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NEW ELEMENTS IN THE REGULATION OF GENETIC COMPATIBILITY OF *FUSARIUM/GIBBERELLA* SPECIES

Abstract of the thesis

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Introduction

Fusarium species are distributed world-wide and pose economically serious problems in many crops. The infection by these fungi causes significant yield losses and the mycotoxin accumulation paralleled by the fungal invasion is harmful to both people and animals. Understanding the reproduction strategies of these fungi could help us to develop more efficient control measures against them. The emergence of new virulence or fungicide resistance genes within a fungal population is greatly influenced by the frequency of sexual and asexual reproduction. In filamentous ascomycetes, mating type and vegetative incompatibility confer self/nonself recognition in the sexual and vegetative reproductive phases, respectively. Sexual interactions are controlled by alleles at the mating type locus (*MAT*) whereas the parasexual interactions are regulated by alleles at the *het* (heterokaryon incompatibility) loci.

The genetic regulation of mating has most extensively been studied in *Neurospora crassa* and *Podospora anserina* among filamentous ascomycetes. Different mating types of *N. crassa* are denoted by A and a. The *mat a* idiomorph contains two genes, including a minor one (Staben and Yanofsky, 1990; Pöggeler and Kück, 2000), whereas the *mat A* idiomorph harbours three genes (Ferreira et al., 1996). The two mating types in *P. anserina* are named as *mat+* and *mat-*. One of the idiomorphs (*mat+*) contains one gene (*FPR1*), the other one (*mat-*) contains also three genes (*FMR1, SMR1* and *SMR2*) (Debuchy, 1999). Results are the same in the case of *Gibberella moniliformis*. The *MAT-1* idiomorph contains three genes and the *MAT-2* idiomorph contains one gene (Yun et al., 2000). Yun and co-workers (2000) also identified these *MAT* idiomorphs in the homothallic species *Gibberella zeae* (*Fusarium graminearum*), as well as in *Fusarium oxysporum* which has not known sexual stage. Structural organization of the *MAT* genes in *F. oxysporum* is similar to that in *G. moniliformis*.

Computer analysis of mating type sequences revealed conserved regions in the MAT proteins of filamentous ascomycetes. The MAT A-1 and FMR1 proteins of *N. crassa* and *P. anserina*, respectively contain an α-domain, whereas the MAT A-2 and SMR1 proteins contain an acidic amphipathic α-helix. The MAT A-3 and SMR2 proteins contain an HMG (high mobility group, HMG-1) domain. The MAT a-1 and FPR1 proteins also contain an HMG domain (HMG-2). The α-domain and HMG-2 genes are necessary and sufficient to confer mating specificity. These genes are highly conserved and interchangeable among different ascomycete species (Shiu and Glass, 2000).

Pheromone signalling is needed to find the appropriate mating partner during mating. Pheromone sensing by the cognate receptor in mating partners initiates signalling events leading to elevated transcription of mating specific genes. The pheromone binds to a cell surface receptor, allowing a contact with a heterotrimeric G protein, which results in a subsequent release of the Gβγ

subunit from the Gα subunit. Either the Gα or Gβγ subunit can transmit the signal via stimulation of effectors such as adenylyl cyclase, phospholipase C, MAP (mitogen-activated protein) kinase cascade or ion channels (Lee et al., 2003).

Mechanism of vegetative incompatibility is quite dissimilar in different organisms and its function has poorly been understood. Vegetative incompatibility is controlled by *het* (heterokaryon) genes. Both allelic and non-allelic systems have been identified. In allelic systems, incompatibility is triggered when two strains, which contain different alleles at one or more *het* loci fuse. In non-allelic interactions the existence of genetic differences at two separate and distinct loci results incompatibility (Glass and Kuldau, 1992).

Most fungi contain several *het* loci: seven, nine and 11 *het* loci have been identified in *Cryphonectria parasitica* (Cortesi and Milgroom, 1998), *P. anserina* (Bégueret et al., 1994), and *N. crassa* (Glass and Kuldau, 1992), respectively. The majority of species examined till now have allelic incompatibility systems. Non-allelic system also exists in a number of fungi, including *P. anserina*, *C. parasitica* and *Heterobasidion annosum* (Chase and Ulrich, 1990). Products of the cloned *het* genes are dissimilar in their sequence, function or localization. The vegetative incompatibility system has been most thoroughly studied in *Fusarium verticillioides* (*G. moniliformis*) within the genus *Fusarium*. Based on examination of vegetative compatibility groups (VCG) at least 10 *het* loci are expected to exist in this species (Leslie, 1993).

The aims of this work were to (1) demonstrate the presence and transcription of mating type genes in *Fusarium* species with no known sexual stage, (2) develop a PCR-based technique for the rapid identification of mating types in a wide range of *Fusarium* species, (3) clone and characterize the adenylate cyclase gene of *Fusarium proliferatum* (*Gibberella intermedia*), and (4) to study polymorphisms and function of *hch*, the homologue of a vegetative incompatibility gene (*het-c*) of *N. crassa* in the *Gibberella fujikuroi* species complex.

Methods

Strains and growth conditions

Fusarium species used for amplifying conserved mating type sequences were: *F. acuminatum ssp. acuminatum* (10 strains); *F. acuminatum ssp. armeniacum* (7 strains); *F. avenaceum* (12 strains); *F. camptoceras* (2 strains); *F. cerealis* (5 strains); *F. chlamydosporum* (3 strains); *F. compactum* (8 strains); *F. culmorum* (18 strains), *F. decemcellulare* (1 strain); *F. equiseti* (8 strains); *F. graminearum* (1 strain); *F. longipes* (1 strain); *F. merismoides* (1 strain); *F. poae* (8 strains); *F.*

scirpi (3 strains); *F. semitectum* (6 strains); *F. solani* (2 strains); *F. sporotrichioides* (10 strains); *F. torulosum* (6 strains); *F. tricinctum* (5 strains); *F. tumidum* (3 strains); *F. verticillioides* (2 strains).

Fusarium species used for examining the hipervariable domain of *fphch* gene were: *F. proliferatum* (76 strains); *F. verticillioides* (2 strains); *F. sacchari* (2 strains); *F. fujikuroi* (2 strains); *F. subglutinans* (2 strains); *F. thapsinum* (2 strains); *F. nygamai* (2 strains).

Strains were maintained on potato dextrose agar under sterile paraffin oil at 4°C.

Fungal mycelium was produced in 100 ml complete medium (LCM) inoculated with 10^8 conidia and grown for three days at 24°C with shaking (120 rpm). Conidia were produced in CMC medium (Cappellini and Peterson, 1965) grown for three days with shaking (23-24°C, 180 rpm). Conidial germination was studied on conidia washed from the surface of mycelia grown on complete medium and used in a final concentration of 10^6 conidia/ml.

Polymerase chain reactions

Polymerase chain reactions (PCRs) were performed in a final volume of 50 μl in Biometra T3 Biocycler. The reaction mixture contained: 1 × PCR buffer (MBI Fermentas), 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, 0.25 μM of each primer, 1 unit of Taq polymerase (MBI Fermentas), about 20 ng of fungal DNA, and sterile distilled water.

Amplification of *MAT* specific regions

Conserved portions of the alpha- or HMG-2 domains of the *MAT1-1-1* or the *MAT1-2-1* genes were amplified from different *Fusarium* species by using the degenerate primers Fα1 and Fα2 or the previously described NcHMG1 and NcHMG2 primers (Arie et al., 1997). The entire *MAT* genes were cloned by inverse PCR technique (Triglia et al., 1988) by using Herculase Taq Polymerase (Stratagene) according to the manufacturer's instructions. Diagnostic PCR primers designed for the rapid identification of mating type of *Fusarium* species were: fusALPHAfor, fusALPHArev, fusHMGfor and fusHMGrev. Transcription of *MAT* genes was demonstrated by RT-PCR experiments. Total RNA was extracted from the mycelia grown on carrot agar plates by using TRI reagent (Sigma) according to the manufacturer's instructions. RNA was treated with RQ1 DNase (Promega), then first strand cDNA was synthetized and used as template.

Construction of genomic library of *Fusarium proliferatum* ITEM 2287

Genomic DNA isolated from *F. proliferatum* strain ITEM 2287 was partially digested with *Sau3A*I. 9-23 kb long fragments were ligated into Lambda DASH II vector and packaged into lambda phages (Gigapack III Gold Cloning Kit, Stratagene). The library was amplified and the titre of the

library (5,5 x 10^7 pfu/ml) was determined. All experiments were done according to the manufacturer's instructions.

Isolation of *fpac1* and *fphch* genes

The entire *fphch* gene, as well as the 3' end of the *fpac1* gene were isolated from the genomic library. Plaque hybridization, phage DNA isolation and restriction mapping were carried out using standard procedures (Sambrook and Russel, 2001). The degenerate oligonucleotide primers used for the isolation of *fpac1* and *fphch* genes were ACkat_for/ACkat_rev and Fs_hetc_for/Fs_hetc_rev, respectively. The amplified fragments were used as radioactive probes to screen the genomic library (Sambrook and Russel, 2001). The 5' end of the *fpac1* gene was isolated by inverse PCR technique (Triglia et al., 1988).

Sequence analysis

DNA sequencing and oligonucleotide synthesis were performed by the Biomi Kft at the Agricultural Biotechnology Center, Gödöllő. Oligonucleotide primers were designed by PrimerSelect programme of the Lasergene software package (DNAStar Inc., Madison, Wis.). BLAST searches (Altschul et al., 1997) were made against the EMBL database, using the GenomeNet website (http://www.genome.jp). DNA sequences were analyzed with the Lasergene (DNAStar Inc., Madison, WI) software packages, and the FGENESH programme (http://www.softberry.com).

Copy number and expression of *fphch*

Genomic DNA isolated from *F. proliferatum* ITEM 2287 strain was digested with different restriction endonucleases. Fragments were separated by electrophoresis in agarose gels and blotted onto Hybond N^+ membranes (Sambrook and Russel, 2001). Southern blots were probed with PCR-amplified fragments of *F. proliferatum* ITEM 2287.

10^6 conidia/ml was inoculated into LCM medium and samples were taken at 0, 5, 10, 24, 48, 72, 96 hours. Total RNA was extracted from the fungal material by using TRI reagent (Sigma) according to the manufacturer's instructions. Northern hybridization was performed according to standard procedures (Sambrook and Russel, 2001). A 900 bp long fragment of *fphch* was used as radioactive probe. For control hybridization a 600 bp long fragment of the actin gene of *F. proliferatum* was labelled with ^{32}P isotope and hybridized to the membrane after washing down the *fphch* probe (Sambrook and Russel, 2001).

PCR-RFLP

DNA was isolated from 76 *F. proliferatum* strains and other *Fusarium* species belong to the *G. fujikuroi* species complex. The putative hypervariable domain of the *hch* homologue was amplified by using Fp_hchHVD_for and Fp_hchHVD_rev primers. The fragments were digested with *Nco*I, *Sal*I, *Hpa*II and *Taq*I (MBI Fermentas) restriction endonucleases and seperated by electrophoresis in agarose gels.

Preparation of disruption constructs and an artificial *fphch* allele; transformation of protoplasts

The putative catalytic domain and its border regions were amplified from *fpac1*. The catalytic domain was then replaced by the 3800 bp long hygromycin phosphotransferase gene (*hph* cassette, Punt et al., 1987). This disruption construct was used to transform *F. proliferatum* ITEM 2287 protoplasts. In the case of *fphch* the entire gene was amplified and a 2299 bp long *Eco*RV fragment was replaced by the *hph* cassette.

To prepare an artificial *fphch* allele, six amino acids (18 bp) were inserted into the putative hypervariable domain of *fphch* by site-directed PCR mutagenesis (Higuchi et al., 1988).

Protoplasts were isolated from exponentially growing mycelial cultures of *F. proliferatum* ITEM 2287 and PEG-mediated transformation was performed according to Proctor et al. (1997). Transformed protoplasts were mixed with 5 ml overlay medium (50 ºC) and spread on the surface of regeneration medium. After six hours the plates were overlaid with hygromycin B containing overlay medium (50 ºC). Final concentration of hygromycin B in the plates was 200 μg ml^{-1}. Transformants were maintained on complete medium containing 200 μg ml^{-1} hygromycin B. DNA was isolated from single-spored, stable transformants (Kerényi et al., 1999) and screened for double homologue recombination events. Results obtained by PCR were confirmed with southern hybridization (Sambrook and Russel, 2001).

Measurement of fumonizin B1 production

Fungi were grown in 100 ml modified Myro medium containing 1% (w/v) corn-hull-extract (Dantzer et al. 1996) for five days with shaking (125 r.p.m.) at 28^{o}C in the dark. The whole cultures were dried and extracted with acetonitrile. After centrifugation (2 min, 12 000 rpm) the supernatants were diluted with phospate-buffered saline (PBS) + Tween 20 (0,1 %) (Barna-Vetró et al., 2000). Fumonisin B1 was measured with an ELISA kit (Toxiklon) according to the manufacturer's instructions.

Sexual crosses and vegetative incompatibility tests

Strains were crossed according to Klittich and Leslie (1988) on carrot agar medium. Strains used as female partners were grown on carrot agar for one week then sprinkled with conidial suspension of the male partner belonging to the opposite mating type. Plates were incubated for 5-6 weeks at 23/24 ^{o}C under a diurnal cycle of 12/12 h light/darkness. Cultures were regularly monitored under stereo-microscope to detect appearance and maturation of perithecia.

Nitrate non-utilizing mutants (*nit*) of *F. proliferatum* and its *fpac1* and *fphch* mutants, selected on potassium-chlorate containing medium were used to assess vegetative compatibility (Puhalla, 1985). Chlorate resistant mutants were assigned to phenotypic classes (*nit1*, *nit3*, and *nitM*) according to Correll et al. (1987). Complementation of the *nit* mutants was performed on basal medium, containing $NaNO_3$ as the sole nitrogen source.

Infection of plants

The upper 5 cm layer of sterile glasshouse soil was mixed with wheat straw colonized by the mycelia of *F. proliferatum* ITEM 2287 and its *fpac1* mutants (Oren et al. 2003). Maize seeds, surface sterilized in 1% (w/v) NaOCl were planted into the soil after inoculation. Plants were grown in glasshouse for 3 weeks at 25-28^{o}C under natural illumination. Leaf and stem samples, collected at weakly intervals were cut into three pieces and surface sterilized by dipping them in 1% (w/v) NaOCl for 2 min. DNA preparations isolated from the plant samples were subjected to PCR, to detect *F. proliferatum* using species specific primers (Waalwijk et al., 2003). The same samples were placed on *Fusarium* selective peptone-pentachlorine-nitrobenzene (PCNB) medium (Papavizas, 1967) and incubated at room temperature for 10 days to confirm the presence of fungal infection.

Results and discussion

Mating type sequences in asexually reproducing *Fusarium* species

A complete sexual cycle has been found for several *Fusarium* species and both homothallic and heterothallic mating has been observed. In heterothallic species mating type is determined by the two idiomorphs (*MAT-1* and *MAT-2*) of the mating type (*MAT*) locus. Two cells can mate when they have different idiomorphs in their mating type loci. Homothallic species contain both idiomorphs linked tightly together. On the other hand there are a number of pathogen species, such as *Fusarium avenaceum, Fusarium cerealis, Fusarium culmorum, Fusarium equiseti, Fusarium*

poae and *Fusarium sporotrichioides* which have no known sexual stage. We supposed that these species also have mating type genes as Yun and co-workers (2000) have already found such sequences in the 'asexual' species, *F. oxysporum*. However, the primers they used were unsuitable to amplify MAT sequences in other members of the genus. Our first aim was to develop semi-degenerate PCR primers that can be used to detect mating type sequences in any species of *Fusarium*. For cloning *MAT* genes from these fungi, sequence information on the α-domain and the HMG-2 domain (conserved regions of the *MAT1-1-1* and the *MAT1-2-1* gene, respectively) were used.

Based on deposited sequences of the *MAT1-1-1* genes of *G. moniliformis* (AF100925), *G. zeae* (AF318048) and *F. oxysporum* (AB011379) we designed degenerate oligonucleotide primers to amplify the conserved α domains. The HMG-2 domains were amplified by using primers described by Arie et al. (1997). Using this approach we could amplify the α- and HMG-2 domains from selected strains of *F. avenaceum*, *F. culmorum*, *F. poae* and *Fusarium semitectum*. DNA sequences of these fragments were deposited to the EMBL database (Accession numbers: AJ535625 – AJ535632). Flanking regions of these conserved domains were amplified by inverse PCR.

Based on *MAT* sequences of the four 'asexual' *Fusarium* species, as well as deposited *MAT* sequences of *F. oxysporum, G. moniliformis* and *G. zeae* we designed new degenerate primer pairs to identify mating type in any member of the genus *Fusarium*. The robustness of these primers was tested on 122 *Fusarium* strains representing 22 species from 9 sections. The primers developed in our laboratory are suitable for the rapid identification of mating type in several *Fusarium* species and facilitates the recognition of potentially compatible strains that could be used in sexual crossing experiments. Degenerate *MAT* specific primers developed in previous studies (Arie et al., 1997; Steenkamp et al., 2000) were unsuitable for this purpose.

Next we examined whether *MAT* genes are transcribed in 'asexual' *Fusarium* species. To achive this we used RT-PCR experiments primed with the α- and HMG-2 domain specific diagnostic primers. We could amplify the expected (150 and 200 bp long) fragments and prove that these genes are transcribed in 'asexual' *Fusarium* species. The size differences between the amplicons obtained by RT-PCR and the fragments generated from genomic DNAs were due to the presence of an intron in the genomic copies of these *MAT* sequences.

Why are the *MAT* genes transcribed in fungi which have no known sexual forms? It is supposed that 'asexual' species are derived from sexual ancestors. If sexuality is lost, it can be predicted that the loss is recorded in the *MAT* genes since the only known function of *MAT* genes is to conduct mating. In 'asexual' fungi, mutations accumulated in the *MAT* genes, that are never used may result in a loss of their function. According to another alternative, 'asexual' species may have a hidden sexual cycle but the sexual structures are not observed because of their rarity of mating.

Alternatively these fungi require special environmental conditions for the induction of the sexual cycle (Turgeon, 1998). Sharon and co-workers (1996) transformed a *Cryptococcus heterostrophus MAT* deletion strain by the MAT-2 gene from *Bipolaris sacchari* and the transformants became fully fertile. However, such a transformation was not successful in reciprocity, indicating that *B. sacchari* lacks factor(s), other(s) than the *MAT* gene required for mating.

Understanding the reproduction strategies of these pathogenic fungi is important, as the high genetic diversity generated by sexual recombination can promote the spread of these organisms. A diverse fungal population is able attack new, resistant plants or tolerates the presence of fungicides.

Isolation and characterization of *fpac1* (adenylate cyclase) gene

Adenylate cyclase plays an important role in several signal transduction pathways. This enzyme catalyzes the conversion of ATP to cyclic AMP on the inner side of the plasmamembrane. cAMP acts as a secondary messenger and transmits signals received from G proteins by activating cAMP-dependent protein kinases. These kinases are able to phosphorilate appropriate transcription factors. The adenylate cyclase signal transduction pathway has a role in vegetative growth and sexual reproduction of fungi, affects pathogenicity and vegetative incompatibility (Choi and Dean, 1997; Flawia et al., 1976; Ivey et al., 2002; Loubradou et al., 1996; Matsumoto et al., 1982; Rocha et al., 2001).

Based on conserved motifs of the catalytic domain of known adenylate cyclase genes of fungal origin, degenerate oligonucleotide primers were designed and used for the amplification of a 200 bp fragment from *F. proliferatum* ITEM 2287(*G. intermedia*). The entire copy of *fpac1* (accession number: DQ067619) was isolated by joining a fragment originated from the genomic library of *F. proliferatum* strain ITEM 2287 and another one amplified by inverse PCR technique. Sequence of *fpac1* is similar to other fungal adenylate cyclase sequences. The FPAC1 protein has a domain structure typical of the currently known fungal adenylyl cyclases. It contains a Ras GTP-binding domain, eleven leucine-rich repeats, a protein phosphatase catalytic domain and an adenylate cyclase catalytic domain. As FPAC1 lacks transmembrane domains, it appears to be peripherally anchored to the cell membrane (Tang and Gilman, 1992).

Disruption of *fpac1* caused phenotypic changes as expected. Vegetative growth of the fungus became reduced and germination of conidia was delayed. Adenylate cyclase defective mutants of *N. crassa* also have reduced vegetative growth and the colonies lack aerial hyphae (Flawia et al., 1976). *MAC1* defective mutants of *Magnaporthe grisea* also have similar features: reduced vegetative growth and delayed conidial germination (Choi and Dean, 1997).

As *F. proliferatum* is one of the main fumonisin producers we examined the effect of the deletion of *fpac1* on fumonisin B1 production. Fumonisin B1 was measured with an ELISA kit (Barna-Vetró et al. 2000). Both the wild type and the *Δfpac1* mutants synthesized high amounts of fumonisin B1 (more than 2000 ng/g), indicating that adenylate cyclase signaling has no influence on toxin production in this fungus.

Previous results suggested a regulatory function of adenylate cyclase in sexual reproduction. Loss of this gene, for example caused sterility in *M. grisea* (Choi and Dean, 1997). *Cr1* mutants of *N. crassa* are able to function as males or females during a sexual cross, but exhibit delayed perithecial and ascospore development compared to the wild type (Ivey et al., 2002). According to our results, disruption of adenylate cyclase in *F. proliferatum/G. intermedia* caused partial female sterility. When Δ*fpac1* mutants were used as female partners in sexual crosses perithecial development was delayed and the amount of perithecia was significantly reduced. This result suggests that adenylate cyclase signal transduction pathway is partially replaceable with another signal transduction pathway during mating, but this supplementary pathway is less efficient than the original one.

Not much data are available on the role of adenynlate cyclase in vegetative incompatibility, but there are some results, suggesting that this enzyme may be involved in self/non-self recognition events. Loubradou et al. (1996) examined *P. anserina* mutants defective in a vegetative incompatibility gene (*mod-D1*). They found that an additional copy of adenylate cyclase gene relieves developmental defects, so mod-D1 could be an activator of adenylate cyclase. To examine the role of *fpac1* in vegetative incompatibility, we selected nitrate non-utilizing mutants (*nit*) of wild type strain and its *fpac1* mutants and paired them in minimal medium containin nitrate as a sole nitrogen source (Correll et al., 1987). *F. proliferatum* ITEM 2287 turned to be a self-incompatible strain. Vegetative self-incompatibility is rare in fungi, therefore the basic mechanism of this trait is poorly understood. Interestingly, vegetative self-incompatibility was abolished in Δ*fpac1* mutants strains: *nit* mutants of the Δ*fpac1* transformants could complement each others, furthermore complementation between *nit* mutants of the Δ*fpac1* transformants and that of the wild type could also be observed. These data clearly indicate that adenylate cyclase signal transduction pathway participates in self/non-self recognition system.

Adenylate cyclase has also an effect on pathogenecity of fungi. *MAC1*$^{-}$ mutants of the plant pathogen *M. grisea* were unable to form appressoria, the mutants had reduced pathogenecity as they were unable to penetrate plant tissues (Choi and Dean, 1997). Adenylate cyclase defective mutants of the human pathogen, *Candida albicans* also showed reduced pathogenecity. The reason of this disability was that these mutants were unable to switch from the yeast to the hyphal form (Rocha et al., 2001). *F. proliferatum* is a common causal agent of *Fusarium* ear rot of maize together with *F.*

verticillioides and *Fusarium subglutinans* (Miller, 1994). *F. verticillioides* is frequently found in symptomless kernels: the fungus is able to colonize plant tissues without any external symptoms (Foley, 1962). As *F. proliferatum* lives together with *F. verticillioides,* we supposed that this species is also able to colonize maize plants. To examine the colonization capability of the fungi we infected soil with the wild type and its *fpac1* mutant strains grown on straw and planted maize kernels into the infected soil. The wild type fungus could be detected in the entire plant after 1 week, whereas the presence of the mutants was restricted to the lower and the middle part of the plants. After two weeks, both the wild type and its mutants appeared only in the middle part of the plants and after three weeks no traces of fungal infection (either by the wild type or the mutants) could be observed. External symptoms weren't observed in any case. We concluded that *F. proliferatum* is able to colonize maize plants in endophytic manner similarly to *F. verticillioides*. Weaker colonization capability of *fpac1* mutants probably can be explained by weaker vegetative growth. These results are important because earlier we had no information on the role of adenylate cyclase in pathogencity in species infecting plants with direct penetration.

Polymorphisms and function of the *hch* (*het-c* homologue) gene in *Fusarium proliferatum*

Mechanism of vegetative incompatibility is quite dissimilar in different livings and its function hasn't been fully understood yet. In filamentous fungi, vegetative incompatibility is controlled by genes in the *het* (heterokaryon) loci. Both allelic and non-allelic systems have been identified. In allelic systems incompatibility is triggered when two strains, that contain different alleles at one or more *het* loci, fuse (Glass and Kuldau, 1992). One of the best studied *het* loci is *het-c* (Saupe és Glass, 1997; Saupe et al., 1994). A homologue of the *N. crassa het-c* gene has also been isolated and characterized from *P. anserina* (Saupe et al., 2000). This gene plays no role in vegetative incompatibility. In the genus *Fusarium* not much data are available on *het* genes. The vegetative incompatibility of *F. verticillioides* is the most intensively studied system. At least ten *het* loci, comprising an allelic system control vegetative incompatibility in this species (Leslie, 1993). To improve knowledge on the function of the *het-c* homologue in *F. proliferatum,* we isolated and characterized the gene.

Screening an EST library of *F. sporotrichioides* we found a sequence similar to *het-c*^{OR} allele of *N. crassa*. Using degenerate oligonucleotide primers, designed for the homologous region, we could amplify a part of the *het-c* homologue (*fphch*) from the genome of *F. proliferatum* ITEM 2287. The entire *fphch* gene (accession number: DQ067618) was isolated from the genomic DNA library of *F. proliferatum* strain ITEM 2287. *Fphch* contains five exons (117, 27, 159, 1251 and 753 bp long), and the encoded protein is 770 amino acid long. Sequence of the FPHCH protein shared

substantial similarity with the HCH and HETC^{GR} proteins from *P. anserina* and *N. crassa*, respectively. Northern and Southern hybridizations showed that *fphch* is a constitutively expressed single copy gene in *F. proliferatum*.

On the *fphch* gene we identified a region homologous to the hypervariable domain (HVD) of *N. crassa het-c*. In *N. crassa* amino acid differences in this domain are responsible for incompatibility reaction triggered by *het-c* alleles (Saupe and Glass, 1997). We assumed that *fphch* may play a role in the incompatibility system of *F. proliferatum*, provided if polymorphisms occur in the putative hypervariable region. 76 strains of *F. proliferatum* and 10 additional strains from five other species of the *G. fujikuroi* complex were examined with PCR-RFLP. We found polymorphisms in six strains that caused amino acid substitutions in the deduced HCH protein, but neither insertions nor deletions (like in *N. crassa het-c*) were found. (Accession numbers of polymorphic HVD regions are: DQ329213-DQ329218.) Based on this highly conserved nature of the *hch* gene in *G. fujikuroi* we concluded that *fphch* plays no role vegetative incompatibility reactions in *F. proliferatum*. Saupe et al. (2000) obtained similar results with *P. anserina hch* gene.

In *P. anserina* all three *N. crassa het-c* alleles are able to trigger incompatibility reaction. There are two different interpretations. One of them is that downstream targets of *het-c* are conserved in *P. anserina*. Another possible reason is that the interaction of HCH and HET-C directly influence the viability of cells. However it is not exluded that if there were polymorphisms in *hch* gene it would function as a *het* gene (Saupe et al., 2000). We mimicked this reaction in *F. proliferatum* by constructing an artificial *fphch* allele by site-directed PCR mutagenesis. We made an artificial *fphch* allele and transformed *F. proliferatum* protoplasts both with the wild type and the artificial allele. There was no difference in the number of viable protoplasts after transformation, consequently the artificial allele didn't trigger incompatibility reaction. This result produce another evidence that *hch* homologues plays no role in vegetative incompatibility in fungi, other than *N. crassa*.

To reveal the function of this highly conserved gene is, we disrupted the *fphch* gene. All Δ*fphch* mutants grew normally with no observable differences in morphology, sporulation or spore germination. Based on cultural characters Δ*fphch* mutants were indistinguishable from the wild type. We examined how the transformants behave in vegetative incompatibility experiments. As *F. proliferatum* strain ITEM 2287 has a heterokaryon self-incompatible phenotype, *nit* mutants derived from this strain do not form heterokaryons when paired on media containing nitrate as sole nitrogen source. *Nit* mutants derived from the Δ*fphch* mutants also failed to form heterokaryons with the wild type strain and did not form heterokaryons when paired with one another. Consequently the absence of *fphch* doesn't abolish the self-incompatibility. There were differences only in sexual fertility. When the Δ*fphch* mutants were used as the female parent, perithecia formed more slowly, and the total number of perithecia significantly decreased. Maturation of perithecia of the wild type

strain and the transformants needed the same time, and in matured perithecia contained asci and ascospores in every case. *Het* genes may also have an influence on sexual compatibility in other ascomycetes, however no data are available on a similar role of the *het-c* homologue. For example the inactivation of the *het-c* (not *hch*) locus *P. anserina* influences the formation of ascospores. *P. anserina het-c2* allele encodes a protein similar to glycolipid transfer proteins and this protein may contribute to maintain the appropriate composition of cell membranes and the transport of vesicles in the cell (Saupe et al., 1994). FPHCH could affect membrane composition organization that is required for formation and differentiation of sexual structures in this fungus.

New scientific results

1. Degenerate oligonucleotide primers were designed and used to amplify mating type sequences from *Fusarium* species which have no known sexual stage; RT-PCR experiments showed that these genes are constitutively transcribed in 'asexual' species of the genus.

2. We designed degenerate oligonucleotide primers for diagnostic purpose and used them for a rapid identification of mating type in 22 species of the genus *Fusarium*.

3. We isolated and sequenced an adenylate cyclase gene (*fpac1*) from *F. proliferatum.*

4. To examine the function of *fpac1,* we produced *fpac1* null-mutant strains. We found that *fpac1* isn't essential for the fungus, but plays an important role in sexual reproduction, vegetative incompatibility and adaptation to new environmental conditions.

5. We isolated a *het-c* homologue gene of *F. proliferatum* and demonstrated that this gene plays no role in the vegetative incompatibility system of the fungus.

6. We produced *fphch* null-mutant strains and found that the miss-function of *fphch* causes reduced female fertility.

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