Protein subcellular targeting in *Trichoderma aggressivum* f. *aggressivum*

by

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Abstract

Trichoderma aggressivum f. *aggressivum* is a filamentous soil fungus. Green mold disease of commercial mushrooms caused by this species in North America has resulted in millions of dollars in lost revenue within the mushroom growing industry. Research on the molecular level of *T. aggressivum* have just begun with the goal of understanding the functions of each gene and protein, and their expression control. Protein targeting has not been well studied in this species yet. Therefore, the intent of this study was to test the protein localization and production levels in *T. aggressivum* with green fluorescent protein (GFP) with an intron and tagged with either nuclear localization signal (NLS) or an endoplasmic reticulum retention signal (KDEL). Two GFP constructs (with and without the intron) were used as controls in this study.

All four constructs were successfully transferred into *T. aggressivum* and all modified strains showed similar growth characteristics as the wild type non-transformed isolate. GFP expression was detected from all modified *T. aggressivum* with confocal microscopy and the expression was similar in all four strains. The intron tested in this study had no or very minor effects as GFP expression was similar with or without it. The GFP signal increased over a 5 day period for all transformants, while the GFP to total protein ratio decreased over the same period for all transformants. The GFP-KDEL transformant showed similar protein expression level and localization as did the control transformant lacking the KDEL retention signal. The GFP-NLS transformant similarly failed to localize GFP into nucleus as fluorescence with this strain was virtually identical to the GFP transformant lacking the NLS. Thus, future research is required to find effective localization signals for *T. aggressivum*.

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Table of contents

	Page
Abstract	**2**
Acknowledgments	**3**
Table of Contents	**4**
List of Tables	**8**
List of Figures	**9**
List of Tables	**8**
1. Introduction	**11**
1.1 Filamentous Fungi	11
1.2 *Trichoderma aggressivum* f. *aggressivum*	11
1.2.1 Classification of *Trichoderma aggressivum*	14
1.2.2 *Trichoderma aggressivum* and green mold disease	15
1.3 Protein Targeting	16
1.3.1 ER Targeting & KDEL Signals	19
1.3.2 Nucleus Targeting and NLS	21
1.4 Green Fluorescent Protein (GFP)	23
1.4.1 GFP expression in fungi	25
1.5 Fungal Transformation Systems	26
1.5.1 Overview of Fungal Transformation	26
1.5.2 *Agrobacterium tumefaciens*	27
1.5.2.1 Ti-plasmid	29
1.5.2.2 Initiation of transformation	29

1.5.2.3 Attachment of *Agrobacterium* to plant cells 29

1.5.2.4 T-DNA Processing 30

1.5.2.5 T-DNA transfer 30

1.5.2.6 T-DNA Integration 31

1.5.3 *Agrobacterium*-Mediated Transformation 32

1.5.4 Binary Vector 33

1.6 Study Objective 34

2. MATERIALS AND METHODS **36**

2.1 Culture Media and Reagents 37

2.2 Bacterial and Fungal Strains 37

2.3 Plasmid Sources 38

2.4 Cloning GFP and Tagged GFP 38

2.4.1 PCR 38

2.4.2 Digestion and Ligation 41

2.4.3 Agarose Gel Electrophoresis 41

2.4.4 PCR Purification and DNA Gel Extraction 42

2.4.5 Transformation of *E. coli* 42

2.4.5.1 Preparation of competent cells 42

2.4.5.2 Transformation of *E. coli.* 42

2.4.6 Plasmid DNA Isolation 43

2.5 Binary Vector Construction 44

2.6 Transformation of *Agrobacterium* 45

2.6.1 Preparation of *Agrobacterium* competent cells 45

2.6.2 *Agrobacterium* transformation with binary vectors 45

2.7 *Agrobacterium*-Mediated Transformation 46

2.8 Fungal Genomic DNA Extraction 47

2.9 Sequence Analysis 47

2.10 Microscopy 47

2.11 GFP Quantification 48

2.12 Bio-Rad Protein Assay 48

2.13 Western blot 49

3. Results **51**

3.1 Plasmid Construction and Gene Transformation 51

3.1.1 PCR 53

3.1.2 GFP Constructions in PUC19 54

3.1.3 Binary Vector 54

3.2 *Trichoderma aggressivum* Transformation 55

3.2.1 *T. aggressivum* Confirmation 55

3.2.2 Mitotic Stability 56

3.2.3 Culture Phenotypes 56

3.3 GFP Analysis 59

3.3.1 GFP Expression 59

3.3.2 GFP Localization 63

3.3.3 GFP Quantification 64

3.3.4 Western Blot Analysis of GFP Production 66

4. Discussion **68**

4.1 Phenotype of Transformed *T. aggressivum* 68

4.2 GFP Quantification 68

4.3 GFP Expression 69

4.3.1 Intron and GFP Expression 69

4.3.2 GFP Expression and KDEL 69

4.3.3 GFP Expression and NLS 70

4.4 Future Research 71

5. Summary 72

References 73

APPENDIX 84

List of Tables

		Page
Table 1.	PCR reaction steps for GFP cloning	39
Table 2.	List of primers used for PCR reactions	40

List of Figures

	Page
Figure 1 - A. Microscopy picture of conidiophores of *Trichoderma aggressivum* f. *aggressivum* B. Simple diagram of asexual life cycle of *Trichoderma aggressivum* f. *aggressivum.*	13
Figure 2 - Overview of sorting of nuclear-encoded proteins in eukaryotic cells.	18
Figure 3 - Schematic diagram of the *Agrobacterium* infection process. ①chem ical signaling; ②Bacterial attachment; ③ vir gene induction; ④ T-DNA processing; ⑤ T-DNA transfer; ⑥ Nuclear t argeting and T-DNA integration.	28
Figure 4 - Simple diagram of PCR of GFP. Primers are composed of a complementary sequence and an overhang. The overhang of front primer contains intron sequence part I or II, *SacI* reco gnition sequence and protection base pairs. The reverse contains KDE L or NLS sequence, *BamHI* recognition sequence and protection base pairs.	39
Figure 5 - Maps of T-DNA constructions contain a hygromycin resistance gen e and GFP/ iGFP/ IGK/ IGN expression cassette. Original GFP coding sequence is in upper case, intron sequence is in lowercase and are underlined. The KDEL and NLS sequence are in uppercase and are underlined.	43
Figure 6 - Diagram of GFP cloning and binary vector construction	51
Figure 7 - Gel picture of first PCR for GFP constructs	52
Figure 8 - Gel picture of second PCR for IGFP, IGK, IGN	52
Figure 9 - Gel picture of GFP constructions released from cloning vector PUC19	53
Figure 10 - Gel picture of GFP constructions in binary vector pFGC1008 which was taken after plasmid DNA isolation from E. coli.	54
Figure 11 - Gel picture of confirmation PCR with *T. aggressivum* specific primer ALAN1 and ALAN2.	55
Figure 12 - Pictures of phenotypic properties of each transformed *T. aggressivum* grown on MEA, BD-MEA, YPD media for 6 days at 28°C	57
Figure 13 - Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 2 days at 28°C	59

Figure 14 - Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 4 days at 28°C 60

Figure 15 - Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 6 days at 28°C 61

Figure 16 - Confocal micrographs of green fluorescent protein: 3 day old mycelia of IGK, IGFP transferred *T. aggressivum* stained with ER-Tracker. 62

Figure 17 - Confocal micrographs of green fluorescent protein: 3 day old mycelia of IGN, IGFP transferred *T. aggressivum* stained with DAPI. 63

Figure 18 - GFP amount in modified *T. aggressivum* sample at different length of culture time 64

Figure 19 - Percentage of GFP in total protein of 4 modified *T. aggressivum* at day 3 to 7 of culture. 65

Figure 20 - Western blot picture of 4 modified *T. aggressivum* with day 3, day 5, and Day 7 broth cultured samples. 66

1. Introduction

1.1 F ilamentous Fungi

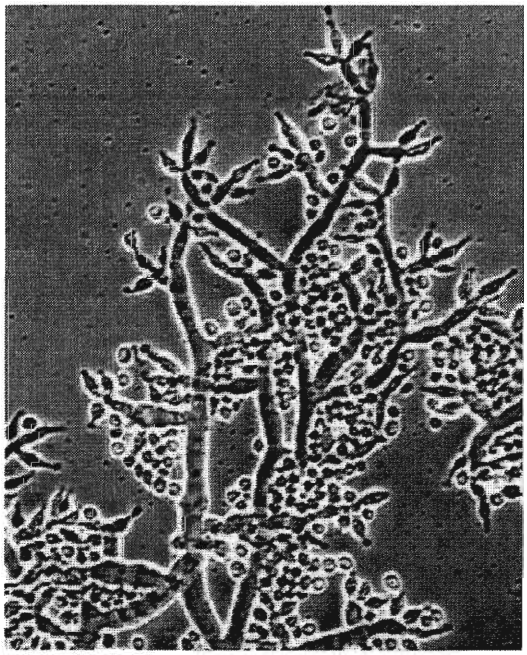
Filamentous fungi include all species of microscopic fungi that grow in the form of multicellular filaments, called hyphae. In contrast, microscopic fungi that grow as single cells are called yeasts. For most fungi, a connected network of these tubular branching hyphae have multiple, genetically identical nuclei and are considered a single organism, referred to as a colony or in more technical terms, mycelium (Smith & Berry, 1978a). Filamentous fungi influence our everyday lives in such diverse areas as industry, medicine, agriculture, and basic science. Some are used for drug and enzyme production, with or without genetic engineering. Many fungi are recognized as important pathogens of humans, plants, animals and even other fungi. Filamentous fungi are responsible for billions of dollars in crop losses annually from disease and post-harvest food spoilage.

1.2 *Trichoderma aggressivu m* f. *aggressivum*

Trichoderma species are asexual filamentous fungi with teleomorphs belonging to the Hypocreales order of the Ascomycota kingdom (Kredics et al., 2005). Many species in this genus can be characterized as avirulent plant symbionts. Cultures in the lab are typically fast growing at 25-30°C. At optimal temperature, mycelium normally takes 5-7 days to cover the whole plate (75mm diameter), conidia then appear on the plate with the color of green or yellow (Samuels et al., 2010). A yellow pigment may be secreted into the agar. *Trichoderma* species have no sexual life cycle but instead produce only asexual spores. Asexual sporulation results in single cell

conidia when the mycelium is experiencing conditions of stress (Smith & Berry, 1978b) (Fig.1). The specialized spore represents a means of propagation, survival and dispersal.

Most fungi in the genus *Trichoderma* are "friendly" to plants and several strains have been developed as biocontrol agents against plant pathogens (Harman, 2006). Once they come into contact with roots, they colonize the root surface or cortex for protection and they are also able to promote the growth of plant roots (Chang et al., 1986). In addition to colonizing roots, *Trichoderma* spp. also provide protection by attacking phytopathogenic fungi. The possible inhibitory mechanisms include antibiosis, parasitism, inducing host-plant resistance, and competition. Although most *Trichoderma* spp. are welcome partners for plants, they can be parasitic with other fungi. *Trichoderma aggressivum* is the causal agent of green mold, a disease of the cultivated button mushroom, *Agaricus bisporus*. *Trichoderma* species can also cause allergies in people with sensitivity to mold.



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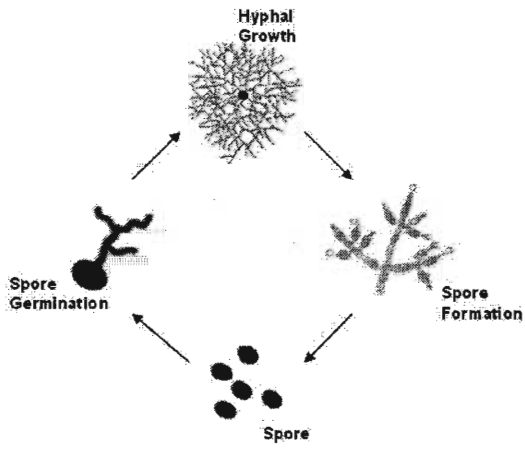


B

Fig.1 A. Microscopy picture of conidiophores of *Trichoderma aggressivum* f. *aggressivum* (Samuels et al., 2002) B. Simple diagram of asexual life cycle of *Trichoderma aggressivum* f. *aggressivum*

1.2.1 Classification of *Trichoderma aggressivum*

Trichoderma aggressivum was initially characterized as a form of *Trichoderma harzianum.* Four biotypes associated with green mold disease were identified as *T. harzianum* Rifai, and referred to as Th1, Th2, Th3 and Th4. All four biotypes were linked to the green mold disease on *Agaricus bisporus* at the beginning because all of them were isolated from the contaminated compost (Muthumeenakshi et al. 1994, Castle et al. 1998). However, later research found only the contamination of Th2 and Th4 are associated with recognizable yield loss while Th1 or Th3 alone does not appear to cause green mold disease (Gams and Meyer 1998). Th2 and Th4 were then identified as the 'aggressive' biotypes, the other two as 'non-aggressive'. Scientists began to question the formal taxonomy of *Trichoderma* spp. because of the strong biological differences between aggressive and non-aggressive biotypes. After further research and the data of molecular sequences, Th1 is now recognized as *T. harzianum* s. str. and has been reported as an inhabitant of most mushroom farms without causing green mold disease (Gams and Meyer 1998), although Krupke et al. (2003) showed that Th1 can also produce antifungal compounds which inhibit *A. bisporus* mycelial growth in the lab. Furthermore, Th3 has been reclassified as *T. atroviride* (Gams and Meyer 1998, Castle et al. 1998). Th2 and Th4 cause green mold disease in commercially grown *A. bisporus* in Europe and North America respectively (Seaby 1996). They were reidentified as *Trichoderma aggressivum* f. *europaeum* (formally Th2) and *Trichoderma aggressivum* f. *aggressivum* (formally Th4) since they are strongly separated from their closest relative, *T. harzianum*, in sequence of the ITS-1 region of nuclear rDNA and an approximately 689bp fragment of the protein coding translation elongation factor gene (EF-1a) (Samuels et al., 2002)

1.2.2 *Trichoderma aggressivum* and green mold disease

Agaricus bisporus, also known as white button mushroom, is an edible basidiomycete and the number one commercial mushroom cultured in more than 70 countries. In 1997, the world production of *A. bisporus* reached 1,955,000 tons, representing 31.8% of all cultivated edible and medicinal mushrooms (Chang, 1999). Since the mid 1980s, commercial production of *A. bisporus* has been seriously affected by 'green mold disease' which leads to enormous economical loss. The first report of this disease was in Ireland and then the UK (Seaby 1987). A similar disease struck North American mushroom farms in the early 1990s (Rinker 1993. cited from Krupke et al. 2003) and then Spain (Hermosa et al. 1999. cited from Samuels et al., 2002). In each case, the causative fungus colonizes compost and results in no mushroom fruiting and significant yield loss. *Trichoderma aggressivum* f. *europaeum* and *Trichoderma aggressivum* f. *aggressivum* are identified as the causal agents of this disease in Europe and North America respectively (Seaby 1996).

Mushroom compost infected with *T. aggressivum* often appears normal even two weeks after inoculation with *A. bisporus* mycelia. After sporulation, a dark green color will appear on the compost surface at the source of infection (Rinker 1996). At this point, green mold proliferates rapidly through the mushroom compost, devastating what appeared to be a healthy crop. Infected areas produce no mushrooms. Mushroom compost contains a large percentage of lignin and little cellulose, which makes it highly selective for basidiomycete (Matcham & Wood 1992). Only basidiomycetes have the extracellular enzyme laccase which is capable of utilizing this lignin as a carbon source. It is not surprising, therefore, to find that *T. aggressivum*, an ascomycete, requires the presence of *A. bisporus* mycelium in order to survive on compost (Seaby 1987). Further evidence shows active *A. bisporus* mycelium can be isolated from infected

compost, indicating that the mushroom mycelium is still viable during and after *Trichoderma* infection (Krupke, 2001).

Many antifungal compounds and enzymes produced were suggested to be involved in the aggressive biotypes' inhibition of *A. bisporus* growth. Mumpuni et al. (1998) published findings suggesting that Th1 and Th2 produce volatile metabolites that inhibit *Agaricus bisporus* growth. Krupke et al (2003) indentified one of two compounds produced by *T. aggressivum* as a methyl-isocoumarin. Later, Guthrie and Castle (2006) showed that *T. aggressivum* chitinases were upregulated during co-cultivation with *A. bisporus* and suggested that these enzymes may be important in green mold disease.

1.3 Pro tein Targeting

Eukaryotic cells evolved membrane-bounded compartments specialized to provide energy, to synthesize lipids, carbohydrates, proteins, and nucleic acids, and to degrade cellular constituents (Pollard et al. 2004). These subcellular compartments, called organelles, have distinctive chemical compositions and hold a monopoly of a given task. A semipermeable membrane surrounds each organelle to allow it function independently. Organelles can provide optimal chemical environments (pH, reactant concentration) for certain reactions and can also protect cells from potentially dangerous activities, such as the degradative enzymes in lysosome. This division has many advantages but also presents cells with challenges of material transfer in or out of individual organelle, which means cross membrane, since these organelles are not autonomous and need to coordinate with other cell compartments.

Protein targeting is the mechanism by which a cell transports proteins to the appropriate positions in the cell or outside of it. The biosynthesis of proteins takes place in the ribosomes

which are located in the cytoplasm. Newly synthesized proteins either remain in the cytoplasm or move to their final destinations in the ER, nucleus, mitochondria, chloroplasts, etc (Fig.2). Günter Blobel was awarded the 1999 Nobel Prize for his findings about protein targeting and protein localization signals. He showed for the first time that the protein itself has short signal peptides functioning like a "postal code" that can recognized by different receptors located on the membrane of different organelles to guide them into their final destinations. Proteins travel to the same organelle process the same or very similar signals. Correct sorting is crucial for the cell, any mistake can lead to disease, even death.

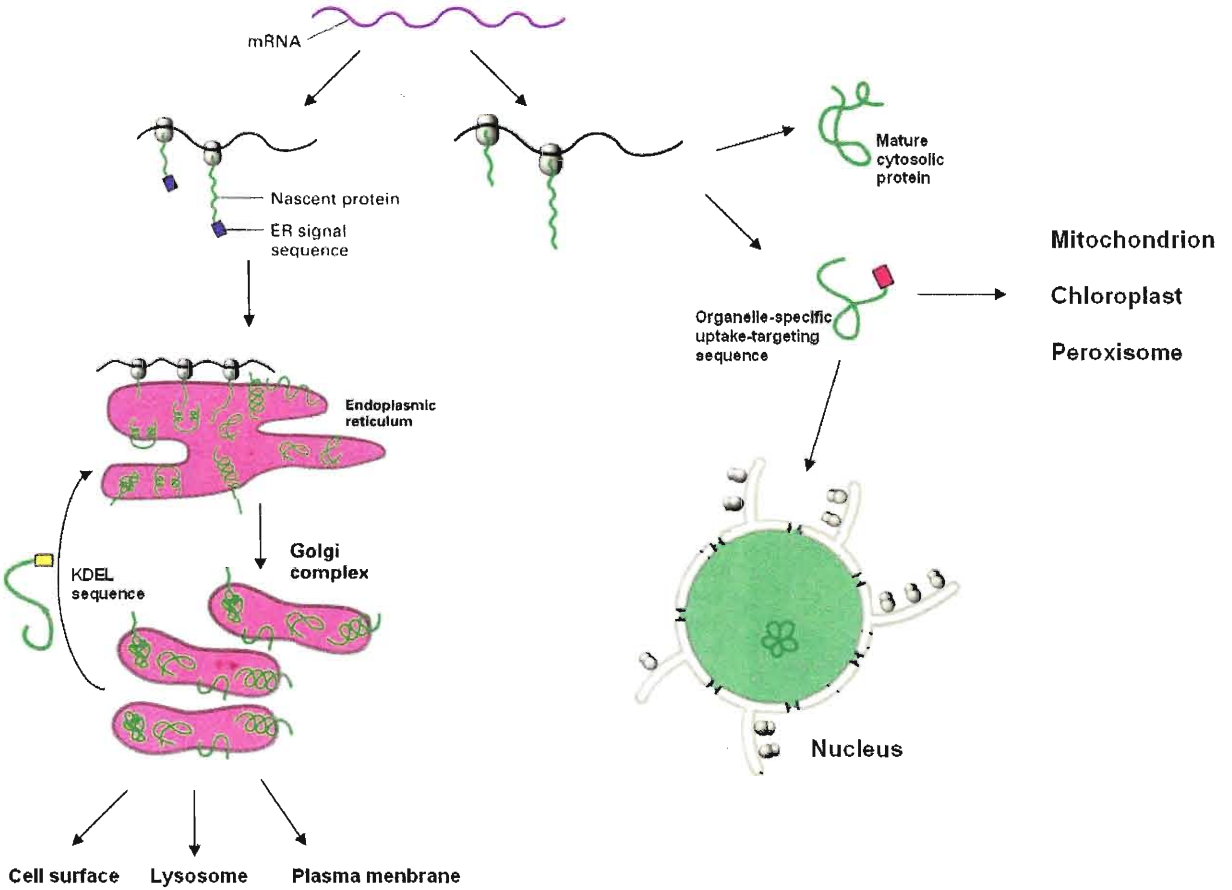


Fig. 2 Overview of sorting of nuclear-encoded proteins in eukaryotic cells.

1.3.1 ER Targeting & KDEL Signals

The endoplasmic reticulum (ER) provides both an expanded membrane surface for carrying out vital cellular functions, including protein and lipid biosynthesis and an internal compartment (lumen) that collects proteins synthesized in the cytoplasm for modification and delivery into the secretory pathway (Pollard et al. 2004). About one third of all cellular proteins are imported into the lumen of the ER or integrated into its membranes.

Proteins transported to the endoplasmic reticulum have a sequence consisting of 5-10 amino acids on the N-terminus, named the ER signal. The protein is guided to the ER by a signal-recognition particle (SRP), which moves between the ER and the cytoplasm (Pollard et al. 2004). As soon as the protein is synthesized, the SRP binds to the signal peptide extruded from the ribosome, then the ribosome-SRP complex binds to the SRP receptor on the ER membrane to transport proteins into ER lumen. ER proteins have two destinations; they may stay in ER lumen or go into the secretory pathway by transporting to the Golgi complex. ER proteins normally have a retention signal to keep it away from the secretory pathway. Proteins with a transport signal will end up in the Golgi apparatus, and proteins that do not have a retention signal nor a transport signal would be consequently exported by default (Harter & Wieland 1996).

Munro and Pelham (1987) first found three soluble ER proteins (grp78, grp94, and protein disulphide isomerase) that share a common carboxy-terminal tetrapeptide sequence, lys-asp-glu-leu (KDEL). One year later, a similar HDEL (his-asp-glu-leu) in yeast was found and the authors proved that this signal sequence is essential for protein ER retention (Pelham et al., 1988). KDEL/HDEL signal peptides can be deleted and will lead to secretion of ER protein, while addition of the tetrapeptide to a normally secreted protein leads to its retention in the ER.

The function of these signals was first thought to be retention of the protein in the endoplasmic reticulum, until a putative sorting receptor was identified on the Golgi membrane in yeast (Semenza et al. 1990). This finding means that ER proteins are carried along the secretory pathway with other proteins, and are recovered by a receptor-mediated process in a post-ER compartment (Pelham 1990). The receptor is the product of the *ERD2* gene, and silencing of this gene leads to ER protein secretion while overexpression improves retention of HDEL-containing proteins. When K/HDEL-containing proteins are present in the Golgi, the receptor recognizes the signal peptide and binds to the protein forming a receptor-ligand complex. The complex is then incorporated into COPI-coated vesicles budding from Golgi that return to the ER where the ER proteins are released (Harter & Wieland 1996). Empty receptors would be returned to their original localization and prepare for the next ligand binding (Wilson et al. 1992). The interaction between KDEL receptor and ligand is very sensitive to pH (Wilson et al. 1993), with a maximal binding between pH 5 and 6. The pH along the secretory pathway becomes increasingly acidic from the ER (pH 7.45) towards the TGN (trans Golgi network) (pH 6.58) (Anderson & Pathak 1985) and may well explain the binding in Golgi and release in ER. Although there is substantial information on how ER resident proteins are captured when they escape, there are still many unsolved mysteries in the functions of K/HDEL receptor in Golgi-ER transport.

The application of KDEL signal to gene transformation has proved functional. Many researchers found that protein constructs tagged with a C-terminal KDEL sequence can not only result in protein ER localization, but also increase the expression of the foreign protein. Wandelt *et al.* (1992) transferred a vicilin coding gene with a novel carboxy-terminal KDEL encoding sequence in the protein being retained in the endoplasmic reticulum and accumulated up to 100 times greater than that of the unmodified vicilin in tobacco. Schouten et al. (1996) showed that

KDEL-tagged single-chain antibody (scFv) had an increased expression level (1%) in transgenic tobacco compared to the unmodified scFv (0.01%). In this study, we will also be expecting the correct ER localization and possible increase of expression of KDEL modified green fluorescent protein in *Trichoderma aggressivum* f. *aggressivum.*

1.3.2 Nucleus Targeting and NLS

The nucleus, the control center of a cell, is a membrane-enclosed organelle found in eukaryotic cells and contains most of the cell's genetic material. The function of the nucleus is to maintain the integrity of these genes and to control the activities of the cell by regulating gene expression. Molecules are frequently required to enter and exit the nucleus during normal cell function, but access is restricted and exchange to and from the nucleus is tightly controlled (Pouton et al. 2007). All transport, both passive and active, into and out of the nucleus, takes place via nuclear membrane-embedded nuclear pore complexes (NPC) (Wente 2000). The NPC was first discovered by Feldherr (1962) when studying the mechanism by which colloidal gold particles enter the nucleus. More than 30 different proteins, named nucleoporins (Nups), are involved in the composition of NPC, and each of these proteins is present in from 8 to 32 copies per NPC (Burczynski et al. 2006). Smaller molecules are able to pass through the NPC via passive diffusion, whereas molecules larger than 40 kDa enter the nucleus through an active transport process (Talcott & Moore 1999).

The active transport of macromolecular cargo between the cytoplasmic and nuclear compartments is facilitated by specific soluble carrier proteins. These carriers are collectively referred to as "karyopherins" (Radu et al. 1995), with those involved in import and export termed "importins" and "exportins", respectively. A large family of transport receptors, importin-β, is

responsible for selective recognition of the most of cargoes, as well as the physical translocation of the cargoes through NPC (Mosammaparast and Pemberton 2004). An N-terminal domain shared by most importin-β is involved in binding to Ran, a small GTPase, and it is believed to regulate the binding/release process between importin-β and cargo protein (Quimby and Dasso 2003). Ran, like all GTPases, cycles between two different states GTP-bound state, RanGTP, and a GDP-bound state, RanGD (McLane and Corbett 2009). RanGTP is primarily in the nucleus and RanGDP state in the cytoplasm because of the differential localization of Ran regulatory proteins (Steggerda and Paschal 2002). During import of proteins into the nucleus, importin-β recognizes and binds cargo in the cytoplasm in the absence of RanGTP (Gorlich and Kutay 1999). Following translocation into the nucleus, an import complex encounters RanGTP, which binds to the importin-β causing a decrease in affinity of the receptor for the cargo resulting in release of the cargo protein into the nucleus (Lee et al. 2005). Although nuclear import can occur through direct binding between cargo and importin-β, the best-studied system, classical nuclear protein import system, in which binding is mediated by importin-α. In classical nuclear protein import system, a specific class of proteins is recognized and bound by importin-α, then complexes with the importin-β95 to finish the translocation (Goldfarb et al. 2004).

Proteins that undergo active transport must possess a specific targeting signal, known as a nuclear localization signal (NLS), or they will be excluded from the nucleus. NLSs are amino acid sequences which act like a 'tag' on the exposed surface of a protein, and they are recognized by receptors from importin family which mediate nuclear transport. NLS will not be removed after the translocation for the possible repeating of this translocation process (Martoglio and Dobberstein 1998). NLSs can be subdivided into classical NLS (cNLS) and non-classical sequences (Jans et al. 1998). cNLSs are characterised by short stretches of basic amino acids

comprised primarily of lysine (K) and arginine (R) residues (Pouton et al. 2007). The classical nuclear import pathway involved cNLS is so far the best understood system for the transport of macromolecules between the cytoplasm and nucleus. cNLS mediate majority of nuclear protein import (Lange et al. 2008), however, there are still many other transport pathways mediated by non-classical NLS, such as proline-tyrosine NLS (PY-NLS). In this study, a NLS sequence was added to the C-terminus of green fluorescent protein to study its nuclear localization ability.

1.4 Gr een Fluorescent Protein (GFP)

Green Fluorescent Protein (GFP) was first discovered in 1962 as a companion protein to aequorin from the jellyfish *Aequorea victoria* (Shimomura et al. 1962). After Prasher et al. (1992) cloned and sequenced the cDNA of GFP, researchers start to realize its utility as a tool for molecular biology. GFP from the jellyfish *Aequorea victoria* is a 238-amino-acid, 27-kDa protein which absorbs blue light with local maxima of 395 and 475 nm and emits green light with a maximum of 508 nm. GFP has a typical beta barrel structure containing the fluorophore running through the center. The fluorophore is formed by posttranslational cyclization and oxidation of the amino acids Ser-65, Tyr-66, and Gly-67 (Lorang et al., 2001).

Formation of the fluorophore in GFP requires molecular oxygen but once formed it absorbs and emits light without any cofactors. Because fluorescence requires no cofactors, the GFP signal can be recorded in living cells with no disruption of cellular integrity. GFP fused to cellular proteins or polypeptides can be used as a subcellular tag that reports not only protein quantity but also subcellular location of individual proteins or whole organelles. The protein is stable and can be functionally expressed in a wide variety of organisms inducing prokaryotes, plants, mammalian cells, parasites, yeasts, and filamentous fungi.

Because GFP fluorophore formation requires oxygen, fluorescence is decreased under anaerobic or reducing conditions. Temperature also affects fluorescence, and at high temperatures (37 °C) GFP may fold into a nonfluorescent, sometimes insoluble form. Several mutants of GFP were developed to address the problem of lack of stability at higher temperature, to increase fluorescence intensity, and also to fit different hosts. The first major improvement was a single point mutation which the Ser-65 in fluorophore was mutated to Thr (S65T) (Heim et al., 1994). This mutation dramatically improved the spectral characteristics of GFP, resulting in increased fluorescence, photostability and a shift of the excitation peak to 488nm with the peak of emission kept at 510 nm. Although the longer excitation wavelength makes it hard to detect under UV light, it has a better match to the standard fluorescence filter sets, increasing the utility by the general researcher. Another mutant, which was a point mutation of Phe-64 to Leu (F64L), was developed by Thastrup et al.(1995).This mutant solved the poor protein folding and fluorescence at higher temperature such as 37 °C and was named enhanced GFP (eGFP). With the stability at 37 °C, eGFP was able to extend the usage of fluorescent proteins to mammalian cells. Pedelacq et al. (2006) reported a superfolder GFP, which is a series of mutations that allow GFP to rapidly fold and mature even when fused to poorly folding peptides.

Many other mutations have been developed which make the fluorescent protein family suit multiple purposes. The most well-known ones are the color mutants, in particular blue fluorescent protein (BFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (Heim et al. 1994; Ormö et al. 1996; Tsien 1998). BFP is a mutant generated from GFP contains an Y66H substitution while the cyan mutation derivatives the Y66W substitution, and YFP is accomplished by the T203Y mutation. Nowadays, fluorescent protein (FP) has formed a big family that is still expanding. New mutants of GFP were developed for different requirements

and new fluorescent proteins were found to complete the palette, especially for the orange and red color. The emission peak of FPs ranged from 383nm to 590nm and emission color from blue to far-red (Shaner et al., 2007).

1.4.1 GFP expression in fungi

Shortly after the cDNA of GFP had been cloned, GFP or its derivatives were successfully expressed and conferred fluorescence to bacteria, yeast, plants, mammal cell, and filamentous fungi. There are three basic requirements for GFP expression in fungi: a reliable transformation system; functional transcriptional control elements to drive expression; and the appropriate GFP-encoding gene (Spear et al., 1999). Several laboratories have reported that the wild-type cDNA of GFP will not yield a functional fluorescent signal in many fungi because it is not efficiently translated (Cormack et al., 1997; Maor et al., 1998). Successful fluorescent signal was detected in filamentous fungi, *Aspergillus nidulans*, when transformed with enhanced GFP (Fernandez-Abalos et al., 1998). Other research groups found that a 56bp intron close to the 5' end is required for efficient GFP expression in *Schizophyllum commune* (Lugones et al., 1999), *Phanerochaete chrysosporium* (Ma et al., 2001), the mushroom *Agaricus bisporus* and *Coprinus cinereus* (Burns et al., 2005). Only the transformants with an intron containing gene express GFP while genes from cDNA which the intron was removed tend to be silenced. Lugone's explanation is that introns and RNA splicing are necessary for mRNA accumulation, and this intron-dependent gene expression phenomenon exists in various homobasidiomycetes and ascomycetes such as *Aspergillus niger*, *Neurospora crassa*, and the species mentioned before. *Trichoderma harzianum* was successfully transformed with GFP and fluorescence signal was detected without any introns (Inglis et al., 1999). Although it is unknown whether or not an intron is necessary for mRNA accumulation or GFP expression in *Trichoderma* spp., it was still

introduced in our GFP constructs in this study to test its affect on GFP expression in *T. aggressivum*.

1.5 Fungal Transformation S ystems

1.5.1 Overview of Fungal Transformation

Nowadays, genetic transformation plays a significant role not only in the understanding of life, but also in the enhancement of particular traits. Transformation techniques are slightly different because of the unique characteristics of filamentous fungi due to their cellular organization and lifecycle. Numerous studies of fungal transformation have been done in the last three decades since the first one was achieved with *N. crassa* (Mishra et al. 1973). Common transformation methods for filamentous fungi include protoplasts/PEG, lithium acetate, electroporation, biolistics and *Agrobacterium* Mediated Transformation (AMT). Although many successful transformation systems have been developed, AMT is the most commonly used because of the inefficiencies of other systems. Protoplasts/PEG involves the preparation of protoplasts which are difficult to obtain for many fungi and is time consuming. High concentration of lithium acetate can induce cell permeability, which allows scientists to transform intact fungal cells, however this method has been so far limited to a few species (Olmedo-Monfil 2004). Expensive equipment is the limiting factor for electroporation and biolistics methods.

Agrobacterium tumefaciens, a Gram-negative, soil-living bacterium, is capable of inducing tumors in plants by transferring a segment of its DNA into plant genome. ATM is well-known and widely used in plant transformation for a long time and has recently been applied on

filamentous fungi. The simple protocol and less equipment requirement makes it useful in any biology lab.

1.5.2 *Agrobacterium tumefaciens*

Smith and Townsend first reported in 1907 that *Agrobacterium tumefaciens* (*Bacterium tumefaciens* at that time) was the cause of crown gall disease in Paris daisy. Smith et al. (1911) indicated *Agrobacterium tumefaciens* is capable of inducing tumors at wound sites on the stems, crowns and roots of hundreds of dicots. Later study found *A. tumefaciens* DNA sequences in host DNA in tumors, while the bacterium itself cannot be detected intracellularly (Schilperoort et al., 1967). This proved that bacterial DNA had been transferred into the plant cells. Since then many attempts have been made to elucidate the possible role of bacterial DNA in this possess. Zaenen et al. (1974) found that *A. tumefaciens* virulence depends on the presence of a large 'tumor-inducing' (Ti) plasmid. Chilton et al. (1977) discovered the transferred-DNA (T-DNA) region of the Ti plasmid is the DNA present in the genome of crown gall tumor cells. After the puzzle was put together, the first plant transformation with a recombinant gene using *Agrobacterium tumefaciens* as a vector was successfully carried out by Zambryski et al. (1983). *A. tumefaciens* has since been used to transform a variety of organisms other than plants, such as yeast, filamentous fungi and even animal cells. *Agrobacterium*-mediated-transformation (AMT) has become one of the most popular and successful transformation tools in molecular biology.

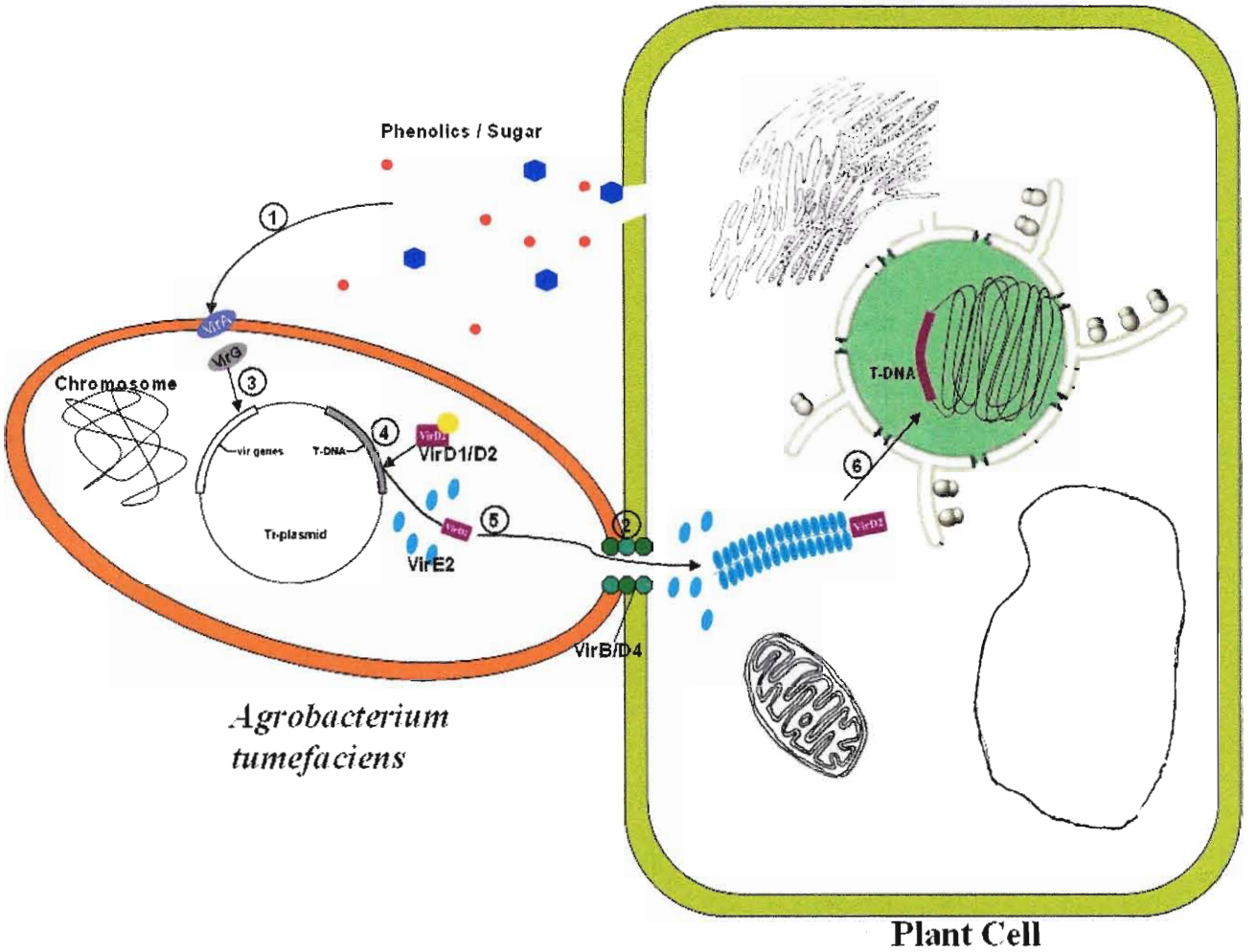


Fig. 3 Schematic diagram of the *Agrobacterium* infection process. ①chemical signaling; ②Bacterial attachment; ③ vir gene induction; ④ T-DNA processing; ⑤ T-DNA transfer; ⑥ Nuclear targeting and T-DNA integration.

1.5.2.1 Ti-plasmid

Two types of DNA were found in *A. tumefaciens* – chromosomal DNA and plasmid. The plasmid is called the tumor-inducing (Ti) plasmid, and is the source of tumorigenesis in plants. There are two main regions on the backbone of the Ti-plasmid that are important for transformation, the first being the transferred-DNA (T-DNA). The T-DNA of wild type *A. tumefaciens* carries the genes for biosynthesis of plant growth hormones and opines, tumor-specific compounds. As a transformation tool, the hormone encoding genes were deleted while the gene of interest is added to this region. T-DNA is flanked by left border (LB) and right border (RB) regions which are composed of 25bp imperfect direct repeats. Any DNA sequence found between these borders can be transferred and integrated into the host cells.

The second region of the Ti-plasmid carries the virulence (vir) genes which are essential for T-DNA transfer. Virulence proteins (virA-H) are encoded in this region and aid in T-DNA transfer, nuclear targeting and integration into the plant genome (Gelvin, 2000).

1.5.2.2 Initiation of transformation

The transfer process initiates when *Agrobacterium* perceives phenolic compounds (mainly acetosyringone) and sugar molecules released by wounded plant cells (Charles et al., 1992). These signaling chemicals are detected by transmembrane sensory protein VirA. Autophosphorylation of VirA and the subsequent transphosphorylation of cytoplasmic VirG protein result in the activation of vir gene transcription by binding to a conserved promoter region, called the vir box. The transcription of all other vir genes initiates T-DNA replication and transformation.

1.5.2.3 Attachment of *Agrobacterium* to plant cells

Agrobacterium attachment to plant cells is necessary for transformation. All non-attaching bacterial mutants are avirulent under normal inoculation conditions (Gelvin 2000). The attachment involves two steps. First, *A. tumefaciens* synthesizes acetylated polysaccharides, which form a slime layer or capsule around the cell. This capsule is proposed to play a role in the initial contact between the bacterium and host cells. Second, this loose contact is tightened and stabilized as cellulose fibrils secure the aggregating bacterial cells, forming a biofilm. T-DNA transfer from *Agrobacterium* to plant cells rapidly follows bacterial attachment.

1.5.2.4 T-DNA Processing

Multiple virulence genes are involved in the processing of T-DNA. First, VirD2 recognize the 25bp directly repeated sequence on the right border and nicks the T-DNA (Filichkin & Gelvin, 1993). After enzymatic cleavage, a single-strand copy of the T-DNA designated the T-strand, distinguished by the borders and marking the RB as the leading end. Multiple VirE2 proteins then bind to the T-strand, acting as protective coat against nucleolytic activity (endo/exonucleases). VirE2 coating proteins also help the T-complex to conform into a thin, unfolded shape, facilitating exportation to the plant cell and importation into the plant nucleus (Christie et al., 1988). The T-complex can form either in the bacteria cell or plant cell since the VirE2 can be exported to plant cells independently with the help of VirE1 protein.

1.5.2.5 T-DNA transfer

T-DNA complex transported into plant cells through a pilus-like transmembrane channel between the bacterium and the host cells, called 'T-pilus'. The mechanism of this interkingdom transfer has remained unclear. VirB operon which encodes eleven proteins and VirD4 were believed to be essential for this transport. A processed form of VirB2 is the polin

protein that helps form the pilus (Lai & Kado, 1998). VirB4, VirB11, VirD4 are ATPases and are believed to provide the energy necessary to drive the protein channel assembly and DNA exportation.

1.5.2.6 T-DNA Integration

Once inside the plant cytoplasm, the T-complex is directed to the nucleus where the T-strand can integrate into the host genome. VirD2 and VirE2 contain plant-active nuclear localization signals, which mediate importation through the nuclear pores (Gelvin, 2000). T-strand is capped with a VirD2 molecule at the RB end and is coated with multiple VirE2 molecules along its length, the T-complex is directly targeted to the plant nucleus after entering the cytoplasm. Finally, the T-strand integrates into the plant genome by illegitimate recombination, a major mode of foreign DNA integration on plants (Gheysen et al., 1991).

Very little is known about the precise mechanism of T-DNA integration into plant genome, or the role specific proteins play in this process. After years of study, the mode for T-DNA integration remains a controversial issue. Two possible modes for T-DNA integration were originally proposed (Mayerhofer, 1991): the double-strand-break repair (DSBR); and single-strand-gap repair (SSGR) integration models. In the DSBR integration model, T-DNA integration initiates with the production of a double-stranded break (DSB) in the target DNA and the conversion of the T-strand to a double strand form. Then the end of both the T-DNA and the DSB in the target DNA can be processed and ligated. In the SSGR model, T-DNA integration initiates with the production of a single strand gap on target DNA by endonucleases. T-DNA then anneal to microhomology areas within the gap. Following the ligation of the T-strand to the target DNA, the upper strand of the target DNA is removed. The gap in the upper strand of the

target DNA is then repaired, using the inserted T-strand as a template. Tinland and Hohn (1995) proposed a microhomology-dependent T-DNA integration mode. In this mode, T-DNA integration initiates by annealing of the T-strand 3'-end to the host DNA that has unwound partially by an unknown mechanism. Then the bottom strand of the target DNA is nicked, followed by anneal of 5'-end of the T-strand to the target DNA. The top strand of target DNA is also nicked and partly removed. The upper strand was repaired using T-strand as template. The differences between these modes are: T-DNA integrate in host genome as single-strand or double-strand; T-DNA replace target DNA or only insert into it. Further research is required to address this issue.

1.5.3 *Agrobacterium*-Mediated Transformation

A. tumefaciens is known for the fact that the only elements from the T-DNA fragment necessary for transfer and integration are the highly conserved right and left borders. Therefore, any DNA sequence found between these borders can be transferred and integrated into the host genome. In other words, the genes encoding the plant growth hormones and opines present on wild type T-DNA can be deleted and replaced with any gene of interest. *A. tumefaciens* is also known for its single, random integration events which give each transformed cells a unique integration site. As a result, the T-DNA may insert into and inactivate a gene of interest, leading to variations in the phenotype. Therefore, large-scale transformation using *A. tumefaciens* can be used to create mutant libraries for functional analysis. It is also known for its ability to transform a variety of organisms, such as plants (Forreiter et al., 1997), yeast (Bundock et al., 1995), filamentous fungi (Gouka et al., 1999) and even animal cells (Kunik et al., 2001). AMT has also been applied on *Trichoderma* species (Mach et al., 1994).

1.5.4 Binary Vector

Binary vector is a Ti-plasmid system in which the vir genes and T-DNA region are on two separate plasmids. The first one is a disarmed T-DNA plasmid which contains a complete T-DNA region including the left and right broader. This plasmid also has origin(s) of replication that could function both in *E. coli* and in *A. tumefaciens*, markers for selection and maintenance in both *E. coli* and *A. tumefaciens.* Second plasmid is a helper Ti plasmid, harbored in *A. tumefaciens*, which lacks the entire T-DNA region but contains an intact *vir* region.

The invention of binary vector systems started with the discovery that the *vir* region essential for DNA transformation and the T-DNA fragment carrying the tumor-inducing agents do not have to be on the same plasmid in order to cause disease. In fact, Hoekema et al. (1983) proved that *A. tumefaciens* harboring both elements on different plasmids induced normal tumorigenesis, although neither plasmid was functional on its own. This breakthrough allowed researchers interested in *Agrobacterium*-mediated transformation to construct a wide variety of T-DNA binary vectors and non-oncogenic *Agrobacterium* strains harboring a disarmed Ti-plasmid, also known as the *vir* helper plasmid.

Lee and Gelvin (2008) list many disarmed *Agrobacterium* strains and T-DNA binary vectors available to the scientific community, with detailed information on their different characteristics. Some examples of the commonly used *Agrobacterium* strains are AGL0, AGL1, LBA4404 and the binary vectors series pBIN, pCAMBIA and pSITE. The main advantage of using binary vectors as opposed to wild type Ti-plasmid is the fact that they are easier to isolate and manipulate due to their smaller size. Furthermore, they can be modified for use in *E. coli* transformation systems, which are used for most gene cloning and DNA manipulations.

1.6 Study Objective

Trichoderma spp. is a genus of fungi present in all soils, where they are the most prevalent culturable fungi. *Trichoderma aggressivum* was initially known as the aggressive biotype of *T. harzianum*, which causes the green mold disease on *Agaricus bisporus*. This disease is responsible for large losses in mushroom farms all over the world. Future molecular biology studies on *Trichoderma aggressivum* will involve the technology of gene transformation to overexpress or silence a domestic gene, or to transfer foreign genes into its genome. The correct translation, expression and targeting of foreign proteins is the top issues in gene transformation. Translation is affected by the presence of specific intron(s), expression level is mainly controlled by the promoter of each gene, and subcellular targeting regulated by targeting signals. All of these attributes are dependent on the manipulation of the foreign gene before transformation. The interest of this study was to examine the subcellular targeting ability of enhanced GFP and signal tagged GFP in the host, *Trichoderma aggressivum* f. *aggressivum*. Green fluorescent protein was chosen for the study because it is one of the most common marker genes used in molecular biology. Enhanced GFP has been tested in various species including filamentous fungi because it is highly expressed in most species, and the results can easily be examined by fluorescence microscopy. KDEL and NLS are the two subcellular signal sequences tested in this study. KDEL is a common ER retention signal. NLS is a nuclear localization signal. The necessary manipulation was done to the GFP gene in order to test expression in *T. aggressivum*. Four GFP constructs, including controls, were generated and transferred into *T. aggressivum* individually. The constructs were: ①GFP control: GFP under the control of a fungal glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter, ②intron-GFP: GFP with an intron close to the 5' end of the gene and regulated as above, ③GFP-KFEL: intron-GFP plus a KFEL encoding region

at the 3' end of the gene, ④GFP-NLS: intron-GFP with an NLS encoding region at the 3' end of the gene. The transformed strains were screened and examined for localization and any difference in expression level.

2. MATERIALS AND METHODS

List of Abbreviations

DNA:	Deoxyribonucleic acid
PCR:	Polymerase chain reaction
EDTA:	Ethylenediaminetetraacetic acid
DMSO:	Dimethyl sulfoxide
SDS:	Sodium dodecyl sulfate
AMP:	Ampicillin
CAR:	Carbenicillin
Cm:	Chloramphenicol
X-Gal:	bromo-chloro-indolyl-galactopyranoside
ER:	Endoplasmic reticulum
NLS:	Nuclear localization signal
Ex/Em:	Excitation/Emission
OD:	Optical density
GFP:	Green fluorescent protein
dH_2O	distilled H_2O
MQ H_2O	MilliQ H_2O

2.1 Culture Media and Reagents

Trichoderma:

MEA broth/agar medium: Malt Extract 20g, dH_2O 1L, (Agar 20g).

BD-MEA agar medium: BD MEA mixture 40g, dH_2O 1L.

YPD medium: Yeast extract 10g, Peptone 20g, Dextrose 20g, dH_2O 1L.

Bacteria:

LB broth/agar medium: NaCl 10g, Tryptone 10g, 5g Yeast extract, dH_2O 1L, (Agar 20g).

LB+AMP broth/agar: NaCl 10g, Tryptone 10g, 5g Yeast extract, dH_2O 1L, (Agar 20g), 100mg/ml Ampicillin stock solution 1ml.

LB+AMP+X-Gal plate: NaCl 10g, Tryptone 10g, 5g Yeast extract, dH_2O 1L, Agar 20g, 100mg/ml Ampicillin stock solution 1ml, 50mg/ml X-Gal stock solution 1ml.

LB+CAR+Cm broth/agar: NaCl 10g, Tryptone 10g, 5g Yeast extract, dH_2O 1L, (Agar 20g), 100mg/ml Carbenicillin stock solution 1ml, 50mg/ml Chloramphenicol stock solution 2ml.

Malt extract and yeast extract for the culture media was purchased from BD. T4 DNA ligase and restriction enzymes was purchased from New England Biolabs Ltd., *Taq* and *Pfu* DNA polymerase were purchased from Fermentas, antibiotics and herbicide were purchased form Sigma-Aldrich Canada Ltd, and chemicals were purchased form Bioshop Canada Inc.

2.2 Bacterial and Fungal Strains

E. coli: DH5α

Agrobacterium tumefaciens: AGL1

Trichoderma aggressivum f. *aggressivum*: T586

2.3 Plas mid Sources

PUC19 was purchased from ChromDB and used regularly as cloning vector. pSITE vector was kindly provided by Dr. Michael Goodin (Department of Plant Pathology, University of Kentucky, Lexington KY, USA) and was used as source of *eGFP*. Commercial binary vector pFGC1008 was purchased from ChromDB. This original RNAi plasmid, pFGC1008 is derived from pCAMBIA1200 and has a chloramphenicol resistance gene for bacterial selection and a hygromycin resistance gene as the plant selectable marker. It was modified by Kamal Abubaker (Brock University) by replacing the 35S promoter with an *Agaricus bisporus GPD* fungal promoter to drive the expression of foreign genes in filamentous fungi.

2.4 Cloning GFP and Tagged GFP

2.4.1 PCR

PCR using *Pfu* DNA polymerase was used to clone GFP from the pSITE vector into pUC19 and tagged GFP fragment. *Pfu* DNA polymerase is able to generate PCR fragments with fewer errors than *Taq* polymerase since *Pfu* possesses a 3' to 5' exonuclease proofreading activity, meaning that it works its way along the DNA from the 5' end to the 3' end and corrects nucleotide misincorporation errors. Reaction system: 16ul MQH_2O, 2.5ul *Pfu* buffer without $MgSO_4$ (10X), 2.5μL $MgSO_4$ (25mM), 0.5μL dNTPs (containing 10 mM each of dATP, dCTP, dGTP, and dTTP), 1μL each primer (5mM), 0.5μL *Pfu* (2.5 u/μL), 1μL DNA template (≈50ng). Primers were designed for all the PCRs and order from Sigma Genosys. Recognition sites of restriction endonuclease *SacI* and *BamHI* were added to one and the other end of all fragments along with several protection base pairs. The intron (Ma et al., 2001) (56 bps) required two PCR reactions to be added to the gene because its length affects primer designs. First front primer contains 15 bps of complementary sequence to the head of GFP ignoring the first 6bp and an

overhang of the first half of the intron. The second front primer contains 15 bps of complementary sequence to the first half of intron, the first 6 bps of GFP sequence, restriction site and some protection base pairs. In total, four clones were produced: GFP, intronGFP, intronGFP-KDEL and intronGFP-NLS.

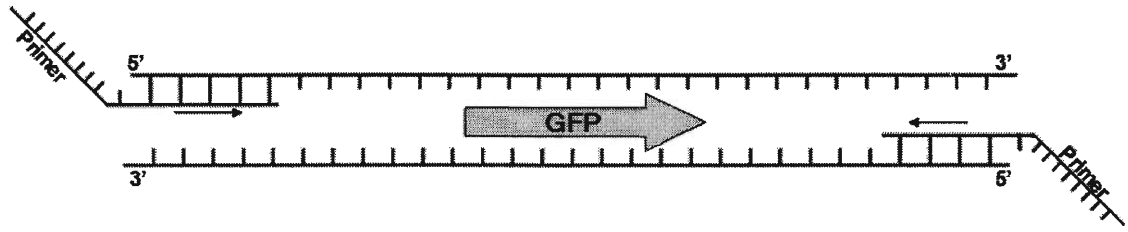


Figure 4. Simple diagram of PCR of GFP. Primers are composed of a complementary sequence and an overhang. The overhang of front primer contains intron sequence part I or II, *SacI* recognition sequence and protection base pairs. The reverse contains KDEL or NLS sequence, *BamHI* recognition sequence and protection base pairs.

Table 1. PCR reaction steps for GFP cloning

Temperature	Time
95°C	1 min
5°C below Annealing temp.	1 min
72°C	2 min
4 more cycles	
95°C	1 min
Annealing temperature	1 min
72°C	2 min
29 more cycles	
72°C	5 min

Table 2. List of primers used for PCR reactions

Primer	Sequence (5'-3')
GFP-F	ACGGATCCAGCCATGGTGAGCAA
GFP-R	GATCTAGACTTGTACAGCTCGTCCA
iGFP-F-I	GACGACCGCGTGCTGACTTCGCTTTCCAGGTCAAAGCAGTGAGCAAGGGCGAG
iGFP-F-II	CGATTCATGCCGGTCAGTACACCACACAGCCCGACCGCGACGACCGCGTGCTG
iGFP-KDEL-R	TCTAGAGAGTTCATCCTTCTTGTACAGCTCGTC
iGFP-NLS-R	TCTAGACTTGATCTTCTTCTTTTCAGGCTTGTACAGCTCGTC
ALAN1	CAGCACCCAGAACAATAAC
ALAN2	CAGATCATCTTGATGAGACG

PCR using *Taq* DNA polymerase was also used to examine the results of each digestion, DNA isolation, gene cloning and transformations. Primers ALAN1 and ALAN2 were designed to specifically target *Trichoderma aggressivum* (Dr. Castle personal communication). Reaction

system: 18.25μL MQH_2O, 2.5μL *Taq* buffer (10X, with KCl), 1.5μL $MgCl_2$ (25mM), 0.5μL dNTPs (containing 10 mM each of dATP, dCTP, dGTP, and dTTP), 1μL each primer (5mM), 1μL *Taq* (5u/μL), 1μL DNA template (≈50ng).

2.4.2 DNA Digestion and Ligation

Double digestion was performed on all four GFP constructions and two kinds of plasmids (PUC19 and binary vector) with restriction enzymes *SacI* and *BamHI*. Reaction system: 0.5μL *SacI*, 0.5μL *BamHI*, 1μL buffer 4 from New England Biolab, 6μL MQH2O, 2μL DNA. The reaction was carried out at 37°C for 2 hours.

To join the inserts with plasmid DNA, ligation with T4 DNA ligase was performed on the digested PCR products with either PUC19 or the binary vector. Reaction system: 1μL binary vector, 9μL PCR GFP fragment, 1μL T4 DNA ligase, 2μL T4 DNA buffer with ATP, 7μL MQH_2O.

2.4.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed after every PCR, digestion, ligation and DNA isolation forverification of results. 1% agarose gel electrophoresis was performed in 1X TAE buffer (2mls 0.5M EDTA (pH 8.0), 4.84g Tris base, 1.142ml glacial acetic acid, adjust pH to 8.0 with glacial acetic acid, then bring volume to 1 Liter with dH_2O) with a Gel System from Bio-Rad Laboratories (Mississauga, Ontario). Ethidium bromide (EB) was used as the nucleic acid stain and was added (1μL /50ml agarose solution) and mixed with the gel prior to solidification. A 5X loading dye (0.25% bromophenol blue, 0.25% xylene xyanol, 40% sucrose) was used to load the DNA sample to the gel. DNA molecular weight marker was the HighRanger 1kb DNA Ladder, MidRanger 1 kb DNA Ladder and PCRSizer 100 bp DNA Ladder from

Norgen Biotek (Thorold, Ontario). The gels were visualized and photographed using Gel Doc 1000 and the Quantity One 1-D Analysis software (version 4.6.2 Basic) from Bio-Rad.

2.4.4 PCR Purification and DNA Gel Extraction

PCR purification and DNA Gel Extraction were performed with QIAquick PCR Purification Kit/ QIAquick Gel Extraction Kit from QIAGEN according to the manufacturer's directions.

2.4.5 Transformation of *E. coli*

2.4.5.1 Preparation of competent cells

Competent cells were prepared by first inoculating a single colony of *E. coli* DH5α into 3ml of LB broth with 20mM $MgSO_4$, followed by shaking (250rpm) at 37°C overnight. 1mL of this culture was inoculated into 50ml of fresh LB broth with 20mM $MgSO_4$ and shaken at 37°C until OD_{600} reached 0.4-0.6. The culture was then centrifuged at 4°C, 1,500×g for 10min. The pellet was resuspended in 20ml of ice-cold TFB solution (5ml 1M MES-KOH pH6.2, 6.0g KCl, 4.5g $MnCl_24H_2O$, 3.5g $CaCl_22H_2O$, 50ml glycerol, volume adjusted to 500ml). After incubation on ice for 30min, the culture was again centrifuged at 4°C, 1,500×g for 10min. 300μL of dimethyl sulfoxide was added to resuspend the pellet and 100ul of cell suspension was placed in 1.5ml microcentrifuge tubes. The cells were frozen by dipping in liquid nitrogen and stored at -80°C.

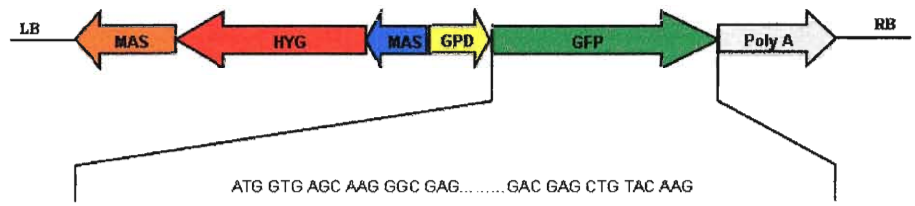
2.4.5.2 Transformation of *E. coli.*

E. coli was transformed with all four constructs by mixing 100ul of competent cells with 10μL of 5ng/ul plasmid DNA, followed by incubation on ice for 30min. Then a heat shock was performed at 42°C for 45seconds and followed by 2min on ice. 800μL of LB medium was added to the suspension followed by 40min incubation at 37°C under constant agitation (250rpm). This culture was then used to inoculate LB plates with 100μg/mL AMP and X-Gal. The plates were cultured at 37°C overnight for blue/white colony selection.

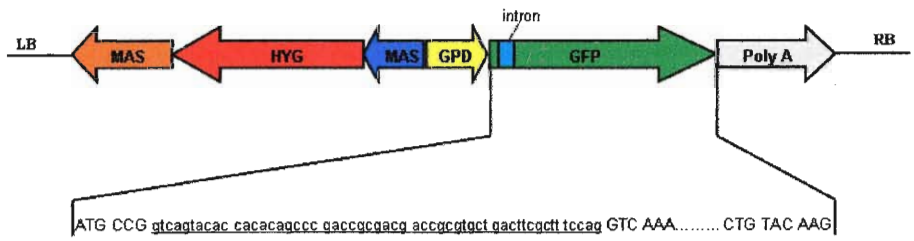
2.4.6 Plasmid DNA Isolation

Bacterial broth culture was transferred into microcentrifuge tube and centrifuged at 3000rpm for 5 min then the supernatant was discarded. 100ul of resuspension solution (100mM Tris pH7.5, 25mM EDTA) was added to the tube and the cells were mixed by vortex. 150μL of freshly prepared lysis solution (0.2N NaOH, 1% SDS) was added and incubated at room temperature for 5min. 150μL of neutralization solution (3M KAc pH5.0) was added and the tube was incubated on ice for 30min. After centrifugation at 13,000rpm for 10min, the supernatant was transferred into a clean tube and 2 vol. of 95% ethanol was added and incubated at -20°C for 30min. The tube was centrifuged again at 13,000rpm for 10min and the supernatant was discarded. 50-100μL of TE buffer was added to dissolve plasmid DNA.

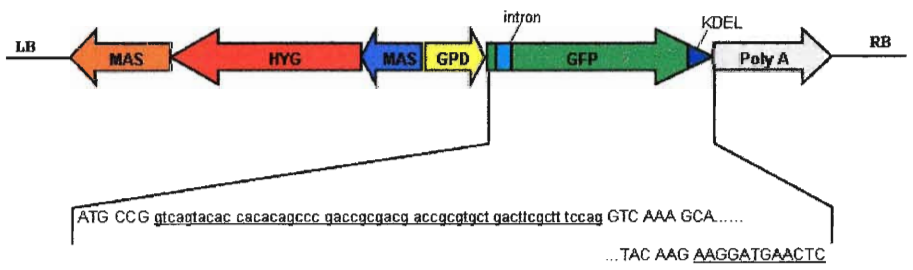
2.5 Binary Vector Construction



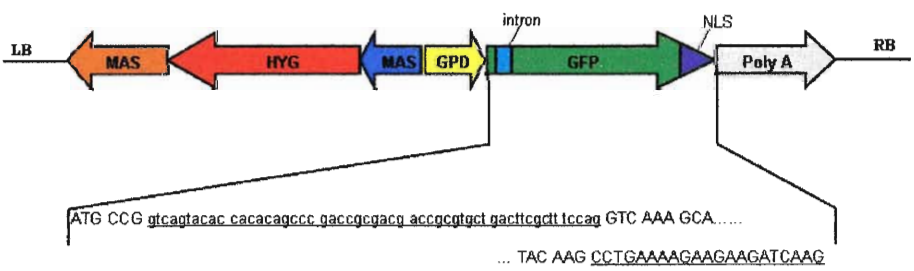
GFP



intron-GFP



intron-GFP-KDEL



intron-GFP-NLS

Fig.5 Maps of T-DNA constructions contain a hygromycin resistance gene and GFP/ iGFP/ IGK/ IGN expression cassette. Original GFP coding sequence is in upper case, intron sequence is in lowercase and are underlined. The KDEL and NLS coding sequences are in uppercase and are underlined.

2.6 Transformation of *Agrobacterium*

2.6.1 Preparation of *Agrobacterium* competent cells

Agrobacterium AGL1 was inoculated into 5mL of LB medium containing 100ug/ml carbenicillin and shaken for two days at 28°C. 1ml of the growing culture was added to 25 mL fresh LB medium containing 100μg/mL CAR and shaken vigorously (250 rpm) at 28°C for 36-48h until the culture reached OD_{600}0.5-1.0. When *Agrobacterium* reached optimum growth, the culture was chilled on ice for 30min and centrifuged at 3000×g for 5min at 4°C. The supernatant was discarded without disturbing the pellet. Cells were resuspended in 1ml of ice-cold 20mM $CaCl_2$ solution and 100ul aliquots were dispensed into 1.5ml microcentrifuge tubes. The cells were frozen by dipping in liquid nitrogen and stored at -80°C or used directly for transformation.

2.6.2 *Agrobacterium* transformation with binary vectors

1ug (max. 5μL) of plasmid DNA was added to the required number of tubes containing frozen cells. An equal volume of sterile dH_2O was added to one representative tube (containing frozen cells) per strain used as a negative control. Cells were thawed for 20min at 37°C in a water bath and then incubated on ice for 30min. 900ul of LB was added and the mix incubated at 28°C for 2h with gentle shaking. Cells were centrifuged at 3000×g for 5min at room temperature. 600μL of the supernatant was carefully discarded and cells were resuspended in the remaining 400μL. For the transformation, the cells were spread on two pre-warmed LB plates (200ul/plate) containing all the appropriate antibiotics, and incubated for two days at 28°C. For the controls, cells were spread on two pre-warmed LB plates with or without antibiotics, as a control for lack of transformation or viability. After two days incubation, growing colonies were observed on positive plates and no antibiotic controls, whereas no colonies on the negative control plates.

Single colonies were picked from plates and inoculated in separate 5mL of LB containing the appropriate antibiotics then grown overnight at 28°C on a rotary shaker. 1ml of the cultures was inoculated into 50mL LB medium in a 250mL flask containing the appropriate antibiotics. Cultures were grown for 48 hours at 28°C with shaking (150 rpm) until OD_{600} of 0.5-1.0.

2.7 *Agrobacte rium*-Mediated Transformation

The strain AGL1 from a 2 day old plate was inoculated into 10 mL of LB broth containing 100µg/mL carbenicillin and 100µg/mL chloramphenicol, followed by incubation at 28°C for 16-20h under constant agitation (250rpm). Once the OD_{600} reached 0.6-0.8, the liquid culture was diluted to an OD_{600} value of 0.15 with induction medium (IMAS) containing 200µM acetosyringone (final concentration) and was incubated at 28°C for 4h to OD_{600} 0.4-0.8 under constant agitation (250rpm). Meanwhile, *Trichoderma aggressivum* conidia were harvested from a 7-day-old plate with 5mL of 0.01% sterile Triton-X 100 and filtered through sterile glass wool to remove the mycelia. The concentration of conidia was calculated with a hemocytometer and was diluted to a final concentration of 10^7conidia/ml with IMAS. A mixture of 100µL of conidia and 100µL of *Agrobacterium* culture was then inoculated onto a 10cm black filter paper deposited on freshly poured IMAS plates. After 2 days of co-culture at 28°C, the filter paper was transferred onto freshly poured M-100 plates containing 400µg/mL cefotaxime and 100µg/µL hygromycin. Cefotaxime was used to kill the remaining *Agrobacterium* while the hygromycin was used as the selective agent for transformed fungi. After 2 days of incubation at 28°C the mycelia can be visualized on the black filter paper and the plates were overlaid with freshly prepared M-100 agar medium containing 100µg/mL hygromycin and the plates were again incubated at 28°C. Putative transformants started to be visible after 3-4 days and were collected to fresh MEA plates (one transformant/plate). As a positive control, transformed *Trichoderma*

aggressivum were applied to plate without selection agent (hygromycin) to show viability. As a negative control, untransformed *Trichoderma aggressivum* was applied to plates with 100μg/mL hyg to indicate the reliability of selection agent (See APPENDIX I for recipe of media and reagents).

2.8 Fungal Ge nomic DNA Extraction

A small piece of mycelium was transferred to a microcentrifuge tube containing 500μL of lysis buffer (400mM Tris-HCl pH 8.0, 60mM EDTA pH 8.0, 150mM NaCl, 1% SDS). The tube was left at room temperature for 10min. After the addition of 150μL of 3M potassium acetate (pH 4.8), the tubes was centrifuged for 5min at 13,000rpm. Supernatant was collected in fresh tube. An equal volume of isopropyl alcohol was added, mixed and centrifuged for 5min at 13,000rpm. The DNA pellet was washed in 300μL of 95% ethanol, centrifuged at 13,000rpm and air dried. The dried DNA was dissolved in 50μL TE buffer (Liu et al., 2000).

2.9 Sequence Analysis

Isolated pUC19 DNA samples carrying each GFP constructs were sent to Core Molecular Biology Facility, York University for DNA sequencing analysis. The cycle sequencing reactions was done with Applied Biosystems BigDye Terminator Cycle Sequencing Kit on the Applied Biosystems 3130xl DNA Sequencer. The sequence information was compared with the GFP sequence found on GenBank with the assay tool MAFFT multiple sequence alignment program version 6.611b.

2.10 Microscopy

Transformed *Trichoderma* mycelia were checked for fluorescence under Nikon confocal microscope C1 which is connected to a Nikon microscope Eclipse E800. The results were analyzed and pictures were taken by using the confocal software EZ-C1. Mycelia were stained

with DAPI (4', 6-diamidino-2-phenylindole) or ER tracker Blue-White DPX dye (Invitrogen) to illustrate the nucleus and endoplasmic reticulum, respectively. DAPI is a fluorescent stain that binds strongly to DNA. DAPI can be used to stain live cells because it can pass through intact cell membranes. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm which appears blue/cyan. The absorption maximum of the ER-Tracker Blue-White DPX is 374 nm in methanol. Since fluorescence of daxoyl dyes is highly environment sensitive, the peak fluorescence emission is variable and ranges from 430 nm to 640 nm (Invitrogen product information).

2.11 GFP Quantification

GFP quantification assay was performed with a GFP quantification kit from BioVision. All genetically modified *Trichoderma aggressivum* strains (GFP, IGFP, IGK, and IGN) were cultured in MEB broth and hyphal samples were taken on different days. These samples were weighed and then lysed with GFP assay buffer on ice for 10min, follow by 5min centrifugation at 14,000rpm. The supernatants were collected into new tubes and stored at -20 °C prior to GFP quantification. The quantification assay was performed on a 96 well plate and a duplicate standard curve was prepared by diluting GFP standard solution (1μg/μL) into 0, 80, 160, 240, 320, 400 ng/well (final volume 100μL). 100ul of each sample was added to different wells and the plate was scanned for fluorescence on BIO-TEK Synergy HT Multi-Detection Microplate Reader Ex/Em=458/522nm. Data were analyzed with BIO-TEK KC4 software.

2.12 Bio-Rad Protein Assay

Protein assay was performed on the same sample used in GFP quantification assay to determine concentration of solubilized protein. 200ul of protein assay buffer (BioRad), 10ul sample and 790μL MQH_2O were mixed in microcentrifuge tube and incubated at room

temperature for 10min. A standard curve was prepared with BSA (bovine serum albumin) protein solution (1mg/mL) by generating samples contain 0, 1, 2, 5, 10, 15, 20μg/mL BSA per assay, 200μL protein assay buffer was added, and the final volume was brought to 1mL with MQH_2O. Absorbances of all samples were measured at 595 nm with a spectrophotometer.

2.13 Western blot

Proteins were separated on a 12% SDS-PAGE gel in a BioRad minigel apparatus. The separating gel consisted of 12% acrylamide (stock: 29.2% w/v acrylamide, 0.8% w/v bis-acrylamide), 0.37M Tris (pH8.8), 0.1% SDS, 0.05% APS, and 0.05% TEMED. After solidification, a gel comb was inserted and stacking gel solution with 4% acrylamide stock, 0.125M Tris (pH6.8), 0.1% SDS, 0.05% APS, and 0.1% TEMED was added to the gel. After solidification, the comb was removed and the gel was load into the tank filled with SDS-PAGE running buffer (0.007M Tris, 1.44% glycine, 0.1% SDS). Protein sample was mixed with sample buffer (0.05M Tris [pH6.8], 2% SDS, 10% glycerol, 0.001% bromophenol blue, 5% 2-mercaptoethanol) and heated at 95°C for 5min, then cooled on ice and loaded into the wells on the gel. The gel was run at 100V for 1.5h, and the gel was removed from the glass plates for transfer. The proteins were eluted to a nitrocellulose membrane in Towbin's transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol; Towbin et al., 1979) for 6h at 30V. The nitrocellulose membrane was blocked in TBST (20mM Tris pH 8, 500mM NaCl, 0.05% Tween-20) + 5% non-fat milk overnight with gentle shaking. The membrane was then washed with TBST + 0.5% non-fat milk for 15min and incubated with primary antibody (monoclonal antibody against full-length GFP; Clontech) in TBST + 0.5% non-fat milk for 1h. Three washes with TBST + 0.5% non-fat milk for 15min were applied to the membrane before it was incubated with the secondary antibody (alkaline phosphatase tagged rabbit anti-mouse IgG; Abcam) for 1h.

The membrane was then washed twice with TBST + 0.5% non-fat milk and once with TBS (TBST lacking Tween-20). The membrane was stained with 100mM Tris (pH8.8), 10mM $MgSO_4$, 150mM NaCl, 0.03% nitroblue tetrazolium, and 0.015% bichloro-indolyl-phosphate for 15min. After colour development, the membranes were washed with dH_2O and stored dry.

3. Results

3.1 Plasmid Construction and Gene Transformation

For plasmid construction, two PCRs were performed to acquire the initial GFP constructs. All four constructs were digested with *BamHI* and *SacI* and then cloned into pUC19 plasmid (digested with *BamHI* and *SacI*). After transformation of *E.coli* and an overnight culture, the plasmid was isolated and GFP constructs were released by the two restriction enzymes used above. The purified GFP constructs were then cloned into binary vector pFGC1008-GPDpro (digested with *BamHI* and *SacI*) for transformation of *Agrobacterium* AGL1 (Fig.6). All steps of PCR, DNA isolation, digestion, and ligation were examined with gel electrophoresis for molecular weight and quantity.

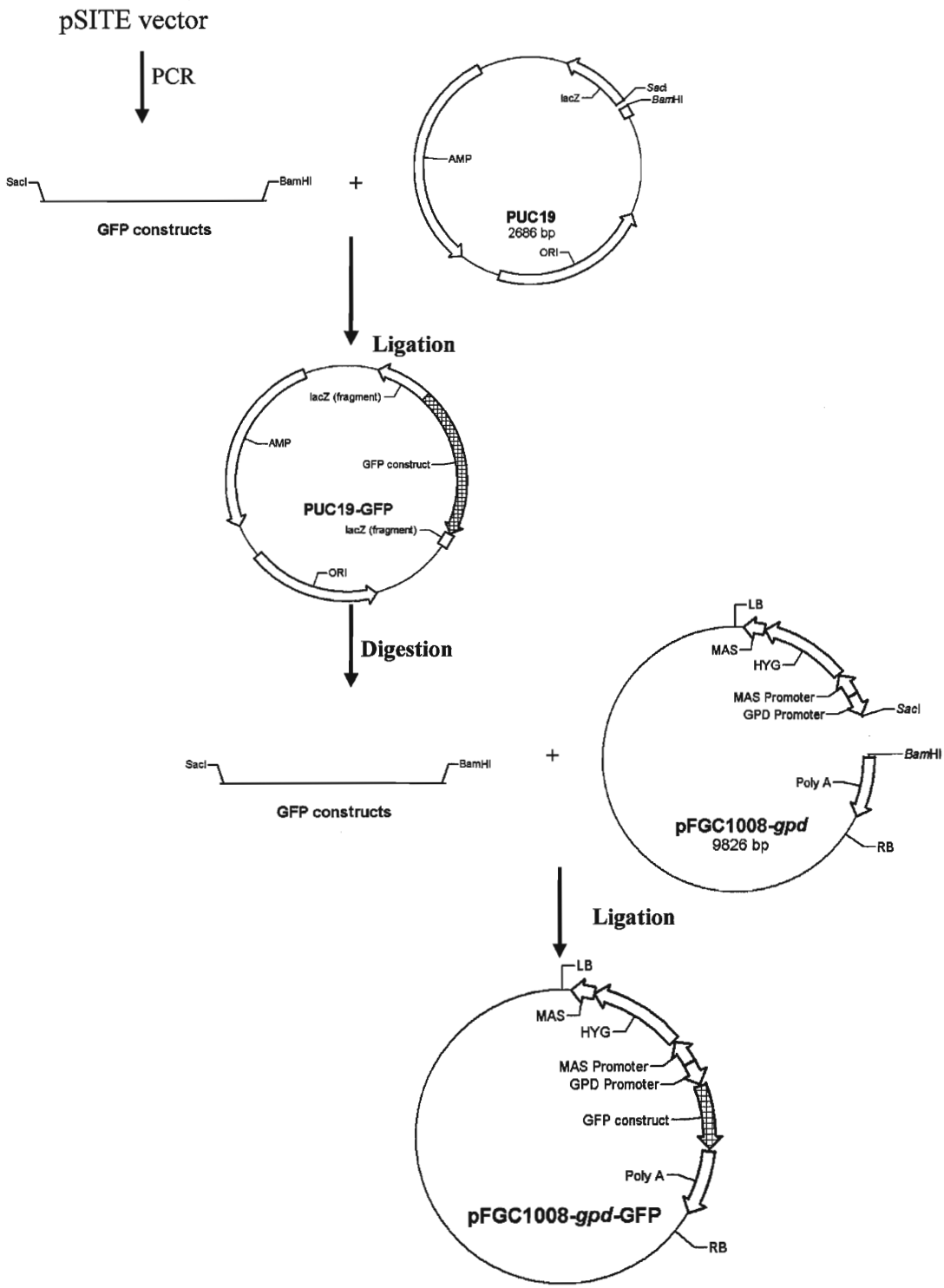


Fig.6 Schematic of GFP cloning and binary vector construction.

3.1.1 PCR

The pSITE vector containing GFP was acquired from Dr. Michael Goodin (University of Kentucky). One PCR reaction was needed for GFP vector construction, and two PCRs were needed for each of the IGFP, IGK, and IGN constructs. The first reaction generated GFP with half of the intron sequence, and the second PCR was used to complete the intron and give localization signal to IGK and IGN. After the first PCR, gel picture showed DNA bands at the size about 700bp by comparing to MidRanger 1kb DNA ladder. The size of GFP gene is 714bp. The products of IGFP, IGK, IGN fragments from the first PCR were purified, diluted and used as DNA templates for the second PCR to generate constructs with intact intron and localization signals. The size of intron is 56bp, while KDEL and NLS signal are 12bp, 21bp respectively. From the gel picture (Fig.8), bands are estimated to be between 700bp and 1000bp as expected.

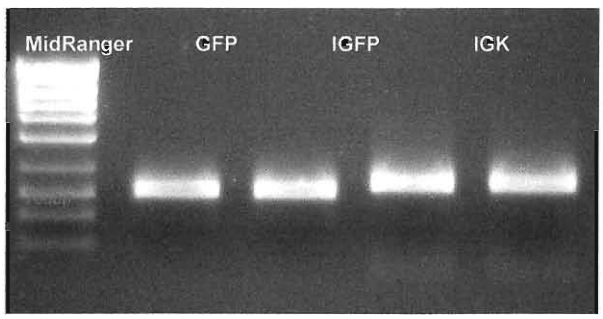


Fig. 7 Gel picture of first PCR for GFP constructs.

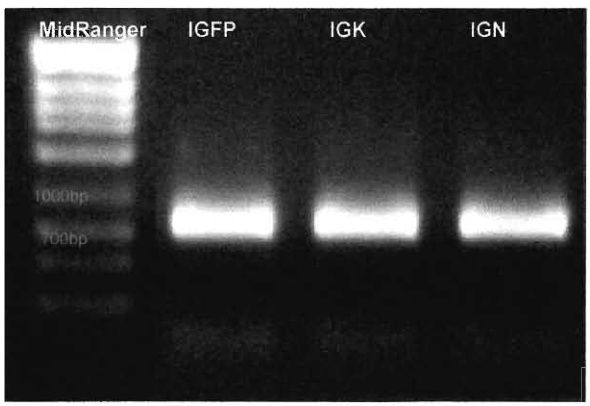


Fig. 8 Gel picture of second PCR for IGFP, IGK, IGN

3.1.2 GFP Constructions in PUC19

After all constructs were cloned into pUC19 and transformed into *E. coli*, plasmid DNAs were isolated after overnight culture and then digested with restriction enzymes *Bam*HI and *Sac*I (Fig.9). The band with approximately 2500bp was considered the open ring pUC19 backbone while the bands at about 700bp were identified as GFP inserts. The GFP bands were cut out from the gel and DNA was extracted for subcloning into the *Agrobacterium* vector.

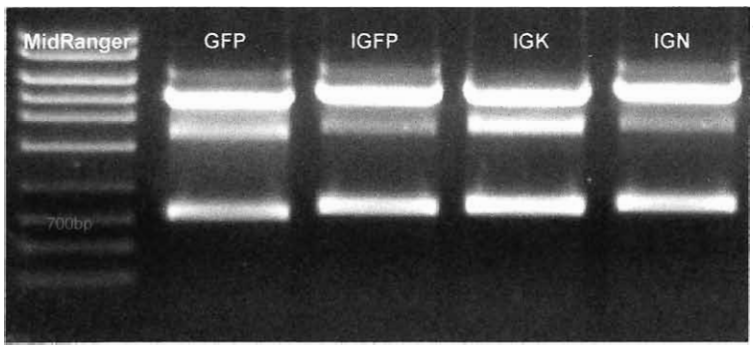


Fig. 9 Gel picture of GFP constructions released from cloning vector PUC19

3.1.3 Binary Vector

The GFP inserts were ligated with open ring binary vector pFGC1008 (9826bp) and then transferred into *E. coli*. Transformed colonies were selected on LB plates containing 100ug/ml chloramphenicol and subcultured in LB broth containing the same antibiotic for 16h. Plasmid DNAs were isolated and used to transform *A. tumefaciens* AGL1. Bacterial colonies were cultured and selected with the same medium used above and plasmid DNA again isolated to confirm the result. DNA bands showed on gel picture at a position higher than the top band of the MidRanger (Norgen) marker with the size of 5000bp (Fig.10). Digestion with *Bam*HI and *Sac*I revealed two bands, one at the position similar to Fig.10 while another one at the size about 700bp similar to the bands released from pUC19.

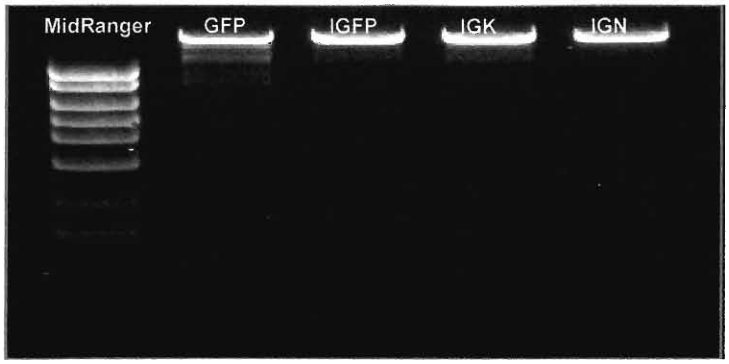


Fig. 10 Gel picture of GFP constructions in binary vector pFGC1008 which was taken after plasmid DNA isolation from *E. coli*.

3.2 *Trichoderma aggressivum* Transformation

Trichoderma aggressivum f. *aggressivum* was transformed with the genetically modified *A. tumefaciens* AGL1 harboring the binary vectors pFGC1008-GFP, pFGC1008-GFP, pFGC1008-IGK, and pFGC1008-IGN respectively. These binary vectors carry a copy of T-DNA construct (Fig.5) and are capable of integration into the genome of the host. Any transformed cells of *T. aggressivum* will express GFP and be resistant to chloramphenicol.

All four different GFP constructions were successfully transformed into *T. aggressivum* and colonies were selected with MEA medium containing 100ug/ml chloramphenicol. All recombinants acquired were examined for both phenotypic properties such as amount of hyphae, conidia production, and pigmentation on different media.

3.2.1 *T. aggressivum* Confirmation

Transformed *T. aggressivum* were screened and subcultured on fresh MEA plates. Genomic DNA of each transformant was isolated for a confirmation PCR with primers ALAN1 and ALAN2 that target an anonymous *T. aggressivum*-specific DNA (Castle, personal communication) and amplified a product of 184bp. The gel picture of all four PCR products show clear bands and position between 100bp and 200bp comparing to PCR Sizer 100bp DNA

Ladder (Norgen). Furthermore, DNA bands of all mutants were at the same position as *T. aggressivum* wild type control. This result confirms that the transformants were *T. aggressivum* and not contaminants.

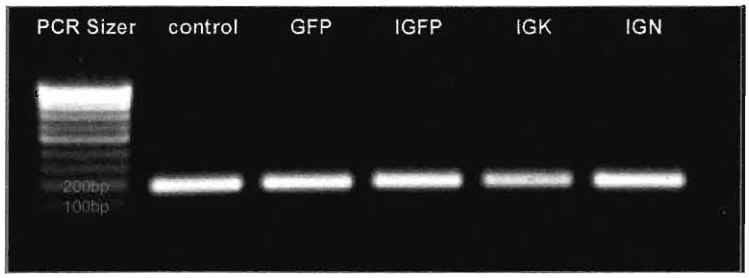


Fig.11 Gel picture of confirmation PCR with *T. aggressivum* specific primers ALAN1 and ALAN2. The expected amplification product of 184bp is observed in all transformants.

3.2.2 Mitotic Stability

The mitotic stability of all transformed *T. aggressivum* was assessed by serially subculturing single-spore isolates on MEA plates and monitoring GFP expression over more than 5 generations. For all recombinants, GFP expression and hygromycin resistance were constant during all experiments indicating stable genomic integration of the T-DNA fragments.

3.2.3 Culture Phenotypes

All four *T. aggressivum* mutants were selected and subcultured on three different media MEA, BD-MEA, and YPD to examine the phenotype variance and GFP expression. No antibiotic was used for the subculture to give a higher growth rate. Picture of the plates were taken after 6 days of culture (Fig.12). All strains had a similar growth rate, and after about 4 days mycelia cover the whole plates and started to produce conidia. Strains behaved differently on three media, mycelia production increased from MEA, BD-MEA to YPD plates for both mutants

and the wild type control. Mycelia on MEA and BD-MEA plates were almost transparent and on YPD plates mycelia showed a white color. Colonies on YPD medium showed much darker color after 6 days of inoculation which indicated that pigment was secreted into the medium. For MEA plates, dark green conidia were concentrated at few points on the surface of the plates, for BD-MEA, conidia were distributed more evenly on the edge of the plates forming a green halo, YPD plates, on the other hand, produced conidia with yellow color and the pattern was similar to BD-MEA. Wild type cultures on YPD produced no conidia after 6 days of culture. No obvious differences were observed between 4 mutants, while from the pictures all mutants produced more conidia than the wild type.

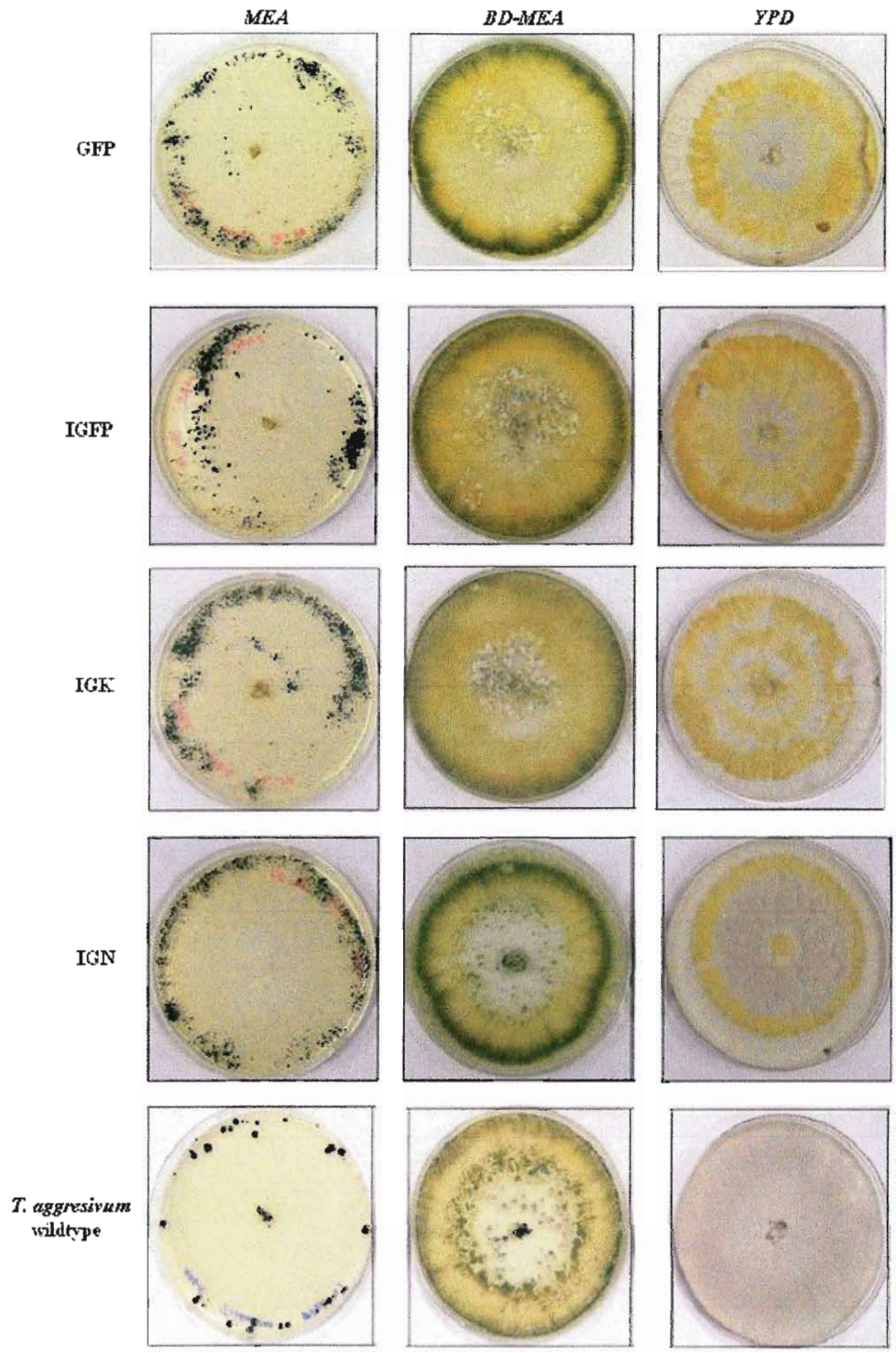


Fig. 12 Pictures of phenotypic properties of each transformed and wildtype *T. aggressivum* grown on MEA, BD-MEA, YPD media for 6 days at 28°C

3.3 GFP Analysis

Mycelia from all transformed *T. aggressivum* were examined under confocal microscope to look for both GFP expression and localization ability.

3.3.1 GFP Expression

Pictures of fluorescent mycelia were taken from all 3 different media at days 2, 4, and 6 of culture to study the effects of media and culture time (Fig.13, 14, 15). At day 2, GFP signals were detected for all four mutants on all three media. Mycelia on MEA and BD-MEA had a similar expression of GFP according to visual estimation. YPD plates showed lower GFP expression than other media, detection sensitivity of the microscope had to be increased in order to observe fluorescence, which resulted in a strong background on the pictures. IGK had overall the strongest fluorescence signal on MEA plate amount all mutants, From the pictures, GFP did not express evenly in all mycelia, some mycelia showed stronger signal than others from the same plate. At day 4, GFP expression on all YPD plates were similar to day 2 which showed weak fluorescent signal. Mycelia on MEA and BD-MEA plates resulted much stronger signal than YPD and also stronger than the same condition at day 2. All four mutants gave a strong fluorescence signal with little difference, except for the picture of IGN that showed slightly lower intensity of fluorescence. The strongest fluorescence signals of all mutants were detected at day 6. MEA plates resulted strong fluorescence yet only a small amount of mycelia can be detected. The picture of IGK on MEA plate showed much lower fluorescence intensity than the other three on the same media. BD-MEA plates showed both high fluorescence signal and large amount of mycelia in pictures while some background signal appeared on pictures. YPD plates showed large amounts of mycelia and very strong fluorescent yet come with fairly strong

background signals. Overall, GFP fluorescent signals can be detected from all mutants on all media.

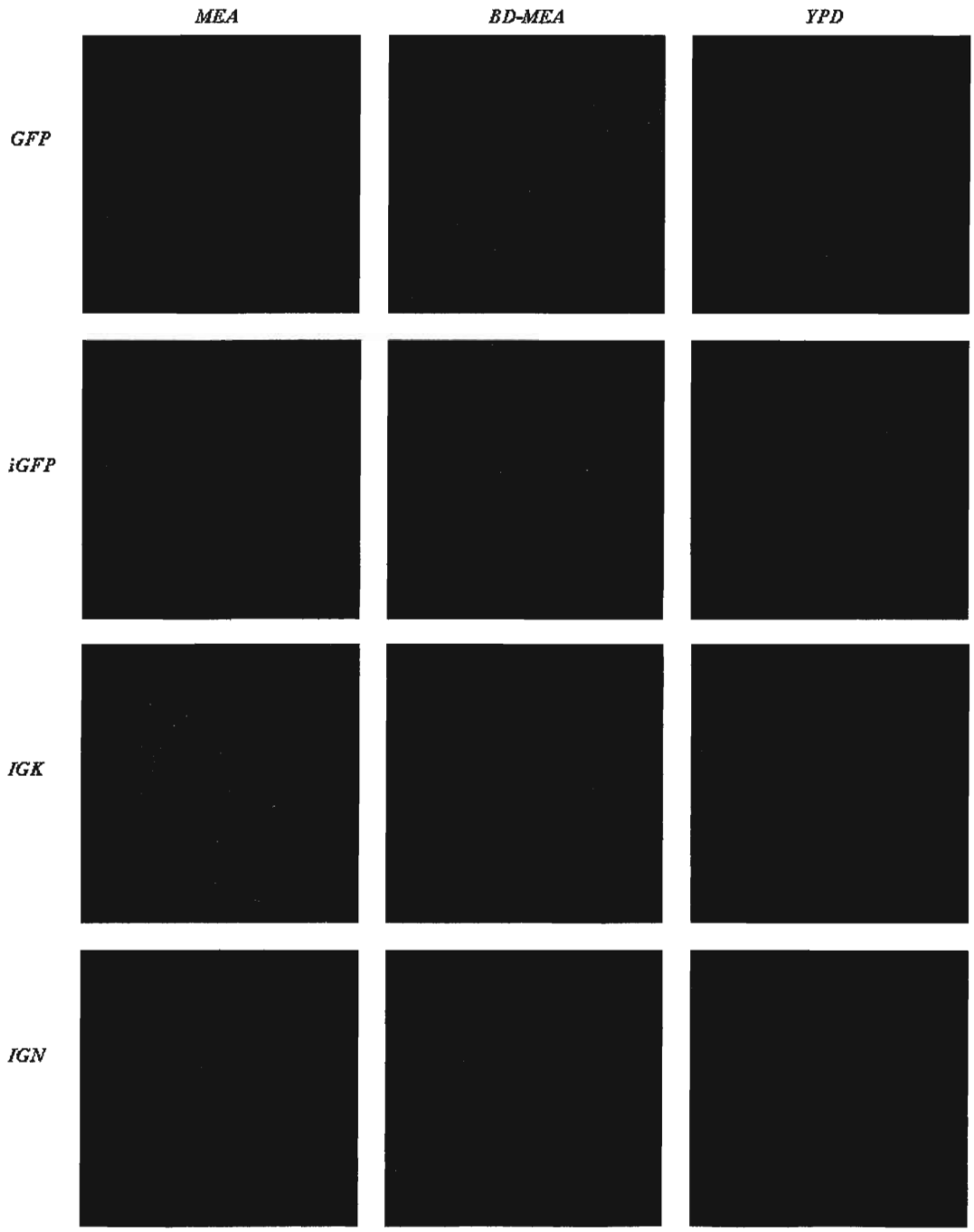


Fig. 13 Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 2 days at 28°C

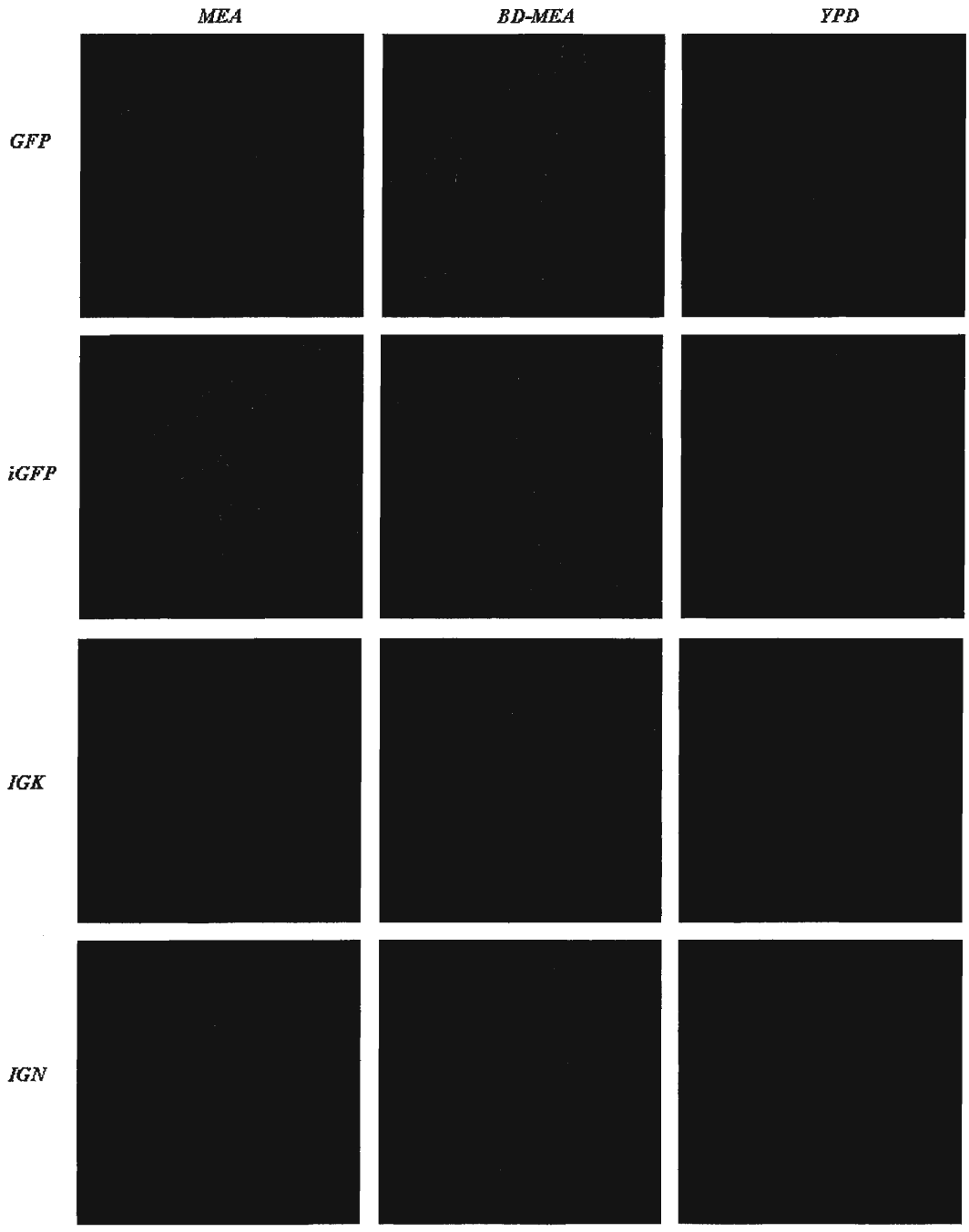


Fig. 14 Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 4 days at 28°C

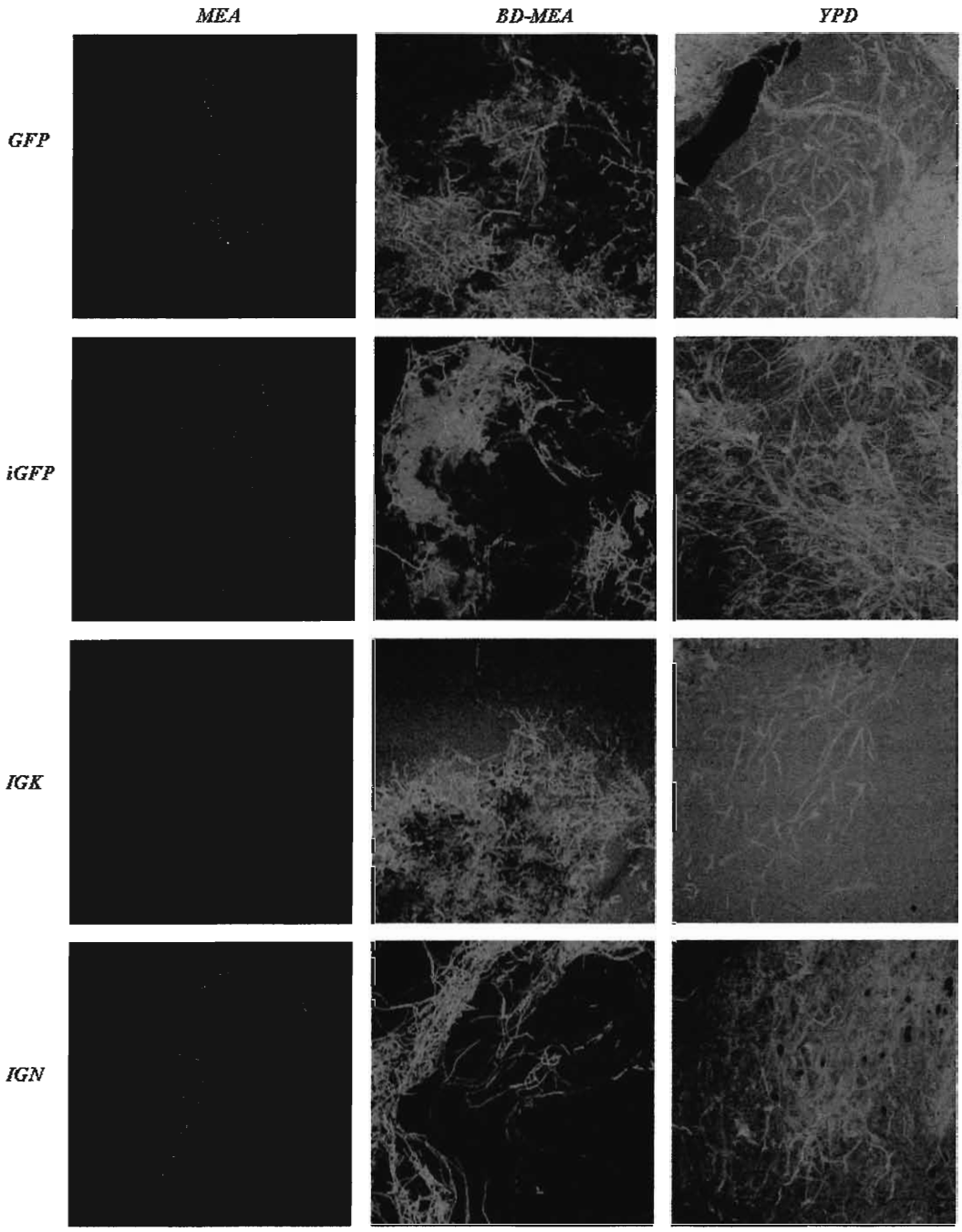


Fig. 15 Pictures of GFP fluorescent signal of each transformed *T. aggressivum* grown on three different media for 6 days at 28°C

3.3.2 GFP Localization

Modified *T. aggressivum* strains IGK, IGN were examined for localization ability when the nuclear dye DAPI and ER tracker dye were used as markers for nuclei and endoplasmic reticulum (ER).

3.3.2.1 KDEL signal and ER localization

Mycelia from *T. aggressivum* transformed with IGK and IGFP were stained with ER-tracker dye to show endoplasmic reticulum in fungal cells, and then examined by confocal microscopy. GFP signals and the blue ER-tracker signal can be seen throughout the mycelium. The overlay of GFP and ER-tracker showed a cyan signal. No localization differences between the IGK and IGFP transformants were apparent.

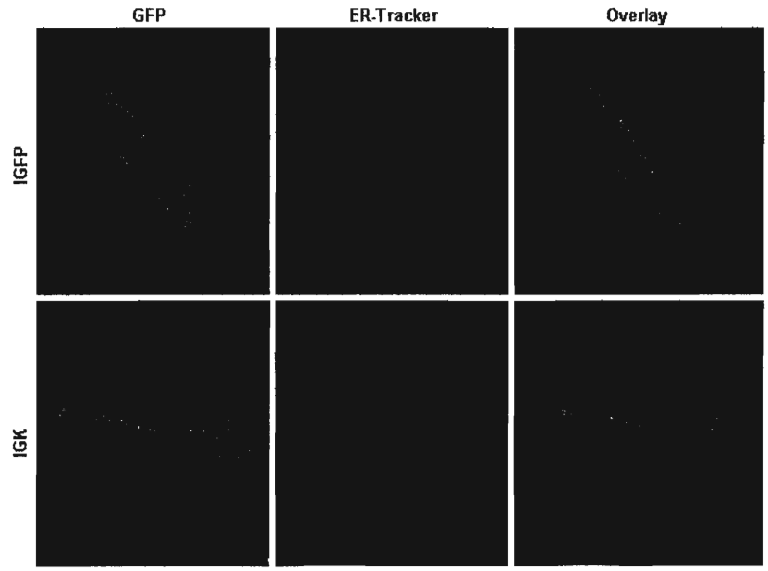


Fig.16 Confocal micrographs of green fluorescent protein: 3 day old mycelia of IGK, IGFP transformed *T. aggressivum* co-stained with ER-Tracker.

3.3.2.2 NLS and nuclear localization

Mycelia from *T. aggressivum* transformed with IGN and IGFP were stained with DAPI to show nuclei in fungal cells, and then examined by confocal microscopy. The picture showed GFP signal all over the mycelia and the DAPI stained nuclei of the mycelia are shown by the arrows. The overlay showed a cyan signal at the position of nuclei and green at other potion of the mycelia. No localization differences between the IGN and IGFP transformants were apparent.

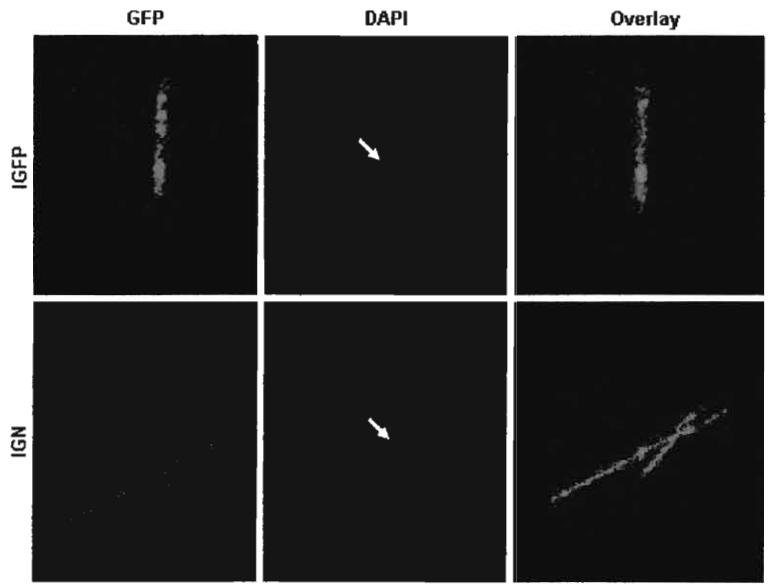


Fig.17 Confocal micrographs of green fluorescent protein: 3-day old hyphae of IGN, IGFP transformed *T. aggressivum* co-stained with DAPI.

3.3.3 GFP Quantification

Modified *T. aggressivum* cultures for protein isolation and analysis were grown in MEB liquid medium at 28°C. Samples were taken on days 3-7 of culture, and used for both GFP and total soluble protein quantification. GFP quantification result showed no significant different GFP protein production for all four mutants, except IGK produced higher GFP (30ng/g fresh

tissue) than the other three mutants (20ng/g fresh tissue) (Fig.16). GFP protein amount decreased from day 2 at 20~30ng/g fresh tissue to day 7 at about 7ng/g fresh tissue.

GFP concentration divided by total soluble protein is presented in Fig.19. From the results, GFP concentrations for all mutants were in the range between 1.5 to 5 mg/g total protein. There was no significant difference between all four mutants. GFP concentrations were high on the day 2 (3~5 mg/g total protein) and decreased to 1.5mg/g total protein on day 7.

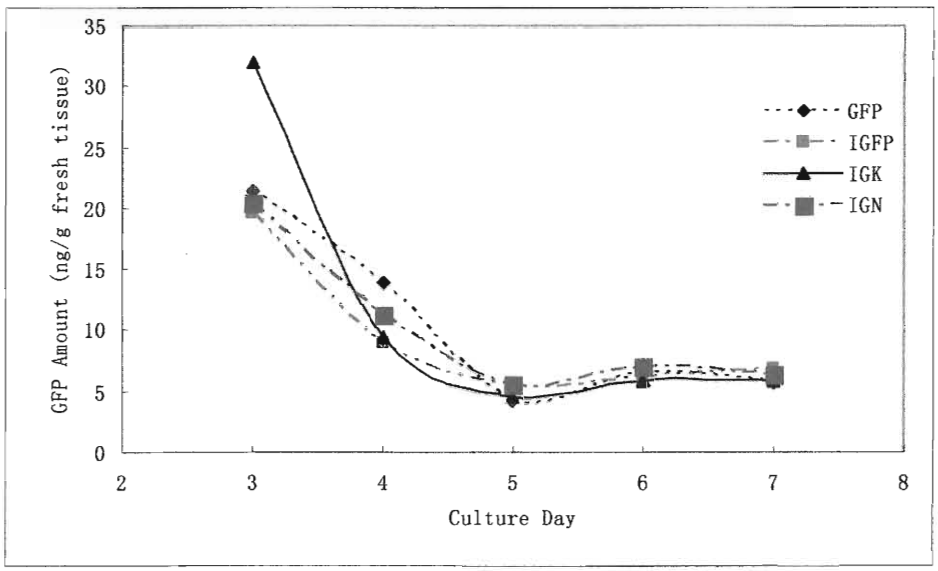


Fig. 18 GFP amount in modified *T. aggressivum* samples at different length of culture time

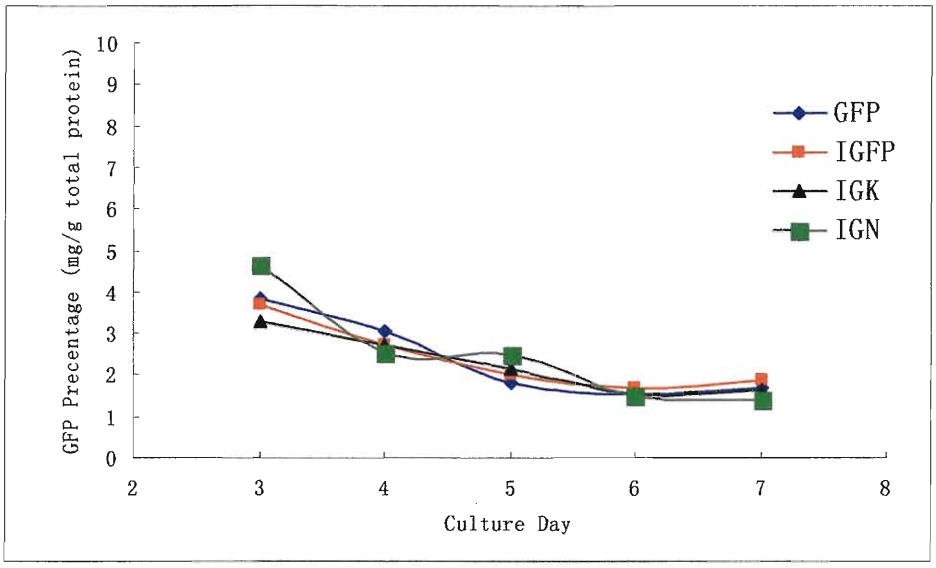


Fig. 19 Amount of GFP as a function of total protein of 4 modified *T. aggressivum* strains at days 3 to 7 of culture.

3.3.4 Western Blot Analysis of GFP Production

Based on the Western blot pictures, the transformants produced proteins with masses of about 30kDa when compared to the protein marker. All four transformants from day 3 and IGK from day 5 showed GFP signals, while no green fluorescent protein was detected from the rest samples. On day 3, IGK showed the highest GFP protein amount and followed by IGFP, both GFP and IGN samples showed the lowest GFP protein amount. GFP band of IGK on day 5 had the lightest color overall sample. GFP bands of all samples had a lighter color than the 2ng standard from the picture, indicating protein amount were lower than 2ng/sample. No GFP protein was detected from wild type *T. aggressivum.*

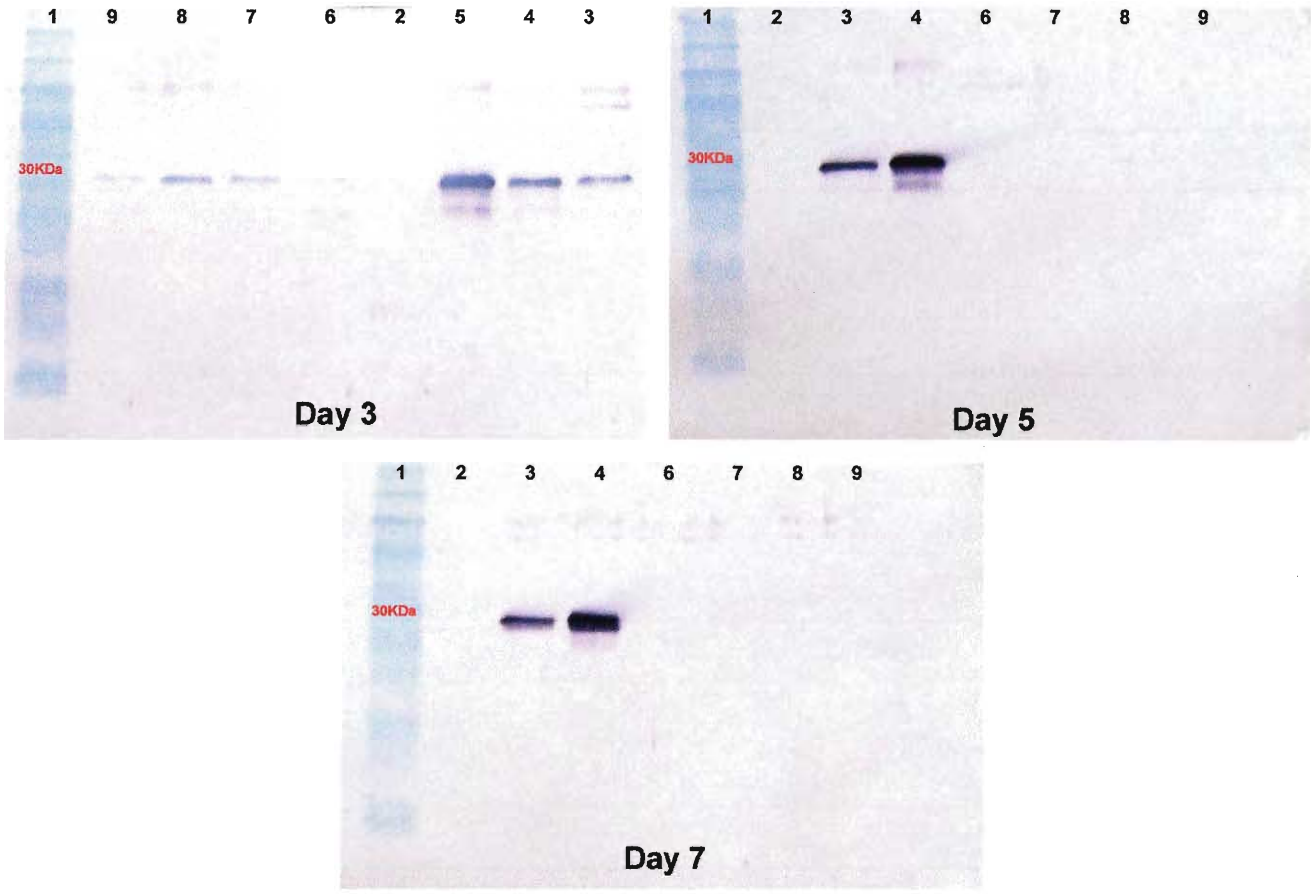


Fig. 20 Western blot picture of 4 modified *T. aggressivum* with day 3, day 5, and Day 7 broth cultured samples. 1. Prestained Protein Marker; 2. wildtype *T. aggressivum*; 3. GFP control (2ng); 4. GFP control (5ng); 5. GFP control (10ng); 6. GFP transformed *T. aggressivum*; 7. IGFP transformed *T. aggressivum*; 8. IGK transformed *T. aggressivum*; 9. IGN transformed *T. aggressivum*.

4. Discussion

4.1 Phenotype of Transf ormed *T. aggressivum*

When comparing the transformed and wild type *T. aggressivum*, we found that the phenotype was not altered by transformation events. Transformed and wild type *T. aggressivum*, appeared similar for growth rate, mycelia colour, conidium colour and distribution, and pigment secretion on all three media. The integration events that occur with *Agrobacterium*-mediated transformation did not apparently alter or disrupt any genes involved in the production of these phenotypes.

4.2 G FP Quantification

Three methods were used to analyze the GFP expression in *T. aggressivum*: microscopy; GFP assay; and Western blot. In general, microscopy results showed increased GFP signal intensity with age in all isolates while the GFP Assay and Western blot analyses showed a consistent decrease of fluorescence and protein amount with culture age in all isolates. The discrepancy between microscopy and the other two techniques are most easily explained by comparing the technical details of each analytical method. Visual estimation of fluorescence with microscopy gives total fluorescent signal given by the mycelium, while GFP assay and Western blot analysis give protein amounts as a function of total soluble protein or fresh weight of the mycelium. Furthermore, newly developed mycelia from the edge of the colonies on agar plates were picked everyday for microscopic observation, while the GFP assay and western blot used the whole broth culture samples. Mycelia start to die and conidia production starts at the late stage of culture development as nutrients are exhausted. Only newly developed hyphae were

examined by microscopy while the GFP and Western blot assays used the whole sample that included both new and aged hyphae. Therefore, we conclude that GFP production does not keep up with biomass production.

4.3 G FP Expression

4.3.1 Intron and GFP Expression

To study the effect of an intron on GFP expression, we compared GFP and IGFP transformants, the only difference of which was the absence of the intron. Both transformants showed GFP expression, which means that an intron is not essential for GFP expression in *T. aggressivum*. Furthermore, the expression level, as assessed by all three analytical methods, appeared similar on different days of culture. These observations proved conclusively that the intron has no affect on GFP expression. Other research groups found that GFP genes without an intron tend to be silenced in *Schizophyllum commune* (Lugones et al., 1999), *Phanerochaete chrysosporium* (Ma et al., 2001), the mushroom *Agaricus bisporus* and *Coprinus cinereus* (Burns et al., 2005). This intron-dependent gene expression phenomenon affects not only the GFP gene but also the expression of any other heterologous genes, and exists in various homobasidiomycetes and ascomycetes (Lugones et al., 1999). It is safe to conclude that gene expression in *T. aggressivum* does not depend on intron presence.

4.3.2 GFP Expression and KDEL

IGK and IGFP recombinants were compared to study the efficiency of KDEL, the ER retention signal. There are two known function of KDEL signals, ER retention of proteins and upregulation of protein expression (Wandelt et al., 1992). From the microscopy pictures, GFP signals from IGFP and IGK transformants occupied a large area of the hypha and only partially

overlapped with the ER-tracker, which indicated GFP still existed in significant amounts outside of the ER. The microscopy pictures did not provide enough resolution for us to make the conclusion that the KDEL signal sequence increased GFP retention in the ER. These results differ from the findings of Shouten et al. (1996) that showed increased ER retention of a single chain antibody in tobacco and of Ma et al. (2009) where inclusion of KDEL in a GFP construct resulted in ER retention in a mammalian cell line. In addition, as noted earlier, the amount of GFP produced by the IGK transformant did not differ from transformants with the other three constructs. Previous research showed the addition of KDEL signal to a target protein had various affects on its expression, as some resulted increased protein amount (Wandelt et al., 1992), while some observed no qualitative differences whether KDEL was added or not (Harholt et al., 2010).

4.3.3 GFP Expression and NLS

IGN and IGFP recombinants were compared to study the effect of a nuclear localization signal on protein targeting and expression. From the confocal micrographs, and protein assays, equivalent GFP signals were detected for both recombinants, which means that GFP expression was not affected by the addition of NLS. GFP signals of both IGN and IGFP recombinants existed all over the mycelia and not just the nucleus as indicated by DAPI staining. These observations proved that GFP was not directed into nucleus in readily observable amounts. Comparing to other research, GFP with a proper nucleus localization signal exhibits significant nuclear accumulation and shows the position of nuclei clearly in the plant *Nicotiana benthamiana* (Chakrabarty et al., 2007), and in the fungus *Aspergillus nidulans* (Leeder & Turner, 2007).

4.4 Future Research

The focus of this study was the subcellular localization of a foreign gene (localization signal-tagged GFP) in *T. aggressivum*. The results showed successful GFP expression, yet the subcellular localization of GFP appeared to be inefficient, at best. It is possible that more refined measurement techniques would provide a more definitive answer. First, the measurement capabilities of our existing equipment could be improved. The confocal microscope used was equipped with a blue laser that emits at 480nm for efficient eGFP detection, yet the excitation peaks of DAPI and ER-tracker dyes is closer to the UV at a wavelength of 355nm. In the present study, DAPI and ER-tracker staining gave very poor fluorescent signals with the blue laser. A UV source is recommended for repeated measurements. Second, fluorescent signals from the endoplasmic reticulum were poorly defined. Higher magnification would give better resolution for locating these structures.

Further manipulation of the signal sequences is also recommended. Since KDEL might function in *T. aggressivum*, we could try to fuse KDEL signal on both C and N termini of GFP to enhance its localization function. HDEL is another common ER-retention signal which can be tested instead of KDEL. There are many other nuclear localization signals can be substituted for the one in this study. The easiest way to find the function NLS is to find it in the target species, *T. aggressivum.* This is feasible by recovering any nuclear proteins in *T. aggressivum* and isolating the coding genes. Then the coding gene, whole or partial, can be fused to the GFP gene to find out the NLS domain. This NLS should function in *T. aggressivum.*

5. Summary

The results shown indicate that GFP and its modifications were successfully integrated into genome of *T. aggressivum* by *Agrobacterium* mediated transformation. All isolates showed similar phenotype as wildtype *T. aggressivum* and the expression of GFP in all constructs was stable for generations. The intron added to GFP was not essential for protein expression in *T. aggressivum*, GFP can express with or without the intron. KDEL signal did upregulate GFP expression on the early culture stage, while microcopy picture showed GFP fail to concentrate to ER. GFP tagged with NLS fail to direct protein into nucleus, microscopy picture showed the same GFP distribution pattern as IGFP control.

With this knowledge, further research will hopefully find the proper ER and nuclear localization signal for *T. aggressivum*. Localization signals for other organelles can be apply to the project, such as Golgi targeting signal. With these signals, we can generate series of vectors ready to transfer GFP or any target genes into different organelle which can greatly aid the future molecular and cell biological study.

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APPENDIX I

Stock solutions

1) 2.5X MM Salts for IMAS

Salts		For 1 liter solution
KH_2PO_4	potassium phosphate monobasic	3.625 g
K_2HPO_4	potassium phosphate dibasic	5.125 g
NaCl	sodium chloride	0.375 g
$MgSO_4$	magnesium sulfate anhydrous	0.610 g
$CaCl_2.2H_2O$	calcium chloride dehydrate	0.165 g
$FeSO_4.7H_2O$	ferrous sulfate	0.0062 g
$(NH_4)_2SO_4$	ammonium sulfate	1.250 g

Dissolve each slat one at a time. Do not autoclave. Store at room temperature. Final solution typically contains a small amount of white precipitate.

2) M-100 Trace Element Solution

Trace Element		For 500 ml solution
H_3BO_3	boric ancid	30 mg
$MnCl_2.4H_2O$	manganous chloride	70 mg
$ZnCl_2.4H_2O$	zinc chloride	200 mg
$Na_2MoO_4.2H_2O$	sodium molybdate dihydrae	20 mg
$FeCl_3$	ferric chloride anhydrous	30 mg
$CuSO_4.5H_2O$	cupric sulfate	200 mg
dH_2O		to 500 ml final volume

3) M-100 Salt Solution

Chemical		For 1 liter solution
KH_2PO_4	potassium phosphate monobasic	16 g
$NaSO_4$	sodium sulfate anhydrous	4 g
KCl	potassium chloride	8 g
$MgSO_4$	magnesium sulfate anhydrous	0.9757 g
$CaCl_2$	calcium chloride anhydrous	0.165 g
M-100 Trace Element Solution		8 ml
dH_2O		to 1 liter final volume

4) 1 M MES

Dissolve 19.52g of low moisture content MES in 80ml dH_2O. Adjust pH to 5.3 with 5M KOH and bring the volume to 100ml. Filter sterilize and store in 10 ml aliquots at -20℃. The salt may precipitate on thawing the frozen stock. If so, put the tube in 65℃ water bath for a while followed by vortexing to dissolve the salt.

5) 1 M Acetosyringone

Dissolve 0.192g of Acetosyringone (AS) in 1ml DMSO. Filter sterilize and store at -20℃. Protect with aluminum foil as AS is lignt sensitive.

6) Antibiotics and Herbicide

Carbenicillin	100mg/ml
Chlroramphenicol	50mg/ml
Cefotaxime	200mg/ml
Hydromycine B	50mg/ml

Media and plates

1) Introduction Liquid Media (IMAS solution)

Ingredients	For 100 ml medium
1X MM Salts	40 ml of 2.5X stock
10mM Glucose (dextrose)	0.18 g
0.5% Glycerol	0.5 ml
dH_2O	to 94 ml final volume

Autoclave, cool to 50℃, then add 4ml of 1M MES (40mM final concentration). Add AS to liquid IMAS as needed just before use.

2) Introduction Agar Media (IMAS plates)

Ingredients	For 100 ml medium
1X MM Salts	40 ml of 2.5X stock
10mM Glucose (dextrose)	0.18 g
0.5% Glycerol	0.5 ml
dH_2O	to 94 ml final volume
1.5% Agar	1.5 g

Autoclave, Prior to pouring the plates, add 4ml of 1M MES (40mM final concentration) and 20ul of 1M AS (200uM final concentration).

3) M-100 Agar Medium (M-100 plates)

Ingredients	For 100 ml medium
Glucose	1 g
KNO3 potassium nitrate	0.3 g
M-100 Salt Solution	6.25 ml
dH_2O	to 100 ml final volume
1.5% Agar	1.5 g

Autoclave.

APPENDIX II

Nucleotide sequences of 4 GFP constructions inserted in PUC19 when sequenced with M13F and M13R sequencing primer.

GFP_M13F.seq

AGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGAC
GTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGG
CAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCAC
CCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACAT
GAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCA
CCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAG
GGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGG
CAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCA
TGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATC
GAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAA
AGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCG
GGATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCTCTAGAGTCGACCTGCAG
GCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCC
GCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCC

GFP_M13R.seq

GCAGGTCGACTCTAGAGGATCCCTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGG
CGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGC
TCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCG
CCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATG
TTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATAT
AGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTC
CTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTT
CACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTG
GACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGG
GTAGCGGCTGAAGCACTGCACGCCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGG
GCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCGTAGGTG
GCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCC

AGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATG
AGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTT
ACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA
GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATG

iGFP_M13F.seq

ACCACACAGCCCGACCGCGACGACCGCGTGCTGACTTCGCTTCCAGGTCAAAGCAG
TGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGAC
GGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCAC
CTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTG
GCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGA
CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGG
AGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAG
TTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA
GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCT
ATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCAC
AACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCAT
CGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCT
GAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCG
CCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCTCTAGAGTCGAC
CTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTG
TTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACAT

iGFP_M13R.seq

CGACTCTAGAGGATCCCTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGG
TCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGG
CGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATG
GGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGG
CGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGT
TGTGGCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAA
GTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTC
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