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Review

What triggers grass endophytes to switch from mutualism to pathogenism?

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ABSTRACT

Symbioses between cool season grasses and fungi of the family Clavicipitaceae are an integral component of both natural and agricultural ecosystems. An excellent experimental model is the association between the biotrophic fungus *Epichloë festucae* and *Lolium perenne* (perennial ryegrass). The fungal partner produces a suite of secondary metabolites that protect the host from various biotic and abiotic stresses. The plant host provides a source of nutrients and a mechanism of dissemination via seed transmission. Crucial mechanisms that maintain a stable mutualistic association include signaling through the stress activated MAP kinase pathway and production of reactive oxygen species by the fungal NADPH oxidase (Nox) complex. Disruption of components of the Nox complex (NoxA, NoxR and RacA), or the stress-activated MAP kinase (SakA), leads to a breakdown in this finely balanced association, resulting in pathogenic infection instead of mutualism. Hosts infected with fungi lacking a functional Nox complex, or the stress-activated MAP kinase, display a stunted phenotype and undergo premature senescence, while the fungus switches from restricted to proliferative growth. To gain insight into the mechanisms that underlie these physiological changes, high throughput mRNA sequencing has been used to analyze the transcriptomes of both host and symbiont in wild-type and a mutant association. In the Δ*sakA* mutant association, a dramatic upregulation of fungal hydrolases and transporters was observed, changes consistent with a switch from restricted symbiotic to proliferative pathogenic growth. Analysis of the plant transcriptome revealed dramatic changes in expression of host genes involved in pathogen defense, transposon activation and hormone biosynthesis and response. This review highlights how finely tuned grass-endophyte associations are, and how interfering with the signaling pathways involved in maintenance of these associations can trigger a change from mutualistic to pathogenic interaction.

Contents

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1. The biology of grass–endophyte interactions

Interactions between plants and fungi play a crucial role in terrestrial ecosystems. These associations span a broad continuum from detrimental pathogens to beneficial symbionts. One of the most ecologically important plant–fungal associations is the mutualistic interaction between fungal endophytes of the *Epichloë* and *Neotyphodium* species (henceforth referred to as epichloë endophytes) and cool season grasses. In these associations, the fungus obtains host nutrients, protection, and a means of transmission via the colonization of host seeds [1]. In return, endophyte infection dramatically enhances plant survival through increased drought tolerance, and protection against insect and mammalian herbivory through the production of bioprotective secondary metabolites (reviewed in Ref. [1]). However, fungal synthesis of these secondary metabolites has important consequences for grazing animals in agricultural ecosystems. Epichloë endophytes produce at least four classes of bioprotective secondary metabolites; lolines, peramine, indole diterpenes and ergot alkaloids. While endophyte synthesis of lolines and peramine is beneficial in agricultural ecosystems because of their insect deterrence properties, ergot alkaloids (despite also having insect deterrence properties) and indole diterpenes are detrimental because of their physiological effects on livestock, including ryegrass staggers and ergot alkaloid toxicoses respectively (reviewed in Ref. [2]).

Interestingly, although epichloë endophyte associations are commonly referred to as mutualistic, this is not entirely accurate. In some *Epichloë* spp. the onset of host flowering induces the fungal sexual cycle, which causes these fungi to switch from a mutualistic asexual lifecycle to an antagonistic pathogenic sexual lifecycle. Hyphae proliferate over the surface of the flag leaf surrounding the host inflorescence and form a stroma that prevents emergence of the inflorescence, a phenomenon known as 'choke' [2]. This begs the question: are epichloë endophytes really mutualistic symbionts, or pathogens whose growth is modulated by the host? Under normal conditions the host is able to suppress or restrict fungal growth. However, when the host begins flowering, resources are mobilized to the inflorescence for reproduction. This change in resource distribution triggers a change in fungal physiology, which in some cases leads to proliferative growth and choke of the host inflorescence. Identifying the genes that trigger this change would be very difficult in natural isolates, but the pathogenic potential of epichloë endophytes can be systematically reconstructed by generating mutants that disrupt the symbiotic interaction. *Epichloë festucae* mutants that cause a stunting of the grass host invariably display a proliferative growth phenotype within the grass leaves. These mutational changes are discussed in more detail below.

Maintenance of endophyte–grass associations requires tightly regulated responses from both host and symbiont, including suppression of host defenses, strict control of fungal growth, and inhibition of fungal production of toxic proteins or metabolites that might elicit a host defense response. In wild-type associations, hyphae systemically colonize the intercellular spaces of host aerial tissues (Fig. 1), are aligned parallel to the leaf axis and seldom branch [3]. Hyphae are rarely found within host vascular tissues and they never penetrate host cells or produce specialized feeding structures such as haustoria, as found in other fungi [2]. Fungal growth in these associations is tightly coordinated with host growth, such that hyphae only grow during periods of leaf growth, resulting in comparable hyphal mass in old and young leaves [3,4]. However, this pattern of growth is inconsistent with the dogma that fungi grow mainly by polarized tip growth [5]. Instead, it has been proposed that epichloë endophytes grow in the leaves by intercalary division and extension [6]. We propose that hyphae initially spread by tip growth in the host shoot apical meristem and form a highly branched hyphal network. The hyphae then enter the developing leaf primordia, where they adhere to host cells undergoing division and extension, thus causing the hyphae to stretch. This stretching is thought to trigger a switch from tip growth to intercalary extension and cell division, thereby avoiding hyphal shear from the rapid leaf growth of >10 mm a day [6]. Above the leaf expansion zone, hyphae stop expanding but remain metabolically active [6,7].

2. Breakdown of mutualism in mutant associations

Given the highly regulated and coordinated nature of endophyte growth *in planta*, signaling between the fungus and its host must control fungal growth and maintain a balanced symbiotic interaction. To gain insight into the genes underlying the signaling required to maintain these associations, a synthetic association between *E. festucae* strain Fl1 and *Lolium perenne* (perennial ryegrass) was developed as a model experimental system [8]. This association provides an excellent framework for studying the symbiotic interaction. *E. festucae* is haploid, grows relatively fast in culture compared to other epichloë endophytes, and has high rates of homologous recombination [8]. The two partners form a stable symbiosis, and it is also relatively easy to inoculate *E. festucae* into perennial ryegrass seedlings, with infection rates of 80–90% for the wild-type strain [8]. In addition, draft genome sequences are now available for *E. festucae* strains E2368 (http://www.endophyte.uky.edu/) and Fl1 (http://csbio-l.csr.uky.edu/ef894/gbrowse/ef/). In a first step to identify fungal genes involved in the signaling required to maintain symbiosis with perennial ryegrass, plasmid insertional mutagenesis was used to create fungal mutants that were then screened for any change in their interaction with perennial ryegrass [9]. This resulted in identification of a mutant that switched from mutualistic to pathogenic growth. In this association, the fungus grew in an unrestricted manner with colonization of host vascular bundles, and dramatically increased biomass. Infected hosts were severely stunted, and precociously senescenced. The mutated gene encoded NoxA, a component of the multi-subunit NADPH oxidase complex, which produces the reactive oxygen species (ROS), superoxide, from molecular oxygen. Using a candidate gene approach, an additional two components of the *E. festucae* Nox complex, NoxR [10] and RacA [11], were found to be essential for maintaining a mutualistic association with perennial ryegrass. This highlights an interesting conundrum, as in the rye endophyte *Claviceps purpurea* deletion of *Cpnox1* [12] or *racA* [13] leads to a loss of pathogenicity. This reduction in pathogenicity is also seen in *Magnaporthe grisea NOX1* and *NOX2* mutants [14], and *Botrytis cinerea bcnoxA* and *bcnoxB* mutants [15], suggesting the Nox complex has evolved to play a role in pathogenicity of phytopathogenic fungi. So why does loss of ROS production induce a switch to pathogenicity in *E. festucae*, whereas pathogenicity is lost in phytopathogenic fungi? One possible explanation arises from the fact that ROS are required for polarized growth and cellular differentiation events (reviewed in Ref. [16]). In phytopathogenic fungi, the loss of pathogenicity is, at least in some systems, linked to either non-differentiation of infection structures such as penetration pegs and/or an inability to establish the polarized growth required for infection [16]. So what is the differentiation event that requires ROS in *E. festucae?* We propose that in *E. festucae*, ROS is needed for the endophyte to switch from proliferative, polarized tip growth in the host shoot apical meristem to intercalary extension in the expanding leaf primordium. Thus, mutants defective in ROS production do not switch to intercalary growth but instead continue proliferating in an unrestricted manner.

However, this hypothesis is complicated by the discovery that the switch from mutualism to pathogenicity is not restricted to *E. festucae* Nox mutants. An iron siderophore, encoded by *sidN*, is

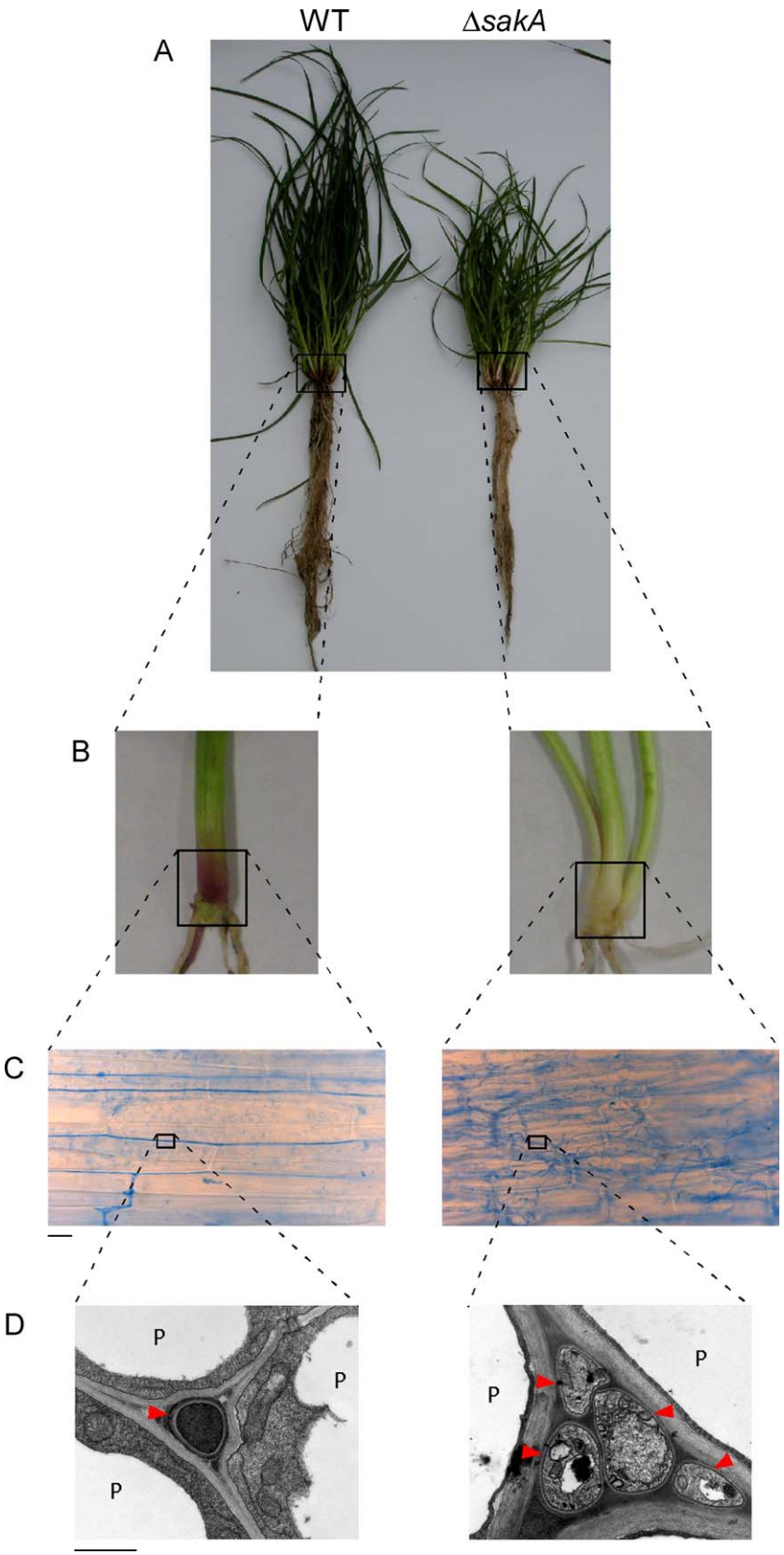

Fig. 1. Host and symbiont growth in a mutualistic, wild-type, *E. festucae*-perennial ryegrass association and in the pathogenic Δ*sakA* mutant association. (A) Phenotype of perennial ryegrass plants infected with wild-type *E. festucae* (WT) and the Δ*sakA* mutant. Photographs were taken 12 weeks after inoculation. (B) Base of tillers infected with WT *E. festucae* and the Δ*sakA* mutant, showing the lack of anthocyanin pigmentation and bulging at the base of Δ*sakA* mutant-infected tillers. (C) Light micrographs showing aniline blue stained hyphae growing in the leaf sheath of WT and Δ*sakA* mutant-infected perennial ryegrass. Bar = 1 mm. (D) Transmission electron micrographs of transverse sections through perennial ryegrass pseudostem tissue infected with WT *E. festucae* and the Δ*sakA* mutant. Arrows indicate fungal hyphae. P, plant cell. Bar = 1 μm.

Box 1: High throughput mRNA sequencing.
Determining rates of gene expression is central to understanding cross-species signaling. Expression rates have conventionally been quantified using suppression subtractive hybridization (SSH) [31], serial analysis of gene expression (SAGE) [32] and cDNA microarrays [33]. High throughput mRNA sequencing is now starting to replace these older technologies [34]. Second generation methods use miniaturized platforms and highly parallel sequencing to generate up to billions of independent sequences per machine run. Sequencing enriched mRNA samples can therefore produce large numbers of cDNA reads. When mapped to known gene (or EST) sequences, the expression of every gene in an organism can be digitally quantified. High throughput mRNA sequencing benefits from its deep coverage (down to one transcript per cell), enhanced dynamic range (high- to low-expression) and improved statistical tests [35]. Further, high throughput mRNA sequencing can be readily generalized, and therefore adapted, to most research questions and study designs. Importantly, gene expression in fungal species and their plant hosts can be quantified simultaneously within the same biological sample [18]. This allows plant–fungal signaling to be tracked over a time series of infection or across different tissue types. High throughput mRNA sequencing is currently leading to a sea change in genomics, and is opening up research on many poorly characterized symbiotic systems.

required for maintenance of the mutualistic association between *E. festucae* and perennial ryegrass [17]. The loss of an extracellular siderophore will potentially result in increased levels of free iron in the intercellular space and its availability to participate in the Fenton reaction, giving rise to increased levels of ROS. Similarly, loss of the *E. festucae* stress-activated MAP kinase, *sakA*, triggers a switch from mutualism to pathogenicity [18] (Fig. 1). Infected hosts are severely stunted and precociously senesce within three months after planting. Their development is also dramatically altered, with the formation of bulb-like structures at the base of infected tillers, which almost completely lack anthocyanin pigmentation. Growth of the Δ*sakA* mutant in these associations is unregulated with increased fungal biomass, hyphal hyper-branching and changes in hyphal morphology. This raises the question: is the mechanism that controls the switch to intercalary extension negatively regulated by physiological stress responses such as iron limitation or oxidative stress, such that under these conditions, the fungus does not switch to intercalary extension but instead continues to proliferate? This would provide an explanation for why the Δ*sidN* mutant, which is subjected to conditions of iron stress, and the Δ*sakA* mutant, which is subjected to osmotic stress, change from mutualists to pathogens.

3. Insights into symbiosis from next generation sequencing

New high throughput sequencing technologies are now opening up new approaches to explore questions such as these (Box 1). To date, high throughput sequencing experiments on grass-fungal systems are sparse – we are currently aware of only one. Here, we describe this study in detail as a potential template for future research. To investigate the molecular basis of the breakdown in symbiosis and the switch to pathogenic growth of these *E. festucae* mutants, second generation high throughput mRNA sequencing was utilized to examine changes in fungal and plant gene expression between a mutualistic, wild-type *Epichloë–Lolium* association and the Δ*sakA* pathogenic association [18]. Using this approach, insights were gained into the changes in gene expression that accompany the switch from mutualist to pathogen. Dramatic up-regulation of fungal hydrolases, transporters and genes involved in translation were observed in the mutant association (Table 1). These are all changes consistent with a switch from restricted to proliferative growth, as the fungus requires increased uptake and breakdown of host-derived nutrients. In this respect, the fungus is acting as a nutrient sink, similar to formation of a stroma, a conidia-producing structure required for production of fungal fruiting bodies upon initiation of the sexual stage in the fungal life cycle. Indeed, we would predict that many of the gene changes observed with the Δ*sakA* mutant will also be observed in the switch from restricted to proliferative growth associated with formation of the stroma. Δ*sakA* mutant hyphae colonize host vascular bundles, where they have direct access to nutrients being transported through the phloem. This increased capacity for nutrient acquisition and degradation likely facilitates the increased biomass *in planta* observed in this mutant association, as evidenced by the detection of ~4-fold as many fungal transcriptome reads in the mutant sample relative to the wild-type control. However, we propose that this increase in fungal biomass alone is probably not the cause of the stunted growth and premature senescence phenotypes of plants infected with the *E. festucae* symbiotic mutants. Wild-type p-endophytes, a class of non-epichloë grass endophytes, have similar, high levels of biomass *in planta*, yet their host plants remain symptomless [3]. A change in signaling between host and symbiont, resulting in changes in plant hormone balance, is probably linked to the dramatic changes in host phenotype observed, as will be described later in this review.

As previously discussed, production of bioprotective secondary metabolites is a defining feature of epichloë endophyte associations. It is therefore of considerable interest that a widespread 'shut-down' in expression of the genes involved in producing these bioprotective secondary metabolites was observed in the Δ*sakA* mutant. This may be a direct consequence of the switch to proliferative growth, as in other species secondary metabolites are generally only made during the stationary phase of growth [19]. This poses an interesting question: in wild-type associations, are secondary metabolites being made in the meristem and in the stroma where fungal growth is highly proliferative? Based on the results of this study, we predict that wherever fungal growth is proliferative, fungal bioprotective secondary metabolite gene expression will be repressed.

4. Plant gene expression

Striking changes in host gene expression were also observed (Table 1). In particular, there was a dramatic up-regulation of host defense related gene expression, including classic pathogenicity response (PR) genes and nucleotide-binding site leucine-rich repeat-type (NBS-LRR) resistance genes [18]. An important requirement for mutualistic associations is the absence of a host defense response to the infecting endophyte, either due to suppression of host defenses by the symbiont or by an inability of the host itself to detect the infecting fungus. A crucial change in the Δ*sakA* mutant association is that the endophyte is now recognized by the host, thus suggesting that there are either chemical changes in the fungal cell wall, or a change in the metabolites or macromolecules released into the apoplast, that elicit this response in the host. It would be interesting to examine whether there is also activation of host defense-related gene expression in stroma tissue, where fungal growth is highly proliferative, similar to the Δ*sakA* mutant association.

Infection of *L. perenne* with the Δ*sakA* mutant also induces activation of host transposases, with a significant increase in their expression relative to the wild-type control. Stress activation of transposition, and the consequential genome mutations and rearrangements it generates, has been proposed as acting as a last-ditch survival mechanism [20–22]. Indeed, activation of both

Table 1
Summary of differentially expressed plant and fungal genes in the Δ*sakA* mutant association organised by GO category.

Fungal genes			Plant genes		
GO category	No. genes up-regulated in mutant association	No. genes down-regulated in mutant association	GO category	No. genes up-regulated in mutant association	No. genes down-regulated in mutant association
Antioxidant	5	2	Antioxidant	18	0
Binding	63	20	Binding	233	107
Catalytic	296	107	Catalytic	562	215
Electron carrier	1	0	Electron carrier	5	5
Enzyme regulator	2	4	Enzyme regulator	12	7
Protein tag	1	1	Protein tag	2	0
Structural	21	1	Structural	27	5
Transcription regulator	1	3	Transcription regulator	8	4
Transducer	2	0	Transducer	6	1
Transporter	64	18	Transporter	78	27
Ef unique	133	41	Lp unique	444	276
Nutrient reservoir	0	1	Nutrient reservoir	–	–
Resistance	–	–	Resistance	47	11
Transposase	–	–	Transposase	49	21
Unknown	305	110	Unknown	301	175
Total[a]	894	308	Total[b]	1792	854

[a] Total number of differentially expressed fungal genes is 1202 out of a total gene set of 11410 (12199 gene models less 789 duplicated models).
[b] Total number of differentially expressed plant genes is 2646 out of a total gene set of 18195 (16002 de novo-assembled EST sequences plus 2193 gene/EST sequences from GenBank).

Type I [23] and Type II [20,24] transposons has been shown to occur under conditions of cold and salt stress [23], when plant cells are cultured or a hypersensitive response (HR) is induced by elicitors [20,24]. It would therefore be interesting to examine whether there is activation of host transposases in natural pathogenic plant–fungal interactions. This also poses the question as to why a stressed physiological state induces activation of these elements? Is this a relaxation of transposon repression, in response to the physiological changes occurring in the plant, that bring about genome rearrangement and changes that promote adaptation to, and survival of, the stress condition? Indeed, Barbara McClintock hypothesized that transposons are a positive evolutionary force for genome rearrangement, which are activated in response to stress conditions [22].

Some of the most important effects on host gene expression induced by the Δ*sakA* mutant were changes in plant hormone related gene expression. Plant hormones play a crucial role in regulating plant growth and development. A complex interplay of auxins, cytokinins and strigolactones determine whether outgrowth will occur from a given leaf axillary bud, the phenomenon of apical dominance (Fig. 2). Cytokinin related gene expression was up-regulated, consistent with an increase in tillering occurring in Δ*sakA* mutant-infected plants, since cytokinins promote outgrowth from axillary buds [25]. Roots of plants infected with the Δ*sakA* mutant grew more slowly, suggestive of a decrease in auxin signaling, given that auxin inhibits outgrowth of axillary buds and promotes growth of lateral roots [25,26]. However, not all auxin responsive genes were down-regulated in the Δ*sakA* mutant [18]. In fact, a considerable number of auxin related genes were up-regulated. A possible explanation for this result was the sampling of tissues of different ages, since mRNA from pseudostem tissue was sequenced, which contains multiple leaves at different stages of development. In any given plant, outgrowth from some meristems will be activated, while at the same time others will be repressed due to the interplay of auxin and cytokinin signaling. Therefore, the balance of transcriptional activators, repressors and co-repressors in each leaf will be different, leading to an apparent inconsistency in auxin-related gene expression. In the future, as the efficiency of library generation for high throughput RNA sequencing improves and the quantity of tissue required for sampling is reduced, it may become possible to use smaller sections of tissue to circumvent these problems of sample heterogeneity. Indeed, high throughput sequencing could be coupled to laser micro-dissection ([27]), such that it would be possible to sequence RNA isolated from just a single hypha or its adjacent host cells. This will allow detection of expression changes only seen in host cells in direct contact with the symbiont, which might otherwise be diluted out. Given the sensitivity of methods for analyzing metabolites, it will also be possible to examine changes in the metabolome in the same samples to establish connections between gene expression and metabolic flux. A number of other classes of plant hormones play crucial roles

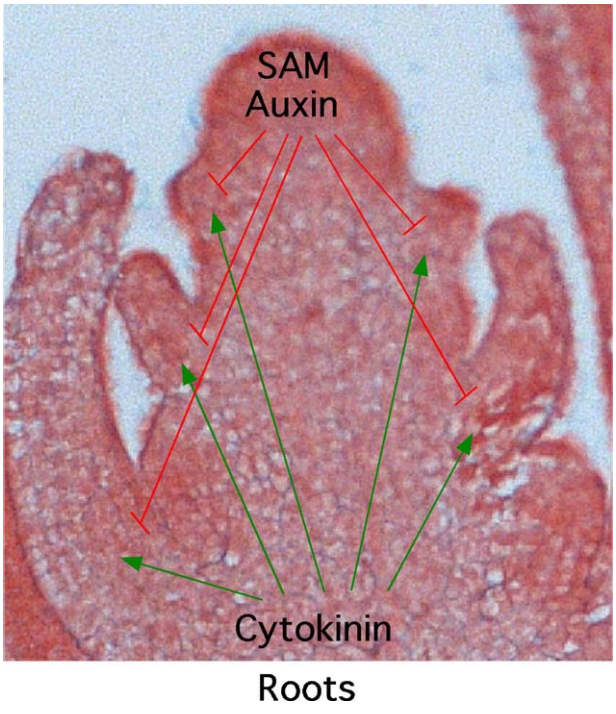

Fig. 2. Schematic showing the action of auxin and cytokinins (CK) in controlling outgrowth from grass axillary shoot meristems. Light micrograph of Alcian blue/Safranin O-stained longitudinal section through the apical meristem of a perennial ryegrass tiller. Auxin is produced in the shoot apical meristem (SAM) and travels basipetally to axillary shoot meristems, where it inhibits their outgrowth. Cytokinins are made in the roots and travel acropetally to axillary shoot meristems, where they promote their outgrowth.

in growth and stress response, including abscisic acid (ABA), ethylene, gibberellins and jasmonic acid. Changes in the expression of genes involved in signaling were observed within all of these classes of hormones. Changes in ethylene related gene expression were of particular interest. Six genes involved in the ethylene response were up-regulated and one gene involved in dampening the ethylene response was down-regulated. This apparent up-regulation or activation of ethylene signaling is likely a major contributing factor leading to precocious senescence of the Δ*sakA* mutant-infected plants. Changes in the abscisic acid and jasmonic acid gene pathways are consistent with the role of these hormones in the host defense response [28,29].

Interestingly, gibberellic acid (GA)-related gene expression was up-regulated. This is of particular interest because GA has long been known to be involved in cell elongation [30]. This is important as in Δ*sakA* mutant-infected plants, host cells just below the shoot apical meristem are disordered compared to the ordered cell files found in uninfected plants and wild-type fungal associations [18]. This effect seems due to cells in this region being elongated and irregular in size and shape [18]. Such disordered growth likely results in the bulging phenotype observed at the base of infected tillers, which gives a spring onion or scallion-like appearance to the host plant.

5. Conclusions

Associations between epichloë endophytes and grasses provide a highly tractable system to study the signaling involved in plant fungal interactions. By utilizing disrupted associations where there has been a switch from a mutualistic to pathogenic interaction, considerable insight can be gained from transcriptome experiments of the molecular mechanisms that underlie mutualism and pathogenicity. Candidate sets of plant and fungal genes can be identified that define each of these physiological states. Further high throughput transcriptome comparisons of various combinations of endophyte infected (E+) and uninfected (E−) tissue and culture mycelium versus infected tissue will allow further refinement of the candidate symbiotic gene set that characterizes the mutualistic symbiotic state, paving the way for detailed functional studies.

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