Engineering Resistance to *Orobanche aegyptiaca*: Evidence of Sarcotoxin IA as an Anti-Parasite Protein and Macromolecule Movement From Host to Parasite

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ABSTRACT

Orobanche species are parasitic weeds that subsist on the roots of many dicotyledonous plants. These parasites form symplastic and apoplastic connections with their hosts and act as strong sinks for the uptake of water, minerals, and photosynthates, often causing severe damage to the hosts. Although the uptake of small molecules such as sugars and herbicides by *Orobanche* has been documented, movement of macromolecules between host and parasite has not been characterized. The objectives of this research were to 1) determine whether, and by what route, host macromolecules can be translocated to the parasite, and 2) engineer host resistance based on inducible expression of sarcotoxin IA, an anti-microbial peptide from the flesh fly (*Sarcophaga peregrina*). To address the first objective, transgenic plants expressing GFP localized to either the host cell cytosol (symplast) or secreted to the extra-cellular space (apoplast) were parasitized by *O. aegyptiaca*. Observations of green fluorescence in *O. aegyptiaca* tubercles growing on these plants indicate that the 27 kDa GFP molecule was translocated to the parasite via both symplastic and apoplastic routes. This work was supported by studies with xylem- and phloem-specific dyes, which showed that fluorescent dextrans as large as 70 kDa moved into the parasite through xylem connections. The second objective was addressed using tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants expressing the sarcotoxin IA transgene under control of the parasite-inducible *HMG2* promoter. In soil experiments, transgenic tobacco plants had greater height and biomass, and showed up to 90% reduction in *O. ramosa* parasitism as measured by the fresh weight of parasite tubercles. In a semi-hydroponic growth system, where *Orobanche* tubercles can be visualized at early stages of growth, *O. aegyptiaca* parasites growing on plants expressing sarcotoxin IA were smaller and had an increased number of senescent tubercles compared to those growing on non-transformed plants. Considering the relatively small size of sarcotoxin IA (4 kDa), it is likely that this peptide moves from host to the parasite, where it accumulates to phytotoxic concentrations. In addition to increasing our knowledge of host-*Orobanche* interactions, this research used an antibiotic peptide to engineer partial *Orobanche* resistance into a highly susceptible crop. This strategy has broad implications for the control of other parasitic weeds.

Dedication

This dissertation is dedicated to my beloved family and to the memory of my father, may the mercy and the blessing of God be upon him.

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Chapter I

LITERATURE REVIEW

I.1. *Orobanche*

Over 3000 species of flowering plants, distributed among 17 families, use a parasitic mode of nutrition (Parker and Riches, 1993). Parasitic angiosperms are generally separated into two broad categories, holoparasites or hemiparasites. Holoparasitic species lack chlorophyll and have little independent capacity to assimilate or fix carbon and/or inorganic nitrogen (Stewart and Press, 1990). Hemiparasites on the other hand, contain chlorophyll and are thought to rely on their host primarily for water and minerals. Parasitic flowering plants are further subdivided on the basis of their site of attachment to the host into stem parasites such as dodders (*Cuscuta* spp.) and mistletoes (*Arceuthobium* and *Viscum* spp.), and root parasites such as broomrapes (*Orobanche* spp.) and witchweeds (*Striga* spp.). Some species have functional roots (e.g. *Rhinanthus* and *Olax* species) and therefore are able to absorb inorganic nutrients from the soil, some have reduced root systems (e.g. *Orobanche*), and others possess nothing that resembles a root (e.g. the Cuscuta and the mistletoes).

The name *Orobanche* derives from the Greek words *Orobos* (= pea) and *Ancho* (= to strangle), referring to the effect the parasites have on their hosts (according to Dioscorides as cited in Koch, 1887). *Orobanche* subsists on the roots of many dicotyledonous plants, connects to the host by a haustorium (see the following section), and draws photosynthates and water from the host, causing significant reductions in crop yield and quality (Sauerborn, 1991). The most economically important weedy *Orobanche* species are *O. crenata* (Forsk.), *O. cumana* (Wallr.), *O. cernua* (Loefl.), *O. ramosa* (L.), *O. aegyptiaca* (Pers.), *O. minor* (Sm.), and *O. foetida* (Poir.). Affected crop species are found in the Solanacae, Fabaceae, Cucurbitaceae, Compositae, Cruciferae, and

Umbelliferae families (Parker and Riches, 1993), but crops in several other families can be parasitized as well.

I.2. *Orobanche* evolution

Parasitism may be the evolutionary result of competition for limited resources in arid and nutrient-poor habitats (Atsatt, 1973). Parasites have a competitive advantage over autotrophic plants, since the host plant delivers nutrients to the parasites enabling them to survive on nutrient-poor sites. Parasites in the Orobanchaceae must have developed the parasitic behavior and morphology from opportunistic root connections during the course of evolution (Kuijt, 1969). Given a certain physiological compatibility, and favorable osmotic gradient, this connection results in a simple parasitic relationship. As specialization occurred, the parasite root system was reduced until the primary haustorium was formed directly from the tip of the germ tube. At the same time, the requirement for germination signals developed and normal plant structures such as leaves were lost or reduced during evolution. Moreover, *Orobanche* evolved roots capable of forming additional host contact and sites of parasitism (Weber, 1980).

I.3. *Orobanche*-host interaction

Parasitic weeds have evolved a complex interaction with their hosts. In addition to the normal germination requirements of angiosperm seeds, *Orobanche* seeds (which are very small at only 0.3 mm long) germinate only in response to chemical signals emitted from a nearby host root (Parker and Riches, 1993). These signals are generally found only in the root exudates of a compatible host plant. Once *Orobanche* seeds germinate, the radicle (which may grow up to 1-2 mm long) emerges and contacts the host. This radicle has no root cap and does not develop procambium or conductive tissue (Joel and

Losner-Goshen, 1994a). The transfer of host solutes and macromolecules into the parasite relies on the formation of a connection between the two organisms. This connection is accomplished by a unique structure, the haustorium (from the Latin, *haurire*, to drink), which is a multi-cellular organ that invades host tissues and serves as a physical and physiological bridge between host and parasite. Intrusive cells of the haustorium penetrate host tissues and reach the conductive system of the host. This penetration is accompanied by the secretion of lytic enzymes, such as pectin methylesterase and polygalacturonase, which digest the middle lamella that holds the cells together (Joel and Losner-Goshen, 1994a; Losner-Goshen et al., 1998).

Once vascular connections are formed, the parasite extracts water, nutrients, and photosynthates from the host vascular tissue. The *Orobanche* radicle outside of the host root swells and develops into a bulbous mass of tissue called a tubercle. Ultimately, the tubercle initiates a floral meristem that develops into a floral spike, the parasite's only above ground structure. Each flowering shoot can produce up to 200,000 seeds (Parker and Riches, 1993).

I.4. *Orobanche* nutrient uptake

The uptake of water and nutrients from the host plant is an essential process for the parasite. Important but incompletely understood aspects of this process include the type of physical connections that are established and the physiological mechanisms that drive uptake. Processes considered here are water, carbon, and nitrogen movement from host to parasite.

1.4.1. Water uptake

The main driving force for water influx into the parasite is a strong osmotic gradient established by the parasite (Solomon, 1952). This is facilitated in some parasites species by a higher transpiration rate due to their stomata remaining open even during dry periods (Musselman, 1980). Those parasitic plants that do not possess transpiring leaves (e.g. *Lathraea*, *Orobanche*) have developed glands that actively secrete water in order to maintain the necessary osmotic gradient. In several host-parasite systems, potassium may play an important role as an osmoticum. Potassium accumulates to high concentrations in the haustoria of *Orobanche* (Singh et al., 1971; Ernst, 1986) and *Cuscuta* (Wallace et al., 1978). Another osmoticum, the sugar-alcohol mannitol, is found in parasitic angiosperms of the *Orobanchaceae* and *Scrophulariaceae* (Stewart et al., 1984).

1.4.2. Movement of carbon and nitrogen from host to parasite

O. aegyptiaca forms symplastic connections with the host and may have xylem connections similar to that seen in *Striga* for water and nutrients uptake (Dörr and Kollmann, 1995; Dörr, 1997). It has been reported that almost 100% of the carbon accumulated from the host derives from the host phloem (Jeschke et al., 1994b; Hibberd et al., 1999), which also supplies the majority of nutrients, even minerals such as nitrogen, magnesium, and potassium, which have larger fluxes in the host xylem.

The extent to which parasitic angiosperms control the nature of solutes received from the host is uncertain. The accumulation of host specific alkaloids in *Castilleja sulphurea* (Scrophulariaceae) parasitizing *Lupinus argentus* (Arslanian et al., 1990) and the movement of glyphosate from host to *O. aegyptiaca* (Rakesh and Foy, 1997) suggest a lack of specificity.

Parasitic angiosperms form soluble carbohydrate "reserves" which differ from the major soluble carbohydrates of the host. *Orobanche* contain mannitol at concentrations of more than 150 mg/g dry weight accounting for more than 75% of total soluble sugars (Press et al., 1986). In some instances, much higher concentrations have been reported, reaching up to 20% of dry weight (Lewis, 1984). One of the reasons why these compounds are present at such high concentrations is that their rate of turnover can be very slow. The precise physiological role of mannitol in angiosperms is unclear, but studies on algae and fungi, where mannitol and other polyols are ubiquitous, suggest its involvement in: 1) storage of carbohydrate and reducing power 2) regulation and stabilization of enzyme systems and 3) osmoregulation (Lewis and Smith, 1967; Bieleski, 1982).

The haustorial cells appear to play an active role in metabolizing acquired nutrients. Evidence for this comes from studies showing that the carbohydrates, amino acids, and organic acids present in the xylem sap of *S. hermonthica* are different from those in that of its host sorghum bicolor (Press, 1989). The carbohydrate concentrations in the parasite's xylem sap are five times those of the host, and the major component is mannitol, which is absent from the host xylem sap. In sorghum, the major nitrogenous solute of the xylem is asparagine, while in *Striga* it is citrulline. There are also some differences in organic acid composition. The main components of sorghum sap are malate and citrate. The latter is absent in *Striga*, but shikimic acid, which is absent from sorghum sap, is present in the sap of the parasite. Differences in metabolic composition of host and parasite xylem saps have also been reported for other species of root hemiparasites (Govier et al., 1967) and mistletoes (Richter and Popp, 1987). Wolswinkel

(1974; 1978a) has shown that the release of solutes from sieve tubes at the site of attachment of *Cuscuta* is a highly specific process, markedly favoring certain solutes over others, an observation supported by the studies of Jeschke and co-workers (1994a; 1994b). Additionally, ultra-structure studies of several species indicate the presence of parenchyma cells with a high density of cell organelles such as mitochondria, ribosomes, dictyosomes, and well-developed endoplasmic reticulum (Visser et al., 1984; Mallaburn and Stewart, 1987; Visser and Dörr, 1987; Kuo et al., 1989).

1.4.3. Mechanism of solute transfer from host to parasite

The mechanism of sugar transfer from host to parasite has been described in *Cuscuta*. This parasite has transfer cells formed from parenchyma cells adjacent to the host vascular tissue (Dörr, 1990). Wolswinkel (1978a; 1978b) has suggested that the transfer cells of *Cuscuta* operate at high efficiency in the absorption of solutes from the apoplast of the host. The author suggests that solute transfer from host to *Cuscuta* may occur in two stages; First, from sieve tube lumena of the host into the free space adjacent to transfer cells of the parasite; Parasite infection strongly stimulates the release of sucrose into the free space, but has little effect on the efflux of either glucose or fructose (Wolswinkel and Ammerlaan, 1983). High free-space acid invertase activity has also been reported in *Cuscuta* (Wolswinkel and Ammerlaan, 1983). This helps create a gradient across the apoplast that favors phloem unloading. Hydrolysis of unloaded sucrose leads to the generation of sink activity because accumulation of hexoses in the apoplast lowers the water potential of the free space, leading to movement of water out of the phloem, and so lowering phloem turgor pressure. The resulting lower sieve tube

osmotic potential then leads to further movement of sucrose along the phloem toward the sink.

1.5. Physiology of infected hosts

The response of host plants to infection varies from profound growth abnormalities to an almost complete absence of visible symptoms (Stewart and Press, 1990). It appears that the additional sink generated by the parasite induces an increase in host photosynthesis. In general, the type and extent of the impact are determined by four factors: 1) the size of the parasite, 2) the rate of growth and metabolic activity of the parasite, 3) the degree of dependency on the host for resources, and 4) the stage of development of the host.

Competition for water, inorganic ions, and metabolites is the simplest explanation for losses in host production. However, according to Graves (1995), *Orobanche*-induced yield reductions are not primarily due to competition for water, but rather due to carbohydrate loss to the parasite. As a consequence, the capacity for host-root water uptake is reduced. Competition for water can be regarded as a secondary cause of yield reduction.

Although *Orobanche* undoubtedly acts as a strong sink for inorganic ions, evidence for nutrient deficiency in hosts is uncommon. One of the few examples was found in tobacco infected with *O. ramosa*, in which the phosphorus concentration was reduced by more than 50 % in roots of infected plants and leaf potassium concentration was reduced by 60 % (Ernst, 1986). These changes in the nutrient budget of infected plants were considered to be the principal reason for a 30% reduction in host growth.

I.6. *Orobanche* control strategies

Orobanche control is difficult because the parasite is closely associated with the host root and is concealed underground for most of its life cycle. Mechanical control is inefficient because it is laborious and the parasite causes significant damage to the host before the *Orobanche* floral shoot emerges from the soil. The most effective chemical control method has been soil fumigation to kill seeds, but this is costly and hazardous to the environment. Herbicide-resistant crop cultivars provide an excellent opportunity to control *Orobanche* because they allow a herbicide to be translocated through the host to the parasite (Joel et al., 1995), but this is dependent on the generation and commercialization of herbicide-resistant crop varieties, and may be countered by the development of herbicide-resistant populations of *Orobanche* (Gressel et al., 1996). Breeding programs have developed resistant sunflower lines, but resistance has been repeatedly overcome by new physiological races of *Orobanche* (Encheva and Shindrova, 1994). The development of new control strategies is urgently needed to protect crops from *Orobanche*, with the best control approach being the development of host crops that can resist parasitism.

1.7. Host resistance

Resistant varieties of host crops have been sought for many years, but with little success. Even for those cases of resistance that exist, the mechanism of resistance in not always clear. For sunflower that is resistant to *O. cumana*, the resistance was attributed to lignifications of host root cells following penetration. Resistance mechanisms in host-*Orobanche* relationships, as far as they are known or suspected, are listed in Table 1.

Breeding for resistance has concentrated mainly on low stimulant production by the host or on mechanical barriers to penetration by the parasite. It is assumed that not just one, but several factors play a role in conferring resistance and that they often affect host resistance in combination. According to El Hiweris (1987), three defense mechanisms act together in Framida, a sorghum variety resistant to *Striga*: low production of stimulant, thickening of the root cells, and an increase of phenol compounds in the host. It was reported that the low infestation of the faba bean variety F402 from Egypt is based on the morphology of the host root. Root architecture, consisting of deep rooting with little branching in top soil, inhibited the attachment of *Orobanche* and illustrates an example of an indirect mechanism of resistance.

I.8. Engineering resistance to *Orobanche*

Biotechnology provides an additional approach to complementing the efforts in traditional breeding to control parasitic weeds such as *Orobanche.* Genetic engineering strategies for resistance to *Orobanche* require two elements: (1) a parasite-responsive gene promoter, and (2) a parasite-inhibitory gene product.

I.8.1. *HMG2* promoter: an *Orobanche* inducible promoter

Plants, under constant threat of infection by pathogens, have evolved sophisticated mechanisms of pathogen detection and defense. Upon pathogen detection, plants activate a number of responses that lead to the production of a broad spectrum of defensive molecules called phytoalexins. Among these classes of phytoalexins are those derived from the isoprenoid pathway.

Table 1. Summary of resistance mechanisms of some host crops against *Orobanche* (updated from Sauerborn, 1991)

Host-Parasite	Mechanism	Reference
Faba bean/*O. crenata* (F 402)	Root morphology (little branching in top soil)	(Nassib et al., 1982)
Faba bean/*O. crenata*	Thicker root, bark	(Nassib et al., 1984)
Faba bean/*Orobanche* spp	Low stimulant production	(Cubero, 1973)
Sunflower/*O. cumana*	Storage of lignin related substances in the host root cells.	(Antonova, 1978)
Sunflower/*O. cumana*	Production of phytoalexins	(Wegmann et al., 1989)
Tobacco/*O. ramosa*	Low stimulant production	(Racovitza, 1973)
Tomato/*O. aegyptiaca*	Haustorium penetration handicapped or delayed	(Avdeev and Shcherbinin, 1978)
Vetch /*O. aegyptiaca*	Mechanical and possibly. chemical barriers	(Goldwasser et al., 2000)

*HMG*2 is one of four differentially regulated genes in tomato that encode the 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*), considered the rate-limiting step in the isoprenoid biosynthetic pathway (Chappell, 1995). This gene is normally not expressed in most tissues, but is induced in response to wounding or pathogen attack (Cramer et al., 1993). Using *GUS* as a reporter gene, Westwood et al. (1998) demonstrated that parasitism by *O. aegyptiaca* induces expression of *HMG*2 in transgenic tobacco. Expression was detected within one day following penetration by *O. aegyptiaca*, and was localized to the region around the site of parasite ingress. This expression intensified during early parasite growth and continued over the course of parasite development, indicating that *HMG*2 expression does not represent a transient response to host injury. The expression pattern of the *HMG2* promoter makes it an efficient regulator for a parasite-resistance gene; an *Orobanche*–specific toxin under the control of the *HMG2* promoter should not be expressed in most healthy tissue, but would accumulate specifically at the site of parasite attachment following host root penetration. Ultimately, young and actively growing *Orobanche* tubercles should absorb the toxin and die.

I.8.2. Sarcotoxin IA : a potential toxin to *Orobanche*

Sarcotoxin IA, a peptide of 39 residues, is one of four cecropin-type proteins encoded by the sarcotoxin IA gene cluster in the flesh fly, *Sarcophaga peregrina* (Kanai and Natori, 1989). The mature peptide consists of two amphiphilic helical regions, helix I (Leu3-Gln23) and Helix II (Ala28-Ala38), with a hinge region (Gly24-Ile27) (Figure 1). The N-terminal half of this molecule is rich in positively charged amino acids and is hydrophilic, whereas the C-terminal half is hydrophobic (Okada and Natori, 1985). This toxin functions as anti-microbial peptides for the fly, which feeds on decaying flesh. The

N-terminal half of the toxin interacts with acidic phospholipids on the bacterial membrane surface, while the C-terminal half penetrates into the membranes, causing disruption of the membrane potential (Natori, 1994). In plants, similar bactericidal peptides with two helices have not been found (Broekaert et al., 1997). In the insect, expression of sarcotoxin is induced in response to injury and infection (Okada and Natori, 1983). This peptide is a component of the self-defense system of a fly against pathogens and has a broad spectrum against both bacterial and fungal pathogens (Mitsuhara et al., 2000).

In a recent study, expression of sarcotoxin under a root-specific promoter (Tob promoter) in tobacco and tomato plants enhanced host resistance to *O. aegyptiaca* and did not appear to adversely affect the growth of the host plant (Aly et al., unpublished observations). However, resistance was incomplete and we hypothesized that this was due to the low, basal expression of sarcotoxin IA driven by the Tob promoter. Intensifying the expression of the toxin in just the area of *Orobanche* attachment may increase the efficacy of the toxin to inhibit the parasite. The *HMG2* promoter would serve this goal. First, the inducibility of the *HMG2* promoter will drive the expression of the toxin only after *Orobanche* attack, and second, the toxin will be accumulated at the site of attachment where it is will be most available for uptake by the growing parasite.

$^{+}$NH3-Gly-Trp-Leu-Lys-Lys-Ile-Gly-Lys-Lys-Ile-Glu-Arg-Val-Gly-Gln-His-Thr-Arg-Asp-Ala-Thr-Ile-Gln-Gly-Leu-Gly-Ile-Ala-Gln-Gln-Ala-Ala-Asn-Val-Ala-Ala-Thr-Ala-Arg-COO^{-}

(Amino acids in red are positively charged)

(Amino acids in blue are negatively charged)

(Amino acids in green are hydrophobic)

(Amino acids in black are polar and non-charged)

(Underlined amino acids mark the hinge region)

Figure 1. Amino acid sequence of sarcotoxin IA. Sarcotoxin IA consists of two domains. The amino-terminal half (position 1-19) contains 9 charged residues, of which 7 are basic, while the carboxyl-terminal half of the molecule (position 20-39) is rich in non-polar amino acid residues, and the only basic amino-acid residue is the carboxyl-terminal Arginine (Arg). The amino-terminal half of the molecule is hydrophilic and is believed to associate with bacterial membrane surfaces, whereas the carboxyl-terminal half is hydrophobic and embeds in the membrane.

I.9. Safety of sarcotoxin IA to humans

Several lines of evidence suggest that sarcotoxin does not pose a threat to human health. The primary target site of sarcotoxin IA exhibits specific bactericidal activity with less toxicity to eukaryotic cells. Ohshima et al. (1999) tested the toxicity of sarcotoxin IA against plants using suspension cells from tobacco and rice and found that sarcotoxin IA was not toxic to these dicot and monocot plant cells at less than 25 μM when present outside the cells. In comparison, antibiotics such as kanamycin and tetracycline inhibited the growth of these cells by about 40 % at 10 μM. Sarcotoxin IA has a minimum inhibitory concentration of 0.2 to 0.3 μM against *E. coli* (Nakajima et al., 1987) which is comparable to that of other antibiotics (Kunin, 1967; Heijzlar et al., 1969). Furthermore, homologs of sarcotoxin IA have been reported in a broad range of insects (Boman and Hultmark, 1987), mammals (Lee et al., 1989) and tunicates (Zhao et al., 1997), suggesting that they help to protect many organisms against attack by pathogenic microbes and are widely occurring in nature. Finally, sarcotoxin IA has been explored for use in clinical situations and is effective against a wide variety if important causal pathogens of human diseases such as *Staphylococcus aureus* and *Diplococcus pneumonia* (Natori, 1988). The half-life of sarcotoxin IA in artificial gastric juice is less than 30 seconds.

Such evidence points to cecropin-type bactericidal peptides as potential new classes of antibiotics (Nakajima et al., 1997). In a recent study, Mitsuhara et al. (2001) tested sarcotoxin IA against 13 human intestinal bacteria including both beneficial and harmful bacteria *in vivo* and found that sarcotoxin IA suppressed the growth of bacteria that have a detrimental effect on human health, such as *Clostridium ramosum, C.*

paraputrificum and *E. coli* O157, but had no effect on *Befidobacterium adolescentis , B. longum* and *Lactobacillus acidophilus,* which are known to benefit human health. This finding implies that of sarcotoxin IA may have a positive effect on human health through the maintenance of a balance of intestinal flora.

I.10. Cauliflower mosaic virus (CaMV) 35S promoter

Cauliflower mosaic virus (CaMV) has a double-stranded DNA genome within which two distinct promoters produce 19S and 35S transcripts (Franck et al., 1980). Initial studies with transformed tobacco revealed that the 35S promoter appears to be active in most, if not all, plant tissues (Odell et al., 1985), suggesting that the 35S promoter can be used as a constitutive promoter in plants. The 35S promoter is a DNA sequence about 400 base pairs in length. A variant of the cauliflower mosaic virus 35S promoter, named double enhanced 35S (de35S), with transcriptional activity approximately tenfold higher than that of the natural promoter was constructed by tandem duplication of the first 250 base pairs of the promoter. The duplicated region acts as a strong enhancer, increasing the activity of an adjacent transferred DNA gene several hundredfold (Kay et al., 1987). This optimized enhancer element has been very useful for obtaining high levels of expression of foreign genes in transgenic plants. In this study the de35S promoter was used to achieve high expression of the green fluorescent protein.

I.11. Protein targeting

The first sorting event for all proteins made in the cytosol separates them into two groups. Proteins in the first group are released in the cytosol and may either remain in that compartment or are targeted to plastids, peroxisomes, mitochondria or nuclei. In

contrast, proteins in the second group are targeted to the endoplasmic reticulum (ER) by signal peptides.

Signal peptides are short peptides of 16 to 30 amino acids. They are found on nearly all secreted and vacuolar proteins as well as on proteins that reside in the lumen of the ER or the Golgi complex. Signal peptides of proteins that enter the secretory pathway may differ from one another, but share important structural features. A signal peptide typically consists of one or more positively charged amino acids followed by a stretch of 6 to 12 hydrophobic amino acids and then several additional amino acids. The signal peptides of different secretory proteins are interchangeable, not only among plant proteins, but also among plant, animal, and yeast proteins. Furthermore, the presence of a signal peptide is sufficient to direct the secretion of any stable and correctly folded protein. In this study, we used the signal peptide of potato patatin gene, which has been shown to effectively direct fused heterologous proteins to the endomembrane system (Bevan et al., 1986).

As the signaling sequence emerges from the ribosomes, they are recognized and bound by a signal recognition particle (SRP). This binding inhibits translation and targets the complex to the ER. The SRP is then released and the ribosome binds to a protein translocation complex in the ER membrane; the signal sequence is inserted into a membrane channel, translation is resumed and the growing polypeptide chain is moved across the membrane into the ER. As translocation proceeds, the signal sequence is cleaved by signal peptidase and the polypeptide is released into the ER lumen. Protein synthesized in the ER enters the secretory pathway, an intracellular system of vesicles and cisternae that includes the ER, Golgi complex, tonoplast, and plasma membrane. The

proteins can then be secreted from the cell or targeted to the various compartments within the cell.

I.12. Green Fluorescent Protein

The Green Fluorescent Protein (GFP) of the jellyfish *Aequorea victoria* (Chaflie et al., 1994) is one member of a small but important class of proteins that exhibit strong visible fluorescence without the requirement of cofactors or other enzymes. It has emerged as a novel genetic marker that can be directly visualized in living cells of many organisms (Haseloff and Amos, 1995; Sullivan and Kay, 1999). The fluorescence of the protein is due to a unique, covalently attached chromophore that is formed post-translationally within the protein upon cyclization and oxidation of residues 65-67, Ser-Tyr-Gly. There are intrinsic advantages in using GFP over other reporter systems. GFP is encoded by a small DNA sequence, thus facilitating construction of gene fusions. Also, GFP fluorescence does not require the addition of an exogenous substrate, allowing real-time imaging, and avoiding fixation artifacts.

When studying higher plants, most researchers employ GFP variants in which the codon usage has been altered to be closer to the plant consensus, which has the additional effect of eliminating aberrant mRNA processing due to false recognition of cryptic introns by some plants species such as *Arabidopsis* and tobacco (Haseloff et al., 1997). The GFP used in this study has been modified for criptic intron splicing, mutations V163A and S175G enhances folding (Siemering et al., 1996), while mutation I167T changes the UV and blue light maxima to equal amplitudes (Heim et al., 1994).

A variety of applications for GFP have been developed in higher plants. It has been used as a general reporter gene (Galbraith et al., 1995; Chiu et al., 1996; Reichel et

al., 1996; Rouwendal et al., 1997) and for other purposes including studies of virus invasion and spread (Oparka et al., 1997), visualization of cellular protein targeting (Haseloff et al., 1997; Grebenok et al., 1997b), characterization of cell type specific gene regulation (Haseloff, 1999), and for visualizing vesicle trafficking to the endoplasmic reticulum, Golgi, and plasma membrane (Horn and Banting, 1994; Girotti and Banting, 1996; Presley et al., 1997). GFP protein can move through plasmodesmata from companion cells into sieve elements, is freely mobile within the phloem, and is translocated together with the stream of assimilates (Imlau et al., 1999).

These characteristics make GFP a good marker for studies of protein movement from host to parasite. GFP expression under the control of *Orobanche*-inducible promoters (e.g. *HMG*2) would provide insights into fundamental aspects of the plant-plant union and will be useful in targeting the sarcotoxin IA to be most effective in inhibiting *Orobanche* growth. Two different GFP constructs were generated to study the two possible routes for protein movement from host to *Orobanche*, symplastic and apoplastic. Two possible routes for protein movement from host to *Orobanche,* apoplastic and symplastic, we investigated using two different GFP-containing gene constructs. In one construct, GFP was put under the control of the *HMG2* promoter without a signal peptide. This should result in the retention of GFP protein in the host cell cytosol, and thus be useful for tracing protein movement from host to *Orobanche* cells through symplastic connections. In the other construct, patatin signal peptide was introduced at the N-terminus of GFP. In this case, GFP should enter the secretory pathway and be secreted to the intercellular space. GFP thus produced will reveal macromolecule movement to *Orobanche* cells through xylem connections.

I.13. Fluorescent probes

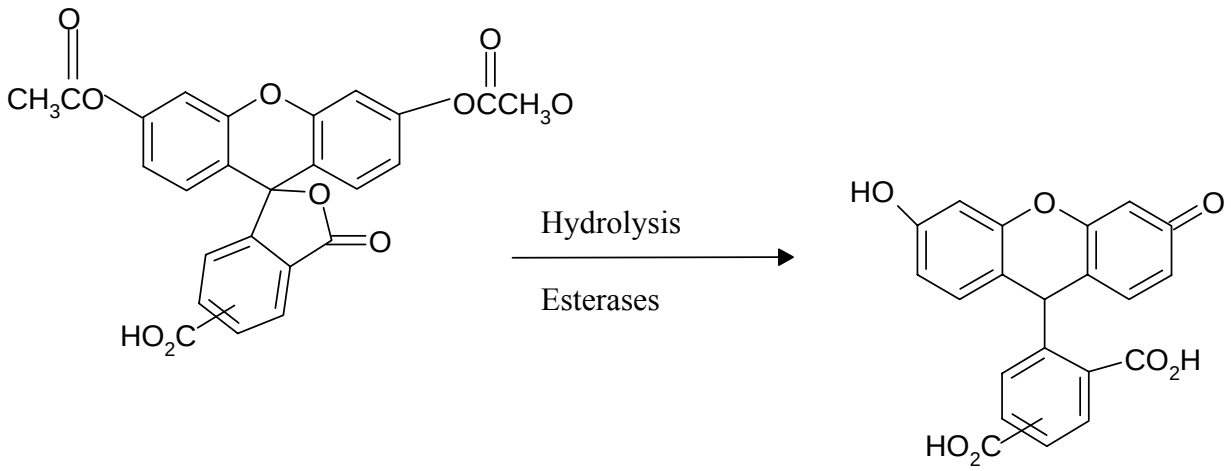
Experiments using the electron dense tracers lanthanum or uranium ions have been used to demonstrate apoplastic continuity between host and dwarf mistletoe *Korthalsella lindsayi* (Coetzee and Fineran, 1987), and between host and *Olax phyllanthi* (Kuo et al., 1989). However, such experiments require tissue fixation and sectioning. Moreover, these tracers can be very toxic to host roots (Robards and Robb, 1972).

The increasing use of fluorescence techniques over the last 10 years has been facilitated by the introduction of a range of fluorescent probes, several of which have physico-chemical properties that are ideally suited to trace movement within the phloem. The use of the relatively permeant dye, fluorescein, once a standard phloem-transport tracer (Oparka, 1991; Oparka et al., 1997), has become largely superseded by the use of the probe 5(6) carboxyfluorescein (CF), which possesses an optimal combination of dissociation constant (pKa) and oil/water partition coefficient to allow "trapping" within the sieve element (Grignon et al., 1989). A related molecule to CF, carboxyfluorescein diacetate (CFDA) is uncharged at apoplastic pH and thus membrane permeant. Once inside the cell, esterases remove the acetyl groups, releasing CF, which is membrane impermeant, get trapped in the cell (Figure 2) (Thomas et al., 1979). Thus, CFDA behaves as an ideal phloem-mobile xenobiotic.

The studies of Grignon and colleagues (1989) have demonstrated that CF remains confined to the phloem and follows a pattern of movement very similar to that of ^{14}C assimilates. Subsequent studies, utilizing the ester form of the probe (CFDA) have effectively monitored the post phloem unloading pathways in sink tissues (Wang et al., 1994; Wang and Fisher, 1994). Monitoring long-distance movement of fluorescent

probes following the loading of wounded cells in one region of the plant, and then observation at distant sites is a technique that has been utilized to monitor symplastic tracer movement following phloem unloading both in leaves and in root meristems (Oparka et al., 1994; Roberts et al., 1997), and to identify symplastic fields within these regions. This method of loading has distinct advantages in that symplastic tracers can be loaded in amounts sufficient to label a whole plant (Oparka et al., 1994; Gisel et al., 1999), and that probe movement can be assessed in tissues that are undisturbed, and only regions symplastically connected with the phloem can be easily visualized.

Dextrans are another class of compounds that have been effectively utilized to assess transport within complex organisms. They are uncharged, very hydrophilic polysaccharides (glucose homo-polymers), which do not interact significantly with intracellular components. They are non-toxic and are not degraded in cells (Thorball, 1981). Beside these properties, their broad size range renders them very valuable as tracer molecules when linked to fluorescent tags in transport studies. Texas Red-labeled dextrans have been used to trace xylem network in importing leaves (Roberts et al., 1997). In this study, texas-red dextrans of 3, 10, 40, and 70 kDa are used to track and elucidate apoplastic continuity between host and parasite.



5-(and–6)-Carboxyfluorescein diacetate

Carboxyfluorescein

Figure 2. Molecular structures of 5-(and-6)-carboxyfluorescein diacetate and carboxyfluorescein.

1.14. REFERENCES

Antonova TS (1978) Reaction of root cells of sunflower to penetration by broomrape and the downy mildew pathogen. Plant Breeding Abstracts **58:** 3944

Arslanian RL, Harris GH, StermItz FR (1990) New quinolizidine alkaloids from *Lupinus argentus* and its hosted root parasite *Castilleja sulphurea*. Stereochemistry and conformation of some naturally occurring cyclic carbinolamides. J. Org. Chem. **55:** 1204-1210

Atsatt PR (1973) Parasitic flowering plants: how did they evolve? Am. Nat. **107:** 502-510

Avdeev YJ, Shcherbinin BM (1978) Investigating tomato resistance to broomrape and the inheritance of this trait. Weed Abstracts **27:** 3074

Bevan M, Barker R, Goldsbrough A, Jarvis M, Kavanagh T, Iturriaga G (1986) The structure and transcription start site of a major potato tuber protein gene. Nucl. Acids **14:** 4625-4636

Bieleski RL (1982) Sugar alcohols. Pages 158-192 in: Encycl. Plant Physiol. New Series 13A, Plant Carbohydrates I, Intracellular Carbohydrates. F.A. Loewus and W. Tanner, eds., Springer Verlag, Berlin, Germany.

Boman J, Hultmark D (1987) Cell-free immunity in insects. Annu. Rev. Microbiol. **41:** 103-126

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K (1997) Antimicrobial peptides from plants. Crit. Rev. Plant Sci. **16:** 297-323

Chaflie M, Tu Y, Euskirchen G, Ward WW, Prashe DC (1994) Green fluorescent protein as a marker for gene expression. Science **263:** 802-805

Chappell J (1995) Biochemistry and molecular biology of the isoprenoid biosynthesic pathway in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**

Chiu WL, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineering GFP as a vital reporter in plants. Curr. Biol. **6:** 325-330

Coetzee J, Fineran BA (1987) The apoplastic continuum, nutrient absorption and plasmatubules in the dwarf mistletoe *Korthalsella lindsayi* (Viscaceae). Protoplasma **136:** 145-153

Cramer CL, Weissenborn D, Cottingham CK, Denbow CJ, Eisenback JD, Radin DN, Yu X (1993) Regulation of defense-related gene expression during plant-pathogen interactions. J. Nematol. **25:** 507-518

Cubero JI (1973) Resistance to *Orobanche crenata* Forsk. in *Vicia faba* L. Pages 205-217 in: Symp. Parasitic Weeds, Euro. Weed Res. Council, Malta.

Dörr I (1990) Sieve elements in haustoria of parasitic angiosperms. Pages 239-256 in: Sieve elements-comparative structure, induction and development. H.D. Behnke and R.D. Sjolund, eds., Berlin, Heidelberg, New York: Springer-Verlag

Dörr I (1997) How *Striga* parasitizes its hosts: a TEM and SEM study. Ann. Bot. **79:** 463-472

Dörr I, Kollmann R (1995) Symplastic sieve element continuity between *Orobanche* and its host. Bot. Act. **108:** 47-55

El Hiweris SO (1987) Nature of resistance to *Striga hermonthica* (Del.) Benth. parasitism in some *Sorghum vulgare* (Pers.) cultivars. Weed Res. **27:** 305-311

Encheva V, Shindrova P (1994) Broomrape (*Orobanche cumana* Wallr.)- a hindrance to sunflower production in Bulgaria. Pages 619-622 in: Proc. 3rd Int. Worksh. on *Orobanche* and related *Striga* Res. A.H. Pieterse, J.A.C. Verkleij and S.J. ter Borg, eds., Royal Tropical Institute, Amsterdam.

Ernst WHO (1986) Mineral nutrition of *Nicotiana tabacum* cv Bursana during infection by *Orobanche ramosa*,. Pages 80-85 in: Proc. Worksh on the Biology and Control of *Orobanche*, S.J. ter Borg, ed., LH/VPO, Wageningen, The Netherlands.

Franck A, Jonard G, Richrds K, Hirth L, Guilley H (1980) Nucleotide sequence of cauliflower mosaic virus DNA. Cell **21:** 285-294

Galbraith DW, Grebenok RJ, Lambert GM, Sheen J (1995) Flow cytometric analysis of transgene expression in higher plants: Green Fluorescent Protein. Methods Cell Biol. **50:** 3-14

Girotti M, Banting G (1996) TGN38-Green Fluorescent Protein hybrid proteins expressed in stably transfected eukaryotic cells provide a tool for the real-time, *in vivo* study of membrane traffic pathways and suggest a possible role for rat TGN38. J. Cell Sci. **109:** 2915-2926

Gisel A, Barella S, Hempel FD, Zambryski PC (1999) Temporal and spatial regulation of symplastic trafficking during development in *Arabidopsis thaliana* apices. Development **126:** 1879-1889

Goldwasser Y, Plakhin D, Kleifeld Y, Zamski E, Rubin B (2000) The Differential Susceptibility of Vetch (*Vicia* spp.) to *Orobanche aegyptiaca*: Anatomical studies. Ann. Bot. **85:** 257-262

Govier RN, Nelson MD, Pate JS (1967) Hemiparasitic nutrition in angiosperms I. The transfer of organic compounds from host to *Odontites verna* (Bell.) Dum. (Scrophulariaceae). New Phytologist **66:** 285-297

Graves JD (1995) Host-Plant responses to parasitism. Pages 206-225 in: Parasitic Plants. M.C. Press, and J.D. Graves, eds.,

Grebenok RJ, Pierson E, Lambert GM, Gong FC, Afonso CL, Haldeman-Cahill R, Carrington JC, Galbraith DW (1997b) Green Fluorescent Protein fusions for efficient characterization of nuclear targeting. Plant J. **11:** 573-586

Gressel J, Segel L, Ransom JK (1996) Managing the delay of evolution of herbicide resistance in parasitic weeds. Int. J. Pest Manag. **42:** 113-129

Grignon N, Touraine B, Durand M (1989) 6(5) carboxyfluorescein as a tracer of phloem sap translocation. Am. J. Bot. **76:** 871-877

Haseloff J (1999) GFP variants for multispectral imaging of living cells. Methods Cell Biol. **58:** 139-151

Haseloff J, Amos B (1995) GFP in plants. Trends Genet. **11:** 328-329

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Nat. Acad. Sci. **94:** 2122-2127

Heijzlar M, Paroubek M, Semidubsky V, Sanfankova M, Hajkova L, Jerabkova V, Simerkova V, Vestakova H, Machkova O, Vymola F, Jedlickva Z, Lochman O, Lonska V, Duben J, Neubauer M, Nadvornik P, Kutalek E, Bolchova S, Budal K, Udrzal J, Stehlik J (1969) Effect of carbenicillin on various biotypes of the genus *Proteus*. Int. J. Clin. Pharmacol. Ther. Toxicol. Suppl. **5:** 8

Heim R, Prasher DC, Tsien RY (1994) Wavelength mutations and post-translational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA **91:** 12501-12504

Hibberd JM, Quick WP, Press MC, Scholes JD, Jeschke WD (1999) Solutes fluxes from tobacco to the parasitic angiosperm *Orobanche cernua* and the influence of infection on host carbon and nitrogen relations. Plant, Cell and Envi. **22:** 937-947

Horn M, Banting G (1994) Okadaic acid treatment leads to a fragmentation of the TGN and an increase in expression of TGN38 at the cell surface. Biochem. J. **301:** 69-73

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11:** 309-322

Jeschke WD, Rath N, Baumel P, Czygan FC, Proksch P (1994a) Modelling the flow and partitioning of carbon and nitrogen in the holoparasite *Cuscuta reflexa* Roxb. and its host *Lupinus albus* L. I. Methods for estimating net flows. J. Exp. Bot. **45:** 791-800

Jeschke WD, Rath N, Baumel P, Czygan FC, Proksch P (1994b) Modelling the flow and partitioning of carbon and nitrogen in the holoparasite *Cuscuta reflexa* Roxb. and its host *Lupinus albus* L. II. Flows between host and parasite and within the parasitized host. J. Exp. Bot. **45:** 801-812

Joel DM, Kleifeld Y, Losner-Goshen D, Herzlinger G, Gressel J (1995) Transgenic crops against parasites. Nature **374:** 220-221

Joel DM, Losner-Goshen D (1994a) The attachment organ of the parasitic angiosperms *Orobanche cumana* and *O. aegyptiaca* and its development. Can. J. Bot. **72:** 564-574

Kanai A, Natori S (1989) Cloning of gene cluster for sarcotoxin IA, antibacterial protein of *Sarcophaga peregrina*. FEBS Lett. **258:** 199-202

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236:** 1299-1302

Koch L (1887) Die Entwicklungsgeschichte der Orobanchen mit desonderer Berucksichtgung ihrer Beziehungen zu den kulturpflanzen. Carl Winters, Heidelber, FRG.

Kuijt J (1969) The Biology of Parasitic Flowering Plants. University of California Press, Berkeley

Kunin CM (1967) A guide to use of antibiotics in patients with renal disease. Annu. Int. Med. **67:** 151-158

Kuo J, Pate JS, Davidson NJ (1989) Ultrastructure of the haustorial interface and apoplastic continiuum between host and the root hemiparasite *Olax phyllanthi* (Labbill.) R. Br. (Olacaceae). Protoplasma **150:** 27-39

Lee JY, Boman A, Chaunxin S, Andersson M, Jornvall H, Mutt V, Boman HG (1989) Antibacterial peptides from pig intestine: Isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA **86:** 9159-9162

Lewis DH (1984) Physiology and metabolism of alditols. Pages 157-179, Storage carbohydrates in vascular plants. D.H. Lewis, ed., Cambridge University Press, Cambridge, UK.

Lewis DH, Smith DC (1967) Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. New Phytologist **66:** 143-184

Losner-Goshen D, Portnoy VH, Mayer AM, Joel DM (1998) Pectolytic activity by the haustorium of the parasitic plant *Orobanche* L. (Orobanchaceae) in host roots. Ann. Bot. **81:** 319-326

Mallaburn PS, Stewart GR (1987) Haustorial function in *Striga*: Comparative anatomy of *S. asiatica* (L.) Kuntze and *S. hermonthica* (Del.) Benth. (Scrophulariaceae). Pages 523-536 in: Proc. 4th Int. Symp. on Parasitic Weeds. H.C. Weber and W. Forstreuter, eds., Philipps-Universistat, Marburg, Germany.

Mitsuhara I, Matsufuru H, Ohshima M, Kaku H, Nakajima Y, Murai N, Natori S, Ohashi Y (2000) Induced expression of sarcotoxin IA enhanced host resistance against both bacterial and fungal pathogens in transgenic tobacco. Mol. Plant-Microbe Interact. **13:** 860-868.

Mitsuhara I, Nakajima Y, Natori S, Mistuoka T, Ohashi Y (2001) In vivo growth inhibition of human intestinal bacteria by sarcotoxin IA, an insect bactericidal peptide. Biotech lett. **23:** 569-573

Musselman LJ (1980) Translocation of glyphosate in the host/parasite system *Vicia faba* and *Orobanche crenata*. Pages 226-233 in: Progress in *Orobanche* research. K. Wegmann, L.J. Musselman, eds., Eberhard-Karls-Universitat, Tubingen, FRG.

Nakajima Y, Alvarez-Bravo J, Cho J, Homma K, Kanegasaki S, Natori S (1997) Chemotherapeutic activity of synthetic antimicrobial peptides: correlation between chemotherapeutic activity and neutrophil-activating activity. FEBS Lett. **415:** 64-66

Nakajima Y, Qu X, Natori S (1987) Interaction between liposomes and sarcotoxin IA, a potent antimicrobial protein of *Sarcophaga peregrina* (flesh-fly). J. Biol. Chem. **262:** 1665-1669

Nassib AM, Hussein AHA, El Rayes FM (1984) Effect of variety, chemical control, sowing date, and tillage on *Orobanche* spp. infestation of faba bean yield. FABIS Newsletter **10:** 11-15

Nassib AM, Ibrahim AA, Khalil SA (1982) Breeding for resistance to *Orobanche*. Pages 199-206 in: Faba bean improvement. G. Hawtin, and C. Webb. Martinus Nijhoff, The Hague, The Netherlands.

Natori S (1988) Future drugs mimicking insect defense proteins *Sarcophaga* lectin and sarcotoxin I. Drugs Future **13:** 59-68

Natori S (1994) Function of antimicrobial proteins in insects. Pages 123-134 in: Antimicrobial Peptides. H. G. Boman, ed., John Wiley and Sons, New York.

Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature **313:** 810

Ohshima M, Mitsuhara I, Okamoto M, Sawano S, Nishiyama K, Kaku H, Natori S, Ohashi Y (1999) Enhanced resistance to bacterial disease of transgenic tobacco plants overexpressing sarcotoxin IA, a bactericidal peptide of insect. J. Biochem. **125:** 431-435

Okada M, Natori S (1983) Purification and characterization of an antibacterial protein from haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae. Biochem. J. **211:** 727-734

Okada M, Natori S (1985) Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh-fly) larvae. J. Biol. Chem. **260:** 7174-7177

Oparka KJ (1991) Uptake and compartmentation of fluorescent-probes by plant-cells. J. Exp. Bot. **42:** 565-579

Oparka KJ, Duckett CM, Prior DAM, Fisher DB (1994) Real-time imaging of phloem unloading in the root-tip of *Arabidopsis*. Plant J. **6:** 759-766

Oparka KJ, Roberts AG, Cruz SS, Boevink P, Prior DAM, A. S (1997) Using GFP to study virus invasion and spread in plant tissues. Nature **388:** 893-900

Parker C, Riches CR (1993) Parasitic Weeds of the world: Biology and Control. CAB Int., Wallingford, UK.

Presley J, Cole N, Schroer K, Hirschberg K, Zaal K, Lippincott-Swartz J (1997) ER-to-Golgi transport visualized in living cells. Nature **389:** 81-85

Press MC (1989) Autotrophy and heterotrophy in root hemiparasites. Trends Ecol. Evol. **4:** 258-263

Press MC, Shah N, Stewart GR (1986) The parasitic habit: trends in metabolic reductionism. Pages 96-106 in: Biology and control of *Orobanche*. S.J. ter Borg, ed., Wageningen, The Netherlands.

Racovitza A (1973) Recherches sur la sensibilité à l'attaque par *Orobanche ramosa* de diverses espèces de *Nicotiana* et de divers cutlivars de tabac. Pages 194-204 in: Proc. Int. Symp. on Parasitic Weeds. Europ. Weed Res. Council, Malta

Rakesh J, Foy CL (1997) Translocation and metabolism of glyphosate in Egyptian broomrape (*Orobanche aegyptiaca*)-infested tomato (*Lycopersicon esculentum*) plants. Plant Growth Regulator Society of America (PGRSA) Quarterly **26:** 1-7

Reichel C, Mathur J, Eckes P, Langenkemper K, Reiss B, Koncz C, Schell J, Mass C (1996) Enhanced green fluorescence by the expression of an *aeqourea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc. Natl. Acad. Sci. USA **93:** 5888-5893

Richter A, Popp M (1987) Patterns of organic acids and solutes in *Viscum album* L. on 12 different hosts. Pages 709-714 in: Parasitic Flowering Plants. Proc. 4th Int. Symp. on Weeds. H.C. Weber and W. Forstreuter, eds., Phyilipps-Universistat, Marburg. Germany.

Robards AW, Robb ME (1972) Uptake and binding of uranyl ions by barley roots. Science **78:** 980-982

Roberts AG, Cruz SS, Roberts IM, Prior DAM, Turgeon R, Oparka KJ (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: Comparison of a fluorescent solute with a fluorescent virus. Plant Cell **9:** 1381-1396

Rouwendal GJA, Mendes O, Wolbert EJT, DeBoer AD (1997) Enhanced expression in tobacco of the gene encoding green fluorescent protein by modification of its codon usage. Plant Mol. Biol. **33:** 989-999

Sauerborn J (1991) The economic importance of the phytoparasites *Orobanche* and *Striga*. Pages 137-143 in: Proc. Int. Symp. on Parasitic Weeds, 5th. J.K. Ransom, L.J. Musselman, A.D. Worsham, and C. Parker, eds., CIMMYT, Nairobi, Kenya.

Siemering KR, Golbig R, Sever R, Haseloff J (1996) Mutations that supress the thermosensitivity of green fluorescent protein. Curr. Biol. **6:** 1653-1663

Singh JN, Singh JN, Rai TB (1971) Studies on the physiology of host parasite relationship in *Orobanche*. II. Growth and mineral nutrition of host and parasite. Physiol. Plant **25:** 425-431

Solomon S (1952) Studies in the physiology and phanerogamic parasitism with special reference to *Striga lutea* Lour. and *S. densiflora* Benth. on *Andropogon sorghum* Hack. The osmotic pressure of the host and parasite in relation to the nutrition of the host. Proc. Indian Acad. Sci. **35:** 122-131

Stewart GR, Nour J, McQueen M, Shah N (1984) Aspects of the biochemistry of *Striga*. Pages 161-178 in: *Striga* Biology and Control. E.S. Ayensu, H. Dogett. R.D. Keynes, J. Marton-Lefevre, L. Musselman, C. Parker, A. Pickering, eds., ICSU Press, Paris, France.

Stewart GR, Press MC (1990) The physiology and biochemistry of parasitic angiosperms. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41:** 127-151

Sullivan KF, Kay SA (1999) Green Fluorescent Proteins. Methods in cell biology. Academic Press. San Diego, USA

Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry **18:** 2210-2218

Thorball N (1981) FITC-dextran tracers in microcirculatory and permeability studies using combined fluorescence stereo-microscopy, fluorescence light microscopy and electron microscopy. Histochemistry **71:** 209-233

Visser JH, Dörr I, Kollman R (1984) The "hyaline body" of the root parasite *Alectra orobanchoides* Benth. (Scrophulariaceae) - its anatomy, ultrastructure and histochemistry. Protoplasma **121:** 146-156

Visser JH, Dörr J (1987) The haustorium. Pages 91-106 in: Parasitic Weeds in Agriculture Vol. I. *Striga.* L. J. Musselman, ed., CRC Press, Boca Raton, Florida, USA

Wallace A, Romney EM, Alexander GV (1978) Mineral composition of *Cuscuta nevadensis* (dodder) in relation to its hosts. Plant Soil **50:** 227-231

Wang Hl, Offler CE, Patrick JW, Ugalde TD (1994) The cellular pathway of photosynthate transfer in the developing wheat grain. I. Delineation of a potential transfer pathway using fluorescent dyes. Plant, Cell and Envi. **17:** 257-267

Wang N, Fisher DB (1994) The use of fluorescent tracers to characterize the post-phloem transport pathway in maternal tissues of developing wheat grains. Plant Physiol. **104:** 17-27

Weber HC (1980) Zur Evolution des Parasitismus bei den Scrophulariaceae und Orobanchaceae. Pl. Syst. Evol. **136:** 217-232

Wegmann K, Harloff HJ, von Elert E, Bergmann C (1989) Biochemistry of *Orobanche*-host systems. Pages 619-624 in: Proc. Interg. Pest Manag in Tropical and Subtropical Cropping Systems Vol. II, Bad Dürkheim, FRG.

Westwood JH, Yu X, Foy CL, Cramer CL (1998) Expression of a defense-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. Mol. Plant-Microbe Interact. **11:** 530-536

Wolswinkel P (1974) Complete inhibition of setting and growth of fruits of *Vicia faba* L. resulting from draining of the phloem system by *Cuscuta* species. Act. Bot. Neerlandica **23:** 48-60

Wolswinkel P (1978a) Phloem unloading in stem parts parasitized by *Cuscuta*: the release of ^{14}C and K^+ to the free space at 0^oC and 25^oC. Physiol. Plant **42:** 167-172

Wolswinkel P (1978b) Accumulation of phloem mobile mineral elements at the site of attachment of *Cuscuta europaea* L. Zeitschrift fur Pflanzenphysiologie **86:** 77-84

Wolswinkel P, Ammerlaan A (1983) Sucrose and hexose release by excised stem segments of *Vicia faba* L. The sucrose specific stimulating influence of *Cuscuta* on sugar release and the activity of acid invertase. J. Exp. Bot. **34:** 1516-1527

Zhao C, Liaw L, Lee IH, Lehrer IH (1997) cDNA cloning of three cercopin-like antimicrobial peptides (Styelins) from the tunicate, *Styela clava*. FEBS Lett. **412:** 144-148

Chapter II

Use of Green Fluorescent Protein and Fluorescent Tracers to Study Macromolecule Movement Across the Host/*Orobanche* Interface.

II.1 INTRODUCTION

Parasitic plants form connections with their hosts in order to gain access to host water and solutes. However, the physiological mechanisms and limitations of these connections are not well understood. The extent of this contact varies among parasitic species and ranges from adjacent xylem vessels, as in the association of the parasite *Olax phyllanthi* with its host (Pate et al., 1990), to direct lumenal contact between xylem vessels as in the association of *Striga* and its host (Dörr, 1997). Variation is even more pronounced with respect to phloem. *Striga asiatica* possesses no phloem links to its host, although phloem-like cells were reported in the haustorium (Rogers and Nelson, 1962). In contrast, *S. gesnerioides* parasitizing *Pisum sativum* appears to develop interspecific plasmodesmata (Dörr, 1996). For *Orobanche crenata*, it has been proposed that interspecific plasmodesmata develop into sieve pores between adjacent sieve elements of host and parasite (Dörr and Kollmann, 1995). Transfer cells linking the phloem of host and parasite have been reported in both *Cuscuta* and *Orobanche*, and in some cases these appear to be associated with interspecific pores between host and parasite (Dörr, 1996). Although, *Orobanche* uptake of small molecules such as sugar and herbicides has been documented (Aber et al., 1983; Muller and Distler, 1989), the movement of macromolecules between host and parasite has not been characterized.

Gene markers and tissue-specific promoters have recently made it possible to reevaluate the connections between hosts and parasites. For instance, symplastic continuity between *Cuscuta* and its hosts has been documented using transgenic tobacco plants expressing GFP under the control of a companion cell-specific promoter (Haupt et al., 2001). GFP moves in the translocation stream of the host and is transferred to the

Cuscuta phloem via the absorbing hyphae of the parasite. Moreover, the pattern of GFP transfer was identical to the movement of the low molecular-weight phloem specific probe carboxyfluorescein, indicating that *Cuscuta* takes up both solutes and macromolecules through the symplastic pathway. However *Cuscuta* has very different haustorial anatomy than *Orobanche*.

In this study, we investigated movement of macromolecules from a tobacco host to *O. aegyptiaca.* GFP was produced in host tissue and targeted to either the extracellular space using a construct containing the patatin signal peptide, or retained within the host cell cytosol. Thus we were able to study GFP uptake via xylem and phloem, respectively. We also used fluorescent probes representing a range of molecular sizes to further explore the extent of vascular continuity between host and *Orobanche*.

This research contributes to our understanding of host-*Orobanche* interactions in general, and also provides insight that will be valuable in optimizing delivery and targeting of protein toxins to the parasite as discussed in Chapter III.

II.2 MATERIALS AND METHODS

II.2.1 Analysis of protein movement using green fluorescent protein

II.2.1.1 *HMG2:GFP* construct

The GFP used in this study has been modified for cryptic intron splicing, mutations V163A and S175G in the gene enhances folding of GFP protein (Siemering et al., 1996), while mutation I167T changes the UV and blue light maxima to equal amplitudes (Heim et al., 1994). The excitation and emission of this GFP variant are 400/496 nm, and 512 nm, respectively. The gene encoding GFP variant was provided by

Dr. Carole Cramer (originally provided by Dr. Jim Haseloff) in a plasmid vector. The *HMG2* promoter was obtained from Crop Tech Corporation (Blacksburg, VA) in a plasmid vector designated pCT151. PCR amplification of *GFP* was performed to generate flanking restriction sites for the enzymes *Xba*I (5'underlined) and *Sst*I (3'underlined) using the following primers:

(a) 5'-CGTCTCTAGAATGAGTAAAGGAGAAG-3'

and (b) 5'-TGCGAGCTCTCATTTGTATAGTTCATCCAT-3'.

PCR was conducted using a PTC-100^{TM} Programmable Thermal Controller (MJ Research, Inc., Watertown, MA. U.S.A). Reactions were run for 35 cycles and consisted of the following sequence: 94^{o}C for 2 min, 60^{o}C for 1 min, and 72^{o}C for 1 min. The cycles were preceeded by a 94^{o}C denaturation period for 4 min and followed by 72^{o}C final extension period for 7 min. A PCR product of 0.7 kb was digested with *Xba*I and *Sst*I and gel purified.

The pCT151 plasmid containing the *HMG2* promoter was digested with *Hind*III and *Xba*I to isolate the promoter. The resulting 0.4 kb fragment and the previously obtained *GFP* fragment (Figure 3) were subcloned into *Hind*III-*Sst*I digested pBC plasmid. Clones with the expected 1.1 kb insert were selected using *Hind*III-*Sst*I restriction analysis and the identity and fidelity of the gene constructed was confirmed by sequencing (Virginia Bioinformatics Institute / DNA sequencing facility, Blacksburg, VA).

II.2.1.2 *HMG2:PSP:GFP* construct

The signal peptide of the potato patatin gene (*PSP*) was provided by Dr. Medina-Bolivar (Virginia Tech) and was introduced at the 5'end of the *GFP* open reading frame.

The pCT151 plasmid containing the *HMG*2 promoter was digested with *Xba*I. The cut ends were filled in using the Klenow polymerase, and then digested with *Hind*III and gel purified.

Similarly, the pBC plasmid harboring the *PSP:GFP* insert was digested first with *Kpn*I, filled in to obtain blunt end, and then digested with *Sst*I. The resulting *PSP:GFP* 0.77 Kb blunt/*Sst*I fragment was gel purified and ligated to the *HMG*2 promoter in a pBC plasmid (Figure 3). The insertion of *PSP:GFP* in-frame with the *HMG*2 promoter was confirmed by sequencing.

In preparation for plant transformation, gene constructs (Figure 3) were subcloned into the *Agrobacterium tumefaciens* vector $pBIB_{hyg}$ (Becker, 1990). This vector contains the appropriate border sequence to aid in the transfer of T-DNA into the plant genome and hygromycin-resistance selectable marker to allow selection of generating transgenic plants on hygromycin-containing selective medium. $pBIB_{hyg}$ vectors containing GFP constructs were subsequently introduced into *A. tumefaciens* strain LBA4404 by electroporation.

II.2.1.3. *de35S:PSP:GFP* construct

Seed from transgenic tobacco previously transformed with a *de35S:PSP:GFP* construct (Figure 3) were provided by Medina-Bolivar (Virginia Tech, Blacksburg, VA). In these plants, the *GFP* has been shown to be constitutively expressed and GFP protein secreted to the extracellular space (Medina-Bolivar and Cramer, 2004).

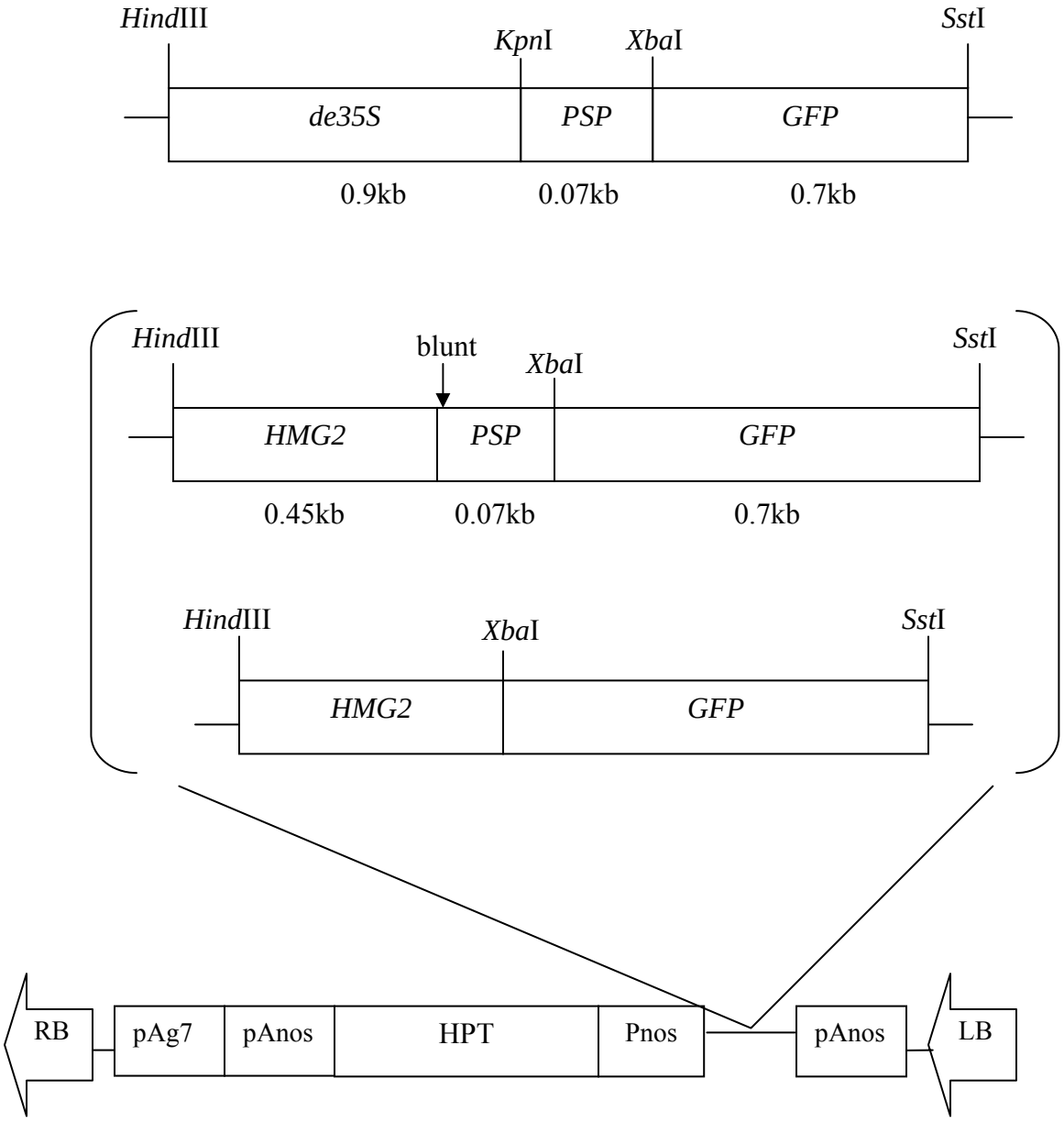


Figure 3. Diagrams of gene constructs used in this study and a map of the T-DNA from *Agrobacterium tumefaciens* plant transformation vector pBIB-hyg. *HMG2* is the promoter from *HMGR*; PSP is the patatin signal peptide; *GFP* encodes green fluorescent protein. The *de35S:PSP:GFP* was generated by F. Medina-Bolivar. The vector contained a hygromycin resistance gene (*HPT*), plant promoter (pAg7, Pnos), plant terminators (pAnos), and the T-DNA border sequences (LB, RB) that define the region to be transferred into the plant genome.

II.2.1.4. Plant transformation

Gene constructs were introduced into tobacco plants (*Nicotiana tabacum* var. Xanthi nc) via *Agrobacterium*-mediated transformation using the petiole method (Medina-Bolivar et al., 2003). A colony of *A. tumefaciens* containing the desired vector was picked with a scalpel and this blade was used to cut the lower part of petiole of aseptically grown tobacco plants. The severed leaves were put on MS medium (Murashige and Skoog, 1962), containing MS salts (GibcoBRL, Rockville, MA), MS vitamins (Sigma, St Louis, MO), 3% sucrose, and 0.4 g/L $MgSO_4.7H_2O$) for 2-3 days, and then transferred to similar MS media containing 0.1 mg/L NAA (α-naphthalene acetic acid), 1mg/L BAP (6-benzyl amino purine), 500 mg/L carbenicillin, and 100mg/L hygromycin for selection and induction of new shoots. After three weeks, plantlets were excised at the stem and transferred to rooting medium (MS medium containing carbenicillin and hygromycin).

II.2.1.5. DNA extraction and PCR

To identify putatively transformed tobacco plants, total DNA was extracted from plant leaves of putative transgenic and non-transformed plants as described by Edward et al. (1991). PCR was performed using the GFP primers and PCR protocol described above, or primers specific for the hygromycin selectable marker. Amplification of the hygromycin gene was performed using the following primer pair: HPT-5': (ATGAAAAAGCCTGAACTCACC) and HPT-3': (CTATTCCTTTGCCCTCGGAC). Samples were run for 30 cycles in the following sequence: 94^{o}C for 2 min, 52^{o}C for 1 min, and 72^{o}C for 1 min. The cycles were preceeded by a 94^{o}C denaturation period for 4 min and followed by 72^{o}C final extension period for 7 min.

II.2.1.6. RNA extraction and RT-PCR to confirm GFP gene expression

To induce the *HMG2* promoter, fully expanded leaves from plants containing the *GFP* gene constructs were wounded by passage through a pasta-maker. Leaf strips (2 mm wide) were incubated in a petri dish at room temperature for 0 and 6 hrs. Total RNA was extracted from the wounded leaves with RNeasy Mini kit (QIAgen, Valencia, CA) according to the manufacturer's directions. RNA samples were treated with DNase (DNA free, Ambion, Austin, TX, U.S.A), and 1 μg of the RNA was reverse-transcribed with the GFP-specific primer as described previously using Superscript^{TM} Rnase H^{-} Reverse Transcriptase (Invitrogen Corp. Carlsbad, CA, U.S.A), according to the manufacturer's instructions. The resulting cDNA was used as a template in PCR reactions to amplify the GFP gene. The primers and PCR conditions used were as described above.

II.2.1.7. Protein extraction and western blot to confirm presence of GFP

To confirm the presence of GFP protein, fully expanded tobacco leaves (1 g) were wounded by passage through a pasta-maker**,** producing uniform leaf strips approximately 2 mm in width, and incubated at room temperature for 48 hrs. Samples were homogenized with 4 ml of extraction buffer (0.1 M K-phosphate buffer, pH 7.0, 5 mM dithiothreitol and protease inhibitor cocktail Complete^{TM} Mini (Roche Diagnostics, Mannheim, Germany). The extract was clarified by centrifugation at 10,000 rpm for 15 min. The supernatant was filtered through Miracloth (EMD Bioscience, Inc. Darmstadt, Germany) and concentrated using a Centricon YM-10 column (Millipore, Bedford, MA, U.S.A). Protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard and measured at OD_{595} with a spectrophotometer (BIO-RAD SmartSpec^{TM}3000).

The protein immuno-blotting protocol was as described by Sambrook et al. (1989). Samples were boiled in SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS. 10% glycerol, 5% ß-mercaptoethanol (v/v), 0.025% bromophenol blue] for 5 min, and resolved (30 µg of total protein per lane) by 12% SDS-polyacrylaminde gel electrophoresis (Mighty Small II, Hoefer, San Francisco, CA) for 1.5 hr (100 V at pH 8.3). The BenchMark Prestained Protein Ladder (Invitrogen) was used as molecular standards for protein size determination. Separated proteins were transferred to a 0.2 µm nitrocellulose membrane (Trans-Blot Transfer Medium, BioRad) overnight [25 V, 4^{o}C) in transfer buffer [25 mM Tris, 192 mM glycine, and 20 % (v / v) methanol (pH 8.3)] using a BioRad mini-blot apparatus. The membrane was blocked with 3 % BSA in PBST (80 mM Na_2HPO4, 100 mM NaCl, 0.3% Tween, pH 7.5) for 1 hr at room temperature and then incubated in primary antibody (rabbit anti-GFP Living Color^{TM} polyclonal antibody, diluted 1:100) (Clontech, Palo Alto, CA) in 3 % BSA in PBST for 1 hr, and washed three times 15 min in PBST. The membrane was then incubated in secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugated (diluted 1: 3000 in PBST, Promega, Madison, WI). CDP-star^{TM} (Boehringer Mannhein, Indianapolis, IN) was used as a chemiluminescent substrate for alkaline phosphatase. The membrane was incubated two times for 5 min in detection buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) and then incubated for 5 min in 4 ml CDP-Star^{TM} solution (1:100 dilution in detection buffer) containing 200 µl Nitroblock enhancer II (Tropix, Bedford).

II.2.1.8. *Orobanche aegyptiaca* inoculation

Transformed host plants were germinated and grown initially on selective medium (50 mg/L hygromycin, 500 mg/L carbenicillin). Surviving plantlets were

transferred to polyethylene bags containing a moist glass fiber filter paper (GFFP) sheet, such that their roots are in contact with the GFFP, while their shoots project from the top of the bag (Figure 4). The bags hold a reservoir of half-strength nutrient solution (Hoagland and Arnon, 1950) and were suspended in boxes to exclude light from the root systems.

Surface-sterilized *Orobanche* seeds were brushed gently onto the host roots. After seven days of preconditioning, 10 ml of a 2 mg/L solution of GR-24 (a synthetic strigolactone analogue seed germination stimulant (Jackson and Parker, 1991) was added to each bag in order to synchronize germination of the *Orobanche* seeds.

II.2.1.9. Fluorescence Microscopy

GFP fluorescence was monitored using either a fluorescence microscope (Olympus, Dulles, Virginia) equipped with a filter set (Excitation 465/30x, Emission 530/50m), or a fluorescence phase microscope (Zeiss Axioscope; Carl Zeiss, Jena, Germany) equipped with a filter set (Excitation 480/40x, Emission 535/50m) suitable for the detection of green fluorescence protein.

II.2.2. Analysis of macromolecules movement using fluorescent dyes

II.2.2.1. Plant material

Seeds of tobacco (*N. tabacum* cv. Xanthi) were grown in soil. Small plantlets were transferred to GFFP and inoculated with *Orobanche* seeds as described above. *Orobanche* seeds were allowed to germinate and attach to tobacco roots. After *Orobanche* tubercles had developed, host plants were subjected to tracer introduction into the xylem or phloem system

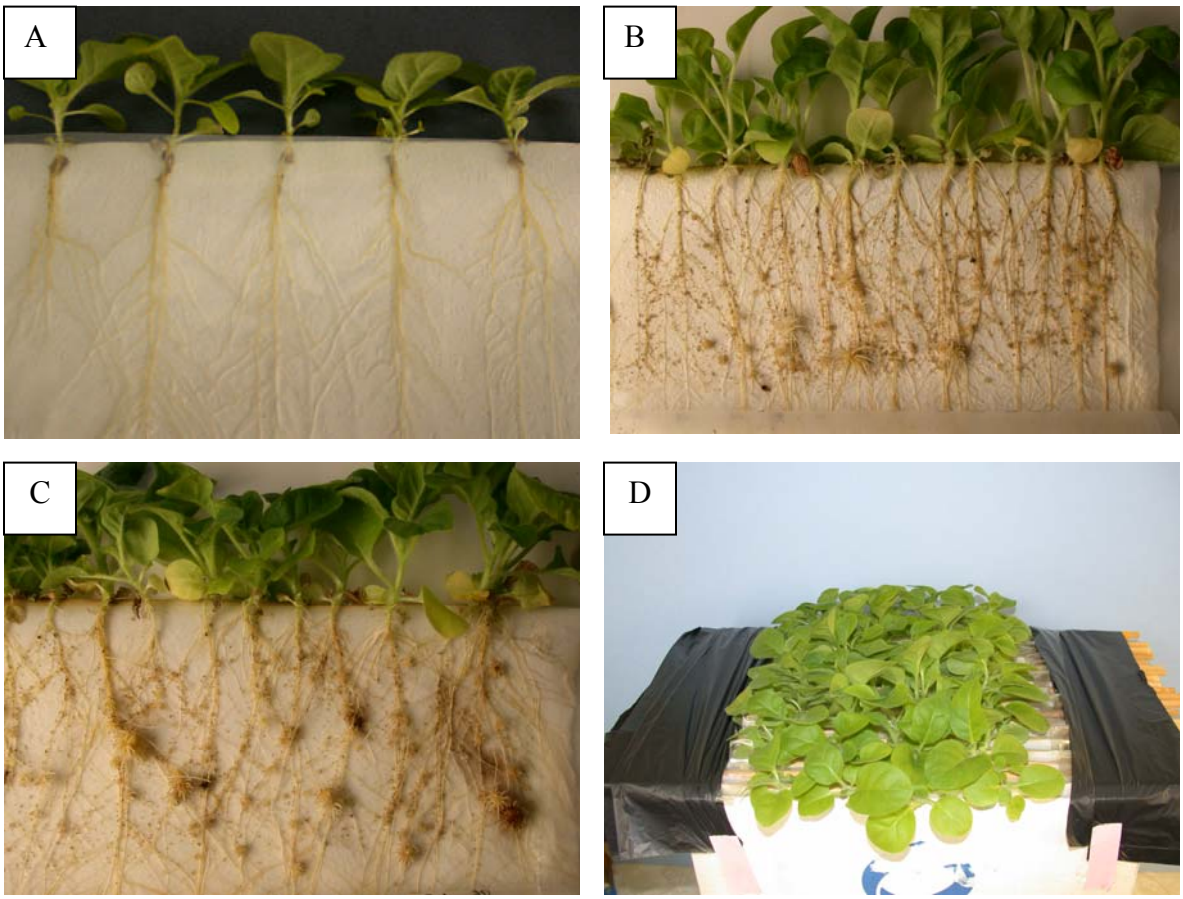


Figure 4. Polyethylene bag growth system used to monitor *Orobanche* growth on tobacco roots. A, tobacco plantlets growing on glass fiber filter paper sheet prior to inoculation with *Orobanche* seeds; B and C, *Orobanche* tubercles growing on tobacco roots; D, bags suspended in a box to exclude light from root systems.

II.2.2.2. Xylem transport

Texas Red-labeled dextrans of various sizes (Mr 3, 10, 40, and 70 kDa) were obtained from Molecular Probes Inc., (Eugene, OR, USA). These dye were purified using a sizing column with a 3 kDa cut-off (Amicon, YM-3 Microcon filters, Bedford, MA, USA), to remove low molecular mass contaminant that may be present in commercially prepared Texas-red dextrans. Dyes were applied to host root xylem by severing the roots of intact tobacco plants (3-4 cm below the tubercle), and placing them in a solution containing 1 mg/ml of Texas-red dextrans (Figure 5). Plants were loaded for 10 to 30 min, after which translocation of the dye was confirmed by observing tobacco leaves. When the dye reached the host leaf, *Orobanche* tubercles with the host root were visualized under Zeiss Axioscope equipped with a filter set (Excitation 545/30x, Emission 610/75m) suitable for the detection of Texas red dye.

II.2.2.3. Phloem transport

To trace movement through the phloem, two to three exporting leaves of tobacco were cut at the base of the blade. A section of gel loading pipette tip approximately 1.5 cm long was filled with 15 µl of 5(6) carboxyfluorescein diacetate (Biotium, Hayward, CA, USA) at a concentration of 60 µg/ml in distilled and was inserted into cut petioles (Figure 5). The tracer was allowed to move through the host plant overnight. Dye accumulation in *Orobanche* was monitored under Zeiss Axioscope equipped with a filter set (Excitation 480/40x, Emission 535/50m) suitable for the detection of carboxyfluorescein dye.

II.2.3. Anatomy of *O. aegyptiaca* tubercles

Pieces of host root of 3–5 mm long with attached *Orobanche* tubercles were fixed and embedded according to Vaughn (2003) with some modifications. Tissues were fixed in 3% (v/v) glutaraldehyde in 0.05 M PIPES buffer (pH 7.4) in 5 ml vials at 4^{o}C for 4 hrs. The samples were washed in two exchanges of PIPES buffer, 30 min each, and dehydrated in an ethanol series at 4^{o}C. After two exchanges in absolute ethanol at 4^{o}C, the samples were transferred to a –20^{o}C freezer and embedded in L.R. White resin, by increasing the concentration of resine by 25% increments each day. The samples were left in 100% resin for 2 days at –20^{o}C, allowed to warm at room temperature, and then placed on a rocking shaker for 24 hrs. The segments were then transferred to BEEM capsules containing resin. The capsules were sealed and placed at 58^{o}C overnight to affect polymerization.

Blocks containing the specimens were cut into 0.3 µm sections using a historange microtome (Ted Pella Inc. Redding, CA. U.S.A) equipped with a glass knife. Sections were placed in a drop of water on glass slides, and dried on a 60^{o}C hot plate. The sections were stained as follows; 1% methylene blue for 30 s, 1% sodium azide II for 30 s, 0.005% sodium borate for 30 s, and then with 0.5% basic fuschin. Slides were dried on a 60^{o}C hot plate, overlaid with a covering glass, and then examined under Zeiss Axioscope (Carl Zeiss, Jena, Germany).

II.2.4. Histochemical analysis of lignifications in *O. aegyptiaca*

To stain xylem tissue in the host root and parasite, *Orobanche* tubercles attached to tobacco roots were hand sectioned and treated with 1 % (w/v) phloroglucinol-hydrochloric acid solution for 1-2 min at room temperature, and then washed with water.

This solution stains lignified cells red upon reaction with hydroxy-cinnamaldehyde groups present in the polymer (Clifford, 1974). Sections were placed on a glass slide and immediately examined under Olympus dissecting scope (OPELCO, Dulles, VA, USA).

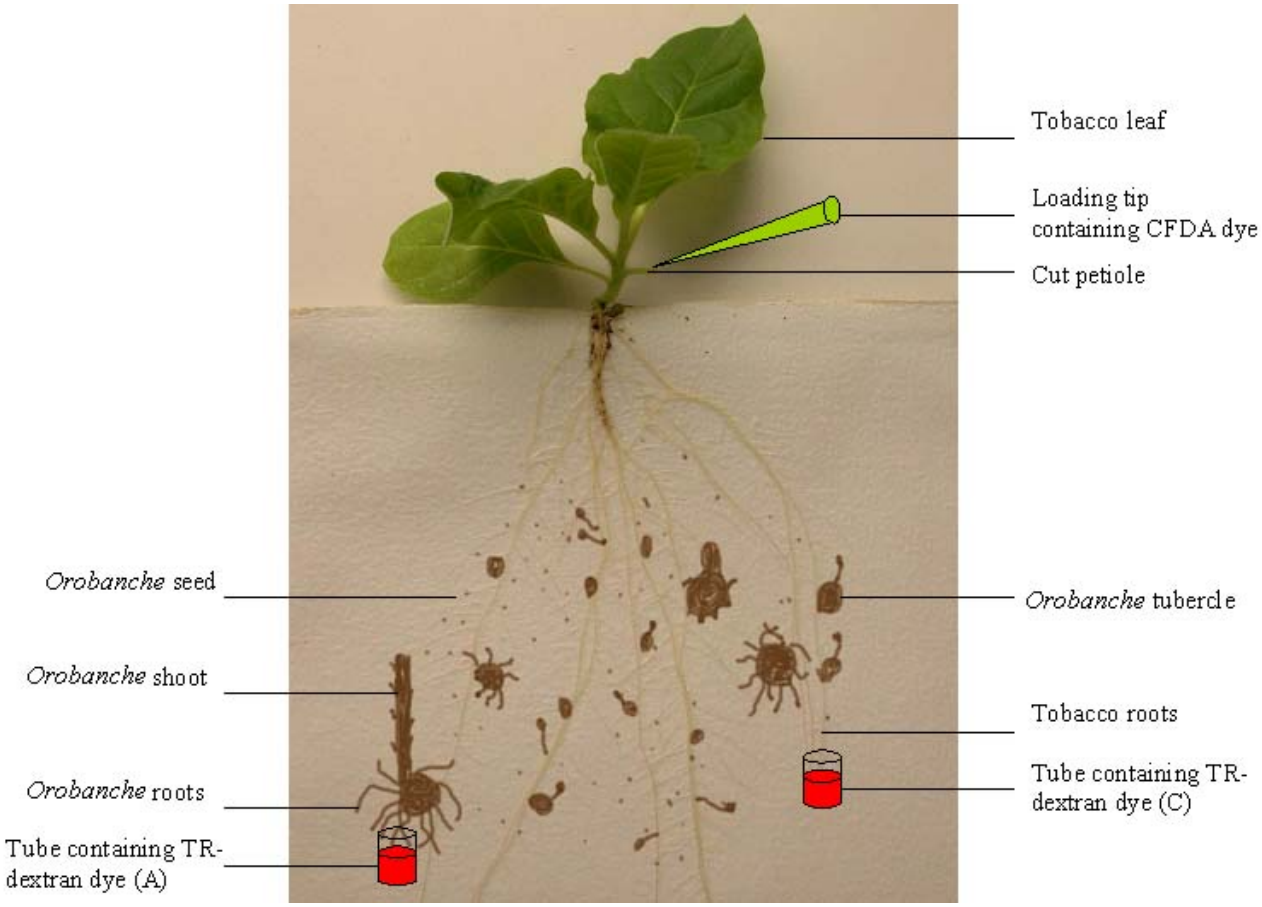


Figure 5. Diagram illustrating loading of fluorescent tracers into tobacco and *Orobanche* plants. Each of the following sites was used for dye loading in individual experiment. A, Tobacco roots were cut and inserted into a tube containing Texas-Red (TR) labeled dextrans; B, CFDA loaded into tobacco petiole; C, *Orobanche* root was cut and inserted into TR-labeled dextrans.

II.3. RESULTS

II.3.1. Expression of GFP in transgenic tobacco plants

Experiments used transgenic tobacco plants expressing GFP either with or without a transit peptide to target the protein to the cytosol or apoplast of the host. For the cytosolic-targeted protein, the GFP gene was fused to the *HMG2* promoter and transformed into *Arabidopsis* and tobacco plants via *A. tumefaciens*. Putatively transgenic lines were selected on hygromycin-containing medium at 50 mg/L. Table 2 summarizes the number of transgenic plants generated for each construct. The presence of the *GFP* gene in tobacco was supported by PCR amplification of the *GFP* gene from genomic DNA isolated from plants transformed with the *HMG2*:*GFP* construct. The expected 700 bp DNA fragment was present in all transgenic plants tested, whereas no band was present in the control (Figure 6). To address the possibility that residual *Agrobacterium* remaining in the transformed plant tissue could cause a false positive, a primer pair was designed for the kanamycin resistance gene (*npt*II), which lies outside of the T-DNA, and thus provides an *Agrobacetrium*-specific, but not T-DNA-specific marker. These primers did not amplify the *npt*II gene from those transformed plants where *HPT* primers resulted in a positive signal (data not shown), suggesting that bands amplified with *HPT* primers derived from a tobacco genome-integrated copy of *HPT*.

In contrast to the *HMG2:GFP* construct, we were unable to amplify the *GFP* gene from tobacco plants transformed with the *HMG2:PSP:GFP* construct (data not shown). Because those plants were regenerated on hygromycin-selective medium at 50 mg/L, we used PCR to check for the presence of *HPT* gene using specific primers. The expected 1 kb fragment was successfully amplified from all transgenic lines (Figure 7). To

compensate for the lack of tobacco plants with this construct, we obtained tobacco plants previously transformed with a *de35S:PSP:GFP* construct. In these plants, the gene construct is constitutively expressed and the GFP protein has been shown to be secreted to the extracellular space (Medina-Bolivar and Cramer, 2004). Because our main question is whether, and by what route, proteins move from host to parasite, the use of a different promoter should not affect the outcome of the experiment.

Expression of GFP gene was tested using RT-PCR. To induce GFP expression as regulated by the wound-inducible *HMG2* promoter, fully expanded leaves were wounded by passage through a pasta maker and incubated at room temperature for 6 hours. Total RNA was analyzed by RT-PCR using GFP-specific primers, and results showed GFP expression in three transgenic lines (L08, L18, and L24) (Figure 8). No GFP message was detected in untransformed plants.

Transgenic plants were further tested at the protein level to confirm the presence of GFP protein. Protein immunobloting was used for this because tobacco roots have high levels of auto-fluorescence that obscures detection of GFP under fluorescence microscopy. To induce production of GFP protein, fully expanded leaves were wounded by passage through a pasta maker and incubated at room temperature for 48 hours. Proteins were analyzed by SDS-PAGE and immunobloting using anti-GFP antibodies. Protein extracts from untransformed plants were used as negative controls. Figure 9 shows that anti-GFP antibodies detected a band at the expected 27 kDa size for GFP in transgenic tobacco plants wounded for 48 hours. No protein was detected in non-transformed plants on in transformed plants wounded for 0 hours.

Table 2. Summary of *GFP* constructs and transgenic tobacco plants generated.

Construct	Rational	Transgenic tobacco plants
HMG2:GFP	Cytsolic GFP (without signal peptide) for monitoring protein movement via symplastic connections	15 lines
HMG2:PSP:GFP	*PSP* (patatin signal peptide) targets GFP to extracellular space to reveal protein movement via apoplastic connections	0 lines*

* 14 lines were transformed and showed hygromycin resistance, but all lacked the *GFP* element of the construct. Transgenic plants with the constitutive cauliflower mosaic virus 35S promoter fused to *PSP:GFP* have been used instead.

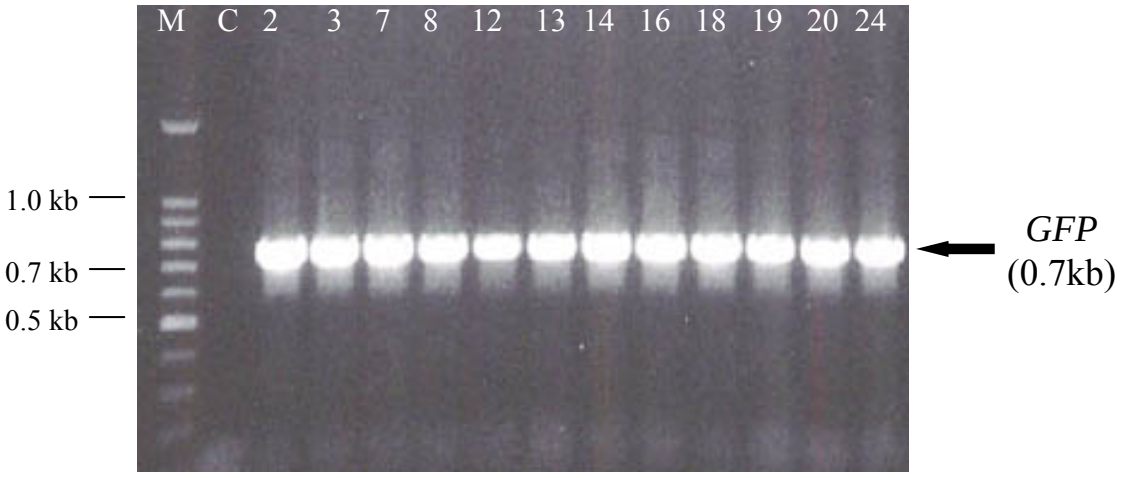


Figure 6. Confirmation of the presence of *GFP* in putatively transformed tobacco lines. Ethidium bromide-stained agarose gel showing products of PCR amplification from *HMG2:GFP* tobacco plants using GFP-specific primers. M, 100 bp DNA ladder; C, non-transformed tobacco plant. Remaining lines designate putatively transformed tobacco lines.

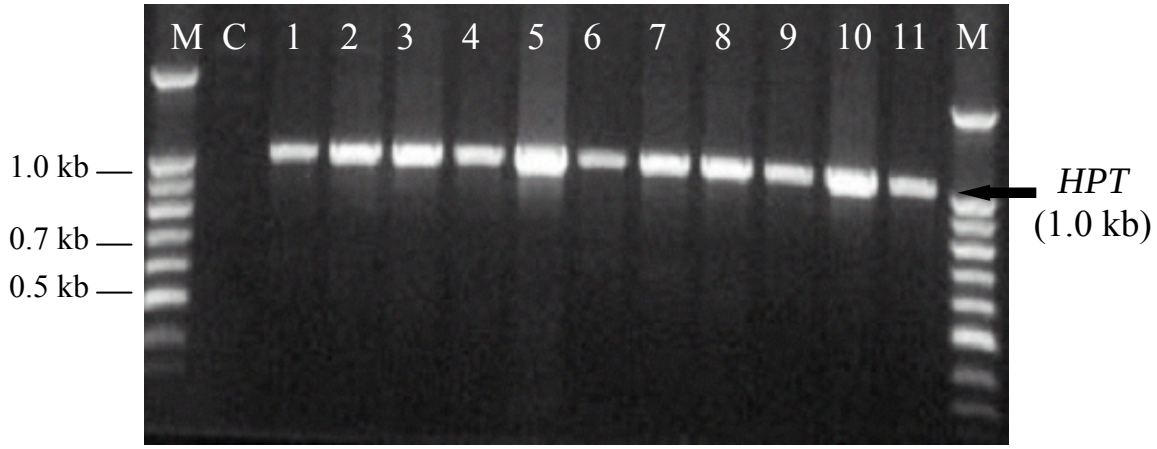


Figure 7. Confirmation of the presence of the hygromycin resistance gene (*HPT)* in putatively transformed tobacco lines. Ethidium bromide-stained agarose gel showing products of *HPT* amplification from *HMG2:PSP:GFP* tobacco plants using *HPT*-specific primers. M, 100 bp DNA ladder; C, non-transformed tobacco plant. Remaining lines designate putatively transformed tobacco lines.

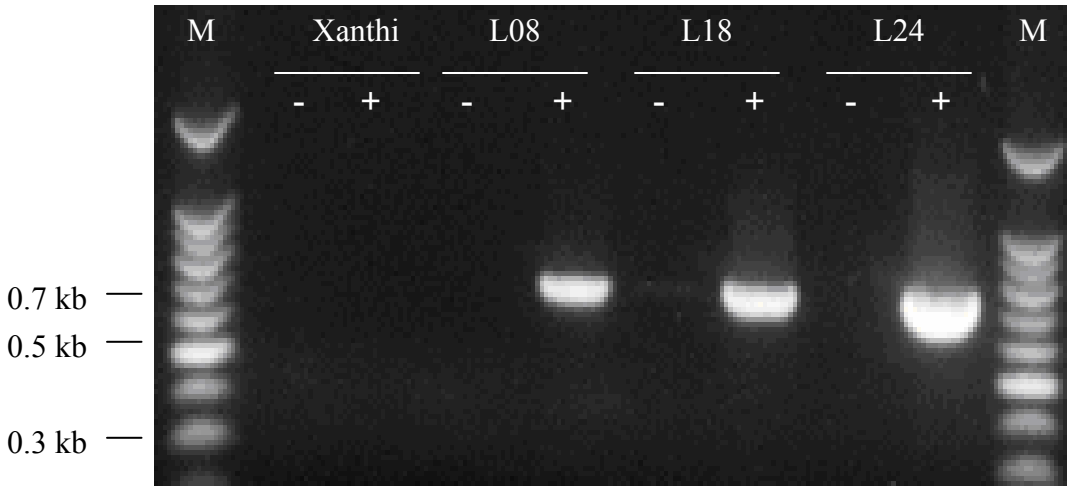


Figure 8. Wound-inducible expression of GFP in leaves of transformed tobacco lines. RT-PCR was conducted using GFP-specific primers and total RNA from transgenic tobacco lines L08, L18, and L24. All plants were wounded for 6 hrs to induce the *HMG2* promoter. M, 100 bp DNA ladder; Xanthi, non-transgenic tobacco plants; (-), PCR reaction without reverse-transcriptase; (+), PCR reaction with reverse-transcriptase.

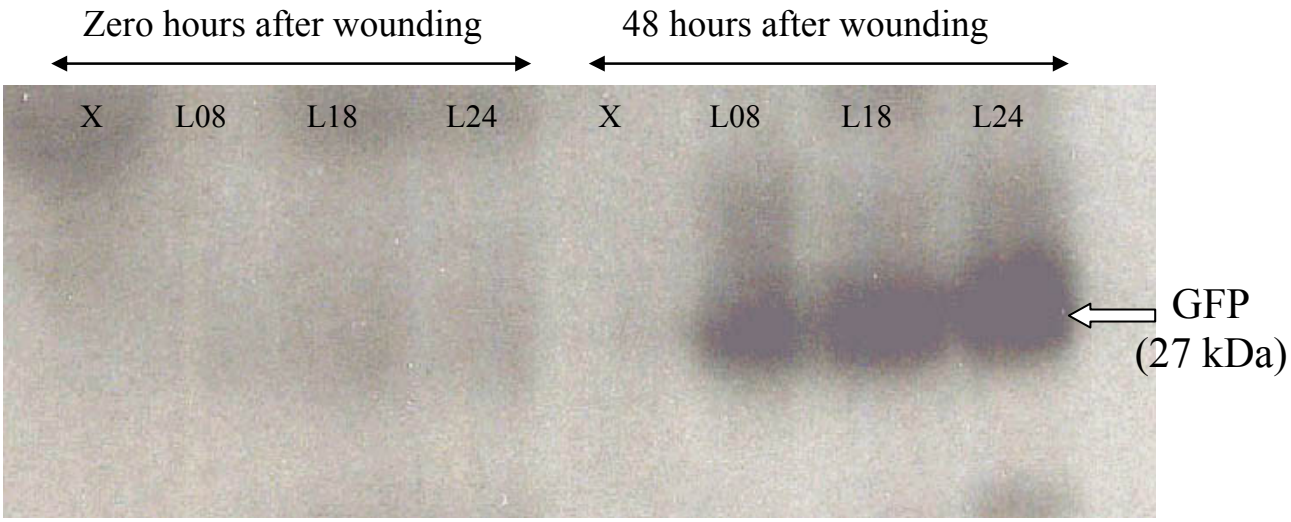


Figure 9. Wound-inducible expression of GFP in transformed tobacco. Immunoblot analysis of tobacco lines L08, L18, and L24 containing the *HMG2*:*GFP* gene. Non-transformed Xanthi (X) protein was used as a control. Total soluble protein extracted from leaves at 0 hours and 48 hours after wound induction was denatured and separated on 12% SDS-PAGE gels. Thirty micrograms of total proteins were loaded for each sample. GFP protein was detected using rabbit anti-GFP Living Color^{TM} (Clontech, CA) antibodies at 1:100 dilution. Commercial goat anti-rabbit secondary antibodies IgG were used at 1:3000 dilution.

To determine if GFP is also produced in tobacco roots parasitized by *Orobanche*, immunoblot analysis was performed on total proteins extracted from transgenic tobacco roots parasitized by *Orobanche* tubercles. Protein from *HMG2:GFP* tobacco roots grown in the absence of *Orobanche* tubercles was used as a negative control. The anti-GFP antibodies detected a 27 kDa protein in transgenic tobacco roots infested with *O. aegyptiaca* (Figure 10). A faint band, of the size of GFP, was observed in the non-parasitized plants. This could be a product of cross-reactivity of anti-GFP antibodies. Such cross-reactivity was not observed when using protein extract from tobacco leaves.

II.3.2. Movement of GFP from tobacco to *Orobanche*

Transgenic tobacco plants expressing GFP were parasitized with *O. aegyptiaca* in order to assess whether tobacco-synthesized GFP was taken up by the parasite. Visualization of GFP fluorescence at the site of the parasite attachment was obscured by high levels of auto-fluorescence in tobacco roots (See Figure 14 B). Furthermore, the *Orobanche* tubercle is opaque and GFP concentrations were not high enough to see by external observation. To overcome this limitation, we examined GFP fluorescence inside *Orobanche* tubercles, which do not present high auto-fluorescence. Tubercles were carefully detached from host roots, squashed between two microscope slides and immediately visualized with a fluorescence microscope. Figure 11 shows green fluorescence in tubercles growing on both *HMG2:GFP* and *de35S:PSP:GFP* hosts, but no similar fluorescence was seen in *Orobanche* tubercles attached to non-transformed tobacco plants. To achieve better resolution of GFP, tubercles were cut by hand with a razor blade. Sections were placed on a microscope slide and visualized with

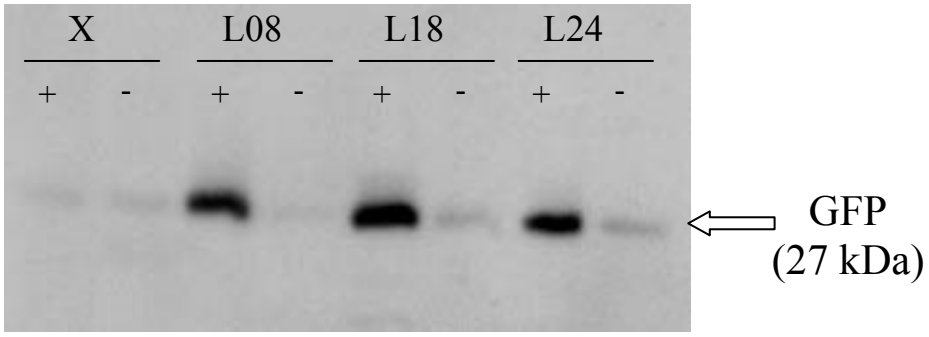


Figure 10. Immunoblot analysis of three tobacco lines containing the *HMG2:GFP* gene showing expression of GFP in response to *O. aegyptiaca* parasitism. Total protein from non-transformed Xanthi (X) parasitized with *Orobanche* was used as a negative control. Twenty micrograms total soluble protein extracted from tobacco roots either non-parasitized (-) or parasitized with *Orobanche* tubercles (+) was loaded in each lane.

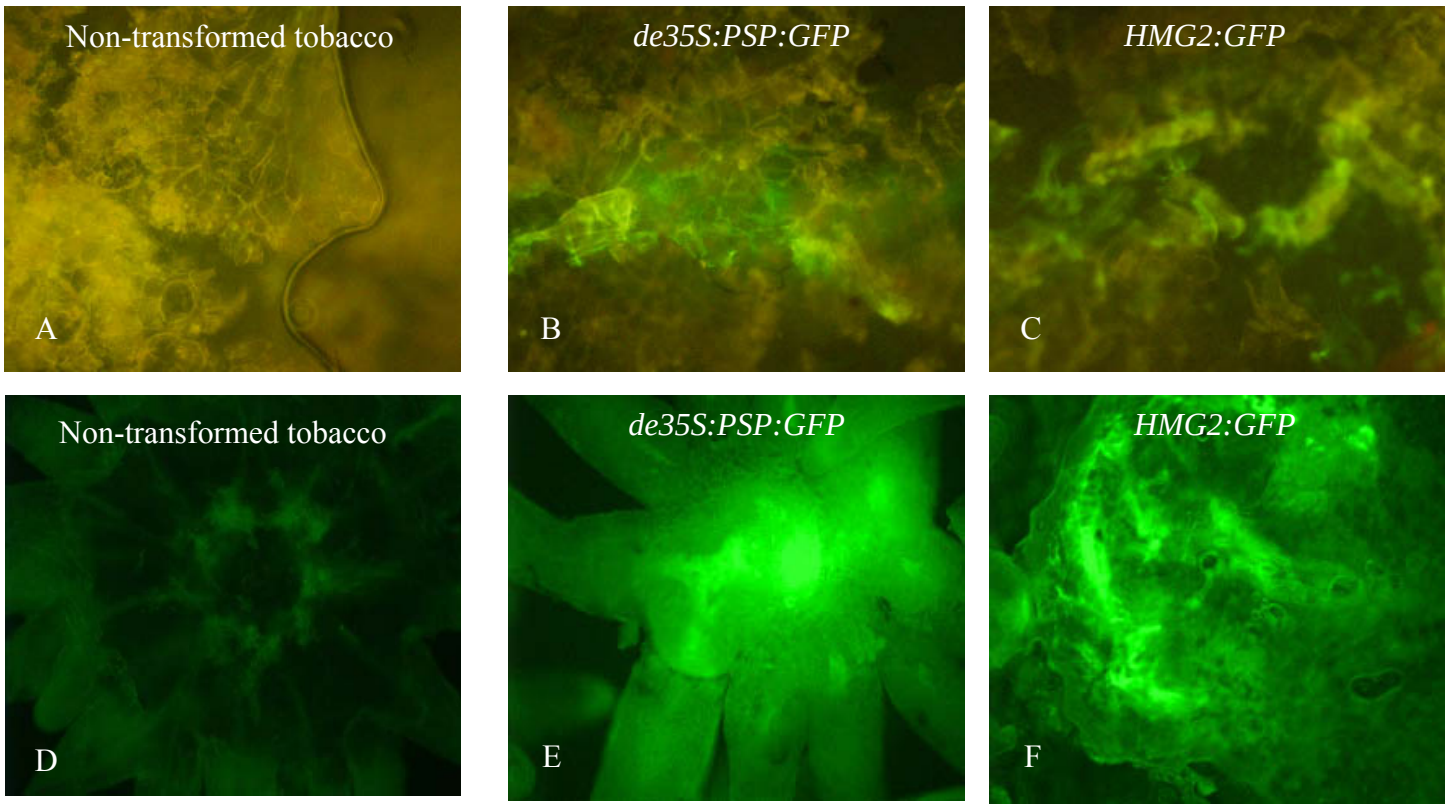


Figure 11. Presence of green fluorescence in *O. aegyptiaca* parasitizing GFP-expressing tobacco. A-C, Fluorescence micrographs of squashed *Orobanche aegyptiaca* tubercles; D-F, Hand sections through tubercles. A and D, Tubercle developed on non-transformed tobacco plant; B and E, Tubercle developed on tobacco transformed with the *de35S:PSP:GFP*; C and F, Tubercle developed on tobacco plant transformed with the *HMG2:GFP*.

fluorescence microscope. Figure 11 E-F shows strong green fluorescence inside *Orobanche* tubercles.

II.3.3. Xylem unloading of macromolecules from tobacco roots by *Orobanche*

Macromolecule movement between the host xylem and *Orobanche* was investigated by placing the cut end of tobacco roots parasitized with *Orobanche* tubercles in a solution of fluorescent dextrans representing a range of molecular weight (3, 10, 40, and 70 kDa). The cut ends of roots were at least 4 cm from the nearest tubercle, and fluorescent dyes were allowed to translocate until observed in tobacco leaves, about 10-30 min (Figure 12). Then, *Orobanche* tubercles and their associated host roots were observed under a fluorescence microscope. All sizes of probes moved rapidly up the host xylem and reach the host leaves within minutes (Figure 12). Observations of red fluorescence in the tubercles indicated that all dyes were translocated into *Orobanche* (Figure 12). These results suggest that size exclusion limit for movement of macromolecules from host to parasite through xylem connections is larger than 70 kDa.

In addition to examining movement of macromolecules from the host to the parasite, we also looked at possible movement of macromolecules from the parasite to the host. Therefore, we introduced the 3 kDa Texas-Red Dextran into *Orobanche* through a cut in *Orobanche* roots, and allowed it to translocate for several hours. Not only did the tracer move up the *Orobanche* shoot, but unexpectedly, it moved to the host vascular tissue and reached the veins of the host leaves (Figure 13). Similarly, when carboxyfluoresein was introduced into *Orobanche* roots, the tracer moved to the host and was detected in the veins of the host leaves (data not shown).

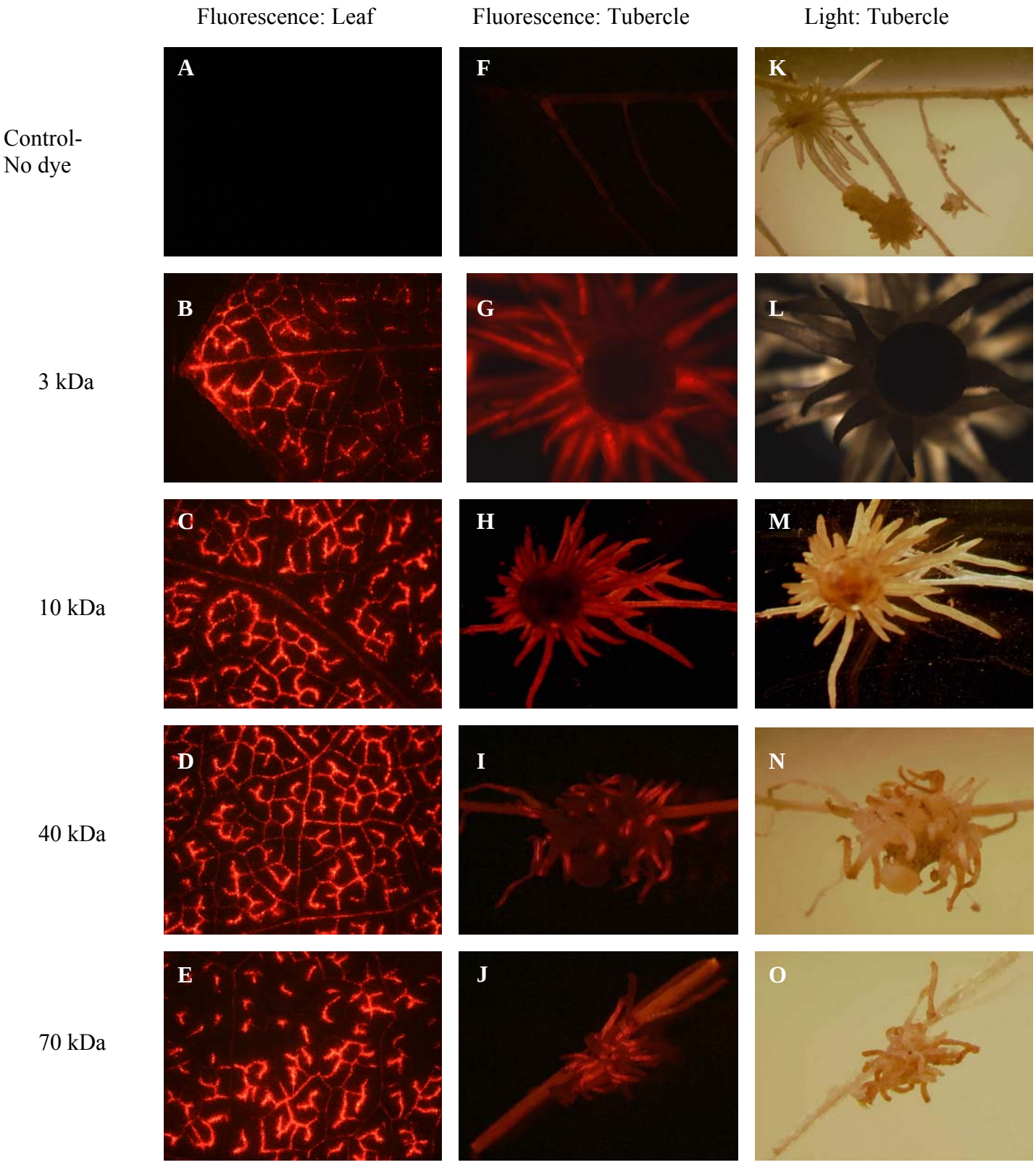


Figure 12. Movement of Texas-red-labeled dextrans from tobacco roots into *O. aegyptiaca*. A-E, fluorescence images of tobacco leaves; F-J, fluorescence images of tobacco roots and associated tubercles; K-O, light images corresponding to fluorescent images in F-J. Dextrans of different sizes were applied to cut tobacco roots below tubercles: A, F, K, water control; B, G, L, 3 kDa; C, H, M, 10 kDa; D, I, N, 40 kDa; E, J, O, 70 kDa.

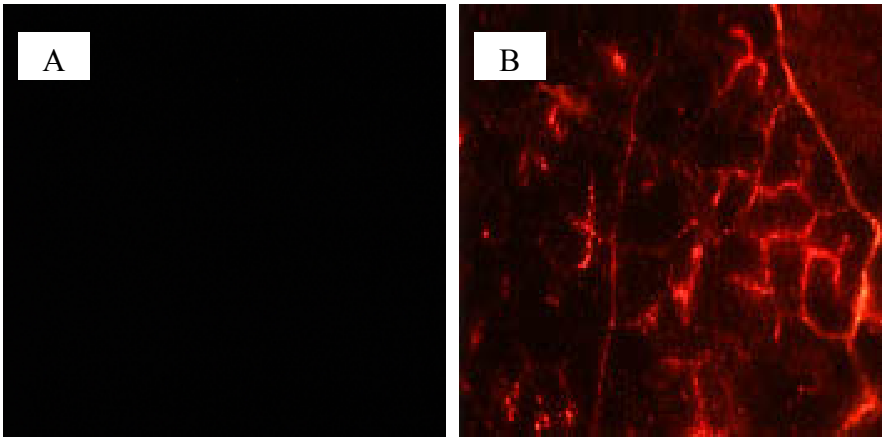


Figure 13. Fluorescence micrograph showing 3 kDa Texas-red dextran movement from *O. aegyptiaca* roots into the host plant. A, tobacco leaf in the absence of the dye; B, Texas-red dextran in the veins of tobacco leaf in which the dye was introduced into cut roots of the parasite.

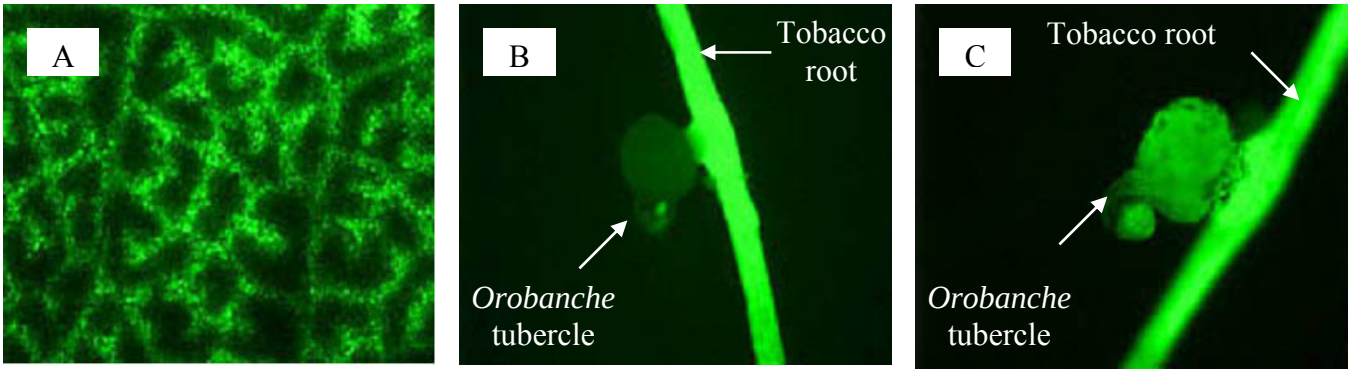


Figure 14. Systemic movement of the phloem probe CFDA in a tobacco-*O. aegyptiaca* interaction. Fluorescence images of A, CFDA within the veins of host leaf; B, Negative control using water rather than dye (Note that tobacco root auto-fluoresces green); C, Tubercle on plant to which CFDA was loaded into a petiole.

II.3.4. Induction of phloem unloading in tobacco root by *Orobanche*

In a separate experiment, the phloem network between host and parasite was traced using the phloem-mobile probe CFDA. The probe was applied to cut petioles, and its translocation pattern was monitored in *Orobanche* tubercles. In the absence of CF application, tobacco roots auto-fluoresce green. In contrast, *Orobanche* tubercles do not show any green auto-fluorescence. However, *Orobanche* tubercles turned green after the application of CF into tobacco. This indicates that the CF has been translocated from the site of application, through the phloem to the host roots, and then uploaded by *Orobanche* (Figure 14).

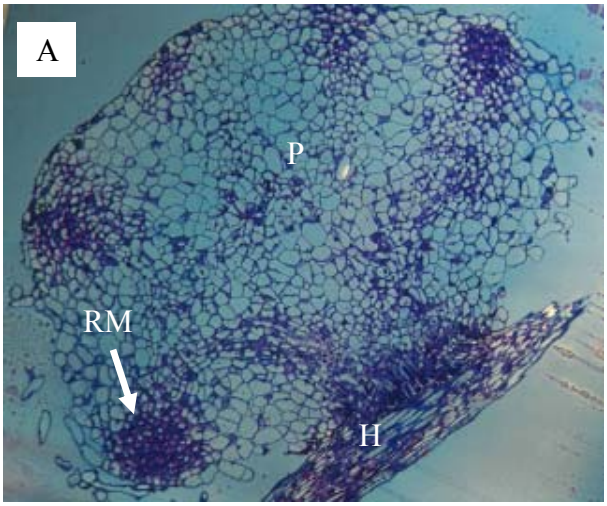
II.3.5. Developmental stages of *Orobanche aegyptiaca*

After establishment of the haustorium in the host root, *Orobanche* develops a globular structure called tubercle. The tubercle is encircled by crown root meristems, which are seen as areas of dense staining in Figure 15. When investigating sections through primary haustoria, an extremely close association between host and parasite cells becomes evident. The intricate arrangement of different tissues, consisting of cells of different shapes and sizes and probably of diverse function, makes a distinction between the two plants very difficult (Figure 15).

II.3.6. Histochemical analysis of lignifications in *Orobanche aegyptiaca*

Hand-sections of *Orobanche*-host root were stained with phloroglucinol-hydrochloric acid solution to visualize xylem connections between the host and the parasite. The solution stains lignin red. Sections through the haustorium clearly indicate

the xylem bridge that *Orobanche* forms with the host xylem (Figure 16, B. and C). In addition to a primary haustorium, *Orobanche* forms secondary attachments from roots. Those roots are able to develop xylem connections with the host root (Figure 16, B). Stained xylem tissues in cross sections of *Orobanche* shoots and a root are also presented in Figure 16.



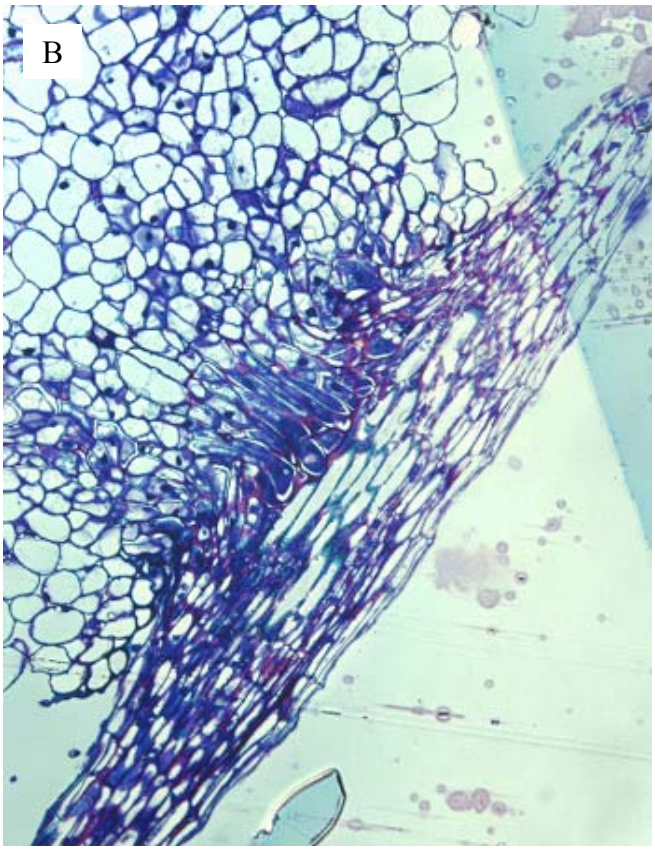


Figure 15. Light microscopy images of longitudinal sections through a young *O. aegyptiaca* (P) parasitizing the root of a host tobacco (H). A, Tubercle showing arrangement of densely staining *Orobanche* root meristems (RM); B, High magnification of the haustorial region of the parasite-host connection.

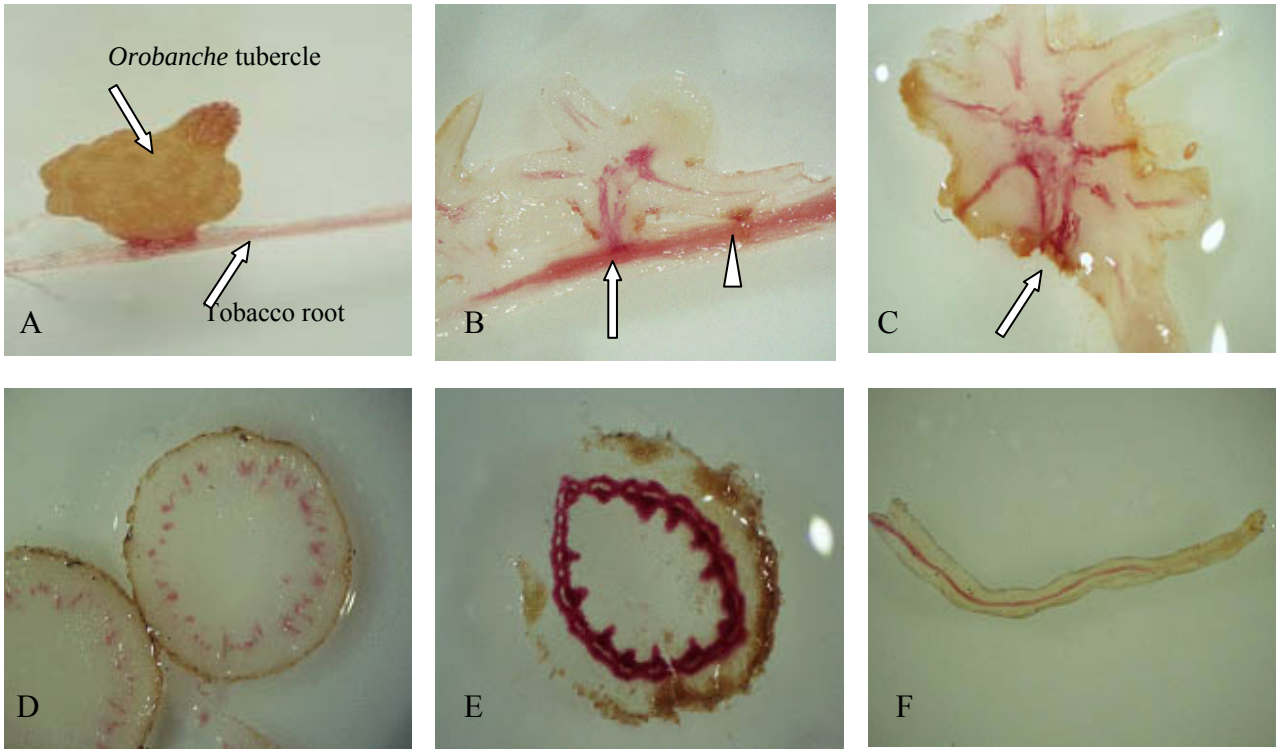


Figure 16. Stereo-micrographs of *O. aegyptiaca* xylem tissues after phloroglucinol staining. A, *Orobanche* tubercle before sectioning showing stained interface; B, longitudinal section through host and *Orobanche* [notice the xylem bridge between host and parasite in primary (arrow) and secondary (arrow head)]; C, cross-section through the host-*Orobanche* connection (arrow indicates the location of the host root); D, cross section of a young *Orobanche* shoot showing early stage of xylem tissue formation; E, cross section of a mature *Orobanche* shoot; F, xylem tissue of *Orobanche* root; G, cross section of older tubercle.

II.4. DISCUSSION

Although xylem and phloem connections between host and *Orobanche* have been documented (Dörr and Kollmann, 1975), little attention has been paid to the capacity for macromolecule translocation between host and parasite. In this study, GFP and other fluorescent tracers have been used to characterize the pathways for transport of macromolecules from host to *Orobanche*.

Evaluation by RT-PCR and immunoblot analysis indicated successful expression of the GFP gene under control of the *HMG2* promoter (Figures 8 and 9). Plants harboring the *HMG2:GFP* and *de35S:PSP:GFP* transgenes were parasitized by *O. aegyptiaca* and used to ask the question of not only whether a protein molecule would move into the parasite, but by which route. The reason why we used *de35S:PSP:GFP* transformed tobacco plants in this study is because our tobacco plants transformed with the *HMG2:PSP:GFP* construct lacked the *GFP* gene, even though they contained the hygromycin selectable marker gene. Because our main question is whether, and by what route, proteins move from host to parasite, the use of a different promoter should not affect the outcome of the experiment.

In the *de35S:PSP:GFP* plants, GFP is targeted for secretion to the host cell extracellular space, and reveals movement of macromolecules through the apoplastic pathway. In contrast, in the *HMG2:GFP* transformed plants, GFP is produced in the host cell and should remain in the cytosol as a marker for macromolecule movement through symplastic connections with the parasite. In both cases, GFP fluorescence was detected in *Orobanche* tubercles (Figure 11), indicating that the 27 kDa protein was translocated to *Orobanche* regardless of original localization. This research provides evidence that

Orobanche may use both symplastic and apoplastic routes for the uptake of host macromolecules.

To strengthen these observations, we introduced fluorescent probes of differing molecular masses into the host system and examined their movement into *Orobanche*. First, CFDA (500 Da) was used to track movement through the symplast between the host and the parasite. The tracer was introduced into the host phloem by application through cut petioles and was observed in attached *Orobanche* tissue (Figure 14). CDFA is a common marker for phloem translocation in plants (Thomas et al., 1979; Grignon et al., 1989; Roberts et al., 1997) because it is initially capable of crossing membranes and entering the phloem. However, once inside a plant cell, it is hydrolyzed by esterases to produce a CF molecule that is membrane impermeant and thus trapped inside the cell. This impermeant form of CF is a valuable marker because those molecules that enter phloem cells become trapped and move around the plant through symplastic connections.

The presence of CF inside *Orobanche* indicates that CF movement took place through cytoplasmic continuity between the host and the parasite. This is not entirely surprising because plasmodesmata have been reported in the *Orobanche*/host union (Dörr and Kollmann, 1975) and have been shown to allow passage of GFP between cells (Imlau et al., 1999). The largest molecule previously documented to move from host to *Orobanche* is sucrose, a 342 Da molecule (Whitney, 1972; Aber et al., 1983), so movement of CF, at 500 Da, and of GFP, at 27 kDa establish new upper limits for the size of molecules that *Orobanche* is able to take up from the host phloem.

A second set of tracers, Texas-red labeled dextrans of 3, 10, 40, and 70 kDa, were used to study macromolecule uptake via the apoplastic continuity between the host and

the parasite. These dyes were used to determine the exclusion limit of macromolecule movement through the apoplastic connections. The labeled dextrans were introduced into the host xylem through cut ends of host roots and rapidly moved through the host. Red fluorescence of the dyes was visible in *Orobanche* tubercles (Figure 12) indicating that macromolecules of up to 70 kDa can move to *Orobanche* through the xylem connections.

Taken together, the present study provides the first evidence that host-derived macromolecules can move to *O. aegyptiaca* through either symplastic or apoplastic routes. Moreover, *Orobanche* is able to withdraw macromolecules at least up to 70 kDa through the xylem. Haupt et al. (2001) has documented movement of GFP move from host to *Cuscuta*. *Cuscuta* and *Orobanche* represent different lineages of parasitic plant evolution, and have different haustorial anatomy. Although both species are able to absorb macromolecules from host phloem, the upper limits of molecule size likely differ. For example, *Cuscuta* absorbs and transmits viruses with the host, but efforts to transmit virus from host to *O. aegyptiaca* suggested no movement (Westwood and Tolin, unpublished data).

Future studies of host-parasite translocation could attempt to localize molecules to specific tissue and cells within the parasite. Experiments using the CF and TR-dextrans tracer could involve simultaneous injection of dyes to host phloem and xylem. This would help refine the pattern of macromolecule movement and ultimate accumulation through the xylem and phloem routes. Moreover, TR-dextrans of various molecular sizes may be introduced to the host phloem to examine the size limit of molecule movement to *Orobanche* via the phloem.

It was reported that *Orobanche* selectively takes certain molecules over others from the host (Whitney, 1972). However, movement of CF, TR, and GFP indicates that *Orobanche* non-selectively withdraws molecules from the host. This information could be used to control *Orobanche* by engineering plants to express parasite toxins such as sarcotoxin discussed in Chapter III. This research was initiated to address the question of whether the 4 kDa sarcotoxin IA protein could move into the parasite and our results demonstrate that it could move easily.

II. 5 REFERENCES

Aber M, Fer A, Salle G (1983) Etude du transfer des substances organiques de l'hote (*Vicia faba*) vers le parasite (*Orobanche crenata* Forsk.). Z. Pflanzenphysiol. **112:** 297-308

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. **18:** 203

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248-254

Clifford MN (1974) Specificity of acidic phloroglucinol reagents. J. Chromat. **94:** 321-324

Dörr I (1996) New results on interspecific bridges between parasites and their hosts. Pages 195-201 in: Advances in parasitic plant research. M.T. Moreno, J.I. Cubero, D.K. Berner, D.M. Joel, L.J. Musselman , and C. Parker, eds., Junta de Andalucia, Cordoba, Spain

Dörr I (1997) How *Striga* parasitizes its hosts: a TEM and SEM study. Ann. Bot. **79:** 463-472

Dörr I, Kollmann R (1975) Structural features of parasitism of *Orobanche* II. The differentiation of assimilate conducting elements within the haustorium. Protoplasma **83:** 185-199

Dörr I, Kollmann R (1995) Symplastic sieve element continuity between *Orobanche* and its host. Bot. Act. **108:** 47-55

Edward K, Johnstone C, Thomson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349

Grignon N, Touraine B, Durand M (1989) 6(5) carboxyfluorescein as a tracer of phloem sap translocation. Am. J. Bot. **76:** 871-877

Haupt S, Duncan GH, Holzberg S, Oparka KJ (2001) Evidence for symplastic phloem unloading in sink leaves of barley. Plant Physiol. **125:** 209-218

Heim R, Prasher DC, Tsien RY (1994) Wavelength mutations and post-translational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA **91:** 12501-12504

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Citrc. **347:** 1-32

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11:** 309-322

Jackson MB, Parker C (1991) Induction of germination by a strigol analog requires ethylene action in *Striga hermonthica* but not in *S. forbesii*. J. Exp. Physiol. **138:** 383-386

Medina-Bolivar F, Cramer CL (2004) Production of recombinant proteins in hairy roots cultured in plastic sleeve bioreactors. In: Recombinant Gene Expression: Reviews and Protocols. P. Balbas and A. Lorence, eds., Humana Press **(in press)**

Medina-Bolivar F, Wright R, Funk V, Sentz D, Barroso L, Wilkins TD, Petri W, Cramer CL (2003) A non-toxic lectin for antigen delivery of plant-based mucosal vaccines. Vaccine **21:** 997-1005

Muller F, Distler B (1989) Translocation of glyphosate in the host/parasite system *Vicia faba* and *Orobanche crenata*. Pages 226-233 in: Progress in *Orobanche* Res. K. Wegmann and L.J. Musselman, eds., Obermarchtal, FRG.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15:** 473-497

Pate JS, Kuo J, Davidson NJ (1990) Morphology and anatomy of the haustorium of the root hemiparasite *Olax phyllanthi* (Olacaceae), with special reference to the haustorial interface. Ann. Bot. **65:** 425-436

Roberts AG, Cruz SS, Roberts IM, Prior DAM, Turgeon R, Oparka KJ (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: Comparison of a fluorescent solute with a fluorescent virus. Plant Cell **9:** 1381-1396

Rogers WE, Nelson RR (1962) Penetration and nutrition of *Striga asiatica*. Phytopath. **52:** 1064-1070

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, N.Y.

Siemering KR, Golbig R, Sever R, Haseloff J (1996) Mutations that supress the thermosensitivity of green fluorescent protein. Curr. Biol. **6:** 1653-1663

Thomas JA, Buchsbaum RN, Zimniak A, Racker E (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry **18:** 2210-2218

Vaughn KC (2003) Dodder hyphae invade the host: a structural and immunocytochemical characterization. Protoplasma **220:** 189-200

Whitney PJ (1972) The carbohydrate and water balance of beans (*Vicia faba*) attacked by broomrape (*Orobanche crenata*). Ann. Appl. Biol. **70:** 59-66

CHAPTER III. Engineering Resistance to Egyptian Broomrape (*Orobanche aegyptiaca* Pers.) Based on Inducible Expression of an Antimicrobial Peptide From the Flesh Fly (*Sarcophaga peregrina*)

III.1. INTRODUCTION

Parasitic weeds of the genus *Orobanche* (broomrapes) are obligate holoparasites that attack the roots of many economically important crops throughout the semiarid regions of the world, especially the Mediterranean and Middle East where they are endemic. The parasites act as strong sinks for the uptake of water, nutrients and photosynthates, causing severe losses in crop yield and quality (Parker and Riches, 1993).

Orobanche control is difficult because they are closely associated with the host root and are concealed underground for most of their life cycle. These parasites are not controlled effectively by traditional cultural or herbicidal weed control strategies (Foy et al., 1989). The best control method is soil fumigation with methyl bromide (Jacobsohn, 1994), but this is expensive and hazardous to the environment, and methyl bromide is being phased out by international agreement to protect the global environment. The development of herbicide-resistant crops offers another *Orobanche* control strategy and is based on herbicide translocation through the host plant to the parasite (Joel et al., 1995; Surov et al., 1998). However, this approach depends on commercial availability of herbicide resistant crops, and is likely to be countered by the development of herbicide resistant parasite populations (Gressel et al., 1996). The best long-term strategy for limiting damage by *Orobanche* is the development of *Orobanche*-resistant crops (Cubero, 1991; Ejeta et al., 1991).

Aly and coworkers (unpublished data) demonstrated that constitutive expression of sarcotoxin IA under a root-specific promoter (Tob) in roots of transgenic tobacco plants reduced parasitism by *Orobanche*. However, this resistance was incomplete, and

we hypothesized that this was due to a low level of expression driven by the Tob promoter.

Independently, Westwood et al. (1998) identified the promoter from *HMG2*, a defense-specific isogene of 3-hydroxy-3-methylglutaryl CoA reductase, as being responsive to *O. aegyptiaca* parasitism in tobacco. The expression pattern of the *HMG2* promoter in response to *O. aegyptiaca* represents many desirable features of an optimal promoter for engineering resistance because it is induced immediately following parasite penetration of the host root, expression occurs specifically in the area immediately surrounding the point of attachment, and expression continues through at least four weeks of parasite development. Developmental expression of this gene is limited to cotyledons, trichomes of young leaves, sites of lateral root initiation, and developing anthers so it should have minimal expression in healthy tissue (Cramer et al., 1993).

Sarcotoxin IA is an anti-microbial peptide from the flesh fly (*Sarcophaga peregrina*). The peptide interacts with the bacterial cell membrane causing a loss of electrochemical potential (Iwai et al., 1993; Nakajima et al., 1997). Sarcotoxin IA has been expressed in tobacco plants and conferred resistance to both bacterial and fungal pathogens (Ohshima et al., 1999; Mitsuhara et al., 2000).

We proposed that expression of sarcotoxin IA under the control of the *HMG2* *Orobanche*-inducible promoter should increase the efficacy of the toxin to inhibit *Orobanche*. To address this, we have generated transgenic tobacco and challenged them with *Orobanche*.

III.2. EXPERIMENTAL METHODS

III.2.1. *HMG2:SSP:SARCO* construct

The sarcotoxin IA gene was provided by Dr. Radi Aly (Newe Ya'ar Research. Center, Israel) in a pET-3 plasmid (Stratagene, La Jolla, California). The gene was excised from the pET-3 plasmid by digestion with *Xba*I and *Sst*I. A pBC plasmid containing the *HMG2* promoter (courtesy of CropTech Corp., Blacksburg, VA) was first digested with *Xba*I and *Sst*I, and then gel extracted. The resulting linear plasmid was used to subclone the sarcotoxin IA gene to the 3'end of the *HMG2* promoter resulting in an *HMG2:SSP:SARCO* construct (Figure 17). The identity, orientation, and junctions of this gene construct were confirmed by sequencing. Sarcotoxin IA has its own signal peptide (SSP) that targets the mature peptide for secretion from cells in the hemolymph of the insect.

III.2.2. *HMG2:SSP:SARCO-HIS* construct

The rationale for adding a 6x-histidine (HIS) tag at the C-terminal end of sarcotoxin IA was to facilitate analysis of plants by using commercially available poly-histidine antibodies. The HIS sequence was added by using pET-3 plasmid containing the sarcotoxin IA gene as a template in a PCR reaction with the following primer pair:

SARCO-1, 5'-GCAGGTACCATATGAATTTCCAGAAC-3'

SARCO-2, 5'-CTAGAGCTCTCAG**TGATGATGGTGATGGTGA**CCTCTG

GCTGTAGCAGC-3'

The 6x-histidine epitope sequence is in bold and the flanking *Kpn*I and *Sst*I restriction sites are underlined. The PCR program used to amplify sarcotoxin IA was as follows: 1 cycle at 94^{o}C for 4 min: 30 cycles at 94^{o}C for 1 min, 52^{o}C for 1 min, and 72^{o}C

for 2 min, and a final extension period of 4 min at 72^{o}C. The resulting sarcotoxin-HIS fragment (approx. 0.18 kb) was digested with *Kpn*I, end-filled with Klenow polymerase, and cut with *Sst*I. Similarly, the pBC plasmid harboring the *HMG*2 promoter was digested with *Xba*I, end-filled, cut with *Hind*III and the resulting *HMG2* promoter was gel purified. The *HMG2* and sarcotoxin-HIS fragments obtained were then subcloned into the pBC plasmid cut with *Hind*III and *Sst*I (Figure 17). The identity, orientation, and junctions of this gene construct were confirmed by sequencing. A summary of the sarcotoxin IA gene constructs generated is presented in Table 3.

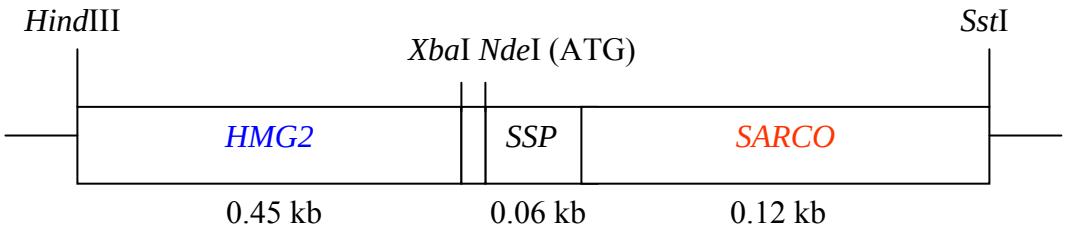
III.2.3. Plant transformation

Gene constructs were subcloned into the *Agrobacterium tumefaciens* vector pBIB$_{hyg}$ (Becker, 1990) using *Hind*III and *Sst*I restriction sites. Tobacco (*N. tabacum* cv. Xanthi) plants were transformed with *A. tumefaciens* strain LBA4404 harboring the gene constructs as described above (see Chapter II for details).

III.2.4. DNA extraction and PCR analysis

To screen regenerated plants for transformants, genomic DNA was extracted as described by Edward et al. (1991). PCR was performed using primers specific for the hygromycin gene as described earlier. Additionally, the following primers were used to check for the presence of the *HMG2:SSP:SARCO*: The *HMG2* primer, AAGTCCAGCGCGGCAACCGC, which anneals within the *HMG2* promoter and the SARCO-1 primer described above, which anneals at the 3'end of the sarcotoxin IA gene. PCR was conducted for 35 cycles in the following sequence: 94^{o}C

HMG2:SSP:SARCO



HMG2:SSP:SARCO-HIS

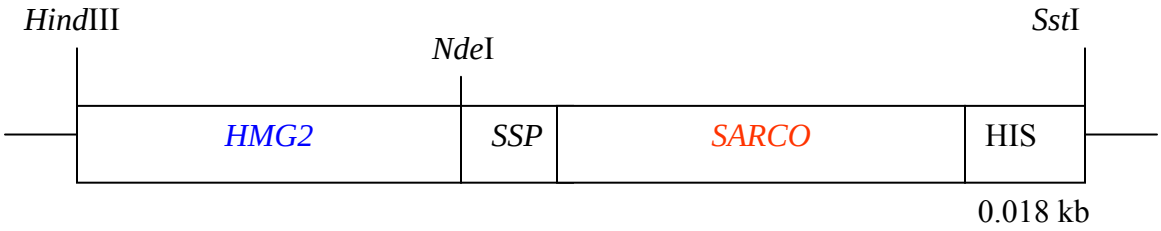


Figure 17. Gene constructs containing the sarcotoxin IA gene. *HMG2* is the promoter from the tomato *HMGR; SSP* is the sarcotoxin IA signal peptide; *SARCO* encodes sarcotoxin IA; HIS is the 6x histidine tag.

Table 3. Summary of sarcotoxin IA gene constructs and transgenic tobacco plants generated.

Construct	Rationale	Transgenic tobacco plants
HMG2:SSP:SARCO	Test efficacy of sarcotoxin IA for *Orobanche* resistance using the endogenous fly signal peptide	9 lines
HMG2:SSP:SARCO-HIS	As above, but allows localization of sarcotoxin IA using antibodies for the HIS tag; may also increases sarcotoxin stability	19 lines

for 2 min, 62°C for 1 min, and 72°C for 1 min. The cycles were preceeded by a 94°C denaturation period for 4 min and followed by 72°C final extension period for 7 min. A 4°C hold followed the cycles.

III.2.5. DNA blot hybridization

DNA blot hybridization analysis was performed on total genomic DNA from the *HMG2:SSP:SARCO* transgenic plants to confirm the genomic incorporation and to determine transgene copy number. Genomic DNA (30 µg) was digested overnight at 37°C with the restriction enzyme *Hind*III. The digested DNA was separated on a 0.8% agarose gel, transferred to a Hybron-N+ charged nylon membrane (Amersham Bioscience, Piscataway, NJ) according to Sambrook and Russell (2001) with some modifications. The gel was soaked in 250 ml alkaline transfer buffer (0.4 M NaOH and 1 M NaCl) with gentle agitation on a rotary shaker for 15 min at room temperature, followed by an additional 20 min in fresh alkaline transfer buffer. DNA was transferred from the gel to the membrane by capillary action overnight. Following transfer, the membrane was soaked in neutralization buffer (0.5 M Tris-HCl, pH 7.2 and 1 M NaCl) for 15 min at room temperature, and the DNA was immobilized by irradiating the membrane at 254 nm in a Spectrolinker XL-1000 UV crosslinker (Spectronics Corp., Westbury, New York) at 1200 x 100 µJ/cm^2.

The hygromycin resistance selectable marker gene (1 kb) gene was used as a probe. The pBIB$_{hyg}$ vector was used as a template for amplification of the hygromycin gene by PCR. The probe was labeled using the Prime-It® Random Primer Labeling Kit (Stratagene, La Jolla, California) with ^{32}P-labeled dCTP (PerkinElmer Life Sciences, Boston, MA) and 50-100 ng of the PCR product. After labeling, the probe was separated

from unincorporated radioactive nucleotides using a Sephadex G-25 TE spin column according to the manufacturer's instructions (Millipore, Bedford, Massachusetts).

The membrane was pre-hybridized at 65^{o}C with 15 ml of hybridization buffer and 200 µl salmon testes DNA (Sigma). The hybridization buffer contained 5X Denhardt's reagent (Denhardt, 1966; Sambrook and Russell, 2001) [(0.01% (w/v) Ficoll, 0.01% (w/v) Polyvinylpyrrolidone and 0.01% (w/v) acetylated BSA)] with 5X SSC buffer and 0.5% sodium dodecyl sulfate (SDS). The membranes were pre-hybridized for one hour prior to addition of the probe. After blocking, pre-hybridization buffer was removed and fresh buffer was added. The probe was added directly to the buffer and replaced in the hybridization oven (National Lab Net, Edison, NJ) at 65^{o}C overnight. The membrane was washed twice with 1X SSC and 0.1% SDS for 20 min, and then twice with 0.1X SSC and 0.1% SDS for 15 min at 65^{0}C. Kodak X-Omat AR-5 Scientific Imaging Film (Eastman Kodak Co., Rochester, NY) was used to visualize radioactive areas on the membrane.

III.2.6. RNA extraction and RT-PCR of the sarcotoxin IA gene

Induction of the *HMG2* promoter and subsequent RNA extraction was performed as described in Chapter II for GFP. Total RNA (1 µg) was reverse-transcribed with an oligo dT primer using Superscript^{TM} RNase H^{-} Reverse Transcriptase according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). PCR amplification of sarcotoxin from *HMG2:SSP:SARCO* transformed tobacco plants was performed using the following internal sarcotoxin primers:

5'-GCAGGTACCATATGAATTTCCAGAAC-3' and

5'-CTGAGCTATACCCAAACCTTGTATG-3'.

Samples were run for 35 cycles in the following sequence: 94^{o}C for 1 min, 48^{o}C for 30 s, and 72^{o}C for 1 min. The cycles were preceeded by 94^{o}C denaturation period for 4 min and followed by 72^{o}C final extension period for 7 min.

Amplification of the sarcotoxin gene from *HMG2:SSP:SARCO-HIS* transformed tobacco plants was performed using the HMG2 and SARCO-1 primer pair described earlier. PCR conditions were as described above.

III.2.7. Interaction of sarcotoxin-expressing plants and *Orobanche*

Transgenic plants expressing sarcotoxin IA were exposed to broomrape seeds and were evaluated for resistance. The experiments were conducted in two separate systems.

a) Tests in soil

Transgenic and non-transgenic tobacco plants were initially grown in soil without *Orobanche* seeds. Two to three weeks after germination, they were each transplanted into a 11 cm x 11 cm plastic pot containing Metro-Mix 360 growing medium (Scotts-Sierra Horticultural, Marysville, Ohio) inoculated with *Orobanche* seeds (400 mg/L) in a band as follows; in each pot, the first quarter of volume was filled with non-inoculated growing medium, the second two quarters contained medium inoculated with *Orobanche* seeds, and the top quarter contained non-inoculated medium. The pots were watered as needed to maintain healthy plant growth. The temperature in the green house ranged from 22 to 30^{o}C. Six to eight weeks later, tobacco plants were removed from the pots and roots were washed to remove the soil. Parameters measured include tobacco shoot dry weight (determined after drying for 24 hrs in an oven at 80^{o}C), and number and fresh weight of *Orobanche* shoots. The experimental design was a randomized complete block. The *HMG2:SSP:SARCO* transformed plants, lines L03, L05, and L07, were challenged once

with *O. aegyptiaca* using three replications of each line, and once with *O. ramosa* using five replications of each line. The *HMG2:SSP:SARCO-HIS* transformed plant, line L21, was challenged with *O. aegyptiaca* with two replications. The data were subjected to analysis of variance and standard errors of means were calculated

b) Tests in polyethylene bags (PEB)

Transgenic tobacco plants were germinated and grown initially on selective medium containing 50 mg/L hygromycin and 500 mg/L carbenicillin. Surviving plantlets were transferred to polyethylene bags containing moist glass fiber filter papers (GFFP) such that their roots were in contact with the GFFP while their shoots projected from the top of the bag. The GFFP sheets were kept moist with half-strength nutrient solution (Hoagland and Arnon, 1950) and were suspended in boxes to exclude light from the root systems. For the *HMG2:SSP:SARCO* transformed plants, three plants in one bag were used, while for the *HMG2:SSP:SRACO-HIS* line L21 seven plants were grown in two bags were used. Surface-sterilized *O. aegyptiaca* seeds were brushed gently onto the host roots. Care was taken to achieve even inoculation in all plants. After seven days of preconditioning, 10 ml of a 2 mg/L solution of GR-24, a synthetic strigolactone analogue seed germination stimulant (Jackson and Parker, 1991), was added to each bag in order to synchronize germination of *Orobanche* seeds. After 2-3 weeks, parasitism was evaluated by counting the number of dead tubercles on each plant.

III.3. RESULTS

III.3.1. Evaluation of transformed tobacco

Transgenic tobacco plants were developed to contain either the *HMG2:SSP:SARCO* or the *HMG2:SSP:SARCO-HIS* gene constructs. Initial screening of putatively transformed tobacco was conducted by PCR using primers specific for the *HPT* gene. PCR produced a product of the expected size (1 kb) from the transformed tobacco DNA but no product was amplified from non-transformed tobacco DNA (Figures 18A and 19A). The presence of the *HMG2:SSP:SARCO* and *HMG2:SSP:SARCO-HIS* transgenes was supported by additional PCR using the SARCO-1 and HMG2 primers described earlier. Transgenic plants showed products of the expected size, but no such product was amplified from DNA of non-transformed tobacco (Figures 18B and 19B). To address the possibility that residual *Agrobacterium* remaining in the transformed plant tissue could cause a false positive, a primer pair was designed for kanamycin resistance gene (*npt*II), which lies outside of the T-DNA, and thus provides an *Agrobacterium*-specific, but not T-DNA-specific marker. These primers did not amplify the *npt*II gene from those transformed plants where *HPT* primers resulted in a positive signal (data not shown), suggesting that bands amplified with *HPT* primers derived from a tobacco genome-integrated copy of *HPT*.

To determine the number of copies of the *HMG2*:*SSP*:*SARCO* transgene that were integrated into the genome of transformed tobacco plants, DNA hybridization analysis was conducted using the *HPT* gene as a probe. The *Hind*III restriction enzyme was used to digest the genomic DNA. This restriction enzyme was chosen because it cuts only once in the T-DNA region. The *HPT* probe detected one copy of the transgene in

genomic DNA extracted from transformed tobacco line L03, one to two copies in L05, and three copies in L07 (Figure 20), whereas no signal was detected in DNA extracted from non-transformed tobacco.

To demonstrate that the transgenes are being expressed, we performed RT-PCR analysis on total RNA extracted from transgenic leaves that had been wounded for 6 hrs to induce the *HMG2* promoter. RT-PCR reactions were performed using sarcotoxin IA-specific primers in two separates tubes; one tube contained the reverse-transcriptase and the other tube did not (see materials and methods). In tubes containing the RT enzyme, PCR amplification yielded a band of the expected size (0.17 kb) in all transgenic lines (Figure 21). No amplification was detected in tubes lacking the RT enzyme, indicating that PCR products amplified by the RT-PCR were amplifications from sarcotoxin IA mRNA, rather than from genomic DNA contamination.

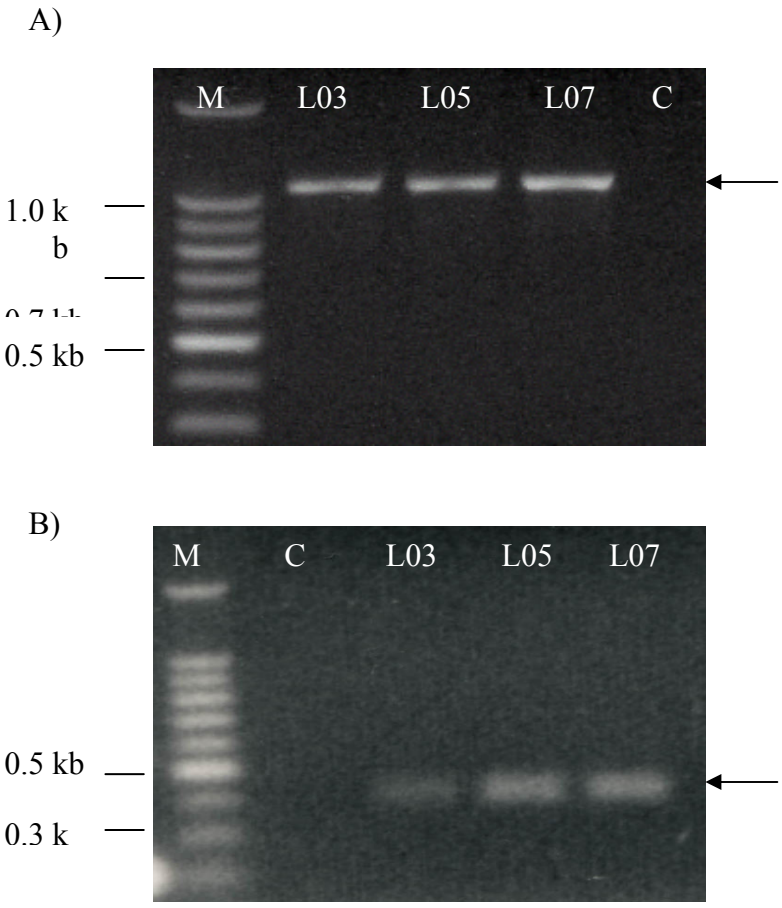


Figure 18. Confirmation of transgene presence in *HMG2:SSP:SARCO* transgenic lines by PCR. Ethidium bromide-stained agarose gels showing products of PCR amplification using specific primers to *HPT* (A) and *HMG2:SSP:SARCO* genes (B) from *HMG2:SSP:SARCO* transformed tobacco lines L03, L05, and L07. M, 100 bp DNA ladder; C, non-transformed tobacco plant

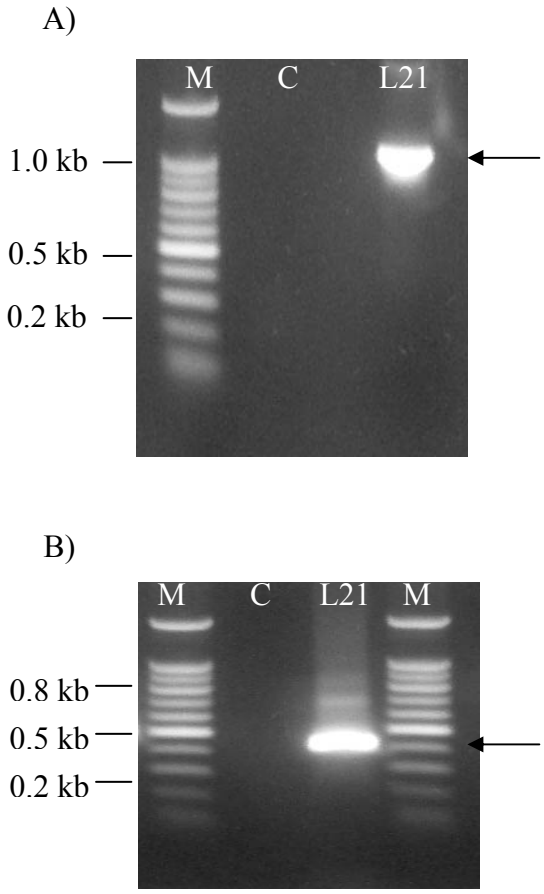


Figure 19. Confirmation of transgene presence in *HMG2:SSP:SARCO-HIS* transgenic plant line L21 by PCR. Ethidium bromide-stained agarose gels showing products of PCR amplification using primers specific to *HPT* (A) and *HMG2:SSP:SARCO-HIS* genes (B) from *HMG2:SSP:SARCO-HIS* putatively transformed tobacco plant line L21. M, 100 bp DNA ladder; C, non-transformed tobacco plant.

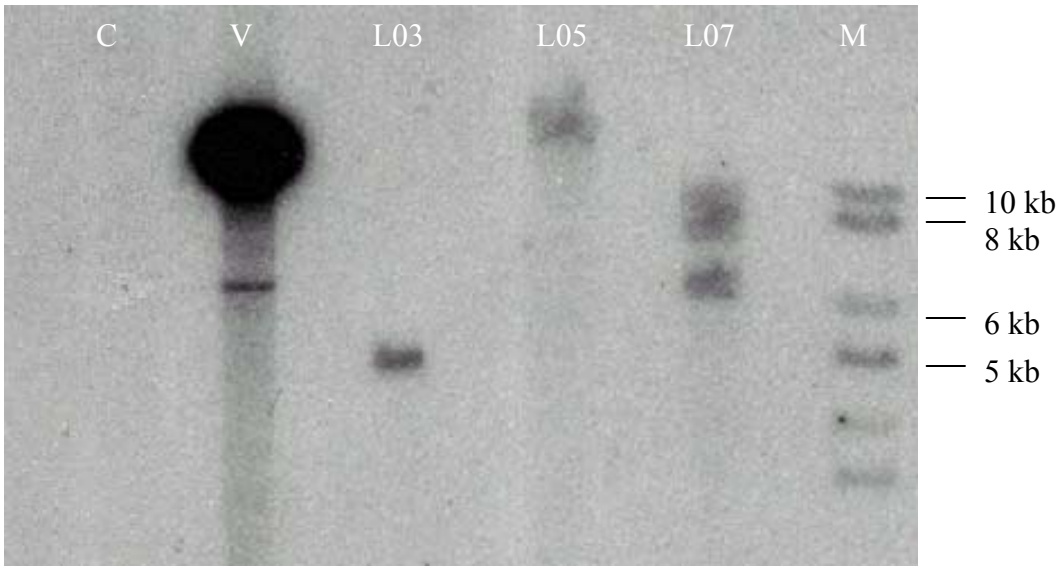


Figure 20. DNA blot hybridization analysis showing transgene incorporation and copy number in tobacco genomes. 30 µg of genomic DNA of each plant was hybridized with ^{32}P-labeled probe for the *HPT* of the T-DNA insert. Since *Hind*III is a unique restriction at the 3' end of the gene construct, digestion of genomic DNA with this enzyme reveals the number of T-DNA insertions in each transgenic line. C, non-transformed tobacco DNA; V, the binary vector used for plant transformation; L03, L05, and L07 are *HMG2:SSP:SARCO* transgenic lines. M, DNA molecular weight marker.

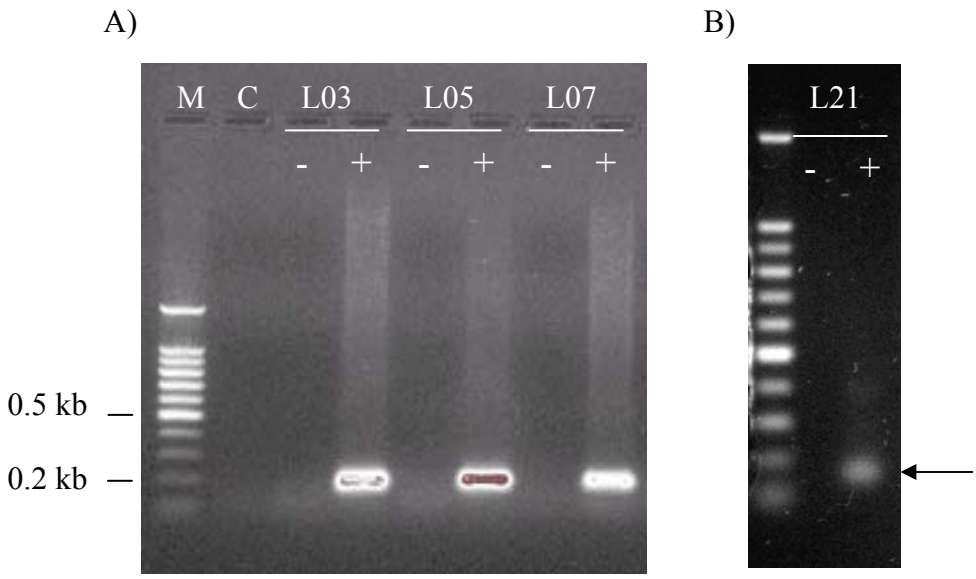


Figure 21. Gene-specific RT-PCR analysis of sarcotoxin IA. RT-PCR with sarcotoxin IA gene-specific primers was used to examine sarcotoxin IA transcript presence in total RNA samples prepared from wounded leaves of *HMG2:SSP:SARCO* (A) and *HMG2:SSP:SARCO:HIS* (B) transgenic tobacco. RT-PCR products were separated on 1.8% (w/v) agarose gel and visualized with ethidium bromide. M, 100 bp DNA ladder; C, PCR reaction with no template; (-), PCR reaction on total RNA without reverse-transcriptase; (+), PCR reaction on cDNA with reverse-transcriptase.

III.3.2. Impact of *HMG2*-driven sarcotoxin IA expression on host resistance to *Orobanche*

In order to evaluate the effect of sarcotoxin IA on host resistance to parasitism by *Orobanche*, experiments were conducted in soil and in a PEB system. The PEB system is advantageous because it allows visualization of the phenotype of *Orobanche* tubercles at their early stages of development. However, experiments in pots more closely approximate field conditions.

HMG2:SSP:SARCO transformed tobacco lines were grown in soil containing *O. aegyptiaca* seeds (400 mg/L) and were evaluated for *Orobanche* development. Results show that transgenic plants were healthy and grew taller than non-transformed plants (Figure 22A). *HMG2:SSP:SARCO* transformed plants did not show complete resistance because *Orobanche* did attach to these plants and shoots had emerged from the soil. However, transformed plants accumulated more dry weight compared to the non-transformed plants (Figure 22B). In this experiment, statistical differences were not observed for *Orobanche* shoot number or fresh weight (Figure 22C,D)

To follow up on results from experiments in pot, we conducted an experiment using the same *HMG2:SSP:SARCO* transformed tobacco lines in the PEB system. High levels of necrotic and dead tubercles were observed in plants expressing sarcotoxin IA, especially in line L03, as compared to the non-transformed plants (Figure 23).

Tobacco lines were also challenged against a different *Orobanche* species, *O. ramosa.* In this experiment the effect of *HMG2:SSP:SARCO* transformed plants was more obvious. *Orobanche* shoots were unable to emerge from the soil, and tubercles

attached to transformed plants were necrotic and dead (Figure 24A). In contrast, *Orobanche* shoots attached to non-transformed plants were healthy.

An *HMG2:SSP:SARCO-HIS* transformed plant (L21) was also challenged with *Orobanche* in soil and in the PEB system. In soil experiment, statistical differences were not observed for transformed tobacco dry weight or for *Orobanche* shoot number and fresh weight (Figure 25). In the PEB system, *Orobanche* tubercles were smaller in the *HMG2:SSP:SARCO-HIS* transformed plants compared to non-transformed plants (Figure 26A). Moreover, the number of dead tubercles was significantly higher in the *HMG2:SSP:SARCO-HIS* line (Figure 26B).

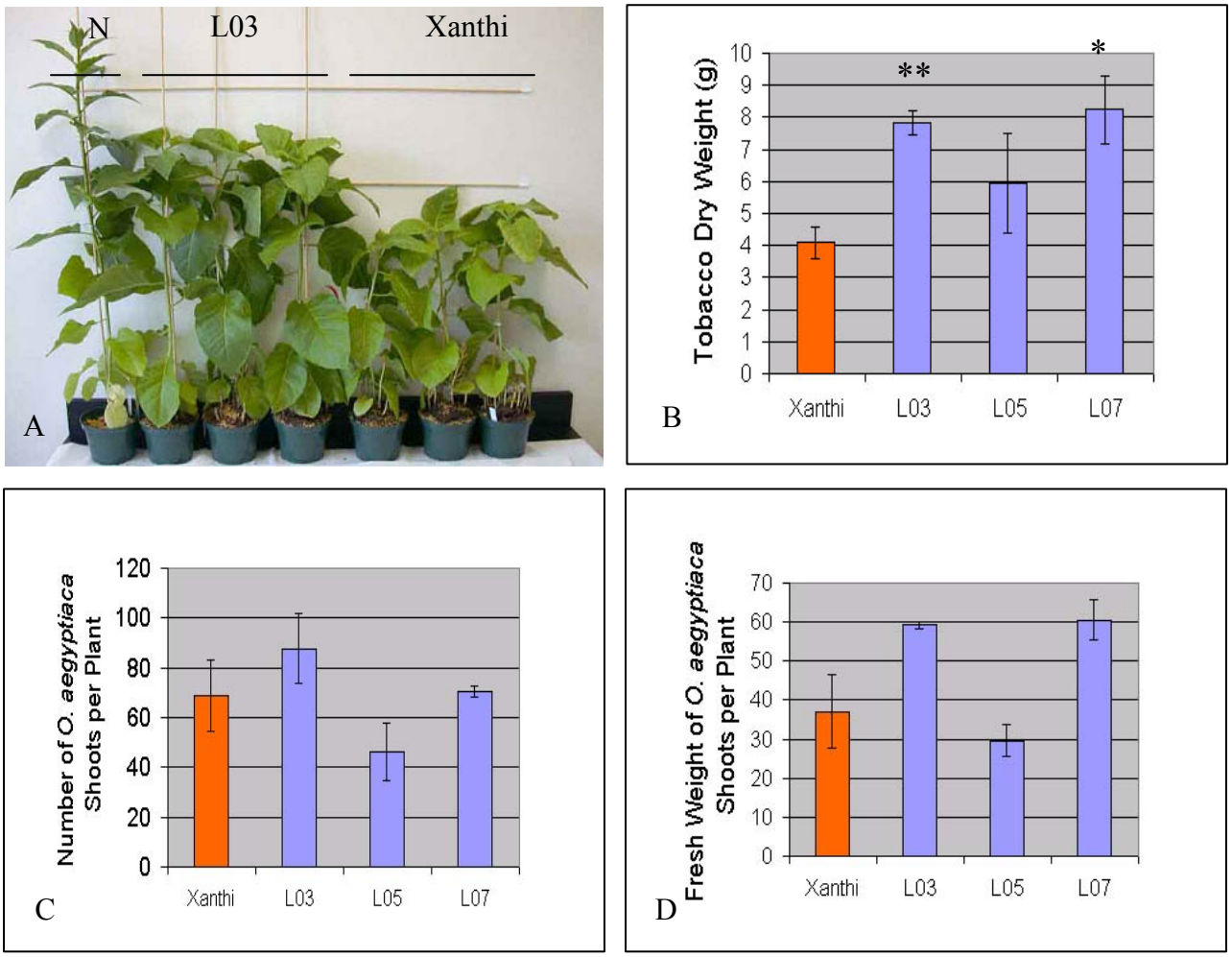


Figure 22. Response of *HMG2:SSP:SARCO* transformed tobacco plants to *O. aegyptiaca* in soil. A, Phenotype of *HMG2:SSP:SARCO* transformed line L03 and untransformed xanthi, growing in soil inoculated with *O. aegyptiaca* seeds (N is line L03 in non-inoculated soil); B, dry weight of tobacco shoots; C, number of *Orobanche* attached to tobacco plants; D, fresh weight of *Orobanche* shoots. Xanthi is the non-transformed line while L03, L05, and L07 are *HMG2:SSP:SARCO* transformed plants. Bars represent the mean of three plants with vertical lines indicating SE. * indicate means different from xanthi as determined by student T-test with $\alpha = 0.05$.

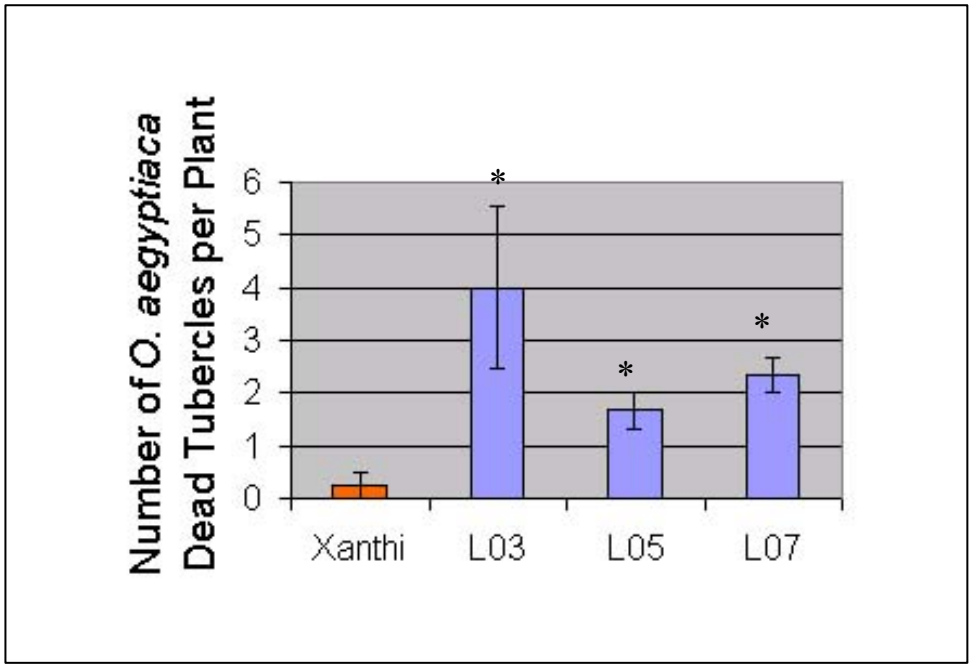


Figure 23. Response of *HMG2:SSP:SARCO* transformed plants to *O. aegyptiaca* in the PEB growth system. L3, L05, and L07 are transformed tobacco lines; Xanthi is untransformed plant. Bars represent the mean of three plants with vertical lines indicating SE. * indicate means different from xanthi as determined by student T-test with $\alpha = 0.05$.

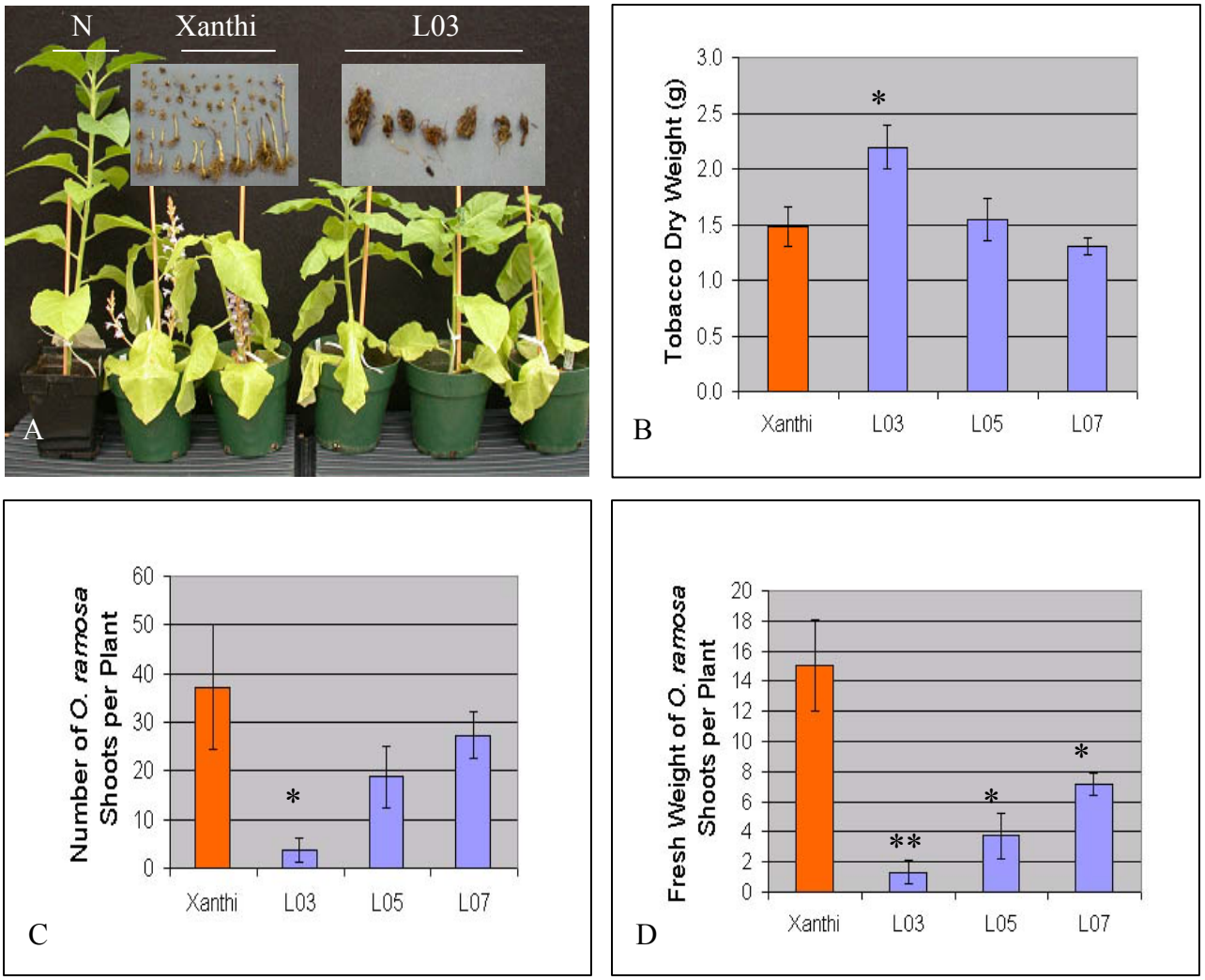


Figure 24. Response of *HMG2:SSP:SARCO* transformed tobacco plants to *O. ramosa* in soil. A, Phenotype of *HMG2:SSP:SARCO* transformed line L03 and non-transformed xanthi, growing in soil inoculated with *O. ramosa.* Inserts show phenotype of *Orobanche* plants (N is line L03 in non-inoculated soil); B, dry weight of tobacco shoots; C, number of *Orobanche* attached to tobacco plants; D, fresh weight of *Orobanche*. Xanthi is the non-transformed plant while L03, L05, and L07 are *HMG2:SSP:SARCO* transformed plants. Bars represent the mean of five plants with vertical lines indicating SE. * indicate means different from xanthi as determined by student T-test with $\alpha = 0.05$.

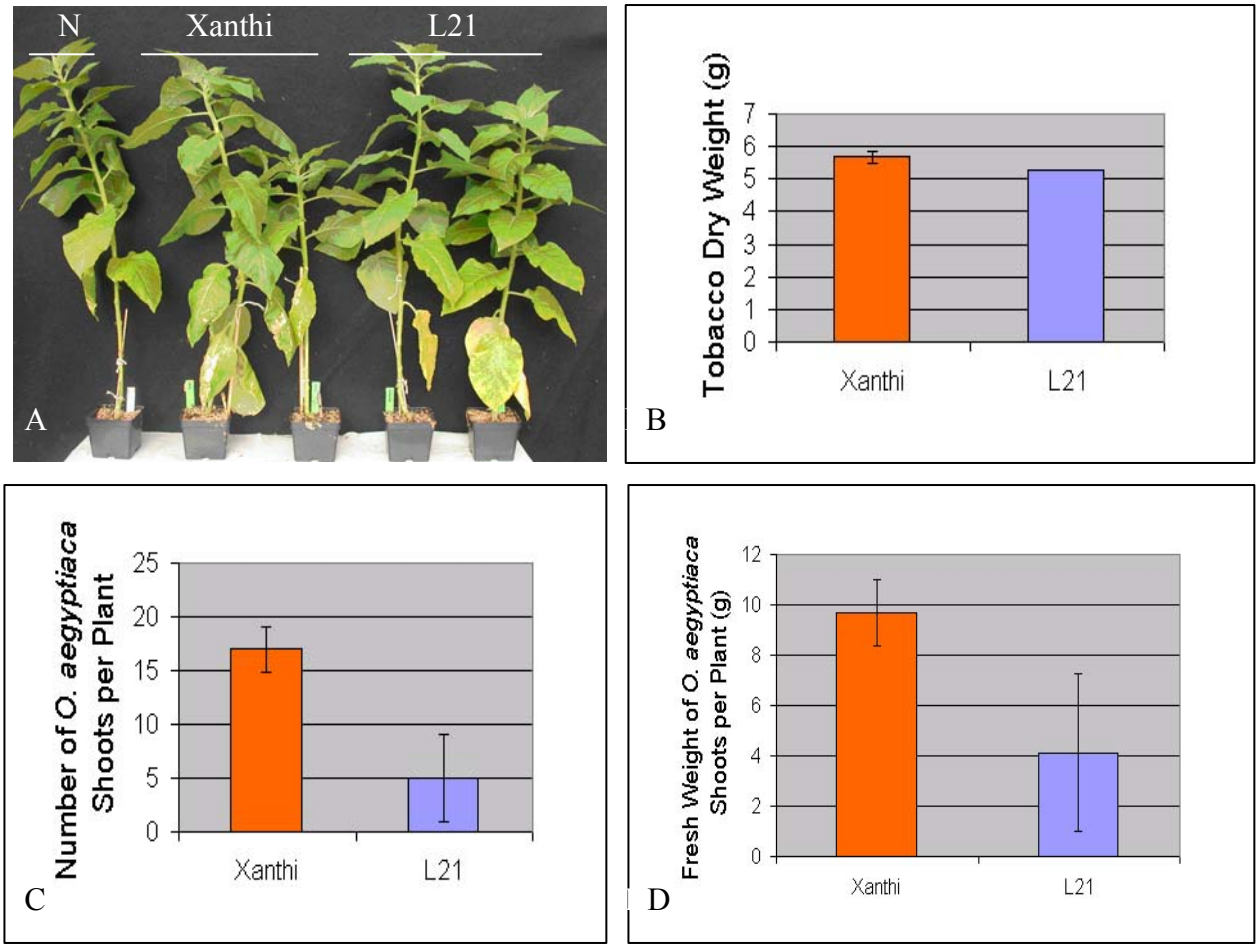


Figure 25. Response of *HMG2:SSP:SARCO-HIS* transformed tobacco plants to *O. aegyptiaca* in soil. A, phenotype of *HMG2:SSP:SARCO-HIS* transformed line L21 and non-transformed Xanthi growing in soil inoculated with *O. aegyptiaca* (N is line L21 in non-inoculated soil); B, dry weight of tobacco shoots; C, number of shoots attached to tobacco plants; D, fresh weight of *Orobanche*. Xanthi is the non-transformed plant while L21 is *HMG2:SSP:SARCO-HIS* transformed plant. Bars are means of two replicates with vertical lines indicating SE.
* indicate means different from xanthi as determined by student T-test with $\alpha = 0.05$.

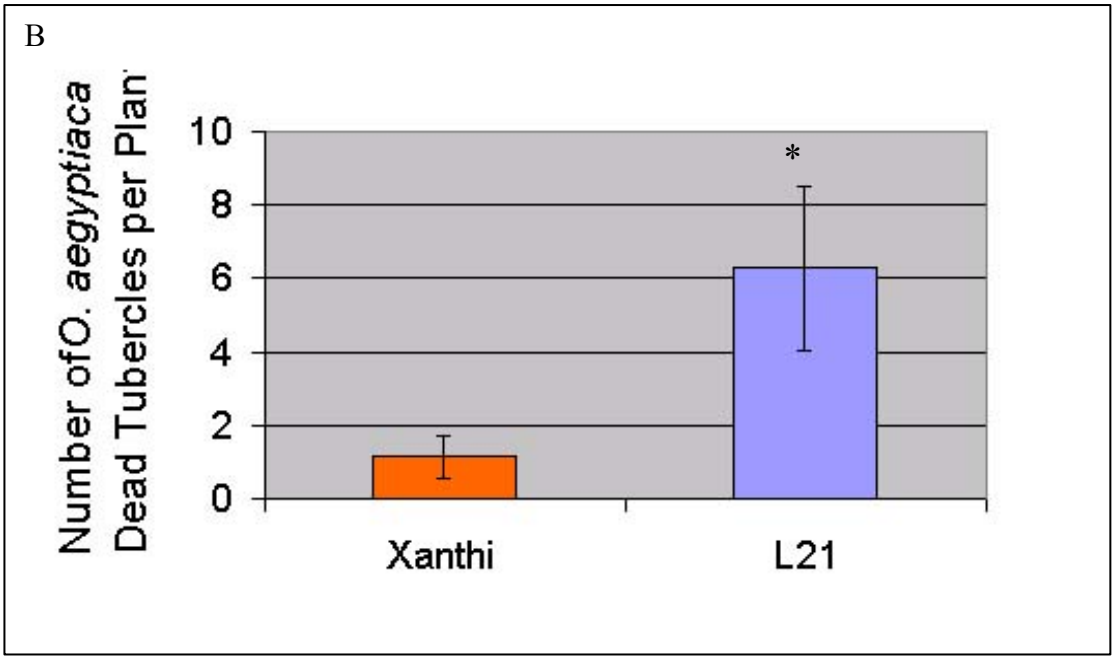


Figure 26. Response of *HMG2:SSP:SARCO-HIS* transformed tobacco plants to *O. aegyptiaca* in the PEB growth system. A, phenotype of *Orobanche* tubercles attached to *HMG2:SSP:SARCO*-HIS transformed line L21 and non-transformed xanthi; B, number of dead *Orobanche* tubercles in transformed and non-transformed plants. Bars are means of three replicates with vertical lines indicating the SE. * indicate means different from xanthi as determined by student T-test with $\alpha = 0.05$.

III.4. DISCUSSION

Biotechnology provides a complementary approach to traditional breeding for controlling parasitic weeds such as *Orobanche*. For example, genetic engineering enables the use of resistance mechanisms that are not present in the germplasm of a host. Here we report progress toward development of a new *Orobanche* resistance strategy based on the inducible expression of an anti-microbial peptide, sarcotoxin IA, which derives from the flesh fly.

This project was initiated by observations that constitutive expression of sarcotoxin IA under Tob, a root-specific promoter, reduced *Orobanche* growth (Aly et al., unpublished results). However resistance in these plants was incomplete, possibly due to a low level of expression by the promoter. We hypothesized that strong and localized expression of sarcotoxin IA at the site of *Orobanche* attachment would increase the level of resistance.

Tobacco plants have been generated that express the sarcotoxin IA or sarcotoxin IA–HIS under control of the *HMG2* promoter. Part of the rationale of using the HIS tag is that expression of sarcotoxin as a fusion protein may confer stability to the sarcotoxin, which is relatively unstable protein. Sarcotoxin IA expressed as a fusion protein to GUS has been documented to increase stability of the peptide (Okamoto et al., 1998). Another advantage of the HIS tag is that anti-HIS antibodies could be used to track the sarcotoxin in the host root, haustorium, or shoots of *Orobanche*.

Sarcotoxin IA seems to negatively affect the growth and development of *Orobanche*. In soil highly infested with *O. aegyptiaca* seeds, transgenic tobacco expressing *HMG2:SSP:SARCO* accumulated more biomass compared to non-transformed

plants, suggesting that sarcotoxin may play a role in protecting the host from *Orobanche* parasitism (Figure 22). However, we observed *Orobanche* parasitizing both non-transformed and transformed plants, indicating that plants expressing sarcotoxin IA did not completely resist *Orobanche* parasitism.

The expression pattern of *HMG2* in response to *Orobanche* parasitism indicates that this promoter is activated shortly after parasite attachment and continues for up to four weeks (Westwood et al., 1998). Therefore, we expect sarcotoxin IA to be effective at the early stages of *Orobanche* tubercle development. To test the hypothesis that sarcotoxin is killing young *Orobanche* tubercles at very early stages, we conducted experiments in a PEB system. This system permits visualization of all tubercles, and the effect of sarcotoxin on young tubercles could be more easily observed than in soil where tiny and/or dead tubercles are difficult to find. Results from the PEB system clearly indicate that transgenic tobacco plants expressing the sarcotoxin had smaller tubercles, and more of them were necrotic, compared to the non-transformed plants (Figure 23). In agreement with these observations, our collaborators in Israel, Dr. R. Aly and coworkers, conducted an experiment testing resistance of these transgenic lines against *O. aegyptiaca* and achieved similar results (Data not shown).

To expand the scope of utility of this resistance mechanism, the *HMG2:SSP:SARCO* plants were also tested in soil against a different species of *Orobanche*, *O. ramosa.* The parasite shoots collected from transformed plants showed 50-90% reduction in *Orobanche* fresh weight compared to non-transformed plants (Figure 24). Shoots of parasites on sarcotoxin-expressing hosts were fewer, smaller, and appeared malformed as compared to parasites on non-transformed hosts.

Resistance of *HMG2:SSP:SARCO:HIS* transformed plants to *O. aegyptiaca* was evaluated in soil and the PEB growth system. The soil experiment indicated that these hosts had reduced parasite growth as compared to non-transformed plants (Figure 25). The number and fresh weight of *Orobanche* shoots on tobacco expressing *HMG2:SSP:SARCO-HIS* were 60% and 70% less compared to non-transformed, respectively. Resistance of *HMG2:SSP:SARCO-HIS* transformed plant to *O. aegyptiaca* was also evident in the PEB growth system. Tubercles parasitizing plants expressing the sarcotoxin IA were smaller and had higher mortality than those on control plants (Figure 26). It seems that the His tag did not interfere with sarcotoxin activity.

Sarcotoxin IA specifically attacks the bacterial membrane (Nakajima et al., 1987; Iwai et al., 1993), yet our results suggest that it also interferes with *Orobanche* development. Although we do not know the exact mechanism of this inhibition, one simple explanation could be that, *Orobanche*, acting as a strong sink, accumulates the toxin to an inhibitory level. Because very large macromolecules (up to 70 kDa) can move from the host to the parasite, as concluded from experiments using the Texas-Red-labeled dextrans, smaller molecules of the size of sarcotoxin IA could also move.

It is important to mention that the transformed tobacco plants analyzed in this study are not homozygous lines. They were not brought to the homozygous stage but were selected on antibiotic-containing medium and then transferred to soil inoculated with *O. aegyptiaca* seeds. Thus, although all plants tested contained the transgene, some variability in response to *Orobanche* may be due to differences in zygosity level. The partial resistance of the transgenic plants tested could also be caused by low level of expression of sarcotoxin IA and/or protein instability. We were unable to detect

sarcotoxin IA by immunoblot analysis (data not shown). This might be due to either reduced level of gene expression or rapid degradation of the peptide by plant proteases. It may also be due to low levels of sarcotoxin-specific antibodies in the serum we used to detect the protein. Moreover, it is possible that sarcotoxin had been degraded rapidly before it accumulates to toxic levels in *Orobanche* tubercles. Sarcotoxin IA has been documented to be very susceptible to proteases present in the intercellular fluid of tobacco plants (Mitsuhara, personal communication).

Another explanation of the observed incomplete resistance observed in plants expressing sarcotoxin could be that the *HMG2* promoter is not expressed at sufficiently high levels after *Orobanche* attachment and/or may not be expressed at every attachment. Therefore, a stronger promoter may be needed for increasing levels of sarcotoxin IA.

In summary, this strategy utilizes an anti-microbial peptide to inhibit growth of *O. aegyptiaca*. The current results indicate that the resistance was incomplete, but show promise for strategies utilizing sarcotoxin IA. There are many aspects of this approach that remain to be optimized. It is possible that the resistance could be further enhanced by modifying the *HMG2:SSP:SARCO* construct, and we have generated several transgenic tobacco and *Arabidopsis* plants containing slight modifications of these constructs. Studies to improve sarcotoxin efficiency will lead to greater understanding of *Orobanche* and ultimately to the generation of crops with high-level of resistance to parasites (see Appendix 2).

III.5. REFERENCES

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. **18:** 203

Cramer CL, Weissenborn D, Cottingham CK, Denbow CJ, Eisenback JD, Radin DN, Yu X (1993) Regulation of defense-related gene expression during plant-pathogen interactions. J. Nematol. **25:** 507-518

Cubero JI (1991) Breeding for resistance to *Orobanche* species: A review. Pages 257-277 in: Progress in *Orobanche* Research. K. Wegmann and L.J. Musselman, eds., Eberhard-Karls-Universitat, Tubingen, Germany.

Denhardt DT (1966) A membrane filter technique for determination of complementary DNA. Biochem. Biophys. Res. Commun **23:** 641-646

Edward K, Johnstone C, Thomson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349

Ejeta G, Butler LG, Hess DE, Vogler RK (1991) Genetic and breeding strategies for *Striga* resistance in sorghum. Pages 539-544 in: Proc 5th Int. Symp. Parasitic Weeds. J.K. Ransom, L.J. Musselman, A.D. Worsham, and C. Parker, eds., CIMMYT, Nairobi, Kenya

Foy CL, Jain R, Jacobsohn R (1989) Recent approaches for chemical control of broomrape (*Orobanche* spp.). Rev. Weed Sci. **4:** 123-152

Gressel J, Segel L, Ransom JK (1996) Managing the delay of evolution of herbicide resistance in parasitic weeds. Int. J. Pest Manag. **42:** 113-129

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Citrc. **347:** 1-32

Iwai H, Nakajima Y, Natori S, Arata Y, Shimada I (1993) Solution conformation of an antibacterial peptide, sarcotoxin IA, as determined by H-NMR. Eur. J. Biochem. **217:** 639-644

Jackson MB, Parker C (1991) Induction of germination by a strigol analog requires ethylene action in *Striga hermonthica* but not in *S. forbesii*. J. Exp. Physiol. **138:** 383-386

Jacobsohn R (1994) The broomrape problem in Israel and an integrated approach to its control. Pages 652-658 in: Proc. 3rd Int. Worksh. on *Orobanche* and related *Striga* Res., A.H. Pieterse, J.A.C. Verkleij, and S.J. ter Borg, eds., Royal Tropical Institute, Amsterdam, The Netherlands.

Joel DM, Kleifeld Y, Losner-Goshen D, Herzlinger G, Gressel J (1995) Transgenic crops against parasites. Nature **374:** 220-221

Mitsuhara I, Matsufuru H, Ohshima M, Kaku H, Nakajima Y, Murai N, Natori S, Ohashi Y (2000) Induced expression of sarcotoxin IA enhanced host resistance against both bacterial and fungal pathogens in transgenic tobacco. Mol. Plant-Microbe Interact. **13:** 860-868.

Nakajima Y, Alvarez-Bravo J, Cho J, Homma K, Kanegasaki S, Natori S (1997) Chemotherapeutic activity of synthetic antimicrobial peptides: correlation between chemotherapeutic activity and neutrophil-activating activity. FEBS Lett. **415:** 64-66

Nakajima Y, Qu X, Natori S (1987) Interaction between liposomes and sarcotoxin IA, a potent antimicrobial protein of *Sarcophaga peregrina* (flesh-fly). J. Biol. Chem. **262:** 1665-1669

Ohshima M, Mitsuhara I, Okamoto M, Sawano S, Nishiyama K, Kaku H, Natori S, Ohashi Y (1999) Enhanced resistance to bacterial disease of transgenic tobacco plants overexpressing sarcotoxin IA, a bactericidal peptide of insect. J. Biochem. **125:** 431-435

Okamoto M, Mitsuhara I, Ohshima M, Natori S, Ohashi Y (1998) Enhanced expression of an antimicrobial peptide sarcotoxin IA by GUS fusion in transgenic tobacco plants. Plant Cell Physiol. **39:** 57-63

Parker C, Riches CR (1993) Parasitic Weeds of the world: Biology and Control. CAB Int., Wallingford, UK.

Sambrook J, Russell DW (2001) Molecular cloning, 3rd ed. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, N.Y.

Surov T, Aviv D, Aly R, Joel DM, Goldman-Guez T, Gressel J (1998) Generation of transgenic assulam-resistant potatoes to facilitate eradication of parasitic

broomrapes (*Orobanche* spp.) with the sul gene as the selectable marker. Theor. Appl. Genet **96:** 132-137

Westwood JH, Yu X, Foy CL, Cramer CL (1998) Expression of a defense-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. Mol. Plant-Microbe Interact. **11:** 530-536

APPENDICES

Appendix 1

Transformation of *Arabidopsis* Plants with GFP-Containing Constructs

The same *HMG2*:*GFP* and *HMG2*:*PSP*:*GFP* gene constructs that were introduced into tobacco were also introduced into *Arabidopsis* plants (*Arabidopsis thaliana* var. Columbia). *Arabidopsis* is, like tobacco, a good host for *Orobanche* parasitism.

Materials and Methods

Plant transformation

GFP-containing gene constructs described in Chapter II were subcloned into *Agrobacterium tumefaciens* vector $pBIB_{hyg}$ (Becker, 1990). This vector contains the appropriate border sequence to aid in the transfer of T-DNA into the plant genome and a hygromycin resistance selectable marker, which allows selection of transgenic plants on hygromycin-containing medium. $pBIB_{hyg}$ vectors containing *GFP* constructs were subsequently introduced into the GV3101 strain of *A. tumefaciens* for *Arabidopsis* transformation.

The procedure for *Arabidopsis* transformation is as follows: Ten milligrams of wild-type Columbia seeds were first scattered on a Whatman filter paper disk pre-moistened with autoclaved double distilled water in a plastic petri dish. The plate was then sealed with parafilm and placed at 4^oC for 2-5 days for stratification. *Arabidopsis* seeds were resuspended in 2 ml of 0.1% agarose solution and dispersed evenly among 3 small pots (6 X 7 cm). For the first two weeks, the seedlings were grown under 8 h days 150 μE of white light, at 22^oC. The plants were then switched to 16 h days at 120 μE, at 22^oC to induce bolting. The following week, the plants were thinned to 20 individuals per pot. These were allowed to develop for 5 d after emergence, and then clipped to

encourage the development of multiple secondary bolts. Six days after clipping, the plants were placed in a pan of water overnight. The next day, siliques, flowers, and partially-opened buds were removed just prior to vacuum infiltration of the remaining closed buds with *A. tumefaciens*.

Transformation was performed using vacuum infiltration as described in Bechtold et al. (1993). *Agrobacterium* containing the GFP constructs in $pBIB_{hyg}$ were grown in 25 ml 2XTY media with 34 mg/L rifampicin, 25 mg/L gentamycin, and 50 mg/L kanamycin for two days at 28^{o}C with vigorous shaking. 400 ml fresh 2XTY medium with antibiotics was added to the culture and incubation was continued under the same conditions. After about 16 hrs, the cells were pelleted by centrifugation at 5,000 RPM for 10 min and then resuspended in approximately 500 ml of infiltration medium (0.5X MS salts, 1X B5 vitamin solution, 5% sucrose, 0.004 μM benzylaminopurine, 0.03% Silwet-77, 0.5% (w/v) thiamine-HCl, 0.05% nicotinic acid, 0.05% pyridoxine-HCl, 5% myoinositol) to an OD_{600} of 0.8. The solution was poured into a plastic dish, on which the plants had been placed in an inverted position with inflorescences displayed on the dish surface. The inverted pots were suspended above the dish surface using tube caps as supports to keep leaves out of the infiltration medium. Vacuum was applied for 15 min, and then rapidly released to aid infiltration of the solution into *Arabidopsis* buds. The plants were then allowed to recover overnight in a dark chamber. The following day, a 16 hrs day cycle was resumed. Seeds were harvested as soon as siliques turned brown or started opening, approximately 2-3 weeks after infiltration. These T1 seeds were surface-sterilized as previously described (Kubasek et al., 1992), spread on MS-agar plates containing 25 mg/L hygromycin and 500 mg/L carbenicillin to kill any remaining *Agrobacterium*,

vernalized as described above, and placed in a 22^{o}C incubator under continuous white light (150 μE). T1 transformants were identified based on survival after 2 weeks under hygromycin selection.

Results and Discussion

Several putatively transformed plants were regenerated (Table 4). PCR was used to screen those plants for the presence of GFP gene using the GFP specific primers and the PCR protocol described above (See Chapter II, Materials and Methods). PCR results indicated that the *Arabidopsis* plants regenerated contained the GFP gene (data not shown). Moreover, when *Arabidopsis* roots were visualized under fluorescent microscope (OPELCO, Dulles, Virginia), they showed green fluorescence at the branching of secondary roots (Figure 27), which is consistent with the expression pattern of *HMG2* promoter. However, analysis of GFP expression using RT-PCR or western blot was not conclusive as to the presence of GFP expression or proteins (data not shown). Expression of the *HMG2* promoter in *Arabidopsis* has not been reported before, so this data suggests that its expression pattern is similar across some species.

Although *Arabidopsis* roots show low level of green auto-fluorescence compared to tobacco plants, which may be advantageous for visualizing GFP fluorescence, the problem of confirming expression of *HMG2* in *Arabidopsis* plants by RT-PCR or an immunoblot analysis lead us to choose tobacco as the plant system in which to study macromolecule movement.

It is worth noting that due to the low level of green auto-fluorescence of *Arabidopsis* plants (unlike tobacco plants), we detected green fluorescence at the attachment surface of *Orobanche* (Figure 28). This auto-fluorescence could be due either

to phenolic compounds secreted by the host as a defense mechanism, or to fluorescent substances produced by the *Orobanche* that may play a role in successful *Orobanche* attachment.

Table 4. Summary of *GFP* constructs and transgenic *Arabidopsis* plants generated.

Construct	Rational	Transgenic *Arabidopsis* plants
HMG2:GFP	Cytsolic GFP (without signal peptide) for monitoring protein movement via symplastic connections	77 lines
HMG2:PSP:GFP	*PSP* (patatin signal peptide) targets GFP to extracellular space to reveal protein movement via apoplastic connections	105 lines

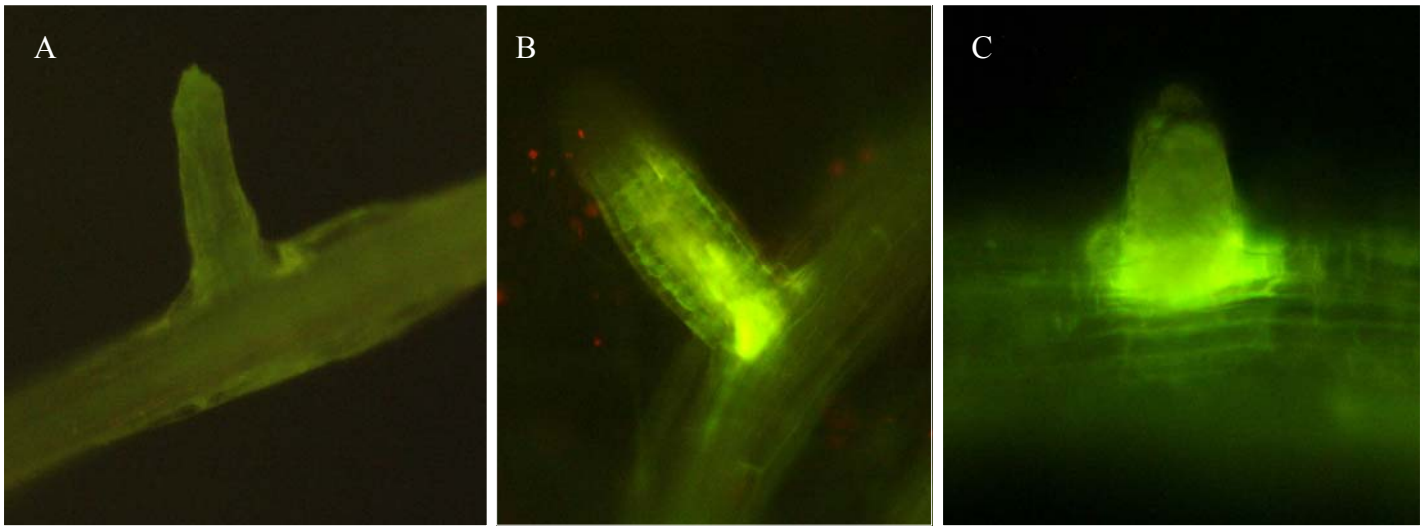


Figure 27. Fluorescence micrographs of *Arabidopsis* roots showing green fluorescence at the branching points of secondary roots. A, non-transformed *Arabidopsis* plant; B, *HMG2:GFP* putatively transformed plant line L14; C, *HMG2:SSP:GFP* putatively transformed plant line L60. This is consistent with known expression pattern of *HMG2*.

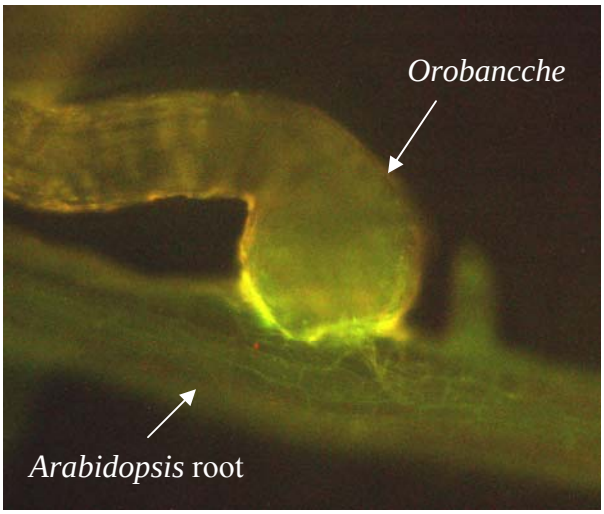


Figure 28. Fluorescence micrograph of wild type *Arabidopsis* root showing green fluorescence at the site of *Orobanche* attachment.

Appendix 2

Additional Gene Constructs Containing Sarcotoxin IA

Additional gene constructs containing the sarcotoxin IA gene were created because they may be useful for future research. Because sarcotoxin IA localized in the plant cytosol may be toxic to the plant, it is important to target the sarcotoxin IA protein for secretion to the extra-cellular space. Thus, additional variants of the *HMG2:SSP:SARCO* construct were made using the potato patatin signal peptide (*PSP*), which functions well in plants (Medina-Bolivar and Cramer, 2004). A summary of the additional sarcotoxin IA gene constructs generated is presented in Table 5 and Figure 29.

Materials and Methods

The previously generated *HMG2:PSP:GFP* construct, described in Chapter II, was digested with *Xba*I and *Sst*I to remove the *GFP* gene, which was replaced by the *SSP:SARCO* gene isolated as an *Xba*I-*Sst*I fragment from the pET-3 plasmid. This resulted in an *HMG2:PSP:SSP:SARCO* construct.

The *HMG2:PSP:SSP:SARCO* gene construct contained two signal peptides, each with a start codon, which may interfere with translation, so we designed two additional gene constructs. The first one lacks the *SSP* start codon and has only the *PSP* start codon, while the second one contains the *PSP* but lacks the *SSP*. In the first case sarcotoxin IA was amplified using the following primer pair:

SARCO-3, 5'- CTAGAGCTCTCAACCTCCTCTGGCTGTAGCAGC-3' and

SARCO-4, 5'-ACGTCTAGAGGTTGGTTGAAAAAG-3'

These primers generate flanking restriction sites for the enzymes *Sst*I and *Xba*I (underlined). This gene construct was named *HMG2:PPS:SSP:SARCO-A*. In the second

case, the fly signal peptide was removed by PCR amplification of sarcotoxin IA using the SARCO-3 primer and the following primer:

SARCO-5, 5'-ACGTCTAGAAATTTCCAGAAC-3', which generates a restriction site for *Xba*I (underlined). This second gene construct was named *HMG2:PSP:SARCO.* The PCR program was as described in Chapter III (see Materials and Methods). PCR products were digested with *Xba*I and *Sst*I, and then gel purified using the QIAgen gel purification kit according to the manufacturer's instructions. The pBC containing *HMG2:PSP:SSP:SARCO* gene construct was digested with *Xba*I and *Sst*I to remove the *SSP:SARCO* gene. The resulting linear plasmid was gel purified and then used to subclone the previously digested PCR products. The identities, orientation, and junctions of these gene constructs were confirmed by sequencing.

Finally, and in order to be obtain sufficient amount of sarcotoxin IA peptide that we can use to generate anti-sarcotoxin IA antibodies, we generated an additional gene construct that would allow constitutive expression of sarcotoxin IA by fusing the sarcotoxin IA gene to the *de35S* constitutive promoter. We also added a 6x-Histidine tag to the N-terminal end of sarcotoxin IA. The HIS tag will allow purification of sarcotoxin IA peptide using His-bind chromatography columns. This gene construct was named *de35S:SARCO-HIS* and was designed as follows: An R8-2 plasmid containing the *de35S* promoter was digested with *Hind*III and *Kpn*I to isolate the *de35S* promoter. The sarcotoxin IA gene was amplified using pET-3 containing sarcotoxin IA gene as a template. The primer pair and the PCR program used are as described in Chapter III (see Materials and Methods). This primer pair generated flanking restriction sites for the enzymes *Sst*I and *Xba*I. A PCR product corresponding to the sarcotoxin IA-HIS gene was

digested with *Sst*I and *Xba*I and then gel purified using the QIAgen gel purification kit according to the manufacturer's instructions. The previously isolated *de35S* promoter and the digested sarcotoxin IA were then subcloned into a pBC plasmid digested with *Hind*III and *Sst*I enzymes. The identity, orientation, and junctions of this gene were confirmed by sequencing.

Plant Transformation

Gene constructs containing sarcotoxin IA gene were subcloned into *A. tumefaciens* vector $pBIB_{hyg}$ (Becker, 1990). This vector contains the appropriate border sequence to aid in the transfer of T-DNA into the plant genome and hygromycin resistance gene to allow selection of putatively transgenic plants on hygromycin-containing medium. $pBIB_{hyg}$ vectors containing sarcotoxin IA constructs were subsequently introduced into LBA4404 and GV3101 strains of *A. tumefaciens* to be used for tobacco and *Arabidopsis* transformation, respectively. Tobacco plant transformation was performed using the method described in Chapter II (see Materials and Methods), while *Arabidopsis* plants were transformed according to the protocol described in Appendix 1.

Results and Discussion

To screen putatively regenerated plants for the presence of the sarcotoxin IA gene, PCR was conducted using the *HPT* specific primers and the PCR protocol described in Chapter III (see Materials and Methods). PCR results indicated that the tobacco and *Arabidopsis* plants regenerated contained the sarcotoxin IA gene (data not shown). A summary of the gene constructs and the putatively transformed tobacco and *Arabidopsis* plants generated are presented in Figure 29 and table 5.

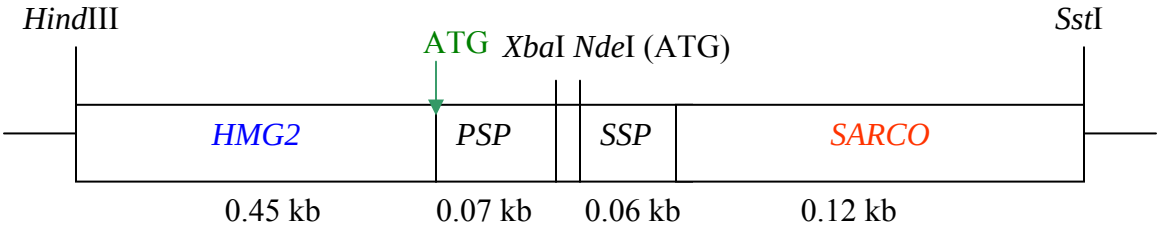
The *de35S:SSP:SARCO-HIS* gene construct was successfully introduced into *A. tumefaciens*. However plant transformation with *Agrobacterium* containing this construct was unsuccessful and putatively transformed tobacco and *Arabidopsis* plants could not be recovered even after multiple attempts. This suggests that constitutive expression of sarcotoxin IA may be lethal to plants.

Table 5. Summary of sarcotoxin IAgene constructs and transgenic plants generated, including those not characterized.

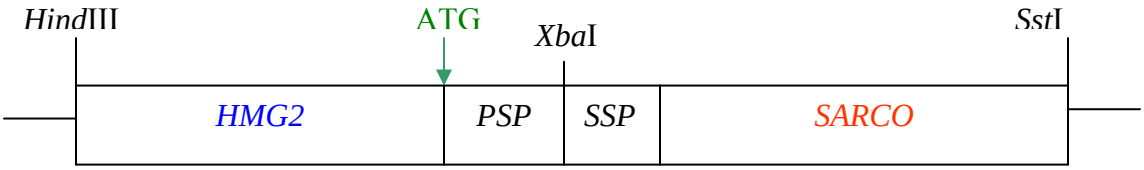
Construct	Rationale	Transgenic plants	
		Tobacco	*Arabidopsis*
HMG2:SSP:SARCO	Test efficacy of sarcotoxin IA for *Orobanche* resistance using the endogenous fly signal peptide	9 lines	30 lines
HMG2:SSP:SARCO-HIS	As above but allows localization of sarcotoxin IA using antibodies for the HIS tag; may also increase sarcotoxin stability	19 lines	Many (not evaluated)
HMG2:PSP:SSP:SARCO	Adds the plant signal peptide to that of the fly for targeting sarcotoxin IA to apoplast	65 lines	Many (not evaluated)
HMG2:PSP:SSP:SARCO-A	Removes the start codon of the sarcotoxin IA signal peptide; This may optimize production and export of sarcotoxin IA from plant cells	25 lines	Many (not evaluated)
HMG2:PSP:SARCO	Replace the fly signal peptide with the plant signal peptide; A modification of the *HMG2:PSP:SSP:SARCO* above	24 lines	Many
de35S:SSP:SARCO-HIS	Constitutive expression of sarcotoxin IA gene to obtain sufficient amount of sarcotoxin-His that could be further purified using histidine columns	0 Lines*	0 Lines*

* This gene construct was successfully introduced into *A. tumefaciens.* However, putatively transformed plants could not be recovered.

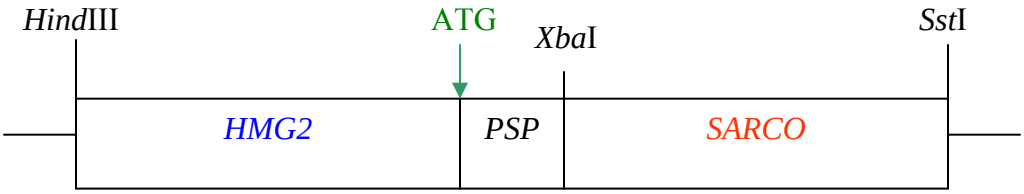
HMG2:PSP:SSP:SARCO



HMG2:PSP:SSP:SARCO-A



HMG2:PSP:SARCO



de35S:SSP:SARCO-HIS

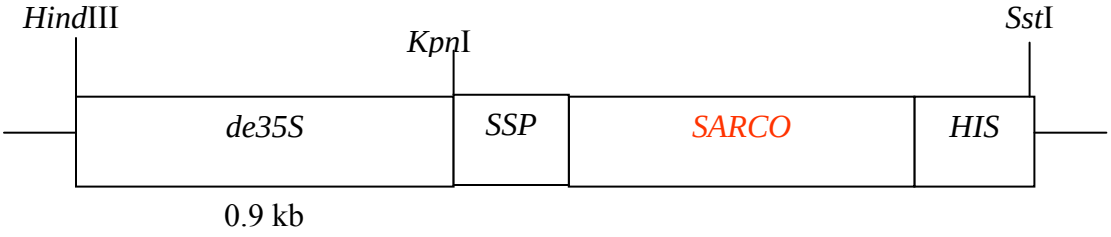


Figure 29. Additional gene constructs containing sarcotoxin IA generated, but not characterized during this project. *HMG2* is the promoter from the tomato *HMGR; de35S* is the constitutive promoter from the Cauliflower Mosaic Virus*; SARCO* encodes sarcotoxin IA gene; *PSP* is the patatin signal peptide; *SSP* is the sarcotoxin IA signal peptide.

Appendix 3

Optimization of *Orobanche* Infection and Analysis in Pot Studies.

Resistance experiments conducted in Chapter III used Metro-Mix 360 potting medium. In this type of medium, *Orobanche* successfully attached to host plants and grew and developed shoots. However, when we try to harvest *Orobanche* shoots, it was very difficult and tedious to clean tobacco roots off the potting medium. To facilitate and accelerate the process of harvesting *Orobanche* shoots in the future experiments, we grew untransformed tobacco plants in mixes of several growing medium and we examined the effort required to harvest *Orobanche* shoots. Potting media used are: Metro-Mix 360 (Scotts-Sierra Horticultural, Marysville, Ohio), Profile (PROFILE Products LLC, Buffalo Grove, Illinois), and Loam soil. *Orobanche* inoculum in each pot was 400 mg/L. Results are presented in Table 6.

Results indicate that a combination of potting medium and Loam soil (1:2) is advantageous over other types of potting media because not only did *Orobanche* grow and develop normally but *Orobanche* shoots were easily harvested, without even the need to use water to wash the potting media off tobacco roots. Moreover, the time required to harvest *Orobanche* shoots was very short (less than 5 min) when using the Loam soil: profile mix (1:2), compared to other types of potting media.

Soil combination	***Orobanche* growth**	**Difficulties to harvest *Orobanche* shoots**	**Time needed to harvest *Orobanche* shoots from one pot**
Profile only	None	N/A	N/A
Metromix 350 only	Good	Difficult	30 min
Metromix 350 : profile (1:1)	Good	Medium	20-30 min
Metromix 350 : profile (2:1)	Good	Medium	20-30 min
Metromix 350 : profile (1:2)	Good	Medium	20-30 min
Loam soil : profile (1:2)	Good	Easy	Less than 5 min

Table 6. *O. aegyptiaca* growth and harvest in different mixtures of potting media.

Optimization of *Orobanche* Inoculum in Pot Studies.

For any resistance evaluation, inoculum level is an important parameter, so we conducted an additional experiment to optimize inoculation rates of *O. aegyptiaca* seeds for our studies. Non-transformed tobacco plants were grown in Loam soil : profile (1:2) potting medium containing various inoculum levels of *Orobanche* seeds. Five weeks later, plants were harvested and development of *Orobanche* shoots was observed. Results are presented in Table 7.

Results indicate that soil inoculated with 10 or 30 mg/L produced very few *Orobanche* shoots were observed and sometimes no shoot could be observed. This may cause misleading conclusions when conducting *Orobanche*-resistance experiments because the absence of detectable *Orobanche* shoots may not be the result of host resistance, but due to low number of *Orobanche* seeds in the vicinity of host roots.

On the contrary, potting medium inoculated with 400 mg/L of *Orobanche* seeds showed high levels of *Orobanche* shoots. In this case, tobacco plants with partial resistance (e.g. those expressing sarcotoxin IA) may be unable to tolerate a high number of parasites, which may explain why were could not see complete resistance when we tested resistance of the *HMG2:SSP:SARCO* lines L03, L05, and L07 against *Orobanche*. Although we do not know the level of *Orobanche* infestation in a typical field, a 400 mg/L inoculum used in these experiments far exceeds what other researchers have used. For example, Aly and coworker used 10 mg/L.

In summary, we suggest that future experiments involving evaluation of tobacco for resistance to *Orobanche*, an inoculum rate of 60 to 100 mg/L should be used.

Inoculum	**Development of *O. aegyptiaca* shoots**
400 mg/L	High
100 mg/L	Medium
60 mg/L	Medium
30 mg/L	Low (very few shoots, sometimes no shoot)
10 mg/L	Low (very few shoots, sometimes no shoot)

Table 7. Development of *O. aegyptiaca* in Loam soil : profile (1:2) potting media containing different inoculum levels.

References

Bechtold N, Ellis J, Pelletier G (1993) In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris Life Sci. **316:** 1194-1199

Becker D (1990) Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. **18:** 203

Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. Plant Cell **4:** 1229-1236

Medina-Bolivar F, Cramer CL (2004) Production of recombinant proteins in hairy roots cultured in plastic sleeve bioreactors. In: Recombinant Gene Expression: Reviews and Protocols. P. Balbas and A. Lorence, eds., Humana Press **(in press)**

VITA

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Noureddine Hamamouch