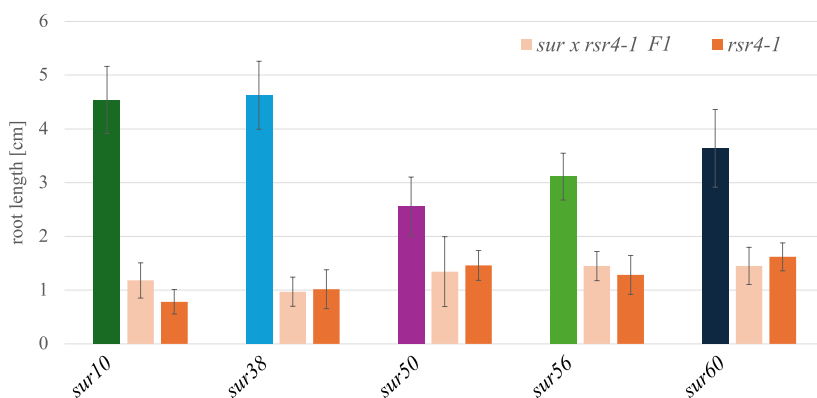


Fig. 3. Backcrossing analysis with *sur* mutants. The different *sur* mutants were backcrossed into their parent mutant *rsr4-1*. Seedlings were grown in sterile culture for 10 days on vertical plates. In the F1 generation, all *sur* seedlings had short roots comparable to *rsr4-1* indicating the recessive nature of the different suppressor mutations. *rsr4-1*, *n* = 10; *sur38*, *n* = 13; *sur10*, *sur56*, *n* = 14; *sur50*, *sur60* and *rsr4-1* × *sur*, *n* = 15. Error bars indicate standard deviation.

Table 1. Mean root length in [cm] of F1 generation *sur* × *sur* crosses compared to C24 wild-type. All suppressor mutant crosses developed in the F1 generation a short, *rsr4-1*-like root, indicating that the suppressor mutants are not allelic to each other. SD, standard deviation.

	F1 root length	C24 root length	<i>p</i> -value
mean	0.674	3.208	< 0.01
SD	0.176	0.379	
<i>n</i>	17	16	
mean	1.394	3.714	< 0.01
SD	0.633	0.424	
<i>n</i>	6	9	
mean	0.578	4.107	< 0.01
SD	0.136	0.267	
<i>n</i>	15	10	
mean	1.441	4.008	< 0.01
SD	0.366	0.809	
<i>n</i>	19	15	
mean	1.018	3.824	< 0.01
SD	0.206	0.186	
<i>n</i>	9	6	
mean	1.262	3.351	< 0.01
SD	0.35	0.844	
<i>n</i>	31	14	
mean	0.816	4.003	< 0.01
SD	0.249	0.543	
<i>n</i>	23	15	
mean	0.913	3.426	< 0.01
SD	0.28	0.389	
<i>n</i>	18	10	
mean	1.807	3.819	< 0.01
SD	0.644	0.587	
<i>n</i>	5	13	
mean	2.088	4.203	< 0.01
SD	0.388	0.709	
<i>N</i>	34	13	

PDX2 was significantly reduced in *rsr4-1*. In *sur10* and *sur38*, *PDX1.1* and *PDX1.2* expression levels were also similar to the wild-type. In contrast, all other mutants exhibited significant upregulation of *PDX* genes, with the strongest increases observed in *sur50* and *sur56* (Fig. 5A–D). Notably, *PDX2* expression was nearly 800-fold higher than C24 in *sur50* and approximately 400-fold higher in *sur56*. Analysis of the two salvage pathway genes *PPOX* and *SOS4* showed comparatively modest changes relative to the *PDX* genes (Fig. 5E,F). *SOS4* expression was slightly downregulated in *rsr4-1* and *sur60* compared to C24, upregulated in *sur38*, *sur50*, and *sur56*, and unchanged in *sur10*. *PPOX* expression was significantly altered only in *sur10* (decreased) and *sur50* (increased), while remaining similar to C24 in the other mutants. Overall, RT-qPCR analysis supports a link between the *sur*

mutations and vitB₆ metabolism, as substantial transcriptional changes were observed in the *sur* mutants.

Comparative analysis of biological pathways impacted in *rsr4-1* versus C24

rsr4-1 plants have only been described phenotypically [15,19,22], and the biological pathways that are impacted in the mutant are currently unknown. To better understand underlying mechanisms that are potentially responsible for phenotypic changes in *rsr4-1*—and which we hypothesized may also be beneficial for better understanding the *sur* mutants—we performed a comparative proteomic analysis of the *rsr4* mutant's roots and shoots relative to the wild-type's tissues from 14-day-old seedlings. This analysis identified 3211 proteins in the roots and 2334 in the shoots (Fig. S2A, Fig. S3A), of these, 353 and 254, respectively, were identified as differentially expressed (Fig. S2B,C; Data S1). As expected, abundance of *PDX1.3* was significantly less in *rsr4-1* relative to wild-type in both tissues (Fig. S2D). To identify global pathways and processes that may be important for the phenotypes, ClueGO [30] was used to explore enrichment of gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with proteins that statically differed in abundance between *rsr4-1* and WT (Fig. 6). For clarity, each dataset (root or shoot) was divided into proteins of higher abundance in *rsr4-1* or higher abundance in WT.

Here, the top GO terms and KEGG pathways identified as enriched in *rsr4-1* roots were 'branched-chain amino acid biosynthesis (leucine, isoleucine, and valine) and transmembrane water transport' and negative regulation of protein biosynthesis, modification, or degradation (annotated as 'negative regulation of protein metabolic processes') (Fig. 6A). In contrast, terms associated with peroxisomal activities and sulfur compound and toxin catabolic processes were less enriched in the *rsr4-1* roots compared to WT which are linked, for example, to lipid metabolism, detoxification processes, redox homeostasis, and stress responses (Fig. 6B).

In shoots, major pathways that were more pronounced in *rsr4-1* were 'glycosyl compound catabolic processes' which include breakdowns of glucose and glycosides as well as storage compounds, such as starch. Processes related to actin filament depolymerization, which impacts the cytoskeleton and secretory pathways [31], were also identified as enriched. Interestingly, also 'P-bodies' or 'Processing-bodies' were more abundant in *rsr4-1* shoot dataset (Fig. 6C).

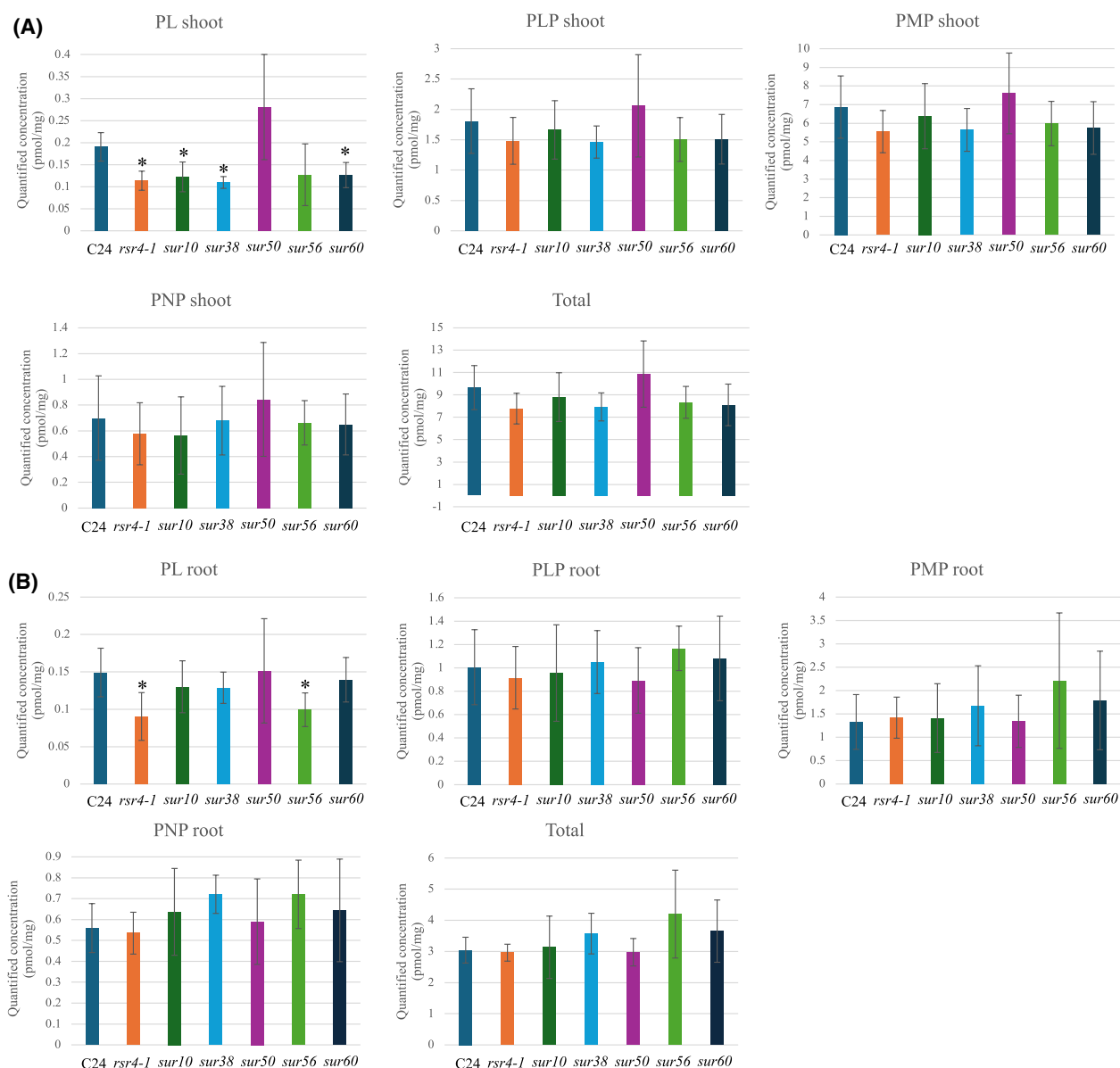


Fig. 4. VitB₆ measurements in C24, *rsr4-1*, and *sur* mutants. Quantitative measurements for pyridoxal (PL), pyridoxal 5'-phosphate (PLP), pyridoxamine 5'-phosphate (PMP), pyridoxine 5'-phosphate (PNP), and their summed-up amounts are shown for (A) shoot and (B) root in 2-week-old seedlings. Measurements are based on $n = 5$ biological replicates. Significance in this graph is calculated for *rsr4-1* and *sur* mutants versus C24 wild-type (*, $P < 0.05$). Significance was calculated using a Kruskal–Wallis test. Error bars indicate standard deviation.

P-bodies are nonmembranous granules in the cytoplasm of eukaryotic cells that contain mRNA molecules that are not being translated, as well as the proteins and enzymes required for mRNA metabolism [32] (Fig. 6C). Enriched terms identified in the WT shoots dataset were 'ribonucleotide triphosphate metabolic processes' which relates to potential heightened needs for energy (ATP), cell signaling, or RNA synthesis. In addition, photosynthetic related processes were represented higher in WT compared to *rsr4-1* which

agrees with the mutant's reduced chlorophyll content [15]. In addition, 'antioxidant activities' were lower in *rsr4-1* which implies a potentially reduced stress tolerance in these plants (Fig. 6D). Overall, the enriched GO terms and KEGG pathways associated with the proteins identified as differentially expressed are generally in agreement with a suggested role of vitB₆ in the broader cellular metabolism and as an important metabolite that impacts stress tolerance and photosynthetic capacities in plants [1,33]. The data also showed

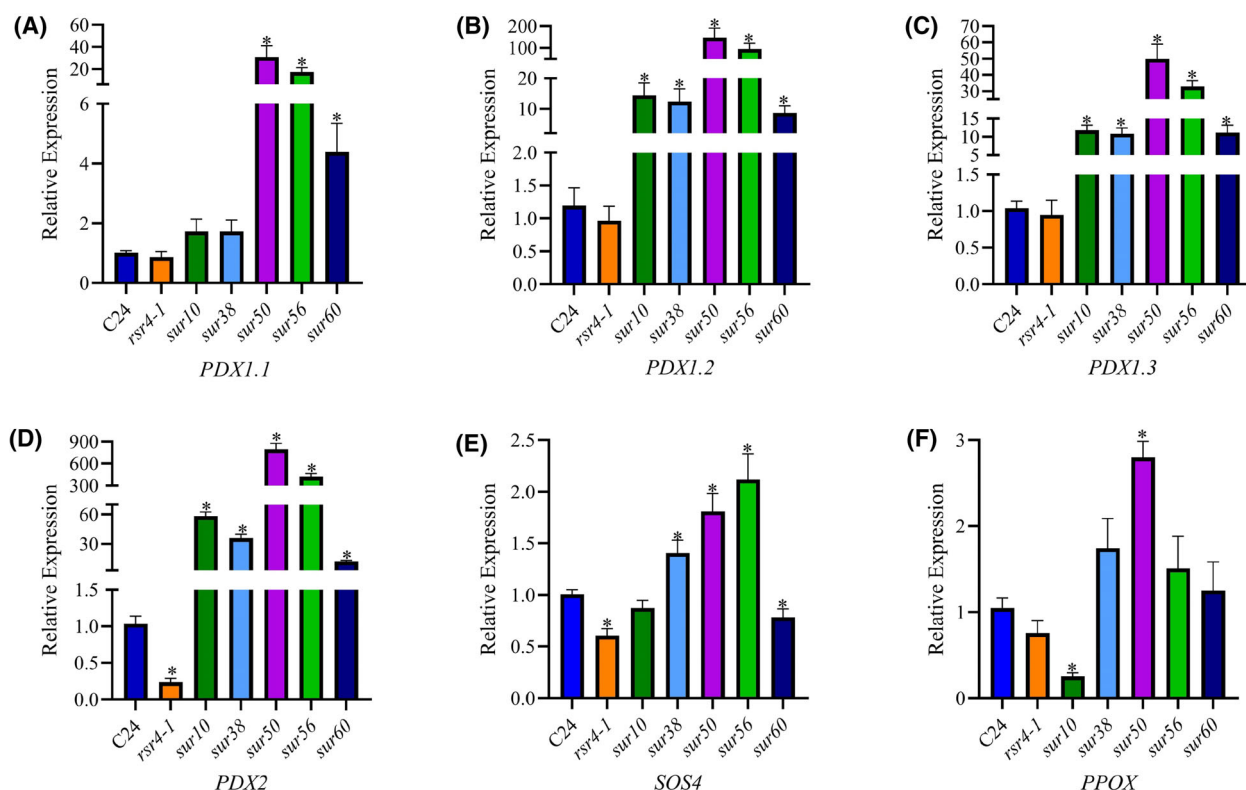


Fig. 5. RT-qPCR analysis in roots of C24, *rsr4-1*, and the different *sur* mutants. (A) *PDX1.1*, (B) *PDX1.2*, (C) *PDX1.3*, (D) *PDX2*, (E) *SOS4*, and (F) *PPOX* gene expression. Data were normalized to *ACTIN2*. All expressions were plotted relative to C24 (= 1). Data are based on $n = 3$ biological replicates. Error bars show standard error. Significance was calculated with a Student's *t*-test (*, $P < 0.05$).

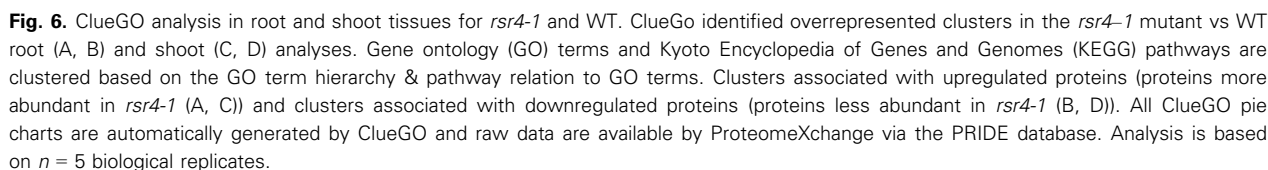
that root and shoot tissues are differently impacted by the loss of *PDX1.3* function.

Cluster analysis to identify proteins of interest in *rsr4-1* with C24 comparison

To identify potential key proteins with significantly altered abundances, we performed comparative analyses of peptides identified in the roots and shoots of the five *sur* mutants relative to C24 (Fig. S2A–D) and *rsr4-1* (Fig. S3A–D; Data S1). Except for *sur50*, all lines had significantly less *PDX1.3* than the WT line (Fig. S2D); while not significant, *PDX1.3* was also less abundant in *sur50*. Differences in *PDX1.3* abundance between *rsr4* and the *sur* lines were mostly not significantly different, apart from the shoots of *sur38* and *sur50* and the roots of *sur60*, all of which had more abundant *PDX1.3* (Fig. S3D).

We performed a network and cluster analysis on comparative proteomic data from root and shoot tissues for C24, *rsr4-1*, and the *sur* mutants to identify potential key candidate proteins. All proteins identified across all analyses were compiled, including

proteins whose peptides were identified in at least one sample but not quantified due to the limitations of mass spectrometry. The comprehensive list was then used as input for the STRING Cytoscape application [34] to generate a network based on high confidence protein–protein associations. The network consisted of 5708 proteins with 75 153 high confidence edges. Clustering of the STRING network identified five RSR4/*PDX1.3* containing clusters (network can be accessed at NDex, Table S1). Of these, two were of particular interest: Cluster 4, because it contained the largest number of unique proteins, and Cluster 2, because it contained vitB₆ salvage pathway proteins (Figs. 7 and 8). These figures were colored to visualize the expression data for all comparative proteomic analyses (Data S1). Because clusters represent groups of proteins likely involved in related biological functions, the overlap observed between clusters 2 and 4 suggests that they play complementary roles in vitB₆ biosynthesis. The proteins shared by both clusters—*PDX1.1*, *PDX1.2*, RSR4/*PDX1.3*, and *PDX2*—are all key components of the *de novo* vitB₆ biosynthesis pathway, directly linking both clusters to this process.



As expected, the abundance of PDX1.3/RSR4 was significantly lower in both root and shoot in *rsr4-1* compared to the C24 WT. Our cluster analysis highlighted several differentially expressed proteins identified across our comparisons, as visualized in Figs 7 and 8. In the *rsr4-1* vs C24 comparison,

ribokinase, RBSK, which catalyzes the phosphorylation of ribose to R5P [35], as well as At1g63290, a predicted ribulose-phosphate 3-epimerase, that is involved in the Calvin-Benson cycle and the oxidative pentose phosphate (OPP) pathway [36,37] were identified as less abundant in the roots and shoots of *rsr4* relative to C24. Only two proteins, PGD1 and 2, were higher in abundance in *rsr4-1* root and shoot relative to C24: PGD1 and 2. Both are 6-phosphogluconate dehydrogenases that facilitate the oxidative decarboxylation of 6-phosphogluconate to R5P and CO₂ and are located either in the cytosol and chloroplast (PGD1) or strictly in the cytosol (PGD2) [38].

In roots, four proteins were reduced in *rsr4-1*: the salvage pathway enzyme SOS4, as well as EMB3119, RPE, and At3g01850 (Fig. 7). EMB3119 is a plastid R5P isomerase that catalyzes R5P conversion to ribulose-5-phosphate (Ru5P) [39]. RPE (ribulose-5-phosphate-3-epimerase) is a plastid protein required for the reversible epimerization of Ru5P to D-xylulose 5-phosphate [40], and At3g01850, like At1g63290, is a predicted ribulose-phosphate 3-epimerase [36,37]. Three proteins were present in higher abundance in root: PDX1.2, RSW10, and PGM3. As mentioned above, PDX1.2 is proposed to impact PLP synthase stability and activity [12,27]. RSW10 (Radial Swelling 10) encodes for a cytosolic R5P isomerase required for the biosynthesis of uridine and UDP-sugars [41]. PGM3 is a cytosolic phosphoglucomutase that participates in the breakdown and synthesis of glucose [42]. VitB₆ has also been brought in context with phytohormone homeostasis, specifically auxin and ethylene, and root development in the past [6,7]. However, we did not see clear correlations in *rsr4-1* or the *sur* mutants in our cluster analysis here.

Of the proteins specifically altered in shoot, four proteins were less abundant in *rsr4-1* (TKL-1 and TKL-2, RPI2, and the vitB₆ salvage enzyme PPOX), and one was found to be in higher abundance in the mutant (PRK) (Fig. 8). TKL-1 and TKL-2 are both plastid-localized transketolases crucial for carbon flow in the Calvin-Benson cycle and OPP pathway to yield xylulose-5-phosphate and erythrose-4-phosphate or R5P [43]. RPI2 (Ribose-5-Phosphate Isomerase 2) is a cytosolic enzyme that, like EMB3119, catalyzes the reversible conversion of R5P to Ru5P [44].

Cluster analysis to compare *sur* mutants with *rsr4-1* and C24

Comparing the expression profiles of proteins in clusters 2 and 4 in our *rsr4-1* vs C24 analysis with the *sur* vs C24 and *sur* vs *rsr4-1* analyses, one can still observe

reduced RSR4/PDX1.3 amounts in the different *sur* mutants. However, the two clusters also revealed a few differences in root and shoot that we will specifically focus on (Figs. 7 and 8).

In *sur50* and *sur60* roots, we observe an increase in the *de novo* biosynthesis protein PDX1.1. This also correlated with higher amounts of RPE and PRK. PRK, like RPE, is plastid-localized and involved in the Calvin-Benson cycle where it functions as a phosphoribulokinase in the conversion of Ru5P to ribulose-1,5-bisphosphate [45]. In *sur10* a general trend appears to be that a range of proteins are less abundant compared to C24, such as PDX1.2, RSW10, PGM3, or PGI1 and 2 (Figs. 7 and 8). On the other hand, SOS4 and PDX1.1 appear to be normalized relative to WT in the *sur10*, while PPOX is reduced, indicating shifts in the *de novo* and salvage pathway activities. In *sur38*, the most striking change is a further increase in the R5P isomerase RSW10; otherwise, the expression profile looks similar to *rsr4-1*. Likewise, *sur56* does not show any dramatic changes relative to *rsr4-1*, but we see a normalization in SOS4 and RPE abundance to C24 levels, and a reduction in RBSK.

In shoots, several *sur* mutants exhibit changes in the *de novo* and/or salvage pathway enzymes. For example, normalization in PDX1.1 levels relative to C24 is present in *sur56* and *sur60* and elevated abundances are detectable in *sur10*, *sur38*, and *sur50*; with *sur50* even having an increased abundance of PDX2 (Fig. 8). Likewise, *sur10*, *sur50*, and *sur56* show increased levels in SOS4, while *sur50* and *sur56* also have higher PPOX amounts. Otherwise, noteworthy is a shift toward increased At1g63290 abundance in *sur38*, *sur50*, and *sur56* (Fig. 8).

Phenotypic description of *sur* mutants

The *sur* mutants not only varied in their primary root length but also showed significant differences with respect to their shoots (Fig. 9A). *rsr4-1* leaf blades were overall shorter and slimmer compared to C24, and in that respect *sur50*, *sur56*, and *sur60* had a similar phenotypical appearance. Only *sur10* and *sur38* leaves had wider, C24-like blades, although the total leaf area was only normalized for *sur38* (Fig. 9B).

rsr4-1 was originally described as a late flowering mutant [15]. This phenotype was only reversed in *sur10*, while all other mutants were still significantly delayed in flowering time compared to C24, with *sur38* having the longest delay compared to *rsr4-1* (Fig. 10A). This flowering phenotype correlated with rosette leaf numbers. Whereas C24 had on average around 50 leaves at the time of flowering, *rsr4-1* plants

rsr4-1 vs *sur* mutants

WT vs mutants

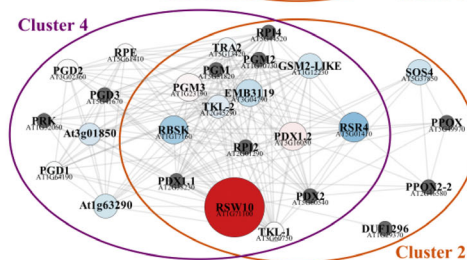
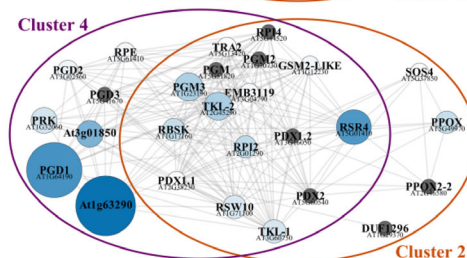
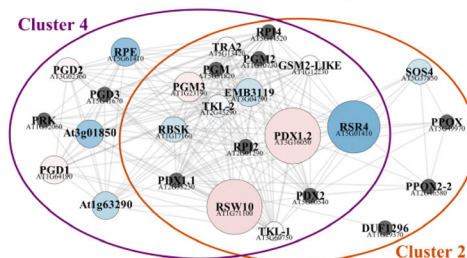
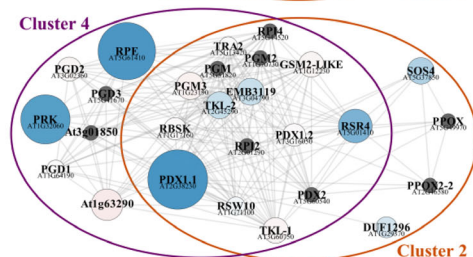
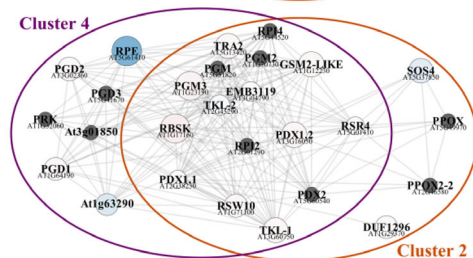
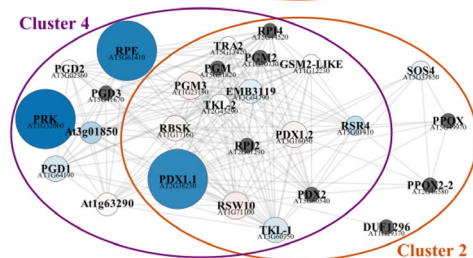
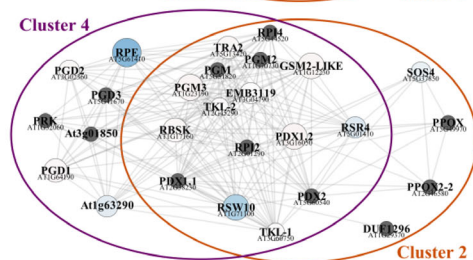
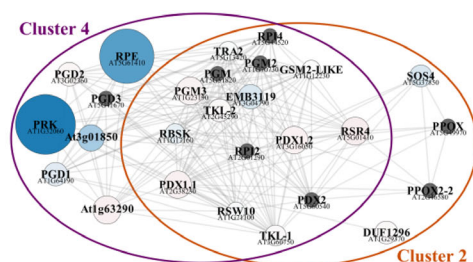
*rsr4-1**sur10**sur38**sur50**sur56**sur60*

Fig. 7. Cluster analysis for root-based proteomics in 12-day-old C24, *rsr4-1*, and *sur* mutant seedlings. Comparison of protein abundance in the roots of the *rsr4-1* line relative to the *sur* mutants (*rsr4-1*/mutant; left) and mutant lines relative to wild-type (mutant / WT; right). Nodes are colored to represent abundance, white: abundance = 1, shades of blue: abundance < 1, shades of red: abundance > 1. Gray nodes represent proteins that were not quantified due to the limitations of MS. Size of nodes inversely represent *p*-value. Data are based on *n* = 5 biological replicates.

reached about 75 leaves (Fig. 10B). As was the case for the normalized flowering time, *sur10* rosette leaf numbers were comparable to C24. *sur56* and *sur60* were in the range of *rsr4-1*, but *sur38* and *sur50* reached close to 100 leaves per plant, a significant increase even compared to *rsr4-1* (Fig. 10B). Finally, measuring total heights based on inflorescence shoot length showed that *rsr4-1* was significantly shorter than C24. This phenotype was only reversed in *sur38*, whereas the other *sur* mutants developed shoot heights comparable to *rsr4-1* (Fig. 10C).

Overall, these data demonstrate that the impact of the different suppressor mutations on *sur* development is quite variable. While the root growth phenotype was normalized to C24 levels in all *sur* mutants, suppression of the aberrant shoot phenotypes characteristic for *rsr4-1* was not consistent in all *sur* mutants. Largely, *sur10* appears to be the mutant that showed the most normalized root and shoot development comparable to the C24 wild-type. Surprisingly, this was not the case for *sur50*, which was the only mutant with normalized PL levels in the shoot, and had even slightly increased total vitB₆ levels.

Photosynthetic parameters

It was previously described that *pdx1.3* mutants with reduced vitB₆ levels are also impacted in their photosynthetic capacity [21]. Consequently, we analyzed C24, *rsr4-1*, and the *sur* mutants for the maximum quantum efficiency of photosystem II (Fv/Fm). In agreement with data from *pdx1.3*, the C24 Fv/Fm values were significantly higher compared to *rsr4-1*, and this was also the case for *sur38* and *sur56*. However, values were normalized close to C24 for *sur10*, *sur50*, and *sur60* (Fig. 11A and B), which was surprising since PL shoot levels in *sur10* and *sur60* were reduced to *rsr4-1* levels (Fig. 3). We did not see any change in nonphotochemical quenching (NPQ) levels for *rsr4-1* or the different *sur* mutants (Fig. S4).

Because heat shock has been linked with *PDX* expression and protein stability [28,29,46], we applied a 2-h heat shock treatment (45 °C) to see whether sensitivity and recovery from this stress is different among C24, *rsr4-1*, and the *sur* mutants. As expected, the

overall Fv/Fm values plummeted in all genetic backgrounds, and all plants showed recovery from this stress over the following three days (Fig. 11C). However, when overall reduction of maximum quantum efficiency of photosystem II was compared before and after heat shock treatment with the *before* values set to 100%, one could observe that C24 and *sur60* showed the highest reduction in Fv/Fm values (~19% reduction 24-h poststress treatment), while *rsr4-1* and the other *sur* mutants had a less pronounced reduction (Fig. 11C). Only *sur38*, with an average reduction of ~13%, was significantly different from C24 (Fig. 11D). Still, this is noteworthy, since differences in maximum quantum efficiency among the mutants did not strictly correlate with the degree of Fv/Fm reduction after heat shock application.

Abiotic stress sensitivities of *rsr4-1* and *sur* mutants

VitB₆ contents and mutations in *PDX1.3* have repeatedly been linked to abiotic stress tolerance [5,18,20,21,28,46,47]. We assessed heat stress sensitivity by exposing 10-day-old seedlings to 45°C for different durations (0, 30 min, 1 h, 1.5 h, 2 h, and 2.5 h) and looked for heat stress survival over the next couple of weeks. However, these heat stress experiments did not yield conclusive results when comparing the *sur* mutants to C24 or *rsr4-1*.

In the following, we focused on germination rates when seeds were exposed to salt (NaCl) and osmotic stress (mannitol). Under control conditions (ATS medium without supplements), no aberrant germination behavior was observed (Fig. 12). *sur50* and *sur60* tended to germinate slightly slower than the other lines; however, these differences were not statistically significant.

In the presence of 125 mM NaCl, *rsr4-1* and all *sur* mutants, except *sur10*, were hypersensitive to the salt treatment (Fig. 12B). Germination was slowed down and overall reduced, with *sur60* showing a tendency of being even more sensitive to NaCl compared to *rsr4-1* (Fig. 12B). In contrast, salt-sensitivity was fully normalized in *sur10* with germination comparable to C24 (Fig. 12C).

rsr4-1 vs *sur* mutants

WT vs mutants

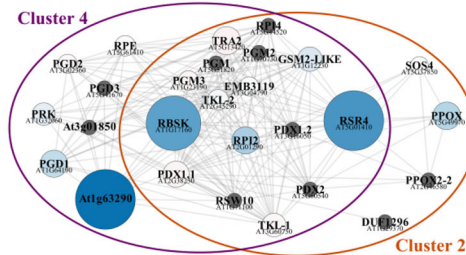
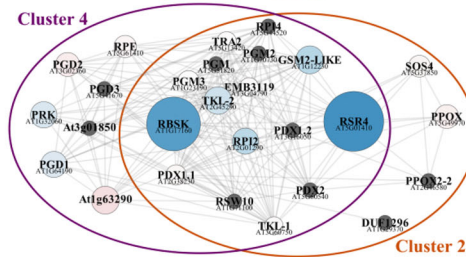
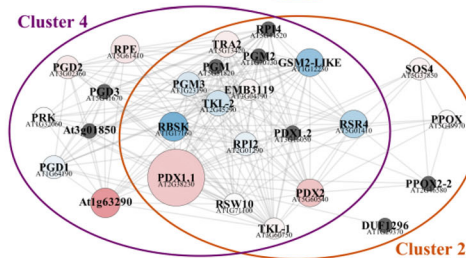
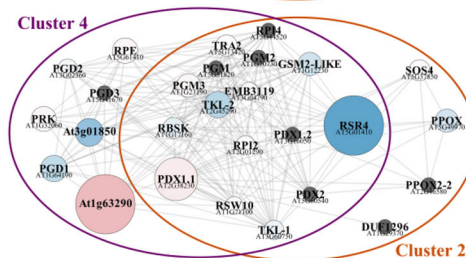
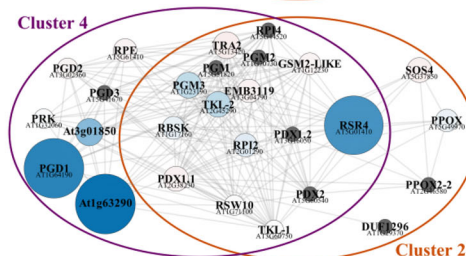
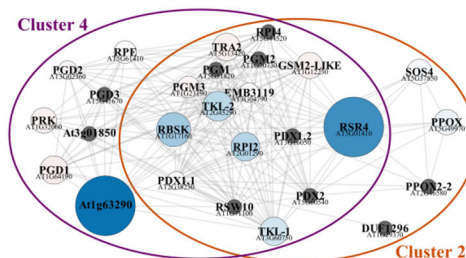
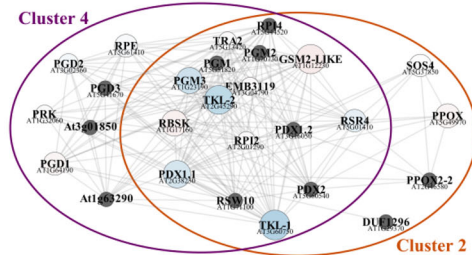
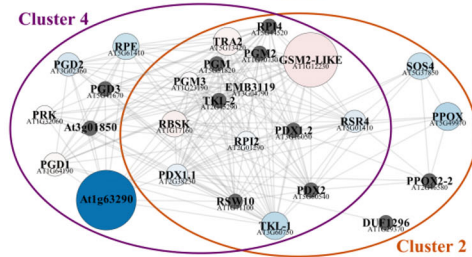
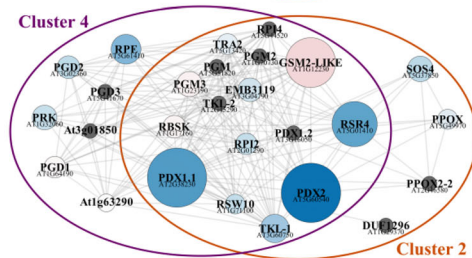
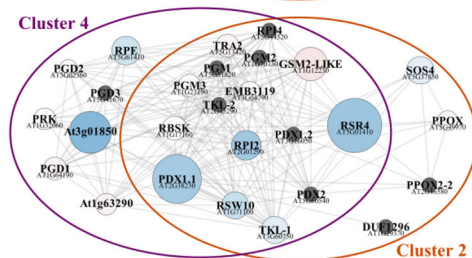
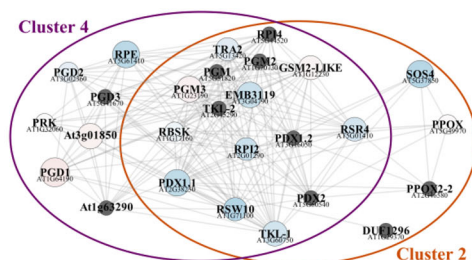
*rsr4-1**sur10**sur38**sur50**sur56**sur60*

Fig. 8. Cluster analysis for shoot-based proteomics in 12-day-old C24, *rsr4-1*, and *sur* mutant seedlings. Comparison of protein abundance in the shoots of the *rsr4-1* line relative to the *sur* mutants (*rsr4-1*/mutant; left) and mutant lines relative to wild-type (mutant/WT; right). Nodes are colored to represent abundance, white: abundance = 1, shades of blue: abundance < 1, shades of red: abundance > 1. Gray nodes represent proteins that were not quantified due to the limitations of MS. Size of nodes inversely represent *P*-value. Data are based on *n* = 5 biological replicates.



Fig. 9. Shoot phenotype of C24, *rsr4-1*, and the different *sur* mutants. (A) Photo of three 4-week-old representative plants grown in soil (scale bar = 1 cm), and (B) quantified leaf area. Data in (B) are based on the average of the three largest leaves of *n* = 5 plants. Significance in this graph is calculated using a Student's *t*-test for *rsr4-1* and *sur* mutants versus C24 wild-type (*, *P* < 0.05). Error bars show standard deviation.

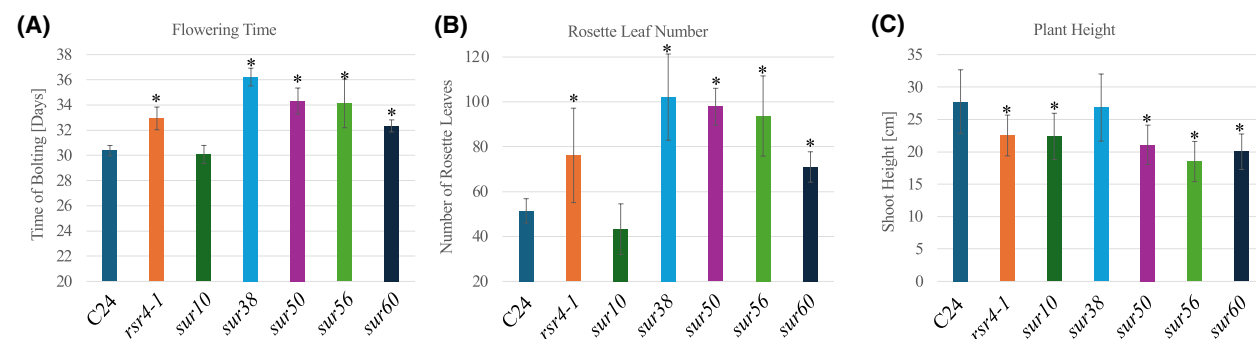


Fig. 10. Flowering time, rosette leaf number, and plant height for C24, *rsr4-1*, and the different *sur* mutants. (A) Average time of first floral bud appearance (time of bolting). The flowering time of *n* = 20 plants/biological replicate was measured for each suppressor mutant, *pdx1.3*, and C24 wild-type. Data show the average of *n* = 3 biological replicates. (B) Average number of rosette leaves for *n* = 7 plants. (C) Average height of *n* = 12 plants at the end of the flowering stage. Asterisks indicate significant differences (*P* < 0.05) to the C24 genotype. Significance in this graph is calculated using a Student's *t*-test for *rsr4-1* and *sur* mutants versus C24 wild-type. Error bars show standard deviation.

More variation was observed when the *sur* mutants were exposed to 250 mM mannitol. Here, *rsr4-1* showed hypersensitive behavior (Fig. 12A), but three of the *sur* mutants, *sur10*, *sur50*, and *sur56*, displayed an intermediate phenotype with germination speed being in between C24 and *rsr4-1* (Fig. 12D). In the

first days of germination, *sur60* showed an increased hypersensitivity to osmotic stress but ultimately reached the same germination rate as *rsr4-1* (~80%) (Fig. 12E), while *sur38* was the only *sur* mutant that showed a fully normalized germination rate that was indistinguishable from the C24 wild-type (Fig. 12F).

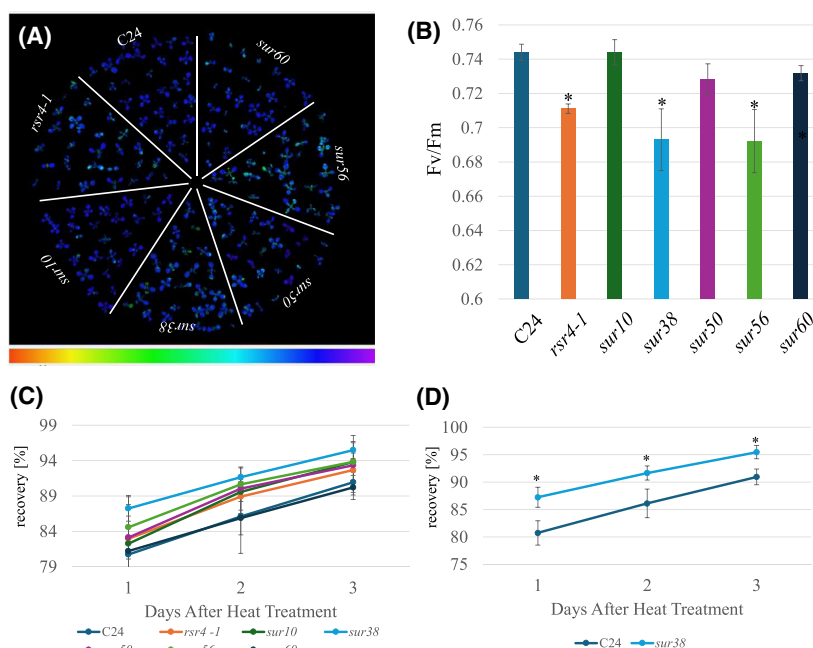


Fig. 11. Maximum quantum efficiency of photosystem II measurements on C24 and the different mutant lines. (A) Example plate of C24, *rsr4-1*, and *sur* seedlings. The image was taken with the IMAGING-PAM chlorophyll fluorescence system camera, and the seedlings are colored by the machine's ImagingWin software based on the calculated Fv/Fm ratios. (B) Data show average Fv/Fm values from six independent plates with $n = 5$ seedlings from each plate were used in the calculations. (C) Average percent recovery of Fv/Fm compared to prestress baseline measurement for all mutants and C24 wild-type. (D) Average percent recovery of Fv/Fm compared to prestress baseline measurement for *sur38* and C24 wild-type. Significance in this graph is calculated using a Student's *t*-test for *rsr4-1* and *sur* mutants versus C24 wild-type. Asterisks indicate significant differences ($P < 0.05$) to the C24 genotype. Error bars indicate standard error.

The salt and osmotic stress treatments strongly point out that the different suppressor mutations have very different impacts on stress tolerance, and further support that the *sur* mutants are impacted in different alleles.

Discussion

In this work, we describe five novel mutants carrying suppressor mutations that alleviate the short root phenotype of *rsr4-1*. Analysis of their vitB₆ content revealed normalized PL levels in roots for four of the five mutants, along with changes in other B₆ vitamers in both root and shoot tissues. Expression analysis of *de novo* and salvage pathway genes revealed significant changes compared to the wild-type and *rsr4-1* in the *sur* mutants. A proteomic analysis identified major processes affected in *rsr4-1* relative to the wild-type, while cluster analysis across all mutant backgrounds and C24 provided insights into changes in proteins functionally linked to RSR4/PDX1.3. Together with a detailed phenotypic characterization of the five *sur* mutants, these data show that while suppressor mutations can normalize *rsr4-1* root growth, they exert

highly diverse effects at both molecular and whole-plant levels.

ClueGO analysis highlighted major pathways affected in *rsr4-1* but also revealed broad pleiotropic changes, consistent with the wide-ranging roles of vitB₆ in cellular metabolism [1]. In shoots, the most notable changes involved photosynthetic processes, matching the *rsr4-1* phenotype and its reduced chlorophyll content [15], and aligning with previous findings that vitB₆ biosynthesis mutants show increased light sensitivity [21]. Also noteworthy were decreased 'antioxidative activities' and increased 'water transmembrane transporter activity' in *rsr4-1* shoots, which may contribute to the heightened salt and osmotic stress sensitivities first described in this work. In roots, the general downregulation of 'protein metabolic processes' likely reflects a disturbance in amino acid metabolism, supported by the upregulation of 'branched-chain amino acid biosynthesis' and processes involving the 'glutamine family' and 'nitrogen metabolism'. Although measured only in shoots, earlier work showed many amino acids are elevated in *rsr4-1* [15], indicating that the mutant is affected in general amino acid metabolism. But surprisingly, not necessarily in a way that

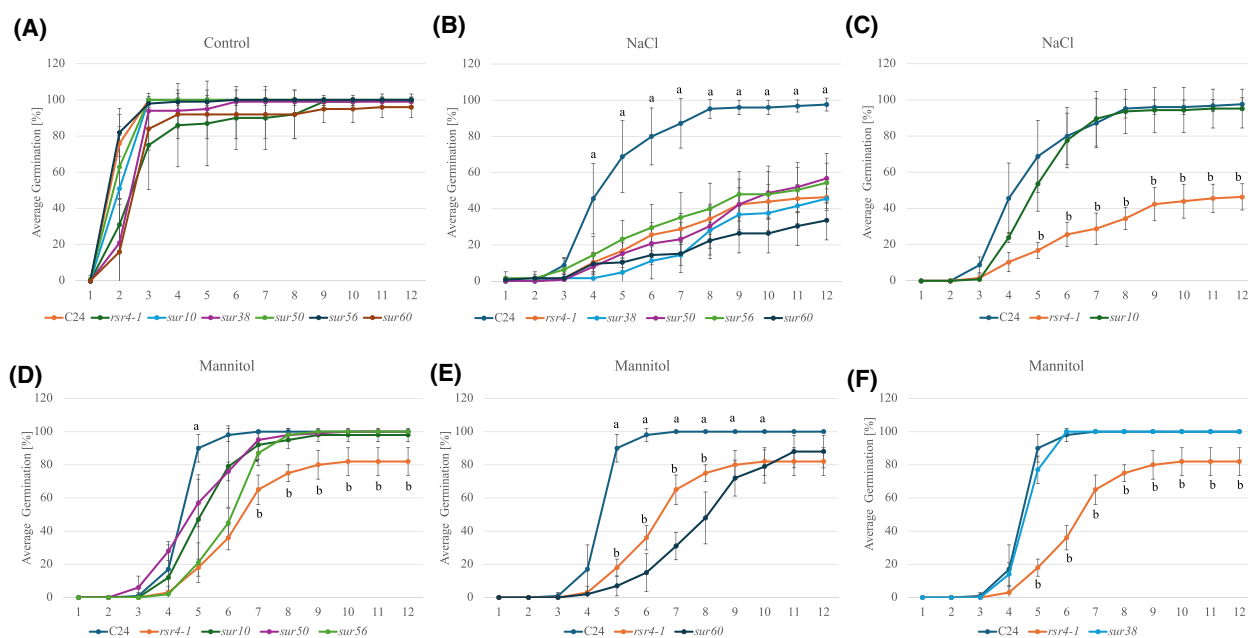


Fig. 12. Germination assays in relation to salt and osmotic stress. Germination of C24, *rsr4-1*, and the *sur* mutants on (A) control plates that lack any additional supplements, (B, C) plates containing NaCl (150 mM) and (D–F) plates supplemented with mannitol (250 mM). Data are based on $n = 5$ biological replicates with at least 20 seeds per replicate. The 'a' letters indicate significant differences ($P < 0.05$) of the *sur* mutants to the C24 wild-type, while 'b' letters indicate significant differences ($P < 0.05$) of *sur* mutants to *rsr4-1*. Significance was calculated using a Student's *t*-test. Error bars indicate standard deviation.

would result in a downregulation of amino acid biosynthesis, despite the critical role of many PLP-dependent enzymes that contribute to this process [48]. However, other processes—such as 'peroxisome', 'actin filament depolymerization' or 'P-body' for example—lack clear connections to PDX proteins or vitB₆ function and may represent indirect or pleiotropic effects.

The vitB₆ contents are consistent with Wagner *et al.* [15], who reported reduced PL levels in *rsr4-1*. Only *sur50* showed restored PL levels in both shoot and root, while the other mutants displayed increased PL levels exclusively in roots. *sur56* was notable for elevated total vitB₆ in roots due to increases in PLP, PMP, and PNP, while PL itself remained unchanged relative to *rsr4-1*. The strong correlation between normalized vitB₆ levels and improved root growth in the *sur* mutants supports the idea that altered root growth—used here as the selection criterion—is an effective strategy for discovering regulatory factors in vitB₆ homeostasis. However, cases like *sur50*, which exhibited increased vitB₆ levels without fully rescuing the *rsr4-1* phenotype, indicate that vitB₆ levels alone cannot explain the *rsr4-1* developmental defects. Notably, although *rsr4-1* is affected in PDX1.3, a key *de novo* enzyme required for PLP production, levels of PLP and other B₆ vitamers were not significantly different

from wild-type. This raises the possibility that PDX1.3, alone or within PDX complexes, may play additional roles in plants beyond vitB₆ biosynthesis.

Cluster analysis, together with vitB₆ measurements, revealed that: (i) root phenotype normalization generally coincides with normalized PL content; and (ii) this correlates with changes in PDX1.1, PDX2, and/or salvage pathway enzyme abundance. It remains unclear whether these changes arise from altered gene expression or altered protein stability. Since all PDX proteins were previously shown to be unstable in cell-free degradation assays [29], altered abundance may reflect changes in proteolytic activity. This includes PDX1.3^{G54S}, which was consistently reduced in *rsr4-1* and the various *sur* mutants. Interestingly, many proteins related to carbohydrate metabolism—especially enzymes involved in the production of the PLP precursors R5P and Ru5P—also changed in abundance across several *sur* mutants. Several of these proteins are localized to plastids, where *de novo* B₆ biosynthesis does not occur [10], suggesting that plastidic metabolic processes may nonetheless exert a significant influence on vitB₆ homeostasis.

An interesting finding was the extreme upregulation of *PDX* gene expression in some of the *sur* mutants.

However, the magnitude of these changes did not correlate with vitB₆ levels in the mutants, nor were they consistent with the differences in protein abundance observed in the proteomic analysis. This suggests that transcript levels do not directly correspond to protein activity and/or that additional regulatory mechanisms contribute to vitB₆ homeostasis. Previous studies have shown that increased *PDX* expression in rice, potato, and *Arabidopsis* can lead to elevated levels of individual B₆ vitamers [16] as well as an overall increase in vitB₆ content [49–51]. However, the findings in the *sur* mutants indicate that additional layers of regulation are involved, and that simple overexpression may not be sufficient to control vitB₆ levels in plants. This is further supported by recent evidence highlighting the importance of *PDX* protein stability [29] and the potential role of *PDX1.2* as a regulator of PLP synthase activity [12].

Phenotypic analyses further showed that the suppressor mutations have diverse effects and usually do not restore the shoot phenotype. Regarding stress responses, *rsr4-1* is shown here for the first time to be hypersensitive to salt and osmotic stress, and only *sur10* and *sur38* showed normalization of salt and mannitol sensitivity, respectively, while the other *sur* mutants were still hypersensitive to the applied stressors. Overall, these findings, again, suggest that vitB₆ levels alone cannot explain the severity of the *rsr4-1* phenotypes.

The next essential step in this research will be to clone the affected genes and analyze how they influence plant growth and vitB₆ homeostasis in *Arabidopsis*, in order to better understand why the PDX1.3^{G54S} point mutation in *rsr4-1* causes such pronounced developmental changes.

Materials and Methods

Plant material and growth

Arabidopsis thaliana (C24 ecotype) was used for all experiments. For sterile culture, surface-sterilized seeds were cold-treated at 4 °C, then plated on *Arabidopsis* growth medium (ATS) as described in [52] without additional sugar supplementation if not otherwise mentioned. Plants were grown at 20 °C under long-day conditions (16 h light: 8 h dark) either under sterile conditions on ATS medium in a growth chamber (Model TC-19, Encornair Ecological Chambers, Winnipeg, Canada) or in soil (Sunshine Mix #1, SunGro Horticulture, MA) in a standard greenhouse. Soil-grown plants were fertilized weekly with Water-Soluble All-Purpose Plant Food, Miracle-Gro, OH.

EMS mutagenesis

For ethyl methane sulfonate (EMS) -based mutagenesis around 50 000 *rsr4-1* seeds (~ 1 g) were incubated for 16 h in a 0.25% EMS solution (Sigma-Aldrich, MO) at room temperature. After 16 h incubation time, seeds (=M1) were thoroughly washed with distilled water. All flow-through and the original EMS solution were treated with 1 M NaOH (Sigma-Aldrich, MO) for 24 h to break down any remaining active EMS. M1 seeds were brought out on soil in 60 different pots, each containing ~ 800 seeds. After selfing, the resulting M2 seeds were brought out in horizontal lines on 10 × 10 cm square culture plates (Corning, NY) containing ATS medium in 1% agar w/o pyridoxine and sugar supplement. Plates were kept vertically to easily identify seedlings with longer root growth. Any seedlings displaying longer roots 10 days after germination were picked, transferred into individual pots in soil, and named (1) *sur* for suppressor of *rsr4-1*, and (2) after the pool they were found in, for example, *sur38*. All *sur* mutants were backcrossed at least twice into the *rsr4-1* parental background to outcross any additional mutations that are not responsible for the primary *sur* root phenotype before any further phenotypical analysis was performed.

Genomic DNA extraction, and polymerase chain reaction and sequencing

Genomic DNA was extracted using standard procedures as we described before [22]. PCR was done on a DNA Engine cycler (Bio-Rad Laboratories, Hercules, CA) (35 cycles, denaturing 15 s, 95 °C; annealing 30 s, 55 °C; elongation 1 min, 72 °C) using Platinum Taq polymerase (Invitrogen, Waltham, MA). PCR products were run on a 0.8% agarose gel and purified using a Qiagen 1x QIAEX II Gel Extraction Kit (Hilden, Germany) according to the manufacturer's instructions. Sequencing samples was outsourced to WSU's sequencing core facility. For a complete list of primers used, see Table S2.

Germination assays

For germination assays, sterilized seeds were plated on ATS medium containing 0.8% agar, supplemented either with or without NaCl (150 mM) or mannitol (250 mM). Plates were divided into seven sections: C24, *rsr4-1*, and the five suppressor mutants. Germination was defined as the time when radicles first emerged from the seed coat. The number of germinated seeds was recorded daily for 12 days after plating.

Root length assays and leaf area measurements

For root length assays, sterilized seeds were plated on ATS medium supplemented with 1% agar in 10 × 10 cm square

culture plates (Thermo Scientific; Waltham, MA) and grown vertically under standard growth conditions for easy analysis of root length. Primary root length was marked at 10-day postgermination and measured using ImageJ software [53]. Each plate was divided into three sections containing a *sur* mutant as well as *rsr4-1* and C24 wild-type as controls for root length comparison. For leaf measurements, plants were grown in soil under standard conditions for three weeks before representative mature sink leaves were selected for area analysis using ImageJ software [53].

Determination of flowering time

The beginning of flowering time was defined as the time when the first floral bud emerged from the rosette. C24, *rsr4-1*, and the five suppressor mutants were grown side by side in soil (groups of 20 per biological replicate) and under long-day conditions (see above). Plants were checked daily, and the day of first floral bud appearance was recorded for each plant.

Photosynthetic measurements

Heat stress assays were conducted on 10-day-old seedlings on sterile plates divided into seven sections: C24, *rsr4-1*, and the five suppressor mutants. At least 20 seeds were plated individually in each section. Plates were placed in a 45 °C incubator for two hours and then plates were returned to the previously mentioned growth chambers for 3 days. Controls were left at normal growth temperature. Fv/Fm was measured one day prior to heat stress, and once per day for three days after the heat stress. Fv/Fm was measured using the IMAGING-PAM MAXI chlorophyll fluorescence system from Heinz Walz GmbH (Effeltrich, Germany).

Extraction and quantification of B₆ vitamers

Extraction of vitB₆ vitamers was performed according to [23]. Plant tissue was finely ground in liquid nitrogen, to which 8 µL/µg of 5% trichloroacetic acid (TCA) was added. Samples were homogenized, centrifuged, and then passed through a 0.45 µm syringe filter (Omicron, Alpha-retta, GA). These steps were done in darkness as vitB₆ is susceptible to light-induced degradation. HPLC was performed to separate the B₆ vitamers using a Cadenza CD-C18 column (Imtakt, Portland, OR) and measured with a Waters 2475 fluorescence detector (Waters Corporation, Milford, MA). The mobile phase contained 0.1 M potassium dihydrogen phosphate and 0.1 M sodium perchlorate, pH 3.0, as described by [54]. For the phosphorylated vitamers, 0.5 g/L of the derivatizing agent sodium bisulfite was added to the above mobile phase. The excitation wavelength was 300 nm, and the emission wavelength was

400 nm. PM, PN, PL, and PLP standards were ordered from Sigma-Aldrich (St. Louis, MO), PMP was obtained from MedChemExpress (Monmouth Junction, NJ), and PNP was ordered from Biosynth (Compton, England).

mRNA isolation and RT-qPCR analysis

Total Arabidopsis RNA from root tissue (14-day-old seedlings grown in sterile culture on 1% ATS medium) was isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, MO). About 100 ng of total RNA was added to an AzuraQuant Green Fast qPCR Mix LoRox Kit (Azura Genomics, MA) and directly used for RT-qPCR. Reactions were done under standard conditions on a 7500 Fast Real-Time PCR system (Thermo Fisher, MA) as described earlier [55]. Gene expression analysis was done using *actin* as the internal control gene. To calculate relative gene expression, the 2^{-ΔΔC_T} method was used [56]. Three biological replicates were performed for each individual experiment. Primers used for RT-qPCR are listed in Table S2.

Proteomic analysis

For proteomic analysis, seedlings grew vertically for 14 days on ATS medium before they were harvested and shipped on dry ice to the University of Wisconsin – Madison for proteomic analysis. Tissue was ground in 150 µL of cold protein extraction buffer 230 mM sorbitol, 50 mM Tris/HCl pH 7.5, 10 mM KC, 1 mM PMSF, and 2 mM sodium vanadate, and 1 Pierce protease inhibitor mini tablet (catalog: A32953, Thermo Fisher Scientific, Madison, Wisconsin, USA). Root samples were briefly sonicated (< 1 s). Samples were centrifuged for 2 min (15 °C) and ground for a second time—this is an optional step that increases protein extraction efficiency. Samples were centrifuged for 5 min (15 °C), and the supernatant was transferred to a new low-bind tube. Protein was isolated from supernatant using a standard chloroform-methanol extraction protocol and resuspended in 50–150 µL of 8 M urea and 50 mM ammonium bicarbonate as determined by size of the protein pellet.

Protein concentrations were quantified using a BCA assay (catalog: #23225, Thermo Fisher Scientific) and a Thermo Fisher Scientific Nanodrop 2000. About 20 µg of protein was added to an overnight Trypsin/Lys-C digest following the manufacturer's protocol (catalog: #V5071, Promega, Madison, Wisconsin, USA). Peptides were cleaned using Sep-Pak C18 cartridges and following the manufacturer's protocol (catalog: WAT054955, Waters, Milford, Massachusetts, USA). Peptides were analyzed using a Thermo Fisher Scientific Orbitrap Fusion Lumos. Spectra files were analyzed using Proteome Discoverer (v.2.4.1.15). Five replicates were performed for each sample except for *sur60* which had four replicates. Default settings

were used for all programs. Student's *t*-test was used to calculate all significance values. Peptides were aligned to the *Arabidopsis thaliana* (Col) proteome (Araport11, 2025 release; [57]). Raw spectra files, Proteome Discoverer methods and results have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol *et al.*, 2022) partner repository with the dataset identifier PXD070834.

Computational analysis of proteomic data

Cytoscape v.3.10.3; [58] was used for STRING, ClueGO, and NCMine analyses and network visualization. Empirically supported interaction networks were generated using the STRING Cytoscape app v.2.2.0; [59], default settings were used with the exception of the confidence score, which was raised to 0.75. The produced network was subsequently clustered using NCMine v1.3.0; [60] cliqueness was set to 0.65, all other settings were left as default. Networks and subnetworks were uploaded to NDex [61], corresponding NDex IDs can be found in supplemental Table S1.

Only significantly differentially expressed proteins were used for GO and KEGG analyses. ClueGO was used to identify and cluster enriched GO terms and KEGG pathways v2.5.10; [62]. The following settings were adjusted: GO tree interval 3–20, kappa score 0.5, and 'show only pathways with pV' was set to true and a *P*-value of < 0.05 was provided; all other settings were left at default.

Author contributions

M.P. performed most of the experiments in this work. C.B. and S.R. participated in HPLC analyses. P.M.H. performed mass spectrometry and comparative and computational proteomic analyses. H.H. participated in experimental design and writing. All authors read and approved the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

Raw tandem mass spectrometry files and Proteome Discoverer results that support the findings of this study are openly available in ProteomeXchange at <https://www.proteomexchange.org> reference number PXD070834. STRING network and clusters that support the findings of this study are openly available in NDex at <https://www.ndexbio.org/#/networkset/a330fb76-c3d2-11f0-a218-005056ae3c32?accesskey=baeccc00b38d40eaa8bc56c3d8754add257c6d6e279a6f22c2200c2cf129cf3c>, reference numbers are included in Table S1. Otherwise, all the data incorporated in this study are available from the corresponding author, hellmann@wsu.edu, upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Compiled results for all comparative proteomic analyses used during this study.

Fig. S1. Partial alignment of C24, *rsr4-1*/*PDX1.3*^{G162A}, and *sur* mutant sequences.

Fig. S2. Comparison of protein abundance between wildtype and mutant lines.

Fig. S3. Comparison of protein abundance between the *rsr4-1* vs *sur* and individual mutant lines vs WT.

Fig. S4. NPQ values for 12-day old C24, *rsr4-1*, and the different *sur* mutant seedlings grown in sterile culture.

Table S1. NDex IDs for Networks and clusters generated during this analysis.

Table S2. Primers used for amplification of *PDX1* genes from genomic DNA.