

Endophytic fungi, characteristics and their potential for genetic manipulation

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Abstract

Unique benign endophytes from Ascomycets have wide distribution among grass species. The symbiotic fungi enhance plant characters including performance, insect and mammalian deterrence, nematode resistance and tolerance to drought, salt and other biotic and abiotic stresses. Endophytes from genus *Neotyphodium* (*Acremonium*) are of the major focus than their ancestors, and *Epichloe* species, because the formers have lost their sexual reproduction. Therefore they should be genetically stable, and most importantly, they cannot disassociate from host tissues, and are transferred vertically. They are maternally inherited and are therefore attractive for genetic transformation without the concern about gene escape. Some marker genes have been successfully transferred to endophyte *Neotyphodium coenophialum* and *Neotyphodium lolii* existing in *Festuca arundinacea* Schreb. and *Lolium perenne* L., respectively. Furthermore, gene silencing has been proved to be feasible for eliminating traits, which are economically harmful. Methods of direct DNA uptake using polyethylene glycol (PEG) and electroporation have been found to be useful in transformation of these fungi. Transgenic fungi can be reinserted into the host without need to tissue culture. The endophytic genes responsible for a specific trait can be isolated and transferred to grass species or other microorganisms for direct exploitation of secondary metabolites and endophytic enzymes. Considering advancements in this filed, endophytes can open new horizons faced to

scientists and biotechnologist to use them as a surrogate target of transformation.

Keywords: Endophyte, Molecular marker, *Neotyphodium*, Transformation

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INTRODUCTION

The well-known biological agents of plant protection in recent years are endophytic fungal symbionts, which predominantly are found in wild species of cool-season grasses (Schardl and Phillips, 1997; Miles *et al.*, 1998). Fungal endophytes, defined as fungi that spend their life cycle within the aerial parts of host plant, form non-pathogenic fungi that systemically occupy intercellular open spaces (Bacon and De Battista, 1991 and Schardl, 1996) and have key roles for ecological success of host plants (Selosse *et al.*, 2004). The well-known grass endophytes are classified in the tribe Balansieae of the family Clavicipitaceae (Ascomycets). In the grass family Poaceae, fungal endophytes have been found in Poaeae tribe in the genera *Festuca*, *Lolium*, and *Poa* (Bacon, 1995), Bromaeae

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tribe in the genus *Bromus* (White Jr, 1987), Stipeae tribe in the genus *stipa* (White Jr and Morgan –Jones, 1987; Bruehl *et al.*, 1994; Kaiser *et al.*, 1996), Meliceae tribe (White Jr, 1987), Aveneae tribe (White Jr, 1994), and Triticeae tribe. In Triticeae, the fungal endophytes have been found in *Elymus* (White Jr, 1987), *Hordeum* (Wilson *et al.*, 1991b) and wild species of *Triticum* (Marshall *et al.*, 1999). The most widely known and demonstrated endophytes from genus *Neotyphodium* are *N. coenophialum* (Morgan-Jones and Gams) Glenn, Bacon and Hanlin, *N. lolii* (Latch, Christensen, and Samuels) Glenn, Bacon and Hanlin and *N. uncinatum* (Gams, Petrini and Schmidt) Glenn, Bacon and Hanlin that infect tall fescue (*Festuca arundinacea* Schreb.) perennial ryegrass (*Lolium perenne* L.) and meadow fescue (*F. pratensis* Huds.), respectively.

It also seems that endophytes discovered in *Hordeum* and *Triticum* should be from genus *Neotyphodium*. Meanwhile endophytes have been found in some Iranian species such as *Bromus tomentellus* and *Melica persica*, beside species of *Festuca* (Khayyam Nekouei *et al.*, 2000; Khayyam Nekouei *et al.*, 2001; Mohammadi and Mirlohi, 2003) and *Lolium* that possibly belong to *Neotyphodium* (Mirlohi and Sabzalian, 2002) (Table 1). Interaction between *Neotyphodium* endophytes and cool-season grasses culminates a defensive mutualism in which endophytes produce a range of alkaloids or stimulate the host grass to synthesize alkaloids and other secondary metabolites that protect macrosymbiont (host) from mammalian, insect, and nematode herbivores (Malinowski and Belesky, 2000). Thus the ecologically diverse grasses and their successful adaptations may be due to this association (Bacon *et al.*, 1997). They have no sexual reproduction and even sometimes do not produce asexual spores. For this reason, *Neotyphodium* endophytes are dependent on the host for their life and instead they produce protective alkaloids to support host species. Four alkaloid classes have so far been associated with the fungal endophytes; the ergot alkaloids (e.g., lysergic acid and ergovaline), the indole diterpenes (e.g., paxilline and lolitrem B), a pyrrolopyrazine (peramine) and the saturated aminopyrrolizidines (loline, norloline, N-acetylloline, and N-formylloline, which are all called “Lolines”). Other compounds such as ergosterol, harmaline, norharmaline and halostachin and plant growth regulators have been reported in some endophyte researches, but their role and mechanism is under investigation (Porter, 1994). Three of the four mentioned alkaloid classes have been produced by endo-

phytes grown in culture except of Lolines that merely have been detected in host plants. Apparently, plant is a bio-enhancer for production of lolines, however, Porter (1994) poses an idea that lolines may be an endophyte-induced production of plant. All four classes have anti-insect activities but peramine is the most potent alkaloid in this respect. Lolitrem B is associated with the disorder ryegrass staggers commonly observed in sheep grazing perennial ryegrass and ergovaline can cause maladies among cattle grazed on infected tall fescue called fescue toxicosis. Ergovaline can reduce lactation and fertility, cause miscarriage and stillborn offspring (Scharndl and Phillips, 1997).

The approaches for mitigating or eliminating toxicosis are: 1) production and replanting with endophyte-free cultivars, 2) alleviating toxic symptoms by vaccination against ergot alkaloids with Metoclopramide, Domperidone and Prazosin, 3) selecting endophyte genotypes with low toxic alkaloid accumulation, and 4) genetic engineering of the endophytes (Scharndl and Phillips, 1997; Thompson and Garner, 1994). Because of the natural role of *Neotyphodium* in biological protection, the approach of removing the endophyte is neither rational nor practical (Tsai *et al.*, 1992). Other ways are available but genetic engineering involves extra advantages. Cloning of genes involved in the ergot alkaloid biosynthetic pathway and their eventual manipulation simultaneously via gene knockout or anti-sense strategies, would provide a means for identifying roles for ergot alkaloids and genetic analysis of biosynthetic pathways (Panaccione *et al.*, 2001). Finally this technique provides conditions for introducing exotic encoding genes that produce insecticide delta toxins, proteinase inhibitors etc.

Endophytes as bio-control agents: The most important effects of endophytic fungi have been observed against wild life, particularly insects feeding on green tissues of the plant. The first of this, was Argentine stem weevil (*Listronotus bonariensis*), when endophyte-free plants were heavily damaged by stem weevil larvae, but damage on endophyte-infected plants was negligible (Rowan and Latch, 1994). Later, sod web worms (*Crambus* spp.), billbugs (*Sphenophorus parvulus*), fall armyworms (*Spodoptera frugiperda*) and hairy chinch bugs (*Blissus leucopterus hirtus*) were found to be affected by endophytes and now this list has been fully prolonged (Clement *et al.*, 1994; Sabzalian *et al.*, 2003; Sabzalian *et al.*, 2004). Evidence suggesting protection against root-feeding insects are rare but authors have shown that roots of

Table 1- List of plant species that have been so far identified to be harboring endophyte in Iran (data not published).

Species*	Distribution
<i>Festuca arundinacea</i> Schreb.	Chahar mahal, Isfahan, Khorasan, Azarbayejan, Lorestan, Hamedan
<i>Festuca pratensis</i> Huds.	Chahar mahal, Gorgan
<i>Lolium perenne</i> L.	Chahar mahal, Azarbayejan, Kordestan
<i>Lolium persicum</i> Boiss. & Hohen. ex Boiss.	Chahar mahal, Isfahan
<i>Bromus tomentellus</i> Boiss.	Isfahan, Chahar mahal, Khorasan, Azarbayejan
<i>Melica persica</i> Kunth.	Isfahan, Chahar mahal

*. Species and regions have been arranged based on priority of identification.

endophyte-free tall fescue plants can be severely attacked by insects and this is not the case in endophyte infected plants (Fig. 1), probably due to alkaloid accumulation in rhizosphere (data not published). Four classes of fungal metabolites are responsible for insect resistance in endophyte infected *Festuca* and *Lolium* species. These are indole diterpenes, ergot alkaloids, peramine, and lolines (Rowan and Latch, 1994). Indole diterpenes include lolitrems, lolitriol, and paxilline that are basically neurotoxin and tremorgenic in mice and sheep but might have anti-insect activity. Out of the ergot alkaloids, ergovaline is the most active against stem weevil and also cause heat stress in cattle. Others are less important in this respect. Loline alkaloids and peramine have the most potent anti-insect properties. They can also contribute to make some other known and unknown benefits. Presumably loline alkaloids were shown to reduce germination rate of competitor weed seeds (Malinowski and Belesky, 2000).

It seems that mechanisms of resistance to insects through endophyte infection consist both antixenosis and antibiosis via alkaloid production. Resistance to insects is so important that may serve as bio-indicator for presence of endophyte. Wilson *et al.* (1991a) in a bioassay with Russian wheat aphid (*Diuraphis noxia*) reported significantly fewer aphids on perennial ryegrass plants containing endophyte as compared to those lacking endophyte.

Beside endophyte influence on insects, there are several reports of endophyte activity against fungal diseases, viral pathogens and nematode herbivores. Gwinn and Gavin (1992) have shown that in germina-

tion of tall fescue seeds highly infected with endophyte, presence of *Rizoctonia zea* can be easily ignored, as if it is not present. In other hand, resistance to fungal pathogens has not been crucial. For instance, authors have not found significant difference between endophyte-free and endophyte infected meadow fescue and tall fescue attacked by *Blumeria graminis* (data not published). Endophytes can indirectly diminish viral diseases by host insect deterrence. West *et al.* (1993) have shown fewer incidence of barley yellow dwarf virus (BYDV) on tall fescue infected with *Neotyphodium coenophialum* possibly through reduction in insect population.

Effect of endophytic fungi on endoparasitic nematodes has been taken the most attention after insects. *Meloidogyne marylandi*, a root knot nematode can be



Figure 1. Influence of endophyte on root colonization by mealy bugs. Endophyte-infected plant of genotype 83 is free from white clonies of mealybug *Phenacoccus solani* (left).

deterred when endophyte exists in above ground tissues of perennial ryegrass (Ball *et al.*, 1997). There are similar results about *Pratylenchus scribneri* (Kimmons *et al.*, 1990). Mechanisms of resistance to nematodes have yet to be approved, but could be due to some alkaloids or indirectly by maintaining general health.

Having known of endophyte effects on biological control, they may serve as genetic sources of insect resistance and tolerance to biotic stresses.

Genetic diversity of endophytes based on molecular markers:

Advances in molecular biology including those for molecular hybridization, cloning, restriction endonuclease digestions, protein profiling and nucleic acid sequencing have provided many new tools for the investigation of phylogenetic relationship particularly amongst microorganisms like endophytes. Depending on the information required, the most appropriate techniques can be used. Lauchtmann and Clay (1990) used isozyme variation for grouping of endophyte from *Epichloe* and asexual derivatives (*Neotyphodium*). The electrophoretic profiles did not reveal much difference between sexually and asexually reproducing endophytes, but endophytes from different populations and different host species showed distinct enzymatic banding patterns. Multibanded electromorph was observed that might be due to multiple copies of the gene with different allozymes via heterokaryosis and/or aneuploidy (Lauchtmann and Clay, 1990). Such conclusion has been resulted in a hypothesis that some endophytes have been evolved from hybridization between two separate species (Tsai *et al.*, 1994). They realized that most tall fescue endophytes have two or three distinct copies of β -tubulin (*tub-2*) gene, while *Epichloe* isolate and meadow fescue endophyte (*Neotyphodium uncinatum*) had only one *tub-2* gene. It is theorized that endophytes when coexist in a single host can be vegetatively fused and produce a new species after subsequent events such as inter-chromosomal interchange and deletion of extra chromosomes. Christensen *et al.* (1993) also used isozyme analysis to discriminate endophytic isolates and found taxonomic grouping peculiar to any of three grass species, *F. arundinacea*, *F. pratensis* and *L. perenne*.

Beside biochemical markers, PCR-based markers have been used to distinguish and detect endophytic fungi of grasses. Arbitrary and specific primers have been designed to detect endophyte purely or within host-endophyte DNA mixture (Ganjali *et al.*, 2004). Liu *et al.* (1995) showed that a decamer primer (5'-CCGAGGTGAG-3') amplifies various fragments

in *Neotyphodium lolii* and *Neotyphodium coenophialum* and thus can distinguish these two species but does not produce any band from the minus DNA control. Specific primers have also been developed to detect endophytes in plants. This technique can be used for detecting endophytes in seed lots and to measure percentage of endophyte infection (Doss and Welty, 1995 and Doss *et al.*, 1998).

Tredway *et al.* (1999) used amplified fragment length polymorphism (AFLP) analysis to estimate phylogenetic relationships among *Epichloe* and *Neotyphodium* fungi and observed that clades of *Epichloe festucae* correspond to the host species from which they have been isolated. This may be due to coevolution in grass-endophyte combinations. Schardl and Siegel (1993) posed that if co-evolution is faithful, the branching order of the endophytes on the phylogenetic tree should correspond to the branching order of the host from which they were obtained, but the result showed discrepancy in this theory. Most of this discrepancy pertains to *Epichloe typhina* biotypes that can cross-inoculate several grass species.

Microsatellites or simple sequence repeats (SSR), have been identified in endophytic fungi that the core sequence of these repeats consists of (CA)_n, (GA)_n, (CAA)_n, (GAA)_n, or (ATC)_n. Primers targeted to flanking sequences of these microsatellites have been constructed that can be used for identification of endophytes both in culture and in plants (Moon *et al.*, 1998 and Van Zijll de Jong *et al.*, 2003). Presence of multiple bands in some samples has confirmed the hybrid nature of the endophytes. Meanwhile the presence of more than one band may be due to coexistence between two or more endophytes as shown (Fig. 2) for amplification with ITS (Internal Transcribed Spacers) primers (Khayyam Nekouei, 2001). These primers can be attached to the conserved sequences of *rrn* locus and amplify the internal transcribed spacers of this region.

Recently, a genomics program has been focused on EST (Expressed Sequence Tagged) of *Neotyphodium* endophytes. The aim of this program is to discover the genes contributing in host colonization, nutrient supply to the endophytic fungus, biosynthesis of active secondary metabolites and physiological mechanisms leading to enhanced plant vigor and tolerances to stresses (Spangenberg *et al.*, 2001; Van Zijll de Jong *et al.*, 2002). More information may help about grass/endophyte association to use this system to increase plant performance and improve grass tolerance to biotic and abiotic stresses (Spangenberg *et al.*, 2001).

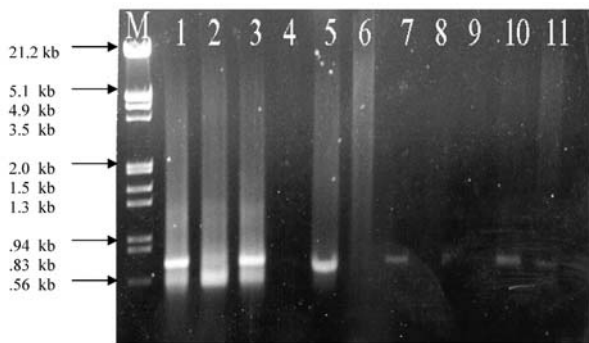


Figure 2. PCR amplification product obtained by using ITS1 and ITS4 primers from tall fescue genotypes containing more than one endophyte (lane 1 and lane 3) (Khayyam Nekouei, 2001).

Transformation of endophytes by marker genes: An important advancement in biotechnological applications of endophytic fungi is the advent of transformation systems that can deliver the novel genes into these organisms (Scharidl, 1994). Methods have been developed to introduce foreign genes into hyphae of endophytes. These techniques have been linked with inoculation of endophyte-free plants by transgenic endophytes. Such inoculation technique was firstly reported by Latch and Christensen (1985). They placed endophyte mycelium into the coleoptile tissue of sterile seedling growing on water agar (Latch and Christensen, 1985; Johnson-Cicalese *et al.*, 2000 and Bouton *et al.*, 2002). Other technologies consist of infection of somatic embryos (Kearney *et al.*, 1991) and inoculation of callus culture (Johnson *et al.*, 1986). The first method has been widely used mainly due to its independent nature to callus formation.

Murray *et al.* (1992) conducted the first experiment to produce transformed endophyte. They utilized *Neotyphodium* sp. strain 187 BB that could not produce neurotoxin lolitrem B in perennial ryegrass. They obtained protoplasts of fungus by means of Novozym 234 application and used a buffer containing a 40% polyethylene glycol (PEG) 4000 solution and added plasmid vectors which individually carried hygromycin resistance (*hph*) and β -glucuronidase (*uidA*) genes. Transformed hyphae were successfully transferred to host plant as described by Latch and Christensen (1985). Tsai *et al.* (1992) also used *hph* gene for transformation of *Neotyphodium coenophialum*. After protoplast production, they used gene pulser in an electroporation system to deliver marker gene into competent protoplast. They concluded that transformation of endophyte does not affect host compatibility compared with non-transformed type. Mikkelsen *et al.*, (2001) in Denmark, performed

another transformation experiment. They used green fluorescent protein gene (*gfp*) to expressed it in *Neotyphodium lolii*, as an endophyte of *Lolium perenne*. They also used PEG solution as proposed by Murray *et al.* (1992), but the efficiency of transformation was lower than Murray's report. Expression of *gfp* could be visualized by epifluorescence microscope. With transfer of this gene there was no need to add substrate, which is required for β -glucuronidase (GUS) activity (Mikkelsen *et al.*, 2001).

The transformation works of Murray and co-workers provided the idea of utilization of these microorganisms as a surrogate carrier for exotic genes instead of plant genomes. The importance of this system basically relates to inability of genes inserted into endophytic genome to flow among plants due to cross-pollination. This is due to maternal inheritance of endophyte and inhibited gene escaping.

Gene knocking out and elimination of undesirable traits: Widespread application of endophytic fungi in forages and agronomic crops depends on elimination of alkaloids contributing to mammalian toxicity. Among the approaches suggested for remedial treatment of livestock toxicity, genetic alteration of the fungus plays an important role in elimination or reduction of toxic factors (Tsai *et al.*, 1992). Two strategies have been suggested for this purpose. The first one relies on disruption of effective genes involved in biosynthetic pathway (Panaccione *et al.*, 2001 and Wang *et al.*, 2004). In another strategy, scientists are looking for microorganisms (bacterial or fungal) that can symbiotically metabolize toxic agents (Panaccione *et al.*, 2001). In the first strategy, it was used a gene fragment namely CP. 605 from *Claviceps purpurea* and found a similar peptide synthetase gene in *Neotyphodium* sp., endophytic fungi of *Lolium perenne*. The *hph* gene was inserted into this fragment and used for transformation of *Neotyphodium* sp. Knockout construct was added to protoplasts as had been described by Murray *et al.* (1992). After transformation, fungus was used for inoculation of perennial ryegrass and transformants, established compatible association with grass host (Panaccione *et al.*, 2001). In this experiment, *lpsA* gene that is essential for ergopeptide biosynthesis in *Neotyphodium* was disrupted. Extraction of surrogate transformed plant and detection of ergovaline by fluorescence HPLC showed that knockout containing symbiote could not produce ergovaline (Panaccione *et al.*, 2001) and some other ergot alkaloids (Panaccione *et al.*, 2003).

Future Technologies by using gene replacement

and gene disruption techniques or expression of anti-sense RNA will be beneficial for alleviating disadvantageous traits (but ecologically advantageous) and help breeders use this association in agronomically important crops.

Future Prospects: With respect to grass species, endophytes can provide resistance to mammalian, insects and nematode herbivores as well as drought tolerance and improved competition and persistence. In the past, the selectable options for remediation of animal toxicities were treatment with vaccines, selection of plant and endophyte genotypes, which were low in toxic alkaloids, or necessarily eradication of endophyte. Nowadays, genetic manipulation of endophyte can produce infected grass cultivars with stress resistance and insect deterrence, without affecting animal life (Porter, 1994). Endophytes may provide unique sources of natural plant growth regulators, insecticides, herbicides and pharmaceutical useful compounds (Strobel, 2003). Production of anti-tumor, anti-cancer agents such as the leucinostatins from some *Acremonium* endophytes has been reported (Strobel *et al.*, 1997). Also ergot alkaloids with medicinal applications and several unknown antibiotics have been found (Porter, 1994; and Mantle, 1987), but unfortunately, progress in this respect is very slow and research may reveal an endless treasure trove of medicinal agents.

Endophytes can also have industrial applications (Stone *et al.*, 2000). For example they can produce enzymes such as xylanase and β -glucanase (Sotu *et al.*, 2002; Moy *et al.*, 2002) probably useful in food and animal feed industries. Furthermore, some *Acremonium* species have genes encoding phenol oxidase that can be used for production of enzymatic bleach (Gouka *et al.*, 2001).

Endophytes transformed with exogenous DNA can be used as surrogates for transgenic grass plants. They may encode insecticide delta toxins from *Bacillus thuringiensis* strains or proteinase inhibitors (Scharndl, 1994). Endophytes transformed with fungicide and herbicide resistance genes will have ecologically advantages for host plant and themselves. It may be possible to isolate some of genes responsible for insect or nematode resistance from endophyte and introduce them into plant genome. These genes may be altered so that to be more effective or have broader range for protection.

Because of their importance for human, preservation of these microorganisms is worth considering. Making scientists informed about endophyte proper-

ties should expedite reaching to this goal. The use of endophytes in agriculture, industry and medicine will be extensive and they will have an especial place in future biological research.

Concluding remarks: In summary, molecular technology with genetically modifying endophytic fungi offers approach to express exotic genes in plant host. Use of this technique removes difficulties in regeneration of genetically modified plants particularly in recalcitrant species and concerns about gene flow into wild species. Methods available for manipulating grass endophytes provide an efficient technique for exploiting novel traits in indigenous plant species. Also it is conceivable to isolate the endophytic genes responsible for desirable traits and transfer them into plant genome. However, reaching to this goal we need to improve our knowledge of biochemical and physiological basis of endophyte-host interactions based on present biodiversity of endophytes. Unfortunately, this knowledge is still superficial and neither biochemical pathways nor endophyte biodiversity are well understood. Toxic effect of endophyte on mammalian consumption is another problem that solutions for elimination of this unwanted trait have been suggested. Overall, promising future for wide use of endophytes in different branches of science is predicted.

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