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RESEARCH ARTICLE

A strategy for identification and characterization of genic mutations using a temperature-sensitive chlorotic soybean mutant as an example

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Abstract

Screening a transposon-mutagenized soybean population led to the discovery of a recessively inherited chlorotic phenotype. This "y24" phenotype results in smaller stature, weaker stems, and a smaller root system. Genome sequencing identified 15 candidate genes with mutations likely to result in a loss of function. Amplicon sequencing of a segregating population was then used to narrow the list to a single candidate mutation, a single-base change in *Glyma.07G102300* that disrupts splicing of the second intron. Single cell transcriptomic profiling indicates that this gene is expressed primarily in mesophyll cells, and RNA sequencing data indicate that it is upregulated in germinating seedlings by cold stress. Previous studies have shown that mutations to *Os05g34040*, the rice ortholog of *Glyma.07G102300*, produced a chlorotic phenotype that was more pronounced in cool temperatures. Growing soybean y24 mutants at lower temperatures also resulted in a more severe phenotype. In addition, transgenic expression of wild-type *Glyma.07G102300* in the knockout mutant of the Arabidopsis ortholog *At4930720* rescues the chlorotic phenotype, further supporting the hypothesis that the mutation in *Glyma.07G102300* is causal of the y24 phenotype. The variant analysis strategy used to identify the genes underlying this phenotype provides a template for the study of other soybean mutants.

KEYWORDS
chloroplast, genome resequencing, mutation, oxidoreductase, virescent

1 | INTRODUCTION

Pressure to increase agriculture yields is increasing as human populations grow, even as arable land and fertilizer resources remain limited. The genetic improvement of crop species to overcome these challenges will be facilitated by the identification of the genes controlling crop yield, quality, and stress tolerance (Thomson et al., 2010). This is a challenging task because plant genomes contain thousands of genes, many of which are essential and cannot be deleted, or are functionally redundant with other genes. Identifying gene function using traditional genetic mapping has been a slow and labor-intensive process, requiring thousands of plants to generate the recombination events needed to pinpoint the gene(s) of interest. Thus, faster methods for identifying the genes responsible for altered or mutant phenotypes are needed. Here, we describe an example in which advances in genomic sequencing and analysis facilitated the identification of a gene responsible for a chlorotic mutant soybean. Uncovering the pathways responsible for healthy chloroplast production has the potential to increase photosynthetic capacity and unlock additional yield potential.

Soybeans are a source of both oil and protein, making them valuable for the food supply, animal production, and renewable fuels. Scientists are developing soybean gene discovery resources to identify targets for breeding and genome editing. These include mutagenic populations made with traditional chemical (i.e., EMS or NMU [Cooper et al., 2008, Espina et al., 2018]) and radiation (i.e., fast neutron [Bolon et al., 2011]) mutagens as well as the development of transposon tagging resources based on the *mPing*, *Tnt1*, and *Ac/Ds* transposable elements (Cui et al., 2013; Hancock et al., 2011; Johnson et al., 2021; Mathieu et al., 2009). In addition, site-directed mutagenesis techniques that can target specific sequences have been developed for soybean (Du et al., 2016; Jacobs et al., 2015; Liu et al., 2024). As all these methods result in off-target mutations, it is important to develop an efficient pipeline for whole genome sequence analysis to complement these studies so that plants having the fewest off-target mutations may be selected for downstream analysis and breeding.

Virescent mutants, in which young leaves fail to develop the normal green color, are an easily observable phenotype. They have been identified in rice (Gong et al., 2014; Sugimoto et al., 2004; Sun et al., 2017; Yoo et al., 2009), tobacco (Archer & Bonnett, 1987), cotton (Benedict et al., 1972), maize (Chollet & Paolillo, 1972), cucumber (Miao et al., 2016), Arabidopsis (Brusslan & Tobin, 1995; Koussevitzky et al., 2007; Zhou et al., 2009), and peanut (Alberte et al., 1976). Evaluation of the underlying genes causing virescent phenotypes commonly shows that these genes are generally associated with chloroplast development. The ability of plants to efficiently synthesize and maintain chlorophyll is an important indicator of plant health and is one of the most common phenotypes collected by agronomists and growers to monitor the irrigation and fertilization status of crops. Thus, identifying the genes important for soybean chloroplast development is economically significant.

This report describes the discovery and genomic analysis of a virescent soybean mutant, y24, discovered in a mutagenic population. This is an example of how the advances in sequencing technology allowed for the gene underlying the mutant phenotype to be identified in a relatively small plant population. The description of these techniques provides a potential template to help plant scientists solve the complex problem of sorting through genome sequencing data to identify specific mutations. The causal gene identified from this analysis can be used to further understand the requirements for healthy chloroplast development.

2 | MATERIALS AND METHODS

2.1 | Plant transformation and screening

Transformation of "Jack" (Nickell et al., 1990) soybeans with *mPing*-based mutagenesis plasmids was performed as previously described (Hancock et al., 2011). The resulting plants were screened for phenotypes in research plots at the University of Georgia for 3 years under standard growing conditions, which includes at least 6 weeks of supplemental lighting to prevent early flowering. In 2019, chlorotic plants (Shot 125-94A-3-27-2-7-2) were crossed as the male to control Jack plants. The resulting F_2 seedlings (Cross 19-1) were phenotyped in the greenhouse before transplanting into the field. A chlorotic F_2 plant was crossed as a male to Williams 82 to produce the Cross 20-7 F_2 population. F_3 progeny from green seedlings were grown to identify lines segregating for the y24 phenotype.

2.2 | Genome sequencing and bioinformatics

Genomic DNA was purified from immature leaves using a scaled up version of the CTAB (cetyltrimethylammonium bromide) method as described previously (Johnson et al., 2021). The RNA and other contaminants were removed by a second purification with the E.Z.N.A. Plant DNA DS Mini Kit (Omega Bio-Tek). The sequencing libraries were prepared using Illumina TruSeq DNA PCR-Free LP kits and were sequenced on an Illumina HiSeq.

The sequence analysis pipeline was based on https://gencore.bio.nyu.edu/variant-calling-pipeline-gatk4/. Briefly, the raw reads were assembled to the Williams 82 reference genome (Gmax_275_v2.0.fa [Schmutz et al., 2010]), duplicates were marked and sorted with Picard, and variant single nucleotide polymorphisms (SNPs) and indels were identified with GATK (genomic analysis toolkit). BCFtools was used to compare the variants found in the different genomes. SnpEff was used to identify SNPs and indels that are likely to cause a loss in protein function. Annotated scripts are available at https://github.com/cnhancock/Plant-Genome-Resequencing/.

The raw sequence reads are available at https://submit.ncbi.nlm.nih.gov/subs/sra/SUB13607166/overview.

2.3 | Amplicon sequencing

DNA from six y24 progeny plants from Cross 20-7 and Cross 20-8 (Williams 82 × y24) was pooled and amplified with primers that flank known polymorphisms in the candidate genes and contain the Illumina adapter (IA) sequence (Table S1). The resulting fragments were purified and sent for Illumina sequencing.

2.4 | Genotyping

Purified genomic DNA was used for PCR with Chr07:9757891 For and Chr07:9758683 Rev primers (Table S1). The resulting PCR products were digested with *Dra*I and cleaned and concentrated before agarose gel electrophoresis.

2.5 | Single cell expression analysis

The average expression of *Glyma.07G102300* was extracted from all cell clusters from the tissues composing the soybean single cell atlas (unpublished data; Libault lab) by importing the RDS files using Seurat (Hao et al., 2021). The results were visualized as a transcriptomic profile using a heatmap plot from yellow (low expression) to red (high expression) across the entire soybean single cell atlas.

2.6 | RNA analysis

RNA was purified from leaf tissue from plants at the V3 stage. The tissue (.1 g) was ground in 800 µL of Trizol using ZR Bashing Bead Lysis Tubes (Zymo Research). The Direct-zol RNA Miniprep Plus Kit (Zymo Research) was then used for RNA isolation according to the manufacturer's recommendations (Zymo Cat# R2070S). cDNA was generated from 1 µg of RNA using the iScript Reverse Transcription Supermix for RT-qPCR kit (BioRad). PCR with Chr07:9757891 For and Chr07 9758683 Rev primers was performed using APEX master mix. The resulting bands were gel purified and cloned into pGEM T-easy (Promega) and sent out for Sanger sequencing.

2.7 | Arabidopsis overexpression

The PCR fragment produced by high fidelity PCR of wild-type soybean with the Glyma.07G102300 Rev attb and Glyma.07G102300 For attb primers (Table S1) was cloned into pDONR Zeo, sequenced, then transferred into a modified version of the pEarleyGate 100 plasmid (Earley et al., 2006) with the 35S promoter replaced with the constitutive Arabidopsis ribosomal RSP5a promoter (pEG 100R) (Renken et al., 2023). This construct was transformed into *Agrobacterium* GV3101 by triparental mating with a pRK2013 helper strain and selecting on YM supplemented with rifampicin and kanamycin.

The Arabidopsis T-DNA mutant CS883546 was genotyped by performing PCR (Figure S1) with the At4G30720 For and At4G30720 Rev primers (Table S1). Seeds from a line identified as homozygous null for *At4G30720* were transformed using the floral dip method (Clough & Bent, 1998).

2.8 | Image analysis

LED strip lighting was added to incubators to create identical growth chambers at different temperatures. Soybean seeds were inoculated with commercial inoculant, then planted 3 cm deep in Sungrow Professional Growing Mix with no additional fertilizer. The first fully opened trifoliolate leaf from each plant was digitally imaged from 23 cm above the leaf. The images were cropped to only include the region with the leaf and processed through Batch Load Image Processor (BLIP) software (Renfroe-Becton et al., 2022) to identify and characterize leaf pixels. Statistical analysis was performed with JMP statistical software.

2.9 | Transcriptome analysis

Transcriptome sequencing data were downloaded from the National Center for Biotechnology Information (NCBI) database and were analyzed as previously described (Goettel et al., 2022). The transcript accumulation of each gene was normalized and indicated in transcripts per million (TPM) based on the *Glycine max* reference genome (Wm82.a2.v1 [Schmutz et al., 2010]). Linear modeling was used to analyze the expression data from Bioproject PRJNA999924. For the other datasets, a two-tailed *t*-test was performed to identify treatments with >2-fold change in average transcript accumulation and *p*-value ≤ .05 in comparison with their controls.

3 | RESULTS

3.1 | Identification of the y24 mutant

A chlorotic mutant was discovered in a soybean population (Jack cultivar) transformed with an *mPing*-based activation tag and the ORF1 and Transposase genes required for its mobilization (Hancock et al., 2011; Johnson et al., 2021). This "y24" phenotype was especially apparent in young plants (early in the growing season) and when grown in the greenhouse (during the winter [20–30°C], inoculated, no additional fertilizer) (Figure 1a). In addition to chlorotic leaves, the y24 plants had a smaller root system (Figure 1b) and fewer nodules (Figure S2). The observed changes in root architecture suggested that nutrient uptake or nitrogen fixation may be responsible for chlorosis. However, leaf nutrient analysis, supplemental fertilization, and grafting experiments did not produce any clear results. Analysis of 165 seedlings from a segregating F_3 population showed that 45 (27.3%) were chlorotic (Figure S2), consistent with a 3:1 ratio caused by a single recessive allele ($\chi^2 = .455$, $p = .5002$). When grown

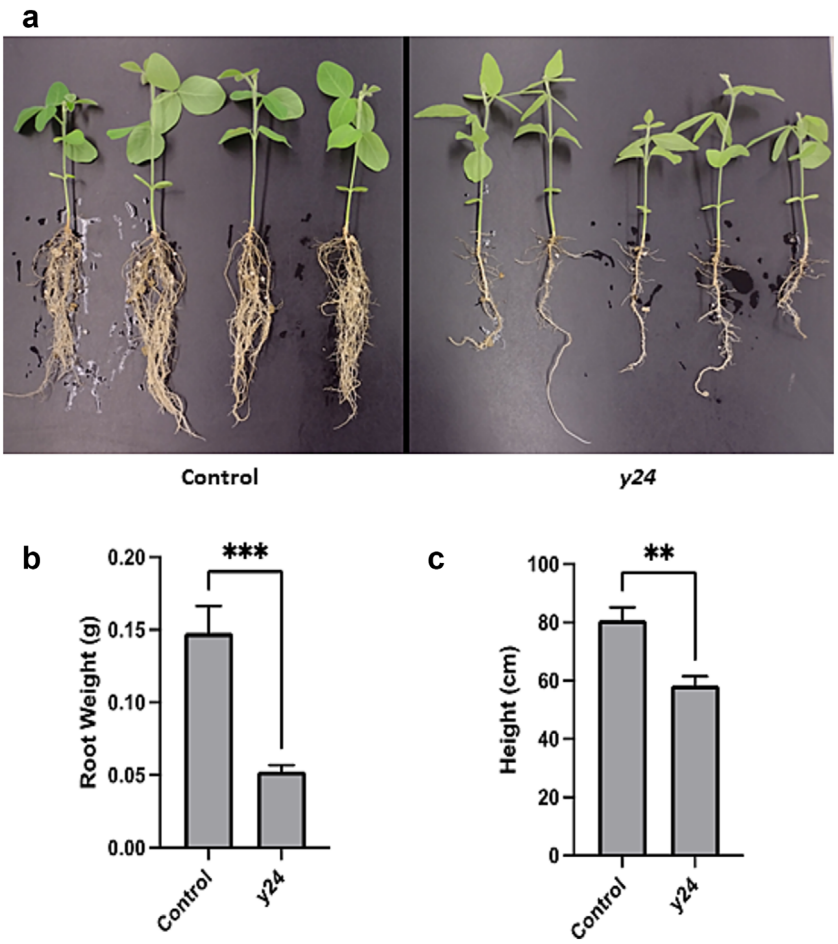

FIGURE 1 Phenotype of y24 soybeans. (a) Images of greenhouse grown control (left) and y24 (right) seedlings. (b) Average root weight for the greenhouse grown plants shown in (a). (c) Average height of mature field grown plants. Error bars represent the standard error of the mean. Asterisks indicate a significant difference based on a two-tailed *t*-test.

in field conditions, the y24 plants were significantly shorter at maturity (Figure 1c), and more plants were observed to lodge due to broken stems. Together, these observations are consistent with the y24 plants having decreased overall photosynthetic capacity.

PCR analysis of these small segregating populations indicated that the y24 phenotype was not associated with the transgenes present in the population (i.e., they potentially resulted from random, *de novo* mutation). To identify the causal gene, we sequenced the genome of a y24 plant and assembled it to the Williams 82 (a2.v1) reference genome (Schmutz et al., 2010). The variants present in the y24 genome were identified using a modified version of a previously developed bioinformatics pipeline (Khalfan, 2020). Using sequences from two other Jack samples, we identified the SNPs and indels that were unique to the y24 plant. Using SnpEff, we identified 15 variants that were predicted to disrupt gene function (Table 1). Five of these putative knockout variants were in genes that had previously been reported in the *G. max* haplotype map list of genes with known loss of function alleles (Torkamaneh et al., 2021). Because the cultivars used for the haplotype map were homozygous and not chlorotic, these five variants were unlikely to be causal for the y24 phenotype. To narrow the list further, we designed primers flanking the 10 remaining candidate variants that would amplify 300-bp fragments including the variant locus. We then amplified these fragments from a pool of six F_3 plants exhibiting the y24 phenotype (a bulk segregant analysis) and sequenced the amplicons. Analysis of these sequences indicated that the variant co-segregating with the y24 phenotypes was a single-base polymorphism in *Glyma.07G102300* (Table 1). The finding that the mutant allele for *Glyma.07G102300* was only present in 86.6% of the reads from the pool suggests that one of the plants in the pool was misidentified as chlorotic. This misidentification was likely the effect of other environmental factors, such as fertility or disease, that also contribute to chlorophyll production.

The mutation in the mutant allele of *Glyma.07G102300* contains a *Dra*I site that is absent from the wild-type allele. We developed a cleaved amplified polymorphic sequence (CAPS) marker by using PCR and digestion with *Dra*I enzyme to genotype plants from an F_3 population segregating for y24 (Figures 2, S2). Results confirmed that the chlorotic phenotype is observed in plants that are homozygous for the variant but not in plants that are heterozygous for the mutation. This result suggests that the homozygous recessive Glyma.07G102300 allele is 100% correlated with the y24 phenotype. Consequently, the CAPS marker is a perfect (functional) co-dominant marker for the y24 trait.

3.2 | *Glyma.07G102300* mRNA analysis

The protein coding sequence of *Glyma.07G102300* is highly conserved across a range of plant species (Figure 3a), suggesting that it

TABLE 1 Variants present in *y24*.

Chromosome position	Reference sequence	Variant sequence	Haplotype map loss of function	% mutant in pool	Gene
Chr02:15034955	G	A		20	*Glyma.02G145600*
Chr03:45596156	TG	T	Yes	-	*Glyma.03G262600*
Chr06:15970458	C	A		44.6	*Glyma.06G184400*
Chr06:37844984	C	A	Yes	-	*Glyma.06G234900*
Chr07:9758094	C	T		86.6	*Glyma.07G102300*
Chr07:39528933	A	T		18	*Glyma.07G220700*
Chr08:3701874	C	T		78	*Glyma.08G047300*
Chr09:3336685	G	A		40.9	*Glyma.09G040000*
Chr10:51324749	G	A	Yes	-	*Glyma.10G296400*
Chr12:35671633	ATTG	A	Yes	-	*Glyma.12G195200*
Chr14:44340827	G	A		21	*Glyma.14G180600*
Chr18:4953932	C	T		10.4	*Glyma.18G056600*
Chr18:12258084	C	T		2	*Glyma.18G107900*
Chr19:47236307	G	A	Yes	-	*Glyma.19G220100*
Chr19:48663487	G	A		5.7	*Glyma.19G237800*

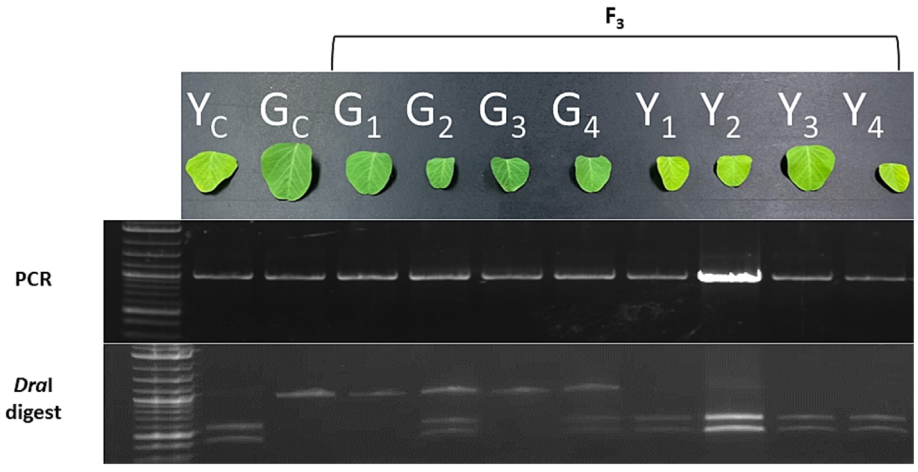

FIGURE 2 Association of the *Glyma.07G102300* variant with the y24 phenotype. PCR and *Dra*I digestion analysis of *Glyma.07G102300* from an F_3 population segregating for the y24 phenotype. Yc, y24 control; Gc, green control (Jack); G, green; Y, yellow.

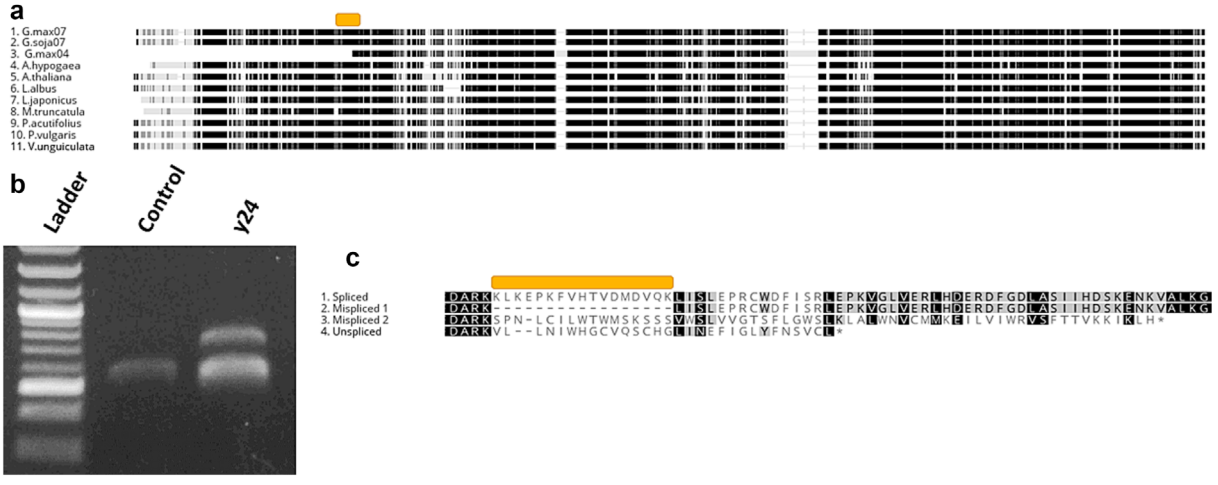

FIGURE 3 *Glyma.07G102300* mRNA analysis. (a) Protein alignment of *Glyma.07G102300* orthologs from various dicot species. Black regions are conserved, orange box indicates region missing from the misspliced 1 mRNA. (b) Gel of reverse transcription PCR amplicons from *Glyma.07G102300*. Ladder = 1 kb+ (New England Biolabs). (c) Protein alignment of the translated cDNAs cloned from y24 shown in (b).

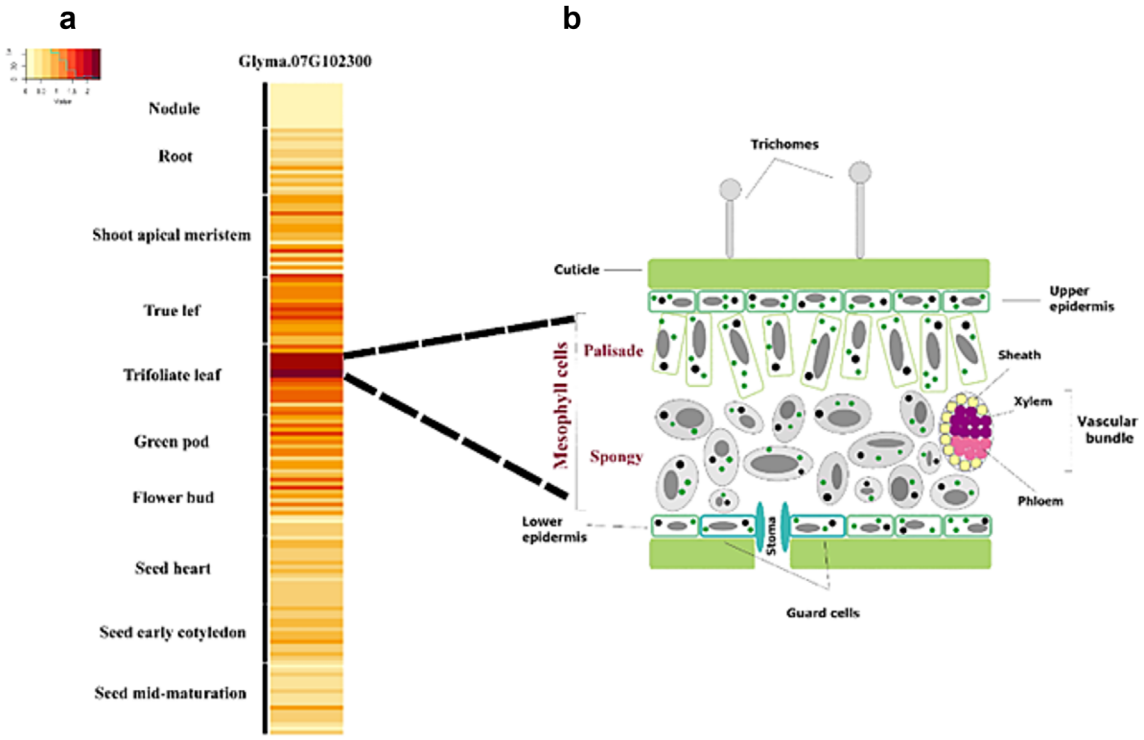

FIGURE 4 Single cell expression analysis of *Glyma.07G102300* in soybean. (a) Heatmap of the average expression of *Glyma.07G102300* in each soybean cell type (y-axis). (b) Diagram representing the cellular diversity of the soybean trifoliate leaf. The palisade and spongy mesophyll cells both show high *Glyma.07G102300* expression activity.

plays an essential role in plant biology. Soybean is an ancient tetraploid, so it was not surprising to find that *Glyma.07G102300* has a closely conserved paralog, *Glyma.04G155100*. However, the predicted coding of the chromosome 4 sequence is missing a large portion of the N-terminal domain due to the presence of a large transposable element insertion (Figure 3a). This likely makes the *Glyma.04G155100* version nonfunctional, suggesting that *Glyma.07G102300* is the only functional copy in the soybean genome.

The knockout allele of *Glyma.07G102300* described herein contains a single C to T change at position 9758094 (Table 1) that is predicted to disrupt the second intron splice acceptor site. We compared the splicing of *Glyma.07G102300* in wild-type and y24 mutants by isolating RNA, making cDNA, and performing PCR with primers that flank the second intron (Figure 3b). Our results show that the wild-type allele of *Glyma.07G102300* mRNA is spliced as expected, giving a single band. However, the y24 allele mRNA resulted in two bands. Sequencing the two bands showed that the upper band was an RNA with an unspliced intron 2 that results in a frame shift and early termination (Figure 3c). Sequencing the lower bands showed that they were aberrantly spliced in two ways, one version resulting in the loss of 17 conserved amino acids and the other producing a frame shift and early stop (Figure 3c). This result is consistent with the hypothesis that y24 is caused by disrupting the function of *Glyma.07G102300*.

To further elucidate the function of *Glyma.07G102300*, we took advantage of the soybean single cell atlas (unpublished data) to determine the expression levels across the cell clusters. Figure 4 shows a heatmap indicating expression levels across a wide range of cell types. The highest expression of wild-type *Glyma.07G102300* is found in four clusters composing the mesophyll cells in trifoliolate leaves. This is consistent with our phenotypic observations in leaves and also points to a potential role in chloroplasts, which are abundant in these tissues. This also matches prior proteome analysis results showing that the wild-type Arabidopsis ortholog, *At4g30720*, was present in chloroplast stroma (Zybailov et al., 2008). Expression of an N-terminal GFP fusion to *At4930720* was also reported in the chloroplast (Vlad et al., 2010). The rice ortholog, *Os05g34040*, has a chloroplast targeting signal, and both C-terminal green flourescent protein (GFP) and N-terminal cyan florescent protein (CFP) fusions were localized to chloroplasts (Morita et al., 2017; Sun et al., 2017). Analysis of the N-terminal amino acid sequence of Glyma.07G102300 using TargetP-2.0 (Armenteros et al., 2019) indicated the presence of a chloroplast signal sequence peaking at residue 39 (.836511). Together, these results suggest that Glyma.07G102300 protein is likely localized to the chloroplast.

3.3 | Functional analysis

The protein produced by *Glyma.07G102300* is predicted to be an oxidoreductase or electron carrier-like protein. Previous studies have identified chlorotic mutants caused by disruption of *Glyma.07G102300* orthologs. The Arabidopsis ortholog, *At4930720*, was

FIGURE 5 Rescue of the *At4g30720* loss of function mutant with a *Glyma.07G102300* transgene. (a) Images of the CS883546, the *At4g30720* loss of function mutant, with (bottom) and without (top) the *RPS5A:Glyma.07G102300* expression construct. (b) Average plant size of eight seedlings of each type measured by the number of plant pixels present in the images. Error bars represent the standard error of the mean. Asterisks indicate a significant difference based on a two-tailed *t*-test.

identified as a pigment defective (pde327-1) SALK line (SALK_059716, CS883546 [Meinke, 2020]). Variation in *At4930720* between Col-0 and Bur-0 ecotypes was found to be the underlying cause of a chlorotic and 70% smaller phenotype observed in recombinant inbred lines generated from those parents (Vlad et al., 2010). This study showed that some ecotypes contain two functional copies of *At4930720*, but at least one functional copy is required for normal pigmentation and growth (Vlad et al., 2010). To test if the soybean and Arabidopsis versions of these genes were functionally the same (protein sequence identity = 66.8%, similarity = 87.6%), we cloned and overexpressed a wild-type genomic *Glyma.07G102300* gene in CS883546, the knockout mutant of *At4930720* (Figure 5). We observed an increase in plant size in the hemizygous T_1 generation and used the T_2 seeds segregating for a single copy of the transgene to quantitate the increase. Figure 5 shows representative plants with and without the transgene. The number of pixels per plant (background removed) was significantly higher when the *RPS5a:Glyma.07G102300* transgene was present. Similar results were obtained when the rice ortholog, *Os05g34040*, was overexpressed in SALK_059716, the parental line for CS883546 (Wang et al., 2016), indicating that it is functionally conserved across dicot and monocot plant species.

Multiple groups have reported that the chlorotic phenotype from loss of function mutations in *Os5G34040* was more pronounced in cool temperatures (20°C) than in warmer temperatures (30°C) (Morita et al., 2017; Sun et al., 2017; Wang et al., 2016). Based on this information, we tested if temperature influenced the phenotype of the soybean y24 mutants (Figures 6, S5). We observed small differences in leaf color and shape between the control and y24 mutants at 25 and 30°C. However, when we grew the mutant at 18°C, we observed a drastic difference in leaf color, shape, and area (Figure 6). This temperature sensitivity further supports the hypothesis that the mutation we identified in *Glyma.07G102300* is causal of the y24 phenotype.

Previous reports on the expression of the rice ortholog *Os05g34040* indicate that lower temperatures increased expression (Sun et al., 2017), but a similar study observed no change in expression (Morita et al., 2017). To gain additional insight into the expression pattern of *Glyma.07G102300*, we analyzed leaf RNA expression data from publicly available data sets. The most relevant data set we identified was Bioproject # PRJNA999924 that includes replicated control (20°C) and low temperature (6°C) treatments of germinating seeds (two genotypes [Zheng et al., 2023]) over 48 h (Figure 7). Linear modeling of *Glyma.07G102300* expression from this dataset showed that there was a significant interaction between time and treatment ($t = 4.687$, $p < .001$), so the control and low temperature treatments were modeled separately. The slope of TPM versus time for all of the control data showed no significant difference from zero ($t = 7.364$, $p < .001$). However, we detected a significant positive relationship between TPM versus time for the low treatment ($t = 9.021$, $p < .001$). Thus, this analysis suggests that *Glyma.07G102300* expression is induced by cold treatment in germinating soybean seeds. In addition to this result, the public data indicated other treatments that significantly alter *Glyma.07G102300* expression (Table 2). A leaf development experiment using the Hedou12 cultivar (Feng et al., 2021) showed that unexpanded trifoliolate leaves had less *Glyma.07G102300* expression than fully expanded trifoliolate leaves. This correlates with the normal greening observed during leaf development. In addition, multiple experiments showed a decrease in *Glyma.07G102300* expression under drought stress (Table 2), consistent with lower photosynthetic activity and growth. Similarly, an RNA expression dataset comparing mock and Soybean Mosaic Virus

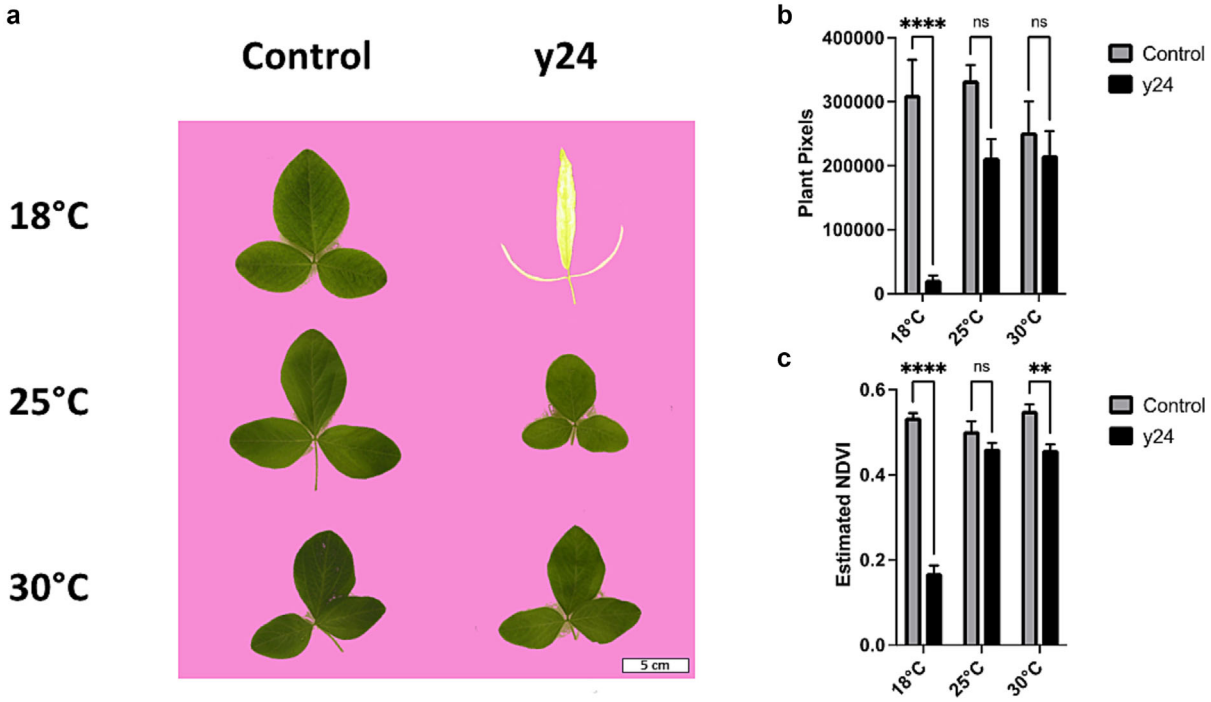

FIGURE 6 Temperature effect on leaf size and color. (a) Example images of first trifoliate leaves grown at three temperatures with identical lighting. Background pixels have been replaced with pink to indicate the plant pixels used for image analysis. (b) Leaf size measured as the average number of plant pixels. (c) Average predicted leaf normalized difference vegetation index (NDVI) based on the color of the plant pixels. Averages represent five or six trifoliate leaves. Error bars represent the standard error of the mean. Asterisks indicate a significant difference based on a two-way ANOVA with Šídák's multiple comparisons test. ns, not significantly different.

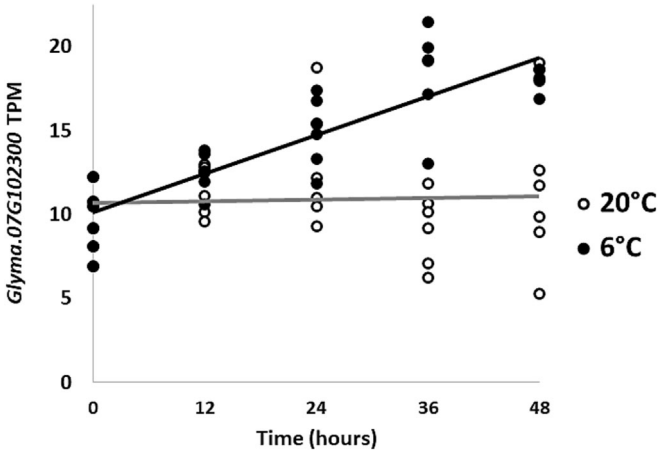

FIGURE 7 Expression of *Glyma.07G102300* under cold stress. Plot of *Glyma.07G102300* expression in germinating seeds measured in transcripts per million (TPM) from Bioproject #PRJNA999924 under control (20°C) and low temperature (6°C) conditions. Each time point includes six data points (three each from chromosome fragment substitution line R48 and R89 [Zheng et al., 2023]). The linear regression for 20 and 6°C are represented by a gray and black line, respectively.

inoculated leaves (Chen et al., 2016) indicates that viral infection results in a drop in *Glyma.07G102300* expression. Surprisingly, an increase in *Glyma.07G102300* expression was observed 120 h after inoculation with *Phakopsora pachyrhizi*, the fungal agent responsible for Asian soybean rust. Additional expression studies, including cold stress treatment, will be helpful in elucidating the role of the *Glyma.07G102300*.

4 | DISCUSSION

Taken together, our results suggest that the knockout allele of *Glyma.07G102300* is the cause of the y24 phenotype. Prior soybean mutant studies identified a number of chlorophyll-deficient mutants named y1 through y23 (Palmer et al., 2004; Sandhu et al., 2018). The y6, y8, and y10 phenotypes are reported to be more severe in young

TABLE 2 Gene expression changes for *Glyma.07G102300* in trifoliate leaves.

Control	Control mean expression	Treatments	Treatment mean expression	Fold* change	Study (Bioproject #)
Unexpanded	13.12	Fully expanded	27.33	2.08	PRJNA660896
V3/watered	21.70	V3/drought	5.39	4.03	PRJNA694374 (Wang et al., 2021)
V2/watered	35.70	V2/mild drought	17.42	2.05	PRJDB11892
Watered	10.32	Dehydrated	4.35	2.37	PRJNA386934 (Wei et al., 2019)
Mock inoculated	13.69	Soybean mosaic virus (G7) inoculated	4.43	3.08	PRJNA308211 (Chen et al., 2016)
Non-inoculated	8.95	*P. pachyrhizi* inoculated	19.90	2.22	PRJNA855551 (Wei et al., 2023)

*All expression changes are significant based on two-tail test at *p*-value of .05.

plants and segregate in a 3:1 ratio (Probst, 1950; Woodworth & Williams, 1938), consistent with the y24 phenotype. Unfortunately, no mapping data are available for the y6, y8, and y10 mutants, and they are no longer available to the soybean research community due to the lack of a soybean stock center. Thus, to our knowledge, y24 is the only virescent soybean mutant with a known locus. The lack of other virescent mutants in soybean may be due to the gene redundancy present in this ancient tetraploid. In fact, the y24 mutant would probably have not been observed if the homoeologous *Glyma.04G155100* gene was functional (Figure 3a). The fact that soybean and its wild ancestor *Glycine soja* only have one functional copy of this conserved oxidoreductase gene could be due to random loss, or it could be due to selection for moderate expression. We did not detect any adverse effects from overexpression of the soybean *Glyma.07G102300* gene in Arabidopsis (Figure 5), and some Arabidopsis accessions naturally have two copies of the *At4g30720* ortholog (Vlad et al., 2010). Our analysis of publicly available data indicates that cold stress upregulates *Glyma.07G102300* expression in germinating seedlings (Figure 7). However, additional experiments are needed to clarify if expression also increases in fully expanded leaves that already have higher levels of *Glyma.07G102300* transcript (Table 1).

Our results provide a successful example of using whole genome resequencing followed by bulk segregant analysis of targeted amplicons to identify the causal mutation for an economically impactful plant mutant phenotype. This technique should be applicable to a wide range of plant species that have an available reference genome. This project required whole genome sequencing a single y24 mutant plant and comparing it to a pool of wild-type plants with the same genetic background (Jack). The need for wild-type whole genome sequences would have been reduced if the mutation had occurred in the reference Williams 82 cultivar. However, detailed characterization of Williams 82 plants has also shown that intercultivar genetic heterogeneity is pervasive (Haun et al., 2011), which could complicate candidate gene discovery.

Identification of the y24 mutant was likely facilitated by the fact that it occurred in a transposon tagging population. First, large populations of plants were phenotyped throughout the growing season. Second, this population had increased frequency of mutations due to *mPing* transposition. Also, previous studies have shown that tissue culture associated with soybean transformation can result in increased levels of mutation (Anderson et al., 2016); however, the levels are low compared to what is caused by other mutagens. Similarly, insertion and subsequent excision of *mPing* from the genome create double-stranded breaks that could result in mutations during DNA repair, though *mPing* excision sites are primarily but not exclusively repaired precisely (Gilbert et al., 2015). Ultimately, we cannot pinpoint the exact cause of the y24 mutation based on the available data.

One factor that facilitated this study was the ease with which the y24 phenotype could be scored. Even with this benefit, our amplicon sequencing suggests that we mischaracterized one of the plants included in our pool. The phenotypic selection was performed with plants grown at about 25°C, where the phenotype is less severe. We now know that this mistake could have been avoided by growing the plants at even cooler temperatures (i.e., 18°C). Despite this mistake, our methods clearly identified the causal mutation. This suggests that application of this technique to other less obvious phenotypes should be feasible if bulk segregant analysis is based on amplicon sequencing of pools of extreme phenotypes. We acknowledge that a single-gene trait in a diploid species (lacking a functional copy from its ancient genome duplication) is the easiest scenario for developing and sequencing extreme phenotypic pools. In the case of more quantitative traits, or in polyploid species, a similar approach would still be effective at capturing large-effect loci to quickly narrow down the list of candidate genes (reviewed in Wang et al., 2023).

The dramatic expression of the y24 phenotype under cool temperatures (Figures 6, S5) suggests that the gene is critical to chloroplast development under cold stress. One possible explanation is that this oxidoreductase plays a role in protecting the chloroplasts from reactive oxygen species (ROS). Photosynthesis produces additional ROS under cold stress conditions, resulting in oxidative damage (Banerjee & Roychoudhury, 2019; Tambussi et al., 2004). The loss of

this oxidoreductase protein may exacerbate ROS stress to the point of inhibiting chloroplast development. Another possibility is that *Glyma.07G102300* is important for the expression of essential chloroplast genes. Previous study of the rice ortholog *Os05g34040* indicated that plastid-encoded RNA polymerase activity is decreased in the knockout plants (Sun et al., 2017). However, it is not clear if this is causal, or simply a downstream effect of chloroplast stress.

To our knowledge, this is the first study to report decreased root biomass (Figure 1) and decreased nodulation (Figure S2) associated with a virescent mutant in soybean. We hypothesize that the reduced root biomass could exacerbate the leaf phenotype by reducing nutrient uptake from the soil. Nitrogen deficiency is known to cause leaf chlorosis, so any reduction in nodulation is likely to exacerbate chlorosis. While our results point to a role for *Glyma.07G102300* in the leaf, additional studies on the function of this important oxidoreductase protein's role in roots are warranted.

AUTHOR CONTRIBUTIONS

C. Nathan Hancock: Conceptualization; experimental design; data acquisition; drafting; editing; funding acquisition. **Tetandianocee Germany, Jack Timmons, Jeffery Lipford, Samantha Burns, Lisa Kanizay, Melinda Yerka**: Data acquisition and editing. **Priscilla Redd, Sergio Alan Cervantes-Perez, Marc Libault, Wenhao Shen, Yong-qiang Charles An**: Data analysis and editing. **Wayne A. Parrott**: Conceptualization; editing; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Alberte, R. S., Hesketh, J. D., & Kirby, J. S. (1976). Comparisons of photosynthetic activity and lamellar characteristics of virescent and normal green peanut leaves. *Zeitschrift für Pflanzenphysiologie*, *77*(2), 152–159. https://doi.org/10.1016/S0044-328X(76)80178-X

Anderson, J. E., Michno, J.-M., Kono, T. J., Stec, A. O., Campbell, B. W., Curtin, S. J., & Stupar, R. M. (2016). Genomic variation and DNA repair associated with soybean transgenesis: A comparison to cultivars and mutagenized plants. *BMC Biotechnology*, *16*, 1–13.

Archer, E. K., & Bonnett, H. T. (1987). Characterization of a virescent chloroplast mutant of tobacco. *Plant Physiology*, *83*(4), 920–925. https://doi.org/10.1104/pp.83.4.920

Armenteros, J. J. A., Salvatore, M., Emanuelsson, O., Winther, O., Von Heijne, G., Elofsson, A., & Nielsen, H. (2019). Detecting sequence signals in targeting peptides using deep learning. *Life Science Alliance*, *2*(5), e201900429. https://doi.org/10.26508/lsa.201900429

Banerjee, A., & Roychoudhury, A. (2019). Cold stress and photosynthesis. In *Photosynthesis, productivity and environmental stress* (pp. 27–37). Wiley. https://doi.org/10.1002/9781119501800.ch2

Benedict, C., McCree, K., & Kohel, R. (1972). High photosynthetic rate of a chlorophyll mutant of cotton. *Plant Physiology*, *49*(6), 968–971. https://doi.org/10.1104/pp.49.6.968

Bolon, Y.-T., Haun, W. J., Xu, W. W., Grant, D., Stacey, M. G., Nelson, R. T., Gerhardt, D. J., Jeddeloh, J. A., Stacey, G., & Muehlbauer, G. J. (2011). Phenotypic and genomic analyses of a fast neutron mutant population resource in soybean. *Plant Physiology*, *156*(1), 240–253. https://doi.org/10.1104/pp.110.170811

Brusslan, J. A., & Tobin, E. M. (1995). Isolation and initial characterization of virescent mutants of *Arabidopsis thaliana*. *Photosynthesis Research*, *44*, 75–79. https://doi.org/10.1007/BF00018298

Chen, H., Arsovski, A. A., Yu, K., & Wang, A. (2016). Genome-wide investigation using sRNA-Seq, degradome-Seq and transcriptome-Seq reveals regulatory networks of microRNAs and their target genes in soybean during soybean mosaic virus infection. *PLoS ONE*, *11*(3), e0150582. https://doi.org/10.1371/journal.pone.0150582

Chollet, R., & Paolillo, D. J. Jr. (1972). Greening in a virescent mutant of maize: I. Pigment, ultrastructural, and gas exchange studies. *Zeitschrift für Pflanzenphysiologie*, *68*(1), 30–44. https://doi.org/10.1016/S0044-328X(72)80007-2

Clough, S. J., & Bent, A. F. (1998). Floral dip: A simplified method for agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant Journal*, *16*(6), 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x

Cooper, J. L., Till, B. J., Laport, R. G., Darlow, M. C., Kleffner, J. M., Jamai, A., El-Mellouki, T., Liu, S., Ritchie, R., & Nielsen, N. (2008). TILLING to detect induced mutations in soybean. *BMC Plant Biology*, *8*, 1–10.

Cui, Y., Barampuram, S., Stacey, M. G., Hancock, C. N., Findley, S., Mathieu, M., Zhang, Z., Parrott, W. A., & Stacey, G. (2013). *Tnt1* retrotransposon mutagenesis: A tool for soybean functional genomics. *Plant Physiology*, *161*(1), 36–47. https://doi.org/10.1104/pp.112.205369

Du, H., Zeng, X., Zhao, M., Cui, X., Wang, Q., Yang, H., Cheng, H., & Yu, D. (2016). Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *Journal of Biotechnology*, *217*, 90–97. https://doi.org/10.1016/j.jbiotec.2015.11.005

Earley, K. W., Haag, J. R., Pontes, O., Opper, K., Juehne, T., Song, K. M., & Pikaard, C. S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. *Plant Journal*, *45*(4), 616–629. https://doi.org/10.1111/j.1365-313X.2005.02617.x

Espina, M. J., Ahmed, C. S., Bernardini, A., Adeleke, E., Yadegari, Z., Arelli, P., Pantalone, V., & Taheri, A. (2018). Development and phenotypic screening of an ethyl methane sulfonate mutant population in soybean. *Frontiers in Plant Science*, *9*, 394. https://doi.org/10.3389/fpls.2018.00394

Feng, X., Yang, S., Zhang, Y., Zhiyuan, C., Tang, K., Li, G., Yu, H., Leng, J., & Wang, Q. (2021). *GmPGL2*, encoding a pentatricopeptide repeat protein, is essential for chloroplast RNA editing and biogenesis in soybean. *Frontiers in Plant Science*, *12*, 690973. https://doi.org/10.3389/fpls.2021.690973

Gilbert, D. M., Bridges, M. C., Strother, A. E., Burckhalter, C. E., Burnette, J. M., & Hancock, C. N. (2015). Precise repair of *mPing* excision sites is facilitated by target site duplication derived microhomology. *Mobile DNA*, *6*, 15. https://doi.org/10.1186/s13100-015-0046-4

Goettel, W., Zhang, H., Li, Y., Qiao, Z., Jiang, H., Hou, D., Song, Q., Pantalone, V. R., Song, B.-H., & Yu, D. (2022). *POWR1* is a domestication gene pleiotropically regulating seed quality and yield in soybean. *Nature Communications*, *13*(1), 3051. https://doi.org/10.1038/s41467-022-30314-7

Gong, X., Su, Q., Lin, D., Jiang, Q., Xu, J., Zhang, J., Teng, S., & Dong, Y. (2014). The rice *OsV4* encoding a novel pentatricopeptide repeat protein is required for chloroplast development during the early leaf stage under cold stress. *Journal of Integrative Plant Biology*, *56*(4), 400–410. https://doi.org/10.1111/jipb.12138

Hancock, C. N., Zhang, F., Floyd, K., Richardson, A. O., LaFayette, P., Tucker, D., Wessler, S. R., & Parrott, W. A. (2011). The rice miniature inverted repeat transposable element mPing is an effective insertional mutagen in soybean. *Plant Physiology*, *157*(2), 552–562. https://doi.org/10.1104/pp.111.181206

Hao, Y., Hao, S., Andersen-Nissen, E., Mauck, W. M., Zheng, S., Butler, A., Lee, M. J., Wilk, A. J., Darby, C., & Zager, M. (2021). Integrated analysis of multimodal single-cell data. *Cell*, *184*(13), 3573–3587. https://doi.org/10.1016/j.cell.2021.04.048

Haun, W. J., Hyten, D. L., Xu, W. W., Gerhardt, D. J., Albert, T. J., Richmond, T., Jeddeloh, J. A., Jia, G., Springer, N. M., & Vance, C. P. (2011). The composition and origins of genomic variation among individuals of the soybean reference cultivar Williams 82. *Plant Physiology*, *155*(2), 645–655. https://doi.org/10.1104/pp.110.166736

Jacobs, T. B., LaFayette, P. R., Schmitz, R. J., & Parrott, W. A. (2015). Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology*, *15*, 1–10.

Johnson, A., McAssey, E., Diaz, S., Reagin, J., Redd, P. S., Parrilla, D. R., Nguyen, H., Stec, A., McDaniel, L. A. L., Clemente, T. E., Stupar, R. M., Parrott, W. A., & Hancock, C. N. (2021). Development of *mPing*-based activation tags for crop insertional mutagenesis. *Plant Direct*, *5*(1), e00300. https://doi.org/10.1002/pld3.300

Khalfan, M. (2020). "*Variant Calling Pipeline using GATK4*." Genomics Core at NYU CGSB, https://gencore.bio.nyu.edu/variant-calling-pipeline-gatk4/

Koussevitzky, S., Stanne, T. M., Peto, C. A., Giap, T., Sjögren, L. L., Zhao, Y., Clarke, A. K., & Chory, J. (2007). An *Arabidopsis thaliana* virescent mutant reveals a role for ClpR1 in plastid development. *Plant Molecular Biology*, *63*, 85–96.

Liu, P., Panda, K., Edwards, S. A., Swanson, R., Yi, H., Pandesha, P., Hung, Y. H., Klaas, G., Ye, X., Collins, M. V., Renken, K. N., Gilbertson, L. A., Veena, V., Hancock, C. N., & Slotkin, R. K. (2024). Transposase-assisted target-site integration for efficient plant genome engineering. *Nature*, *631*(8021), 593–600. https://doi.org/10.1038/s41586-024-07613-8

Mathieu, M., Winters, E. K., Kong, F., Wan, J., Wang, S., Eckert, H., Luth, D., Paz, M., Donovan, C., & Zhang, Z. (2009). Establishment of a soybean (*Glycine max* Merr. L) transposon-based mutagenesis repository. *Planta*, *229*, 279–289. https://doi.org/10.1007/s00425-008-0827-9

Meinke, D. W. (2020). Genome-wide identification of *EMBRYO-DEFECTIVE (EMB)* genes required for growth and development in Arabidopsis. *New Phytologist*, *226*(2), 306–325. https://doi.org/10.1111/nph.16071

Miao, H., Zhang, S., Wang, M., Wang, Y., Weng, Y., & Gu, X. (2016). Fine mapping of virescent leaf gene v-1 in cucumber (*Cucumis sativus* L.). *International Journal of Molecular Sciences*, *17*(10), 1602. https://doi.org/10.3390/ijms17101602

Morita, R., Nakagawa, M., Takehisa, H., Hayashi, Y., Ichida, H., Usuda, S., Ichinose, K., Abe, H., Shirakawa, Y., & Sato, T. (2017). Heavy-ion beam mutagenesis identified an essential gene for chloroplast development under cold stress conditions during both early growth and tillering stages in rice. *Bioscience, Biotechnology, and Biochemistry*, *81*(2), 271–282. https://doi.org/10.1080/09168451.2016.1249452

Nickell, C., Noel, G., Thomas, D., & Waller, R. (1990). Registration of "Jack" soybean. *Crop Science*, *30*(6), 1365.

Palmer, R. G., Pfeiffer, T. W., Buss, G. R., & Kilen, T. C. (2004). Qualitative genetics. *Soybeans: Improvement, Production, and Uses*, *16*, 137–233.

Probst, A. H. (1950). The inheritance of leaf abscission and other characters in soybeans. *Agronomy Journal*, *42*(1), 35–45. https://doi.org/10.2134/agronj1950.00021962004200010007x

Renfroe-Becton, H., Kirk, K. R., & Anco, D. J. (2022). Using image analysis and regression modeling to develop a diagnostic tool for peanut foliar symptoms. *Agronomy*, *12*(11), 2712. https://doi.org/10.3390/agronomy12112712

Renken, K., Mendoza, S. M., Diaz, S., Slotkin, R. K., & Hancock, C. N. (2023). Pol V produced RNA facilitates transposable element excision site repair in *Arabidopsis*. *microPublication Biology*, *2023*. https://doi.org/10.17912/micropub.biology.000793

Sandhu, D., Coleman, Z., Atkinson, T., Rai, K. M., & Mendu, V. (2018). Genetics and physiology of the nuclearly inherited yellow foliar mutants in soybean. *Frontiers in Plant Science*, *9*, 320838.

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J. X., Mitros, T., Nelson, W., Hyten, D. L., Song, Q. J., Thelen, J. J., Cheng, J. L., Xu, D., Hellsten, U., May, G. D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M. K., Sandhu, D., Valliyodan, B., … Jackson, S. A. (2010). Genome sequence of the palaeopolyploid soybean. *Nature*, *463*(7278), 178–183. https://doi.org/10.1038/nature08670

Sugimoto, H., Kusumi, K., Tozawa, Y., Yazaki, J., Kishimoto, N., Kikuchi, S., & Iba, K. (2004). The virescent-2 mutation inhibits translation of plastid transcripts for the plastid genetic system at an early stage of chloroplast differentiation. *Plant and Cell Physiology*, *45*(8), 985–996. https://doi.org/10.1093/pcp/pch111

Sun, J., Zheng, T., Yu, J., Wu, T., Wang, X., Chen, G., Tian, Y., Zhang, H., Wang, Y., & Terzaghi, W. (2017). TSV, a putative plastidic oxidoreductase, protects rice chloroplasts from cold stress during development by interacting with plastidic thioredoxin Z. *New Phytologist*, *215*(1), 240–255. https://doi.org/10.1111/nph.14482

Tambussi, E., Bartoli, C., Guiamet, J., Beltrano, J., & Araus, J. (2004). Oxidative stress and photodamage at low temperatures in soybean (*Glycine max* L. Merr.) leaves. *Plant Science*, *167*(1), 19–26. https://doi.org/10.1016/j.plantsci.2004.02.018

Thomson, M. J., Ismail, A. M., McCouch, S. R., & Mackill, D. J. (2010). Marker assisted breeding. In A. Pareek, S. K. Sopory, & H. J. Bohnert (Eds.), *Abiotic stress adaptation in plants* (Vol. 4) (pp. 451–469). Springer.

Torkamaneh, D., Laroche, J., Valliyodan, B., O'Donoughue, L., Cober, E., Rajcan, I., Vilela Abdelnoor, R., Sreedasyam, A., Schmutz, J., & Nguyen, H. T. (2021). Soybean (*Glycine max*) Haplotype Map (GmHapMap): A universal resource for soybean translational and functional genomics. *Plant Biotechnology Journal*, *19*(2), 324–334. https://doi.org/10.1111/pbi.13466

Vlad, D., Rappaport, F., Simon, M., & Loudet, O. (2010). Gene transposition causing natural variation for growth in *Arabidopsis thaliana*. *PLoS Genetics*, *6*(5), e1000945. https://doi.org/10.1371/journal.pgen.1000945

Wang, X., Han, L., Li, J., Shang, X., Liu, Q., Li, L., & Zhang, H. (2023). Next-generation bulked segregant analysis for Breeding 4.0. *Cell Reports*, *42*(9), 113039.

Wang, Y., Zhang, J., Shi, X., Peng, Y., Li, P., Lin, D., Dong, Y., & Teng, S. (2016). Temperature-sensitive albino gene *TCD5*, encoding a monooxygenase, affects chloroplast development at low temperatures. *Journal of Experimental Botany*, *67*(17), 5187–5202. https://doi.org/10.1093/jxb/erw287

Wang, Z. Q., Yu, T. F., Sun, G. Z., Zheng, J. C., Chen, J., Zhou, Y. B., Chen, M., Ma, Y. Z., Wei, W. L., & Xu, Z. S. (2021). Genome-wide analysis of the *Catharanthus roseus* RLK1-like in soybean and *GmCrRLK1L20* responds to drought and salt stresses. *Frontiers in Plant Science*, *12*, 614909. https://doi.org/10.3389/fpls.2021.614909

Wei, W., Liang, D. W., Bian, X. H., Shen, M., Xiao, J. H., Zhang, W. K., Ma, B., Lin, Q., Lv, J., Chen, X., Chen, S. Y., & Zhang, J. S. (2019). GmWRKY54 improves drought tolerance through activating genes in abscisic acid and Ca(2+) signaling pathways in transgenic soybean. *The Plant Journal*, *100*(2), 384–398. https://doi.org/10.1111/tpj.14449

Wei, W., Wu, X., Garcia, A., McCoppin, N., Viana, J. P. G., Murad, P. S., Walker, D. R., Hartman, G. L., Domier, L. L., & Hudson, M. E. (2023). An NBS-LRR protein in the *Rpp1* locus negates the dominance of *Rpp1*-mediated resistance against *Phakopsora pachyrhizi* in soybean. *The Plant Journal*, *113*(5), 915–933. https://doi.org/10.1111/tpj.16038

Woodworth, C. M., & Williams, L. F. (1938). Recent studies on the genetics of the soybean. *Agronomy Journal*, *30*, 125–129. https://doi.org/10.2134/agronj1938.00021962003000020006x

Yoo, S.-C., Cho, S.-H., Sugimoto, H., Li, J., Kusumi, K., Koh, H.-J., Iba, K., & Paek, N.-C. (2009). Rice *virescent3* and *stripe1* encoding the large and small subunits of ribonucleotide reductase are required for chloroplast biogenesis during early leaf development. *Plant Physiology*, *150*(1), 388–401. https://doi.org/10.1104/pp.109.136648

Zheng, L., Xie, J., Sun, X., Zheng, Y., Meng, F., Fan, X., Li, G., Zhang, Y., Wang, M., & Zhou, R. (2023). QTL mapping and candidate gene analysis of low-temperature tolerance at the germination stage of soybean. *Plant Breeding 142*(6), 758–768.

Zhou, W., Cheng, Y., Yap, A., Chateigner-Boutin, A. L., Delannoy, E., Hammani, K., Small, I., & Huang, J. (2009). The Arabidopsis gene *YS1* encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth. *The Plant Journal*, *58*(1), 82–96. https://doi.org/10.1111/j.1365-313X.2008.03766.x

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., & van Wijk, K. J. (2008). Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE*, *3*(4), e1994. https://doi.org/10.1371/journal.pone.0001994

SUPPORTING INFORMATION

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