Analyzing The Role of Gibberellin in Dwarfism and Shade Tolerance in Perennial Ryegrass (Lolium perenne, L.) as Exhibited by the shadow-1 Mutant Lineage

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Analyzing The Role of Gibberellin in Dwarfism and Shade Tolerance in Perennial Ryegrass (*Lolium perenne*, L.) as Exhibited by the *shadow-1* Mutant Lineage

Lorenzo Katin-Grazzini, PhD

University of Connecticut, 2018

Abstract

Turfgrass is cultivated across the globe as an aesthetically pleasing and functional groundcover. One of the most popular turfgrass species is perennial ryegrass (*Lolium perenne* L.), which is valued for its fast establishment, dark green color, and adaptability to different soil types. In 2011, the Li lab initiated a mutation breeding program focused on developing new perennial ryegrass cultivars with desirable traits for the turfgrass industry. This project first centered around identifying dwarf mutant plants and later evolved into an exploration of beneficial secondary phenotypes associated with dwarfism, such as shade tolerance. One mutant, called *shadow-1*, was analyzed in detail over the course of multiple years of greenhouse and field studies. These studies have determined that *shadow-1* plants possess leaves which elongate slowly compared to wild type and are significantly resistant to the impact of shade stress. These traits were found to be stably inherited in *shadow-1* progeny and segregate together. Both the dwarfism and shade tolerance exhibited by *shadow-1* plants could be abolished through the exogenous application of the phytohormone gibberellin. Hormone analysis revealed that endogenous gibberellin levels were decreased in shade-stressed *shadow-1*, but increased in light-grown *shadow-1*, compared to wild type under each respective condition. Through transcriptome analysis it was determined that shade stress altered the expression of a greater number of genes
than those altered by the mutation(s) found in *shadow-1*. Transcriptome analysis also uncovered downregulation of gibberellin biosynthesis genes in *shadow-1* plants, although this was more severe in shade-stressed *shadow-1*. Additionally, the gibberellin degradation gene GA2ox was downregulated in *shadow-1* kept under light conditions while DELLA, the negative regulator of gibberellin response was upregulated. Taken together, these data provide evidence that dwarfism in *shadow-1* is caused by partial gibberellin insensitivity while shade tolerance is caused by gibberellin deficiency and both of these phenotypes are caused by a single mutation which can impact both pathways. These findings provide valuable information to geneticists and breeders who are interested in developing dwarf and/or shade tolerant plant cultivars.
Analyzing The Role of Gibberellin in Dwarfism and Shade Tolerance in Perennial Ryegrass

(*Lolium perenne*, L.) as Exhibited by the *shadow*-1 Mutant Lineage

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Doctor of Philosophy Dissertation

Analyzing The Role of Gibberellin in Dwarfism and Shade Tolerance in Perennial Ryegrass

(Lolium perenne, L.) as Exhibited by the shadow-1 Mutant Lineage

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Chapter 1 Background Information

1.1 Perennial ryegrass and shade stress

Turfgrass is the most widely cultivated ornamental crop in the United States, and as of 2005 it was estimated that managed turf swards covered ~2% of the land area of the continental US, totaling around 40 million acres (Emmons and Rossi, 2016). Turfgrass is distinguished from forage and crop grass not by its identity, but rather by its usage. Turfgrasses are used for ornamental purposes, while forage and crop grasses are used as animal feed and agricultural products, respectively. In fact many turfgrass species, like perennial ryegrass, can be used in both turf and forage contexts.

Turfgrass use falls into two broad categories, aesthetic and recreational, which together represent a $100 billion industry (Emmons and Rossi, 2016). Aesthetic uses of turfgrass revolve mainly around its appealing green color and uniform appearance when grown in well-maintained swards. However, there are also practical aspects to aesthetic turfgrass cultivation, such as oxygen production. It only takes ~25 ft² of turf to produce enough oxygen to sustain a human being for an entire day (Watschke, 1990). Additionally, the long, intertwining root systems of turf swards keep soil in place, reducing soil erosion. Soil erosion can be a serious issue any place where there are large areas of exposed soil, especially on river banks and in agricultural areas where plowing is the main method of weed management (Emmons and Rossi, 2016). In the 1930’s, decades of soil erosion caused a series of devastating dust storms in the United States which have come to be known as the Dust Bowl. Recreational uses of turfgrass center around their use on athletic fields and golf courses. For high-impact sports, like football and rugby,
turfgrass acts as a cushion, reducing the hardness of the ground during tackling (Gramckow, 1968; Rogers and Waddington, 1992). Turfgrass also provides a durable playing surface for sports and can recover quickly from traffic-related injury, compared to wild grass groundcovers (Emmons and Rossi, 2016, Brosnan, et al., 2014). Turfgrasses cover the vast majority of golf courses, and variations in turfgrass height maintenance is an important contributor to the variability of golf play, from putting greens to fairways to roughs. There are ~775,000 athletic fields and ~16,000 golf courses in the United States (Emmons and Rossi 2016).

Perennial ryegrass (*Lolium perenne* L.) is one of the most widely cultivated cool-season turfgrass species in the world (Jiang and Huang, 2001; Chen J. et al., 2016, Grinberg et al., 2016). It can be found in all manner of ornamental contexts, from small residential lawns to sprawling golf courses. Perennial ryegrass is also popular as a forage grass as it can easily tolerate repeated defoliation from grazing animals (Wilkins, 1991; Pearson et al., 2011). However, the cultivars that are used as turfgrass tend to be distinct from those used for forage, with the former being predominantly diploid and the latter tetraploid (Peterson, 2002). Perennial ryegrass is known for its fast establishment, which is useful for both its turf and forage applications (Grinberg et al., 2016). As a turfgrass, perennial ryegrass is valued for its adaptability to a wide range of soil types and its dark green color (Hannaway et al., 1999; Cen et al., 2016). While perennial ryegrass is incorporated into many seed mixtures due to its positive traits, it is seldom grown by itself because of its sensitivity to a number of environmental stresses, such as heat, drought, and shade stress (Gardner and Taylor, 2002; Peterson, 2002; Tegg and Lane, 2004).

Shade stress in plants is caused by a lack of adequate sunlight to drive photosynthesis and other light-related processes, like phytochrome signaling. Shade stress can be broken down into two
broad categories: artificial and natural. Artificial shade originates from man-made materials, such as buildings, and causes an overall reduction in light intensity. Natural shade comes from overhead vegetation, like forest canopies and not only reduces the overall light intensity but also changes the light quality. Because natural shade originates from photosynthetically active materials, wavelengths of light that are absorbed during photosynthesis are underrepresented in the resultant shade, compared to direct sunlight. Additionally, natural shade tends to create heterogeneous areas of shade, as gaps and inconsistencies exist in leaf coverage within forest canopies that are exacerbated by movement due to wind (Wayne and Bazzaz, 1993). Plants get their energy from the sun, so reducing the amount of photosynthetically active radiation can considerably hamper the growth and development of plants. When plants undergo shade stress they present a number of symptoms which are collectively known as the shade avoidance response (SAR). SAR encompasses many physiological changes to plants, but can be broadly characterized by: weak growth, overly elongated stems and/or leaves, and chlorosis (Franklin and Whitelam, 2005). These symptoms positively correlate with decreasing light intensity as more severe shade stress elicits a more debilitating SAR, ultimately leading to plant death with prolonged exposure to severe shade stress (Nozue et al., 2015; Li et al., 2016). The increased tissue elongation element of SAR is believed to be adaptive as it is generally conserved across plant taxa and the increased growth allows plants to rise above competing vegetation to gain access to unobstructed sunlight (Schwinning and Weiner, 1998). In cereal crop plants, such as maize, shade can inhibit lateral branching, leading to a reduction in overall vegetative biomass (Kebrom et al., 2006; Whipple et al., 2011). Shade has also been shown to reduce grain and seed production, such as maize kernels or Brassica rapa seeds (Page et al., 2010; Procko et al., 2014). Some species are more adapted to shade stress than others are.
Perennial ryegrass has a more severe SAR than many other turf species, exhibiting exaggerated chlorosis and leaf elongation in shade environments (Jiang et al., 2004; Faigón-Soverna et al., 2006). In fact, shade stress has been shown to increase total leaf elongation of perennial ryegrass by 55% while significantly reducing the rate of tiller production (Bahmani et al., 2000). SAR is particularly detrimental in mowed perennial ryegrass swards, where SAR depletes available carbohydrate reserves and contributes to the decline of sward health and appearance. Moreover, SAR is associated with increased vulnerability to disease (Turgeon, 1991). Under shade conditions turfgrass species such as hard fescue (Festuca brevipila) and Supina bluegrass (Poa supina), are recommended over perennial ryegrass due to their improved shade tolerance (Stier, 1999). However, while other turf species can outperform perennial ryegrass under shade conditions, they lack many of the positive traits possessed by perennial ryegrass. Therefore, it is desirable to produce shade tolerant mutants of perennial ryegrass in order to increase its applications in low-light conditions.
1.2 Mutation breeding to produce dwarf and shade tolerant perennial ryegrass mutants

There are a number of techniques for creating new plant traits, ranging from traditional breeding to genetic engineering. Genetic engineering is the most powerful and effective way of introducing new traits to plants, but with it comes concerns regarding the undesirable spread of transgenes through pollen and seeds (Hu et al., 2006; Kausch et al., 2010; Khan et al., 2016). Mutation breeding, one form of traditional breeding, can be effective in creating new plant traits without gene flow concerns, and may be useful for developing shade tolerance (Ahloowalia and Maluszynski, 2001; Shu et al., 2012). Mutation breeding involves the use of a mutagen (ethyl methanesulfonate, x-rays, etc…) to introduce new traits into a germplasm, followed by multiple generations of artificial selection to identify and isolate mutant lines of interest.

Mutation breeding has been widely applied to myriad plant cultivars to improve their traits, including increasing tolerance to a number of abiotic stresses. For example, in one mutation-breeding program for centipedegrass (*Eremochloa ophiuroides*), breeders were able to improve cold tolerance (Dickens et al., 1981). As of 2000, the Food and Agriculture Organization of the United Nations (FAO), working alongside the International Atomic Energy Agency (IAEA) estimated that over 2,600 plant cultivars had been modified using mutation breeding techniques (Maluszynski et al., 2000). Mutation breeding can employ a variety of mutagenesis strategies, from chemical (ethyl methanesulfonate) to irradiation (x-ray, gamma-ray, etc…) and even particle bombardment (fast neutron). These mutagenesis strategies can have drastically different impacts on genomes, ranging from point mutations to large deletions and/or rearrangements. For example, ethyl methanesulfonate (EMS) mostly produces point mutations in the form of nucleotide substitutions, insertions, and deletions ( Sega, 1984), while gamma-ray irradiation results in large (80-6,000 kb) deletions (Naito et al., 2005).
Due to the inherently random nature of mutation breeding, efficient high-throughput screens are required to identify mutant lines presenting phenotypes of interest, and mutation-breeding programs often require the screening of hundreds of thousands of mutant plants. In order to utilize mutation breeding for producing shade-tolerant perennial ryegrass, it is first necessary to devise an effective screening protocol. The Li lab has been working on a mutation breeding program for perennial ryegrass to induce traits deemed important for ornamental applications, chief among them shade tolerance. However, direct screening for shade tolerance can be cumbersome because due to the difficulties of creating a large area with consistent low-light conditions.

To overcome these difficulties, it was proposed that mutant plants first be screened for dwarfism. Putatively dwarf plants would then be screened for shade tolerance (Figure 1.1). Dwarfism, as characterized by reduced leaf elongation is easy to screen for just by visually identifying short seedlings, thereby circumventing the difficulties involved in procuring large areas of consistent shade, as the shade screen step would only be performed on putative dwarf mutants, a much smaller population. The reasoning behind this two-step screening approach is twofold. Firstly, since the main symptom of shade stress, SAR, is characterized by increased leaf elongation it is possible that dwarf plants could prove to also be tolerant to the negative symptoms of shade stress. Secondly, dwarf perennial ryegrass has utility in its own right as dwarfism reduces many of the costs associated with ornamental turf maintenance, such as mowing, fertilizer application, and irrigation.

Towards this end, the lab has treated, irradiated, andbombarded hundreds of thousands of perennial ryegrass seeds, mainly using the cultivar ‘Fiesta 4’ as a background. While three mutagenesis techniques were utilized (EMS, gamma-ray, and fast neutron), gamma-ray
irradiated mutants have since showed the highest frequency of some of the most promising traits for ornamental turf breeding, including dwarfism. Beginning in 2010, the Li lab began irradiating ‘Fiesta 4’ seeds with 9.0 kr doses of gamma rays. This dose corresponded to a 50% lethality rate (lethal dose 50, LD50), as shown by a 50% reduction in germination rates. A LD50 was deemed suitable for mutation breeding purposes as it strikes a good balance between mutation frequency and germination. Irradiated seeds gave rise to the first generation of mutant plants (M1). However, M1 plants are not suitable to be screened for traits of interest, due to a preponderance of background mutations. Therefore, M1 plants were planted in the field and were allowed to randomly cross in order to produce second generation mutants (M2), which were used for trait screening as background mutations would be segregated from mutations of interest (Chen et al., 2016). Perennial ryegrass is self-incompatible therefore only heterozygous mutant plants were produced in the M2 generation.

In order to test the two-step screening method for shade tolerance, the Li lab first tested a direct screen for shade tolerance to act as a comparison. In the fall of 2011, 150,000 M2 perennial ryegrass mutant seeds were directly screened for shade tolerance. The seeds were germinated in the greenhouse under frames covered with black polyfiber cloth that blocked ~95% of incoming sunlight (~100 µmol/m²/s PAR on a sunny day). After two weeks of shade growth, putatively shade-tolerant mutants were identified by a lack of etiolation, a process where seedlings undergo SAR resulting in elongated coleoptiles. This direct screen for shade tolerance identified 305 putatively shade tolerant mutant plants (Table 1.1). These plants were allowed to recover before being rescreened under 6 weeks of 95% shade to eliminate plants that were false positive for shade tolerance. Following this rescreening, only 4 of 305 (1.3%) putative shade tolerant mutants were confirmed to be shade tolerant.
In the spring of 2012, members of the Li lab initiated a two-step screen on a population of 150,000 M2 perennial ryegrass seedlings (Li et al., 2016). M2 seedlings were visually screened for dwarfism at the three-leaf stage (~2 weeks after seeding). Dwarf mutants were identified by a ≥30% reduction in leaf height compared to wild type seedlings (Figure 1.2). The screen identified 51 dwarf mutants from 150,000 M2 seeds, which were grown to maturity and then screened for shade tolerance. Of the 51 dwarf plants, 29 were identified as shade tolerant based on the absence of SAR symptoms after 6 weeks under 95% shade in the greenhouse. The two-step screen was repeated over the summer of 2012 with an additional 150,000 M2 seeds, yielding 85 more dwarf mutants, 36 of which were shade tolerant (Table 1.1).
Figure 1.1 Two-step screen for identifying dominant shade-tolerant mutants of perennial ryegrass. A population of mutagenized seeds (M1) is grown and M2 seeds are harvested. M2 plants are screened for dwarfism at an early stage and dwarf mutants are then screened for shade tolerance. Shade tolerant plants are confirmed by rigorous testing. M2 plants are then crossed with wild type to produce M3 plants, which are then confirmed to be shade tolerant.
Table 1.1 A two-step screen is more effective than a direct screen at identifying shade-tolerant mutants of perennial ryegrass

<table>
<thead>
<tr>
<th>Screen</th>
<th>Time</th>
<th>Seeds used</th>
<th>Putative shade-tolerant mutants(^a)/dwarf mutants(^b)</th>
<th>Confirmed shade-tolerant mutants(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct screen</td>
<td>Fall, 2011</td>
<td>150,000</td>
<td>305</td>
<td>4 (1.3%)(^d)</td>
</tr>
<tr>
<td>Two-step screen</td>
<td>Spring, 2012</td>
<td>150,000</td>
<td>51</td>
<td>29 (56.9%)</td>
</tr>
<tr>
<td></td>
<td>Summer, 2012</td>
<td>150,000</td>
<td>85</td>
<td>36 (42.4%)</td>
</tr>
</tbody>
</table>

\(^a\)M2 seedlings were used for both the direct screen and the two-step screen.
\(^b\)For the direct screen, seeds were germinated under 95% artificial shade in the greenhouse, and seedlings with short coleoptiles, emergent true leaves, and overall reduced heights were considered putatively shade tolerant.
\(^c\)For the two-step screen, dwarf screening was performed at the three-leaf stage, with dwarf mutants identified by a 30% reduction in height compared to wild type, dwarf plants were considered to be potentially shade tolerant.
\(^d\)Confirmation of shade tolerance was performed on mature plants by placing them under 95% artificial shade in the greenhouse.
\(^d\)Percentage of putative shade tolerant or dwarf seedlings that were confirmed to be shade tolerant.
Figure 1.2 M2 seedlings were screened for dwarfism at the three-leaf stage. Dwarf plants (center and right) were identified as those with at least 30% reduction in plant height compared to WT (left).
Chapter 2 Identification and Characterization of the Dwarf, Shade-Tolerant Perennial Ryegrass Mutant *shadow-1*

2.1 Abstract

When subjected to shade, plants undergo rapid shoot elongation, which often makes them more prone to disease and mechanical damage. Shade-tolerant plants can be difficult to breed; however, they offer a substantial benefit over other varieties in low-light areas. Although perennial ryegrass (*Lolium perenne* L.) is a popular species of turfgrasses because of its good appearance and fast establishment, the plant normally does not perform well under shade conditions. Previously, the Li lab developed a two-step procedure for isolating shade-tolerant mutants of perennial ryegrass by first screening for dwarf mutants, and then screening dwarf plants for shade tolerance. One shade-tolerant mutant, *shadow-1*, was characterized in detail and was demonstrated to retain its dwarf and shade tolerant phenotypes after multiple years under both greenhouse and field conditions. Analysis of *shadow-1*’s sexual progeny revealed that the dwarf and shade-tolerant phenotypes segregate together, suggesting that these phenotypes are either caused by the same genetic mutation, or are the result of more than one highly linked mutations. Exogenous applications of gibberellin and gibberellin biosynthesis inhibitors suggested that both of these phenotypes are related to gibberellin signaling. However, hormone content analysis revealed that endogenous gibberellins were increased in light-grown *shadow-1* plants while they were decreased in shade-stressed *shadow-1*, when compared to wild-type plants under the same conditions. These results suggest that two distinct gibberellin-related mechanisms are responsible for dwarfism and shade tolerance in the *shadow-1* mutant line. In the light, *shadow-1* plants appear to be partially insensitive to gibberellins, causing dwarfism,
while in the shade *shadow-I* plants appear to be deficient in gibberellin content, conferring shade tolerance.
2.2 Introduction

Ornamental turfgrass is used around the world to act as a ground cover in open areas, creating a beautiful green landscape in both commercial and residential areas. Turfgrasses are also used to provide a playing surface for many sports. Perennial ryegrass (*Lolium perenne*, L.) is one of the most widely cultivated cool-season turfgrass species, and is valued for its dark green color and its fast establishment. Maintaining large swards of perennial ryegrass, as well as other turfgrass species, can be costly and difficult. In order to maintain the visual appeal of turfgrass swards it is necessary to mow them frequently, to keep them well irrigated, and to apply fertilizer consistently. All of these requirements have large costs associated with them. One way to reduce these costs is to use dwarf turfgrass varieties. These plants have shorter (and/or slower-growing) leaves than non-dwarf varieties and as a result require less frequent mowing, as well as less frequent irrigation and fertilizer applications due to the reduced vegetative biomass (Hanna and Elsner, 1999; Lu *et al.*, 2008, 2009).

Dwarf turfgrass could also have utility for its ability to resist the effect of shade stress. When exposed to shade stress, turf species like perennial ryegrass undergo the shade avoidance response (SAR). SAR is a group of shade-stress symptoms, the most obvious of which are increased leaf-cell elongation and chlorosis. SAR is especially detrimental under mowed conditions where the increased leaf growth results in excessive tissue removal (Casal, 2012). It is possible that the inhibition of leaf elongation caused by dwarfism could conserve carbohydrate and chlorophyll reserves in these tissues, resulting in tolerance to low-light conditions. In fact, it has been reported that chemically-induced dwarfism improves the performance of turfgrass in the shade (Qian and Engelke, 1999; Ervin *et al.*, 2002; Goss *et al.*, 2002; Studzinska *et al.*, 2012), providing circumstantial evidence that some dwarf cultivars could be shade tolerant.
As reported in the previous chapter, identifying dwarfism is simple at the early stages of seedling development, making it possible to be done in a relatively small space over a short time frame. It has been well documented that many dwarf mutants across plant taxa are dominant or semi-dominant (Busov et al., 2008). Other beneficial traits, such as prostrate growth, are also associated with dwarfism in perennial ryegrass (Chen et al., 2016), providing extra utility to dwarf cultivars of this species.

In the late 1990s, researchers began to examine the mutant genes responsible for the various dwarf cultivars of maize, wheat, and rice that were vital to what is now known as the ‘green revolution’ (Peng et al., 1997; Peng et al., 1999; Sasaki et al., 2002). These researchers discovered that in all cases, the dwarfism was due to deficiencies in either the biosynthesis or perception of the phytohormone gibberellin (GA). GAs, which were first identified in fungus and were later successfully isolated from plants, are notable for their ability to stimulate cell division and cell elongation in the stems and leaves of almost all plant taxa (Hedden and Thomas, 2016). GAs are involved in myriad plant processes, but are mainly known for their role in growth and seed germination.

In the previous chapter, it was detailed how the Li lab used a two-step screening process to identify putatively shade-tolerant perennial ryegrass mutant plants. In this chapter, one of these mutant plants, shadow-1, was categorized in its performance under both greenhouse and field conditions. It was found that shadow-1’s dwarfism was maintained through maturity across multiple years. The shade tolerance identified in shadow-1 during its initial shade screening was also verified. The dwarf and shade-tolerant phenotypes were found to segregate together in the progeny of shadow-1 crossed with wild-type perennial ryegrass. Lastly, experiments revealed evidence that GA plays a role in both phenotypes, albeit through different pathways. These
results provide insight into the molecular mechanisms controlling leaf elongation in perennial rye grass under various environmental conditions, and could prove useful to plant breeders interested in introducing dwarfism and/or shade tolerance into their cultivars.
2.3 Results

2.3.1 Evaluating the dwarfism of the shadow-1 perennial ryegrass mutant

As described in Chapter 1, the Li lab produced 136 dwarf mutant perennial ryegrass lines as part of an extensive mutation-breeding program. Of these, 65 lines were also putatively shade tolerant. One dwarf, putatively shade-tolerant mutant, shadow-1, was chosen for detailed characterization based off its degree of shade tolerance during initial screening. In the greenhouse, shadow-1 plants had significantly shorter canopy heights compared to wild type (WT), reaching only 64% of WT height after two months of growth (Figures 2.1A, B).

In order to determine whether the dwarfism of shadow-1 was the result of reduced leaf elongation rates, reduced leaf elongation duration, or both, the growth rates of shadow-1 plants were compared to those of WT. Plants were initially cut to a 5 cm height and the leaf elongation was recorded for both shadow-1 and WT plants over a four-week period, at which point both shadow-1 and WT had achieved maximal leaf growth. shadow-1 leaves elongated at a slower rate than WT leaves, achieving only 85% of WT elongation after 2 days of growth (Figure 2.2). At the end of the four-week period, shadow-1 plants only reached 77% of WT height. These results demonstrate that the dwarfism exhibited by shadow-1 mutant plants is characterized by a reduced leaf elongation rate but not a reduced leaf elongation duration. Interestingly, over this four-week period the leaves of both shadow-1 and WT plants elongated to a greater degree than when previously characterizing canopy heights in two-month-old plants (Figure 2.1B). It is possible that seasonal differences in day length contributed to these differences, as the latter experiment was performed over the winter while the former was performed in the spring.
In the field, *shadow-1* maintained its dwarf phenotype, growing to only 76% of WT height, which was consistent with greenhouse experiments. *shadow-1* also exhibited no reduction in tiller number or root:shoot biomass ratios, compared to WT (Figures 2.1C, D; Table 2.1). This suggests that, while leaf lengths of *shadow-1* mutants were decreased, this was not due to an overall weakness in plant growth and development, but was rather due to differences in a leaf-elongation-specific mechanism.
Figure 2.1 *shadow-1* plants have reduced canopy height compared to the wild-type perennial ryegrass (WT) under full light conditions. (A) Two-month-old, vegetatively propagated WT (left) and *shadow-1* plants (right). (B) Canopy heights of 2-month-old WT and *shadow-1* mutant plants. Data in (B) represent the mean of six replicates; each replicate was one representative plant. Bars represent the standard error. Asterisk represents a significant difference when compared to wild type using two-tailed Student’s *t*-test with pooled variance (*P* ≤ 0.05). (C, D) Field performance of WT (C) and *shadow-1* (D).
**Figure 2.2** *shadow-I* has a reduced leaf elongation rate, but equal leaf elongation duration compared to wild type. Open symbols represent *shadow-I* plants, closed symbols represent wild-type plants. Plants were initially cut to a height of 5 cm. Plants were kept well irrigated and were fertilized every 2 weeks. Asterisk represents a significant difference when compared to wild type using two-tailed Student’s *t*-test (*P* ≤ 0.05).
Table 2.1 *shadow-1* plants are dwarf compared to wild type under full light in the field.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Canopy height (cm) (mean ± SE)</th>
<th>Tiller number (mean ± SE)</th>
<th>Dry root:shoot biomass (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>19.84 ± 0.32</td>
<td>436.33 ± 31.67</td>
<td>0.21 ± 0.050</td>
</tr>
<tr>
<td><em>shadow-1</em></td>
<td>15.12 ± 0.45*</td>
<td>422.00 ± 40.50</td>
<td>0.22 ± 0.026</td>
</tr>
</tbody>
</table>

Each value represents the mean of six replicates. Each replicate consisted of one representative plant. Measurements were taken on May 13, 2013, after 8 months in the field. Asterisks represent a significant difference when compared to wild type using two-tailed Student’s t-test ($P \leq 0.05$). SE, standard error.
2.3.2 Evaluating the shade tolerance of shadow-1

Although initially identified as shade tolerant, shadow-1 plants were screened a second time in order to verify their resistance to shade stress. To this end, shadow-1 plants were subjected to 95% shade (~100 µmol/m²/s PAR on a sunny day) in the greenhouse. After 3 weeks, shadow-1 displayed a reduced shade avoidance response (SAR), as indicated by healthy leaf color and reduced canopy height compared to wild-type (WT) controls (Figures 2.3A, B). To test their degree of shade tolerance, both sets of plants were maintained under shade for an additional 3 weeks. At the end of this 6-week treatment, WT plants suffered severe damage while shadow-1 plants maintained a darker color while suffering less leaf die-off, confirming that shadow-1 plants were indeed shade tolerant under greenhouse conditions (Figure 2.3C).

To test whether shade tolerance could be maintained under field conditions, in a more natural shade setting, shadow-1 was planted alongside WT in a densely wooded section of the field behind the Li lab. Beginning in the spring, after shade reached ~95% (~100 µmol/m²/s PAR on a sunny day), plants were maintained at a 5 cm canopy height, and were cut whenever they reached 7.5 cm. Over a 2-month period, the leaf elongation rate of shadow-1 was significantly reduced compared to WT, as evidenced by a reduced cutting frequency (41.6%; Table 2.2). Under these conditions shadow-1 plants displayed healthy growth, while the leaves of the WT plants suffered severe die off (Figure 2.3D). shadow-1 plants also had more tillers (149.3%) and greater root biomass (125.5%) compared to WT plants (Figure 2.3E; Table 2.2). Similar results were obtained when the experiment was repeated in the subsequent year, with shadow-1 requiring 46.0% cutting frequency compared to WT while maintaining 123.5% tiller number and 145.4% root biomass.
The long-term performance of shadow-1 under shade stress was evaluated by planting shadow-1 plants alongside WT in 85% natural shade in the field (~300 µmol/m²/s PAR on a sunny day). One month after planting, WT plants began to deteriorate, while shadow-1 plants maintained healthy growth (Figure 2.3F). After 30 months (two winters), WT plants had completely died while shadow-1 plants maintained a healthy, green color and produced a number of new tillers (Figure 2.3G).
Figure 2.3 *shadow-1* plants display shade tolerance under both artificial- and natural-shade conditions compared to wild type (WT) under the same conditions. (A) WT (left) and *shadow-1* plants (right) after 3 weeks under 95% artificial shade in the greenhouse. (B) Canopy heights of WT and *shadow-1* plants after 3 weeks under either full light or 95% artificial shade from two separate years. Data represent the average of six replicates. Each replicate consisted of the average height within each pen pack. Error bars represent the standard error. (C) WT (left) and *shadow-1* plants (right) after 6 weeks under 95% artificial shade. (D) WT (left) and *shadow-1* (right) plants after 2 months under 95% natural shade in the field. (E) The root system of WT (left) and
shadow-1 (right) plants after 2 months under 95% natural shade in the field. (F, G) WT (left) and shadow-1 (right) plants under 85% natural shade in the field after 1 month (F) and 30 months (G).
Table 2.2 *shadow-1* plants outperform wild type under 95% natural shade in the field.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tiller number (mean ± SE)</th>
<th>Root biomass (g) (mean ± SE)</th>
<th>Cutting frequency (per month) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Year 2013</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>25.67 ± 2.52^a</td>
<td>0.47 ± 0.03^a</td>
<td>2.67 ± 0.67^a</td>
</tr>
<tr>
<td>shadow-1</td>
<td>38.33 ± 1.53^b</td>
<td>0.59 ± 0.05_b</td>
<td>1.11 ± 0.39^b</td>
</tr>
<tr>
<td><strong>Year 2014</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>28.33 ± 2.08^a</td>
<td>0.44 ± 0.05^a</td>
<td>2.89 ± 0.39^a</td>
</tr>
<tr>
<td>shadow-1</td>
<td>35.00 ± 5.57^b</td>
<td>0.64 ± 0.03^b</td>
<td>1.33 ± 0.39^b</td>
</tr>
</tbody>
</table>

Each value represents the mean of six replicates. Each replicate consisted of one representative plant. Data were collected in July of 2013 and 2014, after plants spent 10 months in the field. Values in the same column followed by the same letter are not significantly different from each other according to Fisher’s protected LSD (*P* = 0.05). SE, standard error.
2.3.3 Confirming dwarfism, shade tolerance, and phenotypic dominance in *shadow-1* progeny

*shadow-1* plants were backcrossed to wild type (WT), generating a population of progeny plants, in order to determine the inheritance of the dwarf and shade-tolerance phenotypes exhibited by *shadow-1*. It was hypothesized that these phenotypes were dominant, as they were identified in second-generation (M2) mutants, and since perennial ryegrass is self-incompatible it is highly improbable that these phenotypes would be recessive as self-crossing is required for recessive traits to present in progeny. It was also hypothesized that the dwarf and shade-tolerant phenotypes were caused by a single mutation, as *shadow-1* was identified using a two-step screening method which relies upon screening for dwarfism prior to screening for shade tolerance on the assumption that dwarfism confers shade tolerance to plants by virtue of its reduced leaf elongation.

A random sample of 200 progeny plants, produced from backcrossing *shadow-1* to WT, were screened and it was found that 106 were dwarf (53%), which demonstrated that dwarfism was dominant in *shadow-1* plants according to Mendelian inheritance patterns. The dwarf progeny were determined to have the same degree of dwarfism as parental *shadow-1* plants, while the non-dwarfs were of a similar height to WT (Figure 2.4A).

The dwarf progeny were then subjected to shade stress, to see whether these progeny also displayed the shade tolerant phenotype found in parental *shadow-1* plants. Following exposure to shade stress, dwarf progeny were found to be shade tolerant, as evidenced by reduced chlorosis and reduced leaf elongation under 95% shade stress in the greenhouse (Figure 2.4B). These results suggest that the dwarf and shade tolerant phenotypes exhibited by *shadow-1* segregate
together, meaning that both are likely caused by the same mutation. However, it is possible, albeit unlikely, that these phenotypes are the result of two or more tightly linked mutations.
Figure 2.4 *shadow-1* plants successfully pass down dominant dwarfism and shade tolerance to progeny. (A) Canopy heights of 2-month-old WT, *shadow-1*, Non-dwarf progeny, and dwarf progeny under full light. Bars represent the standard error. Bars with the same letter above them are not significantly different from each other according to Fisher’s protected least significant difference (*P* = 0.05). (B) Appearances of WT (left), *shadow-1* (center), and dwarf progeny (right) after 2 weeks under 95% artificial shade. Non-dwarf progeny and dwarf progeny were from crosses between *shadow-1* plants and wild-type plants.
2.3.4 Determining the role of gibberellin (GA) in the dwarfism and shade tolerance exhibited by shadow-1

It has been well documented that many dominant dwarf mutants have defects in GA pathways (signaling or metabolic; Hedden, 2016), therefore mutant plants were treated with exogenous gibberellic acid (GA3) in an attempt to characterize the mechanism(s) behind shadow-1’s phenotypes. The exogenous GA treatment experiment was conducted by spraying wild type (WT) and shadow-1 plants with a 50 mg/L GA3 solution. This dose was sufficient to restore the canopy height of shadow-1 to that of untreated wild-type plants (Figures 2.5A, B, E). A dwarf phenotype could also be recreated in WT plants through the application of a GA biosynthesis inhibitor, trinexapac-ethyl (TE). WT treated with TE showed a significant reduction in canopy height, mirroring the dwarf phenotype exhibited by shadow-1 (Figures 2.5C–E).

To further study the involvement of GA in the dwarfism displayed by shadow-1, shadow-1 and WT plants were treated with one of a 50, 100, or 150 mg/L solution of GA3. The heights of shadow-1 and WT plants were then measured following each treatment (Figure 2.6D). Doses of exogenous GA3 in excess of 100 mg/L had no additional effect on the heights of either shadow-1 or wild-type plants. However, at all GA3 concentrations the canopy heights of shadow-1 were lower than those of the wild-type plants. In other words, the canopy heights of shadow-1 could not reach those of the GA3-treated WT plants even at the highest GA concentration used (i.e., 150 mg/L), even though canopy heights of both WT and shadow-1 had individually plateaued (Figure 2.6D). These results suggest that shadow-1 plants have a reduced response to GA compared to WT, and thus reduced GA sensitivity could play a role in the dwarfism exhibited by shadow-1 plants.
Exogenous GA applications were also used to abolish shade tolerance in *shadow-1* plants, in a similar way to the chemical’s ability to abolish light-grown *shadow-1*’s dwarfism. Following GA$_3$ treatment, *shadow-1* plants lost shade tolerance – as evidenced by their increased leaf elongation and chlorosis, compared to untreated *shadow-1* (Figures 2.7A, B). Conversely, when TE was used to block GA biosynthesis in WT plants, they gained shade tolerance comparable to *shadow-1* plants (Figures 2.7A, B). These results provide additional evidence that the dwarfism and shade tolerance displayed in *shadow-1* are due to impaired GA signaling.

However, these experiments present an inherent contradiction. It was demonstrated that exogenous GA applications are sufficient to abolish both dwarfism and shade tolerance in *shadow-1* plants, which suggests that GA deficiency could be the root cause of these phenotypes (Figures 2.5 and 2.7). However, evidence was also presented suggesting that GA insensitivity could play a role in at the dwarfism presented by *shadow-1* plants (Figure 2.6). Additionally, progeny analyses demonstrated that dwarfism and shade tolerance segregate together, making it likely that the two share the same genetic mechanism (Figure 2.4). In order to clarify these seemingly contradictory results, it is important to gain insight into endogenous GA levels within *shadow-1* and WT plants.

Towards that end, the endogenous GA$_1$ content of both *shadow-1* and wild-type plants was analyzed under both full-light and 95% shade conditions. GA$_1$ is one of the main bioactive GAs in higher plants, and is the predominant bioactive form in monocots. GA$_1$ serves as a good marker for total bioactive GA content in plants as it shares the same biosynthesis pathway as the other major bioactive from of GA (GA$_4$) (Davière and Achard, 2013; Hedden and Thomas, 2016). Additionally, GA$_1$ is structurally very similar to GA$_3$, containing only one less double bond on one of its two carbon rings, meaning that the binding properties of the two chemicals are
almost identical (Hedden and Thomas, 2016). Endogenous GA\textsubscript{1} content analysis uncovered an interesting phenomenon in \textit{shadow-1} plants. Under shade-stress conditions, GA\textsubscript{1} content was decreased in \textit{shadow-1} plants, compared to WT, suggesting that GA deficiency is the main cause of shade tolerance in these plants (Figure 2.8). However, for light-grown \textit{shadow-1} GA\textsubscript{1} content was actually increased compared to WT controls, suggesting that partial GA insensitivity is indeed responsible for the dwarfism exhibited by \textit{shadow-1} plants (Figure 2.8). These data are consistent with the results presented in Figure 2.6 that suggest that light-grown \textit{shadow-1} plants are partially insensitive to GA. Not surprisingly, GA insensitive plant mutants of other species, such as the \textit{gai-1} mutant of \textit{Arabidopsis}, have also been reported to contain increased levels of endogenous GA (Peng \textit{et al}., 1997). These results demonstrate that the dwarf and shade-tolerant phenotypes exhibited by \textit{shadow-1} plants have different mechanisms, although both appear to involve GA and are likely caused by the same mutation.
Figure 2.5 Applications of gibberellic acid (GA$_3$) to *shadow-I* and trinexapac-ethyl (TE) to WT reveal a connection between GA and dwarfism. (A) WT plant grown under full light with no chemical treatment. (B) *shadow-I* plant grown under full light, treated with 50 mg/L GA$_3$. (C) *shadow-I* plant grown under full light with no chemical treatment. (D) WT plant grown under full light, treated with 200 mg/L TE. (E) Canopy heights of treated and untreated WT and *shadow-I* plants. Data represent the average of six replicates under that treatment. Each replicate consisted of one plant. All photographs were taken 3 weeks after chemical application. Bars represent the standard error. Bars with the same letter above them are not significantly different from each other according to Fisher’s protected least significant difference ($P = 0.05$).
Figure 2.6 Application of exogenous GA and detection of endogenous GA reveal GA insensitivity in *shadow-1* plants. (A–C) WT (left) and *shadow-1* (right) plants treated with 50 mg/L GA$_3$ (A), 100 mg/L GA$_3$ (B), or 150 mg/L GA$_3$ (C). (D) Comparison of canopy heights for *shadow-1* and WT treated with varying concentrations of GA$_3$. Each data point represents the average height of six replicates. Each replicate consisted of one plant. All photographs and data were taken 3 weeks after GA$_3$ application. Data represent the average of two replicates under each treatment. Each replicate consists of the pooled leaf samples from 10 plants. Error bars represent the standard error.
Applications of gibberellic acid (GA$_3$) to *shadow-1* and TE to WT demonstrate a link between shade tolerance and GA. (A) Untreated WT (far left), GA$_3$-treated *shadow-1* (center left), untreated *shadow-1* (center right), and TE-treated WT (far right) after 2 weeks under 95% artificial shade. (B) Canopy height comparisons between all lines and treatments shown in (A). Plants were allowed to grow in full light for 3 weeks after chemical application before they were placed under artificial shade. All photographs were taken 2 weeks after shade treatment. Data represent the average of six replicates. Each replicate consisted of one plant. Bars represent the standard error. Bars with the same letter above them are not significantly different from each other according to Fisher’s protected least significant difference ($P = 0.05$).
Figure 2.8 GA1 content was elevated in *shadow-1* under full-light conditions, and reduced in *shadow-1* under shade conditions. Error bars represent standard error. Asterisk represents a significant difference when compared to wild type using two-tailed Student’s *t*-test (*P* ≤ 0.05). WT-L, wild type plants kept under full-light conditions, S1-L, *shadow-1* plants kept under full-light conditions, WT-S, wild type plants treated with shade; S1-S, *shadow-1* plants treated with shade.
2.4 Materials and Methods

**Greenhouse and Field Evaluation of shadow-1 Plants under Full Light**

*shadow-1* and wild-type plants were vegetatively propagated by cutting the roots and shoots to a 2.5 cm length to insure uniformity, after which two tillers were placed in each plug of a 50-plug tray containing Pro-Mix soil. After growing for 2 months, plants were photographed and height data were taken. Six plugs of 2-month-old *shadow-1* and wild type were transferred to the field in September of 2012 after which they were fertilized and irrigated as needed. Canopy height was measured and photographs were taken on May 13, 2013, after which they were removed from the field. The plants were then dried at 70°C for 10 days. Dry plants were cut below the crown so that the roots and shoots of each plant could be weighed separately. Data were reported as the mean of the six replicates. Comparisons of means between *shadow-1* and wild-type data collected from greenhouse- and field-grown plants were conducted using two-tailed Student’s *t*-test with the pooled variance (Steel *et al*., 1997).

**Evaluation of shadow-1 Plants under 95% Shade in Greenhouse**

*shadow-1* and wild-type plants were vegetatively propagated in rectangular pots (15 cm × 11 cm × 5 cm). Plants were first cut to a 2.5 cm root and shoot length, and six groups (two tillers each) were evenly spread within each of six pots for both *shadow-1* and wild type. Plants were maintained at a 5 cm height in full light for 6 weeks and then placed in a 95% shade environment (~100 μmol/m²/s PAR on a sunny day, verified with a MQ-100 Quantum Meter, Apogee Instruments, Logan, Utah, USA) in the greenhouse which was created by the use of black polyfiber cloth. Leaf lengths were recorded after 3 weeks of shade treatment as the average within each pot. These lengths were then combined and averaged between the six replicates.
Evaluation of shadow-1 Plants under 95% Shade and 85% Shade in Field

shadow-1 and wild-type plants were propagated in 50-plug trays as described above and were subsequently allowed to grow in full light for 6 weeks. Six plugs from both shadow-1 and wild type were planted in the field at the beginning of September in 2012 and 2013, in a wooded area where shade was measured to be, on average, a 95% reduction in full light. In late May, 2013 and 2014, plants were cut to 5 cm and maintained at that height for the next 7 weeks (plants were mowed to 5 cm whenever they reached a height of 7.5 cm). At the beginning of July in each year, plants were dug up from the field and their tiller numbers were counted. Plants were then left to dry at 70°C for 10 days after which plants were cut below the crown and the dry root mass was weighed. Data were reported as a mean of the six replicates. Analysis of variance was performed on data collected 2013 and 2014 using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences ($P < 0.05$) were observed, Fisher’s protected least significant difference test ($P = 0.05$) was performed to calculate differences between treatments.

Six shadow-1 and wild type plugs were planted in a wooded area on May 10, 2013, where shade was measured to be a ~85% reduction in full light. The plants were left in that area and a representative replicate was photographed on June 11, 2013 and again on October 24, 2015.

Evaluation of shadow-1 Progeny Plants for Dwarfism and Shade Tolerance

shadow-1 and wild-type plants were vegetatively propagated in 50-plug trays as described above. On September 25, 2012, five plugs of shadow-1 and four plugs of wild type were planted in the field in a 3-plug by 3-plug square. Plugs were randomly arranged within each square. Plugs were spaced 46 cm apart in each row, and 18 cm apart in each column. A plastic-wrapped cage was
placed over the square to prevent undesired cross-pollination. Seeds were harvested separately from each plant on June 30, 2013, air-dried at room temperature, and stored at 4°C.

Two hundred shadow-1 progeny seeds were planted in a 28 cm × 56 cm tray of Pro-Mix soil and were cold treated at 4°C for 2 weeks before being moved into the greenhouse. At the three-leaf stage, 200 individuals were randomly selected and transferred to plug trays, along with 10 wild-type and shadow-1 M2 plants, and were allowed to grow for 2 months, after which height data were recorded. Progeny were divided into two groups: Non-dwarf progeny and dwarf progeny. Analysis of variance was performed on data collected from each set of plants using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences (P < 0.05) were observed, Fisher’s protected least significant difference test (P = 0.05) was performed to determine differences between groups. Six representative individuals were selected from the shadow-1 dwarf progeny, in addition to six shadow-1 M2 and six wild-type plants. These plants were vegetatively propagated in 50-plug trays as described above. Plants were maintained at a 5 cm height in full light for 6 weeks, and then placed in a 95% shade environment within the greenhouse for 2 weeks, after which photographs were taken.

**Application of GA₃ to shadow-1 Plants and TE to Wild-Type Plants**

shadow-1 and wild-type plants were vegetatively propagated in 50-plug trays as described above. Plants were maintained for 6 weeks after which they were cut down to a height of 5 cm. The plants were then separated into four groups, each containing six plugs of both shadow-1 and wild type. The plants were sprayed with a GA₃ solution, with different concentrations for each group (50, 100, and 150 mg/L, and water control). Plants were allowed to grow in the greenhouse under full light for 3 weeks, after which pictures and height data were taken. When testing GA₃-treated shadow-1 for shade tolerance, plants were prepared in the same manner as for full light
GA₃ application and separated into two groups. The first consisted of *shadow-1* plants treated with a 50 mg/L GA₃ solution. The second consisted of wild-type plants treated with water, as a control. Three weeks after treatment, plants were cut to 5 cm and placed in a 95% shade environment within the greenhouse. After 2 weeks, photographs and height data were taken.

Wild-type and *shadow-1* plants were vegetatively propagated in 50-plug trays as described above. After 6 weeks, plants were cut to a height of 5 cm. Six plugs of both wild type and *shadow-1* were selected. Wild-type plants were treated with a 200 mg/L trinexapac-ethyl (TE) solution. *shadow-1* plants were treated with water, as a control. Plants were allowed to grow under full light in the greenhouse for 3 weeks, after which pictures and height data were taken. When testing TE-treated wild-type plants for shade tolerance, six wild type and *shadow-1* plugs were prepared and treated as described above. Three weeks after treatment, plants were cut to 5 cm and placed in a 95% shade environment within the greenhouse. After 2 weeks, photographs and height data were taken.

Analysis of variance was performed on height data collected from wild-type and *shadow-1* plants for both treatments as well as a non-treatment control under either full light or 95% shade using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences (*P* < 0.05) were observed, Fisher’s protected least significant difference test (*P* = 0.05) was performed to determine differences between groups.

**Quantification of GA₁ Content**

Wild type and *shadow-1* plants were vegetatively propagated in 50-plug trays as described above and kept in the greenhouse. Plants were allowed to grow for 6 weeks before the experiment was initiated. The elongation zone (where active cell division/elongation occurs) of tillers were
collected from wild type and _shadow-1_ plants kept under either full light or 95% shade for 3 weeks. Leaf samples from 10 plants were pooled for each replicate. Three biological replicates were analyzed for each genotype and treatment. GA extractions were handled in the same manner as described for Kentucky bluegrass (_P. pratensis_) with modifications to include GA isoforms (Krishnan and Merewitz, 2015; Krishnan _et al._, 2016). About 200 mg of frozen leaf samples were freeze dried and then ground to a fine powder. Prior to extraction, 100 nmol of deuterium-labeled GA1 was added as an internal standard for liquid chromatography (LC) analysis. GA1 content analysis was carried out using an ultra-high-performance LC-tandem mass spectrometer (UPLC/MS/MS) (Quattro Premier XE ACQUITY Tandem Quadrupole; Waters, Milford, MA, USA). Data were reported as a mean of three biological replicates. Analysis of variance was performed on GA1 content data collected from wild-type and _shadow-1_ plants under both full light and 95% shade using IBM SPSS 19.0 (IBM Corp., Somers, NY, USA). When sufficient differences (_P_ < 0.05) were observed, Fisher’s protected least significant difference test (_P_ = 0.05) was performed to calculate differences between groups.
2.5 Discussion

In this chapter *shadow-1* mutant perennial ryegrass was demonstrated to be both dwarf and shade tolerant. Under full-light conditions *shadow-1* plants have reduced leaf elongation rates and reduced overall leaf lengths compared to wild-type (WT) kept under the same conditions. This reduction in leaf elongation does not appear to be accompanied by negative traits, such as reduced tiller number or reduced proportional root biomass. Following exposure to shade stress, *shadow-1* plants are resistant to the effects of the shade avoidance response (SAR), compared to shade-stressed WT, with reductions in both leaf elongation and chlorosis. *shadow-1* plants are also able to survive better than WT when exposed to long-term shade stress, be it from artificial or natural sources. These traits can be stably inherited by the sexual offspring of *shadow-1* in a dominant manner and appear to segregate together, with half of *shadow-1* progeny displaying the same degree of dwarfism and shade tolerance as parental plants. These results suggest that *shadow-1* has utility for turf breeding programs aimed at introducing dwarfism and/or shade tolerance into perennial ryegrass cultivars.

By itself, dwarfism can be a highly desirable trait for turfgrass because dwarf cultivars require less frequent mowing and can therefore reduce costs associated with lawn maintenance (Johnson, 1994). It also seems possible that dwarf mutants may require less frequent irrigation and fertilizer application, as they have reduced vegetative biomass. There are other beneficial phenotypes associated with dwarfism in turfgrass, such as drought tolerance (Lu *et al.*, 2009). Additionally, previous reports have shown that, in perennial ryegrass prostrate growth can be associated with dwarfism (Chen *et al.*, 2016a). Prostrate turf varieties are desirable because of their potentially increased heat resistance, traffic resistance, ground coverage, and tolerance to short mowing heights, compared to upright varieties (Youngner, 1969). The dwarfism exhibited
by *shadow-1* is characterized by a reduced leaf elongation rate (LER), without any impact on leaf elongation duration (LED). This means that *shadow-1* grows slowly and has a reduced canopy height compared to WT perennial ryegrass. Because LER is decreased, the metabolic requirements during leaf elongation is substantially decreased, making *shadow-1* an excellent candidate for dwarf breeding programs centered around reducing fertilizer and/or water consumption.

Shade occurs on almost all lawns, and growing healthy lawns under shade conditions is often a challenge for both residential and commercial lawn owners (Koh *et al*., 2003). Shade can be formed by either artificial (e.g., buildings and awnings) or natural (e.g., trees) sources. While artificial shade sources simply reduce light intensity natural sources can also change light quality, as leaves and other green tissues preferentially absorb certain wavelengths of light as a part of photosynthesis (Wherley *et al*., 2005; Takano *et al*., 2009). The *shadow-1* mutant line is highly tolerant to extreme shade (85–95%) from both artificial and natural sources. This means that *shadow-1* can be used in both urban (where artificial shade is prevalent) and rural (where natural shade is prevalent) settings to reduce the negative impact of shade on lawn health and appearance.

*shadow-1* has reduced canopy height compared to wild type, while retaining healthy root mass and tiller number. Reduction in canopy height can reduce mowing frequencies under both normal-light and shade conditions, reducing costs for both landscapers and homeowners. The sexually propagated progeny that inherit the dwarf and shade tolerance phenotypes of *shadow-1* display the same degree of dwarfism and shade tolerance as parental plants. Because these progeny were produced by backcrossing *shadow-1* to wild type, this demonstrates that the mutations responsible for these traits are dominant. Due to perennial ryegrass’s self-
incompatibility, this fact is important for *shadow-1* plants to be readily and easily incorporated into turf breeding programs.

Gibberellin has been shown to play a key role in regulating canopy height in monocots (Ervin and Koski, 1998; Ordonio *et al*., 2014; Ma and Huang, 2016). The data presented in this chapter indicate that the canopy heights of *shadow-1* plants can be artificially restored to those of wild-type controls through the application of exogenous GA₃, suggesting that *shadow-1* mutant plants are GA deficient. However, endogenous GA content analysis revealed that *shadow-1* plants actually had elevated levels of GA under full-light conditions, compared to wild type. This result is consistent with the finding that, following exogenous GA₃ application under full-light conditions, *shadow-1* canopy heights were significantly lower than those of the wild type at the three GA₃ concentrations used. Even at the highest GA₃ concentration used, after the canopy heights plateaued, there remained a height difference between *shadow-1* and wild-type plants. Taken together, these data strongly suggest that *shadow-1* plants are partially insensitive to GA in the light, and this is likely the mechanism behind their dwarfism under these conditions. The phenomenon of higher endogenous bioactive GA contents in GA-insensitive dwarf mutants has also been reported for a number of other plant species, including *Arabidopsis*, maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.) (Talon *et al*., 1990; Dill *et al*., 2001). Furthermore, *shadow-1* plants treated with the GA biosynthesis inhibitor trinexapac-ethyl (TE) displayed no additional reduction in height, under full-light conditions, compared to untreated controls. This suggests that reducing endogenous GA content does not influence dwarfism in these plants. These data further support the hypothesis that partial GA insensitivity is responsible for the dwarfism of *shadow-1* mutants.
The data reveal that exogenous GA application can reduce shade tolerance in *shadow-1* mutants. Meanwhile, wild-type perennial ryegrass treated with TE gained shade tolerance similar to that observed in *shadow-1*. All of these data demonstrate that GA levels can be an important factor for determining a plants ability to tolerate low-light conditions, and that it is possible to manipulate shade tolerance by controlling GA levels in perennial ryegrass. Furthermore, quantification of endogenous GA$_1$ content in *shadow-1* plants revealed a decrease in GA content under shade conditions, suggesting that GA deficiency is the root cause of shade tolerance in these plants.

The results presented in this chapter demonstrate that it is likely that the phenotypes exhibited by *shadow-1* are due to multiple mutations in these plants, with the dwarfism resulting from partial GA insensitivity and the shade tolerance resulting from GA deficiency. While at face value these phenotypes appear to only be tangentially related (they both involve GA), it is possible that they are related. It has been reported that GA can influence its own biosynthesis through negative-feedback loops (Hedden and Thomas, 2016). Therefore, it is possible that the partial insensitivity of *shadow-1* plants could have some impact on altered GA levels under different environmental conditions. Further analysis is required to shed light on the dual genetic mechanisms that give rise to dwarfism and shade tolerance in the *shadow-1* mutant lineage. In the next chapter, the transcriptome of *shadow-1* plants will be examined under both 95% shade and full light conditions. This will provide valuable insight into the genetic mechanisms and hormone pathways involved in the phenotypes exhibited by the *shadow-1* lineage.
Chapter 3 Transcriptome Analysis Reveals Differential Gene Expression and a Possible Role of Gibberellins in *shadow-1*

3.1 Abstract

The molecular basis behind shade tolerance in plants is not fully understood. In the previous chapter, a possible connection was established between shade tolerance and dwarfism via the phytohormone gibberellin (GA). While both likely stem from the same mutation, it appears as though there are different mechanisms that lead to each phenotype with shade tolerance caused by GA deficiency and dwarfism caused by partial GA insensitivity. In order to clarify the connection between these phenotypes, the transcriptome of *shadow-1* was analyzed. 2,200 differentially expressed genes (DEGs) were identified (1,096 upregulated and 1,104 downregulated) in *shadow-1* mutants, compared to wild type, following exposure to shade stress. Of these DEGs, 329 were unique to *shadow-1* plants kept under shade and were not found in any other comparisons. 2,245 DEGs (1,153 upregulated and 1,092 downregulated) were found in *shadow-1* plants, compared to wild-type, under light, with 485 DEGs unique to *shadow-1* plants under light. To elucidate the role of gibberellin in the dwarfism and shade tolerance exhibited by *shadow-1* plants, the expression of GA biosynthesis and response genes were analyzed. In the shade, GA biosynthesis genes were downregulated in *shadow-1* plants, notably gibberellin 20 oxidase (*GA20ox*) which was downregulated to 3.3% (96.7% reduction) of the wild-type expression level. Interestingly, these genes were also downregulated in light-grown *shadow-1*. However, under light conditions *GA20ox*, which is responsible for deactivating bioactive forms of GA, was also downregulated, combatting the impact of decreased GA biosynthesis and leading to an overall increase in bioactive GA content. Under light conditions,
DELLA, a key negative regulator of GA response, was upregulated in shadow-1 plants (283.9% of wild-type expression), which helps explain why these plants were dwarf even though shadow-1 had higher GA levels under these conditions. These data provide valuable insight into a role that GA plays in dwarfism and shade tolerance, as exemplified by shadow-1 plants, and could serve as a guide for plant breeders interested in developing new cultivars with either of these traits.
3.2 Introduction

In plants, shade stress symptoms are collectively categorized as the shade avoidance response (SAR). SAR has been described in previous chapters, with a focus on how it impacts perennial ryegrass (*Lolium perenne*, L.). However, SAR can manifest in different ways depending on species, tissue type, and developmental stage. For example, in seedlings SAR generally takes the form of etiolation, which is characterized by elongation of hypocotyls and petioles and, in some cases, inhibition of cotyledon expansion and reduction in lateral roots (Procko *et al.*, 2014). Petiole elongation is also a symptom of shade stress in adult plants (Kozuka *et al.*, 2005; Sasidharan *et al.*, 2010). Although slightly variable in how they present, these various shade responses are thought to be regulated by light-sensing pigments called phytochromes. Shade conditions reduce the activity of phytochromes, of which phytochromes A and B (PhyA, PhyB) have been shown to be especially important. Phytochromes are responsible for repressing the DNA binding activity of phytochrome interacting factors (PIFs; Park *et al.*, 2012) as well as controlling biosynthesis of gibberellins (GAs; Ogawa *et al.*, 2003). In many plant species, shade response is controlled through various phytohormone response pathways, most notably the GA response pathway (Yamaguchi, 2008; Colebrook *et al.*, 2014).

PIFs are basic helix-loop-helix transcription factors, some of which (PIF3 and PIF4) are mainly responsible for enacting the growth-promoting effects associated with phytochrome suppression (Huq and Quail, 2002; Kim *et al.*, 2003). Some PIFs can act to decrease levels of bioactive GAs, like GA$_1$ and GA$_4$ (Hedden and Thomas, 2016). Once free of PhyB repression, PIFs are able to activate various shade-associated physiological responses, such as stem and petiole elongation, through their activity as transcription factors (Lorrain *et al.*, 2008). DELLA proteins, through
protein–protein interaction, also repress PIF activity (De Lucas et al., 2008; Feng et al., 2008), and some PIFs (PIF1) can upregulate DELLA expression, forming a negative feedback loop. DELLA proteins are degraded after binding to the GA receptor GID1, in the presence of GA, via the E3 ubiquitination pathway (Sun, 2008). The interaction between phytochromes, PIFs, GA, and DELLA, is complex, especially in regards to the activity of PIFs, which can act as positive and/or negative regulators of shade response. In spite of these complexities, it is possible to piece together a rough picture of shade response. Phytochromes suppress GA biosynthesis in light, and DELLA proteins further suppress GA response by subduing the transcription-factor activity of PIFs. The suppression of phytochrome activity in the dark leads to an upregulation of GA biosynthesis. Bioactive GAs are sensed by GID1, which bind to DELLAs, leading to their degradation. Freed from DELLA suppression, PIFs are free to act as transcription factors, ultimately leading to plant presentation of SAR symptoms, notably increased tissue elongation.

GAs are terpenoid products, and GA biosynthesis begins when geranylgeranyl pyrophosphate (GGPP) is catalyzed into ent-copalyl pyrophosphate by ent-copalyl diphosphate synthase. This product is then modified by a number of upstream biosynthesis enzymes, namely: ent-kaurene synthase (KS), ent-kaurene oxidase (KO), and ent-kaurenoic acid oxidase (KAO). The final steps of bioactive GA biosynthesis are catalyzed by gibberellin 20 oxidase (GA20ox) and gibberellin 3 oxidase (GA3ox). The process of deactivation of bioactive GAs is governed by gibberellin 2 oxidase (GA2ox; Hedden and Phillips, 2000; Chen S. et al., 2016).

In the previous chapter, GA content was found to have a potential impact on the shade tolerance exhibited by shadow-1 mutant plants, while partial GA insensitivity appeared to be the major factor influencing dwarfism in these plants under light. In an attempt to uncover the genetic
mechanisms behind dwarfism and shade tolerance in *shadow-1* perennial ryegrass, *shadow-1* and wild-type plants were treated with 95% shade and their transcriptomes were compared against plants kept under full light. Through examination of overall differential gene expression, as well as differential gene expression within the GA biosynthesis and response pathways of *shadow-1* mutant plants, two GA-related mechanisms were implicated in the phenotypes found in *shadow-1*. Under full light conditions, increased expression of *DELLA* is likely responsible for producing a dwarf phenotype, even in the presence of elevated GA levels. Under shade conditions, decreased GA biosynthesis in conjunction with increased GA degradation leads to an overall decrease in bioactive GA levels. This decrease in bioactive GA levels appears to be the mechanism responsible for producing a shade-tolerant phenotype in *shadow-1* plants. These results help to provide insight into the roles that GA biosynthesis and signaling play in reducing leaf elongation in turf species, under both full light and severe shade stress conditions. These insights could provide possible strategies for breeding dwarf and/or shade tolerant plants.
3.3 Results

3.3.1 Sequencing and mapping of the shadow-1 transcriptome

As described in Chapter 2, shadow-1 plants carry two distinct phenotypes, dwarfism and shade tolerance. In order to further examine the genetic mechanisms surrounding these phenotypes, leaf tissues were collected for transcriptome analysis. For this purpose, vegetatively propagated shadow-1 and wild-type (WT) plants were grown in the greenhouse and were then either subjected to two weeks of 95% shade stress, or were kept under full light conditions for two weeks (Figure 3.1). Three biological replicates were collected for shadow-1 and WT under each treatment, for a total of 12 samples. Transcriptome sequencing data were deposited in the NCBI SRA database under the accession number SRP102018. Through sequencing, a total of 657,122,180 raw reads and 633,014,566 clean reads were generated. The average Q20 and Q30 scores for clean reads among all 12 samples were 95.88 and 90.40%, respectively. For these reads, the average GC content was 50.42% (Table 3.1). The perennial ryegrass genome assembled by Byrne et al. (2015) was used as a reference against which the clean reads from each sample were mapped. Around 75% of the clean reads for each sample group were able to be mapped to the reference genome (Table 3.2).

Gene expression among the three biological replicates were compared for all four sample types: light-grown WT, light-grown shadow-1, shade-stressed WT, and shade-stressed shadow-1. The similarity of gene expression profiles between the replicates was determined by a Pearson correlation coefficient analysis. This analysis showed that the three biological replicates for each sample type were highly correlated ($r > 0.96$), demonstrating consistency between replicates in
regards to differential gene expression. This comparison also revealed that plants kept under the same conditions had greater similarities in regards to gene expression, regardless of genotype, than did plants of the same genotype kept under different conditions (Figure 3.2).
Figure 3.1 *shadow-1* plants exhibit a dual phenotype of dwarfism and shade tolerance prior to tissue sampling for transcriptome analysis. (A) Eight-week-old, wild type (left) and *shadow-1* plants (right) grown under full light in the greenhouse. (B) Wild type (left) and *shadow-1* plants (right) after 2 weeks under 95% shade in the greenhouse.
Table 3.1 Summary of sequencing quality

<table>
<thead>
<tr>
<th>Sample</th>
<th>Raw reads</th>
<th>Clean reads</th>
<th>Q20 (%)</th>
<th>Q30 (%)</th>
<th>GC (%)</th>
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<tbody>
<tr>
<td>WT-light-1</td>
<td>56,806,386</td>
<td>57,391,990</td>
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<td>90.95</td>
<td>51.07</td>
</tr>
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<td>WT-light-2</td>
<td>55,033,008</td>
<td>53,234,472</td>
<td>95.81</td>
<td>90.43</td>
<td>50.92</td>
</tr>
<tr>
<td>WT-light-3</td>
<td>56,770,090</td>
<td>54,313,758</td>
<td>95.92</td>
<td>90.28</td>
<td>50.40</td>
</tr>
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<td>shadow-1-light-1</td>
<td>55,896,890</td>
<td>53,696,162</td>
<td>95.96</td>
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<td>50.22</td>
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<td>50.06</td>
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<tr>
<td>shadow-1-light-3</td>
<td>58,432,782</td>
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<tr>
<td>WT-shade-1</td>
<td>52,736,878</td>
<td>50,437,952</td>
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<td>50.66</td>
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<tr>
<td>WT-shade-2</td>
<td>50,532,670</td>
<td>49,147,900</td>
<td>95.89</td>
<td>90.75</td>
<td>50.59</td>
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<td>WT-shade-3</td>
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<td>53,980,982</td>
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<td>55,026,380</td>
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<td>shadow-1-shade-2</td>
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<td>46,160,156</td>
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<td>46,468,404</td>
<td>95.57</td>
<td>89.52</td>
<td>49.90</td>
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<tr>
<td>Total</td>
<td>657,122,180</td>
<td>633,014,566</td>
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<td></td>
</tr>
</tbody>
</table>

Q20, percentage of bases with a Phred value >20; Q30, percentage of bases with a Phred value >30.
Table 3.2 Clean reads were mapped at high percentage to the perennial ryegrass genome

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Total clean reads</th>
<th>Mapped reads (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type light</td>
<td>164,940,220</td>
<td>75.80</td>
</tr>
<tr>
<td>shadow-1 light</td>
<td>166,852,572</td>
<td>75.18</td>
</tr>
<tr>
<td>wild type shade</td>
<td>153,566,834</td>
<td>74.84</td>
</tr>
<tr>
<td>shadow-1 shade</td>
<td>147,654,940</td>
<td>76.64</td>
</tr>
</tbody>
</table>
Figure 3.2 Tissue samples from each shadow-1 and wild type, under full-light or shade conditions, show a high degree of consistency between replicates. Comparisons of gene expression between replicates on the x-axis and those on the y-axis. Pearson correlation
coefficient, as well as a color value (whiter—less similar; redder—more similar), are given for each comparison. WT = wild type; S1 = shadow-1; L1-3 = light-grown samples 1-3; Sh1-3 = shade-stressed samples 1-3.
3.3.2 Differential gene expression

There were 4,022 DEGs identified in shade-stressed wild type (WT) plants, compared to light-grown WT, with 1,392 upregulated and 2,630 downregulated. Similarly, there were 4,067 DEGs (1,374 upregulated and 2,693 downregulated) in shade-stressed shadow-1 plants compared to light-grown shadow-1 (Figure 3.3). There were 2,245 DEGs (1,153 upregulated and 1,092 downregulated) uncovered in light-grown shadow-1 plants, compared to WT under the same conditions. Similarly, there were 2,200 DEGs (1,096 upregulated and 1,104 downregulated) uncovered in shade-stressed shadow-1 plants, compared to WT under the same conditions (Figure 3.3). These results present two sets of striking similarities. For both WT and shadow-1 plants, there were similar differential gene expression patterns when comparing shade-stressed plants to light-grown controls (~2,600 upregulated genes and ~1,400 downregulated genes). On the other hand, when comparing plants of the same genetic background (WT or shadow-1) under shade stress, to light-grown controls there were ~1,100 upregulated genes and ~1,100 downregulated genes. These results, in conjunction with the Pearson correlation coefficient analysis, suggest that the genetic differences between shadow-1 and WT plants result in relatively minor differential gene expression compared to the differential gene expression caused by the physiological processes involved in shade response.

2,668 DEGs (820 upregulated and 1,848 downregulated) were shared between shade-stressed shadow-1 and shade-stressed WT plants, when each were compared to their light-grown counterparts (Figure 3.4A). It is likely that many of these genes are not involved in the shade tolerance exhibited by shadow-1 plants, but instead are representative of the general shade response of perennial ryegrass. 1,240 DEGs (624 upregulated and 616 downregulated) were
shared by *shadow-1* and WT plants when comparing those subjected to shade stress to their respective light-grown controls (Figure 3.4B). It is likely that the expression of the majority of these genes are altered by background mutations and do not represent differences that are linked to *shadow-1*’s shade tolerance. Some of these genes may be involved in *shadow-1*’s dwarf phenotype, assuming that the mutations causing this phenotype result in differential gene expression that is unaffected by differences in light intensity.

Conversely, 1,005 DEGs (529 upregulated, 476 downregulated) were found in light-grown *shadow-1* (compared to wild type), but not shade-stressed *shadow-1*, representing genes whose expression are the most likely to have been impacted by the mutation(s) resulting the dwarfism exhibited by *shadow-1* plants (Figure 3.4B). 960 DEGs (472 upregulated, 488 downregulated) were found in shade-stressed *shadow-1* (compared to wild type), but not light-grown *shadow-1*, representing genes whose expression are the most likely to have been impacted by the mutation(s) resulting the shade tolerance exhibited by *shadow-1* plants (Figure 3.4B).

Alternatively, there were 1,354 DEGs (572 upregulated and 782 downregulated) which were differentially expressed between shade-stressed WT (compared to light-grown controls), but not shade-stressed *shadow-1* (Figure 3.4A). It is possible that the mutation(s) responsible for shade tolerance in *shadow-1* interrupted the WT shade-response by halting differential expression of these genes. 1,399 DEGs (554 upregulated and 845 downregulated) were differentially expressed in shade-stressed *shadow-1* (compared to light-grown controls) but not in shade-stressed WT (Figure 3.4B). It is likely that some of these genes are involved in the shade tolerance exhibited by *shadow-1* plants.
While simple comparisons between pairwise analyses of differential gene expression can help give insight into general trends within the shadow-1 transcriptome under shade stress, or under full-light conditions, a more thorough analysis is required to differentiate between phenotypically-relevant transcriptomic changes and those resulting from background mutations. Therefore, differential gene expression was simultaneously compared between all four pairwise comparisons of transcriptomes (shade-stressed vs light-grown shadow-1, shade-stressed vs light-grown WT, shade-stressed shadow-1 vs shade-stressed WT, and light-grown shadow-1 vs light-grown WT) (Figure 3.4C). This four-way comparison exposed 329 DEGs that were unique to shade-stressed shadow-1 (compared to shade-stressed WT), and 485 DEGs that were unique to light-grown shadow-1 (compared to light-grown WT). There were also 820 DEGs that were unique to shade-stressed WT plants (compared to light-grown WT), and 889 DEGs that were unique to shade-stressed shadow-1 plants (compared to light-grown shadow-1). There were 87 DEGs that were shared between all four pairwise comparisons.

The examination of these DEGs gives us some insight into raw numbers of genes involved in the regulating the dwarf and/or shade-tolerant phenotypes exhibited by shadow-1, compared to those resulting from background mutations. In simple pairwise comparisons there appears to be well over 2,000 DEGs when comparing the transcriptomes of shadow-1 and WT under either shade-stress or light-grown conditions (Figure 3.3). However, the two-way and four-way analysis of these pairwise comparisons (Figure 3.4) demonstrate that the vast majority of these differences result from background mutations, and are not involved in either shadow-1’s dwarfism nor its shade tolerance. Direct exploration of the function of the DEGs uncovered through these analyses, through examination of functional annotation, proved unproductive. This was mainly due to the fact that the perennial ryegrass genome is very poorly annotated, and even when
annotation is present it is based on predicted molecular function (kinase, hydrolase, etc…) rather than involvement in specific physiological processes.

In an effort to better explore the function of DEGs identified in *shadow-1* plants (compared to WT) under shade-stress and light-grown conditions, gene ontology (GO) enrichment analysis was performed. The enriched GO distributions were similar for *shadow-1* plants subjected to shade stress and those kept in the light (compared to WT under the same conditions), speaking to the relatively minor impact of *shadow-1*’s mutations compared to natural shade-response processes. However, there were a few notable differences. Some of the genes from the GO groups that showed differences were analyzed and the associated proteins were queried against the NCBI non-redundant protein database. DEGs involved in “biological adhesion” and “receptor activity” for *shadow-1* plants kept under light were absent in shade-stressed *shadow-1* plants (Figure 3.5). The “biological adhesion” group included a gene that coded for ERECTA, a receptor-like kinase, and was upregulated (985%) in *shadow-1* plants compared to WT. The “receptor activity” group included a gene that coded for phytochrome A (PhyA), a light receptor, which was also upregulated (724%) in *shadow-1* plants under light. For another GO group, “extracellular region part,” light-grown *shadow-1* plants contained only downregulated DEGs, while shade-stressed *shadow-1* plants had both up- and downregulated DEGs (Figure 3.5). These results suggest the involvement of the phytochrome-signaling pathway in at least the dwarf phenotype exhibited by *shadow-1*, which also provides some evidence for the involvement of the phytohormone gibberellin (GA), as phytochrome and GA signaling are closely related.
Figure 3.3 Comparisons of differentially expressed genes (DEGs) between *shadow-1* and wild type (WT) under 95% shade-stress and light-grown conditions. Each set of bars represents a comparison between two sample types. WT-L = light-grown WT; WT-S = WT treated with 95% shade stress; S1-L = light-grown *shadow-1*; S1-S = *shadow-1* treated with 95% shade stress.
Figure 3.4 Overlap of differentially expressed genes (DEGs) across treatments (shade-stressed vs light-grown) and genotypes (shadow-1 vs wild type (WT)). (A) Venn diagram comparing DEGs identified in WT following shade stress (left), to those identified in shadow-1 following shade stress (right). The overlapping region represents DEGs shared between shadow-1 and WT following shade stress. (B) Venn diagram comparing DEGs identified in light-grown shadow-1 (left) to those identified following shade stress (right). The overlapping region represents DEGs shared between light-grown and shade-stressed shadow-1 plants. (A, B) Up-arrows signify upregulated DEGs and down-arrows signify downregulated DEGs. (C) Four-way Venn diagram including all pairwise comparisons from (A) and (B). WT-L = light-grown WT; WT-S = WT treated with 95% shade stress; S1-L = light-grown shadow-1; S1-S = shadow-1 treated with 95% shade stress.
Figure 3.5 Functional gene classification of differentially expressed genes (DEGs). (A) Gene Ontology (GO) distribution of DEGs identified in shadow-1 plants compared to wild type kept under full light. (B) GO distribution of DEGs identified in shadow-1 plants compared to wild type after shade treatment. Black boxes highlight differences between specific GO terms in (A) and (B). WT-L = light-grown WT; WT-S = WT treated with 95% shade stress; S1-L = light-grown shadow-1; S1-S = shadow-1 treated with 95% shade stress.
3.3.3 Differentially expressed genes in the gibberellin pathway

In chapter 2, the dwarfism and shade-tolerance phenotypes displayed in shadow-1 were both connected to gibberellin (GA), albeit through different mechanisms. For light-grown shadow-1 plants, endogenous GA levels were elevated when compared to wild type (WT), and there was some evidence that these plants were partially insensitive to the effects of exogenously applied GA. On the other hand, shade-stressed shadow-1 plants contained decreased levels of endogenous GA compared to shade-stressed WT. In the previous section of this chapter, gene ontology (GO) enrichment analysis suggested that expression of phytochrome A (PhyA) was altered in light-grown plants, providing an additional link between GA signaling and the mutations found in shadow-1 plants. Therefore, the expression levels of GA-related genes were explored, including those involved in GA biosynthesis and bioactive GA degradation. The expression of DELLA, the gene which is responsible for repression of GA signaling, was also examined.

As stated previously, the perennial ryegrass genome is poorly annotated, therefore in most cases direct analysis of differentially expressed genes (DEGs) involved in GA-related processes is difficult. However, GA-related genes are well characterized in bread wheat (Triticum aestivum, L.), a close relative of perennial ryegrass. To uncover DEGs within the GA biosynthesis pathway, protein sequences for enzymes catalyzing key steps of GA biosynthesis were identified in bread wheat (Table 3.3). These proteins were used in conjunction with a basic local alignment search tool (BLAST) to search for homologs within the translated perennial ryegrass reference genome. Using this technique, it was possible to identify putative homologs for most of the upstream GA biosynthesis genes in perennial ryegrass. As shown in Figure 3.6, GA biosynthesis genes were downregulated in shadow-1 plants (compared to WT) under both light and shade.
conditions. Under light, the GA biosynthesis genes, \textit{CPS}, \textit{KS}, \textit{KO}, and \textit{KAO}, were
downregulated to 24.4–84.7\% of their levels in WT plants. Under shade stress, these genes were
more severely downregulated in \textit{shadow-1} plants (17.4–61.4\% of WT expression). The
penultimate step of GA biosynthesis is governed by GA20ox, and unlike the upstream GA
biosynthesis genes \textit{GA20ox} has been identified in perennial ryegrass making it significantly
easier to identify in the transcriptome data (Table 3.3). In shade-stressed \textit{shadow-1} plants
\textit{GA20ox} expression was reduced to 3.3\% of its WT expression, while in light-grown \textit{shadow-1} it
was only reduced to 39.0\% relative to WT expression. Putative homologs of \textit{GA3ox}, which is
responsible for governing the final step of GA biosynthesis, were not to be identified in perennial
ryegrass.

Based on previous endogenous GA content analyses, GA biosynthesis genes were expected to be
be upregulated in light-grown \textit{shadow-1} plants. Therefore, it was surprising to see that they were
actually downregulated light-grown \textit{shadow-1}. This seems to directly contradict the GA content
analysis presented in Chapter 2. However, GA biosynthesis is only one factor determining
bioactive GA content in plants. The other major factor is the rate of degradation of bioactive GA
molecules, which is performed by the protein GA2ox. Luckily, \textit{GA2ox} has been identified in
perennial ryegrass (Table 3.3). When the expression of \textit{GA2ox} was examined in \textit{shadow-1} plants,
it was found to be downregulated in light-grown \textit{shadow-1} plants (11.8\% of WT expression
levels, Figure 3.7A). The downregulation of \textit{GA2ox} in light-grown \textit{shadow-1} plants likely
compensates for the downregulation of GA biosynthesis genes under these conditions, leading to
an overall increase in GA content. Conversely, \textit{GA2ox} was highly upregulated in \textit{shadow-1}
plants under shade conditions (472.1\% of WT expression), which further decreases the bioactive
GA content of shade-stressed \textit{shadow-1} plants (Figure 3.7A).
While these results help to illuminate the factors causing divergence in bioactive GA levels between light-grown and shade-stressed shadow-1 plants, they do not explain why shadow-1 plants are dwarf regardless of increased GA$_1$ content. Therefore, the expression of DELLA was examined, which has been identified in perennial ryegrass (Table 3.3), in an effort to explain this phenomenon. The DELLA protein is a negative regulator of GA response, therefore an increase in DELLA expression could explain why shadow-1 plants are GA insensitive. Also, because DELLA protein is degraded in the presence of bioactive GA, increased DELLA expression should not lead to complete GA insensitivity, only partial insensitivity, which would align with the exogenous GA application experiments performed in Chapter 2. DELLA was upregulated in light-grown shadow-1 plants (283.9% of WT expression), while expression of this gene under shade-stress conditions was virtually identical between shadow-1 and WT plants (Figure 3.7B).
**Table 3.3** Accession numbers of bread wheat proteins and perennial ryegrass genes.

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<thead>
<tr>
<th>Protein name</th>
<th>Abbreviation</th>
<th>Genbank Accession #</th>
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<tr>
<td><em>ent</em>-copalyl diphosphate synthase</td>
<td>CPS</td>
<td>BAH56558.1</td>
</tr>
<tr>
<td><em>ent</em>-kaurene synthase</td>
<td>KS</td>
<td>ADZ55290.1</td>
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<td>KO</td>
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<td><em>ent</em>-kaurenoic acid oxidase</td>
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<table>
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<td><strong>DELLA</strong></td>
<td><strong>DELLA</strong></td>
<td>KP954694.1</td>
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Figure 3.6 The gibberellin biosynthesis pathway was downregulated in *shadow-1*. Arrow boxes represent areas of the pathways catalyzed by specific proteins. Unboxed text represents terpene products at each step in GA biosynthesis pathway. White boxes to the left of each gene show the expression for each gene in light-grown *shadow-1* plants, compared to wild type (WT) plants under the same conditions, with WT expression normalized to 1. Black boxes to the right of each gene show the expression for each gene in *shadow-1* plants treated with shade stress, compared to WT plants under the same conditions, with WT expression normalized to 1. CPS = ent- copalyl diphosphate synthase; KS = ent-kaurene synthase; KO = ent-kaurene oxidase; KAO = ent-kaurenoic acid oxidase.
Figure 3.7 GA-deactivation and -signaling genes were differentially regulated in *shadow-1* compared to wild type (WT). (A) *GA2ox*, which is responsible for deactivating bioactive forms of GA was downregulated in *shadow-1* plants under light conditions, but was upregulated in *shadow-1* under shade conditions. (B) *DELLA*, the negative regulator of GA signaling was upregulated in *shadow-1* under light conditions. Gene expression levels were calculated using reads per kilobase of transcript per million mapped reads (RPKM). Data represent means from three independent biological replicates. WT-L = light-grown WT; WT-S = WT treated with 95% shade stress; S1-L = light-grown *shadow-1*; S1-S = *shadow-1* treated with 95% shade stress.
Asterisk represents a significant difference when compared to wild type under the same conditions using two-tailed Student’s $t$-test ($P \leq 0.05$).
3.3.4 Verification of differentially expressed genes via qRT-PCR

The accuracy of the transcriptome data was verified by selecting two genes (KAO and GA20ox) for qRT-PCR analysis, using mRNA extracted from shade-treated wild type (WT) and shadow-1 plants. The results of qRT-PCR analysis showed similar expression patterns to those obtained from our transcriptome analysis (Figure 3.8). Transcriptome analysis demonstrated that, KAO was downregulated in shadow-1 to 43.3% of its wild-type expression, while qRT-PCR showed downregulation to 49.7%. For shade-treated shadow-1, GA20ox was downregulated to 3.3% of its expression in wild type. The expression of GA20ox in shade-stressed shadow-1 plants corresponds to only 0.03 mapped reads (RPKM) over millions of sequencing reactions, which is considered barely detectable. Consistently, it was not possible to detect expression of this gene in shade-stressed shadow-1 plants using qRT-PCR analysis. In summary, the expression patterns uncovered via transcriptome analysis were consistent with those uncovered via qRT-PCR analysis, demonstrating that the transcriptome data from Illumina sequencing analysis are reliable.
Figure 3.8 qRT-PCR data verified the accuracy of transcriptome analysis. Expression levels of KAO (A) and GA20ox (B) were identified through transcriptome (left) and qRT-PCR (right) analyses. For transcriptome analysis, gene expression levels were calculated using reads per kilobase of transcript per million mapped reads (RPKM). qRT-PCR expression levels in each sample were normalized using the expression level of the internal control, LpGAPDH, in the
same sample, and wild-type expression levels were normalized to 1. Transcriptome expression levels were calculated using reads per kilobase of transcript per million mapped reads (RPKM). The qRT-PCR data presented are the means from three independent biological replicates. Error bars represent the standard error. WT-S = WT treated with 95% shade stress; S1-S = shadow-1 treated with 95% shade stress.
3.4 Materials and Methods

Plant Treatment and Tissue Sampling

*shadow*-*1* and wild type (WT) plants were vegetatively propagated in rectangular pots (15 cm × 11 cm × 5 cm). Plant roots and shoots were cut to 2.5 cm and six groups of two tillers were evenly spread within each pot. Plants were maintained at a 5 cm height in full light for 6 weeks. Individuals selected for shade-stress treatment were placed in a 95% shade environment in the greenhouse, which was created by the use of black polyfiber cloth as described in Chapter 2. Those selected for full-sunlight treatment were left out in the open in the greenhouse. After growing for an additional 2 weeks under either light or 95% shade, leaf tissue was collected from six pots (one biological replicate) for each genotype (WT or *shadow*-*1*) under each treatment (light or shade). A total of three replicates were collected for each genotype under each treatment. Tissue was collected by cutting young leaves directly into a beaker of liquid nitrogen in an effort to preserve mRNA. For shade-treated plants, this was done in a darkroom environment to avoid light contamination.

RNA Extraction and Library Preparation

Total plant RNA was extracted using the RNeasy Plant Mini Kit, including RNase-Free DNase set (Qiagen, Valencia, CA, United States), according to the manufacturer’s protocol. RNA purity and concentration were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). To further assess RNA quality, total RNA was analyzed on the Agilent TapeStation 2200 (Agilent Technologies, Santa Clara, CA, United States) using the RNA High Sensitivity assay. Ribosomal Integrity Numbers (RINe) were recorded for each sample. Only samples with RINe values above 7.0 were used for library
preparation. Total RNA samples were prepared for mRNA-Sequencing using the Illumina TruSeq Stranded mRNA Sample Preparation kit following the manufacturer’s protocol (Illumina, San Diego, CA, United States). Libraries were validated for length and adapter dimer removal using the Agilent TapeStation 2200 D1000 High Sensitivity assay (Agilent Technologies, Santa Clara, CA, United States) and were then quantified and normalized using the dsDNA High Sensitivity Assay for Qubit 2.0 (Life Technologies, Carlsbad, CA, United States). Libraries were prepared for the Illumina HiSeq 2500 (v.4 chemistry) in High Output mode (2 × 100 bp). A total of 12 libraries were sequenced across two lanes.

**Differential Expression Analysis and Functional Annotation**

Clean reads were obtained by first removing adapter sequences, and then filtering out reads with over 20% low-Q-value (≤20) bases, as well as reads with more than 5% ambiguous “N” bases. The clean reads were then aligned to the perennial ryegrass genome assembled by Byrne et al. (2015) using default parameters in Tophat2 software (Kim et al., 2013). Gene expression levels were calculated as reads per kilobase of transcript per million mapped reads (RPKM). Differentially expressed genes (DEGs) were defined as genes having a false discovery rate (FDR) ≤0.05 and an absolute log$_2$ fold change value ≥1. To further characterize the function of DEGs, they were mapped to Gene Ontology (GO) classifications using Blast2GO (Conesa and Götz, 2008). Three categories of GO annotations were analyzed: biological process, molecular function, and cellular component. To uncover upstream GA biosynthesis genes in perennial ryegrass, BLASTP was performed against the translated perennial ryegrass reference genome for each gene of interest, using bread wheat (*Triticum aestivum*) sequences as a query. Top hits with an $E$-value <10$^{-4}$ were aligned using ClustalX 2.0 (Larkin et al., 2007). A phylogenetic tree was constructed for all selected hits by PHYML version 3.0 using the maximum likelihood method.
(Guindon et al., 2010) under the JTT evolutionary model. The closest neighbor for each protein was designated as the putative homolog for that protein in perennial ryegrass.

Quantitative Real-Time PCR Analysis

Two genes: KAO and GA20ox were analyzed using quantitative real-time PCR (qRT-PCR). New plant material was harvested and RNA was extracted as previously described. The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Richmond, CA, United States) was used to synthesize cDNA, and cDNA products were utilized for qRT-PCR assays using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Richmond, CA, United States) on a CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Richmond, CA, United States). The native glyceraldehyde-3-phosphate dehydrogenase (LpGAPDH) gene was used as the internal control (Petersen et al., 2004; Kovi et al., 2016). Primer sequences for all genes analyzed are as follows: KAO forward: 5′-CAGGAAGATGGAGTACCTCT-3′, KAO reverse: 5′-ATGTGCACAGTCCTGTACCA-3′, GA20ox forward: 5′-GACTTCACGCAGAAGCACTA-3′, GA20ox reverse: 5′-GCAGATGCAGAGAAGCAGAA-3′, LpGAPDH forward: 5′-CATCACCATTGTCTCCAACG-3′, LpGAPDH reverse: 5′-AACCTTCAACGATGCCAAAC-3′. Data were analyzed using CFX Manager™ software version 2.0. The expression levels in each sample was normalized using the expression level of LpGAPDH in the same sample. Three biological replicates were performed with each type of sample.
3.5 Discussion

In this chapter, the transcriptomes of the dwarf, shade tolerant perennial ryegrass (*Lolium perenne*, L.) mutant *shadow-1*, under light-grown and 95% shade-stress conditions, were analyzed to explore the genetic mechanisms behind both dwarfism and shade tolerance in these plants. Through Illumina sequencing, millions of clean reads were generated, representing gigabytes of sequencing data, which were mapped to a reference genome. There was high consistency between the biological replicates used for RNA sequencing ($r \geq 96\%$), demonstrating the reliability of the data produced. The accuracy of the transcriptome analysis was verified via qRT-PCR analysis. These data demonstrate the reproducibility of the transcriptome analysis. The transcriptome data acquired from light-grown and shade-stressed *shadow-1* plants proved to be valuable resources and provided a great deal of insight into the dual phenotypes exhibited by *shadow-1* plants.

Through pairwise transcriptome comparisons, noteworthy differences in gene expression were discovered between light-grown *shadow-1* and wild type (WT) plants, in the form of 2,245 differentially expressed genes (DEGs). There was a similar degree of differential gene expression between these two genotypes after both were subjected to shade stress (2,200 DEGs). When DEGs were compared across genotypes and across treatments, 485 DEGs were found to be unique to light-grown *shadow-1* (compared to light-grown WT), and 329 DEGs were unique to shade-stressed *shadow-1* (compared to shade-stressed WT). Additionally, 87 DEGs were differentially expressed in all samples using a four-way comparison of pairwise differential gene expression analyses. By comparing the number of DEGs uncovered in simple pairwise transcriptome analyses to those which were unique (or ubiquitous) across multiple pairwise analyses, phenotypically-relevant *shadow-1* mutation(s) were found to have a relatively small
impact on the transcriptomes of *shdadow-1* plants. This is in direct contrast to the relatively massive impact that shade-stress has on both the transcriptomes of both WT and *shadow-1* plants, as well as the large impact of background (non-phenotypically-relevant) mutations on *shadow-1* gene transcription. Although it is not surprising, these data demonstrate the overwhelming complexity of the shade avoidance response (SAR) in perennial ryegrass, and highlight the need for continuing research on the subject. Additionally, these data demonstrate that multiple generations of backcrossing to WT is necessary before the *shadow-1* mutant line can be utilized for commercial purposes, due to the preponderance of background mutations present in the M2 generation. Once this has been accomplished the *shadow-1* mutant line will serve as a good model for studying dwarfism and shade tolerance in monocots. Both of these traits have utility for plant breeders, in areas ranging from agricultural to ornamental (Wilkins, 1991). For example, dwarf plants can have increased crop yields and could have reduced requirements for nutrients (Monna *et al.*, 2002). Additionally, shade-tolerant plants are able to thrive in environments that are traditionally unconducive to healthy plant growth, such as under tree canopies or in dense urban areas (Jiang *et al.*, 2004).

Due to the poor functional annotation of the perennial ryegrass genome, analysis was focused on pathways of interest, rather than simply assessing the function of the most differentially-expressed genes. This approach led to the exploration of GA-biosynthesis and –response genes in an effort to clarify the somewhat confusing exogenous GA application and endogenous GA content analyses presented in the previous chapter. This was accomplished, in part, by using bread wheat proteins to track down putative homologs of upstream GA biosynthesis genes (*CPS, KS, KO*, and *KAO*) in the transcriptome data. For other key genes in the GA biosynthesis and response pathways (*GA20ox, GA2ox*, and *DELLA*), well-annotated mRNA sequences could be
found within the Genbank database. Downregulation of all GA biosynthesis genes was observed in both light-grown and shade-stressed shadow-1 plants compared to WT under the same conditions, although these genes were more severely downregulated in shade-stressed shadow-1 plants. Most notable was GA20ox, which governs the penultimate step of GA biosynthesis, and was downregulated to 3.3% of WT expression in shade-stressed shadow-1, but only to 39.0% of WT expression in the light. Based on the analysis of GA biosynthesis, in conjunction with the GA content analysis presented in Chapter 2, it seems likely that shadow-1 plants rely on reduced production of GA as a key part of their exhibited shade tolerance.

The elucidation of downregulation of every GA biosynthesis gene in light-grown shadow-1 plants seemingly contradicts the GA content analysis presented in Chapter 2, which showed an increase in GA content in these plants, compared to WT under the same conditions. However, GA2ox, which is responsible for de-activating bioactive forms of GA, was found to be steeply downregulated (11.8% of WT expression) in light-grown shadow-1 plants. This helps to explain why GA1 (the major bioactive form of GA in monocots) levels were lower in light-grown shadow-1 plants, even though GA biosynthesis genes were also downregulated in these plants. In other words, while GA biosynthesis was decreased in light-grown shadow-1 plants (24.4-84.7% of WT expression), GA degradation was decreased to a greater degree, and it appears as though the latter had a larger impact on GA content than the former leading to an overall increase in GA1 content. It is also interesting to note that, following shade stress, GA2ox was steeply upregulated in shadow-1 plants (472.1%), providing even further reasoning for decreased bioactive GA content in these plants. It is known that GA biosynthesis is a key part of the WT response to shade stress in both monocots and dicots (Hedden and Thomas, 2016), therefore it is not wholly surprising that reducing GA biosynthesis can induce shade tolerance. However, there
are major implications to this finding. For turf and other ornamental plants, exogenously-applied GA-biosynthesis inhibitors, like trinexapac-ethyl, has been shown to induce temporary shade tolerance, although this approach could prove costly (Qian and Engelke, 1999; Ervin et al., 2002; Goss et al., 2002; Studzinska et al., 2012). Alternatively, engineering or breeding low-GA cultivars, either by downregulating GA biosynthesis or upregulating GA2ox, could permanently introduce shade tolerance to plants.

While analysis of GA biosynthesis genes can explain the increased bioactive GA content in light-grown shadow-1 plants, they do not explain how these plants could be dwarf in spite of elevated GA content. In plants, elevated GA content is associated with a tall phenotype, which includes longer hypocotyls and petioles as well as accelerated stem elongation (Huang et al., 1998; Croker et al., 1999). In Chapter 2, it was hypothesized that partial GA insensitivity was the root cause for dwarfism in light-grown shadow-1 plants, making them resistant to the effects of elevated bioactive GA levels. Therefore, the transcription of DELLA, a key negative regulator of GA response, was examined within the transcriptome data of light-grown shadow-1 plants. DELLA proteins suppress the activity of transcription factors, such as the phytochrome interacting factor (PIF) family, which in turn are responsible for activating and suppressing genes which lead to downstream GA signaling. DELLA proteins bind to the GA receptor GID1 in the presence of GA, a process which leads to their ubiquitination and subsequent degradation via the 26S proteasome. The degradation of DELLA, and subsequent activation of GA responses, requires the physical interaction of DELLA and GID1 in the presence of GA. Therefore, increasing DELLA protein levels could elicit reduced sensitivity to GA, as higher quantity of DELLA protein, without an equivalent increase in GID1, should lead to a relatively increased amount DELLA protein and an overall suppression of GA response. It is important that the
DELLA protein is degraded in the presence of bioactive GA because it means that increased *DELLA* transcription should only confer partial GA insensitivity, making it an ideal candidate gene to explain the dwarf phenotype exhibited by light-grown *shadow-1* plants.

The transcriptome data revealed that *DELLA* was upregulated to 283.9% of WT expression in light-grown *shadow-1* plants. This datum is key for understanding why *shadow-1* plants are dwarf in the light, despite having elevated GA levels. An attempt was made to determine the expression of *GID1*, the other key factor of post-transcriptional regulation of GA response, but this gene could not be identified in perennial ryegrass. These results are not wholly surprising, as *DELLA* (dis)function has long been associated with dwarfism in plants. In fact, *DELLA* was first identified by analyzing dwarf mutants of *Arabidopsis thaliana*, which were also deemed to be GA insensitive (Peng *et al.*, 1997). In these plants, *DELLA* was truncated on the N-terminal end, preventing *GID1* binding while maintaining its ability to bind to PIFs and other transcription factors, rendering the plants dwarf and GA insensitive. Later analysis of dwarf wheat and maize cultivars produced during the ‘green revolution’ found that these plants had similar mutations, which were deemed to be the root cause of their dwarf phenotypes (Peng *et al.*, 1999). *DELLA* in *shadow-1* was non-mutagenized during the mutation breeding process, however its upregulation should have a similar effect in plants, with the exception of rendering *shadow-1* only partially, rather than wholly, insensitive to GA. This finding could have utility for dwarf breeding and/or genetic engineering programs as exogenously-applied GA has utility in turf to promote fast and uniform germination. If one were to induce dwarfism by truncating *DELLA* it would prevent this technique from being used, especially in monocots which only contain a single copy of *DELLA* (Hedden and Thomas, 2016). Upregulating *DELLA*, on the other
hand, has the potential to induce dwarfism while leaving plants partially sensitive to exogenously-applied GA.

It was not possible to identify the actual mutation(s) causing the dwarf and shade tolerant phenotypes in *shadow-1* through transcriptome analysis due to the preponderance of background mutations. However, the progeny analysis revealed in Chapter 2 strongly suggests that these phenotypes are caused by a single mutation. It is possible that these phenotypes are caused by separate, albeit highly linked, mutations. There is small likelihood that there are two highly linked mutant genes, of which one controls GA biosynthesis and the other controls *DELLA* expression. It seems more likely that there is a single mutation which has the ability to control both GA biosynthesis and *DELLA* expression. One candidate gene is phytochrome interacting factor 1 (PIF1/PIL5). This transcription factor has been shown to regulate *DELLA* expression, while also being regulated by *DELLA* through protein-protein interaction (Oh *et al.*, 2007). Additionally, there is evidence that PIF1/PIL5 can control the levels of bioactive GAs by manipulating GA biosynthesis (Oh *et al.*, 2006). A PIF1/PIL5 homolog could not be identified in the transcriptome data for *shadow-1*.

There were around 2,200 DEGs between *shadow-1* and wild type under both light-grown and shade-stressed conditions. In theory, if one backcrosses *shadow-1* to wild type and screens the progeny for dwarfism and shade tolerance, it should segregate out half of the background mutations from these progeny. It is impossible to know how many DEGs are the result of background mutations versus those resulting from phenotypically-relevant mutations, however after four generations of backcrossing, the background mutations will be decreased by 93.75%, making it possible to identify the phenotypically-linked mutation(s) in the *shadow-1* lineage. Because of the nature of gamma-irradiation-mediated mutations (mostly large deletions) a
knockout mutation is the most likely cause of *shadow-1*’s phenotypes. Therefore, to identify the mutant gene(s) responsible for *shadow-1*’s phenotypes one must look for genes which are expressed in wild type but are absent in the transcriptome of *shadow-1*. 
References


