Histidine Domain-Arabinogalactan Proteins (HD-AGPs) in the Solanaceae

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HISTIDINE DOMAIN-ARABINO GALACTAN PROTEINS (HD-AGPS)

IN THE SOLANACEAE

By

Jenna Kristine Brooks

Accepted in Partial Completion
Of the Requirements for the Degree
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MASTER’S THESIS

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Date: November 14th, 2016
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A Thesis
Presented to
The Faculty of
Western Washington University

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Of the Requirements for the Degree
Master of Science

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Jenna Kristine Brooks

November 2016
ABSTRACT

Arabinogalactan Proteins (AGPs) are a large family of hydroxyproline-rich glycoproteins found in the extracellular matrix of diverse cell types in plants. These glycoproteins are proposed to play a role in cell wall development, cell-cell interactions, and pattern formation. The goal of this research was to compare Histidine-Domain Arabinogalactan Proteins (HD-AGPs) in the Solanaceae and to examine the patterns of expression in vegetative and reproductive tissue. HD-AGP cDNA clones were isolated from pistils of Petunia inflata (PifPRP1), P. axillaris parodii (Pa2PRP1), and P. exserta (PePRP1). HD-AGPs from the two-interbreeding species, P. axillaris parodii and P. exserta, are 99.9% identical at the nucleotide level and identical at the amino acid level. HD-AGPs from P. inflata (PifPRP1) and P. axillaris parodii (Pa2PRP1), two reproductively isolated species, show that PifPRP1 is 98.5% identical to Pa2PRP1, with a 12 bp deletion in the hypervariable domain that is specific to the P. axillaris complex. PCR using genomic DNA yielded a single amplicon from each of these species, but two bands from Petunia hybrida and from Nicotiana tabacum. Comparison of intron sequence yielded some interesting insights about the evolutionary trajectory of sister species in these clades. Immunoblots using P. axillaris axillaris revealed discrete glycoforms of HD-AGPs in vegetative tissues contrasting with the tremendous size heterogeneity displayed by HD-AGPs expressed in the pistil. Etiolated seedlings accumulate higher levels of the protein, including significant amounts of an unglycosylated isoform. These findings suggest an important role for HD-AGPs in rapid elongation of cell types in both reproductive and vegetative tissues.
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INTRODUCTION

The Solanaceae is a large family of plants with more than 2,800 species, including many agriculturally-important species (D’Arcy 1986). Solanum tuberosum (potato), Solanum lycopersicum (tomato), Solanum melongena (eggplant), and Nicotiana tabacum (tobacco) are the most important solanaceous crops. Petunia and Calibrachoa are dominant genera in ornamental horticulture. Petunia species were among the first bedding plants to be cultivated in European and North American gardens, and this ornamental use is known to date back to 1823 (Gerats 2005). Two of my research organisms, Petunia axillaris and Petunia integrifolia, are the wild progenitors of Petunia hybrida; this synthetic species is the most popular bedding plant in the United States.

With their large, animal-pollinated flowers, Petunia species have been model organisms for the study of plant reproduction for more than 100 years. Charles Darwin (1859, 1862, 1876, 1877) studied fertility and self-sterility in Petunia violacea (today considered equivalent to P. integrifolia). Petunia has been a model organism for developmental genetics, especially in the analysis of petal pigmentation genes (Spelt et al., 2000, Sheehan et al., 2016). It was one of the first organisms in which the phenomenon of gene silencing was described (Van der Krol et al., 1990), leading to the discovery of RNAi (interfering RNA). Solanaceous flowers have diverse morphologies, and evolutionary mechanisms such as pollinator-driven speciation have been described in Petunia (Venail et al., 2010) other taxa in the family.

Members of the Solanaceae are model organisms for investigating pollen-pistil interactions as well. The pistil and pollen genes responsible for self-incompatibility in taxa that shed bicellular pollen (the overwhelming majority of flowering plants) were first cloned,
sequenced, and analyzed in *Nicotiana* and *Petunia* species (Takayama and Isogai 2005). Understanding pollen-pistil interactions in this family, and other flowering plants, is valuable for both basic and applied research. The manipulation of inter-specific breeding barriers can facilitate introgression of desired traits (such as cold resistance, salt tolerance, and disease resistance) from wild species to crop plants. The investigation of genes and proteins involved in breeding barriers may enable us to develop strategies for protecting the genomic integrity of native flora by preventing gene flow between genetically modified plants and their wild relatives.

**The Genus Petunia**

Depending on the authors, anywhere from 11 to 14 species of *Petunia* have been described. The genus is endemic to southern South America, with a center of diversity around southern Brazil, Bolivia, Uruguay, Paraguay, and northern Argentina. All wild petunias are diploid with n=7. There is strong evidence that speciation in the genus has been driven mainly by divergence in pollination syndromes, which is the suite of floral characteristics that influence visitation by specific pollinators (Galliot et al., 2006; Sheehan et al., 2016). Thus, *Petunia* species vary chiefly in flower color, size, shape, and scent and nectar production. Some species are pollinated mainly by moths, others mainly by bumblebees, and *P. exserta* is the only hummingbird-pollinated *Petunia* (Venail et al., 2010).

The *Petunia axillaris* complex consists of three allopatric subspecies, *P. axillaris axillaris*, *P. axillaris parodii*, and *P. axillaris subandina*. All members of the *P. axillaris* complex produce scented white flowers that have long corolla tubes and are pollinated by nocturnal hawkmoths. Most *P. axillaris axillaris* populations display self-incompatibility (are
self-sterile), while most populations of the other two species appear to be self-compatible (set seed after self-fertilization). All three subspecies are completely inter-fertile with each other.

The *P. axillaris* complex are believed to have evolved recently from a clade of unscented purple-flowered *Petunia* that is pollinated mostly by bumblebee. Two subspecies are commonly included in the *P. integrifolia* complex: *P. integrifolia integrifolia* and *P. integrifolia depauperata*. Wijsman (1982) placed a third subspecies in this complex, *P. integrifolia inflata*. However, based on morphological differences and DNA divergence Ando et al. (2005) give full species status to *P. inflata* and that designation is followed in most of the recent literature on *Petunia*. Accordingly, *P. inflata* is treated as a species in this study. Strong self-incompatibility is seen in all wild populations of *P. integrifolia integrifolia* and *P. inflata*.

*P. exserta* is an endangered species that is currently restricted to the shaded regions of 9 sandstone towers in the Serro do Sudeste region of southern Brazil (Lorenz-Lemke et al., 2006). It has unscented red flowers and is pollinated mainly by hummingbirds. *P. exserta* is self-compatible.

In the lab, members of the *P. axillaris* complex are completely inter-fertile with *P. exserta*, although in the wild, specialized pollinators are largely effective in maintaining the integrity of each species. *P. inflata* and *P. integrifolia* complex cannot be fertilized by pollen from the *P. axillaris* complex. Reciprocal pollinations done in the lab are somewhat successful but the rate of capsule set (fruit formation) is much lower than in congruous (within-species) pollination. Thus, there are strong pre-zygotic reproductive barriers between the *P. axillaris* complex and the purple-flowered *Petunia* (*P. integrifolia* complex and *P.
inflata), but the P. axillaris complex is not reproductively isolated from the morphologically very distinct P. exserta.

The garden petunia, *Petunia hybrida*, is a synthetic species generated by European horticulturists in the 19th century most likely by hybridizing *P. axillaris axillaris* and *P. integrifolia integrifolia*. In later decades, the genomes of *P. axillaris parodii*, and possibly *P. inflata* as well, were likely introgressed into the garden petunia.

**The Genus Nicotiana**

*Petunia* and *Nicotiana* are distantly related within the Solanaceae, whose evolutionary origin is estimated to about 49 million years ago based on fossil and molecular evidence (Sarkinen et al., 2013). *Nicotiana* is the fifth largest genus in the Solanaceae, with 75 species (Clarkson et al., 2004). *Nicotiana* has a broader geographic distribution than *Petunia*. Some 75% of the species are native to the Americas, and almost all the rest are Australian species, with a few in the South Pacific and one in southern Africa (Namibia). *Nicotiana* is divided into 13 different sections (Knapp et al., 2004), with haploid chromosome varying from 9 to 24. Inter-specific hybrids have been common in the evolutionary history of *Nicotiana*, and subsequent polyploidy has resulted in many amphiploid species.

The cultivated tobacco, *Nicotiana tabacum*, is such an amphidiploid (n=24). Its historical parents have been placed in two divergent sections by Knapp et al. (2004): the putative male progenitor, *N. tomentosiformis* in section Tomentosae; and the maternal parent, *N. sylvestris*, in section Sylvestres.
Pollen-Pistil Interactions

The reproductive success of a flowering plant is dependent on pollen-pistil interactions, which encompass extensive and complex cellular cross-talk between the sperm-carrying male gametophyte (pollen) and the pistil, the female reproductive organ in a flower. A solanaceous pistil is composed of stigma, transition zone, style, and ovary. The stigma is the receptive surface at the apex of the pistil. The solanaceous stigmatic surface is wet, as opposed to the well-documented dry stigma in plants such as Arabidopsis and rice. The epidermis that makes up the stigmatic surface is papillate and secretes copious amounts of a viscous fluid; this exudate plays a key role in hydrating the pollen and is thought to be important for directing pollen tube emergence and directional growth into the sub-epidermal layer of the stigma (Quiapim et al., 2009; Heath, 1990). In both tobacco and Petunia, the exudate is lipidic and contains both proteins and sugars that supply necessary nutrients to the pollen grains that adhere to the papillae of the stigmatic surface (Sanchez et al., 2004). After hydration occurs, the emergent pollen tube extends through tip growth in the nutrient-rich extracellular matrix (ECM) of the stigma and then the style.

The style is the columnar segment of the pistil that lies between the apical stigma and the basal ovary, and is the longest part of the pistil in most genera in the Solanaceae. At the central core of the pistil is a specialized tract of tissue, —the transmitting tissue (tt)— a continuous pollen tube pathway from the stigma to the ovary. The transmitting tissue is funnel-shaped at the apex of the pistil, being widest in the stigma and narrowing gradually through the transition zone, which lies below the stigma and above the style proper; the transmitting tissue is essentially cylindrical through the length of the style. In the style, pollen tubes grow within the pectin-rich extracellular matrix of the transmitting tissue. After
emerging from the base of the style, pollen tubes grow along the surface of the funiculus, presumably following chemical cues emanating from the ovules, similar to the short-range signals such as gamma-amino butyric acid (GABA) and species-specific defensin-like peptides that guide a pollen tube into the ovule in *Arabidopsis* (Palanivelu and Preuss 2006) and in *Torenia* (Takeuchi and Higashiyama 2012). The entry of a single pollen tube into an ovule triggers a block to ingression by other pollen tubes. The synergids that flank the egg cell degenerate upon the arrival of the pollen tubes and the release of cytoplasmic calcium is thought to stimulate the double fertilization, in which one sperm cell delivered by the pollen tube fuses with the egg cell and the other sperm cell fuses with the two haploid polar nuclei to form the triploid nucleus of the endosperm.

The pistil ECM is composed of rich secretory materials, including free sugars, polysaccharides, lipids and glycosylated proteins that support the rapid growth of pollen tubes as they make their way to the ovary. Upon pollination, the carbohydrate content of the ECM as well as the starch content of the transmitting tissue cells is consumed to near depletion. A very high metabolic demand is put forth by pollen tube growth; this is met by biomolecules stockpiled in both the ECM and adjacent cells (Cheung et al., 2000).

Pollen tube growth within the pistil (style) of the Solanaceae has been described as biphasic, consisting of an initial slow phase followed by a phase of very rapid growth rates. The slow growth phase manifests in the first few hours after pollen germination, lasting up to 7-8 hours after pollination in *P. hybrida* (Mulcahy 1982). During this initial slow phase, pollen tubes traverse the stigma to reach the base of the transition zone (TZ), which is defined as the pistil segment immediately superior to the cylindrical style (Lubliner et al., 2004).
The TZ has been shown to be anatomically and functionally distinct from both the stigma and the style (Dickinson and Moriarty 1982).

As the pollen tubes leave the TZ and enter the style proper, they exhibit a marked acceleration in growth rate. In this second phase of growth, pollen tube growth rates increase 2- to 5-fold. The time point at which the pollen tube begins this rapid phase of growth is referred to as the pollen tube growth transition (PGT). It is proposed that the PGT signifies the transition from a largely autonomous mode of growth to a highly pistil-dependent mode of growth by pollen tubes (Lubliner et al., 2003). The mechanistic basis of the PGT remains a mystery and is a focus of our research.

Arabinogalactan proteins are a highly diverse class of cell surface glycoproteins thought to be involved in many biological processes including cell wall development, pattern formation and growth as well as some plant microbial interactions (Seifert and Roberts, 2007). One key feature that aids in the identification of AGPs is the ability to bind to and be precipitated by a class of phenylglycoside known as Yariv reagents. A class of highly glycosylated AGPs are known to be play a critical role in supporting pollen tube growth in the pistil ECM. This family of proteins is thought to help in pollen recognition, adhesion to the stigmatic surface and ECM, nutritional support, and possibly directional growth in the style. The transmitting tissue-specific proteins (TTS-1 and TTS-2 from N. tabacum, and NaTTS from N. alata) are among the most extensively studied pistil AGPs (reviewed by Cheung et al., 2000).

Transmitting Tissue-Specific (TTS) Proteins within the Pistil

TTS proteins were first described in 1993 by Alice Cheung and her collaborators from tobacco, Nicotiana tabacum (Cheung et al., 1993). An ortholog from Nicotiana alata,
NaTTS, was described later (Cheung et al., 2000). TTS proteins were described as a transmitting tissue-specific protein, unique to the pistil EMC. Two cDNAs, encoding a proline-rich extracellular protein, were characterized from tobacco: TTS-1 and TTS-2. These TTS proteins have been shown to promote in vitro pollen tube growth in a minimal medium lacking added sucrose, and attract pollen tubes in a semi-in vivo system using the minimal medium solidified with agarose (Cheung and Wu 1995; Wang and Cheung 1993; Wu and Cheung 1995).

TTS proteins are glycoproteins secreted into the intercellular regions of the transmitting tissue of the style; they are absent in the stigma and not abundant in the TZ. The polypeptide backbone is deglycosylated during pollination as well as if the purified protein is incubated with pollen tubes growing in vitro (Cheung, 1999; Cheung and Wu, 1995). This suggests that deglycosylases associated with the pollen tube surface (cell wall and/or plasma membrane) remove sugar moieties from the protein and the released sugars may be taken up by the pollen tubes.

Cheung et al. propose that in addition to serving as a source of sugars for growing pollen tubes, TTS proteins generate a sugar gradient that guides pollen tubes to the base of the pistil. TTS proteins display enormous heterogeneity in glycosylation but the degree of glycosylation increases from the stigma end of the style toward the ovary in Nicotiana. In addition to its role in nutrition and guidance, these authors propose also that the protein helps adhere pollen tubes to the ECM surface (Cheung and Wu, 1995; Wu et al., 2000).

Because of the great heterogeneity in the degree of glycosylation of TTS proteins, their molecular weight estimate by SDS-PAGE ranges from 40-100 kDa, depending on developmental stage of the flower and position in the pistil from the apex near the TZ to base
near the ovary (Wang and Cheung, 1993). The lowest relative molecular mass \( (M_r) \) for TTS proteins extracted from the pistil is around 30kDa; when the high \( M_r \) purified protein is left to incubate in pollen tube growth cultures, most of the fraction acquires a molecular mass of 30 kDa. Native TTS proteins subjected to chemical deglycosylation runs at about 28kDa when analyzed by SDS-PAGE (Cheung et al., 1996; Wu and Cheung, 1995).

**Histidine-Domain Arabinogalactan Proteins (HD-AGPs)**

Three TTS proteins have been described by Cheung et al. (1999) from *Nicotiana tabacum* (TTS-1 and TTS-2, accession numbers Z16403.1 and Z16404.1, respectively) and from *Nicotiana alata* (*Na*TTS, accession number X70441.1). TTS-2 was also found in *N. sylvestris*, which is one of the ancestors of the amphidiploid cultivated tobacco, *N. tabacum* (Cheung and Wu, 1999). Sommer-Knudsen, Jens (1996, 1998) described a galactose-rich stylar glycoprotein (GaRSGP) from *N. alata* that is identical to *Na*TTS in its amino acid sequence, but that fails to promote pollen tube growth *in vitro*. According to Cheung et al. (2000), GaRSGP constitutes a minor under-glycosylated subpopulation of *Na*TTS that is more tightly bound to the ECM than is the major *Na*TTS fraction.

Based on sequence similarity and conservation of domain architecture, orthologs of TTS proteins have been identified in taxa other than *Nicotiana*. Graduate student Megan Twomey (Master of Science, WWU, 2012) described an extracellular arabinogalactan protein, *Ph*PRP1, from *Petunia hybrida* pistils. The cDNA encoding the protein (accession number FJ19032.1) is 82% identical to *Na*TTS (Twomey et al., 2013). The amino acid sequence, deduced from the cloned cDNA, predicts a 27 kDa polypeptide. \( \beta \)-glucosyl Yariv reagent, a diagnostic test for arabinogalactan proteins, readily stains the purified protein.
Megan Twomey (2012) compared the sequence of PhPRP1, the three TTS proteins from Nicotiana, and CaPRP1 from Capsicum annuum. The multiple alignment revealed a conserved histidine-rich domain and two hypervariable domains. The longer of the two hypervariable domains is proline-rich and contains noncontiguous XKPP repeats that vary in number and location among these solanaceous taxa.

Immunoblots show that TTS-1 and PhPRP1 are also expressed in vegetative tissues of tobacco and petunia respectively (Twomey et al., 2013). In contrast to the molecular mass heterogeneity displayed by the pistil proteins, the isoforms found in seedlings, roots, and leaves each has a discrete size (37, 80, 160, and 200 kDa) on SDS-PAGE gels (Twomey et al., 2013). Based on their chemistry, distinctive domain architecture, and the unique pattern of expression, we have named this group of proteins HD-AGPs (histidine domain-arabinogalactan proteins). Going by its domain structure and pattern of expression, AGP31 (accession number Q9FZA2) from Arabidopsis thaliana (Liu and Mehdy, 2007) is also a member of the HD-AGP family of proteins. The C-terminal PAC domain (Baldwin et al., 2001) is conserved in all the solanaceous HD-AGPs and also in AGP-31 from Arabidopsis.

Nicotiana, the TTS proteins were first described as unique to the transmitting tissue (Cheung et al., 1993; Wu et al., 2000). However, Twomey et al. (2013) showed that the TTS proteins in Nicotiana tabacum, and PhPRP1 in Petunia hybrida, are expressed in vegetative tissue as well. Furthermore, this is true of all the probable HD-AGP orthologs mentioned above. For example, CaPRP1 from Capsicum annuum has been found not only in the pistil tissue but has been described from a cDNA library from very young roots (Mang et al., 2004). We have found sequences highly similar to HD-AGPs in tomato, potato, and eggplant;
expressed sequence tag (EST) databases reveal that the gene is expressed in transmitting tissue, roots, leaves and stems in all three taxa.

Although HD-AGPs have now been identified in several taxa, their precise function and mode of action remains unclear. It is apparent that the proteins promote pollen tube growth in *Nicotiana*, and presumably in the other taxa as well since they are among the most abundant proteins in the pistils of these species. Their presence in vegetative tissues was a surprise and their role in non-reproductive tissue remains a mystery.

My research objectives were to examine HD-AGPs in several *Petunia* and *Nicotiana* species with a view to (1) compare the sequence diversity of their coding and intronic regions by cloning and sequencing pistil cDNA encoding HD-AGP from *P. axillaris parodii* (Figure 1), *P. inflata* (Figure 2), and *P. exserta* (Figure 3); and genomic HD-AGPs from *P. hybrida* (Figure 4), *P. inflata* and *N. tabacum* (Figure 5); and (2) investigate the developmental and tissue-specific pattern of HD-AGP expression in *Petunia axillaris axillaris* (Figure 1), including a comparison of HD-AGP levels in light-grown and etiolated seedlings. HD-AGPs seem to be prevalent in tissues with rapidly expanding cell types (seedlings, developing vasculature, pollen tubes).
METHODS

Plant Material and Growth Conditions

Plants were grown from seed in the research greenhouse at Western Washington University. *P. axillaris* subspecies *parodii* seeds were sourced from the University of California Botanical Gardens, Berkeley, California. *P. axillaris* subspecies *axillaris* seeds were purchased from Diane’s Seeds (Ogden, Utah). *Petunia inflata* seeds were kindly provided by Professor Teh-hui Kao, Penn State University, University Park, Pennsylvania. *Petunia exserta* seeds were obtained from the USDA Ornamental Plant Germplasm Center (accession number OPGC 943, Lot 09ohci01). The accessions of *Petunia hybrida* var. Silver Wave and *Nicotiana tabacum* have been described by Twomey (2012) in her Master of Science thesis (WWU, 2012). *Petunia axillaris axillaris, Petunia axillaris parodii* and *Petunia integrifolia* were grown and maintained in the Biology Department Research Greenhouse, on the main campus of Western Washington University, Bellingham, Washington. The other plants, needed in smaller quantities, were maintained in a walk-in grow room, as described later.

All planting equipment, including pots and trays, were sterilized by soaking for approximately an hour in 20% bleach followed by rinsing with distilled water. A general-use potting mix (Blackgold, Sungrow Horticulture, Agawam, Massachusetts) was sterilized for 45 minutes in a ProGrow Electric Soil Sterilizer (Model SST-15) at 185°C. Over the course of the year, greenhouse temperatures ranged from 15-30°C. Supplemental lighting was provided in autumn and winter (September to April) with a combination of high pressure sodium vapor and metal halide overhead lamps set to a 16 hour photoperiod. Plants were watered 3 times a week for 10 minutes with an overhead sprinkler system.
The other plants used in this thesis research—*P. hybrida, P. exserta* and *Nicotiana tabacum*—were grown from seed in a walk-in grow room in the Biology Department, Western Washington University. The plants were grown under continuous illumination provided by full-spectrum lights (Sungrow T20, Vancouver, British Columbia) set 0.1 to 0.3 meters above the growing plants. The growth room temperature was 20°C, ± 1°C. The potted plants were watered twice a week until the soil was completely moist.

To grow seedlings for protein analysis, 0.1 g of *P. axillaris* seeds was germinated on sterilized medium-grade horticultural sand in small petri-dishes (55mm x 15mm) and watered twice a week with 5.0 mL distilled water. Seedlings were grown under the Sungrow lights described above, or in a dark cupboard in the laboratory (BI334, Biology Building), at a temperature of 20°C ± 1°C. For growth regulator experiments, the following solutions were prepared in double-distilled water: 10 µM potassium salt of gibberellic acid (Sigma, G1025); 1µM abscisic acid (ABA, Sigma A1049); and 1.5 mM ethephon (diluted from a 21.9% stock solution, Carolina Biological Supply).

**Nucleotide Sequence Analysis**

**Genomic DNA Extraction**

Leaves from *N. tabacum* and five petunia species were collected in the same manner: 2-3 young leaves, each less than 2 cm in length, were harvested from the shoot apex. The leaf samples were placed in 1.5 mL microfuge tubes and genomic DNA extracted using a protocol described by Xin et al. (2003). 50µL of Buffer A (100mM NaOH and 2% Tween® 20) was added to each tissue sample and the leaf tissue was homogenized thoroughly using a tube pestle. The homogenates were incubated for 10 minutes at 95°C, then 50 µL Buffer B (100mM Tris-HCl and 2mM EDTA) was added, followed by gentle mixing in a vortexer.
The subsequent PCR reactions were set up without further purification of genomic DNA template.

**RNA Extraction**

Pistils from *Petunia* and *Nicotiana* species were collected from open flowers that had been emasculated at the bud stage approximately 3 days prior to anthesis. Pistils were flash-frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. RNA was extracted using the TÖTALLY RNA Total RNA Isolation Kit (Ambion, Austin, Texas) following manufacturer’s instructions with a few minor changes. Denaturing solution was added to the still-frozen ground pistils (3.0 mL denaturing solution for each 0.3g of pistil tissue). After further grinding, the thawed homogenate was centrifuged to remove cellular debris.

The supernatant was transferred to microfuge tubes and measured to estimate the starting volume. Phenol:Chloroform:isoamyl alcohol (25:24:1) buffered to pH 7.9 was added (typically, 600 µL per tube). After 1 minute of vigorous shaking, the tubes were stored on ice for 5 minutes, then centrifuged for 5 minutes at 16,000 x g. The aqueous phase (top layer) was transferred to a new tube. The extraction with alkaline phenol-chloroform:IAA was repeated until the interphase between the organic and aqueous layers seemed clear and free of flocculent debris; typically, two additional extractions were necessary. Next, the supernatant was extracted once with acid phenol: chloroform:IAA (pH 4), with 1 minute of vigorous shaking and a five-minute incubation on ice, followed by centrifugation for 5 minutes at 16,000 x g. This step yielded an aqueous phase enriched in RNA over DNA, because double-stranded DNA is denatured by the acidified organic solvents and consequently partitions preferentially with the organic phase. Finally, the RNA-enriched aqueous phase was
extracted with chloroform to ensure removal of trace amounts of phenol contaminating the aqueous phase. One starting volume of chloroform was added, mixed by inversion, and centrifuged for 5 minutes at 16,000 x g.

The aqueous phase from the last organic extraction was transferred to a fresh tube and 1/10 aqueous phase volume of sodium acetate (3M, pH 4.5) was then added and the solution mixed by inversion. An equal volume of isopropanol was added to each tube, mixed well, and the preparation stored at -20°C overnight to facilitate precipitation of RNA. The precipitate was centrifuged at 16,000 x g for 15 minutes, and supernatant removed by aspiration. The pellet was air-dried in a fume hood for 10 minutes to evaporate any remaining isopropanol, and the dried pellet was re-suspended in 50 µL DEPC-water (water containing diethyl pyrocarbonate, an inhibitor of RNases). Heat was applied at 70°C for 3 minutes, with intermittent vortexing, to aid the solubilization of the pellet. RNA yield was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). *P. exserta* RNA yield was 671.8 ng/µL with a 260/280 ratio of 1.87 AU. RNA was then visualized by gel electrophoresis using a 0.8% agarose gel containing 50mL MOPS buffer (3-morpholino propane-1-sulfonic acid buffered to pH 6.0) and 100 µg/mL ethidium bromide. The gel was run at 85V using a high-voltage power supply (VWR-135, E-C Apparatus). Gel images were recorded with an AlphaImager Mini gel documentation system (Alpha Innotech, San Leandro, California).

**Reverse-Transcription (cDNA Synthesis)**

The ThermoScript™ RT-PCR system (Invitrogen Inc., California) was used for cDNA synthesis from total RNA, following the manufacturer’s instructions. The following components were assembled in a PCR tube: 50 µM oligo d(T)20 primer (Table 1), 3 µg total
RNA, and 10 mM dNTP for a final volume of 20 µL. The total RNA was denatured by incubation at 65°C for 5 minutes, in the presence of the oligo d(T) primer and dNTPs, before the other components of the reverse transcription were added. A master reaction mix was assembled for each reverse transcription reaction using these stock solutions: 5x cDNA Synthesis Buffer, 0.1 M DTT, RNaseOUT (40 U/µL), and ThermoScript RT (15 units/µL). The master reaction mix was added to the denatured total RNA solution (containing also the oligo primer and dNTPs) in 0.2mL PCR tubes kept on ice. First strand cDNA synthesis was carried out by incubating the assembled reverse transcription reactions in a thermocycler (Applied Biosystems 2720 Thermo Cycler) at 52°C for 60 minutes. The cDNA reaction was terminated by incubating at 85°C for 5 minutes. To hydrolyze the RNA template, 1.0 µL of RNase H was added and the tubes and incubated for 20 minutes at 37°C. The cDNA samples were stored at -20°C until second-strand synthesis and amplification by PCR (polymerase chain reaction).

**Primers for PCR Amplification**

A gene-specific forward primer, 3PhTTS F (Table 1) (Callaway, 2012) was used in PCR reactions to amplify both genomic DNA and cDNA from Petunia species. This primer was based on the full-length cDNA sequence of PhPRP1 from P. hybrida (Genbank accession number: FJ719032) (Twomey, 2012). To amplify genomic DNA from Petunia species, 3PhTTSR as the reverse primer (Table 1) was used. This reverse primer was predicted to be downstream of the single intron located in the approximate center of the coding region based on my preliminary experiments for Petunia species. Nicotiana genomic DNA sequences were amplified using forward and reverse primers (Table 1) designed using conserved domains of cDNA sequences encoding TTS-1 and TTS-2 (Cheung et al., 1993).
Forward and reverse primers corresponding to a *Petunia* actin gene (Genbank accession number: JQ012917) were used as a positive control in amplification of genomic DNA by PCR (see Table 1). *Petunia* cDNAs were amplified using 2PhTTS-F and oligo-d(T). M13F, T3, and SP6 were used for DNA sequencing of genomic or cDNA amplicons cloned into a suitable plasmid vector (described later).

**Table 1. Primers used for cDNA synthesis, PCR amplification, and sequencing.**

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>ANNEALING TEMP. (°C)</th>
<th>SIZE (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3PhTTS F</td>
<td>(5’ CAG TTT TAT TAC TCA GCT CAT TCA CAG TTC 3’)</td>
<td>63.3</td>
<td>30</td>
</tr>
<tr>
<td>3PhTTS R</td>
<td>(5’ GGC ACC TTS RTT GAG GCT TCG 3’)</td>
<td>65.5</td>
<td>21</td>
</tr>
<tr>
<td>NTTS-1 F</td>
<td>(5’ CCA CCC CAA ACA TTC CCC AG 3’)</td>
<td>64.5</td>
<td>20</td>
</tr>
<tr>
<td>NTTS-1 R</td>
<td>(5’ TCC ACC ATG TAA TCT GTT GGA CA 3’)</td>
<td>61.0</td>
<td>23</td>
</tr>
<tr>
<td>Actin F</td>
<td>(5’ ACA GGT ATT GTG TTG GAC TC 3’)</td>
<td>58.4</td>
<td>20</td>
</tr>
<tr>
<td>Actin R</td>
<td>(5’ CTG TAC TTT CTC TCT GGT GG 3’)</td>
<td>60.4</td>
<td>20</td>
</tr>
<tr>
<td>M13 F</td>
<td>(5’ TGT AAA ACG ACG GCC AGT 3’)</td>
<td>59.0</td>
<td>18</td>
</tr>
<tr>
<td>T3</td>
<td>(5’ ATT AAC CCT CAC TAA AGG GA 3’)</td>
<td>57.0</td>
<td>20</td>
</tr>
<tr>
<td>SP6</td>
<td>(5’ ATT TAG GTG ACA CTA TAG 3’)</td>
<td>55.0</td>
<td>18</td>
</tr>
<tr>
<td>Oligo d(T)20</td>
<td>(5’ TTT TTT TTT TTT TTT TT 3’)</td>
<td>55.0</td>
<td>20</td>
</tr>
</tbody>
</table>

**PCR Amplification using Go Taq® Hot Start Polymerase**

To amplify genomic DNA using Go Taq® Hot Start Polymerase (Promega, Madison, WI), the reactions were set up in a final volume of 20 µL. Each PCR reaction contained the following: 2.25 µL MgCl₂ (2.8mM working), 2 µL Go Taq® 5X Buffer, 1 µL dNTPs (1.25mM working), 1 µL forward primer, 1 µL reverse primer, 1 µL DNA template, 1 µL 5% PVP (polyvinyl pyrrolidone), 10.5 µL bovine serum albumin (BSA, 0.53% working), and 0.25 µL Go Taq® Hot Start Taq Polymerase. PCR reaction was run following parameters in Table 2.
Table 2. PCR cycling parameters for DNA amplification. Cycle #: 40

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Cycle Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 (Initial denaturation)</td>
<td>15 minutes</td>
</tr>
<tr>
<td>94 (Denaturation)</td>
<td>15 seconds</td>
</tr>
<tr>
<td>62 (Annealing)</td>
<td>45 seconds</td>
</tr>
<tr>
<td>72 (Extension)</td>
<td>90 seconds</td>
</tr>
<tr>
<td>72 (Final extension)</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

**PCR Amplification with Phusion® High Fidelity Taq Polymerase**

When PCR reactions were set up using Phusion® High Fidelity DNA polymerase (New England BioLabs Inc, Ipswich, MA), each PCR reaction included the following: 5X Phusion® High Fidelity Buffer, 200 µM dNTPs, 0.02 U/µL Phusion® High Fidelity DNA Polymerase, 0.5 µM for each forward and reverse primer, and DEPC-treated water to a final volume of 50µL. For amplification of genomic DNA, the reaction mixture was initially denatured at 98°C for 30 seconds and then amplified for 20 cycles as follows: denaturing at 98°C for 5 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 30 seconds. Final extension was done at 72°C for 7 minutes. The reaction was held at 4°C in the thermocycler until it was retrieved following completion of the PCR.

Amplicons were visualized on a 1.5% agarose gel (SeaKem® GTG® Agarose, Lonza Rockland Inc, Rockland, ME) with 100 µg/mL ethidium bromide and sized by comparison with a 1 kb DNA ladder (Bionexus HI-LO size marker, Invitrogen, Carlsbad, CA). When the Phusion® High Fidelity enzyme was used as the active Taq polymerase, it was necessary to purify the amplicons away from this proofreading DNA polymerase before enzymatic addition of 3’-A overhangs prior to T/A cloning.
**Purification of Amplicons (Phusion® Products) using Phenol-Chloroform Extraction**

The PCR reaction was brought to a total volume of 200 µL (starting volume) with PCR grade water (Sigma-Aldrich, St. Louis, Missouri) in a 1.5 mL microfuge tube. Phenol-chloroform: IAA (Ambion® Thermoscientific) was added at two times the starting volume of each diluted PCR reaction. The preparation was mixed by inversion for 1-3 minutes and then centrifuged for 10 minutes at 16,000 x g. The aqueous phase (top layer) was removed to a clean tube. To remove any residual phenol, one starting volume of chloroform (VW1430-3, VWR) was added and mixed by inversion for 1-3 minutes, followed by centrifugation for 10 minutes at 16,000 x g. The aqueous layer was removed to a fresh tube, and 1/10th volume of sodium acetate was added along with two volumes of absolute ethanol. After precipitation of the nucleic acids overnight at -20°C, the sample was centrifuged for 20 minutes at 16,000 x g. The supernatant was decanted, and the pellet centrifuged once more to collect remaining fluid, which was removed by aspiration with a pipette tip. The pellet was air-dried on ice in a fume hood for 30 minutes. The amplicon pellet was then re-dissolved in 50 µL of nuclease-free water.

**Addition of 3’-A Overhangs**

Purified amplicons (25 µL) were pipetted into a 1.5 mL microfuge tube and kept on ice while the reaction was set up. The following components were added to each tube: 3µL 5X Go Taq® PCR buffer, 1µL dATPs (10mM stock), and 1µL GoTaq® polymerase to a final volume of 30µL. After mixing, and centrifugation to collect the contents, the reaction was incubated at 68°C on a heat block (Fisher Scientific-Isotemp®) for 30 minutes. After the incubation period, the tubes were held on ice until the A-tailed amplicons could be ligated into pENTR 5’TOPO vector using the T/A cloning strategy.
PCR Cloning into the pENTR 5’-TOPO® TA Vector

Each ligation reaction was set up in a 0.2 mL PCR tube with following components from TOPO® TA Cloning® kit for sequencing (Invitrogen, Carlsbad, CA): 4 µL amplicons (with A-overhangs), 1 µL salt solution, and 1 µL pENTR 5’-TOPO® TA vector. After gentle mixing, the reaction was incubated at room temperature for 15 minutes, and then held on ice until competent bacteria could be transformed with the ligation reaction.

One Shot® TOP10 Chemically Competent cells were used for bacterial transformation. The bacterial cells were thawed on ice prior to reaction. 4 µL of each ligation reaction was added to the competent cells and incubated on ice for 5 minutes. The cells were heat-shocked for 30 seconds in a 42\(^\circ\)C water bath, then immediately placed on ice for 2 minutes. Room temperature S.O.C medium (2% w/v bacto- tryptone, 0.5% w/v yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM MgSO\(_4\) and 20 mM glucose) was added to the heat-shocked cells and incubated at 37\(^\circ\)C for 1 hour on a horizontal shaking platform at 250 rpm. Prior to plating the transformed cells, 20 µL of S.O.C. medium was spread on plates containing LB (Luria-Bertoni) agar and ampicillin (50µg/mL); the plates incubated at 37\(^\circ\)C for 30 minutes to warm them and to allow the S.O.C to soak into medium.

Cells were plated (10 to 100 µL per plate) and incubated overnight at 37\(^\circ\)C. Bacterial colonies that appeared on the selective plates were streaked on ‘patch plates’ (LB agar plus ampicillin at 50µg/mL) using a sterile pipette tip. The bacterial cells on the pipette tip were also inoculated into 5 mL LB broth containing kanamycin (50 µg/mL) and ampicillin (50 µg/mL). The liquid cultures were incubated at 37\(^\circ\)C for approximately 17 hours on a horizontal shaker at 200 rpm. Cells were pelleted at 3500 rpm for 12 minutes using IEC|Centra®CL2 Centrifuge. The supernatant was decanted, the bacterial pellet re-suspended
in P1 buffer, and plasmid DNA purified using the QIAprep Spin Miniprep Kit (Valencia, CA). The manufacturer’s protocol was followed and DNA was eluted with nuclease-free water and stored at -20°C.

Plasmids were screened for the presence of the insert (cDNA or genomic amplicon) using PCR with M13F and primers (Table 1). Screening PCR reactions contained 50 ng plasmid DNA and the reactions were set up with GoTaq® DNA Polymerase as described earlier. Amplicons were separated on 1% agarose gels stained with ethidium bromide as described earlier.

**Sanger Sequencing of HD-AGP Genomic and cDNA Clones**

Recombinant plasmids were also analyzed by digestion with restriction enzymes. Restriction digests were set up as follows for each plasmid preparation: 2 µL Buffer H (10X stock), 3 µL plasmid DNA (about 200 ng/µL in most cases), 1.0 µL EcoR1 (10 U/µL stock, New England Biolabs, Massachusetts), brought to 20 µL with nuclease-free water. The reaction was gently mixed, centrifuged for 10 seconds, and then incubated at 37°C for 1 hour. The restriction digests were analyzed on 1% agarose gels stained with ethidium bromide, as described earlier. Recombinant plasmids (300 ng/µL) that were confirmed to harbor the amplicon of interest (generated by PCR from cDNA or genomic DNA templates) were shipped to Nevada Genomics (Reno, NV) for DNA sequencing. Reactions were sent in duplicate to ensure sequence consensus. Each gene was sequenced a total of four times using two different primers, forward primer M13F and reverse primer SP6, for each sample and it’s duplicate. Sequence read quality was monitored by Nevada Genomics and returned only reads with a Phred score of 20 or higher per base, indicating ≥99% accuracy for each base call.
The electropherograms returned by Nevada Genomics were inspected using BioEdit to confirm sequence read quality and remove vector primers off the ends of the sequences. Sequence end trimming was done by evaluating the quality of the electropherogram peaks and by alignment with a known ortholog sequence, *PhPRP1* (Genbank accession FJ719032) as a reference. Open source software was also used, DNA Baser Sequence Assembler v4.x (2014), to assemble the genomic DNA contigs, which were longer and more difficult to assemble, compared to the cDNA sequences, due to the presence of a single intron whose sequence had not been reported in the literature and that was not available from DNA databases. Genomic and cDNA sequences were aligned using open source software programs, including MUSCLE and SnapGene®. Clustal Omega v1.2 was used for multiple alignment of cDNA sequences encoding HD-AGPs from *Nicotiana tabacum* and the three *Petunia* species. The same program was used to align the introns from the two genomic DNA bands amplified from *N. tabacum* (gTTS-1 and gTTS-2), as well as a ~1.3 kb HD-AGP amplicon from *P. hybrida* (gPhPRP1.1300) and the ~1.4 kb HD-AGP amplicon from *P. inflata* (gPifPRP1).

**Protein Analysis**

**Protein Extraction**

Plant tissues (pistil, seedlings, whole leaf, root, leaf lamina and leaf veins) were collected, weighed and homogenized in Tris-buffered saline (100 mM NaCl in 40 mM Tris-HCl, pH 8.0) in microfuge tubes. Extracts from all the vegetative tissues were prepared at a ratio of 100 mg tissue fresh weight per 100 µL of buffer. Pistil extracts were prepared at a ratio of 10 mg tissue fresh weight per 200 µL of buffer. Homogenate was centrifuged for 5
minutes to pellet cellular debris, and the supernatant was used directly in SDS-PAGE and immunoblot analyses.

**SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)**

Tissue samples were prepared by mixing 40 µL of supernatant and 10 µL of 5X loading buffer stock (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, and 0.02% bromophenol blue). The sample-loading buffer mixture was boiled for 5 minutes at 80 °C. Samples were then immediately loaded on 1.5 mm thick discontinuous SDS-PAGE gels prepared and run according to Laemmli (1970).

The MiniProtean gel system (Biorad, Hercules, CA) with 1.5 mm plates was used for SDS-PAGE. The plates were cleaned with 70% ethanol (w/v) and rinsed with double-distilled water (Nanopure) prior to casting the polyacrylamide gels. An 8% resolving gel was made containing these stock solutions: 4.4 mL 4X Tris-HCl (1 M pH 8.8), 3.5 mL 40% acrylamide, 1.0 mL 10% SDS, 150 µL 10% AP (ammonium persulfate) and 30 µL TEMED (tetramethylethylenediamine) and 6.1 mL double-distilled water for a final volume of 15.2 mL. The resolving gel was overlaid with water to enhance polymerization. A stacking gel solution was made using these stock solutions: 1.88 mL 4X Tris-HCl (1 M, pH 6.8), 825 µL 40% acrylamide, 250 µL 10% SDS, 75 µL 10% AP, 15 µL TEMED, and 4.58 mL double-distilled water with a minute amount of bromophenol blue dye to aid in the visualization of the wells during gel loading. The water overlay was poured off the resolving gel after it was fully polymerized, the stacking gel was poured directly on top and an 8-well 1.5 mm thick comb was inserted.

After the stacking gel was fully polymerized, the comb was removed and the gel plates clamped in place in the gel tank. The running buffer was diluted fresh from a 10X
running buffer stock (30.2 g Tris base, 144 g glycine, and 10 g SDS brought to 1.0 L with
double-distilled water).

A directly visible protein size marker was used to track band separation during
electrophoresis and to estimate the $M_r$ of proteins; in immunoblots, this marker also helped
gauge the efficiency of the electrotransfer. The visible protein marker used was Prosieve™
Quadcolor™ Protein Marker (Lonza Rockland Inc, Rockland, ME). In gels intended for
immunoblots, MagicMark XP (Invitrogen, Carlsbad, CA) was loaded, a chemiluminescent
molecular size marker that was used to estimate the $M_r$ of protein bands detected by
chemiluminescence and to serve as a positive control for the secondary antibodies and the
chemiluminescence detection reagents. The MagicMark XP and Prosieve protein markers
were loaded in the first and last wells, respectively, on the opposite sides of the gel, to aid
also in determining the orientation of the gel and thus the loading order on immunoblots.

Samples were loaded, 50µL of each, into the remaining lanes and electrophoresed at 95V
until the dye front migrated from the stacking gel into the resolving gel. After the samples
entered the resolving gel, the voltage was reduced to 80V for the remainder of the run, which
was terminated when the dye front was about one cm from the bottom of the gel; typically,
two hours from the start of electrophoresis. The polyacrylamide gels were removed from the
plates and either stained with Coomassie Brilliant G-250 for visualizing proteins or processed
further for immunoblot analysis.

**Protein Immunoblots**

Proteins separated on SDS-PAGE gels were transferred to a 0.45 µM nitrocellulose
membrane (Thermo Scientific™ Pierce™) at a constant voltage of 120V for two hours at
$4^\circ$C using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories Inc.,
CA). The transfer buffer was diluted with double-distilled water from a 10X stock (containing 30.2 g Tris base, 144 g glycine, 1 g SDS) to contain 20% methanol. When transfer was complete, the gel was removed and discarded, and the nitrocellulose membrane was blocked for at least one hour in PBST buffer (0.1% Tween®20 in phosphate-buffered saline solution) containing 2% non-fat milk powder. The membrane was incubated overnight in anti-TTS primary antibody solution (PBST buffer with 2% nonfat milk and 1:1000 dilution of an anti-TTS antibody). The anti-TTS antibody was a gift from Professor Bruce McClure (University of Missouri, Columbus, Missouri). The antibody, raised in rabbits, recognizes a 12-amino acid epitope (NNTKKTLVEQGK/T) that is almost perfectly conserved among the *Nicotiana* TTS proteins and *PhPRP1* from *Petunia hybrida* (Twomey, 2012).

After incubation with primary antibody, the nitrocellulose membrane was washed three times (10 minutes per wash) using PBST buffer to remove non-specifically bound antibody. The membrane was then probed by incubation in an anti-rabbit IgG secondary antibody solution (PBST buffer with 2% nonfat milk and a 1:10,000 dilution of horseradish peroxidase-linked secondary antibody against rabbit IgG) for at least one hour. As before, the nitrocellulose membrane was washed three times, 10 minutes per wash, using PBST buffer to remove any secondary antibody bound non-specifically to the membrane. To detect the horseradish peroxidase signal, the membrane was submerged in 2 mL chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, ThermoScientific) for at least five minutes prior to imaging. Images of immunoblot were captured using an AlphaInnotech FluorChem Imager. Exposure time varied from one to twenty minutes, depending on the
strength of signal. The relative intensity of the chemiluminescent signal in seedling extracts was compared using an open source computer program, ImageJ.

All immunoblot experiments were repeated a minimum of three times yielding the same data trends.
RESULTS

HD-AGP Intron from P. hybrida is Nearly Identical to Intron Identified in P. inflata

Because P. hybrida is a synthetic species, created by crossing members of the P. axillaris complex and the P. intergrifolia complex (Sink, 1984), I predicted that the P. hybrida genome would harbor an HD-AGPs from each ancestral species. I used genomic DNA extracted from leaves of Petunia as template to PCR-amplify HD-AGPs, using primers expected to amplify a 727 bp cDNA fragment encoding PhPRP1 (Twomey, 2012). As seen in Figure 6, a single approximately 1,300 bp amplicon was amplified from P. inflata genomic DNA, and a single amplicon, approximately 1,900 bp in length, was amplified from P. axillaris parodii genomic DNA. In contrast, P. hybrida genomic DNA yielded two amplicons: an approximately 1,300 bp amplicon (gPhPRP1.1300) and an approximately 2,000 bp amplicon (gPhPRP1.2000).

The smaller of the two amplicons from P. hybrida was cloned, and sequencing revealed its length as precisely 1,338 bp. Alignment with the P. hybrida HD-AGP cDNA (Twomey, 2012) showed the presence of a single 596 bp intron in gPhPRP1.1300 (Appendix: Figure 16). The coding region of gPhPRP1.1300 is 99.2% identical the PhPRP1 cDNA described by Twomey (2012) from P. hybrida. There is 98.9% identity between the coding region of gPhPRP1.1300 and the cDNA encoding Pa2PRP1 from P. axillaris parodii (Figure 7).

The 1,366 bp band amplified from P. inflata genomic DNA was named gPifPRP1. Sequence analysis reveals a single 603 bp intron, flanked by 763 bp of coding sequence (Appendix: Figure 17). The coding region of this genomic fragment from P. inflata, (gPifPRP1) is 99.3% identical to the HD-AGP cDNA from P. inflata, PiPRP1 (Figure 7).
The intron in gPhPRP1.1300 and in gPifPRP1 were found to be 99.5 % identical, with 10 variant nucleotides, including a 7 bp indel that is present in gPifPRP1 but absent in gPhPRP1.1300 (Figure 7).

**HD-AGP Sequences from *N. tabacum* Vegetative Tissue Matches Previously Published cDNA from Pistil Tissue**

As noted in the introduction, *Nicotiana tabacum* is a natural allotetraploid descended from *N. tomentosiformis* and *N. sylvestris*. I amplified genomic DNA extracted from young seedlings of *N. tabacum*, and PCR amplification using TTS-1/TTS-2 primers generated two amplicons: a 1,344 bp fragment and a 1,163 bp fragment (Figure 8). Each fragment was cloned and sequenced. Sequence analysis of the 1,344 bp amplicon revealed an 873 bp intron, flanked by a total of 471 bp of coding sequence (Appendix: Figure 18); because the coding regions had a 99.35 % identity to the cDNA sequence encoding TTS-1, this amplicon was named gTTS-1. Sequence analysis of the 1,163 bp amplicon revealed a 666 bp intron, flanked by a total of 497 bp of coding region (Appendix: Figure 19); because the coding regions had a 99.20 % identity to the cDNA sequence encoding TTS-2, this amplicon was named gTTS-2. Sequence alignment shows 69.38% identity between the introns in gTTS-1 and gTTS-2 (Figure 9).

**The cDNA and Deduced Amino Acid Sequences of *P. exserta* and *P. axillaris* are Identical; *P. inflata* HD-AGP is Divergent**

An 877 bp cDNA sequence, named PePRP1, was cloned and sequenced from *P. exserta* pistils. This cDNA has a 14 bp 5’-untranslated region, a 768 bp coding region, and a 95 bp 3’-untranslated region (Figure 10). The deduced amino acid sequence reveals a highly conserved motif, MAKAFVL, in the 12 bp signal sequence. The motif is 100% conserved between PePRP1 and PhPRP1 (FJ719032.1) from *P. hybrida* (Twomey et al., 2013), as well
with \textit{PaPRP1} from \textit{P. axillaris parodii} and \textit{PiPRP1} from \textit{P. integrifolia integrifolia} (Callaway, 2012).

I also cloned and sequenced cDNA encoding HD-AGP orthologs from \textit{P. axillaris parodii} (\textit{Pa2PRP1}) and \textit{P. inflata} (\textit{PiPRP1}) pistils. \textit{Pa2PRP1} is 877 bp with a 14 bp 5’-untranslated region, a 768 bp coding region, and a 95 bp 3’-untranslated region (Figure 10). \textit{PiPRP1} is 889 bp with a 14 bp 5’-untranslated region, a 780 bp coding region, and a 95 bp 3’-untranslated region (Figure 10). Sequence analysis of \textit{Pa2PRP1} from \textit{P. axillaris parodii} reveals 99.35\% sequence identity with \textit{PaPRP1} from \textit{P. axillaris parodii} (Callaway, 2012), and \textit{PiPRP1} from \textit{P. inflata} is 99.24\% identical to \textit{PiPRP1} from \textit{P. integrifolia intergrifolia} (Callaway, 2012). These sequence comparisons show that HD-AGPs are virtually identical between members of the \textit{Petunia axillaris} complex and \textit{P. exserta} on the one hand, and between \textit{P. integrifolia} and \textit{P. inflata} on the other hand.

An alignment of \textit{PePRP1}, \textit{Pa2PRP1}, and \textit{PiPRP1} cDNA sequences shows 99.89\% identity between \textit{PePRP1} and \textit{Pa2PRP2} with only one nucleotide substitution between the two (Figure 10). There are 25 nucleotide substitutions between the three sequences including a 12 bp indel starting at nucleotide position 309 in \textit{PiPRP1} that is not found in \textit{PePRP1} or \textit{Pa2PRP1}. An alignment of the deduced amino acid sequence shows a 4-amino acid indel in \textit{PiPRP1}, and 5 amino acid substitutions among \textit{PePRP1}, \textit{Pa2PRP1}, and \textit{PiPRP1} (Figure 11). The one nucleotide difference between \textit{PePRP1} and \textit{Pa2PRP1} did not result in a change in the amino acid sequences, indicating that it is a synonymous substitution.

\textbf{Tissue-Specific HD-AGP Glycoforms Found among Various Tissue Types and Developmental Stages in \textit{P. axillaris} and \textit{N. tabacum}}

The profile of HD-AGPs was examined in a variety of \textit{P. axillaris} tissue types using protein immunoblots. An anti-TTS antibody cross-reacted with proteins in extracts made
from one week-old whole seedlings, two week-old young plants, major veins of leaves, minor veins of leaves, leaf lamina, root tips, whole roots from young plants and pollinated pistil.

One week-old whole seedlings show two glycoforms of the protein that migrate at 80 and 37 kDa, respectively (Figure 12). The cDNA sequence of Pa2PRP1 predicts a protein backbone with a molecular mass of 27.1 kDa. Extracts from two week-old young plants have three cross-reacting bands at 200, 150 and 80 kDa. Extracts from leaf veins and whole roots display the same bands (with Mr at 200, 150 and 80 kDa).

The profile of HD-AGP glycoforms is different in extracts made from minor veins, and from the leaf lamina, compared to the profile seen in other vegetative tissues described above. While the 80 kDa band is seen in all extracts from vegetative tissues, the highest Mr glycoform migrates at 220 kDa (instead of 200 kDa), and the 150 kDa band was not present, in extracts made from minor leaf veins or leaf lamina (Figure 12). The deglycosylated form of the protein (~27 kDa) was not present in extracts from any of the vegetative tissues.

The pistil HD-AGP profile shows a smear of glycoforms starting at 120 kDa and ending at 55 kDa. There is a single, faint band observed at 37 kDa. The smear is not homogenous, however: distinct bands are present within the smear that appear darker suggesting discrete glycoforms in higher abundance than the more heterogeneous minor components.

To compare the profile of HD-AGPs expressed in vegetative tissues of P. axillaris with those expressed in vegetative tissues of N. tabacum, I made extracts from two week-old seedlings, and root tips, leaves, and leaf veins of six month-old plants. As with P. axillaris seedlings, the seedling extracts from N. tabacum displayed two glycoforms that
migrated at 80 and 37 kDa (Figure 13). Extracts from root tips showed the same profile (two bands, migrating at 80 and 37 kDa). Extracts made from leaf lamina of six month-old plants, and one month-old whole roots, each showed only one band migrating at 37 kDa. Extracts from leaf veins of six month-old plants showed bands migrating at 200, 150 and 80 kDa, a profile identical to that seen in the leaf vein extracts from *P. axillaris* (compare Figure 12 and 13).

**HD-AGP Glycoforms Present in Etiolated Seedlings are More Abundant than in Light-Grown Seedlings of *P. axillaris axillaris***

Seedlings grown in poor light or darkness are well known to display etiolation, a set of phenotypes that includes yellowing, persistent apical hooks, lack of leaf expansion, and a tall and spindly appearance. These phenotypes are readily observed in *P. axillaris* seedlings grown in darkness (Figure 14A and B). Figure 14C shows that etiolated seedlings were approximately 3-fold taller than light-grown seedlings, in addition to being spindly and yellow, with unexpanded leaves and persistent apical hooks (Figure 14B).

To compare the profile and abundance of HD-AGPs in light-grown and etiolated seedlings, I made protein extracts from *P. axillaris* seedlings that had been grown for one week under bright light or total darkness. Extract from light-grown seedlings showed two cross-reacting bands at 80 and 37 kDa. The profile of dark-grown seedlings proved to be different with two highly glycosylated forms at 200 and 150 kDa as well as one small band at 27 kDa (Figure 15A). Coomassie staining of SDS-PAGE gels was used for qualitative confirmation that equivalent protein levels were loaded in each well. Relative density of bands was calculated and the total density of the bands from dark-grown seedling was found to be 51% higher than the total density of bands from light-grown seedlings (Figure 15B). This experiment had been conducted twice before with one-week old seedlings, yielding the
same data trend, except that the total HD-AGP signal was weaker because the bands are less intense in extracts made from one-week old seedlings. This experiment has been repeated at least three times by other members of the lab who observed the same trend (stronger HD-AGP signal from etiolated seedling extracts).

**HD-AGP cDNA and Genomic Sequence: Major Findings**

The major findings related to HD-AGP nucleotide sequence diversity are the following: (1) a single HD-AGP amplicon was observed when gene-specific primers were used to amplify genomic DNA from four wild species and subspecies of *Petunia*, suggesting a single HD-AGP copy in the haploid genome of these species; (2) two distinct amplicons were observed in the synthetic species, *P. hybrida* var. Tidal Wave Silver, with the smaller of the two (~1.3 kb) hypothesized to have been inherited from the *P. axillaris axillaris* progenitor and the larger (~2.0 kb) of the two bands likely inherited from the *P. axillaris parodii* ancestor; (3) two distinct amplicons were observed also in the other hybrid species, *N. tabacum*, with the smaller of the two bands (*gTTS-2*) clearly inherited from *N. tomentosiformes* and the larger band (*gTTS-1*) from the *N. sylvestris* lineage; (4) intron sequences from *P. hybrida* and *P. inflata* HD-AGPs are strikingly similar (99.5% identity), underscoring the quite low genetic differentiation within this genus; (5) the introns of *TTS-1* and *TTS-2* are more divergent (69.4% nucleotide identity) compared to the *Petunia* introns.

**HD-AGP Protein Expression Pattern: Major Findings**

The major findings related to HD-AGP expression patterns are the following: (1) in contrast to the tremendous size heterogeneity of pistil HD-AGPs on SDS-PAGE gels, vegetative tissues display two to four discrete bands depending on the tissue and developmental stage, in both *P. axillaris axillaris* and *Nicotiana tabacum*; (2) two discrete
bands are seen in one-week old seedlings, but high M, bands characteristic of leaf tissue begin to appear in two-week old seedlings; (3) HD-AGP levels are upregulated in etiolated (dark-grown) seedlings compared to light-grown seedlings, and a 27 kDa unglycosylated isoform that is not seen in other tissues becomes abundant in etiolated seedlings.
DISCUSSION

This study focused on histidine domain-AGPs (HD-AGPs), a subfamily of chimeric or non-classical AGPs that bind Yariv reagent (a phenylglycoside) and have a distinctive domain architecture including a histidine-rich domain near the N-terminus. TTS-1, TTS-2, and NaTTS from Nicotiana are the best characterized members of this subfamily of AGPs (Cheung et al., 2000). Although these Nicotiana AGPs were described as unique to the pistil transmitting tissue (Cheung et al., 1993; Cheung et al., 2000), our investigations show that TTS-1 and TTS-2 are expressed in some vegetative tissues as well (Twomey et al., 2013; Callaway, 2012). Because the proteins are not exclusive to the transmitting tissue, histidine-domain-AGP (HD-AGP) has been proposed as a more inclusive name for this subfamily of non-classical AGPs (Twomey et al., 2013) and that nomenclature is adhered to in this thesis.

**HD-AGPs in Wild Petunia and Nicotiana Species Reveal a Single Intron**

Gene-specific primers corresponding to highly conserved regions of Petunia HD-AGPs (Twomey et al., 2013; Callaway, 2012), or highly conserved regions of TTS-1 and TTS-2 from Nicotiana (Cheung et al., 2000), were used to PCR-amplify genomic DNA from Petunia and Nicotiana species, respectively. My experiments consistently yielded a single band from genomic DNA of four wild species and subspecies of Petunia (*P. axillaris axillaris*, *P. axillaris parodi*, *P. exserta*, and *P. inflata*). An undergraduate student (Suman Panwar) also observed a single band when she conducted the experiment using *P. integrifolia integrifolia* genomic DNA as template (data not shown). Other members of the lab amplified genomic DNA from these species using other primer pairs, including nested primers that flank the intron, and in every case a single amplicon was observed. From these observations,
I infer that a single copy of HD-AGP is present in the haploid genome of the wild species of *Petunia*.

We have proposed that AGP31, an AGP that is expressed in pistils and vegetative tissues in *Arabidopsis thaliana* (Liu and Mehdy, 2007), is an HD-AGP ortholog. The TAIR database shows that *AGP31* occurs as a single copy, located on Chromosome 1, in the *A. thaliana* genome. The annotated genome of *Nicotiana benthamiana* is available at the SolGenomics network, and using a BLAST search with the *TTS-2* sequence as the query term, I located the HD-AGP ortholog of *N. benthamiana* as a single copy on Chromosome 2. Thus, there appears to be little to no redundancy in gene copies per haploid genome as far as solanaceous and *Arabidopsis* HD-AGPs are concerned.

In contrast to the single band amplified from wild *Petunia* species, genomic DNA from the synthetic species, *P. hybrida*, yielded two discrete amplicons. The data strongly support the hypothesis that the 1.3 kb band is derived from the *P. axillaris axillaris* ancestor of *Petunia hybrida*. The larger of the two amplicons matches the *P. axillaris parodii* amplicon in size, which suggests that the larger of the two HD-AGP genes in *P. hybrida* was contributed by a *P. axillaris parodii* progenitor, rather than the other two presumed contributors to *P. hybrida* ancestry, *P. integrifolia integrifolia* and *P. inflata*. The homologous gene copies contributed by *P. integrifolia integrifolia* and/or *P. inflata* may have been lost through genetic segregation in extant *P. hybrida* cultivars or at least in this popular cultivar of *P. hybrida* (Tidal Wave Silver). Zhang et al. (2008) used PCR-RFLP analysis to compare the *Chs-j* chalcone synthase gene of wild *Petunia* species and the garden petunia, and they too concluded that both *P. axillaris axillaris* and *P. axillaris parodii* have made a major genomic contribution to modern cultivars of *P. hybrida*. 
Although the HD-AGP coding regions are near-identical between the two subspecies, PCR with genomic DNA produced strikingly different amplicon from *P. axillaris axillaris* versus *P. axillaris parodii*. A single ~1.3 kb amplicon was amplified from *P. axillaris axillaris*, and from the genome of *P. inflata* and *P. exserta* (Figure 6). In contrast, the genomic template from *P. axillaris parodii* yielded a ~1.9 kb band, indicating an intron roughly twice as large as the intron in HD-AGPs of the other *Petunia* species.

My attempts to clone and sequence the ~1.9 kb amplicon from *P. axillaris parodii* genomic DNA were unsuccessful. I propose that there was a duplication of the ancestral ~600 bp intron in the *P. axillaris parodii* HD-AGP, generating a ~1.2 kb intron with tandem repeats. The presence of repeat elements in the *P. axillaris parodii* intron sequence may be the reason that the ~1.9 kb band amplified from genomic DNA failed to produce any recombinant plasmids. It is striking that I could not clone the similar-sized ‘parodii-type’ band amplified from *P. hybrida* genomic DNA, despite repeated attempts. Repeat sequences are known to produce instability in cloning in *E. coli*. Inserts may be recombined or deleted as a consequence of recombination-mediated DNA repair that takes place during DNA replication (Cromie et al., 2000).

Cheung et al. (2000) suggested that there is a single copy of *TTS-1* and *TTS-2* in the *N. tabacum* genome, each copy inherited from one of the two ancestors of this allotetraploid. A digital northern analysis of the *Nicotiana tabacum* pistil transcriptome found that *TTS-1* mRNA (the second-most abundant transcripts in the pistil) is 2.6 times more abundant than *TTS-2* mRNA (Table 1 in Quiapim et al. 2009). The downregulation of one of the two copies, *TTS-2* in this case, may be adaptive in that conspecific pollen tubes are likely selected to interact optimally with the predominant TTS (*TTS-1*). Thus, reduction in TTS-2 levels likely
minimizes incongruous interactions between pollen and pistil that would result in poor pollen performance leading to poor seed set.

**Petunia Intron Sequences are Similar but Nicotiana Introns are Highly Divergent**

The sequence of the intron embedded in the HD-AGP amplicon (gPifPRP1) from *P. inflata* was compared with the ‘axillaris-type’ of intron in gPhPRP1.1300 from *P. hybrida*. As seen in Figure 7, there is very little divergence between the intron of *P. inflata* and the ‘axillaris-type’ intron sequenced from *P. hybrida*. Intron sequences are usually not under the large purifying selection that operates on the coding region of a typical gene, and are generally expected to show greater sequence variation than the coding sequences of a pair of genes in sister taxa.

The observed conservation of intronic sequences is concordant with the observation that there is very little genetic differentiation among *Petunia* species because of the very recent origins of these species. For example, Ando et al. (2005) found only 8 site mutations in RFLP analysis comparing chloroplast DNA of *P. inflata* and the *P. integrifolia* group. Guo et al. (2015) compared transcriptomes of *P. axillaris*, *P. exserta*, and *P. integrifolia*, and they report SNP (single nucleotide polymorphism) frequencies of 1/2056 bp between *P. axillaris* and *P. exserta*, and 1/726 between *P. axillaris* and *P. integrifolia*. Kulcheski et al. (2006) found low sequence diversity when they compared nuclear ribosomal DNA internal transcribed spacer (ITS) regions among 11 *Petunia* species. Chen et al. (2007) compared the *HflI* gene, which encodes a flavonoid hydroxylase, among 19 natural taxa of *Petunia* and found a 1bp difference in the size on *HflI* intron II between *P. inflata* and *P. integrifolia*. *HflI* intron II size was similar between *P. axillaris axillaris* (2,344 bp) and *P. exserta* (2,354 bp) but much more divergent in *P. axillaris parodii* (2,041 bp). This size deviation of the *P.*
*axillaris parodii* *HfII* intron parallels the size difference I observed between the HD-AGP intron of *P. axillaris parodii* and all other natural species of *Petunia* in this study (Figure 6).

It is striking that the sequence variation in the coding region of *gPhPRP1.1300* (from *P. hybrida*) and *gPifPRP1* (from *P. inflata*) is greater than that between the intron embedded in each of these sequences (Figure 7). The coding region of HD-AGPs likely experiences strong positive selection during incipient speciation between closely-related sympatric taxa. The resulting sequence variation in the HD-AGPs, especially in the proline-rich hypervariable region, likely isolated diverging incipient species reproductively by reducing or blocking gene flow (Callaway, 2012). In this scenario, the introns would evolve at the background mutation rate, but the accumulation of non-synonymous changes in the coding region of HD-AGPs (particularly in the hypervariable region) would be strongly favored as a result of positive selection.

In contrast to the strong sequence conservation in introns of *Petunia* HD-AGPs, the introns of *TTS-1* (inherited from *N. sylvestris*) and *TTS-2* (inherited from *N. tomentosiformis*) are only 69.38% identical (Figure 9). Although these two genes are believed to play a similar role in *N. tabacum*, stimulating growth as well as attracting pollen tubes (Cheung et al., 1996), sequence identity between their coding region is 82.9%. In contrast to the incipient speciation between subspecies of *Petunia* and the relatively recent radiation of species within the genus *Petunia*, the historical ancestors of *N. tabacum*--*N. tomenforensis* and *N. sylvestris*—likely diverged much earlier in the evolutionary history of *Nicotiana* as indicated by their placement in divergent sections of the Solanacea (Knapp et al., 2004).
The cDNA and Deduced Amino Acid Sequences of PePRP1, Pa2PRP1 and PifPRP1

The cDNA sequences I characterized have the domain architecture of previously described HD-AGPs (Cheung et al., 2000; Twomey et al., 2013; Callaway, 2012). Like the other HD-AGPs, the cDNA I cloned all exhibit a multi-domain organization consisting of a short variable motif at the N-terminus of the mature protein followed by a short histidine-rich domain, a proline-rich region, and a C-terminal PAC domain with 4 conserved cysteines (Hijazi et al., 2012).

The HD-AGPs of P. axillaris parodii and P. axillaris axillaris are 99% as might be expected of two subspecies that readily hybridize in the lab and appear to retain their subspecies identity mainly through allopatry. P. exserta has tubular red bird-pollinated flowers (Figure 3) and a highly restricted distribution in southern Brazil. However, despite the morphological distinctions and striking differences in pollination syndrome (P. axillaris flowers are white, scented, and moth-pollinated), I predicted strong similarity in HD-AGPs between P. exserta and the P. axillaris group because these species are completely inter-fertile. I found a 1 bp nonsynonymous substitution in the cDNA between these two species, and an identical amino acid sequence. For comparison, the coding region of the FLS gene, which codes for flavonoid synthase, is 99% identical between P. axillaris and P. exserta, and 97% identical between P. axillaris and P. inflata.

HD-AGPs are Expressed in Rapidly Expanding Tissues in the Solanaceae

Twomey et al. (2013) showed that PhPRP1 found in Petunia hybrida reacted with Yariv reagent and concluded that it belongs to the AGP gene family. We demonstrated that PhPRP1 is an ortholog of the previously described TTS-1 and TTS-2 proteins (Genbank accession Z16403.1 and Z16404.1; Cheung et al. 1993) of N. tabacum. The finding that
PhPRP1 and TTS-1 are expressed in vegetative tissue, in addition to being abundant in the pistil transmitting tissue, lead to the designation of this subfamily of non-classical AGPs as histidine-domain arabinogalactan proteins (HD-AGPs).

Twomey et al. (2013) found that protein extracts from one month-old whole plantlets of Nicotiana tabacum or Petunia hybrida cross-reacted with an anti-TTS antibody raised to a 14-amino acid epitope conserved in both the Nicotiana and Petunia HD-AGPs. Furthermore, cDNA encoding TTS-1 and TTS-2 were isolated from N. tabacum seedling extract and sequencing confirmed that their nucleotide sequence was the same as the nucleotide sequence of the pistil cDNA encoding TTS-1 and TTS-2 (Twomey et al., 2013). I extended this investigation to examine HD-AGP expression in additional tissue types, at different developmental stages, and in response to etiolation versus growth in light.

Twomey et al. (2013) showed that TTS proteins are abundant in both young leaf and terminal portions of roots of six month-old N. tabacum plants, as well as in two week-old whole seedlings. Preliminary results produced by Callaway (2012) showed the presence of HD-AGPs in leaf tissue of P. integrifolia integrifolia. This prompted me to investigate further the role HD-AGPs play in vegetative tissues and more specifically, rapidly expanding tissues, by looking at the protein profile of various tissues and at different developmental stages.

In contrast to the tremendous size heterogeneity of pistil HD-AGPs, vegetative tissues displayed one to a few discrete bands on the immunoblots (Figure 12). Because the genomic DNA analysis strongly suggests that HD-AGPs occur as a single copy in the haploid genome of wild solanaceous species (Figure 6), the multiple bands seen on immunoblots likely represent different glycoforms that vary in the number and length of the glycan sidechains.
attached to the protein backbone through primarily O-linkages in the proline-rich domain and one N-linkage in the C-terminal PAC domain of the polypeptide.

HD-AGPs expressed in pistil tissue of *Nicotiana* and *Petunia* are both strikingly heterogeneous with regard to molecular mass (Wu et al., 2000; Twomey et al., 2013; lane 6 in Figure 12 in this thesis). The enormous size heterogeneity of pistil HD-AGPs may be a reflection of their role in nutritional support of pollen tubes growing in the pistil. Cheung et al. (2000) have reported that TTS proteins are deglycosylated in the presence of pollen tubes and that the released glycans may have a nutritive function in that they may serve as an energy source for pollen tubes. However, it is unlikely that supplying metabolic fuel is sole function of HD-AGPs. Cheung et al. (2000) have suggested that these proteins aid in the adhesion of pollen tubes to the pistil extracellular matrix, and this is more likely to be the chief function of HD-AGPs in pistil tissue. It is possible that deglycosylation by pollen-bound glycanases enables HD-AGPs to interact more effectively with a pollen ligand.

Solanaceous HD-AGPs in vegetative tissues are strikingly different from those in pistil tissue in that vegetative tissue HD-AGPs appear as one to a few discrete tissue-specific glycoforms on immunoblots (Twomey et al., 2013; Figure 12, this thesis). It may be that HD-AGPs in vegetative tissues lack extensive heterogeneity in glycosylation because it is not their function to furnish sugars via deglycosylation of their protein backbone. Instead, their glycan chains may be involved in cell-cell adhesion or other cell wall functions such as regulating patterns of differentiation in vascular tissue.

Because the HD-AGP protein backbone is the same in all the different vegetative and reproductive organs, the tissue-specific variation in glycosylation patterns are mostly likely generated by tissue-specific O-glycosyltransferases. Two small families of
glycosyltransferases (GTs) add glycan chains to AGPs in *Arabidopsis* (Showalter and Basu, 2016). Variant isoforms of glycosyltransferases may be differentially active in different tissue types and at different developmental stages (in leaf veins versus pistils, for example; or in mature roots versus root tips), resulting in variation in the number and size of glycan chains linked to the polypeptide backbone in the different tissue types (Twomey et al., 2013). The remodeling of these sidechains by glycoside hydrolases such as galactanase and arabinase (reviewed by Knoch et al., 2014) might account for the change in the HD-AGP glycoprofile during development (seedling to mature shoot, for example).

**HD-AGPs are Upregulated in Etiolated Seedlings Compared to Light-Grown Seedlings of *Petunia axillaris***

Etiolated seedlings look yellow because they lack chlorophyll and their apical hook remains closed and primary leaves remain unexpanded in the absence of light (Figure 14A and B). As an adaptive light-seeking response, etiolation induces rapid cell expansion of the hypocotyls, which is why etiolated seedlings grow considerably taller compared to dark-grown seedlings. AGPs have been known to be localized in the hypocotyls of *P. vulgaris* (Samson et al., 1983), as is AGP31 in *A. thaliana* (Liu and Mehdy, 2007) and HD-AGPs in the Solanaceae (Figure 15A). I decided to compare HD-AGP abundance in two week-old seedlings of *P. axillaris axillaris* to test the hypothesis that HD-AGPs accumulate at higher levels in etiolated seedlings, compared to light-grown seedlings, to facilitate the rapid growth of etiolated hypocotyls.

An approximately two-fold upregulation of HD-AGPs was observed in etiolated seedlings compared to light-grown seedlings (Figure 15), underscoring the importance of this protein in rapid cell elongation. Although the function of HD-AGPs in vegetative tissue is
not unknown, it is striking that these proteins are expressed in tissues containing cell types that undergo an extraordinary increase in linear dimensions.

The role of HD-AGPs in pollen tube elongation in the extracellular matrix of the pistil has been demonstrated convincingly (Cheung et al., 1995). Further, Liu and Mehdy (2007) have shown that AGP31 is expressed in phloem elements and in protoxylem elements in vascular bundles in the root, stem, and leaves of Arabidopsis. Phloem and xylem conducting elements are both highly elongate cells: phloem sieve tube elements can exceed 1mm in length, xylem tracheids longer than 10mm are known, and phloem and xylem fibers that exceed several centimeters are used in commercial cordage. Cell elongation on this scale may require special mechanisms, such as the adhesion-based (haptotactic) growth that has been suggested to play a role in pollen tube growth by Elizabeth Lord (Chae and Lord, 2011).

HD-AGPs in vegetative tissues may assist in the growth of elongating cells by helping to anchor cell regions proximal to growth foci at the apical ends. According to this model of adhesion-assisted growth, rapid expansion of seedlings would require upregulation of HD-AGPs to support the increased elongation rate of the developing vascular system in the hypocotyl. I predict that growth inhibitors will down-regulate solanaceous HD-AGPs, as has already been demonstrated for AGP31, which is transcriptionally repressed in Arabidopsis upon wounding or upon treatment with abscisic acid (ABA) or methyl jasmonate (Liu and Mehdy, 2007).

Response of P. axillaris Seedlings to Growth Regulators and Future Directions

The growth of eudicot seedlings is known to be sensitive to abiotic and biotic cues, and to growth regulators such as the hormones GA (gibberellin) and ABA (abscisic acid). As a first step toward investigating how HD-AGPs levels might be altered by growth regulators,
I set up an experiment to measure the height of one-week old seedlings of *P. axillaris* *axillaris*, kept in continuous light or continuous dark, and treated with various plant regulators or distilled water (control) two days after seeds were inoculated in sterile horticultural sand. As seen in Appendix: Figure 20, GA treatment boosted seedling growth in both light and dark-grown plants compared to distilled water controls (compare Figure 14C with the bar graph in Figure 20). ABA treatment did not significantly alter growth rates, in either light-grown or etiolated seedlings, compared to controls. In contrast, ethylene treatment was so severely inhibitory that there was almost no discernable seedling growth in the seeds imbibed in the dark and very poor growth in the light-grown seedlings (Appendix: Figure 20).

Based on this preliminary experiment, I would predict that GA will upregulate HD-AGPs in light-grown seedlings as well as dark-grown seedlings, whereas ethylene treatment (a lower concentration should be tried) will likely repress HD-AGP expression in a manner like that observed by Liu and Mehdy (2007) in *Arabidopsis* seedlings treated with methyl jasmonate.

**Conclusions**

The main conclusions of my thesis research are that (1) *Petunia* HD-AGPs are essentially identical between interbreeding species, but they are at least somewhat divergent in non-hybridizing species within the genus and much of that divergence involves XKPP motifs that lie in the proline-rich hypervariable domain; (2) there is very little sequence diversity in the noncoding regions of *Petunia* HD-AGPs, including the introns, suggesting a very recent radiation of this taxon, driven mainly by geographical isolation and variation in genes controlling pollination syndromes (Sheehan et al., 2016); in contrast, *TTS-1* and *TTS-2*
introns are highly divergent, which is consistent with the placement of the two progenitor species in highly divergent clades within *Nicotiana*; (3) discrete glycoforms of HD-AGPs are expressed in seedlings, roots, and leaf vasculature; (4) abiotic factors affect HD-AGP levels, such that etiolation upregulates HD-AGP accumulation.

Successful cloning and sequencing of the ~2,000 bp genomic bands from *P. hybrida* and *P. axillaris parodii* will help round-out the evolutionary history of *Petunia hybrida* and clarify relationships among the subspecies of the *P. axillaris* group. The upregulation of HD-AGP in etiolated seedlings should be confirmed, and it will be exciting to learn how HD-AGP levels are altered by hormones such as GA and methyl jasmonate. The most enlightening next step is understanding the function of HD-AGPs in vegetative tissue, and I am hopeful that my elucidation of HD-AGP profiles in vegetative tissue of *Petunia* has moved us forward in that quest.
Figure 1. Photographs of study species. (A) Lateral and apical views of *Petunia axillaris parodii* and (B) *Petunia axillaris axillaris* flowers.
Figure 2. Photographs of study species. (A) Lateral and apical view of *Petunia inflata*. (B) Lateral and apical view of *Petunia integrifolia integrifolia*. 
Figure 3. Photographs of study species. Lateral and apical view of *Petunia exserta*.
Figure 4. Photographs of study species. Lateral and apical view of *Petunia hybrida* var. Tidal Wave Silver.
Figure 5. Photograph of study species. One-month old plant of *Nicotiana tabacum* on the left, with a 2-week old plantlet on the right.
Figure 6. PCR amplification with HD-AGP primers and genomic DNA extracted from young leaves of Petunia species. (A) Lane M: Hi-Lo DNA size marker. Lane 1: *P. hybrida*; Lane 2: *P. axillaris parodii*; Lane 3: *P. inflata*. (B) Lane M: Hi-Lo DNA size marker. Lane 1: Empty; Lane 2: Empty; Lane 3: *P. axillaris axillaris*. (C) Lane M: Hi-Lo DNA size marker. Lane 1: *P. exserta*. 
Figure 7. MUSCLE alignment of genomic fragments sequenced from *P. hybrida* (*gPhPRP1.1300*) and *P. inflata* (*gPifPRP1*) with cDNA sequences from *P. axillaris parodii* (*Pa2PRP1*) and *P. inflata* (*PifPRP1*). Identical nucleotides are indicated by an asterisk (*). A 12 bp indel in the coding region of all sequences is highlighted. Intron sequences are in red, and a 7 bp indel within the intron is highlighted in grey. Nucleotide substitutions between genomic sequences are indicated by red arrows.
Figure 8. PCR amplification with TTS-specific primers using genomic DNA from one month-old seedlings of *N. tabacum*. Lane M: Hi-Lo DNA size marker. Lane 1: *N. tabacum*. A. 1.35 kb band identified as gTTS-1; B. 1.16 kb band identified as gTTS-2.
Figure 9. Clustal Omega alignment of introns identified in the two genomic fragments (gTTS-1 and gTTS-2) PCR-amplified from N. tabacum genomic DNA. Nucleotide substitutions are indicated by a dash (-). Identical nucleotides are indicated by an asterisk (*).
Figure 10. Clustal Omega alignment of cDNA encoding HD-AGP from *P. integrifolia inflata* (PiPRP1), *P. exserta* (PePRP1), and *P. axillaris parodii* (Pa2PRP1). The cDNA was isolated from pistil tissue of these species. Identical nucleotides are indicated by an asterisk (*). A gap in the nucleotide sequence is indicated by a dash (-). A 12 bp indel unique to the *P. integrifolia inflata* cDNA is highlighted in yellow. Nucleotide substitutions are indicated by red arrows.
| PePRP1 | MAKAFVLFHLSELSSSFFTLLSHGEGLMNGSLTKIHEDLHPAQAPKPHKGHHFPKHSPAP | **                               | PePRP1 | RPVAURGLVYCKPKYRGVGTNLATPLQGAIVKLACNNTKKTLVEQGTTDKNGFLILL | **                               |
| Pa2PRP1 | MAKAFVLFHLSELSSSFFTLLSHGEGLMNGSLTKIHEDLHPAQAPKPHKGHHFPKHSPAP | **                               | Pa2PRP1 | RKPAVURGLVYCKPKYRGVGTNLATPLQGAIVKLACNNTKKTLVEQGTTDKNGFLILL | **                               |
| PifPRP1 | MAKAFVLFHLSELSSSFFTLLSHGEGLMNGSLTKIHEDLHPAQAPKPHKGHHFPKHSPAP | **                               | PifPRP1 | RNPAVURGLVYCKPKYRGVGTNLATPLQGAIVKLACNNTKKTLVEQGTTDKNGFLILL | **                               |

Figure 11. Clustal Omega alignment of deduced amino acid sequences of HD-AGP cDNAs isolated from pistils of *P. exserta* (*PePRP1*), *P. axillaris parodii* (*Pa2PRP1*), and *P. inflata* (*PifPRP1*). Identical amino acids are indicated by an asterisk (*). A gap in the sequence is indicated with a dash (-); a colon (:) indicates a one-nucleotide difference among the translated sequences; a period (·) indicates that two or more nucleotides are different among the translated sequences.
Figure 12. Protein immunoblot analysis of *P. axillaris* HD-AGPs. Total protein was extracted from different tissue types and resolved by SDS-PAGE, prior to transfer to a nitrocellulose membrane and probing with a primary antibody that recognizes an epitope conserved among all *Petunia* HD-AGPs as well as the TTS proteins from *Nicotiana*. Coomassie staining of SDS-PAGE gel was used for qualitative confirmation that equivalent protein levels were loaded in each well. Lane M: Size standard; Lane 1: Protein extract from one week-old whole seedling; Lane 2: Extract from two week-old seedling; Lane 3: Extract from leaf major veins from one month-old plant; Lane 4: Extract from leaf lamina (includes minor veins) of one month-old plant; Lane 5: Whole root extract from one month-old plant. Lane 6: Extract from unpollinated pistil.
Figure 13. Protein immunoblot of HD-AGPs in vegetative tissues of *N. tabacum*. Coomassie staining of SDS-PAGE gels was used for qualitative confirmation that equivalent protein levels were loaded in each well. Lane M: Size standard; Lane 1: Protein extract from two week-old seedling; Lane 2: Extract from terminal portion of roots from six month-old plant; Lane 3: Extract from leaf veins of six month-old plant (main rib from 2-3cm long leaves); Lane 4: Extract from leaf lamina of six month old plant (includes minor veins, from 2-3cm leaves); Lane 5: Extract from whole roots of one month-old plant. The protein blot was probed with the anti-TTS antibody.
Figure 14. Response of one-week old *P. axillaris* seedlings to light and dark. Seedlings were grown on sterile sand for one-week under either 24-hour light or complete dark (etiolated). (A) Light-grown seedlings. (B) Etiolated (dark-grown) seedlings. (C) Bar graph showing mean height (mm) of week-old *P. axillaris* seedlings grown in light or dark. Error bars represent standard error, where n=50.
Figure 15. (A) Protein immunoblot using total protein extracts from *P. axillaris* seedlings grown in light or dark. Coomassie staining of SDS-PAGE gels was used for qualitative confirmation that equivalent protein levels were loaded in each well. Lane M: Size standard; Lane 1: Extract from light-grown seedlings; Lane 2: Extract from etiolated (dark-grown) seedlings. (B) Immunoblot densitometry. The table shows relative density pooled from all the bands seen in the extracts of light-grown seedlings (Lane 1) and of dark-grown seedlings (Lane 2). The pooled density of HD-AGP bands from dark-grown seedlings is 51% higher than that of the bands from light-grown seedling.

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<td>Total Density of Bands</td>
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APPENDIX

cTGCaTTAcTACACGcTTTgCAGGcATAGTGAAGTGTTGcTACgGTACg

Figure 16. Nucleotide and deduced amino acid sequence of a 1.35 kb fragment (gPhPRP1.1300) amplified from P. hybrida genomic DNA. Black letters represent coding regions; red portion represents the intron.

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Figure 17. Nucleotide and deduced amino acid sequences from a genomic fragment of *P. integrifolia inflata* (*gPifPRP1*) whole leaves. Black portions represent coding regions; red portion represents the intron.
Figure 18. Nucleotide and deduced amino acid sequence of a 1.35 kb band (gTTS-I) amplified from genomic DNA of *N. tabacum*. Red letters represents the identified intron.
Figure 19. Nucleotide and deduced amino acid sequence of a 1.16 kb band (gTTS-2) amplified from genomic DNA of *N. tabacum*. Red letters represent the identified intron.
Figure 20. Average height (mm) of week-old *P. axillaris* treated with various growth regulators. Seedlings were grown on sterile sand for one-week under either 24-hour light or complete dark (etiolated) and watered twice a week with distilled water or distilled water containing various growth regulators (10µM gibberellin; 1µM abscisic acid, ABA; and 1.5 µM Ethephon). Ethephon (chloroethylphosphonic acid) degrades in the presence of water to release the plant hormone ethylene. Error bars represent standard error of the mean, where n=50 for all treatments.