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Global Gene Expression Analysis of the Reversed Gravitropic Response of the *lazy-2* Mutation in Tomato (*Solanum lycopersicum*)

Talitha Wilson

ABSTRACT

The *lazy-2* mutation (*lz-2*) in tomato plants (*Solanum lycopersicum*) is exhibited as a phenotype of light-dependent reversed-gravitropism. The mutation's centromeric location has only allowed for speculation on the identity of the *lz-2* gene. In order to investigate the relationship and affect of the *lz-2* mutation on signaling pathways linked to auxin growth hormone regulation, global gene activity was assessed. Modern molecular biology tools including microarray technology and various PCR techniques were utilized to generate a list of significantly differentially regulated genes in *lz-2* mutant plants compared to wild-type plants. Gene ontology functional analysis indicated that genes involved in protein ubiquitination constituted the largest percentage of differentially expressed genes, while signal transduction genes were also a significant fraction of all genes studied. Top gene candidates were tested by qPCR to verify statistically significant up- or down-regulation in *lz-2* mutant plants. Two such candidates, a gene encoding an f-box family protein and the ATCUL1 gene encoding a cullin protein have been implicated as major components of the SCF ubiquitin-ligase complex, which degrades auxin repressor proteins. This study suggests a potential relationship between differential expression of ubiquitin-ligase proteins causing altered auxin signaling and the downward growth of *lz-2* mutant tomato plants in response to gravity.

INTRODUCTION

Environmental cues such as light, nutrition, gravity and temperature affect a multitude of responses in living plants. Historical research of how plants respond to their external environment provides a strong foundational understanding, but the advancement of molecular biology techniques has revolutionized the study of plant physiology. One particular stimuli-response relationship that has received broad attention is gravitropism, which describes how gravity affects the orientation and direction of growth (Correll and Kiss, 2002). This nuanced perception of an unseen force necessarily dictates how a plant should be positioned to optimally utilize all available resources and protect against damage (Perrin et al., 2005). The gravitropic response plays a critical role in numerous biological systems through intricate signal transduction pathways to regulate growth and maintain homeostasis. Such signaling patterns have been described using the Cholodny-Went theory, which proposes that gravistimulation allows for the lateral redistribution of the growth hormone auxin, to form an auxin gradient across an organ (Perrin, et al., 2005). Thus gravity-perceiving cells and molecules must create a biochemical signal that activates auxin-dependent transduction pathways to influence physiological elongation.

The *lazy-2* (*lz-2*) mutation in tomato (*Solanum lycopersicum*) provides an interesting biological context from which to study the interaction between gravitropism, phototropism, and signal transduction. Mutants in this gene exhibit a phenotype of light-dependent reversed gravitropism that is not simply a failure to grow upright, but rather a directed reaction to gravity (Behringer and Lomax, 1999; Gaiser and Lomax, 1993). Previous molecular studies have shown that this unusual response is mediated by phytochromes but not through an ethylene-dependent pathway (Madlung, et al., 1999).

The *lz-2* gene of interest has been mapped to the centromeric region of chromosome 5, but due to the nature of chromatin at that region, complete isolation and specific genetic studies has been historically unattainable (Behringer and Lomax, 1999). Some speculate that the mutation is involved with signal transduction, such as a transport protein like a membrane channel, but definitive evidence still originates from mostly physiological studies (Bio404 Lab Manual).

However, recently developed molecular biology tools allow for assessment of global gene transcription activity by comparing wild-type (wt) organisms to those mutated in a gene of interest. Microarray technology, which has been established within the last two decades, allows for vast analysis of the diversity of the entire genome. Additionally, the advent of PCR allows for quantification of the expression of a specific gene as well as reverse-transcription to convert RNA transcripts into cDNA for further experiments. Using a wide variety of quantitative and qualitative analyses derived from these technologies, the underlying biological and genetic mechanisms behind the *lz-2* mutation can be elucidated. Specifically, the link between the light-dependent gravitropic response of *lz-2* mutation in tomato plants (*S. lycopersicum*) and auxin signal transduction can be understood in terms of differential gene expression.

MATERIALS AND METHODS

Materials. All plant material was obtained from the University of Puget Sound Biology Department. Seeds were germinated in darkness at 24°C for 5 days and exposed to white light at a concentration of approximately 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 30 minutes prior to freezing in liquid nitrogen (Lab Manual). Protocols were provided by the Bio 404 Fall

2012 Laboratory Manual. Quantitative PCR reactions (qPCR) were run on a BioRad 1000 cycler with BioRad CFX Manager software.

RNA extraction. Approximately 100 mg of frozen seedling tissue from wild type or *lz-2* plants was ground into a powder using liquid nitrogen. RNA was then extracted using a Qiagen RNeasy Plant Mini kit. The quality of the isolated RNA was analyzed by gel electrophoresis on a 1% weight/volume (wt/vol) agarose gel with 5X TBE buffer and 3 μL of ethidium bromide. The quantity and quality of extracted RNA was assessed using a Nanodrop 2000 spectrophotometer from the concentration, 260/280 ratio, and 260/230 ratio.

Production of cDNA and Hybridization. Extracted wt and *lz-2* RNA was reverse transcribed into cDNA using a Genisphere Array 900 kit. The green Cy5 primer was mixed with 2.5 μg wt RNA and the red Cy3 primer was mixed with 2.5 μg *lz-2* RNA. Reaction master mixes were prepared for two reactions and included SuperScript II 5X first-strand buffer and enzyme (200 units μL^{-1}). Reverse-transcribed cDNA was stored at -20°C for one week. The slides were incubated in a prehybridization solution of 5XSSC, 0.1% SDS, and BSA for 25 minutes at 43°C in a hybridization oven.

The microarray slide was assembled using 58 μL of hybridization mixture on top of the pre-warmed slide, which was covered with the prepared cover glass. The slide was inserted into a Corning Hybridization Chamber with 15 μL DEPC water. Hybridization was performed on the slide within a container in a hybridization incubator at 44°C overnight. The post cDNA hybridization wash, DNA hybridization and wash, and post 3DNA hybridization wash were performed according to the Genisphere Array 900

protocol. Mixtures were prepared corresponding to a 24x60 mm cover slip and incubations were set to a hybridization temperature of 42°C.

Microarray Analysis. Microarrays from all students were scanned using an Axon 4100A gene chip reader and statistically analyzed with GenePix Pro6.1 and Acuity using the TOM2 26x26 Array list. All features were aligned and assessed for intensity. Data was normalized using a ratio-based Lowess Slide Normalization. The array data was quantified using the log ratio 635/532 and median. A differentially expressed gene list was generated by finding the subset of genes that were at least 2-fold up- or down-regulated in the *lz-2* mutant versus control. Statistical analysis of 8 arrays (TOM2v1q.4.358-TOM2v1q.4.365) was performed using one and two sample significant tests and adjusted using the Bonferroni correction. Significant genes were selected based on corrected p-values less than 0.005. A similar analysis was performed using 6 arrays and Benjamini Hochberg corrected p values. The top eight differentially expressed genes from both analyses were chosen for future experiments. GenBank and Solgenomics were used to obtain the gene name for best hit in the tomato genome.

Primer Design. Primer pairs for RT-PCR and qPCR were designed for 5 top differentially expressed genes using Primer3 and BLAST search engines such that they would fit the constraints of a product size of 150-200 nt and optimal product melting temperature of 59°C.

RNA Purification and cDNA Synthesis. Extracted wt and *lz-2* RNA was subjected to DNase treatment using a Qiagen RNeasy DNase Mini kit (79254). Purification was performed on 6 µg of wt or *lz-2* RNA, which was used to generate cDNA by reverse transcription with unlabeled 6-mer primers (100 µM) and MMLV reverse transcriptase.

PCR was performed according to the parameters 10 minutes at 25°C, 45 minutes at 39°C, and 5 minutes at 85°C. The quality of the synthesized cDNA was assessed by control RT-PCR of the RPL2 gene. The cycler was set to the LC-R program (35 cycles of 94°C for 1 minute, 57°C for 30 seconds, 72°C for 45 seconds, and initial melting at 94°C for 1 minute). Results from the PCR reaction were visualized on a 1% w/v agarose gel using settings previously stated.

Q-PCR optimization. The optimal annealing temperature for each primer was determined from an annealing temperature gradient qPCR run. The efficiency of the primers was assessed by qPCR of cDNA serial dilutions with SYBR Green supermix.

RESULTS

Pooled seedling tissue from *S. lycopersicum* was processed in order to extract total RNA. Purified RNA from *lz-2* mutant plants was visualized by agarose gel electrophoresis as sharp and strong 28S and 18S rRNA bands with no visible fragments or contaminant smears (Figure 1). Visual assessment of RNA quality by the 28S/18S rRNA band intensity ratio indicated high quality of the purified sample by an approximate intensity ratio of 3 compared to a nominal value of 2. Spectrophotometry of the purified RNA sample verified band intensity quality analysis with a 260/280 nm ratio of 2.1, suggesting very low protein contamination compared to a benchmark value of 2 (Table 2). The concentration of purified *lz-2* mutant RNA within the pooled sample was determined to be 0.4743 µg/mL by spectrophotometry (Table 2).

Genome-wide differences in gene expression between wild-type and *lz-2* mutant plants were assessed using microarray technology with a tomato microarray slide with transcript analysis potential of approximately 12,600 genes. A subset of 31 genes were

found to be significantly up- or down regulated (more than 2-fold) by analysis of 8 arrays with Acuity and GenePix Pro6.1 using Loess normalization (Bonferroni corrected p value < 0.005). From the complete gene list, the twenty most highly differentially expressed genes along with two other genes found to be differentially expressed using similar microarray analyses were functionally categorized by TAIR gene ontology (Figure 2). Genes involved with cellular signaling pathways constituted 18% of analyzed genes, which confirms expectations about a link between transduction pathways and the mutation of interest; however, other functions were found to be equally associated, including genes involved with translational regulation and metabolic processes (Figure 2). Additionally, genes related to protein ubiquitination comprised the largest percentage of 23% of all analyzed genes, a function that has not been extensively associated with the *lz-2* mutation in previous studies (Figure 2). Microarray analysis of the *lazy-2* mutation samples compared to wild type plants indicates that genes within broad spectrum of functions were significantly differentially regulated and provides preliminary data for further studies on specific functional genes.

From statistical analysis of the eight hybridized microarrays, two lists of all significantly differentially expressed genes were generated and pooled together to create an abbreviated list of the eight most up- and down-regulated genes (Table 2). A total of three differentially regulated genes on the short list were purported to play a role in a ubiquitin-proteasome pathway, which corresponds to the gene ontology functional analysis results within the larger category of protein ubiquitination (Table 2, Figure 2). Only one gene, PRP4, belonging to the hypothesized *lz-2* mutation genome-wide effect of signal transduction was included on the list of most differentially regulated genes, which

encodes a signal transduction kinase and was found to be the most down-regulated gene by 7.5-fold (Table 2). The other genes found to be most up- or down-regulated are associated with transcriptional regulation or phototransduction functional categories, confirming gene ontology results (Table 2). The PRP4 gene, as well as DCL1, F-box family, and DEAD-box genes were chosen as top gene candidates that may have significantly altered expression in *lz-2* mutant plants.

Primers were designed for the top gene candidates in order to verify microarray results with PCR analytical techniques using cDNA synthesized by real-time PCR (RT-PCR). The cDNA products were then assessed for quality by control PCR with RPL2 housekeeping gene. Gel electrophoresis of the RT-PCR results with a 1 kb and 100 bp ladder showed strong, solid bands for wild-type and *lz-2* cDNA, indicating that both samples of synthesized cDNA were high quality and non-fragmented (Figure 3). Band migration of all cDNA samples indicated a RT-PCR product size of 200 bp, which is less than 50 bp larger than the predicted product size of 154 bp (Figure 3). The control PCR using the RPL2 gene validated the quality and purity of synthesized cDNA that was utilized in future studies.

Designed primers for the top candidate genes were then tested in a series of optimization experiments to determine the optimal annealing temperature and template dilution. A temperature gradient with seven annealing temperatures was used to perform qPCR reactions with each designed primer set and cDNA from wild-type or *lz-2* mutant plants. The qPCR products from the gene candidate DCL1 were analyzed using a generated melt curve, which showed no major contamination of nonspecific products or primer-dimers (Figure 4). However, amplification yielded weak product peaks below the

threshold (220 derivative fluorescence units) with dissimilar melting temperatures for six of the eight tested conditions (Figure 4). The optimal annealing temperature was selected to be 59° C because it produced the strongest melt peak with no contaminants and low C_t value of 35.23 compared to other temperatures tested (Figure 4). Although the experimentally determined optimal annealing temperature is equivalent to the designed annealing temperature for the DCL1 primer set, weak amplification of most annealing temperatures tested indicates that the primers were not effectively designed. Further qPCR analyses for the DCL1 gene expression were discontinued in favor of other gene candidates.

Optimization of annealing temperatures by temperature gradient qPCR was also performed for the PRP4 gene candidate (SGN-U218215). Analysis of the generated melt peak shows no contamination by secondary products and that each experimental condition had approximately the same melting temperature (Figure 5). Compared to the melt peak analysis for the DCL1 gene, the PRP4 gene melt peak indicates higher amplification consistency and purity with effectively designed primers. The annealing temperature of 63.3°C generating the tallest melt peak and a C_t value of 30.5 was chosen for qPCR experiments of the PRP4 gene.

The top gene candidates were tested by qPCR using a series of three ten-fold serial dilutions of cDNA template to construct a standard curve for template concentration optimization. The PRP4 gene was chosen for optimization because of previous microarray results indicating it was the most down-regulated gene and functional analysis implying a role in signal transduction. Amplification by qPCR yielded a standard curve with a linear regression R^2 value of 0.997 and amplification efficiency

of 74.6% (Figure 6). While the experimentally derived standard curve for PRP4 resulted in 15% less efficiency than the ideal amplification efficiency (90-110%), the strong linear regression indicates very low variability between replicates and an overall decent assay. Another candidate gene, encoding an f-box family protein (SGN-U227748) was also chosen for further optimization to verify differential expression within the protein ubiquitination category. The gene was similarly analyzed by qPCR amplification of cDNA template serial dilutions to produce a standard curve with a linear regression R^2 value of 0.926 and amplification efficiency of 119.4% (Figure 7). The F-box gene standard curve exhibited an efficiency percentage closer to the ideal range than the curve for the PRP4 gene, indicating a more robust assay. The respective efficiency value for each gene was later used in delta delta C_t analysis for differential expression verification.

The synthesized cDNA from wild-type and *lz-2* mutant *S. lycopersicum* plants was used to confirm the expression of optimized top gene candidates by qPCR. The PRP4 gene found to be 7.5-fold down-regulated in *lz-2* mutant plants by microarray analysis was tested by qPCR with normalization against the RPL2 housekeeping gene. Amplification by qPCR generated contradictory results to the microarray data, indicating no statistically significant difference in gene expression between *lz-2* mutant and wild-type plants (Figure 8). The f-box gene was also tested by qPCR analysis, which revealed a statistically significant up-regulation of expression by 2.36-fold in *lz-2* mutant sample versus wild-type plants (Figure 9). Thus the microarray data of differential regulation of the f-box gene in *lz-2* mutant plants was verified by the qPCR analytical technique, specifically designating an up-regulation of the gene encoding an f-box family protein.

DISCUSSION

Global gene activity analysis of the *lazy-2* mutation was performed in order to study the diversity of downstream effects of this unique reversed-gravitropic response. Previous molecular and physiological studies of this tomato plant mutation have elucidated valuable interactions between phytochrome, a major light sensing complex, and the light-dependent response to gravity (Gaiser and Lomax, 1993). Phototropism also utilizes phytochromes to cause physiological changes in structure or orientation and most likely contributes to the *lz-2* mutant plant's reaction to certain types of light (Gaiser and Lomax, 1993). Both phototropism and gravitropism involve the ubiquitous plant hormone auxin, which mediates downstream morphological changes through several signaling transduction cascades (Correll and Kiss, 2002). The *lz-2* mutation provides a distinctive physiological model with unidentified genetic origins to explore this foundational understanding of the connection between light and gravity sensation with subsequent growth response regulated by auxin.

The main biological question probed by this study investigated how the *lz-2* mutation affects genes involved in auxin signal transduction and more specifically, which signaling pathway genes are most differentially expressed in mutant versus wild-type tomato plants. Research to explore this hypothesis utilized microarray technology to perform a preliminary genome-wide assessment followed by verification of differential expression of top gene candidates using RT-PCR and qPCR techniques. Gene ontology functional analysis of the microarray data from eight arrays categorized a subsection of regulated genes to provide a more comprehensive understanding of the genome-wide effects of the *lz-2* mutation. Genes involved in signaling pathways constituted a

significant percentage of differentially expressed genes, although these results cannot be necessarily correlated to auxin transduction genes specifically without further study of each constituent gene.

One gene in the cellular signaling function category, PRP4, was found to be most down-regulated by microarray data and was examined by additional qPCR tests as a top gene candidate. The PRP4 gene has been characterized within the *Arabidopsis thaliana* genome as a cell wall protein kinase shown to have temporal specificity during morphogenesis and localization within budding appendages, suggesting a possible link between PRP4 expression and the growth response mediated by auxin (Fowler et al., 1999). Down-regulation of the PRP4 gene in *lz-2* mutants as indicated by preliminary microarray data could cause decreased signal transduction in developing aerial tissues. If such transduction pathways were linked to auxin signaling, irregular auxin transport could contribute to the atypical gravitropic response seen in *lz-2* mutants.

While the microarray data indicated strong differential regulation of the PRP4 gene in *lz-2* mutants, verification by qPCR showed contradictory results of no statistical difference in expression of the PRP4 gene. Microarray technology allows for exceptionally effective assessment of genome-wide expression, but the quality of data can be affected by hybridization procedures or errors in spot intensity analysis (Morey et al., 2006). Though conflicting evidence of PRP4 differential expression does not negate PRP4 as a potential gene candidate nor signal transduction as a viable function significantly affected by the *lz-2* mutation, future research should focus on resolving the divergence of results. In order to uncover the actual role of PRP4 in the tomato genome

and a potential interaction with auxin, targeted knock down of PRP4 with subsequent phenotypic and genetic analysis should be performed.

Gene ontology results showed that genes involved in signaling were not in fact the largest functional category; rather, genes involved in protein ubiquitination constituted the highest percentage, suggesting a protein degradation pathway at work in *lz-2* mutant plants. Microarray results indicated that the most up-regulated gene was ATCUL1, a cullin protein in *A. thaliana* that is a major component of the ubiquitin ligase complex, SCF (del Pozo and Estelle, 1999). Protein degradation by ubiquitin-proteasome pathways is a widely-employed mechanism to regulate intracellular proteins such as those involved in the cell-cycle, metabolic processes and stress responses (del Pozo and Estelle, 1999; Gray et al., 1999). In particular, the auxin response depends on the ubiquitin-ligase SCF complex for regulated proteolysis of repressor proteins (Gray et al., 1999). Thus up-regulation of the ATCUL1 gene in *lz-2* mutants may cause over-expression of the SCF complex, leading to misregulated auxin protein degradation.

In addition to ATCUL1, the microarray results show that several other top gene candidates are potentially associated with ubiquitin-proteolysis in *lz-2* mutant plants. The PUB18 gene found to be a differentially expressed gene is purported to encode a U-box domain containing protein that functions as an ubiquitin ligase in the ubiquitin-proteasome pathway, Ub-26S (Yee and Goring, 2009). However, the specific role of the PUB18 gene in the Ub-26S pathway in the *S. lycopersicum* genome is not well understood and microarray data suggested the PUB18 gene was significantly down-regulated (Yee and Goring, 2009). The PUB18 gene could function in a different ubiquitin-proteolysis cascade than ATCUL1, or alternatively, the *lz-2* mutation may

cause diverse over-expression and down-regulation of several proteins in a ubiquitination pathway. Future studies should explore these possibilities through verification of ATCUL1 and PUB18 gene expression by qPCR.

In conjunction with ATCUL1 and PUB18, a gene encoding an f-box family protein was found to have high differential expression by microarray analysis. Confirmation by qPCR showed a statistically significant up-regulation in *lz-2* mutants compared to wild-type plants. This protein has been described as another major component of the SCF complex that interacts with target proteins and regulates activity of the complex (del Pozo and Estelle, 2000). The N-terminal of the F-box protein mediates interactions between the major proteins of the SCF complex, while the C-terminus binds to the ubiquitination target for degradation (Leyser, 2002). Interestingly, previous studies have shown that over-expression of an f-box protein, TIR1, in transgenic plants led to enhanced auxin response and several morphological changes (Gray et al., 1999). With evidence presented here of possible up-regulation of an f-box protein in *lz-2* mutants, the study by Gray et al. (1999) implies that the *lz-2* mutation may cause downstream effects on the activity of the SCF complex resulting in altered auxin response and growth changes.

Further analyses should investigate whether the top gene candidates involved in protein ubiquitination are in fact components of the SCF complex mediating the response of auxin by employing affinity chromatography purification. The cullin protein encoded by the ATCUL1 gene from *lz-2* mutant tissue could be tagged with a polyhistidine-tag and then bound on a Ni²⁺ column and incubated with whole cell lysate. Sequential washes of the proteins encoded by the PUB18 gene and f-box gene would allow either

protein to bind to the cullin protein if, in fact, it has binding affinity for the cullin protein, as in the SCF complex. Elution of bound proteins and analysis by SDS-PAGE would illustrate if these endogenously derived proteins interact endogenously within the same ubiquitin pathway.

CONCLUSION

By employing a wide variety of molecular tools and previous research on the physiological response of plants to environmental cues, this study investigated the intricate connections between gravitropism, signaling, and auxin regulation through genome-wide analysis of *lazy-2* mutant tomato plants. Microarray data highlighted potential differential expression of key functional genes including those involved in signal transduction. This preliminary data confirmed the hypothesis that mis-regulation of signaling cascades linked to auxin transport could be a contributing biological factor in the observed reversed-gravitropic response.

Global gene activity assessment did elucidate an intriguing trend that the *lz-2* mutation was associated with significant differential expression of genes involved in protein ubiquitination. The cullin protein ATCUL1, known to be a major component of the SCF complex regulating auxin activity by inhibitor degradation, was found to be strikingly up-regulated along with an f-box protein, purported to be another major component of the SCF complex by microarray data. Expression of the f-box protein gene in *lz-2* mutant plants was confirmed by qPCR to have statistically significant up-regulation compared to wild-type tomato plants. An additional member of a ubiquitin-ligase pathway, PUB18, was also found to be a differentially regulated gene candidate, suggesting that a diverse network of up- and down-regulated ubiquitin-related genes

altering auxin regulation may be underlying the reversed-gravitropic phenotype of the *lz-2* mutation in *S. lycopersicum* plants.

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FIGURES AND TABLES

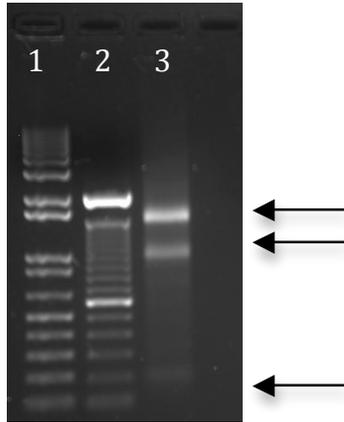


Figure 1. Gel electrophoresis of extracted *lz-2* mutant RNA from *S. lycopersicum*. RNA was extracted from *S. lycopersicum* seedling tissue and analyzed by agarose gel (lane 3) with a 1 kb ladder (lane 1) and 100 bp ladder (lane 2). An intact 28S rRNA band (1,650 bp, top arrow) and 18S rRNA band (1,000 bp, middle arrow) with no visible fragments indicated the sample contained high quality RNA.

Table 1. Quantity and quality analysis of extracted *lz-2* RNA from *S. lycopersicum*. RNA extracted from *lz-2* mutant *S. lycopersicum* seedling tissue was analyzed by a Nanodrop Spectrophotometer. The extracted RNA was determined to be pure by a 260 nm/280 nm ratio of 2.1 compared to a benchmark value of 2.

Sample Name	Concentration	
	($\mu\text{g/mL}$)	260 nm/280 nm
<i>lazy-2</i> Mutant	0.4743	2.1

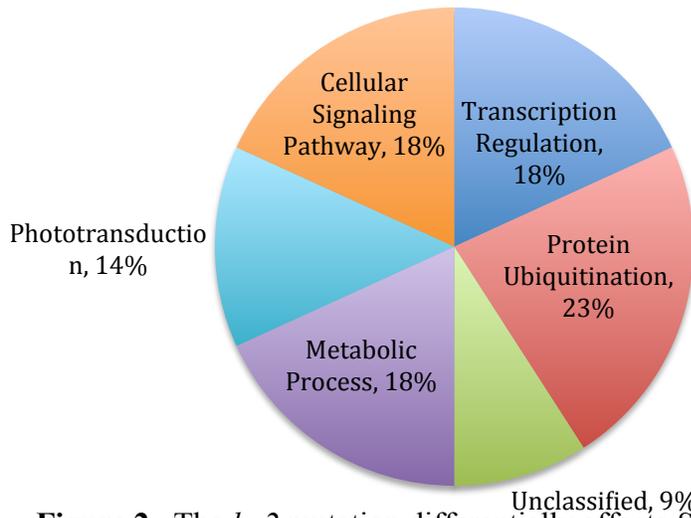


Figure 2. The *lz-2* mutation differentially affects *S. Lycopersicum* gene expression across functional categories. Global gene activity of wt and *lz-2* mutant plants was analyzed by microarray (8 arrays; $p_{\text{corrected}} < 0.005$) to obtain 31 most differentially regulated genes. From the complete list, twenty most differentially expressed genes and two genes from a similar microarray analysis were functionally categorized as described in the Arabidopsis TAIR gene ontology database.

Table 2. Abbreviated list of most differentially expressed genes between *lz-2* mutant and wt *S. lycopersicum* plants from genome-wide expression analysis. From two similar microarray analyses (6 or 8 microarrays), a combined short list of most differentially regulated genes was generated ($p_{\text{corrected}} < 0.005$).

Differentially Regulated Gene	SGN Number	Average Fold Expression ^a	P-Value ^b	Description ^c
ATCUL1	SGN-U236733	6.246	$p_c < 0.005$	Ubiquitin-proteasome pathway
DCL1	SGN-U236515	5.777	$p_c < 0.005$	RNAi gene silencing
CCB1	SGN-U220615	5.609	$p_c < 0.005$	Cytochrome b6f complex
PRP4	SGN-U218215	-7.512	$p_c < 0.005$	Signal transduction kinase
Cu2+ Plastocyanin	SGN-U242499	-6.865	$p_c < 0.005$	Electron carrier in photosynthesis
PUB18	SGN-U219566	-5.823	$p_c < 0.005$	Ubiquitin-proteasome pathway
F-box family	SGN-U227748		$p_c < 0.005$	Ubiquitin-proteasome pathway
DEAD-box protein	SGN-U236839		$p_c < 0.005$	Transcription Regulation

^a Value determined from analysis of 6 or 8 arrays using one and two-sample significant test

^b The Bonferroni correction was used to determine corrected p values

^c Gene descriptions were obtained from Solgenomics

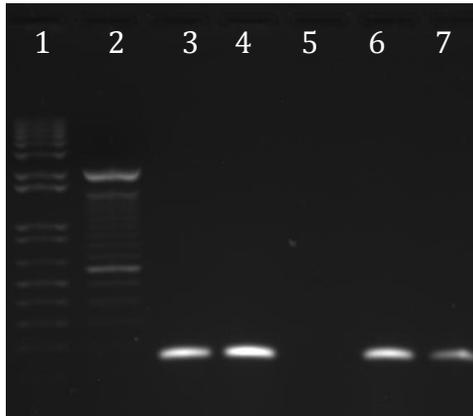


Figure 3. Gel electrophoresis of control RT-PCR of wild-type and *lz-2* mutant cDNA from *S. lycopersicum*. cDNA was synthesized from wt and *lz-2* extracted RNA and subjected to a control RT-qPCR with a primer set for RPL2 (left primer CAGCGGATGTCGTGCTATGAT, right primer GGGATGCTCCACTGGATTCA). A 1kb ladder (lane 1) and 100 bp ladder (lane 2) was run alongside wt cDNA (lane 3 and 6) and *lz-2* cDNA (lane 4 and 7). Lane 5 contained no sample. The PCR product was assessed to be intact and of high quality based on strong band intensity and size of 200 bp compared to a predicted product size of 154 bp.

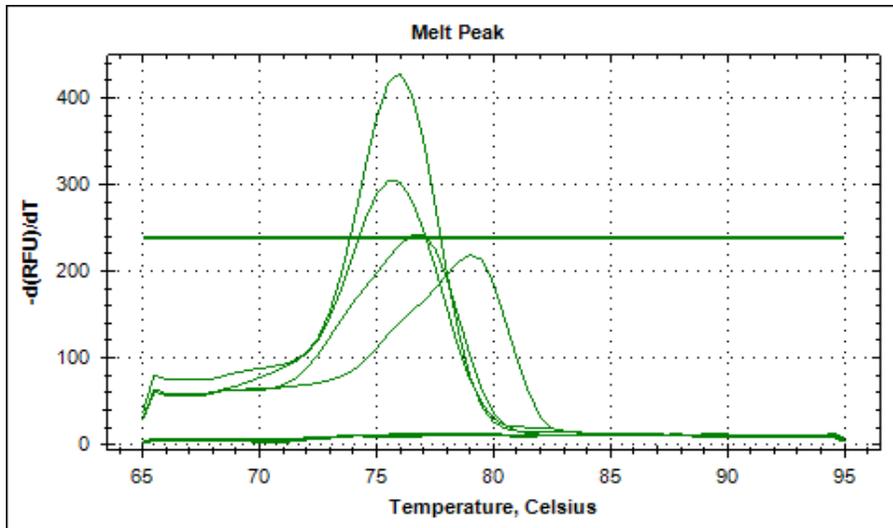


Figure 4. Primer annealing temperature optimization using melt peak analysis of qPCR for the DCL1 gene (SGN-U236515). Synthesized cDNA from wt *S. lycopersicum* was analyzed by qPCR using seven annealing temperatures to maximize primer binding (left primer GCTGGCAGTTTTTCTGGTTC; right primer TGATAGCCAGTTTGCTCACG). The optimal annealing temperature was found to be 59 °C based on the highest melt peak with no contaminating side peaks and a C_t value of 35.23.

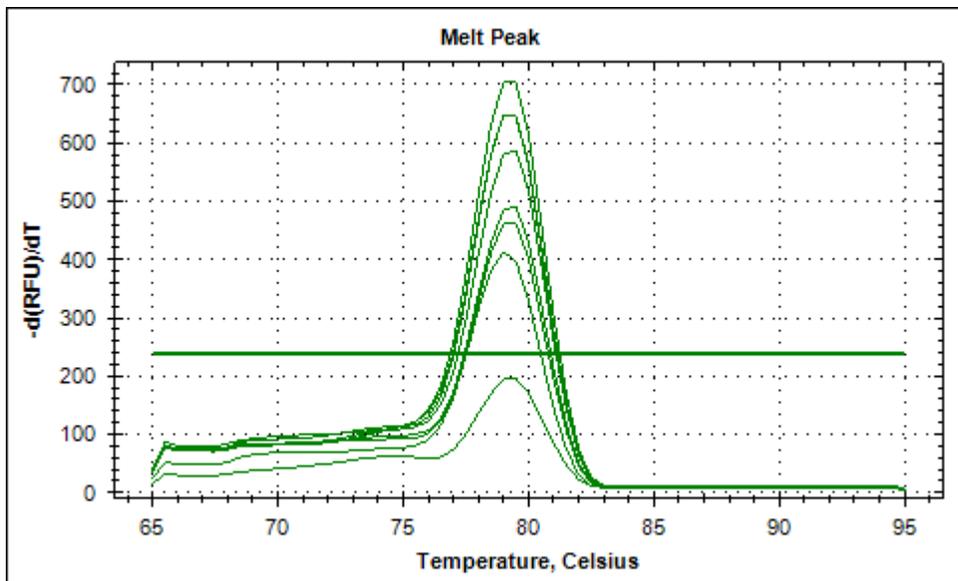


Figure 5. Primer annealing temperature optimization using melt peak analysis of qPCR with the PRP4 gene (SGN-U218215). Synthesized cDNA from extracted RNA of *S. lycopersicum* seedling tissue was analyzed by qPCR for optimal annealing temperature of designed primers. The temperature of 63.3°C produced a C_t value of 30.5 and the tallest melt peak with no contaminating side peaks.

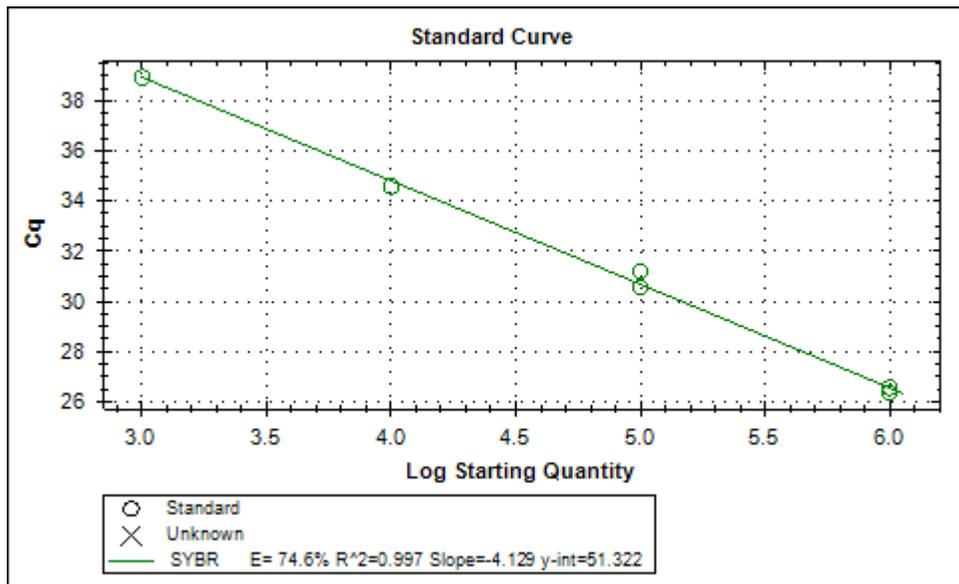


Figure 6. Standard curve based on amplification of three ten-fold dilutions of the PRP4 candidate gene (SGN-U218215) for template dilution optimization. The designed primer set for the PRP4 gene in triplicate was analyzed by qPCR for determination of optimal template concentration by comparing C_q value to log of starting concentration. The amplification efficiency for the samples was 74.6% with a linear regression R^2 value of 0.997, indicating a decent assay.

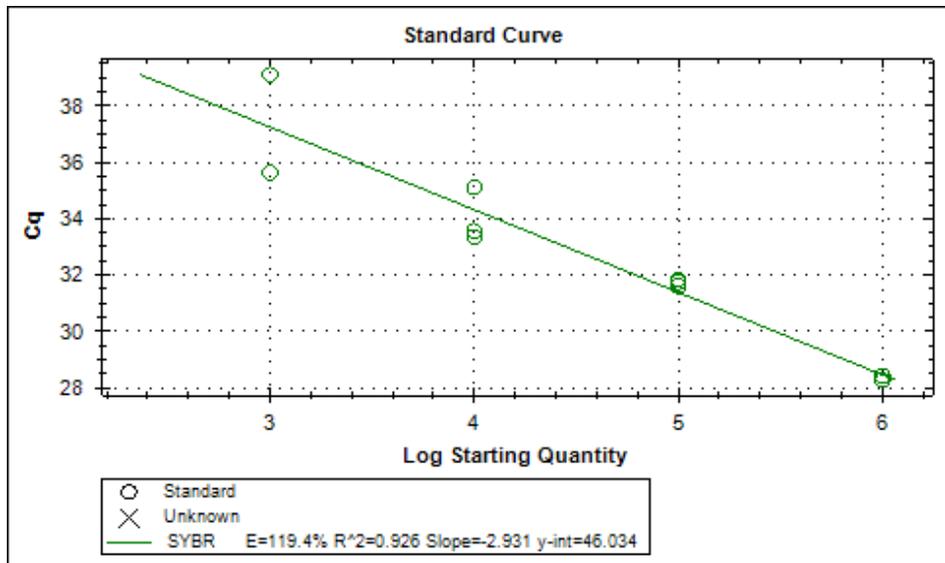


Figure 7. Standard curve based on amplification of three ten-fold dilutions of the f-box family candidate gene (SGN-U227748) for template dilution optimization. The designed primer set for the gene encoding an f-box family protein was tested by qPCR in triplicate to determine the optimal template concentration for the candidate gene. The amplification efficiency for the samples was 119.4% with a linear regression R² value of 0.926, indicating a robust assay.

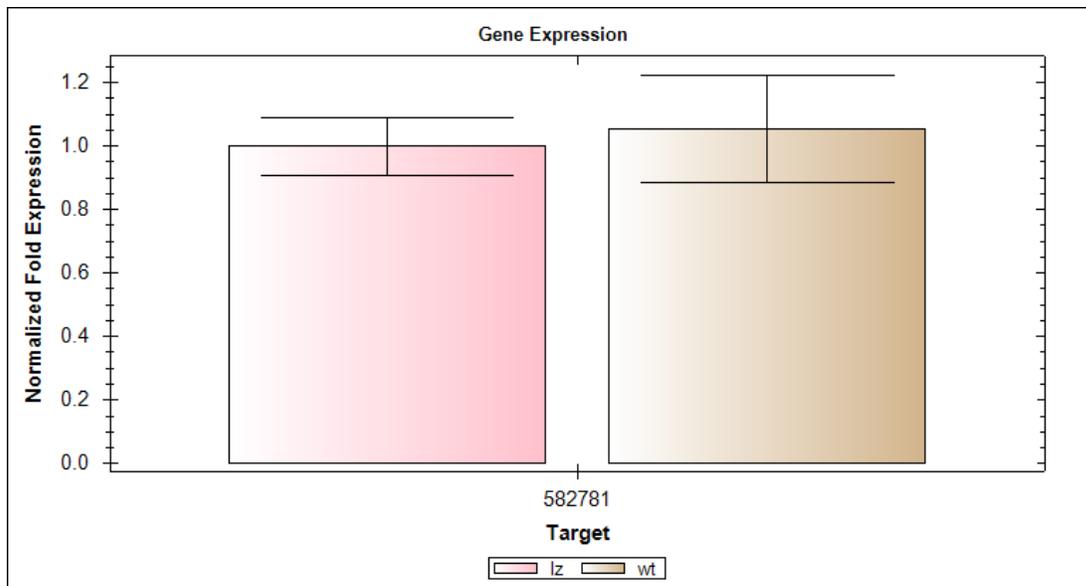


Figure 8. Relative quantification of expression of the PRP4 gene in wt and *lz-2* mutant *S. lycopersicum* plants by qPCR. The $\Delta\Delta C_t$ method was used to determine fold expression with normalization against the RPL2 housekeeping gene. Error bars represent the standard deviation between three technical replicates. No statistical difference in PRP4 gene expression is seen between wild-type (1-fold expression) and *lz-2* mutant plants (1.05-fold expression) by qPCR analysis.

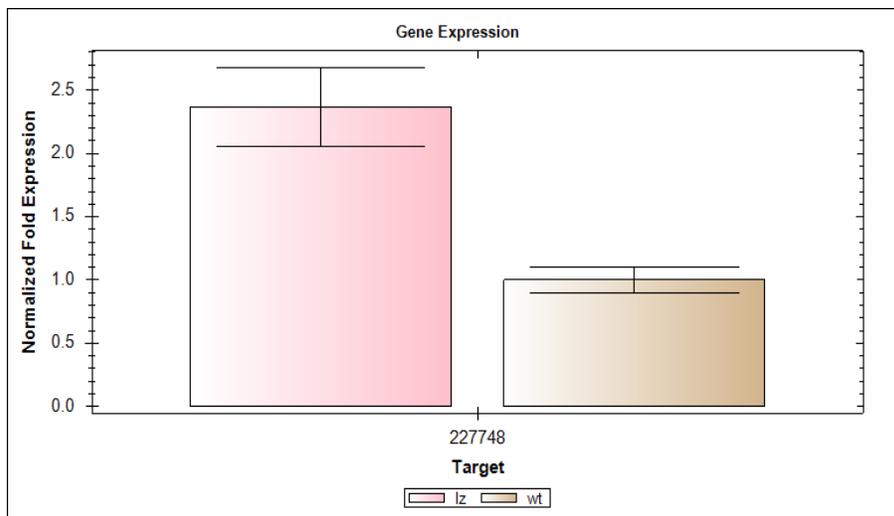


Figure 9. Relative quantification of the f-box family gene (SGN-U227748) in wt and *lz-2* mutant *S. lycopersicum* plants by qPCR. Fold expression was determined using the $\Delta\Delta C_t$ method with normalization against the RPL2 housekeeping gene. Error bars represent the standard deviation between three technical replicates. A statistically significant difference in f-box gene expression was found by qPCR analysis, with up-regulation of 2.36-fold expression in the *lz-2* mutant samples compared to wild-type plants (1-fold expression).