FUNCTIONAL GENOMICS OF PLANT COMPENSATORY RESPONSES

BY

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DISSERTATION

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Abstract

Numerous studies have shown that some plant genotypes can compensate for tissues lost with little or no decrement in fitness relative to those that are undamaged (see Stowe et al. 2000 for a review); such plants are termed as tolerant. This trait motivated many empirical studies demonstrating that herbivore damage can, under certain circumstances, increase, rather than decrease, plant reproductive success (a specialized case termed overcompensation, i.e., increased flower, fruit, and seed production following herbivory). Specifically, studies by Paige and Whitham (1987) showed that when mule deer and elk removed 95% or more of the aboveground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, the lifetime seed production, seed germination, and seedling survival averaged 3.0 times that of uneaten controls (Paige 1992, 1994, 1999). Despite evidence that genetic variation for tolerance exists, the underlying genetic basis of compensation is not known. My research focuses on understanding the molecular basis of plant compensatory responses using suite of quantitative and molecular techniques. As a first step, QTL mapping was combined with results from a microarray analysis to identify potential candidate genes viz., *G6PDH1* and invertase. These genes were then characterized using recombinant DNA techniques.

A set of recombinant inbred lines developed from a cross between Columbia X Landsberg *erecta*, of the annual plant *Arabidopsis thaliana* were screened initially to identify QTLs. A total of three QTL located on chromosomes 1, 4 and 5 explaining 48.2% of the variation in fitness compensation were found. The microarray experiment revealed 109 genes that were differentially expressed between clipped and unclipped plants of the overcompensating ecotype Columbia. Combining the results from the QTL and microarray data, two genes were uncovered that appeared to play a significant role in the phenomenon of overcompensation, a cytosolic glucose-6-phosphate-1-dehydrogenase (*G6PDH1*) and an invertase. *G6PDH1* T-DNA knockout studies of the overcompensatory accession Columbia-4 accession showed patterns of equal/under-compensation verifying its role in the compensatory response. *G6PDH1* is a key enzyme in the oxidative pentose-phosphate pathway that plays a central role in plant metabolism, converting glucose to ribose-5-phosphate. The role of *G6PDH1* in plant compensation was further verified by complementing *G6PDH1* to reinstate its function in a *G6PDH1* knockout and to localize where it is expressed by creating chimeric promoter-reporter (GUS) fusion constructs.

Results from one of four complementation lines showed a partial rescue effect of *G6PDH1*, showing patterns more similar to the overcompensating Columbia line than either Landsberg *erecta* or the knockout line. Furthermore, results of our promoter-reporter fusion studies (*G6PDH1* promoter: β-glucuronidase (GUS)) and subsequent histochemical staining revealed that *G6PDH1* is expressed in virtually all tissues rather than localized to any specific tissue. These results are consistent with patterns of regrowth observed following clipping in *Arabidopsis*, reconstituting the entire plant with greater biomass and higher fitness.

The importance of invertase isoenzymes in the compensatory response of the two *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta* (overcompensating and undercompensating genotypes, respectively) were also determined. Invertases represent one class of enzymes that shunt glucose to activate the oxidative pentose phosphate pathway, therefore expression of invertase isoenzymes over developmental time and fitness analysis of T-DNA knockout mutants was examined. Results showed differences in plasticity in the expression of invertases following the removal of apical dominance. In Columbia, an overcompensating genotype, nine of twelve invertase isoenzymes were significantly up-regulated one to five days after the removal of apical dominance. In, Landsberg *erecta*, an undercompensating genotype, only two neutral invertases showed a decline in expression at 15 days post-clipping. These results were consistent with patterns observed for *G6PDH1*, showing up-regulation at five days post-clipping in Columbia. This is possibly due, in part, to an increase in glucose fed from invertase isoenzymes into the OPP pathway, facilitating the rapid regrowth and greater biomass accumulation observed in the overcompensating genotype Columbia. Furthermore, there was a general trend toward higher expression at 50% flowering for both clipped and unclipped plants (with no significant differences in expression between treatments or between genotypes) in six of twelve Columbia isoenzymes and three of twelve for Landsberg *erecta*. These results suggested Columbia and to a lesser degree Landsberg *erecta* may up-regulate gene expression over earlier time periods in order to facilitate flower and fruit development. These results were also consistent with the patterns observed for *G6PDH1*, showing greater up-regulation at 50% flowering post-clipping in Columbia (i.e., twice the number of invertases up-regulating to supply the added glucose for increased flower and fruit production in the overcompensating genotype, Columbia versus the undercompensating genotype, Landsberg *erecta*). The T-DNA knockout experiments on the two invertase genes, a vacuolar invertase and a neutral invertase, and their

isoforms confirmed their importance in plant growth and fitness in *Arabidopsis thaliana* following the removal of apical dominance. Of particular note, there did not appear to be any functional redundancy of other invertases or any of the sucrose synthases. Thus, all invertase isoenzymes seem to be necessary for normal growth, development and reproduction and, most importantly here, for growth and fitness compensation following apical damage.

Based on these results, it is proposed that plants with the capability of overcompensating reprogram their transcriptional activity in three important ways: a) through defensive mechanisms, b) through increased expression of genes involved in energy metabolism and c) through an increase in DNA content (via endoreduplication) that feed metabolites to pathways involved in defense through transcriptional programming (Scholes and Paige, 2011). Collectively, these results, indicate the direct or indirect importance of *G6PDH1*and invertase isoenzymes in regulating the compensatory response following the removal of apical dominance.

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION ……...1

CHAPTER 2: MOLECULAR BASIS OF OVERCOMPENSATION: THE ROLE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND THE OXIDATIVE PENTOSE-PHOSPHATE PATHWAY…… 7

CHAPTER 3: THE ROLE OF INVERTASE ISOENZYMES IN PLANT COMPENSATION…… ..29

CHAPTER 4: THE ROLE OF GLUCOSE-6-PHOSPHATE-1-DEHYDROGENASE (*G6PDH1*) IN PLANT COMPENSATION: COMPLEMENTATION AND GENE LOCALIZATION OF *G6PDH1* ……...78

CHAPTER 1: GENERAL INTRODUCTION

Plants have evolved numerous traits that mitigate damage caused by pests and pathogens. Although insect pests do cause damage (usually on the order of 10% tissue loss, depending upon severity of the incidence), a single bout of mammalian herbivory is comparatively devastating, as a plant can lose ≥90% of its aboveground biomass. Nonetheless, many plants can tolerate or even take advantage of such high levels of herbivory, e.g., the classic example of overcompensation (increased fitness) following ungulate herbivory in *Ipomopsis aggregate* (Paige and Whitham1987).

Ecologists and evolutionary biologists became interested in overcompensation in the mid-1970's when several authors (Chew 1974, Dyer 1975, Owen and Wiegert 1976) reported that herbivory may result in an increase rather than a decrease in the growth and reproductive success of some plant species (Whitham et al. 1991). This observation was initially dismissed as the result of reallocation of belowground resources to aboveground structures, eventually resulting in a net fitness decrement (Belsky 1986, Verkarr 1986). Studies by Paige and Whitham (1987) provided the first convincing evidence that herbivory can, under some circumstances, lead to enhanced plant fitness. Their choice of a monocarpic plant (i.e., one that reproduces only once and then dies) simplified the estimation of lifetime fitness and eliminated the possibility that apparent overcompensation came at the expense of future reproduction (Vail 1992). They showed that when ungulate herbivores remove 95% or more of the aboveground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, the product of lifetime seed production, seed germination, and seedling survival averaged 3.0 times that of the uneaten controls (Paige and Whitham 1987, Paige 1992, 1994, 1999, Anderson and Paige 2003). The increase in relative fitness was largely because of an architectural change in the plant. Ungulate removal of scarlet gilia's single inflorescence resulted in the production of multiple flowering stalks due to the release of apical dominance and an overall increase in both above- and below-ground biomass. Many researchers have since uncovered additional examples of overcompensation (Maschinski and Whitham 1989, Alward and Joern 1993, Lowenberg 1994, Lennartsson et al, 1997, Weinig et al. 2003a,2003b Rautio et al. 2005), thus, the apparently paradoxical phenomenon of overcompensation could no longer be summarily dismissed (Stowe et al. 2000).

There is evidence that genetic variation for compensation exists. Specifically, some families exhibit overcompensation tolerance, whereas others express incomplete compensation (Mauricio et al. 1997, Tiffin and Rausher 1999, Juenger and Bergelson 2000). Heritability of traits associated with compensation has been demonstrated in one population of scarlet gilia as well (Juenger and Bergelson 2000). In addition, recent studies comparing historically grazed and ungrazed populations of the plant *Gentianella campestris* indicate that *repeatedly grazed populations can overcompensate tolerance*, while ungrazed populations remain completely intolerant (Lennartsson et al. 1997).

Although these observations provide evidence that genetic variation for compensation exists, little is known about the genetic mechanisms leading to enhanced growth and reproduction in scarlet gilia, or for that matter, any other plant species exhibiting growth compensation. To uncover potentially important genes and gene pathways involved in the phenomenon of overcompensation I propose to use a suite of quantitative and molecular techniques. Specifically, I propose to identify potential candidate genes by uncovering QTLs (Quantitative Trait Loci) for compensation in combination with data from a microarray analysis to narrow down the number of genes to a few promising candidate genes. This was followed by knockout, expression assays, and transgenic complementation studies for verification of the potential role of the gene in growth compensation.

I chose to work with *Arabidopsis* because it responds to apical damage in much the same way *Ipomopsis* does, is monoecious, is predominately a selfer such that silique production is the product of male and female fitness, and it has many advantages for genome analysis over other plants. Most importantly, the full genome sequence has recently been published - the first complete sequence for any plant (The Arabidopsis Genome Initiative 2000). *Arabidopsis* is also *the* model system for identifying plant genes and gene function. Overall, the *Arabidopsis* genome is relatively small, estimated to contain approximately 26,751 protein-coding genes of which some 17,250 have been classified according to sequence similarity to proteins of known function (The Multinational Coordinated *Arabidopsis thaliana* Functional Genomics Project 2006). Some 1,250 of these have known phenotypic effects (Alonso-Blanco and Koornneef 2000). Most of the known protein-coding genes have been assigned to functional categories including defense, photomorphogenesis and photosynthesis, gene regulation, development, metabolism, transport, and DNA repair (The Arabidopsis Genome Initiative 2000). In addition,

the availability of Recombinant Inbred Lines (RILs), knockout mutants, BAC, cDNA clones and standardized techniques for characterization and understanding of the functional role of genes underpinning ecological traits, makes *Arabidopsis* a model system for studies of the genetic basis of fitness compensation.

In this study I specifically proposed to:

1) Characterize fitness variation for compensatory responses among RILs (Lister and Dean, 1993) from a cross between Landsberg *erecta* X Columbia, identify QTL and map the QTL data with microarray data to identify candidate genes.

2) Experimentally assess the importance of candidate genes – *G6PDH1* using a suite of molecular techniques including:

   a. An assessment of the phenotypic response of knockout mutants for candidate genes of interest.
   b. mRNA quantification of Columbia and Landsberg *erecta* for clipped and unclipped plants
   c. Complementation of the gene in knockout plants to restore the phenotype.
   d. Histochemical analysis by creating chimeric promoter (*G6PDH1*) – reporter (GUS gene) fusion

3) Experimentally assess the importance of invertase isoenzymes using suite of molecular techniques

   a) An assessment of the phenotypic response of knockout mutants for candidate genes of interest,
   b) mRNA quantification of Columbia and Landsberg *erecta* for clipped and unclipped plants.

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CHAPTER 2: MOLECULAR BASIS OF OVERCOMPENSATION: THE ROLE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND THE OXIDATIVE PENTOSE-PHOSPHATE PATHWAY [1]

Abstract

The idea that some plants can benefit from being eaten is counterintuitive, yet there is now considerable evidence demonstrating enhanced fitness following herbivory (i.e., plants can overcompensate). Although there is evidence that genetic variation for compensation exists, little is known about the genetic mechanisms leading to enhanced growth and reproduction following herbivory. We took advantage of the known compensatory variation in a set of recombinant inbred lines of the annual plant *Arabidopsis thaliana*, combined with microarray and QTL analyses to assess the molecular basis of overcompensation following apical damage. We found a total of three QTL located on chromosomes 1, 4 and 5 explaining 48.2% of the variation in fitness compensation and 109 differentially expressed genes between clipped and unclipped plants of the overcompensating ecotype Columbia. From the QTL/microarray screen we uncovered one gene that plays a significant role in the phenomenon of overcompensation, a cytosolic glucose-6-phosphate-1-dehydrogenase gene. Knockout studies of T-DNA insertion lines of *G6PDH1* showed patterns of equal/under-compensation rather than overcompensation verifying the role of *G6PDH1* in the compensatory response. *G6PDH1* is a key enzyme in the oxidative pentose-phosphate pathway that plays a central role in plant metabolism, converting glucose to ribose-5-phosphate. We propose that plants with the capability of overcompensating reprogram their transcriptional activity in three important ways: through defensive mechanisms, through increased expression of genes involved in energy metabolism and feeding back on pathways involved in defense and metabolism through increased expression through an increase in DNA content (via endoreduplication) with the increase in DNA content feeding back on pathways involved in defense and metabolism through increased expression.

Keywords: overcompensation, *G6PDH1*, endoreduplication, QTL mapping, microarray, oxidative pentose-phosphate pathway

[1] This chapter along with chapter 3 is currently in review in the journal Genetics, as Siddappaji, M.H. Scholes, D.R., Bohn, M.O. and Paige, K.N. Molecular Basis of Overcompensation in *Arabidopsis thaliana*: the Role of Glucose-6-Phosphate Dehydrogenase and the Oxidative Pentose-Phosphate Pathway

Introduction

Numerous studies have shown that some plant genotypes can compensate for tissues lost with little or no decrement in fitness relative to those that are undamaged (see Stowe et al. 2000 for a review); such plants are termed tolerant. Interest in tolerance was motivated by empirical studies demonstrating that herbivore damage can, under certain circumstances, increase, rather than decrease, plant reproductive success (a specialized case termed overcompensation, i.e., increased flower, fruit, and seed production following herbivory). Specifically, studies by Paige and Whitham (1987) showed that when mule deer and elk removed 95% or more of the aboveground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, the product of lifetime seed production, seed germination, and seedling survival averaged 3.0 times that of uneaten controls (Paige 1992, 1994, 1999). Evidence for increased flower, fruit and seed production following herbivory has also been found for numerous plant species since the initial study of Paige and Whitham (1987) including *Ipomopsis arizonica* (Maschinski and Whitham, 1987), *Gentianella campestris, G. amarella* (Nilsson et al. 1996, Lennartson et al. 1997), *Arabidopsis thaliana* (Mauricio et al. 1997, Wenig et al. 2003) and *Erysimum strictum* (Rautio et al. 2005) to name but a few. There is also evidence that genetic variation for tolerance/overcompensation exists. For example, studies comparing historically grazed and ungrazed populations of the plant *Gentianella campestris* indicate that repeatedly grazed populations overcompensate, while ungrazed populations remain completely intolerant (Lennartson et al. 1997). Furthermore, numerous plant families exhibit repeatable patterns of overcompensation, whereas others express only patterns of equal- or undercompensation (Mauricio et al. 1997, Weinig et al. 2003a, 2003b, Rautio et al. 2005, Juenger and Bergelson 2000). Although these observations provide evidence that genetic variation for compensation exists, little is known about the genetic mechanisms leading to enhanced growth and reproduction in plant species exhibiting growth compensation.

A recent study by Scholes and Paige (2011) showed that different ecotypes of *Arabidopsis thaliana* differ in the degree of endoreduplication following the removal of apical dominance. Endoreduplication is the replication of the genome without mitosis, leading to endopolyploidy, an increase in cellular chromosome number. Scholes and Paige (2011) showed that the degree of endopolyploidy achieved is positively correlated with measures of fitness (i.e., the higher the DNA content the higher the fitness following the removal of apical

dominance). Removal of apical dominance reduces the level of auxin leading to axillary bud break and stem regeneration; high levels of auxin are known to repress the endocycle and by contrast, lower levels of auxin trigger an exit from mitotic cycles and an entry into endocycles (Ishida et al. 2010). Thus, there is a direct link between endoreduplication and the removal of apical dominance. However, the variation seen among genotypes in the degree of endoreduplication following the removal of apical dominance suggests that there are genetic differences in triggering this pathway that will require future investigation (Scholes and Paige, 2011). Endoreduplication may have genetic and/or nucleotypic effects (an effect based on DNA content alone) that could lead to rapid regrowth and enhanced fitness by increasing gene expression and/or greater nutrient and water transport. Although we know a great deal about the genetic basis of endoreduplication per se (Vlieghe et al. 2005, Imai et al. 2006, Yoshizumi et al. 2006) and have evidence that it plays a role in fitness compensation (Scholes and Paige, 2011) we still don't know the underpinning genes affecting fitness compensation following endoreduplication in *Arabidopsis* (or any other plant species exhibiting growth compensation). As a first step, we have taken advantage of the known compensatory variation in the annual plant *Arabidopsis thaliana*, combined with commercially available microarrays and QTL analyses to begin to assess the molecular basis of overcompensation (increased fitness) following apical damage. In addition, we use a gene knockout approach to assess the phenotypic effects of one promising candidate gene uncovered from the microarray/QTL screen. Specifically, we (a) characterize fitness variation among recombinant inbred lines (RILs) of a cross between *Landsberg* erecta X Columbia following the removal of apical dominance, (b) determine seasonal variation in the compensatory response, (c) identify QTL responsible for the variation in compensation, (d) quantify differential gene expression underlying clipped and unclipped individuals of the Columbia ecotype using a commercially available microarray platform, (e) combine QTL and microarray data to narrow the genes responsible for the compensatory response, (f) evaluate the compensatory response of T-DNA knockout lines of a promising candidate gene, glucose-6-phosphate-1-dehydrogenase (*G6PDH1*, At5g35790.1), and (g) perform qRT-PCR on *G6PDH1* to verify differences in expression between overcompensating (Columbia) and undercompensating (Landsberg *erecta*) plants.

Materials and Methods

Fitness Variation

A total of 96 recombinant inbred lines (RILs; Lister and Dean, 1993) of *A. thaliana* developed from a cross between Columbia (an overcompensating genotype) and Landsberg *erecta* (an undercompensating genotype) were used to assess fitness variation following the removal of apical dominance (to simulate mammalian herbivory). The 96 F1 lines were advanced through eight generations of inbreeding by single-seed descent (Lister and Dean, 1993) and are available through TAIR (The Arabidopsis Information Resource). The RILs and their parental lines (Columbia and Landsberg *erecta*) were grown for two seasons (Spring 2007 and Fall 2008) in a greenhouse on the campus of the University of Illinois, Champaign under 12 hours of light and dark. Plants were grown in individual pots using LI Sunshine® mix. Ten plants per line (960 plants) were grown from seed and half (five per line) were randomly clipped from 6 cm inflorescence height to ground level, to simulate mammalian herbivory; the remaining five served as undamaged controls. At the end of the flowering season the numbers of siliques per plant were recorded. The seeds collected during the first season were used to generate the second season plants.

Potential differences in fitness were assessed using an Analysis of Variance (Systat 13) comparing plants with apical meristem damage to undamaged controls for each recombinant inbred line. Comparisons were made both within and between years to assess fitness variation among recombinant inbred lines and within line repeatability across the two years. Silique counts were square-root transformed to approximate normality. Plants within a line and over each year were classified as under- (silique production significantly lower than the undamaged control), equal- (silique production not statistically different from the undamaged control) or over-compensators (silique production significantly higher than the undamaged control) based on an Analysis of Variance for each line and year.

QTL Analysis

QTL were identified by importing phenotypic (fitness data) and genotypic data sets (Nottingham Arabidopsis Stock Centre, AtEnsembl, http://atensembl.arabidopsis.info) into QTL Cartographer version 2.5 (Wang et al 2010). Fitness data were pooled from two years using the average response across years. There were 14 of 96 lines for which we had only one year's data; these were used in our QTL mapping study as well. A total of 141 markers equally

distributed on all chromosomes with an average interval of ~4.5cM were selected (Zeng 1994). The data were initially analyzed using Composite Interval Mapping (CIM; Zeng 1994, Jansen and Stam, 1994) to find QTL. Cofactors for Composite Interval Mapping were selected from the forward and backward regression option. QTL were calculated following 1000 permutations at a threshold significance of $p<0.05$. Significant QTL (LOD score of 2.5 and above, Zeng 1994) from CIM were used to find other significant QTL and interactions among QTL elsewhere in the genome using Multiple Interval Mapping (MIM). All the analyses for MIM were performed at an Akaike Information Criteria (AIC) with a penalty of 1 to find the most parsimonious QTL region responsible for compensatory responses. The putative QTL were confirmed by a Chi-square test for the corresponding marker to the QTL. This gives us additional confidence in the results obtained and also helps in assessing the contribution of each parent to the compensatory response. The proportion of the total fitness variation explained by each QTL was also calculated in QTL Cartographer 2.5.

Although QTL can help in identifying regions of the genome responsible for compensation, it is difficult to identify specific candidate genes, as a single QTL likely contains hundreds of genes (a single QTL ranges from10-20cM in size with ~1cM of *Arabidopsis* covering 210kb of the genome (Peters et al. 2001) of which some may and some may not be responsible for observed patterns of fitness compensation. Considering the number of QTL obtained, we combined QTL mapping with microarray expression data to help in identifying potential candidate genes. Wayne and McIntyre (2002), for example, successfully combined data from QTL and microarrays to identify genes responsible for ovariole number in *Drosophila melanogaster*.

Microarray Analysis

To identify potential candidate genes located within a QTL region we carried out a microarray analysis on the Columbia ecotype (one that exhibits patterns of overcompensation) comparing clipped and unclipped individuals. Axillary tissue was collected six days after clipping from both clipped and unclipped plants; 3 Affymetrix oligonucleotide arrays, with 8 pooled clipped plants/chip and 2 Affymetrix oligonucleotide arrays, with 8 pooled unclipped plants/chip were compared in this experiment. We used the *Arabidopsis* Affymetrix GeneChip containing more than 22,500 probe sets representing approximately 24,000 gene sequences. This array is based on information from the International Arabidopsis Sequencing Project

completed in December, 2000 and is constructed by light-directed synthesis of oligonucleosides directly onto a glass "chip" approximately the size of glass cover slip. Each gene is represented on the array by a set of 20 oligonucleotide probes representing 25mer sequences from some portion of the gene. Gene expression in a target sample is assessed by hybridization.

Total RNA was extracted with standard TRIzol (Life Technologies, Carlsbad, California) protocols from clipped (n=3 chips) and unclipped (n=2 chips) plants. The quality of the RNA was checked at 260 and 280 nm for determination of sample purity and concentration. Messenger RNA was reverse transcribed and labeled with the MessageAmp kit (Ambion, Austin, Texas) and biotin labeled dCTP and dGTP (ENZO Diagnostics, Farmingdale, New York). Affymetrix *Arabidopsis* GeneChip Arrays (Version 2.0) were hybridized at the University of Illinois Keck Center. Feature intensities on each chip were quantified with MAS 5.0 software. Following hybridization, the Perfect Match (PM) probes for all arrays were initially quantile normalized with the Affy package in Bioconductor to remove nonbiological variation among arrays. Only the PM data were used for the remainder of the analysis, MM (mismatch) probes were ignored because they tend to increase random noise in the data. Data were analyzed using a t-test for each gene comparing clipped and unclipped plants. We controlled for multiple testing with a false discovery rate (FDR) of $p < 0.01$. Genes with significant overexpression upon clipping based on the microarray analysis were then analyzed for gene ontology. Biological process and molecular function information for each gene was obtained via AmiGo Slimmer Tool (v.1.8) analysis of the Gene Ontology (http://www.geneontology.org)database using the Plant GO Slim term set.

qRT-PCR was also performed on Columbia and Landsberg *erecta* plants to verify differences in expression between overcompensating and undercompensating ecotypes, respectively. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) from rosette/cauline leaf material from clipped and unclipped Columbia and Landsberg *erecta* plants. Leaf samples were collected during five time points (1 day before the inflorescence reached 6 cm (the height threshold for clipping), 1 day after, 5 days after, 15 days after and at 50% flowering) in both clipped and unclipped plants to capture the time course of variation in gene expression patterns. The rationale for choosing samples before clipping was to check the actual change in gene expression following clipping and assess any inherent differences

between the genotypes in gene expression. The first strand cDNA was synthesized using reverse transcriptase (SuperScript III - Invitrogen, CA). The reverse transcription was carried out as recommended by the manufacturer. Three biological replicates and three technical replicates (i.e., three reads from each sample) were used for each ecotype and time interval. qRT-PCR was performed on each ecotype (Columbia and Landsberg *erecta*) and a reference gene from microarray data (ubiquitin) using SYBR green (a fluorescent dye). The data were analyzed using the approach of Pfaffl (2001), followed by an Analysis of Variance and a Fishers LSD Test. Expression data were square-root transformed to approximate normality.

T-DNA knockout evaluation

To assess the role of the candidate gene (see results below) uncovered in our combined QTL mapping and microarray experiment above, we used a T-DNA knockout approach. A gene knockout experiment gives first hand information on the role of a candidate gene in the response of interest (in this case the degree of compensation) and a direct way of measuring the function of the gene product *in situ*. In *Arabidopsis*, T-DNA knockout plants are available for nearly all genes identified to date. A T-DNA inserted within the gene (in the 5`UTR, ORF, or 3`UTR) silences the gene and plants harboring a T-DNA on both chromosomes are devoid of any gene product (or most of the gene product depending upon the position of the insert) for the gene of interest. Mutant knockouts included G6PDH-1 (Sail_1252) , G6PDH-2 (Salk_019323), and G6PDH-3 (Gabi_86405A). T-DNA inserts (Figure 2.1) were confirmed by designing primers for the genomic region and T-DNA insertion using the T-DNA Primer Design Tool (Salk Institute Genomic Analysis Laboratory, http://signal.salk.edu/ tdnaprimers.2.html). The primers LP – TGCCATTCATTTTTAAGCTGG, RP-AGATGCAAGGTAATGTGCACC and LB – ATATTGACCATCATACTCATTGC were used to genotype plants. The PCR reactions produced diagnostic banding patterns for homozygous, heterozygous, and wild type individuals. Three homozygous knockout lines with differing T-DNA insertions were compared for fitness differences among clipped and unclipped plants. Columbia (an overcompensating plant which shares identical genetic background to the knockout plants except for the knocked-out gene) served as a control on the effects of the gene knockout.

Plants were grown under 12 hours of light and dark in individual pots with LI Sunshine® mix. A total of 40 plants per line were grown from seed and half (20 per line) were

randomly clipped to just above ground level at approximately 6 cm of growth to simulate mammalian herbivory; the remaining 20 served as undamaged controls. Fitness comparisons were made in terms of the number of siliques produced. The data were analyzed using an Analysis of Variance (Systat 13) followed by a Games-Howell Test for unequal variances comparing clipped to unclipped plants within each treatment group so that we could assess whether knockout treatments altered the compensatory outcome from that of overcompensation observed in the Columbia wild- type.

Results

Fitness variation among RILs

The RILs used from a cross between Columbia and Landsberg *erecta* showed variation in compensatory responses ranging from undercompensation to overcompensation within both seasons/years (Spring 2007, treatment X line interaction, $F= 1.53$, $df = 92, 654$, $p<0.002$ and Fall 2008, treatment X line interaction, $F =3.88$, $df = 84, 641$, $p<0.0001$; Figure 1). Although there was a significant treatment X line X year effect ($F = 1.29$, $df = 80, 1191$, $p<0.05$) the majority (68%) of lines had similar compensatory responses across years (48 lines equally compensated in both years, 4 overcompensated and 2 undercompensated; $p<0.05$ for those that changed category). Of the remaining lines for which we had two years of data, 19 shifted from overcompensation to equal compensation, 5 shifted from equal compensation to undercompensation and only 1 shifted from overcompensation to undercompensation ($p<0.05$ for those that changed category). There were 14 lines for which we had only one year's data, of which 13 equally compensated and 1 overcompensated.

QTL / Microarray analyses

A total of three main QTL for compensation was found on chromosomes 1, 4 and 5 explaining 11.4%, 10.1% and 26.7% of the variation in compensation, respectively (Table 2.1). Collectively they explain 48.2% of the variation. The three QTL did not show any evidence for epistatic interactions, suggesting that each QTL contributed additively. All QTL were from the Columbia ecotype (Table 2.1), although the compensatory response distribution suggests contributions from Landsberg *erecta* (i.e., evidence for transgressive segregation, see Figure 2.2).

From the microarray analysis a total of 109 genes were found to be differentially

expressed between clipped and unclipped plants of Columbia . A total of 30, 19, 17, 16 and 27 differentially expressed genes were located on chromosomes 1 through 5, respectively between clipped and unclipped plants. Based on the gene ontology analysis, these genes can be generally classified into stress response genes, metabolic genes, and growth/reproductive genes (Table 2.2). When mapped with the QTL data, only a single gene co-localizes within one of the QTL markers (QTL 3 located on chromosome 5, at 87.78 cM, Table 1), a glucose-6-phosphate-1-dehydrogenase (*G6PDH1*: EC 1.1.1.49, hereafter referred to as *G6PDH1*).

T-DNA knockout fitness analyses and gene expression patterns

T-DNA knockout experiments verified the role of *G6PDH1* in the compensatory response, whether direct or indirect (Figure 2.3). Overall, results show a significant clipping treatment X line interaction ($F= 2.65$, $df = 4,114$, $p = 0.037$). Results indicate that Landsberg *erecta* equally compensated with a trend toward undercompensation ($p = 0.194$, see Figure 2.3) while all three knockouts of *G6PDH1* showed patterns of equal compensation ($p = 0.98$, $p = 0.95$, and $p = 0.99$ for knockouts 1 (Sail 1252), 2 (Salk 019323) and 3 (Gabi_86405A), see Figure 2.2), respectively, with a trend toward undercompensation, whereas Columbia overcompensated following clipping ($p = 0.008$). *G6PDH1* expression data through time comparing Landsberg *erecta*, an undercompensating ecotype, and Columbia, an overcompensating ecotype, showed higher levels of expression (1.4- to 2.2-fold) in Columbia following the removal of apical dominance at all time points following clipping (overall expression differences between ecotypes, Columbia 0.907 $\pm$ 0.064 and Landsberg *erecta* 0.684 $\pm$ 0.064, $F = 5.99$, $df = 1,20$, $p = 0.024$; ecotype X time, $F = 3.83$, $df = 4,20$, $p = 0.018$; Figure 2.4).

Discussion

Although there is evidence that genetic variation for fitness compensation exists, little is known about the genetic underpinnings leading to enhanced growth and reproduction in species exhibiting growth compensation following herbivory. Here, using a combination of QTL and microarray analyses we have uncovered one gene that appears to play a significant role in the phenomenon of overcompensation, glucose-6-phosphate 1 dehydrogenase (*G6PDH1*, At5g35790.1). *G6PDH1* is the key regulatory enzyme in the oxidative pentose phosphate pathway (OPPP) that plays a central role in plant metabolism by converting glucose to ribose-

5-phosphate. In non-photosynthetic cells, the OPPP is a primary source of the reductant NADPH for biosynthetic processes such as the assimilation of nitrogen into amino acids, fatty-acid synthesis and resistance to oxidative damage. Intermediates, such as ribose-5-phosphate, are also withdrawn from the OPPP pathway for phenylpropanoid production via the shikimate pathway (Figure 2.5; Kruger and von Schaewen, 2003, Scharte et al. 2009).

Knockout studies of three T-DNA insertion lines of *G6PDH1* (sharing the same genetic background as Columbia) showed patterns of equal compensation, with a trend toward undercompensation, rather than overcompensation as observed in the Columbia wild-type (Figure 2.3). Two of the three T-DNA knockout mutants (*G6PDH1*-1, Sail 1252 and *G6PDH1*-2, Salk 019323, Figure 2.2) showed overall lower levels of fitness (i.e., both clipped and unclipped plants), while the third, *G6PDH1*-3, Gabi_86405A, showed higher overall levels of fitness that were within the range of the wild-types, suggesting potential positional effects of T-DNA insertion resulting in partial knockdown of *G6PDH1*-3 (see Figure 5 for sites of T-DNA insertion). Nonetheless, *G6PDH1*-3 plants also equally compensated with a trend toward undercompensation as in the other two knockout mutants.

In addition, *G6PDH1* expression data through time comparing Landsberg *erecta*, an undercompensating ecotype, and Columbia, an overcompensating ecotype, showed higher levels of expression (1.4- to 2.2-fold) in Columbia following the removal of apical dominance, data consistent with our knockout experiments wherein lowering or knocking out *G6PDH1* resulted in equal to undercompensation instead of overcompensation (Figure 3). There is considerable sequence variation in *G6PDH1*, with three non-synonymous substitutions, each causing a change in an amino acid, between Columbia and Landsberg *erecta* that may explain the differential patterns of expression in *G6PDH1* following apical damage and regrowth and perhaps the differences in compensation (Max Planck Institute for Developmental Biology, POLYMORPH Project, http://polymorph- clark20.weigelworld.org/cgi-bin/retrieve_cds_snp.cgi). Collectively, these results indicate the direct or indirect importance of *G6PDH1* in regulating the compensatory response following the removal of apical dominance.

We propose that plants with the capability of overcompensating (increasing both biomass and fitness when compared to undamaged controls) reprogram their transcriptional activity in at least three important ways: through a suite of defensive mechanisms, through an

increase in expression of genes involved in energy metabolism and through an increase in DNA content (via endoreduplication, see Scholes and Paige, 1994), with the increase in DNA content feeding back on pathways involved in defense and metabolism through increased expression. Initially, following apical damage, the *G6PDH1* gene elicits a suite of defensive reactions that are likely associated with cellular damage from herbivory. These may include reactive oxygen species to ward off infection and induced chemical defenses, such as glucosinolates, via the shikimate pathway (Scharte et al. 2009). When analyzing genes that were significantly differentially expressed (from our microarray data), several of the genes affected were found to be enzymes (e.g., a suite of invertase genes, *G6PDH1*, and galactinol synthase) involved in carbohydrate metabolism and these genes were significantly up-regulated and likely play a significant role in overcoming tissue loss. In addition, up-regulation of *G6PDH1* ultimately leads to the biosynthesis of nucleic acids (see Figure 2.4), consistent with the significant increase in DNA content (through endoreduplication) observed in overcompensating ecotypes of *Arabidopsis thaliana* when compared to undercompensating ecotypes . As noted in the introduction, removal of apical dominance reduces the level of auxin leading to axillary bud break and stem regeneration and low levels of auxin trigger an exit from mitotic cycles into the endocycle (Ishida et al. 2010).

Weinig et al. (2003b) previously mapped QTL for resistance and tolerance (compensation) to apical meristem damage by rabbits under natural conditions of the field over two seasons in RILs from a Columbia X Landsberg *erecta* cross (Lister and Dean 1993) of *Arabidopsis*. Although QTLs for resistance were found within each seasonal cohort, no QTLs for tolerance were detected. This is in contrast to our study here, wherein we uncovered three QTL. We surmise that the differences in our findings can be attributed to the differences in natural herbivory versus artificial herbivory. Natural herbivory resulted in wide variance in regrowth and fitness within any given line whereas our clipping experiments resulted in far less variance, resulting in higher repeatability in fitness compensation making it easier to uncover QTL. Whereas Weinig et al. (2003b) interpreted this to mean that there were many genes of small effect involved in tolerance (compensation) our results indicate fewer genes of larger effect i.e., in our study we have uncovered a single gene of major effect (when knocked out plants equally compensated with a trend toward undercompensation following apical damage, contributing significantly to the phenomenon of overcompensation). In both studies there were

also significant environmental effects (G X E interactions) detected, with 25 of 79 lines for which we had two seasons of data responding differently in fitness compensation from one season to the next (all shifting to lower fitness levels). The remaining 54 lines all maintained the same level of fitness compensation.

Results here confirm the utility of using a combinatorial approach of QTL mapping and microarray data in uncovering potential candidate genes. As Wayne and McIntyre (2002) pointed out "The use of microarray technology allows an efficient, objective, quantitative evaluation of genes in the QTL and has the potential to reduce the overall effort needed in identifying genes causally associated with quantitative traits of interest." Using these combined approaches we uncovered a single differentially expressed gene co- located within one of three QTL regions in the recombinant inbred Lister-Dean lines created from a cross between Landsberg *erecta* and Columbia. Knockout studies of this candidate strongly suggest an important role of this gene and the pathway in which it resides in the compensatory response of *Arabidopsis*. Gene complementation studies are currently being carried out to further confirm the role of *G6PDH1* in the compensatory response.

Gaining an understanding of the genetic basis of overcompensation (increased seed yield after damage), in particular, following apical damage should be of great interest to agriculturists who, through recent advents in genetic technology and selective breeding, might incorporate these traits into crop plants such as oilseed rape (*Brassica napus*), a close relative of *Arabidopsis*. The isoform of *G6PDH1* could also be engineered in crops such as sugarcane or rice where ratoon cropping is conducted (ratoon cropping resembles simulated herbivory where the apical meristem is removed leading to increased plant yields through regrowth). Thus, our findings should be of great value in that the results of this study set the stage for genetically engineering or selecting plants that not only tolerate apical damage, but, actually increase seed yield from such damage. Furthermore, from an evolutionary perspective, the genetic basis of overcompensation uncovered here in the model system *Arabidopsis thaliana* may be readily applied to natural systems, improving our understanding of plant regrowth following herbivory and the complexities of plant- animal interactions. With the results of this study, we are beginning to gain significant insights as to the underpinning genetic basis contributing to the phenomenon of overcompensation.

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Table 2.1 Estimates of QTL positions, effects and interactions. QTL analysis performed on Columbia wild-type. Significant QTL determined at LOD > 2.5.

QTL	Chromosome	Marker	Position (cM)	LOD	% Variation Explained
1	1	10	37.85	2.76	11.4
2	4	22	79.99	3.05	10.1
3	5	23	87.78	3.41	26.7

Note: All QTL are from the Columbia ecotype

Table 2.2 Gene ontology analysis for 109 overexpressed genes in Columbia wild-type after clipping. Shown are a subset of biological processes and a selection of important genes. Superscripts indicate molecular function.

Biological Process	# of Genes	Selected Genes						
Response to stress	19	ATP1[1,2,3]	CGL1[4]	FNR1[1]	GOLS2[5]	PDE345[5]	TCH4[2,4]	WR3[3]
Reproduction	9	AGL8[1,6]	GRH1[7]	GSH1[5]	MPK6[8,9]	RP1[10]		
Carbohydrate metabolic process	9	CGL1[4]	CINV1[2,7]	G6PD1[1,7]	GALAK[1,4,8]	GOLS2[5]	IAR4[5]	
Transport	9	ATP1[1,2,3]	GDI2[11]	WR3[3]				
Response to biotic stimulus	6	CYP38[5]	FNR1[1]	GSH1[5]	MPK6[8,9]	WIN1[4]		
Generation of precursor metabolites and energy	5	FNR1[1]	IAR4[5]	ORF291[3]	PDE345[5]			
Flower development	4	AGL8[1,6]	GRH1[7]	GSH1[5]	MPK6[8,9]			
Secondary metabolic process	2	GSH1[5]	MPK6[8,9]					
Photosynthesis	1	FNR1[1]						
Cell differentiation	1	AGL8[1,6]						
Growth	1	GRH1[7]						

[1]nucleotide/DNA/RNA binding, [2]hydrolase activity, [3]transporter activity, [4]transferase activity, [5]catalytic activity, [6]sequence-specific DNA binding transcription factor activity, [7]protein binding, [8]kinase activity, [9]signal transducer activity, [10]structural molecule activity, [11]enzyme regulator activity

Figure 2.1 Schematic representation of *G6PDH1* showing the position of each of the three T-DNA insertions. Exons (dark shading), introns (light shading) and start and stop codons are shown. The T-DNA inserts are represented by inverted triangles. See text for a discussion of potential positional effects.

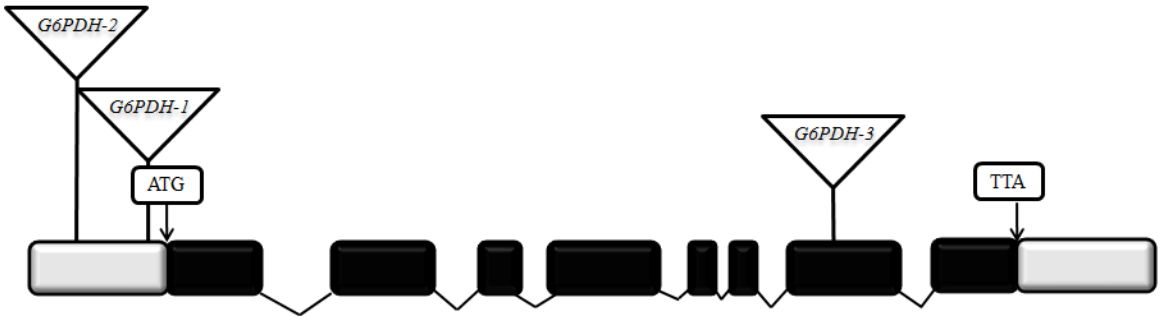


Figure 2.2 Percent change in number of siliques per line between clipped and unclipped plants for Columbia (right arrow) and Landsberg *erecta* (left arrow) parental lines and 93 recombinant inbred lines from a cross between Columbia and Landsberg *erecta* over two years, 14 lines had only one year of data.

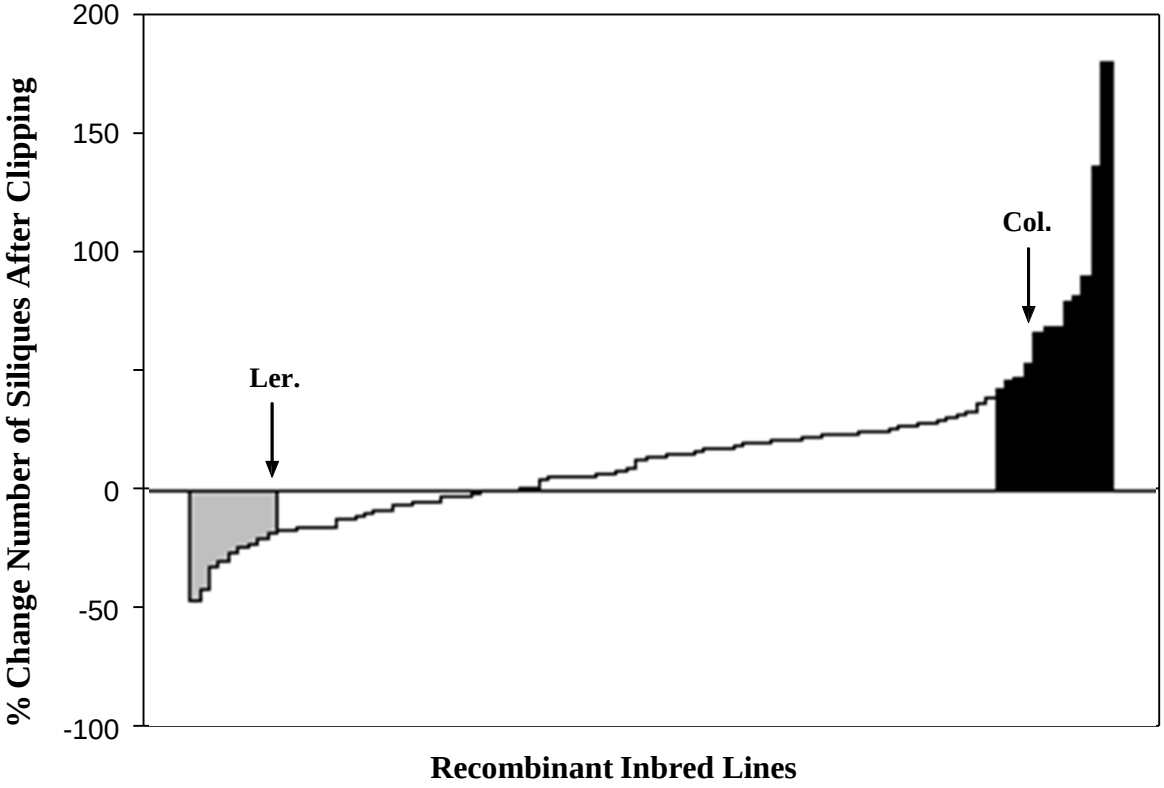


Figure 2.3 Silique production for clipped and unclipped T-DNA knockout lines of *G6PDH1* of the Columbia wild-type and the two ecotypes Landsberg *erecta* (Ler.) and wild- type Columbia (Col.). Shown are means ± 1 SE. Asterices indicate significance at p<0.05.

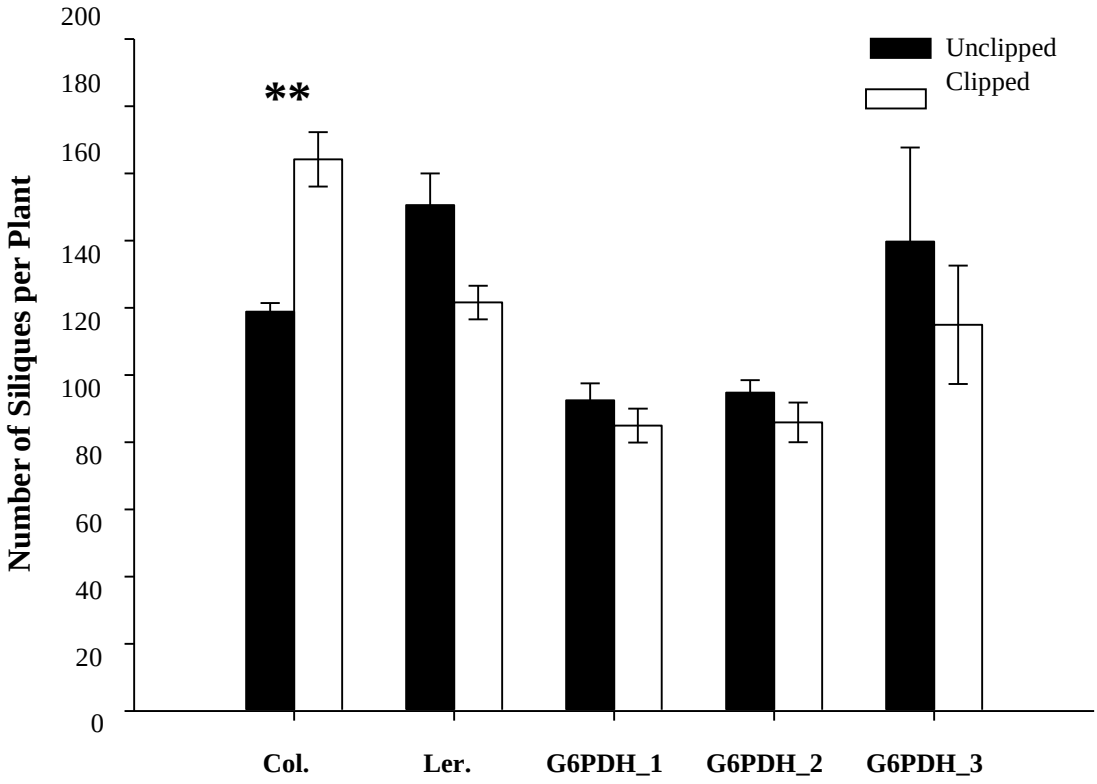


Figure 2.4 *G6PDH1* gene expression through time before and after the removal (clipping) of the plants' apical meristem, simulating mammalian herbivory, for Columbia wild- type (Col.) and Landsberg *erecta* (Ler.). Shown are means ± 1 SE. Asterices indicate significance at **p<0.05 and *p<0.10. Gene expression was adjusted with a housekeeping gene, ubiquitin following Pfaffl (2001).

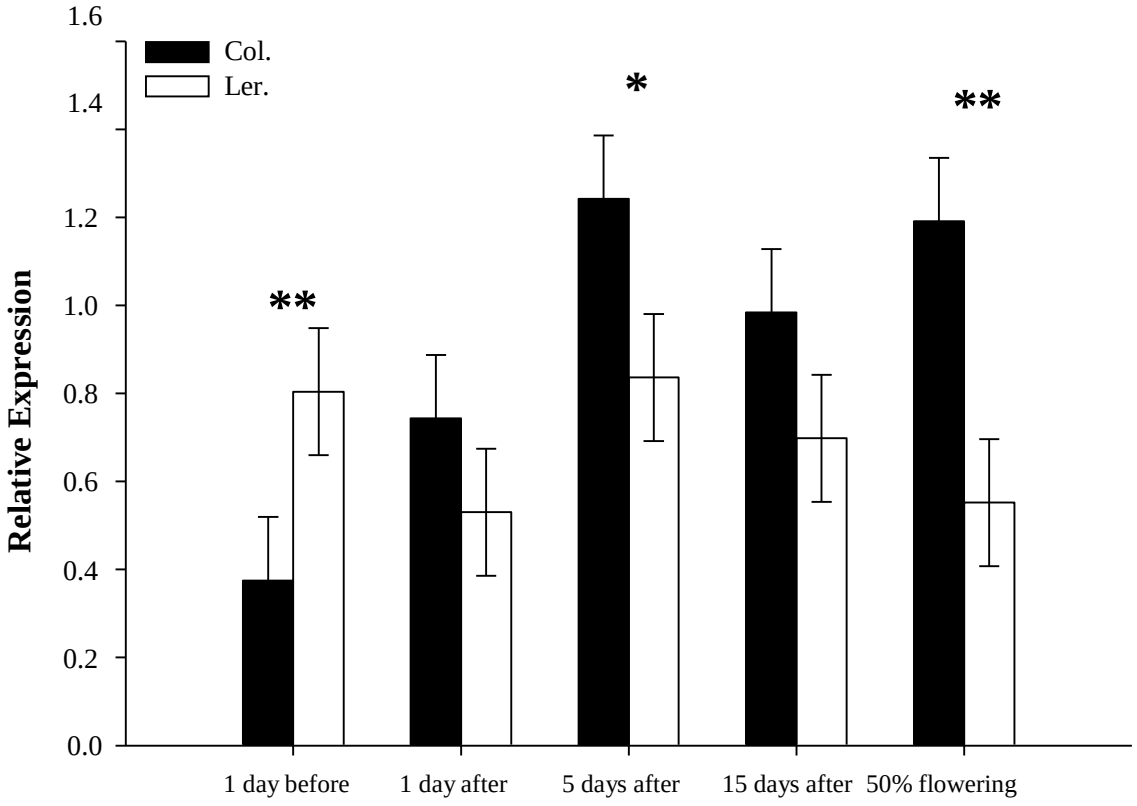
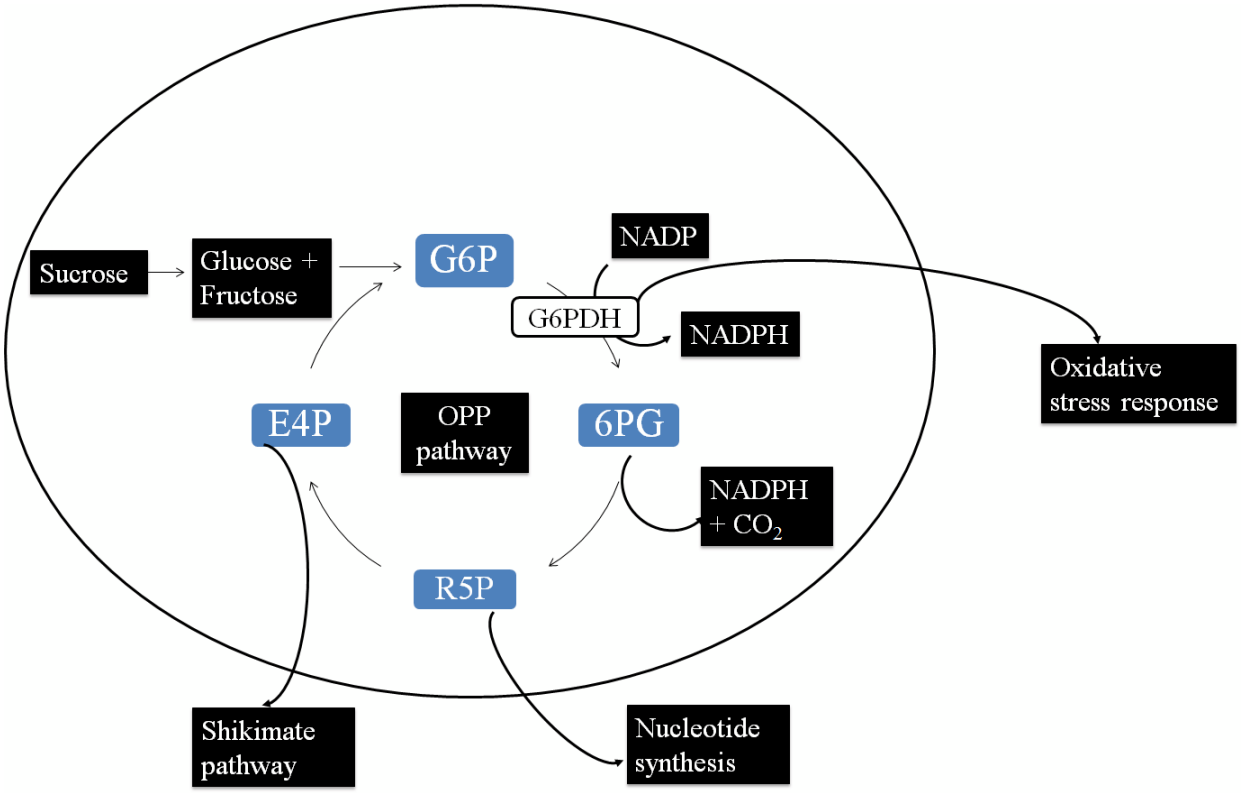


Figure 2.5 Schematic representation of the cytosolic oxidative pentose-phosphate pathway, adapted from Hauschild and von Schaewen (31). G6P (Glucose-6-phosphate) is oxidized by G6PDH to yield 6 phospho gluconate (6PG), and in the process reducing NADP to NADPH and functioning in reducing oxidative damage, and eventually leading to the production of ribulose-5-phosphate (R5P) and erythrose-4-phosphate (E4P), which are used for nucleotide synthesis (essential in the synthesis of aromatic amino acids) and defensive chemistry (such as glucosinolates) via the shikimate pathway, respectively.



CHAPTER 3: THE ROLE OF INVERTASE ISOENZYMES IN PLANT COMPENSATION

Abstract

Although it is clear that genetic variation for fitness compensation exists, little is known about the genetic underpinnings leading to enhanced growth and reproduction in species exhibiting growth compensation following herbivory. In a previous study (Chapter 2) we uncovered that a key enzyme of the oxidative pentose phosphate (OPP) pathway, glucose-6-phosphate-1-dehydrogenase (*G6PDH1*), appears to play a significant role in fitness compensation in *Arabidopsis thaliana*. Here we determined the compensatory response of ecotypes Columbia and Landsberg *erecta,* also involves the enzyme that shunts glucose into the OPP pathway, the enzyme invertase. . To gain insight as to the role of an individual invertase isozymes in compensation fitness in *Arabidopsis*, we analyzed 12 different invertase isozyme using knockout mutants and a suite of expression assays. In the assay involving the removal of apical dominance in Columbia, an overcompensating genotype, eight of 12 invertase isoenzymes were significantly up-regulated one to five days after clipping. In Landsberg *erecta*, an undercompensating genotype, there was no significant increase in the expression of invertases; in constrast, two neutral invertases showed a significant decline in expression at 15 days post-clipping. These results are consistent with the patterns observed in Chapter 1 for *G6PDH1*, showing up-regulation at five days post-clipping in Columbia. Together, this data suggests that an increase in glucose fed from invertase isoenzymes into the OPP pathway is facilitating the rapid regrowth and greater biomass accumulation observed in the overcompensating genotype Columbia. Furthermore, at 50% flowering for both clipped and unclipped plants there was a general trend toward higher expression in six of 12 Columbia invertases and three of 12 for Landsberg *erecta*. These results suggest that Columbia, and to a lesser degree Landsberg *erecta,* may up-regulate gene expression over earlier time periods in order to facilitate flower and fruit development. This greater up-regulation at 50% flowering post-clipping in Columbia are again consistent with the patterns observed in Chapter 2 for *G6PDH1*. The T-DNA knockout experiments on a vacuolar invertase and a neutral invertase, confirm their importance in plant growth and fitness in *Arabidopsis following* the removal of apical dominance. Of particular note, there does not appear to be any functional redundancy of other invertases or any of the sucrose

synthases, supporting that all invertase isoforms appear to be necessary for normal growth, development and reproduction and, most importantly here, for growth and fitness compensation following apical damage.

Keywords: *Arabidopsis,* invertase, isoenzymes, overcompensation, gene expression, *G6PDH1,* Sucrose, OPP pathway.

Introduction

That some plants benefit from being eaten is counterintuitive, yet there is now considerable evidence demonstrating enhanced fitness following herbivory (i.e., plants can overcompensate). Ecologists and evolutionary biologists became interested in overcompensation to herbivory in the mid-1970's when several authors (Chew 1974, Dyer 1975, Owen and Wiegert 1976) reported that herbivory may result in an increase rather than a decrease in the growth and reproductive success of some plant species (Whitham et al. 1991). This observation was initially dismissed as the result of reallocation of below-ground resources to above-ground structures in perennial plants, eventually resulting in a net fitness decrement (Belsky 1986, Verkarr 1986). Studies by Paige and Whitham (1987) provided the first convincing evidence that herbivory can, under some circumstances, lead to enhanced plant fitness. Their choice of a monocarpic plant (i.e., one that reproduces only once and then dies) simplified the estimation of lifetime fitness and eliminated the possibility that apparent overcompensation came at the expense of future reproduction (Vail 1992). They showed that when ungulate herbivores remove 95% or more of the above-ground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, the product of lifetime seed production, seed germination, and seedling survival averaged 3.0 times that of the uneaten controls (Paige and Whitham 1987, Paige 1992, 1994, 1999, Anderson and Paige 2003). The increase in relative fitness was largely because of an architectural change in the plant. Ungulate removal of scarlet gilia's single inflorescence resulted in the production of multiple flowering stalks due to the release of apical dominance and an overall increase in both above- and below-ground biomass. Many researchers have since uncovered additional examples of overcompensation (Maschinski and Whitham 1989, Alward and Joern 1993, Lowenberg 1994, Lennartsson et al, 1997, Weinig et al. 2003a, Rautio et al. 2005), thus, the apparently paradoxical phenomenon of overcompensation in response to herbivory could no longer be summarily dismissed (Stowe et al. 2000).

There is evidence that genetic variation for overcompensation exists. Specifically, some families exhibit overcompensation, whereas others express equal- or under-compensation (Mauricio et al. 1997, Tiffin and Rausher 1999, Juenger and Bergelson 2000). Heritability of traits associated with tolerance has been demonstrated in one population of scarlet gilia as well (Juenger and Bergelson 2000). In addition, recent studies comparing historically grazed and ungrazed populations of the plant *Gentianella campestris* indicate that repeatedly grazed populations can evolve overcompensation while ungrazed populations remain completely intolerant (Lennartsson et al. 1997).

Although there is evidence that genetic variation for compensation exists, little is known about the genetic mechanisms leading to enhanced growth and reproduction following herbivory.

In a recent study (Chapter 1 of this dissertation; Siddappaji et al. submitted) we took advantage of the compensatory variation in RILs of *Arabidopsis thaliana*, combined with microarray and QTL analyses to assess the molecular basis of overcompensation. We found three QTL explaining 48.2% of the variation in fitness compensation and 109 differentially expressed genes between clipped and unclipped plants of the overcompensating ecotype Columbia. From the QTL/microarray screen we uncovered one gene that appeared to play a significant role in overcompensation; glucose-6-phosphate-1-dehydrogenase. Knockout studies of T-DNA insertion lines (Chapter 1) and complementation studies (Chapter 3) of *G6PDH1* verified its role in compensation. *G6PDH1* is a key enzyme in the OPP pathway that plays a central role in plant metabolism. It is likely that plants capable of overcompensating reprogram their transcriptional activity adapt by increasing expression of genes involved in energy metabolism as well as by an increase in cellular DNA content. Scholes and Paige (2011) showed that cells may increase their cellular DNA content by as much as 16 fold in response to herbivory, in a process termed endoreduplication.

Here we determined the importance of the invertases in the compensatory responses of the Columbia and Landsberg *erecta* accessions (overcompensating and undercompensating genotypes, respectively) of *Arabidopsis thaliana*, given that invertases are highly polymorphic glycoproteins that hydrolyze sucrose to glucose and fructose and shunt glucose to initiate the OPP pathway. There are three forms of invertase *viz*., neutral/cytoplasmic, cell wall and vacuolar invertases with similar catalytic function. The glucose produced through hydrolysis of sucrose by invertase is used by *G6PDH1* the key regulatory enzyme in the OPP pathway to produce

ribulose-5-phosphate and erythrose-4-phosphate, which in turn serve as intermediates for nucleotide synthesis and plant defensive chemistry through the shikimate pathway (Kruger and vonSchaewen, 2003, Scharte et al 2009, Eicks et al. 2002). In this study we evaluate the role of invertase genes in plant compensatory responses using gene expression and T-DNA knockouts. Specifically, we assess a) the role/expression of different invertase isoenzymes in the plant compensatory response through development and b) the effects of knocking out the function of a given invertase on plant compensation.

Materials and Methods

Gene Expression/Growing Conditions

Two accessions, Columbia, an overcompensating genotype, and Landsberg *erecta*, an undercompensating genotype, were selected for assessing whether there were differences in the expression of the invertases between the two accessions and their compensatory responses by comparing clipped and unclipped individuals within each accession. Seeds of each accession were vernalized at 4^0C for 3 days to obtain uniform germination and see sown on Sunshine LC1 Mix (Sun Gro Horticulture Canada Ltd., Canada) in 3.5 inch pots to obtain 120 plants of each accession. Plants were grown in a growth chamber at 16:8 hours of light:dark. On the same day, typically when plants were at a height of approximately 6 cm, half of the plants of each accession were clipped to a height of approximately 1 cm. Rosettes or secondary meristematic tissues from three plants were pooled for each of the biological treatments. Overall, three biological and three technical replicates were conducted for each of the treatments/genes. Tissue samples for expression analysis were collected: 1 day before clipping, 1, 5, and 15 days after clipping and at 50% flowering. Rosette leaves were collected 1 day before and 1 day after treatments and secondary meristems were collected for the remaining time points. The rationale for collecting secondary meristems at later time points was to determine affects of translocation of nutrients to the developing tissues (secondary meristems, cauline leaves, siliques).

Total RNA was extracted using TRIzol and purity was measured using a Nanodrop ND1000 (manufacturer and city/state). Approximately 2 µg of RNA was reverse transcribed to obtain cDNA using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). Non-quantitative reverse transcribed PCR (RT-PCR was performed using Arabidopsis invertase-specific primers (Table 3.1) for both genotypes as suggested in (Barratt et al. 2009). For

quantitative RT-PCR (qRT-PCR) the cDNA was diluted to obtain a concentration of 30 ng/µl, PRC was run on 60 ng cDNA in a 10 µl reaction volume following the protocol of (Radwan et al 2012). The reaction and product quantification were conducted with an ABI 7300 quantitative PCR machine (Applied Biosystems CA, USA). All expression data is a ratio of invertase amplicon abundance over that of a reference gene, ubiquitin (At4G27960). The average C_T values were used to calculate the gene expression of each invertase isozyme in relation to ubiquitin expression according to Pfaffl (2001).

Knockouts and Fitness

To further understand the role of invertase genes on fitness compensation we used wild-types Columbia and Landsberg *erecta* along with T-DNA knockouts of two invertase genes in the overcompensating Columbia genotype. The T-DNA knockout lines represent two of the three categories of invertase isoenzymes – neutral and vacuolar. As we were unable to isolate homozygous T-DNA knockout lines of cell-wall invertase genes and a few of the neutral invertase genes, we conducted the study with only one of six neutral, and one of two vacuolar invertases each with two T-DNA knockout lines with differing insertions. The mutant alleles for vacuolar invertase (*V_Inv1* – SAIL_637_C02 and *V_Inv2* – WiscDsLox450D11) and neutral invertase (*NInv_1* - SAIL_441_G04, *NInv_1* - SAIL_518_D02), were used (figures 3.1a and 3.1b). T-DNA inserts were confirmed by designing primers for the genomic region and T-DNA insertion following the protocol from the Salk Institute (http://signal.salk.edu/ tdnaprimers.2.html). The primers used for confirming the genotypes of the mutant alleles are presented in Table 3.2.

Knockout lines and wild-type Columbia and Landsberg *erecta* were grown in the greenhouse in 3 inch circular pots at 16:8 hours of light and dark cycle and clipped during the bolting stage to simulate herbivore browsing, similar to that observed for *Ipomopsis aggregate* . A total of 40 plants per line were grown from seed and half (20 per line) were randomly clipped to ground level at approximately 6 cm of growth to simulate mammalian herbivory; the remaining 20 served as undamaged controls. We counted the number of siliques for each T-DNA insertion line and wild types. The data were analyzed using an analysis of variance in SAS 9.1 (SAS, NC, USA).

Results

Transcriptional Response Of Different Invertase Isoforms

Single amplicons of the predicted sizes were obtained from all 12 invertase primer pairs tested, except primer pair At1g22650 which produced non-specific amplication from Landsberg *erecta* . The 11 specific primer pairs were used in qRT-PCR analyses of expression of each specific invertase in reference to ubiquitin expression. No significant treatment (clipped versus unclipped) effects on average gene expression were observed for Landsberg *erecta* (Tables 3.4 and 3.5). There were however, significant developmental timing effects on gene expression. Specifically, eight of twelve invertase isoenzymes were significantly upregulated in gene expression in clipped plants of Columbia on day 5 following clipping (figures 3.4a – 3.4l and Tables 3.3 and 3.5). In addition, significant expression differences were also found for two cell-wall invertases (At1g12240 and At3g13790) 1 day after clipping in Columbia (Table 3.3, figures 3.4c and 3.4e). One cell-wall invertase (At1g12240) showed significantly higher expression in the unclipped plants and the other (At3g13790) showed significantly higher expression in clipped plants. Landsberg *erecta* showed no significant timing effects on gene expression for any of the invertases on days 1 or 5 after clipping. But on day 15, significant differences were found for two neutral invertases (At1g56560 and At4g09510); in both cases unclipped plants significantly up-regulated gene expression (Table 3.4, figures 3.8h and 3.4j).

In addition, six of 12 invertase isoenzymes of Columbia showed an overall increase in expression at 50% flowering and three of 12 in Landsberg *erecta* (see Tables 3.6 and 3.7). No treatment (clipped versus unclipped) by time (days after clipping) interactions were observed except for one cell-wall invertase (At3g13790) in Columbia (Table 3.3, figure 3.4e). The interaction was due either to the high expression of clipped plants 1 day after clipping and/or the similarity of response at 50% flowering.

Knockouts and Fitness

Results of our knockout experiments on the vacuolar (At1g35580) and neutral (At4g09510) invertase genes and their isoforms each showed a reduction in fitness following clipping. Knocking out the function of the vacuolar invertases reduced fitness by approximately 20 to 32% following clipping (equal compensation with a trend toward undercompensation in *VInv_1*, p =0.111 and significant undercompensation in *VInv_2*, =0.010) and 33 to 47% in the neutral invertases following clipping (with significant undercompensation in both *NInv_1*,

=0.003 and *NInv_2*, = 0.010). As expected, clipping led to an approximate 28% increase in fitness in the wild-type Columbia (p=0.001) and a 20% decrease in fitness in Landsberg *erecta* (p=0.009; see figure 3.2).

Discussion

In a previous study (Chapter 2) we uncovered a key enzyme of the OPP pathway, glucose-6-phosphate-1-dehydrogenase (*G6PDH1*) that appears to play a significant role in fitness compensation. That microarray study also suggested a possible role of invertase, the enzyme that feeds glucose into the OPP pathway. Invertase was one of the highest differentially expressed genes, showing a 9 fold increase in expression in response to clipping in Columbia. Even though no invertase mapped within one of the QTLs associated with the overcompensation response, one vacuolar invertase gene did map just adjacent to QTL located on chromosome 1. Together, these data pointed to invertase as being a high-priority candidate as a player in over compensation.

Here we determined the importance of invertase isoenzymes in the compensatory response of the Columbia and Landsberg *erecta* accessions (overcompensating and undercompensating genotypes, respectively). Plants use sucrose and its metabolites glucose and fructose for growth and development. Sucrose is metabolized by sucrose synthase (EC 2.4.1.1.3) and invertase (EC 3.2.1.26) to yield glucose and fructose. Isozymes of different location (cell wall, cytoplasm and vacuoles), have been shown to have different functional roles. Cell wall invertases are involved in phloem unloading and sink strength, promoting embryo growth, enhanced branching, and flower and pollen development by supplying hexoses to the developing anthers and ovaries (Zhang et al. 2006). Vacuolar invertases play an important role in cell division essential for seed filling (Ruan et al. 2010), hexose accumulation during fruit set and ripening (Jin et al. 2009), tissue expansion in tubers (Ross et al. 1994) and root development (Lou et al. 2007). Similarly, neutral invertases are involved in plant growth and development through their involvement in respiration and the biosynthesis of primary and secondary compounds (Jia et al. 2008, Welham et al. 2009, Lou et al. 2007).

Of particular interest here is the relationship between the invertase isoenzymes and the OPP pathway given that we have uncovered a key enzyme, glucose-6-phosphate-1-dehydrogenase (*G6PDH1*) that plays a significant role in explaining patterns of fitness compensation, including patterns of overcompensation (see Chapter 2; Siddappajji et al., in

review). The glucose produced through hydrolysis of sucrose by invertase, is used by *G6PDH1* in metabolism and plant growth by converting glucose to ribose-5-phosphate. In non-photosynthetic cells, where plants are devoid of above-ground biomass following herbivory and thus lack any substantial photosynthetic capacity, the OPP pathway becomes the primary source of the reductant NADPH for biosynthetic processes to be carried out (including the assimilation of nitrogen into amino acids, fatty-acid synthesis and antioxidant production). Intermediates, such as ribose-5-phosphate, can also be withdrawn from the OPP pathway for phenylpropanoid production via the shikimate pathway (Kruger and von Schaewen 2003, Scharte et al. 2009).

Overall, results show differences in plasticity in the expression of invertases following the removal of apical dominance. Columbia, an overcompensating genotype, showed that nine (two vacuolar, two cell wall and five neutral invertases) of twelve invertase isoenzymes were significantly up-regulated one to five days after the removal of apical dominance whereas, Landsberg *erecta*, an undercompensating genotype, showed only a significant decline in two neutral invertases at 15 days post-clipping. These results are consistent with the patterns observed for *G6PDH1*, showing up-regulation at five days post-clipping in Columbia, possibly due, in part, to an increase in glucose fed from invertase isoenzymes into the OPP pathway, facilitating the rapid regrowth and greater biomass accumulation observed in the overcompensating genotype Columbia. Thus, these results demonstrate a significant timing effect of invertase activity following clipping consistent with the observed differences in the degree of compensation.

Furthermore, there was a general trend toward higher expression at 50% flowering for both clipped and unclipped plants (with no significant differences in expression between treatments or between genotypes) in six of twelve Columbia isoenzymes and three of twelve for Landsberg *erecta* (see Table 3.2). These results suggest that Columbia, and to a lesser degree Landsberg *erecta*, may up-regulate gene expression over earlier time periods in order to facilitate flower and fruit development. Two of the three isoenzymes were significantly up-regulated in both Columbia and Landsberg *erecta* (a cell wall invertase, At1g12240 and a neutral invertase, At1g06500). These results are also consistent with the patterns observed for *G6PDH1*, showing greater up-regulation at 50% flowering post-clipping in Columbia (i.e., twice the number of invertases up-regulating to supply the added glucose for increased flower and fruit production in

the overcompensating genotype, Columbia versus the undercompensating genotype, Landsberg *erecta*).

No average differences in expression (average effects across all developmental time points) between clipped and unclipped plants were found in Landsberg *erecta* and only one invertase isoenzyme showed a significant difference in expression in Columbia (At1g12240), with unclipped plants showing greater average expression (Table 3.5). Similarly, only one invertase (neutral invertase, At1g06500) in Columbia and one invertase (neutral invertase, At4g34860) in Landsberg *erecta* showed significant overall cumulative differences in gene expression between clipped and unclipped plants, with clipped plants in Columbia showing greater overall expression and unclipped plants in Landsberg *erecta* (Tables 3.8 and 3.9). Thus, it is unlikely that differences in compensation can be explained by the average or overall effects of gene expression.

The T-DNA knockout experiments on the two invertase genes, the vacuolar invertase (*VInv_1*- At1g35580.1) and the neutral invertase (*NInv_1* – At4g09510.1), and their isoforms confirm their importance in plant growth and fitness in *Arabidopsis thaliana* following the removal of apical dominance. In three of four cases, fitness was significantly reduced following clipping in the knockout mutants (both neutral invertases and one of the vacuolar invertases) and in the remaining case there was a non-significant trend toward a reduction in fitness (i.e., toward undercompensation; figure 3.2). All four of the mutant knockout lines share the same genetic background as Columbia, therefore, the difference should be due to knocking out the function of the particular invertase. The reduction in overall size in all four of the mutant lines is likely due to the role these invertases play in plant growth and development in general as well as specific effects on root development, with roots typically being shortened by the knockout of the vacuolar invertases (Barratt et al. 2009). Interestingly, there does not appear to be any functional redundancy by other invertases or by any of the sucrose synthases (of which there are six isoenzymes). Thus, all appear to be necessary for normal growth, development and reproduction and most importantly here, for growth and fitness compensation following simulated herbivory.

Gaining an understanding of the genetic basis of overcompensation (increased seed yield), in particular, following apical damage should be of great interest to agriculturists who, through recent advents in genetic technology and selective breeding, might incorporate traits into crop plants. For example, ratoon cropping (harvesting the above-ground biomass and allowing

the plant to regenerate the following season) is a common practice in rice, sugarcane and sorghum. This type of cropping pattern reduces the cost of cultivation. By understanding the genetic basis of plant compensatory response we can engineer plants (such as overexpression promoters) to increase yield. From an evolutionary perspective this study represents an important contribution to the field of plasticity demonstrating a clear example where we have been able to uncover genes involved in a plastic response to changing environmental conditions.

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Table 3.1 Primers used for gene expression evaluation of invertase isoenzymes

Sl. No.	Primer Name	Invertase type	Sequence
1	1g_12240F	CW-Inv	CAGCCAAGCTCTTCTTGTTC
	1g_12240R		GCTTCGTCAGAGTAAGGATGA
2	1g_55120F	CW-Inv	TCTTCAACAAAGGCACTCAA
	1g_55120R		TTCACGCTCAATAAATGGTG
3	3g_13790F	CW-Inv	AGCATGCATTACCTCAAGAGT
	3g_13790R		TCATGCTCCAAGCATTTAAG
4	5g_11920F	CW-Inv	TGGACTGCCCTAAAACAAAT
	5g_11920R		GACAGCATAAACCACACCAA
5	1g_22650F	N-Inv	GAAATCAGGAAGGTTCATCG
	1g_22650R		TCCAATGAGATCATTCCAAG
6	1g_56560F	N-Inv	CCTCCTAGCAAATCCAGAGA
	1g_56560R		TTTCTTCCGGTCAGATTTTC
7	3g_06500F	N-Inv	GCTCGAGACTTGTGTCTGTG
	3g_06500R		ATGGACTTTGGTTCGTGAGT
8	4g_34860F	N-Inv	AAGATGATGCTGGAAGATCC
	4g_34860R		CAACGTTTTCAACAAGTCCA
9	5g_22510F	N-Inv	AGAAACGCATTCTCTTGCAT
	5g_22510R		CCATGGCTCACTTTGAAATA
10	4g_09510F	N-Inv	AGATCTGCTTCATGGACTTG
	4g_09510R		GCCTCAAGTGAAAATAAACC
11	1g_35580F	V-Inv	GGTTCTAATATTTGCCACGA
	1g_35580R		GCAAAGCAAGAAAGAAAGC
12	1g_62660F	V-Inv	TCATCATGTGAGTGAAGAGAAG
	1g_62660R		AATCGGTGACGTTGTTCTTT
13	4G_27960F	Ubiquitin	TCACAATTTCCAAGGTGCTGC
	4G_27960R		TCATCTGGGTTTGGATCCGT

Table 3.2 Primers used for genotyping the t-dna knockout lines obtained from *Arabidopsis* Biological Resource Center

Primer Name	Sequence
LP_V_Inv_1	GTCTCCCTGTCTTAATGCACG
RP_V_Inv_1	CTTCATGGCTTTGAGATCTGC
LB_V_Inv_1	TAGCATCTGAATTTCATAACCAATCTCGATACAC
LP_V_Inv_2	CAATCGACCAAATGAGTGAGG
RP_ V_Inv_2	CGCTAGACCTAGCCATTAGGG
LB_V_Inv_2	TAGCATCTGAATTTCATAACCAATCTCGATACAC
LP_N_Inv_1	TTCTCTCGTGACTCAATTGCC
RP_N_Inv_1	TCCATGAGAACGAACCAGATC
LB_N_Inv_1	ATTTTGCCGATTTCGGAAC
LP_N.INv_2	TGGGAGCCACATAATTCAAAG
RP_N.Inv_2	CATAGCCAGTCGATAACTCGC
LB_ V.Inv_2	AACGTCCGCAATGTGTTATTAAGTTGTC

Table 3.3 P values between clipped and unclipped plants in ecotype Columbia at different growth stages following removal of apical dominance (NS – non significant , * significant at p ≤ 0.05, ** significant at p ≤ 0.01)

Columbia	Invertase class	1 day after	5 days after	15 days after	50% flowering	Treatment	Days	Days vs treatment
At1g35580	V-Inv1	0.06 NS	0.05 *	0.96 NS	0.56 NS	0.61 NS	0.11 NS	0.67 NS
At1g62660	V-Inv2	0.89 NS	0.02 *	0.37 NS	0.39 NS	0.40 NS	0.44 NS	0.42 NS
At1g12240	CW-Inv1	0.002 **	0.55 NS	0.15 NS	0.14 NS	0.05 *	0.0008 **	0.06 NS
At1g55120	CW-Inv2	0.95 NS	0.19 NS	0.87 NS	0.73 NS	0.82 NS	0.0003 **	0.77 NS
At3g13790	CW-Inv3	0.05 *	0.17 NS	0.28 NS	0.85 NS	0.01 NS	0.01 *	0.04 *
At5g11920	CW-Inv4	0.23 NS	0.05 *	0.73 NS	0.61 NS	0.70 NS	0.001 *	0.49 NS
At1g22650	NInv-1	0.28 NS	0.33 NS	0.33 NS	0.29 NS	0.16 NS	0.004 **	0.33 NS
At1g56560	NInv-2	0.24 NS	0.02 *	0.91 NS	0.59 NS	0.98 NS	0.32 NS	0.12 NS
At1g06500	NInv-3	0.65 NS	0.01 **	0.46 NS	0.65 NS	0.39 NS	0.0005 **	0.95 NS
At4g09510	NInv-4	0.48NS	0.05 *	0.54 NS	0.51 NS	0.27 NS	0.0002 **	0.50 NS
At4g34860	NInv-5	0.79 NS	0.008 **	0.38 NS	0.39 NS	0.39 NS	0.44 NS	0.42 NS
At5g22510	NInv-6	0.59 NS	0.03 *	0.34 NS	0.38 NS	0.39NS	0.47 NS	0.40 NS

Table 3.4 P values between clipped and unclipped plants in ecotype Landsberg *erecta* at different growth stages following removal of apical dominance (NS – non significant , * significant at p ≤ 0.05, ** significant at p ≤ 0.01)

Landsberg *erecta*	Invertase class	1 day after	5 days after	15 days after	50% flowering	Treatment	Days	days vs trt
At1g35580	V-Inv1	0.41 NS	0.50 NS	0.34 NS	0.71 NS	0.72 NS	0.57 NS	0.62 NS
At1g62660	V-Inv2	0.37 NS	0.92 NS	0.39 NS	0.21 NS	0.35 NS	0.34 NS	0.39 NS
At1g12240	CW-Inv1	0.38 NS	0.54 NS	0.25 NS	0.85 NS	0.60 NS	0.001 **	0.77 NS
At1g55120	CW-Inv2	0.30 NS	0.70 NS	0.82 NS	0.96 NS	0.61 NS	0.01 **	0.59 NS
At3g13790	CW-Inv3	0.39 NS	0.09 NS	0.66 NS	0.28 NS	0.42 NS	0.05 *	0.26 NS
At5g11920	CW-Inv4	0.31 NS	0.73 NS	0.41 NS	0.40 NS	0.11 NS	0.04 **	0.61 NS
At1g22650	NInv-1	0.26 NS	0.83 NS	0.54 NS	0.91NS	0.19 NS	0.02 **	0.42 NS
At1g56560	NInv-2	0.39 NS	0.95 NS	0.04 *	0.70 NS	0.31 NS	0.33 NS	0.48 NS
At1g06500	NInv-3	0.93 NS	0.99 NS	0.18 NS	0.58 NS	0.72 NS	0.0006 **	0.76 NS
At4g09510	NInv-4	0.25 NS	0.22 NS	0.02 *	0.33 NS	0.11 NS	0.41 NS	0.52 NS
At4g34860	NInv-5	0.22 NS	0.66 NS	0.18 NS	0.51 NS	0.06 NS	0.11 *	0.78 NS
At5g22510	NInv-6	0.41 NS	0.21 NS	0.20 NS	0.53 NS	0.44 NS	0.04 *	0.48 NS

Table 3.5 Pooled gene expression of invertase isoenzymes between clipped and unclipped plants for ecotype Columbia and Landsberg *erecta*

	Invertase class	Columbia		Landsberg *erecta*	
		Clipped	Unclipped	Clipped	Unclipped
At1g35580	V-Inv1	1.28 ± 0.52 [A]	1.00 ± 0.22 [A]	2.4 ± 0.98 [A]	3.13 ± 1.45 [A]
At1g62660	V-Inv2	0.58 ± 0.11 [A]	2.53 ± 2.55[A]	0.21 ± 0.04 [A]	0.41 ± 0.20 [A]
At1g12240	CW-Inv1	2.12 ± 0.63 [A]	3.35 ± 1.21 [A]	1.75 ± 0.50 [A]	2.10 ± 0.46 [A]
At1g55120	CW-Inv2	0.08 ± 0.01 [A]	0.07 ± 0.01 [A]	0.12 ± 0.02 [A]	0.13 ± 0.01 [A]
At3g13790	CW-Inv3	0.16 ± 0.03 [A]	0.08 ± 0.01 [B]	0.17 ± 0.05 [A]	0.12 ± 0.03 [A]
At5g11920	CW-Inv4	0.16 ± 0.02 [A]	0.17± 0.02 [A]	0.11 ± 0.03 [A]	0.21 ± 0.05 [A]
At1g22650	NInv-1	0.19 ± 0.03 [A]	0.27 ± 0.07 [B]	0.16 ± 0.04 [A]	0.24 ± 0.05 [A]
At1g56560	NInv-2	0.59 ± 0.05 [A]	0.59 ± 0.03 [A]	0.36 ± 0.03 [A]	0.58 ± 0.21 [A]
At1g06500	NInv-3	0.64 ± 0.19 [A]	0.52 ± 0.14 [A]	0.36 ± 0.09 [A]	0.34 ± 0.07 [A]
At4g09510	NInv-4	0.22 ± 0.02 [A]	0.19± 0.02 [A]	0.26 ± 0.04 [A]	1.29 ± 0.83 [A]
At4g34860	NInv-5	0.60 ± 0.16 [A]	2.62 ± 2.31 [A]	0.14 ± 0.05 [A]	0.28 ± 0.05 [A]
At5g22510	NInv-6	1.75 ± 0.34[A]	7.45 ± 6.41[A]	0.95 ± 0.14 [A]	1.31 ± 0.50 [A]

* means with same letter are not significantly different

Table 3.6 Pooled gene expression of invertase isoenzymes in clipped and unclipped plants at different growth stages between ecotypes Columbia and Landsberg *erecta.*

At1g35580 (vacuolar invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	1.01 ± 0.15 B	2.92 ± 1.75 A
5 days after	0.63 ± 0.08 B	1.17 ± 0.28 A
15 days after	0.59 ± 0.11 B	2.29 ± 1.53 A
50% flowering	2.35 ± 1.00 A	4.81 ± 2.60 A

At1g62660 (vacuolar invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.51 ± 0.12 A	0.58 ± 0.42 A
5 days after	0.44 ± 0.10 * A	0.09 ± 0.03 A
15 days after	0.42 ± 0.22 A	0.16 ± 0.02 A
50% flowering	4.87 ± 4.48 A	0.43 ± 0.03 A

At1g12240 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	1.13 ± 0.07 ** B	2.37 ± 0.52 A
5 days after	1.42 ± 0.12 B	0.73 ± 0.08 B
15 days after	0.75 ± 0.18 B	0.72 ± 0.19 B
50% flowering	7.65 ± 1.43 A	3.73 ± 0.73 A

At1g55120–(Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.04 ± 0.01 B	0.07 ± 0.03 C
5 days after	0.04 ± 0.01 B	0.19 ± 0.02 A
15 days after	0.09 ± 0.01 A	0.10 ± 0.01 BC
50% flowering	0.14 ± 0.02 A	0.14 ± 0.03 AB

At3g13790 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.21 ± 0.07 * A	0.11 ± 0.06 B
5 days after	0.08 ± 0.02 AB	0.07 ± 0.02 B
15 days after	0.07 ± 0.01 B	0.11 ± 0.03 B
50% flowering	0.14 ± 0.03 B	0.30 ± 0.10 A

Table 3.6 (continued)

At5g11920 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.09 ± 0.01 ^{B}	0.18 ± 0.10 ^{AB}
5 days after	0.19 ± 0.02 * ^{A}	0.06 ± 0.01 ^{B}
15 days after	0.12 ± 0.02 ^{B}	0.10 ± 0.03 ^{B}
50% flowering	0.25 ± 0.04 ^{A}	0.31 ± 0.06 ^{A}

At1g22650 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.15 ± 0.02 ^{B}	0.23 ± 0.10 ^{AB}
5 days after	0.11 ± 0.02 ^{B}	0.09 ± 0.01 ^{B}
15 days after	0.19 ± 0.03 ^{B}	0.14 ± 0.04 ^{B}
50% flowering	0.49 ± 0.11 ^{A}	0.37 ± 0.05 ^{A}

At1g56560 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.67 ± 0.09 ^{A}	0.82 ± 0.42 ^{A}
5 days after	0.59 ± 0.05 * ^{A}	0.25 ± 0.02 ^{A}
15 days after	0.52 ± 0.05 ^{A}	0.38 ± 0.05 * ^{A}
50% flowering	0.61 ± 0.04 ^{A}	0.46 ± 0.10 ^{A}

At1g06500 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.39 ± 0.08 ^{B}	0.36 ± 0.05 ^{B}
5 days after	0.32 ± 0.02 ** ^{B}	0.14 ± 0.01 ^{C}
15 days after	0.21 ± 0.03 ^{B}	0.17 ± 0.02 ^{BC}
50% flowering	1.43 ± 0.24 ^{A}	0.74 ± 0.12 ^{A}

At4g09510 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.17 ± 0.02 ^{B}	1.08 ± 0.71 ^{A}
5 days after	0.15 ± 0.02 * ^{B}	0.39 ± 0.09 ^{A}
15 days after	0.20 ± 0.02 ^{B}	0.30 ± 0.09 * ^{A}
50% flowering	0.32 ± 0.03 ^{A}	2.14 ± 1.59 ^{A}

Table 3.6 (continued)

At4g34860 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.29 ± 0.07 A	0.20 ± 0.09 B
5 days after	0.52 ± 0.07 A	0.12 ± 0.04 B
15 days after	0.67 ± 0.32 ** A	0.16 ± 0.06 B
50% flowering	4.99 ± 4.62 A	0.37 ± 0.09 A

At5g22510 (Neutral invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	1.54 ± 0.32 A	2.38 ± 0.88 A
5 days after	1.71 ± 0.41 * A	0.52 ± 0.05 B
15 days after	1.46 ± 0.64 A	0.61 ± 0.08 B
50% flowering	13.72 ± 12.85 A	1.02 ± 0.17 B

Asterisk in the Columbia/Landsberg column indicate significant gene expression between Columbia vs Landsberg *erecta*.

Means with the same letter are not significantly different.

Table 3.7 Relative gene expression difference between clipped and unclipped plants between ecotypes Columbia and Landsberg *erecta.*

At1g12240

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.98 ± 0.03	1.28 ± 0.02	**49.5 / 0.002**	1.86 ± 0.14	2.87 ± 1.02	**0.94 / 0.38**
5 days after	1.50 ± 0.20	1.33 ± 0.16	**0.41 / 0.55**	0.79 ± 0.11	0.67 ± 0.13	**0.44 / 0.54**
15 days after	0.48 ± 0.22	1.02 ± 0.21	**3.09 / 0.15**	0.49 ± 0.15	0.95 ± 0.31	**1.77 / 0.25**
50% flowering	5.52 ± 0.93	9.79 ± 2.17	**3.24 / 0.14**	3.88 ± 1.45	3.58 ± 0.70	**0.03 / 0.85**
Cumulative gene expression	**8.49 ± 1.38**	**13.42 ± 2.56**		**7.02 ± 1.85**	**8.07 ± 2.16**	

At1g22650

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.13 ± 0.02	0.17 ± 0.01	**1.53 / 0.28**	0.11 ± 0.01	0.36 ± 0.19	**1.70 / 0.26**
5 days after	0.12 ± 0.02	0.09 ± 0.02	**1.19 / 0.33**	0.09 ± 0.02	0.09 ± 0.01	**0.04 / 083**
15 days after	0.16 ± 0.04	0.22 ± 0.02	**1.17 /0.33**	0.11 ± 0.01	0.16 ± 0.07	**0.43 / 0.54**
50% flowering	0.37 ± 0.01	0.62 ± 0.20	**1.45 / 0.29**	0.36 ± 0.09	0.38 ± 0.05	**0.01 / 0.91**
Cumulative gene expression	**0.79 ± 0.09**	**1.10 ± 0.24**		**0.67 ± 0.13**	**1.00 ± 0.22**	

Table 3.7 (continued)

At1g55120

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.04 ± 0.01	0.04 ± 0.01	**0.003 / 0.95**	0.03 ± 0.003	0.10 ± 0.06	**1.40 / 0.30**
5 days after	0.06 ± 0.01	0.03 ± 0.007	**2.42/ 0.19**	0.20 ± 0.02	0.19 ± 0.02	**0.17 / 0.70**
15 days after	0.09 ± 0.01	0.09 ± 0.01	**0.03 / 0.87**	0.10 ± 0.006	0.10 ± 0.01	**0.05 / 0.82**
50% flowering	0.13 ± 0.03	0.15 ± 0.02	**0.13 / 0.73**	0.15 ± 0.05	0.14 ± 0.03	**0.002 / 0.96**
Cumulative gene expression	**0.32 ± 0.06**	**0.31 ± 0.05**		**0.48 ± 0.08**	**0.53 ± 0.12**	

At3g06500

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.43 ± 0.05	0.35 ± 0.16	**0.23 / 0.65**	0.36 ± 0.05	0.35 ± 0.10	**0.008 / 0.93**
5 days after	0.37 ± 0.01	0.27 ± 0.01	**16.09 / 0.01**	0.14 ± 0.01	0.14 ± 0.02	**0.0008 / 0.99**
15 days after	0.24 ± 0.05	0.19 ± 0.02	**0.64 / 0.46**	0.14 ± 0.03	0.20 ± 0.02	**2.50 / 0.18**
50% flowering	1.56 ± 0.48	1.31 ± 0.17	**0.23 / 0.65**	0.81 ± 0.17	0.67 ± 0.17	**0.34 / 0.58**
Cumulative gene expression	**2.59 ± 0.59 ***	**2.11 ± 0.36**		**1.39 ± 0.26**	**2.16 ± 0.31**	

Table 3.7 (continued)

At3g13790

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.33 ± 0.08	0.09 ± 0.03	**7.52 / 0.05**	0.05 ± 0.02	0.17 ± 0.12	**0.88 / 0.39**
5 days after	0.11 ± 0.02	0.06 ± 0.02	**2.66 / 0.17**	0.10 ± 0.02	0.04 ± 0.007	**4.94 / 0.09**
15 days after	0.08 ± 0.01	0.05 ± 0.01	**1.55 / 0.28**	0.13 ± 0.06	0.10 ± 0.03	**0.21 / 0.66**
50% flowering	0.15 ± 0.06	0.13 ± 0.01	**0.03 / 0.85**	0.42 ± 0.16	0.19 ± 0.07	**1.54 / 0.28**
Cumulative gene expression	**0.66 ± 0.17**	**0.33 ± 0.07**		**0.70 ± 0.26**	**0.50 ± 0.23**	

At3g34860

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.26 ± 0.06	0.31 ± 0.13	**0.07 / 0.79**	0.07 ± 0.03	0.32 ± 0.16	**2.01 / 0.22**
5 days after	0.67 ± 0.03	0.37 ± 0.04	**23.4 / 0.0008**	0.10 ± 0.07	0.14 ± 0.03	**0.22 / 0.66**
15 days after	0.99 ± 0.60	0.36 ± 0.22	**0.95 / 0.38**	0.09 ± 0.01	0.24 ± 0.09	**2.60 / 0.18**
50% flowering	0.51 ± 0.25	9.46 ± 9.30	**0.92 / 0.39**	0.30 ± 0.18	0.44 ± 0.04	**0.52 / 0.51**
Cumulative gene expression	**2.43 ± 0.40**	**10.50 ± 3.69**		**0.56 ± 0.29 ***	**1.14 ± 0.32**	

Table 3.7 (continued)

At1g35580

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.74 ± 0.06	1.28 ± 0.20	**6.20 / 0.06**	1.31 ± 0.12	4.53 ± 3.57	**0.081 / 0.41**
5 days after	0.78 ± 0.03	0.48 ± 0.10	**7.46 / 0.05**	0.96 ± 0.58	1.39 ± 0.04	**0.52 / 0.50**
15 days after	0.60 ± 0.24	0.58 ± 0.06	**0.002 / 0.96**	3.91 ± 2.99	0.67 ± 0.21	**1.16 / 0.34**
50% flowering	3.01 ± 2.00	1.69 ± 0.74	**0.38 / 0.56**	3.67 ± 2.92	5.94 ± 4.89	**0.15 / 0.71**
Cumulative gene expression	**5.13 ± 2.28**	**4.03 ± 1.20**		**9.85 ± 6.61**	**12.53 ± 8.71**	

At4g09510

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.16 ± 0.009	0.19 ± 0.04	**0.59 / 0.48**	0.21 ± 0.02	1.95 ± 1.31	**1.74 / 0.25**
5 days after	0.18 ± 0.02	0.12 ± 0.001	**7.10 / 0.05**	0.28 ± 0.07	0.51 ± 0.14	**2.09 / 0.22**
15 days after	0.22 ± 0.04	0.19 ± 0.02	**0.42 / 0.54**	0.13 ± 0.009	0.47 ± 0.09	**11.59 / 0.02**
50% flowering	0.34 ± 0.04	0.30 ± 0.02	**0.50 / 0.51**	0.43 ± 0.08	3.84 ± 3.11	**1.20 /0.33**
Cumulative gene expression	**0.89 ± 0.11**	**0.79 ± 0.08**		**1.04 ± 0.18**	**6.77 ± 3.65**	

Table 3.7 (continued)

At5g11920

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.08 ± 0.01	0.11 ± 0.01	**1.93 / 0.23**	0.06 ± 0.01	0.29 ± 0.19	**1.30 / 0.31**
5 days after	0.22 ± 0.01	0.17 ± 0.01	**0.79 / 0.05**	0.05 ± 0.02	0.06 ± 0.007	**0.12 / 0.73**
15 days after	0.11 ± 0.03	0.13 ± 0.03	**0.13 / 0.73**	0.07 ± 0.02	0.13 ± 0.05	**0.82 / 0.41**
50% flowering	0.23 ± 0.06	0.27 ± 0.03	**0.29 / 0.61**	0.25 ± 0.09	0.36 ± 0.05	**0.85 / 0.40**
Cumulative gene expression	**0.64 ± 0.11**	**0.67 ± 0.08**		**0.44 ± 0.14**	**0.84 ± 0.30**	

At5g22510

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	1.35 ± 0.44	1.74 ± 0.51	**0.32 / 0.59**	1.57 ± 0.11	3.19 ± 1.79	**0.80 / 0.41**
5 days after	2.49 ± 0.44	0.94 ± 0.18	**10.28 / 0.03**	0.58 ± 0.07	0.45 ± 0.03	**2.14 / 0.21**
15 days after	2.14 ± 1.15	0.78 ± 0.49	**1.16 / 0.34**	0.51 ± 0.09	0.72 ± 0.09	**2.33 / 0.20**
50% flowering	1.07 ± 0.53	26.38 ± 2.58	**0.96 / 0.38**	1.14 ± 0.16	0.90 ± 0.31	**0.45 / 0.53**
Cumulative gene expression	**7.04 ± 2.56**	**29.84 ± 3.76**		**3.80 ± 0.43**	**5.26 ± 2.22**	

Table 3.7 (continued)

At1g56560

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.56 ± 0.16	0.78 ± 0.03	**1.86 / 0.24**	0.41 ± 0.02	1.22 ± 0.84	**0.91 / 0.39**
5 days after	0.68 ± 0.04	0.50 ± 0.02	**12.98 / 0.02**	0.25 ± 0.03	0.25 ± 0.03	**0.003 / 0.95**
15 days after	0.51 ± 0.11	0.52 ± 0.02	**0.01 / 0.91**	0.29 ± 0.04	0.47 ± 0.04	**8.35 / 0.04**
50% flowering	0.64 ± 0.08	0.59 ± 0.03	**0.33 / 0.59**	0.50 ± 0.08	0.41 ± 0.20	**0.16 / 0.70**
Cumulative gene expression	**2.38 ± 0.39**	**2.39 ± 0.10**		**1.45 ± 0.17**	**2.36 ± 1.11**	

At1g62660

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.53 ± 0.17	0.49 ± 0.20	**0.01 / 0.89**	0.16 ± 0.02	1.00 ± 0.83	**1.01 / 0.37**
5 days after	0.64 ± 0.10	0.24 ± 0.03	**12.76 / 0.02**	0.10 ± 0.02	0.09 ± 0.05	**0.01 / 0.92**
15 days after	0.65 ± 0.42	0.20 ± 0.12	**1.00 / 0.37**	0.14 ± 0.02	0.18 ± 0.02	**0.91 /0.39**
50% flowering	0.53 ± 0.26	9.20 ± 9.03	**0.91 / 0.39**	0.48 ± 0.03	0.39 ± 0.03	**2.18 / 0.21**
Cumulative gene expression	**2.35 ± 0.87**	**10.14 ± 1.66**		**0.87 ± 0.09**	**1.66 ± 0.93**	

* indicates significant cumulative gene expression differences between clipped and unclipped plants.

Table 3.8 Pooled gene expression of invertase isoenzymes in clipped and unclipped plants at different growth stages between ecotypes Columbia and Landsberg *erecta.*

At1g35580 (vacuolar invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	1.01 ± 0.15 B	2.92 ± 1.75 A
5 days after	0.63 ± 0.08 B	1.17 ± 0.28 A
15 days after	0.59 ± 0.11 B	2.29 ± 1.53 A
50% flowering	2.35 ± 1.00 A	4.81 ± 2.60 A

At1g62660 (vacuolar invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.51 ± 0.12 A	0.58 ± 0.42 A
5 days after	0.44 ± 0.10 * A	0.09 ± 0.03 A
15 days after	0.42 ± 0.22 A	0.16 ± 0.02 A
50% flowering	4.87 ± 4.48 A	0.43 ± 0.03 A

At1g12240 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	1.13 ± 0.07 ** B	2.37 ± 0.52 A
5 days after	1.42 ± 0.12 B	0.73 ± 0.08 B
15 days after	0.75 ± 0.18 B	0.72 ± 0.19 B
50% flowering	7.65 ± 1.43 A	3.73 ± 0.73 A

At1g55120 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.04 ± 0.01 B	0.07 ± 0.03 C
5 days after	0.04 ± 0.01 B	0.19 ± 0.02 A
15 days after	0.09 ± 0.01 A	0.10 ± 0.01 BC
50% flowering	0.14 ± 0.02 A	0.14 ± 0.03 AB

At3g13790 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.21 ± 0.07 * A	0.11 ± 0.06 B
5 days after	0.08 ± 0.02 AB	0.07 ± 0.02 B
15 days after	0.07 ± 0.01 B	0.11 ± 0.03 B
50% flowering	0.14 ± 0.03 B	0.30 ± 0.10 A

At5g11920 (Cell wall invertase)	**Columbia #**	**Landsberg *erecta* #**
1 day after	0.09 ± 0.01 B	0.18 ± 0.10 AB
5 days after	0.19 ± 0.02 * A	0.06 ± 0.01 B
15 days after	0.12 ± 0.02 B	0.10 ± 0.03 B
50% flowering	0.25 ± 0.04 A	0.31 ± 0.06 A

Table 3.8 (Continued)

At1g22650 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	0.15 ± 0.02 B	0.23 ± 0.10 AB
5 days after	0.11 ± 0.02 B	0.09 ± 0.01 B
15 days after	0.19 ± 0.03 B	0.14 ± 0.04 B
50% flowering	0.49 ± 0.11 A	0.37 ± 0.05 A

At1g56560 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	0.67 ± 0.09 A	0.82 ± 0.42 A
5 days after	0.59 ± 0.05 * A	0.25 ± 0.02 A
15 days after	0.52 ± 0.05 A	0.38 ± 0.05 * A
50% flowering	0.61 ± 0.04 A	0.46 ± 0.10 A

At1g06500 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	0.39 ± 0.08 B	0.36 ± 0.05 B
5 days after	0.32 ± 0.02 ** B	0.14 ± 0.01 C
15 days after	0.21 ± 0.03 B	0.17 ± 0.02 BC
50% flowering	1.43 ± 0.24 A	0.74 ± 0.12 A

At4g09510 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	0.17 ± 0.02 B	1.08 ± 0.71 A
5 days after	0.15 ± 0.02 * B	0.39 ± 0.09 A
15 days after	0.20 ± 0.02 B	0.30 ± 0.09 * A
50% flowering	0.32 ± 0.03 A	2.14 ± 1.59 A

At4g34860 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	0.29 ± 0.07 A	0.20 ± 0.09 B
5 days after	0.52 ± 0.07 A	0.12 ± 0.04 B
15 days after	0.67 ± 0.32 ** A	0.16 ± 0.06 B
50% flowering	4.99 ± 4.62 A	0.37 ± 0.09 A

At5g22510 (Neutral invertase)	Columbia #	Landsberg *erecta* #
1 day after	1.54 ± 0.32 A	2.38 ± 0.88 A
5 days after	1.71 ± 0.41 * A	0.52 ± 0.05 B
15 days after	1.46 ± 0.64 A	0.61 ± 0.08 B
50% flowering	13.72 ± 12.85 A	1.02 ± 0.17 B

Asterisk in the Columbia/Landsberg column indicate significant gene expression between Columbia vs Landsberg *erecta*.

Means with the same letter are not significantly different.

Table 3.9: Relative gene expression difference between clipped and unclipped plants between ecotypes Columbia and Landsberg *erecta.*

At1g12240

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.98 ± 0.03	1.28 ± 0.02	**49.5 / 0.002**	1.86 ± 0.14	2.87 ± 1.02	**0.94 / 0.38**
5 days after	1.50 ± 0.20	1.33 ± 0.16	**0.41 / 0.55**	0.79 ± 0.11	0.67 ± 0.13	**0.44 / 0.54**
15 days after	0.48 ± 0.22	1.02 ± 0.21	**3.09 / 0.15**	0.49 ± 0.15	0.95 ± 0.31	**1.77 / 0.25**
50% flowering	5.52 ± 0.93	9.79 ± 2.17	**3.24 / 0.14**	3.88 ± 1.45	3.58 ± 0.70	**0.03 / 0.85**
Cumulative gene expression	**8.49 ± 1.38**	**13.42 ± 2.56**		**7.02 ± 1.85**	**8.07 ± 2.16**	

At1g22650

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.13 ± 0.02	0.17 ± 0.01	**1.53 / 0.28**	0.11 ± 0.01	0.36 ± 0.19	**1.70 / 0.26**
5 days after	0.12 ± 0.02	0.09 ± 0.02	**1.19 / 0.33**	0.09 ± 0.02	0.09 ± 0.01	**0.04 / 083**
15 days after	0.16 ± 0.04	0.22 ± 0.02	**1.17 /0.33**	0.11 ± 0.01	0.16 ± 0.07	**0.43 / 0.54**
50% flowering	0.37 ± 0.01	0.62 ± 0.20	**1.45 / 0.29**	0.36 ± 0.09	0.38 ± 0.05	**0.01 / 0.91**
Cumulative gene expression	**0.79 ± 0.09**	**1.10 ± 0.24**		**0.67 ± 0.13**	**1.00 ± 0.22**	

Table 3.9 (continued)

At1g55120

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.04 ± 0.01	0.04 ± 0.01	**0.003 / 0.95**	0.03 ± 0.003	0.10 ± 0.06	**1.40 / 0.30**
5 days after	0.06 ± 0.01	0.03 ± 0.007	**2.42/ 0.19**	0.20 ± 0.02	0.19 ± 0.02	**0.17 / 0.70**
15 days after	0.09 ± 0.01	0.09 ± 0.01	**0.03 / 0.87**	0.10 ± 0.006	0.10 ± 0.01	**0.05 / 0.82**
50% flowering	0.13 ± 0.03	0.15 ± 0.02	**0.13 / 0.73**	0.15 ± 0.05	0.14 ± 0.03	**0.002 / 0.96**
Cumulative gene expression	**0.32 ± 0.06**	**0.31 ± 0.05**		**0.48 ± 0.08**	**0.53 ± 0.12**	

At3g06500

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.43 ± 0.05	0.35 ± 0.16	**0.23 / 0.65**	0.36 ± 0.05	0.35 ± 0.10	**0.008 / 0.93**
5 days after	0.37 ± 0.01	0.27 ± 0.01	**16.09 / 0.01**	0.14 ± 0.01	0.14 ± 0.02	**0.0008 / 0.99**
15 days after	0.24 ± 0.05	0.19 ± 0.02	**0.64 / 0.46**	0.14 ± 0.03	0.20 ± 0.02	**2.50 / 0.18**
50% flowering	1.56 ± 0.48	1.31 ± 0.17	**0.23 / 0.65**	0.81 ± 0.17	0.67 ± 0.17	**0.34 / 0.58**
Cumulative gene expression	**2.59 ± 0.59 ***	**2.11 ± 0.36**		**1.39 ± 0.26**	**2.16 ± 0.31**	

* indicates significant difference between clipped and unclipped plants

Table 3.9 (continued)

At3g13790

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.33 ± 0.08	0.09 ± 0.03	**7.52 / 0.05**	0.05 ± 0.02	0.17 ± 0.12	**0.88 / 0.39**
5 days after	0.11 ± 0.02	0.06 ± 0.02	**2.66 / 0.17**	0.10 ± 0.02	0.04 ± 0.007	**4.94 / 0.09**
15 days after	0.08 ± 0.01	0.05 ± 0.01	**1.55 / 0.28**	0.13 ± 0.06	0.10 ± 0.03	**0.21 / 0.66**
50% flowering	0.15 ± 0.06	0.13 ± 0.01	**0.03 / 0.85**	0.42 ± 0.16	0.19 ± 0.07	**1.54 / 0.28**
Cumulative gene expression	**0.66 ± 0.17**	**0.33 ± 0.07**		**0.70 ± 0.26**	**0.50 ± 0.23**	

At3g34860

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.26 ± 0.06	0.31 ± 0.13	**0.07 / 0.79**	0.07 ± 0.03	0.32 ± 0.16	**2.01 / 0.22**
5 days after	0.67 ± 0.03	0.37 ± 0.04	**23.4 / 0.0008**	0.10 ± 0.07	0.14 ± 0.03	**0.22 / 0.66**
15 days after	0.99 ± 0.60	0.36 ± 0.22	**0.95 / 0.38**	0.09 ± 0.01	0.24 ± 0.09	**2.60 / 0.18**
50% flowering	0.51 ± 0.25	9.46 ± 9.30	**0.92 / 0.39**	0.30 ± 0.18	0.44 ± 0.04	**0.52 / 0.51**
Cumulative gene expression	**2.43 ± 0.40**	**10.50 ± 3.69**		**0.56 ± 0.29 ***	**1.14 ± 0.32**	

* indicates significant difference between clipped and unclipped plants

Table 3.9 (continued)

At1g35580

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.74 ± 0.06	1.28 ± 0.20	**6.20 / 0.06**	1.31 ± 0.12	4.53 ± 3.57	**0.081 / 0.41**
5 days after	0.78 ± 0.03	0.48 ± 0.10	**7.46 / 0.05**	0.96 ± 0.58	1.39 ± 0.04	**0.52 / 0.50**
15 days after	0.60 ± 0.24	0.58 ± 0.06	**0.002 / 0.96**	3.91 ± 2.99	0.67 ± 0.21	**1.16 / 0.34**
50% flowering	3.01 ± 2.00	1.69 ± 0.74	**0.38 / 0.56**	3.67 ± 2.92	5.94 ± 4.89	**0.15 / 0.71**
Cumulative gene expression	**5.13 ± 2.28**	**4.03 ± 1.20**		**9.85 ± 6.61**	**12.53 ± 8.71**	

At4g09510

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.16 ± 0.009	0.19 ± 0.04	**0.59 / 0.48**	0.21 ± 0.02	1.95 ± 1.31	**1.74 / 0.25**
5 days after	0.18 ± 0.02	0.12 ± 0.001	**7.10 / 0.05**	0.28 ± 0.07	0.51 ± 0.14	**2.09 / 0.22**
15 days after	0.22 ± 0.04	0.19 ± 0.02	**0.42 / 0.54**	0.13 ± 0.009	0.47 ± 0.09	**11.59 / 0.02**
50% flowering	0.34 ± 0.04	0.30 ± 0.02	**0.50 / 0.51**	0.43 ± 0.08	3.84 ± 3.11	**1.20 /0.33**
Cumulative gene expression	**0.89 ± 0.11**	**0.79 ± 0.08**		**1.04 ± 0.18**	**6.77 ± 3.65**	

Table 3.9 (continued)

At5g11920

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.08 ± 0.01	0.11 ± 0.01	**1.93 / 0.23**	0.06 ± 0.01	0.29 ± 0.19	**1.30 / 0.31**
5 days after	0.22 ± 0.01	0.17 ± 0.01	**0.79 / 0.05**	0.05 ± 0.02	0.06 ± 0.007	**0.12 / 0.73**
15 days after	0.11 ± 0.03	0.13 ± 0.03	**0.13 / 0.73**	0.07 ± 0.02	0.13 ± 0.05	**0.82 / 0.41**
50% flowering	0.23 ± 0.06	0.27 ± 0.03	**0.29 / 0.61**	0.25 ± 0.09	0.36 ± 0.05	**0.85 / 0.40**
Cumulative gene expression	**0.64 ± 0.11**	**0.67 ± 0.08**		**0.44 ± 0.14**	**0.84 ± 0.30**	

At5g22510

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	1.35 ± 0.44	1.74 ± 0.51	**0.32 / 0.59**	1.57 ± 0.11	3.19 ± 1.79	**0.80 / 0.41**
5 days after	2.49 ± 0.44	0.94 ± 0.18	**10.28 / 0.03**	0.58 ± 0.07	0.45 ± 0.03	**2.14 / 0.21**
15 days after	2.14 ± 1.15	0.78 ± 0.49	**1.16 / 0.34**	0.51 ± 0.09	0.72 ± 0.09	**2.33 / 0.20**
50% flowering	1.07 ± 0.53	26.38 ± 2.58	**0.96 / 0.38**	1.14 ± 0.16	0.90 ± 0.31	**0.45 / 0.53**
Cumulative gene expression	**7.04 ± 2.56**	**29.84 ± 3.76**		**3.80 ± 0.43**	**5.26 ± 2.22**	

Table 3.9 (continued)

At1g56560

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.56 ± 0.16	0.78 ± 0.03	**1.86 / 0.24**	0.41 ± 0.02	1.22 ± 0.84	**0.91 / 0.39**
5 days after	0.68 ± 0.04	0.50 ± 0.02	**12.98 / 0.02**	0.25 ± 0.03	0.25 ± 0.03	**0.003 / 0.95**
15 days after	0.51 ± 0.11	0.52 ± 0.02	**0.01 / 0.91**	0.29 ± 0.04	0.47 ± 0.04	**8.35 / 0.04**
50% flowering	0.64 ± 0.08	0.59 ± 0.03	**0.33 / 0.59**	0.50 ± 0.08	0.41 ± 0.20	**0.16 / 0.70**
Cumulative gene expression	**2.38 ± 0.39**	**2.39 ± 0.10**		**1.45 ± 0.17**	**2.36 ± 1.11**	

At1g62660

Growth stages	**Columbia Clipped**	**Columbia Unclipped**	**F value / p value**	**Landsberg *erecta* Clipped**	**Landsberg *erecta* Unclipped**	**F value / p value**
1 day after	0.53 ± 0.17	0.49 ± 0.20	**0.01 / 0.89**	0.16 ± 0.02	1.00 ± 0.83	**1.01 / 0.37**
5 days after	0.64 ± 0.10	0.24 ± 0.03	**12.76 / 0.02**	0.10 ± 0.02	0.09 ± 0.05	**0.01 / 0.92**
15 days after	0.65 ± 0.42	0.20 ± 0.12	**1.00 / 0.37**	0.14 ± 0.02	0.18 ± 0.02	**0.91 /0.39**
50% flowering	0.53 ± 0.26	9.20 ± 9.03	**0.91 / 0.39**	0.48 ± 0.03	0.39 ± 0.03	**2.18 / 0.21**
Cumulative gene expression	**2.35 ± 0.87**	**10.14 ± 1.66**		**0.87 ± 0.09**	**1.66 ± 0.93**	

* indicates significant cumulative gene expression differences between clipped and unclipped plants.

Figure 3.1a Schematic representation of neutral invertase gene (At4g09510.1) showing the position of T-DNA insertions. Exons (dark shading), introns (lines), 5` UTR and 3` UTR (light shading) along with start and stop codons are shown. The T-DNA are represented as inverted triangles. See table 3.1 for the primers used for isolating homozygous knockout lines.

Figure 3.1b Schematic representation of vacuolar invertase gene (At1g35580.1.) showing the position of T-DNA insertions. Exons (dark shading), introns (lines), 5` UTR and 3` UTR (light shading) along with start and stop codons are shown. The T-DNA are represented as inverted triangles. See table 3.1 for the primers used for isolating homozygous knockout lines.

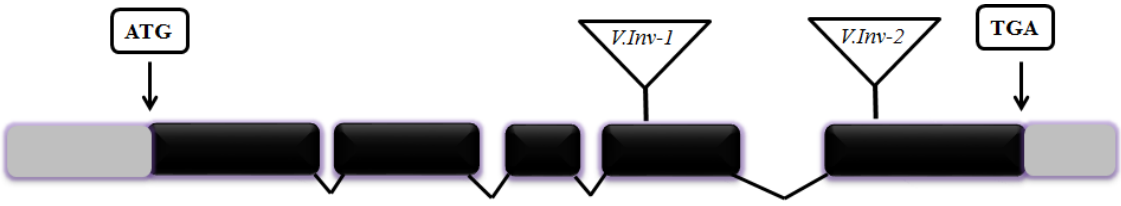


Figure 3.2 Fitness variation for the clipped and unclipped T-DNA knockout lines of At1g35580.1 (SAIL_637_C02 and Wisc450D11) and At4g09510.1 (SAIL_441_G04 and SAIL_518_D02) and ecotypes columbia and Landsberg *erecta*

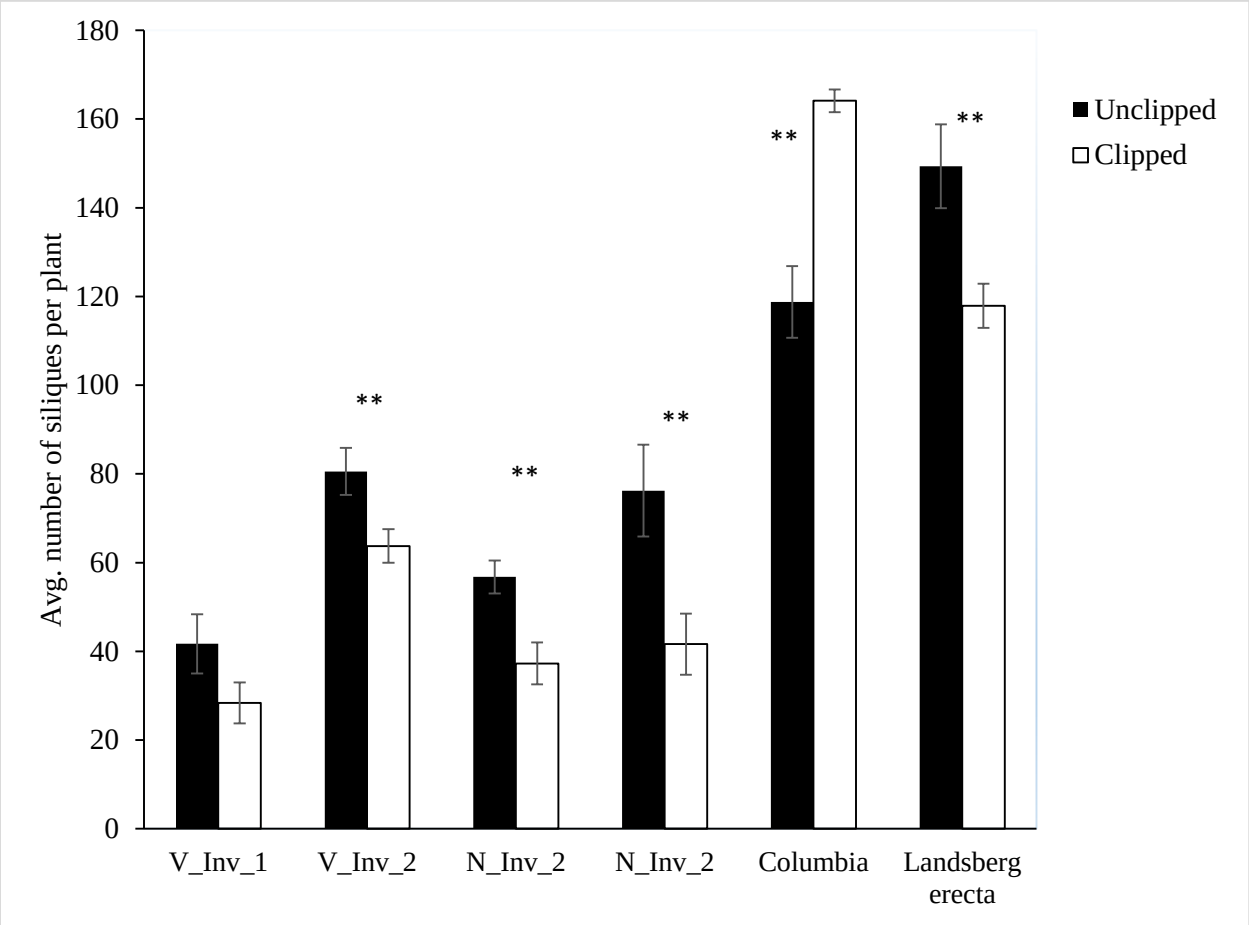


Figure 3.3 Simplified model representing function of all form of invertase enzymes - cell wall (CWINV), neutral/cytoplasmic (NINV) and vacuolar (VINV). Sucrose is hydrolyzed by invertase to yield glucose and fructose. Fructose is phosphorylated by hexokinase (HXK) adding phosphate group, which is eventually used in glycolytic pathway. Similarly glucose is phosphorylated by HXK which adds phosphate group converting glucose to Glucose-6-phosphate (G6P), to be used in oxidative pentose phosphate pathway (OPPP). Glucose-6-phosphate-1-dehydrogenase (EC 1.1.1.49) present in cytosol is activated for plant defense (Scharte et al. 2009) which oxidizes G6P to yield 6-phosho gluconate (6PG) and in the process reducing NADP to NADPH. G6PDH helps in production of NADPH, the reduction power required for anabolic biosynthesis and assimilatory processes. The 6PG is eventually converted to ribulose-5-phosphate (R5P) and erythrose -4-phosphate (E4P), which provide major intermediates for shikimate pathway and nucleic acid biosynthesis (Eicks et al. 2002).

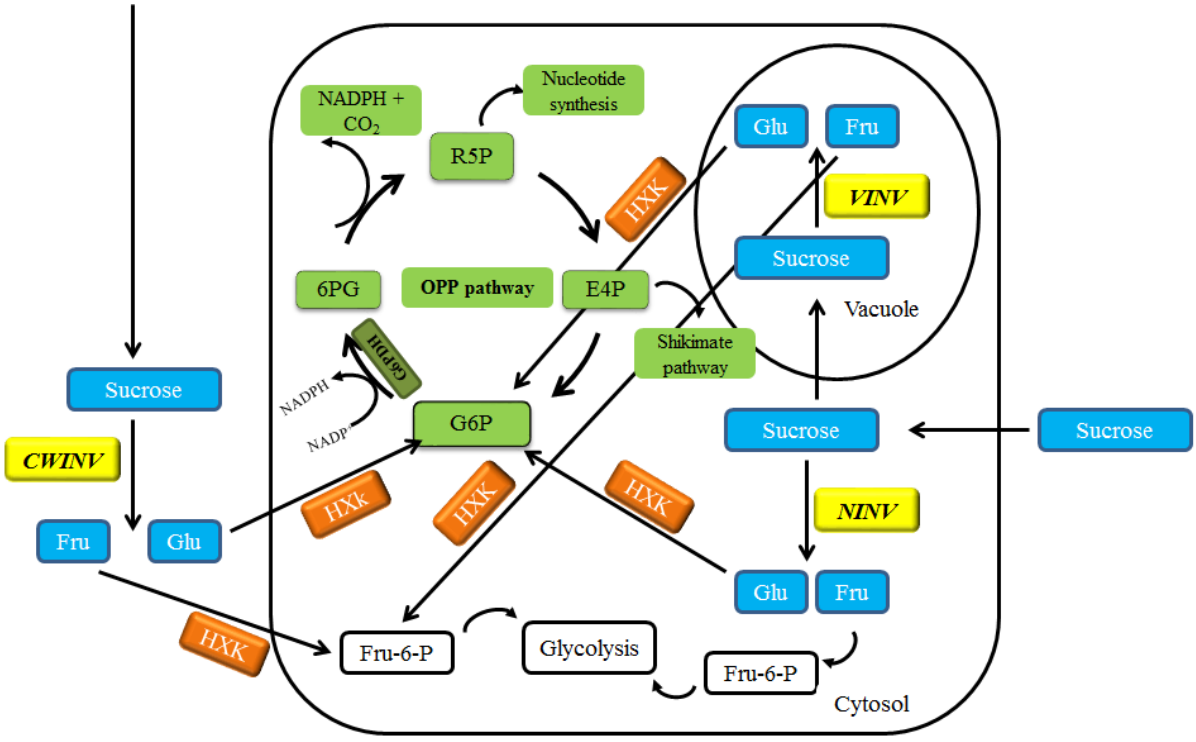
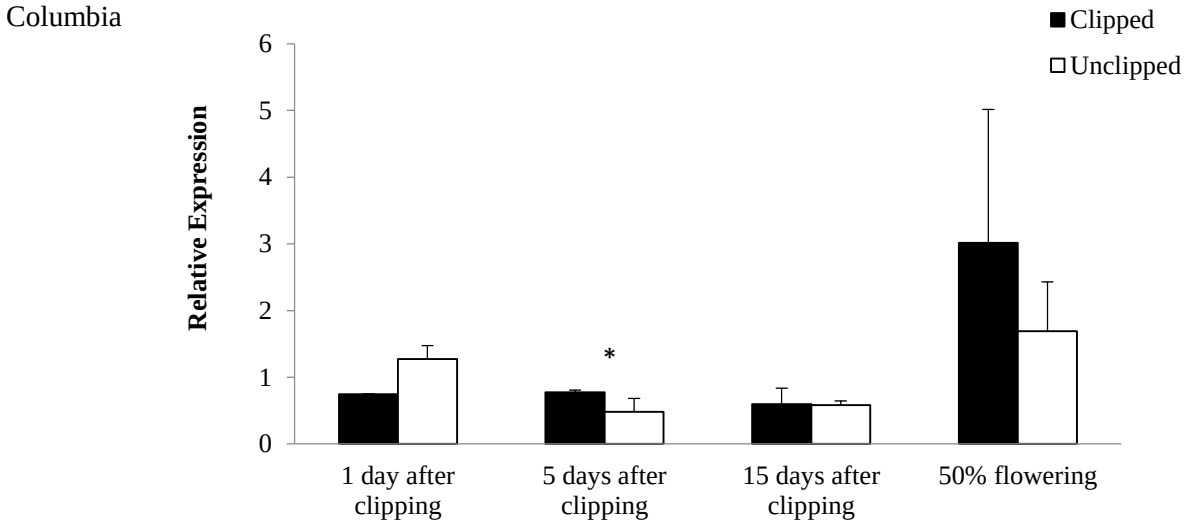


Figure 3.4a: Relative expression of the vacuolar invertase gene (At1g35580.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



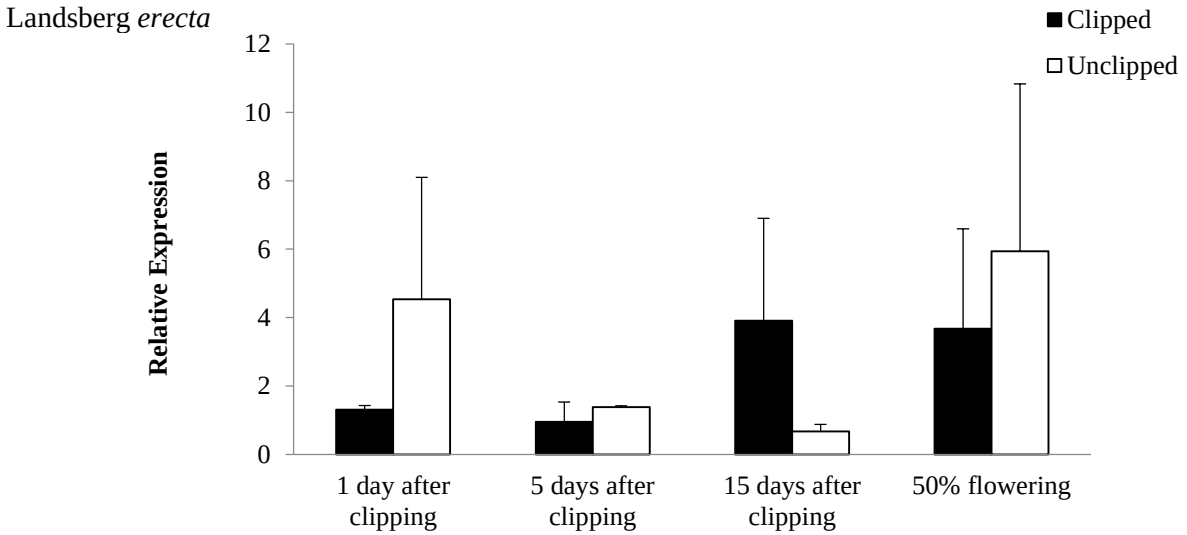
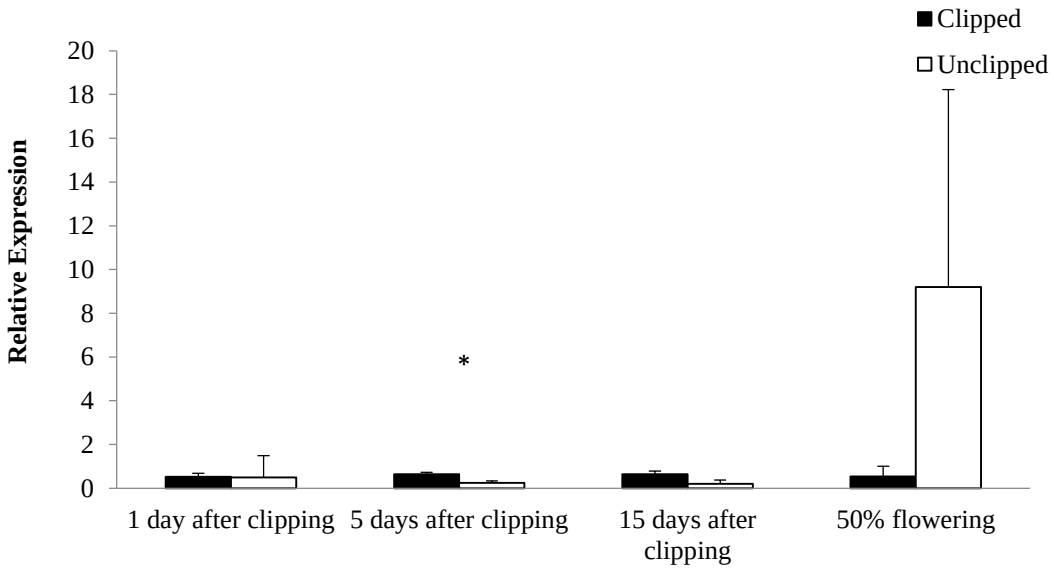


Figure 3.4b: Relative expression of the vacuolar invertase gene (At1g626600.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.

Columbia



Landsberg *erecta*

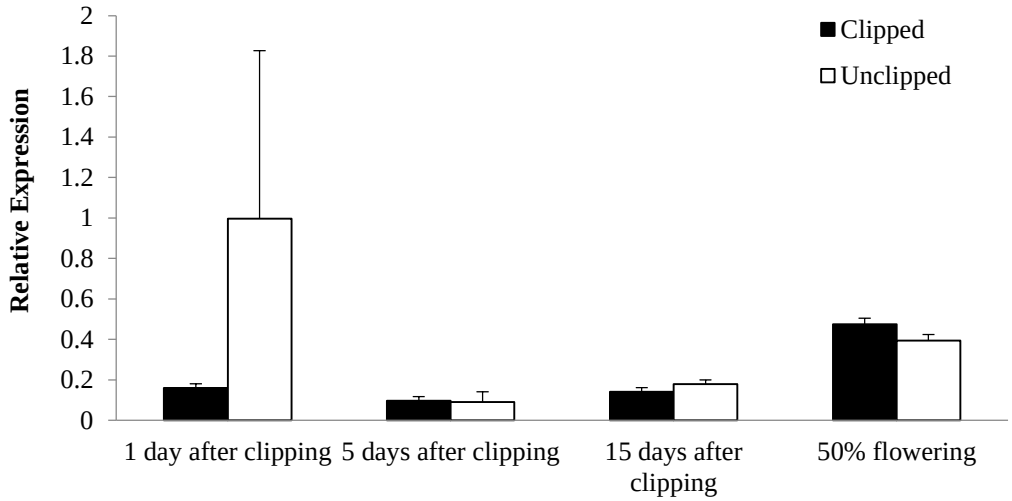
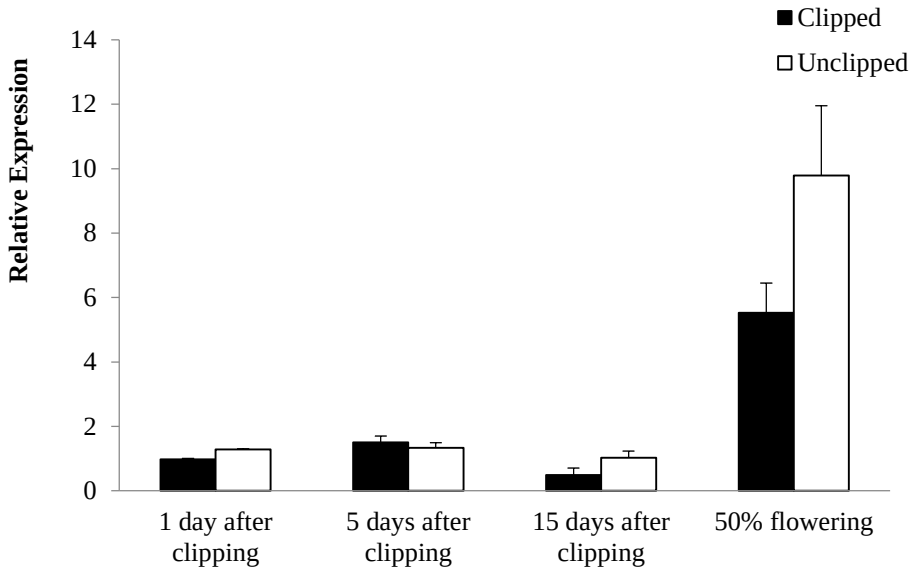
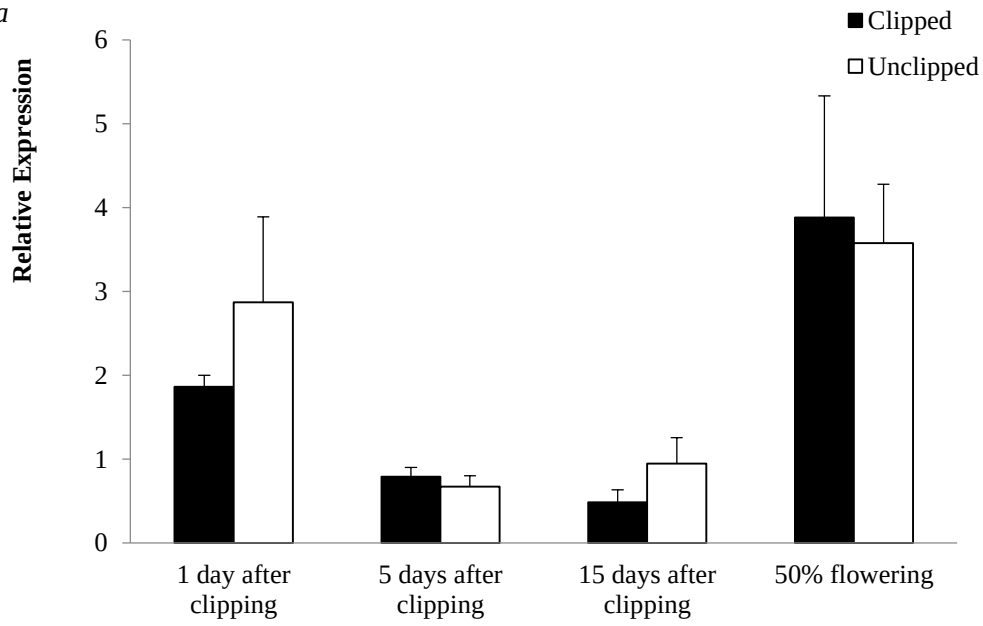


Figure 3.4c Relative expression of the cell wall invertase gene (At1g12240.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.

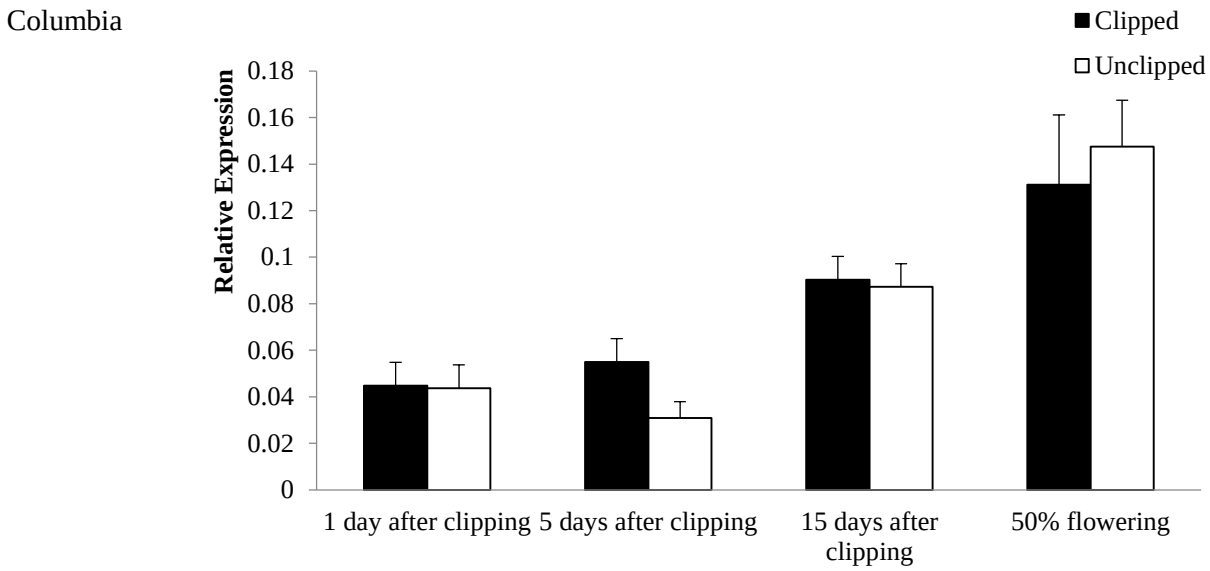
Columbia

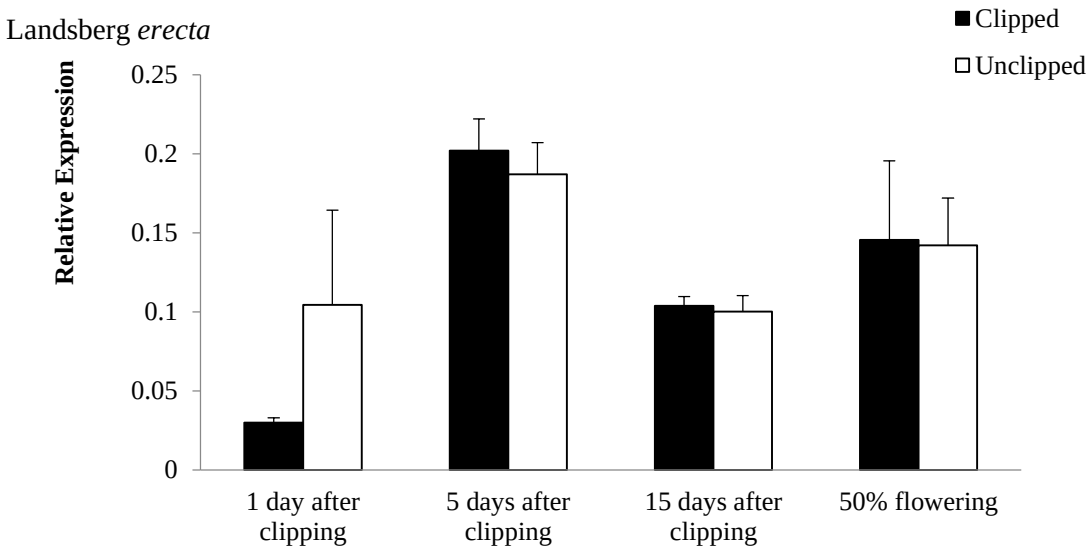


Landsberg *erecta*



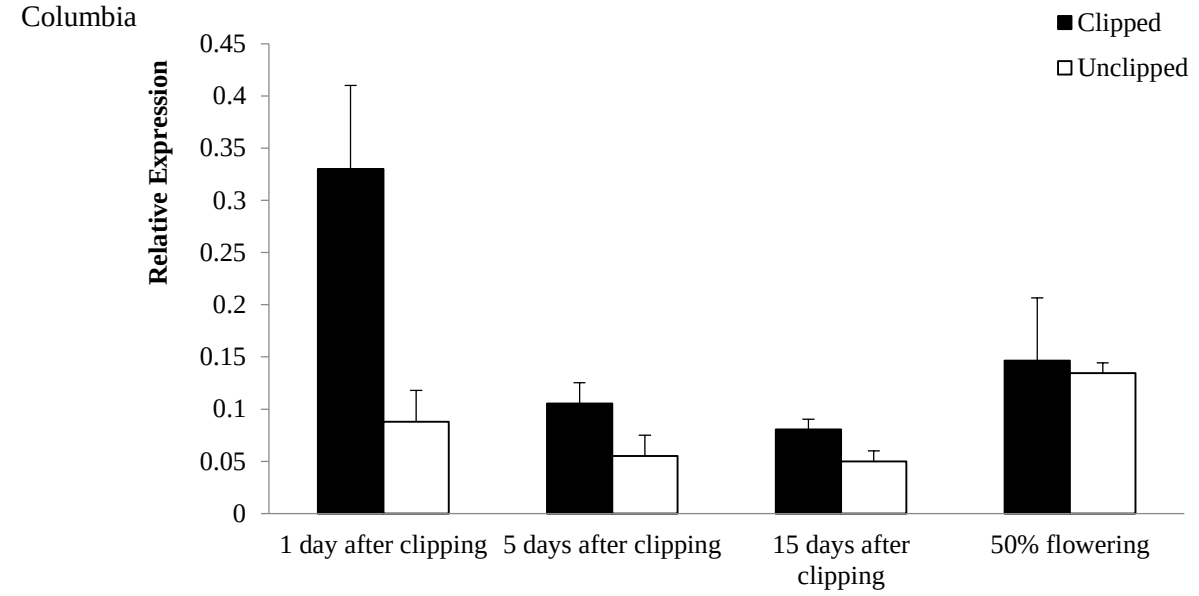
3.4d Relative expression of the cell wall invertase gene (At1g55120.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.





Figure

Figure 3.4e Relative expression of the cell wall invertase gene (At1g13790.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



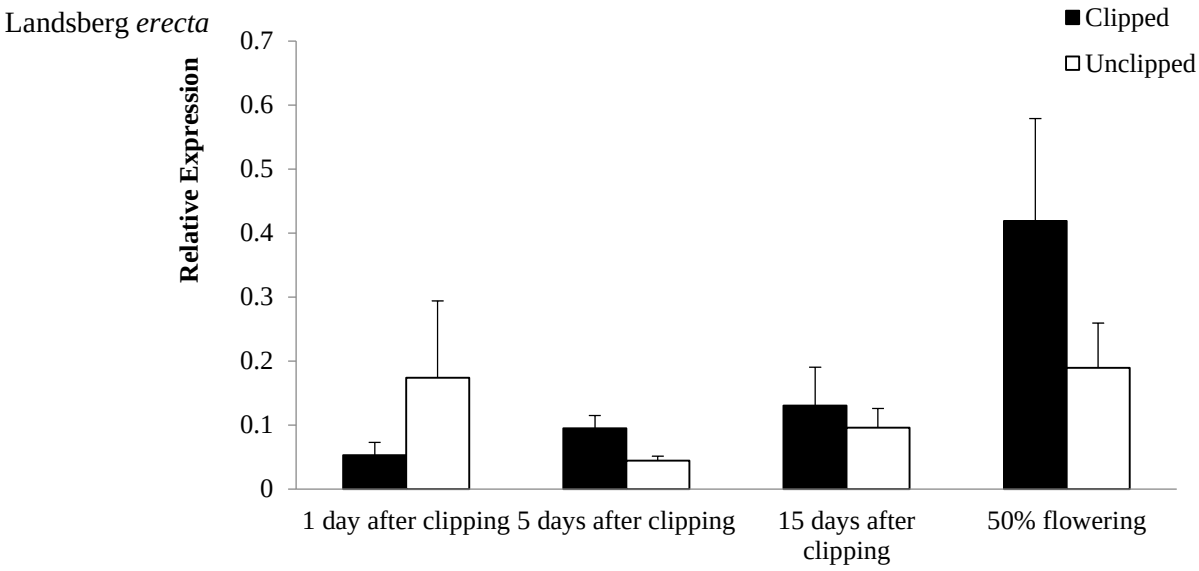
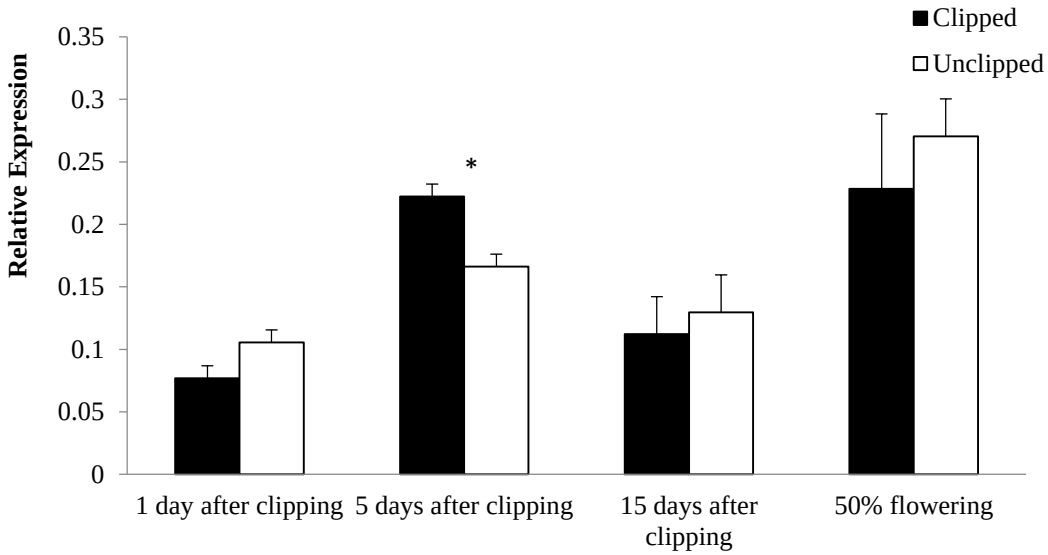


Figure :3.4f Relative expression of the cell wall invertase gene (At1g11920.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



Landsberg *erecta*

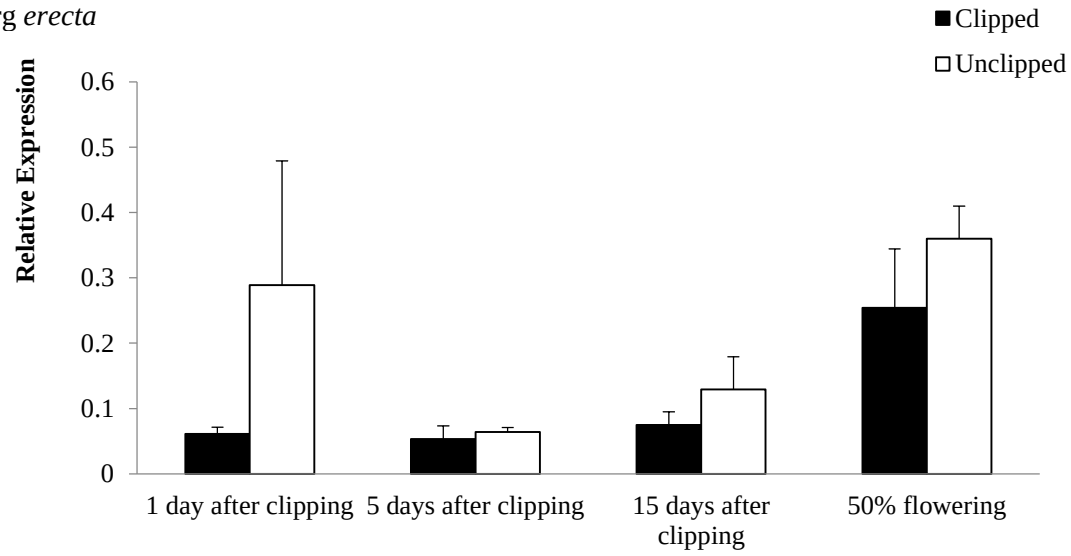
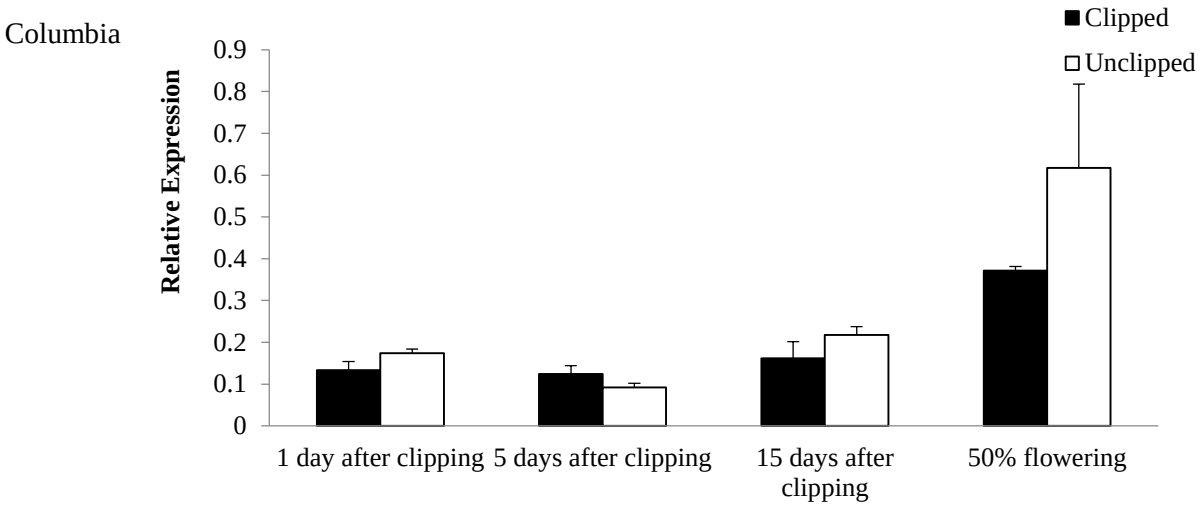


Figure 3.4g Relative expression of the neutral invertase gene (At1g22650.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



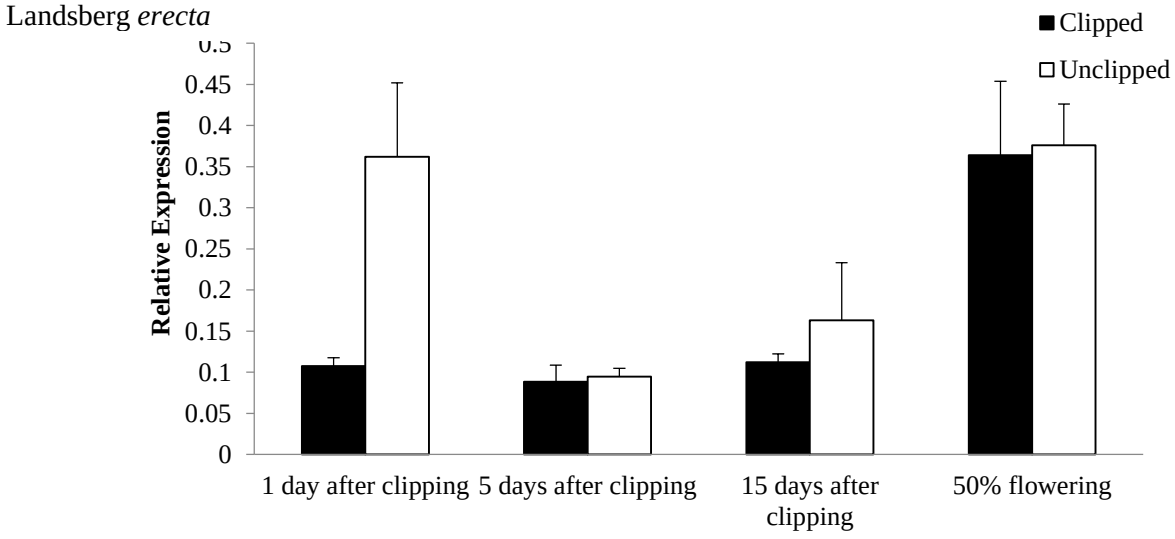
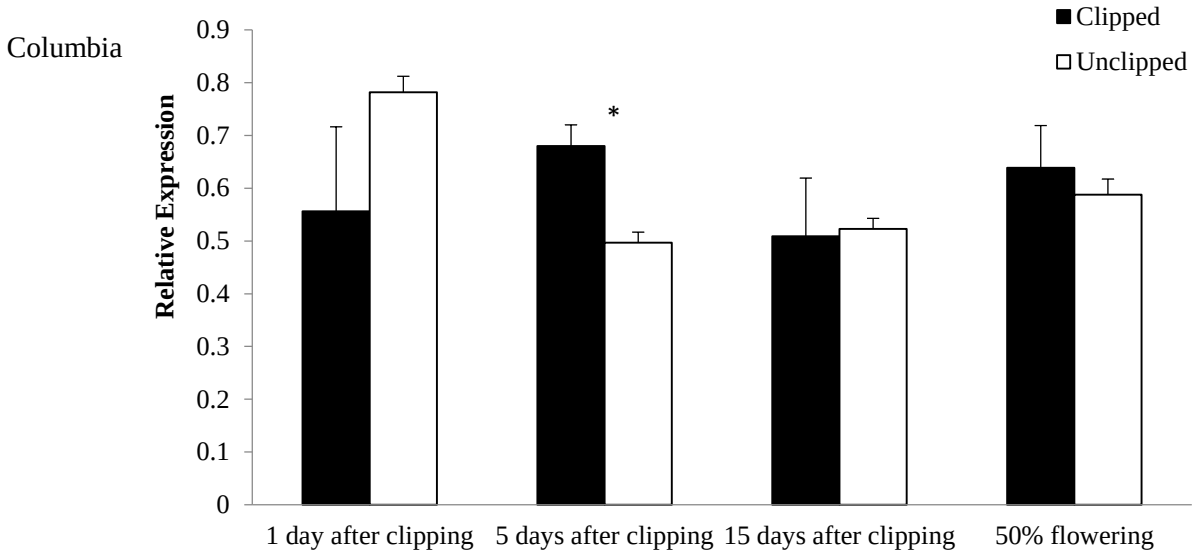


Figure 3.4h Relative expression of the neutral invertase gene (At1g56560.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



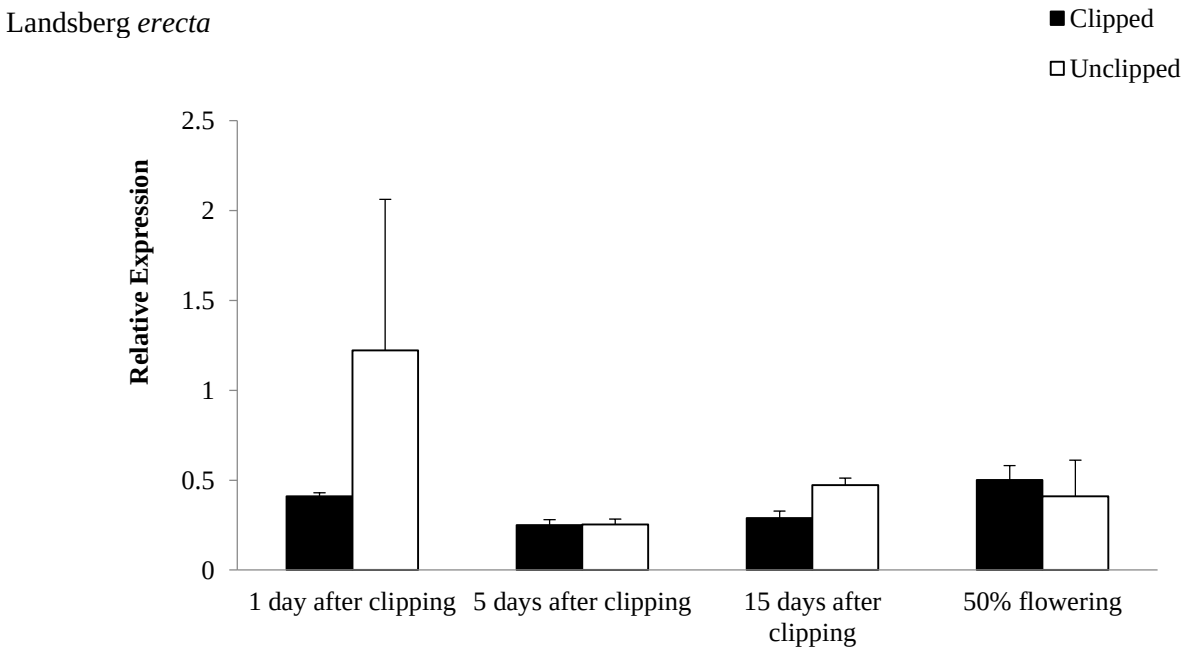
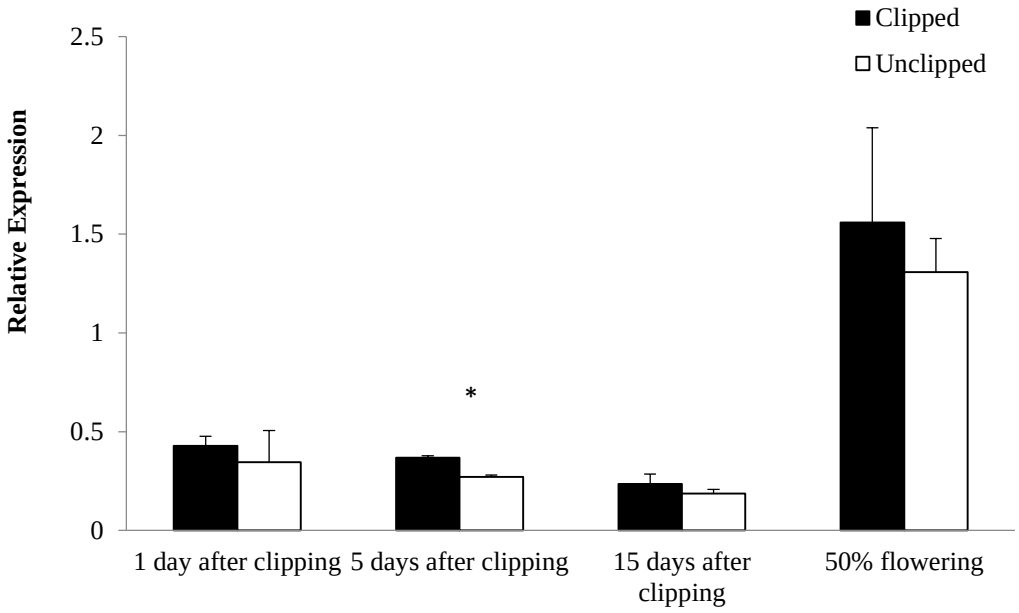


Figure 3.4i Relative expression of the neutral invertase gene (At3g06500.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.

Columbia



Landsberg *erecta*

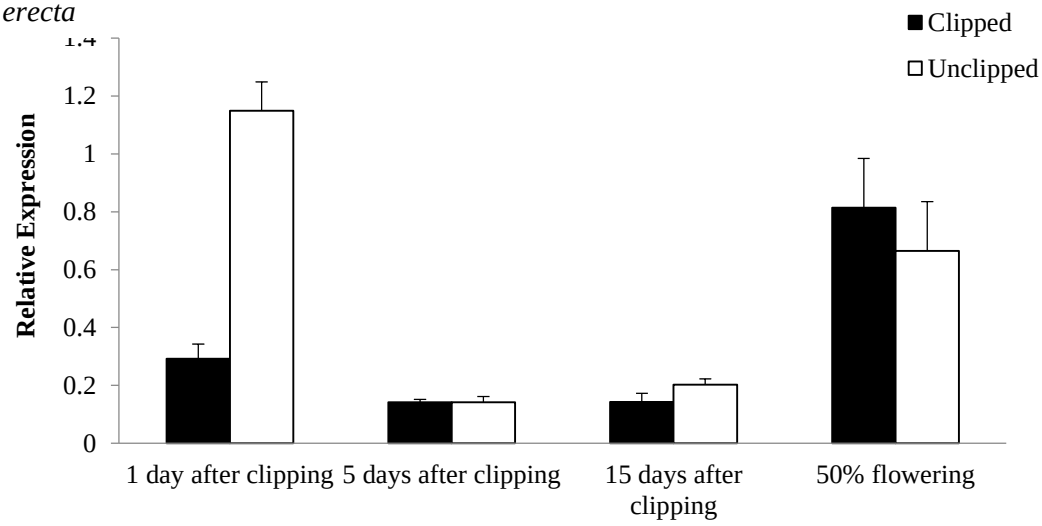
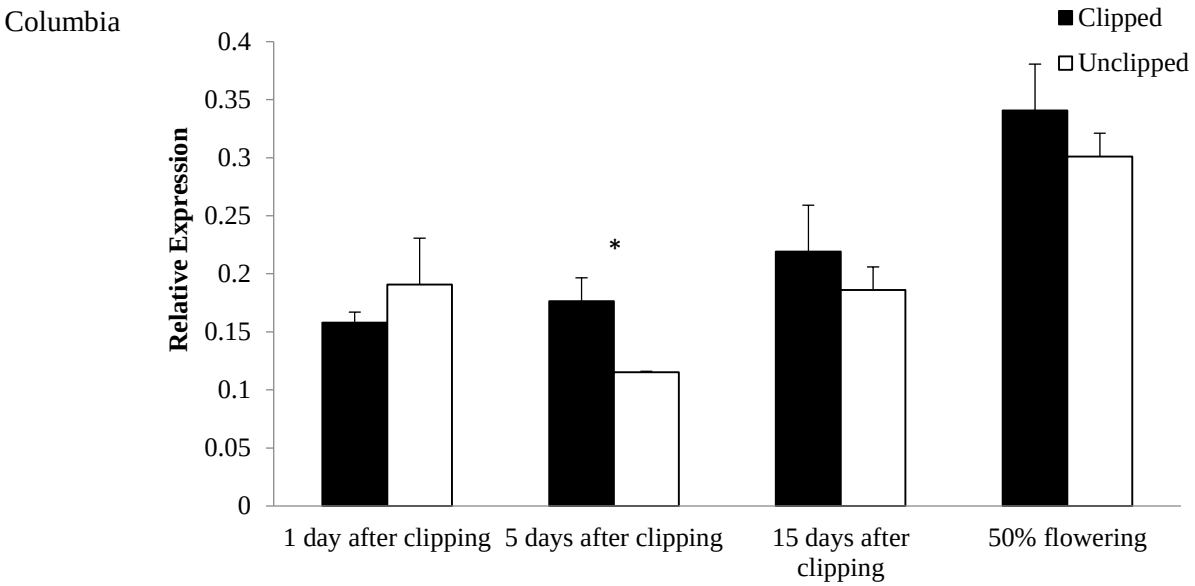


Figure 3.4j Relative expression of the neutral invertase gene (At4g09510.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



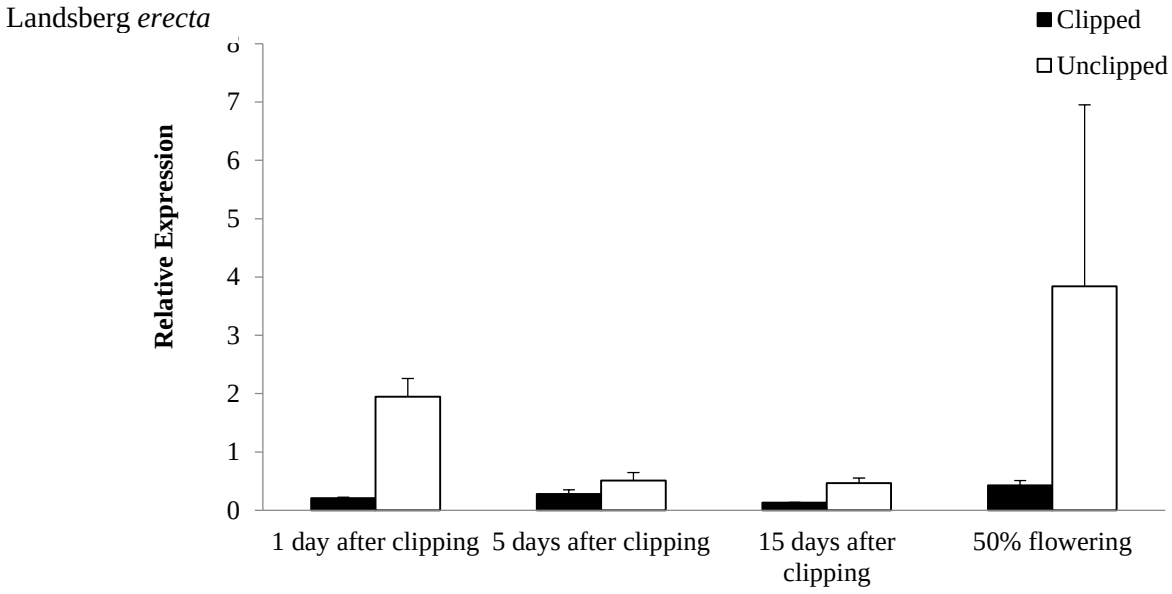
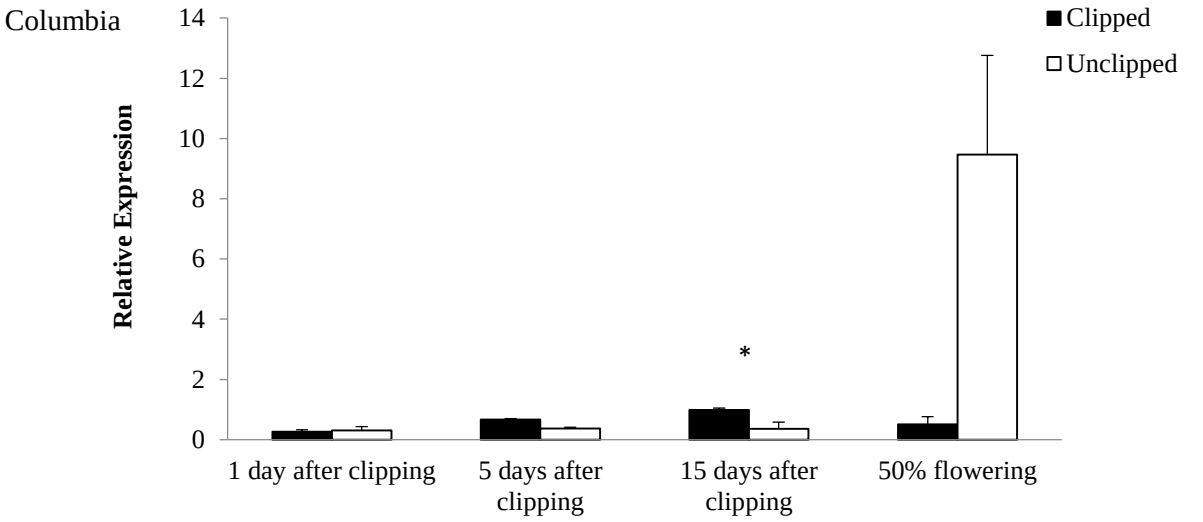


Figure 3.4k Relative expression of the neutral invertase gene (At4g34860.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



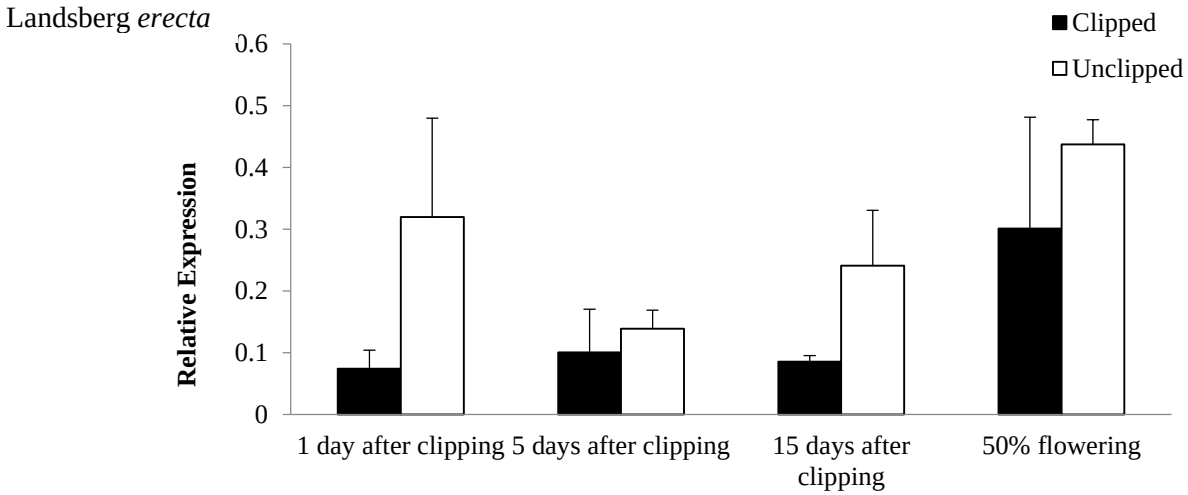
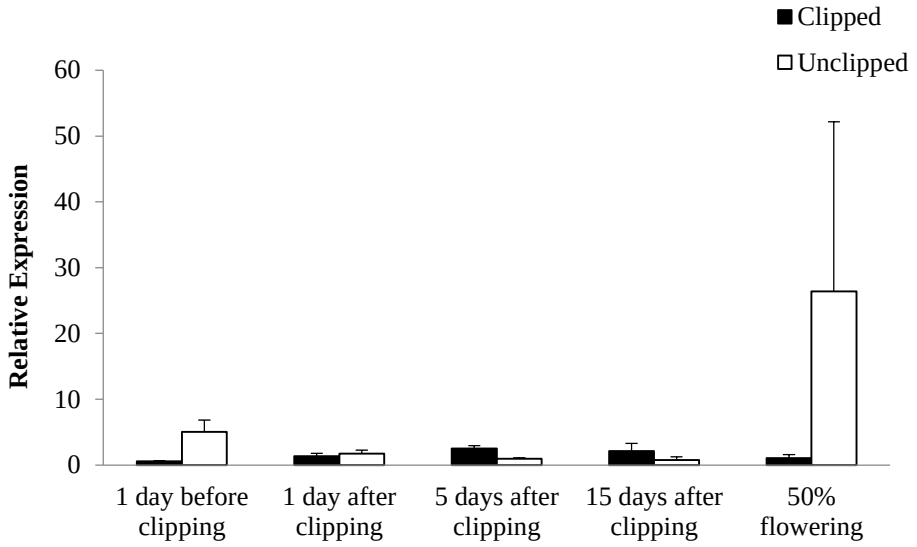
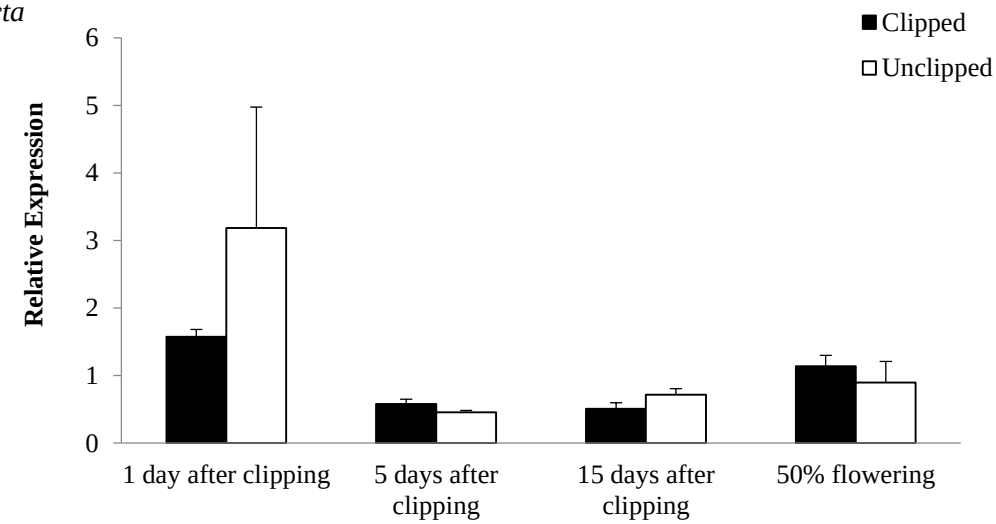


Figure 3.4l Relative expression of the neutral invertase gene (At5g22510.1) through time. Shown are relative gene expression values ± SE. Gene expression was adjusted with the housekeeping gene, Ubiquitin following Pfaffl.



Landsberg *erecta*



CHAPTER 4 :THE ROLE OF GLUCOSE-6-PHOSPHATE-1-DEHYDROGENASE (*G6PDH1*) IN PLANT COMPENSATION:COMPLEMENTATION AND GENE LOCALIZATION OF *G6PDH1* [1]

Abstract

Many plant species have adopted rapid regrowth strategies to overcome the detrimental effects of herbivory; commonly referred as plant tolerance. Interest in plant tolerance was motivated by empirical studies demonstrating that herbivore damage can, under certain circumstances, increase, rather than decrease, plant reproductive success; a specialized case termed overcompensation, i.e., increased flower, fruit, and seed production following herbivory. Although it is clear that genetic variation for fitness compensation exists, little is known about the genetic underpinnings leading to enhanced growth and reproduction in species exhibiting growth compensation following herbivory. In a previous study we uncovered a key enzyme, glucose-6-phosphate-1-dehydrogenase (*G6PDH1*) that appears to play a significant role in fitness compensation. Here, we further verify the role of glucose-6-phosphate-1-dehydrogenase in plant compensation by complementing *G6PDH1* to reinstate its function in a *G6PDH1* knockout and localize where it is expressed by creating chimeric promoter-reporter (GUS) fusion constructs. Results from one of four complementation lines showed a partial rescue effect of *G6PDH1*, showing patters more similar to the overcompensating Columbia line than either Landsberg *erecta* or the knockout line. Furthermore, results of our promoter-reporter fusion studies (*G6PDH1* promoter: β-glucuronidase (GUS)) and subsequent histochemical staining revealed that *G6PDH1* is expressed in virtually all tissues rather than localized to any specific tissue. These results are consistent with patterns of regrowth observed following clipping in *Arabidopsis*, reconstituting the entire plant with greater biomass and higher fitness. Collectively, these results, along with those uncovered in Chapters 1 and 2 of this dissertation, including fitness assays, QTL mapping, microarray analysis, gene expression assays, knockout mutants,

[1] This chapter along with chapter 1 is currently in review in the journal Genetics, as Siddappaji, M.H. Scholes, D.R., Bohn, M.O. and Paige, K.N. Molecular Basis of Overcompensation in *Arabidopsis thaliana*: the Role of Glucose-6-Phosphate Dehydrogenase and the Oxidative Pentose-Phosphate Pathway

previous work on chromosome amplification (Scholes and Paige 2011) and now complementation studies, all indicate the direct or indirect importance of *G6PDH1* in regulating the compensatory response following the removal of apical dominance.

Keywords : *G6PDH1*, complementation, GUS histochemistry, fitness, T-DNA knockout lines,

Introduction

The evolution of plant defense mechanisms in response to herbivory has been a central theme of many evolutionary studies. Most plant tissues defend either structurally (by spines or pubescent leaves) or chemically (production of toxic chemicals) to deter herbivores to lessen their detrimental effects. However, some plant species experience intense and seasonally predictable levels of herbivory, wherein they lose >90% of their aboveground biomass. In response many species have adopted rapid regrowth strategies to overcome such drastic impacts; commonly referred to as tolerating herbivory or plant tolerance (e.g., see Stowe et al. 2000). Interest in plant tolerance was motivated by empirical studies demonstrating that herbivore damage can, under certain circumstances, increase, rather than decrease, plant reproductive success (a specialized case termed overcompensation, i.e., increased flower, fruit, and seed production following herbivory). Specifically, studies by Paige and Whitham (1987) showed that when mule deer and elk removed 95% or more of the aboveground biomass of the monocarpic biennial scarlet gilia, *Ipomopsis aggregata*, six of nine traits directly related to fitness were significantly greater compared to uneaten controls (see also Paige 1992, 1994, 1999). In fact, evidence for increased fitness following herbivory has been found for numerous plant species since the initial study of Paige and Whitham in 1987 (see e.g., Stowe et al. 2000, Pilson and Decker, 2002, Rautio et al. 2005, Maschinski and Whitham, 1989, Weinig et al 2003a, 2003b, Lennartson et al. 1997).

In a recent study (Chapter 2 of this dissertation; Siddappaji et al. in review) we took advantage of the compensatory variation in RILs of *Arabidopsis thaliana*, combined with microarray and QTL analyses to assess the molecular basis of overcompensation. We found three QTL explaining 48.2% of the variation in fitness compensation and 109 differentially expressed genes between clipped and unclipped plants of the overcompensating ecotype Columbia. From the QTL/microarray screen we uncovered one gene that appeared to play a

significant role in overcompensation; glucose-6-phosphate-1-dehydrogenase. Knockout studies of T-DNA insertion lines (Chapter 2) of *G6PDH1* verify its role in compensation. *G6PDH1* is a key enzyme in the oxidative pentose-phosphate pathway (OPPP) that plays a central role in plant metabolism. It is likely that plants capable of overcompensating reprogram their transcriptional activity through defensive mechanisms, increased expression of genes involved in energy metabolism and an increase in DNA content (via endoreduplication; see Scholes and Paige 2011), with the increase in DNA content feeding back on pathways involved in defense and metabolism through increased gene expression.

Here, we further verify the role of glucose-6-phosphate-1-dehydrogenase in plant compensation by complementing *G6PDH1* to reinstate its function and localize where it is expressed by creating chimeric promoter-reporter (GUS) fusion constructs.

Materials and Methods

Plant Material, Growth Conditions

Growth of the *Arabidopsis thaliana* ecotype Columbia and the T-DNA knockout line (GABI86405A) was carried out in a greenhouse on the campus of the University of Illinois. In brief, the seeds were vernalized at 4^{o}C for 3 days to obtain uniform germination and planted in 3-inch circular pots using sunshine soil mix. The plants were grown at 16:8 hours of light:dark. At bolting, the apical meristem was clipped at ground level to simulate mammalian herbivory.

Complementation of G6PDH1

To assess the effects of *G6PDH1* on fitness compensation we used a gene complementation approach wherein we replaced *G6PDH1* in a T-DNA knockout line (of G6PDH_3; Gabi_86405A; with the same genetic background as the Columbia wildtype). We used the following forward and reverse primers, For1 – CACCCGTGTCGACCTC CACTATTGCCTCAAGTTGATGTTGAGTTCCG and Rev1 – CCAATCTTCA TCTTCGTCTTCATGGTACCTAACG, to amplify 6032bp of *G6PDH1* using *Kod* polymerase. The region included ~2.0 kb of the upstream promoter, exons and introns and ~1.1kb of the downstream region. The PCR product was subcloned into a pENTR/d-TOPO vector as per the manufacturer's recommendation and later cloned to pMDC 123 (Curtis and Grossniklaus, 2003) using the gateway LR reaction. As both entry clone and binary vector had the same

selection marker (kanamycin), the entry clone was linearized using the restriction enzyme *MluI*. This enzyme linearizes without affecting the gene or the gateway site-specific recombination sites. The gateway site specific recombination yielded the binary vector (pMHS 207) which was transformed to *Agrobacterium tumefaciens* strain GV3101:pMP90 by the freeze-thaw method (Holsters et al. 1978). Plant transformation was done using Clough and Bent's (1998) floral dip protocol. Primary transformants were selected by spraying glufosinate @250mM concentration. Four transgenic seed lines were carried through to the T2 generation for subsequent fitness analyses.

Fitness Analysis of T-DNA, Wildtypes and Complementation Lines

The ecotypes Columbia, Landsberg *erecta*, T-DNA knockout lines and complementation lines were grown in 3 inch pots in LI Sunshine® mix at 12 hours light and dark. A total of 40 plants per line were grown from seed and half (20 per line) were randomly clipped to ground level at approximately 6 cm of growth to simulate mammalian herbivory; the remaining 20 served as undamaged controls. We chose 4 independent transgenic event lines for analyzing fitness reversals after complementation. Given that the transgenic lines were grown in a separate experiment, under conditions similar to those used in comparing Columbia, Landsberg *erecta* and three t-DNA knockout mutants to one another, a separate ANOVA (in SYSTAT 13.0) was used to compare clipped to unclipped transgenic plants to see if restoring gene function led to a pattern of overcompensation as one would predict if *G6PDH1* played a significant role in overcompensation. Fitness comparisons were made in terms of the number of siliques produced. Siliques were log transformed to meet assumptions of normality. A Fishers LSD Test was run following the ANOVA to look at individual line effects of the clipping treatment.

Promoter-Reporter Fusion Studies and Fixed Material Staining

Primers For1-CACCCGTGTCGACCTCCACTATTGCCTCAAGTTGAT GTTGAGTTCCG and Rev1–GATCATAGAATGTGTCGCCATGAAATTTT CCATGGAGCG were used to amplify the upstream (~2.1kbp) portion of the *G6PDH1* gene using *Kod* polymerase. The PCR product was subcloned to pENTR/d-TOPO vector as per manufacturer's recommendation and later cloned to pMDC 162, with GUS included, using the gateway LR reaction. As both entry clone and binary vector had the same selection marker (kanamycin), the

entry clone was linearized by restriction digestion with *MluI* before transferring the gene to the binary vector. The linearized circular plasmid contained the gene and the gateway site- specific recombination sites. The linearized DNA was ligated with the binary vector pMDC123 using the gateway LR reaction (Curtis and Grossniklaus, 2003) to obtain the destination clone (pMHS207) which was eventually transferred to the *Agrobacterium tumefaciens* strain GV3101:pMP90 by the freeze-thaw method. Plant transformation was done using Clough and Bent's (1998) floral dip protocol. Transgenic plants were selected using hygromycin as a selectable marker (20µg/ml). The transgenic seeds were carried through T_2 generation for subsequent analyses using histochemistry.

GUS assays were performed as described in Arnaud et al. (2010) with slight modification. In brief, plants were fixed in 90% acetone on ice for 10 min, then rinsed with a buffer containing 2 mM of K-ferrocyanide (Sigma-Aldrich), 2 mM of K-ferricyanide (Sigma-Aldrich), and 0.2% Triton X-100 in 50 mM of sodium phosphate buffer, pH 7.0. Samples were then incubated for 24h at 37°C in a buffer containing 2 mM of 5-bromo-4-chloro-3-indolyl β-D-glucuronide (Gold Biotechnology). The samples were later washed with an ethanol series (20%, 35% and 50%) to remove the staining buffer and finally the tissues were fixed in FAA (10 ml of 3.7% formaladehyde, 5mL acetic acid, 50mL of ethyl alcohol with 35ml of water). The samples were stored in 70% ethanol at 4^{o}C for later photography.

Results

Complementation of G6PDH1

Results from one of four complementation lines showed a partial rescue effect of *G6PDH1*, showing patters more similar to the overcompensating Columbia line than either Landsberg *erecta* or the knockout line (see Figure 4.1). Clipped plants of this transgenic line (Event_1; see Figure 1) complemented with *G6PDH1* tended to produce more siliques/plant than unclipped plants ($p=0.065$; clipped plants produced 127.7 ± 5.0 siliques per plant whereas unclipped plants produced 113.3 ± 5.3 siliques per plant). However, the remaining three lines showed no significant effects of complementation ($p \geq 0.55$ in all three cases; clipped versus unclipped plants produced 108.4 ± 6.0 versus 108.9 ± 4.9 siliques/plant, 129.7 ± 5.7 versus 132.4

± 5.2, and 146.7 ± 7.8 versus 140.3 ± 5.8 siliques/plant for the remaining transgenic lines; Figure 1, Events 2-4).

In a separate experiment, wildtype Columbia overcompensated as expected, producing more siliques/plant than unclipped plants (clipped plants produced 164.1 ± 8.1 siliques /plant whereas unclipped plants produced 117.9 ± 5.0 siliques/plant; p=0.024). Landsberg *erecta* and the knockout mutant also performed as expected showing a pattern of equal compensation with a trend toward undercompensation, producing fewer siliques/plant following clipping (clipped plants produced 118.8 ± 2.6 siliques/plant whereas unclipped plants produced 149.3 ± 9.5 siliques/plant for Landsberg and 114.8 ± 15.7 siliques/plant for clipped individuals and 139.7 ± 24.2 siliques/plant for unclipped individuals of the knockout mutant, *G6PDH*_3; p=0.129 and 0.191, respectively).

G6PDH1 Tissue Expression

Results of our promoter-reporter fusion studies (*G6PDH1* promoter: β-glucuronidase (GUS)) and subsequent histochemical staining revealed that *G6PDH1* is expressed in virtually all tissues rather than localized to any specific tissue. Gene expression of *G6PDH1* was observed in rosettes, roots, leaf mid-veins, secondary and tertiary veins, secondary meristems and in flower sepals, filaments, and pedicels of siliques and eventually in the siliques (Figure 4.2 A-G). Most interestingly they were expressed during the seed development and seed filling stages.

Discussion

The results of our complementation studies support the role of *G6PDH1* in plant compensation (most notably the phenomenon of overcompensation). Clipped plants of the transgenic line, Event_1 (Figure 1), complemented with *G6PDH1* produced more siliques/plant than unclipped plants. Thus, this transgenic line complemented with *G6PDH1* restored the compensatory response from equal compensation, with a trend toward undercompensation, in the knockout line (*G6PDH1*_3; Gabi_86405A) to overcompensation. However, we suspect that positional effects of the transgene or unmeasured environmental influences may have constrained the magnitude of the compensatory response typically observed in Columbia. In addition, only a single line appeared to rescue *G6PDH1*, the remaining three did not. This is likely due to positional effects caused by the random insertion of the gene into the genome.

Often gene transformations will result in the insertion of the gene within another gene knocking out its expression leading to results that are inconsistent with the expected outcome. Thus, a number of lines need to be assessed, as we did here, to see if any of the insertion events behave in the expected fashion. Thus, the observation of a single event is informative whereas negative results are often inconclusive.

Results of our promoter-reporter fusion studies (*G6PDH1* promoter: β-glucuronidase (GUS)) and subsequent histochemical staining revealed that *G6PDH1* is expressed in virtually all tissues rather than localized to any specific tissue. These results are consistent with patterns of regrowth observed following clipping in *Arabidopsis*. Virtually the entire plant is reconstituted with production of new meristems, increased flower production, increased root production and ultimately increased silique and seed production. We have previously shown that the suite of invertases that supply glucose to the OPP pathway and ultimately to *G6PDH1*, are localized across all tissues within the plant (e.g., see Barratt et al. 2009). Thus, increased expression of the variety of invertases and *G6PDH1* in the overcompensating genotype Columbia appears to facilitate the rapid regrowth, increased biomass and ultimately the enhanced fitness following the removal of apical dominance, as evidenced by GUS staining and plant-wide localization of *G6PDH1*.

Collectively, these results, along with those uncovered in Chapters 1 and 2 of this dissertation, including fitness assays, QTL mapping, microarray analysis, gene expression assays, knockout mutants, previous work on chromosome amplification (Scholes and Paige 2011) and now complementation studies, all indicate the direct or indirect importance of *G6PDH1* in regulating the compensatory response following the removal of apical dominance. These results demonstrate one of few examples supporting the adaptive plasticity hypothesis of Dudley and Schmitt (1996) that phenotypic plasticity has evolved to maximize fitness in variable environments (in this case the response to the removal of apical dominance, simulating natural patterns of herbivory in Arabidopsis and other plant species with similar compensatory responses).

Acknowledgements

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Figure 4.1. Fitness differences between ecotypes Columbia, Landsberg *erecta*, a knockout mutant of *G6PDH1* and four independent transgenic lines complemented with *G6PDH* gene (Events 1-4). Means ± 1 S.E. of mean are shown.

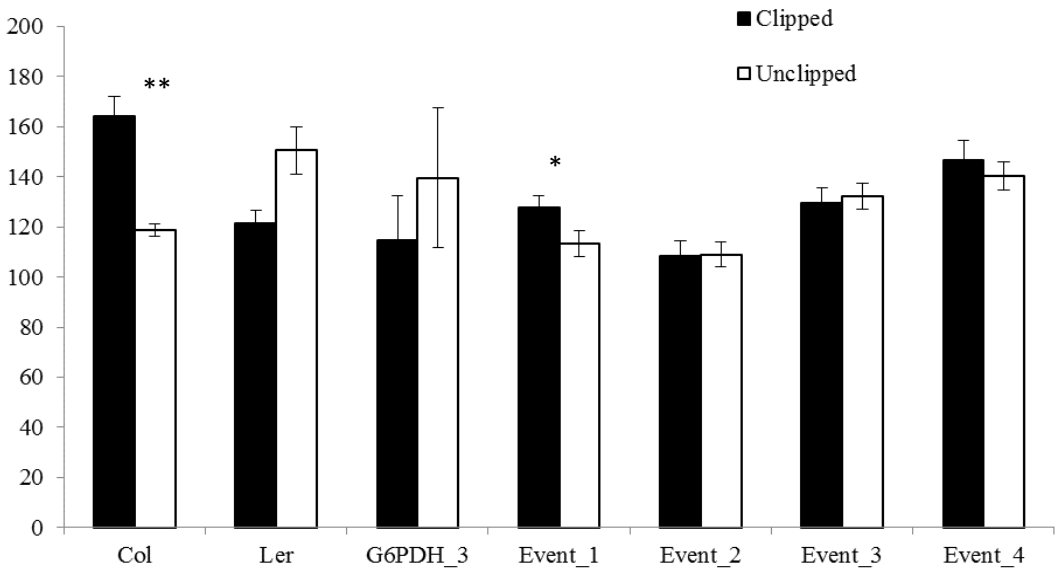

Figure 4.2 Localization of GUS activity in *Arabidopsis* plants transformed with *G6PDH1* promoter fusions.

A – Developing flower
B – Flower cluster with silique development with petiole
C – Flower development with stem
D – Flower development with siliques
E – Fully developed flower
F (a-c) – different stages of silique development indicating seed filling and development
G- 12 day old seedling grown on soil. GUS expression in mid vein, primary and secondary veins, roots, lower part of the cotyledons.