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Research review paper

Parasitic fungus *Claviceps* as a source for biotechnological production of ergot alkaloids

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ABSTRACT

Ergot alkaloids produced by the fungus *Claviceps* parasitizing on cereals, include three major groups: clavine alkaloids, D-lysergic acid and its derivatives and ergopeptines. These alkaloids are important substances for the pharmatech industry, where they are used for production of anti-migraine drugs, uterotonics, prolactin inhibitors, anti-Parkinson agents, etc. Production of ergot alkaloids is based either on traditional field cultivation of ergot-infected rye or on submerged cultures of the fungus in industrial fermentation plants. In 2010, the total production of these alkaloids in the world was about 20,000 kg, of which field cultivation contributed about 50%. This review covers the recent advances in understanding of the genetics and regulation of biosynthesis of ergot alkaloids, focusing on possible applications of the new knowledge to improve the production yield.

Contents

1. Introduction

The *Clavicipitaceae* family includes, among others, a fungal species called ergot that is important for humankind, in terms of both contributions and losses. In medieval history, these fungi parasitizing in cereals and producing a range of ergot alkaloids caused many mass poisonings that resulted in painful deaths of tens of thousands of people (Schiff, 2006). Nowadays, mass poisonings with ergot alkaloids are sporadic and occur only in developing countries. For example, an epidemic of ergotism with a high mortality rate occurred in 1977 in Ethiopia (Demeke et al., 1979). Contrary to mass poisonings, the recent cases of individual poisonings are usually connected with overdoses of medical drugs based on these alkaloids.

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For thousands of years, people tried to take advantage of substances produced by ergot without knowing their chemical structure, proper dosage or side effects, but scientific research on ergot alkaloids started only with the beginning of modern pharmacy in the early 20th century. Nowadays, specific types of ergot alkaloids are widely used as a basic drug-stock for the production of various therapeutic substances, e.g. for the treatment of migraine, in gynecology for its uterotonic effects, as prolactin inhibitors, antiparkinsonian drugs, etc. (de Groot et al., 1998).

The *Claviceps* genus is not only interesting for its ability to produce secondary metabolites usable in pharmaceutical industry, but also in its life strategy, mainly the specificity of invaded host organs, which is in the center of interest of many scientific groups. Last, but not least, its negative influence in agriculture should be mentioned. Some of the *Claviceps* species cause significant losses in cereal production. Recently, there was an infection of the fifth most important cereal crop in the world, *Sorghum bicolor* (Haarmann et al., 2009), by *Claviceps africana*.

2. Ergot alkaloids

2.1. History of poisoning and use of ergot alkaloids

First references to ergot use can be found in early history. The oldest documentation of the positive effects of ergot alkaloids in obstetrics appeared in China in approximately 1100 BC (Schiff, 2006). In one of the sacred books of the Parsees (400 BC to 300 BC), ergot is mentioned as "noxious grasses that cause pregnant women to drop the womb and die in childbirth" (Thoms, 1931). In the Middle Ages, there were many documented cases of mass intoxication with ergot caused by contaminated cereals, usually referred to as ergotism. There are two types of ergotism, which differ in symptoms. The first one, called "convulsive ergotism", was typical for the area located east of the Rhine river in Europe and was accompanied by muscle spasms, hallucinations and fever. These symptoms are typical for serotonergic stimulation of the central nervous system, caused by the activation of serotonin receptors by ergot alkaloids, due to their structural similarity to the neurotransmitter serotonin (Eadie, 2003). The second type of ergotism, typical for the area west of Rhine, is called "gangrenous ergotism", and is accompanied by violent burning and shooting pain of the affected acral part of the human body. Related to the saint who suffered horrible visions sent by the devil, this type of ergotism has been called St. Anthony's fire (Lee, 2009). The first documented epidemic of ergotism is dated 944–945 AD and caused the death of about 10,000 people in France. Some 50 years later, intoxication with ergot alkaloids again killed about 40,000 people in this area. It is likely that ergot alkaloid intoxication was also connected with the well-known witch trials of 1692 in Salem, Massachusetts, USA (Caporael, 1976; Spanos and Gottlieb, 1976) and in Finnmark, Norway in the 17th century (Alm, 2003). A correlation between the symptoms of ergotism and ergot consumption was understood finally in the 1850s, due to the findings of Louis René Tulasne, a French mycologist, who first fully described the life cycle of ergot (Tulasne, 1853). Modern ergot alkaloid research started in 1918 with ergotamine isolation by the Swiss biochemist Arthur Stoll (Stoll, 1945). In 1926, Swiss psychiatrist Hans Maier suggested that ergotamine might be useful in the treatment of vascular headaches of the migraine type (Silberstein et al., 2001). LSD, a synthetic derivative of lysergic acid, is one of the components of ergot alkaloid blend that was first synthesized in 1938 by the Swiss chemist, Alfred Hoffman, but its effect on nervous system was not discovered until he accidentally contaminated himself and experienced the hallucinogenic reaction in 1943 (Minghetti and Crespi-Perellino, 1999). The drug became popular in the mid-1960s when its sense-altering properties were reputed to offer a window into enhanced creativity and self-awareness.

2.2. Chemistry and occurrence of ergot alkaloids

Ergot alkaloids belong to the class of indole derivatives. They can be divided into three major groups: clavine alkaloids, D-lysergic acid and its simple derivatives and ergopeptines. Structures of typical ergot alkaloids are shown in Fig. 1. Species within the genus *Claviceps* differ in their capability to produce diverse types of alkaloids. Only a few species (e.g. *Claviceps purpurea* and *C. africana*) can produce ergopeptines as final products of their ergot alkaloid biosynthetic pathway. For example, the biosynthetic pathway of *Claviceps fusiformis* ends with elymoclavine production that has been explained as a loss of the late pathway genes in the ergot alkaloid gene cluster (Lorenz et al., 2007).

Most of ergot alkaloids contain a tetracyclic ergoline structure, although some of the naturally occurring clavine alkaloids are tricyclic, e.g. chanoclavine-I, chanoclavine-II and isochanoclavine-I (for a review see Buchta and Cvak, 1999). Of these, only chanoclavine-I can serve as a precursor for biosynthesis of other ergot alkaloids. Tetracyclic clavine

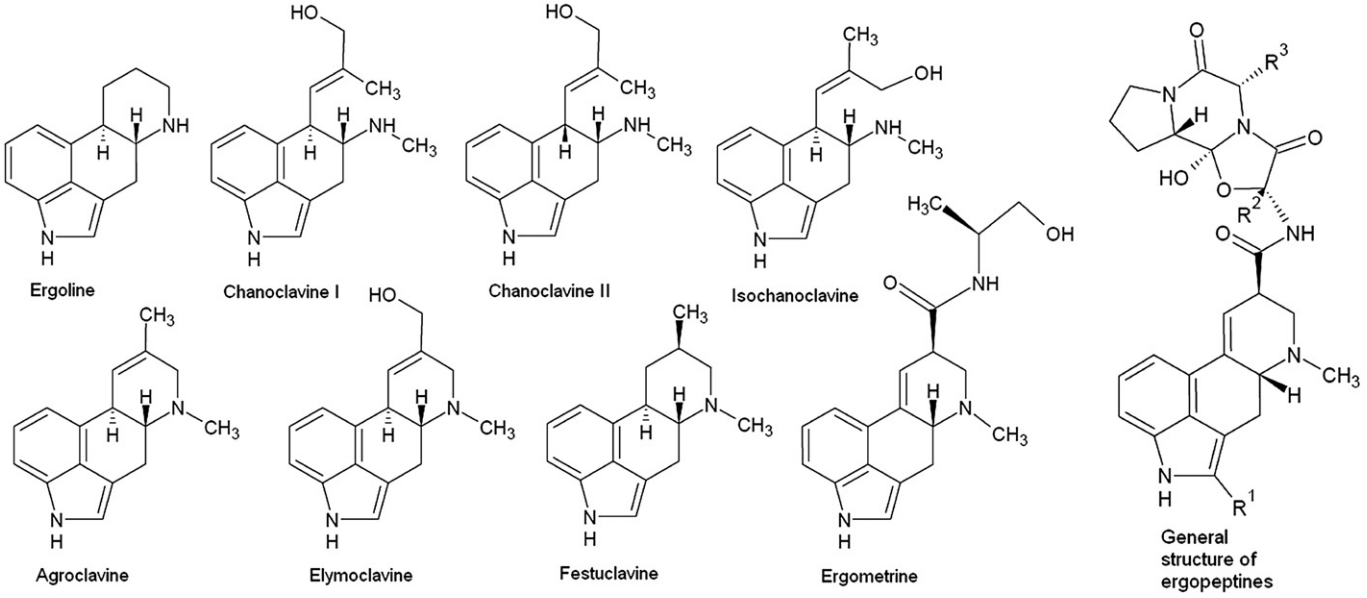

Fig. 1. Structures of ergot alkaloids.

alkaloids can be found in various species of fungi. Agroclavine and elymoclavine are intermediates of the pathway leading to D-lysergic acid production, but in most ergot alkaloid producing fungi, this pathway ends with clavine alkaloids as final products (see Fig. 2A). Besides the genus *Claviceps*, ergot alkaloids were also once reported in other fungi, such as *Botrytis fabae* (Naim, 1980) and *Geotrichum candidum* (El-Refai et al., 1970). However, their occurrence was later unconfirmed by more advanced analytical methods. Similarly, ergopeptines were mentioned to be produced by *Aspergilus fumigatus* (Abe et al., 1967; Cole et al., 1977; Narayan and Rao, 1982; Spilbury and Wilkinson, 1961), but this was not confirmed more recently.

Derivatives of D-lysergic acid can be divided into simple amides and tripeptides. One of the representatives of D-lysergic amides is ergometrine (alternatively also called ergonovine or ergobasine) derived from D-lysergic acid and 2-aminopropanol. Ergometrine is produced by *C. purpurea* together with ergopeptines (Dudley and Moir, 1935), which are built from the lysergic acid fragment and a tripeptide group composed of diverse amino acids, where proline is always in the third position. Ergopeptines are the end products of the biosynthetic pathway of ergot alkaloids. The main producer of ergopeptines is *C. purpurea*, but dihydro-α-ergosine is also produced by *C. africana* (Mantle and Waight, 1968).

The presence of clavine alkaloids and ergopeptines had been for a long time also associated with dicotyledonous plant species of the family *Convolvulaceae*, also known as the bindweed or morning glory. In these plants, the alkaloids are produced by different species of endophytic fungi that are mostly seed-transmitted and may have shared their alkaloid producing genes by hybridization or horizontal transfer (Clay and Schardl, 2002). Microscopic clavicipitalean fungi were recently detected residing on leaves and seeds of *Ipomoea asarifolia* and *Turbina corymbosa* (Steiner et al., 2006). This epibiotic fungi that contains genes involved in ergot alkaloid biosynthesis (Markert et al., 2008) was recently described as a new genus, *Periglandula* (Steiner et al., 2011).

2.3. Biosynthesis of ergot alkaloids: enzymology and genetics

Essential steps of the ergot alkaloid biosynthetic pathway were analyzed by feeding *Claviceps* liquid cultures with isotopically labeled precursors. The general biosynthetic pathway of ergot alkaloids is shown in Fig. 2A,B. As precursors of the ergoline system, three primary metabolites were found: tryptophan, a product of the mevalonate pathway dimethylallylpyrophosphate (DMAPP) as a donor of the isoprene unit, and methionine as a methyl group donor. The first specific step of the biosynthetic pathway is the isoprenylation of tryptophan resulting in 4-(γ,γ-dimethylallyl)tryptophan (DMAT). This step, catalyzed by 105 kDa homodimer enzyme DMAT synthase (Gebler and Poulter, 1992), is positively regulated by tryptophan and negatively feedback-regulated by agroclavine and elymoclavine, further products of the biosynthetic pathway (Cheng et al., 1980). *DmaW* gene coding for DMAT synthase, originally cloned and sequenced from *C. fusiformis*, was the first discovered gene of the ergot alkaloid synthesis (EAS) cluster (Tsai et al., 1995). The 1517 bp ortholog of *dmaW* containing two introns was found in *C. purpurea*, proving a common biosynthetic

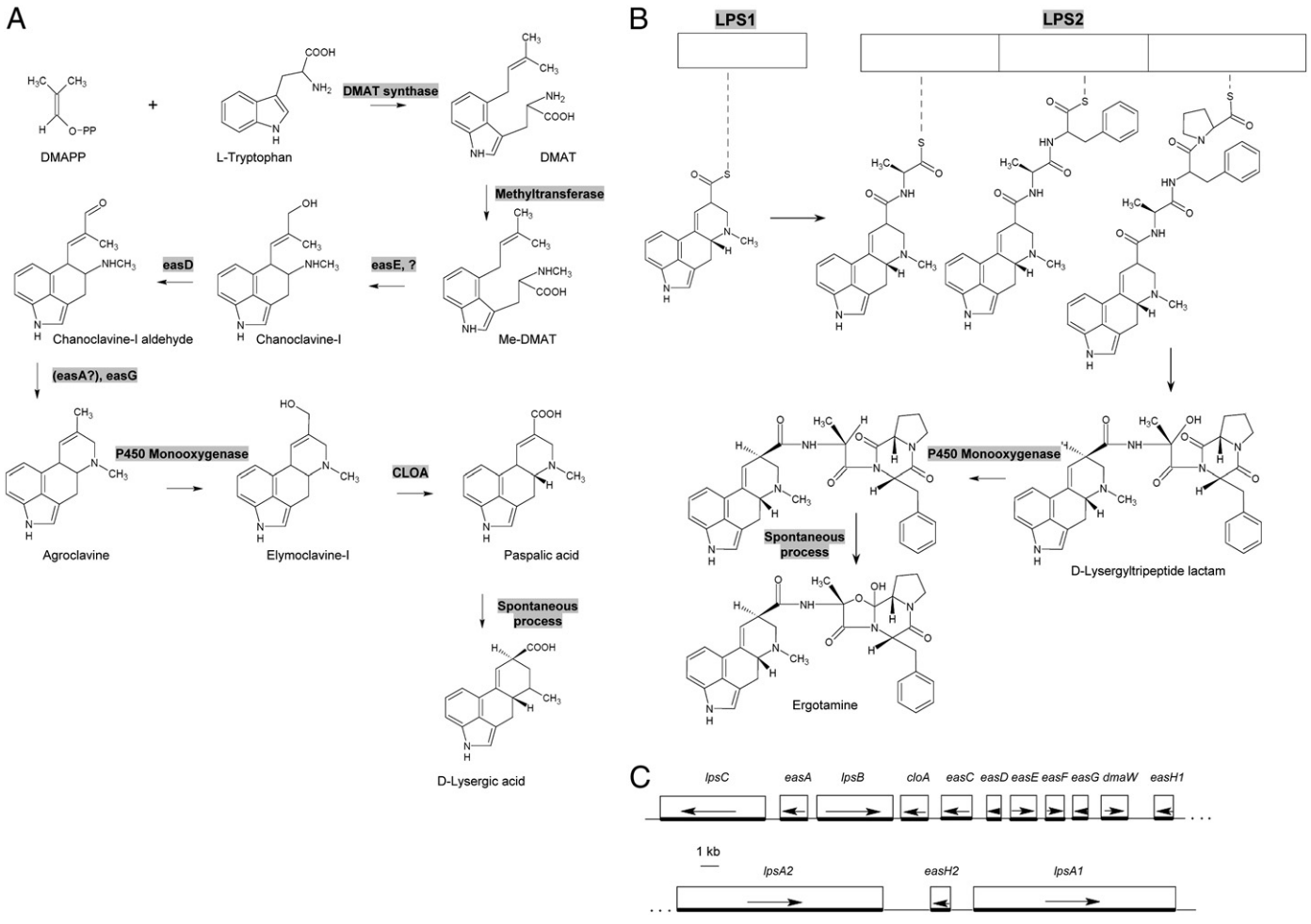

Fig. 2. Biosynthesis of ergot alkaloids in *Claviceps purpurea*: A) scheme of the biosynthetic pathway leading to D-lysergic acid, B) biosynthesis of ergopeptines on two-component non-ribosomal peptide synthetase (NRPS), and C) scheme of the ergot alkaloid synthesis (EAS) gene cluster.

origin of clavines and D-lysergic acid-derived alkaloids (Arntz and Tudzynski, 1997). Analyses of 3′ flanking region of *dmaW* showed a presence of another EAS related gene named *cpps1* (Tudzynski et al., 1999). By chromosome walking, a whole 68.5 kb EAS cluster, consisting of 14 genes (Fig. 2C), was described by Paul Tudzynski's group (Haarmann et al., 2005).

The second step of the EAS pathway is the methylation of DMAT to *N*-methyl-dimethylallyltryptophan (MeDMAT). Otsuka et al. (1980) first isolated an enzyme that is responsible for catalyzing the methylation of DMAT using S-adenosylmethionine as a donor of the methyl group. The biosynthetic pathway continues with several oxidation and reduction steps resulting in the formation of chanoclavine I, chanoclavine I aldehyde and then agroclavine. Chanoclavine I is the only one of the four stereoisomers of chanoclavine, which can be further converted into tetracyclic ergolines (Gröger et al., 1966). Chanoclavine I synthase, a FAD-containing oxidoreductase, is involved in the oxidation of MeDMAT to chanoclavine I, but there are still some other enzymes needed to complete the conversion. The mechanism of this reaction was proposed by Gröger and Floss (1998). In *C. purpurea*, chanoclavine synthase is encoded by *ccsA* gene of 1503 bp, originally named *easE*. The coding region of *ccsA* gene is interrupted by one intron (Lorenz et al., 2010). Chanoclavine-I is then further oxidized to chanoclavine-I aldehyde by an alcohol dehydrogenase encoded by *easD* (Wallwey and Li, 2011).

Closure of D ring of tetracyclic ergot alkaloids proceeds via *cis-trans* isomerization on C2 of the mevalonate group (Floss, 2006) in a series of enzymatic steps, collectively called chanoclavine cyclase. Chanoclavine-I aldehyde first undergoes double bond isomerization to isochanoclavine-I aldehyde, originally thought to be catalyzed by the product of *easA* gene. It was shown recently that the reaction also proceeds non-enzymatically by reduction with glutathione or 2-mercaptoethanol (Matuschek et al., 2011). Isochanoclavine-I aldehyde then forms an iminium ion compound, which is subsequently reduced to agroclavine by the enzyme easG (agroclavine synthase) in the presence of NADPH. The monomeric easG of 31.9 kDa consists of 290 amino acids and is encoded by *easG* gene of 1079 bp containing two introns (Matuschek et al., 2011).

The biosynthetic pathway then continues with a P450 monooxygenase catalyzed oxidation of agroclavine on C17 atom, leading to formation of elymoclavine. Elymoclavine is oxidized to paspalic acid by another P450 monooxygenase, clavine oxidase (CLOA). This enzyme connects the formation of two groups of ergot alkaloids, the clavine alkaloids and D-lysergic acid derived alkaloids, such as simple D-lysergic acid amides and ergopeptines. CLOA protein is encoded by *cloA* gene of 2120 bp interrupted with eight introns (Haarmann et al., 2006). Paspalic acid is converted to D-lysergic acid spontaneously, as described by Gröger and Floss (1998).

Elymoclavine monooxygenase and all subsequent enzymes are not encoded in fungal genomes that produce the clavine type of alkaloids. The following metabolic steps lead to amides of D-lysergic acid and ergopeptines, crucial metabolites of *C. purpurea*. D-lysergic acid is captured and activated by monomodular non-ribosomal peptide synthetase (NRPS) LPS2, one of the two subunits of D-lysergyl peptide synthetase with a molecular mass of 141 kDa. D-lysergic acid is, after binding to LPS2 as a thioester, transferred to trimodular LPS1 NRPS, the second subunit of D-lysergyl peptide synthetase with a size of 370 kDa. Here it is condensed with three bound amino acids, resulting in formation of a D-lysergyl tripeptide lactam (Walzel et al., 1997). LPS2 and LPS1 NRPS are encoded by *cpps2* and *cpps1* genes, respectively (Correia et al., 2003; Tudzynski et al., 1999). *Cpps2*, also named *lpsB*, is a gene of 3991 bp containing one intron, while *cpps1*, also named *lpsA*, consists of 10,850 bp and contains two introns.

Finally, D-lysergyl peptide lactam is oxidized by another P450 monooxygenase and the product is spontaneously converted to ergopeptines (Haarmann et al., 2006). The structure of final ergopeptine depends on the amino acids used for the condensation with D-lysergic acid, where the first two positions are occupied by non-polar amino acids and the third one exclusively by proline (Keller, 1999).

C. purpurea can also synthesize D-lysergylalkanolamides, alkylamides of D-lysergic acid, such as ergometrine. In these compounds, D-lysergic acid is linked to an aminoalcohol derived from alanine. The reaction is catalyzed by LPS2 as the D-lysergic acid activator and another monomodular NRPS subunit, ergometrine synthetase, in the presence of NADPH. LPS3 enzyme (ergometrine synthetase) is encoded by *cpps3* gene (also called *lps3*) located in the EAS gene cluster (Ortel and Keller, 2009).

The EAS cluster contains one more NRPS gene, *cpps4* (also called *lps4*), which encodes LPS4 protein with a function similar to LPS1. The two enzymes differ in their capability to bind different amino acids used for ergopeptine formation. LPS4 is responsible for α-ergocryptine production in P1 strain of *C. purpurea*, while LPS1 catalyzes the production of ergotamine (Haarmann et al., 2005).

2.4. Biological activity of ergot alkaloids

Specific types of ergot alkaloids serve as a basic drug-stock for production of various therapeutic substances to treat migraine headaches, Parkinson's disease, hypertension and diverse sexual disorders (de Groot et al., 1998). Biological activity of ergot alkaloids relates to their structural similarities with neurotransmitters noradrenalin, dopamine and serotonin (Fig. 3). The major pharmaceutical effects of ergot alkaloids are smooth muscle stimulation, central sympatholytic activity and peripheral α_1-adrenergic blockade. Smooth muscle stimulation is the most evident as a vasoconstriction and uterine contraction (Innes, 1962). Since the ergot alkaloids are dopamine receptor agonists, some of them (bromocriptine, cabergoline, pergolide) can be used as inhibitors of prolactin release (Nasr and Pearson, 1975) and anti-Parkinson agents. However, they are not recommended as a first-line antiparkinsonian medication because of the risk of fibrotic reaction (Bonuccelli et al., 2009). Effects of the ergot alkaloids on dopamine receptors are described in more detail in Emilien et al. (1999). Anti-migraine effects of many ergot alkaloids are facilitated by interactions with serotonin receptors, 5-HTs. First compound used for the treatment of this disorder was ergotamine in 1926 (Maier, 1926). The main disadvantage of natural ergot alkaloids is a lack of selectivity for each individual 5-HT receptor, which stimulated the development of semisynthetic serotonergic ligands that are more selective (Pertz and Eich, 1999). Interactions of the ergot alkaloids and their derivatives with serotonin receptors are reviewed in Pertz and Eich (1999). Ergot alkaloids also interact with α_1-adrenoceptors, which mediate vascular contractility. Many ergot alkaloids and their derivatives are partial antagonists or agonists of

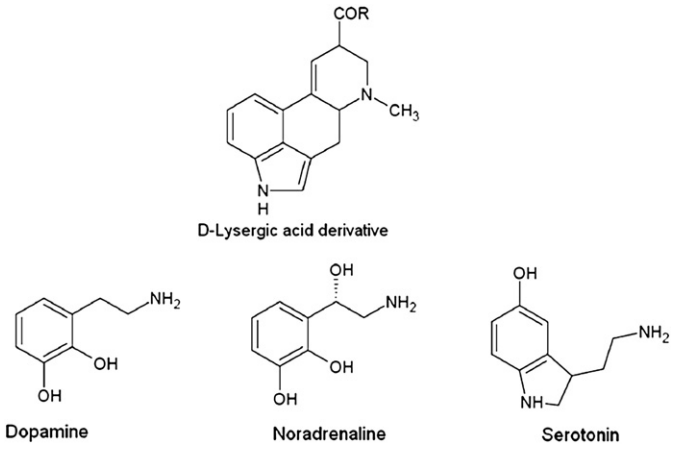

Fig. 3. Structural similarities between the derivatives of D-lysergic acid and neurotransmitters dopamine, noradrenalin and serotonin.

these receptors, which may be the cause of their cardiovascular side effects. The effect of ergot alkaloids on α_1 receptors is described in Görnemann et al. (2008). Agroclavine was also mentioned to show antibacterial effects (Schwarz and Eich, 1983) and cytostatic effects in LSI78Y mouse lymphoma cells (Glatt et al., 1987), but these finding are not supported by more recent studies.

3. Production of ergot alkaloids for pharmaceutical industry

3.1. The fungus Claviceps

There are 36 members of genus *Claviceps*, which can infect about 600 species of monocotyledonous plants. The *Claviceps* genus includes some economically important species because they infect agronomically valuable plants (Bové, 1970). A prominent member, *C. purpurea*, is spread worldwide and has the widest host range of any *Claviceps* species. It can infect about 400 species of grasses (Taber, 1985), but its most typical hosts are rye, wheat and barley (Loveless, 1971). *C. africana*, *Claviceps sorghi* and *Claviceps sorghicola* infect *S. bicolor* (Haarmann et al., 2009) grown in eastern and southern Africa, Southeast Asia, Japan, South America and Australia, *Claviceps gigantea* is found on *Zea mays* in central Mexico, *Claviceps paspali* on *Paspalum* species and *C. fusiformis* on *Pennisetum americanum* (Pažoutová and Parbery, 1999). In all *Claviceps* species, the infection is strictly targeted to the host ovaries (Parbery, 1996).

C. purpurea exhibits a life cycle shown in Fig. 4, which is typical for the *Claviceps* species. The sexual life cycle starts with an infection of unfertilized rye ovaries (Fig. 5A). Specialized penetration structures are not known in *C. purpurea*, indicating that the cell wall is passed due to enzyme secretion (Haarmann et al., 2009). Conidia are transported to the plant by wind or insects, the plant cuticle is penetrated and fungal hyphae colonize ovarian tissues. After 5 to 7 days post infection, the first macroscopically visible sign of infection, production of honeydew, can be detected (Fig. 5B). This liquid contains plenty of sugars attractive for insects and is filled with conidia, which enables secondary infection (Swan and Mantle, 1991; Tenberge, 1999; Tulasne, 1853). *C. purpurea* honeydew also contains inhibitors of conidial germination of many other fungi (Cunfer, 1976). The honeydew production stops after about 2 weeks being followed by the development of a rigid stage sclerotium, composed of a compact mass of hardened fungal mycelium (Tenberge, 1999; Tudzynski and Scheffer, 2004) as shown in Fig. 5C. After 5 weeks post infection, sclerotia mature (Kirchhoff, 1929). Sclerotia can survive unfavorable conditions in winter and germinate after a temperature increase in the spring (optimally at about 20 °C) on or beneath the soil surface (Kirchhoff, 1929), forming stromata composed of stalks with spherical capitula that grow in a phototropic manner to reach the air

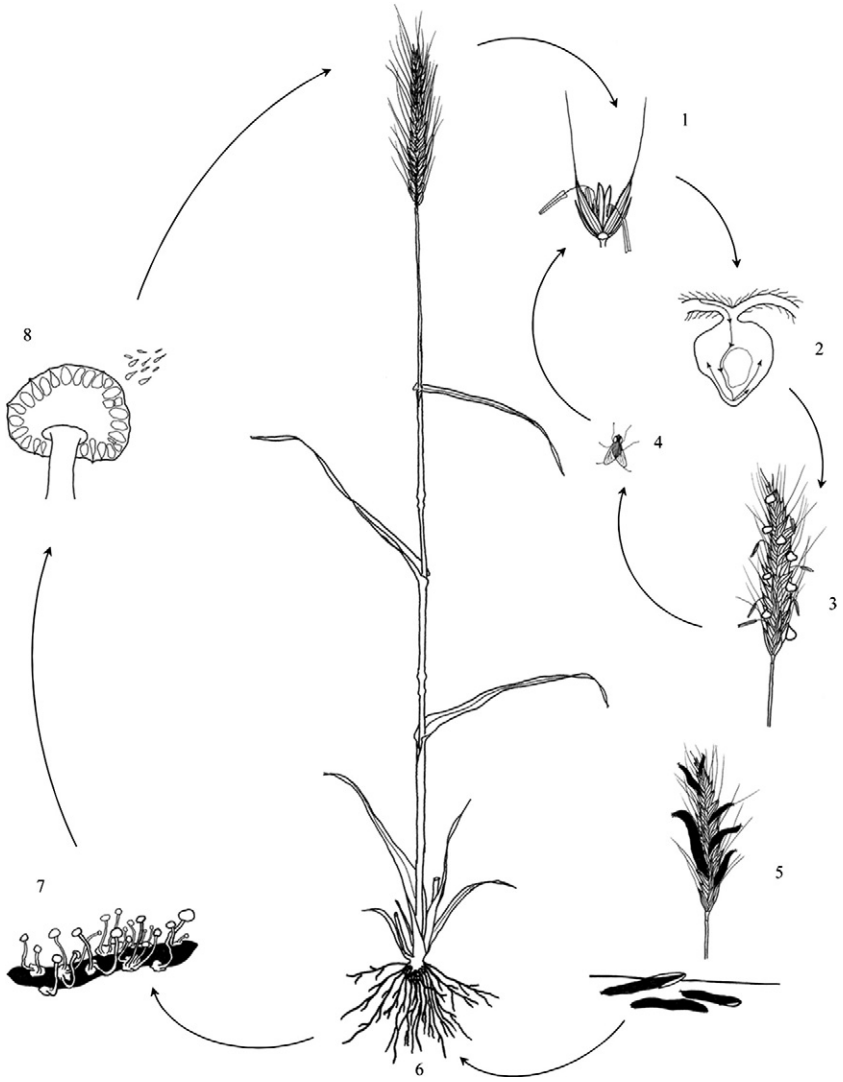

Fig. 4. Life cycle of the fungus *Claviceps purpurea*: 1) opened rye floret, 2) hyphae invasion of the rye ovary, 3) honeydew production, 4) reinfection by an insect, 5) mature sclerotia formation, 6) overwintering of sclerotia, 7) germinating sclerotia, 8) release of ascospores.

(Hadley, 1968). In nature, sclerotia are the unique structures, which produce all types of ergot alkaloids (Ramstad and Gjerstad, 1955).

3.2. Field production on rye

The fungus *C. purpurea* is a species widely used in the pharmaceutical industry for its ability to produce ergot alkaloids. Clavine alkaloids, being derivatives of tetracyclic ergoline ring structure, are produced by various types of fungi. On the other hand, ergopeptines, peptidyl derivatives of D-lysergic acid amides, are produced only by some members of *Clavicipitaceae* family and their preparation by organic synthesis is economically unprofitable. The world production of ergot alkaloids reaches thousands of kilograms annually that include both ergopeptines and semisynthetic ergot alkaloid derivatives, e.g. cabergoline and pergolide (Cvak, 1999). In 2010, the total world production was about 20,000 kg, into which the field cultivation contributed about 50% (Vít Kubesa, Teva Czech Industries, Opava, Czech Republic; personal communication).

The majority of characterized strains of *C. purpurea* and other *Claviceps* species are able to synthesize ergot alkaloids only during the parasitic part of their life. The infection of the plant host by fungal spores initiates the sexual part of the *Claviceps* life cycle. Emerging hyphae invade ovary in the florets and then start to produce masses of new spores and sugar-rich exudates. Approximately 2 weeks later, affected ovaries are transformed into sclerotia where plectenchyma fungal cells initiate the ergot alkaloid biosynthesis (Tudzynski and Scheffer, 2004). Long-term breeding by random mutagenesis and the selection of highly-producing strains of *C. purpurea* allowed an increase in the ergot yield from 400 kg per hectare in the 1940s to over 1 ton per hectare nowadays in addition to increasing the alkaloid content as much as 1.5% of the sclerotia dry mass.

The traditional method of ergot production is based on spraying a conidial suspension onto a field-cultivated rye (*Secale cereale*). From the 1990s, hybrid rye lines with induced male-sterility, such as *Hyclaro* (Rentschler Biotechnologie, Laupheim, Germany), were introduced as the host because their unpollinated florets stay open longer and thus extend the period of susceptibility to ergot infection, which leads to a significantly better yield of generated sclerotia (Németh, 1999). Moreover, under natural conditions, *C. purpurea* usually attacks unfertilized ovaries and the process of infection obviously mimics the course of pollination (Tudzynski and Scheffer, 2004). An important benefit of the field ergot production is the wide variability of strains available for the production of specific types of alkaloids and their better genetic stability compared to the mutant strains used for submerged cultures. The yield of field production can reach 1–2 tons of sclerotia and from 10 kg (Tudzynski et al., 2001) to 20 kg (TEVA Czech Industries, in present) of ergot alkaloids per hectare. However, the field production can be dramatically influenced by climatic conditions of the particular year as well as by the quality and uniformity of used hybrid rye. Hence, unfavorable combinations of these factors may even result in a seasonal failure of the production (Vít Kubesa, personal communication).

3.3. Production in fermentation plants

Regarding the industrial production of ergot, there are reports on the usage of stationary surface cultivations and submerged cultures, the latter being nowadays a dominant method how to grow the *Claviceps* fungus independently of its host. A stationary cultivation using plastic bags was developed in the 1970s and used for both alkaloid production (Kybal and Vlček, 1976) and preparation of inoculums for field cultivation (Malinka, 1999).

Only some selected mutant strains have the ability to produce alkaloids in submerged cultures; the production strategies were extensively reviewed by Malinka (1999). Cells with this ability, which are called sclerotia-like cells, are shorter and thicker than the normal ones with a robust cell wall and large vacuoles (Spalla, 1973). However, a degeneration process that leads to a loss of the sclerotia-like cell

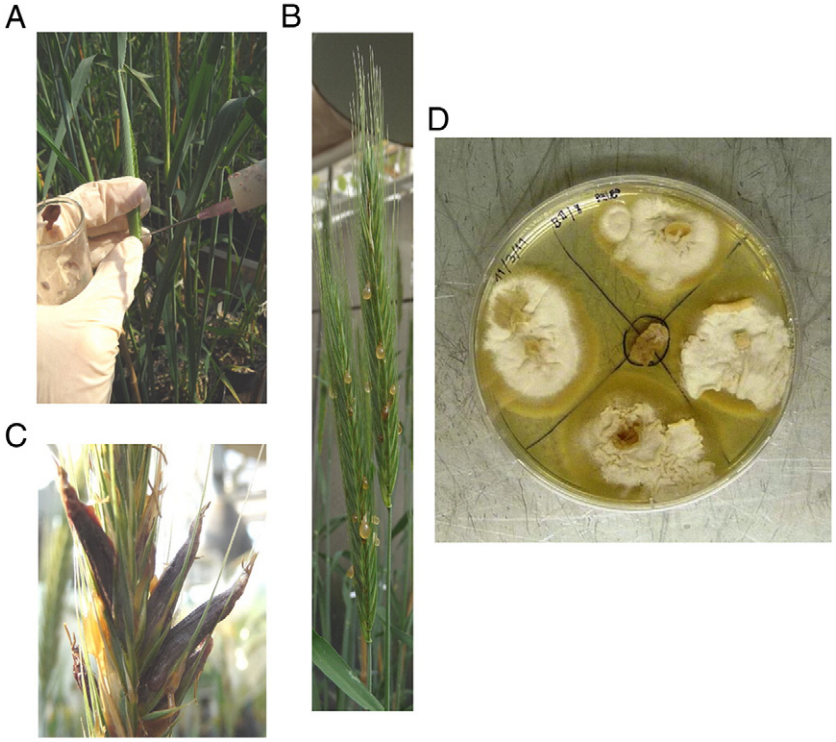

Fig. 5. Various stages of the growth of *Claviceps purpurea*: A) inoculation of rye with conidia suspension, B) honeydew production on rye, C) matured sclerotia growing on rye, D) selection of *Claviceps purpurea* transformants on a Petri dish: protoplasts of the industrial strain GAL404 were transformed with a linearized plasmid bearing *ble* gene conferring phleomycin resistance; a selective growth of transformed mycelia (on the plate periphery) contrary to untransformed mycelia (in the center) on BII agar plate with 100 μg/ml phleomycin.

morphology and lowered alkaloid production is relatively frequent; therefore a continuous selection is needed to maintain a good production strain (Malinka, 1999). Fermentation is nowadays used mostly to produce paspalic acid, which isomerizes to D-lysergic acid, and some derivatives of paspalic acid that serve as a starting material for preparation of semisynthetic alkaloid derivatives. D-lysergic acid can be also obtained by alkaline hydrolysis of simple amides, ergometrine and even ergopeptines (Rucman, 1976).

Production of ergopeptines by fermentation is much more complicated and requires specific conditions. Cultivation media induce the formation of sclerotial-cell like morphology by two mechanisms, substrate limitation and favoring oxidative metabolism. Alkaloid production requires a non-inhibitory, slowly metabolized carbon source such as mannitol, sorbitol or sucrose at a high concentration (sucrose 300 g/l). Moreover, a high osmotic pressure (10–20 bar) of the medium is prerequisite for the formation of sclerotia-like cells while inhibiting conidiation (Kobel and Sanglier, 1986). *Claviceps* converts sucrose, which is the main sugar in plant phloem sap and serves as a natural nutrient of the fungus, to fructofuranosyl-oligosaccharides. These oligosaccharides are used as an energy source in the late stage of the fermentation, which is the key period for the production of ergopeptines. Production media usually also contain an organic acid of the tricarboxylic acid cycle as a carbon source, which under low phosphate promotes the high level of oxidative metabolism in the *Claviceps* cells that is necessary for the biosynthesis of secondary metabolites.

Biosynthesis of ergot alkaloids in axenic cultures is positively regulated by tryptophan, which acts as a precursor and inducer, and negatively by phosphate and ammonium, which repress biosynthesis (Sočič and Gaberc-Porekar, 1992). Typically, alkaloid synthesis starts after depletion of phosphate from the medium, whereas an excess of phosphate leads to undesired growth-linked repression of alkaloid production. General reviews about ergot alkaloid production in submerged cultures have been published by many authors including Esser and Düvell (1984); Robbers (1984); Křen et al. (1994); Sočič and Gaberc-Porekar (1992), and Flieger et al. (2004).

3.4. Genetic transformation of Claviceps

The possibility of genetic transformation of *Claviceps* brought a new tool, not only for obtaining useful knowledge about the genetic principles of ergot alkaloid formation and attractive parasitic lifestyle of the fungus by novel studies of knock-out mutants, but also for having an effect on the quality and/or quantity of ergot alkaloid production for industrial purposes.

Undoubtedly, the most popular method for the genetic transformation of *Claviceps* is protoplast transformation in a medium of high osmotic pressure. A successful method based on protoplast preparation using lytic enzymes was first described in 1989 (Engelenburg et al., 1989) and was later improved (Mey et al., 2002). There are many commercially available enzymes or their mixtures suitable for *C. purpurea* protoplast preparation, such as Lysing enzyme and Driselase (Mey et al., 2002) or β-glucuronidase (Keller et al., 1980). Ca^{2+} ions are universal component of the transformation mixture, while the high osmotic pressure can be adjusted by adding PEG (Engelenburg et al., 1989; Mey et al., 2002). Transformants can then be selected based on acquired antibiotic resistance (Engelenburg et al., 1989; Mey et al., 2002) or by using pyrimidine auxotrophy (Smit and Tudzynski, 1992). Hygromycin (1.0 mg/ml; Comino et al., 1989) and phleomycin or bleomycin (0.1 mg/ml; Mey et al., 2002) are the most often used selection antibiotics. The genes responsible for acquired resistance have been described as *Hph*, encoding hygromycin B phosphotransferase, and *ShBle* gene, coding for a protein binding to bleomycin and inhibiting its DNA cleavage activity (Dumas et al., 1994). Selective growth of transformed vs. untransformed mycelia on a medium containing phleomycin is shown in Fig. 5D (Hulvová et al., 2009).

Technical procedures for *C. purpurea* transformation with the usage of both homologous and non-homologous recombination have been developed (Comino et al., 1989; Smit and Tudzynski, 1992). For the expression of transgenes, constitutive fungal promoters, such as *trpC* (Engelenburg et al., 1989) and *gpdA* from *Aspergillus nidulans* (Lorenz et al., 2010; Scheffer et al., 2005), are used.

Transformation of *C. purpurea* by *Agrobacterium tumefaciens* has been attempted, but no successful procedure has been described yet, although many procedures of *Agrobacterium*-mediated transformation of filamentous fungi are already known (Groot et al., 1998; Meyer et al., 2003; Michielse et al., 2005; Mullins et al., 2001). Because of the advantages of this method, such as one copy insertion or stable transformant preparation, development of the procedure for *Claviceps* transformation would be very useful.

4. Biotechnological prospectives of production improvement

Improvement of biotechnological processes relies on various transformation approaches used during the last several decades. Up to now, only randomly mutated strains of *C. purpurea* have been exploited in the industrial production of ergot-stock. A recently sequenced EAS cluster of fourteen genes sheds more light on the metabolic pathways and regulations of ergot alkaloid production. Thus, a directed overexpression of certain genes or targeted up-regulation of the whole cluster could significantly increase the yield of ergot alkaloids.

Production amounts and a qualitative composition of synthesized alkaloids vary significantly among different isolates of the species *C. purpurea* (Pažoutová et al., 2000). Long terminal repeat sequences and nonautonomous transposons were detected within the ergot alkaloid cluster indicating that spontaneous rearrangements can occur very frequently. Moreover, the cluster for ergot alkaloid production, in *A. fumigatus*, was found to lie in the telomeric region, which is often subject to recombinations, chromosomal breaks and duplications (Coyle and Panaccione, 2005). A recent evolutionary study revealed that the *dmaW* gene passed through many duplications and losses (Liu et al., 2009). The genes of ergot alkaloid cluster are probably also frequently subjected to loss of function mutations, deletions etc. Such an event can be demonstrated on a pseudogene of *dmaW* that was found in an unspecified strain of *C. purpurea* (GenBank AJ312752).

4.1. Manipulation of EAS cluster of Claviceps

Genes encoding all the enzymes participating in ergot alkaloid biosynthesis are likely to be located within the EAS cluster (Fig. 2B), but not all the genes involved in the pathway have been identified and several of detected ORFs still have putative or unknown functions. Recently, an evolutionary study of the alkaloid gene cluster has been accomplished including *Claviceps* species that produce different spectra of alkaloids (Lorenz et al., 2007). *A. fumigatus* cluster, whose end-products are clavine alkaloids, contains all ORFs present in the EAS cluster of *C. purpurea* except for the three genes coding for non-ribosomal peptide synthases, *cloA* gene and a functional form of *easH*. Moreover, frameshifts and partial truncations were found in two ORFs for monooxygenases that are responsible for the final production of D-lysergic acid from a chanoclavine precursor. The comparison of *Claviceps hirtella* and *C. fusiformis* is particularly interesting. Despite their very close relationship, the former fungus can synthesize lysergic acid derivatives, while the latter one lacks this ability. The *C. hirtella* cluster encodes an additional functional monomodular non-ribosomal peptide synthase and two monooxygenases, allowing the production of ergometrine, a single amino acid derivative of D-lysergic acid (Lorenz et al., 2009). In general, the overall organization of the cluster is highly conserved; however rearrangements and point mutations dramatically change the expression and functionality of the enzymes involved in the pathway and consequently both quality and

quantity of produced alkaloids. A detailed characterization of alkaloid gene clusters in other members of the genus *Claviceps* is needed as for instance main products of *C. africana* and *C. gigantea* are unique dihydroclavine derived alkaloids. Hence, a combination of different production strains can improve the quality of produced ergot-stock in biotechnological applications.

The gene *dmaW* is common to all *Claviceps* species described to date; its product catalyzes the first specific step in ergot alkaloid biosynthesis and has the key regulatory function in the pathway (Wang et al., 2004). Feeding experiments with tryptophan led to increased activity of DMAT synthase, which resulted in increased total amount of produced alkaloids (Krupinski et al., 1976). Thus, one can expect that ubiquitous overproduction of DMAT synthase enzyme can result in a higher accumulation of dimethylallyltryptophan and reinforcement of further metabolic steps. The actual concentrations of L-tryptophan and dimethylallylpyrophosphate do not seem to be a limiting factor as they are products of the primary metabolism.

The *EasC* gene, with a high homology to catalases, is another gene of interest in the ergot alkaloid cluster. A knock-out mutant of *C. purpurea* did not produce any alkaloids and the transcripts of other cluster genes were down-regulated (Haarmann and Tudzynski, 2006). An elimination of the *easC* gene in *A. fumigatus* led to the same effects and an exogenous addition of chanoclavine to the growth medium restored the alkaloid production. Hence, the *easC* gene product plays an essential role in the metabolic pathway upstream of chanoclavine (Goetz et al., 2011). Hypothetical catalase easC can either consume hydrogen peroxide generated by chanoclavine synthase or function as a general regulation factor of the whole metabolic cascade (Goetz, 2008).

Unlike other secondary metabolic clusters in fungi, no transcription factor gene has been identified among the ergot alkaloid cluster ORFs yet. Originally, the protein encoded by the *easG* gene was thought to be a good candidate since it shows a homology to Nmr proteins that are involved in regulation of nitrogen assimilation (Tomsett et al., 1981). However, easG was very recently found to catalyze the final step of chanoclavine-I aldehyde conversion to agroclavine (Matuschek et al., 2011).

Based on the published finding, overexpression of either *easC* or *easG* gene was predicted to promote ergot alkaloid biosynthesis in *C. purpurea*. Recently, we used a protoplast method to prepare *C. purpurea* mutants overexpressing genes *easC* and *easG* under a constitutive promoter of glycerol-3-phosphate dehydrogenase (GAPDH) from *A. nidulans* (Hulvová et al., 2010). Confirmed transformants of each gene were applied on rye growing in a greenhouse, but chemical analysis showed no significant increase in the production of ergot alkaloids. However, the native expression of the two genes in the wild type *C. purpurea* was already very strong and reached the level of GAPDH promoter-driven overexpression 20 days after infection (Hulvová et al., unpublished results).

Genetic engineering of the proteins involved in related signaling cascades appears to be another promising way on how to influence ergot alkaloid biosynthesis. Since ergot alkaloid production is dependent on a high osmotic pressure in the culture medium or under natural condition on the production of honeydew, one can expect that receptors and transmitters active in osmoregulation can be good candidates. Pioneering research at this area has been already initiated (Lorenz et al., 2009; Mey et al., 2002).

In fungi, secondary metabolite clusters are often located in a heterochromatin, near the telomere region (Galagan et al., 2005; Nierman et al., 2005; Rehmeyer et al., 2006). Thus, it is speculated, that the EAS cluster of *C. purpurea* may be also located in the subtelomeric area (Lorenz et al., 2007). In *Aspergillus* spp., expressions of many biosynthetic gene clusters are regulated by histone methyltransferase LaeA. An overexpression of the *laeA* gene in *A. nidulans* led to an increased expression of the cluster genes and product formation (Bok and Keller, 2004). The overexpression of *laeA* gene from *A. nidulans* in the *C. purpurea* P1 strain did not lead to increased expression of EAS cluster genes or ergot alkaloid production (Lorenz et al., 2009). Nevertheless other genes coding for the enzymes participating in methylation and acetylation/deacetylation of histones can be also good candidates for improvement of EAS cluster expression. Recently, a formation of heterotrimeric complex of LaeA with two other proteins, VelB and VeA, was shown to be essential for its activity in nucleus to control secondary metabolism gene transcription. Deletion of both genes led to defects in fungal sexual reproduction and ceased production of some secondary metabolites (Bayram et al., 2008). Furthermore, expression of VeA is down-regulated and its accumulation in nucleus ceases after illumination (Stinnett et al., 2007). On the other hand, VelB protein lacks any nuclear targeting signal and its transfer to nucleus depends solely on VeA. Thus, an overexpression of VeA under a constitutive (light independent) promoter and/or expression of VelB with a specific nucleus targeting signal could lead to increased production of ergot alkaloids under natural light conditions (Bayram et al., 2008).

4.2. Improvement of rye hybrid lines with male-sterility

Today, hybrid rye lines with induced cytoplasmic male sterility are widely used in different breeding programs and for heterosis seed production. Fertility can be restored by a suitable parent line bearing restorer gene(s) (Geiger and Miedaner, 2009). Commercial hybrid rye lines with cytoplasmic male-sterility are currently the only suitable host for ergot stock field production. The most crucial factor biasing the quantity of annual ergot yield, besides the weather conditions which influence the progression of the infection in flowering rye plants, is the purity of the rye line. To keep the cytoplasmic male sterility line, regular crossing with the restorer line has to be performed with vigorous selection of male-sterile seed stock. This procedure is very agronomically demanding and significantly elevates production costs. Even though, maintenance of high-quality seed stock offering 100% male-sterile plants for ergot production is almost impossible because rye is an effective wind pollinator and transfer of pollen containing restorer gametes to long distances can occur on either ergot production field or hybrid seed production field. To avoid losses in ergot production by unexpected leakage of pollen, fields for production of hybrid seed and ergot stock should be located out of traditional rye-growing areas.

Ectopic expression of the barnase gene, ribonuclease from *Bacillus amyloliquefaciens*, in tapetum cells of developing anthers leads to destruction of pollen and therefore to male-sterile plants. A system utilizing the barnase gene and the fertility-restorer gene, barstar, has been introduced to many crops and is one of the most widely used transgenic approaches in modern agriculture (Williams, 1995). Due to the fact that rye is one of the most recalcitrant plant species for tissue culture and genetic transformation (Popelka and Altpeter, 2003), and low economic impact of rye cultivation in global agriculture, transgenic rye lines bearing barnase–barstar system have not yet been prepared. Thus, introduction of the barnase gene into the rye genome together with the linked selection gene (e.g. herbicide resistant) can produce an easy, selective and inexpensive system for obtaining a pure male-sterile rye line suitable for ergot production in the future. Seed stock of the sterile line can be maintained by pollination crossing with wild type plants. The barnase gene, as well as a selective marker, act as dominant alleles in the transgenic genome and thus a 100% sterile population of rye plants can be selected every year by proper application of herbicide.

4.3. Susceptibility of rye ovaries to the infection by different Claviceps strains, pathogenicity markers

Susceptibility of rye plants to the infection by ergot is another key determinant of a good yield in the field production of ergot alkaloids. The infection depends on many agronomical parameters, but primarily on the fertility of the host cultivar, its pollen shedding and

Table 1
Virulence and pathogenicity-related proteins of *Claviceps*.

Gene	Protein	Function	Reference
Pg1/2	Polygalacturonase	Pectin degradation in rye ovary	Oeser et al. (2002)
Rac	GTPase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Xyl2	Endo-1,4-β-xylanase	Hydrolysis of xylans	Tudzynski and Scheffer (2004)
Cdc42	GTPase	Sensing of polarity and sporulation	Scheffer et al. (2005a)
Cla4	P21-activated kinase	Sensing of polarity and sporulation	Rolke and Tudzynski (2008)
Cot1	Ser/Thr kinase	Sensing of polarity and branching	Scheffer et al. (2005b)
Mid1	Ca^{2+} channel	Signaling Ca^{2+} uptake after membrane distension	Bormann and Tudzynski (2009)
Mk1/2	MAP kinase	Pathogenicity related signaling	Mey et al. (2002a) Mey et al. (2002b)
Tf1	CREB-like TF	Oxidative stress responses	Nathues et al. (2004)
Hk2	His kinase	Signaling of oxidative stress and osmosensing	Nathues et al. (2007)
Nox1	NADPH-oxidase	ROS generation	Giesbert et al. (2008)

duration of flower opening. For direct testing of the level of pathogen virulence, male-sterile hybrids grown in the greenhouse conditions are used. Several pathogen knock-out transformants were prepared to study the molecular aspects of virulence and host–pathogen interaction. In general, virulence associated genes can be divided into three main categories, those encoding the enzymes participating in degradation of host cell wall; enzymes scavenging reactive oxygen species that are produced by the host as a pathogen protection response; and various regulatory proteins involved in signaling pathways or functioning as transcription factors. *Claviceps* proteins known to be important for fungal virulence and pathogenicity are listed in Table 1.

As the fungus mimics pollen tube growth during the infection, only mild or no host defense reaction is usually observed (Tudzynski and Scheffer, 2004). On the other hand, recent high-throughput screening of EST clone libraries from infected rye tissues revealed expression of various proteins related to general pathogen defense responses (Oeser et al., 2009). Nevertheless, actual pathogen-induced expression of these genes was not shown and none of them was yet confirmed to be involved in the resistance to the pathogen infection. Since the whole genome sequencing of *C. purpurea* strain P1 has been recently accomplished and a rough draft will soon be publically available (Paul Tudzynski, personal communication), a future transcriptomic study shall reveal presumptive host resistance related genes or other genes specifically switched during the ovary invasion as potential easy-to-detect markers of the pathogenicity process. Genome sequencing of other *Claviceps* species is in progress worldwide, among them *C. africana* is being sequenced using a high-throughput shotgun technology in our laboratory.

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