

Chemical Ecology Mediated by Fungal Endophytes in Grasses

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Abstract Defensive mutualism is widely accepted as providing the best framework for understanding how seed-transmitted, alkaloid producing fungal endophytes of grasses are maintained in many host populations. Here, we first briefly review current knowledge of bioactive alkaloids produced by systemic grass-endophytes. New findings suggest that chemotypic diversity of the endophyte-grass symbiotum is far more complex, involving multifaceted signaling and chemical cross-talk between endophyte and host cells (e.g., reactive oxygen species and antioxidants) or between plants, herbivores, and their natural enemies (e.g., volatile organic compounds, and salicylic acid and jasmonic acid pathways). Accumulating evidence also suggests that the tight relationship between the systemic endophyte and the host grass can lead to the loss of grass traits when the lost functions, such as plant defense to herbivores, are compensated for by an interactive endophytic fungal partner. Furthermore, chemotypic diversity of a symbiotum appears to depend on the endophyte and the host plant life histories, as well as on fungal and plant genotypes, abiotic and biotic environmental conditions, and their interactions. Thus, joint approaches of (bio)chemists, molecular biologists, plant physiologists, evolutionary biologists, and ecologists are urgently needed to fully understand the endophyte-grass symbiosis, its coevolutionary history, and ecological importance. We propose that endophyte-grass symbiosis provides an excellent model to study microbially mediated multitrophic interactions from molecular mechanisms to ecology.

Keywords Defensive mutualism · Herbivory · Defense mechanisms · Alkaloids · Hormone pathways

Introduction

Defensive mutualism has been the predominant framework for studies on endophyte-grass symbiosis since the mid 1970s when Bacon *et al.* (1977) found that livestock disorders in tall fescue variety “Kentucky 31” were attributable to alkaloids produced by fungal endophytes (Cheplick and Faeth 2009; Clay 1988, 2009; Saikkonen *et al.* 2006, 2010). Today, these epichloid fungi comprising *Neotyphodium* endophytes and their sexual antecedents in the genus *Epichloë* are known to form a lifelong systemic infection throughout the above-ground parts of many pooid grasses (Cheplick and Faeth 2009; Clay and Schardl 2002; Saikkonen *et al.* 2006; Schardl *et al.* 2012).

In the symbiosis, fungal hyphae grow asymptotically and intercellularly throughout the above-ground host plant parts, including developing inflorescences and seeds. By growing into host seeds, the fungus is vertically transmitted from mother plant to offspring (Clay and Schardl 2002; Saikkonen *et al.* 2004). This promotes stable interaction between the fungal genotype and the host lineage until one or the other partner loses its viability. Thus, vertical transmission and the interdependence of fungal and host fitness generally are thought to align the interests of partners so that the fungus–host association becomes mutualistic (Ewald 1987; Saikkonen *et al.* 2004). Because of many demonstrated reciprocal benefits to the partners, ‘grass-endophyte’ has become synonymous with ‘mutualist’ in biological lexicon (Clay 1990; Clay and Schardl 2002; Saikkonen *et al.* 2004, 2006, 2010), and remains one of the text book examples of microbial mediated terrestrial plant-herbivore interactions (Thompson 2005). Accumulating evidence suggests that the ecological consequences of grass-endophyte symbiosis can further extend from plant-herbivore interactions to plant, microbial and animal communities as well (Clay and Holah 1999; Lemons *et al.* 2005; Omacini *et al.* 2001, 2004;

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Rudgers *et al.* 2010; Saari *et al.* 2010a,b; Saikkonen 2000; Saikkonen *et al.* 2006, 2013; Wäli *et al.* 2006).

Ultimately, the ecological role of fungal endophytes originates in fungal- and plant-origin bioactive alkaloids, hormones, and other metabolites that regulate fungal and plant responses to abiotic and biotic environments separately and/or as a phenotypic unit (Bacon *et al.* 1977; Hamilton *et al.* 2012; Leuchtman *et al.* 2000; Panaccione *et al.* 2013; Rasmussen *et al.* 2012; Saikkonen *et al.* 2004; Siegel and Bush 1996; Siegel *et al.* 1990). Although the literature of endophyte driven chemical ecology has blossomed in the past 35 years, the majority of studies have focused on the toxicity of fungal origin bioactive alkaloids to herbivores. The mounting body of recent literature suggests that in addition to fungal origin defensive alkaloids, other fungal and plant products may play roles in endophyte-grass symbiosis such as chemical cross-talk between endophyte and host cells, or signaling between plants, herbivores and their natural enemies (Cheplick and Faeth 2009; Eaton *et al.* 2010, 2011; Gundel *et al.* 2012; Hamilton *et al.* 2012; Moon *et al.* 2004; Nanda *et al.* 2010; Pieterse and Dicke 2007; Pineda *et al.* 2013; Rasmussen *et al.* 2012; Rodriguez and Redman 2005, 2008; Schardl *et al.* 2012; Simons *et al.* 2008; Tanaka *et al.* 2006, 2008; White and Torres 2010).

In this paper, we first present a summary of bioactive alkaloids produced by systemic grass-endophytes. We then discuss the current knowledge of other chemotypic diversity of endophyte-grass interactions, the ecological and evolutionary consequences of the diversity, and finally present potential gaps in knowledge and testable hypotheses for future studies.

Epichloae Alkaloids

Several distinguished papers have thoroughly reviewed bioactive alkaloids in epichloae endophytes (see *e.g.*, Leuchtman *et al.* 2000; Panaccione *et al.* 2013; Schardl *et al.* 2012; Siegel and Bush 1996; Siegel *et al.* 1990), with the following conclusions:

First, alkaloids with detected anti-herbivore effects fall into four classes: pyrrolizidines (lolines), ergot alkaloids, indole-diterpenoids (including lolitrems), and a pyrrolopyrazine alkaloid (peramine). Although epichloae endophytes can produce high levels of alkaloids, both inter- and intraspecific variation in amount and composition of chemicals in infected plants is remarkable (Siegel and Bush 1996). Peramine appears to be the most commonly detected alkaloid. Endophyte colonized grasses usually contain two or three chemical classes, but all four classes have not been detected in any examined symbiont. Strictly vertically transmitted asexual *Neotyphodium* endophytes appear to produce high levels of alkaloids while sexual *Epichloë* species tend to produce smaller amounts of alkaloids, typically peramines (Schardl *et al.* 2012).

Second, the ergot alkaloids have been demonstrated to provide plant defense against both vertebrate and invertebrate herbivores. In contrast, the lolitrems appear to have only anti-vertebrate properties, and lolines and peramines only anti-invertebrate properties (Schardl *et al.* 2012). Accumulating evidence suggests that the sensitivity of both invertebrate and vertebrate herbivores to different alkaloid patterns may vary among species and/or feeding guilds (Huitu *et al.* 2008; Miranda *et al.* 2011; Saari *et al.* 2010b; Saikkonen *et al.* 2010; Vesterlund *et al.* 2011). In addition to anti-herbivore effects, for instance, some ergot alkaloids such as agroclavine have antimicrobial activity (*e.g.*, Eich and Pertz 1999), thus potentially having direct effects on the host plant and indirect effects on other trophic levels.

Third, alkaloid content tends to vary within an individual plant, with highest levels in young leaves, stems, panicles (or spikes), and seeds (Ball *et al.* 1995; Justus *et al.* 1997). However, the distribution and variation in alkaloid concentrations within and among the plant parts depend on the particular alkaloid (Hovermale and Craig 2001; Spiering *et al.* 2005).

Fourth, present knowledge and analytical methods have the potential to reveal more about the chemotypic diversity and genetic regulation of endophyte-plant phenotypes. For example, peramine is a single chemical derived from three precursors (Tanaka *et al.* 2005). In contrast, ergot alkaloids and lolines are products of complex but linear pathways, and lolitrem B biosynthesis is based on metabolic grid (Panaccione *et al.* 2013; Schardl *et al.* 2012). Novel steps, precursors, associated enzymes and end-products are continuously characterized in new studies (see *e.g.*, Jensen and Popay 2004; Panaccione *et al.* 2013; Popay and Gerard 2007; Schardl *et al.* 2012; Stewart 2005) and genes and gene clusters for biosynthesis of the alkaloids are partly described (Schardl *et al.* 2012; Tanaka *et al.* 2005). However, the effects of environmental conditions on gene regulation are still poorly understood (Zhang *et al.* 2009).

Finally, although recent literature well recognizes the diversity of these alkaloids (Leuchtman *et al.* 2000; Panaccione *et al.* 2013; Schardl *et al.* 2012), ecological and evolutionary causes and consequences of variability within fungal and plant species as well as in their genetic combinations are largely unexplored (but see *e.g.*, Faeth and Shochat 2010; Jani and Faeth 2010).

Variability and the Driving Forces of Alkaloid Production

Presence of genes involved in alkaloid synthesis dictates potential for alkaloid production. Collectively, past studies suggest, however, that the profile and amount of alkaloids depend on the endophyte and the host plant life histories, as well as the interplay between fungal and plant genotypes, abiotic and biotic environmental conditions and their interactions (Boning

and Bultman 1996; Bultman *et al.* 2004; Schardl *et al.* 2012; Sullivan *et al.* 2007; Vázquez de Aldana *et al.* 2003).

We propose that the temporal stability of fungus-plant interaction selects for defensive mutualism. For example, Schardl *et al.* (2012) recently proposed that strict vertical transmission of the fungus selects for enhanced host protection by alkaloids of fungal origin. Empirical evidence supports this idea. Plants infected by vertically transmitted asexual endophytes produce substantially larger amounts of lolines than plants infected by sexually reproducing endophytes (Leuchtman *et al.* 2000; Zhang *et al.* 2010). Similarly, the long life cycle of perennial grasses should provide more opportunities for systemic growth and asexuality of the fungi. The question is whether the length of host life cycle selects for a *specific* or a more *diverse* chemotypic defensive arsenal of endophytes in the given environment. Relatively few annual grasses have been surveyed for their chemotypic diversity, but a few empirical examples support the idea that the endophyte of annual ryegrasses (*Lolium* spp.), *Neotyphodium occultans*, produces only loline alkaloids, while the endophyte of tall fescue (*Schedonorus phoenix*), *Neotyphodium coenophialum*, also produces ergovaline and peramine (see Schardl *et al.* 2012). However, it is noteworthy that selection for defensive mutualism depends not only on the fungal and the host life cycle, and the stability of the interaction, but also on herbivore pressure and its predictability as well as on abiotic environmental conditions (Faeth 2002).

As nitrogen-rich compounds, alkaloid production should depend on resource availability in soils (Faeth and Fagan 2002). A positive response of alkaloid production to the amount of available soil nitrogen and phosphorus has been reported (Arechavaleta *et al.* 1992; Belesky *et al.* 1988; Lyons *et al.* 1986; Malinowski *et al.* 1998). However, more recent research has revealed a negative effect of nitrogen on the concentration of endophyte biomass and alkaloid quantities in plants (Rasmussen *et al.* 2007). This may indicate different, non-linear responses of plants and fungi to the resource gradients (*e.g.*, nitrogen) or interaction between nutrients in soils and other environmental factors. For example, the level of alkaloids parallels the growth dynamics of host plant in response to seasons (Ball *et al.* 1995; Justus *et al.* 1997). The endophyte growth follows the seasonal growth of the host plant (di Menna and Waller 1986) suggesting a positive relationship between the amount of endophyte hyphae and the alkaloid concentration (Ball *et al.* 1995; Rasmussen *et al.* 2007; but see also Spiering *et al.* 2005).

Complexity of Chemical Interplay Between the Endophytic Fungus and the Host Grass

The vast majority of the literature on the chemical ecology of endophyte-grass symbiosis has focused on mycotoxins and defensive mutualism (Leuchtman *et al.* 2000; Panaccione

et al. 2013; Schardl *et al.* 2012). Chemical interplay between the partners is, however, more complex and multifaceted, ranging from cell level signaling (Eaton *et al.* 2011; Hamilton *et al.* 2012; Rasmussen *et al.* 2012) to chemical signaling and crosstalk between players involved in multi-trophic interactions (see *e.g.*, Pineda *et al.* 2013).

For example, recently Hamilton *et al.* (2012) emphasized that reactive oxygen species (ROS) and antioxidants are likely to have importance in endophyte-grass symbiosis. Reactive oxygen species are multifunctional by-products of normal aerobic metabolism such as photosynthesis and respiration, and are produced in both plant and fungal cells. Reactive oxygen species act in programmed cell death, stress responses, plant defense, and systemic long distance signaling within and among plant tissues in conjunction with antioxidants (Apel and Hirt 2004; Foyer and Noctor 2005; Mittler 2002). Oxidative balance is suggested to play a crucial role in the evolution of endophyte-plant interactions, beginning with the endophyte invasion into the host and including the establishment of asymptomatic infection by modulating the recognition and defense responses against the fungus (Gundel *et al.* 2012; Hamilton *et al.* 2012; Nanda *et al.* 2010; Rodriguez and Redman 2005, 2008; Tanaka *et al.* 2006, 2008; White and Torres 2010). Recent evidence also has demonstrated that the disruption of these signaling pathways can lead to a breakdown of the mutualistic interaction (Eaton *et al.* 2010, 2011; Tanaka *et al.* 2006, 2008). The key questions are: 1) whether oxidative balance of endophyte-grass symbiosis has been modulated during their coevolution from pathogenic to endophytic interactions, and 2) whether the oxidative balance affects the endophyte-grass symbiont's ability to cope with prevailing selection pressures (Hamilton *et al.* 2012).

Endophytes also may alter amount of available nutrients, sugars, and water, which also affect herbivore fitness (Agee and Hill 1994; Easton *et al.* 2002; Latch 1994; Leuchtman *et al.* 2000; Liu *et al.* 2011; Rasmussen *et al.* 2007, 2012; Vázquez de Aldana *et al.* 2009). They also alter plant hormones that modulate interactions between plants, herbivores, and their natural enemies. Endophytes can enhance host photosynthesis and potentially increase carbon reserves in host plants (Marks and Clay 1996; Richardson *et al.* 1993), and regulate host carbohydrate metabolism and utilization (Liu *et al.* 2011; Rasmussen *et al.* 2007). In a collaborative study with James Blande and Tao Li, we found that endophytes reduce constitutive emissions of green leaf volatile organic compounds (VOCs) but enhance the induction of volatiles upon herbivore feeding. However, endophyte-mediated volatile emission varied among grass species and genotypes (Li *et al.* unpublished manuscript). Accumulating evidence has demonstrated that volatiles may play a role in plant-to-plant communication and serve as foraging cues for herbivores and their natural enemies (Dicke and Baldwin 2010; McCormick *et al.* 2012; Xiao *et al.* 2012).

Thus, endophytes may have either positive or negative effects on herbivores. They may be an important source of within plant variation that makes host plant quality more unpredictable for herbivores depending on the complexity of trophic interactions.

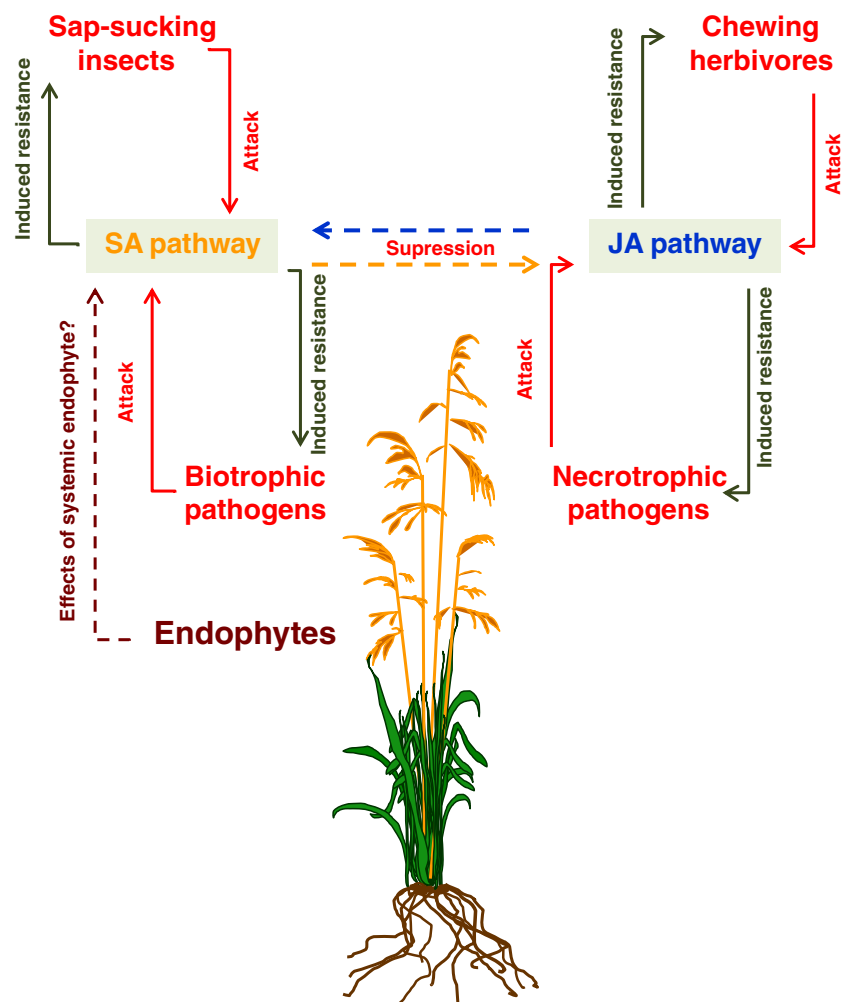
Interaction Between the Endophyte-Mediated Anti-Herbivory Mechanism and Plant Defense Mechanisms

Compared to many other types of plants, grasses are particularly well adapted to tolerate herbivory. Grasses have coevolved with vertebrate grazers since their early evolution (Prasad *et al.* 2005), and intensive ungulate grazing often plays a major role in maintaining grassland ecosystems by preventing succession (McNaughton 1984; Vicari and Bazely 1993). Because of strong selection by grazers, grasses quickly replace the lost photosynthetic tissue, sometimes overcompensating their growth after grazing. However, grasses appear to be less resistant to herbivory than broad leaf plants, supporting the

resistance-tolerance trade-off hypothesis (Agrawal 2011; Agrawal and Fishbein 2008; Coley *et al.* 1985; Van Der Meijden *et al.* 1988). Although grasses produce some defensive chemicals (*e.g.*, hydroxamine acids, phenolics and polyphenolics, and cyanogenic glycosides), silica is suggested as the principal chemical defense in grasses (Massey and Hartley 2006; Vicari and Bazely 1993). Tolerance and multiple resistance traits may evolve together under spatially varying and/or dynamic ecological and evolutionary conditions (Agrawal 2011; Romeo *et al.* 1996) and can be reinforced with coevolved symbiotic micro-organisms such as endophytic fungi.

We propose that an ecophysiological perspective is required for a more comprehensive understanding of the endophyte-grass symbiosis, and grass defense mechanisms, and particularly their relation to the endophyte-mediated defense. Plant responses to herbivores, pathogens, and beneficial microbes such as endophytic fungi are mainly regulated by two evolutionary conserved phytohormone signaling pathways, the salicylic acid (SA) and the Jasmonic acid (JA) pathways (Fig. 1.) (Karban and Baldwin 1997; Pieterse and Dicke 2007; Pineda *et al.* 2013). The SA pathway

Fig. 1 Salicylic acid (SA) and jasmonic acid (JA) pathways, their inducers and targets in a grass colonized by systemic endophytic fungus. The two pathways are suggested to be mutually antagonistic and consequently suppress each other when induced



mediates plant defense against biotrophic pathogens and some sap-sucking insects, while the JA pathway is involved mainly in defense against necrotrophic pathogens and chewing herbivores (Fig. 1)(Thaler *et al.* 2012). The two pathways are suggested to be mutually antagonistic (Ballaré 2011; Pineda *et al.* 2013; Thaler *et al.* 2012). For example, if the SA pathway is induced by a biotrophic pathogen and consequently suppresses the JA pathway, it might increase susceptibility to necrotrophic pathogens or chewing herbivores or both. Because many endophytes are biotrophic pathogens and strictly asexual (*Neotyphodium* endophytes may be derived from biotrophic pathogens), endophyte infection can be expected to induce or modulate the SA pathway in its host plant.

Thus, we can assume that the evolution from pathogenic plant interactions to grass-endophyte symbiosis involved mutual recognition and adaptive plant responses. These adaptations allowed asymptomatic fungal invasion, establishment of symbiosis, and acquisition of the alkaloids conferring resistance to herbivores (Cheplick and Faeth 2009; Moon *et al.* 2004; Pieterse and Dicke 2007; Schardl *et al.* 2012), and has probably had consequences for the plant defense *via* phytohormone signaling pathways. For example, heavy vertebrate grazing should decrease grass resistance to biotrophic microbes by hindering the SA pathway, thus opening a window for systemic endophytes. We can also assume that the endophyte as a biotrophic parasite hinders the JA pathway in endophyte-infected host grasses by inducing the SA pathway, thus decreasing grass resistance to necrotrophic pathogens and chewing herbivores. In some cases, crosstalk between symbiotic partners may lead to “compensated trait loss” when the lost function is provisioned by the interacting partner (Ellers *et al.* 2012). A study by Simons *et al.* (2008) found that although methyl jasmonate treatment hindered tall fescue’s endophyte-mediated resistance against aphids, the magnitude of endophyte-mediated defense is higher compared to the JA-mediated defense.

Concluding Comments

Multiple plant resistance traits and alkaloid production by symbiotic endophytic fungi are not necessarily redundant or mutually exclusive (Agrawal 2011). However, the balance between antagonistic signaling pathways that determine plant responses to different aggressors and endophyte-mediated grass defenses may reflect dynamic selection pressures (Saikkonen *et al.* 2004; Thompson 2005). This may explain detected plant adaptations to systemic endophyte infection, variability in grass-endophyte frequencies, and chemotypic diversity of symbiota. For example, we may expect strong herbivore pressure to select for high plant tolerance to herbivory and defensive endophyte mutualism.

However, recent advances in chemical ecology, molecular mechanisms of induced plant responses, ecology of biotic interactions, and coevolutionary processes suggest that, similar to other biotic interactions, endophyte-grass interactions are far more complex and have far-reaching impacts on other interactive species in communities.

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