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CHARACTERIZATION OF TRANSGENIC PLUM LINES EXPRESSING GASTRODIA ANTIFUNGAL PROTEIN (GAFP)-1

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CHARACTERIZATION OF TRANSGENIC PLUM LINES EXPRESSING *GASTRODIA* ANTIFUNGAL PROTEIN (GAFP)-1

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ABSTRACT

The *Gastrodia* antifungal protein (GAFP)-1 is a mannose-binding lectin originating from the Asiatic orchid *Gastrodia elata*. It has potential for conferring resistance to fungal and non-fungal pathogens in other plants which is currently being investigated. The goals of this research project were to determine (i) the potential movement of GAFP-1 protein from transgenic rootstocks into the non-transgenic scion of chimeric-grafted trees (ii) the levels of GAFP-1 protein in lines of the cultivar 'Bluebyrd' expressing the gene *gafp-1* under the control of the polyubiquitin promoter *bul*409, and (iii) the susceptibility of selected lines to the root pathogens *Phytophthora cinnamomi* Rands and *Meloidogyne incognita* Kofoid & White in greenhouse experiments.

Wild-type (WT) plum (*Prunus domestica* cultivar 'Stanley') tissue was budded onto transgenic plum lines 'Stanley' 4J and 4I to create chimeric-grafted trees. Tissues from chimeric-grafted trees were analyzed for protein (leaf, soft shoot (non-woody shoot), and root) by immunodetection. The GAFP-1 lectin was identified within the roots, but not in the soft shoot or leaf tissues of the grafted, WT scions. These results suggest that GAFP protein is not moving into the WT scion tissues of chimeric-grafted plum trees, a feature that would likely appeal to consumers who are concerned about GMO in their food.

Only 9 out of 17 'Bluebyrd' plum lines containing the *gafp-1* gene produced GAFP-1 protein and only 1 of these 9 lines exhibited increased tolerance to Phytophthora root rot (PRR) caused by *P. cinnamomi*. This same line (BB-1) was also significantly more tolerant to infection by the root-knot nematode (RKN) *Meloidogyne incognita*. BB-1 was superior in resistance to PRR and equal in resistance to RKN compared to the previously

characterized ‘Stanley’ 4J line, which expresses the *gafp-1*gene under the control of the CaMV35S promoter. The levels of GAFP-1 synthesized in BB-1 were not elevated at 30 days after inoculation by *M. incognita* and at 5 days after inoculation by *P. cinnamomi*, suggesting that *bul*409 is not an inducible promoter. This study confirms the potential usefulness of incorporating the *gafp-1* gene in creating disease resistant rootstocks for stone fruit cultivars and suggests that the *gafp-1* gene provides comparable resistance to PRR and RKN irrespective of the promoter (*bul*409 or CaMV35S) being utilized for controlling gene expression.

DEDICATION

I dedicate this dissertation to my beloved husband, Bharat Bhut, for his support and encouragement throughout my study; my mother, Mrs. Jayshree Kalariya; my brother, Bhavesh Kalariya; my sister in-law, Pravina Kalariya; and my husband's family for their support.

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CHAPTER ONE

INTRODUCTION

Peaches, plums, apricots, nectarines, almonds, and cherries are "stone fruit" species bearing a hard pit, or "stone," as seed. Today peaches are being produced commercially in 29 states of the United States with a value of $461 million in 2004 (Borris and Brunke, 2006). Peach production in the USA is 8.7% of the world's total production and was approximately 1.1 million tons in August 2008 (Brunke and Chang, 2010; United States Department of Agriculture National Agricultural Statistics Service, 2009). South Carolina and Georgia are well known for the production of high quality fresh fruit and rank 2nd and 3rd, respectively, behind California in total production. In South Carolina approximately 18,000 acres are currently associated with peach production (South Carolina Department of Agriculture, 2010).

In the warm, humid climate of the southeastern United States pests and diseases tend to thrive. A number of pathogens attack the fruit, branches, trunk, and roots of peach trees. Among the most important root diseases are Armillaria root rot (ARR), Phytophthora root rot (PRR), and root-knot nematode (RKN). All can either weaken or even kill the root system.

ARR is also known as shoestring root rot, oak root rot, honey fungus, or Hallimasch (Fox, 2000a). The disease is caused by various *Armillaria* species, including *A. gallica*, *A. ostoyae*, *A. mellea*, and *A. tabescens* (Fox, 2000a). There is little host specificity among *Armillaria* species and approximately 700 plant species have been identified as

hosts; most of which are woody plants. The pathogen moves relatively slowly from host to host through rhizomorphs or root-to-root contact. In the Southeastern US, *A. tabescens* is the most prevalent species causing severe damage to peach trees (Adaskaveg et al., 2008). It was estimated that ARR caused $3.86 million in damage for South Carolina between 1987 and 1992 (Miller, 1994). In many cases, the pathogen successfully invades healthy tissue after it establishes itself saprophytically. Symptoms and signs of ARR include: the appearance of honey colored mushroom at the base of the tree (Fig 1.1A), poor terminal growth and curled, undersized leaves on all scaffold limbs (Fig.1.1B). White mycelial fans grow under the bark of the tree and their presence can be used to easily identify the disease (Fig.1.1C). Although older trees are more commonly affected, trees as young as 3 years are killed on replant sites (Adaskaveg et al., 2008). Management of this disease is difficult. Chemical control is largely ineffective. Fumigants such as carbon disulphide have good soil penetration but do not kill the fungus directly (West, 2000). Some fumigants, such as methyl bromide, have been banned due to the adverse effect on the environment and particularly on the ozone layer. "Armillatox" (active ingredient polyalkyl phenolics) was marketed for the control of ARR and it did show some effect on rhizomorphs but little effect on eradication of mycelium within wood pieces (West, 2000). The primary impediment to biological and chemical control of ARR is the inability of materials to reach the fungus inside roots (Fox, 2000b). Cultural control of ARR such as complete removal of wood debris is not economically feasible (Fox, 2000c). Biological control with wood-inhabiting organisms can prevent or inhibit growth of the pathogen mycelium by substrate competition (Hagle and Shaw, 1991), but direct

inoculation of the roots is difficult (Raziq, 2000). Additionally, there are no peach rootstocks resistant to this disease.

PRR is a soil-borne disease causing destructive damage to stone fruit orchards. This disease is caused by *Phytophthora* species, occurs at any age of a tree, and results in tree death. *Phytophthora* species are fungal-like organisms classified into the kingdom Stramenopila (Adaskaveg et al., 2008). The cell wall of *Phytophthora* species is composed of cellulose and β-glucans not chitin, which is the cell wall material in true fungi (Adaskaveg et al., 2008). *Phytophthora* species have coenocytic mycelium with few or no septa. In the presence of water the mycelium produces zoospores, which are biflagellate and can move in free water and infect healthy trees (Erwin and Ribeiro, 1996). *P. cinnamomi* causes PRR in southeastern peach orchards and can cause disease in over 3000 other plant species including crop plants such as avocado, pineapple, chestnut, and macadamia (Hardham, 2005). Excessive irrigation and rainfall contribute to disease development, but the most important factor is duration of free water in soil. Free water enables the rapid formation of sporangia (Fig.1.2A) and zoospores (Erwin and Ribeiro, 1996), which can move to new hosts. *P. cinnamomi* can survive in moist soil for as long as 6 years (Hardham, 2005). Estimated loss due to *P. cinnamomi* is $30 million annually in avocado in California and $5 million annually in pine plantations in forests from Virginia to Mississippi (Hardham, 2005). Losses of a comparable magnitude due to infection by *P. cinnamomi* are speculated to occur in other crop species. Disease symptoms include poor terminal growth with small and chlorotic leaves on the entire canopy of the tree (Fig. 1.2B). The tree may exhibit dieback and will decline either

progressively or suddenly depending on disease severity (Fig. 1.2C). *Phytophthora* species survive as chlamydospores, oospores, or hyphae in the root debris. Zoospores are considered the primary spore associated with infection. Management by chemical control using fungicides can be useful but modern fungicides are mostly fungistatic. Phenylamides (e.g. metalaxyl) are the most effective for the control of *P. cinnamomi* but their effectiveness varies with different *P. cinnamomi* isolates (Hardy et al., 2001) and environmental conditions, such as the phosphorus levels in soil (Guest and Grant, 1991). Beside fumigants, phosphites (e.g. fosetyl-Al) are used for PRR control. They are xylem–mobile systemic fungicides shown to reduce the production of zoospores in infected plant material (Hardham, 2005). Recent research, however, suggests that phosphites may cause phytotoxicity producing effects such as foliar damage, reduction of pollen viability, pollen tube growth, and decrease in root growth (Hardham, 2005). Host resistance may be the best management strategy especially for the management of soil-borne diseases like PRR. Currently there are no *Prunus* rootstocks that are resistant to PRR.

Nematodes are true roundworms, non-segmented microscopic animals. They have bilateral symmetry and look like threads under low power magnification. The size of plant parasitic nematodes ranges from 0.4 mm to 5 mm in length. Nematodes are often ignored and underestimated due to their localization in soil and the non-specific symptoms that they cause in plants above ground but, they are economically important. If nematodes are not properly managed they can reduce yield and plant vigor and even cause tree death (Nyczepir and Esmenjaud, 2008). Two nematodes cause major problems on peach trees, *Meloidogyne* species (root knot nematodes) and *Mesocriconema xenoplax*

(Raski) Loof & de Grisse (ring nematodes). *Meloidogyne* species cause root knots on plant roots (Fig. 1.3A) and are commonly referred to as root knot nematodes (RKN). RKN are some of the most economically important plant parasitic nematodes because of their wide host range and widespread distribution (Nyczepir and Esmenjaud, 2008). Above-ground symptoms are generally seen as stunting of growth, yellowing of leaves, while below-ground symptoms include root galls. The nematodes feed on the roots by puncturing and sucking cell contents (The California Tree Fruit Agreement and The California Minor Crops Council, 2006) and the second stage juvenile is the infective stage (Fig. 1.3B). *M. incognita, M. javanica* and *M. arenaria* are considered to be the most damaging species in peach trees. These nematodes species are common on peach in the USA, especially when plants are cultivated in sandy soils (Stirling, 1975). Ring nematodes are migratory ectoparasites. They are short, and easily distinguished by a long stylet and distinctive coarse annulations around the body (Nyczepir and Esmenjaud, 2008). They complete their life cycle in 25 to 35 days. Ring nematodes are associated with Peach Tree Short Life (PTSL) in peach. Currently, Guardian rootstocks are commonly used due to their tolerance to ring nematodes and their ability to reduce the incidence of PTSL in sandy sites. Nematode damage also interferes with nutrient and water uptake and nematodes themselves transmit viral diseases such as Prunus stem pitting disease caused by Tomato ringspot virus (TmRSV; Hoy, 1983). They may also predispose roots of plum and peach to infection by *Agrobacterium tumefaciens* which causes crown gall disease (Rubio Cabetas et al., 2001; Dhanvantari et al., 1975). Crop rotation, the use of resistance rootstocks, and chemical control are major methods for

nematodes management. Crop rotation is difficult, because of the wide host range of *Meloidogyne* species and the longevity of the peach tree, which is not replanted every year but remains in the ground for up to 20 years. Some effective nematicides such as organophosphates and carbamates are non-specific neurotoxins with poor environmental profiles and high toxicity to mammals (Haydock et al., 2006; Risher et al., 1987). Resistant rootstocks may be the most economic and environmentally safe method for management of *Meloidogyne* species in *Prunus* species. In the southeastern US, as started earlier 'Guardian' is the most commonly used rootstock due to its resistance to peach tree short life (PTSL) which is associated with the presence of the ring nematode *M. xenoplax*. However, the rootstock is not immune/resistant to *Meloidogyne* species (Nyczepir and Esmenjaud, 2008, Nyczepir et al., 2009) 'Lovell' and 'Nemaguard' are also important and commonly used peach rootstocks in the USA. 'Lovell' usually outlives 'Nemaguard' on sites in the Southeast where *M. xenoplax* is most prevalent (Nyczepir and Esmenjaud, 2008). In a recent study, 'Halford' rootstock, which is frequently used in the Northern US was found to be highly susceptible to *Meloidogyne species* (Nyczepir et al., 2008). Although, an advanced line of "Guardian^{TM}, SC 3-17-7, showed reduced numbers of root galls, and reduced numbers of eggs per gram of dry weight root due to infestation with *Meloidogyne* species as compared to "Lovell" and SL2891 (another advanced line of "Guardian") (Nyczepir et al., 2006). There is no peach rootstock available that is completely resistance to RKN.

ARR, PRR, and RKN share the commonality of being difficult-to-control, soilborne diseases. The lack of effective chemical, biological, or cultural management strategies

necessitates investigation of alternative methods. Recently a protein was identified in the orchid *Gastrodia elata* which may possibility be used a biological control mechanism. *G. elata* is an achlorophyllous orchid dependent on *A. mellea* for the completion of its life cycle. The plant allows the fungus to invade its primary corm before arresting and digesting the fungal hyphae in the cortical layer of the tuber for its nutritive values. It is speculated that after digestion nutrients are transported from the tuber to the terminal corm for further development of the plant such as the formation of flowers. The terminal corm of this plant, which is completely resistant to fungal infection (Yang and Hu, 1990), releases large amounts of *Gastrodia* anti-fungal protein (GAFP) upon infection by *A. mellea*. The protein was purified from the terminal corm of *G. elata*, and was shown to inhibit the growth of multiple phytopathogenic fungi *in vitro*, including *Valsa ambiens*, *Gibberella zeae, Botrytis cinerea, Armillaria mellea, Rhizoctonia solani*, and *Ganoderma lucidum* (Hu et al. 1988; Xu et al. 1998). Because of the broad spectrum anti-fungal activity of the protein the gene for this protein merits investigation for the possibility of being transferred to susceptible plants which may then provide an effective management of various fungal diseases, especially *Armillaria.*

The first transgenic crop plants expressing GAFP protein utilized the gene under control of the CaMV35S promoter. The Cauliflower mosaic virus (CaMV) is a double-stranded DNA virus that infects a wide range of plants such as cabbage, cauliflowers, and mustard. This virus has two promoters 35S and 19S in front of its genes. The 35S promoter is a strong, constitutive, and widely used promoter in biotechnology (Cummins, 1994). This promoter (CaMV35S) was first isolated by Chau and collaborators at

Rockefeller University (Cummins, 1994) and is patented by Monsanto. Transgenic plants containing the *gafp-1* gene under the control of CaMV35S were generated about a decade after GAFP was discovered in 1988 (Hu et al., 1988). Transgenic cotton, containing the GAFP has displayed resistance against Verticillium wilt disease caused by *Verticillium dahliae* (Wang et al., 2004). In transgenic tobacco, GAFP, isoform VNF (GAFP-1) (Wang et al., 2001) has shown resistance against *M. incognita*, and the basidiomycete fungus *Rhizoctonia solani* (Cox et al., 2006). In 2008, Nagel et al. (2008) showed that GAFP-1 is effective against PRR and RKN in transgenic plum plants when associated with CaMV35S promoter. The best performing transgenic tobacco and plum lines however were not completely resistant to infection and subsequent decline (Cox et al., 2006 and Nagel et al., 2008), leaving room for research focusing on performance improvement. Armillaria has been identified as a major problem of peach in the Southeast (Miller, 1994). No studies have investigated the effectiveness of GAFP incorporation with relation to the management of *Armillaria* due to the absence of a good disease testing assay.

The generation of disease-resistant plants through traditional breeding and the production of bioengineered plants is a key component to meet the demands of food for the increasing world population. It is estimated that the food supply should increase by 60% to meet the world food demand by the year 2020 (Skinner et al., 2004). Since crop land cannot increase endlessly, the yield per acre must be improved, possibly through more effective management of pests and diseases. In this respect, the production of genetically engineered (GE) crops with pest management traits such as herbicides

resistance, fungal resistance or nematode resistance has risen dramatically since the mid-1990s. When adopting bioengineered crops, farm impact, consumer acceptance, and environmental safety are concerns. The primary motivations, however, are profitability and reduction of pesticide use (Fernandez-Cornejo and McBride, 2002). The increasing applications of pesticides in agricultural land has created a number of problems such as insecticide and fungicide resistance, adverse effects on humans and environment, and pest outbreaks due to large amount of pesticides used (Kos et al., 2009). The most commonly available insect-resistant, transgenic plants carry the gene for *Bacillus thuringiensis* (Bt) δ-endotoxins (called crystal proteins or Cry proteins) (Kos et al., 2009). As of June 30, 2005, the U.S. Department of Agriculture and the National Agriculture Statistics Service (USDA/NASS) reported that three transgenic varieties of soybean, corn, and cotton comprised 87, 52, and 79 percent of the acreage planted in the US (Schahczenski and Adam, 2006). As of January 6, 2006, papaya (two varieties), potato, squash, sugar beet, sweet corn, and tomato have been granted deregulated status by the USDA Animal and Plant Health Inspection Service (APHIS) (Schahczenski and Adam, 2006).

There are two major approaches widely used to transform transgenic crop plants, one is biolistic bombardment and another is *Agrobacterium*-mediated transformation. In the latter, more popular approach, the gene of interest is placed under the control of a promoter sequence and markers are inserted in the vector between the left and right border of the *Agrobacterium* plasmid. The commonly used promoter for plant

transformation has been the CaMV35S. The promoter is recognized by RNA polymerase to initiate and regulate transcription in the plant genome.

As mentioned above, transgenic tobacco and transgenic plum showed disease resistance however, there was room for improvement. One way to increase transgenic plant performance (in regard to resistance) is to increase expression of the transgene, and in the case of *gafp-1*, by using a different promoter. A promoter derived from polyubiquitin gene has been widely used to drive various transgenes and it has been shown to increase the level of expression in different transgenic plants (Lu et al., 2008). Ubiquitin is a small protein that contains 76 amino acids and is highly conserved in all eukaryotes. This gene contains two different types of structures, polyubiquitin and the ubiquine gene. The promoter element derived from the polyubiquitin gene has been widely characterized in many transgenic plants such as maize (Christensen and Quail, 1996), potato (Garbarino et al., 1995), tobacco (Plesse et al., 2001), rice (Wang et al., 2000), gladiolus (Joung and Kamo, 2006), and sunflower (Binet et al., 1991). The polyubiquitin *bul*409 promoter derived from the polyubiquitin gene encodes a hexameric polyprotein. It was first isolated from a *Solanum bulbocastanum* BAC library (Rockhold et al., 2008). *bul*409 has shown enhanced expression of the reporter gene (GUS) in transgenic potato lines (Rockhold et al., 2008). Expression was 30-fold higher in transgenic potato lines compared to lines generated using the CaMV35S promoter (Rockhold et al., 2008). In transgenic rice the *rubi*3 polyubiquitin promoter gene showed higher expression of two different reporter genes GUS (β-glucuronidase) and GFP (green fluorescent protein) (Lu et al., 2008). However, the polyubiquitin promoter has not been

studied in transgenic plum plants. Transgenic 'Stanley' plum lines 4I and 4J were generated using the CaMV35S promoter and these showed increased tolerance to RKN and reduced disease symptoms of PRR (Nagel et al., 2008). These lines were generated at the USDA Fruit Research Station in Kearneysville, WV by Dr. Ralph Scorza laboratory.

Whether or not a transgenic rootstock will be successfully implemented for food production hinges on consumer acceptance. One concern needing investigation is potential of *gafp-1* transcripts and GAFP protein to migrate from the rootstock to a non-transgenic scion. Scions with favorable fruit quality traits are routinely grafted onto rootstocks which in turn provide the scion with desirable rootstock characteristics such as tolerance of winter cold, disease resistance, seasonal flooding and summer droughts, or reducing tree size (Merwin, 1999). Grafting techniques have been used in agriculture for centuries for woody species, such as apples, peaches and grapes, and for annual plants as well. In early 1900s, grafting was used for the first time to control Fusarium wilt on watermelons and afterwards grafting was often used to reduce *Ralstonia solanacearum* (bacterium wilt) in tomato (Rivard and Louws, 2006) and has more recently been applied to manage root-knot nematodes (RKN) in tomato crops worldwide (Cortada et al., 2008; Rivard et al., 2010). Using transgenic rootstocks for non-transgenic scions will be more acceptable for consumers if the transgene products do not move upward into scion tissue.

A long term goal is to investigate the potential of *gafp-1* as genetic determinant for ARR control, which currently causes most of the peach tree decline in the Southeast. Those tests are outside the scope of this study and will be performed in the field due to the lack of a suitable ARR pathogenicity test under controlled conditions. Pathogenicity

tests for PRR and RKN diseases are established, however, and were used in this study to screen *gafp-1* expressing transformants. Until a suitable genetic transformation system for peach is developed, plums are being used as a model system to investigate *gafp-1* expressing rootstocks for disease resistance. Many plum varieties, including the two used in this study (Schnabel, unpublished data) can be used as a rootstocks for peach (Layne, 1987). The objectives of this study were (i) to determine the potential movement of GAFP-1 protein across the grafted scion of chimeric-grafted plum, (ii) to characterize *gafp-1*-expressing transgenic 'Bluebyrd' (BB) lines in regard to GAFP-1 protein expression, and (iii) to evaluate the resistance to PRR and RKN susceptibility of 'Bluebyrd' lines that express high levels of GAFP-1 protein.

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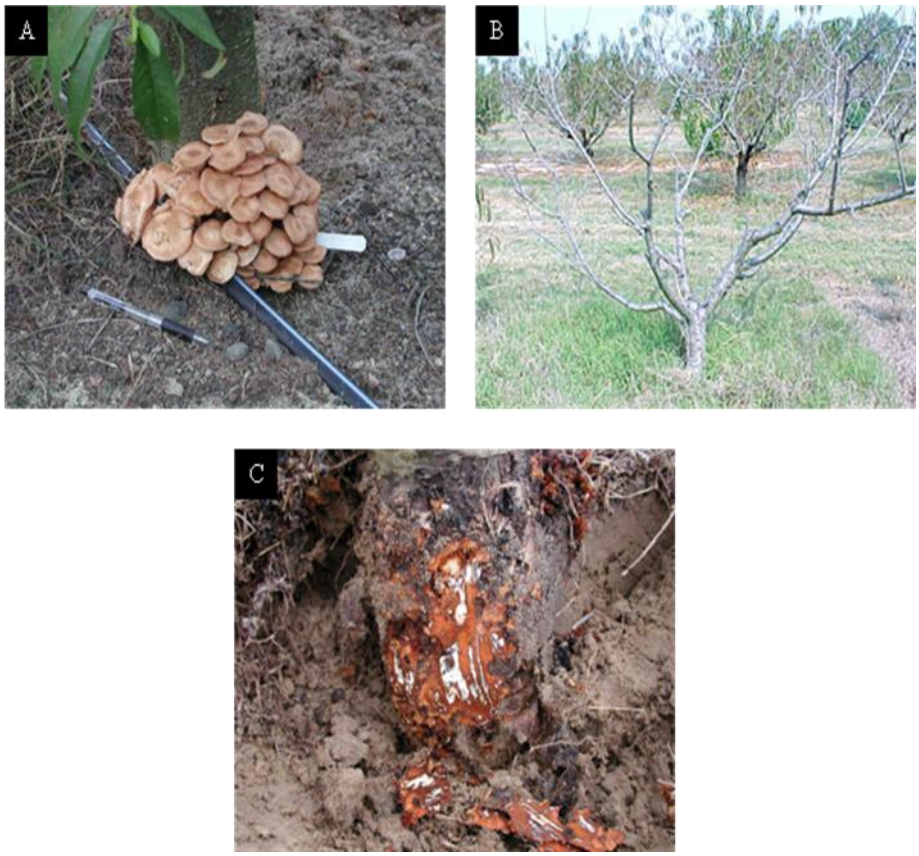

Fig. 1.1. *Armillaria tabescens* basidiocarps produced at infected peach roots (A). Above-ground symptoms of an infected peach tree displaying wilted foliage and dead scaffold limbs (B). White mycelial sheets of *Armillaria* inside the bark of an infected tree (C) Pictures provided by Dr. Schnabel.

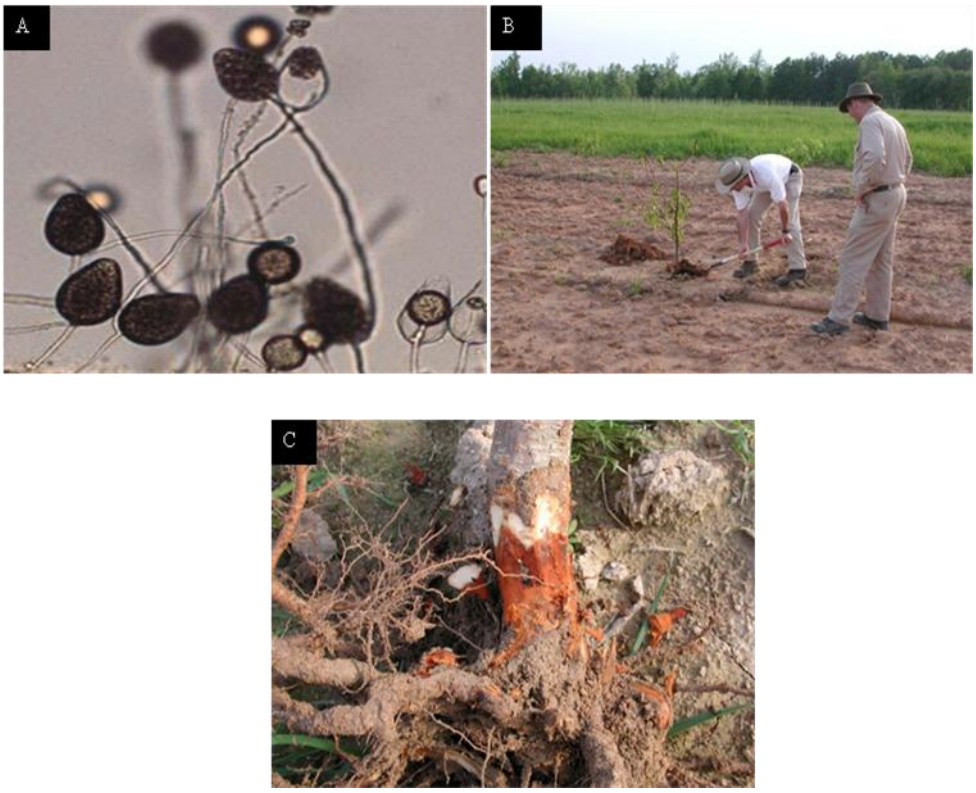

Fig. 1.2. Reproductive structures (sporangia) of *Phytophthora* species (A; Wilcox and Biggs, 2005). Young tree killed by *Phytophthora* species on poor draining site (B). Peach roots and lower trunk infected with *Phytophthora cinnamomi* (C) Pictures provided by Dr. Schnabel.

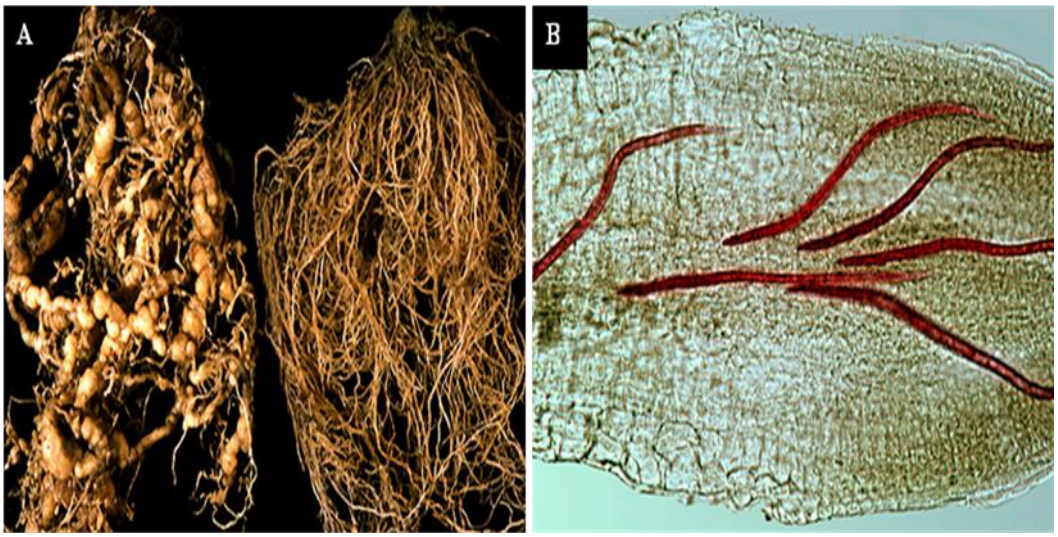

Fig. 1.3. Root-knot nematode infected root system of corn and non-infected root system (A). Root with second stage juveniles of *Meloidogyne* species (B) Tiwari et al., 2009.

CHAPTER TWO

THE *GASTRODIA* ANTIFUNGAL PROTEIN (GAFP-1) AND ITS TRANSCRIPT ARE ABSENT FROM SCIONS OF CHIMERIC-GRAFTED PLUM

[Alexis K. Nagel, Hetal Kalariya, and Guido Schnabel. HortScience 45:188-192, 2010. Tree grafting and detection of *gafp-1* gene transcripts were performed by Alexis Nagel; detection of GAFP-1 protein in grafted and non-grafted tissue was performed by Hetal Kalariya]

INTRODUCTION

Development of genetically modified (GM) agricultural crops has given producers new options to combat pests and diseases. These transgenic options can be limited, however, depending on the type of crop and the nature of the affliction. For instance, despite the important economic impacts that root diseases can have on fruit production, only a few GM fruit tree species have been engineered for resistance to root-associated pathogens (Petri and Burgos, 2005). Transgenic plum (*Prunus domestica* cultivar. 'Stanley') lines (designated 4J and 4I) expressing an isoform of the *Gastrodia* anti-fungal protein, GAFP-1-VNF (hereafter referred to as GAFP-1), are one example of such an engineered fruit tree system. These lines displayed significantly reduced symptom severity when challenged with the stramenopile pathogen *Phytophthora cinnamomi* and both lines trended towards increased tolerance to the root-knot nematode *Meloidogyne incognita* (Nagel et al., 2008).

The presence of foreign gene products in consumables is a controversial issue. Perceptions about the safety of GM food are a factor in the consumer's willingness to purchase such items (Boccaletti and Moro, 2000; Bukenya and Wright, 2007; Burton et al., 2001). Grafting cultivar scions to rootstocks with desirable attributes is already common practice in fruit tree propagation, therefore a potentially more consumer-friendly way to utilize GM technology would be to combine a transgenic, disease-resistant rootstock with a non-transgenic scion. Ideally, foreign gene products expressed in the root tissues would remain in the rootstock and not enter the fruit produced on a grafted scion. Whether the GAFP-1 lectin or its transcripts can move across a graft union into non-transformed scion tissues is not known.

The goal of this study was to determine if *gafp-1-vnf* (hereafter referred to as *gafp-1*) transcripts and protein were moving into grafted, WT scion tissues. Chimeric-grafted plum trees were created, consisting of wild-type (WT) scion tissue budded onto *gafp-1* expressing rootstocks. Root and scion tissues were analyzed for *gafp-1* mRNA and protein.

MATERIALS AND METHODS

Generation of chimeric-grafted and auto-grafted tree (Performed by A. Nagel). Transgenic plum (*Prunus domestica* cultivar 'Stanley') lines 4J and 4I (Nagel et al., 2008) and non-transformed WT plum lines were used in this study. Both transgenic 4J and 4I lines express the *gafp-1* gene under the control of the CaMV-35S promoter sequence (Plant Genetic Systems N. V., Gent, Belgium). Trees from 4J and 4I lines were clonally propagated from their respective mother (T_0) lines. WT trees, however,

originated from different ‘Stanley’ seeds, and thus represented some, albeit limited, inherent genetic variation within the WT population.

WT scion tissue was chip-budded (hereafter referred to as ‘budded’) onto three transgenic 4J and 4I trees and three non-transformed WT trees (Fig. 2.1). Briefly, dormant bud tissue was excised from the scions of donor (WT) and recipient (4J, 4I, or WT) rootstocks. The donor bud was then placed onto the chipped area of the recipient rootstock stem. Buds from 1-year-old WT scions were budded onto the stems of 1-year-old 4J and 4I lines to create chimeric-grafted (CG) trees. Buds from 1-year-old WT plum were budded onto stems of 1-year-old WT plum (originating from different seeds) to create auto-grafted (AG) trees. AG trees served as negative controls for the detection of *gafp-1* mRNA and protein. Three tree-replicates received 2 buds each for a total of 6 budding attempts per line. Buds were wrapped in Parafilm® for 2 weeks. After this time the Parafilm® was removed. Four weeks after the budding event the WT scion was truncated just above the uppermost bud graft. If two buds flushed on the same tree, they were both allowed to develop on the stem. Non-grafted (NG) trees were kept as additional controls for the detection of *gafp-1* molecular products in tissues. Trees were maintained in a biosafety level 2 greenhouse under constant temperature (27 ± 5°C) and light conditions (16/8 h day/night).

Tissue sampling. Leaf, shoot and root tissues were sampled from CG, AG and NG trees for molecular analysis. Sampling of both grafted and non-grafted tissues continued over a 24 month period. During this time, trees were pruned every six months. The grafted scions were truncated about 3-5 inches above the graft union.

Newly-emerged leaves were sampled from just below the apical meristem beginning 2 weeks after maintenance pruning, and partially lignified root tissue was sampled from the tips of the plum tree roots. Non- lignified, soft shoots were sampled from trees between 2 and 6 weeks after maintenance pruning. Two weeks was the minimum amount of time it took for axillary buds to flush new, expanded leaves exhibiting non-curled edges. For each line, leaf tissues were taken from a total of three grafted scions on at least two different CG and AG trees. Root tissues were sampled from corresponding trees. When possible, leaf tissue was sampled from grafted scions on separate trees. Leaf and root tissues were also sampled from three NG 4J, three NG 4I, and three NG WT trees. Soft shoots were sampled from two grafted scions on separate CG, AG, and NG trees from each line.

Detection of *gafp-1* transcripts (Performed by A. Nagel). The reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine if *gafp-1* transcripts were present in leaf and root tissues of CG, AG, and NG trees. Plant tissue (100 mg) was ground in liquid nitrogen and total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed to cDNA using the SuperScript^{TM} First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Control reactions were performed for all samples in which dIH_20 (de-ionized water) was substituted for the RT enzyme. These were included to verify that genomic DNA contamination was not present. Otherwise parameters for cDNA synthesis remained the same. RNA concentration was determined using a GeneQuant *pro* spectrophotometer at

260 nm. The RT procedure is documented to produce cDNA from as little as 1 ng of total RNA (Invitrogen).

Root and leaf tissue-derived cDNAs were selectively amplified using gene specific primers (Table 1). Tissues were analyzed for *gafp-1* transcripts by amplifying cDNA (5 µl first-strand synthesis) with primers 1 and 2, which are specific to the *gafp-1* transcript sequence (NCBI accession number AJ277786). As *gafp-1* transcripts were expected to be absent in WT tissues, additional reactions had to be conducted to verify proper cDNA amplification. Leaf tissue cDNA synthesis was confirmed by PCR amplification with primers 3 and 4, which are specific to the *catalase 2* (*cat2*) transcript sequences from peach (*Prunus persica* L. Batsch) (Accession number AJ496419). Root tissue cDNA was PCR amplified with primers 5 and 6, specific to the *α-tubulin* (*α-tub*) transcript sequence from almond (*Prunus dulcis* (Mill.) D. A. Webb) (NCBI accession number X67162). PCR amplification of all samples was performed with a Bio-Rad iCycler Version 4.006 (Bio-Rad Laboratories, Hercules, CA, USA). Cycling parameters were as follows: initial denaturation at 95ºC for 2 min, 35 cycles of 94ºC for 30 s, 65ºC for 30 s, and 72ºC for 40 s; final elongation was at 72ºC for 10 min. The entire procedure (mRNA isolation and RT-PCR analysis) was repeated for all tissues.

Detection of GAFP-1. Immunoblot analysis was used to determine if the GAFP-1 lectin (expected size 12 kDa) was present in leaf and root tissues of CG, AG, and NG trees. Total protein was extracted from leaf, shoot, and root tissues. Plant tissues (300 mg) were homogenized in liquid nitrogen and total cellular protein was extracted with TRIzol Reagent© (Invitrogen) according to standard methods (Chomczynski, 1993).

Soluble protein was dissolved in 1% sodium dodecyl sulfate (SDS). The total protein concentration for each sample was determined with the DC (Detergent Compatible) Protein Assay (Bio-Rad) according to manufacturer's instructions. Bovine serum albumin was used as a standard. Sample absorbance was quantified at 650 nm using an Emax® precision microplate reader.

Total protein (20 μg) was loaded onto a 15% Tris-HCl gel and separated by SDS-PAGE. Protein molecular weight markers were included in all analyses (Precision Plus Protein™ duel color standard, Bio-Rad). Proteins were tank-transferred to a PVDF membrane (Bio-Rad laboratories, Hercules, CA) for 18 h at 30 V. Membranes were blocked with 5% dry non-fat milk in TBST [(20 mM Tris-HCl, 140 mM NaCl, pH 7.5) + 0.1% Tween 20], rinsed twice, and then incubated with rabbit anti-GAFP-1 polyclonal antisera (1:1000 dilution; Zymed® Laboratories (Invitrogen, Carlsbad, CA) in 1% dry non-fat milk + TBST. Membranes were rinsed three times and then incubated with goat, anti-rabbit alkaline-phosphatase (AP) -conjugated secondary antibodies (1:2500 dilution) (Promega Corp., Madison, WI) in 1% dry non-fat milk + TBST. Membranes were developed with BCIP/NBT solution (Sigmafast™, Sigma Aldrich, St. Louis, MO, USA). The entire procedure (protein isolation and immunoblot analysis) was repeated for all tissues. The AP-conjugated secondary antibody is documented to detect as little as 10 pg of protein (Bio-Rad).

RESULTS

With one exception at least one out of the two budding attempts became successfully established on each replicate tree. One individual from line 4J failed to yield any

successful bud-grafts. Successfully grafted buds began to flush about 3 to 4 weeks after budding.

The expected 367-bp *gafp-1* and 498-bp *α-tub* fragments were successfully amplified from root tissue cDNAs of CG 4J and CG 4I trees (Fig. 2.2A). However, even after 35 cycles of amplification we were not able to detect *gafp-1* transcripts by RT-PCR in leaf tissues taken from WT scions of CG 4J or CG 4I trees (Fig. 2B). Successful amplification of the expected 572-bp *cat2* fragment confirmed the quality of the mRNA extracted from these leaf tissues (Fig. 2.2 B). Transcripts of *gafp-1* were not detected in the leaf or root tissues of NG WT or AG trees, but *gafp-1* fragments were consistently amplified from leaf and root tissue cDNAs of transgenic NG 4J and NG 4I trees (Table 2.2). *Cat2* and *α-tub* transcripts were detected in leaf and root tissues, respectively, from all CG, AG, and NG trees. Duplicates of every sample were created at the RNA extraction stage and subjected to the entire procedure without the inclusion of the RT enzyme. This confirmed that cDNA-derived amplicons were not a result of genomic DNA contamination (Figs. 2.2A and B).

GAFP-1 (expected size 12 kDa) was detected in roots of CG 4J and CG 4I trees but not in the leaf or soft shoot tissues of grafted WT scions (Figs. 3A and B). In contrast, the lectin was detected consistently in the leaf and root tissues of NG 4J and NG 4I trees (Fig. 3C). A GAFP-1 signal was not detected in leaf, soft shoot, or root tissues from NG WT or AG trees (Table 2.2, Figs. 2.3A-C). Lignified tissue from grafted, WT scions of CG trees was not analyzed for *gafp-1* products in this study.

GAFP-1 antibodies showed cross-reactivity with other proteins on the immunoblots, however cross-reaction was not observed at the 12 kDa position. Antibody cross-reaction occurred with an unknown 14 kDa protein in protein extracts taken from leaf, shoot and root tissue, and with a 15 kDa protein in protein extracts from shoot and root tissues. While the binding specificity of the GAFP-1 polyclonal antibody may have been optimized by loading a smaller amount of protein on the gels, we chose to load higher amounts of total protein (20 μg) in an effort to resolve small amounts of GAFP-1 that may have been moving from the rootstock into the leaf tissues of the grafted, WT scion. On the immunoblots the intensity of GAFP-1 bands varied among protein samples taken from different tissues. This occurred despite the fact that the amount of total protein loaded on the gel remained constant. This variation in band density was likely due to some inconsistencies in the homogenization of the fibrous plant tissues.

DISCUSSION

Many phloem-mobile macromolecules have been shown to traverse a graft union formed between compatible plant tissues. *In situ* RT-PCR studies demonstrated that pumpkin-derived *CmNACP* mRNA was present within the functional sieve elements (SE) of grafted cucumber scions (Ruiz-Medrano et al., 1999). Gomez et al. (2005) showed that an RNA-binding phloem lectin from melon, CmmLec17, could be detected within the phloem-exudate of heterografted pumpkin tissues. Grafting experiments between transgenic and WT tissues have demonstrated that transcripts may move through graft junctions and elicit responses in plant cells that do not contain the causal gene.

Transcripts of the tomato *PFP-LeT6* gene, a sequence fusion found exclusively in the dominant mutant *Mouse ears*, were able to move across a graft union and induce changes in leaf pinnation in WT tissues (Kim et al., 2001). St *BEL5* transcripts were translocated across grafts made between potato over-expression lines and WT rootstocks, and localization of St *BEL5* in stolon tips resulted in a two-fold increase in tuber yields (Banerjee et al., 2006). Macromolecules have even been observed to move across graft unions established in host-parasite relationships. After colonization of transgenic tobacco by the adventitious plant species *Cuscuta reflexa*, green-fluorescent protein expressed within tobacco companion cells was detectable within the SE's of the associated parasite (Haupt et al., 2001).

Conversely, this study provides evidence that *gafp-1* transcripts and protein may not be moving into the grafted, WT scions of a CG tree species. These results contradict previous research that supports the phloem mobility of GAFP-1 within its host, the achlorophyllic orchid *Gastrodia elata*. Immunofluorescence studies provided evidence that GAFP is present within the vascular bundle, and it was proposed that the lectin is transported from the primary to the secondary corm of the orchid, as well as into the developing flower stem, via the SE's (Hu and Huang, 1994). There is no data, however, indicating that *gafp-1* transcripts are phloem-mobile in *G. elata*.

As emerging leaves develop on the scion they make the transition from 'sink' to 'source,' and at this point they begin to contribute phloem-assimilates to the scion translocation stream (Haywood et al., 2005). By routinely pruning the trees we strived to keep the grafted scions in a 'sink' state. There was not a single instance in which our

detection procedures gave any indication of *gafp-1* mRNA or protein in the grafted, WT leaf tissues of CG trees, even when tissues were sampled shortly (2 weeks) after maintenance pruning. Similarly, soft shoots, which were sampled from scions of CG grafted trees between 2 and 6 weeks post-pruning, never showed a protein signal at the 12 kDa position. Immunoblots performed on protein extracts from WT leaves of CG 4J and CG 4I trees eight weeks after budding (four weeks post-bud flush) did not show GAFP-1 protein signals (data not shown).

We began to sample leaves and soft shoots for the detection of *gafp-1* mRNA and protein 2 weeks following maintenance pruning. This should have been an adequate amount of time for the hypothetically phloem-mobile *gafp-1* molecular products to move into tissues. Several studies have demonstrated that macromolecules utilizing phloem channels will spread relatively quickly within the plant. It has been reported that phloem-mobile gene silencing signals are distributed systemically within a few days in tomato (Voinnet et al., 1998), and in herbaceous heterografts three weeks has been sufficient for the detection of various imported phloem-mobile transcripts and proteins (Haywood et al., 2005; Gomez et al., 2005; Ruiz-Medrano et al., 1999). Certain phloem-mobile plant viruses, which are thought to travel through the translocation stream as ribonucleoprotein complexes (Santa Cruz, 1999), are capable of spreading systemically in a matter of hours (Ismail et al., 1987; Gal-On, 1994) or days (Bennett, 1940; Capoor, 1949; Helms and Wardlaw, 1976; Más and Pállas, 1996). If assessing virus movement in a woody system by symptom emergence, fruit tree seedling double-budding experiments have shown that virus particles can move from infected to non-infected tissues in 4 weeks (Fridlund,

1980). Most grafting studies conducted with woody plant material do not determine on a molecular level the amount of time it takes for phloem-mobile virus particles to move into budded tissues (S. Scott, personal communication), however it has been shown in herbaceous systems that manifestation of disease symptoms in grafted tissues is preceded by the delivery of virus RNA (Más and Pállas, 1996).

It is possible that the GAFP-1 lectin may not be compatible with the phloem transportation machinery of *P. domestica*. Being in a different genetic background, the GAFP-1 lectin may move into the translocation stream only to be quickly degraded, or the protein may not be entering the translocation stream at all. In higher plants, the non-cell autonomous activity of signaling proteins and transcripts has a significant impact on the coordination of complex developmental and physiological events (Nakajima et al., 2001; Palauqui et al., 1997; Ruiz-Medrano et al., 1999; Yoo et al., 2004; Xoconostle-Cázares et al., 1999), and is likely subject to a certain degree of regulation. Indeed, targeted transport of macromolecules as well as non-specific diffusion has been observed within symplasmically-connected cells and SE's (Crawford et al., 2000; Itaya et al., 2002; Lucas et al., 1995; Stadler et al., 2005). The concept of a surveillance system controlling the movement of transcripts into specific tissues has been supported by studies on the selective entry of virus-derived post-transcriptional gene silencing signals into the plant shoot apex (Foster et al., 2002). Haywood et al. (2005) showed that expressed cucurbit *Cmgaip* transcripts were able to move into the WT leaf and flower tissues of CG tomato, but not into the fruits produced on the WT scions. Analogous mechanisms likely exist for regulating the delivery of phloem-associated proteins to plant tissues. This statement is

congruent with the observation that phloem proteins from *Cucurbita maxima* have the ability to interact with and increase the size exclusion limit of the plasmodesmata in cotyledon mesophyll cells (Balachandran et al., 1997). Likewise, the melon RNA-binding phloem protein CmmPP2 was not detected in scion phloem exudates of intergeneric grafted tissues of pumpkin, despite the successful translocation of other phloem-mobile melon proteins such as CmmLec17 (Gomez et al., 2005).

Our results suggest that *gafp-1* transcripts and protein are not phloem-mobile in CG plum. It remains to be determined whether *gafp-1* products expressed in transgenic rootstocks can accumulate in non-transgenic branches and leaves after several years of establishment in the field, or in flowers or fruits following significant physiological changes such as the onset/breaking of dormancy and fruiting. Most fruit tree crops are propagated by grafting cultivar tissue onto rootstocks with desirable attributes, such as enhanced tolerance to root diseases. Thus, a CG strategy such as we have described could have broader applications for a range of plant systems engineered for root-associated disease resistance. The ability of different engineered rootstocks to retain foreign gene products would depend highly on the nature of the expressed protein or transcript and its compatibility with the translocation machinery of the host plant species to which transgenic resistance was being applied.

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Table 2.1. Primers used for amplification of cDNAs from leaf and root tissues

Primer	Target gene	Size	Orientation	Sequence
1	*gafp-1*	367-bp	forward	5'–CCTGTTCTTTCGCGTGACAACAGT–3'
2	*gafp-1*		reverse	5'–GTGTGGGTTGCCCAAATCGCATT–3'
3	*catalase 2*	572-bp	forward	5'–AGGCACATGGAAGGCTCTAGTGTT–3'
4	*catalase 2*		reverse	5'–ACCTCCTCATCCCTGTGCATGAAA–3'
5	α-*tubulin*	498-bp	forward	5'–TTGACATTGAGCGACCCACCTACA–3'
6	α-*tubulin*		reverse	5'–TGGTCGAGTTGGAGATCATGCACA–3'

Table 2.2. Detection of *gafp-1* mRNA and protein in tissues of auto-grafted (AG), non-grafted (NG), and chimeric-grafted (CG) trees

Tree			Plant Tissue[z]					
			Leaf		Root		Shoot	
Rootstock	Graft type	Observations[y]	mRNA	Protein	mRNA	Protein	Observations[x]	Protein
WT	NG	n = 3	-	-	-	-	n = 2	-
WT	AG	n = 2	-	-	-	-	n = 2	-
4J	NG	n = 3	+	+	+	+	n = 2	+
4J	CG	n = 2	-	-	+	+	n = 2	-
4I	NG	n = 3	+	+	+	+	n = 2	+
4I	CG	n = 3	-	-	+	+	n = 2	-

[z]Leaf, shoot, and root tissues were analyzed twice for the presence (+) or absence (-) of *gafp-1* transcripts or protein.
[y]Number of trees from which leaf and root tissues were sampled.
[x]Number of trees from which soft shoots were sampled.

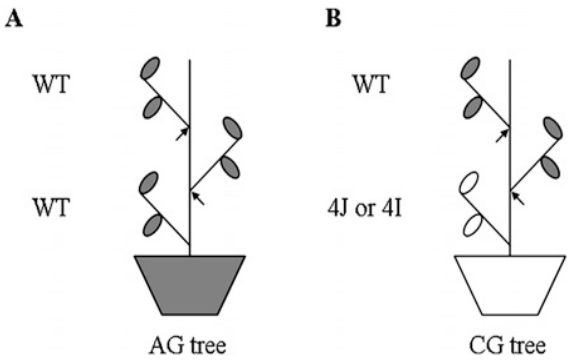

Fig. 2.1. A schematic diagram of the trees used in grafting experiments. (A) Wild-type (WT) plum tissue was budded onto WT rootstocks to create auto-grafted (AG) trees. AG trees served as negative controls. (B) WT plum tissue was budded onto rootstocks from transgenic plum lines 4J and 4I to create chimeric-grafted (CG) trees. WT rootstock and scion tissues are indicated in grey, transgenic tissues are indicated in white. Two buds were budded per rootstock. Black arrows indicate the graft junction occurring on the rootstock stem.

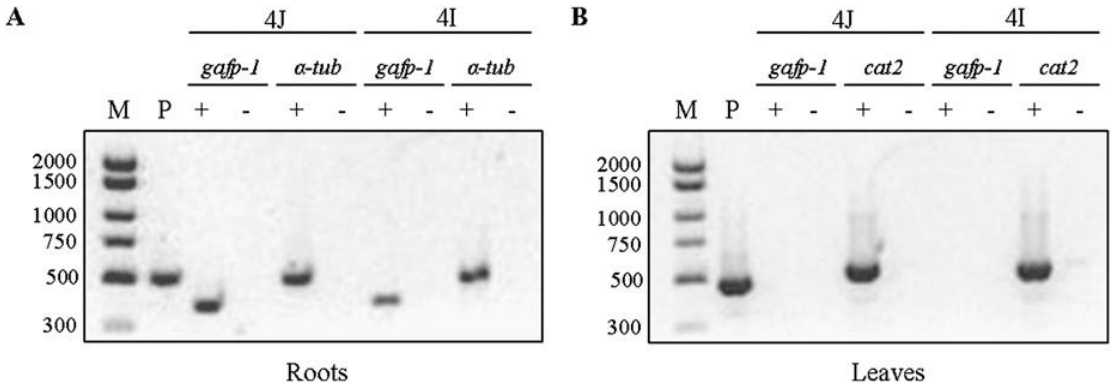

Fig. 2.2. RT-PCR analysis of (A) root and (B) leaf tissues from rootstocks and grafted, wild-type scions of chimeric-grafted trees, respectively. *Gafp-1* (expected size 367-bp) and *α-tubulin* (*α-tub*; expected size 498-bp) or *catalase* (*cat2*; expected size 572-bp) transcripts were reverse-transcribed and PCR-amplified with sequence-specific primers. A plus (+) or minus (-) sign indicates the inclusion or exclusion, respectively, of the reverse transcriptase enzyme during cDNA synthesis. Chloramphenicol acetyltransferase (P; expected size approximately 500-bp) RNA was reverse-transcribed and amplified with gene-specific primers (provided by SuperScript^{TM} First-Strand Synthesis System, Invitrogen) as a positive control for cDNA synthesis and amplification. The numbers to the left of the figures indicate the size of the DNA markers (M) in base pairs (exACTGene® Low Range DNA Ladder, Fisher Scientific, Pittsburgh, PA, USA).

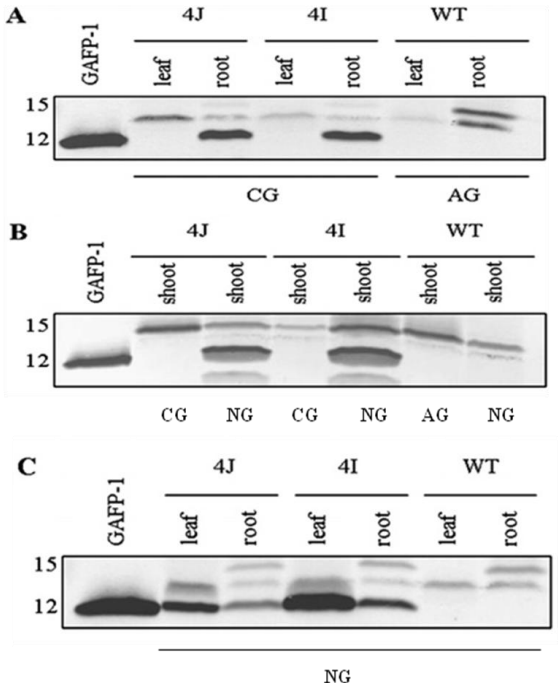

Fig. 2.3. Immunoblot analysis of protein extracts from chimeric-grafted (CG), auto-grafted (AG), and non-grafted (NG) trees. (A) Leaves and roots from CG and AG trees, (B) soft shoots from CG, NG, and AG trees, and (C) leaves and roots from NG trees were analyzed for the presence of the GAFP-1 lectin. Recombinant GAFP-1-VNF (GAFP-1; expected size 12 kDa) cleaved from a maltose-binding protein fusion was included as a standard (140 ng total protein). Protein extracts from leaf and root tissues of AG and NG wild-type (WT) trees served as negative controls. The numbers to the left of the figures indicate the size of the protein bands in kilodaltons.

CHAPTER THREE

CHARACTERIZATION OF TRANSGENIC PLUM LINES EXPRESSING *GAFP-1* WITH THE *BUL*409 PROMOTER

INTRODUCTION

In the Southern United States, peach production is an important segment of the economy. South Carolina and Georgia are major peach producing states in the Southern United States. Several soil-born organisms cause significant problems on peach in the South Carolina including Phytophthora root rot, Root knot nematode. Phytophthora root rot (PRR) is a disease with economic impact on peach (*P. persica*) production worldwide mainly due to tree mortality (Kephart and Dunegan, 1948; Kouyeas, 1971; Stylianides et al., 1985; Haygood et al., 1986). In the southeastern United States, where a significant portion of US peaches are produced, the disease is primarily caused by *P. cinnamomi* (Erwin and Ribeiro, 1996). Excessive soil moisture, moderate temperature, and rootstock susceptibility contribute to disease development (Browne and Mircetich 1996). The soil borne pathogen is hard to control even with fungicide treatments. Current chemical control options include the application of fosetyl-aluminum and mefenoxam. Fumigants 1,3-dichloropropen and chloropicrin are recommended for management of soil borne pathogens (Methyl Bromide Technical Options Committee, 1994). The efficiency of fumigants is dependent on some environmental factors and soil types such as soil moisture, soil temperature are also important considerations (Horton, 2010). Methyl

isothiocyanate requires a long waiting period prior to planting and has been limited in use and distribution because of its stability in the environment and production of corrosive fumes when mixed with water (Methyl Bromide Technical Options Committee, 1994).

Nematodes also impact crop productivity. Crop damage due to plant parasitic nematodes is estimated at about $157 billion worldwide annually (Abad et al., 2008). Among the most devastating nematodes are *Meloidogyne* species. Members of this genus cause root knot disease and have a wide host range of more than 2000 plant species (Sasser, 1977; Lamberti, 1979). *M. incognita*, *M. javanica* and *M. arenaria* are the most damaging species in tropical regions (Triantaphyllou, 1985). In the United States, *M. incognita* is a widespread pathogen of tomatoes, cotton, and soybeans (Ortiz et al., 2010; Castagnone-Sereno, 2006) and one of the most common species reducing fruit production in peach and other stone fruit orchards (Nyczepir et al., 1997). Pre-plant fumigation with 1,3-dichloropropene and metam sodium are still used for RKN control in southeastern peach orchards (Horton et al., 2010), but plants can be killed by 1,3-dichloropropene if planted too soon after fumigation. Control of nematodes is extremely difficult once an orchard is established because an orchard may be in existence for 15 to 25 years. As a result of problems in the management of nematodes, utilizing rootstocks with resistance and/or tolerance to nematodes is the best management option.

Creating rootstocks for fruit trees with resistance to fungal root pathogens and nematodes is a desirable component of IPM management practices. Classical breeding has in the past been the only method available to develop disease-resistant rootstocks. Progress has been made in the example of the peach rootstocks KID I, PR204, GF305

and GF677 which showed some resistance against *Phytophthora cactorum* and *P. megasperma* (Thomidis et al., 2001). Nevertheless, progress has been slow and the number of resistance genes available for conventional breeding is limited. Genetic engineering offers a complementary method of developing resistance that can greatly expand the pool of resistance genes and offers a way to test these genes in a shorter time frame. The first genetically engineered plant was produced in 1983 (Schahczenski and Adam, 2006). Since 1983 a large number of transgenic crops have been introduced for various purposes including disease resistance, high pH tolerance, and several nutritional, taste, texture, and shelf-life characteristics (Skinner et al., 2004). In 2001, the estimated global area occupied by transgenic crops such as soybean, corn and cotton was 52.6 million hectares. Most, currently available, genetically engineered (GE) crops were designed to control insects and weeds (Schahczenski and Adam, 2006). Roundup Ready soybeans and *Bacillus thuringiensis* (*Bt*) gene-containing corn and cotton are some prominent examples. In May 2010, EPA registered C5 HoneySweet Plum, the first transgenic stone fruit variety with resistance to plum pox virus (Environmental Protection Agency, 2010).

The genes that express mannose binding lectins in monocotyledonous species have been used to generate transgenic plants resistant to a wide range of pathogenic and pest organisms (Peumans et al., 1995). The *Gastrodia* anti fungal protein (GAFP) is a mannose binding lectin with anti-fungal activity *in vitro* to *Valsa ambiens* (Wang et al., 2001) and other fungal pathogens including *Armillaria mellea, Rhizoctonia solani, Gibberella zeae, Ganoderma lucidum, and Botrytis cinerea* (Hu and Huang, 1994; Xu et

al., 1998). GAFP increased resistance to *Verticillium wilt* in transgenic cotton (Wang et al., 2004) and, in contrast to other lectins, GAFP showed efficacy against RKN and PRR in transgenic tobacco and plum (Cox et al., 2006, Nagel et al., 2008). Furthermore, transgenic 'Stanley' plum line 5D, expressing the *gafp-1* gene, resulted in the development of fewer eggs and juveniles of ring nematode, *M. xenoplax* (Nyczepir et al., 2009). Even though GAFP-1 protein expression in tobacco and plum showed increased resistance to fungal and non-fungal pathogens (Cox et al., 2006; Nagel et al., 2008), the resistance needs to be improved.One way to increase resistance in the transgenic plant is to increase the expression of the resistance gene by utilizing a more powerful promoter. The polyubiquitin promoter *rubi3* promoter with the 5'-UTR intron was examined and supported enhanced level of constitutive expression of reporter genes *GUS* and *GFP* than the maize Ubi-1 promoter in transgenic rice (Lu et al., 2008). Similarly, promoter *bul*409 was shown to be more active in transgenic potato compared to the CaMV35S promoter lines and expression level of GUS in transgenic potato plants with the polyubiquitin promoter *bul*409 was found 30-fold higher when compared to lines with the CaMV35S promoter (Rockhold et al., 2008). Whether the expression of *gafp-1* and more importantly the synthesis of GAFP-1 can be increased in transgenic woody plants using a polyubiquitin promoter is not known.

The objectives of this study were to identify 'Bluebyrd' plum lines expressing high levels of GAFP-1 protein, compare plum lines with *bul*409 promoter with lines with the CaMV35S promoter to determine their susceptibility to *P. cinnamomi* and *M. incognita* causing PRR and RKN disease, and to determine the inducibility of the *bul*409 promoter.

MATERIALS AND METHODS

Generation of transgenic plum lines expressing *gafp-1* under the control of the *bul*409 promoter (performed by Dr. Ralph Scorza laboratory, USDA, Kearneysville, WV). The pBINPLUS/ARS vector (Belknap et al., 2008), a modified pBINPLUS vector, was used for the transformation of 'Bluebyrd' plum seeds. The *gafp-1* gene was inserted at the multiple cloning sites between HindIII and SacI sites. The *bul*409 promoter was downstream of the insert and the Ubi3terminator was upstream of the insert (Rockhold et al., 2008). *Agrobacterium tumefaciens* strain EHA 105 (Hood et al., 1993) was transformed with the pBINPLUS/ARS vector (Fig.3.1) and prepared for gene transfer as described previously (Gonzalez- Padilla et al., 2003). Transformation of 'Bluebyrd' plum seeds were carried out using a method described by Cesar et al. (2008). Mature seed hypocotyl slices were selected as a primary source of explants, endocarp of the seed was removed and seeds were soaked in a 1% sodium hypochlorite solution for 30 min followed by rinsing with distilled water. Epicotyle and radicle were removed and the hypocotyl was sliced into three cross sections for transformation. Media for transformation and shoot regeneration was used as described by Gonzalez-Padilla et al. (2003). Explants were grown with *A. tumefaciens* in shoot regeneration medium without antibiotics but supplemented with 2,4-D. When shoots started to grow, they were transferred to shoot growing medium containing kanamycin and timentin. When shoots became long enough they were separated from clusters and placed in rooting medium containing antibiotics. Plants were transferred from artificial medium to pots in the greenhouse as described in Cesar et al. (2008). The presence of *gafp-1* in root and leaf

tissues was confirmed by gene-specific PCR as described previously (Cox et al., 2006). Because all transgenic lines derived from seeds, they are technically not 'Bluebyrd' plum any longer. However for simplicity reasons they are referred to as 'Bluebyrd' lines in this study. A total of 18 'Bluebyrd' transgenic lines, 2 empty vector control lines, and 5 wild type control lines were supplied by Dr. Scorza, USDA, Kearneysville, WV. For comparison, the previously characterized 4J 'Stanley' plum line was included in this study (Nagel et al., 2008). In this line the *gafp-1* gene is driven by the CaMV35S promoter instead of the bul409 promoter.

Determination of *gafp-1* gene copy numbers in transgenic plum lines (conducted by Dr. Ralph Scorza laboratory, USDA, Kearneysville, WV). DNA was isolated from young and fully expanded leaves of transformed and non-transformed 'Bluebyrd' lines as described previously (Kobayashi et al., 1998). Briefly, 10-15 µg DNA was digested with BamHI (New England Biolabs, Ipswich, MA), separated on a 1% (w/v) agarose gel and blotted to a positively charged nylon membrane (Roche Diagnostics Corporation, Indianapolis). The membrane was hybridized with a Digoxigenin-11-dUTP alkali-labile (Roche Diagnostics Corporation, Indianapolis) labeled probe coding for *gafp-1* cDNA. The probe was generated by PCR using *gafp-1*-specific primers (Wang et al., 2001).

Detection of GAFP-1 protein in transgenic plum lines. *Gastrodia* anti-fungal protein (GAFP-1; anticipated size 12 kDa) was detected using immunoblot analysis from root tissue of transformed, and non-transformed plum lines from 9-month-old trees as described previously (Nagel et al., 2010). 'Stanley' line 4J and a 'Stanley' control line were included as reference lines (Nagel et al., 2008). Briefly, total protein was extracted

from root tissue of transformed and non-transformed plum plant using TRI reagent® (Sigma-Aldrich, St. Louis, USA). Total protein (20 µg) was used to perform SDS-PAGE using 15% Tris-HCl ready Gels (Bio-rad Laboratories, Hercules, CA). Protein was transferred to an immunoblot PVDF membrane (Bio-Rad laboratories, Hercules, CA), and immunoblotting was performed using rabbit anti-GAFP-1 polyclonal antisera developed by Zymed® Laboratories and goat antirabbit alkaline phosphatase conjugated antibodies (Promega Corp., Madison, WI). Bands detection was accomplished using solution of BCIP/NBT tablet. This experiment was performed twice for each transgenic, empty vector and wild type control lines. Results were reproducible so, and they were combined. Expression of GAFP-1 was measured and scored relative to the expression of GAFP-1 in the 'Stanley' 4J line. Equal to (+++), up to 50% less (++), and less than 50% (+).

Selection and propagation of transgenic plum lines. Transgenic lines revealing a strong, consistent GAFP-1 protein signal on immunoblots were utilized for further experiments. The performance of 'Bluebyrd' lines was compared with previously characterized 'Stanley' line 4J expressing GAFP under the CaMV35S promoter (Nagel et al., 2008). 'Bluebyrd' and 'Stanley' control lines and empty vector control lines were included as negative controls. Vegetative propagation of the plant material was carried out in a biosafety level 2 greenhouse under constant temperature (27 $\pm$ 5°C) and light conditions (16/8 h day/night). Original transformed lines (T0 lines) were pruned every four weeks to stimulate shoot growth. Shoots (15-20 cm) were pruned off the T0 lines, gently scraped at the cut end and dipped into "ROOTECH" Original Cloning Gel

(Technaflora plant product LTD., Port Coquitlam, B.C., Canada). Shoots were placed 3 to 4 cm deep in sterile vermiculite in 36 well plastic trays (25 x 50 cm) and covered with a lid to prevent dehydration. Plants were misted and watered daily and fertilized once per week. Fungicide applications (Pristine 0.019 % active ingredient; BASF, Research Triangle Park, Raleigh, NC) were applied with a mister as needed to control fungal colonization of emerging leaves due to humid condition during propagation.

Disease susceptibility screening. Bluebyrd lines BB-1, BB-3, BB-17, BB-18, BB-21, BB-8, non-transformed lines BB-OP, BB-OP 30, BB-OP 31, 'Stanley' line 4J, and 'Stanley' control were investigated for their the susceptibility to PRR following a protocol as described previously (Nagel et al., 2008). *P. cinnamomi* isolate 05-1127 was obtained from naturally infected peach and had been used for similar studies (Nagel et al., 2008). Disease symptoms were evaluated every other day. Shoot symptoms were rated as: 0 = healthy plant, 1 = less than 25% of the plant showing chlorosis and necrosis, 2 = 25% to 50% of the plant showing chlorosis and necrosis, 3 = 50% to 75% of the plant showing chlorosis and necrosis, and 4 = greater than 75% of the plant exhibiting chlorosis and necrosis (Fig. 3.2). The experiment was concluded after 30 days, when the majority of inoculated control plants had severe wilting. Random root pieces were sampled from inoculated seedlings, surface sterilized, and plated on PARPH [PARP + 50 mg 5-methylisoxazol- 3-ol (hymexazol)] selective medium (Jeffers, 2006) to confirm the presence of *P. cinnamomi*. The disease severity score was calculated as described previously (Nagel et al., 2008). The entire experiment was performed twice with three replicates per experiment.

Three month-old plants of 'Bluebyrd' line BB -1, 'Stanley' 4J, 'Stanley' control, and BB-OP (Bluebryd control) were used to assess susceptibility to *M. incognita* as described previously (Nagel et al., 2008), except that a different method was used to stain the root system. BB-1 was selected for the RKN assay out of five BB lines used for the PRR assay based on reduced number of disease severity score in PRR assay compared to other transgenic lines. After weighing the roots, they were stained using 20% (v/v) solution of McCormick Schilling red food color (Thies et al., 2002) for 25 minutes, after which the roots were rinsed with tap water and blotted dry. Egg masses were observed under ×20 magnification. The number of galls and egg masses were determined per plant and normalized using gram fresh root weight to calculate the numbers per gram fresh root weight. *M. incognita* populations were originally isolated from infected peach in Georgia. The experiment was performed twice with five replicates.

GAFP-1 synthesis in roots of transgenic lines before and after inoculation. To determine whether the *bul*409 promoter is pathogen-stress inducible, total protein was extracted as described above from roots of BB-1 1 day before and 5 days after inoculation with *P. cinnamomi* and 30 days after inoculation with *M. incognita*. Immunoblot analysis was conducted as described previously.

Statistical analysis. Bartlett's test for homogeneity of variances was performed for repeated experiments. Data sets with homogeneous variances were combined and analyzed for significant difference between each line. For error control, all treatments were in a randomized complete block design. Values were analyzed using general linear

model (GLM) or analysis of variance and least significant difference (LSD) mean separation procedures of SAS (version 9.2; SAS Institute, Cary, NC).

RESULTS

A total of 18 plum lines were obtained from individual 'Bluebyrd' (BB) seeds (referred to as 'Bluebyrd' plum lines in this study) and 17 tested positive for the presence of *gafp-1* DNA (with the exception of the empty vector lines; data not shown). In addition, 5 non-transformed 'Bluebyrd' plants from seeds (BB-OP, BB-OP 30, BB-OP 31, BB-OP 32, and BB-OP 33) and two empty vector control lines (BB-7 and BB-8) were included in this study (Table 3.1). 'Bluebyrd' lines were not phenotypically different from each other or from the non-transformed or empty vector control lines (data not shown).

Southern hybridization was used to determine the copy number of the *gafp-1* gene in 'Bluebyrd' lines. Out of 20 transformed lines, 3 yielded no signal for *gafp-1* (2 of them were the empty vector control lines), 2 contained 1 copy, 9 contained 2 copies, 1 contained 3 copies, 3 contained 4 copies, and 2 contained 5 copies (Table 3.1). None of the non-transformed lines yielded a *gafp-1* signal (Table 3.1).

GAFP-1 synthesis was determined by immunoblot analysis. Only nine of the 20 'Bluebyrd' lines revealed a GAFP-1 signal. The consistently strongest signals (data from at least 2 independent experiments) were found for lines BB-1, -3, -17, -18, and -21 (Table 3.1; Fig. 3.3A and 3.3B). No GAFP-1 signal was detected in either the empty vector controls or the non-transformed control lines. Based on more than 4 different

immunoblot assays, the GAFP-1 signal strength of BB-3, which exhibited consistently one of the highest GAFP-1 signals, was comparable in intensity to 'Stanley' line 4J.

The 5 lines with the highest GAFP-1 signals (BB-1, -3, -17, -18, and -21) were selected for PRR disease tests. Included were also one empty vector line (BB-8) and a mixture of untransformed control lines BB-OP, BB-OP-30, and BB-OP-31 (designated BB-control) to account for potential natural genetic variation in disease susceptibility. The results of two independent experiments were not significantly different ($P= 0.3044$, $\alpha=0.05$) and the datasets were combined. BB-1 was significantly more resistant to PRR disease compared to other 'Bluebyrd' lines and compared to the control lines (Fig. 3.4). Interestingly, none of the other 'Bluebyrd' lines were statistically different from the BB-control lines. In addition, disease severity of 'Stanley' line 4J was numerically but not significantly different from the 'Stanley' control line. ($P= 0.3088$, at $\alpha=0.05$; Fig. 3.4).

Based on the PRR test results, the best performing 'Bluebyrd' line (BB-1) was subjected to RKN disease tests and compared with 'Bluebyrd' control line BB-OP, 'Stanley' control and 'Stanley' line 4J. BB-OP was chosen as the sole control line because it represented an average level of susceptibility to PRR among 'Bluebyrd' control lines. Between the independent experiments, no statistical differences were found ($P = 0.6687$ for eggs/g of root; $P = 0.5145$ for egg mass/g of root; and $P = 0.6154$ at $\alpha =0.05$) for galls/g of root, thus the combined dataset is shown. For all parameters tested (eggs/g of root, egg mass/g of root, and galls/g of root) 'Bluebyrd' line BB-1 as well as 'Stanley' 4J performed significantly better than the corresponding controls (Fig. 3.5). No statistical differences were found between the two transgenic lines 'Stanley' 4J and BB-1

plum lines (P=0.2782 for eggs/g of root; P=0.8221 for egg mass/g of root; P=0.3377 at α =0.05 for galls/g of root; Fig. 3.5).

The signals for GAFP-1 in immunoblot analyses were not noticeably higher in root tissue of line BB-1 five days after inoculation with *P. cinnamomi* (Fig. 3.6A) or 30 days after inoculation with *M. incognita* (Fig. 3.6B) compared to the non-infected control root tissue.

DISCUSSION

Among 17 'Bluebyrd' lines that tested positive for the presence of *gafp-1* DNA, only 9 were found to express the GAFP-1 protein in immunoblot studies. The failure of some transgenic plant lines to produce heterologous protein despite successful insertion of the target gene has been described before in other systems including transgenic potato using the potato leafroll virus replicase transgene (Ehrenfeld et al., 2004) and transgenic walnut using the *cryl*A(c) gene (Dandekar et al., 1998). In addition, variation in the level of transgene expression is common among transformed plants. It is not completely understood why inserted transgenes do not function in some transgenic plants, but it is possible that the insertion of the transgene into the plant genome occurs at locations which do not support gene expression. Kumar and Fladung (2001) showed that AT-rich regions in the genome of transgenic aspen *(Populus)* may be involved in the defense against foreign gene insertions. In studies of transgenic tobacco and tomato ATTTA sequences and A+T-rich regions affected the protein expression level in plants (Perlak et

al., 1991). Another possible explanation is that mutations in the promoter or the *gafp-1* gene occurred during transformation.

Bluebyrd line BB-1 showed significantly less severe disease symptoms in PRR and RKN tests, even though it did not exhibit the highest GAFP-1 synthesis level among transgenic lines. While BB-3 had a greater number of copies of the *gafp-1* gene compared than BB-1 and expressed higher levels of GAFP, it was more susceptible than BB-1 to *P.cinnamomi*. Similarly, transgenic *Arabidopsis thaliana* lines with high levels of disease resistance did not correspond to the ones with the highest expression of the insecticidal lectin GNA (*Galanthus nivalis agglutinin*) in roots (Ripoll et al., 2003) Expression of GAFP-1 in transgenic plum line 'Stanley' 5D was higher than that of 'Stanley' lines 4J and 4I, but 5D was more susceptible in PRR and RKN disease tests (Nagel et al., 2008). It is possible that the multiple insertions of *gafp-1* copies in many of the 'Bluebyrd' lines had a negative effect on the physiology of the plant. Some insertions may have impaired inherent disease resistance, erasing the beneficial effect of the transgene. Also, multiple copies of a transgene may increase the chances of pre- and post-transcriptional gene silencing (Stam et al., 1997), a measure the plant uses to protect itself from gene invasion. The increased copy number of the transgene may increase the chances of inactivation.

In immunoblot analyses, proteins with higher molecular weight compared to GAFP-1 (12 kDa) reacted with our GAFP-1 probe. The additional bands were observed in both transgenic and control plants and likely signaled nonspecific binding of the polyclonal antibody. Similar nonspecific binding has been observed in previous studies on tobacco

(Cox et al. 2006) and plum (Nagel et al., 2008). Nonspecific binding of a polyclonal antibody is not uncommon, as shown for apple shoot-extracted Vfa1 and Vfa2 proteins (Malnoy et al., 2008).

BB-1 displayed resistance against RKN, but its performance in contrast to the PRR experiment was not superior to the 'Stanley'-derived 4J line. Both lines had reduced numbers of galls, egg masses, and eggs compared to inoculated control lines. Effects that had previously been noted for the 'Stanley' 4J line (Nagel et al., 2008). The *gafp*-1-expressing lines under the control of *bul*409 did not result in more effective disease management when compared to the lines ones using the CaMV35S promoter. Polyubiquitin promoters such as the *bul*409 have shown enhanced expression of the reporter gene (GUS) in various transgenic plants such as transgenic potato (Rockhold et al., 2008) and rice (Lu et al., 2008). The performance of the *bul*409 promoter may be dependent on the host plant. For example, the polyubiquitin promoter GUBQ1 did not elevate the expression of the GUS reporter gene compared to the CaMV35S promoter in gladiolus, tobacco, rose, rice, and the floral monocot freesia (Joung and Kamo, 2006). In transgenic wheat, the expression of the insecticidal lectin GNA under control of an ubiquitin promoter was significantly lower compared to its expression in transgenic rice (Stoeger et al., 1999).

The *bul*409 promoter had been shown to be wound inducible in transgenic potato lines (Rockhold et al., 2008), but inducibility was not demonstrated in this study. Expression of *bul*409 promoter-driven GUS mRNA levels was higher in wounded tubers and leaves (Rockhold et al., 2008). Neither inoculation with *P. cinnamomi* nor

inoculation with *M. incognita* increased GAFP-1 synthesis, in this study suggesting that there was no inducibility in *gafp-1* gene expression. The two studies however cannot be directly compared since the mRNA level, but not protein expression, was measured in potato tubers and leaves while only the protein production was measured in this study. It is possible that no increase of GUS protein levels occurred in potatoes despite the increase of mRNA levels. It is unlikely that the inducibility of *bul*409 may occur in wounded but not in pathogen- induced tissue since wounding and pathogen responses share a number of components in their signaling pathways (Maleck and Dietrich, 1999).

This study established that the *gafp-1* gene is stable in transgenic plum lines. Line 'Stanley' 4J was developed from 'Stanley' seed for an earlier study in 2006 (Nagel et al., 2008) and has since been grown in the greenhouse under conditions allowing continuous, vegetative growth. After 4 years, GAFP-1 synthesis and pathogen resistance was consistent with that previously described (Nagel et al., 2008) with the exception that resistance to PRR was only increased numerically but was not statistically significant. In the present study fewer replicates were used compared to the earlier study. Stability of GAFP-1 synthesis was also confirmed in transgenic tobacco lines, which were generated in 2004 (Cox et al., 2006) and had been used for GAFP-1 isolation continuously until 2010 (Nagel et al., 2010).In conclusion, this study confirms the potential for *gafp-1* as a disease resistance gene in woody plants. Disease resistance has now been demonstrated in two different cultivars of plum and the long-term stability of GAFP-1 synthesis in transgenic lines was confirmed. The suitability of ubiquitin promoter *bul*409 for *gafp-1* expression plum was established and in one transgenic plant a significant increase of

PRR resistance was observed compared to the best performing ‘Stanley’ plum line containing *gafp-1* under the control of the CaMV35S promoter.

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Table 3.1. Number of *gafp-1* copies and GAFP-1 protein expression in roots of plum lines used in this study

Plum line	Transformation status[a]	*gafp-1* gene copy no.[a]	Relative GAFP protein expression in roots[b]
BB-1	Transformed	2	++
BB-2	Transformed	2	+
BB-3	Transformed	4	+++
BB-4	Transformed	0	—
BB-5	Transformed	5	—
BB-6	Transformed	1	—
BB-7 EV	Empty vector	0	—
BB-8 EV	Empty vector	0	—
BB-10	Transformed	5	+
BB-13	Transformed	2	+
BB-14	Transformed	1	—
BB-15	Transformed	3	—
BB-17	Transformed	4	++
BB-18	Transformed	2	++
BB-19	Transformed	2	+
BB-21	Transformed	4	++
BB-23	Transformed	2	—
BB-27	Transformed	2	—
BB-28	Transformed	2	—
BB-29	Transformed	2	—
BB OP-30	Untransformed	0	—
BB OP-31	Untransformed	0	—
BB OP-32	Untransformed	0	—
BB OP-33	Untransformed	0	—
BB OP	Untransformed	0	—
Stanley control	Untransformed	0	—
Stanley 4J	Transformed	2	+++

[a] Research conducted by Dr. Ralph Scorza's laboratory, USDA, Kearneysville, WV.
[b] Expression of GAFP-1 was measured and scored being equal (+++), up to 50% less (++), and less than 50% (+) relative to the expression in the 'Stanley' 4J line.

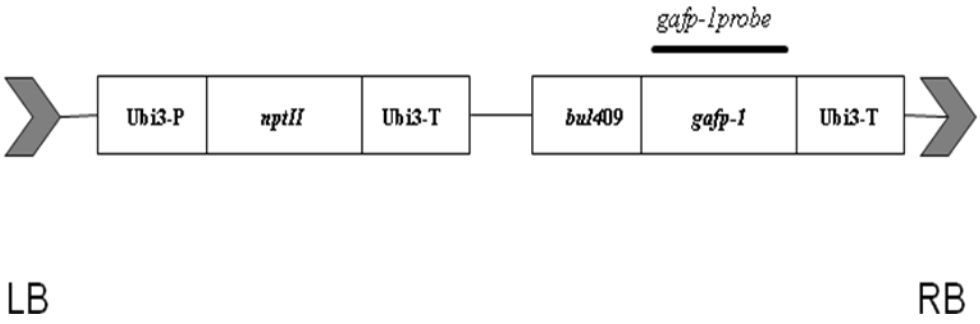

Fig. 3.1. Schematic diagram of pBINPLUS/ARS vector with insertion of the *gafp-1* gene placed under *bul*409 promoter and Ubi3-T (terminator).

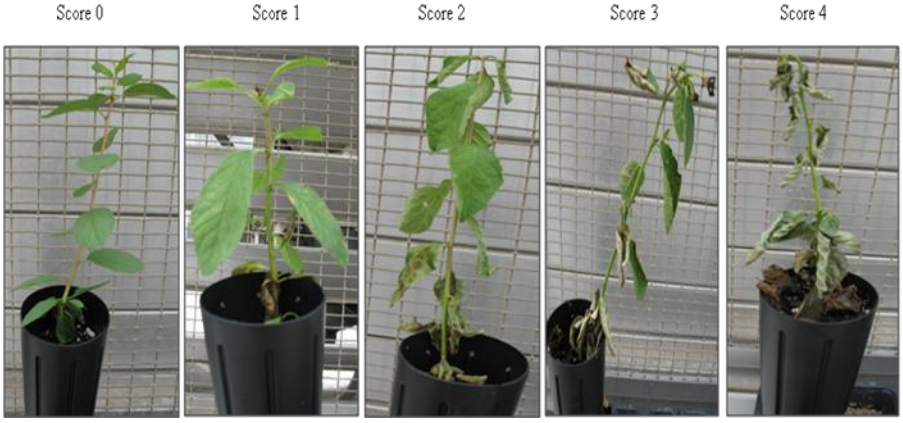

Fig. 3.2. Disease symptoms of ‘Bluebyrd’ plum lines 30-days after *Phytophthora cinnamomi* inoculation. Score 0 = asymptomatic plant (not inoculated); score 1 = less than 25% wilted; score 2 = 25% to 50% wilted; score 3 = more than 50% to 75% wilted; score 4 = more than 75% wilted.

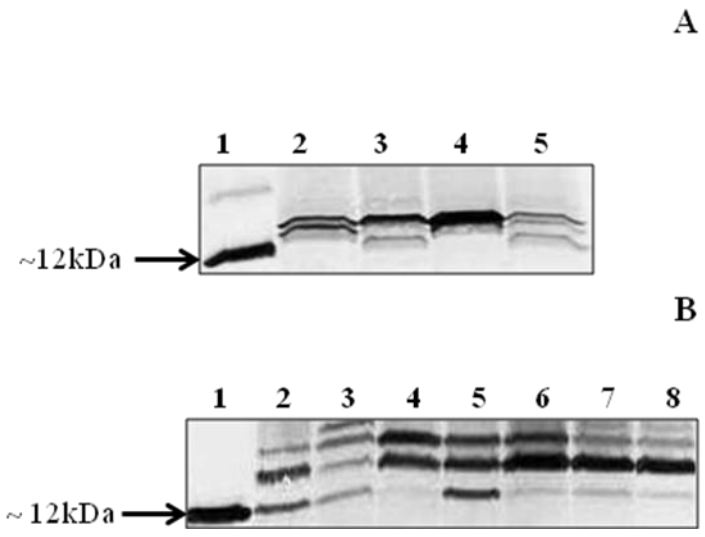

Fig. 3.3. Immunoblot analysis of total protein extracts (20 µg) from root tissue; (A) Lane 1: purified GAFP-1 (~12 kDa); Lanes 2 and 4: nontransformed 'Stanley' control and 'Bluebyrd'-OP respectively; Lanes 3 and 5: transgenic 4J (Stanley plum) and BB-3 (Bluebyrd plum) lines respectively. (B) Lane 1: GAFP-1 (~12 kDa); Lane 2: 4J and Lanes 3-8: transgenic 'Bluebyrd' plum lines BB-1, BB-2, BB-3, BB-17, BB-18, and BB-21, respectively.

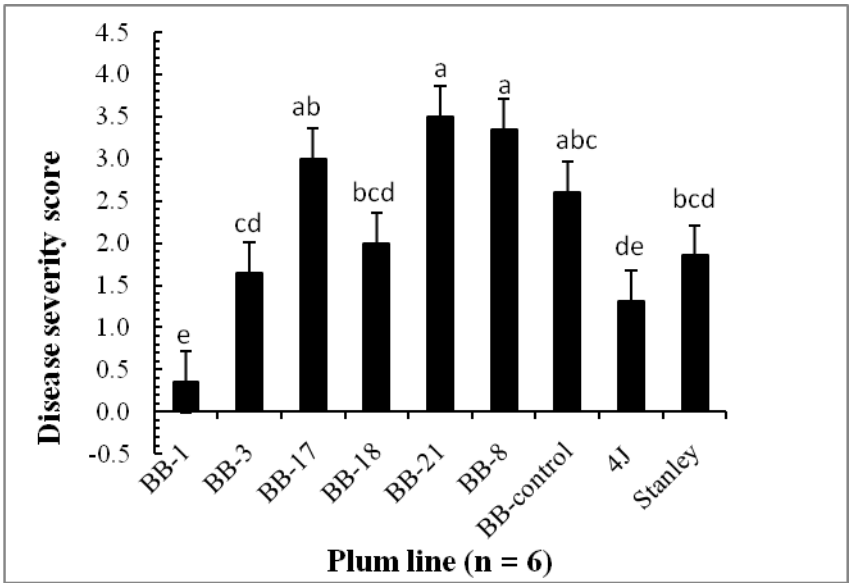

Fig. 3.4. Disease severity of 3-month-old plum lines BB-1, BB-3, BB-17, BB-18, BB-21, BB-8, 'Bluebyrd' control (mixture of lines BB OP, BB OP-30, and BB-OP 31), 4J, and 'Stanley' control 30 days after inoculation with *Phytophthora cinnamomi*. Bars represent the average of two experiments with 3 replicates each. Bars with the same letter are not significantly different (α=0.05)

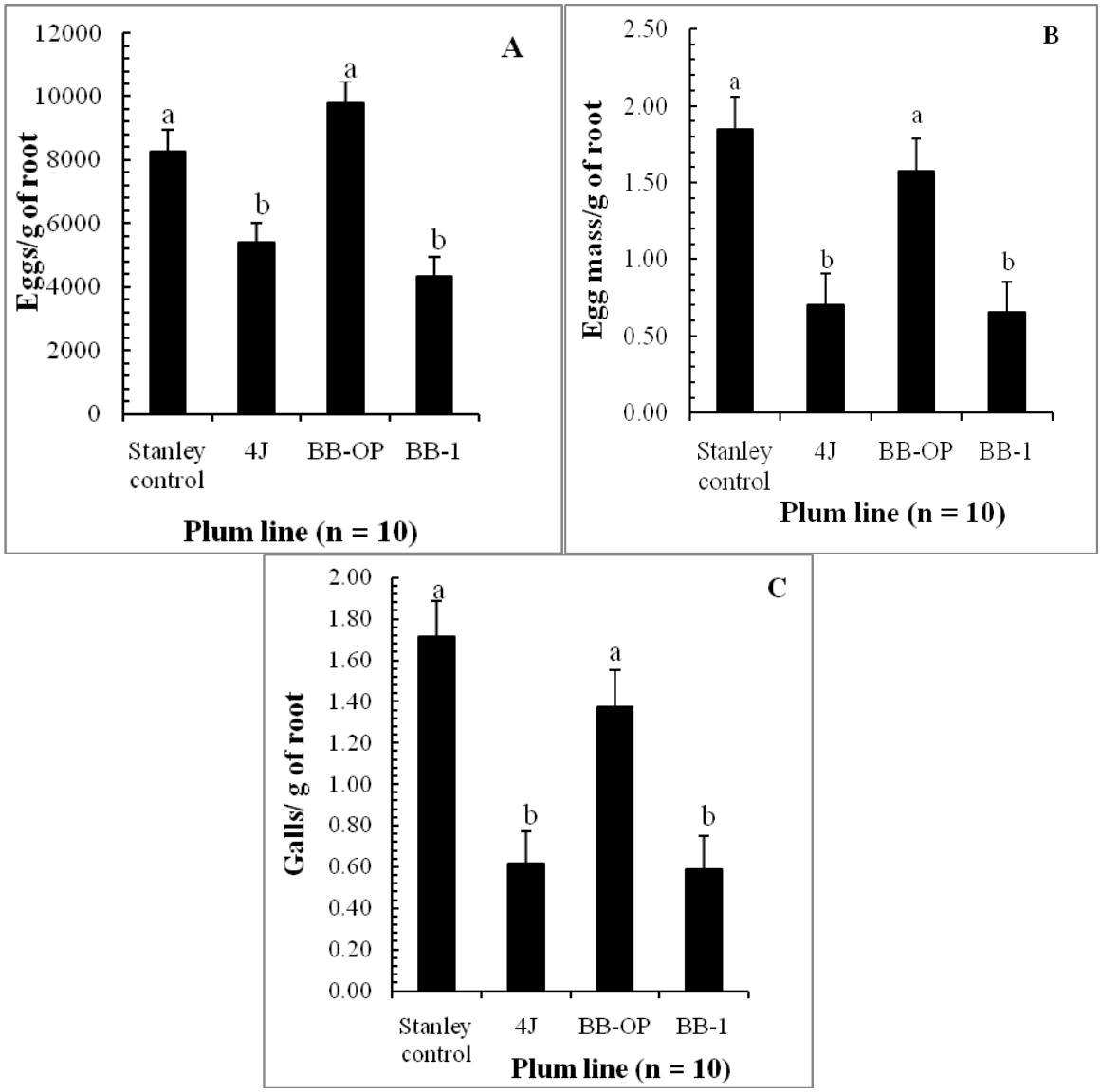

Fig. 3.5. Reproduction of *Meloidogyne incognita* on roots of 'Stanley' control line, 'Bluebyrd' control line (BB- OP), transgenic lines 4J, and BB-1. (**A**) Number of eggs per gram of root; (**B**) Egg mass (egg mass per gram of fresh root), and; (**C**) Gall formation (galls per gram of root). Shown is the combined dataset of two independent experiments. Bars represent the average of two experiments with 5 replicates each. Bars with the same letter are not significantly different ($\alpha = 0.05$).

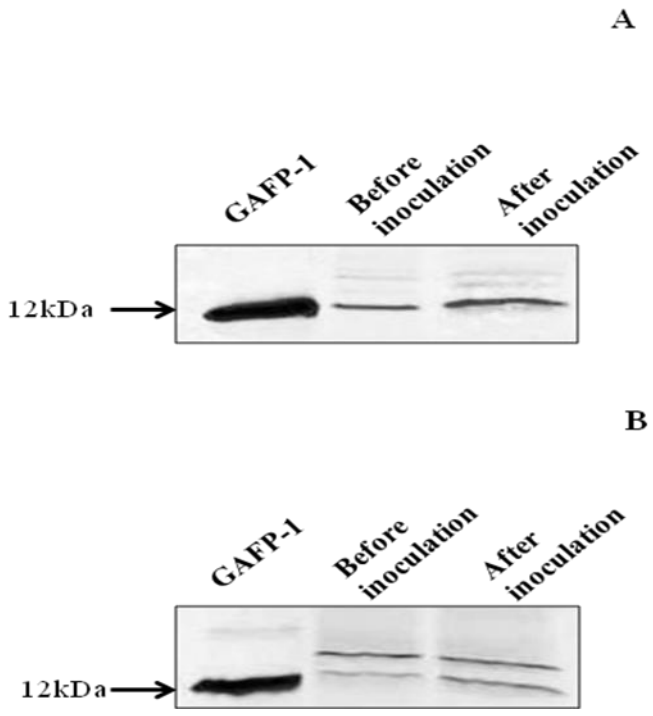

Fig. 3.6. Immunoblot analysis showing GAFP-1 in 20 µg of total protein from root tissue of transgenic line BB-1 (**A**) before (lane 2) and 5 days after (lane 3) inoculation with *Phytophthora cinnamomi* and (**B**) before (lane 2) and 30 days after (lane 3) inoculation with *Meloidogyne incognita*.

CHAPTER FOUR

CONCLUSIONS

In this study, GAFP-1 protein was absent in non transgenic leaf and shoot tissue grafted on *gafp-1* expressing 'Stanley' 4J and 4I line roots indicating that GAFP-1 is not moving across the graft union into the scion. Thus, fruit from chimeric-grafted trees with transgenic rootstocks and non-transgenic scions should be free of GAFP molecules. Further research is needed to confirm this hypothesis in field-grown trees with special emphasis on the fruit. Even if there were traces of GAFP-1 protein in fruit, the risk of harming consumers may still be minimal. That is because the corm of the orchid *Gastrodia elata*, which produces large amounts of GAFP-1 protein (up to 50% of the total protein) upon infection by *Armillaria mellea*, has been consumed by Chinese for centuries for medicinal purposes. In fact, corm extracts containing GAFP are being sold over the internet with health benefit claims (http://www.shop-china.co.uk/en/cp/Gastrodia). It remains to be determined if a chimeric-grafted tree producing fruit with no or minor traces of the foreign gene would be acceptable to GMO-opposed consumers.

'Bluebyrd' plum line BB-1 was largely comparable to the previously characterized 'Stanley' 4J line in regard to the level of GAFP-1 protein expression and disease resistance. These results are promising for the following reasons. The *bul*409 promoter is a plant-derived promoter, which may be an advantage over the virus-derived CaMV35S promoter when discussions arise about whether to register *gafp-1* for crop plants. The

*bul*409 promoter was developed by USDA scientists and thus is not protected by a patent owned by the private industry unlike the CaMV35S whose rights are held by Monsanto. A pathogen-induced increase in GAFP-1 protein expression in *bul*409 plants was not observed.

Future studies should be designed to verify that the results reported in this study hold up under field conditions. Thus future research should investigate whether BB-1 and 'Stanley' 4J provide field resistance to ARR, PRR, and RKN. Considering the ultimate goal to make a transgenic rootstock suitable for peach and resistant to ARR, future research should investigate the potential use of transgenic 'Bluebyrd' and 'Stanley' plum lines as rootstocks for commercial peach production. Although preliminary results suggest that both plum varieties are compatible with peach (Schnabel, unpublished data), a more detailed investigation needs to be implemented to verify long term compatibility, rootstock suitability for southeastern conditions (e.g. drought resistance, nematode resistance, soil type preference), and rootstock impact on fruit quality (e.g. size, shape, and susceptibility to split pit). It would be extremely helpful to develop an assay that could be used for the assessment of resistance to *Armillaria* in greenhouse/laboratory tests. This would speed up the development of rootstocks used with peaches which were resistant to *Armillaria.*