Fungal Biology

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Genetic Transformation Systems in Fungi, Volume 2

Springer

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ISSN 2198-7777 ISSN 2198-7785 (electronic)
ISBN 978-3-319-10502-4 ISBN 978-3-319-10503-1 (eBook)
DOI 10.1007/978-3-319-10503-1
Springer Cham Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014952786

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Fungi are a highly versatile class of microorganisms and their habitats are as diverse. In nature, fungi play a crucial role in a range of degradation processes, enabling recycling of valuable raw materials by wood decaying fungi like the white rot fungus *Phanerochaete chrysosporium*. On the other hand, fungi can be pests to food production like the rice blast fungus *Magnaporthe oryzae*. Furthermore, mankind exploits the enzymatic opportunities of fungi through classical industrial processes as ethanol production by the yeast *Saccharomyces cerevisiae* and heterologous enzyme production by filamentous fungi as *Trichoderma reesei*. All these stimulated an enormous number of studies trying to understand as well as exploit the metabolic capabilities of various fungal species.

One of the game-changing breakthroughs in fungal research was the development of genetic transformation technology. This enabled researchers to efficiently modify the gene content of fungi and study the functional relevance. Interestingly, the first available method (protoplast or spheroplast transformation) evolved from an existing classical method called protoplast fusion, a process which also introduces DNA into a receiving cell however in an uncontrolled way. This publication aims to give an overview of all existing transformation methods used for yeasts and fungi.

Volume I describes in detail the different classical methods as electroporation, protoplast, Agrobacterium mediated, lithium acetate and biolistic transformation as well as more recently developed methods. Transformation methods do not describe the whole story; DNA must enter the cell, the nucleus, and finally integrate in the genome, if required also at predetermined positions. Several chapters will update on the current insights in these processes.

Volume II describes transformation-associated methods and tools as cell fusion, repetitive elements, automation, analysis, markers, and vectors; this volume reflects the many relevant elements at hand for the modern fungal researcher.

This publication is meant not only as reference material for the experienced researcher, but also as introduction for the emerging scientist. Therefore, all methods are supported by several illustrative example protocols from various fungal species and laboratories around the world, which will be a good starting position to develop a working protocol for other fungal species being studied.

Delft, The Netherlands — Marco A. van den Berg
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Part I

Endogenous DNA: Cell Fusion

Anastomosis and Heterokaryon Formation

1

Martin Weichert and André Fleißner

1.1 Introduction

In filamentous ascomycete fungi, vegetative cell fusion or anastomosis formation constitutes a common growth feature promoting the establishment and expansion of mycelial colonies. Fusion occurs at different developmental stages, where anastomosis might serve various physiological functions (Köhler 1930; Craven et al. 2008; Ruiz-Roldan et al. 2010; Roca et al. 2012). In many fungal species, colony initiation includes the fusion of germinating vegetative spores into supracellular networks, which further develop into the mycelium (Roca et al. 2005a; Fleißner 2012). This merger of numerous individuals into one functional unit appears to provide a competitive advantage and promotes successful habitat colonization (Richard et al. 2012). Within established mycelial colonies, fusion between hyphal branches increases interconnectedness, thereby supporting homeostasis and other vital functions of the mycelium (Hickey et al. 2002).

Recent years have seen a revival of scientific interest in the mechanism and physiological outcomes of anastomosis formation. The vast majority of these studies employed the red bread mold *Neurospora crassa* as a model system (Read et al. 2012). However, other species, including important plant pathogenic fungi, have begun to emerge as experimental objects in fusion research and to make significant contributions to the field (Engh et al. 2007; Craven et al. 2008; Ruiz-Roldan et al. 2010; Roca et al. 2012; Charlton et al. 2012).

While the physiological role of anastomosis is just beginning to surface, the ability of fungal hyphae to fuse has long been experimentally exploited. Examples include genetic mapping in asexual fungi using parasex (Debets et al. 1990, 1993), testing allelism in heterokaryons, or combining strain features by fusing different individuals (Todd et al. 2007; Fleissner et al. 2009b; Roca et al. 2010; Dettmann et al. 2012). On a larger scale, fungal heterokaryons have also been employed in biotechnological applications (Stuart 1997)—a strategy which might still hold much untapped potential for production strain improvement.

1.2 Vegetative Fusion

1.2.1 Germling Fusion

Fusion of genetically identical conidia or conidial germlings during colony initiation has been described in numerous filamentous ascomycete species. A literature survey concluded that fusion at this early developmental stage has been reported in more than 70 species covering more

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M.A. van den Berg and K. Maruthachalam (eds.), *Genetic Transformation Systems in Fungi, Volume 2*, Fungal Biology, DOI 10.1007/978-3-319-10503-1_1,

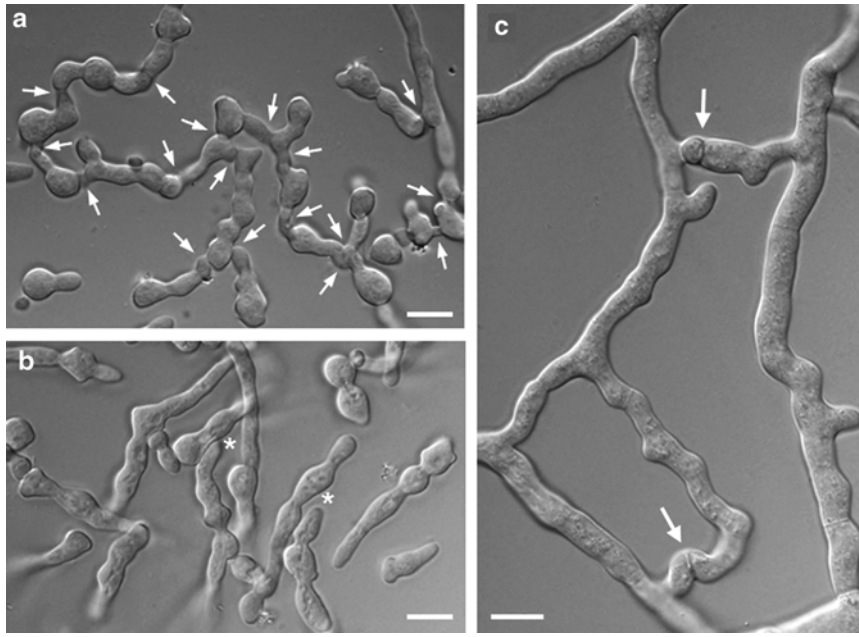

Fig. 1.1 Vegetative cell-to-cell fusion in *Neurospora crassa*. (**a**) Germinating conidia in close proximity readily undergo chemotropic interactions, establish physical contact and finally fuse with each other (*arrows*). As a result, a cellular network is formed, which further develops into the mycelial colony. (**b**) Deletion of the gene *so* (*soft*) completely abolishes intercellular communication. In contrast to wild-type, mutant cells grow in parallel and do not attract each other. Random contact (*asterisks*) of Δ*so* germlings does not result in fusion. (**c**): In the interior part of a mature mycelium, hyphae interconnect via fusion bridges (*arrows*). Hyphal fusion promotes the distribution of nutrients and organelles within the colony

than 20 genera (Roca et al. 2005a). Fusion of spores and/or germlings often involves the formation of specialized hyphal structures termed *c*onidial *a*nastomosis *t*ubes (CATs). These cell protrusions possess characteristic features distinguishing them from germ tubes. They are of significantly thinner diameter, exhibit only limited extension over shorter distances, and do not branch. In contrast to germ tubes, which typically avoid each other, CATs exhibit positive autotropism, resulting in physical contact and fusion (Roca et al. 2005b). Germling fusion is a highly orchestrated multistep process, which comprises a carefully regulated succession of cellular events. These include fusion competence, cell-cell signaling, directed growth, cell adhesion, cell wall breakdown, and finally plasma membrane merger. Formation of the fusion pore allows cytoplasmic mixing and the exchange of organelles, including nuclei, between the two fusion partners. Repeated fusion events within a spore population eventually results in the merger of most individuals into a supracellular network (Fig. 1.1a), which further develops into a mycelial colony.

1.2.2 Hyphal Fusion

Within the mycelium, hyphae fuse via short branches, resulting in hyphal cross-connections, which stimulate the interconnectedness within the fungal colony (Fig. 1.1c). The two processes of germling and hyphal fusion appear to be mechanistically related, since so far all mutant strains affected in conidial fusion were, when tested, also deficient in hyphal fusion. The formation of fusion bridges between hyphae typically occurs in the colony interior, while the periphery of the colony lacks hyphal fusion, indicating differences in fusion competence. Determinants establishing this competence are so far unknown. Fusion-competent hyphal branches actively

attract each other via an unknown interhyphal signaling mechanism and re-orientate their growth direction. After establishing physical contact, polar (apical) tip extension turns into non-polar (isotropic) growth, resulting in the swelling of the opposing hyphal tips. Upon growth arrest, the formation of a fusion pore is initiated, which is followed by the exchange of cytoplasm and organelles between the fused hyphal compartments (Hickey et al. 2002). During pore formation, the Spitzenkörper of the two fusion hyphae remain associated with the fusion point. In actively growing hyphae, this organelle serves as a vesicle supply center, which controls the transfer of vesicles to the plasma membrane of the growing tips. Its presence at the forming fusion pore suggests specific functions during the fusion process, such as the controlled delivery of vesicles containing cell wall degrading enzymes or the plasma membrane fusion machinery.

1.3 The Role of Hyphal Fusion in Colony Establishment and Development

It has long been proposed that vegetative fusion and network formation in filamentous fungi promote genetic and nutritional exchange, thereby increasing the fitness and competitiveness of mycelial colonies (Ward 1888; Rayner 1996). Recently, these hypotheses were supported by experimental evidence. In *N. crassa*, significant translocation of nutrients takes place within wild-type mycelia, thereby allowing the expansion of the colony also in heterogeneous environments. In mutants deficient in hyphal fusion, however, translocation of nutrients within individual growing mycelial units is significantly reduced (Simonin et al. 2012). The growth front of these fusion mutants typically appears frayed and is comprised of individual hyphae of various lengths. In contrast, wild-type colonies possess very even boundaries, suggesting an equal resource distribution to individual hyphae (Fleissner et al. 2005; Richard et al. 2012). The reduced translocation capacity of fusion mutants appears to be not only caused directly by the lack of cross-connection within the colony. In *N. crassa* wild-type hyphae, cytoplasmic flow can occur at high speeds of up to 60 μm/s (Lew 2005). In fusion mutants, the cytoplasmic streaming appears to be restricted (Roper et al. 2013), suggesting that pressure differences between fusing hyphae contribute to the driving forces of cellular flows.

Interestingly, exchange of resources and nuclei between different *N. crassa* individuals via hyphal fusion was only observed in germlings or young undifferentiated colonies, which had not developed clear morphological differences between the colony interior and the periphery, but not between mature mycelia (Simonin et al. 2012). Similarly, in arbuscular mycorrhizal fungi, anastomosis appears to be restricted to certain stages of the life cycle, suggesting that developmental windows of fungal cooperation exist (Purin and Morton 2013). As an outcome of cooperation, fused *N. crassa* germlings gained a competitive advantage, depending on the habitat structure and the initial spore density. While in wild-type strains the number of spores positively correlates with growth rates, in a fusion mutant, increased spore concentrations were neutral or even constituted a disadvantage (Richard et al. 2012). Merging individuals into one functional unit might support coordinated growth and resource consumption, while a lack of fusion creates a population of independent individuals competing for space and nutrients.

In addition to forming functional units, anastomosis between non-clonal individuals results in heterokaryotic mycelia. Such an exchange of genetic material can be followed by parasexual recombination, which is considered to contribute to genetic richness in many fungal species. Genera for which this type of reproduction has been reported include the parasex model *Aspergillus*, but also important plant pathogens, such as *Magnaporthe*, *Alternaria*, or *Colletotrichum* (Stewart et al. 2013; da Silva Franco et al. 2011; Noguchi et al. 2006). During parasex, karyogamy in heterokaryotic hyphae results in instable polyploid nuclei, allowing the recombination of genetical material. During the following mitotic

divisions, surplus chromosomes are successively and randomly lost, until the stable, original ploidity state is restored. Parasexual recombination is nowadays considered to be a driving force of fungal evolution, diversity, fitness, and the emergence of new virulent strains (Roper et al. 2011; Baskarathevan et al. 2012; Clay and Schardl 2002). In *A. nidulans*, isogenic diploid strains undergoing parasexual recombination had a significantly higher fitness than haploid isolates (Schoustra et al. 2007), illustrating the potential of parasex to promote competitiveness and adaptation.

Anastomosis formation appears not only to enable parasex, but also to contribute to the maintenance of genetic diversity in heterokaryotic mycelia by mixing flows. In *N. crassa* fusion mutants, different nucleotypes segregate out into colony sectors and the genetic richness is quickly lost, while wild-type colonies maintain their nuclear diversity (Roper et al. 2013).

In nature, however, hyphal fusion between different colonies bears the risk of transmitting infectious agents or resource plundering by aggressive genotypes (Glass and Kaneko 2003). Anastomosis appears to be the main route of mycovirus transmission and has been used experimentally as well as a strategy to control phytopathogenic species (Dawe and Nuss 2013; Ghabrial and Suzuki 2009). An extreme example of anastomosis followed by taking over by one genotype is illustrated in *Fusarium oxysporum*. Here, fusion between germlings results in the quick degradation of the nucleus in the receptive hyphae, thereby extinguishing the recipient's genotype (Ruiz-Roldan et al. 2010).

In order to prevent such deleterious effects of anastomosis formation, many species restrict non-self-fusion by a genetic vegetative incompatibility system. If genetically incompatible hyphae fuse, the merged compartments are quickly sealed and cell death is induced (Glass and Dementhon 2006). This mechanism can efficiently restrict mycovirus transmission, as shown, for example, in the chestnut blight fungus *Cryphonectria parasitica* (Choi et al. 2012). As indicated by the term "vegetative incompatibility", this control mechanism is differentially active at the various developmental stages of the fungal life cycle, thereby providing temporal windows for genetic mixing. In *N. crassa,* for example, no incompatibility is observed during the sexual fusion of mating partners, while vegetative fusion of strains of opposite mating type is quickly followed by an efficient cell death response (Glass and Kuldau 1992). In the plant pathogen *Colletotrichum lindemuthianum*, heterokaryon incompatibility is suppressed during fusion of germinating conidia, allowing a window of cooperation even during vegetative growth. As a result, heterokaryotic strains are formed, whose conidia grow into cultures with phenotypic features different from the parental strains. Temporal suppression of heterokaryon incompatibility in order to allow parasex therefore provides an asexual way of increasing genetic diversity (Ishikawa et al. 2012).

Exceptional temporal suppression of incompatibility during the fusion between conidia or hyphae of different species has also been discussed as a route for horizontal gene transfer (Richards et al. 2011; van der Does and Rep 2007). So far, the contribution of interspecies fusion to the generation of genetic diversity is not assessable. While an early study indicated that fusion between different species is uncommon (Köhler 1930), conidial anastomoses between two different Colletotrichum species have been documented. Strains originating from these hybrids exhibited intermediate phenotypes, suggesting that they were recombinants (Roca et al. 2004). Therefore, further investigations will be essential to determine the significance of interspecies germling and/or hyphal fusion in creating genetic richness in fungi.

In addition to promoting the exchange of nutrients and genetic material, anastomosis formation can serve various other functions during fungal growth and development, such as the formation of specific three-dimensional structures or hyphal repair. For example, in nematode trapping fungi, such as *Arthrobotrys oligospora*, the individual loops of the net-like traps are formed by the fusion of a hyphal branch and a small peg emerging from the same parental hypha (Nordbring-Hertz et al. 1989). In *Trichophyton*,