THE ADAPTIVE POTENTIAL OF PLANT ENDOPOLYPLOIDY IN RESPONSE TO HERBIVORY

BY

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DISSERTATION

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ABSTRACT

Damage caused by herbivores is a potentially strong selective agent of plant phenotypes, including selecting for many defensive traits, though not all plant-herbivore interactions are strictly negative for the plants. Overcompensation is the increase in components of fitness (flower, fruit, and/or seed yield) following herbivory. Studies by Paige and Whitham (1987) showed that when ungulate herbivores removed 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 uneaten controls (see also Paige 1992, 1994, 1999). The increase in relative fitness was largely due to architectural changes in the plant; 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.

Given that the release of apical dominance undoubtedly alters both genetic and physiological processes to maximize rapid regrowth (particularly in axillary bud break and stem elongation), recent studies have targeted possible mechanisms for facilitating rapid regrowth in overcompensating organisms. One possible mechanism is the replication of the genome without mitotic cell division via an alternative cell cycle under genetic regulation, termed endoreduplication (Brodsky and Uryvaeva 1977, Nagl 1976). Removal of apical dominance by herbivory reduces the level of auxin in the remaining above-ground tissues and leads to axillary bud break and stem regeneration—high levels of auxin are also known to repress the endocycle, and by contrast, a reduction in levels of auxin triggers an exit from mitotic cycles and

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an entry into endocycles (Ishida et al. 2010), providing a physiological mechanism for the promotion of endoreduplication following herbivory.

While much is known about endoreduplication at the cellular level, little is known regarding the role of endoreduplication in determining organismal fitness, let alone the compensatory response. To investigate the potential role of endoreduplication in fitness compensation following herbivory, two accessions of *Arabidopsis thaliana* were grown under greenhouse conditions and the bolting inflorescences of half of the plants of each genotype were clipped with scissors, simulating natural herbivory (Chapter 2 of this dissertation). At the induction of senescence, when fitness and endopolyploidy are fully determined, endopolyploidy was measured by flow cytometry on a set of plants from each treatment group. Clipped plants of *A. thaliana* Columbia, a genotype that historically overcompensates following clipping, displayed greater silique and seed yield than unclipped controls, while clipped plants of *A. thaliana* Landsberg *erecta*, a genotype that typically undercompensates, displayed an equal number of siliques but lower total seed yield than unclipped controls. Columbia-4's overcompensation in fitness measures correlated with an overall increase in nuclear DNA content due to endoreduplication. Landsberg *erecta*, however, experienced no change in its nuclear DNA content. These results provided the first correlative evidence of endoreduplication's role in mitigating the stress of herbivory.

After the initial discovery of the correlation between endopolyploidy and fitness compensation following herbivory in *A. thaliana*, I examined the role of endoreduplication as a generalized mitigator of herbivory. To investigate the generality of this relationship, I grew eight recombinant inbred lines produced by a Columbia-4 × Landsberg *erecta* mating under

greenhouse conditions (Chapter 3 of this dissertation). Attributes of fitness and endopolyploidy

were measured from clipped and unclipped plants of these eight genotypes and a significant

positive relationship between the change in seed yield and the change in endopolyploidy

following clipping was observed. This relationship suggests that endopolyploidy and fitness

compensation following herbivory are in some way directly linked, since fitness and

endopolyploidy are both polygenic traits, and so any chance association between compensation

genes and endopolyploidy genes should be broken by recombination upon crossing of the

parental genotypes. This result also suggests that the relationship exists for the Columbia

family of genotypes generally, since both Columbia-4 and Landsberg *erecta* originated from the

natural Columba population. To further investigate the generality of this relationship, I grew

nine globally-distributed natural ecotypes of *A. thaliana* under greenhouse conditions,

encompassing much of its wide geographic range across the Northern Hemisphere (Chapter 3

of this dissertation). Attributes of fitness and endopolyploidy were measured from clipped and

unclipped plants of these nine ecotypes. Although no significant relationship was observed

between fitness compensation and endopolyploidy following herbivory among these global

ecotypes by themselves, little variation in compensation was measured for these ecotypes. The

global ecotypes did, however, still fit within the range of values present in the Columbia-family

genotypes.

To investigate the direct, causal genetic link between the positive relationship observed

between endopolyploidy and fitness compensation following herbivory in the Columbia family

of genotypes, I grew cell-cycle mutant genotypes to determine if the change in endopolyploidy

with the genetic manipulation affected fitness compensation. Specifically, I used T-DNA

insertion and CaMV 35S overexpression mutants of *INCREASED LEVEL OF POLYPLOIDY1*, *ILP1*, a gene that regulates DNA replication of the endocycle, as well as the Columbia-0 genotype that served as the genetic background for the mutants. The Columbia-0 wildtype displayed decreased seed production, above-ground biomass, and endopolyploidy when clipped relative to when unclipped, providing further evidence of the relationship between endoreduplication and fitness compensation following damage. The two mutant lines, however, did not display changes in any of these measures following clipping damage, possibly due to the loss of natural plasticity in endoreduplication upon *ILP1* expression manipulation. Additionally, the *ILP1* knockout line, which should have lower endopolyploidy than the Columbia-0 wildtype, actually had greater levels of endopolyploidy, possibly due to an endoreduplication compensatory response induced upon the loss of *ILP1* action. Further, the *ILP1* overexpression did not have increased levels of endopolyploidy relative to the wildtype as expected. Phosphorus fertilization of these genetic lines improved fitness compensation measures, but only significantly for the Columbia-0 wildtype, further indicating a potential lack of plasticity tied to the experimental gene manipulation.

These studies collectively show that fitness compensation following herbivory may be influenced by the organism's ability to generate endopolyploidy following damage, potentially contributing to regrowth and organismal fitness by increased cell size, increased gene expression, increased water and nutrient transport, and other genetic and/or physiological mechanisms (Lee et al. 2009). While fitness compensation and endopolyploidy have thus far been researched extensively independently, these are the first studies that provide evidence for a relationship between the two phenomena.

ACKNOWLEDGEMENTS

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CHAPTER 1:
ENDOREDUPLICATION AS A GENERALIZED MITIGATOR OF ENVIRONMENTAL STRESS ACROSS TAXA

1.1 Abstract

Endopolyploidy, the condition of having multiple ploidy levels within a single individual, is produced by endoreduplication, in which the genome is successively replicated without mitosis. The condition is relatively common in a variety of plant and animal taxa and is associated with a conserved suite of nucleotypic and genetic effects. These effects can impact cell, tissue, and organism size, structure, and function. However, their influence on organismal fitness remains largely unknown. Because of its commonality and important role in metabolism and development, endopolyploidy has been linked to stress tolerance in a number of taxa and environmental conditions, though no compilation of these examples have been made to investigate the generality of endoreduplication as an adaptive response to stress. Here we review the role of endopolyploidy as a generalized mitigator of environmental stress across taxa.

1.2 Introduction

Endoreduplication is the process of genome replication without mitotic division, such that replicated chromosomes remain within a single nucleus. This process can occur numerous times in succession, creating nuclei with up to 24,576 times the gametic genome compliment (Nagl 1976, Traas 1998). The number of endoreduplication cycles tends to be relatively consistent among cells of the same tissue type, but may differ substantially from other cell

types, creating a mosaic of nuclear ploidy levels within a single organism (Barow 2006). This condition, termed endopolyploidy, is relatively common in eukaryotes, particularly in flowering plants where an estimated 90% of members exhibit endoreduplication in the majority of their tissues (D'Amato 1984). Endoreduplication also occurs in many animals, although it is typically limited to only a few highly specialized cell types (Lee et al. 2009).

Due to the breadth of taxa and cell types exhibiting endopolyploidy, endopolyploidy is suspected to play important generalized roles in cellular development and function (Nagl 1976). Because cell size is positively correlated with nuclear DNA content (Bradley 1954, Epstein 1967), it is hypothesized that endoreduplication is important in cellular and organismal growth, as well as cell differentiation (Nagl 1976). Due to the increase in the number of nuclear genome copies, metabolic demands may possibly be supported by an increase in transcriptional output of the genome, whether by increases in whole genome expression or by differential expression of genes within the metabolic pathway (D'Amato 1984, Larkins et al. 2001).

Given that endopolyploidy is known to play important roles in cell growth, development, and function, the process of endopolyploidization may also be important in the response to environmental stress by providing the increased cell volume, metabolic output, and/or gene expression to combat the stress through the production of secondary defense compounds, compound detoxification, rapid organismal growth, increased metabolism, or even reproductive output (Nagl 1976, Lee et al. 2009). Here we review the literature to understand the potential role of endopolyploidy as a generalized mitigator of environmental stress.

1.3 Plants

Endoreduplication has been studied extensively in plants, providing both observational and experimental evidence to its influence on stress tolerance and organismal fitness across numerous taxa. Endopolyploidy in response to environmental stresses may be particularly important in plants due to their largely sessile nature; endopolyploidy allows them a degree of cellular optimization to local conditions that may benefit survival and organismal fitness. Plants, perhaps in part for the same reason, also tend to tolerate major chromosomal and genomic aberrations which may allow them to employ endoreduplication widely across their tissues and in various environmental conditions. While endoreduplication is extremely common in angiosperms (Nagl 1976), the process is rare in gymnosperms although it has been observed in certain cell types of a few select families (Avanzi and Cionini 1971, Pichot and Maâtoui 1997).

1.3.1 Angiosperms

1.3.1.1 Abiotic stresses

One environmental variable that seems to directly influence endopolyploidy in plants is light availability. Etiolated hypocotyls of *Lupinus albus*, *Raphanus sativus*, *Glycine max*, *Pisum sativum*, *Arabidopsis thaliana*, *Brassica oleracea* L., and others all show greater numbers of endoreduplication cycles than hypocotyls of light-grown seedlings (Giles and Myers 1964, van Oostveldt and Parijs 1975, Galli 1988, Gendreau et al. 1998, Barow 2006). The rapid elongation of the dark-grown hypocotyls appears to be an adaptive light-searching strategy, likely developed to combat deep seed burying or crowding in nature. Similar examples of low-light

induction of endopolyploidy include *Kalanchoe blossfeldiana* and *Triticum durum* Creso, which both display enlarged leaf epidermal cells by endoreduplication under low light, seemingly to increase photosynthetic cell surface area for improved light capture (Cavallini et al. 1995, von Witsch and Flügel 1951).

Drought is another environmental stressor that can be particularly damaging to plants, including repressing endoreduplication under water stress in certain species. For example, under drought conditions, the maize endosperm remains primarily mitotic, which limits its size and thus its ability to support the metabolic demands of the developing embryo (Lee et al. 2009). Similarly, increased endoreduplication in leaf mesophyll cells of *A. thaliana* maintains leaf area via ploidy-mediated expansion under water stress (Cookson et al. 2006). Many drought-adapted succulents, however, exhibit constitutive endoreduplication widely throughout their tissues (de Rocher et al. 1990). The ability of highly drought-adapted species to maintain high levels of endopolyploidy under severe water stress shows the value of endoreduplication in maintaining cell size, and thus the associated advantageous nucleotypic effects (Bennett 1971, 1972), while less adapted species suffer presumably due to their reliance on water-mediated acid growth (Rayle and Cleland 1992). Succulents may thus benefit from the sequestration of water in their vacuoles during times of water availability while continuing to grow by endoreduplication during times of water limitation.

Potentially drastic seasonal changes in temperature can also have profoundly negative effects on cellular and physiological processes as protein-mediated reactions depart from their temperature optima. Although endoreduplication is generally repressed by both very high and very low temperatures (Tshermak-Woess and Hasitschka 1954, Engelen-Eigles et al. 2001),

endoreduplication at moderately low temperatures allows cell expansion at temperatures that more strongly repress cell division, allowing continued growth through a short period of cold (Barow and Meister 2003). Long periods of moderate cold likewise reduce the rate of endoreduplication and overall plant growth of two species of orchid, but a longer growth period results in no significant difference in floral organ endopolyploidy of control and cold-grown plants at the end of the growth period (Lee et al. 2007). This result suggests that endoreduplication plays an important role in normal floral development, even in cold temperatures that tend to slow the rate of both the mitotic and endomitotic cell cycles. Similar effects have been observed in tomato fruits, where an interplay of growth time and cell expansion by endoreduplication served to maintain final fruit size under a variety of day/night temperatures (Bertin 2005).

Due to plants' sessile nature, poor soil quality, whether by unsuitable pH, high salt, or adulteration by heavy metals, is a particularly damaging stressor to their survival. Tolerant varieties of *Sorghum bicolor*, however, endoreduplicate in their roots following exposure to NaCl, while non-salt tolerant genotypes do not (Ceccarelli et al. 2006), indicating that the increased root cell volume following endoreduplication may serve in the sequestration or cellular processing of excess soil salt. *Echium vulgare* has also been shown to endoreduplicate up to 96C in the endosperm in populations grown in soil polluted with a variety of heavy metals, including copper, zinc, lead, cadmium, cobalt, mercury, and arsenic (Malecka 1975, Biskup and Izmaiłow 2004), with less endoreduplication in the endosperms of plants on non-polluted soils. Increasing ploidy in response to cadmium has also been observed in roots of *Pisum sativum* L. cv. Frisson (Fusconi et al. 2006). Endoreduplication is presumed to improve

the absorptive capacity for sequestration of these heavy metals and the metabolic activity and transport efficiency with fewer, larger cells (Biskup and Izmaiłow 2004).   Heavy metals are also known to cause a variety of genomic aberrations (Coen et al. 2001), which may in part be protected against via the production of numerous additional genomic copies by endoreduplication. Endoreduplication may also mitigate the negative impacts, particularly of reactive oxygen species, caused by soil-borne organic molecules in a wide variety of plants via similar means (Galbraith et al. 1991, Ding et al. 2007, Zhang et al. 2009).

1.3.1.2 Biotic interactions

Not only may aspects of the abiotic environment influence endoreduplication in plants, but interactions with biota may do so as well. Arbuscular mycorrhizal fungi associate widely with a number of plant families, occupying cellular space within plant root tissue. A survey of 25 species across 16 plant families (including species that are not typically endopolyploidy) found that the roots of 22 species increased in endopolyploidy when associated with arbuscular mycorrhizal fungi. These included the presence of endopolyploidy in species not previously known to endoreduplicate (Bainard et al. 2011). Furthermore, the increase in endopolyploidy was strongly positively correlated with the proportion of root length colonized by fungi. Due to the generally accepted symbiotic relationship between plants and arbuscular mycorrhizal fungi, endoreduplication of plant cells is proposed to support fungal associations by providing the necessary cell volume for hosting the fungi, as well as increases in metabolism and protein synthesis (Bainard et al. 2011). Plant endoreduplication also occurs with symbiotic nitrogen-

fixing rhizobia in the root nodules of legumes, but with bacterial endoreduplication further

aiding the transfer of metabolites (Mergaert et al. 2006).

Endoreduplication can also occur in association with parasites. Part of the infection

mechanism of cyst nematodes includes the formation of root nodules, similar to those

stimulated by rhizobia, which occurs in part due to parasite-mediated changes in the expression

of plant cell cycle regulators (Cebolla et al. 1999, Roudier et al. 2003). The promotion of

endoreduplication in infected cells by the parasite serves to provide the cell volume and

metabolic products necessary for the parasite to survive within its plant host. Similar results

have been shown for the infection of *Arabidopsis thaliana* by cabbage leaf curl virus (CaLCuV), a

geminivirus that modulates the host cell's cell cycle machinery (Ascencio-Ibáñez et al. 2008),

which led to the significant generation of high level ploidy (i.e., 8C, 16, and 32C; Ascencio-

Ibáñez et al. 2008). While the authors conclude that the shift to endocycles is likely by the

action of the virus to create an environment conducive to the replication of its own genome,

numerous genes involved in DNA repair and genotoxic stress response were also

overexpressed, leaving open the possibility that endoreduplication could serve as the plant's

effort to conserve undamaged gene copies, particularly given the promotion of

endoreduplication by genotoxic stress of abiotic stressors in numerous taxa (e.g. see Thust and

Bach 1985, Lee et al. 1997, Maralhas et al. 2006).

Gall-forming insects, such as those that feed on the nutritive cells of *Andricus*

quadrilineatus, may induce great increases in host somatic polyploidy within the gall, producing

large cells that are high in lipids and proteins while low in starch and secondary compounds

(Bronner 1992, Harper et al. 2004). Endopolyploidy decreases with distance from the gall-

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feeding larva, indicating that the larva is responsible for the increases in nuclear DNA content, although the direct and indirect mechanisms by which this occurs are not well understood (Nagl 1978). Interactions of numerous symbionts and parasites, including various fungi, bacteria, and nematodes, have been observed to also induce local endopolyploidy, with a decrease in biotroph growth and/or development with a decrease in host endopolyploidy (Wildermuth 2010). This result, along with transcriptomic identification of ploidy-impacted metabolic processes, strongly suggests that endopolyploidy is necessary to support the increased metabolic demands of the nutritive host cells.

Recent discoveries have additionally implicated endoreduplication in the mitigation of damage from herbivory. *Arabidopsis thaliana*, which endoreduplicates widely in nearly all of its tissues (Sugimoto-Shirasu and Roberts 2003), has been shown to endoreduplicate more following herbivory than undamaged plants (Scholes and Paige 2011). This may help the plant overcome stress and increase seed production relative to undamaged plants in a phenomenon termed overcompensation (Paige and Whitham 1987). Regrowth following herbivory is achieved in part due to endoreduplication's role in rapid tissue generation, accelerating stem elongation and tissue expansion by drastic increases in DNA content and cell volume. While plant regrowth following large-scale herbivory may be aided by endoreduplication, the process also serves in the prevention of small-scale herbivory. Trichomes, which protect the plant surface from feeding by small insects, as well as from evaporative water loss, UV radiation, frost damage, and other stressors (Lee et al. 2009), endoreduplicate up to 256C (Sinnott and Trombetta 1936) to achieve the great volume and specialized shape needed to serve this purpose (Barow 2006).

1.3.2 Gymnosperms

Endopolyploidy in gymnosperms is most well described in the endosperms of

Cupressaceae—the cypresses, junipers, and thujas (Pichot and Maâtaoui 1997). The process of

endopolyploidization in these taxa is not merely by endoreduplication, however, as ploidy

levels between those produced by successive doubling are also observed. Cytological analysis

suggests that these ploidy levels are produced via a combination of endoreduplication,

endomitosis, nuclear fusion, and polynucleation (Pichot and El Maâtaoui 1997). The increase in

cellular DNA content by these processes is presumed to support the transcriptional and

translational output necessary to support the embryo and serve as nutrient-storage tissue (Nagl

1978). In gymnosperms, true endoreduplication has so far only been described extensively in

Ginkgo biloba, which exhibits endopolyploidy up to 64C (Avanzi and Cionini 1971).

1.3.2.1 Abiotic stresses

While clear evidence of endoreduplication in gymnosperms remains limited, some

chromosomal abnormalities under environmental stress have been reported. Along with

increased frequency of various chromosome mutations, mixoploidy has been reported in *Pinus*

sylvestris L. and others in Pinaceae growing under the extreme hydromorphic conditions of

eutrophic bogs (Muratova and Kruklis 1982, Ahuja 2005, Sedełnikova and Pimenov 2010).

While not technically endoreduplication due to the lack of complete genomic replication, a

single giant polytene chromosome was detected in *P. sylvestris* L., formed through repeated

replication without sister chromatid segregation (Sedełnikova and Pimenov 2010). The authors

consider this chromosome to be an adaptive element formed to support the very intensive cell

metabolism required to grow in such conditions, playing a role similar to that suggested of full genome endoreduplication.

1.3.3 Non-vascular plants

Endoreduplication has been observed in *Laminaria saccharina* and *Alaria esculenta*, both multicellular brown algae which undergo haploid and diploid phases of their life cycles (Garbary and Clarke 2002). Endoreduplication of the vegetative sporophytes of these species promotes growth by increased cell size, while endoreduplication of the 1C haploid zoospores and gametophytes of *Ectocarpus siliculosus* can likewise undergo endoreduplication, which allows it to produce meiospores in an alternative method than the typical meiosporogenesis from diploid cells following gamete union (Bothwell et al. 2010). This process involves a reductive meiotic division, which would be impossible in the haploid state but may be achieved via endoreduplication. Increasing the number of genome copies in the hemizygous embryo not only allows for parthenogenetic reproduction but also provides gene redundancy, thus protecting against epigenetic defects, allowing double-strand break repair (Aylon and Kupiec 2004), and balancing nuclear DNA content with organellar DNA quantity as more organelles are produced (Sugimoto-Shirasu and Roberts 2003). These numerous effects ultimately enhance the metabolic capacity of the cell (Bothwell et al. 2010). Overall, these numerous protective effects and developmental plasticity provided by endoreduplication may be important to the survival and reproductive success of *Ectocarpus* as it lives in the highly variable and frequently stressful intertidal zone (Bothwell et al. 2010).

1.4 Animals

Endopolyploidy in animals, in contrast to plants, primarily occurs in highly specialized cell types with high metabolic output (Edgar and Orr-Weaver 2001). These include cell types within each of the major animal organ systems, including specific digestive, respiratory, circulatory, reproductive, and nervous system cells. Endoreduplication is particularly common among each of these systems in insects but is much less widely observable in mammals (Edgar and Orr-Weaver 2001), and relatively few other examples of animal endopolyploidy have been reported. Even fewer examples of endopolyploidy in animals in relation to environmental stresses have been observed, perhaps due to the ability of most animals to avoid many environmental stresses due to their relatively mobile nature. Furthermore, many examples of stress-mediated endoreduplication in animals are suggested to be the result of generalized genomic instability and disruption of normal cell cycle progression (Edgar and Orr-Weaver 2001), rather than the adaptive induction of endoreduplication. Thus, in many animals, endoreduplication may possibly be more often a symptom of stress rather than a concerted response by the cell or organism. Here we discuss endopolyploidy in animals in relation to environmental stress with emphasis on examples of endoreduplication as a beneficial response.

1.4.1 Insects

Using experimental overexpression of a particular S-G2 checkpoint regulator in *Drosophila melanogaster*, it has been shown that the absence of the checkpoint, leading to increased DNA content by endoreduplication, enables endocycling cells to tolerate DNA

damage, avoid apoptosis, and generally tolerate genotoxic stress (Hong et al. 2007, Lilly and

Spradling 1996, Mehrotra et al. 2008).

Endoreduplication in *Drosophila* is also influenced by nutrition, where endoreduplicating

cells of newly hatched *Drosophila* larvae cease replication and enter a quiescent state upon the

withdrawal of nutrition (Britton and Edgar 1998). Mitotically active cells, however, remain

unaffected and continue normal proliferation. Why endoreduplicating cells are negatively

affected but mitotic cells are not is not well understood, given the vast overlap of cell cycle

regulators between the mitotic and endocycles, but these results highlight the particularly close

association of the endocycle with external stimuli.

1.4.2 Mammals

Endoreduplication has been studied in several mammalian systems, but most notably in

the mouse, *Mus musculus*, and human heart and liver cells. An analysis of ploidy-induced

changes in gene expression within these tissues, along with phenotypic analysis, indicates that

endopolyploidy is associated with a variety of beneficial effects, including an increase in vitality

due to the induction of sirtuin-pathways that improve stress resistance, an increase in ATP

production, and improved efficiency of tissue-specific function (Anatskaya and Vinogradov

2010). Heart cells specifically became more efficient with greater polyploidy due to the

replacement of alpha myosin contractile chains with the more ATP-efficient beta type. Liver

cells showed greater immunity, improved detoxification efficiency of reactive oxygen species, a

resistance to xenobiotic stress, and numerous long-term metabolic modifications to improve

efficiency (Anatskaya and Vinogradov 2010). The authors suggest changes in gene expression,

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and thus changes in phenotype, with endoreduplication are important for the rapid response to a wide range of stresses, allowing for adaptation to new environments and a balance between cellular function, stress, and metabolism.

Another example of endoreduplication in mammalian liver cells is in response to DNA damage (Denchi et al. 2006). Many types of stressors can cause abnormalities in replication and mitotic division, including telomere dysfunction that may induce senescence or apoptosis (Hemann et al. 2001, Herbig et al. 2004). Liver hepatocyte cells, upon experiencing stress-induced genomic damage, endoreduplicate to continue growth when normal cell proliferation is prevented by genomic instability. This response supports the maintenance of normal liver function under stress and has also been observed in aging mouse hepatocytes (Funk-Keenan et al. 2008). These results show the beneficial effects of increasing genome copy number by endoreduplication even when genome stability is compromised by stress.

Similar effects of endoreduplication are present in response to xenobiotic stress. The injection of a pesticide contaminant in mice resulted in the increased endoreduplication of hepatocyte cells, leading to increased DNA content and reduced apoptosis of these cells (Huang et al. 2005). Because liver hepatocyte cells are involved in detoxification of body fluids, these results suggest that endoreduplication is important in the maintenance of these cells under great toxic stress.

2-Methoxyestradiol, a natural metabolite of estradiol that is additionally being developed as a cancer-combatting drug, induces endoreduplication in differentiated nasopharyngeal carcinoma cells by the activation of ERKm JNK and p38 MAPK stress signaling pathways (Ting et al. 2010). Endoreduplication was also induced after treatment with a

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superoxide donor, as well as the overexpression of mitochondrial superoxide dismutase. These results collectively indicate that endoreduplication in these cells is induced by oxidative stress, although whether endoreduplication is employed to mitigate oxidative damage or results as a byproduct of disruption of the cell cycle pathway is not clear.

Endoreduplication can also be stimulated in humans by exposure to benzene (Ji et al. 2009). When human lymphoblastoid cells were treated with the benzene metabolite hydroquinone, endoreduplication was stimulated by the disruption of microtubule assembly and inhibition of DNA topoisomerase II chromosome segregation activity. This effect has similarly been observed in Chinese hamster ovary cells to a wide host of anti-topoisomerase II chemicals (Pastor et al. 2005), as well as plant secondary metabolites (Neukam et al. 2008). The inhibition of topoisomerase II due to plant metabolites, namely tea flavanols, are suggested to be beneficial in the inhibition of carcinogenesis by promoting apoptosis and decreasing cell proliferation (Neukam et al. 2008), limiting the frequency of cell division and the severity of chromosome aberrations caused by cancerous cell proliferation.

1.4.3 Fish

Endoreduplication in fish has been observed in the gill tissue of *Hypophthalmicthys molitrix* as a result of water pollution by heavy metals, with particularly high levels of cadmium and mercury (Rose et al. 2010). Although a definitive function is unclear, the authors note that protein banding patterns vary in the endoreduplicated tissues, such that gene expression and protein production may be affected by endopolyploidy in response to heavy metals and/or associated genotoxic stress.

Another potential stress, although not directly environmentally-induced, is the increase in diffusion distance of nuclear products throughout the cytoplasm due to the increase in cell volume upon rapid cell growth. Muscle fiber hypertrophy occurs rapidly in many animals, and compensation for this stress was found to occur in a wide range of fish species by endoreduplication (Jimenez and Kinsey 2012). In 11 of 17 species examined, within-species increases in muscle fiber diameter were associated with increases in the myonuclear domain (the volume of cytoplasm that the nucleus services), with 9 of 17 species exhibiting a significant relationship between endopolyploidy and muscle fiber size. These results suggest that endoreduplication is an important process in muscle fiber hypertrophy and for overcoming increased diffusion distances throughout the cellular volume (Jimenez and Kinsey 2012), perhaps by increases in relative nuclear volume within the cell and/or increased nuclear output by an increase in nuclear DNA content.

1.4.4 Others

An increase in endopolyploidy with muscle fiber diameter and size of nuclear domains within fibers has also been observed in a number of decapod crustaceans (Jimenez et al. 2010). Muscle fiber cells, which are multinucleated, increase in their number of nuclei as fiber size increases in all eight species examined, with a significant increase in nuclear ploidy level (ranging from 4C to 32C) in four species. These results suggest that muscle fibers in crustaceans, as with fish discussed above, compensate for decreases in diffusion efficiency within the increased cell volume by increasing nuclear ploidy (Jimenez et al. 2010). Three species, however, exhibited lower muscle fiber ploidy levels in adults than juveniles, indicating

that the increase in the DNA content of each nucleus with muscle fiber growth may be offset by or take place in conjunction with other mechanisms during the transition from juvenile to adult.

1.5 Conclusions

Endopolyploidy may serve as a generalized stress response across taxa with some fundamental, conserved functions. Perhaps the most basic effect of endopolyploidization is the direct increase in cell size, owing to the increase in bulk DNA content and thus nuclear and cell volume (Nagl 1976). This nucleotypic effect, although basic in nature, may stimulate an increase in cytoplasmic constituents and organelles that serve some more complex functions (Bennett 1971, 1972). For example, the differentiation of a cell often entails the permanent change in a cell's basic properties to support a gain in specialized function (Nagl 1976)— endopolyploidy may thus stimulate the cell size and internal composition required for a cell to achieve its fully differentiated state. Specialized cell functions may then be supported by increases in metabolism, driven by increases in gene expression and greater numbers of metabolic organelles, as suggested by numerous examples here. Through this mechanism, increases in nuclear DNA content by endoreduplication leads to increases in cell size, increases in water and nutrient transport, increased gene expression, and/or increased cellular metabolic capacity (Nagl 1976, Kowles and Phillips 1988, Barow 2006, Lee et al. 2009). It therefore appears that increased endopolyploidy may be adaptive in many organisms by mitigating the negative impacts of environmental stressors on organismal survival and reproduction.

CHAPTER 2:
CHROMOSOMAL PLASTICITY: MITIGATING THE IMPACTS OF HERBIVORY[1]

2.1 Abstract

Endoreduplication, the replication of the genome without mitosis, leads to endopolyploidy, an increase in cellular chromosome number. Although endoreduplication is widespread among angiosperms and other groups of eukaryotes, the degree to which this process is plastic under varying environmental conditions and its potential adaptive significance are not known. Here, using flow cytometry, we measured plasticity in chromosome number following the removal of apical dominance (simulating natural herbivory) in two ecotypes of *Arabidopsis thaliana*: Columbia-4 and Landsberg *erecta*. We report that endopolyploidy of clipped Columbia-4 plants was significantly different than unclipped controls following the removal of apical dominance and regrowth, and that cellular ploidy is positively associated with attributes of fitness (biomass, flower, fruit, and seed production). In contrast, clipped Landsberg *erecta* showed no significant differences in endopolyploidy and a decrease in seed production compared to unclipped controls, representing a significant genotype × environment interaction between ecotypes. Altering ploidy via endoreduplication adds a previously unknown way in which plants may be able to cope with environmental stress: enhancing regrowth rates and fitness following plant damage.

2.2 Introduction

Plant tissue loss to herbivores is an important selective agent shaping plant phenotypes. To date, most studies of plant adaptation have focused on the evolution of defensive traits that reduce or prevent tissue damage by herbivores (Berenbaum et al. 1986, Mauricio and Rausher 1997, Agrawal 1998). However, herbivores may also select for traits that allow plants to maintain fitness in the face of tissue loss (Stowe et al. 2000). Plant genotypes that can compensate for tissues lost with little or no decrement in fitness relative to those that are undamaged represent such an example and are termed tolerant (see Stowe et al. 2000 for a review). Interest in tolerance was stimulated by empirical studies demonstrating that herbivore damage can, under certain circumstances, increase, rather than decrease, plant reproductive success (a specialized case of tolerance, termed overcompensation, i.e., increased flower, fruit, and seed production following herbivory). Specifically, studies by Paige and Whitham (1987) showed that when ungulate herbivores 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 (see also Paige 1992, 1994, 1999). The increase in relative fitness was largely due to architectural changes in the plant; 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 belowground biomass.

With an increasing number of investigators seeking evidence for overcompensation, more supportive evidence is being uncovered. For example, evidence for increased flower, fruit, and seed production following herbivory has been found for a number of plant species

including two species of *Ipomopsis*, *I. aggregata* and *I. arizonica* (Paige and Whitham 1987, Maschinski and Whitham 1989), and several unrelated to *Ipomopsis* including *Gentianella campestris*, *G. amarella* (Nilsson et al. 1996, Lennartsson et al. 1997), *Sanicula arctopoides* (Lowenberg 1994), *Bouteloua gracilis* and *Bouteloua hirsute* (Alward and Joern 1993), *Ipomoea purpurea* (Hougen-Eitzman and Rausher 1994), *Arabidopsis thaliana* (Mauricio et al. 1997, Weinig et al. 2003), and *Erysimum strictum* (Rautio et al. 2005).

There is also evidence that genetic variation for tolerance exists. Specifically, some families exhibit overcompensating tolerance, whereas others express incomplete tolerance (Mauricio et al. 1997, Tiffin and Rausher 1999, Juenger and Bergelson 2000, Weinig et al. 2003). 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 overcompensating tolerance, while ungrazed populations remain completely intolerant (Lennartsson et al. 1997). Although these observations provide evidence that genetic variation for tolerance exists, little is known about the genetic mechanisms that may lead to enhanced growth and reproduction in plant species exhibiting growth compensation.

Here we take the first steps toward testing a novel idea: that endoreduplication leads to enhanced growth and reproduction following herbivory, explaining the phenomenon of tolerance/overcompensation in plants. Endoreduplication is the replication of the genome without mitotic cell division, leading to endopolyploidy, where individual cells within an organism produce higher nuclear DNA content from consecutive doublings (Nagl 1976, Brodsky

and Uryvaeva 1977, Melaragno et al. 1993). This process is common in many groups of

eukaryotes and nearly the rule in angiosperms (Nagl 1976, Sugimoto-Shirasu and Roberts

2003). Endoreduplication may have genetic and/or nucleotypic effects (an effect based on DNA

content alone) that could lead to rapid regrowth and enhanced fitness (Bennett 1971, 1972,

Nagl 1976). Interestingly, removal of plant apical dominance, which causes a reduction in the

level of auxin and leads to axillary bud break and stem regeneration, is known to trigger an exit

from mitotic cycles and an entry into endocycles (Ishida et al. 2010). In contrast, high levels of

auxin repress the endocycle (Ishida et al. 2010). Thus, there appears to be a direct link between

endoreduplication and the removal of apical dominance. However, the degree to which

endoreduplication might be plastic following herbivory, or its adaptive significance, is unknown.

In this study we assess (1) whether there is plasticity in endoreduplication following the

removal of apical dominance and (2) whether there is a positive relationship between

endoreduplication and fitness compensation.

Here we use the model system *Arabidopsis thaliana* to address these issues. Our

previous studies established that ecotypes of *A. thaliana* differ in their ability to compensate for

tissue loss due to herbivory, ranging from lowered fitness after damage (undercompensation;

e.g., Landsberg *erecta*) to increased fitness after damage (overcompensation; e.g., Columbia-4).

Landsberg *erecta* (hereafter Ler) shares a common genetic background with Columbia-4

(hereafter Col-4), given that Col-4 was derived from the nonirradiated Laibach Landsberg

population (from the Nottingham Arabidopsis Stock Centre; description available at

http://www.arabidopsis.info). *A. thaliana*, a diploid with five chromosomes, has been shown

to endoreduplicate extensively in nearly all of its tissues (Sugimoto-Shirasu and Roberts 2003),

producing nuclei with DNA content as high as 64C (Melaragno et al. 1993) which represents five

endoreduplication cycles. In addition, endopolyploidy in *Arabidopsis* likely represents a form of

polyteny (i.e., the quantity of DNA doubles with each endocycle but the number of

chromosomes remain the same due to sister chromatid cohesion; Melaragno et al. 1993,

Sugimoto-Shirasu and Roberts 2003).

2.3 Materials and Methods

2.3.1 Growth and experimental clipping

To assess the relationship between plant compensation and the degree of plasticity in

endoreduplication following the removal of apical dominance, we planted a primary set of 170

individuals each of Ler and Col-4 and grew them under greenhouse conditions. At 2.5 weeks,

prior to bolting, 10 rosettes of each ecotype were harvested and analyzed for DNA content to

establish baseline ploidy. When inflorescences reached 6 cm (~3.5 weeks), 80 plants of each

ecotype were clipped, leaving approximately 1 cm of inflorescence (comparable to mammalian

herbivory). At 4.5 weeks, 20 plants of each ecotype (10 clipped, 10 unclipped) were analyzed

for DNA content, independently analyzing rosettes and inflorescences (stems, flower buds,

leaves). At approximately 6.5 weeks (nearing senescence), 30 additional inflorescences (stems,

leaves, flowers, flower buds, and valves of siliques) of each ecotype (15 clipped, 15 unclipped)

were analyzed for DNA content.

A second set of plants, composed of 30 Col-4 and 30 Ler plants (20 clipped, 20 unclipped

of each genotype) were grown as a separate experiment under the same environmental and

experimental regimen. Measures of stem height were recorded every day beginning at the

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induction of stem elongation (when the inflorescence bud reached approximately 0.25 cm in height, Day = 1) of 30 plants of each genotype (15 clipped, 15 unclipped; actual *n* = 12-15) and ending at the completion of elongation (4 weeks after elongation began, approximately 6-7 weeks after planting). For clipped plants, stem heights were measured daily only for the first newly elongating stem induced by clipping. The first induced stem was also the tallest stem at the end of life for all clipped plants of both genotypes.

2.3.2 Cytometric analysis

Tissue for flow cytometric analysis was prepared via standard protocols (see Galbraith et al. 1983). In brief, plant tissue was chopped with a razor blade, matched for tissue type and biomass (e.g. main stems, lateral stems, leaves, etc. to prevent bias from disproportionately distributed tissues between treatments), sheared in a nuclear isolation buffer (sodium citrate, MOPS, magnesium chloride, Triton X-100; Galbraith et al. 1983), filtered for debris removal, and stained with propidium iodide. Suspended nuclei were analyzed via a BD Biosciences FACScanto flow cytometer (San Jose, California, USA) for measurement of nuclear DNA content. Background correction and nuclei population gating were performed using De Novo Software FCS Express (v.3; Los Angeles, California, USA) to measure the proportion of nuclei at each ploidy level (2C, 4C, 8C, 16C) per plant sample. Mean nuclear DNA content per cell per plant was estimated via calculating a weighted average fluorescence value per plant based on the number of nuclei and average fluorescence of each ploidy level. The 2C ploidy level of each plant was used as the internal standard for DNA content calculation for the 4C, 8C, and 16C levels of each plant (*A. thaliana* genome size = 0.32 pg DNA/2C nucleus).

2.3.3 Fitness measures

Upon senescence (~8 weeks), the remaining plants of each treatment of the primary planting were analyzed for fitness (48–54 plants per treatment). Fitness included number of flowers, siliques, average number of seeds per silique, seed mass/100 seeds, number of main stems, number of lateral stems, total stem length (total length of all lateral and main stems combined), plant height, and total aboveground dry biomass at senescence. Because flower production is highly correlated with silique production (R^2 = 0.841, p < 0.0001; Paige, unpublished), only the more ultimate assessments of fitness are discussed.

2.3.4 Statistical analysis

Statistical analyses were conducted in Systat (v.13; Systat, Chicago, Illinois, USA). Analyses for the primary set of plants included independent t-tests followed by a sequential Bonferroni adjustment (Rice 1989) for number of variables measured within each ecotype and experiment. As noted above, fitness was measured from a separate set of plants for each ecotype due to the destructive sampling necessary for obtaining nuclear DNA content. Thus, separate analyses were applied to DNA content (including separate analyses for basal ploidy [rosettes] and time points of 4.5 weeks [rosettes and inflorescences from same plants] and 6.5 weeks of age [inflorescences only]) and fitness measures for each ecotype. A genotype × environment (G × E) interaction was estimated using a multifactorial ANOVA for DNA content (combining 4C, 8C, and 16C) per cell at 6.5 weeks of age.

For the second set of plants, stem heights for each day were analyzed in SAS (v.9.2; SAS, Cary, North Carolina, USA) via non-linear regression as repeated measures over a time series.

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Daily stem measurements were converted to percentage of maximum height achieved by each stem to control for variation in final stem height among plants. Data were fit to the logistic function:

Percent of maximum stem height = $\beta_1 / (1 + \beta_2 \bullet e^{-\beta_3 \bullet day})$

where β_1, β_2, and β_3 are parameters by which the logistic function is fit, e is Euler's number, and *day* is the number of days after the induction of stem elongation that the stem measurement was taken. To test for significant differences among growth curves, parameters were tested for significance by comparing pairwise differences of each β_1, β_2, and β_3 against zero among genotype × treatment groups (Col-4 unclipped, Col-4 clipped, Ler unclipped, Ler clipped). Comparisons were not made among β_1, β_2, and β_3 within or among genotype × treatment groups. To better visualize the rate of change and timing of stem elongation for each genotype × treatment group, the first derivative of the logistic function, representing the change in the percent of maximum stem height per day, was calculated and plotted.

2.4 Results

2.4.1 Endopolyploidy

At 2.5 weeks of age, prior to elongation of the inflorescence and thus before clipping, ploidy did not differ between rosettes of the two ecotypes, Col-4 and Ler (p = 0.659 at each of the two ploidy levels, 2C and 4C), each representing a basal diploid (2C) level with 4C cells likely representing cells with replicated nuclear DNA prior to mitotic cell division (Table 2.1A). At

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approximately 4.5 weeks of age, 1 week after clipping, clipped inflorescences of Col-4 differed from unclipped inflorescences of Col-4 at 2C, 4C, and 8C (Table 2.1B), whereas clipped individuals of Ler showed no significant differences from unclipped individuals at any ploidy level (Table 2.1B). In Col-4 the percentage of nuclei at the 2C level was significantly higher for clipped plants than unclipped plants. As a result, clipped inflorescences had lower proportions of cells at higher ploidy levels than unclipped plants (Table 2.1B), representing a delay in the degree of endopolyploidy achieved via endoreduplication during the regeneration period. Ploidy of clipped rosettes did not differ from unclipped rosettes for either Col-4 or Ler at any level of ploidy (all $p > 0.05$; at this point in time plants were beginning to endoreduplicate with ploidy levels of 2C, 4C, 8C, and 16C present in rosette tissues of both ecotypes; Table 2.1A). Because only inflorescence tissue was removed during clipping, rosettes were not expected to differ between treatments within an ecotype, and may serve as verification that changes in ploidy in the inflorescences were due to the clipping treatment.

At 6.5 weeks of age, 3 weeks after clipping, Col-4 clipped plants differed from unclipped plants at the 2C, 4C, 8C, and 16C levels (Table 2.1B, Fig. 2.1A). Clipped plants of Col-4 had a lower proportion of cells at the 2C level than unclipped plants, indicating higher endopolyploidy in clipped plants. This is confirmed at the 4C, 8C, and 16C levels, where clipped plants had a significantly greater proportion of cells at these higher ploidy levels than did unclipped plants (Table 2.1B, Fig. 2.1A) with an overall higher DNA content (Fig. 2.1C, $p < 0.01$). Although the degree of endoreduplication was initially set back in Col-4 clipped plants at 4.5 weeks, DNA content not only recovered three weeks after clipping but was significantly higher than in unclipped control plants. In contrast, at 6.5 weeks after clipping, clipped plants of Ler showed

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no significant differences in ploidy relative to unclipped plants (Table 2.1B, Fig. 2.1B). There

was also a significant genotype × environment interaction between Ler and Col-4 for higher

level ploidy ($p < 0.01$; Fig. 2.2).

2.4.2 Fitness

As expected, clipped plants of Col-4 produced significantly greater numbers of siliques

and seeds (1.51- and 1.4-fold greater, respectively; Fig. 2.3A and B, $p < 0.0001$ and $p < 0.0001$,

Table 2.2) than plants that were not clipped while clipped plants of Ler produced an equal

number of siliques and fewer seeds when compared to those that were not clipped (Fig. 2.3A

and B, $p = 0.453$ and $p < 0.01$, respectively; Table 2.2). No significant differences were found for

seed masses between clipped and unclipped plants of Col-4 (1.162 mg/100 seeds for clipped

plants and 1.159 mg/100 seeds for unclipped plants; $p = 0.382$) or Ler (1.161 mg/100 seeds for

clipped plants and 1.159 mg/100 seeds for unclipped plants; $p = 0.94$, Table 2.2). Lateral stem

production was significantly greater for clipped individuals of both Col-4 and Ler ($p < 0.0001$)

but the magnitude was slightly higher in Col-4 than Ler (1.75-fold higher for clipped plants of

Col-4 and 1.62-fold higher for clipped plants of Ler over unclipped controls; Table 2.2). Main

stem production was also significantly higher in clipped plants of both Col-4 and Ler ($p <$

0.0001) but the magnitude was almost twice as high in Ler as in Col-4 (2.60-fold higher for

clipped plants of Ler and only 1.43-fold higher for clipped plants of Col-4 over unclipped

controls; Table 2.2). Total stem lengths combined was also significantly higher in clipped plants

of both Col-4 and Ler compared to their unclipped controls ($p < 0.0001$) but the magnitude was

slightly higher in Ler (1.46-fold higher for clipped Ler and 1.2-fold higher for clipped Col-4 over

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unclipped controls; Table 2.2). Plant height was significantly reduced in clipped plants of both

Ler and Col-4 compared to their unclipped controls (each by approximately 7%; Table 2.2).

Furthermore, aboveground biomass was significantly greater for both clipped plants of Col-4 (p

< 0.0001) and Ler (p < 0.0001) when compared to their unclipped controls, although the

magnitude of increase was greater for Col-4 (1.49- vs. 1.24-fold greater, respectively, Fig. 2.3C).

Stem elongation curves did not differ between unclipped and clipped Columbia-4 plants

(all parameters equal; Table 2.3, Figure 2.4A), with a 0.95%/day difference in maximum growth

rate (unclipped: 7.62%/day, clipped: 6.67%/day) and a 1.6 day difference at which the

maximum elongation rate was achieved (unclipped: day 9.4, clipped: day 11.0; Figure 2.4B)

between unclipped stems and those stimulated by damage. There was a difference in the stem

elongation curves between treatments in Landsberg *erecta* (β_3 unclipped = 0.3415, β_3 clipped =

0.2901; Table 2.3, Figure 2.4C), however, where stems of clipped plants had a 1.31%/day

reduction in maximum rate of elongation (unclipped: 8.34 %/day, clipped: 7.04%/day) and a 2.4

day difference at which the maximum elongation rate was achieved (unclipped: day 7.7,

clipped: day 10.1; Figure 2.4D).

2.5 Discussion

The fitness consequences of endoreduplication have not been previously assessed.

Here we show that there is a correlation between fitness and endoreduplication—higher levels

of endoreduplication, following the removal of apical dominance, are positively associated with

greater biomass and higher levels of silique and seed production (relative to their undamaged

controls). As an aside, total DNA content in Col-4 is likely greater than that reported here given

that tissues were matched for biomass and biomass is significantly greater in clipped Col-4 than in clipped Ler; thus, our estimates are at best conservative. Based on these results, we suggest that increasing ploidy within an individual during its lifetime may add a previously unknown way in which some plants may be able to cope with herbivory and may help to explain ways in which some plant species are able to overcome and take advantage of being eaten, i.e., increasing fitness following damage (see Paige and Whitham 1987 and Lennartsson et al. 1997 for examples). In other words, we suggest that there may be a direct tie between plant compensation and endoreduplication. The adaptive plasticity hypothesis (Dudley and Schmitt 1996) suggests that phenotypic plasticity has evolved to maximize fitness in variable environments. Our results suggest that this may be the case: higher ploidy is correlated with rapid regrowth rates and higher fitness following the removal of apical dominance. Increasing chromosome number, and thus gene copy number, may provide a means of increasing gene expression, likely by the up-regulation of selected genes or gene families. Over one hundred genes have been shown to be differentially expressed when comparing clipped and unclipped plants of the Col-4 ecotype, genes predominantly associated with metabolism (Siddappaji et al., unpublished) that may facilitate rapid regrowth. In addition, increasing chromosome number may also increase vast numbers of sequences that encode microRNAs, small interfering RNAs, and long noncoding RNAs; these factors have been shown to play major roles as post-transcriptional regulators (Taft et al. 2010) and their activities may be influenced by endoreduplication.

Furthermore, the longstanding hypothesis is that a certain quantity of DNA is necessary for the maturation of a cell, and so endoreduplication may provide the cell volume and

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transcriptional output needed to sustain a fully differentiated cell's activities (Barow 2006).

Increasing chromosome number increases the total DNA content and hence cell size, leading to

extensive cell growth through endoreduplication, suggested by some to be the primary

consequence of endoreduplication (Barow 2006). Our results here show that while Columbia-4

is able to maintain comparable rates of stem elongation upon clipping (i.e. there are no

significant differences between parameters of Col-4 unclipped and clipped logistic growth

functions; Table 2.3), likely aided by its ability to increase its nuclear DNA content by

endoreduplication, Landsberg *erecta*'s inability to generate increases in endopolyploidy may

underlie its significant reduction in stem elongation rate and delay of maximal growth following

clipping. While stem elongation measures may not be entirely telling of endoreduplication's

role in tissue generation and ultimately fitness, they do provide evidence of the differing

regrowth responses of Col-4 and Ler. Growth by cell division along with growth by cell

expansion through endoreduplication may be faster than growth by cell division alone (Barow

2006). Endoreduplication also occurs predominantly among plants adapted to habitats that

require fast growth and development (Barow and Meister 2003, Barow 2006). This correlation

between life history and endoreduplication suggests a possible functional relationship between

enhanced chromosome production and rapid regrowth following the removal of apical

dominance, and our stem elongation results support this notion.

Previous work also points to a tie between endoreduplication and seed development, as

endoreduplication has been seen to occur widely in suspensor cells, which connect the embryo

to the surrounding nourishing tissue and ensure nutrient transfer. Furthermore,

plasmodesmata of the cell walls connecting the cytoplasm of adjacent cells slow down nutrient

transport; thus, faster transport is facilitated by producing larger and fewer cells with fewer cell walls via endoreduplication (Barow 2006). Kowles and Phillips (1988) suggested that extra DNA produced by endoreduplication is important in maize endosperm development and kernel filling through increased gene expression and protein synthesis. Engelen-Eigles et al. (2001) and Lee et al. (2009) found that high temperatures or water deficits can cause the endosperm of developing seeds to remain primarily mitotic, reducing endoreduplication, leading to smaller endosperm that are ill suited in supporting the embryo. These results are consistent with our findings that lower levels of endoreduplication in clipped plants of Ler are associated with lower seed production (in spite of equal silique production) whereas in clipped plants of Col-4 higher levels of endoreduplication are associated with higher seed filling and seed production.   These results suggest a tie between at least one aspect of fitness and endoreduplication: seed development.

Of particular interest is the fact that allocation patterns/life histories are quite different between ecotypes following the removal of apical dominance. The allocation of the proportional mass of total seed produced to the total biomass produced following clipping in Col-4 is on average 6.0% lower than unclipped controls (15.1% vs. 16.0% biomass allocated to seed for clipped vs. unclipped plants), whereas the allocation of the proportional mass of total seed produced to the total biomass produced following clipping in Ler is on the average 38% lower than unclipped controls (21.0% vs. 29.0% biomass allocated to seed for clipped vs. unclipped plants). Recall that while the number of seeds produced following clipping was significantly greater in Col-4 and significantly fewer in Ler, seed masses did not differ within or between Col-4 and Ler for either treatment. Above-ground biomass was significantly greater

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for both Col-4 and Ler following clipping but the magnitude was about 20% greater biomass for Col-4 (see Table 2.2). The rate at which that biomass was produced, inferred from our stem elongation measures, was also equal between unclipped and clipped Col-4, indicating that clipped Col-4 generated more biomass at the same rate as the smaller Col-4 unclipped plants (see Figure 2.4A,B). Thus, we suggest that endoreduplication may contribute or altogether explain the differences we see in allocation patterns given the genetic and/or nucleotypic effects discussed above—the studies proposed below should help us in addressing this issue from an experimental standpoint.

We also observe that the magnitude and direction of the change in ploidy differs between ecotypes (i.e., there is a significant genotype × environment interaction). Phenotypic plasticity, the ability of an organism to express different phenotypes depending upon the biotic or abiotic environment, has been observed in the physiology, development, morphology, chemistry, and behavior of organisms in response to environmental cues (Agrawal 2001). Although plants are widely known to endoreduplicate, there are few examples of plasticity in the degree of endoreduplication or genotype × environment interactions in endopolyploidy. A noteworthy exception is the study by Ceccarelli et al. (2006) showing that roots of salt tolerant *Sorghum bicolor* endoreduplicate following exposure to NaCl, whereas roots of nonsalt-tolerant genotypes of *Sorghum bicolor* do not endoreduplicate. Here we show that individuals of *Arabidopsis* can plastically alter ploidy during the course of their lifetimes in response to herbivore damage (in this case the removal of apical dominance). As noted above, removal of apical dominance in *Arabidopsis* is known to lower levels of auxin triggering an exit from mitotic cycles causing an entry into the endocycle (Ishida et al. 2010). Of course the variation that we

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see between these two 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.

If endoreduplication leads to enhanced reproduction as these studies suggest, such phenotypic plasticity may be of an adaptive nature and widespread among plants in mitigating the detrimental effects of herbivory. To substantiate this claim, we have conducted a wider screen of *Arabidopsis* ecotypes with similar compensatory responses to substantiate the broad-scale relationship between endoreduplication and fitness compensation in the face of herbivore impacts (see Chapter 3 of this dissertation). In addition, we have now analyzed knockout and overexpression mutants in order to make more direct connections between endoreduplication and fitness following the removal of apical dominance (see Chapter 4 of this dissertation). Lastly, endoreduplication may have important effects beyond mitigating the impacts of herbivory as indicated by the studies of Ceccarelli et al. (2006) on *Sorghum* and salt tolerance noted above. Cookson et al. (2006) also recently showed experimentally, using a *DEL1* mutant of *Arabidopsis*, that an increase in the extent of endoreduplication reduced the impact of water deficit on cell size, leaf expansion rate, final leaf size, and, by inference, fitness. In light of these results, an increase in endopolyploidy may provide an additional level of regulation of the genome, whether by transcriptional or nucleotypic effects (an effect based on DNA content alone; Bennett 1971, 1972), ultimately optimizing organismal fitness under ever-changing environmental stresses.

CHAPTER 3:
ENDOREDUPLICATION MITIGATES THE IMPACTS OF HERBIVORY IN *ARABIDOPSIS THALIANA*: AN ASSESSMENT OF RECOMBINANT INBRED LINES AND GLOBALLY-DISTRIBUTED ECOTYPES

3.1 Abstract

Endopolyploidy has been suggested to play a role in overcompensation (the increase in plant fitness following herbivory) generally, though no studies have yet investigated its role in other genotypes. Endoreduplication, the replication of the genome without mitosis, has been shown to be important in the regrowth and fitness of the Landsberg *erecta* (Ler) and Columbia-4 (Col-4) genotypes of *Arabidopsis thaliana* following damage (Scholes and Paige 2011). Here we provide evidence for the generalizability of this relationship and suggest that endoreduplication is directly related to the compensatory abilities of *A. thaliana*. We grew eight recombinant inbred lines from a cross between Col-4 and Ler, as well as nine globally-distributed naturally-collected ecotypes and clipped the bolting inflorescences of half of the plants, to simulate natural mammalian herbivory. After regrowth, at the induction of senescence, we measured the endopolyploidy of the aboveground tissue of each genotype. We report that the recombinant inbred lines, derived from the genotypes of Scholes and Paige (2011), show a positive relationship between endopolyploidy and compensation (inferred by seed yield and aboveground biomass) following clipping, suggesting direct causality between the phenomena due to recombination of the parental haplotypes in the recombinant inbred offspring. This positive relationship was retained upon inclusion of the globally-distributed ecotypes, although a relationship was not observed in the global ecotypes alone. These results

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suggest that, at least in the Columbia family of genotypes and perhaps beyond, endopolyploidy plays a direct role in the regrowth and compensation of these plants following herbivory.

3.2 Introduction

Overcompensation, the increase in plant fitness following tissue loss, has been shown to occur in numerous plant species, including, among others, *Ipomopsis aggregata* and *I. arizonica* (Paige and Whitham 1987, Maschinski and Whitham 1989), *Gentianella campestris* and *G. amarella* (Nilsson et al. 1996, Lennartson et al. 1997), *Sanicula arctopoides* (Lowenberg 1994), *Ipomoea purpurea* (Hougen-Eitzman and Rausher 1994) and *Arabidopsis thaliana* (Mauricio et al. 1997, Weinig et al. 2003). While these examples represent a wide range of angiosperm families and differ greatly in the nature of the damage stimulus, all show the ability of certain plant genotypes to either maintain or increase flower, fruit, and seed production following herbivory. Yet the phenomenon of overcompensation remains a controversial one. Alternative hypotheses to overcompensation are largely directed at the plants' abilities to reallocate stored resources to the rapid regrowth of aboveground biomass (replacement of lost tissues, but also induced production of secondary defense chemicals). If valid, this model would result in a net fitness decrement in perennial plant (Belsky 1986, Berenbaum et al. 1986, Mauricio and Rausher 1997, Agrawal 1998). Paige and Whitham (1987) showed that herbivory of certain genotypes of the monocarpic biennial *Ipomopsis aggregata* can lead to increased plant fitness while also increasing belowground stored resources during its shortened reproductive stage (Paige 1992, 1994, 1999). These increases are likely due to the release of apical dominance; the removal of the apical meristem by herbivores eliminates apical auxin production, leading to

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rapid axillary bud break and subsequent stem elongation which changes plant architecture, increases photosynthetic capacity, and in some cases enhances fitness (e.g. Paige and Whitham 1987).

Given that the release of apical dominance likely alters both genetic and physiological processes to maximize rapid regrowth and fitness compensation, recent studies have targeted integrative mechanisms for facilitating rapid regrowth in overcompensating organisms. Herbivory of the primary shoot axis undoubtedly results in the reduction of apical auxin production. Because of auxin's role in maintaining a cell's undifferentiated state, the removal of apical dominance likely triggers axillary cell expansion and differentiation via an exit from mitotic cycles and an entry into endocycles (Ishida et al. 2010). The endocycle is characterized by alternation of DNA synthesis and cellular growth phases, a process termed endoreduplication (Brodsky and Uryvaeva 1977, Nagl 1976). Because of its commonality in eukaryotes, and particularly in angiosperms (Nagl 1976, Sugimoto-Shirasu and Roberts 2003), endoreduplication is suspected to play critical roles in cellular development, namely in cell expansion and differentiation. The longstanding hypothesis is that a certain quantity of DNA is necessary for the maturation of a cell, and so endoreduplication may provide the cell volume and transcriptional output needed to sustain a fully differentiated cell's activities (Barow 2006). For example, endoreduplication is a fundamental component of proper trichome development, where increases in cell volume are associated with up to four rounds of endoreduplication (a ploidy increase to 32C; Melaragno et al. 1993). Additionally, experimental induction of endoreduplication in *Arabidopsis thaliana* has been shown to mitigate the effects of water deficit on cell size, reducing the impact on leaf expansion rate and final leaf size (Cookson et al.

2006). Endoreduplication has also been shown to play critical roles in endosperm development and seed-filling of fruits, suggested to be due in part to increased gene expression and protein synthesis given the increase in the number of DNA templates (Kowles and Phillips 1988). Likewise, the reduction of endoreduplication due to high temperatures results in smaller endosperm in maize, which are subsequently ill-suited to support the developing embryo (Engelen-Eigles et al. 2001). Given these demonstrated roles in cell expansion, determination of final organ size, and differentiation of important reproductive structures, endoreduplication could thus play a generalized role in rapid regrowth and fitness compensation as well— endoreduplication, stimulated by the release of apical dominance, increases nuclear DNA content and supports extensive cell expansion, rapid stem elongation, and development of fruits and seeds via increased cell volume and transcriptional output, and may ultimately provide the basis of overcompensation in plants following herbivory.

Our previous work (Scholes and Paige 2011) provided a correlation between endoreduplication and compensation following herbivory in *A. thaliana*. This correlation is suspected to be due to an interplay between hormonal status of the plant following herbivory (the release of apical dominance imposed by auxin) and the genetic background by which architecture, regrowth patterns, and characteristics of endoreduplication are based. While the correlation demonstrated is strongly suggestive of causality, given the known role of endoreduplication in influencing cell, organ, and final plant size in *A. thaliana* and other systems (Cookson et al. 2006, Barow and Meister 2003), this initial study does not provide a direct link to precise mechanisms by which endoreduplication may enhance a plant's capacity for rapid regrowth and increased fitness following herbivory. Likewise, Scholes and Paige (2011)

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compared only two genetic lines of *A. thaliana*, and so the genetic variation necessary to make more substantive claims regarding the role of endoreduplication in fitness compensation and its generalities in *A. thaliana* is lacking.

Here we investigate the generality of the correlation observed between endoreduplication and compensation by Scholes and Paige (2011), and seek to provide an additional data in support of the idea that endoreduplication is directly related to regrowth and compensatory abilities of *A. thaliana*. Because only Columbia-4 and Landsberg *erecta* were used in our previous studies, it is possible that the relationship between endoreduplication and fitness compensation is specific to the unique genotypes of each line—that is, there is no direct mechanistic link between endoreduplication and compensation, but rather the pattern exists by chance or linkage. To address this issue, we specifically assess: 1) whether recombinant inbred lines from a cross of Columbia-4 and Landsberg *erecta* parental lines (those used in Scholes and Paige 2011) show a similar correlation between endoreduplication and fitness compensation and 2) whether the relationship remains when various globally distributed ecotypes of *A. thaliana* are subjected to herbivory. The inclusion of a wide variety of related and unrelated genotypes should provide insights to both the generality and direct causality of the relationship between endoreduplication and compensation previously observed.

3.3 Materials and Methods

3.3.1 A. thaliana *accessions*

Recombinant inbred lines (RILs) were produced through eight generations of single-seed descent from the crossing of Columbia-4 (Col-4; CS933) and Landsberg *erecta* (Ler; CS20)

accessions (Lister and Dean 1993). Landsberg *erecta* and Columbia-4 originate from the Laibach

Landsberg population (Nottingham Arabidopsis Stock Center, http://www.arabidopsis.info),

and thus the parental genotypes are likely closely related. Because endoreduplication and

fitness compensation are both polygenic traits, crossing the Columbia-4 and Landsberg *erecta*

accessions used previously should break up any chance association between endoreduplication

genes and compensation genes in these parental genotypes, and thus the use of RILs should

result in the loss of the correlation if there is no direct link between endoreduplication and

fitness compensation. Seven RILs were selected for analysis from the Col-4 × Ler cross (TAIR

stock numbers: CS1906, CS1913, CS1941, CS1942, CS1948, CS1968, CS1985, CS1999; The

Arabidopsis Information Resource, http://www.arabidopsis.org), as well as the Col-4 and Ler

parental genotypes. These specific RILs were selected due to their range in compensatory

abilities, as measured in a preliminary analysis of 96 Col-4 × Ler RILs (Siddappaji et al.,

unpublished).

Genetic distance generally increases with geographic distance in *A. thaliana* (Sharbel et

al. 2000), and so widely distributed global ecotypes of *A. thaliana* should display a wide range

of genetic variability for both endoreduplication and fitness compensation following herbivory.

Nine field-collected, globally distributed ecotypes were inbred through eight generations of

single-seed descent and selected for analysis based on geographic origin (Table 3.1), with

ecotypes within and outside of *A. thaliana*'s natural range (Figure 3.1). A significant positive

relationship between the two traits among globally distributed genotypes would provide

additional evidence that endoreduplication acts directly in a plant's ability to rapidly regrow

and compensate with regard to seed yield.

3.3.2 Growth and experimental clipping

Ninety individuals of each accession (Col-4, Ler, seven RILs and nine global ecotypes) were grown under greenhouse conditions. At 4 weeks after planting, prior to bolting, rosette tissue of ten plants of each accession was analyzed for baseline nuclear DNA content. When inflorescences reached 6 cm in height (ranging from 5 weeks to approximately 10 weeks after planting), half (40) of the remaining plants were clipped, leaving 1 cm of inflorescence tissue (removing approximately 85% of the inflorescence, comparable to natural mammalian herbivory). At the induction of senescence (ranging from 9 weeks to approximately 15 weeks after planting), all inflorescence tissue (stems, leaves, flowers, flower buds, and valves of siliques) of 20 plants of each accession (10 clipped, 10 unclipped) was analyzed for nuclear DNA content.

3.3.3 Cytometric analysis

Nuclear DNA content was estimated by flow cytometry. Tissue for cytometric analysis was prepared by standard protocols (Galbraith et al. 1983). In brief, fresh tissue was matched for tissue type and mass, chopped with a razor blade, sheared in a nuclear isolation buffer (sodium citrate, MOPS, magnesium chloride, Triton X-100; Galbraith et al. 1983), filtered for debris removal, and stained with propidium iodide. Suspended nuclei were analyzed for DNA content via a BD Biosciences (San Jose, California, USA) FACScanto flow cytometer. Background correction and nuclei population gating were performed using De Novo Software FCS Express (v.3; Los Angeles, California, USA) to measure the number of nuclei at each ploidy level (2C, 4C,

8C, 16C) for each plant sample. The cycle value, calculated as the mean number of

endoreduplication cycles per nucleus and thus an overall measure of endoreduplication (Barow

and Meister 2003), was calculated from the number of nuclei at each ploidy level for each

sample by the equation:

Cycle value = $(0 \bullet n_{2C} + 1 \bullet n_{4C} + 2 \bullet n_{8C} + 3 \bullet n_{16C}) / (n_{2C} + n_{4C} + n_{8C} + n_{16C})$

where the cycle value is the sum of the number of nuclei at each ploidy level multiplied by the

number of endocycles required to achieve each corresponding ploidy level, divided by the total

number of nuclei measured. Here we use the cycle value as a measure of endopolyploidy that

is directly comparable across genotypes that may differ slightly in their genome size (pg DNA /

2C nucleus).

3.3.4 Phenotypic measures

At the completion of senescence, 24 phenotypic characters were measured from the

remaining plants (60) of each accession (30 clipped, 30 unclipped; actual $n = 12 - 29$, average n

= 23). Phenotypic measures included the number and length of primary, secondary, and

tertiary stems, the length of each stem, and the number of siliques for each stem, inflorescence

dry biomass, and rosette diameter at 6 cm inflorescence height, among others (see Table 3.2

for a complete list). The most ultimate fitness measure used in this study is total seed number,

estimated for each plant by multiplying its total number of siliques by the average number of

seeds per silique for its respective genotype × treatment group (Col-4 unclipped, Col-4 clipped, Ler unclipped, Ler clipped).

3.3.5 Statistical analyses

Statistical analyses were conducted with SAS (v.9.2; Cary, North Carolina, USA). Comparisons between clipped and unclipped plants of each accession for seed yield and cycle values were performed by ANOVA followed by linear contrasts. To assess the relationship between endoreduplication and fitness compensation, the percent change cycle value (the mean value of clipped plants as a relative percent of unclipped plants) and the percent change of fitness measures were related by least squares linear regression.

To identify the phenotypic measures that are significantly related to fitness compensation following clipping, unclipped plants of each Columbia-family and global genotype were randomly paired to clipped plants of the same genotype with the same rosette diameter at 6 cm stem height to match plants for size (n = 10 – 19 pairs for each genotype). The percent changes (clipped vs. unclipped) of the 24 measures were calculated for each pair. The percent changes of 23 phenotypic characters (see Table 3.2) were related to the percent change total number of seeds for each pair via stepwise multiple regression, analyzing Columbia-family genotypes separately from globally-distributed ecotypes as the Columbia-family is considered a distinct subset of the wider global population.

To infer genetic relationships between genotypes, a phenogram of Columbia-family and globally-distributed genotypes was constructed based on the 24 phenotypic measures (see Table 3.2) in Mesquite (v.2.75, Maddison and Maddison 2011) using heuristic parsimony by

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subtree pruning and regrafting methods. Branch lengths were calculated in Mesquite to infer

relative genetic distance among genotypes.

To validate the assumption that genetic distance increases with geographic distance, the

total branch length separation was calculated pairwise for all global ecotypes and related to the

distance (km) between each pair of ecotypes' location of collection. Branch lengths between

each pair of ecotypes were summed from the terminal location of each ecotype through their

closest commonly-shared node. The geographic distance between each pair of global ecotypes

was estimated by Google Maps (Google Inc., Mountain View, California, USA) using a direct

estimation application (Google Maps Distance Calculator, Daft Logic,

http://www.daftlogic.com). A Mantel test (Mantel 1967) was conducted to test for a significant

relationship between phenotypic distance and geographic distance matrices in XLSTAT 2013

(Addinsoft, Paris, France) by Pearson correlation coefficient estimation with 10,000

permutations.

3.4 Results

3.4.1 Cycle values

The ANOVA of Columbia-family cycle values revealed significant line (i.e. genotype;

$F(9,179) = 3.75$, $p < 0.001$), clipping (i.e. unclipped, clipped; $F(1,179) = 37.24$, $p < 0.0001$), and

line × clipping effects ($F(9,179) = 4.19$, $p < 0.0001$). Linear contrasts following the overall

ANOVA indicated that cycle values of four of the Columbia-family genotypes (Col-4, CS1913,

1948, 1985) differed significantly between clipped and unclipped plants, each with an increase

in endoreduplication following clipping (Col-4: $t(179) = 4.65$, $p < 0.0001$; CS1913: $t(179) = 7.01$,

p < 0.0001; CS1948: *t*(179) = 5.47, *p* < 0.0001; CS1985: *t*(179) = 3.51, *p* < 0.001; Figure 3.2A).

There were no significant differences between cycle values of clipped and unclipped plants of

the remaining six Columbia-family genotypes (all *p* > 0.05, Figure 3.2A).

For globally-distributed ecotypes, the ANOVA of cycle values indicated a significant

effect of line (*F*(8,159) = 6.08, *p* < 0.0001) and a significant line × clipping interaction (*F*(8, 159) =

2.15, *p* < 0.05), but no significant effect of clipping (*F*(1,159) = 0.52, *p* = 0.4727). Cycle values of

two globally-distributed ecotypes (CS22652 and CS8020) were significantly greater for

unclipped plants than clipped plants (CS22652: *t*(159) = 2.04, *p* < 0.05; CS8020: *t*(159) = 2.31, *p*

< 0.05; Figure 3.2B), indicating a decrease in endoreduplication following clipping. Cycle values

for one global ecotype, CS28796, were marginally greater for clipped plants than unclipped

plants (*t*(159) = 1.7, *p* = 0.0913; Figure 3.2B). Cycle values for the remaining six global ecotypes

did not differ significantly between treatments (all *p* > 0.05, Figure 3.2B).

3.4.2 Fitness

The ANOVA of seed yield of Columbia-family genotypes indicated significant line

(*F*(9,380) = 149.33, *p* < 0.0001), clipping (*F*(1,380) = 184.00, *p* < 0.0001), and line × clipping

(*F*(9,380) = 187.03, *p* < 0.0001) effects. Total seed yield of five of the Columbia-family

genotypes (Col-4, CS1913, CS1941, CS1948, CS1985) was greater for clipped plants than

unclipped plants (Col-4: *t*(380) = 8.85, *p* < 0.0001; CS1913: *t*(380) = 23.24, *p* < 0.0001; CS1941:

t(380) = 5.24, *p* < 0.0001; CS1948: *t*(380) = 21.93, *p* < 0.0001; CS1985: *t*(380) = 17.34, *p* <

0.0001; Figure 3.3A), indicating overcompensation. Seed yield of three of the Columbia-family

genotypes (Ler, CS1968, CS1999) displayed a significant decrease for clipped plants relative to

unclipped controls (Ler: $t(380) = 11.26$, $p < 0.0001$; CS1968: $t(380) = 15.85$, $p < 0.0001$; CS1999: $t(380) = 7.79$, $p < 0.0001$; Figure 3.3A). There was no significant difference in seed yield between clipped and unclipped plants of the remaining two genotypes (all $p > 0.05$, Figure 3.3A).

The ANOVA of seed yield of globally-distributed ecotypes revealed a significant effect of line ($F(8,391) = 30.94$, $p < 0.0001$) and a significant line × clipping interaction ($F(8, 391) = 3.12$, $p < 0.01$), but no significant effect of clipping ($F(1,391) = 1.89$, $p = 0.1705$). Seed yield of one globally-distributed ecotype, CS1284, was significantly greater for clipped plants than unclipped plants ($t(391) = 3.53$, $p < 0.001$; Figure 3.3B). Seed yield of two global ecotypes (CS1564 and CS28850) differed marginally between clipped and unclipped plants, with each trending toward undercompensation (CS1564: $t(391) = 1.81$, $p = 0.0709$; CS28850: $t(391) = 1.79$, $p = 0.0741$; Figure 3.3B), with no other significant differences among global ecotypes in seed yield between treatments (all $p > 0.05$, Figure 3.3B).

The ANOVA of inflorescence dry biomass, a measure of overall plant size, of Columbia-family genotypes indicated significant effects of line ($F(9,380) = 387.81$, $p < 0.0001$), treatment ($F(1,380) = 1759.73$, $p < 0.0001$), and line × treatment ($F(9,380) = 171.91$, $p < 0.0001$). Specifically, inflorescence biomass was greater for clipped plants than unclipped plants for 9 of the 10 Columbia-family genotypes (Col-4, Ler, CS1906, CS1913, CS1941, CS1948, CS1985, CS1999: $p < 0.0001$; CS1942: $p < 0.01$), with one genotype, CS1968, displaying a significant reduction in inflorescence dry biomass upon clipping ($t(380) = 8.54$, $p < 0.0001$). Regression of the change in seed yield versus the change in inflorescence dry biomass upon clipping revealed a significant positive relationship ($F(1,8) = 19.89$, $p < 0.01$, $R^2 = 0.7132$). The biomass ANOVA of

globally-distributed ecotypes revealed a significant line effect ($F_{(8,389)}$ = 20.99, $p < 0.0001$), but no significant effect of clipping or a line × clipping interaction ($F_{(1,389)}$ = 0.001, p = 0.9543, and $F_{(8,389)}$ = 1.88, p = 0.0623, respectively). Inflorescence dry biomass did not differ significantly between clipped and unclipped plants for any of the globally-distributed ecotypes, yet there is a significant positive relationship between the change in seed number and the change in inflorescence dry biomass following clipping ($F_{(1,7)}$ = 13.96, $p < 0.01$, R^2 = 0.6661). The relationship between the change in seed number and the change in inflorescence biomass following clipping is also significantly positive for Columbia-family genotypes and globally-distributed ecotypes together ($F_{(1,17)}$ = 36.94, $p < 0.0001$, R^2 = 0.6848).

3.4.3 Cycle values × fitness measures

There is a significant positive relationship between the percent change cycle value and the percent change seed yield following clipping for the Columbia-family genotypes ($F_{(1,8)}$ = 60.79, $p < 0.0001$, R^2 = 0.8837), but there is no significant relationship for the globally-distributed ecotypes ($F_{(1,7)}$ = 0.2, p = 0.667, R^2 = 0.028). There is a significant positive relationship across all the genotypes studied ($F_{(1,17)}$ = 17.54, $p < 0.001$, R^2 = 0.5078; Figure 3.4A).

There is a significant positive relationship between the change in cycle value and the change in inflorescence dry biomass for the Columbia-family genotypes ($F_{(1,8)}$ = 10.54, $p <0.05$, R^2 = 0.5685), but not for the globally-distributed ecotypes ($F_{(1,7)}$ = 0.05, p = 0.8303, R^2 = 0.007). There is, however, a significant positive relationship across all genotypes ($F_{(1,17)}$ = 13.34, $p < 0.01$, R^2 = 0.4397; Figure 3.4B).

3.4.4 Phenotype analysis

Stepwise multiple regression for the Columbia-family genotypes determined that seven of 23 phenotypic characters were significantly related to the percent change in total seed yield (Table 3.2), with a positive relationship with the change in total number of siliques most significant among them ($F_{(1,155)} = 238.45$, $p < 0.0001$). The total number of stems ($F_{(1,155)} = 11.31$, $p < 0.001$), above-ground dry biomass ($F_{(1,155)} = 7.88$, $p < 0.01$), average number of siliques on secondary stems ($F_{(1,155)} = 13.69$, $p < 0.001$), and total number of siliques on primary stems ($F_{(1,155)} = 74.99$, $p < 0.0001$) were also significantly positively related to compensation in the Columbia-family. The total length of all stems and the total number of secondary stems were negatively related ($F_{(1,155)} = 41.85$, $p < 0.0001$, and $F_{(1,155)} = 12.37$, $p < 0.001$, respectively; Table 3.2). These seven factors combined to explain nearly 97% of the variation in fitness compensation in the Columbia-family of genotypes ($F_{(7,155)} = 653.55$, $p < 0.0001$, adjusted $R^2 = 0.9657$).

Five phenotypic characters were related to the percent change in total seed yield of the globally-distributed ecotypes (Table 3.2), with a positive relationship with the percent change in total number of siliques most significant among them ($F_{(1,111)} = 1967.6$, $p < 0.0001$). Also significantly positively related to compensation were the total length of all secondary stems ($F_{(1,111)} = 22.88$, $p < 0.0001$), the total number of tertiary stems ($F_{(1,111)} = 16.06$, $p < 0.0001$), and the total number of tertiary siliques ($F_{(1,111)} = 8.00$, $p < 0.01$, Table 3.2). The total length of all tertiary stems was significantly negatively related to fitness compensation ($F_{(1,111)} = 13.17$, $p < 0.001$, Table 3.2). These four characters explained nearly 98% of the variation in

fitness compensation in the globally-distributed ecotypes ($F(5,111) = 1097.78$, $p < 0.0001$,

adjusted $R^2 = 0.9793$).

Genetic relatedness among the Columbia-family and globally-distributed genotypes was

inferred via phenogram (Figure 3.5). Columbia-family genotypes form a monophyletic group

with globally-distributed ecotypes basally related (Figure 3.5). There is no evidence of

phenotypic grouping of globally-distributed ecotypes by geographic location, nor by

compensatory or cycle value responses to herbivory, although Columbia-family genotypes with

increased seed yield and cycle value following clipping generally group together (Figure 3.5).

The Mantel test, which relates phenotypic distance (inferred from phenogram branch lengths)

and geographic distance between all pairs of globally-distributed ecotypes, revealed no

significant relationship between the two measures ($r = -0.037$, $p = 0.835$, Figure 3.6).

3.5 Discussion

Here we show that the correlation persists despite the recombination of the Col-4 and

Ler haplotypes—higher levels of endoreduplication of recombinant inbred lines are positively

correlated with greater biomass and seed production following damage. We additionally report

that natural ecotypes, encompassing wide geographic, and likely genetic (though not significant

by our estimates, Figure 3.6; but see also Sharbel et al. 2000), variation show similar rates of

endoreduplication as the recombinant inbred lines, although without sufficient variation in

endopolyploidy and fitness compensation to definitively conclude a relationship in these

genotypes.

The positive relationship between endoreduplication and compensation of Columbia-4 and Landsberg *erecta* is retained in their offspring suggests endoreduplication influences the regrowth of these genotypes. Because the parental lines are homozygous at all genetic loci, the recombinant inbred lines formed from their crossing have at most two alleles per locus among them, although most loci are likely fixed among parental lines due to their common ancestry from the Laibach Landsberg population (The Nottingham Arabidopsis Stock Centre, http://www.arabidopsis.info). Despite starting with only two genotypes, estimates of nuclear DNA content and fitness measures of the Col-family genotypes show wide variation around the parental values (Figures 3.2A and 3.3A). This suggests, as expected, that fitness and cell cycle regulation are polygenic traits, and the quantitative inheritance demonstrated here is a testament to the genetic complexity of each pathway. The pathways do not appear to operate independently, however, since the recombination of the parental haplotypes should break up any chance association between the genes important for endoreduplication and fitness compensation. Retaining a positive association despite recombination suggests that the action of one set of genes is dependent on the other, and the endoreduplication pathway is therefore directly integrated within the framework of rapid regrowth and fitness compensation following herbivory.

We are not able to make a definitive conclusion to the relationship between endoreduplication and compensation in the globally-distributed ecotypes. We emphasize that the global ecotypes occupy the low range of both endoreduplication and compensation relative to the recombinant inbred lines (Figure 3.4A), and thus greater variation in both measures, and particularly overcompensators, would need to be sampled to make a definitive conclusion to

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the significance of the relationship in these natural ecotypes. The pattern observed here is not

entirely unexpected, however—the vast majority of recombinant inbred lines, and likely natural

ecotypes as well, are equal compensators with little change in fitness or endopolyploidy after

herbivory (Siddappaji et al., unpublished). Because natural ecotypes were selected almost

entirely based on their country of origin, with little *a priori* information on the compensatory

capability of the plants or levels of endopolyploidy, it was expected that most genotypes would

display little change in either measure, as seen here. The lack of an observed relationship

however does not eliminate the possibility of one with greater sampling, given that the global

ecotypes fit within the range of points produced by the Columbia-family genotypes (see Figure

3.4A).

Overall, these results indicate that the positive relationship between endoreduplication

and fitness compensation following herbivory is generalizable at least to the Columbia-family of

genotypes (Col-4, Landsberg *erecta*, and their offspring). While the role of endoreduplication in

compensation has only recently been investigated (see Scholes and Paige 2011), the

generalized role of endoreduplication in the rapid production of plant tissue has been described

previously. Plant growth primarily occurs via two processes: cell division and cell expansion.

While proliferative cell divisions increase size through increasing cell number, cell expansion

can increase size via two distinct mechanisms. In most plant tissues, particularly in those

species that do not systemically endoreduplicate, cell expansion is achieved by acid-growth

(Hager et al. 1971, Rayle and Cleland 1992), where auxin-mediated movement of hydrogen ions

to the extracellular space helps temporarily degrade the cell wall matrix. The cell may then

import water into the vacuole, causing an increase in cell volume before the acidic extracellular

space is neutralized and the cell wall regains rigidity. In the absence of adequate water, as for

many succulents (de Rocher et al. 1990), or in plants that endoreduplicate systemically, like

Arabidopsis thaliana (Galbraith et al. 1991), endoreduplication can provide an efficient means

of increasing cellular volume to facilitate growth. Growth by endoreduplication-mediated cell

expansion is particularly important following herbivory since the removal of the apical

meristem eliminates the primary site of auxin production and reduces the potential of auxin-

mediated acid growth of the inflorescence (Rayle and Cleland 1992). Upon clipping, there was

a significant relationship between the change in cycle value and the change in inflorescence dry

biomass for the Columbia-family genotypes. This relationship suggests that endoreduplication

was important in the generation of tissue generally, as *A. thaliana* endoreduplicates

systemically (Galbraith et al. 1991), as well as in the specific tissues promoting fitness through

seed yield (e.g. the suspensor cells; Nagl 1978, Barow 2006). The global ecotypes showed no

significant relationships between endoreduplication and seed yield or biomass, but they also

showed little variation in fitness compensation and so clear relationships are not easily

observable. Both the Columbia-family and the global ecotypes showed significant positive

relationships between fitness compensation and biomass regeneration by regression of these

measures, however, and the increase in inflorescence dry biomass upon clipping was identified

as significantly related to compensation in the Columbia-family by stepwise regression of

numerous phenotypic characters (Table 3.2). These results indicate that those genotypes that

could efficiently regenerate biomass after clipping could also effectively increase seed yield

beyond unclipped controls, such that a process that is involved in both processes, like

endoreduplication, could be an important component of the generalized response to the release of apical dominance.

The reduction in auxin additionally releases lateral buds from dormancy (Thimann and Skoog 1933, Sachs and Thimann 1967), in part by eliminating auxin-mediated inhibition of endoreduplication (Ishida et al. 2010). The growth of numerous lateral buds following herbivory, and thus endopolyploidy, may be important in plants' responses to damage (see Scholes and Paige 2011). By similar means, endoreduplication has been linked to accelerated growth in response to light stress. The etiolated seedlings of numerous species, including *Glycine max*, *Pisum sativum*, and *Arabidopsis thaliana*, among others, endoreduplicate as part of the rapid elongation of the hypocotyl to break the soil surface (van Oostveldt and van Parijs 1975, Galli 1988, Gendreau et al. 1998, Barow 2006). In dark-grown hypocotyls of *A. thaliana*, auxin is nearly absent (Bhalerao et al. 2002, Halliday et al. 2009), promoting endoreduplication by the mechanisms previously discussed. The importance of endoreduplication in the rapid growth and development of plants is also evident by the commonality of the process among herbaceous angiosperms (Barow and Meister 2003), where their annual or biennial life history strategies necessitate development through a short growing season. Endoreduplication, stimulated by integrated physiological and genetic pathways, thus provides a generalized mechanism for the rapid generation of tissue, as the results of this study suggest.

The rapid generation of tissue following herbivory is ultimately critical to the generation of fitness, and our results indicate seed yield of unclipped plants is significantly influenced by a number of phenotypic characters. In both the Columbia-family and global genotypes, the total number of siliques was overwhelmingly the most significant driver of whole-plant fitness, with

an assortment of other measures playing minor, though significant, roles (Table 3.2). The

variety of characters deemed influential by these analyses indicates a variety of ways in which

plants may ultimately generate fitness—compensation is positively related to a combination of

increases in the total number and lengths of secondary and tertiary stems, and the average and

total number of siliques on primary, secondary, and tertiary stems (Table 3.2), though few

genotypes are likely able to maximize each of these characters simultaneously. Four of the

seven characters deemed significantly related to compensation in the Columbia-family

genotypes are whole plant measures—total number of stems, total length of all stems, total

number of siliques, mass of all inflorescence dry tissue—while significant characters of globally-

distributed ecotypes largely consisted of a variety of secondary and tertiary stem measures.

This in part could be a due to the relatively wide range of fitness compensation observed in the

Columbia-family, such that true relationships could be determined, while those of the globally-

distributed ecotypes could be truly important or merely artifactual given the low variation in

compensation observed. Analysis of these characters in unclipped plants, however, confirmed

that the Columbia-family and globally-distributed genotypes represent distinct groups (Figure

3.5), and thus may have truly different compensatory strategies as well. While the Columbia-

family genotypes group together via analysis of these phenotypic characters, as expected, the

globally-distributed ecotypes show no clear grouping by geographic region (Figure 3.5), nor via

genetic distance and geographic Mantel correlation (Figure 3.6), possibly due to the very high

rates of self-fertilization of *A. thaliana* naturally (Abbott and Gomes 1987), their lab-

propagation to a fully-homozygous state before analysis, or simply their great divergence

and/or localization over this wide geographic range such that geographic patterns have been

lost. Given this, it is not surprising that a wide variety of characters were determined to

influence compensation in seed yield—a wide variety of genotypes would be expected to

employ a wide variety of strategies to maximize their fitness under their local conditions.

Endoreduplication may be directly involved in supporting plant fitness via roles in

endosperm development and seed filling. The endosperm is the nutritive tissue surrounding

the embryo and is thus critically important in the germination and initial growth of the embryo.

Endoreduplication is suspected to support endosperm development in numerous ways,

including providing increased cell volume for nutrient storage, enhancing rates of nutrient

transport, and serving as a nutritive process itself—endoreduplication of the endosperm

provides a great resource of nucleotides, proteins, and their derivative components to the

embryo for development (Barow 2006). Engelen-Eigles et al. (2001) and Lee et al. (2009)

suggested this role of endoreduplication is particularly important under high temperature or

drought conditions, in which rates of endosperm endoreduplication are greatly reduced and the

endosperm thus is ill-suited to nourish the developing embryo. Rates of endoreduplication are

also particularly high in suspensor cells, which connect the developing embryo to the

surrounding endosperm (Barow 2006). The suspensor thus serves as the channel through

which nutrients must pass for transfer to the embryo; endoreduplication enhances the rate of

nutrient and water transfer by providing greater volume for intracellular transport rather than

cell-to-cell transport through narrow plasmodesmata (Barow 2006). Among the Columbia-

family and globally-distributed genotypes, the differences between unclipped and clipped

plants in their total number of primary, secondary, tertiary, and whole-plant siliques were

deemed significantly positively related to fitness compensation (Table 3.2)—certainly the

number of siliques produced by a plant is important for fitness as the site of seed production and maturation, and perhaps the levels of endoreduplication in the siliques is an important factor in seed production upon clipping damage, as our results here suggest.

These results collectively suggest that endoreduplication is a generalized facilitator of regrowth and fitness compensation following herbivory. In order to understand the generality of the relationship between endoreduplication and compensation observed here, an understanding of the links between the genetic pathways of each process is needed. Work in this area is currently underway, with recent advances in understanding the molecular basis of overcompensation in *A. thaliana* (Siddappaji et al., unpublished). Specifically, a glucose-6-phosphate dehydrogenase has been shown to play important roles in both pathways, contributing to increased plant metabolism after clipping through the oxidative pentose phosphate pathway. This pathway also supplies raw materials for nucleotide synthesis (Kruger and von Schaewen 2003), which may support the drastic increase in nuclear ploidy by endoreduplication during plant regrowth. Further work may target genes of large effect in the compensation and/or endoreduplication pathways to better elucidate the integration of these gene pathways. Given the results of this study, and the various roles endoreduplication plays in the growth, development, and fitness of most angiosperms, endoreduplication appears to be an important process in the generalized stress response to herbivory.

CHAPTER 4:
IS ENDOREDUPLICATION RELATED TO FITNESS COMPENSATION FOLLOWING HERBIVORY IN *ARABIDOPSIS THALIANA* CELL-CYCLE MUTANT LINES?

4.1 Abstract

Endoreduplication, the replication of the genome without mitosis that increases genome-copy number of the cell (generating endopolyploidy), is suggested to be directly related to plant regrowth and fitness compensation following damage by herbivory (Chapters 2 and 3 of this dissertation; Scholes and Paige 2011). However, the mechanistic basis of this relationship remains unknown. In this study, we investigate the direct causal relationship between endoreduplication and fitness compensation following herbivory in *A. thaliana* Columbia. We grew knockdown and overexpression mutants of an endoreduplication regulator, *INCREASED LEVEL OF POLYPLOIDY1* (*ILP1*), and the wildtype Col-0 and clipped half of the plants of each type upon bolting. To test for nutritive limitation of endoreduplication and compensation in these genotypes, we also administered a phosphorus-fertilization treatment. For each genotype × clipping × fertilization combination, we measured endopolyploidy of the above-ground tissue at the induction of senescence and seed yield after senescence. We found that Col-0 showed a decrease in both endopolyploidy and fitness compensation following clipping, but that Col-0 experienced no changes in either measure upon clipping when phosphorus fertilized. The knockdown and overexpression mutants also experienced no changes in either measure following clipping, regardless of phosphorus fertilization, though total seed yield of each genotype increased with fertilization. The gene manipulation lines did, however, show unintended effects on endoreduplication, as the *ILP1* knockout line had greater

endopolyploidy relative to the Col-0 wildtype and the *ILP1* overexpression mutant did not have

an increase in endopolyploidy as expected. The gene mutants also give indications of a lack of

plasticity in the endopolyploidy response to clipping but without a clear explanation for the

unintended ploidy effects. These results shed light on the interaction of gene regulation, apical

damage, and nutrient availability while providing opportunities for further mechanistic

investigation in the future.

4.2 Introduction

Overcompensation is the increase in reproductive output following exposure to some

stress. The first clear evidence of overcompensation was demonstrated in *Ipomopsis aggregata*

in response to herbivory, where a 3-fold increase in seed yield was observed following the

removal of 95% of the total above-ground biomass of the flowering biennial (Paige and

Whitham 1987). While initially controversial, these results have been confirmed and numerous

studies in other systems have shown similar results, providing substantial evidence that

overcompensation does indeed occur in certain situations (e.g. Paige and Whitham 1987,

Maschinski and Whitham 1989, Nilsson et al. 1996, Lennartson et al. 1997, Lowenberg 1994,

Hougen-Eitzman and Rausher 1994, Mauricio et al. 1997, Weinig et al. 2003), although there is

a strong genotype × environment interaction (namely the genotype interacting with the

intensity and frequency of herbivory) that typically restricts overcompensation to certain

populations of a given species. While the nature of herbivory is arguably the most important

environmental factor influencing a genotype's potential for overcompensation, numerous

studies have determined a strong influence of the abiotic environment on compensatory ability

as well. For example, *I. aggregata* plants that typically demonstrate overcompensation have a reduced compensatory response (but still overcompensate) when grown in soils with low nitrogen and phosphorus, although experimental fertilization with these nutrients can improve the magnitude of fitness compensation of these plants following herbivory (Scott and Paige, unpublished; see Maschinski and Whitham 1989, Wise and Abrahamson 2008 for reviews). Water relations also significantly impact the plant-herbivore interaction, where drought conditions change the nature of herbivory and result in a drastic reduction in fitness of browsed plants (Levine and Paige 2004).

While the ecology of overcompensation has been studied in much depth, the genetic basis of overcompensation has only begun to be elucidated. Studies by Siddappaji et al. (unpublished), using transcriptomic, quantitative trait loci mapping, and candidate gene knockout and complementation methodologies with *Arabidopsis thaliana*, uncovered one key gene with strong evidence supporting its role in influencing compensation as well as over 100 others that are overexpressed in experimentally clipped plants. Major questions in overcompensation research remain, including additional genes and perhaps additional gene pathways that underlie compensatory ability and the interaction of these genes with their biotic and abiotic environments.

The interaction between compensation and one particular gene pathway, cell cycle regulation, have found correlative evidence suggesting a relationship (Scholes and Paige 2011; Chapters 2 and 3 of this dissertation). Endoreduplication is the process by which nuclear ploidy is increased by repeated genome replication without mitosis, leading to a variety of somatic ploidy levels within an individual organism (termed endopolyploidy)(Lee et al. 2009).

Specifically, Scholes and Paige (2011) demonstrated for the first time that the induction of endopolyploidy following experimental clipping is significantly related to fitness compensation following herbivory, where clipped plants of an overcompensating genotype (Col-4) experience a significant increase in endopolyploidy relative to unclipped controls during their regrowth, while clipped plants of a genetically-related undercompensating genotype (Ler) illicit no such increase. This pattern appears to be generalizable, at least among this family of genotypes, as offspring from a Col-4 × Ler mating demonstrate a wide range of compensation and endopolyploidy with a positive relationship between them (see Chapter 3 of this dissertation). These studies suggest the direct role of endoreduplication in regrowth and compensation following herbivory, although a direct test of causality has not yet been conducted.

In this study, we investigated the direct causal relationship between endoreduplication and fitness compensation following herbivory in *A. thaliana* Columbia. Specifically, we determined if the experimental manipulation of endopolyploidy, achieved by overexpressing and down-regulating a key endocycle gene, causes increases and decreases, respectively, in compensatory capability following the removal of apical dominance by simulated herbivory. Additionally, we tested whether the addition of phosphorus, a major component of DNA and often limiting in natural soils, promotes increased endopolyploidy and compensation following clipping in these genotypes. Results that confirm the predicted patterns would confirm the direct and causal relationship between increased endopolyploidy and increased fitness following herbivory in *A. thaliana* Columbia, providing a partial explanation for the phenomenon of overcompensation in these plants.

4.3 Materials and Methods

4.3.1 Genetic lines

To investigate the direct relationship between fitness compensation and the change in endopolyploidy following herbivory, we selected cell-cycle mutant lines with modified levels of endopolyploidy to determine the effect on compensation. Specifically, we targeted *INCREASED LEVEL OF POLYPLOIDY1* (*ILP1*; At5G08550), a transcriptional repressor of the cyclin A2 (CYCA2) family of cyclins, which collectively inhibit the entry into the DNA replication phase of the endocycle. An *ILP1* reduced expression line was obtained from The Arabidopsis Information Resource Center (http://www.arabidopsis.org; SALK_030650), created from a T-DNA insertion in the fifth intron of *ILP1* on a Columbia-0 (Col-0; TAIR accession CS70000) genetic background. An *ILP1* increased expression line was obtained from the lab of Minami Matsui at RIKEN (Plant Functional Genomics Research Group, Plant Science Center), created from the addition of a *CaMV 35S* promoter to *ILP1* on a Col-0 genetic background as described by Yoshizumi et al. (2006). Col-0 was also selected for analysis, and we note that it is related to, yet genetically distinct from, the Columbia-4 (CS933) accession used in our previous studies (Scholes and Paige 2011).

4.3.2 Phosphorus fertilization

All plants were grown in 4" diameter pots of LC1 Sunshine potting mix (SunGro Horticulture Inc., Bellevue, Washington, USA). Before planting, potting mix nutrient profiles of control (LC1) and phosphorus fertilized (+P) media were determined by the saturated paste extract method (U.S. Salinity Laboratory Staff 1954). Phosphorus content of LC1 measured 14.3

ppm. Pelletized triple superphosphate (P_2O_5, NPK: 0-45-0) was ground to a powder for even

application and was amended into the LC1 potting mix at a rate of approximately 1.2 g triple

superphosphate per 1 liter of LC1 potting mix. Phosphorus content of +P media measured 26.4

ppm, nearly doubling the phosphorus content relative to LC1. We note that the pH of both LC1

and +P mixes measured 6.5, which represents a mix of inorganic phosphate in the forms of

H_2PO4^- and HPO_4^{2-}, the two forms most available for plant uptake (Schachtman et al. 1998).

4.3.3 Growth and experimental clipping

To assess the relationship between plant fitness compensation and the change in

endopolyploidy following herbivory, we planted 140 individuals each of Columbia-0, *ilp1-1*, and

ILP1-ox (70 LC1, 70 +P) and grew them under greenhouse conditions. At approximately 3 weeks

after planting, prior to bolting, 20 rosettes of each line (10 LC1, 10 +P) were harvested and

analyzed for nuclear DNA content to establish baseline endopolyploidy. When inflorescences

reached 6 cm (~4 weeks), the inflorescences of 60 plants of each line (30 LC1, 30 +P) were

clipped, such that approximately 1 cm of inflorescence tissue remained, simulating natural

mammalian herbivory. At the induction of senescence (~8 weeks after planting), 40 plants of

each line (10 LC1 clipped, 10 LC1 unclipped, 10 +P clipped, 10 +P unclipped) were analyzed for

nuclear DNA content.

4.3.4 Cytometric analysis

Tissue for flow cytometric analysis was prepared via standard protocols (see Galbraith et

al. 1983). In brief, plant tissue was chopped with a razor blade, matched for tissue type (main

stems, lateral stems, leaves, etc.) and biomass, sheared in a nuclear isolation buffer (sodium

citrate, MOPS, magnesium chloride, Triton X-100; Galbraith et al. 1983), filtered for debris

removal, and stained with propidium iodide. Suspended nuclei were analyzed via a BD

Biosciences FACScanto flow cytometer for measurement of nuclear DNA content. Background

correction and nuclei population gating were performed using De Novo Software FCS Express

(v.3; Los Angeles, California, USA) to measure the proportion of nuclei at each ploidy level (2C,

4C, 8C, 16C) per plant sample. The cycle value, calculated as the mean number of

endoreduplication cycles per nucleus and thus an overall measure of endoreduplication, was

calculated from the number of nuclei at each ploidy level for each flow cytometric sample by

the equation described by Barow and Meister (2003):

Cycle value = $(0 \bullet n_{2C} + 1 \bullet n_{4C} + 2 \bullet n_{8C} + 3 \bullet n_{16C}) / (n_{2C} + n_{4C} + n_{8C} + n_{16C})$

where the cycle value is the sum of the number of nuclei at each ploidy level multiplied by the

number of endocycles required to achieve each corresponding ploidy level, divided by the total

number of nuclei measured.

4.3.5 Fitness measures

Upon senescence (~8 weeks), the remaining plants (20) of each treatment were

analyzed for fitness. Fitness measures included the number of siliques, the number of seeds

per silique, and inflorescence dry biomass at senescence. The primary fitness measurement

examined was the total number of seeds, estimated for each plant by multiplying the number

of siliques by the average number of seeds per silique for the corresponding genotype ×

clipping × fertilization combination.

4.3.6 Statistical analysis

Statistical analyses were conducted with SAS (v.9.2; Cary, North Carolina, USA) to test

for effects on seed number, above-ground dry biomass, and cycle value. Effects of line (Col-0,

ilp1-1, *ILP1*-ox), clipping (unclipped, clipped), phosphorus fertilization (LC1, +P), and their

interactions were analyzed by ANOVA. Specific comparisons between combinations of line,

clipping treatment, and phosphorus fertilizaton for fitness measures and cycle values were

made via linear contrasts. The percent in change cycle value (the mean value of clipped plants

as a relative percent of unclipped plants) and the percent change of seed number and

inflorescence dry biomass were related by least squares linear regression.

4.4 Results

4.4.1 Cycle values

The ANOVA of cycle values revealed a significant line effect (*ilp1-1* vs. Col-0, vs. *ILP1*-ox;

$F(2,108) = 54.96$, $p < 0.0001$), but only marginally significant ($p < 0.1$) overall effects of clipping

and line × clipping interaction ($F(1,108) = 2.78$, $p = 0.0986$, and $F(2,108) = 2.72$, $p = 0.0703$,

respectively). When grown in the control potting mix (LC1), cycle values of unclipped plants

were greatest for *ilp1-1*, the *ILP1* knockout mutant, with no difference between Col-0 and *ILP1*-

ox (LC1 unclipped *ilp1-1* > Col-0 = *ILP1*-ox; *ilp1-1* vs. Col-0: $t(108) = 3.7$, $p < 0.001$; *ilp1-1* vs.

ILP1-ox: $t(108) = 5.35$, $p < 0.0001$; Col-0 vs. *ILP1*-ox: $t(108) = 1.65$, $p = 0.1009$). Cycle values

were significantly greater for unclipped Col-0 plants than clipped plants ($t(108) = 2.61$, $p < 0.05$; Figure 4.1A), but did not differ between treatments of *ilp1-1* or *ILP1*-ox plants ($t(108) = 0.17$, $p = 0.8625$, and $t(108) = 0.22$, $p = 0.8286$, respectively; Figure 4.1A).

There was a significant effect of phosphorus fertilization on cycle value ($F(1,108) = 10.31$, $p < 0.01$), but without significant line × phosphorus ($F(2,108) = 1.13$, $p = 0.3278$), clipping × phosphorus ($F(1,108) = 0.62$, $p = 0.4344$), or line × clipping × phosphorus ($F(2,108) = 0.49$, $p = 0.6133$) interaction effects. Specifically, cycle values were reduced at marginal significance upon phosphorus fertilization of unclipped Col-0 and *ilp1-1* plants relative to unclipped plants grown in LC1 potting mix (LC1 unclipped vs. +P unclipped; Col-0: $t(108) = 1.83$, $p = 0.0702$; *ilp1-1*: $t(108) = 1.87$, $p = 0.0638$), but did not differ upon fertilization of unclipped *ILP1*-ox plants ($t(108) = 1.19$; $p = 0.2357$). Cycle values of clipped *ilp1-1* plants were significantly reduced upon phosphorus addition ($t(108) = 2.37$, $p < 0.05$), with no significant change for Col-0 or *ILP1*-ox clipped plants ($t(108) = 0.51$, $p = 0.6077$, and $t(108) = 0.09$, $p = 0.2357$). Overall, the mean change of DNA content between clipping treatments was decreased for Col-0 but increased in *ilp1-1* and *ILP1*-ox (by absolute value, Figure 4.2A) when phosphorus fertilized relative to when unfertilized. Additionally, cycle values did not differ between fertilized clipped and unclipped plants of any of the genotypes (Col-0: $t(108) = 1.3$, $p = 0.1974$; *ilp1-1*: $t(108) = 0.67$, $p = 0.5035$; *ILP1*-ox: $t(108) = 0.89$, $p = 0.3761$; Figure 4.1B).

4.4.2 Fitness measures

The ANOVA of seed yield revealed significant line and clipping effects ($F(2,212) = 107.93$, $p < 0.0001$, and $F(1,212) = 3.89$, $p < 0.05$, respectively), but no significant line × clipping

interaction ($F(2,212) = 2.61$, $p = 0.0761$). Among LC1-grown unclipped plants, seed yield was greatest for Col-0, followed by *ILP1*-ox, and *ilp1-1* with the lowest seed yield (LC1 unclipped Col-0 > *ILP1*-ox > *ilp1-1*; Col-0 vs. *ILP1*-ox: $t(212) = 2.21$, $p < 0.05$; Col-0 vs. *ilp1-1*: $t(212) = 8.5$, $p < 0.0001$; *ILP1*-ox vs. *ilp1-1*: $t(212) = 6.33$, $p < 0.0001$; Figure 4.3A). Seed yield was significantly greater for unclipped Col-0 plants than clipped plants ($t(212) = 3.85$, $p < 0.001$), but did not differ between treatments of *ilp1-1* or *ILP1*-ox plants ($t(212) = 0.81$, $p = 0.4179$, and $t(212) = 0.47$, $p = 0.6378$, respectively; Figure 4.3A).

We found a significant effect of phosphorus addition on seed yield ($F(1,212) = 39.61$, $p < 0.0001$) and a significant clipping × phosphorus interaction ($F(1,212) = 4.84$, $p < 0.05$), a marginally significant line × phosphorus effect ($F(2,212) = 2.63$, $p = 0.0744$), and no significant line × clipping × phosphorus interaction effect ($F(2,212) = 1.40$, $p = 0.2501$). Specifically, seed yield of fertilized unclipped *ilp1-1* plants was significantly greater than LC1-grown plants ($t(212) = 2.74$, $p < 0.01$), but no such differences were found for Col-0 or *ILP1*-ox ($t(212) = 1.62$, $p = 0.106$, and $t(212) = 0.75$, $p = 0.4542$, respectively). Fertilized clipped plants of all genotypes had significantly greater seed yield than LC1-grown clipped plants (Col-0: $t(212) = 4.99$, $p < 0.0001$; *ilp1-1*: $t(212) = 2.66$, $p < 0.01$; *ILP1*-ox: $t(212) = 2.35$, $p < 0.05$). Overall, the compensatory response improved substantially for Col-0, *ilp1-1*, and *ILP1*-ox when fertilized, with a change from significant undercompensation for Col-0 when LC1-grown to nearly no difference in seed yield between treatments when fertilized (Figure 4.2B). Upon clipping, seed yield of phosphorus fertilized plants of all genotypes did not differ from unclipped controls (Col-0: $t(212) = 0.07$, $p = 0.9471$; *ilp1-1*: $t(212) = 0.54$, $p = 0.5866$; *ILP1*-ox: $t(212) = 1.05$, $p = 0.2954$; Figure 4.3B).

The ANOVA of inflorescence dry biomass, a measure of overall plant size, revealed a significant line effect ($F_{(2,212)} = 29.45$, $p < 0.0001$), no significant clipping effect ($F_{(1,212)} = 0.32$, $p = 0.5696$), but a marginally significant line × clipping interaction ($F_{(1,212)} = 2.83$, $p = 0.0611$). Specifically, biomass of LC1-grown unclipped plants was greatest for Col-0, followed by *ILP1*-ox, and finally *ilp1-1* (Col-0 > *ILP1*-ox > *ilp1-1*; Col-0 vs. *ILP1*-ox: $t_{(212)} = 2.70$, $p < 0.01$; *ILP1*-ox vs. *ilp1-1*: $t_{(212)} = 4.00$, $p < 0.0001$; Col-0 vs. *ilp1-1*: $t_{(212)} = 6.57$, $p < 0.0001$; Figure 4.4A). Furthermore, biomass was significantly greater for unclipped plants than clipped plants for Columbia-0 when grown in LC1 control potting mix, ($t_{(212)} = 3.97$, $p < 0.0001$), but did not differ between treatments for *ilp1-1* and *ILP1*-ox plants (both $p > 0.05$; Figure 4.4A), nor for any genotype grown in phosphorus fertilized potting mix (all $p > 0.05$; Figures 4.2C, 4.4B). Further, there was a significant effect of phosphorus fertilization on inflorescence dry biomass ($F_{(1,212)} = 17.99$, $p < 0.0001$) and a significant clipping × phosphorus interaction ($F_{(1,212)} = 4.97$, $p < 0.05$), with significant increases in biomass upon fertilization of Columbia-0 clipped plants ($t_{(212)} = 3.36$, $p < 0.001$) and *ilp1-1* clipped and unclipped plants ($t_{(212)} = 2.58$, $p < 0.05$, and $t_{(212)} = 2.66$, $p < 0.01$, respectively), relative to their unfertilized controls. There was a marginally significant line × clipping × phosphorus effect ($F_{(2,212)} = 2.91$, $p = 0.0566$).

We report no significant relationship between the percent change in seed yield and the percent change in inflorescence dry biomass following clipping of Col-0, *ilp1-1*, and *ILP1*-ox ($F_{(1,4)} = 0.69$, $p = 0.4516$, $R^2 = 0.1479$), and a significant positive relationship when combined with the Columbia-family and global genotypes ($F_{(1,23)} = 21.7$, $p < 0.0001$, $R^2 = 0.4855$; see Chapter 3 of this dissertation).

4.4.3 Cycle values × fitness measures

There is no significant relationship between the percent change in cycle value and the percent change in seed yield following clipping for Col-0, *ilp1-1*, and *ILP1*-ox grown in LC1 and +P potting mix ($F(1,4) = 0.69$, $p = 0.4516$, $R^2 = 0.1479$), though the significant positive relationship of the Columbia-family and globally-distributed ecotypes (from Chapter 3 of this dissertation; $F(1,17) = 17.54$, $p < 0.001$, $R^2 = 0.5078$) is strengthened upon inclusion of Col-0 and the *ILP1* mutant genotypes grown in LC1 and +P potting mix ($F(1,23) = 24.04$, $p < 0.0001$, $R^2 = 0.5111$; Figure 4.5A).

Additionally, there is a marginally significant positive relationship between the change in cycle value and the change in inflorescence biomass following clipping of LC1- and +P-grown Col-0, *ilp1-1*, and *ILP1*-ox plants ($F(1,4) = 5.05$, $p = 0.0880$, $R^2 = 0.5579$). We note that this marginal relationship is driven by the Col-0 wildtype (see Figure 4.5B). Overall, inclusion of these genotypes strengthens the relationship present among the Columbia-family and global genotypes ($F(1,23) = 21.7$, $p < 0.0001$, $R^2 = 0.4855$; Figure 4.5B, also see Chapter 3 of this dissertation).

4.5 Discussion

We examined if endoreduplication is causally involved in fitness compensation under simulated herbivory. By directly manipulating the expression of an endocycle regulator, *INCREASED LEVEL OF POLYPLOIDY1* (*ILP1*), we sought to experimentally modify the degree to which an *A. thaliana* genotype endoreduplicated to measure the manipulation's effect on fitness compensation following apical damage. We additionally sought to determine the effect

of phosphorus fertilization on endoreduplication and fitness compensation given that phosphorus is an important component of a wide range of molecules, including DNA, and is often limiting in natural soils (Schachtman et al. 1998). The wildtype, Columbia-0, experienced significant decreases in seed yield, inflorescence biomass, and endopolyploidy following clipping, as expected if endoreduplication is related to compensation to clipping damage. Columbia-0 therefore undercompensates, in contrast to the Columbia-4 genotype studied previously (Scholes and Paige 2011, Chapters 2 and 3 of this dissertation). We also conclude that phosphorus fertilization had a positive effect on the compensation of Col-0. Col-0 undercompensates when unfertilized but changes to equal compensation with supplementary phosphorus (Figures 4.2B, 4.3). Phosphorus fertilization also increased the relative level of endopolyploidy between unclipped and clipped Col-0 plants, such that the significant reduction in cycle value upon clipping of LC1-grown plants was lost when plants were supplied additional phosphorus. In total, the Col-0 wildtype appears to behave as expected if endoreduplication is directly involved in the compensatory response following apical damage by clipping. While there are a few conclusive results from our studies, the gene manipulation had unintended effects on endoreduplication and failed to resolve the mechanistic link between endoreduplication and fitness compensation.

The manipulation of *ILP1*, however, had some unpredicted effects on endoreduplication that leave definitive conclusions regarding endoreduplication and compensation elusive. There were no significant differences between unclipped and clipped *ilp1-1* and *ILP1*-ox plants in seed yield, biomass, or cycle value regardless of phosphorus treatment. There were some differences among lines, however, with LC1-grown unclipped plants of both *ilp1-1* and *ILP1*-ox

having significant reductions in seed yield and biomass relative to unclipped Col-0, and *ilp1-1*

dramatically so (Figures 4.3A, 4.4A). The fact that the gene knockout had substantially lower

fitness and biomass, which were reduced even further upon clipping (though non-significantly),

is readily explained by the nature of disrupting a pathway (endoreduplication) that is suspected

to play important roles in both fitness (via supporting seed production) and biomass (via cell

expansion). The reductions observed in the overexpression line further demonstrate the

complexity of these gene networks and suggest a sensitive orchestration of gene regulation

such that even overexpression of a gene has negative consequences for fitness. The primary

unexpected result of gene manipulation, however, was of the cycle values—*ILP1*-ox, the *ILP1*

overexpression mutant, did not have greater endopolyploidy than the Col-0 wildtype as

expected, and *ilp1-1*, the *ILP1* knockout, actually had significantly greater levels of

endopolyploidy than either the Col-0 wildtype or *ILP1*-ox.

The cell cycle is certainly an important component of normal growth, development, and

function, and is thus under tight and complex regulatory processes. Progression through the

cell cycle largely depends on the presence and abundance of numerous classes of cyclins,

cyclin-dependent kinases, their targets, and their regulators, as well as on the relative

proportions of auxins, cytokinins, and giberellins (see Inzé 2007 for an extensive review). These

components together influence the mitotic and endocycle balance, directly influencing the

entry, duration, and number of endocycles in an endoreduplicating cell (Vlieghe et al. 2005,

Imai et al. 2006). For example, the "A" class of cyclins (CYCA) regulates the checkpoint between

the G2 phase and the M phase of cells in the mitotic cell cycle by activating cyclin-dependent

kinase B (CDKB), an important component of the theoretical mitosis-inducing factor that

promotes the induction of mitotic cell division (Boudolf et al. 2004). Entry into the endocycle, however, requires the suppression of mitosis, typically by anaphase-promoting complex (APC) ubiquitin-dependent proteolysis of CYCA/CDKB or transcriptional repression of CYCA proteins stimulated by a low concentration of auxin relative to cytokinin (Vlieghe et al. 2007).

ILP1, the gene manipulated here, is a transcriptional repressor of the "A2" variety of cyclins (CYCA2)(Yoshizumi et al. 2006). The CYCA2 family, and in particular CYCA2;3, is a key component of the interaction with CDKB and ultimately the regulation of ploidy in *A. thaliana* (Imai et al. 2006). Reducing *ILP1* expression by T-DNA insertion as in the *ilp1-1* mutant reduces the repression of CYCA2 and therefore induces mitosis via increased activation of CDKB; conversely, overexpressing *ILP1* by CaMV 35S promotion causes tighter repression of CYCA2 and therefore represses mitotic division, increasing cellular ploidy by continued S-phase DNA replication (Yoshizumi et al. 2006). Because *ILP1* acts as a transcriptional repressor of the CYCA2 family of cyclins, which is a large gene family that is likely under the influence of a suite of positive and negative regulators, it is probable that compensation of the endoreduplication pathway could be achieved through the redundant action of a number of genes if the pathway is disrupted by the genetic manipulation of *ILP1*, as performed here. We note that Yoshizumi et al. (2006) examined endoreduplication of etiolated and control Col-0, *ilp1-1*, and *ILP1*-ox hypocotyls, whereas our measurements were of fully-mature plants, and thus there may have been unintended changes in gene action over the lives of the plants.

Through the action of *ILP1*, Yoshizumi et al. (2006) further demonstrated that *ILP1*-mediated endoreduplication is important in *A. thaliana* stem and root elongation, where *ilp1-1* knockdown mutants displayed inhibited hypocotyl and root elongation while *ILP1*

overexpression mutants had faster hypocotyl and root elongation via endoreduplication-driven increases in cell volume (Yoshizumi et al. 2006). Endoreduplication is also known to be important in seed production and maturation (Kowles and Phillips 1988, Engelen-Eigles et al. 2001, Lee et al. 2009), and thus we expected the manipulation of *ILP1* to have clear effects on the fitness compensation and biomass generation of the Col-0 genotype. Because we obtained seed stocks directly from Yoshizumi et al. (2006) and verified their identity, we believe that *ILP1* expression was modified as intended. One possibility for why no changes in either seed production or biomass upon clipping were observed in the gene mutants may be that the gene manipulation disrupted the natural plasticity of the endoreduplication pathway, limiting the plants' abilities to increase fitness through adjusting cell size, nutrient transport, transcriptional output, and other presumed endoreduplication-mediated attributes (Nagl 1978, Barow and Meister 2003, Barow 2006). Even if the manipulation of *ILP1* expression had produced the intended effect on endoreduplication, however, the Col-0 genotype experienced a reduction in seed production and endopolyploidy following clipping and thus falls within the range of values where the relationship between the change in seed number and the change in cycle value is not easily discernible (see Figure 4.5A and note the dispersed scatter of points for negative values of both measures).

Gene regulation of endoreduplication is further impacted by the physiological status of the plant. The removal of the apical meristem by clipping eliminates the primary site of auxin production in the inflorescence, which typically inhibits lateral bud elongation in part via inhibition of the endocycle (Ishida et al. 2010). The removal of auxin thus allows the induction of endoreduplication as part of the elongation of lateral buds. A cell's entry into the endocycle

is therefore a complex interplay between the environmental and hormonal influence on genetically-based cell cycle regulation. Here we additionally tested the effect of phosphorus fertilization on Col-0, *ilp1-1*, and *ILP1*-ox to determine if phosphorus availability limited endoreduplication, fitness compensation, or both. Upon phosphorus fertilization, the average seed yield for unclipped plants of each genotype increased, though only significantly for *ilp1-1* (Col-0: +15.3%, *ilp1-1*: +163.2%, *ILP1*-ox: +8.4%). The compensatory ability (i.e. the relative change in seed yield from unclipped to clipped plants) of each genotype likewise increased, remediating Col-0's natural undercompensatory response to equal compensation while the *ILP1* mutants improved their relative compensation as well (*ilp1-1* improving compensation by approximately 40%, and *ILP1*-ox displaying a non-significant increase in seed yield following clipping; Figure 4.2B). This indicates that compensation, and perhaps fitness more generally, may be phosphorus-limited in the LC1 control potting mix.

Phosphorus is also commonly limiting in natural soils (Schachtman et al. 1998), and the phosphorus fertilization of field-grown *Ipomopsis aggregata*, the classic example of overcompensation (Paige and Whitham 1987), results in increased compensatory ability following natural ungulate herbivory (Scott and Paige, unpublished). Studies are underway to determine if the increase in fitness compensation may in part be due to increases in endoreduplication since *I. aggregata* is known to endoreduplicate (Paige and Scholes, unpublished). In this study, a marginally-significant trend of reduced endoreduplication in phosphorus-fertilized Col-0 unclipped plants suggests that this genotype likely assimilated the surplus-supplied phosphorus into seed production, both when unclipped and following clipping, allowing greater fitness and better compensatory ability relative to when grown in LC1 control

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media. Because phosphorus is a structural component of ribo- and deoxyribonucleotides (RNA

and DNA), adenosine-5'-triphosphate (ATP), phospholipids, and numerous other molecules

critically important in cellular metabolism (including ribulose-1,5-bisphosphate [RuBP] and

phosphoenolpyruvate [PEP]), the stress of experimental clipping likely created great demand

for phosphorus. *ilp1-1*, the *ILP1* knockout line, and *ILP1*-ox, the *ILP1* overexpression line,

experienced no changes in either fitness or endoreduplication following herbivory regardless of

fertilization, and so no definitive conclusions can be made regarding the link between

endoreduplication and fitness compensation in these genotypes.

Although we did not observe a relationship between endoreduplication and fitness

compensation among these genotypes, we do, however, note a marginally significant positive

relationship (p = 0.0882, R^2 = 0.5574) between the change in cycle value and the change in

inflorescence dry biomass for LC1- and +P-grown plants together. This suggests that even

though endoreduplication may not be directly associated with compensation for seed yield

following clipping of these genotype × treatment groups, it may be an important process in the

generation of biomass generally, as also suggested by our previous studies (Scholes and Paige

2011, Chapter 3 of this dissertation) and in other taxa, including *Caenorhabditis elegans*

(Flemming et al. 2000, Lozano et al. 2006), *Oikopleura dioica* (Ganot and Thompson 2002), and

a number of decapod crustaceans (Jimenez et al. 2010). For the *A. thaliana* genotypes studied

here, however, the efficient regeneration of biomass does not appear to increase fitness since

no relationship between the change in seed yield and biomass could be detected (though the

limited number of genotypes here do fall within the expected range of fitness and

endoreduplication, see Figure 4.5). We again stress that no changes in seed production,

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inflorescence dry biomass, or cycle values following clipping were demonstrated in the *ILP1*

gene expression mutant lines, and therefore no definitive conclusions, supporting or refuting

the possible link between endoreduplication and fitness compensation, can be made from

these data.

If a link does indeed exist between endoreduplication pathway described above and

overcompensation, as our previous studies suggest (Scholes and Paige 2011, Chapters 2 and 3

of this dissertation), it may be through the oxidative pentose phosphate pathway (OPPP). The

OPPP is composed of a series of carbohydrate metabolism reactions, catalyzed in part by

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (*G6PD1*), which is up-regulated in *Arabidopsis*

thaliana following experimental clipping (Siddappaji et al., unpublished), as performed here.

This pathway, which is a component of the generalized oxidative stress response, supplies

intermediate molecules for further modification into induced defensive compounds such as

alkaloids, flavonoids, and glucosinolates via the shikimate pathway (Scharte et al. 2009,

Herrmann and Weaver 1999). The OPPP also supplies ribose-5-phosphate for nucleotide

synthesis, which helps generate the tremendous quantity of nucleotides necessary for

increased endoreduplication following herbivory. The increased number of DNA templates may

then serve to further increase expression of *G6PD1* and the OPPP in a positive feedback loop

(Siddappaji et al., unpublished). Removal of the apical meristem therefore stimulates

endoreduplication via the removal of auxin inhibition of the endocycle (Ishida et al. 2010) and

stimulation of the OPPP, which provides a mechanism for induced defensive chemistry,

remediation of oxidative damage, increased carbohydrate metabolism, and further stimulation

of endoreduplication and the associated beneficial nucleotypic and genetic effects.

In this study, we investigated the interplay of hormonal and genetic regulation of mitosis by experimental clipping, thereby reducing inflorescence auxin concentration of cell-cycle mutant lines. These experiments sought to extend our previous studies (Scholes and Paige 2011, Chapters 2 and 3 of this dissertation) demonstrating a potential mechanistic link between endoreduplication and fitness compensation following simulated herbivory. The correlation between endoreduplication and compensation observed in Columbia-4, Landsberg *erecta*, and their offspring, as well as globally-distributed ecotypes (Chapter 3 of this dissertation), suggested direct causality between the pathways due to the pattern existing despite recombination of the parental genotypes and the inclusion of geographically widespread ecotypes. The inclusion of a wide range of genetic variability should have broken up any association between the polygenic pathways of endoreduplication and compensation and produced no discernible relationship if the two pathways are not causally linked. Here we note that Columbia-0, a related but distinct Columbia genotype from the Columbia-4 accession used previously, further supports the relationship between endoreduplication and fitness compensation by responding in the predicted fashion upon clipping (Figures 4.2, 4.5A; also see Chapter 3 of this dissertation). We note, however, that manipulating the expression of an important endocycle regulator did not have the intended effect on endoreduplication; namely, knocking out *ILP1* seemed to cause a gene regulatory cascade that actually increased endopolyploidy relative to the Col-0 wildtype. To better understand the interaction of endocycle regulators in the *ILP1* mutants, full-transcriptome sequencing is currently underway and should yield great insights into the important genes regulating the endocycle and their relationship with fitness and compensation. Future studies that further investigate the link

between these two genetic pathways, including the manipulation of additional

endoreduplication and/or compensation genes, could also be very telling of the complexity by

which plant regrowth, fitness compensation, and endoreduplication respond to herbivory.

Table 2.1. (A) Percentage of rosette nuclei at each ploidy level for Columbia-4 and Landsberg *erecta* of *Arabidopsis thaliana* prior to bolting at 2.5 weeks (*n* = 10 rosettes per ecotype) and at one week after clipping, i.e., at 4.5 weeks of age (*n* = 10 rosettes per treatment per ecotype). (B) Percentage of inflorescence (stems, leaves, flowers, flower buds, and valves of siliques) nuclei at each ploidy level for clipped and unclipped Columbia-4 and Landsberg *erecta* at 4.5 weeks (*n* = 10 plants per treatment per ecotype) and 6.5 weeks after germination (*n* = 15 plants per treatment per ecotype). Values are means ± SE. Data were analyzed using independent *t*-tests followed by a sequential Bonferroni adjustment to correct for multiple comparisons. Asterisks (*) indicate a significant difference following adjustment (α = 0.05). Rosettes at 2.5 weeks, rosettes and inflorescences at 4.5 weeks, and inflorescences at 6.5 weeks for each ecotype were analyzed separately given that they represented independent sets of plants. Daggers (†) denote *p* values for comparisons of the two ecotypes.

Time	Ploidy	Columbia-4			Landsberg *erecta*		
		Unclipped	Clipped	*p*	Unclipped	Clipped	*p*
A) Rosettes							
2.5 weeks	2C	63.23 ± 3.47			61.29 ± 2.40		0.652†
(Baseline)	4C	36.77 ± 3.47			38.71 ± 2.40		0.652†
4.5 weeks	2C	46.37 ± 1.79	48.03 ± 1.73	0.514	41.20 ± 2.93	41.27 ± 2.39	0.986
	4C	32.27 ± 0.91	29.24 ± 0.83	0.024	33.92 ± 1.43	32.14 ± 1.88	0.460
	8C	14.78 ± 1.16	14.50 ± 0.94	0.855	17.56 ± 1.43	19.85 ± 1.04	0.155
	16C	6.59 ± 0.63	8.24 ± 0.81	0.126	7.32 ± 1.06	6.75 ± 0.59	0.645
B) Inflorescences							
4.5 weeks	2C	46.35 ± 0.75	51.88 ± 0.79	0.0001*	46.97 ± 1.95	51.12 ± 1.04	0.085
	4C	34.69 ± 0.51	31.93 ± 0.35	0.0001*	33.88 ± 0.66	31.64 ± 0.84	0.054
	8C	14.55 ± 0.54	12.15 ± 0.45	0.003*	14.67 ± 1.31	13.14 ± 0.43	0.294
	16C	4.42 ± 0.24	4.04 ± 0.27	0.319	4.48 ± 0.43	4.10 ± 0.42	0.540
6.5 weeks	2C	60.48 ± 1.74	50.02 ± 1.69	0.0001*	54.98 ± 1.78	55.47 ± 1.63	0.839
	4C	26.67 ± 0.78	31.75 ± 1.04	0.001*	30.46 ± 1.46	29.03 ± 1.22	0.461
	8C	10.72 ± 1.00	14.52 ± 0.78	0.006*	11.78 ± 1.06	13.01 ± 0.57	0.316
	16C	2.13 ± 0.58	3.71 ± 0.48	0.046*	2.79 ± 0.51	2.49 ± 0.28	0.609

Table 2.2. Fitness traits of clipped and unclipped plants of Columbia-4 and Landsberg *erecta* at senescence (*n* = 48–54 plants per treatment per ecotype). Means ± SE are shown. Data were analyzed using independent *t*-tests followed by a sequential Bonferroni adjustment to correct for multiple comparisons. Asterisks (*) indicate significance following adjustment (α = 0.05).

Plant Trait	Columbia-4			Landsberg *erecta*		
	Unclipped	Clipped	*p*	Unclipped	Clipped	*p*
Number of siliques	88.00 ± 4.06	132.76 ± 4.06	0.0001*	205.13 ± 4.30	200.33 ± 4.26	0.453
Number of seeds	3728 ± 145	5221 ± 145	0.0001*	6699 ± 154	6065 ± 152	0.002*
Seed mass (mg/100 seeds)	1.159 ± 0.001	1.162 ± 0.003	0.382	1.159 ± 0.003	1.161 ± 0.003	0.940
Number of lateral stems	2.91 ± 0.14	5.09 ± 0.08	0.0001*	3.29 ± 0.08	5.33 ± 0.21	0.0001*
Number of main stems	2.02 ± 0.11	2.89 ± 0.09	0.0001*	1.38 ± 0.08	3.57 ± 0.10	0.0001*
Total stem length (cm)	60.39 ± 1.81	72.37 ± 1.98	0.0001*	146.8 ± 2.78	214.4 ± 5.69	0.0001*
Aboveground biomass (g)	0.270 ± 0.012	0.403 ± 0.011	0.0001*	0.272 ± 0.005	0.338 ± 0.006	0.0001*
Plant height (cm)	20.84 ± 0.260	19.57 ± 0.271	0.001*	42.80 ± 0.424	39.89 ± 0.582	0.0001*

Table 2.3. Logistic growth parameters. Parameters were fit to a logistic function (see Methods) of stem elongation measurements over time for each genotype × treatment group ("Group"; Col-4 unclipped, Col-4 clipped, Ler unclipped, Ler clipped). Letters indicate significance at α = 0.05 for each parameter among the genotype × treatment groups for each β_1, β_2, and β_3 upon comparison of their pairwise differences to zero. Comparisons were not made among β_1, β_2, and β_3 within or among genotype × treatment groups.

Parameter	Group	Estimate	Std. Error	95% Confidence Limits	Significance
β_1	Col-4 unclipped	96.61	1.06	94.54 – 98.69	A
	Col-4 clipped	95.42	1.29	92.90 – 97.95	A
	Ler unclipped	97.75	0.93	95.92 – 99.58	A
	Ler clipped	97.78	1.27	95.30 – 100.30	A
β_2	Col-4 unclipped	19.38	2.48	14.51 – 24.25	AB
	Col-4 clipped	21.48	2.77	16.04 – 26.91	A
	Ler unclipped	14.52	1.74	11.10 – 17.93	B
	Ler clipped	18.11	2.34	13.51 – 22.70	AB
β_3	Col-4 unclipped	0.32	0.014	0.287 – 0.344	AB
	Col-4 clipped	0.28	0.013	0.254 – 0.305	A
	Ler unclipped	0.34	0.016	0.311 – 0.372	B
	Ler clipped	0.29	0.014	0.260 – 0.315	A

Figure 2.1. Measures of DNA content. The percentage of cells at each of four nuclear DNA content levels in (A) Columbia-4 (Col-4) and (B) Landsberg *erecta* (Ler) ecotypes of *Arabidopsis thaliana* clipped and unclipped plants at 6.5 weeks after germination. ''Gated events'' refers to fluorescence events measured by flow cytometry of propidium-iodide-stained DNA within suspended nuclei. Shown are means ± SE (n = 15 plants per treatment). Asterisks (*) indicate a significant difference following a sequential Bonferroni adjustment (α = 0.05). (C) Nuclear DNA content (pg) per cell of Columbia-4 and Landsberg *erecta* clipped and unclipped plants. Shown are means ± SE (n = 15 plants per treatment). Clipped Columbia-4 plants have higher nuclear DNA content per cell than unclipped plants ($p < 0.01$).
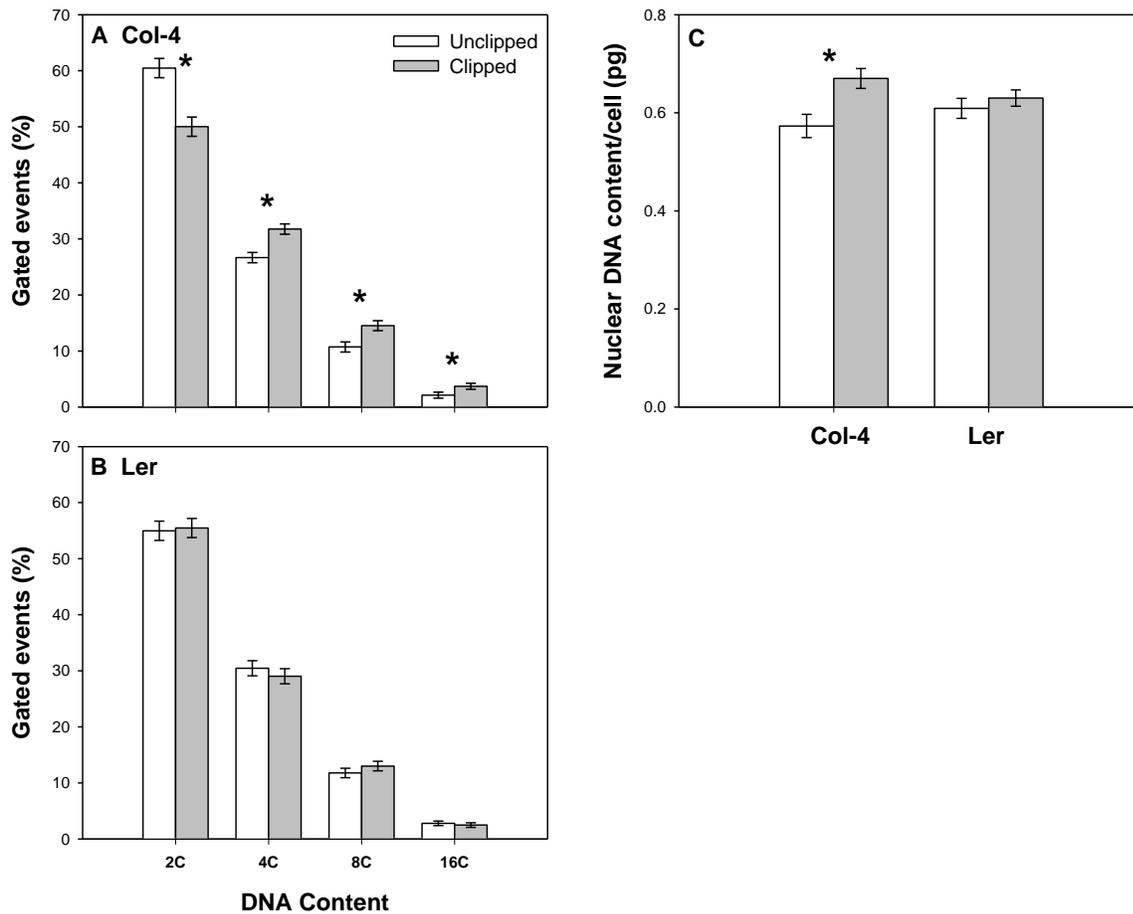
Figure 2.2. Norm of reaction. Combined (4C, 8C, 16C) nuclear DNA content per cell of Columbia-4 and Landsberg *erecta* clipped and unclipped plants at 6.5 weeks after germination. Shown are means ± SE (*n* = 15 plants per treatment). There is a significant genotype × environment interaction (*p* < 0.01).
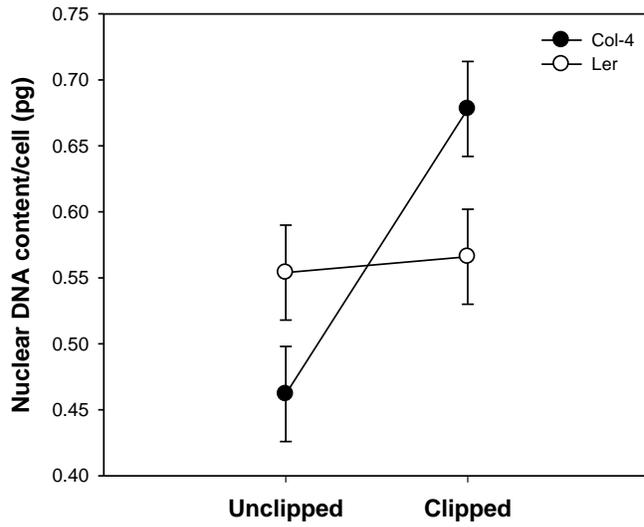
Figure 2.3. Measures of fitness. Number of (A) siliques and (B) seeds per plant for Columbia-4 and Landsberg *erecta* clipped and unclipped treatments at senescence. Shown are means ± SE (n = 48–54). Asterisks (*) indicate significance following a sequential Bonferroni adjustment (α = 0.05). (C) Aboveground plant biomass (g) for Columbia-4 and Landsberg *erecta* clipped and unclipped treatments at senescence. Shown are means ± SE (n = 48–54). Clipped plants of Columbia-4 and Landsberg *erecta* have significantly higher biomass than unclipped plants.
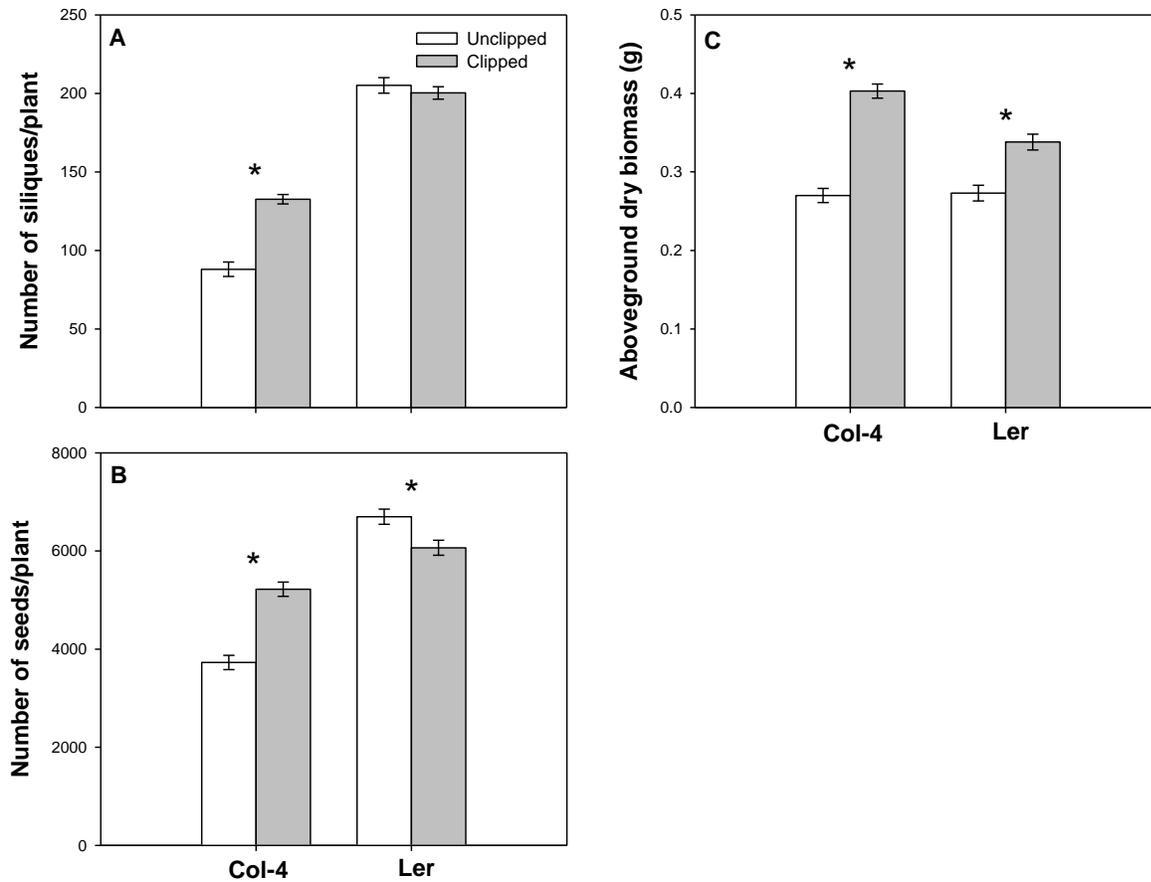
Figure 2.4. Stem elongation curves. Percentage of maximum height through time and stem elongation rates for Columbia-4 (A, C) and Landsberg *erecta* (B, D) unclipped and clipped plants. Shaded regions around logistic growth curves (A, B) are 95% confidence bands for the mean of each genotype × treatment group.
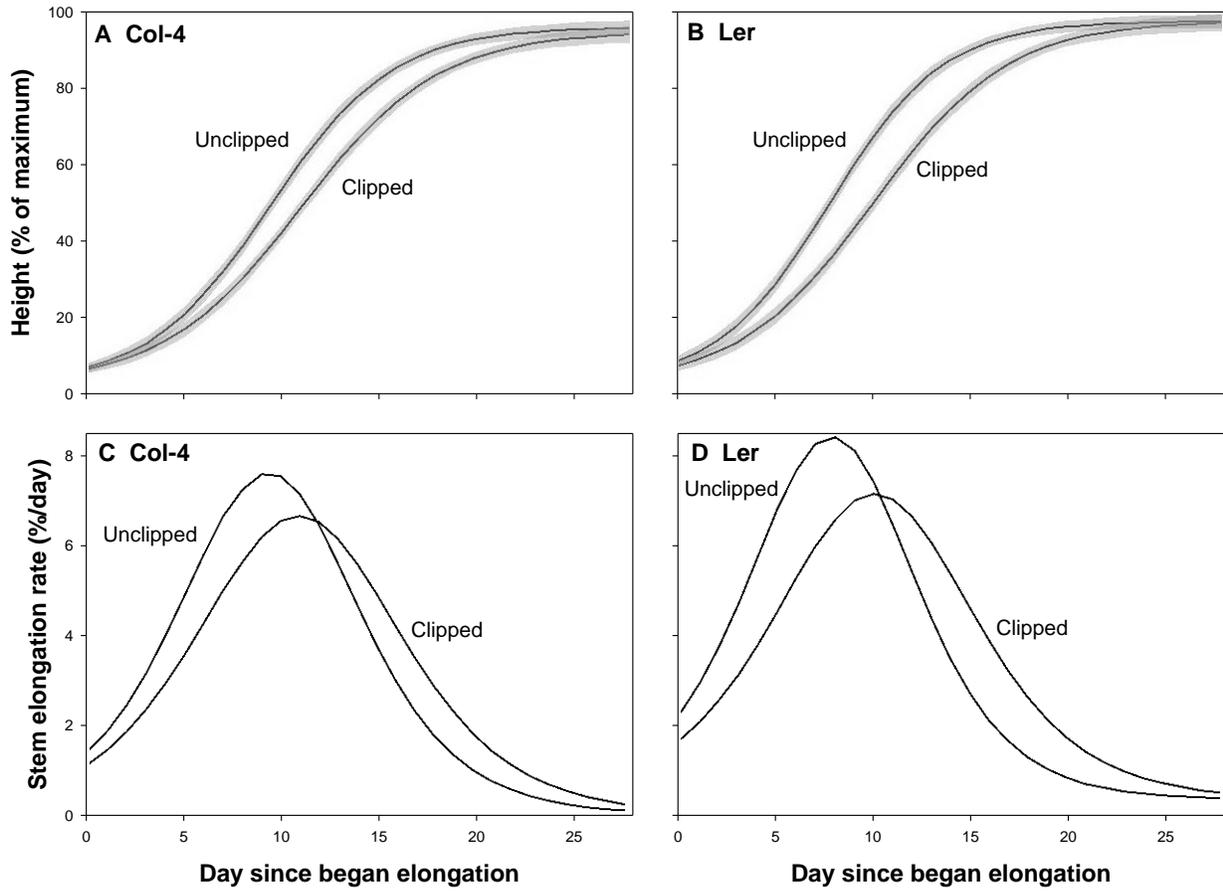
Table 3.1. Accession information for globally-distributed ecotypes. TAIR is The Arabidopsis Information Resource (http://www.arabidopsis.org). NASC is the Nottingham Arabidopsis Stock Centre (http://www.arabidopsis.info).

TAIR Stock #	NASC Stock #	Common Name	Abbreviated Name	Country of Origin
CS1074	N1074	Chisdra	Chi-1	Russia
CS1084	N1084	Coimbra	Co-1	Portugal
CS1284	N1284	Köln	Kl-5	Germany
CS1564	N1564	Tsu	Tsu-0	Japan
CS22610	N22610	Rennes	Rennes-1	France
CS22652	N22652	Shakdara	Sha	Tadjikistan
CS28796	N28796	Vancouver	Van-0	Canada
CS28850	N28850	Gara de Nord	Gdn-0	Romania
CS8020	N8020	Toledo	Tol-0	USA

Table 3.2. Phenotypic characters. Twenty-four phenotypic characters measured for pairs of unclipped and clipped plants of all genotypes after senescence (see Methods for complete description). The percent changes of 23 characters were tested for significant relationships with the percent change total number of seeds (fitness compensation). Probability (*p*) values are listed for characters significantly related to total seed yield upon stepwise multiple regression for Columbia-family and globally-distributed genotypes (α = 0.05). Plus (+) and minus (−) signs for each significant character denote a positive or negative relationship with compensation, respectively.

Significance (*p*)		Character
Col-family	Global	Total number of seeds =
		Number of days after planting at which the inflorescence measured 6 cm in height
		Rosette diameter (cm) at the time the inflorescence measured 6 cm in height
0.0056 +		Mass (g) of the dry inflorescence biomass after senescence
		Total number of primary stems
		Total length (cm) of all primary stems
		Average length (cm) per primary stem
<0.0001 +		Total number of siliques on all primary stems
		Average number of siliques per primary stem
0.0006 −		Total number of secondary stems
		Average number of secondary stems per primary stem
	<0.0001 +	Total length (cm) of all secondary stems
		Average length (cm) per secondary stem
		Total number of siliques on all secondary stems
0.0003 +		Average number of siliques per secondary stem
	0.0001 +	Total number of tertiary stems
		Average number of tertiary stems per secondary stem
	0.0004 −	Total length (cm) of all tertiary stems
		Average length (cm) per tertiary stem
	0.0055 +	Total number of siliques on all tertiary stems
		Average number of siliques per tertiary stem
0.001 +		Total number of all stems
<0.0001 −		Total length of all stems
<0.0001 +	<0.0001 +	Total number of all siliques

Figure 3.1. Approximate locations of collection of globally-distributed ecotypes. Countries where *A. thaliana* is naturally distributed are depicted in gray.

Figure 3.2. Endoreduplication response to herbivory for (A) Columbia-family genotypes and (B) globally-distributed ecotypes. Asterisks (*) indicate that cycle values differed significantly between treatments (α = 0.05).
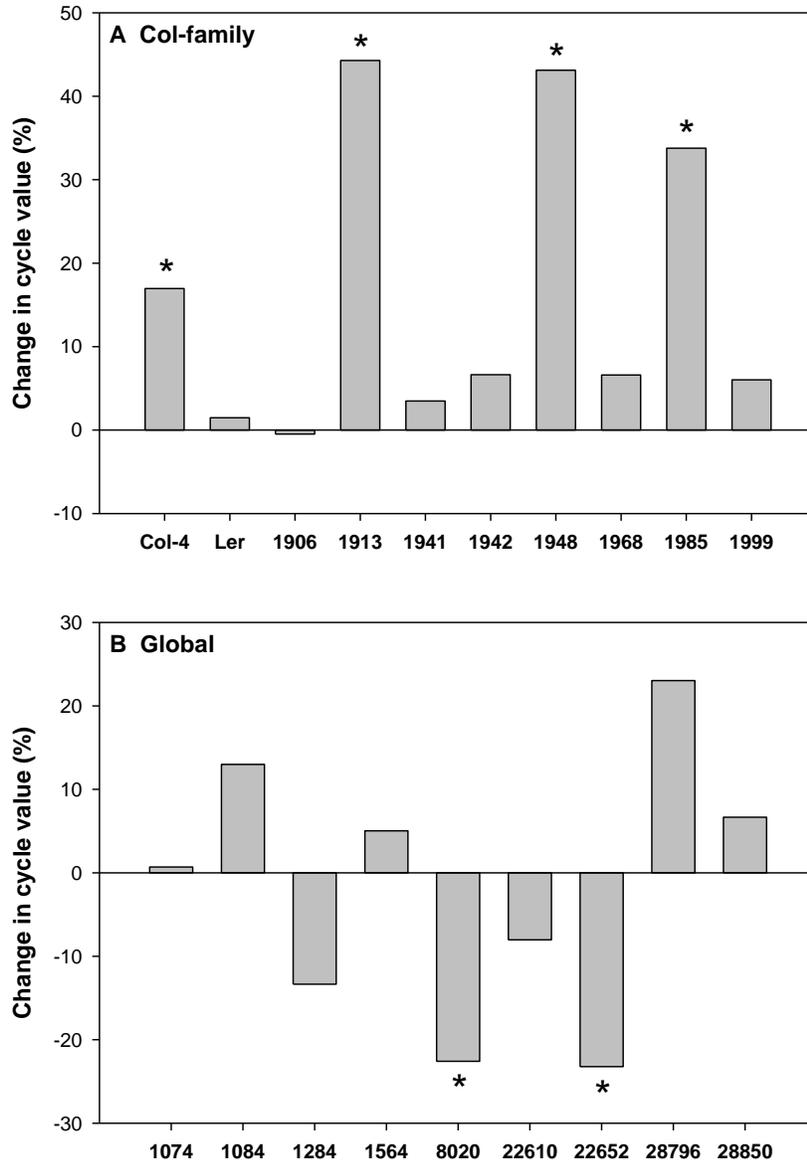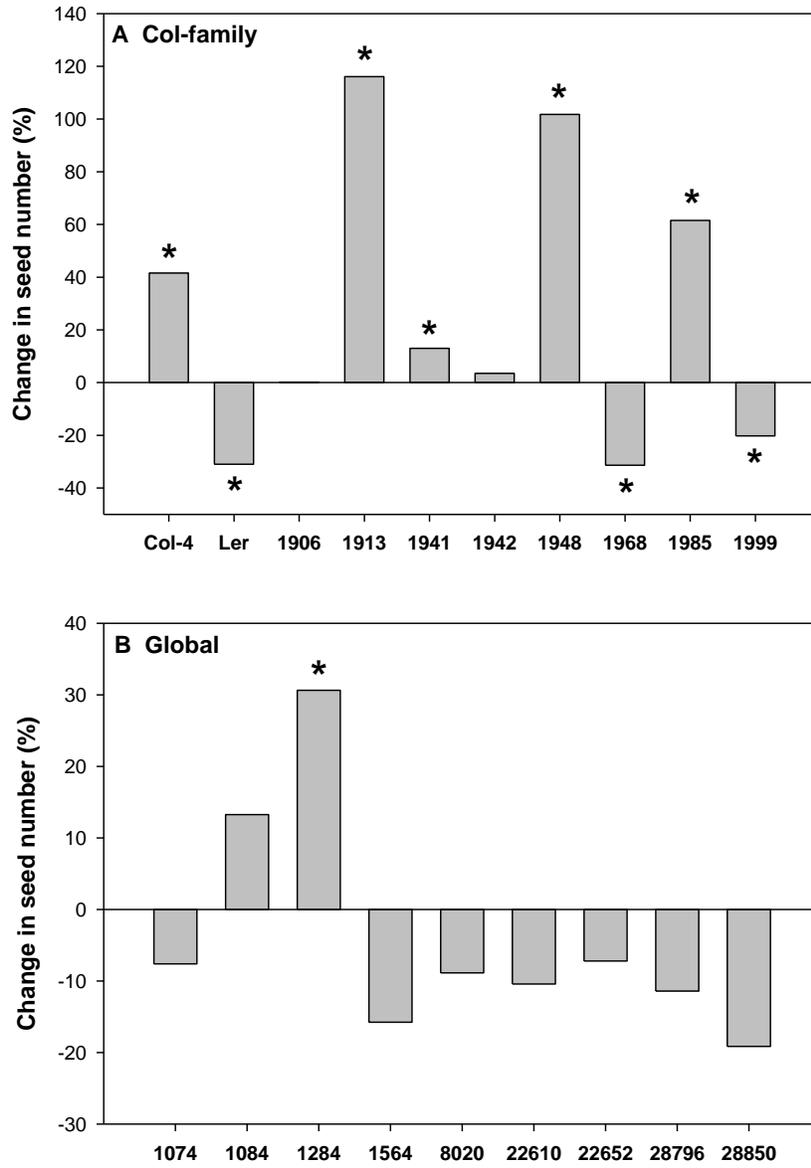
Figure 3.3. Compensatory response to herbivory for (A) Columbia-family genotypes and (B) globally-distributed ecotypes. Asterisks (*) indicate that seed yield differed significantly between treatments (α = 0.05).

Figure 3.4. The change in cycle value regressed against (A) the change in seed yield and (B) the change in inflorescence dry biomass for Columbia-family genotypes (black circles) and globally-distributed ecotypes (white circles). There is a significant positive relationship between differential endoreduplication and seed yield compensation following herbivory across all genotypes ($F(1,17) = 17.54$, $p < 0.001$, $R^2 = 0.5078$), as well as between differential endoreduplication and differential inflorescence biomass production ($F(1,17) = 13.34$, $p < 0.01$, $R^2 = 0.4397$).
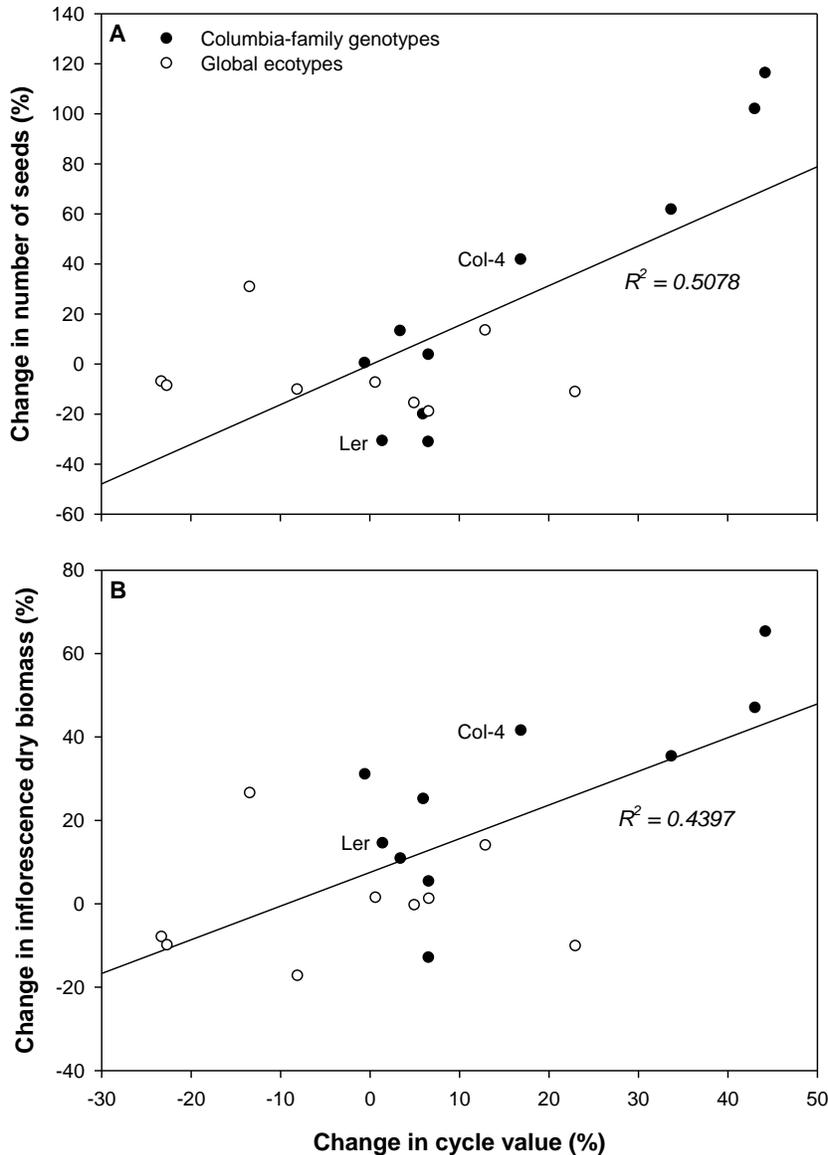
Figure 3.5. Phenogram of unclipped plants of Columbia-family and globally-distributed genotypes based on 24 phenotypic characters (see Table 3.2 for a complete list). Numbers on branches indicate branch lengths calculated for the inference of genetic distance among globally-distributed ecotypes. Genotypes that significantly ($\alpha = 0.05$) increased seed yield (+), decreased seed yield (‡), increased cycle value (×), and decreased cycle value (#) following clipping are indicated.
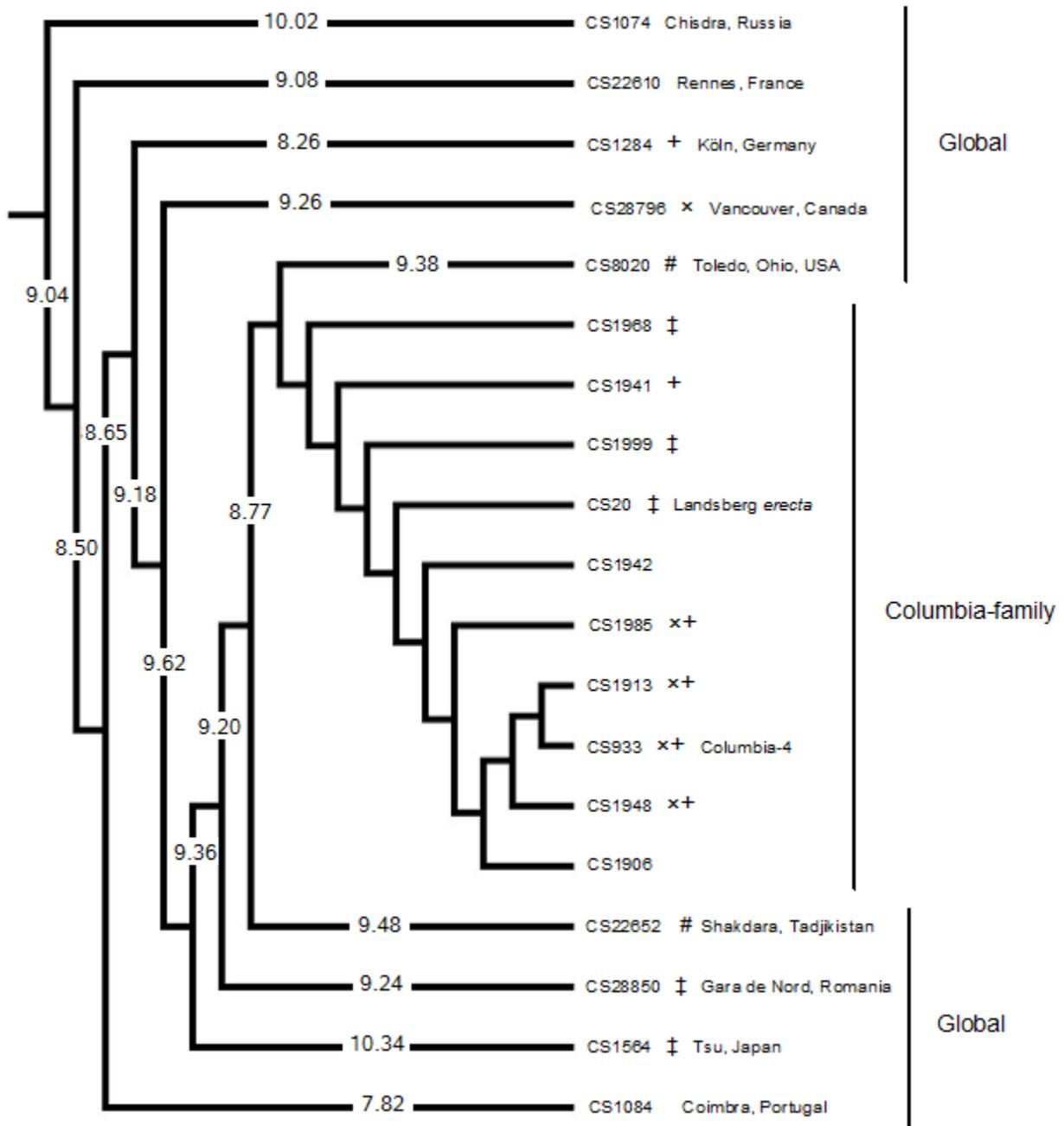
Figure 3.6. Phenotypic distance regressed against geographic distance for all pairwise combinations of globally-distributed ecotypes. Phenotypic distance was measured as the sum of the phenogram branch lengths (see Fig. 3.5) separating each pair of ecotypes through their closest commonly-shared node. Geographic distances were calculated for all pairwise combinations of ecotype collection locations. There is no significant relationship between phenotypic distance and geographic distance (Mantel test, p = 0.835, r = -0.037).
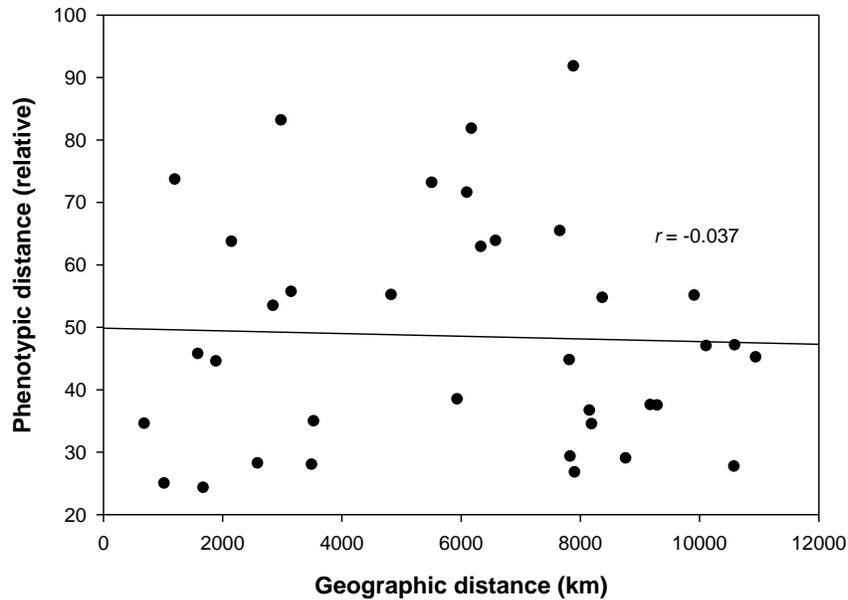
Figure 4.1. Cycle values of Col-0, *ilp1-1*, and *ILP1*-ox grown in (A) LC1 control and (B) phosphorus fertilized (+P) potting mix. Shown are means ± SE for unclipped (white bars) and clipped (gray bars) plants of each genotype. Asterisks (*) indicate that treatment means differed significantly ($\alpha = 0.05$).
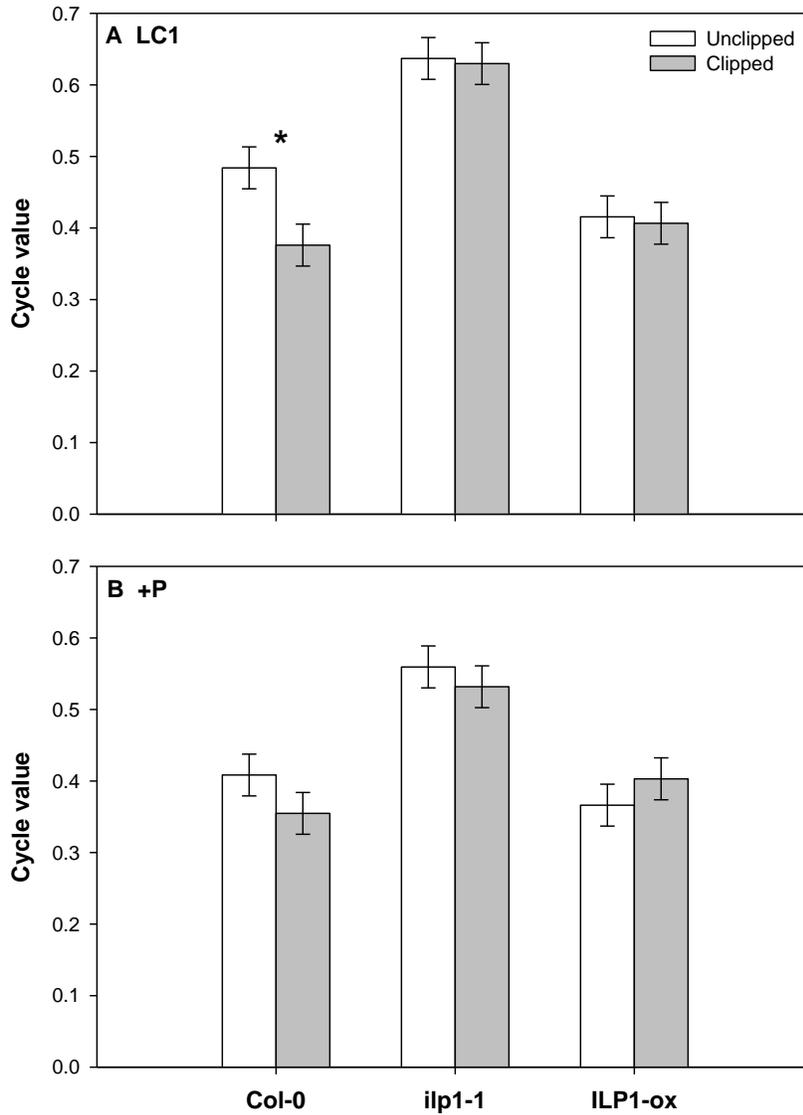
Figure 4.2. Response to herbivory in (A) endoreduplication, (B) fitness compensation, and (C) biomass for Col-0, *ilp1-1*, and *ILP1*-ox grown in control (LC1, white bars) and phosphorus fertilized (+P, gray bars) potting mix. Asterisks (*) indicate significant differences between measures of unclipped and clipped plants ($\alpha = 0.05$).
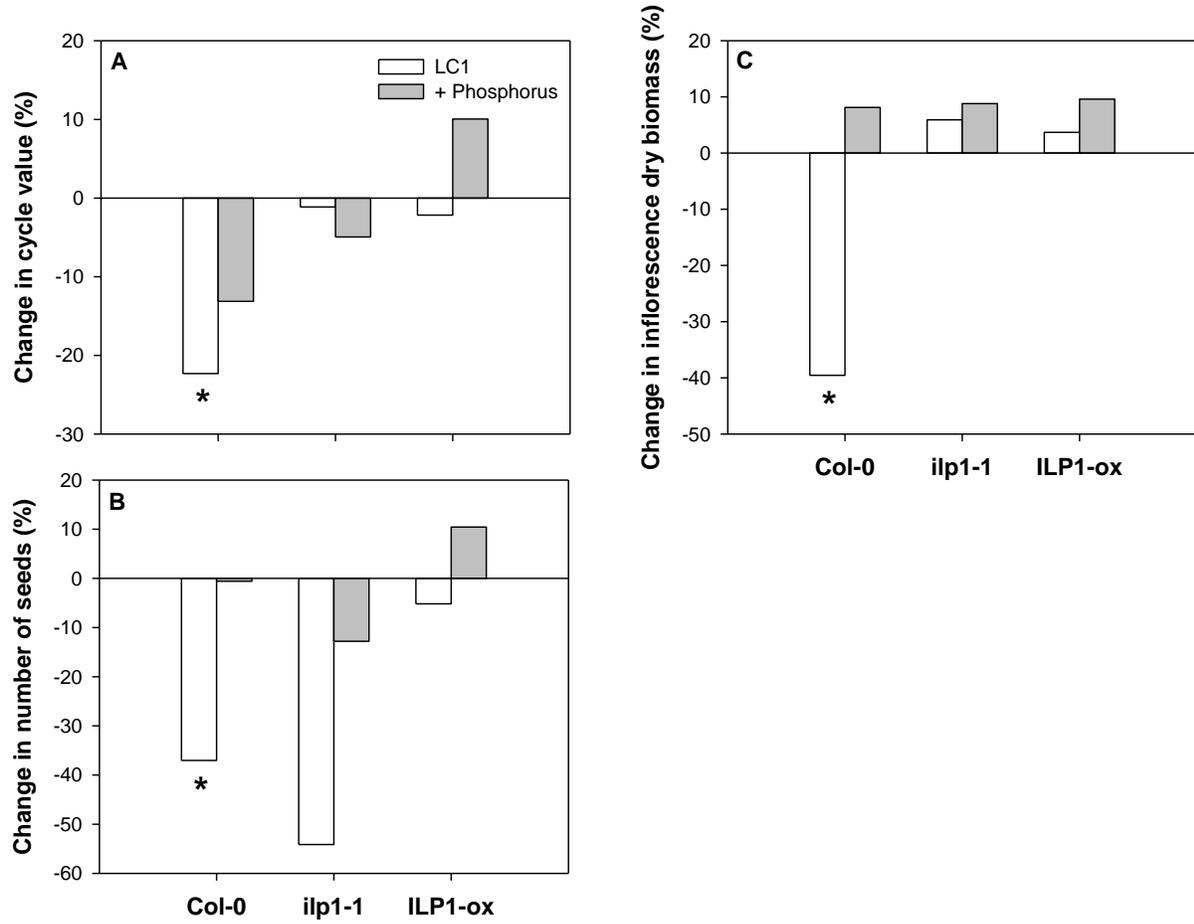
Figure 4.3. Seed yield of Col-0, *ILP1*-ox, and *ilp1-1* grown in (A) LC1 control and (B) phosphorus fertilized (+P) potting mix. Shown are means ± SE for unclipped (white bars) and clipped (gray bars) plants of each genotype. Asterisks (*) indicate that treatment means differed significantly (α = 0.05).
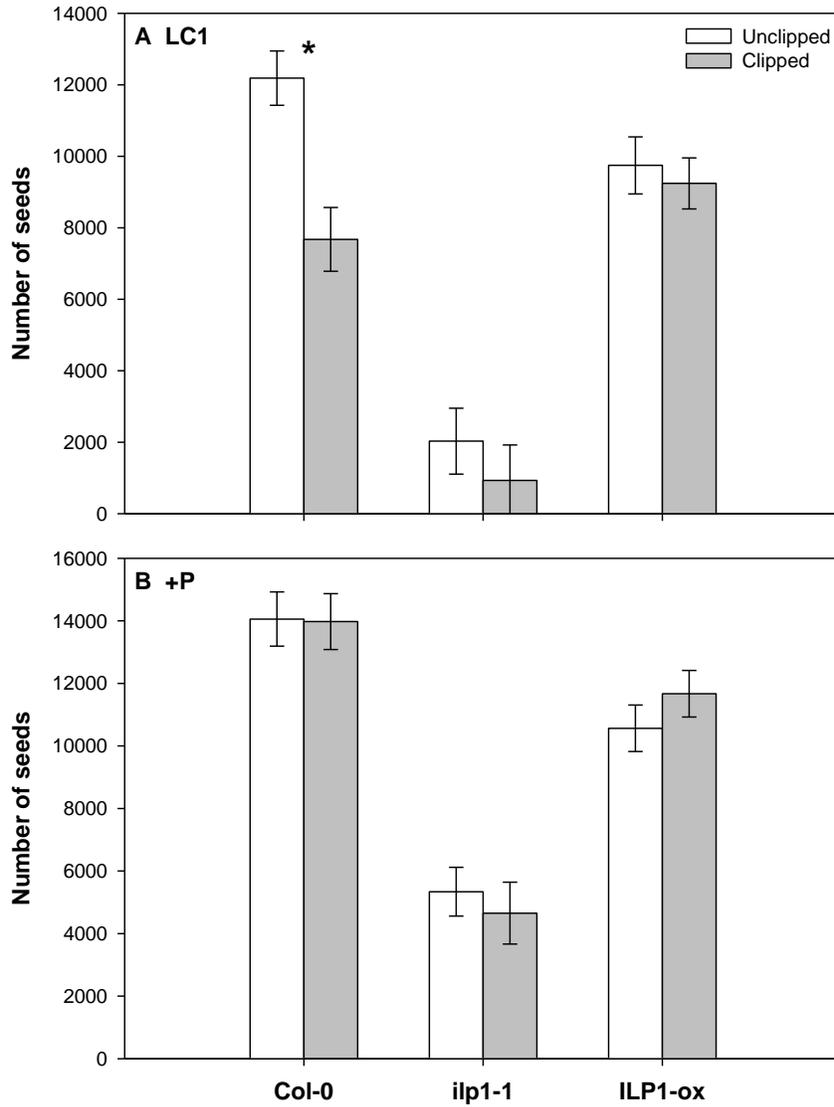
Figure 4.4. Inflorescence dry biomass of Col-0, *ILP1*-ox, and *ilp1-1* grown in (A) LC1 control and (B) phosphorus fertilized (+P) potting mix. Shown are means ± SE for unclipped (white bars) and clipped (gray bars) plants of each genotype. Asterisks (*) indicate that treatment means differed significantly (α = 0.05).
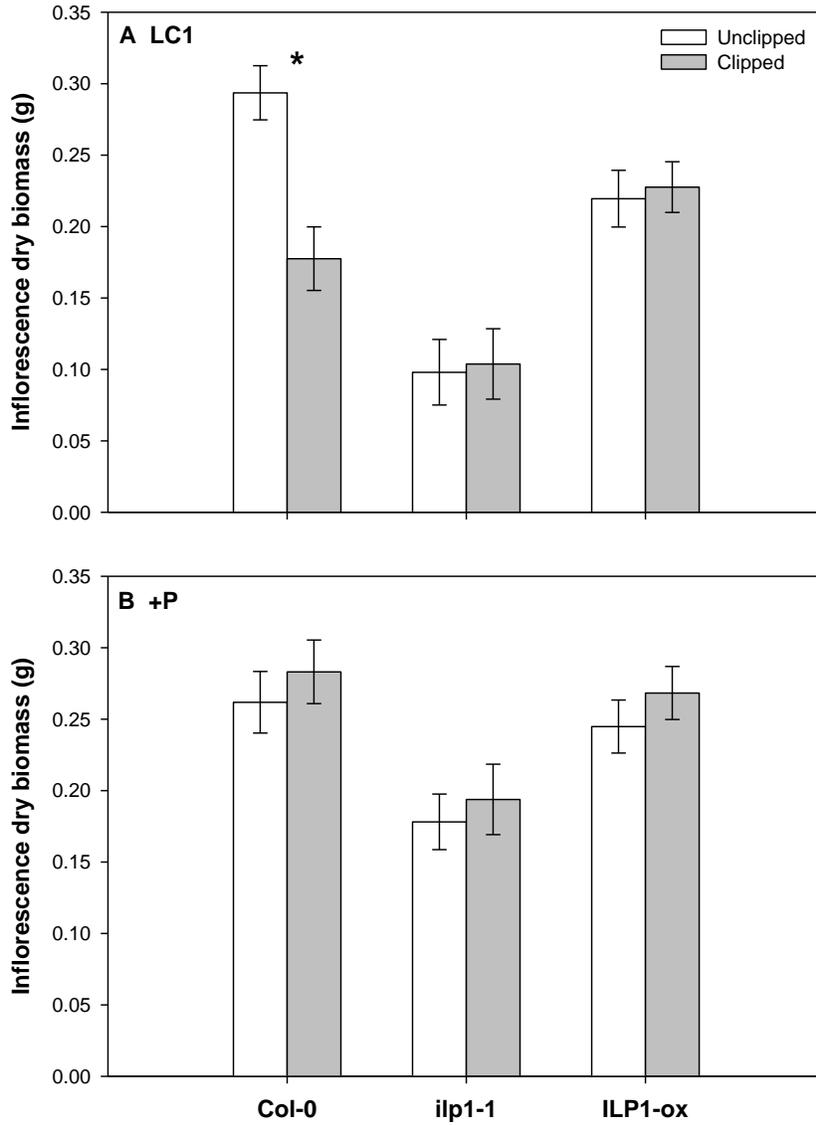
Figure 4.5. The change in seed number regressed against (A) the change in cycle value and (B) the change in inflorescence dry biomass for Columbia-family and globally-distributed genotypes (black circles, from Chapter 3 of this dissertation), and Col-0 and *ILP1* mutants grown in control (LC1, white circles) and phosphorus fertilized (+P, gray circles). There is a significant positive relationship between the fitness compensatory and endoreduplication responses to herbivory across all genotypes ($F(1,23) = 24.04$, $p < 0.0001$, $R^2 = 0.5111$), as well as between endoreduplication and inflorescence regrowth ($F(1,23) = 21.7$, $p < 0.0001$, $R^2 = 0.4855$).
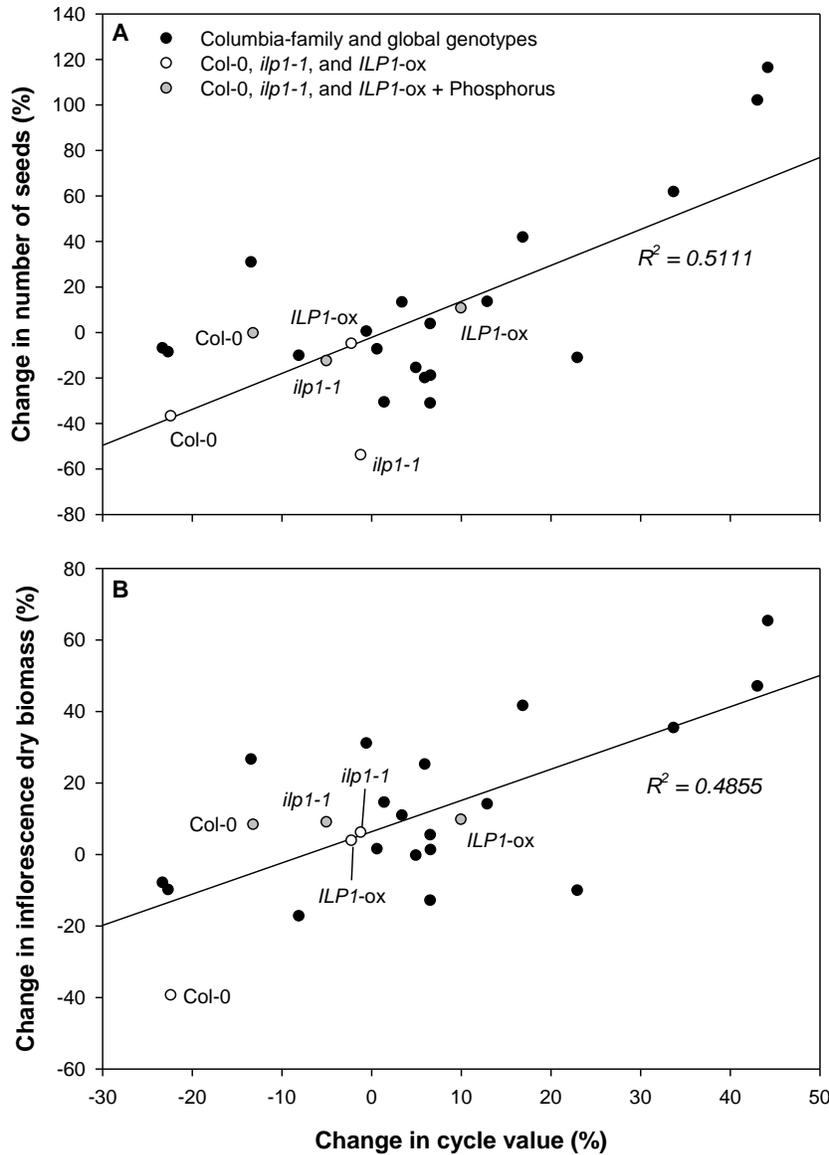
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